nature portfolio

Peer Review File

Paired blood-skin transcriptomic profiling during Dupilumab treatment of SPINK5-FLG-mutated Netherton Syndrome.

Corresponding Author: Professor Catalina Sanz

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Netherton syndrome (NS) is characterized by a triad of clinical manifestations - ILC, atopy, bamboo hair (Tricorrhexis invaginata, TI) - and is inherited as an autosomal recessive trait. I was wondering whether the two siblings here studied are affected by NS. In fact, they carry a heterozygous SPINK5 mutation and do not show TI. The lack of these features calls the NS diagnosis into question. Authors claim that a gene-gene modulated continuum of NS/atopic dermatitis/ichthyosis vulgaris severity could affect these siblings. However, true digenic inheritance in NS (disease manifests when both SPINK5 and FLG genes carry heterozygous variants) has never been reported. Rather, the two papers that the Authors cited as refs 6 and 7 describe individuals affected by NS caused by biallelic SPINK5 mutations, with a phenotype that is exacerbated by a heterozygous FLG variant. It is possible that your siblings have a severe atopic dermatitis (AD) or ichthyosis vulgaris (IV, which is inherited as semidominant trait with variable penetrance) aggravated by a heterozygous SPINK5 variant. You may therefore be treating AD/IV patients rather than NS patients with dupilumab.
To support NS diagnosis, Authors must examine LEKTI expression (which should be absent or significantly reduced) in a patient skin biopsy using immunohistochemistry. Alternatively, they could consider performing a genomic investigation to unmask the second recessive SPINK5 allele in the form of large intragenic deletion/duplication/complex rearrangement using methods such as Multiplex Ligation-dependent Probe Amplification (MLPA), XON assay, or SOPHiA DDM. It is vital to the study that these checks are carried out.

Reviewer #2

(Remarks to the Author)
Cristina Bajo-Santos and collaborators report the analysis of two sibs affected by Netherton syndrome. After identifying heterozygous mutations in SPINK5 and FLG, they carried out blood and skin RNA-sequencing, before and after treatment with an IL4R blocker (dupilumab). This uncovered genes and pathways that are potentially modulated by the drug.

The results of this study are potentially interesting, but the presentation is often confused. In particular:

What is the difference between the protein structures reported in Fig 1 and Fig S1? Are WT and mutant LEKT1 in Fig S1 shown from the same angle? How was flexibility (pTM) calculated?
Mutant FLG structure is predicted at very low confidence, so comments on the impact of the mutation should be avoided as highly speculative.

The authors consistently refer to 18,205 differentially expressed genes (DEG), but their volcano plot and tables show a much smaller number.
The threshold for declaring differential expression is also unclear: is it FC>1.5 (Methods) or log2FC>1.5 (Supplementary Tables). If log2FC was used, then RN7SL4P is upregulated by 8,192-fold! (213; Table S3). What is the Bonferroni correction factor reported in Table S3?
In lines 132-135: the authors state “Twelve mRNAs were significantly upregulated in the pre-treatment (PreDup)state, whereas key mRNAs—including OLAH, IQGAP3, IGKV3-20, and miR-320C1—were significantly downregulated after treatment. In contrast, 40 mRNAs were significantly upregulated post-dupilumab (PostDup)”. What is the difference between upregulated pre-treatment and downregulated after treatment?

Are the correlations in lines 157-159 statistically significant?

The authors report an upregulation of lymphocyte migration pathways after dupilumab treatment (clusters 5 and 10). As this is a rather unexpected finding, they should list the DEG that are driving the enrichment signal.

The discussion is very long (nearly 6 pages) and some of the most speculative sections could be cut. Conversely a paragraph reporting the limitations of the study should be added. The authors should acknowledge the small size of their dataset and the relatedness between study participants. They should also consider the possibility that their results may have been influenced by the differential abundance of various leukocyte populations before after treatment (Table 1).

Minor
1. The ethnicity of study participants is not reported.
2. It is not clear whether this is the first case of NS caused by a single heterozygous mutation. If so, the authors should discuss the possibility that the two sibs carry a second SPINK5 mutation that could not be detected by whole-exome sequencing (e.g. a large deletion or non-coding variant).
3. The differential expression of FURIN is discussed at some length, but the authors do not mention the function of this gene. While a post-treatment increase in skin FURIN expression is reported

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have provided additional clinical findings that support the diagnosis of Netherton syndrome. Furthermore, they have conducted a more in-depth molecular SPINK5 analysis, ruling out complex rearrangement and identifying a compound heterozygous missense variation. As this missense variant (p. Glu748Gly) is categorised as 'benign strong" and has been found in homozygosity in two healthy individuals (gnomAD population database), it is still possible that the true pathogenic second variant has not been identified. Another possibility is that the compound heterozygosity for the identified SPINK5 variants combined with the FLG variant caused the NS phenotype.
While the study is worthy of publication, it still contains some ambiguities:
Minor concerns:
- Please state in the discussion that it is still possible that the true pathogenic second SPINK5 variant has not been identified, being the p.Glu748Gly substitution classified as benign.
- Please, delete the phrase "…heterozygous carriers generally exhibit milder phenotype" in the Discussion. Heterozygous carriers are healthy individuals. In fact, depending on the variant position within protein domains, compound heterozygous individuals usually show milder phenotypes than homozygous individuals (ref. 27).

I suggest adapting the first part of the discussion according to the above comments.

Reviewer #2

(Remarks to the Author)
The authors have addressed most of my queries and concerns. However, I remain confused by the description of >18,000 differentially expressed genes.
The volcano plot in Fig 3 seems to show a total of 18,205 genes. Of these, 52 (purple + blue dots) meet the authors' definition of differential expression ($log2FC>|1.5|$; $P<0.05$). There are a few more that do not reach the Fold Change threshold, but still yield a $P<0.05$ (grey dots above the horizontal dotted lines). The remaining genes cannot be considered differentially expressed, as they do not meet the significance threshold of $P<0.05$.

The Discussion is in my view too long, but I defer to editor's opinion on that.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed my minor concerns appropriately.

Reviewer #2

(Remarks to the Author)

Paired blood-skin transcriptomic profiling during Dupilumab treatment for SPINK5-FLG mutated NS syndrome

We want to thank the reviewers for taking the time to assess and improve our paper. Here, we provide a point-by-point response to the reviewer's comments.

Reviewer 1

We thank Reviewer 1 for highlighting the need for additional evidence supporting the diagnosis of Netherton syndrome (NS) in these patients. We agree that careful distinction from severe atopic dermatitis (AD) or ichthyosis vulgaris (IV) is essential.

Both patients were diagnosed in childhood (1994) and classified as having NS by expert dermatologists based on a characteristic clinical phenotype, including severe atopy, ichthyosis linearis circumflexa, and pronounced hair shaft abnormalities. The extent of hair involvement—marked hair fragility, severe alopecia, and near absence of eyebrows—is not typically encompassed within the clinical spectrum of isolated AD or IV, even when associated with a heterozygous *SPINK5* variant.

Regarding trichological findings, historical (non-digitized) medical records document the presence of trichorrhexis invaginata ("bamboo hair") at the time of the original diagnosis (Fig.1). In contrast, more recent hair shaft analysis identified pili torti. This difference likely reflects temporal variability in hair shaft morphology and/or differences in diagnostic approaches over time. Importantly, pili torti have been reported as part of the hair shaft abnormality spectrum in NS and therefore do not exclude the diagnosis (PMID: 24778540; 7648886**;** 40899446). We have revised the manuscript to explicitly clarify the historical and recent hair findings (Lines 93 -98).

We also acknowledge that interpreting the molecular data was challenging because a second *SPINK5* variant was not initially identified, introducing uncertainty during the early genetic evaluation of these cases. To address this, and following Reviewer 1's suggestion, we expanded the molecular analysis. Copy number variation analysis of *SPINK5* revealed no large deletions or duplications. Subsequent targeted

Sanger sequencing identified a c.2243A>G (p. E748G) variant on the second allele. Reanalysis of the whole-exome sequencing data confirmed the presence of this variant in both siblings. Although this variant has been reported in the literature as benign or of uncertain significance, its presence in compound heterozygosity, together with a heterozygous *FLG* mutation, may contribute to impaired skin barrier function and the severity of the observed phenotype.

Taken together, the clinical presentation, historical and recent hair shaft findings, and expanded genetic data support classifying these patients as having NS rather than AD or IV. The manuscript has been revised accordingly, including clarification of hair shaft abnormalities (Lines 93 to 98) and an updated description of the heterozygous *SPINK5* variant (Lines 31; 113–118; 127-129; 261 to 271 and Table 1).

Figure 1. Tricorrexis Invaginate (picture from one of the patients when she was a child)

Reviewer 2

1. What is the difference between the protein structures reported in Fig 1 and Fig S1? Are WT and mutant LEKT1 in Fig S1 shown from the same angle? How was flexibility (pTM) calculated? Mutant FLG structure is predicted at very low confidence, so comments on the impact of the mutation should be avoided as highly speculative.

We thank the reviewer for these important clarifications and fully agree with the need to avoid speculative interpretation when structural confidence is limited.

Figure 1 presents the predicted three-dimensional structure of filaggrin (FLG) rendered as a Gaussian surface representation. In this figure, domains are colored according to secondary structure elements (α-helices and β-sheets) to illustrate the overall domain organization and highlight features such as the putative Ca^{2+}-binding region. In contrast, Supplementary Figure S1 displays the predicted structures with domains colored according to the AlphaFold-predicted local confidence score (pLDDT), which reflects per-residue reliability of the predicted atomic positions. Higher pLDDT values indicate greater local confidence, whereas lower values typically correspond to flexible, disordered, or poorly constrained regions.

With respect to orientation, wild-type and mutant LEKTI structures shown in Supplementary Figure S1 are displayed from the same angle. This is indicated by the coordinate axes shown in the lower corner of each image, allowing direct visual comparison between the two structures.

Regarding the pTM score, this metric is automatically generated by the AlphaFold3 prediction pipeline and represents the predicted TM-score, a global measure of confidence in the overall fold and domain arrangement rather than a descriptor of molecular flexibility. The pTM score estimates how closely a predicted structure would match a hypothetical experimentally determined structure, based on internally predicted alignment errors (PAE). It should therefore be interpreted as an indicator of expected global accuracy rather than intrinsic conformational dynamics.

We acknowledge that the use of the term “flexibility” in the original text may have caused confusion. We intended to describe the visually apparent and inferred large-scale conformational variability of the full-length FLG model, rather than local structural

uncertainty. To avoid misinterpretation, we have revised the manuscript to clarify this distinction and explicitly stated that the pTM score reflects only global model confidence (Lines 121–129).

We fully agree with the reviewer that the low confidence in the predicted FLG structure—particularly given the protein's repetitive nature and large size—precludes any definitive conclusions about the structural impact of specific mutations. Accordingly, we have removed speculative statements about the effects of mutations from the manuscript. The revised text now emphasizes that the FLG structural model should be considered exploratory and is presented solely as a conceptual framework, noting that, to our knowledge, no full-length structural model of filaggrin has been reported previously (Lines 121 and 135).

2. The authors consistently refer to 18,205 differentially expressed genes (DEG), but their volcano plot and tables show a much smaller number.

In Fig. 2A, it is shown the Volcano Plot with the total of 18,205 differentially expressed genes (n=18,205 bottom right of the figure) when we comparing between PreDup and PostDup; nevertheless, we set a threshold a -1.5 <log2FC>1.5 and a representative value of p-value <0.05; and therefore, from there onwards, we focused on those which are significantly over expressed (pink dots in Fig2A in our volcano plot) or downregulated (blue dots in volcano plot) or the specified genes in the tables based on our threshold.

3. The threshold for declaring differential expression is also unclear: is it FC>1.5 (Methods) or log2FC>1.5 (Supplementary Tables).

Thank you for spotting the typo. As correctly stated in the figure legend, the threshold was set at $-1.5 < \log_2$ fold change (FC) < 1.5, with a p-value < 0.05. This has now been corrected and clearly specified in the Methods section (line 497).

4. If log2FC was used, then RN7SL4P is upregulated by 8,192-fold! (213; Table S3). What is the Bonferroni correction factor reported in Table S3?

Regarding RN7SL4P, we confirm that the reported value corresponds to a log2fold change of 13, which mathematically translates to an approximately 8,192-fold increase when converted to a linear scale (2^{13}). We acknowledge that this value may appear striking; however, it reflects the logarithmic transformation applied during differential expression analysis rather than an isolated or amplified biological effect. Importantly, given the very limited sample size (two patients and two controls), such extreme fold-change estimates should be interpreted with caution. In small datasets, fold-change values can be disproportionately influenced by individual variability and low baseline expression levels, and therefore should be regarded as descriptive rather than definitive indicators of biological significance. We have added this as limitation in Lines 420-443.

5. What is the Bonferroni correction factor reported in Table S3?

Thank you for highlighting this. The *B* statistic corresponds to the log-odds of differential expression as implemented in the limma package as described in the Methods section (lines 492-494), and does not refer to Bonferroni correction. This was a labeling error on our part. The table legends have now been corrected accordingly, as reflected in Supplementary Tables 3 and 4.

6. In lines 132-135: the authors state “Twelve mRNAs were significantly upregulated in the pre-treatment (PreDup)state, whereas key mRNAs—including OLAH, IQGAP3, IGKV3-20, and miR-320C1—were significantly downregulated after treatment. In contrast, 40 mRNAs were significantly upregulated post-dupilumab (PostDup)”. What is the difference between upregulated pre-treatment and downregulated after treatment?

We recognize that the original wording of this paragraph may have caused confusion, as the transcripts described as upregulated prior to treatment are the same transcripts that are downregulated following treatment. To clarify this point and avoid ambiguity, we have rephrased the paragraph accordingly. The revised text can be found in lines 143–147.

7. Are the correlations in lines 157-159 statistically significant?

We thank the reviewer for this question. Yes, the Spearman correlation coefficients reported in lines 157–159 are statistically significant; in all cases, the associated p-values computed by the software were < 0.001. However, these p-values should be interpreted with caution, as the correlations were calculated across a large number of differentially expressed genes. In this high-dimensional context, statistical significance is largely driven by the number of ranked observations rather than by biological replication. Accordingly, we report the Spearman correlation coefficients primarily as descriptive measures of rank-based transcriptomic concordance to illustrate relative similarity between conditions, rather than as standalone evidence of biological effect size or causality. We have rephrased the relevant section discussing Spearman correlation values (Lines 165–174 and have additionally addressed this limitation in the Limitations section of the revised manuscript (Lines 420-443).

7. The authors report an upregulation of lymphocyte migration pathways after dupilumab treatment (clusters 5 and 10). As this is a rather unexpected finding, they should list the DEG that are driving the enrichment signal.

This information is presented in Supplementary Figure 2C as a CNET plot, while gene-specific log2 fold-change values for each comparison are provided in Supplementary Tables 3 and 4.

8. The discussion is very long (nearly 6 pages) and some of the most speculative sections could be cut. Conversely a paragraph reporting the limitations of the study should be added. The authors should acknowledge the small size of their dataset and the relatedness between study participants. They should also consider the possibility that their results may have been influenced by the differential abundance of various leukocyte populations before after treatment (Table 1).

We sincerely thank the reviewer for this constructive comment and for emphasizing the importance of balancing depth with conciseness in the Discussion. In response, we have shortened and revised the discussion to improve focus and transparency. Specifically, we have added a dedicated Limitations section that explicitly addresses key constraints, including the small cohort size, familial relatedness, the use of

bulk rather than single-cell transcriptomics, and the need for cautious interpretation of large effect sizes (Lines 420-443).

We acknowledge that the Discussion might remain still relatively extensive. However, we deliberately retained an integrative discussion for several reasons consistent with the scope and aims of *Communications in Medicine*. First, this study examines a rare, intensively phenotyped cohort with paired skin and blood transcriptomic profiling in Netherton syndrome associated with combined *SPINK5–FLG* variation. Such datasets are exceedingly limited, and we believe that a detailed integrative framework is valuable for contextualizing these findings within existing biological and translational knowledge.

Second, the revised Discussion is structured to transparently link observed pathway-level patterns to established mechanisms of epidermal barrier dysfunction and type 2 inflammation, rather than to infer definitive causality. Given the translational focus of *Communications in Medicine*, we felt it was important to articulate how these molecular signatures may inform future mechanistic investigations and therapeutic stratification in rare and genetically complex diseases.

Finally, as biologic treatment responses in genetically defined Netherton syndrome remain poorly characterized, we believe that retaining an integrative discussion across tissues and pathways enhances the manuscript's value as a resource for clinicians and researchers working at the interface of dermatology, immunology, and precision medicine.

We hope that these revisions adequately address the reviewer's concerns while preserving the conceptual and translational relevance of the work in line with the mission of *Communications in Medicine*.

Minor

1. The ethnicity of study participants is not reported.

We have now added the ethnicity of the study cohort, which is presented in Table 1 together with the other demographic and clinical parameters (table 1).

2. It is not clear whether this is the first case of NS caused by a single heterozygous

mutation. If so, the authors should discuss the possibility that the two sibs carry a second SPINK5 mutation that could not be detected by whole-exome sequencing (e.g. a large deletion or non-coding variant).

We have performed additional copy number variation (CNV) analysis and targeted Sanger DNA sequencing to investigate the possibility of a second pathogenic *SPINK5* variant. CNV analysis did not identify any clinically relevant deletions or duplications. Subsequent targeted sequencing revealed a missense variant in the second *SPINK5* allele (c.2243A>G; p.E748G). Although this variant has previously been reported in the literature as benign or of uncertain significance, its presence in *trans* with the previously identified *SPINK5* variant supports a compound heterozygous *SPINK5* genotype in these patients.

Furthermore, the coexistence of a pathogenic *FLG* variant is likely to contribute to the increased severity of the clinical phenotype observed in these siblings with Netherton syndrome. Collectively, these findings provide a more comprehensive genetic explanation for the disease manifestation and support the classification of these cases as the first reported siblings with this specific *SPINK5* compound heterozygosity in combination with an *FLG* mutation.

The manuscript has been updated accordingly, and the revised information can be found in the relevant section (Lines 31; 113–118; 127-129; 261 to 271 and Table 1).

3. The differential expression of FURIN is discussed at some length, but the authors do not mention the function of this gene. While a post-treatment increase in skin FURIN expression is reported.

In response to the reviewer's comment, a brief description of FURIN function has now been added to the manuscript and can be found in Lines 307-309.

Paired blood-skin transcriptomic profiling during Dupilumab treatment for SPINK5-FLG mutated NS syndrome

We want to thank the reviewers for taking the time to assess and improve our paper. Here, we provide a point-by-point response to the reviewer's comments.

Reviewer 1

Minor concerns:

- Please state in the discussion that it is still possible that the true pathogenic second SPINK5 variant has not been identified, being the p.Glu748Gly substitution classified as benign.

We thank the Reviewer for this valuable suggestion. In accordance with this recommendation, we have revised the Discussion (Lines 259-263) to explicitly acknowledge that the pathogenic role of the *SPINK5* p.Glu748Gly variant remains uncertain. We now state that, given its current classification as benign/likely benign, the possibility that a second pathogenic *SPINK5* variant has not yet been identified cannot be excluded. To facilitate consultation of the available evidence, the corresponding ClinVar accession (ClinVar: 449103) has also been included in the manuscript.

- Please, delete the phrase "…heterozygous carriers generally exhibit milder phenotype" in the Discussion. Heterozygous carriers are healthy individuals. In fact, depending on the variant position within protein domains, compound heterozygous individuals usually show milder phenotypes than homozygous individuals (ref. 27).

We thank the Reviewer for pointing out this imprecision. We agree that heterozygous carriers of a single *SPINK5* variant are generally healthy and that the original wording could be misleading. The sentence has been corrected to refer specifically to compound heterozygous individuals with Netherton syndrome, which was our intended meaning. In accordance with the Reviewer's suggestion and reference 27, the Discussion has been revised (Lines 253-254) to indicate that disease severity may vary in compound heterozygous individuals depending on the location of the variants within LEKTI functional domains, and that these patients may exhibit milder phenotypes than individuals carrying homozygous pathogenic variants.

Reviewer 2

The authors have addressed most of my queries and concerns. However, I remain confused by the description of >18,000 differentially expressed genes. The volcano plot in Fig 3 seems to show a total of 18,205 genes. Of these, 52 (purple + blue dots) meet the authors' definition of differential expression (log2FC>|1.5|; P<0.05). There are a few more that do not reach the Fold Change threshold, but still yield a P<0.05 (grey dots above the horizontal dotted lines). The remaining genes cannot be considered differentially expressed, as they do not meet the significance threshold of P<0.05.

We thank the Reviewer for this careful observation and for highlighting a potential source of confusion in the description of the transcriptomic results.

We would like to clarify that we did not intend to imply that all 18,205 genes were statistically significant differentially expressed genes according to our predefined thresholds. Rather, this number referred to the total set of genes detected and evaluated in the differential expression analysis, a metric that is frequently reported in transcriptomic studies to provide readers with an overview of dataset coverage and analytical scope. In the RNA-seq field, it is common practice to report the total number of genes included in the analysis separately from the subset that meets the selected statistical and fold-change criteria. We recognize, however, that our wording may have inadvertently suggested that all 18,205 genes fulfilled the definition of differential expression.

As correctly noted by the Reviewer, only a limited subset of genes met our predefined significance criteria (log2FC > |1.5| and $P < 0.05$), as shown in Figure 3. We agree that the original text could be misinterpreted and appreciate the opportunity to improve its clarity.

Accordingly, to avoid any ambiguity, we have revised the description of the RNA-seq results throughout the manuscript. Specifically, the text in the Results section (Line 136), Discussion section (Line 327), and the Figure 3 legend (Line 946) has been modified, and the previously reported number has been removed to prevent confusion between the total number of genes analyzed and the subset meeting the differential-expression thresholds.

We thank the Reviewer for this valuable suggestion, which has improved the clarity and accuracy of the manuscript.

The Discussion is in my view too long, but I defer to editor's opinion on that.

We thank the Reviewer for this comment. In response, we have further shortened and streamlined the Discussion, including after incorporating the additional points requested during revision. While we recognize that the Discussion remains relatively detailed, we believe this level of interpretation is justified by the rarity of the condition, the combined genomic and transcriptomic analyses performed, and the translational scope of the study. Importantly, the revised manuscript contains approximately **3,252 words**, which remains well below the **5,000-word limit** specified by *Communications Medicine*. We therefore believe that the current Discussion provides an appropriate balance between conciseness and adequate contextualization of the findings.