

A Search for Biomarkers as Diagnostic Tools for Food Allergy: A Pilot Study in Peanut-Allergic Patients

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Key Words

Peanut allergy • Diagnosis • Metabolite levels • ¹H nuclear magnetic resonance spectroscopy • Multivariate data analysis

Abstract

Background: The impact of peanut allergy is large and accidental ingestion of peanut can lead to severe reactions. Currently used diagnostic tests, such as skin prick tests (SPT) and determination of specific immunoglobulins (IgE) have, however, limited sensitivity and specificity. Therefore, new tools have to be developed to improve the accuracy of the diagnostic work-up of food-allergic patients. Comprehensive metabolite analysis may provide biomarkers for diagnosing food allergy as metabolite levels reflect actual physiological conditions. We investigated whether metabolites can be found that discriminate between peanut-allergic patients and non-peanut-allergic subjects. Such metabolites may be used for future diagnostic purposes. **Methods:** Plasma and saliva samples were obtained from 23 participants (12 peanut allergic and 11 peanut tolerant) prior to and after a peanut challenge and measured with ¹H nuclear magnetic resonance (NMR) spectroscopy with subsequent multivariate

data analysis. **Results:** Clear differences were observed between NMR spectra of peanut-allergic and peanut-tolerant subjects in plasma as well as saliva. Allergic patients already showed aberrant metabolite levels prior to peanut ingestion, thus before the onset of allergic reactions. **Conclusion:** This pilot study shows that aberrant metabolite levels as determined by NMR in combination with multivariate statistics may serve as novel biomarkers for food allergy.

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Introduction

Peanut is one of the most common foods responsible for severe allergic reactions [1]. Careful clinical histories, in vivo skin prick tests (SPT) and in vitro serological assays (for example ImmunoCAP) are often used by clinical practitioners to assist in the diagnosis of peanut allergy. SPT provides a rapid method with a negative predictive accuracy generally greater than 95% [2]. However,

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the test in general has a relatively low specificity of about 50%. Moreover, the positive predictive value is only 20–50% [3], depending the food extracts used which differ in their content of individual allergens, vary between manufacturers, and even between batches [4]. In vitro tests for specific IgE are broadly used in evaluating IgE-mediated food allergy [5, 6], but they also have a limited predictive value. Therefore, the double-blind placebo-controlled food challenge (DBPCFC) remains the gold standard for diagnosing food allergy [7, 8]. The DBPCFC is, however, very expensive, laborious and burdensome for the patient. Moreover, it requires dedicated hospital facilities and trained personnel and is currently only available in specialized allergy centers. Therefore, there is a need for new diagnostic tools. More refined methods to detect IgE to specific allergens (components) are currently investigated. Although such component-resolved diagnosis [9, 10] is promising, future diagnostics should include other factors that are more patient related or patient specific.

Nowadays, it is possible to screen biological fluids and tissues in a holistic approach discovering novel biomarkers. Metabolic fingerprinting research uses analytical techniques like ^1H nuclear magnetic resonance (NMR) spectroscopy or liquid chromatography mass spectrometry in combination with multivariate statistics in order to identify differences in levels of metabolites in biological fluids and tissues of patients and healthy controls [11, 12]. Ultimately, this should result in biomarkers that are specific for certain diseases. For metabolic fingerprinting, NMR is an attractive approach because a wide range of metabolites can be measured simultaneously without extensive sample preparation. In this way a metabolic fingerprint of urine was found that reflects the osteoarthritic changes both in guinea pigs and humans [13, 14]. Plasma is the body fluid that is often studied, but saliva is attractive as well, since it is a readily attainable biological fluid that contains among others many hormones and antibodies [15]. Saliva may contain mediators of allergy, since the immunological reaction to peanut may already start in the oral mucosa, the first tissue to be in contact with food allergens [16].

We performed a pilot study to investigate whether differences could be observed in metabolites in plasma and saliva of patients with peanut allergy and subjects tolerant to peanut by NMR in combination with multivariate statistics, both before and after a challenge with peanut.

Materials and Methods

Study Population

Twelve peanut-allergic patients (8 male and 4 female, mean age 25.4 years) were included. The diagnosis of peanut allergy was confirmed in all patients by DBPCFC with the following increasing amounts of peanut flour used: 0.01, 0.1, 0.5, 1, 10, 100, 300 mg, 1 and 3 g [17]. The severity of symptoms by history was classified according to Mueller [18], a scoring system which was originally designed for the classification of allergic reactions to insect venom and previously used to classify symptoms of food allergy. Symptoms of the oral cavity were classified as Mueller grade 0, symptoms of the skin and mucous membranes (urticaria, angioedema, rhinitis, conjunctivitis) as grade 1, gastrointestinal symptoms (diarrhea, vomiting, nausea, abdominal pain) as grade 2, respiratory symptoms (asthma, hoarseness) as grade 3 and cardiovascular symptoms as grade 4.

Eleven non-peanut-allergic persons (6 male and 5 female, mean age 27.3 years) that tolerated peanut without any symptoms were included as controls.

Specific IgE levels to peanut, grass pollen and birch pollen were determined in all subjects by ImmunoCAP (Phadia, Uppsala, Sweden). An IgE level of ≥ 0.35 kU/l was regarded positive.

This study was reviewed and approved by the Medical Ethical Committee of the University Medical Center Utrecht. All subjects gave written informed consent before enrollment in the study.

Sample Collection

Peanut-allergic patients were allowed to have a light breakfast consisting of low-fat foods and a cup of tea up to 2 h before the diagnostic peanut challenge. Just before the administration of the first dose of peanut, plasma and saliva were collected. When convincing oral allergy symptoms occurred during this challenge at a certain dose, saliva was directly collected and stored. A second plasma sample was drawn 1 h after the consumption of the last dose of peanut. The samples were stored at -80°C .

The peanut-tolerant persons were also allowed to have a light breakfast up to 2 h before the peanut administration. Blood and saliva were collected before peanut ingestion at the same time of the day as was used for sample collection from the peanut-allergic patients to rule out possible variations in metabolite levels throughout the day. Five minutes after peanut ingestion of the peanut-tolerant individuals, saliva was collected and 1 h later plasma was obtained.

NMR Analysis of Plasma and Saliva Samples

NMR is based on the principle that protons act like a magnet when placed in a magnetic field. The energy needed to flip a proton from a stable to a less stable state depends on the applied magnetic field and the environment of the proton. The energy needed is measured and the result is an NMR spectrum showing frequencies at which the various protons of a molecule absorb energy [11]. However, biological fluids contain many metabolites. Consequently, NMR spectra are very complex for visual inspection. Therefore, multivariate analysis is used, in which sophisticated mathematical and statistical methods are applied for the analysis of chemical data. A large set of related variables, such as, for instance, hundreds of signals in numerous NMR spectra, is con-

verted to a smaller set of uncorrelated variables. In this way it is possible to express differences between NMR spectra, which reflect metabolite contents of biological fluids, between patients and healthy controls. The result is a score plot in which each complete NMR spectrum is visualized by a single point. In a factor spectrum, henceforth named metabolic fingerprint, the contribution of each variable to the differences observed in a score plot is visualized.

The NMR procedure we applied in this study is described in detail in previous articles [13, 14]. Sample measurements were carried out in random order and in triplicate in a fully automated manner using a 600-MHz NMR spectrometer (Avance; Bruker BioSpin GmbH, Rheinstetten, Germany).

NMR Data Preprocessing and Multivariate Data Analysis

Each NMR spectrum in the data file was segmented into regions of 0.04 parts per million (ppm) and the integral value for each segment was calculated. The region of the spectrum giving rise to water peaks (4.7–5.2 ppm) was removed. The NMR data reduction file was imported into Winlin (version 2.3; TNO, Zeist, The Netherlands). Data were mean scaled in such a way that small and large peaks contribute similarly to the final study result. Subsequently, principal component discriminant analysis (PCDA) was chosen as the multivariate data analysis technique. For this analysis, the NMR data sets were randomly divided into a training data set and a test data set. The multivariate models were built upon the training data set. Subsequently, the test data set was used to test the reliability of the training model. Health status (peanut-allergic versus peanut-tolerant) and sex were used as a priori information for carrying out PCDA. Models were built to visualize differences between NMR spectra of peanut-allergic and control cases and the scores of the samples were plotted. This procedure was followed for NMR data obtained before as well as after ingestion of peanut. The distinction between scores of peanut-allergic versus peanut-tolerant cases was correlated to the original features (expressed in ppm) in the NMR spectra which provides insight into metabolite identities.

Statistics

PCDA was performed with the NMR data. Each study population and each biological fluid was subjected to a separate PCDA. Mean values of the scores per group were calculated for each PCDA. The unpaired *t* test was performed to evaluate the statistical significance of the difference between the scores of different patient groups in each PCDA analysis. The paired *t* test was used for evaluation of differences before and after challenge in the same group of patients (Excel 2003; Microsoft Corporation, Redmond, Wash., USA). *p* values <0.05 were considered statistically significant.

Results

Study Population

Twelve peanut-allergic patients, proven by DBPCFC, were included in this study. The lowest dose of peanut flour that elicited a convincing subjective reaction (eliciting dose) ranged from 0.1 to 300 mg peanut flour.

Three peanut-allergic patients (25%) reported mild symptoms as most severe symptoms by history (Mueller grade 0 and 1), 2 (17%) reported moderate symptoms (grade 2) and 7 patients (58%) reported severe symptoms (grade 3 and 4). Ten patients (83%) also reported pollinosis symptoms, 6 patients (50%) suffered from concomitant asthma and 6 patients (50%) from atopic eczema. Specific IgE to peanut was present in 92% of the peanut-allergic patients, to grass pollen in 75% and to birch pollen in 67%.

Eleven peanut-tolerant persons entered this study, 2 of which were sensitized to peanut but tolerated the highest dose of 10 g peanuts during the challenge and therefore were considered not to be peanut allergic. Three subjects (27%) reported pollinosis symptoms, of which 1 also suffered from concomitant asthma and atopic eczema. Specific IgE to peanut was present in 18% of the peanut-tolerant subjects, to grass pollen in 36% and to birch pollen in 27%. The subject characteristics are summarized in table 1.

Differences in Plasma NMR Spectra between Peanut-Allergic and Peanut-Tolerant Subjects

Figure 1a shows the NMR spectra of the study population before peanut ingestion. The NMR spectra of the allergic patients were in a significantly different position on the score plot compared to the peanut-tolerant subjects ($p < 0.01$), which means that the peanut-allergic patients already demonstrated a significantly different NMR spectrum before peanut ingestion compared to peanut-tolerant persons. Elevated metabolites in the plasma of peanut-allergic patients that could be identified using our in-house database were lactate, creatinine and glutamine, whereas lipids and nicotinic acids were decreased.

The NMR spectra after peanut ingestion were also studied. Figure 1b shows that the position of the NMR spectra on the score plot of the peanut-allergic patients was significantly different from the position of the peanut-tolerant subjects ($p < 0.01$), demonstrating differences in NMR spectra of plasma between peanut-allergic and peanut-tolerant subjects after peanut ingestion. The metabolites that were identified were similar to the ones obtained before ingestion of peanut, with increasing levels of lactate, creatinine and glutamine. In addition, an increase in tyrosine and tryptophan was identified in peanut-allergic patients after peanut consumption compared to peanut-tolerant subjects.

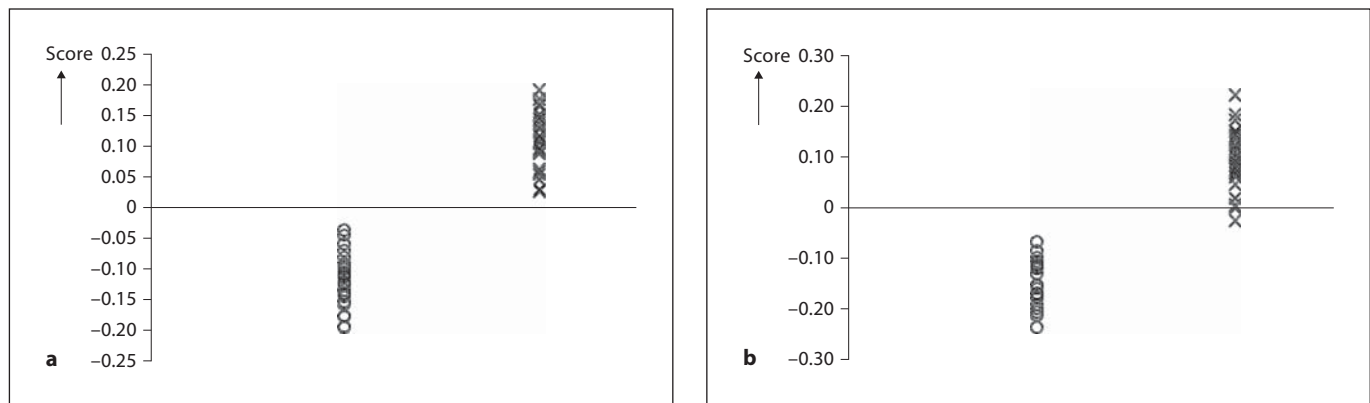


Fig. 1. Differences between plasma metabolite contents of peanut-allergic patients and peanut-tolerant subjects before and after ingestion of peanut. **a** Scores of NMR spectra of peanut-allergic subjects (x) and peanut-tolerant controls (o) before challenge. The difference in scores between peanut-allergic and peanut-tolerant subjects before ingestion of peanut is statistically significant ($p < 0.01$). **b** Scores of NMR spectra of peanut-allergic (x) and peanut-tolerant (o) subjects after ingestion of peanut. The difference between scores of peanut-allergic and peanut-tolerant persons is statistically significant ($p < 0.01$).

Table 1. Subject characteristics

Group	Subject	Sex	Age	Atopy	IgE grass pollen ¹	IgE birch pollen ¹	IgE peanut ¹	Severity (Mueller) ²	Subjective ED, mg
Peanut allergic	1	male	21	AR, AE	39	44	5.37	1	300
	2	male	32	AR	8.1	2.4	1.8	2	100
	3	male	25	AR, AA	34.2	5.8	49.5	4	0.1
	4	male	20	AR	36	68	5.07	1	0.1
	5	female	27	AR, AA, AE	>100	>100	12.9	2	10
	6	male	21	AR, AE	1.2	59	<0.35	0	10
	7	male	33	AR, AA, AE	>100	<0.35	>100	3	1
	8	female	24	AR, AA	<0.35	<0.35	18.3	4	10
	9	male	18	AR, AA, AE	>100	79	7.76	3	0.5
	10	female	29	AR, AE	<0.35	36	4.15	3	100
	11	female	28	–	1	<0.35	16.1	4	0.1
	12	male	27	AA	<0.35	<0.35	41	3	1
Peanut tolerant	1	male	24	–	<0.35	<0.35	<0.35	–	–
	2	male	28	–	<0.35	<0.35	<0.35	–	–
	3	male	22	–	1.7	<0.35	<0.35	–	–
	4	male	22	–	<0.35	<0.35	<0.35	–	–
	5	male	25	–	<0.35	<0.35	<0.35	–	–
	6	male	23	–	<0.35	<0.35	<0.35	–	–
	7	female	27	–	<0.35	<0.35	<0.35	–	–
	8	female	23	–	<0.35	<0.35	<0.35	–	–
	9	female	28	AR	1.6	15	<0.35	–	–
	10	female	21	AR, AE, AA	55	>100	33.5	2	no
	11	female	57	AR	0.6	7.7	0.63	2	no

ED = Eliciting dose; AR = allergic rhinitis; AE = allergic eczema; AA = allergic asthma.

¹ ImmunoCAP.

² Most severe symptoms by history scored according to Mueller [18].

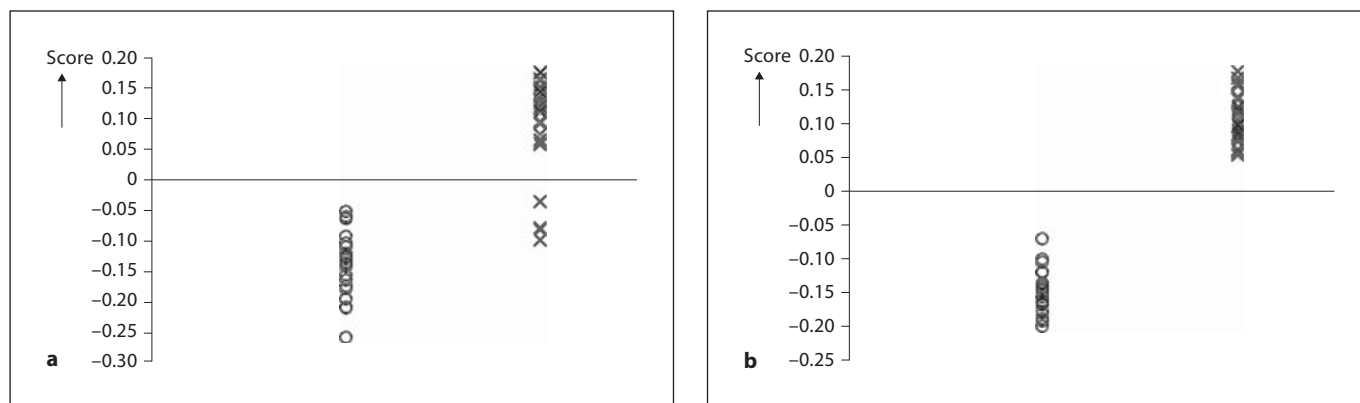


Fig. 2. Differences between saliva metabolite contents of peanut-allergic patients and peanut-tolerant subjects before and after ingestion of peanut. **a** Scores of NMR spectra of peanut-allergic patients (x) and peanut-tolerant controls (o) before challenge. The difference between scores of peanut-allergic and peanut-tolerant subjects is not statistically significant ($p > 0.1$). **b** Scores of NMR spectra of peanut-allergic (x) and peanut-tolerant (o) subjects after ingestion of peanut. The difference between scores of peanut-allergic and peanut-tolerant persons is significantly different ($p < 0.01$).

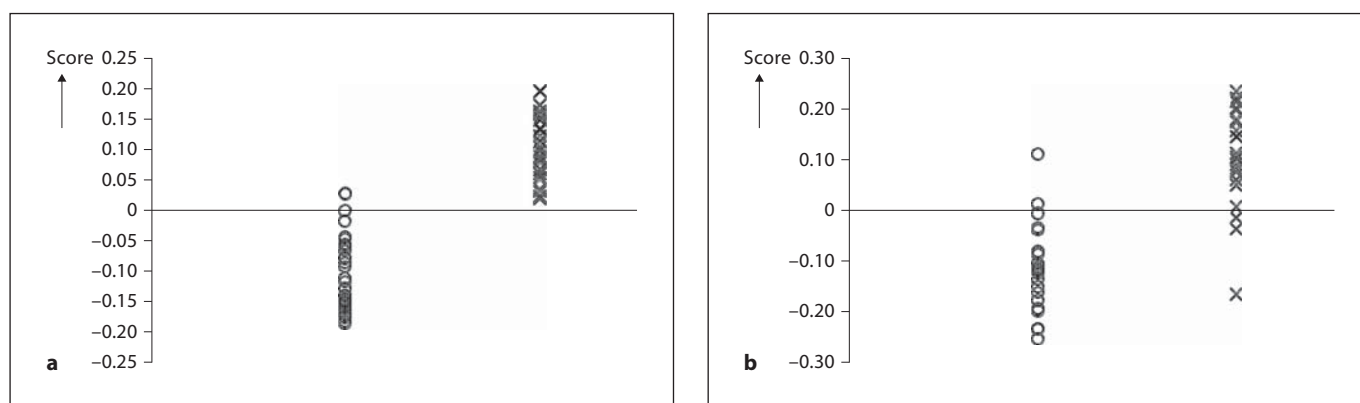


Fig. 3. Differences in plasma NMR spectra in peanut-allergic and peanut-tolerant subjects before and after peanut ingestion. **a** Scores of NMR spectra of peanut-allergic subjects before (o) and after (x) ingestion of peanut. **b** Scores of NMR spectra of peanut-tolerant patients before (o) and after (x) ingestion of peanut. The difference in scores before and after peanut ingestion was not significant in both the peanut-allergic patients and peanut-tolerant subjects ($p > 0.1$).

Differences in Saliva NMR Spectra between Peanut-Allergic and Peanut-Tolerant Subjects

The NMR spectra of saliva of peanut-allergic patients before peanut ingestion were not significantly different compared to peanut-tolerant controls ($p < 0.1$; fig. 2a). Regarding the saliva samples after peanut ingestion, the NMR spectra from peanut-allergic patients and peanut-tolerant subjects were in a remarkably different position on the score plot (fig. 2b; $p < 0.01$).

Identification of metabolites was not possible with the in-house database, since this was dedicated only to the analysis of plasma samples.

Differences in Plasma NMR Spectra in Peanut-Allergic and Peanut-Tolerant Subjects before and after Peanut Ingestion

To study the effect of peanut ingestion in plasma of peanut-allergic and peanut-tolerant subjects, NMR spec-

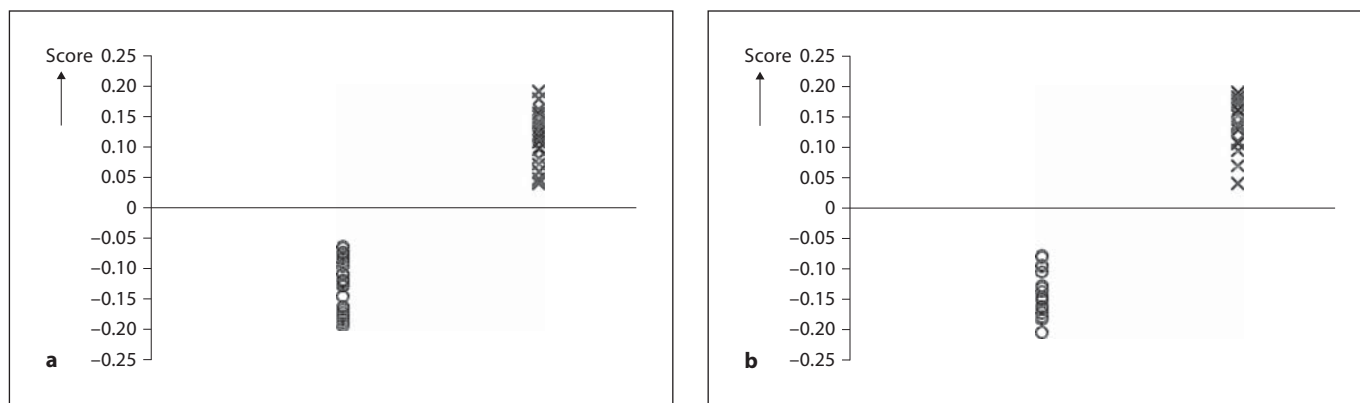


Fig. 4. Differences in saliva NMR spectra in peanut-allergic and peanut-tolerant subjects before and after peanut ingestion. **a** Scores of NMR spectra of peanut-allergic subjects before (o) and after (x) ingestion of peanut. **b** Scores of NMR spectra of peanut-tolerant patients before (o) and after (x) ingestion of peanut. The difference in scores before and after peanut ingestion was statistically significant in both the peanut-allergic patients and peanut-tolerant subjects ($p < 0.01$).

tra before ingestion were compared to NMR spectra after peanut ingestion in peanut-allergic as well as in peanut-tolerant subjects.

The NMR spectra of plasma of peanut-allergic patients before peanut ingestion were not significantly different compared to the NMR spectrum of plasma after peanut ingestion ($p < 0.1$; fig. 3a). Also in peanut-tolerant subjects no difference was observed in NMR spectra before and after peanut ingestion ($p < 0.1$; fig. 3b).

Differences in Saliva NMR Spectra in Peanut-Allergic and Peanut-Tolerant Subjects before and after Peanut Ingestion

Regarding the effect of peanut ingestion on metabolic fingerprints in saliva, samples of saliva before peanut ingestion were compared to samples of saliva after peanut ingestion in peanut-allergic as well as in peanut-tolerant patients.

NMR spectra of saliva before peanut ingestion were in a remarkably different position on the score plot compared to NMR spectra after peanut ingestion in both groups (fig. 4a, b; both $p < 0.001$).

Discussion

Current food allergy diagnosis heavily relies on detection of IgE in serum or by SPT. It is generally accepted that this leads to considerable overdiagnosis, since more than half of the foods to which patients are sensitized are

eaten without any problem. Moreover, there is no good correlation between the degree of sensitization and the severity of the allergy. This clearly illustrates the need for new diagnostic tools.

Comprehensive analysis of metabolites can lead to the discovery of new biomarkers for diagnosis of diseases. In this pilot study we investigated whether we could find differences in plasma and saliva metabolites between patients with peanut allergy and peanut-tolerant subjects using NMR and subsequent multivariate data analysis.

We were able to visualize differences in plasma metabolite levels of patients with peanut allergy compared to peanut-tolerant subjects, before as well as after ingestion of peanut (fig. 1, 2). In addition, a clear difference was found in the saliva of peanut-allergic patients before and after peanut challenge (fig. 3, 4). We hypothesize that the differences found between peanut-allergic and peanut-tolerant patients indicate the presence of biomarker(s) for food allergy. Allergies to other foods (especially foods belonging to the pollen-food syndrome) were present both in the peanut-allergic and in the peanut-tolerant group. However, peanut allergy is associated with more severe symptoms than the pollen-food syndrome. Although the amount of peanut consumption was different between peanut-allergic patients and peanut-tolerant volunteers, it is highly unlikely that the differences found were due to the amount of peanut consumption itself, since the differences found were qualitative and not quantitative.

Many of the observed NMR signals in the fingerprints could not be annotated with our database. Therefore, many metabolites remain unidentified. For identification of these compounds, liquid chromatography and mass spectrometry will be an indispensable tool in future studies. In this study we were only able to identify elevated levels of lactate, creatinine and glutamine in the peanut-allergic patients before as well as after peanut ingestion. This suggests that these are possible biomarkers for the allergic status. Especially creatinine and lactate are commonly encountered metabolites in metabolomics studies [11]. Glutamine is an important nutriment for the enterocytes that are abundantly present in the epithelial lining of the gastrointestinal tract. It therefore plays a role in the maintenance of gut mucosal integrity and function. Food allergy may result in a change in intestinal permeability through cytokine release in response to food allergens [19, 20].

Lipid and nicotinic acid levels were lowered in the peanut-allergic patients. Lipids, mainly polyunsaturated fatty acids, are known to be involved in regulating immune function. It has been shown that levels of high-density lipoprotein cholesterol are positively associated with atopy, suggesting that fat consumption and metabolism play a role in the manifestation of allergy [21]. It remains unclear why lipid levels are decreased instead of increased in peanut-allergic patients.

The differences found before as compared to after peanut challenge might reflect changes in metabolites induced by the food-allergic reaction itself. The fact that the differences found were only significant in saliva and not in plasma might be due to the fact that metabolites in saliva are likely to be present in higher concentration (less diluted) than in plasma. On the other hand the presence of metabolites in plasma might follow a different time course and we might have missed the optimum.

After peanut ingestion an elevation in levels of tyrosine and tryptophan was observed in the peanut-allergic subjects, something that was not observed before consumption. Tyrosine is a precursor to the biologically active catecholamines dopamine, norepinephrine and epinephrine [22]. These compounds are produced by the body in response to stress.

It is beyond the scope of this pilot study to identify the metabolites involved in peanut allergy and to fully explain the biology. Only relatively common and easily recognized metabolites have been identified from the NMR spectra. Future studies should put strong effort into the identification of all metabolites with other techniques than NMR, such as liquid chromatography and mass spectrometry, which are more suitable for compound identification. Moreover, adequate control groups should be included, to be able to identify whether the differences found are specific for atopy [the characteristic to make specific IgE, to allergy (having allergic symptoms), to food allergy or to peanut allergy (specific for a given food)].

In conclusion, using NMR, differences between metabolite levels of patients with and without peanut allergy were detected as well as differences before and after challenge with peanut. This indicates the presence of potential biomarkers for food allergy in plasma and saliva. Metabolomics has to be further explored for its potential in future food allergy diagnosis.

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