LUND UNIVERSITY
Faculty of Medicine

LUP

Lund University Publications
Institutional Repository of Lund University

This is an author produced version of a paper published in International archives of occupational and environmental health. This paper has been peer-reviewed but does not include the final publisher proof-corrections or journal pagination.

Citation for the published paper:

Lena S Jönsson, Karin Broberg, Karin Paulsson,
Kerstin Kronholm Diab, Jörn Nielsen

"Gene expression in nasal lavage from hairdressers
exposed to persulphate."

International archives of occupational and environmental health,
2009 Mar 31

http://dx.doi.org/10.1007/s00420-009-0415-8

Access to the published version may require journal subscription.

Published with permission from:
Springer Verlag

The original publication is available at www.springerlink.com

Gene expression in nasal lavage from hairdressers exposed to persulphate

Deleted: Short title: Gene expression in nasal lavage from hairdressers

Lena S. Jönsson, PhD; Karin Broberg, PhD; Karin Paulsson, Biomedical Scientist; Kerstin Kronholm Diab, RN; Jørn Nielsen MD, PhD

Division of Occupational and Environmental Medicine, Department of Laboratory Medicine, Lund University Hospital, SE-221 85 Lund, Sweden

Corresponding to: Lena Jönsson, Division of Occupational and Environmental Medicine, University Hospital, SE-221 85 Lund, Sweden

Phone no. +46 46 173192, Fax no. +46 46 143702, E-mail: lena_s.jonsson@med.lu.se

Objectives

Many hairdressers experience work-related symptoms from the airways caused by bleaching powder. This contains persulphates, which could be irritating to the mucous membrane and also may evoke an allergic reaction. However, specific IgE antibodies are difficult to detect. We found earlier that hairdressers with work-related bleaching powder-associated nasal symptoms reacted to persulphate, but that atopics also did and that the mechanism appeared to be similar in the two groups. In this study, we analysed gene expression of cytokines in the nose in order to further investigate the mechanism for work-related bleaching powder-associated nasal symptoms.

Methods

The study subjects belonged to either hairdressers with work-related bleaching powder-associated nasal symptoms (S; n=6), hairdressers without work-related bleaching powder-associated symptoms (WS; n=7) or atopics (A; n=6). Nasal lavage was performed before and during (up to 4 hours after the last challenge) provocation with potassium persulphate. Expression of two genes involved in allergic inflammation [interleukin 5 (*IL5*) and *IL13*] and one involved in cell-mediated immunity (interferon-gamma; *IFNG*) were analysed in nasal lavage with quantitative PCR.

Results

The change of *IL5* in the S group differed when compared to the WS group (P=0.051), in the A group when compared to the WS group (P=0.014), but not in the S group when compared to the A group (P=0.82). The change of *IL13* in the A group differed when compared to the S (P=0.041) and WS (P=0.014) groups, but no difference was noticed between the S and WS

groups (P=0.30). The relative level of *IFNG* increased from before challenge to during challenge in the S group (P=0.031).

Conclusions

Symptomatic hairdressers showed increased expression of *IL5* and *IFNG*, but not *IL13*, during challenge. Hairdressers without work-related bleaching powder-associated nasal symptoms showed no markedly changed reaction. Atopics showed increased expression of *IL5* and *IL13*. Thus, this may indicate a difference in the mechanism of symptoms between symptomatic hairdressers and atopics. However, due to the low number of participants, further studies are needed to elucidate the mechanism for persulphate-associated nasal symptoms.

Keywords Cytokines, Gene expression, Interferon, Interleukin, Persulphate

Introduction

Work-related airways symptoms, especially rhinitis, are very common in hairdressers (Albin et al. 2002; Brisman et al. 2003; Leino et al. 1997, 1998). Among hairdressers who could point out a causative agent for their airway symptoms, 80 – 90% named bleaching powder (Albin et al. 2002; Leino et al. 1998).

Bleaching powder contains persulphates known to elicit hypersensitive reactions from the airways (Moscato and Galdi 2006) and persulphate may be associated with nasal symptoms in hairdressers (Kronholm Diab et al. 2009). However, the mechanism is largely unknown. Skin prick testing reveals few cases of sensitisation (Aalto-Korte and Mäkinen-Kiljunen 2003; Hytönen et al. 1997; Leino et al. 1998; Moscato et al. 2005; Muñoz et al. 2003), and even among sensitised hairdressers only one study reports records of specific immunoglobulin (Ig) E against persulphate in sera (Aalto-Korte and Mäkinen-Kiljunen 2003).

Changes in gene expression are quick and sensitive responses to the environment. For assessment of changes associated with symptoms of the nose, expression in this organ will probably be more pronounced compared to changes in the circulation. Also, it has been suggested that the nasal cavity is useful for assessing the levels of biomarkers that may affect the respiratory system.

The aim of this study was to evaluate whether or not nasal lavage could be collected and used for gene expression analysis on an individual basis. In order to elucidate which type of reaction is involved in work-related bleaching powder-associated nasal symptoms, we analysed two genes, which are involved in allergic inflammation [interleukin 5 (*IL5*) and *IL13*], and one gene, which is involved in cell-mediated immunity (interferon-gamma; *IFNG*), before and during challenge with potassium persulphate among hairdressers and atopics.

Materials and methods

Study subjects

Forty-one women were included in the original study by Kronholm Diab et al. 2009. In this study, the inclusion criteria were successful RNA extraction and amplification, which was achieved from 19 of the subjects (Table 1) from the original study by Kronholm Diab et al. 2009. Those 19 subjects belonged to three groups: the target group consisted of female hairdressers with work-related nasal symptoms mainly related to bleaching powder (S). Two comparison groups were employed, one of female hairdressers without work-related bleaching powder-associated symptoms (WS), another of atopic (by history) females not earlier exposed to persulphates and thus not sensitised to persulphates. None of the study subjects had asthma and none of the hairdressers had a history of atopy. Nasal symptoms were scored according to Malm et al. (1981) as a marker of effect. In brief, nasal blockage and secretions were rated and the number of sneezes was scored. A total score was calculated by addition of the score for each symptom. The study subjects gave their informed written consent and the study was approved by the Regional Ethical Committee of Lund University.

Samples and RNA extraction

We performed a nasal challenge with potassium persulphate [0.001 and 0.01% (w/v)] in isotonic saline solution and collected five nasal lavages from each study subject [0h, 1h (immediately before first challenge), 1.5h (25 min after second challenge), 3h and 5h]. In order to obtain sufficient number of cells from the nose, 45 ml saline solution was at each time transmitted to the nasal cavity using a syringe. The nasal lavage was sampled in a pre-chilled test tube and the cells were spun down (2,000 x g, 5 min, 4°C). The cells were then homogenized through a homogeniser (Invitrogen; 12 000 x g, 2 min, 25°C) and total RNA was extracted by Micro-to-Midi Total RNA Purification System (Invitrogen) according to the

manufacturer's protocol. The samples were pooled into two samples; (I) before challenge and (II) during and after challenge; due to limited amount of cells. The extracted RNA was DNase-treated by DNA-free™ kit (Ambion), concentrated by precipitation with Linear Polyacrylamide (Sigma), 3 M NaAc and 100% ethanol, washed with 75% ethanol, and eluted in 11 µl DEPC-treated water.

Amplification and cDNA synthesis

It is difficult to obtain enough RNA for the analysis from nasal lavage. Therefore, in order to achieve more of messenger RNAs, the samples were amplified by one round of linear amplification by MessageAmp™aRNA Kit (Ambion) according to the manufacturer's instructions. The samples were transcribed in vitro during 14 hours and DNase-treated once more. This procedure results in amplification of all messenger RNAs in a sample.

Study subjects giving rise to I and II samples containing at least 1 µg each of amplified RNA, as measured by Bioanalyzer (Agilent), were further analysed. For subsequent quantitative PCR analysis, RNA was transcribed into cDNA as follows: amplified RNA, maximum 5 µg of each sample, was transcribed into cDNA by Superscript™ II RNase H$^-$ Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions, except the following alterations: usage of random hexamers (10 µM, Amersham Biosciences) as primers, RNAguard (Amersham Pharmacia Biotech) as RNase inhibitor, and 40 mM of deoxynucleotide triphosphates (Amersham Pharmacia Biotech).

Quantitive PCR analysis

Quantitative PCR allows you to quantify the amount of RNA in one sample relative to another sample (the calibrator). To normalise for differences in the amount of RNA added to each PCR reaction, an endogenous control is used. This is an active reference (the signal is

generated as a result of the PCR amplification) that constitutes a gene with a stable expression during the challenge with persulphate. In order to find a reliable endogenous control, we examined different housekeeping genes during nasal challenge. The genes were glyceraldehyde-3-phosphate dehydrogenase, *GAPDH*; succinate dehydrogenase flavoprotein subunit A, *SDHA*; ubiquitin C, *UBC*; and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide, *YWHAZ*. *YWHAZ* showed the most stable expression (data not shown) and thus were used as endogenous control. Analysis of three genes of interest, *IFNG*, *IL5* and *IL13*, was performed. Primers were designed at the 3' end due to biased linear amplification of the 3' end (Table 2). In order to only amplify and quantify genes that are expressed, concern was taken not to include pseudogenes.

For the analysis of *IL5*, *IFNG* and *YWHAZ*, the optimised primer concentration was 0.3 µM for forward and reverse primers (Invitrogen). SybrGreen Master Mix containing uracil-N-glycosylase and Rox (Applied Biosystems) and 2 µl cDNA template in a total volume of 25 µl, was used. The PCR program for amplification was: 50°C for 2 minutes, 95°C for 10 minutes and thereafter 50 cycles consisting of 95°C for 15 s and 60°C for 1 minute (ABI 7000; Applied Biosystem).

For the analysis of *IL13*, the optimised primer concentration was 0.1 µM for forward and reverse primers. Platinum SuperMix-UDG containing 6 mM $MgCl_2$ and 1x Rox (Invitrogen) and 2 µl cDNA template in a total volume of 25 µl, was used. The PCR program for amplification was: 50°C for 2 minutes, 95°C for 2 minutes and thereafter 50 cycles consisting of 95°C for 15 s, 61°C for 30 s and 72°C for 30 s (ABI 7000; Applied Biosystem).

Data analysis

Relative expression for each sample was calculated by dividing the mean quantity of the gene of interest by the mean quantity of the endogenous control (each derived from the standard

curve described below), as described by the manufacturer (Applied Biosystems; User Bulletin #2, updated 10/2001). Thereafter, the relative expression in the sample I was compared to the relative expression in the sample II (the calibrator) for each study subject. Thus, a difference in expression between before and during challenge could be detected. The same dilution of cDNA was used for the same person for both the I and II samples, but the dilutions differed by study subject and gene.

A standard curve was included in each run to validate the efficiency of the run and for quantifying the amount of gene of interest/endogenous control. RNA for the standard curve was obtained from phytohemagglutinin-stimulated lymphocytes and monocytes. The RNA was transcribed into cDNA in the same way as the RNA from the study subjects and the cDNA was repeatedly diluted 1:10 in ddH_20. Five to six dilutions were used in each analysis and each dilution, as well as all samples, were analysed in triplicates. The correlation coefficient describing the linearity of the standard curve (the R^2 value) was ≥ 0.99 in all runs.

The sample was reanalysed if the standard deviation was larger than 0.3 and no replicate could be assigned to be deviant. Samples below the detection limit (two samples for each gene of interest) were assigned a value corresponding to half the value of the last point in the standard curve.

Statistical analysis

Differences between groups were analysed with Mann-Whitney *U* test. Differences between samples I and II were analysed with Wilcoxon signed ranks test. For all statistical analyses, exact tests in SPSS v.13.0 (SPSS Inc., Chicago, IL, USA) were used.

Results

Hairdressers with work-related bleaching powder-associated symptoms showed increased symptom score during challenge, whereas the WS group did not react (Table 3). Atopics also showed a response but to a lesser extent than the symptomatic hairdressers.

The change in the sample II compared to the sample I, regarding expression of *IL5* and *IL13*, normalised to *YWHAZ*, differed between the groups (Fig. 1). There was no difference in relative expression of *IFNG* during challenge when comparing the groups.

When comparing the relative level (median) in samples I and II in different groups (Fig. 2), an increase in *IL5* was seen in the S and the A group, but not in the WS group. With regard to *IL13*, an increase in the A group, but not in the S or the WS group, was observed. However, those changes were not statistically significant. The relative level of *IFNG* was increased in sample II when compared to sample I in the S group (P=0.031), whereas no statistically significant changes were observed in neither the WS group nor the A group.

Discussion

The aim of this study was the analysis of gene expression in the target organ for the symptoms, i.e. directly in cells from the nasal cavity. Moreover, in many studies on different tissues, the limited amount of RNA is a problem, which results in pooling of individual samples. However, the individual variations may be significant and thus blur the associations. In this study, changes in individual samples were studied. However, few individuals were included and few genes were studied due to difficulties in obtaining good quality RNA from nasal lavage (contains few cells and probably much RNase) both before and during the challenge. Possibly, a selection may have occurred for individuals with an on-going immunologic reaction, which could result in more cells in the nose and hence more RNA. Still, this would not explain the differences between the groups studied.

Alterations in cytokine expression due to persulphates have not been studied before. We only challenged subjects with potassium persulphate. Hairdressers are often exposed to other persulphates as well, and thus, some reactions may have been missed. However, immunologic differences between used persulphates have not been indicated (Pang and Fiume 2001). The results of our study, with an increased expression of *IL5* among symptomatic hairdressers compared to hairdressers without work-related bleaching powder-associated symptoms, suggest an immune-dependent mechanism. However, the results do not indicate an IgE-dependent response since the symptomatic hairdressers neither demonstrated increased levels of *IL13* nor had signs of specific IgEs towards persulphate in skin prick testing and immunoblotting (Kronholm Diab et al. 2009). This is in accordance with a challenge study on individuals with isocyanate-related asthma (Jones et al. 2006). The authors reported increased numbers of cells expressing *IL5* in the lower airways after challenge, but neither *IL13* nor *IL4* changed. Corresponding to the present study, very few individuals displayed specific IgE antibodies. However, in contrast to their findings, symptomatic

hairdressers also had increased expression of *IFNG* during the challenge. Our earlier study showed a mobilization of Th1 cells in the blood circulation, indicating a Th1-related response in symptomatic hairdressers (Kronholm Diab et al. 2009). In another study on isocyanates, Johnson et al. (2007) reported a mixed Th1/Th2 immune response in mice exposed to toluene diisocyanate. Increased RNA expression of *IL4*, *IL5*, *IL13* and *IFNG* were detected in the nasal mucosa.

Among the atopics in the present study, an increase in relative expression of *IL5* and *IL13*, but no change in *IFNG* expression, was seen during provocation. This indicates that the atopics reacted with a Th2-related response (Till et al. 1997) although they were not sensitised to persulphates.

The limited numbers of individuals prevent us from drawing final conclusions regarding the mechanism behind the work-related bleaching powder-associated symptoms and we suggest further studies. This study indicates that nasal lavage can be used in order to analyse gene expression, however, on a limited number of genes.

Acknowledgement

We thank Anna Axmon for her statistical assistance. The study was supported by the Swedish Council for Working Life and Social Research, Skane county council's research and development foundation, and the Medical Faculty at Lund University, Sweden.

References

Aalto-Korte K, Mäkinen-Kiljunen S (2003) Specific immunoglobulin E in patients with immediate persulfate hypersensitivity. Contact Dermatitis 49:22-25. doi:10.1111/j.0105-1873.2003.00134.x

Albin M, Rylander L, Mikoczy Z, Lillienberg L, Dahlman Höglund A, Brisman J et al (2002) Incidence of asthma in female Swedish hairdressers. Occup Environ Med 59:119-123. doi:10.1136/oem.59.2.119

Brisman J, Albin M, Rylander L, Mikoczy Z, Lillienberg L, Dahlman Höglund A et al (2003) The incidence of respiratory symptoms in female Swedish hairdressers. Am J Ind Med 44:673-678. doi:10.1002/ajim.10293

Hytönen M, Leino T, Sala E, Kanerva L, Tupasela O, Malmberg H (1997) Nasal provocation test in the diagnostics of hairdressers' occupational rhinitis. Acta Otolaryngol Suppl 529:133-136. doi:10.3109/00016489709124104

Johnson VJ, Yucesoy B, Reynolds JS, Fluharty K, Wang W, Richardson D et al (2007) Inhalation of toluene diisocyanate vapor induces allergic rhinitis in mice. J Immunol 179:1864-1871

Jones MG, Floyd A, Nouri-Aria KT, Jacobson MR, Durham SR, Taylor AN et al (2006) Is occupational asthma to diisocyanates a non-IgE-mediated disease? J Allergy Clin Immunol 117:663-669. doi:10.1016/j.jaci.2005.09.053

Kronholm Diab K, Truedsson L, Albin M, Nielsen J (2009) Persulphate challenge in female hairdressers with nasal hyperreactivity suggests immune cell, but no IgE reaction. Int Arch Occup Environ Health. doi:10.1007/s00420-008-0392-3

Leino T, Tammilehto L, Luukkonen R, Nordman H (1997) Self reported respiratory symptoms and diseases among hairdressers. Occup Environ Med 54:452-455. doi:10.1136/oem.54.6.452

Leino T, Tammilehto L, Hytönen M, Sala E, Paakkulainen H, Kanerva L (1998) Occupational skin and respiratory diseases among hairdressers. Scand J Work Environ Health 24:398-406

Malm L, Wihl JÅ, Lamm CJ, Lindqvist N (1981) Reduction of metacholine-induced nasal secretion by treatment with a new topical steroid in perennial non-allergic rhinitis. Allergy 36:209-214. doi:10.1111/j.1398-9995.1981.tb01836.x

Moscato G, Galdi E (2006) Asthma and hairdressers. Curr Opin Allergy Clin Immunol 6:91-95. doi:10.1097/01.all.0000216850.72452.43

Moscato G, Pignatti P, Yacoub MR, Romano C, Spezia S, Perfetti L (2005) Occupational asthma and occupational rhinitis in hairdressers. Chest 128:3590-3598. doi:10.1378/chest.128.5.3590

Muñoz X, Cruz MJ, Orriols R, Bravo C, Espuga M, Morell F (2003) Occupational asthma due to persulfate salts: diagnosis and follow-up. Chest 123:2124-2129. doi:10.1378/chest.123.6.2124

Pang S, Fiume MZ (2001) Final report on the safety assessment of ammonium, potassium, and sodium persulfate. Int J Toxicol 20 Suppl 3:7-21. doi:10.1080/10915810152630710

Till S, Durham S, Dickason R, Huston D, Bungre J, Walker S et al (1997) IL-13 production by allergen-stimulated T cells is increased in allergic disease and associated with IL-5 but not IFN-gamma expression. Immunology 91:53-57. doi:10.1046/j.1365-2567.1997.00218.x

Table 1 Inclusion criteria and some characteristic of the study subjects.

Study subjects	No of subjects for gene expression analysis	Age, median (range), years	Period of employment, median (range), years	Inclusion criteria
Hairdressers with bleaching powder-related nasal symptoms (S)	6	35 (23-47)	14 (5-30)	Only work-related nasal symptoms from bleaching powder
Hairdressers without bleaching powder-related nasal symptoms (WS)	7	29 (23-58)	9 (2-31)	No work-related airway symptoms
Atopics defined by history (A)	6	30 (22-52)	NA	No exposure to bleaching powder Nasal symptoms Atopy by history

NA denotes Not Applicable

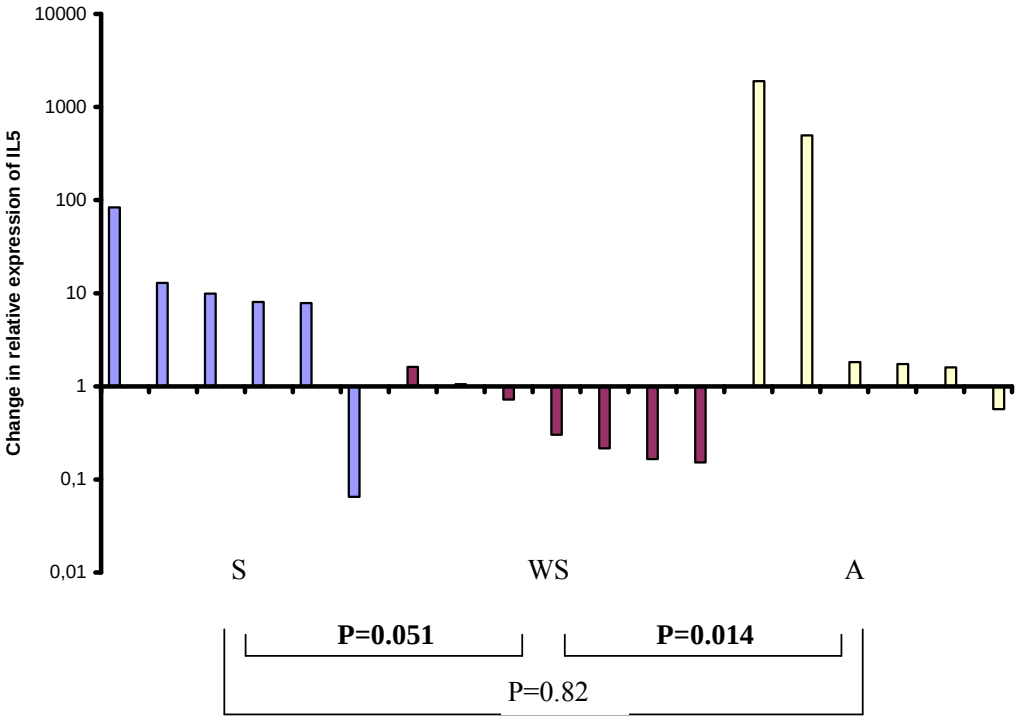
Table 2 The nucleotide sequence (5' to 3') of the primers used for quantitative PCR analysis.

Gene	Forward primer	Reverse primer
YWHAZ	TGGAAAAAGGCCGCATGAT	GTGGGATGCAAGCAAAGGAA
IL5	GACGGAGAGTAAACCAATTCCTAGAC	TCTATTATCCACTCGGTGTTCATTACA
IL13	CATCCCTGACCCTGCCTCGGA[FAM]G	GCACTAAAGCAGTGGACACCAG
IFNG	CCATGGGTTGTGTGTTTATTTCAC	TCAAACCGGCAGTAACTGGATAG

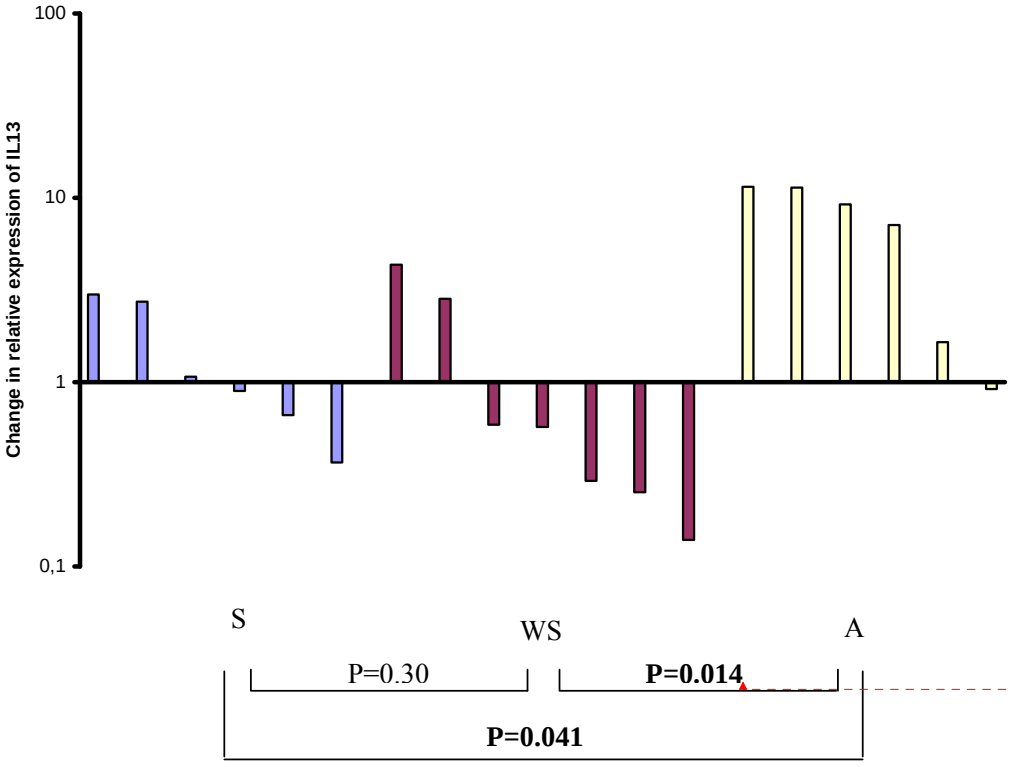
Table 3 Mean (range) of total symptom score [i.e. nasal blockage, secretion and number of sneezes (Kronholm Diab et al. 2009)] before and during challenge with persulphate in each of three examined groups.

Time	Hairdressers with bleaching powder-related nasal symptoms (S)	Hairdressers without bleaching powder-related nasal symptoms (WS)	Atopics defined by history (A)
0 hour	2.8 (1-6)	0.3 (0-1)	0.2 (0-1)
1.5 hours	7.0 (1-14)	0.1 (0-1)	2.7 (0-8)
5 hours	6.3 (1-12)	0.1 (0-1)	1.7 (0-7)

a



b



Formatted

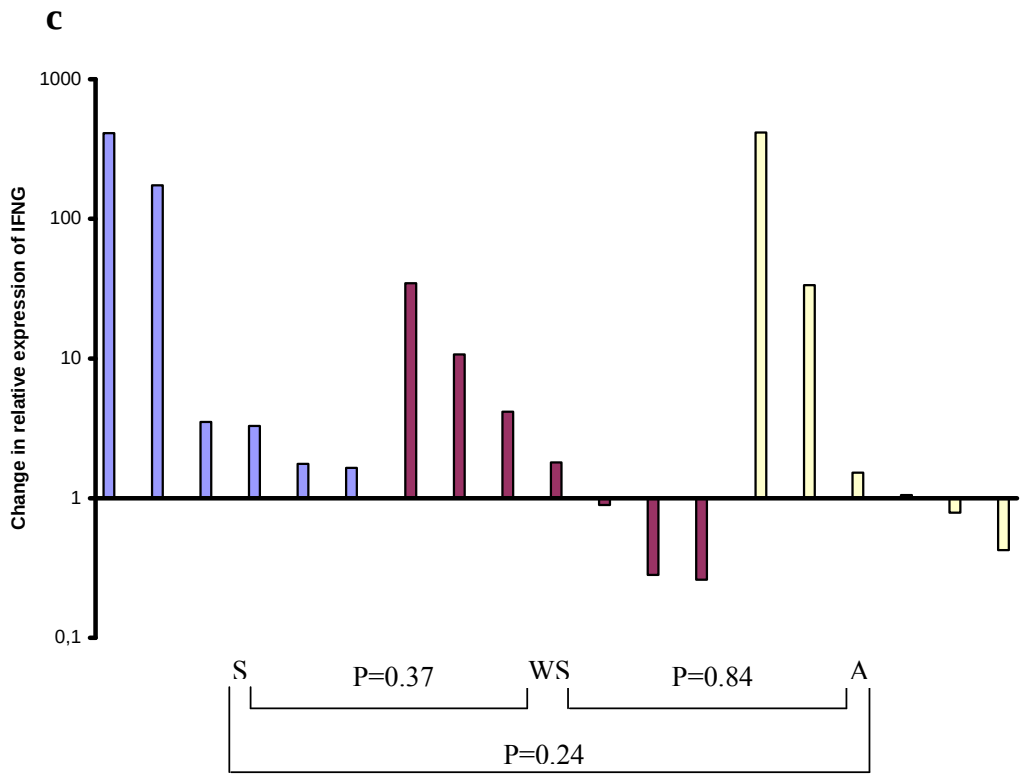
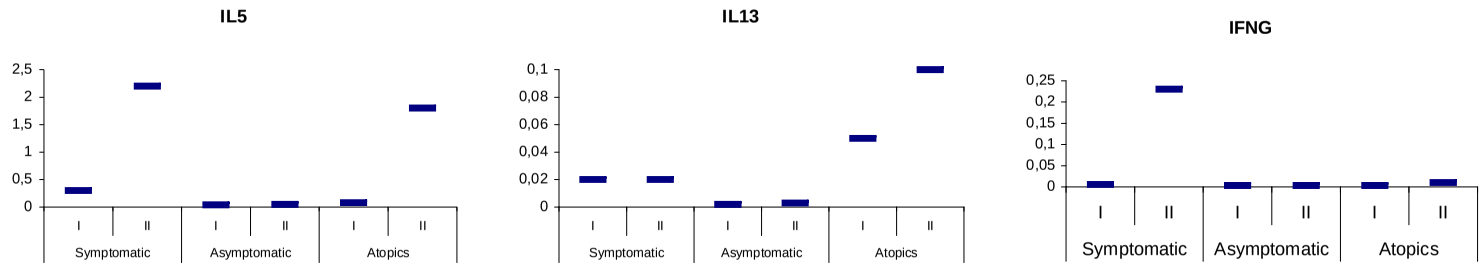


Fig. 1 The change in expression of *IL5* (**a**), *IL13* (**b**) and *IFNG* (**c**) normalised to *YWHAZ* in nasal lavage from samples obtained before challenge with potassium persulphate compared to samples obtained during challenge. Each *column* represents one study subject. Statistically significant (P≤0.05) values are indicated in *bold*. *S* hairdressers with work-related bleaching powder-related nasal symptoms, *WS* hairdressers without work-related bleaching powder-related nasal symptoms and *A* atopics defined by history



P=0.22 P=0.11 P=0.31 P=0.84 P=0.38 P=0.16 **P=0.031** P=0.69 P=0.31

Fig. 2 The relative expression (median) of *IL5*, *IL13* and *IFNG* in the three groups of study subjects before (I) and during (II) challenge. *P* values were calculated to describe the difference between I and II and statistically significant (P≤0.05) values are indicated in *bold*.

Symptomatic denotes "hairdressers with work-related bleaching powder-related nasal symptoms", *Asymptomatic* denotes "hairdressers without work-related bleaching powder-related symptoms" and *Atopics* denotes "atopics defined by history"