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Functional iron-deficiency in women with allergy rhinitis is associated with symptoms after nasal provocation and lack of iron-sequestering microbes

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To the Editor,

It is still unclear which underlying factors in atopic subjects contribute to their heightened immune response and lead to increased IgE production. Iron-deficiency and anemia is clearly associated with the onset of allergic diseases with a poor iron status at birth increasing the risk of developing an atopic disease, whereas an improved iron-status seems to prevent the onset of allergy ¹. Importantly, in our preclinical studies we were able to confirm that iron can be selectively delivered to immune cells with

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beta-lactoglobulin carrying iron complexes as ligands, resulting in immune resilience and allergy prevention². As a lack of iron is also a danger-signal for immune cells leading to Th2-activation², we hypothesized that atopy may be linked with functional iron deficiency, in which the iron stores are not accessible, thus facilitating allergy development and amplifying the clinical symptom burden in allergy sufferers. As iron parameters and reference ranges differ between gender and with age, we eliminated confounding factors (Suppl. Materials, exclusion criteria) and in this study focused only on premenopausal women with or without respiratory allergies

Here, we show for the first time that women with allergic rhinitis and without anemia- though having similar dietary habits as non-allergic ones (**Table S1, Fig S1**) - have decreased levels of metabolically active iron in the circulation with lower serum iron, transferrin saturation and elevated ceruloplasmin-levels compared to non-allergic women, while significantly more hepcidin is produced (**Fig 1 A and more parameters at Table S2 and Fig S2, sensitization profile are listed in Table S3 and Table S4**). Despite increased iron mobilization efforts by the copper-containing ferroxidase ceruloplasmin, which also acts as an acute-phase reactant, allergic women had lower serum iron. However, greater amount of urinary hepcidin, which blocks cellular iron release and mobilization, were present in our allergic rhinitis cohort and may point towards greater immune activity inherent in allergics compared to non-allergic individuals. The increased hepcidin excretion also suggests that in women with allergic rhinitis dietary intestinal iron-absorption and/or cellular iron-release is inhibited to a greater degree than in non-allergic individuals.

Furthermore, for the first time ceruloplasmin-levels in serum ($r = 0.53$, $p = 0.0007$) and nasal secretions ($r = 0.59$, $p = 0.000089$) were linked with symptoms after nasal provocation (**Fig 1 B+C and Table S5, S6, S7**). Gut iron appeared to be beneficial and was associated with reduced symptom burden ($r = -0.78$, $p = 0.000042$) (**Fig 1 B+D**) that even showed linearity in regression analysis in allergic rhinitis subjects that underwent graded nasal provocation. Gut iron also seemed to contribute to an improved iron status in women (**Fig 1E**).

Indeed, elevated levels of ceruloplasmin have already been described in atopic dermatitis and asthma patients and were suggested as a sputum marker for plasma-protein leakage in subjects suffering from asthma and chronic obstructive pulmonary disease ³. Our data also link ceruloplasmin with clinical reactivity and thus with the allergy effector cells, the mast cells.

The presence of iron in stool extracts reflects the occurrence of commensal microbial communities relying on and solubilizing iron. Bacteria associated with gut iron (green), such as members of the genus *Bacteroides*-that also support immune iron repletion and resilience⁴-and *Ruminococcus* were associated with reduced symptom burden (Amplicon sequence variants, ASV_qzd_als, $r=-0.61$, $p=0.00007$; ASV_jhl_q2w, $r=-0.51$, $p=0.0017$) and were negatively associated with serum ceruloplasmin (**Fig 2**). Importantly, the same ASV of the genus *Ruminococcus* was also relatively enriched in our non-allergic cohort (**Fig 2E**), emphasizing its relation to a healthy gut⁵. Similar to a study with atopic dermatitis subjects⁶, only one ASV of the genus *Faecalibacterium* (red, ASV_k3p_vr6, $r=0.34$, $p=0.04$) showed an association with symptom severity and ceruloplasmin ($r=0.3$) and was negatively associated with gut iron ($r=-0.45$).

In summary, in this association study, we linked the hyper-active state in allergic rhinitis subjects with decreased iron mobilization. Ceruloplasmin was the sole protein marker that was linked with clinical reactivity probably due to its increased release by mast cells, whereas gut iron-levels seemed beneficial to reduce symptom burden. The microbial taxa *Bacteroides*, which contribute in immune iron repletion and resilience⁴ and *Ruminococcus*, which was also relatively enriched in non-allergics, were linked to gut iron and reduced clinical reactivity.

Our findings underline the existence of functional iron deficiency in subjects with allergy. The knowledge may be exploited to combat the allergy epidemic by developing a new generation of therapeutic and prophylactic approaches. To replenish immune iron may thus become a way to achieve immune resilience.

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AUTHOR CONTRIBUTIONS

L.M.P. performed major parts of experiments, analyzed data and contributed to writing. S.A.J. acquired major parts of human samples, helped during nasal provocations, analyzed data and contributed to writing; S.S. and M.S. acquired human samples, performed experiments and contributed in writing. T.B. performed nasal provocations and contributed to writing, E.U. contributed to human sample acquisition and contributed in writing, E.V. provided support and contributed in writing, P.P. and B.H. performed 16S rRNA gene amplicon sequencing and analysis, and contributed to writing, K.H., S.M.-A., G.H., L.F. helped with experiments and analysis and contributed to the writing; J. E.-D. provided help in the study design, provided equipment and contributed in writing, E.J.-J. supported the study and contributed to writing, C.G.K. and G.A.R. provided material and contributed to writing, E.R. conducted blood analyzes and contributed to writing, S.H. helped in the study design, performed experiments and trace element analyses and contributed to manuscript writing. F.R-W. conceived, directed, designed the study, analyzed data and wrote the manuscript.

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CONFLICT OF INTEREST

EJJ declares shareholdership in Biomedical Int. R+D GmbH, Vienna, Austria. FRW and EJJ are inventors of EP2894478, owned by Biomedical International R+D GmbH, Vienna, Austria. The other authors declare no relevant conflict of interest in relation to this publication.

Short title: Functional iron-deficiency in allergics correlates with symptoms

Supplementary Materials

Materials and Methods

Table S1. Baseline demographics and trace element intake after dietary questionnaire.

Table S2 Blood parameters of non-allergic and allergic women

Table S3 Sensitization pattern and total IgE in allergic (n=27) and non-allergic women (n=24)

Table S4 Sensitization pattern and total IgE of subjects (n=21) that underwent nasal provocation

Table S5 Unadjusted analysis of factors associated with total nasal symptom scores in allergic women

Table S6 Unadjusted analysis of factors associated with visual analogue scale scores in allergic women

Table S7 Unadjusted analysis of factors associated with nasal fluid weight in allergic women

Fig. S1. Dietary iron intake correlates with hemopexin and is similar in allergics and non-allergic

Fig. S2. Similar trace element composition in the gut of allergic and non-allergic women.

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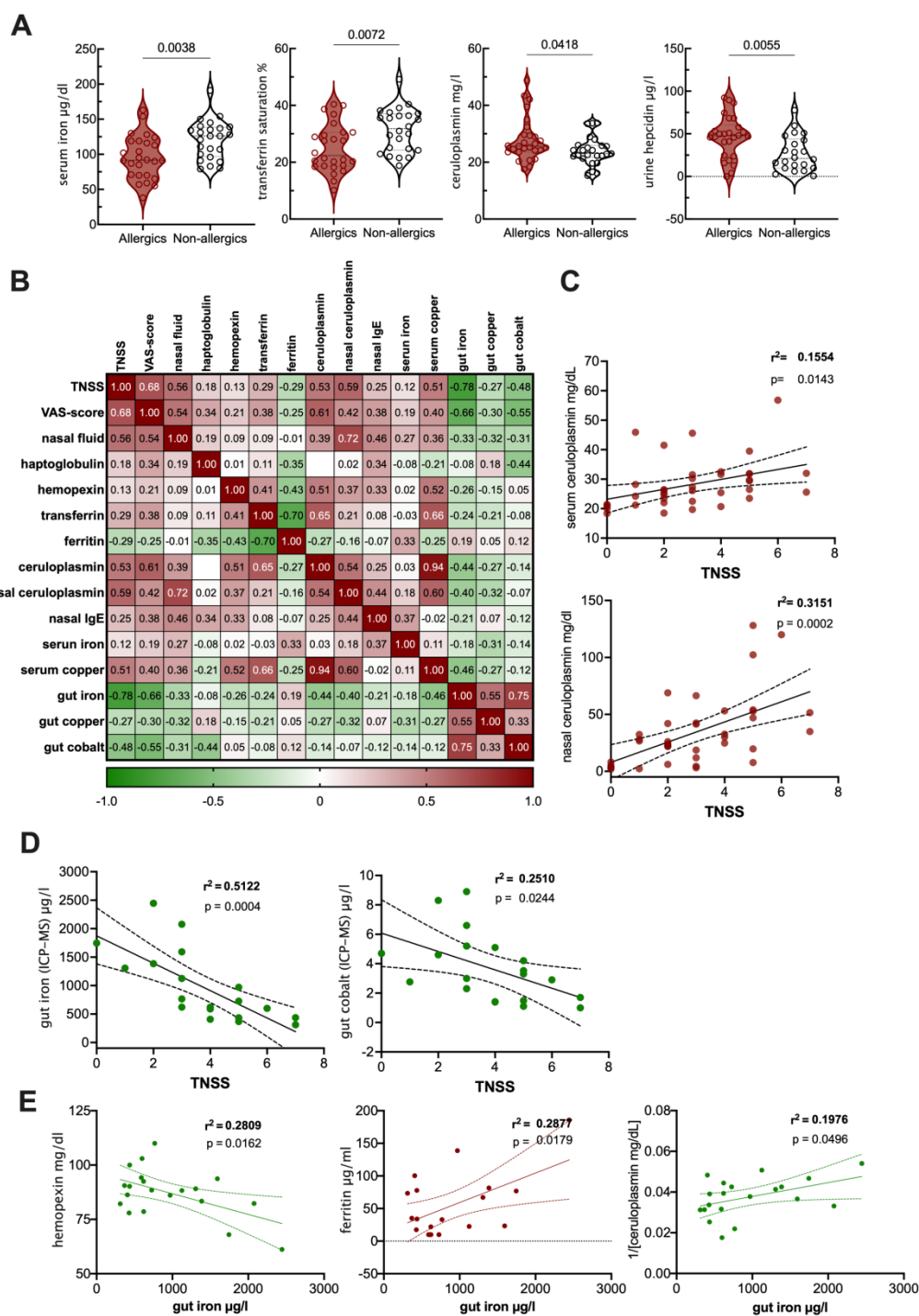
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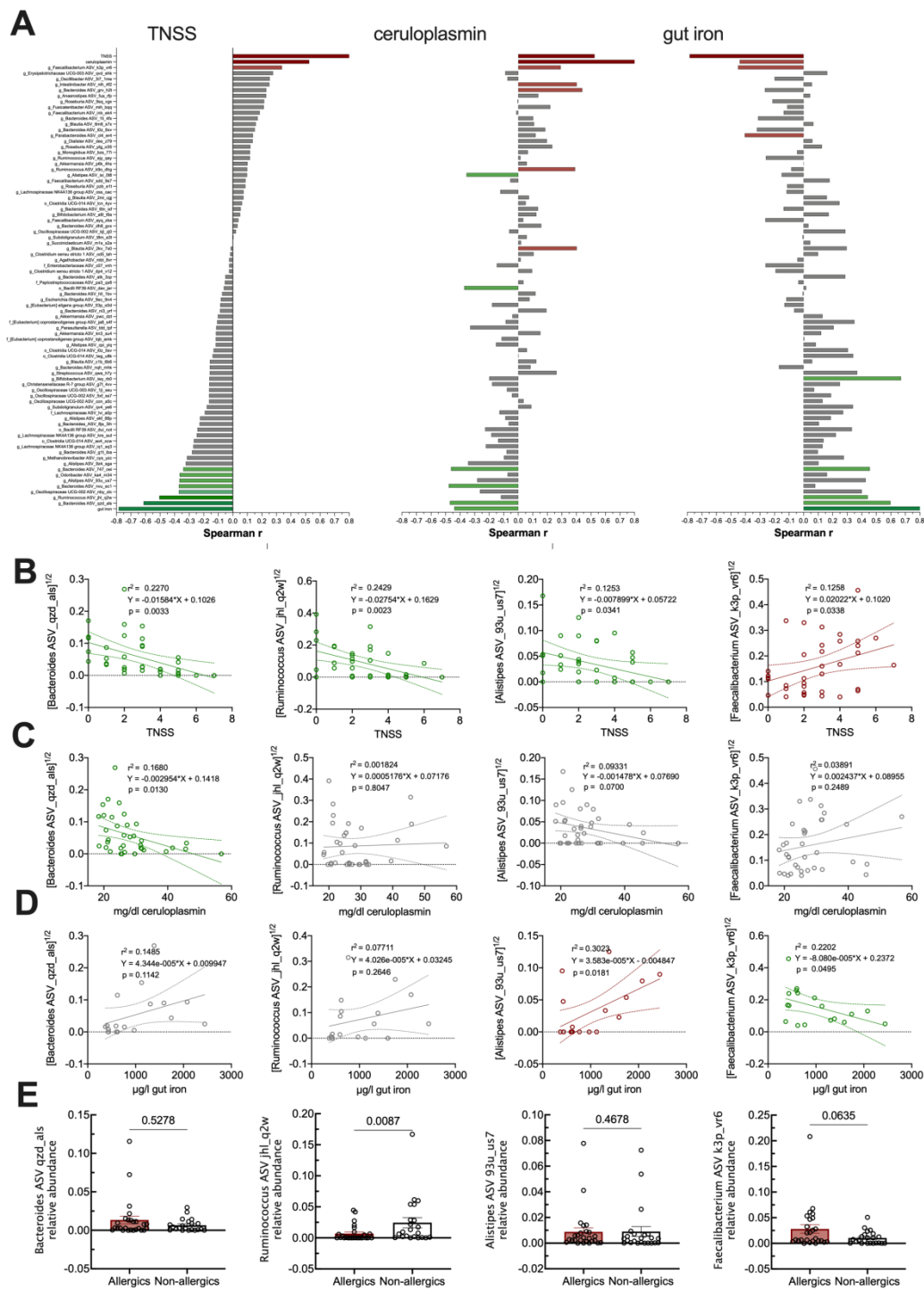
Clinical trials registration # NCT03815981 and NCT03816800

Fig 1. Functional iron-deficiency and elevated ceruloplasmin as a marker for clinical reactivity in allergic women. **A.** Serum iron, transferrin saturation, serum ceruloplasmin and urinary hepcidin-levels of sera from allergic (n=27) and non-allergic (n=24) pre-menopausal women. **B.** Spearman Correlation matrix of total nasal symptom score (TNSS), visual analogue scale (VAS), weight of nasal secretions - as a measure of clinical reactivity -with blood, nasal fluid parameters as well as gut iron, gut copper and gut cobalt of stool extracts of women that underwent nasal provocation with grass- or birch pollen extract (n=38). **C.** Linear regression analysis of TNSS with ceruloplasmin from serum and nasal secretions, **D.** Linear regression analysis of TNSS with gut iron and gut cobalt. **D.** Linear regression of gut iron with hemopexin, ferritin and the reciprocal value of ceruloplasmin. Positive and significant dependencies in linear regression analysis are depicted in red, significant negative ones in green dots. Prior analysis normality and Lognormality -tests were employed. Student's t-test was used for parametric, Mann Whitney U test was used for non-parametric data. *P < 0.05, **P < 0.01.

Fig 2. Members of the genera *Ruminococcus* and *Bacteroides* show a negative correlation with clinical reactivity with *Ruminococcus* ASV being decreased in allergies **A.** Side-by-side comparison of Spearman correlations of gut microbiome ASVs to total nasal symptom score (TNSS), serum ceruloplasmin and gut iron. (dark green and dark red, significant after FDR adjustments $q < 0.05$, light green and red are not significant trends). **B.** Linear regressions to TNSS (n=38), **C** to serum ceruloplasmin (n=38), and **D** to gut iron (n=20) of the relative abundance of ASV of the genera *Bacteroides*, *Ruminococcus* and *Alistipes* negative associated with TNSS as well as relative abundance of ASV of *Faecalibacterium* positive associated with TNSS (green when negatively associated and significant, grey when not significant, red when positive associated and significant). **E.** Comparison of relative abundance of ASVs from the genera *Ruminococcus*, *Bacteroides*, *Alistipes* and *Faecalibacterium* in allergies (n=26) and non-allergics (n=22) as well as the summarized relative abundance of ASVs positively associated with TNSS. Data were non-parametric and Mann Whitney U test were used for statistical analysis. **P<0.01



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SUPPLEMENTARY INFORMATION

MATERIALS AND METHODS

Study design

The data are derived from two clinical studies. In the first study iron parameters were assessed in non-allergic and allergic individuals (ethical approval number #1972/2017, clinical, Trials.gov: NCT03815981). Here, 73 subjects were recruited, of which all men (n=11) and all anemic women (n=9) and menopausal women (n=2) were excluded leaving 27 allergic and 24 non-allergic women without anemia and allowing interpolation of data with the second clinical trial (ethical approval number #1370/2018, clinical, Trials.gov: NCT03816800) in which only premenopausal women were recruited. Subjects filled out a dietary questionnaire and provided a stool sample. Urine and blood samples were collected after overnight fasting as the serum iron concentration is subjected to a strong circadian rhythm. 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 malignancy or if they used medications (e.g., antibiotics, proton-pump inhibitors/PPIs) that influence the iron, inflammatory or microbiome status. Further exclusion criteria were pregnancy, lactation, veganism, zinc and iron supplementation and smoking. Samples were collected in the fall and the winter season.

Subjects were defined as allergic or non-allergic based on anamnesis and testing for specific IgE by ALEX®-tests (Macroarray Diagnostics, Austria). Allergic subjects were considered allergic, when in the ALEX®-test specific IgEs against aeroallergen were at least 1 kU/l. Subjects with no specific IgE > 1kU/l and a total IgE < 20kU/l were considered non-allergic. Iron-deficient anemia was defined according to the World Health Organization using hemoglobin-levels below 12 g/dL and/or ferritin-levels below 15µg/l [1, 2] for premenopausal women as cut-off.

Data from the second trial included allergic premenopausal women with the same exclusion criteria as in the first trial and neither underwent allergen immunotherapy within the last five years prior enrollment nor received anti-IgE therapy. Allergic rhinitis was determined based on medical history, physical examination, specific IgE and a positive nasal provocation test with a TNSS of ≥ 3 considered positive. They were enrolled 2019 in a randomized,

placebo-controlled, double-blind single center trial (ethical approval number #1370/2018, ClinicalTrials.gov: NCT03816800), in which subjects were dietary supplemented with a placebo lozenge – only containing the lozenge vehicle being sorbitol combined with mountain herb flavor or verum dietary lozenge, containing additionally whey proteins combined with zinc, vitamin A and iron-flavonoid complexes.

Blood after overnight fasting and stool samples were collected before nasal provocation and were assessed. Subsequently, subjects received the verum or placebo lozenge for 6 months. Thereafter, a second blood draw, stool sample collection and a second nasal provocation took place. Irrespective of treatment outcome clinical reactivity was correlated with clinical score. In total 38 provocations were conducted, of which 4 were performed in non-allergic subjects.

All participants of both studies provided written informed consent under institutional review board approved protocols before participation.

Dietary questionnaire

Dietary habits were assessed by a questionnaire. Subjects were asked to assess their weekly intake of fruits, legumes, meat, fish, milk products, chocolate, staple foods (rice, pasta, potatoes, soja, flour-containing food and bread) and beverages (tea, coffee, fruit juices, alcohol, etc). Average weekly amounts of trace elements and vitamins were calculated per individual based on the U.S. Department of agriculture food composition data resources FoodData Central.

Nasal provocation

Nasal provocations were conducted as recommended [3] at the ENT facility of the Medical University of Vienna. In short, after a rhinological examination and evaluation of the nasal flow, the nasal provocation was carried out using Pump-Aerosol Spray (50µl/puff) and directing the puffs 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 subjects as well as the doctor evaluated symptoms 10 minutes after each challenge. Subjects evaluated symptoms using a horizontal 0-100 mm scale as Visual Analogue Scales (VAS). The doctor evaluated the symptoms as total nasal symptom score

(TNSS) after the scoring system in the manual of the nasal provocation solution (Leti pharma, Madrid, Spain), but without the use of a rhinomanometer, giving points on 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 TNSS and VAS. After the last challenge, nasal secretions were collected by inserting a pre-weighted sponge (Nasosorption, in the nasal cavity for 1 minute and weighing the sponge thereafter.

Blood analysis

Complete blood cell analysis with iron metabolism marker (hemoglobin, serum iron, reticulocytes, mean reticulocyte haemoglobin, ceruloplasmin, haptoglobin, hemopexin, transferrin, soluble transferrinreceptor, transferrin saturation, serum ferritin), creatinine, C-reactive protein (CRP), lactate dehydrogenase (LDH) were measured in a certified diagnostic laboratory. Hepcidin was measured in diluted serum and urine (both 1:150 in 0.1% bovine serum albumin/PBS) following the manufacture's instruction (R&D systems DY8307, Minneapolis) by ELISA.

16S rRNA gene analyses

DNA extraction and sequencing were performed at the Joint Microbiome Facility of the Medical University of Vienna and the University of Vienna (project ID JMF-1902-6). Stool microbiome composition was determined using amplicon sequencing targeting the V4 region of the 16S rRNA gene using widely applied primer pairs which target both bacteria and archaea [4], as described previously[5-7].

Stool samples were collected within the same week of blood collections for each subject and stored at -20°C until processing. Total DNA was extracted from all samples on the QiaCube (automated extraction) using the Qiagen QIAamp Fast DNA Stool Minikit. Amplicon pools were extracted from the raw sequencing data using the FASTQ workflow in BaseSpace (Illumina) with default parameters. Demultiplexing was performed with the python package

demultiplex (Laros JFJ, github.com/jfjlaros/demultiplex) allowing one mismatch for barcodes and two mismatches for linkers and primers.

Amplicon sequence variants (ASVs) were inferred using the DADA2 R package [8] applying the recommended workflow [5]. FASTQ reads 1 and 2 were trimmed at 220 nt and 150 nt with allowed expected errors of 2 and 2, respectively.

ASV sequences were subsequently classified using SINA version 1.6.0 [9] and the SILVA database SSU Ref NR 99 release 138 [10]. Data were normalized by total sum scaling of each sample and only ASVs with an average relative abundance of 0.25% were considered. Spearman correlation was employed to assess associations. Subsequently, the False Discovery Rate approach for stack of p-Values from Spearman correlations for TNSS, ceruloplasmin and gut iron to microbial ASVs were further investigated using two-stage step-up method of Benjamini, Krieger and Yekutieli as implemented in GraphPad version 9.01 (128). A FDR $q < 0.05$ was considered significant.

Legendplex analysis of nasal fluids

Nasal secretions were eluted from nasal sponge (Nasosorption, Hunt Development, UK) by vortexing for 30 sec in 600 μ l 0.5% BSA in 0.9% NaCl followed by incubation for 15min on a shaker at 300 rpm. For measurements of ceruloplasmin a further 1:50 dilution in 0.1% BSA / 0.9% NaCl was necessary, before performing analysis according to the manufacture's instruction (Biolegend, San Diego, CA). Custom-made panel were for human TSLP, TNF α , IFN γ , NGAL, IL5, IL13 and IL10. Single-plex were performed for nasal IgE (Bead A7, Legendplex 740650) and Ceruloplasmin (bead ID B3, Legendplex 741008). Flow cytometric analysis were conducted with FACS Canto II (BD Bioscience) and using FACSDiva Software 6.0.

ICP-SFMS-analysis

Inductively coupled plasma-sector field mass spectrometry (ICP-SFMS) was used for the analysis of trace elements in human serum, urine and aqueous stool extracts (100 mg stool/ml 0.9% NaCl with 0.2% NaN₃). Stool extracts were prepared by diluting solid stool samples at a concentration of 100 mg stool/ml in 0.9% NaCl with 0.2% NaN₃, before 2 min vortexing and centrifugation at 2000 g for 5 minutes. 0.1g of each sample (serum, urine and stool extracts) was accurately weighed into 1.5 ml PFA vials and diluted with 1ml of double

sub-boiled HNO_3 and 200 μl H_2O_2 . Subsequently, samples were mineralized by open vessel digestion on a hotplat at 100°C for two hours. For quality control, the certified matrix reference material Seronorm™ Trace Elements Serum L-1 and L-2 were processed. The digests were transferred in 12 ml PP-vials and filled up to 10 g with 5% (v/v) double sub-boiled HNO_3 . 100 μL of an internal standard (Indium/Rhenium; final concentration in the sample: 1 $\mu\text{g/l}$) was added to each sample. Quantification was performed by external calibration with internal standardization with multi-element standard solutions obtained by serial gravimetric dilution of a certified multi-element standard (IV-STOCK-6, Inorganic Ventures). The certified reference material TM 35 (Lake Ontario water) was used to check the trueness of the calibration.

Measurements were conducted by ICP-SFMS (Element 2, Thermo Scientific Inc, Bremen, Germany) in low, medium and high resolution using the following settings (Plasma power 1250 W, Nebulizer gas flow 1.0 – 1.1 L/min, Auxiliary gas flow 0.8 L/min, Plasma gas flow 16 L/min; Masses monitored were: Low resolution (LR, $m/\Delta m$ 300): ^{82}Se , ^{95}Mo , ^{98}Mo , ^{115}In ; Medium resolution (MR, $m/\Delta m > 4500$): ^{52}Cr , ^{55}Mn , ^{56}Fe , ^{59}Co , ^{60}Ni , ^{65}Cu , ^{66}Zn , ^{111}Cd , ^{115}In ; High resolution (HR, $m/\Delta m > 10.000$): ^{75}As , ^{115}In .

Statistical analysis

Data were examined for their normal and log-normal distribution using Anderson-Darling and D'Agostino Pearson test. When possible, data were transformed to reach normal distribution.

Normally distributed data were compared with Student's test, when data were non-parametric, Mann Whitney U-tests were applied to determine differences between allergics and non-allergics. Correlation was performed using Spearman r and linear regressions to predict the relationship between two factors. A p-Value below 0.05 was considered significant. Data and graphs were analyzed with GraphPad Software Prism 9.

Data accessibility

The 16S rRNA gene amplicon sequencing data has been deposited at the Sequence Read Archive under the BioProject accession PRJNA704289.

Table S1**Table 1. Baseline demographics and trace element intake after dietary questionnaire**

	Allergics	Non-Allergics	p-Value
Women	27	24	
Age (y), Median	27	35	0.128
95% CI of median	(25.00; 32.00)	(24.00; 46.00)	
Diet (omnivore/vegetarian)	23/4	18/4	
contraception (no/hormonal/Cu-coil)	17/7/3	16/7/1	
BMI, Median	21.4	20.8	0.877
95% CI of median	(19.6; 23.50)	(20.40; 22.30)	
Dietary intake			
Fe, iron (mg), Median	66.53	57.84	0.529
95% CI of median	(50.41; 86.45)	(48.40; 83.55)	
Cu, copper (mg), Median	5.82	6.3	0.992
95% CI of median	(4.75; 11.03)	(4.01; 8.97)	
Zn, zinc (mg), Median	37.7	35.67	0.822
95% CI of median	(40.78; 70.67)	(38.46; 60.38)	
Vitamin B12 (µg), Median	19.4	23.63	0.397
95% CI of median	(13.73; 26.69)	(14.9; 23.36)	

Table S2: Blood parameters of non-allergic and allergic women

	Allergics			Non-Allergic			p-Value
	n	Mean	SD	n	Mean	SD	
age/ year	27	29.89	8.27	24	35.21	11.56	0.177
body mass index	27	22.33	3.73	24	21.74	2.43	0.877
red blood cells T/L	27	4.58	0.22	24	4.60	0.23	0.824
hemoglobin g/dL	27	13.46	0.72	24	13.72	0.75	0.205
hematocrit %	27	39.20	2.10	24	40.00	2.14	0.187
platelets G/L	27	262.1	47.11	24	272.80	60.16	0.684
reticulated platelet fraction %	22	5.31	3.99	15	6.57	4.03	0.458
white blood cells G/L	27	6.12	1.34	24	5.54	1.39	0.131
neutrophils G/L	26	3.57	1.07	24	3.03	1.11	0.086
neutrophil fraction %	27	56.07	6.51	24	53.56	8.72	0.245
lymphocytes G/L	26	1.98	0.54	24	1.95	0.45	0.598
lymphocyte fraction %	27	32.61	5.46	24	35.87	7.50	0.080
monocytes G/L	26	0.42	0.15	24	0.42	0.11	0.885
monocyte fraction %	27	7.40	1.82	24	7.66	1.61	0.261
eosinophils G/L	26	0.18	0.11	24	0.13	0.08	0.022
eosinophil fraction %	27	3.15	1.88	24	2.28	1.50	0.075
basophils G/L	26	0.03	0.05	24	0.03	0.04	0.757
basophil fraction %	26	0.68	0.45	24	0.63	0.30	0.935
reticulocytes G/L	27	49.20	12.47	24	43.58	17.97	0.197
reticulocyte fraction %	27	1.07	0.26	24	0.95	0.38	0.166
low fluorescence fraction %	27	96.18	2.68	24	96.63	2.71	0.177
medium fluorescence fraction %	27	3.56	2.35	24	3.25	2.51	0.282
high fluorescence fraction %	27	0.27	0.41	24	0.13	0.27	0.048
serum iron ug/dL	27	97.85	32.29	24	120.90	27.39	0.009
lactate dehydrogenase U/L	27	177.1	26.43	24	173.70	27.38	0.646
creatinine mg/dL	27	0.75	0.12	24	0.76	0.09	0.698
C-reactive protein mg/dL	27	0.44	0.93	24	0.47	0.98	0.989
transferrin mg/dL	27	272.8	38.35	24	276.70	37.57	0.797
transferrin saturation %	27	25.87	9.45	24	31.36	7.63	0.028
ferritin ug/L	27	51.05	37.48	24	46.32	37.03	0.698
soluble transferrin receptor mg/L	27	1.13	0.20	24	1.13	0.22	0.892
haptoglobin mg/dL	27	90.68	31.46	24	87.84	31.79	0.750
ceruloplasmin mg/dL	27	27.87	7.84	24	23.91	5.08	0.045
hemopexin mg/dL	10	82.28	9.24	12	85.13	8.21	0.452

Table S3: Sensitization pattern and total IgE in allergic (n=27) and non-allergic women (n=24)

Summary of the Alex[®] array: The highest measured IgE concentration per allergen group were further classified into 0: <0.3 kUA/L; 1: 0.3-1 kUA/L; 2: 1-5 kUA/L; 3: 5-15 kUA/L; 4: >15 kUA/L and categorized into following groups: pollen/ microbes (mites, fungi, yeast)/plant food/animal-derived food/animals/insects/others. Various sensitization (e.g. grass and tree pollen sensitization) within the same category were summed up and are listed with the total measured IgE-levels in the table.

Allergic women	pollen (grass, tree, weed)	microbes (mites, fungi, yeast)	plant food	animal-derived food	animals/pets	insects	others	total IgE (kU/L)	Non-Allergic women	pollen (grass, tree, weed)	microbes (mites, fungi, yeast)	plant food	animal-derived food	animals/pets	insects	others	total IgE (kU/L)
A_1	5	0	0	0	0	0	0	10	N_1	0	0	0	0	0	0	0	<20
A_2	5	0	4	0	0	1	1	327	N_2	0	0	0	0	0	0	0	10
A_3	4	0	8	0	0	0	0	161	N_3	0	0	1	1	0	0	0	5
A_4	2	3	0	0	4	0	0	60	N_4	0	0	0	1	0	1	0	2.63
A_5	3	0	3	0	0	0	0	13	N_5	0	0	1	0	0	0	0	1.58
A_6	0	6	0	1	0	0	0	790	N_6	0	0	1	0	0	0	0	1.69
A_7	2	2	0	0	2	0	0	7	N_7	0	0	0	0	0	0	0	<20
A_8	6	4	7	2	5	1	0	264	N_8	0	0	0	0	0	0	0	1.37
A_9	4	0	5	0	4	0	0	79	N_9	0	0	0	0	0	0	0	10
A_10	0	0	1	0	3	1	0	11	N_10	0	0	0	0	0	0	0	1.37
A_11	3	0	0	1	5	0	0	51	N_11	0	0	1	0	0	0	0	1.98
A_12	10	0	0	1	0	2	0	218	N_12	0	0	0	0	0	0	0	<20
A_13	4	0	0	0	0	0	0	59	N_13	0	0	0	0	0	0	0	<20
A_14	3	0	4	2	0	1	2	11	N_14	0	0	0	0	0	0	0	0.85
A_15	10	0	0	0	0	1	0	7	N_15	0	0	0	0	0	0	0	1.49
A_16	6	4	4	0	0	0	0	127	N_16	0	0	1	0	0	0	0	2.16
A_17	9	4	6	0	3	0	2	160	N_17	0	0	1	0	0	0	0	1.88
A_18	1	3	0	0	0	0	0	6	N_18	0	0	0	0	0	0	0	12
A_19	7	3	4	1	0	1	2	112	N_19	0	0	0	0	0	0	0	4
A_20	5	0	0	0	0	0	0	13	N_20	0	0	0	0	0	0	0	3
A_21	2	4	1	0	0	0	0	61	N_21	0	0	0	0	0	0	0	5
A_22	5	0	3	0	0	0	0	14	N_22	1	0	0	0	0	0	0	4
A_23	2	2	2	1	0	3	2	23	N_23	0	0	1	1	0	0	0	3.68
A_24	7	6	1	2	1	3	0	120									
A_25	2	4	0	0	1	0	0	145									
A_26	2	0	1	1	0	0	0	3									
A_27	4	2	7	0	0	0	0	131									
MW	4.19	1.74	2.26	0.44	1.04	0.52	0.33	110.48	MW	0.04	0.00	0.38	0.13	0.00	0.04	0.00	3.83
STDEV	2.68	2.01	2.55	0.68	1.69	0.88	0.72	157.84	STDEV	0.20	0.00	0.56	0.33	0.00	0.20	0.00	3.12
p-Value allergic vs non-allergics									2.10 ⁻⁹ 0.0000160.0000970.00410.00430.0120.0260.002								

Table S4: Sensitization pattern and total IgE of subjects (n=21) that underwent nasal provocation (n=38)

Summary of the Alex[®] array: The highest measured IgE concentration per allergen group were further classified into 0: <0.3 kUA/L; 1: 0.3-1 kUA/L; 2: 1-5 kUA/L; 3: 5-15 kUA/L; 4: >15 kUA/L and categorized into following groups: pollen/ microbes (mites, fungi, yeast)/plant food/animal-derived food/animals/insects/others. Various sensitization (e.g. grass and tree pollen sensitization) within the same category were summed up and are listed with the total measured IgE-levels in the table.

Allergic women	pollen (grass, tree, weed)	microbes (mites, fungi, yeast)	plant food	animal-derived food	animals/pets	insects	others	total IgE (kU/L)
S1	8	2				1		7
S2	1							4
S3	10		12	2	5			467
S4	3	4	1			1		164
S5	6	4	1		2	2		358
S6	5		4			1	1	327
S7		3						14
S8	5							49
S9	3	4	2		1			19
S10	2	3						6
S11	7	1						35
S12	6	3	3		2			58
S13	4	3						11
S14	7	3	4			1	2	112
S15	8						1	113
S16	5							10
S17	5							13
S18	5		3					14
S19								5
S20	7		2		2			38
S21	4							59
MW	5.26	3.00	3.56	2.00	2.40	1.20	1.33	89.67
STDEV	2.27	0.89	3.17	0.00	1.36	0.40	0.47	129.05

Patients demographic that underwent nasal provocation

Subjects' demographics	n
Gender (f/m)	21/0
Age (years, Mean $\pm$ STD)	29.8 $\pm$ 2.1
BMI (Mean $\pm$ STD)	20.7 $\pm$ 0.6
Diet style (omnivore/vegetarian)	19/2
Contraception (no/hormonal/ copper coil)	8/8/5

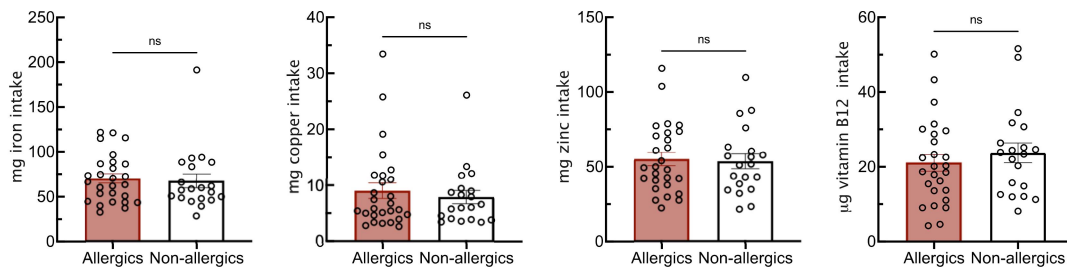
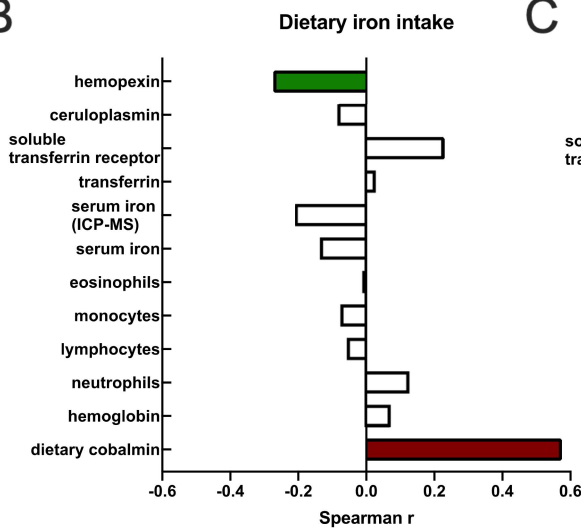
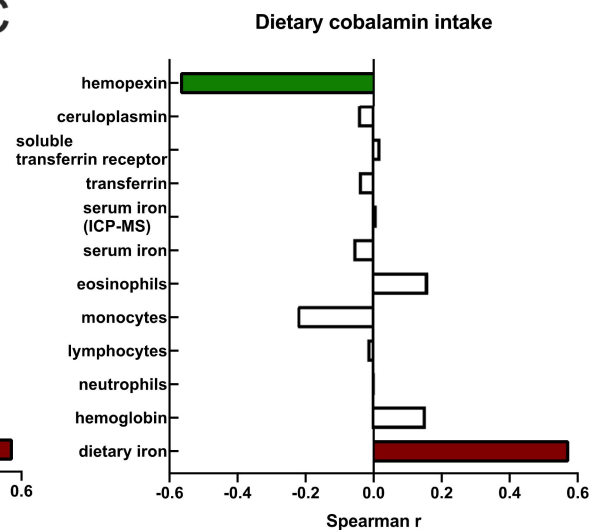
Suppl Fig 1 Dietary iron intake does not differ between allergics and non-allergics**A****B****C**

Fig S1. Dietary iron intake correlates with hemopexin and is similar in allergics and non-allergics **A.** Subjects characteristics: healthy, non-anemic, non-smoking subjects filled out a dietary questionnaire. Blood samples were collected after overnight fasting. Patients' intake of fruit, legumes, meat, fish, dairy products, chocolate, staple foods, beverages and sweets were recorded. Average weekly amounts of iron, copper and zinc and cobalamin (vitamin B12) was calculated based on the USDA food data base in allergics (red) and non-allergics (white) subjects. **B.** Spearman correlation of dietary iron and **C.** vitamin B12 intake vs. selected blood parameters are depicted. Bars in green represent negative, significant correlations, bars in red, positive, significant correlations. Data were tested for normality. Dietary zinc was compared with Student's t -test, others were non-parametric and Mann Whitney U tests were used. *P < 0.05.

Suppl Fig 2 Similar trace elements in the gut of allergics and non-allergics

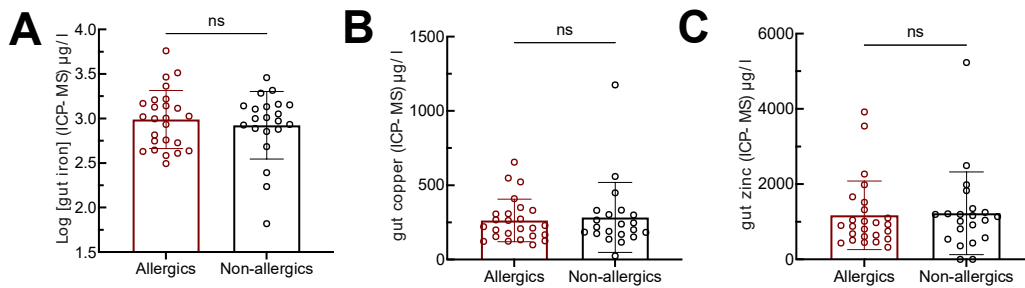


Fig S2. Similar trace-element composition in the gut of allergic and non-allergic women.

The trace elements **A.** iron, **B.** copper, and **C.** zinc were analyzed in aqueous stool extracts from allergic (n=27) and non-allergic (n=24) women *via* inductively coupled plasma mass spectrometry (ICP-MS). Iron-values were log-transformed for parametric distribution and compared with Student's t test, others were non-parametric distributed and compared using Mann-Whitney U-test. A p-Value < 0.05 was considered significant.

Table S5 Unadjusted analysis of factors associated with total nasal symptom scores in allergic women

TNSS correlates with	Kendall's τ -b	p-value	N	Spearman's ρ	p-value	N
Scores and reactivity parameters						
total nasal symptom score	1.000	.	38	1.000	.	38
visual analogue scale	.535**	.000010	38	.683**	.000002	38
nasal secretion mg	.436**	.000275	38	.557**	.000283	38
Nasal fluid parameters						
nasal ceruloplasmin	.434**	.000289	38	.592**	.000089	38
nasal IgE	.183	.126	38	.248	.133	38
nasal NGAL/LCN2	-.038	.750	38	-.042	.801	38
Blood parameters						
hemoglobin	.008	.949	38	.031	.855	38
neutrophils	-.219	.073	37	-.295	.077	37
monocytes	-.056	.672	37	-.065	.702	37
lymphocytes	.003	.979	37	.017	.922	37
eosinophils	.076	.569	37	.087	.607	37
basophils	.181	.216	37	.206	.221	37
lactate dehydrogenase	-.208	.087	37	-.285	.088	37
reticulocytes	-.223	.062	38	-.313	.056	38
serum iron	.139	.245	38	.185	.266	38
serum iron (ICP-MS)	.093	.578	21	.116	.617	21
transferrin	.200	.094	38	.294	.073	38
ferritin	-.206	.087	38	-.293	.074	38
soluble transferrin receptor	.008	.949	38	-.010	.954	38
ceruloplasmin	.404**	.00075	38	.525**	.00071	38
hemopexin	.099	.407	38	.128	.443	38
haptoglobulin	.133	.267	38	.184	.268	38
serum hepcidin in ng/ml	-.136	.256	38	-.191	.250	38
Trace elements in aqueous gut content						
gut iron	-.616**	.000304	20	-.784**	.000042	20
gut cobalt	-.360*	.030	21	-.477*	.029	21
gut copper	-.175	.293	21	-.275	.228	21

** . Correlation is significant at the 0.01 level (2-tailed).

* . Correlation is significant at the 0.05 level (2-tailed).

Table S6 Unadjusted analysis of factors associated with visual analogue scale scores in allergic women

VAS correlates with	Kendall's τ -b	p-value	N	Spearman's ρ	p-value	N
Scores and reactivity parameters						
total nasal symptom score	.535**	.000010	38	.683**	.000002	38
visual analogue scale	1.000	.	38	1.000	.	38
nasal secretions	.388**	.000730	38	.542**	.000445	38
Nasal fluid parameters						
nasal ceruloplasmin	.288*	.012	38	.424**	.008	38
nasal IgE	.279*	.015	38	.384*	.017	38
nasal NGAL/LCN2	.110	.338	38	.156	.349	38
Blood parameters						
hemoglobin	-.157	.177	38	-.190	.252	38
neutrophils	.040	.733	37	.038	.825	37
monocytes	.111	.380	37	.153	.367	37
lymphocytes	.188	.114	37	.271	.105	37
eosinophils	.054	.673	37	.060	.722	37
basophils	.074	.598	37	.088	.605	37
lactate dehydrogenase	-.127	.276	37	-.159	.346	37
reticulocytes	-.207	.072	38	-.294	.073	38
serum iron	.131	.256	38	.180	.280	38
serum iron (ICP-MS)	.125	.432	21	.189	.411	21
transferrin	.246*	.032	38	.379*	.019	38
ferritin	-.185	.109	38	-.248	.133	38
soluble transferrin receptor	.151	.190	38	.240	.146	38
ceruloplasmin	.453**	.0000797	38	.615**	.0000404	38
hemopexin	.145	.208	38	.208	.210	38
haptoglobin	.236*	.040	38	.345*	.034	38
serum hepcidin in ng/ml	-.072	.529	38	-.105	.530	38
Trace elements in aqueous gut content						
gut iron	-.457**	.00517	20	-.656**	.00169	20
gut cobalt	-.375*	.018	21	-.550**	.010	21
gut copper	-.183	.250	21	-.296	.193	21

** . Correlation is significant at the 0.01 level (2-tailed).

* . Correlation is significant at the 0.05 level (2-tailed).

Table S7 Unadjusted analysis of factors associated with nasal fluid weight in allergic women

Nasal secretions correlates with	Kendall's τ -b	p-value	N	Spearman's ρ	p-value	N
Scores and reactivity parameters						
total nasal symptom score	.436**	.0002748	38	.557**	.0002832	38
visual analogue scale	.388**	.001	38	.542**	.0004449	38
nasal secretions	1.000	.	38	1.000	.	38
Nasal fluid parameters						
nasal ceruloplasmin	.530**	.0000029	38	.720**	.0000003	38
nasal IgE	.313**	.006	38	.456**	.004	38
nasal NGAL/LCN2	-.041	.715	38	-.051	.759	38
Blood parameters						
hemoglobin	-.071	.537	38	-.117	.485	38
neutrophils	-.018	.875	37	-.043	.802	37
monocytes	.154	.216	37	.205	.224	37
lymphocytes	.072	.537	37	.110	.517	37
eosinophils	.057	.653	37	.083	.625	37
basophils	.130	.345	37	.157	.353	37
lactate dehydrogenase	-.071	.539	37	-.094	.582	37
reticulocytes	-.111	.327	38	-.169	.311	38
serum iron	.073	.521	38	.124	.458	38
serum iron (ICP-MS)	.200	.205	21	.273	.232	21
transferrin	.070	.538	38	.086	.609	38
ferritin	-.033	.772	38	-.023	.891	38
soluble transferrin receptor	.000	1.000	38	-.029	.863	38
ceruloplasmin	.288*	.011	38	.391*	.015	38
hemopexin	.067	.555	38	.095	.571	38
haptoglobin	.123	.280	38	.193	.245	38
serum hepcidin in ng/ml	-.075	.505	38	-.075	.656	38
Trace elements in aqueous gut content						
gut iron	-.242	.136	20	-.332	.152	20
gut cobalt	-.200	.205	21	-.312	.169	21
gut copper	-.238	.131	21	-.318	.160	21

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

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