The impact of protein quantity during energy restriction on genome-wide gene expression analysis in adipose tissue of obese humans

Van Bussel, I.P.G.[1], Backx, E.M.P.[1,2], De Groot, C.P.G.M.[1,2], Tieland, M.[1,2], Müller, M.[1,3], Afman, L.A.[1,4]

[1] Division of Human Nutrition, Wageningen University & Research centre, Stippeneng 4, 6708 WE, Wageningen, The Netherlands;

[2] Top Institute Food and Nutrition, Wageningen, The Netherlands;

[3] Current: Norwich Medical School, University of East Anglia, NR4 7TJ, Norwich, UK;

[4] Corresponding author: Dr. L.A. Afman: Division of Human Nutrition, Wageningen University & Research centre, PO BOX 17, NL-6700 AA, Wageningen, The Netherlands; lydia.afman@wur.nl; Tel. +31 317 485789; Fax +31 317 483342

CONFLICT OF INTEREST

The project is funded by TI Food and Nutrition, a public–private partnership on precompetitive research in food and nutrition. The researchers are responsible for the study design, data collection and analysis, decision to publish and preparation of the manuscript. The authors declare no conflict of interest.

ABSTRACT

BACKGROUND: Overweight and obesity is a growing health problem worldwide. The most effective strategy to reduce weight is energy restriction (ER). ER has been shown to be beneficial in disease prevention and it reduces chronic inflammation. Recent studies suggest that reducing the protein quantity of a diet contributes to the beneficial effects by ER. The organ most extensively affected during ER is white adipose tissue (WAT).

OBJECTIVE: The first objective was to assess changes in gene expression between a high protein diet and a normal protein diet during ER. Secondly, the total effect of ER on changes in gene expression in WAT was assessed.

METHODS: In a parallel double-blinded controlled study, overweight older participants adhered to a 25% ER diet, either combined with high protein intake (HP-ER, 1.7 g/kg per day), or with normal protein intake (NP-ER, 0.9 g/kg per day) for 12 weeks. From 10 HP-ER participants and 12 NP-ER participants subcutaneous WAT biopsies were collected before and after the diet intervention. Adipose tissue was used to isolate total RNA and to evaluate whole genome gene expression changes upon a HP-ER and NP-ER diet.

RESULTS: A different gene expression response between HP-ER and NP-ER was observed for 530 genes. After NP-ER a downregulation in expression of genes linked to immune cell infiltration, adaptive immune response, and inflammasome was found whereas no such effect was found after HP-ER. HP-ER resulted in upregulation in expression of genes linked to cell cycle, GPCR signalling, olfactory signalling and nitrogen metabolism. Upon 25% ER, gene sets related to energy metabolism and immune response were decreased.

CONCLUSIONS: Based on gene expression changes, we concluded that consumption of normal protein quantity compared to high protein quantity during ER has a more beneficial effect on inflammation-related gene expression in WAT.

INTRODUCTION

Overweight and obesity is a growing health problem worldwide[1, 2, 3]. One of the most effective strategies to lose weight is energy restriction (ER): restriction of food intake without malnutrition[4]. ER is also an effective strategy to diminish age-related diseases in rodents[5] and non-human primates[6]. Recent studies in rodents suggest that not the reduction of calories itself, but the reduction of dietary protein quantity contributes to the health benefits of ER[7]. In mice for example, an *ad libitum* low protein diet seemed to be equally beneficial for health as an energy restricted diet. Low-protein, high-carbohydrate, fed mice showed improved insulin, triglyceride, and high density lipoprotein cholesterol (HDLC) levels and improved Homeostasis Model Assessment (HOMA), similar to ER fed mice, while *ad libitum* fed mice did not show this improvement[8]. Contrary to animal studies, human studies showed less consistent findings. A meta-analysis on protein diets of periods longer than 12 weeks on health outcomes such as blood pressure, LDL, HDL and total cholesterol, triglycerides, and fasting blood glucose showed inconsistent results[9]. Other shorter, but also newer long-term intervention studies mainly focussed on insulin sensitivity and observed increased insulin sensitivity upon high protein ER diets[10, 11, 12]. Based on these studies and this meta-analysis, no definitive conclusion can be drawn on the effect of protein versus other macronutrient ratios in an ER diet on markers of metabolic health. Markers of metabolic health are systemic markers reflecting the total response in the body. One of the organs largely affected by ER is the white adipose tissue (WAT). Despite the important role of visceral WAT in the pathology of obesity, the role of subcutaneous WAT is becoming more clear, especially due to the use of omics tools such as transcriptomics. It has for example been shown that subcutaneous WAT of obese individuals is

characterised by hyperplasia and hypertrophy and that expression of genes involved in fat uptake and cellular differentiation are decreased in obese individuals[13]. This likely limited ability of subcutaneous WAT to store excess energy[14] leads to the compensatory ectopically storage in organs such as liver. Nutrition may play a role in the preservation and improvement of the adequate functioning of subcutaneous WAT. Therefore subcutaneous WAT is an interesting target to study. Transcriptomics has also been used to identify differences and overlap between the different fat depots. It has been shown that deep and superficial subcutaneous WAT depots have overlapping but also site-specific gene expression profiles[15]. Also between epigastric and subcutaneous WAT and VAT overlap is found in expression of genes involved in inflammation, cell cycle and growth, cancer and development[16]. In addition, comparison between subcutaneous WAT and VAT gene expression revealed that macrophage-specific markers were visible in both[17]. During ER, not only the size of the adipocytes is reduced but also expression of genes involved in inflammation is decreased[18-20] which likely also affects other organs and the whole body metabolic health status.

In this manuscript we aimed to elucidate the effects of an exchange of carbohydrates for protein during an ER diet on pathways and signalling routes in human adipose tissue by examining changes in whole genome gene expression on subcutaneous WAT. Participants of this study were older overweight healthy men and women, following either a 12-week completely controlled normal protein ER diet (NP-ER), or a high-protein ER diet (HP-ER), in which carbohydrates of the NP-ER diet were partly replaced by protein. The diets were similar in ER, which allowed us to study potential additional effects of protein quantity over ER on gene expression changes in subcutaneous WAT.

MATERIALS AND METHODS

Study design

The current study was part of a previously published double-blind randomized study[21]. Power calculation has been described in the original study[21] and was based on the primary outcome lean body mass (LBM). Subjects were excluded if they suffered from renal insufficiency (MDRD estimated glomerular filtration rate >60 mL/min per 1.73 m^2), type 1 or type 2 diabetes (fasting glucose levels $\geqslant$7 mmol/L), cancer, chronic obstructive pulmonary disease, allergy to milk products or underwent a gastric bypass. Subjects were also excluded if they had severe loss of appetite, participated in a weight loss or heavy resistance-type exercise program three months before the intervention or if they used supplements or drugs known to interfere with energy balance. Women could only participate if they were postmenopausal (last period 1 year previous to study start). Randomization was carried out with permuted blocks, stratified by gender and BMI. This intervention study was highly controlled, as 90% of the daily energy intake was provided by the University. Sixty-one overweight and obese healthy women (n=25) and men (n=36), aged 55-70 years, were randomly assigned to either a high-protein diet (HP-ER; 1.7 g protein/kg per day) or normal protein diet (NP-ER; 0.9 g protein/kg per day), during a 12-week 25% energy intake restriction. A subcutaneous adipose tissue biopsy was taken before and after the intervention from 22 participants. The study protocol was approved by the Medical Ethical Committee of Wageningen University and written informed consent was obtained before study participation. The study was registered at clinicaltrials.gov as NCT01915030.

Adipose tissue biopsy

Abdominal subcutaneous white adipose tissue biopsies (~1 g) were collected by needle biopsy, 6 to 8 cm lateral from the umbilicus under local anaesthesia (2% lidocaine) in 22 fasted participants. For each person, the second biopsy upon the intervention was taken on the contralateral side opposite to the first biopsy taken before the intervention. After immediate washing with PBS, the tissue was snap-frozen in liquid nitrogen and stored at -80°C until analysis.

RNA isolation and microarray processing

Total RNA was extracted from frozen adipose tissue specimens using TRIzol reagent (Invitrogen, Breda, The Netherlands) and purified on columns using the Qiagen RNeasy Micro Kit (Qiagen, Venlo, The Netherlands). RNA integrity was checked with Agilent 2100 bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). Total RNA (500 ng/sample) was labelled using a one-cycle cDNA labelling kit (MessageAmpTM II-Biotin Enhanced Kit; Ambion Inc, Nieuwekerk aan de IJssel, The Netherlands). Sample labelling, hybridization to chips, and image scanning were performed according to the manufacturer's instructions. Total RNA (100 ng per sample) was labelled by Whole-Transcript Sense Target Assay and hybridized to human whole-genome Affymetrix® Human Gene 1.1 ST arrays targeting 19715 unique genes (Affymetrix, Santa Clara, CA, USA).

Microarray data analysis

Microarray quality control and normalization were performed using Bioconductor software packages integrated in an on-line pipeline called MADMAX[22]. Microarray signals were normalized using robust multichip average (RMA)[23]. Genes with normalized signals >20 on at least 6 arrays were defined as expressed and selected for further analysis. Significant different expression of individual genes were tested using the LIMMA R library[24]. Changes were considered significant

when p-value was <0.05 in a paired t-test with Bayesian correction. Data were further analysed with gene set enrichment analysis (GSEA) using pre-ranked lists based on the t-statistic[25]. Gene sets with a false discovery rate (FDR-q-value) <0.25 were defined as significantly regulated. A transcription factor analysis was performed on the differentially expressed genes (P-value <0.05) with Ingenuity Pathway Analysis (June 2012, Ingenuity Systems, Redwood City, CA, USA). Array data have been submitted to the Gene Expression Omnibus under accession number GSE84046.

Statistical Analysis of clinical measurements

Data is presented as mean ± standard deviation (SD). To check if there were baseline differences between the groups, an independent sample t-test was used. A paired t-test was used to check if parameters changed within groups over time. An unpaired t-test was used to check if changes in parameters were significant different between groups. Data were analyzed using SPSS version 22 (SPSS Inc. Chicago, IL). Results were considered statistically significant below the 0.05 level.

RESULTS

Baseline characteristics of the 22 participants volunteering a white adipose tissue biopsy are summarised in table 1. None of the participant characteristics differed between the two intervention groups (P>0.05). The effect of 12 weeks of 25% ER is seen in the decrease of 9.4 kg (±3.2) body weight on average in all participants. HP-ER and NP-ER both resulted in a decrease in body weight and BMI in both groups (supplementary table 1). Protein quantity of the diets had no effect on weight (P=0.45) or BMI change (P=0.52).

Effect of ER on gene expression: up- and downregulation

To identify the effect of 25% ER on whole genome gene expression in adipose tissue, we first analysed the two ER intervention groups as one group. A total of 1858 genes showed a significant change in expression upon 12 weeks 25% ER (supplementary figure 1).
To identify potential pathways and signalling routes, Gene Set Enrichment Analysis (GSEA) was performed. A total of 353 gene sets was enriched upon ER (supplementary table 2) of which 72 up- and 281 downregulated. To merge overlapping and similar pathways, gene sets were clustered using Cytoscape. Clusters of gene sets are summarized in table 2. Clusters of gene sets involved in energy metabolism, such as lipid metabolism and PPARα targets, NRF2 targets, glucose metabolism, and TCA cycle, as well as gene sets in oxidative phosphorylation, adaptive immune response, immune cell infiltration, and cell cycle were decreased. RNA translation and processing-related gene sets were increased.

As energy metabolism-related pathways turned out to be quite prominently regulated, we visualized the robustness of the ER-induced individual changes in expression of genes related to energy metabolism by creating a heatmap, showing gene expression changes per gene per individual (figure 1). Three out of 22 participants had a different pattern in their gene expression profiles. To evaluate whether these differences in response were due to weight loss differences, BMI/weight loss change was also plotted, below this heatmap. Weight loss or BMI change did no show consistent change with responders or non-responder profile changes. To further analyse whether correlations were present between the gene expression changes and between weight and BMI change, we created a correlation heatmap (supplementary figure 2). Correlations were observed between most genes related to energy metabolism, with the strongest correlation between expression changes of genes involved in lipid metabolism. Not many correlations were observed between gene expression changes and weight or BMI change. To visualize the genes involved in energy metabolism of which the expression was changed upon ER a schematic adipocyte was created (figure 2).

Effect of ER on gene expression: IPA Upstream Regulator analysis

To identify potential upstream transcriptional regulators of genes of which expression changed upon 25% ER, IPA Upstream Regulator Analysis was used (supplementary table 3ab). Many upstream regulators known to control lipid metabolism were significantly predicted to be inhibited and included peroxisome proliferator-activated receptor gamma (PPARG: z=-3.491, P=8.14E-12) and sterol regulatory element-binding proteins 1 and 2 (SREBF1: z=-3.683, P=3.33E-06; SREBF2: z=-3.478, P=7.41E-07). These findings fit with the strong

correlation between changes in expression of their lipid-related target genes upon 25% ER (supplementary figure 2). Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), known to regulate oxidative phosphorylation, was predicted to be inhibited as well.

Protein quantity

Effect of protein quantity on gene expression during ER

To identify the effect of a carbohydrate-for-protein exchange in addition to ER on molecular level, we compared gene expression changes upon HP-ER with gene expression changes upon NP-ER. Flowchart of selection of genes is shown in figure 3. HP-ER resulted in a significant different expression of 1869 genes and NP-ER resulted in a significant different expression of 1690 genes. A number 530 genes showed a significantly different expression change between the HP-ER and NP-ER and 500 genes showed an overlap between both diets.

Effect of ER and protein quantity on gene expression: pathway analysis

To identify in what pathways and signalling routes these genes were involved, GSEA was performed. A total of 371 gene sets were enriched in the NP-ER group, of which 46 up- and 325 downregulated. A total of 241 gene sets were enriched in the HP-ER group, of which 69 up- and 172 downregulated (supplementary table 4a-d). Comparing the response of the two diets, a number of 123 gene sets showed a significant difference between the two groups (supplementary table 5). Gene sets were clustered using Cytoscape as described above. A summary of the identified clusters with a differential change between the diets and a significant

change upon at least one of the diets, is provided in table 3. NP-ER diet showed a downregulation of pathways involved in inflammasome, adaptive immune response, immune cell infiltration, and cell cycle, while HP-ER diet did not result in a downregulation of inflammatory pathways and resulted in an upregulation of cell cycle and GPCR-signalling, olfactory, and nitrogen metabolism-related pathways.

To visualize the individual changes in expression of genes that belonged to the downregulated clusters of pathways, we selected the genes from these clusters: immune cell infiltration, inflammasome, adaptive immune response and cell cycle. Genes with a significant different expression (P-value <0.05) between the HP-ER and NP-ER diet and a significant change in expression in either the HP-ER or the NP-ER diet group, were incorporated in a heatmap (supplementary figure 3a). To identify potential correlations between those genes, a correlation heatmap was made (supplementary figure 3b). Positive correlations were observed for genes involved in immune cell infiltration and cell cycle, as is seen by the purple triangles.

Effect of protein quantity on gene expression: IPA Upstream Regulator analysis

IPA Upstream Regulator Analysis was used to identify potential upstream transcriptional regulators of genes significantly different expressed between HP-ER and NP-ER diet. Only 26S proteasome was predicted to be upregulated comparing the two groups, but was not predicted to be significantly upregulated or inhibited within the HP-ER group or in the NP-ER group.

DISCUSSION

Within this study we aimed to investigate the effect of a change in protein quantity in an ER diet on the regulation of pathways and signalling routes in human white adipose tissue. Although parameters such as weight loss, glucose, and waist circumference did not change due to altered protein quantity in the 25% ER diet, whole genome adipose tissue gene expression did change due to the difference in protein quantity. Only the normal protein ER diet (NP-ER), and not the high protein ER diet (HP-ER), resulted in a downregulation of expression of genes involved in inflammasome, immune cell infiltration, adaptive immune response, and cell cycle-related pathways in human adipose tissue. To the best of our knowledge, no studies are known that explored the effect of an exchange of protein for carbohydrates in ER diets on whole genome gene expression in human adipose tissue. Only one study could be identified that compared the effect of a high-protein, low glycaemic index and soluble fibre ER diet with a standard ER diet on white adipose tissue whole-genome gene expression, but this study showed no significant differences between the two diets. Changes in gene expression were only observed if both ER groups were combined[26]. This is partly in line with findings in our study, in which more changes in gene expression due to ER than due to protein quantity were observed. However, in contrast to the above mentioned study, we could define a clear nutrient-specific set of genes that were either more affected upon HP-ER or more affected in the NP-ER. This deviation in outcomes can be due to differences in study design. In the before mentioned study 13 persons were included, while we had a larger number of 22 participants. In our study, diets were followed for a period of 12 weeks, in contrast to only 4 weeks, which could account for a more persisting effect of the diets on gene expression. Furthermore, the cross-over design had a

300 washout period of 8 weeks between both ER diets. This period might have been
301 too short for adipocytes to recover from ER and may have caused a carryover-
302 effect on gene expression.
303 The observed reduction in expression of genes involved in immune response
304 pathways and inflammasome-related pathways upon a normal protein diet which
305 was not observed on a high-protein diet, is interesting with respect to their role
306 in inflammation in adipose tissue. Adipose tissue in obese individuals is
307 characterized by increased expression of genes involved in the inflammasome
308 and immune response[27 28]. Several studies have observed that caloric restriction
309 or exercise-mediated weight-loss resulted in a reduced expression of these
310 genes[29]. Interestingly, we only observed those effects for the normal protein
311 diet. When the protein quantity of the ER diet increased, the beneficial effects on
312 inflammation–related gene expression were not observed. This observation
313 points towards the potential importance of dietary macronutrient composition
314 during ER on adipose tissue health. Despite the findings of macronutrient-specific
315 effects, the impact of ER on gene expression was much greater. Moreover,
316 strong correlations between changes in gene expression were observed,
317 suggesting a potential upstream regulator responsible for this accurate regulation
318 in expression. Especially a strong correlation between changes in expression of
319 lipid-related genes upon ER was found. In line with this, upstream regulators
320 PPARG and SREBPs, strong regulators of lipid metabolism, were predicted to be
321 inhibited. Furthermore, oxidative phosphorylation related-pathways were
322 downregulated upon ER, which is in line with the finding that PGC-1α was
323 predicted to be inhibited. In addition to the pathway analysis, which identified
324 only downregulated pathways related to energy metabolism upon ER, expression
325 of several genes were found to be upregulated upon CR that were related to

energy metabolism pathways as well. For example, phosphoenolpyruvate carboxykinase 2 (PCK2) and pyruvate dehydrogenase kinase 4 (PDK4) were upregulated. PCK2 catalyses the rate-limiting step in gluconeogenesis, when glucose is formed from lactate and other precursors derived from the TCA-cycle. Upregulation of PCK2 could be due to shortage of glucose intake and the subsequent increased need of glucose formation from different precursors. PDK4 is a key inhibitor in glucose metabolism[30] and its upregulation may be explained by a decreased need for glucose oxidation. PDK4 gene expression is also known to be upregulated in human PBMCs upon fasting[31]. Genes involved in lipid metabolism were also upregulated. For example angiopoietin-like 4 (ANGPTL4), an inhibitor of lipoprotein lipase (LPL), was upregulated and in line with this LPL was downregulated. This inhibition of LPL during ER can be explained by the assumption that fat storage is not of primary importance during ER. Also cell death activator (CIDEA), important for lipolysis, was upregulated upon ER which can be explained by a higher demand for stored lipids as energy source. PCK2, PDK4, ANGPTL4, and CIDEA are all well-known PPAR targets pointing to two kind of effects on PPAR-target-genes, either up- or downregulation, depending on their functional role in adipose tissue during ER.

One problem in changing one macronutrient in a diet is the consequential change of another macronutrient. In our study, protein was exchanged for carbohydrates. The effects could therefore also be due to the higher amount of carbohydrates in the NP-ER diet, or due to the lower amount of carbohydrates in the HP-ER diet. Especially the downregulation observed in glucose metabolism may be related to the decrease in carbohydrate quantity. One clear finding that has also been found in other transcriptome studies is the variation in response. In three out of 22 participants we observed a different gene expression response

upon ER. We could not explain this variation by the amount of weight loss or weight gain. Genetic background could be important in explaining some of the variation in gene expression, as was shown before[32], but sample sizes within this study were too small to adequately measure such effects.

A strength of our study is the highly controlled food intake. Participants were provided with all meals and always consumed their hot meal at the University, leading to a high compliance. Furthermore, effects of participants' habitual diets were largely ruled out before the start of the intervention due to one week of standardised meals for the participants. However, the number of adipose biopsies was small: 10 and 12 participants per intervention arm. The aim of this study was explorative and therefore an FDR q-value of <0.25 was selected. However, examining the data with an FDR q-value of <0.1 resulted in the same clusters of gene sets and conclusion. Although findings are quite robust and provide some interesting leads, care should be taken in interpretation and translation of the findings, since results have not yet been replicated independently. Protein quantity has been studied to investigate its effect on health parameters related to muscle mass but findings show discrepancies [21]. As our findings are based on adipose tissue gene expression, caution should be taken when translating this to health advice. Further studies are needed that explore our findings in a larger population.

In conclusion, 25% ER induces a decrease in lipid and energy metabolism-related pathways, likely partly regulated via PPARG and PGC1α, in white adipose tissue in humans. The consumption of normal protein quantity compared to a high protein quantity during ER has a more beneficial effect on inflammation-related

gene expression in adipose tissue, as reflected by a decrease in inflammasome and adaptive immunity response-related pathways.

ACKNOWLEDGEMENTS

We thank the participants, and Shohreh Keshtkar, Mechteld Grootte-Bromhaar, Jenny Jansen, the nurses, and dieticians for their practical work during the study. Furthermore, we thank Philip de Groot for helping with microarray analysis. Funding was provided by NutriTech, which is financed by the European Commission in the 7th Framework Programme FP7, Grant agreement no: 289511 Version date: 2012-11-30.

AUTHORS

IB: Van Bussel, I.P.G.;

EB: Backx, E.M.P.;

CG: De Groot, C.P.G.M.;

MT: Tieland, M.;

MM: Müller, M.;

LA: Afman, L.A.;

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: EB, MT, CG. Performed the experiments: IB. Analyzed the data: IB, LA. Wrote the paper: IB. Critically revised the manuscript for important intellectual content: EB, MT, CG, MM, LA.

SUPPLEMENTARY INFORMATION

Supplementary files are available at Internal Journal of Obesity's website.

REFERENCES

1. Ogden CL, Carroll MD, Flegal KM. Prevalence of obesity in the United States. *JAMA : the journal of the American Medical Association* 2014; **312**(2)**:** 189-90.

2. Ogden CL, Carroll MD, Kit BK, Flegal KM. Prevalence of childhood and adult obesity in the United States, 2011-2012. *JAMA : the journal of the American Medical Association* 2014; **311**(8)**:** 806-14.

3. von Ruesten A, Steffen A, Floegel A, van der AD, Masala G, Tjonneland A *et al.* Trend in obesity prevalence in European adult cohort populations during follow-up since 1996 and their predictions to 2015. *PloS one* 2011; **6**(11)**:** e27455.

4. Krieger JW, Sitren HS, Daniels MJ, Langkamp-Henken B. Effects of variation in protein and carbohydrate intake on body mass and composition during energy restriction: a meta-regression 1. *The American journal of clinical nutrition* 2006; **83**(2)**:** 260-74.

5. Weindruch R, Kayo T, Lee CK, Prolla TA. Microarray profiling of gene expression in aging and its alteration by caloric restriction in mice. *The Journal of nutrition* 2001; **131**(3)**:** 918S-923S.

6. Mattison JA, Roth GS, Beasley TM, Tilmont EM, Handy AM, Herbert RL *et al.* Impact of caloric restriction on health and survival in rhesus monkeys from the NIA study. *Nature* 2012.

7. Pamplona R, Barja G. Mitochondrial oxidative stress, aging and caloric restriction: the protein and methionine connection. *Biochimica et biophysica acta* 2006; **1757**(5-6)**:** 496-508.

8. Solon-Biet SM, Mitchell SJ, Coogan SC, Cogger VC, Gokarn R, McMahon AC *et al.* Dietary Protein to Carbohydrate Ratio and Caloric Restriction: Comparing Metabolic Outcomes in Mice. *Cell reports* 2015.

9. Naude CE, Schoonees A, Senekal M, Young T, Garner P, Volmink J. Low carbohydrate versus isoenergetic balanced diets for reducing weight and cardiovascular risk: a systematic review and meta-analysis. *PloS one* 2014; **9**(7)**:** e100652.

10. Piatti PM, Monti F, Fermo I, Baruffaldi L, Nasser R, Santambrogio G *et al.* Hypocaloric high-protein diet improves glucose oxidation and spares lean body mass: comparison to hypocaloric high-carbohydrate diet. *Metabolism: clinical and experimental* 1994; **43**(12)**:** 1481-7.

11. Mehrabani HH, Salehpour S, Amiri Z, Farahani SJ, Meyer BJ, Tahbaz F. Beneficial effects of a high-protein, low-glycemic-load hypocaloric diet in overweight and obese women with polycystic ovary syndrome: a randomized controlled intervention study. *Journal of the American College of Nutrition* 2012; **31**(2)**:** 117-25.

12. de Luis DA, Izaola O, Aller R, de la Fuente B, Bachiller R, Romero E. Effects of a high-protein/low carbohydrate versus a standard hypocaloric diet on adipocytokine levels and insulin resistance in obese patients along 9months. *Journal of diabetes and its complications* 2015.

13. Rodriguez-Acebes S, Palacios N, Botella-Carretero JI, Olea N, Crespo L, Peromingo R *et al.* Gene expression profiling of subcutaneous adipose tissue in morbid obesity using a focused microarray: Distinct expression of cell-cycle- and differentiation-related genes. *Bmc Med Genomics* 2010; **3**.

14. Danforth E. Failure of adipocyte differentiation causes type II diabetes mellitus? *Nature genetics* 2000; **26**(1)**:** 13-13.

15. Cancello R, Zulian A, Gentilini D, Maestrini S, Della Barba A, Invitti C *et al.* Molecular and morphologic characterization of superficial- and deep-subcutaneous adipose tissue subdivisions in human obesity. *Obesity* 2013; **21**(12)**:** 2562-2570.

16. Gerhard GS, Styer AM, Strodel WE, Roesch SL, Yavorek A, Carey DJ *et al.* Gene expression profiling in subcutaneous, visceral and epigastric adipose tissues of patients with extreme obesity. *International journal of obesity* 2014; **38**(3)**:** 371-378.

17. Klimcakova E, Roussel B, Kovacova Z, Kovacikova M, Siklova-Vitkova M, Combes M *et al.* Macrophage gene expression is related to obesity and the metabolic syndrome in human subcutaneous fat as well as in visceral fat. *Diabetologia* 2011; **54**(4)**:** 876-887.

18. Ye J, Keller JN. Regulation of energy metabolism by inflammation: a feedback response in obesity and calorie restriction. *Aging* 2010; **2**(6)**:** 361-8.

19. Clement K, Viguerie N, Poitou C, Carette C, Pelloux V, Curat CA *et al.* Weight loss regulates inflammation-related genes in white adipose tissue of obese subjects. *Faseb Journal* 2004; **18**(14)**:** 1657-1669.

20. Johansson LE, Danielsson AP, Parikh H, Klintenberg M, Norstrom F, Groop L *et al.* Differential gene expression in adipose tissue from obese human subjects during weight loss and weight maintenance. *The American journal of clinical nutrition* 2012; **96**(1)**:** 196-207.

21. Backx EM, Tieland M, Borgonjen-van den Berg KJ, Claessen PR, van Loon LJ, de Groot LC. Protein intake and lean body mass preservation during energy intake restriction in overweight older adults. *International journal of obesity* 2015.

22. Lin K, Kools H, de Groot PJ, Gavai AK, Basnet RK, Cheng F *et al.* MADMAX - Management and analysis database for multiple ~omics experiments. *Journal of integrative bioinformatics* 2011; **8**(2)**:** 160.

23. Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. Summaries of Affymetrix GeneChip probe level data. *Nucleic acids research* 2003; **31**(4)**:** e15.

24. Smyth GK. Limma: linear models for microarray data. *In: Gentleman R, Carey V, Huber W, Irizarry R, Dudoit S, editor. Bioinformatics and Computational Biology Solutions Using R and Bioconductor. Springer* 2005**:** 397–420.

25. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 2005; **102**(43)**:** 15545-50.

26. Rizkalla SW, Prifti E, Cotillard A, Pelloux V, Rouault C, Allouche R *et al.* Differential effects of macronutrient content in 2 energy-restricted diets on cardiovascular risk factors and adipose tissue cell size in moderately obese individuals: a randomized controlled trial. *The American journal of clinical nutrition* 2012; **95**(1)**:** 49-63.

27. Stienstra R, van Diepen JA, Tack CJ, Zaki MH, van de Veerdonk FL, Perera D *et al.* Inflammasome is a central player in the induction of obesity and insulin resistance. *Proc Natl Acad Sci U S A* 2011; **108**(37)**:** 15324-9.

28. Rainone V, Schneider L, Saulle I, Ricci C, Biasin M, Al-Daghri NM *et al.* Upregulation of inflammasome activity and increased gut permeability are associated with obesity in children and adolescents. *International journal of obesity* 2016.

29. Vandanmagsar B, Youm YH, Ravussin A, Galgani JE, Stadler K, Mynatt RL *et al.* The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. *Nature medicine* 2011; **17**(2)**:** 179-88.

30. Palomer X, Alvarez-Guardia D, Rodriguez-Calvo R, Coll T, Laguna JC, Davidson MM *et al.* TNF-alpha reduces PGC-1alpha expression through NF-kappaB and p38 MAPK leading to increased glucose oxidation in a human cardiac cell model. *Cardiovascular research* 2009; **81**(4)**:** 703-12.

31. Bouwens M, Afman LA, Muller M. Fasting induces changes in peripheral blood mononuclear cell gene expression profiles related to increases in fatty acid beta-oxidation: functional role of peroxisome proliferator activated receptor alpha in human peripheral blood mononuclear cells. *The American journal of clinical nutrition* 2007; **86**(5)**:** 1515-23.

32. Matualatupauw JC, Radonjic M, van de Rest O, de Groot LC, Geleijnse JM, Muller M *et al.* Apolipoprotein E genotype status affects habitual human blood mononuclear cell gene expression and its response to fish oil intervention. *Molecular nutrition & food research* 2016; **60**(7)**:** 1649-60.

FIGURE LEGEND

Figure 1. Heatmap of individual gene expression changes upon 25% ER of genes involved in energy metabolism. Each column represents the signal log ratio of one person; each row represents one gene. Legend:

Colours in heatmap:
Blue = downregulated, Orange = upregulated;
Red: genes in Oxidative phosphorylation;
Green: genes in NRF2 targets;
Purple: genes in Lipid metabolism;
Pink: genes in TCA cycle;
Light blue: genes in Glucose metabolism.

Figure 2. Schematic visualisation of adipocyte with up- or downregulated expression of genes involved in energy metabolism that changed upon 25% ER.

Figure 3. Stepwise selection of genes for microarray analysis: first, genes were selected for their signal intensity (≥20 in >6 arrays), and second, for a change in expression upon either normal protein energy restriction (NP-ER) diet, or upon high protein energy restriction (HP-ER) diet ($P<0.05$). The last block shows the number of genes that have a significantly different change in expression between HP-ER and NP-ER.

TABLES

Table 1. Baseline characteristics of participants included in the microarray analysis of adipose tissue biopsies.

Legend belonging to table 1: Data represent mean and (SD), or median and [range]. HP-ER: High protein-energy restriction; NP-ER: Normal protein-energy restriction; ♂: men; ♀: women.

Table 2. Summary of changes in main clusters of pathways in white adipose tissue of the total study population upon 12 weeks of 25% energy restriction (supplementary table 1).

Legend belonging to table 2: Significantly changed pathways are determined with GSEA and clusters are based upon Cytoscape ↑: gene sets in this pathway-cluster were upregulated; ↓: gene sets in this pathway-cluster were downregulated. [1]Selection of these clusters based on: gene sets with a significantly different response between HP-ER and NP-ER, and significantly changed upon HP-ER (left) or NP-ER (right).

Table 3. Summary of changes in main clusters of pathways in white adipose tissue upon 12 weeks of 25% energy restriction with either high protein (HP-ER) or normal protein (NP-ER).

Legend belonging to table 3: Significantly changed pathways are determined with GSEA and clusters are based upon Cytoscape ↑: gene sets in this pathway-cluster were upregulated; ↓: gene sets in this pathway-cluster were downregulated; - pathway cluster was not changed. [1]Selection of these clusters based on: gene sets with a significantly different response between HP-ER and NP-ER, and significantly changed upon HP-ER (left) or NP-ER (right).

Table 1. Baseline characteristics of participants included in the microarray analysis of adipose tissue biopsies.

	HP-ER n=12	**NP-ER n=10**
Age (y)	62.3 [56, 69]	61.6 [57, 68]
Gender	8♂ / 4♀	7♂ / 3♀
Height (m)	1.72 (0.11)	1.74 (0.068)
Weight (kg)	93.2 (10.2)	91.9 (6.1)
Body mass index (kg/m^2)	31 (3)	30 (2)
Glucose (mmol/L)	6.0 (0.59)	5.9 (0.54)
Waist circumference (cm)	110 (9.30)	110 (6.41)

Data represent mean and (SD), or median and [range]. HP-ER: High protein-energy restriction; NP-ER: Normal protein-energy restriction; ♂: men; ♀: women.

Table 2. Summary of changes in main clusters of pathways in white adipose tissue of the total study population upon 12 weeks of 25% energy restriction (supplementary table 1).

Pathway cluster[1]	25% ER (n = 22)
Lipid metabolism and PPARα targets	↓
NRF2 targets	↓
Glucose metabolism	↓
TCA cycle	↓
Oxidative phosphorylation	↓
Adaptive immune response	↓
Immune cell infiltration	↓
Cell cycle	↓
RNA translation and processing	↑

Significantly changed pathways are determined with GSEA and clusters are based upon Cytoscape ↑: gene sets in this pathway-cluster were upregulated; ↓: gene sets in this pathway-cluster were downregulated.

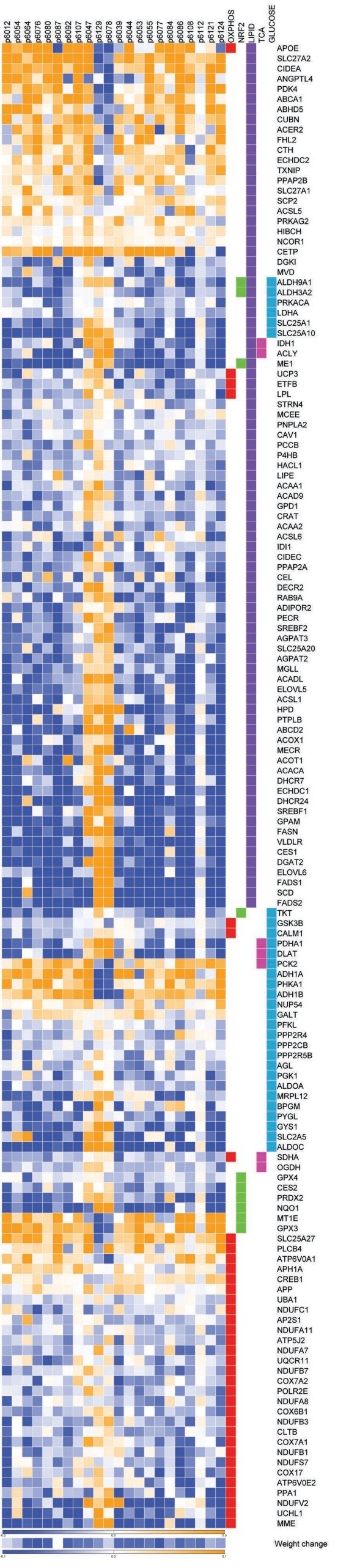
[1]Selection of these clusters based on: gene sets with a significantly different response between HP-ER and NP-ER, and significantly changed upon HP-ER (left) or NP-ER (right).

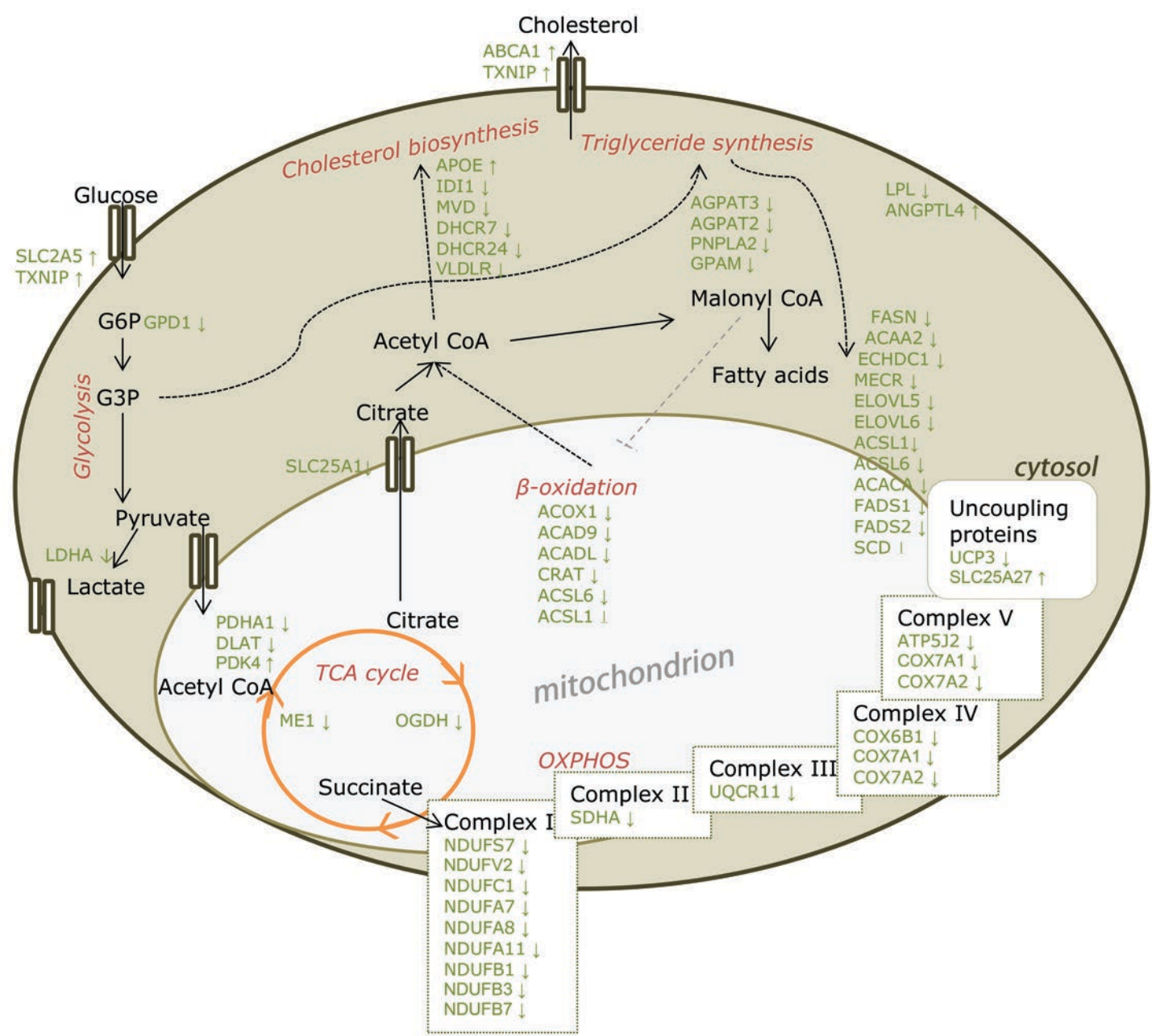
Table 3. Summary of changes in main clusters of pathways in white adipose tissue upon 12 weeks of 25% energy restriction with either high protein (HP-ER) or normal protein (NP-ER).

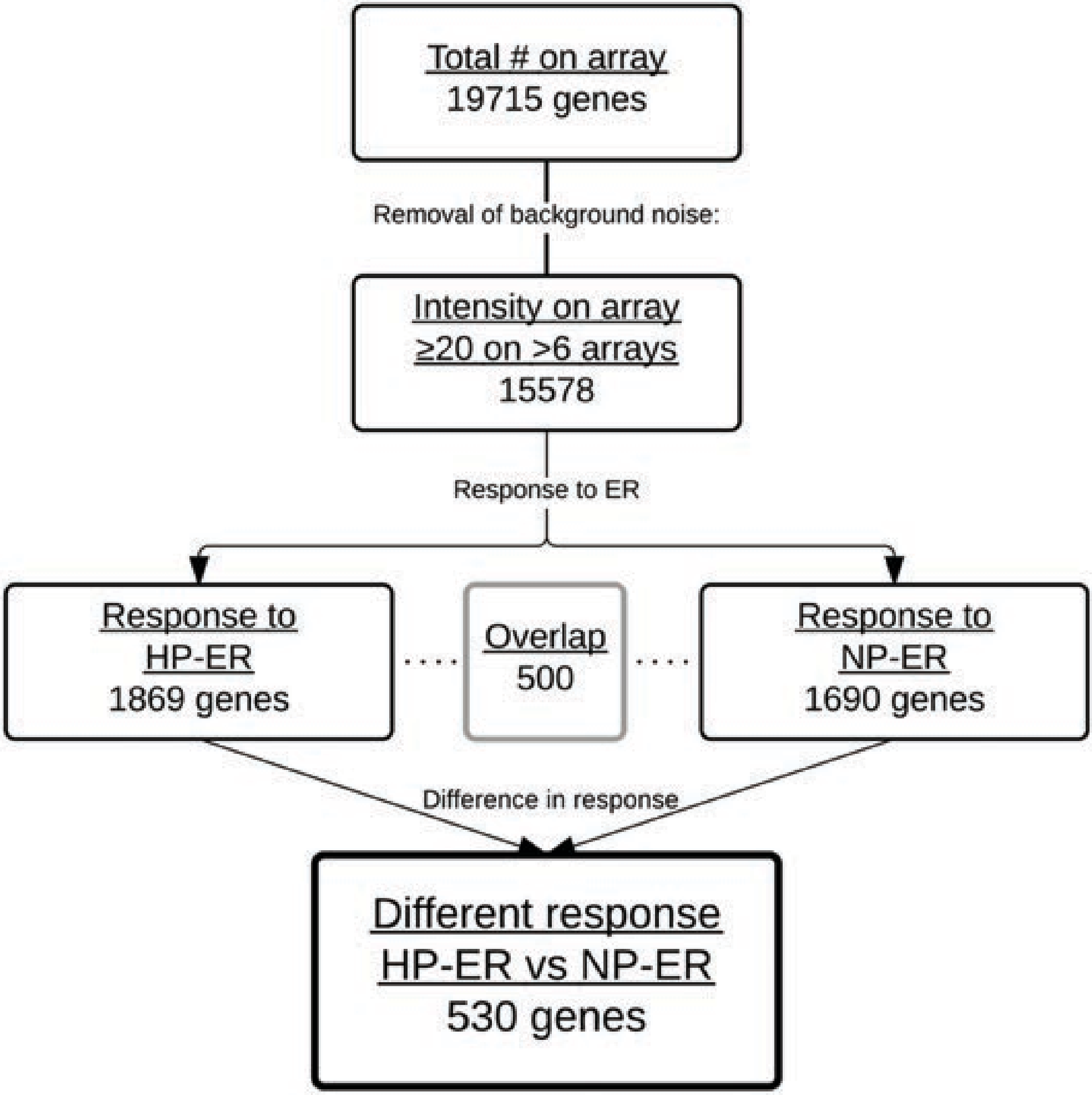
Pathway cluster[1]	**HP-ER (n = 12)**	**NP-ER (n = 10)**
Immune cell infiltration	-	↓
Inflammasome	-	↓
Adaptive immune response	-	↓
Cell cycle	-/↑	↓
GPCR Signalling	-/↑	↓
Including olfactory signalling	↑	-
Nitrogen metabolism	↑	-

Significantly changed pathways are determined with GSEA and clusters are based upon Cytoscape ↑: gene sets in this pathway-cluster were upregulated; ↓: gene sets in this pathway-cluster were downregulated; - pathway cluster was not changed.

[1]Selection of these clusters based on: gene sets with a significantly different response between HP-ER and NP-ER, and significantly changed upon HP-ER (left) or NP-ER (right).


p6012
p6054
p6064
p6076
p6080
p6087
p6092
p6107
p6047
p6129
p6078
p6039
p6044
p6053
p6055
p6077
p6084
p6086
p6108
p6112
p6121
p6124
OXPHOS
NRF2
LIPID
TCA
GLUCOSE
APOE
SLC27A2
CIDEA
ANGPTL4
PDK4
ABCA1
ABHD5
CUBN
ACER2
FHL2
CTH
ECHDC2
TXNIP
PPAP2B
SLC27A1
SCP2
ACSL5
PRKAG2
HIBCH
NCOR1
CETP
DGKI
MVD
ALDH9A1
ALDH3A2
PRKACA
LDHA
SLC25A1
SLC25A10
IDH1
ACLY
ME1
UCP3
ETFB
LPL
STRN4
MCEE
PNPLA2
CAV1
PCCB
P4HB
HACL1
LIPE
ACAA1
ACAD9
GPD1
CRAT
ACAA2
ACSL6
IDI1
CIDEC
PPAP2A
CEL
DECR2
RAB9A
ADIPOR2
PECR
SREBF2
AGPAT3
SLC25A20
AGPAT2
MGLL
ACADL
ELOVL5
ACSL1
HPD
PTPLB
ABCD2
ACOX1
MECR
ACOT1
ACACA
DHCR7
ECHDC1
DHCR24
SREBF1
GPAM
FASN
VLDLR
CES1
DGAT2
ELOVL6
FADS1
SCD
FADS2
TKT
GSK3B
CALM1
PDHA1
DLAT
PCK2
ADH1A
PHKA1
ADH1B
NUP54
GALT
PFKL
PPP2R4
PPP2CB
PPP2R5B
AGL
PGK1
ALDOA
MRPL12
BPGM
PYGL
GYS1
SLC2A5
ALDOC
SDHA
OGDH
GPX4
CES2
PRDX2
NQO1
MT1E
GPX3
SLC25A27
PLCB4
ATP6V0A1
APH1A
CREB1
APP
UBA1
NDUFC1
AP2S1
NDUFA11
ATP5J2
NDUFA7
UQCR11
NDUFB7
COX7A2
POLR2E
NDUFA8
COX6B1
NDUFB3
CLTB
COX7A1
NDUFB1
NDUFS7
COX17
ATP6V0E2
PPA1
NDUFV2
UCHL1
MME
-0.5
0.0
0.5
-0.1
0.0
0.1
Weight change


Cholesterol
ABCA1 ↑
TXNIP ↑
Cholesterol biosynthesis
Triglyceride synthesis
APOE ↑
IDI1 ↓
MVD ↓
DHCR7 ↓
DHCR24 ↓
VLDLR ↓
AGPAT3 ↓
AGPAT2 ↓
PNPLA2 ↓
GPAM ↓
LPL ↓
ANGPTL4 ↑
Glucose
SLC2A5 ↑
TXNIP ↑
G6P
GPD1 ↓
Glycolysis
G3P
Acetyl CoA
Malonyl CoA
Fatty acids
FASN ↓
ACAA2 ↓
ECHDC1 ↓
MECR ↓
ELOVL5 ↓
ELOVL6 ↓
ACSL1↓
ACSL6 ↓
ACACA ↓
FADS1 ↓
FADS2 ↓
SCD ↓
Citrate
SLC25A1↓
cytosol
β-oxidation
ACOX1 ↓
ACAD9 ↓
ACADL ↓
CRAT ↓
ACSL6 ↓
ACSL1 ↓
Uncoupling proteins
UCP3 ↓
SLC25A27 ↑
Pyruvate
LDHA ↓
Lactate
PDHA1 ↓
DLAT ↓
PDK4 ↑
Citrate
Acetyl CoA
TCA cycle
ME1 ↓
OGDH ↓
mitochondrion
Complex V
ATP5J2 ↓
COX7A1 ↓
COX7A2 ↓
Complex IV
COX6B1 ↓
COX7A1 ↓
COX7A2 ↓
OXPHOS
Complex III
UQCR11 ↓
Succinate
Complex II
SDHA ↓
Complex I
NDUFS7 ↓
NDUFV2 ↓
NDUFC1 ↓
NDUFA7 ↓
NDUFA8 ↓
NDUFA11 ↓
NDUFB1 ↓
NDUFB3 ↓
NDUFB7 ↓


Total # on array
19715 genes
Removal of background noise:
Intensity on array
≥20 on >6 arrays
15578
Response to ER
Response to
HP-ER
1869 genes
Overlap
500
Response to
NP-ER
1690 genes
Difference in response
Different response
HP-ER vs NP-ER
530 genes