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Associations between dietary patterns and gene expression pattern in peripheral blood mononuclear cells: A cross-sectional study

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WGCNA

Abstract *Background and aims:* Diet may alter gene expression in immune cells involved in atherosclerotic cardiovascular disease susceptibility. However, we still lack a robust understanding of the association between diet and immune cell-related gene expression in humans. Therefore, we examined associations between dietary patterns (DPs) and gene expression profiles in peripheral blood mononuclear cells (PBMCs) in a population of healthy, Norwegian adults (n = 130 women and 105 men).
Methods and results: We used factor analysis to define *a posteriori* DPs from food frequency questionnaire-based dietary assessment data. In addition, we derived interpretable features from microarray-based gene expression data (13 967 transcripts) using two algorithms: CIBERSORT for estimation of cell subtype proportions, and weighted gene co-expression network analysis (WGCNA) for cluster discovery. Finally, we associated DPs with either CIBERSORT-predicted PBMC leukocyte distribution or WGCNA gene clusters using linear regression models. We detected three DPs that broadly reflected *Western*, *Vegetarian*, and *Low carbohydrate* diets. CIBERSORT-predicted percentage of monocytes associated negatively with the *Vegetarian* DP. For women, the *Vegetarian* DP associated with a large gene cluster consisting of 600 genes mainly involved in regulation of DNA transcription, whereas for men, the *Western* DP inversely associated with a smaller cluster of 36 genes mainly involved in regulation of metabolic and inflammatory processes. A subsequent protein–protein interaction network analysis suggested that genes within these clusters might physically interact in biological networks.
Conclusions: Although the present findings are exploratory, our analysis pipeline serves as a useful framework for studying the association between diet and gene expression.
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Abbreviations: DP, dietary pattern; PBMC, peripheral blood mononuclear cell; WGCNA, weighted gene co-expression network analysis; GO, gene ontology; PPI, protein–protein interaction.

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Introduction

Atherosclerotic cardiovascular disease (ASCVD) is one of the main causes of death worldwide [1]. It is mainly caused by life-long exposure to classical risk factors such as obesity, hypertension, dyslipidemia and dysglycemia [2]. Diet affects these risk factors and thereby contributes to the rate of disease progression [3]. Diet can also influence gene expression in immune cells directly and thus potentially affect ASCVD susceptibility [4–6]. However, we still lack a thorough understanding of the association between diet and immune cell-related gene expression.

Free-living humans consume a variety of foods in combination. To capture this variation meaningfully, we often define so-called dietary patterns (DPs). *A posteriori* DPs are data-driven; they are defined based on the co-consumption of foods in the population under study [7]. Naturally, *a posteriori* DPs reflect local food culture and have high internal validity. As such, *a posteriori* DPs may constitute robust measures of global diet exposure, and could be used to strengthen the reliability of associations between diet and biomarkers within a population. This may be especially relevant in order to examine high-variance biomarkers such as gene expression profiles.

Peripheral blood mononuclear cells (PBMCs) are directly involved in the underlying pathophysiology of ASCVD [8]. They represent a mixture of cells that are transiently part of a specialized niche in the circulation, of which some move to sites of inflammation. Affected by a number of input signals, PBMCs adapt to their environment; dietary metabolites, interleukins and chemokines, classical risk factors, and a host of other factors all influence the PBMC transcriptome [9].

Many previous studies in humans that have associated diet with PBMC gene expression have used a classical gene expression-wide association (gxWA) strategy [10,11]. The underlying correlation structure of the transcriptome, however, provides an opportunity to improve upon gxWA methods. Biologically relevant dimensionality reduction algorithms, such as CIBERSORT and weighted gene co-expression network analysis (WGCNA), simplify whole-genome gene expression matrices into interpretable features [12,13]. These methods also increase the signal-to-noise ratio and thereby robustness of the features, while simultaneously reducing the multiple testing burden [14].

The objective of the present study was to examine the associations between a posteriori-defined DPs and derived gene expression features in PBMCs in a population of healthy, Norwegian adults. We hypothesized that DPs would associate with PBMC gene expression, and that the associations would point to specific biological mechanisms that potentially mediate the effects of diet on ASCVD.

Methods

Study design and participants

The present study is based on cross-sectional data from the screening visit of a randomized controlled dietary intervention trial, presented in detail elsewhere [15]. In short, subjects were interviewed by phone, and those considered eligible were subsequently invited to a screening visit for clinical and dietary assessment, and blood sampling (Supplementary Fig. 1). All those meeting for the screening visit were included in the present study independent of whether they met the inclusion and exclusion criteria for the intervention trial. Briefly, inclusion criteria for the intervention trial were healthy women and men aged 25–70 years with moderate hypercholesterolemia (age-specific range for total cholesterol, and LDL cholesterol $\geq$ 3.5 mmol/L), fasting triglycerides $\leq$ 2.6 mmol/L, and BMI between 20 and 35 kg/m^2. Key exclusion criteria were use of lipid-lowering and certain other drugs, or blood biomarkers indicating liver, kidney or endocrine disease [15]. The study was conducted according to the Declaration of Helsinki guidelines. All subjects gave their written informed consent, and the Regional Ethics Committee for Medical Research in South East Norway approved the study. The study was registered at ClinicalTrials.gov (ClinicalTrials.gov Identifier: NCT 01679496).

We included all participants from whom we had both dietary assessment data and PBMC gene expression data, in addition to standard clinical and biochemical measurements, collected at the screening visit. After excluding four participants with self-reported energy intake above 25 MJ/d, we included 235 participants in the analyses (n = 130 women, n = 105 men) (Supplementary Fig. 1).

The subject characteristics are presented in Table 1. Briefly, the men were younger than the women, but had an unhealthier body composition and subsequent clinical sequelae. Both genders had moderate hypercholesterolemia.

Table 1 Study sample: clinical characteristics.

	Women		Men	
	Median (IQR)	Min-max	Median (IQR)	Min-max
Age, years	58 (13)	24–70	50 (15)	24–69
BMI, kg/m^2	23.7 (4.2)	17.9–36.1	26.4 (4.1)	19.5–36.4
Fat mass, kg	34.2 (7.9)	17.7–45.8	23.4 (9.2)	3.8–44.7
Fat free mass, kg	65.8 (7.9)	54.2–82.3	76.7 (9.2)	55.3–96.2
Creatinine, mmol/L	69 (12)	49–96	85.5 (13)	65–117
ASAT, U/L	20 (9)	4–61	22 (11)	12–80
ALAT, U/L	22 (8)	8–82	28 (13)	13–179
TG, mmol/L	0.9 (0.6)	0.4–3.1	1.2 (0.9)	0.1–4.7
TC, mmol/L	6.6 (1.2)	3.9–8.6	6.2 (1.4)	3.9–8.6
HDL-C, mmol/L	1.8 (0.6)	1–3	1.4 (0.4)	0.8–2.3
LDL-C, mmol/L	3.9 (1)	1.8–5.8	3.9 (1.1)	1.8–5.8

ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides.

Table 2 Groupings of food items used as input in the dietary pattern analysis.

Food group	Food items
Butter	Butter, butter-based margarines, Melange margarine
Margarine	Standard and low-fat margarine, standard and low-fat Vita, standard and low-fat Soft Flora
Cheese, high-fat	Regular Norwegian brown cheese, regular hard cheese, regular cheese spread, regular cream cheese
Cheese, low-fat	Low-fat Norwegian brown cheese, low-fat hard cheese, low-fat cheese spread, low-fat cream cheese
Dairy, high-fat	High-fat milk, flavoured milk, sour cream, ice cream, whipped cream, high-fat yogurt
Dairy, low-fat	Skimmed and semi-skimmed milk, cultured/probiotic low-fat milk, low-fat yogurt drink, low-fat yogurt
Dairy, semi low-fat	Cultura, light milk, natural yoghurt, fruit yoghurt, Go Morgen yoghurt, Biola, chocolate milk
Coffee	Boiled coffee, espresso, filter coffee, instant coffee
Tea	Black tea, green tea, herb tea
Sweet beverages	Artificially sweetened soft drinks, artificially sweetened ice tea, fruit juices with added sugar, squash with sugar, sugar-sweetened soft drinks, iced tea with sugar, orange juice, apple juice, Mana juice
Beer	Non-alcoholic beer, light beer, regular beer, alcopops
Wine	Wine, red wine, white wine
Chocolate and sweets	Chocolate, dark chocolate, extra dark chocolate, sweets/jelly sweets, sweet pastilles/candy
Desserts and snacks	Cookies, wheat bun, pastry, pastry bun, waffles, chocolate cake, cream cake, muffins, pudding, potatoe chips
Sweet spreads	Regular and low-sugar jam, honey, chocolate/nut spread, other sweet spreads
Processed meat	Meat balls, minced meat sauce, taco, kebab, lasagne, grilled/wiener sausage, minced meat sausage, bacon, pork chops, regular and low-fat liver paste, regular and low-fat saveloy, salami
Red meat	Roast of lamb/beef/pork, beef
Poultry	Chicken
Eggs	Eggs in dishes, whole eggs
Fish and shellfish	Salmon, herring, mackerel, mackerel in tomatoe (spread), achovy, caviar, cod, fish balls, fish cakes, tuna, shellfish
Legumes	Legumes
Vegetables	Carrot, cabbage, swede, cauliflower, broccoli, Brussels sprout, onion, spinach, sweet pepper, avocado, tomato, maize, frozen vegetables, mixed salad (with lettuce, cucumber, tomato and sweet pepper), vegetables as spread
Fruits and berries	Apple, pear, banana, orange, clementine, peach/nectarine, kiwi, grapes, melon, pomegranate, fruit as spreads, fresh fruit salad, prune, raisins, other dried fruits, blackberry, blueberry, raspberry, strawberry, cherry, cloudberry, rose hips, redcurrant, blackcurrant, cowberry
Rice	Rice
Potatoes	Potatoes, potato powder
Pasta	Spaghetti, nudles
Whole grains	Semi- and whole-grain bread, crisp bread (whole meal), oatmeal porridge and cereal, unsweetened muesli/breakfast cereal
Nuts and seeds	Almonds, hazelnuts, walnuts, peanuts, peanut butter, pine nuts, linseeds, sunflower seeds, nut mix, cashew nuts
Oil and oil products	Vegetable oils, oil-based dressings, dressing mix, mayonnaise-based salads, pesto
Mixed meals	Pizza, various mixed meals, including wraps and spring rolls
Potatoes, fat-rich	Pommes frites
Refined grains	White bread, crisp bread (wheat flour), hot dog bun, sweet muesli/breakfast cereal, pancake, rice porridge, waffle, sweet bun
Supplements, fish oil	Fish oil supplements, Tran

Assessment of dietary intake

See Supplemental Methods for detailed description of assessment of dietary intake. Briefly, we used a food-frequency questionnaire (FFQ) to assess habitual food intake from the preceding year [16]. We then grouped the 282 food items into 33 food groups based on food category and nutrient content (Table 2). Self-reported intake of foods and nutrients are presented in Supplementary Table 1, Supplementary Table 2, and Supplementary Table 3.

PBMC gene expression analysis pipeline

See Supplemental Methods for detailed description of PBMC isolation, RNA extraction, microarray analysis and gene expression pre-processing steps. Briefly, we collected PBMCs and extracted RNA according to standardized protocols [6]. The subsequent microarray gene expression analyses were performed using HumanHT-12 Expression BeadChips (Illumina Inc., CA, USA), and followed standard Illumina protocol (Illumina Inc., CA, USA). Finally, the gene expression probe level intensity values were subjected to pre-processing in R (version 3.6.0) using standard bioinformatic tools [17].

Statistical and bioinformatics analyses

In the following, we describe in detail the statistical and bioinformatic analyses related to DPs, gene expression clusters, and statistical modeling. All analyses were performed in R version 3.6.2 [17]. We refer to R packages and functions where appropriate, and using the following notation: `package::function`. Important deviations from default function setting are written in parentheses.

The flow of the analysis pipeline is outlined in Fig. 1. Women and men were analyzed separately, as preliminary analyses suggested a strong gender-related signal in both the DPs and gene expression dataset.

Dietary patterns

We used a combination of principal component analysis (PCA) and factor analysis to determine DPs. Factor analysis is a dimensionality reduction method similar to PCA, but it results in more interpretable features. However, because factor analysis is informed by the same covariance matrix as PCA, we used PCA-derived component variances (`stats::prcomp`) to determine a meaningful number of factors to retain; the results are presented in Supplementary Fig. 2. For both genders, the eigenvalue-one criterion suggested around 12 principal components (PCs), but there was little change between components from component 3–5 and outwards; the scree test suggested around 3–5 components; the per-component variance explained suggested that about 7–17% of the variance could be explained until about three components, and then stabilized at 4–5% at 4–6 components, with little change thereafter [18]. We decided to extract three components using factor analysis (`stats::factanal`).

Gene expression features

Two main mechanisms are central in studies of diet-related associations with ASCVD mechanisms in PBMCs: dietary effects on leukocyte subset distributions, and biological modulation independent of leukocyte subset distribution. As a result, we performed analyses to examine each of these aspects, as outlined in the upper right corner of Fig. 1.

Leukocyte subsets We used CIBERSORT to perform *in silico* flow cytometry [13]. This method uses support vector regression to conduct robust deconvolution of a heterogenous cell population, and returns predicted relative levels of various cell subsets. We used the batch-corrected, raw, untransformed, whole-genome gene expression data matrix as input. Although the algorithm provides 22 leukocyte subsets, we filtered on the topmost relevant cell types for the PBMC population, mainly monocyte and lymphocytes subsets, and thereby retained 12 cell subsets (Supplementary Fig. 3). Note that although we had standard blood cell differential counts available, CIBERSORT results in a richer set of cell subsets unique to the gene expression profile of each sample.

Gene expression clusters We used WGCNA to identify highly correlated ("co-expressed") clusters of genes [19]. The `WGCNA` package (CRAN, Bioconductor) provides a well-established and popular framework to perform the WGCNA analysis [12]. The details of the implementation can be found in Ref. [12]; in the following we briefly describe the key steps performed for the present work.

To avoid confounding by cell types, we removed the main effect of monocytes and lymphocytes with the residual method. In short, we subjected the batch-corrected, raw gene expression features ($p = 13\,967$ variables) to linear regression analyses adjusting for percentage monocytes and lymphocytes (by standard differential count) and extracted the residuals. These were then used as input for WGCNA (Fig. 1).

First, we determined the "soft thresholding power β" using the `WGCNA::pickSoftThreshold` function. This function creates a co-expression matrix and raises this to the power β to get the adjacency matrix. Balancing the approximate scale-free network properties and network connectivity, we chose $\beta = 3$ for both genders. Next, we used the high-level `WGCNA::blockwiseModules` function to create the gene expression clusters in blocks of 5000 mRNA using unsigned networks. Any genes that affiliated with a cluster with fewer than 20 members were assigned to the so-called grey cluster. Each cluster was then summarized using the first principal component (the "cluster eigengene"), and genes with low cluster membership were reassigned to another cluster. Finally, by default, cluster eigengenes that strongly correlated ($r > 0.85$) were merged to avoid redundancy.

To examine stability and validity of the resulting gene expression clusters between genders, we calculated module preservation statistics [20]. To do this, we used the `WGCNA::modulePreservation` function; we used women's cluster affiliations as reference, and men's as test, and extracted the median rank preservation and median rank quality, as well as the corresponding Z scores. In addition, we extracted the actual cross-tabulation between women's and men's clusters, and the associated P values.

To highlight a few of the more important genes within interesting clusters, we performed a *driver gene* analysis. First, we calculated *cluster membership*, which we defined as the absolute correlation between gene expression and cluster eigengene; this feature can be interpreted as the degree to which each gene contributes to that cluster's overall behavior, and contributes to its variation. Secondly, we calculated *DP significance*, which is the absolute correlation between gene expression and DP score; this feature is similar to a gxWA for all DP and single gene combinations. A positive correlation between cluster membership and DP significance indicates that those genes that drive the variation in the cluster eigengene are the same that drive the association with the specific DP (*driver genes*). Finally, to rank driver genes, driver gene estimates were calculated as the sum of the cluster membership and DP significance.

We performed gene ontology (GO) analyses to describe relevant WGCNA gene expression clusters biologically. The GO Consortium provides a comprehensive, computational model of biological systems, and is among the largest resources of gene-specific information [21,22]. We used the biomaRt::useMart(`host = "http://jan2019.archive.ensembl.org", dataset = "hsapiens_gene_ensembl"`) function to set up a connection to Ensembl, and then the `biomaRt::getBM` to retrieve various gene annotation, including chromosome, start and end, strand, and GO identifier. We then created a background annotation object for our specific gene set ($p = 13967$ genes), and used this to compile topGOdata objects using the `topGO` package. We did this for all three GO classes: biological process (BP), cellular compartment (CC) and molecular function (MF). Finally, we ran enrichment tests on the topGOdata objects and compiled the results into data tables, using the high-

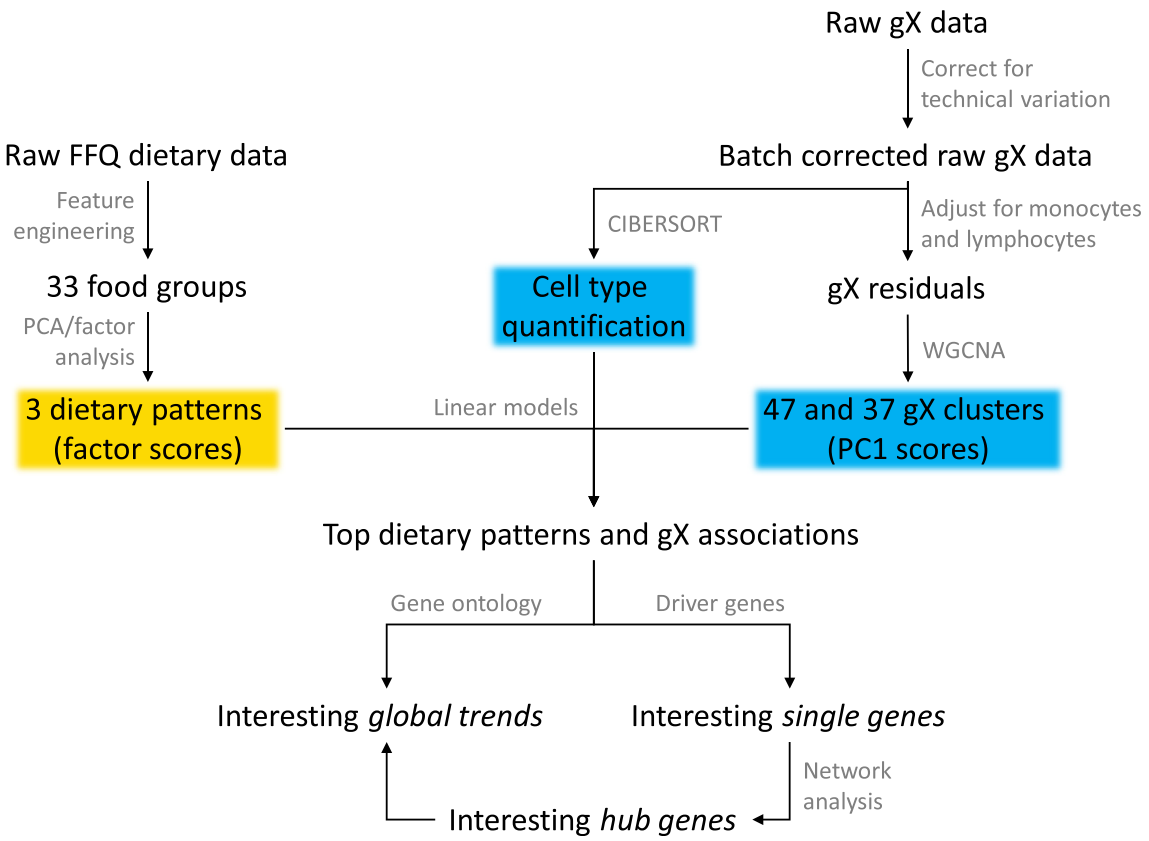

Figure 1 *Analysis pipeline*. The analysis pipeline consisted of two arms that converged in the center. The first arm (left-hand side of figure) involved feature engineering and dimension reduction analyses for the dietary data, particularly the creation of three dietary patterns. The second arm concerned work related to the gene expression data, and both the creation of 47 and 37 gene expression clusters for women and men, respectively, and an *in silico* flow cytometry cell type quantification. We used linear models and pre-specified DAGs (Supplementary Fig. 4) to evaluate the associations between the dietary and gene expression sides. Abbreviations: FFQ, food frequency questionnaire; gX, gene expression; PC1, principal component 1; WGCNA, weighted gene correlation network analysis.

level `topGO::runTest(algorithm = "classic", statistic = "fisher")` and `topGO::genTable` functions.

We performed protein–protein interaction (PPI) network analyses using The Protein Interaction Network Analysis (PINA) 2.0 database to link statistical findings with existing biological knowledge. We downloaded manually curated protein–protein interaction data from PINA (http://omics.bjcancer.org/pina/), and created networks based on input of a smaller set of driver genes defined in upstream analyses. Finally, to rank the importance of the proteins, we calculated and applied the betweenness centrality measure of nodes in the resulting networks, using the `tidygraph::centrality_betweenness` function.

Linear models

We associated DPs with the two main outcomes: CIBERSORT-predicted cell counts, and the eigengenes from the gene expression clusters, using linear models (Fig. 1). Supplementary Fig. 4 shows the directed acyclic graphs (DAGs) that guided model development. We used the open-access web-resource Dagitty (dagitty.net/dags) to evaluate these relationships. Minimal sufficient adjustment sets for estimating the *total effect* of dietary pattern on gene expression were age and education (three levels: lower, middle, higher); this is the adjustment level we report for all associations throughout the present work. For CIBERSORT-predicted cell counts, we additionally adjusted for adiposity (total fat mass, measured by bioelectrical impedance analysis) in sensitivity analyses (reported in text). Also, in sensitivity analyses for the gene expression clusters, we estimated the *direct effect* (see Supplemental Methods). The results were similar (data not shown).

Note that for all models, technical covariates (microarray chip and plate) were considered in upstream batch correction, as described in Supplemental Methods (Fig. 1). Note also that percentage of total leukocyte count of monocytes and lymphocytes (which make up the pool of PBMC subsets) were adjusted for in the gene expression pre-processing pipeline, prior to WGCNA only (Fig. 1). Finally, to aid interpretation of the results, we normalized (`base::scale`) both DP scores and cluster eigengenes to a standard normal distribution (mean = 0, SD = 1) before modeling.

Miscellaneous

The trial was originally powered to detect a clinically relevant and significant change in LDL-C [15]; however, this does not apply to the present work. Because the present work is exploratory, we did not evaluate associations by standard significance level cut-offs. Instead, we evaluated the strength and direction of associations, and their interrelations. However, to indicate the power of our analysis, we performed a simple power calculation for the general linear model, yielding the following result. Given six degrees of freedom, for an association quantified by an R^2 (explained variance) of 0.10 (which corresponds to our top findings), to have 80% power, we would have needed a sample size of approximately 150, 200 and 270 participants, for P value thresholds of 0.05, 0.01 and 0.001, respectively.

Results

Construction and description of study features

Dietary patterns

First, we constructed gender-specific DPs from self-reported FFQ data, yielding three DPs (Fig. 2 and Supplementary Table 4). We considered the patterns to reflect typical *Western* (DP1), *Vegetarian* (DP2), and *Low carbohydrate* (DP3) diets. These three DPs explained 14.1, 8.0 and 6.6%, and 16.6, 9.3 and 7.0% of the variance, for women and men, respectively. Although there was some overlap, the *Vegetarian* and *Low carbohydrate* DPs were more unique to each gender compared to the *Western* DP. This was also supported by the DP loading for various foods (Supplementary Fig. 5). For both genders, the *Western* DP associated with intake of meat and eggs, fast food, snacks, dairy, and fiber-poor carbohydrate sources. The *Vegetarian* DP associated positively with several foods perceived as healthy, including plant foods, whole grains, nuts and seeds, and tea. Additionally, the association with animal products, fast food, dairy, and fiber-poor carbohydrate foods was low or negative. For women, the association with high-fat dairy and snacks was slightly positive. The *Low carbohydrate* DP was generally a mixture of the two former DPs, reflected in positive associations for both plants and animal products. The association with fast foods, snacks and carbohydrate-rich foods, however, was negative. Wine associated positively, whereas sweet beverages associated negatively with the *Low carbohydrate* DP for women and men, respectively.

In addition to the direct link with food intake, the DP scores correlated with both macronutrient intake (Supplementary Fig. 6) and clinical variables (Supplementary Fig. 7). The *Western* DP correlated with energy intake and negatively with fiber intake in both genders. The *Vegetarian* DP correlated positively with fiber and negatively with saturated fat intake in men. In women, the *Vegetarian* DP correlated weakly, but positively, with energy, healthy fats, fiber and sugar. The *Low carbohydrate* DP was negatively correlated with carbohydrate and sugar intake in both genders, and with higher protein and fat intake in men.

For the clinical variables, the negative association between *Western* DP and age was most notable, which indicates that the younger part of the study sample adhere

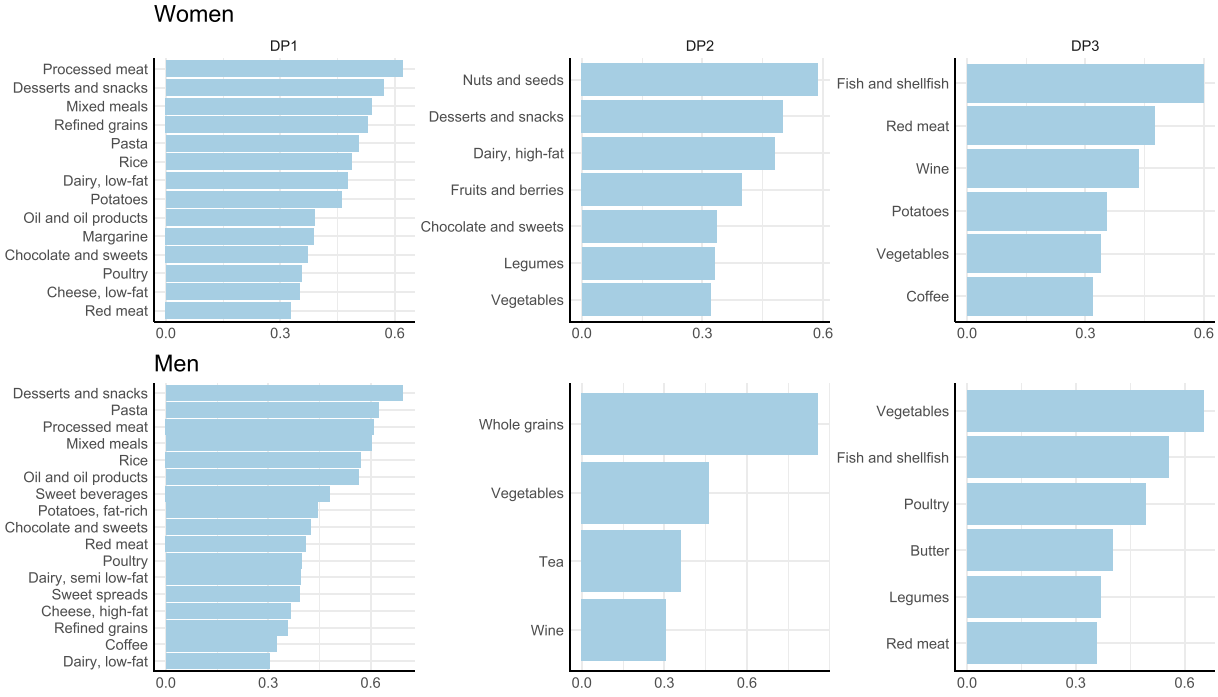

Figure 2 *DPs for women and men*. The figure shows factor loadings for all foods with a loading >0.3. See Supplementary Fig. 5 and Supplementary Table 4 for an overview of factor loading for all foods. DP1, DP2 and DP3 can be considered to reflect typical *Western*, *Vegetarian*, and *Low carbohydrate* DPs, respectively. Abbreviations: DP, dietary pattern.

to a more unhealthy diet. Additionally, The *Vegetarian* DP associated negatively with multiple obesity-related markers, including immune cells and CRP. Again, the *Low carbohydrate* DP was a mixture of the two, with positive correlations for age and lipids.

Leukocyte subsets

We used the CIBERSORT algorithm to computationally estimate the distribution of 12 leukocyte subsets (13). As expected, predicted leukocyte cell proportions associated with multiple clinical variables, although most notably for the differential count measures and obesity-related measures (Supplementary Fig. 8).

Gene expression clusters

Using the WGCNA algorithm, we detected 45 and 37 unique gene expression clusters for women and men, respectively, which by default were named different colors [12]. Although there were large differences in cluster size (range = 67–307 and 85–438 genes for women and men, respectively), most clusters explained a large proportion of the variance of the genes they comprised (range = 32–39 and 33–40% for women and men, respectively) (Supplementary Fig. 9A and B, and Supplementary Table 5). For men, explained variance inversely associated with cluster size (Supplementary Fig. 9C). In addition, genes in all clusters were generally distributed over all chromosomes, with certain exceptions, such as chromosome 1 and 19 (Supplementary Fig. 9D). The gene expression clusters displayed some correlation within each gender, but they could largely be considered unique features (Supplementary Fig. 10). Between genders, the module preservation was acceptable for most medium- and large-sized clusters, and poor for the smaller clusters (Supplementary Figs. 11–13).

Numerous gene expression clusters correlated with clinical phenotypes (Supplementary Fig. 14). Most prominent were the global associations with body composition- and lipid-related markers.

Associations of derived gene expression features with dietary patterns

Dietary patterns and leukocyte subsets

Predicted percentage of monocytes associated negatively with the *Vegetarian* DP for both women ($\beta = -0.21$, $P = 0.02$) and men ($\beta = -0.33$, $P = 0.0008$) (Fig. 3 and Supplementary Fig. 15), suggesting a link between this particular cell subset and diet. Interestingly, when adjusting for adiposity, this association was attenuated only for women ($\beta = -0.15$, $P = 0.11$ for women, and $\beta = -0.33$, $P = 0.001$ for men).

Dietary patterns and gene expression clusters

In general, relatively few associations were evident between DP scores and gene expression cluster eigengenes (Fig. 4). For women, the positive association between the *Vegetarian* DP and the yellow cluster was strongest. The yellow cluster contained 600 genes involved in regulation

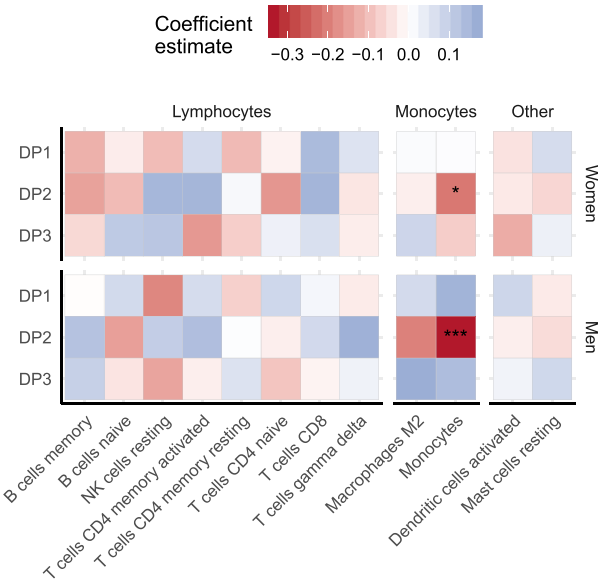

Figure 3 *DPs associate with CIBERSORT-predicted cell types*. The figure displays heatmaps of linear regression β coefficients between DP scores (as the exposure variable, shown in rows), and CIBERSORT-predicted cell types (as the outcome variable, in columns), for both women and men. Models were adjusted for age and education; in addition, the raw gene expression data were adjusted for technical variation in upstream batch correction. Asterix indicate significance level: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. See Fig. 1, Supplementary Fig. 4 and Methods for a thorough explanation of the flow of analyses and adjustment levels. DP1, DP2 and DP3 can be considered to reflect typical *Western*, *Vegetarian*, and *Low carbohydrate* DPs, respectively. Abbreviations: DP, dietary pattern.

of transcription (Supplementary Fig. 16). For men, the *Western* DP associated with multiple clusters, of which the association with the darkmagenta cluster was strongest. This cluster contained 36 genes related to metabolic and inflammatory processes, including sterol/cholesterol transport (Supplementary Fig. 16). Similarly, both the pink and greenyellow clusters associated negatively with the *Western* DP, although not as strongly as darkmagenta. The pink cluster consisted of 475 genes involved in regulation of viral processes, endosome/vacuolar transport, UDP-GlcNAc metabolism, and monocyte and lymphocyte stimulation. On the other hand, the greenyellow cluster consisted of 338 genes involved in regulation of protein synthesis and degradation, and acyl carnitine transport. The top 20 most enriched GO terms (for all three ontologies) for the topmost significant cluster for each gender are listed in Supplementary Table 6.

Identification of driver genes

Next, we examined the most relevant gene expression clusters more in detail, using a driver gene analysis to identify genes with both high DP significance and high cluster membership. Interestingly, DP significance and cluster membership associated strongly (Fig. 5A and B, and Supplementary Table 7), which suggests that genes that associated with DPs were also among the most important parts of the clusters that associated with that DP.

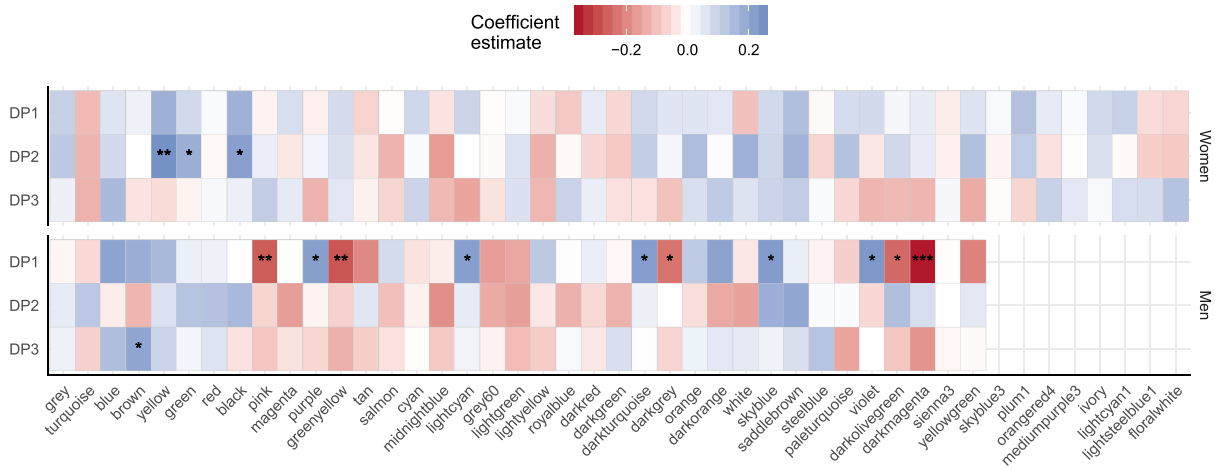

Figure 4 *DPs associate with gene expression clusters*. The figure displays heatmaps of linear regression β coefficients between DP scores (as the exposure variable, shown in rows), and gene expression cluster eigengenes (as the outcome variable, in columns), for both women and men. The clusters are sorted by size. Models were adjusted for age and education; in addition, the raw gene expression data were first adjusted for technical variation in upstream batch correction, and subsequently adjusted for relative abundance of monocytes and lymphocytes using linear regression prior to cluster discovery. Asterisks indicate significance level: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. See Fig. 1, Supplementary Fig. 4 and Methods for a thorough explanation of the flow of analyses and adjustment levels. DP1, DP2 and DP3 can be considered to reflect typical *Western*, *Vegetarian*, and *Low carbohydrate* DPs, respectively. Abbreviations: DP, dietary pattern.

The five top driver genes for the association between the *Vegetarian* DP and the yellow cluster in women were GIMAP7 (GTPase, IMAP family member 7), ZNF200 (zinc finger protein 200), LCMT2 (leucine carboxyl methyltransferase 2), GPR18 (G protein-coupled receptor 18), ASTE1 (asteroid homolog 1) (Fig. 5A). Proteins from these genes regulate aspects of biosynthetic processes, including cell signaling, DNA transcription and repair, and protein synthesis (20,21). For these genes, the correlation coefficients with DP2 score were in the range 0.19–0.26 ($P = 0.03$–0.003), and with the cluster eigengene in the range 0.83–0.90 ($P < 0.001$) (Supplementary Table 7). This means that women who consumed a *Vegetarian* DP tended to have *higher* expression of these genes in PBMCs.

The five top driver genes for the association between the *Western* DP and darkmagenta cluster in men were DMWD (DM1 locus, WD repeat containing), SYTL3 (synaptotagmin like 3), ABCA2 (ATP binding cassette subfamily A member 2), TSEN54 (tRNA splicing endonuclease subunit 54) and C9orf142/XLS (XRCC4-like small protein) (Fig. 5B). Proteins from these genes are involved in lysosomal transport, cholesterol homeostasis, mRNA processing and DNA repair (20,21). The correlation coefficients with DP1 score were in the range −0.29 to −0.22 ($P = 0.03$–0.003), and with the cluster eigengene in the range 0.75–0.83 ($P < 0.001$) (Supplementary Table 7). This means that men who consumed a *Western* DP tended to have *lower* expression of these genes in PBMCs.

Identification of hub proteins

Finally, to examine if these driver genes were part of physically interacting biological networks, we filtered them through the PINA database (22). For the strongest associations for each gender, we then created protein–protein interaction (PPI) networks (Fig. 5C and D, and Supplementary Table 8). These proteins can be considered *hub proteins*; they likely exert a higher degree of control over the protein network, as more proteins physically interact with this hub in order to influence signaling pathways. For women and men, key hub proteins included PPARGC1B (PPARG coactivator 1 beta) and UBC (ubiquitin C), respectively.

Discussion

In the present study of 235 Norwegian adults, we detected novel associations between DPs and gene expression features in PBMCs. Our results suggest that diet may affect a number of specific cell types and pathways, of which the most pronounced were predicted proportion of monocytes, regulation of transcription, and regulation of metabolic and inflammatory processes. The findings and approaches presented herein may be relevant for further understanding the role of diet on ASCVD and other lifestyle-related diseases.

We detected three DPs commonly consumed in Norway

Using data-driven analyses, we extracted three DPs (Fig. 2). These DPs made sense from a dietary perspective, matching typical patterns representing *Western*-type, *Vegetarian*-type and *Low carbohydrate*-type diets; thus, they met the interpretability criterion [7]. These DPs were neither unexpected nor surprising: Norwegian adults follow trends, and this includes the vegetarian and low carbohydrate trends. In previous studies, similar names have been used to characterize

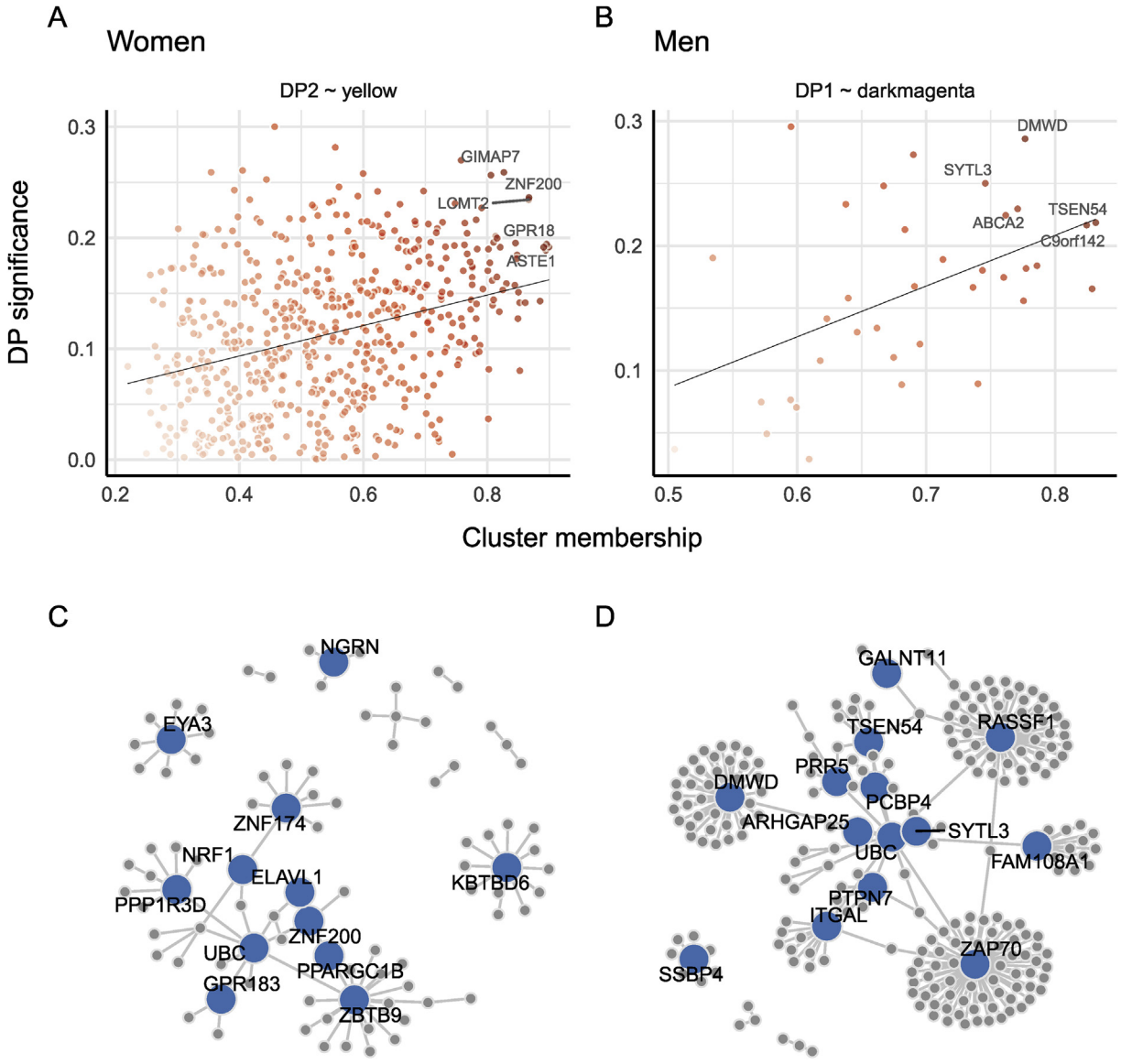

Figure 5 *Identification of driver genes and hub proteins*. A) and B) display the unadjusted, univariate association between *cluster membership* and *DP significance* for the strongest DP and gene expression cluster associations, for each gender. Cluster membership is defined as the absolute correlation between gene expression and cluster eigengene, and can be interpreted as the degree to which each gene belongs in that certain cluster, and contributes to its variation. DP significance is the absolute correlation between gene expression and DP score. A positive correlation between cluster membership and DP significance indicates that those genes the drive the variation in the cluster eigengene are the same that associate with the specific DP (*driver genes*). Finally, to rank driver genes, driver gene estimates were calculated by the sum of the cluster membership and DP significance. The darker the color, the higher the driver gene estimate; the top five genes driving this association are annotated. Note strong positive correlations for both comparisons, as is also evident from the linear regression line. C) and D) show networks of protein–protein interactions (PPI) for the same DP and gene expression cluster associations as above. Each network was created by the top 20 driver genes identified by the driver gene plot. The figures display *hub proteins* that are of particular interest to the gene regulatory network. DP1 and DP2 can be considered to reflect typical *Western* and *Vegetarian* DPs, respectively. Abbreviations: DP, dietary pattern (see Supplementary Table 7 and Supplementary Table 8 for all abbreviations) (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

detected DPs. In a cohort of Norwegian postmenopausal women, Markussen and co-workers found four DPs, including the *Western* and *Vegetarian* DPs [18]. In addition, they found a *High-protein* pattern that resembled our *Low carbohydrate* pattern. Their DPs, similar to ours, share characteristics and therefore also names, with DPs throughout Europe and the US. This emphasizes an important point: although the DPs retained in factor analyses are never exactly equal (in contrast to those from *a priori* methods), our three DPs share characteristics with many other DPs both in Norway and elsewhere [7,18,23,24].

The three DPs associated with food items, nutrient intake and clinical parameters to give a consistent picture: in general, the *Low carbohydrate* DP appeared neutral while the *Western*-type and *Vegetarian*-type DPs associated with a number of unhealthy or healthy behaviors, respectively.

Vegetarian DP associated with monocytes

The *Vegetarian* DP associated with CIBERSORT-predicted levels of monocytes (Fig. 3), suggesting that gene expression related to monocyte differentiation and activity may be affected by diet. These results are corroborated by previous reports by others and us [25–28]. Craddock and co-workers recently reviewed the evidence that vegetarian diets affect inflammatory and immune biomarkers, concluding that they associate with lower CRP, fibrinogen, and total leukocyte concentrations [25]. Similarly, Eichelmann showed that plant-based diets cause reductions in obesity-related inflammatory biomarkers such as CRP, IL6 and sICAM [26]. Indeed, our observed association between diet and monocyte levels might be related to the degree of obesity in the population; however, we found only a slight attenuation of the association for women when adjusting for adiposity. In previous work, we have shown that both diet and risk factors may affect PBMC leukocyte distribution [27,28]. We found that plasma omega 6 fatty acid level, as a marker of dietary intake of omega 6 fatty acids, was associated with predicted leukocyte distribution [27]. Vegetarian diets tend to have a high amount of vegetable oils, which may have also affected our present results. Similarly, we recently showed that children with familial hypercholesterolemia displayed an altered leukocyte distribution [28].

Most studies that examine the association between diet and immune cells use a modest number of established biomarkers, such as standard differential count or protein biomarkers. In the current analysis, however, we used approximately 14 000 mRNA transcripts from PBMCs, potentially making it a more sensitive test of associations with immune cell type distribution specifically, and inflammation in general [13]. Additionally, our finding is important since it adds to the evidence that cell type distribution in cell mixtures can influence the association between diet and gene expression. This must be taken into account when interpreting PBMC gene expression results.

DPs associated with few gene expression clusters

Few WGCNA-based gene expression clusters were associated with DPs after correction for variation in monocytes and lymphocytes number (Fig. 4). This indicates that most of the co-variation between diet and gene expression in PBMCs can be attributed to leukocyte cell type distribution. Nevertheless, in women, the *Vegetarian* DP associated most strongly with a cluster of genes involved in regulation of transcription, and in men, the *Western* DP associated most strongly with a cluster of genes related to metabolic and inflammatory processes, including sterol/cholesterol transport.

In previous reports, dietary intake of a healthy Nordic diet or omega-3 associated with expression of genes related to mitochondrial function, cell cycle, endoplasmic reticulum stress, apoptosis, and inflammatory processes [29–32]. *Regulation of transcription* is another such unspecific term; although highly unspecific, it may relate to age-related global or pathway-specific DNA methylation and gene expression [33–35].

Sterol/cholesterol transport, on the other hand, is a highly specific biological process that is dramatically affected by diet and that affects disease risk [35,36]. Plasma LDL-C is mainly determined by cellular sterol status and the functionality and activity of the LDL receptor; LDL-C in turn is a key determinant of disease risk [36]. Although cholesterol metabolism in liver and monocytes are tightly coupled and similarly regulated, our observed association likely results from molecular events occurring within the pool of PMBCs as they deal with cholesterol-related metabolic challenges. Nevertheless, PBMC expression of genes related to sterol/cholesterol transport could prove a robust marker of dietary variation [10].

Interestingly, the second and third most significant clusters that associated with the *Western* DP in men contained genes related to other metabolic processes, such as UDP-GlcNAc and acyl carnitine metabolism. While UDP-GlcNAc is involved in cellular glucose sensing, acyl carnitines are involved in fatty acid transport into the mitochondria [37,38]. Indeed, in previous work, we found that plasma levels of acyl carnitines of specific chain lengths may be directly altered by changes in fatty acid quality of the diet [6]. Taken together, these processes may be particularly sensitive to variation in dietary intake; therefore, they could potentially be biomarkers of dietary intake, and also potentially predict future risk [39,40]. However, more prediction research is needed before this can be realized.

We identified top driver genes and hub proteins

Finally, the WGCNA cluster analysis detected top driver genes that have been shown to physically interact in PPI networks (Fig. 5) [41]. This is an important finding, as it provides further biological meaning to the statistical associations, and strengthens our belief that the top driver genes may be more than just spurious associations [39]. The network analysis highlighted a few hub proteins that may act as central communicators within each cluster, such as UBC and PPARGC1B. The UBC protein is a key cell signaling molecule, especially related to ubiquitination, cytokine signaling, toll-like receptors, and nuclear factor kappa B (NFκB); in mouse models, knock-down of the ubiquitin system shows protection from diet-induced obesity [42]. Furthermore, PPARGC1B is a transcriptional co-regulator involved in a number of biological processes, including thermogenesis, bone turnover and regulation of energy expenditure by fat and glucose oxidation. For example, Yin and co-workers recently showed that PPARGC1B affects PPAR alpha to protect against cardiomyopathy [43].

Strengths and limitations

To the best of our knowledge, nobody has used CIBERSORT and WGCNA to study molecular associations with DPs. We believe these dimension reduction algorithms may be well

suited to examine diet-related effects on PBMC cell type distribution using sensitive gene expression data. Still, the present work has important limitations. First, this study has a cross-sectional, observational design, and we can therefore neither infer causality nor rule out residual confounding. Furthermore, although we have taken steps to minimize the probability of chance findings, we cannot rule this out. Our study sample is relatively small compared to, for example, Lin and co-workers [39], which increases the risk of false positive and negative findings (see power calculation in Subjects and methods). Moreover, our study sample represents a highly selected part of the Norwegian population (see inclusion and exclusion criteria in Subjects and methods), limiting the generalizability of our results. Also, the PINA analysis is limited to the last update of the background database, and it does not cover potentially relevant non-PPIs. Considering these limitations, our results should not be overinterpreted.

Conclusions

In conclusion, we detected novel associations between DPs and gene expression features in PBMCs. Our results suggest that DPs may affect monocyte proportions and the regulation of biological processes related to transcription, metabolism, and inflammation. Although the present findings are exploratory, our analysis pipeline serves as a useful framework for future studies on the association between diet and gene expression. More research is needed before our results can be translated into clinically meaningful biomarkers.

Authorship

Conception and design: LFA, KBH, SMU. Data analysis: JJC, MT, KW. Data interpretation: JJC, SMU, MT, KBH, KW, LFA. Wrote paper: JJC, SMU, MT, KW, KBH, LFA. In addition, the microarray hybridization and gene expression data preprocessing were performed at the Genomics Core Facility at NTNU, Trondheim, Norway.

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Declaration of Competing Interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.numecd.2020.06.018.

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