

## INVESTIGATIVE REPORT

# Scalp Stratum Corneum Histamine Levels: Novel Sampling Method Reveals Association with Itch Resolution in Dandruff/Seborrhoeic Dermatitis Treatment

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**Dandruff and seborrhoeic dermatitis are accompanied by bothersome itch. We have established a novel non-invasive methodology to sample histamine levels in the stratum corneum in order to facilitate an understanding of pruritogenesis in this condition. Histamine levels were assessed in two groups of subjects with dandruff before and after 3 weeks of treatment with a commercial potentiated zinc pyrithione shampoo. A comparative population without dandruff was also studied. Itch self-perception was quantified on a visual analogue scale. The histamine level in subjects with dandruff was more than twice that in those who did not have dandruff. Under conditions known to resolve flaking symptoms, the shampoo led to a reduction in histamine in subjects with dandruff to a level that was statistically indistinguishable from those who did not have dandruff. This reduction in histamine was accompanied by a highly significant reduction in the perception of itch intensity. These findings suggest an association between the subjective perception of itch in the scalp and the level of histamine in the skin.**  
*Key words: histamine; dandruff; seborrhoeic dermatitis; pruritus; stratum corneum: scalp.*

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Dandruff and seborrhoeic dermatitis (D/SD) are common chronic relapsing scalp skin disorders that share some clinical features with psoriasis and atopic dermatitis. While seborrhoeic dermatitis can affect sebum-rich areas other than the scalp, we routinely refer to these conditions on the scalp collectively as “dandruff” (1). Like psoriasis and atopic dermatitis, the pathogenesis of dandruff is complex, and appears to be the result of interactions between scalp skin, cutaneous microflora and the cutaneous immune system (1, 2). The key clinical features of dandruff include flaking and itch, and although much descriptive work has been done, the precise underlying events that provoke these symptoms are incompletely understood.

Itch is the most frequently described symptom among disorders affecting the scalp (3), and although the precise cause of pruritus in dandruff is not known, it is quite possible that a cascade of events at the surface of the scalp leads to a release of histamine that then results in the perception of itch. Histamine is well known as a chemical mediator that plays important roles in allergic inflammatory and immune reactions (4, 5), and its role in itch has been particularly well established in urticarial reactions, poison ivy and insect bites (6). However, for skin disorders that result from more complex aetiopathological mechanisms, such as atopic dermatitis, the association between histamine and itch is not definitively established (7). Nevertheless, increased levels of histamine have been associated with itching in skin disorders ranging from ordinary dry skin to psoriasis (8, 9).

The role of histamine as a key mediator of itch in the skin is supported by studies involving the administration of anti-histamines, as well as studies involving the induction of itching from superficial injections of histamine (10). In the skin, histamine is synthesized by mast cells that reside in the dermis, as well as by the keratinocytes themselves (11, 12). To our knowledge, histamine and its association with itch have not been studied in dandruff beyond investigations of scalp barrier function that involved topical administration of histamine to subjects with dandruff (13). In addition to demonstrating the poor barrier quality in dandruff, this work showed that scalp skin is responsive to exogenous histamine in the form of increased itch perception. On this basis, endogenous histamine becomes a very reasonable potential biomarker for the itch experienced in dandruff.

We have been successful in non-invasively monitoring various biomarkers in scalp stratum corneum (SC) that accurately reflect biochemical events taking place within the viable epidermis that are causally linked to dandruff pathogenesis (14). Accordingly, building on this experience, we asked whether histamine could be measured in tape strip extracts from scalp SC to determine whether there are any differences in histamine content in dandruff lesional areas vs. subjects who do not have

dandruff. We also asked whether SC histamine levels could be associated with the perception of itch by means of subjective assessments. To do this, we placed subjects with dandruff on a treatment regimen with a potentiated zinc pyrithione (ZPT)-containing shampoo (15) that is known to resolve all of the key symptoms of dandruff, including flaking, inflammation, and restoration of barrier integrity (15), and monitored how histamine levels changed as a function of treatment, and how itch perceptions changed under the same conditions. Insights developed from this novel capability are enabling the evaluation of the hypothesis of the role of histamine in mechanisms of puritogenesis associated with D/SD.

## MATERIALS AND METHODS

### Study designs, populations and test product

Two similar clinical studies were designed and designated "Study 1" and "Study 2." Both were double-blind randomized studies and were carried out under good clinical practice guidelines in accordance with the US 21CFR 312.66, the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Harmonized Tripartite guidelines, and in compliance with federal, state and local government regulations, guidelines and standards applicable to such studies including, but not limited to, those relating to an Institutional Review Board (IRB), the *Health Insurance Portability and Accountability Act of 1996* (HIPAA) compliant informed consent, and good clinical practice. Informed consent was provided by all study participants. Both studies were reviewed and cleared by ethics committees (the boards used by each clinical research organization involved are as follows: ERF (Essex IRB, Lebanon, NJ, USA), Bower (Bower IRB, Colorado Springs, CO, USA), Hilltop (Ontario IRB, Toronto, Canada) and CRG (Independent IRB, Plantation, FL, USA for Study 1 and CRG-IRB, St Anthony, MN, USA for Study 2).

**Study design.** Both Studies 1 and 2 employed the same study design (except where noted), as summarized in Fig. 1. Subjects underwent a 2-week pre-treatment equilibration period, during which they shampooed their hair at least 3 times a week using a placebo-type cosmetic shampoo without conditioning agents. Baseline measurements were made 72 h after the last use of equilibration shampoo and included dandruff severity (Adherent Scalp Flake Score; ASFS) and histamine level (both described in detail below). To be eligible to participate in the subsequent treatment phase of the study, the subjects must have an ASFS of 24 or greater. Upon enrolment, patients were randomized to a treatment group prior to skin surface sampling for histamine analysis (see below). The randomization was stratified by gender, baseline ASFS (24–28, 29–38 and 39–80) and frequency of styling product usage (< than once a week and ≥ once a week). Subjects were instructed to use only the assigned treatment and to use it three times a week for 3 weeks. They then refrained from shampooing their hair for ap-

proximately 72 h, prior to assessment of the Week 3 ASFS value and subsequent skin surface sampling for histamine analysis. In addition to the subjects with dandruff, Study 1 also contained subjects who did not have dandruff, defined by ASFS ≤ 12, who were evaluated at baseline only (they were selected based on the lack of flakes rather than itch self-perception). All subjects were instructed not to use any scalp treatments, scalp sunscreens, hair relaxers, permanent waves, bleaches, hair conditioners or hair colorants during the study period. Test product usage was carried out at home, the product was supplied as blinded bottles of shampoo for Study 1 (used at least three times per week), and as 10 ml unit doses for Study 2 (used three times a week).

**Patient populations.** Healthy male and female subjects, age range 18–75 years, who had dandruff were recruited from databases maintained by the clinical research organizations utilized. See Table I for a summary of the patient population statistics, base size and completion rates for each study. Study populations were drawn from the general populations of Colorado Springs, Colorado, Minneapolis, Minnesota, Lynchburg, Virginia, in the USA, and Winnepeg, in Canada.

**Test product.** The test product was a commercial potentiated (15) zinc pyrithione (ZPT)-based anti-dandruff shampoo (Head & Shoulders Classic Clean, manufactured by Procter & Gamble).

### Measures

**Severity of dandruff.** Dandruff severity was assessed as adherent scalp flaking score (ASFS) by published procedures (16). Assessments were performed by a qualified grader trained by a board-certified dermatologist trained in the method. Briefly, severity is judged on a 0–10 scale (none to severe) for each of eight regions of the scalp, yielding a total ASFS ranging from 0 to 80.

**Histamine sampling.** D-Squame® tapes (CuDerm, Dallas, TX, USA) were collected from each subject at each time-point (baseline and week 3), except for the subjects who did not have dandruff, who were sampled at baseline only. The site of sampling was determined individually by the distribution of severity of flaking; samples were taken from the scalp octant with the highest flaking score at baseline and from an active flaking region. This area was recorded for each individual in order to enable the same site to be sampled at Week 3. The tape samples were collected by exposing the scalp skin by parting the hair (using a comb and clips), the tape was placed on the part area and rubbed repeatedly (15–20 strokes) with the blunt end of a forceps, with sufficient pressure to ensure good contact while not inducing a negative sensation (training involved pushing hard enough on a balance to achieve a 100 g reading). The tape strip sampling was repeated additional times at the same site by placing each D-Squame® tape disc on top of the prior sampled area. The tapes were stored in labelled 12-well plates at –80°C until analysis. In Study 1, the second sequential tape from a given site was used for analysis, whereas in Study 2, the initial tape sample was used.

**Histamine analysis.** Two different analytical methods were employed, Method 1 was based on high-performance liquid

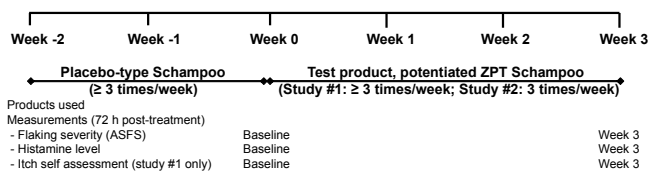


Fig. 1. Overview of protocols used in Study 1 and Study 2. For details, see Materials and Methods.

Table I. Patient population summary

Study	Group (n)	Age, years Mean (SD)	Gender (M/F) %	Completion %
Study 1	Dandruff (315)	45.5 (13.4)	44.7/55.3	99
	Non-dandruff (121)	48.4 (13.0)	33.9/66.1	N/A
Study 2	Dandruff (125)	44.0 (13.7)	39.8/60.2	94

SD: standard deviation; M: male; F: female; N/A: not applicable.

chromatography tandem mass spectrometry (HPLC/MS/MS) and used to quantify histamine in the tape strips of Study 1; Method 2 was based on enzyme-linked immunoassay (ELISA) and used for histamine quantitation of Study 2.

**Histamine method 1: gradient reversed-phase HPLC/MS/MS.** Tape strips were placed into individual polypropylene vials, each vial was spiked with a stable isotope-labelled histamine (D4-histamine) internal standard (ISTD) and then extracted with acidified water (0.1% formic acid in distilled-deionized water) using sonication for 10 min. Each extract solution was isolated from the tape strip and an aliquot of each sample was placed into a specified position of a 96-well polypropylene plate. A set of histamine standards was prepared in the 96-well polypropylene plate over an appropriate calibration range in the acidified water and spiked with ISTD. The standards and the extracts of the scalp tape strips were analysed using gradient reversed-phase HPLC/MS/MS. Histamine and the ISTD were monitored by positive ion electrospray (ESI) using multiple reaction monitoring with the precursor ions of 112 m/z (histamine) and 116 m/z (ISTD) and product ions 95 m/z (histamine) and 99 m/z (ISTD). A standard curve was constructed by plotting the signal, defined here as the peak area ratio (peak area histamine/peak area ISTD), for each standard vs. the mass of histamine for the corresponding standard. The mass of histamine in the calibration standards and human scalp extract samples were then back-calculated using the generated regression equation. The result was reported as the mass of histamine (ng)/ $\mu$ g of protein that was found in the tape strip extract as determined with the BCA™ Protein Assay Kit (Pierce Biotechnology/Thermo Scientific, Rockford, IL, USA). The detection limit was 100 pg/tape strip.

**Histamine method 2: ELISA.** Tape strips were extracted with phosphate-buffered saline (PBS) containing an additional 0.25 M sodium chloride (NaCl) and a commercially available protease inhibitor cocktail containing a mixture of protease inhibitors with broad-spectrum inhibitory specificity (Roche Applied Science, Indianapolis, IN, USA) for 30 min with sonication on ice. The extracts were then centrifuged for 5 min at 2100 rpm to remove skin solids that may interfere in the assay. Aliquots of these extracts were then analysed for soluble protein using the BCA™ Protein Assay Kit (Pierce Biotechnology/Thermo Scientific, Rockford, IL, USA) using bovine serum albumin (BSA), as a standard. After protein analysis, extracts were supplemented with 2% BSA, transferred into 96-well polypropylene deep-well plates and frozen at  $-80^{\circ}\text{C}$  for histamine analysis. Histamine was quantitated using a High Sensitivity Enzyme Immunoassay kit (US distributor, Cayman Chemical Ann Arbor, Michigan, SPIbio manufacturer). Histamine concentrations were reported as histamine standardized to the amount of soluble protein in the extract as determined by the BCA protein assay, and data are reported as ng/ $\mu$ g soluble protein.

**Itch self-perception assessment.** In Study 1, patients were asked to respond to the question “How would you rate the severity of your scalp itch today?” at Baseline and at the end of 3 weeks of treatment. A visual analogue scale (VAS) was used in which “None” represented zero and “Severe” scored as ten. No other descriptors were present on the VAS.

#### Statistical analysis

**Dandruff severity.** The change from baseline for ASFS was analysed using an analysis of covariance (ANCOVA) model. The model included treatment, study site, gender, baseline measurement, and age as covariates.

**Histamine level.** To minimize the variability of histamine levels both between subjects as well as within subject (baseline

to week 3), the biomarker data was transformed to the log10 scale before analysis. Statistical analysis was carried out on the equivalent log10 Ratio values (i.e. log10 (Week 3/Baseline)). Final reported biomarker results were then converted back to their original scale (or % change from baseline values), for ease of interpretation. All statistical analyses were carried out using the SAS JMP (Version 7.0.2) software. A *p*-value of  $\leq 0.05$  (two-sided) was used to determine statistical significance.

**Itch self-perception.** Data was analysed using an ANOVA model, which included the following model terms: treatment (fixed), study site (fixed), and study by site interaction. In addition, baseline data was added to the model as a covariate for the change from baseline data.

## RESULTS

### *Resolution of scalp flaking following treatment with 1% ZPT shampoo*

Table II summarizes the magnitude of reduction in flake appearance as assessed by the ASFS parameter. Statistically significant ( $p < 0.05$ ) reductions, of 17.7 and 11.9 units, were observed in Studies 1 and 2, respectively.

### *Reduction in histamine content of the stratum corneum*

The reductions in SC histamine levels observed in both Studies 1 and 2 are summarized in Table III. Significant reductions as a result of 3 weeks of use of the potentiated ZPT shampoo were observed in both cases. When expressed as relative reduction from baseline (Table III), 46% and 45% reductions were observed in Studies 1 and 2, respectively. Study 1 also included a comparative population without dandruff. Using the basal histamine level found in the population without dandruff in Study 1, the magnitude of reduction due to treatment is shown to almost completely normalize the histamine in the SC (Fig. 2). This substantial normalization of histamine levels occurred under the same conditions that bring about a resolution of all of the major symptoms of dandruff, i.e. flaking, itching, inflammation, barrier integrity (data not shown).

### *Reduction in itch self-perception*

The data from an itch self-perception question in Study 1 are summarized in Table III. A significant reduction in the severity of itch was observed, coinciding with the reductions in flaking severity and histamine levels in this study.

Table II. Reduction in flake appearance as adherent scalp flake score (ASFS)

Study	Baseline ASFS score (SE)	Week 3 ASFS score (SE)	Change from baseline score (SE)
Study 1	33.01 (0.46)	15.19 (0.47)	17.7 (0.47)
Study 2	32.47 (0.75)	20.55 (0.93)	11.9 (0.93)

SE: standard error.

Table III. Summary of measurement parameter statistics

Study	Time or Group	Histamine (ng/μg protein)					Itch self-perception			
		Median	Min.	Max.	Mean	Δ Baseline (SE)	Min.	Max.	Mean (SE)	Δ Baseline
1	Week 0	0.803	0.004	24.8	0.497		0 <sup>a</sup>	10	5.107 (0.180)	
	Week 3	0.290	0.003	20.0	0.267	46% (4.7)	0	10	3.060 (0.171)	2.243 <sup>b</sup>
	Non-dandruff	0.230	0.004	11.1	0.207					
2	Week 0	0.792	0.003	25.8	0.490					
	Week 3	0.190	0.007	14.8	0.263	45% (12.0)				

<sup>a</sup>Five subjects out of 224 had a baseline itch severity of zero, all were included in the analysis.

<sup>b</sup>Does not represent a simple difference of means due to baseline adjustment; see Methods;  $p < 0.0001$ .

Min.: minimum; Max.: maximum; SE: standard error.

## DISCUSSION

The purpose of this study was to investigate the importance of histamine in scalp D/SD using a novel non-invasive sampling technique, and to assess its relationship to itch perception. Itch is the most frequently observed symptom associated with an unhealthy scalp and the one that has the largest negative impact on quality of life (3).

The perception of itch represents the end of a complex physiological pathway initiated at the skin surface by a number of stimuli that can cause the release of histamine from mast cells in the dermis (6). Using invasive sampling techniques, higher tissue histamine levels have been previously found in eczema (17), psoriasis (18, 19) and dry skin (9), and, importantly, itch frequently accompanies these conditions. While histamine levels in D/SD have not been investigated in the past, topical application of exogenous histamine to subjects with dandruff increases the severity of itch (13), suggesting an association between itch perception and histamine levels, whether exogenously supplied or endogenously stimulated.

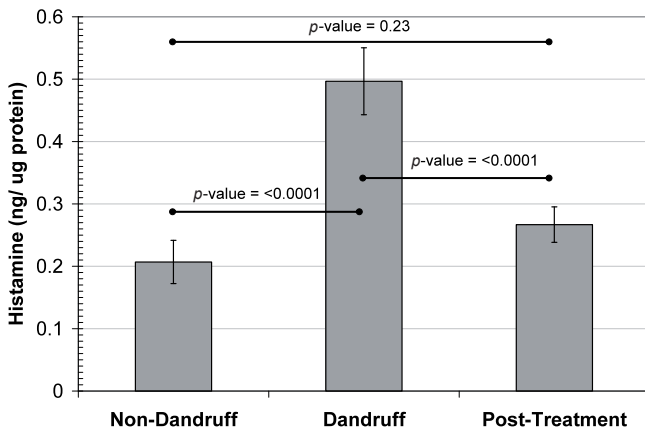


Fig. 2. Specific content of stratum corneum (SC) histamine in normal and dandruff and seborrhoeic dermatitis (D/SD), and its reduction following treatment with potentiated zinc pyrithione (ZPT)-containing shampoo: subjects taking part in Study 1 (see Materials and Methods) used a 1% potentiated ZPT-containing shampoo three times per week for 3 weeks and were assessed for SC histamine level at baseline and after 3 weeks of product use. Additionally, non-dandruff subjects (Adherent Scalp Flake Score (ASFS)  $\leq 12$ ) were assessed at baseline. The levels of the histamine are standardized to soluble SC protein and reported as ng/μg protein ( $\pm$  standard error).

We found, for the first time, that histamine could be detected in scalp SC and that dandruff SC displayed a more than two-fold higher specific content of histamine compared with scalp SC from subjects who did not have dandruff. These findings are consistent with the known symptomatology of dandruff (20). We also demonstrated that a potentiated ZPT-containing shampoo, when used in a treatment regimen known to resolve the symptoms (15) of dandruff, led to an almost complete normalization of SC histamine to basal levels, which was accompanied by a highly significant reduction in the subjective perception of itch quantified by VAS.

While scalp histamine levels in dandruff is elevated, it is not clear what the source of histamine is in the SC samples as we have quantified mast cells and their state of degranulation in the scalp and showed no evident differences between baseline and 3 weeks of treatment of subjects with dandruff (K. J. Mills, unpublished observations) although the scalp contains abundant mast cells (21). It is possible that the source is the viable epidermis (12), but additional work will be needed to definitively address this possibility. This raises interesting questions regarding the role of histamine in normal skin homeostasis. It is clear that there is abundant histamine present in the scalp SC of subjects who do not have dandruff, and most do not complain of any itching sensations. Whether the histamine is pre-packaged in the SC, or is simply diffusing from the viable epidermis is not known, but it is interesting to note that a number of recent studies show that histamine may play an important role in innate immunity and in the amplification of inflammatory cascades that are secondary to breach of the epidermal barrier, wounding, cutaneous infection, and inflammatory dermatoses, of which dandruff is one (22). It is interesting to speculate that, in dandruff, histamine release may exceed a certain threshold, above which itch perception becomes significant.

These findings suggest a relationship between scalp histamine and itch in dandruff, but do not prove a mechanistic linkage as there are many other known mediators of itch in the skin, such as acetylcholine, tryptase, serotonin, substance P, prostaglandin E, leukotrienes, and interleukin-31 (23), and these were not measured.

The findings reported in the present study do, however, provide a basis to ask many new mechanistically-oriented questions regarding scalp itch in dandruff. The results also provide a way forward in the development of agents that could contribute to therapeutic efficacy by more effectively treating one of the key symptoms of dandruff. A logical follow-up study is to evaluate the impact of anti-histamines on detected skin histamine levels and resultant itch perception. Most dandruff therapies are primarily focused on flake reduction, which is a key to, but by no means the only important symptom of, this disorder. The discovery of new anti-itch technologies has been challenged by the difficulties associated with the objective measurement of itch and its mitigation, and although this is being investigated using techniques such as positron emission tomography (PET) scanning and functional magnetic resonance imaging (fMRI) (24), such techniques are not amenable to high throughput, cost-effective screening. If histamine proves to be causally associated with the induction of itch in dandruff then the two-pronged approach we have developed, i.e. non-invasive determination of SC histamine at baseline and end of treatment, together with VAS determination of subjective itch perception could be a useful means to discover new anti-itch technologies in the clinic. Similar approaches are showing promise in simultaneously following key chemokines and itch perception associated with atopic dermatitis (25).

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*Conflict of interest:* KK, JRS, TF, AF, KW and KJM are employees of The Procter & Gamble Company, which paid 100% of the costs of this work. JCS received compensation for activities as an expert consultant.

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