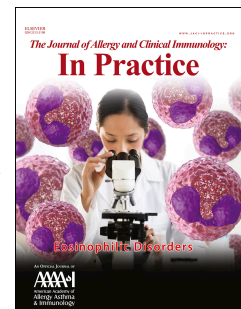


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Ameliorating Atopy by Compensating Micronutritional Deficiencies in Immune cells: a Double-Blind Placebo-Controlled Pilot Study

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Ameliorating Atopy by Compensating Micronutritional Deficiencies in Immune cells: a Double-Blind Placebo-Controlled Pilot Study

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Disclosure of potential conflicts of interest:

F.R-W. and E.J-J. are inventors of EP2894478, EP 14150965.3, Year: 01/2014; US 14/204,570, underlying the holoBLG lozenge, which is owned by Biomedical International R+D GmbH, Vienna, Austria. E.J-J. declares shareholdership in Biomedical Int. R+D GmbH, Vienna, Austria. S.M.A. was supported by grants of Biomedical Int. R+D GmbH and Bencard Allergie GmbH/ Allergy Therapeutics. S.G. was, A.G. and M.F.K. are employees of Bencard Allergie GmbH/ Allergy Therapeutics. The other authors declare no relevant conflict of interest in relation to this publication

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What is already known about this topic?

Iron-deficiency is linked with atopic diseases, while an improved iron-status is associated with decreased risk. Preclinical data and one non-controlled study suggest that whey protein beta-lactoglobulin provide micronutrients to immune cells leading to immune resilience.

What does this article add to our knowledge?

Evidence is given that improving the micronutritional status of immune cells seems to have a similar impact in ameliorating allergic symptoms as specific allergen immunotherapy. In the current practice micronutritional dietary recommendations should be included.

How does this study impact current management guidelines?

Allergen immunotherapy is considered the only causative treatment option to ameliorate atopic diseases. However, providing micronutrients to immune cells seems to have similar efficacy, providing evidence that targeted micronutrition may be another causative cure against allergies.

Keywords: atopy; targeted micronutrition; iron, vitamins, innate immunity, protective farm effect, beta-lactoglobulin, holoBLG lozenge, dietary supplement, Food for special medical purposes

Abbreviations

- BLG, beta-lactoglobulin
- FSMP, food for special medical purposes
- HoloBLG, beta-lactoglobulin with flavonoid-iron complexes and retinoic acid
- Th, T helper
- CRP, C-reactive protein
- LDH, lactate dehydrogenase
- PBMCs, Peripheral blood mononuclear cells
- ITT, intention-to-treat
- TNSS, Total Nasal Symptom Score
- CSMS, Combined symptom medication score
- dSS, daily Symptom Score

Abstract

BACKGROUND: Functional iron deficiency facilitates allergy development and amplifies the symptom burden in allergy sufferers. Previously micronutrients were selectively delivered to immune cells with beta-lactoglobulin as carrier (holoBLG), resulting in immune resilience and allergy prevention.

OBJECTIVE: The clinical efficacy of a FSMP (food for special medical purposes) lozenge containing beta-lactoglobulin with iron, polyphenols, retinoic acid, and zinc (holoBLG lozenge) were assessed in allergic women.

METHODS: In a randomized, double-blind placebo-controlled pilot study, grass- and/or birch pollen allergic women (n=51) were supplemented with holoBLG (verum) or placebo lozenges over 6 months. Prior and after dietary supplementation, participants were nasally challenged, and their blood was analyzed for immune and iron parameters. Daily symptoms, medications, pollen concentrations and well-being were recorded by an eHealth application.

RESULTS: Total nasal symptom score after nasal provocations improved by 42% in the verum versus 13% in the placebo group. Combined symptom medication score during the birch peak and entire season as well as entire grass pollen season improved in allergic subjects supplemented with the holoBLG lozenge by 45, 31 and 40% compared to the placebo arm. Participants ingesting the holoBLG lozenge improved their iron status with increased hematocrit values, decreased red cell distribution width and higher iron-levels in circulating CD14+cells compared to the placebo-group.

CONCLUSIONS: Targeted micro-nutrition with the holoBLG lozenge seems to be effective in elevate the label iron levels in immune cells and to reduce the symptom burden in allergic women in this pilot study. The underlying allergen-independent mechanism provides evidence that dietary nutritional supplementation of the immune system is one of the many cornerstones to combat atopy.

Trial registration NCT03816800, Iron and Pollen Allergy in Women

INTRODUCTION

Nearly a third of the population in the westernized world suffers from allergy. Influencing factors are still under debate; a decreased microbial exposure, a personal predisposition and/or intrinsic features of allergens considered relevant.

The professional term that defines this heightened immune response and the tendency to generate IgE antibodies is called atopy¹. Micro-nutritional deficiencies of the trace element iron²⁻⁴ and vitamin A, D and E are associated with atopy^{5,6}, with mutual relationships existing for all these vitamins^{7,8} with iron⁹⁻¹². Consequently, individuals lacking these micronutrients are prone to develop anemia^{13,14}.

We have previously shown that the whey protein beta-lactoglobulin, BLG, known as one of the major allergens in cow's milk, and loses its allergenic properties when it carries micronutrients such as flavonoid-bound iron^{15,16}, vitamin A¹⁷ and vitamin D¹⁸. Indeed, dietary uptake of the digestion- and protease-resistant BLG occurs via the lymphatic vessels^{19,20}, is receptor-mediated²¹⁻²³ -with different receptors described binding to it²⁴⁻²⁹, probably also via clathrin-dependent³⁰ mechanisms and is thus associated with immune cells in the lamina propria of the intestine within minutes upon ingestion^{15,16,20,29,31,32}. Its iron-binding properties have been explored in the food industry, and the BLG-iron complex is used as a carrier for iron supplementation in *in vitro*, *in vivo* and in clinical trials³³⁻³⁶. HoloBLG specifically targets myeloid cells, thereby evokes immune resilience and tolerance^{15,16,31}, similarly as observed in the "protective farm effect"¹⁵.

In the "protective farm effect" there are two factors contributing independently to the prevention of atopy: components of the "farm dust"^{37,38} as well as the protein-content in the whey fraction of raw cow's milk³⁹. Of note, up to 60% of the whey protein content consists of BLG that is able to carry micronutrients and, in this context, we demonstrated in preclinical studies, that it can evoke immune resilience when carrying micronutrients. In line, Pali-Schöll *et al.* demonstrated that BLG is also present in substantial amounts in "farm dust"⁴⁰ as it is also excreted by cattle and thus represents an unifying entity that could account for the protective capacities of the farm environment and raw milk.

Our research is driven by the hypothesis that iron deficiency leads to immune hyper-reactivity and allergy. Indeed, micronutritional deficiencies are common in atopic individuals, with allergic children having an up to 8-fold higher risk of anaemia^{2,3}. Functional iron deficiency, defined by a lack of mobilised iron due to the inaccessibility to the iron stores, has also been found in non-anaemic allergic individuals⁶. As an elevated immune activity –intrinsic in allergic individuals- always result to a certain degree in a functional iron-deficiency, mucosal iron uptake is impaired ('mucosal block'). Consequently, an adequate iron-supply by the dietary route is hampered.

Though the hyper-activated state of allergics is well recognized, allergen immunotherapy trials usually aim to modulate the course of disease by changing the type of antigen-specific hyper-reactivity, e.g. from T helper (Th) 2 to Th1.

Here, we aimed to correct the primary cause for allergy, i.e. the exaggerated immune response inherent in pollen allergic individuals, by compensating micronutritional deficiencies in myeloid cells via BLG in an unspecific manner during the pollen season. By supplementation with the holoBLG lozenge we aim to induce immune resilience – a mechanism mainly leveraging at the innate branch of the immune system.

METHODS

Participants and study design

We designed a randomized, double-blind, placebo-controlled study (approved by the ethic committee, #1370/2018, of the Medical University of Vienna; the study was prospectively registered at ClinicalTrials.gov: NCT03816800), in which blood and stool samples (for later analysis) were collected and nasal provocation was conducted before and after six months supplementation with a placebo or holoBLG lozenge twice daily. As of the date of taking the lozenge, patients reported their daily symptoms and medication use via an eHealth application provided by the Austrian Pollen Information Service (<https://www.pollenwarndienst.at/en.html>). They were also instructed to record missed intake of lozenges in the eHealth application as well as document any event that affected their wellbeing in the eHealth application. All participants provided written informed consent under institutional review board approved protocols before participation and the study was carried out in accordance with the provisions of the Declaration of Helsinki and Good Clinical Practice guidelines.

71 allergic premenopausal women were screened in winter 2019 (n=20) and 2020 (n=51). Due to lack of birch- and/or grass specific IgE, 13 participants (4 in 2019, 9 in 2020) were excluded. 58 participants were randomized and assigned into two arms (n=28 verum supplemented with holoBLG lozenge, n=30 placebo supplemented with the placebo lozenge). After the first nasal provocation, participants received the lozenges in sealed containers with a randomized number and consumed their first lozenge under supervision of the medical personnel.

Before start of supplementation, 3 participants in the verum (2020) and 4 in the placebo-group (two in 2019, two in 2020) failed to register in the electronic pollen diary and were lost during the follow up, resulting in 25 participants in the verum and 26 in the placebo arm. In the placebo-arm, 4 participants discontinued supplementation and electronic diary entries of the lozenges (one in 2019 due to hospitalization, three in 2020: one started an allergen immunotherapy against house dust mite and birch in August '20, one was lost in follow up and one moved to the country-side during the pandemic, living between two farms and drinking on regular basis raw cow's milk). All pollen-diary entries until the time point of discontinuation were used for our analyses. The study was heavily affected by the COVID-19 pandemic. During the enrollment and recruitment process in 2020, two lock-downs stopped further recruitment and hampered data acquisition of already enrolled study participants. Entering the hospital was prohibited except for emergency cases and thus impeded the visits for nasal provocations and blood drawings. Enrolled participants, who wished to continue the study, but missed one visit due to the lockdown, were sent a package with instructions and the lozenges, and were followed during the season without provocation tests. The electronic diary entries of all participants (n=25 verum, n=26 placebo) were analyzed, whereas - due to the pandemic - matched samples (before and after supplementation) for each study arm were n=20 (total 40) for the blood parameters and n=17 (total n=34) for the nasal provocations.

Randomisation and masking

An investigator without contact to participants or the clinical team managed the randomization process with a computerized system. Block randomization (block of 4 design) was employed to generate the randomized numbers for the vials containing the verum or placebo lozenges and the randomization list. The blinded clinical study team assigned the randomized vials to blinded participants. Assigned randomization number of study participants was recorded on a study case report form by the medical team and was kept in a locked cabinet. Participants in 2020 who did want to continue the study, but were not able to enter the clinics due to the COVID-19 pandemic lockdowns, received their vials via post and the investigator recorded the randomization numbers on a study case report form. Participants and the personnel administering the study interventions, assessing outcomes and the individuals analyzing the data during the study were blinded to the group assignment. All treatment assignments were kept securely and unblinding was performed after the last participants completed the study and samples were processed in 2019 and 2020, respectively. At the end of the study, a list with all the randomization numbers corresponding to study supplement assignments with the study code of the participants was provided as an excel file to the investigators for further analyses. No cases of accidental unblinding occurred.

Inclusion and exclusion criteria

As iron parameters and reference ranges differ between gender and with age, we eliminated confounding factors and focused on premenopausal women. Subjects were not considered for the study if they had co-morbidities such as diabetes mellitus, disorders of the liver including hemochromatosis or kidney, autoimmune or metabolic diseases and malignancies or if they had severe bleeding in the last two years (due to surgery, trauma) or blood transfusions. Further exclusion criteria were pregnancy, lactation, zinc and iron supplementation, allergen immunotherapy within the last five years prior enrollment or anti-IgE therapy.

Allergic rhinitis was determined based on medical history, physical examination, allergen-specific IgE, and a positive nasal provocation test with a total nasal symptom score (TNSS) ≥ 3 .

HoloBLG- and Placebo lozenge

The lozenge was designed as food for special medical purposes (produced and provided by Biomedical International R+D GmbH, Austria). The holoBLG-lozenges contains whey concentrate, iron ($<1\text{mg/lozenge}$), catechins, retinoic acid, zinc and sorbitol as carrier material, with mountain herbs used as flavour. The placebo lozenge is formulated without active ingredients, consists of sorbitol, mountain herbs flavour and karo-coffee providing a similar appearance as the verum lozenge. All ingredients in the lozenge are food-grade and comply with the requirements given by the Austrian Agency for Health and Food Safety, and with the European Food Safety Authority EFSA criteria for FSMPs, respectively.

Nasal provocation

Nasal provocations were conducted out of the birch and grass pollen season (End of January to March; End of September till Mid-November 2019 and 2020; at least two weeks before the respective season started) using the same scoring system as already described before⁶ at the ENT facility of the Medical University of Vienna and recommended by Riechelmann H et al^{41 42}. In brief, after a rhinological examination and evaluation of the nasal flow, the nasal allergen provocation was carried out using a bidose nasal delivery device (50µl/puff) (Aptar, Crystal Lake, Illinois, USA) directing the puffs unilaterally to the inferior meatus or middle turbinate. First, saline solution, followed by 1/100 and 1/10 dilutions of grass- or birch pollen nasal provocation solution (Leti pharma, Madrid, Spain) as recommended by the manufacturer, were applied in 10 minutes intervals. The physician scored symptoms 10 minutes after each challenge following the scoring system in the manual of the nasal provocation solution (Leti pharma, Madrid, Spain): secretion (no secretion – 0 points, little secretion – 1 point, much secretion – 2 points), irritation (sneezing: 0-2 x sneezing – 0 point, 3-5 x sneezing – 1point, >5 x – 2 points), and remote symptoms (no remote symptoms – 0 points, lacrimation and/or palate itching and/or ear itching – 1 point, conjunctivitis and/or chemosis and/or urticaria and/or cough and/or shortness of breathes- 2 points). Sums were calculated and subtracted from baseline scores to calculate the total nasal symptom score (TNSS). After the last challenge, nasal secretions were collected by inserting a pre-weighted sponge (Nasosorption, FX-I, Hunt Developments Limited, Midhurst, West Sussex, United Kingdom) as previously described⁴³ and weighing the sponge thereafter. The patients were provided with the verum/placebo lozenge after the nasal provocation. The first lozenge was taken on-site and participants were observed for 30 min to exclude any immediate adverse events.

Blood analyses

Complete blood cell analysis with iron metabolism marker (hemoglobin, serum iron, reticulocytes, mean reticulocyte hemoglobin, ceruloplasmin, haptoglobin, hemopexin, transferrin, soluble transferrin receptor, transferrin saturation, serum ferritin), creatinine, C-reactive protein (CRP), lactate dehydrogenase (LDH) were measured in the certified diagnostic laboratory of the general hospital of Vienna.

Pollen season

The start and stop dates of the pollen season of the Greater Vienna area (Pannonian lowland) were based on daily pollen concentrations after recommended definitions⁴⁴⁻⁴⁶. The start and the end of the birch pollen season was defined as the first or last five days of 7 consecutive days with at least 10 pollen grains/m³ and having a sum of at least 100 pollen. The start and end of the birch peak season was defined as the first or last 3 consecutive days with ≥ 100 pollen/ m³. The start and end of the grass season was defined by the first and last series of 5 days within 7 consecutive days, in which ≥ 10 pollen/ m³ were counted. The start and end of the grass peak season was defined with the start and end of at least ≥ 50 pollen grains/ m³ on 3 consecutive days having a sum of ≥ 100 pollen/ m³⁴⁴⁻⁴⁶.

Symptom medication score during the pollen season

The evaluation of 4 different symptom score calculations for 2 countries (Austria and Germany) and 2 pollen seasons (birch and grass) over the last decade showed that the choice of the calculation method is not that critical⁴⁷. Here, the raw symptom medication score of the Austrian Pollen Information Service was used as described before⁴⁷. Eyes, nose, and lungs were assessed for their general level of discomfort (0-no, 1-mild, 2-moderate, 3 strong discomfort) resulting in a maximum of 9 points. Further four specific symptoms per organ were rated (0-symptom did not occur, 1-symptom occurred): Eyes were assessed for itching, foreign body sensation, redness, and watering; nose for itching, sneezing, running, and blocked nose; and lung for wheezing, shortness of breath, cough, and asthma, resulting in a maximum of 12 points for specific symptoms and combined with the rating of the general discomfort of each organ, a maximum total of 21 points. Medication scores were weighted and resulted in a maximum of 4.65 points, in which eye medication were given a maximum of 1.8 points composed of 1 point for eye drops or tablets, 0.5 for others, and 0.3 for homeopathic medicine. Nose medication was given a maximum of 2.05 points composed of 1 point for nose drops or tablets, 0.25 for eye drops, 0.5 for others, and 0.3 for homeopathic medicine and lung medication was given a total of 0.8 points composed of 0.25 for tablets or others and 0.3 for homeopathic medicine. The maximum achievable points for the symptom medication score is 25.65. See references^{44, 46, 47} describing these established scores in detail.

Combined symptom medication score

The symptom medication scores of the Austrian Pollen Information service were dissected and transformed into the combined symptom medication score (CSMS) as recommended for allergen immunotherapy studies by the European Academy for Allergy and Clinical Immunology⁴⁸. Here, the daily symptom score was assessed by summing up 4 nasal symptoms (itchy nose, sneezing, runny nose, blocked nose) and 2 eye symptoms (itchy/red eye, watery eye), each reaching a maximum of 3 points (0-no, 1- mild, 2- moderate, 3- severe symptoms), finally resulting in a maximum of 18 points. The score reached was subsequently divided by the 6 specific symptoms, resulting in a maximum of 3 points for the daily symptom score (dSS). Daily medication score (dMS) was determined by giving 1 point for the use of oral and/or topical non-sedative H1 antihistamines (H1A), 2 points for intranasal corticosteroids with or without H1A and 3 points for oral corticosteroids irrespective whether intranasal corticosteroids or H1A were used or not, resulting in a maximum score of 3. The Combined symptom medication score (CSMS) was the sum of dSS and dMS, with a maximum of 6 points possible – mirroring the recommendations of the European Academy of Allergy and Clinical Immunology (EAACI) established as the gold standard measure of efficacy in allergen immunotherapy trials⁴⁸.

Flow cytometric analysis

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficon-Paque (GE Healthcare), and washed using 0.9% NaCl, before PBMCs were incubated in media neither containing phenol red nor fetal calf serum for 18 hours³⁵. Only a single time-point was measured due to the technical limitations that were encountered when working with iron, as media and buffers had to be iron-free, though iron is essential for cell survival.

Cells were stained with combinations of Calcein violet 450 AM (Thermo-Fisher 65-0854-39), CD14-APC (Biolegend, San Diego, Calif, USA; clone M5EZ), HLADR-PE (Biolegend, clone L243PC), and CD86-PE-CY7 (Biolegend, clone IT2.2). A second set was stained with Calcein violet 450 AM and CD3-APC-Cy7 (Biolegend, clone SK7), CD4-PE-Cy7 (Biolegend, clone SK3), CD25-APC (Biolegend, clone BC96), CD127-PE (Biolegend, clone A019D5) for flow cytometric analysis. Calcein-AM violet was used as a living marker as well as to determine the iron load in living cells¹⁶.

Doublets were excluded, before gating on the living cells as Calcein+, further gating in the FSC/SSC plot to the “lymphocytic gate” for plotting CD3+CD4+ cells, respectively and gating the “monocytic” gate for plotting CD14+cells. Cells were acquired by flow cytometry (BD Bioscience Canto II machine). Acquired cells were analyzed using the FACSDiva Software 6.0.

Outcomes

The primary endpoint was defined by assessing total nasal symptom score at baseline and after supplementation via controlled nasal provocation. The secondary outcomes included mean daily combined symptom medication score during the peak of the pollen season, and assessment of iron status. Further, exploratory endpoints for later analysis include the microbial composition at baseline and after supplementation.

Role of the funding source

Biomedical International R+D GmbH was involved in the study design and writing of the report. Bencard Allergie GmbH was involved in interpretation of the data and writing of the report. No other funders were involved. FRW had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Data availability and sharing

The study protocol and statistical analysis plan are provided as Supplementary Note. The trial dataset will be available to appropriate academic parties on request from the corresponding author, in accordance with the data sharing policies of the University of Veterinary Medicine Vienna and the Medical University of Vienna, with input from the investigator group where applicable, subject to submission of a suitable study protocol and analysis plan, on publication of all initial trial results.

Statistical analysis

The study and analysis plan were designed before enrolment of participants. Data were examined for their normal and log-normal distribution using Anderson-Darling and D'Agostino Pearson test. Normally distributed data sets were compared with Student's t-test, the non-parametric Mann Whitney U-tests was applied for not normally distributed data sets to determine differences between the two study arms in the intention-to treat groups. Matched non-parametric parameters were compared with Wilcoxon matched-pairs signed rank test, parametric data sets were compared with paired t-test. All tests were two-sided and a p-value below 0.05 was considered significant. Data and graphs were analyzed with GraphPad Software Prism 9.

RESULTS

Study participants

Of the 71 screened subjects in total, 58 were eligible and randomized (Table E1 details trial profile per year) into two arms (n=28 allocated to verum; n=30 allocated to placebo). Details on patient characteristics are given in Figure 1. All randomized patients receiving at least one dose of the verum/placebo lozenge and registered successfully in the electronic diary were designated as intention-to-treat ITT population (n=25 verum, n=26 placebo). The trial was completed by 47 patients. All subjects in the verum-arm completed the study (n=25), whereas 4 in the placebo-arm discontinued supplementation and 22 completed the study. The baseline demographics and clinical characteristics were comparable in the placebo- and verum-arm (Table 1 and Figure E1 for pre-birch seasonal symptom medication score) including only premenopausal women with a mean age of about 31 years, being comparable in weight and having a total IgE over 100 kU/L on average. Patients were either allergic to birch and/or grass pollen (68% to birch and 88% to grass pollen allergic in the verum-arm, 77% to birch and 81% to grass in the placebo-arm). Specific antibodies did neither significantly differ between the ITT-arms nor in the matched samples (Table E3).

Study participants Total Nasal Symptom Score

Subjects were nasally challenged before and after 6-month supplementation with birch or grass pollen extract. Total nasal symptom score, TNSS, did not differ before, but was significantly lower after supplementation with the holoBLG lozenge (Figure 2A) compared to placebo. Mean TNSS of the ITT population before and after supplementation was 4.37 and 3.82 for the placebo-arm and 4.78 and 2.58 for the verum-arm, respectively. As depicted in Figure 2B, matching the data revealed a significant reduction in the verum group ($p=0.0005$) resulting in an average individual improvement of 42.4% (Table 2), whereas no significant changes in matched samples were visible after placebo-supplementation. Placebo effects were nevertheless measureable as the symptoms improved on average by 12.9% (Figure 2C, Table 2). A similar picture was apparent by the collection of the nasal secretions (Figure 2D-F). Furthermore, the two arms did not differ at randomization, but after holoBLG supplementation significantly lower nasal secretion amounts were collected (Figure 2D). Regarding the individual nasal outcome before and after supplementation, both groups had significantly less nasal discharge after 6-month supplementation, irrespective of the lozenge taken. However, the reduction in the nasal outflow was significantly larger in the group receiving the holoBLG lozenge (Figure 2F). Summing up, as observed in the vast majority of placebo-controlled allergy trials, a certain placebo-effect was apparent in the nasal provocation tests which led to an improvement of the symptoms by $\sim 13\%$. HoloBLG supplementation significantly exceeded this placebo-effect resulting in a general reduction of the nasal symptom burden of $\sim 42\%$ for the primary endpoint of the study. The observed difference in nasal allergen provocation between verum and placebo reached statistical significance ($p=0.04$).

Symptom burden during the birch and grass pollen seasons.

Participants recorded their daily symptoms and medication use daily in the pollen diary provided by the Austrian Pollen Information Service – as described before^{46, 47, 49}. Those data were analyzed for the entire and peak pollen season with the caveat that neither the 2019 nor the 2020 grass pollen season met the EAACI criteria of a peak pollen season because of the respective climatic conditions. Results of the ITT-statistical analyses of the daily symptom burden as assessed by combined symptom medication score (EAACI CSMS definition)⁴⁸ and daily symptom score dSS during the entire and peak birch-pollen season 2019 and 2020 for birch pollen-allergic subjects as well as the entire grass pollen season 2019 and 2020 for grass-pollen allergic participants are depicted in Figure 3. Detailed analyses of the year 2019 and 2020 are depicted in Figure E2 and Figure E3. The raw symptom medication scores, SMS, as provided by the Austrian Pollen Information Service are shown in Figure E4. The analysis of this secondary outcomes demonstrates a statistically significant improvement in the average CSMS by 45% and the dSS by 39% and by 41% for SMS in the holoBLG-lozenge group during the birch pollen peak season relative to placebo (Figure 3A, Table 2), thereby meeting this predefined endpoint. The absolute mean difference was 0.23 for CSMS and 0.16 for dSS and 2.1 for the SMS ($p < 0.0001$ for all) for the birch peak season. Over the whole birch season average CSMS improved by 31% and the dSS by 35% (Figure 3B, Table 2). Over the entire grass pollen season, a relative improvement of 40% for CSMS, 26% for dSS and SMS by holoBLG supplementation compared to placebo was achieved (all $p < 0.0001$) (Figure 3C, Table 2). All analyses of key secondary endpoints supported the primary analysis results. Thus, supplementation with micronutrients via holoBLG was effective in reducing the symptom burden in an allergen-unspecific manner.

Total and specific IgE antibodies

Immune resilience is a non-allergen specific phenomenon elicited by modulation of the innate immune system. Despite that fact, we also looked at allergen-specific parameter in the context of that trial: Allergen-specific and total IgE levels were comparable at baseline (Table 1) and in matched samples (Figure 4 and Table E3). Allergen-specific IgE increased in both groups (Figure 4B-D), whereas total IgE increased in the placebo-supplemented group only (Figure 4A), illustrative of the well described changes of IgE titers caused by natural pollen exposure. There was no statistically significant difference in allergen-specific IgE levels observed between both arms.

Changes in blood parameters

Blood parameters were compared before and after dietary intervention with the holoBLG or placebo lozenge. Results of the ITT-statistical analyses (Table E2) revealed that all parameters were within the normal range, though after supplementation the CRP-values were significantly higher in the placebo-arm and a trend towards less absolute lymphocyte counts were apparent compared to participants consuming the holoBLG lozenge. Data were analyzed by Mann-Whitney or Student's t test and were not adjusted for the numbers of parameters measured. However, on the basis of these parameters, one can readily appreciate that key and not purely

coincidental blood values have changed. Due to the low n-number and the amount of iron supplied we consider the changes nevertheless considerable.

As depicted in Table E4, detailed analyses of the matched blood parameters, in which before and after values were individually assessed, revealed that supplementation with holoBLG lozenge significantly improved the hematocrit-levels and reduced the red cell distribution width. This indicates that particularly the myeloid cell lineage was affected by 6-month supplementation with holoBLG compared to the placebo-arm.

Hence, in this study supplementation with the holoBLG lozenge in particularly seemed to strengthen cellular components of the myeloid lineage.

Immunological changes

In previous preclinical studies, holoBLG was able to increase intracellular iron levels in monocytic cells¹⁶. Hence in a next step, we isolated and analysed unstimulated peripheral mononuclear cells of both arms before and after supplementation. As depicted in Figure 5, no significant differences in the relative numbers of monocytic CD14⁺ cells were apparent before or after supplementation in the two arms. However, an increase of the intracellular iron content in the circulating CD14⁺ monocytes could be demonstrated after 6-month supplementation with the holoBLG-lozenge, but not in subjects given the placebo lozenge.

This iron supply was restricted to the monocytic CD14⁺ compartment and did not extend to T cells. The relative numbers and the iron status of T cells, Th2 and Tregs were comparable in both arms and remained unchanged after supplementation (Figure E5).

Hence, we demonstrate here that supplementation with the holoBLG lozenge was effective in delivering its micronutritional cargo to monocytes, but not lymphocytes. An improved iron-status in the monocytes was achieved upon holoBLG supplementation, but not upon placebo supplementation.

Safety, tolerability and adherence

In this study, the holoBLG lozenge was designed as food for special medical purposes and all ingredients were food grade. In accordance, the tolerability was excellent and no adverse events were reported; only two participants of the verum-arm commented to dislike the taste. Adherence to the lozenge was high in general; patients who forgot single doses were advised to still finish all lozenge doses provided and had therefore a slightly increased intake period.

DISCUSSION

Atopy is associated with micronutritional deficiencies such as iron and vitamin A, D, E and a hyperresponsive immune system^{2-5, 15, 16, 31}. Particularly the lack of iron acts as a danger signal for immune cells leading to their activation. Here, we hypothesized that the inherent exaggerated immune response of atopic individuals can be abrogated when these micronutritional deficiencies are restored. Indeed, a study conducted by Fortes et al.⁵⁰ reported that children in which mothers used both iron and folic acid supplementation had a fourfold decreased risk of developing atopic dermatitis.

Our previous preclinical studies suggested that holoBLG, as the major whey component which is also linked to the so-called protective farm effect, could be exploited to target micronutrient to myeloid cells and induce immune resilience^{15, 16, 31, 33, 51}. Indeed, in a first observational trial conducted in house dust mite allergic rhinoconjunctivitis patients, in which participants were challenged in an standardized and validated allergen exposure chamber before and after supplementation with the holoBLG lozenge for 3 months, a significant reduction of median TNSS at 120min by 60 %⁵² could be demonstrated emphasizing its antigen-independent potential. Indeed, 3-months supplementation with the holoBLG lozenge already showed a long-lasting impact, as seven to eight months after cessation of supplementation, a sustained long-term reduction by 40% of the total symptom score compared to prior supplementation was reported⁵³.

Here, we went one step further and performed a randomized, double-blind, placebo-controlled trial as a proof-of-concept, in which particular attention was given to immune and iron-parameters.

The holoBLG lozenge demonstrated a statistically significant and clinically relevant impact in reducing the total nasal symptom score by 42% measured during nasal allergen provocation and reducing the combined symptom medication score and daily symptom score in both the birch and the grass pollen season.

Supplementation with holoBLG resulted in a substantial and significant improvement for average CSMS, dSS and SMS in the birch- and grass pollen season (relative difference to placebo for CSMS, dSS, SMS in the birch pollen peak season: 45%, 39%, 41% and for the grass pollen season 40%, 26% and 26%, respectively). In fact, using the "golden standard" (= the EAACI CSM score⁴⁸) the observed efficacy was comparable if not exceeding efficacy rates reported for allergen immunotherapy^{54, 55}, all in an antigen-independent manner, via achievement of innate immune resilience. Such comparison however, is of course weakened by the fact that our proof-of-concept pilot study was a relatively small and mono-center trial.

Our data emphasize that micronutritional deficiencies are indeed present in atopic individuals^{56, 57, 2, 58, 59} and that iron-deficiency per se is sufficient to generate a Th2-milieu in vitro as well as in the preclinical^{33, 51, 60, 61} and clinical setting⁶² which is the prerequisite for allergy development.

Immune resilience is a non-allergen specific phenomenon elicited by modulation of the innate immune system. Despite that fact, we also sought to understand allergen-specific parameters in the context of the

trial, an increase of specific IgE was observed in both study arms, which might simply reflect seasonal allergen exposure leading to this effect. An increase in specific IgE has also been reported after the first year of sublingual allergen immunotherapy, but not subcutaneous therapy^{63, 64, 65}.

Interestingly, total IgE-levels differed within the groups. In line with reports of other clinical studies showing an increase of total IgE in the placebo-arm⁶⁶ as well as a seasonal increase of total IgE⁶⁷, also in this study total IgE was significantly elevated after 6-month supplementation in the placebo-arm. Whatsoever, no significant changes in the total IgE-levels were observed in the group that received holoBLG, which emphasizes again that holoBLG acts antigen-unspecific and that immune resilience is established. Interestingly, also after successful allergen immunotherapy⁶⁸ a significantly decline of total IgE over time has been reported.

The holoBLG lozenge was initially developed to investigate whether supplying micronutrients could change the atopic state. As immune activation always leads to different degrees of functional iron-deficiency, and consequently iron absorption and mobilization is reduced, all kinds of immune activation will affect the iron parameters^{6, 51}. Thus, allergic individuals with their increased immune response, are in a vicious cycle, in which they have to exploit different nutritional strategies to compensate their immune iron requirements as otherwise their immune system remains hyper-activated and prone to the Th2 milieu.

The amount of iron present in the lozenge (less than 1mg) was made available in a form that seem to particularly target cells from the myeloid lineage - hence from the bone marrow. The myeloid lineage includes a number of morphologically, phenotypically and functionally distinct cell types such as granulocytes (neutrophils, eosinophil and basophils), monocytes, macrophages, erythrocytes, megakaryocytes and mast cells⁶⁹.

Indeed, several studies demonstrated that providing mast cells with iron - suitable for uptake- hamper their readiness to degranulate^{16, 70, 71} and thus, the reduced symptom burden observed in this study after holoBLG supplementation may point out that holoBLG also provides micronutrition to effector cells such as mast cells.

As such, holoBLG supplementation improved the hematocrit – the volume percentage of red blood cells-, decreased the red cell distribution width (increased RDW are observed in iron-, folate- and Vitamin B12-deficiencies), and significantly more iron could be measured in the circulating monocytes after 6-month supplementation. In contrast, T cells were not the primary target and again distinguish this nutritional approach from specific allergen immunotherapy, in which the innate lymphoid and T-cell repertoire are changed^{72, 73}.

In this study, we used a small, very well-defined cohort which is the strength and the limitation of the study at the same time. Only non-smoking, otherwise healthy premenopausal women with no co-morbidities such

as diabetes mellitus, disorders of the liver or kidney, autoimmune or metabolic diseases and malignancies, were included in this study and thus may exaggerate the impact of the holoBLG lozenge in ameliorating atopic rhinitis under real life conditions. In particular as functional iron-deficiency is a common problem not only for atopic^{2-4, 6, 74} individuals, but also extend to autoimmune diseases^{75, 76}, chronic and metabolic diseases such as chronic kidney diseases⁷⁷⁻⁷⁹, congestive heart failure⁸⁰⁻⁸², chronic pulmonary diseases⁸³⁻⁸⁵, and obesity^{86, 87}. In the real-life situation several comorbidities, a vegan life-style and so on may reduce the observed efficacy of the FSMP-lozenge. As in the first observational trial in house dust mite allergic rhinoconjunctivitis patients⁵², also men were included, we expect that supplementation in men will similarly calm the immune system.

Summing up, we demonstrate in this single-center, proof-of-concept study, that the underlying BLG-related mechanisms of the protective farm effect seems to be translatable into the clinical setting via the holoBLG lozenge and to improve the symptoms of allergic individuals. By formulating this holoBLG lozenge, we show in this novel approach that atopy may be ameliorated by targeted micronutrition. Compensating these deficiencies seems to rebalance immune cells and establish immune homeostasis which we termed immune resilience^{16, 88}. Our concept revolutionizes the understanding of allergy and the mechanisms of resilience to fight allergies.

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Author contributions:

T.B. performed and supervised nasal provocations and acquisitions of human samples, provided support and contributed in data analyses and to writing. SAJ under supervision performed nasal provocations and acquisition of human samples, contributed in data analyses and to writing. S.M.A, R.B. K.H., G. H. performed experiments and contributed to writing. MBe, M.Ba and U.B. provided the pollen diary platform, acquired data and contributed in data analysis and writing. E.R. and K.S. conducted blood analyses and contributed to writing. J. E.-D. provided help in the study design, provided equipment and contributed in writing. E.V. and F.F.B. helped in nasal provocations and sample acquisition, and contributed to writing. S.G. and A.G. provided support and contributed in manuscript writing. M.F.K. helped in data interpretation and analysis, provided support and contributed in manuscript writing. E.J-J. financed research, helped in sample acquisition, contributed to the study design and manuscript writing. F.R-W. conceived, directed the research, designed the experiments, analyzed and interpreted the data and wrote the manuscript. **Competing interests:** F.R-W. and E.J-J. are inventors of EP2894478, EP 14150965.3, Year: 01/2014; US 14/204,570, underlying the holoBLG lozenge, which is owned by Biomedical International R+D GmbH, Vienna, Austria. E.J-J. declares shareholdership in Biomedical Int. R+D GmbH, Vienna, Austria. S.M.A. was supported by grants of Biomedical Int. R+D GmbH and Bencard Allergie GmbH/ Allergy Therapeutics. S.G. was, A.G. and M.F.K. are employees of Bencard Allergie GmbH/ Allergy Therapeutics. The other authors declare no relevant conflict of interest in relation to this publication. **Data and materials availability:** The trial dataset will be available to appropriate academic parties on request from the corresponding author, in accordance with the data sharing policies of the University of Veterinary Medicine Vienna and the Medical University of Vienna, with input from the investigator group where applicable, subject to submission of a suitable study protocol and analysis plan, on publication of all initial trial results.

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Table 1. Patient's characteristics at baseline

	holoBLG lozenge			Placebo lozenge			
	n	Mean	CI	n	Mean	CI	P
Age (y)	25	31.96	(27.65/36.27)	26	30.78	(26.58/34.97)	0.53
BMI		22.30	(20.88/23.73)		22.62	(21.06/24.28)	0.95
total IgE (kU/L)	25	100.96	(21.04/216.7)	26	118.88	(57.14/144.8)	0.41
birch pollen-specific IgE (kUA/L)							
Bet v 1-spec. IgE	17	14.95	(8.40/21.49)	20	14.03	(7.94/20.13)	0.55
grass pollen -specific IgE (kUA/L)	22			21			
Phlp 1 - spec. IgE		6.99	(4.0/9.97)		15.39	(8.31/22.46)	0.16
Phlp 5 - spec. IgE		13.56	(8.08/19.03)		13.19	(7.33/19.06)	0.99
total nasal symptom score, TNSS before supplementation	18	4.78	(3.72/5.84)	21	4.33	(3.65/5.01)	0.45
blood parameters							
hemoglobin g/dL		13.31	(12.98/13.63)		13.50	(13.13/13.86)	0.43
hematocrit %		39.18	(38.55/39.82)		39.85	(38.83/40.89)	0.27
ferritin µg/l		54.44	(35.93/72.96)		54.83	(34.78/75.07)	0.99
transferrin mg/dL		306.50	(285.4/327.6)		287.75	(262/312.9)	0.25
soluble transferrin receptor mg/L		1.45	(1.16/1.73)		1.20	(1.09/1.30)	0.27
serum iron µg/dL		99.37	(78.33/120.4)		94.83	(77.5/112.2)	0.73
hemopexin mg/L		91.85	(87.46/96.25)		91.67	(85.67/97.66)	0.96
Haptoglobulin mg/dL		27.09	(24.25/29.93)		34.21	(27.34/41.07)	0.17
Ceruloplasmin mg/dL		27.09	(24.25/29.93)		30.95	(26.38/35.53)	0.35
Inclusion and exclusion criteria:							
Non-smoking pre-menopausal women with no co-morbidities such as diabetes mellitus, disorders of the liver or kidney, autoimmune or metabolic diseases and malignancies. They had no severe bleeding in the last two years (due to surgery, trauma) or blood transfusions prior to enrollment. Further exclusion criteria were pregnancy, lactation, zinc and iron supplementation, allergen immunotherapy within the last five years prior enrollment or anti-IgE therapy							

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Table 2. Analytical results for the primary (TNSS) and key secondary endpoint (CSMS)

Endpoints	Treatment group	n	Mean (SD)	Difference between Mean ± SEM (95% CI) or #Difference between Medians (95% CI exact)	p
Total nasal symptom score TNSS					
TNSS before supplementation	Placebo	19	4.37 (1.57)	—	0.509
	holoBLG	18	4.78 (2.13)	0.41 ± 0.61 (-0.83 to 1.65) # 0.50 (-1.00 to 1.00)	
TNSS after supplementation	Placebo	22	3.82 (2.36)	—	0.033
	holoBLG	24	2.58 (2.26)	1.24 ± 0.68 (-0.14 to 2.61) #1.00 (0.00 to 2.00)	
Individual TNSS improvement in %	Placebo	17	12.89 (51.33)	—	0.045
	holoBLG	17	42.39 (25.97)	29.50 ± 13.95 (1.08 to 57.92) # 16.67 (0.00 to 40.00)	
Combined symptom medication score CSMS					
Absolute difference to placebo ± SEM (95% CI)					
Birch pollen peak season 2019 and 2020	Placebo	20	0.50 (0.15)	—	<0.0001
	holoBLG	17	0.28 (0.10)	0.23 ± 0.042 (0.14 to 0.31)	
CSMS improvement in %	Placebo	20	0 (30.26)	—	<0.0001
	holoBLG	17	45.02 (20.33)	45.02 ± 8.36 (61.99 to 28.06)	
Birch pollen entire season 2019 and 2020	Placebo	20	0.42 (0.13)	—	<0.0001
	holoBLG	17	0.29 (0.11)	0.13 ± 0.03 (0.08 to 0.18)	
CSMS improvement in %	Placebo	20	0.00 (32.18))	—	<0.0001
	holoBLG	17	30.73 (26.43)	30.73 ± 6.42 (17.95 to 43.51)	
Grass pollen season 2019 and 2020	Placebo	20	0.28 (0.07)	—	<0.0001
	holoBLG	21	0.17 (0.06)	0.11 ± 0.01 (0.09 to 0.13)	
CSMS improvement in %	Placebo	20	0.00 (26.46)	—	<0.0001
	holoBLG	17	39.89 (21.63)	39.89 ± 3.42 (33.15 to 46.64)	
Daily symptom score dSS					
Birch pollen peak season 2019 and 2020	Placebo	20	0.42 (0.12)	—	<0.0001
	holoBLG	17	0.26 (0.09)	0.16 ± 0.03 (0.09 to 0.23)	
dSS improvement in %	Placebo	20	0.01 (27.55)	—	<0.0001
	holoBLG	17	38.58 (21.85)	38.58 ± 8.07 (22.22 to 54.94)	
Birch pollen entire season 2019 and 2020	Placebo	20	0.37 (0.12)	—	<0.0001
	holoBLG	17	0.24 (0.10)	0.13 ± 0.03 (0.08 to 0.19)	
dSS improvement in %	Placebo	20	0.00 (30.98)	—	<0.0001
	holoBLG	17	35.20 (25.64)	35.20 ± 7.34 (20.51 to 49.89)	
Grass pollen season 2019 and 2020	Placebo	20	0.21 (0.07)	—	<0.0001
	holoBLG	21	0.16 (0.07)	0.05 ± 0.01 (0.04 to 0.07)	
dSS improvement in %	Placebo	20	0.00 (31.74)	—	<0.0001
	holoBLG	17	25.62 (26.43)	25.62 ± 3.97 (17.78 to 33.44)	

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FIGURE legends

FIGURE 1. Trial profile.

FIGURE 2. Total nasal symptom score and nasal secretions after nasal allergen challenge with birch- or grass pollen-extract. **A.** Total nasal symptom score before and after supplementation, **B.** Matched TNSS in the placebo and holoBLG supplemented group **C.** TNSS improvement in % of the placebo and holoBLG supplemented group. **D.** Nasal secretions in mg before and after supplementation, **E.** Matched secretions in the placebo and holoBLG supplemented group **F.** Improvement in the nasal outflow in % of the placebo and holoBLG supplemented group. ns. Not significant. * $P < 0.05$, *** $P < 0.001$.

FIGURE 3. EAACI Combined Symptom Medication Score and daily Symptom Score over the A. peak and B. entire birch and C. the entire grass pollen season 2019 and 2020. Mixed-effect model (REML) using uncorrected Fisher's LSD test with a single pooled variance were used for analysis over birch season. Normality in the groups was tested via Anderson-Darling. Comparison of placebo- and the holoBLG-arm was performed with unpaired t-test for parametric data, whereas non-parametric data were compared with Mann Whitney U-test. ns. Not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ **** $P < 0.0001$

FIGURE 4 Total and specific IgE antibodies of matched samples before and after 6 months-supplementation with placebo- or holoBLG lozenge. Individual levels of **A**, total **B**, Bet v 1-specific **C**, Phl p 1-specific and **D**, Phl p 5-specific IgE-levels within the placebo- and verum-group as well as compared to each other. Normality in the groups were tested via Anderson-Darling. Matched data were compared with Wilcoxon-matched pairs signed rank test. Placebo- and the verum-arm was compared with unpaired t-test for total IgE, whereas non-parametric data were compared with Mann Whitney U-test (specific IgEs); ns. Not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

FIGURE 5. Supplementation with the holoBLG lozenge increases the intracellular iron status of monocytes. Peripheral blood mononuclear cells were isolated by density gradient and incubated overnight in iron-free media, before staining for CD14 and measuring the intracellular iron-content by means of quenching of the calcein-signal. **A**, After excluding doublets and gating the monocytic population, further gating was performed on CD14 expression following by measuring the mean fluorescence intensity of the calcein-signal. Summary of the relative numbers of CD14+ cells and the respective calcein signal **B**, before and **C**, after 6 months supplementation with the placebo or holoBLG lozenge. Normality in the groups were tested via Anderson-Darling. Normal distributed data were compared with unpaired t-test, non-parametric data were compared with Mann Whitney U-test; ns. Not significant, ** $P < 0.01$

Ameliorating Atopy by Compensating Micronutritional Deficiencies in Immune cells: a Double-Blind Placebo-Controlled Pilot Study

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- 9.

Table E1 Trial profile

participants	2019	2020	sum	per arm
Enrolled	20	51	71	
Excluded	4	9	13	
Included	16	42	58	
Placebo lozenge	8	22		30
holoBLG lozenge	8	20		28
Sum	16	42		
never registered nor took supplementation				
Placebo lozenge	2	2	4	26
holoBLG lozenge	0	3	3	25
sum never	2	5	7	
ITT included	14	37	51	
discontinued				
Placebo lozenge	1	3	4	22
holoBLG lozenge	0	0	0	25
sum discontinued	1	3	4	
Completed study	13	34	47	
Placebo lozenge	5	17	22	
holoBLG lozenge	8	17	25	

1 Table E2 Blood parameter, intention to treat: before and after supplementation

	before supplementation					After supplementation				
	holoBLG lozenge n=22		Placebo lozenge n=24		p	holoBLG lozenge n=23		Placebo lozenge n=24		p
	Mea n	CI	Mea n	CI		Mea n	CI	Mea n	CI	
red blood cells T/L	4.609	(4.51/4.71)	4.58	(4.46/4.71)	0.74	4.56	(4.45/4.67)	4.42	(4.29/4.56)	0.10
hemoglobin g/dL	13.3	(13.0/13.6)	13.5	(13.1/13.9)	0.43	13.3	(13.0/13.7)	13.0	(4.2/4.6)	0.59
hematocrit %	39.18	(38.5/39.8)	39.8	(38.8/40.9)	0.27	39.4	(38.7/40.2)	38.7	(37.5/40.0)	0.31
MCV fL	85.38	(83.1/88)	87.2	(84.3/90)	0.34	86.5	(84.6/88.5)	87.7	(37.5/40.0)	0.68
MCH pg	28.99	(28.1/29.9)	29.5	(28.9/30.2)	0.42	29.3	(28.4/30.2)	29.5	(28.7/30.4)	0.86
MCHC g/dL	33.94	(33.4/34.5)	33.8	(33.4/34.3)	0.83	33.8	(33.4/34.2)	33.6	(33.4/33.9)	0.54
Red cell distribution width %	13.25	(12.7/13.8)	12.7	(12.5/12.9)	0.07	12.7	(12.4/13.1)	12.9	(12.7/13.2)	0.34
platelets G/L	283	(252/314)	260.	(239/282)	0.22	288.	(255/321)	270	(245/295)	0.37
mean platelet volume fL	10.48	(10.1/10.9)	10.7	(10.3/11.2)	0.42	10.5	(9.74/11.3)	10.8	(10.4/11.35)	0.62
white blood cells G/L	5.445	(4.79/6.10)	5.68	(4.95/6.43)	0.67	6.89	(6.12/7.66)	7.43	(6.79/8.07)	0.27
neutrophils G/L	2.895	(2.38/3.41)	3.12	(2.64/3.61)	0.49	4.16	(3.59/4.72)	4.42	(3.91/4.93)	0.47
neutrophils relative %	53.4	(49.9/56.9)	56.4	(52.3/60.6)	0.35	58.0	(54.2/61.8)	57.5	(54.1/61.0)	0.87
lymphocytes G/L	1.83	(1.61/2.05)	1.72	(1.50/1.95)	0.50	2.20	(1.94/2.46)	2.63	(2.25/3.01)	0.06
lymphocytes relative %	34.87	(31.7/38.1)	32.6	(28.9/36.5)	0.38	31.2	(28.3/34.2)	33.4	(30.2/36.7)	0.31
monocytes G/L	0.385	(0.33/0.44)	0.39	(0.34/0.44)	0.92	0.49	(0.41/0.56)	0.46	(0.41/0.52)	0.77
monocytes relative %	7.50	(6.73/8.27)	7.00	(6.14/7.86)	0.25	6.97	(6.25/7.69)	5.87	(5.17/6.59)	0.08
eosinophils G/L	0.19	(0.14/0.24)	0.17	(0.14/0.21)	0.70	0.21	(0.16/0.28)	0.17	(0.12/0.23)	0.24
eosinophils relative %	3.418	(2.6/4.23)	3.20	(2.28/4.13)	0.52	2.92	(1.88/3.98)	2.21	(1.53/2.89)	0.54
basophils G/L	0.04	(0.02/0.06)	0.02	(0.01/0.04)	0.52	0.04	(0.02/0.06)	0.02	(0.00/0.04)	0.33
basophils relative %	0.777	(0.61/0.94)	0.64	(0.50/0.79)	0.21	0.63	(0.51/0.75)	0.52	(0.35/0.69)	0.10
reticulocytes	43.3	(38.2/48.4)	45	(39.5/50.5)	0.64	44.0	(38.1/50.0)	44.2	(39.9/48.5)	0.96
reticulocytes relative	0.941	(0.84/1.04)	0.98	(0.87/1.10)	0.57	0.96	(0.84/1.09)	1.00	(0.91/1.10)	0.63
reticulocytes, low fluorescence rel.	96.5	(95.6/97.4)	97.1	(96.5/97.8)	0.22	95.6	(94.5/96.7)	96.5	(95.8/97.2)	0.15
reticulocytes, middle fluorescence rel.	3.24	(2.35/4.12)	2.73	(2.08/3.37)	0.53	4.01	(3.08/4.94)	3.12	(2.45/3.79)	0.11
reticulocytes, high fluorescence rel.	0.27	(0.14/0.41)	0.17	(0.09/0.25)	0.39	0.37	(0.16/0.59)	0.35	(0.16/0.56)	0.94
lactate dehydrogenase U/L	160.9	(146/176)	167.	(154/180)	0.52	170.	(153/189)	175.	(163/189)	0.65
c-reactive protein mg/dL	0.118	(0.07/0.16)	0.29	(0.11/0.48)	0.11	0.07	(0.04/0.11)	0.18	(0.08/0.28)	0.04
serum iron µg/dL	100.3	(79/122)	94.8	(77/112)	0.68	87.2	(69/105)	86.3	(68/105)	0.75
transferrin mg/dL	306.5	(285/328)	288	(263/313)	0.25	309.	(288/330)	296.	(274/320)	0.41
transferrin saturation %	24.0	(18.3/29.8)	24.4	(19.8/29.0)	0.92	20.7	(15.9/25.7)	22.8	(17.5/28.2)	0.59
ferritin µg/L	38.44	(25.1/51.7)	52.9	(34.2/71.7)	0.30	42.7	(27.0/58.5)	49.2	(29.3/69.2)	0.65

soluble transferrin receptor mg/L	1.45	(1.16/1.73)	1.19	(1.09/1.3)	0.27	1.41	(1.17/1.65)	1.25	(1.1/1.4)	0.47
haptoglobulin mg/dL	94.4	(77/112)	109.3	(92/126)	1	91.1	(74/108)	104.4	(89/120)	0.23
hemopexin mg/L	91.85	(87/96)	91.6	(86/98)	0.9	90.3	(85/96)	93.2	(87/99)	0.28
ceruloplasmin mg/dL	27.09	(24/30)	30.9	(26/35)	0.3	27.1	(24/30)	30.9	(27/35)	0.15

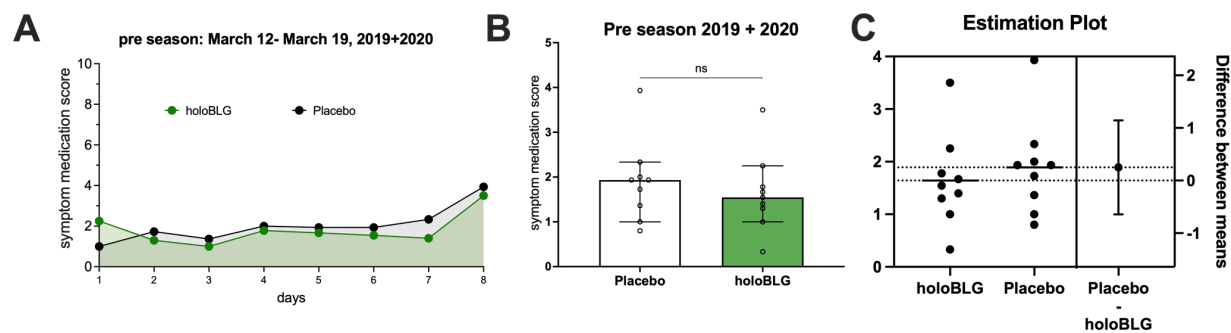
Table E3 Total and specific IgE of matched samples: before and after supplementation

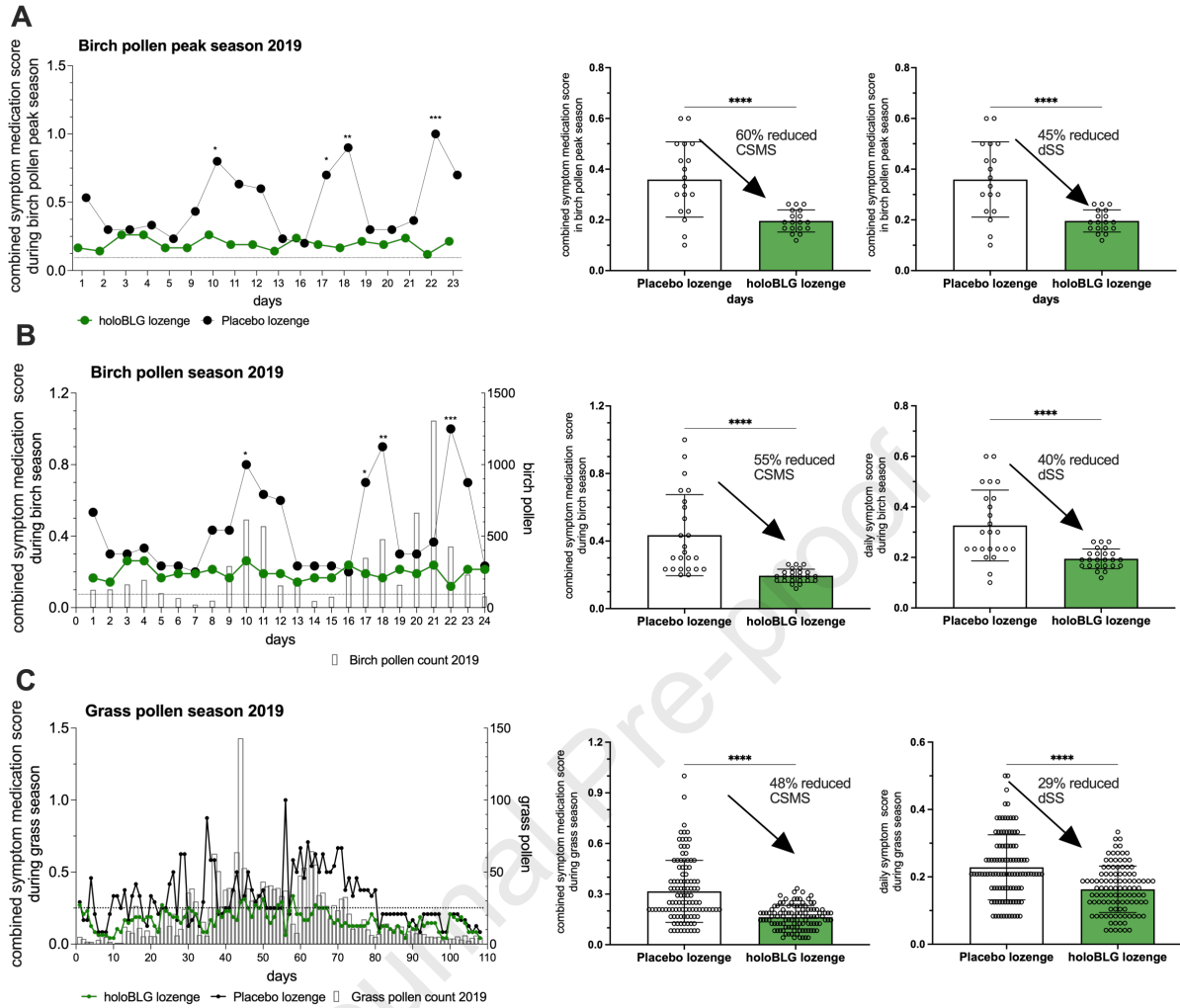
	holoBLG lozenge			Placebo lozenge			p
	n	Mean	CI	n	Mean	CI	
Total IgE (kU/L)	20			20			
before		110.2	(55.84/164.6)		50.3	(33.82/66.78)	0.08
after		97.1	(69.75/124.5)		92	(70.85/113.1)	0.96
change (after-before)		-13.1	(93.15/20.83)		41.7	(19.78/63.62)	0.02
birch pollen-specific IgE (kUA/L)							
Bet v 1-spec. IgE, before	15	14.50	(7.73/21.28)	14	11.26	(5.23/17.29)	0.40
Bet v 1-spec. IgE, after		17.00	(10.26/23.74)		16.69	(8.34/25.04)	0.91
Delta (after-before)		18.00	(10.26/23.74)		17.69	(8.34/25.04)	1.91
grass pollen -specific IgE (kU/L)	17			18			
Phlp 1 - spec. IgE, before		6.47	(3.52/9.41)		11.39	(4.99/17.80)	0.23
Phlp 1 - spec. IgE, after		11.79	(6.62/16.95)		14.83	(7.77/21.89)	0.63
Delta (after-before)		7.18	(3.21/11.16)		3.43	(0.82/6.05)	0.13
Phlp 5 - spec. IgE, before		10.85	(4.60/17.11)		11.58	(6.04/17.12)	0.51
Phlp 5 - spec. IgE, after		16.98	(9.38/24.58)		15.32	(7.98/22.66)	0.73
Delta (after-before)		4.83	(1.45/8.21)		5.6	(2.72/8.47)	0.59
total nasal symptom score, TNSS	17			17			
before		4.77	(3.63/5.89)		4.29	(3.47/5.12)	0.71
after		3.00	(1.74/4.26)		3.65	(2.53/4.77)	0.22
% improvement		42.39	(29.04/55.75)		11.91	(-14.2/38.0)	0.03

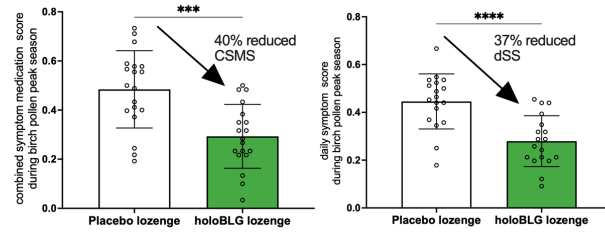
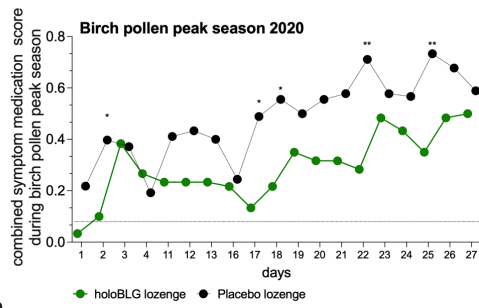
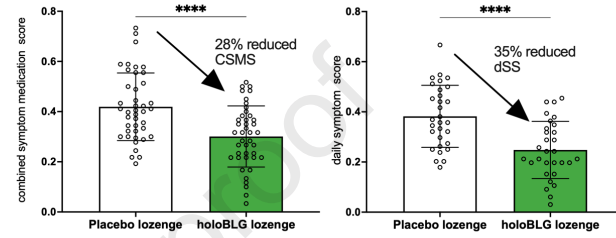
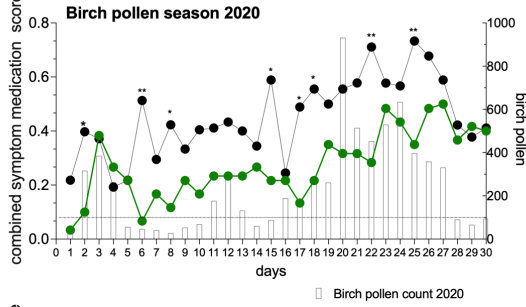
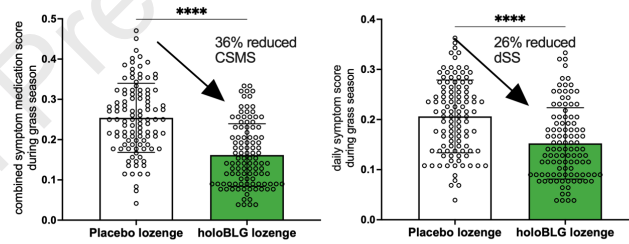
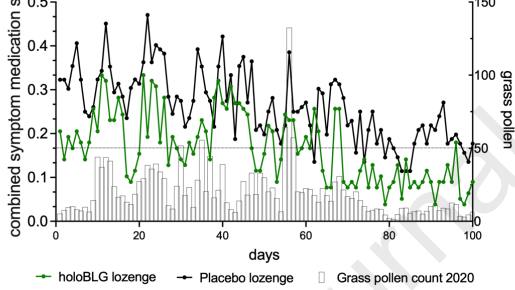
Table E4. Paired blood parameters: before and after supplementation

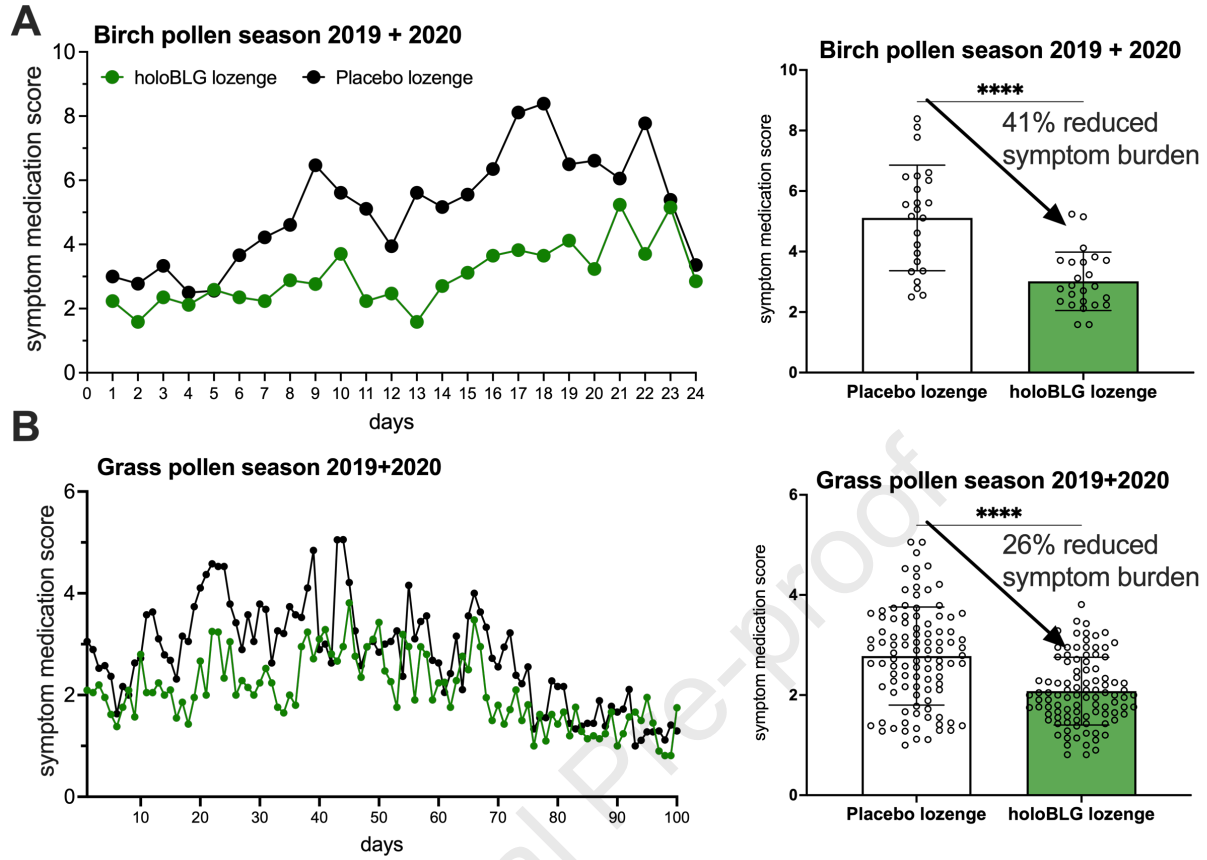
	before supplementation					After supplementation					Changes after supplementation				
	holoBLG lozenge		Placebo lozenge		p	holoBLG lozenge		Placebo lozenge		p	holoBLG lozenge n=20		Placebo lozenge n=20		p
	Mean	CI	Mean	CI		Mean	CI	Mean	CI		Mean	CI	Mean	CI	
red blood cells T/L	4.58	4.48/4.68	4.58	4.44/4.71	0.52	4.57	4.45/4.69	4.43	4.30/4.55	0.09	-0.01	-0.11/0.09	-0.15	-0.27/-0.02	0.08
hemoglobin g/dL	13.31	13.00/13.61	13.47	13.03/13.90	0.53	13.34	12.93/13.74	13.12	12.72/13.51	0.60	0.03	-0.28/0.34	-0.35	-0.72/0.02	0.11
hematocrit %	39.22	38.58/39.86	39.88	38.66/41.10	0.32	39.49	38.64/40.34	38.85	37.75/39.85	0.34	0.27	-0.48/1.02	-1.08	-2.14/-0.03	0.04
MCV fL	85.98	83.99/87.97	87.50	84.81/90.19	0.35	86.61	84.37/88.84	87.89	85.51/90.27	0.61	0.63	-0.81/2.06	0.39	-0.84/1.62	0.84
MCH pg	29.16	28.42/29.90	29.53	28.74/30.31	0.50	29.25	28.20/30.29	29.70	28.85/30.54	0.64	0.09	-0.39/0.56	0.17	0.01/0.33	0.44
MCHC g/dL	33.92	33.39/34.44	33.78	33.26/34.29	0.69	33.74	33.27/34.21	33.78	33.51/34.05	0.88	-0.18	-0.54/0.18	0.01	-0.44/0.45	0.52
Red cell distribution width %	13.12	12.59/13.64	12.72	12.45/12.99	0.17	12.67	12.28/13.06	12.77	12.54/12.99	0.66	-0.45	-0.81/-0.08	0.045	-0.18/0.27	0.02
plateles G/L	278.4	246.7/310.1	261.0	238.2/283.7	0.36	287.8	249.9/325.6	270.2	240.1/300.2	0.45	9.35	-14.16/32.86	9.20	-6.35/24.74	0.85
mean platelet volume fL	10.47	10.01/10.93	10.51	10.04/10.97	0.91	10.79	10.22/11.35	10.65	10.13/11.16	0.70	0.32	0.07/0.56	0.14	-0.11/0.39	0.30
white blood cells G/L	5.41	4.70/6.11	5.92	5.09/6.75	0.29	6.98	6.17/7.80	7.29	6.54/8.04	0.56	1.58	1.03/2.13	1.37	0.46/2.28	0.69
neutrophils G/L	2.93	2.39/3.47	3.25	2.77/3.72	0.29	4.21	3.61/4.80	4.44	3.81/5.08	0.57	1.28	0.81/1.75	1.21	0.52/1.91	0.87
neutrophils relative %	53.54	49.78/57.30	57.25	53.36/61.14	0.16	57.76	53.47/62.05	58.33	54.46/62.20	0.84	4.48	1.12/7.85	1.08	-3.10/5.26	0.20
lymphocytes G/L	1.82	1.59/2.06	1.82	1.58/2.06	0.97	2.26	1.98/2.54	2.55	2.12/2.98	0.24	0.39	0.23/0.55	0.54	0.28/0.80	0.29
lymphocytes relative %	34.78	31.34/38.21	32.88	29.14/36.61	0.44	31.48	28.06/34.90	32.88	29.23/36.52	0.56	-3.25	-5.73/-0.76	0.00	-3.94/3.94	0.16
monocytes G/L	0.38	0.32/0.44	0.38	0.32/0.44	0.92	0.49	0.41/0.57	0.45	0.39/0.50	0.50	0.10	0.03/0.17	0.07	0.02/0.12	0.44
monocytes relative %	7.49	6.67/8.31	6.51	5.84/7.19	0.06	6.89	6.17/7.62	5.76	4.93/6.59	0.07	-0.60	-1.50/0.31	-0.75	-1.75/0.25	0.88
eosinophils G/L	1.90	0.13/0.24	0.16	0.13/0.20	0.57	0.23	0.16/0.29	0.17	0.10/0.23	0.13	0.02	-0.03/0.06	0.01	-0.05/0.08	0.61
eosinophils relative %	3.44	2.53/4.34	2.74	1.97/3.50	0.26	2.96	1.80/4.12	2.07	1.29/2.84	0.32	-0.49	-1.19/0.21	-0.68	-1.43/0.01	0.71
basophils G/L	0.04	0.02/0.07	0.03	0.01/0.05	0.74	0.05	0.02/0.07	0.02	0.00/0.05	0.18	0.01	-0.02/0.03	-0.01	-0.04/0.01	0.33
basophils relative %	0.76	0.58/0.95	0.62	0.46/0.79	0.24	0.66	0.52/0.79	0.52	0.31/0.73	0.08	-0.15	-0.29/-0.01	-0.11	-0.34/0.13	0.80
reticulocytes	41.45	36.96/45.94	45.11	39.00/51.22	0.32	43.81	37.17/50.44	46.02	41.37/50.66	0.57	2.34	-3.53/8.24	0.90	-4.73/6.54	0.71
reticulocytes relative	0.92	0.82/1.01	0.99	0.86/1.13	0.37	0.96	0.82/1.10	1.04	0.94/1.15	0.32	0.05	-0.08/0.18	0.05	-0.07/0.17	0.96
reticulocytes, low fluorescence rel.	96.67	95.89/97.44	97.39	97.73/98.04	0.15	95.62	94.44/96.79	96.74	96.03/97.45	0.10	-1.05	-2.41/0.31	-0.65	-1.22/-0.06	0.57
reticulocytes, middle fluorescence rel.	3.06	2.36/3.75	2.41	1.77/3.05	0.16	4.05	3.03/5.06	2.90	2.24/3.56	0.06	0.99	-0.22/2.21	0.49	-0.09/1.07	0.44
reticulocytes, high fluorescence rel.	0.28	0.13/0.43	0.21	0.12/0.29	0.72	0.34	0.10/0.58	0.36	0.12/0.60	0.65	0.06	-0.20/0.32	0.16	-0.09/0.40	0.65
lactate dehydrogenase U/L	159.8	144.0/175.6	166.7	152.8/180.5	0.73	166.8	146.8/186.7	175.9	160.6/191.1	0.45	-1.35	-27.10/24.40	9.20	0.62/17.78	0.27
c-reactive protein mg/dL	0.11	0.07/0.16	0.34	0.12/0.56	0.08	0.07	0.03/0.11	0.21	0.08/0.33	0.01	-0.04	-0.08/-0.00	-0.13	-0.29/0.02	0.53
serum iron µg/dL	102.6	80.11/125.1	97.80	78.86/116.7	0.79	87.35	66.55/108.1	96.95	78.91/115.0	0.23	-15.25	-39.95/9.45	-0.85	-21.96/20.26	0.21
transferrin mg/dL	304.0	283.2/324.7	282.1	257.0/307.2	0.17	308.3	284.6/332.0	293.7	266.4/320.9	0.40	4.35	-9.42/18.12	11.55	-3.22/26.32	0.46
transferrin saturation %	24.56	18.55/30.56	25.25	20.39/30.11	0.85	21.02	15.44/26.60	25.07	19.28/30.86	0.29	-3.54	-9.97/2.89	-0.18	-5.64/5.28	0.26
ferritin µg/L	39.33	24.94/53.71	58.63	37.05/80.21	0.15	46.48	28.82/64.14	55.03	31.78/78.28	0.62	7.15	-4.61/18.92	-3.60	-11.35/4.15	0.24
soluble transferrin receptor mg/L	1.39	1.13/1.65	1.15	1.04/1.26	0.13	1.43	1.16/1.71	1.18	1.04/1.32	0.21	0.04	-0.14/0.22	0.03	-0.04/0.09	0.72
haptoglobin mg/dL	91.96	75.62/108.3	113.2	93.11/133.4	0.09	91.26	72.72/109.8	108.8	90.72/126.9	0.16	-0.73	-14.76/13.30	-4.43	-13.72/4.87	0.64
hemopexin mg/L	91.37	86.87/95.86	91.64	84.63/98.64	0.95	90.93	84.58/97.27	94.32	87.28/101.4	0.24	-0.44	-5.27/4.39	-2.04	-13.37/9.30	0.78
ceruloplasmin mg/dL	27.25	24.14/30.35	30.94	26.40/35.48	0.17	27.28	23.70/30.85	31.32	27.23/35.40	0.08	0.03	-2.34/2.40	0.38	-2.11/2.86	0.29

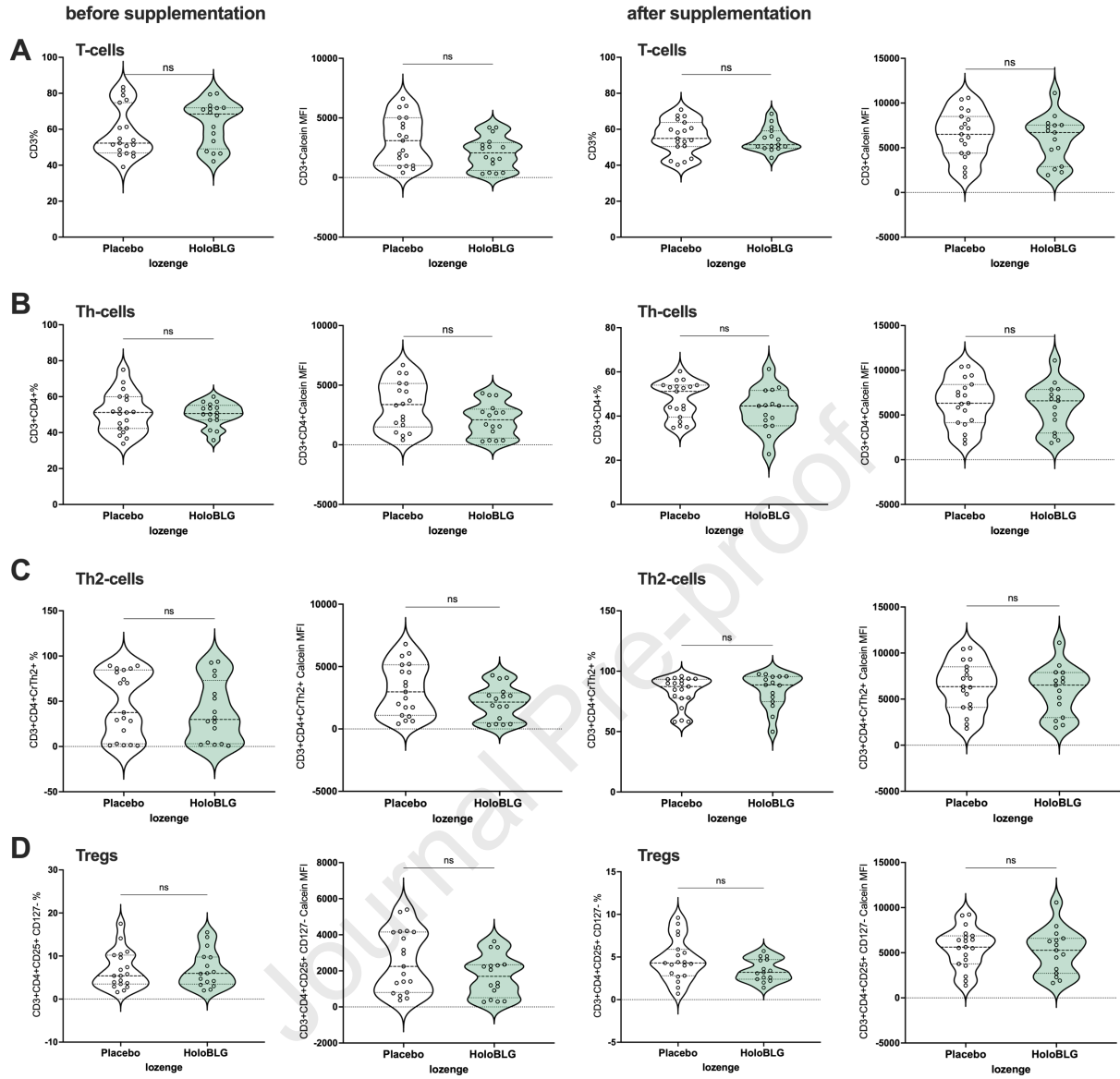
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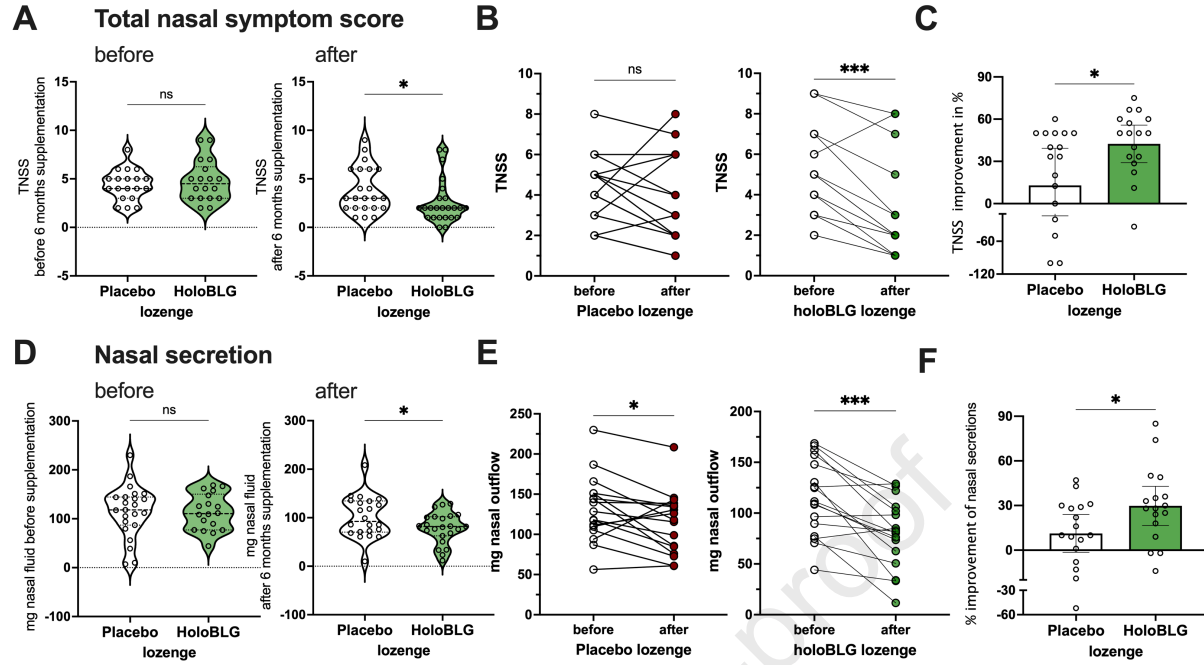


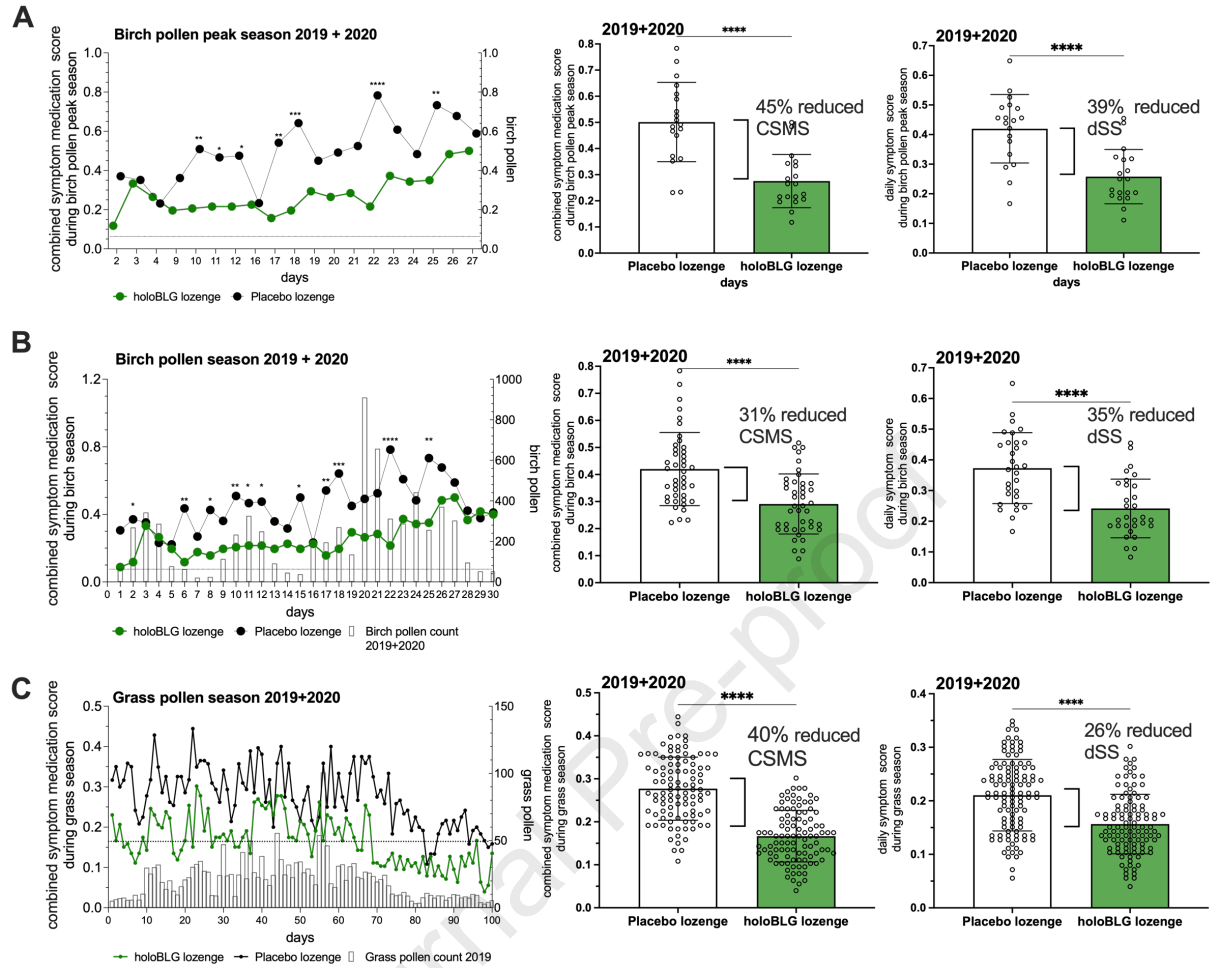


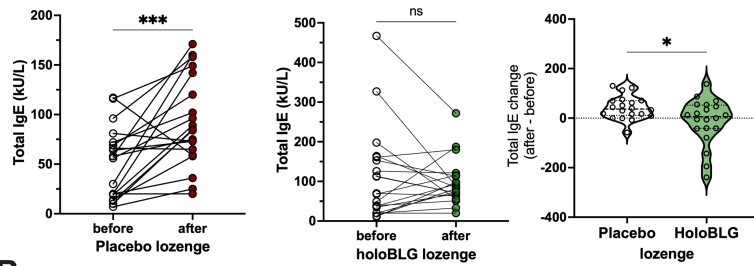
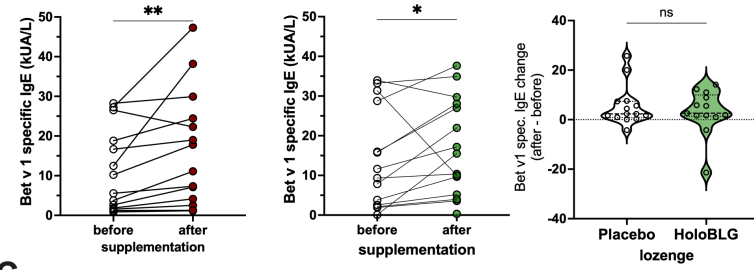
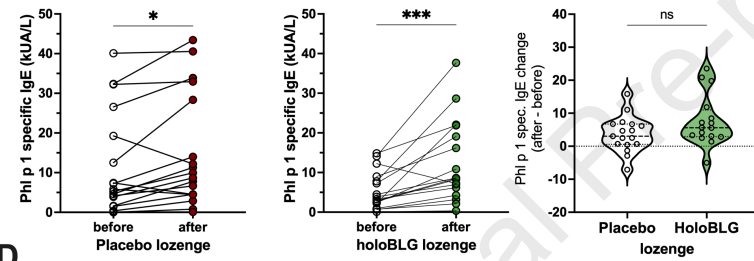
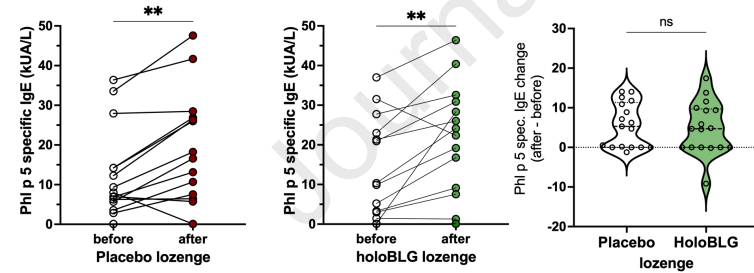
A**B****C**



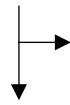






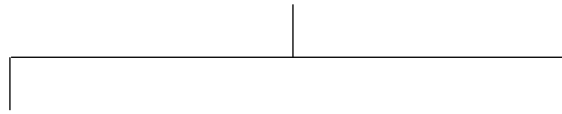
A**B****C****D**

71 screened



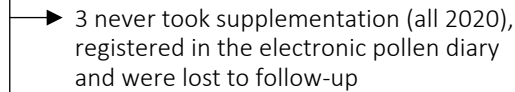
13 excluded (4 in 2019, 9 in 2020):
not birch pollen or grass pollen allergic after
component-resolved diagnostic

58 randomized



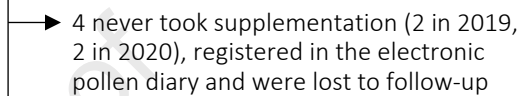
28 assigned to holoBLG supplementation

30 assigned to placebo supplementation



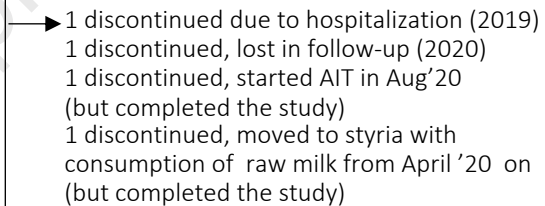
3 never took supplementation (all 2020),
registered in the electronic pollen diary
and were lost to follow-up

25 verum

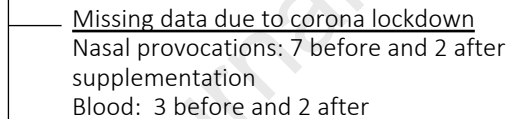


4 never took supplementation (2 in 2019,
2 in 2020), registered in the electronic
pollen diary and were lost to follow-up

26 placebo



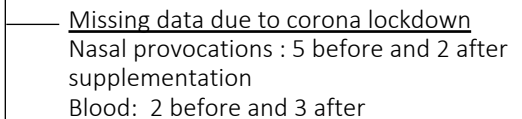
1 discontinued due to hospitalization (2019)
1 discontinued, lost in follow-up (2020)
1 discontinued, started AIT in Aug'20
(but completed the study)
1 discontinued, moved to styria with
consumption of raw milk from April '20 on
(but completed the study)



Missing data due to corona lockdown
Nasal provocations: 7 before and 2 after
supplementation
Blood: 3 before and 2 after

25 completed supplementation

Complete pollen diary entries of 25
Complete blood analyses (before & after): 20
Complete nasal provocations (before & after): 17



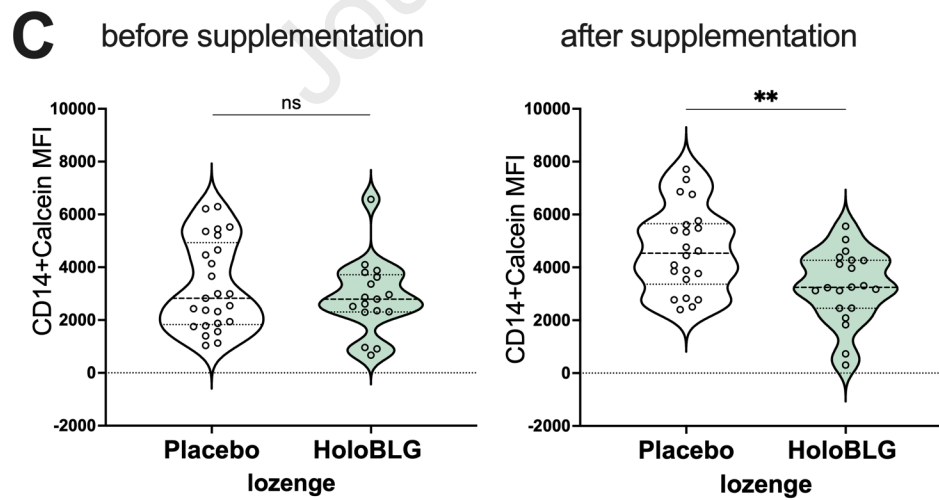
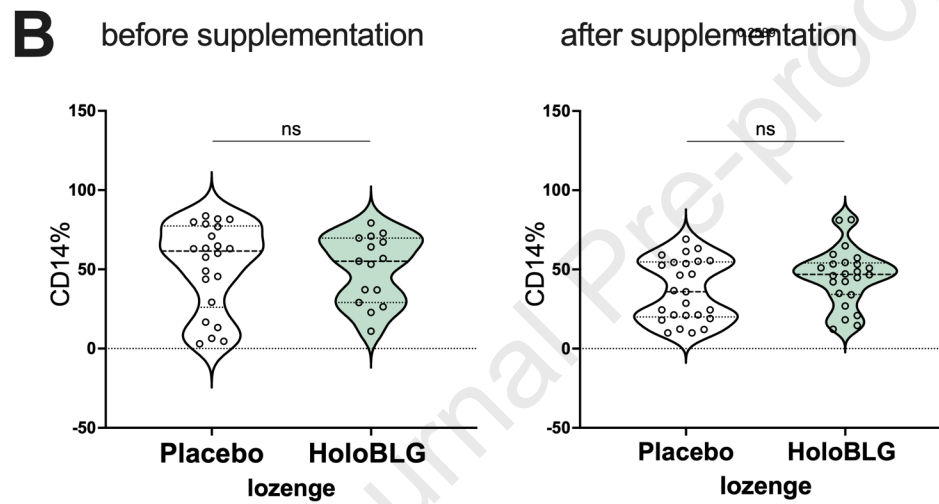
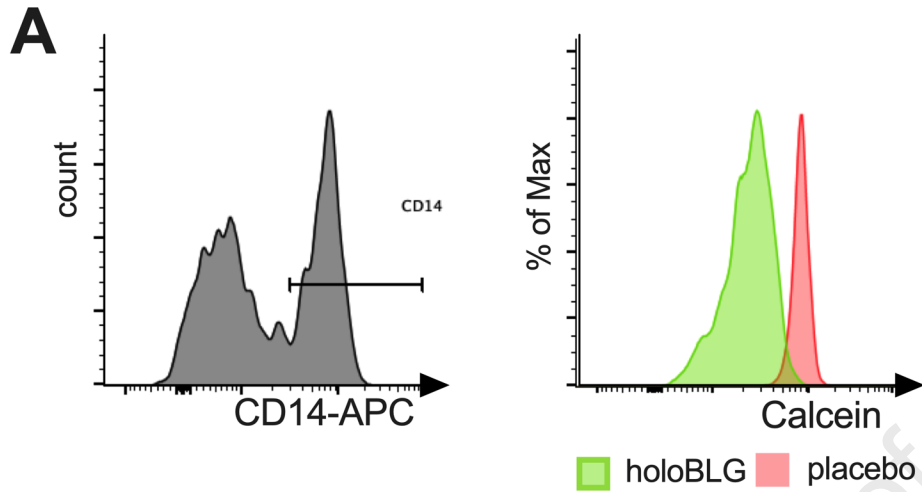
Missing data due to corona lockdown
Nasal provocations : 5 before and 2 after
supplementation
Blood: 2 before and 3 after

22 completed supplementation

Complete pollen diary of 26
Complete blood analyses (before & after): 20
Complete nasal provocations (before & after): 17

25 included in intention-to-treat analysis

26 included in intention-to-treat analysis



Journal Pre-proof

Ameliorating Atopy by Compensating Micronutritional Deficiencies in Immune cells: a Double-Blind Placebo-Controlled Pilot Study

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7. Allergy Therapeutics Ltd., Worthing, United Kingdom
8. Biomedical International R+D GmbH, Vienna, Austria

FIGURE E1. Preseasonal assessment of the raw symptom medication score of birch-pollen allergic subjects at the beginning of the supplementation (day 1 to day 30) with the placebo or holoBLG lozenge **A.** daily symptom medication score, **B.** summary of medians with 95% CI, **C.** estimation plot of the means in the placebo- and holoBLG-arm during the pre-birch season 2019 and 2020. Normality in the groups were tested via Anderson-Darling. Comparison of placebo- and the holoBLG-arm were compared with Mann Whitney U-test. ns. not significant

FIGURE E2. EAACI Combined Symptom Medication Score and daily Symptom Score over the **A.** peak and **B.** entire birch and **C.** entire grass pollen season 2019. Mixed-effect model (REML) using uncorrected Fisher's LSD test with a single pooled variance were used for analysis over birch season. Normality in the groups were tested via Anderson-Darling. Comparison of placebo- and the holoBLG-arm were compared with unpaired t-test for parametric data, whereas non-parametric data were compared with Mann Whitney U-test. ns. Not significant, *P < 0.05, **P < 0.01, ***P < 0.001****P < 0.0001

FIGURE E3. EAACI Combined Symptom Medication Score and daily Symptom Score over the **A.** peak and **B.** entire birch and **C.** entire grass pollen season 2020. Mixed-effect model (REML) using uncorrected Fisher's LSD test with a single pooled variance were used for analysis over birch season. Normality in the groups were tested via Anderson-Darling. Comparison of placebo- and the holoBLG-arm were compared with unpaired t-test for parametric data, whereas non-parametric data were compared with Mann Whitney U-test. ns. Not significant, *P < 0.05, **P < 0.01, ***P < 0.001****P < 0.0001

FIGURE E4. Raw symptom medication score over the **A.** birch and **B.** grass pollen season 2019 and 2020. Normality in the groups were tested via Anderson-Darling. Comparison of placebo- and the holoBLG-arm were compared with unpaired t-test for parametric data, whereas non-parametric data were compared with Mann Whitney U-test. ns. ****P < 0.0001

FIGURE E5. Supplementation with the holoBLG lozenge do not change intracellular iron levels in lymphocytes. Peripheral blood mononuclear cells isolated by density gradient and were incubated overnight in iron-free media, before staining for CD3, CD4, CrTh2, CD25 and CD127 and measuring the intracellular iron-content by means of quenching of the calcein-signal. Summary of the relative numbers and their respective calcein signal before and after 6-month supplementation with placebo or holoBLG lozenge of **A.** CD3+ cells, **B.** CD3+CD4+ cells, **C.** CD3+CD4+CrTh2+ cells and CD3+CD4+CD25+CD127- cells. Normality in the groups were tested via Anderson-Darling. Normal distributed data were compared with unpaired t-test, non-parametric data were compared with Mann Whitney U-test; ns. Not significant.