Clinical, Cosmetic and Investigational Dermatology

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ORIGINAL RESEARCH

Unveiling the Effect of Age and IgE Level on Alopecia Areata: Insights from Comparative RNAseq Analysis

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Background: Alopecia areata (AA) is a common autoimmune disease, causes sudden hair loss on the scalp, face, and sometimes other areas of the body. Previous studies have suggested more severe manifestations and higher recurrence rates in children than in adults. Moreover, pediatric AA patients with atopic predisposition often exhibit elevated IgE levels, early onset, and a poor prognosis.
Purpose: This study aimed to investigate the impact of age and IgE levels on AA by conducting RNA sequencing on scalp samples from AA patients with atopic predisposition, age-matched healthy controls, and AA samples with varying IgE levels.
Patients and Methods: We employed the single-sample Gene Set Enrichment Analysis (ssGSEA) algorithm in conjunction with gene expression analysis to assess immune infiltration. Differential gene expression analysis was performed using the DESeq package in R. Immunohistochemical staining and qPCR was performed to validate these findings.
Results: Our results revealed a more pronounced inflammatory immune infiltration in AA patients across all age groups compared to healthy controls. Pediatric AA was characterized by an upregulation of genes controlling inflammatory responses, such as the IFN-γ pathway and JAK-STAT cascade, contrasting to adult AA. Compared to age-matched healthy controls, pediatric AA patients exhibited a significant increase in the infiltration of B cell subtypes, mast cells, and regulatory T cells. Additionally, high IgE levels in AA patients led to the upregulation of IFN-γ pathway genes, compared to AA patients with normal IgE levels.
Conclusion: In summary, the heightened immune and inflammatory responses, along with the more significant infiltration of immune cells in pediatric AA with atopic predisposition, may explain the increased clinical severity and recurrence rates. Dissecting these molecular mechanisms sheds some light on the contributions of age and IgE to the pathogenesis and progression of AA, revealing potential age-specific and allergy-related therapeutic targets.
Keywords: alopecia areata, age, IgE, immune response, inflammatory response

Introduction

Alopecia areata (AA) is a prevalent autoimmune dermatological condition characterized by non-scarring hair loss affecting the scalp and body[1,2]. The pathogenesis of AA involves a complex interplay of inflammatory responses, with contributing factors ranging from genetic predispositions to psychological stress, emotional distress, and infections, presenting significant therapeutic challenges. The etiology of AA remains incompletely understood. However, it is currently believed to be primarily due to the loss of immune privilege of the hair follicle, autoimmune-mediated follicular destruction, and upregulation of inflammatory pathways. Interferon-gamma (IFN-γ) plays a pivotal role in the collapse of hair follicle immune privilege, inducing the upregulation of major histocompatibility complex (MHC) class I molecules in cells and recruiting Natural killer group 2 member D ($NKG2D^+$) in $CD8^+$ T cells, leading to follicular damage.[3,4]

Received: 11 October 2024
Accepted: 6 January 2025
Published: 13 January 2025

AA is not confined to any specific age group but is particularly prevalent among children and adolescents, ranking as the third most common dermatological condition in this demographic. Epidemiological evidence suggests that pediatric AA may exhibit more aggressive features and a higher recurrence rate than adult AA,[5–9] implying that the maturity of the immune system and its interaction with environmental factors may play a crucial role in disease manifestation. Despite the significant impact of AA, particularly on younger patients, there is a notable deficiency in clinical and epidemiological research targeting this population. A comprehensive review of the existing literature reveals extensive sequencing studies on AA, which primarily fall into three categories: those comparing AA patients with healthy controls,[10–12] those examining differences among AA patients with varying degrees of severity,[10] and those assessing changes pre- and post-treatment with various JAK inhibitors and other inhibitors.[12–16] However, there is a conspicuous absence of sequencing studies focused on pediatric AA patients, hindering our understanding of the molecular basis of this disease in children.

Furthermore, it has been observed that there is a close relationship between AA and atopic diseases. This was evident in a study where 32.5% of patients with chronic severe AA exhibit elevated serum IgE levels.[17] Therefore, it would be interesting to explore the impact of IgE levels on AA's molecular and cellular effects. Reports suggest that AA patients, regardless of atopic disease presence, have increased serum IgE levels, highlighting the potential efficacy of antihistamine therapy in alleviating the severity of atopic hair loss and underscoring the relevance of allergic processes in the pathophysiology of the disease.[18] Additionally, pediatric AA patients with an atopic disposition often present with elevated IgE levels, early onset, and poor prognosis.[19,20]

Given these considerations, the present study aims to investigate the gene expression profiles in scalp samples from children and adults with severe AA, all of whom exhibit an atopic predisposition but have varying levels of IgE. Our primary objective is to elucidate the impact of age and IgE levels on the pathogenesis and progression of AA, thereby identifying potential therapeutic targets that are specific to age and allergy-related mechanisms.

Materials and Methods

Patients and Specimens

The investigation was executed at the Dermatology Hospital of Southern Medical University and entailed the procurement of 5 mm punch biopsies from the lesional skin regions of 28 patients who were histopathologically confirmed to have severe AA, with a Severity of Alopecia Tool (SALT) score exceeding 50%. All patients exhibited an atopic predisposition. The patient cohort was stratified into two age-based groups. The pediatric subset included 6 patients under the age of 18, all manifesting elevated IgE levels. The adult contingent comprised 22 individuals over the age of 18 diagnosed with AA, 5 of whom presented with increased IgE levels. For comparative analysis, normal skin samples were also acquired from 8 healthy subjects (comprising 5 pediatric and 3 adult individuals) who were recipients of plastic surgery procedures. These specimens constituted the control group for the investigation. The specific information of AA patients and their age-matched healthy controls in this study are detailed in Supplementary Table S1–S3. The study protocol was reviewed and subsequently approved by the Ethics Committee of the Dermatology Hospital of Southern Medical University. This ensured the adherence to ethical guidelines and the safeguarding of the rights and welfare of all participants.

RNA-seq

Illumina/TruSeq stranded mRNA library preparation kit (RS-122-2102; Illumina, USA) was utilized for library preparation. The sequencing reads were aligned to the reference genome GRCh38 using Hisat2 (v2.0.1). The alignment results were then converted into Sequence Alignment/Map (SAM) files using the SAM tool. Featurecount (v2.0.1) was employed to obtain read counts, with the union option selected for this process. Differential gene expression analysis was conducted using the DESeq2 package in R/Bioconductor. The criteria for significant differential expression were set at an adjusted P-value of <0.05 and an absolute log2 fold change of >1.

Immune Infiltration Analysis

The gene signatures corresponding to 28 distinct types of immune cells were procured from the TISIDB database (http://cis.hku.hk/TISIDB/). This comprehensive list included a variety of cells, such as Activated CD8 T cells, Activated CD4 T cells, T follicular helper cells, and Gamma delta T cells. To evaluate the immunological characteristics of each sample included in the study, we employed the single-sample Gene Set Enrichment Analysis (ssGSEA) algorithm.[21] This analysis was conducted using the GSVA package (v1.40.1) in the R software environment (v4.1.0). The ssGSEA algorithm allowed for a robust and comprehensive evaluation of the immunological landscape of each sample.

Immunohistochemical Staining

Immunohistochemistry (IHC) staining was conducted utilizing a concentration of 1:50 for CD117 and 1:200 for CD20. Skin biopsies, embedded in paraffin, were sectioned meticulously to a thickness of 5 μm. These sections were then subjected to an overnight incubation at 4°C with primary antibodies specifically targeting CD117 and CD20. Following primary antibody incubation, the sections were treated with secondary antibodies at a dilution of 1:500. The final step involved scanning the treated sections using a NanoZoomer S6 (Hamamatsu, Japan).

Hematoxylin and Eosin

Hematoxylin and Eosin (H&E) staining was meticulously executed following standardized procedures. Paraffin-embedded skin biopsy specimens were precisely sectioned to a uniform thickness of 5 μm. Subsequently, these sections underwent a deparaffinization process in xylene, and a graded alcohol series was employed for rehydration. The slides were immersed in hematoxylin for a duration of 5–10 minutes for nuclear staining, followed by a swift rinse in distilled water. Eosin was applied thereafter for 1–3 minutes to provide cytoplasmic and extracellular matrix staining. Post-staining, the sections were subjected to a dehydration sequence using increasing concentrations of alcohol and cleared in xylene. The final step entailed the application of a resin-based mounting medium to the stained sections. The completed slides were scrutinized under a microscope, with image acquisition facilitated by a NanoZoomer S6 (Hamamatsu, Japan).

qRT- PCR

Total RNA was extracted from skin biopsies using the TRIzol reagent (Invitrogen). This RNA was then reverse-transcribed into complementary DNA (cDNA) using the PrimeScript™ RT Kit (RR047A, TAKARA). Quantitative real-time PCR (qRT-PCR) was conducted using a LightCycler® 96 real-time PCR system (5815916001; Roche, USA). The specific primers employed in this study are detailed in Supplementary Table S4.

Data Analysis and Statistics

We employed the ssGSEA algorithm to evaluate the enrichment scores of immune-related gene sets in individual samples, enabling the assessment of pathway-specific activity based on the gene expression profile.[21] Differential gene expression analysis was conducted using the DESeq package in R, leveraging linear models and empirical Bayes methods to identify differentially expressed genes (DEGs) across distinct groups. P-values were adjusted via the Benjamini-Hochberg method to control the false discovery rate (FDR). Principal Component Analysis (PCA) was utilized for an unsupervised exploration of sample patterns and interrelationships based on gene expression data, facilitating the visualization of sample clustering and the identification of key sources of variation. IHC quantification was performed using bioinformatics tools to estimate the composition of immune cell types from gene expression data, offering insights into the immune landscape disparities between pediatric and adult patients with AA, as well as among patients with varying IgE levels. Statistical analyses were conducted using Prism v8.0 software, with the Student's *t* test employed for pairwise group comparisons and one-way analysis of variance with Bonferroni posthoc testing for multiple group comparisons. Quantitative outcomes were expressed as mean ± standard deviation (SD), with statistical significance set at $P \leq 0.05$.

Result

Immune-Inflammatory Pathway in Pediatric AA is More Active Than in Adult AA

Clinical studies have indicated that AA tends to manifest more severely in pediatric patients, exhibiting a higher recurrence rate compared to adults.[9] The samples were stratified into four distinct cohorts: pediatric AA, adult AA, pediatric healthy controls, and adult healthy controls. Among the pediatric AA participants, the mean age ± standard deviation was 10.0 ± 3.6 years; 83.3% (5/6) were male and 16.7% (1/6) were female. The mean age of onset was 7.8 ± 3.3 years, and the median duration of disease was 2 years (IQR: 1–11). For the adult AA participants, the mean age ± standard deviation was 39.4 ± 12.3 years; 59.1% (13/22) were female and 40.9% (9/22) were male. The mean age of onset was 34.6 ± 12.9 years, and the median duration of disease was 2 years (IQR: 0.5–20). The most common comorbidities in the overall cohort included allergic rhinitis (42.9%, 12/28), atopic dermatitis (21.4%, 6/28), and Hashimoto thyroiditis (14.3%, 4/28). Laboratory results indicated that 39.2% (11/28) had high IgE levels, and 28.6% (8/28) tested positive for anti-TG (Table 1). Notably, all samples were collected from the scalp.

The Principal Component Analysis (PCA) of the RNA sequencing unveiled distinct clustering of pediatric AA samples, segregating them from their healthy counterparts. This suggests the existence of divergent gene expression profiles between these two groups (Figure 1A). Moreover, the gene expression patterns underscored phenotypic differences among the groups, thereby highlighting the potential influence of age and disease status on the molecular

Table 1 Demographic Characteristics of the Patients at Baseline

Characteristic	Value
Sex	
Male, n (%)	14 (50.0%)
Female, n (%)	14 (50.0%)
Age, mean (SD)	30.6 (17.7)
Age of onset, mean (SD)	25.1 (17.6)
Duration of disease, median (IQR)	3 (1–20)
Clinical subtypes	
Alopecia Universalis (AU), n (%)	3(10.7%)
Others, n (%)	25 (89.3%)
Disease course	
Acute, n (%)	4 (14.3%)
Chronic, n (%)	24 (85.7%)
Comorbidities	
Allergic rhinitis, n (%)	12 (42.9%)
Atopic dermatitis, n (%)	6 (21.4%)
Neurosis, n (%)	1 (3.6%)
Eczema, n (%)	3 (10.7%)
Urticaria, n (%)	3 (10.7%)
Asthma, n (%)	2 (7.1%)
Atopic conjunctivitis, n (%)	2 (7.1%)
Androgenetic alopecia, n (%)	2 (7.1%)
Vitiligo, n (%)	0 (0.0%)
Pituitary disease, n (%)	1 (3.6%)
Hashimoto thyroiditis	4 (14.3%)
Laboratory Results	
ANA positive, n (%)	3 (10.7%)
Anti-dsDNA positive, n (%)	0 (0.0%)
Anti-TG positive, n (%)	8 (28.6.1%)
Anti-TPO positive, n (%)	4 (14.3%)
High IgE levels, n (%)	11,(39.2%)

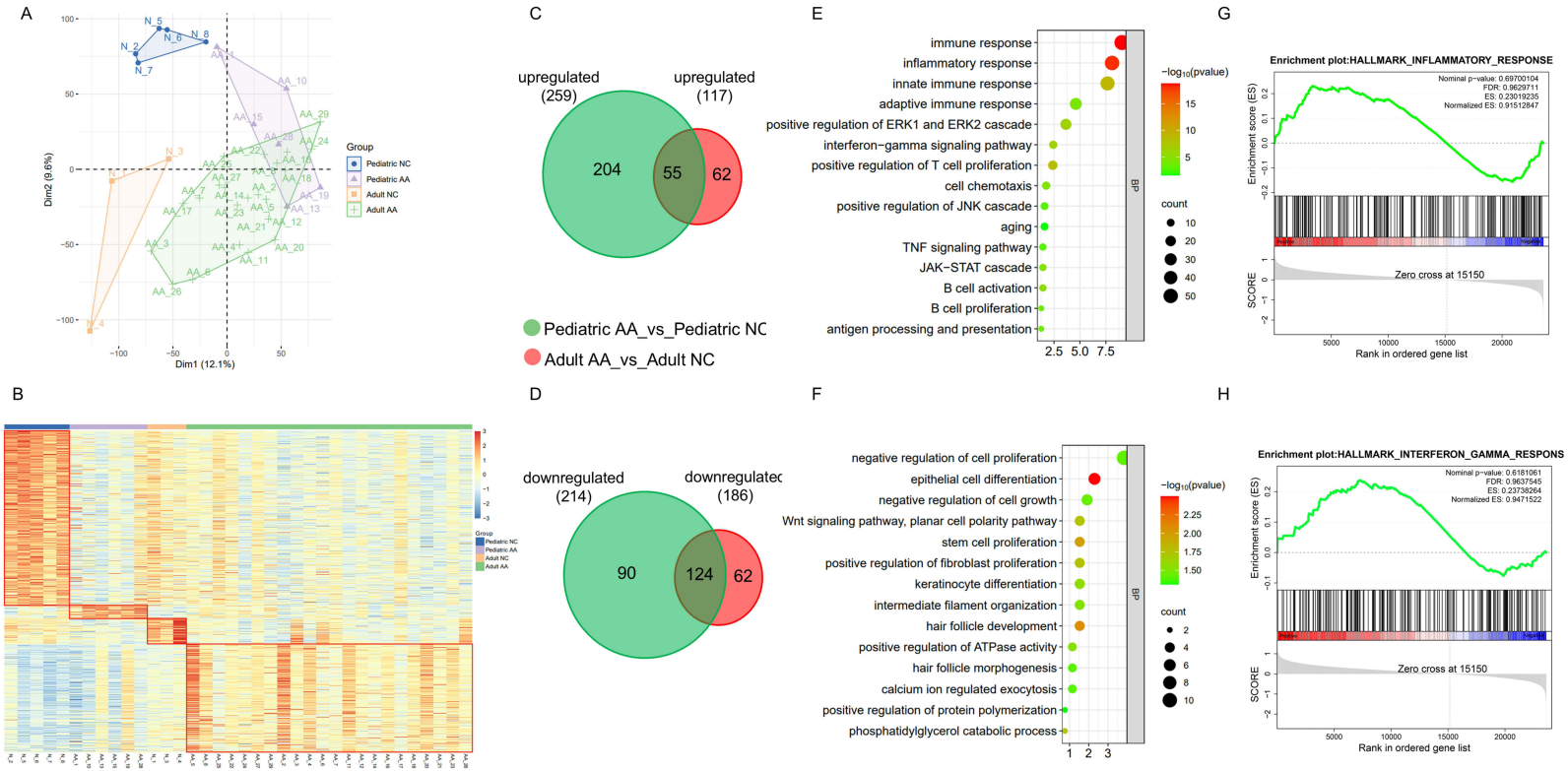

Figure 1 Comprehensive genomic profiling of alopecia areata across different age groups and controls. (**A**) Principal component analysis (PCA) of pediatric alopecia areata, adult alopecia areata, pediatric healthy controls, and adult healthy controls. (**B**) Heatmap illustrating differentially expressed genes across the four sample groups. (**C**) Venn diagram displaying up-regulated genes in pediatric alopecia areata (green) and adult alopecia areata (red) compared to their respective healthy control groups, with the intersection representing genes commonly up-regulated (n=55). (**D**) Venn diagram showing downregulated genes in pediatric alopecia areata (green) and adult alopecia areata (red) compared to their respective healthy control groups, with the intersection indicating commonly downregulated genes (n=124). (**E**) Gene Ontology (GO) Biological Process (BP) enrichment analysis for genes uniquely up-regulated in pediatric alopecia areata (n=204). (**F**) GO BP enrichment analysis for genes uniquely downregulated in pediatric alopecia areata (n=90). (**G**) Gene Set Enrichment Analysis (GSEA) snapshot comparing pathway enrichment between pediatric and adult alopecia areata: inflammatory response. (**H**) GSEA snapshot comparing pathway enrichment between pediatric and adult alopecia areata: IFN-γ signaling.

https://doi.org/10.2147/CCID.S493584

landscape of AA. This comprehensive analysis provides a foundation for further investigations into the age-specific pathophysiology of AA. (Figure 1B).

Gene expression profiling of pediatric and adult AA patient groups and their respective healthy controls revealed differential gene regulation. In pediatric AA, 259 genes were up-regulated, and 106 genes were down-regulated compared to healthy children. A similar trend was observed in the adult AA cohort, with 214 genes up-regulated and 228 genes down-regulated compared to healthy adults (Figure 1C and D). Across both pediatric and adult AA, up-regulated genes were primarily associated with the immune-inflammatory pathway, while down-regulated genes were involved mainly in keratinization and hair follicle development (Supplementary Figure S1A-F). Previous research has highlighted the activation of various immune cells in AA, characterized by extensive cytokine secretion. Our sequencing and qPCR data consistently showed significant elevations of CXCL9 and CXCL10 in AA-affected areas (Supplementary Figure S2). Both pediatric and adult AA patients exhibited significantly higher levels of CXCL9, CXCL10, and CCL13 than their healthy counterparts (Supplementary Figure S2A-B and S2E-F). This is well aligned with previous AA studies, which reinforces our findings' validity and emphasizes AA's common pathogenic mechanisms across age groups. AA is fundamentally an immune-inflammatory disorder, irrespective of age.

Further comparative analysis of gene expression between pediatric and adult AA patients revealed that 55 genes were consistently up-regulated, while 124 genes were down-regulated across both age groups. Up-regulated genes were predominantly associated with immune responses (Supplementary Figure S1G), while downregulated genes were linked to keratinization (Supplementary Figure S1H).

Interestingly, 204 genes were uniquely up-regulated in pediatric AA, and 90 genes were uniquely down-regulated. These age-specific gene regulatory patterns were characterized by up-regulation of genes related to immune responses, aberrant activation of the ERK1/2 and IFN-γ pathways, and immune response and down-regulation of genes involved in keratinization and hair morphogenesis (Figure 1E–H). The aberrant activation of ERK1/2 may disrupt the hair follicle growth cycle, leading to the activation of immune cells and the release of inflammatory cytokines.[22,23] Simultaneously, the production of IFN-γ may trigger an immune attack on hair follicles, resulting in hair loss.[12] Apart from the differences in enriched pathways of differential genes, CCL21 and CCL19 were both up-regulated in pediatric AA compared to pediatric controls, whereas these chemokines showed no difference between adult AA and adult controls (Supplementary Figure S2C-D and S2G-H). These findings suggest an intensified immune-inflammatory response in pediatric AA, potentially leading to abnormal dermal and hair follicle development. This might provide insight into the observed higher recurrence rates and more severe hair loss in pediatric AA patients.

Cell Infiltration Disparities in Pediatric AA is More Significant Than in Adult AA

AA is a chronic inflammatory condition distinguished by a complex interplay between immune cell infiltration and the disruption of the hair follicle cycle. Our comprehensive analysis affirmed the heightened infiltration of $CD8^+$ T cells in AA patients compared to healthy subjects, a finding consistent with previous research. Further stratified analysis by age revealed that $CD8^+$ T cell infiltration was a consistent characteristic across all age groups in AA patients compared with their respective age-matched controls, emphasizing the presumptive crucial role of $CD8^+$ T cells in the pathogenesis of AA. Importantly, our data suggest that the infiltration of $CD4^+$ T cells, B-cell subtypes, mast cells, and regulatory T cells is significantly higher in pediatric AA patients than in pediatric controls ($p<0.05$). However, no significant difference was observed in the infiltration levels of these cells between adult AA patients and their adult counterparts (Figure 2A).

Furthermore, HE staining consistently demonstrated an increase in lymphocytic infiltration around the dermal hair follicles and blood vessels in the AA cohort, regardless of age or serum IgE levels, compared to the control group. Moreover, we detected lymphocytes, eosinophils, and mast cells in the peribulbar region of AA patients. We also observed melanin casts, melanin-containing vascular connective tissue columns, and inflammatory cells in hair follicles beneath graded hair follicles in AA patients. These observations suggest a degenerative or dormant state, indicating that the number of telogen hair follicles exceed the number of anagen hair follicles in AA patients. (Supplementary Figure S3). IHC results showed a significant increase in $CD117^+$ (mast cells) and $CD20^+$ (B cells) cells in the epidermis, perivascular, perifollicular, and deep dermal regions of patients with various subtypes of AA compared to healthy individuals. However, we observed a differential pattern in the presence of CD20+ cells when comparing pediatric and adult AA samples. Notably, CD20+ cells

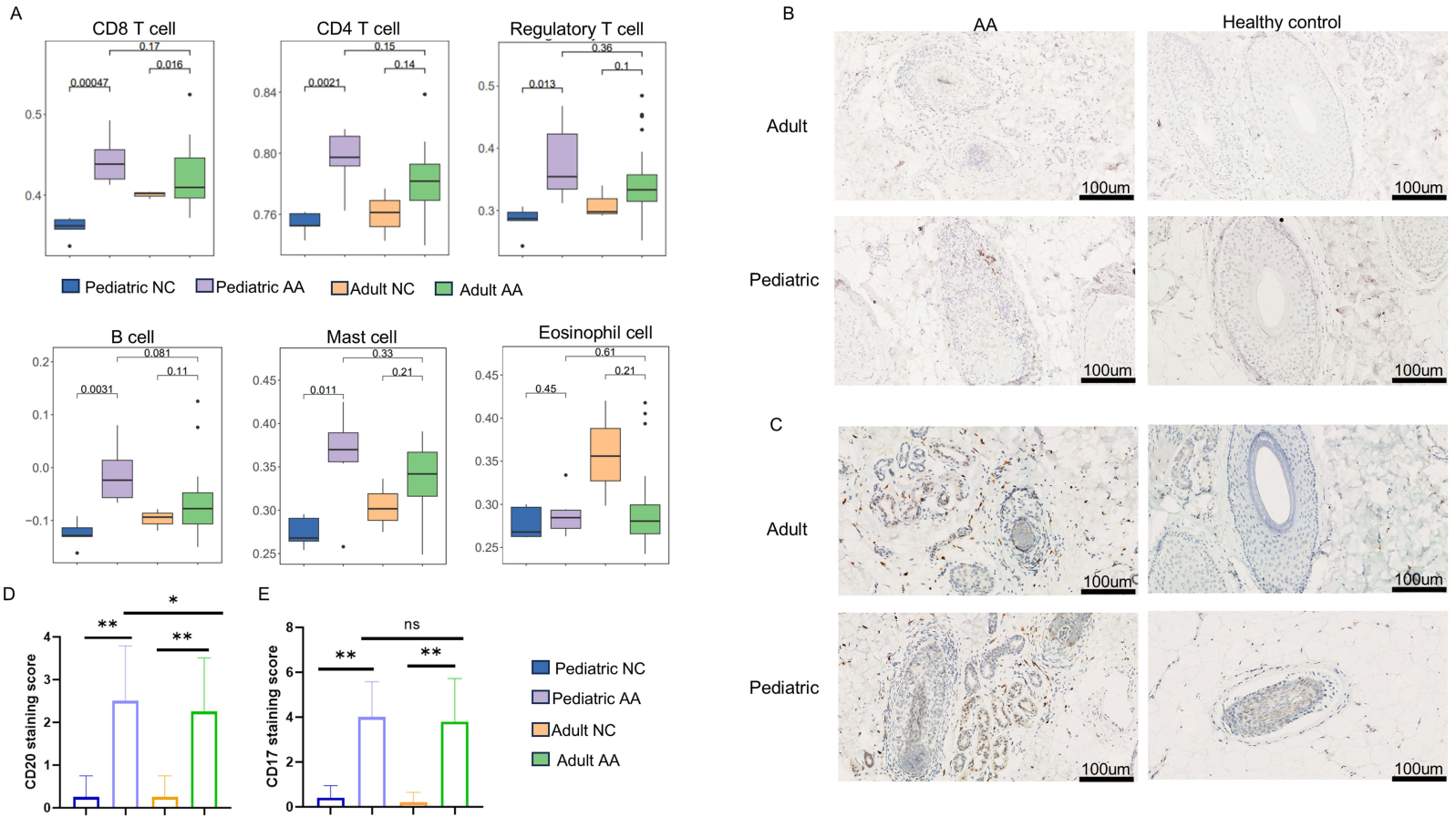

Figure 2 Immunological profiling and immunohistochemical characterization of alopecia areata and controls. (**A**) Single-sample GSEA (ssGSEA) for comprehensive immunological feature assessment across groups. (**B**) Immunohistochemical staining for CD20 in adult alopecia areata, adult healthy controls, pediatric alopecia areata, and pediatric healthy controls (n = 3, n = 3). (**C**) Immunohistochemical staining for CD117 across the same groups as in (**B**) (n = 3, n = 3). (**D**) Statistical analysis of CD20 staining scores in the aforementioned groups. (**E**) Statistical analysis of CD117 staining scores in the aforementioned groups. *P < 0.05, **P < 0.01, ns indicates no statistical significance (P > 0.05) (1-way analysis of variance).

https://doi.org/10.2147/CCID.S493584

appeared more prevalent in the pediatric AA specimens (Figure 2B and D). In contrast, the prevalence of CD117+ cells did not exhibit a noticeable difference between the pediatric and adult AA groups (Figure 2C and E), suggesting a consistent distribution across these age categories. The discrepancy between the IHC analysis and staining results may be because RNA sequencing typically involves mixed RNA from the entire sample, which might not accurately capture the specific immune cell distribution in localized regions.

Immune-Inflammatory Pathway in Pediatric AA with High IgE is More Intense Than in Adult AA with High IgE

Numerous studies have indicated that individuals suffering from AA frequently display elevated levels of IgE, implying that allergic reactions could potentially instigate the disease's onset and recurrence.[24,25] To scrutinize the association between IgE concentrations and AA, we segregated patients into two categories: adults with escalated IgE levels and adults with IgE levels within the normal range. We omitted samples from pediatric AA patients from the analysis due to their uniformly high IgE levels.

PCA and heatmap visualizations indicated a somewhat blurred distinction between adult patients with high and normal IgE levels and healthy controls based solely on IgE levels, suggesting a degree of overlap (Supplementary Figure S4A and B). Our data pointed towards a muted activation of the conventional inflammatory pathways in the context of adult AA patients with elevated IgE levels (Supplementary Figure S4C-F). Interestingly, GSEA did not identify a significant enrichment of inflammatory pathways. Instead, it highlighted the involvement of the IFN-γ pathway, thereby suggesting that escalated IgE levels may not directly reflect the degree of inflammation but could potentially be associated with specific immunomodulatory pathways (Supplementary Figure S4G and S4H). This inference was further corroborated by the absence of a significant difference in the infiltration of immune cells such as $CD8^+$ T cells, $CD4^+$ T cells, B cell subtypes, and mast cells between adults with high and normal IgE levels (Supplementary Figure S5).

Given the frequent occurrence of in pediatric AA patients with an atopic predisposition, we embarked on a comparative study of the gene expression profiles of pediatric AA patients and adult AA patients with high IgE levels. Our investigation unveiled distinct gene expression signatures between children with AA and adults with AA who had high IgE levels (Figure 3A and B). Specifically, we identified 219 genes that were exclusively up-regulated in children with high IgE (Figure 3C, supplementary Table S5), which were predominantly enriched in immune-inflammatory response, innate immunity, and the ERK1/2 and IFN-γ signaling pathways, among others (Figure 3E). Conversely, the 79 genes that were exclusively down-regulated (Figure 3D) were primarily associated with intermediate filamentation, keratinization, and hair follicle development (Figure 3F). GSEA further underscored the enrichment of inflammatory pathways in children with high IgE and AA (Figure 3G and H).

By juxtaposing these findings, it becomes apparent that while elevated IgE levels in adult AA patients may not directly correlate with heightened inflammatory activity, pediatric AA patients with similar IgE elevations exhibit a more robust activation of immune-inflammatory pathways. This distinction underscores the intricate pathophysiology of AA and the potential influence of age on the disease's immune response dynamics.

Cell Infiltration Disparities in Pediatric AA with High IgE is More Active Than in Adult AA with High IgE

Immune infiltration analysis revealed a significantly higher infiltration of CD4 cells, B cell subtypes, mast cells, and regulatory T cells in pediatric AA patients with high IgE compared to normal pediatric controls (Figure 4A). However, there was no significant difference in the infiltration of these cells when compared to adult patients and their corresponding adult controls. IHC demonstrated a substantial increase in the number of CD117-positive cells (mast cells) and CD20-positive cells (B cells) in the epidermis, perivascular, perifollicular, and deep skin areas of pediatric AA and adult AA with high IgE compared to healthy adults of the corresponding age group (Figure 4B). Nevertheless, there was no difference in the number of $CD117^+$ cells (Figure 4C and E) and $CD20^+$ cells (Figure 4B and D) between children with high IgE and adults with AA.

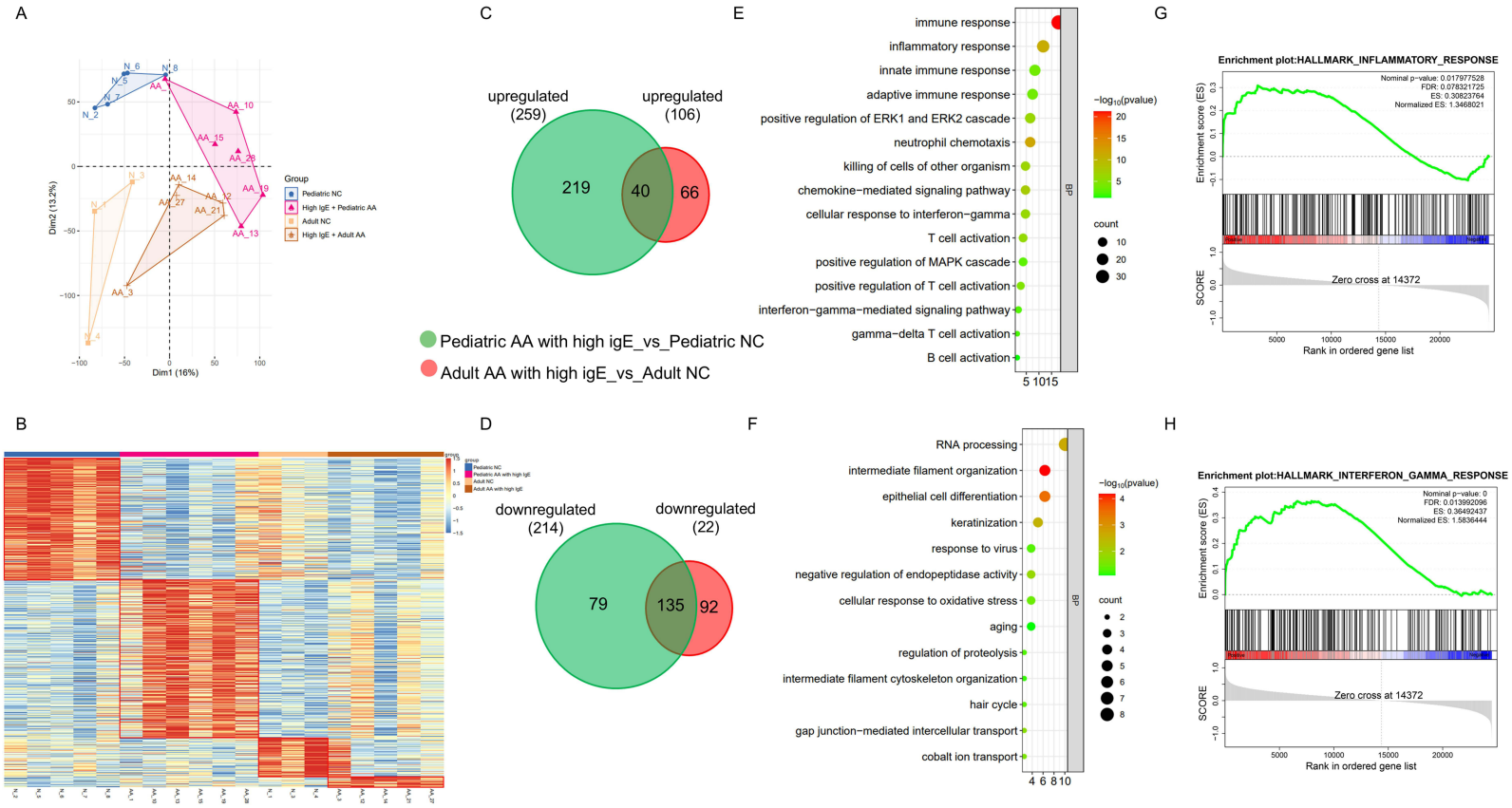

Figure 3 Comparative genomic analysis of pediatric and adult alopecia areata with high IgE levels. (**A**) PCA of high IgE pediatric alopecia areata, high IgE adult alopecia areata, pediatric healthy controls, and adult healthy controls. (**B**) Heatmap showing differentially expressed genes across the four sample groups. (**C**) Venn diagram of up-regulated genes in high IgE pediatric alopecia areata (green) and high IgE adult alopecia areata (red) compared to their respective healthy controls, with the intersection representing commonly up-regulated genes (n=40). (**D**) Venn diagram of downregulated genes in high IgE pediatric alopecia areata (green) and high IgE adult alopecia areata (red) compared to their respective healthy controls, with the intersection representing commonly downregulated genes (n=135). (**E**) GO BP enrichment analysis for genes uniquely up-regulated in high IgE pediatric alopecia areata (n=204). (**F**) GO BP enrichment analysis for genes uniquely downregulated in high IgE pediatric alopecia areata (n=204). (**G**) GSEA snapshot comparing pathway enrichment between high IgE pediatric and adult alopecia areata: inflammatory response. (**H**) GSEA snapshot comparing pathway enrichment between high IgE pediatric and adult alopecia areata: IFN-γ signaling.

https://doi.org/10.2147/CCID.S493584

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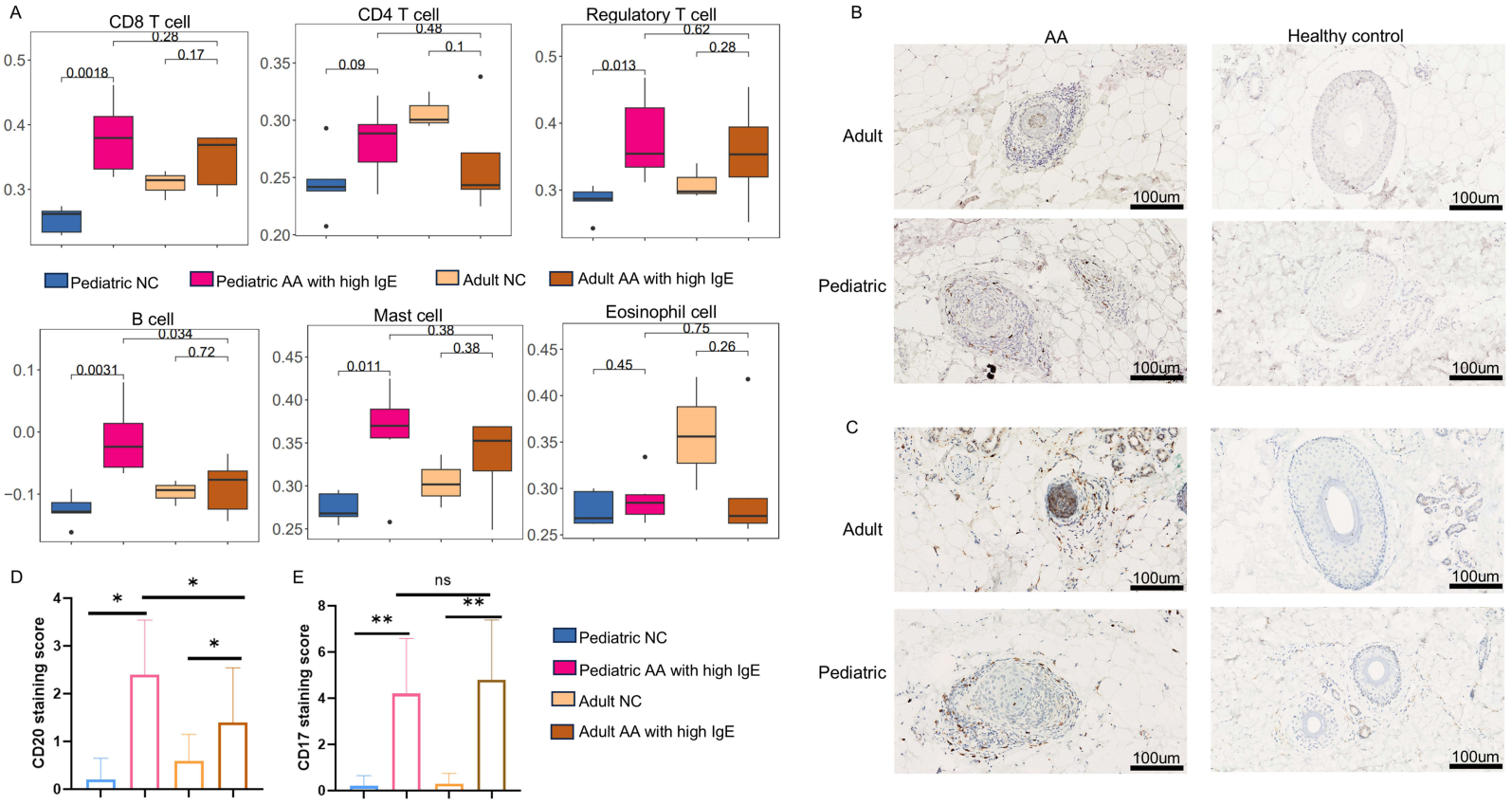

Figure 4 Immunological assessment and immunohistochemical analysis of high IgE alopecia areata. (**A**) ssGSEA for comprehensive immunological feature assessment across adult alopecia areata with high IgE, adult alopecia areata with normal IgE, and adult healthy controls. (**B**) Immunohistochemical staining for CD20 in adult alopecia areata with high IgE, pediatric alopecia areata with high IgE, pediatric healthy controls, and adult healthy controls (n = 3, n = 3, n = 3, n = 3). (**C**) Immunohistochemical staining for CD117 across the same groups as in (**B**) (n = 3, n = 3, n = 3, n = 3). (**D**) Immunohistochemical staining for CD20 in the aforementioned groups. (**E**) Immunohistochemical staining for CD117 in the aforementioned groups. *P < 0.05, **P < 0.01, ns indicates no statistical significance (P > 0.05)(1-way analysis of variance).

In summary, our findings suggest distinct pathological features between pediatric and adult patients with AA, particularly in the context of high IgE levels. Patients with pediatric AA exhibit a more active immune response, which may be correlated with higher recurrence rates and more aggressive disease characteristics.

Discussion

In this study, we conducted RNAseq on samples from AA patients of different ages and IgE levels to comprehensively investigate the pathophysiological basis of AA in relation to age and IgE levels.

Pediatric vs Adult AA (in Inflammatory Response and Analysis Gene Expression)

Our findings reveal a heightened immune-mediated inflammatory response in pediatric AA patients. In comparison to adult AA, pediatric AA patients exhibited up-regulated genes enriched in pathways such as ERK1/2 signaling, IFN-γ signaling, JAK-STAT signaling, antigen presentation, TNF signaling, JNK, and NF-kappaB signaling. Aberrant activation of ERK1/2 may disrupt the hair follicle growth cycle, thereby impacting normal hair growth and shedding.[22] Additionally, ERK1/2 activation may lead to an overproduction of inflammatory cytokines. The ERK1/2 signaling pathway may regulate the activation of these immune cells and the release of inflammatory cytokines, influencing the intensity and duration of inflammation. This pathway may also impact autoimmune responses, including the activation and differentiation of T cells, which are believed to be the primary immune cells attacking hair follicles in AA.[26] IFN-γ, an up-regulator of MHC-I during the anagen phase in vivo, has been previously considered to trigger the collapse of hair follicle immune privilege and subsequent immune-mediated damage to hair follicle epithelial stem cells.[27] The TNF and JNK signaling pathways can induce inflammatory responses and cell apoptosis, potentially contributing to the apoptosis of hair follicle stem cells in pediatric AA.[28] The JAK-STAT signaling pathway is crucial for hair follicle immune escape and inflammation.[29,30] The interaction of these signaling pathways intensifies the immune attack and inflammatory response to hair follicles, leading to a large number of hair follicles transitioning from anagen to catagen and telogen, and hair follicle miniaturization.

These findings also suggest that the immune system of children, still in the process of development and maturation, may lead to more active or abnormal immune responses to self-tissues. The highly active state of the immune system in children may lead to a more vigorous attack on hair follicles, triggering a more significant inflammatory response in pediatric AA. Currently, several JAK inhibitors, including tofacitinib,[16] ruxolitinib,[12,14] and baricitinib,[29] are used to treat AA. Our data provide a foundation for treating pediatric AA with JAK inhibitors.

Down-regulated genes are primarily enriched in the positive regulation of ATPase activity, intermediate filament organization, epithelial cell differentiation, hair follicle development, and the Wnt signaling pathway. The down-regulation of these pathways may reflect the suppression of hair follicle growth and regeneration ability in AA patients, potentially due to the inflammatory environment caused by autoimmune responses or inherent defects in signaling pathways caused by genetic factors.[31,32]

Cell Infiltration Analysis

A previous study on non-cicatricial alopecia found that $CD103^+CD69^+$TRM (tissue-resident memory T cells) cells were up-regulated in the skin lesions of AA patients. Similar to what this study suggested that TRM cells may be involved in AA recurrence,[33] our Cell infiltration analysis also well corroborates the heightened immune-mediated inflammatory response in pediatric AA patients compared to adult AA. In our study, various immune cells infiltrate more prominently in pediatric AA compared to adult AA, especially in the pediatric patient subgroup. Significant immune infiltration characterized by increased B cells and CD4 cells and mast cell proliferation in the affected scalp area is one of the key findings of our study. B cells play a crucial role in maintaining autoimmune responses through antigen presentation and autoantibody production. When re-encountering the same antigen, B cells can rapidly proliferate, differentiate into effector cells, and quickly initiating an immune response. $CD4^+$ T cells can attract other immune cells (such as $CD8^+$ T cells, macrophages, and natural killer cells) in the hair follicle area by secreting cytokines such as IFN-γ, and chemokines, which enhancing the immune attack on hair follicles. Mast cells in AA patients' skin lesions exhibit a pro-inflammatory phenotype, promoting the inflammatory response through degranulation and increased contact with $CD8^+$

T cells.[34] This could explain why pediatric AA often presents as a more severe course and higher recurrence rate. The strong immune response is reflected by the severity of the disease, and B cell infiltration may be an immunological manifestation of this severity.

IgE Levels and Immune Response

Approximately one-third of individuals with severe alopecia areata (AA) exhibit elevated IgE levels, a phenomenon well-documented in the literature[17.] Atopic predisposition manifests in two primary forms: external (extrinsic) and internal (intrinsic).[35–37] External atopic predisposition is commonly characterized by heightened IgE levels and a medical history inclusive of allergic conditions such as asthma, allergic rhinitis, and eczema.[36] Conversely, internal atopic predisposition presents with normal IgE levels despite the presence of atopic symptoms.[37] In our investigation, we stratified AA patients based on their IgE levels, distinguishing between those with elevated IgE (reflecting external atopic predisposition) and those with normal IgE (indicative of internal atopic predisposition). Our genomic analysis unveiled a notable enrichment of the IFN-γ pathway in adult AA patients exhibiting elevated IgE levels, as opposed to the anticipated inflammatory pathway activation. This intriguing finding suggests that heightened IgE levels may not necessarily exacerbate the inflammatory cascade in adult AA patients. Furthermore, our assessment of immune cell infiltration patterns revealed no significant alterations in the adult AA cohort with elevated IgE, implying that elevated IgE levels may not directly correlate with heightened inflammation but rather with the modulation of specific immune regulatory pathways.

Normally, the elevation of IgE is associated with Th2-type immune responses, while IFN-γ is a key cytokine of Th1-type responses. Ideally, there should be a balance between Th1 and Th2 responses, but this balance may be disrupted in certain disease states.[26,38] Our sequencing results showed that the inflammatory pathway was not enriched in adult AA patients with high IgE but in the interferon-γ (IFN-γ) pathway. This suggests that the pathogenesis of AA is complicated by involving multiple immune pathways and factors. In addition, the elevation of IgE levels may not directly reflect the degree of inflammation, but may be related to specific immune regulatory pathways.

Pediatric AA with High IgE

In pediatric AA patients with high IgE (external atopic predisposition), we found up-regulated inflammatory immune pathways and IFN-γ signaling, in addition to a significant increase in CD4 cells, B cells, mast cells, and other infiltrations compared to adult AA patients with high IgE. These findings suggest that the balance between Th1 and Th2 may be disrupted in pediatric AA patients with high IgE, leading to the simultaneous up-regulation of inflammatory immune responses. The increase in immune cell infiltration in pediatric AA patients may reflect the severity and progression of the disease. This may mean pediatric AA patients need earlier or more aggressive treatment interventions. In contrast, pediatric AA patients with normal IgE levels (internal atopic predisposition) also showed significant immune cell infiltration, but the patterns were different. The immune response in these patients was characterized by a more balanced Th1/Th2 response, indicating that internal atopic predisposition may lead to a different immune regulatory mechanism compared to external atopic predisposition. This suggests that the underlying immune mechanisms in pediatric AA may vary depending on the type of atopic predisposition, which could have implications for targeted therapies.

Rationale for Choosing IgE Levels: The decision to categorize AA patients based on their IgE levels stems from the well-documented association between elevated IgE levels and allergic diseases.[19,24,25] Numerous studies have indicated that individuals suffering from AA frequently display elevated levels of IgE, suggesting that allergic reactions could potentially instigate the disease's onset and recurrence.[19] By segregating patients into groups with elevated and normal IgE levels, we aimed to elucidate the role of IgE-mediated immune responses in the pathogenesis of AA. This stratification allows for a more nuanced understanding of how IgE levels influence disease severity, immune cell infiltration, and gene expression profiles.

Limitations

While our study contributes significantly to the understanding of the impact of age and IgE levels on AA pathogenesis, it is essential to recognize and address several limitations. Firstly, the exclusive focus on IgE levels may present an oversimplified view of AA's pathogenesis. Future research endeavors should aim to incorporate additional biomarkers

and clinical parameters, such as specific IgE levels, comprehensive atopic history, and other immune markers, to offer a more nuanced perspective on the disease. Longitudinal studies that track IgE levels alongside disease progression over time would provide valuable insights into the dynamic role of IgE in AA development. Moreover, the study's limitations encompass the inadequate sample size in subgrouping and the oversight of considering the acute and chronic disease trajectories of the patients. To mitigate these limitations in forthcoming investigations, we intend to expand the sample size and integrate the patients' disease progression profiles into our analytical framework. This approach will facilitate a more robust and comprehensive evaluation of the relationship between IgE levels and AA pathogenesis, offering a more detailed understanding of the disease mechanisms.

Conclusion

This study represents a comprehensive investigation into the pathophysiology of AA across diverse age groups with varying levels of IgE. Through RNA sequencing of patient samples, we have identified key differences in the immune response mechanisms between pediatric and adult AA patients.

The findings underscore a pronounced immune-mediated inflammatory response in pediatric AA with elevated IgE levels, characterized by an upregulation of genes in pathways such as ERK1/2, IFN-γ, JAK-STAT, and TNF signaling. This aberrant activation suggests a disruption in the hair follicle growth cycle and an intensified inflammatory state, particularly in pediatric cases. The data indicates that the immature immune system in children may contribute to more robust and atypical responses to self-antigens, leading to aggressive hair follicle targeting. These insights provide a molecular basis for the observed clinical severity in pediatric AA and highlight the potential of JAK inhibitors as a therapeutic strategy.

In conclusion, our study elucidates the significant impact of IgE levels and age on the pathogenesis and progression of AA. By integrating gene expression profiles and immune cell infiltration patterns, we have provided a deeper understanding of the molecular mechanisms underlying AA. These findings pave the way for age-specific and allergy-related therapeutic targets, offering new avenues for personalized treatment strategies in AA.

Abbreviations

AA, Alopecia areata; IgE, immunoglobulin E; IFN-γ, Interferon-gamma; IHC, Immunohistochemistry; HE, Hematoxylin and Eosin; GSEA, Gene Set Enrichment Analysis; SALT, Severity of Alopecia Tool.

Data Sharing Statement

The accession number for the RNA sequencing data reported in this paper is GEO: GSEXXX.

Ethics Approval

The study, examining human subjects with AA and matched controls, was conducted with the approval of the Institutional Review Board of the Dermatology Hospital, Southern Medical University (approval no. 2023129). Written informed consent has been obtained from their parents or legal guardians for all minor patients participating in the study. Prior to participation, informed consent was obtained from all subjects, ensuring ethical compliance and participant understanding of the study's scope and potential implications. This study adheres to the ethical standards of the Declaration of Helsinki and has been approved by the relevant ethics committees.

Acknowledgments

We would like to thank all the doctors and nurses in the Department of Hair Diseases, Dermatology Hospital, Southern Medical University for their support of this study. The study was funded by the Hair Group's Specialized Disease Construction Fund. At the same time, we would like to thank Prof. Jun Liu, Prof. Yingping Xu, Prof. Jiajian Zhou and Prof. Zhili Rong for their guidance.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically

reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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