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ENDOKANABINOIDNI SISTEM PRI BOLNIKIH Z ASTMO IN VPLIV KANABINOIDOV NA MODULACIJO VNETNEGA ODZIVA

THE ENDOCANNABINOID SYSTEM IN ASTHMA PATIENTS AND THE EFFECT OF CANNABINOIDS IN THE MODULATION OF INFLAMMATORY RESPONSE

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I dedicate this work to my loving parents.

ABSTRACT

Asthma is a chronic inflammatory condition characterised by intermittent and reversible airflow obstruction caused by inflammation, bronchospasm, and increased airway secretions. Questions about the endocannabinoid system's function in asthma pathogenesis have arisen as evidence grows, demonstrating it is a native modulator of immune functions.

The main goal of this study was to genetically characterise the endocannabinoid system in naive asthma patients and determine if there is a relationship between endogenous cannabinoids and their inflammatory response. We studied a case-control cohort of 353 patients with mild/moderate persistent asthma and 276 controls. The mRNA expression levels of the selected genes were quantified in peripheral blood mononuclear cells (PBMCs) and N-acylethanolamines (NAEs) quantified from plasma samples. Our results revealed that the genes for the cannabinoid receptor 1 (CNR1) and 2 (CNR2), along with genes for the enzymes N-arachidonoyl phosphatidylethanolamine - phospholypase D (NAPEPLD), α,β-hydrolase 4 (ABHD4) and monoacylglycerol lipase (MGLL) were up-regulated in asthma patients and associated with their clinical and inflammatory condition. In addition, two of the genotyped polymorphisms located in the CNR2 gene were also associated with worse clinical symptoms. Palmitoylethanolamide (PEA) levels were lower and significantly different between allergic asthma patients and the control group and associated with worse clinical symptoms. Furthermore, our findings indicate that asthma patients with high CNR1 mRNA expression levels at the time of diagnosis, treated with leukotriene receptor antagonist (LTRA), have better treatment response, while asthma patients with high CNR1 mRNA expression levels, treated with inhaled corticosteroids (ICS), had worse treatment response. Long-term ICS or LTRA therapy reduced mRNA expression of CNR1 together with IL4 and IL5.

It is evident from these findings that the endocannabinoid system plays a role in asthma, but it is not possible to determine whether this up-regulation is a cause or a result of the condition. Nonetheless, our findings add to a better understanding of the endocannabinoid system's significance in asthma pathogenesis.

KEYWORDS: Asthma, Endocannabinoid system, Cannabinoids, Inflammation, Molecular genetics

POVZETEK

Endokanabinoidni sistem pri bolnikih z astmo in vpliv kanabinoidov na modulacijo vnetnega odziva.

Uvod

Astma je dolgotrajna kronična vnetna bolezen dihalnih poti, ki omejuje pretok izdihanega zraka in je ena najpogostejših nenalezljivih bolezni. Za astmo je značilna občasna in reverzibilna obstrukcija zračnega toka zaradi vnetja, bronhospazma in povečanega izločanja v dihalne poti z blagimi ali hudimi kliničnimi manifestacijami.

Farmakološko zdravljenje astme zahteva postopen pristop pri odmerjanju obstoječih zdravil ali vključitev drugih, če se ugotovi, da obvladovanje astme ni dobro nadzorovano v okviru trenutnega načrta zdravljenja. Zato zdravljenje hujše astme je pogosto potrebna kombinacija zdravil, ki skupaj vplivajo na več mehanističnih poti [1].

Človeški endokanabinoidni sistem uravnava tako centralne kot periferne organe, pri čemer vpliva na širok nabor bioloških funkcij, kot so spanje, razpoloženje, zaznavanje bolečine in užitka ter apetit. Za raziskave astme je posebej zanimiv terapevtski potencial endokanabinoidnega sistema kot nova farmakološka tarča za modulacijo imunskega sistema.

CB1 je med vrstami evolucijsko zelo ohranjen [2]. Pri ljudeh je protein CB1 kodiran z genom CNR1, ki se nahaja na kromosomu 6 [3]. Zdi se, da ima CB2 več medvrstnih variacij in je pri ljudeh kodiran z genom CNR2, ki se nahaja na kromosomu 1 [2, 3].

Endokanabinoidi so bioaktivni lipidi, ki prek kanabinoidnih receptorjev signalizirajo modulacijo funkcionalnih aktivnosti celic. Prvi odkriti endokanabinoid je bil anandamid (AEA), ki se z visoko afiniteto veže na receptor CB1 [4]. Temu je sledilo odkritje 2-arahidonoil glicerol (2-AG) s podobno afiniteto [5]. Znano je, da se AEA in 2-AG sintetizirata po potrebi in nista shranjena v sekretornih veziklih, kot so klasični nevrotransmiterji [6, 7]. Prekinitev endokanabinoidne signalizacije naj bi bil dvostopenjski proces. Prvi korak je transport endokanabinoidov čez plazemsko membrano. V celici jih nato hidrolizirajo specifični encimi.

Endokanabinoidi delujejo kot naravni modulatorji imunskih funkcij, kar pomeni, da lahko endokanabinoidni sistem služi kot terapevtska tarča pri avtoimunskih ali vnetnih boleznih. V imunskih celicah izražanje kanabinoidnih receptorjev sprožijo različni vnetni dejavniki (npr. LPS), enako pa lahko trdimo tudi o proizvodnji endokanabinoidov v imunskih celicah [8, 9].

Karakterizacija endokanabinoidnega sistema je pri bolnikih z astmo ključnega pomena, saj lahko razlike, kot so genetske spremembe (npr. genski polimorfizmi, diferencialno izražanje ali alternativno izrezovanje intronov) v receptorjih ali encimih endokanabinoidnega sistema, vplivajo na njegovo normalno delovanje in so tako delno prispevajo k patogenemu imunskemu odzivu opaženem pri bolnikih z astmo [10]. Cilj tega dela je genetsko okarakterizirati še neraziskan endokanabinoidni sistem pri naivnih bolnikih z astmo in ugotoviti, ali obstaja povezava med endogenimi kanabinoidi in njihovim vnetnim odzivom, ki ga tukaj predstavljajo bolniki z blago do zmerno persistentno astmo med starostjo 5 do 18 kavkaškega porekla.

Osrednja hipoteza tega dela je, da so spremembe, opažene v regulaciji endokanabinoidnega sistema, ki se odražajo v izražanju njegovih genov v plazemskih ravneh PBMC in NAE, pomembne determinante posameznega kliničnega profila, ki ga predstavljajo bolniki z astmo, in zagotavljajo novo terapevtsko perspektivo.

V ta namen smo si zastavili naslednje cilje:

1. *Kvantificirati nivoje izražanja genov, ki so del endokanabinoidnega sistema (receptorjev: CNR1 in CNR2; ter encimov: NAPEPLD, ABHD4, FAAH, DAGLA in MGLL), v celicah PBMC naivnih bolnikov z astmo, in jih primerjati s kontrolno skupino.*

2. *Raziskati povezavo izražanja teh genov s kliničnimi simptomi in biomarkerji vnetja ob diagnozi ter uporabnost analize izražanja genov pri napovedovanju odziva na zdravljenje z ICS ali LTRA.*

3. *Ugotoviti, kako na raven izražanja teh genov vpliva dolgotrajno zdravljenje z ICS ali LTRA.*

4. *Ugotoviti, ali genetske različice rs4237, rs2229579 in rs35761398 (ki se nahajajo v ali blizu kodirajoče regije gena CNR2) ter rs13197090 (ki se nahaja v bližini kodirajoče regije gena CNR1) prispevajo k nastanku astme, resnosti simptomov ali izidu zdravljenja.*

5. *Izvesti prvo farmakogenetsko analizo z namenom ugotoviti ali izbrane genetske različice vplivajo na odziv na zdravljenje z ICS ali LTRA.*

6. *Ugotoviti, ali so izbrane genetske variante v eQTL z ustreznim sosednjim preiskovanim genom.*

7. *Kvantificirati plazemske ravni NAE (AEA, PEA in OEA) pri naivnih bolnikih z astmo in jih primerjati s povprečno populacijo.*

8. *Raziskati korelacije plazemskih ravni AEA, PEA in OEA ter kliničnih simptomov in biomarkerjev vnetja ob diagnozi ter njihovo uporabnost pri napovedovanju odziva na zdravljenje z ICS ali LTRA.*

9. *Ugotoviti, kako je na plazemske ravni teh NAE vplivalo dolgotrajno zdravljenje z ICS ali LTRA.*

Metode

Med letoma 2008 in 2012 so bili rekrutirani bolniki z astmo iz Klinike za pediatrično medicino Splošne bolnišnice Murska Sobota in UKC Maribor v Sloveniji, kjer so se zdravili. Udeleženci (n = 353) so bili kavkazijci slovenskega porekla, stari od 5 do 18 let, z blago ali zmerno persistentno astmo, izbrani ob izpolnjevanju strogih meril za vključitev in izključitev.

Bolniki, vključeni v študijo, so bili razvrščeni v dve skupini na podlagi rezultatov alergijskega testiranja (kožni vbodni test in specifične vrednosti IgE). V podskupini alergijske astme so bili bolniki, ki so bili pozitivni na vsaj en aeroalergen (n = 235), tisti z negativnimi testi na aeroalergene pa so bili v podskupini nealergijske astme (n = 102). Kot kontrolno skupino so bili zdravi kavkazijci (brez alergij ali astme) podobnega etničnega porekla kot astmatiki, naključno izbrani iz slovenske populacije (n = 276).

Od udeležencev je bilo 64,9 % bolnikov na novo diagnosticiranih z astmo v skladu z merili Nacionalnega programa za izobraževanje in preprečevanje astme in Ameriškega torakalnega združenja (ATS) [11, 12]. Preostalih 35,1 % (n= 124) udeležencev so bili že zdravljeni bolniki, ki pa se še niso zdravili z ICS. Ti bolniki so bili vključeni samo v študijo ocenjevanja genetskega tveganja. Od 229 naivnih bolnikov, vključenih v to študijo, je 103 začelo zdravljenje z ICS in 116 je začelo zdravljenje z LTRA, kot je odločil njihov zdravnik. Klinični podatki so bili zbrani ob vključitvi v to študijo in ponovno 4-6 tednov pozneje, da bi ocenili resnost astme in učinkovitost zdravljenja. Ob vključitvi v študijo so bili odvzeti vzorci krvi in, ob soglasju, še en vzorec krvi dve leti kasneje z namenov določanja učinka dolgotrajnega zdravljenja na izražanje genov.

Geni, ki smo jih preiskovali v tej študiji, so bili: CNR1 (kanabinoidni receptor 1), CNR2 (kanabinoidni receptor 2), NAPEPLD (N-arahidonoil fosfatidiletanolamin - fosfolipaza D), ABHD4 (α, β-hidrolaza 4), FAAH (amidna hidrolaza maščobnih kislin), DAGLA (diacilglicerol lipaza α), MGLL (monoacilglicerol lipaza), IL4 (interlevkin-4), IL5 (interlevkin-5) in IL13 (interlevkin-13).

Genske transkripte smo kvantificirali s kvantitativno verižno reakcijo polimeraze v realnem času (qPCR). Genotipizacija analiziranih polimorfizmov je bila izvedena

z analizo krivulje visoke ločljivosti (HRM) po pomnoževanju s PCR. Kvantifikacijo amidov maščobnih kislin, AEA, PEA in OEA v človeški plazmi smo izvedli s tekočo kromatografijo-tandemsko masno spektrometrijo (LC-MS/MS) po ekstrakciji v trdni fazi (SPE).

Rezultati

Pred zdravljenjem je imela skupina z astmo mediano relativne ravni izražanja mRNA CNR1 in CNR2, ki je bila med 1,4 in 1,9-krat višja kot v kontrolni skupini (slika 3.2). Stopnje izražanja mRNA CNR1 so pokazale zelo šibko negativno korelacijo z FEV1/FVC (r_s = -0,147, P = 0,036). Bolniki z astmo z višjo stopnjo izražanja mRNA CNR1 so imeli povečano obstrukcijo dihalnih poti, kar je bilo bolj poudarjeno v podskupini bolnikov z alergijsko astmo (r_s = -0,263, P = 0,003).

Podobno kot ravni izražanja mRNA CNR1 so tudi ravni izražanja mRNA CNR2 pri bolnikih z astmo pokazale zelo šibko negativno korelacijo z FEV1/FVC. Bolniki z astmo z višjimi ravnmi izražanja mRNA CNR2 so imeli tudi povečano obstrukcijo dihalnih poti (r_s= -0,159, P = 0,024), kar je bilo spet bolj poudarjeno v podskupini z alergijsko astmo (r_s = -0,242, P= 0,007). Poleg tega so stopnje izražanja mRNA CNR2 pri bolnikih z astmo pokazale zelo šibko negativno korelacijo s hiperodzivnostjo dihalnih poti (r_s = -0,150, P = 0,034). Bolniki z astmo z višjimi ravnmi izražanja mRNA CNR2 so imeli povečano odzivnost dihalnih poti glede na vrednost logPC20.

Ugotovljeno je bilo tudi, da so ravni izražanja mRNA CNR1 in CNR2 povezane z ravnmi vnetja in alergijskimi markerji. Dodatne analize primerjave razmerja izražanja genov CB1 in CB2 z izražanjem genov citokinov IL-4, IL-5 kot bioloških označevalcev vnetja kažejo, da se CNR1 in delno CNR2 so-izražata (Sliki 3.3 in 3.4).

Ugotovili smo, da imajo stopnje izražanja mRNA CNR1 pri bolnikih z astmo pred zdravljenjem z ICS šibko negativno korelacijo z vrednostmi ΔFEV1 (r_s = -0,281, P = 0,014) (tabela 3.2). Za razliko pa so imele ravni izražanja mRNA CNR1 pri bolnikih z astmo pred zdravljenjem z LTRA šibko pozitivno korelacijo z vrednostmi ΔFEV1/FVC (r_s = 0,229, P = 0,019) in so ostale značilne le v podskupini nealergijske astme (r_s = 0,344, P = 0,034) po stratifikaciji v fenotipe (tabela 3.3).

Po recesivnem modelu asociacijske študije je bila frekvenca genotipa rs4237 (CNR2) CC v skupini z astmo bistveno nižja kot v kontrolni skupini (P = 0,017) (slika 3.7, tabela 3.4 in tabela 3.5). Genotip rs35761398 (CNR2) je bil po splošnem genetskem modelu povezan tudi s tveganjem za astmo (P = 0,040) in alergijsko astmo (P = 0,039), ne pa z nealergijsko astmo (P = 0,140) (slika 3.8, tabela 3.6 in tabela 3.7).

Naivni bolniki z astmo z genotipom rs4237 CC so imeli višji FEV1 kot tisti, ki so imeli genotip TT (96,1 % proti 89,0 %, P = 0,012), kar je potrdil GLM s prilagoditvijo glede na starost in spol (P_{GLM} = 0,006) (Slika 3.10). Povezava med polimorfizmom rs35761398 in funkcijo pljuč je bila značilna tudi v skupini z astmo, kjer so imeli bolniki, ki so nosilci alela Q, višji FEV1 (92,7±0,9%) kot tisti, ki nosijo alel R (90,1±0,7%, P = 0,018) (slika 3.11). Vendar ta povezava ni bila potrjena s prilagoditvijo GLM glede na starost in spol (P_{GLM} = 0,432).

Podobno naši rezultati kažejo, da imajo bolniki z astmo, ki nosijo alel T rs2229579, večjo stopnjo disfunkcije dihalnih poti, kvantificirano s testiranjem bronhoprovokacije (logPC20; -0,549±0,088), kot tisti, ki so nosilci alela C (-0,3747±0,026) (slika 3.12). Vendar pa ta povezava ni bila potrjena s prilagajanjem GLM glede na starost in spol (P_{GLM} = 0,076).

Pred zdravljenjem je imela skupina z astmo mediano relativne ravni izražanja mRNA NAPEPLD in ABHD4, ki so bile med 1,22 in 1,35-krat višje od kontrolne skupine (slika 3.19). Medtem so bile stopnje izražanja mRNA FAAH v vseh skupinah podobne. Mediana relativne ravni izražanja mRNA MGLL je bila med 1,21 in 1,33-krat višja od kontrolne skupine (slika 3.23), medtem ko je bila mediana relativne ravni izražanja mRNA DAGLA v skupini z astmo 0,85-kratna od kontrolne skupine.

Bolniki z astmo so imeli mediane ravni izražanja mRNA IL4 in IL5, ki so bile 0,48 oziroma 0,35-kratne ravni, izmerjene pred zdravljenjem. Ravni izražanja mRNA CNR1 so se prav tako zmanjšale na 0,75-krat od ravni, izmerjene pred zdravljenjem. Prav tako je je bilo po dolgotrajnem zdravljenju z ICS nižje (za 0,75-krat) izražanje gena, ki kodira NAPE-PLD, enega izmed glavnih encimov, ki sodelujejo pri sintezi AEA. V nasprotju se je izražanje gena ABHD4 povečala za 1,25-krat. Drugi encimi, katerih izražanjem genov se je povečala, so bili FAAH (za 1,27-krat) in MGLL (za 1,35-krat), oba sodelujeta pri presnovi endokanabinoidov.

Po dolgotrajnem zdravljenju z LTRA je prišlo do pomembnih sprememb v ravneh izražanja mRNA kvantificiranih genov (slika 3.27). Bolniki z astmo so imeli mediane ravni izražanja mRNA IL4, IL5 in IL13, ki so bile 0,23, 0,07 oziroma 0,53-kratne v primerjavi z ravnmi izmerjenimi pred zdravljenjem. Ravni izražanja mRNA CNR1 so se prav tako zmanjšale na 0,19-krat od ravni, izmerjene pred zdravljenjem. Kar zadeva gensko izražanjem analiziranih encimov, se je po dolgotrajnem zdravljenju z LTRA le NAPEPLD zmanjšal na 0,45-kratno vrednost.

Mediane plazemske koncentracije PEA pred zdravljenjem so se v skupini z astmo zmanjšale za 45 % v primerjavi s kontrolami (P = 0,034, slika 3.28). Plazemske ravni PEA so pokazale zmerno negativno korelacijo s FeNO (r_s = -0,431, P = 0,01). Po zdravljenju z LTRA smo s pomočjo parne analize ugotovili, da so se plazemske

ravni PEA v podskupini alergijske astme še dodatno znižale (slika 3.30). Nasprotno, čeprav pa povezava ni statistično značilna, so se plazemske ravni PEA po zdravljenju z LTRA povečale v podskupini nealergijske astme.

Diskusija

Odkrili smo, da je bila v PBMC bolnikov z astmo (alergičnimi in nealergičnimi) v času diagnoze povečana raven izražanja mRNA genov za oba kanabinoidna receptorja (CNR1 in CNR2) ter njihovih beljakovin (CB1 in CB2) v primerjavi z zdravimi kontrolami. Pri nekaterih kroničnih boleznih lahko povečanje ravni izražanja kanabinoidnih receptorjev zmanjša simptome in/ali zavira napredovanje bolezni. IL-4 je povečal raven izražanja mRNA CNR1 in beljakovin v primarnih človeških T-107 celicah in Jurkat T-celicah [13]. IFN-γ in GM-CSF sta povišala ravni izražanjem mRNA CNR2 v mikroglialnih celicah miši [14].

Polimorfizmi, ki se nahajajo v območju gena CNR2, so bili povezani s tveganjem za astmo, resnostjo in odzivom na zdravljenje na ICS in LTRA. Nosilci T alela rs4237 imajo 1,7-krat večjo verjetnost, da bodo razvili alergijsko astmo, kar kaže na tveganje, ki daje vlogo tega SNP. Ker rs35761398 povzroči spremembo aminokislinske strukture CB2, je verjetno, da je rs35761398 vzročna varianta, rs4237 pa le reprezentativni SNP haplobloka.

Ugotovili smo, da so bile ravni izražanja genov NAPE-PLD (NAPEPLD) in Abhd4 (ABHD4) višje pri naivnih bolnikih z astmo kot v kontrolni skupini. Ko je NAPE-PLD v celicah prekomerno izražen, zmanjša NAPE, hkrati pa poveča ravni AEA [15]. Povečane ravni izražanja ABHD4 pri bolnikih z astmo, izmerjene v naši študiji, so lahko posledica prisotnosti kroničnega vnetja v dihalnih poteh in kot odziv na poškodovane epitelijske celice. Pokazalo se je, da farmakološko ali genomsko blokiranje FAAH povzroči zvišanje bazalnih ravni AEA in preprečuje hidrolizacijo eksogeno oskrbljene AEA [16, 17, 18, 19]. Vendar pa so bile ravni izražanja genov FAAH in AEA v plazmi podobne med bolniki z astmo in kontrolnimi skupinami. V prisotnosti dražljaja se AEA večinoma tvori in sprošča lokalno ter ga sosednje celice takoj ponovno prevzamejo, da se hidrolizira, kar lahko pojasni, zakaj plazemske ravni AEA pri bolnikih z astmo ostanejo primerljive brez dražljaja s tistimi v kontrolni skupini.

Naša študija kaže, da se je izražanje DAGLa zmanjšalo, vendar se je izražanje MAGL povečalo pri bolnikih z astmo v primerjavi s kontrolno skupino. Zaradi nejasne vloge 2-AG pri vzdrževanju imunske homeostaze [20] je težko ugibati, ali ravnovesje med izražanjem DAGLA in MGLL prispeva k astmi ali je posledica astme.

Prvič smo dokazali, da so plazemske koncentracije PEA pri bolnikih z astmo znižane, nižje ravni PEA in OEA pa so povezane z višjimi ravnmi FeNO, kar je označevalec vnetja, značilnega za alergijsko astmo. Šteje se, da PEA nastane kot odziv na celično poškodbo kot pro-homeostatski zaščitni odziv in je dokazano, da ima protivnetne, analgetične in nevroprotektivne lastnosti [21].

Povezava med glukokortikoidnimi in endokanabinoidnimi signalnimi potmi je bila dokazana v prejšnjih študijah. Ugotovljeno je bilo, da lahko glukokortikoidi mobilizirajo endokanabinoidni sistem [22, 23, 24], številni dokazi pa kažejo, da je za različne učinke, posredovane z glukokortikoidi, potrebna nedotaknjena endokanabinoidna signalizacija [25, 26, 27, 28, 29].

V nasprotju s terapevtskim odzivom, ugotovljenim pri ICS, so imeli bolniki z astmo z visokimi ravnmi izražanja mRNA CNR1 ali FAAH v času diagnoze, zdravljeni z LTRA 4-6 tednov, najboljši odziv (ΔFEV1/FVC). Naši rezultati kažejo, da niso imeli samo tisti bolniki z astmo z visokim CNR1 boljši odziv na zdravljenje z LTRA, ampak tudi tisti, ki so nosilci manjšega alela SNP rs2229579 (CNR2), so imeli slabši odziv na LTRA. Kolikor nam je znano, je to prvič, da CB1 in CB2 igrata vlogo pri odzivu na LTRA.

Po dolgotrajnem zdravljenju z ICS so bile ravni izražanja IL4 in IL5 v PBMC bolnikov z astmo, ki smo jih spremljali v naši študiji, znatno zmanjšali, kar je v skladu z dolgo znanimi učinki kortikosteroidov [30, 31, 32, 33, 34]. Dolgotrajno zdravljenje z ICS je zmanjšalo ravni izražanja CNR1 in NAPEPLD, hkrati pa je povečalo ABHD4, FAAH in MGLL. Nedavna študija in vitro je pokazala, da je kortikosteron zmanjšal izražanja mRNA in beljakovin CB1 v celicah glioblastoma in zaviral ugodne učinke aktivacije CB1 s kanabinoidi [35]. Dolgoročni vpliv kortikosteroidov na endokanabinoidni sistem pa še ni določen.

Dolgotrajno zdravljenje z LTRA je zmanjšalo ravni izražanja CB1 in NAPEPLD. Pri bolnikih z alergijsko astmo je LTRA povečal tudi nivoje izražanja FAAH, kar bi lahko po dolgotrajnem zdravljenju dodatno zmanjšalo njihove plazemske koncentracije PEA.

Zaključek

Rezultati kažejo, da je endokanabinoidni sistem pri bolnikih z astmo povečan v povezavi z resnostjo bolezni. Ni jasno, ali je povečana regulacija endokanabinoidnega sistema vzrok ali posledica astme. Edini namig za to uganko prihaja iz genetskih povezav, najdenih v tej študiji. Dolgotrajno zdravljenje z ICS ali LTRA je zmanjšalo izražanje mRNA CNR1, kar kaže na prevladujočo vlogo CB1 kot odgovor na katero koli zdravljenje.

Kljub temu ugotovitve te študije prispevajo k boljšemu razumevanju vloge endokanabinoidnega sistema v patologiji astme, posamezni geni pa lahko služijo kot biomarkerji in/ali nove molekularne tarče za zdravljenje otroške astme.

Ključne besede: Astma, endokanabinoidni sistem, kanabinoidi, vnetje, molekularna genetika

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ABBREVIATIONS

2-AG 2-arachidonoyl glycerol

5-LOX 5-lipoxygenase

CNR1 Human cannabinoid receptor 1 gene

CNR2 Human cannabinoid receptor 2 gene

AA Arachidonic acid

Abhd4 α,β-hydrolase 4

AEA Anandamide

ATS American Thoracic Society

BALF Bronchoalveolar lavage fluid

CB1 Cannabinoid receptor 1

CB2 Cannabinoid receptor 2

CBD Cannabidiol

CC Calibration curve

CCL (C-C motif) ligand

CCR C-C chemokine receptor

COX Cyclooxygenase

Cq Quantification cycle

DAG Sn-1-acyl-2-arachi-donoylglycerol

DAGLα Diacylglycerol lipase α

DAGLβ Diacylglycerol lipase β

EMT Endocannabinoid membrane transporter

eQTL Expression quantitative locus

ERS European Respiratory Scociety

FAAH Fatty acid amide hydrolase

FeNO Fractional exhaled nitric oxide

FEV1 Forced expiratory volume in 1 second

FVC Forced vital capacity

GDE1 Glycerolphosphodiesterase 1

GLM generalized linear model

GM-CSF Granulocyte-macrophage colony-stimulating factor

GpAEA Glycerophospho-arachidonoyl ethanolamine

GPCRs G protein-coupled receptors

GR Glucocorticoid receptors

GWAS Genome-wide association studies

HRM High-resolution melting

ICS Inhaled corticosteroids

IFN-γ Interferon-gamma

IgE Immunoglobulin E

IL Interleukin

IQR Inter-quartile range

LD Linkage disequilibrium

LPA Lysophosphatidic acid

LPS lipopolysaccharide

LTRA Leukotriene receptor antagonists

LTs Leukotrienes

lyso-PLD Lyso-phospholipase D

MAGL Monoacylglycerol lipase

MAPK Mitogen-activated protein kinase

MHC major histocompatibility complexes

NAEs N-acylethanolamines

NAPE N-arachidonoyl phosphatidylethanolamine

NAPE–PLD N-arachidonoyl phosphatidylethanolamine - phospholipase D

NCBI National Center for Biotechnology Information

OEA oleoylethanolamide

pAEA Phosphoanandamide

PAMPs pathogen-associated molecular patterns

PBMCs Peripheral blood mononuclear cells

PC20 Provocative concentration that causes a 20% fall in FEV1

PEA Palmitoylethanolamide

PEFR Peak expiratory flow rate

PG Prostaglandin

PI Phosphatidylinositol

PIP_2 Phosphatidylinositol 4,5-bisphosphate

PLA1 Phospholipase A1

PLA2 Phospholipases A2

PLC Phospholipase C

PLCβ Phospholipase C-β

PPARγ Peroxisome proliferator-activated receptor gamma

ppb parts per billion

PRRs Pattern recognition receptors

PTPN22 Putative tyrosine phosphatase N22

QC Quality control

qPCR Quantitative polymerase chain reaction

SD Standard deviation

SNP Single-nucleotide polymorphism

sPLA2 Secretory phospholipase A2

TFH T follicular helper

Th1 T helper 1

Th2 T helper 2

TNFα Tumour necrosis factor alpha

TRP Transient receptor potential

TSLP Thymic stromal lymphopoietin

Δ9-THC Δ9-tetrahydrocannabinol

1 INTRODUCTION

Asthma is a long-term chronic inflammatory disorder of the airways, limiting expiratory airflow, and is one of the major non-communicable diseases. According to the Global Burden of Disease Study report from 2017 [36], an estimated 273 million people in the world were affected by asthma at the time. Asthma is characterised by intermittent and reversible airflow obstruction due to inflammation, bronchospasm and increased airway secretions with clinical manifestations that can range from mild to severe.

The word *asthma* is old, and initially, it was not associated with a disease, it meant "noisy breathing" [37]. It was not until the 17^{th} century that medicine viewed asthma as a condition in its own right [38]. Since then, our understanding of the elements involved in asthma pathogenesis has grown dramatically, particularly during the last few decades, as we gain greater insight into links between clinical features of asthma and genetic patterns [39].

The pharmacological treatment of asthma requires a step-wise approach in the dosage of existing medications or the inclusion of others if the management of asthma is found to be not well controlled under the current treatment plan. Therefore, the treatment of more severe asthma frequently requires a combination of drugs addressing multiple mechanistic pathways [1]. In order to attain treatment optimization, it is becoming plainly evident that a deep understanding of the basic mechanisms of a particular patient's asthma phenotype is crucial in directing their care.

Given the increasing amount of evidence showing the endocannabinoid system's involvement in regulating the immune system, questions arise on its role in asthma pathogenesis. This emergent research topic can unveil new therapeutic targets by elucidating how the endocannabinoid system participates in asthma onset or the development of recurrent symptoms. There is also increasing interest in cannabinoid-based compounds for treating chronic inflammatory disease given their anti-inflammatory properties. Furthermore, as cannabis becomes legalised for medical or recreational purposes and cannabinoid-based products grow in popularity across the general population, it becomes crucial to elucidate potential beneficial or harmful effects of manipulating the endocannabinoid system in asthma patients.

1.1 Asthma

In 1991, the National Asthma Education and Prevention Program (National Heart, Lung, and Blood Institute) published the first *Expert Panel Report: Guidelines for the Diagnosis and Management of Asthma* [40]. In this publication, experts propose the following definition of asthma (later reviewed in 1997 [41] and 2007 [1]):

"*Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role: in particular, mast cells, eosinophils, T lymphocytes, macrophages, neutrophils, and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. The inflammation also causes an associated increase in the existing bronchial hyper-responsiveness to a variety of stimuli. Reversibility of airflow limitation may be incomplete in some patients with asthma.*"

1.1.1 Clinical presentation

Diagnosis of asthma requires assessing the patient's clinical history, a physical examination, and some laboratory studies [40]. A history of exacerbation and symptoms including shortness of breath, chest tightness, wheeze and cough is expected. Asthma is a chronic disease with acute exacerbations (triggered by various stimuli) that can be fatal. It can also vary significantly in terms of onset (i.e. early-onset and late-onset) and response to current asthma treatments [1]. Symptoms can be mild or severe, and they can occur as a result of a variety of pathogenic mechanisms and primary cause, including immunoglobulin E (IgE)-mediated allergic responses, pollution exposure, exercise, stress, or airway infections [1].

Studies have demonstrated that symptom reported by patients do not always reflect the extent of airflow obstruction [42]. Therefore, objective measures of lung function are essential to determine the severity of asthma and make appropriate therapeutic recommendations [40].

Lung Function

The most common test used to determine the degree of airway obstruction is the spirometry. Spirometry measures the forced expiratory volume in 1 second (FEV1), the forced vital capacity (FVC), and peak expiratory flow rate (PEFR). FVC is the

total volume of air exhaled as rapidly as possible after a maximal expiration. The FEV1 is the volume of air exhaled within the first second of the FVC manoeuvre and is considered the best pulmonary function measure to assess severity [40]. In obstructive lung diseases such as asthma, the FEV1, and the FEV1/FVC ratio are often decreased. FEV1/FVC ratio is considered as a principal measure of obstruction, which is typically intermittent and reversible in asthmatics. Therefore, the increase of FEV1 >12% after the administration of a broncho-dilating drug (such as short acting beta2-agonist) is considered diagnostic for asthma. PEFR is the maximum velocity of airflow during the expiration that can be obtained starting with fully inflated lungs and requires maximum effort for accuracy. PEFR can be measured at home and is therefore best used as a tool for ongoing monitoring, not a diagnosis [1]. In clinical studies, PEFR values have been used as a major outcome indicator to track both short-term [43] and long-term [44] asthma control and treatment responses.

Bronchial Hyperresponsiveness

Airway responsiveness is determined by administering serially increased doses of a provocative agent, usually methacholine, and calculating the "provocative concentration" that causes a 20 percent fall in FEV1 (PC20). The bronchial challenge test is useful to establish an initial diagnosis and quantify the severity of airway hyperresponsiveness in patients with asthma [40].

Biomarkers of Inflammation

Fractional exhaled nitric oxide (FeNO) and blood or sputum eosinophils are biomarkers of type 2 inflammation, both accessible and easy to use.

Nitric oxide (NO) is a gaseous signalling molecule formed in response to an inflammatory process [45]. It plays an essential role in the immune system regulation by controlling the vascular and bronchial constriction [46]. This molecule is measured in the patient's breath using the FeNO test. This test can help the clinician distinguish asthma from other lung disorder and monitor patient response to therapy [47]. A FeNO level less than 25 parts per billion (ppb) (< 20 ppb, for children under 12 years old) is considered normal, more than 50 ppb (> 35 ppb, for children under 12 years old) is regarded as elevated and considered a sign of eosinophilic inflammation characteristic of allergic asthma [46].

A substantial number of severe asthma patients have eosinophilic inflammation, linked to decreased lung function and inadequate pharmacological control [48]. Since patients with moderate-to-severe asthma are significantly more likely to have ele-

vated baseline eosinophil counts than those with mild disease, blood eosinophils can be a useful biomarker [49]. A notable cohort study investigating the link between blood eosinophil counts and asthma-related outcomes found that patients with elevated baseline blood eosinophil count (> 400 cells/µl) underwent more severe subsequent exacerbations and had worse asthma control [50].

FeNO has been suggested as a surrogate biomarker for eosinophilic inflammation [51]. However, recent studies support that FeNO [52, 53] and blood eosinophils levels [54, 55, 56] are dependent on the production of different cytokines and may therefore represent distinct parts of the inflammation seen in asthma.

Allergy testing

Allergy skin prick testing offers proof of sensitization and can assist in diagnosing a suspected type I allergy. It is a reliable procedure to identify asthma patients whose underlying condition is IgE-mediated and identify possible environmental triggers [57].

Additionally, patients can have their serum IgE levels measured to confirm atopy. Asthma often has an allergic basis and is associated with some IgE-related reaction [58]. Allergic sensitisation often contributes to airway inflammation in asthmatics. Studies have shown that increased serum IgE levels are characteristic of allergic diseases, such as asthma [59, 60], are correlated with airway hyperresponsiveness in adults [58, 61] and children [62] and are associated with the severity of asthma [59].

1.1.2 Phenotypes and endotypes

Currently, asthma diagnostics require defining asthma cases as allergic or non-allergic. Rackemann proposed the phenotypes over 70 years ago, and they are based on age of onset and the presence or absence of an environmental trigger [63]. Allergic asthma usually develops in childhood and is associated with sensitization to one or more airborne allergens, such as animal dander, pollen, mold, or house dust mite. Non-allergic asthma, on the other hand, is typically late-onset, has no link to allergen sensitization, and is thought to be caused by an as-yet unidentified intrinsic and environmental asthma triggers [64].

So far, it has been challenging to link molecular mechanisms to these clinical phenotypes of asthma. Allergic asthma is reported to represent 70-90 percent of asthma patients, making it the most commonly diagnosed asthma phenotype [65]. Thus, the standard asthma assumption is that airway hyperresponsiveness and inflammation

are related to increased T helper 2 (Th2) cell responses and specific IgE. While this accurately describes allergic asthma's main mechanisms, the term "asthma" now represents a group of diseases, all of which have intermittent symptoms of wheezing and shortness of breath to cough and chest tightness, and are characterised by variable airflow obstruction. Clinically, the recognition that asthma is a heterogeneous disease has been apparent for decades. Guidelines noted this aspect of asthma and divided patient groups into categories of intermittent and persistent, with the latter being further subdivided into mild, moderate, and severe [1]. Currently, a patient's asthma is typically described in terms of disease phenotypes. In the context of asthma, a phenotype describes "observable characteristics" such as clinical, physiological, morphological, and biochemical characteristics, as well as the response to various therapies [66]. Allergic asthma was diagnosed using this method based on symptoms triggered by allergen exposure and confirmation of allergy by measuring specific IgE levels in the blood and/or testing skin prick reactivity to common allergens [66, 67]. Despite the fact that phenotypes are usually clinically relevant in terms of presentation, triggers, and treatment response, they do not always correspond to or provide insight into the underlying disease processes. The classification strategy, however, is now evolving to link molecular mechanisms to phenotype. Asthma endotypes distinguish these distinct pathophysiological pathways at the cellular and molecular levels [66].

1.1.3 Asthma pathophysiology

There are two core areas in the pathology of asthma. Airway inflammation, including a broad number of inflammatory cells and signal mediators [68], and airflow obstruction due to smooth muscle contraction and hypertrophy, mucus secretion and remodeling of airways (figure 1.1) [11]. Still, what determines the clinical manifestations and asthma severity is the interaction between inflammation, airflow obstruction, and bronchial hyper-responsiveness [69] (figure 1.2). This relationship can be highly variable among patients and over time within patients.

Airflow obstruction in asthma is intermittent and is generated by a number of changes in the airway [1], which include:

Bronchoconstriction

Bronchoconstriction is a result of smooth muscle contraction in the airway. It is an element of our airway defence reflexes that defend the lungs and the human body from inhaled toxic substances [70]. In asthma, bronchoconstriction occurs due to

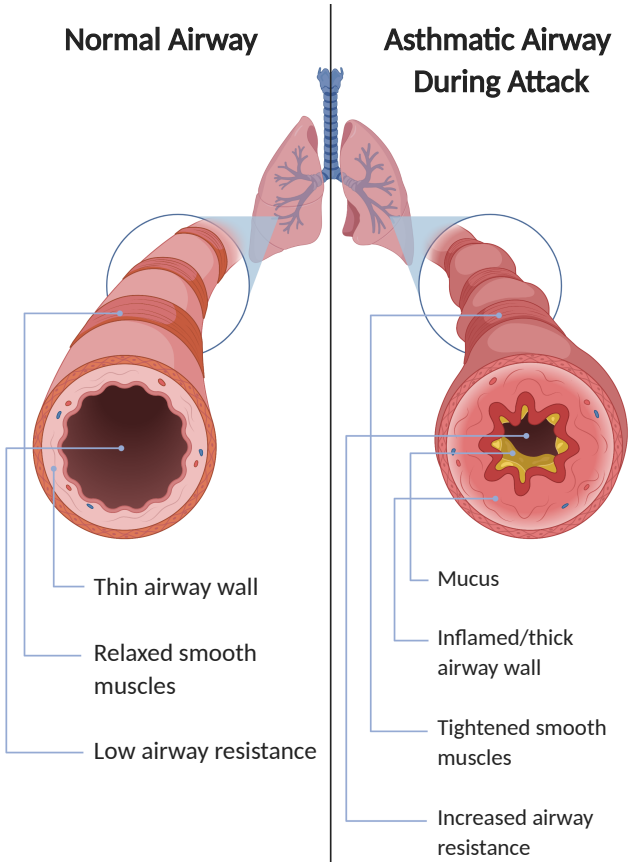

Figure 1.1: Normal airway versus asthmatic airway during attack.

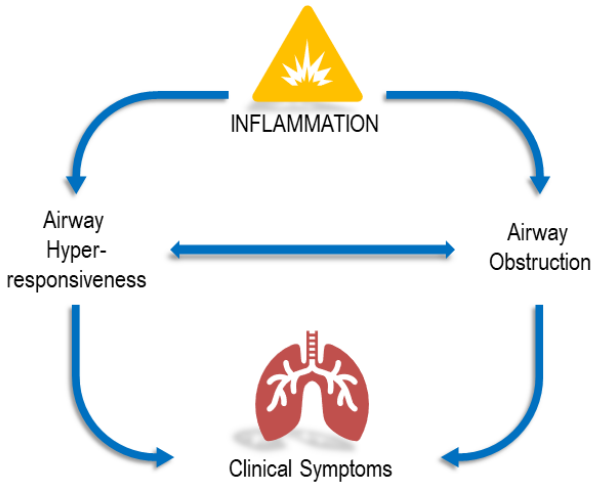

Figure 1.2: The interplay and interaction between airway inflammation, the clinical symptoms and the pathophysiology of asthma [1].

an intrinsic abnormality. Airway narrowing and the resulting airflow obstruction is the main physiological event that leads to the clinical symptoms observed in these patients. With persistent inflammation and as the disease develops, other elements further contribute to airflow obstruction, such as edema, mucus hypersecretion, inflammation, and hypertrophy and hyperplasia of the airway smooth muscle [1].

Airway hyperresponsiveness

Airway hyperresponsiveness is a functional abnormality characteristic of asthma. It refers to the exaggerated bronchoconstrictor response that occurs in response to various nonspecific stimuli. The bronchus begins to spasm and induces a pronounced narrowing of the airway lumen typical of asthma and coincides with the condition's clinical severity [71].

Airway remodeling

In asthma patients, structural changes occur in the airways that are not found to happen in healthy subjects. These structural changes are referred to as airway remodeling. In asthma, the airway wall thickens in proportion to disease severity and duration [72]. In some patients, airflow obstruction is only partially reversible due to permanent structural changes, which are associated with progressive loss of lung function [73].

Structural changes in the airways of asthmatics include, increased airway vascularity [74, 75], increased smooth muscle mass [76], decreased cartilage integrity [77], subepithelial fibrosis [78], loss of epithelial integrity [79], thickening of basement membrane [80], and goblet cell and submucosal gland hyperplasia [76, 81].

1.1.4 Allergic asthma pathogenesis

The most common form of asthma is allergic (or atopic) asthma, which is associated with sensitization to environmental allergens. The first defence mechanism, referred to as innate or non-specific immunity, consists of physical barriers (e.g. skin, cilia and mucus) and immediate response to foreign particles by some immune cells. The second mechanism, the adaptive or specific immune response, requires information from the innate immunity to be activated. A specific group of immune cells "adapt" to a particular antigen presented to them. It is, therefore, slower to respond. However, because it holds a memory to protect the host from the same type of pathogen, it will be efficient and quick to respond in future re-exposures. Both innate and adaptive immune responses play a role in asthma's pathogenesis

and contribute to chronic airway inflammation.

Inhaling allergens is relatively harmless to the general population. It is usually followed by low-grade immune responses characterised by the production of allergen-specific IgGs and the differentiation and proliferation of T helper 1 (Th1) cells that release interferon-gamma (IFN-γ) [82, 83]. However, in individuals genetically predisposed to atopy, an excessive immune response may occur after being exposed to one or more inhalatory allergens. The abnormal adaptive immune response directed against non-infectious environmental substances (allergens), in this case, is associated with a Th2-driven immune response and the production of allergen-specific IgE [58].

Allergen sensitisation typically occurs during early childhood following first exposure to the allergen (figure 1.3) . After sensitisation, the primary clinical symptoms of allergic asthma commonly and quickly arise due to the individual's subsequent exposure to the same allergens.

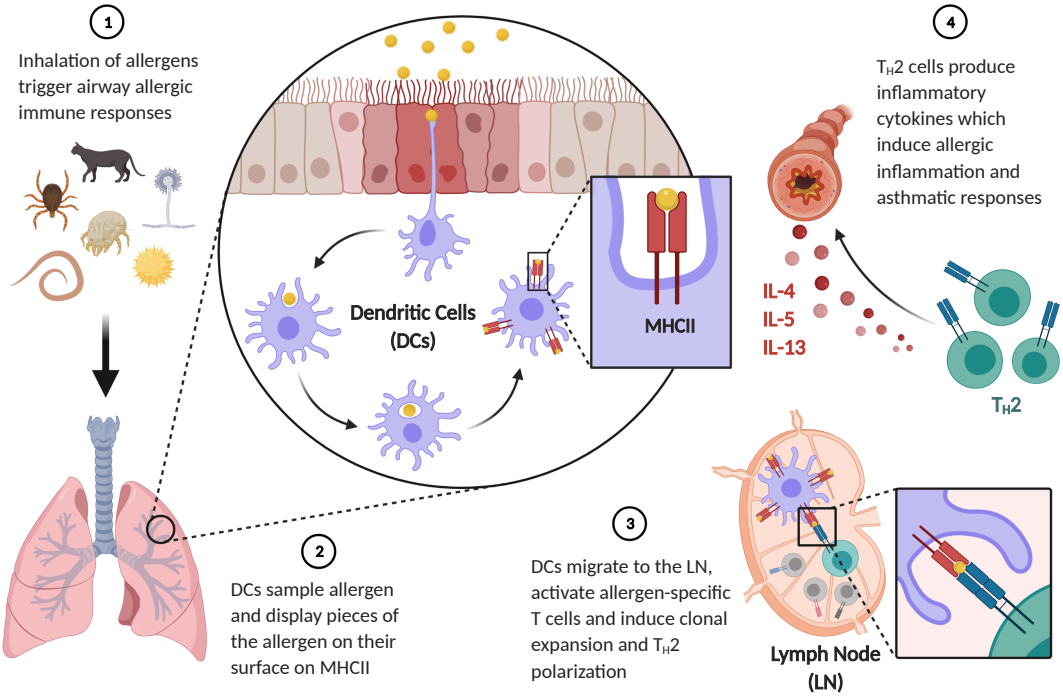

Figure 1.3: Allergen sensitisation phase in asthma [1].

Some allergens have proteolytic activity. Once in the airways, they can disrupt epithelial tight junctions and trigger receptors that detect protease activity on den-

dritic cells' surface [84]. Additionally, allergens can be detected by airway epithelial and dendritic cells that detect unique molecular patterns in their structure. These patterns, known as pathogen-associated molecular patterns (PAMPs), are distinct from those of the host [85]. They do this using pattern recognition receptors (PRRs) that bind to a PAMP and stimulate the production of various mediators that initiate non-specific responses in the form of inflammation and trigger the adaptive immune system [86].

Airway epithelial cells

Airway epithelium cells are the most abundant cell type in the lung and essential part of innate immunity [87]. They play a central role in the inflammatory response acting as a primary interface between the external environment and the host, exposed to numerous stimuli [88]. In asthma, the airway epithelium is an important source of cytokines known as "alarmins," such as interleukin (IL)-25 , IL-33, and thymic stromal lymphopoietin (TSLP), as well as chemokines such as C-C motif ligand (CCL) 5 , CCL17, CCL11, and CCL22, which induce Th2 cell polarisation in response to allergens, pollutants, and other pathogenic components [89, 90, 91, 92]. Other mediators released by airway epithelium cells include inflammatory cytokines, such as tumour necrosis factor alpha (TNFα), IL-1β and IL-6, produced in large quantities and have a wide variety of effects in cells of the immune system [93].

Dendritic cells

Foreign antigens are continuously sampled from the environment by dendritic cells strategically located in the lung mucosa. They are the most important players in the initiation of allergen-specific Th-cell mediated immune responses in asthma and are considered to be the most important antigen-presenting cell and a key link between innate and adaptive immunity [94, 95]. When dendritic cells come into contact with airborne allergens in the airways, they break them down into small peptides, create major histocompatibility complexes (MHC), and transport them to the mediastinal lymph nodes, where they present allergen components to naive T cells [96]. Depending on the type and dose of allergen, as well as the cytokine microenvironment, the interaction of allergen-loaded dendritic cells with naive Th cells results in differentiation of the latter into Th1, Th2, Th9, or Th17 cells [97].

Lymphocytes

T lymphocytes play a key role in asthma pathogenesis: a shift towards a Th2-cytokine release profile results in the recruitment and survival of eosinophils and

maintaining the airways' mast cell population [98]; Th17 cells promote the clearance of extracellular pathogens such as fungi and bacteria, but over-expression of these cells and IL-17 is present in asthma development [98]; and the number and function of regulatory T cells, which are essential in modulating and regulating immune responses by promoting tolerance, counterbalancing aggressive inflammatory reactions, and maintaining homeostasis, is impaired or altered in allergic patients compared with healthy individuals [99]. The precise mechanisms by which regulatory T cells prevent the allergic inflammation are still largely unknown. However, a recent study showed that these cells produce a large amount of neuritin, a protein which targets B cells. Neuritin was taken up by B cells, where it caused phosphorylation of numerous proteins and inhibited IgE class switching [100]. When type 2 cytokines are present during activation by antigen-presenting cells, allergen-specific Th2 or T follicular helper (TFH) cells are produced. Th2 cells migrate to the inflammation site and primarily produce IL-4, IL-5, IL-9, and IL-13 in response to allergen challenge. While TFH cells produce IL-4, which stimulates IgE antibody production when they interact with allergen-specific B cells [95].

B lymphocytes produce IgE in allergic asthma when stimulated by IL-4 and IL-13 but elevated IgE levels also been found even in non-atopic asthmatics [101]. Inhaled allergens cross-link membrane-bound IgE on mast cells and basophils, inducing the activation and release of proinflammatory mediators, and driving type-2 inflammation and clinical symptoms manifestation [102]. Because B cells are the only cells that produce IgE, it is clear that they play a key role in the initiation of allergen-induced inflammatory processes.

Eosinophils

Eosinophilic inflammation is characteristic of asthmatic airways [103]. Eosinophils are recruited from the bloodstream to inflammation sites and consequently activated in response to Th2-mediated inflammation. Once they reach the airways, activated eosinophils release pro-inflammatory mediators that contribute to sustained inflammation and tissue damage [104]. Eosinophils can also influence the actions of other leukocytes. Eosinophils can stimulate T cells' proliferation and cytokine production in an antigen-specific manner and regulate the recruitment of Th2 cells in response to allergen sensitization and exposure by acting together with dendritic cells [105, 106]. Furthermore, eosinophil can regulate the immune response by releasing cytokines, like IL-10 and IL-14, which help maintain homeostasis [107, 108].

Eosinophilic inflammation can occur in both allergic and non-allergic asthma. However, the recruitment pathway of eosinophils is different. In allergic asthma,

activation of Th2 cells leads to IgE class switch in B cells, airway eosinophilia and hypersecretion [109]. While in non-allergic asthma, the airway epithelial cells release cytokines in response to air pollutants or microbes that bind to and activate receptors on type-2 innate lymphoid cells (ILC2s), which results in the recruitment of eosinophils, mucous hypersecretion and airway hyperreactivity [109].

Neutrophils

Neutrophils act as the first line of defence against infections. They respond to inflammatory cytokines and chemokines, phagocytizing infectious agents, undergoing respiratory burst to produce reactive oxygen species, and releasing their DNA in the form of neutrophil extracellular traps (NETs) [110]. Their role in the pathophysiology of asthma remains uncertain, but recent evidence shows that neutrophils actively contribute to both tissue damage and clinical signs in asthma [111, 112]. Neutrophilic inflammation is more common in patients who do not respond to inhaled corticosteroids, a condition known as severe asthma [113].

Mast cells

In asthma, mast cells are recruited to the airways' mucosa by epithelial cells [114]. When mucosal mast cells are activated by cross-linking of allergen-specific IgE antibodies bound to the high-affinity FcεR on the surface of mast cells, they release several bronchoconstrictor mediators. These mediators include histamine and AA (AA) metabolites such as leukotrienes (LTs) and prostaglandin (PG)-D2 [115]. Mast cells also release several cytokines linked to allergic inflammation (IL-4, IL-5 and IL-13) and other pro-inflammatory mediators [116]. Therefore, mast cells are critical players in the development of asthma and their presence in the airway smooth muscle is associated with airway hyperresponsiveness [117].

Macrophages

Despite the fact that macrophages are the most abundant immune cell on the respiratory mucosal surface and can either promote or suppress inflammatory responses in the airways, their role in allergic asthma is still poorly understood [118, 119]. Recent studies showed that macrophage polarisation has a significant impact of asthma pathogenesis. Recruited macrophages can be polarized into either classically activated (or M1) or alternatively activated (or M2) phenotypes after being exposed to local micro-environments. M1 macrophages activated by IFN-γ and lipopolysaccharide (LPS) stimulate the expression of genes important in pathogen clearance and drive inflammation in response to intracellular pathogens. M2 macrophages, on

the other hand, are activated by IL-4 and IL-13 and up-regulate the expression of genes implicated in wound healing, the clearance of dead or dying cells, and anti-inflammatory responses [120, 121]. These two macrophage states are comparable to the Th1-Th2 polarisation of T cells [120, 122]. Asthma was associated with increased M2 macrophage polarisation and activation, and its hypothesised to play an important role in allergic asthma-[123, 124].

1.1.5 Non-allergic asthma pathogenesis

With the exception of higher levels of tissue macrophages in the non-allergic patients' bronchial mucosa, early analyses of epithelial cellular infiltrates revealed striking similarities in allergic and non-allergic asthma patients [125, 126]. This increase in macrophages was linked to increased numbers of cells expressing the α-subunit of the GM-CSF receptor observed in the bronchial mucosa of non-allergic asthma patients compared to the levels recorded in samples from allergic asthma patients [127]. However, more recently, mucosal epithelial eosinophil counts were shown to be significantly higher in allergic asthmatics compared to non-allergic asthmatics, while airways epithelial neutrophils were reported to be elevated in non-allergic asthmatics compared to allergic asthmatics and non-asthmatic control subjects [128].

Additionally, there seems to be identical, higher production not only of the major eosinophil-active cytokine IL-5, but also of the two B cell IgE-switching cytokines IL-4 and IL-13 in the bronchial mucosa of both allergic and non-allergic asthmatics [129, 130, 131]. Despite the fact that there are slight variations between allergic and non-allergic asthma at the mucosal and submucosal levels, the similarities often exceed the differences. Having said that, many elements of the pathophysiology of non-allergic asthma, including the involvement of IgE, remains unresolved.

1.1.6 Inflammatory mediators in asthma

In response to the inflammatory process, inflammatory cells and injured tissue release a variety of specialized substances that actively contribute to and regulate the inflammatory response [132].

Lipid mediators

Eosinophils and mast cells are the leading producers of AA-derived lipid mediators, such as LTs and PGs. When exposed to allergens, they secrete phospholipases

A2 (PLA2), responsible for the AA release from membrane phospholipids [133]. AA is the precursor for eicosanoid generation from which LTs and PGs derive.

The metabolism of AA through the 5-lipoxygenase (5-LOX) pathway leads to the production of leukotriene A4 (LTA4). However, LTA4 is unstable, so it is further converted into leukotriene B4 (LTB4) or conjugated with glutathione to form leukotriene C4 (LTC4). LTB4 is a potent chemoattractant for neutrophils, macrophages, and other inflammatory cells and induces chemokinesis and adhesion of these cells to the vascular endothelium [134]. Leukotriene C4, together with its metabolites leukotriene D4 (LTD4) and E4 (LTE4), are referred to as cysteinyl LTs. These increase vascular permeability and contract smooth-muscle cells, contributing to the bronchoconstriction seen in asthma [135].

PGs and thromboxane A2 (TXA2), collectively called prostanoids, are generated from AA metabolism by cyclooxygenases (COX), and their biosynthesis can be inhibited by nonsteroidal anti-inflammatory drugs [136]. Humans produce four bioactive PGs: prostaglandin E2 (PGE2), prostacyclin (PGI2), prostaglandin D2 (PGD2) and prostaglandin F2α (PGF2α) [136]. Their production is widespread (each cell type makes one or two dominant PGs) and is generally very low in healthy tissues where they sustain local homeostatic functions in the body. Once an inflammatory response is initiated, both the profile and the levels of PGs production changes dramatically. PG production increases immediately in acute inflammation before the recruitment of leukocytes [137].

COX catalyzes the initial cyclooxygenase reaction to generate prostaglandin G2 (PGG2) and transform it into PGH2, the precursor for PGD2, PGE2, PGF2α, PGI2, and TXA2 [138]. Humans have two homologous COX enzymes, COX-1 and COX-2. COX-1 is expressed ubiquitously in most tissues, where it facilitates the production of homeostatic PGs [139]. On the other hand, COX-2 expression is usually temporary and induced by cellular stress or inflammatory reactions after stimulation by LPS or cytokines, such as IL-1, IL-2, and TNFα [139]. All PGs elicit their biological effects by activating cell surface G protein-coupled receptors (GPCRs). Mast cells predominantly release PGD2, a potent bronchoconstrictor, in response to IgE. The PGD2 produced contributes to the vascular and cellular changes associated with early and late allergic response phases. Additionally, PGD2 recruits leukocytes, eosinophils, and mast cells by binding to DP2 receptors on the surface of these cells [140].

Cytokines

In asthma, multiple cytokines coordinate the inflammatory response. Allergic

inflammation is mediated mostly by Th2 cytokines:

- IL-5 is responsible for eosinophil differentiation and recruitment to airways [141].
- IL-4 induces IgE isotype switch by B cells, promotes eosinophilic inflammation, and can drive naive T cells' differentiation into Th2 cells [142].
- IL-9 prepares mast cells to respond to allergens by increasing its cell surface expression of the IgE receptor and the production of inflammatory cytokines such as IL-6 [143].
- IL-13 induces many biological responses relevant to asthma, such as IgE production, eosinophil recruitment, maturation of mucus-secreting goblet cells, and up-regulation of airway smooth muscle cells contractility [144].

Other key cytokines include IL-1β and TNFα that amplify the inflammatory response and granulocyte-macrophage colony-stimulating factor (GM-CSF), which increases eosinophil survival in the airways. Some cytokines, such as IL-10 and IL-12, are anti-inflammatory and may be deficient in asthma [116]. Th17 cells release IL-17A/F and IL-22, which is increased in severe asthma and contribute to neutrophilic inflammation [145].

Airway epithelial-derived cytokines such as interleukin IL-25, IL-33 and TSLP are released from stressed or infected cells. They act solely or in concert to stimulate an immune response. Alarmins released from asthmatics' epithelial cells coordinate the chemokine release that selectively attracts Th2 cells [146].

Chemokines

Chemokines play a crucial role in attracting inflammatory cells from the circulation into the lungs in asthma, a link between early innate immune responses and adaptive immunity. The primary stimuli for chemokines' secretion are early signals such as an increase in pro-inflammatory cytokines IL-1 and TNFα. They are mainly expressed in airway epithelial cells and act through GPCRs [147]. C-C motif chemokine 11 (CCL11) selectively attracts eosinophils via C-C chemokine receptor (CCR) type 3 and is expressed by epithelial cells of asthmatics. At the same time, CCL17 and CCL22 from denditric cells attract Th2 cells via CCR4 [116].

Immunoglobulin E

The process of sensitization to allergens is triggered by the activation of Th2 and B lymphocytes. Cytokines released by activated Th2 cells (e.g., IL-4 and IL-13) stimulate IgE antibodies' production in B-cells [148]. These antibodies, in turn, bind

to specific receptors for IgE on the surface of other resident cells. There are two types of IgE receptors: high-affinity (Fcε RI) surface receptors on mast cells, smooth muscle cells, and dendritic cells, or low-affinity receptors (Fcε RII, also known as CD23) on B lymphocytes, T lymphocytes and eosinophils [149].

IgE is the antibody mediating allergic reactions and plays a role in the pathogenesis of allergic diseases and the development and persistence of inflammation. Mast cells are particularly abundant in IgE receptors that, when activated, release a range of signalling molecules to initiate acute bronchospasm and pro-inflammatory cytokines to perpetuate underlying airway inflammation [150].

1.1.7 Asthma management

Many pharmacologic therapies have been developed to prevent and control asthma symptoms, decrease the occurrence and severity of asthma exacerbations, and reverse airflow obstruction. Short-term or reliever medications include short-acting β2-agonists, anticholinergics, and systemic corticosteroids. Long-term medications available to treat asthma are inhaled corticosteroids, cromolyn sodium, nedocromil, long-acting β2-agonists, methylxanthines, leukotriene modifiers, and IgE antibody blocker (omalizumab) [1]. Of interest to the present study are corticosteroids and leukotriene modifiers.

Corticosteroids

Corticosteroids (also known as glucocorticoids or simply steroids) are a class of medications used to treat various inflammatory and immune diseases. However, they are often used in the treatment of asthma. Inhaled corticosteroids (ICS) with topical activity was introduced in 1972, and this was the breakthrough that revolutionised asthma treatment [151]. To date, ICS are the most potent and reliable anti-inflammatory agents for long-term asthma management. ICS significantly reduce airway inflammation, hyperresponsiveness, successfully prevent acute exacerbations, increase lung function, and reduce symptom severity [1].

Corticosteroids move quickly across the cell membrane and bind to cytoplasmic glucocorticoid receptors (GR). These receptors are usually bound to proteins, called molecular chaperones, such as the heat shock protein-90 (hsp90) and FK-binding protein, necessary to block its transport through the nuclear membrane into the nucleus [152]. When corticosteroids are bound to GR, it dissociates the molecular chaperones exposing its nuclear localisation signals, which results in the rapid transport of the GR-corticosteroid complex into the cell nucleus. Once inside the nucleus,

two GR molecules form a homodimer that binds to specific DNA sequences known as glucocorticoid response elements (GRE) in the promoter region of genes responsive to corticosteroids [153]. Corticosteroids directly inhibit pro-inflammatory signalling by activating the transcription of genes that hinder the synthesis of inflammatory mediators and suppressing the transcription of genes that lead to the activation of immune cells. As a result, ICS can decrease the number of inflammatory cells in asthmatic airways, such as eosinophils, T-lymphocytes, mast cells, and dendritic cells [154].

Current guidelines recommend ICS as the first-line therapy for mild persistent asthma at low doses, and it is the preferred therapy for moderate asthma in combination with long-acting beta-agonists [155]. High doses of ICS are only recommended for patients with uncontrolled persistent asthma [1]. Because of the well-known adverse effects of systemic corticosteroids, there is widespread awareness of the potential for adverse systemic effects with ICS, particularly when administered at high doses for extended periods of time. High-dose ICS has been linked to systemic side effects such as osteoporosis, slowed growth in children, skin thinning, cataracts, and glaucoma. Other side effects such as oropharyngeal candidiasis, dysphonia, reflex cough and bronchospasm, and pharyngitis are common local side effects of ICS [156]. Another disadvantage of ICS is the vast heterogeneity in both efficacy and systemic safety of ICSs among individuals with asthma. This response variability is multifactorial, encompassing environmental and genetic factors [157].

Leukotriene modifiers

As already mentioned, LTs are potent lipid mediators that contract airway smooth muscle, increase vascular permeability, increase mucus secretions, and attract and activate inflammatory cells in the airways of patients who have asthma [158]. LTs manifest their physiological and pathogenic effects through analogous receptors, such as cysteinyl leukotriene receptor type 1 (CysLTR1), CysLTR2, G-protein coupled (GPR) receptor 17, GPR99 and purinergic receptors (P2Y12R) [159]. Asthma severity is associated with increased LT levels in the sputum [160]. However, treatment with ICS did not significantly reduce LTs, suggesting that the LT pathway is relatively unaffected by corticosteroids [161].

Considering the role of the LTs/CysLTR pathway in asthma pathogenesis, efforts were made to attenuate this axis effectively. Currently, there are two types of pharmacologic compounds that act as leukotriene modifiers: 5-LOX pathway inhibitors (e.g., zileuton) and Leukotriene receptor antagonists (LTRA) (e.g., montelukast and zafirlukast). LTRA specifically blocks CysLTR1 on the surface of airway epithelial

cells [162] and only montelukast (for children as young as one year old) and zafirlukast (for children as young as seven years old) have been approved for pediatric use [1].

Several studies have shown that LTRAs have broncho-dilating and anti-inflammatory effects, making them ideal candidates for asthma treatment [163]. The discussion of the LTRA's functional role in asthma management is still ongoing because of their relatively recent introduction and the shortage of fully published comparative studies [164]. However, some studies support oral montelukast as a first-line control therapy for mild asthma in children. It offers broncho-protection in some preschool children with allergic asthma and reduces airway inflammation measured by nitrogen oxide [165]. LTRAs improve lung function and result in fewer asthma symptoms, especially by night in patients with mild-to-moderate chronic asthma.

Although treatment with ICS is more effective at improving lung function, the additional benefit from ICS treatment may be offset by the greater compliance attained with oral treatment. [166]. The decision to choose between ICS and LTRA is made by the physician and the patient, based on ICS' higher superior efficacy versus the expected higher compliance associated with leukotriene modifiers. LTRA is an alternative treatment for asthma patients who cannot control their symptoms with ICS therapy, are unsatisfied with it, or decline to take it [167].

It is worth noting that current biologic therapies have shown positive outcomes in the Th2-high patient group and directly target inflammatory modulators, which have been implicated in the pathophysiology of asthma [168]. With deeper knowledge of the immunopathogenesis of asthma, new inflammatory pathways are identified that can be used as targets for biological treatments. Since the endocannabinoid system is widely believed to modulate the immune system, it has the potential to become such a target.

1.1.8 Genetics of asthma

Asthma is a polygenic and truly multifactorial disease caused by the interaction of multiple environmental factors and an individual's genetic makeup [169]. Furthermore, unlike single-gene disorders, asthma phenotype is non-linear and highly variable. These qualities make predicting asthma status for a specific genotype or genotype combination difficult.

According to twin studies, asthma has a heritability of 60–70%, meaning that determining the genetic cause of asthma may help identify multiple disease-causing

mechanisms [170]. This possibility has fed an interest in discovering more about asthma's genetic risk factors in recent decades. These genetic risk factors are predominantly single base-pair mutations present in more than 1% of the general population, known as genetic variants or single-nucleotide polymorphisms (SNPs) . Currently, there are 1473 genes reported to be associated with asthma in the Human Genetic Epidemiology Navigator database [171].

Asthma susceptibility genes fall into four broadly divided functional groups [172]. Below is an overview of currently identified genes whose function have been shown to contribute to asthma.

Epithelial barrier function

Studies have linked asthma susceptibility to mutations in genes of the epithelial barrier's structural components, such as filaggrin (FLG), which encodes a large protein called profilaggrin that binds to keratin fibres in epithelial cells [173]. Asthma has also been linked to changes in genes that play essential roles in epithelial innate immune activity and recruiting adaptive immune responses. These genes include defensin-b1 (an antimicrobial peptide), uteroglobin/Clara cell 16-kD protein (CC16) (an inhibitor of Th2-cell differentiation), and multiple chemokines involved in T-cell and eosinophil recruitment (CCL-5, CCL-11, CCL-24, and CCL-26) [172].

Interaction with environment

Variation in a separate group of genes, essential for detecting and recognizing potentially harmful environmental substances, has been linked to asthma. These genes encode PRRs such as toll-like receptor 2 (TLR2), TLR4, TLR6, and TLR10 and intracellular receptors such as NOD1/CARD4 [174, 175, 176, 177]. Additional research has also linked variants in the HLA class II genes to asthma and allergen-specific IgE responses [177].

Th2-mediated cell response

As expected, genetic variants in many of the genes involved in Th2-cell differentiation and function are linked to asthma. Variations in the genes *TBX21*, *GATA3*, *STAT6*, *IL12B*, *IL4/IL4RA*, *IL13*, and *FCER1* affect asthma susceptibility, likely altering the Th2-mediated cell response, which is critical in asthma pathogenesis [172, 174, 177].

Tissue response

Different genes that mediate the tissue response to allergic inflammation and ox-

idative stress seem to be significant contributors to asthma susceptibility. Genetic variations in genes encoding structural proteins (COL29A1), involved in smooth muscle contractility control (PDE4D, NOS1), airway remodelling (ADAM33), free radical metabolism (GSTP1/GSTM1), and leukotriene synthesis (LTC4S, ALOX-5) have all been identified to modify the lung tissue's reaction to allergic inflammation [172].

1.1.9 Discovery of new genetic risk factors

In the past, genetic studies conventionally employed two strategies to identify genes and genetic variants associated with asthma: positional cloning [178, 179, 180, 181] and candidate gene association studies [182, 183]. Positional cloning (also known as genome-wide linkage studies) is a hypothesis-free approach that focuses on families affected by the disease, where the entire genome is screened for marker and disease co-transmission. On the other hand, candidate gene association studies analyse a small number of genes for association with the disease based on their known function and likelihood of being involved in any disease process. A significant portion of the genes associated with asthma so far have resulted from studies that used one of these two approaches, and several recent reviews address these findings [172, 184, 185, 186, 187].

Because candidate gene association studies are hypothesis-based, genes that act through non-classical disease pathways are more likely to be excluded for association in these studies. Not surprisingly, many candidate genes are selected based on the current immunologic understanding of asthma, feeding into the idea that most asthma genes are directly involved in the immune response [188].

However, the genome-wide association studies (GWAS) have recently emerged, allowing screening of the entire genome for hundreds of thousands of gene polymorphisms, from thousands of individuals, with decreased cost and time consumption. It is a powerful method that can detect truly novel disease candidate genes, especially those associated with moderate risks and common variants [189, 190]. GWAS have rapidly become popular for use across a wide range of phenotypes, and the number of publications in the GWAS Catalogue has increased from two in 2005 to more than 300 in 2010 [191]. At the time of writing, the GWAS Catalog has 4961 publications and 251401 associations, out of which 141 publications and 2711 associations are listed under the trait label "asthma" [192]. Significant associations are often presented in a Manhattan plot where each genetic variant is plotted against its chromosomal position and statistical significance reached for a study. The GWAS

Catalog has pulled together their data from GWAS on asthma and produced the Manhattan plot shown below (figure 1.4).

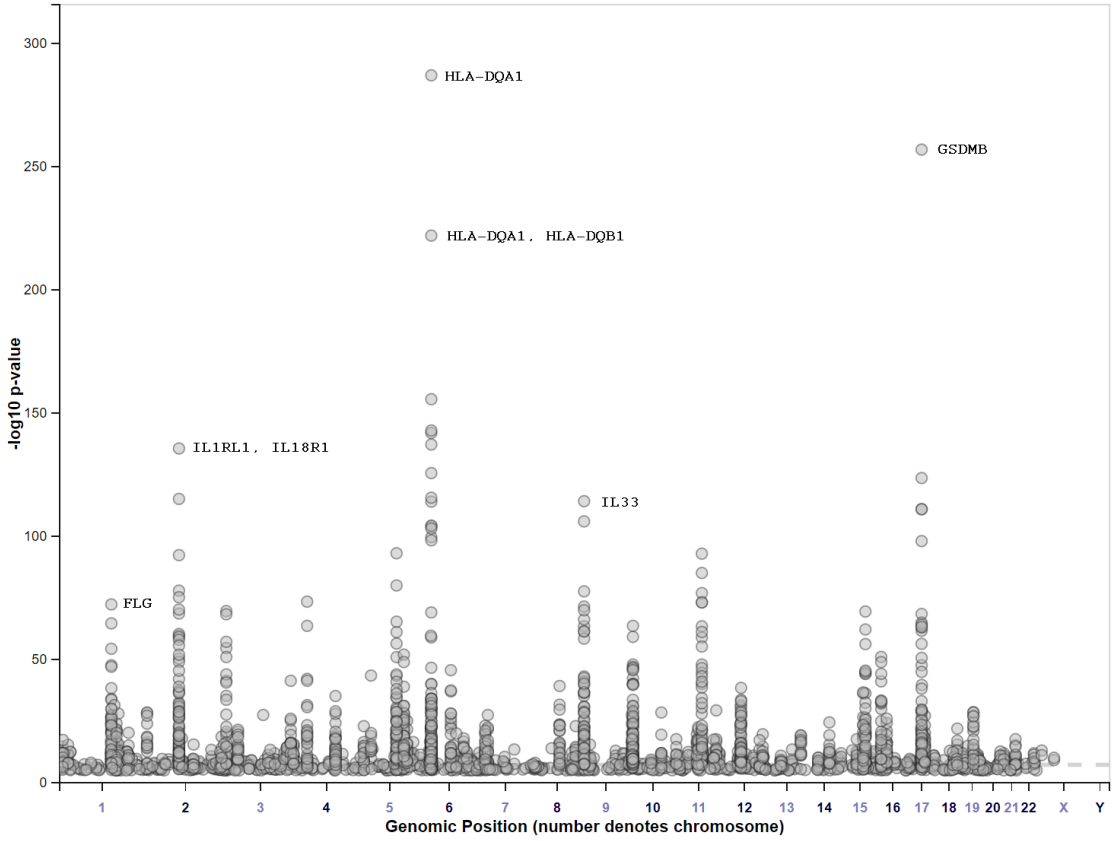

Figure 1.4: A Manhattan plot of the GWAS Catalogue genetic variants associated with asthma and respective mapped genes [192].

With the use of GWAS so far, it was possible to confirm the association of genes already known to contribute to asthma and to discover new genes unsuspected of association, such as *ORMDL3* and *GSDMB*. Increased *ORMDL3* and *GSDMB* expression is associated with a genetic variant linking chromosome 17q21 to asthma. Mice with increased levels of human *ORMDL3* or human *GSDMB* develop asthma with increased airway responsiveness, increased airway remodelling (increased smooth muscle and fibrosis) in the absence of airway inflammation [193].

Although, as with any other technique, GWAS have its faults and limitations [194]. Altogether, GWAS only detect a few per cent of the estimated heritability for complex human traits and diseases. In GWAS, the traditional P value of 0.05 is divided by the number of tests performed to assess whether the newly identified associations are true associations and to avoid the report of false-positive associations. Using this strategy usually means that only P values lower than 5.0 x 10^{-8} are considered

significant [185]. Genes listed in the GWAS Catalogue database also reflect this approach and do not include those with P values higher than 1.0 x 10^{-5}. As a result, if the individual effects of genetic risk variants are too small to pass rigorous tests, they are left hidden below the threshold for genome-wide associations and are not reported as significant (figure 1.5) [195].

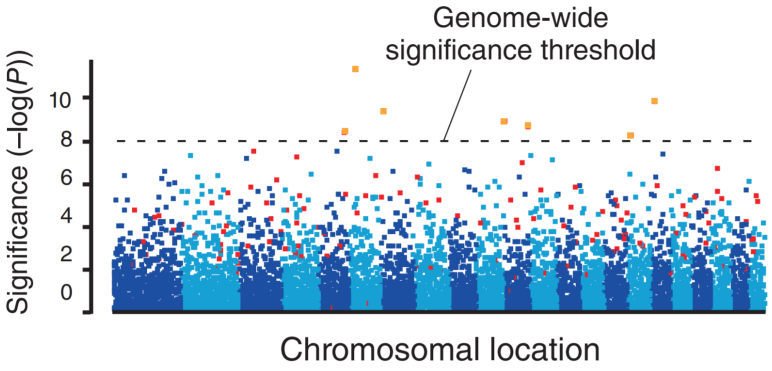

Figure 1.5: Genome-wide significance threshold. A Manhattan plot of significance against chromosomal location, as shown here for a generic common disease, is used in GWAS to visualize the genome-wide association of genetic variants. Genetic variants in gold have been detected in current GWAS; genetic variants in red may be detectable in larger sample size GWAS (Adapted from: [196]).

Another limitation of GWAS is that, for a considerable proportion of genetic variants associated with asthma, it is unclear what their downstream effects are, on account of many residing outside of coding regions and having no known or evident functional effects. Several expression quantitative trait loci (eQTLs) studies have already been carried out to address this issue. eQTLs studies identify genetic variants that affect gene regulation. One of the predominant observation from eQTL studies is that most eQTLs are cis-eQTLs as they map to the approximate location, i.e. on the same DNA molecule (defined by some as within 100 kb of the transcription start site), of the gene they regulate [197, 198, 199, 200, 201, 202]. The remainder of the eQTLs map far from the location of the gene they regulate, often on different chromosomes (referred to as trans-eQTL or as being in trans-regulatory elements). However, the genetic marker's precise location is usually limited to 10-20 kb of resolution in humans due to strong linkage disequilibrium (LD).

If genetic variants are in close physical proximity (typically, 50 kb apart or closer), they are likely to have alleles that travel together in a block when passed from parent to offspring (figure 1.6). This phenomenon, termed LD, allows one genetic variant in such a block to serve as an indicator for the presence of other genetic variants.

It eliminates the need for direct (and expensive) individual testing for the presence of every genetic variant [203]. The combination of genetic variants in a block on a single chromosome is called a haplotype. Any new mutation arising in a block region will travel along with other block members for hundreds of generations, surrounded by many co-inherited genetic variant alleles [203].

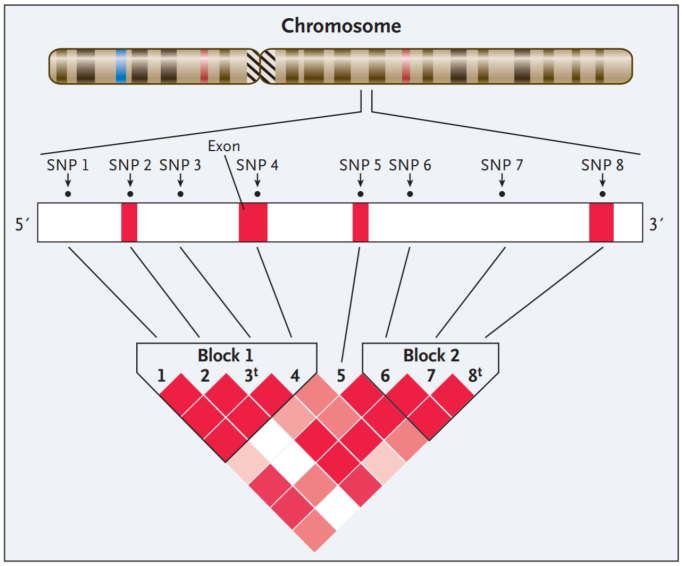

Figure 1.6: Mapping the relationships among genetic variants. Each dot above the magnified chromosome (labelled 5' to 3') represents one genetic variant. At the intersection between any two of these genetic variants, the associations between their variants are shown in shades from white to red, with the deepest red indicating the strongest association [204].

Data gathered from these studies (GWAS and eQTLs) offer an opportunity to select potentially disease-associated genetic variants more successfully for future studies, which may also regulate the expression of the gene of interest.

Not only can genetic variants affect gene expression levels, but they can also disrupt the normal splicing of mRNA [205]. Before mRNA (pre-mRNA) is spliced, it includes several introns and exons. Some exons are variably included or skipped during the splicing process to produce mature mRNA, which will give rise to different protein isoforms. This process is highly controlled, involving trans-acting splicing factors (repressors and activators) and cis-acting regulatory sites (silencers and enhancers) [206]. Mutations within these factors or regulatory signals may disrupt the process of mRNA alternative splicing regulation, giving rise to changes in the peptide sequence of the encoded protein. Subsequently, unwanted changes in the peptide sequence can alter ligand binding, enzymatic activity, allosteric regulation, protein

localization, or even render a protein inactive [205]. Errors in alternative splicing have been associated with different diseases [207, 208] and can be heritable [209].

1.2 The endocannabinoid system

Throughout evolution, animals have developed a wide range of protective mechanisms against situations or stimulus that disrupt the body's normal condition or function. An example, is the endocannabinoid system is a complex biological system found in just about any animal (vertebrates and invertebrates), except for the Phyla Protozoa and Insecta [210]. In humans, the endocannabinoid system regulates both central and peripheral organs, affecting a vast range of biological functions, such as sleep, mood, pain and pleasure perception, and appetite. Of particular interest to the topic of asthma is the therapeutic potential of the endocannabinoid system as a new pharmacological target to modulate the immune system.

Cannabis sativa was one of the first plants to be used by man in preparations for medical purposes [211]. For centuries, many of the available herbal and plant medicine texts have contained information on the medical properties of *Cannabis*. Though it was not until the discovery of Δ9-tetrahydrocannabinol (Δ9-THC) [212], the major psychoactive and psychotropic constituent of *Cannabis*, in 1964, that modern science took an interest on the effects of phytocannabinoids (the active constituents of *Cannabis*). Only recently, many of the therapeutic properties of *Cannabis* have been experimentally verified with pure, natural or synthetic cannabinoids revealing its therapeutic potential [213, 214, 215]. While a reasonably clear picture of phytocannabinoid pharmacology had emerged by the mid-1980s, the mechanism of these effects remained unclear. The discovery of the first cannabinoid receptor in the late-1980s naturally stimulated the search for its endogenous ligands, and the first endogenous agonist of these receptors known as endocannabinoids [216], was soon discovered. The enzymes involved in synthesis and hydrolysis of these ligands were also identified, and thus an endocannabinoid system was established.

With the increased interest in the endocannabinoid system, the number of studies exploring its regulatory function in health and disease increased significantly. The endocannabinoid system has been shown to be involved in the modulation of a wide range of physiological and pathological functions (e.g. in the nervous [217], cardiovascular [218], digestive [219], metabolic [220], excretory [221], endocrine [222], reproductive [223], musculoskeletal [224], respiratory [225], and immune systems [226]). The potential for the therapeutic use of the endocannabinoid system is therefore

wide and has been reviewed by several authors [213, 227, 228]. Here the focus will be on the endocannabinoid system role in asthma.

1.2.1 Cannabinoid receptors

In 1988, Devane *et al.* [229] discovered a new receptor in the brain that displayed high affinity for Δ9-THC, and it was named the cannabinoid receptor. This finding was indicative that membrane receptors mediated at least some of the effects of the cannabinoids. Soon after, a second receptor was discovered by Munro *et al.* [230], who described this as a peripheral receptor and proposed that the brain receptor is called CB1 and their newly discovered receptor, CB2.

CB1 is highly conserved across species [2]. In humans, the CB1 protein is encoded by the *CNR1* gene located on chromosome 6 [3]. CB2 appears to have more cross-species variation and in humans is encoded by the *CNR2* gene located on chromosome 1 [2, 3]. According to data from an RNA-seq project of 27 different tissues from 95 healthy human individuals [231], submitted to the National Center for Biotechnology Information (NCBI) the most common location for CB1 receptors is in brain tissue, whereas CB2 is more abundant in organs with an immune function such as lymph node tissue and spleen (figure 1.7).

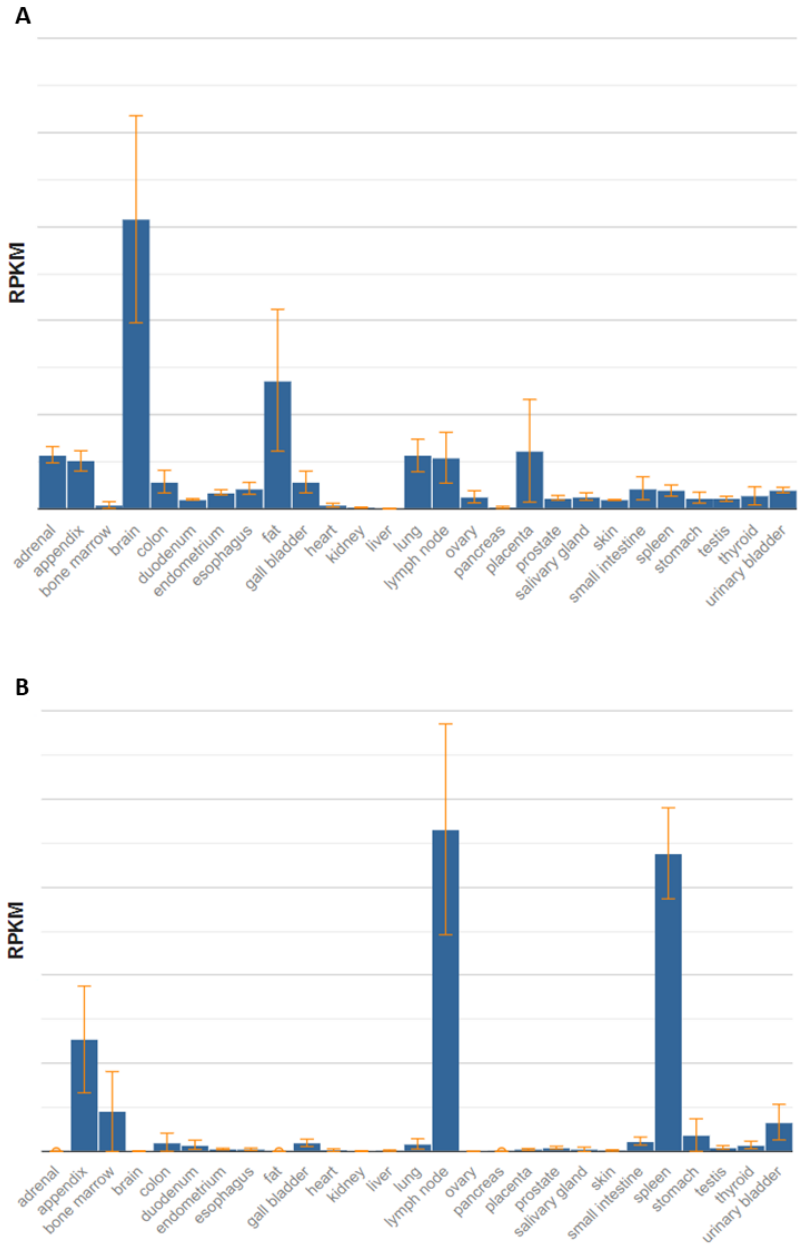

Figure 1.7: RNA distribution of A) CB1 and B) CB2 in 27 different tissues from 95 healthy human individuals [231].

CB1 is, in fact, the most abundant receptor of its type within the adult central nervous system [232], particularly in the basal ganglia nuclei, hippocampus, cortex and cerebellum [233, 234, 235]. However, CB1 is also present in many other cell types and tissues, including B-cells, T-cells and monocytes [236] leading to both central and peripheral effects [213, 237]. CB2, which exhibits 44% homology with CB1, was initially found in immune cells [230]. CB2 expression is higher in the lymph nodes and spleen than in peripheral blood cells and is different in different types

of immune cells (B cells>NK cells>monocytes>neutrophils>CD8 T-cells>CD4 T-cells) [238, 239]. However, CB2 can also be found in other tissues, including the brain [237, 240] where it plays a critical immune role in the central nervous system [241, 242]. Though initial it was suggested that CB2 was absent in the healthy brain [243, 244], it was later shown CB2 expression in diseased brain cells [245, 246], which indicate that the CB2 is up-regulated in response to immune cell activation and inflammation [247, 248]. Even though CB2 effects appear to be mainly protective (e.g. anti-inflammatory), they are not exclusively immunological as reviewed recently by Pacher and Mechoulam [249].

To date, only these two receptors meet strict pharmacological and biochemical characterisation for designation as endocannabinoid receptors. Both cannabinoid receptors, CB1 and CB2, are seven-transmembrane-spanning GPCRs (figure 1.8). The CB1 receptor is a larger protein with an additional 72 amino acid residues in the N-terminal portion, 13 additional residues in the third intracellular loop, and 14 additional residues in the C-terminal segment [250].

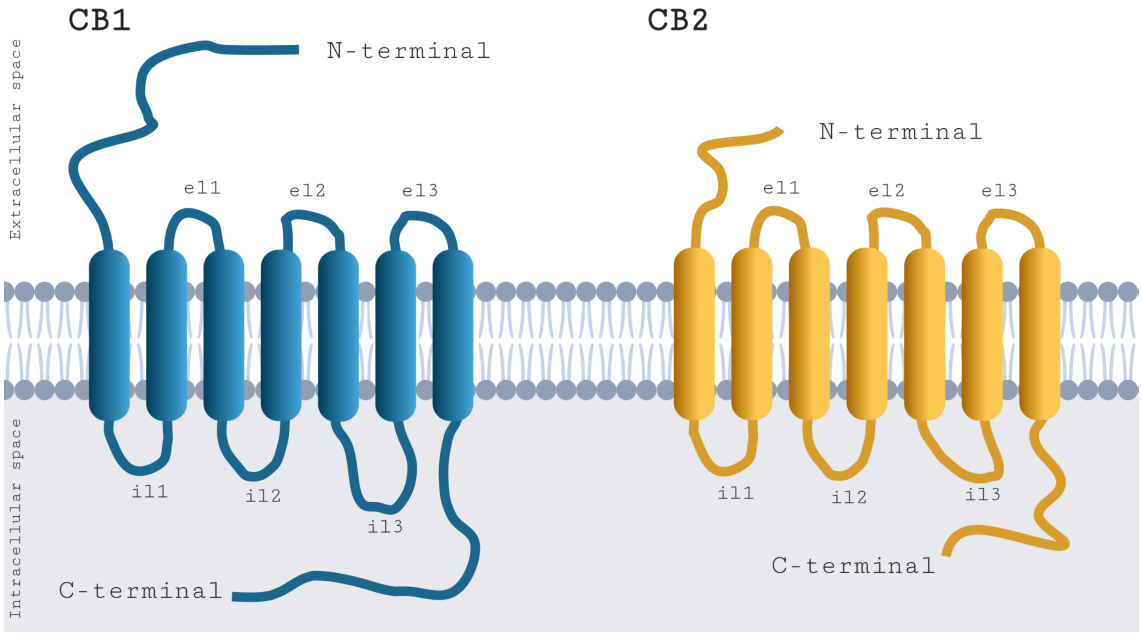

Figure 1.8: Schematic representation of the cannabinoid CB1 and CB2 receptors. *TM*, transmembrane; *el*, extracellular loop; *il*, intracellular loop [250].

GPCRs are the largest cell-surface receptor superfamily and have a vital role in the cellular response to ligands as diverse as hormones, neurotransmitters, odours and light [251]. Upon agonist binding, the CB1 and CB2 receptors regulate effectors such as adenylyl cyclase and ion channels, mitogen-activated protein kinase

(MAPK), and other signalling pathways using the $G_{i/o}$ family and other G proteins as signal transducers [232].

1.2.2 Endogenous cannabinoid signaling molecules

It is now well known that endocannabinoids are produced by all body parts and tissues as part of a homeostatic mechanism that operates at virtually every level of biological life to manage numerous physiopathological states and preserve human health [252].

Endocannabinoids are bio-active lipids that signal via the cannabinoid receptors to modulate the functional activities of cells. To date, numerous endocannabinoid-like compounds have been discovered [253, 254]. The first endocannabinoid to be discovered was anandamide (AEA), shown to bind to the CB1 receptor with high affinity [4], followed by 2-arachidonoyl glycerol (2-AG) [5]. Other endocannabinoids identified include virodhamine [255], noladin [256] and N-arachidonoyl-dopamine [257]). However, AEA and 2-AG, both derived from AA, are currently the most well-studied endocannabinoid signalling molecules [253]).

In addition to the endocannabinoids listed above, several other endogenous compounds that interact with the endocannabinoid system can interfere with their actions. These can be endocannabinoid-like compounds, which are structurally similar to true endocannabinoids, and can engage the same synthesizing and degrading enzymes but do not bind to cannabinoid receptors [258].

Like AEA, some classical endocannabinoid-like compounds are NAEs, such as palmitoylethanolamide (PEA) and oleoylethanolamide (OEA). PEA does not bind to cannabinoid receptors directly. However, it can promote the effects of phyto- and endocannabinoids by serving as an agonist of the transient receptor potential vanilloid type 1 (TRPV1), peroxisome proliferator-activated receptor alpha (PPARα) [259]. OEA is derived from the omega-9 monounsaturated fatty acid oleic acid and is formed in the gastrointestinal tract. OEA has many unique homeostatic properties, including anti-inflammatory properties, lipolysis stimulation, and fatty acid oxidation [260].

1.2.3 Endocannabinoid biosynthesis

Both AEA and 2-AG are known to be synthesised on-demand, instead of being stored in secretory vesicles like classical neurotransmitters [6, 7]. Their formation

and release from the cells is thought to occur following Ca^{2+} influx, which causes activation of Ca^{2+}-dependent biosynthetic enzymes (figure 1.9-1) [6].

Although both endocannabinoids originate from membrane glycerophospholipids, their metabolic pathways are very different. AEA belongs to the family of NAEs, and the canonical view is that AEA is mainly generated from its membrane precursor, N - arachidonoyl phosphatidylethanolamine (NAPE), through cleavage by NAPE-phospholipase D (NAPE–PLD)(figure 1.8-2) [6, 261]. Other pathways for AEA biosynthesis have been proposed following evidence that conversion of NAPE to AEA was not suppressed in tissues from NAPE-PLD knockout mice [262], and that in macrophages, decreased gene expression of NAPE-PLD induced by LPS treatment was observed, despite the increase in AEA production [263].

Three other parallel pathways have been described so far:

- NAPE conversion to 2-lyso-NAPE by a secretory phospholipase A2 (sPLA2), followed by conversion to AEA by a selective lyso-phospholipase D (lyso-PLD) through a Ca^{2+}-independent mechanism (sensitive to inhibition by methyl arachidonoyl fluorophosphate) (figure 1.8-3) [264].
- NAPE conversion to glycerophospho-arachidonoyl ethanolamine (GpAEA) by α,β-hydrolase 4 (Abhd4) followed by conversion to AEA by the glycerolphosphodiesterase 1 (GDE1)(figure 1.8-4) [265].
- NAPE conversion to phosphoanandamide (pAEA) by a phospholipase C (PLC), followed by dephosphorylation by the putative tyrosine phosphatase N22 (PTPN22) (figure 1.8-5) [263].

Although NAPE-PLD has a dominant role in AEA production, PLC was shown to be important in the rapid initial synthesis of this endocannabinoid [266].

The most important precursors of 2-AG biosynthesis are the sn-1-acyl-2-arachidonoylglycerols (DAGs) that are formed from the hydrolysis of either phosphatidylinositol 4,5-bisphosphate (PIP_2) catalysed by a phospholipase C-β (PLCβ) (figure 1.8-6), or of phosphatidic acid, catalysed by the phosphatidic acid-selective phosphohydrolase [267, 268]. Two sn-1 selective-DAG lipases isoenzymes (DAGLα and DAGLβ) have been cloned and characterised, and are thought to be responsible for converting DAGs to 2-AG (figure 1.8-6) [269]. Both DAGLα and DAGLβ are localised in the plasma membrane, stimulated by calcium and inhibited by glutathione, and although they exhibit strong selectivity for DAGs, they do not appear to prefer DAGs with any particular fatty acyl chain in the 2 or sn-1 position [269]. This

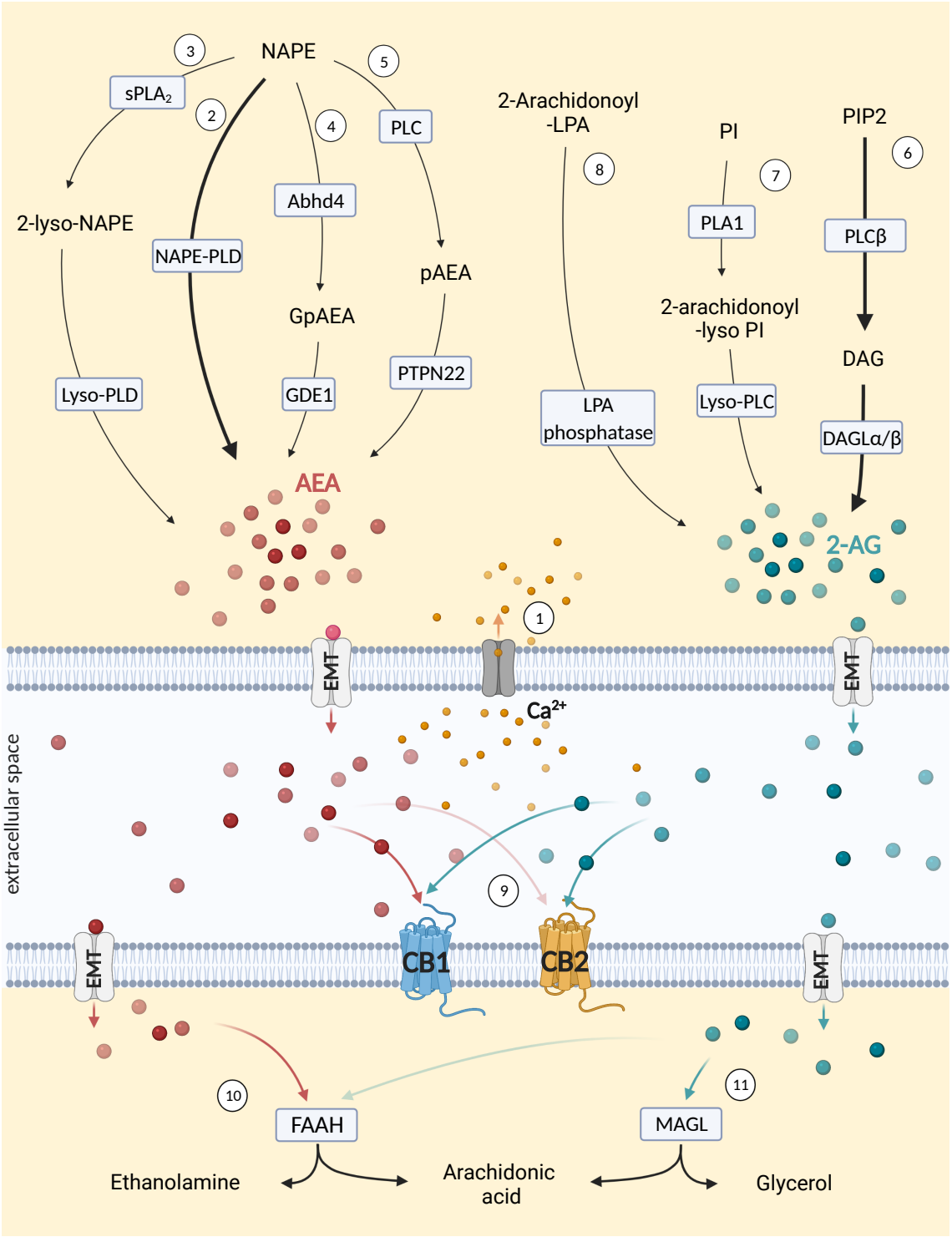

Figure 1.9: Schematic representation of the cannabinoid CB1 and CB2 receptors. In *blue*, the CB1 receptor; in *green*, the CB2 receptor; *TM*, transmembrane; *el*, extracellular loop; *il*, intracellular loop [250].

pathway is, however, not unique for 2-AG biosynthesis. When PLCβ activation was suppressed in mice brain, no reduction of 2-AG was observed [270].

Two other alternative pathways have been proposed for 2-AG synthesis. The first is the production of 2-AG via a two-step process, starting with the conversion of phosphatidylinositol (PI) to 2-arachidonoyl-lyso PI, by a phospholipase A1 (PLA1), and then to 2-AG by lyso-PLC [271] (figure 1.8-7). The second pathway involves lysophosphatidic acid (LPA) hydrolysis by LPA phosphatase [272] (figure 1.8-8).

Following their biosynthesis, AEA and 2-AG are released from the cell (figure 1.8-9). This is hypothesised to occur through action of the same putative membrane transporter referred to as endocannabinoid membrane transporter (EMT), proposed to also facilitate endocannabinoid cellular uptake [273]. Once in the extracellular medium, endocannabinoids act mostly, and with varying selectivity, on cannabinoid receptors. AEA is a partial or full cannabinoid receptor agonist, depending on the tissue and biological response measured, and with a slight higher affinity for CB1 than CB2 [274]. 2-AG, however, is a full non-selective agonist, so it binds to both receptors with similar affinity [5].

In addition to CB1 and CB2, endocannabinoids have also been reported to target several other receptors and channels, such as several transient receptor potential (TRP) ion channels, GPCRs such as GPR55, GPR18, GPR119, γ-aminobutyric acid A (GABA), glycine receptors, and the nuclear receptor peroxisome proliferator-activated receptor-gamma (PPARγ) [275, 276]. The endocannabinoids AEA and 2-AG and their structural analogs have, therefore, an extended network of cellular signaling pathways beyond the two cannabinoid receptors.

1.2.4 Endocannabinoid metabolism

Termination of endocannabinoid signalling is thought to be a two step process. First step consist of endocannabinoids transport across the plasma membrane. Inside the cell, these are then hydrolyzed by specific enzymes.

It is still not clear what exact mechanism is in place for endocannabinoid cellular uptake. Four models for AEA re-uptake have been proposed [277]. In three, hydrolysis of AEA by fatty acid amide hydrolase (FAAH) is the driving force of the process. It appears, however, to be a facilitated transport process [278] since AEA transport meets four criteria of a carrier-mediated process: saturabilty, fast rate, temperature dependence and substrate selectivity [279]. Not much is known regarding 2-AG cellular uptake and only a few studies have suggested a specific 2-AG transporter [280].

Once inside the cell, AEA is hydrolysed to AA and ethanolamine mainly by FAAH [215, 281, 282], an integral membrane bound protein whose activity is associated with microsomal and mitochondrial membranes (figure 1.8-10) [253]. Even though FAAH can also hydrolyse 2-AG to AA and glycerol [283], in FAAH knockout mice 2-AG levels increase is not observed as seen with AEA levels [284]. The main enzyme responsible for 2-AG hydrolysis is monoacylglycerol lipase (MAGL) (figure 1.8-11). These enzymes are usually found in both membrane and cytosolic fractions, and can also recognise other unsaturated monoacylglycerols, (e.g. monooleoyl-glycerol, which is a competitive inhibitor of 2-AG hydrolysis) [285, 286].

Overall, it is apparent that these metabolic enzymes regulate the in vivo biological availability of endocannabinoids, which altogether are responsible for keeping the endocannabinoid tone [16, 287].

Both AEA and 2-AG are also substrates for eicosanoid synthesizing enzymes such as LOX, COX-2 and cytochrome P450 epoxygenases (CYP450) to form new bioactive molecules [288].

1.2.5 The endocannabinoid system role in immunity

Endocannabinoids act as native modulators of immune functions, which means that the endocannabinoid system can serve as a therapeutic target in auto-immune or inflammatory diseases. Several studies with cannabinoids in disease models such as multiple sclerosis, septic shock, rheumatoid arthritis and allergic asthma have demonstrated that cannabinoids modulate the immune response during inflammatory processes [289, 290, 291, 292].

Cannabinoids [e.g. Δ9-THC and cannabidiol (CBD)] may modulate the homeostatic immune balance, regulated by the endocannabinoid system, by perturbing the balance of Th1 pro-inflammatory versus Th2 anti-inflammatory cytokines [293]. CB2 is currently intensively studied as a mediator of cannabinoid related effects to avoid the psychotropic complications involved with activating CB1.

In immune cells, the number of CB2 receptors is higher than CB1. CB2 receptors are expressed in the following rank order of mRNA levels: B cells > NK cells > monocytes > PMNs > T cells [8].

In immune cells, the expression of cannabinoid receptors is triggered by various inflammatory agents (e.g. LPS), and the same can be said about the production of endocannabinoids in immune cells [8, 9]. LPS has been reported to trigger a significant increase in biosynthesis of AEA in macrophages [294]. Cannabinoids exert their immunosuppressive properties mainly by regulating cytokine and chemokine

production, inhibiting cell proliferation, induction of apoptosis, and induction of regulatory T cells [226, 295, 296, 297]. Additionally, cannabinoids can regulate the expression of nitric oxide synthase, nitric oxide and reactive oxygen species production in immune cells, important in protection against pathogens [298].

Cannabinoids can influence T cell number and proliferation. However, studies on Δ9-THC (non-selective CB1/CB2 receptor agonist) and CBD (no activity at CB1 or CB2) effect on Th1 and Th2-specific cytokine production are contradictory [reviewed by [297, 299]. In case of allergic reactions, cannabinoids are thought to negatively regulate Th2-mediated production of interleukin-4, which is critical for immunoglobulin class switching to IgE by B-cells [300, 301]. In an animal model of allergen-induced airway inflammation, treatment with either cannabinol (CBN) or Δ9-THC for 3 days decreased IL-2, IL-4, IL-5 and IL-13 mRNA in the lungs [290]. CBN also inhibited IgE increase and overproduction of mucus in the lungs. AEA has been shown to be synthesized in lung tissue [225] but was reported to have only mild anti-inflammatory properties and failed to prevent effects of A23187-induced airway constriction in guinea pigs [302]. Furthermore, initial Δ9-THC studies have shown bronchodilator effects in asthmatic patients [303]. Although still a controversial topic, other studies have proposed a role for the endocannabinoid system in the modulation of airway smooth muscle relaxation [reviewed in [213]].

The effects of cannabinoids in the immune system are complex, and evidence of the endocannabinoid system being a possible useful target for treatment of asthma is coming to light. The characterization of the endocannabinoid system in asthma patients is, however, of crucial importance since differences such as in genetic factors (e.g. gene polymorphisms, expression, or alternative splicing) of receptors or enzymes involved in the endocannabinoid system can affect its normal function, could be partially responsible for the immune response seen in asthma patients [10]. Furthermore, this characterisation could help to point the direction of future research to develop targeted pharmacologic strategies as it can aid to elucidate the role of the endocannabinoid system in modulating the organism's immune reaction.

1.3 Aims and objectives

The necessity of being able to discriminate between the many asthma phenotypes and endotypes is becoming increasingly obvious as our understanding of asthma grows. Greater knowledge of the various aetiologies and pathologies will allow for more accurate disease diagnosis and the identification of novel treatment targets.

Because of the endocannabinoid system role in maintaining immune homeostasis in the human body, there is an unmet need for characterising this system in the asthmatic population. Understanding how different parts of the endocannabinoid system are expressed in asthma patients and exploring how it is associated with different parameters, such as clinical characteristics and outcomes, treatment response and genetic susceptibility offers the opportunity for new insights into asthma pathogenesis, potential drug targets, and predicting response to therapy.

genetic variants associated with disease that are located in the non-coding regions, most likely alter the individual's disease risk through their effect on gene expression in different tissues.

Studies have shown that the missense polymorphisms rs2229579 (Tyr316His) and rs35761398 (Arg63Gln) in the *CNR2* gene that codes for CB2 introduce functional changes to the receptor and reduce ligand efficacy to inhibit cAMP accumulation [10]. Moreover, rs35761398 can reduce endocannabinoid immune modulation in peripheral blood mononuclear cells (PBMCs) [304]. Additionally, a 1p36 polymorphism (rs4237) located in the vicinity of the *CNR2* gene region and a 6q16 polymorphism (rs13197090) located in the vicinity of the *CNR1* gene region were reported to be in expression quantitative trait loci regions that contribute to variation in expression of *CNR2* and *CNR1*, respectively [197, 305].

This thesis, therefore, aims to address the unmet need to genetically characterise the endocannabinoid system in naive asthma patients and determine if there is a relationship between endogenous cannabinoids and their inflammatory response, here represented by patients with mild to moderate persistent asthma between the ages of 5 to 18 of Caucasian origin.

In order to do so, we aimed to undertake the following:

1. Quantify the level of mRNA expression of genes (receptors: *CNR1* and *CNR2*; and enzymes: *NAPEPLD*, *ABHD4*, *FAAH*, *DAGL* and *MGLL*) that comprise the endocannabinoid system in PBMCs from naive asthma patients and compare them with the control group.

2. Investigate the relationship of the mRNA expression levels of these genes with clinical symptoms and biomarkers of inflammation at the time of diagnosis, and their usefulness in predicting treatment response with ICS or LTRA.

3. Determine how the mRNA expression levels of these genes were affected by long-term treatment with ICS or LTRA.

4. Determine if genetic variants rs4237, rs2229579 and rs35761398, (located in or near the *CNR2* gene coding region) and rs13197090 (located in the vicinity of the *CNR1* gene coding region) contribute to the onset of asthma, symptom severity or treatment outcome.

5. Perform the first pharmacogenetic analysis to determine whether the selected genetic variants affect treatment response to ICS or LTRA.

6. Determine if the selected genetic variants are in eQTL for their corresponding neighbouring gene of interest.

7. Quantify plasma levels of NAEs (AEA, PEA and OEA) in naive asthma patients and compare them with the average population.

8. Investigate the relationship of the plasma levels of AEA, PEA and OEA with clinical symptoms and biomarkers of inflammation at the time of diagnosis, their usefulness in predicting treatment response with ICS or LTRA.

9. Determine how the plasma levels of these NAEs were affected by long-term treatment with ICS or LTRA.

The central hypothesis of this thesis is that changes seen in regulation of the endocannabinoid system, reflected in the expression of its genes in PBMCs and NAEs plasma levels, are important determinants of the individual clinical profile presented by asthma patients and provide a new therapeutic perspective.

2 METHODS

2.1 Subject description

Asthmatic patients were recruited between 2008 and 2012 from the Department of Pediatric Medicine, General Hospital Murska Sobota and the University Medical Centre Maribor in Slovenia, where they were treated. Participants (n = 353) were Caucasian children of Slovenian origin, aged between 5 and 18, with mild or moderate persistent asthma, selected by fulfilling strict inclusion and exclusion criteria.

Patients included in the study were stratified into two groups based on their allergy testing results (skin prick test and specific IgE values). Subjects who tested positive to at least one aeroallergen were selected for the allergic asthma subgroup (n = 235), and those with negative tests to aeroallergens were selected for the non-allergic asthma subgroup (n = 102). The age, sex and asthma severity of both subgroups were matched. Allergy testing was not successful in 16 patients (lack of response to histamine, dermographism or skin inflammation and borderline specific IgE values), who were subsequently not stratified. All subjects with other chronic inflammatory diseases, except the allergic diseases of the upper airways, were excluded from the study. Caucasian, non-allergic and non-asthmatic subjects of similar ethnic background as asthmatic subjects, randomly selected from the Slovene population, were used as a control group (n = 276).

2.2 Study design and demographic data

Clinical data were collected at the time of enrollment in this study and again 4-6 weeks later for the purpose of evaluating asthma severity and efficacy of treatment.

Blood samples were also collected at the time of enrollment to quantify the initial gene expression, protein expression and fatty acid amides concentration in PBMCs, and to determine the patient genotype for the selected genetic polymorphisms. If

consent was given, blood samples were collected again two years later in order to establish the effect of long-term treatment in gene expression. Patients were free from any acute diseases or asthma exacerbation when blood samples and clinical measurements were taken.

Of the recruited participants, 64.9% were patients newly diagnosed with asthma, according to the National Asthma Education and Prevention Program and the American Thoracic Society (ATS) criteria [11, 12]. The other 35.1% (n= 124) of participants were not treatment-naive patients and were already receiving ICS treatment. These patients were included only in the genetic risk assessment study.

Of the 229 naive patients enrolled in this study, 103 began treatment with ICS and 116 began treatment with LTRA, as decided by their clinical physician. For 10 participants, no data was collected regarding treatment, and so these were excluded from analyses related to this topic. Patients receiving ICS treatment were prescribed 200 µg of fluticasone dry powder (Flixotide diskus®, GSK Pharmaceuticals S. A., Poland) per day if younger than 12 years of age and 400 µg daily if older. Patients receiving LTRA treatment were treated with montelukast, 5 mg tablets for patients under 12 years of age and 10 mg tablets for older patients.

To summarize, we generated the asthma subgroups shown in figure 2.1 for the purpose of individual statistical analyses (see Table 2.1 for demographic data).

Patients included in gene expression analyses

In this section of the study, 229 therapeutically naive asthma patients were included, of which 143 were allergic asthmatics, 73 were non-allergic asthmatics and 13 patients with undetermined allergic status. The mean age of this asthma group was 11.16 ± 3.04 years, of the allergic asthma subgroup, was 11.39 ± 3.00 years, and of the non-allergic asthma subgroup was 10.95 ± 2.93 years.

Differences in the clinical parameters of the two clinical phenotypes included in this study are shown in Table 2.2. Patients with allergic asthma had higher levels of total IgE (615.1 ± 554.8 IU/mL) compared to patients with non-allergic asthma (132.7 ± 180.2 IU/mL), consistent with their clinical phenotype. In addition, both FeNO levels and blood eosinophil count were higher in allergic asthma (FeNO: 46.57 ± 30.99 ppb, eosinophils: 591.8 ± 379.1 /mm^3) /than in non-allergic asthma subgroups (FeNO: 22.91 ± 20.98 ppb, eosinophils: 220.3 ± 157.4 /mm^3).

In terms of lung function, FVC/FEV1 and FEV1 measurements were significantly lower ($P = 0.0002$) for non-allergic asthma (FEV1/FVC: 88.42 ± 6.49, FEV1: 92.10 ± 11.56%) than allergic asthma patients (FEV1/FVC: 90.63 ± 6.17, FEV1: 96.08 ± 14.43%).

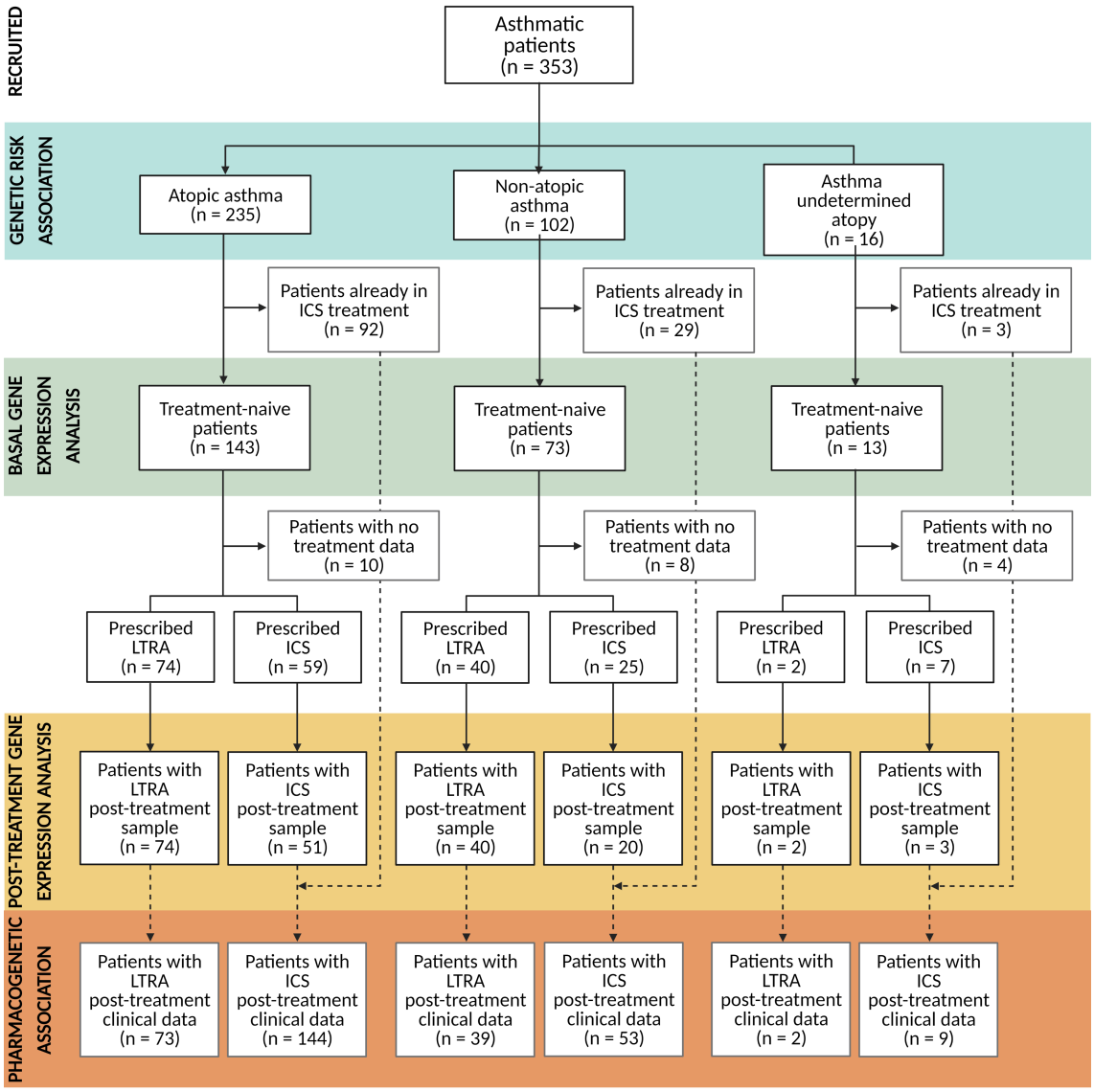

Figure 2.1: Composition of asthma patients enrolled in the study.

Finally, at the time of diagnosis, patients with allergic asthma had higher bronchial hyper-reactivity (logPC20: -0.565 ± 0.653) than those with non-allergic asthma (-0.199 ± 0.577).

Patients included in genetic association analyses

Because some patients were already receiving anti-asthmatic treatment at the time of recruitment, they were excluded from the gene expression analyses and included only in the genetic associations section of this study.

The asthma group used for these analyses included 353 asthma patients, of which 235 were allergic asthmatics, 102 were non-allergic asthmatics and 16 patients with undetermined allergic status.

Table 2.1: Demographic data of subjects enrolled in the study

		Total	Treated with ICS	Treated with LTRA
Control group	n	276	-	-
	Sex, M/F	111/165	-	-
	Age (yr, mean ± SD)	33.06±12.87	-	-
Asthma group[1]	n	353*	103 naive + 124 receiving treatment[2]	116 naive
	Sex, M/F	196/157	127/100	64/52
	Age (yr, mean ± SD)	10.99±3.22	10.83±3.40	11.26±2.91
Allergic asthma subgroup	n	235*	64 naive + 92 receiving treatment[2]	74 naive
	Sex, M/F	132/103	90/66	40/34
	Age (yr, mean ± SD)	11.06±3.13	10.90±3.28	11.36±2.87
Non-allergic asthma subgroup	n	102	28 naive + 29 receiving treatment[2]	40 naive
	Sex, M/F	54/48	28/29	23/17
	Age (yr, mean ± SD)	10.84±3.26	0.75±3.53	10.93±2.97
Ashma (undetermined atopy)	n	16	11 naive + 3 receiving treatment[2]	2 naive
	Sex, M/F	10/6	9/5	1/1
	Age (yr, mean ± SD)	10.88±4.26	10.00±3.61	14.05±2.83

ICS - Inhaled corticosteroids; LTRA - Leukotriene receptor antagonist.
[1]Asthma group is composed of allergic asthma, non-allergic asthma and asthma with undetermined allergic status.
[2]Asthmatic subjects that were already under ICS treatment at the time blood sample was collected.
*These subjects were only included in the genetic association part of the study. No post treatment clinical data was collected from 10 asthma patients (5 patients from the allergic asthma subgroup and 5 patients from the non-allergic asthma subgroup).

Table 2.2: Clinical parameters at the time of diagnosis of asthma patients included in the gene expression analyses.

Clinical parameter	**Asthma group (I)**			**Allergic asthma subgroup (II)**			**Non-allergic asthma subgroup (III)**				**Inter-group comparisons *P* value**		
	Mean	SD	n	Mean	SD	n	Mean	SD	n	*P* value	I vs. II	I vs. III	II vs. III
FEV1/FVC	89.86	3.04	229	90.63	6.17	143	88.42	6.49	73	**0.0075**	0.4204	0.0867	**0.0054**
FEV1	94.03	6.65	229	96.08	14.43	143	92.10	11.56	73	**0.0260**	0.3155	**0.0059**	**0.0002**
logPC20	-0.466	0.651	226	-0.565	0.653	142	-0.199	0.577	73	**0.0004**	0.1473	**0.0021**	**$8.3\text{x}10^{-5}$**
FeNO	38.74	30.61	223	46.57	30.99	141	22.91	20.98	69	**5.2×10^{-7}**	**0.0414**	**0.0003**	**2.3×10^{-7}**
Total IgE	447.9	492.5	47	615.1	554.8	27	132.7	180.2	15	**0.0095**	**0.4523**	**0.0860**	**0.0070**
Eosinophils	466.9	353.4	45	591.8	379.1	26	220.3	157.4	14	**0.0059**	0.4149	0.0591	**0.0042**

FEV1, forced expiratory volume in 1 s (%); FVC, forced vital capacity; logPC20, base 10 logarithm of provocative methacholine concentration causing a drop in FEV1 of 20%; FeNO, fractional exhaled nitric oxide (ppb); Total IgE (IU/mL); Eosinophils (/mm^3). One-way ANOVA followed by Bonferroni multiple comparisons correction; significant correlations (P <0.05) are shown in bold.

The mean age of the asthma group was 10.99 ± 3.22 years, of the allergic asthma subgroup, was 11.06 ± 3.13 years, and of the non-allergic asthma subgroup was 10.84 ± 3.26 years. Differences in the clinical parameters of the two clinical phenotypes included in this study are shown in Table 2.3. Patients with allergic asthma had higher levels of total IgE (683.3 ± 724.8 IU/mL) compared with patients with non-allergic asthma (203.1 ± 237.0 IU/mL), consistent with their clinical phenotype. Both FeNO levels and blood eosinophil count were also higher in allergic asthma (FeNO: 47.75 ± 30.88 ppb, eosinophils: 547.2 ± 327.5 /mm^3) than in non-allergic asthma (FeNO: 23.81 ± 21.14 ppb, eosinophils: 377.8 ± 314.8 /mm^3). Additionally, at the time of diagnosis, patients with allergic asthma had higher bronchial hyper-reactivity, measured as logPC20 (-0.504 ± 0.654), than those with non-allergic asthma (-0.178 ± 0.586). In terms of lung function, no significant difference was found between the FVC/FEV1 or FEV1 measurements from allergic and non-allergic asthma patients.

Patients included in the ICS treatment gene expression analyses.

The number of asthma patients that received ICS and continued in the study after 2 years was 74, of which 51 were allergic asthmatics, 20 were non-allergic asthmatics and 3 patients with undetermined allergic status. The mean age of this asthma group was 11.54 ± 3.08 years, of the allergic asthma subgroup, was 11.82 ± 3.04 years, and of the non-allergic asthma subgroup was 11.05 ± 3.15 years.

Differences between the asthma phenotypes in the clinical parameters of patients that received ICS and continued in the study are shown in Table 2.4. At the time of diagnosis, patients with allergic asthma had higher levels of total IgE (610.5 ± 528.8 IU/mL) compared to patients with non-allergic asthma (130.4 ± 207.4 IU/mL), consistent with their clinical phenotype. In addition, both FeNO levels and blood eosinophil count were higher in the allergic asthma (FeNO: 57.72 ± 33.24 ppb, eosinophils: 553.9 ± 289.5 /mm^3) than in non-allergic asthma subgroup (FeNO: 25.30 ± 28.74 ppb, eosinophils: 225.5 ± 178.9 /mm^3).

In terms of lung function (FVC/FEV1 and FEV1 measurements) there were no significant differences found between the allergic and non-allergic asthma subgroups However, at the time of diagnosis, patients with allergic asthma had more increased bronchial hyper-reactivity (logPC20: -0.717 ± 0.617) than those with non-allergic asthma (-0.228 ± 0.607).

Table 2.3: Clinical parameters at the time of diagnosis of asthma patients included in the genetic association analyses.

Clinical parameter	**Asthma group (I)**			**Allergic asthma subgroup (II)**			**Non-allergic asthma subgroup (III)**				**Inter-group comparisons *P* value**		
	Mean	SD	n	Mean	SD	n	Mean	SD	n	*P* value	I vs. II	I vs. III	II vs. III
FEV1/FVC	88.71	7.72	345	89.00	7.54	228	87.88	7.69	102	0.4672	0.8945	0.6021	0.4353
FEV1	90.70	14.93	345	91.65	15.56	228	89.72	13.56	102	0.5277	0.7346	0.8291	0.5217
logPC20	-0.420	0.653	332	-0.504	0.654	220	-0.178	0.586	99	**0.0002**	0.2920	**0.0032**	**0.0001**
FeNO	40.09	30.55	279	47.75	30.88	181	23.81	21.14	84	**1.1×10^{-8}**	**0.0182**	**3.1×10^{-5}**	**4.6×10^{-9}**
Total IgE	545.8	651.0	154	683.3	724.8	106	203.1	237.0	41	**0.0003**	0.2068	**0.0071**	**0.0002**
Eosinophils	503.8	329.5	149	547.2	327.5	103	377.8	314.8	39	**0.0257**	0.5540	0.0830	**0.0170**

FEV1, forced expiratory volume in 1 s (%); FVC, forced vital capacity; logPC20, base 10 logarithm of provocative methacholine concentration causing a drop in FEV1 of 20%; FeNO, fractional exhaled nitric oxide (ppb); Total IgE (IU/mL); Eosinophils (/mm^3). One-way ANOVA followed by Bonferroni multiple comparisons correction; significant correlations (P <0.05) are shown in bold.

Table 2.4: Clinical parameters of asthma patients included in the ICS treatment gene expression analyses.

Clinical parameter	**Asthma group (I)**			**Allergic asthma subgroup (II)**			**Non-allergic asthma subgroup (III)**				**Inter-group comparisons *P* value**		
	Mean	SD	n	Mean	SD	n	Mean	SD	n	*P* value	I vs. II	I vs. III	II vs. III
FEV1/FVC	89.57	7.60	74	90.51	7.651	51	87.95	6.057	20	0.4216	0.999	0.999	0.581
FEV1	91.76	12.79	74	93.43	12.33	51	89.40	12.98	20	0.4687	0.999	0.999	0.688
logPC20	-0.601	0.651	74	-0.717	0.617	51	-0.228	0.607	20	**0.015**	0.573	0.053	**0.011**
FeNO	49.03	35.30	73	57.72	33.24	50	25.30	28.74	20	**0.002**	0.489	**0.018**	**0.001**
Total IgE	450.6	489.9	28	610.5	528.8	18	130.4	207.4	9	0.053	0.801	0.248	**0.048**
Eosinophils	449.0	294.6	27	553.9	289.5	18	225.5	178.9	8	**0.028**	0.669	0.157	**0.024**

FEV1, forced expiratory volume in 1 s (%); FVC, forced vital capacity; logPC20, base 10 logarithm of provocative methacholine concentration causing a drop in FEV1 of 20%; FeNO, fractional exhaled nitric oxide (ppb); Total IgE (IU/mL); Eosinophils (/mm^3). One-way ANOVA followed by Bonferroni multiple comparisons correction; significant correlations (P <0.05) are shown in bold.

Patients included in the LTRA treatment gene expression analyses.

The number of asthma patients that received LTRA and continued in the study after 2 years was 116, of which 74 were allergic asthmatics, 40 were non-allergic asthmatics and 2 patients with undetermined allergic status. The mean age of the asthma group that received LTRA was 11.26 ± 2.91 years, of the allergic asthma subgroup, was 11.36 ± 2.87 years, and of the non-allergic asthma subgroup was 10.93 ± 2.97 years. Differences between the clinical phenotypes in the clinical parameters of patients that received LTRA and continued in the study are shown in Table 2.5.

Similar to the group of asthma patients included in the ICS treatment analysis, at the time of diagnosis the FeNO levels were higher in the allergic asthma subgroup (40.18 ± 27.44 ppb) than in the non-allergic asthma subgroup (23.55 ± 17.27 ppb), and patients with allergic asthma had more increased bronchial hyper-reactivity (logPC20: -0.522 ± 0.608) than those with non-allergic asthma (-0.182 ± 0.566).

In terms of lung function (FVC/FEV1 and FEV1 measurements) there were no significant differences found between the allergic and non-allergic asthma subgroup No eosinophil count or quantification of total IgE were performed for these patients.

Patients included in Fatty acid ethanolamides analyses

A small group of patients were selected from those who had agreed to another blood withdrawal to be included in this study. In an effort to reduce the difference in the mean age of the asthma patients and healthy individuals, the criteria for selection was their age. The oldest 37 asthma patients were included in this study of which 24 were allergic asthmatics, 12 non-allergic asthmatics and 1 patients with undetermined allergic status. The mean age of this asthma group was 15.49 ± 1.50 years, of the allergic asthma subgroup, was 15.75 ± 1.45 years, and of the non-allergic asthma subgroup was 14.92 ± 1.56 years. From the control group, the youngest 17 healthy individuals were selected and the mean age was 21.35 ± 0.99 years.

No differences were found between the clinical phenotypes in the clinical parameters of patients included (Table 2.6).

Table 2.5: Clinical parameters of asthma patients included in the LTRA treatment gene expression analyses.

Clinical parameter	**Asthma group (I)**			**Allergic asthma subgroup (II)**			**Non-allergic asthma subgroup (III)**				**Inter-group comparisons *P* value**		
	Mean	SD	n	Mean	SD	n	Mean	SD	n	*P* value	I vs. II	I vs. III	II vs. III
FEV1/FVC	89.28	5.79	116	89.92	5.06	74	88.43	6.70	40	0.411	0.999	0.999	0.558
FEV1	96.88	13.50	116	98.35	15.11	74	94.45	9.85	40	0.340	0.999	0.983	0.427
logPC20	-0.410	0.610	116	-0.522	0.608	74	-0.185	0.566	40	**0.019**	0.431	0.105	**0.013**
FeNO	35.10	26.76	114	40.18	27.44	74	23.55	17.27	38	**0.006**	0.559	**0.051**	**0.004**
Total IgE	-	-	0	-	-	0	-	-	0	-	-	-	-
Eosinophils	-	-	0	-	-	0	-	-	0	-	-	-	-

FEV1, forced expiratory volume in 1 s (%); FVC, forced vital capacity; logPC20, base 10 logarithm of provocative methacholine concentration causing a drop in FEV1 of 20%; FeNO, fractional exhaled nitric oxide (ppb); Total IgE (IU/mL); Eosinophils (/mm^3). One-way ANOVA followed by Bonferroni multiple comparisons correction; significant correlations (P <0.05) are shown in bold.

Table 2.6: Clinical parameters of asthma patients included in the NAEs plasma analyses.

Clinical parameter	**Asthma group (I)**			**Allergic asthma subgroup (II)**			**Non-allergic asthma subgroup (III)**				**Inter-group comparisons *P* value**		
	Mean	SD	n	Mean	SD	n	Mean	SD	n	*P* value	I vs. II	I vs. III	II vs. III
FEV1/FVC	89.24	5.81	37	88.96	6.66	24	89.83	4.17	12	0.916	0.999	0.999	0.999
FEV1	97.62	9.36	37	99.58	9.72	24	93.92	8.07	12	0.239	0.999	0.703	0.267
logPC20	-0.331	0.549	37	-0.378	0.583	24	-0.211	0.494	12	0.694	0.945	0.790	0.671
FeNO	44.35	32.47	37	54.29	34.56	24	27.17	17.87	12	0.056	0.693	0.311	0.051
Total IgE	297.2	198.9	5	321.8	220.7	4	199.0	0.000	1	0.873	0.999	0.999	0.999
Eosinophils	351.2	356.8	5	401.8	390.8	4	149.0	0.000	1	0.835	0.999	0.999	0.999

FEV1, forced expiratory volume in 1 s (%); FVC, forced vital capacity; logPC20, base 10 logarithm of provocative methacholine concentration causing a drop in FEV1 of 20%; FeNO, fractional exhaled nitric oxide (ppb); Total IgE (IU/mL); Eosinophils (/mm^3). One-way ANOVA followed by Bonferroni multiple comparisons correction; significant correlations (P <0.05) are shown in bold.

2.3 Sample collection

Six to twelve millilitres of venous blood were collected from all subjects, in the morning period (9 am - 11 am), into tubes with ethylenediaminetetraacetic acid and processed on the same day.

2.4 Clinical parameters

Several clinical parameters were measured in asthmatic subjects and handled as quantitative variables: forced vital capacity (FVC), forced expiratory volume in 1 sec expressed as a percent of the predicted value for sex, height and age before treatment and after treatment (FEV1), FEV1/FVC ratio, the provocative concentration of methacholine causing a drop in FEV1 of 20% (PC20) and its base ten logarithm (logPC20), total IgE concentration, eosinophil count in peripheral blood and fractional exhaled nitric oxide (FeNO) measured in parts per billion (ppb).

Allergic status was determined with the skin prick tests (Allergopharma) and specific IgE to the most common aeroallergens (CAP-RAST Pharmacia&Upjohn, Freiburg, Germany). Pulmonary function (FEV1, FVC) was measured with a Vitalograph 2150 spirometer (Compact, Buckingham, UK), according to the European Respiratory Society (ERS) and ATS guidelines [306]. Asthma patients treated with ICS or LTRA repeated the spirometry test after 4-6 weeks of treatment, and ΔFEV1 value was used as a primary measure of treatment response.

Bronchial hyperreactivity was assessed with a methacholine bronchoprovocation challenge test with dosimeter Provojet (Ganshorn Medizin Electronic, Niederlauer, Germany) and according to ATS guidelines [307]. For online measurement of the FeNO we used a Niox analyzer (Aerocrine, Inc., New Providence, NJ, USA) using the chemiluminescence method for gas analysis. The measurement was done according to ATS/ERS guidelines [308].

2.5 PBMCs and plasma isolation

PBMCs are white blood cells with round nuclei and include lymphocytes (T cells, B cells, and NK cells), monocytes, and dendritic cells. The most typical approach for isolating PBMCs requires a density gradient medium (e.g., Ficoll) and centrifugation. PBMCs and plasma were isolated using Ficoll-Plaque Plus (GE Healthcare,

Uppsala, Sweden) gradient centrifugation according to the manufacturer's instructions. Peripheral blood (6 - 12 mL) was diluted with an equal volume of PBS solution (see Appendix A). Ficoll-Plaque Plus was added to a centrifuge tube, and the diluted blood was layered over the Ficoll-Plaque Plus (figure 2.2).

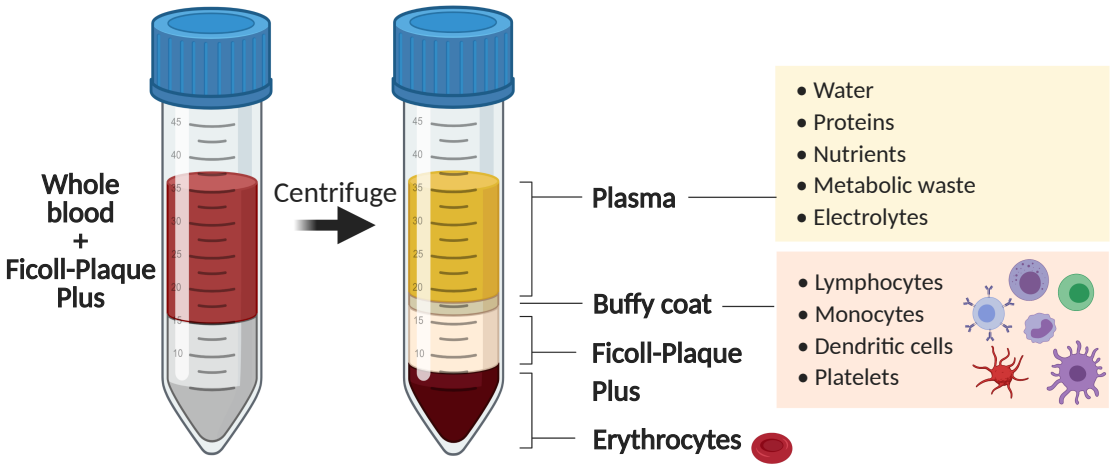

Figure 2.2: Isolation of PBMCs and plasma with Ficoll-Plaque PLUS. Peripheral blood was layered over the Ficoll-Plaque PLUS and, following centrifugation, the blood components are separated into (1) plasma, (2) PBMCs, and (3) granulocytes and erythrocytes.

Tubes were then centrifuged at 400 x g for 30 min at 18-20°C. The upper layer (plasma) was transferred into new tubes containing 100 µM phenylmethylsulfonyl fluoride for FAEs quantification, and stored at -80°C. The next layer, which contained the PBMCs, was transferred into new centrifuge tubes and washed three times with PBS and pelleted by centrifuging at 100 x g for 10 minutes at 18-20°C. After the last wash, the supernatant was removed, and the pellet of cells was stored at -80°C.

2.6 RNA and DNA isolation from PBMCs

Total RNA and genomic DNA from PBMCs were isolated using QIAzol Lysis Reagent (QIAgen, Valenica, CA, USA) according to the manufacturer's instructions. QIAzol Lysis Reagent (1.5 mL) was added to the PBMCs and cells were lyzed by pipetting and incubated for 5 min at room temperature. The lyzed cells were transferred into 2mL tubes, chloroform (300 µL) was added to the tubes and these were shaken vigorously for 15 s and then allowed to stand at room temperature for 2-3 min. Tubes were centrifuged at 12,000 x g for 15 min at 4°C.

The aqueous upper phase (figure 2.3, which contains the RNA, was transferred into a new tube where 750 µL of isopropanol was then added. The tube was inverted and allowed to stand at room temperature for 5-10 min and after centrifuged at 7,500 x g for 5 min at 4°C to precipitate the RNA. After washing with 1.5mL of 75% ethanol and centrifuging at 12,000 x g for 10 min at 4°C, the RNA pellet was allowed to air-dry for 5 min and dissolved in 60 µL of diethylpyrocarbonate (DEPC) treated water.

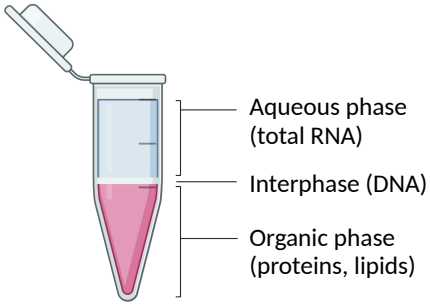

Figure 2.3: Phase separation using QIAzol Lysis Reagent. Genomic DNA, total RNA and protein from PBMCs were isolated using QIAzol Lysis Reagent.

Genomic DNA was precipitated by adding 450 µL of 100% ethanol to the interphase and organic phase, mixed by inversion and allowed to stand for 2-3 min at room temperature. After centrifuging at 5,000 x g for 5 min at 4°C, the supernatant containing the protein fraction was collected into a new tube, and the DNA pellet was washed twice with 1.5 mL of sodium citrate/ ethanol solution (0.1 M sodium citrate in 10% ethanol, pH 8.5). The DNA pellet was incubated for 30 min at room temperature with the sodium citrate/ ethanol solution and centrifuged at 2,000 x g for 5 min at 4°C. The last step of washing the DNA pellet included adding 2 mL

of 75% ethanol, incubating for 10 min at room temperature and centrifuged at at 2,000 x g for 5 min at 4°C. The DNA pellet was then allowed to air-dry for 5 min and dissolved in 50 µL of RNase free water.

The protein fraction was precipitated by adding 1.5 mL of isopropanol and mixed by inversion for 15 sec and incubated at room temperature for 10 min. After centrifuging at 12,000 x *g* for 10 min, at 4°C the supernatant was discarded and the pellet containing the protein was washed 3 times by adding 2 mL of a guanidine-ethanol solution to the pellet, incubating for 20 min at room temperature and centrifuged at 7,500 x *g* for 5 min at room temperature. After the final wash, the supernatant was 2 mL of 100% ethanol was added to the pellet and incubated for a further 20 min at room temperature. A final centrifugation was performed at 7,500 x *g* for 5 min at room temperature to remove the supernatant, after which the pellet allowed to air-dry for 5-10 min. Protein extracts were dissolved in 1% sodium dodecyl sulphate and the amount of protein was determined using Bradford reagent and BSA (Sigma, Steinheim, Germany) as standard, according to the manufacturer instructions.

DNA and RNA concentrations were determined by a ND1000 spectrophotometer and NanoDrop 3.0.1 software (NanoDrop Technologies, Wilmington, DE, USA). DNA was dissolved in water at a final concentration of 2.5 ng/µL, RNA concentrations ranged from 0.1–1.17 µg/µL, and 260/280 ratios ranged from 1.7 to 2.0. The integrity of RNA samples was analyzed by electrophoresis on a 2% agarose gel, where the 28S rRNA band was approximately twice as intense as the 18S rRNA band. DNA and RNA samples that did not pass these high-quality control standards were excluded from further analyses. All samples were immediately frozen and stored at -80°C.

2.7 Gene expression quantification

Gene transcripts (mRNA expression levels) were quantified by quantitative real-time polymerase chain reaction (qPCR). An important factor in producing reliable and consistent qPCR data is the methods used to perform qPCR experiments, from sample preparation to data and statistical analysis. For this reason, in our study, we adhered to the MIQE (minimum information for publication of quantitative real-time PCR experiments) guidelines to the best of our abilities [309].

2.7.1 cDNA synthesis

First-strand cDNA was generated by reverse transcription of 1 µg total RNA per sample with random primers and MultiScribeTM Reverse Transcriptase (50U/reaction) using High-Capacity cDNA Reverse Transcription kit (Cat. #4368813, Applied Biosystems, USA) in a final reaction volume of 20 µL. The first step of reverse transcriptase reaction was performed at 25°C for 10 min followed by a 2h incubation period at 37°C and finalized with 5 min incubation at 85°C, according to the manufacturer's instructions. The cDNA was diluted 1:20 with RNase-free water and stored at -80°C.

2.7.2 Selection of reference genes

With qPCR it is necessary to normalize gene expression data of the target gene with the gene expression data of one or more reference genes from the same sample [310, 311]. Normalization to reference genes ensures that changes in target gene expression are selective and not reflective of variations in extraction yield, reverse transcription yield, and efficiency of amplification [312].However, the identification of a reference gene whose mRNA copy number per cell remains constant must be experimentally validated [310, 311].

The use of β-actin (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) or 18S ribosomal RNA (*18S rRNA*) as reference genes is very common. However, several studies have shown that the expression levels of classical reference genes can vary extensively in different tissues, different cell types and different disease stages and are therefore unsuitable for normalization purposes [311, 313, 314, 315, 316].

Analyses of gene expression from leukocytes of asthma patients are common because they are easier to collect than specimens from the lower airways and share most abnormalities with T cells in bronchial mucosa [317]. Therefore, we analyzed a panel of 7 candidate reference genes in isolated total blood leukocytes of a limited but representative number of asthmatic patients before and after anti-asthma treatment with ICS or LTRA, and control subjects. The selected candidate reference genes included *18S rRNA*, *ACTB*, *B2M*, *GAPDH*, polymerase (RNA) II (DNA directed) polypeptide A (*POLR2A*), ribosomal protein L13a (*RPL13A*) and and ribosomal protein L32 (*RPL32*). These are commonly used as endogenous controls and good candidates for normalization of gene expression data for pharmacogenetic studies that were reported to be among the most stable reference genes in a wide variety of studies [311, 313, 318, 319, 320, 321, 322, 323, 324]. Gene stability was evalu-

ated using geNorm software, version 3.5, according to instructions. The software is available for free from `http://medgen.ugent.be/genorm/`.

We have determined that for the conditions used in this study, the most reliable strategy is the use of the geometric mean of *ACTB*, *B2M* and *GAPDH* for normalization of data [325].

2.7.3 Primer design

Primers for qPCR were designed using the Universal ProbeLibrary Assay Design Center from Roche Applied Science (`https://www.roche-applied-science.com/sis/rtpcr/upl`), selecting a set of forward and reverse primers in two separate adjacent exons.

All primers were manufactured by Sigma (Steinheim, Germany), with the exception of primers for *18S rRNA*. For *18S rRNA*, TaqMan® Endogenous Controls hydrolysis probes (Cat. #4319413E, Applied Biosystems, USA) were used (see [325] for reference gene primers). Details of primers for the genes of interest can be found in Table 2.8 and for the reference genes in Table 2.7.

Table 2.7: Reference gene's primers used for Real-Time qPCR

Gene	Accession number	Primer sequence (5'-3')	Primer (nM)	PCR efficiency (E)	Slope
ACTB	NM_001101.3	CATCGAGCACGGCATCGTCA TAGCACAGCCTGGATAGCAAC	400	1.996	-3.331
B2M	NM_004048.2	TTCTGGCCTGGAGGCTATC TCAGGAAATTTGACTTTCCATTC	500	2.005	-3.310
GAPDH	NM_002046.5	GAAGGTGAAGGTCGGAGTC GAAGATGGTGATGGGATTTC	200	2.025	-3.263

2.7.4 qPCR standard curves

To determine the optimal primer concentration for qPCR, standard curves were generated using 7 serial dilutions from a random cDNA sample. Duplicate qPCR reactions were carried out for each gene at each dilution. The LightCycler® 480 software automatically calculates efficiency ($E = 10^{(-1/slope)}$; E, efficiency and 'slope' the slope of the line generated in the efficiency plot) using the mean quantification cycle (Cq) plotted against the $\log_{10}$ of the cDNA input.

Table 2.8: Genes of interest primers used for Real-Time qPCR

Gene	Accession number	Primer sequence (5'-3')	Primer (nM)	PCR efficiency (E)	Slope
IL4	NM_000589.2	CTTTGTCAGCATTGCATCGT GATTTGCAGTGACAATGTGAGG	400	1.996	-3.331
IL5	NM_000879.2	CACTGAAGAAATCTTTCAGGGAAT CCGTCTTTCTTCTCCACACTTT	200	2.005	-3.310
IL13	NM_002188.2	AGCCCTCAGGGAGCTCAT TGATGCTCCATACCATGCTG	350	2.025	-3.263
CNR1	NM_016083.4	GCTCTCGAGATACCCAAGCA GCCTTAGAGCGTGAACCGTA	250	2.004	-3.312
CNR2	NM_001841.2	GGGAGAGGACAGAAAACAACTG GAGCTTGTCTAGAAGGCTTTGG	350	2.001	-3.319
NAPEPLD	NM_001122838.1	GCAGTGTTCCAAGTTCTAAAGAGG TGTGACTCTTAAGCCAGCTTCC	350	1.986	-3.355
ABHD4	NM_022060.2	AATCCATTGGCTGTTCTTCG GAATCGCTGCACCAGACC	350	2.002	-3.318
FAAH	NM_001441.2	GCCTGAAGGGCTGTGTCTAT CATGTCCTCGCACAGCAG	500	1.992	-3.342
DAGLA	NM_006133.2	GAGGTGGACCTGACTCCTGA AGACTGGGACTTGCTCCTGA	300	2.002	-3.316
MGLL	NM_001003794.1	CGTGCTCTCTCGGAATAAGAC AGTTGGATGCCGAAGCAC	250	2.008	-3.303

2.7.5 qPCR

The expression study was performed using a 96 multi-well white-plate (Cat. # 04729692001, Roche Applied Science, Germany) on a Roche LightCycler® 480 detection system (Roche Applied Science, Germany) with Maxima® SYBR Green qPCR Master Mix (Fermentas, Lithuania). Samples were amplified in reactions containing 2 µL of cDNA, 5 µL of 2x SYBR Green master mix, primers (concentration according to optimized standard curve of each target gene) and RNase-free water in a final reaction volume of 10 µL.

The PCR program was initiated at 95°C for 10 min to activate Taq DNA polymerase, followed by 40 thermal cycles of 15 s at 95°C, 30 s at 60°C and 30 s at 72°C. The specificity analysis of the PCR products (melting curve analysis) was performed after the real-time PCR. The temperature range used for the melting curve generation was from 65°C to 95°C.

With *18S rRNA* hydrolysis probes, samples were amplified in reactions containing 2 µL of cDNA, 5 µL of 2x LightCycler® 480 Probes Master (Roche Applied Science, Germany), 0.5 µL of hydrolysis probe and 2.5 µL RNase-free water in a final reaction volume of 10 µL. The PCR program was initiated at 95°C for 10 min, followed by 50 thermal cycles of 15 seconds at 95°C and 60 seconds at 60°C. Samples

were analyzed in duplicate wells and each PCR run included a no-template control using water instead of cDNA.

Data was retrieved using the LightCycler® 480 software release 1.5.0 (Roche Applied Science, Germany).The target gene relative expression was calculated using the following equation:

$$-\Delta\Delta Cq = -\left(Cq_{target} - Cq_{reference}\right)_{sample} - \text{average}\left(Cq_{target} - Cq_{reference}\right)_{control}$$

2.8 Genotyping

Genotyping of polymorphisms analyzed was performed using the High Resolution Melting (HRM) curve analysis following touchdown PCR amplification.

2.8.1 Primer design

Primers for HRM analysis were designed using Primer3 tool (available from `http://primer3plus.com/primer3web/primer3web_input.htm`) for amplicon sizes between 60-100 bp. All primers were manufactured by Sigma (Steinheim, Germany). Details of primers used for genotyping genetic variants can be found in Table 2.9.

Table 2.9: Details on the primers used for High-Resolution Melting (HRM)

Gene	**rs number**	**Primer sequence (5'-3')**	**Amplicon size (bp)**	**Primer (nM)**	**$MgCl_2$ (mM)**
CNR2	rs4237	CATGGCCACATTAACTGGAA TGGTTCTCTAGGGCGCTGT	93	200	3.0
CNR2	rs2229579	TGCTCTACGGAGTGGAGAGAT AGGCCCCTCACACACTTCT	75	200	2.5
CNR2	rs35761398	CAAGCTGCCAATGAACAGGT AACGTGGCTGTGCTCTATCT	78	200	3.5
CNR1	rs13197090	TGCCTCTGAAATGTCTAAATCGG TGGTAGTCAGAGGGCTAGGC	95	200	2.5

2.8.2 Touchdown PCR amplification

The touchdown PCR amplification was performed using a 96 multi-well white-plate (Cat.#04729692001, Roche Applied Science, Mannheim, Germany) on a Roche LightCycler® 480 detection system (Roche Applied Science, Mannheim, Germany).

Samples were amplified in reactions containing 2 µL of genomic DNA (2.5 ng/µL), 5 µL of 2x LightCycler® 480 High Resolution Melting Master mix (Roche Applied Science, Mannheim, Germany), primers (200 nM final concentration), $MgCl_2$ (final concentration according to optimization), and RNase-free water in a final reaction volume of 10 µL. The touchdown PCR program was initiated at 95°C for 10 min, followed by 45 thermal cycles of 10 s at 95°C, 15 s at 63°C (secondary target temperature 53°C, with 0.5°C steps) and 10 s at 72°C.

2.8.3 HRM curve analysis

The HRM curve analysis was performed with a temperature range used for the melting curve generation from 65°C to 95°C with 25 signal acquisitions per °C.Data was retrieved and genotypes were determined using the LightCycler® 480 software release 1.5.0 (Roche Applied Science, Germany).

2.9 Protein expression

CB1 and CB2 protein levels were measured in protein extractions from lysed blood leukocytes by indirect enzyme-linked immunosorbent assay (ELISA). Samples (50µL, 20µg/mL) were incubated overnight at 4°C in Nunc-Immuno MicroWell MaxiSorp 96 well plates (Nunc, Roskilde, Denmark). Plates were washed 3x with PBS followed by blocking with 5% FBS in PBS and incubating at room temperature for 2h, after which they were washed 2x with PBS and incubated with 100µL of rabbit anti-CB1 (Abcam, Cambridge, UK; ab75165; 1:4000 dilution) or goat anti-CB2 (Abcam; ab77265; 1:600 dilution) primary antibody solution overnight at 4°C. Washing of plates with PBS was repeated 4x and then incubated with 100µL of conjugated secondary antibody to rabbit IgG (Abcam; ab97064; 1:1000 dilution) or goat IgG (Abcam; ab6741; 1:1000 dilution) for 2h at room temperature. Following a final 4x wash with PBS, 100µL of 3,3'5,5'-tetramethylbenzidine peroxidase substrate solution (Sigma) was added to each well and incubated for 30 min. An equal volume of 2M H_2SO_4 (Sigma) was added to each well and the optical density read at 450nm. The A405 values in unknown samples were within the linearity range of a calibration curve drawn with different amounts of homogenates (in the range 0 – 40 µg/mL protein per well). The results are expressed as median percentages ± (IQR) of control subjects.

2.10 AEA, PEA and OEA quantification

The quantification of the fatty acid amides, AEA, PEA and OEA in human plasma was performed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) following solid-phase extraction (SPE). Samples were quantified in two analytical runs. Each run included a set of controls and the pre and post-treatment samples for a set of subjects.

2.10.1 Reference items

Anandamide (Cat. #A0580, batch 076k5202), Palmitoylethanolamide (Cat. #P0359, batch 037K1316) and Oleoylethanolamine (Cat. #O0383, batch 067K5200) were purchased from Sigma-Aldrich.

2.10.2 Test method

The analysis of samples extracts for AEA, PEA and OEA, was performed using triple quadrupole liquid chromatogragy-mass spectrometry (TQ LC-MS/MS), G6460 from Agilent with electrospray ionisation and Agilent jet stream. Separation was performed on Agilent Poroshell 120 Phenyl-Hexyl, 4.6 x 50 mm; 2.7 µm, using water (A) and acetonitrile 0.1% formic acid (B) as mobile phase and a stop time of 12 min. The flow rate was 0.3 mL/min. Samples were maintained at 4ºC throughout. The ionisation mode was electrospray, polarity positive. Electrospray jetstream conditions were as follows: capillary voltage, 3500 V; drying gas flow, 10 L/min nitrogen; drying gas temperature, 300°C; nebuliser pressure, 30 psi; sheath gas temperature, 400°C; and sheath gas flow, 11 L/min. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. The multiple reaction monitoring pair was m/z 326.29→62, for OEA; m/z 300.26→62, for PEA; m/z 328.5→62, for OEA-d2; m/z 304.5→62, for PEA-d4; m/z 348→62, for AEA; m/z 356.6→62, and for AEA-d8. The collision energy used for all compounds is 12 eV; however, depending on the equipment conditions, it can change. Injection volumes for samples and standards were 5 µL. Peaks from standards and analytes were integrated using MassHunter Workstation software version B.04.00. MassHunter Workstation software calculated the concentration of each compound using calibration curves of concentration against relative response.

2.10.3 Other equipment used

ASPEC-XL4 was used to extract the compounds by SPE.

2.10.4 Sample extraction

Aliquots of human plasma (100 µL) were added to 400 µL of an internal standard working solution containing AEA-d8, PEA-d4 and OEA-d2 (33 ng/mL) in MilliQ water. Samples placed into (16*125 mm) glass culture tubes were vortex-mixed and loaded (400 µL) into Oasis cartridges (HLB, 30 mg, 1mL Waters) previously conditioned with 1 mL of methanol and with 1 mL of water. After loading with a sample, the cartridges were washed twice with 0.5 mL of 40% aqueous methanol, and after the second wash, the cartridges were flushed with an air push of 2 mL at 1 mL/min. The samples were eluted twice with 500 µL of acetonitrile with an air push of 2 mL at 1 mL/min. The eluate was dried under vacuum until dryness for up to 2 hours and reconstituted in 100 µL of acetonitrile. The samples were injected (5 µL) into the LC-MS/MS.

2.10.5 Quality control of the assay

An analytical run to quantify AEA, PEA and OEA consisted of a calibration curve (CC), quality control (QC) samples, and all processed samples analyzed as one batch. A chromatographic run was considered acceptable if the CC had at the minimum four calibration points and the r 0.990; a set of QC samples running at the beginning and the end of the analytical run and at least 50% of the total QC samples were within an accuracy of 25%.

2.10.6 Data acquisition

The data acquisition was performed using MassHunter B.04.01, Agilent. The Quantitative Analysis was performed using MassHunter Workstation Software (version B.04.00/ Build 4.0.225.19, Agilent Technologies).

2.10.7 Data analysis

The software calculated the estimation of concentration in unknown samples by interpolating from standard curves, and the results are given in ng/mL of plasma. The concentration of the analyte was calculated using the internal standardization method.

2.11 Statistical analysis

Data analysis was carried out using GraphPad Prism 9 (GraphPad Software, Inc., La Jolla, CA, USA). Normal distribution of continuous variables was tested using D'Agostino-Person omnibus normality test.

When data was determined to be normally distributed, the ANOVA test followed by Bonferroni multiple comparison correction test or t-test for two independent samples was used to determine if there is a significant difference between the means of the groups analysed. Data are presented as mean ± standard deviation (SD) when parametric tests were used. When data was determined to be not normally distributed, the nonparametric equivalent was used (Kruskal–Wallis test followed by Dunn's multiple comparison test). Data are presented as median ± inter-quartile range (IQR) when nonparametric tests were used.

Spearman's correlation, the non-parametric version of Pearson's correlation test, was used to analyse associations between two continuous variables, such as levels of mRNA expression and clinical data. In table 2.10 shows the relationship strength used as a guideline when interpreting correlation coefficients.

Table 2.10: Interpretation of Spearman's r_s values

r_s value	Strength of the Relationship
0.000 - 0.100	Negligible
0.101 - 0.200	Very weak
0.201 - 0.400	Weak
0.401 - 0.600	Moderate
0.601 - 0.800	Strong
0.801 - 1.000	Very Strong

Genetic associations

The X^2 test and two-sided Fisher's exact test were used to calculate the significance of differences in allele and genotype frequencies (according to general genetic, dominant and recessive models) between asthmatic and control subjects. We calculated odds ratio (OR) for asthma with 95% confidence interval (95% CI). The X^2 test was also used for assessment of Hardy–Weinberg equilibrium.

The power of the association study for rs4237 was calculated to be 0.61 for the dominant model and 0.26 for the recessive model and for rs35761398 it was 0.68 for the dominant model and 0.12 for the recessive model of case-control genetic associations at $\alpha = 0.05$ (determined using the tool available at `http://pngu.mgh.harvard.edu/~purcell/gpc/`). Linkage disequilibrium (LD) was calculated using Haploview software version 4.2 [326]. We analyzed the influence of genotype on some clinical parameters that are quantitative traits: FeNO, logPC20, blood eosinophil count, total serum IgE, FEV1, FEV1/FVC ratio and their change after ICS or LTRA treatment (ΔFEV1, ΔFEV1 /FVC). All genetic associations with clinical parameters were confirmed with generalized linear model (GLM) adjusting for age and gender, and respective P values are indicated by P_{GLM}. When ΔFEV1 is used as a primary measurement of treatment response, the results will depend on the level of initial airway obstruction, therefore, we confirmed the significant associations with GLM adjusting for FEV1 before treatment in addition to age and gender as potential confounders. GLM analysis was carried out using IBM SPSS Statistics software version 23. Genotype influence on gene expression was assessed with Kruskal–Wallis test followed by Dunn's multiple comparison test.

Statistical significance

P values <0.05 were considered to indicate statistical significance. Bonferroni correction of the multiple analysis performed in the entirety of the study was not performed to avoid unnecessary increase in type II errors [327].

2.12 Ethical approval

The study was carried out in accordance with the Helsinki declaration of the World Medical Association (1975) and approved by the Slovenian National Medical Ethics Committee (KME 88/02/15). All patients and healthy individuals agreed to participate prior to inclusion in the study with signed informed consent. For patients younger than 16 years informed consent was given by their caregivers.

3 RESULTS

3.1 The cannabinoid receptors in naive asthma patients

3.1.1 IL-4, IL-5 and IL-13 gene expression as biomarkers of inflammation

Because naive patients were not receiving any anti-asthmatic treatment before samples were collected, it was possible to use the mRNA expression levels of *IL4*, *IL5* and *IL13* as additional biomarkers of inflammation and results are shown in Figure 3.1. The increased inflammatory response in asthma patients was confirmed by elevated mRNA expression levels of *IL4* (1.25 ± 3.1 fold, relative to control), *IL5* (0.535 ± 2.34 fold, relative to control) and *IL13* (1.29 ± 1.79 fold, relative to control) compared to healthy subjects (*IL4*: -0.52 ± 1.2+1.293, *IL5*: -0.13 ± 0.685+0.9825, *IL13*: 0.22 ± 0.945+0.76 fold, relative to control), and it did not differ significantly between both clinical phenotypes, allergic and non-allergic asthma.

Because the average age of participants in the control group is higher than in the asthma group, we analyzed gene expression according to age to test the possibility that the observed difference was age associated. No significant correlation was found between the subjects' age and mRNA expression levels of *IL4* (r_s = -0.043, P = 0.593), *IL5* (r_s = 0.142, P = 0.076) or *IL13* (r_s = 0.119, P = 0.509).

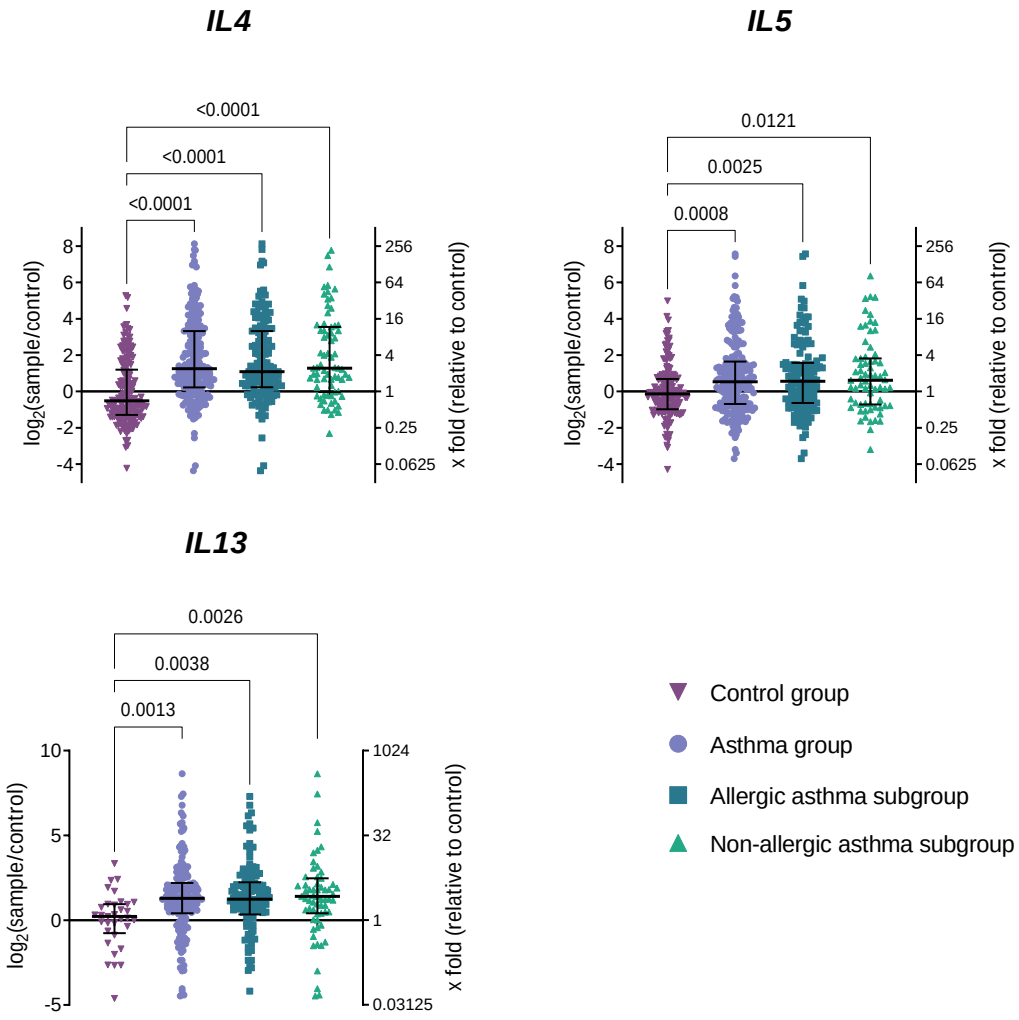

Figure 3.1: IL-4, IL-5 and IL-13 gene expression in PBMCs from asthmatic children before treatment. *IL4* mRNA expression levels [control group (n = 162), asthma group (n = 218), allergic asthma subgroup (n = 135), non-allergic asthma subgroup (n = 71)]. *IL5* mRNA expression levels [control group (n = 162), asthma group (n = 219), allergic asthma subgroup (n = 136), non-allergic asthma subgroup (n = 71)]. *IL13* mRNA expression levels [control group (n = 33), asthma group (n = 183), allergic asthma subgroup (n = 117), non-allergic asthma subgroup (n = 63)] Each dot represents a data point, horizontal lines display the median value and whiskers illustrate the IQR. Kruskal-Wallis test followed by Dunn's multiple comparisons test.

3.1.2 CB1 and CB2 gene expression in naive asthma patients and correlation with asthma severity

Before treatment, the asthma group, as well as the allergic and non-allergic subgroups, had median relative mRNA expression levels of *CNR1* and *CNR2* that were between 1.4 and 1.9-fold higher than the control group (Fig. 3.2). Because the average age of participants in the control group is higher than in the asthma group, we analyzed gene expression according to age to test the possibility that the observed difference was age associated. No significant correlation was found between the subjects' age and mRNA expression levels of *CNR1* ($r_s = 0.051$, $P = 0.537$) or *CNR2* ($r_s = 0.113$, $P = 0.158$).

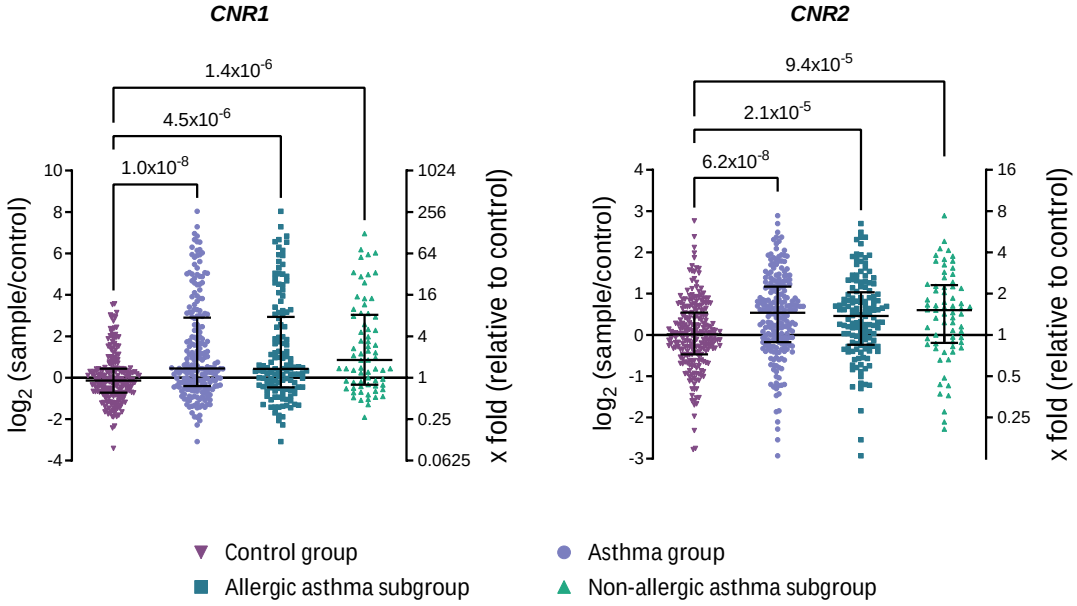

Figure 3.2: CB1 (*CNR1*) and CB2 (*CNR2*) gene expression in PBMCs from naive asthma patients. *CNR1* mRNA expression [control group (n = 237), asthma group (n = 204), allergic asthma subgroup (n = 127), non-allergic asthma subgroup (n = 66)]. *CNR2* mRNA expression [control group (n = 235), asthma group (n = 206), allergic asthma subgroup (n = 130), non-allergic asthma subgroup (n = 65)]. Each dot represents a data point, horizontal lines display the median value and whiskers illustrate the IQR. Kruskal-Wallis test followed by Dunn's multiple comparisons test.

Both *CNR1* and *CNR2* mRNA expression levels were significantly correlated with several clinical parameters and these are summarised in Table 3.1 (see Table 1 for all correlation results).

The mRNA expression levels of *CNR1* showed a very weak negative correlation with FEV1/FVC (r_s = -0.147, P = 0.036). Asthma patients with higher levels of *CNR1* mRNA expression had increased airway obstruction, which was more evident in the subgroup of patients with allergic asthma (r_s = -0.263, P = 0.003).

In a similar trend to *CNR1* mRNA expression, the levels of *CNR2* mRNA expression asthma patients showed a very weak negative correlation with FEV1/FVC. Asthma patients with higher *CNR2* mRNA expression levels also had increased airway obstruction (r_s = -0.159, P = 0.024), which was again more evident in the allergic asthma subgroup (r_s = -0.242, P = 0.007). Additionally, the mRNA expression levels of *CNR2* from asthma patients showed a very weak negative correlation with airway hyper-responsiveness (r_s = -0.150, P = 0.034). Asthma patients with higher levels of *CNR2* mRNA expression had increased airway responsiveness, as measured by logPC20.

Table 3.1: Significant correlations of CB1 (*CNR1*) and CB2 (*CNR2*) gene expression with clinical data before anti-asthmatic treatment.

Gene	**Asthma phenotype**	**Clinical parameter**	**n**	**r_s**	***P* value**
CNR1	Asthma	FEV1/FVC	204	-0.147	0.036
	Allergic asthma	FEV1/FVC	127	-0.263	0.003
	Non-allergic asthma	FeNO	62	0.346	0.006
CNR2	Asthma	FEV1/FVC	200	-0.159	0.024
		logPC20	201	-0.150	0.034
		Total IgE	43	0.323	0.035
		Eosinophils	41	0.378	0.015
	Allergic asthma	FEV1/FVC	124	-0.242	0.007

FEV1, forced expiratory volume in 1 s (%); FVC, forced vital capacity; logPC20, base 10 logarithm of provocative methacholine concentration causing a drop in FEV1 of 20%; FeNO, fractional exhaled nitric oxide (ppb); Total IgE (IU/mL); Eosinophils (/mm^3); r_s, Spearman's correlation coefficient, P <0.05 was considered significant.

The mRNA expression levels of *CNR1* and *CNR2* were also found to be correlated with levels of inflammation and allergic markers measured at the clinic. In the non-allergic asthma subgroup the mRNA expression levels of *CNR1* showed a weak correlation with FeNO ($r_s = 0.346$, $P = 0.006$). In this subgroup, patients with higher *CNR1* mRNA expression levels had higher FeNO values. While in the asthma group *CNR2* mRNA expression levels showed a weak correlation with eosinophil count ($r_s = 0.378$, $P = 0.015$) and with total IgE levels($r_s = 0.323$, $P = 0.035$). Asthma patients with higher levels of *CNR2* mRNA expression had higher number of eosinophils in the blood and higher levels of total IgE.

Additional analyses of *CNR1* and *CNR2* gene expression relationship with the gene expression of *IL4*, *IL5* as biomarkers of inflammation indicate that *CNR1*, and to some extent *CNR2*, are co-expressed (Figure 3.3 and 3.4). In naive asthma patients, *CNR1* mRNA expression levels strongly correlated with the mRNA expression levels of *IL4* ($r_s = 0.668$, $P = 2.2\text{x}10^{-27}$) and *IL5* ($r_s = 0.668$, $P = 4.0\text{x}10^{-27}$) (Figure 3.3). A similar pattern of co-expression was found when patients were stratified according to their phenotype.

The mRNA expression levels of *CNR1* strongly correlated with the mRNA expression levels of *IL4* and *IL5* in both allergic and non-allergic asthma subgroups. On the other hand, the correlation of mRNA expression levels of *CNR2* with the mRNA expression levels of *IL4* ($r_s = 0.218$, $P = 0.002$) and *IL5* ($r_s = 0.282$, $P = 6.4\text{x}10^{-5}$) in naive asthma patients was weak (Figure 3.4). When asthma patients were stratified into their phenotypes, this co-expression was stronger in the non-allergic asthma subgroup than in the allergic asthma subgroup.

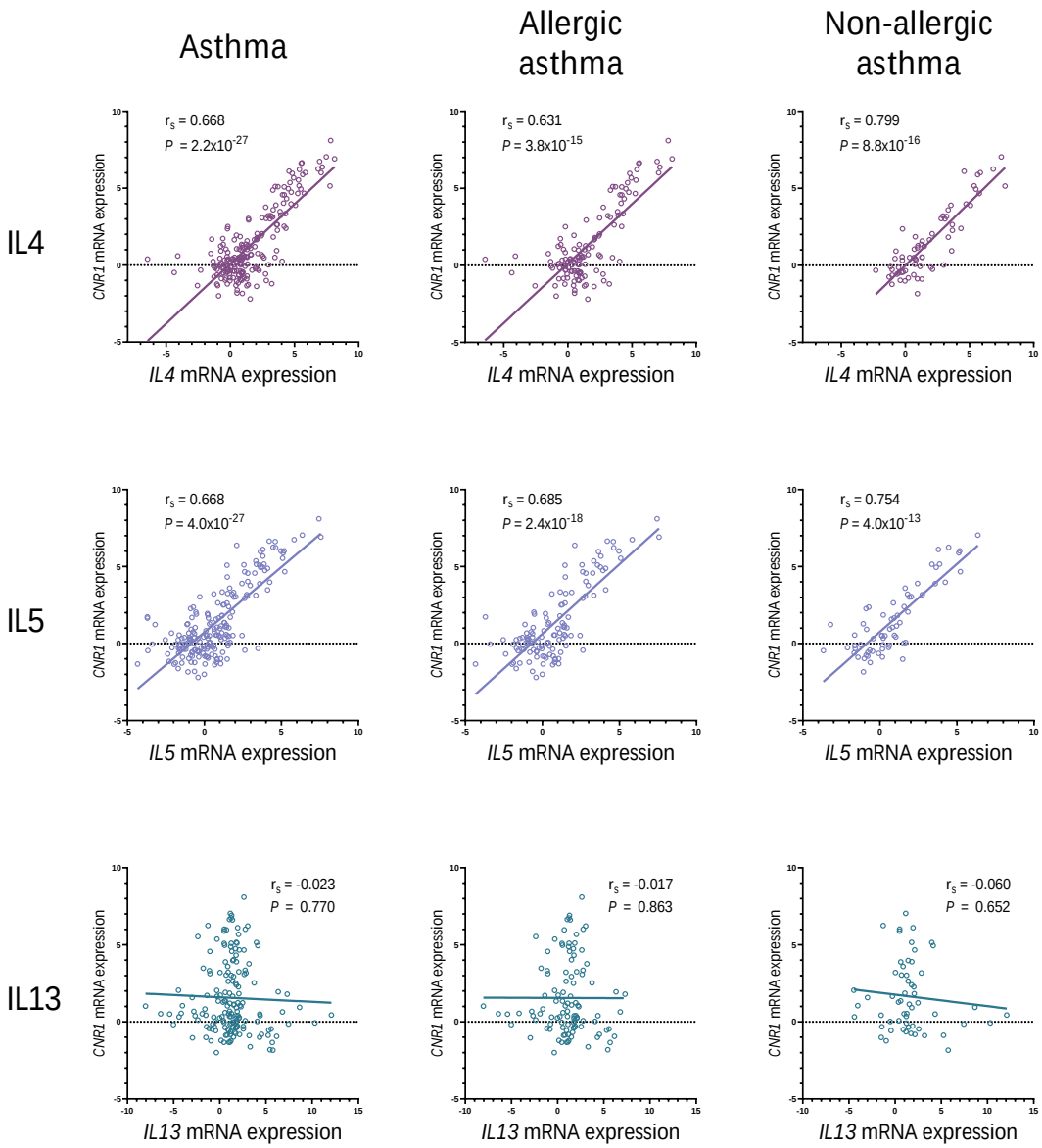

Figure 3.3: CB1 (*CNR1*) gene expression in PBMCs from naive asthma patients. IL-4 (*IL4*): asthma group (n = 201), allergic asthma subgroup (n = 124), non-allergic asthma subgroup (n = 66); IL-5 (*IL5*): asthma group (n = 199), allergic asthma subgroup (n = 123), non-allergic asthma subgroup (n = 65); IL-13 (*IL13*): asthma group (n = 168), allergic asthma subgroup (n = 107), non-allergic asthma subgroup (n = 58) Each dot represents a data point, lines display the trend. r_s, Spearman's correlation coefficient, P <0.05 was considered significant.

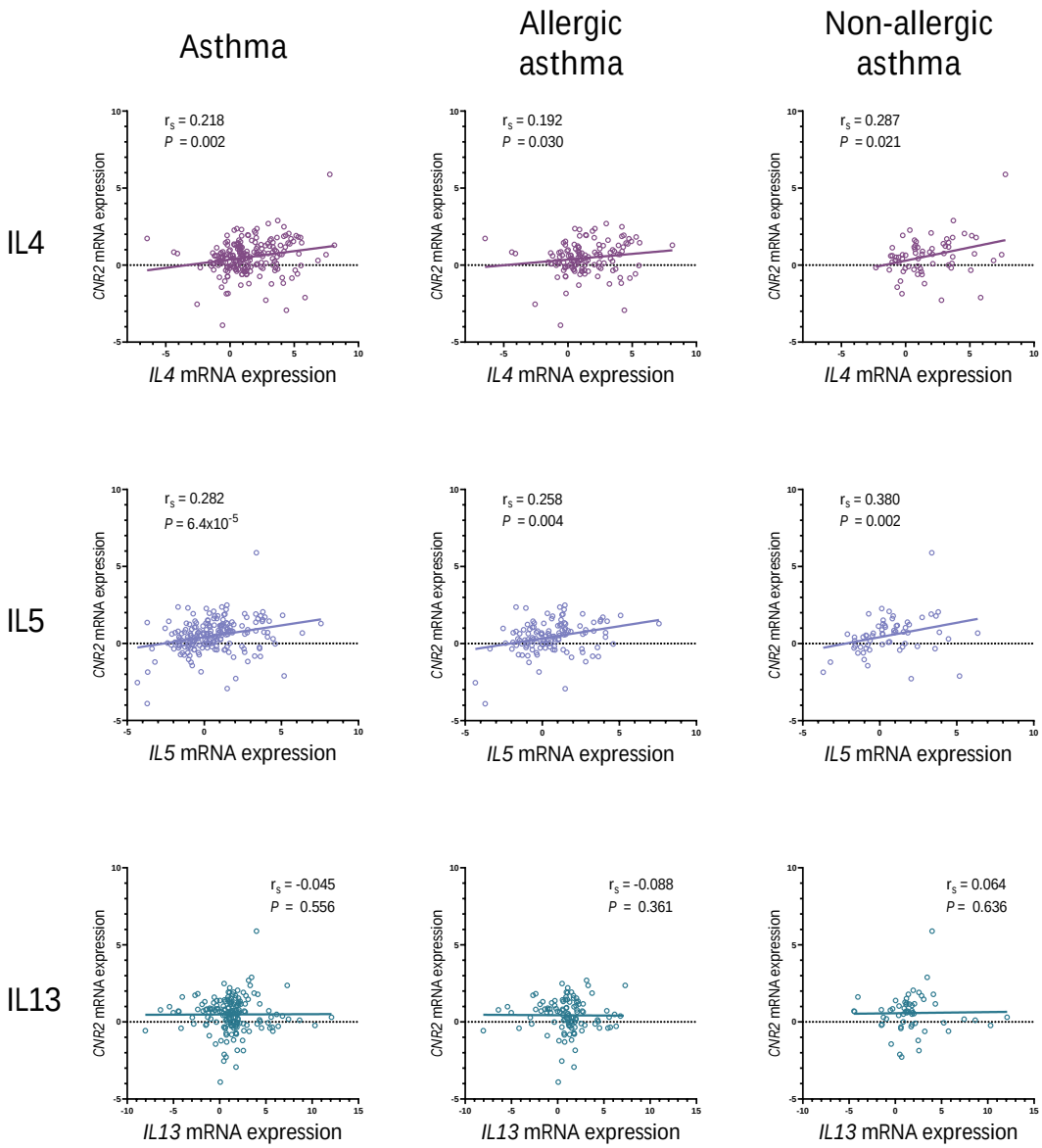

Figure 3.4: CB2 (*CNR2*) gene expression in PBMCs from naive asthma patients. IL-4 (*IL4*): asthma group (n = 203), allergic asthma subgroup (n = 127), non-allergic asthma subgroup (n = 65); IL-5 (*IL5*): asthma group (n = 195), allergic asthma subgroup (n = 122), non-allergic asthma subgroup (n = 62); IL-13 (*IL13*): asthma group (n = 169), allergic asthma subgroup (n = 109), non-allergic asthma subgroup (n = 57) Each dot represents a data point, lines display the trend. r_s, Spearman's correlation coefficient, P <0.05 was considered significant.

3.1.3 *CNR1* and *CNR2* mRNA expression levels before treatment as predictors of ICS and LTRA treatment response

We analyzed the correlation of *CNR1* and *CNR2* gene expression before treatment with changes of the FEV1/FVC ratio and FEV1 after ICS and LTRA treatment.

We found that the *CNR1* mRNA expression levels of asthma patients before treatment with ICS had a weak negative correlation with ΔFEV1 values (r_s = -0.281, $P = 0.014$) (Table 3.2). Asthma patients with higher levels of *CNR1* mRNA expression before treatment and who received ICS treatment had smaller change in lung function indicated by smaller ΔFEV1 values.

Table 3.2: Correlation of CB1 (*CNR1*) and CB2 (*CNR2*) gene expression of naive asthma patients with their response to ICS treatment.

Phenotype	Treatment response	*CNR1*			*CNR2*		
		n	r_s	P value	n	r_s	P value
Asthma	ΔFEV1/FVC	76	-0.024	0.840	78	0.034	0.771
	ΔFEV1	**76**	**-0.281**	**0.014**	78	0.016	0.892
Allergic asthma	ΔFEV1/FVC	52	0.092	0.517	50	0.039	0.788
	ΔFEV1	52	-0.203	0.149	50	0.103	0.479
Non-allergic asthma	ΔFEV1/FVC	19	-0.035	0.887	23	-0.045	0.839
	ΔFEV1	19	-0.234	0.336	23	-0.127	0.563

ICS, inhaled corticosteroids; *CNR1*, CB1 gene expression; *CNR2*, CB2 gene expression; FEV1, forced expiratory volume, 1 s; FVC, forced vital capacity; r_s, Spearman's correlation coefficient, significant correlations (P <0.05) are shown in bold.

On the other hand, the levels of *CNR1* mRNA expression of asthma patients before treatment with LTRA had a weak positive correlation with ΔFEV1/FVC values (r_s = 0.229, P = 0.019), and remained significant only in the non-allergic asthma subgroup (r_s = 0.344, P = 0.034) after stratification into phenotypes (Table 3.3). Asthma patients with higher levels of *CNR1* mRNA expression before treatment and who received LTRA treatment had a more significant improvement of lung function indicated by higher ΔFEV1/FVC values.

Similarly, the *CNR2* mRNA expression levels of asthma patients after treatment with LTRA also had a weak positive correlation with ΔFEV1/FVC values (r_s = 0.202, P = 0.042), which remained significant only in the non-allergic asthma subgroup (r_s = 0.427, P = 0.011) after stratification into phenotypes (Table 3.3). Asthma patients with high *CNR2* mRNA expression levels before treatment and who received LTRA treatment had a more significant improvement of lung function indicated by higher ΔFEV1/FVC values.

Table 3.3: Correlation of CB1 (*CNR1*) and CB2 (*CNR2*) gene expression of naive asthma patients with their response to LTRA treatment.

Phenotype	Treatment response	*CNR1*			*CNR2*		
		n	r_s	P value	n	r_s	P value
Asthma	ΔFEV1/FVC	**104**	**0.229**	**0.019**	**102**	**0.202**	**0.042**
	ΔFEV1	103	0.115	0.247	101	0.150	0.135
Allergic asthma	ΔFEV1/FVC	64	0.156	0.219	66	0.072	0.567
	ΔFEV1	63	0.116	0.366	65	0.040	0.752
Non-allergic asthma	ΔFEV1/FVC	**38**	**0.344**	**0.034**	**35**	**0.427**	**0.011**
	ΔFEV1	38	0.164	0.324	35	0.306	0.074

LTRA, leukotriene receptor antagonist; *CNR1*, CB1 gene expression; *CNR2*, CB2 gene expression; FEV1, forced expiratory volume, 1 s; FVC, forced vital capacity; r_s, Spearman's correlation coefficient, significant correlations (P <0.05) are shown in bold.

3.1.4 Relationship between CB1 and CB2 gene expression and protein expression levels

CB1 and CB2 protein expression levels were quantified from the PBMCs samples of all subjects participating in this study. Correlation analysis revealed that for both CB1 and CB2 receptor expression, there is a weak positive correlation between the gene expression and the protein levels (Figure 3.5).

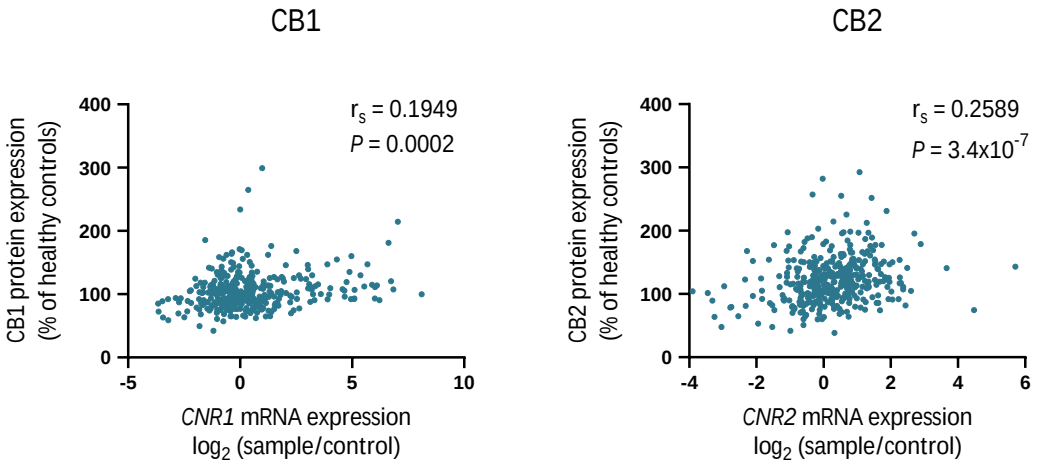

Figure 3.5: Spearman´s correlation analysis of gene and protein expression levels of CB1 (n = 361) and CB2 (n = 378). Each dot represents a data point, P <0.05 considered significant.

This correlation is reflected on a pattern of protein expression across the different groups similar to their respective gene expression (Figure 3.6). Before treatment, the asthma group, as well as the allergic and non-allergic subgroups, had median relative mRNA expression levels of *CNR1* and *CNR2* that were between 15.5 and 16.5% higher than the control group (P <0.0001, Figure 3.6).

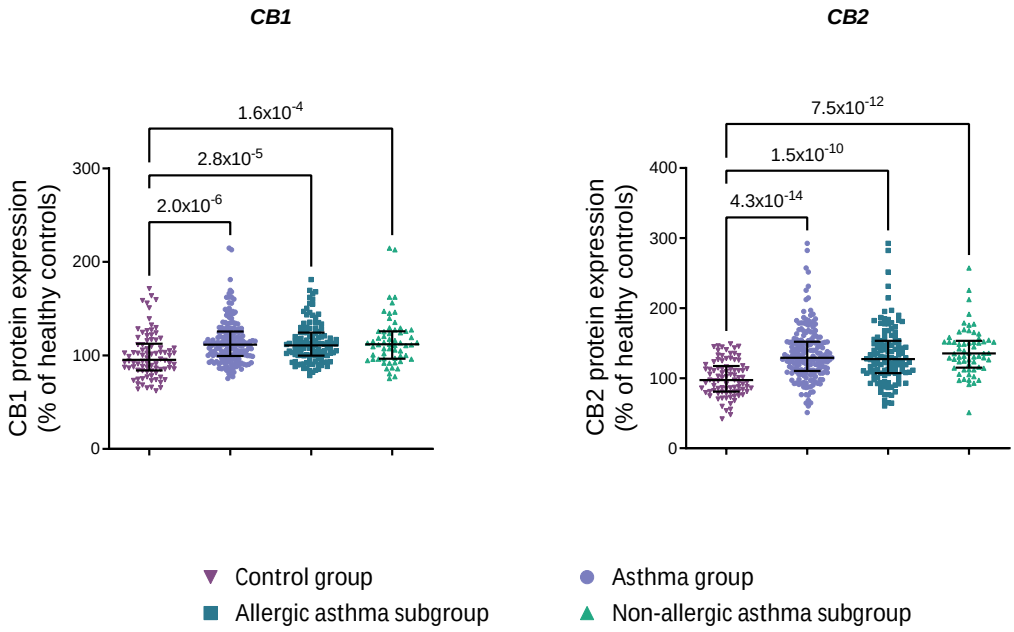

Figure 3.6: CB1 and CB2 protein expression in PBMCs from naive asthma patients. CB1: control group (n = 86), asthma group (n = 181), allergic asthma subgroup (n = 118), non-allergic asthma subgroup (n = 59). CB2: control group (n = 88), asthma group (n = 187), allergic asthma subgroup (n = 122), non-allergic asthma subgroup (n = 61). Each dot represents a data point, horizontal lines display the median value and whiskers illustrate the IQR. Kruskal-Wallis test followed by Dunn's multiple comparisons tests.

3.1.5 Association of the CB1 and CB2 genetic variants with risk of developing asthma

According to the recessive model of genetic association, the frequency of the rs4237 (*CNR2*) CC genotype in the asthma group was significantly lower than in the control group ($P = 0.017$) (Figure 3.7, Table 3.4 and Table 3.5). When the genetic association of rs4237 with asthma risk was analyzed in the allergic and non-allergic asthma subgroups it only remained significant in the allergic asthma subgroup where the frequency of the rs4327 CC genotype was lower than in the control group ($P = 0.028$).

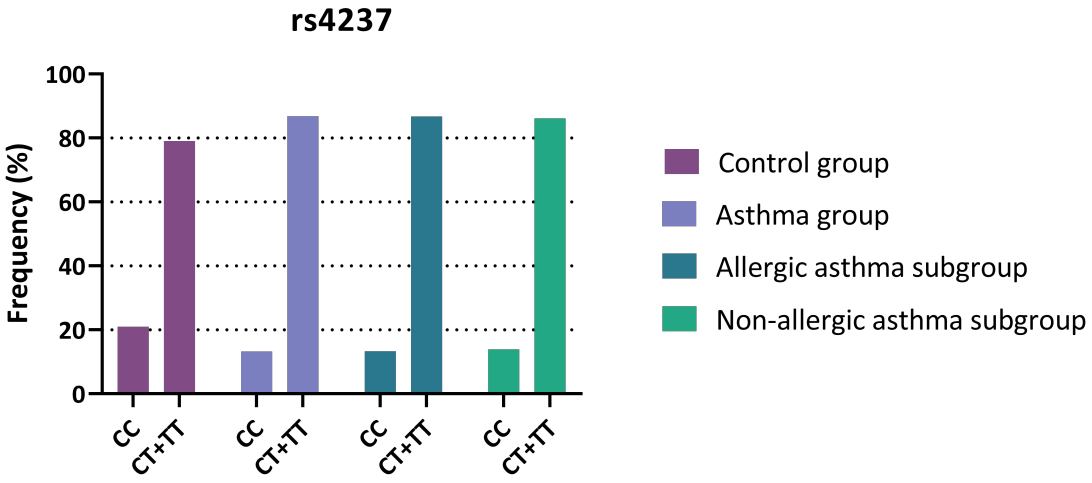

Figure 3.7: Genotype frequency distribution of rs4237 in the *CNR2* gene region. Graph shows frequency distribution in percentage according to the dominant model. Control group, CC (n = 51), CT+TT (n = 192); Asthma group, CC (n = 45), CT+TT (n = 295); Allergic asthma subgroup, CC (n = 30), CT+TT (n = 196); Non-allergic asthma subgroup, CC (n = 14), CT+TT (n = 87)

Table 3.4: Genotype frequency distribution of rs4237 (C>T) in the *CNR2* gene region.

Phenotype	Genotype, n (%)		
	CC	CT	TT
Asthma	45 (13.2%)	173 (50.9%)	122 (35.9%)
Allergic asthma	30 (13.3%)	115 (50.9%)	81 (35.8%)
Non-allergic asthma	14 (13.9%)	55 (54.5%)	32 (31.7%)
Control	51 (21.0%)	99 (40.7%)	93 (38.3%)

Table 3.5: Disease risk association analysis of rs4237 (*CNR2*).

Phenotype	**CC vs. CT+TT[1]**		**CC+CT vs. TT[2]**		**C vs. T**	
	P value	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)
Asthma	**0.017**	**1.74 (1.13-2.70)**	0.602	0.90 (0.64-1.56)	0.363	1.12 (0.88-1.42)
Allergic asthma	**0.028**	**1.74 (1.06-2.87)**	0.633	0.90 (0.62-1.62)	0.424	1.12 (0.86-1.45)
Non-allergic asthma	0.133	1.65 (0.88-3.08)	0.270	0.75 (0.46-1.22)	1.000	1.01 (0.72-1.41)

[1]Dominant model, [2]Recessive model; Odds ratio (OR) with 95% Confidence Interval (CI) from cross tabulation of genotype frequencies. Significant associations (P <0.05) are shown in bold.

The rs35761398 (*CNR2*) genotype was also linked with asthma risk ($P = 0.040$) and allergic asthma ($P = 0.039$), but not with non-allergic asthma ($P = 0.140$), according to the general genetic model (Figure 3.8, Table 3.6 and Table 3.7).

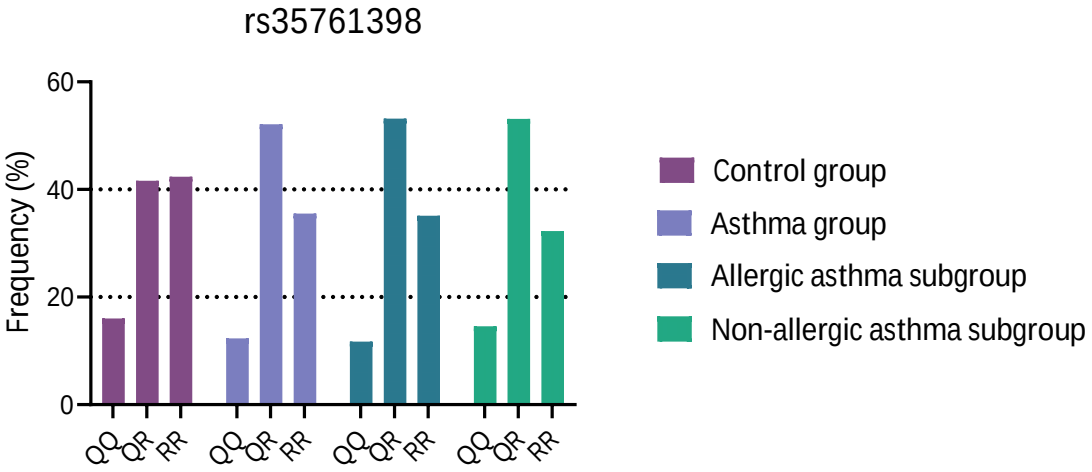

Figure 3.8: Genotype frequency distribution of rs35761398 (*CNR2*). Graph shows frequency distribution in percentage according to the general genetic model.

Table 3.6: Genotype frequency distribution of rs35761398 (Q>R)in the *CNR2* gene region.

Phenotype	Genotype, n (%)		
	QQ	QR	RR
Asthma	41 (12.3%)	173 (52.1%)	118 (35.5%)
Allergic asthma	26 (11.7%)	118 (53.2%)	78 (35.1%)
Non-allergic asthma	14 (14.6%)	51 (53.1%)	31 (32.3%)
Control	40 (16.0%)	104 (41.6%)	106 (42.4%)

Table 3.7: Disease risk association analysis of rs35761398 (*CNR2*).

Phenotype	**QQ vs. QR+RR[1]**		**QQ+QR vs. RR[2]**		**Q vs. R**	
	P value	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)
Asthma	0.102	1.34 (0.95-1.87)	0.227	0.74 (0.46-1.18)	0.583	1.07 (0.84-1.36)
Allergic asthma	0.109	1.36 (0.94-1.97)	0.187	0.70 (0.41-1.18)	0.638	1.07 (0.82-1.39)
Non-allergic asthma	0.088	1.54 (0.94-2.53)	0.869	0.90 (0.46-1.73)	0.295	1.20 (0.85-1.69)

[1]Dominant model, [2]Recessive model; Odds ratio (OR) with 95% Confidence Interval (CI) from cross tabulation of genotype frequencies. Significant associations (P <0.05) are shown in bold.

No significant association was found between the rs2229579 (*CNR2*) genotype and disease risk. (Table 3.8 and Table 3.9).

Table 3.8: Genotype frequency distribution of rs2229579 (*CNR2*).

Phenotype	Genotype, n (%)		
	CC	CT	TT
Asthma	277 (81.2%)	64 (18.8%)	0 (0.0%)
Allergic asthma	182 (80.5%)	44 (19.5%)	0 (0.0%)
Non-allergic asthma	83 (83.0%)	17 (17.0%)	0 (0.0%)
Control	204 (83.3%)	41 (16.7%)	0 (0.0%)

Table 3.9: Disease risk association analysis of rs2229579 (*CNR2*).

Phenotype	**CC vs. CT+TT[1]**		**CC+CT vs. TT[2]**		**C vs. T**	
	P value	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)
Asthma	0.585	1.15 (0.75-1.77)	1.000	0.72 (0.01-36.39)	0.605	1.13 (0.75-1.88)
Allergic asthma	0.473	1.20 (0.75-1.93)	1.000	1.20 (0.75-1.93)	0.494	1.23 (0.80-1.88)
Non-allergic asthma	1.000	1.02 (0.55-1.90)	1.000	2.44 (0.05-124.10)	1.000	1.02 (0.56-1.83)

[1]Dominant model, [2]Recessive model; Odds ratio (OR) with 95% Confidence Interval (CI) from cross tabulation of genotype frequencies. Significant associations (P <0.05) are shown in bold.

Because genetic variants that are located near each other tend to be inherited together, we analysed the linkage disequilibrium (LD) between rs4237, rs35761398 and rs2229579 (Figure 3.9).

The genetic variants rs4237 and rs35761398 were relatively close to a "perfect LD" with each other as both calculated r^2 and D' are high ($r^2 = 0.76$, D' = 0.91) indicating that the two alleles of these genetic variants are co-inherited the majority of time.

The LD between rs2229579 and both rs4237 and rs35761398 is conflicting. Even though the calculated D' are high (rs4237 - rs2229579: D' = 0.84, rs35761398 - rs2229579: D' = 0.86), the r^2, which is the correlation between a pair of alleles, was very low (rs4237 - rs2229579: $r^2 = 0.10$, rs35761398 - rs2229579: $r^2 = 0.11$).

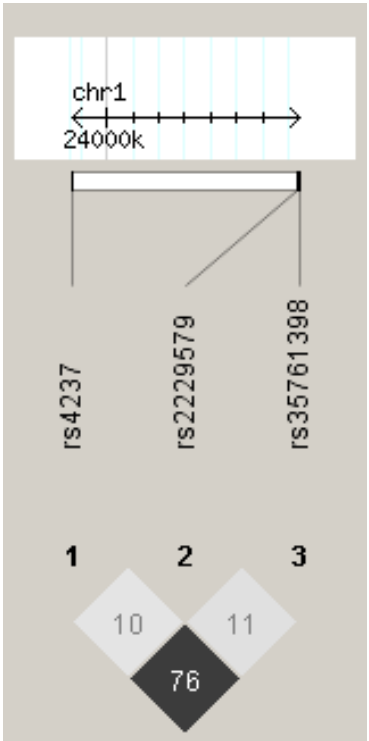

Figure 3.9: Linkage disequilibrium plot of genetic variants analyzed in the *CNR2* gene region. Values indicate r^2 between each pair as calculated by Haploview software version 4.2, according to data obtained in the present study.

No significant association was found between the rs13197090 (*CNR1*) genotype and disease risk. (Table 3.10 and Table 3.11).

Table 3.10: Genotype frequency distribution of rs13197090 (*CNR1*).

Phenotype	Genotype, n (%)		
	TT	CT	CC
Asthma	297 (90.5%)	28 (8.8%)	2 (0.6%)
Allergic asthma	198 (89.6%)	21 (9.5%)	2 (0.9%)
Non-allergic asthma	87 (93.5%)	6 (6.5%)	0 (0.0%)
Control	232 (88.2%)	31 (11.8%)	0 (0.0%)

Table 3.11: Disease risk association analysis of rs13197090 (*CNR1*).

Phenotype	**TT vs. CT+CC[1]**		**TT+CT vs. CC[2]**		**T vs. C**	
	P value	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)
Asthma	0.418	0.78 (0.46-1.32)	0.505	4.04 (0.19-84.48)	0.5208	0.85 (0.51-1.40)
Allergic asthma	0.666	0.87 (0.49-1.54)	0.208	6.00 (0.29-125.80)	0.8911	0.96 (0.56-1.65)
Non-allergic asthma	0.170	0.52 (0.21-1.28)	1.000	2.82 (0.06-143.20)	0.1823	0.53 (0.21-1.30)

[1]Dominant model, [2]Recessive model; Odds ratio (OR) with 95% Confidence Interval (CI) from cross tabulation of genotype frequencies. Significant associations (P <0.05) are shown in bold.

3.1.6 Association of genetic variants with asthma severity

All analyzes of genetic associations with clinical data obtained at the time of diagnosis that reflect the severity of asthma are shown in Tables 2, 3, 4 and 5.

Naive asthma patients with the rs4237 CC genotype presented higher FEV1 than those who had the TT genotype. (96.1% vs. 89.0%, $P = 0.012$), which was confirmed by GLM adjusting for age and gender as confounders ($P_{GLM} = 0.006$) (Figure 3.10).

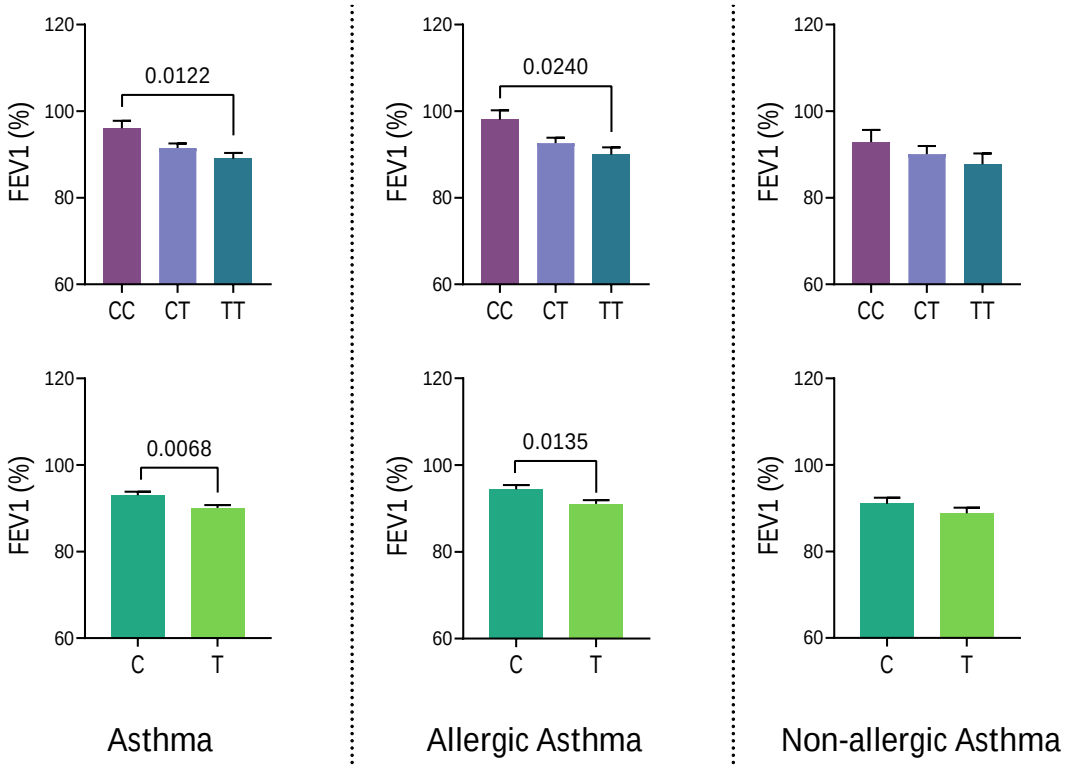

Figure 3.10: Forced expiratory volume in 1 sec (FEV1) at the time of diagnosis according to rs4237 (*CNR2*) genotype and alleles. Asthma group: CC (n = 42), CT (n = 167), TT (n = 117), C (n = 251) and T (n = 401); allergic asthma subgroup: CC (n = 27), CT (n = 109), TT (n = 77), C (n = 163) and T (n = 263); non-allergic asthma subgroup, CC (n = 14), CT (n = 55), TT (n = 32), C (n = 163) and T (n = 263)]. Bars represent mean ± SEM. P <0.05 considered statistically significant (unpaired t-test for two groups and one-way ANOVA with Bonferroni correction for three groups comparison).

When data was analysed according to the rs4237 allele, asthma patients who are carriers of the C allele had higher FEV1 (93.03 ± 12.84%) than those who are carriers of the T allele (90.06 ± 14.02%) ($P = 0.007$, $P_{GLM} = 0.013$). After asthma patients were stratified according to their phenotype this trend remained significant in the allergic asthma subgroup. Allergic asthma patients with the rs4237 genotype CC had higher FEV1 than those with the TT genotype ($P = 0.028$, $P_{GLM} = 0.017$), and C allele carriers had higher FEV1 (94.41 ± 12.61%) than those who are carriers of the T allele (91.06 ± 14.11%, $P = 0.014$, $P_{GLM} = 0.036$).

The genetic association between the rs35761398 genetic variant and lung function was also significant in the asthma group where patients who are carriers of the Q allele had higher FEV1 (92.7 ± 0.9%) than those who carry the R allele (90.1 ± 0.7%, $P = 0.018$) (Figure 3.11). However, this association was not confirmed with GLM adjusting for age and sex as confounders ($P_{GLM} = 0.432$). After stratifying into phenotypes, the genetic association of the rs35761398 genetic variant with FEV1 remained significant only in the allergic asthma subgroup ($P = 0.0421$).

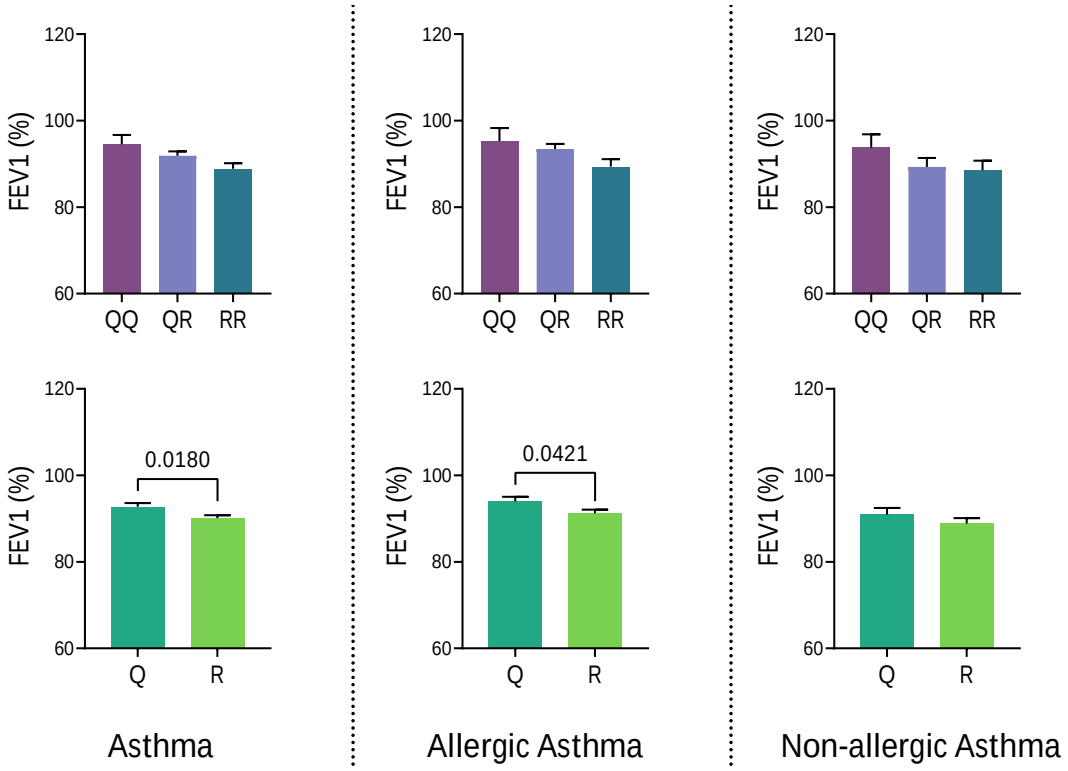

Figure 3.11: Forced expiratory volume in 1 sec (FEV1) at the time of diagnosis according to rs35761398 (*CNR2*) genotype and alleles. Asthma group: QQ (n = 117), QR (n = 166), RR (n = 39), Q (n = 400) and R (n = 244); allergic asthma subgroup: QQ (n = 76), QR (n = 112), RR (n = 77), Q (n = 265) and R (n = 160); non-allergic asthma subgroup, QQ (n = 33), QR (n = 50), RR (n = 14), Q (n = 116) and R (n = 78)]. Bars represent mean ± SEM. P <0.05 considered statistically significant (unpaired t-test for two groups and one-way ANOVA with Bonferroni correction for three groups comparison).

These findings imply that the major alleles of rs4237 and rs35761398 (both in LD) may have a protective function in asthma patients. Similarly, our results show that asthma patients carrying the rs2229579 minor allele T have a greater degree of airway dysfunction, quantified by the bronchoprovocation testing (logPC20; -0.549 ± 0.088), than those who are carriers of the major allele C (-0.3747 ± 0.026) (Figure 3.12). However, this association was not confirmed with GLM adjusting for age and sex as confounders ($P_{GLM} = 0.076$).

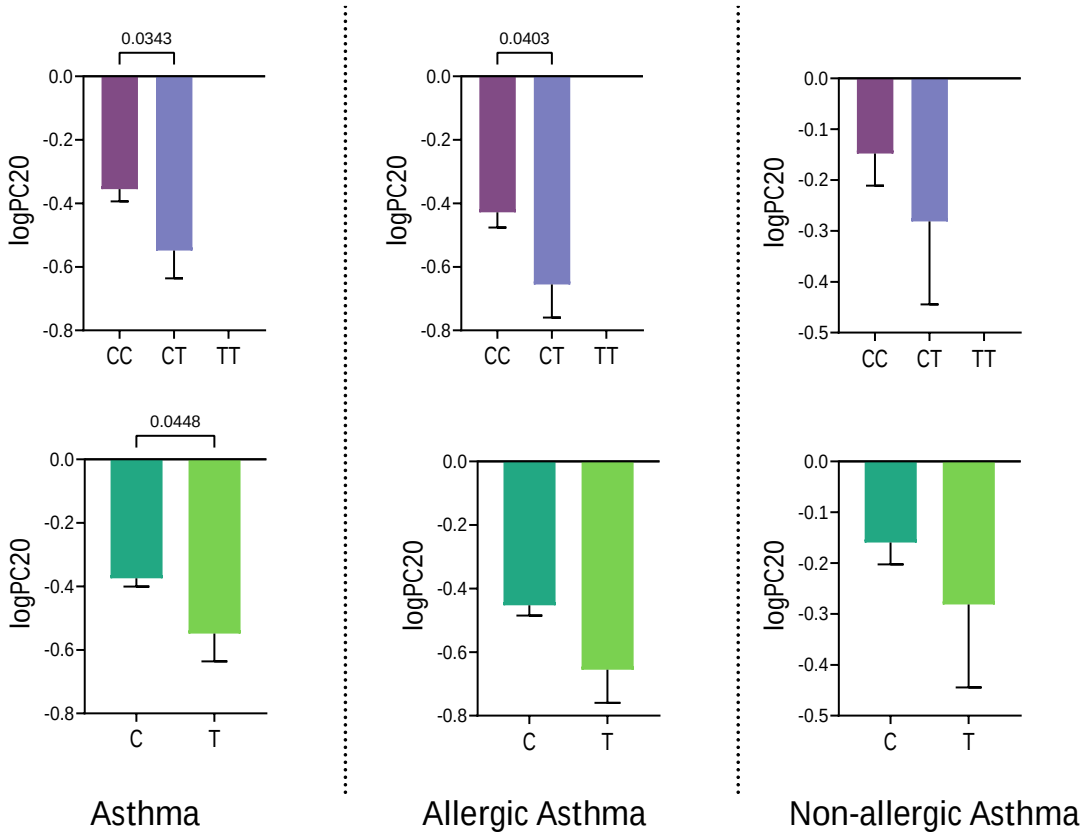

Figure 3.12: logPC20 at the time of diagnosis according to rs2229579 (*CNR2*) genotype and alleles. Asthma group: CC (n = 261), CT (n = 59), TT (n = 0), C (n = 581) and T (n = 59); allergic asthma subgroup: CC (n = 27), CT (n = 109), TT (n = 77), C (n = 163) and T (n = 263); non-allergic asthma subgroup, CC (n = 14), CT (n = 55), TT (n = 32), C (n = 163) and T (n = 263)]. Bars represent mean ± SEM. P <0.05 considered statistically significant (unpaired t-test for two groups and one-way ANOVA with Bonferroni correction for three groups comparison).

In addition to having a higher degree of airway dysfunction, asthma patients carrying the rs2229579 minor allele T also displayed increased inflammation according to the blood eosinophil count (Figure 3.13) and FeNO levels (Figure 3.14) at the time of diagnosis. Blood eosinophil count of asthma patients carrying the rs2229579 CT genotype was significantly higher (659.7 ± 67.63) than patients with the CC genotype (468.9 ± 29.1; $P = 0.012$, $P_{GLM} = 0.008$). After stratifying patients into their phenotype, the increased number of blood eosinophils in carriers of the CT genotype remained significant only in the non-allergic asthma subgroup (P <0.0001, P_{GLM} = 1.36x10^{-6}).

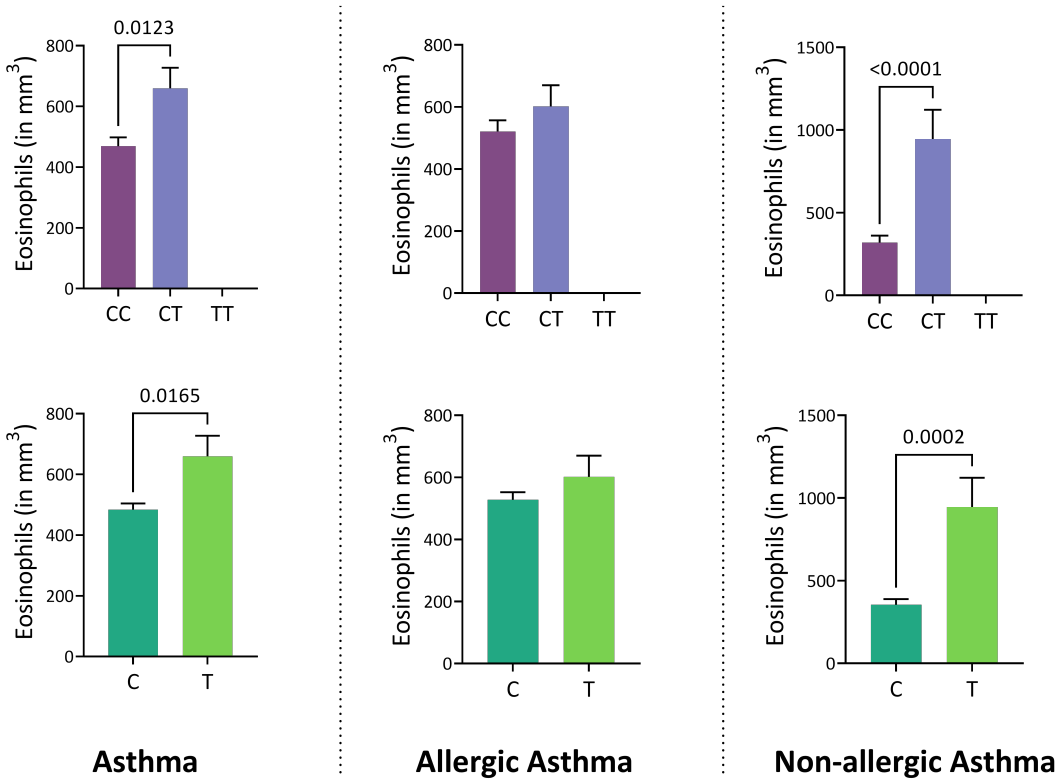

Figure 3.13: Eosinophils at the time of diagnosis according to rs2229579 (*CNR2*) genotype and alleles. Asthma group: CC (n = 121), CT (n = 21), TT (n = 0), C (n = 263) and T (n = 21); allergic asthma subgroup: CC (n = 82), CT (n = 16), TT (n = 0), C (n = 180) and T (n= 16); non-allergic asthma subgroup, CC (n = 33), CT (n = 4), TT (n = 0), C (n = 70) and T (n = 4)]. Bars represent mean ± SEM. P <0.05 considered statistically significant (unpaired t-test for two groups comparisons).

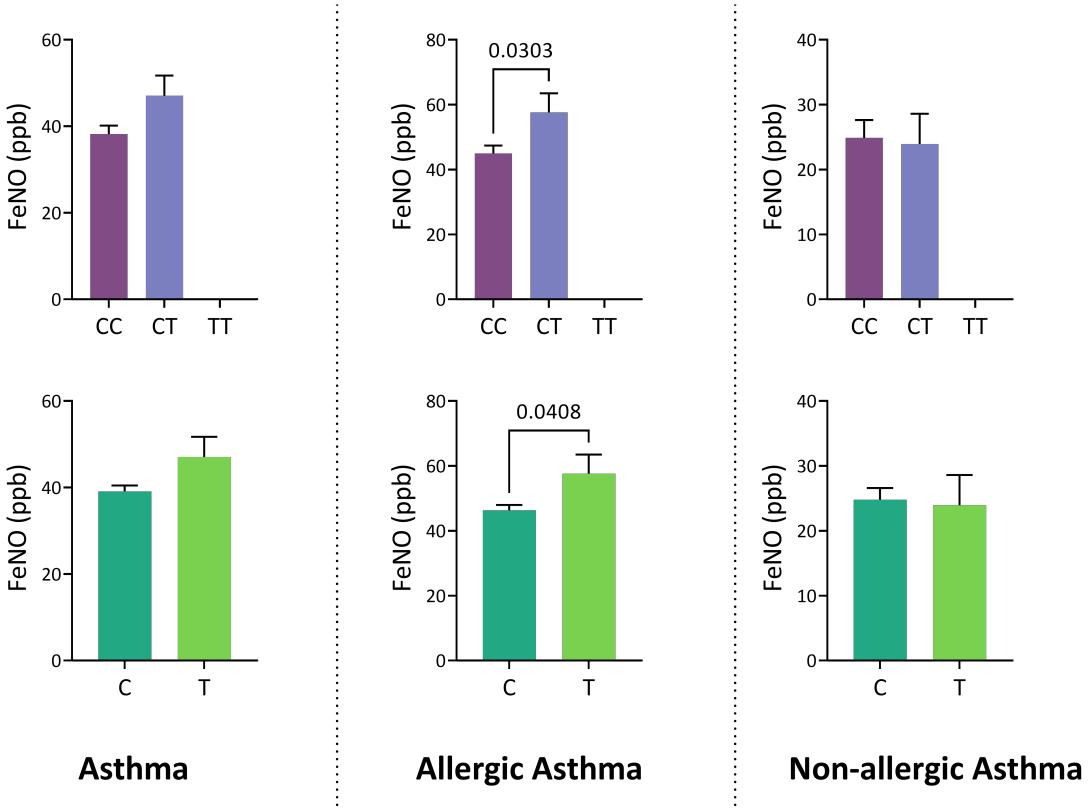

Figure 3.14: FeNO at the time of diagnosis according to rs2229579 (*CNR2*) genotype and alleles. Asthma group: CC (n = 220), CT (n = 51), TT (n = 0), C (n = 491) and T (n = 51); allergic asthma subgroup: CC (n = 141), CT (n = 34), TT (n = 0), C (n = 316) and T (n = 34); non-allergic asthma subgroup, CC (n = 69), CT (n = 14), TT (n = 0), C (n = 152) and T (n = 14)]. Bars represent mean ± SEM. P <0.05 considered statistically significant (unpaired t-test for two groups and one-way ANOVA with Bonferroni correction for three groups comparison).

Similar to the association of rs2229579 with eosinophil count, asthma patients carrying the rs2229579 minor allele T had higher FeNO levels (47.1 ± 4.7) than those who are carriers of the C allele (39.1 ± 1.4). However, while the rs2229579 allele association with eosinophil count was only significant in the non-allergic subgroup, the rs2229579 allele association with FeNO levels was significant only in the allergic asthma subgroup ($P = 0.030$, $P_{GLM} = 0.009$).

The rs2229579 genetic variant was also associated with the level of total IgE in the blood of asthmatic patients, at the time of diagnosis. Asthma patients carrying the rs2229579 minor allele T had higher total IgE levels (806.8 ± 189.3 IU/mL) than those who are carriers of the C allele (468.4 ± 30.4 IU/mL) ($P = 0.004$, $P_{GLM} = 0.034$)

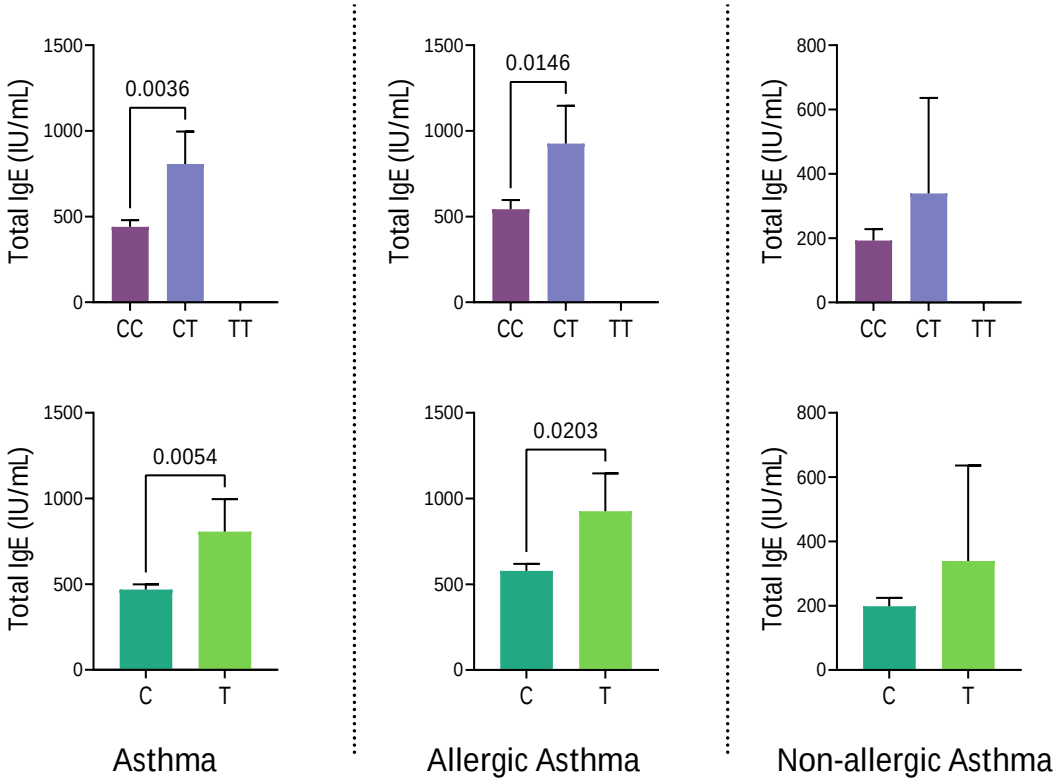

Figure 3.15: Total IgE at the time of diagnosis according to rs2229579 (*CNR2*) genotype and alleles. Asthma group: CC (n = 124), CT (n = 21), TT (n = 0), C (n = 269) and T (n = 21); allergic asthma subgroup: CC (n = 82), CT (n = 17), TT (n = 0), C (n = 181) and T (n = 17); non-allergic asthma subgroup, CC (n = 36), CT (n = 3), TT (n = 0), C (n = 75) and T (n = 3)]. Bars represent mean ± SEM. P <0.05 considered statistically significant (unpaired t-test for two groups and one-way ANOVA with Bonferroni correction for three groups comparison).

No significant association between the rs13197090 genetic variant located near *CNR1* and asthma severity was found.

3.1.7 Association of CB1 and CB2 genetic variants with ICS and LTRA treatment response

A summary of the significant genetic associations with ICS is presented in Table 3.12 [see Tables 6, 7, 8, 9 for all results].

In the allergic asthma subgroup, patients with the rs4237 genotype TT had a greater increase of FEV1 after treatment with ICS according to the dominant model ($P = 0.032$). Allergic asthma patients carrying the QQ genotype in rs35761398 also had a better response to treatment with ICS (measured by ΔFEV1) than those with RR or QR genotype ($P = 0.049$).

In addition, we found that patients with allergic asthma carrying the less functional allele T in rs2229579 had a worse response to treatment with ICS than patients homozygous for the C allele ($P = 0.044$). However, none of these associations were confirmed with GLM when adjusting for FEV1 before treatment.

Table 3.12: Significant associations between analyzed genetic variants and response to ICS treatment.

SNP	Asthma phenotype	Assessment of treatment response[1]	Mean (± SD)		n	Genotype	Genotype with worse response	*P* value[2]	P_{GLM} value[3]
rs4237 (*CNR2*)	Allergic asthma	ΔFEV1	10.64	(±11.69)	47	TT	TT	0.032	0.101
			6.80	(±10.76)	70	CT			
			4.07	(±9.73)	15	CC			
rs35761398 (*CNR2*)	Allergic asthma	ΔFEV1/FVC	5.36	(±7.46)	47	QQ	RR	0.049	0.038
			3.28	(±7.80)	72	QR			
			-0.07	(±4.71)	14	RR			
		ΔFEV1	10.49	(±12.30)	47	QQ	RR and QR	0.048	0.272
			6.81	(±10.45)	72	QR			
			4.43	(±11.75)	14	RR			
rs2229579 (*CNR2*)	Allergic asthma	ΔFEV1	4.19	(±7.72)	109	CC	CT	0.044	0.262
			0.85	(±6.73)	26	CT			
			-	-	0	TT			

FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity. [1]Treatment response was measured after 4-6 weeks of treatment with ICS. [2]ANOVA followed by Newman–Keuls multiple comparison test or t-test for two independent samples. [3]Generalized linear model adjusting for age, gender and FEV1 values measured before treatment. $P < 0.05$ was considered statistically significant.

Regarding the response to treatment with LTRA, we found that patients with asthma carrying the less functional T allele in rs2229579 had a worse response to treatment measured by ΔFEV1/FVC ($P = 0.029$, $P_{GLM} = 0.018$) (figure 3.16). However, this difference did not remain significant after stratifying patients into their phenotypes.

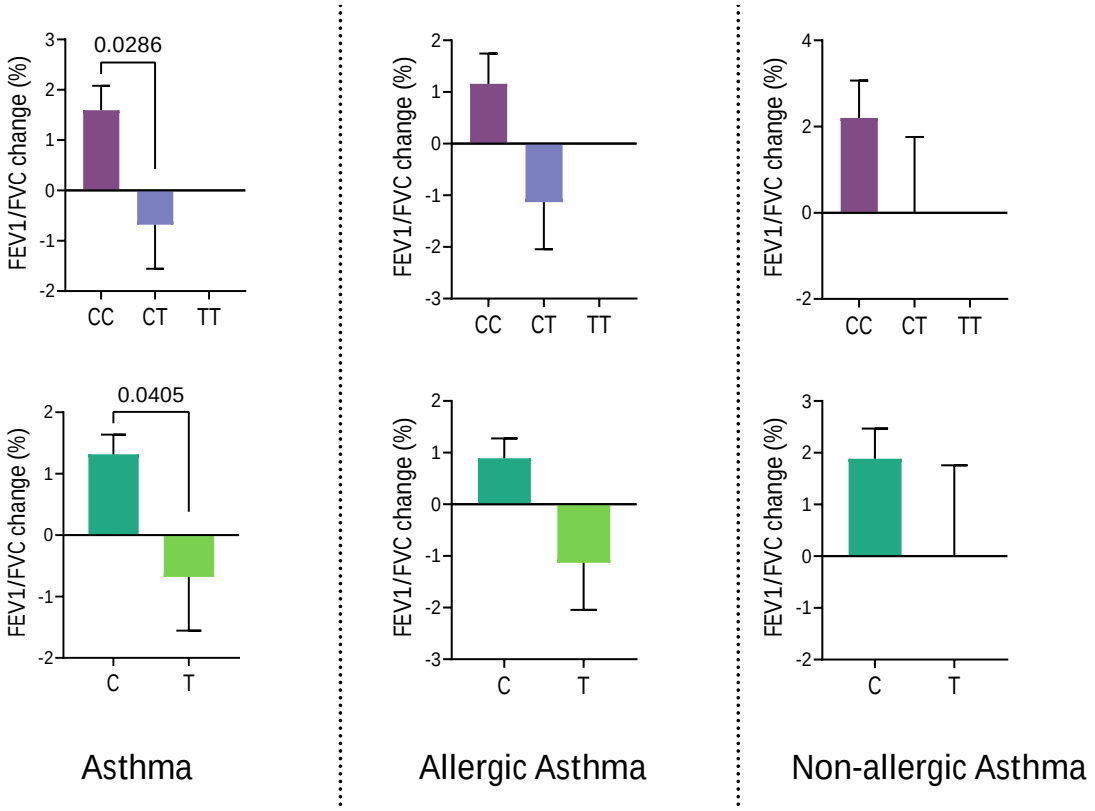

Figure 3.16: Change in FEV1/FVC (%) in response to LTRA treatment according to rs2229579 (*CNR2*) genotype and alleles. Asthma group: CC (n = 89), CT (n = 25), TT (n = 0), C (n = 203) and T (n = 25); allergic asthma subgroup: CC (n = 57), CT (n = 15), TT (n = 0), C (n = 129) and T (n = 15); non-allergic asthma subgroup, CC (n = 30), CT (n = 10), TT (n = 0), C (n = 70) and T (n = 10)]. Bars represent mean ± SEM. P <0.05 considered statistically significant (unpaired t-test for two groups comparisons).

3.1.8 Association of genetic variants with *CNR1* and *CNR2* gene expression

The gene expression data from different genotype groups according to genetic variants rs4237, rs35761398 and rs2229579 (*CNR2*) and rs13197090 in (*CNR1*) are provided in Tables 10, 11, 12, 13.

Only in the allergic asthma subgroup the rs4237 genetic variant was significantly associated with *CNR2* mRNA expression levels. In the allergic asthma subgroup,

carriers of the rs4237 C allele had lower median *CNR2* mRNA expression levels before treatment than carriers of the T allele ($P = 0.007$, Fig. 3.17).

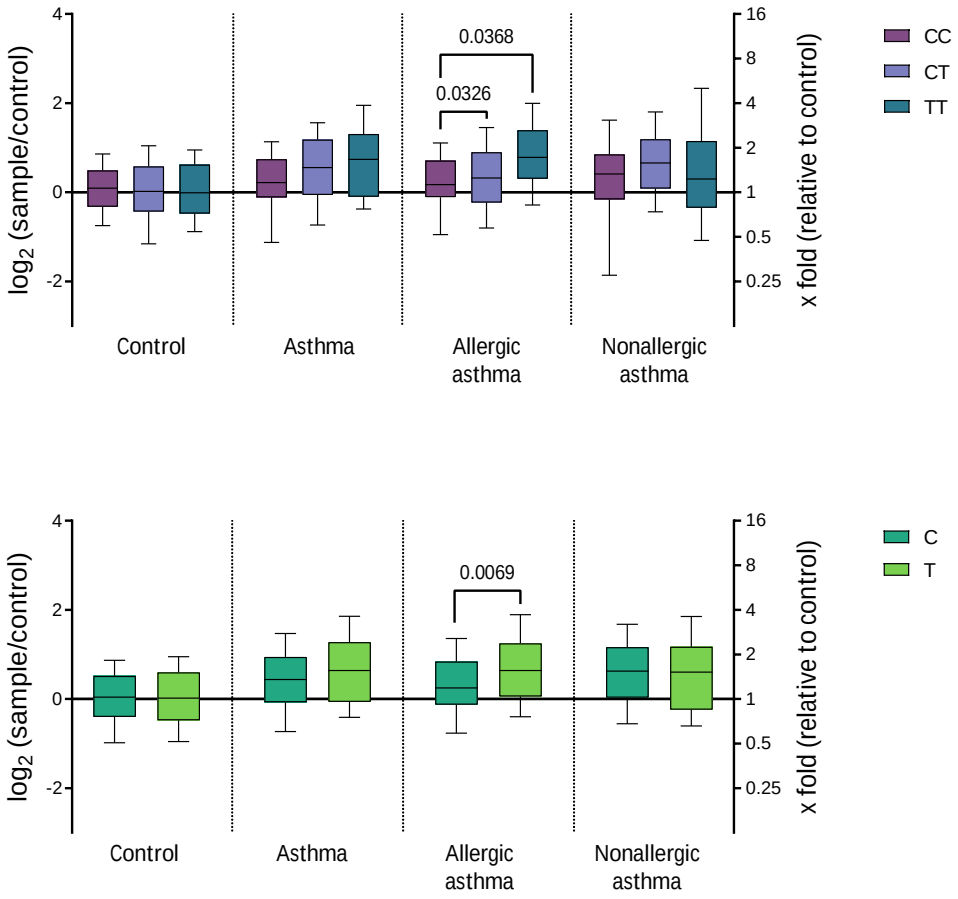

Figure 3.17: CB2 (*CNR2*) gene expression in PBMCs from allergic asthmatic children before treatment association with rs4237. Control group, T (n = 244) and C (n = 174); asthma group T (n = 232) and C (n = 150); allergic asthma subgroup T (n = 141) and C (n = 95); non-allergic asthma subgroup T (n = 74) and C (n = 52). Boxes represent interquartile range with medians; whiskers illustrate the 10–90 percentiles of samples. P <0.05 considered significant (Mann-Whitney test).

The genetic variant rs13197090 was significantly associated with *CNR1* mRNA expression levels only in the control group ($P = 0.009$) (Figure 3.18). Control subjects with the rs13197090 TT genotype had lower *CNR1* mRNA expression levels (0.82 ± 0.82) compared to subjects with the CT genotype (1.27 ± 3.32).

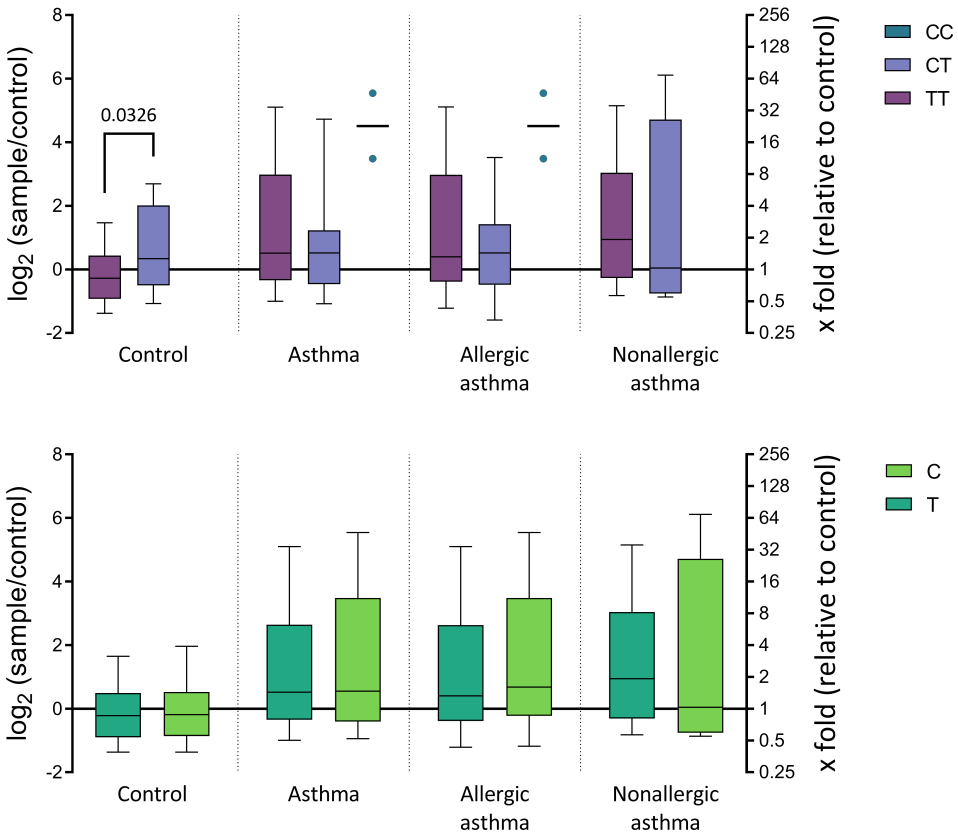

Figure 3.18: CB1 (*CNR1*) gene expression in PBMCs from allergic asthmatic children before treatment association with rs13197090. Control group, T (n = 244) and C (n = 174); asthma group T (n = 232) and C (n = 150); allergic asthma subgroup T (n = 141) and C (n = 95); non-allergic asthma subgroup T (n = 74) and C (n = 52). Boxes represent interquartile range with medians; whiskers illustrate the 10–90 percentiles of samples; dots represent individual measurements. P <0.05 considered significant (Mann-Whitney test).

No significant associations were found between genetic variants rs35761398 and rs2229579 and the mRNA expression levels of *CNR2*.

3.2 The endocannabinoid enzymes in naive asthma patients.

3.2.1 NAPE-PLD, Abhd4 and FAAH gene expression in naïve asthma patients and correlation with asthma severity

Before treatment, the asthma group, as well as the allergic and non-allergic subgroups, had median relative mRNA expression levels of *NAPEPLD* and *ABHD4* that ranged between 1.22 and 1.35-fold higher than the control group (figure 3.19). The mRNA expression levels of *FAAH*, on the other hand, were similar across all groups. Because the average age of participants in the control group is higher than in the asthma group, we again analyzed gene expression according to age to test the possibility that the observed differences were age associated. No significant correlation was found between the subjects' age and mRNA expression levels of *NAPEPLD* (r_s = -0.036, $P = 0.648$), *ABHD4* (r_s = -0.010, $P = 0.897$), or *FAAH* ($r_s = 0.038$, $P = 0.635$).

Both *ABHD4* and *FAAH* mRNA expression levels were significantly correlated with several clinical parameters and these are summarised in Table 3.13 (see Table 14 for all correlation results). The mRNA expression levels of *ABHD4* showed a very weak positive correlation with FEV1 ($r_s = 0.175$, $P = 0.010$). Asthma patients with higher *ABHD4* mRNA expression levels had better lung function.

In addition, the mRNA expression levels of *ABHD4* showed a weak positive correlation with FeNO in the non-allergic asthma subgroup ($r_s = 0.321$, $P = 0.008$).

The mRNA expression levels of *FAAH* were found to be correlated with levels of allergic inflammation. In asthma patients, the mRNA expression levels of *FAAH* showed a weak correlation with eosinophil count ($r_s = 0.327$, $P = 0.037$), that after stratification into phenotypes remained significant only in the allergic asthma subgroup ($r_s = 0.471$, $P = 0.020$). In the non-allergic asthma subgroup the mRNA expression levels of *FAAH* also showed a weak correlation with FeNO ($r_s = 0.263$, $P = 0.037$).

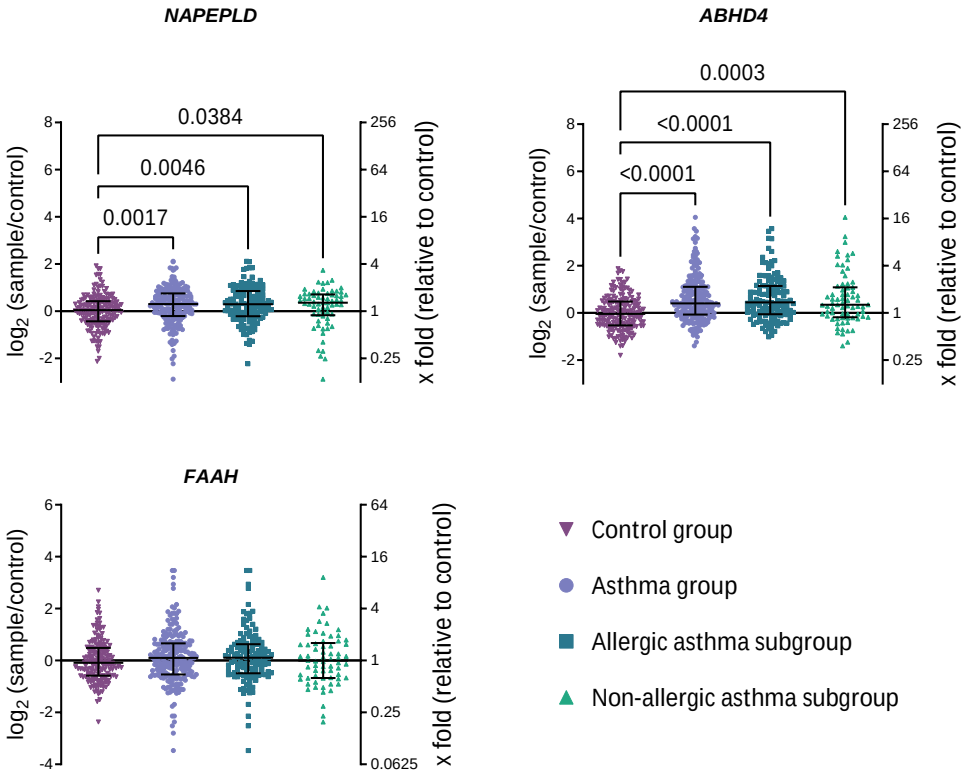

Figure 3.19: NAPE-PLD (*NAPEPLD*), Abhd4 (*ABHD4*) and FAAH (*FAAH*) gene expression in PBMCs from naive asthma patients. *NAPEPLD* mRNA expression [control group (n = 166), asthma group (n = 206), allergic asthma subgroup (n = 130), non-allergic asthma subgroup (n = 65)]. *ABHD* mRNA expression [control group (n = 174), asthma group (n = 219), allergic asthma subgroup (n = 137), non-allergic asthma subgroup (n = 71)]. *FAAH* mRNA expression [control group (n = 165), asthma group (n = 207), allergic asthma subgroup (n = 130), non-allergic asthma subgroup (n = 66)]. Each dot represents a data point, horizontal lines display the median value and whiskers illustrate the IQR. Kruskal-Wallis test followed by Dunn's multiple comparisons test.

In regards to the biomarker for allergy, the mRNA expression levels of *FAAH* showed weak positive correlation with total IgE in asthma patients ($r_s = 0.355$, $P = 0.020$), but after stratification into phenotypes, it only remained significant in the non-allergic asthma subgroup ($r_s = 0.631$, $P = 0.018$).

Table 3.13: Significant correlations of Abhd4 (*ABHD4*) and FAAH (*FAAH*) gene expression with clinical data before anti-asthmatic treatment.

Gene	Asthma phenotype	Clinical parameter	n	r_s	*P* value
ABHD4	Asthma	FEV1	219	0.175	0.010
	Non-allergic asthma	FeNO	67	0.321	0.008
FAAH	Asthma	Total IgE	43	0.355	0.020
		Eosinophils	41	0.327	0.037
	Allergic asthma	FEV1/FVC	124	-0.177	0.049
		Eosinophils	24	0.471	0.020
	Non-allergic asthma	FeNO	62	0.263	0.039
		Total IgE	14	0.631	0.018

FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; logPC20, base 10 logarithm of provocative methacholine concentration causing a drop in FEV1 of 20%; FeNO, fractional exhaled nitric oxide; r_s, Spearman's correlation coefficient, $P < 0.05$ was considered significant.

In addition, the mRNA expression levels of *FAAH* were found to have a very weak negative correlation with FEV1/FVC in the allergic asthma subgroup (r_s = -0.177, $P = 0.049$). Allergic asthma patients with higher mRNA expression levels of *FAAH* had lower FEV1/FVC values.

Additional analyses of NAPE-PLD, Abhd4 and FAAH gene expression indicate that all are co-expressed with cytokine IL-5, and to some extent, with cytokine IL-4 biomarkers of inflammation.

In naive asthma patients, *NAPEPLD* mRNA expression levels showed a weak positive correlation with the mRNA expression levels of *IL5* ($r_s = 0.219$, $P = 0.002$) (figure 3.20). However, *ABHD4* and *FAAH* mRNA expression levels showed a moderate positive correlation with the mRNA expression levels of *IL5* (*ABHD4*: $r_s = 0.452$, $P = 8.8\text{x}10^{-12}$; *FAAH*: $r_s = 0.390$, $P = 1.5\text{x}10^{-8}$) (figure 3.21 and 3.22). Asthma patients with higher levels of IL5 gene expression have higher levels of NAPE-PLD, Abhd4 and FAAH gene expression.

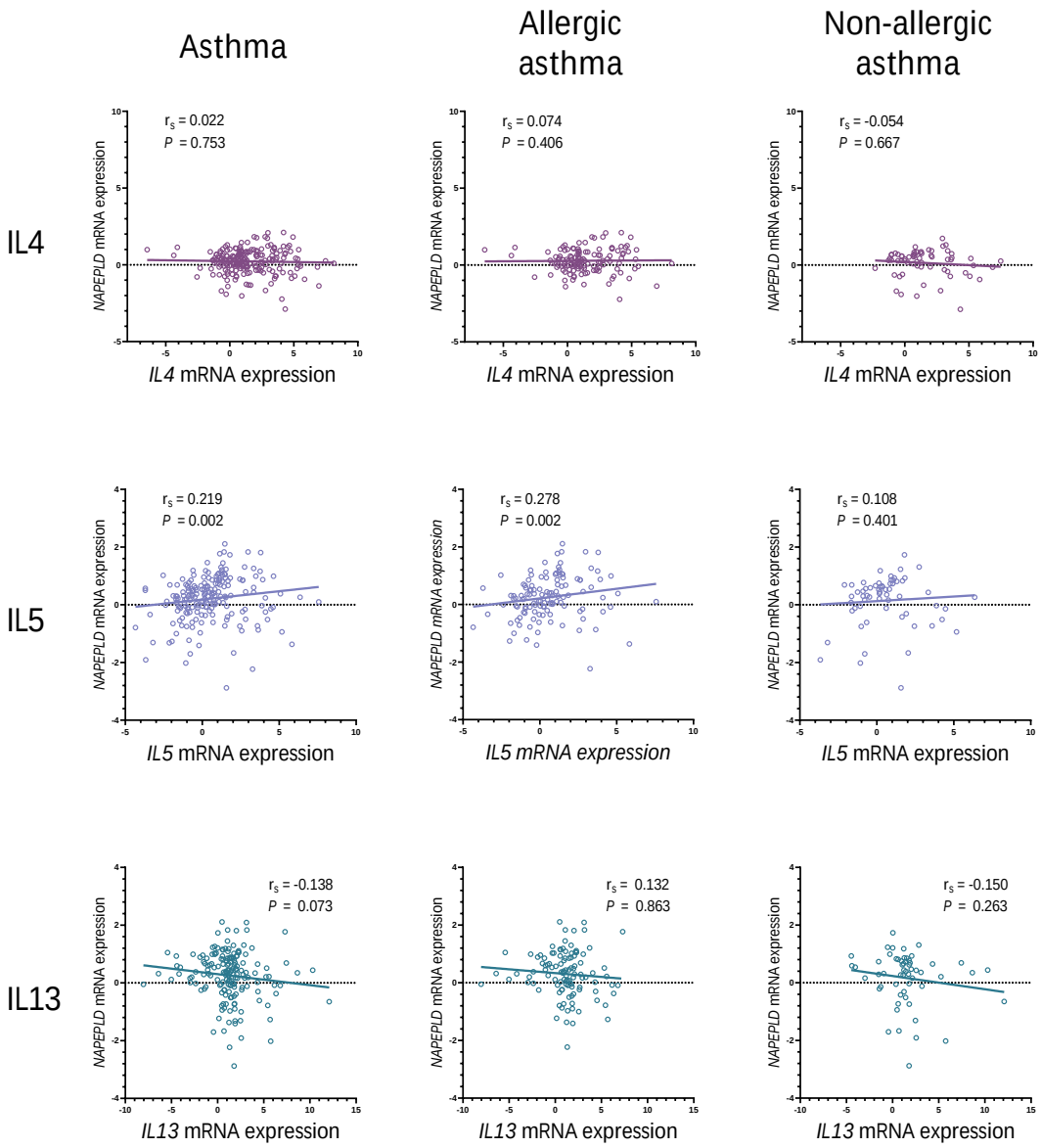

Figure 3.20: NAPE-PLD (*NAPEPLD*) gene expression in PBMCs from naive asthma patients. IL-4 (*IL4*): asthma group (n = 201), allergic asthma subgroup (n = 124), non-allergic asthma subgroup (n = 66); IL-5 (*IL5*): asthma group (n = 199), allergic asthma subgroup (n = 123), non-allergic asthma subgroup (n = 65); IL-13 (*IL13*): asthma group (n = 168), allergic asthma subgroup (n = 107), non-allergic asthma subgroup (n = 58) Each dot represents a data point, lines display the trend. r_s, Spearman's correlation coefficient, P <0.05 was considered significant.

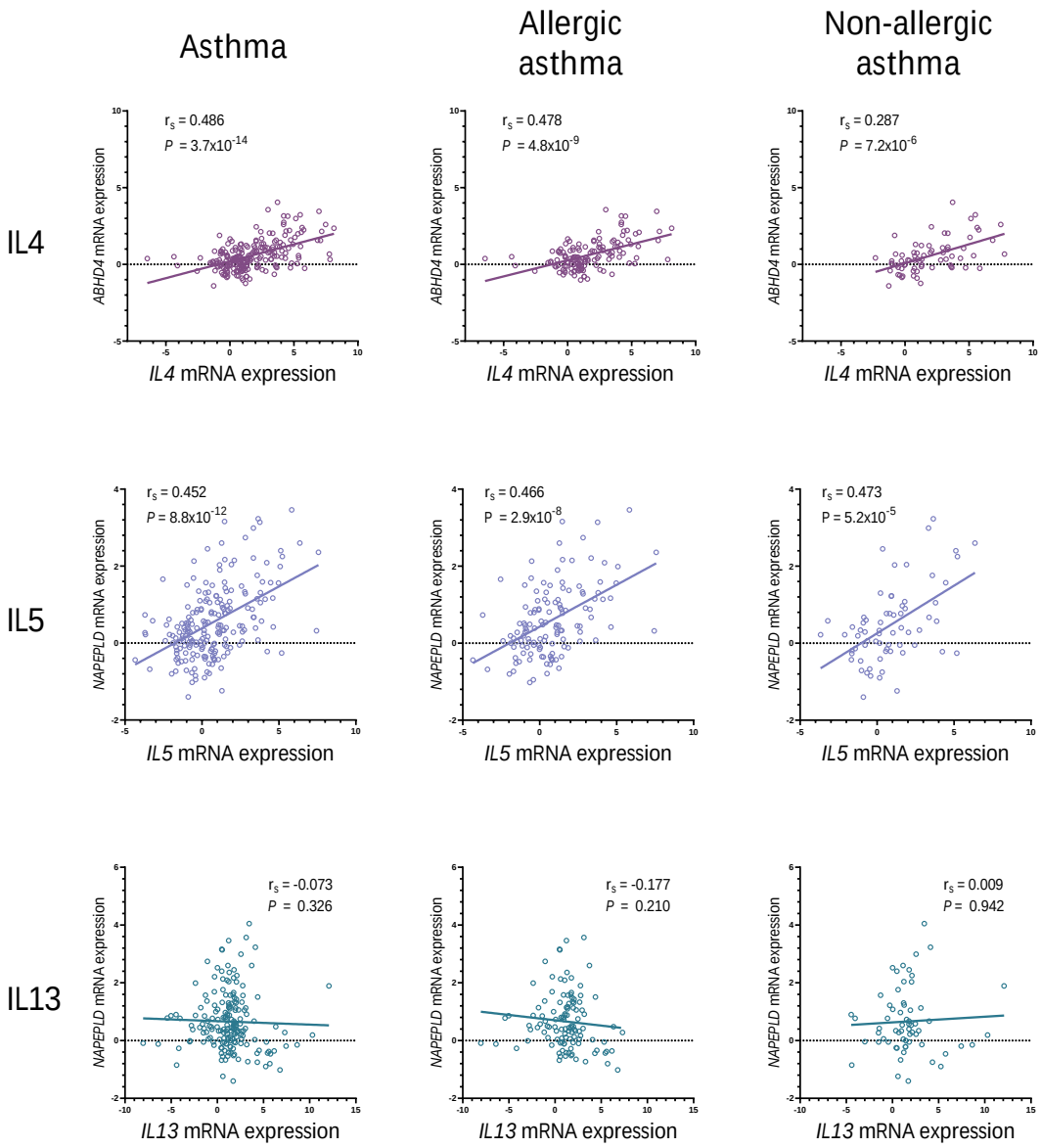

Figure 3.21: Abhd4 (*ABHD4*) gene expression in PBMCs from naive asthma patients. IL-4 (*IL4*): asthma group (n = 203), allergic asthma subgroup (n = 127), non-allergic asthma subgroup (n = 65); IL-5 (*IL5*): asthma group (n = 195), allergic asthma subgroup (n = 122), non-allergic asthma subgroup (n = 62); IL-13 (*IL13*): asthma group (n = 169), allergic asthma subgroup (n = 109), non-allergic asthma subgroup (n = 57) Each dot represents a data point, lines display the trend. r_s, Spearman's correlation coefficient, P <0.05 was considered significant.

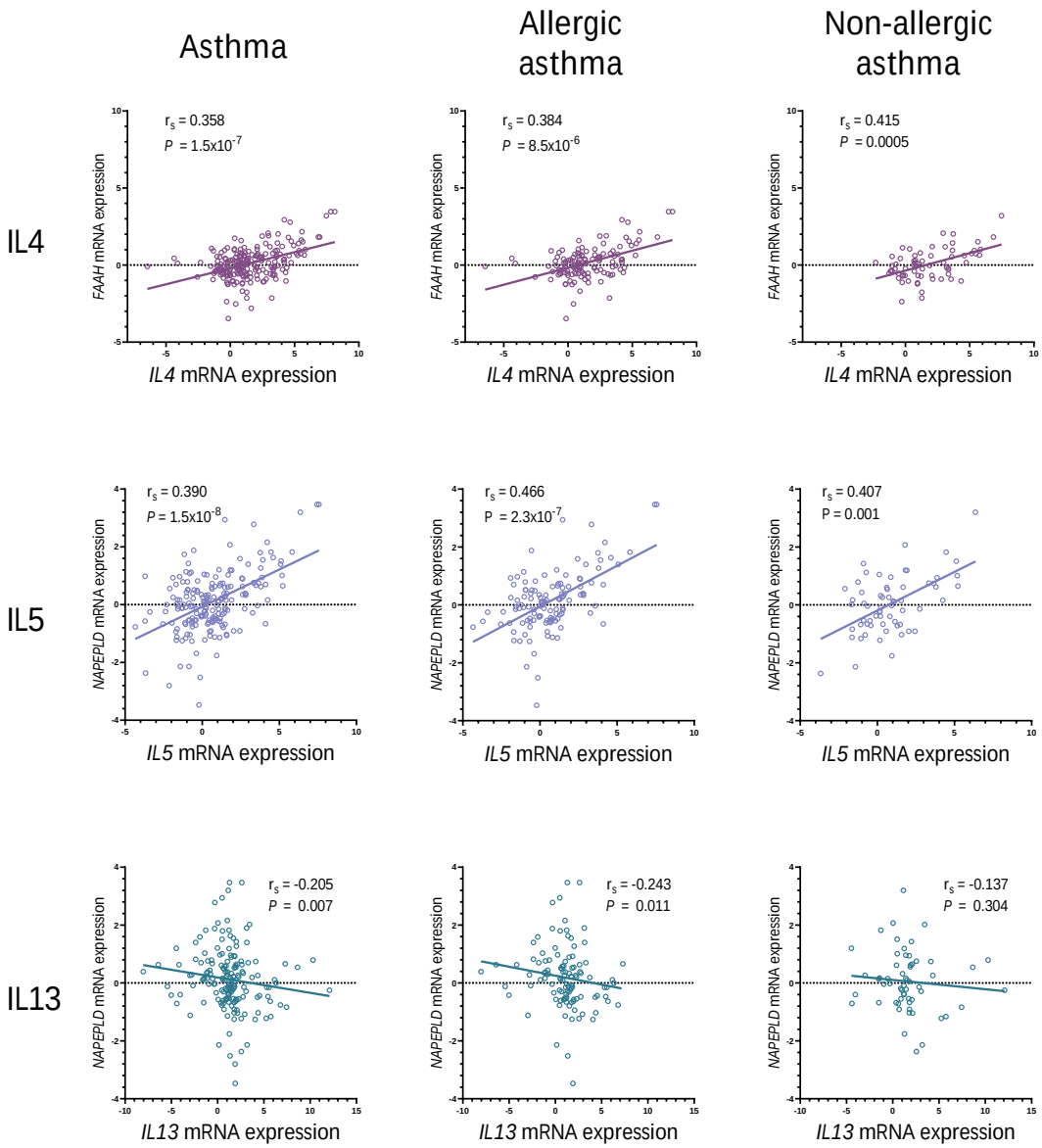

Figure 3.22: FAAH (*FAAH*) gene expression in PBMCs from naive asthma patients. IL-4 (*IL4*): asthma group (n = 203), allergic asthma subgroup (n = 127), non-allergic asthma subgroup (n = 65); IL-5 (*IL5*): asthma group (n = 195), allergic asthma subgroup (n = 122), non-allergic asthma subgroup (n = 62); IL-13 (*IL13*): asthma group (n = 169), allergic asthma subgroup (n = 109), non-allergic asthma subgroup (n = 57) Each dot represents a data point, lines display the trend. r_s, Spearman's correlation coefficient, P <0.05 was considered significant.

In addition, the *ABHD4* and *FAAH*, mRNA expression levels also showed a moderate positive correlation with the mRNA expression levels of *IL4* (*ABHD4*: r_s = 0.486, $P = 3.7\text{x}10^{-14}$; *FAAH*: r_s = 0.358, $P = 1.5\text{x}10^{-7}$). Asthma patients with higher levels of IL-4 gene expression have higher levels of Abhd4 and FAAH gene expression, but not of NAPE-PLD. A similar pattern of co-expression was found when patients were stratified according to their phenotype.

On the other hand, analyses of NAPE-PLD, Abhd4 and FAAH gene expression indicate that IL-13 and FAAH are oppositely co-expressed. In naive asthma patients, *FAAH* mRNA expression levels showed a weak negative correlation with the mRNA expression levels of *IL13* (r_s = -0.205, P = 0.007) (Figure 3.22). Asthma patients with higher levels of IL-13 gene expression have lower levels of FAAH gene expression. When asthma patients were stratified into their phenotypes, this correlation remained significant only in the allergic asthma subgroup.

3.2.2 *NAPEPLD*, *ABHD4* and *FAAH* mRNA expression levels before treatment as predictors of ICS and LTRA treatment response

We analyzed the correlation of *NAPEPLD*, *ABHD4* and *FAAH* mRNA expression levels before treatment with changes of the FEV1/FVC ratio and FEV1 after ICS and LTRA treatment.

We found no correlation between the mRNA expression of *NAPEPLD*, *ABHD4* and *FAAH* from naive asthma patients with response to treatment with ICS (Table 3.14).

Table 3.14: Correlation of NAPE-PLD (*NAPEPLD*), Abhd4 (*ABHD4*) and FAAH (*FAAH*) gene expression with response to ICS treatment.

Gene	Phenotype	Treatment response	n	r_s	*P* value
NAPEPLD	Asthma	ΔFEV1/FVC	82	0.108	0.336
		ΔFEV1	82	-0.064	0.571
	Allergic asthma	ΔFEV1/FVC	56	0.050	0.717
		ΔFEV1	56	0.003	0.984
	Non-allergic asthma	ΔFEV1/FVC	21	0.369	0.100
		ΔFEV1	21	-0.277	0.322
ABHD4	Asthma	ΔFEV1/FVC	85	0.002	0.988
		ΔFEV1	85	-0.109	0.319
	Allergic asthma	ΔFEV1/FVC	57	0.023	0.863
		ΔFEV1	57	-0.049	0.719
	Non-allergic asthma	ΔFEV1/FVC	23	-0.068	0.759
		ΔFEV1	23	-0.287	0.189
FAAH	Asthma	ΔFEV1/FVC	82	-0.093	0.407
		ΔFEV1	82	-0.003	0.980
	Allergic asthma	ΔFEV1/FVC	55	0.027	0.843
		ΔFEV1	55	0.164	0.231
	Non-allergic asthma	ΔFEV1/FVC	22	-0.287	0.195
		ΔFEV1	22	-0.211	0.345

ICS, inhaled corticosteroids; *NAPEPLD*, NAPE-PLD gene expression; *ABHD4*, Abhd4 gene expression; *FAAH*, FAAH gene expression; FEV1, forced expiratory volume, 1 s; FVC, forced vital capacity; r_s, Spearman's correlation coefficient, significant correlations (P <0.05) are shown in bold.

However, the *FAAH* mRNA expression levels of asthma patients showed a weak positive correlation with ΔFEV1 values after treatment with LTRA (r_s = 0.214, P = 0.030), which after stratification into phenotypes remained significant only the the non-allergic asthma subgroup. Asthma patients with higher *CNR1* mRNA expression levels before treatment and who received LTRA treatment had a more significant improvement of lung function indicated by higher ΔFEV1 values.

No correlation between the mRNA expression of *NAPEPLD*, *ABHD4* and response to treatment with LTRA was found (Table 3.15).

Table 3.15: Correlation of NAPE-PLD (*NAPEPLD*), Abhd4 (*ABHD4*) and FAAH (*FAAH*) gene expression with response to LTRA treatment.

Gene	Phenotype	Treatment response	n	r_s	*P* value
NAPEPLD	Asthma	ΔFEV1/FVC	103	0.091	0.359
		ΔFEV1	103	-0.037	0.712
	Allergic asthma	ΔFEV1/FVC	64	0.054	0.670
		ΔFEV1	64	-0.010	0.935
	Non-allergic asthma	ΔFEV1/FVC	37	0.214	0.203
		ΔFEV1	37	-0.133	0.433
ABHD4	Asthma	ΔFEV1/FVC	112	0.085	0.373
		ΔFEV1	112	-0.039	0.685
	Allergic asthma	ΔFEV1/FVC	70	0.084	0.488
		ΔFEV1	70	0.034	0.781
	Non-allergic asthma	ΔFEV1/FVC	40	0.112	0.493
		ΔFEV1	40	-0.088	0.587
FAAH	Asthma	ΔFEV1/FVC	**102**	**0.214**	**0.030**
		ΔFEV1	102	0.171	0.089
	Allergic asthma	ΔFEV1/FVC	64	0.211	0.092
		ΔFEV1	64	0.233	0.064
	Non-allergic asthma	ΔFEV1/FVC	**36**	**0.355**	**0.034**
		ΔFEV1	36	0.058	0.738

LTRA, leukotriene receptor antagonist;*NAPEPLD*, NAPE-PLD gene expression; *ABHD4*, Abhd4 gene expression; *FAAH*, FAAH gene expression; FEV1, forced expiratory volume, 1 s; FVC, forced vital capacity; r_s, Spearman's correlation coefficient, significant correlations ($P < 0.05$) are shown in bold.

3.2.3 DAGLα and MAGL gene expression in naïve asthma patients and correlation with asthma severity

Before treatment, the asthma group, as well as the allergic and non-allergic subgroups, had median relative mRNA expression levels of *MGLL* that ranged between 1.21 and 1.33-fold higher than the control group (figure 3.23). While the median relative mRNA expression level of *DAGLA* in the asthma group, on the other hand, was 0.85-fold of the control group. Once again we analyzed the gene expression

according to age to test the possibility that the observed difference was age associated. No significant correlation was found between the subjects' age and mRNA expression levels of *DAGLA* (r_s = -0.062, P = 0.439) or *MGLL* (r_s = -0.148, P = 0.059).

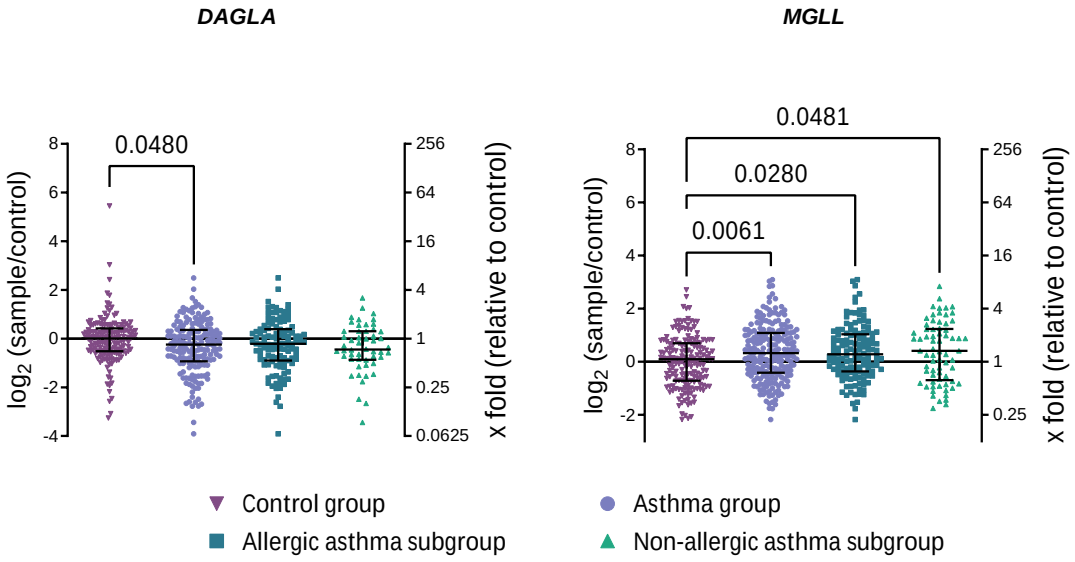

Figure 3.23: DAGα (*DAGLA*) and MAGL (*MGLL*) gene expression in PBMCs from naive asthma patients. *DAGLA* mRNA expression [control group (n = 160), asthma group (n = 177), allergic asthma subgroup (n = 115), non-allergic asthma subgroup (n = 52)]. *MGLL* mRNA expression [control group (n = 168), asthma group (n = 214), allergic asthma subgroup (n = 134), non-allergic asthma subgroup (n = 69)]. Each dot represents a data point, horizontal lines display the median value and whiskers illustrate the IQR. Kruskal-Wallis test followed by Dunn's multiple comparisons test.

Both *DAGLA* and *MGLL* mRNA expression levels were significantly correlated with several clinical parameters and these are summarised in Table 3.16 (see Table 15 for all correlation results).

The mRNA expression levels of *DAGLA* showed a strong positive correlation with serum levels of total IgE but only in the non-allergic asthma subgroup (r_s = 0.724, P = 0.005). Non-allergic asthma patients with higher levels of the allergic biomarker had higher levels of *DAGLA* mRNA expression.

Table 3.16: Significant correlations of DAGα (*DAGLA*) and MAGL (*MGLL*) gene expression with clinical data before anti-asthmatic treatment.

Gene	Asthma phenotype	Clinical parameter	n	r_s	*P* value
DAGLA	Non-allergic asthma	Total IgE	14	0.724	0.005
MGLL	Asthma	FEV1	214	0.151	0.027
	Non-allergic asthma	logPC20	69	0.242	0.045
		FeNO	65	0.294	0.018

FEV1, forced expiratory volume in 1 s; logPC20, base 10 logarithm of provocative methacholine concentration causing a drop in FEV1 of 20%; FeNO, fractional exhaled nitric oxide; r_s, Spearman's correlation coefficient, $P < 0.05$ was considered significant.

The mRNA expression levels of *MGLL*, on the other hand, showed a very weak positive correlation with FEV1 in the asthma group. Asthma patients with lower airway obstruction had higher *MGLL* mRNA expression levels (r_s = 0.151, P = 0.027). This correlation lost significance after stratifying patients into their phenotype.

Additionally, the mRNA expression levels of *MGLL* from non-allergic asthma patients showed a weak positive correlation with airway hyper-responsiveness (r_s = -0.150, P = 0.034). Non-allergic asthma patients with lower airway responsiveness, measured by logPC20, had higher levels of *MGLL* mRNA expression. However, these same patients had higher levels of airway inflammation reflected on higher FeNO levels (r_s = 0.294, P = 0.018)

Additional analyses of *DAGLA* mRNA expression levels with the levels of cytokine mRNA expression indicate that DAGLA had a weak negative correlation with the levels of *IL13* mRNA expression in the asthma group (r_s = -0.340, P = 3.4×10^{-5}). After stratifying patients into their phenotypes, this correlation only remained significant in the allergic asthma subgroup (figure 3.24).

On the other hand, the levels of *MGLL* mRNA expression showed a moderate positive correlation with the levels of *IL4* mRNA expression (r_s = 0.486, P = 7.6×10^{-14}) and a weak positive correlation with the levels of *IL5* mRNA expression (r_s = 0.372, P = 5.4×10^{-8}). Asthma patients with higher levels of *IL4* and of *IL5* mRNA expression had higher levels of *MGLL* mRNA expression (figure 3.25). A similar pattern of *MGLL* co-expression with *IL4* and *IL5* was found when patients were stratified according to their phenotype.

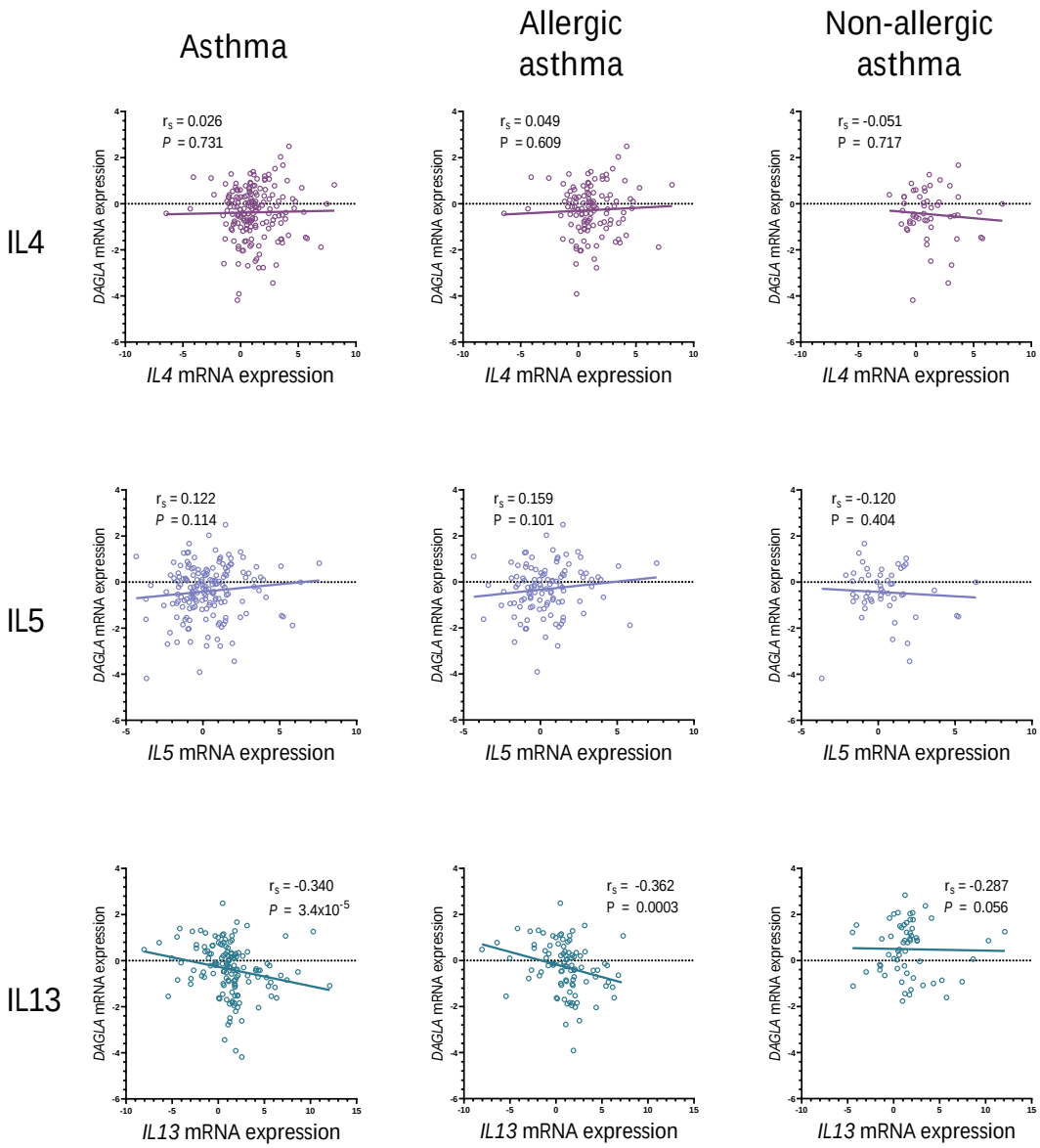

Figure 3.24: DAGLα (*DAGLA*) gene expression in PBMCs from naive asthma patients. IL-4 (*IL4*): asthma group (n = 175), allergic asthma subgroup (n = 113), non-allergic asthma subgroup (n = 52); IL-5 (*IL5*): asthma group (n = 169), allergic asthma subgroup (n = 108), non-allergic asthma subgroup (n = 51); IL-13 (*IL13*): asthma group (n = 142), allergic asthma subgroup (n = 94), non-allergic asthma subgroup (n = 45) Each dot represents a data point, lines display the trend. r_s, Spearman's correlation coefficient, P <0.05 was considered significant.

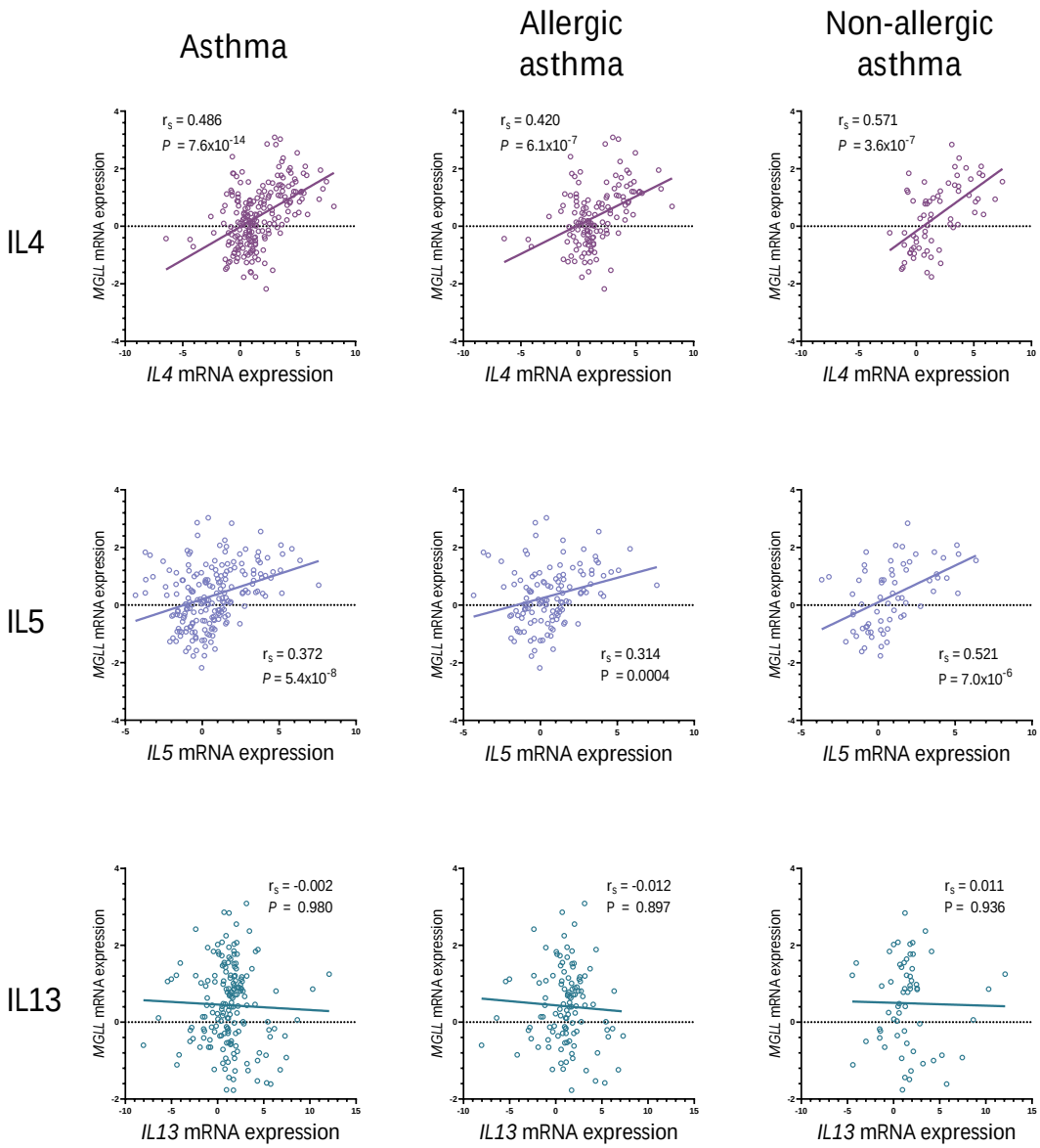

Figure 3.25: MAGL (*MGLL*) gene expression in PBMCs from naive asthma patients. IL-4 (*IL4*): asthma group (n = 210), allergic asthma subgroup (n = 131), non-allergic asthma subgroup (n = 68); IL-5 (*IL5*): asthma group (n = 201), allergic asthma subgroup (n = 124), non-allergic asthma subgroup (n = 66); IL-13 (*IL13*): asthma group (n = 176), allergic asthma subgroup (n = 112), non-allergic asthma subgroup (n = 61) Each dot represents a data point, lines display the trend. r_s, Spearman's correlation coefficient, P <0.05 was considered significant.

3.2.4 *DAGLA* and *MGLL* mRNA expression levels before treatment as predictors of ICS and LTRA treatment response

We analyzed the correlation of *DAGLA* and *MGLL* mRNA expression levels before treatment with changes of the FEV1/FVC ratio and FEV1 after ICS and LTRA treatment.

We found no correlation between the mRNA expression of *DAGLA* and *MGLL* from naive asthma patients with response to treatment with ICS (Table 3.17 and 3.18.

Table 3.17: Correlation of DAGα (*DAGLA*) and MAGL (*MGLL*) gene expression with response to ICS treatment.

Gene	Phenotype	Treatment response	n	r_s	*P* value
DAGLA	Asthma	ΔFEV1/FVC	72	-0.035	0.772
		ΔFEV1	72	0.043	0.720
	Allergic asthma	ΔFEV1/FVC	49	0.082	0.573
		ΔFEV1	49	0.109	0.455
	Non-allergic asthma	ΔFEV1/FVC	18	-0.256	0.306
		ΔFEV1	18	0.098	0.670
MGLL	Asthma	ΔFEV1/FVC	84	-0.064	0.562
		ΔFEV1	84	-0.207	0.060
	Allergic asthma	ΔFEV1/FVC	57	-0.093	0.493
		ΔFEV1	57	-0.220	0.101
	Non-allergic asthma	ΔFEV1/FVC	22	0.143	0.525
		ΔFEV1	22	-0.099	0.661

ICS, inhaled corticosteroids; *DAGLA*, DAGα gene expression; *MGLL*, MAGL gene expression; FEV1, forced expiratory volume, 1 s; FVC, forced vital capacity; r_s, Spearman's correlation coefficient, significant correlations ($P < 0.05$) are shown in bold.

Table 3.18: Correlation of DAGα (*DAGLA*) and MAGL (*MGLL*) gene expression with response to LTRA treatment.

Gene	Phenotype	Treatment response	n	r_s	*P* value
DAGLA	Asthma	ΔFEV1/FVC	89	0.167	0.116
		ΔFEV1	89	0.032	0.769
	Allergic asthma	ΔFEV1/FVC	59	0.226	0.082
		ΔFEV1	59	0.067	0.616
	Non-allergic asthma	ΔFEV1/FVC	28	0.220	0.262
		ΔFEV1	28	-0.088	0.655
MGLL	Asthma	ΔFEV1/FVC	107	0.096	0.323
		ΔFEV1	107	0.094	0.334
	Allergic asthma	ΔFEV1/FVC	66	-0.025	0.843
		ΔFEV1	66	-0.011	0.931
	Non-allergic asthma	ΔFEV1/FVC	39	0.199	0.224
		ΔFEV1	39	0.231	0.157

LTRA, leukotriene receptor antagonist;*DAGLA*, DAGα gene expression; *MGLL*, MAGL gene expression; FEV1, forced expiratory volume, 1 s; FVC, forced vital capacity; r_s, Spearman's correlation coefficient, significant correlations (P <0.05) are shown in bold.

3.3 Change in gene expression after treatment with ICS or LTRA.

3.3.1 Change in gene expression after treatment with ICS

After long-term treatment with ICS, there were significant changes in the levels of mRNA expression of the genes quantified (figure 3.26).

Asthma patients had median levels of *IL4* and *IL5* mRNA expression that were 0.48 and 0.35-fold, respectively, of the levels measured before treatment. The levels of mRNA expression of *CNR1* were also reduced to 0.75-fold of the levels measured before treatment.

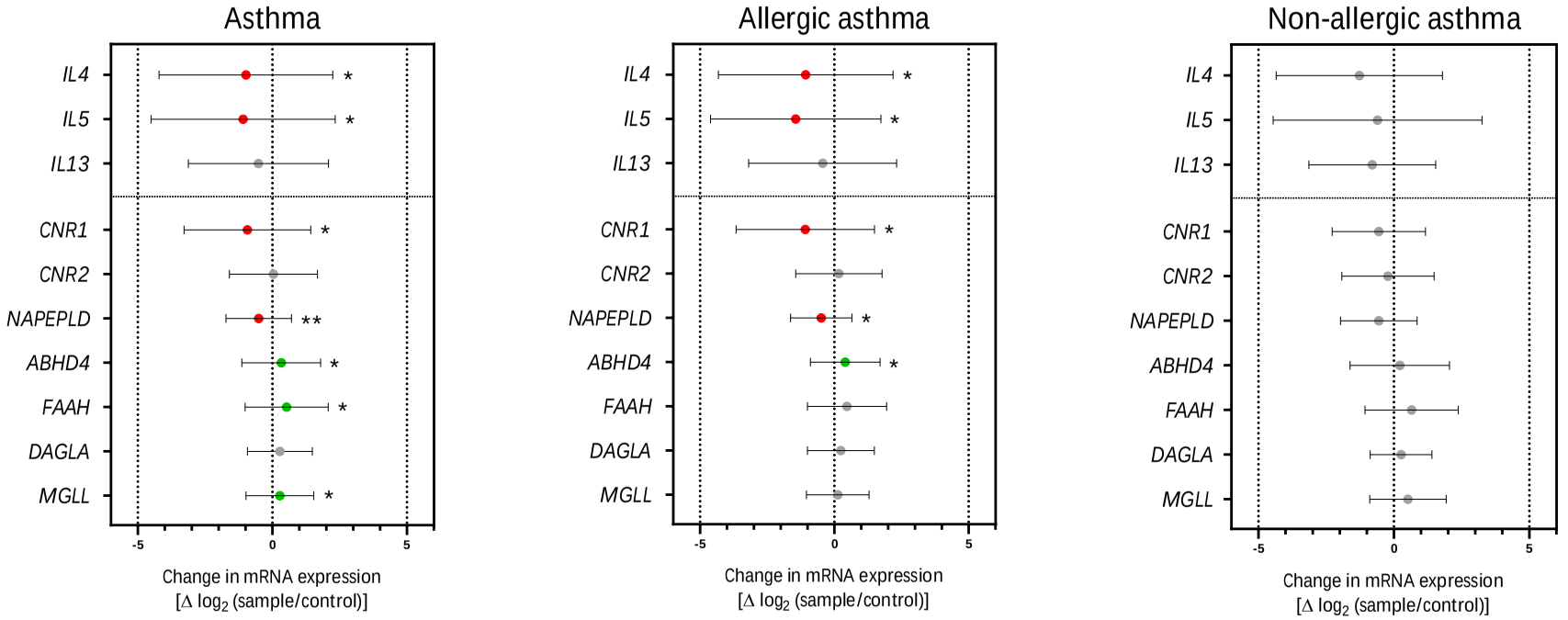

Figure 3.26: Differential gene expression analysis in PBMCs from asthma patients after long-term treatment with ICS. Asthma: *IL4* (n = 60), *IL5* (n = 50), *IL13* (n = 56), *CNR1* (n = 45), *CNR2* (n = 54), *NAPEPLD* (n = 55), *ABHD4* (n = 60), *FAAH* (n = 53), *DAGLA* (n = 43), *MGLL* (n = 60). Allergic asthma: *IL4* (n = 41), *IL5* (n = 35), *IL13* (n = 38), *CNR1* (n = 32), *CNR2* (n = 36), *NAPEPLD* (n = 38), *ABHD4* (n = 41), *FAAH* (n = 36), *DAGLA* (n = 31), *MGLL* (n = 41). Non-allergic asthma: *IL4* (n = 17), *IL5* (n = 14), *IL13* (n = 17), *CNR1* (n = 13), *CNR2* (n = 18), *NAPEPLD* (n = 17), *ABHD4* (n = 18), *FAAH* (n = 17), *DAGLA* (n = 11), *MGLL* (n = 18). Dots display the median-fold change of the gene's mRNA expression levels and whiskers illustrate the IQR. Wilcoxon matched-pairs signed rank t-test, $^{*}P$ <0.05, $^{**}P$ <0.01.

The remaining gene whose expression was lower after long-term treatment with ICS encodes NAPE-PLD (to 0.75-fold), one of the main enzymes involved in the synthesis of AEA, as opposed to Abhd4, whose gene expression was increased to 1.25-fold. Other enzymes whose gene expression was increased include FAAH (to 1.27-fold) and MGLL (to 1.35-fold), both involved in the metabolism of endocannabinoids.

After stratifying patients into their phenotype allergic asthma patients had median levels of *IL4* and *IL5* mRNA expression that were 0.44 and 0.30-fold, respectively, of the levels measured before treatment. The levels of *CNR1* mRNA expression were also reduced to 0.74-fold of the levels measured before treatment.

Similar to what was observed in the asthma group, in allergic asthma patients the levels of *NAPEPLD* mRNA expression were reduced to 0.76-fold, as opposed to Abhd4, whose gene expression was increased by 1.38-fold. No other changes reached statistical significance in the remaining genes analysed. In contrast to the allergic asthma subgroup, no significant change in gene expression after treatment with ICS was observed in the non-allergic asthma subgroup.

3.3.2 Change in gene expression after treatment with LTRA

After long-term treatment with LTRA, there were significant changes in the levels of mRNA expression of the genes quantified (figure 3.27). Asthma patients had median levels of *IL4*, *IL5* and *IL13* mRNA expression that were 0.23, 0.07 and 0.53-fold, respectively, of the levels measured before treatment. The levels of mRNA expression of *CNR1* were also reduced to 0.19-fold of the levels measured before treatment. As for the gene expression of the enzymes analysed, only *NAPEPLD* was reduced to 0.45-fold after long-term treatment with LTRA.

After stratification into phenotypes, patients in the allergic asthma subgroup had median levels of *IL4* and *IL5* mRNA expression that were 0.28 and 0.05-fold, respectively, of the levels measured before treatment. However, the change in IL13 gene expression did not remain significant. The levels of *CNR1* mRNA expression, were also reduced in this subgroup to 0.39-fold of the levels measured before treatment. Similar to what was observed in the asthma group, in allergic asthma patients the levels of *NAPEPLD* mRNA expression were reduced to 0.44-fold. The only gene whose expression was increased after LTRA is FAAH that increased 1.28-fold. No other changes reached statistical significance in the remaining genes analysed.

In the non-allergic subgroup, in contrast to the lack of a significant effect on

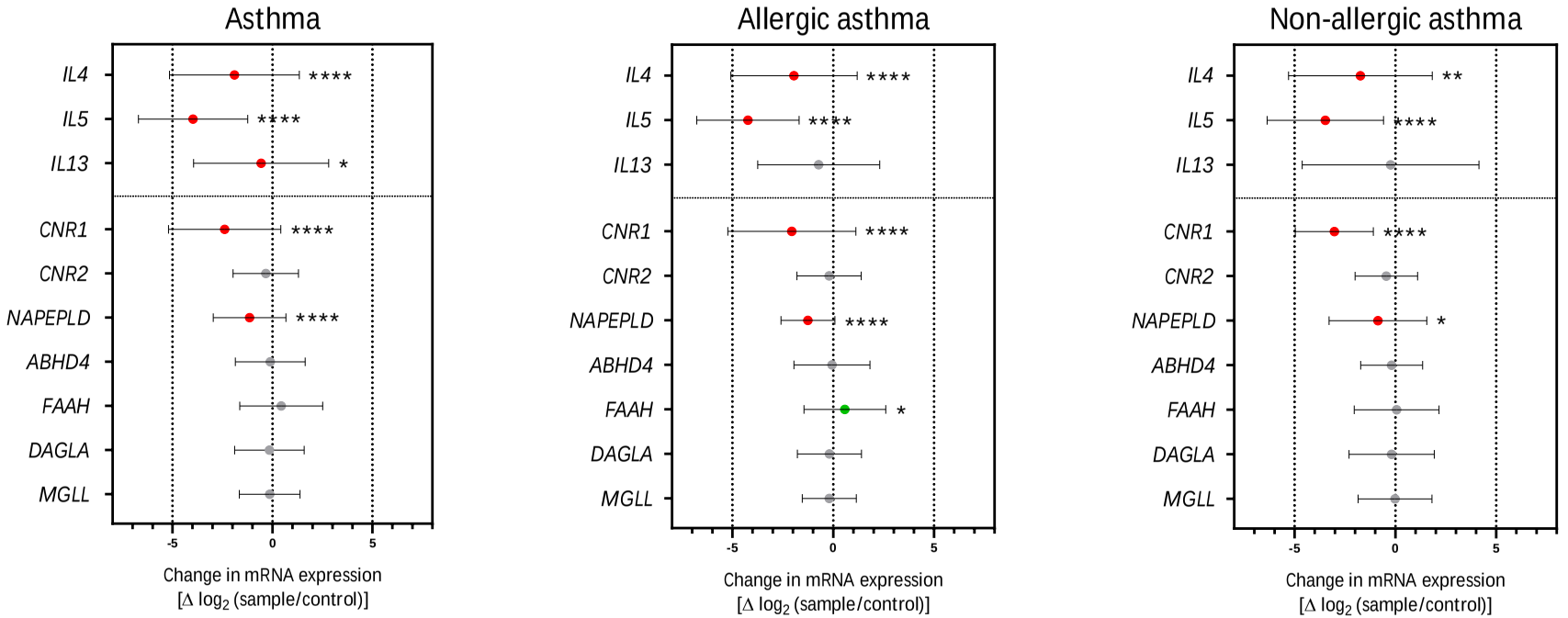

Figure 3.27: Differential gene expression analysis in PBMCs from asthma patients after long-term treatment with LTRA. Asthma: *IL4* (n = 98), *IL5* (n = 73), *IL13* (n = 52), *CNR1* (n = 75), *CNR2* (n = 80), *NAPEPLD* (n = 80), *ABHD4* (n = 96), *FAAH* (n = 82), *DAGLA* (n = 65), *MGLL* (n = 97). Allergic asthma: *IL4* (n = 62), *IL5* (n = 43), *IL13* (n = 37), *CNR1* (n = 48), *CNR2* (n = 53), *NAPEPLD* (n = 50), *ABHD4* (n = 62), *FAAH* (n = 53), *DAGLA* (n = 44), *MGLL* (n = 60). Non-allergic asthma: *IL4* (n = 34), *IL5* (n = 28), *IL13* (n = 14), *CNR1* (n = 26), *CNR2* (n = 25), *NAPEPLD* (n = 28), *ABHD4* (n = 32), *FAAH* (n = 27), *DAGLA* (n = 19), *MGLL* (n = 35). Dots display the median-fold change of the gene's mRNA expression levels and whiskers illustrate the IQR. Wilcoxon matched-pairs signed rank t-test, $^{*}P$ <0.05, $^{**}P$ <0.01,$^{***}P$ <0.001, $^{***}P$ <0.0001.

gene expression of long-term treatment with ICS, treatment with LTRA reduced the levels of *IL4* and *IL5* mRNA expression to 0.20 and 0.09-fold, respectively. The levels of *CNR1* mRNA expression, were also reduced in this subgroup to 0.11-fold. As for the gene expression of the enzymes analysed, only *NAPEPLD* was reduced to 0.52-fold after long-term treatment with LTRA.

3.4 Fatty acid ethanolamides in asthma

3.4.1 AEA, PEA and OEA plasma levels in naïve asthma patients and correlation with asthma severity

The median PEA plasma levels before treatment were decreased by 45% in the asthma group when compared to controls ($P = 0.034$, figure 3.28). After stratifying patients into their phenotypes the median PEA plasma levels were decreased by 65% in the allergic asthma subgroup ($P = 0.0\ 13$) while in the non-allergic asthma group they remained similar to the control group. No significant differences were found in the medial plasma levels of AEA and OEA in asthma patients.

Only PEA and OEA plasma levels were significantly correlated with clinical parameters [Figure 3.29 (see Table 16 for all correlation results)]. The plasma levels of PEA showed a moderate negative correlation with FeNO (r_s = -0.431, $P = 0.01$). Asthma patients with higher levels of inflammation measured by FeNO had lower plasma levels of PEA. This correlation did not remained significant after stratifying patients into their phenotypes. The plasma levels of PEA were also correlated with airway hyper-responsiveness in the non-allergic asthma subgroup (r_s = -0.431, $P = 0.01$).

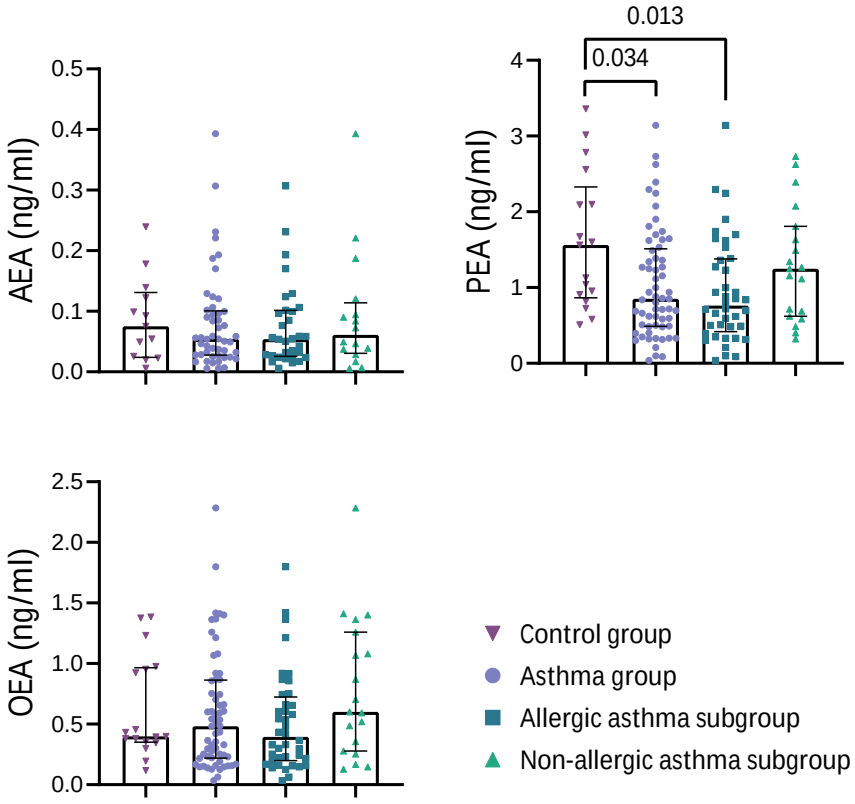

Figure 3.28: AEA, PEA and OEA plasma levels from naive asthma patients. AEA plasma levels [control group (n = 13), asthma group (n = 49), allergic asthma subgroup (n = 32), non-allergic asthma subgroup (n = 16)]. PEA plasma levels [control group (n = 17), asthma group (n = 61), allergic asthma subgroup (n = 40), non-allergic asthma subgroup (n = 19)]. OEA plasma levels [control group (n = 17), asthma group (n = 61), allergic asthma subgroup (n = 40), non-allergic asthma subgroup (n = 19)]. Bars displays median plasma levels, and whiskers illustrate the IQR. Each dot represents a data point.

Similar to PEA, OEA median plasma levels of asthma patients showed a weak negative correlation with FeNO (r_s = -0.349, P = 0.04). Asthma patients with higher levels of FeNO had lower plasma levels of OEA. This correlation did not remained significant after stratifying patients into their phenotypes. No significant correlation was found between AEA plasma levels and the clinical parameters measured at the time of diagnosis.

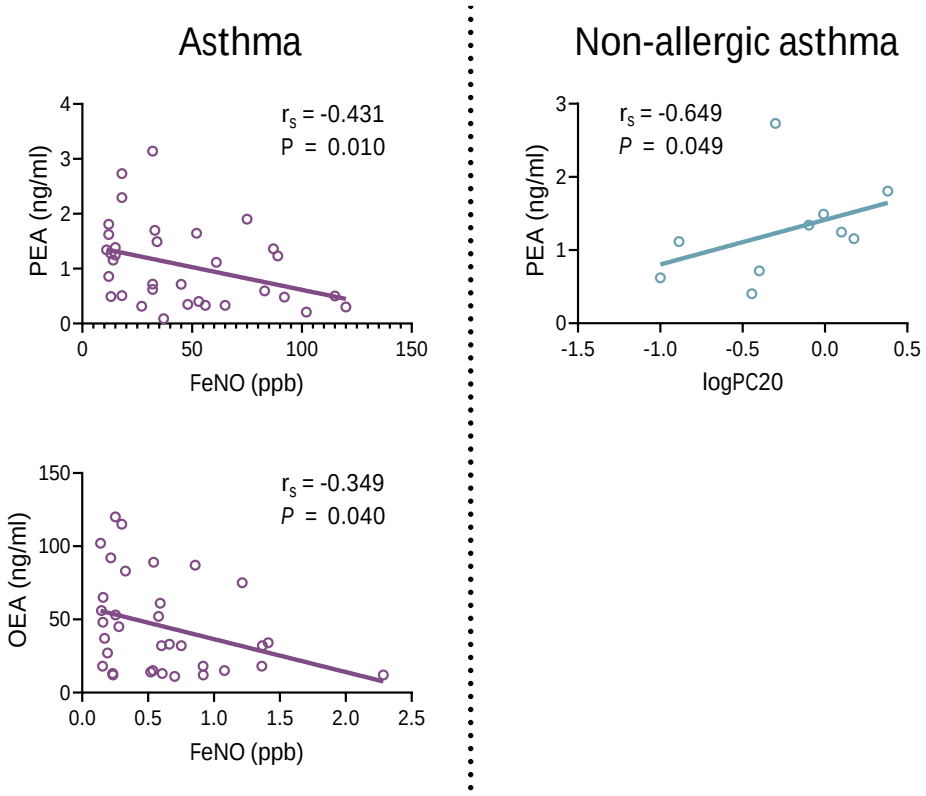

Figure 3.29: Significant correlations of PEA and OEA plasma levels with clinical data before anti-asthmatic treatment. Asthma group: PEA (n = 35) and OEA (n = 34). Non-allergic asthma subgroup: PEA (n = 10). Each dot represents a data point, lines display the trend. r_s, Spearman's correlation coefficient, P <0.05 was considered significant.

3.4.2 AEA, PEA and OEA plasma levels correlation with gene expression in naive asthma patients

No correlation was found between AEA, PEA, and OEA plasma levels, and the mRNA expression levels of the genes measured in this study.

All correlation results are shown in Table 17.

3.4.3 Change in AEA, PEA and OEA plasma levels after treatment with ICS or LTRA

Only PEA plasma levels were significantly different after long-term treatment with LTRA. After LTRA treatment, using paired analysis we found that PEA plasma

levels decreased further in the allergic asthma subgroup (figure 3.30). In contrast, although statistical significance was not reached, PEA plasma levels appear to be increased after LTRA treatment in the non-allergic asthma subgroup. No statistically significant changes in AEA, PEA or OEA plasma levels were found after treatment with ICS in the asthma group and in the allergic asthma subgroup. Statistical analysis regarding the change of AEA, PEA or OEA plasma levels in the non-allergic subgroup was not possible due to low number of paired results.

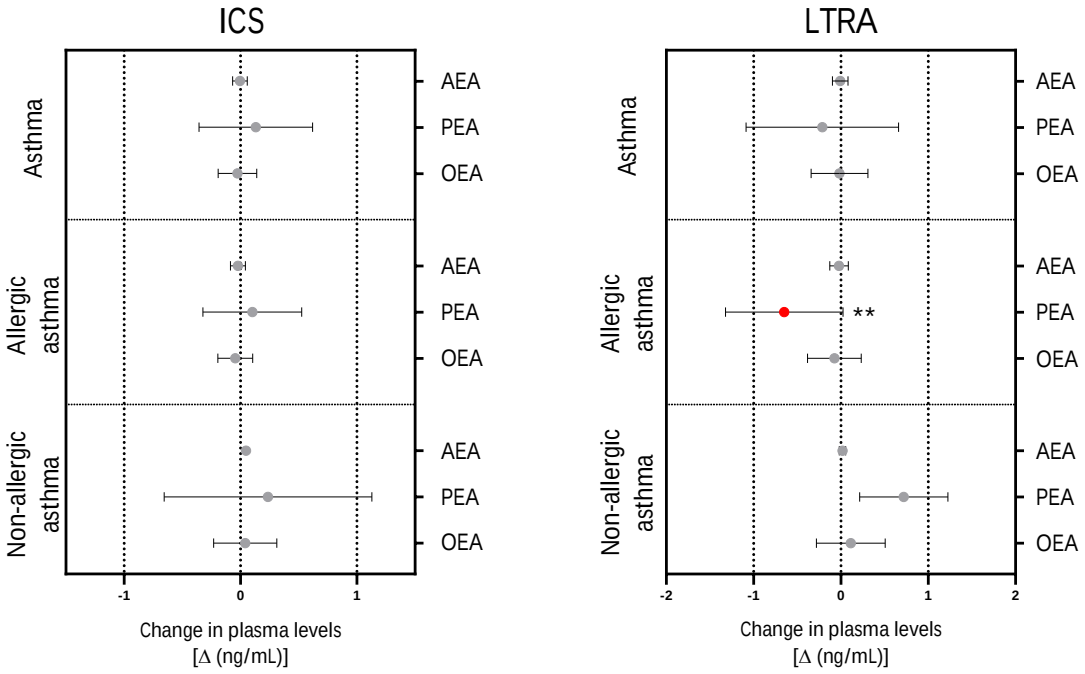

Figure 3.30: Differential gene expression analysis in PBMCs from asthma patients after long-term treatment with LTRA. Treated with ICS: Asthma [AEA (n = 4), PEA (n = 9), OEA (n = 9)], Allergic asthma [AEA (n = 3), PEA (n = 7), OEA (n = 7)], Non-allergic asthma [AEA (n = 1), PEA (n = 2), OEA (n = 2)]. Treated with LTRA: Asthma [AEA (n = 12), PEA (n = 16), OEA (n = 16)], Allergic asthma [AEA (n = 8), PEA (n = 10), OEA (n = 10)], Non-allergic asthma [AEA (n = 4), PEA (n = 5), OEA (n = 5)]. Dots display the median-fold change of the gene's mRNA expression levels and whiskers illustrate the IQR. Wilcoxon matched-pairs signed rank t-test, $^{**}P < 0.01$

4 DISCUSSION

The primary goals of asthma management are widely acknowledged to be attaining and maintaining asthma control. However, despite significant advancements in the knowledge of asthma epidemiology, physiopathology, and therapy, there is compelling evidence that asthma control remains poor in many individuals, especially those with severe asthma [328]. Therefore, there is a significant need to investigate novel methods of controlling and treating asthma. The therapeutic use of Cannabis is currently gaining widespread clinical and political acceptance. Therefore, it is not surprising that a considerable proportion of the asthmatic population is now attempting to relieve their symptoms with cannabinoids. Currently, about 18% of adults, members of the Allergy and Asthma Network and who answered an online survey, use Cannabis, primarily for medical purposes [329]. With every new scientific study published, it is becoming increasingly evident that cannabinoids significantly influence the immune system. They modulate immune responses during inflammatory processes, and their anti-inflammatory effects have been studied in several diseases, including rheumatoid arthritis, diabetes, and multiple sclerosis [289, 291, 330]. However, our knowledge of how the endocannabinoid system works to regulate the human immune system is still in its early stages, particularly regarding asthma. It is, therefore, crucial to elucidate how the different components of the endocannabinoid system act in humans who suffer from asthma.

In this study, the mRNA expression levels of the most significant elements from the endocannabinoid system were quantified in the PBMCs from 5-18 years old asthma patients, Caucasians of Slovene origin, and the relationship between these levels and the severity of asthma was investigated. Additionally, genetic variants near or in the coding region of CB1 and CB2 were genotyped to examine their contribution as a risk factor for developing asthma.

Our results show that the endocannabinoid system is regulated differently in asthma patients compared to the general population. The main findings are schematically represented in figure 4.1. Only minor differences were found between the allergic and non-allergic phenotypes.

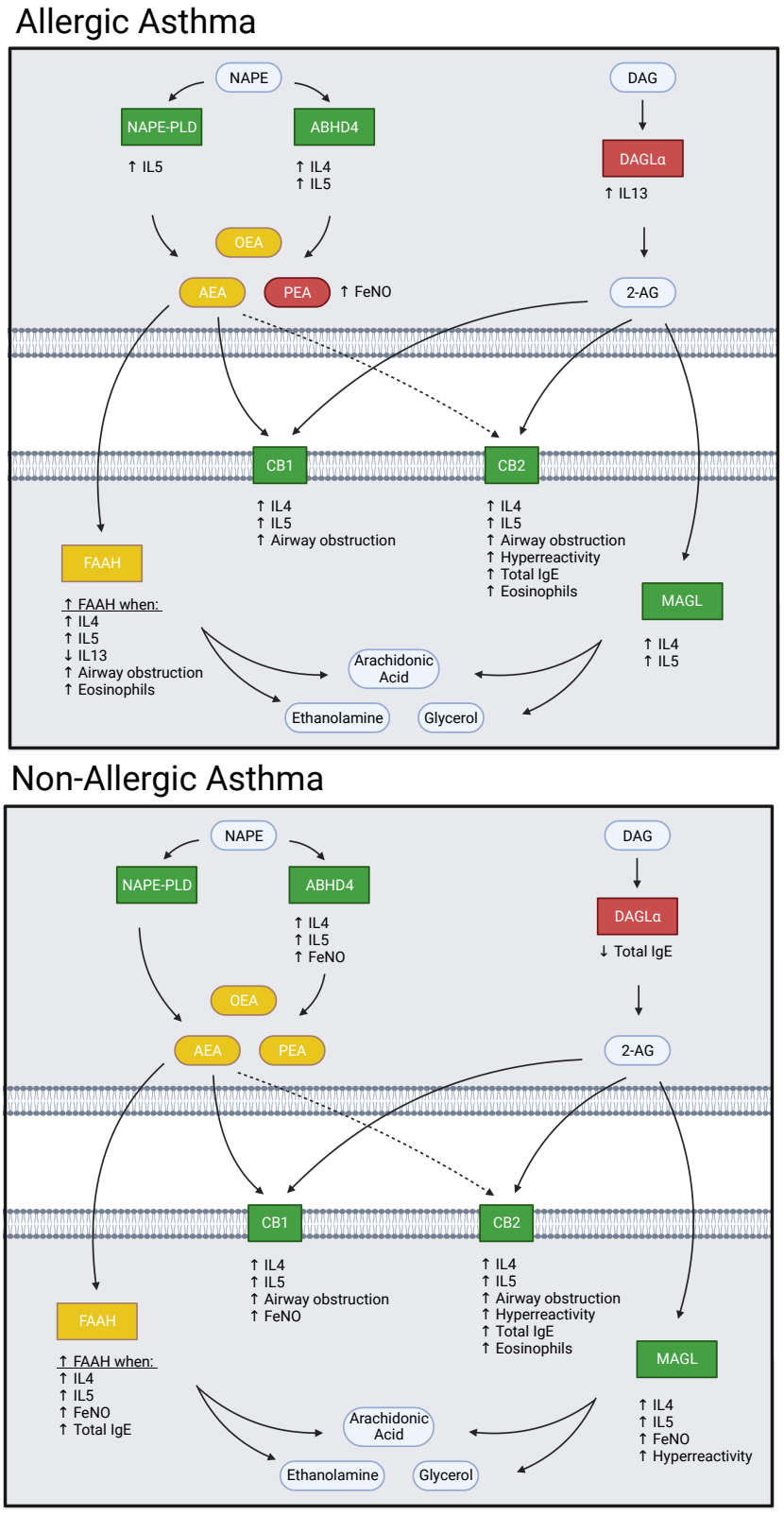

Figure 4.1: Change in the levels of core components of the endocannabinoid system in allergic and non-allergic asthma alongside associated clinical symptoms and inflammatory biomarkers. Green - up-regulated; yellow - normal; red - down-regulated.

The cannabinoid receptors

We discovered that the PBMCs from asthma patients (allergic and non-allergic) had increased mRNA expression levels of the genes for both cannabinoid receptors (*CNR1* and *CNR2*) and of their corresponding proteins (CB1 and CB2) at the time of diagnosis as compared to healthy controls. The correlation between mRNA expression levels with the levels of protein quantified was only weak (r_s between 0.19 and 0.25). According to several comparative studies, the correlation between mRNA and protein levels can be weak or moderately positive. However, despite breakthroughs in high-throughput genomic and proteomic technologies, the connection between gene expression and protein content remains unknown [331]. Patients with allergic asthma that presented with a higher degree of airway obstruction (FEV1/FVC) had the highest levels of mRNA expression of *CNR1* and *CNR2*. In patients with non-allergic asthma, higher mRNA expression levels of *CNR1* were measured in those who had the highest level of inflammation measured by FeNO. The mRNA expression levels of *CNR2* were also highest when asthma patients presented with higher bronchial hyper-reactivity (logPC20), higher levels of allergic marker (Total IgE) or higher eosinophil count.

The up-regulation of CB1 and CB2 receptors is a surprising finding as one would expect that suppression of the endocannabinoid system would be associated with uncontrolled airway inflammation in asthma. However, this is not the first time this pattern of expression has been reported, and it is in agreement with a recent pilot study that reported CB2 and CB1 expression to be up-regulated in nasal polyps of patients with aspirin-induced respiratory disease [332].

The endocannabinoid system frequently responds to stress by changing the expression of CB1 and CB2. In some chronic diseases, increases in cannabinoid receptor expression are thought to reduce symptoms and/or inhibit disease progression, such as neuropathic pain and multiple sclerosis, and thus are considered to play a protective role [333]. While changes in receptor expression can be inadequate in other diseases, such as CB1 up-regulation in liver fibrosis and down-regulation in colorectal cancer [334, 335]. Even though the list of diseases associated with changes in cannabinoid receptor expression keeps growing, little is known about the underlying mechanisms governing these changes. According to the few research that has investigated this field, factors released locally in response to disease can cause an increase in CB1 and CB2 expression [336]. For example, cytokines, which are released locally in response to inflammation to regulate neighbouring immune cells, have been linked to CB1 and CB2 up-regulation in immune cells. IL-4 was shown to directly increase *CNR1* mRNA and protein expression levels in primary human

T-cells and Jurkat T-cells [13]. At the same time, IFN-γ and GM-CSF up-regulated the mRNA expression levels of *CNR2* in microglial cells of mice [14]. In agreement with these findings, the levels of CB1 and CB2 expression reported here were also correlated with the levels of *IL4* and *IL5* expression, which were higher in the asthma patients compared to the general population. Therefore, the up-regulation of CB1 and CB2 expression seen in these asthma patients could be partially explained as a response to increased cytokine levels. Changes in CB1 and CB2 expression can also be a result of autoregulation mechanisms. For example, endocannabinoids and exogenous cannabinoids were shown to induce CB1 up-regulation in hepatocytes and T-cells, respectively [337, 338]. In another study performed on donors with allergic airway inflammation, it was also shown that surface CB2 receptor expression in peripheral blood eosinophils is higher in symptomatic allergic donors and that the CB2 receptor mediates eosinophil migration in the presence of IL5 [339, 340].

We also show for the first time that genetic variants located in the *CNR2* gene region are associated with asthma risk, severity and treatment response to ICS and LTRA. The rs4237 (C→T) lies in a 3' UTR region of the *PIHD1* gene located in the vicinity of *CNR2*, and it was reported to be in an expression quantitative trait locus to *CNR2* [197]. We found that the rs4237 genetic variant was associated with the risk of asthma and allergic asthma onset. Moreover, carriers of the rs4237 minor allele T are 1.7 times more likely to develop allergic asthma, suggesting a risk conferring role of this genetic variant. Allergic asthma patients who are carriers of the rs4237 (*CNR2*) minor allele T had lower lung function when compared with carriers of the major allele C. Furthermore. In addition, our results show that in allergic asthma patients, carriers of the rs4237 C allele have lower *CNR2* mRNA expression levels than carriers of the T allele. These findings are in agreement with the previous report that the rs4237 genetic variant is associated with *CNR2* gene expression in lymphoblastoid cell lines (C allele: effect = -0.413, H2 = 8.15, LOD = 7.153) [197].

The nonsynonymous genetic variant rs35761398 is located in exon 2 of the *CNR2* coding region. The rs35761398 variant (also referred to as Q63R), which occurs in amino acids 188–189, is created by an AA→GG polymorphism that leads to the substitution of the polar, uncharged glutamine (Q) at position 63 with a positively charged arginine (R) at the beginning of the first intracellular loop. According to our data, rs35761398 and rs4237 were in LD (most subjects with the rs35761398 QQ genotype also had the rs4237 CC genotype). Because rs35761398 causes a change in the amino acid structure of CB2, it is more likely that rs35761398 is the causal variant and rs4237 to be a tag genetic variant (a genetic variant that represents

a region of the genome with high LD and represents a group of genetic variants called a haplotype). However, the association between the rs35761398 genotype and asthma or atopic asthma in our study was only marginal, probably due to the low sample size. In line with our study, the rs35761398 Q allele has been reported as less frequent in subjects with celiac disease [341]. Several other association studies of the rs35761398 genetic variants with immune-mediated diseases, in which the rs35761398 RR genotype frequency was higher compared to control subjects, have been reported [304, 342, 343, 344]. Our results also suggest that rs35761398 may influence lung function in children with atopic asthma (carriers of the rs35761398 R allele had lower lung function). These results agree with other reports that suggest the rs35761398 genotype is associated with the degree of pathological outcomes from other diseases. It has been reported that the rs35761398 RR genotype is associated with increased inflammation that occurs during non-alcoholic fatty liver disease [342]. While the rs35761398 QQ genotype has been associated with persistently normal alanine aminotransferase in subjects with chronic hepatitis C [345]. Taken together, all evidence regarding the rs35761398 genetic variant points towards a protective role of the CB2 receptor with the QQ genotype and increased disease risk of the RR form of the receptor.

The genetic variant rs2229579 (also referred to as H316Y) in exon 2 of *CNR2* is another nonsynonymous polymorphism (C→T), which results in the substitution of histidine (H) at position 316 with tyrosine (Y) at the C-terminal tail. According to our data, rs2229579 was not in LD with rs4237 or rs35761398. In addition, we found that asthma patients carrying the rs2229579 minor allele T had increased levels of asthmatic/allergic inflammation markers. To the best of our knowledge, this is the first time that rs2229579 is associated with markers of allergy and inflammation in an immune-mediated disease.

Because both rs2229579 and rs35761398 genetic variants change amino acids in the part of the CB2 protein that is in the cytoplasmic side of the plasma membrane, they are not expected to have a significant effect on ligand binding [346]. Indeed, Carrasquer and colleagues reported that the binding affinities of several cannabinoid agonists were similar in HEK293 cells expressing the minor allele for rs2229579 or rs35761398 compared with cells expressing the major allele, except for 2-AG that had lower affinity for cells expression the rs2229579 minor allele [10]. The same authors suggested that activation of the CB2 receptor was compromised in cells expressing the minor allele for rs2229579 or rs35761398 because cannabinoid ligands had reduced ability to decrease forskolin-stimulated cAMP accumulation. Furthermore, they showed that although the constitutive activity of the CB2 receptor of

cells expressing the minor allele rs35761398 was similar to that of the major allele, the cells expressing the minor allele rs2229579 displayed higher constitutive activity than the cells expressing the major allele. GPCR variants with increased constitutive activity result from changes in GPCR coupling, receptor desensitization, or receptor recycling [347]. Several disease-causing GPCR variants with increased constitutive activity have been identified [348]. The investigation of these genetic variants is critical for developing drugs targeted at GPCRs variants in personalized medicine. Another study using transfected CHO cells has demonstrated the same effect of the rs35761398 minor allele. A reduced response to 2-AG, AM630 and JWH-015 was observed in CHO cells expressing the rs35761398 minor allele [349]. Furthermore, it has been shown that in humans carrying the rs35761398 RR genotype, the cannabinoid inhibition of antigen-nonspecific T cell proliferation was reduced compared to the rs35761398 QQ genotype [304]. These reports thus suggest that the rs2229579 and rs35761398 genetic variants result in reduced activity of CB2 receptor after ligand binding and consequently diminish its ability to inhibit immune responses, potentially contributing to more severe asthma / asthmatic inflammation, as observed in our study.

The endocannabinoid levels are also known to fluctuate in several inflammatory disorders, such as brain injury [350], cerebral ischemia [351], hepatic ischemia-reperfusion injuries [352], Huntington disease [353], multiple sclerosis [354], rheumatoid arthritis [355], atherosclerosis [356], sepsis [357], ulcerative colitis [358], contact dermatitis [359], and inflammatory pain [360]. The levels of endocannabinoids in circulation should be tightly controlled by a balance of biosynthetic and degradative enzymes [361]. Biosynthesis of AEA and 2-AG is usually observed in immune cells, following cell stress, activation with GPCR agonists or Ca^{2+} ionophores [362].

AEA biosynthesis and inactivation

We analysed the gene expression levels of NAPE-PLD (*NAPEPLD*) and Abhd4 (*ABHD4*) (AEA synthesising enzymes) and discovered that they were higher in naive asthma patients (allergic and non-allergic) than in the control group. The higher expression levels of both *NAPEPLD* and *ABHD4* were associated with higher expression levels of *IL5*. However, higher expression levels of *ABHD4* were also associate with higher expression levels of *IL4*. Additionally, higher expression levels of *ABHD4* were associated with higher FeNO levels in non-allergic asthma patients. These results suggest a correlation between the up-regulation of *NAPEPLD* and *ABHD4* with the degree of inflammation observed in asthma patients. NAPEs

can be converted to NAEs through several enzymatic pathways, including direct phospholipase D-mediated catalysis by NAPE-PLD, which is considered to be the principal enzyme responsible for the calcium-dependent conversion of NAPEs to NAEs -[261, 262, 363, 364]. When NAPE-PLD is overexpressed in cells, it decreases NAPE while increasing AEA levels [15]. In NAPE-PLD knock-out mice, brain tissue analysis revealed that most NAE species were significantly decreased coupled with increased levels of NAPEs, supporting the importance of NAPEPLD in the NAE biogenesis *in vivo*. In addition, PEA and OEA significantly decreased in these mice. However, one study reported that polyunsaturates NAEs such as AEA were not reduced NAPE-PLD knock-out mice [262]. The discovery of minor but measurable levels of NAEs indicated that alternative pathways to create NAEs exist.

Indeed, Abhd4 was shown to be involved in the primary pathway of long-term AEA synthesis [266]. However, the function of Abhd4 *in vivo* is still largely unknown. So far, it has been identified as a necessary and sufficient mediator for the elimination of pathologically detached cells, a process known as anoikis, during the embryonic cerebral cortex development of mice [365]. This function of Abhd4 was explicitly associated with pathological insults and absent in cells detached under normal physiological conditions. In agreement with this finding *ABHD4* knockdown in RWPE-1 prostate cells was shown to provide resistance to anoikis [366]. Using a cell model that faithfully replicates *in vivo* airway epithelial structure and electrophysiological function, Vermeer and colleagues demonstrated that treatment with MMP9, a protein associated with asthma, involved in the breakdown of extracellular matrix, induced anoikis [367]. As a result of cell loss and extrusion, the surviving epithelial cells grew in size, stretching and flattening to fill the space left by extruded, dead, and dying cells, similar to remodelling changes associated with asthma. Anoikis has also been reported in the airways of patients with severe asthma, where the remodelling of the airways becomes irreversible over time [368]. However, it was proposed that the dysregulated epithelium in severe asthma is caused by increased epithelial proliferation rather than anoikis, leading to a thicker remodelled epithelium. The increased *ABHD4* expression levels in asthma patients measured in the current study could, therefore, be a result of the presence of chronic inflammation in the airways and as a response to damaged detached epithelial cells.

Different enzymatic routes to AEA synthesis might have developed to preserve an AEA signalling division of tasks in time and place. AEA signalling is likely to require different regulatory mechanisms in the context of different biological phenomena, which can be accomplished by independently adjusting gene expression and enzymatic activity of the different molecular components of the endocannabi-

noid system [365].

The pharmacological or genomic deletion of FAAH was shown to also result in increased basal levels of AEA and prevent exogenously supplied AEA from being hydrolyzed [16, 17, 18, 19]. In addition, inhibition of FAAH in the airways of guinea pigs suppressed citric acid-evoked cough paired with increased levels of AEA, PEA and OEA [369]. However, the gene expression levels of *FAAH* were similar between asthma patients and the control group which, taken together with the up-regulation of *NAPEPLD* and *ABHD4*, should result in higher AEA plasma levels in asthma patients. Also essential to keep in mind is that NAPE-PLD is not exclusive to the synthesis of AEA. NAPE-PLD is also involved in the formation of other NAEs such as PEA and OEA. Similarly, FAAH is the primary enzyme responsible for the catabolism of AEA but can also metabolize PEA and OEA [16, 282]. Therefore, we performed a small preliminary study to determine the plasma concentration of AEA, PEA and OEA in asthma patients. Results from this study indicate that not only there is no systemic increase in the levels of these NAEs, but in fact, a decrease in PEA plasma levels was detected in samples from asthma patients compared to the control group (discussed below). Nonetheless, the variation of FAAH gene expression in the asthma group was associated with the patient's condition when the samples were collected. Asthma patients with higher *FAAH* expression levels had higher levels of IgE, eosinophils, FeNO (non-allergic asthma subgroup), or higher airway obstruction (allergic asthma subgroup). Additionally, higher expression levels of *FAAH* were associated with higher expression levels of *IL5* and *IL4*, suggesting even minor adjustments in *FAAH* expression are correlated with the degree of inflammation observed in asthma patients. However, higher expression levels of *FAAH* were also weakly associated with lower expression levels of *IL13*, remaining significant only in the allergic asthma subgroup after patient stratification. IL-13 induces many biological responses relevant to asthma, such as enhanced allergen-induced airway hyperresponsiveness, allergic inflammation, tissue eosinophilia, IgE antibody production, and tissue remodelling [370]. However, IL-13 blockage was so far shown to be ineffective in preventing the development of symptoms in clinical studies [144]. Even though *IL13* was up-regulated in asthma patients in our study, similar to *IL4* and I*IL5*, its expression was not correlated with *IL4* expression levels (results not shown). IL-4 and IL-13 possess overlapping biological roles, which is explained mainly by their capability to signal via the type 2 IL-4 receptors expressed primarily in non-hematopoietic cells [370]. Because IL-4 and IL-13 compete for type II receptor binding, the ratio of IL-4/IL-13 determines which of the two cytokines drives the inflammatory response and could be why the correlation of *FAAH*

expression was positive with *IL4* and negative with *IL13*.

Asthma patients, when exposed to allergens, were shown to have higher AEA concentrations in their bronchoalveolar lavage fluid (BALF) [371]. This study was able to show that the endocannabinoid system has a role in the pathogenesis of allergic asthma and was the first to show that endocannabinoids are involved in human asthma. In a recent animal study, AEA was shown to induce pulmonary vasoconstriction in a dose-dependent manner [372]. However, it is still unclear whether the elevated AEA concentrations are a cause or a result of the pathophysiology of asthma in humans. Cannabinoids have many anti-inflammatory properties. Nevertheless, the endocannabinoid system may elicit both pro- and anti-inflammatory responses [373, 374]. It has been shown that AEA concentrations are required to be in the micromolar range to cause tissue damage in a study performed on cardiomyocytes. Thus, the increase of AEA seen in BALF of asthma patients when exposed to allergens, from 5 pmol/L to 30 pmol/L, could be an indicator that it is a protective mechanism rather than a pathological component [375]. In our study, asthma patients were free from acute diseases or asthma exacerbation when blood samples were taken. In the presence of a stimulus, AEA is mainly generated and released locally and promptly re-uptaken by neighbouring cells to be hydrolyzed, which may explain why AEA plasma levels from asthma patients stay comparable in the absence of a stimulus to those in the control group. Another factor to take into account is the recent findings that have significantly challenged the long-held notion that AEA is synthesized on demand [376]. It has been shown that once AEA enters cells, it is quickly redirected to adiposomes [377, 378]. These adiposomes contain not only intracellular AEA but also the degradation enzyme FAAH, making these lipid particles modulators of intracellular AEA storage and breakdown [377].

Another way in which AEA levels are decreased is through conversion into other compounds by several alternative pathways [379]. One of these pathways involves oxidation of AEA into compounds that are structurally related to prostaglandins by COX-2, an enzyme not specific to the endocannabinoid system [380]. If in asthma patients, the dominant route responsible for reducing AEA plasma levels after its release is through oxidation by COX-2, then it could indicate a clear contribution to asthma pathology. Evidence in support of this view comes from a recent study that showed AEA has a pro-inflammatory-like action on bronchial epithelial permeability, which can be mediated via cyclooxygenase metabolites, and this indicates that inhibiting AEA degradation may offer a unique strategy to address airway inflammation [381].

2-AG biosynthesis and inactivation

2-AG is another major endocannabinoid with immunomodulatory effects, for which DAGL is considered the primary synthesising enzyme and MAGL the primary metabolising enzyme. 2-AG, but not AEA, was shown to induce minimal eosinophil migration, which was enhanced by IL-5 in a CB2-dependent manner [340], implying a role in asthma pathogenesis. Our study shows that DAGLα expression was reduced, but MAGL expression was increased in asthma patients compared with the control group.

Expression levels of *DAGLA* were strongly correlated with total IgE levels in the non-allergic subgroup. Higher expression levels of DAGLα were seen in non-allergic asthma patients with higher total IgE levels. Despite negative skin prick tests and negative testing for allergen-specific IgE in blood, some individuals might have high amounts of allergen-specific IgE antibodies in the airways [382, 383]. As a result, patients labelled as 'non-allergic' based on skin prick tests and measures of specific IgE antibodies in blood might be allergic to an unidentified allergen, potentially due to a local allergic response in the airways [384]. Higher *DAGLA* expression levels were also associated with lower *IL13* expression levels. In a mouse model of non-allergic asthma, an anti-IL-13 antibody was shown to improve bronchial hyperresponsiveness and mucus production [385], implying IL-13 in the development of symptoms in this asthma phenotype. These results suggest a more prominent role for DAGLα in the pathogenesis of non-allergic asthma versus allergic asthma. The expression levels of *MGLL* were also correlated with inflammation (FeNO) and hyper-reactivity (logPC20) of the airways of non-allergic asthma patients, which suggests MAGL's activity and upstream/downstream compounds are also more relevant in this asthma phenotype. Thus it is possible that 2-AG has a more dominant role in the pathogenesis of non-allergic asthma.

However, the balance between the expression of *DAGLA* and *MGLL* observed here could result in significantly reduced plasma levels of 2-AG due to a higher rate of 2-AG metabolism than biosynthesis. Additionally, 2-AG is also a substrate for COX-2, so with higher expression of COX-2, more 2-AG can be oxidised through this route. Unfortunately, we did not measure 2-AG plasma levels as it was outside the scope of this work. However, a recent study focused on the mechanism by which PEA suppressed mast cell degranulation, and histamine formation demonstrates for the first time that the effects of PEA are due to the stimulation of 2-AG biosynthesis by DAGLs [386]. This evidence further supports the hypothesis that 2-AG plasma levels might be reduced in asthma patients since our measurements of PEA plasma levels were found to be decreased in asthma patients compared to the control group.

There is conflicting evidence regarding the role of 2-AG in the modulation of immune responses as it was shown to possess both anti and pro-inflammatory effects [20]. For example, in studies on the modulation of macrophage/microglia responses, 2-AG was shown to inhibit the production of TNFα and IL-6. However, it also boosted iNOS-dependent nitric oxide and chemokine generation, as well as migration and cell adhesion [20]. It has been proposed that differences in 2-AG effects may be due to their conversion into bioactive COX-2 metabolites [387]. In addition, a unique pathway in which MAGL hydrolyses the 2-AG to create a significant arachidonate precursor pool for neuroinflammatory prostaglandins was described [388]. In our study, high *MGLL* expression levels were also associated with high *IL4* and *IL5* in asthma patients, regardless of their phenotype. Therefore, it is clear that DAGLα and MAGL are expressed differently in asthma patients, which appears to be more relevant to the non-allergic asthma phenotype. However, due to the unclear role of 2-AG in maintaining immune homeostasis, it is difficult to speculate whether the balance between *DAGLA* and *MGLL* expression contributes to or is a result of asthma.

Fatty acid ethanolamines

Other substances in the NAE class with similar structural properties to AEA have an impact on the endocannabinoid system. In this study, we demonstrate for the first time that PEA plasma levels are decreased in asthma patients and lower PEA and OEA levels are associated with higher FeNO levels, a marker of inflammation characteristic of allergic asthma.

PEA, which is produced alongside AEA, is an endocannabinoid-like compound. Much like AEA, PEA is synthesised on-demand from membrane phospholipids that act on a variety of molecular targets in both peripheral and central organs and systems [21, 389, 390]. PEA is considered to be generated in response to cellular damage as a pro-homeostatic protective response and has been shown to have anti-inflammatory, analgesic and neuroprotective properties [21]. PEA's diverse effects are attributed to its distinct modes of action, which influence various pathways at different locations [391]. However, the predominant route of action of PEA is by activating the nuclear receptor peroxisome proliferator-activated alpha (PPARα) [259]. In addition, PEA can enhance AEA activity by increasing receptor binding affinity or decreasing AEA hydrolysis, in a process known as the "entourage effect." [392]. PEA metabolism is disrupted during inflammation, pain, and neurodegeneration, causing a change in its levels in disease-affected tissues [21, 393].

Our results are in agreement with previous reports that show PEA and OEA lev-

els are reduced in other inflammatory conditions [355] and that PEA reduces nitric oxide production in macrophages after LPS stimulation *in vitro* [394]. Other studies have shown that inflammatory triggers reduce PEA levels in animals [395, 396]. In addition, PEA has been shown to reduce levels of TNFα in BALF of mice [397] and inhibit IL-4, IL-6 and IL-8 release from human PBMCs [398]. Similarly, OEA was reported to decrease TNFα gene expression in LPS stimulated neuroinflammation in rats [399]. More importantly, a recent animal study has shown that allergen sensitisation reduces PEA bronchial levels and propose that its supplementation may help avoid the onset of asthma-like symptoms [400].

The endocannabinoid system as a predictor of treatment response

Successful prediction of treatment response can provide several health benefits to patients while also lowering healthcare expenditures associated with ineffective therapies. This is a core focus of the personalised medicine concept [401]. However, treatment response prediction is a difficult task since many distinct variables will influence individual results. Because of the significant advancements in pharmacogenomic research, genetic markers have attracted significant attention in personalised medicine [402].

ICS are used as a first-line treatment in adults and children with persistent asthma [403]. Even though most asthma patients treated with ICS see a reduction in their symptoms [404], there are significant variations in asthma treatment response among individuals and communities [405, 406]. These findings imply that genetics may play a significant influence in asthma therapy responsiveness [406, 407]. As a result, the identification of unique pharmacogenetic profiles may be improved in the future by the characterisation of multiple genetic markers affecting therapeutic responses to asthma medications. This would allow for the clinical identification of asthma patients who do not respond satisfactorily to these treatments or who experience side effects [408].

As the results presented here show, the endocannabinoid system is regulated differently in asthma patients compared to the general population represented in this study. Thus, the components that make up the endocannabinoid system are potential biomarkers of disease and treatment response. Currently, clinicians may choose to initiate treatment with ICS, with LTRA as an alternative to ICS or in combination with ICS in managing asthma symptoms. Asthma patients recruited in this study were prescribed either ICS or LTRA.

Our results show that after 4-6 weeks of ICS treatment, asthma patients with higher *CNR1* mRNA expression levels at the time of diagnosis had a lower response

to treatment (ΔFEV1). The connection between glucocorticoid and endocannabinoid signalling pathways has been demonstrated in previous studies. For instance, the CB1 receptor is abundantly expressed in biological tissues and neuroanatomical areas of the brain associated with glucocorticoids function [409, 410]. Moreover, it has been found that glucocorticoids can mobilise the endocannabinoid system [22, 23, 24], and numerous lines of evidence indicate that an intact endocannabinoid signalling is required for various glucocorticoid-mediated effects [25, 26, 27, 28, 29]. However, only a few studies have looked into the connection between glucocorticoid and endocannabinoid signalling in controlling inflammatory pathways. For example, glucocorticoids, alongside endocannabinoids, have been found to modulate cell adhesion, favouring an anti-inflammatory phenotype [411, 412, 413, 414]. In a study using human synovial tissue, glucocorticoids induced cell adhesion via a GR-dependent mechanism, which was suppressed by blocking either CB1, CB2, or TRPV1 receptor signalling [412]. Furthermore, using both FAAH and COX-2 inhibitors, this work revealed that AEA levels control the effects of glucocorticoids on cell adhesion. Regrettably, the precise role of each of these receptors in the glucocorticoid-mediated effects remains to be established. Nonetheless, assuming that glucocorticoids exert their anti-inflammatory effects in part through the synthesis of AEA, FAAH inhibition may enhance the therapeutic benefits of glucocorticoids [412].

In contrast to the therapeutic response found with ICS, asthma patients with high *CNR1* or *FAAH* mRNA expression levels at the time of diagnosis, treated with LTRA for 4-6 weeks, had the best response (ΔFEV1/FVC). The complex metabolism of endocannabinoids means that they can further contribute to producing a diverse range of pro- and anti-inflammatory bioactive lipids. Both AEA and 2-AG hydrolysis can drive the production of leukotrienes by providing AA for its biosynthesis. Therefore, it stands to reason that treatment with LTRA can block at least some of the pro-inflammatory effects of endocannabinoids resulting from the activation of leukotriene receptors. While a previous animal study had shown that AEA inhalation prevented leukotriene-induced bronchospasm [415], a more recent animal study showed that AEA induced pulmonary vasoconstriction, dependent on the production of AA by FAAH and was blocked by LTRA treatment [372]. AEA has also been found to enhance barrier permeability by lowering trans-epithelial resistance in airway cells, a response mediated by AEA conversion to one or more LOX and COX metabolites [416]. Thus, increased AEA levels in asthma patients may contribute to epithelial permeability via degradation to AA metabolites. Thus, inhibiting AEA hydrolysis in the airways may aid in the prevention of asthmatic epithelial permeability. On the other hand, an *in vitro* study has shown that 2-AG

can activate human neutrophils as a consequence of its hydrolysis into AA and subsequent leukotriene biosynthesis. However, this effect on human neutrophils was not affected by LTRA treatment [417].

Our results show that not only those asthma patients with high *CNR1* had a better response to LTRA treatment, but also those who are carriers of the minor allele of genetic variant rs2229579 (*CNR2*) had a worse response to LTRA. To the best of our knowledge, this is the first time that CB1 and CB2 are reported to play a role in the response to LTRA.

ICS and LTRA long-term treatment effect on the endocannabinoid system

After long-term ICS treatment, the expression levels of *IL4* and *IL5* were significantly reduced in the PBMCs of the asthma patients followed in our study, which is in line with the long-known effects of corticosteroids [30, 31, 32, 33, 34]. Additionally, long-term ICS treatment reduced the expression levels of *CNR1* and *NAPEPLD* while simultaneously increasing *ABHD4*, *FAAH* and *MGLL*. A recent *in vitro* study has shown that corticosterone down-regulated the mRNA and protein expression of CB1 in glioblastoma cells suppressing the beneficial effects of CB1 activation by cannabinoids [35]. However, the long-term impact of corticosteroids on the endocannabinoid system is yet to be determined.

After long-term LTRA treatment, the expression levels of *IL4*, *IL5*, *IL13* were significantly reduced in the PBMCs of the asthma patients followed in our study. This reduction is in agreement with other studies reporting similar effects of LTRA on the expression of these cytokines [418, 419, 420]. In addition, similar to long-term ICS treatment, long-term LTRA treatment reduced the expression levels of CB1 and *NAPEPLD*. In allergic asthma patients, LTRA also increased the expression levels of *FAAH*, which could further reduce their PEA plasma levels after long-term treatment.

5 CONCLUSION

The main focus of this thesis was to genetically characterise the endocannabinoid system in naive asthma patients and determine if there is a relationship between endogenous cannabinoids and their inflammatory response. In order to address this, work was divided into the nine goals specified under section 1.3, the results of which are summarized below:

1. We found that the genes for CB1 (*CNR1*) and CB2 (*CNR2*), along with genes for the enzymes NAPE-PLD (*NAPEPLD*), Abhd4 (*ABHD4*) and MAGL (*MGLL*) were up-regulated in asthma patients, regardless of their phenotype. Given that the expression of DAGLα (*DAGLA*) was the only gene found to be down-regulated in asthma patients suggests that the balance between AEA and 2-AG has shifted in favour of AEA biosynthesis.

2. Overall, the up-regulation of the genes listed above was associated with several inflammatory biomarkers and clinical symptoms. Asthma patients that presented worse clinical symptoms and higher levels of inflammatory biomarkers also had the highest expression of these genes. The mRNA expression of *FAAH* was similar between asthma patients and the control group, but still, within the asthma group was found to be higher in asthma patients with worse clinical symptoms and higher levels of inflammatory biomarkers. These results suggest that the endocannabinoid system is up-regulated in asthma patients in association with disease severity. This study has also uncovered a potential role for the endocannabinoid system in personalised medicine. Our results show that asthma patients with high *CNR1* mRNA expression levels at the time of diagnosis have better response to treatment with LTRA, while asthma patients with high *CNR1* mRNA expression levels, treated with ICS, had worse treatment response. Furthermore, long-term treatment with ICS or LTRA reduced the mRNA expression of *CNR1* alongside *IL4* and *IL5*, suggesting a dominant role of CB1 in the individual response to either treatment.

3. In addition to reducing *IL4* and *IL5* expression levels, long-term ICS and LTRA treatment also reduced the expression levels of *CNR1* and *NAPEPLD*. In allergic asthma patients, long-term treatment with LTRA also resulted in increased *FAAH* mRNA expression levels, possibly contributing to a further reduction in their PEA plasma levels.

4. When the genetic association of rs4237 with asthma risk was analyzed we found that in the allergic asthma subgroup the frequency of the rs4327 CC genotype was significantly lower than in the control group, according to the recessive model. We also show that carriers of the rs35761398 or rs2229579 genetic variants have worse clinical symptoms than those with the wild-type CB2. Although *CNR1* and *CNR2* transcription rate appears elevated in asthma, their role in asthma pathogenesis remains to be determined.

5. Our findings demonstrate that not only did asthma patients with high *CNR1* responded better to LTRA therapy, but also those who carry the minor allele of the rs2229579 genetic variant (*CNR2*) did not. As far as we are aware, this is the first time CB1 and CB2 have been implicated in the response to LTRA.

6. Only in the allergic asthma subgroup the rs4237 genetic variant was significantly associated with *CNR2* mRNA expression levels.

7. In this study, we demonstrate for the first time that PEA plasma levels are decreased in asthma patients compared to the control group, while the plasma levels of AEA and OEA remain similar between the groups.

8. We found that PEA and OEA plasma levels in asthma patients are associated with higher FeNO levels, a marker of inflammation characteristic of allergic asthma.

9. Following prolonged LTRA treatment, only PEA plasma levels showed a significant change. We discovered that the PEA plasma levels in the allergic asthma subgroup continued to drop after LTRA treatment.

Even though the total sample size in our study gives us enough power to detect major associations, some small effects could have been missed. Further work should confirm our observations in different populations with larger sample sizes and understand the mechanism by which the endocannabinoid system modulates asthma inflammation and its clinical consequences.

In addition, several studies have found that a variety of individual factors, including gender, age, BMI, food consumption and circadian rhythms, can impact circulating endocannabinoid concentrations [421, 422, 423, 424]. The subjects enrolled in this study were not screened for the presence of exogenous cannabis metabolites, which were not disclosed through the initial patient questionnaire. Therefore, objective testing should be included in future studies to control for this possible confounding factor.

Nonetheless, the findings of this study contribute to a better understanding of the endocannabinoid system's role in the pathology of asthma, with individual genes potentially serving as biomarkers and/or new molecular targets for the treatment of childhood asthma.

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Appendix

Supplemental data

Table 1: Correlation of CB1 (*CNR1*) and CB2 (*CNR2*) gene expression with clinical data before anti-asthmatic treatment.

	Clinical parameter	*CNR1*			*CNR2*		
		n	r_s	*P* value	n	r_s	*P* value
Asthma	FEV1/FVC	**204**	**-0.147**	**0.036**	**200**	**-0.159**	**0.024**
	FEV1%	204	0.119	0.089	200	0.034	0.630
	logPC20	201	-0.095	0.181	**201**	**-0.150**	**0.034**
	FeNO	198	0.004	0.960	200	-0.001	0.987
	Total IgE	43	0.081	0.606	**43**	**0.323**	**0.035**
	Eosinophils	41	0.011	0.948	41	0.378	0.0148
Atopic asthma	FEV1/FVC	**127**	**-0.263**	**0.003**	**124**	**-0.242**	**0.007**
	FEV1%	127	0.097	0.279	124	0.055	0.542
	logPC20	126	-0.153	0.087	123	-0.161	0.075
	FeNO	125	-0.086	0.339	122	-0.025	0.786
	Total IgE	25	0.068	0.746	25	0.153	0.467
	Eosinophils	24	0.002	0.994	24	0.296	0.160
Non-atopic asthma	FEV1/FVC	66	-0.039	0.753	65	0.023	0.858
	FEV1%	66	0.161	0.198	65	0.102	0.418
	logPC20	66	-0.073	0.559	65	-0.162	0.198
	FeNO	**62**	**0.346**	**0.006**	61	0.210	0.104
	Total IgE	14	0.534	0.052	14	0.508	0.067
	Eosinophils	13	0.126	0.683	13	0.374	0.210

FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; logPC20, base 10 logarithm of provocative methacholine concentration causing a drop in FEV1 of 20%; FeNO, fractional exhaled nitric oxide (ppb); Total IgE (IU/mL); Eosinophils (in mm_3). r_s, Spearman's correlation coefficient, Significant associations (P <0.05) are shown in bold.

Table 2: Genetic association between rs4237 (*CNR2*) and clinical parameters from before treatment in asthmatic children.

		Asthma					**Allergic asthma**					**Non-allergic asthma**				
		TT	CT	CC	CT+CC[1]	CT+TT[2]	TT	CT	CC	CT+CC[1]	CT+TT[2]	TT	CT	CC	CT+CC[1]	CT+TT[2]
FEV1/FVC (%)	n	118	168	42	210	286	78	110	27	137	188	32	55	14	69	87
	Mean (±SD)	88.73 (±8.51)	88.99 (±7.37)	89.55 (±6.18)	89.10 (±7.14)	88.88 (±7.85)	88.97 (±8.37)	89.38 (±7.18)	90.70 (±6.15)	89.64 (±6.99)	89.21 (±7.68)	87.16 (±8.55)	88.42 (±7.52)	87.57 (±6.07)	88.25 (±7.21)	87.95 (±7.89)
	P value	0.837			0.670	0.601	0.589			0.532	0.336	0.751			0.507	0.863
FEV1 (%)	n	**117**	**167**	**42**	**209**	**284**	**77**	**109**	**27**	136	**186**	32	55	14	69	87
	Mean (±SD)	**89.03 (±14.38)**	**91.50 (±13.45)**	**96.07 (±11.07)**	**92.42 (±13.11)**	**90.48 (±13.87)**	**89.96 (±14.86)**	**92.61 (±12.96)**	**98.04 (±11.21)**	93.69 (±12.78)	**91.52 (±13.80)**	87.75 (±14.15)	90.07 (±13.87)	92.86 (±10.54)	90.64 (±13.24)	89.22 (±13.94)
	P value	**0.015**			**0.032**	**0.013**	**0.028**			0.055	**0.020**	0.485			0.321	0.353
logPC20	n	115	162	41	203	277	76	107	26	133	183	32	53	14	67	85
	Mean (±SD)	-0.41 (±0.65)	-0.43 (±0.66)	-0.27 (±0.53)	-0.40 (±0.64)	-0.42 (±0.66)	-0.49 (±0.62)	-0.55 (±0.68)	-0.30 (±0.56)	-0.50 (±0.66)	-0.53 (±0.65)	-0.16 (±0.69)	-0.15 (±0.54)	-0.24 (±0.50)	-0.17 (±0.53)	-0.15 (±0.60)
	P value	0.346			0.816	0.149	0.197			0.904	0.091	0.890			0.932	0.631
FeNO (ppb)	n	92	141	34	175	233	57	91	24	115	148	27	47	9	56	74
	Mean (±SD)	37.22 (±27.14)	40.39 (±32.63)	45.62 (±30.07)	41.41 (±32.13)	39.14 (±30.56)	43.02 (±25.44)	50.10 (±33.27)	49.83 (±33.02)	50.04 (±33.07)	47.37 (±30.60)	27.89 (±27.67)	21.17 (±18.15)	33.67 (±19.37)	23.18 (±18.75)	23.62 (±22.15)
	P value	0.381			0.287	0.248	0.374			0.161	0.718	0.196			0.364	0.197
Total IgE (IU/ml)	n	57	69	18	87	126	38	49	11	60	87	15	19	6	25	34
	Mean (±SD)	512.0 (±615.5)	537.8 (±620.5)	532.8 (±857.4)	536.8 (±670.6)	526.2 (±615.9)	630.3 (±698.4)	683.4 (±669.2)	714.9 (±1062)	689.2 (±745.5)	660.2 (±678.6)	190.5 (±243.5)	186.8 (±246.6)	257.2 (±231.4)	203.7 (±240.2)	188.4 (±241.5)
	P value	0.975			0.823	0.968	0.920			0.697	0.815	0.816			0.868	0.522
Eosinophils (in mm3)	n	54	70	16	86	124	37	49	10	59	86	13	20	5	25	33
	Mean (±SD)	485.4 (±319.0)	487.5 (±329.2)	509.3 (±271.3)	491.5 (±317.8)	486.6 (±323.5)	545.4 (±331.6)	515.1 (±305.0)	569.4 (±298.1)	524.3 (±301.9)	528.2 (±315.1)	284.2 (±212.1)	434.1 (±386.5)	402.0 (±227.0)	427.7 (±356.4)	375.0 (±333.3)
	P value	0.964			0.912	0.789	0.840			0.749	0.695	0.424			0.192	0.863

FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; logPC20, base 10 logarithm of provocative methacholine concentration causing a drop in FEV1 of 20%; FeNO, fraction of nitric oxide in exhaled air; IgE, immunoglobulin class E; ICS, Inhaled corticosteroid; LTRA, leukotriene receptor antagonist. [1]Dominant model, [2]Recessive model; ANOVA followed by Newman–Keuls multiple comparison test or t-test for two independent samples. Significant associations (P <0.05) are shown in bold.

Table 3: Genetic association between rs35761398 (*CNR2*) and clinical parameters from before treatment in asthmatic children.

		Asthma					**Allergic asthma**					**Non-allergic asthma**				
		RR	QR	QQ	QR+RR[1]	QR+QQ[2]	RR	QR	QQ	QR+RR[1]	QR+QQ[2]	RR	QR	QQ	QR+RR[1]	QR+QQ[2]
FEV1/FVC (%)	n	116	168	39	284	207	77	113	24	190	138	31	51	14	82	65
	Mean (±SD)	88.38 (±8.51)	88.95 (±7.34)	89.64 (±6.47)	88.71 (±7.83)	89.08 (±7.18)	88.58 (±8.28)	89.16 (±7.23)	90.58 (±6.43)	88.93 (±7.66)	89.38 (±7.07)	86.77 (±8.72)	88.22 (±7.34)	88.29 (±6.68)	87.67 (±7.87)	88.23 (±7.15)
	P value	0.648			0.481	0.434	0.525			0.311	0.456	0.690			0.784	0.388
FEV1 (%)	n	115	167	39	282	206	76	112	24	188	**136**	31	51	14	82	65
	Mean (±SD)	89.38 (±14.23)	91.90 (±13.29)	94.56 (±13.34)	90.88 (±13.71)	92.41 (±13.31)	89.79 (±15.33)	93.45 (±12.29)	95.29 (±14.73)	91.97 (±13.68)	**93.77 (±12.72)**	89.45 (±12.29)	89.43 (±14.62)	93.79 (±11.46)	89.44 (±13.70)	90.37 (±14.03)
	P value	0.091			0.115	0.058	0.110			0.268	**0.044**	0.540			0.265	0.756
logPC20	n	113	165	37	278	202	75	113	22	188	135	31	49	14	80	63
	Mean (±SD)	-0.41 (±0.65)	-0.42 (±0.65)	-0.31 (±0.54)	-0.42 (±0.65)	-0.40 (±0.63)	-0.47 (±0.64)	-0.52 (±0.67)	-0.37 (±0.58)	-0.50 (±0.66)	-0.49 (±0.65)	-0.17 (±0.66)	-0.18 (±0.56)	-0.24 (±0.49)	-0.17 (±0.60)	-0.19 (±0.54)
	P value	0.612			0.334	0.964	0.608			0.378	0.819	0.931			0.715	0.837
FeNO (ppb)	n	91	141	32	232	173	56	95	22	151	117	26	44	9	70	53
	Mean (±SD)	36.81 (±26.75)	40.17 (±31.79)	48.28 (±31.61)	38.85 (±29.90)	41.67 (±31.92)	42.36 (±25.87)	48.07 (±32.31)	55.68 (±33.62)	45.95 (±30.12)	49.50 (±32.55)	28.92 (±25.91)	22.73 (±20.39)	29.78 (±19.19)	25.03 (±22.61)	23.92 (±20.19)
	P value	0.181			0.098	0.215	0.207			0.165	0.152	0.446			0.549	0.350
Total IgE (IU/ml)	n	54	69	16	123	85	37	49	10	86	59	13	19	5	32	24
	Mean (±SD)	524.9 (±625.0)	541.6 (±615.1)	617.7 (±964.5)	534.3 (±617.0)	555.9 (±688.0)	622.3 (±706.2)	672.8 (±669.7)	881.7 (±1156.0)	651.1 (±682.0)	708.2 (±764.7)	226.2 (±287.5)	196.5 (±244.3)	176.6 (±135.1)	208.6 (±258.6)	192.4 (±223.5)
	P value	0.887			0.637	0.788	0.621			0.354	0.583	0.915			0.790	0.694
Eosinophils (in mm3)	n	51	71	13	122	84	36	50	8	86	58	11	20	4	31	24
	Mean (±SD)	516.2 (±310.8)	500.1 (±329.6)	540.5 (±374.4)	506.8 (±320.6)	506.3 (±334.8)	558.5 (±330.4)	514.5 (±303.8)	635.6 (±430.8)	532.9 (±314.1)	535.2 (±322.3)	353.0 (±208.4)	440.1 (±381.5)	374.5 (±252.4)	409.2 (±329.3)	429.1 (±359.4)
	P value	0.906			0.723	0.865	0.576			0.394	0.694	0.762			0.841	0.520

FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; logPC20, base 10 logarithm of provocative methacholine concentration causing a drop in FEV1 of 20%; FeNO, fraction of nitric oxide in exhaled air; IgE, immunoglobulin class E; ICS, Inhaled corticosteroid; LTRA, leukotriene receptor antagonist. [1]Dominant model, [2]Recessive model; ANOVA followed by Newman–Keuls multiple comparison test or t-test for two independent samples. Significant associations (P <0.05) are shown in bold.

Table 4: Genetic association between rs2229579 (*CNR2*) and clinical parameters from before treatment in asthmatic children.

		Asthma					**Allergic asthma**					**Non-allergic asthma**				
		CC	CT	TT	CT+TT[1]	CT+CC[2]	CC	CT	TT	CT+TT[1]	CT+CC[2]	CC	CT	TT	CT+TT[1]	CT+CC[2]
FEV1/FVC (%)	n	272	62	0	62	334	178	42	0	42	220	83	17	0	17	100
	Mean	88.63	89.82	-	89.82	88.85	88.86	90.26	-	90.26	89.13	87.87	88.18	-	88.18	87.92
	(±SD)	(±7.98)	(±6.07)	-	(±6.07)	(±7.66)	(±7.94)	(±5.31)	-	(±5.31)	(±7.52)	(±7.74)	(±7.54)	-	(±7.54)	(±7.67)
	P value	0.269			0.269	-	0.278			0.278	-	0.881			0.881	-
FEV1 (%)	n	271	61	0	61	332	177	41	0	41	**218**	83	17	0	17	100
	Mean	90.91	91.18	-	91.18	90.96	92.05	91.63	-	91.63	**91.97**	89.18	92.29	-	92.29	89.71
	(±SD)	(±14.68)	(±11.82)	-	(±11.82)	(±14.18)	(±15.12)	(±11.61)	-	(±11.61)	**(±14.50)**	(±14.14)	(±10.56)	-	(±10.56)	(±13.60)
	P value	0.892			0.892	-	0.871			0.871	**-**	0.393			0.393	-
logPC20	n	**262**	**59**	0	**59**	321	**169**	**41**	0	**41**	210	82	16	0	16	98
	Mean	**-0.36**	**-0.55**	-	**-0.55**	-0.40	**-0.43**	**-0.66**	-	**-0.66**	-0.47	-0.15	-0.28	-	-0.28	-0.17
	(±SD)	**(±0.63)**	**(±0.67)**	-	**(±0.67)**	(±0.64)	**(±0.63)**	**(±0.66)**	-	**(±0.66)**	(±0.64)	(±0.57)	(±0.65)	-	(±0.65)	(±0.53)
	P value	**0.034**			**0.034**	-	**0.040**			**0.040**	-	0.405			0.405	-
FeNO (ppb)	n	220	51	0	51	271	**141**	**34**	0	**34**	175	69	14	0	14	83
	Mean	38.20	47.06	-	47.06	39.86	**44.94**	**57.62**	-	**57.62**	47.40	24.87	23.93	-	23.93	24.71
	(±SD)	(±29.42)	(±33.35)	-	(±33.35)	(±30.34)	**(±29.39)**	**(±34.32)**	-	**(±34.32)**	(±30.72)	(±22.90)	(±17.50)	-	(±17.50)	(±21.99)
	P value	0.060			0.060	-	**0.030**			**0.030**	-	0.885			0.885	-
Total IgE (IU/ml)	n	**124**	**21**	0	**21**	145	**82**	**17**	0	**17**	99	36	3	0	3	39
	Mean	**439.8**	**806.8**	-	**806.8**	492.9	**542.5**	**926.0**	-	**926.0**	608.3	192.3	339.0	-	339.0	203.6
	(±SD)	**(±445.5)**	**(±867.6)**	-	**(±867.6)**	(±539.3)	**(±487.3)**	**(±909.6)**	-	**(±909.6)**	(±593.7)	(±213.2)	(±514.4)	-	(±514.4)	(±239.5)
	P value	**0.004**			**0.004**	-	**0.015**			**0.015**	-	0.315			0.315	-
Eosinophils (in mm3)	n	**121**	**21**	0	**21**	142	82	16	0	16	98	**33**	**4**	0	**4**	37
	Mean	**468.9**	**659.7**	-	**659.7**	497.1	520.7	601.8	-	601.8	533.9	**318.7**	**945.3**	-	**945.3**	386.4
	(±SD)	**(±319.6)**	**(±309.9)**	-	**(±309.9)**	(±324.3)	(±329.8)	(±273.2)	-	(±273.2)	(±321.3)	**(±243.8)**	**(±353.7)**	-	**(±353.7)**	(±319.6)
	P value	**0.012**			**0.012**	-	0.358			0.358	-	**¡0.0001**			**¡0.0001**	-

FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; logPC20, base 10 logarithm of provocative methacholine concentration causing a drop in FEV1 of 20%; FeNO, fraction of nitric oxide in exhaled air; IgE, immunoglobulin class E; ICS, Inhaled corticosteroid; LTRA, leukotriene receptor antagonist. [1]Dominant model, [2]Recessive model; ANOVA followed by Newman–Keuls multiple comparison test or t-test for two independent samples. Significant associations (P <0.05) are shown in bold.

Table 5: Genetic association between rs13197090 (*CNR1*) and clinical parameters from before treatment in asthmatic children.

		Asthma					**Allergic asthma**					**Non-allergic asthma**				
		TT	CT	CC	CT+CC[1]	CT+TT[2]	TT	CT	CC	CT+CC[1]	CT+TT[2]	TT	CT	CC	CT+CC[1]	CT+TT[2]
FEV1/FVC (%)	n	292	28	2	30	320	193	20	2	22	213	87	6	0	6	93
	Mean	89.24	87.46	88.5	87.53	89.08	89.65	87.70	88.5	87.77	89.47	87.76	89.83	-	89.83	87.89
	(±SD)	(±7.44)	(±7.14)	(±0.71)	(±6.90)	(±7.42)	(±7.26)	(±6.05)	(±0.71)	(±5.76)	(±7.16)	(±7.49)	(±9.60)	-	(±9.60)	(±7.60)
	P value	0.478			0.230	0.912	0.499			0.242	0.849	0.521			0.521	-
FEV1 (%)	n	290	28	2	30	318	191	20	2	22	**211**	87	6	0	6	93
	Mean	91.52	91.07	93.5	91.23	91.48	92.43	92.70	93.5	92.77	**92.46**	90.60	87.00	-	87.00	90.37
	(±SD)	(±13.83)	(±14.43)	(±17.68)	(±14.32)	(±13.86)	(±14.12)	(±16.54)	(±17.68)	(±16.20)	**(±14.33)**	(±13.14)	(±6.87)	-	(±6.87)	(±12.83)
	P value	0.966			0.914	0.838	0.992			0.917	**0.919**	0.510			0.510	-
logPC20	n	**281**	**27**	2	**29**	308	**186**	**19**	2	**21**	205	85	6	0	6	91
	Mean	**-0.41**	**-0.45**	-0.88	**-0.48**	-0.42	**-0.49**	**-0.52**	-0.88	**-0.55**	-0.49	-0.22	0.02	-	0.02	-0.20
	(±SD)	**(±0.63)**	**(±0.67)**	(±0.25)	**(±0.66)**	(±0.64)	**(±0.65)**	**(±0.64)**	(±0.25)	**(±0.62)**	(±0.65)	(±0.58)	(±0.55)	-	(±0.55)	(±0.58)
	P value	**0.576**			**0.602**	0.311	**0.698**			**0.690**	0.405	0.331			0.331	-
FeNO (ppb)	n	235	22	1	23	257	**153**	**14**	1	**15**	167	71	6	0	6	77
	Mean	40.84	34.95	15.00	34.09	40.33	**48.54**	**39.86**	15.00	**57.62**	47.40	25.99	13.17	-	13.17	24.99
	(±SD)	(±30.39)	(±30.45)	(±0.00)	(±30.03)	(±30.38)	**(±31.30)**	**(±24.29)**	(±0.00)	**(±24.27)**	(±30.80)	(±22.06)	(±11.25)	-	(±11.25)	(±21.64)
	P value	-			0.310	-	**-**			**0.216**	-	0.165			0.165	-
Total IgE (IU/ml)	n	**122**	**13**	0	**13**	135	**86**	**9**	0	**9**	95	30	3	0	3	33
	Mean	**577.8**	**423.0**	-	**423.0**	562.9	**699.7**	**592.2**	-	**592.2**	689.5	236.3	31.0	-	31.0	217.6
	(±SD)	**(±680.2)**	**(±529.3)**	-	**(±529.3)**	(±667.1)	**(±758.2)**	**(±561.5)**	-	**(±561.5)**	(±740.1)	(±259.2)	(±6.6)	-	(±6.6)	(±253.9)
	P value	**0.428**			**0.428**	-	**0.681**			**0.681**	-	0.186			0.186	-
Eosinophils (in mm3)	n	**119**	**13**	0	**13**	132	83	9	0	9	92	**30**	**3**	0	**3**	33
	Mean	**524.3**	**394.3**	-	**394.3**	511.5	542.6	513.9	-	513.9	539.8	**452.9**	**99.7**	-	**99.7**	420.8
	(±SD)	**(±328.7)**	**(±272.2)**	-	**(±272.2)**	(±325.0)	(±334.7)	(±238.2)	-	(±238.2)	(±325.5)	**(±319.2)**	**(±67.9)**	-	**(±67.9)**	(±321.4)
	P value	**0.172**			**0.172**	-	0.803			0.803	-	**0.069**			**0.069**	-

FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; logPC20, base 10 logarithm of provocative methacholine concentration causing a drop in FEV1 of 20%; FeNO, fraction of nitric oxide in exhaled air; IgE, immunoglobulin class E; ICS, Inhaled corticosteroid; LTRA, leukotriene receptor antagonist. [1]Dominant model, [2]Recessive model; ANOVA followed by Newman–Keuls multiple comparison test or t-test for two independent samples. Significant associations (P <0.05) are shown in bold.

Table 6: Genetic association between rs4237 (*CNR2*) and clinical parameters from after treatment in asthma patients.

Phenotype	Genotype	ICS treatment								LTRA treatment							
		ΔFEV1/FVC				ΔFEV1				ΔFEV1/FVC				ΔFEV1			
		n	Mean (± SD)		*P value*	n	Mean (± SD)		*P value*	n	Mean (± SD)		*P value*	n	Mean (± SD)		*P value*
Asthma	TT	72	3.28	(±7.41)		71	8.28	(±13.57)		35	1.14	(±4.80)		35	-0.94	(±7.41)	
	CT	96	2.95	(±7.27)	0.605	96	6.82	(±10.56)	0.424	64	0.44	(±4.77)	0.328	63	1.32	(±11.30)	0.544
	CC	24	1.58	(±6.03)		24	4.79	(±9.43)		15	2.47	(±5.19)		15	0.07	(±7.49)	
	CT+CC[1]	120	2.68	(±7.04)	0.574	120	6.42	(±10.34)	0.286	79	0.82	(±4.88)	0.746	78	1.08	(±10.64)	0.312
	CT+TT[2]	168	3.09	(±7.31)	0.337	167	7.44	(±11.92)	0.298	99	0.69	(±4.77)	0.185	98	0.51	(±10.10)	0.871
Allergic asthma	TT	48	4.44	(±7.78)		47	10.64	(±11.69)		26	1.00	(±4.55)		26	-0.73	(±7.77)	
	CT	70	3.11	(±7.93)	0.250	70	6.80	(±10.76)	0.070	36	-0.47	(±5.02)	0.243	35	0.31	(±10.29)	0.744
	CC	15	0.73	(±5.33)		15	4.07	(±9.73)		10	2.00	(±3.16)		10	-2.00	(±4.74)	
	CT+CC[1]	85	2.69	(±7.57)	0.209	**85**	**6.32**	**(±10.58)**	**0.032**	46	0.07	(±4.76)	0.419	45	-0.20	(±9.34)	0.807
	CT+TT[2]	118	3.65	(±7.86)	0.165	117	8.34	(±11.26)	0.163	62	0.15	(±4.85)	0.247	61	-0.13	(±9.24)	0.535
Non-allergic asthma	TT	20	0.15	(±5.86)		20	6.40	(±13.96)		8	0.50	(±5.10)		8	-1.25	(±7.05)	
	CT	24	2.79	(±5.28)	0.321	24	7.75	(±9.98)	0.901	27	1.67	(±4.30)	0.606	27	2.26	(±12.65)	0.669
	CC	8	1.88	(±6.75)		8	6.00	(±10.00)		5	3.40	(±8.39)		5	4.20	(±10.66)	
	CT+CC[1]	32	2.56	(±5.58)	0.143	32	7.31	(±9.85)	0.783	32	1.94	(±5.00)	0.473	32	2.56	(±12.22)	0.405
	CT+TT[2]	44	1.59	(±5.65)	0.899	44	7.14	(±11.82)	0.800	35	1.40	(±4.44)	0.408	35	1.46	(±11.61)	0.621

FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; ICS, Inhaled corticosteroid; LTRA, leukotriene receptor antagonist. [1]Dominant model, [2]Recessive model; ANOVA followed by Newman–Keuls multiple comparison test or t-test for two independent samples. Significant associations (P <0.05) are shown in bold.

Table 7: Genetic association between rs35761398 (*CNR2*) and clinical parameters from after treatment in asthma patients.

Phenotype	Genotype	ICS treatment								LTRA treatment							
		ΔFEV1/FVC				ΔFEV1				ΔFEV1/FVC				ΔFEV1			
		n	Mean (± SD)		*P value*	n	Mean (± SD)		*P value*	n	Mean (± SD)		*P value*	n	Mean (± SD)		*P value*
Asthma	QQ	22	0.82	(±5.78)		22	5.32	(±10.84)		15	1.93	(±5.54)		15	0.80	(±8.24)	
	QR	97	3.01	(±7.25)	0.157	97	7.50	(±11.23)	0.670	62	0.53	(±4.76)	0.562	61	1.53	(±11.34)	0.301
	RR	69	4.15	(±7.30)		68	7.96	(±13.52)		37	1.19	(±4.75)		37	-1.62	(±7.03)	
	QR+RR[1]	166	3.48	(±7.27)	0.101	165	7.69	(±12.19)	0.388	99	0.78	(±4.74)	0.392	98	0.34	(±10.01)	0.865
	QR+QQ[2]	119	2.61	(±7.03)	0.155	119	7.09	(±11.15)	0.638	77	0.81	(±4.92)	0.694	76	1.38	(±10.75)	0.125
Allergic asthma	QQ	**14**	**-0.07**	**(±4.71)**		14	4.43	(±11.75)		9	1.89	(±3.33)		9	-0.44	(±7.00)	
	QR	72	3.28	(±7.80)	**0.049**	72	6.81	(±10.45)	0.062	38	-0.29	(±4.94)	0.326	37	0.22	(±10.02)	0.765
	RR	**47**	**5.36**	**(±7.46)**		46	11.02	(±11.87)		26	1.08	(±4.60)		26	-1.31	(±7.25)	
	QR+RR[1]	**119**	**4.10**	**(±7.70)**	**0.050**	**118**	**8.45**	**(±11.17)**	**0.208**	64	0.27	(±4.82)	0.333	63	-0.41	(±8.95)	0.992
	QR+QQ[2]	86	2.73	(±7.47)	0.054	**86**	**6.42**	**(±10.64)**	**0.025**	47	0.13	(±4.73)	0.410	46	0.09	(±9.44)	0.517
Non-allergic asthma	QQ	7	1.00	(±6.78)		7	7.00	(±10.36)		6	2.00	(±8.25)		6	2.67	(±10.25)	
	QR	23	2.70	(±5.41)	0.557	23	9.13	(±11.84)	0.417	23	1.91	(±4.32)	0.780	23	3.22	(±13.27)	0.466
	RR	18	0.78	(±6.20)		18	4.11	(±12.57)		10	0.60	(±4.72)		10	-2.20	(±7.12)	
	QR+RR[1]	41	1.85	(±5.78)	0.726	41	6.93	(±12.27)	0.988	33	1.52	(±4.41)	0.832	33	1.58	(±11.91)	0.835
	QR+QQ[2]	30	2.30	(±5.68)	0.390	30	8.63	(±11.38)	0.206	29	1.93	(±5.18)	0.478	29	3.10	(±12.54)	0.215

FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; ICS, Inhaled corticosteroid; LTRA, leukotriene receptor antagonist. [1]Dominant model, [2]Recessive model; ANOVA followed by Newman–Keuls multiple comparison test or t-test for two independent samples. Significant associations (P <0.05) are shown in bold.

Table 8: Genetic association between rs2229579 (*CNR2*) and clinical parameters from after treatment in asthma patients.

Phenotype	Genotype	ICS treatment								LTRA treatment							
		ΔFEV1/FVC				ΔFEV1				ΔFEV1/FVC				ΔFEV1			
		n	Mean (± SD)		*P value*	n	Mean (± SD)		*P value*	n	Mean (± SD)		*P value*	n	Mean (± SD)		*P value*
Asthma	CC	161	3.29	(±7.19)		162	7.56	(±12.36)		**89**	**1.60**	**(±4.58)**		90	0.76	(±10.47)	
	CT	33	1.79	(±7.08)	0.274	33	6.46	(±10.43)	0.631	**25**	**-0.68**	**(±4.37)**	**0.029**	24	-0.79	(±6.23)	0.491
	TT	0	-	-		0	-	-		0	-	-		0	-	-	
	CT+TT[1]	33	1.79	(±7.08)	0.274	33	6.46	(±10.43)	0.631	**25**	**-0.68**	**(±4.37)**	**0.029**	24	-0.79	(±6.23)	0.491
	CT+CC[2]	194	3.04	(±7.18)	-	195	7.37	(±12.03)	-	114	1.10	(±4.61)	-	138	0.22	(±9.21)	-
Allergic asthma	CC	**109**	**4.19**	**(±7.72)**		110	8.41	(±11.62)		57	1.16	(±4.42)		58	0.07	(±9.32)	
	CT	**26**	**0.85**	**(±6.73)**	**0.044**	26	5.92	(±10.79)	0.322	15	-1.13	(±3.52)	0.068	14	-2.43	(±5.10)	0.338
	TT	**0**	**-**	**-**		0	-	-		0	-	-		0	-	-	
	CT+TT[1]	**26**	**0.85**	**(±6.73)**	**0.044**	26	5.92	(±10.79)	0.322	15	-1.13	(±3.52)	0.068	14	-2.43	(±5.10)	0.338
	CT+CC[2]	135	3.55	(±7.63)	-	136	7.93	(±11.47)	-	72	0.68	(±4.33)	-	72	-0.42	(±8.69)	-
Non-allergic asthma	CC	46	1.24	(±5.47)		46	6.52	(±11.91)		30	2.20	(±4.75)		30	1.90	(±12.60)	
	CT	5	5.40	(±8.17)	0.130	5	12.60	(±5.08)	0.267	10	0.00	(±5.56)	0.232	10	1.50	(±7.18)	0.925
	TT	0	-	-		0	-	-		0	-	-		0	-	-	
	CT+TT[1]	5	5.40	(±8.17)	0.130	5	12.60	(±5.08)	0.267	10	0.00	(±5.56)	0.232	10	1.50	(±7.18)	0.925
	CT+CC[2]	51	1.65	(±5.81)	-	51	7.12	(±11.54)	-	40	1.65	(±4.99)	-	40	1.80	(±11.40)	-

FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; ICS, Inhaled corticosteroid; LTRA, leukotriene receptor antagonist. [1]Dominant model, [2]Recessive model; ANOVA followed by Newman–Keuls multiple comparison test or t-test for two independent samples. Significant associations (P <0.05) are shown in bold.

Table 9: Genetic association between rs13197090 (*CNR1*) and clinical parameters from after treatment in asthma patients.

Phenotype	Genotype	ICS treatment								LTRA treatment							
		ΔFEV1/FVC				ΔFEV1				ΔFEV1/FVC				ΔFEV1			
		n	Mean (± SD)		*P value*	n	Mean (± SD)		*P value*	n	Mean (± SD)		*P value*	n	Mean (± SD)		*P value*
Asthma	TT	169	2.66	(±7.43)		168	6.96	(±12.10)		103	0.98	(±4.64)		102	0.62	(±9.88)	
	CT	15	4.07	(±5.68)	0.405	15	4.80	(±9.63)	0.713	10	0.60	(±7.12)	0.917	10	0.00	(±8.96)	0.666
	CC	1	7.00	(±0.00)		1	21.00	(±0.00)		1	3.00	(±0.00)		1	-8.00	(±0.00)	
	CT+CC[1]	16	3.09	(±5.53)	0.405	16	5.81	(±10.15)	0.713	11	0.82	(±6.79)	0.917	11	-0.73	(±8.83)	0.666
	CT+TT[2]	184	2.68	(±7.30)	-	183	6.79	(±11.91)	-	113	0.95	(±4.87)	-	112	0.56	(±9.76)	-
Allergic asthma	TT	120	2.94	(±7.87)		119	7.56	(±11.59)		65	0.60	(±4.49)		64	-0.06	(±8.69)	
	CT	11	5.27	(±5.99)	0.291	11	6.82	(±8.77)	0.898	7	-1.14	(±6.52)	0.488	7	-2.57	(±9.18)	0.331
	CC	1	7.00	(±0.00)		1	21.00	(±0.00)		1	3.00	(±0.00)		1	-8.00	(±0.00)	
	CT+CC[1]	12	5.42	(±5.73)	0.291	12	8.00	(±9.31)	0.898	8	-0.63	(±6.21)	0.488	8	-3.25	(±8.71)	0.331
	CT+TT[2]	131	3.14	(±7.74)	-	130	7.49	(±11.36)	-	72	0.43	(±4.70)	-	71	-0.31	(±8.70)	-
Non-allergic asthma	TT	42	1.67	(±6.14)		42	6.62	(±11.01)		37	1.41	(±4.74)		37	1.92	(±11.76)	
	CT	3	1.00	(±4.00)	0.855	3	-2.00	(±13.08)	0.201	2	7.00	(±9.90)	0.128	2	3.50	(±4.95)	0.853
	CC	0	-	-		0	-	-		0	-	-		0	-	-	
	CT+CC[1]	3	1.00	(±4.00)	0.855	3	-2.00	(±13.08)	0.201	2	7.00	(±9.90)	0.128	2	3.50	(±4.95)	0.853
	CT+TT[2]	45	1.62	(±5.99)	-	45	6.04	(±11.20)	-	39	1.69	(±5.04)	-	39	2.00	(±11.48)	-

FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; ICS, Inhaled corticosteroid; LTRA, leukotriene receptor antagonist. [1]Dominant model, [2]Recessive model; ANOVA followed by Newman–Keuls multiple comparison test or t-test for two independent samples. Significant associations (P <0.05) are shown in bold.

Table 10: eQTL analysis of rs4237 with *CNR2* gene expression from naive asthma patients.

Phenotype	rs4237	*CNR2* mRNA expression			
		n	Median	(± IQR)	*P* value
Asthma	TT	63	1.67	(±1.55)	
	CT	105	1.45	(±1.30)	0.108
	CC	22	1.16	(±0.75)	
	CT+CC[1]	127	1.40	(±1.24)	0.092
	CT+TT[2]	168	1.53	(±1.38)	0.098
Allergic asthma	TT	38	1.72	(±1.40)	
	CT	65	1.25	(±1.01)	0.011
	CC	15	1.13	(±0.71)	
	CT+CC[1]	**80**	**1.21**	**(±0.95)**	**0.004**
	CT+TT[2]	103	1.52	(±1.26)	0.116
Non-allergic asthma	TT	18	1.23	(±1.43)	
	CT	38	1.57	(±1.23)	0.594
	CC	7	1.33	(±0.91)	
	CT+CC[1]	45	1.56	(±1.22)	0.453
	CT+TT[2]	56	1.54	(±1.37)	0.633
Control	TT	80	0.99	(±0.82)	
	CT	84	1.01	(±0.76)	0.855
	CC	45	1.06	(±0.61)	
	CT+CC[1]	129	1.03	(±0.69)	0.971
	CT+TT[2]	164	1.01	(±0.79)	0.602

mRNA expression levels expressed as x fold relative to control. [1]Compared with TT genotype (dominant model), [2]Compared with CC genotype (recessive model), ANOVA followed by Newman–Keuls multiple comparison test or t-test for two independent samples. Significant associations (P <0.05) are shown in bold.

Table 11: eQTL analysis of rs35761398 with *CNR2* gene expression from naive asthma patients.

Phenotype	rs35761398	*CNR2* mRNA expression			
		n	Median	(± IQR)	*P* value
Asthma	RR	65	1.61	(±1.58)	
	QR	108	1.43	(±1.42)	0.866
	QQ	24	1.60	(±1.19)	
	QR+QQ[1]	173	1.48	(±1.47)	0.711
	QR+RR[2]	132	1.45	(±1.45)	0.786
Allergic asthma	RR	39	1.62	(±1.36)	
	QR	71	1.25	(±1.11)	0.332
	QQ	15	1.57	(±1.21)	
	QR+QQ[1]	110	1.44	(±1.34)	0.803
	QR+RR[2]	86	1.31	(±1.21)	0.177
Non-allergic asthma	RR	19	1.06	(±1.29)	
	QR	34	1.58	(±1.38)	0.540
	QQ	9	1.62	(±1.56)	
	QR+QQ[1]	53	1.45	(±1.45)	0.265
	QR+RR[2]	43	1.60	(±1.35)	0.756
Control	RR	94	0.98	(±0.98)	
	QR	86	0.92	(±0.92)	0.562
	QQ	35	1.09	(±0.61)	
	QR+QQ[1]	180	0.97	(±0.75)	0.296
	QR+RR[2]	121	1.01	(±1.01)	0.845

mRNA expression levels expressed as x fold relative to control. [1]Compared with QQ genotype (dominant model), [2]Compared with RR genotype (recessive model), ANOVA followed by Newman–Keuls multiple comparison test or t-test for two independent samples. Significant associations (P <0.05) are shown in bold.

Table 12: eQTL analysis of rs2229579 with *CNR2* gene expression from naive asthma patients.

Phenotype	rs2229579	*CNR2* mRNA expression			
		n	Median	(± IQR)	*P* value
Asthma	CC	164	1.52	(±1.46)	
	CT	38	1.55	(±1.26)	0.534
	TT	0	-	-	
	CT+TT[1]	38	1.55	(±0.67)	0.534
	CT+CC[2]	202	1.52	(±0.64)	-
Allergic asthma	CC	102	1.44	(±1.28)	
	CT	24	1.37	(±1.37)	0.674
	TT	0	-	-	
	CT+TT[1]	24	1.37	(±1.37)	0.674
	CT+CC[2]	126	1.44	(±1.29)	-
Non-allergic asthma	CC	53	1.56	(±1.48)	
	CT	12	1.43	(±1.14)	0.539
	TT	0	-	-	
	CT+TT[1]	12	1.43	(±1.14)	0.539
	CT+CC[2]	65	1.53	(±1.37)	-
Control	CC	177	0.97	(±0.80)	
	CT	37	1.09	(±0.57)	0.212
	TT	0	-	-	
	CT+TT[1]	37	1.09	(±0.57)	0.212
	CT+CC[2]	214	1.01	(±0.73)	-

mRNA expression levels expressed as x fold relative to control. [1]Compared with CC genotype (dominant model), [2]Compared with TT genotype (recessive model), ANOVA followed by Newman–Keuls multiple comparison test or t-test for two independent samples. Significant associations (P <0.05) are shown in bold.

Table 13: eQTL analysis of rs13197090 with *CNR2* gene expression from naive asthma patients.

Phenotype	rs13197090	*CNR2* mRNA expression			
		n	Median	(± IQR)	*P* value
Asthma	TT	177	1.42	(±7.10)	
	CT	18	1.43	(±1.62)	0.123
	CC	2	22.785	(±35.37)	
	CT+CC[1]	20	1.43	(±3.02)	0.731
	CT+TT[2]	195	1.43	(±5.41)	0.067
Allergic asthma	TT	110	1.31	(±7.10)	
	CT	13	1.43	(±1.96)	0.173
	CC	2	22.785	(±35.37)	
	CT+CC[1]	15	1.49	(±3.11)	0.870
	CT+TT[2]	123	1.32	(±5.02)	0.070
Non-allergic asthma	TT	59	1.92	(±7.40)	
	CT	4	1.03	(±25.64)	0.616
	CC	0	-	-	
	CT+CC[1]	4	1.03	(±25.64)	0.616
	CT+TT[2]	62	1.91	(±7.62)	-
Control	TT	**202**	**0.82**	**(±0.82)**	
	CT	**25**	**1.27**	**(±3.32)**	**0.009**
	CC	0	-	-	
	CT+CC[1]	**25**	**1.27**	**(±3.32)**	**0.009**
	CT+TT[2]	227	0.86	(±0.89)	-

mRNA expression levels expressed as x fold relative to control. [1]Compared with TT genotype (dominant model), [2]Compared with CC genotype (recessive model), ANOVA followed by Newman–Keuls multiple comparison test or t-test for two independent samples. Significant associations (P <0.05) are shown in bold.

Table 14: Correlation of NAPE-PLD (*NAPEPLD*), ABHD4 (*ABHD4*) and FAAH (*FAAH*) gene expression with clinical data before anti-asthmatic treatment.

	Clinical parameter	*NAPEPLD*			*ABHD4*			*FAAH*		
		n	r_s	*P* value	n	r_s	*P* value	n	r_s	*P* value
Asthma	FEV1/FVC	201	-0.125	0.078	213	-0.120	0.081	201	-0.117	0.097
	FEV1 (%)	206	0.098	0.163	**219**	**0.175**	**0.010**	207	-0.014	0.841
	logPC20	203	-0.069	0.325	214	-130	0.057	203	-0.127	0.070
	FeNO (ppb)	200	-0.019	0.790	213	0.089	0.196	201	0.053	0.458
	Total IgE	43	-0.041	0.795	43	0.240	0.121	**43**	**0.355**	**0.020**
	Eosinophils	41	0.011	0.947	41	0.175	0.273	**41**	**0.327**	**0.037**
Allergic asthma	FEV1/FVC	125	-0.103	0.253	131	-0.148	0.091	**124**	**-0.177**	**0.049**
	FEV1 (%)	130	0.096	0.277	137	0.141	0.101	130	-0.008	0.932
	logPC20	129	-0.049	0.583	136	-0.105	0.223	129	-0.085	0.337
	FeNO (ppb)	128	-0.125	0.159	135	-0.033	0.703	128	-0.048	0.589
	Total IgE	25	0.071	0.737	25	0.002	0.994	25	0.125	0.552
	Eosinophils	24	0.181	0.396	24	0.026	0.903	**24**	**0.471**	**0.020**
Non-allergic asthma	FEV1/FVC	65	-0.063	0.619	71	-0.115	0.340	66	-0.106	0.398
	FEV1 (%)	65	0.131	0.299	71	0.228	0.056	66	0.051	0.682
	logPC20	65	-0.082	0.519	71	-0.157	0.191	66	-0.115	0.357
	FeNO (ppb)	61	0.176	0.175	**67**	**0.321**	**0.008**	**62**	**0.263**	**0.039**
	Total IgE	14	0.253	0.380	14	0.481	0.084	**14**	**0.631**	**0.018**
	Eosinophils	13	0.173	0.569	13	0.505	0.081	13	0.231	0.448

FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; logPC20, base 10 logarithm of provocative methacholine concentration causing a drop in FEV1 of 20%; FeNO, fractional exhaled nitric oxide (ppb); Total IgE (IU/mL); Eosinophils (in mm_3). r_s, Spearman's correlation coefficient, Significant associations (P <0.05) are shown in bold.

Table 15: Correlation of DAGLα (*DAGLA*) and MAGL (*MGLL*) gene expression with clinical data before anti-asthmatic treatment.

	Clinical parameter	*DAGLA*			*MGLL*		
		n	r_s	*P* value	n	r_s	*P* value
Asthma	FEV1/FVC	177	-0.103	0.171	214	-0.090	0.191
	FEV1	177	0.057	0.454	**214**	**0.151**	**0.027**
	logPC20	174	-0.121	0.111	211	-0.047	0.500
	FeNO	171	-0.002	0.978	208	0.069	0.322
	Total IgE	44	-0.084	0.587	44	0.023	0.880
	Eosinophils	42	-0.079	0.618	42	-0.095	0.548
Allergic asthma	FEV1/FVC	115	-0.090	0.341	134	-0.109	0.210
	FEV1	115	-0.028	0.766	134	0.116	0.182
	logPC20	114	-0.043	0.653	133	-0.110	0.209
	FeNO	113	-0.091	0.339	132	0.020	0.822
	Total IgE	26	-0.117	0.570	26	-0.243	0.231
	Eosinophils	25	0.023	0.914	25	-0.155	0.459
Non-allergic asthma	FEV1/FVC	52	-0.072	0.612	69	-0.085	0.490
	FEV1	52	0.140	0.324	**69**	**0.242**	**0.045**
	logPC20	52	-0.207	0.141	69	-0.003	0.981
	FeNO	48	0.018	0.906	**65**	**0.294**	**0.018**
	Total IgE	**14**	**0.724**	**0.005**	14	0.402	0.155
	Eosinophils	13	0.231	0.444	13	-0.060	0.849

FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; logPC20, base 10 logarithm of provocative methacholine concentration causing a drop in FEV1 of 20%; FeNO, fractional exhaled nitric oxide (ppb); Total IgE (IU/mL); Eosinophils (in mm_3). r_s, Spearman's correlation coefficient, Significant associations (P <0.05) are shown in bold.

Table 16: Correlation of anandamide (AEA), palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) plasma levels with clinical data before anti-asthmatic treatment.

Phenotype	Clinical parameter	AEA			PEA			OEA		
		n	r_s	P value	n	r_s	P value	n	r_s	P value
Asthma	FEV1/FVC	31	0.026	0.891	35	-0.040	0.823	34	0.020	0.912
	FEV1	31	0.034	0.855	35	0.197	0.258	34	-0.012	0.945
	logPC20	31	-0.092	0.623	35	0.129	0.459	34	0.142	0.423
	FeNO	31	0.047	0.803	**35**	**-0.343**	**0.044**	**34**	**-0.340**	**0.049**
	Total IgE	5	-0.300	0.683	5	-0.300	0.683	5	0.000	0.999
	Eosinophils	5	-0.800	0.133	5	-0.800	0.133	5	-0.400	0.750
Allergic asthma	FEV1/FVC	22	0.115	0.612	24	0.020	0.927	24	0.041	0.849
	FEV1	22	0.096	0.672	24	0.282	0.182	24	0.177	0.407
	logPC20	22	-0.063	0.779	24	-0.034	0.875	24	0.000	0.998
	FeNO	22	0.101	0.656	24	-0.289	0.170	24	-0.190	0.375
	Total IgE	4	-0.400	0.750	4	-0.400	0.750	4	0.600	0.417
	Eosinophils	4	-0.800	0.333	4	-0.800	0.333	4	-0.800	0.333
Non-allergic asthma	FEV1/FVC	8	-0.359	0.381	10	0.134	0.713	10	0.140	0.700
	FEV1	8	-0.180	0.672	10	0.304	0.391	10	0.243	0.496
	logPC20	8	-0.333	0.428	**10**	**0.649**	**0.049**	10	0.527	0.123
	FeNO	8	0.214	0.619	10	-0.576	0.088	10	-0.503	0.144
	Total IgE	1	n/a	n/a	1	n/a	n/a	1	n/a	n/a
	Eosinophils	1	n/a	n/a	1	n/a	n/a	1	n/a	n/a

FEV1, forced expiratory volume, 1 s; FVC, forced vital capacity; logPC20, base 10 logarithm of provocative methacholine concentration causing a drop in FEV1 of 20%; FeNO, fractional exhaled nitric oxide (ppb); Total IgE (IU/mL); Eosinophils (in mm_3); r_s, Spearman's correlation coefficient, significant correlations (P <0.05) are shown in bold.

Table 17: Correlation of anandamide (AEA), palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) plasma levels with gene expression in naive patients.

	Gene	AEA			PEA			OEA		
		n	rs	*P* value	n	rs	*P* value	n	rs	*P* value
Asthma	*IL4*	31	0.027	0.884	35	0.061	0.730	35	0.057	0.746
	IL5	31	0.064	0.732	35	0.058	0.742	35	0.136	0.436
	IL13	30	0.165	0.384	34	0.147	0.407	34	0.003	0.988
	CNR1	30	0.114	0.549	34	0.308	0.077	33	0.270	0.129
	CNR2	31	-0.134	0.472	35	0.173	0.320	34	0.109	0.540
	NAPEPLD	30	0.006	0.975	34	0.009	0.960	33	0.143	0.427
	ABHD4	31	-0.032	0.863	35	0.230	0.183	34	0.202	0.253
	FAAH	30	0.276	0.140	34	0.165	0.352	33	0.116	0.520
	DAGLA	26	-0.154	0.452	29	0.050	0.798	28	0.041	0.838
	MGLL	31	0.154	0.408	35	0.200	0.250	34	0.068	0.703
Allergic asthma	*IL4*	22	-0.127	0.573	24	-0.170	0.428	24	-0.236	0.268
	IL5	22	0.002	0.994	24	-0.167	0.436	24	-0.110	0.610
	IL13	21	0.110	0.636	23	0.171	0.435	23	0.033	0.883
	CNR1	21	0.133	0.567	23	0.134	0.542	23	0.200	0.36
	CNR2	22	-0.278	0.210	24	0.073	0.736	24	0.033	0.878
	NAPEPLD	22	-0.254	0.255	24	-0.206	0.334	24	-0.014	0.949
	ABHD4	22	0.037	0.871	24	0.061	0.778	24	0.142	0.509
	FAAH	22	0.285	0.199	24	0.022	0.918	24	0.026	0.904
	DAGLA	20	-0.186	0.431	21	0.016	0.947	21	0.008	0.973

Table 17 continued from previous page

	Gene	AEA			PEA			OEA		
		n	r_s	P value	n	r_s	P value	n	r_s	P value
	MGLL	22	0.228	0.307	24	0.127	0.556	24	0.148	0.489
Non-allergic asthma	*IL4*	7	-0.321	0.498	9	0.617	0.086	9	0.617	0.086
	IL5	8	-0.262	0.536	10	0.588	0.081	10	0.588	0.081
	IL13	8	0.500	0.216	10	0.103	0.785	10	-0.115	0.759
	CNR1	8	-0.310	0.462	10	0.224	0.537	10	0.273	0.448
	CNR2	8	0.310	0.462	10	0.006	0.999	10	0.030	0.946
	NAPEPLD	7	0.750	0.066	9	0.117	0.776	9	0.233	0.552
	ABHD4	8	-0.667	0.083	10	0.079	0.838	10	0.200	0.584
	FAAH	7	-0.107	0.840	9	0.250	0.521	9	0.200	0.613
	DAGLA	5	0.000	0.999	7	0.250	0.595	7	0.429	0.354
	MGLL	8	-0.381	0.360	10	-0.467	0.179	10	-0.491	0.155

r_s, Spearman's correlation coefficient, significant correlations (P <0.05) are shown in bold.

Curriculum Vitae

Curriculum Vitae

Name: Carina Esteves Pinto Kozmus **Nationality:** Portuguese

E-mail address: carina.kozmus@gmail.com **Date of birth:** 27.08.1980

ACADEMIC DEGREES

2010 – Present **PhD in Biomedical Technology**
University of Maribor, Slovenia
Thesis tittle: "The endocannabinoid system in asthma patients and the effect of cannabinoids in the modulation of inflammatory response"

2001 – 2002 **MSc Biomedical basis of disease**
Sheffield Hallam University, United Kingdom
Thesis tittle: "Effect of Progesterone on the Expression of *EGR-1* and *Reprimo* in HEC-1-B and U87MG Cell Lines"

1998 – 2001 **BSc (Hons) Biology** – 2.2 grade
University of Glamorgan, United Kingdom

WORK EXPERIENCE

2007 – 2010 **Research Fellowship**
Institute of Pharmacology and Therapeutic, Faculty of Medicine, University of Porto, Portugal
Main researcher in two projects: 1) the *in vivo* effect of two prebiotics in the hepatic redox state; and 2) the *in vivo* effect of tetrahydrocannabinol in the hepatic redox state. Collaboration in the study of the relationship between the inhibitory mechanism over adrenaline release from the adrenal medulla mediated by alpha2C-adrenoceptors and the development of heart failure.
Also involved in the study of the cannabinoid receptors' role in melanin production in a cellular model of human skin.

2006 – 2007 **Research Assistant** (Voluntary)
Biological Chemistry Research Group, Department of Chemistry, University of Minho, Portugal

2005 – 2006 **Advanced Level Biomedical Support Worker**
Biochemistry Department, Bristol Royal Infirmary, United Kingdom

2003 – 2005 **Medical Laboratory Assistant**
Biochemistry Department, Bristol Royal Infirmary, United Kingdom

2002 – 2003 **Research Assistant**
Biomedical Research Centre, Sheffield Hallam University, United Kingdom

2000 **Technical Officer** (Trainee)
Medical Genetics Institute Jacinto Magalhães, Porto, Portugal

GRANTS AND AWARDS

2016 Student Travel Award – International Cannabinoid Research Society

2011 – 2019 PhD Scholarship – Fundação para a Ciência e a Tecnologia (Portugal), scholarship SFRH/BD/79804/2011.

2012 Student Travel Award - International Cannabinoid Research Society

ADITIONAL EDUCATION

Sep/2008 **"Real-time PCR and analysis of mRNA and miRNA expression data"**. Molecular Genomics 2008 Advanced course series. University of Aveiro, Portugal

Jun/2008 **"Enzymatic activities determination. Metabolism – Clinic and Trial".** University of Porto, Portugal

May/2008 **"Real Time PCR: New Tools for Gene Expression".** Bio-Rad, Portugal

Feb/2008 **"Animal cell and tissue cultures: From Basic Principles to Advanced Techniques"** International Postgraduate Programme. University of Minho, Portugal

May/2007 **Workshop "Exploring molecular approaches to cancer research".** University of Minho, Portugal

Set/2005 **PADI SCUBA Diver.** Master Diver with the following specialities: Dry suit, Night diver, Deep diver, Underwater Navigation and Turtle diver.

Jun/2000 **First Certificate in English.** Cambridge University, United Kingdom

PROFESSIONAL MEMBERSHIPS

The American Society of Cell Biology – Student Member
International Cannabinoid Research Society – Predoctoral Member
British Pharmacological Society – Undergraduate Member

PUBLICATIONS

- **Papers in international scientific periodicals:**

1. Berce V, Kozmus CE, Potočnik U. *CTLA4 expression in childhood asthma and the effect of treatment with inhaled corticosteroid and leukotriene receptor antagonist*. Annual Research & Review in Biology, 2016. 10(2):1-13.

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11. Pinto CE, Moura E, Serrao MP, Martins MJ, Vieira-Coelho MA. *Effect of (-)-Delta(9)-etrahydrocannabinol on the hepatic redox state of mice.* Brazilian Journal of Medical and Biological Research, 2010. 43(4):325-9.

12. Vieira-Coelho MA, Serrao MP, Afonso J, Pinto CE, Moura E. *Catecholamine synthesis and metabolism in the central nervous system of mice lacking alpha(2)-adrenoceptor subtypes. British Journal of Pharmacology, 2009. 158(3):726-37.*

- **Abstracts in international scientific periodicals:**

1. Kozmus CEP, Deželak M, Berce V, Potočnik U. A missense SNP in CNR2 gene is associated with childhood asthma severity and treatment outcome. European Journal of Human Genetics. 2015; 23(S1):145.

2. Berce V, Kozmus CEP, Potocnik U. Expression of ORMDL3 and CTLA4 genes in childhood asthma and their association with the antiasthmatic effect of inhaled corticosteroids. Allergy. 2013; 68:245.

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- **Abstracts in conference books:**

1. Kozmus CEP, Berce V, Potočnik U. Cannabinoid receptor 2 gene polymorphisms are associated with childhood asthma severity and treatment outcome. Abstract book from the *Genetika 2015: 7th Congress of the Genetic Society of Slovenia and 7th Meeting of the Slovenian Society of human Genetics*, Rogaška Slatina, Slovenia, September 20-23, 2015, pp. 42.

2. Kozmus CEP, Berce V, Potočnik U. Cannabinoid receptor 2 gene polymorphisms are associated with asthma onset, disease severity and treatment outcome. Abstract book from the *Cannabinoid Conference 2015: 7th European Workshop on Cannabinoid Research and IACM 8th Conference on Cannabinoids in Medicine*, Sestri Levante, Italy, September 17-19, 2015, pp.111.

3. Kozmus CEP, Deželak M, Berce V, Potočnik U. A missense SNP in CNR2 gene is associated with childhood asthma severity and treatment outcome. Abstract book from the *European Human Genetics Conference 2015*, Glasgow, Scotland, United Kingdom, June 6-9, 2015, PS07.01, pp. 145.

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5. Berce V, Kozmus CEP, Potocnik U. Expression of ORMDL3 and CTLA4 genes in childhood asthma and their association with the antiasthmatic effect of inhaled corticosteroids. Abstract book from the *European Academy of Allergy and Clinical Immunology and World Allergy Organization World Allergy and Asthma Congress*, Milan, Italy, June 22-26, 2013, pp. 245.

6. Potočnik U, Kozmus CEP, Berce V. Expression of CTLA4 isoforms is altered in patients with childhood asthma and is changing during treatment with inhaled corticosteroids and

leukotriene receptor antagonist. Abstract book from the *European Human Genetics Conference 2013*, Paris, France, June 8-11, 2013, P16.006, pp. 380.

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8. Kozmus CEP, Berce V, Potočnik U. CB1 and CB2 gene expression in atopic and non-atopic asthma - expression levels before and after treatment with inhaled corticosteroids or leukotriene receptor or leukotriene receptor antagonist. Abstract book from the *6th European Workshop on Cannabinoid Research*, Dublin, Ireland, April 18-20, 2013, P022, pp. 50.

9. Berce V, Homšak M, Tomazin M, Kozmus CEP, Potočnik U. Genetics and pharmacogenomics of asthma and the influence of the ORMDL3 gene on the effect of inhaled corticosteroids. Abstract book from the *Bolezni in simptomi otrok kot odraz slabega družinskega ravnotežja. Genetika v pediatriji. Zastrupitve pri otrocih: zbornik predavanj*. Maribor, Slovenia, 2013, pp. 67.

10. Berce V, Pinto-Kozmus CE, Potocnik U. Pharmacogenetics of inhaled corticosteroids in childhood asthma - ORMDL3 and CTLA4 as candidate genes. Abstract book from the *BSACI 2012 Annual Meeting - New Frontiers in Allergy*, Nottingham, UK, July 2-4, 2012, P12, pp. 56.

11. Kozmus CEP, Potocnik U. Stable reference genes for real-time qPCR for normalization of target genes in pharmacogenomics studies in asthma patients. Abstract book from the *International symposium at 40th anniversary of Institute of Biochemistry and 20th anniversary of Medical Centre for Molecular Biology, University of Ljubljana*, Ljubljana, Slovenia, June 27-29, 2012, P33, pp. 89.

12. Kozmus CEP, Potocnik U. Evaluation of gene expression normalization strategy for real-time qPCR in leukocytes from asthmatic patients before and after treatment. Abstract book from the *European Human Genetics Conference 2012*, Nurnberg, Germany, June 23-26, 2012, P09.020, pp. 222.

13. Kozmus CEP, Potocnik U. Selection of most stable reference genes for real-time qPCR to normalise gene expression in leukocytes from asthmatic patients before and after anti-asthma treatment. Abstract book from the *3rd Congress of Croatian Geneticists*, Krk, Island of Krk, Croatia, May 13-16, 2012, P25, pp. 88.

14. Kozmus CEP, Berce V, Potočnik U. Gene expression of proteins that compose the endocannabinoid system is up-regulated in asthma patients. Abstract book from the *Genetika 2012: 6th Congress of the Genetic Society of Slovenia and 6th Meeting of the Slovenian Society of human Genetics*, Maribor, Slovenia, September 26-29, 2012, PS1-26, pp. 132.

15. Savić T, Kozmus CEP, Potočnik U. Optimization and accuracy of the high resolution melting analysis for genotyping of selected DNA polymorphisms associated with chronic immune diseases. Abstract book from the *Genetika 2012: 6th Congress of the Genetic Society of Slovenia and 6th Meeting of the Slovenian Society of human Genetics*, Maribor, Slovenia, September 26-29, 2012, PS4-6, pp. 162.

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PRESENTATIONS

- **Oral presentations:**

1. Kozmus CEP, Berce V, Potočnik U. Cannabinoid receptor 2 gene polymorphisms are associated with childhood asthma severity and treatment outcome. *Genetika 2015: 7th Congress of the Genetic Society of Slovenia and 7th Meeting of the Slovenian Society of human Genetics*, Rogaška Slatina, Slovenia, September 20-23, 2015.

2. Kozmus CEP, Berce V, Potočnik U. Polymorphism on chromosome 1p36 is associated with atopic childhood asthma onset, severity, treatment outcome with inhaled corticosteroids, and with the expression of the cannabinoid receptor 2 (CNR2) gene. *4th Colloquium of Genetics*, Piran, Slovenia September 19, 2014.

3. Pinto CE, Martins MJ, Moura E, Serrão MP, Vieira-Coelho MA. The Effect of (-)-delta9-tetrahydrocannabinol on hepatic redox state. *XXXIX Reunião Anual da Sociedade Portuguesa de Farmacologia, XXVI Reunião de Farmacologia Clínica, VIII Reunião de Toxicologia,* Lisbon, Portugal, 3-5 December, 2008

- **Poster presentations:**

1. Kozmus CEP, Berce V, Potočnik U. Cannabinoid receptor 2 gene polymorphisms are associated with asthma onset, disease severity and treatment outcome. *Cannabinoid Conference 2015: 7th European Workshop on Cannabinoid Research and IACM 8th Conference on Cannabinoids in Medicine*, Sestri Levante, Italy, September 17-19, 2015.

2. Kozmus CEP, Deželak M, Berce V, Potočnik U. A missense SNP in CNR2 gene is associated with childhood asthma severity and treatment outcome. *European Human Genetics Conference 2015*, Glasgow, Scotland, United Kingdom, June 6-9, 2015.

3. Kozmus CEP, Berce V, Potočnik U. A 1p36 polymorphism in the cannabinoid receptor 2 gene region is associated with CNR2 gene expression, atopic childhood asthma onset, severity, and treatment outcome with inhaled corticosteroids. *European Human Genetics Conference 2013*, Paris, France, June 8-11, 2013.

4. Kozmus CEP, Berce V, Potočnik U. CB1 and CB2 gene expression in atopic and non-atopic asthma - expression levels before and after treatment with inhaled corticosteroids or leukotriene receptor or leukotriene receptor antagonist. *6th European Workshop on Cannabinoid Research*, Dublin, Ireland, April 18-20, 2013.

5. Kozmus CEP, Potocnik U. Stable reference genes for real-time qPCR for normalization of target genes in pharmacogenomics studies in asthma patients. *International symposium at 40th anniversary of Institute of Biochemistry and 20th anniversary of Medical Centre for Molecular Biology, University of Ljubljana*, Ljubljana, Slovenia, June 27-29, 2012.

6. Kozmus CEP, Potocnik U. Evaluation of gene expression normalization strategy for real-time qPCR in leukocytes from asthmatic patients before and after treatment. *European Human Genetics Conference 2012*, Nurnberg, Germany, June 23-26, 2012.

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8. Kozmus CEP, Berce V, Potočnik U. Gene expression of proteins that compose the endocannabinoid system is up-regulated in asthma patients. *Genetika 2012: 6th Congress of the Genetic Society of Slovenia and 6th Meeting of the Slovenian Society of human Genetics*, Maribor, Slovenia, September 26-29, 2012.

9. Savić T, Kozmus CEP, Potočnik U. Optimization and accuracy of the high resolution melting analysis for genotyping of selected DNA polymorphisms associated with chronic immune diseases. Abstract book from the *Genetika 2012: 6th Congress of the Genetic Society of Slovenia and 6th Meeting of the Slovenian Society of human Genetics*, Maribor, Slovenia, September 26-29, 2012, PS4-6, pp. 162.

10. Berce V, Kozmus CEP, Perin P, Potočnik U. Pharmacogenetics of inhaled corticosteroids in childhood asthma - ORMDL3 and CTLA4 as candidate genes. Abstract book from the *Genetika 2012: 6th Congress of the Genetic Society of Slovenia and 6th Meeting of the Slovenian Society of human Genetics*, Maribor, Slovenia, September 26-29, 2012, pp. 40.

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ANNEX 4: STATEMENT OF AUTHORSHIP AND IDENTITY BETWEEN PRINTED AND ELECTRONIC FORM OF THE DOCTORAL DISSERTATION

UNIVERSITY OF MARIBOR
FACULTY OF MEDICINE

STATEMENT OF AUTHORSHIP AND IDENTITY BETWEEN PRINTED AND ELECTRONIC FORM OF THE DOCTORAL DISSERTATION

Name and surname of the student: Carina Esteves Pinto Kozmus

Study programme: Biomedical Technology

Title of the doctoral dissertation: The endocannabinoid system in asthma patients and the effect of cannabinoids in the modulation of the inflammatory response.

Supervisor: Prof. Uroš Potočnik

Co-supervisor: Prof. Maria Augusta Vieira Coelho and Dr. Vojko Berce

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