Received: 6 December 2023 | Revised: 25 March 2024 | Accepted: 14 April 2024

DOI: 10.1111/all.16148

Allergy EUROPEAN JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY EAACI WILEY

ORIGINAL ARTICLE

The impact of high-salt diet on asthma in humans and mice: Effect on specific T-cell signatures and microbiome

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Funding information
Welsh Government; British Heart Foundation; Cancer Research UK; Diabetes UK; Wellcome Trust; Medical Research Council; NIHR; Helmholtz Munich: intramural funding (FA); SFB1054,

Abstract

Background: The rise in asthma has been linked to different environmental and lifestyle factors including dietary habits. Whether dietary salt contributes to asthma incidence, remains controversial. We aimed to investigate the impact of higher salt intake on asthma incidence in humans and to evaluate underlying mechanisms using mouse models.

Methods: Epidemiological research was conducted using the UK Biobank Resource. Data were obtained from 42,976 participants with a history of allergies. 24-h sodium excretion was estimated from spot urine, and its association with asthma incidence was assessed by Cox regression, adjusting for relevant covariates. For mechanistic studies, a mouse model of mite-induced allergic airway inflammation (AAI) fed with high-salt diet (HSD) or normal-salt chow was used to characterize disease development. The

Abbreviations: AAI, allergic airway inflammation; BAL, bronchoalveolar lavage; HDM, house dust mite; HSD, high-salt diet; NaCl, sodium chloride; PAS, Periodic acid–Schiff; PBS, Phosphate-buffered saline; T_H, T helper; Treg, regulatory T-cell.

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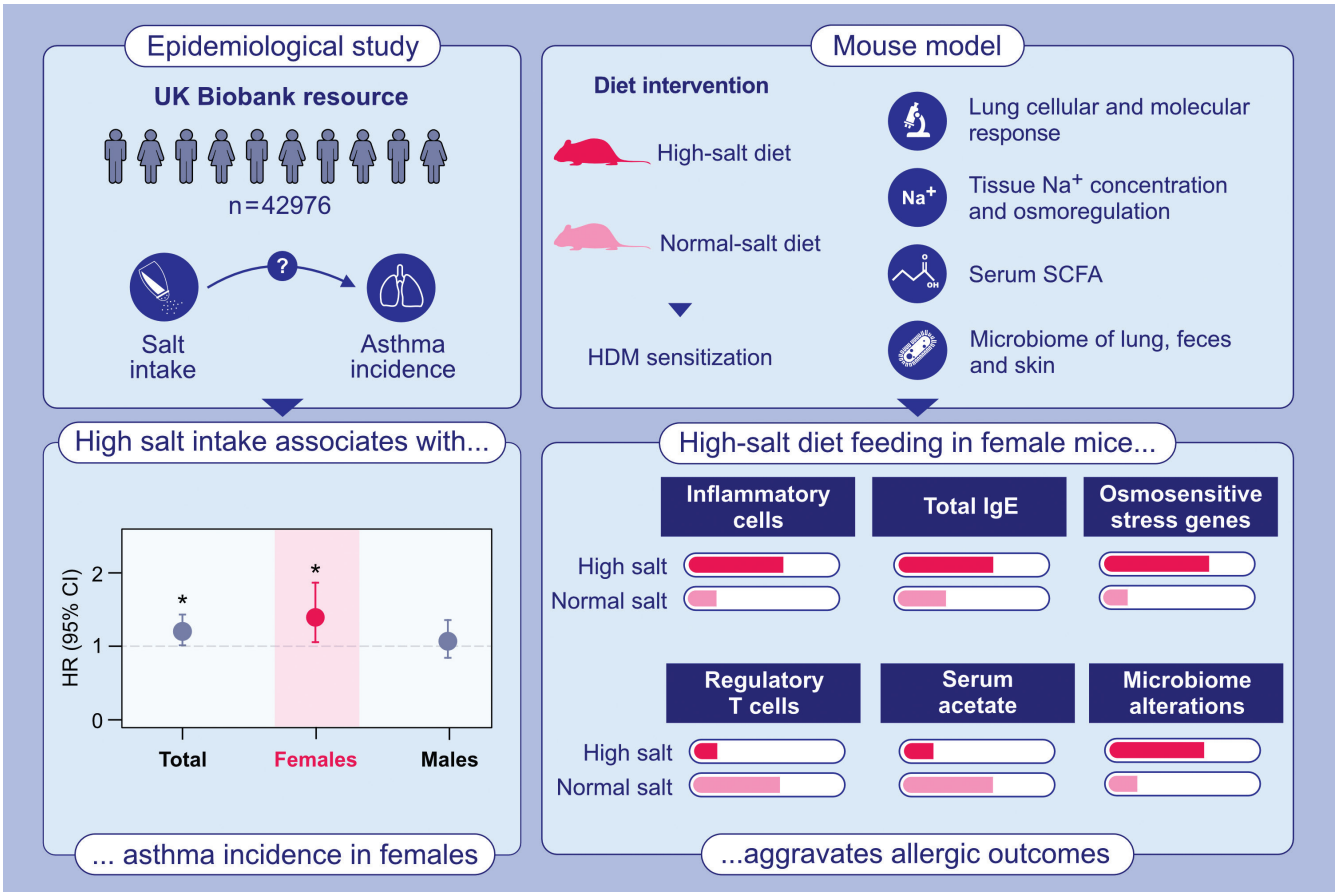
FungiNet TR124, Excellence Cluster Balance of the Microverse (CEZ)

microbiome of lung and feces (as proxy for gut) was analyzed via 16S rRNA gene based metabarcoding approach.

Results: In humans, urinary sodium excretion was directly associated with asthma incidence among females but not among males. HSD-fed female mice displayed an aggravated AAI characterized by increased levels of total IgE, a T_H2-T_H17-biased inflammatory cell infiltration accompanied by upregulation of osmosensitive stress genes. HSD induced distinct changes in serum short chain fatty acids and in both gut and lung microbiome, with a lower *Bacteroidetes* to *Firmicutes* ratio and decreased *Lactobacillus* relative abundance in the gut, and enriched members of *Gammaproteobacteria* in the lung.

Conclusions: High dietary salt consumption correlates with asthma incidence in female adults with a history of allergies. Female mice revealed HSD-induced T-cell lung profiles accompanied by alterations of gut and lung microbiome.

KEYWORDS
allergic airway inflammation, asthma, dietary salt, microbiome



GRAPHICAL ABSTRACT

- Epidemiological study discovers novel associations between high intake of dietary salt and asthma incidence in females but not in males.
- High-salt diet feeding in female mice aggravates allergic outcomes: serum IgE, lung inflammatory cell infiltration, T_H2-T_H17 profiles, reduced Tregs and increased expression of lung osmosensitive stress genes.
- High-salt consumption induces alterations of SCFA in serum and of gut and lung microbiome.

1 | INTRODUCTION

The incidence of asthma and other allergic diseases has risen over the past decades, whereby environmental factors, including changes in lifestyle and dietary habits, play an important role.[1,2] A dietary risk factor which rapidly emerged together with the "western diet" is the consumption of high amounts of salt.[3] Although the World Health Organization (WHO) recommends to consume not more than 5 g of salt per day,[4] some countries highly exceed these daily recommendations.[3]

While, under normal conditions, the extracellular fluid volume and Na^+ homeostasis are constantly regulated by the circulating volume and by osmotically active Na^+ excretion through the kidneys, intake of large amounts of Na^+ leads to increased plasma concentrations, osmolality, and its accumulation in parts of the body without significant changes in body water content.[5–7] Notably, the ionic composition of the microenvironment has an impact on the immune system, affecting elements of both innate and adaptive immune responses, as NaCl was shown to promote chemotaxis of macrophages,[8] to stimulate the differentiation of T_H17 cells and to inhibit the suppressive function of $Foxp3^+$ regulatory T cells (Tregs) in rodents and humans.[9–12] Moreover, NaCl was recently identified as an ionic checkpoint in atopic dermatitis, being able to stimulate type 2 immunity[13,14] and to shape the pathogenicity of human Th17 cells dependently on local cytokine microenvironments.[15] Besides the direct effects of dietary salt on host immunity, also salt-induced changes in the microbiome might impact the development of asthma, due to the close host-microbiome interplay.[16]

Whilst the association between dietary salt consumption and asthma symptoms is still under discussion,[17–21] human intervention studies set out to clarify whether higher levels of dietary salt have an impact on asthma have produced overall positive results, although dependent on the length of treatment.[22–27] In this study we investigated the epidemiological association of urinary sodium excretion levels with the incidence of allergic asthma in humans, taking advantage of a large cohort of 42,976 individuals. Our analysis revealed a direct association of salt intake with asthma incidence among females, but not males. To obtain mechanistic insights into this association, we used female mice to perform a detailed immunological characterization of lung allergic response using an aeroallergen-induced murine model of allergic airway inflammation (AAI) following high-salt diet (HSD)- or normal-salt chow feeding. HSD consumption aggravated the development of AAI and led to distinct shifts in key lung inflammatory cell populations as well as in gut and lung microbiome. Gut microbiome shifts comprised a lower *Bacteroidetes* to *Firmicutes* ratio and decreased relative abundance of *Lactobacillus* and in the lung we observed a higher relative abundance of *Gammaproteobacteria*. To which extent the observed changes in microbiome might have causative effects on the altered pulmonary immunologic response remains for future investigations. Overall, these findings may lay the foundation to disentangle the complexity of mechanisms supporting the aggravation of allergic disorders induced by high dietary salt consumption.

2 | METHODS

2.1 | Epidemiological data

Epidemiological analyses were conducted using the UK Biobank Resource (application number 70262). Data from 42,976 participants with a diagnosed allergic disease (allergic rhinitis, allergy to house dust mite (HDM), eczema) were obtained through questionnaire, interviews, clinical assessments, measurements of urinary biomarkers, and linked hospital admission and death registries. UK Biobank has approval from the North West Multi-centre Research Ethics Committee as a Research Tissue Bank (RTB) approval (REC reference: 21/NW/0157). This approval means that researchers do not require separate ethical clearance and can operate under the RTB approval. Consent in relation to the Data Protection Act 1998 and (where applicable) the Human Tissue Act 2004 has been obtained from the relevant UK Biobank participants. Details of the study population, data collection, inclusion criteria, covariates, and statistical analysis are provided in the Data S1.

2.2 | Murine experimental protocol

Seven-eight-week-old female C57Bl/6J mice were fed a HSD or kept on normal chow for 4 weeks. In the last 2 weeks of experiment an established HDM model was used to induce AAI.[28] At sacrifice, a detailed analysis of lung inflammatory cell infiltration, cytokine release, airway hyperresponsiveness, serology, as well as tissue sodium and potassium concentration and expression of osmosensitive genes was performed and combined to a thorough microbiome analysis of lung and feces. The study was conducted according to the European Convention for Animal Care and Use of Laboratory Animals and was approved by local ethics committee and government authorities (ROB-55.2-2532. Vet_02–18-94). For methodological details, see the Data S1.

2.3 | Data analysis and statistics

Epidemiological analyses were performed in the total population and stratified by sex using R (https://www.R-project.org/), version 4.2.2. Murine data was analyzed by GraphPad Prism (GraphPad Software, La Jolla, CA, USA) and the microbial data in R, version 4.2.1. Methodological details are provided in the Data S1.

3 | RESULTS

3.1 | Higher levels of urinary sodium are associated with increased asthma incidence in female adults

The study population for epidemiological analyses comprised 42,976 individuals (25,706 females, 17,270 males). Their descriptive characteristics are provided in Table S1. The mean urinary 24-h sodium excretion was higher in males (3.74 g/day) than in

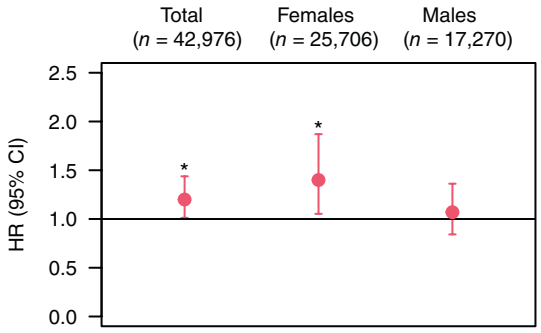


FIGURE 1 Association of 24-h sodium excretion with asthma incidence. Effects presented are hazard ratio (HR) and 95% confidence intervals (95% CI) for a 1 g/day increase in sodium excretion in the total population, females only and males only. *Significant associations ($p < .05$).

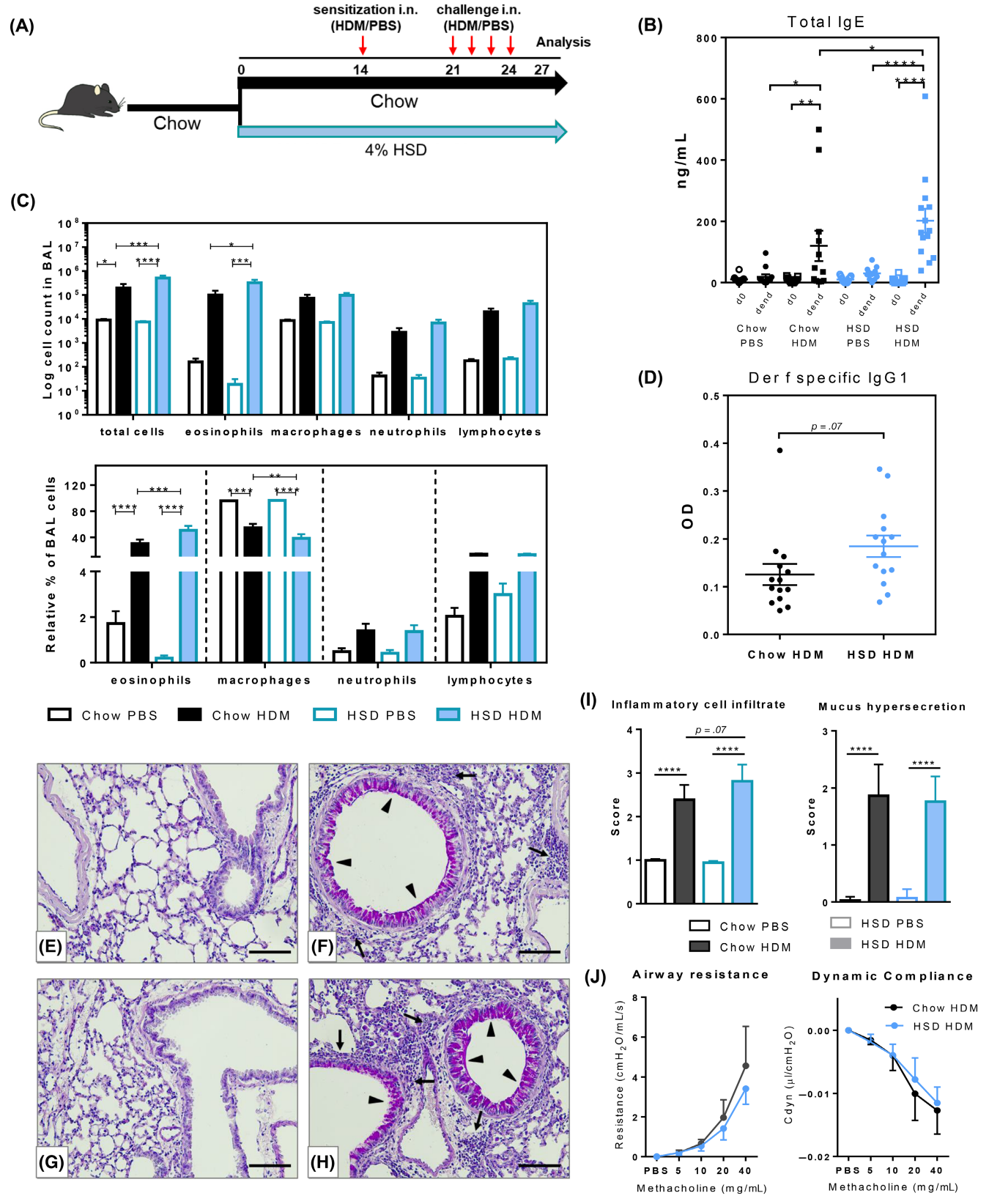
females (2.67 g/day). The average follow-up time was 12.4 years, within which 2.4% of females and 1.9% of males developed asthma. Urinary 24-h sodium excretion was directly associated with asthma incidence in the total population (HR [95% CI] = 1.206 [1.012; 1.438], $p = .037$). Interestingly, sex-stratified analyses revealed an even stronger association among females only (1.403 [1.053; 1.870], $p = .021$). In contrast, no significant associations were observed in males (1.071 [0.842; 1.362], $p = .579$) (Figure 1). These results were confirmed by additional analyses using alternative exposure variables: (1) absolute sodium concentrations; (2) reported added salt. Results from both these analyses showed a significant direct association with asthma among females but not among males (Table S4).

3.2 | A diet rich in NaCl enhances HDM-induced AAI in female mice

To mechanistically explore the epidemiological associations observed in our female cohort, we employed a mouse model of AAI combined to HSD feeding (treatment scheme, Figure 2A) in female C57BL/6J mice.

Allergic mice fed with HSD developed an aggravated disease phenotype compared to allergic mice fed with chow. In fact, serum total IgE levels, which were increased in both groups following sensitization, reached higher levels in HSD-HDM compared to chow-HDM (Figure 2B) and bronchoalveolar lavage (BAL) cellular infiltration, in particular concerning eosinophils and to a lesser extent neutrophils and lymphocytes, was higher in HSD-HDM compared to chow-HDM (Figure 2C). *Der f*-specific IgG1 measured at the end of experiment was slightly higher in HSD-HDM compared to chow-HDM, although the difference did not reach statistical significance (Figure 2D). Similar results were obtained for *Der f*-specific IgE (data not shown). Histopathologic scoring of lung inflammatory infiltrate was near-to-significantly ($p = .068$) higher in HSD-HDM compared to chow-HDM, whereas mucus hypersecretion and airway hyperresponsiveness were similar in the two groups (Figure 2E–J). Analysis of BAL fluid following AAI revealed increased levels of signature cytokines of T_H2, T_H17 cells and pro-inflammatory cytokines in mice fed with HSD compared to mice fed with chow, whilst no effect was detected for IFN-γ (Figure 3). IL-17A, IL-17F (but not IL-6) in BAL fluid significantly correlated with neutrophils in BAL, whereas IL-4, IL-5, and IL-13 with eosinophils; TNF-α correlated with both granulocytes (Figure S4). To further characterize the type of lung immune response in our experimental setting, we analyzed per flow cytometry the expression of master transcription factors in lung T cells. Hereby, we revealed increased T_H2 and T_H17 cells following AAI in HSD-fed compared to chow-fed animals, whereas Tregs were significantly decreased in HSD-fed challenged animals (Figure 4). Additionally, uncoventional lymphocytes, the γδT and specifically the IL-17-committed γδT cells, being particularly enriched at barrier sites, represent an innate source of IL-17 which can rapidly contribute to the inflammatory response.[29,30] In response to HSD we detected increased concentrations of γδT cells in the lung of mice which slightly decreased following AAI regardless of diet. The response of γδT cells to HSD was most pronounced in the functional subset committed to an IL-17A effector type (γδT17) (Figure 4). To evaluate if T_H9 cells may also play a role in high-salt-induced enhancement of allergic response, we evaluated lung IL-9 mRNA expression, IL-9 protein levels in BAL fluid and the percentage of the IL-9 transcription factor PU.1 in lung tissue. Our results do not indicate a role of IL-9 in our experimental context, since dietary salt did not affect Th9 cells beyond their known impact on AAI[31] (Figure S5). FACS analysis of lung draining lymph nodes revealed a slightly increased infiltration of eosinophils and a significant augmentation of neutrophils in HSD-HDM compared to chow-HDM, whereby both cell types where increased in HSD-HDM compared to HSD-PBS (Figure S6, left). Additionally, and in line with the lung data, cervical lymph nodes showed an increased T_H2 response in HSD-HDM compared to PBS control, enhanced

FIGURE 2 Impact of HSD feeding on HDM-induced AAI in mice. Female C57Bl/6J mice were fed with HSD or kept on control chow for 4 weeks and sensitized to HDM or sham sensitized to PBS last 2 weeks of experiment. (A) Treatment scheme. (B) Total immunoglobulin E (IgE) in serum samples at the beginning (d0) and end (dend) of experiment. (C) BAL absolute cell numbers (top) and relative percentages of BAL eosinophils, macrophages, neutrophils and lymphocytes (bottom). (D) *Der f*-specific immunoglobulin G (IgG) in serum samples at the end of experiment. (E–H) Lung histology (PAS staining) of (E) Chow-PBS, (F) Chow-HDM, (G) HSD-PBS, (H) HSD-HDM. Arrows, inflammatory infiltrate; arrowheads, mucus hypersecretion; scale bar: 100 μm. (I) Histological scoring of inflammatory cell infiltrate (left) and mucus hypersecretion (right). (J) Measurement of airway hyperresponsiveness. (B–D) $n = 6$–14, mean ± SEM; (I) $n = 5$; (J) $n = 6$, mean ± SD. Results were analyzed by two-way analysis of variance (ANOVA) with Tukey's multiple comparison test (B, J), one-way ANOVA with Bonferroni test (C, I) or Student's unpaired *t*-test (D). *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$.



T_H17 response in HSD-fed compared to chow-fed animals and no variations in Tregs (Figure S6, right). On the other hand, the observed increase of HSD-dependent lung γδT cells, in particular γδT17 cells, was only minimally detected in the lymph nodes (Figure S6, right). Altogether, these data demonstrate that HSD aggravates an HDM-induced lung inflammatory response with combined T_H2-T_H17 mechanisms and shows for the first time a γδT17 cells response triggered by dietary high-salt conditions.

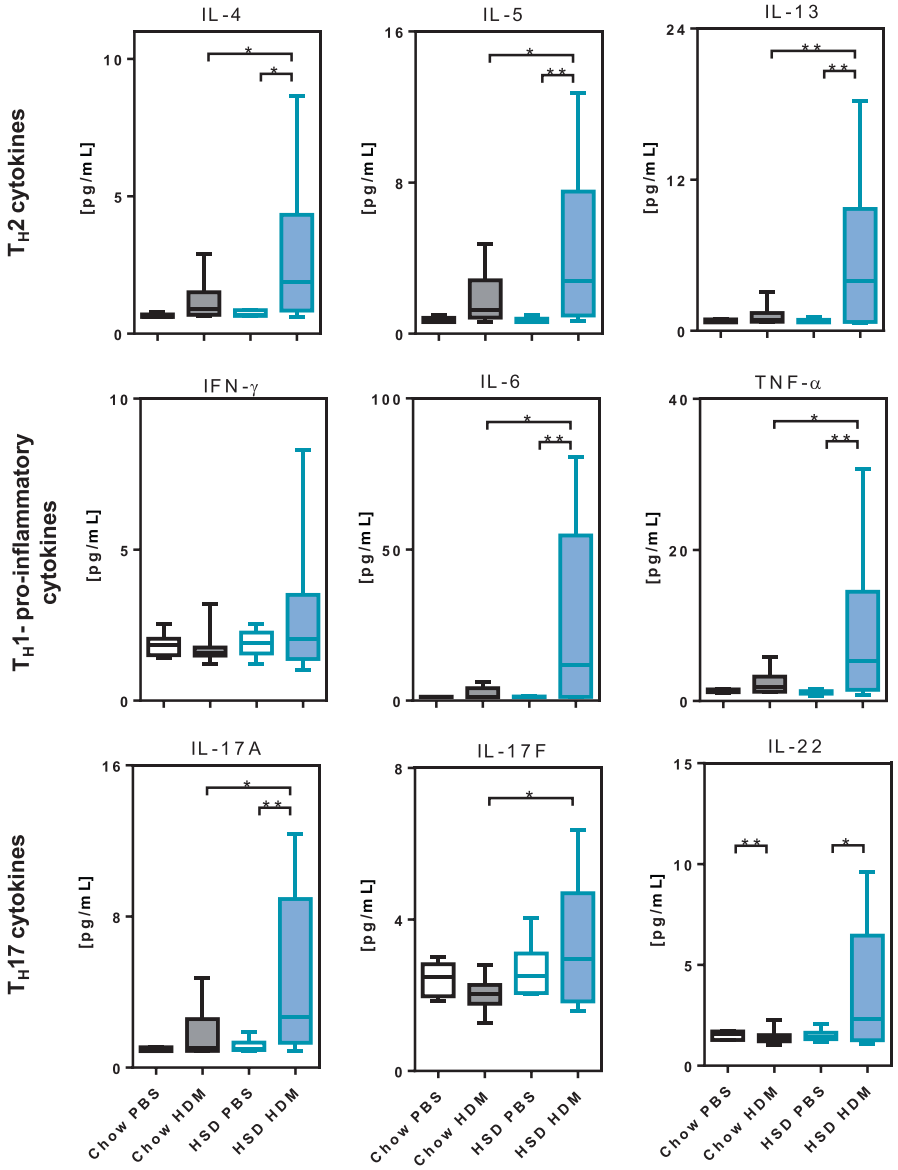


FIGURE 3 Impact of HSD feeding on cytokine levels in BAL fluid. Female C57Bl/6J mice were fed with HSD or kept on control chow for 4 weeks and sensitized to HDM or sham sensitized to PBS last 2 weeks of experiment. Cytokines representative for T_H1, T_H2, T_H17, and pro-inflammatory response were assessed by Legendplex in BAL fluid. $n = 6–13$. Boxplots indicate minimum, 25th percentile, median, 75th percentile, and maximum. Results were analyzed by one-way analysis of variance (ANOVA) with Bonferroni test. $^*p < .05$, $^{**}p < .01$.

3.3 | Consumption of HSD enhances pulmonary Na^+ concentration

Measurements of Na^+ and K^+ concentrations in lung and skin samples (used as reference) detected higher Na^+ concentrations and lower K^+ concentrations in lung compared to skin samples, independently of the diet employed (Figure 5A,B). Interestingly, lung Na^+ concentration was higher following HSD compared to chow feeding, albeit only in sham-sensitized animals (Figure 5A). Due to the slight increased Na^+ concentrations in chow-fed allergic animals in line with,[32] no difference in Na^+ concentration was found in the lungs of allergic mice. Noticeably, HSD feeding per se did not affect mouse weight (Figure S7A). Therefore, increased dietary sodium intake alone had an impact on lung Na^+ concentration independently of variations in body weight. Analysis of ion concentrations excreted in feces revealed only a minor increase of Na^+ concentration in HSD-fed animals (Figure S7B).

3.4 | HSD feeding enhances pulmonary NFAT5 and SGK1 signatures in allergic mice

Having established that HSD feeding has considerable effects on the induction and enhancement of the T_H2 and T_H17 signature in the lung, we sought to investigate the underlying molecular changes in key osmoregulated genes known to affect both T_H2 and T_H17 cells,[9,10,33,34] which may potentially drive the observed immunological alterations. First, we tested whether the expression of nuclear factor of activated T cells 5 (NFAT5), a key osmosensitive transcription factor, which is involved in protecting mammalian cells from hyperosmotic stress,[35] was induced in the lung upon HSD consumption in our mouse allergy model. Our results show an up-regulation of lung expression of *NFAT5* in allergic animals compared to sham sensitized, whereby only following HSD feeding reached statistical significance. Moreover, the increased *NFAT5* expression detected in HDM-sensitized animals was significantly higher

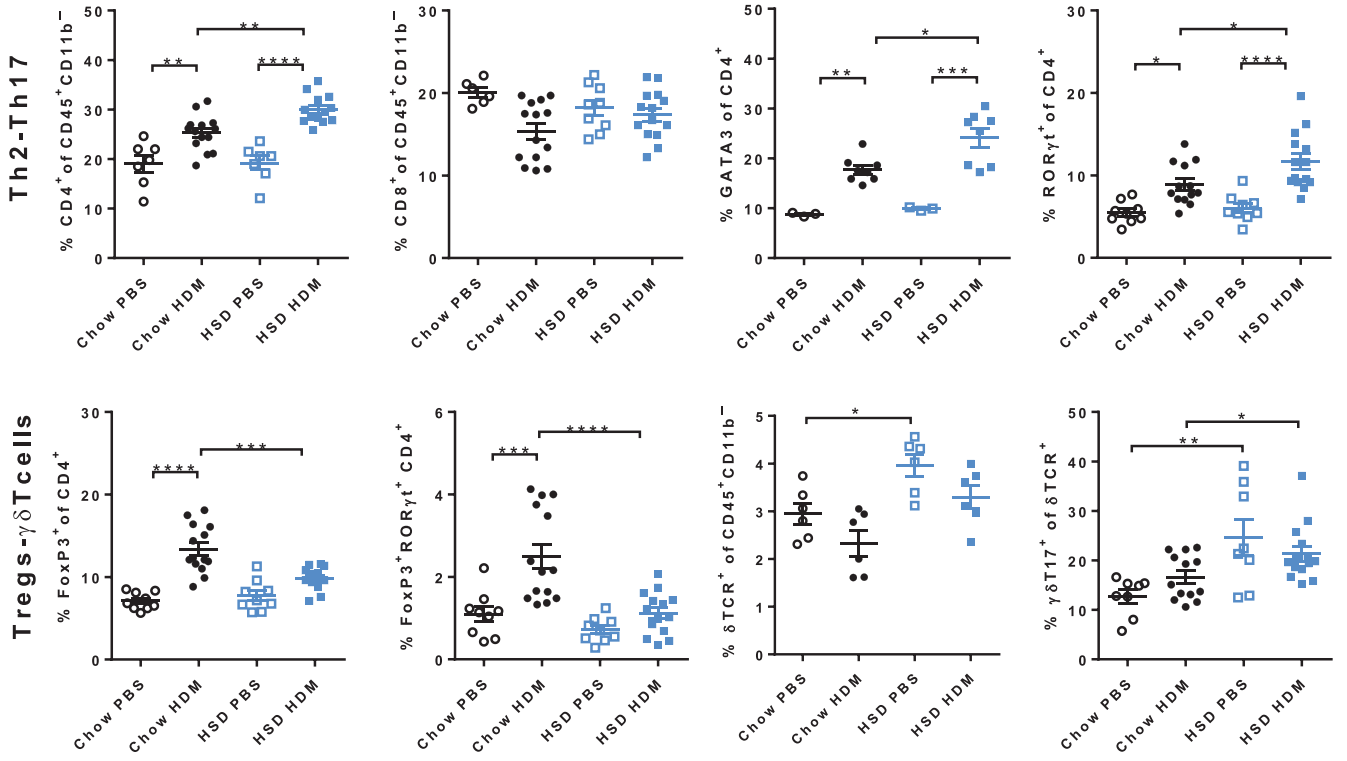


FIGURE 4 Impact of HSD feeding on lung T-cell populations. Flow cytometric analysis of lungs was performed 4 weeks after HSD or chow feeding and induction of AAI/sham sensitization in C57Bl/6J female mice. Cells were pre-gated on single cells/live-dead and analyzed for different T cells subsets (T_H2, T_H17, Tregs, γδT cells). $n = 6–14$; each data point represents an individual mouse; bars indicate mean ± SEM. Results were analyzed by one-way analysis of variance (ANOVA) with Bonferroni test. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$.

in HSD-compared to chow-fed mice (Figure 5C, left). Similarly, the lung expression of the salt-sensing serum glucocorticoid–regulated kinase (SGK1)[10,33] was increased only in allergic animals fed with HSD (Figure 5C, right). Furthermore, the main epithelial Na^+ channel *SCNN1a*[36] was mildly, near-to-significantly, upregulated in the lungs by HSD feeding. The lung expression of its regulator prostasin[37] was increased by HSD feeding, but in turn attenuated by HDM challenge (Figure 5D). Taken together, we show that HSD feeding increases the expression of both *NFAT5* and *SGK1* in the lungs of allergic female mice.

3.5 | Consumption of HSD decreases the level of acetate in mouse serum

Given the important immune modulating properties of short chain fatty acids (SCFA) which are produced by bacterial fermentation of specific diet components,[38,39] we measured the levels of three key SCFA in mouse serum. Our results show a significant decrease of both acetate and propionate in control animals and of acetate only in allergic animals following HSD feeding (Figure 5E). Additionally, serum levels of acetate were in tendency lower in allergic mice vs controls, whereby in HSD-fed animals the difference was close to significant ($p = .06$). Contrarily, no difference in butyrate were observed (Figure 5E, right).

3.6 | HSD affects gut and lung microbiome composition to different extents

Changes in diet are known to affect the composition and function of the gut microbiome, which not only strongly impacts local and systemic immune responses[40,41] but was also described to influence the lung microbial community via the so-called gut-lung axis.[42] Therefore we investigated microbial changes in feces (as proxy for gastro-intestinal tract) and in lung in response to HSD feeding alone, or to HSD combined to AAI in female mice. While alpha diversity (analyzed via Richness, Shannon and Simpson index) of gut was not affected by HSD and/or HDM challenge (Figure 6A, Figure S8), bacterial community composition showed a significant response to high-salt consumption (Figure 6B). In lung samples, alpha diversity was increased by HSD in sham-sensitized animals, whereas the diversity remained unchanged after HDM sensitization (Figure 6A, Figure S8). In contrast to the gut, the bacterial community composition of the lung was only slightly altered by HSD (Figure 6B).

To identify bacterial biomarkers responding to HSD and/or HDM challenge we had a more detailed look on the abundances of the observed taxa. The gut microbiome was dominated by members of *Bacteroidetes* and *Firmicutes*, with *Bacteroidetes* being decreased and *Firmicutes* being enriched in relative abundance after HSD intake (Figure 6C, Figure S9A, Table S5). Especially members of *Muribaculaceae*, *Prevotellaceae* (UCG-001, NK3B31) and *Lactobacillus*

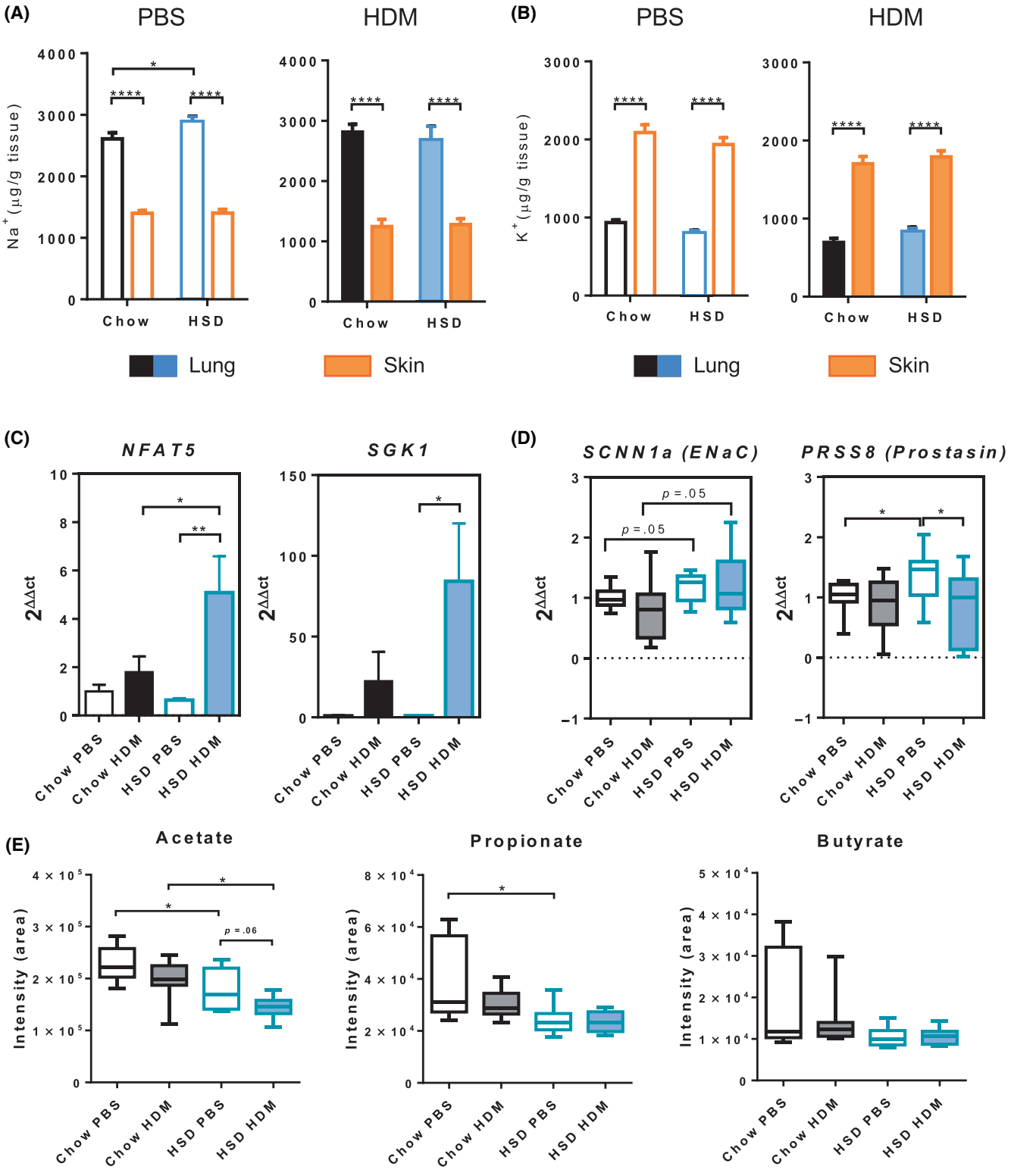


FIGURE 5 Analysis of ion concentration and of osmosensitive markers in lung tissue. Female C57Bl/6J mice were fed with HSD or kept on control chow for 4 weeks and sensitized to HDM or sham sensitized to PBS last 2 weeks of experiment. (A) Na^+ and (B) K^+ concentration in lung and skin tissue determined by neutron activation analysis. (C, D) Real-time PCR analysis of osmosensitive markers nuclear factor of activated T cells 5 (*NFAT5*), serum glucocorticoid–regulated kinase (*SGK1*) and of the main subunit of the epithelial Na^+ channel *SCNN1a* and its regulator prostasin (*PRSS8*). (E) Short chain fatty acids (SCFA) measured in mouse serum by reversed phase analysis (RP) coupled to tandem mass spectrometry (LC–MS/MS). (A, B) $n = 8–11$; (C, D) $n = 8–12$; (E) $n = 8$. (A–C) mean ± SEM; (D, E) boxplots indicating minimum, 25th percentile, median, 75th percentile, and maximum. Results were analyzed by one-way analysis of variance (ANOVA) with Bonferroni test. $^*p < .05$, $^{**}p < .01$, $^{****}p < .0001$.

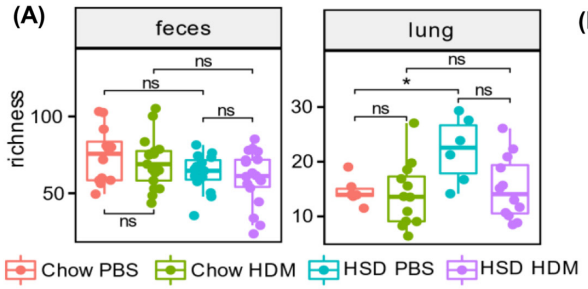
were reduced in relative abundance in HSD samples, whereas various members of *Clostridia* (*Blautia*, *Tuzzerella*, *Roseburia*, *Romboutsia*) and *Faecalibacterium* were increased (Figure 6C, Figure S9B, Table S5). Only two genera of *Proteobacteria* were detected in feces: whereas *Parasutterella* was negatively affected by HSD, *Escherichia-Shigella* was increased in relative abundance. Although HSD caused a pronounced shift in the gut microbiome, solely *Bifidobacterium* was affected by AAI, with reduced relative abundance after HDM challenge (Figure 6C, Figure S9B). The lung microbiome was dominated by *Proteobacteria* (mainly *Acidovorax*, *Pseudomonas*) > *Fusobacteriota* (mainly *Fusobacterium*) > *Firmicutes* (mainly *Staphylococcus*) > *Actinobacteria* (mainly *Corynebacterium*, *Rothia*). As expected, the effects of diet and treatment on the lung microbial community were less pronounced compared to the effects described on feces. However, HSD led to a relative increase of *Pseudomonas* and *Acinetobacter* whereas HDM challenge showed no effect, also in conjunction with HSD feeding (Figure 6D, Figure S9A,B, Table S5).

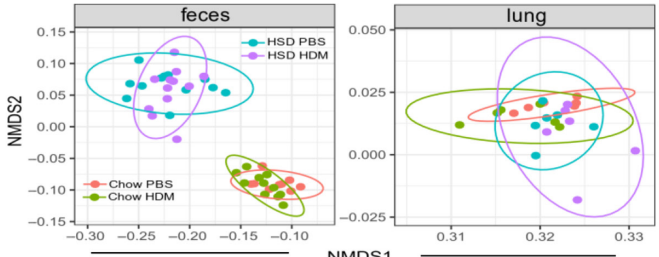
Since the skin represents a critical target organ for Na^+-induced immunologic and antimicrobial responses,[32] we sought to investigate whether HSD induces microbiome changes also in this organ. The results show that HSD feeding induces significant changes in skin microbiome compared to chow, as depicted in the NMDS plot (Figure S10A). The shifts were accompanied by a significant loss of microbial diversity expressed as richness or Simpson index (Figure S10B). Investigation of taxa relative abundances showed that the *Firmicutes* Phylum dominated the HSD microbiome landscape, with significant increases of representative genera as the *Jeotgalicoccus*, *Aerococcus*, *Mammaliicoccus*, *Sporosarcina*, and *Lederbergia*. Yet, two other *Firmicutes* members, namely the *Staphylococcus* dominating the skin microbiome of chow-fed mice and *Fusimonas*, exhibited an opposite trend. On the other hand, key members of the *Bacteroidetes* (*Muribaculum*, *Muribaculaceae RIAY* and *Prevotella*) and *Proteobacteria* (*Thiolapillus HQ191085*) Phyla, more abundant following chow feeding, were completely depleted from the skin of HSD-fed mice (Figure S10C–E). Taken together, whilst AAI impacted solely the relative abundance of *Bifidobacterium* in the gut, HSD feeding led to distinct changes in the gut, lung, and skin microbiome, with a lower *Bacteroidetes* to *Firmicutes* ratio in gut and skin, a decreased relative abundance of *Lactobacillus* in the gut, and enriched members of *Gammaproteobacteria* in the lung.

4 | DISCUSSION

This study uses epidemiological data from a large, population-based prospective cohort study of adults to evaluate associations of dietary salt intake with allergic asthma incidence, and a mouse AAI model to investigate underlying mechanisms.

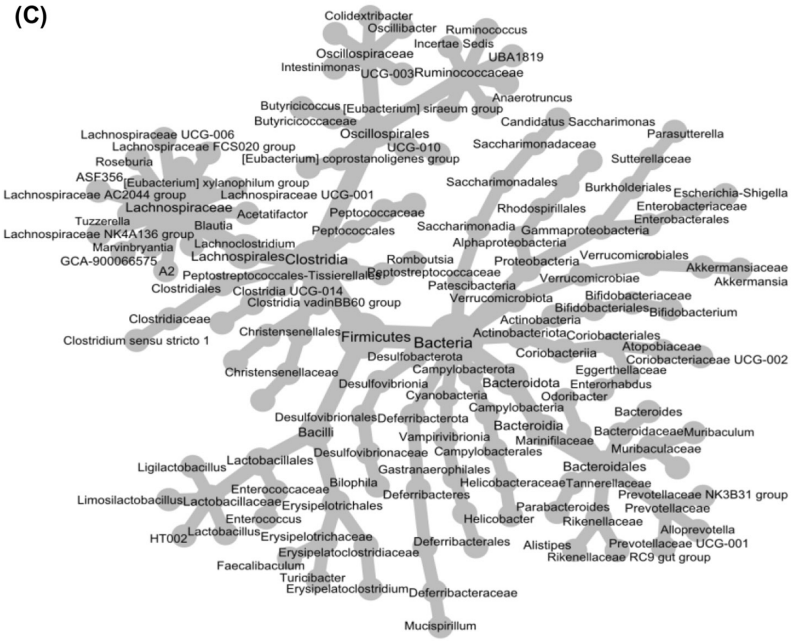
Our epidemiological analyses show that dietary salt is directly associated with asthma incidence among female but not male adults. The observed positive association is in line with several clinical trial reports on disease severity in asthmatics[22,23,25]; however, the detrimental effects of salt observed in these studies are not necessarily restricted to females. Comparability with the available epidemiological evidence is limited due to the scarcity of studies and differences in study designs, often focusing on asthma control rather than prevention, with mixed findings, as summarized by Pogson and McKeever.[43] While an ecological study reported higher mortality only among males with higher salt purchases,[19] others observed a positive association of salt intake with mild bronchial hyperresponsiveness in females but not in males.[44] From the present study data, we are unfortunately not able to determine the underlying reasons for the observed sex-specific association of sodium and asthma. This calls for human intervention trials investigating the effect-modifying roles of sex steroid hormones, anatomical, or other physiological differences. On the other hand, sex-specific lifestyle, occupational or environmental aspects could also be involved.[45] For example, some foods that can alter urinary sodium excretion levels are rich in other compounds that have been previously linked to asthma. In our study population, females were more likely to adhere to fruit and vegetable guidelines (rich in protective antioxidants[46]), whereas males consumed more processed meats (high in saturated fat associated with increased asthma risk[47]). Since we adjusted our analyses for both dietary behaviors, the observed association between sodium and asthma can be assumed to be independent of these. However, despite adjusting our models for these and several other covariates identified in the literature, we cannot entirely exclude the possibility of residual confounding, a limitation inherent to the epidemiological study design. We nevertheless highlight the value of observational data in assessing the prospective effects of habitual dietary behaviors on disease incidence, as such evidence could not otherwise be obtained through randomized clinical trials or using mouse models.[45] To our knowledge, the present study is the first to report on the long-term effects of dietary salt using a prospective design considering only incident asthma cases, and excluding individuals with previous related diseases, thus largely reducing the possibility of reverse causation. The reduced sample size and statistical power resulting from such selection criteria is a potential reason for the lack of similar studies to date. Hence, the large study population available from the UK Biobank Resource is a key strength of our analyses. In contrast, given that the UK Biobank is an adult cohort (>40 years), and given the predominance of healthy participants in the selected study population (excluding those with, e.g., hypertension), the generalizability of our findings is limited. Furthermore, the lack of repeated 24-h urine collections in the UK Biobank (the gold standard method for assessing habitual salt intake), is an important study limitation. Considering the many challenges of assessing salt intake by means of nutritional tools, such as reporting bias and difficulties in sodium quantification,[48] we opted for using the available spot urine biomarkers, which are objectively measured yet highly dependent on diurnal and within-person variability.[49] Systematic bias has been reported in the estimation of 24-h urinary sodium excretion using spot urine, with overestimation at lower levels and underestimation at higher levels of sodium intake.[50] We would expect the impact of such bias to be a loss of statistical power, and to alter the target estimate toward the null value, causing an attenuation of the estimated





	p value
diet	.001
treat	.286
diet*treat	.286

	p value
diet	.032
treat	.169
diet*treat	.151



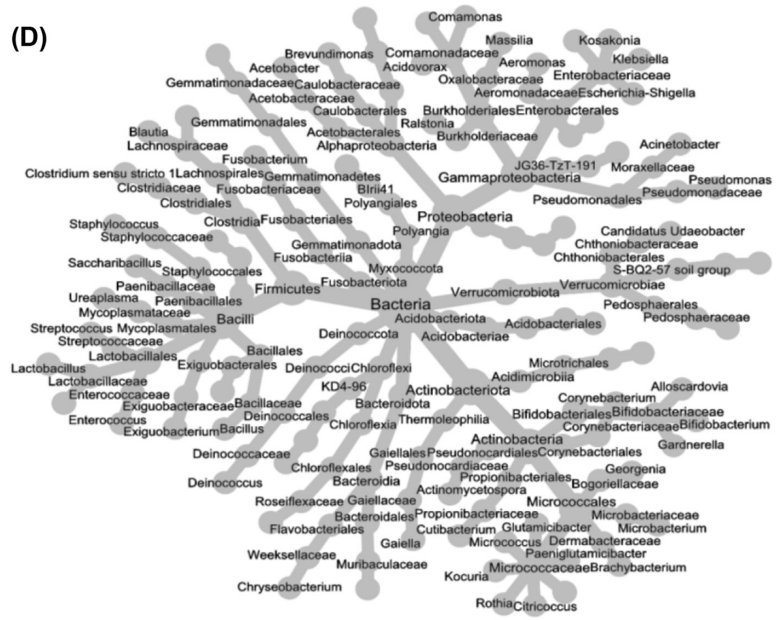


FIGURE 6 Gut and lung microbiome analysis. Microbiome analysis of mouse feces and lungs was performed following 4 weeks of feeding with HSD or control chow and treatment with HDM or PBS last 2 weeks of experiment. (A) Alpha diversity of feces and lung microbiome expressed as microbial richness. Significance was calculated by Wilcoxon-Rank-Sum test with Benjamini Hochberg correction. (B) NMDS plot of beta diversity of feces and lung microbiome based on weighted Unifrac distances. Differences were calculated by PERMANOVA with Benjamini Hochberg correction. (C, D) Heat tree of feces and lung microbiome, respectively, including genera >=0.3% of all reads. The labeled trees (left panels) show the taxonomic information (domain to genus), key for the unlabeled smaller trees (right panels), which represent the experimental comparisons. Colored taxa are significantly ($p < .05$ based on Wilcoxon–Rank Sum test with Benjamini–Hochberg correction) more abundant (based on log2-transformed ratio of median proportions) in the samples indicated in the respective column or row. Red colored taxa are enriched in HSD and HDM, whereas blue colored taxa are enriched in chow and PBS. (A) n=44 (feces) n=36 (lungs); (B-D) n=44 (feces) n=24 (lungs).

effects. Thus, although the herewith applied INTERSALT formulas were found to be the least prone to bias when comparing different predictive equations,[51] the true association of sodium and asthma incidence is likely underestimated in the present study. These data are therefore not adequate for informing dietary recommendations on salt intake. Nonetheless, we believe the association observed in females is robust enough to warrant further study into its potential role in asthma pathophysiology. It has been shown that spurious associations with health outcomes can arise from the use of predictive equations of 24-h sodium excretion, generated by their incorporation of age, sex, weight, and creatinine, which can be associated with the health outcome independently of sodium intake.[52,53] We confirmed that this was not the case in our study, as the same direct association was observed in females, when running analyses using absolute sodium concentrations as the main exposure. We further corroborated our findings by assessing the effects of self-reported added salt (based on a simple categorical question, which is not dependent on recalling all foods consumed, nor on sodium quantification). Again, asthma incidence was greatest among females who reported always adding salt to their food, while no association was present among males. Thus, despite the methodological limitations, our data speak for a clear role of dietary salt in the development of late-onset asthma among females.[51]

To evaluate the mechanistic underpinnings of the direct association between high-salt consumption and allergic asthma incidence in females, we used a mouse model of AAI combined to HSD feeding in female mice. HSD-fed mice demonstrated aggravated AAI as characterized by serum immunoglobulins, BAL and lung cellular infiltrate and release of inflammatory cytokines, in accordance with an analysis of induced sputum following dietary salt manipulation in humans.[54] Contrarily, mucus hypersecretion and airway hyperresponsiveness were not affected by HSD, likely due to the fairly short diet intervention in a relatively low hyperresponsive strain.[55] FACS analysis of lung tissue confirmed a HSD-driven increase in T_H2-T_H17-skewed lung allergic inflammatory milieu and inhibition of Tregs, in line with established effects of NaCl on T-cell subsets in various inflammatory conditions.[9,11–13,15] Interestingly, also the percentage of γδT17 cells was slightly increased in the lymph nodes following AAI and, most fascinating, significantly increased in the lungs following HSD feeding alone. This unexpected result identifies γδT17 cells as novel players in immune regulations developing under HSD conditions. To shed light on mechanisms responsible for the T_H2/T_H17 profile following AAI in HSD- fed mice, we investigated the expression of both NFAT5 and its downstream target SGK-1,[56] which are critical for sensing and regulating Na^+ transport, NaCl homeostasis, and for inducing T_H2 and T_H17 cell differentiation and activation.[9,10,13,33] SGK-1 expression is notably activated in hypertonic conditions, where it was found to stabilize IL-23R and thus reinforces the Th17 phenotype.[10] In our study, exposure to HSD alone was not sufficient to increase the lung expression of NFAT5 and SGK-1. Conversely, AAI induced a mild upregulation of both genes, which was significantly enhanced upon HSD regimen, consequently boosting the inflammatory response. The activation of SGK-1 is also known to activate a variety of epithelial ion channels, which regulate the liquid clearance in various body sites including the gas exchange region of the lungs.[36,57] Important in our context is the epithelial sodium channel with its main subunit *SCNN1A*, regulated by the membrane-bound channel-activating protease 1 (*PRSS8*).[37] Here we display mild changes in lung *SCNN1A* and *PRSS8*, likely because of their relatively low expression in whole lung homogenates.

Recent research has greatly enhanced our understanding of the complicated cross-talk between the microbiome and the immune system. An intriguing finding of our study was the effect of HSD on the lung microbiome with enrichment of members of *Gammaproteobacteria* (*Pseudomonas* and *Acinetobacter*), as it has been previously shown for human asthmatic lungs.[58–60] Interestingly, *Pseudomonas* colonization in the lungs, together with *Lactobacillus*, was shown to promote IL-17 response in a model of chronic lung inflammation, establishing a possible link to the observed IL-17-driven signature in our study.[61] Considering the HSD-induced aggravated disease phenotype, it was not surprising that *Gammaproteobacteria*, which typically utilize inflammatory byproducts for their growth thereby outcompeting other residential microbes, were enriched.[62] Also inflammation-driven mucosal metabolic changes offer terminal electron acceptors for anaerobic respiration, which in turn provide a further selective advantage for *Gammaproteobacteria* (e.g. *E.coli* and *Pseudomonas*), as they often encode for denitrifying pathways rather than using fermentation for their energy needs.[63,64] In contrast, we did not detect HDM-induced expected alterations[59] in lung microbiome. Likely, the mild sensitization protocol employed in our study accounts for this deficiency. However changes on the transcript level and phenotypic differences even at this low level of exposure cannot be excluded.

SCFAs, the main end products of bacterial dietary fiber fermentation, are known to have immune modulating properties.[38,65,66] An attenuated production of SCFAs from the gut microbiome caused

by antibiotic treatment leads to increased susceptibility to AAI, which in turn is inhibited by dietary SCFA supplementation.[39,65] In these studies, an increase in *Bacteroidetes* and decrease in *Firmicutes* were associated with augmented SCFA levels and suppression of AAI. Following this lead, we show that HSD feeding induces a lower *Bacteroidetes* to *Firmicutes* ratio in the gut, concomitantly to a decrease in serum acetate and propionate and an increased lung allergic response. Notably, a similar picture was also observed when analyzing skin microbiome shifts upon HSD feeding, where a loss of *Bacteroidetes* members was paralleled by an expansion of taxa belonging to *Firmicutes* already 1 week after onset of diet (Figure S10). Nevertheless, if these similarities are triggered by typical mouse behavioral patterns in a cage environment cannot be excluded. On genus level, HSD was shown to reduce *Lactobacillus* relative abundance in gut,[12,67] which was confirmed by our study. A decrease in *Lactobacillus* spp., known for their ability to process non-digestible dietary fibers and produce SCFAs, might promote AAI by inducing a Th17-type response.[12] Interestingly, sera of allergic mice tended to have less acetate compared to controls, which is in line with recent data pointing to protective effects of acetate in a mouse asthma model.[68] In addition to *Lactobacillus* spp., allergen challenge significantly reduced *Bifidobacterium* in the gut. The genus *Bifidobacterium*, which is decreased in the gut of long-term asthma patients,[69] improves asthmatic symptoms in a murine AAI model[70] and, when supplemented together with *Lactobacillus*, also in children,[71] implicating potential therapeutic effects of these two genera in the context of AAI.

However, to ascertain the existence of a causative link between the observed alterations in lung and gut microbial communities associated to HSD feeding or allergen challenge and the pathological outcome, further studies using gnotobiotic models with fecal microbiota transplantation implementing transcriptomics, proteomics, and metabolomics approaches are needed.

Taken together, our study demonstrates that high consumption of dietary salt increases asthma incidence in female adults with a history of allergies and mice, driving specific lung T-cell subsets in response to aeroallergens and impacting lung and gut microbiome. Changes in gut microbiota could be used as biomarkers to define dietary recommendations related to salt consume for the prevention or therapy of asthma.

AUTHOR CONTRIBUTIONS

Epidemiological study design: CPH and FA. Epidemiological data analysis: CPH, SCB. Mouse experimental design: SM, SG, and FA. Conduction of experiments: SM, AB, BS, DR, and FA. Experimental data analysis: SM, SG, AB, YA, DR, and FA. Supervision: CPH, CBS-W, CEZ, FA. Writing original draft: SM, CPH, SG, and FA. Review&Editing: All Authors. Funding acquisition: MS, CBS-W, CEZ, and FA.

ACKNOWLEDGMENTS

The authors are grateful to the UK Biobank participants. Epidemiological research has been conducted using the UK Biobank Resource under application number 70262. For the experimental study, we wish to thank the animal caretakers of the Helmholtz Center Munich and Johanna Grosch for technical assistance. We are also grateful to Dr. Annette Schuhmacher, Ssniff Spezialdiäten GmbH, for her assistance in choosing the diets used in the study. LC–MS/MS analysis was performed by the Metabolomics Facility at Vienna BioCenter Core Facilities, member of the Vienna BioCenter, Austria and funded by the Austrian Federal Ministry of Education, Science & Research and the City of Vienna. Open Access funding enabled and organized by Projekt DEAL.

FUNDING INFORMATION

Epidemiological study: UK Biobank was established by the Wellcome Trust medical charity, Medical Research Council, Department of Health, Scottish Government, and the Northwest Regional Development Agency. It has also had funding from the Welsh Government, British Heart Foundation, Cancer Research UK, and Diabetes UK. UK Biobank is open to bona fide researchers anywhere in the world, including those funded by academia and industry. The medical research project is a non-profit charity which has been awarded core funding of around £180 Million. Core funding continues to be received from the Wellcome Trust, the MRC, and more recently, from Cancer Research UK and NIHR. The present study was designed, conducted, and interpreted by the authors entirely independently of the funding sources. Mouse studies: Mouse studies were supported by the institutional funding for allergy research from the Helmholtz Munich (FA) and by SFB1054, FungiNet TR124, Excellence Cluster Balance of the Microverse (CEZ).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Musiol S, Harris CP, Gschwendtner S, et al. The impact of high-salt diet on asthma in humans and mice: Effect on specific T-cell signatures and microbiome. *Allergy*. 2024;00:1-14. doi:10.1111/all.16148