ELUCIDATION OF CLINICAL, ENVIRONMENTAL AND T HELPER 2 FACTORS ON TIGHT JUNCTION EXPRESSIONS IN ALLERGIC RHINITIS

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by

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TABLE OF CONTENTS

APPENDICES

LIST OF PUBLICATIONS

LIST OF AWARDS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AJCs	Apical junctional complexes
ALI	Air-liquid interface
AMPs	Antimicrobial peptides
APCs	Antigen presenting cells
AR	Allergic rhinitis
ARIA	AR and its Impact on Asthma
B. tropicalis	*Blomia tropicalis*
cDNA	Complementary DNA
CLDN	Claudin
D. farinae	*Dermatophagoides farinae*
D. pteronyssinus	*Dermatophagoides pteronyssinus*
DCs	Dendritic cells
DEPs	Diesel exhaust particles
dsDNA	Double-stranded DNA
DSG	Desmoglein
ECP	Eosinophils cationic protein
ENT	Ear, nose and throat
ETD	Eustachian tube dysfunction
FcεR	Fc receptor for IgE
GEP	Gene expression profiling
GM-CSF	Granulocyte-macrophage colony stimulating factor
GO	Gene ontology
HDAC	Histone deacetylase

HDACi	Histone deacetylase inhibitor
HDM	House dust mite
HMGB1	High-mobility group box 1
HNECs	Human nasal epithelial cells
Hospital USM	Hospital Universiti Sains Malaysia
IDST	Intradermal (intracutaneous) skin tests
IF	Immunofluorescence
IFN-γ	Interferon gamma
IgE	Immunoglobulin E
IL	Interleukin
IL-13Rα1	IL-13 receptors alpha 1
IL-13Rα2	IL-13 receptors alpha 2
IL-4Rα	IL-4 receptors alpha
ILC2	Type 2 innate lymphoid cells
IR	Idiophatic rhinitis
JAK1	Janus kinase 1
JAMs	Junctional adhesion molecules
JC	Japanese cheddar
LED	Light-emitting diodes
LPS	Lipopolysaccharide
LT	Leukotriene
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MDD	Major depressive disorder
MHC	Major histocompatibility complex
MUC5AC	Mucin 5AC

NAC	Nasal allergen challenge
NECs	Nasal epithelial cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NKT	Natural killer T
OCLN	Occludin
ORL-HNS	Otorhinolaryngology, Head and Neck Surgery
PARs	Protease-activated receptors
PCR	Polymerase chain reaction
PE	Phycoerythrin
PG	Prostaglandin
PKC	Protein kinase C
PM2.5	Particulate matter 2.5
QOL	Quality of life
RANTES	Regulated on activation normal T cell expressed and secreted
rh	Recombinant human
RHEC	Reconstructed human epidermis cells
ROS	Reactive oxygen species
RT	Reverse transcription
sIgE	Specific immunoglobulin E
siRNA	Silencing RNA
SLIT	Sublingual immunotheraphy
SNP	Small nucleotide polymorphism
SoB	Sodium butyrate
SP	Surface protein

SPT	Skin prick test
ssDNA	Single-stranded DNA
ssIgE	Serum allergen-specific IgE
STAT6	Signal transducer and activator of transcription 6
TARC	Thymus and activation regulated chemokine
TER	Transepithelial resistance
Th2	T helper 2
TJ	Tight junction
TLR	Toll-like receptor
Tm	Melting temperature
TNF	Tumor necrosis factor
TSA	Trichostatin A
TSLP	Thymic stromal lymphopoietin
TSLPR	TSLP receptor
TYK2	Tyrosine kinase 2
UA	Ursolic acid
VAS	Visual analogue scale
VCAM-1	Vascular cell adhesion molecule 1
ZO	Zonula occludens

LIST OF APPENDICES

PENENTUAN FAKTOR KLINIKAL, PERSEKITARAN DAN T PEMBANTU 2 TERHADAP EKSPRESI SIMPANG KETAT DALAM RINITIS ALAHAN

ABSTRAK

Kegagalan penghalang epitelia hidung melalui kerosakan molekul sel simpangan termasuk simpangan ketat (TJ) dan desmosom menjadi faktor penyebab dalam patogenesis rinitis alergi (AR). Walau bagaimanapun, perkaitan antara TJ epitelia hidung dan ekspresi desmosom dengan ciri demografi, klinikal dan persekitaran, serta dengan sitokin T pembantu 2 (Th2) dan reseptor sitokin Th2 tetap tidak jelas. Oleh itu, kajian ini bertujuan untuk menyiasatnya dalam kumpulan pesakit AR berbanding kawalan bukan alergi. Tiga puluh pesakit AR sederhana/teruk yang disebabkan oleh hama debu rumah (HDM) dan 30 kawalan bukan alergi direkrut dalam kajian ini, dan sensitiviti HDM dinilai melalui ujian tusukan kulit (SPT). Tahap ekspresi mRNA molekul TJ (*OCLN*, *CLDN3* dan *CLDN7*), desmosom (*DSG1* dan *DSG3*), TSLP (sitokin yang berasal dari epitelium) dan reseptor sitokin Th2 (*IL4R*, *IL5RA*, *IL6R* dan *IL13RA1*) dalam sel epitelia hidung pesakit AR berbanding kawalan bukan alergi disiasat melalui tindak balas rantai polimerase transkripsi terbalik kuantitatif (RT-qPCR). Tahap serum sitokin Th2 (IL-4, IL-5, IL-6 dan IL-13) pesakit AR berbanding kawalan bukan alergi disiasat menggunakan Magnetic Luminex® Assay dan hubungan antara heterodimer reseptor IL-4/IL-13 dengan ekspresi gen TJ ditentukan melalui analisis bioinformatik. Untuk pertama kalinya diperhatikan bahawa ukuran pemekaan SPT *D. farinae* mempunyai hubung kait yang ketara dengan skor keterukan gejala hidung dan gejala bukan hidung yang lebih tinggi, dan perkaitan ini tidak diperhatikan pada saiz pemekaan SPT *D. pteronyssinus* dan *B. tropicalis*. Dari segi TJ, tahap ekspresi transkrip *OCLN*, *CLDN3* atau *CLDN7* (tetapi tidak *DSG1*, *DSG3* atau *TSLP*) jauh lebih rendah

pada pesakit AR berbanding dengan kawalan bukan alergi. Perkaitan yang ketara antara lokasi bandar dengan ekspresi *OCLN* yang lebih rendah, atau pendedahan kepada asap rokok dengan ekspresi *CLDN7* yang lebih rendah dijumpai pada pesakit AR. Seterusnya, peranan paksi IL-4/IL-13 dalam AR diperhatikan di mana tahap IL-4, IL-5, IL-6 dan IL-13 serum yang lebih tinggi, dan peningkatan ekspresi *IL13RA1* didapati pada pesakit AR berbanding kawalan bukan alergi. Tahap serum IL-4 dan IL-13 berkorelasi positif dengan ekspresi *IL13RA1* pada pesakit AR tetapi tidak dalam kawalan bukan alergi. Pada asasnya, analisis korelasi set data profil ekspresi gen (GSE44037; 12 pesakit AR berbanding enam kawalan bukan alergi) menunjukkan bahawa enam TJ (*CLDN4*, *CLDN7*, *CLDN12*, *CLDN15*, *TJP1* dan *TJP2*) dan dua isyarat JAK/STAT (*STAT2* dan *STAT3*) ekspresi gen masing-masing berkorelasi positif dan negatif, dengan ekspresi reseptor heterodimerik IL-4Rα/IL-13Rα1 pada pesakit AR. Ini tidak diperhatikan pada sampel kawalan bukan alergi. Terakhir, analisis motif pengikat DNA STAT menunjukkan bahawa masing-masing dari enam gen TJ ini mengandungi urutan konsensus pengikat STAT dalam linkungan pengawal atur DNA mereka. Secara kolektif, ini menunjukkan bahawa isyarat paksi IL-4/IL-13 melalui jalan JAK/STAT menahan ekspresi TJ pada pesakit AR. Kesimpulannya, mensasarkan epitelia hidung dengan memulihkan ekspresi TJs serta mensasarkan paksi IL-4/IL-13 dan jalan JAK/STAT hilirnya mungkin mewakili pendekatan baru dalam mengembangkan terapi yang disasarkan untuk pesakit AR.

ELUCIDATION OF CLINICAL, ENVIRONMENTAL AND T HELPER 2 FACTORS ON TIGHT JUNCTION EXPRESSIONS IN ALLERGIC RHINITIS

ABSTRACT

The breakdown of nasal epithelial barrier via the impairment of cell junction components including tight junctions (TJs) and desmosomes plays causative roles in the pathogenesis of allergic rhinitis (AR). However, the associations between nasal epithelial TJs and desmosomes expression with demographical, clinical and environmental characteristics, as well as with T helper 2 (Th2) cytokines and Th2 cytokine receptors remain unclear. Therefore, this study aimed to investigate these in a cohort of AR patients vs non-allergic controls. Thirty house dust mite (HDM)-induced moderate/severe AR patients and 30 non-allergic controls were recruited in this study, and HDM sensitisations were assessed through skin prick test (SPT). mRNA expression levels of TJ genes (*OCLN*, *CLDN3* and *CLDN7*), desmosomes (*DSG1* and *DSG3*), *TSLP* (an epithelium-derived cytokine) and Th2 cytokine receptors (*IL4R*, *IL5RA*, *IL6R* and *IL13RA1*) in nasal epithelial cells of AR patients vs non-allergic controls were investigated via quantitative reverse transcription polymerase chain reaction (RT-qPCR). Serum levels of Th2 cytokines (IL-4, IL-5, IL-6 and IL-13) of AR patients vs non-allergic controls were investigated using Magnetic Luminex® Assay and the correlations between IL-4/IL-13 receptor heterodimer with TJ genes expressions were determined via bioinformatics analysis. It was observed, for the first time, that SPT wheal sizes of *D. farinae* sensitisation were significantly associated with higher severity scores of nasal and non-nasal symptoms, and these associations were not observed in SPT wheal sizes of *D. pteronyssinus* and *B. tropicalis* sensitisation. In terms of TJs, the expression levels of *OCLN*, *CLDN3* or *CLDN7* (but not *DSG1*, *DSG3* or *TSLP*)

transcripts were significantly lower in AR patients compared with non-allergic controls. A significant association between urban locations and lower *OCLN* expression, or exposure to second-hand smoke with lower *CLDN7* expression was found in AR patients. Next, the roles of IL-4/IL-13 axis in AR were observed where significantly higher levels of serum IL-4, IL-5, IL-6 and IL-13, and increased *IL13RA1* expression were found in AR patients compared to non-allergic controls. Serum IL-4 and IL-13 levels were positively correlated with *IL13RA1* expression in AR patients but not in non-allergic controls. Essentially, correlation analyses of a gene expression profiling dataset (GSE44037; 12 AR patients vs six non-allergic controls) showed that six TJ (*CLDN4*, *CLDN7*, *CLDN12*, *CLDN15*, *TJP1* and *TJP2*) and two JAK/STAT signaling (*STAT2* and *STAT3*) genes expressions were positively and negatively correlated, respectively, with IL-4Rα/IL-13Rα1 heterodimeric receptor expression in AR patients. These were not observed in non-allergic control samples. Lastly, STATs DNA binding motif analysis showed that each of these six TJ genes contains STATs binding consensus sequence within their DNA regulatory regions. Collectively, these suggest that the IL-4/IL-13 axis signalling via the JAK/STAT pathway represses the expression of TJs in AR patients. In conclusion, targeting nasal epithelial through restoring the expression of TJs as well as targeting IL-4/IL-13 axis and its downstream JAK/STAT pathway may represent a novel approach in developing targeted therapies for AR patients.

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Allergic rhinitis (AR) is a common disease affecting approximately 400 million people worldwide (Greiner et al., 2011; R. Pawankar, 2014). The disease significantly impairs the quality of life, school or work performance, and with high cost of treatment, leading to major social-economic consequences. In addition, AR is usually accompanied with comorbidities such as asthma, conjunctivitis and sinusitis, complicating treatment and management of AR patients.

Epithelial barrier serves as the first line defense of the immune system where an intact mucosal barrier is crucial in protecting the host immune system from the exposure of harmful pathogens. Recently, it has been observed that impairment of nasal epithelial barrier is one of the underlying causes of AR pathogenesis (Steelant et al., 2018; Vareille et al., 2011). Breakdown of nasal epithelial barrier integrity is attributable to reduced expression of tight junction (TJ) observed in AR patients compared with non-allergic controls (Steelant et al., 2018). T helper 2 (Th2) cytokines such as interleukin (IL)-4 are involved in suppressing the expression of TJs components in nasal epithelial cells of AR patients (Steelant et al., 2018).

Desmogleins, a group of calcium-dependent anchoring junctions distinct from TJ, consist of desmosomal cadherin desmoglein (DSG)1, DSG2, DSG3 and DSG4. Desmogleins are involved in maintaining epithelial homeostasis where they display

spatially distinct expression patterns at various levels among different stratified epithelia (Kowalczyk & Green, 2013; Najor, 2018; Rubsam et al., 2017; Saito et al., 2012). The DSG family members have not been investigated in AR.

Activation of Th2 cytokines not only enhances inflammatory cell activation but also regulates epithelial cell barrier in allergic disease. Th2 cytokines maintain a continuous inflammation in the nasal mucosa and infiltrate within the sinonasal microenvironment that alter the composition of epithelial TJ (Capaldo & Nusrat, 2009; London et al., 2016). It has been shown that Th2 cytokines signal through their respective receptors on nasal epithelial cells leading to breakdown of nasal epithelial barrier in AR (Steelant et al., 2018).

IL-4 binds to type I (consisting of IL4R and common γ-chain) and type II (consisting of IL-4Rα and IL-13Rα1) IL-4 receptors. The IL-4Rα/13Rα1 heterodimer receptor is bound by either IL-4 or IL-13 (Tan et al., 2016) , and such shared binding of this heterodimeric receptor has been termed as the IL-4/IL-13 axis which has also been implicated in AR pathogenesis.

1.2 Problem Statement

House dust mites (HDM) have been identified as the major triggering allergen in most of Asia. The aforementioned studies have focused on moderate/severe HDM-induced AR patients where the main allergens patients sensitised to were HDMs including *Dermatophagoides pteronyssinus* (*D. pteronyssinus*), *Dermatophagoides farinae* (*D. farinae*) and *Blomia tropicalis* (*B. tropicalis*) determined by skin prick test (SPT) or allergen-specific immunoglobulin E (sIgE). Approximately 80% of AR

patients in Malaysia are sensitised to these three HDM species (Azid et al., 2019; Ho et al., 1995; Lim et al., 2015; Majdiah, 2011; Sani et al., 2019). AR is a major risk factor for poor asthma control and markedly impairs quality of life, sleep, social life, school, and work performance, leading to a huge socioeconomic burden, with medical costs greater than those of diabetes, coronary heart disease and asthma.

Understanding the underlying pathomechanisms is central to developing better and more targeted therapies. However, the mechanism responsible for the development of AR is not fully understood yet. Currently there are two, partially different basic theories on the pathogenesis of allergy. For decades the primary assumption has been that allergy is caused by unbalanced and overactive immunological responses against allergens, mostly driven by activated Th2 cells and due to aberrant T- regulatory cells. The second more recent hypothesis which is gaining more attention now relies on the dysregulation of the epithelial barrier, which might result in the allergen uptake as a primary defect in the pathogenesis of allergic reactions.

To date, there is a lack of literature on the expression profile of TJ genes in HDM-induced AR patients. The expression levels of Th2 cytokine receptors in HDM-induced AR have not been reported, and the potential involvement of Th2 cytokines signalling to regulate TJs expression in epithelial cells remains unclear. In addition, no study has been conducted on SPT wheal size's association with AR characteristics. Hence, this study focuses to address these in moderate/severe HDM-induced AR patients attending Otorhinolaryngology, Head and Neck Surgery (ORL-HNS) clinic in Hospital Universiti Sains Malaysia (Hospital USM) in Kelantan.

1.3 Theoretical Framework

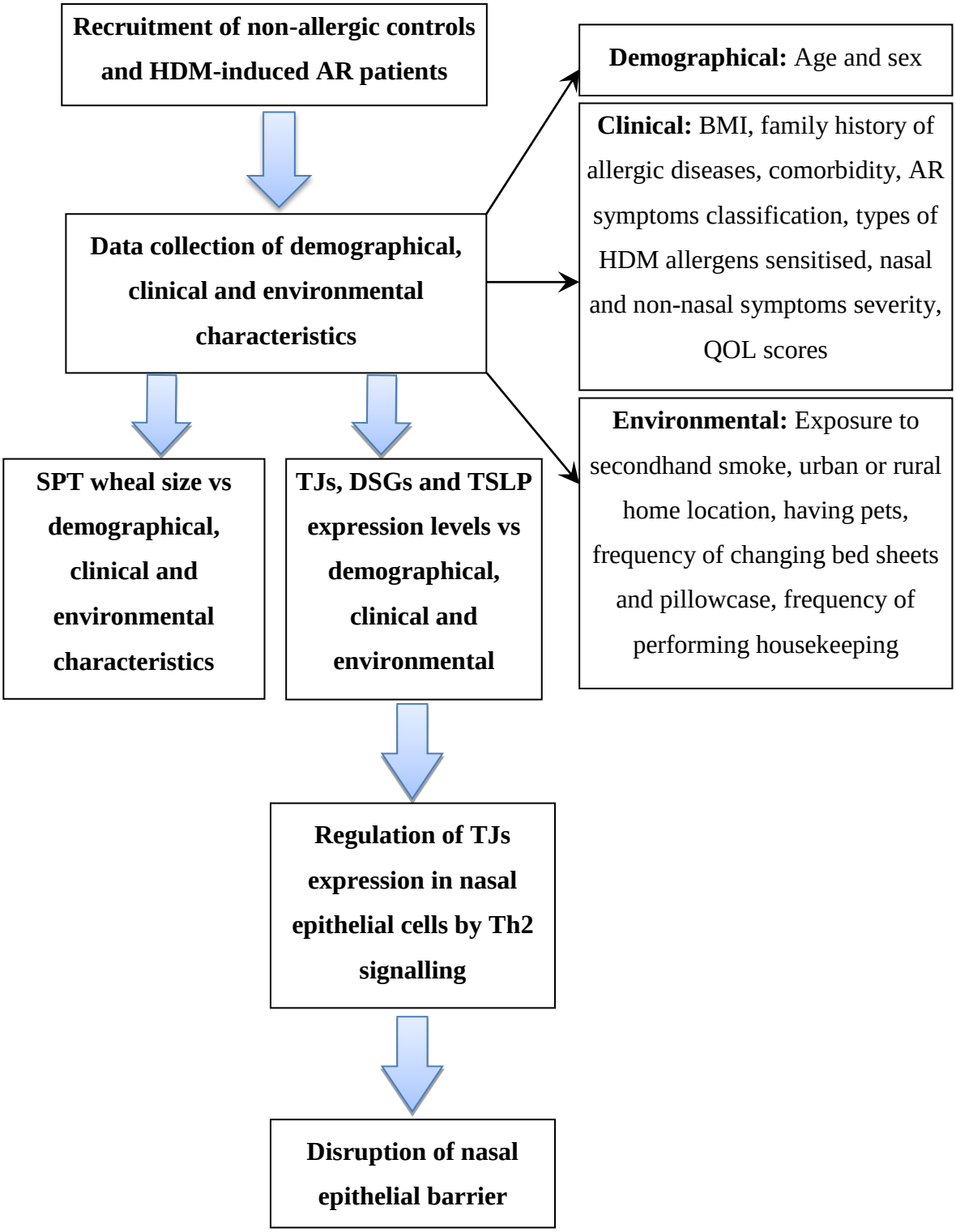

Figure 1.1: Theoretical framework of the PhD project. BMI: Body mass index; QOL: Quality of life.

1.4 Objectives of the Study

1.4.1 General objective

To evaluate clinical and immunological characteristics on TJ gene expression in allergic rhinitis.

1.4.2 Specific objectives

1. To determine and compare the demographical, clinical and environmental characteristics of AR patients and non-allergic controls.
2. To determine the association of SPT wheal sizes with demographical, clinical and environmental characteristics of AR patients and non-allergic controls, to determine the association of nasal and non-nasal symptoms severity scores with the number of HDM allergens sensitisation of AR patients and to correlate the nasal and non-nasal symptoms severity scores with SPT wheal sizes of HDM allergens of AR patients.
3. To determine and compare the mRNA expression levels of TJ genes (*OCLN*, *CLDN3* and *CLDN7*), desmosome genes (*DSG1* and *DSG3*) and epithelium-derived cytokine (*TSLP*) in nasal epithelial cells of AR patients vs non-allergic controls and to determine their association with demographical, clinical and environmental characteristics of AR patients and non-allergic controls.
4. To determine, compare and correlate the levels of serum Th2 cytokines (IL-4, IL-5, IL-6 and IL-13) and mRNA expression levels of Th2 cytokine receptors (*IL4R*, *IL5RA*, *IL6R* and *IL13RA1*) in nasal epithelial cells of AR patients vs non-allergic controls and to correlate IL-4/IL-13 receptor heterodimer with TJ genes expressions via bioinformatics analysis.

1.5 Hypothesis

1. There are differences in the demographical, clinical and environmental characteristics of AR patients compared with non-allergic controls.
2. There are association between SPT wheal sizes and demographical, clinical and environmental characteristics of AR patients and non-allergic controls, and between nasal and non-nasal symptoms severity scores with the number of HDM allergens sensitisation of AR patients and the nasal and non-nasal symptoms severity scores correlated positively with SPT wheal sizes of HDM allergens of AR patients.
3. The mRNA expression levels of TJ genes (*OCLN*, *CLDN3* and *CLDN7*), desmosome genes (*DSG1* and *DSG3*) and epithelium-derived cytokine (*TSLP*) in nasal epithelial cells of AR patients are reduced compared with non-allergic controls and there are associations of certain demographical, clinical and environmental characteristics of AR patients and non-allergic controls with mRNA expression levels of TJ genes (*OCLN*, *CLDN3* and *CLDN7*), desmosome genes (*DSG1* and *DSG3*) and epithelium-derived cytokine (*TSLP*) in AR patients and non-allergic controls.
4. The levels of serum Th2 cytokines (IL-4, IL-5, IL-6 and IL-13) and mRNA expression levels of Th2 cytokine receptors (*IL4R*, *IL5RA*, *IL6R* and *IL13RA1*) in nasal epithelial cells of AR patients are increased compared with non-allergic controls, increased levels of serum Th2 cytokines (IL-4, IL-5, IL-6 and IL-13) correlate positively with the mRNA expression of Th2 cytokine receptors (*IL4R*, *IL5RA*, *IL6R* and *IL13RA1*) in the nasal epithelial cells of AR patients compared with non-allergic controls and expression of

IL-4/IL-13 receptor heterodimer is inversely correlated with TJ genes expressions.

CHAPTER 2

LITERATURE REVIEW

2.1 Allergy

2.1.1 Type I hypersensitivity

Type I hypersensitivity is an allergic reaction mediated by immunoglobulin E (IgE) antibody in response to normally non-pathogenic antigens commonly called allergens (Wheatley & Togias, 2015). Type I hypersensitivity reactions occur rapidly within approximately 20 minutes after the exposure of allergen termed as immediate hypersensitivity reactions. It is characterised by activation of mast and inflammatory cells, and tissue infiltration (Gangwar et al., 2016).

Cross-linking of the Fc receptor for IgE (FcεR) on mast cells, triggered by the interaction of multivalent allergen-specific IgE bound to their high affinity FcεRI, causes release of allergic mediators. These mediators consist of histamine, proteases and lipid mediators such as leukotriene (LT) C4, and prostaglandin (PG) D2 that cause vascular leak, bronchoconstriction, inflammation, and intestinal hypermotility (Finkelman et al., 2016; He et al., 2015; Justiz Vaillant & Zito, 2018; Moon et al., 2014). These mediators are responsible for the development of signs and symptoms in allergic diseases.

The hypersensitivity reactions can be observed in AR, bronchial asthma, allergic conjunctivitis, allergic dermatitis, food allergy and anaphylactic shock (Justiz Vaillant

& Zito, 2018). Over 30% of the population suffer from symptoms of allergy which can lead to severe disability and life-threatening conditions such as anaphylaxis (Valenta et al., 2018). In severe cases, intense bronchospasm, laryngeal edema, cyanosis, hypotension and shock can occur (Justiz Vaillant & Zito, 2018).

2.2 Allergic Rhinitis (AR)

2.2.1 Definition and pathophysiology of AR

AR is clinically defined as a symptomatic disorder of the nose induced by an IgE-mediated inflammation after allergen exposure of the membranes lining the nose, and it is usually accompanied by classical symptoms such as nasal itching, sneezing, rhinorrhoea, and nasal congestion (J. Bousquet et al., 2008). Other symptoms include conjunctivitis, itching of the palate, postnasal drip, and cough (Brozek et al., 2017). The symptoms of AR usually cause impairment in quality of life by causing sleep disturbances, alter school and work performance as well as daily activities.

The allergic response in AR can be divided into two phases *i.e.* the early and late phase. The early phase starts within 20 minutes after exposure to harmful allergens. APCs (*e.g.* dendritic cells [DCs] in the mucosal surface) uptake, process and present peptides from allergens on the major histocompatibility complex (MHC) class II molecule. The antigen complex and the MHC class II molecule serve as a ligand for T cell receptors on naïve $CD4^+$ T cells, resulted in differentiation of naïve $CD4^+$ T cells into allergen-specific Th2 cell. Cytokines (*e.g.* IL-4 and IL-13) released from the activated Th2 cells interact with B cells to produce allergen-specific IgE. This allergen-

specific IgE binds to high-affinity FcεR receptors on mast cells leading to mast cell activation (Tan et al., 2016).

The degranulation of mast cells releases preformed inflammatory mediators such as histamine, tryptase, chymase, kininogenase (generates bradykinin), heparin, and other enzymes. In addition, mast cells secrete several inflammatory mediators *de novo* (*i.e.* not preformed and stored in mast cell granules) including PGD2 and the sulfidopeptidyl LTC4, LTD4, and LTE4 (Skoner, 2001). These mediators induce mucosal oedema and watery rhinorrhoea characteristic of AR by causing the blood vessels to leak. Histamine is the major mediators in AR where it activates H1 receptors on sensory nerve endings and causes sneezing, pruritus, and reflex secretory responses, and it also interacts with H1 and H2 receptors on mucosal blood vessels, leading to vascular engorgement (nasal congestion) and plasma leakage (Sin & Togias, 2011).

After four to six hours of allergens exposure, the late phase of allergic response is initiated. In this phase, nasal mucosal inflammation occurs with the influx and activation of a variety of inflammatory cells (*i.e.* T cells, eosinophils, basophils, neutrophils, and monocytes) into nasal mucosa that mainly depend on chemokines and cytokines such as IL-4 and IL-5 (Sin & Togias, 2011). These cytokines upregulate the expression of adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) on the endothelial cells which facilitate inflammatory cellular influx (R. Pawankar et al., 2000).

The activation of structural cells in the nasal mucosa, such as epithelial cells and fibroblasts induced by other effector cell products, can promote the release of additional

chemokines (*e.g.* eotaxin, RANTES [regulated on activation normal T cell expressed and secreted], and TARC [thymus and activation regulated chemokine]) that facilitate cell influx from the peripheral blood (Plewako et al., 2008). The schematic representation of pathophysiology of AR is illustrated in **Figure 2.1**.

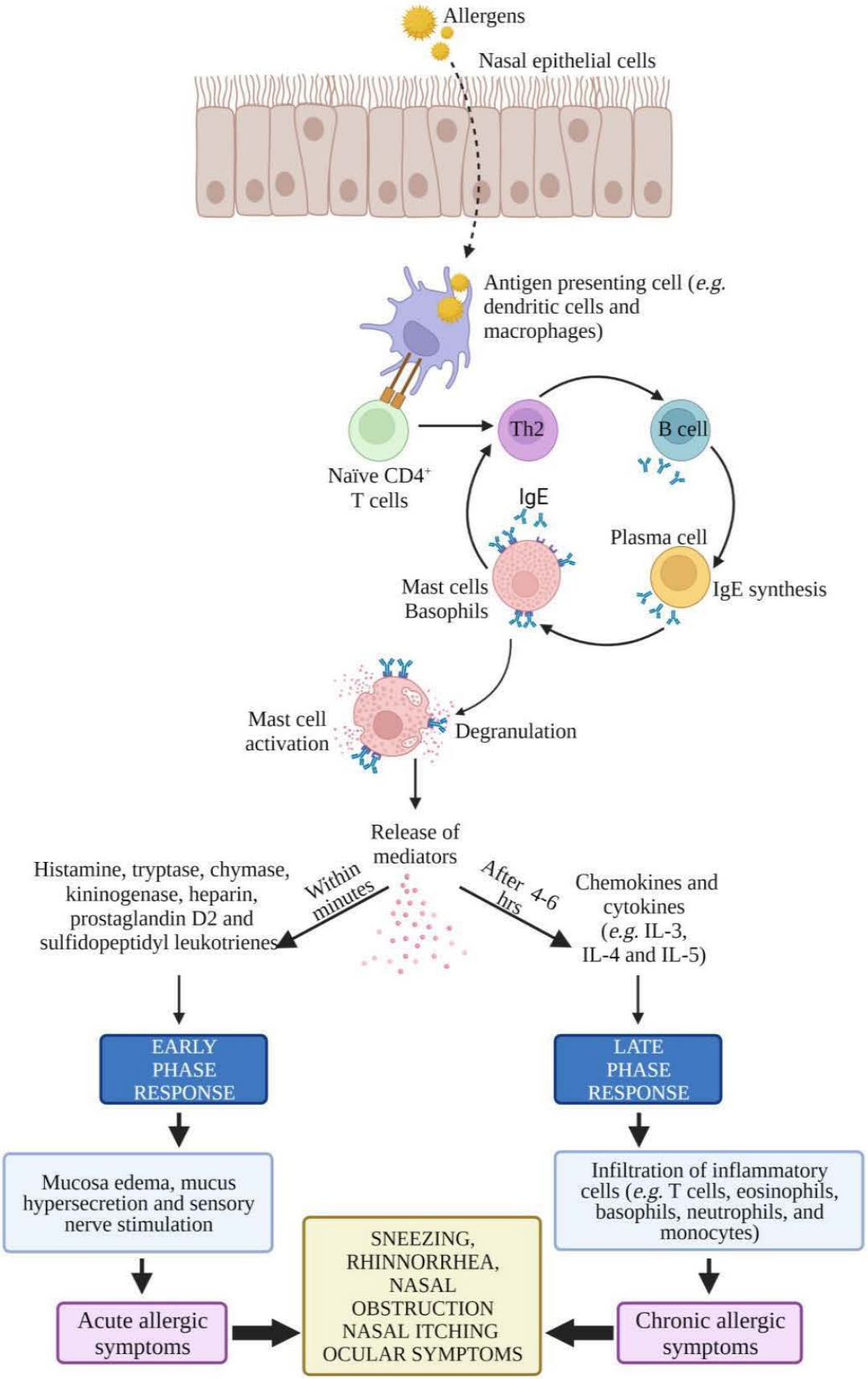

Figure 2.1: Schematic presentation of pathophysiology of AR. See texts for details (Plewako et al., 2008; Sin & Togias, 2011; Skoner, 2001; Tan et al., 2016). Created with BioRender.com.

2.2.2 Allergens

Allergens are proteins with molecular weight ranging from 10 to 40 kDa that induce type I hypersensitivity by reacting with specific IgE antibodies (Baldacci et al., 2015; Justiz Vaillant & Zito, 2018). The common types of allergens include food allergens (*e.g.* shrimp, soybean, crab, clam, wheat, peanut, yolk egg, cow's milk), pet allergens (*e.g.* cat and dog dander), and HDMs (Aalberse, 2000; Hosseini et al., 2014).

HDM-induced AR is the most common allergens causing allergic sensitisation among AR patients including in Malaysia (Ho et al., 1995; J. H. Lee et al., 2018; Liam et al., 2002). Mite sensitisation significantly increased the risk of AR with objective response (OR) of 1.94 (95% CI: 1.46–2.58) (I. J. Wang et al., 2016). The major species of HDM causing allergy in Malaysia include *D. pteronyssinus* and *D. farinae* (Lim et al., 2015; Majdiah, 2011).

HDM allergen is highly associated with the disruption of epithelial barrier where they have proteolytic activity that can cleave the epithelial TJ proteins. *D. pteronyssinus*, Der p 1 (*i.e.* a HDM cysteine proteinase allergen) has been reported to cleave extracellular domain sites of occludin (OCLN) and claudin 1 (CLDN1), resulted in amplified epithelial permeability that allowed the passage of Der p 1 through the epithelial barrier (London & Ramanathan, 2017; Platts-Mills & Woodfolk, 2011; Steelant, Farre, et al., 2016). Inhibition of the protease activity of Der p 1 as a therapeutic approach to reduce HDM-induced barrier dysfunction has been proposed (John et al., 2000). Treatment of cultured primary human nasal epithelial cells (HNECs) *in vitro* with Der p 1 showed markedly decrease in CLDN1 expression, resulted in

significantly increased FITC-labeled 4 kDa dextran (FD4; a fluorescent probe to assess cell permeability) epithelial permeability (M. Wang, Jun et al., 2021).

In this project we focused on investigating the breakdown of nasal epithelial barrier in HDM-induced AR. The HDM allergens tested during SPT were all from group 1 HDM allergens which belong to the cysteine protease family. HDM allergens through their protease activities can also induce Th2-biased immune responses. Der p 1 can activate the production of IL-6 by epithelial cells where this cytokine has pleiotropic effects that can promote IL-4 signalling, leading to Th2 cells differentiation, and IL-6 simultaneously inhibits IFN-γ signalling and therefore Th1 differentiation (Diehl & Rincón, 2002). The protease activity of Der p 1 can affect the polarization of $CD4^+$ T cells by targeting proteins present on either T cells or antigen presenting cells (APCs). The protease activity also stimulates the synthesis of epithelium derived-cytokines [*i.e* IL-33, thymic stromal lymphopoietin (TSLP) and IL-25] that further activate type 2 innate lymphoid cells (ILC2) to produce earliest source of Th2 cytokines prior to the development of adaptive Th2 cells (Halim et al., 2014).

Protease activity of HDM also reduces Th1 polarization. In vitro Der p 1 cleaved CD40 causing a reduction in IL-12 that results in a lower production of interferon gamma (IFN-γ) and an increased production of IL-4 (Ghaemmaghami et al., 2002). Suppressed Th1 polarisation also occurs through ligation of CD40 on APC that affects the production of extracellular thiols (factors promoting Th1 polarization) and decreases the presence of Der p 1, and thus supports a Th2-biased immune response (Hasan et al., 2009).

The protease activity of HDM also causes excessive IgE production. The level of IgE-production by B cells is controlled by a negative feedback mechanism that involves IgE-binding to CD23 (*i.e.* the low-affinity receptor for IgE FceRII). Upon binding of IgE/allergen-complexes to CD23, IgE production was then downregulated by B cells (Reithofer & Jahn-Schmid, 2017). Der p 1 disrupts this IgE-feedback mechanism by selective cleavage of CD23, causing overproduction of IgE by B cells (Hewitt et al., 1995). Lastly, in allergic diseases, pulmonary surfactants [*i.e.* surface protein (SP)-A and SP-D) are vital in the clearance of allergens (Reithofer & Jahn-Schmid, 2017). They bind the allergens and lessen allergic sensitization by allergen removal or interference with IgE-binding (Madan et al., 2001; J. Y. Wang et al., 1996). Der p 1 was found to cleave SP-A and SP-D and bring down lung clearance (Deb et al., 2007). A summary of effects resulting from proteolytic activity of HDM is presented in **Figure 2.2**.

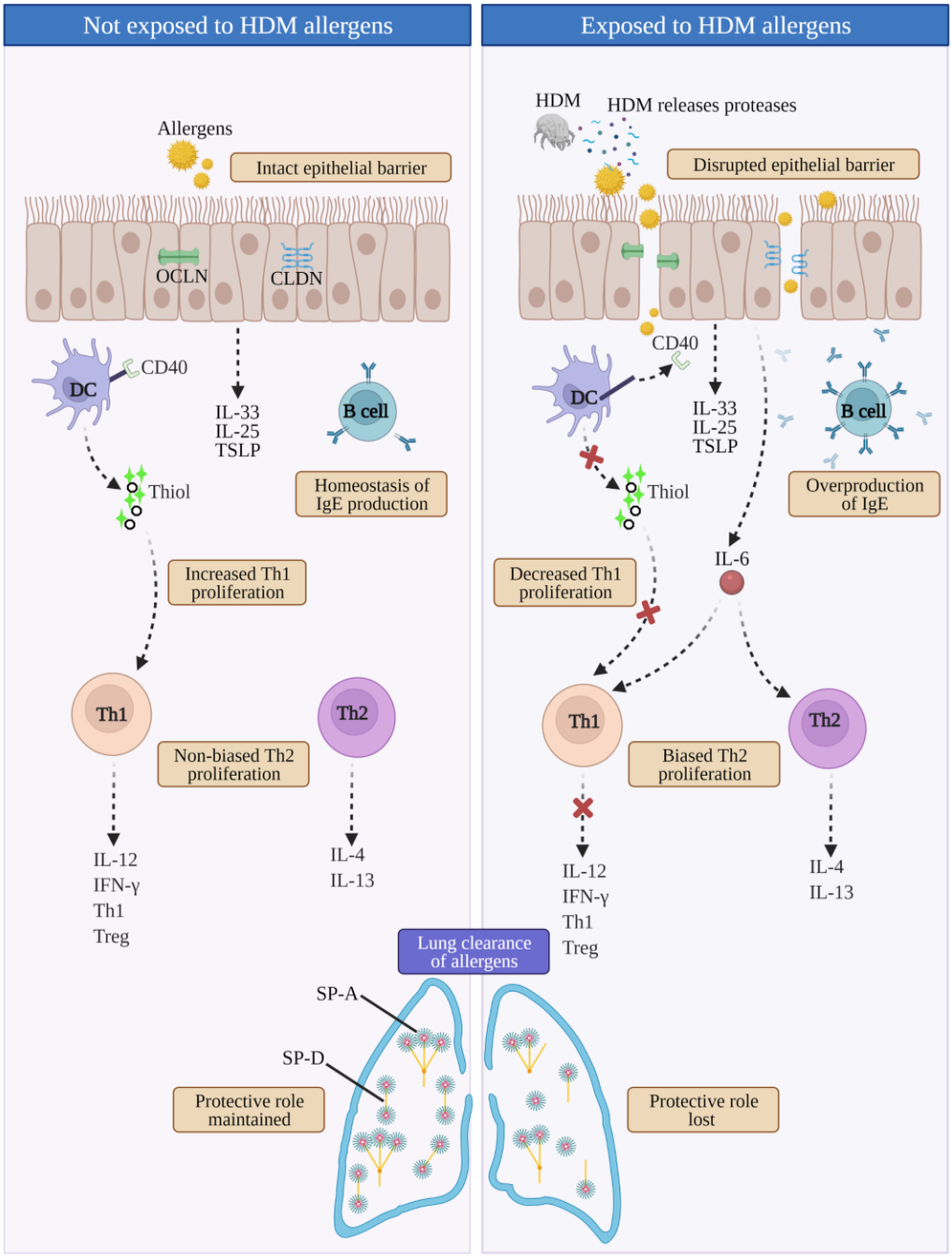

Figure 2.2: In normal physiological state (left panel), intact epithelial barrier prevents allergens infiltration and hence homeostasis of immune components and functions are maintained. In AR such as HDM-sensitized AR (right panel), proteases released by HDMs disrupt tight junctions leading to disrupted epithelial barrier that allows infiltration of allergens. This triggers a cascade of IgE overproduction by B cells, cleaved CD40 on the surface of DCs disrupts the production of thiols by DCs causing decreased Th1 proliferation and collectively with increased IL-6 secretion leads to biased Th2 proliferation. Th2 cells produce the hallmark AR cytokines IL-4 and IL-13. HDM proteases also cleave the pulmonary surfactants SP-A and SP-D, causing decreased lung clearance of allergens. CLDN: Claudin; DC: Dendritic cell; HDM: House dust mite; IL-4: Interleukin 4; IL-12: Interleukin 12; IL-13: Interleukin 13; IL-25: Interleukin 25; IL-33; Interleukin 33; IFNγ: Interferon gamma; OCLN: Occludin; SP-A: Surface protein A; SP-D: Surface protein D; Th1: T helper type 1; Th2: T helper type 2; Treg: Regulatory T cell; TSLP: Thymic stromal lymphopoietin. Created with BioRender.com.

Apart from HDMs, previous reports have also demonstrated that pollen allergens also impair TJ barrier function. Pollens conferred proteolytic activities by degrading OCLN in monolayers of Calu-3 cells (lung cancer cells of epithelial origin), resulted in increased paracellular permeability of the cells (Runswick, 2007). Reduced expression of CLDN1 enhanced Calu-3 cell transepithelial permeability. This has also been reported for pollen allergens widespread in the Mediterranean area (*i.e.* Olive tree, Orchard grass, Italian cypress and Scots pine) where their exposure increased Calu-3 transepithelial permeability by disrupting TJ proteins (Vinhas, 2011).

2.2.3 T helper 2 (Th2) cells in allergy

Th2 cells are derived from differentiated T lymphocytes expressing CD4. Th2 cells activate type 2 responses by stimulating B cells to proliferate and differentiate into plasma cells through the production of Th2 cytokines including IL-4, IL-5, IL-6 and IL-13 (Brzustewicz & Bryl, 2015).

Th2 cells are major contributors of IgE-producing B cells through induction by IL-4 (Guo et al., 2015; Kubo, 2017), and Th2 cells play a predominant role in AR pathogenesis. Together with eosinophils and basophils, Th2 cells infiltrate the nasal mucosa tissue, resulting in late phase allergic response (Eifan & Durham, 2016). IL-4 is a key cytokine in promoting Th2 differentiation from naïve $CD4^+$ T cells (Ansel et al., 2006). The mechanism is dependent on the activation of signal transducer and activator of transcription 6 (STAT6) signalling through the IL-4 receptor.

Th2 cytokines not only enhance inflammatory cell activation but also regulate epithelial cell barrier in allergic disease (*e.g.* AR, eosinophilic esophagitis, asthma and

chronic rhinosinusitis) (Gruber et al., 2015; Heijink et al., 2014; Holgate, 2007; Steelant, Seys, et al., 2016; Steelant et al., 2018; Travers et al., 2016). The cytokines may also be released within the sinonasal microenvironment including sinonasal epithelial cells, causing increased epithelial cell permeability (Capaldo & Nusrat, 2009; London et al., 2016). This is thought to be due to regulation of transmembrane transcription involved in TJ remodelling where the "tight" barrier properties of TJ proteins are switched to "leaky" properties (Capaldo & Nusrat, 2009). Th2 cytokines also hinder the epithelial barrier from resealing which may maintain the inflammation and exposure to inflammatory antigens (London et al., 2016).

As this study focuses on the association between cytokines level with the expression of TJ and DSG genes in AR patients, the descriptions of AR, each Th2 cytokines studied as well as TJs and DSGs are presented later in this Chapter.

2.2.4 Epidemiology

AR represents a global health problem affecting about 10-40% of the population worldwide and it usually persists throughout life (J. Bousquet et al., 2008; Brozek et al., 2017). It is reported to affect approximately 25% and 40% of children and adult globally, respectively. Approximately 80% of AR symptoms develop before the age of 20 years (Skoner, 2001) and peak at age 20-40 years before gradually declining (Wheatley & Togias, 2015). The incidence rate of AR in children over the first five years of life was reported to be 17.2%, with a peak age at diagnosis between 24 and 29 months (2.5%) (Hill et al., 2016). Meta-analysis studies have shown the sex-specific differences in the prevalence of AR with male predominance in childhood and a female predominance in adolescents (Frohlich et al., 2017; Pinart et al., 2017). Children have a

greater likelihood to be diagnosed with AR if both parents have a history of atopy than if only one parent is atopic (Skoner, 2001). Children with higher serum IgE levels (>100 IU/mL before age six) have higher risk of developing AR (deShazo, 2017).

Obesity is often linked with the prevalence of allergic diseases. However, association of body mass index (BMI) with AR has yielded conflicting results. A cross-sectional study on obesity indicators and rhinitis (n=8,165) reported that no association in adults AR, while in children central obesity was associated with reduced odd of AR diagnosis, regardless of sex (Han et al., 2016). In another study, higher BMI was also negatively associated with the prevalence of AR (Sybilski et al., 2015).

Prevalence of AR has increased with years due to several risk factors including global urbanisation as shown by several studies comparing AR prevalence in urban settings with rural areas (Elholm et al., 2016; C. W. Li et al., 2014). This is mainly caused by increased levels of pollutants (*e.g.* traffic-related pollutants and particulate matter 2.5 [PM2.5]) (Leung et al., 2012; I. J. Wang et al., 2016). It has been reported that AR is more prevalent in urban compared with rural areas (C. W. Li et al., 2014).

Smoking, however, did not show a significant association with the severity of nasal symptoms and usually impacted those with chronic rhinitis (P. J. Bousquet et al., 2009; Hisinger-Molkanen et al., 2018). Conversely, maternal smoking conferred the greatest risk in paediatric AR (Singh et al., 2018).

The economic impact of AR is underestimated because the disease often does not induce elevated direct costs. However, indirect cost is substantial with total annual

cost of self-reported AR in Sweden estimated at €1.3 billion (Cardell et al., 2016) and up to $20.9 billion in United States (R. Pawankar, 2014). In addition, AR is a systemic inflammatory disease and often co-morbids with other disease such as asthma, atopic disease, sinusitis, conjuctivitis and otitis media (J. Bousquet et al., 2008), complicating the treatment and management of these patients.

2.2.5 Clinical symptoms

AR is characterised by the presence of nasal symptoms and non-nasal symptoms. Nasal symptoms include anterior or posterior rhinorrhoea, sneezing, nasal blockage and/or itching of the nose (J. Bousquet et al., 2008). Nasal obstruction usually occurs in pre-school children but if the nasal obstruction is the only symptom, it is very rarely associated with allergy. These symptoms may persist for hours after allergic reaction upon the exposure of allergens that cause mucosal inflammation (Wheatley & Togias, 2015). In consequences, the mucosa is rendered more reactive to the triggering allergen (priming) as well as to other allergens and to non-allergenic stimuli (*e.g.* strong odours and other irritants).

Non-nasal symptoms are characterised by ocular symptoms such as allergic rhinoconjunctivitis (*i.e.* itching and redness of the eyes and tearing) which also frequently occurs in AR patients (Brozek et al., 2017). Other symptoms include itching of the palate, postnasal drip and cough. Management of AR symptoms is presented in **Figure 2.3**.

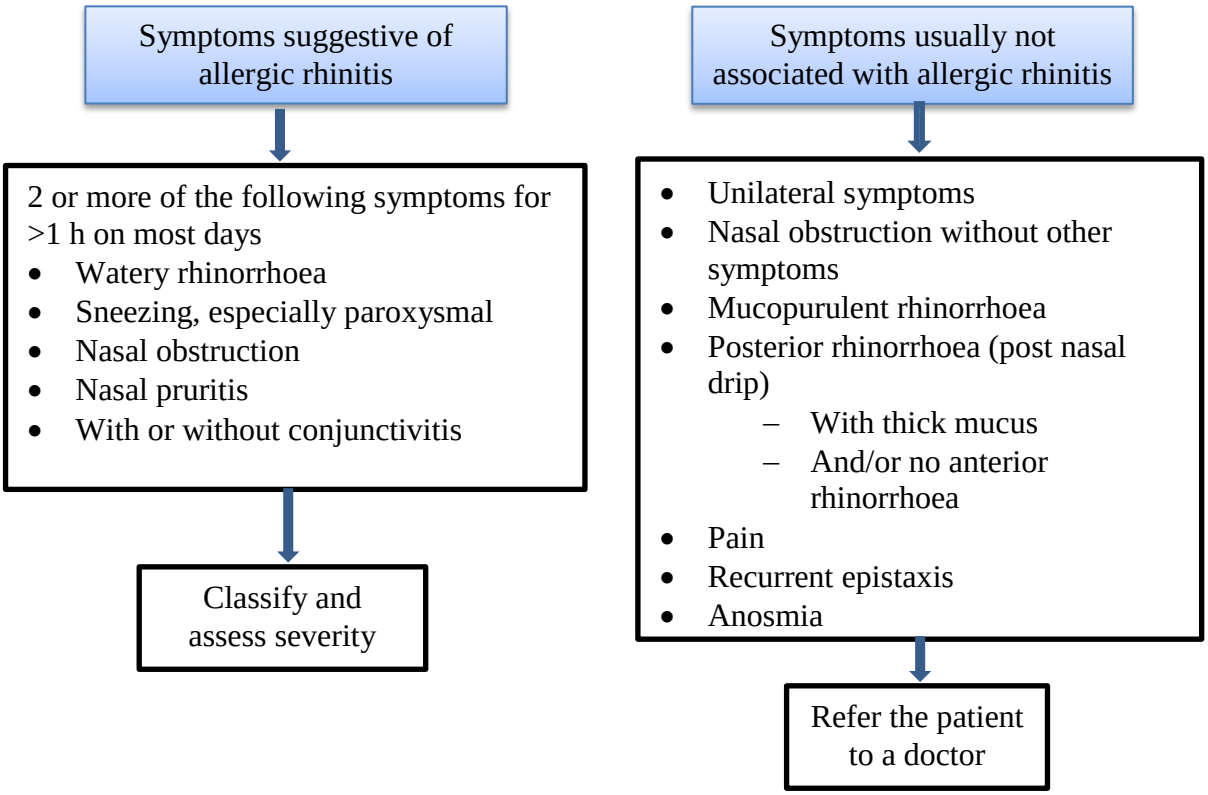

Figure 2.3: Management of AR symptoms. Figure is adapted from Members of the Allergic Rhinitis and its Impact on Asthma (ARIA) Workshops (2004).

The severity of AR can be classified as mild and moderate/severe based on the AR and its Impact on Asthma (ARIA) guidelines (J. Bousquet et al., 2008). The severity of AR is measured based on four items including sleep abnormality, impairment in daily activities, impairment in school or work performance and troublesome symptoms. Patients without the aforementioned items are considered as mild AR while patients with one or more of the items are considered as moderate/severe AR. The ARIA guidelines also classify AR symptoms into intermittent and persistent based on the duration of symptoms present in AR patient. For intermittent symptoms, the symptoms occur in less than four days per week or less than four consecutive weeks while for persistent symptoms, they occur in more than four days per week and more than four consecutive weeks. The summary of AR severity and classification of symptoms based on ARIA guidelines is presented in **Figure 2.4**.

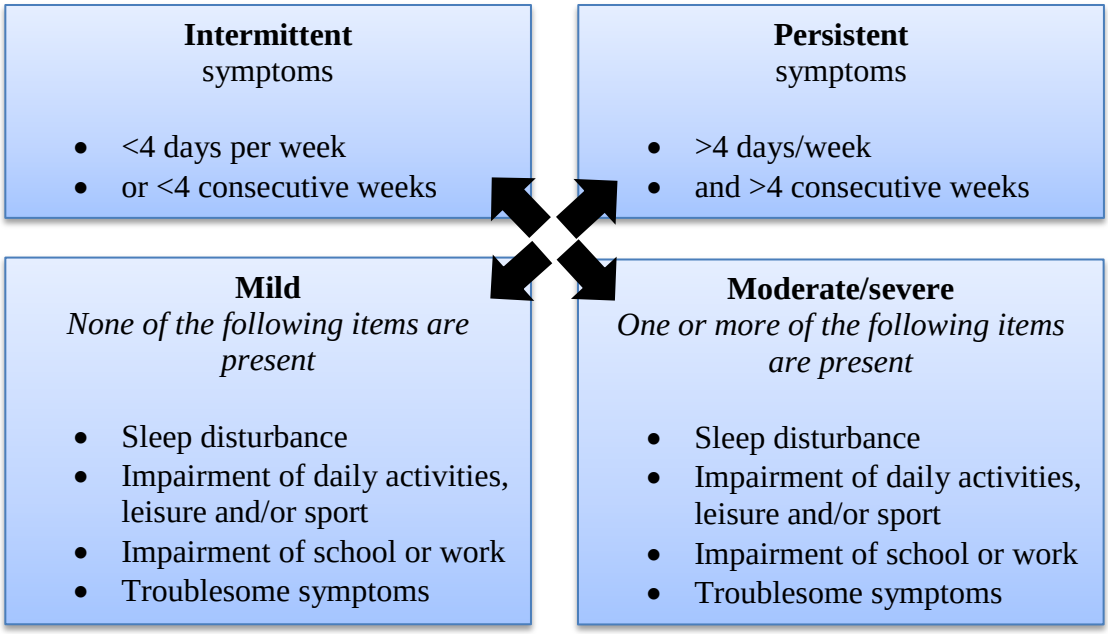

Figure 2.4: Summary of AR severity and classification of symptoms based on ARIA guidelines (J. Bousquet et al., 2008).

2.2.6 Laboratory characteristics

To determine the specific allergen that causes the development of IgE antibody in AR, multiple tests can be done such as *in vivo* skin tests including skin prick test (SPT; percutaneous) and intradermal (intracutaneous) skin tests (IDST), and *in vitro* serum allergen-specific IgE (ssIgE) immunoassay. SPT and ssIgE immunoassay are the most common laboratory tests and have strong correlation with the specificity and sensitivity in the diagnosis of sensitisation to common allergens (Nam & Lee, 2017; Wongpiyabovorn et al., 2018). However, there is no 'gold standard' laboratory test in diagnosing AR but SPT represents the first-line approach in the assessment of allergic sensitivities (J. Bousquet et al., 2008; Erel et al., 2017). SPT presents as a quick and cost-effective methodology in diagnosing any allergic sensitisation (J. Bousquet et al., 2012).

ssIgE immunoassay utilises commercially available test panels which are more costly (J. Bousquet et al., 2012). It is also less sensitive for the diagnosis of allergy due to inhalant allergens compared with SPT. However, ssIgE immunoassay can be useful when skin testing is not available or cannot be performed because patients have extensive skin disease, unable to discontinue antihistamines or other interfering medications, dermatographic, or other issues that complicate skin testing (Cox et al., 2011; Mansfield et al., 2012).

Other alternative or supporting diagnostic tests for AR include IDST in which tiny quantity of allergen is injected into the dermis with a hypodermic needle for the diagnosis of IgE-mediated allergic conditions (Tanno et al., 2016), eosinophils cationic protein (ECP) and the percentage of eosinophils (A et al., 2018; Y. Li et al., 2016), tryptase (*i.e.* marker of mast cell activation) (J. H. Kim et al., 2016), leukotriene B4 (A et al., 2018) and basophil activation test (*i.e.* using flow cytometry) to detect the causative allergen in local AR (Campo et al., 2019; Hoffmann et al., 2015).

2.2.7 Diagnostic criteria

Diagnostic criteria are a set of signs, symptoms, and tests for use in routine clinical care of patients and for clinical research purpose. It is generally broad and must reflect the different features of a disease (heterogeneity), with a view to accurately identify as many people with the condition as possible (Aggarwal et al., 2015). Due to the lack of gold standards in diagnosing AR, diagnostic criteria are difficult to establish. The choice of confirmatory test is a matter of clinical judgement and the results obtained must be considered together with additional risk factors, rather than definitive indicators of disease (Robert R. Rich, 2019).

However, for the patients to be diagnosed with AR, they must have clinical symptoms and possess laboratory characteristics as discussed in the previous section. AR patients must encounter two or more of the following clinical symptoms for more than one hour on most days: 1) Watery rhinorrhoea; 2) Sneezing, especially paroxysmal; 3) Nasal obstruction; 4) Nasal pruritis; 5) With or without conjunctivitis. After the patients are presented with clinical symptoms of AR, allergy laboratory tests are conducted for confirmation. They must exhibit either one of the laboratory characteristics of the allergy tests. In skin tests, positive result is considered when the wheal-and-flare reaction occurs on the skin test site after 20 minutes of exposure to allergens. For SPT, positive result must show wheal (*i.e.* a red and itchy raised bump with surrounding inflammation that indicates presence of allergic antibodies) size in diameter of ≥4 mm (deShazo, 2017; van der Valk et al., 2015). **Figure 2.5** shows a positive SPT on HDM allergen (*D. pteronyssinus*, *D. farinae* and *B. tropicalis)* tested on an AR patient who attended the ORL-HNS clinic of Hospital USM, and each wheal size was greater than 4 mm.