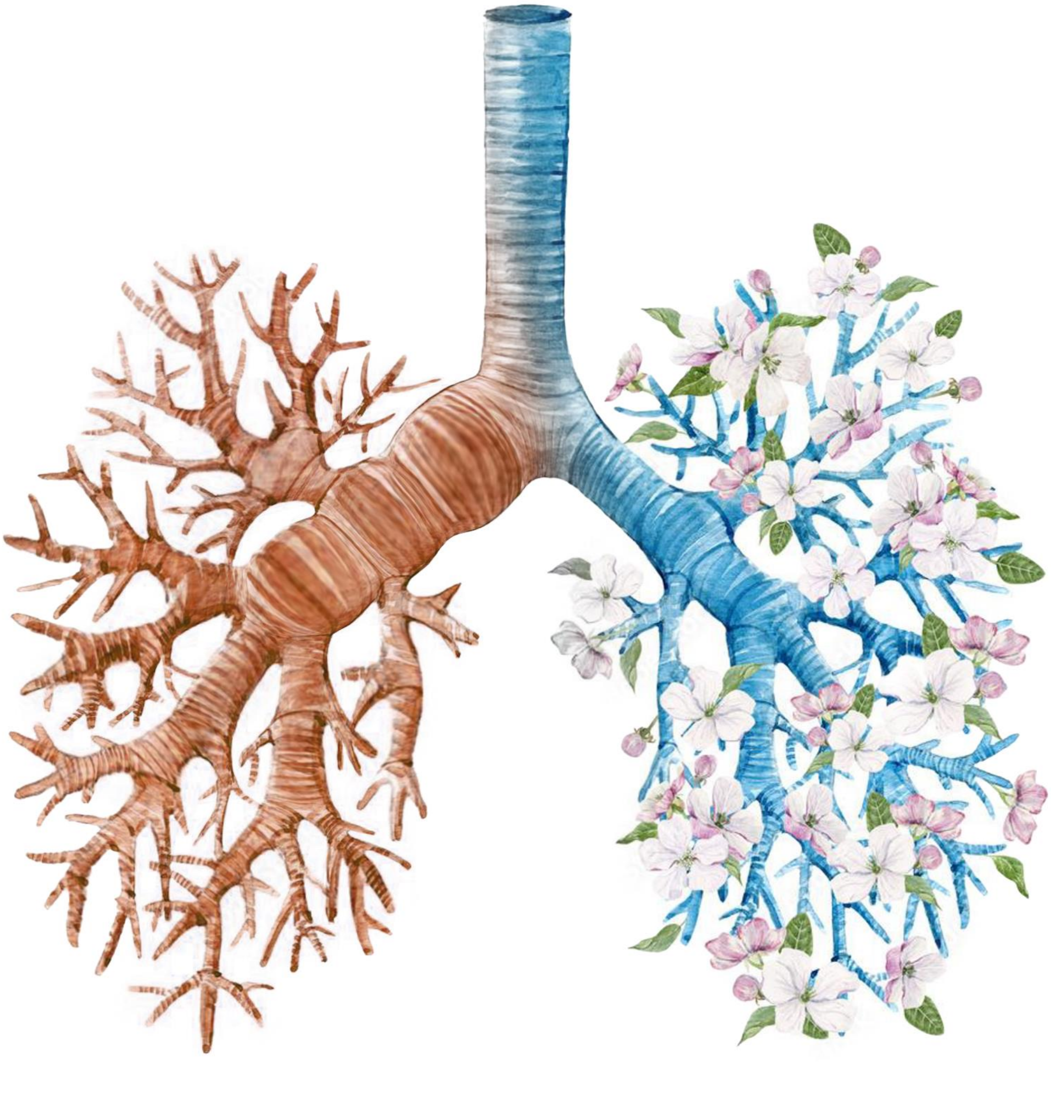
UNIVERSITAT DE BARCELONA

Asthma and obesity. Glucocorticoid sensitivity before and after bariatric surgery. Effect of vitamin D

Marina Bantulà Fonts

ASTHMA AND OBESITY

GLUCOCORTICOID SENSITIVITY BEFORE AND AFTER BARIATRIC SURGERY. EFFECT OF VITAMIN D.



Marina Bantulà Fonts

Doctoral Thesis, 2022

Asthma and obesity. Glucocorticoid sensitivity before and after bariatric surgery. Effect of vitamin D.

(Asma i obesitat. Sensibilitat als glucocorticoides abans i després de cirurgia bariàtrica. Efecte de la vitamina D.)

Tesi presentada per

Marina Bantulà Fonts

Per a l'obtenció del títol de doctora per la Universitat de Barcelona.

Dirigida per

Dra. Ebymar Arismendi Núñez
(Directora)

Dr. Jordi Roca Ferrer
(Director)

Dr. Joan Bartra Tomás
(Tutor)

Programa de Doctorat en Biomedicina
Facultat de Medicina, Universitat de Barcelona
2022

A la meva mare i al meu pare.

AGRAÏMENTS

Per començar, m'agradaria agrair al **Dr Picado** i a la **Dra Arismendi** haver confiat en mi i haver-me donat l'oportunitat de fer el Doctorat amb ells. Gràcies per ensenyar-me el camí a seguir per desenvolupar un projecte, per aportar inspiració i noves idees per resoldre dubtes reals que es plantegen durant la pràctica clínica. Gràcies, per la vostra dedicació i orientació durant aquests cinc anys. Poder treballar en un projecte d'investigació havia sigut moltes vegades un somni.

A l'altra persona a qui li vull agrair la seva dedicació és a en **Jordi Roca**. Gràcies per l'acompanyament amb tots els temes relacionats amb els experiments de laboratori. M'has ensenyat a tenir pensament crític i a fer-me les preguntes necessàries per acabar entenent els resultats, que com bé sabem, no sempre són del tot obvis.

També m'agradaria agrair al **Dr Mullol** l'oportunitat que m'ha donat per participar ens els diferents cursos i xerrades organitzats durant aquets temps, així com la seva disponibilitat i amabilitat a l'hora de resoldre dubtes.

A la **Mireya**, quién des del primer día ha estado allí, haciendo el trabajo más cómodo y viendo la parte positiva a los contratiempos tanto científicos como personales. Por tu sinceridad, cariño, motivación y todos los mediodías compartidos, que no han sido pocos!

A la **Maria**; amb tu recordo dels primers dies al laboratori, aprenent la tècnica juntes i, ja més tard, totes les vegades que has estat al meu costat per ajudar-me amb els centenars d'extraccions de RNA, a fer ELISAs, a rotular tubs o a buscar mostres al congelador. Gràcies per tot això i, sobretot, per les converses compartides entre experiment i experiment.

A **Valeria**, darte las gracias por involucrarte en todos mis proyectos, en muchos has sido el empujón necesario para terminarlos. Gracias por tu cariño, ánimos, comprensión y empatía durante todo este tiempo, des de los primeros días en cultivos, hasta día de hoy.

A la meva amiga **Sonia**, donar-te les gràcies per tots els moments compartits, per escoltar-me, animar-me i ajudar-me a superar en moments on jo no ho veia del tot clar. Em sento afortunada d'haver-te conegut i haver format un bon equip juntes!

També m'agradaria donar les gràcies a la **Mariona**, per acabar trobant un forat i ajudar-me sempre que ho he necessitat, i per les reunions d'al·lèrgia compartides, juntament amb en **Joan**, la **Marga** i la **Rosa**. Gràcies a a tots quatre per haver-me donat, en més d'una ocasió, l'espai per explicar com anaven avançant els resultats de la tesi. Sempre heu tingut alguna pregunta o idea a tenir en compte en el projecte.

A **Lucía**, quería darte las gracias por tus ánimos constantes en esta recta final y, junto con **Laia**, por hacerme ver que el tiempo pasa, por confiar en que yo tendré respuestas a vuestras preguntas y darme cuenta de que sí que sé dónde encontrarlas. Me he dado cuenta de todo lo aprendido durante este tiempo, y eso es reconfortante. Y con esta reflexión me acuerdo de **Borja**, quién fue el primero que me enseñó de qué trataba esto de realizar un doctorado, siempre con buenos consejos para ofrecer.

I ja acabant amb el grup de l'IRCE, agrair-li a la **Conchi** haver pogut conèixer la seva línia de recerca, que no em deixa de fascinar. També a tot l'equip d'infermeres del Centre de Diagnòstic Respiratori, de la

6.0, la seva amabilitat i disponibilitat en rebre pacients i realitzar la recollida de mostres, podent organitzar la feina de forma àgil i còmode.

Fora del propi grup de recerca també he tingut molta sort. A la mateixa planta del Cellex, la 2B, he fet bons amics, hem compartit el dia a dia, els aparells de laboratori, els dubtes i hem acabat celebrant Nadals, estius i aniversaris.

Per començar, A la **Raquel** i a la **Leti**, mis amigas, ha sido un regalo compartir estos años a vuestro lado. Des de los primeros after-curros, hasta día de hoy, acompañándonos en nuestros dilemas de la vida i, sobretodo, riendo, riendo mucho! Os quiero!

Al meu amic **Ruben**, sempre recordaré els inicis al laboratori, puntes i provetes grup amunt i grup avall. Tu què fas? Tu què tens? Què em deixes? i així fins a l'últim dia, aprenent l'un de l'altre i enriquint-nos junts. Per entendre'ns científicament i personalment, gràcies per tot!

A la resta de grupillo de la hora del cafè, a la **Nonna**, a l'**Alba**, a l'**Àlex**, i un llarg etcètera, que fan que els dies a la feina siguin més agradables, ja des de primera hora del matí.

A en **Jordi**, per la teva disponibilitat quan t'he vingut a veure amb dubtes de citometria, i per deixar-me reactius i anticossos sempre que els he necessitat. Agrair també a la **Mari**, la **Natalia**, l'**Elisenda** i la **Marta** el seu coneixement i amabilitat per fer entre totes un laboratori on poder compartir els recursos i, així, fer la feina més fàcil i còmode. Així com també agrair a la **Ruth**, la **Cheles** i la **Lourdes**, per aconsellar-me sempre que ho he necessitat i per tots els dubtes resolts sobre genètica, PCRs i extraccions d'àcids nucleics.

Un especial agraïment a en **Roberto**. Por todas las bromas que hemos ido acumulando, que han hecho que cada día fuese distinto y divertido. Desde el sol solet cubà, a cortar carreteras, el plantón en Madrid, o...brownie!, entre muchas otras.

Tinc la sort d'estar envoltada de persones meravelloses tant a dins com a fora del lab. És per això que m'agradaria agrair als dos grans grups d'amigues i amics el saber-nos cuidar i donar suport amb les decisions que anem prenent a la vida. En especial, a l'**Anna Ripoll**, saber que hi ets em fa feliç. A la **Blanca** i a la **Sandra**, les meves estimades cosines, per fer-me costat sempre. A l'**Andreu**, a la **Ingrid**, a la **Ismat**, i al **Marc Serra**, per no conformar-nos només amb el que ens expliquen. Per buscar altres maneres d'entendre la el cos humà i la medicina. També vull donar les gràcies al **Lluís**, per tots els inicis, i a l'**Aïna** i a la **Marta Martí**, pels confinaments. A tots tres, per viure el Doctorat des de casa.

Per a últim, agrair al **Raul** aquesta mirada amable, calmada i segura. Per mostrar-me l'orgullós que està de mi i per saber valorar la feina ben feta.

Per acabar amb els agraïments, vull donar les gràcies a la meva família. A la meva **mare**, per ajudar-me a complir els meus somnis. "Marina, tu pots!" Gràcies per estar sempre al meu costat, no deixar que m'enfonsi i per tenir recursos per (quasi) tot. Al meu **pare**, pels ànims constants i per aprendre que la vida és per viure-la feliç, pel bon humor i l'optimisme. A la meva germana **Helena**, per ser exemple de constància, dedicació i responsabilitat. Tot esforç té la seva recompensa. I per acabar, m'agradaria recordar els avis, en especial a la **iaia Carmen**. Ella m'ha vist començar aquest camí i sempre em deia que un dia em veuria per la tele fent un gran descobriment.

“Fear tends to come from ignorance.
Once I knew what the problem was,
it was just a problem, nothing to fear.”

Patrick Rothfuss
The Name of the Wind

TABLE OF CONTENTS

SUMMARY / RESUMEN / RESUM

SUMMARY

This thesis has been designed to characterize the low-grade chronic inflammation present in obesity in patients suffering from asthma, and to describe some of the mechanisms involved in the glucocorticoid hyporesponsiveness seen in clinical practice. Moreover, the effects of weight loss through bariatric surgery on the inflammatory status and on glucocorticoid response of the asthma-obesity association have been defined. Finally, the vitamin D effect on cell proliferation and on glucocorticoid sensitivity has been discussed.

It seems quite well demonstrated that obesity is associated with asthma, however, the mechanisms that link the two processes are surely very complex. The inflammatory process underlying both diseases could be one of the potential connections between them. The excessive accumulation of adipose tissue in subjects with obesity results in an increased production of pro-inflammatory cytokines (IL-1β, IL-6, IL-18, TNF-α, MCP-1) and adipokines such as leptin, and a reduction in the release of adiponectin, with anti-inflammatory properties. This low-grade chronic systemic inflammation present in obesity contributes towards increasing the systemic and/or airway inflammatory process underlying asthma.

Inhaled corticosteroids (ICS) are considered the cornerstone of controller therapy for asthmatic patients. ICS have the ability to reduce airway inflammation, airway obstruction, airway hyperresponsiveness, and asthma symptoms. However, asthmatic patients with obesity are more likely to have a poor response to glucocorticoids: they have reduced odds of achieving asthma control, higher risk of asthma hospitalizations, and lower quality of life compared with asthmatics with a normal body mass index. Detrimental effects of obesity on lung function and additive or synergistic effects of obese systemic inflammation on airways inflammation, have been proposed as potential mechanisms to explain glucocorticoid hyporesponsiveness in asthmatic patients with obesity. Moreover, a defective induction of anti-inflammatory genes by glucocorticoids, such as mitogen-activated protein kinase phosphatase-1 (MKP-1), has been described as another possible mechanism of the reduced response to glucocorticoids seen in asthmatic patients with obesity.

Moreover, bariatric surgery is considered the most effective and sustained long-term treatment of severe obesity. Weight reduction significantly improves systemic and adipose tissue inflammatory activity levels. However, the effect of weight loss on specific molecules potentially involved in the inflammatory process in obesity and in obesity-related asthma remains to be examined. Several studies have shown an improvement in asthma control, medication use, hospitalization rate, and lung function after weight loss via bariatric surgery. However, no studies have assessed glucocorticoid sensitivity before and after bariatric surgery.

Finally, vitamin D is a hormone with pleiotropic effects and numerous regulatory mechanisms beyond bone health. Evidence from observational studies suggests that obesity is associated with vitamin D deficiency. Furthermore, low serum vitamin D levels have been associated with asthma exacerbations. Other reports described an association between vitamin D deficiency and the risk of glucocorticoid resistance. There is also *in vitro* evidence for vitamin D increasing glucocorticoid sensitivity. In peripheral blood mononuclear cells from steroid resistant asthmatic patients, the active form of vitamin D (1,25-dihydroxyvitamin D) increases the expression of MKP-1, thus enhancing glucocorticoid inhibition of cell proliferation.

RESUMEN

Esta tesis ha sido diseñada para caracterizar la inflamación crónica de bajo grado presente en sujetos con obesidad que padecen asma y para describir algunos de los mecanismos implicados en la baja respuesta a los glucocorticoides que se observa en estos pacientes durante la práctica clínica. Además, se han definido los efectos de la pérdida de peso mediante cirugía bariátrica sobre el estado inflamatorio y sobre la respuesta a los glucocorticoides en la asociación asma-obesidad. Finalmente, se ha discutido el efecto de la vitamina D sobre la proliferación celular y sobre la sensibilidad a los glucocorticoides.

Existen múltiples evidencias que muestran una asociación entre la obesidad y el asma. Sin embargo, los mecanismos involucrados en la relación entre ambos procesos son de alta complejidad. El proceso inflamatorio subyacente en ambas enfermedades podría ser una de las posibles conexiones entre ellas. En la obesidad, adipoquinas proinflamatorias como la leptina están aumentadas, mientras que hay una reducción en la liberación de adiponectina, con propiedades antiinflamatorias. Además, la acumulación excesiva de tejido adiposo da como resultado una mayor producción de citoquinas proinflamatorias (IL-1β, IL-6, IL-18, TNF-α, MCP-1). Esta inflamación crónica de bajo grado presente en la obesidad contribuye a aumentar el proceso inflamatorio sistémico y/o de las vías respiratorias que subyace al asma.

Los corticosteroides inhalados (ICS) se consideran la piedra angular de la terapia de control para pacientes asmáticos. Los ICS tienen la capacidad de reducir la inflamación, la obstrucción, la hiperreactividad de las vías respiratorias y los síntomas del asma. Sin embargo, es más probable que los pacientes asmáticos con obesidad presenten una respuesta reducida a los glucocorticoides. Este subgrupo de pacientes tiene un mayor riesgo de hospitalizaciones por un peor control del asma y, por lo tanto, una calidad de vida reducida en comparación con los asmáticos con un índice de masa corporal normal. Los efectos perjudiciales de la obesidad sobre la función pulmonar y los efectos aditivos o sinérgicos de la inflamación sistémica crónica sobre la inflamación de las vías respiratorias, se han propuesto como mecanismos potenciales para explicar la baja respuesta a los glucocorticoides en pacientes asmáticos obesos. Además, se ha descrito una inducción defectuosa de genes antiinflamatorios por parte de los glucocorticoides, como por ejemplo, la proteína cinasa fosfatasa-1 activada por mitógeno (MKP-1), como otro posible mecanismo de la respuesta reducida a los glucocorticoides observada en pacientes asmáticos con obesidad.

Además, la cirugía bariátrica se considera el tratamiento a largo plazo más efectivo y sostenido de la obesidad grave. La reducción de peso mejora significativamente los niveles de inflamación sistémica y del tejido adiposo. Sin embargo, queda por examinar el efecto de la pérdida de peso sobre moléculas específicas potencialmente involucradas en el proceso inflamatorio del asma asociado a la obesidad. Varios estudios han demostrado una mejora en el control del asma, el uso de medicamentos, la tasa de hospitalización y la función pulmonar después de la pérdida de peso mediante cirugía bariátrica. Sin embargo, ningún estudio ha evaluado la sensibilidad a los glucocorticoides antes y después de la cirugía bariátrica.

Finalmente, la vitamina D es una hormona con efectos pleiotrópicos y numerosos mecanismos reguladores más allá de la salud ósea. La evidencia de estudios observacionales sugiere que la obesidad está asociada con la deficiencia de vitamina D. También se ha encontrado una asociación entre niveles bajos de vitamina D en suero y exacerbaciones del asma. Otros estudios describieron una asociación entre la deficiencia de vitamina D y el riesgo de resistencia a los glucocorticoides. También hay evidencia

in vitro de que la vitamina D aumenta la sensibilidad a los glucocorticoides. En las células mononucleares de sangre periférica de pacientes asmáticos resistentes a los glucocorticoides, la forma activa de la vitamina D (1,25-dihidroxivitamina D) aumenta la expresión del gen MKP-1, lo que aumenta la inhibición de la proliferación celular por parte de los corticosteroides.

RESUM

Aquesta tesi ha estat dissenyada per tal de caracteritzar la inflamació crònica de baix grau present en persones asmàtiques amb obesitat i per descriure alguns dels mecanismes implicats en la baixa resposta als glucocorticoides que s'observa a la pràctica clínica. A més, s'han definit els efectes de la pèrdua de pes a través de la cirurgia bariátrica sobre l'estat inflamatori i sobre la resposta als glucocorticoides en l'associació asma-obesitat. Finalment, s'han discutit els efectes de la vitamina D sobre la proliferació cel·lular i sobre la sensibilitat als glucocorticoides.

Existeixen múltiples evidències que mostren una associació entre l'obesitat i l'asma. No obstant, els mecanismes involucrats en la relació entre els dos processos són d'elevada complexitat. El procés inflamatori present en les dues malalties podria ser una de les possibles connexions entre elles. En l'obesitat, adipoquines proinflamatòries com la leptina estan augmentades, mentre que hi ha una reducció en l'alliberació d'adiponectina, amb propietats antiinflamatòries. A més, l'acumulació excessiva de teixit adipós té com a resultat una major producció de citocines proinflamatòries (IL-1β, IL-6, IL-18, TNF-α, MCP-1). Aquesta inflamació crònica de baix grau present en l'obesitat contribueix a augmentar el procés inflamatori sistèmic i/o de les vies respiratòries present en l'asma.

Els corticosteroides inhalats es consideren la pedra angular de la teràpia de control per a pacients asmàtics. Els ICS tenen la capacitat de reduir la inflamació, l'obstrucció, i la hiperreactivitat de les vies respiratòries, així com millorar els símptomes de l'asma. No obstant, és més probable que els pacients amb asma i obesitat presentin una resposta reduïda als glucocorticoides. Aquest subgrup de pacients té un major risc d'hospitalitzacions per un pitjor control de l'asma i, per tant, una qualitat de vida reduïda en comparació amb els asmàtics amb un índex de massa corporal normal. Els efectes perjudicials de l'obesitat sobre la funció pulmonar i els efectes additius o sinèrgics de la inflamació sistèmica crònica sobre la inflamació de les vies respiratòries, s'han proposat com a mecanismes potencials per explicar la baixa resposta als glucocorticoides en pacients asmàtics amb obesitat. A més, s'ha descrit una inducció defectuosa de gens antiinflamatoris per part dels glucocorticoides, com per exemple, la proteïna cinasa fosfatasa-1 activada per mitògen (MKP-1), com un altre possible mecanisme de la resposta reduïda als glucocorticoides observada en pacients asmàtics amb obesitat.

Per altra banda, la cirurgia bariátrica es considera el tractament a llarg plaç més efectiu i sostingut per l'obesitat greu. La reducció de pes millora significativament el nivell d'inflamació sistèmica i del teixit adipós. No obstant, falta estudiar l'efecte de la pèrdua de pes sobre les molècules especifiques potencialment involucrades en el procés inflamatori en l'asma associada a l'obesitat. Diferents estudis han demostrat una millora en el control de l'asma, en l'ús de medicaments, en la taxa d'hospitalització i en la funció pulmonar desprès de la pèrdua de pes a través de la cirurgia. Tot i així, no hi han estudis avaluant la sensibilitat als glucocorticoides abans i desprès de la cirurgia bariàtrica.

Finalment, la vitamina D és una hormona amb efectes pleiotròpics i nombrosos mecanismes reguladors més enllà de la salut òssia. L'evidència d'estudis observacionals suggereix que l'obesitat està associada amb la deficiència de vitamina D. També s'ha trobat una associació entre els nivells baixos de vitamina D en sèrum i les exacerbacions de l'asma. Altres estudis han descrit una associació entre la deficiència de vitamina D i el risc de resistència als glucocorticoides. També hi ha evidencia in vitro de que la vitamina D augmenta la sensibilitat als glucocorticoides. En cèl·lules mononuclears de sang perifèric de pacients asmàtics resistents als glucocorticoides, la forma activa de la vitamina D (1,25-

dihidroxivitamina D) augmenta l'expressió del gen MKP-1 i, per tant, augmenta la inhibició de la proliferació cel·lular per part dels corticosteroides.

LIST OF FIGURES

ABBREVIATIONS

$1,25(OH)_2D$	1,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
ACQ	Asthma control questionnaire
ACT	Asthma control test
AHR	Airway hyporesponsiveness
BALF	Bronchoalveolar lavage fluid
BMI	Body mass index
COX-2	Cyclooxygenase-2
CYP	Cytochrome P450
ERM	Ezrin/radixin/moesin
FEV_1	Forced expiratory volume in one second
FRC	Functional residual capacity
GILZ	Glucocorticoid-induced leucine zipper
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GR	Glucocorticoid receptor
GRE	Glucocorticoid response elements
IC_{50}	Dexamethasone half maximal inhibitory concentration
ICS	Inhaled corticosteroids
IFN-γ	Interferon gamma
IgE	Immunoglobulin E
IL	Interleukin
ILC	Innate lymphoid cells
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein
miRNA	microRNA
MKP-1	MAPK phosphatase-1
mRNA	Messenger RNA
NFκB	Nuclear factor kappa B
NLRP3	Nucleotide-binding domain-like receptor protein 3
PBMCs	Peripheral blood mononuclear cells
PHA	Phytohemagglutinin
RCTs	Randomized control trials
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
sST2	Soluble ST2
ST2	Suppression of tumorigenicity 2
T2	Type 2
TGF-β	Transforming growth factor beta
Th	T helper
TLR	Toll-like receptor
TNFR1	TNF-α receptor 1
TNFR2	TNF-α receptor 2
TNF-α	Tumor necrosis factor alpha
Treg	T regulatory
TSLP	Thymic stromal lymphopoietin
TTP	Tristetraprolin
VDR	Vitamin D receptor
VEGF	Vascular endothelial growth factor
YKL-40	Chitinase-3-like protein 1

INTRODUCTION

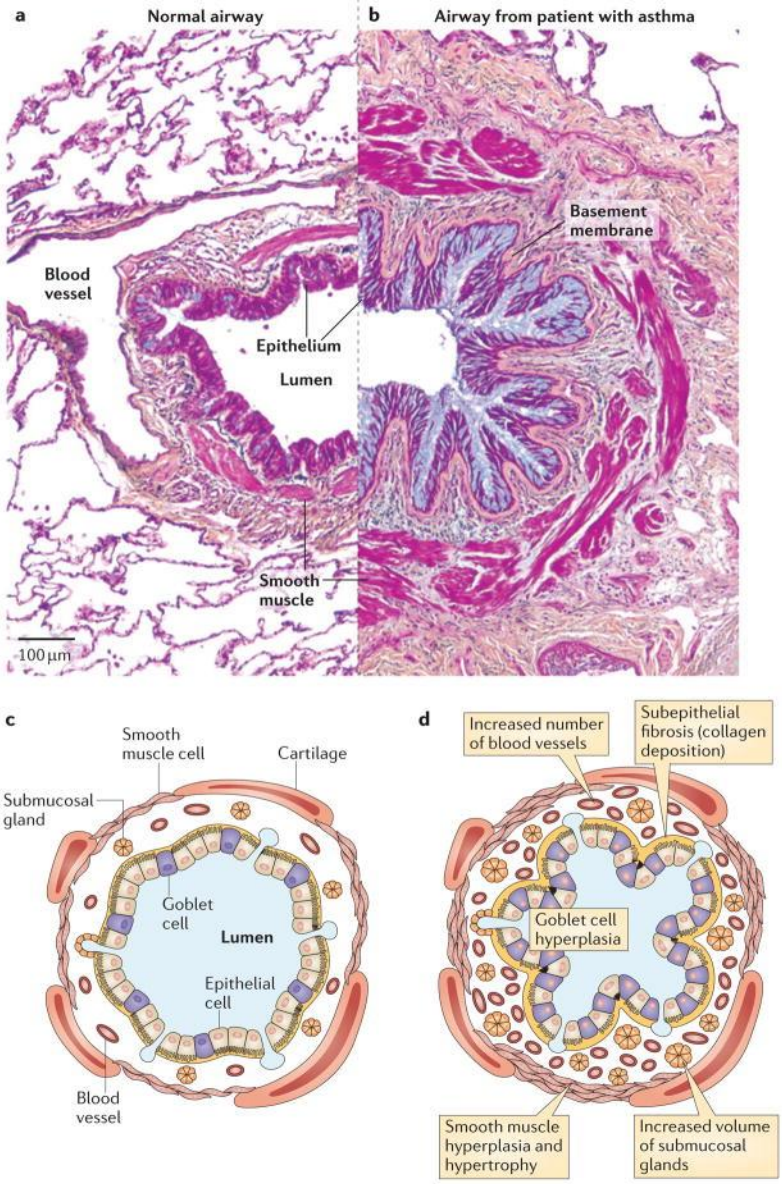
1. State-of-the art

Asthma and obesity are two epidemics affecting the developed world. The relationship between obesity and asthma, appears to be weight-dependent, causal, partly genetic and probably bidirectional. Adipose tissue is an important source of adipokines, such as leptin, which is a pro-inflammatory cytokine and adiponectin with anti-inflammatory effects. The inflammatory process underlying obesity also involves the synthesis of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor alpha (TNF-α), and transforming growth factor beta (TGF-β), and some of them contribute to asthma pathogenesis. The resulting regulatory effects of the immunomodulatory pathways underlying both diseases have been hypothesized to be one of the mechanisms by which obesity increases asthma risk and severity. Moreover, obesity is associated with vitamin D deficiency. The effects of vitamin D on bone health are well stablished, and there is increasing evidence of a role for vitamin D in blood pressure and immune system regulation. Furthermore, several studies have reported an association between serum levels of vitamin D and asthma exacerbations. It is described that glucocorticoids are less effective in obese than in lean subjects with asthma. There is growing literature on the association between vitamin D deficiency and the risk of glucocorticoid resistance. Weight reduction by diet, exercise or bariatric surgery reduces inflammatory activity and improves asthma symptoms and lung function. Airway hyperresponsiveness (AHR), a defining feature of asthma, is decreased after weight-loss, suggesting that obesity was causal for their asthma. Moreover, bariatric surgery improves medication use, hospitalization rate, lung function and inflammatory markers.

2. Asthma

Asthma affected an estimated 262 million people in 2019 - with at least 445,000 deaths — which makes it the most common chronic lung disease [1]. The prevalence of asthma has markedly increased over the last decades, especially in Westernized countries [2,3]. In Spain, the prevalence of asthma ranges between 1.5 and 16.7% in the adult population, and about 10% in the paediatric population [4].

Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough, that vary over time and in intensity, together with variable expiratory airflow limitation [5]. Multiple pathological changes occur in the airway epithelium and submucosa in asthma (Figure 1) and these changes are collectively referred to as airway remodelling [6].



Airway pathology in asthma. Airway structures in medium-sized healthy airways (part a; a schematic representation is depicted in part c) and in a patient with asthma (part b; a schematic representation is depicted in part d). The airways in asthma show considerable structural remodelling, including goblet cell hyperplasia, subepithelial fibrosis and increases in smooth muscle volume.
Fahy et al. *Nature Reviews Immunology*. 2015;15:57–65.

2.1. Asthma phenotypes

Asthma underlies different diseases processes. Traditional asthma phenotyping (description of observable features) classifies patients according to clinical characteristics such as exacerbating factors (allergens, exercise, and infections), age of onset, concomitant comorbidities (sinusitis and obesity), and response to treatment. More recently, unbiased clustering algorithms that have the capacity to incorporate a range of clinical variables (*e.g.* forced expiratory volume in 1 second [FEV_1], body mass index [BMI], asthma control questionnaire [ACQ], atopic status, and blood eosinophils) have been applied to large patient datasets to objectively identify clinical phenotypes of asthma. Therefore, patients have been recently partitioned into up-to five clinical phenotypes [5,7]. These are:

- Allergic asthma: this is the most easily recognized asthma phenotype, which often commences in childhood and is associated with a past and/or family history of allergic diseases such as eczema, allergic rhinitis, or food or drug allergy. Examination of the induced sputum of these patients before treatment reveals eosinophilic airway inflammation. Patients with this asthma phenotype usually respond well to inhaled corticosteroids (ICS) treatment.
- Non-allergic asthma: some patients have asthma that is not associated with allergy. The cellular profile of the sputum of these patients may be neutrophilic, eosinophilic or contain only a few inflammatory cells (paucigranulocytic). Patients with non-allergic asthma often demonstrate less short-term response to ICS.
- Adult-onset (late-onset) asthma: some adults, particularly women, present with asthma for the first time in adult life. These patients tend to be non-allergic, and often require higher doses of ICS or are relatively refractory to glucocorticoids treatment. Occupational asthma (*i.e.* asthma due to exposures at work) should be ruled out in patients presenting with adult-onset asthma.
- Asthma with persistent airflow limitation: some patients with long-standing asthma develop airflow limitation that is persistent or incompletely reversible. This is thought to be due to airway wall remodelling.
- Asthma with obesity: some asthmatic patients with obesity have prominent respiratory symptoms and little eosinophilic airway inflammation.

Based on clinical experience of over 20 years in over 5,000 patients, a patient can be determined to have eosinophilic asthma if there is evidence of elevated sputum (>3% with or without degranulation) and/or blood eosinophils (≥300 cells/µL) on at least two occasions, and if treatment strategies aimed at suppressing eosinophils are effective in controlling symptoms and exacerbations [8–10]. Likewise, a patient can be determined to have neutrophilic asthma if there is evidence of elevated neutrophils (≥64%) but not eosinophils (<3%) with an increased total cell count (≥9.7 million cells/g) on at least two occasions and if treatment strategies aimed at suppressing eosinophils are not effective in controlling symptoms and exacerbations. Patients can exhibit features of both the eosinophilic and neutrophilic phenotype. A patient can be determined to have mixed-granulocytic asthma if there is evidence of both neutrophils and eosinophils, independently or concurrently, on at least two occasions. Finally, a patient can be determined to have paucigranulocytic asthma if there is no evidence of elevated eosinophils (<3%) or neutrophils (<64%) and if treatment strategies aimed at suppressing eosinophils and neutrophils are not effective in controlling symptoms and exacerbations [7,11]. Occasionally, neutrophilic asthma and paucigranulocytic asthma are referred to together as non-eosinophilic asthma [12].

2.2. Inflammatory endotypes

In patients with more severe asthma, some phenotype-guided treatments are available. However, composites of observable characteristics do not provide us insight into mechanisms of persistent symptoms, physiological abnormalities, or inflammation, and therefore are of limited value to choose the appropriate treatment. As opposed to phenotypes, characterizing asthmatics according to their endotype, a term applied to a "subtype of a condition that is defined by a distinct functional or pathophysiological mechanism", may be more useful to directing therapy [13]. This notion is strongly supported by the efficacy of biologic therapies that target-specific inflammatory mediators (*e.g.*, IL-5) in well-defined patients characterized based on inflammatory biomarkers [14,15].

Wenzel *et al*. defined two distinct inflammatory endotypes of severe, glucocorticoid-dependent asthma based on the presence or absence of eosinophils in endobronchial biopsy and lavage [16]. Eosinophilic or type 2 (T2)-high asthma is characterized by the presence of T2 immune responses, whereas non-eosinophilic (or non-T2) asthma is characterized by neutrophilic or paucigranulocytic airway inflammation, and the presence of non-T2 inflammatory mediators, such as type 1 and type 17 [7]. Figure 2 describes the immunopathology of distinct asthma endotypes [17,18].

Approximately 50-70% of adults with asthma have T2 inflammation with airway eosinophilia, which in many individuals is associated with a good therapeutic response to glucocorticoids and biologics targeting T2 cytokines [19]. Non-T2 inflammation is associated with neutrophilic or paucigranulocytic airway inflammation, glucocorticoid insensitivity, and a poor response to current biological therapies [12].

2.2.1. T2-high asthma

T2-high asthma with eosinophilia can occur with and without any demonstrated allergy. Type 2 asthma with allergy is the most common asthma phenotype with an early onset. T2 immune responses involves T helper (Th)-2 cells, innate lymphoid cells (ILC)-2, immunoglobulin E (IgE)-producing B secreting cells, natural killer T cells, mast cells, basophils, eosinophils and their cytokines. Th2 cells produce various cytokines such as IL-4, IL-5, IL-9 and IL-13 [20–24].

T2 eosinophilic non-allergic asthma is in most cases a moderate to severe, late-onset disease frequently associated with chronic rhinosinusitis and nasal polys. Eosinophil non-allergic asthma is an ILC2 predominant process. Cytokine production from ILC2 is controlled by IL-10, TGF-β and alarmins, such as IL-25, IL-33 and thymic stromal lymphopoietin (TSLP). Alarmins are released mainly by airway epithelial cells when triggered by the exposure to toxic agents, pollutants, diesel particles, tobacco smoke and virus, they contribute to the inflammatory airway response in T2 non-allergic asthma. When blood ILC2 are exposed to TSLP, they become steroid resistant. Moreover, dexamethasone cannot suppress cytokine production by ILC2, obtained from patients with severe asthma [25]. It is currently estimated that only about half of asthma patients have evidence of T2 immunity in their airways [6].

2.2.2. Non-T2 asthma

Non-T2 asthma has been described mostly in adults and rarely in children and, as a non-eosinophilic asthma, comprises neutrophilic and paucigranulocytic asthma [26,27].

On the one hand, Th1 and Th17 cells are subsets of cells which produce IL-17, IL-21 and IL-22, the dominant cytokines in neutrophilic asthma [28,29]. IL-17 is associated with neutrophilic inflammation, AHR, and severe asthma with glucocorticoid resistance [30–34]. ILC3, other IL-17 secreting ILCs and pro-inflammatory macrophages also appear to play a key role in neutrophilic glucocorticoid-resistant asthma [35]. Th1 related cytokines such as interferon gamma (IFN-γ) and TNF-α have been found increased in patients with severe neutrophilic asthma [36,37]. The amount of TNF-α is also increased in the airways of patients with severe steroid-resistant asthma [38].

On the other hand, paucigranulocytic inflammation is a common endotype among adolescents and adults with asthma [11,39,40], and with lower rates in severe asthma [11,40]. In some studies, paucigranulocytic inflammation is associated with better lung function and less AHR compared with eosinophilic asthma [11]. The mechanisms underlying paucigranulocytic asthma are unclear, but could be due to asthma in remission, glucocorticoid-induced suppression of airway eosinophilia, glucocorticoid-insensitive airway inflammation involving nerves, smooth muscle, or other cells, and tissue remodelling [11,41]. In the absence of identifiable targetable inflammatory pathways, treatment is largely symptomatic [42].

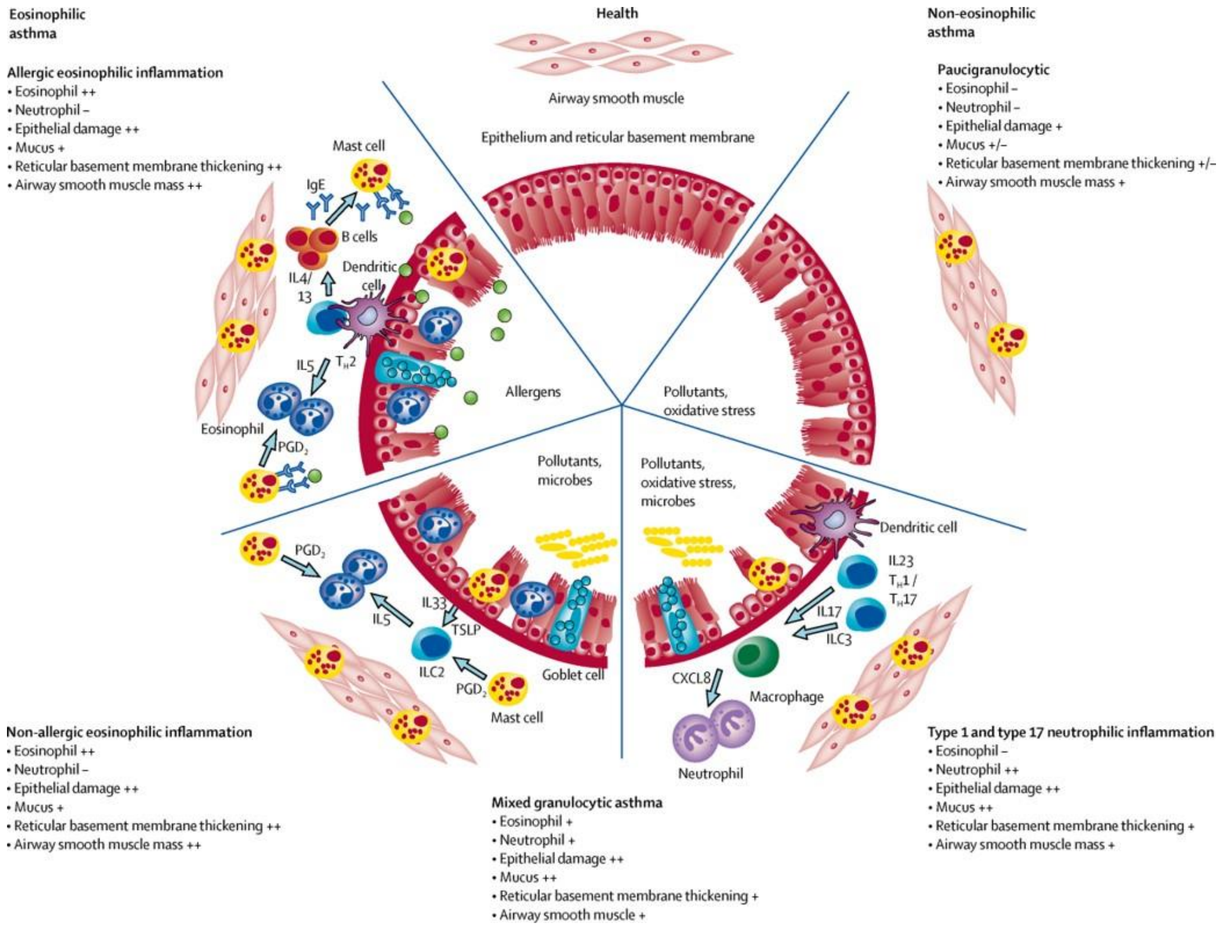


Figure 2. Mechanisms and characteristic pathological features of asthma immunopathology. Features are divided into eosinophilic (allergic and non-allergic), non-eosinophilic (neutrophilic type 1 and type 17 and paucigranulocytic), and mixed granulocytic inflammation. Abbreviations: IL, interleukin; TH, T helper; PDG2, prostaglandin D2; TSLP, thymic stromal lymphopoietin; ILC, innate lymphoid cells; CXCL8, CXC motif chemokine ligand 8; IgE, immunoglobulin E.

Russell et al. *Clin Sci.* 2017;131:1723–35. and Papi et al. *The Lancet*. 2018;391:783–800.

As explained before, there are several cytokine networks that account for asthma inflammation. Some of them are well-recognized cytokines with specific roles in asthma pathogenesis. However, there are other cytokines which their roles have not yet been elucidated, such as ezrin and chitinase-3-like protein 1 (YKL-40). Serum levels of ezrin have been found increased in patients with allergic asthma after allergen exposure [43], whereas YKL-40 has been found to be elevated in asthmatic patients compared to healthy controls [44,45]. In addition, YKL-40 has been linked to severe asthma and there are discrepancies over whether it is associated more with non-allergic asthma than with allergic asthma. More research in needed to clarify their role in asthma inflammation.

3. Obesity

Obesity is a major and raising global health problem and, over the recent decades, has become the most prevalent metabolic alteration, constituting one of the main causes of death and disability [46]. Worldwide obesity has nearly tripled since 1975. In 2016, 13% (11% men and 15% women) were obese [47]. In Spain the age-adjusted prevalence for being obese increased between 1987 and 2012 from 8.0% to 16.5% [48]. Obesity is a major risk factor for non-communicable diseases such as cardiovascular diseases, type II diabetes, musculoskeletal disorders and, some cancers [47].

Obesity is the consequence of an abnormal or excessive body fat accumulation due to an imbalance of energy intake and energy expenditure that may impair health [47,49]. BMI is a simple index of weight-for-height that is commonly used to classify overweight and obesity. In adults, obesity is defined as having a BMI of ≥ 30 kg/m^2, overweight is defined as BMI ≥ 25 to < 30 kg/m^2, and lean weight is defined as BMI < 25 kg/m^2. BMI provides the most useful population-level measure of overweight and obesity. However, it should be considered a rough guide because it may not correspond to the same degree of fatness in different individuals [47].

3.1. Obesity and inflammation

There are two types of adipose tissue in the body whose function appears to be clearly differentiated. White adipose tissue stores energy reserves as fat, whereas the metabolic function of brown adipose tissue is lipid oxidation to produce heat [50]. For decades, white adipose tissue was considered an inert fat depot; however, we now appreciate it as a large, interactive, multi-compartment organ that produces several mediators, collectively called adipokines (able to exert both paracrine and endocrine effects) [51]. Some adipokines such as leptin or resistin are produced in excess in obesity while others, such as adiponectin, are reduced [52].

There is increasing evidence that there is a link between inflammation and obesity, a condition where the secretion of pro-inflammatory adipokines is increased [53]. Visceral adipose tissue in obesity is responsible for the formation of low-grade chronic inflammation in obese individuals, that is not related to infection or tissue damage, called metainflammation (metabolically triggered inflammation) [54]. Systemic inflammation in obesity is characterized for three general aspects: 1) abnormal cytokine production, 2) increased acute-phase reactants and other mediators, 3) activation of a network of inflammatory signalling pathways [55,56]. This network of inflammatory pattern is also called the inflammome, and it is related to pulmonary inflammation and not influenced by sex, smoking status or metabolic syndrome [57].

The mechanisms triggering adipose tissue inflammation have yet to be elucidated; however, the cells that primarily compose adipose tissue, named adipocytes [54], and macrophages are involved in this inflammatory process (Figure 3). As adipose tissue expands and the distance between adipocytes and capillaries increases, hypoxic death of some adipocytes occurs. Moreover, in response to adipocyte death, pro-inflammatory macrophages, called M1 macrophages, surround dead and dying cells and remove debris from the damaged area. During this process, adipocytes and M1 macrophages produce inflammatory cytokines including IL-6, TNF-α, IL-1β, and monocyte chemoattractant protein (MCP-1) [58].

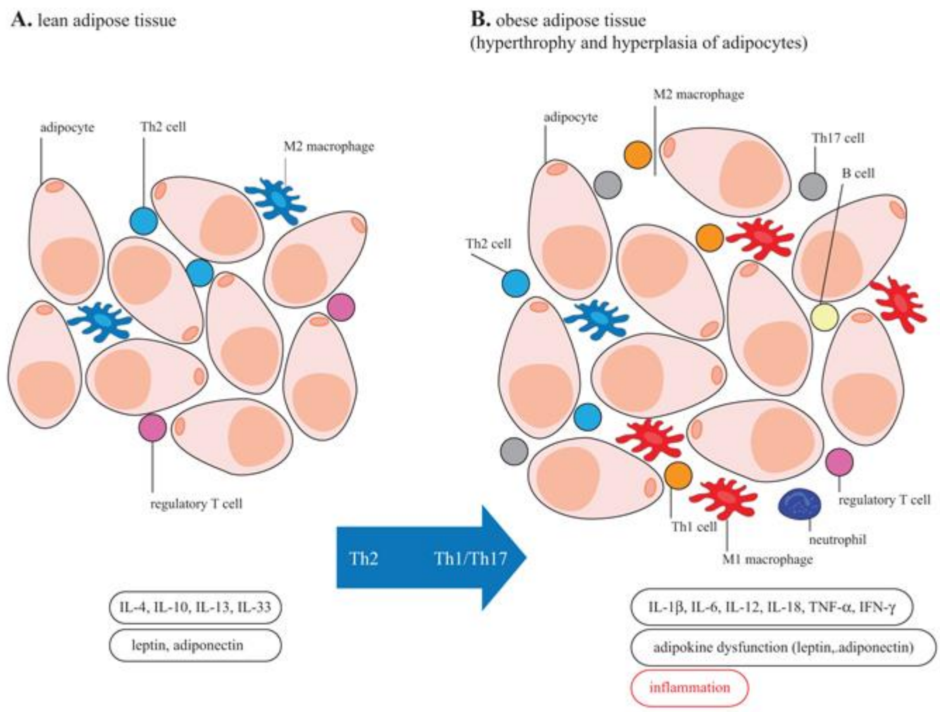


Figure 3. Key changes in immune cell composition of adipose tissue in obesity. Obesity induces adipocyte hypertrophy and changes in stromovascular cell composition with their phenotypic activation to a pro-inflammatory state. Cells from the adaptive immune system interact with adipose tissue macrophages to modify their activation state. In lean adipose tissue, Th2 and Treg lymphocytes promote a M2 macrophage polarization, which maintain an anti-inflammatory state. Eosinophils may also induce a M2 macrophage polarization. In obesity, adipose tissue is characterized by an enrichment of macrophages and T lymphocytes with a shift from an anti- inflammatory to a pro-inflammatory state. Th1 and Th17 lymphocytes stimulate M1 macrophage polarization. Other cells, including B lymphocytes and mast cells, are also increased in obese adipose tissue and may contribute to obesity-induced inflammation. Abbreviations: IL, interleukin; INF-γ, interferon gamma; Th, T helper; Treg, T regulatory; TNF-α, tumor necrosis factor alpha. From Karczewski et al. *Eur Cytokine Netw.* 2018;29:83–94.

Leptin is an adipokine mainly produced by adipocytes with a dual role as a hormone and as a cytokine. As a hormone, it has a key function in the regulation of food intake and energy expenditure and as a cytokine exerts strong pro-inflammatory activities [59]. Leptin stimulates adipocytes to secrete pro-inflammatory mediators such as TNF-α, IL-6 [60,61], MCP-1 [62–64] and IL-12 [65]. An *in vitro* study showed that, in a concentration dependent manner, leptin could activate human peripheral blood mononuclear cells (PBMCs) to induce secretion of IL-6 and TNF-α [66]. Higher levels of TNF-α and IFN-γ have been found in PBMCs culture from individuals with a BMI ≥ 30 kg/m^2 compared with those with BMI < 30 kg/m^2, and this was associated with an increased leptin blood concentration in these obese subjects [67]. Leptin promotes naive T cell survival and facilitates the differentiation and activity of Th1 cells while inhibiting the cytokine production of Th2 cells (IL-4, IL-5, and IL-10) [64,68–70], enhances the proliferation and activation of T cells, and exerts differential effects on the proliferation of naïve *vs* memory T cells or effector T cells *vs* regulatory T (Treg) cells [71,72]. It also promotes the activity of pro-inflammatory Th17 cells [73].

Early studies have shown that adiponectin possesses anti-inflammatory properties [52,74]. On macrophages, adiponectin promotes cellular differentiation of monocytes to M2 macrophages, which maintain the anti-inflammatory state, and suppresses their differentiation to M1 macrophages [74,75]. Adiponectin activates anti-inflammatory IL-10 and reduces pro-inflammatory cytokines such as IFN-γ, IL-6, and TNF-α in human macrophages [76]. Circulating serum levels of adiponectin are decreased in patients with obesity, type 2 diabetes, metabolic syndrome, or cardiovascular disease inflammation, and are associated with an increased release of pro-inflammatory cytokines like IL-6 and TNF-α [77]. It was demonstrated that adiponectin has a protective role in the murine inflammatory response, leading to decreased neutrophil recruitment and decreased expression of cytokines and chemokines, especially IL-17 [78]. After weight loss, the adiponectin serum level increases, and this has a positive relationship with BMI reduction [79].

Interestingly, obesity and metabolic syndrome are distinguished by an increase in circulating leptin concentrations, in parallel to a drop in adiponectin levels. Consequently, adiponectin/leptin ratio has been suggested as a reliable biomarker of adipose tissue dysfunction [80]. Patients with a reduced ratio are characterized by an increased cardiometabolic risk evidenced by raised systemic inflammation and oxidative stress [80–82].

4. Asthma and obesity: two linked diseases

The epidemiological link between asthma and obesity was first suggested in a study involving 85.911 nurses in the United States [83]. The study found that the risk of developing late-onset asthma was significantly increased when the BMI was ≥ 30 kg/m^2 with an odds ratio of 2.6. Subsequently, several studies were carried out and corroborated the existence of an excess risk of developing asthma in obese subjects compared with subjects not overweight, regardless of gender or age [84–91]. The relationship also appears stronger for those with central versus general adiposity [92,93]. Another study reported that increased waist circumference was associated with asthma even among those with BMI's within the normal range [92]. The European Community Respiratory Health Survey found an association between asthma and obesity, but with a greater excess risk in females with respect to males [92,94]. A longitudinal cohort reported the persistence of symptoms into adulthood in obese children with asthma, and also found that being overweight is an independent risk factor for developing post-puberty asthma [95]. Research has also shown associations between mothers' overweight status just before and during pregnancy and offspring's asthma [96,97]. Another study found that fathers who were overweight during adolescence predispose their offspring to develop asthma [98].

Interestingly, a study suggested that asthma may also lead to obesity. The authors followed non-obese children for up to 10 years. Children with an initial diagnosis of asthma were approximately 50% more likely to become obese than children without asthma. The study also showed that the increased risk of obesity with asthma was driven by children who were already overweight at baseline. Since being overweight is a very strong predictor of subsequent obesity, these observations support the possibility that being overweight may drive both asthma and subsequent obesity, and that both obesity and asthma, can interact by complex and multiple mechanisms [99].

Asthma with obesity is one of the most common phenotypes recently described in asthma guidelines [5] and two main sub-phenotypes of obese asthma have been described according to age: early-onset and late-onset asthma. The first phenotype associating asthma and obesity of early onset affects children with asthma under 12 years old [100] with more severe airway obstruction, more marked AHR compared with early non-obese asthma, and inflammation is predominantly eosinophilic [101]. Children are, in the majority of cases, allergic and both sexes are affected equally. The second main phenotype is characterized by delayed onset asthma developing, at least in part, as a consequence of obesity [102]. This asthma is generally non-allergic with pronounced symptomatology despite treatments with high doses of ICS and long-acting bronchodilators [40,103]. Various studies which analysed the profile of mild to severe asthma patients highlighted a subgroup of obese asthma patients, more often female, with late-onset, non-atopic asthma. Asthma in these patients is more difficult to control, lung function is impaired, they undergo more treatments with limited therapeutic effects and suffer from more frequent exacerbations than the non-obese asthma population [40,103–107].

There are some discrepancies with respect to the type of airway inflammation associated with the obese asthmatic phenotype. Some cluster analyses have shown that the association between obesity and neutrophilic inflammation is more common in women [40]. On the contrary, other studies have not found such an association between obesity and neutrophilic airway inflammation in adults with asthma [108,109].

Asthma and obesity share some of the mechanisms responsible for their underlying inflammatory process (Figure 4). This finding raises the possibility that additive or synergistic effects between both inflammatory processes may account. A deeper understanding of how molecular mechanisms interact between asthma and obesity may help us further understanding the complex inflammatory puzzle of asthma associated with obesity.

4.1. Adipokines role in obesity-related asthma

It is well known the role of adipokines in the pathogenesis of obesity. It has also been reported that they may play a role in the mechanisms involved in asthma. Low concentration of serum adiponectin is associated with higher asthma incidence according to a recent meta-analysis of 13 studies [110]. The meta-analysis found that, in the overall study population, the diagnosis of asthma was associated with lower levels of adiponectin in patients with asthma compared with controls. However, borderline association of adiponectin with asthma was seen in adults, but not in children. The study also shows that higher leptin levels were associated with asthma both in adults and children.

Leptin pathway may partly explain the obesity asthma relationship. Sideleva *et al*. found that increased leptin levels are associated with AHR [111]. Leptin treatment augmented allergen-induced AHR but did not affect eosinophil influx or Th2 cytokine expression, suggesting that leptin is capable of augmenting AHR through a mechanism independent of T2 inflammation. Rather than modifying adaptive immunity, leptin could be acting on the innate immune system: exogenous administration of leptin to lean mice increases their subsequent inflammatory response to acute O_3 exposure [112], a response characterized by the release of acute-phase cytokines and chemokines, and dependent to some extent on toll-like receptors (TLR) activation [113].

A few cross-sectional studies have reported positive associations of serum leptin concentration with asthma severity, asthma control, lung function and asthma severity in children and in adults [114,115]. However, the cross-sectional design does not allow for the establishment of whether the modification in the biological marker concentration is a cause or a consequence of the disease. Two recent studies examined the potential role of leptin in obese asthmatics using analytical methods which help elucidate the possible causal role of leptin in the association between obesity and asthma. One study found an association between serum leptin levels and asthma control assessed by the ACQ [116]. The second recent longitudinal study reported an indirect effect mediated by leptin in the association between adiposity and persistent asthma [117]. Collectively, these results support that leptin may be a mediator which contributes to explain the association between obesity and both asthma persistence and control. However, to study the relevance of leptin in the inflammation present in asthma and obesity it should be considered to analyse the adiponectin/leptin ratio. In our knowledge no studies have been reported the association between this ratio and asthma control or glucocorticoid response.

4.2. Cells and cytokines in obesity-related asthma

Several studies have evaluated the cell dominant pattern of airway inflammation in asthma patients with and without associated obesity. Data from these studies support that obesity is associated with a neutrophil dominant rather than eosinophil dominant inflammatory pattern in the airway lumen [99,118–120]. The abundant neutrophil concentration is associated with the presence of greater levels of IL-17A, a cytokine involved in neutrophil recruitment to the airways [99,118,119]. Data from animal models also supports a link between IL-17A and obese asthma. Obese mice typically exhibit innate AHR, but this AHR is not observed when the animals are IL-17A deficient [121]. In the lungs of obese mice, increased IL-17A was found associated with AHR and neutrophilic inflammation [121]. Obese mice with a deletion of the TNF-α receptor 2 (TNFR2) were protected against innate AHR and presented reduced levels of IL-17 in comparison with controls [122].

Interestingly, the relative reduction of eosinophil numbers with respect to neutrophils in the airway secretions of obese asthmatics contrasts with the higher eosinophil counts found in the airway submucosa in obese versus lean severe asthmatics [118,123]. Similarly to IL-17, sputum IL-5 and IL-25 levels have been found to be significantly higher in obese asthmatics compared with their lean counterparts. In contrast, neither IL-4 nor IL-13 sputum levels were found associated with BMI in asthma patients [118]. Two hypotheses have been suggested to explain the apparent paradox represented by the reduced presence of eosinophils in sputum versus elevated eosinophilia in submucosa: 1) Survival of eosinophils in the airway is reduced in obese asthmatics and 2) Eosinophils are retained in the submucosa and do not migrate to the airway lumen. The second hypothesis appears to be supported by studies reporting that more eosinophils are recruited to the lungs of asthmatic patients with obesity compared with non-obese asthmatic patients [124]. Furthermore, it is unlikely that eosinophils fail to survive within the airway lumens of obese asthmatics because IL-5, a well-known eosinophil survival factor, is elevated in the sputum of obese versus lean severe asthmatics [118,123]. This observation is interesting because some severe obese asthmatics may have an eosinophilic non-T2 dominant type of asthma, and therefore may benefit from eosinophil targeted therapeutics that might be excluded if attending only sputum results.

Interestingly, the four cytokines with a prominent pro-inflammatory role in the asthmatic lung -IL-4, IL-5, IL-13 and IL-33- contribute to maintain the lean state. IL-4 and IL-13 are produced by ILC2 cells and eosinophils and trigger M2 macrophages to express two anti-inflammatory cytokines, TGF-β and IL-10. TGF-β inhibits adipogenesis, and IL-10 maintains insulin sensitivity in adipocytes [125,126]. IL-33 maintains ILC2 cells and reduces the risk of metabolic syndrome [127]. In humans, low serum IL-33 levels are associated with high BMI [128]. IL-33 is able to counteract excessive inflammation in adipose tissue responses by targeting immune cells expressing the suppression of tumorigenicity 2 (ST2) receptor. Two isoforms of ST2 have been identified, the full-length receptor and the soluble ST2 (sST2). The sST2 functions as a decoy receptor capable of abrogating IL-33 signalling. Mice lacking ST2 or IL-33 present increased adiposity and worsened metabolic profiles. IL-33 treatment triggered the expansion of a group of Tregs expressing ST2 and attenuated adipose tissue inflammation [128].

The obesity-related asthma phenotype is also associated with the presence of increased interleukins levels, such as TNF-α and IL-1β in the lung, even in the absence of an antigenic challenge [129]. TNF-α expression increased in PBMCs in parallel with BMI increase in subjects with asthma [101].

Interestingly, some studies have found that obesity is associated with higher levels of YKL-40 especially in severe asthma, but due to the fact that severity is also associated with obesity, it is not clear what is most important: severity, obesity, or both have an additive or synergistic effect on serum YKL-40 levels [130–133]. These are findings in studies regarding the relationship between YKL-40 and asthma but, to our knowledge, no progress has been made in studying the interaction between asthma severity, obesity, and the high levels of YKL-40.

Taking into account the discrepancies reported when asthma and obesity cytokine patterns are studied, more research is needed. First of all, by exploring cytokines that account for asthma or for obesity, and studying their implications in the association of asthma and obesity. Secondly, it should be considered that most of the studies include healthy subjects and asthmatic patients with or without obesity. However, studies comparing asthmatic patients with obesity with their non-asthmatic counterparts are lacking.

4.3. Inflammasome in obesity-related asthma

One possible mechanism that accounts for the obesity and asthma association has been identified in the inflammasomes. The nucleotide-binding domain-like receptor protein 3 (NLRP3) is an intracellular multiprotein complex that facilitates the autoactivation of pro-inflammatory cysteine protease caspase-1. Then, the activated caspase-1 cleaves pro-IL-1β and pro-IL-18 into their mature forms [134].

IL-1β is synthesized by blood monocytes, tissue macrophages, and dendritic cell after activation by pro-inflammatory stimuli such as TNF or TLR ligands [135]. Active IL-1β was found to promote Th17 cell dependent inflammation [134]. IL-1β induces the development of IL-17-producing T cells in cooperation with IL-23 [136].

IL-18 is a pro-inflammatory cytokine constitutively expressed by various cell types, including monocytes, macrophages, epithelial, and dendritic cells [137]. In contrast to IL-1β, IL-18 signalling affects IFN-γ producing Th1 cell differentiation in conjunction with IL-12 and with IL-13 [134]. In the absence of IL-12, IL-18 promotes Th2 rather than Th1 immune responses and, in combination with IL-23, it activates the Th17 pathway [138]. Among the multiple lymphocytes subsets, IL-1β and IL-18 in combination with the other cytokines may exert biologic effects on enhancement of Th17 cell differentiation in the lung leading to neutrophilic inflammation [139].

In asthma, NLRP3 is activated by danger-associated molecular patterns and serum amyloid A protein which is produced by epithelial cells exposed to microbes [134]. Gene expression of NLRP3, IL-1β and caspase-1 are detected at high levels in sputum and peripheral blood of asthma patients [140]. In addition, IL-1β levels correlate with sputum IL-8 levels in patients with neutrophilic asthma [141].

Moreover, NLRP3 is activated by saturated fatty acids such as palmitate and stearate via TLR4, as well as free cholesterol and cholesterol crystals, and by oxidative stress, which is also known to be present in adipose tissue in obesity [142]. Consequently, M1 macrophages are able to secrete pro-inflammatory cytokines such as IL-1β, IL-18, MCP-1, TNF-α and IL-6 into the circulation and act together with other secreted inflammatory adipokines, driving inflammation to many organs [65]. IL-1β is found elevated in the blood of obese individuals [143]. Increased sputum concentrations of IL-1β and increased NLRP3 and TLR4 expression in sputum cells has been reported in obese versus non-obese asthmatic patients [144,145].

It has been reported that obesity induced by a high-fat diet in mice triggered the activation of an NLRP3 inflammasome in M1 macrophages resident on adipose tissue and in the lungs. This results in an amplification of IL-1β production, the subsequent ILC3 activation and IL-17 secretion, which in turn facilitates AHR in these patients [65]. This is a novel mechanism that has not been previously linked with airway disease [129].

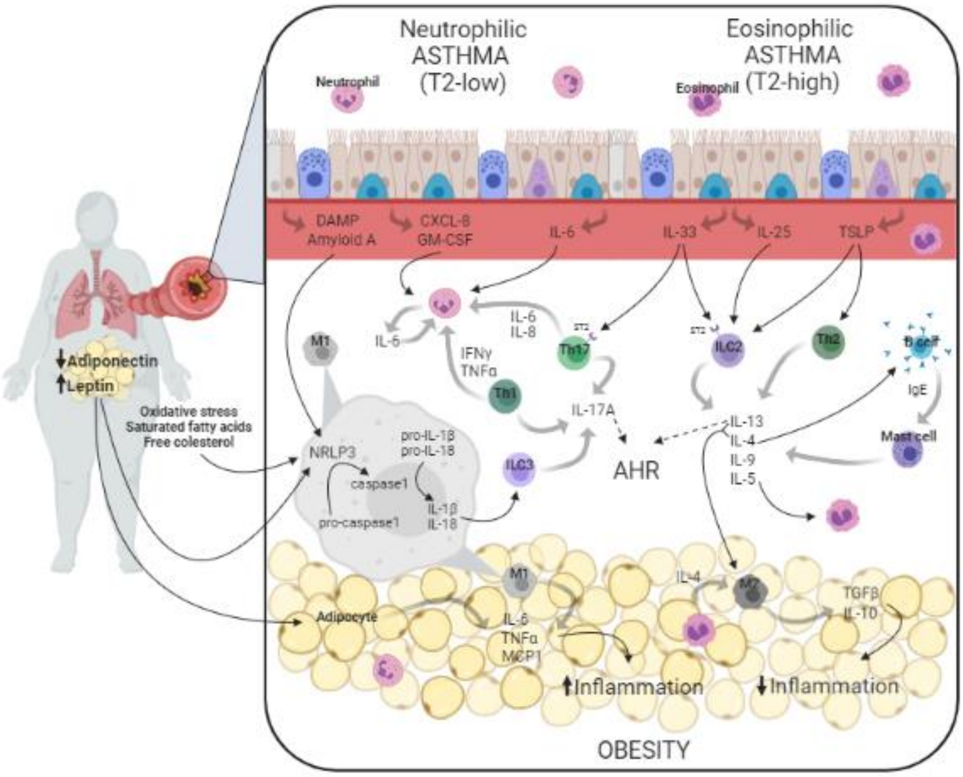


Figure 4. Interrelationship between the inflammatory processes that account for the asthma-obesity phenotype. Eosinophilic asthma is triggered by T2 inflammation where Th2, ILC2 and type 2 cytokines are involved. This could be allergic (when IgE is present) or non-allergic. Neutrophilic asthma, where neutrophils are present in the airway lumen, is mediated by T1 inflammation and Th1, Th17 and ILC3

release T1 cytokines. Inflammation in obesity is due to the imbalance of adipokines. Leptin stimulates adipocytes to release inflammatory mediators and activates M1 macrophages intracellular multiprotein complex, named the NLRP3 inflammasome. The activation of NLRP3 in M1 macrophages resident on adipose tissue and in the lungs, resulting in an amplification in IL-1β and IL-18 production, the subsequent ILC3 activation and IL-17 secretion, which in turn facilitates AHR in this patients. In obesity, pro-inflammatory cytokines such IL-1β, IL-6 and TNFα contribute to asthma pathogenesis. In contrast, cytokines that are pro-inflammatory in asthma such as IL-4, IL-13, and IL-33 contribute to maintain the lean state. Abbreviations: T2, type 2; IL, interleukin; M1, type 1 macrophages; M2, type 2 macrophages; Th, T helper; ILC, innate lymphoid cells; IgE, immunoglobulin E; AHR, airway hyperresponsiveness; NLRP3, NLR family pyrin domain containing 3; DAMP, danger-associated molecular pattern; CXCL8, CXC motif chemokine ligand 8; GM-CSF, granulocyte-macrophage colony-stimulating factor; TSLP, thymic stromal lymphopoietin; IFNγ, interferon gamma; TNFα, tumor necrosis factor alpha; ST2, suppressor of tumorigenicity 2; MCP-1, monocyte chemoattractant protein-1; TGFβ, transforming growth factor beta. From Bantulà et al. *J Clin Med.* 2021;10:1–21.

Asthma and obesity is a recognized phenotype, however, the identification of specific entodypes in asthmatic patients with obesity may help understand why some of these patients present a reduced response to glucocorticoids and, consequently, a non-controlled asthma symptoms and worst quality of life. Moreover, the precise identification of the inflammatory endotype present in asthmatic patients with obesity could be useful to develop more effective treatments.

5. Glucocorticoids

In airway inflammatory disorders, ICS are the mainstay of therapy. However, up to 50% of asthmatics may not respond well to ICS, and up to 25% patients with difficult to control asthma may not respond well to oral glucocorticoids [146]. This makes glucocorticoid hyporesponsiveness in asthma a challenging healthcare problem associated with significant morbidity and life-threatening disease progression. Specifically, in the asthma with obesity phenotype, a reduction in clinical response to inhaled glucocorticoids has been described [147,148]. However, the mechanisms by which this insensitivity to glucocorticoids might occur have not been elucidated.

Glucocorticoids have anti-allergy, anti-inflammatory, and immunosuppressive properties, in addition to regulating the biosynthesis and metabolism of key nutrients such as sugars, fats, and proteins [149]. As one of the most effective medications to prevent and treat asthma, glucocorticoids primarily act on airway epithelium to inhibit airway inflammation [149,150]. At a cellular level, the anti-inflammatory effect of glucocorticoids is exerted through a reduction in both the cell number and the function of inflammatory cells in the airways, including eosinophils, T-lymphocytes, mast cells, and dendritic cells [151,152].

5.1. Glucocorticoids: mechanism of action

In the last few decades, one of the main focuses of research in glucocorticoid resistance has been the glucocorticoid receptor (GR). GRs belong to the super-family of ligand-regulated nuclear receptors that also includes the receptors for mineralocorticoids, thyroid and sexual hormones, vitamin D and retinoic acid [153–155]. The widespread distribution of the GR within the body explains the efficacy of glucocorticoid treatment in most patients and in numerous inflammatory diseases [150,155].

Glucocorticoids diffuse across the cell membrane and bind to GR in the cytoplasm [152]. After binding to glucocorticoid, the GR undergoes two conformational changes: dissociation of heat shock proteins and dimerization of receptor. This allows translocation of hormone receptor complex into the nucleus. In nucleus, the GR binds to palindromic nucleotide sequences in promoter region of target genes (glucocorticoid response elements, GRE) and this interaction activates (transactivation) or inhibits (transrepression) gene transcription depending on type of GRE sequence [146]. Apart from their classic genomic effects, glucocorticoids also have non genomic and post-transcriptional effects. Figure 5 represents the mechanisms of action of glucocorticoids.

5.1.1 Glucocorticoids: genomic actions

Genomic actions are mediated after binding to GRs. Alternate splicing of GR primary transcript produces four different isoforms: GRα, GRβ, GRδ and GRγ of which GRα and GRβ have been studied more extensively. GRα is the only biologically active isoform. It is widely expressed in tissues and mediates genomic action of glucocorticoids. GRβ has non-functional ligand binding domain and therefore is incapable of binding glucocorticoids [156]. Increased expression of GRβ may interfere with binding of GR to DNA and influence sensitivity of glucocorticoids due to its dominant negative inhibitor function [157].

Glucocorticoids induce genes of anti-inflammatory proteins such as IL-10, annexin 1, mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1), inhibitor of nuclear factor kappa B (NFκB), lipocortin-1, IL-1 receptor antagonist, glucocorticoid-induced leucine zipper (GILZ), and the RNA-binding protein tristetraprolin (TTP) [153,158–160].

MKP-1 is a central target of glucocorticoids, whose function may be modulated by other steroid hormones. It is a phosphatase that selectively inactivates p38 MAPK, thus inhibiting the production of pro-inflammatory cytokines. Inactivating p38 MAPK is critical for the anti-inflammatory functions of glucocorticoids [161]. This induction was mediated by the GR and dependent upon ongoing transcription. No other known p38-inactivating phosphatases were significantly induced by dexamethasone, and cells which failed to express MKP-1 also failed to down-regulate p38 activity in response to dexamethasone [162]. Since MKP-1 plays a significant role in the suppression of inflammation by glucocorticoids, glucocorticoid resistance in some inflammatory disease could be related to defects in the expression or function of MKP-1 [163].

Glucocorticoids also function by transrepression, where glucocorticoid/GR complex directly or indirectly inhibits the function of transcription factors. These effects can be mediated by protein–protein interactions by binding of GR to pro-inflammatory transcription factors such as activator protein 1 and NFκB [164,165], inhibiting the transcription of pro-inflammatory genes, such as cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF), TNFα and IL-8 [150]. In addition, glucocorticoids/GR complex can prevent gene transcription by binding to negative GRE, and examples of genes affected by this mechanism include prolactin and osteocalcin [164,165].

5.1.2 Glucocorticoids: non-genomic and post-transcriptional actions

Glucocorticoids have non-genomic actions that are characterized by their rapid (*i.e.* lasting a few minutes) anti-inflammatory and immunosuppressive effects on different cells and tissues. Several basic mechanisms of non-genomic action of glucocorticoids have been proposed. Glucocorticoids may activate secondary messengers by binding to GR also in cytosol, by binding to GR in cell membrane, or by interacting non-specifically with cellular membranes [151,166,167].

In mast cells an additional level of regulation was described, in which glucocorticoids inhibited the degradation of MKP-1 [168]. The precise mechanisms of regulation of MKP-1 expression and the impact of this additional, non-genomic pathway in cells other than mast cells are not yet clear. Nevertheless, it is likely that MKP-1 plays a role in the inhibition of p38 and the consequent destabilisation of pro-inflammatory mRNAs by glucocorticoids. As the MAPK pathways are pleiotropic regulators of gene expression in the immune system [169], MKP-1 may be an important negative regulator of many aspects of the inflammatory response.

Glucocorticoids may also act post-transcriptionally to regulate expression at level of mRNA. GR can act as a RNA-binding protein in the cytoplasm, where it can bind to the mRNA of cytokines and chemokines to regulate their expression. For example, glucocorticoids induce the production of TTP, a protein that is crucial for glucocorticoids effects in airway epithelial cells [170] which have been shown to modulate effects of hormone [171].

More recently, glucocorticoids have been implicated in the expression and function of microRNAs (miRNAs). miRNAs are small, non-coding RNAs which bind to transcripts and repress their translation or promote their degradation. Glucocorticoids can alter the expression of miRNAs involved in pro-inflammatory responses and T-cell differentiation to impart their anti-inflammatory effects [172–174].

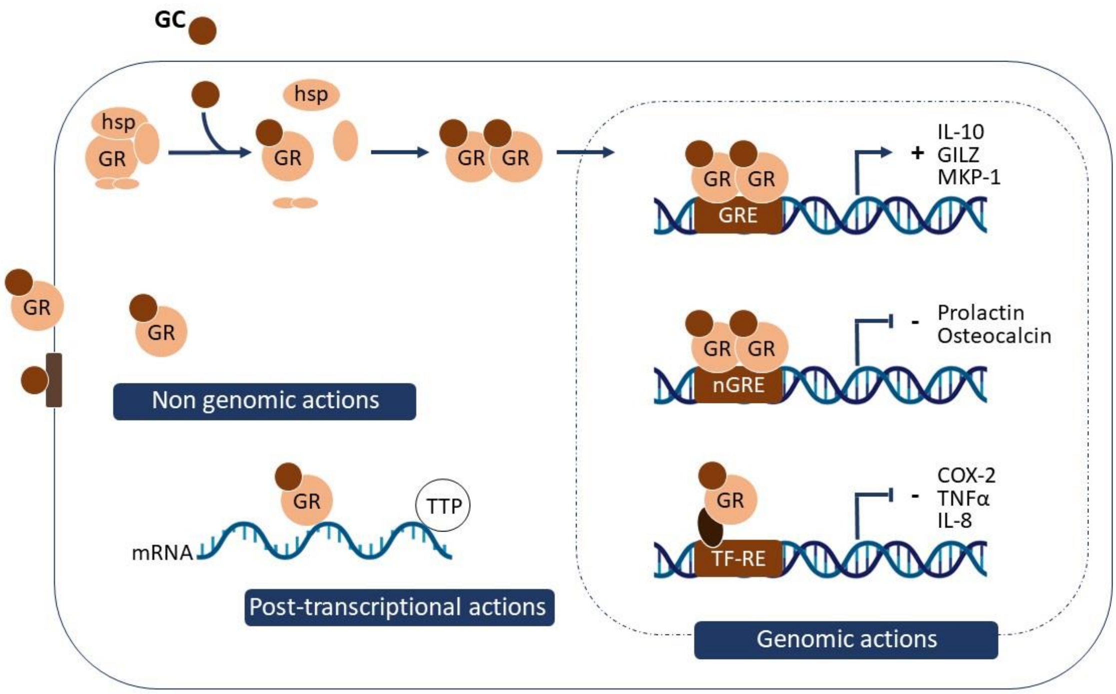


Figure 5. Mechanism of action of glucocorticoids. After passing the cell membrane by passive diffusion, glucocorticoids (GC) bind to glucocorticoid receptor (GR), associated heat shock protein (hsp) are released, and GR dimerizes. This allows hormone receptor translocation into the nucleus. GR can bind to glucocorticoid responsive elements (GRE) on the promoter regions of steroid-sensitive genes and activate transcription. Binding of GR to a negative GRE (nGRE) leads to repression. Protein-protein

interactions between GR and transcription factors response elements (TF-RE), repress the transcription of pro-inflammatory genes. Glucocorticoids can also act via non-genomic and post-transcriptional mechanisms. Abbreviations: IL, interleukin; MKP-1, mitogen-activated protein kinase phosphatase 1; GILZ, glucocorticoid-induced leucine zipper; TNF-α, tumor necrosis factor alpha; TTP, tristetraproline.

5.2. Mechanisms of glucocorticoid hyporesponsiveness

It has now become apparent that both genetic and acquired factors affect glucocorticoid sensitivity and several molecular mechanisms have been proposed to describe hyporesponsiveness to glucocorticoids. Some studies analyse GR abnormalities [175–177], while others evaluate cytokine profile in cultured cells under glucocorticoid treatment [178–180]. Moreover, analysing expression of genes induced by glucocorticoids, such as MKP-1 [108,177] and measuring dexamethasone-suppression of cell proliferation assays [175,181–183] are two of the most used methods to study glucocorticoid hyporesponsiveness.

In proliferation assays, cells are treated with dexamethasone or another glucocorticoid, and the main goal is to obtain the dexamethasone half maximal inhibitory concentration (IC_{50}) (Figure 6B). IC_{50} value can be used as an indirect marker of glucocorticoid sensitivity because it represents the dexamethasone dose that inhibits the 50% of cell proliferation. In order to obtain the IC_{50} value, cells are stained with a fluorescent dye. This dye passively diffuses into cells and stains intracellular proteins. When cell divides, it is equally partitioned between daughter cells and generates a fluorescent signal that progressively halves with each mitosis. Each new generation in a population of proliferating cells is marked by decrements in cellular fluorescence intensity that is readily discriminated by flow cytometry (Figure 6A). These assays involve a mitogen (*e.g.*, phytohemagglutinin, PHA) stimulation and a 3-4 days culture of both unstimulated and stimulated cells [184].

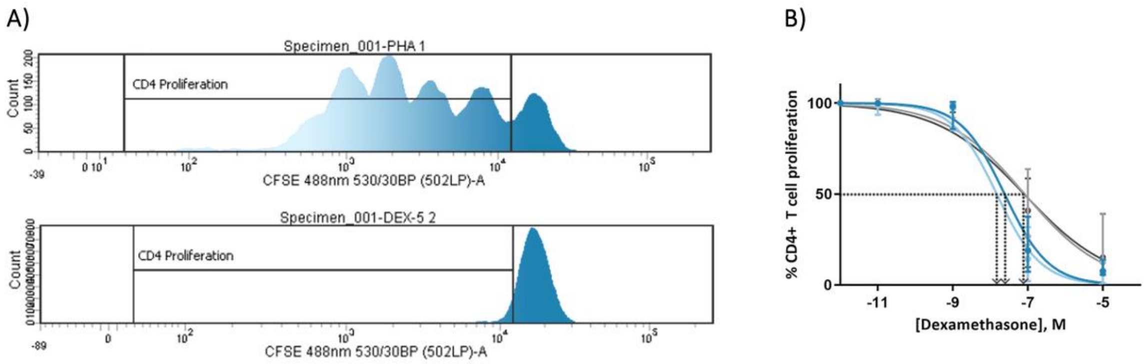


Figure 6. Dexamethasone's inhibition of CD4⁺ T cells proliferation. A) Flow cytometry representation of PHA-induced CD4⁺ T cells proliferation. Cells stained with carboxyfluorescein succinimidyl ester (CFSE) proliferate with PHA stimulation (upper graph). Incubation with dexamethasone reduces CD4⁺ T cell proliferation (lower graph). **B)** Dexamethasone half maximal inhibitory concentration (IC_{50}) measurement. % of CD4⁺ T cell proliferation (y axis) under distinct dexamethasone doses (x axis). Dot lines represent dexamethasone IC_{50} values.

Microarray studies of PBMCs identified 11 genes that discriminate between glucocorticoid hyporesponsiveness and glucocorticoid sensitive asthma patient groups [185]. Several abnormalities in GR have been described in familial glucocorticoid hyporesponsiveness [146]. Splice variants of GR formed by alternative splicing may modulate glucocorticoid sensitivity. Increased expression of GRβ isoform has been reported in patients with glucocorticoid resistant asthma. It is induced by pro-inflammatory cytokines and competes with GRα to bind with GRE, thus acting as dominant negative inhibitor [186,187]. A recent study showed that the GR nuclear translocation in $CD4^+$ T cells from healthy controls and patients with moderate asthma at the baseline was the same without significant difference while almost no GR translocation was detected in $CD4^+$ T cells of patients with uncontrolled severe asthma [188].

Glucocorticoids hyporesponsiveness may also be driven by multiple immune mechanisms. IL-10 is a potent anti-inflammatory and immune-regulatory cytokine which is secreted by Treg cells in response to glucocorticoids [189]. Th17 cells, which secrete IL-17, are increased in patients with severe asthma which is in turn associated with neutrophilic inflammation [190]. Th17 cells appear to be glucocorticoid resistant in mice studies [191].

GRβ up-regulation by IL-17/IL-23 cytokines is associated with induced steroid insensitivity in PBMCs, observed as diminished dexamethasone's effects on cell proliferation, apoptosis and gene regulation. Steroid hyporesponsiveness induced by IL-2/IL-4 is associated with decreased GRα expression. This study supports the possibility that Th17 lymphocytes and associated cytokines play a role in the mechanism of steroid hyporesponsiveness in severe asthmatics [182].

Post translational modification of GR may influence glucocorticoid sensitivity via several mechanisms. It has been theorized that GR nuclear translocation and binding affinity is reduced by GR phosphorylation. Although several kinases can cause GR phosphorylation, p38 MAPK is considered important by its ability to alter GR nuclear translocation and binding [192]. p38 MAPK is expressed in greater degree in alveolar macrophages of glucocorticoid resistant asthma patients [179]. IL-13, IL-2 and IL-4 are also overexpressed in patients with glucocorticoid resistant asthma and thought to reduce GR binding and translocation via p38 MAPK [192–194].

5.2.1. Obesity and glucocorticoid hyporesponsiveness

Patients with obesity could present an altered molecular response to glucocorticoids due to systemic inflammation. Glucocorticoids inhibit pro-inflammatory gene expression, in part through negative regulation of MAPK signalling pathways by molecules such as MKP-1 [163]. Given that pro-inflammatory cytokines, such as IL-1, IL-6, and TNF-α, are increased in many obese individuals, and given that these same cytokines are regulated by and potential regulators of p38 MAPK [163], it is possible that this pro-inflammatory environment might modify glucocorticoid function in asthmatic patients with obesity [108].

These data indicate that *in vitro* biomarkers of glucocorticoid insensitivity increase in both the lung and peripheral blood as body mass increases in individuals with asthma, but not in control subjects without asthma. This effect is manifested by a reduced induction of MKP-1 expression in response to dexamethasone in both PBMCs and bronchoalveolar lavage fluid (BALF) cells, and is related to enhanced expression of TNF-α in both peripheral and lung immune cells as body mass increases, suggesting a scenario in which one or more molecular pathways governing glucocorticoid responses are modified in both the airway and peripheral blood in asthmatic subjects with overweight and obesity [108].

As it is explained above, there are only few studies evaluating *in vitro* glucocorticoid sensitivity in asthmatic patients with obesity, and all of them analyse MKP-1 gene expression. Cell proliferation assays in this population are missing, as they could serve as other reliable methodology to study glucocorticoid resistance in asthmatic patients with obesity.

Moreover, it would be interesting to study whether inflammatory markers that are increased in obesity with and without asthma correlate with glucocorticoid response. In fact, no study has described this correlation, and finding it could help to have a predictive marker of glucocorticoid response as well as explain some of the mechanisms of glucocorticoid hyporesponsiveness present in these patients.

6. Vitamin D effects on asthma and obesity

During the last decades, laboratory and observational studies have demonstrated that different nutrients, such as long chain omega 3 fatty acids, curcumin, resveratrol, and vitamin D, among others, have therapeutical anti-oxidant and anti-inflammatory effects [195–198]. These studies showed that supplementation with these compounds may be associated with reduced airway inflammation [198] and reduced exacerbations of inflammatory diseases including chronic obstructive lung disease or asthma [195].

Vitamin D, first identified as a vitamin early in the 20th century, is now recognized as a prohormone that is obtained from exposure to sunlight, from diet, and from dietary supplements [199]. Vitamin D metabolism and its mechanism of action is explained in Figure 7.

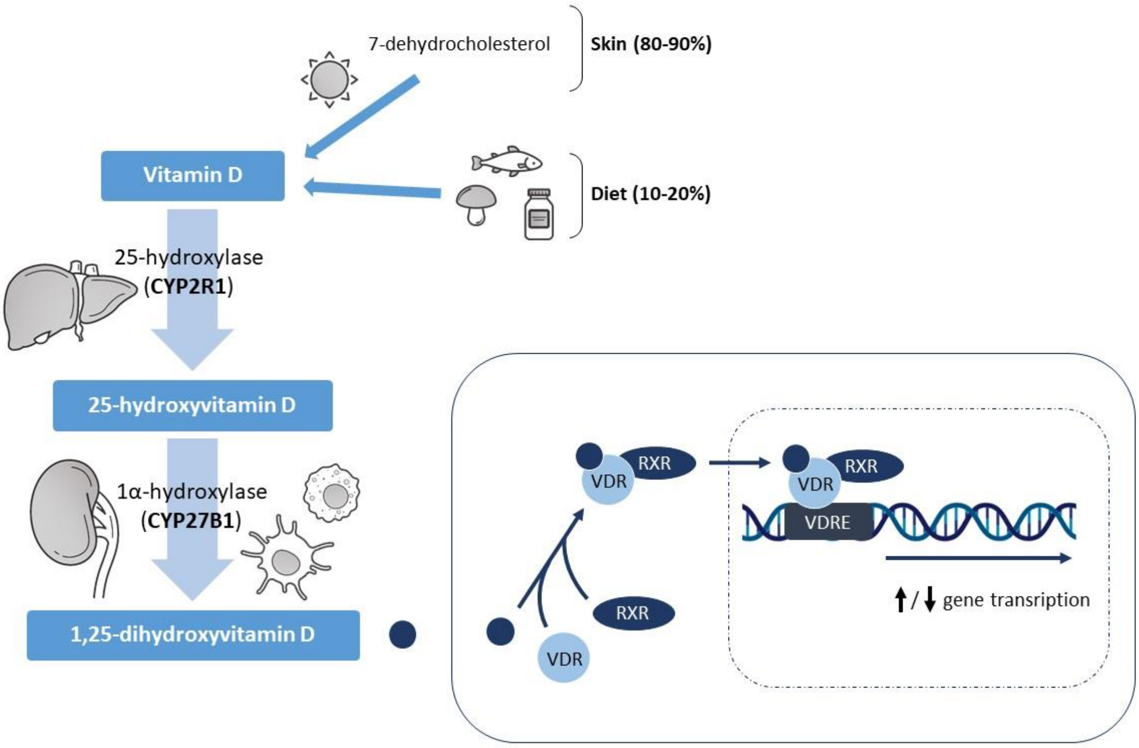


Figure 7. Vitamin D metabolism and receptor binding. Vitamin D is obtained through ultraviolet B exposure, diet and supplement intake [199]. It is activated in two enzymatic steps and begins with 25-hydroxylation in the liver by CYP2R1 producing 25-hydroxyvitamin D [25(OH)D], also named calcidiol or

calcifediol [200]. Circulating 25-hydroxyvitmain D is then hydroxylated by CYP27B1 in the kidneys, although this conversion also occurs locally in immune cells in extra-renal tissues, such as macrophages and dendritic cells [201] which is likely to be important for immunomodulation [202]. In target cells, the active form of vitamin D, called 1α,25-dihydroxyvitamin D, [1,25$(OH)_2$D] or calcitriol, binds to the vitamin D receptor (VDR) in cytoplasm and forms an heterodimer with members of the retinoic X receptor (RXR) family, which subsequently translocates to the nucleus [146,200]. In nucleus, VDR-RXR complex binds to specific DNA sequences on gene promoter region called vitamin D response element (VDRE) [203]. Binding to VDRE recruits co-activators and enzymes with histone acetylation activity, causing structural changes in chromatin, thereby facilitating gene transcription [204].

6.1. Vitamin D action

Vitamin D receptors (VDRs) are expressed in a plethora of tissues and cells, while the pleiotropic actions of vitamin D in non-skeletal outcomes have become increasingly recognized [205]. Vitamin D has been acknowledged to influence different functions with regard to the immune, nervous, cardiovascular and endocrine systems [206,207].

Experimental evidence suggests an effect of the biological active form of vitamin D (1,25-dihydroxyvitamin D; 1,25$(OH)_2$D) on multiple different processes and cell types. In the immune system it leads to a decrease in the Th1 response, thought to be the mechanism involved in the association between low vitamin D levels and a variety of autoimmune diseases. It modulates the Th2 response affecting cytokines such as IL-4, IL-5 and IL-13. This is one possible link between vitamin D and allergy/asthma. It has been shown that vitamin D upregulates Treg cells, leading to an increase in the synthesis of the anti-inflammatory cytokine IL-10. In macrophages, vitamin D also upregulates the synthesis of the antimicrobial peptide cathelicidin which may enhance the ability to fight infections. In airway smooth muscle cells, it has been shown to modulate chemokine release. Vitamin D may play a role in foetal lung development and in the differentiation of type II pneumocytes and surfactant secretion. Vitamin D has also been associated with a lower incidence of and mortality from a variety of cancers [208].

Serum 25-hydroxyvitamin D [25(OH)D] is the major circulating form of vitamin D, with a circulating half-life of 2–3 weeks, and it is the best indicator to monitor for vitamin D status [199,209–211]. The circulating half-life of 1,25$(OH)_2$D is approximately 4 hours. It circulates at 1000 times lower concentration than 25(OH)D, and the blood level is tightly regulated by serum levels of parathyroid hormone, calcium, and phosphate [211].

While there is consensus that serum 25(OH)D concentrations should be used to assess vitamin D status (because it reflects the contribution of both diet and endogenous synthesis), there has been a debate on the suggested thresholds to define low vitamin D status or hypovitaminosis D (deficiency and insufficiency) [212]. Previous guidelines define vitamin D deficiency as 25(OH)D values below 50 nmol/L (20 ng/mL) and vitamin D insufficiency as 25(OH)D values of 50–75 nmol/L (20–30 ng/mL) [211]. However, in a most recent consensus statement, vitamin D deficiency is defined as 25(OH)D values below 30 nmol/L (12 ng/mL) and vitamin D insufficiency as 25(OH)D values between 30 and 50 nmol/L (12–20 ng/mL), while levels between 50–125 nmol/L (20–50 ng/mL) are considered safe and sufficient regarding skeletal health in the general population [213]. These definitions are endorsed by the US National Academy of Medicine, while a concentration above 75 nmol/L (30 ng/mL) is recommended by the US Endocrine Society for optimal health benefit particularly in elderly subjects with augmented risk of fractures as well as osseous, renal and digestive disorders [211,214].

6.1.1. Vitamin D and asthma

VDR is found in all tissues, including bronchial epithelial cells and lung fibroblasts [215]. Several epidemiological and clinical studies have linked vitamin D deficiency to various respiratory diseases such as asthma [177,202,216,217]. Low serum 25(OH)D is correlated with impaired lung function, increased AHR, poor asthma control, reduced glucocorticoid response and increased number of asthma exacerbations [177,218,219].

Korn *et al*. investigated 280 adult asthmatic patients with evaluation of 25(OH)D levels and clinical parameters of asthma. A positive relationship between vitamin D status and FEV_1 was observed and they demonstrated that a higher level of serum vitamin D may have a positive effect on prevention of exacerbation and better treatment of asthma symptoms [220]. Higher vitamin D levels are associated with higher rate of FEV_1 and forced vital capacity (FVC) and better asthma control [219,221].

Mechanisms by which vitamin D protects against asthma are shown in Figure 8.

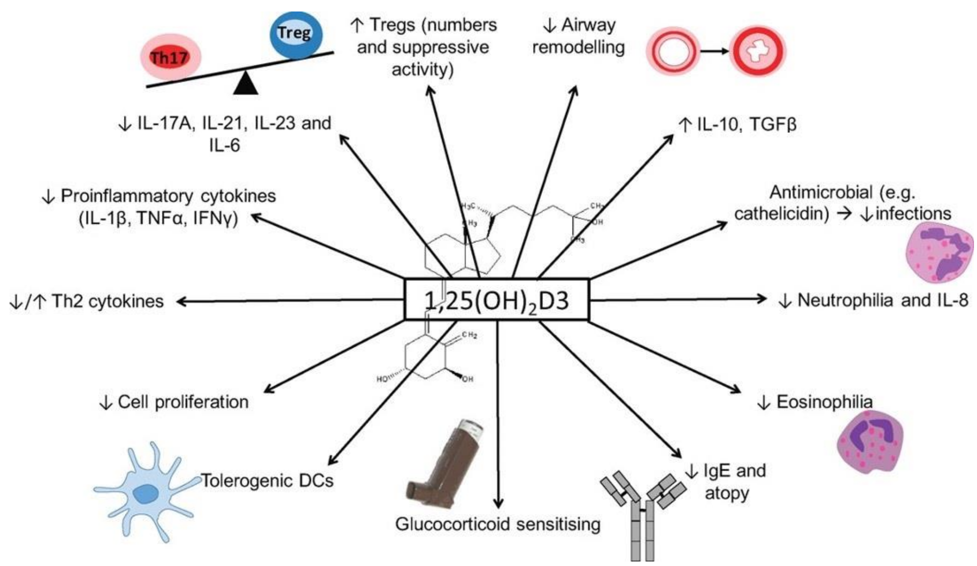


Figure 8. Mechanisms by which vitamin D protects against asthma. Mechanisms by which 1,25-dihydroxyvitamin D3 [1,25(OH)$_2$D3] protects against asthma include increasing glucocorticoid responsiveness, skewing immune cells towards a regulatory phenotype, adjusting the Th1 and Th2 effect, reducing incidence of infections, airway remodelling, eosinophilia, and lowering the levels of IgE [202]. Abbreviations: 1,25(OH)$_2$D3, 1,25-dihydroxyvitamin D3; IL, interleukin; Th, T helper; Treg, T regulatory; TNFα, tumor necrosis factor alpha; IFNγ, interferon gamma; DCs, dendritic cells; IgE, immunoglobulin E; TGFβ, transforming growth factor beta. From Mann et al. *Ann N Y Acad Sci.* 2014;1317:57–69.

In vitro 1,25(OH)$_2$D reduces the production of IgE form peripheral human B cells and increases expression of IL-10 [222,223]. Moreover, serum 25(OH)D inversely correlates with sputum eosinophil count [220,224,225]. Vitamin D can also prevent asthma by adjusting the effect of $CD4^+$ T cells such as, Th1, Th2 and Treg cells. It also makes asthma control better by inhibiting Th17 lymphocytes related to asthma severity and low steroid responsiveness [226]. Interestingly, 1,25$(OH)_2$D potently induced expression of the gene SERPINA1, encoding the anti-protease α-1-antitrypsin. α-1-antitrypsin promotes anti-inflammatory IL-10 synthesis in other immune cell populations [227]. Evidence exists that 1,25$(OH)_2$D controls synthesis of the anti-inflammatory cytokine IL-10, with positive correlations seen *in vivo* [219,228]. Vitamin D has also been shown to induce Treg cell differentiation and IL-10 secretion that helps attenuate the airway smooth muscle cell hypertrophy which underlies severe asthma pathophysiology [219].

As mentioned before, the anti-inflammatory mechanism of vitamin D is well-established regarding asthmatic patients and their disease control. However, it would be interesting to study if the anti-inflammatory effect is still present in asthmatic patients with obesity.

6.1.2. Vitamin D and obesity

Low 25(OH)D levels have been extensively reported in obesity, with a prevalence ranging 40-80% [229–231]. Low vitamin D levels inversely correlated with body weight, BMI, fat mass [207,232,233], and the risk of abdominal obesity [234]. It is well stablished that 25(OH)D levels in subjects with obesity are lower than people of normal weight [235], and a number of clinical studies support the role of obesity as a causal risk factor for the development of a low vitamin D status [236–238]. However, the exact mechanisms responsible for this association remain unclear.

There is an hypothesis based on the potential of adipose tissue to store and retain vitamin D, preventing uncontrolled synthesis of 25(OH)D in the liver and protecting against potential toxicity [236]. When obese and non-obese adults were exposed to simulated sunlight or received an oral dose of 50,000 IU of vitamin D, they were able to raise their blood levels of vitamin D by no more than 50% compared with non-obese adults [211]. The study by Wortsman *et al.* demonstrated that although skin biosynthesis of vitamin D did not differ between subjects with normal weight and obesity, serum 25(OH)D concentrations were lower in those with obesity [232]. These findings suggest that, vitamin D, as a fat-soluble vitamin, is possibly accumulated and sequestered into adipose tissue and is unable to enter the circulation to produce 25(OH)D in the liver. This can lead to lower plasma levels of 25(OH)D in subjects with excess accumulation of adipose tissue [205].

Furthermore, cytochrome P450 (CYP) 2R1 was repressed in mice livers by fasting, obesity, type 2 diabetes, and type 1 diabetes and was associated with the downregulation of vitamin D 25-hydroxylation activity [239]. Similarly, Roizen *et al*. reported that obesity downregulates CYP2R1 and vitamin D 25-hydroxylation in the mouse liver [240]. These results indicate that, in contrast to previous assumptions, CYP2R1 expression and consequent vitamin D 25-hydroxylation are actively regulated and may contribute to the changes in circulating 25(OH)D levels. Currently, there is no information whether energy homeostasis affects CYP2R1 regulation in humans or whether similar regulation occurs in extrahepatic tissues that express CYP2R1 [200].

One of the few studies regarding vitamin D effect in asthmatic patients with obesity found that FEV_1 and functional residual capacity (FRC) were lower among vitamin D deficient obese asthmatic children than their sufficient counterparts, and total lung capacity was lower than their insufficient counterparts. Similar associations were not observed in normal-weight asthmatics and were not influenced by systemic inflammation [241].

6.2. Glucocorticoid sensitizing by vitamin D

Vitamin D alters the anti-inflammatory action of glucocorticoids. Both steroid hormone receptors and vitamin D receptors belong to nuclear receptor super family. Given the structural similarities of steroid hormone molecules and their receptors, their co-localization in cells, and similar mechanisms on gene regulation and signalling, it is possible that cross-talk between these molecules affects glucocorticoid function and glucocorticoid response [146].

Several reports have shown that low level of serum 25(OH)D can lead to a reduced glucocorticoid response and, consequently, a higher demand of daily doses of glucocorticoids [218,219,221]. Moreover, it has been suggested that receiving high doses of 1,25(OH)$_2$D as an add-on therapy taken at monthly intervals may significantly decrease the requirement for glucocorticoid [242] and subsequently enhance the glucocorticoid response [243]. A small prospective study of children with asthma showed that vitamin D added on budesonide treatment reported less number of respiratory infections included asthma exacerbation than the steroid treatment alone [217]. Another epidemiological study in children showed that higher vitamin D levels were associated with lower use of ICS as well as leukotriene antagonists. Moreover, higher levels were also associated with low total IgE levels and blood eosinophil count [244].

These studies suggest that analysing vitamin D serum levels before treating patients with glucocorticoids is crucial to prevent hyporesponsiveness especially in vitamin D deficient patients.

6.2.1. Molecular interactions of vitamin D and glucocorticoids

In addition to the *in vivo* clinical studies illustrating a glucocorticoid-sensitizing effect of vitamin D, evidence also exists *in vitro* studies*.* Interaction of vitamin D with GR is not clearly understood, but studies show that vitamin D has steroid sparing effects enhancing glucocorticoid responsiveness by increasing its anti-inflammatory activity [146,161]. Significantly lower concentration of glucocorticoids was required to suppress pro-inflammatory cytokine production by PBMCs in the presence of vitamin D. This suggests that vitamin D may enhance glucocorticoid responsiveness resulting in a lower dose of glucocorticoid required to reduce inflammation, thereby reducing glucocorticoid-mediated side effects [161].

In patients with glucocorticoid resistant asthma, there appear to be defects in glucocorticoid induced gene transcription of anti-inflammatory mediators such as IL-10 and MKP-1. In an *in vitro* study, PBMCs isolated from asthmatic subjects were treated with dexamethasone alone and dexamethasone with vitamin D. Enhanced induction of MKP-1 and IL-10 was seen in dexamethasone with vitamin D treatment group as compared to dexamethasone alone treatment group [216]. Moreover, Xystrakis *et al*. showed that adding 1,25(OH)$_2$D to CD4$^+$ T cells culture or supplementing steroid refractory asthmatics with 1,25(OH)$_2$D restored the dexamethasone induction of IL-10 [245]. A proposed mechanism for this is that 1,25(OH)$_2$D can overcome the downregulation of the glucocorticoid receptor by dexamethasone [202]. They concluded that supplementation of vitamin D may increase therapeutic response to glucocorticoid in glucocorticoid-resistant asthma [245].

Asthmatic patients synthesized much higher levels of IL-17A and IL-22 than non-asthmatic control subjects, with patients with steroid resistant asthma expressing the highest levels of IL-17A. Glucocorticoids did not inhibit IL-17A cytokine expression in patients and, in contrast, enhanced its production in cell cultures from control subjects. Treatment with 1,25(OH)$_2$D with or without dexamethasone significantly reduced both IL-17A and IL-22 levels [216,246–248]. Chambers *et al.* found that oral calcitriol improved dexamethasone-induced IL-10 production *in vitro* and suppressed IL-17A production [31]. Human studies reveal that 1,25(OH)$_2$D diminished the production of IL-17A via modulation of the VDR pathway [202,247,249]. Cross-talk between vitamin D and glucocorticoids is represented in Figure 9.

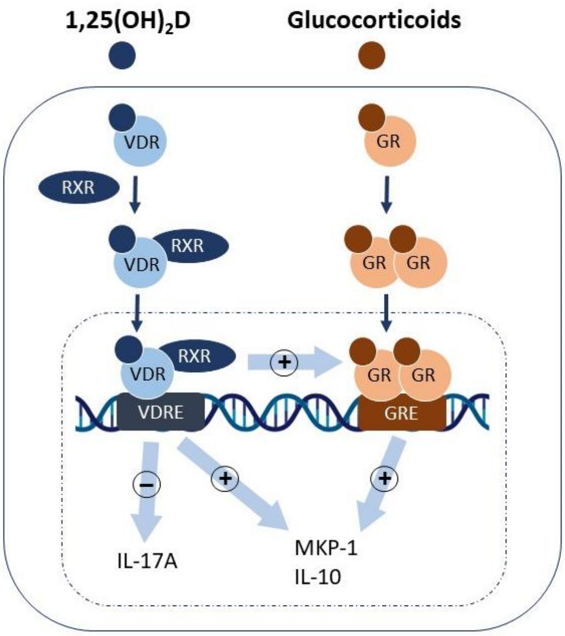


Figure 9. Cross-talk between vitamin D and glucocorticoids. 1,25-dihydroxyvitamin D [1,25(OH)$_2$D], the active form of vitamin D, enhance the effects of glucocorticoids by promoting MKP-1 expression and by producing anti-inflammatory proteins such as IL-10. Moreover, 1,25(OH)$_2$D may reduce the production of IL-17A via modulation of the VDR pathway. Abbreviations: VDR, vitamin D receptor; RXR, retinoic X receptor; VDRE, vitamin D response elements; IL, interleukin; MKP-1, mitogen-activated protein kinase phosphatase 1; GR, glucocorticoid receptor; GRE, glucocorticoid response elements.

As we have just mentioned, a factor to consider when prescribing glucocorticoids in asthmatic patients, whether obese or not, is the possible synergy between vitamin D and glucocorticoids. Interestingly, glucocorticoid interacting with vitamin D inhibit CYP27B1, which converts circulating 25(OH)D into active calcitriol. This observation supports to treat patients directly with calcitriol, to prevent molecular interactions [31]. If the patient is supplemented with vitamin D, it could be possible to decrease the glucocorticoid dose and, therefore, significantly reduce the adverse effects. However, more *in vitro* studies are needed to verify this association and to explain possible mechanisms underlying glucocorticoid and vitamin D synergy.

7. Bariatric surgery

Obesity is a complex and multifactorial disease involving sociocultural, economic and genetic aspects that make it difficult to approach and treat. Weight loss is the main target of all therapeutic options and can be achieved with a conservative management (diet, changes in life style, physical exercise or pharmacotherapy) or surgically. Bariatric surgery is based on the reduction of the caloric intake by means of the modification of the gastrointestinal tract [250]. It is well-known that bariatric surgery is a safe and effective option for the treatment of severe obesity and that it has a profound effect on the low-grade systemic inflammation of these individuals [57,251]. It is currently considered as the recommended treatment for morbid obese subjects (BMI ≥ 40 kg/m^2) as it provides the best results and the longest lasting effects. There are two types of surgery: malabsorptive and restrictive. On the one hand, malabsorptive bariatric surgery derives portions of small intestine in which nutrient absorption occurs. On the other hand, restrictive bariatric surgery limits intake by creating a small gastric reservoir. Restrictive procedures include: 1) gastroplasty; 2) adjustable gastric band; and 3) sleeve gastrectomy [250]. Sleeve gastrectomy, also known as gastric sleeve is not only a bariatric surgery technique used for morbid obese subjects, but also for the treatment of type 2 diabetes and metabolic syndrome in moderate obese subjects [252]. This type of surgery was first performed for gastric tumor resection and later it was adapted for bariatric surgery as it produced an important weight loss. Roux-en-Y gastric bypass is frequently referenced as a combination of restrictive and malabsorptive procedures [250]. This treatment consists of a subtotal gastrectomy leaving a small gastric pouch as the stomach and the distal portion of the small intestine are anatomized with the gastric pouch. The distal portion of the stomach and proximal small intestine is anatomized end-to-side under further down the jejunum [250]. In 1991, the National Institute for Health reached a consensus of gastrointestinal surgery for severe or morbid obesity. The resulting recommendations of this consensus included the criteria listed below for bariatric surgery: 1) BMI ≥ 40 kg/m^2, 2) BMI ≥ 35 kg/m^2 in subjects who present comorbidities that can improve with weight loss or high risk conditions such as severe obstructive sleep apnoea, obesity related heart disease or severe type 2 diabetes and, 3) Other criteria include: failure to lose weight by medical control, in the absence of medical and/or psychological contraindications [253].

7.1. Effects of weight loss on asthma and inflammation

Weight loss obtained thanks to dietetic treatments has a beneficial effect on asthma control, and reduces the use rescue medication, asthma exacerbations, hospitalizations, as well as improves the quality of life, lung function tests [254–256]. However, when assessed, there were no significant changes in markers of inflammation including: fraction exhaled nitric oxide, induced sputum cellularity, leptin, C-reactive protein, eotaxin, and TGF-β [254,255]. Combining diet with exercise is more effective on asthma control than taking these measures alone [257–259]. Inflammatory leptin and IL-6 plasma levels were significantly reduced after the combined interventions [257]. Percentage sputum eosinophil was significantly reduced in subjects who completed the diet-exercise intervention. In contrast, a significant reduction in sputum percentage neutrophils was found in females but not in males [257]. A significant reduction in plasma levels of IL-4, IL-6, TNF-α, and leptin, associated with increased levels of 25-hydroxyvitamin D [25(OH)D], IL-10, and adiponectin has also been reported with the combined therapy [258,259].

The beneficial effect of bariatric surgery on severity, control or therapeutic load was reported in asthma in the late 1990s [260,261]. These seminal studies found significant improvements in asthma control, asthma severity, medications required, hospitalizations, sleep, exercise capacity and lung function tests [260,261].

Sideleva *et al*. sought to determine if adipose tissue inflammation was associated with increased inflammation in the airway of obese asthma patients and whether weight loss after bariatric surgery would simultaneously improve metabolic and airway inflammation. In the asthmatic subjects with obesity, concentrations of the pro-inflammatory cytokines IL-6 and IL-8 decreased with weight loss. Changes in leptin and adiponectin protein levels in airways were similar to those found in adipose tissue: at baseline leptin levels were higher, and adiponectin lower in BALF of participants with asthma compared with control subjects. AHR was more significantly related to visceral fat leptin than to BMI. The authors concluded that the high levels of adipokines produced in visceral adipose tissue in obese asthma are associated with airway reactivity but not with airway inflammation [111]. With respect to markers of allergic inflammation, no changes in submucosal cell counts of eosinophils, neutrophils, B cells, macrophages, $CD4^{+}$ T cells or CD8+ T cells were found with weight loss following bariatric surgery. In contrasts, mast cells decreased significantly in the same patients [262].

In a large retrospective study, bariatric surgery led to the discontinuation of bronchodilators in 39.3% of patients within one year [263]. Similar results, with a 49% reduction in inhaled treatments at 1 year of bariatric surgery, improvement in asthma control assessed by the ACQ score, and reduction of emergency room visits were found in other studies [251,264]. Bariatric surgery also improved lung function and reduced AHR [178,265]. However, in another study, the improved effect on AHR was only found in the group of obese asthmatics with high serum IgE levels [251]. The effect on asthma control, therapeutic reduction and lung function seems to persist after five years of bariatric surgery [266]. Taken together these observations support the use of bariatric surgery in the treatment of severe obese asthma. However, it should be considered after previous failure of medical, nutritional, dietetic, exercise and psychotherapeutic treatment in severe asthma patients with obesity.

7.2. Weight loss and vitamin D levels

Buscemi *et al*. confirmed that patients with obesity have lower vitamin D levels that normalize after significant weight loss induced by a low-calorie ketogenic diet, supporting the hypothesis that vitamin D is stored in the adipose tissue and released following weight loss [267]. However, other authors found that bariatric patients who were found to have vitamin D in their fat (4–320 ng/g) showed no significant change in their serum 25(OH)D levels 3, 6, and 12 months after surgery [211].

Meta-analysis of 10 prospective observational studies, including 344 patients with severe obesity who underwent bariatric surgery (roux-en-Y gastric bypass), demonstrated that 25(OH)D did not change significantly after surgery, while calcium decreased and parathyroid hormone increased [268]. Of note, vitamin D and calcium supplementation postoperatively was reported in all studies, while duration of follow-up was 6–36 months. However, bone mineral density was significantly decreased, indicating bone loss after surgery, despite vitamin D and calcium supplementation. There was a high heterogeneity between studies regarding the doses and the duration of supplemental vitamin D [205]. Vitamin D deficiency was investigated in a meta-analysis of 12 prospective studies of 1285 subjects with obesity after bariatric surgery, who received vitamin D supplementation [269]. Vitamin D supplementation was associated with significant improvement in 25(OH)D levels 1 year after BS, independently of study design, baseline levels, weight loss and vitamin D dosage. Also, vitamin D deficiency decreased from 54% preoperatively to 31% 1 year postoperatively [205]. Finally, a recent meta-analysis of 13 studies published between 2010 and 2018 compared gastric bypass to sleeve gastrectomy regarding bone metabolism and related complications [270]. This meta-analysis showed that circulating 25(OH)D and calcium were significantly lower after gastric bypass compared to sleeve gastrectomy. Interestingly, BMI and bone mineral density after these procedures were similar. The doses of supplemental vitamin D and calcium were not reported and the duration of postoperative follow-up was 12 months in most of the included studies. Overall, the above meta-analyses are inconclusive regarding vitamin D status after bariatric surgery. Besides the high heterogeneity and the small number of selected studies, the variable vitamin D supplementation strategies present the most important confounding factor [205]. The effect of bariatric surgery on vitamin D status is extremely difficult to be evaluated, due to the great variability in vitamin D supplementation strategy after surgery.

Nutritional deficiency is a well-known long-term complication of bariatric surgery and varies according to the type of procedure [271]. Sleeve gastrectomy is a restricted procedure, while gastric bypass is both restricted and malabsorptive. Malabsorptive procedures hinder the absorption of macronutrients and micronutrients, including B vitamins, fat-soluble vitamins (A, D and K) and minerals (calcium, iron, zinc and copper) [271,272]. Therefore, supplementation with vitamin D and calcium is always recommended after surgery according to consensus guidelines [273].

Interestingly, CYP2R1 mRNA expression was significantly upregulated in adipose tissue samples taken after the gastric bypass surgery compared with the samples obtained before surgery. These results indicate that obesity affects CYP2R1 expression in humans and weight loss increases adipose tissue CYP2R1 expression. Furthermore, CYP27B1 expression was decreased by 64% after surgery in all patients. In contrast to CYP2R1, CYP27B1 expression is relatively tissue selective and the expression in predominantly detected in the kidney [200].

Therefore, observation of patients before and after surgery may provide a unique perspective of the relationship between obesity-related comorbidities and their potential improvement after weight loss. However, the main part of the studies has analysed the effect of bariatric surgery in asthmatic subjects with obesity or in obese subjects without asthma, with no comparison between them. To determine the precise effect of bariatric surgery in asthmatic patients with obesity, an obese control group is recommended.

In summary, what we know is that:

1. The prevalence of both asthma and obesity is increasing worldwide due to changes in lifestyle.

2. There is a link between asthma and obesity epidemics, and asthma with obesity is nowadays considered a specific phenotype recognized by international guidelines.

3. Obesity is associated with a chronic low-grade inflammatory state, and asthma is characterized by airway inflammation. Both are considered inflammatory diseases and the coexistence of them increases the inflammatory complexity.

4. Weight loss induced by bariatric surgery improves systemic inflammation and asthma symptomatology.

5. There is growing literature that explains that asthmatic patients with obesity present a reduced response to glucocorticoids, more frequent exacerbations and poor asthma control.

6. Vitamin D has anti-inflammatory properties and vitamin D deficiency is related to asthma exacerbations and obesity.

However, it is not clear whether:

1. Low-grade systemic inflammation present in obesity influences airway inflammation, and thus worsens asthma pathogenesis.

2. Asthma with obesity patients suffer from a specific inflammatory endotype that could explain this association.

3. Glucocorticoid hyporesponsiveness seen in clinical practice can be detected *in vitro* using peripheral blood cells from asthmatic patients with obesity.

4. Some molecules related to asthma with obesity phenotype can be useful biomarkers to predict glucocorticoid response.

5. Weight loss induced by bariatric surgery increases glucocorticoid responsiveness.

6. Vitamin D serum levels are associated with glucocorticoid response, and vitamin D supplementation can increase glucocorticoid sensitivity in asthmatic subjects with obesity.

HYPOTHESIS

Asthma-obesity related phenotype is characterized by poor control of asthma symptomatology, more frequent exacerbations and a decreased response to glucocorticoid treatment. The mechanisms of glucocorticoid hyporesponsiveness in obese asthmatics are not well defined. Understanding the links between asthma, obesity, and vitamin D can help elucidate.

A) In the first study, we hypothesized that the selection of suitable reference genes is the first and crucial step before performing genetic studies. Reference genes should have a stable expression in different samples and should be not affected by experimental conditions, such as incubation with dexamethasone and vitamin D.

B) In the second study, we hypothesized that the spill over of pro-inflammatory substances from adipose tissue results in enhanced systemic inflammation in obese asthma subjects compared with their non-obese asthma counterparts. The activated systemic inflammation accounts for the increased airway inflammation, which in turn contributes towards explaining the poor response of obese asthmatics to anti-inflammatory therapy. Weight loss after bariatric surgery may counteract low-grade systemic inflammation in subjects with obesity.

C) In the third study, we hypothesized that the reduced response to glucocorticoids of asthmatic patients with obesity can be measured *in vitro* through the analysis of PBMCs or $CD4^+$ T cells proliferation incubated with dexamethasone. Moreover, the increased glucocorticoid sensitivity due to weight loss induced by bariatric surgery can be demonstrated by using *in vitro* analysis. Finally, we hypothesized that serum vitamin D serum correlates with the *in vitro* glucocorticoid response and, that in cells (PBMCs and $CD4^+$ T cells) incubated with vitamin D there is an increased glucocorticoid response.

OBJECTIVES

According to this background, this thesis has been directed to investigate the relationship between asthma and obesity inflammation, glucocorticoid resistance, and the vitamin D implications, evaluating asthmatic patients with obesity before and after bariatric surgery. The current investigation was designed with the following specific aims:

A) First manuscript: Reference gene validation for RT–qPCR in PBMCs from asthmatic patients with or without obesity.

General objective: To validate the reliability of eight reference genes which will be used in further studies involving genetic studies in PBMCs from asthmatic patients with obesity treated *in vitro* with dexamethasone and/or vitamin D.

Specific objectives:

- To analyse reference genes stability through reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using four different algorithms in PBMCs from asthmatic patients with and without obesity, compared with heathy subjects.
- To select the most stable reference genes in PBMCs incubated with dexamethasone and vitamin D.

B) Second manuscript: Differences in inflammatory cytokine profile in obesity-associated asthma: effects of weight loss.

General objective: To evaluate the systemic inflammatory pattern present in obese subjects with and without asthma, before and after bariatric surgery.

Specific objectives:

- To assess pro-inflammatory cytokine serum levels in asthmatic subjects with obesity and compare their levels with non-asthmatics and non-obese participants.
- To investigate the possible role of specific inflammatory molecules, present in obesity-related asthma inflammation.
- To compare the systemic inflammation present in obese asthmatic and obese subjects before and after bariatric surgery.

C) Third manuscript: Weight loss and vitamin D improve hyporesponsiveness to corticosteroids in obese asthma.

General objective: To investigate the reduced response of the anti-inflammatory activity of glucocorticoids in asthmatic patients with obesity and the association with vitamin D deficiency, and how both deficiencies are corrected following weight loss-induced bariatric surgery.

Specific objectives:

- To show that asthmatic patients with obesity present glucocorticoid hyporesponsiveness, compared with non-obese asthmatics, measured *in vitro* through dexamethasone-treated PBMCs proliferation assay and determine the implication of $CD4^+$ T cells on glucocorticoid sensitivity.
- To demonstrate that *in vitro* glucocorticoid sensitivity increases after bariatric surgery.
- To show that the expression of the anti-inflammatory gene MKP-1 induced by the action of glucocorticoids is reduced in asthmatic patients with obesity.
- To evaluate the relationship between serum vitamin D levels and glucocorticoid sensitivity measured *in vitro*.
- To identify whether the active form of vitamin D has an anti-inflammatory effect on cells (PBMCs and $CD4^+$ T cells).
- To explore the synergy between the active form of vitamin D and dexamethasone in the inhibition of PHA-induced proliferation assay in PBMCs and $CD4^+$ T cells.

RESULTS

A) First manuscript: Reference gene validation for RT–qPCR in PBMCs from asthmatic patients with or without obesity

methods and protocols

MDPI

Brief Report

Reference Gene Validation for RT–qPCR in PBMCs from Asthmatic Patients with or without Obesity

Marina Bantulà [1,*], Ebymar Arismendi [1,2,3], César Picado [1,2,3], Joaquim Mullol [1,3,4], Jordi Roca-Ferrer [1,3] and Valeria Tubita [1]

1 August Pi i Sunyer Biomedical Research Institute (IDIBAPS), Hospital Clinic, Universitat de Barcelona, 08036 Barcelona, Spain; earismen@clinic.cat (E.A.); cpicado@ub.edu (C.P.); jmullol@clinic.cat (J.M.); jrocaf@clinic.cat (J.R.-F.); v.tubita@gmail.com (V.T.)
2 Department of Pneumology, Hospital Clinic, 08036 Barcelona, Spain
3 Centre for Biomedical Investigation in Respiratory Diseases (CIBERES), 08036 Barcelona, Spain
4 Rhinology Unit and Smell Clinic, ENT Department, Hospital Clinic, 08036 Barcelona, Spain
* Correspondence: bantula@clinic.cat

Abstract: Obesity is known to impair the efficacy of glucocorticoid medications for asthma control. Glucocorticoid-induced gene expression studies may be useful to discriminate those obese asthmatic patients who present a poor response to glucocorticoids. The expression of genes of interest is normalized with respect to reference genes (RGs). Ideally, RGs have a stable expression in different samples and are not affected by experimental conditions. The objective of this work was to analyze suitable RGs to study the role of glucocorticoid-induced genes in obese asthmatic patients in further research. The gene expression of eight potential RGs (*GUSB*, *B2M*, *POLR2A*, *PPIA*, *ACTB*, *GAPDH*, *HPRT1*, and *TBP*) was assessed with reverse transcription–quantitative polymerase chain reaction in peripheral blood mononuclear cells (PBMCs) from asthmatic, obese asthmatic, and healthy individuals. Their stability was analyzed using four different algorithms—BestKeeper, ΔCt, geNorm, and NormFinder. geNorm analysis recommended the use of a minimum of three genes for normalization. Moreover, intergroup variation due to the treatment was calculated by NormFinder, which found that *B2M* was the gene that was least affected by different treatments. Comprehensive rankings indicated *GUSB* and *HPRT1* as the best RGs for qPCR in PBMCs from healthy and asthmatic subjects, while *B2M* and *PPIA* were the best for obese asthmatic subjects. Finally, our results demonstrated that *B2M* and *HPRT1* were the most stable RGs among all groups, whereas *ACTB*, *TBP*, and *GAPDH* were the worst shared ones.

Keywords: reference genes; RT–qPCR; obesity; asthma; BestKeeper; ΔCt; geNorm; NormFinder

check for updates

Citation: Bantulà, M.; Arismendi, E.; Picado, C.; Mullol, J.; Roca-Ferrer, J.; Tubita, V. Reference Gene Validation for RT–qPCR in PBMCs from Asthmatic Patients with or without Obesity. *Methods Protoc.* **2022**, *5*, 35. https://doi.org/10.3390/mps5030035

Academic Editor: Stuart Levine

Received: 7 March 2022
Accepted: 19 April 2022
Published: 22 April 2022

1. Introduction

Synthetic glucocorticoids (GCs) are a large family of anti-inflammatory agents used in asthma as the initial recommended controller treatment [1]. When asthma is associated with obesity (defined as having a body mass index of $\geq$30 kg/m^2), patients present a decreased response to GC treatment [2] that results in poor asthma control with an increase in the frequency of exacerbations and a greater severity of respiratory symptoms [3,4]. Furthermore, obese patients present low levels of vitamin D, and it is known that this is associated with impaired lung function, higher asthma exacerbation frequency, and a reduced response to GCs [5–7].

Currently, there are no biomarkers to predict GC effectiveness; however, genetic testing may help discriminate patients with different sensitivities to GCs. The expression of anti-inflammatory genes induced by GCs in peripheral blood mononuclear cells (PBMCs) represents a useful in vitro method to predict and evaluate clinical responses to GCs. PBMCs are a good and long-lasting source of nucleic acids for this type of study [8]. Comparisons of their gene expression profiles in asthmatic subjects with and without obesity can provide information on the pathophysiological mechanisms underlying the poor response to GC in these patients.

Reverse transcription (RT)—quantitative polymerase chain reaction (qPCR) has become the method of choice for mRNA gene expression analysis [9,10] due to its high sensitivity, specificity, broad quantification range [11], low cost, and reliability [12]. However, the accuracy of the results may be influenced by several factors, including inter-sample variations, sample collection, RNA preparation and quality, and the efficiency of RT and qPCR amplification [13,14]. To mitigate differences in RNA sampling, the normalization of gene expression data against reference genes (RGs) is critical to avoid the misinterpretation of results in gene expression studies [15]. An ideal RG must be stably expressed, non-regulated, and unaffected by biological or experimental conditions [13,16]. However, numerous studies have shown that commonly used RGs considerably vary in different disease processes or different tissue and cell types [17,18]. For this reason, it is essential to validate the suitability of potential RGs for each experimental condition [19].

To date, no studies that have validated RGs in PBMCs treated with GCs from asthmatic patients with or without obesity compared to healthy subjects. Here, we aimed to analyze gene expression stability of eight commonly used RGs via RT–qPCR using four different algorithms—BestKeeper [20], ΔCt [21], geNorm [13], and NormFinder [14]—in order to validate their reliability for further gene expression studies involving PBMCs from obese asthmatic patients treated in vitro with dexamethasone (DEX) and/or the active form of vitamin D (1,25-dihydroxyvitamin D_3) (Vit D_3).

2. Materials and Methods

2.1. Patients and Control Subjects

The study was conducted with PBMCs from diagnosed asthmatic patients (n = 3) and obese asthmatic patients (n = 3) and with age- and gender-matched healthy individuals (n = 3). The patients' clinical features are shown in Supplementary Table S1. Asthmatic and obese asthmatic patients were not under systemic corticosteroid use.

The study was conducted with informed written consent from the participating subjects. The study was approved by the ethics committee of our institution (HCB/2016/0861), and all methods were carried out in accordance with relevant guidelines and regulations.

2.2. Blood Collection and PBMCs Isolation

Twenty milliliters of whole blood was collected from each patient via venipuncture into a vacutainer tube containing an anticoagulant (EDTAK2). PBMCs were isolated using Lymphoprep^{TM} (Stem Cell TM) following the manufacturer's instructions. Whole blood was diluted to 1:1 with a balanced salt solution, and this was then layered on top of the Lymphoprep solution in a 50 mL conical tube and centrifuged at 800× g for 20 min at room temperature. After centrifugation, the white ring containing mononuclear cells was transferred to a new tube and washed twice with balanced salt solution [22].

2.3. Cell Culture Conditions

PBMCs were cultured at 37 °C in a 5% CO_2 atmosphere using an RPMI-1640 medium supplemented with 5% charcoal-stripped fetal bovine serum and antibiotics (100 U/mL of penicillin and 100 µg/mL streptomycin), with a final cell concentration of 2×10^6/mL.

The cell suspension from each patient was divided into four wells according to the following experimental conditions: negative control well (cells with culture medium), DEX at a final concentration of 1×10^{-5} M, Vit D_3 at a final concentration of 1×10^{-7} M, and DEX 1×10^{-5} M with Vit D_3 1×10^{-7} M together. These conditions were repeated in order to harvest cells at two different time-points: 6 and 24 h.

2.4. RNA Extraction and cDNA Synthesis

Total RNA was isolated from PBMCs using the TRIzol (Life Technologies, Paisley, UK) reagent according to the manufacturer's protocol. Total mRNA concentration was measured at 260 nm, and purity was assessed from the 260/280 nm absorbance ratio. RNA was treated with DNase I (Thermo Fisher, Vilnius, Lithuania) for 30 min at 37 °C to remove any potential genomic DNA contamination. We converted 1 µg of RNA into cDNA using the High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions (Thermo Fisher, Vilnius, Lithuania). Samples were incubated for 10 min at 25 °C, 120 min at 37 °C, and 5 min at 85 °C. Final cDNA products were diluted 10-fold prior to use in qPCR.

2.5. Real-Time qPCR

The expression of eight selected RGs (Supplementary Table S2) was analyzed with real-time qPCR. qPCR experiments were carried out using 100 ng of cDNA in the Viia7 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's guidelines. The qPCR reaction consisted of 0.5 µL of TaqMan gene Expression Assay (20X), 5 µL of TaqMan Universal PCR Master Mix (2X), 2.5 µL of RNase-free water, and 2 µL of cDNA in a total volume of 10 µL. The thermal cycler was set to 95 °C for 20 min, followed by 40 reaction cycles of 1 s at 95 °C and 20 s at 60 °C. All qPCR reactions were set up in duplicate.

2.6. Statistical Analysis

Gene expression stability was analyzed using four different, statistically-based methods: BestKeeper, ΔCt, geNorm, and NormFinder. Afterwards, a final ranking based on the results from these algorithms was calculated.

Clinical data were reported as mean ± SD for normally distributed data and median (range) for experimental nonparametric data. Comparisons of gene expression levels between subgroups were performed using the Kruskal–Wallis H test for nonparametric data followed by post hoc Dunn's multiple comparisons test. The Wilcoxon test was used to compare paired samples. All analyses were performed using GraphPad Prism version 6.02 for Windows, (GraphPad Software, La Jolla, CA, USA). Statistical significance was defined as p-value < 0.05.

3. Results

3.1. Quantification Cycle (Cq) Characterization of Candidate RGs

Our results showed a wide range of expression levels (Supplementary Table S3). One of the samples showed a 3-fold intrinsic variance compared to the mean of all RGs, so this sample was excluded [20]. The final analysis contained 24 samples from healthy patients, 24 from asthmatic patients, and 23 from obese asthmatic patients. The high-abundance genes (Cq < 25) were *B2M* < *PPIA* < *ACTB* < *GAPDH* in healthy and obese asthmatic PBMCs and *B2M* < *ACTB* < *PPIA* < *GAPDH* in asthmatic PBMCs. The low-abundance genes (Cq > 25) were *HPRT1* > *TBP* > *GUSB* > *POLR2A* in the three groups studied.

3.2. Influence of Experimental Conditions

First of all, we compared gene expression between 6 and 24 h of incubation time and found no differences in PBMCs from healthy individuals. However, *GAPDH* showed an increased expression after 24 h of incubation in both the asthmatic and obese asthmatic groups ($p < 0.05$ and $p < 0.01$, respectively). Moreover, *PPIA* and *TBP* presented differences in asthmatic PBMCs between 6 and 24 h ($p < 0.05$ and $p < 0.01$, respectively).

Secondly, we assessed the impact of in vitro treatments: DEX with or without the addition of Vit D_3 for 6 and 24 h. In PBMCs from healthy individuals, there were no differences between experimental conditions; however, we found differences in gene expression in asthmatic and obese asthmatic PBMCs. DEX was the treatment that most affected stability: *POLR2A* and *GAPDH* gene expression presented differences at both 6 h ($p < 0.05$ and $p < 0.01$, respectively) and 24 h ($p < 0.01$ and $p < 0.05$, respectively). *HPRT1* and *TBP* showed differences after 6 h of incubation ($p < 0.01$ for both). Vit D_3 treatment only affected *POLR2A* gene expression after 6 h of incubation ($p < 0.05$). No differences were found with Vit D_3 at 24 h or with the co-incubation of DEX and Vit D_3 at 6 h. *GUSB* and *POLR2A* showed differences with the combined treatment of DEX and Vit D_3 after 24 h of incubation ($p < 0.01$ for both).

3.3. Analyses of Gene Expression Stability

To determine the stability values for each candidate RG, we used four different programs (BestKeeper, ΔCt, geNorm, and NormFinder).

3.3.1. BestKeeper Analysis

BestKeeper determines the optimal RGs by performing a pairwise correlation approach and then ranking the RG candidates' stability based on the standard deviation (SD) of Cq values; RGs with SD > 1 are excluded [20]. All candidate RGs showed a strong correlation ($0.69 < r < 0.92$) and were combined into an index. The highest Pearson correlation coefficient (r) value for the relationship between the index and the contributing RGs was obtained for *HPRT1* ($r = 0.89$, $p = 0.001$) in healthy subjects. In obese asthmatic patients, *HPRT1* also presented the highest correlation coefficient ($r = 0.92$, $p = 0.001$), whereas the highest coefficient in asthmatic patients was for *GAPDH* ($r = 0.92$, $p = 0.001$). SD was <1 for all genes when studying groups separately, signifying that all candidate RGs could be considered stable genes. The worst ranked gene was *GAPDH* for all three groups.

3.3.2. ΔCt Analysis

ΔCt generates 'pair of genes' comparisons between each gene and the other RGs within each sample, and then it calculates the average SD against the other RGs [21]. All samples presented good stability values (SD < 1), but the samples that had the lowest SDs were *HPRT1* in healthy PBMCs, *GUSB* in asthmatic PBMCs, and *B2M* in obese asthmatic PBMCs.

3.3.3. geNorm Analysis

geNorm provides a measure of gene expression stability (M) by calculating the average pairwise variation of each RG from all the other RG candidates. A low M value represents stable gene expression [13]. All studied genes had an M value below the default limit of 1.5, demonstrating that all tested genes had high expression stability. Genes with the lowest M value for each group were the same as for the ΔCt method. Furthermore, the least stable RGs also coincided between both algorithms, with *GAPDH* showing the lowest stability value for both healthy and obese asthmatic PBMCs and *B2M* showing the lowest stability value for asthmatic PBMCs.

Additionally, the optimal number of RGs needed to quantify the expression of the gene of interest was also determined using geNorm. Here, the pairwise variation ($V_n/n+1$) between the two sequential normalization factors is calculated, and when the V value is below the 0.15 cut-off value, it is not necessary to include additional RGs [13]. The V3/4 value was found to be below the threshold of 0.15 in healthy and obese asthmatic samples, indicating that three genes were sufficient for normalization. However, in asthmatic PBMCs, the optimal number of RGs for normalization was four.

3.3.4. NormFinder

NormFinder is an add-in for Microsoft Excel that uses a model-based approach to estimate intragroup and intergroup variation for each candidate RG. Both values are combined to give a stability value (S value), where the lowest value indicates the most stable expression [14]. The most stable gene in this study was *HPRT1*, and the ones with the highest S values were *GAPDH* for healthy and obese asthmatic PBMCs and *PPIA* for asthmatic PBMCs.

Moreover, intergroup variation was calculated by NormFinder to assess the influence of different cell culture treatments (Figure 1). The most stable genes with the least intergroup variation were *B2M* for healthy and obese asthmatic and *HPRT1* for asthmatic PBMCs.

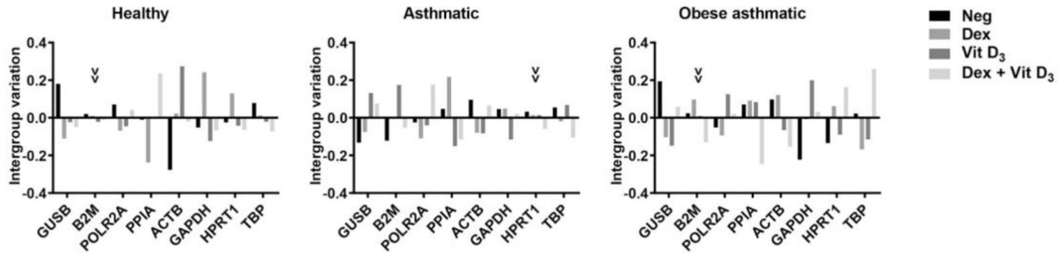


Figure 1. Intergroup variations in PBMCs from healthy subjects, asthmatic subjects, and obese asthmatic subjects. Arrowheads point to the most stable gene with the least intergroup variation. Neg: negative control; Dex: dexamethasone; Vit D_3: 1,25-dihydroxyvitamin D.

3.4. Comprehensive Ranking

An overall final ranking was calculated for each RG as the geometric mean based on their weight according to the used statistical methods (Table 1). RGs were sorted from the most stable gene, having the lowest ranking position, to the least stable one, with the highest number. As shown in Table 1, in healthy and asthmatic PBMCs, *HPRT1* and *GUSB* were the best ranked RGs, whereas *B2M* and *HPRT1* were the most stable ones for the obese asthmatic group.

Table 1. Ranking of potential RGs according to stability values calculated with different algorithms.

Group	Reference Genes	Stability Value					Comprehensive Ranking
		BestKeeper		ΔCt	geNorm	NormFinder	
		SD	r	SD	M	S	
Healthy	*HPRT1*	0.50	0.89	0.58	0.58	0.08	1.32
	GUSB	0.45	0.76	0.63	0.63	0.11	2.06
	B2M	0.54	0.83	0.63	0.63	0.11	2.63
	POLR2A	0.47	0.75	0.64	0.64	0.12	3.36
	ACTB	0.55	0.76	0.69	0.69	0.15	5.48
	TBP	0.75	0.89	0.68	0.68	0.16	5.69
	PPIA	0.67	0.74	0.78	0.78	0.19	6.74
	GAPDH	0.85	0.82	0.87	0.87	0.19	8.00
Asthmatic	*GUSB*	0.76	0.91	0.67	0.67	0.09	1.32
	HPRT1	0.80	0.91	0.70	0.70	0.13	3.16
	PPIA	0.75	0.90	0.72	0.72	0.22	3.46
	B2M	0.64	0.69	0.91	0.91	0.13	3.72
	TBP	0.78	0.84	0.76	0.76	0.13	4.47
	POLR2A	0.82	0.89	0.72	0.73	0.15	4.90
	GAPDH	1.00	0.92	0.81	0.81	0.12	5.29
	ACTB	0.84	0.91	0.77	0.77	0.18	6.48
Obese Asthmatic	*B2M*	0.59	0.91	0.61	0.61	0.10	1.19
	PPIA	0.57	0.86	0.66	0.66	0.15	2.11
	POLR2A	0.60	0.86	0.66	0.66	0.15	3.57
	HPRT1	0.75	0.92	0.66	0.66	0.13	3.87
	TBP	0.65	0.85	0.70	0.70	0.13	4.16
	ACTB	0.73	0.91	0.70	0.70	0.14	5.42
	GUSB	0.71	0.79	0.76	0.76	0.20	6.44
	GAPDH	0.77	0.82	0.79	0.79	0.20	8.00

SD: standard deviation; r: Pearson correlation coefficient; M: geNorm stability value; S: NormFinder stability value.

3.5. Identification of the Best and Worst Shared Scored Genes

Finally, we sought to identify the best and worst shared genes between healthy, asthmatic, and obese asthmatic groups. We selected the four best-scored genes and the four worst-scored genes from the comprehensive ranking for each group of patients, and we found the following coincidences: *B2M* and *HPRT1* were the best RGs among all groups, whereas *ACTB* and *TBP* were the worst shared RGs. The Venn diagram in Figure 2 illustrates these coincidences.

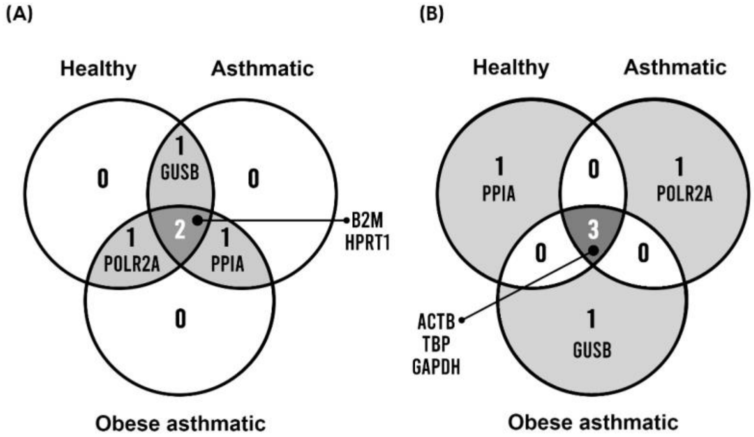


Figure 2. Best (**A**) and worst (**B**) shared genes among healthy, asthmatic, and obese asthmatic groups.

4. Discussion

The authors of the present study aimed to evaluate the expression stability of eight commonly used RGs (*TBP*, *GUSB*, *POLR2A*, *PPIA*, *B2M*, *GAPDH*, *ACTB*, and *HPRT1*) for their use in gene expression analysis with RT–qPCR. We compared the stability of such RGs in PBMCs using four different algorithms. To our knowledge, this is the first study to validate RG stability in PBMCs treated with DEX and Vit D_3 in obese asthmatic patients compared to non-obese patients with and without asthma. Only a few studies have tackled this issue [8,23,24], and, to the best of our knowledge, none have compared RG stability in these diseases.

We found that all analyzed genes were expressed in PBMCs from healthy, asthmatic, and obese asthmatic subjects. *B2M* was the most expressed gene, and *HPRT1* was the least expressed gene. These results are in agreement with those of Oturai et al., who reported a low expression of *HPRT1* in PBMCs from healthy and multiple sclerosis patients [25]. In this study, the most variable gene was *GAPDH,* despite being a frequently used RG. This observation is in line with previous studies reporting high variability for this RG in PBMCs [25,26].

We found differences in RG expression between healthy, asthmatic, and obese asthmatic patients, and we suggest that these differences could have been due to different experimental conditions such as distinct incubation times and/or distinct in vitro treatments. It is known that PBMC gene expression could be modified as a function of waiting time in sample processing or as a function of cell culture incubation time [26]. We found no differences between 6 and 24 h incubations in PBMCs from healthy individuals. However, *GAPDH* presented differences in both asthmatic and obese asthmatic groups, and *PPIA* and *TBP* presented differences in asthmatic PBMCs. Moreover, RG expression could change according to their stimulation [18]. We assessed the impact of in vitro GC and vitamin D treatment on RG stability. PBMCs were treated with DEX with or without Vit D_3 for 6 and 24 h. *POLR2A* was the least stable gene, being expressed differently in PBMCs from asthmatic and obese asthmatic patients treated with DEX, Vit $D_{3,}$ or both treatments combined. In contrast, the *B2M*, *PPIA*, and *ACTB* genes were not affected by the in vitro treatments.

Altogether, these findings suggest that not all RGs retain stability under distinct culture times and in vitro treatments, thus evidencing the importance of measuring their stability under the specific conditions of each study, such as the study population and experimental conditions.

We found that all analyzed RGs could be considered stable according to stability values calculated via the distinct algorithms, although there were some discrepancies according to the patients' characteristics. These different results depending on the disease are in accordance with the work of Usarek et al., who found that the best RG for healthy PBMCs was *B2M*, whereas in PBMCs from patients with amyotrophic lateral sclerosis, it was *HPRT1* [27]. Additionally, Wang et al. found lower basal expression levels of *PPIA* in asthmatics than in healthy controls [15]. The cell type used for the study is also important, as the stability of the possible RGs may be affected. In our case, we used PBMCs, which are a mixture of different cell types, so there may be variability in the stability of RG expression, as others have shown in their studies [22,25].

Consistent with the MIQE (minimum information for publications of quantitative real-time PCR experiments) guidelines, our results support a normalization strategy based on at least two RGs [9], with three genes being the optimal number for healthy and obese asthmatic PBMCs and four genes being the optimal number for asthmatic samples. Furthermore, with the NormFinder algorithm, we investigated whether the stability of RGs was subject to variation according to each experimental treatment. *B2M* was the gene with the least intergroup variation in healthy and obese asthmatic PBMCs; however, *HPRT1* presented the least intergroup variation in the asthmatic samples.

To date, there is no consensus on what method should be used to study RG expression stability, and, because the programs are based on different algorithms, the consensus between them increases the reliability of the results. Unfortunately, we did not identify any RG that was universally stable across these four programs. To overcome the discrepancies and obtain a final ranking, we calculated a comprehensive ranking based on the geometric mean of the positions for each program.

Finally, we identified the best and worst shared genes between the three studied groups. *B2M* and *HPRT1* were the most stable RGs in PBMCs from healthy, asthmatic, and obese asthmatic patients. These results contrast with those of Nakayama et al., who found *B2M* to be an unstable gene for chronic rhinosinusitis patients [28], and Ledderose et al., who stated that *TBP* was the best RG for T cell and neutrophil gene expression analysis [19]. On the other hand, *GAPDH* and *TBP* were the worst studied RGs, which is in line with previous studies that described *GAPDH* as an unstable gene along with *ACTB*, which was also positioned last in our ranking [29,30].

Since obesity is considered to influence the disease process of asthma, exploring the underlying mechanisms is necessary. Therefore, genetic expression assays could serve as a good in vitro model to study GC resistance in PBMCs. However, the selection of suitable RGs is the first and crucial step before we can determine the molecular basis. We identified *HPRT1* and *B2M* as the most stable RGs and *ACTB*, *TBP*, and *GAPDH* as the least stable RGs. This screening is a preliminary analysis for future gene expression studies in PBMCs from obese asthmatic patients treated in vitro with DEX and/or Vit D_3.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/mps5030035/s1. Table S1: Patients' clinical features; Table S2: Reference genes evaluated in this study; Table S3: Quantification cycle values in healthy, asthmatic, and obese asthmatic PBMCs.

Author Contributions: M.B., J.R.-F., E.A., C.P., J.M. and V.T. were involved in the conception and design of the project. M.B. conducted the experiments and analyzed the data. M.B., C.P. and V.T. wrote the paper. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of Hospital Clínic (HCB/2016/0861).

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

Data Availability Statement: Data generated or analyzed during this study are included in this published article or available from the corresponding author on request. All analyzed datasets were sourced by the authors.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Global Initiative for Asthma. 2021 GINA Report, Global Strategy for Asthma Management and Prevention (2021). Available online: https://ginasthma.org/ (accessed on 15 February 2022).
2. Anderson, W.J.; Lipworth, B.J. Does body mass index influence responsiveness to inhaled corticosteroids in persistent asthma? *Ann. Allergy Asthma Immunol.* **2012**, *108*, 237–242. [CrossRef] [PubMed]
3. Dixon, A.E.; Holguin, F.; Sood, A.; Salome, C.M.; Pratley, R.E.; Beuther, D.A.; Celedón, J.C.; Shore, S.A.; Boulet, L.P.; O'Donnell, C.; et al. An official American thoracic society workshop report: Obesity and asthma. *Proc. Am. Thorac. Soc.* **2010**, *7*, 325–335. [CrossRef] [PubMed]
4. Taylor, B.; Mannino, D.; Brown, C.; Crocker, D.; Twum-Baah, N.; Holguin, F. Body mass index and asthma severity in the National Asthma Survey. *Thorax* **2008**, *63*, 14–20. [CrossRef] [PubMed]
5. Sutherland, E.R.; Goleva, E.; Jackson, L.P.; Stevens, A.D.; Leung, D.Y.M. Vitamin D Levels, Lung Function, and Steroid Response in Adult Asthma. *Am. J. Respir. Crit. Care Med.* **2010**, *181*, 699–704. [CrossRef]
6. Cassim, R.; Russell, M.A.; Lodge, C.J.; Lowe, A.J.; Koplin, J.J.; Dharmage, S.C. The role of circulating 25 hydroxyvitamin D in asthma: A systematic review. *Allergy* **2015**, *70*, 339–354. [CrossRef]
7. Vo, P.; Bair-Merritt, M.; Camargo, C.A. The potential role of vitamin D in the link between obesity and asthma severity/control in children. *Expert Rev. Respir. Med.* **2015**, *9*, 309–325. [CrossRef]
8. Goleva, E.; Jackson, L.P.; Gleason, M.; Leung, D.Y.M. Usefulness of PBMCs to predict clinical response to corticosteroids in asthmatic patients. *J. Allergy Clin. Immunol.* **2012**, *129*, 687–693. [CrossRef]
9. Bustin, S.A.; Benes, V.; Garson, J.A.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M.W.; Shipley, G.L.; et al. The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* **2009**, *55*, 611–622. [CrossRef]
10. Thellin, O.; ElMoualij, B.; Heinen, E.; Zorzi, W. A decade of improvements in quantification of gene expression and internal standard selection. *Biotechnol. Adv.* **2009**, *27*, 323–333. [CrossRef]
11. Bustin, S.A. Absolute quantification of mrna using real-time reverse transcription polymerase chain reaction assays. *J. Mol. Endocrinol.* **2000**, *25*, 169–193. [CrossRef]
12. de Jonge, H.J.M.; Fehrmann, R.S.N.; de Bont, E.S.J.M.; Hofstra, R.M.W.; Gerbens, F.; Kamps, W.A.; de Vries, E.G.E.; van der Zee, A.G.J.; te Meerman, G.J.; ter Elst, A. Evidence based selection of housekeeping genes. *PLoS ONE* **2007**, *2*, e898. [CrossRef] [PubMed]
13. Vandesompele, J.; De Preter, K.; Pattyn, F.; Poppe, B.; Van Roy, N.; De Paepe, A.; Speleman, F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **2002**, *3*, 1–12. [CrossRef] [PubMed]
14. Andersen, C.L.; Jensen, J.L.; Ørntoft, T.F. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* **2004**, *64*, 5245–5250. [CrossRef] [PubMed]
15. Wang, T.; Liang, Z.A.; Sandford, A.J.; Xiong, X.Y.; Yang, Y.Y.; Ji, Y.L.; He, J.Q. Selection of Suitable Housekeeping Genes for Real-Time Quantitative PCR in CD4+ Lymphocytes from Asthmatics with or without Depression. *PLoS ONE* **2012**, *7*, e48367. [CrossRef] [PubMed]
16. Schmittgen, T.D.; Zakrajsek, B.A. Effect of experimental treatmentfile on housekeeping gene expression: Validation by real-time, quantitative RT-PCR. *J. Biochem. Biophys. Methods* **2000**, *46*, 69–81. [CrossRef]
17. Radonić, A.; Thulke, S.; Mackay, I.M.; Landt, O.; Siegert, W.; Nitsche, A. Guideline to reference gene selection for quantitative real-time PCR. *Biochem. Biophys. Res. Commun.* **2004**. [CrossRef]
18. Schmid, H.; Cohen, C.D.; Henger, A.; Irrgang, S.; Schlöndorff, D.; Kretzler, M. Validation of endogenous controls for gene expression analysis in microdissected human renal biopsies. *Kidney Int.* **2003**, *64*, 356–360. [CrossRef]
19. Ledderose, C.; Heyn, J.; Limbeck, E.; Kreth, S. Selection of reliable reference genes for quantitative real-time PCR in human T cells and neutrophils. *BMC Res. Notes* **2011**, *4*, 427. [CrossRef]
20. Pfaffl, M.W.; Tichopad, A.; Prgomet, C.; Neuvians, T.P. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pair-wise correlations. *Biotechnol. Lett.* **2004**, *26*, 509–515. [CrossRef]

21. Silver, N.; Best, S.; Jiang, J.; Thein, S.L. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Mol. Biol.* **2006**, *7*, 1–9. [CrossRef]
22. Wang, J.; Wang, Y.; Wang, H.; Hao, X.; Wu, Y.; Guo, J. Selection of reference genes for gene expression studies in porcine whole blood and peripheral blood mononuclear cells under polyinosinic:Polycytidylic acid stimulation. *Asian-Australas. J. Anim. Sci.* **2014**, *27*, 471–478. [CrossRef] [PubMed]
23. Müller, G.C.; Pitrez, P.M.; Lopes, R.F.M.; Souza Pires, P.; Correa, B.L.; Pillat, M.M.; Teixeira, A.L.; Jones, M.H.; Stein, R.T.; Bauer, M.E. Peripheral glucocorticoid sensitivity in children with controlled persistent asthma. *Neuroimmunomodulation* **2010**, *18*, 98–102. [CrossRef] [PubMed]
24. Vazquez-Tello, A.; Halwani, R.; Hamid, Q.; Al-Muhsen, S. Glucocorticoid receptor-beta up-regulation and steroid resistance induction by IL-17 and IL-23 cytokine stimulation in peripheral mononuclear cells. *J. Clin. Immunol.* **2013**, *33*, 466–478. [CrossRef] [PubMed]
25. Oturai, D.B.; Søndergaard, H.B.; Börnsen, L.; Sellebjerg, F.; Romme Christensen, J. Identification of Suitable Reference Genes for Peripheral Blood Mononuclear Cell Subset Studies in Multiple Sclerosis. *Scand. J. Immunol.* **2016**, *83*, 72–80. [CrossRef]
26. Barnes, M.G.; Grom, A.A.; Griffin, T.A.; Colbert, R.A.; Thompson, S.D. Gene expression profiles from peripheral blood mononuclear cells are sensitive to short processing delays. *Biopreserv. Biobank.* **2010**, *8*, 153–162. [CrossRef]
27. Usarek, E.; Barańczyk-Kuźma, A.; Kaźmierczak, B.; Gajewska, B.; Kuźma-Kozakiewicz, M. Validation of qPCR reference genes in lymphocytes from patients with amyotrophic lateral sclerosis. *PLoS ONE* **2017**, *12*, e0174317. [CrossRef]
28. Nakayama, T.; Okada, N.; Yoshikawa, M.; Asaka, D.; Kuboki, A.; Kojima, H.; Tanaka, Y.; Haruna, S.I. Assessment of suitable reference genes for RT-qPCR studies in chronic rhinosinusitis. *Sci. Rep.* **2018**, *8*, 1–9. [CrossRef]
29. Bas, A.; Forsberg, G.; Hammarström, S.; Hammarström, M.L. Utility of the housekeeping genes 18S rRNA, β-actin and glyceraldehyde-3-phosphate-dehydrogenase for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes. *Scand. J. Immunol.* **2004**, *59*, 566–573. [CrossRef]
30. Glare, E.M.; Divjak, M.; Bailey, M.J. β-Actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels. *Thorax* **2002**, *57*, 765–770. [CrossRef]

B) Second manuscript: Differences in inflammatory cytokine profile in obesity-associated asthma: effects of weight loss.

Journal of
Clinical Medicine

MDPI

Article

Differences in Inflammatory Cytokine Profile in Obesity-Associated Asthma: Effects of Weight Loss

Marina Bantulà [1,*], Valeria Tubita [1], Jordi Roca-Ferrer [1,2], Joaquim Mullol [1,2,3], Antonio Valero [1,2,4], Irina Bobolea [1,2,4], Mariona Pascal [5], Ana de Hollanda [1,6,7], Josep Vidal [1,6,8], César Picado [1,2,4] and Ebymar Arismendi [1,2,4]

1 Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), 08036 Barcelona, Spain; v.tubita@gmail.com (V.T.); jrocaf@clinic.cat (J.R.-F.); jmullol@clinic.cat (J.M.); valero@clinic.cat (A.V.); bobolea@clinic.cat (I.B.); amdehol@clinic.cat (A.d.H.); jovidal@clinic.cat (J.V.); cpicado@ub.edu (C.P.); earismen@clinic.cat (E.A.)
2 Centro de Investigaciones Biomédicas en Red de Enfermedades Respiratorias (CIBERES), 28029 Madrid, Spain
3 Rhinology Unit & Smell Clinic, ENT Department, Hospital Clinic, 08036 Barcelona, Spain
4 Pulmonology and Allergy Department, Hospital Clinic, University of Barcelona, 08036 Barcelona, Spain
5 Immunology Department, CDB, Hospital Clinic, University of Barcelona, 08036 Barcelona, Spain; mpascal@clinic.cat
6 Obesity Unit, Endocrinology and Nutrition Department, Hospital Clínic, 08036 Barcelona, Spain
7 Centro de Investigaciones Biomédicas en Red de Fisopatología de la Obesidad y Nutrición (CIBEROBN), 28029 Madrid, Spain
8 Centro de Investigaciones Biomédicas en Red en Diabetes y Enfermedades Metabólicas (CIBERDEM), 28029 Madrid, Spain
* Correspondence: bantula@clinic.cat; Tel.: +34-932275400

Citation: Bantulà, M.; Tubita, V.; Roca-Ferrer, J.; Mullol, J.; Valero, A.; Bobolea, I.; Pascal, M.; de Hollanda, A.; Vidal, J.; Picado, C.; et al. Differences in Inflammatory Cytokine Profile in Obesity-Associated Asthma: Effects of Weight Loss. *J. Clin. Med.* **2022**, *11*, 3782. https://doi.org/10.3390/jcm11133782

Academic Editor: Pierachille Santus

Received: 24 May 2022
Accepted: 27 June 2022
Published: 29 June 2022

Abstract: Obesity and asthma are associated with systemic inflammation maintained by mediators released by adipose tissue and lung. This study investigated the inflammatory serum mediator profile in obese subjects (O) (n = 35), non-obese asthma (NOA) patients (n = 14), obese asthmatics (OA) (n = 21) and healthy controls (HC) (n = 33). The effect of weight loss after bariatric surgery (BS) was examined in 10 OA and 31 O subjects. We analyzed serum markers including leptin, adiponectin, TGF-β1, TNFR2, MCP-1, ezrin, YKL-40, ST2, IL-5, IL-9, and IL-18. Compared with HC subjects, the O group showed increased levels of leptin, TGF-β1, TNFR2, MCP-1, ezrin, YKL-40, and ST2; the OA group presented increased levels of MCP-1, ezrin, YKL-40, and IL-18, and the NOA group had increased levels of ezrin, YKL-40, IL-5, and IL-18. The higher adiponectin/leptin ratio in NOA with respect to OA subjects was the only significant difference between the two groups. IL-9 was the only cytokine with significantly higher levels in OA with respect to O subjects. TNFR2, ezrin, MCP-1, and IL-18 concentrations significantly decreased in O subjects after BS. O, OA, and NOA showed distinct patterns of systemic inflammation. Leptin and adiponectin are regulated in asthma by obesity-dependent and -independent mechanisms. Combination of asthma and obesity does not result in significant additive effects on circulating cytokine levels.

Keywords: asthma; bariatric surgery; cytokines; ezrin; obesity; YKL-40

1. Introduction

Obesity (defined as having a body mass index [BMI] > 30 kg/m^2) is a risk factor for asthma; asthmatic patients with obesity are more likely to have a poor response to corticosteroids (CS) and a severe disease. How obesity affects the pathogenesis and severity of asthma is poorly understood [1,2].

Detrimental effects of obesity on lung function have been proposed as a potential mechanism to explain the deleterious effects of obesity on asthma. A restrictive ventilatory alteration has been described in adults with obesity with a predominant decrease in functional residual capacity (FRC) and expiratory reserve capacity (ERC) associated with obstruction in the distal airways [3–5]. In children, obesity can contribute towards altering the normal development of the lung with a disproportionate growth of the lung parenchyma with respect to the airways, a phenomenon known as dysanapsis, that results in bronchial obstruction [4,6]. In addition, obesity in adults and children is associated with increased airway hyperresponsiveness (AHR) [4]. A recent study reported that the effects of obesity on pulmonary function in children are transitory and changes from the obstructive to the restrictive pattern in adulthood [7].

There is also a hypothesis that states that the low-grade chronic systemic inflammation present in obesity results in a spillover of pro-inflammatory systemic products from the inflamed adipose tissue, which contributes, via additive or synergistic mechanisms, towards increasing the systemic/airway inflammatory process underlying asthma [1]. Roles for the increased release of some pro-inflammatory cytokines and decreased production of some anti-inflammatory agents have been described [1]. Leptin and adiponectin are the markers most frequently used to assess the relationship between the inflammation of obesity and that of asthma. However, changes in these two markers are difficult to interpret as their systemic levels are influenced by asthma, independently of obesity [8–13]. Other makers of systemic inflammation, such as interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), and monocyte chemotactic protein-1 (MCP-1), have also been assessed with some contradictory results [5,13,14]. However, the number of pro- and anti-inflammatory biological products potentially involved in asthma and obesity continuously increases and their potential role in both processes needs to be examined.

Weight loss after either bariatric surgery (BS) or dietetic treatments associated or not with physical exercise may counteract low-grade systemic inflammation in subjects with obesity [1]. The effect of weight loss on the synthesis of some biological products related to asthma and obesity has been assessed in previous studies in asthmatic subjects with obesity. Concentrations of the pro-inflammatory cytokines IL-6 and IL-8 decreased with weight loss [15], but there was no effect on other cytokines such as IL-8, TNF-α, and GM-CSF [5]. However, the effect of weight loss on some other molecular agents also potentially involved in the inflammatory process in obesity and in obese asthma remains to be examined.

A deeper understanding of how molecular mechanisms interact between asthma and obesity may help us further understand the complex inflammatory puzzle of asthma associated with obesity.

The present study aimed to evaluate circulating cytokine levels, before and after BS-induced weight loss, in obese subjects with and without asthma.

2. Materials and Methods

2.1. Subjects

We recruited 35 asthma patients: 21 obese (OA) (BMI $\geq$ 30 kg/m^2) and 14 with non-obese asthma (NOA) (BMI < 25 kg/m^2); 35 obese (O) patients, and 33 age- and gender-matched healthy control (HC) individuals. Forty-one obese (10 OA and 31 O) subjects were evaluated at baseline (V1) and between six and twelve months after they underwent BS (V2). The collaborating endocrinologist presented the objective of the study to the subjects with obesity during the pre-surgery evaluation; those who initially agreed to participate were referred to the Respiratory Department for further studies.

The following criteria were used to select asthmatic patients: (1) a clinical history of asthma; (2) either bronchodilator responsiveness (>12% and 200 mL improvement in forced expiratory volume in 1 s [FEV$_1$] after 180 µg salbutamol metered-dose inhaler) or AHR (PC$_{20}$ methacholine < 8 mg/mL). None of the subjects had received systemic CS for one month or longer prior to evaluation. None of the subjects were current smokers. Forced spirometry was performed according to ERS/ATS standards [16].

2.2. Blood Collection and Serum Extraction

Blood samples were obtained by peripheral venipuncture and collected into BD Vacutainer SST™ II Advance tubes. Serum was obtained by centrifugation and then stored at −80 °C until analysis.

2.3. Measurement of Serum Cytokine Levels and Immunoglobulin E

Serum leptin, adiponectin, tumor necrosis alpha 2 receptor (TNFR2), MCP-1, ezrin, YKL-40, suppression of tumorigenicity 2 receptor (ST2) for IL-33, IL-5, IL-9, and IL-18 levels were quantified by Luminex® multiplex immunoassay using Human ProcartaPlex Mix&Match kits, and the Human Simplex Kit for transforming growth factor-beta1 (TGF-β1) (Thermo Fisher Scientific, Vienna, Austria) on a Luminex 200 analyzer. Ezrin levels were analyzed in serum using an enzyme-linked immunosorbent assay (ELISA) kit (516351 Cayman chemical). The procedure was carried out in accordance with the manufacturer's protocol.

IL-17A, IL-6, IL-8, IL-10, TNFR1, IL-13, IL-33, IL-1β, IL-4, interferon gamma (IFN-γ), GM-CSF, and thymic stromal lymphopoietin (TSLP) were also measured. However, in many samples, the levels were below the detection limit and, therefore, could not be accurately assessed.

Serum levels of total and specific immunoglobulin E (IgE) against common allergens (mites, pollens, cats, dogs, fungi) were measured by immunofluorescence enzyme immunoassay (ImmunoCAP, ThermoFisherScientific, Uppsala, Sweden). Patients with elevated specific IgE against one or more allergens were classified as atopic.

2.4. Statistical Analysis

Clinical and experimental data were reported as the median and 25th to 75th percentile. Differences between two groups were analyzed using nonparametric tests: Mann–Whitney U test (unpaired data), Wilcoxon rank test (paired data), or Kruskal–Wallis H test for multiple comparisons. Correlation coefficients were calculated using the Spearman rank method. All analyses were performed using GraphPad Prism version 8.4 for Windows, (GraphPad Software, La Jolla, CA, USA). Statistical significance was defined as p-value < 0.05.

3. Results

The participants' demographic and clinical characteristics are shown in Table 1. The level of asthma severity (mild, moderate, severe) was established according to the pharmacological treatment used to control the disease [17,18]. The level of control was assessed using the asthma control test (ACT) [15,16]. OA patients presented reduced forced vital capacity (FVC) and FEV_1, compared with HC, whereas the FEV_1/FVC ratio was significantly lower in NOA and OA participants in comparison with O and HC individuals. Blood eosinophilia was higher in NOA subjects, compared with the HC and O groups.

BS was performed in 41 subjects (10 OA and 31 O), in 17 of whom a sleeve gastrectomy was performed (41.5%), while in the remaining 24 (58.5%) a Roux-en-Y gastric bypass was carried out. Table 2 presents the clinical, functional, and inflammatory marker changes after BS. BMI was significantly reduced in both groups of subjects with obesity. Weight loss was associated with a significant improvement in the ACT scores and a reduction in the amount of therapy needed to control the disease. Lung function tests improved in both groups after BS, being statistically significant only in the obese group.

Table 1. Baseline demographic and clinical data of the study population.

	HC (n, 33)	NOA (n, 14)	OA (n, 21)	O (n, 35)
Age, years	43 (37–50)	52 (43–59)	56 (50–61) *	49 (45–58)
Female, n (%)	24 (72)	12 (86)	16 (76)	29 (83)
BMI, kg/m^2	22.5 (21.6–25.0)	23.2 (22.3–24.9)	38.4 (34.9–44.1) *,#	43.4 (39.3–46.9) *,#
Mild asthma, n (%)	-	0 (0)	4 (19.1)	-
Moderate asthma, n (%)	-	6 (42.9)	5 (23.8)	-
Severe asthma, n (%)	-	8 (57.1)	12 (57.1)	-
FVC, % pred	126.6 (122.2–136.3)	132.1 (116.7–141.3)	110.4 (94.0–122.5) *,#	116.0 (102.4–127.0) *,#
FEV$_1$, % pred	101.0 (93.5–109.0)	86.5 (75.2–103.8)	85.0 (61.0–95.5) *	90.5 (84.0–101.0)
FEV$_1$/FVC	79.0 (76.0–83.0)	71.0 (57.0–75.0) *	77.0 (69.5–82.0)	82.0 (79.0–84.0) #,†
ICS §, n (%)	-	13 (92.9)	19 (82.6)	-
Atopia, n (%)	-	8 (57.1)	10 (43.5)	-
IgE, kU/L	26.9 (16.5–80.2)	95.5 (34.8–306.0)	63.7 (14.4–273.0)	48.3 (18.9–110.0)
Eosinophils, %	2.0 (1.3–3.2) #	4.7 (3.6–6.3)	3.3 (2.3–5.0)	2.4 (1.6–3.2) #
Eosinophils, cells/mm^3	100 (100–200)	300 (200–425) *	200 (125–300) *	200 (100–200) #
Neutrophils, %	60.5 (55.2–65.6)	54.8 (51.4–61.0)	56.7 (51.6–64.6)	63.2 (56.4–66.2) #
Neutrophils, cells/mm^3	3.5 (3.0–4.3)	3.6 (3.2–4.2)	3.7 (3.0–4.9)	4.5 (3.7–5.1) *

Data presented as medians (25th–75th percentile). HC, healthy controls; NOA, non-obese asthmatics; OA, obese asthmatics; O, obese subjects; BMI, body mass index; FVC, forced vital capacity; pred, predicted; FEV$_1$, forced expiratory volume in 1 s; ICS, inhaled corticosteroids; IgE, immunoglobulin E. * $p < 0.05$, compared with HC; # $p < 0.05$, compared with NOA; † $p < 0.05$ compared with OA; Kruskal–Wallis followed by Dunn's multiple comparisons test. § For NOA and OA patients who received ICS, the mean ± SD of the ICS dose in budesonide equivalents was 557.1 ± 256.6 and 1222.1 ± 862.9 µg/day, respectively.

Table 2. Demographic and clinical data of asthmatic and non-asthmatic obese patients before (V1) and six to twelve-months after (V2) bariatric surgery.

	Obese Asthmatics (n, 10)		Obese (n, 31)	
	V1	V2	V1	V2
Age, years	53 (47–58)	54 (49–59)	49 (45–59)	50 (47–59)
Female, n (%)	10 (100)	10 (100)	26 (84)	26 (84)
BMI, kg/m^2	44.1 (38.7–47.1)	29.9 (26.4–34.8) *	42.7 (38.7–45.9)	29.0 (27.4–30.8) #
Mild asthma, n (%)	4 (40)	9 (90)	-	-
Moderate asthma, n (%)	4 (40)	1 (10)	-	-
Severe asthma, n (%)	2 (20)	0 (0)	-	-
ACT	18 (18–24)	25 (25–25) *	-	-
FVC, % pred	110.6 (96.9–121.5)	119.0 (111.9–136.3)	116.5 (104.3–129.2)	130.4 (116.7–141.7) #
FEV$_1$, % pred	89.0 (79.5–97.2)	96.5 (92.5–104.8)	93.5 (84.7–101.0)	102.0 (89.0–109.0) #
FEV$_1$/FVC	80.5 (76.5–82.5)	81.5 (77.5–83.0)	82.0 (79.0–84.0)	81.0 (75.0–83.0) #
ICS §, n (%)	6 (60)	2 (20)	-	-
IgE, kU/L	35.4 (17.3–117.5)	35.7 (20.3–64.0)	51.7 (16.8–115.0)	28.3 (14.4–62.8)
Eosinophils, %	2.9 (1.9–3.7)	2.5 (1.4–3.3)	2.4 (1.6–3.4)	2.2 (1.6–2.8)
Eosinophils, cells/mm^3	200 (100–225)	200 (75–225)	200 (100–200)	100 (100–200) #
Neutrophils, %	56.7 (53.5–64.3)	56.2 (54.7–58.5)	62.5 (55.5–65.3)	56.9 (52.3–64.4)
Neutrophils, cells/mm^3	3.6 (2.9–4.4)	3.3 (2.9–4.2)	4.4 (3.7–5.1)	3.1 (2.6–4.2) #

Data presented as medians (25th–75th percentile). BMI, body mass index; ACT, asthma control test; FVC, forced vital capacity; pred, predicted; FEV$_1$, forced expiratory volume in 1 s; ICS, inhaled corticosteroids; IgE, immunoglobulin E. * $p < 0.05$, compared between V1 and V2 in the obese asthmatic group, # $p < 0.05$ compared between V1 and V2 in the obese group; Wilcoxon test. § For OA patients before and after bariatric surgery who received ICS, the mean ± SD of the ICS dose in budesonide equivalents was 643.3 ± 496.1 and 150.0 ± 70.7 µg/day, respectively.

3.1. Leptin, Adiponectin, and Adiponectin/Leptin Ratio before and after BS

Circulating levels of pro-inflammatory leptin were significantly higher in O, OA, and NOA than in HC, but there were no differences between them (Figure 1A). Anti-inflammatory adiponectin levels were significantly higher in NOA compared with HC, O, and OA (Figure 1B). Changes in leptin and adiponectin in A, OA, and NOA subjects resulted in a predominant anti-inflammatory adiponectin/leptin ratio in the NOA group relative to the OA and O groups (Figure 1C).

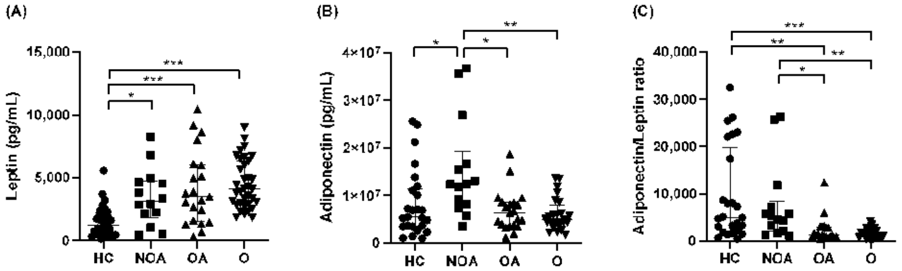


Figure 1. Leptin (**A**), adiponectin (**B**), and adiponectin/leptin ratio (**C**) serum level comparisons between groups. Data presented as individual values and as medians (25th–75th percentile). HC, healthy controls; NOA, non-obese asthmatics; OA, obese asthmatics; O, obese subjects. * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; Kruskal–Wallis followed by Dunn's multiple comparisons test.

Higher leptin values were observed in non-atopic compared with atopic asthma patients (NOA + OA) [median (25th–75th) 4639 (2824–7425) pg/mL, 2859 (1471–4155) pg/mL, respectively; $p = 0.041$].

Weight loss was associated with a decrease in serum leptin in O subjects, which resulted in a further increase in the adiponectin/leptin ratio (Figure 2B). Serum adiponectin levels did not change after BS. In the OA group, similar results were found, although no statistical significance was reached (Figure 2A).

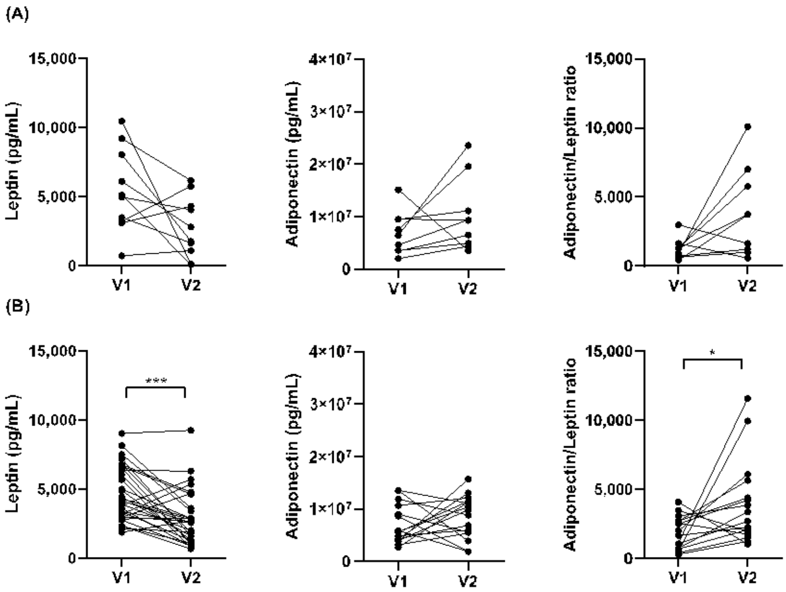


Figure 2. Serum leptin, adiponectin, and adiponectin/leptin ratio values from obese asthmatics (**A**) and obese subjects (**B**) before (V1) and after (V2) bariatric surgery. Data presented as individual values. * $p < 0.05$, *** $p \leq 0.001$; Wilcoxon test for paired data comparisons between V1 and V2.

3.2. Comparison of Serum Inflammatory Markers between HC and the NOA, OA and O Groups

The circulating concentration levels of six molecular products: TGF-β1, TNFR2, MCP-1, ezrin, YKL-40, and ST2 were found to be significantly higher in O subjects with respect to HC. The OA group also showed significantly increased levels of four products compared with HC: MCP-1, ezrin, YKL-40, and IL-18. Finally, in the serum of NOA individuals, four molecular products also had significantly higher concentrations compared with HC: ezrin, YKL-40, IL-5, and IL-18 (Table 3).

Table 3. Serum cytokine level comparison of non-obese asthmatics, obese asthmatics, and obese subjects with healthy controls.

		HC	NOA	OA	O	HC vs. NOA	HC vs. OA	HC vs. O
						p-Value	*p*-Value	*p*-Value
TGF-β1	N, ng/mL 25th–75th	33 361.2 (158.2–516.4)	14 349.1 (255.1–442.0)	21 348.6 (239.2–416.1)	35 495.4 (288.0–746.0)	0.9725	0.7382	0.0155
TNFR2	N, ng/mL 25th–75th	32 32.4 (8.8–56.2)	14 45.5 (25.0–99.2)	21 45.1 (22.4–65.8)	34 70.0 (45.8–96.7)	0.0792	0.1163	<0.0001
MCP-1	N, ng/mL 25th–75th	28 8.6 (6.5–17.8)	14 9.7 (8.1–14.7)	21 14.5 (10.6–28.1)	34 21.3 (11.5–75.7)	0.5388	0.0441	0.0003
Ezrin	N, ng/mL 25th–75th	33 216.9 (160.4–285.4)	14 325.5 (217.2–361.0)	21 322.5 (232.1–471.1)	35 341.7 (251.1–414.0)	0.0025	0.0005	<0.0001
YKL-40	N, ng/mL 25th–75th	33 23,440 (13,720–39,958)	14 58,428 (47,346–103,219)	21 60,240 (44,494–171,396)	34 59,151 (41,590–86,112)	<0.0001	<0.0001	<0.0001
ST2	N, ng/mL 25th–75th	32 999.1 (676.5–1456.0)	14 931.2 (576.0–1308.0)	21 1009.0 (486.2–2229.0)	35 1570.0 (820.8–3207.0)	0.6667	0.7903	0.0238
IL-5	N, ng/mL 25th–75th	33 116.6 (93.6–191.1)	14 294.2 (155.5–949.6)	20 252.1 (72.3–718.2)	35 155.5 (58.1–240.7)	0.0004	0.1443	0.7442
IL-9	N, ng/mL 25th–75th	28 3.2 (2.0–7.5)	8 4.9 (1.8–16.4)	14 12.8 (2.2–19.3)	28 3.4 (1.9–7.1)	0.3530	0.0171	0.7666
IL-18	N, ng/mL 25th–75th	27 40.7 (22.3–81.0)	14 57.8 (30.4–152.6)	21 69.3 (40.9–102.9)	32 56.9 (34.0–99.4)	0.0207	0.0118	0.0648

Data presented as medians (25th–75th). HC, healthy controls; NOA, non-obese asthmatics; OA, obese asthmatics; O, obese subjects. Mann–Whitney U test.

In atopic patients compared with non-atopic patients, we found higher MCP-1 values [median values (25th–75th) 14.5 (10.4–19.7), 9.0 (7.7–15.6) ng/mL, respectively; $p = 0.0472$] and IL-9 values [16.7 (12.0–29.3), 6.1(1.9–13.7), respectively; $p = 0.0346$].

3.3. Comparisons between Obese Subjects with and without Asthma

Obese subjects displayed higher serum levels of TGF-β1 and of TNFR2 than their OA counterparts, while IL-9 was the only cytokine showing significantly higher levels in OA with respect to O subjects (Table 4).

Table 4. Serum cytokine level comparison between obese groups with and without asthma.

		OA	O	OA vs. O *p*-Value
TGF-β1	N, ng/mL 25th–75th	21 348.6 (239.2–416.1)	35 495.4 (288.0–746.0)	0.0103
TNFR2	N, ng/mL 25th–75th	21 45.1 (22.4–65.8)	34 70.0 (45.8–96.7)	0.018
MCP-1	N, ng/mL 25th–75th	21 14.5 (10.6–28.1)	34 21.3 (11.5–75.7)	0.1528
Ezrin	N, ng/mL 25th–75th	21 322.5 (232.1–471.1)	35 341.7 (251.1–414.0)	0.8273
YKL-40	N, ng/mL 25th–75th	21 60,240 (44,494–171,396)	34 59,151 (41,590–86,112)	0.6002
ST2	N, ng/mL 25th–75th	21 1009.0 (486.2–2229.0)	35 1570.0 (820.8–3207.0)	0.1502
IL-5	N, ng/mL 25th–75th	20 252.1 (72.3–718.2)	35 155.5 (58.1–240.7)	0.221
IL-9	N, ng/mL 25th–75th	14 12.8 (2.2–19.3)	28 3.4 (1.9–7.1)	0.0299
IL-18	N, ng/mL 25th–75th	21 69.3 (40.9–102.9)	32 56.9 (34.0–99.4)	0.3049

Data presented as medians (25th–75th percentile). OA, obese asthmatics; O, obese subjects. Mann–Whitney U test.

3.4. Comparisons between NOA, OA, and HC

A higher adiponectin/leptin ratio was the only statistically significant difference between asthma patients with and without obesity (Figure 1C). IL-5 levels were significantly elevated in NOA patients with respect to HC and O subjects without asthma (Figure 3). Weight loss had no significant effect on IL-5 levels (data not shown).

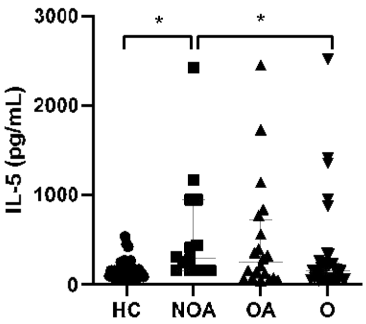


Figure 3. Serum IL-5 level comparisons between groups. Data presented as individual values and as medians (25th–75th percentile). HC, healthy controls; NOA, non-obese asthmatics; OA, obese asthmatics; O, obese subjects. * $p < 0.05$; Kruskal–Wallis followed by Dunn's multiple comparisons test.

3.5. Correlations

The cytokines MCP-1 (r = 0.368, $p = 0.0002$), TNFR2 (r = 0.295, $p = 0.0025$), YKL-40 (r = 0.391, $p < 0.0001$), ST2 (r = 0.216, $p = 0.028$), ezrin (r = 0.482, $p < 0.0001$), leptin (r = 0.517, $p < 0.0001$), and the adiponectin/leptin ratio (r = −0.241, $p < 0.0001$) correlated with BMI. We further explored the relationship between serum cytokine levels and lung function and found that YKL-40 (r = −0.399, $p < 0.0001$), ezrin (r = −0.229, $p = 0.02$), and leptin (r = −0.350, $p = 0.0003$) presented a negative correlation with FEV_1 (%). In contrast, the adiponectin/leptin ratio positively correlated (r = 0.253, $p = 0.01$) with FEV_1 (%). No consistent correlations were observed between blood eosinophilia and serum cytokine levels. Neither were significant differences obtained when asthmatic patients were divided into two groups of more or less than 300 eosinophils/mm^3.

3.6. Impact of Weight Loss on Serum Cytokine Levels

Weight loss had no statistically significant effect on serum cytokine levels in OA subjects (Figure 4A). However, serum TNFR2, ezrin, MCP-1, and IL-18 levels significantly decreased at the six–twelve-month post-BS follow-up in O subjects (Figure 4B). Other biomarkers in the O group: ST2, YKL-40, IL-9, and TGF-β1 were unaltered (data not shown).

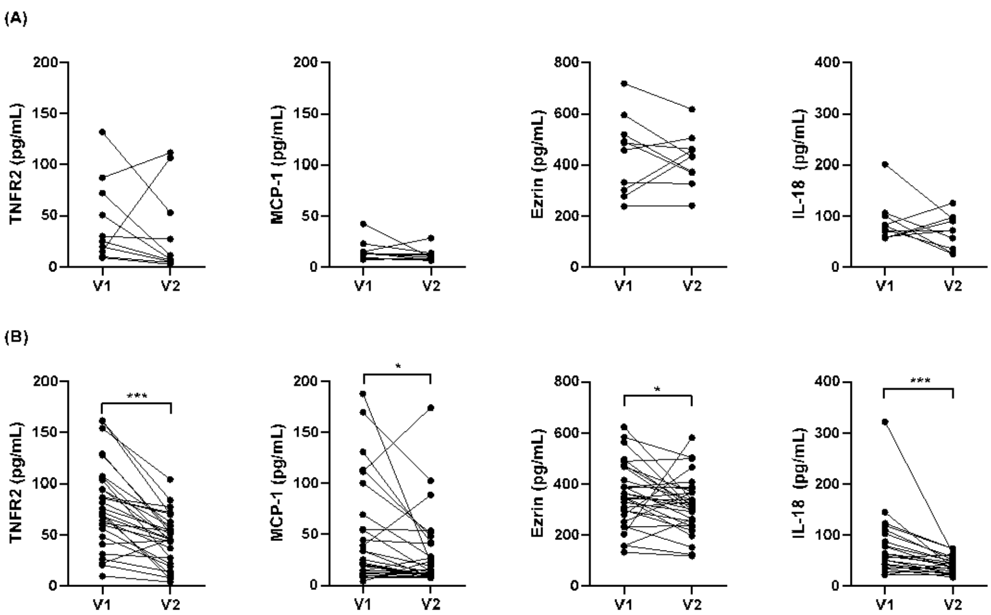


Figure 4. Serum TNFR2, MCP-1, Ezrin, and IL-18 level comparison between asthmatic subjects with obesity (**A**) and obese subjects without asthma (**B**) before (V1) and after (V2) bariatric surgery. Data presented as individual values. * $p < 0.05$, *** $p \leq 0.001$; Wilcoxon test.

4. Discussion

In the present study, we tested the hypothesis that the spillover of pro-inflammatory substances from adipose tissue will result in enhanced systemic inflammation in obese asthma subjects compared with their non-obese asthma counterparts. The activated systemic inflammation would account for the increased airway inflammation, which in turn would contribute towards explaining the poor response of obese asthmatics to anti-inflammatory therapy.

The study presents four main findings: (1) Leptin and adiponectin are regulated in asthma by both obesity-dependent and -independent mechanisms, the adiponectin/leptin ratio being the only statistically significant difference between asthma patients with and without obesity; (2) obesity is associated with a systemic inflammation that is more active than that associated with asthma; (3) the combination of asthma and obesity does not result in additive effects on circulating cytokine levels except for IL-9; (4) IL-5 levels were only found to be significantly elevated in NOA patients with respect to HC.

4.1. Leptin and Adiponectin in Asthma Are Regulated by Obesity-Dependent and -Independent Mechanisms

Leptin is a protein synthesized and secreted mainly by the adipose tissue [19] and plays a key role in the regulation of appetite and body weight [20]. Leptin promotes cytokine release, generates reactive oxygen species, activates natural killer cells, and up-regulates leukotriene release by alveolar macrophages, multiple actions that collectively promote inflammation [21,22]. Interestingly, serum leptin levels have been found elevated in patients with asthma compared with healthy controls [8,9], an observation suggesting that leptin may contribute to the pathogenesis of the disease by obesity-independent mechanisms. It has been reported that leptin enhances chemotaxis, survival, and secretion of pro-inflammatory cytokines by eosinophils [23–27]. These findings may indicate that an excessive production of leptin may contribute towards activating eosinophilic inflammation and, thereby, increase the severity of eosinophilic asthma [28]. However, the mechanism by which asthma appears to induce leptin synthesis remains to be elucidated. In keeping with previous observations [8,9], our study also found higher leptin levels in asthma patients—particularly in non-atopic patients—and obese subjects than in healthy control individuals. However, no clear additive effects of obesity on leptin levels in asthma were detected. Weight loss was associated with a marked decrease in leptin levels in obese subjects, but it had inconsistent effects in obese asthmatics, possibly reflecting, at least in part, the independent role played by asthma in leptin levels.

Adiponectin plays a key role in the regulation of insulin sensitivity and energy homeostasis [29]. The role of adiponectin in inflammation is complex and is in part dependent on the tissue wherein the inflammatory process takes place [29]. Along this line, in the endothelium and adipose tissue, adiponectin has anti-inflammatory effects [30,31], while in the synovial, kidney, and intestine, adiponectin exerts pro-inflammatory actions [32–34]. The mechanisms underlying these different effects seem complex and include the isoforms of adiponectin involved, the type of macrophages (M1 or M2) activated, and the adiponectin receptor to which it binds. Adiponectin has three different molecular weight isoforms: low (LMW), middle (MMW), and high (HMW). LMW exerts anti-inflammatory effects, while HMW activates pro-inflammatory pathways [29]. In M1 macrophages, adiponectin triggers the expression of pro-inflammatory cytokines (IL-6, TNF-α, IL-12), whereas in M2 macrophages, adiponectin induces the expression of IL-10 [29]. Adiponectin binds to four types of receptors AdipoR1, AdipoR2, T-cadherin, and calreticulin, which are differently distributed among cells and also show different isoform binding affinity. Taken together, all these differences can account for the opposite effects that can be induced by adiponectin [29]. The relationship between asthma, with and without obesity, and serum adiponectin levels has been examined in epidemiological and cross-sectional studies, yielding contradictory results that are difficult to interpret due to the heterogeneity of the studies [29]. Our results show that asthma is associated with increased adiponectin levels, a finding previously published in some [10–12] but not all studies [35]. Regarding the inhibitory role of obesity in adiponectin levels in asthma and non-asthmatic subjects, our results partially agree with some works [36–38], but not all [39]. The heterogeneity of the study populations (adults vs. children), as well as the differences in BMI and asthma severity of the subjects enrolled in the studies, could account for the observed differences. Childhood-onset obese asthma and obese asthma in adults share some similarities but also show substantial differences [1,4]. Adiponectin levels decrease in a negative relationship to increases in BMI [38,40]. Poor asthma control [39] and asthma severity [41,42] are both associated with lower adiponectin levels. Studies assessing the effects of bariatric surgery found that weight loss was associated with the recovery of serum adiponectin levels in obese [3] and obese asthma [5,14]. Interestingly, the balance of the adiponectin/leptin inflammatory ratio leaned towards normal in asthmatic patients compared with obese asthmatic and non-asthmatic subjects, in whom the ratio tended towards the pattern favoring inflammation. The adiponectin/leptin ratio negatively correlated with BMI. Weight loss was associated with a normalization of the ratio in obese subjects.

Taken together, all these findings reveal the key role played by adipokines in asthma and their interactions in the regulation of systemic inflammation in obese asthma.

4.2. Obesity Is Associated with a Systemic Inflammation (TGF-β1, TNFR2, MCP-1, Ezrin, YKL-40, and ST2) Which Is More Active Than That Associated with Asthma

TGF-β1 levels increase in parallel with increased adipose body mass tissue, suggesting a role for this cytokine in obesity-related diseases [43,44]. High-fat diet-induced obesity in animal models is associated with increased TGF-β1 signaling expression in the bronchial epithelium resulting in the release of inflammatory mediators and fibrosis [45]. Moreover, serum TGF-β1 levels have been found elevated in asthma [46] and TGF-β1 expression has also been reported as elevated in both structural and inflammatory cells derived from the airways of asthma patients [44,47–49]. Induced expression of the TGF-β pathway in the lungs promotes the recruitment of cells such as eosinophils, neutrophils, macrophages, mast cells, and fibroblasts [44,47,50–52]. TGF-β1 also induces IL-8 production in human airway smooth muscle cells [53]. Collectively, these observations suggest that TGF-β1 may play an amplifying inflammatory and remodeling role in the airways of obese asthma patients. We found higher elevated serum levels in O subjects, but obesity did not increase serum TGF-β1 levels in OA patients, and in contrast to previous studies in patients with severe asthma [46], we did not find any differences between asthma and healthy control individuals, probably because most of our patients had a well-controlled disease.

TNF-α is mainly produced by cells of the immune system, such as macrophages, lymphoid cells, and mast cells, and plays relevant regulatory roles in the process of inflammation and healing [54]. Excessive TNF-α production causes systemic inflammation, which is eventually involved in the development of metabolic diseases [54]. TNF-α can bind to two receptors, TNFR1 and TNFR2, which differ in their cellular expression and inflammatory potential [54,55]. TNF-α and TNFRs are expressed as transmembrane proteins that become soluble after being cleaved [55]. Serum TNF-α has been found to be significantly higher in obese subjects compared with healthy individuals [56]. Similarly, serum TNF-α levels have also been reported as elevated in the serum and bronchoalveolar lavage fluid of patients with severe compared with mild asthma and healthy controls [57]. In addition, previous studies have shown that TNF-α signaling via TNFR2 may promote AHR [58]. We found that the serum levels of TNFR2 were significantly higher in obese subjects than in healthy and asthma individuals, with no additive effect between asthma and obesity. The significant and marked decrease in serum TNFR2 levels after BS suggests that the demonstrated beneficial effects of weight loss on AHR [59] may be due, at least in part, to the reduction in the expression of the receptor mainly involved in TNF-α-related AHR.

IL-18 is a pro-inflammatory cytokine constitutively expressed by various cell types, including monocytes, macrophages, epithelial, and dendritic cells [60]. Immature IL-18 is activated after cleavage by caspase-1 and subsequently released from cells in its bioactive form [61]. The IL-18 receptor (IL-18R) is composed of one binding chain, IL-18Rα, and one signaling chain, IL-18Rβ. [61]. After binding to IL-18, they form a complex that triggers various intracellular pro-inflammatory signals including IRAKs, TRAF6, and NF-κB [62]. In collaboration with IL-12, IL-18 targets CD8+ T-cells and NK cells to stimulate IFN-γ production [63]. In the absence of IL-12, IL-18 promotes Th2 rather than Th1 immune responses and, in combination with IL-23, it activates the Th17 pathway [61]. IL-18 bioactivity is also regulated by the inhibitory effect of IL-18BP [64]. IL-18BP binds mature IL-18 to form high-affinity complexes [64]. In healthy individuals, most of the circulating IL-18 is bound to IL-18BP and, therefore, remains inactivated [65]. Elevated serum levels of free IL-18 have been found in different rheumatological diseases [66]. Previous studies reported that serum IL-18 levels were elevated in obese subjects and were reduced by weight loss [67]. Similarly, high serum IL-18 levels have been found in patients with uncontrolled asthma [68] and in mild and moderate asthma exacerbations [69]. A recent study found that IL-18R was highly expressed in the lung of the most severe asthma patient cluster, supporting that IL-18 signaling may play a key role in the pathogenesis of severe asthma [70]. In line with these observations, we found higher serum IL-18 levels in asthma and obese subjects; however, with no evidence of additive effects. As previously reported [67], weight loss significantly reduced serum IL-18 levels. Collectively, these findings support that the reduction of IL-18 might contribute to the salutary effects of weight loss in asthma.

Monocyte chemotactic protein-1 (MCP-1) is a member of the CC chemokine families and appears to play a relevant role in asthma pathogenesis because of its ability to attract monocytes and eosinophils, as well as activate mast cells and release leukotriene C-4 into the airway, resulting in AHR [71,72]. Elevated MCP-1 expression has been demonstrated in the bronchial epithelium of asthmatic patients [71], which increases even further preceding asthma exacerbation [73]. Neutralization of MCP-1 significantly inhibits T-cell and eosinophil recruitment to the lung and reduces AHR [74]. One of the most common immune cells infiltrating adipose tissues are macrophages, and their recruitment to adipose tissue is promoted by MCP-1 secreted by the adipose tissue itself [75]. In our study, MCP-1 was found to be significantly higher in obese subjects with and without asthma with respect to healthy controls. Moreover, there was a positive correlation between MCP-1 levels and BMI. A significant decrease in MCP-1 levels was seen with weight loss after BS, a finding in keeping with previous observations [76]. Collectively, these findings support the notion that MCP-1 plays an important role in asthma and obesity pathogenesis. We did not observe any additive effects between asthma and obesity; however, the reduced levels of MCP-1 after BS might partly explain the beneficial effects of weight loss in asthma patients.

IL-33 is a member of the IL-1 family which is expressed by epithelial and endothelial cells as an immature form [77]. When epithelial cells are exposed to irritants, allergens, bacteria, or viruses, IL-33 is cleaved into mature forms via a proteolytic mechanism. Once secreted, IL-33 binds to its specific membrane receptor ST2 (four isoforms) expressed on numerous cells, including immune cells such as innate lymphoid cell type 2 (ILC2), regulatory T cells (Tegs), mast cells, and macrophages [78,79]. Among the four ST2 isoforms, the transmembrane ST2 receptor (ST2L), and the soluble, circulating truncated form of the ST2 protein (sST2) are the best known [78,79]. The soluble form (sST2) is abundantly expressed and secreted by many cell types and tissues, and acts as a decoy receptor, neutralizing free IL-33 in biological fluids [78–81]. High sST2 levels are found in the serum of patients with inflammatory diseases. When the levels of IL-33 released into the extracellular environment increase, the expression of the soluble sST2 receptor also increases, probably to prevent the severe effects of excessive IL-33 [78–81]. IL-33 appears to play a major role in the initiation and development of type 2 immune responses involved in the development of asthma. Increased expression of IL-33 has been observed in murine asthma and in human asthma and rhinitis [82–84]. Concerning obesity, IL-33 is able to down-regulate excessive inflammation in adipose tissue by targeting immune cells expressing the ST2 receptor. Mice lacking ST2 or IL-33 develop increased adiposity and worsened metabolic profiles. IL-33 treatment contributes to homeostasis in the adipose tissue by facilitating the differentiation and maintenance of Foxp3+ST2+ Treg cells and ILC2 in visceral adipose tissue, an immunomodulatory effect that can contribute towards down-regulating obesity-associated inflammation [79,85]. Because IL-33 quantification in samples of human biological fluids is currently limited by the use of ELISA kits, which lack sensitivity and specificity, in our study we chose to assess the sST2 receptor. We found significantly higher levels of ST2 in obese individuals with respect to healthy controls and asthma subjects. There was a non-significant trend towards increased levels in obese asthmatics, which might suggest an additive effect. sST2 levels significantly correlated with BMI; however, weight loss was not associated with a decrease in sST2 levels. Nevertheless, without data on IL-33 levels, this observation is difficult to interpret as the measurement of sST2 only provides partial information on the balance between IL-33/sST2.

Ezrin is one of the members of the ezrin/radixin/moesin (ERM) family of proteins [86]. ERM proteins exert numerous physiological functions in morphogenesis, cell polarity, and modulation of cell membrane tension [86]. All these varied roles are carried out by ERM proteins by regulating the assembly of protein complexes at the cell surface. Additionally, ezrin, which is localized at the cytoplasmic surface of cellular membranes, links plasma membrane proteins to the cortical actin cytoskeleton [86]. The role of ezrin in the pathogenesis of asthma is, as yet, poorly understood. After allergen exposure, significant increases in serum levels of ezrin were observed in patients with allergic asthma [87]. In contrast to our results, a recent study found that the ezrin levels measured in serum were lower in asthma patients compared with non-asthma controls. In addition, serum ezrin levels were found to be related to the level of asthma control, the worse the control, the lower the ezrin levels [88]. Based on these observations, Jia et al. [88] proposed using serum ezrin levels to monitor asthma control. Most of our asthma patients had a well-controlled disease, a finding that could partly explain the different results between the studies. Despite this relatively good control, ezrin negatively correlated with FEV_1 in our study, supporting the notion that serum ezrin levels, to some extent, reflect asthma severity.

To the best of our knowledge, the relationship between obesity and ezrin has not been previously assessed. Interestingly, over-expression of ezrin in pancreatic β-cells results in an enhancement of insulin secretion [89]. Whether ezrin contributes towards overcoming insulin resistance usually in obese subjects is as yet unknown. We found higher levels of ezrin in obese subjects and ezrin levels positively and significantly correlated with BMI. Given the key regulatory role played by the ERM family of proteins, further studies are needed to better understand its role in asthma and obesity.

Chitin is a biopolymer that is part of the structure of fungi, molluscs, arthropods, and other invertebrates. Chitin is an enzymatic product of chitin synthases and requires specific enzymes—referred to as chitinases—for its degradation. Chitinases probably represent a defensive mechanism against chitin-containing parasites and fungi. Chitinase-3-like protein 1 (CHI3L1), also named YKL-40, is one of several known human chitinases. Elevated serum YKL-40 levels have been found in asthma patients compared with healthy controls [13,90]. Levels of YKL-40 in serum and bronchoalveolar lavage fluid correlated with subepithelial membrane thickness [90], and circulating levels are a significant independent factor associated with annual FEV_1 decline [91]. Additionally, elevated serum YKL-40 levels have been reported associated with irreversible airway obstruction, poor asthma control, and severe asthma exacerbations [92–95]. In line with these observations, we found that serum YKL-40 concentrations in NOA were significantly higher than those of HC and, in keeping with previous observations, YKL-40 levels negatively correlated with FEV_1%, supporting that high serum YKL-40 might be considered a marker of asthma severity [13,93,94].

High serum levels of YKL-40 correlating with BMI have also been found in obese children and adults [96–98]. The potential additive effects of asthma and obesity on YKL-40 have been evaluated in previous studies with discrepant results. In some studies, OA was associated with significantly higher serum levels compared with NOA, but without differences between NOA and HC [95]. In contrast, other studies found that YKL-40 levels were higher in asthmatics than HC, regardless of obesity [13]. We found the highest YKL-40 levels in OA patients but the difference with respect to NOA was small and non-significant. We did not find any significant effect of weight loss on YKL-40 levels. Similar studies have shown contradictory results, with some studies reporting a decrease in YKL-40 levels [97], while others could not find any significant change [99] after weight loss mediated by the Roux-en-Y gastric bypass.

4.3. IL-9 Was the Only Cytokine Presenting Significantly Higher Levels in OA with Respect to O Subjects

IL-9-producing Th9 cells have been described as a distinct group of CD4+ helper T cells resulting from the combined effects of Interleukin-4 (IL-4) and TGF-β [100,101]. The role of IL-9 in inflammation is a matter of controversy [102–104]. On the one hand, IL-9 has been shown to promote Th17 differentiation and production of IL-17 and IL-6 [103]; in contrast, it has also been shown that IL-9 enhances the suppressive function of Treg [104]. Asthma patients have increased numbers of Th9 cells in peripheral blood mononuclear cells (PBMCs) and increased IL-9 levels in both serum and bronchoalveolar lavage fluid (BALF) [105–109]. Th9 cells and IL-9 promote accumulation and activation of T cells, ILC2s, mast cells, and eosinophils and increase levels of IL-5, IL-13, and IgE in animal asthma models [110]. IL-9 concentrations have been found significantly elevated in male obese mice but not in female obese mice with respect to lean mice, suggesting that sexual dimorphism influences IL-9 production, with dominant expression in males [111]. In our study, serum levels of IL-9 were higher in obese asthma patients with respect to healthy controls and obese subjects, a finding that suggests that asthma and obesity might have additive effects on IL-9 secretion. However, there was no significant effect of weight loss on IL-9 serum levels. Since most of our patients were women and sexual dimorphism could influence the effect of obesity on IL-9 levels, further studies in men are needed to better decipher the role of obesity in regulating IL-9 synthesis.

4.4. IL-5 Levels Were Only Found to Be Significantly Elevated in NOA Patients with Respect to HC

IL-5 is produced by T helper type 2 cells (Th2), mast cells, and ILC2s, which in turn are activated by epithelium-derivate alarmins, including IL-24, IL-33, and thymic stromal lymphopoietin (TSLP) [112]. Activation, recruitment, and survival of eosinophils are largely driven by IL-5. The contribution of eosinophils to airway inflammation is well recognized; however, results regarding the use of blood eosinophil count as a predictor of asthma exacerbation are controversial and depend upon multiple factors. Indeed, a review published in 2018 showed that elevated blood eosinophil counts were associated with a lower incidence of future acute asthma attacks in patients with asthma that required a previous hospital admission [113]. Indeed, obesity was not associated with a higher risk of asthma hospital admission [114]. However, a recent meta-analysis including 155,772 participants demonstrated blood eosinophil count to be a predictor of asthma exacerbations [115]. Sputum IL-5 levels have been found to be significantly higher in obese asthmatics compared with their lean counterparts. Surprisingly, increased IL-5 levels are not associated with a parallel increase in eosinophils in the sputum, which contrasts with the demonstrated augmentation of eosinophil numbers in the airway submucosa, which appears to be due to an enhanced recruitment of eosinophils to the lung in obese asthmatics [116]. We found higher levels of IL-5 in NOA subjects with respect to HC and O individuals, but we could not find any additive effect of obesity in OA patients.

4.5. Study Limitations

The main limitation of our study was the small size of OA subjected to BS, which significantly reduced the capacity to assess the effects of weight loss on serum cytokine levels in this group of patients. Results need to be examined in a larger cohort. A six to twelve-month follow-up may not be long enough to detect significant changes in some cytokines after BS. Furthermore, we only focused on serum levels, without any parallel information regarding cytokine levels in the airways, where inflammation determines the course and severity of asthma. The impact of asthma inflammatory phenotypes (eosinophilic, neutrophilic, paucigranulocytic) on cytokine levels should also be considered in future studies.

In summary, there has been much speculation and debate regarding the role of obese-related low-grade systemic inflammation as a potentiating factor for systemic and airway inflammation in patients with obese asthma in order to explain why patients with this association frequently present a severe disease, resistant to anti-asthmatic treatment. However, the scientific evidence supporting this hypothesis is very scarce and inconsistent. In our study, we found evidence of partially differentiated systemic inflammation in asthma and obesity. We also observed similar increased levels of some cytokines in subjects with asthma and obesity. However, we could only find additive effects for IL-9 in patients with both diseases.

5. Conclusions

It is likely that the scant progress achieved to date in understanding the mechanisms that associate inflammation in obesity and asthma is due to the great complexity of the mechanisms involved in this association. An example of the complexity is supported by the observation that two adipokines associated with obesity, leptin, and adiponectin, are also up-regulated in asthma via a mechanism that remains to be elucidated. Moreover, the excessive synthesis by the adipose tissue of pro-inflammatory cytokines, such as IL-1β, IL-6, TNF-α, and TGF-β, may contribute to asthma pathogenesis. However, in contrast, enhanced synthesis of pro-inflammatory cytokines in asthma, such as IL-4, IL-5, IL-13, and IL-33, can contribute towards maintaining the lean state [113]. The partial counteracting effects of both inflammations make the interpretation of the complex interplay between asthma and obesity difficult, along with other factors such as differences between OA in children and adults and the influence of different asthma phenotypes on their association with obesity. Finally, weight loss achieved via dietary or surgical therapeutic methods can yield different results in the synthesis of pro-inflammatory mediators. Further studies must take into account all these contributory variables.

Author Contributions: Conception, M.B., V.T., J.R.-F., C.P. and E.A.; design, M.B., J.R.-F., M.P., A.d.H., J.V., C.P. and E.A.; analysis, M.B., V.T., J.R.-F., A.d.H., C.P. and E.A.; interpretation of the results, M.B., V.T., J.R.-F., J.M., A.V., I.B., A.d.H., J.V., C.P., E.A.; drafting of the article, M.B. and, C.P.; critical revision for important intellectual content and final approval of article, M.B., V.T., J.R.-F., J.M., A.V., I.B., M.P., A.d.H., J.V., C.P. and E.A. All authors have read and agreed to the published version of the manuscript.

Funding: The study was supported by grants from the Fundación Respira (SEPAR) 167/2016 and 736/2018, Fundació Catalana de Pneumologia (FUCAP) 2018–2021, and by an unrestricted grant from Menarini.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Hospital Clínic of Barcelona (2018/4015).

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

Data Availability Statement: Data generated or analyzed during this study are included in this published article or available from the corresponding author on request. All analyzed datasets were sourced from the authors.

Conflicts of Interest: J. Mullol is a member of national or international advisory boards and has received speaker fees or funding for clinical trials and research projects from Allakos, AstraZeneca, Genentech, GSK, Glenmark, Menarini, Mitsubishi-Tanabe, MSD, Mylan-MEDA Pharma (Viatris), Novartis, Procter & Gamble, Regeneron Pharmaceuticals, Inc., Sanofi-Genzyme, UCB Pharma, and Uriach Group. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. Other authors declare that they have no conflicts of interest.

References

1. Bantulà, M.; Roca-Ferrer, J.; Arismendi, E.; Picado, C. Asthma and Obesity: Two Diseases on the Rise and Bridged by Inflammation. *J. Clin. Med.* **2021**, *10*, 169. [CrossRef] [PubMed]
2. Zhang, X.; Zheng, J.; Zhang, L.; Liu, Y.; Chen, G.P.; Zhang, H.P.; Wang, L.; Kang, D.Y.; Wood, L.G.; Wang, G. Systemic Inflammation Mediates the Detrimental Effects of Obesity on Asthma Control. *Allergy Asthma Proc.* **2018**, *39*, 43–50. [CrossRef] [PubMed]
3. Arismendi, E.; Rivas, E.; Agustí, A.; Ríos, J.; Barreiro, E.; Vidal, J.; Rodriguez-Roisin, R. The Systemic Inflammome of Severe Obesity before and after Bariatric Surgery. *PLoS ONE* **2014**, *9*, e107859. [CrossRef]

4. Arismendi, E.; Bantulà, M.; Perpiñá, M.; Picado, C. Effects of Obesity and Asthma on Lung Function and Airway Dysanapsis in Adults and Children. *J. Clin. Med.* **2020**, *9*, 3762. [CrossRef] [PubMed]
5. Van Huisstede, A.; Rudolphus, A.; Cabezas, M.C.; Biter, L.U.; Van De Geijn, G.J.; Taube, C.; Hiemstra, P.S.; Braunstahl, G.J. Effect of Bariatric Surgery on Asthma Control, Lung Function and Bronchial and Systemic Inflammation in Morbidly Obese Subjects with Asthma. *Thorax* **2015**, *70*, 659–667. [CrossRef]
6. Forno, E.; Weiner, D.J.; Mullen, J.; Sawicki, G.; Kurland, G.; Han, Y.Y.; Cloutier, M.M.; Canino, G.; Weiss, S.T.; Litonjua, A.A.; et al. Obesity and Airway Dysanapsis in Children with and without Asthma. *Am. J. Respir. Crit Care Med.* **2017**, *195*, 314–323. [CrossRef]
7. Huang, L.; Wang, S.T.; Kuo, H.P.; Delclaux, C.; Jensen, M.E.; Wood, L.G.; Costa, D.; Nowakowski, D.; Wronka, I.; Oliveira, P.D.; et al. Effects of Obesity on Pulmonary Function Considering the Transition from Obstructive to Restrictive Pattern from Childhood to Young Adulthood. *Obes. Rev.* **2021**, *22*, e13327. [CrossRef]
8. Sood, A.; Ford, E.S.; Camargo, C.A., Jr. Association between Leptin and Asthma in Adults. *Thorax* **2006**, *61*, 300–305. [CrossRef]
9. Quek, Y.W.; Sun, H.L.; Ng, Y.Y.; Lee, H.S.; Yang, S.F.; Ku, M.S.; Lu, K.H.; Sheu, J.N.; Lue, K.H. Associations of Serum Leptin with Atopic Asthma and Allergic Rhinitis in Children. *Am. J. Rhinol. Allergy* **2010**, *24*, 354–358. [CrossRef]
10. Ozde, C.; Dogru, M.; Erdogan, F.; Ipek, I.O.; Ozde, S.; Karakaya, O. The Relationship between Adiponectin Levels and Epicardial Adipose Tissue Thickness in Non-Obese Children with Asthma. *Asian Pac. J. Allergy Immunol.* **2015**, *33*, 289–295. [CrossRef]
11. Lu, Y.; Van Bever, H.P.S.; Lim, T.K.; Kuan, W.; Goh, D.Y.T.; Mahadevan, M.; Sim, T.B.; Ho, R.; Larbi, A.; Ng, T.P. Obesity, Asthma Prevalence and IL-4: Roles of Inflammatory Cytokines, Adiponectin and Neuropeptide Y. *Pediatr. Allergy Immunol.* **2015**, *26*, 530–536. [CrossRef] [PubMed]
12. Hayashikawa, Y.; Iwata, M.; Inomata, M.; Kawagishi, Y.; Tokui, K.; Taka, C.; Kambara, K.; Okazawa, S.; Yamada, T.; Hayashi, R.; et al. Association of Serum Adiponectin with Asthma and Pulmonary Function in the Japanese Population. *Endocr. J.* **2015**, *62*, 695–709. [CrossRef] [PubMed]
13. Vezir, E.; Civelek, E.; Dibek Misirlioglu, E.; Toyran, M.; Capanoglu, M.; Karakus, E.; Kahraman, T.; Ozguner, M.; Demirel, F.; Gursel, I.; et al. Effects of Obesity on Airway and Systemic Inflammation in Asthmatic Children. *Int. Arch. Allergy Immunol.* **2021**, *182*, 679–689. [CrossRef]
14. Baltieri, L.; Cazzo, E.; de Souza, A.L.; Alegre, S.M.; de Paula Vieira, R.; Antunes, E.; de Mello, G.C.; Claudio Martins, L.; Chaim, E.A. Influence of Weight Loss on Pulmonary Function and Levels of Adipokines among Asthmatic Individuals with Obesity: One-Year Follow-Up. *Respir. Med.* **2018**, *145*, 48–56. [CrossRef] [PubMed]
15. Sideleva, O.; Suratt, B.T.; Black, K.E.; Tharp, W.G.; Pratley, R.E.; Forgione, P.; Dienz, O.; Irvin, C.G.; Dixon, A.E. Obesity and Asthma: An Inflammatory Disease of Adipose Tissue Not the Airway. *Am. J. Respir. Crit. Care Med.* **2012**, *186*, 598–605. [CrossRef]
16. Miller, M.R.; Hankinson, J.; Brusasco, V.; Burgos, F.; Casaburi, R.; Coates, A.; Crapo, R.; Enright, P.; Grinten, C.P.M.; Gustafsson, P.; et al. Standardisation of Spirometry. *Eur. Respir. J.* **2005**, *26*, 319–338. [CrossRef]
17. Papi, A.; Brightling, C.; Pedersen, S.E.; Reddel, H.K. Asthma. *Lancet* **2018**, *391*, 783–800. [CrossRef]
18. Reddel, H.K.; Bacharier, L.B.; Bateman, E.D.; Brightling, C.E.; Brusselle, G.G.; Buhl, R.; Cruz, A.A.; Duijts, L.; Drazen, J.M.; FitzGerald, J.M.; et al. Global Initiative for Asthma Strategy 2021 Executive Summary and Rationale for Key Changes. *Am. J. Respir. Crit. Care Med.* **2022**, *205*, 17–35. [CrossRef]
19. Masuzaki, H.; Ogawa, Y.; Sagawa, N.; Hosoda, K.; Matsumoto, T.; Mise, H.; Nishimura, H.; Yoshimasa, Y.; Tanaka, I.; Mori, T.; et al. Nonadipose Tissue Production of Leptin: Leptin as a Novel Placenta-Derived Hormone in Humans. *Nat. Med.* **1997**, *3*, 1029–1033. [CrossRef]
20. Barrios-Correa, A.A.; Estrada, J.A.; Contreras, I. Leptin Signaling in the Control of Metabolism and Appetite: Lessons from Animal Models. *J. Mol. Neurosci.* **2018**, *66*, 390–402. [CrossRef]
21. Procaccini, C.; De Rosa, V.; Galgani, M.; Carbone, F.; Cassano, S.; Greco, D.; Qian, K.; Auvinen, P.; Calì, G.; Stallone, G.; et al. Leptin-Induced MTOR Activation Defines a Specific Molecular and Transcriptional Signature Controlling CD4 + Effector T Cell Responses. *J. Immunol.* **2012**, *189*, 2941–2953. [CrossRef] [PubMed]
22. Mancuso, P.; Canetti, C.; Gottschalk, A.; Tithof, P.K.; Peters-Golden, M.; Peters, M. Leptin Augments Alveolar Macrophage Leukotriene Synthesis by Increasing Phospholipase Activity and Enhancing Group IVC IPLA 2 (CPLA 2) Protein Expression. *Am. J. Physiol. Lung Cell Mol. Physiol.* **2004**, *287*, 497–502. [CrossRef] [PubMed]
23. Bastien Conus, S.; Bruno, A.; Simon, H.-U. Leptin Is an Eosinophil Survival Factor. *J. Allergy Clin. Immunol.* **2005**, *116*, 1228–1234. [CrossRef]
24. Wong, C.K.; Cheung, P.F.-Y.; Lam, C.W.K. Leptin-Mediated Cytokine Release and Migration of Eosinophils: Implications for Immunopathophysiology of Allergic Inflammation. *Eur. J. Immunol.* **2007**, *37*, 2337–2348. [CrossRef] [PubMed]
25. Kato, H.; Ueki, S.; Kamada, R.; Kihara, J.; Yamauchi, Y.; Suzuki, T.; Takeda, M.; Itoga, M.; Chihara, M.; Ito, W.; et al. Leptin Has a Priming Effect on Eotaxin-Induced Human Eosinophil Chemotaxis. *Int. Arch. Allergy Immunol.* **2011**, *155*, 335–344. [CrossRef] [PubMed]
26. Zheng, H.; Zhang, X.; Castillo, E.F.; Luo, Y.; Liu, M.; Yang, X.O. Leptin Enhances TH2 and ILC2 Responses in Allergic Airway Disease. *J. Biol. Chem.* **2016**, *291*, 22043–22052. [CrossRef]
27. Zheng, H.; Wu, D.; Wu, X.; Zhang, X.; Zhou, Q.; Luo, Y.; Yang, X.; Chock, C.J.; Liu, M.; Yang, X.O. Leptin Promotes Allergic Airway Inflammation through Targeting the Unfolded Protein Response Pathway. *Sci. Rep.* **2018**, *8*, 8905. [CrossRef] [PubMed]

28. Liang, Z.; Liu, L.; Zhao, H.; Xia, Y.; Zhang, W.; Ye, Y.; Jiang, M.; Cai, S. A Systemic Inflammatory Endotype of Asthma with More Severe Disease Identified by Unbiased Clustering of the Serum Cytokine Profile. *Medicine* **2016**, *95*, 3774. [CrossRef]
29. Ruxandra Otelea, M.; Arghir, O.C.; Zugravu, C.; Rascu, A. Molecular Sciences Adiponectin and Asthma: Knowns, Unknowns and Controversies. *Int. J. Mol. Sci.* **2021**, *22*, 8971. [CrossRef]
30. Ajuwon, K.M.; Spurlock, M.E. Adiponectin Inhibits LPS-Induced NF-KB Activation and IL-6 Production and Increases PPARγ2 Expression in Adipocytes. *Am. J. Physiol.* **2005**, *288*, R1220–R1225. [CrossRef]
31. Okamoto, Y.; Folco, E.J.; Minami, M.; Wara, A.K.; Feinberg, M.W.; Sukhova, G.K.; Colvin, R.A.; Kihara, S.; Funahashi, T.; Luster, A.D.; et al. Adiponectin Inhibits the Production of CXC Receptor 3 Chemokine Ligands in Macrophages and Reduces T-Lymphocyte Recruitment in Atherogenesis. *Circ. Res.* **2008**, *102*, 218–225. [CrossRef] [PubMed]
32. Saxena, A.; Baliga, M.S.; Ponemone, V.; Kaur, K.; Larsen, B.; Fletcher, E.; Greene, J.; Fayad, R. Mucus and Adiponectin Deficiency: Role in Chronic Inflammation-Induced Colon Cancer. *Int. J. Colorectal. Dis.* **2013**, *28*, 1267. [CrossRef]
33. Lee, Y.A.; Hahm, D.H.; Kim, J.Y.; Sur, B.; Lee, H.M.; Ryu, C.J.; Yang, H.I.; Kim, K.S. Potential Therapeutic Antibodies Targeting Specific Adiponectin Isoforms in Rheumatoid Arthritis. *Arthritis Res. Ther.* **2018**, *20*, 245. [CrossRef] [PubMed]
34. Guebre-Egziabher, F.; Drai, J.; Fouque, D. Adiponectin and Chronic Kidney Disease. *J. Ren. Nutr.* **2007**, *17*, 9–12. [CrossRef]
35. Aydin, M.; Koca, C.; Ozol, D.; Uysal, S.; Yildirim, Z.; Kavakli, H.S.; Yigitoglu, M.R. Interaction of Metabolic Syndrome with Asthma in Postmenopausal Women: Role of Adipokines. *Inflammation* **2013**, *36*, 1232–1238. [CrossRef]
36. Magrone, T.; Simone, M.; Altamura, M.; Munno, I. Characterization of the Immune Inflammatory Profile in Obese Asthmatic Children. *Endocr. Metab. Immune. Disord. Targets* **2014**, *14*, 187–195. [CrossRef]
37. Jensen, M.E.; Gibson, P.G.; Collins, C.E.; Wood, L.G. Airway and Systemic Inflammation in Obese Children with Asthma. *Eur. Respir. J.* **2013**, *42*, 1012–1019. [CrossRef]
38. De Lima Azambuja, R.; da Costa Santos Azambuja, L.S.E.; Costa, C.; Rufino, R. Adiponectin in Asthma and Obesity: Protective Agent or Risk Factor for More Severe Disease? *Lung* **2015**, *193*, 749–755. [CrossRef]
39. Dogru, M.; Ozde, S.; Aktas, A.; Yuksel Karatoprak, E. The Adiponectin Levels and Asthma Control in Non-Obese Children with Asthma. *J. Asthma* **2015**, *52*, 772–776. [CrossRef]
40. Capelo, A.V.; da Fonseca, V.M.; Peixoto, M.V.M.; de Carvalho, S.R.; Azevedo, C.M.; Elsas, M.I.G.; Marques, B. Visceral Adiposity Is Associated with Cytokines and Decrease in Lung Function in Women with Persistent Asthma. *Rev. Port. Pneumol.* **2016**, *22*, 255–261. [CrossRef]
41. Ma, C.; Wang, Y.; Xue, M. Correlations of Severity of Asthma in Children with Body Mass Index, Adiponectin and Leptin. *J. Clin. Lab. Anal.* **2019**, *33*, e22915. [CrossRef] [PubMed]
42. Tsaroucha, A.; Daniil, Z.; Malli, F.; Georgoulias, P.; Minas, M.; Kostikas, K.; Bargiota, A.; Zintzaras, E.; Gourgoulianis, K.I. Leptin, Adiponectin, and Ghrelin Levels in Female Patients with Asthma during Stable and Exacerbation Periods. *J. Asthma* **2013**, *50*, 188–197. [CrossRef] [PubMed]
43. Alessi, M.C.; Bastelica, D.; Morange, P.; Berthet, B.; Leduc, I.; Verdier, M.; Geel, O.; Juhan-Vague, I. Plasminogen Activator Inhibitor 1, Transforming Growth Factor-Beta1, and BMI Are Closely Associated in Human Adipose Tissue during Morbid Obesity. *Diabetes* **2000**, *49*, 1374–1380. [CrossRef] [PubMed]
44. Lee, M.J. Transforming Growth Factor Beta Superfamily Regulation of Adipose Tissue Biology in Obesity. *Biochim. Biophys. Acta Mol. Basis Dis.* **2018**, *1864*, 1160–1171. [CrossRef] [PubMed]
45. Park, Y.H.; Oh, E.Y.; Han, H.; Yang, M.; Park, H.J.; Park, K.H.; Lee, J.-H.; Park, J.-W. Insulin Resistance Mediates High-Fat Diet-Induced Pulmonary Fibrosis and Airway Hyperresponsiveness through the TGF-B1 Pathway. *Exp. Mol. Med.* **2019**, *51*, 1–12. [CrossRef]
46. Wang, C.H.; Huang, C.D.; Lin, H.C.; Lee, K.Y.; Lin, S.M.; Liu, C.Y.; Huang, K.H.; Ko, Y.S.; Kian, F.C.; Kuo, H.P. Increased Circulating Fibrocytes in Asthma with Chronic Airflow Obstruction. *Am. J. Respir. Crit. Care Med.* **2012**, *178*, 583–591. [CrossRef]
47. Minshall, E.M.; Leung, D.Y.M.; Martin, R.J.; Song, Y.L.; Cameron, L.; Ernst, P.; Hamid, Q. Eosinophil-Associated TGF-1 MRNA Expression and Airways Fibrosis in Bronchial Asthma. *Am. J. Respir. Cell Mol. Biol.* **1997**, *17*, 326–333. [CrossRef]
48. Torrego, A.; Hew, M.; Oates, T.; Sukkar, M.; Kian, F.C. Expression and Activation of TGF-β Isoforms in Acute Allergen-induced Remodelling in Asthma. *Thorax* **2007**, *62*, 307. [CrossRef]
49. Chu, H.W.; Trudeau, J.B.; Balzar, S.; Wenzel, S.E. Peripheral Blood and Airway Tissue Expression of Transforming Growth Factor β by Neutrophils in Asthmatic Subjects and Normal Control Subjects. *J. Allergy Clin. Immunol.* **2000**, *106*, 1115–1123. [CrossRef]
50. Ohno, I.; Nitta, Y.; Yamauchi, K.; Hoshi, H.; Honma, M.; Woolley, K.; O'Byrne, P.; Tamura, G.; Jordana, M.; Shirato, K. Transforming Growth Factor Beta 1 (TGF Beta 1) Gene Expression by Eosinophils in Asthmatic Airway Inflammation. *Am. J. Respir. Cell Mol. Biol.* **2012**, *15*, 404–409. [CrossRef]
51. Kelley, J.; Fabisiak, J.P.; Hawes, K.; Absher, M. Cytokine Signaling in Lung: Transforming Growth Factor-Beta Secretion by Lung Fibroblasts. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **1991**, *260*, L123–L128. [CrossRef] [PubMed]
52. De Boer, W.I.; Van Schadewijk, A.; Sont, J.K.; Sharma, H.S.; Stolk, J.; Hiemstra, P.S.; Van Krieken, J.H.J.M. Transforming Growth Factor B1 and Recruitment of Macrophages and Mast Cells in Airways in Chronic Obstructive Pulmonary Disease. *Am. J. Respir. Crit. Care Med.* **2012**, *158*, 1951–1957. [CrossRef] [PubMed]
53. Fong, C.Y.; Pang, L.; Holland, E.; Knox, A.J.; Yi, C.; Knox TGF-, A.J. TGF-1 Stimulates IL-8 Release, COX-2 Expression, and PGE 2 Release in Human Airway Smooth Muscle Cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **2000**, *279*, L201–L207. [CrossRef] [PubMed]

54. Dostert, C.; Grusdat, M.; Letellier, E.; Brenner, D. The TNF Family of Ligands and Receptors: Communication Modules in the Immune System and Beyond. *Physiol. Rev.* **2019**, *99*, 115–160. [CrossRef]
55. Cabal-Hierro, L.; Lazo, P.S. Signal Transduction by Tumor Necrosis Factor Receptors. *Cell Signal.* **2012**, *24*, 1297–1305. [CrossRef]
56. Eslick, S.; Williams, E.J.; Berthon, B.S.; Wright, T.; Karihaloo, C.; Gately, M.; Wood, L.G. Chain Fatty Acids Reduce Systemic Inflammation in Monocytes and Adipose Tissue Macrophages from Obese Subjects. *Nutrients* **2022**, *11*, 765. [CrossRef]
57. Golikova, E.A.; Lopatnikova, J.A.; Kovalevskaya-Kucheryavenko, T.V.; Nepomnyashih, V.M.; Sennikov, S.V. Levels of TNF, TNF Autoantibodies and Soluble TNF Receptors in Patients with Bronchial Asthma. *J. Asthma* **2013**, *50*, 705–711. [CrossRef]
58. Hurrell, B.P.; Galle-Treger, L.; Jahani, P.S.; Howard, E.; Helou, D.G.; Banie, H.; Soroosh, P.; Akbari, O. TNFR2 Signaling Enhances ILC2 Survival, Function, and Induction of Airway Hyperreactivity. *Cell Rep.* **2019**, *29*, 4509. [CrossRef]
59. Dixon, A.E.; Pratley, R.E.; Forgione, P.M.; Kaminsky, D.A.; Whittaker-Leclair, L.A.; Griffes, L.A.; Garudathri, J.; Raymond, D.; Poynter, M.E.; Bunn, J.Y.; et al. Effects of Obesity and Bariatric Surgery on Airway Hyperresponsiveness, Asthma Control, and Inflammation. *J. Allergy Clin. Immunol.* **2011**, *128*, 508–515.e2. [CrossRef]
60. Mantovani, A.; Dinarello, C.A.; Molgora, M.; Garlanda, C. IL-1 and Related Cytokines in Innate and Adaptive Immunity in Health and Disease. *Immunity* **2019**, *50*, 778. [CrossRef]
61. Dinarello, C.A.; Novick, D.; Kim, S.; Kaplanski, G. Interleukin-18 and IL-18 Binding Protein. *Front. Immunol.* **2013**, *4*, 289. [CrossRef] [PubMed]
62. Sedimbi, S.K.; Hägglöf, T.; Karlsson, M.C.I. IL-18 in Inflammatory and Autoimmune Disease. *Cell. Mol. Life Sci.* **2013**, *70*, 4795–4808. [CrossRef] [PubMed]
63. Caligiuri, M.A.; Suzuki, K.; Wechser, M.; Goodsaid, F.; VanDeusen, J.B.; Whitman, S.P.; Fehniger, T.A.; Cooper, M.A.; Shah, M.H.; Turner, M.J. Innate Immune Response Combination with IL-12: Implications for the Activation with IL-18 or IL-15 in Expression by Human NK Cells Following Differential Cytokine and Chemokine Gene. *J. Immunol. Ref.* **1999**, *4511*, 4511–4520.
64. Kim, S.-H.; Eisenstein, M.; Reznikov, L.; Fantuzzi, G.; Novick, D.; Rubinstein, M.; Dinarello, C.A. Structural Requirements of Six Naturally Occurring Isoforms of the IL-18 Binding Protein to Inhibit IL-18. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 1190–1195. [CrossRef]
65. Novick, D.; Schwartsburd, B.; Pinkus, R.; Suissa, D.; Belzer, I.; Sthoeger, Z.; Keane, W.F.; Chvatchko, Y.; Kim, S.H.; Fantuzzi, G.; et al. A novel IL-18BP ELISA shows elevated serum IL-19BP in sepsis and extensive decrease of free IL-18. *Cytokine* **2001**, *14*, 334–342. [CrossRef]
66. Girard-Guyonvarch, C.; Harel, M.; Gabay, C. The Role of Interleukin 18/Interleukin 18-Binding Protein in Adult-Onset Still's Disease and Systemic Juvenile Idiopathic Arthritis. *J. Clin. Med.* **2022**, *11*, 430. [CrossRef]
67. Esposito, K.; Pontillo, A.; Ciotola, M.; Di Palo, C.; Grella, E.; Nicoletti, G.; Giugliano, D. Weight Loss Reduces Interleukin-18 Levels in Obese Women. *J. Clin. Endocrinol. Metab.* **2002**, *87*, 3864–3866. [CrossRef]
68. Patil, S.P.; Wisnivesky, J.P.; Busse, P.J.; Halm, E.A.; Li, X.-M. Detection of Immunological Biomarkers Correlated with Asthma Control and Quality of Life Measurements in Sera from Chronic Asthmatic Patients. *Ann. Allergy Asthma Immunol.* **2011**, *106*, 205–213. [CrossRef]
69. Tanaka, H.; Miyazaki, N.; Oashi, K.; Teramoto, S.; Shiratori, M.; Hashimoto, M.; Ohmichi, M.; Abe, S. IL-18 Might Reflect Disease Activity in Mild and Moderate Asthma Exacerbation. *J. Allergy Clin. Immunol.* **2001**, *107*, 331–336. [CrossRef]
70. Camiolo, M.J.; Zhou, X.; Wei, Q.; Bittar, H.E.T.; Kaminski, N.; Ray, A.; Wenzel, S.E. Machine Learning Implicates the IL-18 Signaling Axis in Severe Asthma. *JCI Insight* **2021**, *6*, e149945. [CrossRef]
71. Sousa, A.R.; Lane, S.J.; Nakhosteen, J.A.; Yoshimura, T.; Lee, T.H.; Poston, R.N. Increased Expression of the Monocyte Chemoattractant Protein-1 in Bronchial Tissue from Asthmatic Subjects. *Am. J. Respir. Cell Mol. Biol.* **2012**, *10*, 142–147. [CrossRef] [PubMed]
72. Campbell, E.M.; Charo, I.F.; Kunkel, S.L.; Strieter, R.M.; Boring, L.; Gosling, J.; Lukacs, N.W. Monocyte Chemoattractant Protein-1 Mediates Cockroach Allergen-Induced Bronchial Hyperreactivity in Normal but Not CCR2-/- Mice: The Role of Mast Cells. *J. Immunol.* **1999**, *163*, 2160–2167. [PubMed]
73. Kurashima, K.; Mukaida, N.; Fujimura, M.; Schröder, J.M.; Matsuda, T.; Matsushima, K. Increase of Chemokine Levels in Sputum Precedes Exacerbation of Acute Asthma Attacks. *J. Leukoc. Biol.* **1996**, *59*, 313–316. [CrossRef]
74. Gonzalo, J.A.; Lloyd, C.M.; Wen, D.; Albar, J.P.; Wells, T.N.C.; Proudfoot, A.; Martinez-A., C.; Dorf, M.; Bjerke, T.; Coyle, A.J.; et al. The Coordinated Action of CC Chemokines in the Lung Orchestrates Allergic Inflammation and Airway Hyperresponsiveness. *J. Exp. Med.* **1998**, *188*, 157. [CrossRef] [PubMed]
75. Kolattukudy, P.E.; Niu, J. Inflammation, Endoplasmic Reticulum Stress, Autophagy, and the Monocyte Chemoattractant Protein-1/CCR2 Pathway. *Circ. Res.* **2012**, *110*, 174–189. [CrossRef]
76. Sachan, A.; Singh, A.; Shukla, S.; Aggarwal, S.; Mir, I.; Yadav, R. An Immediate Post Op and Follow up Assessment of Circulating Adipo-Cytokines after Bariatric Surgery in Morbid Obesity. *Metab. Open* **2022**, *13*, 100147. [CrossRef]
77. Kakkar, R.; Lee, R.T. The IL-33/ST2 Pathway: Therapeutic Target and Novel Biomarker. *Nat. Rev. Drug Discov.* **2008**, *7*, 827. [CrossRef]
78. Cayrol, C. IL-33, an Alarmin of the IL-1 Family Involved in Allergic and Non Allergic Inflammation: Focus on the Mechanisms of Regulation of Its Activity. *Cells* **2021**, *11*, 107. [CrossRef]
79. Liew, F.Y.; Girard, J.P.; Turnquist, H.R. Interleukin-33 in Health and Disease. *Nat. Rev. Immunol.* **2016**, *16*, 676–689. [CrossRef]

80. Bandara, G.; Beaven, M.A.; Olivera, A.; Gilfillan, A.M.; Metcalfe, D.D. Activated Mast Cells Synthesize and Release Soluble ST2-a Decoy Receptor for IL-33. *Eur. J. Immunol.* **2015**, *45*, 3034–3044. [CrossRef]
81. Hayakawa, H.; Hayakawa, M.; Kume, A.; Tominaga, S.I. Soluble ST2 Blocks Interleukin-33 Signaling in Allergic Airway Inflammation. *J. Biol. Chem.* **2007**, *282*, 26369–26380. [CrossRef] [PubMed]
82. Al-Sajee, D.; Sehmi, R.; Hawke, T.J.; El-Gammal, A.; Howie, K.J.; Watson, R.M.; Londei, M.; Gauvreau, G.M.; O'Byrne, P.M. Expression of IL-33 and TSLP and Their Receptors in Asthmatic Airways after Inhaled Allergen Challenge. *Am. J. Respir. Crit. Care Med.* **2018**, *198*, 805–807. [CrossRef]
83. Préfontaine, D.; Nadigel, J.; Chouiali, F.; Audusseau, S.; Semlali, A.; Chakir, J.; Martin, J.G.; Hamid, Q. Increased IL-33 Expression by Epithelial Cells in Bronchial Asthma. *J. Allergy Clin. Immunol.* **2010**, *125*, 752–754. [CrossRef] [PubMed]
84. Kamekura, R.; Kojima, T.; Takano, K.; Go, M.; Sawada, N.; Himi, T. The Role of IL-33 and Its Receptor ST2 in Human Nasal Epithelium with Allergic Rhinitis. *Clin. Exp. Allergy* **2012**, *42*, 218–228. [CrossRef]
85. Li, C.; Spallanzani, R.G.; Mathis, D. Visceral Adipose Tissue Tregs and the Cells That Nurture Them. *Immunol. Rev.* **2020**, *295*, 114–125. [CrossRef] [PubMed]
86. Kawaguchi, K.; Asano, S. Pathophysiological Roles of Actin-Binding Scaffold Protein, Ezrin. *Int. J. Mol. Sci.* **2022**, *2022*, 3246. [CrossRef]
87. Kalinauskaite-Zukauske, V.; Januskevicius, A.; Janulaityte, I.; Miliauskas, S.; Malakauskas, K.; Katsaounou, P.A. Serum Levels of Epithelial-Derived Cytokines as Interleukin-25 and Thymic Stromal Lymphopoietin after a Single Dose of Mepolizumab in Patients with Severe Non-Allergic Eosinophilic Asthma: A Short Report. *Scand. J. Immunol.* **2019**, *90*, e12820. [CrossRef]
88. Jia, M.; Yan, X.; Jiang, X.; Wu, Y.; Xu, J.; Meng, Y.; Yang, Y.; Shan, X.; Zhang, X.; Mao, S.; et al. Ezrin, a Membrane Cytoskeleton Cross-Linker Protein, as a Marker of Epithelial Damage in Asthma. *Am. J. Respir. Crit. Care Med.* **2019**, *199*, 496–507. [CrossRef]
89. Lopez, J.P.; Turner, J.R.; Philipson, L.H. Glucose-Induced ERM Protein Activation and Translocation Regulates Insulin Secretion. *Am. J. Physiol. Endocrinol. Metab.* **2010**, *299*, E772. [CrossRef]
90. Chupp, G.L.; Lee, C.G.; Jarjour, N.; Shim, Y.M.; Holm, C.T.; He, S.; Dziura, J.D.; Reed, J.; Coyle, A.J.; Kiener, P.; et al. A Chitinase-like Protein in the Lung and Circulation of Patients with Severe Asthma. *N. Engl. J. Med.* **2007**, *357*, 2016–2027. [CrossRef]
91. Kimura, H.; Shimizu, K.; Tanabe, N.; Makita, H.; Taniguchi, N.; Kimura, H.; Suzuki, M.; Abe, Y.; Matsumoto-Sasaki, M.; Oguma, A.; et al. Further Evidence for Association of YKL-40 with Severe Asthma Airway Remodeling. *Ann. Allergy Asthma Immunol.* **2022**, *24*, S1081–S1206. [CrossRef] [PubMed]
92. Gomez, J.L.; Yan, X.; Holm, C.T.; Grant, N.; Liu, Q.; Cohn, L.; Nezgovorova, V.; Meyers, D.A.; Bleecker, E.R.; Crisafi, G.M.; et al. Characterisation of Asthma Subgroups Associated with Circulating YKL-40 Levels. *Eur. Respir. J.* **2017**, *50*, 1700800. [CrossRef] [PubMed]
93. Ilmarinen, P.; Tuomisto, L.E.; Niemelä, O.; Hämäläinen, M.; Moilanen, E.; Kankaanranta, H. YKL-40 and Adult-Onset Asthma: Elevated Levels in Clusters with Poorest Outcome. *J. Allergy Clin. Immunol. Pract.* **2019**, *7*, 2466–2468.e3. [CrossRef] [PubMed]
94. Konradsen, J.R.; James, A.; Nordlund, B.; Reinius, L.E.; Söderhäll, C.; Melén, E.; Wheelock, Å.; Lödrup Carlsen, K.C.; Lidegran, M.; Verhoek, M.; et al. The Chitinase-like Protein YKL-40: A Possible Biomarker of Inflammation and Airway Remodeling in Severe Pediatric Asthma. *J. Allergy Clin. Immunol.* **2013**, *132*, 328–335.e5. [CrossRef]
95. Specjalski, K.; Chełmińska, M.; Jassem, E. YKL-40 Protein Correlates with the Phenotype of Asthma. *Lung* **2015**, *193*, 189–194. [CrossRef]
96. Thomsen, S.B.; Rathcke, C.N.; Jørgensen, N.B.; Madsbad, S.; Vestergaard, H. Effects of Roux-En-Y Gastric Bypass on Fasting and Postprandial Levels of the Inflammatory Markers YKL-40 and MCP-1 in Patients with Type 2 Diabetes and Glucose Tolerant Subjects. *J. Obes.* **2013**, *2013*, 361781. [CrossRef]
97. Hempen, M.; Kopp, H.P.; Elhenicky, M.; Höbaus, C.; Brix, J.M.; Koppensteiner, R.; Schernthaner, G.; Schernthaner, G.H. YKL-40 Is Elevated in Morbidly Obese Patients and Declines After Weight Loss. *Obes. Surg.* **2009**, *19*, 1557–1563. [CrossRef]
98. Kyrgios, I.; Galli-Tsinopoulou, A.; Stylianou, C.; Papakonstantinou, E.; Arvanitidou, M.; Haidich, A.B. Elevated Circulating Levels of the Serum Acute-Phase Protein YKL-40 (Chitinase 3-like Protein 1) Are a Marker of Obesity and Insulin Resistance in Prepubertal Children. *Metab. Clin. Exp.* **2012**, *61*, 562–568. [CrossRef]
99. Catalán, V.; Gómez-Ambrosi, J.; Rodríguez, A.; Ramírez, B.; Rotellar, F.; Valentí, V.; Silva, C.; Gil, M.J.; Salvador, J.; Frühbeck, G. Increased Circulating and Visceral Adipose Tissue Expression Levels of YKL-40 in Obesity-Associated Type 2 Diabetes Are Related to Inflammation: Impact of Conventional Weight Loss and Gastric Bypass. *J. Clin. Endocrinol. Metab.* **2011**, *96*, 200–209. [CrossRef]
100. Veldhoen, M.; Uyttenhove, C.; van Snick, J.; Helmby, H.; Westendorf, A.; Buer, J.; Martin, B.; Wilhelm, C.; Stockinger, B. Transforming Growth Factor-β "reprograms" the Differentiation of T Helper 2 Cells and Promotes an Interleukin 9–Producing Subset. *Nat. Immunol.* **2008**, *9*, 1341–1346. [CrossRef]
101. Dardalhon, V.; Awasthi, A.; Kwon, H.; Galileos, G.; Gao, W.; Sobel, R.A.; Mitsdoerffer, M.; Strom, T.B.; Elyaman, W.; Ho, I.C.; et al. Interleukin 4 Inhibits TGF-β-Induced-Foxp3+T Cells and Generates, in Combination with TGF-β, Foxp3− Effector T Cells That Produce Interleukins 9 and 10. *Nat. Immunol.* **2008**, *9*, 1347. [CrossRef]
102. Deng, Y.; Wang, Z.; Chang, C.; Lu, L.; Lau, C.S.; Lu, Q. Th9 Cells and IL-9 in Autoimmune Disorders: Pathogenesis and Therapeutic Potentials. *Hum. Immunol.* **2017**, *78*, 120–128. [CrossRef] [PubMed]

103. Ciccia, F.; Guggino, G.; Ferrante, A.; Cipriani, P.; Giacomelli, R.; Triolo, G. Interleukin-9 and T Helper Type 9 Cells in Rheumatic Diseases. *Clin. Exp. Immunol.* **2016**, *185*, 125. [CrossRef] [PubMed]
104. Elyaman, W.; Bradshaw, E.M.; Uyttenhove, C.; Dardalhon, V.; Awasthi, A.; Imitola, J.; Bettelli, E.; Oukka, M.; Van Snick, J.; Renauld, J.C.; et al. IL-9 Induces Differentiation of TH17 Cells and Enhances Function of FoxP3+ Natural Regulatory T Cells. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 12885–12890. [CrossRef] [PubMed]
105. Jia, L.; Wang, Y.; Li, J.; Li, S.; Zhang, Y.; Shen, J.; Tan, W.; Wu, C. Detection of IL-9 Producing T Cells in the PBMCs of Allergic Asthmatic Patients. *BMC Immunol.* **2017**, *18*, 38. [CrossRef] [PubMed]
106. Mahdaviani, S.A.; Eskian, M.; Khorasanizadeh, M.; Bashardoost, B.; Nejad, S.T.; Jamaati, H.; Rezaei, A.; Sadr, M.; Aryan, Z.; Rezaei, N. Interleukin 9 Serum Level and Single Nucleotide Polymorphism in Patients with Asthma. *Acta Bio. Med. Atenei Parm.* **2021**, *92*, 2021206. [CrossRef]
107. Erpenbeck, V.J.; Hohlfeld, J.M.; Volkmann, B.; Hagenberg, A.; Geldmacher, H.; Braun, A.; Krug, N. Segmental Allergen Challenge in Patients with Atopic Asthma Leads to Increased IL-9 Expression in Bronchoalveolar Lavage Fluid Lymphocytes. *J. Allergy Clin. Immunol.* **2003**, *111*, 1319–1327. [CrossRef]
108. Yao, W.; Tepper, R.S.; Kaplan, M.H. Predisposition to the Development of IL-9-Secreting T Cells in Atopic Infants. *J. Allergy Clin. Immunol.* **2011**, *128*, 1357–1360.e5. [CrossRef]
109. Huang, M.; Dong, J. Critical Roles of Balanced T Helper 9 Cells and Regulatory T Cells in Allergic Airway Inflammation and Tumor Immunity. *J. Immunol. Res.* **2021**, *2021*, 8816055. [CrossRef]
110. Sehra, S.; Yao, W.; Nguyen, E.T.; Glosson-Byers, N.L.; Akhtar, N.; Zhou, B.; Kaplan, M.H. TH9 Cells Are Required for Tissue Mast Cell Accumulation during Allergic Inflammation. *J. Allergy Clin. Immunol.* **2015**, *136*, 433–440.e1. [CrossRef]
111. Dhanraj, P.; Van Heerden, M.B.; Pepper, M.S.; Ambele, M.A. Sexual Dimorphism in Changes That Occur in Tissues, Organs and Plasma during the Early Stages of Obesity Development. *Biology* **2021**, *28*, 717. [CrossRef] [PubMed]
112. Pelaia, C.; Paoletti, G.; Puggioni, F.; Racca, F.; Pelaia, G.; Canonica, G.W.; Heffler, E. Interleukin-5 in the Pathophysiology of Severe Asthma. *Pathophysiol. Sev. Asthma Front. Physiol.* **2019**, *10*, 1514. [CrossRef] [PubMed]
113. Gonzalez-Barcala, F.J.; San-Jose, M.E.; Nieto-Fontarigo, J.J.; Carreira, J.M.; Calvo-Alvarez, U.; Cruz, M.J.; Facal, D.; Garcia-Sanz, M.T.; Valdes-Cuadrado, L.; Salgado, F.J. Association between Blood Eosinophil Count with Asthma Hospital Readmissions. *Eur. J. Intern. Med.* **2018**, *53*, 34–39. [CrossRef]
114. Gonzalez-Barcala, F.J.; Nieto-Fontarigo, J.J.; Lourido-Cebreiro, T.; Rodríguez-García, C.; San-Jose, M.E.; Carreira, J.M.; Calvo-Alvarez, U.; Cruz, M.J.; Facal, D.; Garcia-Sanz, M.T.; et al. Obesity Does Not Increase the Risk of Asthma Readmissions. *J. Clin. Med.* **2020**, *9*, 221. [CrossRef] [PubMed]
115. Mallah, N.; Rodriguez-Segade, S.; Gonzalez-Barcala, F.J.; Takkouche, B. Blood Eosinophil Count as Predictor of Asthma Exacerbation. A Meta-Analysis. *Pediatr. Allergy Immunol.* **2021**, *32*, 465–478. [CrossRef] [PubMed]
116. Marijsse, G.S.; Seys, S.F.; Schelpe, A.S.; Dilissen, E.; Goeminne, P.; Dupont, L.J.; Ceuppens, J.L.; Bullens, D.M.A. Obese Individualswith Asthma Preferentially Have a High IL-5/IL-17A/IL-25 Sputum Inflammatory Pattern. *Am. J. Respir. Crit. Care Med.* **2014**, *189*, 1281–1284. [CrossRef] [PubMed]

C) Third manuscript: Weight loss and vitamin D improve hyporesponsiveness to corticosteroids in obese asthma.

Weight loss and vitamin D improve hyporesponsiveness to corticosteroids in obese asthma

Short Title: Weight loss improves steroid response in obese asthma

Bantulà M[1], Tubita V[1], Roca-Ferrer J[1,2], Mullol J[1,2,3], Valero A[1,2,4], Bobolea I[1,2,4], Pascal M[5], de Hollanda A[1,6,7], Vidal J[1,6,8], Picado C*[1,2,4], Arismendi E*[1,2,4]

[1]Institut d'Investigacions Biomèdiques August Pi I Sunyer (IDIBAPS), Barcelona, Spain.
[2]Centro de Investigaciones Biomédicas en Red de Enfermedades Respiratorias (CIBERES), Madrid, Spain.
[3]Rhinology Unit & Smell Clinic, ENT Department, Hospital Clinic, Barcelona, Spain.
[4]Pulmonology and Allergy Department, Hospital Clinic, University of Barcelona, Barcelona, Spain.
[5]Immunology Department, CDB, Hospital Clinic, University of Barcelona, Barcelona, Spain.
[6]Obesity Unit, Endocrinology and Nutrition Department, Hospital Clínic, Barcelona, Spain.
[7]Centro de Investigaciones Biomédicas en Red de Fisopatología de la Obesidad y Nutrición (CIBEROBN), Madrid, Spain.
[8]Centro de Investigaciones Biomédicas en Red en Diabetes y Enfermedades Metabólicas (CIBERDEM), Madrid, Spain.
* Both authors contributed equally to this work with senior responsibilities.

Corresponding author:
Marina Bantulà
Institut d'Investigacions Biomèdiques August Pi I Sunyer (IDIBAPS)
Barcelona, Spain.
E-mail: bantula@clinic.cat

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.18176/jiaci.0861

J Investig Allergol Clin Immunol 2023; Vol. 33(6)
doi: 10.18176/jiaci.0861

Abstract

Background: Obesity negatively impacts on asthma response to inhaled corticosteroids by unknown mechanisms.

Objective: To demonstrate that the poor response to inhaled corticosteroids in obese asthma is associated with an impaired anti-inflammatory activity of corticosteroids and vitamin D deficiency, both improved by weight loss.

Methods: 23 obese asthmatics (OA) [18 females; median age (interquartile range) 56 (51-59) years], 14 non-obese asthmatics (NOA) [11 females; 53 (43-60) years], 15 obese (O) [13 females; 47 (45-60) years], and 19 healthy controls (HC) [14 females; 43 (34-56) years] were enrolled. 10 OA and 11 O patients were evaluated at baseline (V1) and six months after (V2) bariatric surgery. Corticosteroid response was measured by dexamethasone inhibition of peripheral blood mononuclear cell (PBMC) proliferation. Lung function, serum levels of leptin, adiponectin, and vitamin D were measured at V1 and V2.

Results: We found a reduced response to dexamethasone in PBMCs of O and OA patients with respect to NOA and HC subjects, that inversely correlated with the adiponectin/leptin ratio and vitamin D levels. Bariatric surgery improved corticosteroid responses in O and OA patients and normalized adiponectin/leptin ratio and vitamin D levels. Exposure of PBMCs to vitamin D potentiated the antiproliferative effects of corticosteroids. Dexamethasone and vitamin D induced similar MKP-1 expression in O and OA patients.

Conclusion: The efficacy of weight loss to improve symptoms and lung function in OA patients can be, at least in part, due to the recovered anti-inflammatory effects of corticosteroids. Vitamin D deficiency may contribute to corticosteroid hyporesponsiveness in OA.

Key words: Asthma. Bariatric surgery. Corticosteroid. Obesity. Vitamin D

doi: 10.18176/jiaci.0861

Resumen

Antecedentes: La obesidad tiene un impacto negativo en la respuesta del asma a los corticosteroides inhalados por mecanismos desconocidos.

Objetivo: Demostrar que la mala respuesta a los corticosteroides inhalados en pacientes obesos asmáticos se asocia con una actividad antiinflamatoria alterada de los corticosteroides, así como también a la deficiencia de vitamina D, ambos mejorados por la pérdida de peso.

Métodos: 23 obesos asmáticos (OA) [18 mujeres; mediana de edad (rango intercuartílico) 56 (51-59) años], 14 asmáticos no obesos (NOA) [11 mujeres; 53 (43-60) años], 15 obesos (O) [13 mujeres; 47 (45-60) años], y 19 controles sanos (HC) [14 mujeres; 43 (34-56) años] fueron incluidos. Se evaluaron 10 pacientes OA y 11 O al inicio (V1) y seis meses después (V2) de cirugía bariátrica. La respuesta a los corticosteroides se midió mediante la inhibición con dexametasona de la proliferación de células mononucleares de sangre periférica (PBMC). La función pulmonar, los niveles séricos de leptina, adiponectina y vitamina D se midieron en V1 y V2.

Resultados: Encontramos una respuesta reducida a la dexametasona en PBMC de pacientes O y OA con respecto a los NOA y HC, que se correlacionó de forma inversamente proporcional con la relación adiponectina/leptina y los niveles de vitamina D. La cirugía bariátrica mejoró las respuestas de los corticosteroides en los grupos de pacientes O y OA y normalizó la relación adiponectina/leptina y los niveles de vitamina D. La exposición de las PBMC a la vitamina D potenció los efectos antiproliferativos de los corticosteroides. La dexametasona y la vitamina D indujeron una expresión similar de MKP-1 en los pacientes O y OA.

Conclusiones: La eficacia de la pérdida de peso para mejorar los síntomas y la función pulmonar en pacientes OA puede deberse, al menos en parte, a los efectos antiinflamatorios restablecidos de los corticosteroides. La deficiencia de vitamina D puede contribuir a la baja respuesta a los corticosteroides en los OA.

Palabras clave: Asma. Cirugía bariátrica. Corticosteroides. Obesidad. Vitamina D

J Investig Allergol Clin Immunol 2023; Vol. 33(6)

doi: 10.18176/jiaci.0861

Introduction

Asthma is a chronic respiratory illness characterized by chronic airway inflammation, chronic airflow obstruction, and airway hyperresponsiveness [1]. Based on the inflammatory pathway involved, chronic inflammation in asthma is broadly divided into two predominant endotypes: type 2 (T2-high) and non-type 2 (T2-low) [2].

Inhaled corticosteroids (ICS) are considered the cornerstone of controller therapy for T2-high asthma, which contrasts with their poor therapeutic efficacy in T2-low asthma [1,2]. ICS have the ability to effectively control asthma by reducing airway inflammation, airway obstruction, airway hyperresponsiveness, and asthma symptoms [1,3]. However, asthma patients often do not follow the prescribed ICS regimen and, among the compliant patients, the response to treatment is highly variable, from those who respond to low doses of ICS to those who are resistant to even very high ICS doses. In both cases the results are similar: increased risk of exacerbations, hospitalization, and mortality [1,3].

Multiple molecular mechanisms have been involved in corticosteroids (CS) hyporesponsiveness. Obesity has been recognized as a risk factor for ICS hyporesponsiveness in asthma patients. Asthmatic patients with obesity have reduced odds of achieving asthma control, higher risk of asthma hospitalizations, and lower quality of life compared with asthmatics with a normal body mass index (BMI) [4–6]. Positive correlation has been found between high BMI with residual asthma symptoms that remained present in OA patients despite intensive treatment with high doses of ICS [6]. The mechanism(s) by which obesity negatively impacts on asthma control by ICS remains to be fully elucidated. Detrimental effects of obesity on lung function and additive or synergistic effects of obese systemic inflammation on airways inflammation, have been proposed as potential mechanisms to explain ICS hyporesponsiveness in OA [7,8]. On the other hand, a defective induction by CS of anti-inflammatory genes such as mitogen-activated protein kinase phosphatase-1 (MKP-1) has been involved [9].

In adults, the increased weight is associated with a relatively small reduction of forced expiratory volume in 1s (FEV_1) and of forced vital capacity (FVC), with the FEV_1/FVC ratio usually remaining

unaltered or slightly increased and associated with a reduction of lung volumes, such as the functional residual capacity, alterations that characterize the so-called restrictive ventilatory defect [7].

J Investig Allergol Clin Immunol 2023; Vol. 33(6)
doi: 10.18176/jiaci.0861

Concerning inflammation, the excessive accumulation of adipose tissue in subjects with obesity results in an increased production of pro-inflammatory cytokines (IL-1β, IL-6, IFN-γ, TNF-α) and adipokines such as leptin, while there is a reduced release of adiponectin, a cytokine with anti-inflammatory properties [8]. Very little is known about the links that may exist between obesity and asthma inflammations, as it has been observed that the high systemic production of adipokines produced in visceral adipose tissue is capable of affecting the reactivity of the airways, without apparently modifying airway inflammation in OA patients [10]. Similarly, the mechanisms responsible for the loss of anti-inflammatory efficacy of CS in OA remain to be elucidated. A single study showed that the ability of dexamethasone (DEX) to induce MKP-1 gene expression in peripheral blood mononuclear cells (PBMCs) is reduced in subjects with obesity and severe asthma compared with normal-weight asthma patients [11].

Vitamin D (VitD) is a hormone with pleiotropic effects and numerous regulatory mechanisms beyond bone health [12]. The active form 1,25-dihydroxyvitamin D (1,25$(OH)_2$D) binds the VitD receptor, present in all tissues including T and B lymphocytes, dendritic cells, and bronchial epithelial cells [12]. VitD inhibits the expression of IL-2, and suppresses T helper 1 cytokines IL-12, IL-6, and TNF, but increases IL-4, IL-5, and IL-10 release [12,13].

The serum level of 25-hydroxyvitamin D (25(OH)D) that is defined as VitD deficiency remains somewhat controversial. Previous guidelines defined VitD deficiency as 25(OH)D values below 20ng/ml [14]. However, in a more recent consensus statement, VitD deficiency is defined as 25(OH)D values below 12 ng/ml [15,16]. Evidence from observational studies suggests that obesity is associated with VitD deficiency [17,18]. Moreover, serum 25(OH)D levels decline with increasing BMI and body fat mass [19,20], and the decreased level of VitD appears to be mainly due to its dilution in the increased adipose tissue mass [19].

Low serum 25(OH)D levels have been found associated with asthma exacerbations [21]. Moreover, an inverse correlation between serum 25(OH)D concentrations with FEV_1 and BMI has been reported [22]. Other reports described an association between VitD deficiency and the risk of CS resistance [23]. In PBMCs from steroid resistant patients, the active form of VitD, 1,25$(OH)_2$D, enhances DEX inhibition of cell proliferation [24], increasing the expression of MKP-1 [25]. Interestingly, leptin levels inversely correlated with the ability of 1,25$(OH)_2$D, to inhibit *in vitro* production of inflammatory cytokines [26].

J Investig Allergol Clin Immunol 2023; Vol. 33(6)
doi: 10.18176/jiaci.0861

Weight reduction significantly improves systemic and adipose tissue inflammatory activity levels [26,27]. Bariatric surgery (BS) is considered the most effective and sustained long-term treatment of severe obesity. Several studies have shown an improvement in asthma control, medication use, hospitalization rate, lung function, systemic inflammatory markers, and decreased mast cell numbers in the airways of OA after weight loss via BS [28–33].

We hypothesized that understanding the links between asthma, obesity, and VitD can help elucidate the mechanisms underlying ICS hyporesponsiveness in OA. A step that can also contribute to the development of therapies with VitD specifically designed for this often difficult to manage asthma phenotype.

The objective of this study is to demonstrate that the poor response to ICS in obese asthma is due to an impaired anti-inflammatory activity of CS associated with a VitD deficiency and that both deficiencies are corrected following BS-associated weight loss with VitD supplementation.

Subjects and methods

Subjects

We recruited thirty-seven asthma patients: 23 obese (all of them(OA) [BMI ≥ 30 kg/m^2] and 14 non-obese asthma (NOA) patients [BMI < 25 kg/m^2]. Fifteen obese (O) patients and 19 age- and gender-matched healthy control (HC) individuals were also recruited. Twenty-one O (10 OA and 11 O) were evaluated at baseline (V1) and six months after undergoing BS (V2). Obese patients were recruited from the Obesity Unit of our Institution. According to the standard procedure the patients were supplemented with VitD after BS.

The following criteria were used to select asthmatic patients: (1) a clinical history of asthma; and 2) either bronchodilator responsiveness (>12 % and 200 ml improvement in FEV$_1$ after 180 µg metered-dose inhaler salbutamol) or airway hyperresponsiveness (PC20 methacholine < 8 mg/ml). Forced spirometry and methacholine test were performed according to the ERS/ATS standards [34,35]. Reference values were those of Roca et al. [36,37]. None of the subjects had received systemic CS for one month or longer prior to evaluation. None of the subjects were current smokers. All of them were nonsmokers or ex-smokers for more than one year with tobacco history <10 packs/years.

J Investig Allergol Clin Immunol 2023; Vol. 33(6)
doi: 10.18176/jiaci.0861

Serum was obtained by peripheral venipuncture followed by centrifugation and stored at -80°C until analysis. Serum adiponectin and leptin levels were quantified by Luminex® multiplex immunoassay using Human ProcartaPlex Mix&Match kits (Thermo Fisher Scientific, Vienna, Austria) on a Luminex 200 analyser. Serum 25(OH)D concentrations were determined by a chemiluminescent immunoassay (Atellica™, Siemens).

The study was performed with written informed consent from participating subjects and approved by the Ethics Committee of Hospital Clínic of Barcelona (2018/4015).

Blood collection and PBMC isolation

Based on previous studies, we used PBMC proliferation to investigate CS response [38]. Ten ml of whole blood was collected from each participant into a vacutainer tube containing heparin. PBMCs were isolated using Lymphoprep™ (Palex Medical) following manufacturer's instructions. Cell viability was determined using Trypan blue staining and final cell concentration was 10 x 10^6 cells/ml.

Proliferation assay

CFSE dilution

Five hundred µl of cell suspension were incubated with 5 µM carboxyfluorescein diacetate succinimidyl ester (CFSE) for 5 min in the dark at room temperature. PBMCs were resuspended in X-Vivo15 medium (Cultek) containing gentamycin and supplemented with 10% charcoal stripped foetal bovine serum (FBS) (Sigma-Aldrich) at a final concentration of $2x10^6$ cells/ml.

Cell culture conditions

To assess cell proliferation suppression by DEX, 500 µl of labelled PBMCs were stimulated with 1 µg/ml of phytohemagglutinin-L (PHA-L) (Sigma-Aldrich) in the presence of a range of DEX (Kern Pharma) concentrations (10^{-11} – 10^{-5}M), as well as in the presence or absence of 1,25$(OH)_2$D at 10^{-7}M. Cultures were incubated at 37°C and 5% CO_2 for four days and the cell proliferation assessed based on CFSE dilution.

Flow cytometer analysis

PBMCs were transferred to flow cytometry round-bottom tubes and stained with Zombie Violet TM Fixable Viability Kit (BioLegend) and anti-CD4 APC antibody (BioLegend). Cells were analysed on a FACSCanto II flow cytometer (BD Biosciences) and % proliferation was recorded for PBMCs and CD4+

doi: 10.18176/jiaci.0861

T cells, strategically gated. Sensitivity to DEX was expressed as the half maximal inhibitory concentration (IC_{50}).

Analysis of gene expression by reverse transcription quantitative PCR (RT-qPCR)

Total RNA was isolated from PBMCs cultured (24 h) in the presence or absence of DEX at 10^{-9} M and with or without $1,25(OH)_2D$ at 10^{-7} M, using the TRIzol (Life Technologies) reagent according to the manufacturer's protocol. RNA was converted into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher).

MKP-1 mRNA expression was analysed by real-time qPCR using TaqMan probes in the Viia7 Real-Time PCR system (Applied Biosystems). The thermal cycler was set to 95°C for 20 min followed by 40 reaction cycles of 1 s at 95°C and 20 s at 60°C. All PCRs were run in duplicate for both target genes and two control genes (B2M, HPRT1).

Statistical analysis

Clinical and experimental data were reported as median and interquartile range. Differences between two groups were analysed using non-parametric tests: Mann-Whitney U test (unpaired data), Wilcoxon rank test (paired data), or Kruskal-Wallis H test for multiple comparisons. Correlation coefficients were calculated using Spearman's Rank method. All analyses were performed using GraphPad Prism version 8.4 for Windows, (GraphPad Software, La Jolla California, USA). Statistical significance was defined as P-value < 0.05.

Results

Subjects' characteristics at baseline

The participants' demographic and clinical characteristics are shown in Table 1. The level of severity of asthma (mild, moderate, severe) was established according to the pharmacological treatment used to control de disease [1,3]. The level of control was assessed using the asthma control test (ACT) [1,3]. OA patients presented reduced FVC and FEV_1, compared with HC, whereas the FEV_1/FVC ratio was significantly lower in NOA and OA participants in comparison with O and HC individuals. Serum 25(OH)D levels showed a tendency to be lower in O subjects than in HC subjects, however the difference did not reach statistical significance. The adiponectin/leptin ratio was significantly reduced in O and OA patients, compared with both HC and NOA individuals.

J Investig Allergol Clin Immunol 2023; Vol. 33(6)
doi: 10.18176/jiaci.0861

Relationship between obesity and CS sensitivity

To examine the link between obesity and CS response, DEX IC_{50} values were calculated in PBMCs and CD4+ T cells. IC_{50} values were significantly higher in OA patients compared with NOA patients when analysing both PBMCs and CD4+ T cells (Figure 1).

There were no differences in CS sensitivity between OA and O subjects. IC_{50} values were higher in O subjects with respect to the HC group. However, not all differences were statistically significant, which in part was dependent on the type of cells selected for the analysis (PBMCs vs. CD4+T cells) (Figure 1).

Relationship of adipokines and VitD with CS response

Analysis of the adiponectin/leptin ratio and CD4+ T cell sensitivity to CS *in vitro* showed a negative correlation between the adiponectin/leptin ratio and the DEX IC_{50} value (r= -0.4113, p= 0.0008). The higher the ratio, the greater the antiproliferative effect of DEX.

Moreover, we studied the association between serum 25(OH)D concentrations and DEX response *in vitro*. We observed a negative correlation between 25(OH)D concentrations and DEX IC_{50} values (r= -0.2512, p= 0.0346). The higher the VitD concentration, the greater the antiproliferative effects of DEX.

There was no correlation between adiponectin/leptin and 25(OH)D (data not shown). Similar results were found in PBMCs (data not shown).

If patients were separated according to the reference values established for serum 25(OH)D levels, a significant decrease in IC_{50} values was observed only in patients who presented VitD deficiency levels (< 12 ng/ml) (n=8, 4 OA and 4 O patients), compared with patients with sufficiency (>20 ng/ml) and, also with insufficiency levels (12 – 20 ng/ml) (Figure 2) [15].

Effects of weight loss after bariatric surgery

BS was performed in 21 subjects (10 OA and 11 O), in 8 of whom a sleeve gastrectomy was performed (38%), while in the remaining 13 (62%) a Roux-en-Y gastric bypass was carried out. Table 2 presents the clinical, functional, and inflammatory marker changes after BS. BMI was significantly reduced in both groups of subjects with obesity. Weight loss was associated with a significant improvement in the ACT scores and a reduction in the amount of therapy needed to control the disease. Lung function tests improved after BS and the adiponectin/leptin ratio increased significantly after BS in O and OA patients.

J Investig Allergol Clin Immunol 2023; Vol. 33(6)
doi: 10.18176/jiaci.0861

Serum 25(OH)D levels increased in both groups with obesity, with no subjects with deficient (<12 ng/ml) reference values, reflecting the effect of both BS and VitD supplementation.

Weight loss and CS sensitivity

To evaluate the impact of weight loss on CS sensitivity we compared the DEX IC_{50} in both groups with obesity before (V1) and after BS (V2) (Figure 3). DEX IC_{50} values diminished in both PBMCs and CD4+ T cells after BS in OA and O patients. However, the reduction was not statistically significant in CD4+ T cells from O subjects.

Effect of 1,25(OH)$_2$D on CD4+ T cell proliferation *in vitro*

The addition of 1,25(OH)$_2$D to culture media resulted in a significant reduction of PHA-stimulated CD4+ T cell proliferation without any statistically significant differences between the four groups. We found similar results in PBMCs (data not shown) (Figure 4).

Impact of 1,25(OH)$_2$D on CS sensitivity

To further assess the effects of VitD on the inhibitory ability of DEX, PBMCs were incubated in the presence of both DEX and 1,25(OH)$_2$D. 1,25(OH)$_2$D significantly increases the antiproliferative effects of DEX in CD4+ T cells. Although the effect appears to be greater in OA and O patients than in HC and NOA subjects, the difference was not statistically significant (Figure 5). We found the same results in PBMCs (data not shown).

MKP-1 gene expression

We analysed the expression of MKP-1 in PBMCs after stimulation with DEX for 24 h. As expected, DEX induced MKP-1 mRNA expression in PBMCs, which correlated with DEX sensitivity in PBMCs expressed as IC_{50} value ($r=0.4860$, $p=0.0075$). However, no differences were found between groups. We also examined the expression of MKP-1 in response to VitD and DEX. MKP-1 expression increased similarly in the four groups (data not shown). However, there were statistically significant differences in the induced expression of MKP-1 when 1,25(OH)$_2$D was added to DEX in subjects with obesity (Figure 6).

Discussion

Our study demonstrates that obese subjects with and without asthma are characterized by a reduced response to CS treatment, assessed by means of a PBMC proliferative assay.

J Investig Allergol Clin Immunol 2023; Vol. 33(6)
doi: 10.18176/jiaci.0861

The reduced response negatively correlates with serum adiponectin/leptin ratio, a marker associated with the level of inflammation in subjects with obesity. A similar negative correlation was observed between the antiproliferative effects of CS with VitD serum levels. Obese patients with VitD deficiency levels (<12 ng/ml) were those with the poorest antiproliferative response to CS.

Weight loss after BS was associated with a marked improvement in the reduced antiproliferative effects of CS in subjects with obesity. Weight loss was also associated with an increase in both the adiponectin/leptin ratio and serum VitD levels. After BS and VitD supplementation, there were no patients with obesity with VitD deficiency levels (<12 ng/ml). These observations suggest that reduction of obesity-related systemic inflammation and increased levels of serum VitD can account for the recovered ability of CS to inhibit PBMCs proliferation.

However, it is well known that association does not necessarily mean a causal relationship. To further investigate the potential causal relationship between VitD deficiency and CS hyporesponsiveness, we conducted an *in vitro* study to assess the effects of VitD on cell proliferation and found that the hormone exerts antiproliferative effects on cells by itself and that it is also capable of potentiating the antiproliferative effects of CS. Collectively these findings support the notion that VitD may play a role in the complex interaction between obesity, inflammation, and CS hyporesponsiveness.

In order to definitely establish the role of VitD deficiency in CS hyporesponsiveness in asthma, randomized clinical trials (RCTs) should be performed to evaluate the clinical efficacy of VitD administration in OA. Numerous RCTs studies, mostly in children, have been carried out to evaluate the efficacy of VitD administration in asthma patients. The trials enrolled patients with significant differences in the selection of participants, baseline VitD levels and dosage of VitD supplementation. Some systematic reviews of RCTs concluded that high-dose VitD may be effective in paediatric asthma [39]; however, others could not find significant evidence favouring the use of this complementary therapy in children and adults [40].

VitD supplementation studies in asthma face many unresolved issues. Thus, for example, studies on the anti-inflammatory and anti-oxidant effects of VitD suggest that the serum level of VitD that should be reached to be effective in asthma may be higher than that generally accepted for bone health [41]. In addition, it is unknown whether, due to the effect of excessive accumulation of fat on the distribution

doi: 10.18176/jiaci.0861

of VitD, OA patients should require a supplemental VitD dose higher than that required by NOA patients with VitD deficiency [41]. RCT studies tailored for this specific group of overweight/obese asthma patients are needed to elucidate the potential therapeutic effect of VitD supplementation that our findings suggest.

The present study also aimed to investigate the mechanisms responsible for CS hyporesponsiveness in asthmatic patients with obesity. Despite some progress made in the field, the mechanisms involved in CS insensitivity in patients with asthma remain to be fully understood [9]. Anti-inflammatory actions of CS are mediated by the glucocorticoid receptor alpha (GRα), which suppresses inflammation through transactivation and transrepression mechanisms [9]. In the presence of CS, GRα translocates into the nucleus to regulate the expression of various anti-inflammatory genes (transactivation) such as MKP-1 [9]. In our study, we examined this gene as previous studies found that the induced expression of MKP-1 by DEX is blunted in overweight/obese asthma patients compared with lean asthma subjects [11]. Interestingly, obesity in non-asthmatic subjects had no effect on MKP-1 expression, suggesting that, due to unclear reasons, the effect of obesity only impacts on MKP-1 expression in asthma patients [11]. In our study we did not find any significant difference in the DEX-dependent induction of MKP-1 in OA versus NOA. This finding suggests that obesity does not affect the capacity of CS to transactivate anti-inflammatory genes in PBMCs. Differences in methods and the characteristics of the populations studied might explain discrepancies between our study and that of Sutherland *et al.* [11]. In that study, the induction of MKP-1 was analysed against a single dose of DEX (10^{-6} M), while in our study it was analysed by means of a dose-dependent response, which is considered most suitable for evaluating pharmacologically-induced responses *in vitro*. Moreover, asthma severity has been shown to be closely related to the level of DEX-induced MKP-1 expression [38], and most patients enrolled in our study suffered from mild/moderate disease. In the Sutherland study [11], pulmonary function showed a more pronounced airway obstruction (FEV_1) in OA than in the present study (70% vs. 77%).

CS anti-inflammatory effects are explained by mechanisms other that activation of MKP-1, such as alterations in the nuclear translocation of GRα, increased expression of the GRβ isoform—a dominant negative regulator of active GRα—or inhibition of proinflammatory gene expression through blockade of proinflammatory transcription factors [9]. Interestingly, a very recent study found that dysregulation of the GRα/GRβ isoform ratio may contribute to CS hyporesponsiveness in OA [42].

J Investig Allergol Clin Immunol 2023; Vol. 33(6)
doi: 10.18176/jiaci.0861

We also examined the effects of VitD on the induction of MKP-1 in subjects with and without obesity, finding that the hormone similarly increases the expression of MKP-1, with some differences between obese and non-obese subjects, a finding that suggests that the efficacy of VitD to increase DEX-induction of anti-inflammatory genes is more pronounced in subjects with obesity.

As a complementary methodological contribution, we used both PBMCs and CD4+T cell proliferation to assess sensitivity to DEX treatment. The two methods have been used to examine CS sensitivity in previous studies [21,38,43]. We found some differences in the results obtained with PHA-induced PBMC and CD4+T cell proliferation in the response to CS, a finding that should be taken into account when results from different studies are compared.

Some of the limitations of the study include the relatively small sample size of patients examined and the short follow-up time of six months after BS. Patients were enrolled from the obesity program of our centre and, in all cases, BS was indicated to treat obesity, not asthma.

In summary, our study has several novel contributions that may help to better understand the links between obesity and poor response to ICS in adults with OA. Our findings support the role of obesity in ICS hyporesponsiveness and demonstrate that the efficacy of weight loss to improve asthma symptoms and lung function can be, at least in part, due to the recovered anti-inflammatory response to CS. Finally, our results suggest that VitD supplementation in asthma patients with obesity and VitD deficiency may contribute to achieving asthma control by improving ICS efficacy.

J Investig Allergol Clin Immunol 2023; Vol. 33(6)
doi: 10.18176/jiaci.0861

Funding

The study was supported by grants from the Fundación Respira (SEPAR) 167/2016 and 736/2018, Fundació Catalana de Pneumologia (FUCAP) and by an unrestricted grant from Menarini.

Conflict of interest

J. Mullol is a member of national or international advisory boards and has received speaker fees or funding for clinical trials and research projects from Allakos, AstraZeneca, Genentech, GSK, Glenmark, Menarini, Mitsubishi-Tanabe, MSD, Mylan-MEDA Pharma (Viatris), Novartis, Procter & Gamble, Regeneron Pharmaceuticals, Inc., Sanofi-Genzyme, UCB Pharma, and Uriach Group. Other authors declare that they have no conflicts of interest.

References

1. Papi A, Brightling C, Pedersen SE, Reddel HK. Asthma. Lancet. 2018;391:783–800.
2. Hammad H, Lambrecht BN. The basic immunology of asthma. Cell. 2021;184:1469–85.
3. Reddel HK, Bacharier LB, Bateman ED, Brightling CE, Brusselle GG, Buhl R, et al. Global Initiative for Asthma Strategy 2021 Executive Summary and Rationale for Key Changes. Am J Respir Crit Care Med. 2022;205:17–35.
4. Boulet LP, Franssen E. Influence of obesity on response to fluticasone with or without salmeterol in moderate asthma. Respir Med. 2007;101:2240–7.
5. Forno E, Lescher R, Strunk R, Weiss S, Fuhlbrigge A, Celedón JC. Decreased response to inhaled steroids in overweight and obese asthmatic children. J Allergy Clin Immunol. 2011;127:741–9.
6. Farah CS, Kermode JA, Downie SR, Brown NJ, Hardaker KM, Berend N, et al. Obesity is a determinant of asthma control independent of inflammation and lung mechanics. Chest. 2011;140:659–66.
7. Arismendi E, Bantulà M, Perpiñá M, Picado C. Effects of Obesity and Asthma on Lung Function and Airway Dysanapsis in Adults and Children. J Clin Med. 2020;9:3762.
8. Bantulà Fonts M, Roca-Ferrer J, Arismendi E, Picado C. Asthma and Obesity: Two Diseases on the Rise and Bridged by Inflammation. J Clin Med. 2021;10:1–21.
9. Barnes PJ. Corticosteroid resistance in patients with asthma and chronic obstructive pulmonary disease. J Allergy Clin Immunol. 2013;131:636–45.
10. Sideleva O, Suratt BT, Black KE, Tharp WG, Pratley RE, Forgione P, et al. Obesity and asthma: An inflammatory disease of adipose tissue not the airway. Am J Respir Crit Care Med. 2012;186:598–605.
11. Sutherland ER, Goleva E, Strand M, Beuther DA, Leung DYM. Body mass and glucocorticoid response in asthma. Am J Respir Crit Care Med. 2008;178:682–7.
12. Bouillon R, Marcocci C, Carmeliet G, Bikle D, White JH, Dawson-Hughes B, et al. Skeletal and Extraskeletal Actions of Vitamin D: Current Evidence and Outstanding Questions. Endocr Rev. 2019;40:1109.

doi: 10.18176/jiaci.0861

13. Dankers W, Colin EM, van Hamburg JP, Lubberts E. Vitamin D in Autoimmunity: Molecular Mechanisms and Therapeutic Potential. Front Immunol. 2017;7:697.
14. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: An endocrine society clinical practice guideline. J Clin Endocrinol Metab. 2011;96:1911–30.
15. Giustina A, Adler RA, Binkley N, Bollerslev J, Bouillon R, Dawson-Hughes B, et al. Consensus statement from 2nd International Conference on Controversies in Vitamin D. Rev Endocr Metab Disord. 2020;21:89–116.
16. Cashman KD. Vitamin D Deficiency: Defining, Prevalence, Causes, and Strategies of Addressing. Calcif Tissue Int. 2020;106:14–29.
17. Afzal S, Brøndum-Jacobsen P, Bojesen SE , Nordestgaard BG. Vitamin D concentration, obesity, and risk of diabetes: a mendelian randomisation study. Lancet Diabetes Endocrinol. 2014;2:298–306.
18. Vimaleswaran KS, Berry DJ, Lu C, Tikkanen E, Pilz S, Hiraki LT, et al. Causal relationship between obesity and vitamin D status: bi-directional Mendelian randomization analysis of multiple cohorts. PLoS Med. 2013;10:e1001383.
19. Drincic AT, Armas LAG, Van Diest EE, Heaney RP. Volumetric dilution, rather than sequestration best explains the low vitamin D status of obesity. Obesity (Silver Spring). 2012;20:1444–8.
20. González-Molero I, Rojo-Martínez G, Morcillo S, Gutierrez C, Rubio E, Pérez-Valero V, et al. Hypovitaminosis D and incidence of obesity: a prospective study. Eur J Clin Nutr. 2013;67:680–2.
21. Szentpetery SE, Han YY, Brehm JM, Acosta-Pérez E, Forno E, Boutaoui N, et al. Vitamin D insufficiency, plasma cytokines, and severe asthma exacerbations in school-aged children. J allergy Clin Immunol Pract. 2018;6:289-91.
22. Sutherland ER, Goleva E, Jackson LP, Stevens AD, Leung DYM. Vitamin D Levels, Lung Function, and Steroid Response in Adult Asthma. Am J Respir Crit Care Med. 2010;181:699–704.

doi: 10.18176/jiaci.0861

23. Devereux G, Wilson A, Avenell A, McNeill G, Fraser WD. A case–control study of vitamin D status and asthma in adults. Allergy. 2010;65:666–7.
24. Searing DA, Zhang Y, Murphy JR, Hauk PJ, Goleva E, Leung DYM. Decreased Serum Vitamin D Levels in Children with Asthma are Associated with Increased Corticosteroid Usage. J Allergy Clin Immunol. 2010;125:995.
25. Zhang Y, Leung DYM, Goleva E. Anti-inflammatory and Corticosteroid Enhancing Actions of Vitamin D in the Monocytes of Steroid Resistant and Steroid Sensitive Asthmatics. J Allergy Clin Immunol. 2014;133:1744.
26. Dias ASO, Santos ICL, Delphim L, Fernandes G, Endlich LR, Cafasso MOSD, et al. Serum leptin levels correlate negatively with the capacity of vitamin D to modulate the in vitro cytokines production by CD4 + T cells in asthmatic patients. Clin Immunol. 2019;205:93–105.
27. Arismendi E, Rivas E, Agustí A, Ríos J, Barreiro E, Vidal J, et al. The Systemic Inflammome of Severe Obesity before and after Bariatric Surgery. Thatcher TH, editor. PLoS One. 2014;9:e107859.
28. Van Huisstede A, Rudolphus A, Cabezas MC, Biter LU, Van De Geijn GJ, Taube C, et al. Effect of bariatric surgery on asthma control, lung function and bronchial and systemic inflammation in morbidly obese subjects with asthma. Thorax. 2015;70:659–67.
29. Maniscalco M, Zedda A, Faraone S, Cerbone MR, Cristiano S, Giardiello C, et al. Weight loss and asthma control in severely obese asthmatic females. Respir Med. 2008;102:102–8.
30. Reddy RC, Baptist AP, Fan Z, Carlin AM, Birkmeyer NJO. The effects of bariatric surgery on asthma severity. Obes Surg. 2011;21:200–6.
31. van Huisstede A, Rudolphus A, van Schadewijk A, Cabezas MC, Mannaerts GHH, Taube C, et al. Bronchial and Systemic Inflammation in Morbidly Obese Subjects with Asthma: A Biopsy Study. Am J Respir Crit Care Med. 2014;190:951–4.
32. Dixon AE, Pratley RE, Forgione PM, Kaminsky DA, Whittaker-Leclair LA, Griffes LA, et al. Effects of obesity and bariatric surgery on airway hyperresponsiveness, asthma control, and inflammation. J Allergy Clin Immunol. 2011;128:508-15.
33. Boulet LP, Turcotte H, Martin J, Poirier P. Effect of bariatric surgery on airway response and lung function in obese subjects with asthma. Respir Med. 2012;106:651–60.

J Investig Allergol Clin Immunol 2023; Vol. 33(6)
doi: 10.18176/jiaci.0861

34. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. Standardisation of spirometry. Eur Respir J. 2005;26:319–38.

35. Coates AL, Wanger J, Cockcroft DW, Culver BH, Carlsen KH, Diamant Z, et al. ERS technical standard on bronchial challenge testing: General considerations and performance of methacholine challenge tests. Eur Respir J. 2017;49:1–17.

36. Roca J, Burgos F, Barberà JA, Sunyer J, Rodriguez-Roisin R, Castellsagué J, et al. Prediction equations for plethysmographic lung volumes. Respir Med. 1998;92:454–60.

37. Roca J, Burgos F, Sunyer J, Saez M, Chinn S, Antó JM, et al. References values for forced spirometry. Eur Respir J. 1998;11:1354–62.

38. Goleva E, Jackson LP, Gleason M, Leung DYM. Usefulness of PBMCs to predict clinical response to corticosteroids in asthmatic patients. J Allergy Clin Immunol. 2012;129:687-93.

39. Martineau AR, Cates CJ, Urashima M, Jensen M, Griffiths AP, Nurmatov U, et al. Vitamin D for the management of asthma. Cochrane Database Syst Rev. 2016;9:CD11511.

40. Kumar J, Kumar P, Goyal JP, Thakur C, Choudhary P, Meena J, et al. Vitamin D supplementation in childhood asthma: a systematic review and meta-analysis of randomised controlled trials. ERJ Open Res. 2022;8:662.

41. O'Sullivan BP, James L, Majure JM, Bickel S, Phan LT, Serrano Gonzalez M, et al. Obesity-related asthma in children: A role for vitamin D. Pediatr Pulmonol. 2021;56:354–61.

42. Al Heialy S, Gaudet M, Ramakrishnan RK, Mogas A, Salameh L, Mahboub B, et al. Contribution of IL-17 in Steroid Hyporesponsiveness in Obese Asthmatics Through Dysregulation of Glucocorticoid Receptors α and β. Front Immunol. 2020;11:1724.

43. Koetzier SC, Van Langelaar J, Blok KM, Van Den Bosch TPP, Wierenga-Wolf AF, Melief M-J, et al. Brain-homing CD4 + T cells display glucocorticoid-resistant features in MS. Neurol Neuroimmunol Neuroinflamm. 2020;7:894.

doi: 10.18176/jiaci.0861

TABLES

Table 1. Baseline demographic and clinical data of the study population.

	HC (*n*, 19)	**NOA (*n*, 14)**	**OA (*n*, 23)**	**O (*n*, 15)**
Age, years	43 (34-56)	53 (43-60)	56 (51-59)	47 (45-60)
Female, *n* (%)	14 (74)	11 (79)	18 (78)	13 (87)
BMI, kg/m2	23.7 (22.2-25-4)	23.2 (22.3-25.3)	37.6 (34.5-45) *#	42.7 (39.5-48.5) *#
Mild asthma, *n* (%)	-	0 (0)	4 (17.4)	-
Moderate asthma, *n* (%)	-	5 (35.7)	5 (21.7)	-
Severe asthma, *n* (%)	-	9 (64.3)	14 (60.9)	-
FVC, % pred	101.0 (95.0-107.0)	97.5 (89.7-107.3)	84.0 (73.0-93.0) *#	93.0 (82.7-96.2)
FEV_1, % pred	102.0 (95.0-109.0)	80.0 (75.2-100.3)	80.0 (62.0-95.0) *	90.0 (86.2-101.0)
FEV_1/FVC	81.0 (76.0-84.0)	65.5 (57.0-75.0) *	77.0 (69.0-82.0)	81.5 (75.0-83.0) #
ICS [§], *n* (%)	-	13 (92.9)	19 (82.6)	-
Eosinophils, %	2.3 (1.6-3.3) #	4.7 (3.6-7.1)	3.5 (2.4-5)	2.3 (3.4-1.2) #
IgE, kU/L	26.9 (16.5-80.2)	122 (34.8-391)	67.1 (19.43-278.8)	38 (15.43-107.5)
25(OH)D, ng/ml	20.8 (17.5-28.8)	20.1 (14.1-24.9)	20.0 (14.7-31.9)	16.4 (11.7-27.5)
Adiponectin, µg/ml	11.9 (5.3-16.0)	12.3 (7.7-21.8)	5.8 (3.6-8.4) #	4.3 (4.0-9.0) #
Leptin, ng/ml	1.7 (1.0-2.5)	3.4 (2.2-4.8)	3.6 (1.8-6.1) *	4.1 (2.7-6.3) *
Adipo/Lept	6.7 (2.1-8.6)	4.6 (1.9-6.5)	1.3 (0.9-2.9) *#	1.1 (0.7-2.7) *#

Data are presented as median (interquartile range). HC, healthy controls; NOA, non-obese asthmatic patients; OA, obese asthmatic patients; O, obese patients; BMI, body mass index; FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 s; ICS, inhaled corticosteroids; IgE, immunoglobulin E; 25(OH)D, 25-hydroxyvitamin D; Adipo/Lept, adiponectin/leptin ratio. *P < 0.05, compared with HC; # P < 0.05, compared with NOA subjects; Kruskal-Wallis followed by Dunn's multiple comparisons test. [§] For NOA and OA patients who received ICS, the mean ± SD of the ICS dose in budesonide equivalents was 557.1 ± 256.6 and 1222.1 ± 862.9 µg/day, respectively.

doi: 10.18176/jiaci.0861

Table 2. Demographic and clinical data of asthmatic and non-asthmatic obese patients before (V1) and six months after (V2) BS.

	Obese asthmatic patients (*n*, 10)		Obese patients (*n*, 11)	
	V1	V2	V1	V2
Female, *n* (%)	10 (100)	10 (100)	10 (91)	10 (91)
BMI, kg/m2	44.1 (38.7-47.1)	30.0 (26.4-34.9) *	41.9 (37.3-45.7)	29.7 (27.4-34.6) #
Mild asthma, *n* (%)	4 (40)	9 (90)	-	-
Moderate asthma, *n* (%)	4 (40)	1 (10)	-	-
Severe asthma, *n* (%)	2 (20)	0 (0)	-	-
ACT	18 (18-24)	25 (25-25) *	-	-
FVC, % pred	85.5 (77.5-96.0)	95.0 (88.3-106.0)	94.5 (85.3-98.0)	99.0 (91.0-106.0)
FEV_1, % pred	89.0 (79.5-97.3)	96.5 (92.5-104.8)	93.5 (87.0-101.0)	101.0 (89.0-109.0) #
FEV_1/FVC	80.5 (76.5-82.5)	81.5 (77.5-83.0)	81.0 (75.0-83.0)	79.0 (76.0-83.0)
ICS [§], *n* (%)	6 (60)	2 (20)	-	-
Eosinophils, %	3.0 (2.0-3.7)	2.6 (1.4-3.3)	2.8 (1.6-3.6)	2.5 (1.8-2.8)
IgE, kU/L	32.3 (12.4-113.3)	33.8 (7.6-52.1)	51.7 (16.8-115)	28.3 (14.4-62.8)
25(OH)D, ng/ml	25.2 (11.9-43.2)	31.7 (20.6-38.3)	18.8 (10.1-30.6)	37.7 (23.2-41.8) #
Adiponectin, µg/ml	7.0 (3.5-9.5)	9.4 (4.8-20.6) *	5.6 (4.0-10.6)	9.8 (6.0-12.1)
Leptin, ng/ml	5.0 (3.2-8.3)	2.3 (0.8-4.7)	4.3 (3.3-7.2)	2.5 (1.3-3.4) #
Adipo/Lept	1.2 (0.7-2.0)	4.7 (1.2-43.2) *	1.1 (0.7-2.7)	4.2 (2.7-6.9) #

Data are presented as median (interquartile range). ACT, asthma control test; BMI, body mass index; FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 s; ICS, inhaled corticosteroids; IgE, immunoglobulin E; 25(OH)D, 25-hydroxyvitamin D3; Adipo/Lept, adiponectin/leptin ratio. *$P < 0.05$, compared between V1 and V2 in obese asthmatic group, # $P < 0.05$ compared between V1 and V2 in obese group; Wilcoxon test. [§] For OA patients before and after bariatric surgery who received ICS, the mean ± SD of the ICS dose in budesonide equivalents was 643.3 ± 496.1 and 150.0 ± 70.7 µg/day, respectively.

J Investig Allergol Clin Immunol 2023; Vol. 33(6)
doi: 10.18176/jiaci.0861

FIGURES

Figure 1. DEX IC_{50} values from HC, NOA, OA, and O participants in both PBMCs and CD4+ T cells. Data are presented as median with interquartile range. PBMCs, peripheral blood mononuclear cells; DEX IC_{50}, dexamethasone half maximal inhibitory concentration; HC, healthy controls; NOA, non-obese asthmatics; OA, obese asthmatics; O, obese. $^*P < 0.05$, Mann-Whitney test.

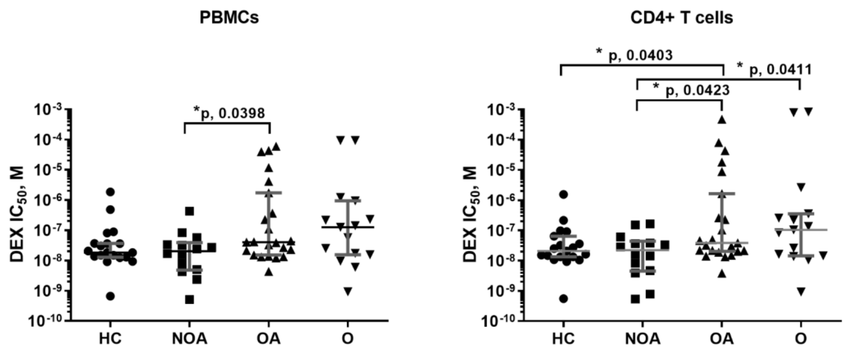


Figure 2. Patients classified accordingly to 25(OH)D serum levels and *in vitro* CS response. DEX IC_{50}, dexamethasone half maximal inhibitory concentration; 25(OH)D, 25-hydroxyvitamin D. ***P < 0.001; Mann-Whitney test.

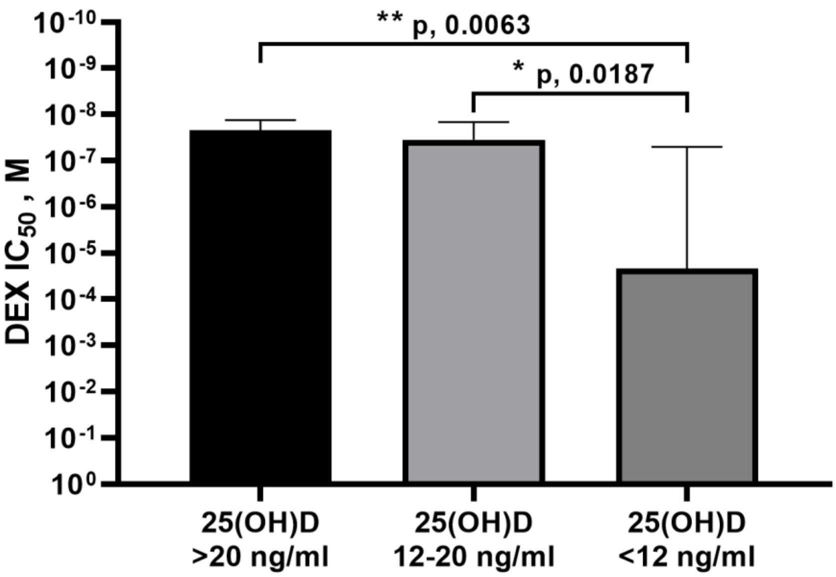


Figure 3. Comparison between DEX IC_{50} values from OA and O groups before (V1) and after (V2) BS in both PBMCs and CD4+ T cells. PBMCs, peripheral blood mononuclear cells; DEX IC_{50}, dexamethasone half maximal inhibitory concentration; OA, obese asthmatic; O, obese. *P < 0.05, **P < 0.01; Wilcoxon test between V1 and V2.

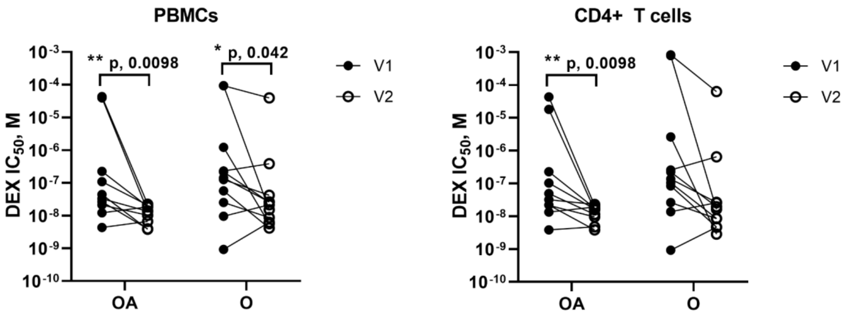
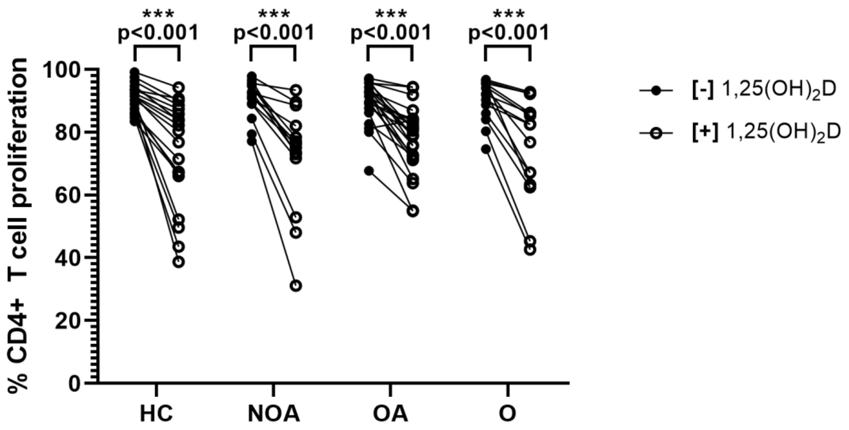
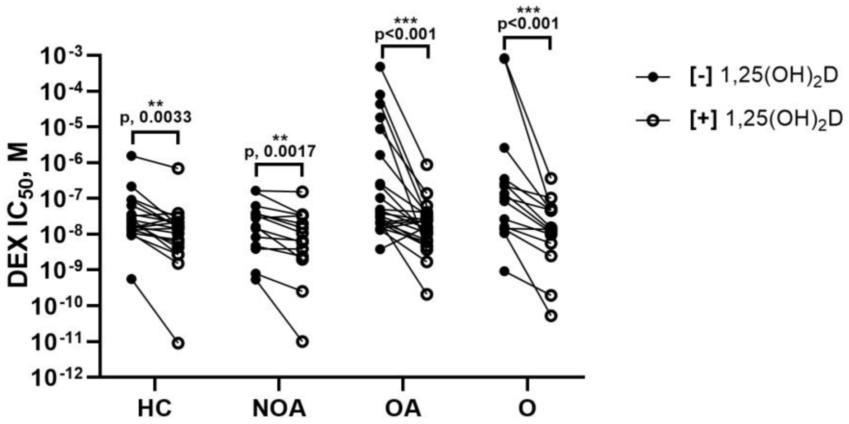


Figure 4. Inhibition of CD4+ T cell proliferation by $1,25(OH)_2D$ in HC, NOA, OA, and O participants. Solid circles represent cells without treatment and open circles represent cells treated *in vitro* with $1,25(OH)_2D$ $1x10^{-7}$ M. $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; HC, healthy controls; NOA, non-obese asthmatics; OA, obese asthmatics; O, obese. ***P < 0.001; Wilcoxon test.



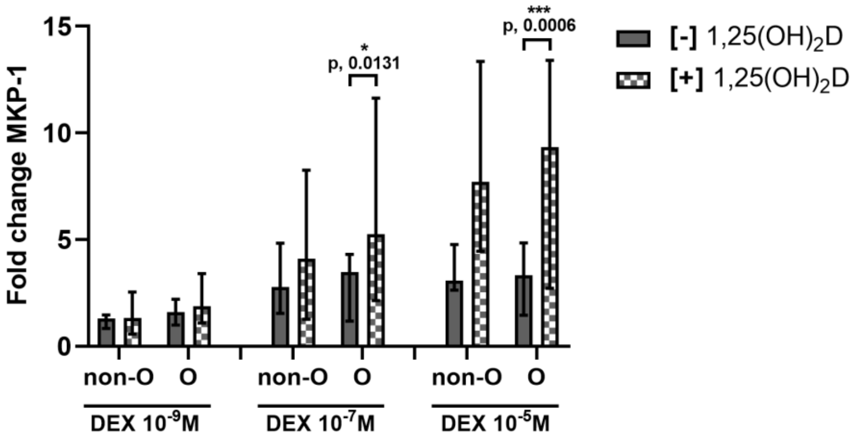
doi: 10.18176/jiaci.0861

Figure 5. Comparison between CS sensitivity in CD4+ T cells with the absence (solid circles) or presence (open circles) of 1,25(OH)$_2$D. 1,25(OH)$_2$D, 1,25-dihydroxyvitamin D; HC, healthy controls; NOA, non-obese asthmatics; OA, obese asthmatics; O, obese. **P < 0.01, ***P < 0.001; Wilcoxon test.



J Investig Allergol Clin Immunol 2023; Vol. 33(6)
doi: 10.18176/jiaci.0861

Figure 6. DEX-induced MKP-1 expression with (chequered columns) and without (solid columns) $1,25(OH)_2D$ comparison in non-obese and obese groups. Data are presented as median with interquartile range. DEX, dexamethasone; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; non-O, non-obese group (healthy controls and non-obese asthmatic subjects); O, obese group (obese asthmatic and obese subjects). *P < 0.05; ***P < 0.001; Wilcoxon test.



J Investig Allergol Clin Immunol 2023; Vol. 33(6)
doi: 10.18176/jiaci.0861

2. Global summary of results

In this doctoral thesis we evaluated systemic inflammation and glucocorticoid responsiveness in asthmatic patients with obesity before and after weight loss induced by bariatric surgery.

On the one hand, the specific objective which aimed to explore the implication of low-grade systemic inflammation in subjects with obesity, resulted in one publication:

Bantula M *et al*. **Asthma and Obesity: Two Diseases on the Rise and Bridged by Inflammation.** J Clin Med. 2021;10(2):169. doi: 10.3390/jcm10020169.

This study investigated the inflammatory serum mediator profile in 21 obese asthmatics, 14 non-obese asthmatics, 35 obese non-asthmatics, and 33 healthy subjects. Compared with healthy subjects, the obese asthmatic group presented increased levels of MCP-1, ezrin, YKL-40, and IL-18, the non-obese asthmatic group had increased levels of ezrin, YKL-40, IL-5, and IL-18, and the obese non-asthmatic group showed increased levels of leptin, TGF-1, TNFR2, MCP-1, ezrin, YKL-40, and ST2. The adiponectin/leptin ratio was higher in non-obese asthmatics with respect to asthmatic subjects with obesity, and IL-9 was the only cytokine with significantly higher levels in asthmatic patients with obesity with respect to their non-asthmatic counterparts.

On the other hand, we analysed the negative impact of obesity on asthma response to glucocorticoids and the vitamin D implications, which resulted in two publications:

Bantula M *et al*. **Weight loss and vitamin D improve hyporesponsiveness to corticosteroids in obese asthma.** J Investig Allergol Clin Immunol. 2023;33(6). doi: 10.18176/jiaci.0861.

In this study, 23 obese asthmatics, 14 non-obese asthmatics, 15 obese non-asthmatics, and 19 healthy subjects were enrolled. Lung function tests and serum levels of leptin, adiponectin, and vitamin D were quantified. Corticosteroid response was measured by dexamethasone inhibition of PBMCs and $CD4^+$ T cell proliferation, and MKP-1 gene expression was also analysed. We found a reduced response to dexamethasone in PBMCs from obese subjects with and without asthma with respect to non-obese asthmatics and healthy subjects, that inversely correlated with the adiponectin/leptin ratio. Moreover, vitamin D deficiency was associated with less i*n vitro* sensitivity to glucocorticoids and exposure of PBMCs to vitamin D potentiated the anti-proliferative effects of corticosteroids. Dexamethasone and vitamin D induced similar MKP-1 expression in both groups with obesity.

Bantulà M, *et al*. **Reference Gene Validation for RT – qPCR in PBMCs from Asthmatic Patients with or without Obesity.** Methods Protoc. 2022;5(3):35. doi: 10.3390/mps5030035.

This study aimed to select the most appropriate reference genes to perform RT-qPCR studies analysing glucocorticoid-induced gene expression in PBMCs from asthmatic patients with obesity. We identified HPRT1 and B2M as the most stable reference genes and ACTB, TBP, and GAPDH as the more unstable ones in PBMCs from asthmatic patients with obesity incubated with dexamethasone and/or vitamin D.

Finally, the effects of weight loss after bariatric surgery on systemic inflammation and on PBMCs sensitivity to glucocorticoids is also discussed in these recently published manuscripts. In both studies we found that BMI was significantly reduced after six to twelve months of surgery. Weight loss was associated with a significant improvement in the asthma control test (ACT) scores and lung function tests. Moreover, a reduction in the amount of therapy needed to control the disease was observed.

In the first study, we found that weight loss had no statistically significant effect on serum cytokine levels in asthmatic subjects with obesity. However, leptin, TNFR2, ezrin, MCP-1, and IL-18 concentrations significantly decreased and adiponectin/leptin ratio increased in non-asthmatic subjects with obesity after bariatric surgery. In the second study, bariatric surgery improved *in vitro* glucocorticoid responses in obese subjects with and without asthma. Furthermore, vitamin D serum levels increased in both groups after bariatric surgery, reflecting the effect of both bariatric surgery and vitamin D supplementation.

The efficacy of weight loss improving symptoms and lung function in asthmatic patients with obesity can be, at least in part, due to the reduction of systemic inflammation, and therefore, recovered anti-inflammatory effects of glucocorticoids.

3. Supervisor reports

Supervisor's report on impact factor

The doctoral thesis *"Asthma and severe obesity. Glucocorticoid sensitivity before and after bariatric surgery. Effect of vitamin D."* is based on the original results obtained by Marina Bantulà Fonts. These results have been published or have been submitted to international peer reviewed journals. The impact factors of these journals demonstrate the quality of the research, as follows:

1. **Reference gene validation for RT–qPCR in PBMCs from asthmatic patients with or without obesity**, published in *Methods and Protocols* journal.

Methods and Protocols is an international, peer-reviewed, open access journal devoted to the publication of new procedural approaches and cutting-edge methodological developments. The ultimate objective of this scientific communication is to provide researchers with an indispensable tool, enabling better use of the latest scientific technologies. It is ranked by Journal Citation Indicator in the 67 position out of a total of 84 on the area of Biochemical Research Methods.

2. **Differences in inflammatory cytokine profile in obesity-associated asthma: effects of weight loss**, published in *Journal of Clinical Medicine*.

Journal of Clinical Medicine is an international, peer-reviewed, open access journal of clinical medicine, published semimonthly online by MDPI. The journal focuses on all topics related to clinical and pre-clinical research. Manuscripts are blindly reviewed. It is indexed in Journal Citation Reports with a current impact factor of 4.964 and classified in the first tercile of the area of Medicine, General and Internal (ranking: 54/172).

3. **Weight loss and vitamin D improve hyporesponsiveness to corticosteroids in obese asthma**, published in *Journal of Investigational Allergology and Clinical Immunology.*

Journal of Investigational Allergology and Clinical Immunology (JIACI) is an official journal of the Spanish Society of Allergology and Clinical Immunology (SEAIC). The Editorial Board includes internationally prominent individuals who are devoted to the optimal treatment of allergies and immunological disorders. It is indexed in Journal Citation Reports with a current impact factor of 8.185 and classified in the first tercile of the area of Allergy (ranking: 5/27), as well as of the area of Immunology (ranking: 37/161).

I hereby confirm the quality of the published and submitted articles.

EBYMAR ARISMENDI NUÑEZ / num:08463622

Firmado digitalmente por EBYMAR ARISMENDI NUÑEZ / num:08463622
Fecha: 2022.10.28 13:20:23 +02'00'

Signed by **Dra Ebymar Arismendi**

Barcelona, September 2022

Supervisor's report on the PhD candidate contribution to the articles

Dra Ebymar Arismendi Núñez, specialist of the Pulmonology Department in the Hospital Clínic, in charge of Pulmonary Function Lab and the Multidisciplinary Severe Asthma Unit and supervisor of the present doctoral thesis by Marina Bantulà Fonts, hereby certifies that none of the coauthors of the three presented articles, have used these publications for a doctoral thesis, and that the participation of the applicant in these articles included the following tasks:

- Participation in the conception and design of the studies.
- Experimental work.
- Statistical analyses and interpretation of data.
- Writing the manuscripts.
- Critical revision of the articles for intellectual content.

EBYMAR ARISMENDI NUÑEZ / num:08463622

Firmado digitalmente por EBYMAR ARISMENDI NUÑEZ / num:08463622
Fecha: 2022.09.21 11:08:53 +02'00'

Signed by Dra **Ebymar Arismendi**

Barcelona, September 2022

DISCUSSION

This doctoral thesis demonstrates that asthmatic and non-asthmatic subjects with obesity, characterized by adipose tissue dysfunction and low-grade systemic inflammation, show a reduced response to glucocorticoid treatment assessed by means of a PBMCs proliferation assay. To our knowledge, this is the first report evaluating *in vitro* glucocorticoid response and vitamin D effects in asthmatic patients with obesity before and after bariatric surgery.

Asthmatic subjects with obesity presented a distinct pattern of systemic inflammation than those without obesity or without asthma, where leptin and adiponectin were also up-regulated in asthma via a mechanism that is independent of obesity. Moreover, we demonstrated that obesity is playing a role in glucocorticoid hyporesponsiveness. Furthermore, vitamin D supplementation in asthma patients with obesity and vitamin D deficiency may contribute to achieve asthma control by improving glucocorticoids efficacy. Weight loss though bariatric surgery reduced systemic inflammation in obese subjects, improved asthma symptoms and lung function and increased *in vitro* glucocorticoid sensitivity. Finally, we found stable reference genes to be able to use them in further studies regarding glucocorticoid sensitivity and vitamin D effect on PBMCs from asthmatic patients with obesity.

1. Systemic inflammation

Although asthma with obesity is a recognized phenotype [5], biomarkers to measure airway inflammation are lacking because the mechanisms underlying this inflammation remain unclear and poorly understood. Biomarkers play an essential role in asthma today, and must be analysed in all patients to determine the inflammatory profile, classify them according to endotypes, and help not only decide between different therapeutic options, but also to predict response to these treatments.

In the first study, we hypothesized that the low-grade chronic systemic inflammation present in obesity results in a spill over of pro-inflammatory systemic products from the inflamed adipose tissue, which contributes, via additive or synergistic mechanisms, towards increasing the systemic/airway inflammatory process underlying asthma. Weight loss after bariatric surgery may counteract low-grade systemic inflammation in subjects with obesity.

This study presented three main findings: 1) Leptin and adiponectin are regulated in asthma by both obesity-dependent and -independent mechanisms, the adiponectin/leptin ratio being statistically significant difference between asthma patients with and without obesity; 2) Obesity is associated with a systemic inflammation that is more active than that associated with asthma; however, in our study, the combination of asthma and obesity does not result in additive effects on circulating cytokine levels except for IL-9; 3) IL-5 levels were found significantly elevated in asthmatic patients without obesity with respect to subjects with obesity and healthy controls.

1.1. Adipokines before and after bariatric surgery

Leptin and adiponectin are biomarkers frequently used to assess the relationship between the inflammation of obesity and that of asthma. However, changes in these two markers are difficult to interpret as their systemic levels are influenced by asthma, independently of obesity [44,274–278]. In line with this observation, we found that leptin and adiponectin in asthma are regulated by obesity-dependent and -independent mechanisms.

Leptin is a protein synthesized and secreted mainly by the adipose tissue [279] and plays a key role in the regulation of appetite and body weight [280]. In keeping with previous observations [274,275], our study also found higher leptin levels in asthma patients and obese subjects than in healthy control individuals. We found significant correlations between leptin serum levels with both BMI and FEV_1 (%). However, no clear additive effects of obesity on leptin levels in asthma were detected. Weight loss was associated with a marked decrease of leptin levels in obese subjects, but it had inconsistent effects in obese asthmatics, possibly reflecting, at least in part, the independent role played by asthma in leptin levels.

Adiponectin plays a key role in the regulation of insulin sensitivity and energy homeostasis [281]. The relationship between asthma, with and without obesity, and serum adiponectin levels has been examined in epidemiological and cross-sectional studies, yielding contradictory results that are difficult to interpret due to the heterogeneity of the studies [281]. Our results showed that asthma was associated with increased adiponectin levels, a finding previously published in some [44,276,277] but not all studies [282]. Regarding the inhibitory role of obesity in adiponectin levels in asthma and non-asthmatic subjects, our results partially agree with some works [282–284], but not all [285]. The heterogeneity of the study populations (adults *vs* children), as well as the differences in BMI and asthma severity of the subjects enrolled in the studies could account for the observed discrepancies between studies. Childhood-onset obese asthma and obese asthma in adults share some similarities but also show substantial differences [286,287]. Serum adiponectin levels negatively correlated with BMI [282,288], and low adiponectin levels were associated with poor asthma control [285] and asthma severity [285,289]. Studies assessing the effects of bariatric surgery found that weight loss was associated with the recovery of serum adiponectin levels in obese [57] and obese asthma [251,277]. Interestingly, the balance of the adiponectin/leptin inflammatory ratio leaned towards normal in asthmatic patients compared with obese asthmatic and non-asthmatic subjects, in whom the ratio tended towards the pattern favouring inflammation as Frühbeck *et al*. recently proposed the adiponectin/leptin ratio as a reliable biomarker of adipose tissue dysfunction [80]. The adiponectin/leptin ratio negatively correlated with BMI and positively correlated with FEV_1 (%), and weight loss was associated with a normalization of the ratio in obese subjects.

Taken together, all these findings reveal the key role played by adipokines in asthma and their interactions in the regulation of systemic inflammation in obese asthma.

1.2. Comparison of serum inflammatory markers between healthy controls and the asthma and obesity groups.

In addition to analysing leptin and adiponectin levels, twenty-one other makers of systemic inflammation have been assessed. Some of them could not be detected in basal serum quantification because their concentration was below the detection limit of the used technique and, therefore, could not be accurately assessed. The non-detected cytokines were IL-17A, IL-6, IL-8, IL-10, IL-13, IL-33, IL-1β, IL-4, IFN-γ, TSLP, granulocyte-macrophage colony-stimulating factor (GM-CSF), and TNF-α receptor 1 (TNFR1). One possible explanation to not detecting these cytokines, is that we used basal serum from peripheral blood instead of adipose tissue samples, bronchial biopsies, or cell culture supernatants from stimulated cells, where the cytokine production is higher. For example, it is known that IL-10 and IL-17 are produced by mucosal-associated invariant T cells, in adipose tissue, but not in circulation [290]. Another possible explanation is that the dilution of the samples we used was too high, we would need to concentrate the samples and try to detect these cytokines. Other authors analysed IL-1β, IL-6, IL-8, TNF-α, GM-CSF, and MCP-1, and found contradictory results [44,251,277].

We found that obesity is associated with a systemic inflammation which is more active than that associated with asthma. The circulating concentration levels of six molecules (TGF-β1, TNFR2, MCP-1, ST2, ezrin, and YKL-40) were found to be significantly higher in subjects with obesity with respect to healthy controls. The asthmatic patients with obesity also showed significantly increased levels of four mediators compared with healthy controls: MCP-1, ezrin, YKL-40, and IL-18. Finally, in the serum of non-obese asthmatics, four molecular products also had significantly higher concentrations compared with healthy controls: ezrin, YKL-40, IL-5, and IL-18.

TGF-β1 levels increase in parallel with increased adipose body mass tissue, suggesting a role for this cytokine in obesity-related diseases [291,292]. High-fat diet-induced obesity in animal models is associated with increased TGF-β1 signalling expression in the bronchial epithelium resulting in the release of inflammatory mediators and fibrosis [293]. Moreover, serum TGF-β1 levels have been found elevated in asthma [294] and TGF-β1 expression has also been reported as elevated in both structural and inflammatory cells derived from the airways of asthma patients [292,293,295,296]. Induced expression of the TGF-β pathway in lungs promotes recruitment of cells such as eosinophils, neutrophils, macrophages, mast cells, and fibroblasts [292,294,295,297,298]. TGF-β1 also induces IL-8 production in human airway smooth muscle cells [299]. Collectively, these observations suggest that TGF-β1 may play an amplifying inflammatory and remodelling role in the airways of obese asthma patients. We found higher elevated serum levels in subjects with obesity, but obesity did not increase serum TGF-β1 levels in asthmatic patients with obesity, and in contrast to previous studies in patients with severe asthma [294], we did not find any differences between asthma and healthy control individuals, probably because most of our patients had a well-controlled disease.

TNF-α plays relevant regulatory roles in the process of inflammation and healing [300]. TNF-α can bind to two receptors, TNFR1 and **TNFR2**, which differ in their cellular expression and inflammatory potential [301,302]. Serum TNF-α has been found to be significantly higher in obese subjects compared with healthy individuals [303]. Similarly, serum TNF-α levels have also been reported as elevated in the serum and bronchoalveolar lavage fluid of patients with severe compared with mild asthma and healthy controls [304]. In addition, previous studies have shown that TNF-α signalling via TNFR2 may promote AHR [305]. We found that the serum levels of TNFR2 were significantly higher in obese subjects than in healthy and asthma individuals, with no additive effect between asthma and obesity. TNFR2 positively correlated with BMI. The significant and marked decrease in serum TNFR2 levels after bariatric surgery suggests that the demonstrated beneficial effects of weight loss on AHR [178] may be due, at least in part, to the reduction of the expression of the receptor mainly involved in TNF-α-related AHR.

MCP-1 is a member of the chemokine families, and appears to play a relevant role in asthma pathogenesis because of its ability to attract monocytes and eosinophils, as well as activate mast cells and release leukotriene C4 into the airway, resulting in AHR [306,307]. Elevated MCP-1 expression has been demonstrated in the bronchial epithelium of asthmatic patients [308], which increases even further preceding asthma exacerbation [309]. Neutralization of MCP-1 significantly inhibits T cell and eosinophil recruitment to the lung and reduces AHR [310]. One of the most common immune cells infiltrating adipose tissues are macrophages, and their recruitment to adipose tissue is promoted by MCP-1 secreted by the adipose tissue itself [311]. In our study, MCP-1 was found to be significantly higher in obese subjects with and without asthma with respect to healthy controls. Moreover, there was a positive correlation between MCP-1 levels and BMI. A significant decrease in MCP-1 levels was seen with weight loss after BS, a finding in keeping with previous observations [312]. Collectively, these findings support the notion that MCP-1 plays an important role in asthma and obesity pathogenesis. We did not observe any additive effects between asthma and obesity, however the reduced levels of MCP-1 after bariatric surgery might partly explain the beneficial effects of weight loss in asthma patients.

IL-33 appears to play a major role in the initiation and development of T2 immune responses involved in the development of asthma. Increased expression of IL-33 has been observed in murine asthma and in human asthma and rhinitis [45,313,314]. Once secreted, IL-33 binds to its specific membrane receptor **ST2** expressed on numerous cells, including immune cells such as ILC2, Tregs, mast cells, and macrophages [309,310]. sST2 is abundantly expressed and secreted by many cell types and tissues, and acts as a decoy receptor, neutralising free IL-33 in biological fluids [309–312]. High sST2 levels are found in the serum of patients with inflammatory diseases. When IL-33 is released into the extracellular environment increase, the expression of the soluble sST2 receptor also increases, probably to prevent the severe effects of excessive IL-33 [309–312]. Concerning obesity, IL-33 is able to down-regulate excessive inflammation in adipose tissue by targeting immune cells expressing the ST2 receptor. Mice lacking ST2 or IL-33 developed increased adiposity and worsened metabolic profiles. IL-33 treatment contributes to homeostasis in the adipose tissue by facilitating the differentiation and maintenance of Treg cells expressing ST2 and ILC2 in visceral adipose tissue, an immunomodulatory effect that can contribute towards down-regulating obesity-associated inflammation [128,315]. We found significantly higher levels of sST2 in obese individuals with respect to healthy controls and asthma subjects. There was a non-significant trend towards increased levels in obese asthmatics, which might suggest an additive effect. sST2 levels significantly correlated with BMI, however, weight loss was not associated with a decrease in sST2 levels. Nevertheless, without data on IL-33 levels, this observation is difficult to interpret as the measurement of sST2 only provides partial information on the balance between IL-33/sST2.

Immature **IL-18** is activated in inflammasomes after cleavage by caspase-1 and subsequently released from cells in its bioactive form [138]. High serum IL-18 levels have been found in patients with uncontrolled asthma [316] and in mild and moderate asthma exacerbations [317]. A recent study found that IL-18R was highly expressed in the lung of the most severe asthma patient cluster, supporting that IL-18 signalling may play a key role in the pathogenesis of severe asthma [318]. Previous studies reported that serum IL-18 levels were elevated in obese subjects and were reduced by weight loss [307]. In line with these observations, we found higher serum IL-18 levels in asthma and obese subjects, however, with no evidence of additive effects. As previously reported [307], weight loss significantly reduced serum IL-18 levels. Collectively, these findings support that the reduction of IL-18 might contribute to the salutary effects of weight loss in asthma.

IL-5 is produced by Th2, mast cells, and ILC2s, which are in turn activated by epithelium-derivate alarmins, including IL-24, IL-33, and TSLP [319]. Activation, recruitment, and survival of eosinophils are largely driven by IL-5. The contribution of eosinophils to airway inflammation is well recognised, with higher blood eosinophil counts predicting future exacerbation risk and poor asthma control in patients [320]. Sputum IL-5 levels have been found to be significantly higher in obese asthmatics compared with their lean counterparts. Surprisingly, increased IL-5 levels are not associated with a parallel increase in eosinophils in the sputum, which contrasts with the demonstrated augmentation of eosinophil numbers in the airway submucosa, which appears to be due to an enhanced recruitment of eosinophils to the lung in obese asthmatics [118]. We found higher serum levels of IL-5 in asthmatic patients without obesity with respect to obese subjects and healthy controls, but we could not find any additive effect of obesity in asthmatic patients with obesity. Weight loss had no significant effect on IL-5 levels.

Finally, we analysed ezrin and YKL-40 serum levels as potential molecules to be involved in the obesity-related asthma inflammation. **Ezrin** is one of the members of the ezrin/radixin/moesin (ERM) family of proteins [321]. ERM proteins exert numerous physiological functions in morphogenesis, cell polarity, and modulation of cell membrane tension [321]. Ezrin, which is localized at the cytoplasmic surface of cellular membranes, links plasma membrane proteins to the cortical actin cytoskeleton [321]. The role of ezrin in the pathogenesis of asthma is poorly understood. After allergen exposure, significant increases in serum levels of ezrin were observed in patients with allergic asthma [43]. In contrast to our results, a recent study found that the ezrin levels measured in serum were lower in asthma patients compared with non-asthma controls. In addition, serum ezrin levels were found to be related to the level of asthma control: the worse the control, the lower the ezrin levels [322]. Based on these observations, Jia *et al*. proposed using serum ezrin levels to monitor asthma control. Most of their asthma patients had a well-controlled disease, a finding that could partly explain the different results between the studies [322]. Despite this relatively good control, ezrin negatively correlated with FEV_1 in our study, supporting the notion that serum ezrin levels reflect asthma severity, to some extent. To the best of our knowledge, the relationship between obesity and ezrin has not been previously assessed. Interestingly, over-expression of ezrin in pancreatic β-cells results in an enhancement of insulin secretion [323]. Whether ezrin contributes towards overcoming insulin resistance usually in obese subjects is still unknown. We found higher levels of ezrin in obese subjects and ezrin levels positively and significantly correlated with BMI. Given the key regulatory role played by the ERM family of proteins, further studies are needed to better understand its role in asthma and obesity.

Another possible biomarker is **YKL-40**, one of several known human chitinases. Chitinases are specific enzymes that degradate a biopolymer that is part of the structure of fungi, molluscs, arthropods, and other invertebrates. Chitinases probably represent a defensive mechanism against chitin-containing parasites and fungi. Elevated serum YKL-40 levels have been found in asthma patients compared with healthy controls [44,45]. Levels of YKL-40 in serum and BALF correlated with subepithelial membrane thickness [324], and circulating levels are a significant independent factor associated with annual FEV_1 decline [325]. Additionally, elevated serum YKL-40 levels have been associated with irreversible airway obstruction, poor asthma control, and severe asthma exacerbations [43,128,321,322]. In line with these observations, we found that serum YKL-40 concentrations in asthmatics without obesity were significantly higher than those of healthy controls and, in keeping with previous observations, YKL-40 levels negatively correlated with FEV_1 (%), supporting that high serum YKL-40 might be considered a marker of asthma severity [43,44,321]. High serum levels of YKL-40 correlating with BMI have also been found in obese children and adults [323–325]. The potential additive effects of asthma and obesity on YKL-40 has been evaluated in previous studies with discrepant results. On the one hand, obese asthma was associated with significantly higher serum levels compared with non-obese asthmatics, but without differences between non-obese asthmatics and healthy controls [326]. On the other hand, other studies found that YKL-40 levels were higher in asthmatics than healthy controls, regardless of obesity [278]. We found the highest YKL-40 levels in asthmatic patients with obesity but the difference with respect to non-obese asthmatics was small and non-significant. We did not find any significant effect of weight loss on YKL-40 levels. Similar studies have shown contradictory results, with some studies reporting a decrease in YKL-40 levels [133], while others could not find any significant change [327] after weight loss mediated by the roux-en-Y gastric bypass.

1.3. Comparisons between obese subjects with and without asthma

We found that obese subjects displayed higher serum levels of TGF-β1 and of TNFR2 that their obesity-associated asthmatic counterparts, while IL-9 was the only cytokine showing significantly higher levels in asthmatic patients with obesity with respect to non-asthmatic patients with obesity.

Th9 cells have been described as a distinct group of $CD4^+$ Th cells that release **IL-9**, resulting from the combined effects of IL-4 and TGF-β [328,329]. The role of IL-9 in inflammation is a matter of controversy [133,326,330]. On the one hand, IL-9 has been shown to promote Th17 differentiation and production of IL-17 and IL-6 [331]; in contrast, it has also been shown that IL-9 enhances the suppressive function of Treg [332]. Asthma patients have increased numbers of Th9 cells in PBMCs and increased IL-9 levels in both serum and BALF [132,327,333–335]. Th9 cells and IL-9 promote accumulation and activation of T cells, ILC2, mast cells, and eosinophils and increase levels of IL-5, IL-13 and IgE in animal asthma models [336]. IL-9 concentrations have been found significantly elevated in male obese mice but not in female obese mice with respect to lean mice, suggesting that sexual dimorphism influences IL-9 production, with dominant expression in males [337]. In our study, serum levels of IL-9 were higher in obese asthma patients with respect to healthy controls and obese subjects, a finding that suggests that asthma and obesity might have additive effects on IL-9 secretion. However, there was no significant effect of weight loss on IL-9 serum levels. Since more than 75% of our study participants were women and sexual dimorphism could influence the effect of obesity on IL-9 levels, further studies in men are needed to better decipher the role of obesity in regulating IL-9 synthesis.

The activated systemic inflammation present in obesity would account for the increased airway inflammation, which in turn would contribute towards explaining the poor response of obese asthmatics to anti-inflammatory therapy. Understanding the molecular mechanisms involved in glucocorticoid insensitivity in these patients may help us to develop new strategies for rating airway inflammatory diseases.

2. Glucocorticoid sensitivity in PBMCs

Despite some progress made in the field, the mechanisms involved in glucocorticoid hyporesponsiveness in patients with asthma and obesity remain to be fully understood. In the second study, we hypothesized that the reduced response to glucocorticoids of asthmatic patients with obesity could be measured *in vitro* through the analysis of PBMCs or specific $CD4^+$ T cell population proliferation under dexamethasone treatment. Moreover, we hypothesized that increased glucocorticoid sensitivity due to bariatric surgery could be confirmed by same *in vitro* analysis.

2.1. Influence of obesity in glucocorticoid response

Obesity is known to impair the efficacy of glucocorticoid medications for asthma control. When asthma is associated with obesity, patients present a decreased response to glucocorticoid treatment [338,339] resulting in poor asthma control with an increase in the frequency of exacerbations and greater severity of respiratory symptoms [340,341].

There are only few studies regarding glucocorticoid sensitivity in asthmatic patients with obesity. Our study is the first one comparing glucocorticoid sensitivity between asthmatics with and without obesity, healthy controls and patients with obesity and without asthma through dexamethasone-inhibition of PHA-induced PBMCs and $CD4^+$ T cells–proliferation assay. There is one study regarding glucocorticoid sensitivity in asthmatic patients with obesity [108] measuring MKP-1 gene expression, discussed below. Moreover, stimulated adipocytes with dexamethasone and IL-17 from asthmatic patients with obesity presented a decreased GRα/GRβ ratio in obese asthmatics in comparison to lean, showing glucocorticoid resistance in asthmatic patients with obesity [176]. More studies are needed analysing how obesity affects glucocorticoid sensitivity in the asthmatic population.

2.1.1. PHA-induced PBMCs proliferation assay

Currently, there are no biomarkers to predict glucocorticoids effectiveness. However, dexamethasone IC_{50} value may help discriminate patients with different sensitivities to glucocorticoids. In the present study, we demonstrated that obese subjects with and without asthma are characterized by a reduced response to glucocorticoid treatment, assessed by means of a PBMCs proliferation assay. This has been reported previously in asthmatic patients [175,181,182], but never in obese asthmatics. As a complementary methodological contribution, we used PBMCs and $CD4^+$ T cells proliferation to assess sensitivity to dexamethasone treatment, as both methodologies have been used to examine glucocorticoid sensitivity in other studies [181,183,342]. We found some differences in the results obtained with PHA-induced PBMCs and $CD4^+$ T cells proliferation in the response to glucocorticoids, a finding that should be taken into account when results from different studies are compared.

In addition, we found that the reduced response to glucocorticoids negatively correlated with serum adiponectin/leptin ratio, a marker associated with the level of inflammation in subjects with obesity [80], and with insulin resistance and other cardiometabolic comorbidities [82].

2.1.2. MKP-1 gene expression study

As we already explained, the expression of anti-inflammatory genes induced by glucocorticoids in PBMCs, such as MKP-1, represents a useful *in vitro* method to predict and evaluate clinical responses to glucocorticoids.

Before performing a dexamethasone-induced MKP-1 gene expression analysis, we carried out another study, presented in this work as Manuscript 3, in order to select the most stable reference genes for our samples and experimental conditions. To date, there are no studies that have validated reference genes in PBMCs treated with glucocorticoids from asthmatic patients with or without obesity compared to healthy subjects. We analysed gene expression stability of eight commonly used reference genes via RT–qPCR using four different algorithms - BestKeeper [343], ΔCt [344], geNorm [345], and NormFinder [346]. Our results demonstrated that B2M and HPRT1 were the most stable reference genes among healthy controls and asthmatics with and without obesity. These results contrast with those of Nakayama *et al.*, who found *B2M* to be an unstable gene for chronic rhinosinusitis patients [347]. On the other hand, ACTB, TBP, and GAPDH were the ones with less stable among our experimental conditions, which is in line with previous studies that described *GAPDH* as an unstable gene along with *ACTB*, which was also positioned last in our ranking [348,349].

Once we had selected the appropriate reference genes, we examined MKP-1 gene expression. Previous studies found that the induced expression of MKP-1 by dexamethasone was reduced in overweight/obese asthma patients compared with lean asthma subjects [108]. Interestingly, obesity in non-asthmatic subjects had no effect on MKP-1 expression, suggesting that, due to unclear reasons, the effect of obesity only impacts on MKP-1 expression in asthma patients [108]. In our study we did not find any significant difference in the dexamethasone-dependent induction of MKP-1 in asthmatics with obesity versus their lean counterparts. This finding suggests that obesity does not affect the capacity of glucocorticoids to activate anti-inflammatory genes in PBMCs. Differences in methods and the characteristics of the populations studied might explain discrepancies between our study and that of Sutherland *et al*. [108]. In that study, the induction of MKP-1 was analysed against a single dose of dexamethasone (10^{-6} M), while in our study it was analysed by means of a dose-dependent response, which is considered most suitable for evaluating pharmacologically-induced responses *in vitro*. Moreover, asthma severity has been shown to be closely related to the level of dexamethasone-induced MKP-1 expression [181], and most patients enrolled in our study suffered from mild/moderate disease. In the Sutherland *et al*. study, pulmonary function showed a more pronounced airway obstruction (FEV_1) in asthmatic patients with obesity than in the present study (70% *vs* 77%) [108].

Anti-inflammatory effects of glucocorticoids are also explained by mechanisms other that activation of MKP-1, such as alterations in the nuclear translocation of GRα, increased expression of the GRβ isoform—a dominant negative regulator of active GRα—or inhibition of pro-inflammatory gene expression through blockade of pro-inflammatory transcription factors [350].

2.2. Influence of bariatric surgery in glucocorticoid response

Airway responsiveness, lung function and asthma severity and control have been reported to markedly improved with weight loss following bariatric surgery in patients with severe obesity [251,261,265]. In our study, lung function tests improved after BS, in agreement with Arismendi *et al*. [57], and weight loss was associated with a significant improvement in the ACT scores et six months' follow-up. This result is in agreement with other authors who conclude that asthma control and quality of life improved at 1-year follow-up [178,351]. Other authors found that bariatric surgery is associated with an improvement of respiratory symptoms, and a 50% reduction in the total number of oral glucocorticoids, daily ICS, or inhaled bronchodilators respiratory medication prescriptions to control asthma during the first postoperative year [261,263,264].

We hypothesized that weight-loss and the reduction in systemic inflammation would increase glucocorticoid sensitivity after bariatric surgery. In this study, it is the first time glucocorticoid sensitivity measured *in vitro* through PBMCs proliferation assay is compared before and six-months after bariatric surgery. We demonstrated that weight-loss after surgery is accompanied with an increase in cellular sensitivity to dexamethasone in obese subjects, with or without asthma. This suggests that the reduction in inflammation after surgery could be the cause of the increased cellular glucocorticoid response. This further supports a key role of obesity in the glucocorticoid sensitivity of asthmatic patients.

2.3. Influence of vitamin D in glucocorticoid response

Patients with obesity present low levels of vitamin D and it is known that this associates with impaired lung function, higher asthma exacerbation frequency, and a reduced response to glucocorticoids [177,352,353].

In the second study, we also hypothesized that vitamin D serum concentrations correlates with *in vitro* glucocorticoid response, and that *in vitro* vitamin D treatment increases glucocorticoid response analysed by PBMCs and $CD4^+$ T cells proliferation assay.

2.3.1. Serum vitamin D effects

It has been previously described in asthmatic children that more usage of glucocorticoids is needed if they had low levels of vitamin D in serum [180,216]. Our study population presented vitamin D serum levels that were on the limit of sufficiency, being insufficient (<20 ng/ml) in non-asthmatic patients with obesity. We found a negative correlation between vitamin D serum concentrations and *in vitro* response to glucocorticoid measured as dexamethasone IC_{50} value, suggesting that our *in vitro* model could be useful to study the mechanisms involved in glucocorticoid resistance present in patients with deficient vitamin D levels. Interestingly, subjects with obesity with the lowest vitamin D levels were those with the poorest anti-proliferative response to glucocorticoids.

There are few other studies regarding the relationship between vitamin D serum concentration and *in vitro* glucocorticoid response. Sutherland *et al*. found a positive correlation between vitamin D serum levels and dexamethasone-induced MKP-1 gene expression; however, they did not find correlation with dexamethasone IC_{50} value [177], as we did. Moreover, vitamin D serum correlated with CYP24, a vitamin D inducible gene, that correlated with *in vitro* glucocorticoid response measured as dexamethasone-suppression of TNF-α and IL-13 only in children, not in adults [180].

Weight loss induced by a low-calorie diet is associated with an increase in serum vitamin D levels, supporting the hypothesis that vitamin D is stored in the adipose tissue and released following weight loss [267]. We found that after weight-loss via bariatric surgery and vitamin D supplementation, there were no patients with obesity with vitamin D deficiency levels. These observations suggest that reduction of obesity-related systemic inflammation and increased levels of serum vitamin D can account for the recovered ability of glucocorticoids to inhibit PBMCs proliferation.

In order to definitely establish the role of vitamin D deficiency in glucocorticoid hyporesponsiveness in asthma, randomized clinical trials (RCTs) should be performed to evaluate the clinical efficacy of vitamin D administration in obesity-associated asthmatic patients. Numerous RCTs studies, mostly in children, have been carried out to evaluate the efficacy of vitamin D administration in asthma patients. The trials enrolled patients with significant differences in the selection of participants, baseline vitamin D levels and dosage of vitamin D supplementation. Some systematic reviews of RCTs concluded that high-dose vitamin D may be effective in paediatric asthma [354]; however, others could not find significant evidence favouring the use of this complementary therapy in children and adults [355].

Vitamin D supplementation studies in asthma face many unresolved issues. Thus, for example, studies on the anti-inflammatory and anti-oxidant effects of vitamin D suggest that the serum level of vitamin D that should be reached to be effective in asthma may be higher than that generally accepted for bone health [356]. In addition, due to the effect of excessive accumulation of fat on the distribution of vitamin D, asthmatic patients with obesity should require a supplemental vitamin D dose higher than that required by non-obese asthmatic patients with vitamin D deficiency [356]. RCT studies tailored for this specific group of overweight/obese asthma patients are needed to elucidate the potential therapeutic effect of vitamin D supplementation that our findings suggest.

2.3.2. In vitro vitamin D effects

After knowing that vitamin D in serum is related with glucocorticoid sensitivity, we studied if 1,25$(OH)_2$D *in vitro* treatment had any effect on PBMCs and $CD4^+$ T cells proliferation. One of the first studies regarding the vitamin D effect on the proliferation of PBMCs dates back to 1984. They found that calcitriol is a potent inhibitor of PHA-induced lymphocyte proliferation (Rigby, 1984). We also found that the hormone exerts anti-proliferative effects on cells by itself, both in PBMCs and specifically in $CD4^+$ T cells. It has been reported that vitamin D diminishes pro-inflammatory cytokines in cell culture [357,358]. Moreover, the effect of vitamin D modulating cytokine production was lower in patients with overweight or obesity, more markedly in those with severe asthma [358]. Moreover, in patients with severe asthma, enhanced production of IL-17A is inhibited by *in vitro* vitamin D in a glucocorticoid-independent manner [247]. In a more recent study, they found that the stimulation of $CD4^+$ T cells with 1,25$(OH)_2$D, suppressed proliferation capacity, enhanced the expression of inhibitory markers on $CD4^+$ T cells, and diminished the percentage of pro-inflammatory cytokines including IFN-γ, IL-17, and IL-22 except IL-4 in $CD4^+$ T cells. The data suggested a potential insight into the consideration of vitamin D in the prevention/control of pro-inflammatory disorders [359].

In order to further investigate the potential causal relationship between vitamin D deficiency and glucocorticoid hyporesponsiveness, we conducted an *in vitro* study to assess the effects of vitamin D on cell proliferation and found that the hormone is capable of potentiating the anti-proliferative effects of glucocorticoids. Other markers have been measured to study the synergy between glucocorticoids and vitamin D, such as measuring PBMCs proliferation stimulated by a toxin to induce glucocorticoid resistance. They found no proliferation inhibition with dexamethasone alone, but when 1,25$(OH)_2$D was added to culture, $CD4^+$ T cell proliferation was suppressed [216]. We also demonstrate that vitamin D has steroid sparing effects, decreasing dexamethasone IC_{50} values in asthmatic patients with and without obesity. Zhang *et al*. found that vitamin D has anti-inflammatory and corticosteroid enhancing effects increasing MKP-1 gene expression in monocytes of steroid resistant and steroid sensitive asthmatics. We also examined the effects of vitamin D on the induction of MKP-1 in subjects with and without obesity, finding that the hormone similarly increases the expression of MKP-1, with some differences between obese and non-obese subjects, a finding that suggests that the efficacy of vitamin D to increase dexamethasone-induction of anti-inflammatory genes is more pronounced in subjects with obesity. These findings support the notion that vitamin D may play a role in the complex interaction between obesity, inflammation, and glucocorticoid hyporesponsiveness, and suggests the relevance of measuring vitamin D patients' serum levels as well as provide an *in vitro* model to study the mechanism involved in this effect.

In summary, there has been much speculation and debate regarding the role of obese-related low-grade systemic inflammation as a potentiating factor for systemic and airway inflammation in patients with obese asthma in order to explain why patients with this association frequently present a severe disease, resistant to anti-asthmatic treatment. However, the scientific evidence supporting this hypothesis is very scarce and inconsistent. In our study we found evidence of partially differentiated systemic inflammation in asthma and obesity. We also observed similar increased levels of some cytokines in subjects with asthma and obesity. However, we could only find additive effects for IL-9 in patients with both diseases. Moreover, our study has several novel contributions that may help to better understand the links between obesity and poor response to ICS in adults with obesity-associated asthma. Our findings support the role of obesity in ICS hyporesponsiveness and demonstrate that the efficacy of weight loss to improve asthma symptoms and lung function can be, at least in part, due to the recovered anti-inflammatory response to glucocorticoids. Finally, our results suggest that vitamin D supplementation in asthma patients with obesity and vitamin D deficiency may contribute to achieving asthma control by improving ICS efficacy.

3. Study limitations

The main limitation of our first study was the small size of asthmatic patients with obesity subjected to BS, which significantly reduced the capacity to assess the effects of weight loss on serum cytokine levels in this group of patients. Results need to be examined in a larger cohort. A six to twelve-months follow-up may not be long enough to detect significant changes in some cytokines after bariatric surgery. Furthermore, we only focused on serum levels, without any parallel information regarding cytokine levels in the airways, where inflammation determines the course and severity of asthma. The impact of asthma inflammatory phenotypes (eosinophilic, neutrophilic, paucigranulocytic) on cytokine levels should also be considered in future studies.

CONCLUSIONS

- B2M and HPRT1 are stable reference genes to perform genetic studies in PBMCs from asthmatic patients with obesity incubated with glucocorticoids.
- Obesity is associated with systemic inflammation that is more active than that associated with asthma.
- Asthma control, pulmonary function, and serum vitamin D levels improve with significant weight loss after bariatric surgery in obese subjects with and without asthma.
- Adiponectin/leptin ratio is reduced after bariatric surgery, and other pro-inflammatory mediators are also decreased (TNFR2, MCP-1, ezrin, and IL-18) in patients with obesity.
- Leptin and adiponectin are regulated in asthma by both obesity-dependent and -independent mechanisms, and adiponectin/leptin ratio is different between asthma patients with and without obesity.
- PBMCs and $CD4^+$ T cells from obese participants with and without asthma show a reduced response to the *in vitro* glucocorticoid treatment.
- Glucocorticoid sensitivity improves significantly after bariatric surgery measured *in vitro* through PBMCs proliferation assay.
- Dexamethasone-induced MKP-1 mRNA expression in PBMCs correlates with glucocorticoid sensitivity expressed as IC_{50} value.
- Only in subjects with obesity, MKP-1 gene expression is higher when cells are treated *in vitro* with the active form of vitamin D in addition to dexamethasone, in comparison with dexamethasone alone.
- Adiponectin/leptin ratio and vitamin D serum levels correlate with glucocorticoid sensitivity measured *in vitro*.
- The active form of vitamin D shows anti-proliferative effects on PBMCs and $CD4^+$ T cells, and significantly increases the anti-proliferative effects of dexamethasone independently of asthma and obesity.

REFERENCES

1. WHO. Asthma; 2022. Available from: https://www.who.int/news-room/fact-sheets/detail/asthma (accessed on June 9, 2022).
2. Beasley R, Semprini A, Mitchell EA. Risk factors for asthma: is prevention possible? *Lancet*. 2015;386(9998):1075–85.
3. Burbank AJ, Sood AK, Kesic MJ, et al. Environmental determinants of allergy and asthma in early life. *J Allergy Clin Immunol.* 2017;140:1–12.
4. GEMA 4.4 - Guía española para el manejo del asma; 2019. Available from: https://www.semg.es/index.php/consensos-guias-y-protocolos/316-gema-4-4-guia-espanola-para-el-manejo-del-asma (accessed on May 1, 2021).
5. Global initiative for asthma. Global Strategy for Asthma Management and Prevention, 2022. Available from: www.ginasthma.org (accessed on May 1, 2021).
6. Fahy J V. Type 2 inflammation in asthma-present in most, absent in many. *Nature Reviews Immunology*. 2015;15:57–65.
7. Svenningsen S, Nair P. Asthma Endotypes and an Overview of Targeted Therapy for Asthma. *Front Med*. 2017;4:158.
8. Aleman F, Lim HF, Nair P. Eosinophilic Endotype of Asthma. *Immunol Allergy Clin North Am*. 2016;36:559–68.
9. Yilmaz I, Turk M. What should be the cutoff value of blood eosinophilia as a predictor of inhaled corticosteroid responsiveness in patients with chronic obstructive pulmonary disease? *Am J Respir Crit Care Med*. 2017;196(9):1229–30.
10. Bakakos A, Loukides S, Bakakos P. Clinical Medicine Severe Eosinophilic Asthma. *J Clin Med.* 2019;8:1375.
11. Ntontsi P, Loukides S, Bakakos P, et al. Clinical, functional and inflammatory characteristics in patients with paucigranulocytic stable asthma: Comparison with different sputum phenotypes. *Allergy*. 2017;72(11):1761–7.
12. Thomson NC. Novel approaches to the management of noneosinophilic asthma. *Ther Adv Respir Dis.* 2016;10(3):211–34.
13. Lötvall J, Akdis CA, Bacharier LB, et al. Asthma endotypes: A new approach to classification of disease entities within the asthma syndrome. *J Allergy Clin Immunol*. 2011;127(2):355–60.
14. Haldar P, Brightling CE, Hargadon B, et al. Mepolizumab and Exacerbations of Refractory Eosinophilic Asthma. *N Engl J Med*. 2009;360:973–84.
15. Nair P, Pizzichini MMM, Kjarsgaard M, et al. Mepolizumab for Prednisone-Dependent Asthma with Sputum Eosinophilia. *N Engl J Med*. 2009;360:985–93.
16. Wenzel SE, Schwartz LB, Langmack EL, et al. Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. *Am J Respir Crit Care Med*. 1999;160(3):1001–8.
17. Russell RJ, Brightling CE. Pathogenesis of asthma: Implications for precision medicine. *Clin Sci*. 2017;131(14):1723–35.
18. Papi A, Brightling C, Pedersen SE, et al. Asthma. *The Lancet*. 2018;391:783–800.
19. Peters MC, Wenzel SE. Intersection of biology and therapeutics: type 2 targeted therapeutics for adult asthma. *The Lancet*. 2020;395:371–83.
20. Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol.* 2015;16(1):45–56.
21. Cohn L, Elias JA, Chupp GL. Asthma : Mechanisms of Disease Persistence and Progression. *Annu Rev Immunol.* 2004;22(1):789–815.
22. Wills-Karp M. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu Rev Immunol*. 1999;17(1):255–81.
23. Holgate ST, Polosa R. Treatment strategies for allergy and asthma. Nat Rev Immunol. 2008 Mar 15;8(3):218–30.
24. Jia L, Wang Y, Li J, et al. Detection of IL-9 producing T cells in the PBMCs of allergic asthmatic patients. *BMC Immunol*. 2017;18(1):38–38.
25. Liu S, Verma M, Michalec L, et al. Steroid resistance of airway type 2 innate lymphoid cells from patients with severe asthma: The role of thymic stromal lymphopoietin. *J Allergy Clin Immunol*.

2018;141(1):257-268.e6.

26. Del Giacco SR, Bakirtas A, Bel E, et al. Allergy in severe asthma. *Allergy.* 2017;72(2):207–20.
27. Green RH, Brightling CE, Woltmann G, et al. Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. *Thorax*. 2002;57(10):875–9.
28. Al-Kufaidy R, Vazquez-Tello A, BaHammam AS, et al. IL-17 enhances the migration of B cells during asthma by inducing CXCL13 chemokine production in structural lung cells. *J Allergy Clin Immunol*. 2017;139(2):696-699.e5.
29. Halwani R, Sultana A, Vazquez-Tello A, et al. Th-17 regulatory cytokines IL-21, IL-23, and IL-6 enhance neutrophil production of IL-17 cytokines during asthma. *J Asthma*. 2017;54(9):893–904.
30. Al-Ramli W, Préfontaine D, Chouiali F, et al. TH17-associated cytokines (IL-17A and IL-17F) in severe asthma. *J Allergy Clin Immunol*. 2009;123:1185–7.
31. Chambers ES, Nanzer AM, Pfeffer PE, et al. Distinct endotypes of steroid-resistant asthma characterized by IL-17Ahigh and IFN-γhigh immunophenotypes: Potential benefits of calcitriol. *J Allergy Clin Immunol*. 2015;136(3):628-637.e4.
32. Oda N, Canelos PB, Essayan DM, et al. Interleukin-17F Induces Pulmonary Neutrophilia and Amplifies Antigen-induced Allergic Response. *Am J Respir Crit Care Med*. 2005;171(1):12–8.
33. Kudo M, Melton AC, Chen C, et al. IL-17A produced by αβ T cells drives airway hyper-responsiveness in mice and enhances mouse and human airway smooth muscle contraction. *Nat Med*. 2012;18(4):547.
34. Medoff BD, Okamoto Y, Leyton P, et al. Adiponectin deficiency increases allergic airway inflammation and pulmonary vascular remodeling. *Am J Respir Cell Mol Biol*. 2009;41(4):397–406.
35. Hekking PP, Loza MJ, Pavlidis S, et al. Pathway discovery using transcriptomic profiles in adult-onset severe asthma. *J Allergy Clin Immunol.* 2018;141(4):1280–90.
36. Raundhal M, Morse C, Khare A, et al. High IFN-γ and low SLPI mark severe asthma in mice and humans. *J Clin Invest.* 2015;125(8):3037–50.
37. Berry MA, Hargadon B, Shelley M, et al. Evidence of a role of tumor necrosis factor α in refractory asthma. *N Engl J Med*. 2006;354(7):697–708.
38. Manni ML, Trudeau JB, Scheller E V., et al. The complex relationship between inflammation and lung function in severe asthma. *Mucosal Immunol.* 2014;7(5):1186–98.
39. Simpson JL, Scott R, Boyle MJ, et al. Inflammatory subtypes in asthma: Assessment and identification using induced sputum. *Respirology*. 2006;11(1):54–61.
40. Moore WC, Hastie AT, Li X, et al. Sputum neutrophil counts are associated with more severe asthma phenotypes using cluster analysis. *J Allergy Clin Immunol.* 2014;133(6).
41. Tliba O, Panettieri RA. Paucigranulocytic asthma: Uncoupling of airway obstruction from inflammation. *J Allergy Clin Immunol.* 2019;143(4):1287–94.
42. Thomson NC. Identifying Subtypes of Paucigranulocytic Asthma: Now There Are 3. *J Allergy Clin Immunol Pract*. 2021;9(6):2356–7.
43. Kalinauskaite-Zukauske V, Januskevicius A, Janulaityte I, et al. Serum Levels of Epithelial-Derived Cytokines as Interleukin-25 and Thymic Stromal Lymphopoietin after a Single Dose of Mepolizumab in Patients with Severe Non-Allergic Eosinophilic Asthma: A Short Report. *Canadian Respiratory Journal.* 2019;8607657.
44. Lu Y, Van Bever HPS, Lim TK, et al. Obesity, asthma prevalence and IL-4: Roles of inflammatory cytokines, adiponectin and neuropeptide Y. *Pediatr Allergy Immunol.* 2015;26(6):530–6.
45. Kamekura R, Kojima T, Takano K, et al. The role of IL-33 and its receptor ST2 in human nasal epithelium with allergic rhinitis. *Clin Exp Allergy.* 2012;42(2):218–28.
46. Bray GA, Frühbeck G, Ryan DH, et al. Management of obesity. *Lancet*. 2016;387(10031):1947–56.
47. WHO. Obesity and overweight; 2021. Available from: https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight (accessed on June 10, 2022).

48. Basterra-Gortari FJ, Bes-Rastrollo M, Ruiz-Canela M, et al. Prevalencia de obesidad y diabetes en adultos españoles, 1987-2012. *Med Clin*. 2017;148(6):250–6.
49. Ping Z, Pei X, Xia P, et al. Anthropometric indices as surrogates for estimating abdominal visceral and subcutaneous adipose tissue: A meta-analysis with 16,129 participants. *Diabetes Res Clin Pract.* 2018;143:310–9.
50. Esteve Ràfols M. Adipose tissue: Cell heterogeneity and functional diversity. *Endocrinol y Nutr.* 2014;61(2):100–12.
51. Richard AJ, White U, Elks CM, et al. Adipose Tissue: Physiology to Metabolic Dysfunction. In: Feingold KR, Anawalt B, Boyce A, et al., editors. South Dartmouth (MA): MDText.com, Inc.; 2000.
52. Muc M, Todo-Bom A, Mota-Pinto A, et al. Leptin and resistin in overweight patients with and without asthma. *Allergol Immunopathol*. 2014;42(5):415–21.
53. Mathieu P, Poirier P, Pibarot P, et al. Brief Review Visceral Obesity The Link Among Inflammation, Hypertension, and Cardiovascular Disease. *Hypertension*. 2009;53:577-584.
54. Hotamisligil GS. Inflammation and metabolic disorders. *Nature*. 2006;444(7121):860–7.
55. Visser M, Bouter LM, McQuillan GM, et al. Low-grade systemic inflammation in overweight children. *Pediatrics*. 2001;107(1):E13.
56. Mraz M, Haluzik M. The role of adipose tissue immune cells in obesity and low-grade inflammation. *Journal of Endocrinology.* 2014;222:113–27.
57. Arismendi E, Rivas E, Agustí A, et al. The Systemic Inflammome of Severe Obesity before and after Bariatric Surgery. *PLoS One*. 2014;9(9):e107859.
58. Karczewski J, Śledzińska E, Baturo A, et al. Obesity and inflammation. *Eur Cytokine Netw*. 2018;29(3):83–94.
59. Kelesidis T, Kelesidis I, Chou S, et al. Narrative review: The role of leptin in human physiology: Emerging clinical applications. *Annals of Internal Medicine*. 2010;152:93–100.
60. Bastard JP, Maachi M, Lagathu C, et al. Recent advances in the relationship between obesity, inflammation, and insulin resistance. *Eur Cytokine Netw*. 2006;17(1):4–12.
61. Loffreda S, Yang SQ, Lin HZ, et al. Leptin regulates proinflammatory immune responses. *FASEB J*. 1998;12(1):57–65.
62. Fantuzzi G. Adipose tissue, adipokines, and inflammation. *J Allergy Clin Immunol*. 2005;115(5):911–9.
63. Xu H, Barnes GT, Yang Q, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest*. 2003;112(12):1821–30.
64. Rastogi D, Fraser S, Oh J, et al. Inflammation, metabolic dysregulation, and pulmonary function among obese urban adolescents with asthma. *Am J Respir Crit Care Med.* 2015;191(2):149–60.
65. Umetsu DT. Mechanisms by which obesity impacts upon asthma. *Thorax.* 2017;72(2):174–7.
66. Agrawal S, Gollapudi S, Su H, et al. Leptin activates human B cells to secrete TNF-α, IL-6, and IL-10 via JAK2/STAT3 and p38MAPK/ERK1/2 signaling pathway. *J Clin Immunol.* 2011;31(3):472–8.
67. Zarrati M, Salehi E, Razmpoosh E, et al. Relationship between leptin concentration and body fat with peripheral blood mononuclear cells cytokines among obese and overweight adults. *Ir J Med Sci.* 2017;186(1):133–42.
68. Martín-Romero C, Santos-Alvarez J, Goberna R, et al. Human leptin enhances activation and proliferation of human circulating T lymphocytes. *Cell Immunol.* 2000;199(1):15–24.
69. Matarese G, La Cava A, Sanna V, et al. Balancing susceptibility to infection and autoimmunity: a role for leptin? *Trends Immunol.* 2002;23(4):182–7.
70. Lord GM, Matarese G, Howard JK, et al. Leptin modulates the T-cell immune response and reverses starvation- induced immunosuppression. *Nature.* 1998;394(6696):897–901.
71. De Rosa V, Procaccini C, Calì G, et al. A Key Role of Leptin in the Control of Regulatory T Cell Proliferation. *Immunity.* 2007;26(2):241–55.
72. Procaccini C, De Rosa V, Galgani M, et al. Leptin-Induced mTOR Activation Defines a Specific Molecular and Transcriptional Signature Controlling CD4 + Effector T Cell Responses . *J Immunol*. 2012;189(6):2941–53.

73. Yu Y, Liu Y, Shi FD, et al. Cutting Edge: Leptin-Induced RORγt Expression in CD4 + T Cells Promotes Th17 Responses in Systemic Lupus Erythematosus . *J Immunol*. 2013;190(7):3054–8.
74. Luo Y, Liu M. Adiponectin: a versatile player of innate immunity. *J Mol Cell Biol.* 2016;8(2):120.
75. Achari AE, Jain SK. Adiponectin, a therapeutic target for obesity, diabetes, and endothelial dysfunction. *Int J Mol Sci*. 2017;18:1321.
76. Wolf AM, Wolf D, Rumpold H, et al. Adiponectin induces the anti-inflammatory cytokines IL-10 and IL-1RA in human leukocytes. *Biochem Biophys Res Commun*. 2004;323(2):630–5.
77. Esser N, Legrand-Poels S, Piette J, et al. Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes. *Diabetes Research and Clinical Practice*. 2014;105:141–50.
78. Kasahara DI, Kim HY, Williams AS, et al. Pulmonary inflammation induced by subacute ozone is augmented in adiponectin-deficient mice: role of IL-17A. *J Immunol.* 2012;188(9):4558–67.
79. Yang WS, Lee WJ, Funahashi T, et al. Weight Reduction Increases Plasma Levels of an Adipose-Derived Anti-Inflammatory Protein, Adiponectin. *J Clin Endocrinol Metab.* 2001;86(8):3815–9.
80. Frühbeck G, Catalán V, Rodríguez A, et al. Adiponectin-leptin Ratio is a Functional Biomarker of Adipose Tissue Inflammation. *Nutrients.* 2019;1;11(2).
81. Frühbeck G, Catalán V, Rodríguez A, et al. Adiponectin-leptin ratio: A promising index to estimate adipose tissue dysfunction. Relation with obesity-associated cardiometabolic risk. *Adipocyte.* 2018;7(1):57–62.
82. Frithioff-Bøjsøe C, Lund MAV, Lausten-Thomsen U, et al. Leptin, adiponectin, and their ratio as markers of insulin resistance and cardiometabolic risk in childhood obesity. *Pediatr Diabetes.* 2020;21(2):194–202.
83. Camargo CA, Weiss ST, Zhang S, et al. Prospective study of body mass index, weight change, and risk of adult-onset asthma in women. *Arch Intern Med*. 1999:159(21):2582–8.
84. Shaheen SO, Sterne JAC, Montgomery SM, et al. Birth weight, body mass index and asthma in young adults. *Thorax.* 1999;54(5):396–402.
85. Beckett WS, Jacobs DR, Xinhua YU,et al. Asthma is associated with weight gain in females but not males, independent of physical activity. *Am J Respir Crit Care Med.* 2001;164(11):2045–50.
86. Chen Y, Dales R, Tang M, et al. Obesity may increase the incidence of asthma in women but not in men: Longitudinal observations from the Canadian National Population Health Surveys. *Am J Epidemiol.* 2002;155(3):191–7.
87. Xu B, Pekkanen J, Laitinen J, et al. Body build from birth to adulthood and risk of asthma. *Eur J Public Health*. 2002;12(3):166–70.
88. Guerra S, Sherrill DL, Bobadilla A, et al. The relation of body mass index to asthma, chronic bronchitis, and emphysema. *Chest.* 2002;122(4):1256–63.
89. Huovinen E, Kaprio J, Koskenvuo M. Factors associated to lifestyle and risk of adult onset asthma. *Respir Med.* 2003;97(3):273–80.
90. Ford ES. The epidemiology of obesity and asthma. *J Allergy Clin Immunol.* 2005;115:897–909.
91. Nystad W, Meyer HE, Nafstad P, et al. Body mass index in relation to adult asthma among 135,000 Norwegian men and women. *Am J Epidemiol.* 2004;160(10):969–76.
92. Von Behren J, Lipsett M, Horn-Ross PL, et al. Obesity, waist size and prevalence of current asthma in the California Teachers Study cohort. *Thorax*. 2009;64(10):889–93.
93. Kronander UN, Falkenberg M, Olle Z. Prevalence and incidence of asthma related to waist circumference and BMI in a Swedish community sample. *Respir Med*. 2004;98(11):1108–16.
94. Antó JM, Sunyer J, Basagaña X, et al. Risk factors of new-onset asthma in adults: A population-based international cohort study. *Allergy Eur J Allergy Clin Immunol*. 2010;65(8):1021–30.
95. Guerra S, Wright AL, Morgan WJ, et al. Persistence of asthma symptoms during adolescence: Role of obesity and age at the onset of puberty. *Am J Respir Crit Care Med*. 2004;170(1):78–85.
96. Harpsøe MC, Basit S, Bager P, et al. Maternal obesity, gestational weight gain, and risk of asthma and atopic disease in offspring: A study within the Danish National Birth Cohort. *J Allergy Clin Immunol.* 2013;131(4):1033–40.
97. Dumas O, Varraso R, Gillman MW, et al. Longitudinal study of maternal body mass index, gestational weight gain, and offspring asthma. *Allergy Eur J Allergy Clin Immunol*.

2016;71(9):1295–304.

98. Johannessen A, Lønnebotn M, Calciano L, et al. Being overweight in childhood, puberty, or early adulthood: Changing asthma risk in the next generation? *J Allergy Clin Immunol*. 2020;145(3):791-799.e4.
99. Chen Z, Salam MT, Alderete TL, et al. Effects of childhood asthma on the development of obesity among school-aged children. *Am J Respir Crit Care Med*. 2017;195(9):1181–8.
100. Dixon AE, Poynter ME. Mechanisms of asthma in obesity pleiotropic aspects of obesity produce distinct asthma phenotypes. *Am J Respir Cell Mol Biol.* 2016;54:601–8.
101. Holguin F, Bleecker ER, Busse WW, et al. Obesity and asthma: An association modified by age of asthma onset. *J Allergy Clin Immunol.* 2011;127(6):1486-93.e2.
102. Sweeney J, Patterson CC, Menzies-Gow A, et al. Comorbidity in severe asthma requiring systemic corticosteroid therapy: cross-sectional data from the Optimum Patient Care Research Database and the British Thoracic Difficult Asthma Registry. *Thorax.* 2016;71(4):339–46.
103. Haldar P, Pavord ID, Shaw DE, et al. Cluster analysis and clinical asthma phenotypes. *Am J Respir Crit Care Med.* 2008;178(3):218–24.
104. Ray A, Oriss TB, Wenzel SE. Emerging molecular phenotypes of asthma. *Am J Physiol Lung Cell Mol Physiol*. 2015;308(2):L130-40.
105. Sutherland ER, Goleva E, King TS, et al. Cluster analysis of obesity and asthma phenotypes. *PLoS One*. 2012;7(5):1–7.
106. Holguin F. Obesity as a risk factor for increased asthma severity and allergic inflammation; cause or effect? Clin Exp Allergy. 2012;42:612–3.
107. Barros R, Moreira P, Padrão P, et al. Obesity increases the prevalence and the incidence of asthma and worsens asthma severity. *Clin Nutr.* 2017;36(4):1068–74.
108. Sutherland ER, Goleva E, Strand M, et al. Body mass and glucocorticoid response in asthma. *Am J Respir Crit Care Med*. 2008;178(7):682–7.
109. Todd DC, Armstrong S, D'Silva L, et al. Effect of obesity on airway inflammation: A cross-sectional analysis of body mass index and sputum cell counts. *Clin Exp Allergy.* 2007;37(7):1049–54.
110. Zhang MY, Dini AA, Yang XK, Li LJ, Wu GC, Leng RX, et al. Association between serum/plasma adiponectin levels and immune-mediated diseases: a meta-analysis. *Arch Dermatol Res*. 2017;309(8):625–35.
111. Sideleva O, Suratt BT, Black KE, et al. Obesity and asthma: An inflammatory disease of adipose tissue not the airway. *Am J Respir Crit Care Med*. 2012;186(7):598–605.
112. Shore SA, Rivera-Sanchez YM, Schwartzman IN, et al. Responses to ozone are increased in obese mice. *J Appl Physiol.* 2003;95(3):938–45.
113. Williams AS, Leung SY, Nath P, et al. Role of TLR2, TLR4, and MyD88 in murine ozone-induced airway hyperresponsiveness and neutrophilia. *J Appl Physiol.* 2007;103(4):1189–95.
114. Tsaroucha A, Daniil Z, Malli F, et al. Leptin, adiponectin, and ghrelin levels in female patients with asthma during stable and exacerbation periods. *J Asthma.* 2013;50(2):188–97.
115. Kattan M, Kumar R, Bloomberg GR, et al. Asthma control, adiposity, and adipokines among inner-city adolescents. *J Allergy Clin Immunol.* 2010;125(3):584–92.
116. Zhang X, Zheng J, Zhang L, et al. Systemic inflammation mediates the detrimental effects of obesity on asthma control. *Allergy Asthma Proc.* 2018;39(1):43–50.
117. Li Z, Leynaert B, Dumas O, et al. Role of Leptin in the Association Between Body Adiposity and Persistent Asthma: A Longitudinal Study. *Obesity.* 2019;27(6):894–8.
118. Marijsse GS, Seys SF, Schelpe AS, et al. Obese individualswith asthma preferentially have a high IL-5/IL-17A/IL-25 sputum inflammatory pattern. *Am J Respir Crit Care Med.* 2014; 189:1284–5.
119. Scott HA, Gibson PG, Garg ML, et al. Airway inflammation is augmented by obesity and fatty acids in asthma. *Eur Respir J.* 2011;38(3):594–602.
120. Telenga ED, Tideman SW, Kerstjens HAM, et al. Obesity in asthma: More neutrophilic inflammation as a possible explanation for a reduced treatment response. *Allergy Eur J Allergy Clin Immunol*. 2012;67(8):1060–8.

121. Kim HY, Lee HJ, Chang YJ, Pet al. Interleukin-17-producing innate lymphoid cells and the NLRP3 inflammasome facilitate obesity-associated airway hyperreactivity. *Nat Med.* 2014;20(1):54–61.
122. Williams AS, Chen L, Kasahara DI, et al. Obesity and airway responsiveness: role of TNFR2. *Pulm Pharmacol Ther*. 2013;26(4):444–54.
123. Desai D, Newby C, Symon FA, et al. Elevated sputum interleukin-5 and submucosal eosinophilia in obese individuals with. *Am J Respir Crit Care Med*. 2013;188(6):657–63.
124. Farahi N, Loutsios C, Tregay N, et al. In vivo imaging reveals increased eosinophil uptake in the lungs of obese asthmatic patients. *J Allergy Clin Immunol*. 2018;142(5):1659-1662.e8.
125. Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J Clin Invest.* 2007;117(1):175–84.
126. Tsurutani Y, Fujimoto M, Takemoto M, et al. The roles of transforming growth factor-β and Smad3 signaling in adipocyte differentiation and obesity. *Biochem Biophys Res Commun.* 2011;407(1):68–73.
127. Brestoff JR, Kim BS, Saenz SA, et al. Group 2 innate lymphoid cells promote beiging of adipose and limit obesity. *Nature.* 2015;519(7542):242.
128. Liew FY, Girard JP, Turnquist HR. Interleukin-33 in health and disease. *Nat Rev Immunol.* 2016;16(11):676–89.
129. Leiria LOS, Martins MA, Saad MJA. Obesity and asthma: Beyond TH2 inflammation. *Metabolism*. 2015;64(2):172–81.
130. Catalán V, Gómez-Ambrosi J, Rodríguez A, et al. Increased Obesity-Associated Circulating Levels of the Extracellular Matrix Proteins Osteopontin, Chitinase-3 Like-1 and Tenascin C Are Associated with Colon Cancer. *PLoS ONE.* 2016;11(9):e0162189.
131. Thomsen SB, Gjesing AP, Rathcke CN, et al. Associations of the Inflammatory Marker YKL-40 with Measures of Obesity and Dyslipidaemia in Individuals at High Risk of Type 2 Diabetes. *PLoS ONE*. 2015;10(7):e0133672.
132. Kyrgios I, Galli-Tsinopoulou A, Stylianou C, et al. Elevated circulating levels of the serum acute-phase protein YKL-40 (chitinase 3-like protein 1) are a marker of obesity and insulin resistance in prepubertal children. *Metab Clin Exp.* 2012;61(4):562–8.
133. Hempen M, Kopp HP, Elhenicky M, et al. YKL-40 is Elevated in Morbidly Obese Patients and Declines After Weight Loss. *Obes Surg.* 2009;19(11):1557–63.
134. Lee TH, Song HJ, Park CS. Role of inflammasome activation in development and exacerbation of asthma. *Asia Pac Allergy*. 2014;4(4):187.
135. Dinarello CA. Immunological and Inflammatory Functions of the Interleukin-1 Family. *Ann Rev Imunol.* 2009;27:519-550
136. Sutton CE, Lalor SJ, Sweeney CM, et al. Interleukin-1 and IL-23 Induce Innate IL-17 Production from γδ T Cells, Amplifying Th17 Responses and Autoimmunity. *Immunity*. 2009;31(2):331–41.
137. Mantovani A, Dinarello CA, Molgora M, et al. IL-1 and related cytokines in innate and adaptive immunity in health and disease. *Immunity.* 2019;50(4):778.
138. Dinarello CA, Novick D, Kim S, et al. Interleukin-18 and IL-18 binding protein. *Front Immunol*. 2013;4:289.
139. Besnard A, Couillin I, Tan Z, et al. Inflammasome – IL- 1 – Th 17 response in allergic lung inflammation. *J Mol Cell Biol.* 2011;4:3–10.
140. Simpson JL, Phipps S, Baines KJ, et al. Elevated expression of the NLRP3 inflammasome in neutrophilic asthma. *Eur Respir J.* 2014;43(4):1067–76.
141. Ozseker F, Buyukozturk S, Depboylu B, et al. Serum amyloid A (SAA) in induced sputum of asthmatics: A new look to an old marker. *Int Immunopharmacol.* 2006;6(10):1569–76.
142. Masters SL, Dunne A, Subramanian SL, et al. Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1β in type 2 diabetes. *Nat Immunol*. 2010;11(10):897–904.
143. Parisi MM, Grun LK, Lavandoski P, et al. Immunosenescence Induced by Plasma from

Individuals with Obesity Caused Cell Signaling Dysfunction and Inflammation. *Obesity.* 2017;25(9):1523–31.
144. Wood LG, Garg ML, Gibson PG. A high-fat challenge increases airway inflammation and impairs bronchodilator recovery in asthma. *J Allergy Clin Immunol*. 2011;127(5):1133–40.
145. Wood LG, Li Q, Scott HA, et al. Saturated fatty acids, obesity, and the nucleotide oligomerization domain–like receptor protein 3 (NLRP3) inflammasome in asthmatic patients. *J Allergy Clin Immunol.* 2019;143(1):305–15.
146. Kalra N, Ishmael FT. Cross-talk between vitamin D, estrogen and corticosteroids in glucocorticoid resistant asthma. *OA Inflamm*. 2014;2(1):2.
147. Boulet LP, Franssen E. Influence of obesity on response to fluticasone with or without salmeterol in moderate asthma. *Respir Med*. 2007;101(11):2240–7.
148. Peters-Golden M, Swern A, Bird SS, et al. Influence of body mass index on the response to asthma controller agents. *Eur Respir J.* 2006;27:495–503.
149. Liu J, Zhang M, Niu C, et al. Dexamethasone Inhibits Repair of Human Airway Epithelial Cells Mediated by Glucocorticoid-Induced Leucine Zipper (GILZ). *PLoS One*. 2013;8(4):1–9.
150. Pujols L, Mullol J, Picado C. Importance of glucocorticoid receptors in upper and lower airways. *Front Biosci.* 2010;15(2):789–800.
151. Stahn C, Löwenberg M, Hommes DW, et al. Molecular mechanisms of glucocorticoid action and selective glucocorticoid receptor agonists. *Mol Cell Endocrinol.* 2007;275(1–2):71–8.
152. Barnes PJ. Corticosteroids. In: Asthma and COPD: Basic Mechanisms and Clinical Management. Elsevier; 2008. pp. 639–53.
153. Duma D, Jewell CM, Cidlowski JA. Multiple glucocorticoid receptor isoforms and mechanisms of post-translational modification. *J Steroid Biochem Mol Biol.* 2006;102:11–21.
154. Lu NZ, Cidlowski JA. Glucocorticoid receptor isoforms generate transcription specificity. *Trends Cell Biol.* 2006;16(6):301–7.
155. Pujols L, Mullol J, Picado C. Alpha and beta glucocorticoid receptors: Relevance in airway diseases. *Curr Allergy and Asthma Rep*. 2007;7:93–9.
156. Nick ZLU, Cidlowski JA. The Origin and Functions of Multiple Human Glucocorticoid Receptor Isoforms. *Ann N Y Acad Sci*. 2004;1024(1):102–23.
157. Oakleyt RH, Jewell CM, Yudt MR, et al. The dominant negative activity of the human glucocorticoid receptor β isoform. Specificity and mechanisms of action. *J Biol Chem*. 1999;274(39):27857–66.
158. Ito K, Chung KF, Adcock IM. Update on glucocorticoid action and resistance. *J Allergy Clin Immunol*. 2006;117(3):522–43.
159. Clark AR. Anti-inflammatory functions of glucocorticoid-induced genes. *Mol Cell Endocrinol.* 2007;275(1–2):79–97.
160. Kassel O, Herrlich P. Crosstalk between the glucocorticoid receptor and other transcription factors: Molecular aspects. *Mol Cell Endocrinol.* 2007;275:13–29.
161. Zhang Y, Leung DYM, Goleva E. Anti-inflammatory and Corticosteroid Enhancing Actions of Vitamin D in the Monocytes of Steroid Resistant and Steroid Sensitive Asthmatics. *J Allergy Clin Immunol*. 2014;133(6):1744.
162. Lasa M, Abraham SM, Boucheron C, et al. Dexamethasone Causes Sustained Expression of Mitogen-Activated Protein Kinase (MAPK) Phosphatase 1 and Phosphatase-Mediated Inhibition of MAPK p38. *Mol Cell Biol.* 2002;22(22):7802–11.
163. Clark AR. MAP kinase phosphatase 1: a novel mediator of biological effects of glucocorticoids? *J Endocrinol*. 2003;178(1):5–12.
164. Sakai DD, Helms S, Carlstedt-Duke J, et al. Hormone-mediated repression: a negative glucocorticoid response element from the bovine prolactin gene. *Genes Dev.* 1988;2:1144-54.
165. Revollo JR, Cidlowski JA. Mechanisms Generating Diversity in Glucocorticoid Receptor Signaling. *Ann N Y Acad Sci*. 2009;1179(1):167–78.
166. Buttgereit F, Scheffold A. Rapid glucocorticoid effects on immune cells. *Steroids.* 2002;67(6):529–34.

167. Cato ACB, Nestl A, Mink S. Rapid Actions of Steroid Receptors in Cellular Signaling Pathways. *Sci STKE.* 2002;2002(138):re9.
168. Kassel O, Sancono A, Krätzschmar J, et al. Glucocorticoids inhibit MAP kinase via increased expression and decreased degradation of MKP-1. *EMBO J.* 2001;20(24):7108–16.
169. Kracht M, Saklatvala J. Transcriptional and post-transcriptional control of gene expression in inflammation. *Cytokine.* 2002;20(3):91–106.
170. Ishmael FT, Fang X, Galdiero MR, et al. Role of the RNA-binding Protein Tristetraprolin in Glucocorticoid-mediated Gene Regulation. *J Immunol.* 2008;180:8342–53.
171. Ishmael FT, Fang X, Houser KR, et al. The Human Glucocorticoid Receptor as an RNA-binding Protein: Global Analysis of Glucocorticoid Receptor-Associated Transcripts and Identification of a Target RNA Motif NIH Public Access. *J Immunol.* 2011;186(2):1189–98.
172. Smith LK, Shah RR, Cidlowski JA. Glucocorticoids modulate microRNA expression and processing during lymphocyte apoptosis. *J Biol Chem*. 2010;285(47):36698–708.
173. Zheng Y, Xiong S, Jiang P, et al. Glucocorticoids inhibit lipopolysaccharide-mediated inflammatory response by downregulating microRNA-155: a novel anti-inflammation mechanism. *Free Radic Biol Med*. 2012;52(8):1307–17.
174. Davis TE, Kis-Toth K, Szanto A, et al. Glucocorticoids suppress T cell function by upregulating microRNA 98 NIH Public Access. *Arthritis Rheum.* 2013;65(7):1882–90.
175. Torrego A, Pujols L, Roca-Ferrer J, et al. Glucocorticoid receptor isoforms alpha and beta in in vitro cytokine-induced glucocorticoid insensitivity. *Am J Respir Crit Care Med*. 2004;170(4):420–5.
176. Heialy A, Gaudet M, Ramakrishnan RK, et al. Contribution of IL-17 in Steroid Hyporesponsiveness in Obese Asthmatics Through Dysregulation of Glucocorticoid Receptors α and β. *Front Immunol*. 2020;11:1724.
177. Sutherland ER, Goleva E, Jackson LP, et al. Vitamin D Levels, Lung Function, and Steroid Response in Adult Asthma. *Am J Respir Crit Care Med*. 2010;181:699–704.
178. Dixon AE, Pratley RE, Forgione PM, et al. Effects of obesity and bariatric surgery on airway hyperresponsiveness, asthma control, and inflammation. *J Allergy Clin Immunol*. 2011;128(3):508-515.e2.
179. Bhavsar P, Hew M, Khorasani N, et al. Relative corticosteroid insensitivity of alveolar macrophages in severe asthma compared with non-severe asthma. *Thorax.* 2008;63:784–90.
180. Goleva E, Searing DA, Jackson LP, et al. Steroid requirements and immune associations with vitamin D are stronger in children than adults with asthma. *J Allergy Clin Immunol*. 2012;129(5):1243-51.
181. Goleva E, Jackson LP, Gleason M, et al. Usefulness of PBMCs to predict clinical response to corticosteroids in asthmatic patients. J *Allergy Clin Immunol*. 2012;129(3):687-693.e1.
182. Vazquez-Tello A, Halwani R, Hamid Q, et al. Glucocorticoid receptor-beta up-regulation and steroid resistance induction by IL-17 and IL-23 cytokine stimulation in peripheral mononuclear cells. *J Clin Immunol*. 2013;33(2):466–78.
183. Koetzier SC, Van Langelaar J, Blok KM, et al. Brain-homing CD4 + T cells display glucocorticoid-resistant features in MS. N*eurol Neuroimmunol Neuroinflamm*. 2020;7:894.
184. Azarsiz E, Karaca N, Ergun B, et al. In vitro T lymphocyte proliferation by carboxyfluorescein diacetate succinimidyl ester method is helpful in diagnosing and managing primary immunodeficiencies. *J Clin Lab Anal.* 2018;32(1):1–7.
185. Hakonarson H, Bjornsdottir US, Halapi E, et al. Profiling of genes expressed in peripheral blood mononuclear cells predicts glucocorticoid sensitivity in asthma patients. *Proc Natl Acad Sci*. 2005;102(41):14789–94.
186. Jeffrey C. Webster, Robert H. et al. Proinflammatory cytokines regulate human glucocorticoid receptor gene expression and lead to the accumulation of the dominant negative mechanism for the generation β isoform: A of glucocorticoid resistance. *Proc Natl Acad Sci*. 2001;84(7):1744–8.
187. Vazquez-Tello A, Semlali A, Chakir J, et al. Induction of glucocorticoid receptor-β expression in

epithelial cells of asthmatic airways by T-helper type 17 cytokines. *Clin Exp Allergy.* 2010;40(9):1312–22.
188. Alizadeh Z, Mazinani M, Mortaz E, et al. Glucocorticoid receptor nuclear translocation in CD4 T cells from severe and moderate asthmatic patients treated with fluticasone/vilanterol. *Iran J Allergy, Asthma Immunol*. 2018;17(1):1–8.
189. Hawrylowicz CM. Regulatory T cells and IL-10 in allergic inflammation. *J Exp Med.* 2005;202(11):1459–63.
190. Alcorn JF, Crowe CR, Kolls JK. TH17 Cells in Asthma and COPD. *Annu Rev Physiol*. 2010;72:495-516.
191. McKinley L, Alcorn JF, Peterson A, et al. TH17 Cells Mediate Steroid-Resistant Airway Inflammation and Airway Hyperresponsiveness in Mice. *J Immunol.* 2008;181(6):4089.
192. Irusen E, Matthews JG, Takahashi A, et al. p38 mitogen-activated protein kinase-induced glucocorticoid receptor phosphorylation reduces its activity: Role in steroid-insensitive asthma. *J Allergy Clin Immunol.* 2002;109(4):649–57.
193. Naseer T, Minshall EM, Leung DYM, et al. Expression of IL-12 and IL-13 mRNA in asthma and their modulation in response to steroid therapy. *Am J Respir Crit Care Med*. 1997;155(3):845–51.
194. Leung DYM, Martin RJ, Szefler SJ, et al. Dysregulation of interleukin 4, interleukin 5, and interferon gamma gene expression in steroid-resistant asthma. *J Exp Med*. 1995;181(1):33.
195. Gold DR, Litonjua AA, Carey VJ, et al. Lung VITAL: Rationale, design, and baseline characteristics of an ancillary study evaluating the effects of vitamin D and/or marine omega-3 fatty acid supplements on acute exacerbations of chronic respiratory disease, asthma control, pneumonia and lung function in adults HHS Public Access. *Contemp Clin Trials.* 2016;47:185–95.
196. Jaiswal A, Dash D, Singh R. Intranasal curcumin and dexamethasone combination ameliorates inflammasome (NLRP3) activation in lipopolysachharide exposed asthma exacerbations. *Toxicol Appl Pharmacol*. 2022;436:115861.
197. Whyand T, Hurst JR, Beckles M, et al. Pollution and respiratory disease: can diet or supplements help? A review. *Respir Res*. 2018;19:79.
198. Tenero L, Piazza M, Zanoni L, et al. Antioxidant supplementation and exhaled nitric oxide in children with asthma. *Allergy Asthma Proc.* 2016;37(1):e8–13.
199. Holick MF. Vitamin D deficiency. *N Eng J Med*. 2007;357:266–81.
200. Elkhwanky MS, Kummu O, Piltonen TT, et al. Obesity Represses CYP2R1, the Vitamin D 25-Hydroxylase, in the Liver and Extrahepatic Tissues. *JBMR Plus.* 2020;4(11):1–14.
201. Miclea A, Bagnoud M, Chan A,et al. A Brief Review of the Effects of Vitamin D on Multiple Sclerosis. *Front Immunol.* 2020;11:781.
202. Mann EH, Chambers ES, Pfeffer PE, et al. Immunoregulatory mechanisms of vitamin D relevant to respiratory health and asthma. *Ann N Y Acad Sci.* 2014;1317(1):57–69.
203. Quack M, Carlberg C. Ligand-triggered stabilization of vitamin D receptor/retinoid X receptor heterodimer conformations on DR4-type response elements. *J Mol Biol*. 2000;296(3):743–56.
204. Kim S, Shevde NK, Pike JW. ,25-Dihydroxyvitamin D 3 Stimulates Cyclic Vitamin D Receptor/Retinoid X Receptor DNA-Binding, Co-activator Recruitment, and Histone Acetylation in Intact Osteoblasts. *J Bone Min Res.* 2004;20:305–17.
205. Karampela I, Sakelliou A, Vallianou N, et al. Vitamin D and Obesity: Current Evidence and Controversies. *Curr Obes Rep*. 2021;10(2):162–80.
206. Zupo R, Castellana F, Sardone R, et al. Hydroxyvitamin D Serum Levels are Negatively Associated with Platelet Number in a Cohort of Subjects Affected by Overweight and Obesity. *Nutrients*. 2020;12(2):474.
207. De Pergola G, Triggiani V, Bartolomeo N,et al. Low 25 Hydroxyvitamin D Levels are Independently Associated with Autoimmune Thyroiditis in a Cohort of Apparently Healthy Overweight and Obese Subjects. *Endocrine, Metab Immune Disord*. 2018;18(6):646–52.
208. Lange NE, Litonjua A, Hawrylowicz CM, et al. Vitamin D, the immune system and asthma.

Expert Rev Clin Immunol. 2009;5(6):693.
209. Bischoff-Ferrari HA, Shao A, Dawson-Hughes B, et al. Benefit - Risk Assessment of Vitamin D Supplementation. *Osteoporos Int.* 2010;21(7):1121.
210. Christakos S, Dhawan P, Liu Y, et al. New insights into the mechanisms of vitamin D action. *J Cell Biochem*. 2003;88(4):695–705.
211. Holick MF, Binkley NC, Bischoff-Ferrari HA, et al. Evaluation, treatment, and prevention of vitamin D deficiency: An endocrine society clinical practice guideline. *J Clin Endocrinol Metab*. 2011;96(7):1911–30.
212. Holick MF. The vitamin D deficiency pandemic: Approaches for diagnosis, treatment and prevention. *Rev Endocr Metab Disord*. 2017;18(2):153–65.
213. Giustina A, Adler RA, Binkley N, et al. Consensus statement from 2nd International Conference on Controversies in Vitamin D. *Rev Endocr Metab Disord.* 2020;21(1):89–116.
214. IOM. Dietary Reference Intakes for Calcium and Vitamin D. Editors: A Catharine Ross, Christine L Taylor, Ann L Yaktine, and Heather B Del Valle.; Washington (DC): National Academies Press (US); 2011 Mar 30.
215. Ramirez AM, Wongtrakool C, Welch T, et al. Vitamin D inhibition of pro-fibrotic effects of transforming growth factor β1 in lung fibroblasts and epithelial cells. *J Steroid Biochem Mol Biol*. 2010;118(3):142.
216. Searing DA, Zhang Y, Murphy JR, et al. Decreased Serum Vitamin D Levels in Children with Asthma are Associated with Increased Corticosteroid Usage. *J Allergy Clin Immunol*. 2010;125(5):995.
217. Majak P, Olszowiec-Chlebna M, Smejda K, et al. Vitamin D supplementation in children may prevent asthma exacerbation triggered by acute respiratory infection. *J Allergy Clin Immunol*. 2011;127(5):1294–6.
218. Einisadr A, Rajabi M, Moezzi H, et al. Impact of rapid correction of vitamin D deficiency in asthmatic patients. *Wien Klin Wochenschr.* 2022;134(1–2):18–23.
219. Gupta A, Sjoukes A, Richards D, et al. Relationship between Serum Vitamin D, Disease Severity, and Airway Remodeling in Children with Asthma. *Am J Respir Crit Care Med*. 2011;184(12):1342.
220. Korn S, Hübner M, Jung M, et al. Severe and uncontrolled adult asthma is associated with vitamin D insufficiency and deficiency. Respir Res. 2013;14(1):25.
221. Gupta A, Bhat G, Pianosi P. What is New in the Management of Childhood Asthma? *Indian J Pediatr*. 2018;85:773–81.
222. Heine G, Niesner U, Chang HD, et al. 1,25-dihydroxyvitamin D3 promotes IL-10 production in human B cells. *Eur J Immunol*. 2008;38(8):2210–8.
223. Hartmann B, Heine G, Babina M, et al. Targeting the vitamin D receptor inhibits the B cell-dependent allergic immune response. *Allergy Eur J Allergy Clin Immunol.* 2011;66(4):540–8.
224. Brehm JM, Celedón JC, Soto-Quiros ME, et al. Serum Vitamin D Levels and Markers of Severity of Childhood Asthma in Costa Rica. *Am J Respir Crit Care Med.* 2009;179(9):765.
225. Rothenberg ME, Hogan SP. The eosinophil. *Ann Rev Immunol*. 2006;24:147–74.
226. Mendy A, Cohn RD, Thorne PS. Endotoxin Exposure, Serum Vitamin D, Asthma and Wheeze Outcomes. *Respir Med*. 2016;114:61–6.
227. Dimeloe S, Rice LV, Chen H, et al. Vitamin D (1,25(OH) 2 D3) induces α-1-antitrypsin synthesis by CD4 + T cells, which is required for 1,25(OH) 2 D3-driven IL-10. *J Steroid Biochem Mol Biol*. 2019;189:1–9.
228. Urry Z, Xystrakis E, Richards DF, et al. Ligation of TLR9 induced on human IL-10-secreting Tregs by 1α,25-dihydroxyvitamin D3 abrogates regulatory function. *J Clin Invest*. 2009;119(2):387–98.
229. Walsh JS, Evans AL, Bowles S, et al. Free 25-hydroxyvitamin D is low in obesity, but there are no adverse associations with bone health. *Am J Clin Nutr.* 2016;103(6):1465–71.
230. Samuel L, Borrell LN. The effect of body mass index on optimal vitamin D status in U.S. adults: The National Health and Nutrition Examination Survey 2001–2006. *Ann Epidemiol*. 2013;23(7):409–14.

231. Park JE, Pichiah PBT, Cha YS. Vitamin D and metabolic diseases: Growing roles of Vitamin D. *J Obes Metab Syndr.* 2018;27(4):223–32.
232. Wortsman J, Matsuoka LY, Chen TC, et al. Decreased bioavailability of vitamin D in obesity. *Am J Clin Nutr*. 2000;72(3):690–3.
233. Asghari S, Hamedi-Shahraki S, Amirkhizi F. Vitamin D status and systemic redox biomarkers in adults with obesity. *Clin Nutr*. 2021;45:292–8.
234. Hajhashemy Z, Shahdadian F, Ziaei R, et al. Serum vitamin D levels in relation to abdominal obesity: A systematic review and dose–response meta-analysis of epidemiologic studies. *Obes Rev*. 2021;22(2):e13134.
235. Barrea L, Muscogiuri G, Laudisio D, et al. Influence of the Mediterranean Diet on 25-Hydroxyvitamin D Levels in Adults. *Nutr.* 2020;12(5):1439.
236. Savastano S, Barrea L, Savanelli MC, et al. Low vitamin D status and obesity: Role of nutritionist. *Rev Endocr Metab Disord.* 2017;18(2):215–25.
237. Barrea L, Frias-Toral E, Pugliese G, et al. Vitamin d in obesity and obesity-related diseases: an overview. *Minerva Endocrinol.* 2021;46(2):177–92.
238. Muscogiuri G, Barrea L, Somma C Di, et al. Sex Differences of Vitamin D Status across BMI Classes: An Observational Prospective Cohort Study. *Nutrients*. 2019;11:34.
239. Aatsinki SM, Elkhwanky MS, Kummu O, et al. Fasting-Induced Transcription Factors Repress Vitamin D Bioactivation, a Mechanism for Vitamin D Deficiency in Diabetes. *Diabetes*. 2019;68(5):918–31.
240. Roizen JD, Long C, Casella A, et al. Obesity Decreases Hepatic 25-Hydroxylase Activity Causing Low Serum 25-Hydroxyvitamin D. *J Bone Miner Res.* 2019;34(6):1068.
241. Lautenbacher LA, Jariwala SP, Markowitz ME, et al. Vitamin D and pulmonary function in obese asthmatic children. *Pediatr Pulmonol*. 2016;51(12):1276–83.
242. Yadav M, Mittal K. Effect of vitamin D supplementation on moderate to severe bronchial asthma. *Indian J Pediatr*. 2014;81(7):650–4.
243. De Groot JC, Van Roon ENH, Storm H, et al. Vitamin D reduces eosinophilic airway inflammation in nonatopic asthma. *J Allergy Clin Immunol.* 2015;135(3):670-675.e3.
244. Brehm JM, Schuemann B, Fuhlbrigge AL, et al. Serum vitamin D levels and severe asthma exacerbations in the Childhood Asthma Management Program study. *J Allergy Clin Immunol*. 2010;126(1):58.e5.
245. Xystrakis E. Reversing the defective induction of IL-10-secreting regulatory T cells in glucocorticoid-resistant asthma patients. *J Clin Invest.* 2005;116(1):146–55.
246. Gupta A, Dimeloe S, Richards DF, et al. Defective IL-10 expression and in vitro steroid-induced IL-17A in paediatric severe therapy-resistant asthma. *Thorax.* 2014;69(6):508-15.
247. Nanzer AM, Chambers ES, Ryanna K, et al. Enhanced production of IL-17A in patients with severe asthma is inhibited by 1α,25-dihydroxyvitamin D3 in a glucocorticoid-independent fashion. *J Allergy Clin Immunol*. 2013;132(2):297-304.e3.
248. Palmer MT, Lee YK, Maynard CL, et al. Lineage-specific effects of 1,25-dihydroxyvitamin D3 on the development of effector CD4 T cells. *J Biol Chem.* 2011;286(2):997–1004.
249. Jeffery LE, Burke F, Mura M, *Zheng Y, Qureshi OS, Hewison M*, et al. 1,25-dihydroxyvitamin D3 and interleukin-2 combine to inhibit T cell production of inflammatory cytokines and promote development of regulatory T cells expressing CTLA-4 and FoxP3. *J Immunol*. 2009;183(9):5458.
250. DeMaria EJ. Bariatric Surgery for Morbid Obesity. *N Engl J Med*. 2007;356:2176-83.
251. Van Huisstede A, Rudolphus A, Cabezas MC, et al. Effect of bariatric surgery on asthma control, lung function and bronchial and systemic inflammation in morbidly obese subjects with asthma. *Thorax*. 2015;70(7):659–67.
252. Jiménez A, Casamitjana R, Flores L, et al. Long-term effects of sleeve gastrectomy and roux-en-y gastric bypass surgery on type 2 diabetes mellitus in morbidly obese subjects. *Ann Surg.* 2012;256(6):1023–9.
253. Gastrointestinal surgery for severe obesity: National Institutes of Health Consensus Development Conference Statement. *Am J Clin Nutr*. 1992;55(2):615S-619S.

254. Dias-Júnior SA, Reis M, De Carvalho-Pinto RM, et al. Effects of weight loss on asthma control in obese patients with severe asthma. *Eur Respir J.* 2014;43(5):1368–77.
255. Stenius-Aarniala B, Poussa T, Kvarnström J, et al. Immediate and long term effects of weight reduction in obese people with asthma: Randomised controlled study. *Br Med J.* 2000;320(7238):827–32.
256. Moreira A, Bonini M, Garcia-Larsen V, et al. Weight loss interventions in asthma: EAACI evidence-based clinical practice guideline. *Allergy.* 2013;68:425–39.
257. Scott HA, Gibson PG, Garg ML, et al. Dietary restriction and exercise improve airway inflammation and clinical outcomes in overweight and obese asthma: A randomized trial. *Clin Exp Allergy*. 2013;43(1):36–49.
258. Freitas PD, Ferreira PG, Silva AG, et al. The role of exercise in a weight-loss program on clinical control in obese adults with Asthma: A randomized controlled trial. *Am J Respir Crit Care Med*. 2017;195(1):32–42.
259. Freitas PD, Silva AG, Ferreira PG, et al. Exercise Improves Physical Activity and Comorbidities in Obese Adults with Asthma. *Med Sci Sports Exerc*. 2018;50(7):1367–76.
260. Dixon JB, Chapman L, O'Brien P. Marked improvement in asthma after Lap-Band® surgery for morbid obesity. *Obes Surg.* 1999;9(4):385–9.
261. Maniscalco M, Zedda A, Faraone S, et al. Weight loss and asthma control in severely obese asthmatic females. *Respir Med*. 2008;102(1):102–8.
262. Van Huisstede A, Rudolphus A, van Schadewijk A, et al. Bronchial and Systemic Inflammation in Morbidly Obese Subjects with Asthma: A Biopsy Study. *Am J Respir Crit Care Med*. 2014;190(8):951–4.
263. Reddy RC, Baptist AP, Fan Z, et al. The effects of bariatric surgery on asthma severity. *Obes Surg.* 2011;21(2):200–6.
264. Sikka N, Wegienka G, Havstad S, et al. Respiratory medication prescriptions before and after bariatric surgery. *Ann Allergy Asthma Immunol.* 2010;104(4):326–30.
265. Boulet LP, Turcotte H, Martin J, et al. Effect of bariatric surgery on airway response and lung function in obese subjects with asthma. *Respir Med*. 2012;106(5):651–60.
266. Maniscalco M, Zamparelli AS, Vitale DF, et al. Long-term effect of weight loss induced by bariatric surgery on asthma control and health related quality of life in asthmatic patients with severe obesity: A pilot study. *Respir Med*. 2017;130:69–74.
267. Buscemi S, Buscemi C, Corleo D, et al. Obesity and Circulating Levels of Vitamin D before and after Weight Loss Induced by a Very Low-Calorie Ketogenic Diet. *Nutrients.* 2021;13(6):1829.
268. Liu C, Wu D, Zhang JF, et al. Changes in Bone Metabolism in Morbidly Obese Patients After Bariatric Surgery: A Meta-Analysis. *Obes Surg.* 2016;26(1):91–7.
269. Li Z, Zhou X, Fu W. Vitamin D supplementation for the prevention of vitamin D deficiency after bariatric surgery: a systematic review and meta-analysis. *Eur J Clin Nutr*. 2018;72(8):1061–70.
270. Tian Z, Fan XT, Li SZ,et al. Changes in Bone Metabolism After Sleeve Gastrectomy Versus Gastric Bypass: a Meta-Analysis. *Obes Surg*. 2020;30(1):77–86.
271. Gletsu-Miller N, Wright BN. Mineral Malnutrition Following Bariatric Surgery. *Adv Nutr*. 2013;4(5):506.
272. Chakhtoura MT, Nakhoul NN, Shawwa K, et al. Hypovitaminosis D in bariatric surgery: A systematic review of observational studies. *Metabolism.* 2016;65(4):574–85.
273. Mechanick JI, Apovian C, Brethauer S, et al. Clinical practice guidelines for the perioperative nutrition, metabolic, and nonsurgical support of patients undergoing bariatric procedures - 2019 update. *Surg Obes Relat Dis*. 2020;16(2):175–247.
274. Sood A, Ford ES, Camargo Jr CA. Association between leptin and asthma in adults. *Thorax*. 2006;61:300–5.
275. Quek YW, Sun HL, Ng YY, et al. Associations of Serum Leptin with Atopic Asthma and Allergic Rhinitis in Children. *Am J Rhinol Allergy.* 2010;24(5):354–8.
276. Ozde C, Dogru M, Erdogan F, et al. The relationship between adiponectin levels and epicardial adipose tissue thickness in non-obese children with asthma. *Asian Pac J Allergy Immunol*.

2015;33:289–95.
277. Hayashikawa Y, Iwata M, Inomata M, et al. Association of serum adiponectin with asthma and pulmonary function in the Japanese population. *Endocr J.* 2015;62(8):695–709.
278. Vezir E, Civelek E, Dibek Misirlioglu E, et al. Effects of Obesity on Airway and Systemic Inflammation in Asthmatic Children. *Int Arch Allergy Immunol*. 2021;182(8):679–89.
279. Masuzaki H, Ogawa Y, Sagawa N, et al. Nonadipose tissue production of leptin: Leptin as a novel placenta-derived hormone in humans. *Nat Med*. 1997;3(9):1029–33.
280. Barrios-Correa AA, Estrada JA, Contreras I. Leptin Signaling in the Control of Metabolism and Appetite: Lessons from Animal Models. *J Mol Neurosci*. 2018;66(3):390–402.
281. Ruxandra Otelea M, Arghir OC, Zugravu C, et al. Adiponectin and Asthma: Knowns, Unknowns and Controversies. *Int J Mol Sci.* 2021;22(16): 8971.
282. Aydin M, Koca C, Ozol D, et al. Interaction of Metabolic Syndrome with Asthma in Postmenopausal Women: Role of Adipokines. *Inflamm*. 2013;36(6):1232–8.
283. Lee YA, Hahm DH, Kim JY, et al. Potential therapeutic antibodies targeting specific adiponectin isoforms in rheumatoid arthritis. *Arthritis Res Ther.* 2018;20(1).
284. Guebre-Egziabher F, Drai J, Fouque D. Adiponectin and Chronic Kidney Disease. *J Ren Nutr.* 2007;17(1):9–12.
285. Dogru M, Ozde S, Aktas A, et al. The adiponectin levels and asthma control in non-obese children with asthma. *J Asthma*. 2015;52(8):772–6.
286. Bantulà M, Roca-Ferrer J, Arismendi E, et al. Asthma and Obesity: Two Diseases on the Rise and Bridged by Inflammation. *J Clin Med*. 2021;10(2):1–21.
287. Arismendi E, Bantulà M, Perpiñá M, et al. Effects of Obesity and Asthma on Lung Function and Airway Dysanapsis in Adults and Children. *J Clin Med*. 2020;9(11):3762.
288. Jensen ME, Gibson PG, Collins CE, et al. Airway and systemic inflammation in obese children with asthma. *Eur Respir J*. 2013;42(4):1012–9.
289. de Lima Azambuja R, da Costa Santos Azambuja LSE, Costa C, et al. Adiponectin in Asthma and Obesity: Protective Agent or Risk Factor for More Severe Disease? *Lung*. 2015;193(5):749–55.
290. Carolan E, Tobin LM, Mangan BA, et al. Altered Distribution and Increased IL-17 Production by Mucosal-Associated Invariant T Cells in Adult and Childhood Obesity. *J Immunol*. 2015;194(12):5775–80.
291. Capelo AV, da Fonseca VM, Peixoto MVM, et al. Visceral adiposity is associated with cytokines and decrease in lung function in women with persistent asthma. *Rev Port Pneumol*. 2016;22(5):255–61.
292. Ma C, Wang Y, Xue M. Correlations of severity of asthma in children with body mass index, adiponectin and leptin. *J Clin Lab Anal*. 2019;33(6):e22915.
293. Park YH, Oh EY, Han H, et al. Insulin resistance mediates high-fat diet-induced pulmonary fibrosis and airway hyperresponsiveness through the TGF-β1 pathway. *Exp Mol Med*. 2019;51–9.
294. Wang CH, Huang C Da, Lin HC, et al. Increased Circulating Fibrocytes in Asthma with Chronic Airflow Obstruction. *Am J Respir Crit Care Med.* 2008;178(6):583-91.
295. Alessi MC, Bastelica D, Morange P, et al. Plasminogen activator inhibitor 1, transforming growth factor-beta1, and BMI are closely associated in human adipose tissue during morbid obesity. *Diabetes.* 2000;49(8):1374–80.
296. Lee MJ. Transforming growth factor beta superfamily regulation of adipose tissue biology in obesity. *Biochim Biophys Acta.* 2018;1864(4):1160–71.
297. Minshall EM, Leung DYM, Martin RJ, et al. Eosinophil-associated TGF-1 mRNA Expression and Airways Fibrosis in Bronchial Asthma. *Am J Respir Cell Mol Biol.* 1997;17(3):326-33.
298. Torrego A, Hew M, Oates T, et al. Expression and activation of TGF-β isoforms in acute allergen-induced remodelling in asthma. *Thorax.* 2007;62(4):307.
299. Fong CY, Pang L, Holland E, et al. TGF-1 stimulates IL-8 release, COX-2 expression, and PGE 2 release in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol.* 2000;279(1):L201-7.

300. Dostert C, Grusdat M, Letellier E, et al. The TNF Family of Ligands and Receptors: Communication Modules in the Immune System and Beyond. *Physiol Rev*. 2019;99:115–60.
301. Ohno I, Nitta Y, Yamauchi K, Hoshi H, et al. Transforming growth factor beta 1 (TGF beta 1) gene expression by eosinophils in asthmatic airway inflammation. *Am J Respir Cell Mol Biol.* 1996;15(3):404-9.
302. Kelley J, Fabisiak JP, Hawes K, et al. Cytokine signaling in lung: transforming growth factor-beta secretion by lung fibroblasts. Am J Physiol. 1991;260:L123-8.
303. Eslick S, Williams EJ, Berthon BS, et al. Chain Fatty Acids Reduce Systemic Inflammation in Monocytes and Adipose Tissue Macrophages from Obese Subjects. *Nutrients*. 2022;14(4):765.
304. Golikova EA, Lopatnikova JA, Kovalevskaya-Kucheryavenko TV, et al. Levels of TNF, TNF autoantibodies and soluble TNF receptors in patients with bronchial asthma. *J Asthma.* 2013;50(7):705–11.
305. Hurrell BP, Galle-Treger L, Jahani PS, et al. TNFR2 Signaling Enhances ILC2 Survival, Function, and Induction of Airway Hyperreactivity. *Cell Rep*. 2019;29(13):4509.
306. Girard-Guyonvarc'h C, Harel M, Gabay C. The Role of Interleukin 18/Interleukin 18-Binding Protein in Adult-Onset Still's Disease and Systemic Juvenile Idiopathic Arthritis. *J Clin Med*. 2022;11(2):430.
307. Esposito K, Pontillo A, Ciotola M, et al. Weight Loss Reduces Interleukin-18 Levels in Obese Women. *J Clin Endocrinol Metab*. 2002;87(8):3864–6.
308. Sousa AR, Lane SJ, Nakhosteen JA, et al. Increased expression of the monocyte chemoattractant protein-1 in bronchial tissue from asthmatic subjects. *Am J Respir Cell Mol Biol.* 2012;10(2):142–7.
309. Kurashima K, Mukaida N, Fujimura M, et al. Increase of chemokine levels in sputum precedes exacerbation of acute asthma attacks. *J Leukoc Biol*. 1996;59(3):313–6.
310. Gonzalo JA, Lloyd CM, Wen D, et al. The Coordinated Action of CC Chemokines in theLung Orchestrates Allergic Inflammation andAirway Hyperresponsiveness. *J Exp Med*. 1998;188(1):157.
311. Kolattukudy PE, Niu J. Inflammation, Endoplasmic Reticulum Stress, Autophagy, and the Monocyte Chemoattractant Protein-1/CCR2 Pathway. *Circ Res.* 2012;110(1):174-89.
312. Sachan A, Singh A, Shukla S, et al. An immediate post op and follow up assessment of circulating adipo-cytokines after bariatric surgery in morbid obesity. *Metab Open*. 2022;13:100147.
313. Al-Sajee D, Sehmi R, Hawke TJ, et al. Expression of IL-33 and TSLP and their receptors in asthmatic airways after inhaled allergen challenge. *Am J Respir Crit Care Med*. 2018;198(6):805–7.
314. Préfontaine D, Nadigel J, Chouiali F, et al. Increased IL-33 expression by epithelial cells in bronchial asthma. *J Allergy Clin Immunol*. 2010;125(3):752–4.
315. Li C, Spallanzani RG, Mathis D. Visceral adipose tissue Tregs and the cells that nurture them. *Immunol Rev.* 2020;295(1):114–25.
316. Patil SP, Wisnivesky JP, Busse PJ, et al. Detection of immunological biomarkers correlated with asthma control and quality of life measurements in sera from chronic asthmatic patients. *Ann Allergy Asthma Immunol*. 2011;106(3):205–13.
317. Tanaka H, Miyazaki N, Oashi K, et al. IL-18 might reflect disease activity in mild and moderate asthma exacerbation. *J Allergy Clin Immunol*. 2001;107(2):331–6.
318. Camiolo MJ, Zhou X, Wei Q, et al. Machine learning implicates the IL-18 signaling axis in severe asthma. J*CI Insight.* 2021;6(21):e149945.
319. Rivellese F, Paloczi J, Heffler E, et al. Interleukin-5 in the Pathophysiology of Severe Asthma. *Pathophysiol Sev Asthma Front Physiol*. 2019;10:1514.
320. Price DB, Rigazio A, Campbell JD, et al. Blood eosinophil count and prospective annual asthma disease burden: a UK cohort study. *Lancet Respir Med*. 2015;3(11):849–58.
321. Kawaguchi K, Asano S. Pathophysiological Roles of Actin-Binding Scaffold Protein, Ezrin. *Int J Mol Sci*. 2022;2022:3246.

322. Jia M, Yan X, Jiang X, et al. Ezrin, a membrane cytoskeleton cross-linker protein, as a marker of epithelial damage in asthma. *Am J Respir Crit Care Med.* 2019;199(4):496–507.
323. Lopez JP, Turner JR, Philipson LH. Glucose-induced ERM protein activation and translocation regulates insulin secretion. *Am J Physiol - Endocrinol Metab.* 2010;299(5):E772.
324. Chupp GL, Lee CG, Jarjour N, et al. A Chitinase-like Protein in the Lung and Circulation of Patients with Severe Asthma. *N Engl J Med.* 2007;357(20):2016–27.
325. Kimura H, Shimizu K, Tanabe N, et al. Further evidence for association of YKL-40 with severe asthma airway remodeling. *Ann Allergy, Asthma Immunol.* 2022;128(6):682-688.e5
326. Specjalski K, Chełmińska M, Jassem E. YKL-40 Protein Correlates with the Phenotype of Asthma. *Lung.* 2015;193(2):189–94.
327. Catalán V, Gómez-Ambrosi J, Rodríguez A, et al. Increased circulating and visceral adipose tissue expression levels of YKL-40 in obesity-associated type 2 diabetes are related to inflammation: impact of conventional weight loss and gastric bypass. *J Clin Endocrinol Metab.* 2011;96(1):200–9.
328. Ilmarinen P, Tuomisto LE, Niemelä O, et al. YKL-40 and adult-onset asthma: Elevated levels in clusters with poorest outcome. *J Allergy Clin Immunol Pract.* 2019;7(7):2466-2468.e3.
329. Konradsen JR, James A, Nordlund B, et al. The chitinase-like protein YKL-40: A possible biomarker of inflammation and airway remodeling in severe pediatric asthma. *J Allergy Clin Immunol.* 2013;132(2):328-35.e5.
330. Thomsen SB, Rathcke CN, Jørgensen NB, et al. Effects of Roux-en-Y gastric bypass on fasting and postprandial levels of the inflammatory markers YKL-40 and MCP-1 in patients with type 2 diabetes and glucose tolerant subjects. *J Obes.* 2013;2013:361781.
331. Ciccia F, Guggino G, Ferrante A, et al. Interleukin-9 and T helper type 9 cells in rheumatic diseases. *Clin Exp Immunol.* 2016;185(2):125.
332. Elyaman W, Bradshaw EM, Uyttenhove C, et al. IL-9 induces differentiation of TH17 cells and enhances function of FoxP3+ natural regulatory T cells. *Proc Natl Acad Sci*. 2009;106(31):12885–90.
333. Veldhoen M, Uyttenhove C, van Snick J, et al. Transforming growth factor-β “reprograms” the differentiation of T helper 2 cells and promotes an interleukin 9–producing subset. *Nat Immunol.* 2008;19;9(12):1341–6.
334. Dardalhon V, Awasthi A, Kwon H, et al. Interleukin 4 inhibits TGF-β-induced-Foxp3+T cells and generates, in combination with TGF-β, Foxp3− effector T cells that produce interleukins 9 and 10. *Nat Immunol*. 2008;9(12):1347.
335. Deng Y, Wang Z, Chang C, et al. Th9 cells and IL-9 in autoimmune disorders: Pathogenesis and therapeutic potentials. *Hum Immunol.* 2017;78(2):120–8.
336. Sehra S, Yao W, Nguyen ET, et al. TH9 cells are required for tissue mast cell accumulation during allergic inflammation. *J Allergy Clin Immunol.* 2015;136(2):433-440.e1.
337. Dhanraj P, Van Heerden MB, Pepper MS, et al. Sexual Dimorphism in Changes That Occur in Tissues, Organs and Plasma during the Early Stages of Obesity Development. *Biology (Basel).* 2021;10(8):717.
338. Anderson WJ, Lipworth BJ. Does body mass index influence responsiveness to inhaled corticosteroids in persistent asthma? *Ann Allergy, Asthma Immunol*. 2012;108(4):237–42.
339. Boulet LP, Franssen E. Influence of obesity on response to fluticasone with or without salmeterol in moderate asthma. *Respir Med*. 2007;101(11):2240–7.
340. Dixon AE, Holguin F, Sood A, et al. An official American thoracic society workshop report: Obesity and asthma. *Proc Am Thorac Soc.* 2010;7(5):325–35.
341. Taylor B, Mannino D, Brown C, et al. Body mass index and asthma severity in the National Asthma Survey. *Thorax*. 2008;63(1):14–20.
342. Szentpetery SE, Han YY, Brehm JM, et al. Vitamin D insufficiency, plasma cytokines, and severe asthma exacerbations in school-aged children. *J allergy Clin Immunol Pract*. 2018;6(1):289-291.e2.
343. Pfaffl MW, Tichopad A, Prgomet C, et al. Determination of stable housekeeping genes,

differentially regulated target genes and sample integrity: BestKeeper - Excel-based tool using pair-wise correlations. *Biotechnol Lett.* 2004;26(6):509–15.

344. Silver N, Best S, Jiang J, et al. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Mol Biol*. 2006 Oct 6;7.
345. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. 2002;3(7):research0034.1.
346. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res*. 2004;64(15):5245–50.
347. Nakayama T, Okada N, Yoshikawa M, et al. Assessment of suitable reference genes for RT-qPCR studies in chronic rhinosinusitis. *Sci Rep*. 2018;8(1):1–9.
348. Bas A, Forsberg G, Hammarström S, et al. Utility of the housekeeping genes 18S rRNA, β-actin and glyceraldehyde-3-phosphate-dehydrogenase for normalization in real-time quantitative reverse transcriptase-polymerase chain reaction analysis of gene expression in human T lymphocytes. *Scand J Immunol*. 2004;59(6):566–73.
349. Glare EM, Divjak M, Bailey MJ. β-Actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels. *Thorax.* 2002;57(9):765-70.
350. Barnes PJ. Corticosteroid resistance in patients with asthma and chronic obstructive pulmonary disease. *J Allergy Clin Immunol*. 2013;131(3):636–45.
351. Dias-Júnior SA, Stelmach R, Pinto RC, et al. Effects Of Weight Reduction In Obese People With Severe Asthma: Randomised Controlled Study. *Am Thorac Soc.* 2011;A4315–A4315.
352. Cassim R, Russell MA, Lodge CJ, et al. The role of circulating 25 hydroxyvitamin D in asthma: a systematic review. *Allergy.* 2015;70(4):339–54.
353. Vo P, Bair-Merritt M, Camargo CA. The potential role of vitamin D in the link between obesity and asthma severity/control in children. *Expert Rev Respir Med.* 2015;9(3):309–25.
354. Martineau AR, Cates CJ, Urashima M, et al. Vitamin D for the management of asthma. *Cochrane Database Syst Rev*. 2016;2016(9):CD011511.
355. Kumar J, Kumar P, Goyal JP, et al. Vitamin D supplementation in childhood asthma: a systematic review and meta-analysis of randomised controlled trials. *ERJ Open Res.* 2022;8(1):662-2021.
356. O'Sullivan BP, James L, Majure JM, et al. Obesity-related asthma in children: A role for vitamin D. *Pediatr Pulmonol.* 2021;56(2):354–61.
357. Zhang Y, Leung DYM, Richers BN, et al. Vitamin D Inhibits Monocyte/macrophage Pro-inflammatory Cytokine Production by Targeting Mitogen-Activated Protein Kinase Phosphatase 1. *J Immunol*. 2012;188(5):2127.
358. Dias ASO, Santos ICL, Delphim L, et al. Serum leptin levels correlate negatively with the capacity of vitamin D to modulate the in vitro cytokines production by CD4 + T cells in asthmatic patients. *Clin Immunol*. 2019;205:93–105.
359. Sheikh V, Kasapoglu P, Zamani A, et al. Vitamin D3 inhibits the proliferation of T helper cells, downregulate CD4+ T cell cytokines and upregulate inhibitory markers. *Hum Immunol*. 2018;79(6):439–45.