

Gene profiling reveals a contact allergy signature in most positive Amerchol L-101 patch-test reactions

Short title: Amerchol L-101-induced contact allergy

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Abbreviations

AD: Atopic dermatitis

AL-101: Amerchol L-101

ACD: Allergic contact dermatitis

CA: Contact allergy

CD: Contact dermatitis

CTL: Cytotoxic CD8⁺ T cell

DEG: Differentially expressed gene

Fc: Fold change

FDR: False discovery rate

GZMB: Granzyme B

GNLY: Granulysin

IFN- γ : Interferon gamma

PCA: Principal Component Analysis

Pet: Petrolatum

PT: Patch test

qRT-PCR: Quantitative reverse transcription polymerase chain reaction

Abstract

Background: Diagnosis of contact allergy (CA) to Amerchol L-101 (AL-101), a marker for lanolin allergy, is problematic. Positive patch-test (PT) reactions are frequently doubtful or weakly positive and difficult to associate with clinical relevance.

Objective: To gain further insight on the allergic or irritant nature of skin reactions induced by AL-101 PT.

Methods: We retested in a dose-response fashion, ten subjects with AL-101 CA and performed comprehensive transcriptomic analysis (gene arrays, qRT-PCR) of samples of their skin reactions.

Results: Eight of the ten CA subjects reacted positively upon retest, whilst two did not react. Most of AL-101 positive PT expressed an allergy signature with strong activation of gene modules associated with adaptive immunity and down-regulation of cornification pathway genes. Besides, the breadth of gene modulation correlated with the magnitude of PT reactions and the concentration of AL-101 applied. However, we observed that some of the positive PT reactions to AL-101 expressed no/few allergy biomarkers, suggesting the induction of an irritant skin inflammation in these samples.

Conclusions: This study confirms that AL-101 is an allergen that can cause both contact allergy and contact irritation. Our results also highlight that molecular profiling might help to strengthen clinical diagnosis.

Keywords: Allergic contact dermatitis, contact allergy, irritant contact dermatitis, irritant reaction, lanolin, patch testing, transcriptomic profiling, Amerchol L-101.

1. Introduction

Lanolin, also called wool wax, is a natural substance derived from secretions of the sebaceous glands of sheep. It serves as a protective coating on sheep wool. Lanolin consists of a complex and variable mixture of alcohols, fatty acids, esters and polyesters of high-molecular-weight alcohols¹⁻³. Because of its emollient and moisturizing properties, lanolin is widely used in cosmetics, such as creams, powders, lipsticks, shampoos, soaps, and shaving creams. Lanolin is also found in ointment bases for topical medications. Other uses are in industrial applications, such as lubricants in ball bearings^{3,4}.

Lanolin is considered to be a weak sensitizer. The prevalence of lanolin sensitization in referred dermatitis patients was reported to range from 1.2% to 6.9%⁵⁻⁸. Although lanolin contact allergy is uncommon on normal skin and with cosmetic usage, several studies have reported that lanolin-containing products may cause allergic contact dermatitis (ACD) after repeated and prolonged topical exposure, or after exposure on compromised skin^{3,9,10}. The diagnosis of lanolin contact allergy (CA) is routinely performed by patch-testing, using lanolin alcohols and Amerchol L-101 (AL-101), two major derivatives of the sheep wool wax refining and purification process¹¹. At the present time the sensitizer/s in lanolin are not identified. However, the alcohol fraction is considered to be the major sensitizing part of lanolin³. The use of AL-101 in patch-test (PT) procedures has been shown to provide additional diagnostic value¹¹.

However, the existence of lanolin allergy has long been a subject of debate. Clinicians often experience difficulties when performing PTs with lanolin derivatives. Positive PT reactions are often doubtful, and the morphologically weak positive reactions are rarely found to have any clinical relevance¹¹. In addition, various studies showed a significant lack of reproducibility in the results of AL-101 and lanolin alcohol PTs¹¹. A compound such as AL-101 has irritant properties, attributable to the mineral oil it contains, which, although resulting in a morphologically positive patch test, might in fact be a false-positive one^{5,12,13}. Therefore, a diagnosis of contact allergy to lanolin based on a positive AL-101 PT is regularly questioned.

We recently conducted an extensive analysis of the transcriptomic signatures of positive PT reactions to several reference chemical allergens (also referred to as haptens) and irritants¹⁴. Our results identified the core signature of chemical allergen-induced reactions providing mainly a framework to improve CA diagnosis, and eventually also ACD.

Using a similar approach, we searched in this study to gain further insight on the nature of skin inflammation induced in patients with clear positive PT reactions to AL-101.

2. Methods

Study population

Ten subjects (A1-A10; including 7 females and 3 males, range 31–74 years, mean age 56.9 years) previously diagnosed with contact allergy to AL-101 at the Department of Occupational and Environmental Dermatology (DOED), Skåne University Hospital, Malmö were enrolled in the study. Only subjects with previous weak positive (+: A1, A3, A5 and A10) and strong positive (+: A2, A4, A6-A9) reactions (read at 72h) to AL-101 [100%] were recruited. In addition, 5 controls (referred to as control C1-C5, including 4 females and 1 male; range 48–74 years, mean age 56.4 years) not diagnosed with CA to AL-101, when previously patch tested because of a suspected ACD, were also included.

Exclusion criteria were subjects undergoing treatment with oral corticosteroids or other systemic treatment, ongoing eczema on the back, phototherapy or topical corticosteroids used on the back two weeks before the study, known or suspected pregnancy.

Patch test procedure and skin samples

All participants were re-tested with a dilution series of AL-101 (Chemotechnique Diagnostics, Vellinge, Sweden) (3% w/w petrolatum (pet.), 10% w/w pet., 30% w/w pet., 50% w/w pet. and 100%), as well as with petrolatum (Vaselin, APL, Stockholm, Sweden) as a control. Solutions from Chemotechnique Diagnostics were used for AL-101 [100%] (Delivery-on Request) and 50% w/w pet. (commercially available), and a serial dilution was then performed at the DOED based on AL-101 as is and further diluted to: 30%, 10%, 3% w/w in petrolatum (pet.). Patch testing was performed with 8-mm Finn Chambers[®] (SmartPractice, Phoenix, Arizona, USA) and covered with Scanpor[®] tape (Norgesplaster A/S, Vennesla, Norway). 20 mg (40 mg/cm²) of test preparation was applied in the test chambers. PT positions (on lower back) were randomized and tests were removed at 48h and read by experienced dermatologists at 72h, in a blinded fashion (both for patient assignment and test concentrations). Test reactions were scored according to the classification of the International Contact Dermatitis Research Group (ICDRG) and European Society of Contact Dermatitis (ESCD)^{15,16}. Skin reactions were clinically graded as negative (-), doubtful (+/?), weak positive (+), strong positive (++) or extreme positive (+++) reactions (**Table 1**).

Punch biopsies (3mm) were collected after test readings from areas tested with AL-101 [100%, 50%, 30% and 10% pet; 3% pet PT areas were not biopsied] and pet. for subjects A1 to A10. Control subjects C1 to C5 were only biopsied from AL-101 [100%] and areas patch tested with pet.

Gene arrays

Affymetrix PrimeView Human Gene Expression arrays (Affymetrix, Inc., Santa Clara, CA, USA) were used for the gene array study. They allowed the analysis of more than 36000 transcripts, including approximately 22 000 genes and unigenes.

Sample preparation for the array hybridization was carried out as described in the Affymetrix GeneChip 3' IVT Express Kit user manual. In brief, total RNA was extracted from skin biopsies using the Qiagen RNeasy Mini Kit (Courtaboeuf, France) and contaminating DNA was removed using the DNA-FreeTM DNA Removal kit (Invitrogen, Cergy-Pontoise, France). The RNA was then reverse-transcribed into double-stranded copy DNA (cDNA), and then underwent *in vitro* transcription to generate biotin-labeled amplified RNA (aRNA). Purified aRNA was hybridized to the Affymetrix GeneChip Human PrimeView arrays for 16 h at 45° C in a GeneChip hybridization oven. Hybridized arrays were washed and stained in an Affymetrix Fluidics Station FS450, and the fluorescent signals were measured with an Affymetrix GeneChip Scanner 3000 7G. Fluidics and scan functions were controlled by the Affymetrix GeneChip Command Console v4.1.3 software.

Bioinformatics and statistical analysis of gene array data

Quality control of the gene arrays was carried out using standard QC metrics and R package quality control. Data were preprocessed (background correction and normalization) by applying (i) function *backgroundCorrect* from the limma package on the perfect match (PM) signals, (ii) the variance stabilizing transformation algorithm (function *justvs* from the vsn package¹⁷), and (iii) a quantile normalization (function *normalize* from the affy package with parameter *method* = "quantiles").

To filter probe sets, they were marked as informative or noninformative based on the result of the I/NI method¹⁸. Noninformative probe sets were excluded from unsupervised analyses. All sets were included when the statistical models were applied, but only the informative sets were considered when correcting for multiple testing.

Single values for the probe signals for each probe set of each sample were obtained using the summarization step of the Robust Multichip Average (RMA¹⁹) approach (function *rmaPLM* from the affyPLM package, with parameters *background*=FALSE and *normalize*=FALSE). *rmaPLM* also returned standard error estimates for each estimated expression level: these were used to incorporate weight inputs into the linear regression models.

The gene array dataset generated in this study were also compared to previous datasets obtained from skin biopsies of positive PT reactions induced by reference chemical allergens and irritants¹⁴.

Expression values were modeled using different mixed linear regression models, with the group of chemicals for the previous dataset (chemical allergens, irritants, controls) and the treatment condition for the new samples (AL-101 [100%], [50%], [30%], [10%], controls AL-101 [100%], petrolatum) as fixed factor and a random effect for each patient. Mixed linear regression models were applied using function `lme` from the `nlme` package²⁰. Comparisons of interest were then analyzed through statistical contrasts, using the `emmeans` package²¹. P-values from paired t-tests were adjusted for multiple hypotheses using the Benjamini and Hochberg false discovery rate (FDR) controlling procedure (function `p.adjust` from package `stats` with parameter `method="BH"`).

Multiple analyses were performed on the gene array data to determine whether global gene expression levels differed between the different treatment conditions (AL-101 [100%], [50%], [30%], [10%] and controls) and to identify genes and pathways preferentially/specifically induced. Comparisons were also performed to investigate the nature of Amerchol-induced response versus those induced by reference chemical allergens and irritants.

In **Table S1**, to determine the effect of AL-101 [100%], the statistical model used was $signal \sim -1 + Condition$ where *Condition* represents the treatment condition. To account for the paired design, an additional random effect was included ($\sim 1 | Donor$). The model estimated the average gene expression level in AL-101 100% condition after correction for the donor effect.

Results from **Table S1** report fold change (Fc), the FDR adjusted p-values and information for each identified gene (Symbol and Description).

In **Figure 1** and **Table S2**, differentially expressed genes (DEGs) identified in AL-101 [100%] samples were included in an input list for the MGSA algorithm (using the `mgsa` function from the `mgsa` package²²). The model assumed that some gene sets were active and that all gene members of active sets were themselves active. Fitting the model amounts to inferring the probability of every set to be active given the input list (i.e., scores reported for each gene set are not p-values and the closer to 1, the better). The approach was applied to Gene Ontology terms (from the Biological Process tree). Only sets with at least 10 genes and a maximum of 1000 genes were considered.

In **Figure 2A**, the PCA of differential expression was performed with probe set Fc neither centered nor scaled, using function `prcomp` from package `stats`. To investigate the type of the response to Amerchol, the inductions for the new samples were projected onto the PCA of the inductions of the previous chemical allergen and irritant samples.

In **Figure 2B**, the venn diagrams (created with `eulerr` package in R) include the number of DEGs (adjusted $p < 0.05$, fold-change > 1.41 or $< 1/1.41$) in each group (allergens, irritants

and AL-101 [100%]) and the intersection represents the DEGs that are positively correlated for 2 or all the groups. **Table S3** reports DEGs specific to the AL-101-induced skin inflammation.

In **Figure 3**, a heatmap was used to illustrate individual variations of 52 allergy DEGs (selected from Lefevre et al. ¹⁴) in the different Amerchol-treated samples.

Gene clustering was performed using the complete agglomerative method, passing the Euclidean distance between scaled probe set signals to function `hclust` from package `stats`.

In **Figure 4**, to determine correlations existing between the magnitude of the allergy signature and the applied Amerchol dose, the value of each gene induction ($\text{Log}_2(\text{Fc})$) was added to obtain an average level for the 52 allergy DEGs.

In **Figure S1**, similar approach was used to establish correlations between the magnitude of the allergy signature and the intensity of the patch test reactions.

qRT-PCR

Total RNA samples were also used for quantitative RT-PCR (qRT-PCR) analysis. qRT-PCR investigations were performed using a StepOnePlus PCR system (ThermoFisher, Villebon-sur-Yvette, France). CD69 and granulysin (GNLY) primers (QuantiTect Primer Assays) were from Qiagen (Courtaboeuf, France). CD8A, interferon gamma (IFNG), granzyme B (GZMB) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were designed using the publicly Universal Probe Library Assay Design Center (https://lifescience.roche.com/en_fr/brands/universal-probe-library.html#assaydesign-center) and produced by Sigma (Saint-Quentin-Fallavier, France).

Primers are listed in **Table S4**.

Analysis of qRT-PCR data

In **Figure S2**, expression values for each mRNA transcript in each sample were normalized to values obtained for housekeeping gene (GAPDH), and results were presented as Fc versus controls.

In **Figure 5**, to determine correlations existing between the magnitude of the allergy signature and the applied Amerchol dose, the value of each gene induction ($\text{Log}_2(\text{Fc})$) was added to obtain an average level for the 5 allergy DEGs.

Data Availability

Expression microarray data have been deposited in Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo>) under accession no. GSE169573.

Study approval

The study was approved by the Regional Ethical Review Board, Lund University, Sweden (Dnr-2017/197), and written informed consent was obtained from each participant prior to inclusion.

3. Results

AL-101 PT results

Ten subjects with previously documented CA to AL-101, and five non-allergic control subjects, were retested with AL-101, before being biopsied for extensive genomic and molecular profiling of their patch-tested skin.

Eight subjects (subjects A3-A10) reacted positively upon re-testing, with weak (+, n=6) and strong positive (++, n=2) reactions at the maximum AL-101 dose [100%]. Two subjects (subjects A1 & A2) did not react. One subject from the control group (C5) showed a doubtful reaction (+/?), while the four others (C1-C4) had negative reactions.

CA subjects were tested with several AL-101 dilutions (50% pet., 30% pet., 10% pet. and 3% pet.). A progressive decrease in the intensity of the PT reactions was observed with dilutions, but with a clear difference between subjects. Four of the eight positive PT subjects (subjects A7-A10) were still positive with doubtful or weak reactions at AL-101 [10%], while subject A3 was negative at the first dilution [50%].

In total, more doubtful (n=11) and weak (n=12) reactions were observed, and no extreme (+++) reactions were noted (**Table 1**). Of note, we mostly observed doubtful (n=5) and weak reactions (n=2) to the commercially available dose of AL-101 [50%, pet], which is used by most clinicians.

Main signaling pathways activated by AL-101 are comparable to that of reference contact allergens

We next searched to define the genes and the signaling pathways activated in Amerchol-induced skin inflammation. We first evaluated the number of differentially expressed genes (DEG) in AL-101 biopsy samples from CA subjects, when compared to their respective controls (biopsy samples collected from the pet-applied skin of the same subject). 1510 DEGs, including 873 upregulated and 637 downregulated genes, were found to be modulated consistently among the ten AL-101 [100%] samples (**Table S1**). By comparison, no statistically significant DEGs were detected in samples from the five control subjects (**data not shown**).

Gene ontology (GO) annotations revealed several upregulated pathways in Amerchol-inflamed skin, mainly related to immune pathways (including “antigen processing and presentation”, “immune effector process”, “response to cytokine”, “leukocyte activation”, “inflammatory response”), epidermal compartment (“peptide cross-linking”) and metabolism

(“cellular response to ketone”). In contrast, a significant down-regulation of genes associated to the “cornification” pathway was observed (**Figure 1** and **Table S2**).

We then compared gene profiling of Amerchol-inflamed skin with the transcriptomic signatures of positive PT reactions to several reference chemical allergens (nickel, methylisothiazolinone, linalool hydroperoxide) and irritants (SLS, nonanoic acid and cantharidin), evaluated in a previous study from our group ¹⁴. To this end, we executed a hypothesis-free principal component analysis (PCA) of data from the full set of genes measured on the arrays for each AL-101 samples. To compensate for donor-induced variability, the gene expression level of the control sample (pet) of each donor was subtracted from the corresponding-treated samples of the same donor. Projecting the resulting data on previous PCA comparing gene expression in chemical allergen (also called hapten) and irritant samples ¹⁴, we found that the gene inductions for positive AL-101 samples (+/?, + and ++) overlapped for the most part with the inductions recorded for hapten but not irritant samples, typifying an allergen-like response to Amerchol (**Figure 2A**, colored symbols).

To further characterize the Amerchol-induced skin inflammation, we next analyzed common and unique DEGs in AL-101, hapten and irritant skin samples. As represented in the Venn diagram depicted in **Figure 2B**, we identified 266 DEGs commonly regulated by the three groups, 1061 DEGs regulated by both AL-101 and haptens, and only 8 DEGs regulated by both AL-101 and irritants (**Figure 2B**). We also found 214 DEGs specific to the AL-101-induced skin inflammation, including CD36 and FOXO1 transcripts, which are associated with energy metabolism, or IGFBP3, IGFBP4 and GIGYF2 transcripts, associated with insulin-like growth factor receptor signaling pathway (**Table S3**).

Collectively, these results therefore demonstrate that molecular signatures of positive patch test reactions to AL-101 are highly similar to those of reference chemical allergens but not of irritants.

The majority of the AL-101-induced PT reactions displays an allergy signature

We have previously identified and selected 52 DEGs as the basis of a hapten core signature. The latter mainly consisted of several T-cell- and cytotoxic-related transcripts ¹⁴, which are up-regulated within the 72 hours following hapten re-test. We then analyzed the induction of these 52 DEGs in the different AL-101-treated skin samples. Illustrated as a heatmap representing individual gene expression variations (**Figure 3**), or as box and whisker plots representing the average level of the 52 DEG inductions, results showed a clear gene induction - dose response effect in the ten CA subjects (**Figure 4A**). In contrast, very low gene induction was observed in the four controls C1-C4 who reacted negatively to AL-101

[100%], and a substantial transcript upregulation was detected for several of the 52 DEGs in the 5th subject (C5) who developed a doubtful reaction (**Figures 3-4**).

We also observed that the average level of the 52 DEG inductions increased with the intensity of the PT reactions (**Figure S1**). However, when individual data were taken into account, a substantial degree of variability was recorded between subjects. Hence, subjects A1 and A2, who developed negative PT reactions upon re-testing, but also subject A3, showed no/weak baseline gene inductions for each of the AL-101 doses tested (**Figure 4, Figure S1**). Comparative analysis demonstrated that gene inductions for these 3 subjects were in the same range as the data obtained from the skin lesions of patients with “positive” PT reactions to reference irritants (**Figure 4**). On the other hand, strong inductions were detected in subjects A4, A5, A6, A8 and A9 at the maximal dose of AL-101, and in subjects A7 and A10 at all doses tested (**Figure 4**). Subjects A5, A6, A8 and A9 developed weak or doubtful positive PT response when tested with AL-101 50%, but a weak allergy core gene signature was observed in their skin biopsies (**Figure 4, Figure S1**).

In aggregate, results thus demonstrate that the majority (7/10), but not all, of AL-101-induced skin reactions (mainly at the maximal AL-101 dose) displays a contact allergy signature (**Table 1**), which is dose-dependent and correlates with the intensity of the patch-test response.

A small set of biomarkers allows for robust detection of AL-101–induced allergy

Finally, we aimed to validate our gene array results by confirming the expression of a small set of allergy biomarkers by qRT-PCR. We selected 5 genes present in our core allergen signature based on their relevance for ACD pathophysiology, and then explored their expression in the respective AL-101 samples. Our results demonstrated that there was an excellent correlation between gene array data and qRT-PCR results for these 5 genes (**data not shown**). They confirmed the strong upregulation of CD8A, CD69, Granzyme B (GZMB), Granulysin (GNLY), and IFNG mRNA transcripts in the different samples tested with AL-101 [100%], comparatively to the data obtained from positive PT reactions to reference irritants (**Figure 5, Figure S2**). No gene induction was detected in subjects A2 and A3 and the 5 control subjects. A weak gene induction was detected this time with subject A1 (**Figure 5, Figure S2**).

Results therefore suggest that there is an infiltration of activated cytotoxic CD8⁺T lymphocytes in a majority of the subjects with positive AL-101 PT reactions.

4. Discussion

The main objective of our study was to better understand the nature of PT reactions induced by the lanolin derivative, AL-101. Our gene profiling study univocally demonstrated that AL-101 is a contact allergen. Indeed, most of the positive PT reactions induced by this chemical displayed a robust allergy signature. Additionally, our results confirmed that the use of AL-101 for the diagnosis of contact allergy to lanolin can lead to questionable results. Positive PT results to AL-101 were not reproduced in some subjects upon re-testing, and our transcriptomic study highlighted a lack of allergic response in a subject (subject A3) with a positive PT reaction. Interestingly, dilution series experiments demonstrated a significant correlation between the concentration of AL-101 applied, the magnitude of positive patch test reactions and the breadth of gene modulation induced after patch-testing, suggesting that molecular profiling approaches could be of major value to monitor the development of PT reactions, and help the clinician to refine his diagnosis.

Test reactivity to AL-101

AL-101 is routinely used as a representative marker for lanolin allergy. This commercial product is manufactured by the hydrolysis of wool fat, and consists of lanolin alcohols, cholesterol and sterols³ with an addition of mineral oil. Analogous to lanolin, the sensitizers in AL-101 are not identified, but are certainly comprised in the alcoholic fraction^{7,23}.

In this study, re-testing with AL-101 induced positive patch tests in 8 of 10 CA subjects. We also observed significant variations in the strength of the PT reactions between the initial response at diagnosis and the present investigation, with only two subjects (instead of six) developing a strong positive (++) PT reaction at the highest AL-101 concentration. Such findings are not unexpected, as several studies have already reported variability in PT results to AL-101 or lanolin alcohol^{3,11,24}. This phenomenon has also been described for other contact allergens like metal salts: gold, cobalt, aluminium, palladium, and nickel^{25–28} but also non-metals such as formaldehyde, fragrance mixes and eugenol^{29–33}. There are multiple methodological reasons which can explain differences in test reactivity over time for the same preparation: (i) differential skin adherence of the PT, (ii) different amounts or distribution of the substance in the chamber¹⁶, (iii) differences in test reading (here, readings were performed at D3 only) and also (iv) seasonal factors³⁴. Alternatively, (v) variation in individual skin sensitivity²⁵ or (vi) the presence of high concentrations of mineral oils or

contaminants (such as detergent) in AL-101 mixture may have caused irritancy, leading to false positive PT reactions during the initial test explorations^{5,12,35,36}.

To diagnose individuals with true allergic reactions, we then retested subjects with dilution series. Serial dilution patch testing is often recommended when the diagnosis of CA is problematic¹⁶. Hence, it is considered that the strength of allergic reactions gradually reduces with decreasing concentration, while a false-positive irritant reaction disappears abruptly when the concentration is lowered. Here, we observed significant variations in PT reactivity during serial dilutions, with one subject (A3) reacting negatively to all dilutions of AL-101 below 100%, whereas four of the subjects (A7-A10) reacted even to the low AL-101 [10%] concentration, supporting the finding of AL-101 as a true hapten in the latter (15).

Of note, the commercially available test preparation for AL-101 is classically [50% pet]. Increasing the maximal AL-101 concentration to [100%] in dilution series allowed us to confirm PT positivity in 5 subjects (A4, A5, A6, A7 and A8) who developed doubtful reactions at [50%, pet], and also to detect reactivity (albeit false positive) in subject A3. Thus, increasing the AL-101 concentration from [50%, pet] to [100%; 40 mg/cm²] increases PT sensitivity, but with the risk to bring false positive reaction, as described in our transcriptomic study. The decision on optimal patch test concentration (actually dose in mg/cm²) is tricky as considerations have to be taken on the significance of a false negative reaction due to a too low test dose versus a false positive reaction due to a too high test dose. Knowledge on the clinical relevance of a positive reaction at patch testing with 100% and negative at 50%, would help the decision-making concerning test concentration for AL-101. This knowledge is presently missing.

The molecular signatures of Amerchol-induced reactions

Our gene profiling study demonstrated that the molecular pathways which are activated in response to Amerchol application largely overlapped with the pathways found in the skin of allergic individuals patch-tested with reference allergens, such as nickel, methylisothiazolinone, linalool peroxide¹⁴ or para-phenylenediamine and epoxy resin-bisphenol A³⁷. Recently, Dhingra et al. suggested that molecule-dependent profiles could exist between contact allergens, and they reported that a significant T_H2 bias with T_H22 polarization characterized the responses induced by molecules such as fragrances, whereas type-1/type-17 phenotypes predominated for molecules like nickel³⁸. Although we detected several DEGs that were most prominently modulated in the Amerchol-induced response, none of these DEGs was related to a specific inflammatory pathway susceptible to typify the Amerchol inflammation. In contrast, several of the pathways and biomarkers common to

Amerchol and other reference allergens were linked to the activation of the adaptive immunity and cytotoxic CD8⁺ T cells (CTLs), suggesting that AL-101-induced inflammation is also characterized by infiltration of activated cytotoxic CD8⁺T lymphocytes in a majority of the patch-tested subjects. Hapten-specific CTLs have long been considered to be the primary effector cells responsible for the formation and recurrences of ACD lesions.

The interest of gene profiling to strengthen clinical diagnosis

Beyond an in-depth characterization of Amerchol-induced inflammation, our results also illustrated the value of gene profiling to help to strengthen clinical diagnosis. Indeed, we observed a significant correlation between the magnitude of patch-test reactivity and the induction (differential gene expression) of our set of core allergy biomarkers. Similar correlations were also noted with the applied dose. Hence, several of the doubtful (+/-) reactions induced in the AL-101 dilution series (notably for subject A7) expressed a clear allergy signature, but with a low intensity (**Figures 3-5, Figures S1-S2**). This indicates that doubtful and weakly positive PT reactions to Amerchol, which are much more prevalent in clinic than the stronger (++) reactions^{8,39} should not be overlooked by clinicians and considered non-allergic, because they can be infiltrated by low numbers of hapten-specific effector T cells.

On the other hand, we detected a clear + positive PT to AL-101 [100%] in subject A3, but with no/very mild allergy signature in subject biopsy. We detected a weak increase in T cell transcripts, such as CD3D, TRAT1, THEMIS, CD2 or CD69, but no upregulation for markers associated to cytotoxicity (Granzyme B, Granzyme A, Granulysin, IFNG) (**Figure 3**). In contrast, in this subject, inflammation may have been induced by the activation of innate immunity, as we detected a significant upregulation of several DEGs associated with the GO term “inflammatory pathways”, including DEGs coding for the chemokines IL-8, CCL17, CCL19, CCL20; the matrix metalloproteinase MMP9 or the S100 family proteins S100A7, S100A7A, S100A8 and S100A9 (**data not shown**). Coupled with the fact that this subject failed to react to dilution series, this suggests that subject A3 is probably not sensitized to AL-101, but she developed irritancy to AL-101 during patch-testing, which translated into a + (yet false-positive) patch test reaction.

The complexity of the diagnosis of lanolin allergy

Uldahl *et al.* recently conducted a repeated-open application test (ROAT) study with 3 different lanolin-containing creams to assess the clinical relevance of Amerchol-induced PT reactions⁴⁰. Twelve of the fifteen subjects analyzed here (with the exception of subjects A2,

A4 and A7) participated in the ROAT study. Confirming previous works^{3,11,24}, it was observed that none of the participants with positive PT to AL-101 had any skin reactions during ROAT. It is always difficult to determine whether the lanolin markers used for patch-testing, such as AL-101, are representative of the lanolin derivatives encountered in daily-life products. Therefore, it would be interesting in a future study to characterize and compare the molecular signatures of contact dermatitis lesions induced by repeated or prolonged topical exposure to lanolin-containing products, with those of AL-101- or lanolin-induced PT reactions. The identification of a CA signature in active lesions could help clinicians to better understand the nature and the clinical relevance of the lanolin-induced inflammation, particularly in subjects with atopic dermatitis (AD), where the differential diagnosis is complicated by the presence of an underlying disease. Recent promising gene profiling works have shown that CA signatures to reference allergens such as nickel segregate from those of other types of dermatoses, such as AD or psoriasis^{37,41}.

Limitations of the study

Despite well-matched recruitment, this study included only a limited number of subjects: 10 subjects with previous positive patch test reactions to AL-101 and 5 control subjects. Thus, it will be interesting to extend our results to a larger cohort.

Another important limitation of our study is related to the fact that the use of molecular profiling for the diagnosis of contact allergy is still in its infancy. Hence, to date, no clinical studies have accurately determined the performance (i.e., sensitivity and specificity) of these approaches. As our study shows, the mean induction of the 52 core allergy genes is sometimes only slightly different from the data obtained from the skin lesions of patients with “positive” PT reactions to reference irritants; as illustrated in subject C5 in **Figure 4**. For this subject, a substantial transcript upregulation was detected for several of the 52 DEGs (**Figure 3**). However, it was difficult to state with certainty, based on mean gene induction level, whether the molecular response was an allergic reaction or not (**Figure 4 & Table 1**). Of note, the 5 genes selected for qRT-PCR validation, and that are relevant for ACD pathophysiology, were negative for this subject (**Figure 5** and **Figure S2**). Thus, it will be crucial to determine in future studies gene induction thresholds that accurately discriminate weak allergic reactions from false positive responses. This will require combined assays of PTs and ROATs performed with the same culprit molecule(s), as well as in-depth molecular profiling.

Conclusion

In conclusion, this study confirms that AL-101 is an allergen that can cause both contact allergy and contact irritation. In addition, our results also highlight that molecular profiling could open important avenues to guide the development of future diagnostic approaches to improve the diagnosis of lanolin allergy.

Author contributions

LLS and MAL carried out the experiments and analysed the data.

SDB, JN and LB provide support for bioinformatic/biostatistics analysis.

LLS, OB, AN, MB and CS managed the clinical study, performed sample collections, obtained consents and/or participated in the interpretation of the data.

JFN, CS and MV designed the experiments and supervised this study.

LLS, MAL, JFN, CS, MB and MV wrote the manuscript.

All authors approved the final submitted and published version.

Co-first authors can prioritize their names when adding this paper's reference to their resumes.

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References

1. Schlossman ML, McCarthy JP. Lanolin and derivatives chemistry: relationship to allergic contact dermatitis. *Contact Dermatitis*. 1979;5(2):65-72. doi:10.1111/j.1600-0536.1979.tb04801.x
2. Matthieu L, Dockx P. Discrepancy in patch test results with wool wax alcohols and Amerchol L-101. *Contact Dermatitis*. 1997;36(3):150-151. doi:10.1111/j.1600-0536.1997.tb00398.x
3. Lee B, Warshaw E. Lanolin allergy: history, epidemiology, responsible allergens, and management. *Dermatitis*. 2008;19(2):63-72.
4. Loden M, Maibach HI. *Dry Skin and Moisturizers: Chemistry and Function*. CRC Press; 1999.
5. Wakelin SH, Smith H, White IR, Rycroft RJ, McFadden JP. A retrospective analysis of contact allergy to lanolin. *Br J Dermatol*. 2001;145(1):28-31. doi:10.1046/j.1365-2133.2001.04277.x
6. Warshaw EM, Nelsen DD, Maibach HI, et al. Positive patch test reactions to lanolin: cross-sectional data from the north american contact dermatitis group, 1994 to 2006. *Dermatitis*. 2009;20(2):79-88.
7. Miest RYN, Yiannias JA, Chang YHH, Singh N. Diagnosis and prevalence of lanolin allergy. *Dermatitis*. 2013;24(3):119-123. doi:10.1097/DER.0b013e3182937aa4
8. Fransen M, Overgaard LEK, Johansen JD, Thyssen JP. Contact allergy to lanolin: temporal changes in prevalence and association with atopic dermatitis. *Contact Dermatitis*. 2018;78(1):70-75. doi:10.1111/cod.12872
9. Kligman AM. Lanolin allergy: crisis or comedy. *Contact Dermatitis*. 1983;9(2):99-107. doi:10.1111/j.1600-0536.1983.tb04314.x
10. Matiz C, Jacob SE. The lanolin paradox revisited. *Journal of the American Academy of Dermatology*. 2011;64(1):197. doi:10.1016/j.jaad.2010.07.047
11. Knijp J, Bruynzeel DP, Rustemeyer T. Diagnosing lanolin contact allergy with lanolin alcohol and Amerchol L101. *Contact Derm*. 2019;80(5):298-303. doi:10.1111/cod.13210
12. Geier J, Uter W, Pirker C, Frosch PJ. Patch testing with the irritant sodium lauryl sulfate (SLS) is useful in interpreting weak reactions to contact allergens as allergic or irritant. *Contact Dermatitis*. 2003;48(2):99-107. doi:10.1034/j.1600-0536.2003.480209.x
13. Geier J, Uter W, Lessmann H, Schnuch A. The positivity ratio--another parameter to assess the diagnostic quality of a patch test preparation. *Contact Dermatitis*. 2003;48(5):280-282. doi:10.1034/j.1600-0536.2003.00033.x
14. Lefevre MA, Nosbaum A, Rozieres A, et al. Unique molecular signatures typify skin inflammation induced by chemical allergens and irritants. *Allergy*. Published online June 26, 2021. doi:10.1111/all.14989

15. Fregert S. Fregert S. Manual of Contact Dermatitis. 2nd ed. Munksgaard, Copenhagen: Year Book Medical Publishers, Inc; 1981. Chapter 10, Patch Testing; pp. 71–81. Accessed June 16, 2021. google scholar
16. Johansen JD, Aalto-Korte K, Agner T, et al. European Society of Contact Dermatitis guideline for diagnostic patch testing - recommendations on best practice. *Contact Dermatitis*. 2015;73(4):195-221. doi:10.1111/cod.12432
17. Huber W, von Heydebreck A, Sültmann H, Poustka A, Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics*. 2002;18 Suppl 1:S96-104. doi:10.1093/bioinformatics/18.suppl_1.s96
18. Talloen W, Clevert DA, Hochreiter S, et al. I/NI-calls for the exclusion of non-informative genes: a highly effective filtering tool for microarray data. *Bioinformatics*. 2007;23(21):2897-2902. doi:10.1093/bioinformatics/btm478
19. Irizarry RA, Hobbs B, Collin F, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*. 2003;4(2):249-264. doi:10.1093/biostatistics/4.2.249
20. Pinheiro JC, Bates DJ, DebRoy S, Sakar D. *The Nlme Package: Linear and Nonlinear Mixed Effects Models, R Version 3*. Vol 6.; 2012.
21. Lenth RV. Least-Squares Means: The R Package **lsmeans**. *J Stat Soft*. 2016;69(1). doi:10.18637/jss.v069.i01
22. Bauer S, Gagneur J, Robinson PN. GOing Bayesian: model-based gene set analysis of genome-scale data. *Nucleic Acids Res*. 2010;38(11):3523-3532. doi:10.1093/nar/gkq045
23. Fregert S, Dahlquist I, Trulsson L. An attempt to isolate and identify allergens in lanolin. *Contact Dermatitis*. 1984;10(1):16-19. doi:10.1111/j.1600-0536.1984.tb00054.x
24. Gollhausen R, Przybilla B, Ring J. Reproducibility of patch tests. *J Am Acad Dermatol*. 1989;21(6):1196-1202. doi:10.1016/s0190-9622(89)70329-7
25. Hindsén M, Bruze M, Christensen OB. Individual variation in nickel patch test reactivity. *Am J Contact Dermat*. 1999;10(2):62-67. doi:10.1016/s1046-199x(99)90001-5
26. Masjedi K, Bruze M, Hindsén M, Minang J, Ahlborg N. Is the variability of nickel patch test reactivity over time associated with fluctuations in the systemic T-cell reactivity to nickel? *Br J Dermatol*. 2009;161(1):102-109. doi:10.1111/j.1365-2133.2009.09182.x
27. Rosholm Comstedt L, Engfeldt M, Svedman C, Åkesson A, Hindsén M, Bruze M. Variation and covariation in patch test reactivity to palladium and nickel salts. *Eur J Dermatol*. 2018;28(5):668-676. doi:10.1684/ejd.2018.3423
28. Siemund I, Mowitz M, Zimerson E, Bruze M, Hindsén M. Variation in aluminium patch test reactivity over time. *Contact Dermatitis*. 2017;77(5):288-296. doi:10.1111/cod.12836

29. Engfeldt M, Tillman C, Hindsén M, Bruze M. Variability in patch test reactivity over time, falsely indicating patch test sensitization, in a patient tested with palladium salts. *Contact Dermatitis*. 2012;67(2):109-111. doi:10.1111/j.1600-0536.2012.02086.x
30. Svedman C, Engfeldt M, Api AM, et al. A pilot study aimed at finding a suitable eugenol concentration for a leave-on product for use in a repeated open application test. *Contact Dermatitis*. 2012;66(3):137-139. doi:10.1111/j.1600-0536.2011.02041.x
31. Hauksson I, Pontén A, Gruvberger B, Isaksson M, Engfeldt M, Bruze M. Skincare products containing low concentrations of formaldehyde detected by the chromotropic acid method cannot be safely used in formaldehyde-allergic patients. *Br J Dermatol*. 2016;174(2):371-379. doi:10.1111/bjd.14241
32. Bruze M, Engfeldt M, Ofenloch R, et al. Validation of a questionnaire algorithm based on repeated open application testing with the constituents of fragrance mix I. *Br J Dermatol*. 2020;182(4):955-964. doi:10.1111/bjd.18224
33. Bruze M, Engfeldt M, Elsner P, et al. Validation of questionnaire algorithm based on repeated open application testing with the constituents of fragrance mix II: the EDEN Fragrance Study. *J Eur Acad Dermatol Venereol*. Published online April 29, 2021. doi:10.1111/jdv.17315
34. Bruze M. Seasonal influence on routine patch test results. *Contact Dermatitis*. 1986;14(3):184. doi:10.1111/j.1600-0536.1986.tb01206.x
35. Carmichael AJ, Foulds IS, Bransbury DS. Loss of lanolin patch-test positivity. *Br J Dermatol*. 1991;125(6):573-576. doi:10.1111/j.1365-2133.1991.tb14797.x
36. Edman B, Möller H. Testing a purified lanolin preparation by a randomized procedure. *Contact Dermatitis*. 1989;20(4):287-290. doi:10.1111/j.1600-0536.1989.tb03147.x
37. Fortino V, Wisgrill L, Werner P, et al. Machine-learning-driven biomarker discovery for the discrimination between allergic and irritant contact dermatitis. *Proc Natl Acad Sci U S A*. 2020;117(52):33474-33485. doi:10.1073/pnas.2009192117
38. Dhingra N, Shemer A, Correa da Rosa J, et al. Molecular profiling of contact dermatitis skin identifies allergen-dependent differences in immune response. *J Allergy Clin Immunol*. 2014;134(2):362-372. doi:10.1016/j.jaci.2014.03.009
39. Löffler H, Becker D, Brasch J, Geier J, German Contact Dermatitis Research Group (DKG). Simultaneous sodium lauryl sulphate testing improves the diagnostic validity of allergic patch tests. Results from a prospective multicentre study of the German Contact Dermatitis Research Group (Deutsche Kontaktallergie-Gruppe, DKG). *Br J Dermatol*. 2005;152(4):709-719. doi:10.1111/j.1365-2133.2004.06465.x
40. Uldahl A, Engfeldt M, Svedman C. Clinical relevance of positive patch test reactions to lanolin- a ROAT study. *Contact Dermatitis*. Published online August 25, 2020. doi:10.1111/cod.13689
41. Quaranta M, Knapp B, Garzorz N, et al. Intraindividual genome expression analysis reveals a specific molecular signature of psoriasis and eczema. *Sci Transl Med*. 2014;6(244):244ra90. doi:10.1126/scitranslmed.3008946

Figure legends

Figure 1: Signaling pathway analysis of Amerchol patch test responses.

Shown are the most significant hits for the GO terms “biological process” for genes predominantly regulated in AL-101 [100%] patch test reactions.

The bar size indicates the level of the estimated frequency for the activation of the respective pathway (from 0 to 1).

Figure 2: Comparison of Amerchol effects with existing datasets from allergen and irritant patch test reactions.

A-Principal component analysis performed on the full set of genes measured on the arrays. Gene inductions in Amerchol samples were projected onto a previous PCA obtained from induction comparisons between allergen and irritant patch test reactions. In this PCA, the gene expression level of the control sample of each subject is subtracted from the corresponding treated sample from the same subject to compensate for donor-induced variability. Results depict sample location in the first two components (PCs), with the controls at coordinate 0. Together, the first 2 components represented approximately 65% of the variance. Subject numbers are reported above each sample.

B-Venn diagram representing common and unique DEGs in AL-101 [100%] and allergens and irritant patch test samples.

Blue circle indicates genes (n=2312) predominantly modulated in the allergen samples.

Red circle indicates genes (n=672) predominantly modulated in the irritant samples.

Grey circle indicates genes (n=1549) predominantly modulated in the AL-101 [100%] samples.

Intersections represent the common DEGs.

Figure 3: Allergy signature in Amerchol samples

A set of 52 DEGs illustrating the core transcriptome of reference chemical allergens was used to analyse AL-101-induced reactions. Results are depicted as a heatmap, in which samples are grouped by subject and arranged by AL-101 doses, from 10% to 100%. Blue, white and red colors represent respectively low, moderate and high signal intensity.

Figure 4: Correlations between allergy DEG inductions and applied Amerchol doses and the magnitude of patch test reactions

Box-and-whisker plots depict the distribution of the average levels of the 52 allergy DEG inductions ($\text{Log}_2(\text{Fold change})$) in samples applied with different AL-101 concentrations. Each symbol represents a sample. Results from previous allergen and irritant reference data sets are shown for comparison.

Figure 5: Validation of small set of segregating allergy biomarkers by qRT-PCR.

Box-and-whisker plots depict the distribution of the average levels of variations in CD8A, GNLY, GZMB, CD69 and IFNG gene inductions ($\text{Log}_2(\text{Fold change})$) as assessed by qRT-PCR, in samples applied with different AL-101 doses.

Each symbol represents a sample. Results from previous allergen and irritant reference data sets are shown for comparison.

Tables

Table 1: Subjects' demographics, clinical history and patch test results.

Patients			Patch tests Occlusion time: 48h Reading: 72h		Previous Patch tests Occlusion time: 48h Reading: 72h	Investigators interpretation <i>based on patch test and gene profiling results</i>
Number	Gender/Age	Clinical history	Dose of AL-101	Results	Results	
A1	M/64	Dermatitis	100% 50% 30% 10% 3% Pet	- - - - - -	+ NT NT NT NT NT	No Contact Allergy suggesting possible Contact Irritation in previous patch tests
A2	F/62	Contact dermatitis	100% 50% 30% 10% 3% Pet	- - - - - -	++ - NT NT NT NT	No Contact Allergy suggesting possible Contact Irritation in previous patch tests
A3	F/54	Hand dermatitis	100% 50% 30% 10% 3% Pet	+ - - - - -	+ NT NT NT NT NT	No Contact Allergy suggesting Contact Irritation
A4	F/36	Atopic and contact dermatitis	100% 50% 30% 10% 3% Pet	+ ?/+ - - - -	++ - NT NT NT NT	Contact Allergy
A5	F/56	Leg dermatitis	100% 50% 30% 10% 3% Pet	+ ?/+ - - - -	+ NT NT NT NT NT	Contact Allergy
A6	F/61	Eyelid dermatitis	100% 50% 30% 10% 3% Pet	+ ?/+ ?/+ - - -	++ ++ NT NT NT NT	Contact Allergy
A7	M/68	Atopic dermatitis, hand dermatitis	100% 50% 30% 10% 3% Pet	+ ?/+ ?/+ ?/+ - -	++ + NT NT NT NT	Contact Allergy
A8	F/31	Hand and lip dermatitis	100% 50% 30% 10% 3% Pet	+ ?/+ ?/+ ?/+ - -	++ NT NT NT NT NT	Contact Allergy
A9	F/53	Dermatitis	100% 50% 30% 10% 3% Pet	++ + + ?/+ - -	++ NT NT NT NT NT	Contact Allergy
A10	M/74	Hand dermatitis	100% 50% 30%	++ + +	+ NT NT	Contact Allergy

			100%	+	NT	
			3%	+	NT	
			Pet	-	NT	
C1	F/50	Hand dermatitis	100%	-	-	Negative
			50%	-	NT	
			30%	-	NT	
			10%	-	NT	
			3%	-	NT	
			Pet	-	NT	
C2	M/74	Atopic dermatitis	100%	-	-	Negative
			50%	-	NT	
			30%	-	NT	
			10%	-	NT	
			3%	-	NT	
			Pet	-	NT	
C3	F/48	Hand dermatitis	100%	-	-	Negative
			50%	-	NT	
			30%	-	NT	
			10%	-	NT	
			3%	-	NT	
			Pet	-	NT	
C4	F/54	-	100%	-	-	Negative
			50%	-	NT	
			30%	-	NT	
			10%	-	NT	
			3%	-	NT	
			Pet	-	NT	
C5	F/56	Hand dermatitis	100%	?/+	-	No Contact Allergy <i>but questionable results</i>
			50%	?/+	NT	
			30%	?/+	NT	
			10%	-	NT	
			3%	-	NT	
			Pet	-	NT	

M=Male. F=Female.

AL-101=Amerchol L-101.

Pet=Petrolatum

NT: not tested

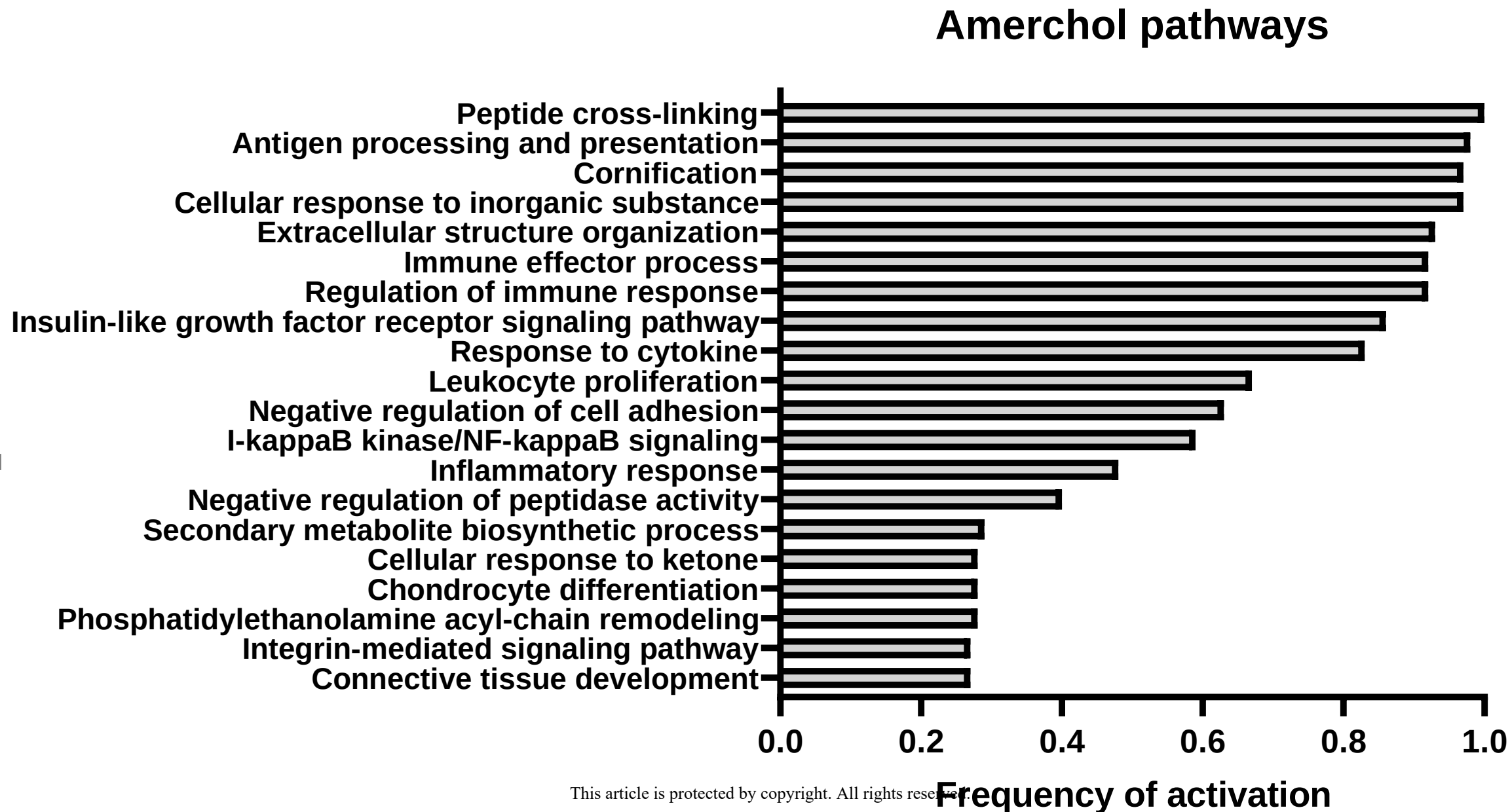
Figure 1

Figure 2a

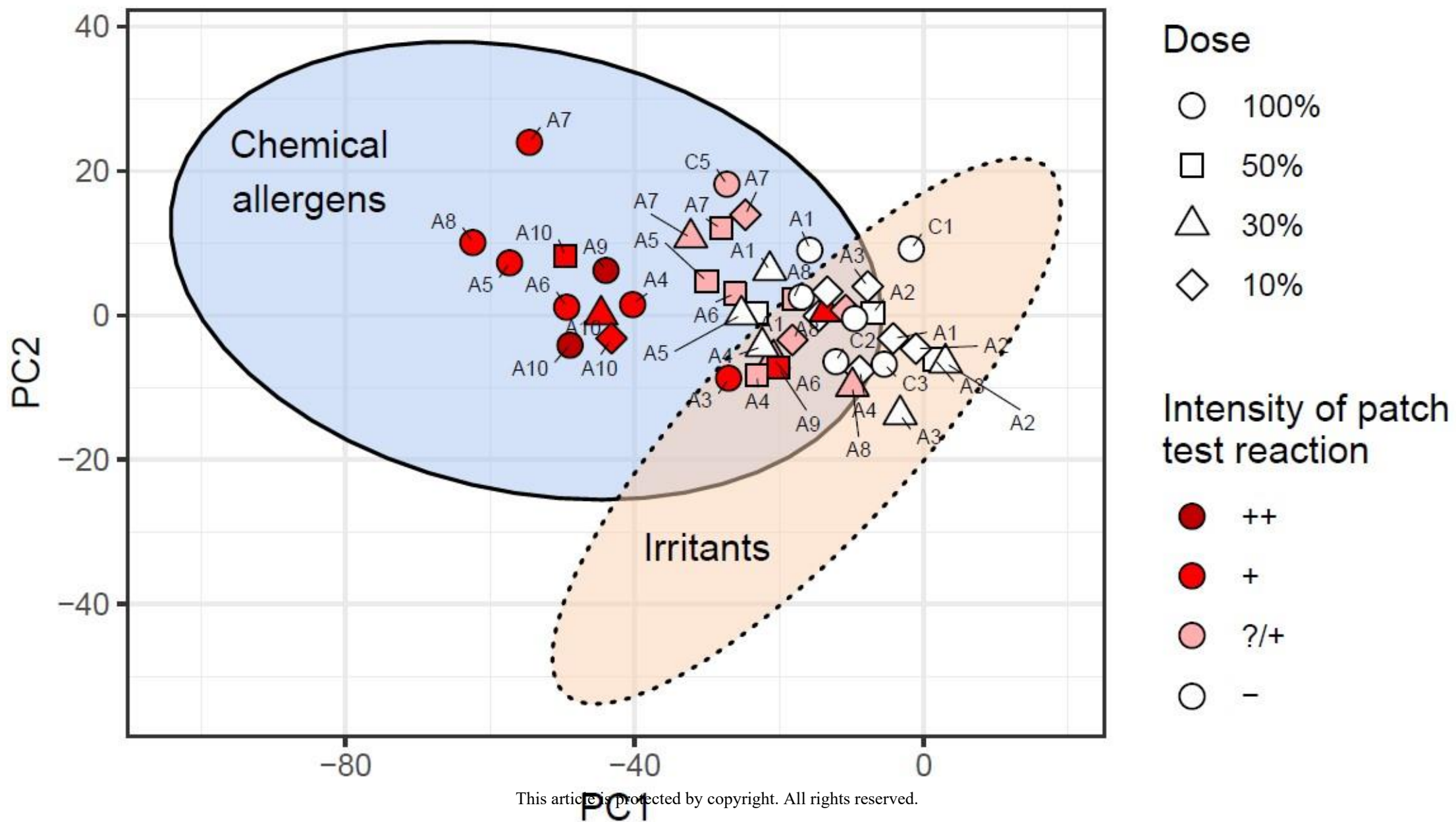


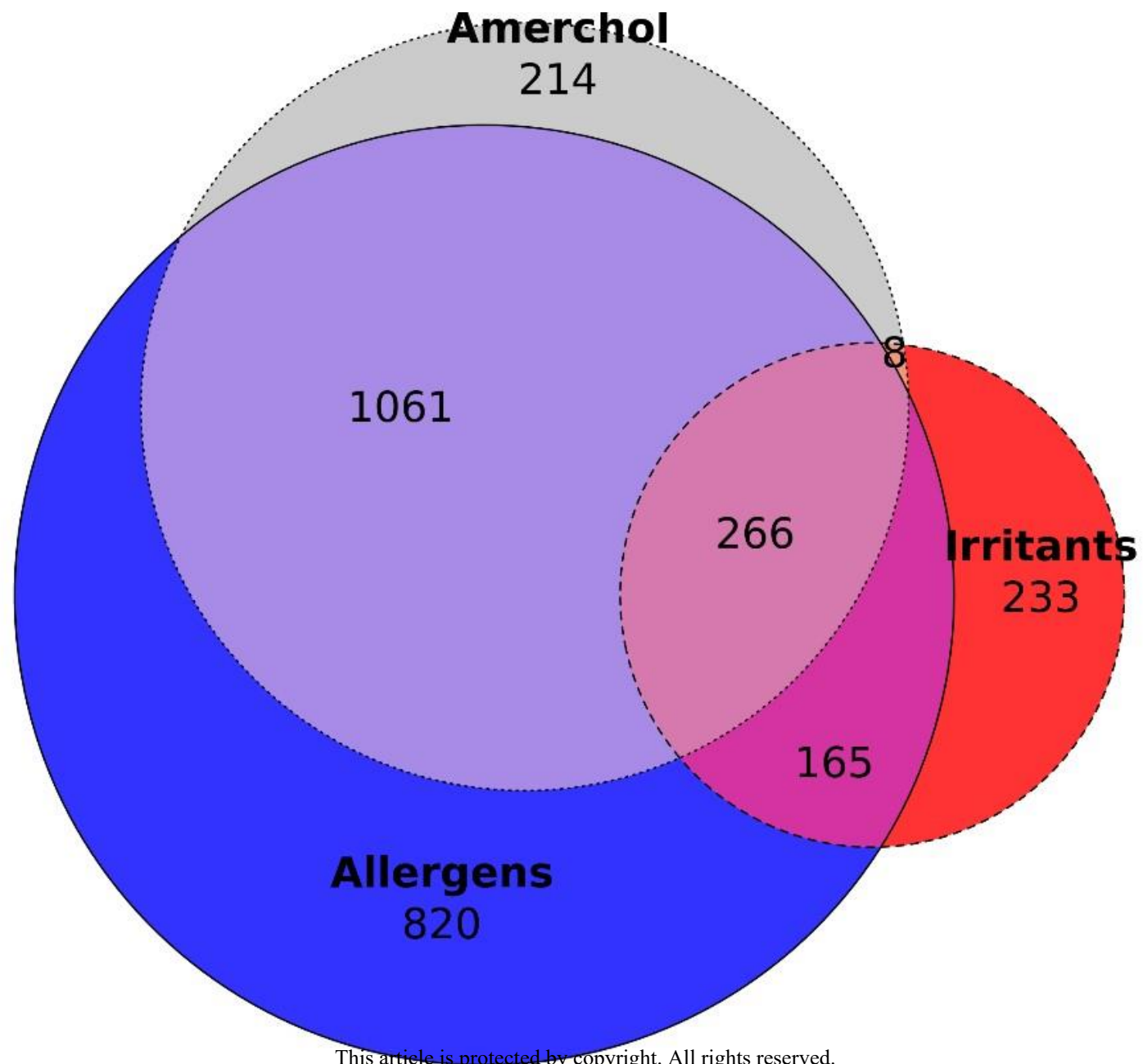
Figure 2b

Figure 3

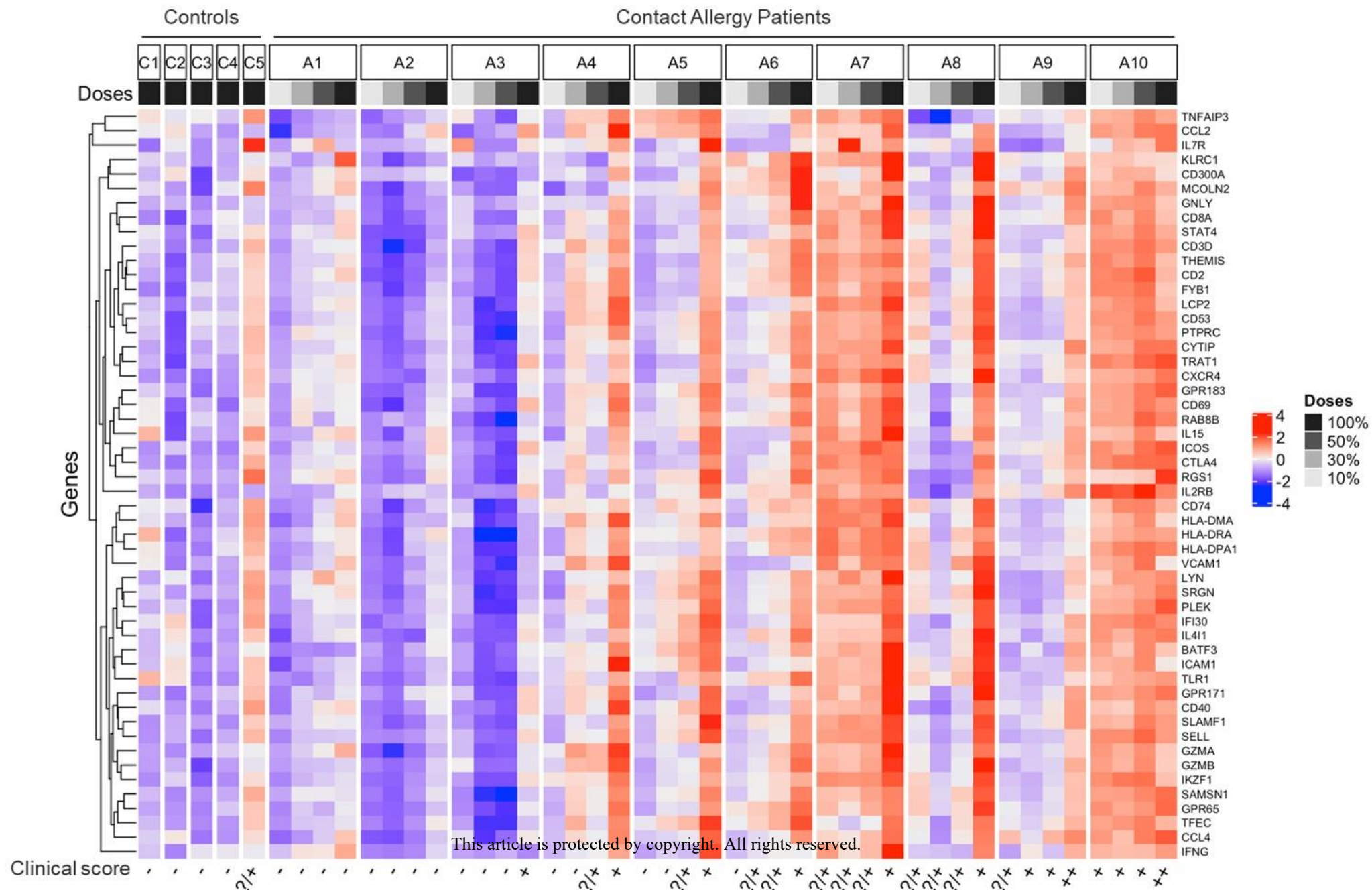


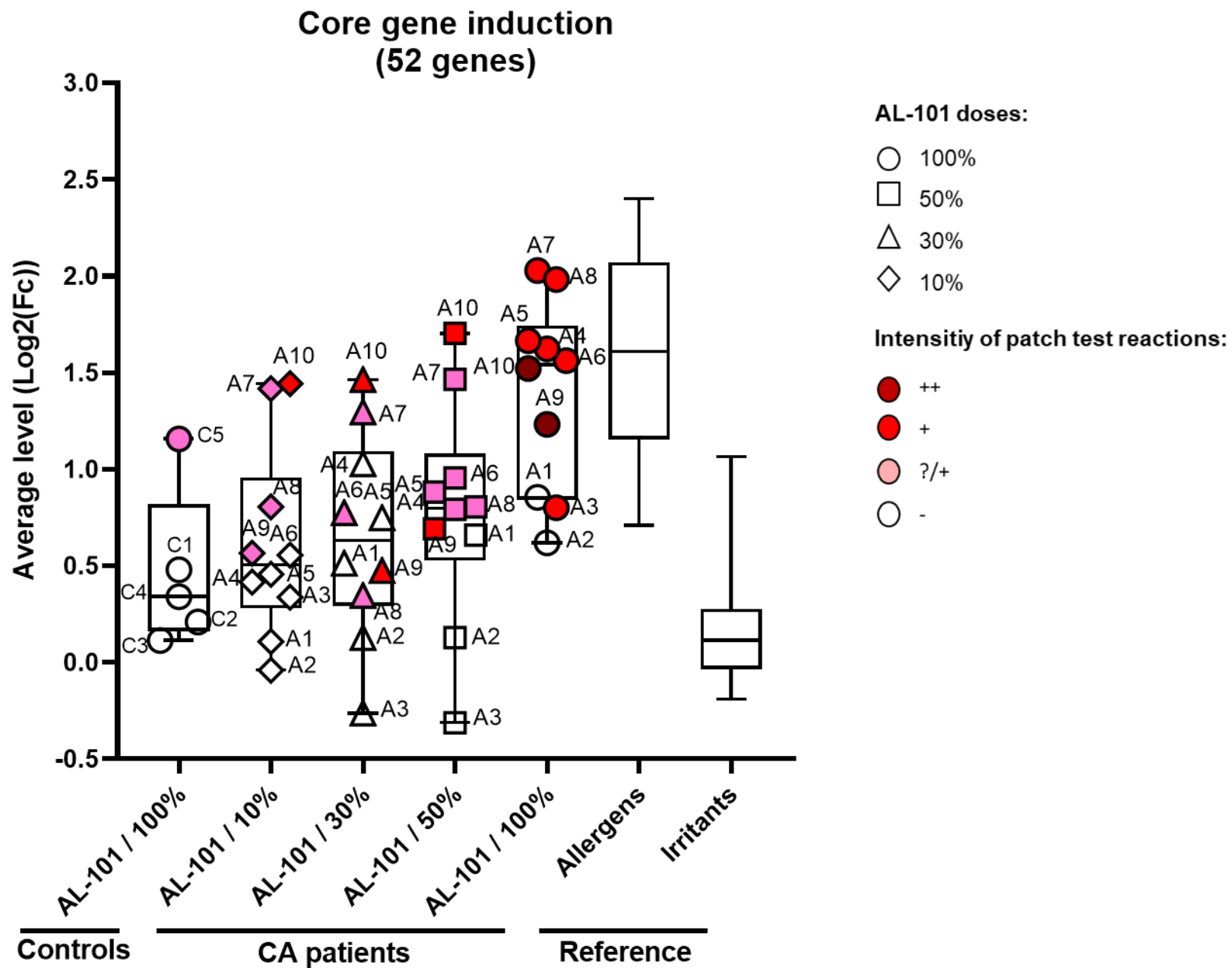
Figure 4

Figure 5

