**From phenotypes to endotypes: Deciphering the immune signatures associated with atopic dermatitis (AD)**

Project Code: BM004

## Introduction

Atopic dermatitis (AD) affects 13.1% of the local population, and is the most common skin disorder in Singapore (Tay, Y. K. *et al.*, 2002). AD is characterized by dry, itchy and inflamed skin that causes pain, distress and loss of sleep. AD arises from the complex interaction of genetics and the environment of patients, and results in a dysregulated immune response.

Loss-of-function mutations in the Filaggrin (FLG) gene have been proven to be a strong genetic causative agent of AD. (Gao *et al.*, 2009). Filaggrin, otherwise known as filament aggregating protein, aggregates keratin in the epidermis. Located in the stratum corneum of the skin, FLG confers the first line of defense against moisture loss, microbes and allergens (Carson G. C. *et al.*, 2012). Hence, FLG mutation increases risk of skin barrier dysfunction. Consequently, an impaired epidermal barrier function increases the skin's susceptibility to environmental triggers such as *Staphylococcus aureus*, which induces high levels of Immunoglobulin E (IgE) antibodies (Seite & Bieber, 2015).

Biomarkers found in blood, including IgE and Th2 cytokines, are involved in the immune response of AD. An immune response of AD would first require exposure to allergens in the environment, resulting in production of allergen-specific IgE antibodies, and would bring about allergen sensitisation. Cytokines are the inflammatory mediators responsible for most biological effects in the immune system, such as cell mediated immunity and allergic type reactions. Th2 cells mostly produce IL4, 5, and 13, which are associated with the promotion of IgE and eosinophilic responses in atopy. (Bojuniewicz *et al.*, 2018). Therefore, there is an overexpression of Th2 cytokines observed in patients with AD (Brunner *et al.*, 2017).

Hitherto, there is no known treatment to cure AD. Current treatments like topical corticosteroid creams focus on treating flares by suppressing the immune system. However, they only address AD at a physiological level, and bring about side effects such as skin thinning, stretch marks and bruising susceptibility. There is a need to go further and address AD at a molecular level so that we can provide targeted, personalised treatments to patients instead of the current generic treatment protocol.

AD patients can be stratified into endotypes, which are subtypes of a disease condition, often defined by distinct pathophysiological mechanisms at the molecular level. Past research has

shown that food allergy is an endotype of AD (Leung *et al*., 2019). However, recent studies have found that AD patients can be further stratified into 2 distinct skin dermotypes - dermotype A and dermotype B. Dermotype B can be characterised by the decrease in microbial richness, increase in virulence genes, and a concomitant abundance of *Staphylococcus aureus,* whereas microbial richness was maintained in dermotype A. In addition, there is a difference in skin hydration levels and transepidermal water loss between both dermotypes (Angeline *et al.,* 2020). Therefore, it is evident that the 2 dermotypes within the AD population display varying properties**.**

Since the microbe-host interactions on skin can influence immune response and contribute to AD, skin microbiomes can be analysed to find biomarkers present, which can then be used to monitor AD progression. Hence, this study seeks to find the immune signatures associated with these two dermotypes.

**Hypothesis**

*FLG* and *S. aureus* - representing genetic and environmental factors, respectively - trigger immune dysregulation, and dermotypes will have different IgE and TH2 cytokine levels.

**Objectives**

The objective of this study was to determine underlying immune phenotypes associated with dermotype-specific patients.

**Methodology**

**Blood phenotyping**

IgE levels were detected using ImmunoCAP; Immune markers using Luminex Technology, and filaggrin genotyping using a customised sequencing panel.

**Isolation of peripheral blood mononuclear cells (PBMC) and Gene expression**

Transcriptional profile of isolated PBMC was determined using next generation sequencing by estimating reads per million transcript. Total RNA was extracted following the double extraction protocol: RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction (TRIzol, Thermo Fisher Scientific, Waltham, MA, USA) followed by a Qiagen RNeasy Micro clean-up procedure (Qiagen, Hilden, Germany). Gene expression was determined using next generation

sequencing by the SMARTSeq v2 protocol using an indexed paired-end sequencing run of 2x151 cycles on an Illumina HiSeq 4000 system (Illumina) (25 samples/lane).

**Dermotype definition:** Previously published data was used to define the dermotype (Angeline et al., 2020) using whole genome metagenomics of skin tape samples from the study cohort.

**Data Analysis**

**(1)** The Mann-Whitney U test (for 2 setups only) or Kruskal-Wallis test (for 3 or more setups) were carried out to find out if there are significant differences in immune markers found in dermotype A and B. The t-test (for 2 setups with more than 15 samples) is carried out to determine if there are significant differences in the means of test setups.

**(2)** R-squared test was conducted to statistically measure the association between two categorical data sets. R value represents the proportion of the variance for a dependent variable, explained by an independent variable or variables in a regression model. We used the R-squared test to correlate immune markers with dermotype A and B.

**(3)** False Discovery Rate (FDR) was used to determine the genes that were significantly expressed in dermotypes. FDR refers to the ratio of number of false positive results to the number of total positive test results and is used in experiments where multiple comparisons are tested to correct for random events that falsely appear significant.

**Results & Discussion**

**(A)** There was a significant difference in IgE levels between dermotype A and dermotype B. Patients with dermotype B had significantly highly IgE levels as compared to patients with dermotype A.

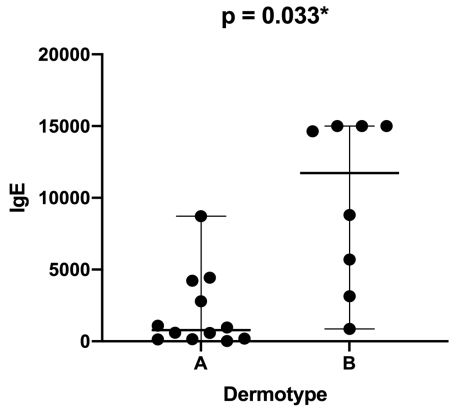


**Fig. 1.1:** Significant differences in IgE levels between dermotypes A and B

| | |
|---|---|
| n | 20 |
| Median for Dermotype A | 776 |
| Median for Dermotype B | 11726 |

**(B)** Of the 56 immune markers analysed, Chemokine Ligand 13 (CCL13/MCP-4) and Chemokine Ligand 17 (CCL17/TARC) displayed a significant difference between dermotype A and dermotype B. For both immune markers, dermotype B had a higher median level as compared to dermotype A.

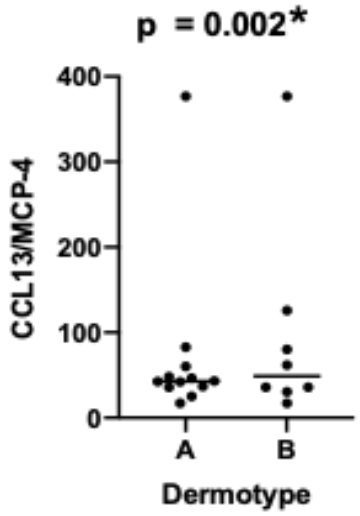


**Fig 1.2:** Significant difference in CCL13 and level between dermotype A and dermotype B.

| | |
|---|---|
| n | 20 |
| Median for Dermotype A | 42.895 |
| Median for Dermotype B | 48.84 |

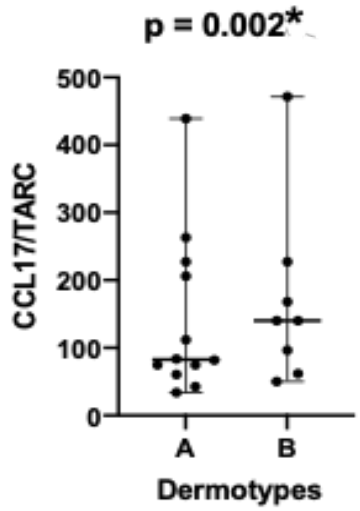


**Fig 1.3:** Significant difference in CCL17 level between dermotype A and dermotype B.

| | |
|---|---|
| n | 20 |
| Median for Dermotype A | 82.605 |
| Median for Dermotype B | 140.2 |

There were elevated levels of CCL13 and CCL17 biomarkers found in Dermotype B. Since these biomarkers are produced by TH2 cells, this suggests that immune signatures associated with dermotype B have strong inflammatory response and Th2 response, thereby reflecting a type 2 inflammation.

**(C)** Among the 33,000 genes screened, only PTCH1, IGHE, NLGN4Y and AC021127.1 were significantly expressed across both dermotypes. Given that NLGN4Y is only present in the Y chromosome and that AC021127.1 is a non-coding gene, the genes selected for analysis were narrowed down to Protein Patched Homolog 1 (PTCH1) and Immunoglobulin heavy constant epsilon (IGHE)n.

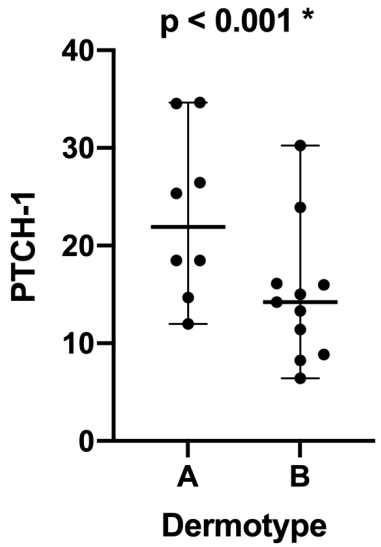


**Fig 1.4:** Patients classified under dermotype A **(median = 21.9143)** exhibited higher expression of PTCH1 genes as compared to that of dermotype B **(median = 14.2142)**, as evident from the higher median level of expression in dermotype A.

Higher expressions of PTCH1 gene were found in patients with dermotype A. PTCH1 produces patched-1 proteins which brings about an increase in regulatory T cells. AD pathology is reduced via the sonic hedgehog pathway which regulates epidermal homeostasis.

In addition, Abe & Tanaka (2017) have found that the sonic hedgehog pathway maintains epidermal stem cells which in turn promotes differential epidermal tissue repair. Evidently, higher expression of PTCH1 gene in dermotype A confers greater skin hydration and barrier function, as previously observed (Angeline *et al.,* 2020).

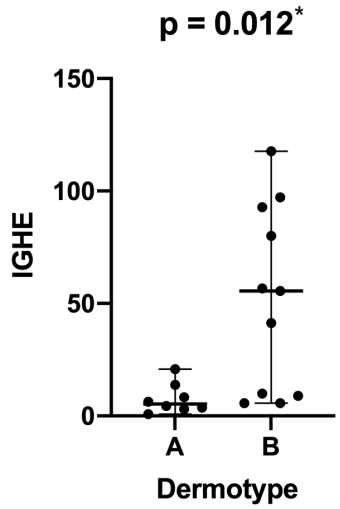


**Fig. 1.5:** Higher levels of IGHE were observed in dermotype B patients **(median = 55.5569)** than those of dermotype A **(median = 5.32781)**.

Patients with dermotype B displayed higher expression of IGHE genes which correlates with higher IgE levels, and elevated levels of CCL13 and CCL17 biomarkers previously observed in section **(A)** and **(B)**. Since these biomarkers are produced by TH2 cells, this further supports the fact that immune signatures associated with dermotype B have a strong inflammatory response and Th2 response, reflecting a type 2 inflammation.

**(D)** Out of all 56 immune markers tested against, FLG status only impacted levels of G-CSF, with significant differences between the means of the two set-ups: FLG mutant and FLG wild-type (WT).

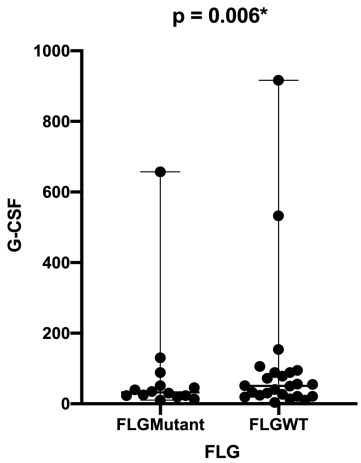


**Fig 1.8: Graph comparing G-CSF against FLG status**

Therefore, this suggests that there is no significant association between immune markers and FLG status.

**Conclusion**

A stratified approach is needed to understand the underlying phenotypes and endotypes of a clinically heterogeneous disease such as AD, which will be useful in defining treatment strategies. The endotype that was investigated in this study was dermotypes and it was found that distinct skin microbiome configurations found on skin dermotypes can be used to stratify AD patients and there were unique immune signatures found on AD patients with different skin dermotypes.

While there have been challenges standardising the size and ethnicity of samples, this study conclusively demonstrates that AD phenotype has different underlying immune signatures. AD patients can be stratified into 2 distinct groups based on their skin dermotype - A or B. Immune signatures associated with dermotype B reflect type 2 inflammation whereas higher expression of PTCH1 gene in dermotype A confers greater skin hydration and barrier function, as previously observed (Angeline *et al.,* 2020).

Dermotype B has shown to promote type 2 inflammatory responses, which are associated with the promotion of IgE and eosinophilic responses in atopy (Bojuniewicz *et al.*, 2018).
Based on our findings we recommend that treatments targeting type 2 inflammations be used to effectively treat AD patients with dermotype B, while patients with dermotype A can be administered with low dosage of drugs tackling IGHE genes or type 2 inflammations. This specialised treatment not only addresses AD at the physical level but also at the molecular immune level. For future work, eosinophil count can be obtained to check if there is a correlation between eosinophils and skin dermotype.

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