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11 **Transcriptome changes during peanut oral immunotherapy and omalizumab** 12 **treatment**

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14 To the Editor,

15 Peanut allergy is a common food allergy and the main cause of anaphylaxis among children¹. In
16 recent years, oral immunotherapy has emerged as a promising treatment for children with different
17 IgE-mediated food allergies, although safety issues must be considered². The main aim of
18 immunotherapy is to induce tolerance or desensitization to an allergen which otherwise causes an
19 allergic reaction. For oral immunotherapy this means ingesting the allergen in a controlled manner
20 with gradually increasing dosages. Specifically, peanut oral immunotherapy (pOIT) is able to
21 induce tolerance/desensitization³. While the pathogenesis of food allergy is relatively well-
22 studied⁴, mechanisms of OIT-induced tolerance are not well understood. Omalizumab (anti-IgE)
23 which is used as treatment for severe allergic asthma and other IgE-driven allergies, can also
24 facilitate OIT initiation⁵, however, little is known about the involved mechanisms, including
25 possible changes at the transcriptional level. We therefore investigated transcriptional changes in
26 whole blood using RNA-sequencing profiles during omalizumab treatment and pOIT in
27 participants from the FASTX (Food Allergen Suppression Therapy with Xolair ®) study
28 previously described^{5,6}.

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17 peanut-allergic adolescents (age 12-18 years) were included in this study (Table S1). Participants were sampled at treatment start (Baseline) and were then treated with omalizumab at recommended dosages for allergic asthma for an initial 8-week period and for some participants additional periods (total of 16 or 24 weeks) until basophil activation test (BAT/CD-sens)⁷ analysis showed suppressed reactivity to peanut. All participants then underwent an open peanut challenge (pOIT start), before starting peanut OIT (pOIT) in combination with continued omalizumab treatment. The peanut-dose was gradually increased until reaching a maintenance dose. Guided by a BAT/CD-sens after 8 weeks on the maintenance dose, participants decreased the omalizumab dose by 50% (Maintenance) and continued to decrease the dose if pOIT was tolerated. Eleven participants were able to tolerate pOIT without omalizumab for >8 weeks and then passed an open peanut food challenge (Final); six participants could not discontinue omalizumab, however blood samples were obtained after 2-3 years on omalizumab (Final); six participants dropped out of the study (**Figure S1**). RNA-sequencing was performed on whole blood (n=17) at Baseline, pOIT start, Maintenance and Final time-points using the NovaSeq 6000 platform. DESeq2 was used for differential expression analysis of the omalizumab effect and a linear mixed-effect model for analyses during pOIT in combination with omalizumab (pOIT+O) after adjustment for treatment outcome and cell types estimates via deconvolution (**Figure S2**). For further details on the treatment protocol, methods and statistical analysis, see the online Appendix. For study participant characteristics at Baseline, see **Table S1**. To elucidate if omalizumab treatment alone induced alterations in peripheral blood gene expression, we investigated the two first timepoints, Baseline and pOIT start, however no significant differences were observed (**Figure S3**). In the longitudinal analysis (pOIT start to Final), 680 genes associated with pOIT+O at nominal $p < 0.005$ (**Table S2**). The Gene Ontology (GO) biological process of these 680 genes are presented in **Figure 1A,B**. Upregulation of 337 genes were linked to GO terms “protein regulation and modification”, while “neutrophil degranulation, immune response, phagocytosis, and metabolic process” were among the top terms for the 343 downregulated genes. Out of the 680 genes, 16 were differentially expressed at false discovery rate (FDR) adjusted $p < 0.05$ (**Table 1, Figure S4**). The three genes with the largest negative and positive coefficients, respectively, are displayed in **Figure 1C,D**; downregulation of *ASGR2*, *GPBAR1* and *HMI3*, and upregulation of *USP44*, *ICOS* and *CDKN2AIP*. Finally, we evaluated the overlap between our 680 pOIT+O-associated genes and, peripheral blood gene expression changes associated with acute peanut allergic reactions in a recently published clinical study by Watson *et al* using the same p-value cut-

off ($p < 0.005$)⁸. Out of our 680 significant genes, 108 genes overlapped with differentially expressed genes found by Watson *et al*⁸, mostly with opposite direction, $P_{\text{enrichment}} = 0.0095$ (**Figure 2**).

Our results demonstrate that omalizumab treatment alone does not induce alterations in whole blood gene expression in patients with severe food allergy. This is not surprising given that these patients were unexposed to peanut allergen at the time of Baseline blood sampling, and any concomitant asthma, rhinitis or eczema were well controlled. However, since omalizumab can facilitate OIT, this drug likely allows for biological changes related to allergen tolerance during OIT. Our longitudinal analysis during pOIT+O identified up- and downregulation of several immune-related genes. CD278/ICOS (Inducible T-cell costimulatory) is expressed on activated T-cells and appears to play a role in directing effector T-cell differentiation and responses during inflammatory conditions⁹. ICOS-expression on T regulatory cells and T follicular helper cells may be involved in the allergic disease mechanism¹⁰. In the pathway analyses, we observed significant enrichment for several GO biological process terms related to T-cell function and immune responses. Notably, we have previously described alterations in T-cell polyclonal *in vitro* activation during pOIT +O in the FASTX study¹¹, and our cell fraction analyses also identified immunological changes (**Figure S2**). Comparing our findings with data described by Watson *et al*⁸, suggests that pOIT+O may alter the expression level of genes that were found affected during an acute peanut allergic reaction.

The main limitations of this study are small sample size and lack of control subjects without omalizumab treatment, which may mask changes only associated with pOIT. Further, whole blood contains a highly heterogeneous cell population which can only be partly accounted for by estimated cell fractions and our analysis may have failed to detect important treatment-related changes connected specifically to peanut antigen-specific cells. In conclusion, omalizumab treatment alone does not alter the transcriptional signature in peripheral blood of peanut allergic patients, but during pOIT+O, several immune-related signatures were observed. These results may provide insights into mechanisms of allergen tolerance.

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94 **CONFLICT OF INTEREST**

95 None.

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97 **ETHICAL APPROVAL**

98 Approved by the ethics committee in Stockholm: 2013/827-31/3, 2014/1980-32, 2016/1390-32,
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101 informed consent.

102

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FIGURE LEGENDS

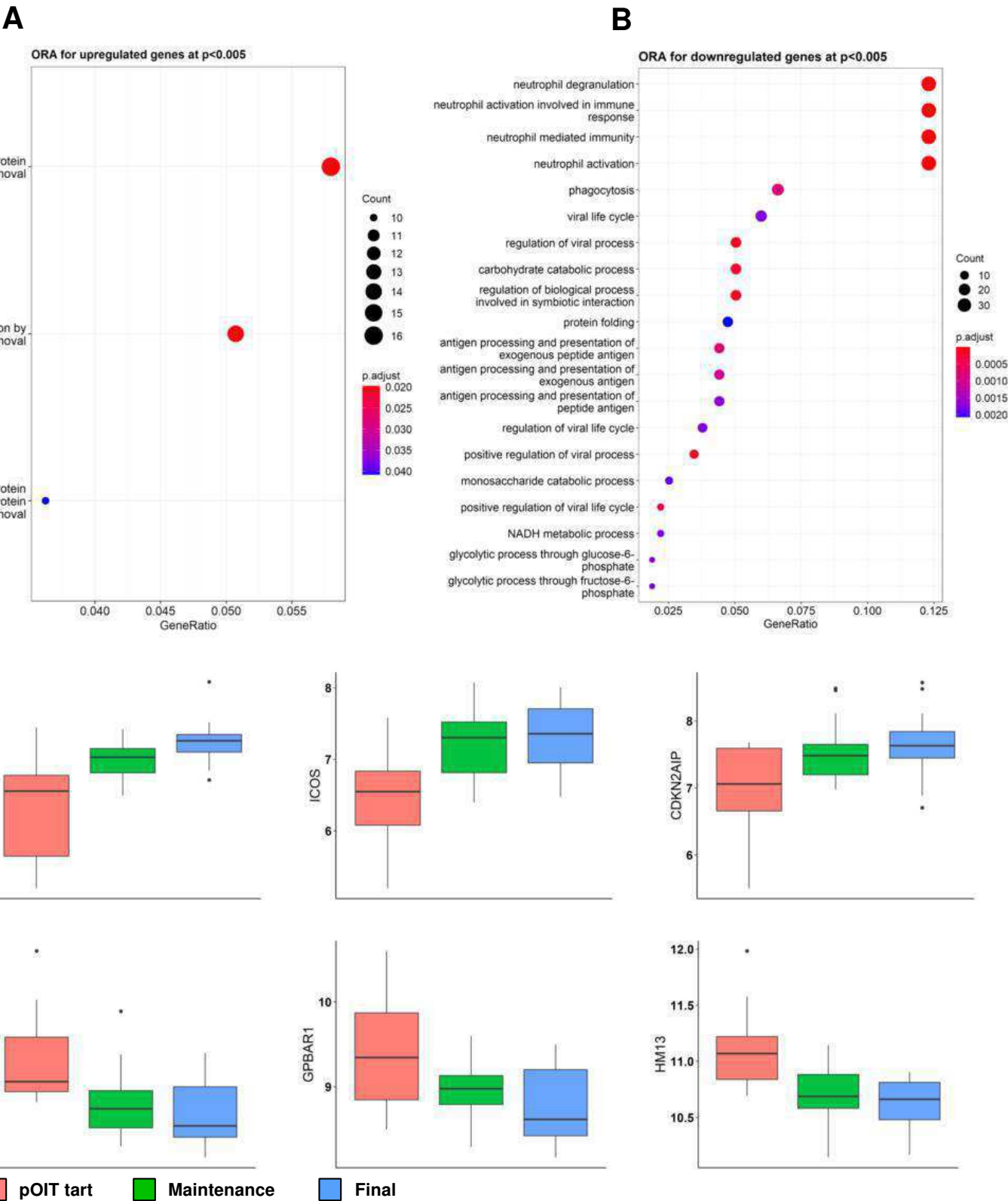
Figure 1. (A, B) Gene ontology (GO) biological process analysis of upregulated (A) and downregulated (B) genes. The x-axis shows the gene ratio of the overlapping genes of our gene list with the pathway gene set. The colour bar represents the adjusted p-value and the circle size is the count of overlapping genes. **(C, D)** Boxplots display log2 expression values for six pOIT genes (C: upregulated, D: downregulated) throughout the treatment protocol at FDR $p < 0.05$. Red box: pOIT start, green box: Maintenance, blue box: Final. ORA = Over-representation analysis.

Figure 2. Overlap of 108 genes from the FASTX pOIT study with the peanut-related genes found in *Watson et al.* at nominal $p < 0.005$. The y-axis represents changes in gene expression between the means at baseline and the four-hour time point of the peanut challenge in *Watson et al.*, and the x-axis is effect size estimates from the mixed-effect model in the FASTX pOIT study. The green colour shows opposite direction, and the pink same direction.

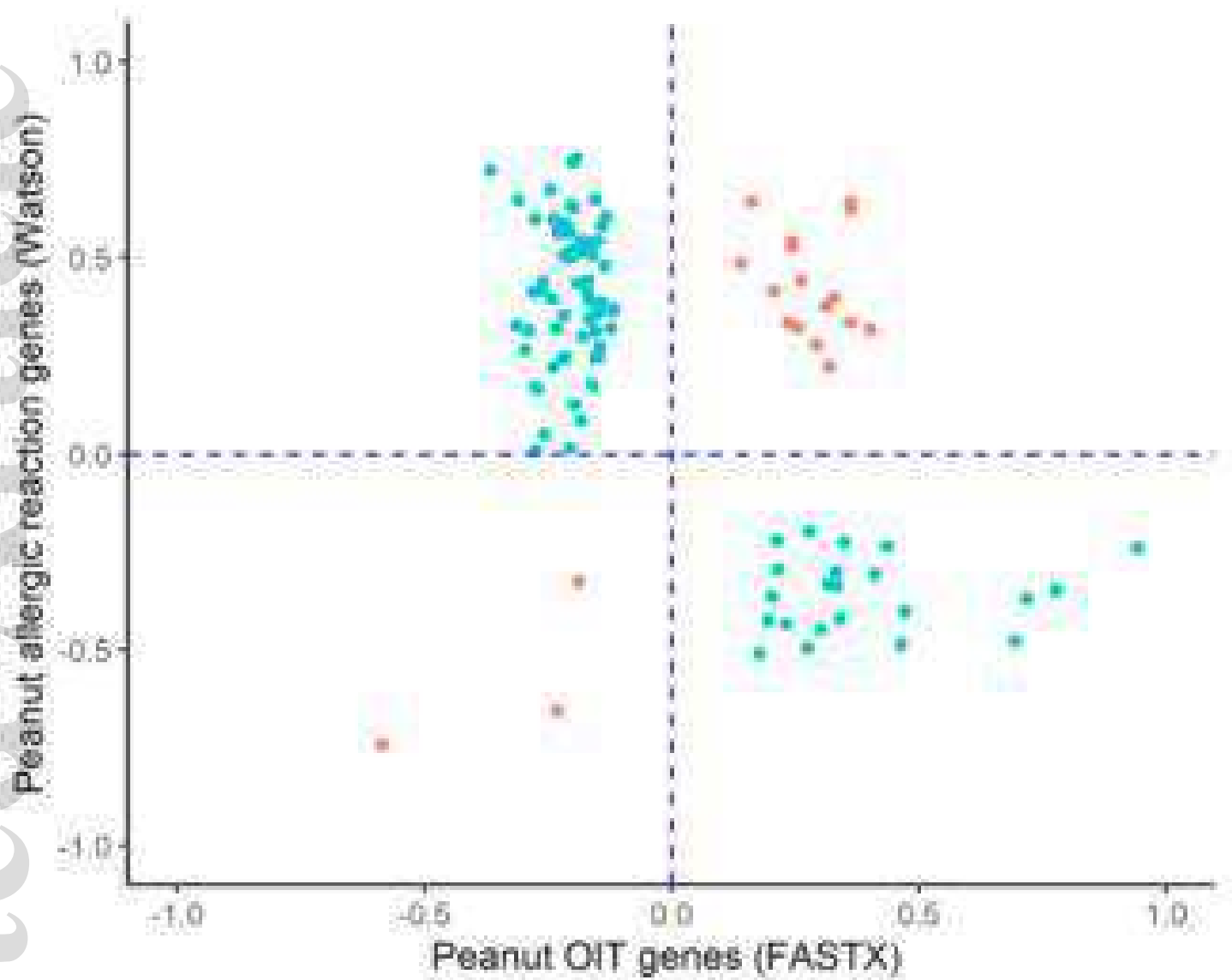
TABLE 1

Gene name	Full name	Ensembl.ID	Coefficient	Standard error	p-value	FDR
<i>ASGR2</i>	Asialoglycoprotein Receptor 2	ENSG00000161944	-0.3908	0.0809	4.68×10^{-05}	0.0496
<i>GPBAR1</i>	G Protein-Coupled Bile Acid Receptor 1	ENSG00000179921	-0.3264	0.0656	3.21×10^{-05}	0.0453
<i>HM13</i>	Minor Histocompatibility antigen H13	ENSG00000101294	-0.2385	0.0461	1.81×10^{-05}	0.0414
<i>CALR</i>	Calreticulin	ENSG00000179218	-0.2120	0.0370	3.67×10^{-06}	0.0386
<i>RASSF4</i>	Ras Associated Domain Family Member 4	ENSG00000107551	-0.1865	0.0370	2.87×10^{-05}	0.0453
<i>CFL1</i>	Cofilin 1	ENSG00000172757	-0.1859	0.0331	5.85×10^{-06}	0.0386
<i>KDELRL1</i>	KDEL Endoplasmic Reticulum Retention Protein Receptor 1	ENSG00000105438	-0.1680	0.0300	6.83×10^{-06}	0.0386
<i>YWHAE</i>	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase	ENSG00000108953	-0.1576	0.0296	1.19×10^{-05}	0.0414
Activation Protein Epsilon						
<i>USP44</i>	Ubiquitin Specific Peptidase 44	ENSG00000136014	1.0998	0.2224	3.48×10^{-05}	0.0454
<i>ICOS</i>	Inducible T Cell Costimulator	ENSG00000163600	0.4596	0.0957	4.69×10^{-05}	0.0496
<i>CDKN2AIP</i>	Cyclin-dependent Kinase Inhibitor 2A Interacting Protein	ENSG00000168564	0.3603	0.0705	2.27×10^{-05}	0.0428
<i>ATM</i>	ATM Serine/Threonine Kinase	ENSG00000149311	0.2913	0.0563	1.95×10^{-05}	0.0414
<i>ADD3</i>	Adducin 3	ENSG00000148700	0.2619	0.0524	3.18×10^{-05}	0.0453
<i>GNG2</i>	G Protein Subunit Gamma 2	ENSG00000186469	0.2436	0.0497	4.01×10^{-05}	0.0486
<i>SEPTIN11</i>	Septin 11	ENSG00000138758	0.1897	0.0358	1.47×10^{-05}	0.0414
<i>OGT</i>	O-Linked N-Acetylglucosamine (GlcNAc) Transferase	ENSG00000147162	0.1859	0.0354	1.56×10^{-05}	0.0414

Figure 1

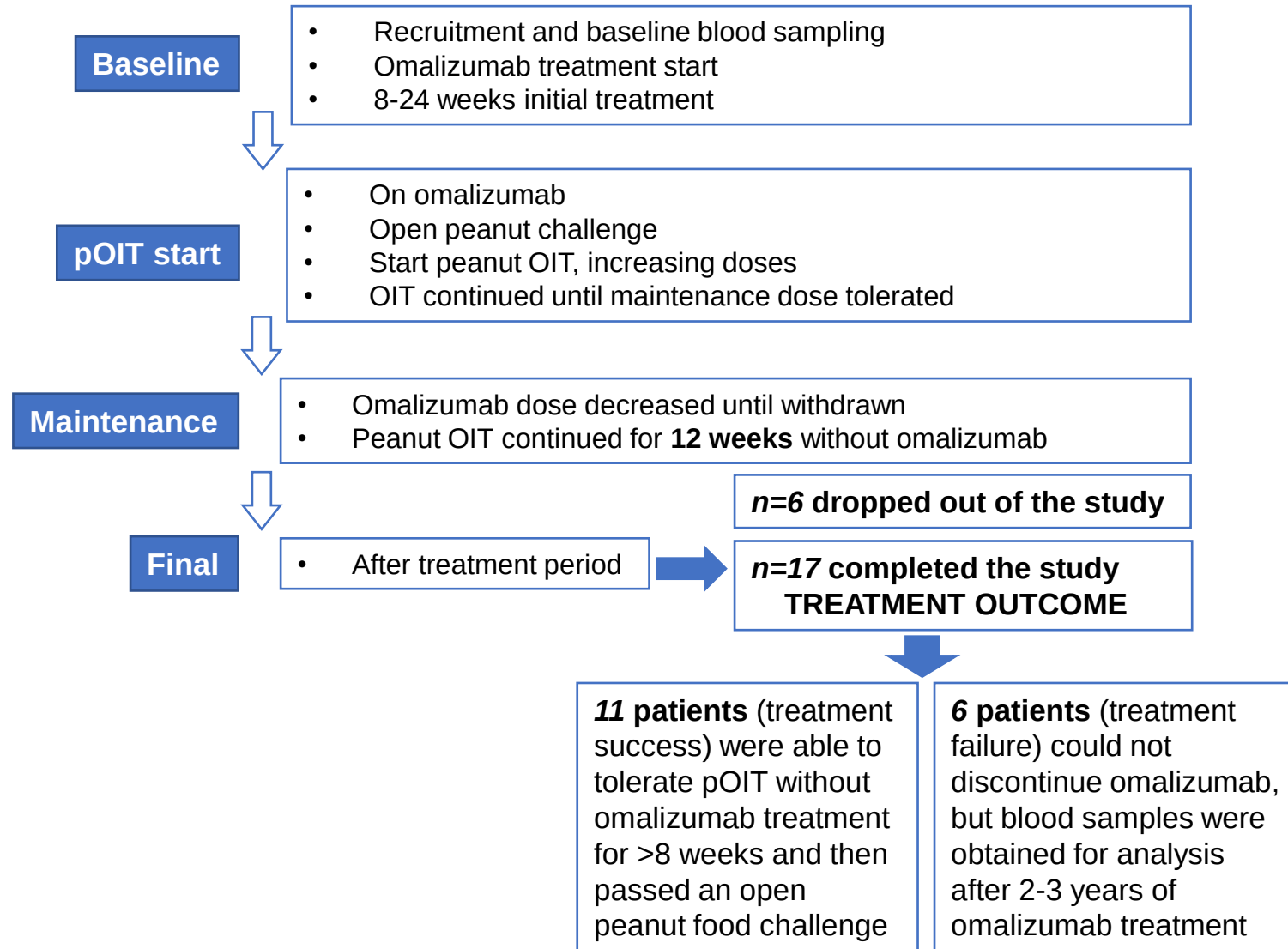


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pai_13682_f2.eps

Treatment Protocol ($n=23$)



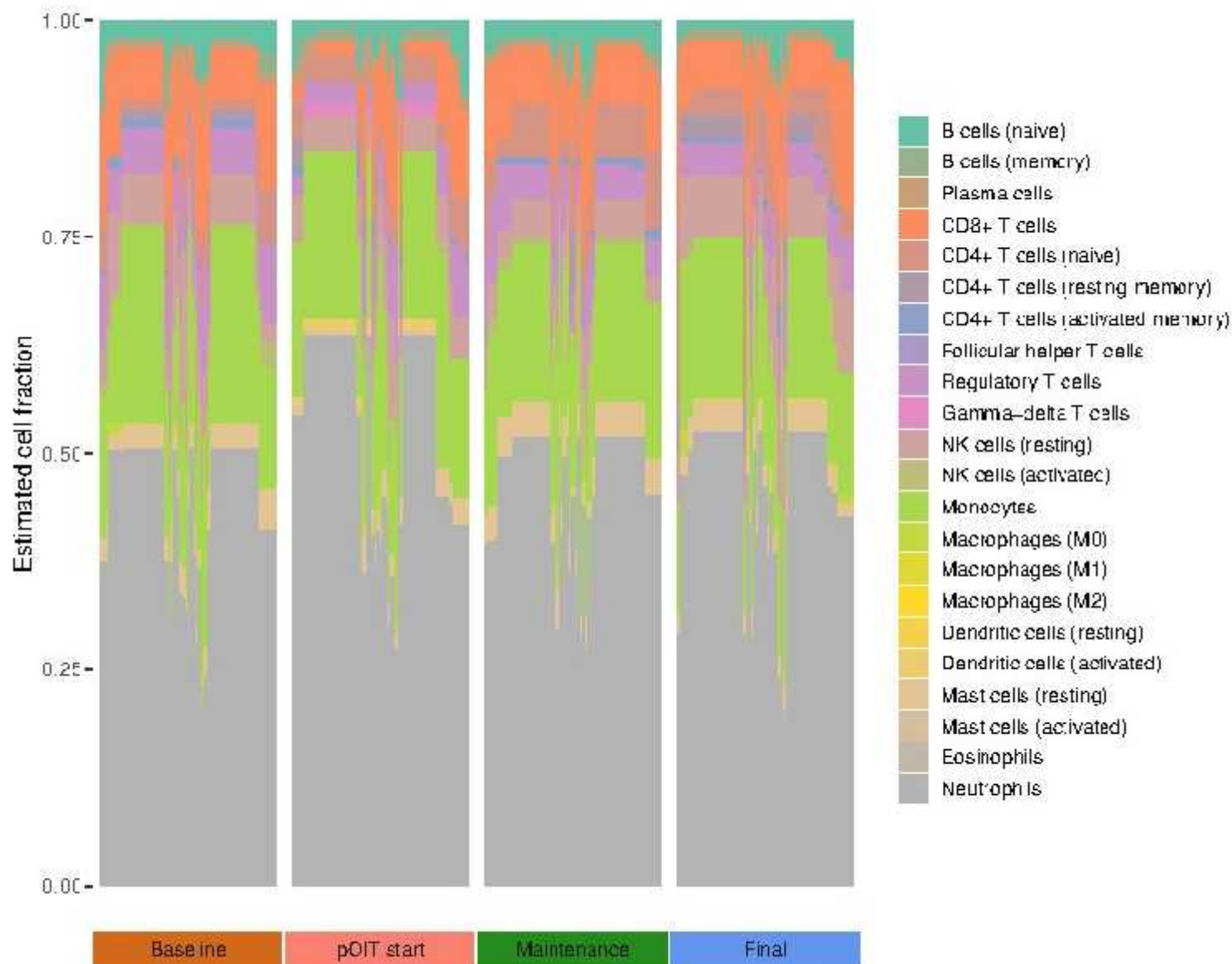


Figure S3

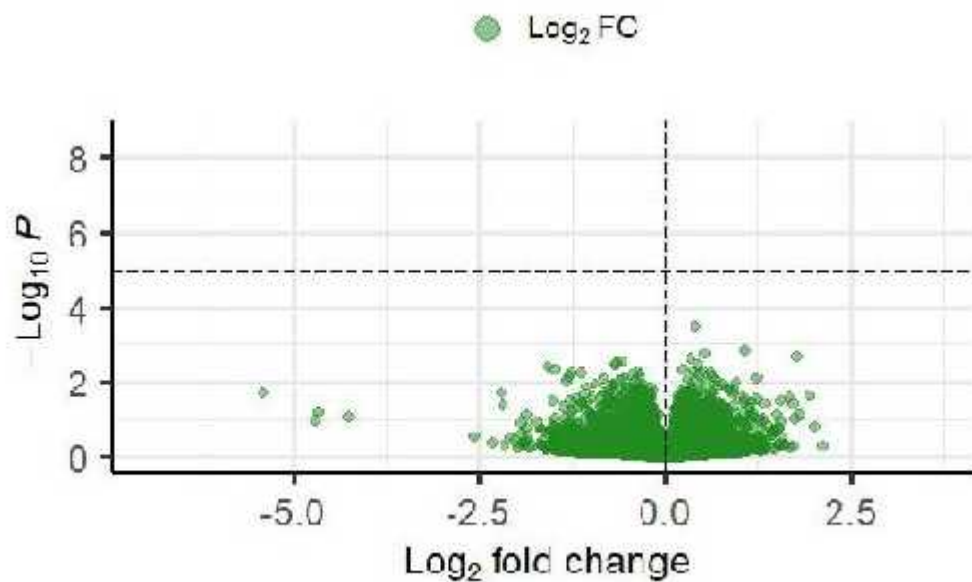
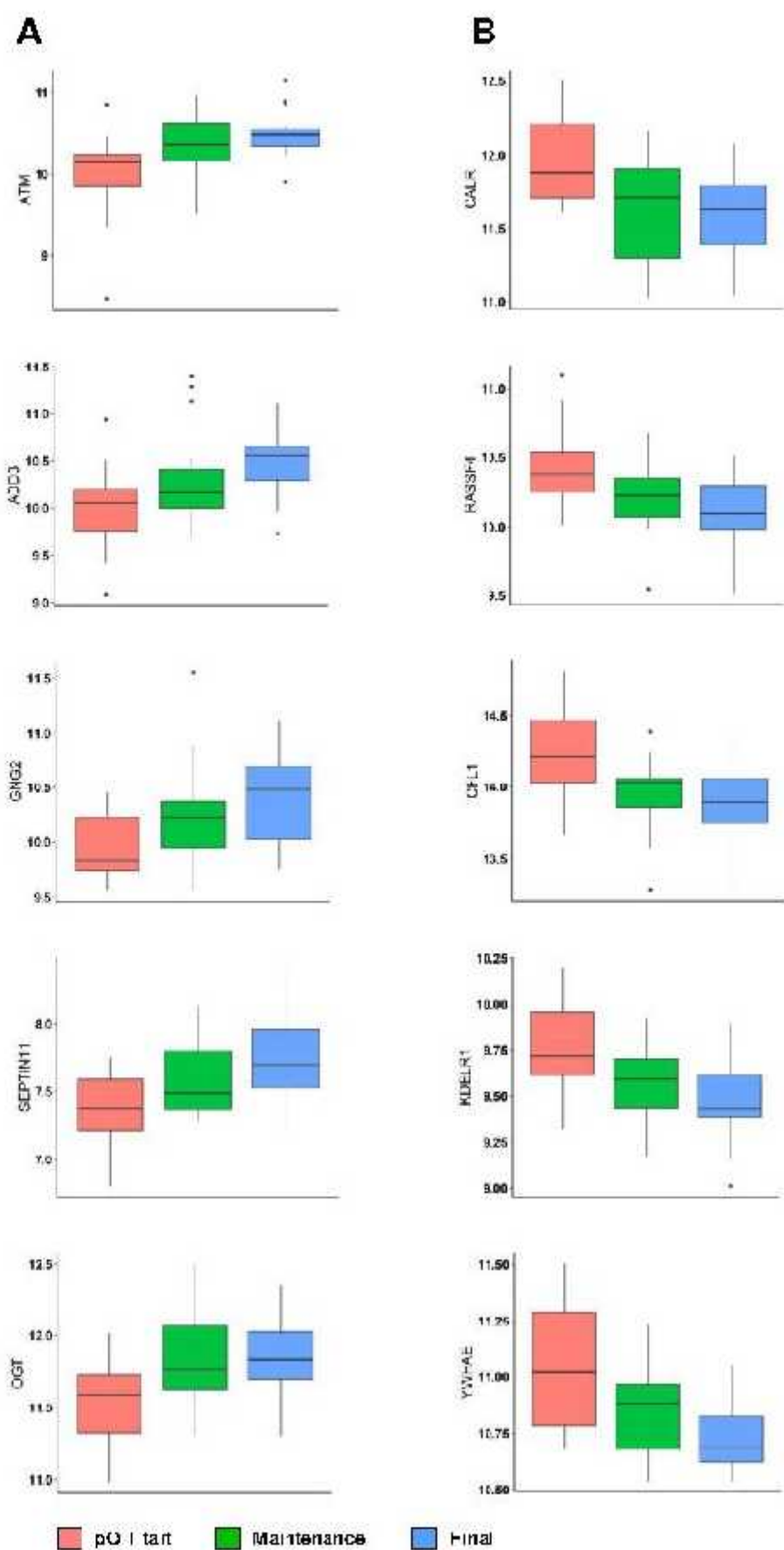


Figure S4



Transcriptome changes during peanut oral immunotherapy and omalizumab treatment

APPENDIX

Extended Methods

Study participants and Omalizumab and peanut oral immunotherapy treatment protocols

The treatment protocol for the FASTX (Food Allergen Suppression Therapy with Xolair®)-study and information regarding included participants and clinical outcomes have previously been described in detail¹⁻³.

In brief, peanut-allergic adolescents (n=23 of whom 17 completed the study, age 12-18 years) with documented anaphylactic reactions to peanut and that were positive for peanut specific basophil allergen threshold sensitivity tests (CD-sens)⁴ were included and sampled at the start of the treatment protocol (baseline sample).

All participants (n=17) were treated with omalizumab at individual recommended dosages (dosages for allergic asthma) for an initial 8-week period and for some participants additional periods (10 participants for 16 weeks, 1 participant for 24 weeks) until the BAT/CD-sens analysis showed suppressed reactivity to peanut^{1,2}.

After reaching suppressed reactivity, all participants underwent an open peanut challenge (pOIT start), before starting peanut OIT (pOIT) at 280 mg peanut protein in combination with omalizumab treatment. The peanut-dose was gradually increased every 2 weeks, if tolerated, until reaching a maintenance dose of 2800 mg. After 8 weeks on the maintenance dose, participants decreased the omalizumab dose by 50% ("Maintenance" sample) and thereafter continued to reduce the omalizumab by 50 % every 8th week if pOIT was tolerated and BAT/CD-sens suppressed. Eleven participants (treatment success) were able to tolerate pOIT without omalizumab protection for >8 weeks and then pass an open peanut food challenge (Final). Six participants (treatment failure) could not discontinue omalizumab, but blood samples were obtained for analysis after 2-3 years of omalizumab treatment (final). Six participants dropped out of the study (*Figure S1*).

RNA-sequencing

Blood samples were collected from study participants in PAX gene blood RNA tubes (PreAnalytix/Qiagen) and stored at -80°C until all samples had been collected to be processed as one batch. RNA was extracted from blood samples using the PAXgene Blood miRNA kit (PreAnalytix, QIAGEN, Inc., Germantown, MD, USA). Purified RNA samples were prepared with TruSeq Stranded Total RNA using the Qiagen Fastselect human rRNA and Globin removal. The RNA sequencing was performed on the Illumina NovaSeq-6000 platform, using 150bp paired ends sequencing, which allowed us to sequence both ends of a gene fragment, generating alignable sequence data. Quality control for the RNA seq raw reads were performed with FASTQC, and adapter trimming was done with Trim Galore (cutadapt 2.8). Spliced alignment for the reads to the human reference genome (GRCh38) was done using STAR 2.6.1 and quantified for gene-level counts using featureCount 1.5.1 method⁵.

Statistical analysis

Differential gene expression analysis of the omalizumab effect was performed using DESeq2 version 1.30.1; DESeq2 provides methods for differential expression testing by employing a negative binomial distribution and a shrinkage estimator for the distribution's variance. P-values were generated using a Wald test provided within DESeq2 adjusted with 2 principal components (PCs) of estimated cell count. For the longitudinal analysis during pOIT we applied linear mixed models with repeated measurement taking into account the within-person time effect. The model was adjusted for treatment outcome (treatment success and treatment failure) and 2 PCs of estimated cell count. Cell composition was estimated through the CIBERSORT method⁶ and the 2 PCs were selected using the significant (permutation p-value $< 10^{-3}$) PCs derived from the estimated cell count. After 1000 permutations 2 PCs were retained. Multiple testing was accounted for by applying Benjamini-Hochberg's false discovery rate (FDR) method. Functional enrichment of Gene Ontology (GO) biological processes were performed using R package enrichplot⁷. We assessed enrichment of our identified genes associated with pOIT with genes associated with peanut allergic reactions in Watson *et al*⁸ at the same p-value cut-off ($p < 0.005$) by using the two-sided doubling mid p-value of the hypergeometric test⁹.

Supplemental Tables

Table S1. Study participant characteristics

Participants	n=17*
Age in years, median (range)	16 (12-18)
Female, n (%)	12 (70.6)
Rhinitis, n (%)	14 (82.4)
Asthma, n (%)	15 (88.2)
Conjunctivitis, n (%)	16 (94.1)
Eczema, n (%)	4 (23.5)

* In the original study by Brandström *et al*^{1,2}, 23 subjects were included. Here, we present data on the 17 subjects who completed the pOIT study protocol. Thus, 6 subjects with incomplete data were excluded from RNA sequencing analyses.

Table S2. The complete list of the 680 significant pOIT+O-related genes identified by linear mixed-effects models at $p < 0.005$. Please see separate supplemental excel-file.

Supplemental figure legends

Figure S1. Flow chart of included subjects and the treatment protocol.

Figure S2. Fractions of cell type estimated from transcriptome-wide RNA-sequencing gene expression signatures from FASTX study, partitioned by treatment protocols.

Figure S3. Volcano-plot of pairwise comparison of Baseline and after omalizumab treatment (pOIT start).

Figure S4. (A, B) Boxplots displaying log₂ expression values for 10 pOIT genes (*A*: up-regulated, *B*: down-regulated) throughout the pOIT treatment protocol at FDR $p < 0.05$. Red box: pOIT start, green box: Maintenance, blue box: Final.

Additional funding

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