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RESEARCH LETTER

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Investigation of novel salivary biomarkers in paediatric food allergy

To the Editor,

Double-blind placebo-controlled food challenge (DBPCFC) is the "gold standard" to diagnose food allergy.[1,2] Challenge outcome is based on reported symptoms and can be difficult to interpret, especially if subjective symptoms occur (e.g. itchy mouth or abdominal discomfort).[3] Previous research showed that the determination of DBPCFC outcome is subject to individual interpretation, even if uniform assessment scores or outcome algorithms would be used.[4] Therefore, it would be valuable to have additional objective biomarkers.

The most common allergic reactions are caused by an immunoglobin E (IgE) immunologic mechanism in which symptoms do occur due to cross-linking of IgE to the IgE-receptor on mast cells, resulting in the release of several mediators such as tryptase and histamine.[5] In search of biomarkers associated with allergic reactions in children, it is desirable to use non-invasive diagnostic methods, especially if serial assessments are needed. Recently, we found that tryptase could be detected in saliva samples (by chewing on a synthetic swab) of children that underwent an oral food challenge.[6] Here, we aimed to gain insight into the kinetics of this biomarker and we investigated if salivary histamine can also be detected, now using a different method of saliva collection (passive drooling) which is broadly used in research with salivary biomarkers and is better suitable for children. We hypothesized that a dose-dependent release of these salivary biomarkers occurs during DBPCFCs with positive outcomes, but not during negative outcomes. This study is recorded in the Dutch trial register (URL: ICTRP Search Portal (who.int)) and approved by the Medical Ethical Committee (MEC) of Martini Hospital, Groningen, The Netherlands (MEC 2020-026). Written informed consent was obtained from children aged 12 years and older and all parents.

Between October 2020 and July 2021, 24 patients from 6 years and older (referred for suspected peanut or tree nut allergy) were included. DBPCFCs were performed according to European Academy of Allergy and Clinical Immunology (EAACI) guidelines.[1] As described previously, both challenge days consist of a maximum of seven steps with increasing dose of allergenic protein (starting dose 3 mg, total amount 4443 mg) and 30 min waiting time between each dose.[6] During the placebo day, no food allergen was administered. Symptoms were registered using the scoring system as proposed in a recent publication by Grabenhenrich et al.[7] In short, symptoms were divided into five categories (skin, respiratory, gastrointestinal, cardiologic/neurologic and other) and classified as mild, moderate and/or severe. Anaphylaxis was registered separately according to European guidelines.[8] Sensitization to the suspected food allergen was defined as serum-specific IgE (sIgE) level ≥0.35 kU/L (Phadia 250, Uppsala, Sweden). Additional information about study methods and findings is available in the following repository: https://doi.org/10.5281/zenodo.7794094.

Saliva samples were collected prior to, during and following both DBPCFC days following a pre-set schedule using the passive drooling method (Salimetrics, USA) to minimize contamination by the administered food as well as increase the possibility to collect sufficient saliva volumes.[9] Prior to collection, the mouth is rinsed with water and saliva is collected 5 min afterwards. If symptoms needed to be treated immediately (oral antihistamine and/or intramuscular adrenaline) at planned collection times, samples were collected directly after drug treatment. Collection vials for histamine were pre-filled with 5 μL 20% chlorhexidinedigluconate. Collected saliva samples were frozen immediately at −20°C for a maximum of 5 h and then stored at −80°C. Two samples (baseline and 30 min after the last dose) per challenge day were analyzed for histamine and methylhistamine (MH) using LC–MS/MS. All other samples were analyzed for tryptase with ImmunoCAP Tryptase immunoassay on the Phadia250 (Phadia, Uppsala, Sweden) using protocols described in our previous report.[6]

All statistical analyses were performed using IBM SPSS Statistics version 25 (IBM, NY, USA). Paired *t*-test was performed if the variables were normally distributed. If the distribution was not normal, Wilcoxon-Signed rank test was performed. To investigate differences between groups, unpaired *t*-test or Mann–Whitney *U* test (or Fisher's Exact) were performed if data were, respectively, skewed or not. A *p*-value of $<.05$ was considered statistically significant. GraphPad Prism Software was used to create all figures.

In total, 24 DBPCFCs were performed, and 3/24 were inconclusive due to (subjective) symptoms on both challenge days. Samples of one patient were excluded due to incorrect labelling. Analysis was limited to the other 20 challenges of which 14 (70.0%) had a positive and six had a negative outcome respectively. Anaphylaxis occurred in four patients. Most challenges were executed for hazelnut (8/20) and peanut (6/20). In general, the majority of patients (57.1%)

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developed symptoms after the first dose (3 mg allergenic protein) was administered and all these were classified as subjective. The dose that induced objective symptoms varied but was found to be most often the sixth (1000 mg) or seventh (3000 mg) dose respectively. Medication was needed in 10/14 DBPCFCs with positive outcomes. Saliva samples were collected 30–35 min after the last dose had been eaten in six of these challenges. The collection of samples was delayed (range: 40–62 min) in the other four challenges due to the occurrence of symptoms.

The patients (55.0% male) had a median age of 10 years (IQR 8.0–15.0). Allergic rhinitis was the most common atopic comorbidity. Proven allergies to other allergens mostly included walnut and cashew. Two patients (9.5%) were not sensitized to the specific food, however, DBPCFC was performed since medical history was suggestive for an allergic reaction. In our experience, challenge outcome could be positive despite sIgE <0.35 kU/L. Therefore, thorough assessment of patient's clinical history is most important to determine whether DBPCFC is needed. More patients with a positive DBPCFC outcome carried an adrenaline auto-injector ($p = .037$); no other significant differences were found between both groups.

An overview of all (methyl)histamine concentrations is provided (Figure 1). Log-transformation was performed because of a skewed distribution. Baseline histamine concentrations of the same patient on both challenge days were comparable. Median period between both days was 7 with a range of 7–28 days. Coefficients of variation were 23.50% (concentrations first day) and 24.39% (concentrations second day) respectively. Histamine concentrations were not different for DBPCFCs with positive and negative outcomes. Interestingly, methylhistamine concentrations were significantly lower at T30 regardless of the challenge outcome. Histamine is inactivated by methyltransferase and converted into methylhistamine. It is unlikely this pathway was influenced by administered medication (e.g. antihistamines) as lower concentrations were also observed after DBPCFCs with negative outcomes. Histamine mediators may be washed away due to rinsing of the mouth before collection. Additionally, it might have been too early to detect histamine metabolites in saliva.

Key messages

- Histamine and methylhistamine can be detected in saliva
- Histamine concentrations increased in 9/13 positive and 2/6 negative food challenges
- Collection of saliva using the passive drooling method was not suitable for the determination of tryptase

Delta histamine values were calculated by the subtraction of salivary histamine concentration of the sample collected 30 min after the last dose had been eaten (T_{30}) with salivary concentration of the baseline sample. Verum day samples of one positive challenge were excluded because the histamine concentration was more than four times standard deviation. Delta histamine values of saliva samples collected on verum days (i.e. allergen administered) showed an increase of histamine concentration in 9/13 positive and 2/6 negative DBPCFCs ($p = .114$). No increase of histamine was found in 14/20 samples collected on placebo days (Figure 2). In addition, delta histamine values were negative (i.e. baseline value higher than T_{30}) in cases with mainly mild symptoms. In contrast, an increase in salivary histamine was observed when reported symptoms were moderate to severe. Solitary objective symptoms

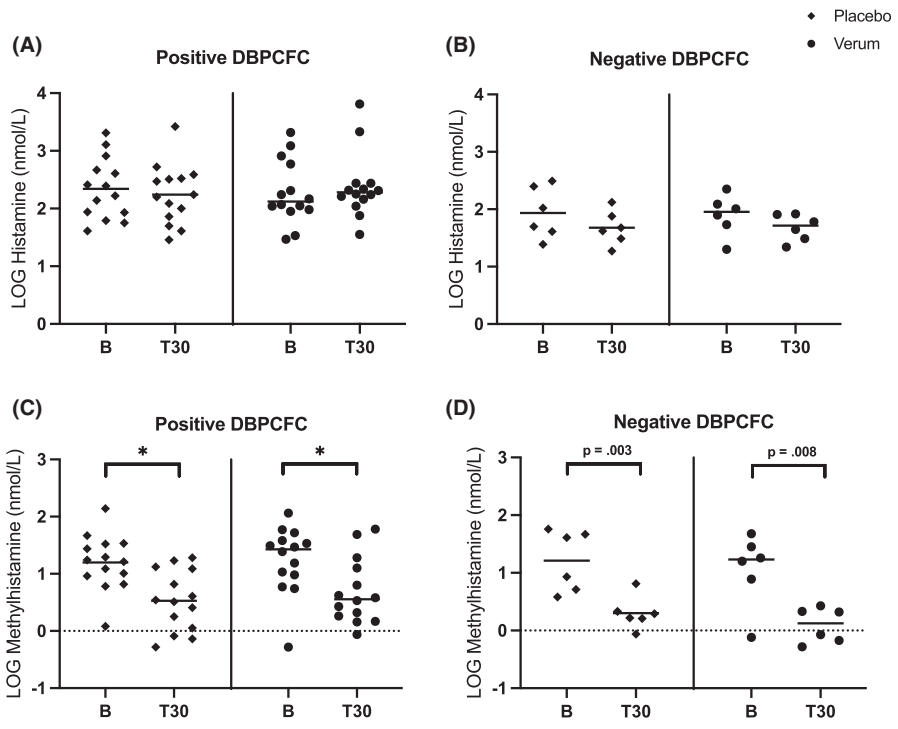

FIGURE 1 (A) LOG Histamine values from saliva samples collected during DBPCFCs with positive outcome. (B) LOG Histamine values from saliva samples collected during DBPCFCs with negative outcome. (C) LOG Methylhistamine values from saliva samples collected during DBPCFCs with positive outcome. (D) LOG Methylhistamine values from saliva samples collected during DBPCFCs with negative outcome. *p-value <.001.

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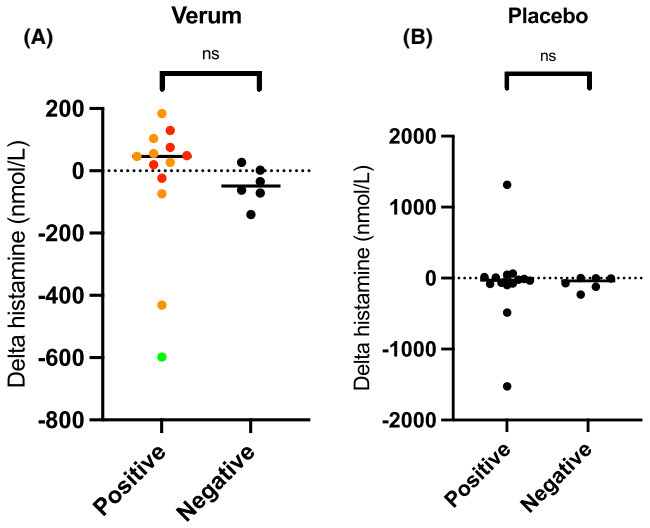

FIGURE 2 Delta histamine in saliva. Data stratified for verum (A) and placebo (B) days of positive and negative DBPCFC outcome respectively. Differences between the groups were tested with Mann–Whitney *U* test and were not significant ($p = .114$ for verum and $p = .539$ for placebo days respectively). Delta values are the result of salivary histamine concentration at T30 minus salivary histamine concentration at baseline (see Table S5, Appendix S1). Colours indicate severity of symptoms according to Table S6 (Appendix S1). DBPCFC, double-blind placebo-controlled food challenge; T30, sample collected 30 min after the last dose had been eaten; ns, not significant; green/orange/red dots indicate mild/moderate/severe symptoms respectively.

were reported once, solitary subjective symptoms occurred during two challenges and in all other cases both objective and subjective symptoms occurred.

This study demonstrates that salivary histamine concentrations can be detected, and differences were found between concentrations at baseline and T30. The majority of these increases were found in DBPCFCs with positive outcomes. We observed an interesting trend of histamine increase during positive DBPCFCs and believe that future studies are needed to address its discriminative power and optimal timing of samples. While our study is the first to measure histamine in saliva from children who underwent DBPCFC, several limitations need to be considered. First, if patients suffer from other atopic comorbidities, this may affect histamine concentrations (e.g. allergic rhinitis during the pollen season). Therefore, it will be necessary to determine baseline salivary histamine and methylhistamine concentrations in healthy subjects to gain information about possible reference values. Second, despite our study design, we were unable to obtain information on kinetics of salivary tryptase. Based on additional validation experiments, we discovered that the passive drooling method according to our protocol is not suitable for salivary tryptase analysis. Given the results of our previous study in which salivary tryptase could be detected in samples collected with synthetic swabs, collection of saliva samples using this method might provide opportunities to investigate kinetics in more detail. Finally, future studies should focus on the kinetics of salivary histamine (by collecting samples at more time points) to reveal optimum time points in order to minimize the number of samples to be collected for implementation in daily healthcare.

In conclusion, the results of our study demonstrated that salivary histamine may be a potential objective biomarker of DBPCFC outcome. Further research is needed to explore possibilities for clinical applications.

AUTHOR CONTRIBUTIONS

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KEYWORDS

biomarker, children, diagnostic, food allergy, histamine, oral food challenge, tryptase

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CONFLICT OF INTEREST STATEMENT

The authors report no proprietary or commercial interest in any product mentioned, concept discussed or personal relationships with other people or organizations that could influence their work and conclusions in this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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