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Alopecia areata is characterised by dysregulation in systemic type 17 and type 2 cytokines, which may contribute to disease-associated psychological morbidity.

Short title: Alopecia areata and cytokine dysregulation


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What’s already known about this topic?
- NKG2D^+ CD8^+ T cells cause hair loss in alopecia areata (AA) but the immunological mechanisms underlying the disease are not fully understood
- AA is associated with changes in levels of IL-6, TNF, IL-1β and type 17 cytokines
- Psychiatric comorbidity is common amongst people with AA

What does this study add?
- People with AA have increased plasma levels of type 2 cytokines, IL-33, IL-31 and IL-17E/25, in addition to type 17 cytokines, IL-17A, IL-21, IL-23, IL-17F
- Levels of IL-17E and IL-22 positively predict depression score

What is the translational message?
- AA is associated with increased levels of multiple inflammatory cytokines, implicating both type 17 and type 2 immune pathways
- Our data indicate that therapeutic strategies for treating AA may need to address the underlying type 17 and type 2 immune dysregulation, rather than focussing narrowly on the CD8^+ T cell response
- An immunological mechanism might contribute directly to the depression observed in people with AA

ABSTRACT
Background Alopecia areata (AA) is a common autoimmune disease, causing patchy hair loss that can progress to involve the entire scalp (totalis) or body (universalis). CD8^+ NKG2D^+ T cells dominate hair follicle pathogenesis, but the specific mechanisms driving hair loss are not fully understood.

Objectives To provide a detailed insight into the systemic cytokine signature associated with AA, and assess the association between cytokines and depression.

Methods Multiplex analysis of plasma cytokines from AA patients, psoriatic arthritis (PsA) patients and healthy controls. We also assessed incidence of depression and anxiety using the Hospital Anxiety and Depression Scale.

Results Our analysis identified a systemic inflammatory signature associated with AA, characterised by elevated levels of IL-17A, IL-17F, IL-21 and IL-23 indicative of a type 17 immune response. Circulating levels of the type 2 cytokines IL-33, IL-31 and IL-17E/25 are also significantly increased in AA. In comparison to PsA, AA was associated with higher levels of IL-17F, IL-17E and IL-23. We hypothesised that circulating inflammatory cytokines may contribute to wider comorbidities associated with AA. We assessed psychiatric comorbidity in AA using the Hospital Anxiety and Depression Scale and found that 18% and 51% of people with AA experienced symptoms of depression and anxiety, respectively. Using linear regression modelling, we identified that levels of IL-22 and IL-17E are positively and significantly associated with depression.

Conclusion Our data highlight changes in both type 17 and 2 cytokines, suggesting that complex
systemic cytokine profiles may contribute both to the pathogenesis of AA and to the associated depression.

INTRODUCTION

Alopecia areata (AA) is a polygenic autoimmune disease characterised by patchy hair loss that can progress to affect the entire scalp (totalis) and body (universalis). AA is one of the most common autoimmune diseases, with a lifetime incidence of 2.1% and prevalence of 0.1 - 0.2% 1,2. AA develops in genetically predisposed individuals, usually with no identifiable trigger factor, although viral infection, vaccination and psychological stress have been reported in association with disease onset 3-5. Hair loss caused by AA is sometimes considered a cosmetic problem, despite being associated with significant psychological distress 6 and increased risk of developing inflammatory co-morbidities 7. Treatment options for AA include topical, intralesional or systemic steroids or contact-sensitisers, but their efficacy is limited and treatment is commonly ineffective for extensive AA 8.

Human genetic, and functional studies using model organisms, have identified pathways critical for AA development, implicating a role for CD8+ T cells and IFN-γ in mediating hair follicle (HF) damage. These data have led to clinical studies targeting the JAK-STAT pathway 9. JAK inhibitors, ruxolitinib and tofacitinib, have demonstrated promising results in promoting hair regrowth (>50%) in 58-75% of patients 10-12. Unfortunately, treatment response is variable and successful regrowth is often reversed following cessation of treatment. Furthermore, study participants may develop infections of the skin, upper respiratory tract or urinary tract. Whilst these data indicate the integral role of JAK-STAT-dependent cytokines in AA, they also exemplify the risks of broadly targeting the immune system, thus highlighting the urgent need for identifying pathways specific to AA.

IFN-γ and IL-15 are crucial for potentiating the activity of CD8+ T cells and natural killer cells in AA 9,13. Concentrations of circulating IL-6, TNF, L-17A, IL-21, IL-22 and IL-23 are elevated in patients with AA 14-17. In the skin, molecular profiling reveals dysregulation in cytokines associated with type 1 and 2 inflammation, whilst also indicating altered expression of IL-23 18. These data and those based on other skin inflammatory disease indicate the central role of cytokines in driving skin pathology 19,20. Furthermore, evidence from a range of autoimmune diseases indicate that inflammatory cytokines are implicated in wider co-morbidities, impacting vascular, metabolic and psychological pathways 21,22.

AA is highly associated with incidence of psychological comorbidities. Between 60 – 70% of people with AA will develop a psychiatric condition 6,23-25. Depression and anxiety are common, as well as death by suicide amongst individuals affected by AA 26. Depressive symptoms are increased in individuals with a number of other inflammatory conditions, including rheumatoid arthritis (RA) 22, inflammatory bowel disease (IBD) 27 and psoriasis 28, which has led to studies exploring the casual link between inflammation and its effect on the brain 29. While it has long been recognised that peripheral inflammation induces sickness behaviour, there is more recent and increasingly compelling data implicating immune-mediated mechanisms in depression 30. Specifically, IL-1β, TNF and IL-6 have been strongly associated with depression and this relationship has now been the subject of several meta-analyses 31-34. Further, antagonism of cytokines such as TNF are currently

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being investigated as a therapeutic tool in tackling treatment resistant depression in a number of randomised controlled trials globally.\textsuperscript{35,36}

We sought to fully characterise the AA cytokine signature by simultaneously measuring type 17, type 2 and pro-inflammatory cytokines in a cohort of AA patients and healthy controls. We hypothesised that deeper analysis of cytokines in people with AA would indicate and confirm the complexity of the AA cytokine signature. We also hypothesised that depression is prevalent in this cohort of people with AA, and may be associated with exposure to circulating pro-inflammatory cytokines.

\textbf{PATIENTS & METHODS}

\textbf{Study participants}
Volunteers with AA were recruited to the Queen Elizabeth University Hospital, Glasgow. Eligible participants did not have a diagnosis of additional inflammatory disease (e.g. IBD, RA, psoriatic arthritis (PsA), psoriasis, ankylosing spondylitis). All participants provided written informed consent in line with research ethics committee approval (West of Scotland REC 1, 17/WS/0029) and ICH good clinical practice. Volunteers with PsA were recruited as controls under separate ethics at the Universities of Manchester and Glasgow. Age and sex-matched healthy control (HC) volunteers were recruited under ethics approved by the College of Medical, Veterinary and Life Sciences Ethics Committee at the University of Glasgow.

\textbf{Cytokine analysis}
Peripheral blood was collected into EDTA coated vacutainers (BD, USA). Plasma was isolated by centrifugation and stored at -80°C prior to analysis. Plasma concentrations (pg/mL) of IFN-\(\gamma\), IL-1\(\beta\), IL-6, IL-8, IL-10, IL-12p40, TNF, IL-17A, IL-17E/IL-25, IL-17F, IL-21, IL-22, IL-23, IL-31, IL-33, IL-27 were determined using U-plex multiplex assay platforms (Meso Scale Discovery, USA).

\textbf{Clinical data}
Depression and anxiety experienced by AA patients was measured using the Hospital Anxiety and Depression Scale (HADS) questionnaire\textsuperscript{37}. HADS scores were interpreted as no depression/anxiety (0-7), mild (8-10), moderate (11-14) and severe (15-20). Severity of Alopecia Tool (SALT)\textsuperscript{38} scores were calculated to assess severity of hair loss and patients were stratified accordingly; inactive disease (S0), patchy hair loss (S3\(\geq\) S4a), severe hair loss (S4b\(\geq\) S5, alopecia universalis and alopecia totalis).

\textbf{Linear regression model}
A linear regression model was fitted, \(Y_i = B_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \epsilon_i\), where \(Y_i\) represents depression or anxiety score. Leaps package was used to identify the top 10 subsets of explanatory variables \(X_i\) in the model by ranking the adjusted R\(^2\) value. The predictive accuracy of the model was assessed by performing 10-fold cross validation using CVlm() from R’s DAAD package, whereby data are randomly assigned to the number of folds, each fold is removed, and in turn, the remaining data is used to re-fit the regression model and to predict at the deleted observations.

\textbf{Statistical analysis}
Differences in cytokine levels were analysed by Mann-Whitney U-test for single comparisons (Fig. 1) or Kruskal-Wallis test for multiple comparisons, followed by a Dunn’s multiple comparison test to
identify which groups were significantly different, and to generate p-values. (Fig. 2, Fig. S1) (*p<0.05, **p<0.01 ***p<0.001, ****p<0.0001). Undetected cytokine levels were reported as zero. All data were analysed using GraphPad Prism (San Diego, USA).

RESULTS

Study participants
Demographics of study participants are described in Table 1. Thirty-nine AA patients were enrolled, of whom 18 had patchy AA (SALT: S1≥ S4a), 17 had severe disease (SALT: S4b≥ S5) and four were in remission/inactive. Twenty patients were atopic and six had thyroid-related conditions. Seventeen AA participants were receiving treatment at time of enrolment (Table 2). Twenty-three PsA patients were enrolled under separate ethics from a cross-sectional study of whom none were receiving biologic treatment.

Circulating levels of proinflammatory cytokines are increased in AA
We first measured pro-inflammatory cytokines in AA patients and healthy controls (Fig. 1). Plasma concentration of IL-1β (0.09 pg/ml, 95% CI 0.08 – 0.10), IL-6 (0.76 pg/ml, 95% CI 0.59 – 0.94), TNF (1.03 pg/ml, 95% CI 0.63 – 1.44), and IL-10 (0.38 pg/ml, 95% CI 0.15 – 0.61) were significantly elevated in AA patients in comparison to HCs (0.08 pg/ml 95% CI 0.05 – 0.1 IL-1β, 0.48 pg/ml 95% CI 0.36 – 0.59 IL-6, 0.70 pg/ml 95% CI 0.53 – 0.87 TNF, 0.27 pg/ml 95% CI 0.03 – 0.5 IL-10, P < 0.05).

Whereas IL-8 was significantly reduced (P < 0.5) in AA (3.37 pg/ml, 95% CI 2.997 – 3.75) compared to HCs (4.28 pg/ml, 95% CI 3.5 – 5). Despite previous reports, we observed no change in the levels of IFN-γ between AA patients and HCs, likely due to the high IFN-γ expression observed in a small number of our HC samples. In addition, no change was observed in the levels of IL-15 or IL-12p40 between AA patients and HCs.

Circulating levels of type 17 cytokines are increased in AA
We also found increased levels of IL-17A (1.57 pg/ml, 95% CI 1.22 – 1.92), IL-17F (254 pg/ml, 95% CI 198 – 311) and IL-21 (7.28 pg/ml, 95% CI 1.29 – 13.3) when compared with HCs (1.15 pg/ml 95% CI 0.53 – 1.76 IL-17A, 225 pg/ml 95% CI 65 – 384 IL-17F, 2.53 pg/ml 95% CI 0.85 – 4.2 IL-21, P < 0.05, Fig. 2). The concentration of IL-23 (1.8 pg/ml, 95% CI 1.1 – 2.5) in AA plasma compared to HCs (0.67 pg/ml, 95% CI 0.24 – 1.1) was also significantly increased. We compared these data with the levels of type 17 cytokines observed in PsA, a disease known to be driven by the IL-23/IL-17 axis on the basis of effective clinical cytokine-specific blockade. Mean concentration of IL-17A (1.95 pg/ml, 95% CI 1.13 – 2.78) in PsA plasma was modestly higher than in AA (1.57 pg/ml, 95% CI 1.22 - 1.92) but this difference was not significant (P < 0.05, Fig. 2). However, we did observe a higher concentration of IL-17A in a proportion of patients. Levels of IL-17F and IL-23 were not significantly increased in PsA in comparison to HCs.

We stratified AA participants to determine whether this cytokine signature is apparent in those who are not receiving treatment (Table 2). When compared to HCs, participants receiving no treatment also had significantly increased levels of IL-17A, IL-21 and IL-23 (data not shown). IL-17F was not significantly different between AA patients receiving no treatment and HCs.

We assessed the levels of type 2-associated cytokines. Plasma concentrations of IL-33 (0.57 pg/ml, 95% CI, 0.27– 0.87), IL-31 (18.7 pg/ml, 95% CI, 15.86 – 21.58) and IL-17E/25 (8.39 pg/ml, 95%...
CI, 6.28 – 10.49) were significantly higher in AA patients than in HCs (0.28 pg/ml 95% CI 0.12 – 0.44 IL-33, 10.42 pg/ml 95% CI 5.03 – 15.81 IL-31, 5.9 pg/ml 95% CI 1.94 – 9.86, P < 0.05, Fig. 2). Similar results were obtained when cytokine concentrations were compared between samples obtained from HCs and people with AA that were not receiving treatment. Group sizes of AA patients receiving specific treatments were too small to evaluate for significant differences from control samples. Type 2 cytokines were not found to be elevated in PsA plasma samples.

We hypothesised that the type 2 cytokine signature may be attributed to history of atopy amongst AA patients, however comparison of type 2 cytokine concentrations between atopic and non-atopic AA patients did not reveal any significant differences (data not shown), indicating that both atopic and non-atopic individuals in our cohort are experiencing cytokine dysregulation, evidenced by their high levels of both type 17 and type 2 cytokines.

**Depression and AA**

Depression and anxiety were assessed using the HADS questionnaire. 18% recorded scores that are associated with depression (8% mild, 3% moderate, and 8% severe) (Fig. 3a) and 51% experienced symptoms of anxiety (Fig. 3b). We then stratified AA patients based on disease severity to determine whether the extent of hair loss correlated with the incidence of depression or anxiety. A higher proportion of patients with patchy hair loss (SALT: S1 ≥ S4a) scored above the threshold for depression; 17%, 6% and 11% scored within mild, moderate and severe ranges, respectively (Fig. 3a). 55% of those with patchy hair loss experienced anxiety (Fig. 3b). Conversely, patients with severe hair loss (S4b ≥ S5, totalis or universalis) experienced depression and anxiety less frequently; one participant was experiencing both severe anxiety and depression, whereas the other participants were not depressed and six recorded signs of mild anxiety. We also assessed whether duration of disease played a role in the incidence of depression amongst AA patients. People with patchy hair loss and with disease onset ≤ 10 years had the highest burden of depression and anxiety. 13% showed severe depression, 13% showed moderate depression, 25% showed mild depression and 50% were not affected by depression. Strikingly, 75% of people in this cohort (patchy hair loss and with disease onset ≤10 years) experienced anxiety.

To test the relationship between depression and circulating cytokine levels we performed subset linear regression (Fig. S1b, summarised in Fig. S1a). IL-22 significantly and positively predicted depression score across 10-fold subset validations (P < 0.01). IL-17E also significantly predicted depression score, in 6 out of 10-fold cross validations. We assessed whether levels of IL-22 and IL-17E were also associated with hair loss severity or disease duration. Comparisons between disease groups did not reveal any relationship between levels of these cytokines and disease duration. However, levels of IL-17E were higher in those with severe hair loss (SALT score >95) than in HCs (Figure S1).

Linear regression analysis also revealed a significant negative association between anxiety and both disease duration and severity of AA (SALT score). Participants’ age was not correlated with levels of depression or anxiety.

**DISCUSSION**

Multiplex cytokine analysis of AA patient plasma revealed a significant type 17 cytokine signature, characterised by an increase in IL-17A, IL-17F, IL-21 and IL-23. To understand the biological
significance of this result, we compared these data with cytokine levels observed in PsA patients. PsA is driven by the IL-23/IL-17 axis and these cytokines directly promote keratinocyte hyperproliferation, contributing to plaque formation in the skin 40. Levels of IL-17A were similar between AA and PsA, however a proportion of PsA patients had a higher concentration. These data and others 15,18,41 indicate that type 17 cytokines are altered in AA. Clinically, IL-17A blockade has failed to demonstrate efficacy 42. In contrast, therapeutically targeting the IL-12/23 p40 subunit has been reported to be effective in promoting hair regrowth 43, indicating that these pathways may operate independently in AA.

Interestingly, AA patients also had increased circulating levels of Th2-associated cytokines, IL-17E/IL-25, IL-33 and IL-31. To our knowledge, these findings have not been reported in serum of AA patients. IL-33 is an alarmin protein released by inflamed tissue 44, while IL-31 is expressed by multiple immune cells. Both are strongly associated with allergic disease 45. Notably, expression of IL-31 is increased in dermatitis skin sections in comparison to AA 46. IL-31 is a member of the IL-6 family and mediates its downstream effects via JAK-STAT signalling 47. Due to the therapeutic efficacy of JAK inhibitors in AA, we suggest that IL-31 may play a role in promoting inflammation against HFs 10. Transcriptome analysis of AA biopsies implicates multiple type 2-associated molecules, supporting the involvement of a type 2 response 18. Further, IL-13 is a susceptibility locus associated with AA that is strongly linked to atopic diseases 48.

Additionally, we identified increased levels of IL-1β, IL-6 and TNF. These findings are consistent with other studies 19, however we did not observe changes in IFN-γ or IL-12. IL-6 acts in synergy with TGF-β to promote differentiation of IL-17+ Th17 cells 49. AA patients also have elevated levels of anti-inflammatory IL-10. Heightened IL-10 is also associated with other autoimmune diseases 50,51, and may here indicate an attempt to regulate HF-associated inflammation.

We observed depression and anxiety in 18% and 51% of participants, respectively. Meta-analysis of studies assessing incidence of depression within the general population reveals a point prevalence of 12.9% 52. Psychiatric comorbidity and inflammatory disease are increasingly considered to be connected 22,27,53,54. Pro-inflammatory proteins, such as TNF, can influence neural pathways involved in neurotransmitter expression, such as glutamate 55. The putative mechanism here is via the upregulation of the enzyme indoleamine 2,3-dioxygenase (IDO). Ultimately, this leads to increased levels of downstream products, such as quinolinic acid, which is glutamatergic, neurotoxic and thus damaging to neural cell function 56.

We hypothesised that cytokines may contribute to driving depression in AA. Linear regression revealed that IL-22 and IL-17E were positively and significantly associated with depression score. IL-17E/IL-25 is produced by innate cells and keratinocytes, and promotes generation of Th2 cells 57, whereas IL-22 is produced by Th17 cells. An association between depression and IL-17E has not previously been reported. However, other cytokines of the IL-17 family are associated with depression in other cohorts, including in patients with psoriasis, and in animal models of disease. Notably IL-17RA, which binds both IL-17A and IL-17E, is expressed by neural cells in humans during inflammation 58. IL-22 has also not been previously associated with depression, but its receptor has been detected on brain endothelial cells 58. IL-22 has also been implicated in neuroinflammatory diseases, such as multiple sclerosis (MS) 59 and Guillain-Barré syndrome 60. Depression is common in MS patients and is considered to be a consequence of the pathogenic mechanisms causing MS 61.

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Our data do not enable us to determine whether there is a direct or indirect relationship between cytokines and depression; it is possible that the correlation occurs because changes in cytokines and depression are caused by similar extrinsic factors.

We did not observe any association between these cytokines and disease duration, but did identify raised IL-17E in participants with severe disease (SALT score >95) (Figure S1). The levels of IL-17E and IL-22 vary widely between individuals, and the largest group within our AA cohort are in the severe hair loss group. The large size of this group gives sufficient statistical power to identify the difference in IL-17E levels from the HCs. However, we are unable to identify a clear relationship between cytokine levels, depression, and disease severity. In future, analyses could be performed with larger cohorts to elucidate these relationships.

Aside from psychological issues, people with AA are often considered to be ‘healthy’. People with AA do not report pain or fatigue that characterise many other chronic inflammatory conditions. The general good health of people with AA suggests that HF-associated inflammation does not extend from the tissue. RA, for instance, is known to be associated with extra-articular manifestations that may affect the lungs, heart and eyes. Paradoxically, we and others have demonstrated elevated levels in type 1, type 17 and type 2 cytokines both in the tissue and circulation of individuals with AA. We propose that the elevated circulating cytokines of these individuals provides a plausible link between pathology in the skin and wider tissue involvement.

We have identified a robust type 17 signature, and report novel data highlighting changes in type 2 cytokines. These data complement previous studies whilst providing further insight into the multifactorial cytokine profile associated with AA. We suggest that, in addition to contributing to the pathogenesis of hair loss, peripheral inflammation may be a key contributor to the increased incidence of depression. Future clinical trials of immune-modulating therapies for AA should measure depression, to evaluate potential additive benefits of combatting AA-associated psychological co-morbidities.

ACKNOWLEDGEMENTS
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**TABLES**

**Table 1.** Demographics of alopecia areata, psoriatic arthritis and healthy control participants

<table>
<thead>
<tr>
<th>ALOPECIA AREATA</th>
<th>PSORIATIC ARTHRITIS</th>
<th>HEALTHY CONTROLS</th>
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</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
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<td>23</td>
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<tr>
<td><strong>Sex F/M</strong></td>
<td>30/9</td>
<td>16/7</td>
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<tr>
<td><strong>Average age (SD)</strong></td>
<td>Male 37 (10.2)</td>
<td>Male 43 (9.2)</td>
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<tr>
<td></td>
<td>Female 45 (13.1)</td>
<td>Female 40 (14.4)</td>
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<td><strong>Mean duration of disease (range)</strong></td>
<td>16.6 years (1-55)</td>
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</table>

**Table 2.** Current treatment information for AA participants

<table>
<thead>
<tr>
<th>No. of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No treatment</strong></td>
</tr>
<tr>
<td><strong>Intralesional steroids</strong></td>
</tr>
<tr>
<td><strong>Diphenycprone (DCP)</strong></td>
</tr>
<tr>
<td><strong>Methotrexate</strong></td>
</tr>
</tbody>
</table>
**Figure Legends**

**Figure 1. Inflammatory plasma cytokine levels.** Cytokines (pg/mL) were measured by multiplex assay from alopecia areata (AA) participants and healthy controls (HCs). Black dotted line represents lower limit of detection for each assay. Mann-Whitney U-test, *P<0.05, **P<0.01.

**Figure 2. Type 17 and 2-associated plasma cytokine levels.** Cytokines (pg/mL) were measured by multiplex assay from alopecia areata (AA) participants, psoriatic arthritis (PsA) participants and healthy controls (HCs). Black dotted line represents lower limit of detection for each assay. Kruskal-Wallis test followed by a Dunn’s multiple comparison, *P<0.05, **P<0.01.

**Figure 3. Hospital Anxiety and Depression Scale (HADS) scores.** The HADS questionnaire was used to assess the extent of depression and anxiety experienced by alopecia areata (AA) patients. (a) Depression scores for total AA participants (n=39), participants with patchy hair loss (n=18), participants with disease onset <10 years (n=8), participants with severe hair loss (AT/AU) (n=17). (b) Anxiety scores for total AA participants (n=39), participants with patchy hair loss (n=18), participants with disease onset <10 years (n=8), participants with severe hair loss (AT/AU) (n=17). HADS scores were considered as no depression/anxiety (0-7), mild (8-10), moderate (11-14) and severe (15-20).

**Table S1. Subset linear regression analyses of alopecia areata circulating cytokine concentration and Hospital Anxiety and Depression scale scores.**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>β-coefficient</th>
<th>Mean Squared Error (MSE)</th>
<th>Adjusted R²</th>
<th>Predictor Influence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-17E</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IL-22</strong></td>
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</tr>
</tbody>
</table>

Predictors that fail to be selected in the model are represented as an empty cell. Significant predictors that positively/negatively influence depression and anxiety scores are highlighted in red and blue. A predictor consistently selected in multiple subsets with consistent positive influence serves as an important predictor.

We have used plot_summs() function from R’s jtools package to summarize the results of subset analyses by using it to draw rescaled normal distributions of the β-coefficients for all the subsets together.

**Figure S1. Levels of IL-17E and IL-22 in AA plasma stratified by hair loss severity.** Cytokines (pg/mL) were measured by multiplex assay from alopecia areata (AA) participants and healthy controls (HCs). AA participants were stratified based on SALT scoring, into four groups: inactive, <50% patchy, >50% patchy and total loss (>95% SALT). Black dotted line represents lower limit of detection for each assay. Kruskal-Wallis test followed by a Dunn’s multiple comparison, *P<0.05, **P<0.01.
Figure 1.
Figure 2.
Figure 3.