Serum omega 6 fatty acids and immunology-related gene expression in peripheral blood mononuclear cells: a cross-sectional analysis in healthy children

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Running title: Omega 6 fatty acids and gene expression

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Abbreviations: AA, C20:4n6 Arachidonic acid; C5, Complement component 5; DE, differentially expressed; GO, gene ontology; GSEA, gene set enrichment analysis; IDO1, Indoleamine 2,3-dioxygenase 1; IFNG/IFNγ, Interferon gamma; LA, C18:2n6 Linoleic acid; NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells; NLRP3, NLR family pyrin domain containing 3; PMBC, peripheral blood mononuclear cells, PTGS2, Prostaglandin-

endoperoxide synthase 2 (Cyclooxygenase 2); TF, transcription factor; TNF, tumor necrosis factor

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ABSTRACT

Scope: Some studies suggest that a high dietary intake of omega 6 fatty acids is pro-inflammatory. However, whether omega 6 fatty acids actually cause pathogenic inflammation in humans is debated. Therefore, we investigated the associations between expression of immunology-related genes in peripheral blood mononuclear cells (PBMCs) and serum total omega 6 PUFA status.

Methods and results: We measured serum fatty acid profile and expression of 460 immunology-related genes in PBMCs from 58 healthy children (6-13 years), and examined the expression differences between children with high or low total omega 6 PUFA status (upper versus lower tertile). Taken together, both univariate analyses and integrated *omics* analyses support that while high omega 6 PUFA level associated with higher expressing of genes related to innate immune responses, it also associated with lower expression of several genes related to adaptive immune responses.

Conclusion: Omega 6 PUFA status associated both positively and negatively with expression of specific immunology-related genes in PBMCs in healthy children. Our results may suggest a nuanced role for omega 6 fatty acids in the interphase of lipids and inflammation, which warrants further examination in gene-environment studies and randomized controlled trials.

INTRODUCTION

Cardiovascular disease (CVD) is a main cause of death and disability worldwide.[1] It is usually caused by atherosclerosis, which is a life-long progressive disease of the arteries mainly driven by close and bi-directional interaction between lipids and the immune system.[2] Accordingly, expanded knowledge on the interrelationship between lipids and inflammation in the early phases of atherosclerosis may have large potential in future prevention of CVD.

Diet affects the classical risk factors for atherosclerosis.[3] As a key strategy to reduce low-density lipoprotein cholesterol (LDL-C) and prevent atherosclerotic CVD, health authorities worldwide recommend reducing the intake of saturated fatty acids (SFAs) and to increase the intake of polyunsaturated fatty acids (PUFAs).[3, 4] Of the latter, omega 6 PUFAs is a major component, in particular the essential linoleic acid (C18:2-n6, LA). However, long-chained PUFAs like omega 6 PUFAs may be more prone to peroxidation that subsequently could contribute to DNA instability, as well as protein and membrane damage. Additionally, the omega 6 fatty acid arachidonic acid (C20:4-n6, AA) is a precursor for eicosanoids. These are molecules with significant inflammatory potential, and include the prostaglandins and leukotrienes, as well as specialized pro-resolving mediators, such as the lipoxins.[5, 6] In other words, the recommended intake of omega 6 PUFAs is a balance between beneficial and harmful effects on processes involved in atherosclerosis.

Still, whether dietary intake of omega 6 PUFAs actually causes *pathogenic* inflammation in humans is debated. First, precisely measuring dietary intake of omega 6 PUFAs is inherently difficult, even with validated questionnaires. Secondly, inflammation is not a simple entity such as C-reactive protein (CRP) or the key pyrogenic interleukins (that is, IL1b, IL6 and

tumor necrosis factor, TNF), but represents a complex set of molecules and interactions that usually includes both pro- and anti-inflammatory signals.[5]

However, serum total omega 6 PUFA level may be used as a biomarker of dietary total omega 6 PUFA intake, since LA, the main contributor of serum total omega 6 PUFA variability, cannot be synthesized in humans, and the conversion of LA into long-chain PUFAs is limited.[7-10] Moreover, the systemic environment may prime leukocytes in circulation; therefore, data obtained from isolated peripheral blood mononuclear cells (PBMCs) could well reflect the *in vivo* situation, and immunology-related mRNA expression in could provide a more comprehensive characterization of inflammation, and at a higher resolution, compared to standard measurements of circulating markers.

On this background, the aim of the present study was to investigate if immunology-related gene expression in PBMCs associated with serum total omega 6 PUFA status in healthy children.

EXPERIMENTAL SECTION

In the present study in healthy children, we measured immunology-related gene expression in PBMCs using Nanostring technology. We adjusted for key covariates, and applied various statistical and bioinformatics analyses to associate these measurements with serum omega 6 PUFA status.

Subjects

We used clinical and biological measurements from 58 children that participated in the Stork children study, thoroughly described previously.[11] Briefly, in 2015, when the children were 6-13 years, we examined them with respect to CVD risk factors, including body composition using dual-energy X-ray absorptiometry (DEXA). We also collected blood samples and isolated PBMCs for mRNA extraction and analyses.

Fatty acid analyses

Total serum fatty acid profile was analyzed at Vitas Ltc (Oslo, Norway), and expressed as percentage of total fatty acids, as previously described.[7] Total omega 6 PUFA level (in percentage) was calculated with the following formula: Total serum omega 6 PUFA level = C18:2n6 Linoleic acid (LA) + C18:3n6 Gamma Linoleic acid + C20:2n6 Eicosadienoic acid + C20:3n6 Dihomo Gamma Linoleic acid + C20:4n6 Arachidonic acid (AA). These fatty acids made up approximately 76 %, 1 %, 1 %, 5 % and 18 % of the total omega 6 PUFA level, respectively.

We split the subjects into tertiles (three groups) of total serum omega 6 PUFA level, and focused on the lower (below 33.7 %) and upper tertile (above 35.9 %), hereafter called *low* and *high omega 6 group*, respectively.

Nanostring gene expression and bioinformatics analyses

PBMCs were isolated, and mRNA was extracted as previously described.[12] RNA expression analysis was run on the nCounter® analysis system, running 12 samples at a time (referred to as one *strip*). The procedure was performed according to the manufacturer´s instructions, applying about 100 ng mRNA. We analyzed a fixed codeset of mRNA target, comprising key genes involved in human immunology (nCounter GX Human Immunology Kit v2, Nanostring Technologies). We used Nsolver Analysis Software for automated quality control, normalization and analysis of nCounter Nanostring data (NanoString Technologies). Detection limit was set to 14 mRNA molecules based on the following formula: average (negative control) + 2SD (negative controls). Of totally measured 579 target genes in the kit, 460 were included in the final analyses (79.4 %) (SuppMat 1). Endogenous controls were analyzed in Normfinder software from MOMA (Microsoft Excel add-in) with strip and group affiliation as factors. Based on this analysis, coefficient of variation (CV) and density of frequency, RPL19, POLR2A, ABCF1, HPRT1 and TBP were selected. Finally, number of mRNA molecules was normalized against negative, positive and endogenous controls, and the resulting data was imported into Partek Genomics Suite 6.6 (NanoString Technologies).

Batch correction

To reduce variation from sources other than the group effect, we batch corrected the gene expression data. To do this, we analyzed the contribution from a set of covariates related to biology as well as technical steps in the pipeline: strip, age, gender, date and time of blood sampling, PBMC processing time, and quality and purity of RNA. Although multiple variables slightly affected the variation in the dataset, only age (binned/categorized), gender and strip were included as covariates in the batch correction adjustment.

Univariate and multivariate analyses

Supplemental figure 1 illustrates the outline of the analysis pipeline. We used 4-way ANOVA (group, gender, strip and age [binned]) to test for differential gene expression, that is, compare gene expression between groups. For further biological insight, we analyzed differentially expressed (DE) genes (based on nominal $p < 0.05$, both higher and lower expressed genes) using standard multivariate pathway analyses and gene ontology (GO) analyses in Partek Genomics Suite 6.6. Using the complete set of available genes, we also performed gene set enrichment analyses (GSEA). These multivariate analyses quantify and test enrichment of genes within some aspect of biology, often presented as enrichment score (such as a Z score) and corresponding significance level (generally using a Chi-squared-type test). Gene set enrichment could for example encompass genes related to *localization: cell membrane* or *molecular function: IL6 signaling*. Hence, they may provide a more holistic understanding of complex gene expression results, compared with single gene investigations. To further examine these results, we also did GO analyses in Metacore (portal.genego.com, Clarivate Analytics, London, UK), which is an accessible bioinformatics tool that enables fast analysis of both whole-genome expression datasets as well as smaller subsets of genes. In Metacore we also did transcription regulation analyses, which specifically build lists of networks for every transcription factor (TF) related to the subset of genes of interest. For these analyses, we used DE genes (based on nominal $p < 0.05$, either higher or lower expressed, or both) and five clusters of DE genes (based on FDR < 0.2; see *Additional statistical analyses*).

In order to estimate the relative proportion of different blood cell types in each sample, we used the predictive CIBERSORT algorithm.[13] This algorithm applies feature selection and

subsequent linear support vector regression (SVR) using a whole-genome gene expression data matrix as input, and returns relative proportions of 22 leukocyte subtypes. Since we ran the analysis using less than 50 % of the genes for each cell type panel (only 460 genes; not whole-genome), the sensitivity of this analysis was expected to be lower than usual. However, we filtered out results with a poor goodness of fit (based on deconvolution result across all cell subsets), and focused on the cell subsets that make up most of the PBMC pool: Monocytes and lymphocytes (mainly resting NK cells, CD8+ T cells, B cells, naïve CD4+ T cells and resting CD4+ memory T cells).

Additional statistical analyses

In sensitivity analyses, we performed least squares linear regression adjusting for visceral fat content according to the following formula: $Gene \sim omega\ 6\ groups + visceral\ fat\ content + \varepsilon$. In an attempt to detect meaningful patterns of gene expression and thereby improve translation of our findings, we correlated all DE genes with Spearman correlation. Correlation was in this case used as a measure of co-expression, which may or may not be biologically relevant. In contrast to previous analyses, here we defined DE genes as FDR less than 0.2 (233 genes); this to include a larger number of genes in the subsequent analyses and thereby increase the robustness of the biological interpretations. Next, we calculated the Euclidean distance on the correlation matrix, and subsequently performed hierarchical clustering (complete linkage) on the distance matrix. Visual inspection of the correlation plot, dendogram, and elbow plot of the total within-cluster sum of squares suggested that the data contained approximately 3-6 clusters; hence, the dendogram was cut into 5 clusters of genes. These candidate genes and gene clusters were subjected to similar multivariate analyses as described for the crude DE analysis.

Additional statistical analyses and visualizations were executed in R version 3.5.0 using the RStudio interface.[14] All packages used in the analyses pipeline are listed in the supplemental material (SuppMat 2).

RESULTS

Mean serum omega 6 PUFA level was 37.6 (SD 1.2) and 30.8 (SD 2.1) % in children in the upper (n=19) and lower (n=19) tertile, respectively, primarily reflecting increased proportion of LA and AA (Table 1). As expected, children in the high omega 6 group had significantly different levels of several saturated and monounsaturated fatty acids, as compared with children in the low omega 6 group, but with no differences in omega 3 levels (SuppTable 1). Also as seen in other studies, children in the high omega 6 group had lower total body fat percentage, visceral fat percentage and triglycerides than children in the low omega 6 group (Table 1, SuppTable 1).[15, 16]

Association between serum omega 6 PUFA status and PBMC gene expression

Forty-six genes (10.0 %) were differentially expressed (DE) between the high and low omega 6 groups at nominal α significance level 0.05 (Figure 1A-B, SuppTable 2). The most DE genes, measured by significance level and fold difference, were HLA-DPB1 (MHC class II DP beta 1), TICAM1 (TLR adaptor molecule 1), RELB (RELB proto-oncogene, NF-kB subunit), C5 (Complement component 5) and FCER1A (Fc fragment of IgE, high affinity I), which were higher expressed in the high omega 6 group, and ZAP70 (TCR zeta-chain associated protein kinase 70kDa), IL11RA (IL11 receptor alpha), and PDGFRB (Platelet-derived growth factor receptor, beta polypeptide), which were lower expressed in the high omega 6 group (Figure 1A-B, SuppTable 2). Also, although they did not reach statistical significance, a number of genes displayed relatively large absolute fold differences between the two groups, including the KIR (killer immunoglobulin-like receptors) family of genes (Figure 1A). Also, PTGS2 (Prostaglandin-endoperoxide synthase 2, also known as Cyclooxygenase 2, COX2), IDO1 (Indoleamine 2,3-dioxygenase 1) and IL1R1 (Interleukin 1 receptor type 1) positively

associated with omega 6 PUFA level, and IFNG (Interferon gamma) and CXCR1 (C-X-C motif chemokine receptor 1) negatively associated with omega 6 PUFA level.

Integrated analyses

In order to understand systematic patterns underlying the gene expression differences between the two omega 6 groups, we performed several integrated analyses of many genes simultaneously. We focused particularly on pathway analyses, GO analyses, GSEA and transcription regulation analyses.

Pathway analysis displayed enrichment for a number of immunity- and infection-related pathways, including *NFkB signaling pathway* and *Influenza A* (Figure 2A, SuppTable 3). Furthermore, the top three GO terms involved *TNF* and *TNF superfamily processes*, but other terms included *serotonin*, *myeloid* and *mast cell processes* (Figure 2B, SuppTable 4). More specifically, a number of GO terms were enriched by DE genes ($P < 0.05$) that were expressed higher in the high omega 6 group (SuppFig 2, SuppTable 5B, 6B and 7B). Among these include the biological process terms *immune* and *inflammatory response*, *defense response* and *cytokine production*, the molecular function terms *immunoglobulin*, *chemokine* and *cytokine receptor binding* and *activity*, and cellular localization related to the *cell membrane*. In contrast, other GO terms were enriched by DE genes ($P < 0.05$) that were lower expressed in the high omega 6 group (SuppFig 2, SuppTable 5C, 6C and 7C). Among these include the biological process terms *leukocyte* and *lymphocyte activation*, and *T cell differentiation*, the molecular function terms *protein tyrosine kinase* and *PI3 kinase*, and cellular localization related to both the *cell membrane* and *cytoplasm*.

GSEA analysis showed enrichment of gene sets that include *TNF* and *IFN production*, *response to bacteria*, *regulation of mitochondrial processes*, and *innate immune responses*

for the high omega 6 group, which further emphasized key biological differences between the two groups (Figure 2C, SuppTable 8).

Finally, transcription regulation analysis detected large numbers of important TFs (Figure 2D, SuppTable 9). The top five were p53, c-Myc, STAT1, STAT3, and SP1, which regulate genes involved in immune response, cytokine production and leukocyte activation (Figure 2D, SuppTable 9). These and other TFs were enriched in groups of genes that were either higher or lower expressed in the high omega 6 group (SuppFig 3, SuppTable 9). However, as for GO terms, the relative enrichment of different TFs varied with the expression of DE genes (SuppFig 3). For example, Bcl-6, RelA, YY1, IRF8 and STAT6 were mainly enriched in the group of higher expressed genes, and STAT5, FOXP3, AML1 and EST1/RUNX1 were mainly enriched in the group of lower expressed genes (SuppFig 3).

Sensitivity analyses

Both total body fat mass and serum triglycerides were inversely correlated with omega 6 PUFA level, and both fat mass and monocyte count were associated with multiple sets of genes, as defined by cluster analysis (data not shown). To remove some of this confounding effect on the association between omega 6 PUFA level and gene expression, we adjusted for visceral fat content using linear models. After adjustment, 233 genes were DE between the two groups (FDR < 0.2). Next, in an attempt to detect and understand biologically meaningful sets of genes, we grouped these 233 genes into 5 gene clusters by calculating Spearman's correlation coefficient, followed by hierarchical clustering (SuppFig 4). Supplemental figures 5A-E, visualize the coefficient estimates (± 95 % CI) for all genes as a function of group, adjusting for visceral fat content. Interestingly, the five gene clusters were different with respect to pattern of GO terms and TFs (SuppFig 6 and 7). Visually, clusters 1

and 5 displayed similarity, as did clusters 3 and 4; cluster 2, on the other hand, was more unique (SuppFig 6 and 7). Cluster 2 consisted of 150 genes that were higher expressed in the high omega 6 group. Biological processes such as *leukocyte activation*, and *immune* and *defense response* were enriched in these genes; so were the molecular function terms *pattern recognition receptor (PRR) activity* and *signal transduction*, and the cellular localizations terms *cell membrane* and *intracellular vesicles* (SuppFig 6). This cluster was also associated with TFs that include NFkB and RelA; again emphasizing the importance of innate immunity for the genes that were higher expressed (SuppFig 7). In contrast, cluster 1, for example, consisted of 42 genes that were lower expressed in the high omega 6 group. These genes mapped to biological processes such as *lymphocyte activation* and *leukocyte cell-cell adhesion*, and molecular functions such as *CD40* and *TNF receptor binding*, and *DNA* and *nucleic acid binding*, mainly located to the *cell membrane* (SuppFig 6). Furthermore, multiple TFs of the STAT family, including STAT3, STAT5A, STAT5, and STAT6, as well as p53 and RelA were enriched by genes in cluster 1 (SuppFig 7).

Cell type-specific gene expression

To attribute gene expression differences to specific PBMC cell types, we used the predictive CIBERSORT algorithm. First, cell type did not differ markedly across serum omega 6 PUFA levels (SuppFig 8). Furthermore, although predicted cell type weakly correlated with leukocytes measured by standard differential count (data not shown), they showed clear discrepancies between the 5 gene clusters (SuppFig 9), which suggests that gene expression differences still may be caused by leukocyte distribution differences. In support of this, in a linear model adjusting for visceral fat, monocyte level was higher in the high omega 6 group (Figure 3).

Fatty acid-specific associations

To shed light on differences between LA and AA in mediating the associations between total omega 6 and gene expression, we performed additional sensitivity analyses. Compared with omega 6 status, the group affiliation changed minimally if splitting of the subjects was based on tertiles of LA status, but it changed markedly if splitting based on tertiles of AA status (SuppFig 10A). Out of the 19 in the high omega 6 group, four (21 %) and nine (47 %) were reassigned when splitting the groups based on LA and AA, respectively. And out of 19 in the low omega 6 group, two (11 %) and eight (42 %) were reassigned when splitting the groups based on LA and AA, respectively. This could be explained by the high correlation between total omega 6 level and LA, and lower correlation between total omega 6 level and *other* specific fatty acids (SuppFig 10B and C), which suggests that variation in LA is the major driver of variation in total serum omega 6. This is further supported by linear models adjusted for either AA or LA level on top of the adjustment for visceral fat; only the latter displayed a significant recession of the Volcano plot (SuppFig 10D). Finally, DE genes based on LA groups enriched pathways and GO terms similar to that based on omega 6 groups, whereas DE genes based on AA groups enriched other pathways (data not shown).

DISCUSSION

In the present study of healthy children, we found that total omega 6 PUFA status was associated with expression of a large number of immunology-related genes in PBMCs. The differences in gene expression may reflect functional differences in innate and adaptive immunity, mediated at least partly by alterations in leukocyte distribution, differentiation or activation.

In main analyses, 46 genes were differentially expressed between the groups. Genes that associated with omega 6 PUFA level that are of particular interest include PTGS2, IDO1, IFNG, and C5.

As expected, PTGS2, more commonly known as COX2, was positively associated with omega 6 PUFA level. PTGS2 is an essential enzyme in prostaglandin synthesis, and in contrast to PTGS1, it is also inducible: PTGS2 expression is directly affected by a number of metabolites, including LA, AA, and the omega 3 PUFAs EPA and DHA.[5] This single finding may support the classical hypothesis that a high omega 6 PUFAs intake can “cause inflammation” via increased PTGS2-mediated eicosanoid production; however, depending on the physiological setting, the final molecular effect on inflammation are probably determined by a whole array of input signals, which complicates interpretation.[5, 6] Nevertheless, this gene is related to multiple diseases including autoimmune diseases, cardiovascular disease and diabetes type 2, indicating its importance in fatty acid-related pathophysiology.[17, 18]

IDO1 and IFNG associated positively and negatively with omega 6 PUFA level, respectively. This is a surprising and interesting finding. IDO1 is the first and rate-limiting step in tryptophan catabolism to form N-formyl-kynurenine, which in monocytes and macrophages is an important cellular signal to regulate T cell behavior. IFNG, on the other hand, is a

cytokine produced in cells from both the innate and adaptive arms of the immune system that among other things facilitates protection against intracellular microbes. Of particular interest in this setting: IFNγ activates the enzyme IDO in regulation of the tryptophan-kynurenine pathway, which has pivotal roles in immuno-metabolism.[19] Indeed, this pathway may be involved in CVD.[20, 21] Therefore, our data suggest that there is an interaction between omega 6 PUFA level and the tryptophan-kynurenine pathway that needs further exploration in randomized controlled trials (RCTs).

C5 was positively associated with omega 6 PUFA level. C5 is a key part of the complement system, and has recently been implicated in cholesterol-crystal-mediated inflammation in atherosclerosis.[22, 23] C5 can be cleaved to C5a, which is a potent mediator of the immune response, for example in activation of Th1 responses via the NLRP3 inflammasome.[24]

Integrated analyses displayed an association between higher omega 6 PUFA level and higher expression of sets of genes that map to the innate immune responses, such as TLR, NFkB and NLRP3-related processes, and cytokine production, as well as gene expression-predicted percentage of monocytes. These findings are unexpected, because compared for example with SFA, omega 6 PUFAs reduce both LDL-C and liver fat content, which causally affects atherosclerosis and non-alcoholic fatty liver disease (NAFLD), respectively, both of which are highly inflammation-dependent diseases.[25-28] Therefore, we anticipated a negative association between omega 6 PUFAs and circulating measures of inflammation.[29-32] One may speculate that omega 6 PUFAs induce training of circulating myeloid cells, potentially via epigenetic mechanisms. Such a mechanism is described for several transient exposures, including a western diet, oxidized LDL, and metabolic signals from the mevalonate

pathway.[33-35] Of note, Christ and co-workers found that training of monocytes following a western diet was mediated via epigenetic modification of NLRP3.[34]

High omega 6 PUFA level associated negatively with expression of genes that map to adaptive immune responses, such as lymphocyte activation and T cell differentiation, potentially mediated via cell-cell interactions and protein tyrosine kinase signaling. The negative association of IFNG with omega 6 PUFA may further support such a notion. This finding, too, is somewhat unexpected. PTGS2 converts AA and the omega 3 fatty acids EPA and DHA into eicosanoids.[5] A number of eicosanoids regulate inflammation, and a common hypothesis states that higher activity in the AA-eicosanoid pathway inhibits the anti-inflammatory effects of omega 3 fatty acids, and hence may contribute to certain immune-related diseases such as asthma, allergy and autoimmune diseases.[36, 37] In contrast, our data support that, as opposed to innate immunity, adaptive immunity-mediated responses may be attenuated with higher serum level of omega 6.

Worldwide, dietary guidelines recommend substituting SFA by PUFA to lower serum LDL-C and thereby the risk of CVD.[3, 4] For the most part, increased PUFA intake is achieved by a consuming a combination of omega 6 and omega 3 PUFAs, of which the former is the main contributor. In this respect, in a recent targeted analysis in humans we showed that plasma omega 6 and omega 3 PUFA level, and SFA to PUFA ratio, associated with PBMC expression of genes related to lipid metabolism, particularly cholesterol metabolism.[38]

The strengths of the present study include the use of DEXA to measure body composition, the high-quality fatty acid profile measurement, as well as the large number of specific immunology-related genes examined in PBMCs using state-of-the-art Nanostring technology. Also, the study of children may reduce the impact of confounding variables such as exposure

to alcohol and smoking, and the cumulative effect of lifestyle over many years; these issues may be more critical in adult subjects. The main limitations include the cross-sectional design and the low number of participants, which increases the probability of type 1 and 2 errors. Specifically, this may have concealed the true association between omega 6 PUFA and immunology-related gene expression. Indeed, omega 6 PUFA level and expression of the LDLR gene in PBMCs was inversely correlated in the present study; opposite of our expectation based on a randomized controlled trial in adult humans that we recently conducted (data not shown). Furthermore, in the CIBERSORT analysis we ran less than 50 % of the genes for each cell type panel, which lowers the sensitivity and specificity of the analysis. Nevertheless, this analysis may potentially be more sensitive in detecting cell distribution differences than standard blood cell types (differential count). Finally, although we have adjusted for several covariates, we cannot exclude that the associations between omega 6 PUFA status and gene expression is affected by residual confounding, for example by specific fatty acids and lipid species, including oxidized compounds.

In conclusion, in healthy children, omega 6 PUFA status in serum was associated with several genes in PBMCs, and integrated analyses revealed that multiple arms of the immune system were involved. Interesting, while high omega 6 PUFA levels associated with increased expressing of genes related to innate immune responses, it also associated with decreased expression of several genes related to adaptive immune responses, suggesting the omega 6 PUFAs may differently affects these pathways. Our results point to specific genes and pathways that may interact with omega 6 PUFA level in homeostatic control of inflammatory processes – candidates that warrant further examination in other epidemiological studies, and well-conducted gene-environment studies and RCTs in humans.

AUTHOR CONTRIBUTION

Conceived and designed research: JJC, SMU, KBH; Conducted research: JJC, SSB, TH, JB; Performed statistical analyses: JJC, SSB, SMU, KBH; Interpreted results: JJC, SSB, SMU, KR, TH, JB, TE, PA, BH, KBH; Wrote paper: JJC, SSB, SMU, KR, TE, PA, BH, KBH; Responsibility for final content: JJC, KBH. All authors read, critically revised and approved the final manuscript.

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CONFLICTS OF INTEREST

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Table 1. Subject characteristics

	Omega 6 status in lower tertile	Omega 6 status in upper tertile	
	n = 19	n = 18-19	P[1]
Female gender, n (%)	8 (42)	10 (53)	0.75[2]
Age, y	9.9 (2.0)	9.8 (2.0)	0.92
Clinical parameters			
Weight, kg	40 (17)	35 (10)	0.29
Height, m	1.43 (0.14)	1.42 (0.14)	0.90
BMI, kg/m2	18.8 (4.1)	16.9 (2.2)	*0.070*
Visceral fat, %	23.8 (12.9)	16.7 (7.1)	***<0.05***
SBP, mmHG	109 (8)	105 (6)	0.16
Biochemistry			
Total cholesterol, mmol/L	4.0 (0.7)	4.1 (0.6)	0.49
LDL-C, mmol/L	1.9 (0.6)	2.2 (0.6)	0.20
HDL-C, mmol/L	1.5 (0.3)	1.7 (0.3)	*0.074*
Triglycerides, mmol/L	1.3 (0.5)	0.6 (0.2)	***<0.001***
Glucose, mmol/L	5.3 (0.7)	5.3 (0.4)	0.88
CRP, mg/L	0.8 (0.4)	0.7 (0.3)	0.46
Omega 6 polyunsaturated fatty acids, %			
Plasma total n-6 level	30.8 (2.1)	37.6 (1.2)	***<0.001***
C18:2n6 Linoleic acid	23.1 (1.8)	28.6 (1.9)	***<0.001***
C18:3n6 Gamma Linoleic acid	0.46 (0.22)	0.34 (0.20)	*0.093*
C20:2n6 Eicosadienoic acid	0.24 (0.04)	0.24 (0.04)	0.56
C20:3n6 Dihomo Gamma Linoleic acid	1.6 (0.4)	1.5 (0.3)	0.70
C20:4n6 Arachidonic acid	5.4 (0.9)	6.9 (1.1)	***<0.001***

Distributions for all continuous variables are described by mean and SD. Gender distribution is described by n and %.

[1]Two sample T test

[2]Pearson's Chi-squared test

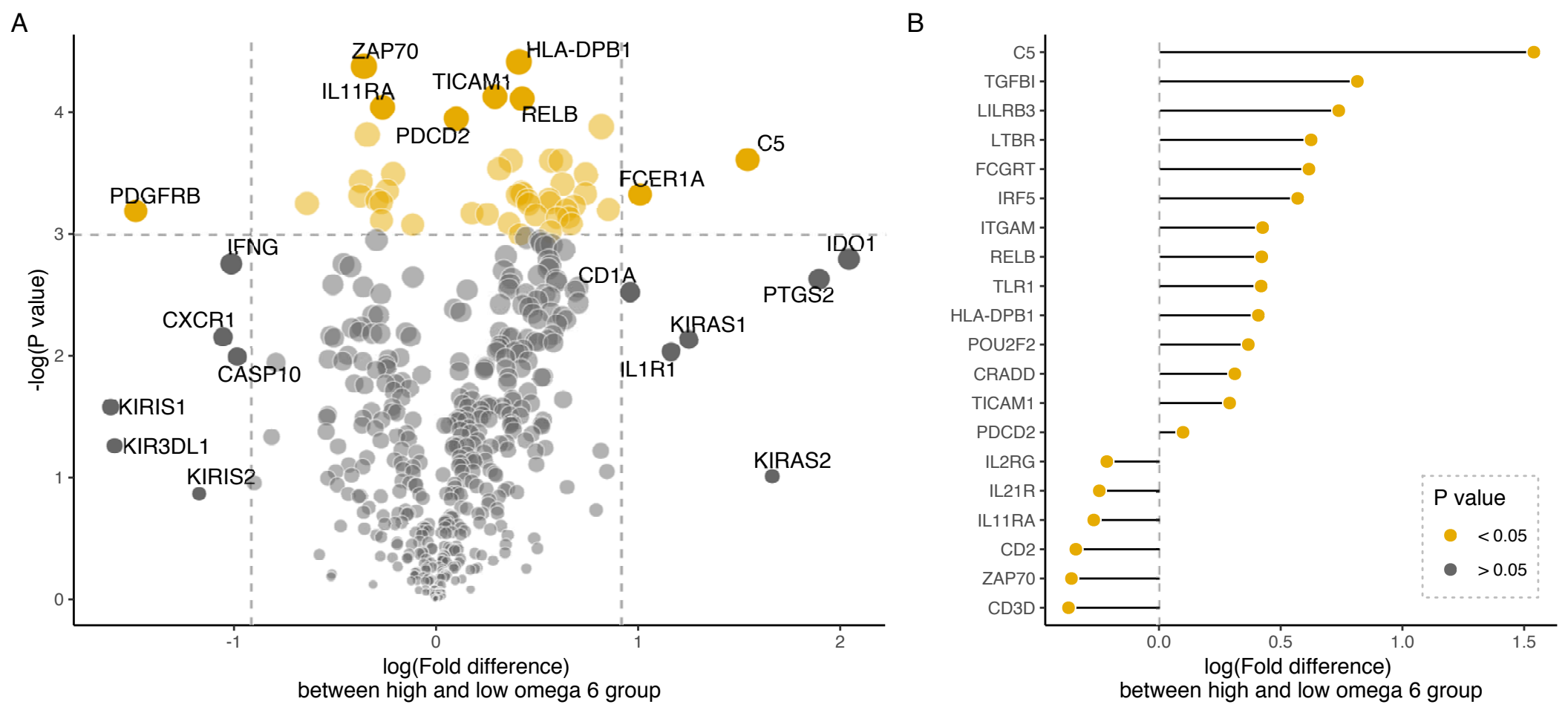
Figure legends

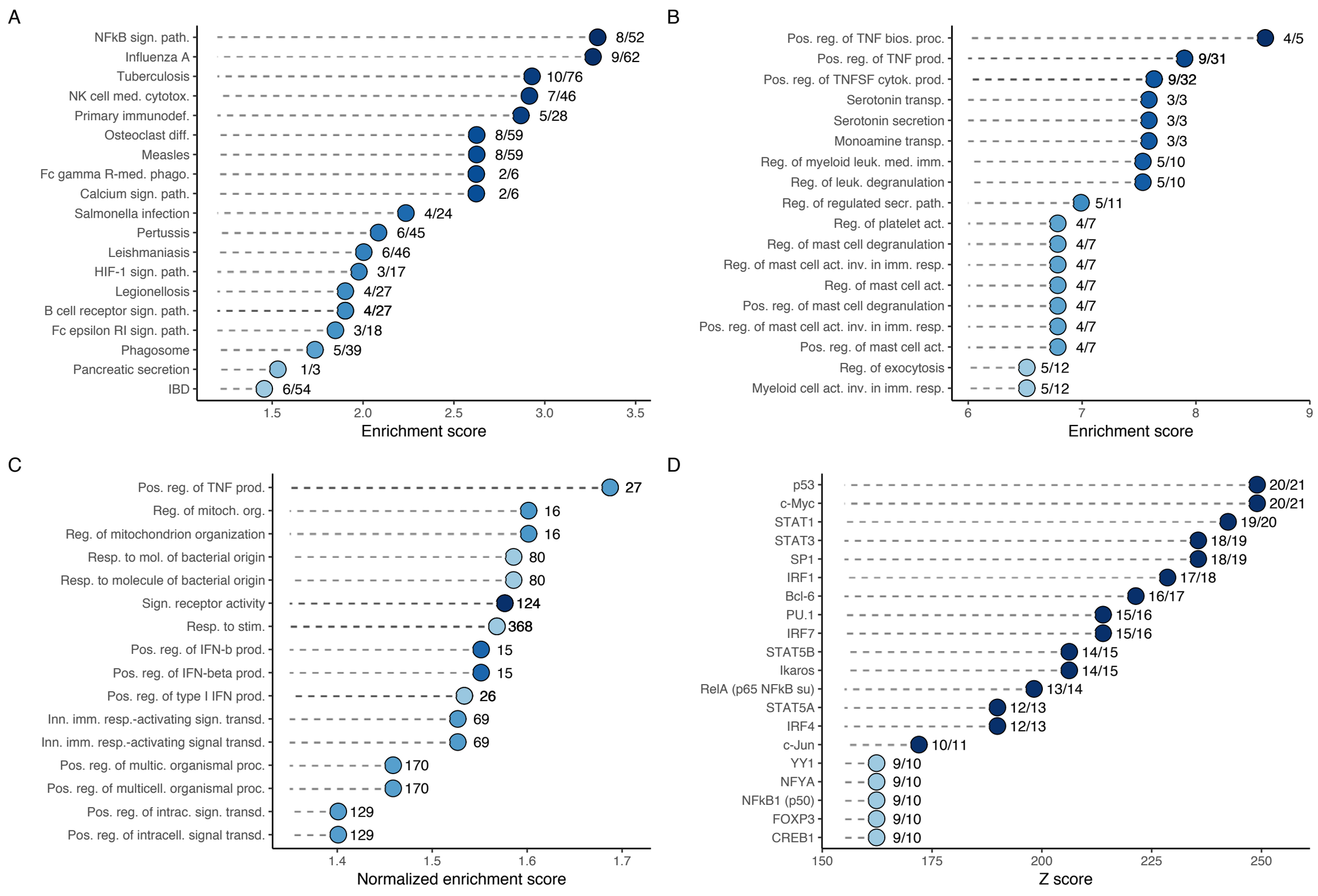
Figure 1. Differential expression analysis. A) Volcano plot highlighting both significant genes and genes with an absolute fold difference above 2.5, and B) top 20 most significantly different genes, sorted by fold difference. Log is natural logarithm; size aesthetic is mapped to y-axis value. The data are based on 4-way ANOVA between groups with high and low omega 6 PUFA level; higher expression means higher in the group with high omega 6 PUFA level. Abbreviations: C5, Complement Component 5; CASP10, Caspase 10, Apoptosis-Related Cysteine Peptidase; CD1A, Cd1a Molecule; CD2, Cd2 Molecule; CD3D, Cd3d Molecule, Delta (Cd3-Tcr Complex); CRADD, Casp2 And Ripk1 Domain Containing Adaptor With Death Domain; CXCR1, Chemokine (C-X-C Motif) Receptor 1; FCER1A, Fc Fragment Of Ige, High Affinity I, Receptor For; Alpha Polypeptide; FCGRT, Fc Fragment Of Igg, Receptor, Transporter, Alpha; HLA-DPB1, Major Histocompatibility Complex, Class Ii, Dp Beta 1; IDO1, Indoleamine 2,3-Dioxygenase 1; IFNG, Interferon, Gamma; IL11RA, Interleukin 11 Receptor, Alpha; IL1R1, Interleukin 1 Receptor, Type I; IL21R, Interleukin 21 Receptor; IL2RG, Interleukin 2 Receptor, Gamma; IRF5, Interferon Regulatory Factor 5; ITGAM, Integrin, Alpha M (Complement Component 3 Receptor 3 Subunit); KIR3DL1, Killer Cell Immunoglobulin-Like Receptor, Three Domains, Long Cytoplasmic Tail, 1; KIRAS1, Killer Cell Immunoglobulin-Like Receptor; KIRAS2, Killer Cell Immunoglobulin-Like Receptor; KIRIS1, Killer Cell Immunoglobulin-Like Receptor; KIRIS2, Killer Cell Immunoglobulin-Like Receptor; LILRB3, Leukocyte Immunoglobulin-Like Receptor, Subfamily B (With Tm And Itim Domains), Member 3; LTBR, Lymphotoxin Beta Receptor (Tnfr Superfamily, Member 3); PDCD2, Programmed Cell Death 2; PDGFRB, Platelet-Derived Growth Factor Receptor, Beta Polypeptide; POU2F2, Pou Class 2 Homeobox 2; PTGS2, Prostaglandin-Endoperoxide Synthase 2 (Prostaglandin G/H Synthase And Cyclooxygenase); RELB, V-Rel

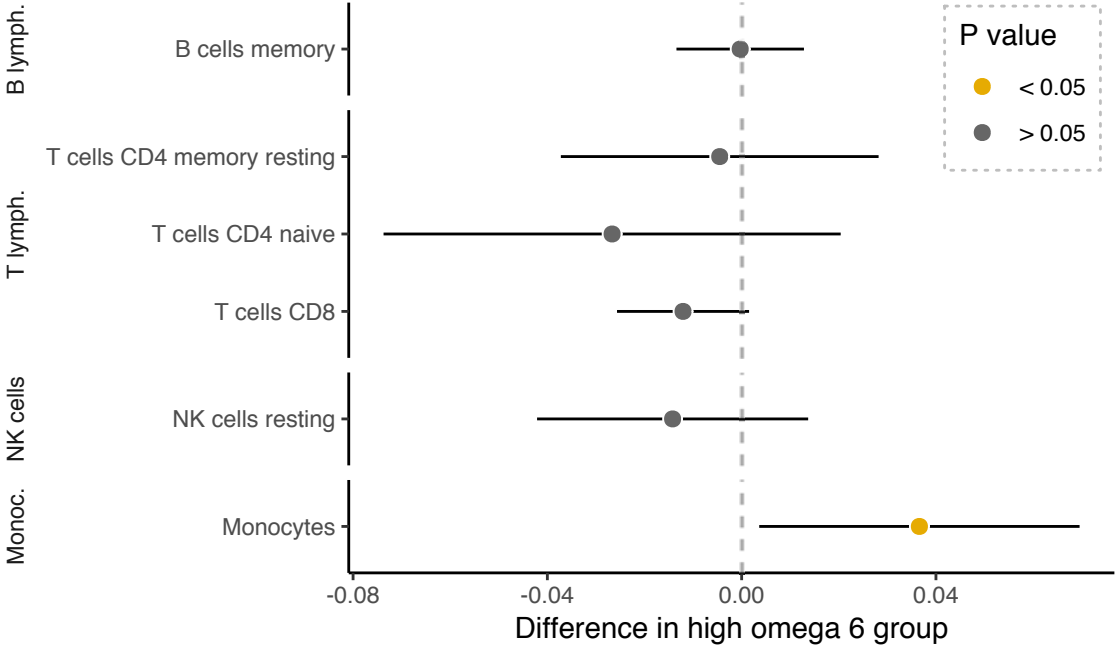
Reticuloendotheliosis Viral Oncogene Homolog B; TGFBI, Transforming Growth Factor, Beta-Induced, 68kda; TICAM1, Toll-Like Receptor Adaptor Molecule 1; TLR1, Toll-Like Receptor 1; ZAP70, Zeta-Chain (Tcr) Associated Protein Kinase 70kda.

Figure 2. Integrated omics analyses. The top 20 most relevant A) pathways, B) gene ontology (GO) terms, C) gene sets, and D) transcription factors (TFs), sorted by enrichment or z score. The numbers to the right represent number of genes *present* and *total number of genes* in that pathway, GO term, or TF. For gene sets, the number represents *total number of genes* in the respective gene set. The P values are extremely low for all, but for the darker the color, the lower the P value; for exact p value, see Supplemental table 3-9. Higher enrichment means higher in the group with high omega 6 PUFA level. Results in panel A, B and C are based on Partek analyses; results in panel D is based on Metacore analyses (see Methods). For abbreviations, see Supplemental tables 3-9.

Figure 3. CIBERSORT-predicted cell types. Association between omega 6 PUFA level and CIBERSORT-predicted leukocyte cell types, based on gene expression pattern. Symbols are regression coefficient estimates (± 95 % CI) of difference between high and low omega 6 groups, adjusted for visceral fat. Higher level means higher in the group with high omega 6 PUFA level.


A
ZAP70
HLA-DPB1
TICAM1
IL11RA
RELB
PDCD2
C5
FCER1A
PDGFRB
IFNG
IDO1
CD1A
PTGS2
CXCR1
KIRAS1
IL1R1
CASP10
KIRIS1
KIR3DL1
KIRAS2
KIRIS2
-log(P value)
log(Fold difference)
between high and low omega 6 group
B
C5
TGFBI
LILRB3
LTBR
FCGRT
IRF5
ITGAM
RELB
TLR1
HLA-DPB1
POU2F2
CRADD
TICAM1
PDCD2
IL2RG
IL21R
IL11RA
CD2
ZAP70
CD3D
P value
< 0.05
> 0.05
log(Fold difference)
between high and low omega 6 group


A
NFkB sign. path.
Influenza A
Tuberculosis
NK cell med. cytotox.
Primary immunodef.
Osteoclast diff.
Measles
Fc gamma R-med. phago.
Calcium sign. path.
Salmonella infection
Pertussis
Leishmaniasis
HIF-1 sign. path.
Legionellosis
B cell receptor sign. path.
Fc epsilon RI sign. path.
Phagosome
Pancreatic secretion
IBD
8/52
9/62
10/76
7/46
5/28
8/59
8/59
2/6
2/6
4/24
6/45
6/46
3/17
4/27
4/27
3/18
5/39
1/3
6/54
1.5
2.0
2.5
3.0
3.5
Enrichment score
B
Pos. reg. of TNF bios. proc.
Pos. reg. of TNF prod.
Pos. reg. of TNFSF cytok. prod.
Serotonin transp.
Serotonin secretion
Monoamine transp.
Reg. of myeloid leuk. med. imm.
Reg. of leuk. degranulation
Reg. of regulated secr. path.
Reg. of platelet act.
Reg. of mast cell degranulation
Reg. of mast cell act. inv. in imm. resp.
Reg. of mast cell act.
Pos. reg. of mast cell degranulation
Pos. reg. of mast cell act. inv. in imm. resp.
Pos. reg. of mast cell act.
Reg. of exocytosis
Myeloid cell act. inv. in imm. resp.
4/5
9/31
9/32
3/3
3/3
3/3
5/10
5/10
5/11
4/7
4/7
4/7
4/7
4/7
4/7
4/7
5/12
5/12
6
7
8
9
Enrichment score
C
Pos. reg. of TNF prod.
Reg. of mitoch. org.
Reg. of mitochondrion organization
Resp. to mol. of bacterial origin
Resp. to molecule of bacterial origin
Sign. receptor activity
Resp. to stim.
Pos. reg. of IFN-b prod.
Pos. reg. of IFN-beta prod.
Pos. reg. of type I IFN prod.
Inn. imm. resp.-activating sign. transd.
Inn. imm. resp.-activating signal transd.
Pos. reg. of multic. organismal proc.
Pos. reg. of multicell. organismal proc.
Pos. reg. of intrac. sign. transd.
Pos. reg. of intracell. signal transd.
27
16
16
80
80
124
368
15
15
26
69
69
170
170
129
129
1.4
1.5
1.6
1.7
Normalized enrichment score
D
p53
c-Myc
STAT1
STAT3
SP1
IRF1
Bcl-6
PU.1
IRF7
STAT5B
Ikaros
RelA (p65 NFkB su)
STAT5A
IRF4
c-Jun
YY1
NFYA
NFkB1 (p50)
FOXP3
CREB1
20/21
20/21
19/20
18/19
18/19
17/18
16/17
15/16
15/16
14/15
14/15
13/14
12/13
12/13
10/11
9/10
9/10
9/10
9/10
9/10
150
175
200
225
250
Z score


B lymph.
T lymph.
NK cells
Monoc.
B cells memory
T cells CD4 memory resting
T cells CD4 naive
T cells CD8
NK cells resting
Monocytes
P value
< 0.05
> 0.05
-0.08
-0.04
0.00
0.04
Difference in high omega 6 group

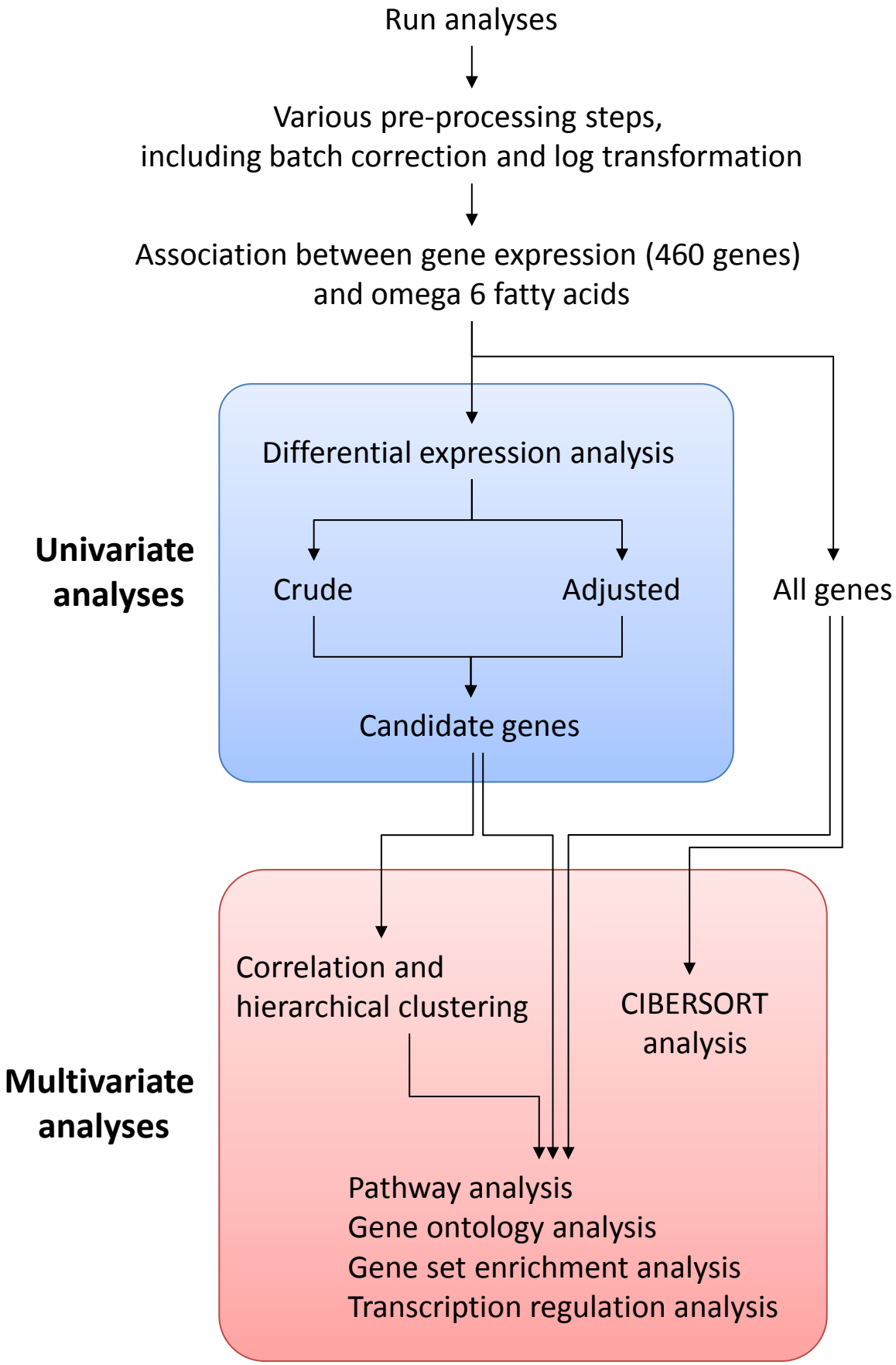
SUPPLEMENTAL INFORMATION

Supplemental figures and figure legends

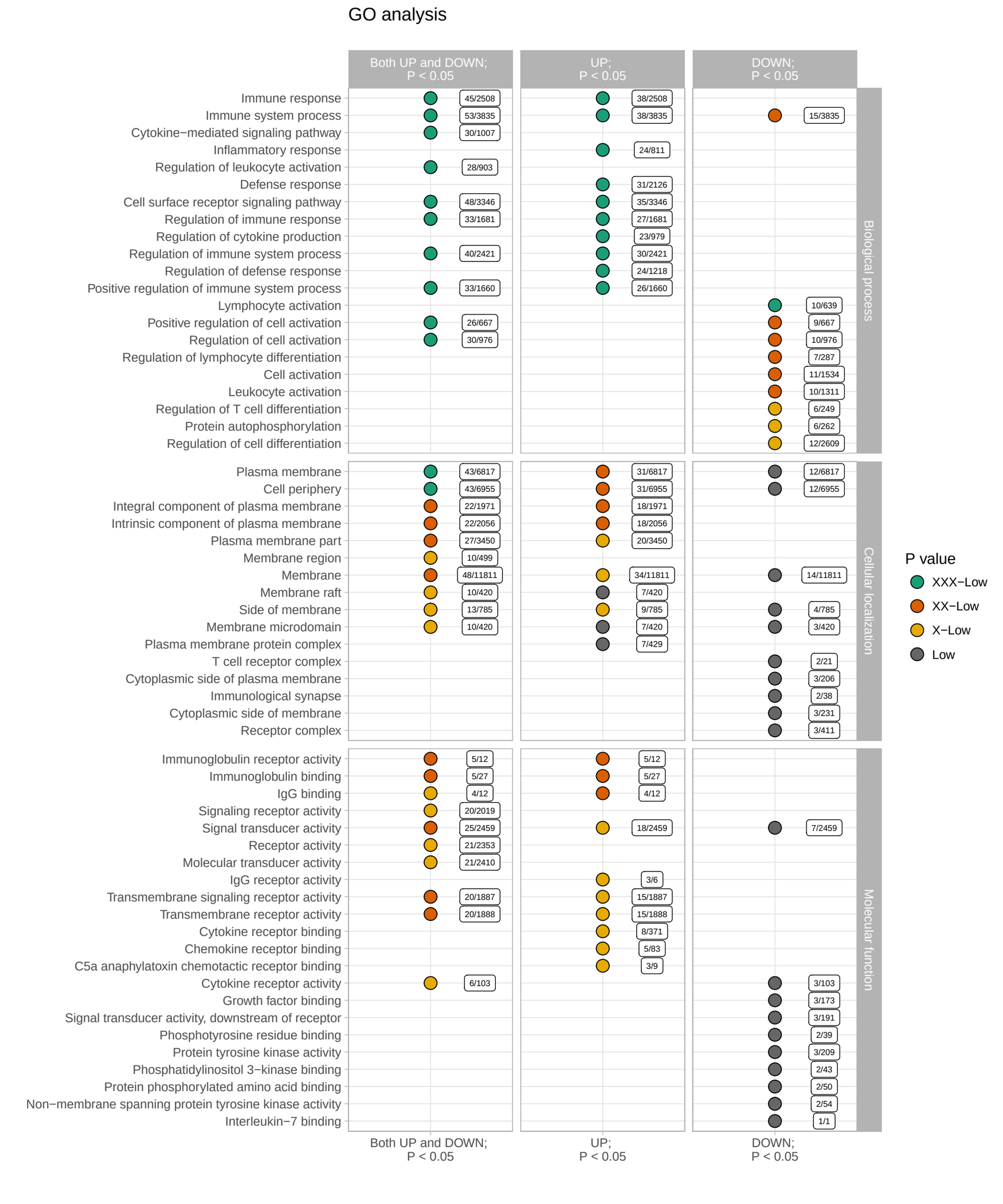
Serum omega 6 fatty acids and immunology-related gene expression in peripheral blood mononuclear cells: a cross-sectional analysis in healthy children

Jacob J. Christensen, Siril S. Bakke, Stine M. Ulven, Kjetil Retterstøl, Tore Henriksen, Jens Bollerslev, Terje Espevik, Pål Aukrust, Bente Halvorsen, Kirsten B. Holven

Supplemental figure 1. Methods flow chart. Methods flow chart that illustrates the main univariate and multivariate approaches used in the statistical and bioinformatics analyses, and their interrelations.

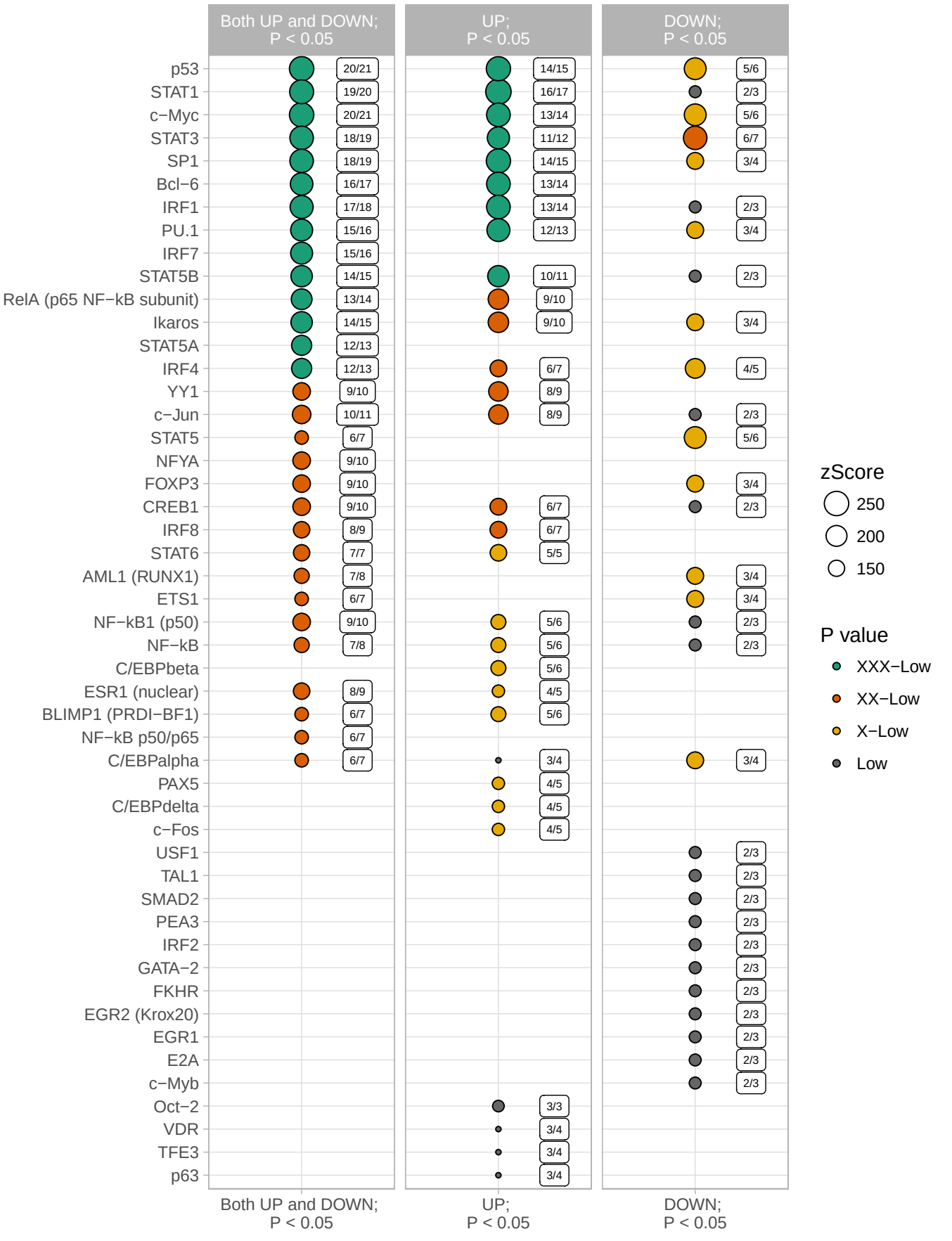

Run analyses
Various pre-processing steps,
including batch correction and log transformation
Association between gene expression (460 genes)
and omega 6 fatty acids
Univariate analyses
Differential expression analysis
Crude
Adjusted
Candidate genes
All genes
Multivariate analyses
Correlation and hierarchical clustering
CIBERSORT analysis
Pathway analysis
Gene ontology analysis
Gene set enrichment analysis
Transcription regulation analysis

Supplemental figure 2. Gene ontology analysis: higher and lower expressed genes. The top gene ontology (GO) terms that display enrichment for differentially expressed genes ($P < 0.05$). Labels represent *in data* and *total data* for that particular GO term, respectively. Data are faceted on type of GO term (rows) and whether genes are higher (UP) or lower (DOWN) in the high omega 6 group, or both (UP and DOWN) (columns). Colors denote strength of significance; for exact p values, see Supplemental table 5-7. Abbreviations: XXX, extremely (low); XX, extremely-very (low); X, very (low).

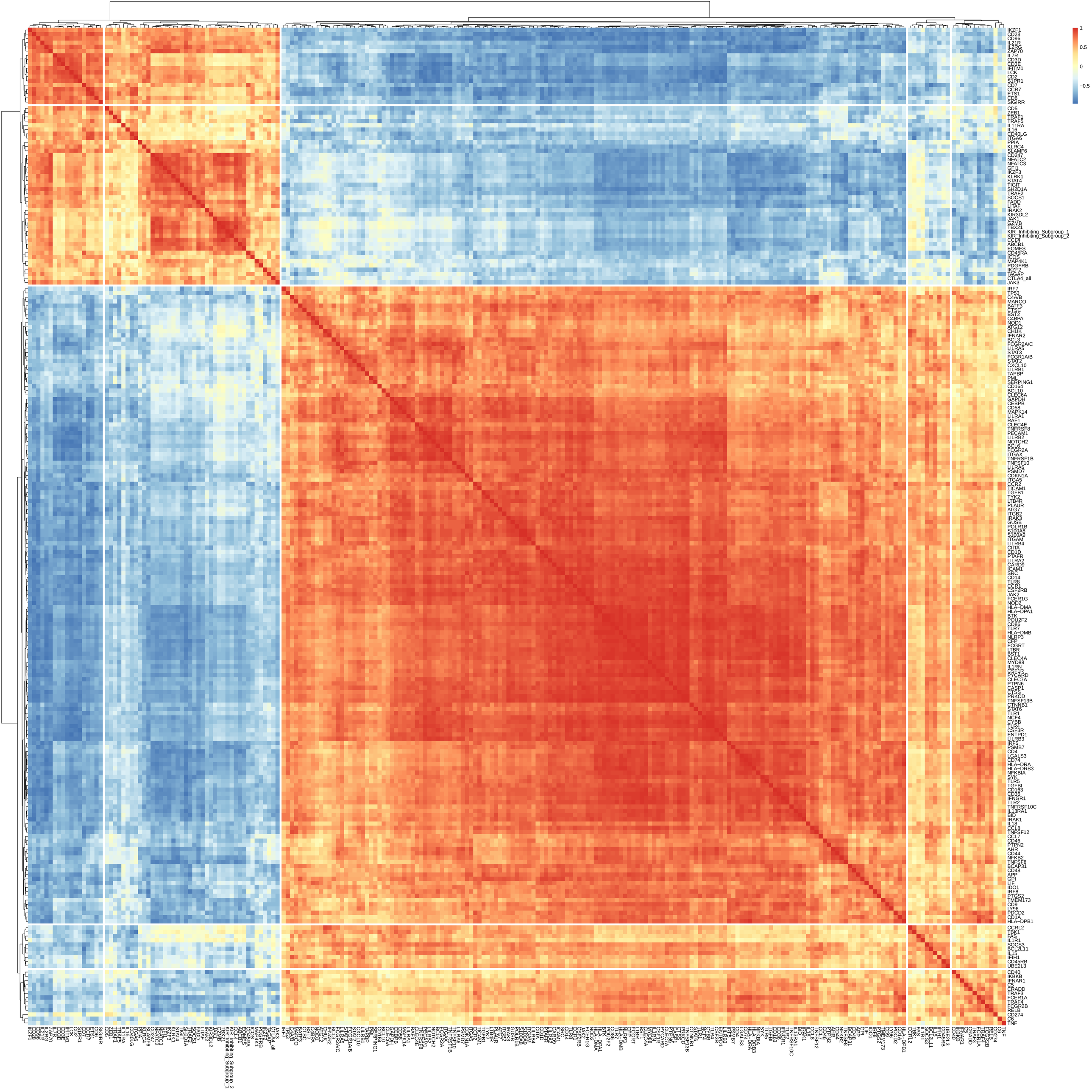

GO analysis
Both UP and DOWN; P < 0.05
UP; P < 0.05
DOWN; P < 0.05
Biological process
Cellular localization
Molecular function
Immune response
Immune system process
Cytokine−mediated signaling pathway
Inflammatory response
Regulation of leukocyte activation
Defense response
Cell surface receptor signaling pathway
Regulation of immune response
Regulation of cytokine production
Regulation of immune system process
Regulation of defense response
Positive regulation of immune system process
Lymphocyte activation
Positive regulation of cell activation
Regulation of cell activation
Regulation of lymphocyte differentiation
Cell activation
Leukocyte activation
Regulation of T cell differentiation
Protein autophosphorylation
Regulation of cell differentiation
45/2508
53/3835
30/1007
28/903
48/3346
33/1681
40/2421
33/1660
26/667
30/976
38/2508
38/3835
24/811
31/2126
35/3346
27/1681
23/979
30/2421
24/1218
26/1660
15/3835
10/639
9/667
10/976
7/287
11/1534
10/1311
6/249
6/262
12/2609
Plasma membrane
Cell periphery
Integral component of plasma membrane
Intrinsic component of plasma membrane
Plasma membrane part
Membrane region
Membrane
Membrane raft
Side of membrane
Membrane microdomain
Plasma membrane protein complex
T cell receptor complex
Cytoplasmic side of plasma membrane
Immunological synapse
Cytoplasmic side of membrane
Receptor complex
43/6817
43/6955
22/1971
22/2056
27/3450
10/499
48/11811
10/420
13/785
10/420
31/6817
31/6955
18/1971
18/2056
20/3450
34/11811
7/420
9/785
7/420
7/429
12/6817
12/6955
14/11811
4/785
3/420
2/21
3/206
2/38
3/231
3/411
Immunoglobulin receptor activity
Immunoglobulin binding
IgG binding
Signaling receptor activity
Signal transducer activity
Receptor activity
Molecular transducer activity
IgG receptor activity
Transmembrane signaling receptor activity
Transmembrane receptor activity
Cytokine receptor binding
Chemokine receptor binding
C5a anaphylatoxin chemotactic receptor binding
Cytokine receptor activity
Growth factor binding
Signal transducer activity, downstream of receptor
Phosphotyrosine residue binding
Protein tyrosine kinase activity
Phosphatidylinositol 3−kinase binding
Protein phosphorylated amino acid binding
Non−membrane spanning protein tyrosine kinase activity
Interleukin−7 binding
5/12
5/27
4/12
20/2019
25/2459
21/2353
21/2410
20/1887
20/1888
6/103
5/12
5/27
4/12
18/2459
3/6
15/1887
15/1888
8/371
5/83
3/9
7/2459
3/103
3/173
3/191
2/39
3/209
2/43
2/50
2/54
1/1
P value
XXX−Low
XX−Low
X−Low
Low
Both UP and DOWN; P < 0.05
UP; P < 0.05
DOWN; P < 0.05

Supplemental figure 3. Transcription factor analysis: higher and lower expressed genes.

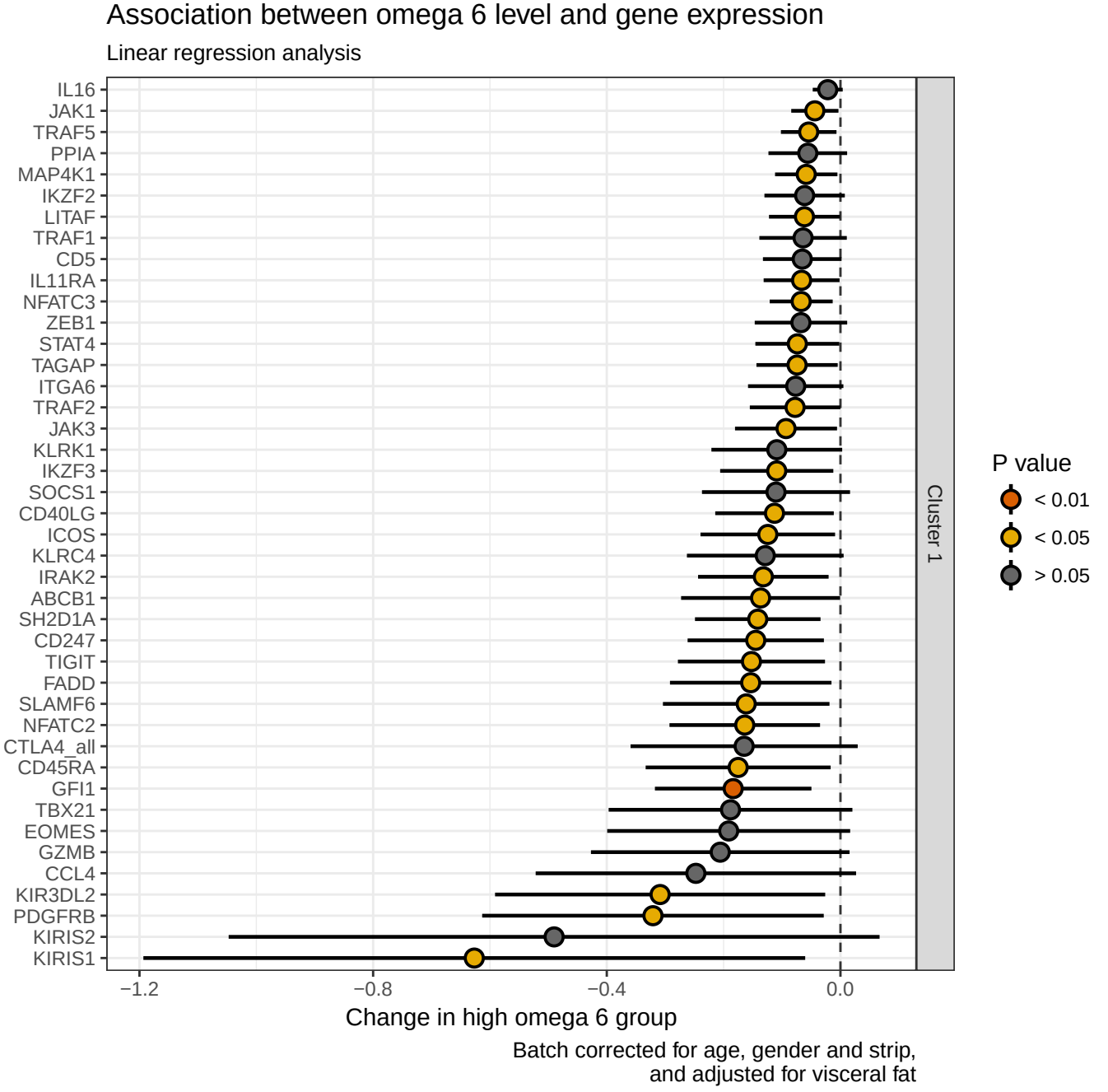
The top transcription factors (TFs) that display enrichment for differentially expressed genes ($P < 0.05$). Labels represent *seed nodes* and *total nodes* for that particular TF, respectively. Data are faceted on whether genes are higher (UP) or lower (DOWN) in the high omega 6 group, or both (UP and DOWN) (columns). Colors and point size denote strength of significance and Z score; for exact values, see Supplemental table 9. Abbreviations as for Supplemental figure 3.

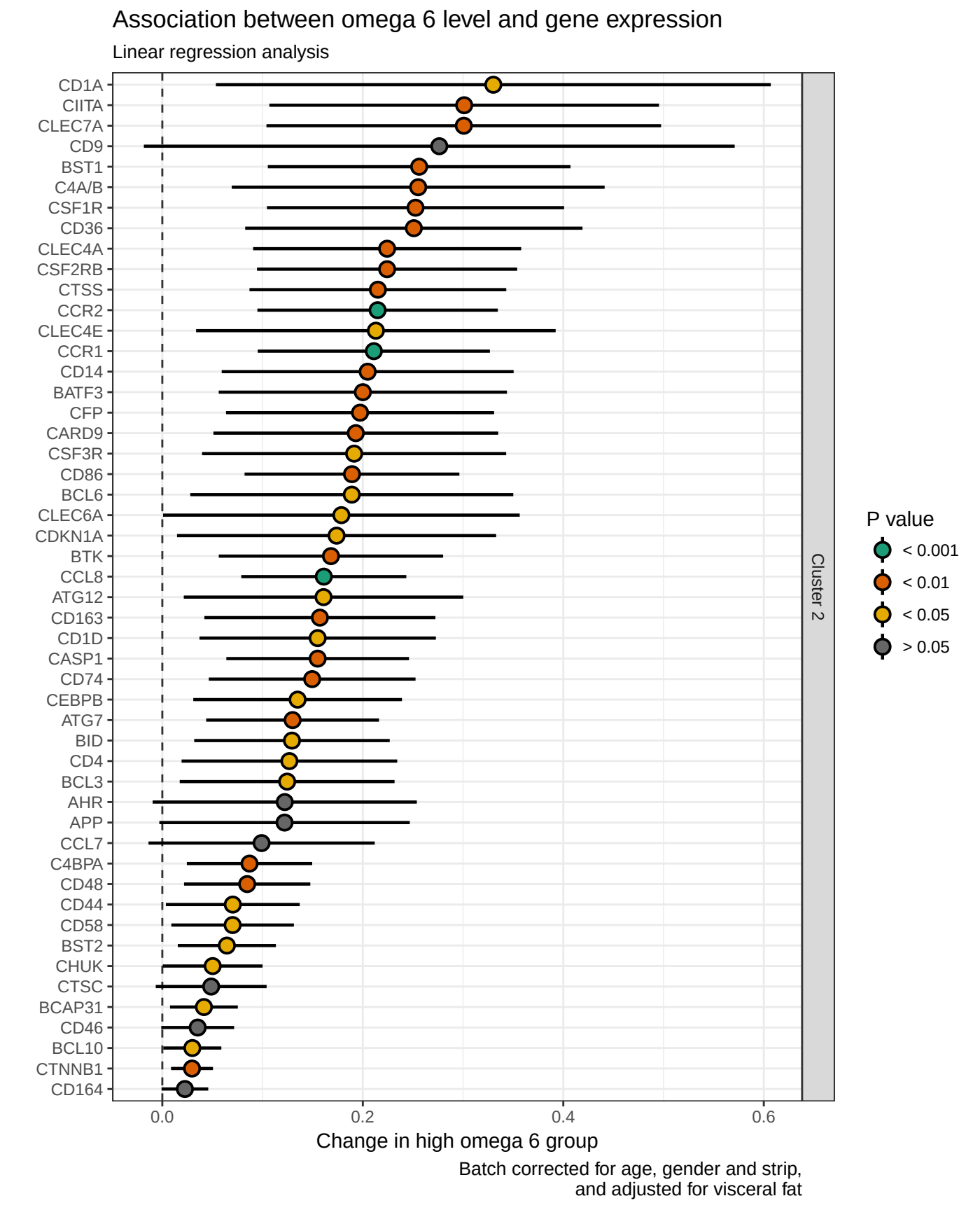

Transcription factor analysis
Both UP and DOWN; P < 0.05
UP; P < 0.05
DOWN; P < 0.05
p53
STAT1
c−Myc
STAT3
SP1
Bcl−6
IRF1
PU.1
IRF7
STAT5B
RelA (p65 NF−kB subunit)
Ikaros
STAT5A
IRF4
YY1
c−Jun
STAT5
NFYA
FOXP3
CREB1
IRF8
STAT6
AML1 (RUNX1)
ETS1
NF−kB1 (p50)
NF−kB
C/EBPbeta
ESR1 (nuclear)
BLIMP1 (PRDI−BF1)
NF−kB p50/p65
C/EBPalpha
PAX5
C/EBPdelta
c−Fos
USF1
TAL1
SMAD2
PEA3
IRF2
GATA−2
FKHR
EGR2 (Krox20)
EGR1
E2A
c−Myb
Oct−2
VDR
TFE3
p63
20/21
19/20
20/21
18/19
18/19
16/17
17/18
15/16
15/16
14/15
13/14
14/15
12/13
12/13
9/10
10/11
6/7
9/10
9/10
9/10
8/9
7/7
7/8
6/7
9/10
7/8
8/9
6/7
6/7
14/15
16/17
13/14
11/12
14/15
13/14
13/14
12/13
10/11
9/10
9/10
6/7
8/9
8/9
6/7
6/7
5/5
5/6
5/6
5/6
4/5
5/6
3/4
4/5
4/5
4/5
3/3
3/4
3/4
3/4
5/6
2/3
5/6
6/7
3/4
2/3
3/4
2/3
3/4
4/5
2/3
5/6
3/4
2/3
3/4
3/4
2/3
2/3
3/4
2/3
2/3
2/3
2/3
2/3
2/3
2/3
2/3
2/3
2/3
2/3
2/3
zScore
250
200
150
P value
XXX−Low
XX−Low
X−Low
Low
Both UP and DOWN; P < 0.05
UP; P < 0.05
DOWN; P < 0.05

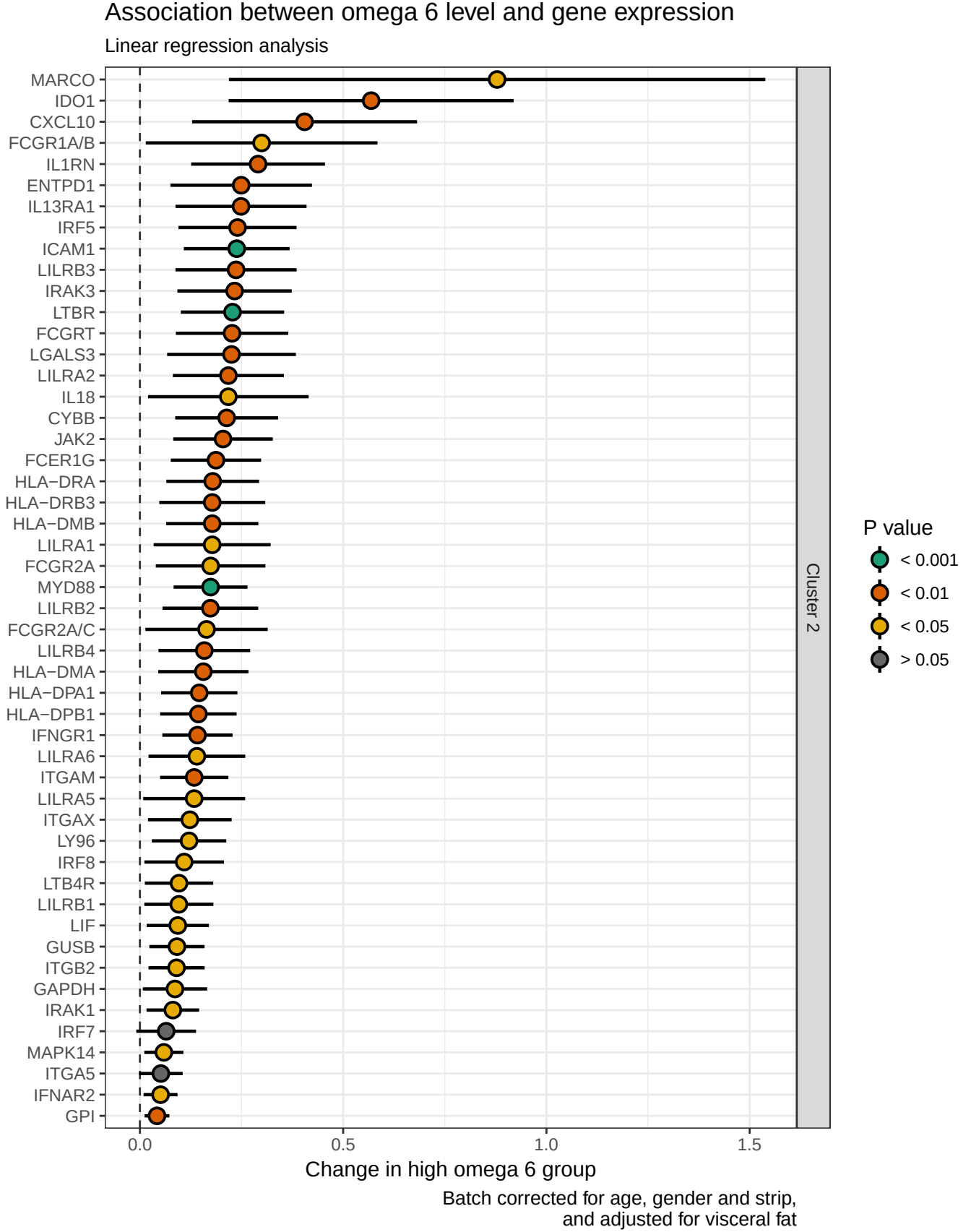
Supplemental figure 4. Gene expression correlation heatmap. Correlation heatmap of 233 genes with FDR < 0.2, after adjusting for visceral fat. Genes are clustered into k = 5 clusters using hierarchical clustering (Euclidean distance, complete linkage). Red and blue color means positive and negative correlation, respectively; color intensity denotes strength of the association. For abbreviations, see Supplemental material 1.

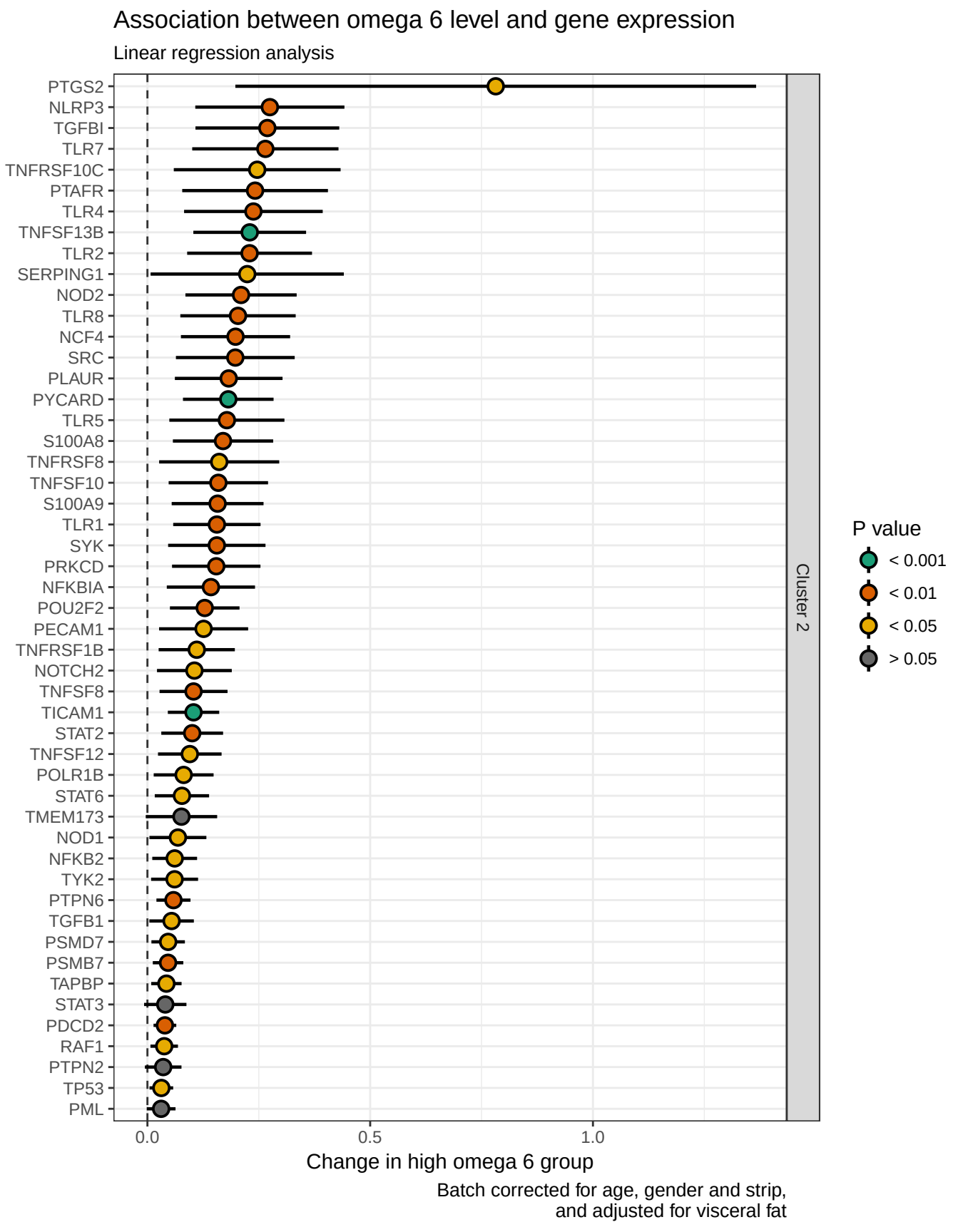

1
0.5
0
−0.5

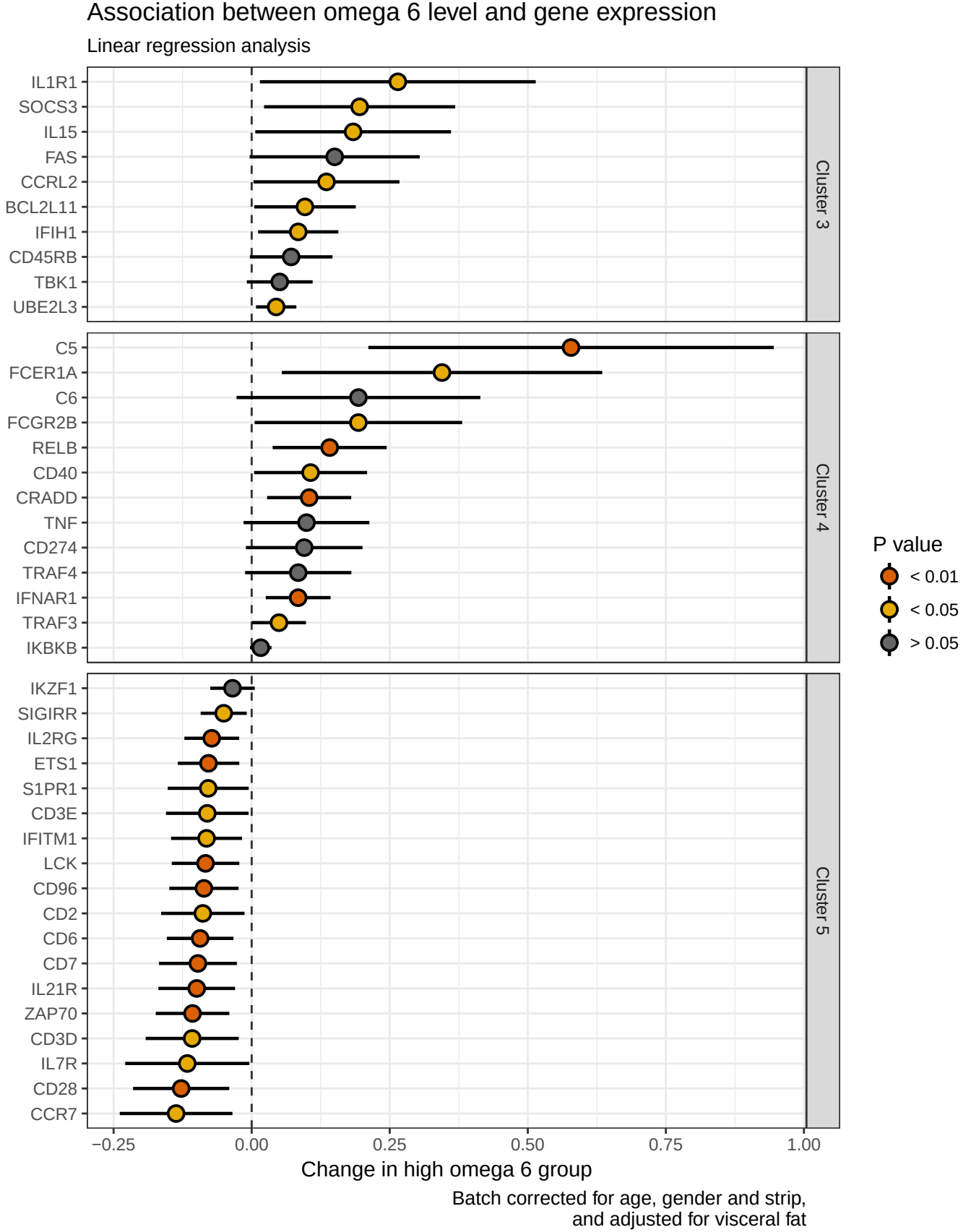
Supplemental figure 5. Forestplot: clusters 1-5. Forestplots that display the differences between high and low omega 6 groups for single genes in A) cluster 1, B-D) cluster 2, and E) clusters 3 through 5. Data are beta estimates ± 95 % confidence interval. The genes were clustered into k = 5 clusters using hierarchical clustering (Euclidean distance, complete linkage) (see Supplemental figure 5). As opposed to data in Figure 1 and Supplemental table 2, these results are adjusted for visceral fat. For abbreviations, see Supplemental material 1.


Association between omega 6 level and gene expression
Linear regression analysis
IL16
JAK1
TRAF5
PPIA
MAP4K1
IKZF2
LITAF
TRAF1
CD5
IL11RA
NFATC3
ZEB1
STAT4
TAGAP
ITGA6
TRAF2
JAK3
KLRK1
IKZF3
SOCS1
CD40LG
ICOS
KLRC4
IRAK2
ABCB1
SH2D1A
CD247
TIGIT
FADD
SLAMF6
NFATC2
CTLA4_all
CD45RA
GFI1
TBX21
EOMES
GZMB
CCL4
KIR3DL2
PDGFRB
KIRIS2
KIRIS1
Cluster 1
−1.2
−0.8
−0.4
0.0
Change in high omega 6 group
P value
< 0.01
< 0.05
> 0.05
Batch corrected for age, gender and strip,
and adjusted for visceral fat


Association between omega 6 level and gene expression
Linear regression analysis
CD1A
CIITA
CLEC7A
CD9
BST1
C4A/B
CSF1R
CD36
CLEC4A
CSF2RB
CTSS
CCR2
CLEC4E
CCR1
CD14
BATF3
CFP
CARD9
CSF3R
CD86
BCL6
CLEC6A
CDKN1A
BTK
CCL8
ATG12
CD163
CD1D
CASP1
CD74
CEBPB
ATG7
BID
CD4
BCL3
AHR
APP
CCL7
C4BPA
CD48
CD44
CD58
BST2
CHUK
CTSC
BCAP31
CD46
BCL10
CTNNB1
CD164
0.0
0.2
0.4
0.6
Change in high omega 6 group
Batch corrected for age, gender and strip,
and adjusted for visceral fat
Cluster 2
P value
< 0.001
< 0.01
< 0.05
> 0.05

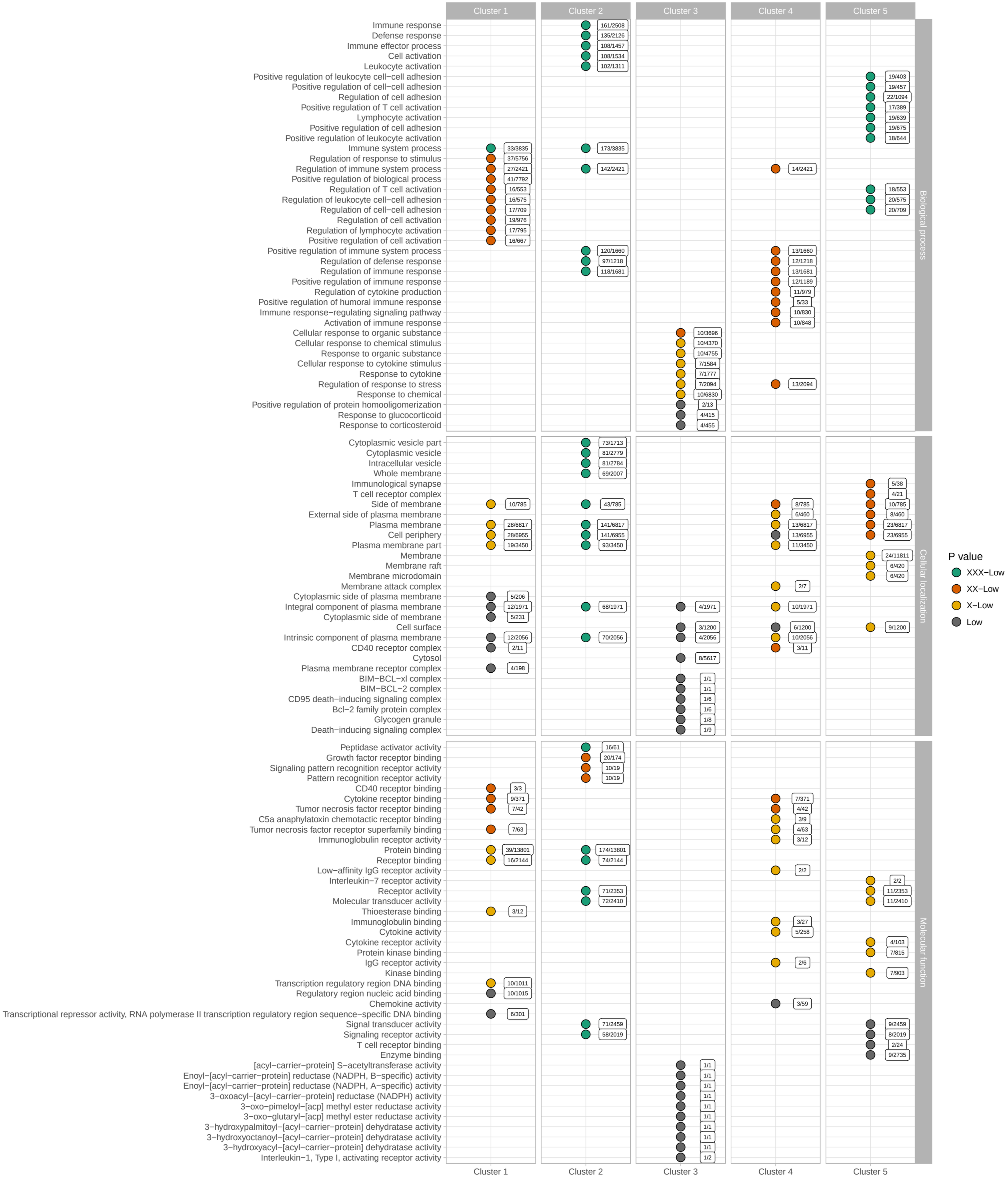

Association between omega 6 level and gene expression
Linear regression analysis
MARCO
IDO1
CXCL10
FCGR1A/B
IL1RN
ENTPD1
IL13RA1
IRF5
ICAM1
LILRB3
IRAK3
LTBR
FCGRT
LGALS3
LILRA2
IL18
CYBB
JAK2
FCER1G
HLA−DRA
HLA−DRB3
HLA−DMB
LILRA1
FCGR2A
MYD88
LILRB2
FCGR2A/C
LILRB4
HLA−DMA
HLA−DPA1
HLA−DPB1
IFNGR1
LILRA6
ITGAM
LILRA5
ITGAX
LY96
IRF8
LTB4R
LILRB1
LIF
GUSB
ITGB2
GAPDH
IRAK1
IRF7
MAPK14
ITGA5
IFNAR2
GPI
Cluster 2
0.0
0.5
1.0
1.5
Change in high omega 6 group
Batch corrected for age, gender and strip,
and adjusted for visceral fat
P value
< 0.001
< 0.01
< 0.05
> 0.05


Association between omega 6 level and gene expression
Linear regression analysis
PTGS2
NLRP3
TGFBI
TLR7
TNFRSF10C
PTAFR
TLR4
TNFSF13B
TLR2
SERPING1
NOD2
TLR8
NCF4
SRC
PLAUR
PYCARD
TLR5
S100A8
TNFRSF8
TNFSF10
S100A9
TLR1
SYK
PRKCD
NFKBIA
POU2F2
PECAM1
TNFRSF1B
NOTCH2
TNFSF8
TICAM1
STAT2
TNFSF12
POLR1B
STAT6
TMEM173
NOD1
NFKB2
TYK2
PTPN6
TGFB1
PSMD7
PSMB7
TAPBP
STAT3
PDCD2
RAF1
PTPN2
TP53
PML
0.0
0.5
1.0
Change in high omega 6 group
Batch corrected for age, gender and strip,
and adjusted for visceral fat
Cluster 2
P value
< 0.001
< 0.01
< 0.05
> 0.05

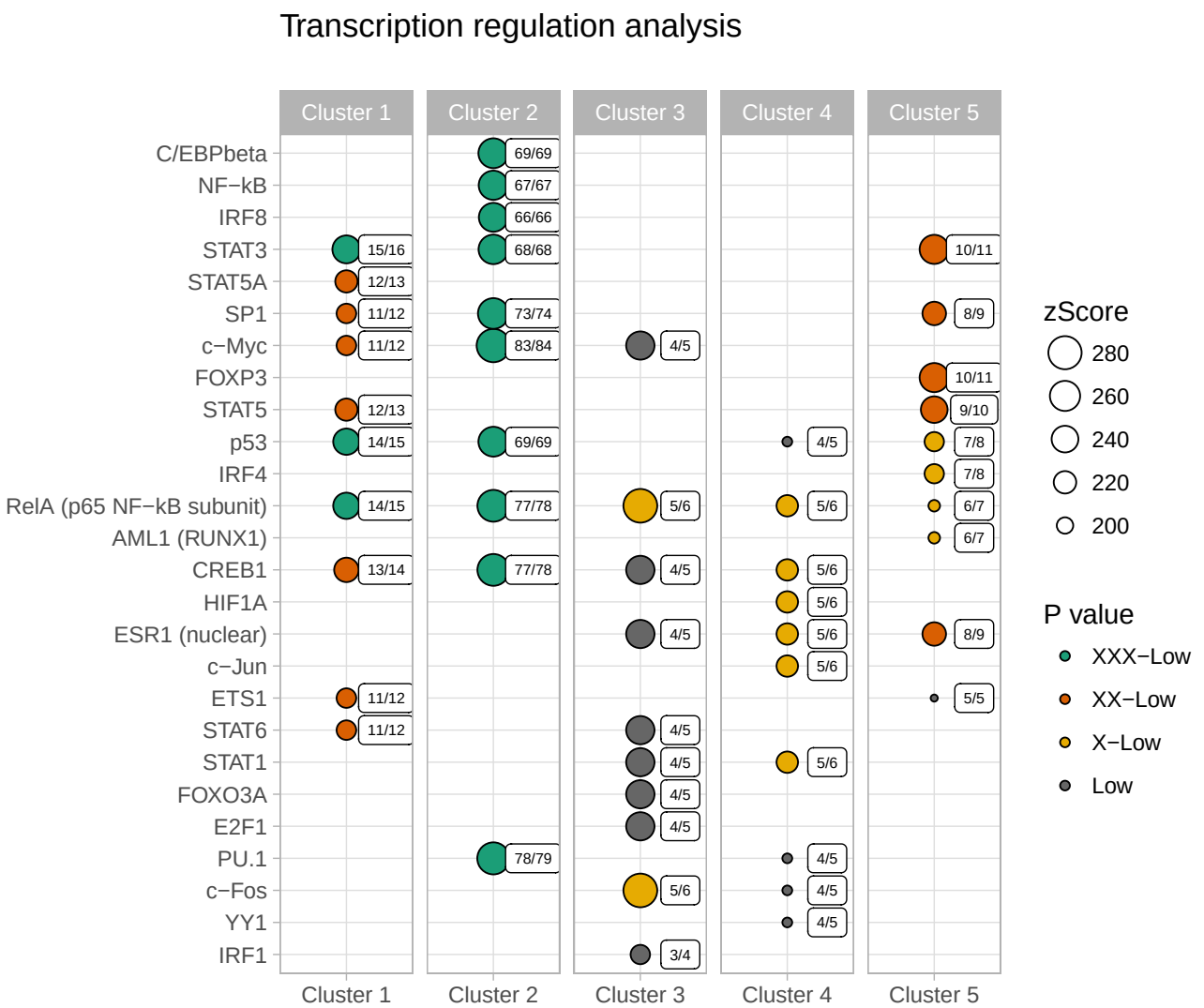

Association between omega 6 level and gene expression
Linear regression analysis
IL1R1
SOCS3
IL15
FAS
CCRL2
BCL2L11
IFIH1
CD45RB
TBK1
UBE2L3
Cluster 3
C5
FCER1A
C6
FCGR2B
RELB
CD40
CRADD
TNF
CD274
TRAF4
IFNAR1
TRAF3
IKBKB
Cluster 4
IKZF1
SIGIRR
IL2RG
ETS1
S1PR1
CD3E
IFITM1
LCK
CD96
CD2
CD6
CD7
IL21R
ZAP70
CD3D
IL7R
CD28
CCR7
Cluster 5
−0.25
0.00
0.25
0.50
0.75
1.00
Change in high omega 6 group
Batch corrected for age, gender and strip,
and adjusted for visceral fat
P value
< 0.01
< 0.05
> 0.05

Supplemental figure 6. Gene ontology analysis: gene clusters. The top gene ontology (GO) terms that display enrichment for differentially expressed genes (FDR < 0.2), after adjusting for visceral fat. Labels represent *in data* and *total data* for that particular GO term, respectively. Data are faceted on type of GO term (rows) and cluster affiliation (columns). Colors denote strength of significance; for exact p values, see Supplemental table 5-7. Abbreviations as for Supplemental figure 3.

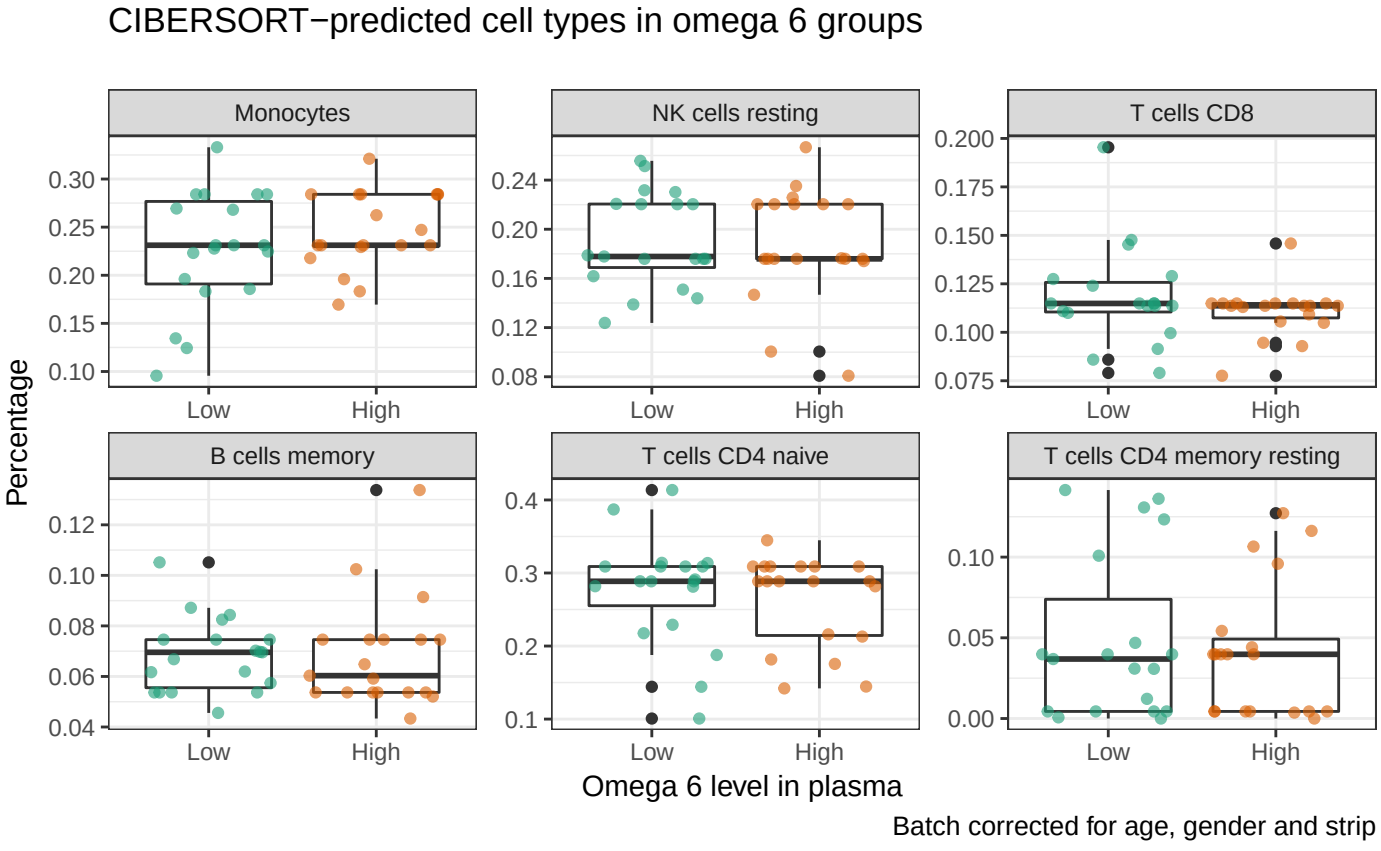
GO analysis


Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Immune response
Defense response
Immune effector process
Cell activation
Leukocyte activation
Positive regulation of leukocyte cell−cell adhesion
Positive regulation of cell−cell adhesion
Regulation of cell adhesion
Positive regulation of T cell activation
Lymphocyte activation
Positive regulation of cell adhesion
Positive regulation of leukocyte activation
Immune system process
Regulation of response to stimulus
Regulation of immune system process
Positive regulation of biological process
Regulation of T cell activation
Regulation of leukocyte cell−cell adhesion
Regulation of cell−cell adhesion
Regulation of cell activation
Regulation of lymphocyte activation
Positive regulation of cell activation
Positive regulation of immune system process
Regulation of defense response
Regulation of immune response
Positive regulation of immune response
Regulation of cytokine production
Positive regulation of humoral immune response
Immune response−regulating signaling pathway
Activation of immune response
Cellular response to organic substance
Cellular response to chemical stimulus
Response to organic substance
Cellular response to cytokine stimulus
Response to cytokine
Regulation of response to stress
Response to chemical
Positive regulation of protein homooligomerization
Response to glucocorticoid
Response to corticosteroid
161/2508
135/2126
108/1457
108/1534
102/1311
19/403
19/457
22/1094
17/389
19/639
19/675
18/644
33/3835
37/5756
27/2421
41/7792
16/553
16/575
17/709
19/976
17/795
16/667
173/3835
142/2421
14/2421
18/553
20/575
20/709
120/1660
97/1218
118/1681
13/1660
12/1218
13/1681
12/1189
11/979
5/33
10/830
10/848
10/3696
10/4370
10/4755
7/1584
7/1777
7/2094
10/6830
2/13
4/415
4/455
13/2094
Biological process
Cytoplasmic vesicle part
Cytoplasmic vesicle
Intracellular vesicle
Whole membrane
Immunological synapse
T cell receptor complex
Side of membrane
External side of plasma membrane
Plasma membrane
Cell periphery
Plasma membrane part
Membrane
Membrane raft
Membrane microdomain
Membrane attack complex
Cytoplasmic side of plasma membrane
Integral component of plasma membrane
Cytoplasmic side of membrane
Cell surface
Intrinsic component of plasma membrane
CD40 receptor complex
Cytosol
Plasma membrane receptor complex
BIM−BCL−xl complex
BIM−BCL−2 complex
CD95 death−inducing signaling complex
Bcl−2 family protein complex
Glycogen granule
Death−inducing signaling complex
73/1713
81/2779
81/2784
69/2007
10/785
43/785
8/785
6/460
5/38
4/21
10/785
8/460
28/6817
28/6955
19/3450
141/6817
141/6955
93/3450
13/6817
13/6955
11/3450
23/6817
23/6955
24/11811
6/420
6/420
2/7
5/206
12/1971
5/231
68/1971
4/1971
10/1971
12/2056
2/11
70/2056
3/1200
4/2056
6/1200
10/2056
3/11
9/1200
4/198
8/5617
1/1
1/1
1/6
1/6
1/8
1/9
Cellular localization
Peptidase activator activity
Growth factor receptor binding
Signaling pattern recognition receptor activity
Pattern recognition receptor activity
CD40 receptor binding
Cytokine receptor binding
Tumor necrosis factor receptor binding
C5a anaphylatoxin chemotactic receptor binding
Tumor necrosis factor receptor superfamily binding
Immunoglobulin receptor activity
Protein binding
Receptor binding
Low−affinity IgG receptor activity
Interleukin−7 receptor activity
Receptor activity
Molecular transducer activity
Thioesterase binding
Immunoglobulin binding
Cytokine activity
Cytokine receptor activity
Protein kinase binding
IgG receptor activity
Kinase binding
Transcription regulatory region DNA binding
Regulatory region nucleic acid binding
Chemokine activity
Transcriptional repressor activity, RNA polymerase II transcription regulatory region sequence−specific DNA binding
Signal transducer activity
Signaling receptor activity
T cell receptor binding
Enzyme binding
[acyl−carrier−protein] S−acetyltransferase activity
Enoyl−[acyl−carrier−protein] reductase (NADPH, B−specific) activity
Enoyl−[acyl−carrier−protein] reductase (NADPH, A−specific) activity
3−oxoacyl−[acyl−carrier−protein] reductase (NADPH) activity
3−oxo−pimeloyl−[acp] methyl ester reductase activity
3−oxo−glutaryl−[acp] methyl ester reductase activity
3−hydroxypalmitoyl−[acyl−carrier−protein] dehydratase activity
3−hydroxyoctanoyl−[acyl−carrier−protein] dehydratase activity
3−hydroxyacyl−[acyl−carrier−protein] dehydratase activity
Interleukin−1, Type I, activating receptor activity
16/61
20/174
10/19
10/19
3/3
9/371
7/42
7/63
39/13801
16/2144
174/13801
74/2144
71/2353
72/2410
3/12
7/371
4/42
3/9
4/63
3/12
2/2
2/2
11/2353
11/2410
3/27
5/258
4/103
7/815
2/6
7/903
10/1011
10/1015
6/301
3/59
71/2459
58/2019
9/2459
8/2019
2/24
9/2735
1/1
1/1
1/1
1/1
1/1
1/1
1/1
1/1
1/2
Molecular function
P value
XXX−Low
XX−Low
X−Low
Low

Supplemental figure 7. Transcription factor analysis: gene clusters. The top transcription factors (TFs) that display enrichment for differentially expressed genes (FDR < 0.2), after adjusting for visceral fat. Labels represent *seed nodes* and *total nodes* for that particular TF, respectively. Data are faceted on cluster affiliation (columns). Colors and point size denote strength of significance and Z score; for exact values, see Supplemental table 9. Abbreviations as for Supplemental figure 3.


Transcription regulation analysis
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
C/EBPbeta
NF−kB
IRF8
STAT3
STAT5A
SP1
c−Myc
FOXP3
STAT5
p53
IRF4
RelA (p65 NF−kB subunit)
AML1 (RUNX1)
CREB1
HIF1A
ESR1 (nuclear)
c−Jun
ETS1
STAT6
STAT1
FOXO3A
E2F1
PU.1
c−Fos
YY1
IRF1
15/16
12/13
11/12
11/12
12/13
14/15
14/15
13/14
11/12
11/12
69/69
67/67
66/66
68/68
73/74
83/84
69/69
77/78
77/78
78/79
4/5
5/6
4/5
4/5
4/5
4/5
4/5
5/6
3/4
4/5
5/6
5/6
5/6
5/6
5/6
4/5
4/5
4/5
10/11
8/9
10/11
9/10
7/8
7/8
6/7
6/7
8/9
5/5
zScore
280
260
240
220
200
P value
XXX−Low
XX−Low
X−Low
Low

Supplemental figure 8. CIBERSORT-predicted cell types. Level of key PBMC cell types predicted by CIBERSORT for serum omega 6 groups.


CIBERSORT−predicted cell types in omega 6 groups
Monocytes
NK cells resting
T cells CD8
B cells memory
T cells CD4 naive
T cells CD4 memory resting
Low
High
Percentage
Omega 6 level in plasma
Batch corrected for age, gender and strip

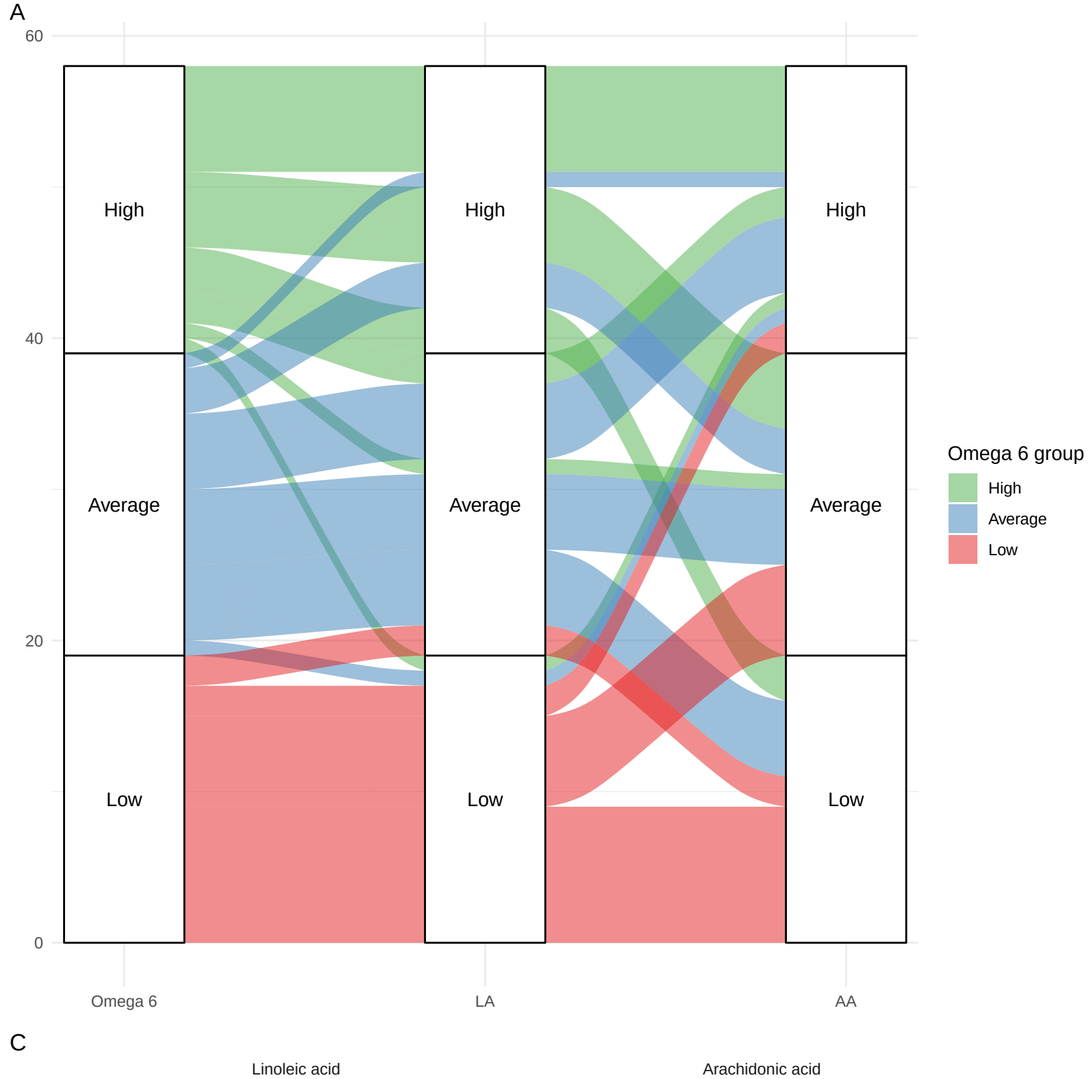
Supplemental figure 9. CIBERSORT-gene expression correlation heatmap. Spearman correlations between CIBERSORT-predicted cell types and gene expression. Data are faceted on immune cell type (rows) and cluster affiliation (columns) (see Supplemental figure 5). Blue and red colors mean positive and negative correlation coefficients, respectively. Color intensity denotes strength of the association. For abbreviations, see Supplemental material 1.

CIBERSORT−predicted cell type distribution in gene expression clusters

Spearman correlation

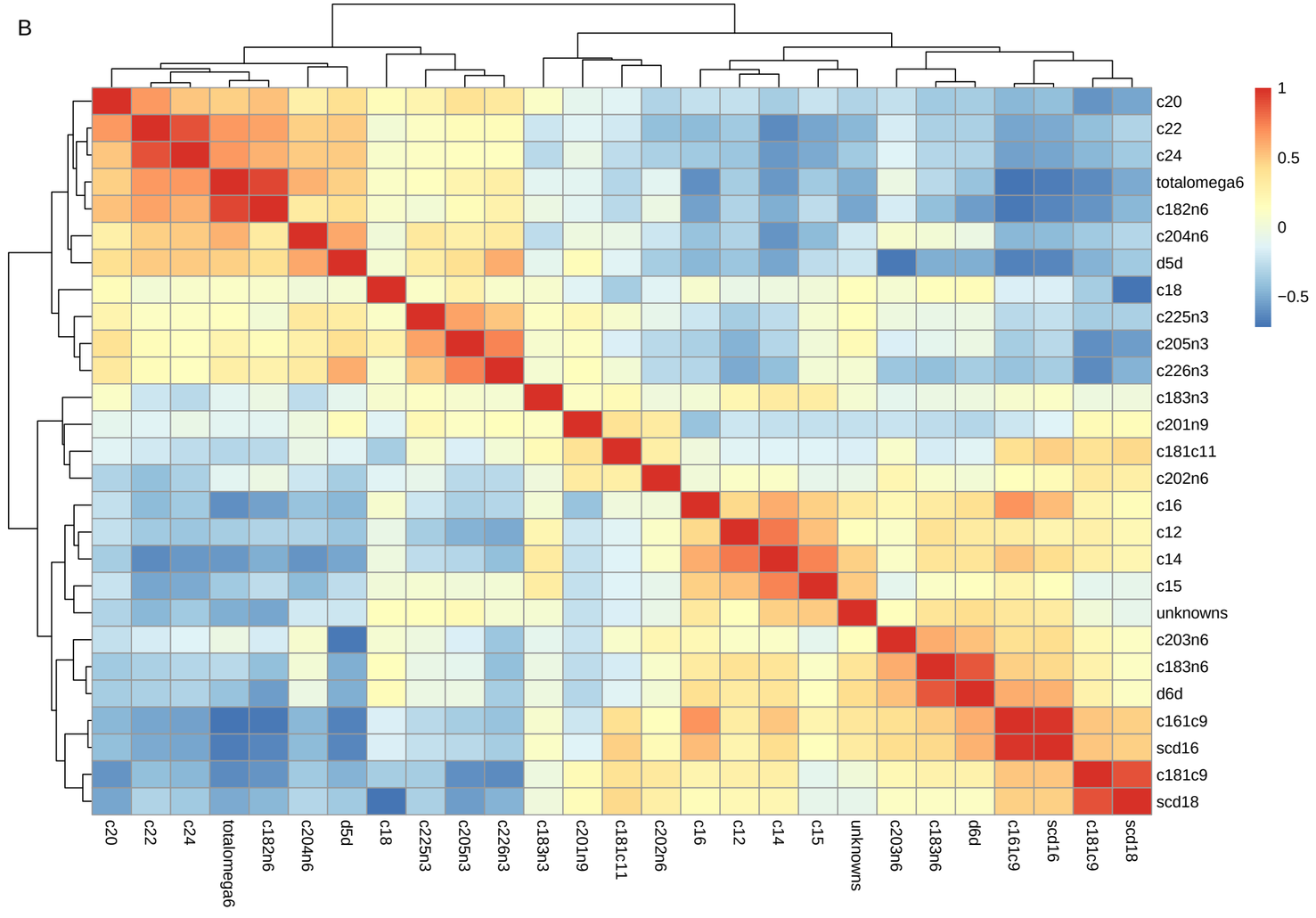
Supplemental Figure 10. Fatty acid-specific associations. Differences between linoleic acid (L) and arachidonic acid (AA) in mediating the associations between total omega 6 and gene expression. A) Alluvial diagram depicting changes in group affiliation when splitting into tertiles based on LA or AA, colored by total omega 6 group affiliation. Note the similarity between the left omega 6 group split (left column) and LA group split (middle column), and the divergence in the AA group split (right column). B) Heatmap displaying Spearman correlation coefficients between all individual fatty acids and desaturase indices, as well as total omega 6 level. Both rows and columns are clustered (Euclidean distance, hierarchical clustering, complete linkage). Note clustering of very long-chained saturated fatty acids (SFAs), LA and AA, and delta 5 desaturase (cluster 1, top left), omega 3 fatty acids (cluster 2, middle), and middle- and long-chained SFAs, monounsaturated fatty acids, and other desaturase indices (cluster 3, bottom right). C) Scatterplots of total omega 6 level versus the specific omega 6 fatty acids. Note a high positive association between total omega 6 level and LA, but lower association between total omega 6 level and *other* specific fatty acids. D) Volcano plots displaying coefficient estimates versus p-values from linear models for all 460 genes, adjusted for visceral fat, or either AA or LA level on top of visceral fat. Note fewer significantly different genes in the latter. Abbreviations: c, carbon; c20, C20:0 (and similar for the other SFAs); c204n6, C20:4-omega 6 (and similar for the other unsaturated fatty acids); d5d/d6d, delta 5/6-desaturase; scd16/scd18, Stearoyl-CoA desaturase (Δ-9-desaturase)-16/18.

A

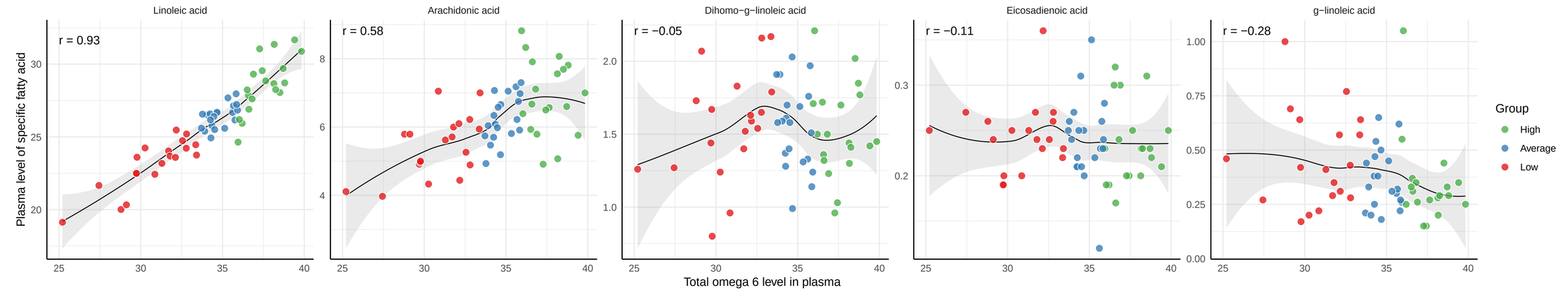

60
40
20
0
High
Average
Low
Omega 6
LA
AA

Omega 6 group
High
Average
Low

B


c20
c22
c24
totalomega6
c182n6
c204n6
d5d
c18
c225n3
c205n3
c226n3
c183n3
c201n9
c181c11
c202n6
c16
c12
c14
c15
unknowns
c203n6
c183n6
d6d
c161c9
scd16
c181c9
scd18
1
0.5
0
−0.5

C


Linoleic acid
Arachidonic acid
Dihomo−g−linoleic acid
Eicosadienoic acid
g−linoleic acid
r = 0.93
r = 0.58
r = −0.05
r = −0.11
r = −0.28
Plasma level of specific fatty acid
Total omega 6 level in plasma
Group
High
Average
Low

D

Visceral fat
Visceral fat + AA
Visceral fat + LA
−log(P value)
Difference in the high omega 6 group compared with low omega 6 group
P value
< 0.001
< 0.01
< 0.05
> 0.05

SUPPLEMENTAL INFORMATION

Supplemental table legends

Serum omega 6 fatty acids and immunology-related gene expression in peripheral blood mononuclear cells: a cross-sectional analysis in healthy children

Jacob J. Christensen, Siril S. Bakke, Stine M. Ulven, Kjetil Retterstøl, Tore Henriksen, Jens Bollerslev, Terje Espevik, Pål Aukrust, Bente Halvorsen, Kirsten B. Holven

Supplemental material 1A. Target genes for nCounter GX Human Immunology Kit v2 (Nanostring Technologies).

Supplemental material 1B. Internal reference genes for nCounter GX Human Immunology Kit v2 (Nanostring Technologies).

Supplemental material 2. R packages used in analyses pipeline.

Supplemental table 1. Extended subject characteristics.

Supplemental table 2. Differential gene expression.

Supplemental table 3. Pathway enrichment analysis of differentially expressed genes.

Supplemental table 4. Gene ontology enriched in differentially expressed genes.

Supplemental table 5A. GO-biological processes enriched in genes that are higher and lower expressed in high omega 6 group.

Supplemental table 5B. GO-biological processes enriched in genes that are higher expressed in high omega 6 group.

Supplemental table 5C. GO-biological processes enriched in genes that are lower expressed in high omega 6 group.

Supplemental table 5D. GO-biological processes enriched in genes in cluster 1.

Supplemental table 5E. GO-biological processes enriched in genes in cluster 2.

Supplemental table 5F. GO-biological processes enriched in genes in cluster 3.

Supplemental table 5G. GO-biological processes enriched in genes in cluster 4.

Supplemental table 5H. GO-biological processes enriched in genes in cluster 5.

Supplemental table 6A. GO-molecular functions enriched in genes that are higher and lower expressed in high omega 6 group.

Supplemental table 6B. GO-molecular functions enriched in genes that are higher expressed in high omega 6 group.

Supplemental table 6C. GO-molecular functions enriched in genes that are lower expressed in high omega 6 group.

Supplemental table 6D. GO-molecular functions enriched in genes in cluster 1.

Supplemental table 6E. GO-molecular functions enriched in genes in cluster 2.

Supplemental table 6F. GO-molecular functions enriched in genes in cluster 3.

Supplemental table 6G. GO-molecular functions enriched in genes in cluster 4.

Supplemental table 6H. GO-molecular functions enriched in genes in cluster 5.

Supplemental table 7A. GO-cellular localizations enriched in genes that are higher and lower expressed in high omega 6 group.

Supplemental table 7B. GO-cellular localizations enriched in genes that are higher expressed in high omega 6 group.

Supplemental table 7C. GO-cellular localizations enriched in genes that are lower expressed in high omega 6 group.

Supplemental table 7D. GO-cellular localizations enriched in genes in cluster 1.

Supplemental table 7E. GO-cellular localizations enriched in genes in cluster 2.

Supplemental table 7F. GO-cellular localizations enriched in genes in cluster 3.

Supplemental table 7G. GO-cellular localizations enriched in genes in cluster 4.

Supplemental table 7H. GO-cellular localizations enriched in genes in cluster 5.

Supplemental table 8. Gene set enrichment analysis.

Supplemental table 9A. Transcription factors enriched in genes that are higher and lower expressed in high omega 6 group.

Supplemental table 9B. Transcription factors enriched in genes that are higher expressed in high omega 6 group.

Supplemental table 9C. Transcription factors enriched in genes that are lower expressed in high omega 6 group.

Supplemental table 9D. Transcription factors enriched in genes in cluster 1.

Supplemental table 9E. Transcription factors enriched in genes in cluster 2.

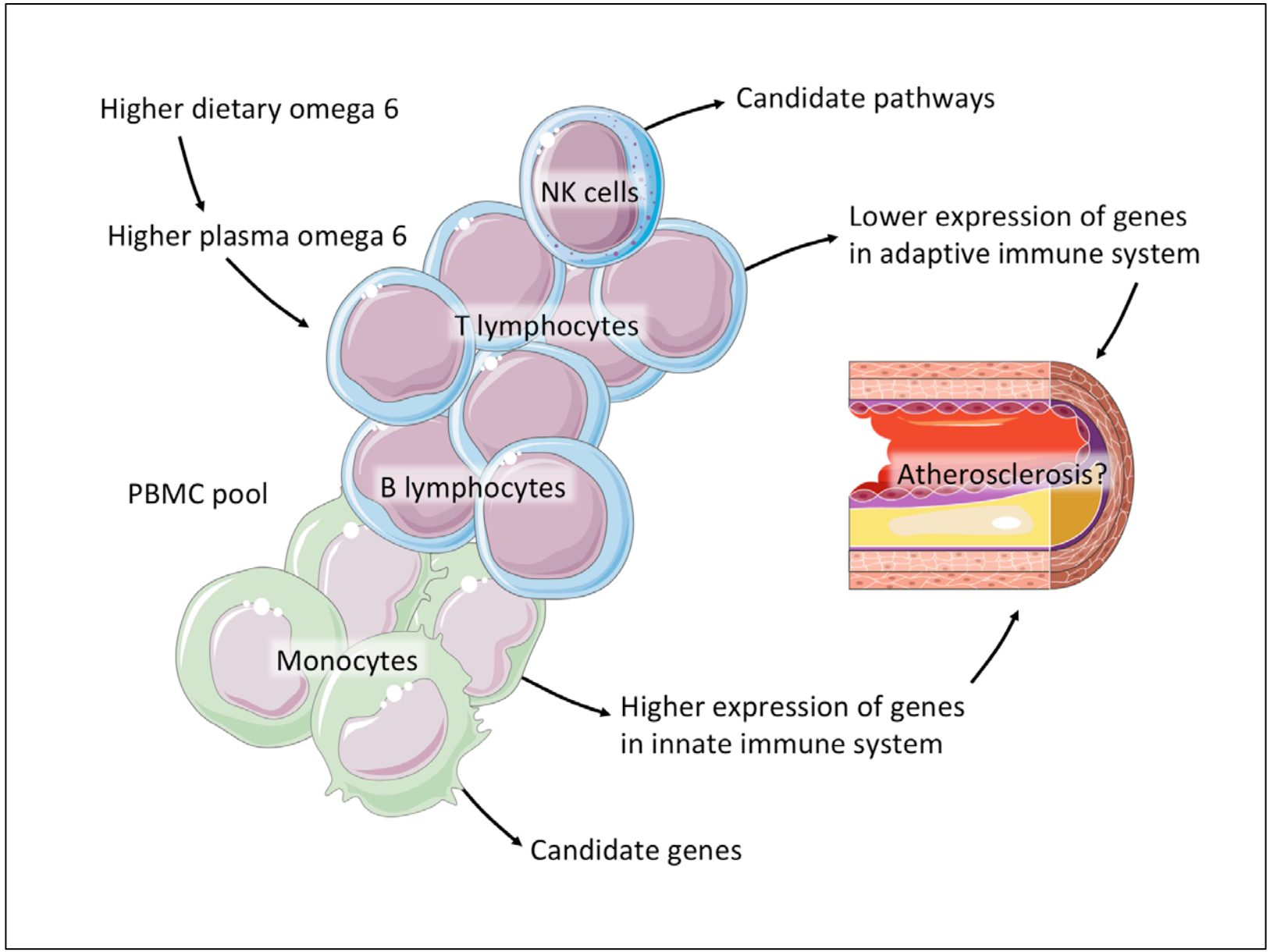
Supplemental table 9F. Transcription factors enriched in genes in cluster 3.

Supplemental table 9G. Transcription factors enriched in genes in cluster 4.

Supplemental table 9H. Transcription factors enriched in genes in cluster 5.

Christensen Mol Nutr Food Res: graphical abstract text

High omega 6 polyunsaturated fatty acids (PUFAs) level, a biomarker of dietary intake of omega 6 PUFAs, associated with higher expressing of genes related to innate immune responses, and with lower expression of genes related to adaptive immune responses in peripheral blood mononuclear cells (PBMCs) in healthy children. A number of specific immunology-related genes and pathways were significantly different between high and low omega 6 PUFA level. These may be further examined in gene-environment studies and randomized controlled trials, in order to better understand how dietary omega 6 PUFAs influence atherosclerosis development via regulation of gene expression.


Higher dietary omega 6
Higher plasma omega 6
PBMC pool
NK cells
T lymphocytes
B lymphocytes
Monocytes
Candidate pathways
Lower expression of genes
in adaptive immune system
Atherosclerosis?
Higher expression of genes
in innate immune system
Candidate genes