

ORIGINAL PAPER

Correlation between immune and neuronal parameters and stress perception in allergic asthmatics

R. A. Joachim*, O. Noga[†], V. Sagach*, G. Hanf[†], H. Fliege*, R. D. Kocalevent*, E. M. Peters* and B. F. Klapp*

Departments of *Internal Medicine and Psychosomatics and [†]Internal Medicine/Infectious and Respiratory Diseases, Charité Center for Internal Medicine and Dermatology, Charité – Universitätsmedizin, Berlin, Germany

Clinical and Experimental Allergy

Summary

Background Asthma is a chronic disease defined by airway inflammation, increased airway hyperresponsiveness and episodes of airway obstruction. Although there are abundant clinical and experimental data showing that stress may worsen asthma, the mechanisms linking stress to asthma are not well understood. By inducing a pro-inflammatory cytokine milieu, stress might enhance airway inflammation in bronchial asthma. We therefore investigated the correlation of stress perception and the cytokine profile of circulating lymphocytes in humans.

Methods Allergic asthmatic patients and healthy controls were evaluated for perceived level of stress, demographic and lung function data. Whole blood cells were obtained and stimulated by mitogen to assess intracellular IL-4, IFN- γ and TNF- α by flow cytometry. Neurotrophins nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) were measured in serum.

Results Asthmatic patients showed significantly higher percentages of TNF- α -producing T cells than healthy controls. Only in asthmatic patients was stress perception correlated with percentages of TNF- α -producing T cells and serum BDNF levels, while forced expiratory volume in 1 s (% predicted) was negatively correlated to BDNF.

Conclusion The results of our study support the hypothesis that stress deteriorates bronchial asthma by inducing a pro-inflammatory cytokine profile in allergic asthmatics. Stress management might provide a supplement therapy of allergic asthma.

Keywords asthma, BDNF, inflammation, lung function, mediator, stress, TNF- α

Submitted 12 June 2006; revised 18 September 2007; accepted 22 October 2007

Correspondence:

Ricarda Joachim, Biomedizinisches Forschungszentrum, R. 2.0549, Charité – Universitätsmedizin Berlin, Campus Virchow, Augustenburger Platz 1, 13353 Berlin, Germany.
E-mail: ricarda.joachim@charite.de

Introduction

Asthma is a chronic disease defined by airway inflammation, increased airway hyperresponsiveness and episodes of airway obstruction. There are broad clinical and experimental data showing that stress can worsen asthma. Patients exposed to acute stressors show altered lung function [1, 2]. Furthermore, an increased airway inflammation was shown by Liu et al., [3] who found that final exams lead to increased eosinophilic airway inflammation in students with asthma.

Stress has been defined as a stimulus that activates both the hypothalamic pituitary adrenal (HPA) axis and the sympathetic nervous system, thereby inducing neuronal and humoral changes that may finally lead to an adaptation of the individual to the changed environment [4].

This activation depends on the appraisal of the individual regarding the threat of a certain situation, and its the ability to cope with it [5]. When these abilities are judged as insufficient, the individual reacts to an impairment of homeostasis by eliciting a stress response. Thus, the personality's perception contributes to the magnitude of the response to a challenging stimulus. A large body of evidence has accumulated showing that stress can alter the immune system in both humans and animals. While acute stress seems to have an activating impact on immune parameters [6–9], chronic stress more often is associated with decreased immune responses [10–13].

The mechanisms linking stress to asthma are not well understood. One possible link might be the influence of stress on inflammatory processes [14]. Several studies indicate that psychological stress can induce the release

of pro-inflammatory cytokines like IL-1, IL-6 and TNF- α [15–17], and it has been shown that stress promotes a local pro-inflammatory cytokine profile in a murine model of abortion [18]. Thus, by inducing a pro-inflammatory cytokine milieu, stress might enhance airway inflammation in bronchial asthma. Several studies have shown that the pro-inflammatory cytokine TNF- α plays an important role in asthmatic airway inflammation. TNF- α levels were elevated in supernatants from cultures of unstimulated and stimulated bronchoalveolar lavage fluid cells [19], and in the lavage fluid itself after challenge with an allergen [20].

Neurotrophins like nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are involved in central stress processing in animal models. Immobilization stress altered BDNF mRNA and protein in rat hypothalamus, pituitary gland and the hippocampal dentate gyrus [21–23]. Von Richthofen *et al.* [24] reported NGF alterations in different rat brain regions after exposure to acute stress. In humans, serum NGF was found to be increased after stress [25], and altered serum BDNF levels were shown in patients with depression or schizophrenia [26–28], diseases that are associated with stress [29, 30].

In recent years, evidence has grown for a role of neurotrophins in allergic diseases and asthma not only in animal models [31, 32] but also in humans. An increased level of circulating NGF has been found in humans with allergic diseases, with the highest levels in allergic asthmatics [33]. After segmental allergen provocation, neurotrophins were increased in the late-phase reaction [34], and Lommatzsch *et al.* [35] found higher serum BDNF levels in asthmatics compared with control subjects. Serum BDNF levels were also increased in patients with atopic dermatitis [36].

Based on the mechanisms identified in the stress-immune interactions reported above, we investigated psycho-neuro-immunological imbalances as a possible cause of asthma exacerbations analysing cytokine-producing T cells and neurotrophins in blood samples of stress-scored asthmatic patients and healthy controls.

The following questions were addressed:

1. Is there an association between stress perception and cytokine or neurotrophin profiles in asthmatic patients?
2. Is there an association between stress perception and lung function in asthmatic patients?

Methods

Participants

A group of 61 volunteers participated in this study. The local ethics committee approved the study. All participants had given their informed consent. Their clinical and

Table 1. Participants data

	Asthmatics	Controls
<i>n</i>	41	20
Mean age (minimum–maximum)	50 (24–75)*	39 (20–77)
Sex (f/m)	34/7	11/9 [†]
Asthma medication (+/–)	30/11	–
Stress score	0.37 $\pm$ 0.03	0.36 $\pm$ 0.03
CD4 ⁺ (%)	52.1 $\pm$ 1.5	49.1 $\pm$ 1.4
CD8 ⁺ (%)	23.2 $\pm$ 1.5	21.5 $\pm$ 1.2
CD4 ⁺ CD25 ⁺ (%)	1.5 $\pm$ 0.2	2.6 $\pm$ 0.4 [‡]
CD3 ⁺ IL-4 ⁺ (%)	0.5 $\pm$ 0.1	0.6 $\pm$ 0.1
CD3 ⁺ IFN- γ ⁺ (%)	10.6 $\pm$ 1.2	9.2 $\pm$ 1.7
CD3 ⁺ TNF- α ⁺ (%)	9.9 $\pm$ 1.1 [§]	6.8 $\pm$ 1.5
BDNF (ng/mL)	16.7 $\pm$ 1.0	17.5 $\pm$ 2.0
FEV ₁ (% predicted)	85.4 $\pm$ 3.0 [¶]	98.9 $\pm$ 0.9

*Asthmatics were significantly older than controls ($P < 0.05$).

[†]Proportion of males was significantly higher in controls compared to asthmatics ($P < 0.05$).

[‡]Percentage of CD4⁺ CD25⁺ cells was significantly higher in controls compared to asthmatics ($P < 0.01$).

[§]Percentage of CD3⁺ TNF- α ⁺ cells was significantly higher in asthmatics compared to controls ($P < 0.05$).

[¶]FEV₁ (% predicted) was significantly lower in asthmatics compared with controls ($P < 0.01$).

BDNF, brain-derived neurotrophic factor; FEV₁, forced expiratory volume in 1 s; m, male; f, female.

demographic data are shown in Table 1. Patients were recruited at the outpatient department for asthma and allergic diseases.

Two different groups were examined:

1. Patients with allergic asthma without treatment (newly diagnosed) or those treated with inhalative corticosteroids for at least 1 year. Bronchodilators were allowed as rescue medication.
2. Healthy controls who had no history of asthma or other atopic diseases and no respiratory symptoms.

Allergy diagnosis

Allergy was diagnosed based on history and a skin prick test with at least one positive reaction (weal greater than the histamine response) to 10 common inhalant allergens including pollen, dust mites and animal dander (Allergopharma, Hamburg, Germany). Total IgE and allergen-specific IgE was detected (Pharmacia CAP, Upsala, Sweden).

Lung function

Forced expiratory volume in 1 s (FEV₁) was determined with body plethysmography (Jaeger, Wuerzburg, Germany).

Blood sampling and flow cytometry

Venous blood was collected between 8:00 and 11:00 hours. For intracellular cytokine staining, 100 µL of heparinized blood was cultured in 900 µL of AIM V culture medium (Gibco BRL, Life Technologies GmbH, Karlsruhe, Germany). Cells were stimulated with Phorbol 12-myristate 13-acetate (PMA, 10 ng/mL, Sigma, Munich, Germany) and ionomycin (1 µg/mL, Sigma) in the presence of monensin (2.0 µM/mL, Sigma) for 4 h (37 °C, 5% CO₂). After culture, the cells were washed once with phosphate-buffered saline (PBS). For surface staining, cells were incubated with 5 µL of anti-CD3-FITC. After a washing step, cells were fixed with 2% paraformaldehyde (Sigma) for 10 min at room temperature. After washing, fixed cells were stored at 4 °C in the dark before intracellular staining on the next day. For permeabilization, cells were incubated in PBS containing 0.5% saponin (Riedel de Haen, Selze, Germany) for 10 min at room temperature. Cells were washed once with 0.5% saponin before incubation with 10 µL of mouse-anti-human PE fluorescence-labelled cytokine antibodies against IL-4, IL-5, IFN-γ and TNF-α in 0.5% saponin. After two further washing steps with PBS containing 0.5% saponin and PBS alone, cells were analysed by flow cytometry. The acquisition was performed using a FACS Calibur (Becton Dickinson). A gate was set on the lymphocyte population on the forward and side scatter plot and 10 000 cells were computed in this gate and analysed with appropriate software (Cell Quest, Becton Dickinson, Heidelberg, Germany). As controls, cells were stained with the corresponding isotype-matched antibodies. The number of CD3⁺ cells staining for each cytokine was expressed as a percentage of CD3⁺ T cells. One tube per probe was incubated with surface markers for CD4, CD8 and CD25 and measured after fixation. All monoclonal antibodies were purchased from BD Biosciences, Heidelberg, Germany.

Serum determination of neurotrophins nerve growth factor and brain-derived neurotrophic factor

Venous blood was drawn between 8:00 and 11:00 hours. After clotting, samples were centrifuged. Serum was immediately frozen at -20 °C. Neurotrophic factors were detected with sandwich ELISAs according to the manufacturer's instructions for NGF and BDNF (R&D Systems, Minneapolis, MN, USA). Assays were performed on 96-well U-bottom plates (Nunc, Wiesbaden, Germany). Wells were developed with tetramethylbenzidine and measured at 450 nm. Neurotrophin content was quantified against a standard curve calibrated with known amounts of protein. The detection limits were 9 pg/mL for NGF and BDNF. Measurements were performed in duplicate and are expressed as means.

Perceived Stress Questionnaire

To assess the individual stress perception, participants were asked to fill out the Perceived Stress Questionnaire (PSQ) modified by Fliege et al. [37]. The PSQ was originally designed to specifically measure stress in clinical psychosomatic research [38]. The German adaptation has been validated in a psychosomatic sample [37] as well as in the general population [39]. Validation studies showed excellent psychometric properties [37, 38, 40], and it has been used in research, demonstrating good predictive value in stress-related diseases such as ulcerative colitis [41, 42] as well as in a sample of women after miscarriage [37]. The PSQ assesses subjectively experienced stress independent of a specific and objective occasion. The German adaptation used here consists of 20 items representing the four dimensions of worries, tension, joy and demands. The PSQ score ranges from 0 (lowest possible level of perceived stress) to 1 (highest possible level of perceived stress).

Statistical analysis

Results were calculated as mean ± SEM. Data were analysed using SPSS, Version 11.5 (SPSS Inc., Chicago, IL, USA). Significance of differences between groups was determined with the non-parametric test for independent values (Mann-Whitney *U*-test). Correlations were calculated using Spearman's ρ . *P*-values <0.05 were considered to be as significant.

Results

The demographic characteristics and the clinical data of the studied population are reported in Table 1. Asthmatic patients were significantly older than controls and the proportion of males was significantly lower. Asthma patients with and without medication did not differ with respect to the investigated parameters lung function (FEV₁), IgE and specific IgE, T cell populations and cytokine production or stress perception (data not shown).

Stress

Perceived stress scores were calculated for asthmatic patients and healthy controls. No differences were found between the groups with regard to the stress level that the individuals experienced (Table 1).

T cell populations in the peripheral blood

No differences were found between asthmatic patients and healthy controls with respect to CD4⁺ and CD8⁺ T cells in the peripheral blood. In contrast, healthy controls showed significantly higher percentages of CD4⁺CD25⁺ T cells

compared with asthmatic patients (Table 1). A positive correlation of CD4⁺ T cells with stress was found in asthmatics ($r = 0.35$, $P < 0.05$; Table 2) while CD8⁺ T cells correlated negatively with stress ($r = -0.38$, $P < 0.05$; Table 2). There was no correlation with T cell populations in healthy controls.

Cytokines

Regarding TNF- α -producing peripheral blood T cells, asthmatic patients showed significantly higher percentages than healthy controls (Table 1, Fig. 1). A significant correlation was found between stress and percentages of TNF- α ⁺ CD3⁺ cells in asthmatics ($r = 0.418$, $P < 0.05$; Fig. 2a). In contrast, there was no correlation between stress perception and TNF- α ⁺ CD3⁺ cells in controls ($r = 0.02$, NS).

Analysis of IFN- γ -producing T cells showed no significant differences between healthy and asthmatic individuals (Table 1), and no correlation with stress. Analysing

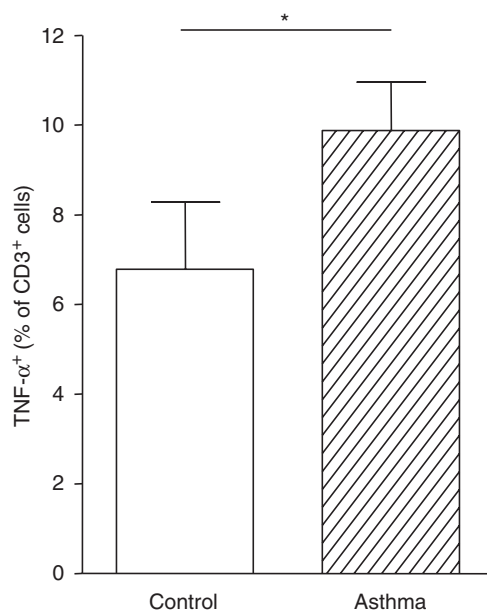


Fig. 1. Percentage of TNF- α producing peripheral blood T cells after phorbol 12-myristate 13-acetate/ionomycin stimulation. Results are indicated as mean and SEM and show percentages of TNF- α ⁺ CD3⁺ cells from gated lymphocyte population (* $P < 0.05$).

the T-helper type 2 (Th2) cytokines in stimulated blood T cells, we found no differences between groups in the frequency of IL-4-producing CD3⁺ T cells (Table 1), and there was no correlation with stress. In the current experimental setting, IL-5-expressing T cells were at the detection limit for most participants (data not shown).

Neurotrophins

All participants showed high levels of BDNF serum levels and no differences were found when comparing healthy controls with asthmatic patients (Table 1).

A significant correlation between stress perception and BDNF levels was found in asthmatic patients ($r = 0.43$, $P < 0.01$; Fig. 2b) but not in healthy controls ($r = -0.08$, NS).

While BDNF was detectable in all participants, NGF was below the detection limit in eight cases of the control group and 24 cases in the asthma patient group. Therefore, NGF data were not further analysed (data not shown).

Lung function

FEV₁ was reduced in asthmatic patients. No significant correlation was found between stress and FEV₁% in asthmatics ($r = -0.177$, $P > 0.05$). However, in asthmatic patients FEV₁ showed a negative correlation with BDNF ($r = -0.34$, $P < 0.05$; Fig. 2c) and CD4⁺ T cells ($r = -0.39$, $P < 0.05$; Table 2).

To evaluate the possible influence of lung function impairment on the investigated parameters, asthmatic patients were divided into mild asthmatics (FEV₁ > 80, $n = 26$) and moderate to severe asthmatics (FEV₁ < 80, $n = 15$). No differences were found for any of the investigated parameters, although a tendency towards higher percentages of TNF- α ⁺ CD3⁺ cells was found in patients with FEV₁ < 80 (data not shown).

Discussion

In the present study, in which allergic asthmatic patients and healthy controls were evaluated for levels of perceived stress, neuro-immunologic and lung function data, we found a significantly higher percentage of TNF- α -producing CD3 T cells in peripheral blood after PMA/

Table 2. Correlation between stress, respectively FEV₁, and blood parameters (T cells and BDNF)

	CD3 ⁺ /TNF- α ⁺	CD3 ⁺ /IFN- γ ⁺	CD3 ⁺ /IL-4 ⁺	CD4 ⁺	CD8 ⁺	CD4 ⁺ /CD25 ⁺	BDNF (serum)
Stress	0.42 $P = 0.017$	0.03	-0.21	0.35 $P = 0.047$	-0.38 $P = 0.034$	-0.07	0.44 $P = 0.006$
FEV ₁ (% predicted)	0.03	-0.10	-0.27	-0.39 $P = 0.029$	0.26	-0.11	-0.34 $P = 0.039$

Values indicate the correlation coefficient and statistical significance. If nothing but the correlation coefficient is shown, it is nonsignificant.

BDNF, brain derived neurotrophic factor; FEV₁, forced expiratory volume in 1 s.

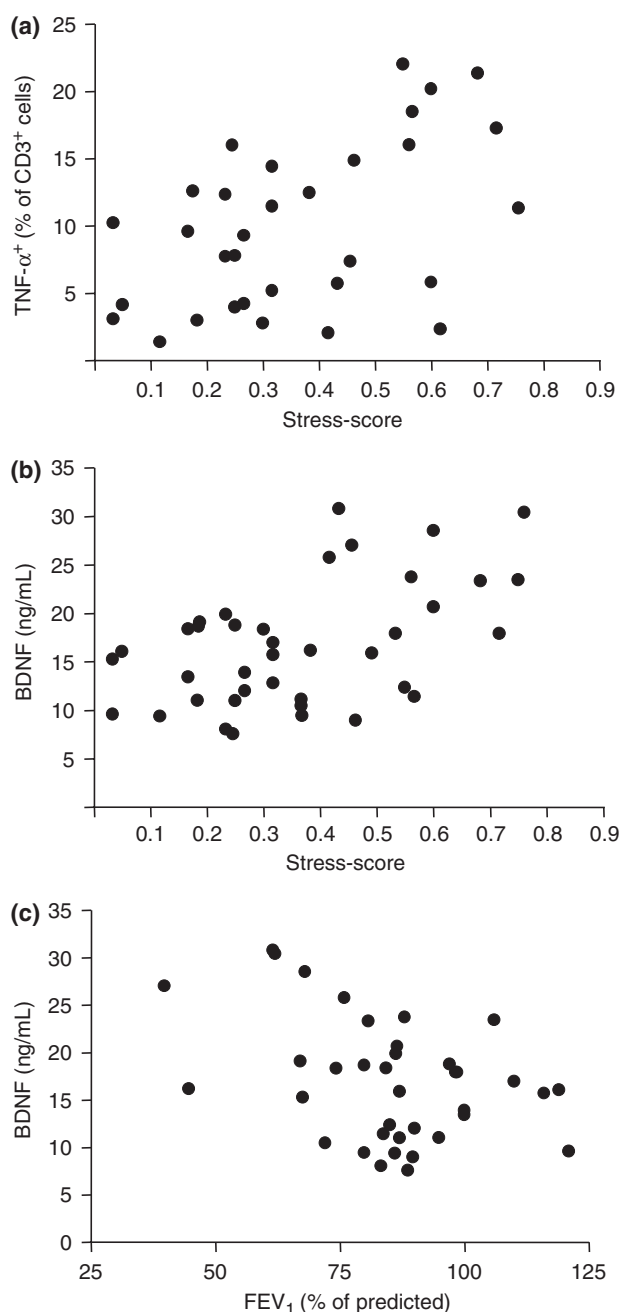


Fig. 2. Correlations between (a) stress score and percentage of TNF- α^+ CD3 $^+$ cells; (b) stress score and brain-derived neurotrophic factor (BDNF) (ng/mL); (c) forced expiratory volume in 1 s (FEV $_1$) (% predicted) and BDNF (ng/mL).

ionomycin stimulation in patients with atopic asthma compared with healthy control subjects. Our results further indicate that there is a correlation between subjective stress perception and the pro-inflammatory cytokine TNF- α in asthmatic patients. Moreover, BDNF serum levels are correlated to stress perception and to lung function in asthmatic patients but not in healthy controls.

Chronic diseases-like bronchial asthma may have psychological consequences by impairing the daily life of the

individual, which might be reflected by higher stress perception. Here, there was no difference in stress perception between asthmatic patients and healthy controls. Using the same tool, Fliege et al. [37] found the highest scores for psychosomatic outpatients (0.59), followed by tinnitus patients (0.48) and patients with inflammatory bowel disease (0.43). The mean stress level scores of patients and healthy controls in our study are comparable with those obtained for students and healthy controls in the above-mentioned study.

Stress has been discussed to induce cytokines and even alter cytokine patterns [14]. Depending on the experimental set-up, some groups found an association between stress and a pro-inflammatory or T-helper type 1 (Th1)-like cytokine profile and increases in cytokines such as TNF- α , IL-1 and IFN- γ [18, 43, 44]; other studies have shown an opposite effect of stress with a shift towards Th2-type responses [45, 46]. Allergic asthma is an inflammatory disease of the airways characterized by an altered Th2 response as a consequence of allergen contact. Increasing evidence indicates that other than Th2 cytokines are also associated with the inflammatory response that characterizes human asthma. In this aspect, there are ample clinical and experimental data tagging TNF- α as an important mediator of allergic airway inflammation. TNF- α is present in the lavage fluid of asthmatic patients [47], and increased TNF- α mRNA expression in bronchoalveolar lavage (BAL) cells from asthmatic patients compared with controls has been reported [48]. Cembrzynska-Nowak et al. [19] were able to show that TNF- α release from BAL leucocytes is increased in asthmatic patients. Most recently, Howarth et al. [49] identified TNF- α as a therapeutic target in severe asthma. Here, we found higher percentages of TNF- α -producing T cells and a correlation with perceived stress in asthmatic patients. The finding that higher stress is related to increased TNF- α expression has been reported previously in children at risk for asthma [50]. Hence, the exacerbation of bronchial asthma in individuals with a high stress level might be spurred by cytokines like TNF- α . Recent data from our lab using a combined murine model of allergic airway inflammation and sound stress have demonstrated that stress-increased airway inflammation is not associated with increases in Th2 cytokines like IL-4 and IL-5 locally [51], but that rather other cytokines like TNF- α play an important role in mediating stress effects on the airways [52].

In recent years, the population of CD4 $^+$ CD25 $^+$ T cells has evoked considerable interest, a population that seems to regulate the function of other immune cells, generally in an inhibitory way. In this aspect, they might also play a critical role in controlling the expression of allergy and asthma. Tolerance to environmental allergens encountered at mucosal surfaces, such as the lungs or the intestinal tract, could be mediated by clonal deletion or anergy of allergen-specific cells or the development of

regulatory T cells. Patients who specifically lack CD25⁺ regulatory T cells show eczema, elevated IgE levels, eosinophilia and food allergy [53]. Therefore, it is very likely that some form of regulatory T cells controls the development of allergic disease and asthma. Here, we found lower percentages of CD4⁺CD25⁺ cells in asthmatic patients, supporting the hypothesis that this cell population might be involved in controlling the expression of allergic diseases.

Neurotrophins are involved in central stress regulation and different groups have elucidated an important role for neurotrophins in asthma in the past decade. Serum BDNF levels are altered in atopic diseases [35, 36] but also in psychological and psychiatric disorders like anorexia and bulimia nervosa [54] and depression [26]. Here, we show for the first time that stress perception is correlated to BDNF serum levels in asthmatic patients. A neuro-immunological interaction can be speculated for this finding. For example, human peripheral blood monocytes have been shown to express BDNF constitutively and to enhance secretion when stimulated with TNF- α [55]. In addition, various other cell types might be involved.

FEV₁ showed no correlation with perceived stress in asthmatic patients. However, the finding that FEV₁ correlates negatively with BDNF and positively with CD4⁺ T cells, whereas an inverse correlation with stress was found for those parameters, might lead to the assumption that complex neuroimmunological interactions – which still have to be revealed – might have impact on airway function in asthma patients.

If stress management has therapeutic potential in asthmatic patients with high stress perception has to be evaluated [56]. This might provide a non-pharmacological additional treatment – without side effects – of allergic asthma.

Acknowledgements

This study was supported by a grant from the German Research Association to Ricarda Joachim (DFG Jo 365/2-2).

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