

Mechanisms of Atopic Dermatitis and Allergy Development in African (AmaXhosa) Children between 12-36 Months of Age

Nonhlanhla Lunjani

LNJNON001

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DEDICATION

This PhD is dedicated to the loving memory of my first teacher-my mom, May Lunjani.

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ABBREVIATIONS

AAD

American Academy of Dermatology

AD

Atopic dermatitis

ADHD

Attention deficit hypersensitivity disorder

AE

Atopic eczema

AEDS

Atopic eczema/dermatitis syndrome

agr

Accessory gene regulator

AhR

Aryl hydrocarbon receptor

AHRR

Aryl hydrocarbon receptor repressor

AMP

Antimicrobial peptide

AMT

Autologous microbiome transplant

AR

Allergic rhinitis

ASD

Autistic spectrum disorder

ATP

Adenosine triphosphate

BDNF

Brain-derived neurotrophic factor

bFGF

basic Fibroblast growth factor

CCD

Cross-reactive carbohydrate determinants

CE

Cornified envelope

CLA

Cutaneous lymphocyte antigen

CNS

Central nervous system

CNV

Copy number variation

CRP

C-reactive protein

DALYs

Disability- adjusted life years

DAMPs

Damage associated molecular patterns

DARC

Danish Allergy Research Centre

DC

Dendritic cell

DCQ

Digital cell quantification

DGE

Differential gene expression

DMSO

Dimethyl sulfoxide

DNA

Deoxyribonucleic acid

EAACI

European Academy of Allergy and Clinical Immunology

EASI

Eczema Area and Severity Index

ECM

Extracellular matrix

ELOVL

Elongation of very long chain fatty acids-like

EPIC

Estimating the Proportion of Immune and Cancer cells

ETFAD

European Task Force on Atopic Dermatitis

FA

Food allergy

FCS

Fetal calf serum

FFA

Free fatty acids

FLG

Filaggrin

FLT1

FMS-like tyrosine kinase

GBD

Global Burden of Disease

GC

Germinal centre

GM-CSF

Granulocyte-macrophage colony stimulating factor

GPCR

G-protein coupled receptor

GWAS

Genome-wide association studies

h β D

human beta defensins

HC

Healthy Control

HDAC

Histone deacetylase

HDC

Histidine decarboxylase

HDM

House dust mite

HOME

Harmonising Outcomes Measures of Eczema

HR

Histamine receptor

HSV

Herpes simplex virus

ICD

International classification of disease

IDEC

Inflammatory dendritic epidermal cell

IGA

Investigator Global Assessment

IgE

Immunoglobulin E

IL

Interleukin

ILC

Innate lymphoid cell

ISAAC

International Study of Asthma and Allergies in Childhood

JAK

Janus kinase

JAM

Junctional adhesion molecule

JDA

Japanese Dermatology Association

KLK

Kallikreins

LC

Langerhans cell

LILR

Leukocyte immunoglobulin like receptor

LoF

Loss-of-function

MAIT

Mucosal-associated invariant T cell

MAPK

Mitogen-activated protein kinase

MCP

Monocyte chemoattractant protein

MDC

Macrophage-derived chemokine

MIP

Macrophage inflammatory protein

MRGPRX2

Mas-Related G Protein-coupled Receptor Member X2

mRNA

messenger Ribonucleic acid

MUPP

Multi PZD-1 protein

Neu5GC

N-glycolneuraminic acid

NFκBNuclear factor kappa B

NGF

Nerve growth factor

NK

Natural killer cell

NOD

Nucleotide-binding oligomerization domains

NPV

Negative predictive value

OFC

Oral food challenge

oSCORAD

objective SCORing Atopic Dermatitis

OSMR

Oncostatin M receptor

OVOL1

Ovo like transcriptional repressor 1

PAR

Protease-activated receptor

PAS

Pern-Arnt-Sim

PBMC

Peripheral blood mononuclear cells

PBS

Phosphate buffered saline

PCA

Pyrrolidone-5- carboxylic acid

pDC

plasmacytoid Dendritic cell

PI3K

Phosphoinositide 3 kinase

PIGF

Placental growth factor

PPV

Positive predictive value

PRR

Pattern recognition receptor

PSM

Phenol soluble modulins

QoL

Quality of life

RANTES

Regulated on activation, normal T cell expressed and secreted

RNA

Ribonucleic acid

SAA

Serum amyloid A

SASSAD

Six area six sign atopic dermatitis

SC

Stratum corneum

SCORAD

SCORing Atopic Dermatitis index

SHS

Second hand smoke

sICAM

soluble Inter-cellular adhesion molecule

SNP

Single nucleotide polymorphism

SPT

Skin prick test

STAT

Signal transducer and activator of transcription

sVCAM

soluble Vascular cell adhesion molecule

TARC

Thymus and activation regulated chemokine

TEWL

Trans epidermal water loss

TJ

Tight junction

TLR

Toll-like receptor

TNF

Tumour necrosis factor

TPM

Transcript per million

TRAP

Traffic-related air pollutants

TRPA1

Transient receptor potential ankyrin 1

TRPV1

Transient receptor potential vallinoid 1

TSLP

Thymic stromal lymphopoietin

UCA

Urocanic acid

UMAP

Uniform manifold approximation and projection

UV

Ultraviolet

VAS

Visual Analogue Scale

VEGF

Vascular endothelial growth factor

VOC

Volatile organic compound

WAO

World Allergy Organization

ZO

Zonula occludens

ABSTRACT

Atopic dermatitis (AD) is a highly complex multifaceted inflammatory cutaneous disease. The specific pathogenic mechanisms of AD cannot be easily distilled as it is a polygenic disorder with environmental factors also contributing to its aetiopathogenesis. Atopic dermatitis disproportionately affects children from an early age and is increasing rapidly in South Africa. Current data suggest that the changing diversity of environmental exposures, socioeconomic factors, dietary and lifestyle factors across African societies may provide unique disease-promoting or protective effects in populations where a low risk of developing AD and allergic disease was previously documented.

In order to identify novel immunological endotypes and responsible environmental exposures that may associate with AD in South African children, we collected data on environmental exposures, lifestyle, and socioeconomic factors and conducted clinical assessments in 217 children (Urban AD, n=56; Rural AD, n=60; Urban Healthy Controls, n=49; Rural Healthy Controls, n=52). Detailed immunological assessments (cell counts, cytokine levels, serology, and RNA-Seq) were performed on 152 of these children. The recruited participants were from the same ethnolinguistic AmaXhosa background.

Strong evidence for persistent T_H2 responses (elevated circulating eosinophils, allergen-specific IgE, TARC, MCP-4, and IL-16 levels) and differential T_H17 responses (reduced IL-17A and IL-23 levels) were seen in South African children with AD irrespective of whether they lived in urban or rural communities. However, independent of AD, a vast array of circulating immune mediators (including proinflammatory cytokines, chemokines, soluble adhesion molecules, and growth factors) were associated with early life dietary practices, medications, animal exposures, indoor environment, sunlight exposure, household size,

household income and, parental education levels. RNA sequencing of peripheral blood mononuclear cells (PBMCs) corroborated the T_H2 adaptive immune profile findings in this AD cohort. Furthermore, the expression of multiple immunological pathways (including immunoregulatory factors, aryl hydrocarbon receptor, and G protein-coupled receptors) differ in rural and urban children, regardless of their atopic status.

In conclusion, the study results described in this thesis fill in some gaps in our understanding of the interactions between atopic dermatitis and environmental exposures in South African children. Focusing my research on this previously understudied population has helped to clarify the relevant AD endotypes, as well as help to quantify the relative contribution of host factors or environment in immune development and immune-mediated diseases. Protective (e.g., outdoor and animal) exposures and potentially detrimental (e.g., viral infection and pollutant) exposures shape the early life innate and adaptive immune response. Lastly, when inflammatory disorders occur in children, we should consider the living environment and its effects on background immune profiles that can interact with immune-targeting therapies and impact treatment efficacy.

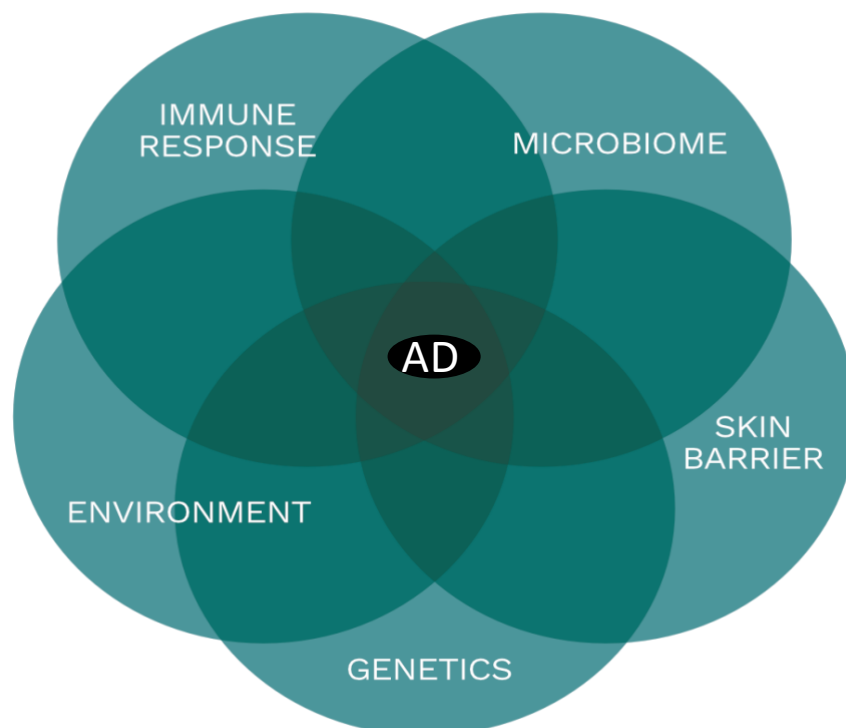
CHAPTER 1

INTRODUCTION

1.1 Definition of AD and its general mechanisms

Atopic dermatitis (AD); atopic eczema (AE) or eczema is a chronic relapsing inflammatory dermatosis.¹ The hallmark clinical feature of the disease is intense itch or pruritus. Notable AD lesions include xerosis, erythema, oedema, excoriations, oozing, crusting, and lichenification, with the characteristic distribution of lesions at predilection sites.² It has a broad range of clinical manifestations that vary with age and ethnicity.³ The development of the AD phenotype involves an intricate interplay between epidermal barrier defects, particularly filaggrin gene defects in a subgroup of patients with filaggrin null genotypes, immune dysregulation, and skin microbiome abnormality. The increasing global prevalence of AD and allergy support the role of environmental factors interacting with host immune responses, microbial composition, and epidermal barrier factors in the aetiopathogenesis of AD.^{4,5}

Figure 1.1. Complex interplay between environmental factors and host factors in AD



1.2 Prevalence of Atopic Dermatitis

This debilitating skin condition affects up to 20% of children and 3% of adults worldwide. 1-3% of the elderly population have AD in industrialized countries, with a 2:1 male preponderance.^{6,7} Globally, the prevalence of AD has been on the rise parallel to industrialization, with a 2-3-fold increase over the past few decades. The increasing trend continues in low-middle-income countries, with notable stabilization in high-income countries.¹ Onset of disease is commonly between 3-6 months of age. AD skin lesions emerge within the first twelve months of life in 60% of children. 90% of children with AD develop eczematous lesions by their fifth birthday. Approximately a third of childhood cases of AD have moderate to severe disease.⁸ 10-30% of patients experience disease persistence into adulthood, and a minority of patients develop new-onset AD as adults.⁹ The International Study of Asthma and Allergies in Childhood (ISAAC) utilized a uniformly validated methodology and produced the most robust global epidemiology data for children and adolescents. ISAAC recorded significant variations in AD prevalence worldwide, with the highest AD prevalence (15%) in both 6-7-year-olds and 13-14-year-olds reported for urban regions in Africa, the Baltics, Australasia, Northern, and Western Europe. They also described a female: male ratio of 1.3:1, consistent with findings elsewhere.¹⁰ There is a sharp decline in AD prevalence after adolescence, which is supported by the Global Burden of Disease (GBD) study, which suggests that adult AD varies between 1.2% and 6.4%.¹¹ The most recent GBD report on AD shows a bimodal curve with the highest peak in early childhood, decreasing in prevalence among young adults, and a second peak in middle-aged and older populations. They also found a moderate positive correlation between a country's gross domestic product and disease burden. In addition, variations in global AD prevalence data may vary in part due to differences in AD diagnostic criteria used in different study

centres. Growing evidence suggests an increased AD prevalence in urban compared to rural settings in developed "Western" countries and developing countries such as Iran and South Africa.^{12,13} Several studies reported an increased prevalence of AD in populations of African ancestry and a significantly higher risk for severe forms of AD compared to their European counterparts.^{5,14}

The increased prevalence of AD in developed or urbanized settings highlights the role of environmental exposures in altering disease risk. Traffic-related air pollutants (TRAP), volatile organic compounds (VOCs,) and second-hand smoke (SHS) exposure predispose children to increased development of atopic dermatitis and other atopic diseases.¹⁵ These pollutants induce oxidative stress, barrier disruption, and immune dysregulation by epigenetic mechanisms such as DNA methylation and microRNA regulations. Environmental oxidants such as air pollutants and solar radiation were shown to cause oxidative protein damage in the upper layers of the stratum corneum compared to the deeper layers. Overexpression of IL-4-producing T cell subsets were observed in children following VOC exposure. Exposure to tobacco smoke is associated with DNA methylation, specifically, hypomethylation of TSLP 5' CpG island resulting in elevated TSLP production and AD development.¹⁶ Several studies have associated an increased prevalence of AD symptoms in areas of increased water hardness, which may impact the epidermal barrier via interactions with detergents in washing products. In contrast, rural and in particular the farming environment has been associated with a lower risk of AD, potentially due to exposure to a more microbially diverse environment which has direct and indirect effects on the immune system and epigenetic regulation. The impact of these environmental exposures and others

on allergy development is complex and variable, likely reflecting differences in timing, dose of exposure, and host genetic and immune factors.

1.3 Societal burden of AD and healthcare costs

The negative impact of AD on the quality of life (QoL) of patients and their caregivers is profound and long-lasting. It imposes limitations on physical, emotional, and social functioning.¹¹ QoL surveys in adults have shown significantly reduced QoL amongst AD patients compared to healthy controls. Increased disease severity was also significantly associated with lower QoL scores. Especially, itch, sleep disturbance, and facial and genital AD lesions can predict poorer QoL outcomes.¹⁷ A more significant impact on QoL impairment has been reported in females.¹⁸ In a study comparing QoL of children with skin conditions and other common childhood chronic disorders with healthy controls, parents reported the greatest negative impact of AD compared to other cutaneous conditions such as urticaria, psoriasis, and alopecia. When comparisons to general chronic conditions were made, the negative impact of AD was second only to cerebral palsy.¹⁹ Another study reported that the negative impact on the family of a child with AD was more significant than that experienced by the family of a child with type I diabetes.¹⁶ Increased risk for non-atopic comorbidities such as Attention-deficit hyperactivity disorder (ADHD), behavioural disorders, autism, depression, anxiety, suicide, and suicidal ideation have been described in children and adolescents with AD.⁸ Moderate to severe AD carries an increased susceptibility to developing atopic comorbidities such as food allergy (FA), allergic rhinitis (AR) and asthma as well as an increased risk of psychological comorbidity.⁸ AD represents a

skin condition with the most significant worldwide cutaneous disease burden regarding disability-adjusted life years (DALYs).²¹

The healthcare costs of AD are associated with direct costs, such as more frequent visits to the doctor and emergency room, prescriptions and higher hospital costs than patients without eczema. AD sufferers have higher healthcare costs than patients without AD per person year.¹¹ In the US, a study conducted in 2004 based on public and private databases estimated the costs of AD treatment to be USD 1,009 billion, excluding the cost of over-the-counter medication.²² More recently, a 2015 report demonstrated substantially high out-of-pocket costs, increased healthcare utilization, and lost workdays for American adults with AD.²³ Indirect costs can be attributed to absenteeism and presenteeism. Absenteeism leads to productivity loss for patients with AD and their families, with an estimated loss of USD 619 million in 2004. A 2006 study reported the monetary value of lost productivity in the European Union to be 2 billion euros annually. Indirect costs of AD have been similarly reported in Japan and Korea.¹¹ More studies need to detail the societal impact and healthcare costs in Africa. Nevertheless, a recent report on the status of AD in Africa showed that AD treatment might cost up to 15% of total monthly income in certain countries.²⁴ Atopic and non-atopic comorbidities add to the costs incurred by patients with AD.⁴

1.4. Features of AD

1.4.1 Itch and its transmission pathway

Itch, or pruritus, forms the primary criterion of AD diagnosis and is described as an uncomfortable sensation that elicits a strong impulse to scratch.²⁵⁻²⁷ The intractable itch of AD remarkably interferes with the quality of life, including sleep disturbance.¹⁷ Complex networks between the disrupted epidermal barrier and neuroimmune system induce pruritogens. Pruritus is mediated by peripheral sensory afferent fibres, especially C-fibres, which innervate itch-sensing tissues. The pruritus signals from the peripheral sensory afferent fibres are transmitted through the spinal cord in the dorsal root ganglia or trigeminal ganglia to the somatosensory cortex, which leads to the perception of itch, which evokes the reflex to scratch, therefore initiating the vicious itch-scratch cycle.^{26,27} Pruritogen receptors promote the opening of the ion channels, such as transient receptor potential vallinoid 1 (TRPV1) and transient receptor potential ankyrin 1 (TRPA1). These receptors are triggered by diverse stimuli, including receptor ligands, extracellular pH, adenosine triphosphate (ATP), prostaglandins, oxidants, capsaicin (a well-known algescic substance), allyl thiocyanate, heat or cold-which contribute to AD pathogenesis.²⁶

Eczematous skin has increased elongation of sensory neurons (with pruritogen receptors) in the epidermis through upregulation of artemin and nerve growth factor (NGF), respectively, and downregulation of Semaphorin 3A, which inhibits keratinocyte innervation.^{26,27} Artemin also plays a role in the skin hypersensitivity reaction to warm sensations, which gives rise to warmth-induced itch in AD. The inflammation, epidermal barrier disruption, and increased pathogen and allergen entry elicit intense pruritis.

Additionally, AD skin has a reduced itch threshold which induces alloknosis and hyperknosis.²⁸ Alloknosis is an abnormal itch sensation in which itch is provoked by otherwise harmless mechanical stimuli such as skin contact with fabrics, whereas

hyperknesis is a stronger itch sensation elicited by pruritogens in AD compared to healthy skin. Notably, noxious stimuli elicit itch rather than pain in AD, unlike psoriasis-related pruritus, which associates with pain and burning sensation.²⁹ Numerous substances induce pruritus in AD skin. Histamine and the cognate histamine receptors, endogenous and exogenous proteases, cytokines -mainly IL-31, thymic stromal lymphopoietin (TSLP), Interleukin (IL)-4, IL-13 and IL-33, neuropeptides and lipids are some of these mediators. Th2 adaptive immune responses are particularly adept at driving the chronic itch of AD.^{25,26}

1.4.2 Phenotypic variation according to age

The disorder has a heterogeneous clinical presentation. Skin lesions are distributed in a typical age-related manner. Rarely will AD present with erythroderma. The disease course is characterized by relapsing eczematous flares. Early onset AD eczematous lesions are often more acute, poorly defined, erythematous, oedematous, vesicular, excoriated lesions, with exudate manifesting as oozing and crusting, emerging on the cheeks and scalp at age 3-6 months. Lesions may be widely distributed and extend to the trunk, with the nappy area usually unaffected.^{2,5}

Eczematous lesions become localized to flexures and extensor surfaces of the limbs beyond infancy. Compared to infancy, childhood lesions may have polymorphous features, with acute, subacute, and chronic lichenified lesions that are less erythematous. Overall, the skin is generally dry.

Adolescent and adult lesions are usually diffuse, predominantly lichenified, may be localized, and affect flexures, eyelids, and head and neck region. Adult AD may present with

pruriginous lesions or chronic hand eczema only.² Palmar-plantar hyperlinearity, Dennie-Morgan infraorbital folds, keratosis pilaris, perifollicular accentuation, orbital hyperpigmentation, and thinning of the lateral third of the eyebrows- known as Hertoghe's sign are also features associated with AD. These signs and numerous others are contained in the more exhaustive AD diagnostic criteria such as Hanifin and Rajka and may be helpful to confirm the diagnosis. Elderly AD (> 60 years) features correspond with adult AD; however, localized lichenification at predilection sites of the knees and elbows is absent, and the notable "reverse sign" of eczematous lichenification surrounding unaffected flexural skinfolds is a common manifestation.⁷

1.4.3 Morphological variations related to ethnicity.

Morphological variations in AD phenotype have been noted with increased frequency in individuals of Asian and African ancestry. AD patients of Asian ancestry may present with parakeratosis-a classical feature of psoriasis- which represents immature nucleated keratinocytes in the stratum corneum. It is an atypical morphological feature of AD and consistent with psoriasis. It has been observed that African individuals may have a pronounced treatment-resistant lichenified type and often present with severe refractory forms of AD. AD, which presents with perifollicular accentuations, is commonly described in darkly pigmented skin.^{3,5,30} Longstanding disease may present with prurigo AD type.⁴ These phenotypic variations have been shown to correspond with specific immune endotypes and filaggrin genotype status.

1.4.4 Atopy

AD is associated with elevated total and allergen-specific immunoglobulin (IgE) and is considered a foreshadowing of the atopic diathesis involving IgE-mediated food allergy, asthma, and allergic rhinitis.⁴ About 20-30% of infants follow the traditional atopic march trajectory. Additionally, AD susceptibility loci, particularly filaggrin gene mutations, predispose to the atopic march.³¹ Some researchers suggest heterogeneity in the chronology of developing symptoms. Early onset AD with later development of airway allergic disease may not represent the typical progression of the atopic march but belong to a distinct phenotype. Modeling longitudinal data from two UK birth cohort studies also suggests that approximately 7% of individuals follow the developmental profiles that resemble the "atopic march" by age 11. Notably, this group of children did not outgrow AD.^{5,32}

1.4.5 Heritability of disease

1.4.5.1 Genetic risk factors

AD represents a complex genetic trait. Parental history of allergic disease is frequently reported and is a well-documented risk factor for AD.³³ Family studies suggest a three to fivefold higher risk of AD development if one or both parents have had AD. Twin studies estimate the heritability of AD to be approximately 75 per cent, with distinctly higher concurrence reported in identical twins compared to non-identical twins.^{32,33} To date, linkage analysis and genome-wide association studies have described the most significant genetic risk factor for AD.³¹ Null mutations of the gene encoding filaggrin (FLG) have been

reported in up to 30% of patients with AD. Filaggrin (FLG) is an epidermal structural protein essential for keratinocyte differentiation, located in the epidermal differentiation complex (EDC) within chromosome 1q21.³²⁻³⁵ Additionally, genome-wide association studies (GWAS) analysis discovered more than 30 genetic risk loci. FLG, OVOL1 (ovo-like transcriptional repressor 1 -a transcriptional factor that regulates FLG expression) at 11q13.1 and IL-13 have the most significant association with AD among the genetic susceptibility loci identified. Notably, some susceptibility loci overlap with known asthma risk loci, highlighting shared 'atopy' risk.³¹ In addition, some AD risk loci overlap with immune-mediated diseases such as inflammatory bowel disease (IBD) and psoriasis. The novel risk loci identified were related to immune regulation.^{31,36} Other AD-associated polymorphisms occur in genes encoding elements of innate immune signaling, e.g., TLR-2 and TLR-4³⁷, as well as T-cell immunity. Variants of Th2 cytokine genes (IL-4; IL-5; IL-9; IL-13 including RAD50) found on chromosome 5q31-33 associating with AD have been described but need confirmation in large studies^{31,36,38-40} These observations underscore the decisive role played by innate and adaptive immunity in addition to barrier defects in the pathogenic mechanisms of AD.

1.4.5.2 Epigenetic risk factors

Epidemiology studies suggest environmental factors are associated with increased AD prevalence. Epigenetic modifications are the most likely mechanism by which the environment modifies gene expression.⁴¹ Epigenetic changes in DNA, such as methylation, histone modification, and regulation of transcription by non-coding RNAs such as microRNA, have been documented in AD. Epigenetic modifications can be inherited transgenerationally from both parents, and multiple environmental exposures in pregnancy and early infancy

may induce changes in the offspring's epigenome that may protect against or confer the risk of AD development.⁴¹⁻⁴³

Reduced expression of DNA methyltransferase 1 in AD with high serum IgE has been reported.⁴² DNA methyltransferase 1 is a key enzyme for maintaining DNA methylation therefore, alterations to this enzyme may lead to differential expression of AD-related genes. Hypomethylation of the promoter region of the gamma subunit of high-affinity IgE receptor on AD monocytes correlated with increased gene expression.⁴³ Overexpression of S100 proteins correlated with CpG hypermethylation of DNA within S100A5.⁴⁴

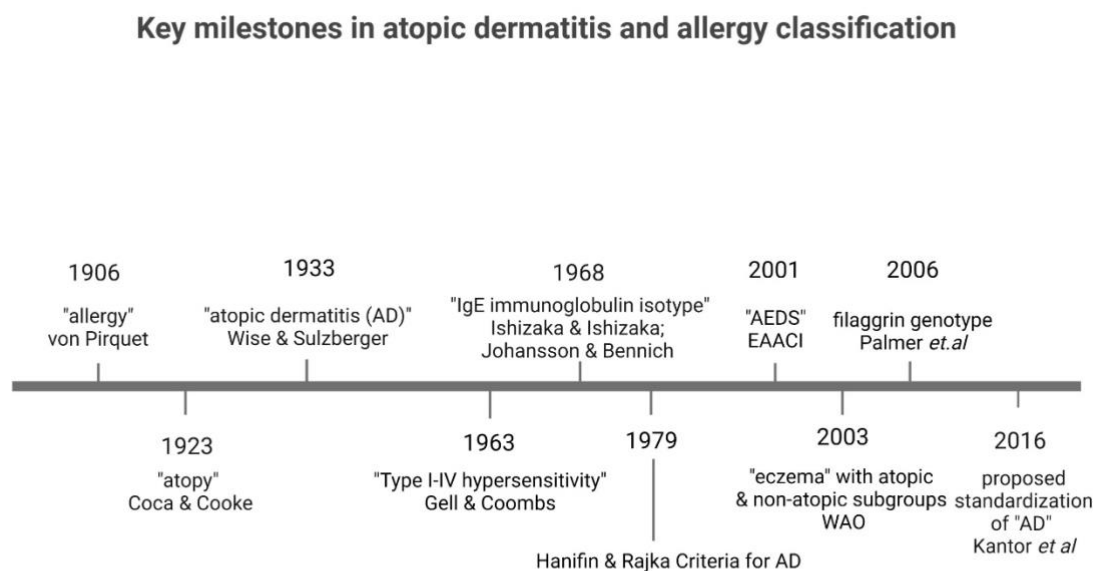
Demethylation of the TSLP gene promoter region contributes to increased expression of TSLP in AD lesions.⁴⁵ Use of butyrate (a *Staphylococcus epidermidis* metabolite known to inhibit histone deacetylase (HDAC) activity) in an AD animal model inhibited *Staphylococcus aureus* overgrowth, a critical pathogenic organism in AD. This intervention augurs well for AD, as glucocorticoids, the cornerstone of AD treatment, also work by similarly reducing HDAC activity.⁴⁶ Butyrate also represents a mechanism by which the microbiome may ameliorate AD. Non-coding molecules such as micro-RNAs regulate post-transcriptional gene expression. Altered expression of several micro-RNAs has been described in AD skin and sera. Increased expression of miR-155 in AD skin leads to increased proliferation of effector T cells. Let-7a-Let7d miRNA molecules, which inhibit IL-13, are reduced in AD and contribute to Th2 polarisation.⁴⁷ Genomic imprinting of the high-affinity IgE receptor has been identified in AD patients.⁴⁷ Exposure to polluted air and maternal cigarette smoking affect the epigenome to influence AD.

1.5 Nomenclature

The term "atopic dermatitis" was established by Sulzberger and Wise in 1933, 10 years after Coca and Cooke coined the term 'atopy' and has been established over time (Fig.1.2).⁴⁹

Following the establishment of the Hanifin and Rajka diagnostic criteria in 1979, 'atopic dermatitis' usage increased concomitantly with the increase in disease prevalence. Several additional terms have been used for AD, such as "childhood eczema," "infantile eczema," "flexural eczema," "atopic neurodermatitis," "atopiform dermatitis" and "Besnier's prurigo." However, only a few publications reflect their usage.⁵⁰ These numerous designations, in part, also reflect divergent opinions on AD pathogenesis.

Figure 1.2. Timeline of key advances in atopic dermatitis research and classifications.⁴⁹⁻⁵³



There has been a concerted attempt to harmonize AD terminology globally. In 2001 the European Academy of Allergy and Clinical Immunology (EAACI) recommended the use of the term “atopic eczema/dermatitis syndrome” (AEDS) to restrict the use of the term “atopy” to describe IgE antibody high-responders. In 2003 the World Allergy Organisation (WAO) proposed the use of “eczema” (with “atopic eczema” and “non-atopic eczema” as subgroups) to replace AEDS.⁵¹ Different terms have been suggested for the group of patients who exhibit the typical syndrome of AD but lack elevated total or specific IgE levels, for example, non-atopic eczema, non-IgE associated dermatitis, atopiform dermatitis, or intrinsic AD.

These proposals have been met with heavy criticism. Using the term eczema represented a step backwards in standardizing AD terminology.⁵² It has also been cautioned that the term "eczema" etymologically connotes an acute inflammatory process and may, therefore, inadequately capture the whole clinical presentation of AD.⁵³ The binary view of atopic dermatitis v non-atopic dermatitis, which hinged on the presence or absence of demonstrable IgE sensitization was considered flawed as IgE is not a prerequisite in AD initiation. The poor efficacy of IgE-inhibiting therapies in AD provides further evidence that IgE sensitization may be an epiphenomenon of the disease. Reliance on elevated serum IgE levels may underestimate disease prevalence.⁵³ Atopy may not be apparent in early infancy as (i) IgE sensitization is expected to develop during the disease course; (ii) the utility of total serum IgE cut-off values to accurately predict disease presence depends on the establishment of population-specific and region-specific reference values for diagnosis⁵⁴; (iii) the limited ability to standardize quantification of the plethora of possible specific-IgE allergens including sensitization to self-protein or microbial protein or as-yet to be identified allergens that are not part of the routine diagnostic work up.⁵³ Moreover, it was also argued

that the majority of the US population (54,3%) was sensitized to at least one of 10 common allergens; therefore, reservation of the term 'atopy' to those who have evidence of high IgE becomes irrelevant.⁵⁵

Also, it is evident that the lack of consensus on AD definition internationally translates into heterogeneous diagnostic criteria for AD. In regions such as China, the absence of atopy/IgE sensitization means the disease is termed "eczema" and, therefore, not included in disease estimates of "atopic dermatitis" regionally.⁵³ It is also fascinating to note that the discussion around AD nomenclature had centred to a great extent on IgE. IgE was discovered in 1968. However, the description of the disease long predates this period. In addition, scientific advances have been made in understanding AD pathogenesis post IgE discovery, and attempts have yet to be made to rename the disease according to emerging evidence. The growing view that AD is an umbrella term for a constellation of subtypes may be more appropriate and better reflects our improved molecular and phenotypic understanding of the disease.

A 2016 systematic review proposed the standardization of "atopic dermatitis" as the most accurate term for communicating in the scientific community, literature, and patient education.⁵⁰ This study looked at publications from 1945-2016 and determined that the term "atopic dermatitis" was frequently used, followed by "eczema" and then "atopic eczema" (AE). The use of "eczema" alone as synonymous with AD/AE was discouraged because it was non-specific and described the morphology of several dermatoses. Further, the use of eczema was predominantly in conjunction with prefixes that were descriptors of non-atopic eczematous disorders, such as 'contact eczema' and 'dyshidrotic eczema,' and these outnumbered the use of 'atopic eczema.' ICD-9 and ICD-10 coding for AD/AE is 691.8

and L20.x, respectively, whereas the coding for eczema is 692.9 and L30.9, respectively.⁵⁰

Therefore, the use of AD or AE compared to eczema has a material impact on accurate disease estimates, patient reimbursements, access to treatment, and patient AD literacy.

1.6 Diagnostic Criteria of atopic dermatitis

The diagnosis of AD is clinical. The earliest formal classification of AD is the comprehensive Hanifin and Rajka diagnostic criteria instituted in 1979. While it may be considered the gold standard, its refinement by The UK Working Party into concise, less cumbersome criteria was necessary.⁵⁶ Both have been well-validated in hospital and population-based studies. In addition, several expert panels have devised diagnostic criteria such as the Kang and Tian criteria, the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire, Danish Allergy Research Centre (DARC), Schultz-Larsen, and Diepgen criteria for various rationales, including addressing the unwieldy nature of the Hanifin and Rajka diagnostic criteria, especially for epidemiology studies.⁵⁷ Other criteria developed by the Japanese Dermatology Association (JDA) criteria and the American Academy of Dermatology (AAD) were specifically developed for greater relevance for AD in the Asian population and childhood AD, respectively.^{9,50,53,57}

Nevertheless, with an improved understanding of disease pathogenesis, precise biomarkers may aid the definition of AD and its diagnosis. Modifying diagnostic criteria to consider ethnicity and regional variations is desirable to improve sensitivity.

1.7 Disease severity and clinical outcomes grading of atopic dermatitis.

There is no established gold standard for assessing AD severity. There are several clinical criteria for estimating the severity of AD for baseline assessment in clinical practice, standardized inclusion criteria in research, and evaluating response to therapeutic interventions in clinical trials.⁵⁸ To date, there are over 60 AD severity criteria, 16 of which have undergone the rigors of validation in at least one study.^{58,59} The Scoring Atopic Dermatitis (SCORAD) index, Visual Analogue Scale (VAS)-pruritis, Investigator Global Assessment (IGA), Eczema Area and Severity Index (EASI) are the most routinely used grading systems in randomized controlled trials. ⁵⁸ Of these, SCORAD is the most commonly used and has been validated thus far in 26 published studies.^{58,59}

Clinical criteria limitations have been reported in several studies. Interobserver variability in assessing lesional extent has been noted with the SCORAD index.⁶⁰ Moderate Interobserver reliability has been noted with the EASI ⁶¹, and despite IGA's common usage and putative role as a proxy gold standard for evaluating other criteria, it has not been formally validated.⁵⁹

Several defining characteristics of severity constitute these criteria. The main parameters include symptoms, clinical signs, the extent of body surface area involved, and AD lesional intensity. However, as many as 19 objective and patient-reported parameters were evaluated by the Harmonising Outcomes Measures of Eczema (HOME) initiative, which sought to identify essential parameters of AD severity for clinical record keeping and clinical trials.⁶² They are weighted and measured differently in the various criteria due to divergent views on their contribution to AD severity. Some studies show no correlation between

patient and physician-assessed parameters of AD severity.⁵⁹ Additionally, the heterogeneity of criteria used in clinical trials precludes comparisons across studies and evidence-based decision-making.

All these factors highlight inadequacies within current clinical criteria that have a material impact on accurate and reproducible AD severity measurements especially given the growing research output and treatment options for AD.

1.8 Pathophysiological mechanisms of AD

The disease mechanisms of AD are not entirely known. However, several studies have shown that a combination of epidermal barrier dysfunction, immune dysregulation, microbiome imbalances, and environmental factors interact to give rise to AD in individuals who may or may not be genetically susceptible. The varied contribution of each of these systems may account for the heterogenous clinical phenotypes and relapsing nature of the disease among patients with AD.⁶³

1.8.1 Epidermal barrier defects in AD

The epidermis is 15- to 30 nm thick. It provides a physical, chemical, microbiome, and immune layer comprised of proteins and lipids with various immune cells and keratinocytes as the main skin cell type.^{63,64} The stratum corneum (SC) is the most superficial layer of the epidermis and serves as the first line of defence against the external environment. It is

organized in 10-20 flattened, interlocking, vertically stacked columns of keratinocytes.⁶⁵ The stratum corneum layer, which lies superficially and loosely, is the stratum disjunctum. The cells in this layer desquamate easily as the inter-corneocyte adhesions decrease. The deeper, condensed layer is called the stratum compactum.⁶⁶ A process of keratinization or cornification continuously regenerates the epidermis. The epidermal cell turnover occurs every 28 days.⁶⁴ The SC comprises two compartments, commonly called a "brick and mortar" structure: terminally differentiated protein-rich keratinocytes and the lipid-rich extracellular matrix. It aids skin hydration and water retention. The anucleated keratinocytes contain keratin fibres embedded in the filaggrin matrix. The corneocytes provide mechanical support, protect actively dividing cells from ultraviolet (UV) damage, modulate the initiation of inflammation by cytokines, and maintain hydration. The lipid-enriched extracellular matrix (ECM) regulates normal permeability barrier function, has antimicrobial peptides, triggers keratinocyte desquamation, excludes toxins, and selectively allows for chemical absorption.^{65,66}

Disrupted epidermal barrier function plays an essential role in AD pathogenesis.^{1,2} It can be mediated by primary defects in the skin, such as genetically predetermined reduced expression of stratum corneum structural protein and secondary mechanisms featuring dominant Th2 immune activation leading to reduced levels of epidermal barrier proteins. Altered lipid content and organisation and reduced proteins such as filaggrin, loricrin, involucrin keratins, transglutaminases, and intercellular proteins corneodesmosin, lead to increased water loss, increased skin pH, a porous permeability barrier facilitating allergen and microbe entry, decreased inflammatory threshold and induction of proinflammatory cytokines.^{2-5,63-65} Epidermal promotion of inflammation by expressing damage-associated

molecular patterns (DAMPs), including IL-1 β , IL-25, IL-33, and TSLP, which deplete epidermal barrier protein levels.⁶⁷

In 2006 filaggrin null mutations (which cause the monogenic disorder ichthyosis vulgaris) emerged as the most significant epidermal risk factor for AD.^{33, 68} Subsequently, FLG-null genotype status has been associated with food allergy, allergic rhinitis, and asthma. Filaggrin (filament aggregating protein) is a histidine-rich structural protein hydrolyzed from profilaggrin during the terminal differentiation of keratinocytes. Filaggrin (FLG) binds keratin filaments into tight keratin bundles to form a matrix that gives rigidity to the keratinocyte. FLG monomers induce the collapse and flattening of keratinocytes, which is a key feature of keratinocytes in the stratum corneum⁶⁹ and mediates stratum corneum hydration by proteolytic processing of FLG into several constituent amino acids and their respective deiminated products such as glutamine $\rightarrow$ pyrrolidone-5- carboxylic acid (PCA); histidine $\rightarrow$ trans-urocanic acid (UCA) and arginine. These metabolites and ions are collectively termed "natural moisturizing factors" (NMF).⁷⁰ In AD, epidermal FLG deficiency or complete absence in the skin may result from heterozygous and homozygous loss-of-function gene mutations, respectively. Acquired epidermal FLG deficiency may be secondary to Th2 immune responses downregulating FLG expression, epidermal irritant exposure, and low ambient humidity, inducing accelerated filaggrin proteolysis.⁷¹ FLG breakdown products are generated as terminally differentiated anucleated keratinocytes migrate apically toward the superficial layer of the skin. FLG depletion can result in the retraction of the keratinocyte's cytoskeleton, which leads to a misshapen keratinocyte, impairing the extrusion of lamellar body contents into the extracellular space. Furthermore, FLG production of UCA is reduced, which results in increased stratum corneum pH. The increased pH activates neutral-pH-

dependent kallikreins (KLK) serine proteases, accelerating SC desquamation.^{72,73} Increased pH may also inhibit regenerative metabolic pathways by deactivating two ceramide-generating enzymes: acidic sphingomyelinase and β -glucocerebrosidase.^{73,74} Increased pH also restricts kallikrein 5, which depends on physiological skin pH. It is responsible for the cleavage of nascent cathelicidin, a major AMP class, into its bioactive form, which has multiple antimicrobial activities, such as direct antimicrobial effects and initiation of a keratinocyte-driven inflammatory cytokine cascade. The AMPs, under normal conditions, regulate keratinocyte migration, proliferation, repair, new vascular formation, and wound healing.⁶⁴ Therefore, FLG deficiency in the skin leads to alterations in skin pH resulting in suboptimal antimicrobial defences.⁷⁵⁻⁷⁷ Trans-UCA is converted to cis-UCA upon ultraviolet irradiation. Cis-UCA exhibits immunomodulatory effects on the skin. PCA is a potent hygroscopic FLG derivative that effectively retains stratum corneum water.⁷⁸ Cytosolic proteins keratin 1 and 10, which are major constituents of the anucleate corneocyte, are inhibited in AD by the activities of *Staphylococcus Aureus*.⁶⁴

FLG deficiency can abrogate barrier hydration via extracellular mechanisms. The extracellular defects are secondary to a decline in quantities of lamellar bilayers resulting from impaired lamellar body secretions.⁷⁹ In infancy, FLG breakdown products are lowest at cheek skin compared to elbow and nasal tip sites, with increased filaggrin degrading enzyme activity noted in cheek skin compared to elbow skin. Cheek skin was shown to have immature corneocytes compared to elbow skin, and while elbow skin NMF content stabilized by four weeks of age, cheek skin NMF content was noted to still increase within the 1-11-month and 12-35-month age bracket. The cheek skin site also had the slowest rate of increase in NMF content.⁸⁰ This comprehensive data highlights the importance of FLG in

AD pathogenesis and the unique susceptibility of cheek skin as a predilection site of the infantile AD phenotype.⁸⁰ Filaggrin breakdown products are significantly reduced in the stratum corneum layer of atopic dermatitis skin, even in the absence of filaggrin gene mutations.⁷⁸

Major lipid classes such as free fatty acids (FFAs), ceramides, and cholesterol make up the lipid matrix. It is organized in intercellular lipid lamellae and between corneocytes.⁸¹

Precursor lipids are stored in lamellar bodies during epidermal differentiation and extruded into the extracellular compartment. Afterwards, enzymatic catalytic processes produce the lipid classes required to maintain an intact epidermal permeability barrier.⁷⁹ Long-chain omega-hydroxy-ceramides coat the surface of each keratinocyte. They are bound to cornified envelope (CE) proteins and form the foundation for subsequently binding FFAs, ceramides, and cholesterol in the cornified envelope.⁶⁵ The extracellular matrix is depleted of all lipid classes secondary to intracellular FLG deficiency states. Th2 immune activation further decreases ceramides, truncation of long and esterified free fatty acids, and downregulation of elongation of very long chain fatty acids-like (ELOVL) elongases such as ELOVL3 and ELOVL6 which elongate LCFAs to provide precursors required for ceramide and sphingolipid synthesis.⁷⁰⁻⁷² In addition, elevated pH causes delays in the processing of lamellar body contents in AD. Altered lipid composition and organization are observed in AD skin lesions and non-lesional skin. Notably, decreased long-chain ceramide subclasses and long-chain FFAs are evident in AD skin.⁷²⁻⁷⁴ All these factors contribute to a bidirectional permeability to steep water loss, ingress of microbes, allergens, and low molecular weight chemicals. This accounts for the prominent xerosis observed in AD and reduced

inflammatory threshold to topical irritants.^{70,79} Depletion of epidermal lipids has been shown to correlate with *S. aureus* abundance in AD skin.⁸²

The cornified envelope (CE) is formed by dense cross-linking of barrier proteins such as loricrin, involucrin, and other epidermal differentiation-linked structural barrier proteins by transglutaminases. Additionally, an external monolayer of ceramides provides mechanical strength for the skin and serves as a scaffold for the deposition of the lipid lamellae.⁸³ In AD skin, loricrin and involucrin, together with other epidermal differentiation-linked proteins, are downregulated by STAT6-mediated signaling of Th2 cytokines.⁸⁴

Keratinocytes undergo several steps in the differentiation process, beginning in basal cell layers and culminating in the stratum corneum.⁶⁶ During this differentiation process, tight junctions are formed in the second layer of the stratum granulosum. In the external layers of the stratum granulosum, keratinocytes are bound by tight junction (TJs) proteins, desmosomes, and adherens junctions. These paracellular proteins aid adjacent cell adhesion and control the intercellular permeability of macromolecules.⁸⁴ Tight junctions are found at the most apical region of the cell-cell adhering junctional complex.⁸⁵ The TJ is formed by occludins, claudins, junctional adhesion molecule-1(JAM1), zonula occludens (ZO1) 1 and (ZO2) 2, and the multi PZD-1 protein (MUPP1), with the claudins proteins chiefly responsible for the cell-cell adhesion complex and the functional properties of the paracellular barrier. Claudin-1 and claudin-4 are detected abundantly in the epidermis and are mainly responsible for forming the epidermal paracellular barrier, whereas claudin-3 and -12 are less abundantly expressed. Claudins-23 and -25 are also detected in the epidermis.⁸⁶ Claudin 1 is depleted in AD lesions and seems to associate with a higher susceptibility to infection with herpes simplex virus (HSV1) in individuals with AD.⁸⁷ Corneodesmosomes, specialized

cell-cell adhesion molecules support corneocyte adhesions and skin barrier integrity. They consist of corneodesmosin, a secreted serine-rich glycoprotein, and cadherins (desmoglein 1 and desmocollin 1). Corneodesmosomes are degraded by the cytokine cascade seen in AD, allergens with protease activity, and surfactants found in soaps and cleansers.^{64,88} Impaired TJ protein expression is part of the epidermal barrier defects in AD corneodesmosomes.

AD animal models have advanced our understanding of the epidermal mechanisms of AD. AD in dogs is the closest naturally occurring model to human AD, with shared clinical features and pathophysiological mechanisms. Diminished epidermal lipid content has also been documented in canine AD.⁶⁹ Several mouse models of AD have been developed to research disease pathogenesis and preclinical therapeutic trials. Although no single murine model fully simulates human AD profiles, filaggrin-deficient flaky tail mice caused by a loss-of-function *flg* mutation recapitulate eczematous lesions, enhanced percutaneous allergen entry and Th2 polarization similar to human AD.⁸⁹⁻⁹¹ Downregulation of filaggrin and loricrin expression characteristic of AD at the epidermal level is best captured by flaky tail mice and *flg*-mutated models of AD.^{90,91}

Loss-of-function (LoF) mutations and copy number variations in the FLG gene located within the epidermal differentiation complex (EDC) on chromosome 1q21 and associated AD risk have been documented. However, FLG mutations are not uniformly distributed globally. FLG LoF mutations are detected in 8% of the general European population.⁹² However, up to 30% European AD patients have FLG LoF mutations.⁹² p.R501X, c.2282del4, R2447X, and S3247X are commonly described variants in European populations with p.R501X, c.2282del4 mutations accounting for 80% of the gene defects identified in Irish individuals with AD.^{93,94} Oriental specific FLG mutations were detected in up to 25% of Japanese individuals with

AD.⁹⁵ Predominant FLG mutations in Japanese populations include 3321delA, S2554X, S2889X, S3296X, S1695, p.R501, p.K4022, and p.Q1701.⁹⁶ Ethno-specific mutations have also been reported in Chinese, Korean and Bangladeshi populations that associate with specific phenotypes such as dryness.^{97,98} In particular, the c.3321delA is specific to the Asian population and is the most commonly described variant in the Chinese Han population.⁹⁹ Earlier studies reported an absence of common FLG gene mutations in AD patients of African ancestry. However, the "common" mutations examined were derived from European populations.^{78, 100-103} In a 2012 US study, 27,5% of European American AD participants displayed any of the common European null genotypes, whereas only 5,8% of African American participants displayed these mutations. Disease persistence was similar in both ethnicities with null mutations. The R501X genotype was associated with poor response to treatment.¹⁰⁴ In a follow-up study using whole exome sequencing, new FLG gene mutations were described-Q570X, R3409X and S3707X, in African American AD patients.¹⁰⁵ An Ethiopian AD study identified a novel heterozygous FLG mutation 632del2.¹⁰¹ In 2018, unique FLG exon 3 LoF variants, namely p.R501X; p.R826X; p.R2447X; p.Q3818X; p.Q570X; p.R3409X; p.S3247X; p.S3316X; p.H440fs were described in AD patients of African ancestry albeit with a lower prevalence of 12,21% compared to previous reports from European descendants with AD. Notably, unlike European AD, no single FLG null genotype predominates, likely reflecting the genetic heterogeneity of African populations.¹⁰⁶ Two novel FLG variants c.488del G; p.S3101 were reported in another African American childhood AD cohort.¹⁰⁷ In a recent study, African and European AD demonstrated shared FLG null genotypes and some ethnicity-specific mutations.⁹²

FLG intragenic copy number variation (CNV) impacts gene dosage and, consequently, the amount of filaggrin produced in the epidermis. CNV has been shown to associate with AD risk in a large study of Irish paediatric AD.¹⁰⁸ FLG CNV notably varies with ethnicity. Studies examining the effects of CNV on AD risk in African populations have yet to make consistent findings.¹⁰⁹⁻¹¹² Several variations in other genes which encode for epidermal barrier proteins located within the epidermal differentiation complex (EDC) have also been reported in African AD, including filaggrin-2 (FLG2), trichohyalin (TCHH), trichohyalin-like 1 (TCHHL1) cornulin (CRNN) and hornerin (HRNR).^{105,113-115}

With FLG LoF mutations being the most vital genetic determinant of epidermal barrier dysfunction in AD and its relevance in only a subtype of AD patients, it only partially explains the pathogenesis of AD and, therefore, the exploration of other mechanisms that contribute to AD pathogenesis is warranted.

Epigenetic modifications also impact the epidermal barrier in AD and may account for the rapid rise in AD and allergic diseases. Heritable modifications to DNA influence gene expression without changes to DNA gene sequencing. Epigenetics refer to reversible chemical reactions that temporarily or permanently turn parts of the genome on or off, impacting how DNA is transcribed.¹¹⁶ Environmental and lifestyle factors can influence epigenetics. Many of these factors affect epidermal barrier integrity.⁴²

The primary mechanisms responsible for epigenetic modifications are DNA methylation, including genome imprinting, histone modifications, and non-coding RNA, such as microRNA.^{42,46,116} DNA methylation commonly represses transcription by silencing genes at cytosine-phosphate-guanine (CpG) sites.⁴⁶ Histone modifications include acetylation,

phosphorylation, methylation, and ubiquitination, which activate or repress cell or tissue-specific transcription. microRNAs (miRNA) post-transcriptionally inhibit target messenger RNA (mRNA).^{42,46,116}

In addition to FLG mutations, DNA methylation of CpG sites within the FLG gene in individuals with a FLG null genotype synergistically increase AD risk.¹¹⁷ DNA methylation changes were identified in AD skin lesions compared to healthy skin. The S100A5 protein was hypermethylated and decreased KRT6A methylation was observed.⁴⁴ Polymorphisms in the kinesin family number 3A (KIF3A) gene are associated with the risk of developing AD by creating novel CpG sites that are highly methylated and associated with higher levels of transepidermal water loss (TEWL). Epidermis-specific KIF3A knockout mice confirmed increased TEWL, skin thickness, decreased adherens and tight junction proteins expression.¹¹⁸ Differential expression of miRNAs targeting HAS3, a keratinocyte proliferation and migration regulator, have been observed in AD. Downregulated hsa-miR-26a-5a and upregulation of miR-10a-5p may impair skin barrier function.¹¹⁹ Overexpression of miR-155-5p is evident in AD skin and is associated with reduced tight junction proteins claudin-1 (CLDN1), claudin domain containing-1 (CLDND1), and occludins. miR-155 targets PKI α which regulates tight junction protein expression.¹²⁰

1.8.2 Immune dysregulation and neuroimmune mechanisms of atopic dermatitis

In addition to genetically and epigenetically predetermined barrier dysfunction (e.g., FLG deficiency), cutaneous inflammation and activation of multiple T cell subsets are central features of AD, which interacts synergistically with other mechanistic drivers to reduce

filaggrin levels in the skin. Inflammation also influences several other parts of disease pathogenesis, such as suppressed antimicrobial peptide (AMP) production by keratinocytes. Typical histological features such as spongiosis and T cell infiltration are pathognomonic of AD and are noted in lesional and non-lesional AD (albeit to a lesser degree).⁵ Despite the well-documented T cell infiltration and Th2 skewing in AD, the precise molecular mechanisms underlying the immune response still need to be fully elucidated. Regardless, the immune mechanisms of disease offer the most promise of identifying pathways amenable to targeting with novel therapies in all patients with AD.

1.8.2.1 AD as a systemic immune disease?

AD is associated with other Immunoglobulin E(IgE)-mediated immune disorders. Among adult patients, AD is associated with inflammatory diseases such as inflammatory bowel disease (IBD), arthritis, neuropsychiatric and cardiovascular disease, including increased heart attacks. These observations have led to the proposal that AD may be a systemic condition.^{121,122} Novel longitudinal data analytical approaches suggest multiple trajectories for childhood AD exist, including the 'atopic march' wherein AD is the putative 'starting point'.³² Furthermore, 20 per cent of paediatric AD persisted beyond eight years of age. Five per cent of young adults had persistent AD at follow-up 20 years post-diagnosis.¹²³ In paediatric AD, associations with non-allergic comorbidities are less well documented. It has recently been reported that children with AD who progress along the atopic march have a 2.7-fold increased risk of developmental delay.¹²⁴ This suggests that Th2-driven inflammation might negatively impact the developing brain. It is thought that systemic

immune activation in AD increases cytokine signaling in the brain and promotes local production of cytokines. CNS production of cytokines influences neurodevelopment by altering developmental pathways and immune function. Astrocytes and microglia modulate immune activity within the brain and are activated by elevated levels of IL-4. IL-4 can also induce brain-derived neurotrophic factor (BDNF). This neurotrophin is elevated in childhood AD and correlates with pruritus and disease severity. The developmental disorders associated with AD include autistic spectrum disorder (ASD), developmental delay, memory impairment, speech disorders, and seizure disorders. AD also associates with attention deficit hyperactivity disorder (ADHD), sleep disturbance, anxiety, depression, suicidal ideation, and suicide attempts.¹²⁵ It will be interesting to assess if systemic therapies such as dupilumab can also resolve developmental and psychological disorders at a young age.

1.8.2.2 The role of keratinocytes

The apical layer of the stratum corneum is made up of dead cells that act as a physical barrier at the interface between the body's internal environment and the external environment. While the initiating events precipitating skin inflammation in AD are not fully understood, keratinocytes can orchestrate complex immune activities in AD, producing alarmin cytokines such as IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), which are induced following mechanical trauma due to scratching.^{64,126,127} The disrupted epidermal barrier leads to steep transepidermal water loss (TEWL), which increases skin dryness and itch. In addition, the disrupted epidermal barrier facilitates transepidermal ingress of allergens, pollutants, bacteria, viruses, and low molecular weight chemicals that further

promote type 2 immune activation, thereby worsening inflammatory responses by keratinocytes and local immune cells.¹²⁸

Keratinocytes also produce additional chemokines and cytokines. These include RANTES (Regulated on activation, normal T cell expressed and secreted/CCL5) that may have a role in the accumulation of Th1 lymphocytes in chronic AD; Macrophage Inflammatory Protein-3 Alpha (MIP-3 α / CCL20) contributes to the recruitment of T cells and dendritic cells in AD. CCL18 and CCL27 are chemotactic for skin-homing T cells;^{129,130} Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) promotes the production of eosinophils, basophils, macrophages, and dendritic cells from the bone marrow and Tumour Necrosis Factor α (TNF α) which is a potent proinflammatory cytokine.^{131,132} Keratinocyte-derived alarmins can further drive cytokine production by resident immune cells. Alarmins IL-33 and TSLP directly activate mast cells, while TSLP stimulates naïve T cells and dendritic cells as well as promoting B cell proliferation. Alarmins activate type 2 innate lymphoid cells (ILC2) to produce T helper 2 cytokines.⁸⁸

1.8.2.3 Innate immune responses in atopic dermatitis

Polymorphisms in Pattern Recognition Receptors (PRRs) such as Toll-Like Receptor 2 (TLR2)- the main receptor responsible for recognizing staphylococcal ligands, Nucleotide-binding oligomerization domains 1 (NOD1), and NOD2 are associated with AD in a way that enables *S. aureus* overgrowth.¹²⁶ This observation in AD patients heterozygous for TLR2 mutations was also associated with a severe disease phenotype and markedly reduced IL-8 cytokine production compared to wildtype AD patients. Low levels of CD14, a multifunctional receptor that induces innate immune cell activation via a TLR-dependent pathway, have

been reported in atopic children.¹³³ In human skin, keratinocytes, sebocytes, eccrine glands, and mast cells produce antimicrobial peptides (AMPs) such as cathelicidin (LL-37), human beta-defensins (hBD2; hBD3), and dermcidin to protect the host from infection.^{64,65} Defensins and cathelicidin induce cytokines and chemoattractants, such as IL-18, IL-6, IL10, MIP3- α , and RANTES by human keratinocytes.^{112,113} Levels of multiple AMPs are reduced in AD patients and have a defective antimicrobial function. AMP dysfunction likely contributes to the observation that over 90% of patients with AD exhibit *Staphylococcus aureus* overabundance in eczematous skin lesions and non-lesional skin (to a lesser extent) and are prone to herpes simplex virus infection.¹³⁶

1.8.2.4 Innate Lymphoid Cells type 2 (ILC2) responses in atopic dermatitis.

Innate immune cells that do not have T cell or B cell rearranged antigen receptors and lack markers of myeloid or lymphoid lineages were identified in 2010 and were subsequently classified into phenotypes similar to their T-cell counterparts.¹³⁷ They contribute to allergic disease pathology. Innate lymphoid cells type 2 (ILC2) rely on continuous GATA-3 expression and respond to NF κ B-activating cytokines such as IL-25 and IL-33 to produce type 2 cytokines in human lungs, gut and skin, predominantly IL-5 and IL-13, including IL-4 and IL-9 under more specific conditions, with TSLP regulating the quality and magnitude of this response.¹³⁸ Interestingly, in healthy human skin, ILC2 activation independent of the classical cytokines IL-25 and IL-33 has been observed. This is elicited by TSLP-TSLP receptor interactions. They interact very closely with epithelial cells, promoting Th2 inflammation. ILC2 cells are restricted to the dermal compartment of the skin and are located close to skin-resident mast cells. Dermal ILC2s are markedly elevated in lesional human skin of AD

patients and are thought to affect disease severity by regulating mast cells and recruiting eosinophils. Data from murine models also support a pathogenic role of ILC2s in AD. Depletion of ILC2s in a mouse model of AD significantly attenuated skin inflammation. ILC2s have also been implicated in IgE sensitization to environmental allergens.¹³⁹

1.8.2.5 Antigen-presenting cells (APCs) in atopic dermatitis.

The increased number of cytokines, chemokines, and growth factors in AD skin are thought to influence the differentiation of precursor cells into dendritic cells (DCs) and the recruitment of activated DCs into the site of inflammation. DCs, macrophages, and B cells are the most well-studied antigen-presenting cells (APCs). Distinct DC subsets include epidermal Langerhans cells; inflammatory dendritic epidermal cells (IDECs); interstitial/dermal dendritic cells, and macrophages eosinophils, mast cells, infiltrate AD epidermal and dermal compartments along with T cell subsets.^{140,141}

Recent data suggests that the Langerhans cells (LCs) have dual identities, as they belong to the macrophage family as a result of originating from the human foetal liver and also display DC functions such as their migratory capacity.^{142,143} LCs are considered to be resident dendritic cells found in the upper epidermal layer, strategically poised to extend dendrites through the tight junction to capture antigen. As a feature of AD, LCs express FcεR1. TSLP regulates LCs in a way that contributes to the manifestation of clinical AD. The inflammatory dendritic epidermal cell (IDEC) subtypes are increased in response to inflammation and are highly activated in AD skin. However, they cannot capture antigen on the surface as they are located in the lower epidermis, and their dendrites extend horizontally.^{144,145} IDECs have been shown to express the high-affinity IgE receptor FcεR1 and high histamine receptor 4

(HR4) levels in lesional skin.^{144,145} Dendritic cell-bound IgE antibodies facilitate allergen uptake and initiate T cell-mediated reactions. Plasmacytoid Dendritic Cells (pDCs) also have a distinct array of pattern recognition receptors for microbial pathogen sensing. Increased type 1 interferon-producing pDCs have been detected in the circulation of patients with AD compared to psoriasis patients. Increased pDCs were noted in a case of eczema herpeticum (EH)- a disseminated skin infection with herpes simplex virus.^{146,147} However, a reduced number of pDCs and pDC dysfunction have been observed in AD skin, in part due to a lack of chemerin, a pDC chemoattractant, and a lack of skin-homing molecule in circulating pDCs.¹⁴⁸ Treatment with tacrolimus and glucocorticoids topically reduced the FcER1 expression on epidermal DCs and the number of DC subtypes in the skin.^{149,150}

1.8.2.6 Mast cells and Basophils

Granulocytes such as mast cells, basophils, and eosinophils are essential effector immune cells in the type 2 inflammation of AD, regulating both innate and adaptive immune responses. Together with eosinophils, they are a significant source of pruritogens.¹⁵¹ Both mast cells and basophils constitutively express high-affinity IgE receptor FcεR1 on their surface, which correlates with high circulating IgE levels observed in AD patients.¹⁵² These cells are found near peripheral nerves and produce potent pruritogens such as histamine, neuropeptides, interleukins, cytokines, lipid mediators, and proteases. An increased number of IL-31-expressing mast cells have been reported in AD patients.¹⁵¹ Mast cells are known for their longevity in the tissues and proliferate in situ in response to locally produced cytokines and stimuli. They are particularly increased in number in chronic lichenified eczematous

lesions compared to acute eczematous lesions, where they are not increased. In contrast, basophils have a short lifespan and are found in the circulation and infiltrate AD skin in response to inflammation.^{151,152}

1.8.2.7 Eosinophils

Early studies of AD identified the pathogenic involvement of eosinophils.¹⁵¹ Elevated circulating eosinophils, eosinophil granule proteins, lesional eosinophilia, and lesional eosinophil granule proteins have been observed in AD patients at any stage of disease and correlate with disease activity.¹⁵³ Keratinocyte-derived chemokines such as CCL5/RANTES contribute to the influx of eosinophils in the skin. T helper 2-related cytokines such as IL-5 promote eosinophil survival, maturation, and differentiation. Other chemokines, including eotaxins, macrophage chemotactic protein (MCP)-3, and MCP-4, also facilitate eosinophil chemotaxis.^{88,153} There is a paucity of studies assessing IL-5 blockade in atopic dermatitis of sufficient duration to demonstrate changes in disease severity parameters as well as eosinophil-related parameters. Nevertheless, in a 2-week AD clinical trial of anti-interleukin 5 therapy (mepolizumab), no clinical benefit was observed in the mepolizumab treatment group compared to placebo. A significant reduction in peripheral blood eosinophil counts was observed in the treatment group with no depletion of eosinophils in the skin within the 2-week time frame. It is expected that eosinophils and eosinophil granule proteins persist in tissues for up to 6 weeks.¹⁵⁴ Another 16-day study assessing the effects of mepolizumab depletion of eosinophils on the development of new eczematous lesions induced by atopy patch test (APT) showed that mepolizumab therapy did not reduce APT skin reactivity. This

represented a persistent skin influx of eosinophils despite a significant reduction in peripheral blood eosinophil numbers. A more recent randomized controlled trial evaluating a longer duration of treatment (16 weeks) with mepolizumab in AD patients with moderate to severe disease, also did not show clinical improvement despite reduced peripheral blood eosinophils. Based on this observation it is thought that mepolizumab may perhaps be effective in preventing AD flares, comparable with findings from a severe eosinophilic asthma clinical trial where patients were phenotyped according to frequency of asthma flares and blood eosinophil levels, however this would require further study.

1.8.2.8 Role of T cells

Proinflammatory mediators support T cell recruitment, proliferation, and survival in the skin. Excessive T helper 2 (Th2) cell-mediated adaptive immune activation contributes to epidermal barrier disruption and depleted levels of skin barrier proteins, including tight junctions in AD lesions and non-lesional AD skin.⁸⁸ CD4+ T cell subsets predominantly infiltrate the skin and are the main drivers of inflammation. CD8+ T cell homing to AD skin is also involved in AD inflammation and amplifies Th2 immune responses by producing Th2 cytokines IL-4, IL-5, IL-9, and IL-13. IL-4 and IL-13, in particular, reduce the levels of cornified envelope molecules hornerin, involucrin, and loricrin and reduce lamellar body contents in the upper epidermal layer.^{84,141} IL-4 and IL-13 also induce local production of kallikrein 7 (KLK7) by keratinocytes, catalyzing skin desquamation and promoting micro epidermal fissuring and transepidermal water loss.¹⁵⁵ Both cytokines can reduce the expression of several genes responsible for forming the extracellular matrix (ECM).¹⁵⁶ IL-4 and IL-13

mediate IgE isotype class switching and production of allergen-specific IgE to food allergens, aeroallergens, microbial allergens, or keratinocyte-derived self-antigens via signal transducer and activator of transcription (STAT) signaling pathway.¹⁵³ IL-5 is produced by Th2 cells and mediates activation, recruitment, maturation, and survival of eosinophils in the skin. IL-9 has been shown to contribute to the allergy endotype by promoting IgE production and eosinophilia.⁸⁸

Genes encoding Th2 and Th22 cytokines and those related to keratinocyte activity are upregulated in AD skin lesions. Th1 and Th17-related cytokines additionally play a role in chronic AD and paediatric and Asian AD phenotypes, respectively.¹⁵⁷ However, the precise role of Th1 and Th17 cellular activity is yet to be fully clarified. Skin-homing adhesion molecules such as cutaneous lymphocyte antigen (CLA) and P-selectin glycoprotein ligand 1 is expressed on infiltrating T cells in AD skin. CCR4, CCR2, and CCR10/CD181 chemoattractant receptors are also expressed by skin-homing T cells. Resident memory T cells (T_{RM}) that are antigen primed have also been observed to deploy rapidly in AD.¹⁵⁸⁻¹⁶⁰ Allergen exposures contribute to AD exacerbation in IgE-sensitized patients, and polyclonal T cell responses, recognizing allergens, have been identified in AD skin.⁸⁸

Epidermal barrier impairment, in turn, further promotes keratinocyte-driven T-cell and immune cell dysregulation. Keratinocytes produce Th2-associated chemoattractants thymus and activation-regulated chemokine (TARC/CCL17) and macrophage-derived chemokine (MDC/CCL22) and release damage-associated molecular patterns (DAMPs), including innate cytokines IL-1 α , IL-25, IL-33 and thymic stromal lymphopoietin (TSLP). Alarmins activate skin-resident group 2 innate lymphoid cells (ILC2s), inflammatory dendritic epidermal cells (IDECs), and Th2 immune responses.^{65,88,126-130}

TSLP mediates OX40 ligand (OX40L), CD80, and CD86 expression by dendritic cells binding to the OX40L receptor on T cells and stimulating IL-4, IL-5 and IL-13.^{63,65,148} Keratinocyte and fibroblast-derived TSLP drive tissue remodeling and fibrosis in conjunction with Th17 and TH22 cells. TSLP promotes dendritic cell proliferation and maturation, Th2 skewing, and B-cell proliferation and differentiation. TSLP, IL-33, and other Th2 cytokines also communicate directly with sensory neurons in the skin to exacerbate pruritus.^{63,88,127}

Innate lymphoid cells type 2 (ILC2) are a potent source of Th2 cytokines IL-4, IL-5, IL-9, and IL-13. The eosinophils are found to express CCR4 and Histamine 4 receptor (H4R). IL-4 and IL-13 activate B cell class switching to IgE, promoting T cell and eosinophil migration to tissues and tight junction disruption. IL-9 promotes IgE production and eosinophilia.^{88,}

^{138,139,151}

1.8.2.9 Role of IgE

A strong association exists between total IgE and IgE-mediated sensitization to common food allergens, aeroallergens sensitization, and AD. The functional mechanisms of IgE in AD need further clarification.^{2,141} Measuring total and specific IgE provides valuable data on the sensitization profile of patients during the natural course of AD and allergy. It has been observed that a significantly large proportion of patients with putative normal total IgE also have high specific IgE to common foods and aeroallergens. Therefore, the ratio of specific IgE to total IgE may provide a more accurate representation of the sensitization profile.⁵³ Additionally, IgE sensitization to self-proteins and skin microbial organisms such as *Staphylococcus Aureus* and *Malassezia furfur* has been described in children and adults with AD, however, these are not quantified in routine clinical practice.¹⁴¹ In a clinical trial of the

anti-IgE biologic Omalizumab, depletion of peripheral blood and skin IgE was not associated with an altered disease course in the adult AD population studied. In a small study ($n=8$) including cases of paediatric AD, the Omalizumab treated group displayed a profound reduction in immune mediators involved in Th2 polarization and improvement in disease severity scores, however, the clinical improvement was comparable with the placebo group. More recently, a clinical trial of 62 participants between 4-19 years of age, reported a positive outcome. The study found that treatment with high dose Omalizumab improved severe childhood AD clinical scores, quality of life and markedly reduced potent topical steroid use. Although there is a paucity of clinical trial data supporting the use of anti-IgE in AD, this data provides indirect evidence that IgE may play a more impactful role in certain phenotypes of AD, which will need to be better defined in future studies.

1.8.2.10 Neuroimmune mechanisms

Epidermal thickening with the sprouting of nerve fibres is noted in acute AD flares with Th2-dominated cellular infiltration, whereas chronic lesions at later stages of the disease exhibit a mixed T cell infiltrate with the expansion of both Th1 and Th2 cells. Th22 and Th17 cells also contribute to skin inflammation, neuroinflammation, and tissue remodeling, albeit to a lesser degree.⁴ Innate immune cells such as mast cells, basophils, and eosinophils play key effector roles in pruritogenesis in AD, producing histamine, cytokines (including IL-31), neuropeptides, proteases, and lipid mediators.^{88,151} TSLP and IL-33 directly stimulate sensory neurons leading to an itch sensation. Histamine is significantly increased in plasma and skin lesions of AD patients compared to healthy donors. It mediates pruritogenesis and

inflammation via several mechanisms.⁶³ Histamine directly stimulates sensory neurons and induces IL-31 production. IL-31 stimulates sensory neurons that express IL31R α and mediate neurite branching in the skin. Via the histamine 1 receptor (H1R), histamine induces nerve growth factor (NGF), which increases innervation of keratinocytes and downregulates Semaphorin 3A, which inhibits the outgrowth of sensory neurons in the epidermis, thus promoting itch, the hallmark feature of AD.¹⁶¹ In human keratinocytes, histamine reduces the expression of filaggrin and tight junction formation, contributing to epidermal barrier disruption. As histamine-dependent itch is well recognized, drugs targeting the TRPV1 activating histamine receptors H₁ and H₄ have been used. H₁ antihistamines are very successful therapies for immediate allergic hypersensitivity reactions and urticaria but are ineffective against the intractable and intense itch of AD.^{127,162} H₄ blockade in preclinical animal models modulated immune responses. A selective H₄ antihistamine clinical trial reported reduced inflammation and scratching in AD; however, the clinical trial was discontinued early because of severe non-fatal off-target effects such as agranulocytosis in a minority of patients.¹⁶³ A more recently completed study of selective H₄ antagonist ZPL-3893787 also showed improvements in AD scoring indices and patient-reported pruritus without circulating agranulocytosis.¹⁶⁴

Histamine-independent immune pathways of itch have been explored as AD therapeutic targets. Overexpression of IL-31 promotes sensory neuron outgrowth and stimulation in the skin and increases itch sensation to minor itch stimuli, contributing to the sustained itch-scratch cycle. Preclinical and clinical studies of IL-31 and its receptor complex-IL-31 receptor A (IL31RA) and Oncostatin M receptor β (OSMR) have examined the cytokine's contribution to cutaneous inflammation, downregulation of tissue filaggrin expression, and acute and

chronic pruritus. Higher expression of IL-31RA and no difference in OSMR expression were discovered in AD keratinocytes compared to healthy participants. Higher IL31RA expression highlights the receptor's key role in disease pathogenesis.¹⁶¹ The therapeutic targeting of IL-31 and IL-31RA has been suggested to ameliorate symptoms in various pruritic diseases and inflammation. The binding of IL-31 to its receptor results in JAK-STAT downstream signaling, including further PI3K/AkT and MAPK activation. Lokivetmab, targeting IL-31 in canine AD, is efficacious and may inform drug development of the human isoform.¹⁶⁵ Nemolizumab, a humanized monoclonal antibody targeting the α -subunit of the IL-31 receptor complex, thereby inhibiting the proinflammatory, barrier modulating, and pruritogenic effects of IL-31.¹⁶⁶ The phase III clinical trial reported improvements in pruritus as a primary endpoint and disease severity scoring as a secondary endpoint, which was sustained up to 64 weeks of the study duration.¹⁶⁷ The success of nemolizumab demonstrates effective targeting of itch-mediating pathways and confirms the substantive pathogenic role of IL-31/IL-31RA in AD.

Collectively the contribution of the epidermal keratinocytes, local and recruited immune cell subsets, and cutaneous sensory nerves highlight the complex and dynamic interaction between immunity, barrier disruption, and itch perception that initiate and sustain disease.

1.8.3 Microbiome

1.8.3.1 Skin Microbiome dysbiosis

Early culture-based methods suggested that *Staphylococcus aureus* is essential in AD pathogenesis.⁵ A growing appraisal of the skin microbiome aided by non-culture-based methods such as 16S ribosomal RNA sequencing and metagenome shotgun sequencing have

clarified skin microbial communities' composition and functional role in human health and disease states. A dynamic and diverse composition of bacteria, fungi, viruses (including bacteriophages), and archaeal communities constitute the healthy human skin microbiome, with bacteria being the most widely studied component.¹³⁶ Bacteria are found superficially and in deeper layers of the epidermis, the dermis, and dermal adipose tissue.¹⁶⁸ Lifestyle factors, UV exposure, climate, age, gender, ethnicity, and skin site influence the composition of the skin microbiome.¹⁶⁹ *Propionibacterium* species thrive in sebaceous areas such as the glabella, *Corynebacterium*, and *Staphylococcus* species, predominantly in moist areas such as the antecubital fossae. *Malassezia* species represent the most abundant fungi in the human integumentary system.¹⁷⁰ *Staphylococcus aureus* (*S. aureus*) has consistently been tightly linked to AD and correlates strongly with disease severity, acute eczematous flares, and increased allergen sensitization. The concomitant decline in commensal species such as *Staphylococcus epidermidis* has also been documented in the lesional and non-lesional of AD skin.^{126,136} The disrupted skin microbial composition acts in tandem with local and systemic inflammatory responses, reduced epidermal barrier proteins, altered skin lipids, and pH changes to give rise to the characteristic epidermal dysfunction of AD.¹⁷¹

High levels of Th2 cytokines IL-4 and IL-13 impair control of pathogenic microbes by depleting antimicrobial peptides (AMPs) such as human β defensin-2 (hBD-2), human β defensin 3 (hBD-3) and cathelicidin LL-37.¹⁷¹ Impaired immune recognition and clearance of pathogenic bacteria may also result from reduced Langerhans cell expression of TLR-2 in AD skin.¹⁷² Alterations in specific lipids, such as increased ceramide AS associated with *S. aureus* abundance at AD predilection sites.¹³⁶

S. aureus isolates from AD skin act in several ways to disrupt the epidermal barrier and induce inflammation.¹²⁶ They produce adhesins that enable bacterial adherence to the skin and downregulate filaggrin and loricrin in the stratum corneum. *S. aureus* expression of proteases disrupts the skin barrier by directly degrading skin barrier proteins and activating protease-activated receptors (PARs), leading to abnormal desquamation and cytokine production.¹⁷³ This facilitates microbial contact with the immune system. Staphylococcal superantigens, including staphylococcal enterotoxins A, B and toxic shock syndrome toxin-1 secreted by *S. aureus*, trigger cytokine activation, leading to the permeability of the epidermal barrier. Toxin-specific IgE also contributes to local inflammatory responses. In addition, *S. aureus* expresses a range of virulence factors. Virulence peptides such as phenol soluble modulins (PSMs) are key to pathogenic staphylococci's mechanisms promoting colonization. They are cytotoxic to keratinocytes, induce alarmin secretion that mediates skin inflammation, and promote biofilm formation on AD skin. Exotoxins such as α -toxin cause keratinocyte damage through cytolytic activity. β -, γ - and δ - toxins elicit inflammation by inducing mast cell degranulation.¹⁷⁴ *S. aureus* exposure leads to inflammatory lipoprotein activation of TLR-2 on keratinocytes. It is also thought that *S. aureus* contributes to eczema herpeticum susceptibility by secreting products that enhance viral replication.¹³⁶

It has been shown that AD patients can be persistently colonized with a single clonal strain of *S. aureus*.¹⁷⁵ Notably, patients with severe AD were shown to be colonized with methicillin-sensitive *staphylococci* in contrast to less severe AD, which were frequently colonized with methicillin-resistant strains.¹³⁶ This has positive implications for response to treatment, particularly when potentially protective staphylococci strains such as *Staphylococcus epidermidis* may withstand antibiotic use and pathogenic *S. aureus* are

antibiotic-sensitive strains. Commensal microbes such as *S. hominis*, *S. lugdunensis*, and *S. epidermidis* have been shown to limit *S. aureus* dominance and biofilm formation.

Commensal staphylococci can augment host defences by producing bacteriocins and autoinducing peptides (AIPs) which inhibit the accessory gene regulator (agr) quorum sensing systems that control the production of *S. aureus* virulence factors.¹⁷³⁻¹⁷⁵ This process is referred to as quorum quenching. Additionally, *S. epidermidis* strains can activate TLR-2, enhancing tight junction formation.¹⁷⁵ *S. epidermidis* also produces specific serine proteases, which disrupt *S. aureus* biofilm formation. *S. hominis* produces *Sh*-lantibiotics α/β -, which are cyclic AMP-containing lanthionine and methyllanthionine. *S. lugdunensis* produces lugdunin, which also has inhibitory effects on *S. aureus*.¹⁷⁶ However, these antimicrobial activities' relative abundance and potency are often reduced in AD skin.

The skin commensal community members, including *Propionibacterium*, *Streptococcus*, *Acinetobacter*, *Corynebacterium*, and *Prevotella* at the genera level and Proteobacteria phylum, are reduced in AD skin.¹³⁶ *Malassezia* is widely considered a healthy skin commensal. However, strain level differences occur between healthy and AD skin, indicative of critical strains with pathogenic potential in AD. Pathogenic *Malassezia* strains are also thought to associate with AD severity and promote skin inflammatory responses. *Malassezia* IgE sensitization has been detected more often in adult AD than in childhood AD and healthy individuals.^{177,178,}

1.8.3.2 Gut-Skin Microbiome axis

The gut microbiome influences local and systemic human immunology and is essential for immune maturation.¹⁷⁹ Various products such as metabolites, short-chain fatty acids

(SCFAs), glycolipids, oligosaccharides, and signaling molecules are derived from the intestinal microbiome, promoting gut homeostasis and sustaining a diverse microbiota composition.¹⁸⁰ Numerous studies have shown that infant gut microbiota composition influences allergic disease risk.¹³⁶ *Bifidobacterium species* are considered beneficial microbes as they have recognized immunoregulatory effects. It has been shown that patients with AD have disturbances in intestinal microbiota composition characterized by reduced levels of *Bifidobacterium* in the gut than healthy controls. *Bifidobacterium* abundance inversely correlates with disease severity.¹⁸¹ Increased abundance of another butyrate-producing microbe, *Coprococcus eutactus*, associated with improved AD symptoms and disease severity.¹⁸² Butyrate and other SCFAs, such as propionate and acetate, have anti-inflammatory and immunomodulatory effects based on their capacity to promote Treg polarization and expansion and enhance regulatory T cell ability to suppress effector T cells.^{179,180} The proportions of *Bacteroidetes* and *Bacteroides genus* are reduced in AD children.¹⁷⁹

The overall microbiome alpha diversity was significantly decreased in infants with AD. Findings from numerous longitudinal studies suggest that gut microbiota alterations detected in the first few months of life predate the development of clinically apparent atopic dermatitis.¹⁸³ Gut colonization with *C. difficile*, *Enterobacteriaceae* and *E. coli* was shown to associate with AD development.¹⁸⁴ Several studies have reported depleted levels of specific microbes such as *Akkermansia muciniphila* in AD children.¹⁸⁵ Whole-metagenome sequencing of the intestinal microbiota in infants with AD demonstrated depletion of mucin-degrading bacteria such as *Akkermansia muciniphila*, *Ruminococcus gnavus*, and *Lachnospiraceae bacteria 2_1_58FAA* which are associated with reduced abundance of

genes related to immune development.¹⁸⁶ These bacteria do not only mediate immune development by influencing signaling pathways, antigen presentation, and processing; they can further lead to microbial diversity as they degrade complex polysaccharides into SCFA, which provide an endogenous source of nutrients that promote intestinal colonization by other diverse microbes.^{186,187} IL-4 and IL-13 dominated Th2 immune microenvironment contributes to intestinal barrier defects and increased intestinal permeability in AD by disrupting tight junction¹³⁶

1.8.3.3 Exposures influence microbiota composition, microbiota metabolism, and immune education.

Establishing specific microbial exposures during a critical development window early in life promotes regulatory immune responses that prevent aberrant inflammatory reactions later in life. Perinatal events, including delivery mode, breastfeeding, maternal health status, and antibiotic use, shape the newborn's acquisition of microbes.^{187,188} The postnatal period seems important for developing immune tolerance as commensal bacteria and Treg cells abundantly populate the neonatal skin.¹⁶⁹ Commensal exposure inhibits proinflammatory immune activation. Several studies have reported the impact of exposome-innate immune system interactions. Dog ownership is associated with reduced AD risk in early life.¹⁸⁹ Antibiotic use in early life, specifically the first two years of life, was associated with AD risk. This is thought to arise from an immunomodulatory effect of antibiotics on the gut microbiota, which may influence systemic immune responses.¹³⁶ Protective effects of farming environments on allergy development have been consistently reported.¹⁹⁰ A

longitudinal study found that the skin microbiota of infants with AD demonstrated an absence of *S.aureus* sequences at multiple time points on eczematous skin, contrary to frequently described observations in longstanding AD. The most commonly detected species were *S. epidermidis* and *S. cohnii*. However, those who developed AD at 1 year had profoundly lower levels of commensal staphylococci detectable at two months compared to unaffected infants. These observations highlight that *S. aureus* dominance may not always precede clinically apparent AD. A low abundance of commensal staphylococci may be the more accurate marker of early preclinical AD. Longitudinal studies may clarify the transition to disrupted microbiota composition in AD.¹⁹¹ Early colonization of the neonatal human skin by *S. epidermidis* is associated with a tolerogenic immune environment characterized by *S. epidermidis*-specific FOXP3+ Treg cell expansion.¹⁸⁸ In a recent study, daycare centres deliberately increased environmental biodiversity with sod, forest floor segments, and planters, and skin and gut microbiota changes were documented. This was accompanied by changes in plasma IL-10 levels and Treg cell frequencies in blood.¹⁹²

1.8.3.4 Animal Models of AD recapitulate the critical pathogenic feature of microbial dysbiosis.

Dogs naturally develop AD and house dust mite IgE sensitization.⁶⁹ Similar to human AD, canine AD is associated with reduced microbial diversity and overexpression of *Staphylococcus pseudintermedius* and *Corynebacterium species*. Canine eczematous lesions improve with antimicrobial treatment. Additionally, reducing specific pathogenic staphylococcus species coincided with restoring bacterial diversity.^{193,194} Mouse AD models

have been developed, advanced our understanding of disease mechanisms, and corroborated findings in human AD.^{136,194,195} Ovalbumin-sensitized filaggrin-deficient flaky tail mice show *S. aureus* abundance, which correlates with Th2 cytokines associated with AD. Inbred DS-Ng mice spontaneously develop dermatitis with eczematous lesions heavily colonized by *S. aureus*. A recently developed model of *S. aureus* skin occupation triggered cutaneous inflammation, which induced *S. aureus* accessory gene regulatory (Agr) virulence systems and *S. aureus* produced δ -toxin molecules that induce Th2 inflammation. Adam 17-deficient mice closely resemble features of human AD.^{136,194} Antimicrobial therapy which specifically target *S. aureus* and *Corynebacterium bovis* in Adam 17-deficient mice improved lesional skin and increased microbial diversity. Discontinuation of targeted antimicrobial treatments caused a reappearance of eczematous lesions and recurrent dysbiosis. Itching is a hallmark feature of AD. Scratching, as a result, induces extracellular molecules, which enhance the adhesiveness of *S. aureus*. A mouse model of scratching has shown that Th17 and Th22 cytokines direct neutrophils to limit the overexpression of *S. aureus* on injured skin.^{136,196} C5aR deficient mice develop decreased microbial diversity, indicating that the complement system modulates skin microbiome composition. A mouse model of AD showed that *Vitreoscilla filiformis*, a bacterial lysate, effectively ameliorated clinical AD-related inflammation. In neonatal mice, immune tolerance to commensal bacteria such as *S. epidermidis* is preferentially established on the skin.¹³⁶ These animal models support the hypothesis that the development of immune system regulatory functions is at least partially dependent on exposure to particular microbes and their metabolites at an all-important period in early life.

1.8.3.5 Therapeutic-targeting of the microbiome

Early intervention, such as emollient to protect the epidermal barrier, might prevent the atopic march in some AD children. Manipulating the skin microbiome community may offer unique therapeutic targets. However, very few studies have demonstrated clinical efficacy.^{136, 175, 188} There is also no universally accepted therapeutic microbe to target the skin. Nevertheless, the topical application of emollients supplemented with *Vitreoscilla filiformis* extract showed more significant benefit than placebo control alone. In another study, the topical application of commensal bacteria *Roseomonas mucosa* showed improvements in clinical disease severity parameters in adult and childhood AD. Autologous microbiome transplant (AMT) of skin commensals such as *S. hominis* and *S. epidermidis* showed efficacy in controlling *S. aureus* overproliferation.^{136, 175, 188, 197}

Oral probiotic supplementation has also been evaluated in AD patients. Far more randomized controlled trials of different probiotic strains have been conducted in AD compared to topical microbial products. However, significant heterogeneity exists between the microbes and clinical study outcomes.¹⁹⁷ Notwithstanding, probiotic use to modify the aberrant gut bacterial composition has been attempted based on the low *Bifidobacteria* or *Lactobacillus* enterotype associated with AD. Antenatal pre-treatment with a *Lactobacillus* strain decreased the risk of AD development in infancy, which was sustained at follow-up. In another study, infants with established AD were weaned onto formula containing a *Lactobacillus* or *Bifidobacteria* strain, which improved disease severity scores and reduced topical steroid requirement.¹⁹⁷ This data indirectly supports the concept of a gut-skin axis and demonstrates that probiotic supplementation may shape the intestinal microbiota in a way that is beneficial for disease prevention and can impact skin inflammation.

1.9 Rationale for the research described in this thesis.

While the understanding of the pathogenesis of AD is incomplete, it is evident that atopic dermatitis is a highly complex multifactorial disease. It is a common refractory inflammatory skin disorder, mainly affecting children from a very young age. It is highly prevalent in industrialized nations, and a rapidly increasing incidence has been noted in developing countries, especially in urbanized areas. The disease is refractory and causes significant impairment in quality of life and poses an enormous financial burden for patients and caregivers. Currently, there is no cure, and treatments are mainly comprised of emollients and glucocorticoids (which globally suppress inflammation and immune cell activities) in addition to systemic immunosuppressive drugs such as methotrexate, azathioprine, cyclosporin for treating moderate to severe disease as well as targeted biologics which are currently prohibitively priced out of reach for many poor communities.

The discovery of the association between filaggrin gene mutations with epidermal barrier disruption in AD has improved our understanding of the disease. However, this genotype features only in a minority of AD cases and is an immutable factor. Furthermore, a similar pattern of immune-mediated dysregulation is observed in patients with or without filaggrin mutations. Recent advances in biologics for AD have ushered in the era of precision medicine owing to a more granular understanding of the immune mechanisms of disease. Modulating appropriately targeted immune mechanisms relevant to specific patients holds the most promise for AD therapies for all AD sufferers. Thus, there is a growing appreciation of population/ethnicity-specific disease phenotypes and corresponding immune endotypes that may be unique to specific geographies and early life exposures.

Given the above considerations, we sought to study AD in South African AmaXhosa children as they represent a population that has thus far been shown to

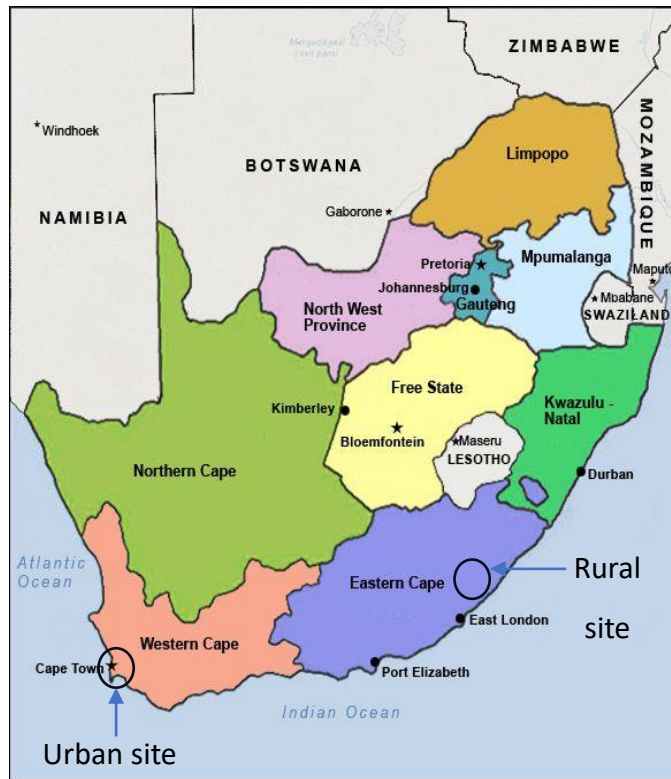
- (i) have a low frequency of commonly identified filaggrin gene mutations⁷⁸
- (ii) be resident in rural and urban settings in a developing country and may therefore have unique exposures that may differentially affect disease risk.
- (iii) be at a high risk of allergic disease and have a high IgE producer genotype¹⁹⁸⁻²⁰⁷
- (iv) have similar IgE-mediated food allergy rates as has been described in industrialized countries. AD patients as a group are known to have higher levels of IgE-mediated food allergy than the general population. In addition, South African AmaXhosa children display different population-specific food sensitization and food allergy profiles compared to published data from predominantly European participants^{208,209}
- (v) belong to an age group that has been shown to have age-specific immune profiles that are substantially distinctive from adult AD.²¹⁰

There is a need for more literature to provide detailed data on the immune mechanisms of AD in populations of African ancestry globally. It is essential to describe immune pathways activated in AD patients of African ancestry to determine if available biologics targeting specific pathways will be effective in this demographic. AD diagnostic criteria must be adequately evaluated in African populations in developed and developing settings.²¹¹ AD severity scoring indices are reliant on the presence of erythema. Erythema is not readily appreciable in darkly pigmented skin, leading to gross underestimation of disease activity and severity. This situation is not mitigated by assessments conducted by well-experienced dermatologists who predominantly treat patients with darkly pigmented skin types.²¹² Therefore, objective immune biomarkers of disease and disease severity are desirable to

accurately diagnose, guide clinical management and monitor response to novel treatments in clinical trials. In addition, studies in diverse populations may lead to identifying novel targets for therapy, ie. population-specific precision medicine.

Furthermore, the rapid rise in allergic disease coincides with urbanization. An urban-rural gradient in allergy prevalence is already evident in South Africa. Unprecedented urbanization is ongoing throughout the African continent and will represent the fastest urban growth in the world. It is estimated that the population of Africa will double by the year 2050, with two-thirds of this growth concentrated in urban areas. Notably, 70 percent of Africans live in urban informal settlements.²¹³ Therefore, it is crucial to study AD and allergy development and healthy immune development in African urban and rural settings; to document the influence of local environmental exposures, identify interventions that may mitigate against adverse health outcomes related to the living environment, and anticipate future healthcare needs.

In this study, settlements were defined as urban when the population was greater than 5,000 people near a high level of industrialisation, while rural settlements had less than 5,000 people with low levels of urbanisation and industrialisation. The urban AmaXhosa study participants reside in urban townships in Cape Town, while the rural AmaXhosa study recruits reside in rural reserves and live in homesteads (cluster of houses with extended family) practicing subsistence crop and pastoral farming in Umthatha (see map).



The specific goal of this thesis:

To investigate the mechanisms of atopic dermatitis and allergy development in South African AmaXhosa children between 12 to 36 months living in urban and rural settings

To achieve this goal, I addressed the following Objectives:

1. Describe detailed clinical characteristics and questionnaire-based environmental exposures of children with atopic dermatitis and corresponding non-allergic healthy children living in urban and rural settings.
2. Quantifying the seven most common food allergen and house dust mite allergen levels in children with atopic dermatitis and healthy non-allergic children living in urban and rural settings using Skin Prick Tests (SPTs) and ImmunoCAP IgE.

3. Quantification of inflammatory responses in plasma samples of children with atopic dermatitis and compare these with healthy non-allergic children living in urban and rural settings using multiplex immunoassays.
4. Describe the immune transcriptome of children with atopic dermatitis and healthy non-allergic children living in urban and rural settings using RNASeq of peripheral blood mononuclear cells (PBMCs).

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CHAPTER 2

CLINICAL CHARACTERISTICS AND LIVING ENVIRONMENT OF SOUTH AFRICAN

CHILDREN WITH AD IN RURAL AND URBAN SETTINGS

INTRODUCTION

Atopic dermatitis (AD) is a highly pruritic and refractory inflammatory skin condition. Global epidemiology data shows that AD affects 15-20% of children and 1-3% of adults. AD frequently precedes other atopic disorders.¹ The disease is multifactorial with a heterogenous clinical presentation.² The heritability of IgE-mediated allergic disease and AD has been reported.³ Susceptible gene loci have also been identified, with filaggrin gene defects representing the most vital link to AD risk in a subset of patients.^{4,5} However, it is likely that several genetic modifications interact with host immunity and environment to contribute to AD pathogenesis. Skin barrier disruption, altered microbial composition with *Staphylococcal aureus* predominance and dysregulated innate and adaptive immune responses characterise this syndrome.³⁻⁶ Several molecular and cellular mechanisms have been shown to lead to similar clinical outcomes. These mechanisms have led to the concept of AD endotypes- subtypes of AD defined by distinct pathophysiological mechanisms.⁷ Systems biology becomes a valuable approach merging numerous clinical features, endotypes and environmental exposures to stratify personalised treatment of distinct immune and inflammatory subgroups.⁸

State of the art in populations of African ancestry

The study of clinical AD, molecular endophenotypes and possibly unique environmental exposures in populations of African ancestry needs to be sufficiently documented.² African populations are the most genetically diverse in the world and will likely benefit profoundly from precision medicine as these therapies will target specific pathways in clinically heterogenous conditions like AD.⁹

African researchers in other disciplines have shown differences in kidney disease prevalence that were found to associate with ancestry.¹⁰ Within the context of pharmacogenetics, biomedical variation in drug response in African populations has also been documented.¹¹ Under the current state-of-the-art, precision medicine efforts, particularly the burgeoning use of biologics, will be less well-targeted in individuals of African ancestry, as they need to be better represented in the experimental data and clinical studies underlying these initiatives.

As early as the mid-nineties, researchers in the UK reported an increased AD prevalence amongst Afro-Caribbean Londoners compared to European children.¹² Ongoing studies emerging from the US and UK also report an increasing prevalence and severity of allergic diseases such as AD and asthma in individuals of African heritage compared with those of European descent. Children of African ancestry were shown to have a six-fold increased risk of severe AD compared to European children. African Americans were reported to have five times more likelihood of fatal outcomes following acute asthma exacerbations than European American patients.¹²⁻¹⁷ However, further studies are needed to clarify the contribution of genetic and epigenetic susceptibility, socioeconomic status, environmental factors and their collective interactions to account for this disparity.

Studies conducted in the 1980s in South Africa reported high serum IgE levels in healthy African adults compared to European adults in the absence of helminth infection. Similarly, higher concentrations of cord blood IgE independent of atopic family history were documented in African newborns than in European newborns.¹⁸ This study suggested that African populations had high basal IgE levels and likely represented a pool of genetically determined IgE "high-responder" phenotypes. Since then, single nucleotide polymorphisms (SNPs) in IL-4R α have been identified and associated with severe asthma exacerbations and

mast cell-related tissue inflammation.¹⁹ These SNPs are reported much more frequently in individuals of African ancestry. A recent South African study also observed IL-10 and IL-4 polymorphisms associated with allergic disease in African children.²⁰ Furthermore, data emerging from the 1000 Genome project identified population-specific allele frequencies (IL-4, IL-4R, IL-13, IL-13R α 2, TGF- β , IFN- γ , IFN- γ R1, IFN- γ R2 and IL-10) for an enhanced Th2 immune response in African populations compared to European descendants in a way that may simultaneously increase susceptibility to allergic disease.²¹

With regards to filaggrin gene mutations, four loss-of-function (LOF) mutations in the filaggrin (FLG) gene are the strongest known genetic risk factor for Atopic Dermatitis and have been robustly reproduced in studies of European populations (R501X, 2282del4, S3247X, R2447X).²² Studies of East Asian populations have observed the 3321delA variant as the most potent variant associated with AD risk in the Asian population.²³ A few studies have yet to be able to reproduce an AD link to these specific variants in individuals of African ancestry.^{24,25,26} However, single studies have suggested that polymorphisms in other barrier-associated genes, such as Filaggrin-2 gene variants²⁷ and uncommon FLG gene loss-of-function mutations, may associate with AD risk in African Americans.²⁸ Admittedly, these are significant findings. However, not detracting from their significance, only 15% of mild-moderate AD cases and 50% of moderate-severe cases can be explained by FLG gene mutations in the studied populations.²⁸ Within the recent report describing uncommon filaggrin mutation in African American children with AD, only 12,2% of the cohort under study displayed the FLG mutations²⁸. FLG intragenic copy number variation (CNV) impacts gene dosage and the amount of filaggrin produced in the epidermis. Thus far, to our knowledge, CNV has yet to be sufficiently studied in African ancestry and has not shown an association with AD in the two studies reported on African populations. Additional studies

will be needed to adequately appraise epidermal barrier genotypes that may associate with AD risk in the African demographic. Furthermore, two reports have demonstrated an AD skin phenotype with regular FLG protein expression in early-onset paediatric AD compared to filaggrin-deficient adult AD skin.^{29,30} This accumulating evidence supports the vital contribution of other factors, including multipolar cytokine axes, in the pathogenesis of AD, as these can deplete epidermal barrier proteins and tight junction component expression^{31,34}.

Environmental and regional considerations

In addition to immune and epidermal risk factors, environmental exposures may influence AD development³⁵. South Africa has had a reportedly lower overall prevalence of IgE sensitisation in rural areas compared with urban areas over the decades, which highlights the importance of environmental factors on allergy risk. However, this rural-urban gradient is rapidly narrowing over time³⁶. The farming environment has been shown to have protective effects in other parts of the world, potentially due to exposure to a more microbially diverse environment³⁷. In addition, a recent intervention study in an urban daycare centre that modified its outdoor environment to include sod, forest floor segments and planters saw skin and gut microbial composition modification coinciding with an increased tolerogenic immune profile.³⁸

A wealth of literature has drawn parallels in the increase in respiratory allergy and environmental factors, specifically ambient air pollutants associated with urbanisation.³⁹ However, a limited number of studies show that air pollution, characteristic of urbanisation, positively associates with AD prevalence. Prenatal exposure to certain air pollutants may link to allergy development through dysregulated immune responses mediated by

microRNA and DNA methylation.⁴⁰ Birth cohort studies suggest indoor and outdoor air pollution is associated with AD development. Longitudinal studies also suggest that various air pollutants are associated with AD exacerbations and disease persistence.^{41,42} Furthermore, region-specific variations in allergic disease presentation have been reported. Colombian researchers have observed that the clinical presentation of allergic disease in their region is inconsistent with the putative 'atopic march', but the natural history of allergy is skewed towards a debut with respiratory symptoms.⁴³ This is possibly influenced by the tropical climate, which supports high mould, cockroach, and house dust mite allergen burden in humid conditions. It has also been noted that urbanisation in developing countries often occurs in the context of economic deprivation, and this promotes exposure to noxious substances that might influence immune function and modify allergy susceptibility.

Collectively, this data suggests that the role of genetic background generally needs careful consideration in terms of the clinical presentation of disease, diagnostic criteria, disease mechanisms, and treatment responses. This consideration is relevant in AD and allergic diseases. While allergy rates might differ in rural South Africa and urban settings, allergic diseases still do arise in these diverse environments.

STUDY RATIONALE

We sought to describe detailed clinical and exposure-related characteristics in a childhood cohort with infantile AD and healthy participants from the same ethnolinguistic African population in urban and rural settings. We hypothesise that unique factors underscore features of disease development that may confer AD risk or protection in both these settings.

OBJECTIVE

To characterise and compare the clinical, demographic, socioeconomic and living environments of infants with atopic dermatitis in rural and urban South African settings.

METHODOLOGY

Study Design

We performed a case-control study of participants from the ethnolinguistic AmaXhosa population. Participants with atopic dermatitis having moderate and severe disease (n=56), ie. oSCORAD 15-40 and oSCORAD >40, were enrolled at the Paediatric Dermatology clinic, Red Cross Children's Hospital, Cape Town. Healthy children without atopic dermatitis and no evidence of allergic sensitisation (n=49) were enrolled from randomly selected crèches in the Cape Town metropole. Similarly, children from a rural setting were enrolled in the study. Participants with AD (n=60) were recruited at the dermatology department at the Nelson Mandela Academic Hospital, Umthatha. Their healthy non-allergic control counterparts (n=52) were randomly selected using a community sampling method at study sites based at local clinics in the Mqanduli district of Umthatha, as crèches are rarely found in this district. All participants were seen at a single time point for evaluation. Detailed questionnaires were completed, Skin Prick Tests (SPTs) for seven common food allergens and clinical assessments were performed, and blood samples were collected at this visit. This clinical study received approval from the University of Cape Town Human Research Ethics Committee (HREC 451/2014). It was conducted per the declaration of Helsinki, and written informed consent was obtained from parents/guardians of all participants prior to inclusion in the study.

Inclusion and Exclusion Criteria

Participants with a dermatologist diagnosis of AD who fulfilled the UK Working Party Diagnostic criteria, with moderate to severe atopic dermatitis, were eligible for inclusion.

44. The minor criteria 'onset before age 2' and 'personal history of other atopic diseases' were not applicable as all the participants were under four years old.

AD was graded according to the objective SCORAD (SCORing Atopic Dermatitis) index.^{45,46}

AD participants were on only soap substitutes, emollients, and topical steroid therapy.

Healthy, non-allergic, age-matched and gender-matched participants were eligible as controls if they reported no personal history of allergy, demonstrated no signs of allergic disease on clinical assessment, and were SPT negative for food allergens. AD and healthy participants were excluded from the study if they had any other skin or systemic disease. Participants with a positive stool test for helminthic infection were excluded from the analysis.

Stool sample helminth analysis

Infant stool samples were examined during the screening visit for the presence of helminths using the Kato-Katz method.

Questionnaire

A detailed standardised questionnaire was completed with the parent/guardian to obtain clinical data on family history of atopy, household size, family income, infant exposures, medication use and environmental exposures, as already described by Basera et al.⁴⁷. The questionnaire is included in Appendix 1 of this thesis.

Skin Prick Test

Participants had skin prick tests (SPTs) with standardised reagents from ALK-Abello for sensitisation to the seven most common allergenic foods - i.e., cow's milk, egg (white), peanut, hazelnut, wheat, soy, fish (cod) with positive and negative controls performed with 10 mg/mL histamine and saline respectively. Additionally, modified SPT of fresh cow's milk, raw egg white, and fresh peanut was performed. The skin prick test was performed on the non-eczematous skin on the forearm or back. The skin was pricked through a drop of allergen extract with ALK-Abelló lancets on the volar aspect of the forearm or the back and the reaction measured 15 minutes later. The average wheal diameter was recorded in millimeters (mm). A wheal size of ≥ 1 mm more than the negative control was considered a positive skin prick test and indicative of sensitisation.^{36,47}

Statistical Analysis

The non-parametric Mann-Whitney test (Prism GraphPad Software version 7TM) was used to determine the statistical significance (two-tailed analysis with gaussian approximation) between the two groups as the data was not normally distributed. The non-parametric Kruskal-Wallis test with p-values adjusted for multiple comparisons (Dunn's multiple comparisons test) was performed to compare differences between multiple groups. The chi-square analysis was used to estimate differences in proportions between groups for the demographic data. Linear regression analysis using 95% confidence intervals was performed to determine the relationship between age and serum immunoglobulin levels. A participant was excluded from the analysis if one of the data points was missing.

RESULTS

Participant Demographics

The urban study participants reside in urban townships comprising formal housing and shanty towns. In contrast, the rural study participants reside in rural reserves and dwell in homesteads (clusters of houses with extended family members) practising subsistence and pastoral farming. There were no significant differences between the groups regarding age, gender, and anthropometric measurements such as height or weight (**Table 2.1**).

Table 2.1. Participant Demographics

	Rural AD	Rural HC	Urban AD	Urban HC
Study population (n)	60	52	56	49
Age [months]	21.0 (14.3 – 25.0)	20.0 (15.3 – 28.0)	23.5 (16.0 – 30.0)	24.0 (16.5 – 30.5)
Gender (F/M)	26/34	22/30	26/30	26/23
Height [cm]	80.0 (76.3 – 85.8)	84.0 (79.0 – 87.8)	82.0 (77.5 – 86.0)	85.0 (78.5 – 89.5)
Weight [kg]	11.7 (10.5 – 13.2)	12.0 (10.9 – 13.0)	12.2 (10.7 – 13.1)	12.5 (10.7 – 14.7)

AD – Atopic Dermatitis; HC – Healthy Control

Results are shown as median with the 25% - 75% percentile range in brackets.

UK working party criteria of AD and AD Severity

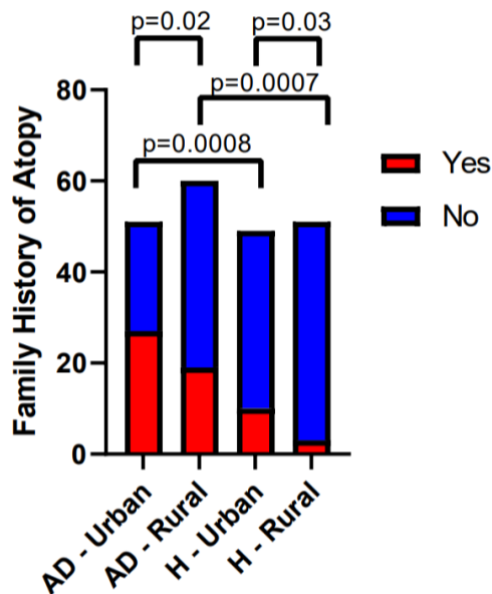
All children with AD fulfilled the major and minor UK working party criteria to be diagnosed as AD (**Table 2.2**), with 100% of children being positive for itch, a history of flexural AD and dry skin and visible flexural dermatitis. However, only 48% or 31% of children with AD from urban or rural backgrounds, respectively, had a first-degree relative with atopy. In addition, a positive family history of atopy was significantly higher ($p=0.02$) in the urban AD group than in the rural AD group (**Figure 2.1**). Significant differences are also notable between AD groups and their corresponding healthy control groups and between urban and rural healthy participants.

Table 2.2: Number of children (under 4) with features used in the UK working party criteria

UK Working Party Criteria	Urban AD	Rural AD
Major feature		
Itchy skin condition	56 (100%)	60 (100%)
Minor features		
History of flexural involvement	56 (100%)	60 (100%)
History of generally dry skin	56 (100%)	60 (100%)
History of any atopic disease in a first degree relative*	27 (48%)	19(31%)
Visible flexural dermatitis	56 (100%)	60 (100%)

* $p=0.02$

Figure 2.1: Differences in family history of atopy (red indicates a positive family history and blue indicates a negative family history). The number of children per group is indicated on the y-axis.



Further examination into which family members have a history of atopy in the urban and rural groups revealed a significant difference that is driven by maternal atopy but no difference in paternal or sibling atopy between urban and rural cohorts (**Figure 2.2**). Also, only two urban and three rural participants reported atopy in both parents. Parental atopy history included similar frequencies of asthma, allergic rhinitis (AR) or AD within urban AD, rural AD and healthy urban groups. Reports of parental asthma, AR and AD were absent in the healthy rural group (**Figure 2.3a**). The most notable differences in parental atopic disease across the different groups were observed for parental allergic rhinitis ($p=0.0004$) and parental atopic dermatitis ($p=0.002$) (**Figure 2.3b**).

Figure 2.2: The contribution of first-degree relatives to familial atopy. The number of family members per group is indicated on the y-axis.

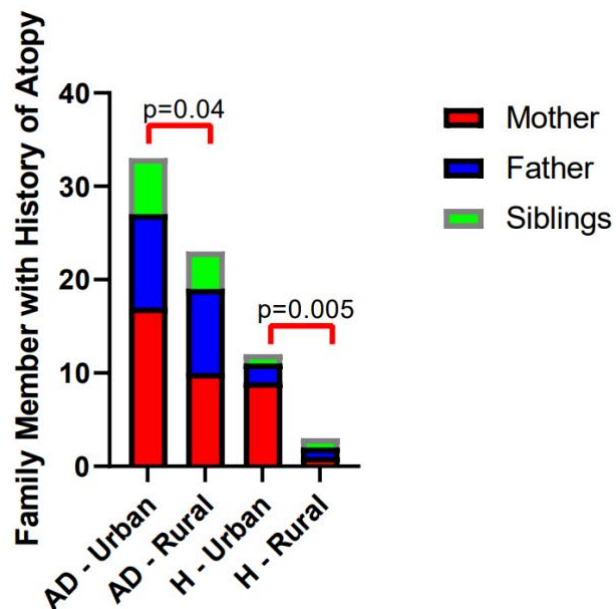


Figure 2.3a Parental atopic disease in AD and healthy children. The number of parents per group is indicated on the y-axis.

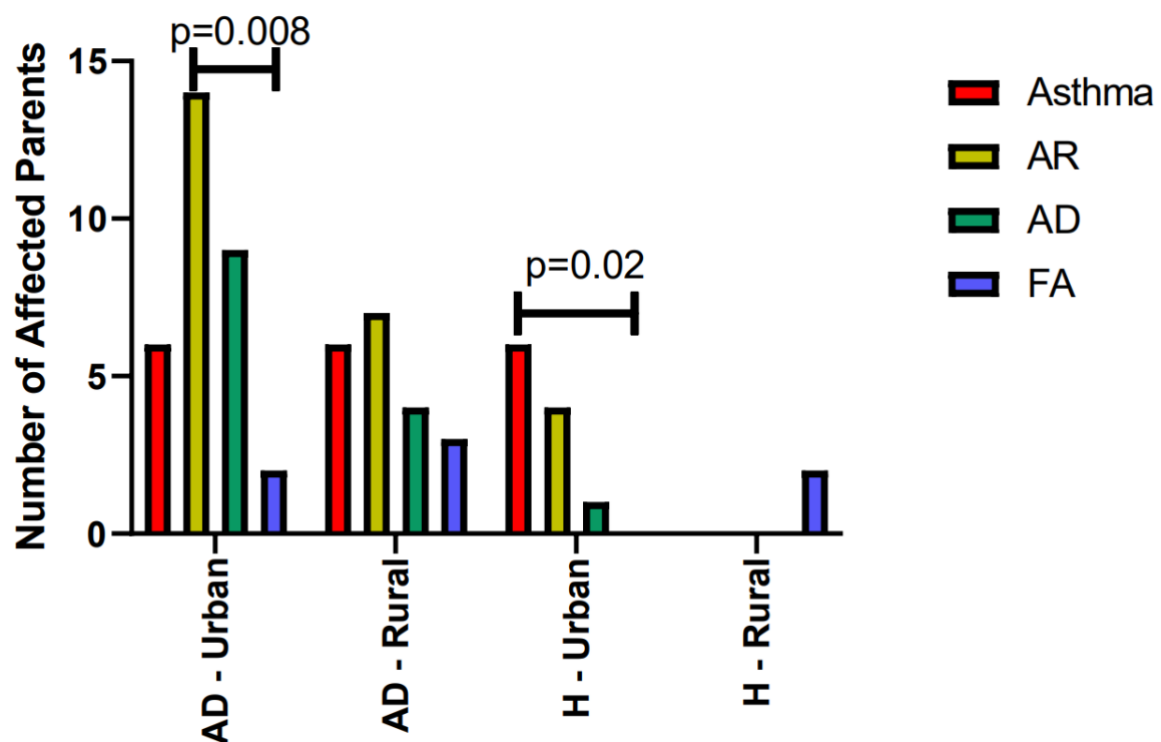
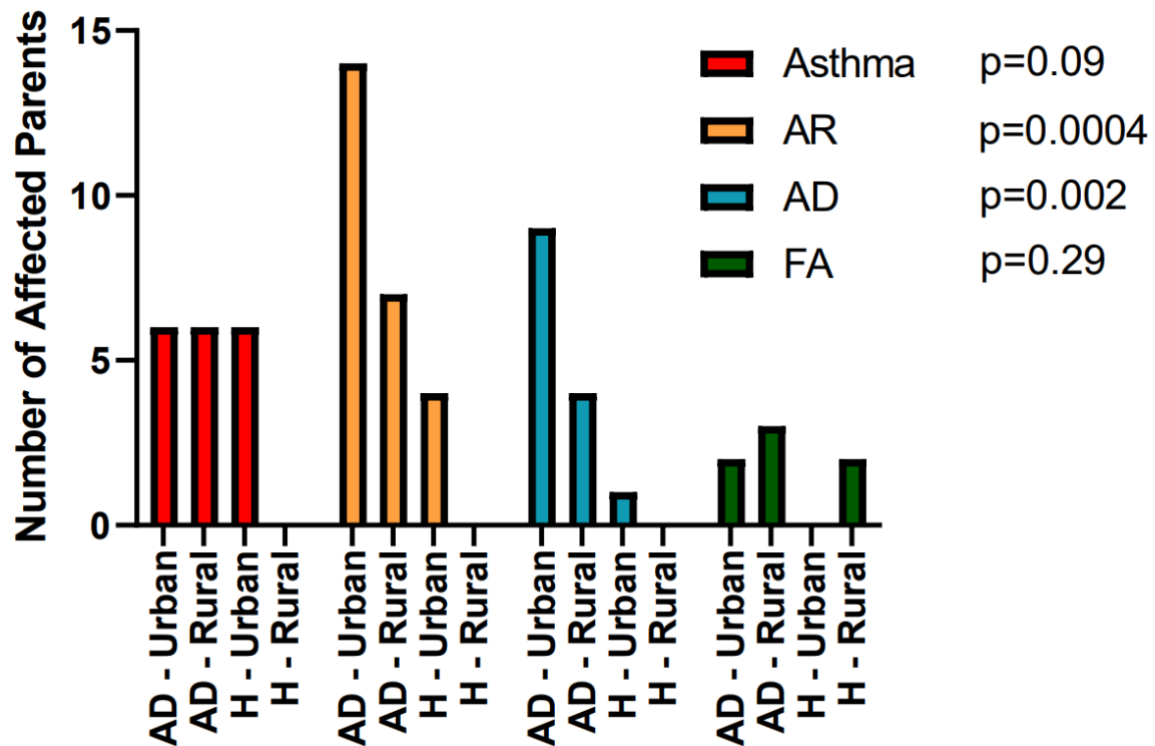


Figure 2.3b Parental atopic disease in AD and healthy children. The number of parents per group is indicated on the y-axis. Parental AR and AD rates are significantly different between the four groups.



Rural and urban children with AD presented with moderate to severe disease, and objective SCORAD scores were comparable between the children with AD from urban and rural settings (**Figure 2.4**). In addition, the age at which AD was first diagnosed did not impact AD severity as measured by SCORAD in both the urban and rural cohorts (**Figure 2.5**).

The median age of onset of AD was six months in the urban area and five months in the rural area. The 25th percentile was 3 months for urban and rural AD, and the 75th percentile was 6.75 months and 7.75 months for urban and rural AD, respectively. The median age of onset of food allergy reactions was 8 months and 8.5 months in the urban AD

and rural AD groups, respectively. The 25th percentile was 6.5 months and 5.3 months in the urban AD and rural AD groups, respectively. The 75th percentile was 16.5 and 11.8 months in the urban AD and rural AD groups, respectively. The median age of allergic rhinitis symptoms was 12 months in urban and rural AD groups. The 25th percentile was 8.5 months and 12 months for urban and rural AD groups, respectively. The 75th percentile was 18 and 24 months for urban and rural AD groups, respectively. There were no statistically significant differences in the distribution of the medians or means of the age of onset of AD, food allergy and AR between the AD groups. However, it is evident that the trend of the age of onset follows the trajectory of the 'atopic march' (**Figure 2.6**).

Figure 2.4. SCORAD scores in AD patients from urban and rural settings. The median is indicated by the dashed line.

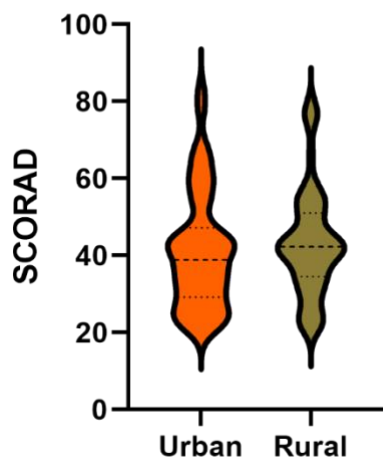


Figure 2.5. SCORAD scores in AD patients from urban and rural settings were similar for children diagnosed earlier (0-5 months) compared to those with a later diagnosis (>6 months). The median is indicated by the dashed line.

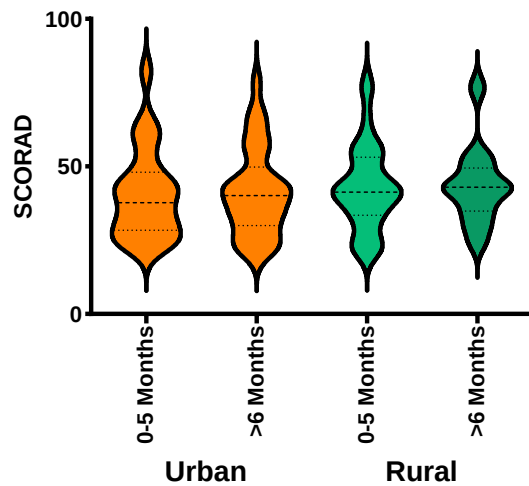
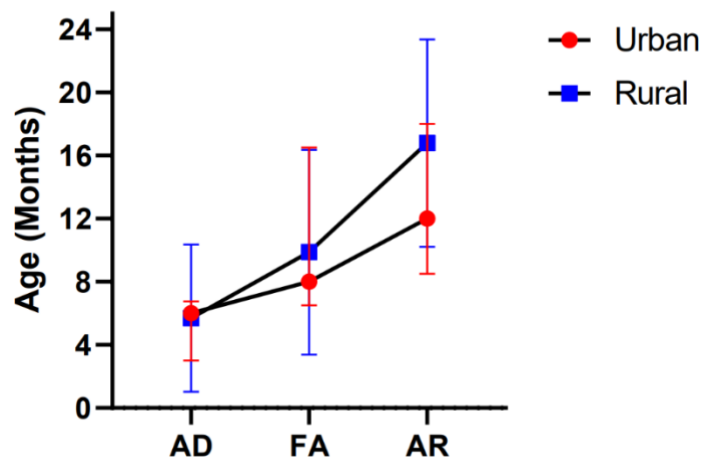


Figure 2.6: Median age of disease onset for atopic dermatitis (AD), food allergy (FA) and allergic rhinitis (AR) in urban and rural children.



Personal atopic comorbidities, Skin Prick Test reactivity and food tolerance

Thirteen urban and eight rural participants with AD reported reacting to 1 or more foods.

There was no difference in the proportion of reported food reactions ($p=0.17$) between the

groups. Four urban participants with AD and one rural participant with AD reported reactions to 2 or more foods. Food reaction frequency to specific food types was similar in urban and rural AD.

A significantly higher proportion of self-reported allergic rhinitis was found in 15 urban AD participants compared to 5 reported by rural AD participants ($p=0.009$). Only 1 urban AD participant reported a diagnosis of asthma, and there were no reports of asthma in the rural AD group.

SPT reactivity rates for rural and urban children with AD were similar for the majority of the allergens tested. However, reactions to fresh egg and egg reagent were more common in urban AD ($p < 0.05$) (**Table 2.3**). Of all AD participants with skin prick testing performed, 57% of rural AD and 74% of urban AD tested SPT positive for any food. Although more urban ADs were sensitised to foods, this was not statistically significant ($p=0.068$). Age of AD diagnosis was not associated with any differences in food allergen SPT positivity when comparing infants with an onset of AD ≤ 6 months and those with an onset of AD > 6 months. There were also no differences in reported food tolerance (i.e., no clinical reaction in the presence of IgE sensitisation) between rural and urban groups. Within those patients with positive SPT reactions, the allergen preparation that induced the largest SPT wheal response was fresh egg, followed by peanut reagent (**Figure 2.7**). None of the urban or rural healthy controls were SPT positive to food antigens, as required for entry into this study. Notably, the wheal diameter in response to the positive control (histamine) was significantly increased ($p < 0.001$; ANOVA) in urban and rural children with AD (median 5.5 mm and 5.0 mm respectively) compared to urban and rural healthy children (median 4.0 mm and 4.0 mm respectively), as seen in Figure 2.8. SCORAD scores correlated with skin prick test

reactivity to egg reagent ($p=0.004$) and fresh egg ($p=0.005$) in urban AD but not rural AD.

(Figure 2.9a, b) No significant correlations were observed with other food allergens.

Figure 2.7. SPT Wheal Diameter to Allergen Challenge

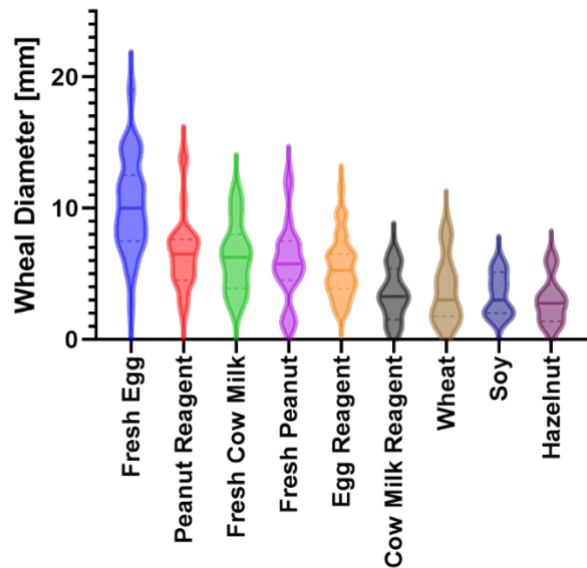


Figure 2.8. Wheal Response to Histamine positive control

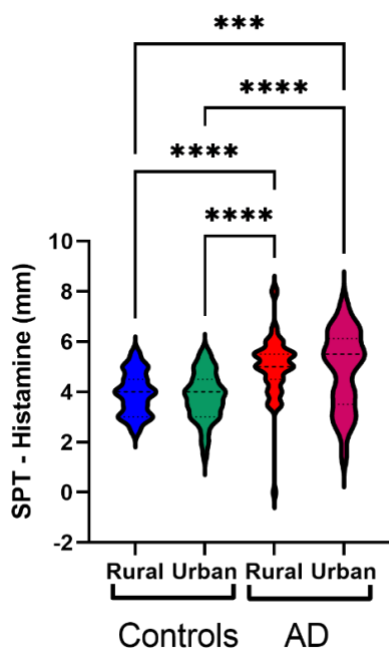


Table 2.3. Food allergy reactions, Skin Prick Test Positivity and allergen tolerance

	Rural AD(n=60) Food reaction	Urban AD(n=56) Food reaction	Rural AD SPT (+)	Urban AD SPT (+)	Rural Tolerant	Urban Tolerant
Fresh Egg*	4 (7%)	8 (14%)	*23 (42%)	34 (63%)	9	10
Egg White Reagent*			*14 (26%)	24 (44%)	9	10
Peanut Reagent			14 (26%)	12 (22%)	4	7
Fresh Peanut	1(2%)	3(5%)	11 (20%)	11 (20%)	4	7
Fresh Cow Milk	5(8%)	1(2%)	4 (7%)	6 (11%)	1	5
Cow Milk Reagent			1 (2%)	3 (5%)	1	5
Wheat	3	1	1 (2%)	5 (9%)	1	3
Hazelnut	1	1	2 (4%)	4 (7%)	0	1
Soy	0	1	1 (2%)	3 (5%)	1	2
Fish	2	3	0	1 (2%)	0	1
Any Food SPT+ Response			31 (57%)	40 (74%)		

*p<0.05 Urban Atopic Dermatitis (AD) versus Rural AD

Figure 2.9a Correlation between SCORAD scores and wheal reaction size to egg reagent skin prick test (SPT). p values estimated using linear regression.

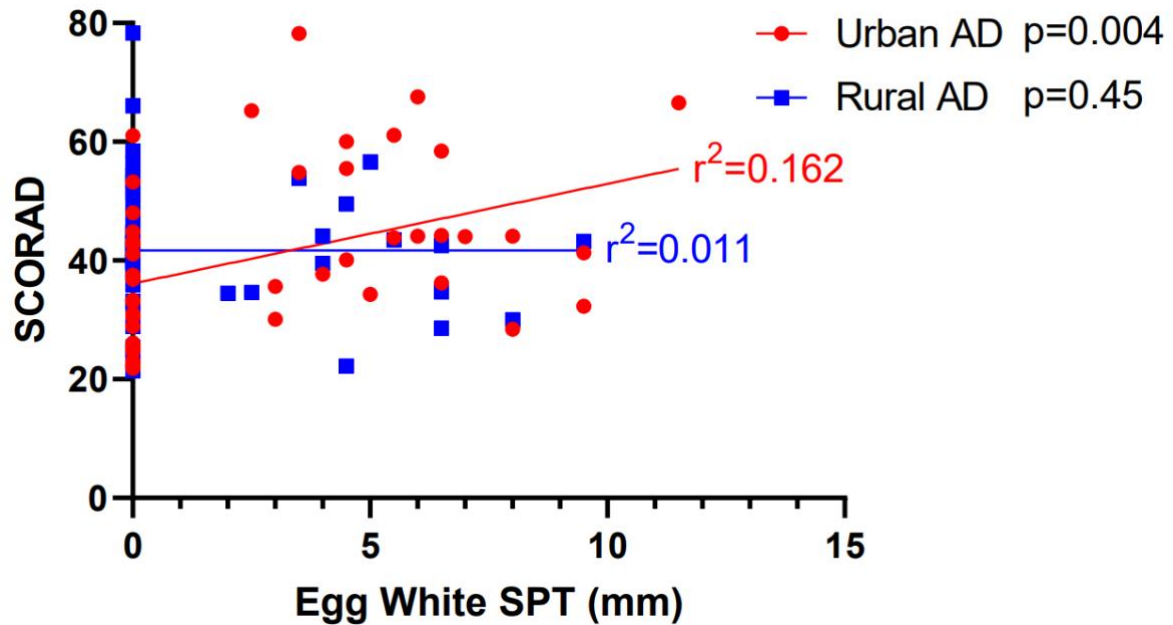
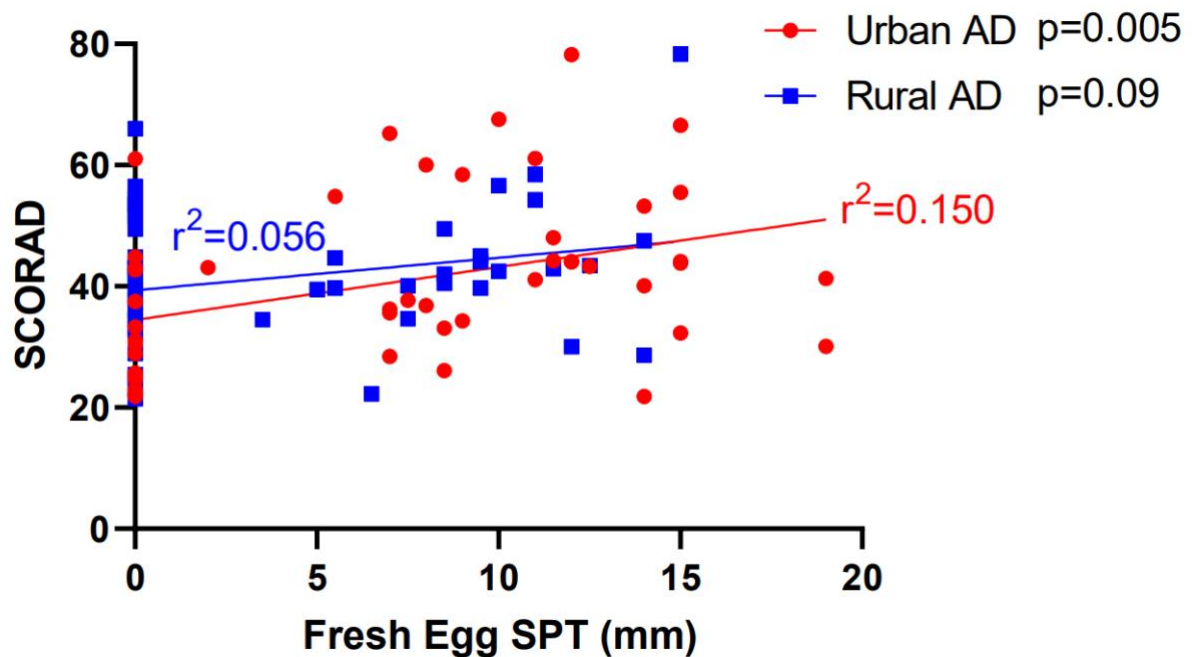


Figure 2.9b Correlation between SCORAD scores and wheal reaction size to fresh egg SPT. p values estimated using linear regression.



Urban and Rural Living Environments

The children's living environments differed depending on their home location (**Table 2.4**).

Parents of healthy rural children had a lower level of formal education, and their children were less frequently consuming Amasi (a fermented cow's milk drink) than all other groups.

For both rural AD and rural healthy children, average household income was lower with a larger average family size, normal vaginal delivery (NVD) was more frequent, they were exposed to more sunlight in summer and winter, received paracetamol at a younger age, were more frequently exposed to cats, dogs, farm animals antenatally and during early childhood, compared to their urban counterparts. Rural AD and healthy control children were less likely to be exposed to cigarette smoke in the home. However, the rural healthy controls were more likely to live in a home using a paraffin stove or an open fire for cooking than their urban counterparts.

Table 2.4. Rural and Urban Living Conditions and Environmental Exposures

	Rural AD (n=60)	Rural HC (n=52)	Urban AD (n=56)	Urban HC (n=49)
Parental Education Level ⁺⁺⁺	14.7 (1.9)	12.1 (3.2)*	14.9 (1.9)	14.4 (1.7)
Household Income (USD per month) ***	499 (474)*	153 (149)*	560 (484)	576 (561)
Household size*	6.1 (2.7)*	6.9 (3.0)*	5.2 (2.0)	5.5 (2.6)
Family history of allergy*	18 (30%)*	4 (8%)*	27 (48%)	12 (24%)
Breastfeeding (Ever)	48 (80%)	45 (86%)	45 (80%)	41 (84%)
Duration of exclusive breastfeeding (Months)	2.5 (2.4)	3.1 (2.5)	3.2 (2.4)	2.6 (2.4)
Duration of breastfeeding (Months)	6.5 (6.4)	7.0 (7.1)	8.5 (8.4)	7.4 (8.6)
Delivery Mode*	45 (75%) NVD*	42 (81%) NVD*	32 (57%) NVD	29 (59%) NVD
Sunlight Exposure	3.1 (2.5)*	4.3 (2.4)*	0.9 (1.0)	1.0 (1.0)

(Hours per day): Winter***				
Sunlight Exposure (Hours per day): Summer***	4.7 (2.8)*	6.3 (3.2)*	2.0 (1.7)	2.1 (1.5)
Antibiotics exposure (Ever)	49 (82%)	42 (81%)	42 (75%)	33 (68%)
Age of first antibiotic exposure (Months)	4.6 (2.2)	5.9 (3.0)	6.7 (2.7)	5.9 (3.3)
Paracetamol exposure (Ever)	58 (97%)	52 (100%)	53 (95%)	42 (85%)
Age of first paracetamol exposure (Months)*	4.1 (1.9)*	4.7 (2.5)*	5.1 (2.1)	6.1 (2.9)
Amasi exposure (Ever) ***	55 (92%)	34 (65%)*	50 (89%)	42 (86%)
Age of Amasi exposure (Months)	11.0 (3.9)	11.4 (4.2)	10.8 (4.1)	12.7 (5.6)
Unpasteurized Milk	1 (2%)	0	2 (4%)	3 (6%)
Cat exposure*	14 (23%)*	40 (36%)*	3 (5%)	5 (10%)
Dog exposure***	34 (57%)*	42 (81%)*	14 (25%)	14 (29%)
Farm animal exposure as a Child***	42 (70%)*	52 (100%)*	2 (4%)	0
Farm animal exposure during Pregnancy***	45 (75%)*	51 (98%)*	4 (7%)	0
Smoking in the Home**	10 (17%)*	11 (21%)*	18 (32%)	23 (47%)
Cooking – Electricity/Gas***	58 (97%)	11 (21%)*	56 (100%)	49 (100%)
Cooking – Paraffin Stove***	6 (10%)	31 (60%)*	0	0
Cooking – Open Fire Outside House***	14 (23%)	37 (71%)*	0	0
Heating – Kerosene/Paraffin***	47 (78%)	20 (38%)*	32 (57%)	36 (73%)
Heating – Wood/Coal***	4 (7%)	24 (46%)*	4 (7%)	0
Heating - Electricity	6 (10%)	3 (6%)	17 (30%)	11 (22%)
Heating – Gas	0	0	3 (5%)	3 (6%)
Heating – None	2 (3%)	4 (8%)	0	2 (4%)

* $p < 0.05$ All Rural Children (AD and healthy) versus All Urban Children (AD and healthy)
** $p < 0.01$ All Rural Children (AD and healthy) versus All Urban Children (AD and healthy)
*** $p < 0.001$ All Rural Children (AD and healthy) versus All Urban Children (AD and healthy)
+++ $p < 0.001$ Rural Healthy Children versus Urban Healthy Children

* Indicates significant data.

NVD – normal vaginal delivery

Continuous data is presented as mean (standard deviation), while categorical data is presented as an n value (% of group).

DISCUSSION

All AD participants in this study fulfilled the UK working party criteria, which suggests that the diagnostic criteria perform reasonably well in a hospital-based setting. However, it had low sensitivity and positive predictive value in a South African study that sought to validate these criteria in a community-based setting.⁴⁸ History of flexural AD and visible flexural AD are universally present. Only 31% of rural and 48% of urban AD reported a positive family history of atopy. Therefore, in the main, these are breakthrough AD cases in families with no previous history of atopy (**Table 2.2**). Significantly more urban participants with AD reported a positive family history of atopy than rural AD participants ($p=0.02$), suggesting heredity played a lesser role in AD development in rural children (**Figure 2.1**). Also, some healthy participants in both settings reported having first-degree relatives with allergic diseases. However, this did not translate into AD or any other allergic disease in the index participant. Urban healthy participants had significantly higher reports of family atopy than rural healthy participants ($p=0.03$). When AD participants in rural and urban settings are compared with their corresponding healthy counterparts, there are significantly higher reports of familial atopy in both the rural and urban AD groups ($p=0.0007$; $p=0.0008$), respectively. This

suggests that the urban environment and familial atopic history independently confer risk for AD, as well as potentially interacting synergistically.

The observation that only two urban AD children and three rural AD children had a history of atopy in both parents suggests that biparental atopy plays a lesser role in AD risk in this cohort, contrary to other studies that have described more atopic disease in offsprings with a history of atopy in both parents.⁴⁹ Maternal atopy was also notably the main difference in familial atopy when urban AD was compared to rural AD ($p=0.04$) and the urban healthy group was compared to the rural healthy group ($p=0.005$). Whilst familial aggregation of allergic disease is well established, the results of various epidemiology studies have reported stronger associations of childhood atopic disease with maternal rather than paternal atopy,⁵⁰ similar to our findings. The epigenetic phenomenon of genomic imprinting such that a gene conferring atopy susceptibility on the offspring is expressed on the maternally acquired chromosome; maternally acquired transgenerational epigenetic transmission and shared mother-and-child environmental exposures, especially in early childhood and maternal influences during pregnancy may account for the apparent more influential maternal pattern of heritability.⁴⁰ Others have also observed that the type of parental allergic disease can be associated with AD in the offspring.⁵¹ A critical limitation of our study is that mothers usually completed questionnaires, and therefore paternal atopic symptoms may be underreported or misclassified.

Of the participants with parental atopy, there were no differences in parental asthma or food allergy rates. However, the groups had significant differences in parental allergic rhinitis rates and parental AD rates. Other reports have also suggested that a history of AD in one or both parents strongly predicts offspring AD⁵¹. However, our results are only partially consistent with previous studies, which suggested that allergic rhinitis in a parent

was a minor factor in AD development in the offspring.⁵¹ Our data further supports the likelihood that even the AD children with parental atopy represent breakthrough atopic disease similar to the AD participants without parental atopy.

AD participants were classified as having moderate to severe AD according to the objective SCORAD. The European Task Force on Atopic Dermatitis (ETFAD) has noted that the SCORAD index may be heavily influenced by parents' and caregivers' divergent gradings of subjective components.⁴⁵ Furthermore, the subjective grading provides a general assessment of disease severity, but for monitoring more granular changes in disease severity, it is, therefore, preferable to concentrate on objective scores. The SCORAD index is the most widely validated and published disease severity scoring tool. However, it has been shown to have limitations in accurately quantifying disease severity in darkly pigmented skin. It has been shown that well-validated and widely used clinical scores such as SCORAD, Six Area Six Sign, Atopic Dermatitis (SASSAD) and EASI, which are reliant on the detection of erythema, grossly underestimate disease severity in darkly pigmented skin compared to those with lighter phototypes.⁵² This limitation is not mitigated by clinicians who are well experienced in assessing patients with Fitzpatrick skin-type V-VI.⁵³ Notwithstanding these limitations, we used SCORAD as we performed this study in a single ethnic population where phototypes would be comparable.

AD is often associated with an increased risk for allergic responses to food and aeroallergens and an increased risk of developing other allergic diseases later in life.⁵⁴ This is likely mediated in part by the defective epidermal barrier of AD. We have observed an atopy trajectory that is in keeping with the established concept of the atopic march, unlike observations made in other geodiverse settings/tropics.⁴³ This suggests that genetic background may play a more critical role than geographic/climate-related factors in

determining disease trajectory in our setting. A large number of the children with AD in this study had positive SPT responses to food allergens. More urban children with AD (40) than rural children with AD (31) were sensitised to at least one food allergen. Whilst there was a statistical trend ($p=0.068$), the potential mitigating effects of rural living on atopy risk could not fully protect AD children from being sensitised to food allergens. The most frequent sensitisation was to fresh egg. Egg SPT also induced the largest wheal diameter in those that were sensitised. Interestingly, the urban AD group was more likely to be sensitised to egg, potentially suggesting differences in egg exposure within the home. This is further supported by SCORAD scores being positively correlated with urban AD egg reactivity but not rural AD, although the SCORAD scores were comparable between the 2 groups. Interestingly, rural and urban children with AD had larger wheal responses to histamine, compared to healthy children, demonstrating the heightened responsiveness of the AD skin to mast cell-derived products such as histamine. This may be mediated via increased expression of histamine and its cognate receptors in AD skin which concurs with previous reports on the role of histamine in AD pathology,⁵⁵ or reduced capacity to degrade histamine enzymatically.

Within this study cohort, it is clear that moderate-severe AD exists in children from both rural and urban locations within South Africa. While disease severity is similar for children with AD recruited to this study, the living environments are vastly different. Exposure to animals, cooking or heating fuel type and lower socioeconomic status were associated with the rural setting. Animal exposure may have critical immune effects due to microbial and non-microbial factors, which will be explored later in this thesis.⁵⁶⁻⁵⁸ Exposure to indoor and outdoor air pollutants and socioeconomic factors may exert themselves on disease development and persistence.^{39,42,59-63} Thus, it will be necessary to follow up and continue

to examine the rural children regarding the development of inflammatory disorders over the coming years.

CONCLUSIONS

AD in South African children resembles the phenotypic hallmark features of itch, dry skin and flexural disease and is associated with food allergen sensitisation. AD in our setting seems to arise from factors other than heritability of disease. Additionally, despite differences in socioeconomic status and environmental exposures, both urban and rural children manifest with clinically similar AD patterns and severity. However, more detailed data may become evident as molecular immune mechanisms of disease are examined in subsequent chapters.

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CHAPTER 3

ALLERGEN-SPECIFIC ANTIBODY SENSITIZATION PATTERNS IN URBAN AND RURAL CHILDREN WITH AD

INTRODUCTION

Allergy represents an inappropriate activation of the immune system to innocuous, non-infectious, or non-damaging antigens, which results in local and systemic responses that significantly impact tissue function and homeostasis. One key molecule the immune system produces that contributes to allergic pathology and symptomatology is immunoglobulin IgE. IgE is a unique subclass of antibodies that do not fix complement. However, it binds to high-affinity receptors (e.g., FcεR1) on effector cells such as mast cells, basophils, and various other cell types.¹ Under the influence of type 2 immune factors (such as IL-4), antigen presentation to B cells produces antigen or allergen-specific IgE.^{2,3} In an allergic individual whose tissue mast cells already have antigen-specific IgE bound to FcεR1, re-exposure to the antigen results in cross-linking of FcεR1-bound IgE on the cell surface, which triggers mast cells and basophils to secrete a diverse range of biologically active products.⁴ Preformed products in cytoplasmic granules (e.g., histamine, proteases, lipid mediators) are released quickly following degranulation, while other effector molecules (e.g., cytokines and chemokines) are newly generated and secreted over a more extended period. Local production of these mediators within tissue results in increased vascular permeability, epithelial barrier degradation, itch, recruitment, and activation of inflammatory cells, while the systemic release of these mediators can result in anaphylaxis.⁵⁻⁸ IgE stabilises the expression of FcεR1 on the mast cell surface and influences long-term mast cell survival. Thus, quantification of allergen-specific IgE is a helpful indicator of sensitisation, whereby an individual has the potential to react to a given allergen.

In contrast to IgE, the IgG4 subclass of antibodies is considered to have anti-inflammatory activity and may protect against anaphylaxis and allergic inflammation. IgG4 has a low

affinity for the classical Fcγ receptors, does not activate complement, and IgG4 cannot cross-link antigens on mast cells.^{9,10} High-dose allergen exposure (e.g., in cat owners or beekeepers) usually results in peripheral immunological tolerance accompanied by increased allergen-specific IgG4 antibodies.¹¹ The production of IgG4 by human B cells is strongly enhanced by IL-10.^{12,13} In addition, increased production of allergen-specific IgG4 antibodies is frequently observed in allergic patients receiving allergen-specific immunotherapy (AIT).¹⁴

Competition between IgE and IgG4 may be critical in determining the effector immune response to allergen challenge. Allergen-specific IgG4 antibodies can act as blocking antibodies by competing with IgE for allergen binding to IgE receptor-expressing mast cells and basophils. IgG4 levels were shown to be highest in cow's milk atopic subjects who could tolerate the consumption of cow's milk. At the same time, the egg white-specific serum IgE/IgG4 ratio in egg-sensitised children was important for predicting reactivity to egg during food challenges.^{15,16}

OBJECTIVE

To evaluate IgE sensitisation patterns to common food and relevant aeroallergens in South African children with AD in rural and urban environments.

To determine if IgG4 allergen-specific responses are similarly induced in rural and urban settings.

To evaluate the positive predictive value of recommended SPT and specific IgE cut off levels for diagnosing food allergy

METHODOLOGY

Allergen-Specific Immunoglobulins

Peripheral blood was drawn from children and collected in lithium heparin tubes. Plasma was obtained following centrifugation and was stored at -80 °C until batched analysis. Total IgE, allergen-specific IgE and allergen-specific IgG4 were measured from participant samples (n=152) using singleplex ImmunoCap, according to manufacturer's instructions (Thermo Fisher Scientific/Phadia, Uppsala, Sweden). Briefly, ImmunoCAP™ is a fluoroenzyme immunoassay for in vitro quantification of circulating antibody levels and can detect a wide range between 2 to 5000 kU/L. Anti-IgE covalently bound to ImmunoCap reacted with IgE antibodies in the plasma samples. The samples were washed, and enzyme-labelled anti-IgE antibodies were added to form a complex. The samples were incubated at 37 °C, unbound anti-IgE antibodies were washed away, and the bound complex was then incubated with a developing agent. Thereafter the reaction was stopped, and the fluorescence of the eluate was measured. The fluorescence is directly proportional to the concentration of IgE in the sample. The reactions generated for each sample were transformed to quantifiable concentrations using a calibration curve.

The samples were run on a fully automated high throughput Phadia Laboratory instrument using 40ul of patient samples in single determinations. ImmunoCAP egg allergen f245; ImmunoCAP hazelnut allergen; ImmunoCAP fish(cod) allergen f3; ImmunoCAP wheat allergen f4; ImmunoCAP milk allergen f2; ImmunoCAP soybean allergen f14; ImmunoCAP peanut allergen f13 and ImmunoCAP house dust mite allergen d1 were used for quantification of specific IgE and IgG4 antibodies. However, Bos d 8 Casein IgG4 antibody was used instead of ImmunoCAP milk allergen f2 as this test does not perform well technically due to the presence of interfering bovine IgG in the allergen preparation

(personal correspondence from ThermoFisher, Uppsala, Sweden). Furthermore, Bos d 8 casein IgG4 was selected as opposed to Bos d 4 or Bos d 5 milk component allergen as it is the most common and persistent allergenic component of cow's milk, especially in children with atopic dermatitis.¹⁷ In addition, evaluation of Bromelain MUXF3 cross-reactive carbohydrate determinants (CCD) IgE was conducted to measure the presence of antibodies to glycoprotein carbohydrates from ubiquitous allergens from plants and invertebrates as these can confound positive specific IgE antibody tests to a wide variety of clinically relevant allergens. The MUXF3 carbohydrate epitope obtained from pineapple bromelain glycoprotein is used as cases of IgE sensitisation reflecting true clinically relevant allergy to the pineapple bromelain glycoprotein are rarely found, and this has become a well-established biomarker for assessing the presence of IgE antibodies that interact with CCD.

Statistical Analysis

Levels of analytes in samples were not normally distributed (Shapiro-Wilks test), and statistical significance between two groups was determined using the Mann-Whitney test (Prism GraphPad Software version 7TM). Multiple group comparisons were analysed using the Kruskal-Wallis test, while differences in proportions between groups were estimated using Fisher's exact test.

RESULTS

IgE Levels

In keeping with the atopic association of AD, total IgE levels were highly elevated in both rural and urban AD groups compared to healthy controls (**Figure 3.1**). Specific IgE levels to food allergens (egg, milk, peanut, soybean, hazelnut, wheat, and fish (cod)) were elevated in

the AD groups compared to their healthy non-allergic counterparts (**Figure 3.2**). In healthy donors, egg-specific IgE levels were higher in healthy urban controls than in healthy rural controls. In comparison, milk-specific IgE levels were higher in rural healthy controls than in urban healthy controls (**Figure 3.2**). The timing of solid food introduction was comparable between children from both rural and urban settings, with solid food introduction occurring between 4-6 months of age. In addition to food allergens, IgE levels specific to the house dust mite (HDM) *Dermatophagoides pteronyssinus* was highly elevated in children with AD (**Figure 3.3**). Finally, we quantified the levels of IgE against cross-reactive carbohydrate determinants (anti-CCD) to determine if cross-reactive IgE to common sugar residues might interfere with interpreting IgE responses to protein allergens. The median anti-CCD IgE levels were higher in children with AD than in their non-AD counterparts. However, the absolute levels remained low and are unlikely to contribute significantly to the IgE sensitisation patterns to specific allergens described above (**Figure 3.4**).

Figure 3.1. Total IgE Levels in Atopic Dermatitis (AD) and Healthy Control (HC). Median values with interquartile ranges are illustrated

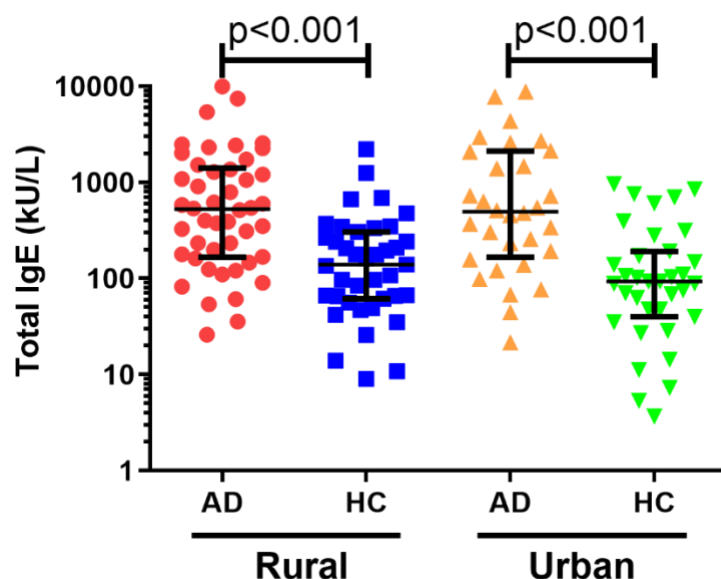


Figure 3.2. Food Allergen-Specific IgE Levels in Atopic Dermatitis (AD) and Healthy Control (HC). Median values with interquartile ranges are illustrated.

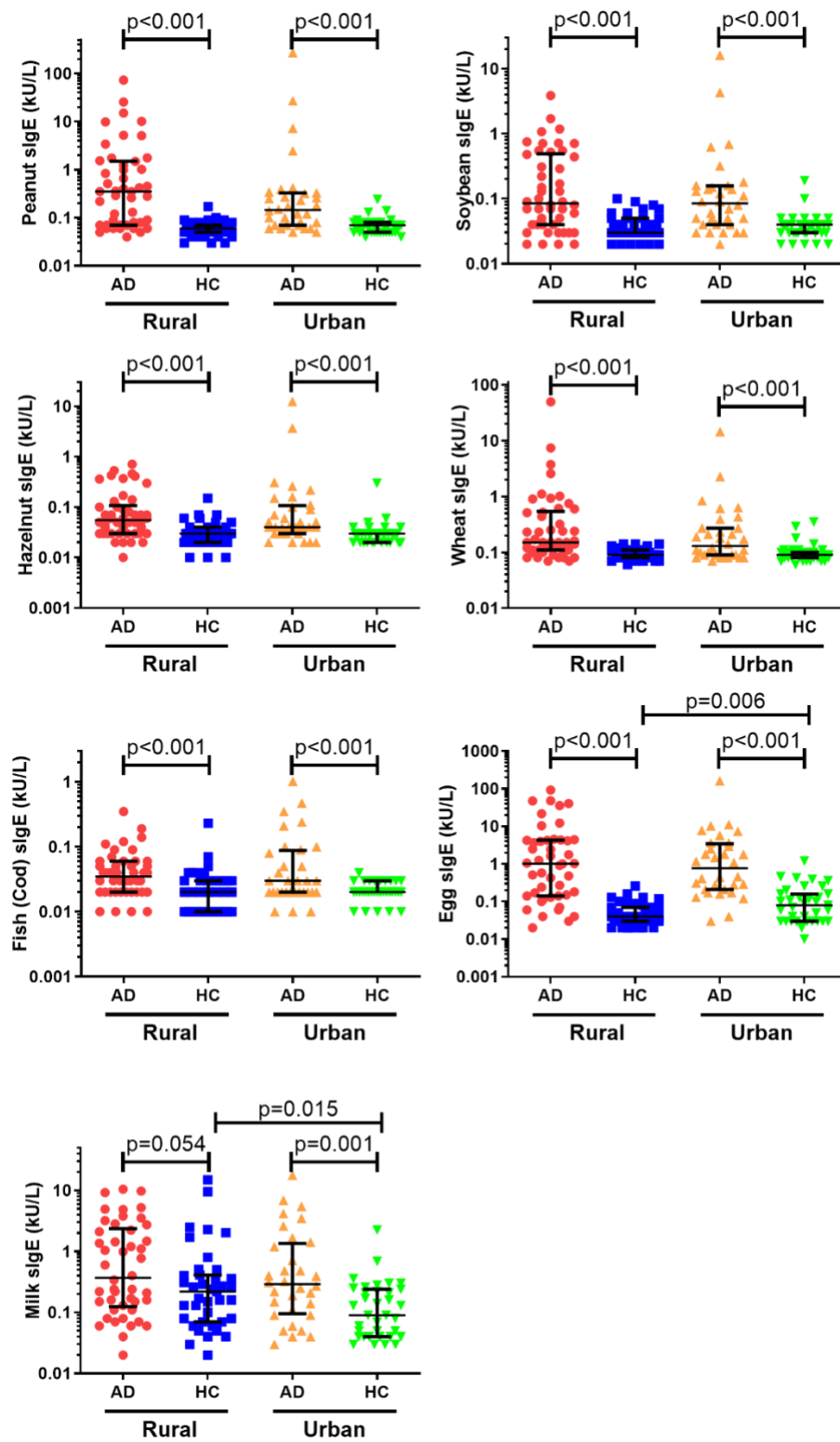


Figure 3.3. *Dermatophagoides pteronyssinus*-specific IgE levels in Atopic Dermatitis (AD) and Healthy Control (HC). Median values with interquartile ranges are illustrated.

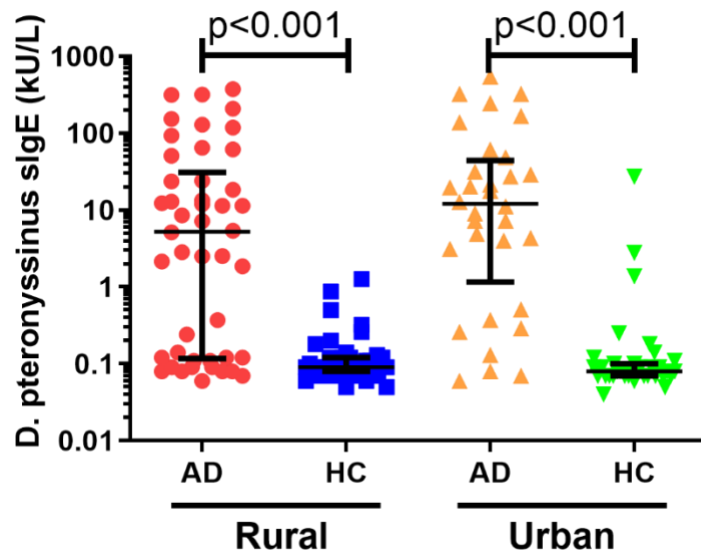
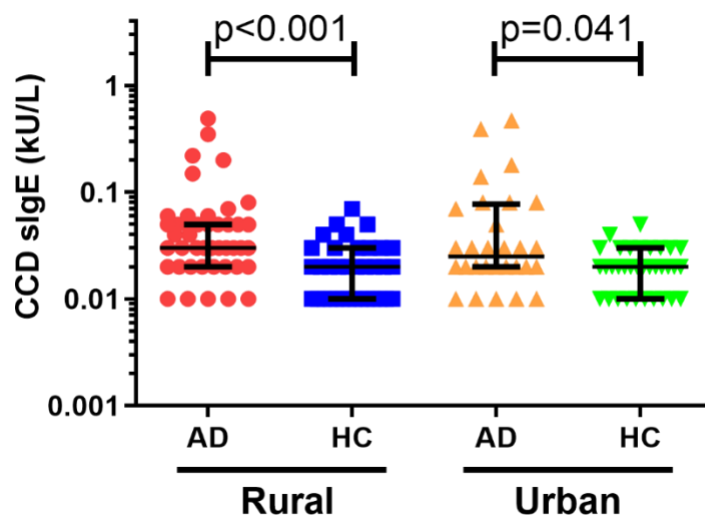


Figure 3.4. Anti- Cross-reactive Carbohydrate Determinants IgE levels in Atopic Dermatitis (AD) and Healthy Control (HC). Median values with interquartile ranges are illustrated.



Using 0.35 kU/L as a cut-off value to indicate sensitisation, the most common food allergens are egg, cow's milk, and peanut, followed by wheat, soybean, and hazelnut in children with AD (**Table 3.1**). Few children displayed an IgE level to fish above the 0.35 kU/L cut-off. Similar sensitisation patterns were seen for children with AD from both locations, except for peanut. Rural children with AD tended to be more frequently sensitised to peanut than urban children with AD (**Table 3.1**). The proportion of children with HDM-specific IgE levels above 0.35 kU/L (65% and 81% of rural and urban AD cases, respectively) was more significant than in any food allergens tested (**Table 3.1**). Urban healthy controls were more likely to be sensitised to egg than their rural counterparts, while rural healthy controls were more likely to be sensitised to cow's milk. However, none of these children had a positive SPT to egg or cow's milk, as described in Chapter 2. Anti-CCD IgE levels were marginally higher than the 0.35 kU/L cut-off in 2 rural and 2 urban children with AD.

Table 3.1. Proportion of children with an IgE result >0.35 kU/L

	Rural AD	Rural HC	Urban AD	Urban HC
Peanut ^{1**}	52%	0%	19%	0%
Soybean ¹	28%	0%	13%	0%
Hazelnut ¹	15%	0%	6%	0%
Wheat ¹	30%	0%	22%	3%
Fish	2%	0%	9%	0%
<i>D. pteronyssinus</i> ¹	65%	8%	81%	9%
Egg ^{1*}	63%	0%	59%	15%
Cow's Milk ^{1*}	50%	31%	44%	9%
Any IgE+ Response	86%	33%	97%	23%

¹p<0.05 Atopic Dermatitis (AD) versus Healthy Control (HC)

**p<0.01 Rural AD versus Urban AD

*p<0.05 Rural HC versus Urban HC

Allergen-Specific IgG4 Levels

All donors had detectable specific IgG4 levels against milk, egg, peanut, hazelnut, soybean and wheat. However, healthy rural control children had the lowest levels of peanut-specific IgG4, soybean-specific IgG4, hazelnut-specific IgG4, wheat-specific IgG4, egg-specific IgG4 and casein bos d8-specific IgG4 compared to all other groups (**Figure 3.5**). Cod-specific IgG4 levels were lower in rural groups than in urban ones (**Figure 3.5**). In contrast to the food allergens, *D. pteronyssinus*-specific IgG4 levels were elevated in both AD groups (**Figure 3.5**).

Allergen Specific IgE: IgG4 Ratio

IgG4 can inhibit IgE binding to mast cells, and a low IgE to IgG4 ratio can indicate immunological tolerance of an allergen, even when an IgE response has been initiated to that allergen. The allergen-specific IgE to IgG4 ratio for egg, milk, hazelnut, soybean, peanut and wheat was lowest in healthy urban controls (**Figure 3.6**). There was no statistically significant difference in the fish-specific IgE: IgG4 ratio between the disease groups or locations. However, two clusters were observed within each group in these fish-specific ratios, which was not observed for the other food allergens examined (**Figure 3.6**). A different pattern was observed for the aeroallergen HDM IgE: IgG4 ratio, where the children with AD in both locations have a higher ratio than their healthy counterparts (**Figure 3.7**).

Specific IgG4 levels over time (age)

It has been noted that the natural course of egg allergy is associated with natural tolerance, where a decline in specific IgE in addition to increasing egg IgG4 occurs.¹⁸ Egg-specific IgG4

levels significantly increased with age in rural AD children ($p=0.02$), and egg-specific IgE levels in this group displayed a decreasing but insignificant trend with age ($p=0.06$). (**Fig 3.8**) This was not observed in rural healthy, urban healthy and in urban AD groups. The relationship between egg-specific IgG4 and egg-specific IgE with age was not significant in these 3 groups. Milk-specific IgG4 and IgE had no significant association with age in all groups. Milk-specific IgE in healthy rural children displayed a marginally increasing trend with age ($p=0.06$). (**Fig 3.9**) Peanut-specific IgG4 and peanut-specific IgE displayed no association with age in all groups. (**Fig 3.10**)

Figure 3.5. Anti- food allergen and *Der. Pteronyssinus* (HDM) IgG4 levels in Atopic Dermatitis (AD) and Healthy Control (HC). Median values with interquartile ranges are illustrated.

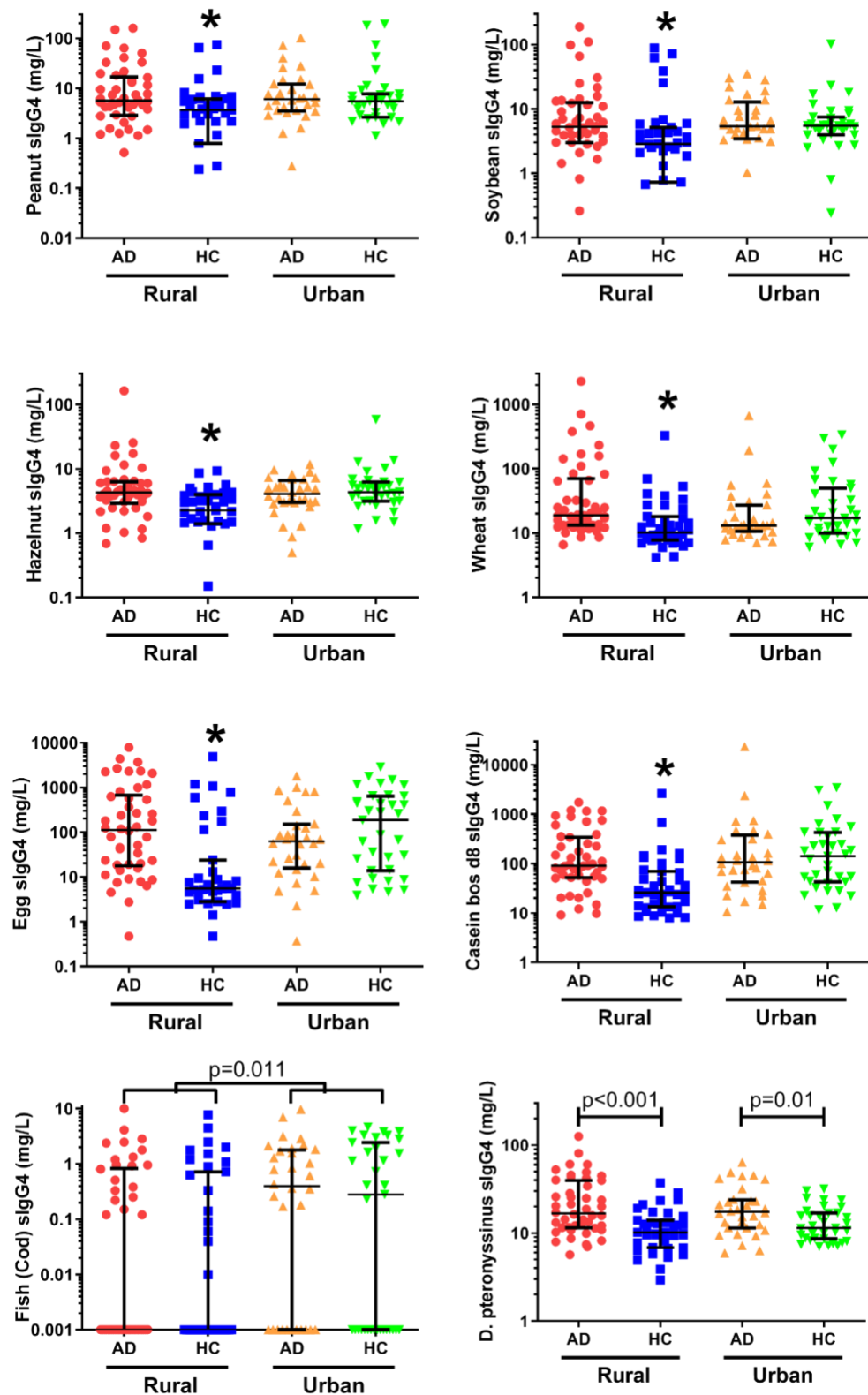


Figure 3.6. Anti-food allergen IgE: IgG4 levels in Atopic Dermatitis (AD) and Healthy Control (HC). Median values with interquartile ranges are illustrated.

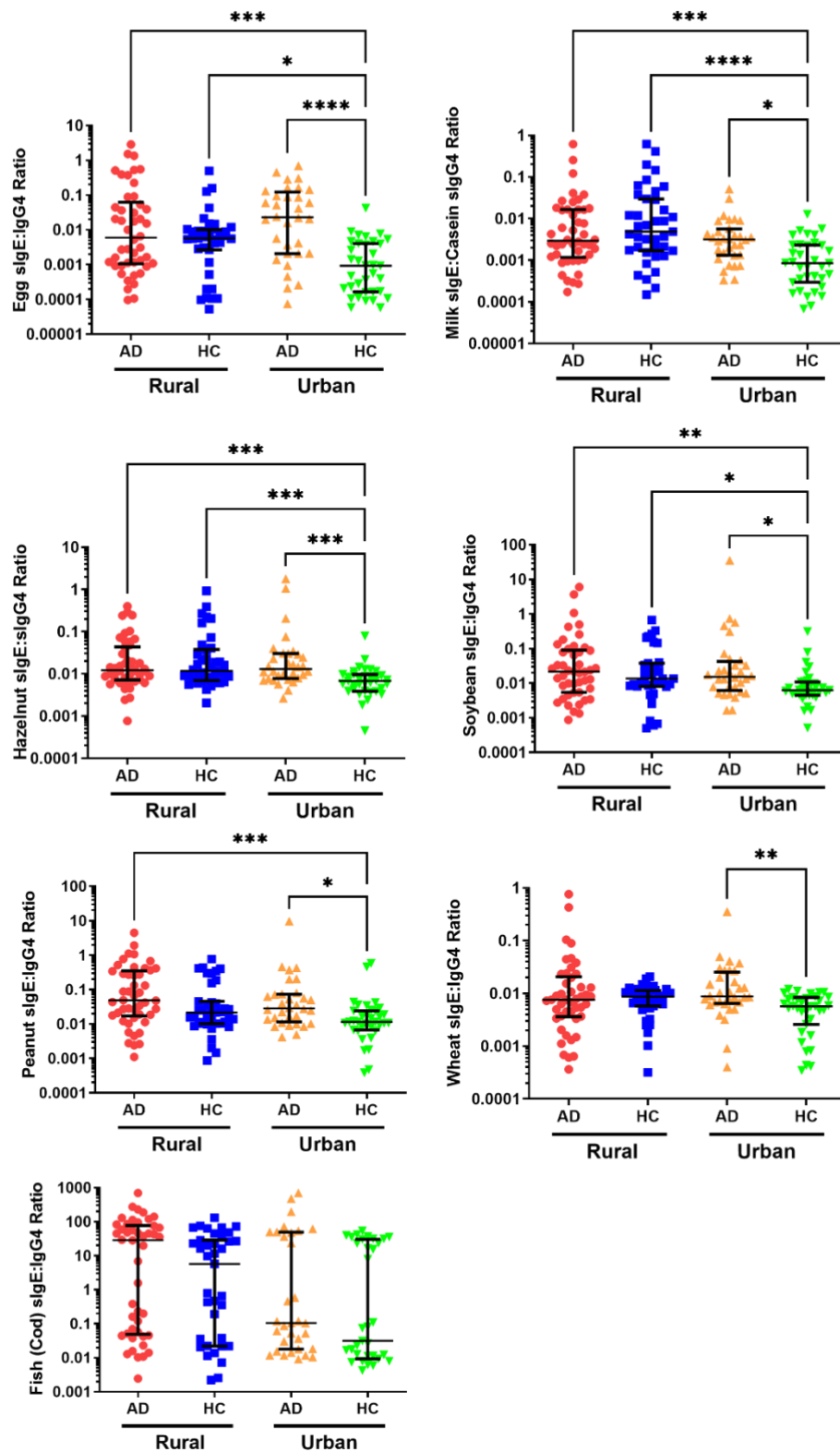


Figure 3.7. Anti-HDM IgE: IgG4 levels.

Median values with interquartile ranges are illustrated.

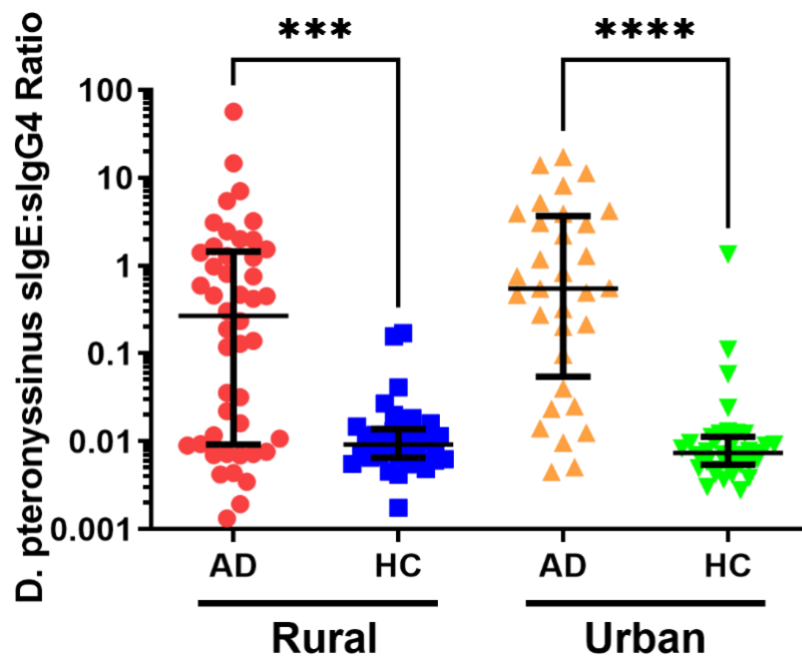


Figure 3.8. Egg-specific IgG4 levels over time (age) and egg-specific IgE over time (age) in each group. *P* value estimated using linear regression.

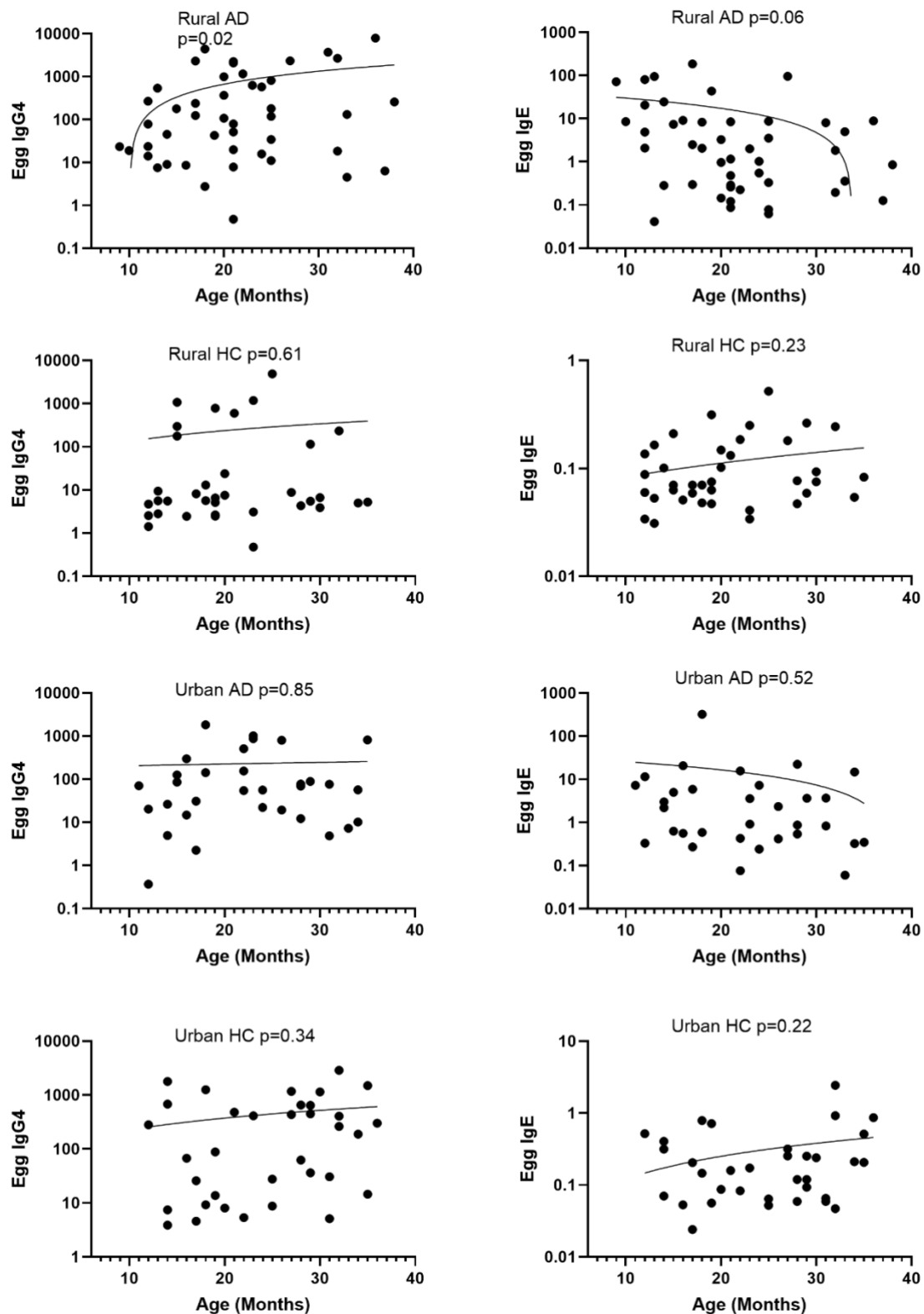


Figure 3.9. Milk-specific IgG4 levels over time (age) and milk-specific IgE over time (age) in each group. *P* value estimated using linear regression.

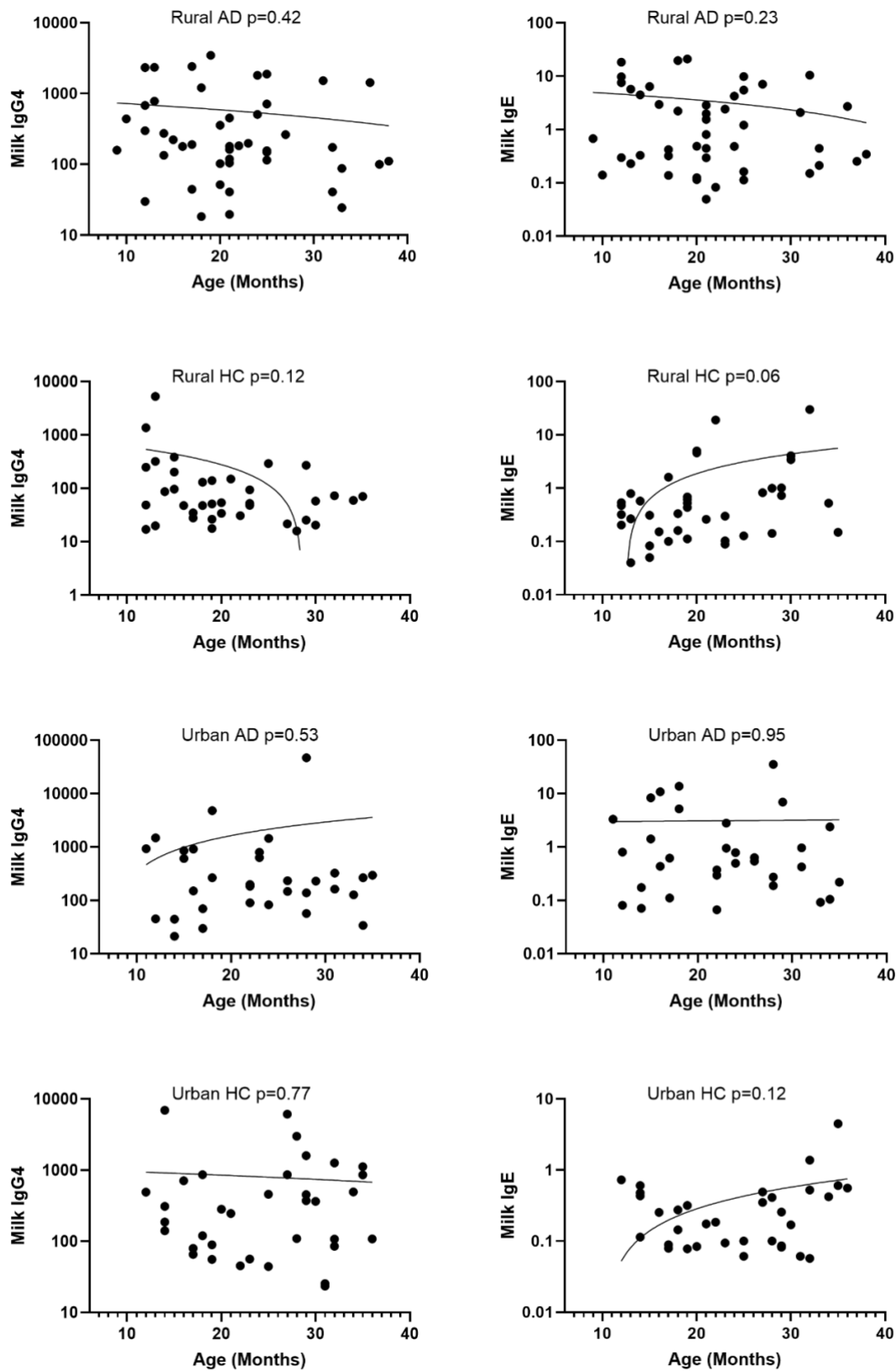
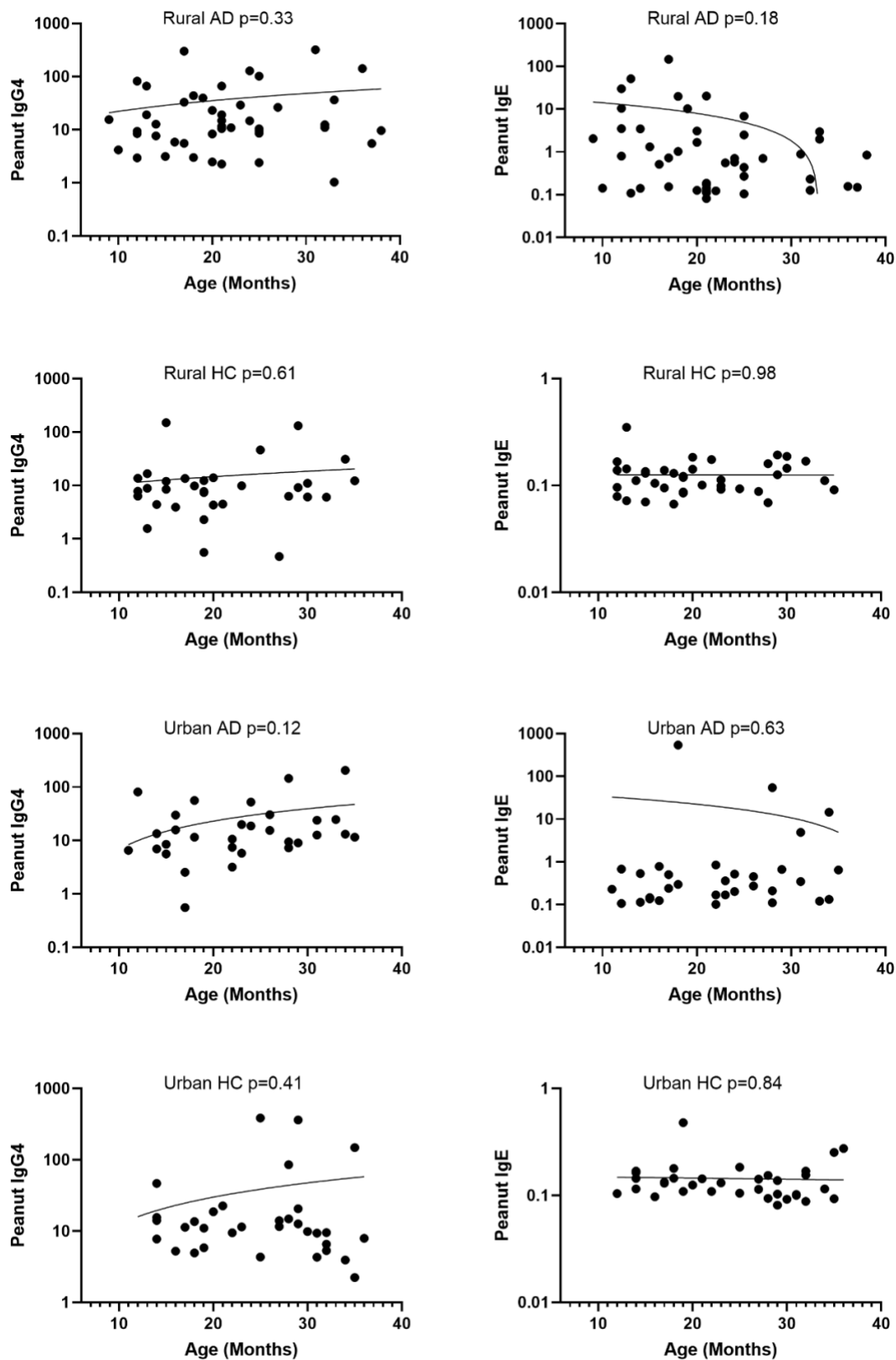


Figure 3.10. Peanut-specific IgG4 levels over time (age) and peanut-specific IgE over time (age) in each group. *P* value estimated using linear regression.



The predictive value of skin prick tests and IgE used to support food allergy diagnosis.

The diagnostic gold standard for food allergies is Oral Food Challenges (OFCs). However, OFCs are time-consuming, and a significant concern is the risk of acute allergic reactions of unpredictable severity. Clinical history is vital to accurately diagnosing food allergies. Improving diagnostic accuracy before referring the patient for an OFC is preferable. The diagnosis of IgE-mediated food allergy can be supported by demonstrable IgE sensitisation by skin prick test (SPT) with a wheal diameter $\geq 3\text{mm}$ greater than the negative control or serum IgE $\geq 0.35\text{kU/L}$, in addition to a clear clinical history. These cut-offs have a high sensitivity and negative predictive value (NPV) but have a low specificity and positive predictive value (PPV). In order to optimise specificity and the probability that a patient has a food allergy, given that SPT/sIgE is positive, diagnostic cut-offs for specific IgE and SPTs with 95% positive predictive values have been reported.

Although it is not within the scope of this study to confirm food allergy status with OFC, based on our detailed clinical history, we were able to classify AD participants with SPT or allergen-specific IgE evidence of IgE sensitisation as "suspected food allergy" if they reported a history of reaction to a specific allergenic food; having never eaten the allergenic food and no longer eating the food. We classified participants as "tolerant" based on a history of regular consumption of age-appropriate portions of the suspected allergenic food.

Positive predictive values for egg

We focused on infants with AD ≤ 2 years as the mean age of the rural and urban AD participants was 21 and 23,5 months, respectively. Furthermore, the utility of positive predictive values in African children over two years of age has been reported elsewhere. Fifty-two infants with AD were ≤ 2 years. Four did not have SPT, which was contraindicated

due to extensive AD/erythrodermic skin. Seventeen infants had positive SPT to egg reagent. Fourteen of these SPTs met the 95% PPV cut-off value of 4-5mm¹⁹ with 9 "suspected egg allergy" and 5 "tolerant" to egg. Three infants had positive SPT that did not meet the cut-off value (1 was classified as "tolerant" to egg, and 2 were classified as having "suspected egg allergy"). Based on this, we conducted the Fisher's exact test and computed a sensitivity of 81%, specificity of 16%, positive predictive value of 64% and negative predictive value of 33%.

26 AD participants had a positive SPT to fresh raw egg. All the positive SPT to raw egg met the 95% PPV cut-off value of 4-5mm. Of these, 11 were "tolerant", and 15 were classified as "suspected egg allergy". Based on this, the SPT 4-5mm yielded a sensitivity of 100% and PPV of 57%.

39 patients with AD, ≤ 2 years of age had positive egg IgE (≥ 0.35 kU/L). 29 AD participants had IgE levels above the 95% PPV cut-off value of 2kU/L for infants ≤ 2 years old¹⁹. Of these, 18 were classified as "suspected egg allergy", and 11 were "tolerant". Ten did not meet the 2kU/L 95% PPV. 2 were classified as "suspected egg allergy" and eight were "tolerant". At the 2kU/L cut-off, these figures yield 90% sensitivity, 80% NPV, 42% specificity and 62% PPV in this cohort.

Positive predictive values for cow's milk

Of the 48 infants with SPTs performed, 1 cow's milk reagent and 4 fresh cow's milk SPTs in AD participants were positive. The 1 positive SPT to cow's milk reagent fell under the 95% PPV of 6mm for infants ≤ 2 years of age¹⁹. The 4 positive SPTs to fresh cow's milk had 1 below and 3 above the cut-off value. However, all these positive SPTs reflect sensitisation as all the participants tolerated cow's milk.

Fifty-two infants had cow's milk-specific IgE measured. 35 of 52 AD cow's milk specific IgE tested were above 0.35kU/L. 11 participants had levels above the 95% cut-off value, i.e. 11 infants ≤ 2 years had cow's milk IgE above 5kU/L¹⁹, and 24 had levels below the cut-off value. All these participants regularly consumed cow's milk and would be classified as tolerant. Therefore, no further clinical investigation was required, and no analysis was done.

Positive predictive values for peanut

11 AD participants of the 48 participants ≤ 2 who had SPTs performed had positive SPT to peanut reagent. Ten had SPT levels above the 95% PPV (4mm in infants ≤ 2 years¹⁹), with 6 of these classified as having "suspected peanut allergy" and 4 "tolerant" participants. 1 SPT was below the cut-off value and was considered "tolerant". This data yield a sensitivity of 100%, NPV of 100%, 20% specificity and 60% PPV.

7 SPTs to fresh peanut were positive, above the 95% PPV cut-off value. Four were classified as suspected peanut allergy, and three as tolerant. This yielded a sensitivity of 100%; and a PPV of 57%.

The recommended 95% PPV for peanut-specific IgE range from 15kU/L to 34kU/L.¹⁹ This is due to different study methodologies. The 15kU/L cut-off value was derived from a highly atopic children and adolescents cohort. In contrast, the upper end of the spectrum (34kU/L) was derived from a population-based study of infants under two years of age. We use the lower cut-off as it represents a similarly atopic population, although it is not matched for age. 28 AD participants ≤ 2 years of age had positive peanut IgE, with 6 participants having levels above the 95% PPV of 15kU/L. Five participants were classified as suspected peanut allergy, and one was classified as tolerant. The 22 participants whose values fell below the cut-off were made up of 16 participants who were classified as suspected peanut allergy and

6 who tolerated peanut. This resulted in a sensitivity of 23%, NPV of 27%, specificity of 85% and 83% PPV.

DISCUSSION

Moderate to severe AD is associated with an increased risk for sensitisation to food and aeroallergens and an increased risk of developing other allergic diseases, particularly food allergies, in infancy. IgE sensitisation is potentially mediated through the disrupted cutaneous barrier.²⁰⁻²² We observed high levels of total IgE and poly sensitisation to multiple foods in South African children with AD, which was not dependent on their urban or rural location (except peanut). House dust mite (HDM) IgE levels were also significantly elevated in rural and urban children with AD, suggesting more significant exposure to HDM allergens via the defective AD skin barrier, combined with the deleterious effects of HDM proteases. Previous observations of high IgE levels to major allergens in tropical countries have been dismissed as epiphenomena and are not indicative of atopic phenotypes. This view was based on presumptions of high parasite infection rates and cross-reactivities to ubiquitous environmental exposures.²³ However, the relatively low anti-CCD IgE reactivity in this cohort suggests genuine IgE sensitisation to food and aeroallergens as antihelminth treatment is regularly administered in South African children. In addition, a recent study assessing the prevalence of reactivity to CCDs in Ugandan children in urban and rural settings reported a higher level of CCD sensitisation in rural participants than urban participants.²² They also observed a higher rate of reactions to major food, house dust mite and cat component allergens in urban participants than rural participants. In contrast, our findings suggest no urban-rural differences in recognising CCDs, and food allergen and HDM sensitisation rates are similar except for peanut sensitisation being higher in rural AD participants.

Food allergen-specific IgG4 levels were lowest in healthy children from the rural community. Low allergen-specific IgG4 is in line with observations that milk-specific IgE and IgG4 responses were lower in Amish (more traditional farming environment) compared to Hutterite children, and there was a similar trend for IgG4 responses to beta-lactoglobulin, bovine serum albumin, caseins, and egg.²⁴ One potential mechanism might be related to the inverse relationship between endotoxin exposure levels in early childhood and aeroallergen sensitisation in these communities, which may also impact the production of antibodies to food allergens. Another potential explanation is that exposure to food allergens is required to promote IgG4 responses, as demonstrated for aeroallergens such as cat Fel d 1,²⁵ and rural healthy children may have a relatively lower level of diverse food allergen exposures compared to their urban counterparts. However, other tolerance-related immunological mechanisms (e.g., IL-10) that impact antibody production might also play a role. The IgE: IgG4 ratio was lowest for healthy urban children, indicating an effective blockade of IgE-mediated responses in these children. This may explain why there was no SPT response or clinical symptoms to milk in healthy urban children, while they could still display (ImmunoCAP) IgE sensitisation to milk. The increased IgE: IgG4 ratio in children with AD, especially against HDM, demonstrates a high relative level of IgE that would outcompete IgG4 for binding to mast cell receptors. The preferential induction of IgG4 by allergen-specific immunotherapy is thought to mediate some of the beneficial effects of this therapy, particularly for this ubiquitous allergen in the indoor environment.^{26,27}

Elevated specific IgE to egg, milk and peanut has been noted in children with AD (**Table 3.1**). Several studies have shown that IgE-mediated allergies to egg and milk usually resolve in childhood, whereas IgE-mediated peanut allergy can often persist.^{28,29} Although food allergy to specific allergens was not confirmed by OFC in our cohort, we observed a pattern of

decline in egg IgE with age ($p=0.06$), whilst IgG4 levels significantly increased with age, but only in rural children with AD ($p=0.02$). (**Fig 3.8**) The increasing IgG4 levels may be similar to the egg-OIT profile, where OIT weakly influences IgE levels but induces robust antigen-specific IgG4 levels similar to non-allergic or naturally tolerant individuals.³⁰ However, urban AD children do not exhibit this pattern of tolerance acquisition. There was no difference in the mean age of egg (including milk or peanut) introduction, and parameters that have been used to predict food allergy resolution, such as sIgE and severity of AD, were comparable between the AD groups for all allergens. Perhaps there may be immune alterations resulting in delayed tolerance acquisition in urban children that may account for this difference, or this may reflect egg avoidance. Milk-specific IgE and IgG4 or peanut-specific IgE and IgG4 levels did not associate with age. Our data is in keeping with findings elsewhere. One longitudinal study showed that 52% of milk-allergic children outgrew their disease at a median age of 63 months.³¹ Conversely, peanut allergy is associated with disease persistence, which might be reflected in sustained IgE levels, while peanut-specific IgG4 levels may reflect exposure rather than tolerance.

AD is a risk factor for egg sensitisation and egg allergy. Egg sensitisation and egg allergy are risk factors for sensitisation to certain aeroallergens, including ubiquitous perennial allergens such as house dust mite and asthma.²⁸ Internationally derived diagnostic cut-offs for specific IgE and SPT with 95% positive predictive value have been reported as 7kU/L (2kU/L in infants $\leq$ 2yr old) and 7mm (4-5mm in infants $\leq$ 2yr old) for egg allergy. Less than 2kU/L egg-specific IgE represents a 50% negative predictive value. A previously published South African study that includes AmaXhosa children with atopic dermatitis reported that the recommended cut-offs might be of limited utility in our setting as SPT $\geq$ 7mm for egg white extract yielded 46% PPV.²⁸ In addition, an SPT of 7mm for fresh egg white had a 52%

PPV. Egg-specific IgE of 7 kU/L had a 75% PPV. They further determined PPV for egg that applied to local populations. A PPV of 100% was reached at 12 mm for egg white extract, 67% PPV for fresh raw egg SPT was reached at 17 mm and 100% PPV at 21mm. Regarding egg-specific IgE, a value of 13 kU/L obtained a PPV of 88%.²⁸ They similarly reported that 95% PPV derived internationally were also less predictive of peanut allergy, with 8mm cut-off for peanut extract SPT producing a PPV of 80%, and the 14kU/L peanut-specific IgE cut-off producing a PPV of 57%. A PPV of 88% with an SPT to peanut reagent of ≥ 11 mm was derived.³² Whilst these are significant and relevant observations, they are not fully generalisable for our cohort as the median age of African participants in that study was 40 months of age, with no age-specific cut-off data reported. While our cohort is predominantly made up of infants under 2 years, we similarly conclude that the 95% PPV that have been established internationally yield low predictive values in our setting. This is evident irrespective of the allergen tested, supporting this discrepancy's population specificity. Therefore, population and age-specific cut-off values must still be developed and validated. Generally, our data and Gray et al. suggest that much higher SPT/sIgE values may be more accurate in predicting food allergy in this South African population. These guidelines are urgently required to reduce the burden of OFCs, especially in settings where this service may not be easily accessible.

CONCLUSION

IgE sensitisation to food and aeroallergens is common in children with AD in South Africa, largely independent of their location. CCD reactivity is not significantly relevant in this age group in our setting. While multiple sensitisations are evident, illustrating the strong atopic

association of this disorder in the infantile phenotype of AD, the possibility that there may be some cross-reactivity in the IgE responses cannot be excluded.

IgG4 responses to food and aeroallergens are observed for the majority of children.

However, healthy children from the rural environment have the lowest IgG4 levels to multiple food allergens for reasons that are yet to be clarified. Differential egg IgG4 responses in rural AD compared to urban AD gives us a glimpse that different environmental exposures may leave a material immune imprint that is allergen-specific and may be associated with acquiring a tolerogenic signature with increasing age.

Our data also supports observations that established food allergy positive predictive values (PPVs) perform poorly in our population and age category, which still necessitates labour-intensive and resource-demanding oral food challenges.

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CHAPTER 4

IMMUNE ENDOTYPES IN URBAN AND RURAL CHILDREN WITH AD

INTRODUCTION

Different molecular and pathogenetic mechanisms in complex diseases such as AD can result in similar manifestations and symptoms. With the advent of personalised medicine and the availability of biologics targeting specific pathways, it becomes crucial to detail the immune endotypes of all AD patients considering age, ethnicity and living environment. In order to better select immune targeting therapies that cover the spectrum of AD endotypes.¹

The different functional properties of almost all immune responses can be characterised by the secretion of cytokines and chemokines that bind to their specific cell surface receptors to induce their effects. These secreted proteins mediate intercellular communication between immune and non-immune cells and regulate activation, differentiation, recruitment, survival, and polarization of the specialised immune cell subsets that are specifically required at that time.^{2,3} This process is particularly well described for CD4+ lymphocytes. They differentiate from naive T cells into multiple potential effector subsets, including TH1, TH2, TH9, TH17, TH22, follicular effector T cells, and regulatory T (Treg) cell subsets. These T cell subsets are influenced by their respective cytokine and chemoattractant repertoires and interactions with other cells to promote the development of different types of protective immune responses or tissue-damaging inflammatory responses.⁴⁻⁶

Atopy is usually associated with effector TH2 cells that produce IL-4, IL-5, IL-9, and IL-13. Secretion of additional cytokines, such as thymic stromal lymphopoietin (TSLP), IL-25, IL-31, and IL-33 from innate and epithelial cells can prolong and increase the intensity of TH2 milieu and inflammation.⁷ Chemokines such as thymus- and activation-regulated chemokine (TARC) can be secreted by activated myeloid cells, such as dendritic cells, leading to the

recruitment of TH2 cells to atopic lesional skin via binding to the chemokine receptor CCR4 present on TH2 cells. These cytokines support allergen-specific IgE production, eosinophilia, and tissue migration of TH2 effector cells and can reduce epithelial barrier integrity.^{8,9} TH1 cells are characterised by their secretion of IFN- γ , which activates anti-viral defence networks.¹⁰ However, a TH1 response can have significant collateral damage, especially in combination with other proinflammatory cytokines, including activation-induced cell death of keratinocytes.¹¹ Inappropriate TH1 responses are associated with many autoimmune disorders. TH17 cells are characterised by their secretion of IL-17A, IL-17F, IL-6, IL-8, TNF- α , IL-22, and IL-26, and TH17 cells coordinate and support immune responses to extracellular infections.¹² TH22 cells and its main effector cytokine IL-22 play a role in skin inflammation.¹³ TH17 cell activation is associated with multiple inflammatory disorders of epithelial barrier tissues, including psoriasis. TH9 cells secrete IL-9, which might represent a clinically relevant T-cell subset in food allergy.¹⁴ However, multiple T cell subsets may be simultaneously activated in chronic inflammatory diseases. Understanding these multipolar immune axes and their potential contribution and amplitude of that contribution is essential as these represent tangible targets amenable to therapeutic manipulation.

A detailed assessment of cytokine levels in South African children with AD has yet to be described. In addition, integrating the immunological findings with environmental conditions, lifestyle and home exposures, and socioeconomic factors described in Chapter 2 will help identify novel and distinct immune endotypes in these children.

OBJECTIVES

To identify immune endotypes and disease biomarkers relevant for South African children with AD.

To determine the early life exposures that influence patterns of immunological differentiation in healthy and atopic South African children.

METHODOLOGY

Peripheral Blood Immune Cells and Cytokines

Blood samples were collected from urban children with moderate to severe AD (n=32), urban healthy controls (n=35), rural children with moderate to severe AD (n=46) and their corresponding healthy controls (n=39). Lithium heparin tubes were used for sample collection. Peripheral blood leukocyte counts, eosinophil, neutrophil, monocyte, and lymphocyte numbers were quantified using an automated cell counter. Plasma samples were obtained following centrifugation and were stored at -80 °C until batched analysis. The ultrasensitive Meso Scale Discovery multi-spot assay system (Meso Scale Discovery, Gaithersburg, MD, USA) was used to quantify 44 plasma analyte levels. Plasma analytes were read using the MESO QuickPlex SQ 120 (Meso Scale Discovery) platform. The measured analytes were Interleukin (IL)-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-12/IL23p40, IL-12p70, IL-13, IL-15, IL-16, IL-17A, IL-21, IL-22, IL-23, IL-27, IL-31, interferon (IFN) γ , interferon-inducible protein (IP)-10, tumour necrosis factor (TNF) α , TNF β , macrophage inflammatory protein (MIP)-1 α , MIP-1 β , MIP-3 α , monocyte chemoattractant protein 1 (MCP-1), MCP-4, macrophage-derived chemokine (MDC), Eotaxin, Eotaxin-3, thymus and activation regulated chemokine (TARC), granulocyte macrophage colony stimulating factor (GM-CSF), soluble inter-cellular adhesion molecule 1 (sICAM-1), soluble vascular cell adhesion molecule 1 (sVCAM-1), serum amyloid A (SAA), c reactive protein (CRP), vascular endothelial growth factor (VEGF)-A, VEGF-C, VEGF-D, Tie-2, FMS-like tyrosine kinase (FLT1), placental growth factor (PIGF) and basic fibroblast growth factor (bFGF).

Statistical analysis

The non-parametric Mann-Whitney test (Prism GraphPad Software version 7TM) was used to determine the statistical significance (two-tailed analysis with gaussian approximation) between the two groups as the data was not normally distributed. The non-parametric Kruskal-Wallis test with p-values adjusted for multiple comparisons (Dunn's multiple comparisons test) was performed to compare differences between multiple groups. Differences in continuous demographic data were analysed using the Mann-Whitney test, while differences in categorical demographic data were estimated using Fishers Test. The cytokine data was log transformed for better visualisation in the figures and to reduce the effect of outliers. Linear regression models examined associations between plasma cytokine levels and environmental exposures. Firstly, nearly zero variance, correlated and linear dependent environmental exposure measurements were discarded to reduce the complexity of the model and facilitate identification of the most important environmental exposures. Then, a recursive feature elimination algorithm was used to further refine the list of the most informative environmental exposures. Finally, a linear regression model was built for each cytokine level to explore their associations. The visualisation in the heatmap was produced by R package corrplot (<https://cran.rproject.org/web/packages/corrplot/index.html>).

RESULTS

AD-related systemic immune changes

Circulating eosinophil and monocyte counts were significantly elevated in children with AD than in healthy children, regardless of study location (Figure 4.1). There were no differences in circulating numbers of neutrophils or lymphocytes when comparing all children with AD versus all healthy children (Figure 4.1). In addition, circulating levels of TH2-associated

cytokines thymus and activation regulated chemokine (TARC), monocyte chemoattractant protein 4 (MCP-4), and interleukin (IL)-16 were significantly elevated in AD patients compared to healthy children (Figure 4.2). In contrast, levels of the TH17 primary effector cytokine IL-17A were significantly reduced with a trend ($p=0.059$) towards reduced IL-17-associated cytokine IL-23 levels in children with AD (Figure 4.3). None of the other cytokines and chemokines measured differed significantly between children with or without AD (Figure 4.4).

Figure 4.1. Immune cell subsets in peripheral blood demonstrating circulating immune biomarkers associated with atopic dermatitis (AD). Circulating eosinophils and monocytes are increased in children with AD compared to healthy controls (HC). No differences were observed in circulating levels of neutrophils and lymphocytes between AD and healthy controls. Median values with interquartile ranges are illustrated.

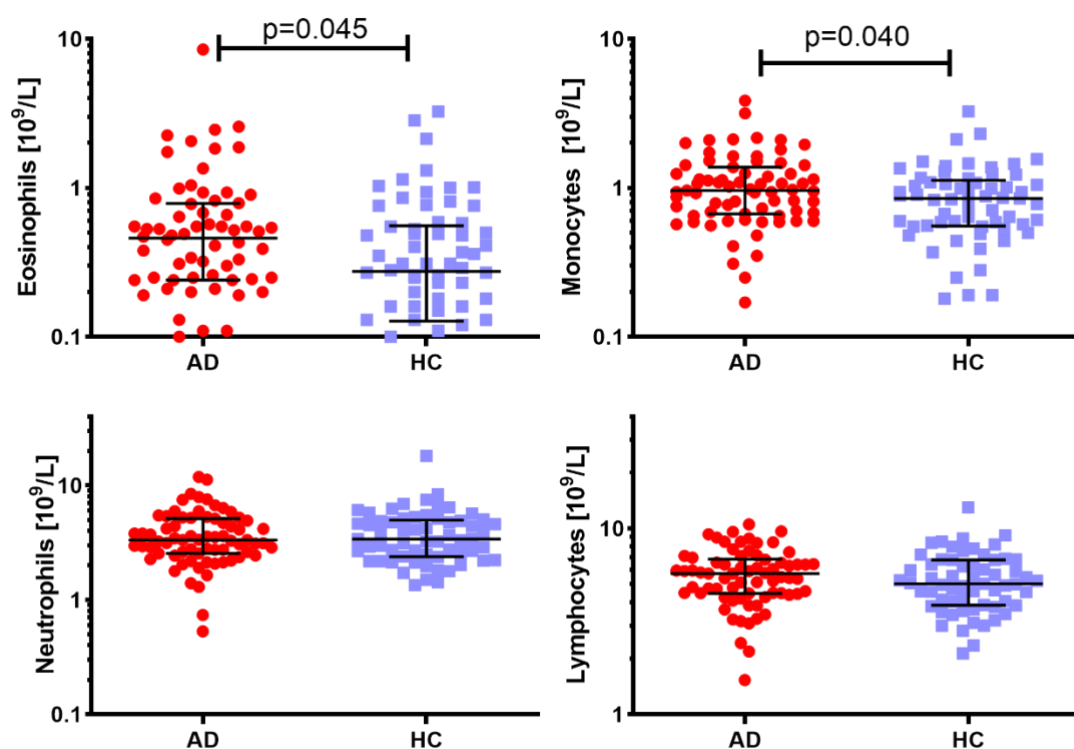


Figure 4.2. Elevated T_H2 cytokines in children with AD. Plasma cytokines levels of TARC, MCP-4 and IL-16 are significantly elevated in children with atopic dermatitis (AD) compared to healthy controls (HC). Results are presented on a log scale and differences analysed using a Mann-Whitney test. Median values with interquartile ranges are illustrated.

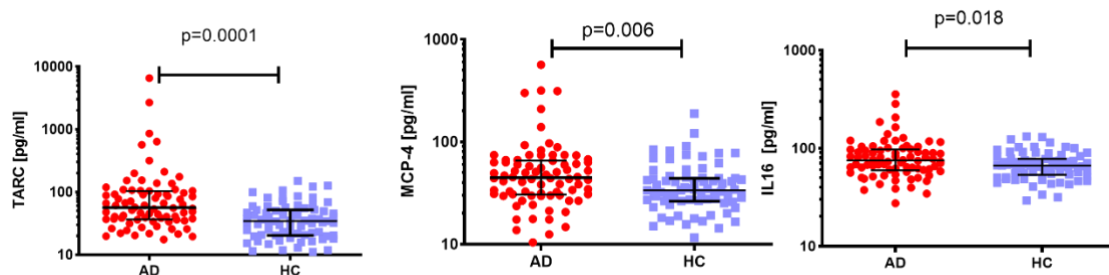


Figure 4.3. Reduced levels of T_H17 cytokines in children with AD. Plasma levels of IL-17A and IL-23 are reduced in children with atopic dermatitis (AD) compared to healthy controls (HC). Results are presented on a log scale and differences analysed using the Mann-Whitney test. Median values with interquartile ranges are illustrated.

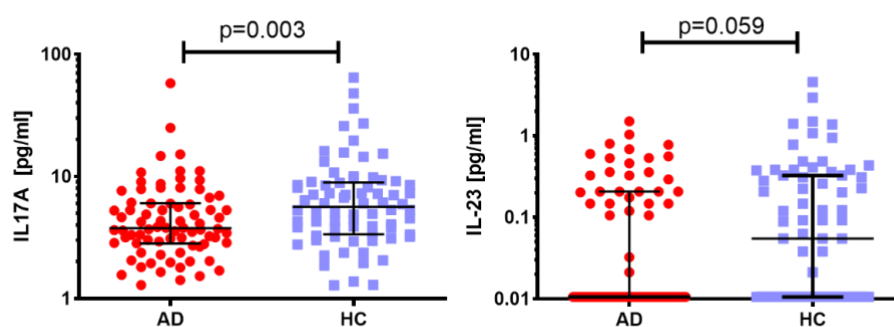


Figure 4.4. Circulating immune biomarkers not associated with AD

Plasma cytokine levels for children with AD or healthy control children (HC). Results are presented on a log scale and differences analysed using a Mann Whitney test.

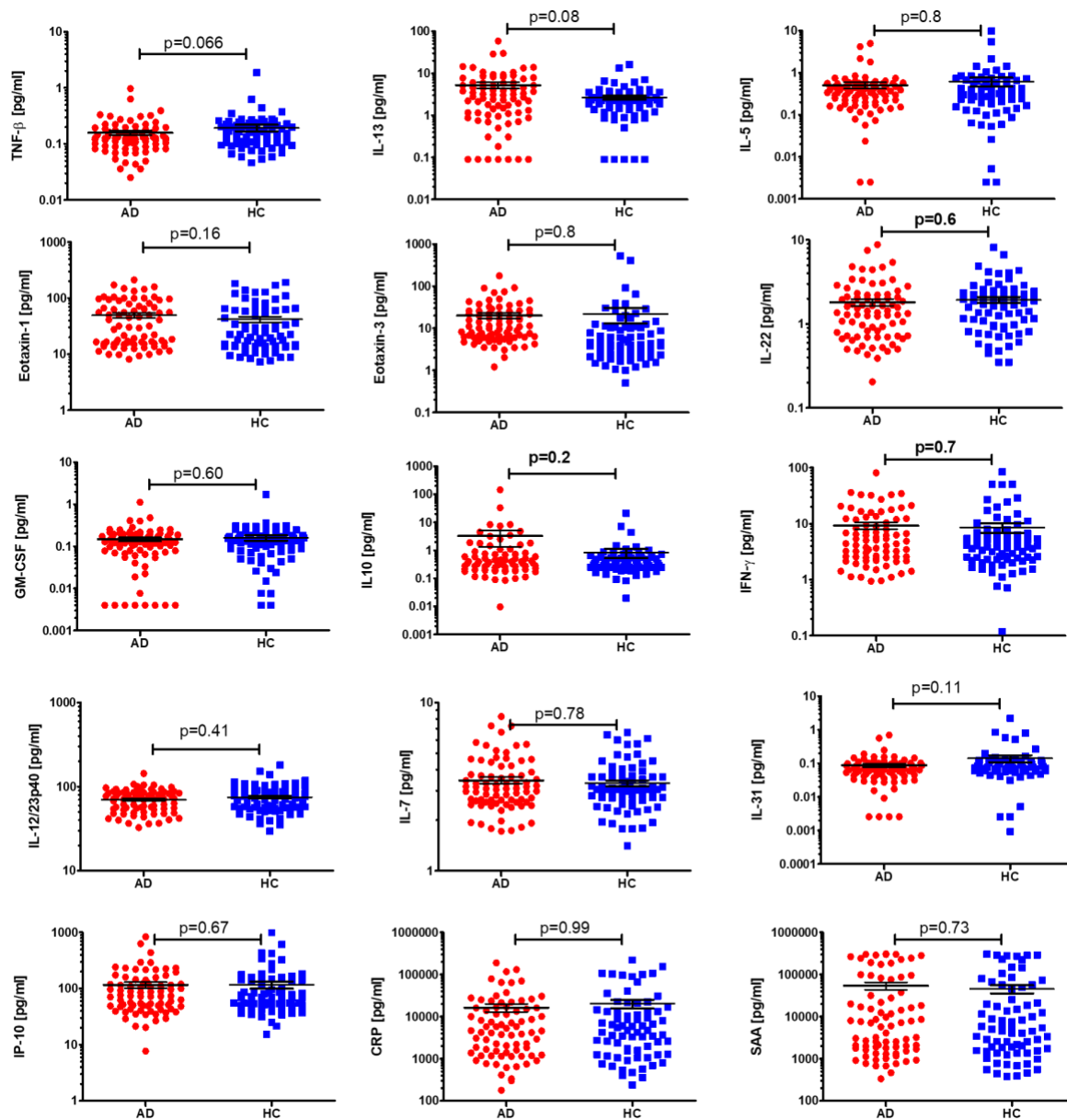
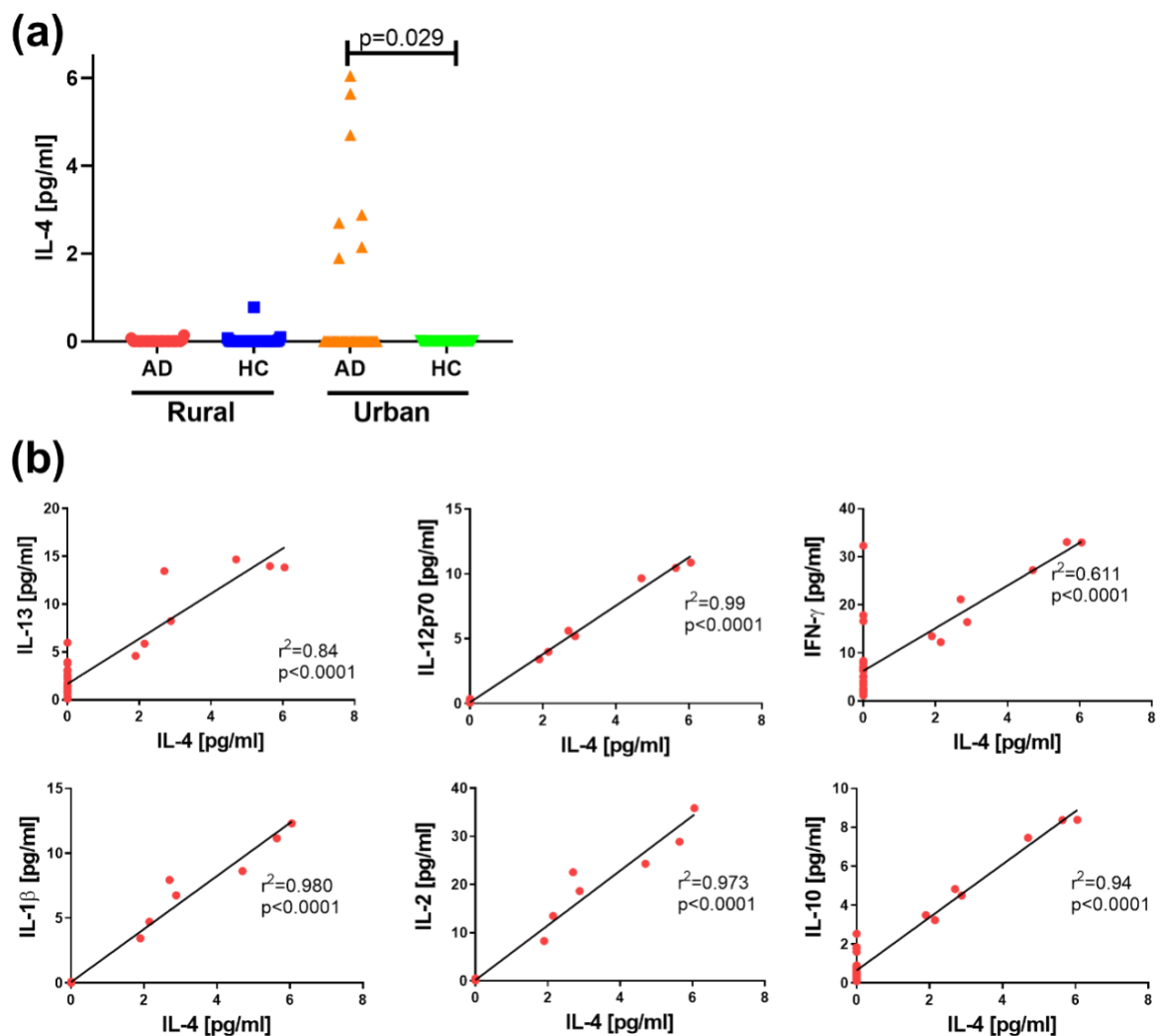


Figure 4.5. Urban cytokine^{high} subgroup of children with atopic dermatitis (AD). (a) Plasma levels of IL-4 are highly elevated in this subgroup. Results are presented on a linear scale and differences analysed using Kruskal-Wallis test with p-values adjusted for multiple comparisons (Dunn's multiple comparisons test). (b) Linear regression plots illustrating that the high levels of IL-4 in the urban AD subgroup correlate significantly with elevated plasma levels of IL-13, IL-12p70, IFN γ , IL-1 β , IL-2 and IL-10. Median values with interquartile ranges are illustrated.



When the data was separated and analysed based on study location, a subgroup of urban children with AD that displayed significantly elevated plasma IL-4 levels were identified. This was not observed for rural children with AD (Figure 4.5(a)). In addition, IL-4 levels were positively correlated with serum levels of IL-13, IL-12p70, IFN- γ , IL-1 β , IL-2 and IL-10 within this subgroup (Figure 4.5(b)). While none of the AD SCORAD, demographic or exposure data was significantly associated with this urban cytokine^{high} subgroup, the majority of this group (6 of 7 children) did have a positive SPT to egg.

Environment-related associations with circulating cytokines

The environment children lived in significantly impacted plasma levels of several immunological markers, irrespective of AD status. Rural children had significantly higher levels of proinflammatory TNF- α and IL-6 than their urban counterparts (Figure 4.6). IL-6 and TNF- α levels were significantly higher in urban children with AD than in healthy urban children but still at levels far lower than those observed in rural children. Rural children had higher levels of IL-8 compared to urban children, with notably higher levels in rural AD compared to rural control children. Children living in the rural environment had significantly higher plasma levels of chemokines such as monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 α (MIP-1 α), MIP-1 β and macrophage derived chemokine (MDC) than in children living in the urban environment (Figure 4.6).

Additionally, circulating IL-1 β , TNF- α , soluble inter-cellular adhesion molecule 1 (sICAM-1), soluble vascular cell adhesion molecule 1 (sVCAM-1), vascular endothelial growth factor-A (VEGF-A), VEGF-D and soluble Tie 2 levels were significantly elevated in the rural children regardless of AD status (Figure 4.6). sICAM-1 and sVCAM-1 are induced by proinflammatory

cytokines. VEGF-A is involved in vascular angiogenesis, VEGF-D is involved in lymphangiogenesis and soluble Tie 2 is involved in VEGF signalling. Plasma IL-21 was detectable only in a subgroup of healthy rural children and those with AD. However, healthy rural controls displayed higher IL-21 levels. Overall rural IL-21 levels were higher than urban groups. IL-21 is the prototype cytokine associated with T follicular helper (Tfh) cells, a specialised CD4+ T cell subset that promotes B cell production of antibodies.

Conversely, children living in the urban environment had several highly elevated markers compared to rural children. Elevated plasma levels of IL-22 and IL-15 (Figure 4.7), as well as significantly elevated levels of growth factors such as FMS-like tyrosine kinase (FLT1), placental growth factor (PIGF) and basic fibroblast growth factor (bFGF), were notable in urban children regardless of AD presence.

Figure 4.6. Elevated plasma cytokines, chemokines, soluble adhesion molecules and growth factors in rural children compared to urban children. Results are presented on a log scale and differences analysed using the Mann-Whitney test. Median values with interquartile ranges are illustrated.

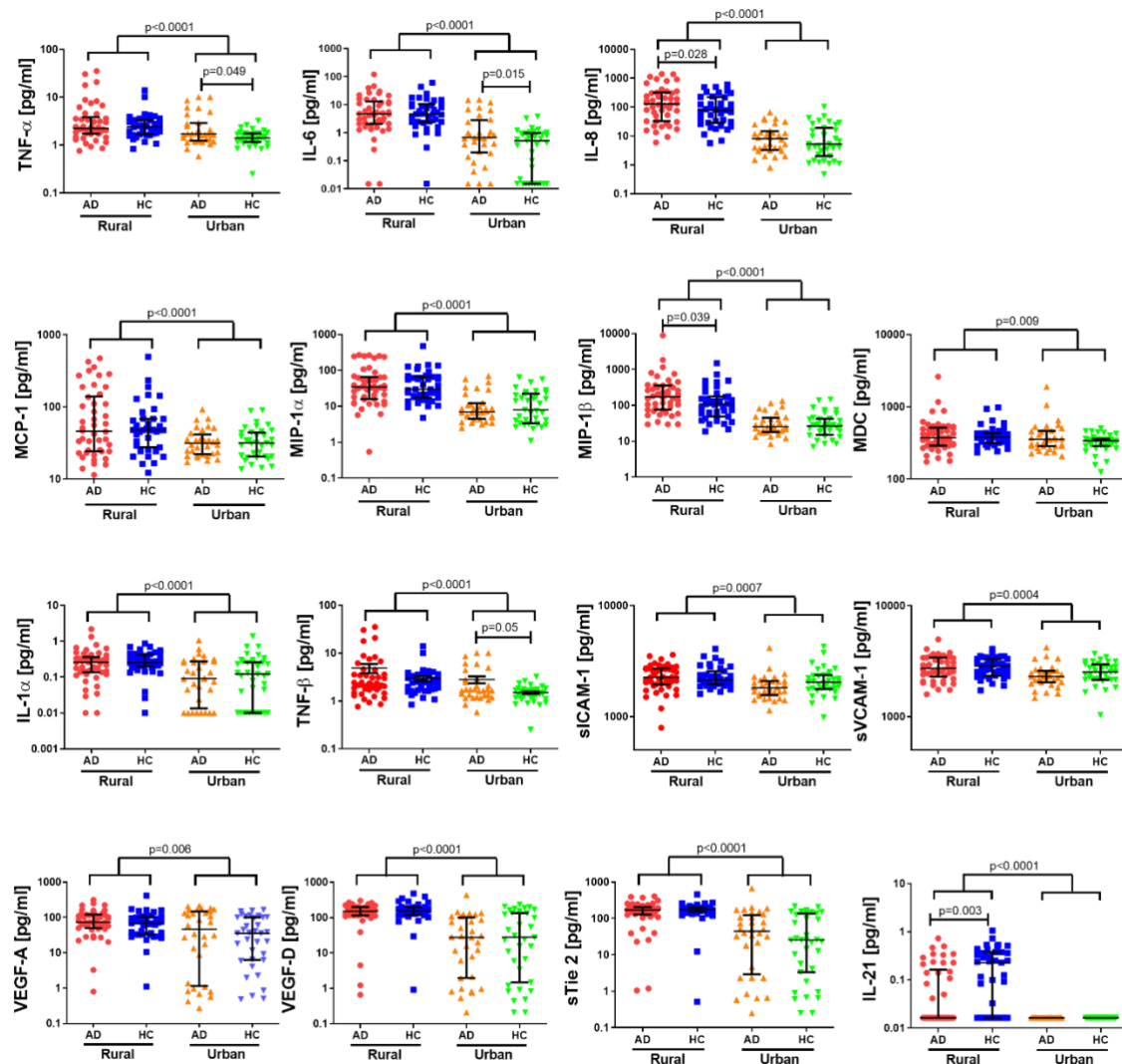


Figure 4.7. Elevated plasma cytokines in urban children compared to rural children.

Cytokines (IL-22 and IL-15) and growth factors (Flt-1, PlGF and bFGF) are significantly elevated in urban children compared to rural children. Results are presented on a log scale and differences analysed using the Mann-Whitney test. Median values with interquartile ranges are illustrated.

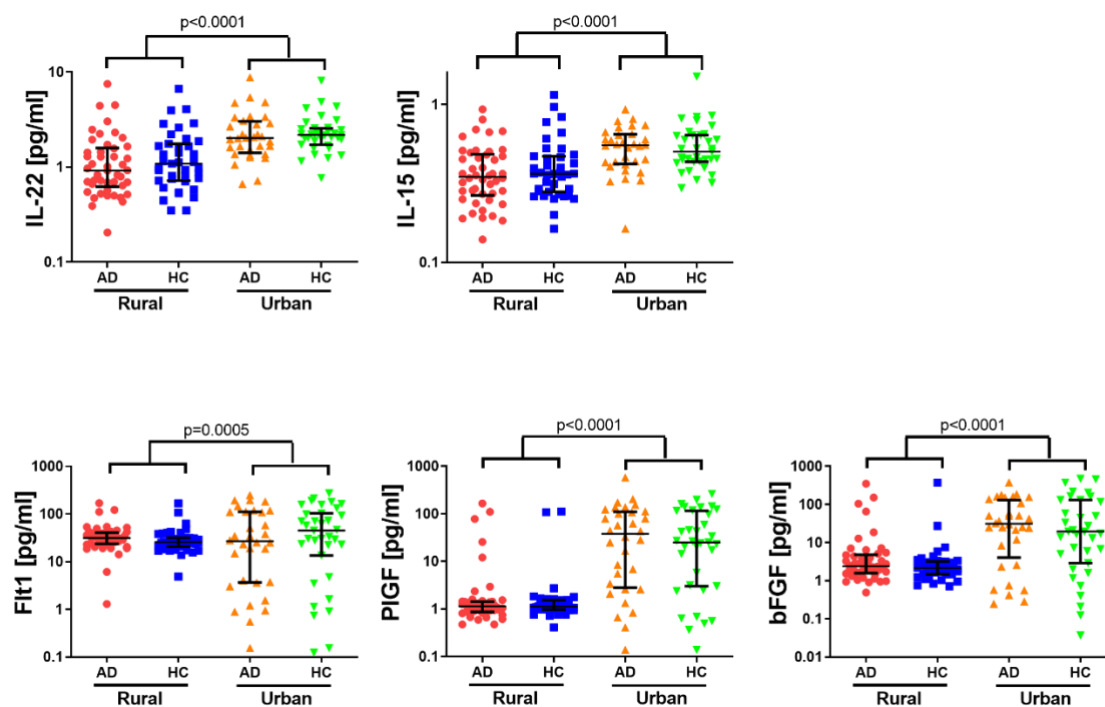


Table 4.1. Biomarker findings across the four subgroups

Increased in AD compared to healthy controls	Eosinophils, Monocytes, TARC, MCP-4, IL-16
Decreased in AD compared to healthy controls	IL-17A, IL-23
Increased in rural compared to urban children	TNF-a, IL-6, IL-8, MCP-1, MIP-1a, MIP-b, MDC, IL-1a, TNF-b, sICAM-1, sVCAM-1, VEGF-A, VEGF-D, sTIE-2, IL-21
Increased in urban compared to rural children	IL-22, IL-15, Flt1, PlGF, bFGF

Dominant environmental factors that influence circulating immune mediators

Many lifestyle factors and environmental exposures were associated with individual circulating cytokine levels (Figure 4.8). These included multiple early childhood exposures, including dietary practices (such as the age of solid food introduction and consumption of the fermented milk drink Amasi), medications use (such as paracetamol use in the first year of life, H1 receptor blocking anti-histamine, and topical steroid cream) and animal exposures (particularly exposure to farm animals during pregnancy, which correlated with multiple molecules such as sVCAM-1, VEGF-D, bFGF, PIGF, Tie 2, IL-15, IFN- γ , IL-12p70, IL-22, IL-4 and MCP-1 levels).

The indoor household environment (including the type of fuel used for heating and cooking), the outdoor environment (including sunlight exposure) and socioeconomic factors (including household size, number of siblings, household income and parental education status) were also quantified and correlated with individual cytokine levels. When each group was separated and analysed by location, many of the associations were related to the environmental setting of the child (i.e., urban versus rural) and AD status also significantly influenced the associations. Notably, urban children with AD had plasma levels of Th2-related cytokines IL-4 and IL-13 which were positively correlated with the age of solid food introduction, use of antibiotics in the first year of life and household family number. In contrast, these cytokines were negatively correlated with cat exposure, farm animal exposure during pregnancy and the age of paracetamol first use (Figure 4.9). None of these factors was associated with IL-4 and IL-13 levels in rural children with AD (Figure 4.10).

Similarly, healthy urban control participants had IL-12/IL-23p40 plasma levels that were positively correlated with Amasi consumption, household income, use of paracetamol in the

first year and smoking in the home. At the same time, significant negative IL-12/IL-23p40 correlations were observed for the type of heating fuel, age of paracetamol first exposure and the use of probiotics (Figure 4.11). However, no significant associations for IL-12/IL-23p40 levels were demonstrated with any measured exposures in healthy rural children (Figure 4.12).

Figure 4.8. Plasma cytokines levels of all children that associate with environment and living conditions Heatmaps illustrate the association between individual exposure and cytokine levels. Red boxes indicate positive associations between plasma cytokines and environmental exposure whereas blue boxes indicate negative associations

* $p < .10$, ** $p < .05$, *** $p < .01$

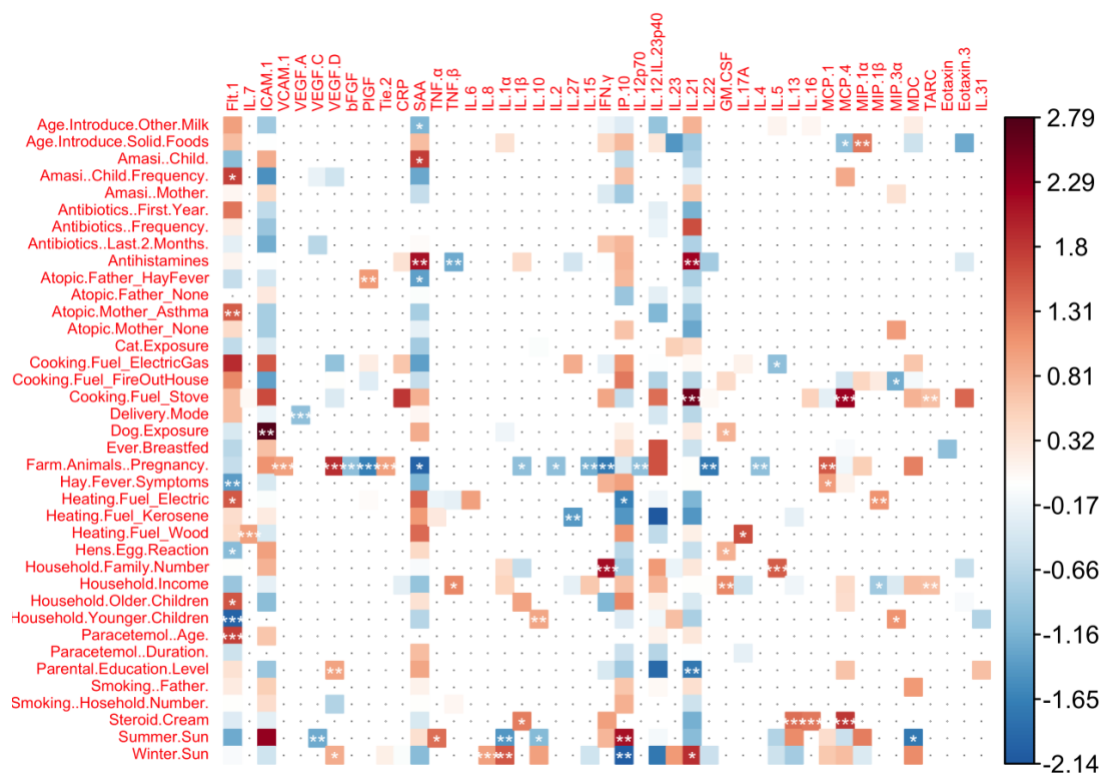


Figure 4.9. Plasma Cytokines Levels of urban children with atopic dermatitis that associate with environment and living conditions. Heatmaps illustrate the association between individual exposure and cytokine levels. Red boxes indicate positive associations between plasma cytokines and environmental exposures whereas blue boxes indicate negative associations * $p < .10$, ** $p < .05$, *** $p < .01$

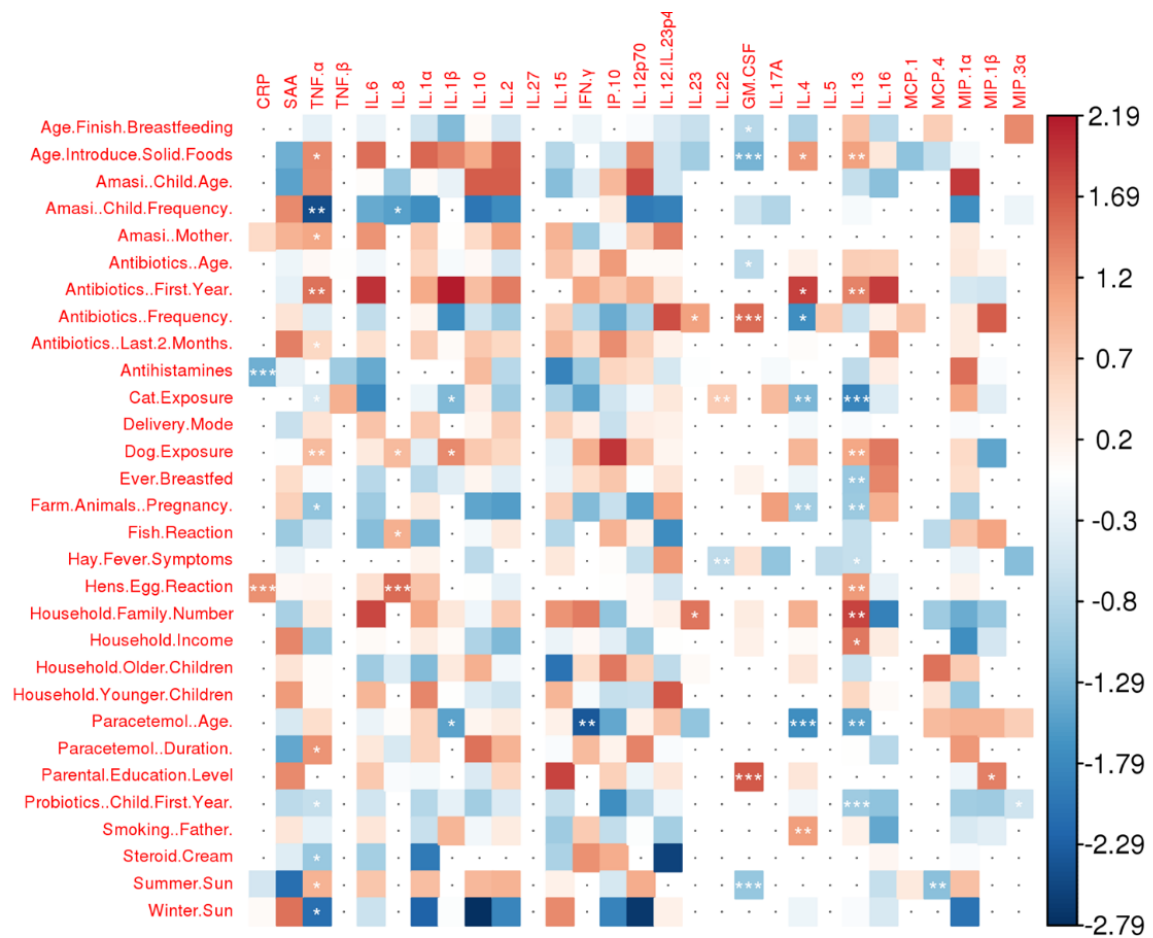


Figure 4.10. Cytokine-environment associations in rural children with AD. Plasma Cytokines

Levels that Associate with Environment and Living Conditions Heatmaps illustrate the association between individual exposure and cytokine levels. Red boxes indicate positive associations between plasma cytokines and environmental exposures whereas blue boxes indicate negative associations $*p < .10$, $**p < .05$, $***p < .01$

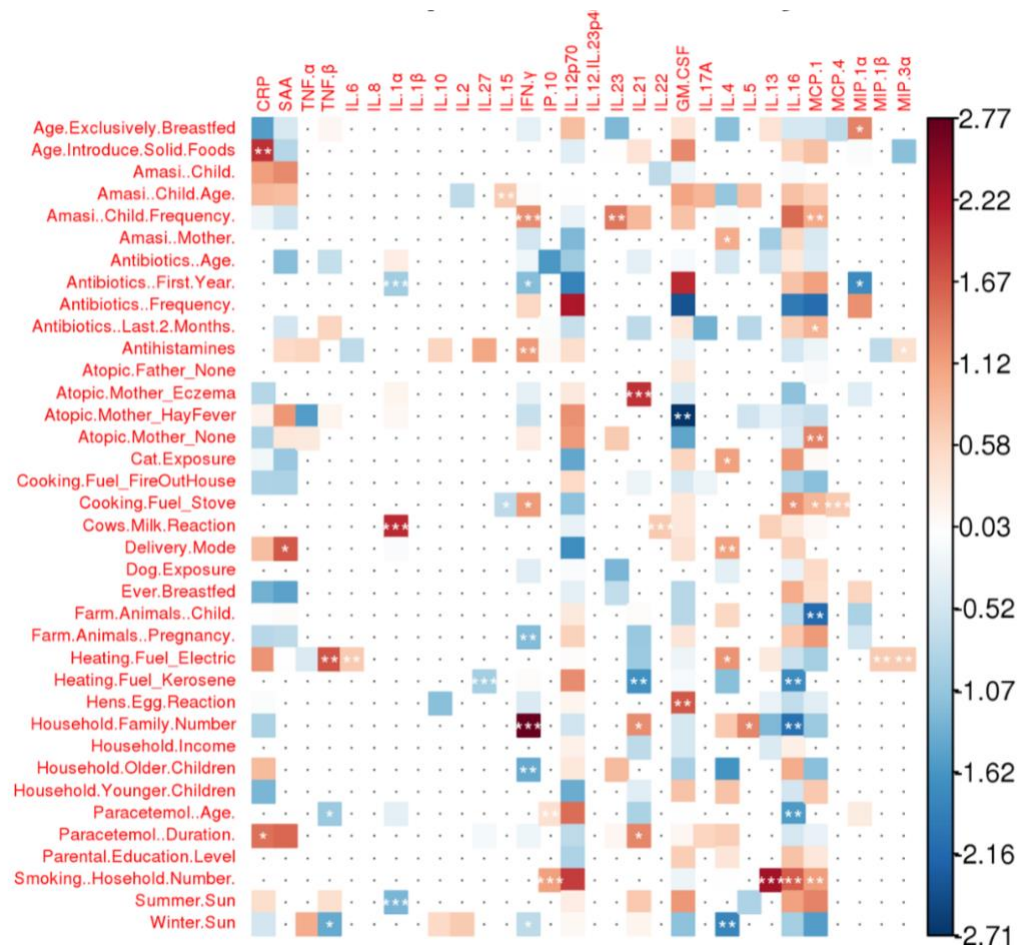


Figure 4.11. Cytokine-environment associations in healthy control urban children Heatmaps

illustrate the association between individual exposure and cytokine levels. Red boxes indicate positive associations between plasma cytokines and environmental exposures whereas blue boxes indicate negative associations $*p < .10$, $**p < .05$, $***p < .01$

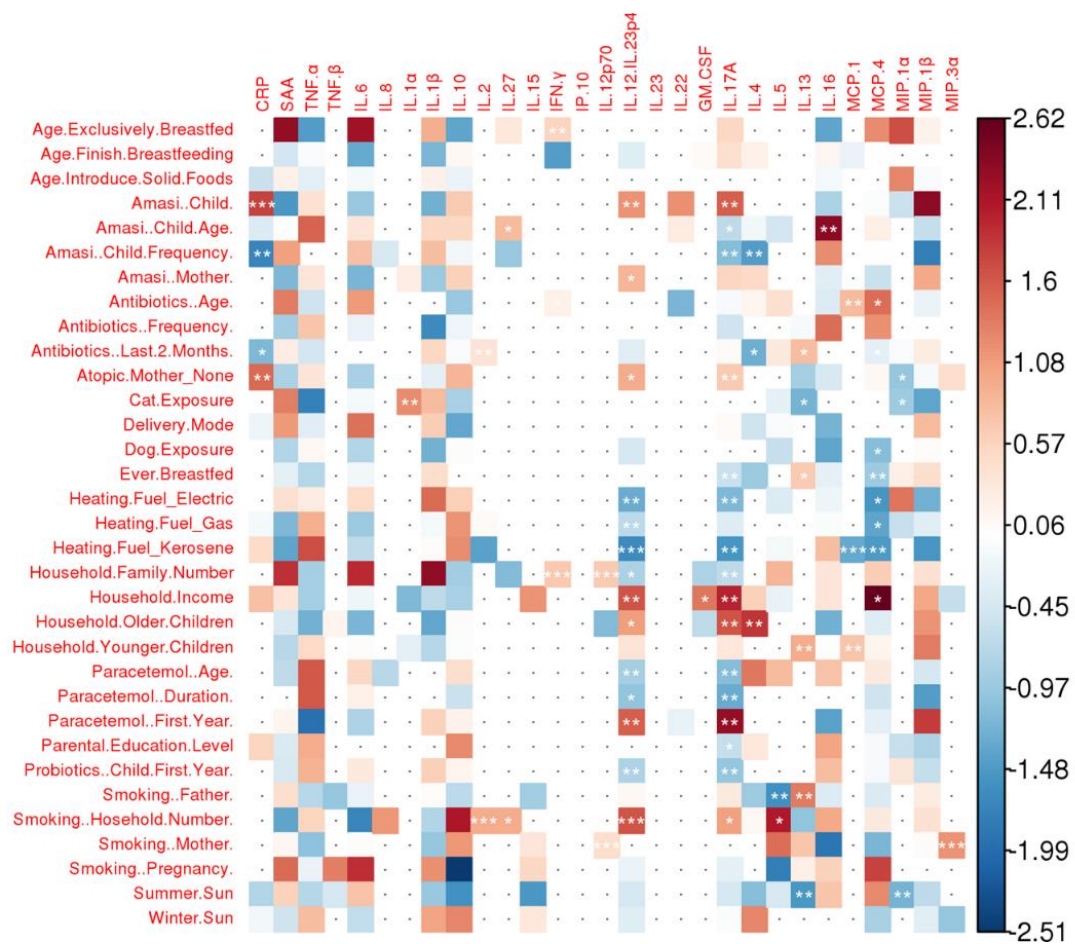
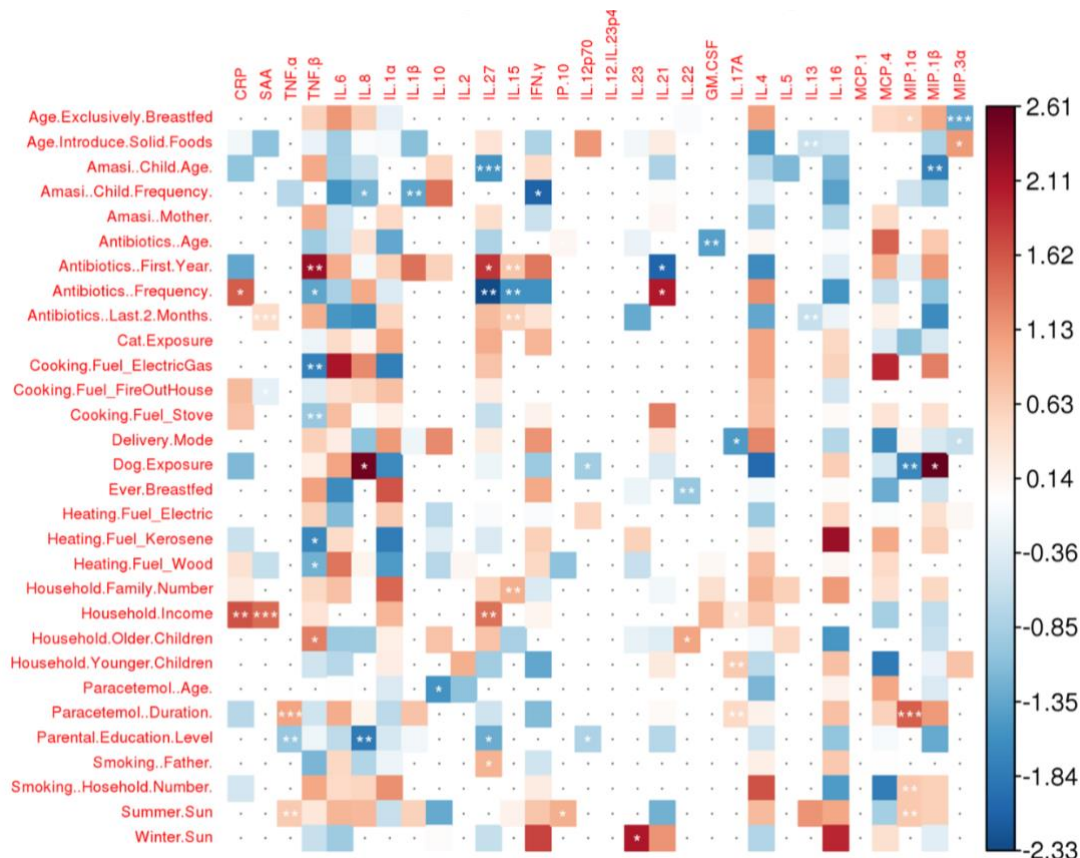


Figure 4.12. Cytokine-environment associations in healthy control rural children Heatmaps illustrate the association between individual exposure and cytokine levels. Red boxes indicate positive associations between plasma cytokine levels and environmental exposures whereas blue boxes indicate negative associations * $p < .10$, ** $p < .05$, *** $p < .01$



DISCUSSION

Several findings in this South African paediatric AD cohort corroborate results observed elsewhere. Elevated eosinophil and monocyte counts in the circulation of AD patients align with the well-described role of eosinophils in atopic dermatitis pathogenesis and monocyte trafficking in skin inflammation.^{15,16} TARC, one of the main chemokines in T cell homing to the skin and IL-16 which is constitutively secreted by T cells, eosinophils and mast cells and

promotes CD4+ T cell chemotaxis to sites of inflammation as well as MCP-4 which induces chemotaxis in eosinophils, monocytes, basophils and T cells and has previously been shown to be elevated in skin biopsies of AD, are all elevated in our AD children compared to healthy children.^{17,18} The detection of TARC, IL-16 and MCP-4 in paediatric AD in plasma may be helpful in clinical practice and clinical trials, as it may be easier to obtain blood samples compared to skin tissue sampling for monitoring responses to treatment. The use of TARC and IL-16 as disease severity biomarkers may be particularly relevant for African AD patients as it has been determined that widely used disease severity scores such as Scoring Atopic Dermatitis Index (SCORAD), Six Area Six Sign Atopic Dermatitis (SASSAD) and Eczema Area Severity Index (EASI), which are reliant on detection of erythema, grossly underestimate disease severity in darkly pigmented skin.¹⁹ This limitation is not mitigated by clinicians who are well experienced and predominantly manage patients with Fitzpatrick skin-type V-VI. Therefore, this population will benefit the most from using accurate molecular disease severity biomarkers.

In contrast to the elevated TH2 cytokine pattern, the significant reduction in levels of IL-17A and IL-23 observed in this African paediatric AD cohort represents a departure from previous observations of elevated TH17 cytokines in AD patients of European or Asian ethnicity, including paediatric AD.^{20, 21} It is possible that reduced IL-17A and IL-23 levels is related to the suppressive effects of TH2-related cytokines on TH17 responses in AD.²² This may be more apparent on a genetically predetermined background of robust, high amplitude TH2 skewed immune responses in populations of African ancestry.^{23,24} In future studies, the antimicrobial role of Th17-related responses in AD at the epithelial barrier level, all the more in moderate to severe AD, which features a marked overrepresentation of extracellular pathogens such as *S. aureus* will need further clarification in addition to

characterising the functional dynamics of Th17 cell skin homing in AD in early childhood. Furthermore, the TH17/IL-17A axis is central to psoriasis pathogenesis, and psoriasis is relatively rare in individuals of African ancestry.^{25,26} Collectively, our observation may reflect population-specific immune responses. It will be interesting to determine in follow-up studies if these cytokine differences are also observed in South African AD patients with mild disease and adult groups with longstanding disease, as our current data is limited to children with moderate to severe AD at the early life initiation phase of the disease.

The cytokine^{high} subgroup described in this cohort represents an urban AD immune endotype with multipolar activation involving TH2 (IL-4 and IL-13), TH1 (IL-2, IL-12p70 and IFN- γ), proinflammatory (IL-1 β) and regulatory (IL-10) immune responses, indicating a mixed inflammatory state. The levels of IL-10 secretion in this inflammatory milieu do not seem sufficient to counteract the hyper-inflamed state. While the critical role of TH2 responses in AD pathogenesis is well established, providing potent itch-inducing cytokines and epidermal barrier-disrupting cytokines,²⁷⁻³⁰ parallel TH1 activation can also be present and associates with AD chronicity.^{31,32} This AD endotype displaying multipolar activation must be carefully clarified from a therapeutic standpoint, as TH2 blockade alone may have limited efficacy. This is in keeping with the observation in clinical trials of dupilumab. While dupilumab has been an enormous breakthrough in AD treatment targeting the IL-4R, it was shown to be efficacious in a third of AD cases with moderate to severe disease in adults and adolescents.^{33,34} This can be juxtaposed with psoriasis, another inflammatory skin disorder. Psoriasis is a unipolar immune disease featuring Th17/IL-17A activation where IL-17A targeting with secukinumab is highly successful with 79 per cent efficacy. In addition, high IL-4 and IL-13 levels in urban children with AD were associated with significantly less exposure to animals but greater antibiotic and paracetamol use within the first year of life,

which suggests that a lack of appropriate immune education at a critical period in early life may predispose to this endotype. Alternatively, infants born predisposed to more pronounced TH2 polarisation may require more frequent antibiotic use due to potentially inadequate antimicrobial defence responses.

The predominant effect of urban and rural environmental exposures on modulating levels of circulating cytokines, independent of AD, is striking. The long-term functional imprinting of innate immune responses evoked by exogenous or endogenous exposures can be mediated via several mechanisms, including epigenetic tuning of cellular reactivity, as recently incorporated in the concept of trained immunity.³⁵ Enhanced innate immune responses were observed in rural participants, consistent with previous studies that linked rural or traditional farm living environments with robust immune activation. Elevated soluble cell adhesion molecules accompany the innate immune activation in these rural children. These mediators should inhibit immune cell adhesion to endothelial cells and their trafficking from the bloodstream to immunologically active tissue. In addition, the associated elevated angiogenic factors suggest differential regulation of endothelial function and homeostasis in children from rural and urban backgrounds. Farm exposures have been shown to associate with higher expression of anti-inflammatory toll-like receptor (TLR) and CD14 genes and enhanced expression of innate immunity-related genes IRAK-4 and RIPK1.^{36,37} High levels of endotoxin exposure in Amish children who lived in close proximity to barns was shown to influence the innate immune response.³⁸ While these studies showed innate immune system activation associated with asthma/allergy protection, our study shows a high level of innate activation in both healthy and AD children within the rural setting. This data may suggest that the protective farm effects observed for respiratory allergy may not be similarly protective for atopic skin inflammation or may not be able to mitigate against putative risk

factors for AD that may exert their effects via non-immune mechanisms, or our observations may be different due to qualitative and quantitative differences in rural/farming environmental exposures in our setting from those previously reported in other regions.

Exposure to animals, cooking or heating fuel type and lower socioeconomic status were associated with the rural immune profile. Both microbes and their products, such as endotoxins and non-microbial factors e.g., N-glycolylneuraminic acid (Neu5GC)-a sialic acid molecule- have been shown to protect farmers' children from airway inflammation, can contribute to the effects of animal exposures on inflammatory immune responses.³⁹⁻⁴¹

Biomass and kerosene fuel combustion can be relevant as indoor and outdoor air pollutants.

Traffic-related air pollution and environmental tobacco smoke exposure have been associated with AD development, AD exacerbations and disease persistence, partly mediated by epigenetic mechanisms.^{42,43} Compared to allergic diseases such as asthma, a limited number of studies show that air pollution, characteristic of urbanisation, positively associates with AD prevalence. Prenatal exposure to certain air pollutants may link to AD development through dysregulated immune responses mediated by microRNA and DNA methylation. Birth cohort studies show indoor and outdoor air pollution is associated with AD development. Longitudinal studies suggest various air pollutants are associated with AD exacerbations and disease persistence.⁴³ Biomass and kerosene fuel emissions are associated with adverse health outcomes, and it has been shown that particles from wood smoke have comparable proinflammatory effects to traffic emissions, albeit mediated by different particle characteristics.⁴⁴ Studies looking at the direct immune effects of kerosene combustion are sparse. However, kerosene particles are demonstrably similar to that of diesel.⁴⁵ Rural children exhibit an increased proinflammatory signature that may be related to their increased exposure to volatile organic compounds (VOCs) from indoor kerosene use

not unlike the Swiss LISA birth cohort study, which showed that emitted VOCs and Semi VOCs during indoor renovations were associated with elevated IL6, IL8, TNF α and MCP1 in the serum of affected children.⁴⁶

Socioeconomic status, or socioeconomic disadvantage (and its correlates), has been consistently associated with higher levels of circulating inflammatory mediators throughout life via multiple interacting mechanisms.⁴⁷⁻⁵⁰ Besides environmental exposures, dietary habits can significantly differ between rural and urban families, which heavily influence the gut microbiome composition, metabolism and subsequent immune function.^{51,52} Rural South African children have been shown to have a *Prevotella*-dominated microbiota associated with high fibre consumption, which diminishes with the transition to urban living.⁵³⁻⁵⁶ In vitro, *Prevotella* strains are potent stimulators of innate immune activation, and a recent study in adults showed that individuals with a *Prevotella*-dominated microbiota had the highest levels of circulating cytokines.^{57,58} The *Prevotella* gut enterotype is an excellent example of microbial factors modulating immune responses. Future studies are needed to determine the precise functional contribution of specific nutritional components, microbiome composition and metabolites on the high levels of circulating cytokines in rural children.

Th22 immune responses are associated with epidermal barrier repair and have been described in cases of AD to the extent that this molecular pathway, specifically IL-22, is considered a possible biomarker of AD.⁵⁹ However, within the urban cohort, relatively higher levels of IL-22, independent of AD, may indicate a more pronounced TH22 response compared to the rural group. TH22 cells have been shown to secrete IL-22 and TNF α and can synergise with IL-17A in host antimicrobial responses in inflammatory skin disease. IL-22

can also independently induce tissue remodelling factors such as FGFs and chemokines involved in angiogenesis and fibrosis.⁶⁰ Interestingly, a higher level of IL-22 was observed in parallel with elevated tissue remodelling factors such as bFGF, PlGF and its soluble receptor FLT-1 in urban children compared to rural children. In addition, IL-15 levels were also higher in urban children. IL-15 is a pleiotropic cytokine that also acts as a growth factor. It supports the proliferation of keratinocytes and inhibits fibroblast apoptosis in psoriasis and rheumatoid arthritis, respectively. It also associates with fibrosis in systemic sclerosis.⁶¹ MDC, an AD biomarker, is elevated in all rural individuals despite its putative role in AD. In this case, environment-dependent factors may be masking this biomarker's allergy/AD specificity.

CONCLUSION

A range of circulating immunological mediators in South African children are altered due to moderate to severe inflammatory atopic skin disease, i.e., AD, or are largely dependent on the child's living environment. Taken together, this data highlights the intersection of various environmental exposures in the manifestation of inflammatory responses, which become particularly important in the context of disease (AD) and selecting appropriate disease-targeting immunological therapies. The environment is a dominant driver of circulating cytokine levels. With the advantage of analysing multiple molecules that span proinflammatory markers, cytokines, chemokines, biomarkers, angiogenesis and vascular injury markers our data has highlighted significant associations between several individual environmental and immune responses rather than a simple dichotomous view that suggests

that one environment is 'superior' or 'beneficial' over another as there may be individual exposures that may be harmful alongside several beneficial exposures in each setting.

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CHAPTER 5

AD AND ENVIRONMENTAL EFFECTS ON THE IMMUNE TRANSCRIPTOME OF CHILDREN IN URBAN AND RURAL SOUTH AFRICA

INTRODUCTION

While AD is associated with a distinct pattern of circulating cytokines (such TARC, MCP-4 and IL-16 as described in Chapter 4) and immunoglobulins (such as allergen-specific IgE as described in Chapter 3), the dominant effect of rural and urban exposures on modifying levels of circulating cytokines is striking in this South African cohort. This is consistent with reports from Europe and North America where rural or farming lifestyles have also been shown to modify innate (e.g., pattern recognition receptors – PRRs) and adaptive (e.g., T_H1 versus T_H2 polarization) immune responses in children¹⁻³. Differences in exposure to microbes, exposure to animals, dietary habits, use of cooking or heating methods that generate pollutants and socioeconomic status have all been shown to influence the development of the early life immune system⁴⁻¹³. However, in addition to circulating cytokines and immunoglobulins, there are many effector molecules, regulatory molecules, receptors, metabolic enzymes, and transcription factors that underpin appropriate functioning of the immune system. Ideally these should also be assessed to provide more extensive unbiased insights for a more comprehensive and granular understanding of the molecular immune mechanisms underlying AD development and the rural/urban immunological gradient. In addition, this analysis may help identify potential novel targets for intervention studies, may help guide diagnosis and will certainly expedite development of treatment strategies in line with principles of precision medicine.

We have therefore conducted high-throughput mRNA sequencing (RNA-Seq) on isolated peripheral blood mononuclear cells from the children's cohort that has already been described in this thesis. This includes rural children with AD (n=46) or healthy (n=45), and urban children with AD (n=31) or healthy (n=30). RNA-Seq is a deep sequencing technology

that has revolutionized transcriptomics as it provides a far more precise measurement of gene transcripts compared to previously available methods such as gene arrays¹⁴.

STUDY RATIONALE

Our previous analysis revealed an AD-related cytokine profile, but also a cytokine profile that was heavily influenced by the living environment of the child. While serum cytokine levels are very useful, one limitation is that the source of the cytokines (e.g., skin-derived versus gut-derived) cannot be determined. In addition, there are many immune regulators and immune mediators that were not measured when focused solely on cytokine quantification. We hypothesized that many other immunological pathways, in addition to cytokines, can be influenced by AD and by children's living conditions in rural and urban environments.

OBJECTIVE

To identify gene expression changes in peripheral blood mononuclear cells from South African children, which are dependent on AD and living conditions.

METHODOLOGY

Cell Isolation & Cryopreservation

Whole blood samples were collected from participants (n=152) with atopic dermatitis from an urban setting- Cape Town and a rural setting-Umtata with corresponding healthy control

counterparts (Rural AD – 46 children; Urban AD – 31 children; Rural healthy – 45 children; Urban healthy – 30 children). These are the same children as described earlier in this thesis. Peripheral blood mononuclear cells (PBMC) were isolated according to a standard protocol. Briefly, PBMC were isolated from freshly collected whole blood which had been collected in lithium Heparin tubes to prevent coagulation. The separation of PBMCs was achieved using Ficoll-Paque 1.077 g/ml density gradient centrifugation which results in the differential migration of cell types into separate layers. 3 ml of Ficoll was dispensed into a 15 ml Falcon tube. Blood was diluted 1:1 with sterile phosphate buffered saline PBS and 10 ml (maximum) of diluted blood carefully layered on top of the Ficoll in each tube. Centrifugation of tubes was performed at 800 g for 20 min with brakes off. The upper layer of plasma was carefully drawn off so as not to disturb the PBMC interface. The PBMC layer was recovered and transferred to a new 15 ml tube. Up to 15 ml PBS was added to the PBMC fraction to wash, performed by centrifugation at 300 g for 10 min (brake on). The supernatant was decanted, while the cell pellet was resuspended and up to 15 ml of PBS was added and the wash step repeated. The supernatant was thereafter removed, and cells were resuspended with RPMI media and counted.

Freezing medium prepared with dimethylsulfoxide (DMSO), heat inactivated fetal calf serum (FCS) and RPMI was added to the cells (following centrifugation and removal of RPMI medium). Cells were transferred in a cryobox into a -80 freezer and thereafter liquid nitrogen the following day.

RNA Isolation & Sequencing

Total mRNA was isolated from PBMCs using the RNeasy Mini Kit (Qiagen, Valencia, CA) and RNA levels quantified by NanoDrop (Thermo Fisher Scientific, Waltham, MA). RNA integrity was confirmed using the Agilent 2100 BioAnalyzer (Agilent, Palo Alto, CA). Libraries were prepared with Illumina's Truseq stranded mRNA library prep kit with polyA enrichment. RNA-Seq analysis was performed on samples from all children (n=152) using HiSeq 4000.

Data Analysis

All the raw fastq files generated following sequencing were processed using the rnaseq pipeline from the nf-core community¹⁵. The pipeline uses Trim Galore for adapter trimming with default options¹⁶. Human reference genome version GRCh38 obtained from Ensembl was used for alignment and read count estimation using aligner option star_salmon provided by the pipeline. Gene level estimated count and TPM (transcript per million) of genes belonging to biotypes namely, protein-coding, lincRNAs, IG gene and TR gene were used for all the downstream analysis. Biotypes can be viewed at <https://www.ensembl.org/info/genome/genebuild/biotypes.html>. Participant demographics data were included in the analysis to identify gene expression changes associated with living environment and AD. Similarity and dissimilarity between the samples are examined by visualizing them in 2-Dimensional space. Dimensionality of the data was first reduced using Uniform Manifold Approximation and Projection (UMAP) for Dimension Reduction using the R package umap v0.2.7.0. TPM normalized gene expression tables were then used for UMAP dimensionality reduction.

Digital cell quantification (DCQ) was used to normalize the gene expression data. DCQ estimates proportion of cell types in the sample based on the bulk RNA-seq data. As the data was generated from PBMCs, differences in cell type abundance will significantly influence and bias gene expression for genes that are expressed by specific cell types. To adjust for this bias, we have used digital cell quantification (DCQ) while performing the differential expression analysis to deduce changes in abundance of specific immune cell types in each sample. The procedure uses cell type specific gene expression data of 18 different cell types downloaded from Human Protein Atlas and signature genes of each cell type collected from CellMarker and PangloDb¹⁷⁻¹⁹. A deconvolution algorithm was then applied, which is adapted from Estimating the Proportions of Immune and Cancer cells (EPIC) method²⁰.

Differential gene expression analysis (DGE) was performed to identify genes up-regulated or down-regulated in the study cohort using Bioconductor package DESeq2 v1.34.0²¹.

Confounding factors such as cell type proportions (which are statistically significant ($\text{padj} < 0.05$) between the cohorts), age and gender were adjusted while performing differential expression analysis. Bioconductor package RUVSeq v1.28.0 was used to compute the factor of unwanted variation and added to the DESeq2 design matrix²². A gene was considered to be significantly regulated if it has $\text{padj} < 0.05$ and $|\log_2 \text{fold change}| > 1$ (two times the expression difference).

Finally, gene-set enrichment analysis was performed using R package piano v2.10.0 to identify pathways of interest²³. KEGG pathway gene-set and gene-level statistics, and $\log_2$ fold change values were used to identify regulated pathways in each of the pair-wise comparisons. KEGG pathways belong to the categories entitled Metabolism, Environmental

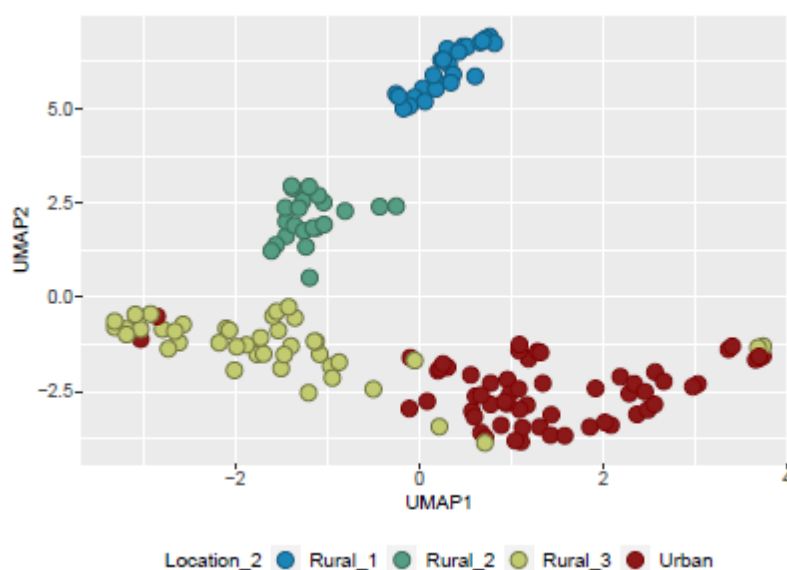
Information Processing and Organismal Systems used for the analysis. Pathways were identified as being significantly regulated pathways when $\text{padj} < 0.05$.

RESULTS

Clustering of children's PBMC gene expression profiles

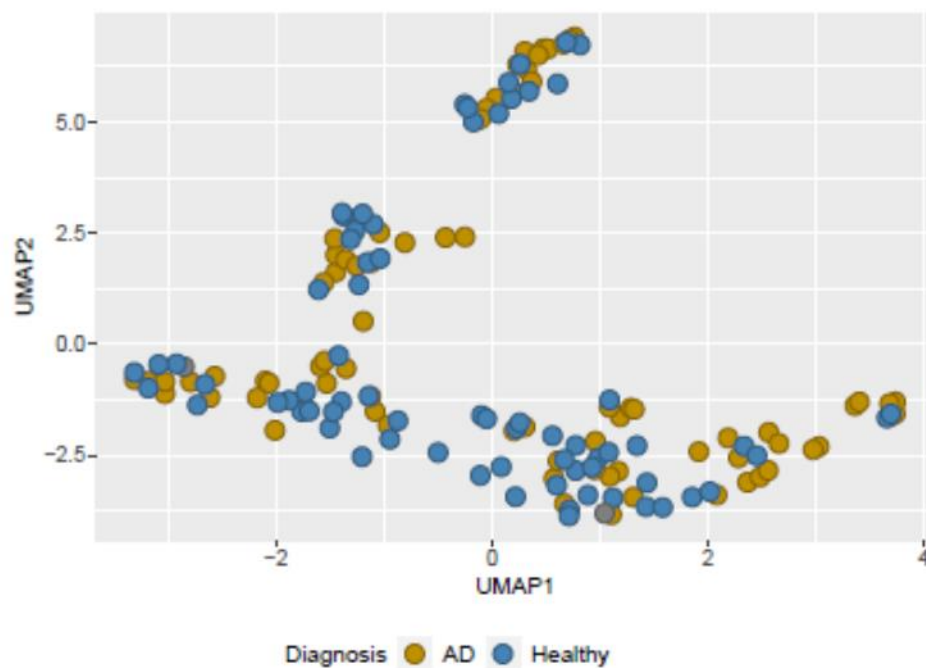
Similarity and dissimilarity in PBMC gene expression between all the children was examined using Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP). All children from the urban group clustered closely together, while there are three sub-clusters of children in the rural setting, termed Rural_1, Rural_2 and Rural_3 (**Figure 5.1**). There were no differences in age or gender between the groups.

Figure 5.1. UMAP based sample distribution of the gene expression data showing three rural clusters and one urban cluster.



Interestingly, this clustering was primarily driven by the location of the child, and not the presence of atopic dermatitis, demonstrating the strong environmental and living conditions effect on the immune system of the growing child (**Figure 5.2**).

Figure 5.2. UMAP based sample distribution with AD diagnosis or healthy child colored differently.



Several exposures were significantly related to the rural and urban gene expression clusters. Using the same questionnaire data as already described in earlier chapters, we found that the most statistically significant differences between rural and urban clusters were related to animal exposures and sunlight exposure during both winter and summer (**Table 5.1**). In addition, there were differences in delivery mode, education level and family income between the rural and urban groups. There were no highly significant differences in the questionnaire data that could explain the three different rural clusters, but there were

trends for differences in cat and dog exposures, summer sun exposure, antibiotic and paracetamol use during the first year of life (**Table 5.1**).

Table 5.1. Exposures that significantly associate with the UMAP clustering are illustrated.

The p-values are shown for those comparisons that are significant, and for those comparisons that show p-value trends between different groups. A p-value greater than 0.20 is shown ($p>0.20$) where there is no statistical significance or trend.

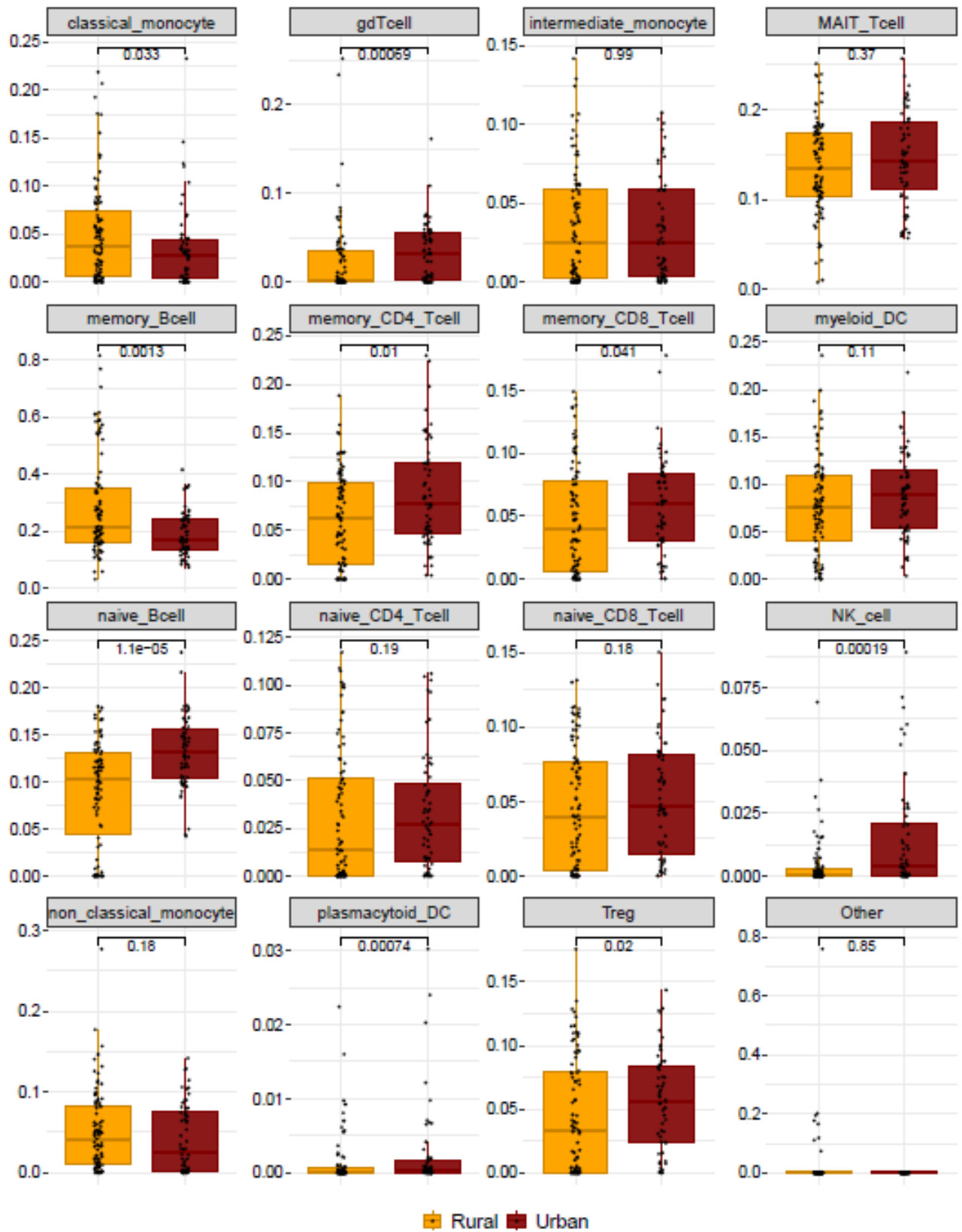
	Rural 1 versus Urban	Rural 2 versus Urban	Rural 3 versus Urban	Rural 1 versus Rural 2	Rural 1 versus Rural 3	Rural 2 versus Rural 3
Any Animal Exposure	9×10^{-9}	6×10^{-8}	4×10^{-8}	$p>0.20$	$p>0.20$	$p>0.20$
Cat Exposure	3×10^{-5}	0.02	0.02	0.14	0.06	$p>0.20$
Dog Exposure	2×10^{-5}	6×10^{-3}	8×10^{-4}	$p>0.20$	0.17	$p>0.20$
Farm Animals – Child	1×10^{-12}	4×10^{-15}	4×10^{-15}	$p>0.20$	$p>0.20$	$p>0.20$
Farm Animals – Mother	8×10^{-14}	4×10^{-14}	8×10^{-15}	$p>0.20$	$p>0.20$	$p>0.20$
Winter Sun	1×10^{-9}	3×10^{-9}	1×10^{-10}	$p>0.20$	$p>0.20$	0.13
Summer Sun	3×10^{-8}	2×10^{-9}	4×10^{-6}	0.03	$p>0.20$	0.06
Income	2×10^{-4}	2×10^{-4}	9×10^{-6}	$p>0.20$	$p>0.20$	$p>0.20$
Education Level	0.03	0.08	0.03	$p>0.20$	$p>0.20$	$p>0.20$
Antibiotics	0.01	$p>0.20$	$p>0.20$	0.02	0.07	$p>0.20$

Paracetamol	0.17	p>0.20	p>0.20	4x10 ⁻¹²	p>0.20	p>0.20
Paracetamol	0.01	0.07	0.15	p>0.20	p>0.20	p>0.20
Age						
Delivery	0.003	p>0.20	0.02	0.09	p>0.20	p>0.20
Mode						

Digital cell quantification (DCQ)

Analysis of the cell type specific gene signatures by DCQ revealed highly significant differences in cell type abundances between rural and urban children (**Figure 5.3**). Rural children had higher circulating levels of classical monocytes and memory B cells. Urban children had significantly higher levels of $\gamma\delta$ T cells, memory CD4 cells, memory CD8 cells, naïve B cells, NK cells, plasmacytoid DCs and T regulatory cells. There were no differences in the levels of intermediate monocytes, MAIT cells, myeloid DCs, naïve CD4 T cells, naïve CD8 T cells and non-classical monocytes.

Figure 5.3. Digital cell quantification identifies changes in cell type specific gene signatures that are associated with living environment.



In order to further characterize the features associated with the three rural sub-groups, we also performed DCQ on all of these groups (**Figure 5.4**). Rural group 1, which was the furthest from the urban cluster in the UMAPs, displayed a unique pattern of cell type abundances that were significantly different to the other rural subgroups and the urban children. These cell types included intermediate monocytes, memory B cells, memory CD4 T cells, memory CD8 T cells, myeloid DCs, naïve B cells, naïve CD4 T cells, naïve CD8 T cells and T regulatory cells (**Figure 5.4**).

In addition to living environment, we examined if there were differences in circulating populations of immune cells in children with AD compared to children without AD (**Figure 5.5**). There were no statistically significant differences in cell type specific gene signatures in children with AD compared to their healthy counterparts.

Figure 5.4. Digital cell quantification identifies changes in cell type specific gene signatures that are associated with living environment.

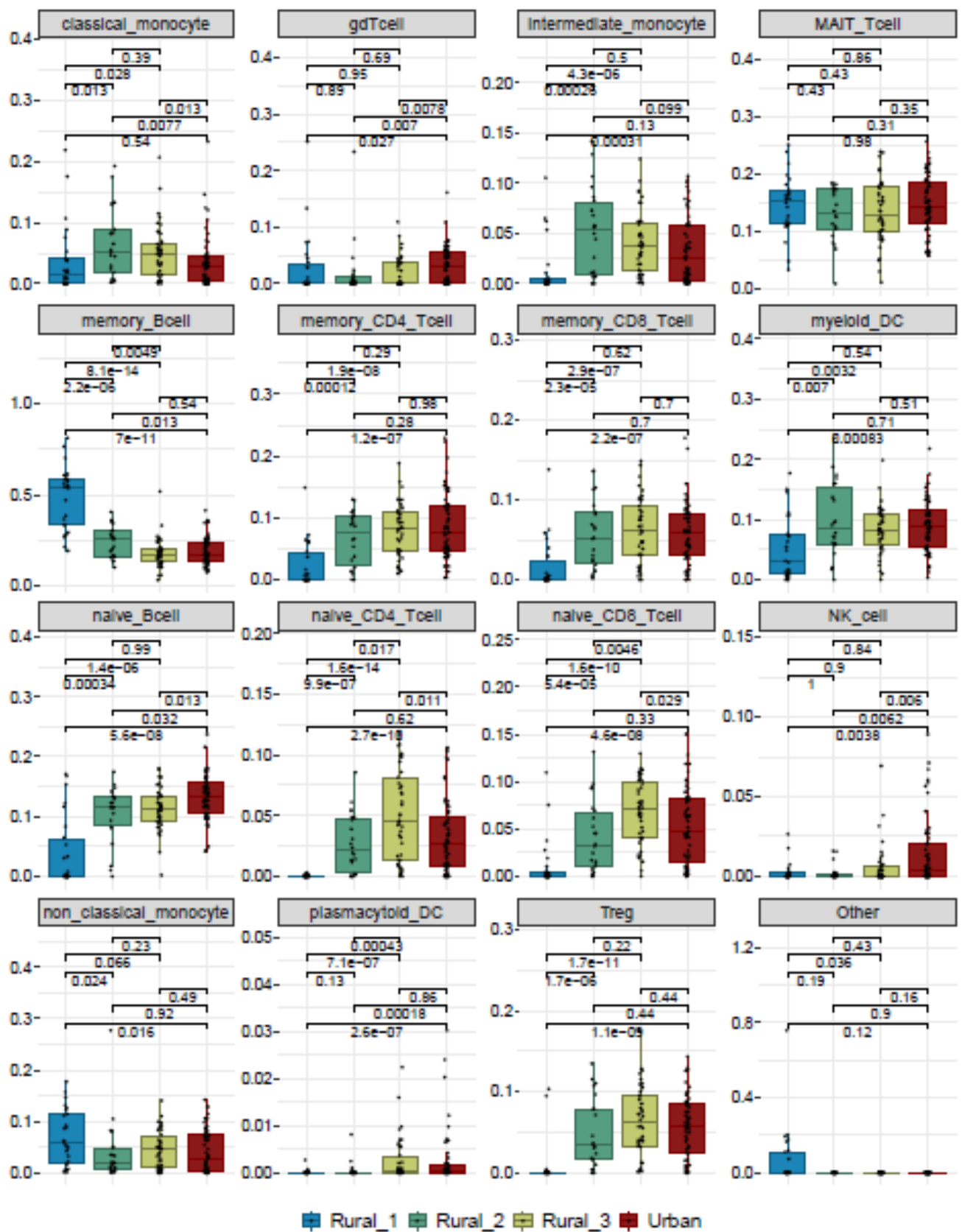
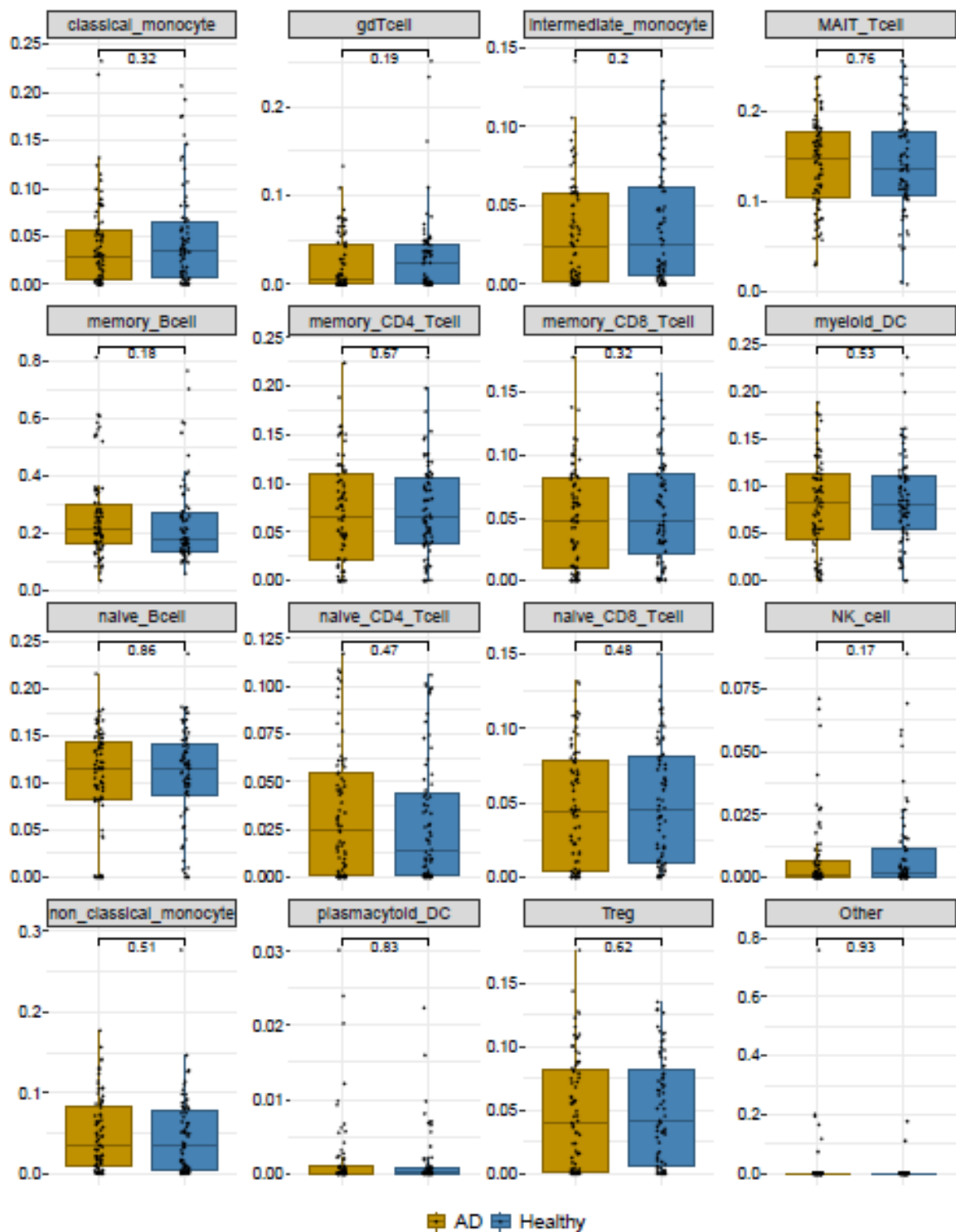


Figure 5.5. Digital cell quantification examines changes in cell type specific gene signatures that are associated with atopic dermatitis.



Differential Gene Analysis

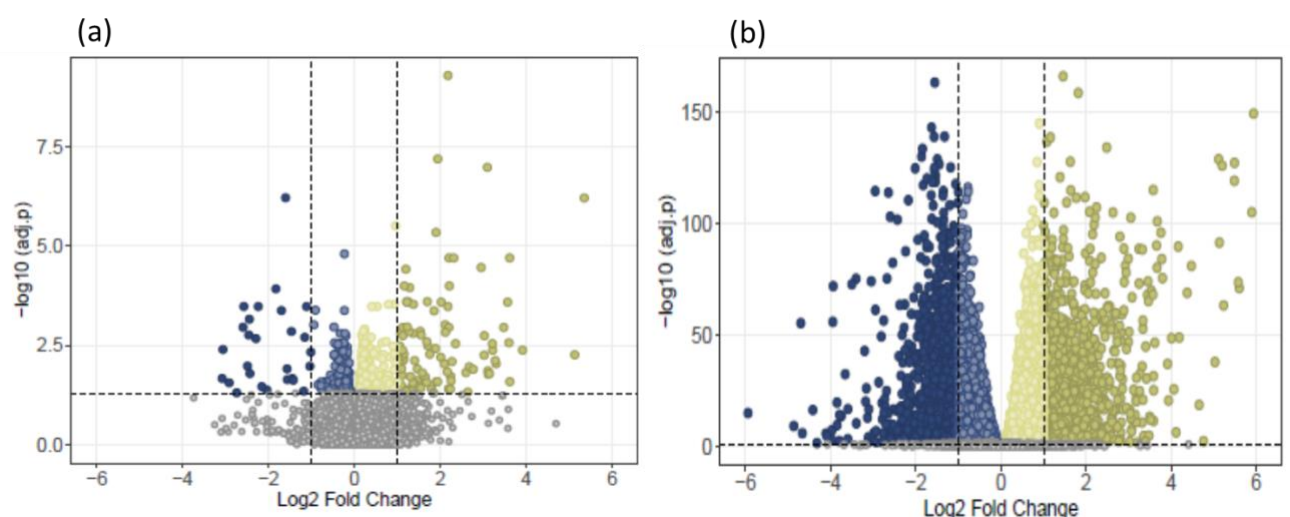
Following adjustments for confounding factors, genes that were up-regulated or down-regulated were identified. A summary of the analysis results is shown in **Table 5.2**. As there are too many genes to list in this thesis, the full list of significantly altered genes can be viewed online at <https://figshare.com/s/b63148eff833b5072f62>.

Table 5.2. Summary of the statistically significant differential expression analysis results for all group comparisons. A gene was considered significantly regulated when $p_{adj} < 0.05$ and greater than two-times difference in fold change between groups.

Comparison	Up regulated genes	Down regulated genes	Total
Healthy vs AD	105	27	132
Urban vs Rural	1321	1436	2757
Urban vs Rural 1	2428	1391	3819
Urban vs Rural 2	1351	1565	2916
Urban vs Rural 3	981	1802	2783
Rural 1 vs Rural 2	23	602	625
Rural 1 vs Rural 3	296	1836	2132
Rural 2 vs Rural 3	85	293	378

As can be clearly seen in this analysis, the primary driver of gene expression changes is the living environment of the child, rather than the presence of AD. This can be even more clearly illustrated when comparing the volcano plots for healthy versus AD and urban versus rural (**Figure 5.6**). However, even though relatively few genes were altered in PBMCs of AD patients, the most highly upregulated gene was IgE ($p_{adj} = 5.2 \times 10^{-10}$), reinforcing the link between AD and atopic mechanisms. In addition, gene expression of AD associated mediators such as IL-21, IL-21 receptor, IL-4, IL-5 and IL-9 receptor are significantly altered in this cohort using unadjusted p values and are just outside statistical significance with adjusted p values.

Figure 5.6. Volcano plots showing upregulated (in yellow) and downregulated (in blue) gene expression changes in **(a)** healthy children versus children with AD and **(b)** children living in an urban versus a rural setting. The x-axis indicates the fold change differences between the groups while the y-axis indicates the level of statistical significance (adjusted p value).



Interleukin and interleukin receptor gene expression were significantly different between urban and rural children (**Figure 5.7** and **Figure 5.8**). PBMCs from rural children had higher gene expression levels of mediators such as IL-6, IL-1 α and TNF α , which correlated well with the sera data for these cytokines in Chapter 4 of this thesis. In addition, rural children displayed significantly elevated levels of IL-1 receptor subtypes (IL-1R1, IL-1R2, IL-1RL2, IL-1RAP and IL-1RN) suggesting higher levels of IL-1 signaling in rural children compared to urban children (**Figure 5.7**). Importantly, IL-10 gene expression was also significantly upregulated in rural children. Each of the rural subgroups displayed significantly higher IL-10 gene expression levels compared to urban children (Rural 1 vs Urban padj = 3×10^{-18} ; Rural 2 vs Urban padj = 1×10^{-21} ; Rural 3 vs Urban padj = 1×10^{-20}). In contrast, urban children had the highest expression levels of IL-2 receptor subunits (IL-2R γ and IL-2R β) and genes related to T_H1 and T_H17 signaling (IL-12 α , IL-12R β 1, IL-23R, IL-17RC, IL-17RE and IL-17RA; **Figure 5.8**).

Figure 5.7. Interleukins and interleukin receptors that are significantly upregulated (padj<0.05) in PBMCs from rural children compared to urban children.

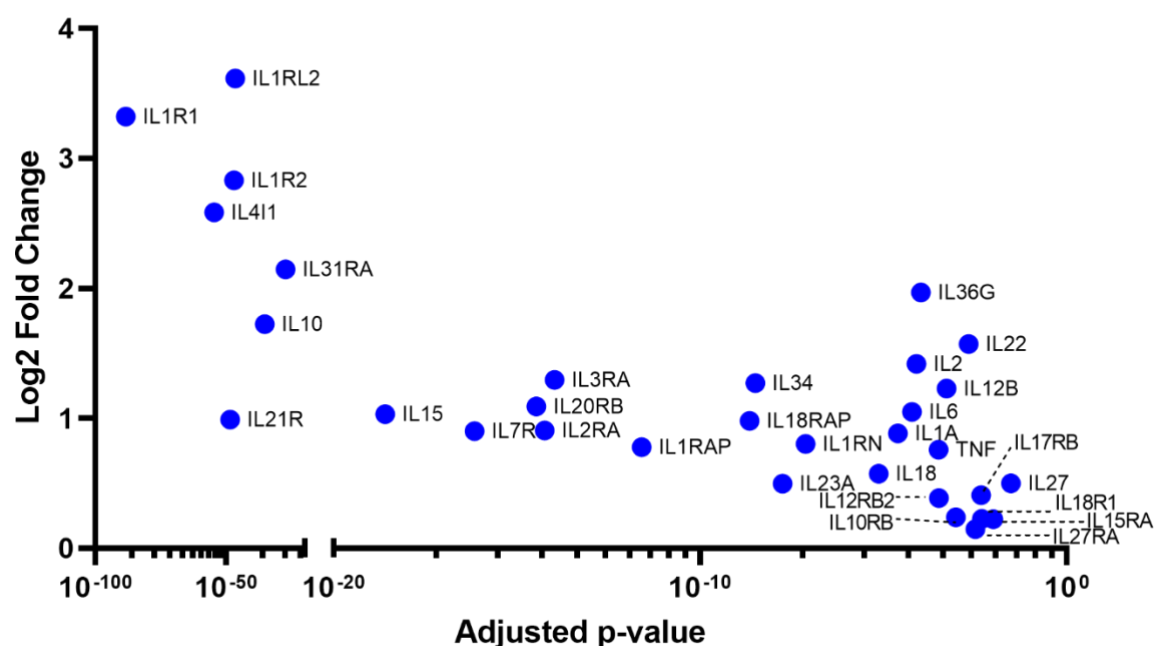
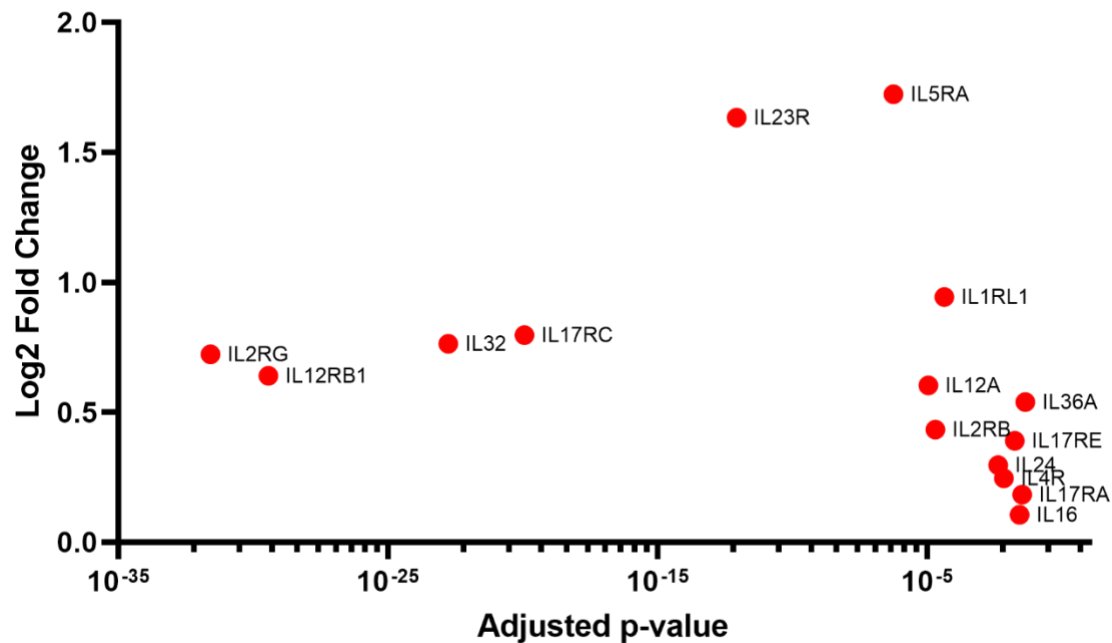


Figure 5.8. Interleukins and interleukin receptors that are significantly upregulated ($p_{adj} < 0.05$) in PBMCs from urban children compared to rural children.



In Chapter 4 of this thesis, levels of many chemokines were significantly elevated in the sera of rural children. Similarly, analysis of PBMC gene expression patterns revealed highly significant upregulation of chemokines and chemokine receptors in PBMCs from rural children (**Figure 5.9**). A substantially smaller number of chemokine and chemokine receptor genes were upregulated in PBMCs from urban children (**Figure 5.10**).

Figure 5.9. Chemokine and chemokine receptors that are significantly upregulated ($p_{adj} < 0.05$) in PBMCs from rural children compared to urban children.

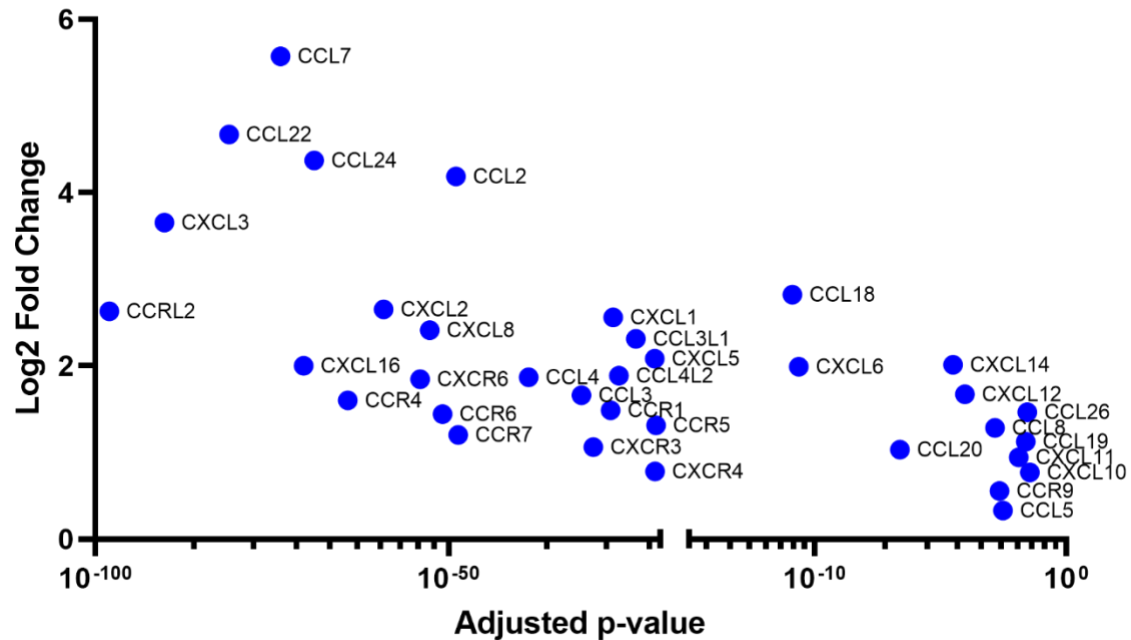
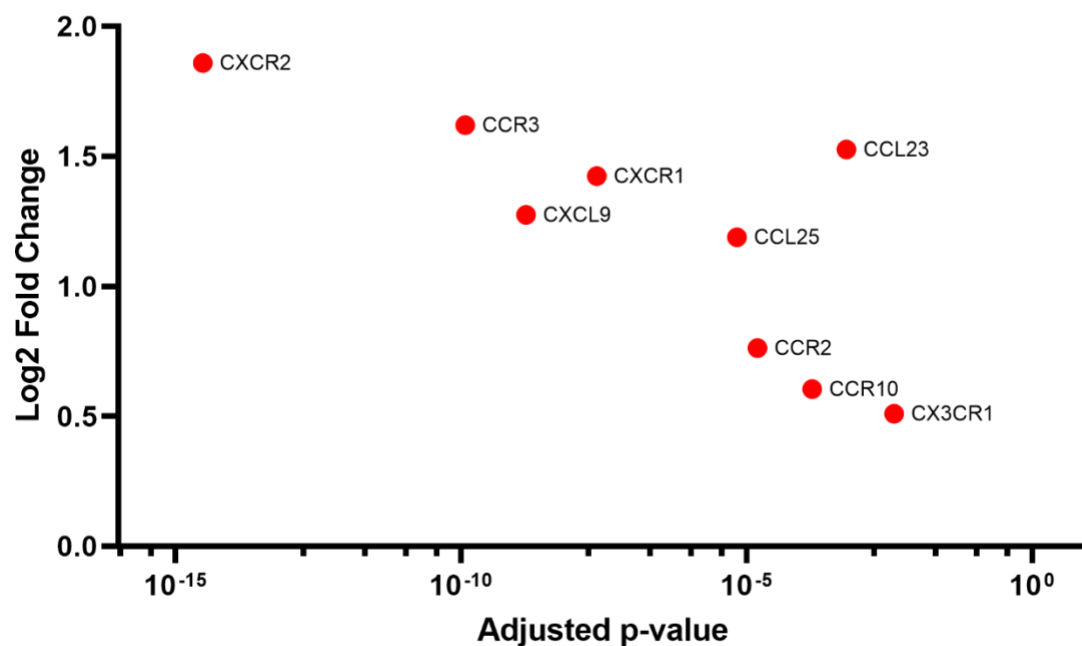


Figure 5.10. Chemokine and chemokine receptors that are significantly upregulated ($p_{adj} < 0.05$) in PBMCs from urban children compared to rural children.



G-protein coupled receptors are the largest family of human cell surface receptors, are ubiquitous in the body and involved in a wide array of physiological roles including regulation of the immune system. Drugs targeting G protein-coupled receptors have been the most successfully exploited class of small molecule therapeutics with 34-50% of FDA approved drugs targeting GPCRs. G protein-coupled receptors (GPCRs) have been shown to exert significant modulatory effects on immune cell activity and polarization. Multiple GPCRs were differentially regulated between rural and urban children with similar numbers of GPCR genes either being upregulated in PBMCs from rural children (**Figure 5.11**) or PBMCs from urban children (**Figure 5.12**). The most significantly upregulated GPCR gene in rural children was GPR132, which has been shown to be critical for blocking autoimmune responses in murine models. Interestingly, many of these cell surface GPCRs that were differentially expressed have been shown to play important roles in regulating immune responses by attenuating inflammation and promoting return to homeostasis or production of inflammatory mediators thereby influencing progression of infectious diseases and non-communicable diseases. Inhibitory leukocyte immunoglobulin like receptors (LILRs) that can block MHC-I induced immune responses (leukocyte immunoglobulin like receptor B1 (LILRB1; $\text{padj} = 7 \times 10^{-13}$), LILRB2 ($\text{padj} = 2 \times 10^{-10}$), LILRB3 ($\text{padj} = 2 \times 10^{-7}$) and LILRB4 ($\text{padj} = 7 \times 10^{-15}$) were also significantly upregulated in PBMCs from rural children (not depicted).

Figure 5.11. GPCR gene expression that are significantly upregulated ($p_{adj}<0.05$) in PBMCs from rural children.

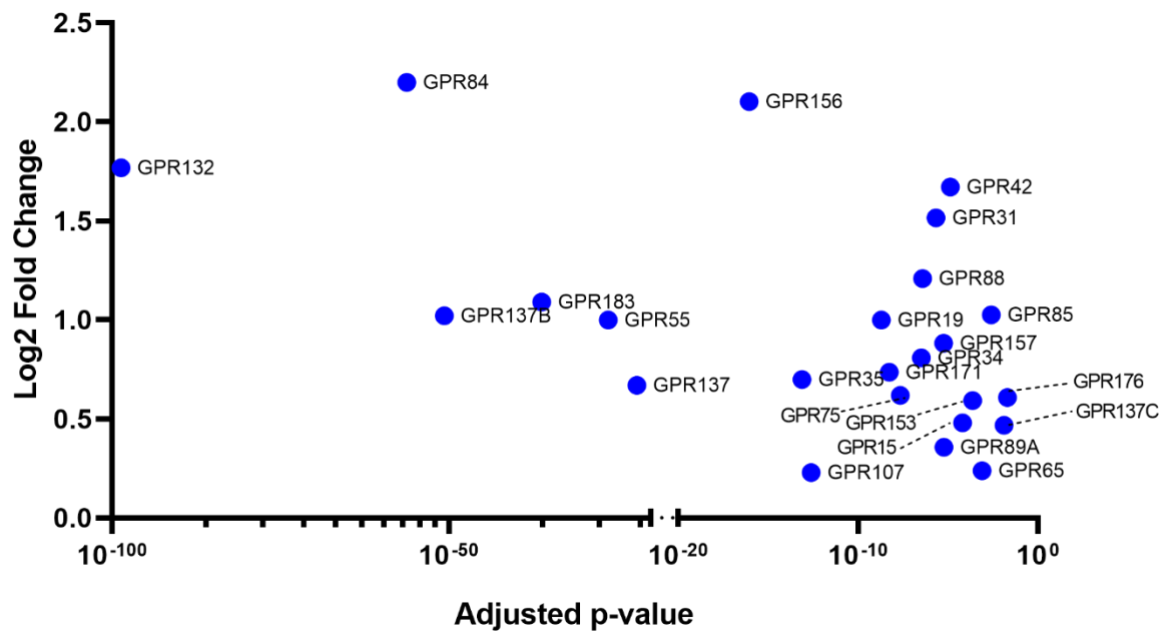
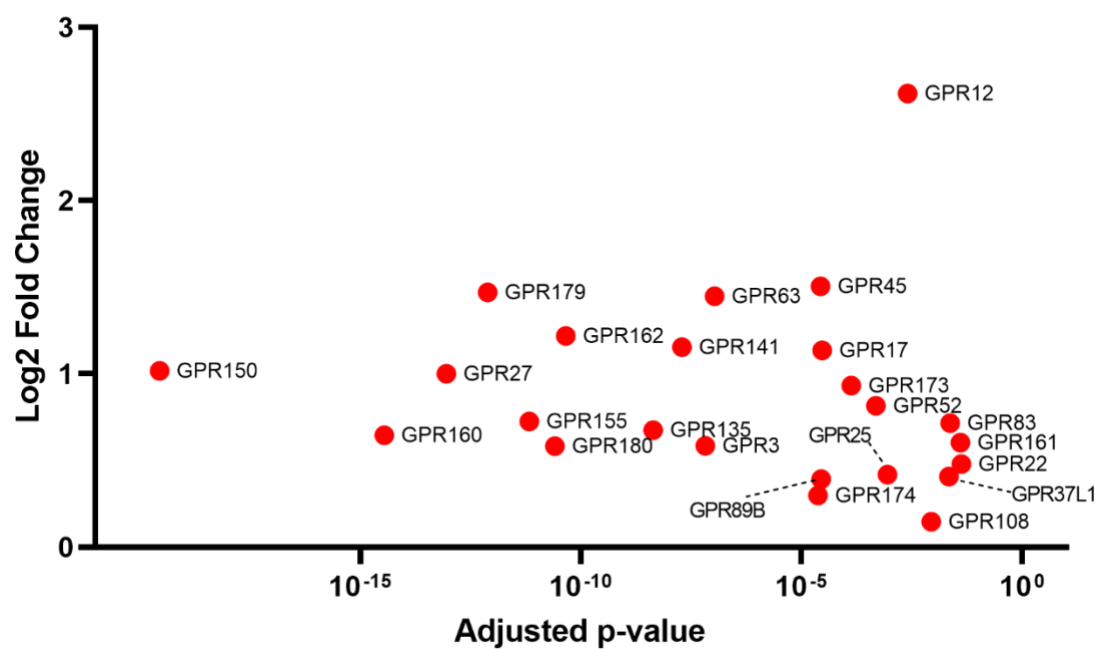


Figure 5.12. GPCR gene expression that are significantly upregulated ($p_{adj}<0.05$) in PBMCs from urban children.



Histamine is an important immunomodulator and expression levels of genes required for histamine synthesis, histamine degradation and histamine receptors were significantly different between rural and urban children. Histidine decarboxylase (HDC; $\text{padj} = 4 \times 10^{-12}$) and histamine receptor 2 (H_2R ; $\text{padj} = 2 \times 10^{-6}$) were significantly elevated in PBMCs from urban children, while H_1R ($\text{padj} = 1 \times 10^{-29}$) and histamine N-methyltransferase (HNMT; $\text{padj} = 1 \times 10^{-36}$) were significantly elevated in PBMCs from rural children (not depicted). Finally, genes related to aryl hydrocarbon receptor (AHR) signaling were significantly increased in PBMCs from rural children (AHR: $\text{padj} = 4 \times 10^{-12}$; AHR repressor (AHRR): $\text{padj} = 8 \times 10^{-17}$) (not depicted). AHR is well known for its important role for sensing xenobiotic agents and its crucial activity in xenobiotic metabolism in the liver. However, it is also expressed within epithelial barrier tissue such as the skin, intestine, lung and associated immune cells, where AHR signaling contributes to regulatory functions and protection from microbial invasion.²⁴ AHRR, on the other hand is expressed in response to AhR activation by its ligands and subsequently functions to counter regulate AHR target genes. It also plays a major role in immune cell differentiation and function.²⁵ AHRR was most significantly different in the Rural 1 subgroup compared to urban children ($\text{padj} = 6 \times 10^{-17}$), than the Rural 2 or Rural 3 subgroups compared to urban children ($\text{padj} = 1 \times 10^{-11}$ and 7×10^{-7} respectively) (not depicted).

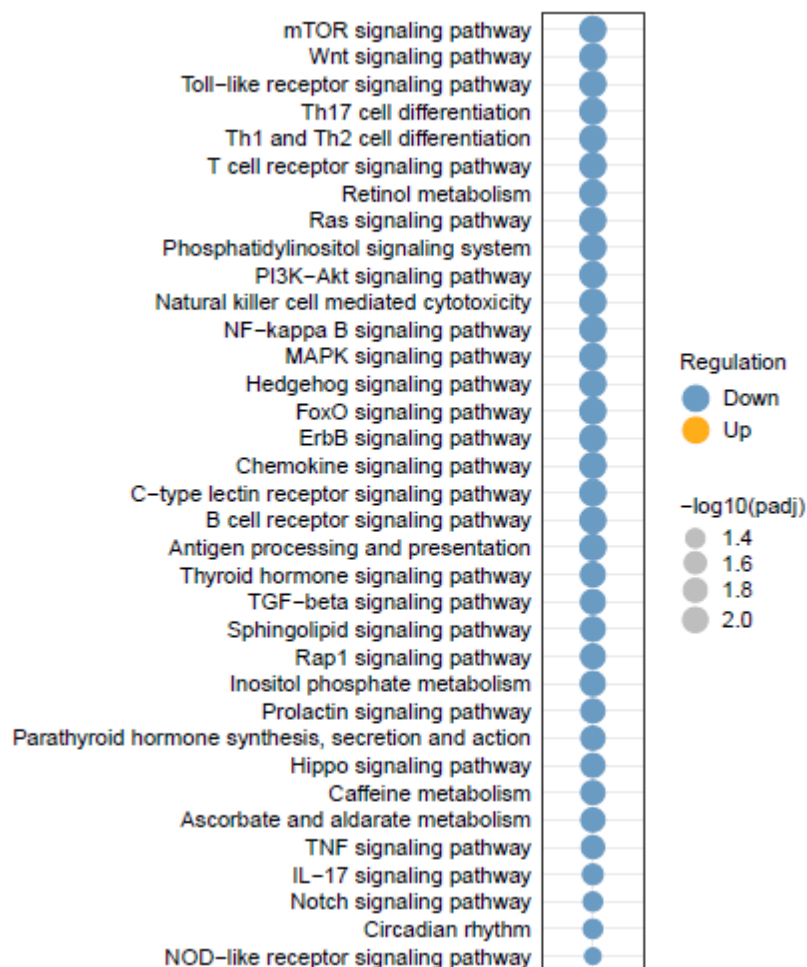
Gene Set Enrichment Analysis

While many individual genes were significantly different between the groups, a more complete understanding of the biological changes can be inferred from analysis of gene sets that group together into specific pathways. KEGG pathways belonging to the categories

Metabolism, Environmental Information Processing and Organismal Systems were used for this analysis.

Firstly, pathway analysis of gene expression in AD compared to healthy children revealed several pathways that were downregulated in PBMCs from children with AD (**Figure 5.13**). This includes pathways related to T cell polarization (e.g., T_H1 , T_H2 and T_H17 responses), innate responses to bacterial ligands (e.g., TLR signaling, NF- κ B), chemokine signaling and antigen processing.

Figure 5.13. Significantly regulated pathways ($p_{adj} < 0.05$) in children with AD compared to their healthy counterparts. Orange denotes upregulated pathways and blue denotes downregulated pathways in AD. Circle size is related to the level of statistical significance.



The pathways that are most significantly upregulated in rural children compared to urban children (**Figure 5.14**) were related to innate activation of the immune system (e.g., TLR signaling, NF- κ B signaling, MAPK signaling), cytokine (including TNF) and chemokine signaling, and changes in immune cell metabolism (i.e., oxidative phosphorylation). When each of the rural subgroups were analyzed separately for pathway level enrichments compared to urban children (**Figure 5.15**), consistent changes were observed for all three rural subgroups for TNF signaling, TLR signaling, cytokine-cytokine receptor interactions and oxidative phosphorylation. The neuroactive ligand-receptor interaction and ECM-receptor interaction pathways are only different in the Rural 1 subgroup, which was suggested to be the most significantly different rural subgroup according to the UMAP plots above.

Figure 5.14. The significantly regulated pathways ($p_{adj} < 0.05$) in rural children compared to urban children are illustrated. Orange denotes upregulated pathways and blue denotes downregulated pathways in AD. The size of the circle is related to the level of statistical significance.

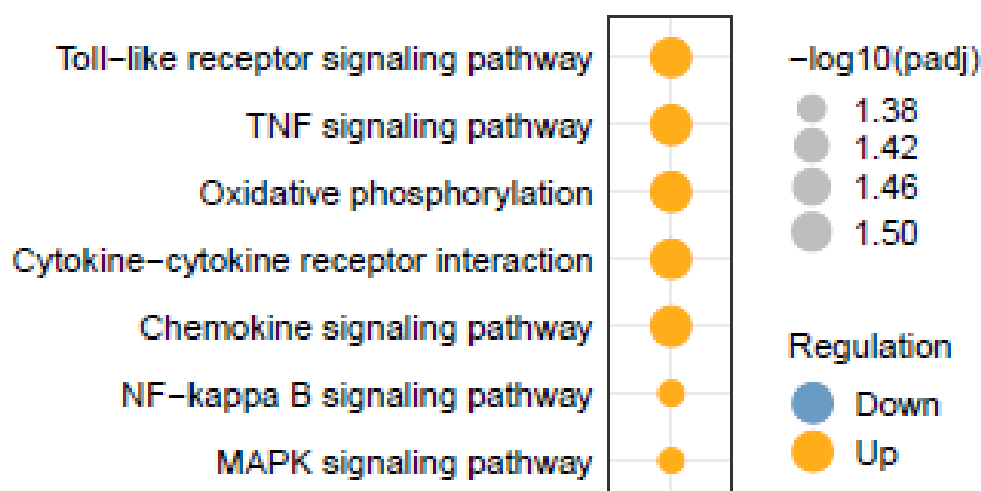
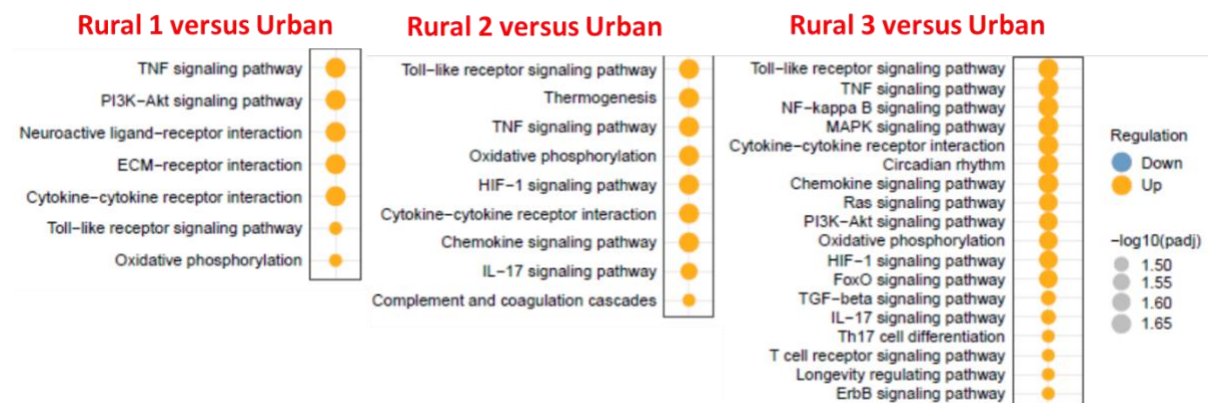


Figure 5.15. The significantly regulated pathways ($p_{adj} < 0.05$) in rural children subgroups compared to urban children are illustrated. Orange denotes upregulated pathways and blue denotes downregulated pathways in AD. The size of the circle is related to the level of statistical significance.



DISCUSSION

Multiple studies have shown that many human immune traits are primarily influenced by environmental factors.^{26,27} In agreement with these findings, our RNA-Seq analysis of PBMCs from this South African children's cohort revealed highly significant changes in immune cell gene expression profiles that were heavily dependent on the rural versus urban environment of the child. However, AD had a relatively minor effect on PBMC gene expression profiles, perhaps due to the inflammatory responses being predominant in the skin. Similar findings were reported in other atopic diseases, such as asthma, where few gene expression changes were observed in peripheral blood cells, although multiple gene expression changes were observed in bronchial biopsies from the disease site.²⁸ In addition, many of the gene expression changes noted in AD lesional skin relate to barrier associated factors and these are not expressed by circulating immune cells.²⁹

Unsupervised clustering of children's PBMC gene expression profiles revealed three rural subgroups that were separated from children within the urban setting. These three rural gene expression patterns may represent the transition from a truly rural gene expression signature (rural group 1) to an intermediate gene expression signature (rural group 2), to a rural group that has a gene expression profile not very distinct from the urban profile (rural group 3). Animal exposures and sunlight exposures were significantly different between rural children and urban children but were not significantly different between the three rural subgroups. However, antibiotic and paracetamol use, and delivery mode were significantly different only for rural group 1 (not for rural group 2 or rural group 3) compared to urban children, suggesting these factors could be related to the separation of PBMC gene expression profiles from all the other groups. Antibiotic and paracetamol use might indicate a higher frequency of early life infections. In addition, antibiotic use and c-section delivery would significantly influence the early life microbiota, thereby impacting immune development.³⁰⁻³²⁻

The innate and adaptive cell phenotypes present within peripheral blood were significantly different for children living within the rural versus the urban environments, while AD did not significantly influence the relative levels of circulating immune cells. In a recent Swedish study, they did not find any significant differences in the proportions of major immune lineages between anthroposophic non-IgE sensitized and non-anthroposophic IgE sensitized children, suggesting that the rural-urban differences in exposures that influence immune cell populations in our South African cohort may be missing in the North European setting.³³ Rural children had relatively higher levels of classical monocytes and memory B cells. Many of the cytokines and chemokines shown to be elevated in rural children (in Chapter 4) can be produced by innate cells such as monocytes.³⁴ Memory B cells develop within germinal

centers of secondary lymph nodes and can circulate in peripheral blood for decades where they respond rapidly and robustly to subsequent encounters with the antigen that first triggered them.^{35,36} Rural group 1 had the highest level of memory B cells, perhaps due to a greater frequency of exposure to microbes or pathogens, as already suggested by their use of antibiotics and paracetamol in the first year of life. During early life naïve T cells undergo cell differentiation mediated by epigenetic changes in response to external stimuli such as the cytokine microenvironment leading to the establishment of a basal immune state that may confer lifelong disease risk or protection. Indeed, Rural group 1 were the most significantly different to urban children for memory and naïve CD4 and CD8 cells, Treg cells and myeloid DCs. However, the relative levels of these cells were lowest in the Rural group 1 children. One possibility is that these cells were rapidly recruited to tissues where there were ongoing immune responses to microbes or pathogens (e.g., in the gut or respiratory tract) and this results in relatively lower levels of these cell subsets within peripheral blood. These cell types will be looked at in more detail in future cohorts.

One notable feature of the rural children's PBMC transcriptome was the high level of multiple pathways that are well described to regulate or suppress aberrant inflammatory immune responses. IL-10 gene expression was highly upregulated in rural children's PBMCs, and IL-10 potentially limits effector functions of antigen presenting cells and lymphocytes.³⁷ Gene expression of additional IL-10 family members (IL-20R and IL-22) were also elevated in rural children.

GPCRs are involved in mediating a large range of cellular events in response to external stimuli and serve as means of communication between the external and internal environments of the cell.³⁸ Recently, the Mas-Related G Protein-coupled Receptor Member

X2 (MRGPRX2) has been implicated in atopic dermatitis pathogenesis via various mechanisms and is expressed by key type 2 effector cells such as mast cells, basophils, and eosinophils. According to RNA sequence data MRGPRX2 is most abundantly expressed in skin tissue. MRGPRX2 has been shown to participate in the induction of human beta defensins 2 h β D-2 and h β D-3 and cathelicidin LL-37, which are important antimicrobial peptides (AMPs) that control *S.aureus* overgrowth in AD. It is also involved in the transduction of itch as well as regulation of sleep pattern - both key components of AD.³⁹ MRGPRX2 expression is restricted to tissues such as the skin and we did not detect MRGPRX2 gene expression in PBMCs. However, we did identify differences in expression for many other GPCRs expressed by PBMCs. Several significantly upregulated GPCR genes were identified in rural children including some that have proven immune regulatory function namely: GPR132; GPR183; GPR55; GPR17; GPR84; GPR35; GPR31; GPR171; GPR75; GPR15 and GPR65. GPR132, has been shown to be critical for blocking autoimmune responses in murine models⁴⁰ as well as GPR183 which has also been shown to be downregulated in another canonical autoimmune disease-systemic lupus erythematosus (SLE).⁴¹ GPR183 plays a role in germinal centre (GC) reactions that contribute greatly to humoral immune responses, which is keeping with our observation that rural children also had notably higher levels of memory B cells than urban children. GPR55 and GPR17 are negative regulators of $\gamma\delta$ T cell migration and negative regulators of inflammatory cell recruitment respectively.⁴² GPR17 also regulates TH2/TH17 cytokine expression in murine models.⁴³ Interestingly, GPR84 which was also highly expressed in rural children has been shown to play a role in regulating Th2 effector cell function. In GPR84 knockout mice, higher IL-4, IL-5, and IL-13 production was observed compared to wild-type mice.⁴⁴ In recent years the contribution of dietary components and their metabolites as regulators of immune function via their effects

on GPCR signaling has been established. For example, GPR35 expressed by dendritic cells can be activated by kynurenic acid, which is a metabolite generated during metabolism of the essential amino acid tryptophan.⁴⁵ Increased expression of GPR35 in rural children lends support to the concept that immune maturation can be influenced by different dietary practices that supply substrates used to generate GPCR targeting metabolites. GPR31, which is also significantly expressed in rural children, is also a metabolite sensing GPCR. Bacterial-derived metabolites such as pyruvate and lactate mediate CX3CR1^{high} myeloid cell sampling of luminal food antigen in a GPR31 dependent manner. GPR31 was shown to be essential in the induction of oral tolerance in response to dietary antigen by maintaining IL-10 producing intestinal RORγt⁺ Foxp3⁺ Treg cells.⁴⁶ GPR171, upregulated in rural children, has previously been shown to mediate suppressor effects on T cell mediated effector immune responses.⁴⁷ The functional consequences of higher levels of GPR75 expression in rural children will be interesting to clarify in future studies as it is known to be negatively regulated by RANTES/CCL5 (a proinflammatory chemokine).⁴⁸ GPR15 is a lymphocyte trafficking receptor abundantly detected in peripheral blood, spleen and colon. GPR15 is critical for preventing pathological inflammation by regulating preferential homing of Foxp3⁺ Treg cells to the lamina propria of the large intestine. Furthermore, the microbiota composition of the gut affects GPR15 expression as evidenced by a reduction in GPR15 expression in broad spectrum antibiotic-treated mice.⁴⁹ GPR65 is a pH sensing GPCR that is widely expressed on immune cells. Signaling via this receptor is important for regulating immune cell migration and maintaining epithelial barrier homeostasis. Diminished GPR65 signaling has been associated with asthma, inflammatory bowel disease and AD, while GPR65 deficiency in murine models results in more severe skin inflammation.⁵⁰ Other less well studied GPCRs such as GPR137; GPR137B; GPR137C; GPR156; GPR107; GPR42; GPR88;

GPR19; GPR85; GPR157; GPR34; GPR153; GPR176; GPR89A were also upregulated in the PBMCs of rural children. Overall, the emerging pattern of GPCR expression in these rural children is in agreement with the regulatory immune profile observed in these children.

Insufficient data has been reported to clarify the functional role of the majority of GPCRs that are upregulated in urban children's PBMCs specifically GPR150; GPR160; GPR27; GPR179; GPR162; GPR155; GPR180; GPR63; GPR141; GPR135; GPR45; GPR12; GPR17; GPR173; GPR52; GPR83; GPR22; GPR25; GPR37L1; GPR89B. However, notable observations have been made with regards to GPR3, GPR174 and GPR108. GPR3 is detected at low levels in naïve T cells and resting Treg cells but its expression is increased in effector T cells and activated Tregs.⁵¹ GPR174 signaling acts on Foxp3+ Treg cells in a manner that restrains their development, activation, and immune regulatory activity. In a mouse model of experimental autoimmune encephalitis (EAE), GPR174 expression on Tregs was associated with significantly higher disease scores compared to GPR174 deficient mice, which were protected from autoimmune pathology.^{52,53} GPR108 may exhibit dual functions where it negatively regulates TLR-triggered innate immune responses through antagonizing MyD88 and is a very potent activator of NF-κB when overexpressed.⁵⁴

Inhibitory leukocyte immunoglobulin like receptors were increased in rural children (LILRB1 to LILRB4). These receptors are expressed on immune cells where they bind to MHC class I molecules on antigen-presenting cells and transduce a negative signal that inhibits stimulation of an immune response. It is thought to control inflammatory responses and cytotoxicity to help focus the immune response and limit autoreactivity.^{55,56} Specifically, LILRB1 is expressed on numerous immune cell subsets and fine tunes key immune and cellular processes by modulating immune cell activation threshold and has a pivotal role in

immune tolerance for self- vs non-self-signaling. LILRB2 can induce tolerogenic DCs that impair effector T cell activation and promotes the induction of T regulatory cells (Tregs). LILRB3 is less well characterized but has demonstrated inhibitory effects on FcεRI or LILRA2-mediated IgE-dependent and -independent allergy. It also acts as a myeloid checkpoint receptor.⁵⁷ Interestingly, LILRB4 was suggested to promote dysfunctional immune tolerance to aeroallergens. The expression of LILRB4 in CD4⁺ CD25⁺ FOXP3⁺ Treg subset reduced suppression activity to aeroallergen.⁵⁸

Given the well-recognized role of histamine as a mediator of type I hypersensitivity reactions, it was surprising to note the diametrically opposed expression profile of related enzymes along environmental site differences. Highest expression of histidine decarboxylase gene was observed in urban children. Histidine decarboxylase is the enzyme responsible for generating histamine from the oxidative decarboxylation of the amino acid histidine whereas histamine N-methyltransferase degrades histamine, and this enzyme was most highly expressed in rural children. On balance, this could lead to higher background levels of histamine in urban children due to the increased rate of generation and decreased rate of degradation. There are several immune cellular sources of histamine with the main ones being mast cells and basophils. The human gut microbiota also synthesizes histamine. Certain fish and fermented foods can contain histamine generated by bacteria. The H1R receptor plays an important role in mediating the allergic reactions associated with histamine and it also has proinflammatory effects by promoting IFN γ secretion. H2R mediates the effect of histamine on acid secretion in the stomach and has been shown to have anti-inflammatory effects by inhibiting IFN γ , IL-12 and TNF α cytokines, accompanied by increased IL-10 secretion.⁵⁹ The differential expression pattern of both histamine receptors and enzymes according to study site, irrespective of their allergy phenotype,

suggests that environmental exposures early in life can remarkably influence responses to histamine. Overall, the combination of regulatory cytokines, GPCRs and inhibitory receptors suggest a higher level of immunoregulatory pathway activation in rural compared to urban children.

The aryl hydrocarbon receptor (AHR) is a ligand-activated cytosolic receptor, which belongs to Pern-Arnt-Sim (PAS) superfamily.⁶⁰ It was originally identified as a sensor of xenobiotic chemicals, in particular aromatic (aryl) hydrocarbons from which the receptor derives its name.⁶¹ The AHR repressor (AHRR) is also upregulated by AHR ligands.⁶² Increased expression of AHR and AHRR by rural children may suggest differences in exposures to pollutants generated by cooking or heating practices within the home. AHRR expression profiles when it comes to the rural subgroup analysis does not mirror AHR expression. This may be due to the selective tissue and immune cell expression of AHRR or induction of AHRR that is not based on environmental xenobiotic stimuli (AHR requiring) or its activation via AHR-independent mechanisms.²⁵ More recently, activation of AHR by microbial and human tryptophan metabolites has been shown to be important for maintenance of regulatory cell function and TH17 differentiation.²⁴ Interestingly, GPR135 was also upregulated in rural PBMCs and GPR135 responds to tryptophan metabolites such as serotonin derivatives and kynurenic acid.^{63,64} Given the importance of tryptophan metabolism for overall immune function, these pathways should be targeted for detailed examination in future studies.

CONCLUSIONS

A distinct AD gene expression signature is evident with relevant genes such as IgE being upregulated. However, the predominant influences on the immune cell transcriptome are related to early life environmental and lifestyle exposures. Protective (e.g., microbiota and animal) exposures and potentially detrimental (e.g., virus infection and pollutant) exposures shape the early life innate and adaptive immune response. Understanding these mechanisms will progress our appreciation for the importance of environment on immune development and prevention of chronic immune mediated disorders. These discoveries will also underpin the development of novel diagnostic markers and translational targets for more specific and safe modulation of immune activity, both within the skin, systemically and within other organs.

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THESIS CONCLUSIONS

The understanding of AD pathogenesis is far from complete. Atopic dermatitis is a highly complex multifactorial disease involving major compartments such as the skin, the immune system, and the microbiome, all of which may be under the influence of host genotype. The chronicity of the skin condition, the young age of affected patients and rapid rise in prevalence make effective treatments urgently needed.

Modulation of targeted immune pathways relevant to specific patient endotypes and disease phenotypes holds the most promise for AD therapies. There is a growing appreciation that population/ethnicity-specific disease phenotypes are mediated by distinct immune endotypes. One important outcome of this thesis is the identification of high levels of T_H2 mediators such as TARC and MCP-4 in South African children with AD. These are important objective immune biomarkers of disease and disease severity that are urgently needed to overcome the limitations of clinical scoring criteria, to aid accurate diagnosis, guide clinical management and monitor response to novel treatments in clinical trials.

Another important finding in this thesis is that clinical disease and disease trajectories are similar in urban and rural children where early childhood AD resembles the phenotypic hallmark features of itch, dry skin and flexural disease and associates with food allergen sensitization. In our setting, the majority of children with AD did not report family history of atopy, suggesting that these are breakthrough cases of AD representing the first generation of children that display allergic phenotypes previously observed in other regions.

Notwithstanding, when familial atopy was reported, this occurred more frequently in families of children with AD compared to those with no AD and was more frequent in urban families compared to their rural counterparts. Taken together this suggests that the urban

environment and familial atopy independently confer risk for AD, as well as potentially interacting in a synergistic manner.

In this thesis I demonstrated that IgE sensitization to food and aeroallergens is common in children with AD in South Africa with similar levels of sensitization in these high-risk groups in both urban and rural settings. This is in direct contrast to previously published epidemiology data that suggested lower levels of sensitization in rural South African communities. The high level of IgE sensitization reflects the unique susceptibility of having a disrupted epidermal barrier through which sensitization is likely mediated, even in otherwise protective environments such as rural lifestyles. The evident poly IgE sensitization pattern also illustrates the strong atopic association of this phenotype of AD. Given the relatively young age of this cohort, their regular anti-helminthic treatment and low CCD IgE, the IgE sensitization data described in this thesis can no longer be dismissed as epiphenomena of cross-reactive carbohydrate epitopes. Importantly, a potentially tolerogenic IgG4 antibody profile was associated with increasing age in rural children with AD and egg sensitization. This may be due to rural exposures that promote regulatory responses but may not be simultaneously evident for other allergens given their individual variations in allergenicity. In addition, data presented in this thesis suggests that available positive predictive values for food allergy are not sufficiently specific for young infants in this South African population.

One of the most notable and remarkable findings is that living environment is the dominant driver of distinct circulating immune and inflammatory mediators in urban and rural children regardless of atopic disease status. This highlights the importance of identifying and understanding the exposures that promote optimal development of immune functions within the first years of life. In addition to circulating mediators, I have performed a detailed

gene expression analysis of immune cells within peripheral blood using RNA sequencing and this is the first study utilizing this powerful technology in South African children with AD.

The immune cell gene expression signatures corroborated the clinical and molecular type 2 immune activation of this AD phenotype, but also better defined the molecular fingerprint for rural and urban living environments. Overall, protective (e.g., outdoor and animal) exposures and potentially detrimental (e.g., virus infection and pollutant) exposures shape the early life innate and adaptive immune response. In addition, when immune-mediated disorders do occur in children, we should consider background living environment for potential interactions with immune-targeting therapies and their impact on treatment efficacy.

In conclusion, the study results described in this thesis fill in some of the gaps in our knowledge on the interactions between atopic dermatitis and environmental exposures in South African children. Focusing my research on this previously understudied African population has helped to clarify the relevant AD endotypes, as well as helping to quantify the relative importance of genetics versus environment in immune development and immune-mediated pathologies. My results also suggest novel basic mechanisms that underpin the gene-environment interactions that lead to atopic disorders. The next phase of this fascinating work should include detailed functional studies in experimental models to clarify the mechanisms engaged to communicate environmental effects on the developing immune system. In addition, we should promote and encourage immune mapping of African AD patients undergoing clinical trials in order to identify variations in the pharmacogenetic responses that will ensure successful targeting of precision medicine efforts to individuals of African ancestry.

While the analyses described in this thesis have provided important mechanistic insights, there are several limitations that may impact the generalizability and interpretation of the findings. Firstly, a larger AD study in both settings may provide more robust data to corroborate the findings described in this thesis. In addition, my findings have been described specifically for a single ethnolinguistic population within South Africa and future studies in additional populations, both within Africa and other parts of the world, should assess if my findings are broadly relevant or if they are applicable only to defined subpopulations. Furthermore, the impact of environmental exposures on AD and immune development should be further explored in a longitudinal study to capture the trajectory of changes, rather than a single time point snapshot of differences between subgroups. Lastly, maternal self-reported lifestyle and environment exposure data can be subject to biases and additional validation measures, such as researcher recorded measurements within the family home, would improve the reliability of the data and enhance confidence in my conclusions.

APPENDIX 1

ALLERGY QUESTIONNAIRE

Participant details

Q1	Participant ID	0000/XX	
Q2	Enrolment date	DD/MM/YYYY	
Q3	Study site	1 = Urban	2 = Rural
Q4	Date of birth	DD/MM/YYYY	
Q5	Age at enrolment	00 months	
Q6	Sex	1 = Male	2 = Female
Q7	Weight	00.0 kg	
Q8	Height/Length	000 cm	

Q9 Immunisations

			Yes	No				Yes	No
Birth	9.1	BCG			14 weeks	9.10	RV(2)		
	9.2	OPV (0)				9.11	DTaPIPv/HiB(3)		
6 weeks	9.3	OPV (1)				9.12	HepB(3)		
	9.4	RV (1)				9.13	PCV7 or 13 (2)		
	9.5	DTaPIPv/HiB (1)			9 months	9.14	Measles vaccine		
	9.6	HepB (1)				9.15	PCV 7 or 13 (3)		
	9.7	PCV7 or 13(1)			18 months	9.16	DTaPIPv/HiB (4)		
10 weeks	9.8	DTaPIPv/HiB (2)				9.17	Measles Vaccine		
	9.9	HepB (2)							

Q10 Vaccination Status

1 = Complete		2 = Incomplete	
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Q11 Paracetamol Exposure

Q11.1	Did your child have paracetamol or medicines containing paracetamol in the first year of life? (Panado, Calpol, Paramed)	0 = No	1 = Yes	
Q11.2	If Yes, How old was your child when they first had paracetamol?			Months
Q11.3	If Yes, How often did your child have paracetamol on the first year of life?	1 = 1-10 days	2 = 10-20 days	3 = More than 20 days

Q12 Childhood infections

Has your child had any of the following childhood infections?									
		0 = No	1 = Yes	999 = don' t know			0 = No	1 = Yes	999 = don't know
12.1	Measles				12.5	Glandular Fever			
12.2	Mumps				12.6	Tuberculosis			
12.3	Rubella				12.7	Hepatitis			
12.4	Chickenpox				12.8	Other (please specify)			

Q13 Antibiotic and anti-helminthic exposure

13.1	Did your child have any antibiotics in the first year of life?	0 = no	1 = Yes	999 = Don't know	
13.2	If Yes, how old were they with the first course??				Months
13.3	How many courses did your child have in the first year of life?	1 = None	2 = 1 to 2 Courses	3 = 3 to 5 courses	4 = > 5 courses
13.4	Did your child have any antibiotics in the last 2 months?	0 = No	1 = Yes	999 = Don't know	

13.5	Has your child ever had medicines for worms?	0= No	1 = Yes	999=Don't Know
13.6	If Yes, at what age were they dewormed?			months
13.7	If Yes, at what age did they last take anti worm medicines?			months
13.8	Has your child had regular (yearly) medicine for worms?	0 = No	1 = Yes	999 = Don't know

Q14 Probiotic Exposure and *Amasi* Exposure (CHILD)

14.1	Did this child have probiotics in food or supplements in the first year of life?	0 = No	1 = Yes	999 = Don't know
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14.2	If Yes , how old was this child when they first had probiotics?			Months
14.3	If Yes , How often did this child have probiotics in the first year of life?	1 = 1-10 days	2 = 10-20 days	3 = >20 days
14.4	If Yes , which probiotic product were they given?			

14.3	Has your child ever have amasi ?	0 = No	1 = Yes	999 = Don't know
14.4	If Yes , how old was this child when they first had amasi ?			Months
14.5	If Yes , how often does your child take amasi?	1 = Less than once a month	2 = 1-4 times per month	3 = more than 4 times per month

Probiotic and *Amasi* exposure (MOTHER)

14.6	Did this child's mother have probiotics during pregnancy?	0 = No	1 = Yes	999 = Don't know
14.7	If Yes, How often did she use probiotics?	1 = 1-10 days	2 = 10-20 d ays	3 = >20 days
14.8	Which yes, which products did she use?			
14.9	Did this child's mother regularly have amasi during pregnancy? (more than once a month)	0 = No	1 = Yes	999 = Don't know

Q15 Delivery mode

Q15	How was this child born?	1 = Normal vaginal delivery	2 = Caesarean section	999 = Don't Know
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Q16 Sunlight Exposure

How much time does your child spend outdoors on an average day? (add crèche daily average X5, to weekend total hours and divide by 7. Report to nearest 0.1)				
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Q16.1 Winter	Q16.2 Summer
00.0 hours	00.0 hours
999 = Don't Know	999 = Don't Know

Q17 Peanut exposure in pregnancy

17.1	Did this child's mother eat peanuts regularly (every week) during pregnancy?	0 = No	1 = Yes	999 = Don't know
17.2	Did this child's mother completely avoid peanuts during pregnancy?	0 = No	1 = Yes	999 = Don't know

Q18 Breastfeeding information

Q18.1	Was this child ever breastfed?	0 = No	1 = Yes	999 = Don't know
Q18.2	If Yes, up to what age was this child exclusively breastfed (i.e. no milk or other fluids via bottle nor given any solids)		Months	999 = Don't know
Q18.3	At what age did you completely stop breastfeeding this child?		Months	999 = Don't know
Q18.4	At what age did you first introduce any other milk (other than breast milk)		Months	999 = Don't know

Q19 Weaning foods

Q19.1	When did you first introduce solids into your child's diet?		Months
Q19.2	Which three types of foods did you introduce first? (see coding chart for food categories)	Free Text	Free text

Q20 Exposure to Unpasteurised milk

Q20	Has this child ever had non-pasteurised (Fresh farm) milk?	0 = No	1 = Yes	999 = Don't know
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Q21 Food exposure

Foods Eaten	Ever Eaten	Age first eaten	Still eating regularly (once a month or more)
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21.1	Peanut e.g. peanut butter, peanut oil, peanuts in cookies or chocolates	21.1 .1	0 = No	1 = Yes	21.1 .2		mont hs	999 = don't know	21.1 .3	0 = No	1 = Yes If yes please contin ue with 21.1.4
If yes to 21.1.4 Please try an remember how many times your child ate the following peanut containing foods in the last day and week		Type of peanuts consumed	Numbe r of times per day	Number of times per week	Usual amount			Total amount in grams			
		Peanut butter on bread				Thin slices	21.1.4				
						Thick slices	21.1.5				
		Peanut butter in porridge				Teaspoons	21.1.6				
		Raw/boiled peanuts				Handfuls	21.1.7				
		Roasted peanuts				Handfuls	21.1.8				
		Peanuts in chocolate or biscuits					21.1.9				
		Total amount of peanut consumed in the last week							21.1.10		
21.2	Other nuts e.g. Cashew, hazelnut, brazil nut, almonds, walnuts, pistachio, macadamia in chocolate and cookies or cakes and breads	21.2 .1	0 = No	1 = Yes	21.2 .2		mont hs	999 = don't know	21.2 .3	0 = No	1 = Yes
21.3	Cow's milk products e.g. fresh milk, cheese, yoghurt, cream	21.3 .1	0 = No	1 = Yes	21.3 .2		mont hs	999 = don't know	21.3 .3	0 = No	1 = Yes
21.4	Cow's milk formula e.g. Nan, Lactogen, Novolac, S26, Infacare, Pelargon	21.4 .1	0 = No	1 = Yes	21.4 .2		Mont hs	999 = don't know	21.4 .3	0 = No	1 = Yes

21.5	Soya products e.g. soya mince, soya sauce, tofu, baby foods, soya in bread	21.5 .1	0 = No	1 = Yes	21.5 .2		Mont hs	999 = don't know	21.5 .3	0 = No	1 = Yes
21.6	Soya milk products e.g. Infasoy, Isomil, Infacare	21.6 .1	0 = No	1 = Yes	21.6 .2		Mont hs	999 = don't know	21.6 .3	0 = No	1 = Yes
21.7	Hen's egg e.g. boiled, scrambles eggs, omelettes, quiches(souttert), cakes, cookies, rusks	21.7 .1	0 = No	1 = Yes	21.7 .2		Mont hs	999 = don't know	21.7 .3	0 = No	1 = Yes
21.8	Wheat e.g. cereal (Wheetabix, All Bran, Tasty Wheat), bread or pasta, cakes, rusks, couscous, semolina	21.8 .1	0 = No	1 = Yes	21.8 .2		Mont hs	999 = don't know	21.8 .3	0 = No	1 = Yes
21.9	Fish (excluding shellfish) Hake, snoek, sardines,pilchards, tuna, kingklip, salmon etc and fish products: Fish paste(Redro), Worcester sauce, fish sauce	21.9 .1	0 = No	1 = Yes	21.9 .2		mont hs	999 = don't know	21.9 .3	0 = No	1 = Yes

Q22 Food Reactions

22.1 PEANUTS			
22.1.1	Has your child ever had any of these reactions below to peanuts or food containing peanuts		0 = No 1 = Ye
22.1.2	If yes, which of the following reactions has this child had? (you can circle more than one)		
	1= none	6= tight throat	11=shock/low blood pressure
	2= itchy Rash	7= wheeze	12= collapse/loss of consciousness
	3=swelling(face/lips/eyes)	8= vomiting	13 = worsening of eczema
	4= flushing	9= diarrhoea	
	5= itchy mouth/throat	10= blue lips	
22.1.3	If Yes, how old was this child when he/she had the first reaction?		Months

22.1.4	If yes, how long after the eating the food did the reaction occur?	1 = < 1 hour	2 = 1-24 hours	3 = >24 hours
22.1.5	If yes, was this reaction confirmed by a doctor?	0 = No		1 = Yes

22.2 OTHER NUTS					
22.2.1	Has your child ever had ny of these reactions below to other nuts or food containing nuts other than peanuts		0 = No	1 = Yes	
22.2.2	If yes, which of the following reactions has this child had? (you can circle more than one)				
	1 = none	6 = tight throat	11 =shock/low blood pressure		
	2 = itchy Rash	7 = wheeze	12 = collapse/loss of consciousness		
	3 =swelling(face/lips/eyes)	8 = vomiting	13 = worsening of eczema		
	4 = flushing	9 = diarrhoea			
	5 = itchy mouth/throat	10 = blue lips			
22.2.3	If Yes, how old was this child when he/she had the first reaction?			Months	
22.2.4	If yes, how long after the eating the food did the reaction occur?		1 = < 1 hour	2 = 1-24 hours	3 = >24 hours
22.2.5	If yes, was this reaction confirmed by a doctor?		0 = No		1 = Yes

22.3 COW'S MILK				
22.3.1	Has your child ever had any of these reactions below to cow's milk or food containing cow's milk	0 = No		1 = Yes
22.3.2	If yes, which of the following reactions has this child had? (you can circle more than one)			
	1 = none	6 = tight throat	11 = shock/low blood pressure	
	2 = itchy Rash	7 = wheeze	12 = collapse/loss of consciousness	
	3 =swelling(face/lips/eyes)	8 = vomiting	13 = worsening of eczema	
	4 = flushing	9 = diarrhoea		
	5 = itchy mouth/throat	10 = blue lips		
22.3.3	If Yes, how old was this child when he/she had the first reaction?	Months		
22.3.4	If yes, how long after the eating the food did the reaction occur?	1 = < 1 hour	2 = 1-24 hours	3 = >24 hours

22.3.5	If yes, was this reaction confirmed by a doctor?	0 = No	1 = Yes
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22.4 SOYA					
22.4.1	Has your child ever had any of these reactions below to soya or food containing soya		0 = No	1 = Yes	
22.4.2	If yes, which of the following reactions has this child had? (you can circle more than one)				
	1 = none	6= tight throat	11=shock/low blood pressure		
	2 = itchy Rash	7= wheeze	12= collapse/loss of consciousness		
	3 =swelling(face/lips/eyes)	8= vomiting	13 = worsening of eczema		
	4 = flushing	9= diarrhoea			
	5 = itchy mouth/throat	10= blue lips			
22.4.3	If Yes, how old was this child when he/she had the first reaction?			Months	
22.4.4	If yes, how long after the eating the food did the reaction occur?		1 = < 1 hour	2 = 1-24 hours	3 = >24 hours
22.4.5	If yes, was this reaction confirmed by a doctor?		0 = No	1 = Yes	

22.5 HEN'S EGG					
22.5.1	Has your child ever had any of these reactions below to hen's egg or food containing egg		0 = No	1 = Yes	
22.5.2	If yes, which of the following reactions has this child had? (you can circle more than one)				
	1 = none	6 = tight throat	11 =shock/low blood pressure		
	2 = itchy Rash	7 = wheeze	12 = collapse/loss of consciousness		
	3 =swelling(face/lips/eyes)	8 = vomiting	13 = worsening of eczema		
	4 = flushing	9 = diarrhoea			
	5 = itchy mouth/throat	10= blue lips			
22.5.3	If Yes, how old was this child when he/she had the first reaction?			Months	
22.5.4	If yes, how long after the eating the food did the reaction occur?		1= < 1 hour	2= 1-24 hours	3= >24 hours
22.5.5	If yes, was this reaction confirmed by a doctor?		0 = No	1 = Yes	

22.6 WHEAT				
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22.6.1	Has your child ever had any of these reactions below to wheat or food containing wheat	0 = No	1 = Yes
22.6.2	If yes, which of the following reactions has this child had? (you can circle more than one)		
	1= none	6= tight throat	11=shock/low blood pressure
	2= itchy Rash	7= wheeze	12= collapse/loss of consciousness
	3=swelling(face/lips/eyes)	8= vomiting	13 = worsening of eczema
	4= flushing	9= diarrhoea	
	5= itchy mouth/throat	10= blue lips	
22.6.3	If Yes, how old was this child when he/she had the first reaction?		Months
22.6.4	If yes, how long after the eating the food did the reaction occur?	1= < 1 hour	2= 1-24 hours 3= >24 hours
22.6.5	If yes, was this reaction confirmed by a doctor?	0 = No	1 = Yes

22.7 FISH (excluding shellfish)			
22.7.1	Has your child ever had any of these reactions below to fish or food containing fish	0 = No	1 = Yes
22.7.2	If yes, which of the following reactions has this child had? (you can circle more than one)		
	1 = none	6 = tight throat	11 =shock/low blood pressure
	2 = itchy Rash	7 = wheeze	12 = collapse/loss of consciousness
	3 =swelling(face/lips/eyes)	8 = vomiting	13 = worsening of eczema
	4 = flushing	9 = diarrhoea	
	5 = itchy mouth/throat	10 = blue lips	
22.7.3	If Yes, how old was this child when he/she had the first reaction?		Months
22.7.4	If yes, how long after the eating the food did the reaction occur?	1= < 1 hour	2= 1-24 hours 3= >24 hours
22.7.5	If yes, was this reaction confirmed by a doctor?	0 = No	1 = Yes

Q23 Asthma

Q23.1	Has your child ever symptoms of asthma without having a cold or chest infection? (e.g. wheeze, persistent cough at night or when exercising, shortness of breath)	0 = No	1 = Yes
Q23.2	If Yes, how old was he/she?		Months

Q23.3	If yes, who diagnosed the asthma?	1 = Self	2 = Nurse	3 = Doctor
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Q24 Hay fever

Q24.1	Has your child ever had symptoms of hay fever (e.g. itchy runny eyes, itchy runny nose, blocked nose, frequent sneezing) without having a “cold” or upper respiratory tract infection?	0 = No		1 = Yes	
Q24.2	If Yes, how old was he/she?			Months	
Q24.2	If yes, who diagnosed the hay fever?	1 = Self	2 = Nurse		3 = Doctor

Q25 Eczema

Q25.1	Has your child ever had symptoms of eczema (e.g. an itchy rash especially in the folds of the elbows, behinds the knees, in front of the ankles, under the buttocks or around the neck, ears or eyes?)	0 = No		1 = Yes	
Q25.2	If yes, how old was your child then?				Months
Q25.3	If yes, who diagnosed the eczema?	1 = Self	2 = Nurse	3 = Doctor	

Q26 Medication use

Q26.1	Is your child on any of the following medication? (see NAEP Chart)							
26.1.1	INHALERS	Relievers (blue) e.g. Asthavent	0 = No	1 = Yes	25.1.5	Nasal corticosteroid spray e.g. Beclate	0 = No	1 = Yes
26.1.2		Controllers (brown/cream) e.g. Budeflam	0 = No	1 = Yes	25.1.6	Antihistamines (If yes, please complete Q26.4 below)	0 = No	1 = Yes
26.1.3		Other (please specify below)	0 = No	1 = Yes	25.1.7	Steroid creams	0 = No	1 = Yes
26.1.4		Home nebuliser	0 = No	1 = Yes	25.1.8	Adrenalin auto injector or pen e.g. Epipen	0 = No	1 = Yes
Q26.2	If your child is on any other oral medication (pills or syrups) for asthma, please specify each one.		Free text					

	(e.g. oral steroids or leukotriene receptor antagonists)			
Q26.3	If your child is on antihistamines, please specify which type/brand?	Free text		
Q26.4	If your child is on antihistamines, how many days since they were last taken?	1 = <2 days	2 = 2-5 days	3 = >5 days
Q27.5	If your child is on any other medication for other illnesses, please specify each one.	Free text		

Q27 Family history of allergic disease

Does anyone in your family have allergic diseases? Please circle. (you can choose more than one option)						
	Family member	None	Asthma	Hay fever	Eczema	Food allergy
Q27.1	Mother	1 = none	2 = asthma	3 = hay fever	4 = eczema	5 = food allergy
Q27.2	Father	1 = none	2 = asthma	3 = hay fever	4 = eczema	5 = food allergy
Q27.3	Full Sibling 1	1 = none	2 = asthma	3 = hay fever	4 = eczema	5 = food allergy
Q26.4	Full Sibling 2	1 = none	2 = asthma	3 = hay fever	4 = eczema	5 = food allergy
Q27.5	Full Sibling 3	1 = none	2 = asthma	3 = hay fever	4 = eczema	5 = food allergy
Q27.6	Full Sibling 4	1 = none	2 = asthma	3 = hay fever	4 = eczema	5 = food allergy

Q28 Child's Medical history

Q28.1	Does your child have any other significant medical problems? (e.g. heart or lung problems, kidney or liver disease, epilepsy, diabetes)	0 = No	1 = Yes
Q28.2	If yes, please specify		

Q29 Home Language and Migration

Q29.1	What language do you mainly speak at home? (Please choose only one)		
	1 = IsiXhosa	2 = English	3 = Afrikaans
	4 = IsiZulu	5 = Sesotho	6 = Setswana
	7 = SiSwati	8 = IsiNdebele	9 = Xitsonga
	10 = Sepedi	11 = Tshivenda	12 = Other
Q29.1.2	If Xhosa speaking, was this child born in Cape Town?	0 = No	1 = Yes

	Province
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29.2.1	If No, where was this child born?	1 = Western Cape 2 = Eastern Cape 3 = Northern Cape 4 = Free state 5 = Gauteng		6 = Kwazulu Natal 7 = Mpumalanga 8 = Limpopo 9 = North West Province 10 = Other	
		Town (specify)		Free text	
29.2.2	When did this child move to Cape Town?		Year		Month

Q29.3	Was this child's mother born in Cape Town?				0 = No	1 = Yes
29.3.1	If No, where was she born?	Province (please circle)				
		1 = Western Cape 2 = Eastern Cape 3 = Northern Cape 4 = Free state 5 = Gauteng		6 = Kwazulu Natal 7 = Mpumalanga 8 = Limpopo 9 = North West Province 10 = Other		
		Town (specify)		Free text		
29.3.2	If no when did she move to Cape Town?		Year		Month	
29.3.3	Was this Child's father born in Cape Town?				0 = No	1 = Yes
	If No, where was he born?	Province				
		1 = Western Cape 2 = Eastern Cape 3 = Northern Cape 4 = Free state 5 = Gauteng		6 = Kwazulu Natal 7 = Mpumalanga 8 = Limpopo 9 = North West Province 10 = Other		
		Town(specify)				

Q30 Ethnicity

Q30	What is your child's ethnic origin? (circle as appropriate)	1 = White/Caucasian	2 = Coloured / Mixed race
		3 = Black African	4 = Asian/Indian
		5 = Other (Specify)	

Q30 Household information

Q31.1	How many people live together in your house?	
Q31.2	How many children (12 years or less) that are OLDER than this child live in the same household?	
Q31.3	How many children that are YOUNGER than this child live in the	

	same household?	
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Q32 Parental Education level

1 = None	11 = Grade 9 / Std 7
2 = Grade R / preschool	12 = Grade 10 / Std 8
3 = Grade 1 / SubA	13 = Grade 11 / Std 9
4 = Grade 2 / Sub B	14 = Grade 12 / Matric
5 = Grade 3 / Std 1	15 = Grade 9,10,11 (Std 7,8,9) & diploma
6 = Grade 4 / Std 2	16 = Grade 12 (Std 10) & Certificate or Diploma
7 = Grade 5 / Std 3	17 = Grade 12 (Std 10) & Degree
8 = Grade 6 / Std 4	18 = Grade 12 (Std 10) & Degree plus Diploma or further degree
9 = Grade 7 / Std 5	19 = Grade 12 (Std 10) & PhD
10 = Grade 8 / Std 6	Other (Specify) : Free text

Q33 Household income

Q33.1	What job does this child's mother/female guardian do?	
Q33.2	What job does this child's father/male guardian do?	
Q33.3	How much money or income does your household receive every month after tax? (incl. money from work, pensions, informal business etc.)	R 999 = Don't know

Q34 Contact with pets and animals

Q34 Contact with pets and animals				
Q34.1	Do you own a cat or have a cat in your home?		0 = No	1 = Yes
Q34.2	Do you own a dog or have a dog in your home?		0 = No	1 = Yes
Q34.3	Does your child have regular (at least once a week) contact with farm animals (e.g. cattle, pigs, goats, sheep or poultry)?	0 = No	1 = Yes	999 = Don't know
Q34.4	Has this child's mother had regular (at least once a week) contact with farm animals (e.g. cattle, pigs, goats, sheep or poultry) while being pregnant with this child?	0 = No	1 = Yes	999 = Don't know

Q35 Fuel exposure

	At your house, what fuel is used for cooking?
1 = Electricity/Gas	4 = Open fires outside the house

Q35.1	2 = Paraffin Stove	5 = Other (specify)
	3 = Open fires in the house	
Q35.2	At your house, what fuel is used for heating?	
	1 = Electricity	4 = Wood/coal
	2 =Gas	5 = Other (specify)
	3 =Kerosene/Paraffin	

Q36 Cigarette smoke exposure

Q36.1	Does this child's mother (or female guardian) currently smoke cigarettes?	0 = No	1 = Yes	3 = Don't know
36.1.1	If YES , about how many cigarettes does the child's mother (or female guardian) smoke each day?	number of cigarettes:		
Q36.2	Does this child's father (or male guardian) currently smoke cigarettes?	0 = No	1 = Yes	3 = Don't know
36.2.1	If YES , about how many cigarettes does the child's father (or male guardian) smoke each day?	number of cigarettes:		
Q36.3	How many people living in the house currently smoke cigarettes, including parents?	No of people:		
Q36.4	Did this child's mother smoke cigarettes while being pregnant with this child?	0 = No	1 = Yes	3 = Don't know