The transcriptome as early marker of diet-related health

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Evidence in energy restriction studies in humans

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Thesis

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Chapter 1 |

General introduction

1

Energy restriction

Energy restriction (ER) is a dietary intervention that reduces energy intake without malnutrition and is used as a dietary strategy to lose weight. Next to ER as a weight loss strategy, it has been implemented as a tool to extend life span, as was shown in *Caenorhabditis elegans* [1], *Saccharomyces cerevisiae* [2], and rodents [3]. In this context it is mostly referred to as caloric restriction (CR). Within this thesis I will use the following nomenclature (1) ER as a reduction in energy intake aimed at inducing weight loss in overweight or obese subjects; and (2) CR as a reduction in energy intake aimed at improving health and life span in non-obese subjects. CR is not directly aimed at weight loss. Nevertheless reduction of calorie intake often leads to weight loss.

Of ER and CR, ER is mostly applied in humans, since it is the most effective strategy to lose bodyweight in overweight and obese people. The resulting loss in bodyweight has many beneficial health effects, including a reduction in blood pressure, low-density lipoprotein cholesterol, triglycerides, fasting plasma glucose, and haemoglobin A1c [4], resulting in a reduced risk of developing cardiovascular disease (CVD), and diabetes type II [5].

In rodents, CR has been shown to be associated with reduced age-related dysfunction of organs [6] and reduced risks of several age-related diseases in rodents [7, 8]. CR is therefore also suggested to extend an organism's 'health span': the period of time when an organism is healthy. Two studies in non-human primates found both an improvement in overall health, but only one study showed an extended life span [9, 10]. Studying the effects of CR on extending life span in humans is difficult as it requires extremely long-term studies. Furthermore studying the effect of a reduction of energy intake in non-obese subjects is also difficult since non-obese subjects are not interested in losing weight as opposed to obese subjects. For these reasons, CR studies in humans focus primarily on the shorter term, i.e. weeks, beneficial health effects that may explain the extension in life span. Although limited studies in humans have been performed, some evidence on health effects of CR comes from studies in non-obese persons, investigating the effect of CR on the risk of several diseases. For example one year of 20% CR in non-obese humans decreased risk factors for coronary heart disease [11] and two years of CR with the last year of weight stability also decreased risk factors for coronary heart disease [12]. Voluntary CR is applied by subjects in the CR Society who follow the Caloric Restriction Optimal Nutrition diet ('CRON'). Subjects in this society voluntarily implement CR into their lifestyle and believe that the CRON diet increases their life and health span. Studies on these weight-stable CRON subjects, with an average of six years of ~20% CR, showed decreased risk factors for atherosclerosis [13]. Core body temperature [14] and serum T3 hormone concentration [15] in these weight-stable CRON subjects are also lower than in matched controls.

Phenotypic traits

How and to what extent ER and CR interventions may affect health is thought to be affected by phenotypic traits including age, gender, lifestyle, body mass index (BMI), and (epi)genetics. For example, for CR, muscle metabolism has been shown to be improved after 14 weeks of CR in mature rats but not in young rats [16]. In addition, extension of lifespan has been shown to be

present when CR is started in both mature mice [17] and in young mice [18, 19]. Although the initiation of CR early in life is beneficial for life span, an extreme percentage of CR at this early life-onset could also lead to possible developmental disturbances as more energy is needed for growth and development. For ethical reasons, studies on early life-onset of CR in humans are not feasible. However, studies in non-human primates on early life-onset of CR do exist. For example, one study in rhesus macaques showed that CR started before puberty or at an advanced age eliminated the beneficial effects of adult onset of CR in view of delayed T-cell aging by CR [20]. The initiation of CR late in life had no effect on longevity in rats [21] and has also been shown to lead to increased mortality in mice [22]. Taken together, these study results are inconclusive and show different beneficial but also detrimental effects of specific ages when CR is implemented. In rodents, it has been shown that the age when CR is implemented can be an important factor in study outcomes, but to what extent this is important also dependents on the study outcome. A comparison of different ages and the study of the influence of age on ER and weight loss outcomes have not been done. Nevertheless, age might be an important factor that should be taken into account in weight loss studies. For example, it has been shown that implementation of excessive ER in overweight or obese elderly subjects is beneficial for weight loss, but also led to detrimental effects such as bone mass loss [23].

An influence of gender on the effect of CR is also likely to exist since females from most mammalian species tend to live longer than males [24]. In fruit flies, CR increased female life span by 60%, while male life span increased by only 30% [25]. This was contrary to findings in rats, in one of the first studies on CR published in 1935 by McCay [26], which showed an increase in CR male life span but not in female life span.
The effect of gender on ER and weight loss has been summarized in a recent review. In the majority of 49 studies that were examined, men had greater weight loss than women [27]. This differences in weight loss could be due to the differences in fat distribution, as women have more fat mass in lower regions while man have more fat mass in the abdominal region [28]. Another factor may be the amount of muscle mass, which is higher in men than in women, leading to a higher energy expenditure. Gender differences in human studies are mostly circumvented by excluding premenopausal women and randomisation of both genders amongst the groups.

The effect of CR on life span extension can also be influenced by genotypes, as was illustrated in mice, where long-term CR increased maximum life span in only two out of three different genetic strains [22]. Genotypic differences can also lead to variation in the response to ER interventions studies in overweight and obese subjects. Several studies have been performed on the effect of genotypes on weight-loss in the obese, including study of the role of genetic variations in the well-known fat mass and obesity-associated (FTO) gene involved [29-31]. However, carriers of the high-weight variant of FTO do not show differences in response to weight loss interventions as compared to carriers of the normal-weight variant [32]. Other genetic variants, including variants in the genes encoding melanocortin 4 receptor (MC4R) and transmembrane protein 18 (TMEM18) could account for differences in diet-induced weight loss between subjects. A recent review on gene-environment interactions emphasises the need for more research

on the relation between genetic variants and diet-induced weight loss and the development of personal weight loss strategies [33]. One example mentioned in this review is the rs1440581 variant, which in subjects given a high fat ER diet leads to less weight loss and less improvement in insulin sensitivity as compared to individuals without the variant [34]. Thanks to high-throughput screening and sequencing of DNA variants, more of these studies are expected to be conducted in the future, hopefully yielding more insight into the role of genetics in weight loss and ER interventions. In addition, epigenetic traits are also thought to play a role in the response to a diet. For example, low methylation levels in promotor region of ATP10A have been linked to a high response to eight weeks of 30% ER [35]. Furthermore, the chance for weight-regain after an ER diet might be epigenetically regulated, as was shown by increased weight regain due to the increased methylation of proopiomelanocortin (POMC) regions and the decreased methylation of neuropeptideY (NPY) [36].

Diet composition

Phenotypic traits are not the only factors that affect the outcome of CR and ER interventions. The percentage of restricted calories and the nutrient composition of a diet are also factors that possibly affect the outcome of these interventions. Macronutrients are often studied in this respect, since each macronutrient has a different metabolic role in energy production [37]. Studying the effect of different macronutrient compositions can be difficult as one should be aware that reaching a low percentage of one macronutrient requires the exchange other macronutrients. This will inevitably increase the percentage of other macronutrients.

Different macronutrient-exchanges have been studied for CR to achieve greater health and for ER to achieve greater weight loss. To achieve greater health by CR, it has been proposed that specific macronutrient restriction without CR might lead to similar beneficial health effects as CR [38]. If this would work, this would increase the feasibility in humans because the exchange of one macronutrient for another might be easier to adhere to than the restriction of total calorie-intake. Protein is one of the macronutrients that has been restricted and shown to have similar health benefits as CR diets in mice [39]. In addition, the origin of protein might be of interest in mimicking the effects of CR. The substitution of animal-based protein for plant-based protein has demonstrated to lead to less tumour growth in human xenograft cancer models [40]. Furthermore, plant-based protein in non-CR diets seemed to extend life span in mice and health span in humans [41]. Besides the origin of protein, reduced intake of specific amino acids such as branched-chain amino acids (BCAAs) improved glucose tolerance in humans and mice as compared to an average diet. This improvement in glucose tolerance was equivalent to a protein-restricted diet [42]. In addition, it has been shown that restriction of methionine intake improved insulin sensitivity, metabolic flexibility and lipid metabolism, and decreased systemic inflammation [43] in rats and mice [44].

For ER, macronutrient-exchange diets have led to different results. In mildly obese subjects, 12 months of a low fat ER diet led to a similar amount of weight loss as a low carbohydrate ER diet [45]. In contrast, in severe obese subjects, with a BMI above 40 kg/m^2, six months of a low carbohydrate ER diet led to greater weight loss and more metabolic improvements than a low

fat ER diet [46]. The exchange of protein for other macronutrients within ER is also extensively studied. A meta-analysis on the exchange of carbohydrates for protein showed a greater decrease in body fat mass upon high protein-low carbohydrate ER diets compared to low protein-high carbohydrate ER diets [47]. One explanation for this difference in body fat mass is the increased need for protein for gluconeogenesis in low-carbohydrate diets, demanding more energy and possible leading to more body fat mass loss and diet-induced thermogenesis [48]. Next to the beneficial loss of body fat mass upon ER, a disadvantage of ER is the loss of muscle mass [49]. An increase in protein has been suggested to be beneficial for muscle mass preservation. However, contrasting study results emerge [50-52] which are possibly due to differences in age, gender, and length of interventions.

Based on the studies on macronutrient-exchanges, the optimal macronutrient composition for metabolic health in humans that ensure comparable health effects as CR, still needs to be elucidated. In addition, results of studies on ER with different macronutrient compositions are inconclusive.

Biomarkers of health

In general, the relation between diet and health is hard to prove, taking in mind that effects of nutrition are subtle, show high interindividual variations in response, and can take long before they become visual. Recently, the definition of health has been redefined as an organism's ability to adapt to physical, emotional, and social challenges of life [53]. This adaptability can also be extended to metabolic health, which has been named 'phenotypic flexibility' [54]. Measuring phenotypic flexibility could reflect the health of an organism, resembled by the ability to cope with a stressor to return to homeostasis.

Nutritional challenge tests

To measure phenotypic flexibility in the human body, a stressor should be exerted on the body. Examples of such stressors in nutrition are nutritional challenge tests, which provide a high load of nutrients that act as a stressor for cells and tissues. This nutritional challenge will elicit a response and metabolic adaptation. The ability to cope with a nutritional stressor, showing an adequate response to return to homeostasis, resembles the phenotypic flexibility and likely reflects the health status of the body. Measuring the phenotypic flexibility shows a more dynamic response than the more static measurement in the fasted state. In addition, the response to a nutritional challenge might be able to magnify the effects of a dietary intervention on health. To assess this dynamic response, blood can be withdrawn multiple times in a row during a challenge test, leading to insight in the response to a challenge test over time.

One well-known nutritional challenge test is the oral glucose tolerance test (OGTT). An OGTT consists of a high load of glucose and measures how well the body is able to clear the glucose from the blood. OGTTs are widely applied in clinic clinical settings to diagnose insulin resistance by measuring plasma glucose and insulin levels [55]. Other nutritional challenges are a high fat challenge test with a high fat load and a mixed meal test (MMT), consisting of protein, fat and

carbohydrates resulting in a high energy density. All challenge tests stress the metabolic capacity of organs and tissues to cope with this high load of one or multiple nutrients. Several of these challenges have been applied to stress the system and to magnify differences between health states. For example, high fat challenges evoked a more pronounced postprandial triglyceride response in obese and obese diabetic subjects when compared to lean subjects [56]. Also, the postprandial triglyceride response has been shown to be superior to fasting levels for the assessment of CVD risk as was shown in large cohort studies [57, 58] and reviewed by Boren et al. [59] and by Pirillo et al. [60].

Transcriptomics

In addition to challenge tests, the use of high-throughput techniques, in particular 'omics' techniques has emerged in the field of nutrition research. Omics represent the study of a large amount of variables in a non-targeted and non-biased manner. Omics can be divided based on the measured variables and can measure for example the genome (genomics), the proteome (proteomics), or the transcriptome (transcriptomics). Transcriptomics is often applied in nutrition research to detect subtle changes in whole genome gene expression due to dietary interventions. Investigating these changes in gene expression could possibly lead to the discovery of mechanisms explaining how nutrition can affect health. The transcriptome is assessed using microarrays or RNA sequencing techniques that have the ability to perform genome-wide surveys of the complete transcriptome.

Nutritional effects on the transcriptome

Nutrients participate in the regulation of the transcriptome by either binding to transcription factors or by secreting other molecules that interact with transcription factors. The most important group of transcription factors that bind nutrients and their metabolites, is the nuclear hormone receptors superfamily of transcription factors [61]. The binding of nutrients to these nuclear hormone receptors leads to either an activation or an inhibition of the expression of specific genes. An example is the peroxisome proliferator-activated receptor alpha (PPARα), which is activated upon fatty acid binding and leads to the regulation of the transcription of numerous genes involved in lipid metabolism [62].

Tissues

A drawback of measuring the transcriptome in human studies is that it requires cellular material and not all tissues are easy to collect. Dietary interventions are mostly performed in healthy individuals making it almost impossible to obtain samples that require surgery. Instead, we have the possibility to use other samples, such as subcutaneous white adipose tissue (WAT), muscle and blood samples. Subcutaneous WAT and muscle samples can be obtained via needle biopsy under local anaesthesia. Blood cells, including peripheral blood mononuclear cells (PMBCs), are easy to obtain in all kinds of dietary intervention studies and are therefore an easy accessible target to study in humans [63, 64]. As PBMCs circulate in the blood [65], they are exposed to both exocrine signals such as nutrients, and to endocrine signals such as metabolites, hormones, chemokines or

cytokines from tissues as the liver and WAT. This makes PBMCs an interesting subset of cells to investigate the effects of nutrition. The transcriptome from PBMCs has been shown to be affected by different dietary interventions including ER [66] and fasting [65] in humans, and CR in rats [67]. Likewise, WAT is an interesting target to study as it is greatly affected by ER. WAT functions as a fat storage organ and plays an important role in the regulation of energy and glucose homeostasis. The response of adipose tissue to ER will lead to metabolic adaptations in WAT which could be reflected in the transcriptome. The transcriptome from WAT has been shown to be affected by dietary interventions including very low ER [68], and more moderate ER [69] in humans.

Transcriptomics during challenge tests

Combining omics with nutritional challenge tests may be a valuable strategy to magnify health effects of dietary interventions. This would make it possible to detect small diet-induced changes in health, which might eventually lead to the discovery of markers of diet-related health. The ability of challenge tests combined with transcriptomics to magnify the difference between health states has already been shown in the comparison of lean and obese individuals. Differences in gene expression between these two groups were more pronounced after a challenge test as compared to the fasted state [70]. The discovery of markers of diet-related health using challenge tests and omics techniques requires easy and repeatedly accessible tissues, such as urine or blood. From blood, PBMCs can easily be collected multiple times in a row making them an interesting target. The PBMC transcriptome has been shown to be affected by nutritional challenge tests such as high fat challenges [71, 72] and the OGTT [73]. This response of the PBMC transcriptome upon a challenge, might be able to reflect a change in phenotypic flexibility upon a dietary intervention. Therefore, the combination of challenge tests and the PBMC transcriptome may have the ability to serve as an early and sensitive marker of diet-related health.

Outline thesis

In this thesis, the effect of CR, ER, and challenge tests on whole genome gene expression were investigated in human PBMCs and in subcutaneous WAT. In chapter 2, the influence of age on the effects of CR on the PBMC transcriptome was investigated by studying the effect of three weeks of 30% CR in young and in old men. In chapter 3, we studied the effect of the macronutrient composition in ER on the subcutaneous WAT transcriptome by exchanging a part of the amount of carbohydrates for protein during an ER diet. In chapter 4, we investigated the added value of measuring the PBMC transcriptome during challenge tests compared to fasting to magnify effects of ER on health. Finally, in chapter 5, we determined differences between a challenge test consisting of glucose (OGTT) and a mixed meal challenge consisting of glucose plus other macronutrients (MMT) on the PBMC transcriptome in diet-related health. An overall evaluation of all the findings can be found in the General discussion in chapter 6.

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Chapter 2

Differences in genome-wide gene expression response in peripheral blood mononuclear cells between young and old men upon caloric restriction

2

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Abstract

Background: Caloric restriction (CR) is considered to increase lifespan and to prevent various age-related diseases in different nonhuman organisms. Only a limited number of CR studies have been performed on humans, and results put CR as a beneficial tool to decrease risk factors in several age-related diseases. The question remains at what age CR should be implemented to be most effective with respect to healthy aging.

Objective: The aim of our study was to elucidate the role of age in the transcriptional response to a completely controlled 30% CR diet on immune cells, as immune response is affected during aging.

Methods: Ten healthy young men, aged 20–28, and nine healthy old men, aged 64–85, were subjected to a 2-week weight maintenance diet, followed by three weeks of 30% CR. Before and after 30% CR, the whole genome gene expression in peripheral blood mononuclear cells (PBMCs) was assessed. ClinicalTrials.gov, NCT00561145

Results & Conclusions: Expression of 554 genes showed a different response between young and old men upon CR. Gene set enrichment analysis revealed a downregulation of gene sets involved in the immune response in young but not in old men. At baseline, immune response-related genes were higher expressed in old compared to young men. Upstream regulator analyses revealed that most potential regulators were controlling the immune response.
Based on the gene expression data, we theorise that a short period of CR is not effective in old men regarding immune-related pathways while it is effective in young men.

Introduction

Caloric restriction (CR), the restriction of food intake without malnutrition, increases longevity in *Caenorhabditis elegans* [1], *Saccharomyces cerevisiae* [2], and rodents [3]. In addition to longevity, CR minimises the age-related dysfunction of organs [4] and lowers risks of several age-related diseases, for example, cancer in rats and mice [5], and age-related aorta sclerosis in rats [6]. CR studies in primates led to less conclusive results.

CR did increase longevity in monkeys at the Wisconsin National Primate Research Centre [7] but did not increase longevity in monkeys at the National Institute of Aging [8]. Factors such as genetics, husbandry, or dietary composition are perhaps more relevant for longevity in these primate studies than the number of calories [8]. Despite contrasting longevity results, both studies documented beneficial health effects of CR, including improved immune function and improved glucose homeostasis [8]. The limited number of studies investigating the effect of a CR diet in humans is, because of long life expectancy [9], solely directed at beneficial health effects and not at longevity [10]. For example, 6 years of CR decreased risk factors for atherosclerosis in humans [11] and 1 year of CR decreased risk factors for coronary heart disease in humans [12]. Also, aging processes seem to be altered by CR: the gene expression profiles from skeletal muscles from humans of the CR Society showed a closer relationship to the gene expression profiles of young subjects than to those of age-matched subjects [13]. The preventive or retardative effect of CR on age-associated changes in gene expression has also been shown in the muscle, brain, heart, and adipose tissue from other species [14].

Mechanisms underlying beneficial effects of CR remain largely unclear. To understand these mechanisms, genes and molecular pathways involved in the effects of CR on longevity and healthy aging have been investigated. Overall, the effects of CR are characterised by the downregulated expression of genes involved in growth hormone signalling and genes involved in immune response [15]. In contrast, aging is characterised by the upregulated expression of genes involved in immune response [16]. The opposing effects of CR and aging on the immune response might be one potential lead for the beneficial effects of CR on healthy aging. In this regard, immune cells, such as peripheral blood mononuclear cells (PBMCs), are an interesting target to study in humans [15, 17]. PBMCs are easily accessible and circulate in the blood [18], exposing them to metabolites, hormones, chemokines, or cytokine from tissues such as the liver and adipose tissue [19], which make them relevant to study. So far, most human CR studies have been executed in middle-aged subjects; the question remains at what age CR should be implemented to be most effective with respect to healthy aging. To approach this question, we aimed to elucidate the effect of age in the response to CR by comparing whole genome gene expression response to three weeks of 30% CR in PBMCs from old and young men.

Materials and Methods

Study population and eligibility criteria

Our study population was a subgroup of participants who participated in a previously reported controlled-feeding trial [20]. Male Caucasian participants were recruited, by publishing advertisements in local newspapers and by sending out general e-mails to an e-mail list of persons who had indicated their interest in participating in studies of our university, at Wageningen University (The Netherlands), in October 2007 till January 2008, and followed up until the 15th of March 2008. Participants were excluded based on the following criteria: body mass index (BMI, kg/m^2) less than 20 or higher than 30, adherence to a weight-reduction or medically prescribed diet, dementia (Mini-Mental State Examination score <21), diabetes, anaemia, gastrointestinal disorders, use of drugs known to interfere with energy balance, or a history of medical or surgical events known to affect the study outcome. Participants were divided based on their age into young (20–40 years) and old (65–85 years) men. Based on these criteria, 15 young and 17 old men were included in the original study at Wageningen University (The Netherlands) [20]. Microarray analyses were performed on high-quality PBMC RNA of ten young men, age range 20–28 years, and nine old men, age range 64–85 years (figure 1).

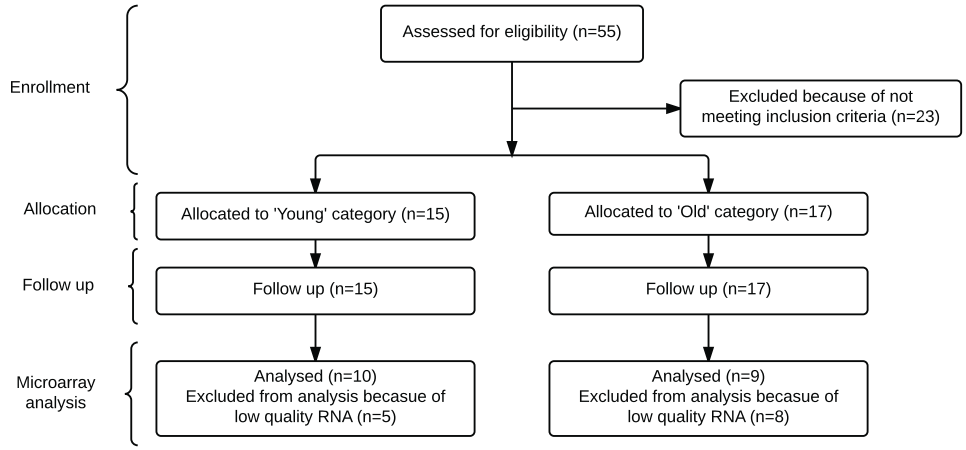


Figure 1. Flow diagram of subject inclusion. Each of the participants was informed about the design and purpose of the study, and each of the participants provided written informed consent. The Medical Ethical Committee of Wageningen University (The Netherlands) approved the study. This clinical study was registered with ClinicalTrials.gov as NCT00561145.

Study design

The original study consisted of three subsequent phases as described previously [20] and was all carried out at the Division of Human Nutrition of Wageningen University (The Netherlands). Only samples collected after phases 1 and 2 are the subject of the current paper. Phase 1 (days 1–14): controlled dietary intervention in which each of the participants had to remain weight stable. Each of the participants was provided with a diet containing approximately 90% of their estimated total daily energy requirement. The remaining 10% was chosen from a list of choice

items. In phase 2 (days 15–35): each of the participants was provided with a diet containing 70% of the energy consumed during the last 3 days of phase 1. Composition of the diets was determined as described in [20]. Blood samples were taken at the end of phase 1, before CR, and at the end of phase 2, after CR.

PBMC RNA isolation and microarray processing

PBMCs were isolated from whole blood using BD Vacutainer® Cell Preparation Tubes™ according to the manufacturer's instructions. Total RNA was isolated from PBMC samples using TRIzol reagent (Invitrogen, Breda, The Netherlands) and purified using 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 (MessageAmp™ II-Biotin Enhanced Kit; Ambion Inc., Nieuwerkerk aan de IJssel, The Netherlands) and hybridised to human whole genome GeneChip arrays encoding 17,054 genes, designed by the European Nutrigenomics Organization and manufactured by Affymetrix (Santa Clara, CA). Sample labelling, hybridization to chips, and image scanning were performed according to the manufacturer's instructions.

Microarray data analysis

Quality control was performed and fulfilled the criteria for array hybridisation suggested by the Tumor Analysis Best Practices Working Group [21]. Microarrays were analysed using reorganised oligonucleotide probes as described by Dai et al. [22]. All individual probes for a gene were combined, allowing the possibility to detect overall transcription activity on the basis of latest genome and transcriptome information, rather than on the basis of Affymetrix probe set annotation. Expression values were calculated with the Robust Multi-array Average (RMA) method and quantile normalised [23, 24]. Only probe sets with normalised signals >5 on ≥ 5 arrays were defined as expressed and selected for analysis. This normalisation level was chosen because of a low microarray intensity level, due to the use of expired microarrays. It has, however, been shown that microarray data generated by microarrays more than 4 years past the manufacturer's expiration date had lower signal intensities but were highly specific and consistent with those from unexpired microarrays [25]. We used microarrays within 2 years of the expiry date. Individual genes were defined as changed when comparison of the average normalised signal intensities showed a P-value <0.05 in a two-tailed paired t-test with Bayesian correction (Limma) [26]. Filtered data were analysed with Gene Set Enrichment Analysis (GSEA; GSEA/ MSigDB website v3.87 released April 4, 2013). Significantly regulated gene sets were defined with a false discovery rate of <0.25. Gene sets were visualised and clustered using Cytoscape [27], which enabled the identification of clusters of gene sets. Ingenuity Pathway Analysis version 8.5 (Ingenuity Systems, Redwood City, CA) was also used for pathway analysis and upstream regulator analysis, but because of similar results, only GSEA results are displayed. Ingenuity Pathway Analysis has been performed based on findings from human experiments.

For upstream regulator analysis at baseline, genes with a significant different expression at baseline were included ($P<0.05$). For young, genes with a significant different response upon CR

in young were included ($P<0.05$), and for old, genes with a significant different response upon CR in old were included ($P<0.05$). For correlation heat maps, target genes of the upstream regulators were included if they also had a significantly different response between old and young ($P<0.05$) upon CR. Array data have been submitted to Gene Expression Omnibus under accession number GSE63117.

Statistical analysis of clinical measurements

The statistical package SPSS (version 15.0; SPSS Inc, Chicago, IL) was used for analysis of the following data: expression changes within age groups were determined by paired t-tests, and differential changes between age groups were determined by unpaired t-tests.

Results

Baseline characteristics for ten young and nine old men of which high quality microarrays were present are summarised in table 1. Besides the lower body mass index (BMI, P=0.04) and lower fasting glucose level (P<0.001) in old compared to young men, no differences were observed.

Table 1. Baseline characteristics of young (n = 10) and old (n = 9) men of whom microarray analysis on PBMCs was performed.

	Young men	Old men	P-value
Age (y)	24 [20, 28]	70 [64, 85]	4.37E−09
Height (m)	1.78 ± 0.06	1.77 ± 0.04	5.26E−01
Weight (kg)	71.1 ± 8.5	76.7 ± 7.4	1.28E−01
Body mass index (kg/m^2)	22.4 ± 2.3	24.6 ± 2.0	3.08E−02
Glucose (mmol/L)	4.5 [3.7, 5.1]	5.2 [4.8, 5.5]	5.17E−04
Haemoglobin (g/L)	9.4 ± 0.4	9.3 ± 0.4	5.69E−01
Haematocrit (%)	45 ± 2	44 ± 2	5.46E−01

Data represent mean and ±SD or median and [range].

Three weeks of CR resulted in a decrease in body weight and BMI in both groups (table 2). Age had no effect on weight (P=0.18) or BMI change (P=0.18).

Table 2. Body weight and body mass index of young and old men before and upon three weeks of 30% CR and significance (P-value).

		Before CR	Upon CR	P-value
Weight (kg)	Young (n = 10)	71.1 ± 8.5	68.7 ± 8.6	2.87E−05
	Old (n = 9)	76.7 ± 7.4	74.9 ± 7.4	6.53E−05
Body mass index (kg/m^2)	Young (n = 10)	22.4 ± 2.3	21.6 ± 2.3	3.64E−05
	Old 9 (n = 9)	24.6 ± 2.0	24.0 ± 2.0	4.21E−05

Data represent mean with ±SD.

Effect on gene expression: old versus young

At baseline, before 30% CR, 696 genes were significantly differently expressed between old and young men (figure 2a). To identify the effect of age on CR-induced gene expression changes, responses to CR were compared between old and young men (figure 2b). A total of 554 genes showed a significantly different expression response between old and young men.

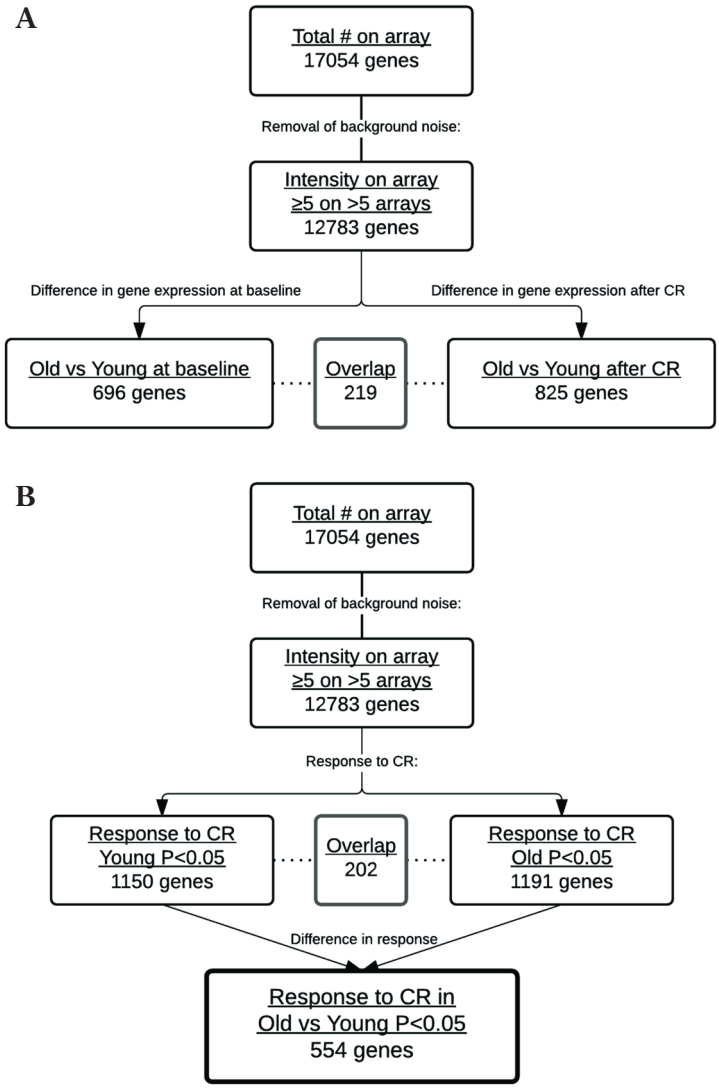


Figure 2. Stepwise selection of genes in microarray analysis of old versus young men upon three weeks of 30% CR: 12,783 genes were selected for signal intensity (≥5 in >5 arrays), **a.** for a difference in expression between old and young ($P < 0.05$) men at baseline (left track) and upon CR (right track), and **b.** a change in expression of genes for young (left track) and old (right track) in response to CR. The last box depicts the number of genes that has a different response to CR in old versus young men.

Effect of CR on gene expression: pathway analysis

Gene Set Enrichment Analysis (GSEA) was used to identify pathways in which gene expression was differentially regulated by age, at baseline, or in response to CR. Before the start of the 30% CR diet, the expression of genes involved in immune response was higher in old compared to young men and the expression of genes involved in RNA processing was lower in old compared to young men (table 3). Upon three weeks of 30% CR, the expression of genes involved in immune response and glucose metabolism was downregulated in young men only, whereas the expression of genes involved in olfactory signalling was downregulated in old men only.

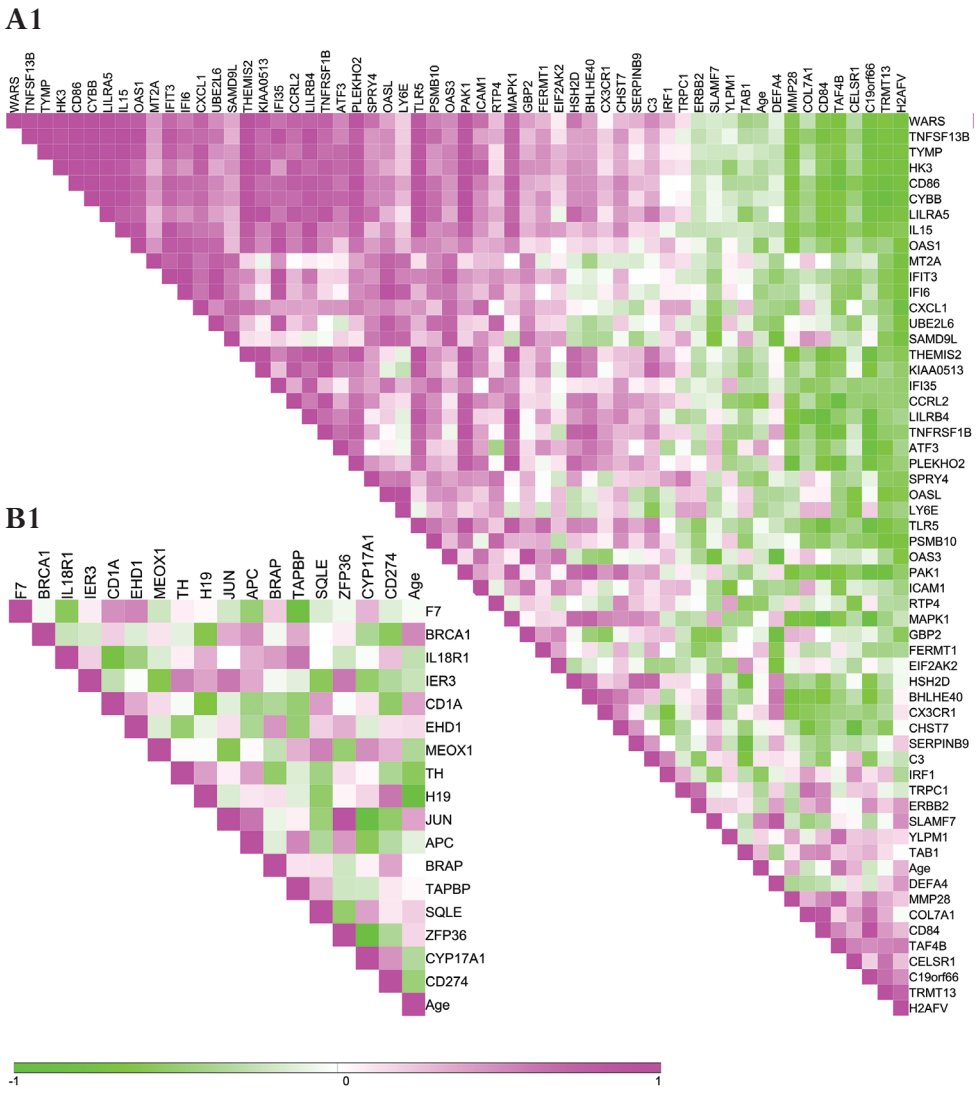
Table 3. Pathways changed in PBMC gene expression profiles of young and old men before and upon three weeks of 30% CR

Pathway cluster[1]	Baseline (old vs young)	Young men	Old men
RNA processing	↓	↑	↑
Cell cycle	-	↑/↓[a]	↑/-[a]
Oxidative stress	-	↓	↓
Immune response	↑	↓	-
Glucose metabolism	-	↓	-
Olfactory signalling	-	-	↓

[1]Significantly changed pathways are determined with GSEA (supplementary table **2**) and clusters are based upon Cytoscape. ↑: upregulated; ↓: downregulated; - no change; [a]Part of gene sets classified under these pathways were upregulated, whereas others where downregulated.

Upstream regulator analysis

Ingenuity Pathway Analysis (IPA) Upstream Regulator Analysis is a tool to find transcription regulators that may explain the observed gene expression. To identify these upstream regulators of genes that had a different expression before CR between old and young, or had a changed expression upon CR, we performed IPA Upstream Regulator Analysis. The regulators that were predicted to be affected at baseline and upon CR are listed in supplementary table1. This list shows immune-related upstream regulators, interferon lambda 1 (INFL1), interferon alpha 2 (IFNA2), and interferon gamma (IFNG), that were predicted to be significantly higher in old compared to young before intervention. IFNA2 and IFNG were inhibited upon CR in young men, but not in old men. To identify correlation between the genes predicted to be regulated, we selected all significantly changed genes targeted by the predicted transcriptional regulators upon CR and created correlation heat maps of the significant changes in expression of these genes for young men (figure 3a1) and old men (figure 3a2). For young men, 57 unique genes were affected by the six identified transcriptional regulators, i.e. IFNA2, IFNG, eukaryotic translation initiation factor 2-alpha kinase 2 (EIF2AK2), mitogen-activated protein kinase 1 (MAPK1), glyceraldehyde-2-phosphate dehydrogenase (GAPDH), and transglutaminase 2 (TGM2) (supplementary table 1). These genes showed a distinct correlation in young men (figure 3a1) which was less strong or absent in old men (figure 3a2). Contrary for old men, many genes regulated by the upstream regulators were overlapping: 15 potential upstream regulators (supplementary table 1) were predicted to affect 17 unique genes, and no specific correlation pattern for old could be identified (figure 3b).


A1
WARS
TNFSF13B
TYMP
HK3
CD86
CYBB
LILRA5
IL15
OAS1
MT2A
IFIT3
IFI6
CXCL1
UBE2L6
SAMD9L
THEMIS2
KIAA0513
IFI35
CCRL2
LILRB4
TNFRSF1B
ATF3
PLEKHO2
SPRY4
OASL
LY6E
TLR5
PSMB10
OAS3
PAK1
ICAM1
RTP4
MAPK1
GBP2
FERMT1
EIF2AK2
HSH2D
BHLHE40
CX3CR1
CHST7
SERPINB9
C3
IRF1
TRPC1
ERBB2
SLAMF7
YLPM1
TAB1
Age
DEFA4
MMP28
COL7A1
CD84
TAF4B
CELSR1
C19orf66
TRMT13
H2AFV
B1
F7
BRCA1
IL18R1
IER3
CD1A
EHD1
MEOX1
TH
H19
JUN
APC
BRAP
TAPBP
SQLE
ZFP36
CYP17A1
CD274
Age
-1
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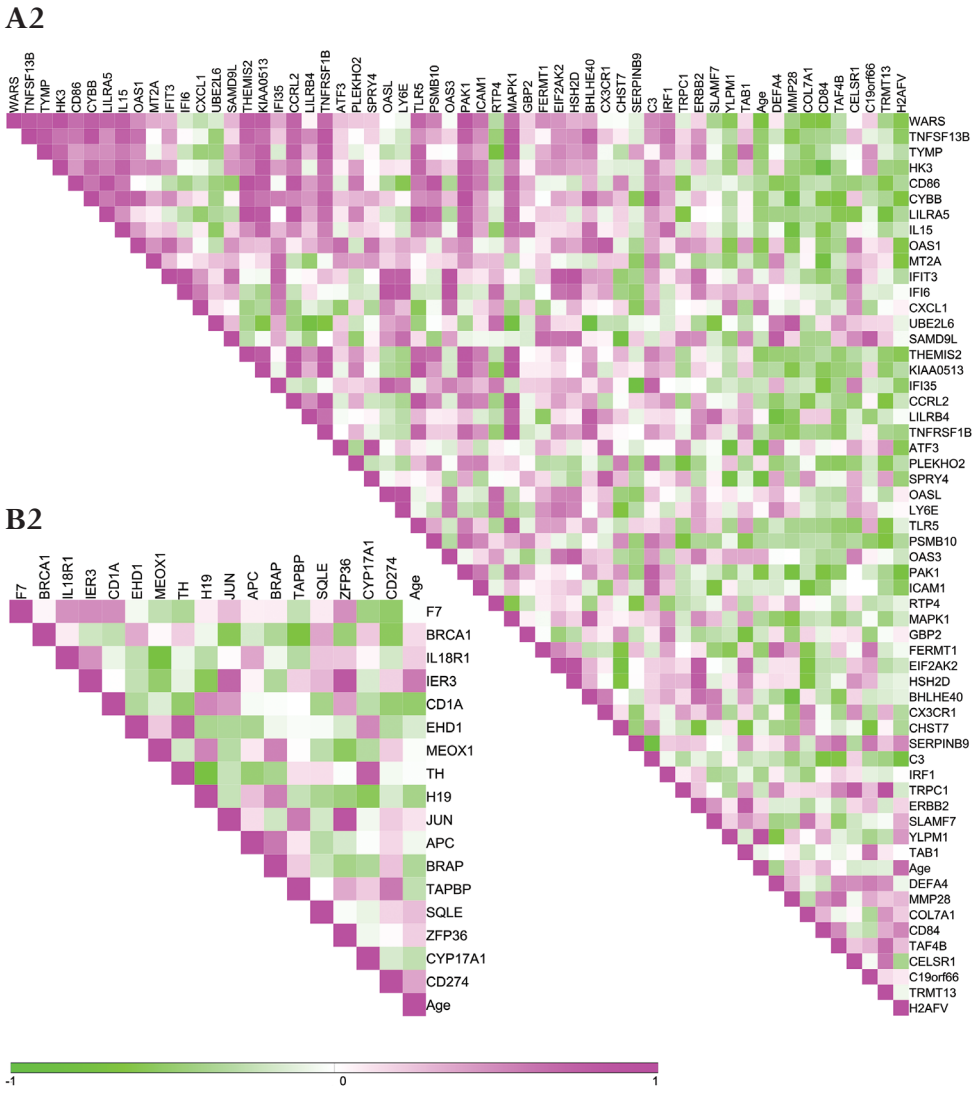

Figure 3. Correlation heat maps of CR-induced significant changes in gene expression of **a.** genes regulated by upstream regulators in young, depicted for the response in young (a1) and old (a2), and of **b.** genes regulated by upstream regulators in old, depicted for the response in young (b1) and old (b2).
Scale: green = correlation score of −1, pink = correlation score of 1

Younger transcriptional profile upon CR

To identify if old men were able to obtain, at least for a subset of genes, a younger gene expression profile upon CR, the following approach was used: selection of genes with a different expression between old and young before CR resulting in 696 genes (figure 4); 96 of these genes also showed a significant different response upon CR between old and young men (figure 4); 55 of these genes had a changed expression in old men only. Figure 5 shows a heatmap of the gene expression per subject and illustrates the different expressed genes at baseline and the change towards a young profile in old men, as is shown by the third part of the heat map where no significant expression differences between old and young upon CR were present.

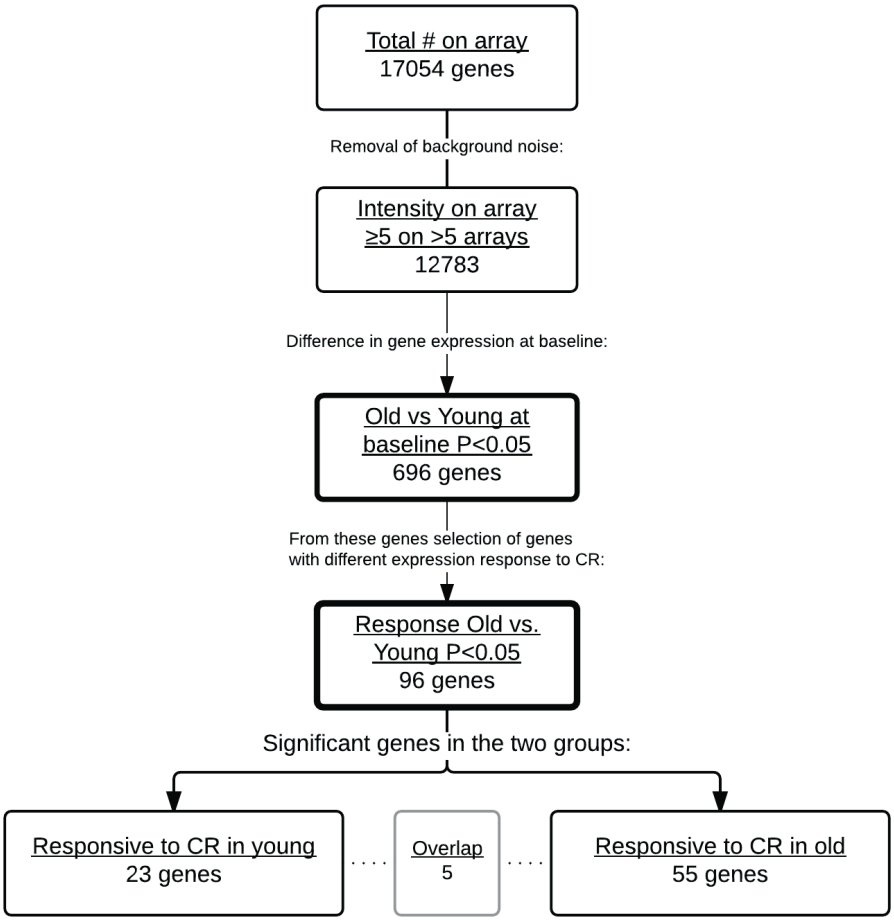

Figure 4. Stepwise selection of genes in microarray analysis to identify genes in which expression changed from an old profile to a young profile upon three weeks of 30% CR. For signal intensity, 12,783 genes were selected (≥5 in >5 arrays); from these, 96 genes were selected that were differently expressed between old and young men before CR (P<0.05); the last part depicts number of genes that show a different response to CR in old versus young men. Finally, 23 genes with a significant change in expression within young (left track) men and 55 genes within old (right track) men are shown.

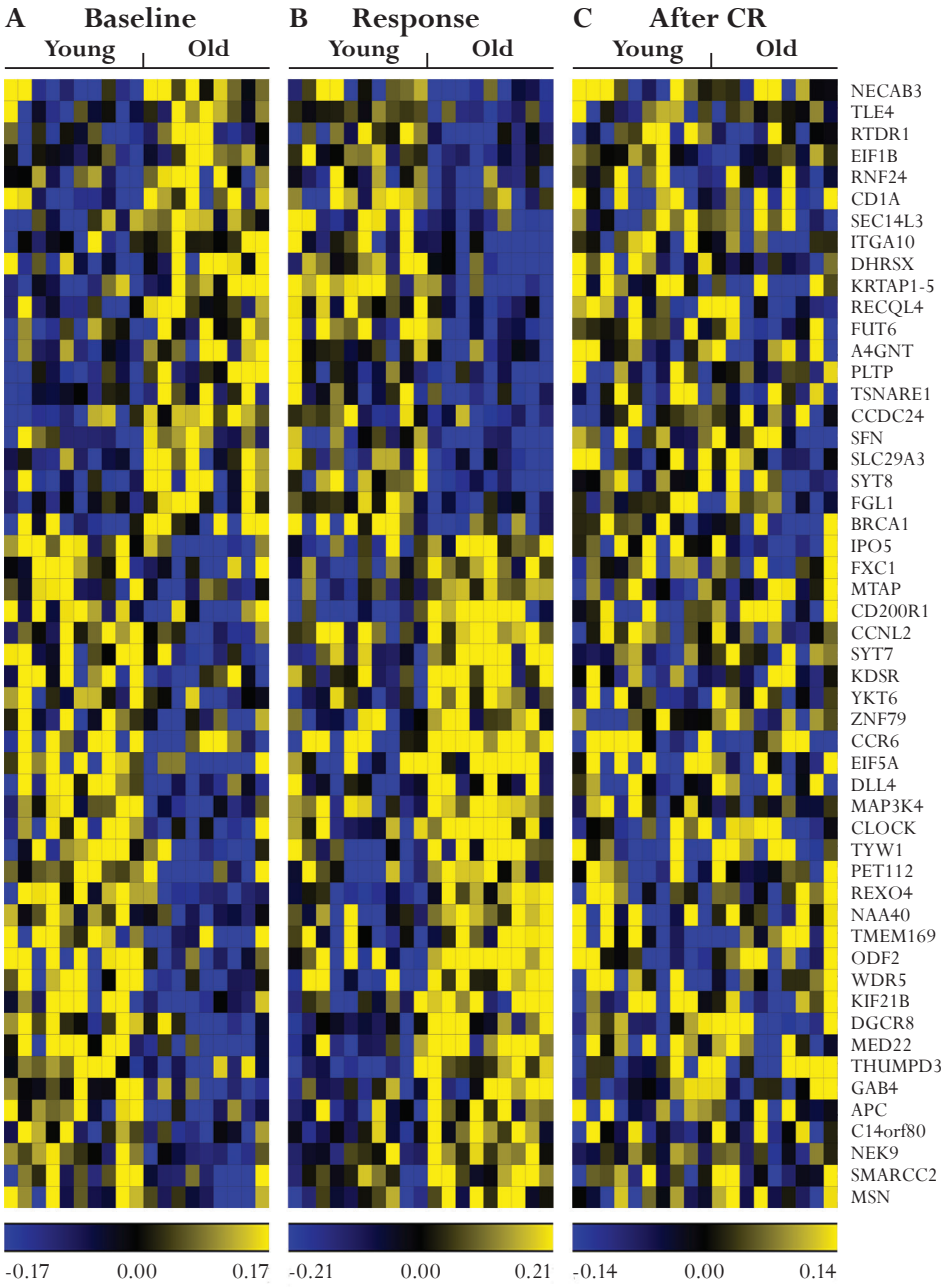

Figure 5. Heatmap of genes of which expression changed from an old profile to a young profile upon CR. **a.** baseline expression level, **b.** response to CR, and **c.** expression levels after CR for young and old men. Each column represents one person; each row represents one gene. Depicted are for (a) the signal log ratio calculated as gene expression values at baseline compared to the average of the whole group, for (b) the signal log ratios calculated as gene expression values upon CR compared to gene expression values at baseline, and for (c) the signal log ratios as gene expression values after CR compared to the average of the whole group.

Discussion

We aimed to investigate the potential relevance of age at which CR should be implemented to be most effective on gene expression changes of pathways important for healthy aging. To achieve this, we compared the gene expression changes in PBMCs of old men with the gene expression changes in PBMCs of young men upon three weeks of 30% CR.

Three weeks of CR resulted in a downregulated expression of genes involved in immune response and glucose metabolism in young but not in old men. Effects of CR on immune response-related gene expression have been shown before; 8 weeks of CR in middle-aged obese men downregulated the expression of genes involved in immune response in PBMCs [28]. The response of these middle-aged obese men is similar to the response of young men in our study. One reason why we do not see the response in old men might be due to 'immunosenescence' [29], known to take place in elderly individuals above 65 years of age. Immunosenescence means the loss of immune functions and is characterised by an increase in the expression of inflammation- and immune response-related genes [16]. Although the overlap between the genes in this paper and in the current study is minimal, we also observed an increase in gene expression and a predicted activation of transcriptional regulators involved in immune pathways, i.e. IFNA2 and IFNG, in old men at baseline when compared to young. Upon CR, a decreased activation of IFNA2 and IFNG was only observed in young men. MAPK1 was activated upon CR. MAPK1 represses the expression of IFNG-induced genes via DNA-binding [30]. An increase in MAPK1 might have affected the decrease in IFNG-induced genes. The potential immunosenescence present at baseline may be the reason why we do not see a response on immune-related pathways upon CR in the old men. This is further illustrated by the correlation heat maps of immune-related genes in which high correlations are observed between gene expression responses in young men and far less pronounced effects are observed in old men. Old men seem to have lost the ability to change gene expression in immune response upon CR. This inability to change expression might be due to a potential advanced aging-related state of epigenetics, keeping the DNA structure in a more rigid structure and making it less likely to change gene expression. Either three weeks of 30% CR is not sufficient to reduce the higher gene expression of immune-related genes in old men or the age-related potential epigenetic changes are too strong to overcome with CR and CR should be started at an earlier age. Three weeks of CR also resulted in a downregulated expression of genes involved in glucose metabolism in young men only. We did not find a difference in the expression of genes involved in glucose metabolism at baseline between old and young men, even though aging is known to have a diminishing effect on adequate glucose metabolism [31]. Aging might, however, have played a role in the nonresponsive effect of our short-term CR on glucose metabolism-related pathways in old men.

The decreased expression of genes related to olfactory signalling pathways in old men has not been described in the literature. However, other studies in fruit flies have been done in which the absence of odorants from nutrients affected the expression of odorant-binding proteins [32].

In addition, it has been described before in yeast that CR has an increasing effect on the expression of genes involved in RNA processing [33] as seen in our study for both young and old men. Although old men did not respond with the same changes on immune response and glucose metabolism, many genes did show a change in expression upon CR. We identified a group of genes that changed from an 'old' expression level towards a 'young' expression level upon CR. This was in line with the finding that the expression of genes from the skeletal muscle tissue of middle-aged subjects of the Caloric Restriction Society matched closer to gene expression profiles of younger subjects than to gene expression profiles of age-matched controls [13]. We could, however, not find any clear signalling route, pathway, or network for these genes.

It should be mentioned that both baseline differences and differences in gene expression changes between young and old can be due to different subpopulations of immune cells in the PBMCs between the groups. During aging, involution of the thymus, responsible for production of naïve T cells, leads to a shift in the T cell population [34]. Unfortunately, we did not have the opportunity to determine PBMC subpopulations. Furthermore, a period of three weeks of CR is short and might not have been long enough to induce changes in the gene expression of old men. A strength of our study design is the completely controlled dietary run-in period of 2 weeks and the completely controlled dietary 30% CR intervention of three weeks which excludes a potential effect of habitual diet differences between the young and old men on gene expression differences at baseline and on gene expression response upon CR.

In our study, the expression of genes involved in immune response pathways was higher in old compared to young men at baseline. Three-week 30% CR did not affect this higher immune-related gene expression in old men whereas it did reduce immune-related gene expression in young men. Due to our small sample size, we cannot draw solid conclusions about the relevance of age on the effect of CR on gene expression changes of pathways important for healthy aging. We hypothesise based on immune-related gene expression changes in men that for a short period of 30% CR a young onset has more potential benefit than an old onset.

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Supplementary information

Availability of supporting data

The data set supporting the results of this article is available in the Gene Expression Omnibus repository, under accession number GSE63117, at http://www.ncbi.nlm.nih.gov/geo/. The online version of this article (doi:10.1186/s12263-016-0528-0) contains supplementary material (Additional file 2: Complete quality control report of microarrays; Additional file 3: Gene expression file with expression analysis (A) baseline, (B) response to intervention in old men compared to response to intervention in young men, and (C) genes in old towards a young expression profile; Additional file 5: Ingenuity Pathway Analysis Upstream regulators output used for Additional file 1: Supplementary table 1 (A) baseline, (B) response to intervention in young men, and (C) response to intervention in old men.), which is available to authorized users.
Additional file 1 (Supplementary table 1. Predicted upstream regulators) and Additional file 4 (Supplementary table 2. Gene Set Enrichment Analysis) are added below.

Supplementary table 1. Predicted upstream regulators. Predicted difference before CR between old and young, in the response of young, the response in old, and the difference upon CR between old and young.

		Baseline (Old vs Young)			Young			Old		
Upstream regulator	**Function**		z-score	P-value		z-score	P-value		z-score	P-value
IFNL1	Cytokine	↑	2.630	4.94E-03	-	-1.929	2.25E-03	-	-	-
IFNA2	Cytokine	↑	3.272	1.68E-04	↓	-3.138	1.08E-03	-	-	-
IFNG	Cytokine	↑	2.934	1.85E-05	↓	-2.052	1.40E-02	-	0.792	3.48E-02
EIF2AK2	Kinase	-	-	-	↓	-2.828	1.10E-02	-	-	-
MAPK1	Kinase	↓	-2.985	1.12E-01	↑	3.63	1.19E-02	-	-	-
GAPDH	Enzyme	-	-	-	↑	2.433	6.81E-03	-	-	-
TGM2	Enzyme	-	0.112	1.11E+00	↓	-4.375	1.23E-03	-	-	-
PDGF BB	Complex	-	-	-	-	-	-	↓	-4.026	5.16E-09
ERK	Group	-	-	-	-	-	-	↓	-3.538	7.29E-04
CCL5	Cytokine	-	-	-	-	-	-	↓	-2.985	6.15E-04
Jnk	Group	-	-	-	-	-	-	↓	-2.960	3.71E-03
F7	Peptidase	-	-	-	-	-	-	↓	-2.772	9.95E-05
CSF2	Cytokine	-	-	-	-	-	-	↓	-2.722	3.14E-04
STAT3	Transcription reg.	-	-	-	-	-	-	↓	-2.598	1.19E-04
ERK1/2	Group	-	-	-	-	-	-	↓	-2.256	2.79E-03
TNF	Cytokine	-	0.870	8.23E-03	-	-1.993	0.261	↓	-2.219	5.28E-03
P38 MAPK	Group	-	-	-	-	-	-	↓	-2.131	1.21E-02
EGF	Growth factor	-	-	-	-	-	-	↓	-2.072	1.53E-02
IL1	Group	-	-	-	-	-	-	↓	-2.000	1.33E-02
COL18A1	Other	-	-	-	-	-	-	↑	3.000	1.70E-02
CD24	Other	-	-	-	↑	2.236	6.20E-02	↑	2.121	6.39E-03
TAB1	Enzyme	-	-	4.05E-02	-	-	-	↑	2.000	4.02E-02

↑: activated; ↓: inhibited; -: not activated/inhibited; Black text: regulator is present in dataset and z-score is below -2 or above 2 with P-value<0.05; Grey text: regulator is present in dataset, but z-score is above -2 or below 2, or P-value is >0.05

Supplementary table 2. Gene Set Enrichment Analysis. Cut-off value FDR q-value 0.25.
Supplementary table 2a. Gene sets differently regulated in PBMCs from old compared to young men.

Gene set	NES	FDR q-value
KEGG_STAPHYLOCOCCUS AUREUS INFECTION	2.174	0.020
KEGG_AFRICAN TRYPANOSOMIASIS	2.127	0.019
REACT_TRAF3-DEPENDENT IRF ACTIVATION PATHWAY	2.114	0.016
WIP_HS_INTERFERON_ALPHA-BETA_SIGNALING	2.111	0.013
KEGG_PERTUSSIS	2.053	0.018
KEGG_LYSOSOME	2.006	0.030
REACT_INTERFERON SIGNALING	2.003	0.026
REACT_CHEMOKINE RECEPTORS BIND CHEMOKINES	1.994	0.027
WIP_HS_TYPE_II_INTERFERON_SIGNALING_(IFNG)	1.965	0.033
REACT_NEGATIVE REGULATORS OF RIG-I_MDA5 SIGNALING	1.959	0.032
REACT_INTERFERON ALPHA_BETA SIGNALING	1.908	0.051
KEGG_MALARIA	1.892	0.056
KEGG_RHEUMATOID ARTHRITIS	1.890	0.052
KEGG_LEISHMANIASIS	1.861	0.062
REACT_RIG-I_MDA5 MEDIATED INDUCTION OF IFN-ALPHA_BETA PATHWAYS	1.861	0.058
KEGG_CYTOKINE-CYTOKINE RECEPTOR INTERACTION	1.859	0.056
WIP_HS_SENESCENCE_AND_AUTOPHAGY	1.855	0.054
NCI_UPA_UPAR_PATHWAY	1.854	0.052
REACT_INTERLEUKIN-1 SIGNALING	1.819	0.066
WIP_HS_COMPLEMENT_AND_COAGULATION_CASCADES	1.794	0.081
REACT_PLATELET DEGRANULATION	1.784	0.085
REACT_RESPONSE TO ELEVATED PLATELET CYTOSOLIC CA2+	1.779	0.084
REACT_TRAF6 MEDIATED IRF7 ACTIVATION	1.728	0.123
WIP_HS_SELENIUM_PATHWAY	1.719	0.127
BIOC_P53HYPOXIAPATHWAY	1.716	0.125
NCI_TAP63PATHWAY	1.706	0.130
NCI_DELTANP63PATHWAY	1.704	0.127
KEGG_COMPLEMENT AND COAGULATION CASCADES	1.701	0.125
REACT_CYTOKINE SIGNALING IN IMMUNE SYSTEM	1.697	0.125
KEGG_INFLUENZA A	1.682	0.136
KEGG_TOLL-LIKE RECEPTOR SIGNALING PATHWAY	1.651	0.168
WIP_HS_APOPTOSIS_MODULATION_BY_HSP70	1.628	0.194
WIP_HS_INTRINSIC_PATHWAY_FOR_APOPTOSIS	1.627	0.190
BIOC_PTDINSPATHWAY	1.624	0.189
REACT_PEROXISOMAL LIPID METABOLISM	1.624	0.184
WIP_HS_VITAMIN_B12_METABOLISM	1.620	0.183
REACT_NCAM1 INTERACTIONS	1.616	0.184
REACT_FORMATION OF FIBRIN CLOT (CLOTTING CASCADE)	1.613	0.183
NCI_TOLL_ENDOGENOUS_PATHWAY	1.605	0.188
WIP_HS_GPCRS,_CLASS_A_RHODOPSIN-LIKE	1.595	0.197
WIP_HS_GPCRS,_OTHER	1.588	0.201
REACT_G ALPHA (I) SIGNALLING EVENTS	1.579	0.210
NCI_INTEGRIN_CS_PATHWAY	1.578	0.207
REACT_GPCR LIGAND BINDING	1.573	0.209
KEGG_CYTOSOLIC DNA-SENSING PATHWAY	1.569	0.211
NCI_IL8CXCR1_PATHWAY	1.567	0.209
REACT_CLASS A_1 (RHODOPSIN-LIKE RECEPTORS)	1.563	0.209
NCI_IL1PATHWAY	1.559	0.211
KEGG_RIG-I-LIKE RECEPTOR SIGNALING PATHWAY	1.558	0.208
WIP_HS_ADIPOGENESIS	1.551	0.214
NCI_P38ALPHABETADOWNSTREAMPATHWAY	1.551	0.210
WIP_HS_REGULATION_OF_TOLL-LIKE_RECEPTOR_SIGNALING_PATHWAY	1.547	0.212
WIP_HS_TOLL-LIKE_RECEPTOR_SIGNALING_PATHWAY	1.539	0.219
WIP_HS_CYTOKINES_AND_INFLAMMATORY_RESPONSE	1.539	0.216
KEGG_SPHINGOLIPID METABOLISM	1.533	0.220

NRF2_TARGETS	1.530	0.221
REACT_G ALPHA (S) SIGNALLING EVENTS	1.526	0.222
WIP_HS_OXIDATIVE_STRESS	1.522	0.225
KEGG_VITAMIN DIGESTION AND ABSORPTION	1.519	0.227
KEGG_TUBERCULOSIS	1.513	0.232
KEGG_CHEMOKINE SIGNALING PATHWAY	1.503	0.243
REACT_SIGNAL AMPLIFICATION	1.500	0.245
REACT_PEPTIDE LIGAND-BINDING RECEPTORS	1.499	0.241
REACT_ACTIVATED TAK1 MEDIATES P38 MAPK ACTIVATION	1.497	0.242
KEGG_PPAR SIGNALING PATHWAY	1.495	0.241
REACT_METABOLISM OF LIPIDS AND LIPOPROTEINS	1.494	0.240
KEGG_PHAGOSOME	1.490	0.241
REACT_NF-KB ACTIVATION THROUGH FADD_RIP-1 PATHWAY MEDIATED BY CASPASE-8 AND -10	1.486	0.244
REACT_INTRINSIC PATHWAY FOR APOPTOSIS	1.485	0.242
NCI_AP1_PATHWAY	1.485	0.239
BIOC_IL1RPATHWAY	1.484	0.237
NCI_NECTIN_PATHWAY	1.481	0.237
REACT_INFLUENZA VIRAL RNA TRANSCRIPTION AND REPLICATION	-2.706	0.000
REACT_EUKARYOTIC TRANSLATION TERMINATION	-2.680	0.000
REACT_EUKARYOTIC TRANSLATION ELONGATION	-2.678	0.000
REACT_FORMATION OF A POOL OF FREE 40S SUBUNITS	-2.650	0.000
REACT_VIRAL MRNA TRANSLATION	-2.645	0.000
REACT_GTP HYDROLYSIS AND JOINING OF THE 60S RIBOSOMAL SUBUNIT	-2.631	0.000
REACT_PEPTIDE CHAIN ELONGATION	-2.626	0.000
REACT_L13A-MEDIATED TRANSLATIONAL SILENCING OF CERULOPLASMIN EXPRESSION	-2.624	0.000
WIP_HS_CYTOPLASMIC_RIBOSOMAL_PROTEINS	-2.604	0.000
REACT_TRANSLATION	-2.604	0.000
REACT_CAP-DEPENDENT TRANSLATION INITIATION	-2.588	0.000
REACT_3 -UTR-MEDIATED TRANSLATIONAL REGULATION	-2.584	0.000
REACT_EUKARYOTIC TRANSLATION INITIATION	-2.582	0.000
REACT_NONSENSE MEDIATED DECAY ENHANCED BY THE EXON JUNCTION COMPLEX	-2.578	0.000
REACT_NONSENSE MEDIATED DECAY INDEPENDENT OF THE EXON JUNCTION COMPLEX	-2.575	0.000
REACT_REGULATION OF GENE EXPRESSION IN BETA CELLS	-2.561	0.000
KEGG_RIBOSOME	-2.550	0.000
REACT_NONSENSE-MEDIATED DECAY	-2.533	0.000
REACT_REGULATION OF BETA-CELL DEVELOPMENT	-2.499	0.000
REACT_INSULIN SYNTHESIS AND PROCESSING	-2.371	0.000
REACT_METABOLISM OF PROTEINS	-2.125	0.001
REACT_FORMATION OF THE TERNARY COMPLEX, AND SUBSEQUENTLY, THE 43S COMPLEX	-2.115	0.001
KEGG_RIBOSOME BIOGENESIS IN EUKARYOTES	-2.058	0.002
REACT_METABOLISM OF MRNA	-2.046	0.002
REACT_TRANSLATION INITIATION COMPLEX FORMATION	-2.039	0.002
REACT_METABOLISM OF RNA	-2.033	0.002
REACT_GENE EXPRESSION	-2.029	0.002
REACT_RIBOSOMAL SCANNING AND START CODON RECOGNITION	-2.027	0.002
REACT_ACTIVATION OF THE MRNA UPON BINDING OF THE CAP-BINDING COMPLEX AND EIFS, AND SUBSEQUENT BINDING TO 43S	-1.962	0.006
KEGG_CYSTEINE AND METHIONINE METABOLISM	-1.901	0.012
KEGG_PRIMARY IMMUNODEFICIENCY	-1.894	0.013
WIP_HS_ELECTRON_TRANSPORT_CHAIN	-1.884	0.015
KEGG_RNA TRANSPORT	-1.854	0.022
REACT_MRNA SPLICING - MAJOR PATHWAY	-1.819	0.031
REACT_TRNA AMINOACYLATION	-1.802	0.037
KEGG_AMINOACYL-TRNA BIOSYNTHESIS	-1.800	0.037
REACT_ACTIVATION OF THE PRE-REPLICATIVE COMPLEX	-1.775	0.047
REACT_MRNA SPLICING - MINOR PATHWAY	-1.772	0.047
REACT_MRNA SPLICING	-1.771	0.046
WIP_HS_TRANSLATION_FACTORS	-1.768	0.046
REACT_MRNA PROCESSING	-1.766	0.046
REACT_PROCESSING OF CAPPED INTRON-CONTAINING PRE-MRNA	-1.753	0.051
BIOC_CTLA4PATHWAY	-1.749	0.052

REACT_DNA STRAND ELONGATION	-1.749	0.051
REACT_RESPIRATORY ELECTRON TRANSPORT, ATP SYNTHESIS BY CHEMIOSMOTIC COUPLING, AND HEAT PRODUCTION BY UNCOUPLING PROTEINS.	-1.744	0.052
KEGG_PROTEIN EXPORT	-1.735	0.056
REACT_FORMATION AND MATURATION OF MRNA TRANSCRIPT	-1.713	0.069
BIOC_VEGFPATHWAY	-1.706	0.072
WIP_HS_TRNA_AMINOACYLATION	-1.704	0.072
REACT_G2_M CHECKPOINTS	-1.701	0.073
REACT_DIABETES PATHWAYS	-1.700	0.072
REACT_RESPIRATORY ELECTRON TRANSPORT	-1.700	0.071
WIP_HS_DNA_REPLICATION	-1.697	0.072
REACT_EXTENSION OF TELOMERES	-1.676	0.084
KEGG_TYROSINE METABOLISM	-1.673	0.086
WIP_HS_MRNA_PROCESSING	-1.670	0.087
KEGG_PARKINSON'S DISEASE	-1.664	0.090
REACT_ASPARAGINE N-LINKED GLYCOSYLATION	-1.663	0.089
WIP_HS_ASPARAGINE_N-LINKED_GLYCOSYLATION	-1.656	0.094
KEGG_MRNA SURVEILLANCE PATHWAY	-1.654	0.094
REACT_MITOCHONDRIAL TRNA AMINOACYLATION	-1.653	0.093
REACT_ACTIVATION OF ATR IN RESPONSE TO REPLICATION STRESS	-1.625	0.116
KEGG_PROGESTERONE-MEDIATED OOCYTE MATURATION	-1.624	0.115
REACT_SYNTHESIS OF SUBSTRATES IN N-GLYCAN BIOSYTHESIS	-1.617	0.121
BIOC_TCRPATHWAY	-1.614	0.121
REACT_INTERACTIONS OF VPR WITH HOST CELLULAR PROTEINS	-1.605	0.129
REACT_CHROMOSOME MAINTENANCE	-1.597	0.136
KEGG_T CELL RECEPTOR SIGNALING PATHWAY	-1.591	0.140
REACT_INTERACTIONS OF REV WITH HOST CELLULAR PROTEINS	-1.590	0.140
WIP_HS_GENERIC_TRANSCRIPTION_PATHWAY	-1.587	0.141
NCI_IL2_STAT5PATHWAY	-1.583	0.144
REACT_ASSOCIATION OF TRIC_CCT WITH TARGET PROTEINS DURING BIOSYNTHESIS	-1.580	0.145
REACT_NUCLEAR IMPORT OF REV PROTEIN	-1.573	0.152
KEGG_SPLICEOSOME	-1.571	0.151
NCI_CD8TCRPATHWAY	-1.566	0.155
REACT_CYTOSOLIC TRNA AMINOACYLATION	-1.562	0.158
REACT_DEADENYLATION-DEPENDENT MRNA DECAY	-1.555	0.166
REACT_ACTIVATION OF CHAPERONES BY IRE1ALPHA	-1.549	0.172
KEGG_RNA POLYMERASE	-1.548	0.170
REACT_REGULATION OF GLUCOKINASE BY GLUCOKINASE REGULATORY PROTEIN	-1.544	0.173
REACT_TELOMERE C-STRAND (LAGGING STRAND) SYNTHESIS	-1.544	0.171
REACT_MITOTIC M-M_G1 PHASES	-1.543	0.170
BIOC_AMIPATHWAY	-1.543	0.168
KEGG_DNA REPLICATION	-1.542	0.168
REACT_LAGGING STRAND SYNTHESIS	-1.540	0.168
REACT_POST-TRANSLATIONAL PROTEIN MODIFICATION	-1.535	0.173
BIOC_CSKPATHWAY	-1.534	0.173
REACT_PREFOLDIN MEDIATED TRANSFER OF SUBSTRATE TO CCT_TRIC	-1.517	0.195
WIP_HS_OXIDATIVE_PHOSPHORYLATION	-1.516	0.194
REACT_METABOLISM OF NON-CODING RNA	-1.515	0.193
REACT_LATE PHASE OF HIV LIFE CYCLE	-1.515	0.192
REACT_HIV LIFE CYCLE	-1.512	0.193
REACT_DNA REPLICATION	-1.509	0.196
REACT_REV-MEDIATED NUCLEAR EXPORT OF HIV-1 RNA	-1.507	0.196
REACT_COOPERATION OF PREFOLDIN AND TRIC_CCT IN ACTIN AND TUBULIN FOLDING	-1.507	0.195
REACT_IMMUNOREGULATORY INTERACTIONS BETWEEN A LYMPHOID AND A NON-LYMPHOID CELL	-1.505	0.195
REACT_FORMATION OF TUBULIN FOLDING INTERMEDIATES BY CCT_TRIC	-1.496	0.206
REACT_SNRNP ASSEMBLY	-1.484	0.223
REACT_GENERIC TRANSCRIPTION PATHWAY	-1.475	0.234
REACT_M PHASE	-1.475	0.233
REACT_GAP-FILLING DNA REPAIR SYNTHESIS AND LIGATION IN GG-NER	-1.465	0.247

Supplementary table 2b. Gene sets upregulated in PBMCs in young men upon three weeks of 30% CR.

Gene set	NES	FDR q-value
WIP_HS_MRNA_PROCESSING	2.203	0.002
WIP_HS_PROCESSING_OF_CAPPED_INTRON-CONTAINING_PRE-MRNA	2.172	0.004
WIP_HS_RNA_POLYMERASE_I,_RNA_POLYMERASE_III,_AND_ MITOCHONDRIAL_TRANSCRIPTION	2.115	0.005
REACT_FORMATION OF A POOL OF FREE 40S SUBUNITS	2.035	0.013
REACT_3 -UTR-MEDIATED TRANSLATIONAL REGULATION	2.021	0.013
REACT_L13A-MEDIATED TRANSLATIONAL SILENCING OF CERULOPLASMIN EXPRESSION	1.995	0.014
REACT_GTP HYDROLYSIS AND JOINING OF THE 60S RIBOSOMAL SUBUNIT	1.982	0.015
REACT_PEPTIDE CHAIN ELONGATION	1.972	0.014
NCI_AR_TF_PATHWAY	1.969	0.014
REACT_MRNA SPLICING - MAJOR PATHWAY	1.962	0.013
REACT_MRNA SPLICING	1.943	0.016
REACT_GENE EXPRESSION	1.939	0.015
REACT_VIRAL MRNA TRANSLATION	1.905	0.021
REACT_GENERIC TRANSCRIPTION PATHWAY	1.900	0.020
REACT_INFLUENZA VIRAL RNA TRANSCRIPTION AND REPLICATION	1.893	0.021
REACT_EUKARYOTIC TRANSLATION ELONGATION	1.893	0.019
REACT_PROCESSING OF CAPPED INTRON-CONTAINING PRE-MRNA	1.893	0.018
REACT_VPR-MEDIATED NUCLEAR IMPORT OF PICS	1.888	0.018
KEGG_RIBOSOME	1.861	0.023
REACT_NONSENSE MEDIATED DECAY INDEPENDENT OF THE EXON JUNCTION COMPLEX	1.854	0.023
REACT_DOUBLE-STRAND BREAK REPAIR	1.832	0.028
REACT_RNA POLYMERASE I TRANSCRIPTION	1.831	0.027
REACT_MRNA PROCESSING	1.811	0.032
REACT_EUKARYOTIC TRANSLATION TERMINATION	1.801	0.034
KEGG_SPLICEOSOME	1.770	0.043
REACT_INTERACTIONS OF VPR WITH HOST CELLULAR PROTEINS	1.768	0.043
WIP_HS_CYTOPLASMIC_RIBOSOMAL_PROTEINS	1.735	0.056
REACT_FORMATION AND MATURATION OF MRNA TRANSCRIPT	1.729	0.057
REACT_RIBOSOMAL SCANNING AND START CODON RECOGNITION	1.701	0.073
REACT_REGULATION OF GLUCOKINASE BY GLUCOKINASE REGULATORY PROTEIN	1.696	0.074
REACT_REGULATION OF GENE EXPRESSION IN BETA CELLS	1.681	0.081
REACT_LATE PHASE OF HIV LIFE CYCLE	1.649	0.105
REACT_TRANSCRIPTION	1.647	0.103
REACT_EUKARYOTIC TRANSLATION INITIATION	1.635	0.111
REACT_REV-MEDIATED NUCLEAR EXPORT OF HIV-1 RNA	1.630	0.112
REACT_TRANSLATION INITIATION COMPLEX FORMATION	1.629	0.109
REACT_REGULATION OF BETA-CELL DEVELOPMENT	1.627	0.109
REACT_TRANSPORT OF MATURE MRNA DERIVED FROM AN INTRON-CONTAINING TRANSCRIPT	1.616	0.115
REACT_RNA POLYMERASE I TRANSCRIPTION TERMINATION	1.614	0.114
REACT_NONSENSE-MEDIATED DECAY	1.600	0.124
REACT_NUCLEAR IMPORT OF REV PROTEIN	1.598	0.123
REACT_CAP-DEPENDENT TRANSLATION INITIATION	1.595	0.123
REACT_INTERACTIONS OF REV WITH HOST CELLULAR PROTEINS	1.593	0.123
REACT_DEADENYLATION-DEPENDENT MRNA DECAY	1.581	0.131
REACT_TRANSLATION	1.581	0.129
KEGG_RNA DEGRADATION	1.577	0.130
REACT_HIV LIFE CYCLE	1.572	0.131
REACT_FORMATION OF THE TERNARY COMPLEX, AND SUBSEQUENTLY, THE 43S COMPLEX	1.562	0.139
REACT_NONSENSE MEDIATED DECAY ENHANCED BY THE EXON JUNCTION COMPLEX	1.561	0.137
REACT_RNA POLYMERASE I PROMOTER CLEARANCE	1.553	0.143
REACT_RNA POLYMERASE I, RNA POLYMERASE III, AND MITOCHONDRIAL TRANSCRIPTION	1.548	0.146
REACT_CLEAVAGE OF GROWING TRANSCRIPT IN THE TERMINATION REGION	1.540	0.151
KEGG_FANCONI ANEMIA PATHWAY	1.538	0.150
REACT_CENTROSOME MATURATION	1.536	0.150
REACT_RECRUITMENT OF MITOTIC CENTROSOME PROTEINS AND COMPLEXES	1.532	0.151

REACT_RNA POLYMERASE I TRANSCRIPTION INITIATION	1.532	0.149
REACT_MRNA 3-END PROCESSING	1.531	0.147
REACT_POST-ELONGATION PROCESSING OF INTRON-CONTAINING PRE-MRNA	1.523	0.153
REACT_TRANSPORT OF MATURE TRANSCRIPT TO CYTOPLASM	1.521	0.153
REACT_G2_M TRANSITION	1.512	0.160
REACT_RNA POLYMERASE II TRANSCRIPTION TERMINATION	1.504	0.167
REACT_LOSS OF NLP FROM MITOTIC CENTROSOMES	1.502	0.166
REACT_LOSS OF PROTEINS REQUIRED FOR INTERPHASE MICROTUBULE ORGANIZATION Å FROM THE CENTROSOME	1.501	0.164
WIP_HS_ENERGY_METABOLISM	1.483	0.183
REACT_MITOTIC G2-G2_M PHASES	1.483	0.180
REACT_POST-ELONGATION PROCESSING OF THE TRANSCRIPT	1.479	0.183
BIOC_CARM_ER PATHWAY	1.475	0.185
BIOC_IL7 PATHWAY	1.470	0.188
REACT_RNA POLYMERASE II TRANSCRIPTION	1.449	0.214
NCI_PI3KCI AKT PATHWAY	1.439	0.226
WIP_HS_MITOCHONDRIAL_GENE_EXPRESSION	1.436	0.227
REACT_ACTIVATION OF THE MRNA UPON BINDING OF THE CAP-BINDING COMPLEX AND EIFS, AND SUBSEQUENT BINDING TO 43S	1.431	0.231
REACT_RNA POLYMERASE II PRE-TRANSCRIPTION EVENTS	1.423	0.240
REACT_TAT-MEDIATED HIV-1 ELONGATION ARREST AND RECOVERY	1.422	0.238
NCI_HDAC_CLASSI_PATHWAY	1.420	0.237
REACT_PAUSING AND RECOVERY OF TAT-MEDIATED HIV-1 ELONGATION	1.413	0.245
REACT_TAT-MEDIATED ELONGATION OF THE HIV-1 TRANSCRIPT	1.412	0.243
REACT_NEPHRIN INTERACTIONS	1.409	0.244
REACT_FORMATION OF HIV-1 ELONGATION COMPLEX IN THE ABSENCE OF HIV-1 TAT	1.409	0.242
REACT_FORMATION OF HIV-1 ELONGATION COMPLEX CONTAINING HIV-1 TAT	1.404	0.246

Supplementary table 2c. Gene sets downregulated in PBMCs in young men upon three weeks of 30% CR.

Gene set	NES	FDR q-value
WIP_HS_TYPE_II_INTERFERON_SIGNALING_(IFNG)	-2.351	0.000
KEGG_LYSOSOME	-2.315	0.000
REACT_INTERFERON GAMMA SIGNALING	-2.240	0.002
REACT_INTERFERON SIGNALING	-2.237	0.001
WIP_HS_PROTEASOME_DEGRADATION	-2.205	0.002
REACT_REGULATION OF ORNITHINE DECARBOXYLASE (ODC)	-2.112	0.009
KEGG_PROTEASOME	-2.105	0.008
REACT_VPU MEDIATED DEGRADATION OF CD4	-2.069	0.013
WIP_HS_LEUKOCYTE_TARBASE	-2.069	0.012
REACT_UBIQUITIN-DEPENDENT DEGRADATION OF CYCLIN D	-2.049	0.014
REACT_UBIQUITIN-DEPENDENT DEGRADATION OF CYCLIN D1	-2.046	0.013
NCI_UPA_UPAR_PATHWAY	-2.040	0.012
REACT_INTERFERON ALPHA_BETA SIGNALING	-2.032	0.012
KEGG_PHAGOSOME	-2.022	0.014
NCI_IL12_2 PATHWAY	-2.004	0.016
REACT_CYTOKINE SIGNALING IN IMMUNE SYSTEM	-1.984	0.019
REACT_CDK-MEDIATED PHOSPHORYLATION AND REMOVAL OF CDC6	-1.980	0.018
KEGG_NATURAL KILLER CELL MEDIATED CYTOTOXICITY	-1.977	0.018
REACT_REGULATION OF ACTIVATED PAK-2P34 BY PROTEASOME MEDIATED DEGRADATION	-1.950	0.026
KEGG_STAPHYLOCOCCUS AUREUS INFECTION	-1.948	0.025
REACT_SCF-BETA-TRCP MEDIATED DEGRADATION OF EMI1	-1.946	0.024
REACT_DEGRADATION OF BETA-CATENIN BY THE DESTRUCTION COMPLEX	-1.943	0.024
REACT_SIGNALING BY WNT	-1.933	0.025
KEGG_GALACTOSE METABOLISM	-1.926	0.026
BIOC_FMLP PATHWAY	-1.924	0.025
KEGG_OTHER GLYCAN DEGRADATION	-1.918	0.026
KEGG_PENTOSE PHOSPHATE PATHWAY	-1.908	0.027

REACT_CDT1 ASSOCIATION WITH THE CDC6_ORC_ORIGIN COMPLEX	-1.902	0.028
REACT_SCF(SKP2)-MEDIATED DEGRADATION OF P27_P21	-1.901	0.027
REACT_AUTODEGRADATION OF THE E3 UBIQUITIN LIGASE COP1	-1.900	0.027
REACT_GLUCOSE METABOLISM	-1.888	0.030
REACT_ASSEMBLY OF THE PRE-REPLICATIVE COMPLEX	-1.883	0.030
NCI_ALPHASYNUCLEIN_PATHWAY	-1.880	0.030
REACT_VIF-MEDIATED DEGRADATION OF APOBEC3G	-1.871	0.032
KEGG_PERTUSSIS	-1.868	0.032
REACT_REGULATION OF APOPTOSIS	-1.867	0.031
BIOC_AT1RPATHWAY	-1.852	0.035
REACT_STABILIZATION OF P53	-1.849	0.036
REACT_P53-INDEPENDENT G1_S DNA DAMAGE CHECKPOINT	-1.842	0.038
REACT_P53-INDEPENDENT DNA DAMAGE RESPONSE	-1.842	0.037
WIP_HS_REGULATION_OF_TOLL-LIKE_RECEPTOR_SIGNALING_PATHWAY	-1.839	0.037
NCI_INTEGRIN2_PATHWAY	-1.837	0.037
REACT_REGULATION OF DNA REPLICATION	-1.832	0.038
NCI_GMCSF_PATHWAY	-1.831	0.037
WIP_HS_TOLL-LIKE_RECEPTOR_SIGNALING_PATHWAY	-1.816	0.042
REACT_UBIQUITIN MEDIATED DEGRADATION OF PHOSPHORYLATED CDC25A	-1.812	0.043
KEGG_OSTEOCLAST DIFFERENTIATION	-1.809	0.043
KEGG_RHEUMATOID ARTHRITIS	-1.807	0.043
KEGG_TUBERCULOSIS	-1.805	0.043
REACT_REMOVAL OF LICENSING FACTORS FROM ORIGINS	-1.804	0.043
WIP_HS_INTRINSIC_PATHWAY_FOR_APOPTOSIS	-1.798	0.044
REACT_ORC1 REMOVAL FROM CHROMATIN	-1.796	0.044
KEGG_LEISHMANIASIS	-1.794	0.044
REACT_APOPTOSIS	-1.793	0.044
WIP_HS_IL-3_SIGNALING_PATHWAY	-1.792	0.044
REACT_DESTABILIZATION OF MRNA BY AUF1 (HNRNP D0)	-1.790	0.044
WIP_HS_OXIDATIVE_STRESS	-1.789	0.043
NCI_IL8CXCR2_PATHWAY	-1.789	0.043
REACT_SWITCHING OF ORIGINS TO A POST-REPLICATIVE STATE	-1.782	0.045
WIP_HS_INTERFERON_ALPHA-BETA_SIGNALING	-1.776	0.047
KEGG_INFLUENZA A	-1.769	0.049
NCI_PRLSIGNALINGEVENTSPATHWAY	-1.768	0.049
NCI_ANTHRAXPATHWAY	-1.764	0.050
KEGG_GLYCOLYSIS _ GLUCONEOGENESIS	-1.759	0.051
NCI_ENDOTHELINPATHWAY	-1.757	0.051
REACT_CYCLIN E ASSOCIATED EVENTS DURING G1_S TRANSITION	-1.757	0.050
KEGG_GRAFT-VERSUS-HOST DISEASE	-1.756	0.050
NCI_ERBB2ERBB3PATHWAY	-1.754	0.050
REACT_P53-DEPENDENT G1 DNA DAMAGE RESPONSE	-1.747	0.053
REACT_P53-DEPENDENT G1_S DNA DAMAGE CHECKPOINT	-1.745	0.053
REACT_SYNTHESIS OF DNA	-1.744	0.052
KEGG_MUCIN TYPE O-GLYCAN BIOSYNTHESIS	-1.744	0.052
NCI_TXA2PATHWAY	-1.740	0.053
NCI_IL12_STAT4PATHWAY	-1.734	0.054
KEGG_TOLL-LIKE RECEPTOR SIGNALING PATHWAY	-1.724	0.058
KEGG_MALARIA	-1.723	0.058
REACT_SIGNALLING TO RAS	-1.719	0.060
NCI_CD8TCRDOWNSTREAMPATHWAY	-1.717	0.060
REACT_G1_S DNA DAMAGE CHECKPOINTS	-1.714	0.061
KEGG_AMINO SUGAR AND NUCLEOTIDE SUGAR METABOLISM	-1.710	0.062
REACT_AUTODEGRADATION OF CDH1 BY CDH1_APC_C	-1.707	0.062
WIP_HS_MUSCLE_CELL_TARBASE	-1.703	0.064
WIP_HS_SQUAMOUS_CELL_TARBASE	-1.701	0.065
WIP_HS_PROSTAGLANDIN_SYNTHESIS_AND_REGULATION	-1.700	0.064
REACT_METABOLISM OF AMINO ACIDS AND DERIVATIVES	-1.698	0.065
REACT_TRANSPORT TO THE GOLGI AND SUBSEQUENT MODIFICATION	-1.684	0.071
REACT_M_G1 TRANSITION	-1.680	0.073
NRF2_TARGETS	-1.680	0.072
KEGG_DRUG METABOLISM - OTHER ENZYMES	-1.678	0.072

REACT_GLUCONEOGENESIS	-1.677	0.073
REACT_CYCLIN A_CDK2-ASSOCIATED EVENTS AT S PHASE ENTRY	-1.677	0.072
REACT_S PHASE	-1.677	0.071
REACT_GPVI-MEDIATED ACTIVATION CASCADE	-1.673	0.072
WIP_HS_INTEGRIN-MEDIATED_CELL_ADHESION	-1.669	0.074
NCI_VEGFR1_2_PATHWAY	-1.669	0.073
KEGG_ANTIGEN PROCESSING AND PRESENTATION	-1.665	0.075
REACT_DNA REPLICATION PRE-INITIATION	-1.664	0.074
REACT_APC_C_CDH1 MEDIATED DEGRADATION OF CDC20 AND OTHER APC_C_CDH1 TARGETED PROTEINS IN LATE MITOSIS_EARLY G1	-1.664	0.073
NCI_S1P_S1P3_PATHWAY	-1.657	0.078
NCI_TCRCALCIUMPATHWAY	-1.654	0.079
WIP_HS_PROTEINS_AND_DNA_SEQUENCES_IN_CARDICAC_STRUCTURES	-1.653	0.079
KEGG_ALZHEIMER'S DISEASE	-1.650	0.080
REACT_PLATELET ACTIVATION TRIGGERS	-1.647	0.081
NCI_AMB2_NEUTROPHILS_PATHWAY	-1.643	0.083
REACT_G1_S TRANSITION	-1.643	0.082
KEGG_CITRATE CYCLE (TCA CYCLE)	-1.638	0.085
KEGG_TYPE I DIABETES MELLITUS	-1.637	0.085
WIP_HS_EICOSANOID_SYNTHESIS	-1.635	0.085
REACT_APC_C_CDC20 MEDIATED DEGRADATION OF MITOTIC PROTEINS	-1.635	0.084
REACT_MITOTIC G1-G1_S PHASES	-1.632	0.086
KEGG_CHAGAS DISEASE (AMERICAN TRYPANOSOMIASIS)	-1.631	0.086
KEGG_ARGININE AND PROLINE METABOLISM	-1.630	0.086
REACT_APC_C_CDC20 MEDIATED DEGRADATION OF SECURIN	-1.630	0.085
KEGG_HEPATITIS C	-1.627	0.086
WIP_HS_MEMBRANE_TRAFFICKING	-1.626	0.086
WIP_HS_LYMPHOCYTE_TARBASE	-1.626	0.085
BIOC_PROTEASOMEPATHWAY	-1.617	0.091
WIP_HS_SIGNAL_TRANSDUCTION_OF_S1P_RECEPTOR	-1.614	0.092
BIOC_RACCYCDPATHWAY	-1.608	0.095
WIP_HS_EPITHELIUM_TARBASE	-1.607	0.095
REACT_TRANSFERRIN ENDOCYTOSIS AND RECYCLING	-1.607	0.095
WIP_HS_ADIPOCYTE_TARBASE	-1.606	0.095
BIOC_SPPAPATHWAY	-1.604	0.095
KEGG_CELL ADHESION MOLECULES (CAMS)	-1.603	0.095
PPARA_TARGETS	-1.602	0.095
REACT_INSULIN RECEPTOR RECYCLING	-1.598	0.097
NCI_AR_NONGENOMIC_PATHWAY	-1.595	0.099
BIOC_TOLLPATHWAY	-1.594	0.098
KEGG_VIBRIO CHOLERAE INFECTION	-1.591	0.099
REACT_ACTIVATION OF APC_C AND APC_C_CDC20 MEDIATED DEGRADATION OF MITOTIC PROTEINS	-1.589	0.100
REACT_INTERLEUKIN RECEPTOR SHC SIGNALING	-1.588	0.100
NCI_THROMBIN_PAR1_PATHWAY	-1.587	0.100
NCI_AVB3_OPN_PATHWAY	-1.585	0.101
NCI_IL8CXCR1_PATHWAY	-1.583	0.102
WIP_HS_GLYCOLYSIS_AND_GLUCONEOGENESIS	-1.581	0.103
NCI_CERAMIDE_PATHWAY	-1.581	0.103
NCI_PDGFRBPATHWAY	-1.575	0.106
BIOC_GSK3PATHWAY	-1.572	0.108
WIP_HS_MAPK_CASCADE	-1.571	0.108
REACT_TRAF6 MEDIATED INDUCTION OF NFKB AND MAP KINASES UPON TLR7_8 OR 9 ACTIVATION	-1.571	0.108
REACT_APC_C-MEDIATED DEGRADATION OF CELL CYCLE PROTEINS	-1.566	0.110
REACT_REGULATION OF APC_C ACTIVATORS BETWEEN G1_S AND EARLY ANAPHASE	-1.566	0.110
KEGG_GLYCEROLIPID METABOLISM	-1.562	0.112
WIP_HS_SIGNALING_OF_HEPATOCYTE_GROWTH_FACTOR_RECEPTOR	-1.560	0.113
REACT_TOLL LIKE RECEPTOR 7_8 (TLR7_8) CASCADE	-1.557	0.114
REACT_INTRINSIC PATHWAY FOR APOPTOSIS	-1.555	0.116
KEGG_MINERAL ABSORPTION	-1.553	0.116
REACT_REGULATION OF MITOTIC CELL CYCLE	-1.550	0.118

REACT_MYD88 DEPENDENT CASCADE INITIATED ON ENDOSOME	-1.543	0.122
REACT_AMINO ACID TRANSPORT ACROSS THE PLASMA MEMBRANE	-1.543	0.122
REACT_TOLL LIKE RECEPTOR 9 (TLR9) CASCADE	-1.534	0.129
KEGG_FC GAMMA R-MEDIATED PHAGOCYTOSIS	-1.533	0.129
REACT_SEMAPHORIN INTERACTIONS	-1.532	0.129
NCI_PTP1BPATHWAY	-1.528	0.132
BIOC_PYK2PATHWAY	-1.523	0.135
REACT_CLASS A_1 (RHODOPSIN-LIKE RECEPTORS)	-1.523	0.135
REACT_CELL SURFACE INTERACTIONS AT THE VASCULAR WALL	-1.521	0.136
REACT_CTLA4 INHIBITORY SIGNALING	-1.520	0.136
BIOC_METPATHWAY	-1.519	0.136
REACT_IRON UPTAKE AND TRANSPORT	-1.518	0.137
KEGG_ALLOGRAFT REJECTION	-1.513	0.140
WIP_HS_COMPLEMENT_AND_COAGULATION_CASCADES	-1.511	0.141
BIOC_ERK5PATHWAY	-1.510	0.142
WIP_HS_TRANSPORT_OF_INORGANIC_CATIONS-ANIONS_AND_ AMINO_ACIDS-OLIGOPEPTIDES	-1.508	0.143
KEGG_FRUCTOSE AND MANNOSE METABOLISM	-1.507	0.142
KEGG_AMYOTROPHIC LATERAL SCLEROSIS (ALS)	-1.502	0.147
KEGG_PATHOGENIC ESCHERICHIA COLI INFECTION	-1.501	0.147
BIOC_VEGFPATHWAY	-1.499	0.148
KEGG_TYPE II DIABETES MELLITUS	-1.499	0.147
REACT_MEMBRANE TRAFFICKING	-1.498	0.147
WIP_HS_NUCLEOTIDE_METABOLISM	-1.496	0.148
REACT_GLYCOLYSIS	-1.496	0.148
KEGG_VEGF SIGNALING PATHWAY	-1.494	0.149
KEGG_BLADDER CANCER	-1.492	0.149
KEGG_AUTOIMMUNE THYROID DISEASE	-1.490	0.150
KEGG_CARDIAC MUSCLE CONTRACTION	-1.490	0.150
REACT_METABOLISM OF CARBOHYDRATES	-1.486	0.153
KEGG_EPITHELIAL CELL SIGNALING IN HELICOBACTER PYLORI INFECTION	-1.484	0.154
KEGG_FAT DIGESTION AND ABSORPTION	-1.481	0.156
WIP_HS_GPCRS,_CLASS_A_RHODOPSIN-LIKE	-1.478	0.158
NCI_MAPKTRKPATHWAY	-1.477	0.159
WIP_HS_FOLATE_METABOLISM	-1.471	0.163
KEGG_FC EPSILON RI SIGNALING PATHWAY	-1.471	0.162
REACT_CDC20_PHOSPHO-APC_C MEDIATED DEGRADATION OF CYCLIN A	-1.471	0.162
REACT_MAP KINASE ACTIVATION IN TLR CASCADE	-1.470	0.162
NCI_LYSOPHOSPHOLIPID_PATHWAY	-1.469	0.162
KEGG_FATTY ACID ELONGATION	-1.469	0.162
KEGG_MEASLES	-1.469	0.161
REACT_INTERLEUKIN-2 SIGNALING	-1.468	0.161
WIP_HS_APOPTOSIS	-1.466	0.163
REACT_LYSOSOME VESICLE BIOGENESIS	-1.465	0.162
WIP_HS_ASPARAGINE_N-LINKED_GLYCOSYLATION	-1.465	0.162
KEGG_REGULATION OF ACTIN CYTOSKELETON	-1.464	0.162
KEGG_CHEMOKINE SIGNALING PATHWAY	-1.463	0.162
KEGG_OXIDATIVE PHOSPHORYLATION	-1.461	0.164
KEGG_PANCREATIC CANCER	-1.460	0.164
REACT_HEMOSTASIS	-1.457	0.166
REACT_AMYLOIDS	-1.456	0.167
KEGG_AFRICAN TRYPANOSOMIASIS	-1.451	0.171
KEGG_ADIPOCYTOKINE SIGNALING PATHWAY	-1.450	0.171
REACT_TRAF6 MEDIATED INDUCTION OF PROINFLAMMATORY CYTOKINES	-1.450	0.171
KEGG_NEUROACTIVE LIGAND-RECEPTOR INTERACTION	-1.448	0.172
KEGG_SHIGELLOSIS	-1.445	0.173
WIP_HS_METABOLISM_OF_CARBOHYDRATES	-1.444	0.175
NCI_P38ALPHABETAPATHWAY	-1.441	0.177
REACT_N-GLYCAN ANTENNAE ELONGATION IN THE MEDIAL_TRANS-GOLGI	-1.439	0.178
REACT_FORMATION OF PLATELET PLUG	-1.439	0.177
REACT_SIGNALLING TO ERKS	-1.439	0.177

NCI_TOLL_ENDOGENOUS_PATHWAY	-1.438	0.177
KEGG_STEROID BIOSYNTHESIS	-1.435	0.180
REACT_PLATELET DEGRANULATION	-1.433	0.182
KEGG_PORPHYRIN AND CHLOROPHYLL METABOLISM	-1.427	0.187
REACT_RESPIRATORY ELECTRON TRANSPORT	-1.425	0.189
REACT_GPCR LIGAND BINDING	-1.423	0.190
NCI_ERBB1_DOWNSTREAM_PATHWAY	-1.422	0.191
KEGG_PANCREATIC SECRETION	-1.419	0.193
KEGG_VALINE, LEUCINE AND ISOLEUCINE DEGRADATION	-1.419	0.193
KEGG_BACTERIAL INVASION OF EPITHELIAL CELLS	-1.417	0.193
NCI_NETRIN_PATHWAY	-1.411	0.200
NCI_FASPATHWAY	-1.411	0.199
REACT_REGULATION OF MRNA STABILITY BY PROTEINS THAT BIND AU-RICH ELEMENTS	-1.411	0.199
KEGG_SPHINGOLIPID METABOLISM	-1.410	0.198
NCI_LYMPHANGIOGENESIS_PATHWAY	-1.405	0.203
WIP_HS_ANGIOGENESIS_OVERVIEW	-1.405	0.203
WIP_HS_IL-5_SIGNALING_PATHWAY	-1.405	0.202
REACT_ACTIVATION OF KAINATE RECEPTORS UPON GLUTAMATE BINDING	-1.403	0.203
KEGG_SALMONELLA INFECTION	-1.403	0.202
BIOC_KERATINOCYTEPATHWAY	-1.400	0.206
WIP_HS_FATTY_ACID,_TRIACYLGLYCEROL,_AND_KETONE_BODY_METABOLISM	-1.398	0.207
KEGG_GLYCEROPHOSPHOLIPID METABOLISM	-1.397	0.208
WIP_HS_IL-4_SIGNALING_PATHWAY	-1.397	0.207
WIP_HS_CELL_SURFACE_INTERACTIONS_AT_THE_VASCULAR_WALL	-1.395	0.208
WIP_HS_IL-6_SIGNALING_PATHWAY	-1.393	0.210
WIP_HS_FLUOROPYRIMIDINE_ACTIVITY	-1.391	0.212
REACT_BASIGIN INTERACTIONS	-1.388	0.214
REACT_PLATELET ACTIVATION	-1.385	0.217
REACT_IMMUNOREGULATORY INTERACTIONS BETWEEN A LYMPHOID AND A NON-LYMPHOID CELL	-1.384	0.218
REACT_TOLL LIKE RECEPTOR 3 (TLR3) CASCADE	-1.383	0.219
KEGG_STARCH AND SUCROSE METABOLISM	-1.381	0.220
WIP_HS_ANGIOGENESIS	-1.381	0.220
KEGG_COMPLEMENT AND COAGULATION CASCADES	-1.380	0.219
WIP_HS_TRYPTOPHAN_METABOLISM	-1.376	0.224
KEGG_METABOLISM OF XENOBIOTICS BY CYTOCHROME P450	-1.375	0.225
NCI_INTEGRIN3_PATHWAY	-1.373	0.227
NCI_EPOPATHWAY	-1.372	0.226
WIP_HS_SELENIUM_PATHWAY	-1.369	0.229
REACT_SIGNAL TRANSDUCTION BY L1	-1.368	0.231
WIP_HS_ESTROGEN_SIGNALING_PATHWAY	-1.367	0.231
NCI_RAC1_REG_PATHWAY	-1.366	0.231
KEGG_VIRAL MYOCARDITIS	-1.366	0.230
KEGG_PARKINSON'S DISEASE	-1.365	0.230
WIP_HS_RANKL-RANK_SIGNALING_PATHWAY	-1.365	0.230
NCI_CXCR3PATHWAY	-1.363	0.231
REACT_TRIGLYCERIDE BIOSYNTHESIS	-1.361	0.233
BIOC_TIDPATHWAY	-1.360	0.234
REACT_INTERLEUKIN-3, 5 AND GM-CSF SIGNALING	-1.357	0.237
WIP_HS_DNA_DAMAGE_RESPONSE_(ONLY_ATM_DEPENDENT)	-1.355	0.238
KEGG_MELANOMA	-1.353	0.240
BIOC_ECMPATHWAY	-1.353	0.240
KEGG_TOXOPLASMOSIS	-1.350	0.243
REACT_CELL JUNCTION ORGANIZATION	-1.345	0.249
WIP_HS_ALPHA_6_BETA_4_SIGNALING_PATHWAY	-1.345	0.248
KEGG_ENDOCYTOSIS	-1.345	0.248
KEGG_DORSO-VENTRAL AXIS FORMATION	-1.344	0.248
NCI_TCPTP_PATHWAY	-1.344	0.247
NCI_HIF1_TFPATHWAY	-1.342	0.249

Supplementary table 2d. Gene sets upregulated in PBMCs in old men upon three weeks of 30% CR.

Gene set	NES	FDR q-value
REACT_INTERACTIONS OF REV WITH HOST CELLULAR PROTEINS	2.134	0.020
REACT_REGULATION OF GLUCOKINASE BY GLUCOKINASE REGULATORY PROTEIN	2.124	0.011
REACT_REV-MEDIATED NUCLEAR EXPORT OF HIV-1 RNA	2.105	0.011
REACT_NUCLEAR IMPORT OF REV PROTEIN	2.092	0.010
NCI_CD8TCRPATHWAY	2.077	0.010
REACT_VPR-MEDIATED NUCLEAR IMPORT OF PICS	2.048	0.012
WIP_HS_TRNA_AMINOACYLATION	2.008	0.018
WIP_HS_MRNA_PROCESSING	1.972	0.026
REACT_INTERACTIONS OF VPR WITH HOST CELLULAR PROTEINS	1.969	0.024
REACT_MITOTIC G2-G2_M PHASES	1.966	0.023
REACT_RECRUITMENT OF MITOTIC CENTROSOME PROTEINS AND COMPLEXES	1.959	0.023
REACT_CENTROSOME MATURATION	1.944	0.026
WIP_HS_PROCESSING_OF_CAPPED_INTRON-CONTAINING_PRE-MRNA	1.928	0.029
KEGG_SPLICEOSOME	1.924	0.028
KEGG_RIBOSOME BIOGENESIS IN EUKARYOTES	1.924	0.026
BIOC_CTLA4PATHWAY	1.921	0.026
REACT_MRNA SPLICING - MAJOR PATHWAY	1.912	0.028
REACT_NONSENSE-MEDIATED DECAY	1.909	0.027
REACT_REGULATION OF GENE EXPRESSION IN BETA CELLS	1.899	0.030
REACT_MRNA PROCESSING	1.896	0.029
REACT_MRNA SPLICING	1.889	0.030
REACT_G2_M TRANSITION	1.888	0.029
REACT_FORMATION OF A POOL OF FREE 40S SUBUNITS	1.881	0.031
KEGG_T CELL RECEPTOR SIGNALING PATHWAY	1.875	0.031
REACT_NONSENSE MEDIATED DECAY ENHANCED BY THE EXON JUNCTION COMPLEX	1.864	0.034
REACT_FORMATION AND MATURATION OF MRNA TRANSCRIPT	1.862	0.033
REACT_GLUCOSE TRANSPORT	1.861	0.033
REACT_COSTIMULATION BY THE CD28 FAMILY	1.855	0.034
REACT_HEXOSE TRANSPORT	1.842	0.037
REACT_LOSS OF PROTEINS REQUIRED FOR INTERPHASE MICROTUBULE ORGANIZATIONÅ FROM THE CENTROSOME	1.842	0.036
REACT_LOSS OF NLP FROM MITOTIC CENTROSOMES	1.837	0.036
REACT_PROCESSING OF CAPPED INTRON-CONTAINING PRE-MRNA	1.836	0.036
NCI_IL2_STAT5PATHWAY	1.822	0.040
WIP_HS_ENERGY_METABOLISM	1.822	0.039
REACT_LATE PHASE OF HIV LIFE CYCLE	1.797	0.050
REACT_EUKARYOTIC TRANSLATION ELONGATION	1.789	0.053
REACT_PEPTIDE CHAIN ELONGATION	1.780	0.057
REACT_CD28 DEPENDENT PI3K_AKT SIGNALING	1.778	0.057
KEGG_MRNA SURVEILLANCE PATHWAY	1.776	0.057
REACT_GENE EXPRESSION	1.774	0.056
REACT_VIRAL MRNA TRANSLATION	1.771	0.057
REACT_INFLUENZA VIRAL RNA TRANSCRIPTION AND REPLICATION	1.770	0.056
KEGG_AMINOACYL-TRNA BIOSYNTHESIS	1.770	0.055
KEGG_PRIMARY IMMUNODEFICIENCY	1.769	0.054
REACT_REGULATION OF BETA-CELL DEVELOPMENT	1.767	0.054
BIOC_IL7PATHWAY	1.764	0.054
REACT_HIV LIFE CYCLE	1.762	0.054
NCI_TCR_PATHWAY	1.756	0.056
WIP_HS_IL-7_SIGNALING_PATHWAY	1.748	0.059
REACT_EFFECTS OF PIP2 HYDROLYSIS	1.737	0.065
REACT_3 -UTR-MEDIATED TRANSLATIONAL REGULATION	1.726	0.070
REACT_GLYCOLYSIS	1.708	0.081
REACT_L13A-MEDIATED TRANSLATIONAL SILENCING OF CERULOPLASMIN EXPRESSION	1.695	0.090
REACT_NONSENSE MEDIATED DECAY INDEPENDENT OF THE EXON JUNCTION COMPLEX	1.690	0.093
WIP_HS_T_CELL_RECEPTOR_SIGNALING_PATHWAY	1.689	0.092
REACT_METABOLISM OF MRNA	1.681	0.097

REACT_CELL CYCLE, MITOTIC	1.671	0.105
NCI_RHOA_REG_PATHWAY	1.668	0.107
REACT_TCR SIGNALING	1.664	0.108
REACT_GTP HYDROLYSIS AND JOINING OF THE 60S RIBOSOMAL SUBUNIT	1.663	0.107
KEGG_RNA TRANSPORT	1.657	0.111
NCI_ATR_PATHWAY	1.656	0.111
REACT_M PHASE	1.653	0.111
REACT_METABOLISM OF RNA	1.651	0.112
KEGG_RIBOSOME	1.638	0.125
KEGG_MEASLES	1.629	0.132
REACT_MITOTIC PROMETAPHASE	1.619	0.141
WIP_HS_ALPHA_6_BETA_4_SIGNALING_PATHWAY	1.618	0.140
KEGG_PHOSPHATIDYLINOSITOL SIGNALING SYSTEM	1.615	0.142
REACT_DARPP-32 EVENTS	1.601	0.156
KEGG_CIRCADIAN RHYTHM - MAMMAL	1.598	0.159
REACT_REGULATORY RNA PATHWAYS	1.597	0.158
REACT_MICRORNA (MIRNA) BIOGENESIS	1.596	0.157
REACT_CAP-DEPENDENT TRANSLATION INITIATION	1.595	0.156
REACT_CTLA4 INHIBITORY SIGNALING	1.595	0.154
REACT_EUKARYOTIC TRANSLATION TERMINATION	1.594	0.152
REACT_EUKARYOTIC TRANSLATION INITIATION	1.582	0.166
REACT_TRANSPORT OF MATURE MRNA DERIVED FROM AN INTRON-CONTAINING TRANSCRIPT	1.580	0.167
REACT_DEADENYLATION-DEPENDENT MRNA DECAY	1.571	0.176
WIP_HS_TCR_SIGNALING	1.566	0.182
WIP_HS_G13_SIGNALING_PATHWAY	1.566	0.180
REACT_NF-KB ACTIVATION THROUGH FADD_RIP-1 PATHWAY MEDIATED BY CASPASE-8 AND -10	1.560	0.186
REACT_MITOTIC M-M_G1 PHASES	1.554	0.192
REACT_MRNA SPLICING - MINOR PATHWAY	1.554	0.191
REACT_DEADENYLATION OF MRNA	1.551	0.191
REACT_MTOR SIGNALLING	1.551	0.189
WIP_HS_IL-2_SIGNALING_PATHWAY	1.549	0.190
WIP_HS_SIGNALING_BY_EGFR	1.545	0.194
KEGG_INOSITOL PHOSPHATE METABOLISM	1.542	0.196
REACT_CD28 CO-STIMULATION	1.542	0.194
NCI_ERBB1_RECEPTOR_PROXIMAL_PATHWAY	1.541	0.193
REACT_RNA POLYMERASE II TRANSCRIPTION	1.541	0.191
REACT_TRANSLATION	1.540	0.191
REACT_HIV INFECTION	1.539	0.189
REACT_MITOCHONDRIAL TRNA AMINOACYLATION	1.536	0.192
WIP_HS_TNF-ALPHA-NF-KB_SIGNALING_PATHWAY	1.535	0.192
NCI_IGF1_PATHWAY	1.532	0.195
REACT_INSULIN SYNTHESIS AND PROCESSING	1.528	0.199
KEGG_LEUKOCYTE TRANSENDOTHELIAL MIGRATION	1.519	0.210
NCI_IL2_PI3KPATHWAY	1.516	0.213
REACT_CLEAVAGE OF GROWING TRANSCRIPT IN THE TERMINATION REGION	1.513	0.216
REACT_ADAPTIVE IMMUNITY SIGNALING	1.512	0.214
REACT_TRANSLATION INITIATION COMPLEX FORMATION	1.512	0.213
WIP_HS_INTEGRATED_BREAST_CANCER_PATHWAY	1.507	0.218
REACT_RNA POLYMERASE II TRANSCRIPTION TERMINATION	1.507	0.216
REACT_TRNA AMINOACYLATION	1.504	0.219
REACT_FORMATION OF THE TERNARY COMPLEX, AND SUBSEQUENTLY, THE 43S COMPLEX	1.503	0.218
REACT_PKB-MEDIATED EVENTS	1.501	0.219
REACT_NUCLEOTIDE-BINDING DOMAIN, LEUCINE RICH REPEAT CONTAINING RECEPTOR (NLR) SIGNALING PATHWAYS	1.495	0.228
REACT_HOST INTERACTIONS OF HIV FACTORS	1.490	0.234
NCI_ERBB1_INTERNALIZATION_PATHWAY	1.489	0.233
NCI_HDAC_CLASSII_PATHWAY	1.487	0.235
REACT_DOWNSTREAM TCR SIGNALING	1.485	0.236
WIP_HS_INFLAMMATORY_RESPONSE_PATHWAY	1.484	0.234
REACT_POST-ELONGATION PROCESSING OF THE TRANSCRIPT	1.480	0.240

Supplementary table 2e. Gene sets downregulated in PBMCs in old men upon three weeks of 30% CR.

Gene set	NES	FDR q-value
REACT_OLFACTORY SIGNALING PATHWAY	-2.324	0.005
KEGG_OLFACTORY TRANSDUCTION	-2.209	0.006
WIP_HS_GPCRS,_CLASS_A_RHODOPSIN-LIKE	-2.004	0.048
NCI_S1P_S1P2_PATHWAY	-1.949	0.066
WIP_HS_CYTOKINES_AND_INFLAMMATORY_RESPONSE	-1.889	0.094
WIP_HS_COMPLEMENT_AND_COAGULATION_CASCADES	-1.833	0.136
WIP_HS_GPCRS,_OTHER	-1.833	0.117
WIP_HS_EICOSANOID_SYNTHESIS	-1.817	0.118
NCI_AP1_PATHWAY	-1.817	0.106
NCI_ATF2_PATHWAY	-1.807	0.104
KEGG_NEUROACTIVE LIGAND-RECEPTOR INTERACTION	-1.802	0.098
REACT_CLASS A_1 (RHODOPSIN-LIKE RECEPTORS)	-1.799	0.093
KEGG_DRUG METABOLISM - OTHER ENZYMES	-1.792	0.090
REACT_BIOLOGICAL OXIDATIONS	-1.767	0.103
WIP_HS_OXIDATIVE_STRESS	-1.714	0.148
KEGG_DRUG METABOLISM - CYTOCHROME P450	-1.695	0.162
REACT_GPCR LIGAND BINDING	-1.665	0.192
NCI_HNF3APATHWAY	-1.657	0.192
REACT_GPCR DOWNSTREAM SIGNALING	-1.657	0.182
REACT_G ALPHA (I) SIGNALLING EVENTS	-1.657	0.173
REACT_PYRIMIDINE METABOLISM	-1.656	0.166
KEGG_STAPHYLOCOCCUS AUREUS INFECTION	-1.644	0.174
REACT_SIGNALING BY GPCR	-1.639	0.174
KEGG_COMPLEMENT AND COAGULATION CASCADES	-1.630	0.178
KEGG_PERTUSSIS	-1.626	0.176
WIP_HS_HEART_DEVELOPMENT	-1.623	0.173
KEGG_METABOLISM OF XENOBIOTICS BY CYTOCHROME P450	-1.617	0.174
BIOC_INFLAMPATHWAY	-1.615	0.170
REACT_PEPTIDE LIGAND-BINDING RECEPTORS	-1.609	0.172
REACT_PHASE II CONJUGATION	-1.604	0.171
WIP_HS_METABOLISM_OF_AMINO_ACIDS_AND_DERIVATIVES	-1.603	0.168
WIP_HS_METAPATHWAY_BIOTRANSFORMATION	-1.587	0.181
KEGG_RHEUMATOID ARTHRITIS	-1.578	0.189
REACT_AMYLOIDS	-1.549	0.224
REACT_PHASE 1 - FUNCTIONALIZATION OF COMPOUNDS	-1.543	0.225
REACT_POTASSIUM CHANNELS	-1.538	0.227
NCI_MAPKTRKPATHWAY	-1.527	0.239

Supplementary table 2f. Gene sets differently regulated in PBMCs of old men compared to young men upon three weeks of 30% CR.

Gene set	NES	FDR q-value
REACT_CTLA4 INHIBITORY SIGNALING	2.179	0.015
REACT_INTERFERON GAMMA SIGNALING	2.174	0.008
NCI_INTEGRIN2_PATHWAY	2.156	0.008
NCI_IL12_2PATHWAY	2.128	0.008
WIP_HS_IL-7_SIGNALING_PATHWAY	2.117	0.008
KEGG_PROTEASOME	2.067	0.011
REACT_INTERFERON SIGNALING	2.066	0.010
NCI_IL12_STAT4PATHWAY	2.058	0.010
NCI_VEGFR1_2_PATHWAY	2.054	0.009
REACT_DEGRADATION OF BETA-CATENIN BY THE DESTRUCTION COMPLEX	2.040	0.011
REACT_SIGNALING BY WNT	2.028	0.012
WIP_HS_LEUKOCYTE_TARBASE	2.009	0.014

KEGG_NATURAL KILLER CELL MEDIATED CYTOTOXICITY	1.998	0.015
NCI_HIVNEFPATHWAY	1.997	0.014
WIP_HS_PROTEASOME_DEGRADATION	1.992	0.014
REACT_REGULATION OF ORNITHINE DECARBOXYLASE (ODC)	1.990	0.014
NCI_ECADHERIN_KERATINOCYTE_PATHWAY	1.982	0.014
BIOC_ECMPATHWAY	1.957	0.018
NCI_IL27PATHWAY	1.951	0.019
REACT_INTERFERON ALPHA_BETA SIGNALING	1.945	0.020
REACT_INTERLEUKIN RECEPTOR SHC SIGNALING	1.943	0.019
REACT_SCF(SKP2)-MEDIATED DEGRADATION OF P27_P21	1.937	0.020
REACT_COSTIMULATION BY THE CD28 FAMILY	1.934	0.020
REACT_CDK-MEDIATED PHOSPHORYLATION AND REMOVAL OF CDC6	1.928	0.021
REACT_CYTOKINE SIGNALING IN IMMUNE SYSTEM	1.917	0.023
NCI_CXCR3PATHWAY	1.915	0.023
BIOC_HIVNEFPATHWAY	1.915	0.022
REACT_ASSEMBLY OF THE PRE-REPLICATIVE COMPLEX	1.913	0.022
REACT_INTERLEUKIN-2 SIGNALING	1.913	0.021
REACT_VPU MEDIATED DEGRADATION OF CD4	1.912	0.020
REACT_SCF-BETA-TRCP MEDIATED DEGRADATION OF EMI1	1.912	0.020
REACT_CDT1 ASSOCIATION WITH THE CDC6_ORC_ORIGIN COMPLEX	1.911	0.020
REACT_ORC1 REMOVAL FROM CHROMATIN	1.910	0.019
REACT_GLUCOSE METABOLISM	1.910	0.019
REACT_STABILIZATION OF P53	1.905	0.019
REACT_SWITCHING OF ORIGINS TO A POST-REPLICATIVE STATE	1.898	0.020
REACT_REGULATION OF DNA REPLICATION	1.894	0.021
REACT_UBIQUITIN-DEPENDENT DEGRADATION OF CYCLIN D1	1.890	0.021
KEGG_AMINOACYL-TRNA BIOSYNTHESIS	1.889	0.021
REACT_GLYCOLYSIS	1.886	0.021
KEGG_MEASLES	1.881	0.022
REACT_UBIQUITIN-DEPENDENT DEGRADATION OF CYCLIN D	1.879	0.022
KEGG_PANCREATIC CANCER	1.876	0.022
REACT_ADAPTIVE IMMUNITY SIGNALING	1.870	0.023
REACT_APOPTOSIS	1.869	0.023
KEGG_HEPATITIS C	1.867	0.023
WIP_HS_TYPE_II_INTERFERON_SIGNALING_(IFNG)	1.866	0.023
REACT_AUTODEGRADATION OF THE E3 UBIQUITIN LIGASE COP1	1.864	0.023
REACT_REMOVAL OF LICENSING FACTORS FROM ORIGINS	1.853	0.025
BIOC_BIOPEPTIDESPATHWAY	1.852	0.025
NCI_FASPATHWAY	1.851	0.025
NCI_TCPTP_PATHWAY	1.845	0.026
KEGG_LYSOSOME	1.842	0.027
KEGG_LEUKOCYTE TRANSENDOTHELIAL MIGRATION	1.834	0.029
REACT_M_G1 TRANSITION	1.833	0.029
REACT_ANTIGEN PRESENTATION_ FOLDING, ASSEMBLY AND PEPTIDE LOADING OF CLASS I MHC	1.830	0.029
WIP_HS_EPO_RECEPTOR_SIGNALING	1.830	0.028
REACT_P53-INDEPENDENT G1_S DNA DAMAGE CHECKPOINT	1.827	0.029
NCI_ERBB1_RECEPTOR_PROXIMAL_PATHWAY	1.827	0.028
NCI_IL2_STAT5PATHWAY	1.826	0.028
WIP_HS_G13_SIGNALING_PATHWAY	1.824	0.028
NCI_ALPHASYNUCLEIN_PATHWAY	1.823	0.028
REACT_REGULATION OF ACTIVATED PAK-2P34 BY PROTEASOME MEDIATED DEGRADATION	1.820	0.028
NCI_IL2_1PATHWAY	1.818	0.028
NCI_GMCSF_PATHWAY	1.818	0.028
REACT_P53-INDEPENDENT DNA DAMAGE RESPONSE	1.818	0.028
REACT_P53-DEPENDENT G1_S DNA DAMAGE CHECKPOINT	1.814	0.028
KEGG_CHRONIC MYELOID LEUKEMIA	1.813	0.028
REACT_DNA REPLICATION PRE-INITIATION	1.810	0.028
KEGG_TOXOPLASMOSIS	1.810	0.028
KEGG_ANTIGEN PROCESSING AND PRESENTATION	1.808	0.028
REACT_REGULATION OF MITOTIC CELL CYCLE	1.804	0.029
WIP_HS_T_CELL_RECEPTOR_SIGNALING_PATHWAY	1.804	0.029

KEGG_FC EPSILON RI SIGNALING PATHWAY	1.802	0.029
REACT_GPVI-MEDIATED ACTIVATION CASCADE	1.802	0.029
REACT_CYCLIN A_CDK2-ASSOCIATED EVENTS AT S PHASE ENTRY	1.801	0.028
REACT_P53-DEPENDENT G1 DNA DAMAGE RESPONSE	1.798	0.029
REACT_UBIQUITIN MEDIATED DEGRADATION OF PHOSPHORYLATED CDC25A	1.798	0.028
REACT_APC_C-MEDIATED DEGRADATION OF CELL CYCLE PROTEINS	1.797	0.028
WIP_HS_IL-2_SIGNALING_PATHWAY	1.796	0.028
WIP_HS_TRNA_AMINOACYLATION	1.796	0.028
REACT_REGULATION OF APC_C ACTIVATORS BETWEEN G1_S AND EARLY ANAPHASE	1.792	0.029
REACT_G1_S DNA DAMAGE CHECKPOINTS	1.785	0.030
BIOC_CASPASEPATHWAY	1.771	0.034
REACT_DARPP-32 EVENTS	1.764	0.036
REACT_ACTIVATION OF APC_C AND APC_C_CDC20 MEDIATED DEGRADATION OF MITOTIC PROTEINS	1.761	0.037
KEGG_GLYCOLYSIS _ GLUCONEOGENESIS	1.750	0.040
REACT_CELL SURFACE INTERACTIONS AT THE VASCULAR WALL	1.750	0.040
REACT_APC_C_CDC20 MEDIATED DEGRADATION OF MITOTIC PROTEINS	1.747	0.040
KEGG_INFLUENZA A	1.744	0.041
REACT_APC_C_CDC20 MEDIATED DEGRADATION OF SECURIN	1.744	0.041
KEGG_VIRAL MYOCARDITIS	1.743	0.041
BIOC_CTLA4PATHWAY	1.742	0.040
REACT_SIGNALLING TO RAS	1.732	0.044
KEGG_PROTEIN PROCESSING IN ENDOPLASMIC RETICULUM	1.724	0.047
REACT_CDC20_PHOSPHO-APC_C MEDIATED DEGRADATION OF CYCLIN A	1.723	0.047
REACT_DESTABILIZATION OF MRNA BY AUF1 (HNRNP D0)	1.718	0.049
REACT_CYCLIN E ASSOCIATED EVENTS DURING G1_S TRANSITION	1.717	0.049
REACT_REGULATION OF APOPTOSIS	1.714	0.049
KEGG_TYPE I DIABETES MELLITUS	1.714	0.049
WIP_HS_FATTY_ACID,_TRIACYLGLYCEROL,_AND_KETONE_BODY_METABOLISM	1.712	0.049
KEGG_T CELL RECEPTOR SIGNALING PATHWAY	1.711	0.049
NCI_IL2_PI3KPATHWAY	1.711	0.049
REACT_VIF-MEDIATED DEGRADATION OF APOBEC3G	1.707	0.050
KEGG_PATHOGENIC ESCHERICHIA COLI INFECTION	1.704	0.051
REACT_GLUCONEOGENESIS	1.702	0.051
NCI_CDC42_PATHWAY	1.701	0.051
BIOC_CHEMICALPATHWAY	1.698	0.052
KEGG_ALLOGRAFT REJECTION	1.697	0.052
KEGG_INOSITOL PHOSPHATE METABOLISM	1.694	0.053
WIP_HS_IL-6_SIGNALING_PATHWAY	1.693	0.052
WIP_HS_IL-4_SIGNALING_PATHWAY	1.692	0.053
KEGG_VEGF SIGNALING PATHWAY	1.691	0.052
REACT_IMMUNOREGULATORY INTERACTIONS BETWEEN A LYMPHOID AND A NON-LYMPHOID CELL	1.685	0.055
WIP_HS_LYMPHOCYTE_TARBASE	1.682	0.056
KEGG_MUCIN TYPE O-GLYCAN BIOSYNTHESIS	1.671	0.061
KEGG_GRAFT-VERSUS-HOST DISEASE	1.668	0.062
NCI_CD8TCRDOWNSTREAMPATHWAY	1.667	0.062
NCI_ERBB2ERBB3PATHWAY	1.665	0.062
WIP_HS_EPITHELIUM_TARBASE	1.665	0.062
REACT_CD28 CO-STIMULATION	1.663	0.062
REACT_DOWNSTREAM TCR SIGNALING	1.663	0.062
NCI_TCR_PATHWAY	1.663	0.062
BIOC_KERATINOCYTEPATHWAY	1.662	0.061
KEGG_CYSTEINE AND METHIONINE METABOLISM	1.652	0.066
BIOC_FMLPPATHWAY	1.647	0.068
WIP_HS_CELL_SURFACE_INTERACTIONS_AT_THE_VASCULAR_WALL	1.646	0.068
REACT_MITOTIC M-M_G1 PHASES	1.643	0.069
NCI_SMAD2_3PATHWAY	1.641	0.070
REACT_APC_C_CDH1 MEDIATED DEGRADATION OF CDC20 AND OTHER APC_C_CDH1 TARGETED PROTEINS IN LATE MITOSIS_EARLY G1	1.640	0.070
REACT_PROTEIN FOLDING	1.640	0.069
WIP_HS_MICRORNAS_IN_CARDIOMYOCYTE_HYPERTROPHY	1.637	0.070

REACT_AUTODEGRADATION OF CDH1 BY CDH1_APC_C	1.636	0.070
REACT_SYNTHESIS OF DNA	1.631	0.073
KEGG_GALACTOSE METABOLISM	1.629	0.073
BIOC_SPPAPATHWAY	1.622	0.077
REACT_HOST INTERACTIONS OF HIV FACTORS	1.621	0.078
REACT_FORMATION OF PLATELET PLUG	1.620	0.078
NCI_PDGFRBPATHWAY	1.619	0.078
REACT_INTERLEUKIN-3, 5 AND GM-CSF SIGNALING	1.618	0.078
REACT_PLATELET ACTIVATION	1.618	0.077
WIP_HS_IL-3_SIGNALING_PATHWAY	1.618	0.077
REACT_CELL CYCLE CHECKPOINTS	1.617	0.077
KEGG_CIRCADIAN RHYTHM - MAMMAL	1.617	0.077
NCI_FCER1PATHWAY	1.616	0.076
REACT_MITOTIC G2-G2_M PHASES	1.616	0.076
BIOC_CXCR4PATHWAY	1.616	0.075
REACT_RECRUITMENT OF MITOTIC CENTROSOME PROTEINS AND COMPLEXES	1.614	0.076
REACT_CD28 DEPENDENT PI3K_AKT SIGNALING	1.614	0.076
REACT_S PHASE	1.613	0.075
REACT_G1_S TRANSITION	1.613	0.075
NCI_TXA2PATHWAY	1.612	0.075
NCI_EPHA2_FWDPATHWAY	1.611	0.075
BIOC_ERK5PATHWAY	1.611	0.075
WIP_HS_MUSCLE_CELL_TARBASE	1.610	0.075
REACT_MITOCHONDRIAL TRNA AMINOACYLATION	1.609	0.075
NCI_ANTHRAXPATHWAY	1.608	0.075
NCI_HNF3BPATHWAY	1.606	0.076
WIP_HS_INTERFERON_ALPHA-BETA_SIGNALING	1.599	0.080
BIOC_IL2RBPATHWAY	1.597	0.081
REACT_METABOLISM OF CARBOHYDRATES	1.596	0.081
WIP_HS_KIT_RECEPTOR_SIGNALING_PATHWAY	1.593	0.082
REACT_CENTROSOME MATURATION	1.593	0.082
REACT_G2_M TRANSITION	1.591	0.083
KEGG_HERPES SIMPLEX INFECTION	1.590	0.083
WIP_HS_SIGNALING_BY_INSULIN_RECEPTOR	1.589	0.083
KEGG_RIBOSOME BIOGENESIS IN EUKARYOTES	1.587	0.084
NCI_P38_MKK3_6PATHWAY	1.587	0.084
NCI_CASPASE_PATHWAY	1.583	0.086
REACT_ASSOCIATION OF TRIC_CCT WITH TARGET PROTEINS DURING BIOSYNTHESIS	1.582	0.086
NCI_AR_NONGENOMIC_PATHWAY	1.582	0.085
REACT_PLATELET ACTIVATION TRIGGERS	1.581	0.085
NCI_NETRIN_PATHWAY	1.581	0.085
KEGG_DORSO-VENTRAL AXIS FORMATION	1.580	0.085
NCI_CD8TCRPATHWAY	1.579	0.085
KEGG_FC GAMMA R-MEDIATED PHAGOCYTOSIS	1.576	0.087
BIOC_PYK2PATHWAY	1.575	0.087
REACT_CELL CYCLE, MITOTIC	1.571	0.090
KEGG_TUBERCULOSIS	1.570	0.090
REACT_HEMOSTASIS	1.569	0.090
KEGG_AUTOIMMUNE THYROID DISEASE	1.564	0.092
WIP_HS_METABOLISM_OF_CARBOHYDRATES	1.557	0.097
KEGG_ENDOMETRIAL CANCER	1.555	0.098
REACT_PD-1 SIGNALING	1.554	0.098
REACT_HIV INFECTION	1.554	0.098
KEGG_CELL ADHESION MOLECULES (CAMS)	1.552	0.098
KEGG_CHAGAS DISEASE (AMERICAN TRYPANOSOMIASIS)	1.550	0.099
BIOC_IL12PATHWAY	1.550	0.099
WIP_HS_ALPHA_6_BETA_4_SIGNALING_PATHWAY	1.548	0.100
KEGG_VIBRIO CHOLERAE INFECTION	1.548	0.099
NCI_THROMBIN_PAR1_PATHWAY	1.547	0.100
REACT_DNA REPLICATION	1.543	0.102
WIP_HS_INTRINSIC_PATHWAY_FOR_APOPTOSIS	1.543	0.102
KEGG_PENTOSE PHOSPHATE PATHWAY	1.541	0.103

REACT_CLASS I MHC MEDIATED ANTIGEN PROCESSING & PRESENTATION	1.539	0.103
WIP_HS_ANGIOGENESIS_OVERVIEW	1.539	0.103
REACT_MITOTIC G1-G1_S PHASES	1.537	0.104
NCI_IL23PATHWAY	1.534	0.106
WIP_HS_GLYCOLYSIS_AND_GLUCONEOGENESIS	1.532	0.107
REACT_ASPARAGINE N-LINKED GLYCOSYLATION	1.531	0.107
WIP_HS_NOTCH_SIGNALING_PATHWAY	1.530	0.107
REACT_CHAPERONIN-MEDIATED PROTEIN FOLDING	1.528	0.109
BIOC_PTENPATHWAY	1.520	0.114
WIP_HS_DNA_DAMAGE_RESPONSE_(ONLY_ATM_DEPENDENT)	1.518	0.116
KEGG_SNARE INTERACTIONS IN VESICULAR TRANSPORT	1.517	0.116
WIP_HS_SREBP_SIGNALLING	1.517	0.115
WIP_HS_FAS_PATHWAY_AND_STRESS_INDUCTION_OF_HSP_REGULATION	1.516	0.115
NCI_IL6_7PATHWAY	1.516	0.115
WIP_HS_ENDOCHONDRAL_OSSIFICATION	1.515	0.115
REACT_NUCLEOTIDE-BINDING DOMAIN, LEUCINE RICH REPEAT CONTAINING RECEPTOR (NLR) SIGNALING PATHWAYS	1.513	0.116
NCI_RAC1_REG_PATHWAY	1.512	0.117
REACT_TCR SIGNALING	1.511	0.117
WIP_HS_RANKL-RANK_SIGNALING_PATHWAY	1.508	0.119
WIP_HS_ADIPOCYTE_TARBASE	1.508	0.118
BIOC_ERKPATHWAY	1.505	0.120
WIP_HS_EBV_LMP1_SIGNALING	1.505	0.119
REACT_INTRINSIC PATHWAY FOR APOPTOSIS	1.503	0.120
KEGG_FRUCTOSE AND MANNOSE METABOLISM	1.502	0.120
REACT_RHO GTPASE CYCLE	1.502	0.120
KEGG_BACTERIAL INVASION OF EPITHELIAL CELLS	1.500	0.122
WIP_HS_SQUAMOUS_CELL_TARBASE	1.500	0.121
BIOC_DEATHPATHWAY	1.497	0.123
REACT_INTEGRIN CELL SURFACE INTERACTIONS	1.496	0.123
REACT_SIGNALING BY RHO GTPASES	1.490	0.128
WIP_HS_TNF-ALPHA-NF-KB_SIGNALING_PATHWAY	1.489	0.128
NCI_ERBB1_INTERNALIZATION_PATHWAY	1.486	0.131
NCI_IFNGPATHWAY	1.485	0.130
REACT_PLATELET DEGRANULATION	1.482	0.133
KEGG_NON-SMALL CELL LUNG CANCER	1.481	0.133
NCI_CXCR4_PATHWAY	1.480	0.133
BIOC_TPOPATHWAY	1.480	0.133
BIOC_IGF1RPATHWAY	1.479	0.133
BIOC_AT1RPATHWAY	1.477	0.135
REACT_APOPTOTIC CLEAVAGE OF CELLULAR PROTEINS	1.475	0.135
REACT_PLATELET AGGREGATION (PLUG FORMATION)	1.472	0.138
NCI_LYMPHANGIOGENESIS_PATHWAY	1.471	0.138
REACT_MICRORNA (MIRNA) BIOGENESIS	1.471	0.138
KEGG_AMYOTROPHIC LATERAL SCLEROSIS (ALS)	1.471	0.137
WIP_HS_AMPK_SIGNALING	1.467	0.141
BIOC_CREBPATHWAY	1.466	0.141
BIOC_GLEEVECPATHWAY	1.465	0.141
NCI_FGF_PATHWAY	1.463	0.143
WIP_HS_ASPARAGINE_N-LINKED_GLYCOSYLATION	1.461	0.144
NCI_IL8CXCR2_PATHWAY	1.461	0.143
REACT_DOWN-STREAM SIGNAL TRANSDUCTION	1.460	0.144
NCI_ERBB4_PATHWAY	1.457	0.146
KEGG_TYPE II DIABETES MELLITUS	1.456	0.146
KEGG_ENDOCYTOSIS	1.451	0.150
KEGG_CITRATE CYCLE (TCA CYCLE)	1.451	0.150
REACT_TRIGLYCERIDE BIOSYNTHESIS	1.447	0.153
KEGG_PROGESTERONE-MEDIATED OOCYTE MATURATION	1.447	0.153
REACT_RECYCLING PATHWAY OF L1	1.446	0.154
KEGG_GNRH SIGNALING PATHWAY	1.445	0.153
REACT_TRNA AMINOACYLATION	1.445	0.153
BIOC_EDG1PATHWAY	1.444	0.154

WIP_HS_TOLL-LIKE_RECEPTOR_SIGNALING_PATHWAY	1.443	0.154
REACT_INTERACTIONS OFTHE IMMUNOGLOBULIN SUPERFAMILY (IGSF) MEMBER PROTEINS	1.443	0.154
NCI_ATR_PATHWAY	1.441	0.155
BIOC_NKCELLSPATHWAY	1.439	0.157
KEGG_DILATED CARDIOMYOPATHY	1.436	0.160
BIOC_GHPATHWAY	1.435	0.160
WIP_HS_IL-5_SIGNALING_PATHWAY	1.432	0.162
NCI_ER_NONGENOMIC_PATHWAY	1.432	0.161
REACT_REGULATORY RNA PATHWAYS	1.430	0.163
REACT_OPIOID SIGNALLING	1.426	0.166
BIOC_P38MAPKPATHWAY	1.425	0.167
REACT_SIGNALING BY INTERLEUKINS	1.420	0.171
NCI_CERAMIDE_PATHWAY	1.419	0.171
NCI_UPA_UPAR_PATHWAY	1.418	0.172
NCI_IGF1_PATHWAY	1.417	0.173
REACT_INTEGRIN ALPHAIIB BETA3 SIGNALING	1.416	0.173
KEGG_CARBOHYDRATE DIGESTION AND ABSORPTION	1.416	0.173
KEGG_CHEMOKINE SIGNALING PATHWAY	1.416	0.173
NCI_PI3KPLCTRKPATHWAY	1.415	0.172
BIOC_TIDPATHWAY	1.415	0.173
KEGG_OTHER GLYCAN DEGRADATION	1.415	0.172
NCI_PTP1BPATHWAY	1.414	0.171
BIOC_PTDINSPATHWAY	1.411	0.175
WIP_HS_REGULATION_OF_TOLL-LIKE_RECEPTOR_SIGNALING_PATHWAY	1.406	0.179
REACT_APOPTOTIC EXECUTION PHASE	1.406	0.179
WIP_HS_INFLAMMATORY_RESPONSE_PATHWAY	1.404	0.180
REACT_REGULATION OF MRNA STABILITY BY PROTEINSTHAT BIND AU-RICH ELEMENTS	1.404	0.179
KEGG_AFRICANTRYPANOSOMIASIS	1.403	0.181
KEGG_FATTY ACID ELONGATION	1.402	0.181
KEGG_GAP JUNCTION	1.400	0.182
KEGG_APOPTOSIS	1.400	0.182
NCI_KITPATHWAY	1.399	0.182
KEGG_ADIPOCYTOKINE SIGNALING PATHWAY	1.398	0.183
REACT_SIGNALING BY EGFR	1.393	0.187
REACT_RESPONSETO ELEVATED PLATELET CYTOSOLIC CA2+	1.393	0.187
REACT_CELL-EXTRACELLULAR MATRIX INTERACTIONS	1.392	0.187
REACT_TRANSPORTTOTHE GOLGI AND SUBSEQUENT MODIFICATION	1.390	0.189
KEGG_SHIGELLOSIS	1.390	0.189
KEGG_TOLL-LIKE RECEPTOR SIGNALING PATHWAY	1.389	0.189
NCI_NEPHRIN_NEPH1_PATHWAY	1.387	0.191
BIOC_TCRPATHWAY	1.384	0.194
KEGG_OSTEOCLAST DIFFERENTIATION	1.384	0.193
NCI_NCADHERINPATHWAY	1.383	0.194
REACT_METABOLISM OF MRNA	1.381	0.196
BIOC_MITOCHONDRIAPATHWAY	1.379	0.198
REACT_LOSS OF PROTEINS REQUIRED FOR INTERPHASE MICROTUBULE ORGANIZATIONÂ FROMTHE CENTROSOME	1.378	0.199
BIOC_PROTEASOMEPATHWAY	1.375	0.202
REACT_POST-TRANSLATIONAL PROTEIN MODIFICATION	1.374	0.202
WIP_HS_TOR_SIGNALING	1.372	0.203
NCI_ENDOTHELINPATHWAY	1.372	0.203
KEGG_REGULATION OF ACTIN CYTOSKELETON	1.371	0.204
REACT_TRANSLOCATION OF ZAP-70TO IMMUNOLOGICAL SYNAPSE	1.371	0.203
WIP_HS_APOPTOSIS_MODULATION_BY_HSP70	1.371	0.203
NCI_ANGIOPOIETINRECEPTOR_PATHWAY	1.371	0.202
BIOC_RASPATHWAY	1.369	0.203
KEGG_PHOSPHATIDYLINOSITOL SIGNALING SYSTEM	1.365	0.208
KEGG_AMINO SUGAR AND NUCLEOTIDE SUGAR METABOLISM	1.364	0.208
REACT_LOSS OF NLP FROM MITOTIC CENTROSOMES	1.364	0.207
KEGG_PHAGOSOME	1.363	0.209
NCI_AMB2_NEUTROPHILS_PATHWAY	1.362	0.209
REACT_N-GLYCAN ANTENNAE ELONGATION INTHE MEDIAL_TRANS-GOLGI	1.360	0.210

REACT_THE ROLE OF NEF IN HIV-1 REPLICATION AND DISEASE PATHOGENESIS	1.358	0.212
REACT_METABOLISM OF PROTEINS	1.358	0.212
KEGG_ONE CARBON POOL BY FOLATE	1.353	0.218
REACT_POST NMDA RECEPTOR ACTIVATION EVENTS	1.350	0.220
WIP_HS_B_CELL_RECEPTOR_SIGNALING_PATHWAY	1.349	0.221
REACT_ANTIGEN PROCESSING_ UBIQUITINATION & PROTEASOME DEGRADATION	1.349	0.221
WIP_HS_MAPK_CASCADE	1.349	0.221
KEGG_NOTCH SIGNALING PATHWAY	1.345	0.224
BIOC_RACCYCDPATHWAY	1.344	0.225
KEGG_OOCYTE MEIOSIS	1.342	0.228
BIOC_PDGFPATHWAY	1.340	0.229
NCI_PRLSIGNALINGEVENTSPATHWAY	1.337	0.233
BIOC_VEGFPATHWAY	1.335	0.235
NCI_NFKAPPABATYPICALPATHWAY	1.333	0.237
NCI_MET_PATHWAY	1.330	0.241
KEGG_ALZHEIMER'S DISEASE	1.329	0.241
BIOC_MTORPATHWAY	1.328	0.241
KEGG_PRIMARY IMMUNODEFICIENCY	1.328	0.241
KEGG_SALIVARY SECRETION	1.328	0.241
REACT_EFFECTS OF PIP2 HYDROLYSIS	1.327	0.241
BIOC_EGFPATHWAY	1.326	0.242
NCI_INTEGRIN3_PATHWAY	1.320	0.249
BIOC_EIF4PATHWAY	1.319	0.250
BIOC_GSK3PATHWAY	1.319	0.249
REACT_ACTIVATION OF KAINATE RECEPTORS UPON GLUTAMATE BINDING	1.318	0.250
REACT_OLFACTORY SIGNALING PATHWAY	-1.895	0.244

2

Chapter 3 |

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

3

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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 & Conclusions: 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. 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 and is therefore 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, 19, 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). Subject 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 ⩾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 (MessageAmp™ 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 analysed using SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). 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).

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 ± 0.6	5.9 ± 0.5
Waist circumference (cm)	110 ± 9	110 ± 6

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

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.

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.

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	↑

[1]Significantly changed pathways are determined with GSEA (supplementary table **2**) and clusters are based upon Cytoscape.
↑: gene sets in this pathway-cluster were upregulated;
↓: gene sets in this pathway-cluster were downregulated.

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).

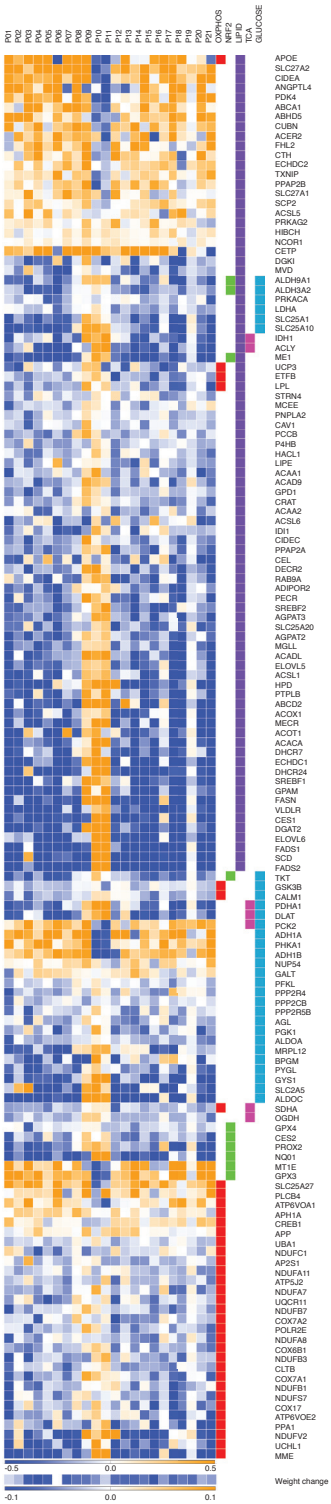

Figure 1. Heatmap of individual gene expression changes upon 25% ER of genes involved in energy metabolism. Each column represents the signal log ratio of one person; each row represents one gene. Scale: blue = downregulated, orange = upregulated.

Colours in heatmap:
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.

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).

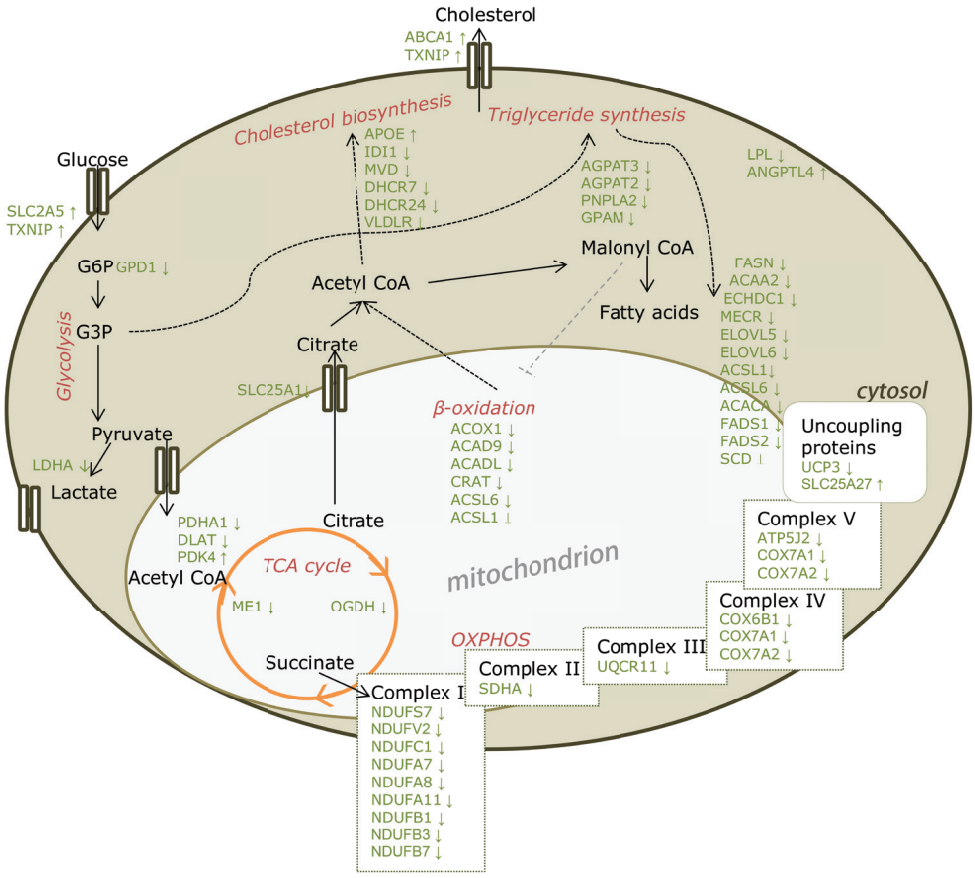

Figure 2. Schematic visualisation of adipocyte with up- or downregulated expression of genes involved in energy metabolism that changed upon 25% ER.

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 3). 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.

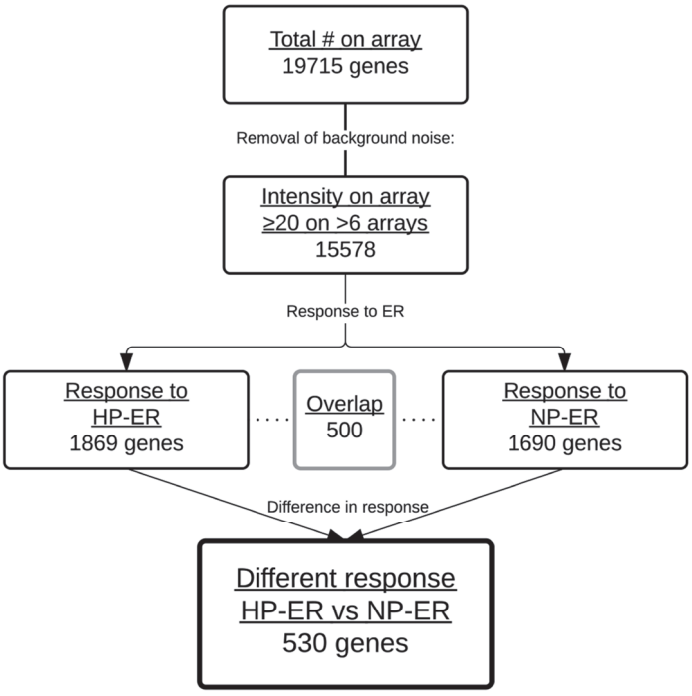

Figure 3. Stepwise selection of genes for microarray analysis: first, genes were selected for their signal intensity (≥20 in >6 arrays), and second, for a change in expression upon either normal protein energy restriction (NP-ER) diet, or upon high protein energy restriction (HP-ER) diet (P<0.05). The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

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 241 gene sets were enriched in the HP-ER group, of which 69 up- and 172 downregulated. A total of 371 gene sets were enriched in the NP-ER group, of which 46 up- and 325 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.

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	↑	-

[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), as determined with GSEA (supplementary table 4,5) and clusters are based upon Cytoscape.
↑: gene sets in this pathway-cluster were upregulated;
↓: gene sets in this pathway-cluster were downregulated;
-: no change in this pathway-cluster.

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 pink 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 washout period of 8 weeks between both ER diets. This period might have been too short for adipocytes to recover from ER and may have caused a carryover-effect on gene expression.

The observed reduction in expression of genes involved in immune response pathways and inflammasome-related pathways upon a normal protein diet which was not observed on a high-protein diet, is interesting with respect to their role in inflammation in adipose tissue. Adipose tissue in obese individuals is characterized by increased expression of genes involved in the inflammasome and immune response [27, 28]. Several studies have observed that caloric restriction or exercise-mediated weight-loss resulted in a reduced expression of these genes [29]. Interestingly, we only observed those effects for the normal protein diet. When the protein quantity of the ER diet increased, the beneficial effects on inflammation-related

gene expression were not observed. This observation points towards the potential importance of dietary macronutrient composition during ER on adipose tissue health. Despite the findings of macronutrient-specific effects, the impact of ER on gene expression was much greater. Moreover, strong correlations between changes in gene expression were observed, suggesting a potential upstream regulator responsible for this accurate regulation in expression. Especially a strong correlation between changes in expression of lipid-related genes upon ER was found. In line with this, upstream regulators PPARG and SREBPs, strong regulators of lipid metabolism, were predicted to be inhibited. Furthermore, oxidative phosphorylation related-pathways were downregulated upon ER, which is in line with the finding that PGC-1α was predicted to be inhibited. In addition to the pathway analysis, which identified only downregulated pathways related to energy metabolism upon ER, expression 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 determine 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.

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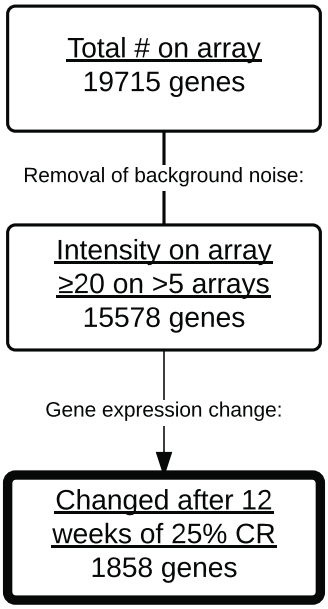
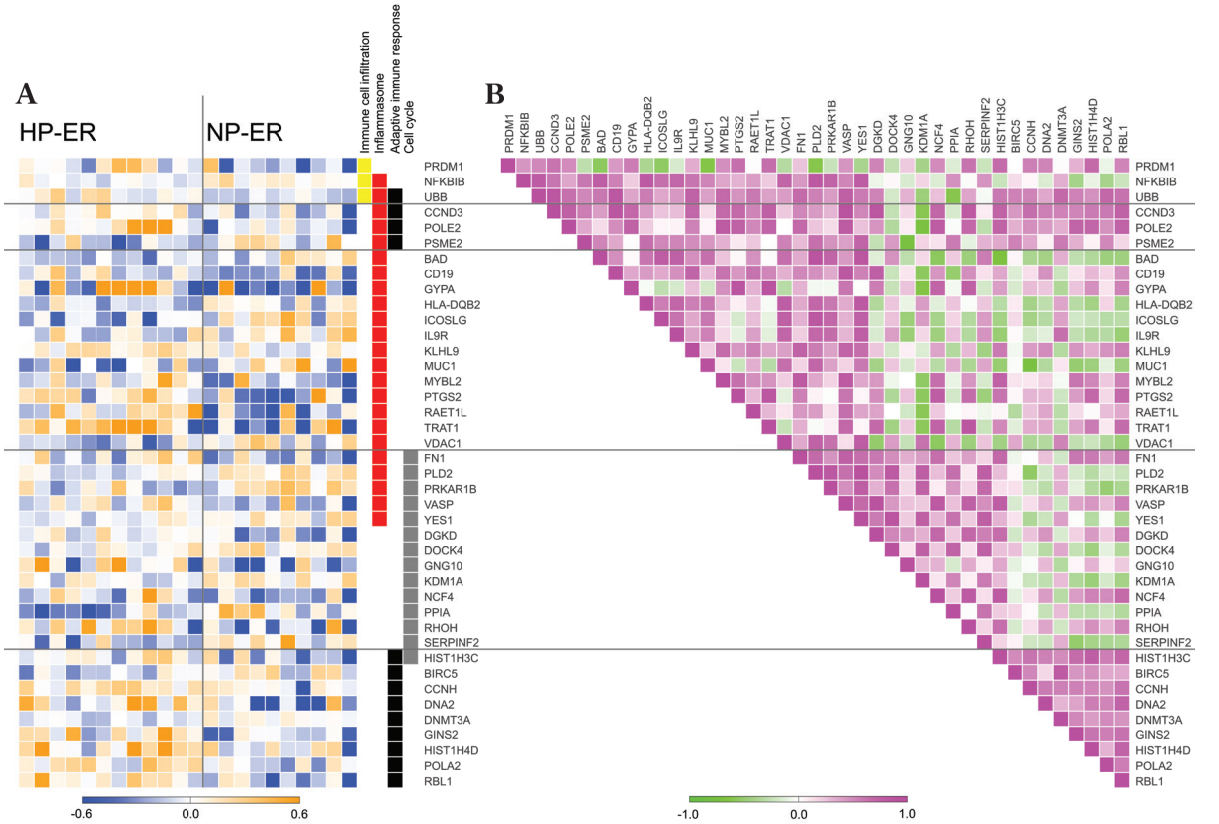
Supplementary information



Supplementary figure 1. Stepwise selection of genes of which the expression was significantly changes upon 25% ER in microarray analysis ($P<0.05$).

Supplementary figure 2. Correlation heatmap of changes in expression of genes upon ER. Genes are selected from gene sets involved in energy metabolism. Scale: green = correlation score of -1, pink = correlation score of 1.

Colours in heatmap:
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.



Colours in heatmap:
Yellow: Immune cell infiltration;
Red: Inflammasome;
Black: Adaptive immune response;
Grey: Cell cycle.

Supplementary figure 3. a. Heatmap of genes which expression changed differently between the HP-ER and NP-ER group. Each column represents the signal log ratio of one person; each row represents one gene. Scale: blue = downregulated, orange = upregulated. **b.** Correlation heatmap of genes which expression changed differently between the HP-ER and NP-ER group. Scale: green = correlation score of -1, pink = correlation score of 1.

Supplementary table 1. Changes in anthropometric and plasma glucose measurements after 12 weeks 25% ER.

	HP-ER (n=12)		NP-ER (n=10)		P-value for changes
	Baseline	Change	Baseline	Change	
Weight (kg)	93.2 ± 10.2	-9.71 ± 3.73	91.9 ± 6.1	-9.08 ± 2.53	0.67
Body mass index (kg/m^2)	31 ± 3	-3.24 ± 0.70	30 ± 2	-3.00 ± 1.27	0.62
Glucose (mmol/L)	5.7 [5.3, 6.8]	-0.31 ± 0.47	5.8 [5.1, 6.6]	-0.13 ± 0.45	0.40
Waist circumference (cm)	110 ± 9	-10.9 ± 4.8	110 ± 6	-10.7 ± 6.1	0.92

HP-ER: High Protein Energy Restriction diet; NP-ER: Normal Protein Energy Restriction diet; Data represent mean with ± SD, P-value depicts statistical significance of 'Change'.

Supplementary table 2. Gene Set Enrichment Analysis. Cut-off value FDR q-value 0.25.
Supplementary table 2a. Gene sets upregulated in white adipose tissue upon twelve weeks of 25%.

Gene set	NES	FDR q-value
REACT_INFLUENZA VIRAL RNA TRANSCRIPTION AND REPLICATION	2.821	0.000
KEGG_RIBOSOME	2.789	0.000
REACT_FORMATION OF A POOL OF FREE 40S SUBUNITS	2.784	0.000
REACT_PEPTIDE CHAIN ELONGATION	2.766	0.000
REACT_VIRAL MRNA TRANSLATION	2.752	0.000
REACT_EUKARYOTIC TRANSLATION ELONGATION	2.713	0.000
REACT_EUKARYOTIC TRANSLATION TERMINATION	2.699	0.000
REACT_L13A-MEDIATED TRANSLATIONAL SILENCING OF CERULOPLASMIN EXPRESSION	2.687	0.000
WIP_HS_CYTOPLASMIC_RIBOSOMAL_PROTEINS	2.658	0.000
REACT_NONSENSE MEDIATED DECAY INDEPENDENT OF THE EXON JUNCTION COMPLEX	2.649	0.000
REACT_NONSENSE MEDIATED DECAY ENHANCED BY THE EXON JUNCTION COMPLEX	2.648	0.000
REACT_NONSENSE-MEDIATED DECAY	2.644	0.000
REACT_3 -UTR-MEDIATED TRANSLATIONAL REGULATION	2.624	0.000
REACT_REGULATION OF GENE EXPRESSION IN BETA CELLS	2.579	0.000
REACT_REGULATION OF BETA-CELL DEVELOPMENT	2.575	0.000
REACT_GTP HYDROLYSIS AND JOINING OF THE 60S RIBOSOMAL SUBUNIT	2.574	0.000
REACT_CAP-DEPENDENT TRANSLATION INITIATION	2.545	0.000
REACT_EUKARYOTIC TRANSLATION INITIATION	2.527	0.000
WIP_HS_PROCESSING_OF_CAPPED_INTRON-CONTAINING_PRE-MRNA	2.504	0.000
REACT_TRANSLATION	2.489	0.000
REACT_INSULIN SYNTHESIS AND PROCESSING	2.323	0.000
REACT_FORMATION OF THE TERNARY COMPLEX, AND SUBSEQUENTLY, THE 43S COMPLEX	2.336	0.000
REACT_TRANSLATION INITIATION COMPLEX FORMATION	2.263	0.000
REACT_ACTIVATION OF THE MRNA UPON BINDING OF THE CAP-BINDING COMPLEX AND EIFS, AND SUBSEQUENT BINDING TO 43S	2.219	0.000
WIP_HS_MRNA_PROCESSING	2.230	0.000
REACT_RIBOSOMAL SCANNING AND START CODON RECOGNITION	2.176	0.000
REACT_MRNA SPLICING - MAJOR PATHWAY	2.117	0.001
REACT_GENERIC TRANSCRIPTION PATHWAY	2.107	0.001
REACT_MRNA SPLICING	2.098	0.001
REACT_PROCESSING OF CAPPED INTRON-CONTAINING PRE-MRNA	2.084	0.001
REACT_TRANSPORT OF MATURE MRNA DERIVED FROM AN INTRON-CONTAINING TRANSCRIPT	2.089	0.001
REACT_OLFACTORY SIGNALING PATHWAY	2.078	0.001
REACT_MRNA PROCESSING	2.037	0.001
REACT_POST-ELONGATION PROCESSING OF INTRON-CONTAINING PRE-MRNA	1.976	0.003
REACT_MRNA 3-END PROCESSING	1.971	0.003

REACT_METABOLISM OF MRNA	1.906	0.008
KEGG_SPLICEOSOME	1.876	0.011
REACT_POST-ELONGATION PROCESSING OF THE TRANSCRIPT	1.869	0.011
REACT_CLEAVAGE OF GROWING TRANSCRIPT IN THE TERMINATION REGION	1.859	0.012
REACT_METABOLISM OF RNA	1.833	0.016
REACT_FORMATION AND MATURATION OF MRNA TRANSCRIPT	1.834	0.016
REACT_RNA POLYMERASE II TRANSCRIPTION TERMINATION	1.803	0.020
REACT_TRANSPORT OF MATURE TRANSCRIPT TO CYTOPLASM	1.804	0.020
WIP_HS_MITOCHONDRIAL_GENE_EXPRESSION	1.792	0.022
BIOC_LAIRPATHWAY	1.770	0.026
KEGG_MINERAL ABSORPTION	1.750	0.031
BIOC_TIDPATHWAY	1.718	0.041
REACT_METABOLISM OF PROTEINS	1.694	0.050
REACT_DIABETES PATHWAYS	1.646	0.073
NCI_AP1_PATHWAY	1.626	0.082
WIP_HS_SEROTONIN_RECEPTOR_4-6-7_AND_NR3C_SIGNALING	1.627	0.083
REACT_COMPLEMENT CASCADE	1.613	0.090
REACT_ACTIVATION OF THE PRE-REPLICATIVE COMPLEX	1.586	0.110
KEGG_OLFACTORY TRANSDUCTION	1.568	0.125
NCI_HIF2PATHWAY	1.561	0.129
WIP_HS_INTEGRATED_BREAST_CANCER_PATHWAY	1.546	0.142
WIP_HS_DNA_REPLICATION	1.535	0.148
KEGG_RIBOSOME BIOGENESIS IN EUKARYOTES	1.537	0.148
BIOC_MTORPATHWAY	1.526	0.155
REACT_NUCLEAR RECEPTOR TRANSCRIPTION PATHWAY	1.513	0.165
NCI_HDAC_CLASSI_PATHWAY	1.515	0.166
NCI_BARD1PATHWAY	1.509	0.167
WIP_HS_STATIN_PATHWAY	1.504	0.171
NCI_RXR_VDR_PATHWAY	1.483	0.194
REACT_INTERACTIONS OF REV WITH HOST CELLULAR PROTEINS	1.467	0.215
KEGG_DRUG METABOLISM - CYTOCHROME P450	1.464	0.216
KEGG_GLYCOSAMINOGLYCAN BIOSYNTHESIS - CHONDROITIN SULFATE	1.448	0.234
REACT_FORMATION OF FIBRIN CLOT (CLOTTING CASCADE)	1.445	0.235
WIP_HS_COMPLEMENT_AND_COAGULATION_CASCADES	1.448	0.237
REACT_REV-MEDIATED NUCLEAR EXPORT OF HIV-1 RNA	1.442	0.237
NCI_AR_PATHWAY	1.434	0.246
REACT_NUCLEAR IMPORT OF REV PROTEIN	1.427	0.249

Supplementary table 2b. Gene sets downregulated in white adipose tissue upon twelve weeks of 25%.

Gene set	NES	FDR q-value
PPARA_TARGETS	-2.339	0.000
REACT_TRIGLYCERIDE BIOSYNTHESIS	-2.323	0.000
KEGG_BIOSYNTHESIS OF UNSATURATED FATTY ACIDS	-2.287	0.000
KEGG_OXIDATIVE PHOSPHORYLATION	-2.525	0.000
REACT_RESPIRATORY ELECTRON TRANSPORT, ATP SYNTHESIS BY CHEMIOSMOTIC COUPLING, AND HEAT PRODUCTION BY UNCOUPLING PROTEINS.	-2.509	0.000
WIP_HS_OXIDATIVE_PHOSPHORYLATION	-2.470	0.000
KEGG_PARKINSON'S DISEASE	-2.429	0.000
WIP_HS_ELECTRON_TRANSPORT_CHAIN	-2.373	0.000
REACT_RESPIRATORY ELECTRON TRANSPORT	-2.326	0.000
REACT_FATTY ACYL-COA BIOSYNTHESIS	-2.240	0.000
REACT_GLUCOSE METABOLISM	-2.180	0.000
WIP_HS_GLYCOLYSIS_AND_GLUCONEOGENESIS	-2.187	0.000
KEGG_ALZHEIMER'S DISEASE	-2.189	0.001
REACT_VIF-MEDIATED DEGRADATION OF APOBEC3G	-2.160	0.001
KEGG_PROTEASOME	-2.153	0.001
KEGG_GLYCEROLIPID METABOLISM	-2.124	0.001
WIP_HS_FATTY_ACID_BIOSYNTHESIS	-2.134	0.002

KEGG_PYRUVATE METABOLISM	-2.096	0.002
KEGG_PENTOSE PHOSPHATE PATHWAY	-2.071	0.003
REACT_PLATELET SENSITIZATION BY LDL	-2.078	0.003
WIP_HS_FATTY_ACID,_TRIACYLGLYCEROL,_AND_KETONE_BODY_METABOLISM	-2.081	0.003
NCI_TCR_PATHWAY	-2.055	0.004
NCI_CD8TCRPATHWAY	-2.048	0.004
REACT_P53-INDEPENDENT G1_S DNA DAMAGE CHECKPOINT	-2.045	0.004
REACT_DEGRADATION OF BETA-CATENIN BY THE DESTRUCTION COMPLEX	-2.043	0.004
REACT_SIGNALING BY WNT	-2.036	0.004
WIP_HS_CHOLESTEROL_BIOSYNTHESIS	-2.028	0.005
REACT_UBIQUITIN MEDIATED DEGRADATION OF PHOSPHORYLATED CDC25A	-2.021	0.005
REACT_P53-INDEPENDENT DNA DAMAGE RESPONSE	-2.019	0.005
REACT_STABILIZATION OF P53	-2.010	0.005
REACT_REGULATION OF ACTIVATED PAK-2P34 BY PROTEASOME MEDIATED DEGRADATION	-2.013	0.005
NCI_INSULIN_PATHWAY	-2.011	0.005
REACT_CHOLESTEROL BIOSYNTHESIS	-1.996	0.006
REACT_POST-CHAPERONIN TUBULIN FOLDING PATHWAY	-1.983	0.006
REACT_REGULATION OF ORNITHINE DECARBOXYLASE (ODC)	-1.981	0.006
REACT_GLYCOLYSIS	-1.983	0.006
BIOC_INTEGRINPATHWAY	-1.987	0.007
WIP_HS_GLYCOGEN_METABOLISM	-1.983	0.007
NCI_CXCR3PATHWAY	-1.987	0.007
REACT_SCF-BETA-TRCP MEDIATED DEGRADATION OF EMI1	-1.971	0.007
WIP_HS_INTEGRATION_OF_ENERGY_METABOLISM	-1.950	0.009
REACT_UBIQUITIN-DEPENDENT DEGRADATION OF CYCLIN D	-1.945	0.010
REACT_CTLA4 INHIBITORY SIGNALING	-1.943	0.010
REACT_AUTODEGRADATION OF CDH1 BY CDH1_APC_C	-1.940	0.010
REACT_GLUCONEOGENESIS	-1.936	0.010
REACT_GAP JUNCTION ASSEMBLY	-1.938	0.010
REACT_AUTODEGRADATION OF THE E3 UBIQUITIN LIGASE COP1	-1.933	0.011
REACT_UBIQUITIN-DEPENDENT DEGRADATION OF CYCLIN D1	-1.920	0.011
KEGG_GLYCOLYSIS _ GLUCONEOGENESIS	-1.920	0.012
REACT_VPU MEDIATED DEGRADATION OF CD4	-1.922	0.012
WIP_HS_PROTEASOME_DEGRADATION	-1.908	0.012
REACT_APC_C_CDH1 MEDIATED DEGRADATION OF CDC20 AND OTHER APC_C_CDH1 TARGETED PROTEINS IN LATE MITOSIS_EARLY G1	-1.909	0.013
REACT_GAP JUNCTION TRAFFICKING	-1.905	0.013
KEGG_CITRATE CYCLE (TCA CYCLE)	-1.909	0.013
KEGG_PROPANOATE METABOLISM	-1.885	0.015
REACT_APC_C_CDC20 MEDIATED DEGRADATION OF SECURIN	-1.886	0.015
KEGG_FATTY ACID ELONGATION	-1.881	0.015
WIP_HS_TRIACYLGLYCERIDE_SYNTHESIS	-1.865	0.018
NCI_GMCSF_PATHWAY	-1.866	0.018
NCI_VEGFR1_2_PATHWAY	-1.830	0.025
NCI_INSULIN_GLUCOSE_PATHWAY	-1.832	0.025
WIP_HS_IL-3_SIGNALING_PATHWAY	-1.822	0.027
REACT_INTERLEUKIN-2 SIGNALING	-1.814	0.029
KEGG_VALINE, LEUCINE AND ISOLEUCINE DEGRADATION	-1.808	0.030
KEGG_HUNTINGTON'S DISEASE	-1.802	0.030
REACT_METABOLISM OF CARBOHYDRATES	-1.804	0.030
REACT_SIGNALING BY PDGF	-1.803	0.030
REACT_FATTY ACID, TRIACYLGLYCEROL, AND KETONE BODY METABOLISM	-1.805	0.030
REACT_CDK-MEDIATED PHOSPHORYLATION AND REMOVAL OF CDC6	-1.792	0.031
REACT_DOWN-STREAM SIGNAL TRANSDUCTION	-1.793	0.031
REACT_INTEGRIN ALPHAIIB BETA3 SIGNALING	-1.795	0.031
NCI_PI3KPLCTRKPATHWAY	-1.776	0.034
REACT_CDC20_PHOSPHO-APC_C MEDIATED DEGRADATION OF CYCLIN A	-1.779	0.034
WIP_HS_FATTY_ACID_BETA_OXIDATION	-1.777	0.035
REACT_CITRIC ACID CYCLE (TCA CYCLE)	-1.772	0.035
REACT_SCF(SKP2)-MEDIATED DEGRADATION OF P27_P21	-1.769	0.036
BIOC_PROTEASOMEPATHWAY	-1.766	0.036
WIP_HS_FOCAL_ADHESION	-1.749	0.040

REACT_GAP JUNCTION TRAFFICKING AND REGULATION	-1.749	0.040
WIP_HS_CELL_JUNCTION_ORGANIZATION	-1.750	0.040
NCI_PI3KCIAKTPATHWAY	-1.750	0.041
REACT_METABOLISM OF LIPIDS AND LIPOPROTEINS	-1.750	0.041
REACT_APC_C_CDC20 MEDIATED DEGRADATION OF MITOTIC PROTEINS	-1.742	0.042
REACT_ACTIVATION OF APC_C AND APC_C_CDC20 MEDIATED DEGRADATION OF MITOTIC PROTEINS	-1.740	0.042
REACT_SYNTHESIS AND INTERCONVERSION OF NUCLEOTIDE DI-AND TRIPHOSPHATES	-1.738	0.042
REACT_COOPERATION OF PREFOLDIN AND TRIC_CCT IN ACTIN AND TUBULIN FOLDING	-1.733	0.044
REACT_KINESINS	-1.728	0.045
REACT_G1_S DNA DAMAGE CHECKPOINTS	-1.726	0.045
REACT_PLATELET AGGREGATION (PLUG FORMATION)	-1.724	0.046
KEGG_GLYOXYLATE AND DICARBOXYLATE METABOLISM	-1.715	0.048
REACT_FORMATION OF TUBULIN FOLDING INTERMEDIATES BY CCT_TRIC	-1.716	0.049
WIP_HS_IL-2_SIGNALING_PATHWAY	-1.710	0.050
REACT_PLATELET ACTIVATION	-1.705	0.051
REACT_P53-DEPENDENT G1_S DNA DAMAGE CHECKPOINT	-1.701	0.051
KEGG_ACUTE MYELOID LEUKEMIA	-1.706	0.051
WIP_HS_IL-5_SIGNALING_PATHWAY	-1.701	0.051
REACT_FORMATION OF PLATELET PLUG	-1.702	0.051
REACT_COSTIMULATION BY THE CD28 FAMILY	-1.699	0.052
KEGG_FOCAL ADHESION	-1.695	0.052
WIP_HS_NETRIN-1_SIGNALING	-1.696	0.052
REACT_PREFOLDIN MEDIATED TRANSFER OF SUBSTRATE TO CCT_TRIC	-1.692	0.053
BIOC_GLEEVECPATHWAY	-1.689	0.054
BIOC_IGF1RPATHWAY	-1.688	0.054
WIP_HS_IL-7_SIGNALING_PATHWAY	-1.680	0.055
REACT_DESTABILIZATION OF MRNA BY AUF1 (HNRNP D0)	-1.684	0.056
REACT_P53-DEPENDENT G1 DNA DAMAGE RESPONSE	-1.682	0.056
KEGG_PATHOGENIC ESCHERICHIA COLI INFECTION	-1.681	0.056
NCI_CXCR4_PATHWAY	-1.674	0.059
BIOC_PYK2PATHWAY	-1.670	0.059
REACT_RECRUITMENT OF NUMA TO MITOTIC CENTROSOMES	-1.671	0.060
NRF2_TARGETS	-1.667	0.061
REACT_PROTEIN FOLDING	-1.665	0.061
REACT_CYCLIN E ASSOCIATED EVENTS DURING G1_S TRANSITION	-1.665	0.061
REACT_CHAPERONIN-MEDIATED PROTEIN FOLDING	-1.661	0.062
NCI_KITPATHWAY	-1.657	0.064
KEGG_REGULATION OF ACTIN CYTOSKELETON	-1.654	0.065
BIOC_SPPAPATHWAY	-1.654	0.065
KEGG_B CELL RECEPTOR SIGNALING PATHWAY	-1.648	0.067
REACT_IMMUNOREGULATORY INTERACTIONS BETWEEN A LYMPHOID AND A NON-LYMPHOID CELL	-1.642	0.070
KEGG_INSULIN SIGNALING PATHWAY	-1.635	0.073
REACT_CYCLIN A_CDK2-ASSOCIATED EVENTS AT S PHASE ENTRY	-1.632	0.074
REACT_NCAM SIGNALING FOR NEURITE OUT-GROWTH	-1.629	0.075
KEGG_STARCH AND SUCROSE METABOLISM	-1.623	0.076
KEGG_ECM-RECEPTOR INTERACTION	-1.623	0.076
WIP_HS_T_CELL_RECEPTOR_SIGNALING_PATHWAY	-1.624	0.076
REACT_CDT1 ASSOCIATION WITH THE CDC6_ORC_ORIGIN COMPLEX	-1.623	0.076
WIP_HS_TRANSPORT_OF_VITAMINS_NUCLEOSIDES_AND_RELATED_MOLECULES	-1.624	0.077
BIOC_IL6PATHWAY	-1.625	0.077
NCI_VEGFR1_PATHWAY	-1.617	0.078
REACT_ADAPTIVE IMMUNITY SIGNALING	-1.615	0.078
REACT_PYRUVATE METABOLISM AND CITRIC ACID (TCA) CYCLE	-1.617	0.078
REACT_PLATELET HOMEOSTASIS	-1.615	0.078
REACT_DARPP-32 EVENTS	-1.609	0.081
NCI_FCER1PATHWAY	-1.606	0.082
NCI_REELINPATHWAY	-1.604	0.083
KEGG_GLYCOSYLPHOSPHATIDYLINOSITOL(GPI)-ANCHOR BIOSYNTHESIS	-1.602	0.084
KEGG_T CELL RECEPTOR SIGNALING PATHWAY	-1.599	0.085
KEGG_FATTY ACID METABOLISM	-1.598	0.085

NCI_ER_NONGENOMIC_PATHWAY	-1.597	0.085
BIOC_IGF1PATHWAY	-1.595	0.086
NCI_WNT_CANONICAL_PATHWAY	-1.587	0.090
REACT_MITOTIC PROMETAPHASE	-1.583	0.092
NCI_SYNDECAN_2_PATHWAY	-1.584	0.092
REACT_M PHASE	-1.570	0.100
KEGG_PORPHYRIN AND CHLOROPHYLL METABOLISM	-1.569	0.100
WIP_HS_GLUTATHIONE_METABOLISM	-1.571	0.100
REACT_TIE2 SIGNALING	-1.572	0.100
REACT_REGULATION OF APOPTOSIS	-1.567	0.101
NCI_RET_PATHWAY	-1.564	0.102
BIOC_SPRYPATHWAY	-1.564	0.102
WIP_HS_IL-6_SIGNALING_PATHWAY	-1.559	0.105
BIOC_INSULINPATHWAY	-1.554	0.109
REACT_CD28 DEPENDENT PI3K_AKT SIGNALING	-1.549	0.111
NCI_IL2_PI3KPATHWAY	-1.549	0.112
WIP_HS_SIGNALING_OF_HEPATOCYTE_GROWTH_FACTOR_RECEPTOR	-1.544	0.115
WIP_HS_TCA_CYCLE	-1.539	0.118
NCI_IL8CXCR2_PATHWAY	-1.537	0.119
NCI_CD8TCRDOWNSTREAMPATHWAY	-1.536	0.119
REACT_CELL-EXTRACELLULAR MATRIX INTERACTIONS	-1.534	0.121
WIP_HS_KEAP1-NRF2_PATHWAY	-1.532	0.121
KEGG_FC EPSILON RI SIGNALING PATHWAY	-1.530	0.122
WIP_HS_MITOCHONDRIAL_LC-FATTY_ACID_BETA-OXIDATION	-1.526	0.123
KEGG_ALPHA-LINOLENIC ACID METABOLISM	-1.525	0.123
NCI_IGF1_PATHWAY	-1.524	0.123
REACT_CHEMOKINE RECEPTORS BIND CHEMOKINES	-1.526	0.124
NCI_THROMBIN_PAR1_PATHWAY	-1.522	0.124
REACT_CD28 CO-STIMULATION	-1.527	0.124
REACT_REGULATION OF APC_C ACTIVATORS BETWEEN G1_S AND EARLY ANAPHASE	-1.519	0.126
KEGG_ENDOMETRIAL CANCER	-1.509	0.127
REACT_ASSEMBLY OFTHE PRE-REPLICATIVE COMPLEX	-1.510	0.127
BIOC_BIOPEPTIDESPATHWAY	-1.510	0.127
NCI_MET_PATHWAY	-1.515	0.127
WIP_HS_NICOTINE_ACTIVITY_ON_DOPAMINERGIC_NEURONS	-1.511	0.127
WIP_HS_KIT_RECEPTOR_SIGNALING_PATHWAY	-1.515	0.128
REACT_EFFECTS OF PIP2 HYDROLYSIS	-1.516	0.128
BIOC_MCALPAINPATHWAY	-1.511	0.128
KEGG_GAP JUNCTION	-1.513	0.128
WIP_HS_WNT_SIGNALING_PATHWAY	-1.511	0.129
REACT_SIGNALING BY INTERLEUKINS	-1.512	0.129
REACT_METABOLISM OF AMINO ACIDS AND DERIVATIVES	-1.502	0.132
NCI_LIS1PATHWAY	-1.502	0.132
REACT_INTEGRIN CELL SURFACE INTERACTIONS	-1.499	0.133
REACT_SHC-RELATED EVENTS	-1.499	0.133
REACT_REGULATION OF MITOTIC CELL CYCLE	-1.500	0.133
WIP_HS_METABOLISM_OF_CARBOHYDRATES	-1.492	0.136
BIOC_PTDINSPATHWAY	-1.493	0.136
REACT_DOWNSTREAM SIGNALING OF ACTIVATED FGFR	-1.494	0.136
BIOC_METPATHWAY	-1.494	0.137
REACT_APC_C-MEDIATED DEGRADATION OF CELL CYCLE PROTEINS	-1.489	0.137
WIP_HS_METABOLISM_OF_WATER-SOLUBLE_VITAMINS_AND_COFACTORS	-1.490	0.137
REACT_HEMOSTASIS	-1.489	0.138
KEGG_PHOTOTRANSDUCTION	-1.486	0.139
REACT_SIGNALING BY FGFR	-1.483	0.141
REACT_MEMBRANETRAFFICKING	-1.478	0.146
NCI_AR_NONGENOMIC_PATHWAY	-1.477	0.146
BIOC_GSK3PATHWAY	-1.469	0.153
REACT_AXON GUIDANCE	-1.470	0.153
WIP_HS_AMPK_SIGNALING	-1.468	0.153
REACT_PEROXISOMAL LIPID METABOLISM	-1.457	0.164
BIOC_G1PATHWAY	-1.450	0.165

WIP_HS_TYPE_II_DIABETES_MELLITUS	-1.452	0.165
WIP_HS_REGULATION_OF_ACTIN_CYTOSKELETON	-1.452	0.165
WIP_HS_INTEGRIN-MEDIATED_CELL_ADHESION	-1.450	0.165
KEGG_PPAR SIGNALING PATHWAY	-1.449	0.165
REACT_NEPHRIN INTERACTIONS	-1.452	0.166
NCI_IL2_STAT5PATHWAY	-1.448	0.166
REACT_G1 PHASE	-1.452	0.166
NCI_AVB3_INTEGRIN_PATHWAY	-1.452	0.167
REACT_INTEGRATION OF ENERGY METABOLISM	-1.453	0.167
WIP_HS_SIGNALING_BY_INSULIN_RECEPTOR	-1.443	0.169
BIOC_RASPATHWAY	-1.442	0.169
KEGG_NON-SMALL CELL LUNG CANCER	-1.441	0.169
REACT_MITOTIC M-M_G1 PHASES	-1.443	0.169
NCI_FAK_PATHWAY	-1.440	0.170
REACT_PI-3K CASCADE	-1.443	0.170
REACT_IRS-MEDIATED SIGNALLING	-1.443	0.170
KEGG_ADIPOCYTOKINE SIGNALING PATHWAY	-1.438	0.171
NCI_PDGFRBPATHWAY	-1.436	0.172
KEGG_PEROXISOME	-1.435	0.173
KEGG_CHEMOKINE SIGNALING PATHWAY	-1.432	0.176
BIOC_IL2PATHWAY	-1.426	0.180
WIP_HS_METABOLISM_OF_AMINO_ACIDS_AND_DERIVATIVES	-1.427	0.181
BIOC_AT1RPATHWAY	-1.424	0.183
NCI_IL8CXCR1_PATHWAY	-1.420	0.187
NCI_BCR_5PATHWAY	-1.418	0.188
WIP_HS_NEURORANSMITTER_RECEPTOR_BINDING_AND_ DOWNSTREAM_TRANSMISSION_IN_THE_POSTSYNAPTIC_CELL	-1.417	0.189
REACT_PLATELET DEGRANULATION	-1.413	0.191
NCI_ERBB2ERBB3PATHWAY	-1.413	0.191
KEGG_STEROID BIOSYNTHESIS	-1.414	0.191
REACT_CYCLIN D ASSOCIATED EVENTS IN G1	-1.409	0.194
REACT_SIGNALLING TO RAS	-1.409	0.194
REACT_MITOTIC G1-G1_S PHASES	-1.407	0.195
KEGG_AMOEBIASIS	-1.407	0.196
WIP_HS_INTRINSIC_PATHWAY_FOR_APOPTOSIS	-1.405	0.196
BIOC_PDGFPATHWAY	-1.399	0.202
REACT_IRS-RELATED EVENTS	-1.399	0.203
BIOC_ERKPATHWAY	-1.396	0.204
WIP_HS_SIGNAL_TRANSDUCTION_OF_S1P_RECEPTOR	-1.396	0.205
KEGG_PROTEIN DIGESTION AND ABSORPTION	-1.394	0.206
BIOC_EGFPATHWAY	-1.391	0.208
REACT_CELL CYCLE, MITOTIC	-1.391	0.209
NCI_ECADHERIN_KERATINOCYTE_PATHWAY	-1.388	0.211
WIP_HS_ENDOCHONDRAL_OSSIFICATION	-1.386	0.212
REACT_DNA REPLICATION	-1.387	0.212
NCI_ERBB1_RECEPTOR_PROXIMAL_PATHWAY	-1.381	0.212
WIP_HS_INSULIN_SIGNALING	-1.387	0.212
WIP_HS_EPO_RECEPTOR_SIGNALING	-1.382	0.213
KEGG_LONG-TERM DEPRESSION	-1.384	0.213
KEGG_BETA-ALANINE METABOLISM	-1.382	0.213
REACT_SYNTHESIS OF GLYCOSYLPHOSPHATIDYLINOSITOL (GPI)	-1.384	0.213
REACT_SWITCHING OF ORIGINS TO A POST-REPLICATIVE STATE	-1.382	0.214
WIP_HS_TRANSPORT_OF_GLUCOSE_AND_OTHER_SUGARS,_BILE_ SALTS_AND_ORGANIC_ACIDS,_METAL_IONS_AND_AMINE_COMPOUNDS	-1.377	0.217
REACT_G ALPHA (12_13) SIGNALLING EVENTS	-1.373	0.217
KEGG_CARDIAC MUSCLE CONTRACTION	-1.375	0.217
REACT_ORC1 REMOVAL FROM CHROMATIN	-1.376	0.218
REACT_SIGNALING BY EGFR	-1.373	0.218
REACT_L1CAM INTERACTIONS	-1.373	0.218
REACT_NGF SIGNALLING VIA TRKA FROM THE PLASMA MEMBRANE	-1.374	0.219
KEGG_FRUCTOSE AND MANNOSE METABOLISM	-1.370	0.219
WIP_HS_ALPHA_6_BETA_4_SIGNALING_PATHWAY	-1.370	0.220

BIOC_FCER1PATHWAY	-1.369	0.220
REACT_CLASS I MHC MEDIATED ANTIGEN PROCESSING & PRESENTATION	-1.368	0.221
REACT_POST-TRANSLATIONAL MODIFICATION_ SYNTHESIS OF GPI-ANCHORED PROTEINS	-1.366	0.223
BIOC_NGFPATHWAY	-1.363	0.226
NCI_NFAT_3PATHWAY	-1.359	0.228
BIOC_TPOPATHWAY	-1.359	0.228
KEGG_GLUTATHIONE METABOLISM	-1.360	0.229
REACT_ANTIGEN PROCESSING_ UBIQUITINATION & PROTEASOMEDEGRADATION	-1.359	0.229
WIP_HS_IL-1_PATHWAY	-1.360	0.229
REACT_REGULATION OF DNA REPLICATION	-1.352	0.236
REACT_SHC-MEDIATED CASCADE	-1.351	0.236
KEGG_HEDGEHOG SIGNALING PATHWAY	-1.350	0.237
REACT_SMOOTH MUSCLE CONTRACTION	-1.348	0.239
REACT_INSULIN RECEPTOR SIGNALLING CASCADE	-1.347	0.239
REACT_REMOVAL OF LICENSING FACTORS FROM ORIGINS	-1.347	0.240
NCI_ENDOTHELINPATHWAY	-1.345	0.240
REACT_APOPTOSIS	-1.345	0.240
KEGG_STEROID HORMONE BIOSYNTHESIS	-1.341	0.245
REACT_INTERLEUKIN-3, 5 AND GM-CSF SIGNALING	-1.340	0.245
REACT_CYTOSOLICTRNA AMINOACYLATION	-1.338	0.248
BIOC_NO1PATHWAY	-1.337	0.248

Supplementary table 3. Predicted upstream regulators for the response to ER of both groups together, and signal log ratio (SLR) of gene expression upon ER with P-value.

Upstream Regulator	Function		z-score	P-value	Average SLR 25% ER	P-value 25% ER
SREBF1	transcription regulator	↓	-3.683	3.33E-06	-0.42	2.76E-03*
PPARG	ligand-dependent nuclear receptor	↓	-3.491	8.14E-12	0.03	5.61E-01
SREBF2	transcription regulator	↓	-3.478	7.41E-07	-0.20	8.89E-03*
Insulin	group	↓	-3.452	9.67E-11	NA[1]	NA
SCAP	other	↓	-3.307	4.19E-08	0.02	6.30E-01
MAPK9	kinase	↓	-3.05	2.41E-02	-0.07	7.33E-02
PPARGC1A	transcription regulator	↓	-2.842	1.18E-04	0.13	2.07E-01
IRS1	enzyme	↓	-2.626	6.21E-05	-0.01	9.58E-01
CASP1	peptidase	↓	-2.607	9.85E-03	-0.11	2.27E-01
PTPN1	phosphatase	↓	-2.577	1.52E-02	0.06	2.27E-01
ERBB2	kinase	↓	-2.567	6.69E-05	0.02	7.57E-01
INS	other	↓	-2.549	3.32E-06	NA	NA
HIPK2	kinase	↓	-2.534	2.95E-02	-0.09	1.51E-01
PI3K (family)	group	↓	-2.425	1.95E-02	NA	NA
INSR	kinase	↓	-2.411	6.91E-07	0.04	4.58E-01
PI3K (complex)	complex	↓	-2.406	5.60E-03	NA	NA
SP3	transcription regulator	↓	-2.334	2.50E-03	0.08	3.15E-02*
GHRL	growth factor	↓	-2.261	2.69E-02	-0.10	1.02E-01
PCDH11Y	other	↓	-2.236	1.96E-02	NA	NA
DGAT2	enzyme	↓	-2.219	5.05E-05	-0.59	2.29E-03*
EP300	transcription regulator	↓	-2.176	5.03E-05	0.02	4.16E-01
MLX	transcription regulator	↓	-2.173	2.26E-04	0.01	7.91E-01
MLXIPL	transcription regulator	↓	-2.124	4.07E-04	-0.10	2.31E-01
ERBB3	kinase	↓	-2.118	9.93E-03	NA	NA
MTORC1	complex	↓	-2.106	4.17E-03	NA	NA
GPER1	g-protein coupled receptor	↓	-2.085	1.00E-02	-0.12	4.43E-02*
KLF15	transcription regulator	↓	-2.06	1.80E-05	0.25	1.43E-02*
NOTCH1	transcription regulator	↓	-2.005	2.34E-02	-0.15	9.19E-03*
MEDAG	other	↓	-2	1.93E-03	0.11	4.14E-01
SIRT2	transcription regulator	↓	-2	3.75E-02	-0.10	5.19E-02
MYCN	transcription regulator	↑	3.844	1.61E-10	-0.15	9.98E-02
OSM	cytokine	↑	3.431	4.32E-06	NA	NA
N-cor	group	↑	3.293	1.09E-04	NA	NA
LDL	complex	↑	3.201	2.39E-02	NA	NA
INSIG2	other	↑	2.934	3.15E-07	0.09	1.77E-01
FGF2	growth factor	↑	2.845	3.90E-04	0.03	6.35E-01
INSIG1	other	↑	2.841	2.04E-03	-0.26	9.04E-03*
ELOVL5	enzyme	↑	2.804	6.79E-05	-0.24	5.55E-04*
MAP4K4	kinase	↑	2.782	1.76E-05	0.02	3.81E-01
POR	enzyme	↑	2.704	3.28E-04	-0.07	3.06E-01
TNF	cytokine	↑	2.676	5.50E-07	-0.42	2.76E-03
FBXW7	transcription regulator	↑	2.621	8.99E-05	0.03	5.61E-01
FOXO3	transcription regulator	↑	2.589	5.27E-03	-0.20	8.89E-03
ABCB6	transporter	↑	2.53	1.34E-03	NA	NA
PLIN1	other	↑	2.425	6.02E-05	0.02	6.30E-01
LOC102724428/SIK1	kinase	↑	2.408	1.45E-05	-0.07	7.33E-02
TNFSF11	cytokine	↑	2.34	1.95E-02	0.13	2.07E-01
Jnk	group	↑	2.309	4.11E-02	-0.01	9.58E-01
ATP2A2	transporter	↑	2.236	4.27E-03	-0.11	2.27E-01
PML	transcription regulator	↑	2.233	4.76E-05	0.06	2.27E-01
Sik1	kinase	↑	2.201	2.75E-06	0.02	7.57E-01
MAPKAPK2	kinase	↑	2.18	4.50E-02	NA	NA
ACACB	enzyme	↑	2.178	9.19E-06	-0.09	1.51E-01
C5	cytokine	↑	2.156	4.13E-02	NA	NA
CD24	other	↑	2.121	3.57E-04	0.04	4.58E-01
UBD	other	↑	2	7.55E-03	NA	NA
ARHGDIG	other	↑	2	4.54E-02	0.08	3.15E-02

↑ activated; ↓ inhibited; [1]NA: not in data set or other compound than gene; *significantly changed (P<0.05)

Supplementary table 4. Gene Set Enrichment Analysis. Cut-off value FDR q-value 0.25.
Supplementary table 4a. Gene sets upregulated in white adipose tissue upon twelve weeks of HP-ER.

Gene set	NES	FDR q-value
KEGG_RIBOSOME	2.744	0.000
REACT_INFLUENZA VIRAL RNA TRANSCRIPTION AND REPLICATION	2.723	0.000
REACT_VIRAL MRNA TRANSLATION	2.720	0.000
REACT_PEPTIDE CHAIN ELONGATION	2.706	0.000
REACT_EUKARYOTIC TRANSLATION ELONGATION	2.700	0.000
REACT_EUKARYOTIC TRANSLATION TERMINATION	2.678	0.000
REACT_NONSENSE MEDIATED DECAY INDEPENDENT OF THE EXON JUNCTION COMPLEX	2.626	0.000
REACT_FORMATION OF A POOL OF FREE 40S SUBUNITS	2.621	0.000
REACT_REGULATION OF GENE EXPRESSION IN BETA CELLS	2.584	0.000
WIP_HS_CYTOPLASMIC_RIBOSOMAL_PROTEINS	2.550	0.000
REACT_L13A-MEDIATED TRANSLATIONAL SILENCING OF CERULOPLASMIN EXPRESSION	2.548	0.000
REACT_3 -UTR-MEDIATED TRANSLATIONAL REGULATION	2.536	0.000
REACT_NONSENSE-MEDIATED DECAY	2.527	0.000
REACT_NONSENSE MEDIATED DECAY ENHANCED BY THE EXON JUNCTION COMPLEX	2.506	0.000
REACT_REGULATION OF BETA-CELL DEVELOPMENT	2.500	0.000
REACT_GTP HYDROLYSIS AND JOINING OF THE 60S RIBOSOMAL SUBUNIT	2.485	0.000
REACT_OLFACTORY SIGNALING PATHWAY	2.479	0.000
WIP_HS_PROCESSING_OF_CAPPED_INTRON-CONTAINING_PRE-MRNA	2.458	0.000
REACT_CAP-DEPENDENT TRANSLATION INITIATION	2.383	0.000
REACT_EUKARYOTIC TRANSLATION INITIATION	2.364	0.000
REACT_TRANSLATION	2.362	0.000
REACT_INSULIN SYNTHESIS AND PROCESSING	2.187	0.000
BIOC_LAIR PATHWAY	2.182	0.000
REACT_FORMATION OF THE TERNARY COMPLEX, AND SUBSEQUENTLY, THE 43S COMPLEX	2.153	0.001
KEGG_OLFACTORY TRANSDUCTION	2.105	0.001
REACT_ACTIVATION OF THE MRNA UPON BINDING OF THE CAP-BINDING COMPLEX AND EIFS, AND SUBSEQUENT BINDING TO 43S	2.098	0.001
REACT_TRANSLATION INITIATION COMPLEX FORMATION	2.032	0.003
REACT_RIBOSOMAL SCANNING AND START CODON RECOGNITION	2.027	0.003
WIP_HS_MRNA_PROCESSING	2.025	0.003
REACT_GENERIC TRANSCRIPTION PATHWAY	2.013	0.003
REACT_POST-ELONGATION PROCESSING OF INTRON-CONTAINING PRE-MRNA	1.916	0.010
REACT_MRNA 3-END PROCESSING	1.883	0.014
REACT_COMPLEMENT CASCADE	1.863	0.017
REACT_TRANSPORT OF MATURE MRNA DERIVED FROM AN INTRON-CONTAINING TRANSCRIPT	1.862	0.016
REACT_MRNA SPLICING	1.834	0.021
REACT_INTERACTIONS OF REV WITH HOST CELLULAR PROTEINS	1.829	0.021
REACT_MRNA SPLICING - MAJOR PATHWAY	1.828	0.021
KEGG_NITROGEN METABOLISM	1.810	0.025
REACT_REV-MEDIATED NUCLEAR EXPORT OF HIV-1 RNA	1.797	0.027
REACT_REGULATION OF GLUCOKINASE BY GLUCOKINASE REGULATORY PROTEIN	1.782	0.031
REACT_PROCESSING OF CAPPED INTRON-CONTAINING PRE-MRNA	1.782	0.030
REACT_MRNA PROCESSING	1.762	0.035
REACT_NUCLEAR IMPORT OF REV PROTEIN	1.757	0.036
KEGG_PRION DISEASES	1.733	0.043
REACT_CLEAVAGE OF GROWING TRANSCRIPT IN THE TERMINATION REGION	1.700	0.055
REACT_METABOLISM OF MRNA	1.699	0.055
WIP_HS_DNA_REPLICATION	1.668	0.071
REACT_POST-ELONGATION PROCESSING OF THE TRANSCRIPT	1.652	0.078
KEGG_SPLICEOSOME	1.650	0.078
REACT_RNA POLYMERASE II TRANSCRIPTION TERMINATION	1.645	0.079
BIOC_TID PATHWAY	1.642	0.080
WIP_HS_MITOCHONDRIAL_GENE_EXPRESSION	1.635	0.083
KEGG_STAPHYLOCOCCUS AUREUS INFECTION	1.627	0.087
NCI_AP1_PATHWAY	1.623	0.088

REACT_ACTIVATION OF THE PRE-REPLICATIVE COMPLEX	1.612	0.094
REACT_VPR-MEDIATED NUCLEAR IMPORT OF PICS	1.607	0.096
REACT_DIABETES PATHWAYS	1.598	0.101
KEGG_MINERAL ABSORPTION	1.586	0.108
REACT_METABOLISM OF PROTEINS	1.577	0.114
REACT_TELOMERE C-STRAND (LAGGING STRAND) SYNTHESIS	1.568	0.121
REACT_TRANSPORT OF MATURE TRANSCRIPT TO CYTOPLASM	1.565	0.121
REACT_METABOLISM OF RNA	1.561	0.123
BIOC_MTORPATHWAY	1.548	0.132
REACT_INTERACTIONS OF VPR WITH HOST CELLULAR PROTEINS	1.515	0.165
REACT_EXTENSION OF TELOMERES	1.495	0.186
REACT_NUCLEAR RECEPTOR TRANSCRIPTION PATHWAY	1.470	0.216
REACT_FORMATION AND MATURATION OF MRNA TRANSCRIPT	1.464	0.222
NCI_SMAD2_3NUCLEARPATHWAY	1.463	0.220
WIP_HS_CELL_CYCLE	1.454	0.230

Supplementary table 4b. Gene sets downregulated in white adipose tissue upon twelve weeks of HP-ER.

Gene set	NES	FDR q-value
KEGG_BIOSYNTHESIS OF UNSATURATED FATTY ACIDS	-2.446	0.000
PPARA_TARGETS	-2.411	0.000
REACT_GLYCOLYSIS	-2.340	0.000
KEGG_OXIDATIVE PHOSPHORYLATION	-2.278	0.000
REACT_GLUCOSE METABOLISM	-2.271	0.000
WIP_HS_OXIDATIVE_PHOSPHORYLATION	-2.250	0.000
KEGG_PARKINSON'S DISEASE	-2.226	0.000
REACT_RESPIRATORY ELECTRON TRANSPORT, ATP SYNTHESIS BY CHEMIOSMOTIC COUPLING, AND HEAT PRODUCTION BY UNCOUPLING PROTEINS.	-2.204	0.000
WIP_HS_GLYCOLYSIS_AND_GLUCONEOGENESIS	-2.176	0.001
WIP_HS_ELECTRON_TRANSPORT_CHAIN	-2.176	0.001
REACT_RESPIRATORY ELECTRON TRANSPORT	-2.101	0.003
REACT_CHOLESTEROL BIOSYNTHESIS	-2.090	0.004
WIP_HS_CHOLESTEROL_BIOSYNTHESIS	-2.082	0.004
WIP_HS_GLYCOGEN_METABOLISM	-2.071	0.004
WIP_HS_FATTY_ACID_BIOSYNTHESIS	-2.071	0.004
REACT_FATTY ACYL-COA BIOSYNTHESIS	-2.063	0.003
KEGG_FATTY ACID ELONGATION	-2.046	0.005
KEGG_CITRATE CYCLE (TCA CYCLE)	-2.017	0.006
KEGG_PYRUVATE METABOLISM	-2.011	0.006
REACT_PYRUVATE METABOLISM AND CITRIC ACID (TCA) CYCLE	-2.007	0.006
WIP_HS_FATTY_ACID_BETA_OXIDATION	-1.999	0.007
WIP_HS_INTEGRATION_OF_ENERGY_METABOLISM	-1.965	0.011
REACT_TRIGLYCERIDE BIOSYNTHESIS	-1.961	0.011
KEGG_ALPHA-LINOLENIC ACID METABOLISM	-1.955	0.012
KEGG_ALZHEIMER'S DISEASE	-1.947	0.012
REACT_VIF-MEDIATED DEGRADATION OF APOBEC3G	-1.945	0.012
KEGG_GLYOXYLATE AND DICARBOXYLATE METABOLISM	-1.944	0.012
KEGG_GLYCEROLIPID METABOLISM	-1.943	0.011
REACT_CITRIC ACID CYCLE (TCA CYCLE)	-1.928	0.013
KEGG_PENTOSE PHOSPHATE PATHWAY	-1.925	0.013
BIOC_INTEGRINPATHWAY	-1.923	0.013
WIP_HS_TRIACYLGLYCERIDE_SYNTHESIS	-1.905	0.015
KEGG_GLYCOLYSIS _ GLUCONEOGENESIS	-1.892	0.017
KEGG_FATTY ACID METABOLISM	-1.884	0.018
WIP_HS_TCA_CYCLE	-1.882	0.018
KEGG_PROPANOATE METABOLISM	-1.882	0.017
WIP_HS_IL-3_SIGNALING_PATHWAY	-1.874	0.018
KEGG_STARCH AND SUCROSE METABOLISM	-1.852	0.022
WIP_HS_IL-7_SIGNALING_PATHWAY	-1.852	0.022

REACT_SIGNALING BY WNT	-1.839	0.025
REACT_REGULATION OF ORNITHINE DECARBOXYLASE (ODC)	-1.836	0.025
REACT_DEGRADATION OF BETA-CATENIN BY THE DESTRUCTION COMPLEX	-1.833	0.025
WIP_HS_FATTY_ACID,_TRIACYLGLYCEROL,_AND_KETONE_BODY_METABOLISM	-1.831	0.025
REACT_FATTY ACID, TRIACYLGLYCEROL, AND KETONE BODY METABOLISM	-1.825	0.026
KEGG_VALINE, LEUCINE AND ISOLEUCINE DEGRADATION	-1.824	0.025
REACT_CTLA4 INHIBITORY SIGNALING	-1.824	0.025
REACT_SIGNALING BY PDGF	-1.816	0.027
BIOC_IGF1RPATHWAY	-1.814	0.027
REACT_METABOLISM OF CARBOHYDRATES	-1.808	0.028
REACT_UBIQUITIN-DEPENDENT DEGRADATION OF CYCLIN D1	-1.808	0.027
REACT_GLUCONEOGENESIS	-1.807	0.027
KEGG_PEROXISOME	-1.805	0.027
KEGG_PROTEASOME	-1.804	0.027
REACT_DOWN-STREAM SIGNAL TRANSDUCTION	-1.787	0.032
WIP_HS_TRANSPORT_OF_VITAMINS_NUCLEOSIDES_AND_RELATED_MOLECULES	-1.786	0.032
REACT_METABOLISM OF LIPIDS AND LIPOPROTEINS	-1.777	0.034
KEGG_PPAR SIGNALING PATHWAY	-1.772	0.036
REACT_UBIQUITIN-DEPENDENT DEGRADATION OF CYCLIN D	-1.772	0.035
KEGG_HUNTINGTON'S DISEASE	-1.761	0.039
WIP_HS_IL-2_SIGNALING_PATHWAY	-1.749	0.044
WIP_HS_IL-5_SIGNALING_PATHWAY	-1.736	0.050
BIOC_PROTEASOMEPATHWAY	-1.736	0.049
BIOC_BIOPEPTIDESPATHWAY	-1.735	0.049
NCI_PI3KPLCTRKPATHWAY	-1.726	0.053
KEGG_ACUTE MYELOID LEUKEMIA	-1.725	0.052
NCI_PI3KCIAKTPATHWAY	-1.718	0.055
REACT_CYTOSOLIC TRNA AMINOACYLATION	-1.709	0.060
NCI_GMCSF_PATHWAY	-1.708	0.059
REACT_VPU MEDIATED DEGRADATION OF CD4	-1.708	0.059
REACT_CDK-MEDIATED PHOSPHORYLATION AND REMOVAL OF CDC6	-1.696	0.064
REACT_REGULATION OF ACTIVATED PAK-2P34 BY PROTEASOME MEDIATED DEGRADATION	-1.692	0.066
REACT_PLATELET AGGREGATION (PLUG FORMATION)	-1.692	0.065
REACT_TRNA AMINOACYLATION	-1.689	0.066
REACT_PLATELET SENSITIZATION BY LDL	-1.684	0.068
WIP_HS_CELL_JUNCTION_ORGANIZATION	-1.679	0.070
NCI_INSULIN_PATHWAY	-1.669	0.076
REACT_SCF-BETA-TRCP MEDIATED DEGRADATION OF EMI1	-1.667	0.076
KEGG_INSULIN SIGNALING PATHWAY	-1.666	0.076
WIP_HS_PROTEASOME_DEGRADATION	-1.664	0.077
WIP_HS_AMPK_SIGNALING	-1.659	0.080
REACT_P53-INDEPENDENT DNA DAMAGE RESPONSE	-1.656	0.080
WIP_HS_EPO_RECEPTOR_SIGNALING	-1.654	0.081
NCI_SYNDECAN_3_PATHWAY	-1.647	0.085
REACT_INTEGRIN ALPHAIIB BETA3 SIGNALING	-1.646	0.085
WIP_HS_MITOCHONDRIAL_LC-FATTY_ACID_BETA-OXIDATION	-1.640	0.088
NCI_ERBB2ERBB3PATHWAY	-1.638	0.089
NCI_CXCR3PATHWAY	-1.637	0.089
REACT_AUTODEGRADATION OF THE E3 UBIQUITIN LIGASE COP1	-1.636	0.089
REACT_PEROXISOMAL LIPID METABOLISM	-1.634	0.089
REACT_UBIQUITIN MEDIATED DEGRADATION OF PHOSPHORYLATED CDC25A	-1.632	0.089
REACT_COSTIMULATION BY THE CD28 FAMILY	-1.632	0.088
REACT_STABILIZATION OF P53	-1.632	0.088
REACT_P53-INDEPENDENT G1_S DNA DAMAGE CHECKPOINT	-1.626	0.091
REACT_INTERLEUKIN-2 SIGNALING	-1.617	0.097
KEGG_NON-SMALL CELL LUNG CANCER	-1.612	0.100
BIOC_PTDINSPATHWAY	-1.611	0.100
NCI_VEGFR1_2_PATHWAY	-1.606	0.103
WIP_HS_METABOLISM_OF_CARBOHYDRATES	-1.606	0.102
KEGG_BETA-ALANINE METABOLISM	-1.606	0.101
WIP_HS_FOCAL_ADHESION	-1.597	0.108
KEGG_GLYCOSYLPHOSPHATIDYLINOSITOL(GPI)-ANCHOR BIOSYNTHESIS	-1.584	0.119

NCI_RET_PATHWAY	-1.570	0.132
KEGG_ENDOMETRIAL CANCER	-1.567	0.134
KEGG_ECM-RECEPTOR INTERACTION	-1.551	0.152
REACT_SYNTHESIS AND INTERCONVERSION OF NUCLEOTIDE DI- AND TRIPHOSPHATES	-1.551	0.151
REACT_AUTODEGRADATION OF CDH1 BY CDH1_APC_C	-1.547	0.153
REACT_SCF(SKP2)-MEDIATED DEGRADATION OF P27_P21	-1.546	0.153
KEGG_STEROID HORMONE BIOSYNTHESIS	-1.545	0.153
REACT_IRS-RELATED EVENTS	-1.543	0.154
NCI_INSULIN_GLUCOSE_PATHWAY	-1.539	0.158
REACT_IRS-MEDIATED SIGNALLING	-1.538	0.157
KEGG_FC EPSILON RI SIGNALING PATHWAY	-1.532	0.164
REACT_SHC-RELATED EVENTS	-1.527	0.168
BIOC_GLEEVECPATHWAY	-1.527	0.166
WIP_HS_FLUOROPYRIMIDINE_ACTIVITY	-1.524	0.169
REACT_MEMBRANE TRAFFICKING	-1.521	0.171
REACT_METABOLISM OF NUCLEOTIDES	-1.520	0.171
REACT_PYRUVATE METABOLISM	-1.519	0.171
REACT_INTEGRATION OF ENERGY METABOLISM	-1.519	0.170
REACT_CDT1 ASSOCIATION WITH THE CDC6_ORC_ORIGIN COMPLEX	-1.516	0.172
BIOC_CERAMIDEPATHWAY	-1.513	0.174
REACT_APC_C_CDH1 MEDIATED DEGRADATION OF CDC20 AND OTHER APC_C_CDH1 TARGETED PROTEINS IN LATE MITOSIS_EARLY G1	-1.510	0.177
KEGG_ARGININE AND PROLINE METABOLISM	-1.509	0.177
WIP_HS_IL-6_SIGNALING_PATHWAY	-1.505	0.180
REACT_POST-CHAPERONIN TUBULIN FOLDING PATHWAY	-1.504	0.181
REACT_SIGNALING BY EGFR	-1.502	0.182
WIP_HS_ERBB_SIGNALING_PATHWAY	-1.501	0.181
BIOC_MCALPAINPATHWAY	-1.485	0.203
REACT_APC_C_CDC20 MEDIATED DEGRADATION OF SECURIN	-1.480	0.210
REACT_P53-DEPENDENT G1_S DNA DAMAGE CHECKPOINT	-1.480	0.209
WIP_HS_INTRINSIC_PATHWAY_FOR_APOPTOSIS	-1.479	0.208
KEGG_FOCAL ADHESION	-1.478	0.207
REACT_DESTABILIZATION OF MRNA BY AUF1 (HNRNP D0)	-1.477	0.208
BIOC_IL6PATHWAY	-1.476	0.208
BIOC_ERKPATHWAY	-1.470	0.215
REACT_RNA POLYMERASE III TRANSCRIPTION INITIATION FROM TYPE 1 PROMOTER	-1.470	0.214
REACT_P53-DEPENDENT G1 DNA DAMAGE RESPONSE	-1.467	0.216
REACT_RNA POLYMERASE II TRANSCRIPTION INITIATION	-1.466	0.216
REACT_G1_S DNA DAMAGE CHECKPOINTS	-1.466	0.214
WIP_HS_SIGNALING_BY_INSULIN_RECEPTOR	-1.464	0.216
KEGG_LONG-TERM DEPRESSION	-1.462	0.216
REACT_ASSEMBLY OF THE PRE-REPLICATIVE COMPLEX	-1.462	0.216
WIP_HS_KIT_RECEPTOR_SIGNALING_PATHWAY	-1.458	0.220
NRF2_TARGETS	-1.455	0.223
REACT_GAP JUNCTION TRAFFICKING	-1.454	0.223
KEGG_STEROID BIOSYNTHESIS	-1.454	0.222
REACT_FORMATION OF PLATELET PLUG	-1.452	0.224
REACT_REGULATION OF APOPTOSIS	-1.451	0.222
REACT_HIV-1 TRANSCRIPTION INITIATION	-1.449	0.224
BIOC_SPPAPATHWAY	-1.443	0.232
NCI_KITPATHWAY	-1.443	0.232
REACT_RNA POLYMERASE II TRANSCRIPTION PRE-INITIATION AND PROMOTER OPENING	-1.439	0.236
BIOC_IL2PATHWAY	-1.437	0.237
WIP_HS_TRYPTOPHAN_METABOLISM	-1.437	0.236
WIP_HS_EUKARYOTIC_TRANSCRIPTION_INITIATION	-1.436	0.237
REACT_TIE2 SIGNALING	-1.435	0.236
BIOC_RASPATHWAY	-1.432	0.240
KEGG_ADIPOCYTOKINE SIGNALING PATHWAY	-1.432	0.238
KEGG_CARDIAC MUSCLE CONTRACTION	-1.431	0.238
REACT_RNA POLYMERASE II HIV-1 PROMOTER ESCAPE	-1.430	0.238
REACT_RNA POLYMERASE II PROMOTER ESCAPE	-1.430	0.237
REACT_INSULIN RECEPTOR SIGNALLING CASCADE	-1.429	0.236

REACT_NCAM SIGNALING FOR NEURITE OUT-GROWTH	-1.429	0.235
KEGG_PORPHYRIN AND CHLOROPHYLL METABOLISM	-1.426	0.239
REACT_PLATELET HOMEOSTASIS	-1.425	0.239
REACT_RNA POLYMERASE II TRANSCRIPTION INITIATION AND PROMOTER CLEARANCE	-1.424	0.239
REACT_SHC-MEDIATED CASCADE	-1.423	0.239
REACT_SIGNALING BY FGFR	-1.423	0.239
NCI_TOLL_ENDOGENOUS_PATHWAY	-1.421	0.239
NCI_ERBB1_RECEPTOR_PROXIMAL_PATHWAY	-1.421	0.239
NCI_MET_PATHWAY	-1.420	0.237
WIP_HS_REGULATION_OF_ACTIN_CYTOSKELETON	-1.415	0.245

Supplementary table 4c. Gene sets upregulated in white adipose tissue upon twelve weeks of NP-ER.

Gene set	NES	FDR q-value
REACT_PEPTIDE CHAIN ELONGATION	2.323	0.000
REACT_FORMATION OF A POOL OF FREE 40S SUBUNITS	2.302	0.000
KEGG_RIBOSOME	2.270	0.000
REACT_INFLUENZA VIRAL RNA TRANSCRIPTION AND REPLICATION	2.261	0.000
REACT_3 -UTR-MEDIATED TRANSLATIONAL REGULATION	2.261	0.000
REACT_VIRAL MRNA TRANSLATION	2.246	0.000
REACT_EUKARYOTIC TRANSLATION ELONGATION	2.240	0.000
REACT_L13A-MEDIATED TRANSLATIONAL SILENCING OF CERULOPLASMIN EXPRESSION	2.235	0.000
REACT_GTP HYDROLYSIS AND JOINING OF THE 60S RIBOSOMAL SUBUNIT	2.226	0.000
REACT_EUKARYOTIC TRANSLATION INITIATION	2.170	0.000
REACT_CAP-DEPENDENT TRANSLATION INITIATION	2.151	0.000
REACT_EUKARYOTIC TRANSLATION TERMINATION	2.139	0.000
REACT_TRANSLATION	2.095	0.001
WIP_HS_CYTOPLASMIC_RIBOSOMAL_PROTEINS	2.088	0.001
REACT_REGULATION OF GENE EXPRESSION IN BETA CELLS	2.069	0.002
REACT_NONSENSE MEDIATED DECAY INDEPENDENT OF THE EXON JUNCTION COMPLEX	2.048	0.002
REACT_REGULATION OF BETA-CELL DEVELOPMENT	2.037	0.002
WIP_HS_PROCESSING_OF_CAPPED_INTRON-CONTAINING_PRE-MRNA	2.008	0.003
REACT_MRNA SPLICING - MAJOR PATHWAY	1.989	0.003
REACT_PROCESSING OF CAPPED INTRON-CONTAINING PRE-MRNA	1.980	0.004
REACT_NONSENSE-MEDIATED DECAY	1.975	0.004
WIP_HS_MRNA_PROCESSING	1.966	0.004
REACT_NONSENSE MEDIATED DECAY ENHANCED BY THE EXON JUNCTION COMPLEX	1.951	0.005
REACT_MRNA SPLICING	1.946	0.005
REACT_MRNA PROCESSING	1.894	0.009
REACT_FORMATION OF THE TERNARY COMPLEX, AND SUBSEQUENTLY, THE 43S COMPLEX	1.864	0.013
KEGG_SPLICEOSOME	1.862	0.013
REACT_TRANSPORT OF MATURE MRNA DERIVED FROM AN I NTRON-CONTAINING TRANSCRIPT	1.819	0.020
REACT_TRANSPORT OF MATURE TRANSCRIPT TO CYTOPLASM	1.815	0.020
REACT_POST-ELONGATION PROCESSING OF INTRON-CONTAINING PRE-MRNA	1.810	0.021
REACT_RIBOSOMAL SCANNING AND START CODON RECOGNITION	1.805	0.021
REACT_MRNA 3-END PROCESSING	1.804	0.020
REACT_GENERIC TRANSCRIPTION PATHWAY	1.799	0.021
REACT_ACTIVATION OF THE MRNA UPON BINDING OF THE CAP-BINDING COMPLEX AND EIFS, AND SUBSEQUENT BINDING TO 43S	1.754	0.035
REACT_TRANSLATION INITIATION COMPLEX FORMATION	1.747	0.036
REACT_INSULIN SYNTHESIS AND PROCESSING	1.714	0.050
REACT_FORMATION AND MATURATION OF MRNA TRANSCRIPT	1.713	0.049
KEGG_ETHER LIPID METABOLISM	1.691	0.060
REACT_CLEAVAGE OF GROWING TRANSCRIPT IN THE TERMINATION REGION	1.674	0.069
REACT_RNA POLYMERASE II TRANSCRIPTION TERMINATION	1.664	0.073
REACT_POST-ELONGATION PROCESSING OF THE TRANSCRIPT	1.641	0.088
KEGG_ASTHMA	1.618	0.106
KEGG_DRUG METABOLISM - CYTOCHROME P450	1.556	0.173

REACT_COMPLEMENT CASCADE	1.555	0.170
BIOC_TIDPATHWAY	1.541	0.185
WIP_HS_STATIN_PATHWAY	1.526	0.204

Supplementary table 4d. Gene sets downregulated in white adipose tissue upon twelve weeks of NP-ER.

Gene set	NES	FDR q-value
NCI_TCR_PATHWAY	-2.641	0.000
NCI_CD8TCRPATHWAY	-2.633	0.000
REACT_RESPIRATORY ELECTRON TRANSPORT, ATP SYNTHESIS BY CHEMIOSMOTIC COUPLING, AND HEAT PRODUCTION BY UNCOUPLING PROTEINS.	-2.584	0.000
WIP_HS_OXIDATIVE_PHOSPHORYLATION	-2.499	0.000
KEGG_OXIDATIVE PHOSPHORYLATION	-2.436	0.000
REACT_RESPIRATORY ELECTRON TRANSPORT	-2.406	0.000
KEGG_PARKINSON'S DISEASE	-2.401	0.000
REACT_VIF-MEDIATED DEGRADATION OF APOBEC3G	-2.345	0.000
REACT_UBIQUITIN MEDIATED DEGRADATION OF PHOSPHORYLATED CDC25A	-2.325	0.000
REACT_P53-INDEPENDENT G1_S DNA DAMAGE CHECKPOINT	-2.305	0.000
KEGG_PROTEASOME	-2.303	0.000
REACT_P53-INDEPENDENT DNA DAMAGE RESPONSE	-2.290	0.000
WIP_HS_ELECTRON_TRANSPORT_CHAIN	-2.266	0.000
REACT_REGULATION OF ACTIVATED PAK-2P34 BY PROTEASOME MEDIATED DEGRADATION	-2.237	0.000
REACT_SIGNALING BY WNT	-2.229	0.000
WIP_HS_GLYCOLYSIS_AND_GLUCONEOGENESIS	-2.212	0.000
REACT_DEGRADATION OF BETA-CATENIN BY THE DESTRUCTION COMPLEX	-2.206	0.000
WIP_HS_T_CELL_RECEPTOR_SIGNALING_PATHWAY	-2.203	0.000
REACT_AUTODEGRADATION OF THE E3 UBIQUITIN LIGASE COP1	-2.192	0.000
REACT_UBIQUITIN-DEPENDENT DEGRADATION OF CYCLIN D	-2.165	0.001
REACT_UBIQUITIN-DEPENDENT DEGRADATION OF CYCLIN D1	-2.163	0.001
REACT_TRIGLYCERIDE BIOSYNTHESIS	-2.150	0.001
REACT_IMMUNOREGULATORY INTERACTIONS BETWEEN A LYMPHOID AND A NON-LYMPHOID CELL	-2.138	0.001
NRF2_TARGETS	-2.137	0.001
REACT_STABILIZATION OF P53	-2.122	0.002
KEGG_PYRUVATE METABOLISM	-2.110	0.002
KEGG_BIOSYNTHESIS OF UNSATURATED FATTY ACIDS	-2.105	0.002
NCI_GMCSF_PATHWAY	-2.090	0.003
REACT_GLUCOSE METABOLISM	-2.073	0.003
REACT_REGULATION OF ORNITHINE DECARBOXYLASE (ODC)	-2.065	0.003
REACT_VPU MEDIATED DEGRADATION OF CD4	-2.059	0.004
KEGG_ALZHEIMER'S DISEASE	-2.058	0.003
REACT_PLATELET SENSITIZATION BY LDL	-2.048	0.004
NCI_IL2_STAT5PATHWAY	-2.047	0.004
KEGG_B CELL RECEPTOR SIGNALING PATHWAY	-2.038	0.004
REACT_SCF-BETA-TRCP MEDIATED DEGRADATION OF EMI1	-2.038	0.004
WIP_HS_TCR_SIGNALING	-2.033	0.005
REACT_CDK-MEDIATED PHOSPHORYLATION AND REMOVAL OF CDC6	-2.030	0.005
REACT_APC_C_CDC20 MEDIATED DEGRADATION OF SECURIN	-2.026	0.005
WIP_HS_CHOLESTEROL_BIOSYNTHESIS	-2.024	0.005
BIOC_INTEGRINPATHWAY	-2.020	0.005
REACT_FATTY ACYL-COA BIOSYNTHESIS	-2.002	0.005
REACT_DESTABILIZATION OF MRNA BY AUF1 (HNRNP D0)	-1.998	0.006
REACT_APC_C_CDH1 MEDIATED DEGRADATION OF CDC20 AND OTHER APC_C_CDH1 TARGETED PROTEINS IN LATE MITOSIS_EARLY G1	-1.986	0.007
REACT_POST-CHAPERONIN TUBULIN FOLDING PATHWAY	-1.984	0.007
NCI_WNT_CANONICAL_PATHWAY	-1.969	0.009
KEGG_GLYCEROLIPID METABOLISM	-1.962	0.009
REACT_GLUCONEOGENESIS	-1.959	0.009
KEGG_T CELL RECEPTOR SIGNALING PATHWAY	-1.954	0.010

REACT_PLATELET ACTIVATION	-1.951	0.010
WIP_HS_WNT_SIGNALING_PATHWAY	-1.950	0.009
REACT_INTERLEUKIN-2 SIGNALING	-1.942	0.010
REACT_CDT1 ASSOCIATION WITH THE CDC6_ORC_ORIGIN COMPLEX	-1.938	0.010
REACT_G1_S DNA DAMAGE CHECKPOINTS	-1.937	0.010
WIP_HS_IL-3_SIGNALING_PATHWAY	-1.936	0.010
REACT_FORMATION OF PLATELET PLUG	-1.933	0.010
REACT_ACTIVATION OF APC_C AND APC_C_CDC20 MEDIATED DEGRADATION OF MITOTIC PROTEINS	-1.932	0.010
REACT_CDC20_PHOSPHO-APC_C MEDIATED DEGRADATION OF CYCLIN A	-1.929	0.011
WIP_HS_GLYCOGEN_METABOLISM	-1.924	0.011
KEGG_PENTOSE PHOSPHATE PATHWAY	-1.921	0.011
REACT_INTERLEUKIN-3, 5 AND GM-CSF SIGNALING	-1.921	0.011
REACT_AMYLOIDS	-1.918	0.011
NCI_CD8TCRDOWNSTREAMPATHWAY	-1.917	0.011
REACT_APC_C_CDC20 MEDIATED DEGRADATION OF MITOTIC PROTEINS	-1.916	0.011
REACT_AUTODEGRADATION OF CDH1 BY CDH1_APC_C	-1.911	0.011
REACT_P53-DEPENDENT G1 DNA DAMAGE RESPONSE	-1.906	0.012
KEGG_PRIMARY IMMUNODEFICIENCY	-1.900	0.012
REACT_SCF(SKP2)-MEDIATED DEGRADATION OF P27_P21	-1.899	0.012
REACT_DNA REPLICATION	-1.899	0.012
REACT_P53-DEPENDENT G1_S DNA DAMAGE CHECKPOINT	-1.893	0.012
REACT_TCR SIGNALING	-1.889	0.013
KEGG_GLYCOLYSIS _ GLUCONEOGENESIS	-1.888	0.013
REACT_GLYCOLYSIS	-1.886	0.013
REACT_CYCLIN A_CDK2-ASSOCIATED EVENTS AT S PHASE ENTRY	-1.876	0.014
WIP_HS_IL-2_SIGNALING_PATHWAY	-1.876	0.014
NCI_KITPATHWAY	-1.865	0.015
WIP_HS_PROTEASOME_DEGRADATION	-1.862	0.015
WIP_HS_FATTY_ACID_BIOSYNTHESIS	-1.860	0.015
WIP_HS_IL-6_SIGNALING_PATHWAY	-1.859	0.015
NCI_BCR_5PATHWAY	-1.854	0.016
WIP_HS_B_CELL_RECEPTOR_SIGNALING_PATHWAY	-1.853	0.016
REACT_CYCLIN E ASSOCIATED EVENTS DURING G1_STRANSITION	-1.851	0.016
WIP_HS_FATTY_ACID,_TRIACYLGLYCEROL,_AND_KETONE_BODY_METABOLISM	-1.851	0.016
NCI_INSULIN_PATHWAY	-1.844	0.017
PPARA_TARGETS	-1.844	0.017
REACT_MITOTIC M-M_G1 PHASES	-1.843	0.017
REACT_SIGNALING BY INTERLEUKINS	-1.843	0.017
NCI_FCER1PATHWAY	-1.832	0.018
WIP_HS_NEURORANSMITTER_RECEPTOR_BINDING_AND_ DOWNSTREAM_TRANSMISSION_IN_THE_POSTSYNAPTIC_CELL	-1.829	0.019
KEGG_FC GAMMA R-MEDIATED PHAGOCYTOSIS	-1.828	0.019
NCI_REELINPATHWAY	-1.826	0.019
NCI_INSULIN_GLUCOSE_PATHWAY	-1.822	0.019
REACT_ADAPTIVE IMMUNITY SIGNALING	-1.813	0.021
REACT_INTEGRIN ALPHAIIB BETA3 SIGNALING	-1.810	0.022
NCI_INTEGRIN5_PATHWAY	-1.805	0.022
NCI_IL8CXCR2_PATHWAY	-1.804	0.022
KEGG_REGULATION OF ACTIN CYTOSKELETON	-1.803	0.022
NCI_CXCR4_PATHWAY	-1.801	0.023
WIP_HS_KEAP1-NRF2_PATHWAY	-1.797	0.023
WIP_HS_TRIACYLGLYCERIDE_SYNTHESIS	-1.790	0.024
REACT_CELL-EXTRACELLULAR MATRIX INTERACTIONS	-1.783	0.026
REACT_METABOLISM OF CARBOHYDRATES	-1.779	0.027
NCI_LYMPHANGIOGENESIS_PATHWAY	-1.779	0.026
REACT_INTEGRIN CELL SURFACE INTERACTIONS	-1.772	0.028
WIP_HS_FOCAL_ADHESION	-1.769	0.028
KEGG_FATTY ACID ELONGATION	-1.764	0.030
BIOC_GSK3PATHWAY	-1.764	0.029
BIOC_GLEEVECPATHWAY	-1.764	0.029
KEGG_FOCAL ADHESION	-1.756	0.031

REACT_REGULATION OF APOPTOSIS	-1.755	0.031
REACT_CHOLESTEROL BIOSYNTHESIS	-1.753	0.031
REACT_HEMOSTASIS	-1.738	0.035
WIP_HS_CELL_JUNCTION_ORGANIZATION	-1.737	0.035
NCI_PI3KPLCTRKPATHWAY	-1.737	0.035
BIOC_PROTEASOMEPATHWAY	-1.736	0.035
NCI_PTP1BPATHWAY	-1.733	0.036
REACT_MITOTIC G1-G1_S PHASES	-1.732	0.036
REACT_CYCLIN D ASSOCIATED EVENTS IN G1	-1.729	0.036
REACT_ASSEMBLY OFTHE PRE-REPLICATIVE COMPLEX	-1.725	0.037
REACT_EFFECTS OF PIP2 HYDROLYSIS	-1.724	0.037
REACT_MITOTIC PROMETAPHASE	-1.723	0.037
BIOC_IL2PATHWAY	-1.721	0.037
REACT_M PHASE	-1.720	0.037
REACT_CELL CYCLE, MITOTIC	-1.711	0.040
REACT_G1 PHASE	-1.711	0.040
REACT_NUCLEOTIDE-LIKE (PURINERGIC) RECEPTORS	-1.705	0.041
KEGG_NATURAL KILLER CELL MEDIATED CYTOTOXICITY	-1.701	0.043
KEGG_CITRATE CYCLE (TCA CYCLE)	-1.699	0.043
KEGG_STARCH AND SUCROSE METABOLISM	-1.699	0.043
WIP_HS_GPCR_LIGAND_BINDING	-1.690	0.045
BIOC_IL2RBPATHWAY	-1.688	0.046
BIOC_IGF1RPATHWAY	-1.687	0.046
REACT_PLATELET AGGREGATION (PLUG FORMATION)	-1.685	0.046
REACT_NUCLEOSOME ASSEMBLY	-1.685	0.046
REACT_CTLA4 INHIBITORY SIGNALING	-1.682	0.047
REACT_DEPOSITION OF NEW CENPA-CONTAINING NUCLEOSOMES AT THE CENTROMERE	-1.681	0.047
REACT_GPVI-MEDIATED ACTIVATION CASCADE	-1.676	0.049
BIOC_CELLCYCLEPATHWAY	-1.673	0.050
REACT_REGULATION OF SIGNALING BY CBL	-1.668	0.051
REACT_INTERLEUKIN RECEPTOR SHC SIGNALING	-1.667	0.051
REACT_NUCLEOTIDE-BINDING DOMAIN, LEUCINE RICH REPEAT CONTAINING RECEPTOR (NLR) SIGNALING PATHWAYS	-1.667	0.051
KEGG_PATHOGENIC ESCHERICHIA COLI INFECTION	-1.663	0.052
KEGG_CHEMOKINE SIGNALING PATHWAY	-1.659	0.053
KEGG_HEMATOPOIETIC CELL LINEAGE	-1.651	0.057
REACT_FORMATION OFTUBULIN FOLDING INTERMEDIATES BY CCT_TRIC	-1.648	0.057
WIP_HS_WNT_SIGNALING_PATHWAY_AND_PLURIPOTENCY	-1.645	0.058
BIOC_NKCELLSPATHWAY	-1.644	0.059
BIOC_BCRPATHWAY	-1.643	0.058
REACT_SYNTHESIS OF DNA	-1.639	0.060
KEGG_PROPANOATE METABOLISM	-1.639	0.060
REACT_PLATELET ACTIVATIONTRIGGERS	-1.636	0.061
REACT_G1_STRANSITION	-1.633	0.061
WIP_HS_INTEGRATION_OF_ENERGY_METABOLISM	-1.630	0.062
NCI_IL12_STAT4PATHWAY	-1.629	0.062
KEGG_ACUTE MYELOID LEUKEMIA	-1.627	0.063
WIP_HS_IL-7_SIGNALING_PATHWAY	-1.623	0.065
KEGG_AMOEBIASIS	-1.621	0.065
BIOC_PYK2PATHWAY	-1.620	0.065
BIOC_SPPAPATHWAY	-1.619	0.065
NCI_AVB3_INTEGRIN_PATHWAY	-1.617	0.066
WIP_HS_G13_SIGNALING_PATHWAY	-1.613	0.068
BIOC_FCER1PATHWAY	-1.611	0.069
REACT_REGULATION OF APC_C ACTIVATORS BETWEEN G1_S AND EARLY ANAPHASE	-1.610	0.068
REACT_HOST INTERACTIONS OF HIV FACTORS	-1.607	0.070
REACT_SWITCHING OF ORIGINSTO A POST-REPLICATIVE STATE	-1.606	0.070
NCI_IL8CXCR1_PATHWAY	-1.604	0.070
REACT_RAP1 SIGNALLING	-1.600	0.072
REACT_CITRIC ACID CYCLE (TCA CYCLE)	-1.600	0.072
REACT_ORC1 REMOVAL FROM CHROMATIN	-1.599	0.072
KEGG_LEUKOCYTETRANSENDOTHELIAL MIGRATION	-1.598	0.072

REACT_REMOVAL OF LICENSING FACTORS FROM ORIGINS	-1.598	0.071
NCI_TOLL_ENDOGENOUS_PATHWAY	-1.594	0.073
REACT_CHAPERONIN-MEDIATED PROTEIN FOLDING	-1.594	0.073
KEGG_FC EPSILON RI SIGNALING PATHWAY	-1.590	0.075
REACT_REGULATION OF DNA REPLICATION	-1.589	0.075
REACT_REGULATION OF MRNA STABILITY BY PROTEINS THAT BIND AU-RICH ELEMENTS	-1.587	0.075
REACT_APC_C-MEDIATED DEGRADATION OF CELL CYCLE PROTEINS	-1.587	0.075
REACT_S PHASE	-1.586	0.075
KEGG_ECM-RECEPTOR INTERACTION	-1.584	0.076
REACT_PROTEIN FOLDING	-1.581	0.077
WIP_HS_METABOLISM_OF_WATER-SOLUBLE_VITAMINS_AND_COFACTORS	-1.581	0.076
REACT_REGULATION OF MITOTIC CELL CYCLE	-1.580	0.077
NCI_IL2_PI3KPATHWAY	-1.576	0.078
REACT_GENERATION OF SECOND MESSENGER MOLECULES	-1.572	0.080
BIOC_AMIPATHWAY	-1.566	0.083
KEGG_PORPHYRIN AND CHLOROPHYLL METABOLISM	-1.564	0.084
NCI_IL12_2PATHWAY	-1.557	0.088
WIP_HS_ESTROGEN_SIGNALING_PATHWAY	-1.554	0.089
WIP_HS_INFLAMMATORY_RESPONSE_PATHWAY	-1.552	0.090
REACT_PLATELET HOMEOSTASIS	-1.547	0.092
REACT_M_G1 TRANSITION	-1.545	0.093
WIP_HS_KIT_RECEPTOR_SIGNALING_PATHWAY	-1.543	0.094
NCI_INTEGRIN_CS_PATHWAY	-1.538	0.097
WIP_HS_INTERFERON_ALPHA-BETA_SIGNALING	-1.536	0.098
REACT_DNA REPLICATION PRE-INITIATION	-1.535	0.098
REACT_CELL JUNCTION ORGANIZATION	-1.535	0.098
BIOC_CSKPATHWAY	-1.535	0.098
REACT_DARPP-32 EVENTS	-1.534	0.098
WIP_HS_MAPK_CASCADE	-1.531	0.099
REACT_PYRUVATE METABOLISM AND CITRIC ACID (TCA) CYCLE	-1.531	0.099
REACT_COSTIMULATION BY THE CD28 FAMILY	-1.530	0.099
KEGG_HUNTINGTON'S DISEASE	-1.526	0.102
WIP_HS_IL-5_SIGNALING_PATHWAY	-1.525	0.101
NCI_VEGFR1_2_PATHWAY	-1.524	0.101
NCI_PI3KCIPATHWAY	-1.524	0.102
REACT_PACKAGING OFTELOMERE ENDS	-1.520	0.103
WIP_HS_NOD_PATHWAY	-1.519	0.104
KEGG_NON-SMALL CELL LUNG CANCER	-1.516	0.106
REACT_PLATELET DEGRANULATION	-1.509	0.111
NCI_CXCR3PATHWAY	-1.508	0.111
REACT_KINESINS	-1.500	0.116
REACT_RECRUITMENT OF NUMA TO MITOTIC CENTROSOMES	-1.500	0.115
REACT_NOD1_2 SIGNALING PATHWAY	-1.498	0.116
WIP_HS_INTEGRIN-MEDIATED_CELL_ADHESION	-1.498	0.116
NCI_NFAT_3PATHWAY	-1.497	0.116
WIP_HS_METABOLISM_OF_CARBOHYDRATES	-1.497	0.116
WIP_HS_TRANSPORT_OF_VITAMINS_NUCLEOSIDES_AND_RELATED_MOLECULES	-1.497	0.115
REACT_GAP JUNCTION ASSEMBLY	-1.495	0.116
REACT_RESPONSE TO ELEVATED PLATELET CYTOSOLIC CA2+	-1.492	0.118
WIP_HS_REGULATION_OF_ACTIN_CYTOSKELETON	-1.491	0.119
NCI_INTEGRIN1_PATHWAY	-1.489	0.119
REACT_INFLAMMASOMES	-1.488	0.120
WIP_HS_TCA_CYCLE	-1.487	0.120
NCI_PDGFRBPATHWAY	-1.483	0.123
WIP_HS_GLUTATHIONE_METABOLISM	-1.481	0.125
REACT_CLASS A_1 (RHODOPSIN-LIKE RECEPTORS)	-1.480	0.125
BIOC_INSULINPATHWAY	-1.475	0.128
REACT_DOWNSTREAM TCR SIGNALING	-1.475	0.128
KEGG_SMALL CELL LUNG CANCER	-1.471	0.130
REACT_TELOMERE MAINTENANCE	-1.470	0.131
NCI_ALPHASYNUCLEIN_PATHWAY	-1.467	0.132
NCI_SYNDECAN_1_PATHWAY	-1.461	0.138

NCI_TCRCALCIUMPATHWAY	-1.461	0.137
BIOC_IL12PATHWAY	-1.456	0.141
NCI_HEDGEHOG_GLIPATHWAY	-1.453	0.143
BIOC_TCRPATHWAY	-1.452	0.143
BIOC_HCMVPATHWAY	-1.452	0.143
KEGG_PANTOTHENATE AND COA BIOSYNTHESIS	-1.451	0.142
KEGG_OSTEOCLAST DIFFERENTIATION	-1.451	0.142
WIP_HS_INSULIN_SIGNALING	-1.448	0.144
REACT_CHEMOKINE RECEPTORS BIND CHEMOKINES	-1.445	0.146
REACT_COOPERATION OF PREFOLDIN AND TRIC_CCT IN ACTIN AND TUBULIN FOLDING	-1.445	0.146
NCI_IGF1_PATHWAY	-1.443	0.147
KEGG_ENDOMETRIAL CANCER	-1.443	0.146
WIP_HS_FATTY_ACID_BETA_OXIDATION	-1.442	0.146
REACT_PREFOLDIN MEDIATED TRANSFER OF SUBSTRATE TO CCT_TRIC	-1.440	0.147
WIP_HS_NICOTINE_ACTIVITY_ON_DOPAMINERGIC_NEURONS	-1.440	0.147
REACT_CHROMOSOME MAINTENANCE	-1.438	0.148
REACT_G ALPHA (I) SIGNALLING EVENTS	-1.438	0.148
REACT_G ALPHA (12_13) SIGNALLING EVENTS	-1.433	0.152
BIOC_NGFPATHWAY	-1.432	0.153
KEGG_SNARE INTERACTIONS IN VESICULAR TRANSPORT	-1.429	0.154
KEGG_BETA-ALANINE METABOLISM	-1.427	0.155
REACT_METABOLISM OF LIPIDS AND LIPOPROTEINS	-1.426	0.156
WIP_HS_GPCRS,_CLASS_A_RHODOPSIN-LIKE	-1.426	0.156
REACT_SYNTHESIS AND INTERCONVERSION OF NUCLEOTIDE DI- AND TRIPHOSPHATES	-1.421	0.159
BIOC_WNTPATHWAY	-1.419	0.161
KEGG_HTLV-I INFECTION	-1.410	0.169
KEGG_BASAL CELL CARCINOMA	-1.409	0.170
REACT_GAP JUNCTION TRAFFICKING	-1.407	0.172
NCI_TXA2PATHWAY	-1.406	0.171
REACT_G BETA_GAMMA SIGNALLING THROUGH PI3KGAMMA	-1.405	0.172
REACT_CD28 CO-STIMULATION	-1.404	0.173
REACT_GPCR LIGAND BINDING	-1.404	0.172
REACT_CYTOKINE SIGNALING IN IMMUNE SYSTEM	-1.404	0.171
KEGG_NOD-LIKE RECEPTOR SIGNALING PATHWAY	-1.403	0.171
REACT_FATTY ACID, TRIACYLGLYCEROL, AND KETONE BODY METABOLISM	-1.401	0.172
NCI_LIS1PATHWAY	-1.400	0.174
REACT_METABOLISM OF AMINO ACIDS AND DERIVATIVES	-1.399	0.174
REACT_MEIOTIC RECOMBINATION	-1.397	0.175
KEGG_MEASLES	-1.395	0.177
REACT_APOPTOSIS	-1.394	0.177
KEGG_GAP JUNCTION	-1.391	0.179
KEGG_COLLECTING DUCT ACID SECRETION	-1.391	0.179
REACT_HIV INFECTION	-1.390	0.179
BIOC_PTDINSPATHWAY	-1.389	0.180
NCI_ILK_PATHWAY	-1.389	0.179
WIP_HS_AMPK_SIGNALING	-1.389	0.179
WIP_HS_CELL_SURFACE_INTERACTIONS_AT_THE_VASCULAR_WALL	-1.386	0.182
REACT_SIGNALING BY GPCR	-1.384	0.182
NCI_INTEGRIN_A9B1_PATHWAY	-1.379	0.187
BIOC_G1PATHWAY	-1.374	0.193
NCI_VEGFR1_PATHWAY	-1.372	0.194
NCI_FASPATHWAY	-1.370	0.195
REACT_CELL SURFACE INTERACTIONS AT THE VASCULAR WALL	-1.370	0.195
NCI_AMB2_NEUTROPHILS_PATHWAY	-1.369	0.195
KEGG_INSULIN SIGNALING PATHWAY	-1.367	0.197
KEGG_MUCIN TYPE O-GLYCAN BIOSYNTHESIS	-1.367	0.197
REACT_PHOSPHORYLATION OF CD3 AND TCR ZETA CHAINS	-1.366	0.196
REACT_THE ROLE OF NEF IN HIV-1 REPLICATION AND DISEASE PATHOGENESIS	-1.365	0.197
NCI_MYC_REPRESSPATHWAY	-1.357	0.206
REACT_SIGNALING BY PDGF	-1.357	0.206
REACT_SIGNAL AMPLIFICATION	-1.355	0.207
NCI_IL2_1PATHWAY	-1.353	0.209

REACT_GPCR DOWNSTREAM SIGNALING	-1.353	0.208
KEGG_PROTEIN DIGESTION AND ABSORPTION	-1.351	0.210
BIOC_IL7PATHWAY	-1.347	0.214
WIP_HS_TGF_BETA_SIGNALING_PATHWAY	-1.347	0.214
BIOC_SPRYPATHWAY	-1.346	0.214
KEGG_GLYCOSYLPHOSPHATIDYLINOSITOL(GPI)-ANCHOR BIOSYNTHESIS	-1.346	0.213
NCI_P38ALPHABETAPATHWAY	-1.345	0.214
REACT_INTERACTIONS OFTHE IMMUNOGLOBULIN SUPERFAMILY (IGSF) MEMBER PROTEINS	-1.344	0.215
REACT_SIGNALING BY EGFR	-1.341	0.217
NCI_SYNDECAN_2_PATHWAY	-1.341	0.217
WIP_HS_IL-4_SIGNALING_PATHWAY	-1.340	0.217
WIP_HS_METABOLISM_OF_AMINO_ACIDS_AND_DERIVATIVES	-1.336	0.221
NCI_THROMBIN_PAR1_PATHWAY	-1.336	0.221
NCI_E2F_PATHWAY	-1.335	0.222
REACT_CLASS I MHC MEDIATED ANTIGEN PROCESSING & PRESENTATION	-1.334	0.223
REACT_G-PROTEIN BETA_GAMMA SIGNALLING	-1.332	0.223
REACT_CELL CYCLE CHECKPOINTS	-1.327	0.230
WIP_HS_TRANSPORT_OF_INORGANIC_CATIONS-ANIONS_AND_ AMINO_ACIDS-OLIGOPEPTIDES	-1.326	0.231
NCI_MET_PATHWAY	-1.320	0.237
BIOC_IGF1PATHWAY	-1.320	0.237
KEGG_JAK-STAT SIGNALING PATHWAY	-1.319	0.237
KEGG_CHAGAS DISEASE (AMERICAN TRYPANOSOMIASIS)	-1.316	0.241
KEGG_GASTRIC ACID SECRETION	-1.315	0.241
WIP_HS_DNA_DAMAGE_RESPONSE_(ONLY_ATM_DEPENDENT)	-1.315	0.241
NCI_EPOPATHWAY	-1.314	0.241
NCI_DELTANP63PATHWAY	-1.313	0.241
REACT_PEPTIDE LIGAND-BINDING RECEPTORS	-1.311	0.243
KEGG_GLYCINE, SERINE AND THREONINE METABOLISM	-1.309	0.246
REACT_ANTIGEN PROCESSING_ UBIQUITINATION & PROTEASOME DEGRADATION	-1.306	0.249
NCI_PI3KCIAKTPATHWAY	-1.306	0.249
REACT_PYRUVATE METABOLISM	-1.306	0.248

Supplementary table 5. Gene Set Enrichment Analysis. Gene sets differently regulated in white adipose tissue upon twelve weeks of HP-ER and NP-ER. Cut-off value FDR q-value 0.25.

Gene set	**NES**	**FDR q-value**
REACT_IMMUNOREGULATORY INTERACTIONS BETWEEN A LYMPHOID AND A NON-LYMPHOID CELL	-2.374	0.000
KEGG_HEMATOPOIETIC CELL LINEAGE	-2.314	0.002
REACT_INFLAMMASOMES	-2.236	0.004
NCI_CD8TCRPATHWAY	-2.217	0.005
WIP_HS_TCR_SIGNALING	-2.208	0.005
NCI_TCR_PATHWAY	-2.134	0.010
KEGG_PRIMARY IMMUNODEFICIENCY	-2.068	0.019
REACT_TCR SIGNALING	-2.064	0.016
BIOC_LAIRPATHWAY	-2.005	0.033
REACT_AMYLOIDS	-1.995	0.031
REACT_CELL SURFACE INTERACTIONS AT THE VASCULAR WALL	-1.972	0.038
REACT_TELOMERE MAINTENANCE	-1.971	0.034
REACT_CHROMOSOME MAINTENANCE	-1.967	0.034
NCI_IL2_STAT5PATHWAY	-1.943	0.039
KEGG_NATURAL KILLER CELL MEDIATED CYTOTOXICITY	-1.914	0.049
REACT_GPVI-MEDIATED ACTIVATION CASCADE	-1.909	0.048
BIOC_RAC1PATHWAY	-1.906	0.047
KEGG_LEUKOCYTE TRANSENDOTHELIAL MIGRATION	-1.904	0.045
REACT_OLFACTORY SIGNALING PATHWAY	-1.903	0.043
REACT_DEPOSITION OF NEW CENPA-CONTAINING NUCLEOSOMES AT THE CENTROMERE	-1.902	0.041
NCI_IL12_2PATHWAY	-1.871	0.053

WIP_HS_INFLAMMATORY_RESPONSE_PATHWAY	-1.853	0.061
REACT_NUCLEOSOME ASSEMBLY	-1.852	0.058
WIP_HS_T_CELL_RECEPTOR_SIGNALING_PATHWAY	-1.843	0.062
WIP_HS_GPCR_LIGAND_BINDING	-1.823	0.071
NCI_LYMPHANGIOGENESIS_PATHWAY	-1.821	0.070
REACT_DNA REPLICATION	-1.821	0.067
BIOC_AMIPATHWAY	-1.817	0.067
NCI_CD8TCRDOWNSTREAMPATHWAY	-1.815	0.066
REACT_GENERATION OF SECOND MESSENGER MOLECULES	-1.812	0.065
BIOC_IL12PATHWAY	-1.809	0.065
BIOC_CSKPATHWAY	-1.806	0.065
REACT_TELOMERE C-STRAND (LAGGING STRAND) SYNTHESIS	-1.805	0.063
NCI_IL8CXCR1_PATHWAY	-1.794	0.068
REACT_DNA STRAND ELONGATION	-1.785	0.071
NCI_TCRCALCIUMPATHWAY	-1.776	0.074
REACT_CYCLIN D ASSOCIATED EVENTS IN G1	-1.767	0.078
WIP_HS_G1_TO_S_CELL_CYCLE_CONTROL	-1.756	0.084
REACT_G1 PHASE	-1.750	0.086
WIP_HS_CELL_CYCLE	-1.748	0.085
REACT_EXTENSION OFTELOMERES	-1.744	0.085
NCI_CXCR4_PATHWAY	-1.737	0.088
NCI_E2F_PATHWAY	-1.732	0.089
BIOC_CELLCYCLEPATHWAY	-1.726	0.092
WIP_HS_CELL_SURFACE_INTERACTIONS_AT_THE_VASCULAR_WALL	-1.722	0.094
REACT_INTEGRIN CELL SURFACE INTERACTIONS	-1.719	0.094
REACT_MEIOTIC RECOMBINATION	-1.716	0.095
NCI_IL8CXCR2_PATHWAY	-1.710	0.098
REACT_MEIOTIC SYNAPSIS	-1.708	0.097
NCI_INTEGRIN2_PATHWAY	-1.708	0.096
KEGG_B CELL RECEPTOR SIGNALING PATHWAY	-1.700	0.099
KEGG_OSTEOCLAST DIFFERENTIATION	-1.698	0.099
REACT_PACKAGING OFTELOMERE ENDS	-1.697	0.098
BIOC_IL7PATHWAY	-1.695	0.098
KEGG_CHAGAS DISEASE (AMERICAN TRYPANOSOMIASIS)	-1.687	0.101
REACT_LAGGING STRAND SYNTHESIS	-1.682	0.104
KEGG_OLFACTORY TRANSDUCTION	-1.681	0.102
REACT_NUCLEOTIDE-BINDING DOMAIN, LEUCINE RICH REPEAT CONTAINING RECEPTOR (NLR) SIGNALING PATHWAYS	-1.676	0.105
WIP_HS_MIRNAS_INVOLVED_IN_DDR	-1.666	0.111
NCI_AMB2_NEUTROPHILS_PATHWAY	-1.666	0.110
REACT_DOWNSTREAM TCR SIGNALING	-1.665	0.108
KEGG_FC GAMMA R-MEDIATED PHAGOCYTOSIS	-1.655	0.114
WIP_HS_B_CELL_RECEPTOR_SIGNALING_PATHWAY	-1.650	0.117
WIP_HS_NOD_PATHWAY	-1.646	0.119
KEGG_T CELL RECEPTOR SIGNALING PATHWAY	-1.639	0.122
WIP_HS_GPCRS,_CLASS_A_RHODOPSIN-LIKE	-1.631	0.128
NCI_MYC_REPRESSPATHWAY	-1.629	0.128
REACT_SYNTHESIS OF DNA	-1.629	0.126
WIP_HS_PEPTIDE_GPCRS	-1.628	0.125
WIP_HS_G13_SIGNALING_PATHWAY	-1.625	0.125
REACT_ADAPTIVE IMMUNITY SIGNALING	-1.622	0.126
REACT_NUCLEOTIDE-LIKE (PURINERGIC) RECEPTORS	-1.621	0.126
WIP_HS_GPCRS,_OTHER	-1.617	0.128
REACT_PLATELET ACTIVATION TRIGGERS	-1.617	0.126
BIOC_FCER1PATHWAY	-1.613	0.129
REACT_S PHASE	-1.608	0.131
REACT_MITOTIC M-M_G1 PHASES	-1.603	0.134
REACT_CELL CYCLE, MITOTIC	-1.601	0.135
KEGG_NOD-LIKE RECEPTOR SIGNALING PATHWAY	-1.600	0.134
REACT_HEMOSTASIS	-1.597	0.135
BIOC_NKCELLSPATHWAY	-1.597	0.133
NCI_BCR_5PATHWAY	-1.587	0.141

NCI_PI3KCIPATHWAY	-1.581	0.145
NCI_INTEGRIN3_PATHWAY	-1.575	0.149
REACT_RAP1 SIGNALLING	-1.574	0.148
REACT_GPCR DOWNSTREAM SIGNALING	-1.569	0.152
NCI_IL12_STAT4PATHWAY	-1.567	0.152
REACT_INTERLEUKIN-3, 5 AND GM-CSF SIGNALING	-1.563	0.154
KEGG_SMALL CELL LUNG CANCER	-1.562	0.154
BIOC_CASPASEPATHWAY	-1.561	0.153
BIOC_WNTPATHWAY	-1.557	0.155
REACT_PHOSPHORYLATION OF CD3 AND TCR ZETA CHAINS	-1.554	0.157
WIP_HS_GENERIC_TRANSCRIPTION_PATHWAY	-1.554	0.155
BIOC_TCRPATHWAY	-1.553	0.155
REACT_M PHASE	-1.551	0.155
REACT_REGULATION OF SIGNALING BY CBL	-1.550	0.155
BIOC_NTHIPATHWAY	-1.550	0.153
KEGG_NITROGEN METABOLISM	-1.546	0.155
REACT_SIGNALING BY GPCR	-1.527	0.175
WIP_HS_TGF_BETA_SIGNALING_PATHWAY	-1.523	0.179
REACT_MITOTIC PROMETAPHASE	-1.521	0.179
REACT_TRANSPORT TO THE GOLGI AND SUBSEQUENT MODIFICATION	-1.511	0.189
NCI_P38ALPHABETAPATHWAY	-1.511	0.188
WIP_HS_EICOSANOID_SYNTHESIS	-1.510	0.187
REACT_PLATELET ACTIVATION	-1.504	0.193
NCI_NFAT_TFPATHWAY	-1.497	0.200
REACT_E2F MEDIATED REGULATION OF DNA REPLICATION	-1.487	0.211
NCI_EPHRINBREVPATHWAY	-1.478	0.221
NCI_AP1_PATHWAY	-1.475	0.224
KEGG_AMOEBIASIS	-1.475	0.222
REACT_SIGNALING BY INTERLEUKINS	-1.473	0.223
KEGG_MEASLES	-1.467	0.230
REACT_TRANSLOCATION OF ZAP-70 TO IMMUNOLOGICAL SYNAPSE	-1.466	0.229
BIOC_NKTPATHWAY	-1.465	0.228
NCI_IL4_2PATHWAY	-1.464	0.228
REACT_EFFECTS OF PIP2 HYDROLYSIS	-1.461	0.230
KEGG_HTLV-I INFECTION	-1.456	0.235
BIOC_P38MAPKPATHWAY	-1.453	0.238
REACT_G1_S DNA DAMAGE CHECKPOINTS	-1.453	0.236
REACT_CELL-EXTRACELLULAR MATRIX INTERACTIONS	-1.447	0.242
KEGG_REGULATION OF ACTIN CYTOSKELETON	-1.443	0.248
BIOC_RACCYCDPATHWAY	-1.443	0.246
REACT_GLUCOSE TRANSPORT	-1.440	0.247

3

Chapter 4 |

Measuring phenotypic flexibility by using transcriptome time course analyses during challenge tests before and after energy restriction

4

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In preparation

Abstract

Background: Nutritional challenge tests may be valuable to magnify the efficacy of dietary interventions on health outcomes by measuring the body's phenotypic flexibility, especially in combination with comprehensive tools such as transcriptomics. A dietary intervention well-known to improve health through weight loss is energy restriction (ER). ER is therefore a good model to examine the potential of challenge tests in combination with transcriptomics to magnify diet-induced effects on health.

Objective: To investigate if the use of challenge tests magnified the effects of ER on health, we measured whole genome gene expression in peripheral blood mononuclear cells (PBMCs) in the fasted state, and during an oral glucose tolerance test (OGTT) and a mixed meal test (MMT), both before and after 12 weeks of 20% ER.

Methods: 72 healthy, overweight men and women, aged 50-65, were subjected to an OGTT and an MMT, before and after 12 weeks of a 20% ER diet or control diet. Whole genome gene expression was performed in the fasted state, during the OGTT at 30, 60, and 120 min and during the MMT at 60, 120, 240, and 360 min before and after the intervention resulting in a total of 1231 microarrays.

Results & Conclusions: In the fasted state, ER induced gene sets involved in oxidative phosphorylation (OXPHOS), cell adhesion, energy metabolism, immune system, and cell cycle. During the OGTT, ER consistently downregulated gene sets involved in OXPHOS, cell adhesion, and DNA replication. The MMT did not show consistent effects at all time points. ER increased phenotypic flexibility as reflected by a faster and more pronounced downregulation of OXPHOS, cell adhesion, and DNA replication during the OGTT. The OGTT combined with transcriptomics can be used to measure increased phenotypic flexibility in an ER-intervention which cannot be determined with a static measurement in the fasted state. Translation of this finding to ER-related health outcomes needs further investigation.

Introduction

Nutrition research is facing several challenges since effects of nutrition on health are subtle, show high interindividual variations in response, and can take decades before they become visual. Recently, the definition of health has been redefined as an organism's ability to adapt and implement control in light of physical, emotional, and social challenges encountered in life [1]. This ability to adapt to challenges can also be extended to metabolic health, and in this metabolic context the ability to adapt to challenges has been named 'phenotypic flexibility' [2].

To measure phenotypic flexibility, a nutritional stressor is needed to stress the metabolic system. Nutritional challenge tests can act as a stressor for cells, tissues and organs as they provide a high load of specific nutrients, requiring an adequate response and adaptation in their metabolism. The measurement of this adaptation response may provide us with knowledge on the flexibility of the cells and tissues and hence the health state. Therefore, nutritional challenge tests are hypothesised to be a valuable tool to magnify the health effects of dietary interventions making it possible to detect changes in health even before these changes are visible in the fasted state.

One well-known nutritional challenge test is the oral glucose tolerance test (OGTT). An OGTT consists of a high load of glucose and measures how well cells are able to clear this glucose from the blood. OGTTs are widely applied in the clinic to diagnose insulin resistance in participants by measuring glucose and insulin levels. Other examples of nutritional challenges are a high fat meal and a mixed meal test (MMT) that accompanies a high energy density. These types of challenges have been applied in research to stress the metabolic system and to magnify differences between health states. This was for example shown using a monounsaturated fatty acid challenge that resulted in a more pronounced triglyceride response in obese and obese diabetic participants compared to lean participants [3]. Also the postprandial triglyceride response has been shown to be superior to fasting levels for the assessment of cardiovascular disease risk as reviewed by Boren et al. [4], and Pirillo et al. [5].

In addition to challenge tests, the development of 'omics' techniques further enables us to more comprehensive characterise differences in health states. We showed that the use of challenge tests in combination with transcriptomics in peripheral blood mononuclear cells (PBMCs) magnified the difference in PBMCs gene expression between lean and obese individuals if compared to the fasting state [6]. It has also been shown that PBMC gene expression patterns are sensitive to longer-term dietary interventions such as three weeks of 30% ER [7], eight weeks of saturated fatty acid with monounsaturated fatty acid diet [8], eight weeks of a low-calorie diet [9], 26 weeks of n-3 polyunsaturated fatty acids [10], and 18-24 weeks of a healthy Nordic diet [11]. The question remains if the effects of an intervention on the health states of participants can be magnified when a dietary intervention is combined with the execution of challenge tests, especially in combination with the use of comprehensive tools such as transcriptomics. As PBMCs circulate in the blood and are exposed to both

exogenous as endocrine signals, the diet-induced changes in PBMC gene expression may also be valuable to reveal signalling routes and pathways responsible for a change in health state. Especially considering the capacity of the main subpopulation of PBMCs, the monocytes and lymphocytes, to shift their metabolism in response to environmental signals. This shift either leads to more use of oxidative phosphorylation (OXPHOS) or more use of glycolysis [12, 13]. Dietary interventions might as well be able to facilitate such a shift in metabolism.

Of all dietary interventions, energy restriction (ER) is the most effective dietary strategy to improve health through weight loss in overweight people. To investigate if challenge tests magnify the health effects of an intervention, we used ER as a model to improve health. We studied phenotypic flexibility by means of whole genome gene expression in human PBMCs in the fasted state, and postprandially upon an OGTT and an MMT before and after 12 weeks of 20% ER or before and after 12 weeks of a weight maintenance diet in a parallel-designed study.

Materials and Methods

Study population

81 healthy, overweight, Caucasian males and women, aged 50-65, with a BMI 25-35kg/m^2 were included in this study. Exclusion criteria included: weight change of ≥3kg in the preceding three months, current smokers, substance abuse, excess alcohol intake, pregnancy, diabetes, cardiovascular disease, cancer, gastrointestinal disease e.g. inflammatory bowel disease or irritable bowel syndrome, kidney disease, liver disease, pancreatitis, use of medications likely to interfere with energy metabolism, appetite regulation and hormonal balance (including: anti-inflammatory drugs or steroids, antibiotics, androgens, phenytoin, erythromycin or thyroid hormones), having any metallic or magnetic implants such as pacemakers, and claustrophobia.

Intervention diets

72 participants completed the study and were randomly assigned to either a 20% ER diet (n=40) or to a control diet (n=32) for 12 weeks. This study was a parallel design. The ER diet consisted of 50% calories from carbohydrates, 35% from fat and 15% from protein, >18g of fibre, and at least five portions of fruit and vegetables a day. The control diet consisted of 40% calories from carbohydrates, 45% from fat and 15% from protein, and at least three portions of fruit and vegetables a day. Both groups completed a seven-day food diary the first week and the last. Diets were based on dietary advice. Compliance was assessed via a weekly telephone interview. Exercise was kept at the habitual level.

Nutritional challenge tests

All participants performed an oral glucose tolerance test (OGTT) and the next day a mixed meal test (MMT) before and after the intervention diet. Insulin, glucose, and triglycerides were assessed in the fasted state and during the OGTT at time points 15, 30, 60, 90, 120,

and 240 min and during the MMT at time point 60, 120, 240, 360 and 480 min. Insulin and glucose were measured by the laboratory of Imperial College London, United Kingdom. Triglycerides were measured by the laboratory of Medical University Varna (MUV, Varna, Bulgaria). Peripheral blood mononuclear cells (PBMCs) were collected in the fasted state, and postprandially during the OGTT at 30, 60, and 120 min and during the MMT at 60, 120, 240, and 360 min. Each participant provided written informed consent. The study was executed at Imperial College London (London, UK) and approved by the Brent Ethics Committee (REC ref: 12/LO/0139) and was registered at clinicaltrials.gov as NCT01684917. All fasting values were based on time point 0 minutes on the day of the OGTT.

PBMC RNA isolation and microarray processing

PBMCs were isolated from whole blood using BD Vacutainer® Cell Preparation Tubes™ according to the manufacturer's instructions. Total RNA was isolated from PBMC samples using Trizol reagent (Invitrogen, Breda, The Netherlands) and purified using Qiagen RNeasy Micro Kit (Qiagen, Venlo, The Netherlands). RNA integrity was checked with Agilent 2100 bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). Microarrays were performed during OGTT at 0, 30, 60, and 120 min and during MMT at 0, 60, 120, 240, and 360 min. 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 Gene 1.1 ST arrays targeting 19 697 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 [14]. Microarray signals were normalized using robust multichip average (RMA) [15]. Genes with normalized signals >20 on at least 100 arrays were defined as expressed and selected for further analysis. Significant different expression of individual genes were tested using the LIMMA R library [16]. Changes were considered significant when P-value was <0.05 in a t-test/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 [17]. Gene sets with a false discovery rate (FDR q-value) <0.25 were defined as significantly regulated.

All data analyses for fasting samples, on gene expression and on gene set level were performed as follows: each fasting sample obtained after the intervention was compared to the fasting sample before intervention [(week 12(T0) - week 0(T0))] within each intervention group. To determine the difference between the ER diet and the control diet these values were compared between the two diets [(ER(week 12 (T0) - week 0 (T0))) - (Control(week 12 (T0) - week 0 (T0)))].

Postprandial data analysis on gene expression and gene set level was performed as follows: (1) each postprandial value was compared to its fasting value [T30-T0, T60-T0, etc.];

(2) these values were compared within each diet group before and after the intervention [(week 12 (T60-T0) - week 0 (T60-T0)], etc.], and (3) to determine the difference in postprandial response between the ER diet and the control diet these values were compared between the two diets [(ER(week 12 (T60-T0) - week 0 (T60-T0)) - Control(week 12 (T60-T0) - week 0 (T60-T0)))], etc.].

Selection of gene sets of interest during the postprandial course was as follows: (1) gene sets had to be significantly different between the ER and control diet for at least two postprandial time points, and (2) had to be significantly different for at least two postprandial time points within the ER diet group. Enrichment Map is a Cytoscape plugin [18] and was used for functional enrichment visualisation and clustering. Array data have been submitted to the Gene Expression Omnibus under accession number GSE88794.

Statistical analysis of biochemical measurements

The statistical package SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) was used for analysis of the biochemical measurements. Statistical analyses of the challenge tests were performed by linear mixed models for repeated measures (covariance type: compound symmetry). These analyses were performed using delta values (changes from fasting) as dependent variables, and 'time(weeks)', 'timepoint(mins)', and 'time(weeks) x timepoint(mins)' as fixed effects. If the fasting values were significantly different upon the intervention, then the these changes were used as covariate. To determine the effect of the different diets, values (subtracted delta-values from before the intervention time with after the intervention time) were used as dependent variables, and fixed effects were 'diet', 'timepoint(minutes)', and 'diet x timepoint(minutes)'. A value of $P<0.05$ was considered significant. If statistical significance was found, post-hoc LSD tests were performed.

Macrophage markers

Macrophage M1 and M2 phenotype markers were selected based on literature [19-22].

Results

Baseline characteristics of all 72 participants are summarised in table 1. None of the baseline characteristics differed between the two intervention groups. 12 weeks of the 20% ER diet resulted in an average weight loss of 5.6 kg (±2.9, P<0.001) and the control diet group remained weight stable (0.1 kg (±2.0), P=0.9). Fasting insulin and glucose levels, and fasting lymphocyte, monocyte numbers and lymphocytes/monocytes-ratio remained unchanged upon the intervention between both groups (P>0.05, table 2). Fasting triglycerides were significantly reduced upon the ER-intervention between the two groups (P=0.031, table 2).

Table 1. Baseline characteristics of participants.

	ER (n=40)	**Control (n=32)**
N (♂ / ♀)	40 (19/21)	32 (16/16)
Age (y)	60 [50-65]	60 [51-65]
Weight (kg)	84.3 ± 12.5	83.4 ± 13.3
BMI (kg/m^2)	29.2 ± 3.0	29.1 ± 2.5
Physical activity level	1.5 ± 0.3	1.5 ± 0.3

Data represent mean and ±SD, or median and [range].
ER: 20% energy restriction; ♂: men; ♀: women.

Table 2. Fasting changes in blood parameters upon 12 weeks intervention.

				P-value	
		Baseline (0 weeks)[1]	**Change** (12 weeks)	Time (weeks)[2]	Time(weeks) x Diet[3]
Glucose (mmol/L)	ER	5.20 ± 0.58	-1.70 ± 3.40	0.012	0.516
	Ctrl	5.13 ± 0.54	-0.50 ± 3.45	0.159	
Insulin (mmol/L)	ER	15.22 ± 5.68	-1.57 ± 6.12	0.113	0.323
	Ctrl	16.46 ± 8.27	-0.17 ± 5.70	0.870	
Triglycerides (mmol/L)	ER	1.31 ± 0.47	-0.17 ± 0.52	0.041	0.031
	Ctrl	1.24 ± 0.49	0.09 ± 0.43	0.290	
Lymphocytes (# giga/L)	ER	1.73 ± 0.43	-0.03 ± 0.30	0.568	0.549
	Ctrl	1.48 ± 0.41	0.01 ± 0.27	0.775	
Monocytes (# giga/L)	ER	0.44 ± 0.12	-0.01 ± 0.12	0.761	0.236
	Ctrl	0.50 ± 0.22	-0.05 ± 0.20	0.159	
Lymphocytes/ monocytes-ratio	ER	4.27 ± 1.50	0.10 ± 1.11	0.586	0.999
	Ctrl	3.44 ± 1.70	0.09 ± 1.23	0.654	

Data represent mean and ±SD, or median and [range]. ER: 20% energy restriction; Ctrl: control.
[1] Baseline is based on fasting value at time point 0 on the day of OGTT;
[2] Time(weeks) = fasting changes within diet group
[3] Time(weeks) x Diet = fasting changes between diet groups

Effect of ER on gene expression in the fasted state

To identify the effect of 12 weeks 20% ER on fasting whole genome PBMC gene expression, we examined the difference in gene expression response upon 12 weeks between the ER and control group. A total number of 468 genes showed a significant different change in expression in the fasted state upon 12 weeks of 20% ER compared to the control diet (figure 1).

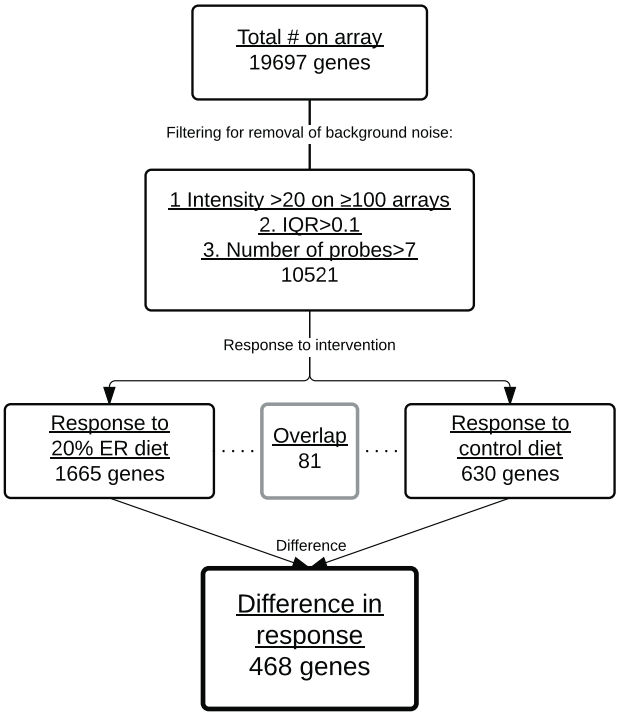


Figure 1. Fasting changes in whole genome PBMC gene expression upon 12 weeks 20% energy restriction. Depicted is the stepwise selection of genes in microarray analysis, based on intensity, IQR, and number of probes and the significant ($P<0.05$) changed gene expression upon 12 weeks of 20% ER (left track) and 12 weeks of control diet (right track). The last box depicts the number of genes that showed a significant different change in expression in the fasted state upon 12 weeks of 20% ER compared to the control diet.

To identify potential gene sets and signalling routes that were changed in the fasted state upon 20% ER, Gene Set Enrichment Analysis (GSEA) was performed. A total of 312 gene sets was significantly upregulated upon ER if compared to the control group with FDR q-value<0.25 (supplementary table 1) and one gene set was downregulated in ER compared to control. Of these 313 gene sets, 134 gene sets were also significantly changed within the 20% ER group. To identify gene sets with overlapping genes, clusters were formed and visualised using the Enrichment map plugin from Cytoscape. These clusters are summarised in table 3. Gene sets involved in OXPHOS, cell adhesion, energy metabolism, immune system and cell cycle were upregulated upon 20% ER.

Table 3. Summary of changes in main clusters of gene sets in PBMCs upon 12 weeks of 20% ER.

Pathway cluster[1]	**20% ER (n = 40)**	**Control (n = 32)**
Oxidative phosphorylation	↑	↓/-
Cell adhesion	↑	-
Glucose metabolism	↑	-
Lipid metabolism	↑	-
Immune system	↑	-
Cell cycle	↑	↓
DNA replication	↑	↓

[1]Significantly changed pathways are determined with GSEA (supplementary table 1) and clusters are based upon Cytoscape.
↑: gene sets in this pathway-cluster were upregulated;
↓: gene sets in this pathway-cluster were downregulated;
- : no change in this pathway-cluster.

Postprandial effect of ER on blood parameters

12 weeks of 20% ER induced a significantly different postprandial response only for glucose during the OGTT (table 4).

Table 4. Changes in blood parameters and PBMC-composition. Depicted are t=0 min values and changes (Δ) from this values for time points during **a.** the oral glucose tolerance test (OGTT) and **b.** the mixed meal test (MMT).

A: OGTT			**TIME**		
			0 min	**Δ15 min**	**Δ30 min**
Glucose (mmol/L)	ER	week 0	5.20 ± 0.58	1.83 ± 0.68	3.12 ± 1.17
		week 12	5.03 ± 0.50	1.78 ± 0.81	2.86 ± 1.18
	Ctrl	week 0	5.13 ± 0.54	1.93 ± 0.84	3.47 ± 1.55
		week 12	5.02 ± 0.51	1.51 ± 0.82	2.81 ± 1.25
Insulin (mmol/L)	ER	week 0	15.22 ± 5.68	40.25 ± 24.87	74.11 ± 52.63
		week 12	13.66 ± 6.78	36.28 ± 31.72	63.92 ± 54.95
	Ctrl	week 0	16.46 ± 8.27	48.68 ± 36.44	69.40 ± 39.44
		week 12	16.29 ± 5.53	34.03 ± 28.21	61.21 ± 37.07
Triglycerides (mmol/L)	ER	week 0	1.31 ± 0.47	-0.04 ± 0.23	-0.02 ± 0.19
		week 12	1.12 ± 0.55	0.02 ± 0.31	0.02 ± 0.29
	Ctrl	week 0	1.24 ± 0.49	0.01 ± 0.23	-0.02 ± 0.10
		week 12	1.31 ± 0.60	-0.07± 0.38	-0.08 ± 0.44
Lymphocytes (# giga/L)	ER	week 0	1.73 ± 0.43	-0.03 ± 0.16	-0.12 ± 0.18
		week 12	1.70 ± 0.43	-0.02 ± 0.17	-0.15 ± 0.19
	Ctrl	week 0	1.48 ± 0.41	-0.04 ± 0.17	-0.13 ± 0.18
		week 12	1.50 ± 0.46	0.03 ± 0.17	-0.05 ± 0.19
Monocytes (# giga/L)	ER	week 0	0.44 ± 0.12	-0.02 ± 0.07	-0.10 ± 0.07
		week 12	0.43 ± 0.16	-0.03 ± 0.09	-0.09 ± 0.12
	Ctrl	week 0	0.50 ± 0.22	-0.05 ± 0.08	-0.10 ± 0.08
		week 12	0.45 ± 0.12	-0.01 ± 0.09	-0.08 ± 0.09
Lymphocytes/ Monocytes-ratio	ER	week 0	4.27 ± 1.50	0.04 ± 0.68	0.97 ± 1.17
		week 12	4.37 ± 1.67	0.07 ± 1.01	0.59 ± 1.28
	Ctrl	week 0	3.44 ± 1.70	0.34 ± 0.73	0.59 ± 1.09
		week 12	3.56 ± 1.28	0.34 ± 0.90	0.73 ± 0.93

Data represent mean and ±SD. ER: 20% energy restriction; Ctrl: control diet.

TIME				P-value	
Δ60 min	Δ90 min	Δ120 min	Δ240 min	Time(weeks) x Timepoint (mins)[1]	Diet x Timepoint (mins)[2]
2.86 ± 2.32	1.96 ± 2.32	0.91 ± 2.07	-1.39 ± 0.70	0.597	0.036
2.51 ± 2.05	1.50 ± 2.14	0.32 ± 1.40	-1.28 ± 0.80		
3.02 ± 2.08	1.96 ± 2.32	1.00 ± 1.86	-1.29 ± 0.56	0.376	
3.06 ± 2.33	1.77 ± 2.75	1.27 ± 2.31	-1.03 ± 0.72		
90.53 ± 68.20	80.05 ± 54.79	54.93 ± 48.74	-2.62 ± 3.98	0.709	0.642
81.74 ± 64.92	66.26 ± 52.66	39.49 ± 40.20	-3.20 ± 7.23		
89.42 ± 52.08	75.44 ± 64.05	54.76 ± 61.64	-1.60 ± 7.17	0.825	
77.45 ± 52.10	72.53 ± 56.77	50.35 ± 42.52	-1.03 ± 15.09		
0.07 ± 0.30	0.00 ± 0.24	-0.08 ± 0.23	0.01 ± 0.37	0.919	0.683†
0.08 ± 0.38	0.05 ± 0.41	0.05 ± 0.55	0.05 ± 0.48		
0.08 ± 0.23	0.12 ± 0.40	-0.02 ± 0.22	0.15 ± 0.27	0.539	
-0.03 ± 0.40	-0.05 ± 0.40	-0.02 ± 0.69	0.01 ± 0.46		
-0.13 ± 0.21	-0.01 ± 0.27	0.08 ± 0.27	0.85 ± 0.81	0.028	0.669
-0.09 ± 0.23	0.02 ± 0.26	0.13 ± 0.22	0.60 ± 0.55		
-0.08 ± 0.24	-0.02 ± 0.27	0.08 ± 0.28	0.57 ± 0.48	0.148	
-0.04 ± 0.23	0.03 ± 0.26	0.09 ± 0.27	0.43 ± 0.50		
-0.08 ± 0.08	-0.04 ± 0.08	0.02 ± 0.11	0.14 ± 0.13	0.129	0.541
-0.08 ± 0.12	-0.04 ± 0.10	0.00 ± 0.10	0.08 ± 0.10		
-0.11 ± 0.12	-0.05 ± 0.10	-0.01 ± 0.09	0.12 ± 0.15	0.839	
-0.08 ± 0.09	-0.02 ± 0.09	0.01 ± 0.09	0.12 ± 0.11		
0.74 ± 1.33	0.39 ± 1.23	0.00 ± 0.86	0.46 ± 1.11	0.759	0.265
0.63 ± 1.31	0.35 ± 1.30	0.17 ± 1.09	0.37 ± 1.04		
0.63 ± 1.11	0.31 ± 1.18	0.22 ± 1.09	0.39 ± 0.95	0.500	
1.18 ± 2.24	0.57 ± 1.01	0.43 ± 1.23	0.19 ± 1.10		

†due to significant changes found in the fasted state, these P-values are calculated using change in fasting value (T0,12wks-T0,0wks) as covariate.

[1]Time(weeks) x Timepoint(mins) = Effect of time within diet on the response to OGTT;

[2]Timepoint(mins) x Diet = Effect of diet on the response to OGTT.

Table 4. Continued.

B: MMT			TIME		
			0 min	Δ60 min	Δ120 min
Glucose (mmol/L)	ER	week 0	5.12 ± 0.57	1.65 ± 1.42	0.37 ± 1.38
		week 12	4.98 ± 0.53	1.25 ± 1.63	0.36 ± 1.24
	Ctrl	week 0	5.01 ± 0.42	1.57 ± 1.65	0.90 ± 1.42
		week 12	5.03 ± 0.51	1.30 ± 1.82	0.51 ± 1.35
Insulin (mmol/L)	ER	week 0	18.46 ± 8.15	99.21 ± 74.23	59.76 ± 45.76
		week 12	14.83 ± 5.18	84.51 ± 58.55	57.90 ± 47.75
	Ctrl	week 0	17.88 ± 6.90	81.02 ± 49.07	61.54 ± 37.88
		week 12	19.17 ± 10.14	80.83 ± 60.12	50.39 ± 33.67
Triglycerides (mmol/L)	ER	week 0	1.32 ± 0.50	0.25 ± 0.54	0.52 ± 0.64
		week 12	1.19 ± 0.44	0.20 ± 0.38	0.39 ± 0.51
	Ctrl	week 0	1.35 ± 0.54	0.19 ± 0.48	0.47 ± 0.72
		week 12	1.26 ± 0.65	0.13 ± 0.56	0.23 ± 0.53
Lymphocytes (# giga/L)	ER	week 0	1.78 ± 0.43	0.00 ± 0.22	0.13 ± 0.35
		week 12	1.69 ± 0.46	-0.05 ± 0.26	0.13 ± 0.29
	Ctrl	week 0	1.55 ± 0.49	-0.08 ± 0.20	0.13 ± 0.28
		week 12	1.53 ± 0.45	-0.07 ± 0.20	0.10 ± 0.26
Monocytes (# giga/L)	ER	week 0	0.41 ± 0.11	-0.04 ± 0.08	0.05 ± 0.10
		week 12	0.39 ± 0.13	-0.06 ± 0.09	0.03 ± 0.10
	Ctrl	week 0	0.45 ± 0.15	-0.07 ± 0.10	0.02 ± 0.11
		week 12	0.46 ± 0.15	-0.06 ± 0.11	0.03 ± 0.11
Lymphocytes/ Monocytes-ratio	ER	week 0	4.63 ± 1.59	0.70 ± 1.41	-0.08 ± 0.92
		week 12	4.77 ± 1.95	0.57 ± 1.33	-0.19 ± 1.36
	Ctrl	week 0	3.69 ± 1.35	0.56 ± 1.23	0.27 ± 1.15
		week 12	3.62 ± 1.38	0.64 ± 1.27	0.17 ± 1.30

Data represent mean and ±SD. ER: 20% energy restriction; Ctrl: control diet.

TIME			P-value	
Δ240 min	Δ360 min	Δ480 min	Time(weeks) x Timepoint (mins)[1]	Diet x Timepoint (mins)[2]
-0.79 ± 0.77	-0.57 ± 0.61	-0.44 ± 0.56	0.648	0.773
-0.77 ± 0.86	-0.56 ± 0.56	-0.37 ± 0.48		
-0.65 ± 0.89	-0.54 ± 0.49	-0.45 ± 0.40	0.738	
-0.62 ± 0.69	-0.51 ± 0.53	-0.44 ± 0.48		
5.43 ± 11.20	-2.83 ± 5.48	-3.47 ± 4.30	0.614	0.384
1.98 ± 6.25	-2.17 ± 4.14	-2.54 ± 3.82		
7.30 ± 14.96	-0.85 ± 7.94	-4.12 ± 3.16	0.854	
4.27 ± 11.07	-3.32 ± 7.18	-4.85 ± 7.28		
0.71 ± 0.69	0.66 ± 0.74	0.13 ± 0.60	0.517	0.480[†]
0.55 ± 0.63	0.69 ± 0.61	0.22 ± 0.62		
0.83 ± 0.80	1.01 ± 1.05	0.50 ± 0.99	0.706	
0.64 ± 0.79	0.81 ± 0.75	0.43 ± 1.12		
0.41 ± 0.34	0.58 ± 0.39	0.58 ± 0.35	0.266	0.617
0.43 ± 0.33	0.49 ± 0.26	0.68 ± 0.32		
0.40 ± 0.45	0.41 ± 0.26	0.50 ± 0.42	0.919	
0.35 ± 0.37	0.43 ± 0.35	0.57 ± 0.39		
0.13 ± 0.10	0.11 ± 0.10	0.08 ± 0.10	0.835	0.819
0.13 ± 0.10	0.09 ± 0.08	0.08 ± 0.08		
0.10 ± 0.12	0.10 ± 0.13	0.07 ± 0.10	0.913	
0.13 ± 0.11	0.11 ± 0.09	0.10 ± 0.08		
-0.34 ± 0.95	0.13 ± 0.94	0.42 ± 0.86	0.965	0.934
-0.41 ± 1.11	0.14 ± 1.15	0.56 ± 1.13		
0.21 ± 0.84	0.10 ± 0.77	0.55 ± 0.99	0.863	
-0.08 ± 0.89	0.08 ± 0.84	0.43 ± 0.74		

[†]due to significant changes found in the fasted state, these P-values are calculated using change in fasting value (T0,12wks-T0,0wks) as covariate.

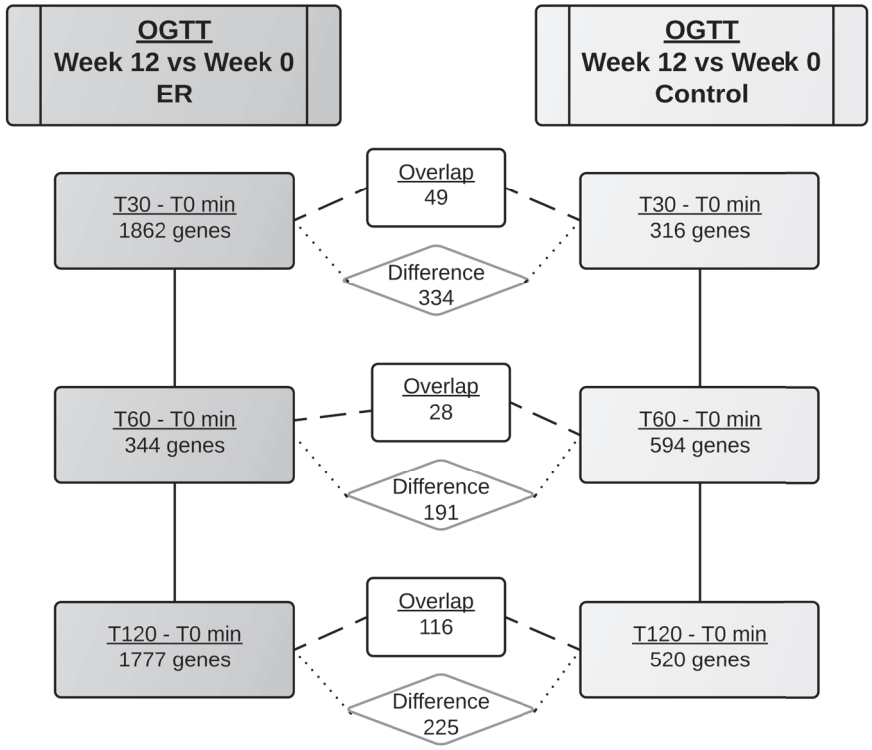
[1]Time(weeks) x Timepoint(mins) = Effect of time within diet on the response to MMT;

[2]Timepoint(mins) x Diet = Effect of diet on the response to MMT.

Effect of ER on PBMC gene expression at postprandial time points

PBMC gene expression changes were analysed upon ER diet and upon control diet during the OGTT and during the MMT and the number of significantly regulated genes is visualized in figure 2. Each postprandial value was first compared to its fasting (T0, 0 minutes) value (data not shown), then within the diet group and finally between the diet groups. A higher number of genes was significantly changed during the OGTT upon ER compared to the control diet and during the OGTT when compared to the MMT within the diet groups.

A



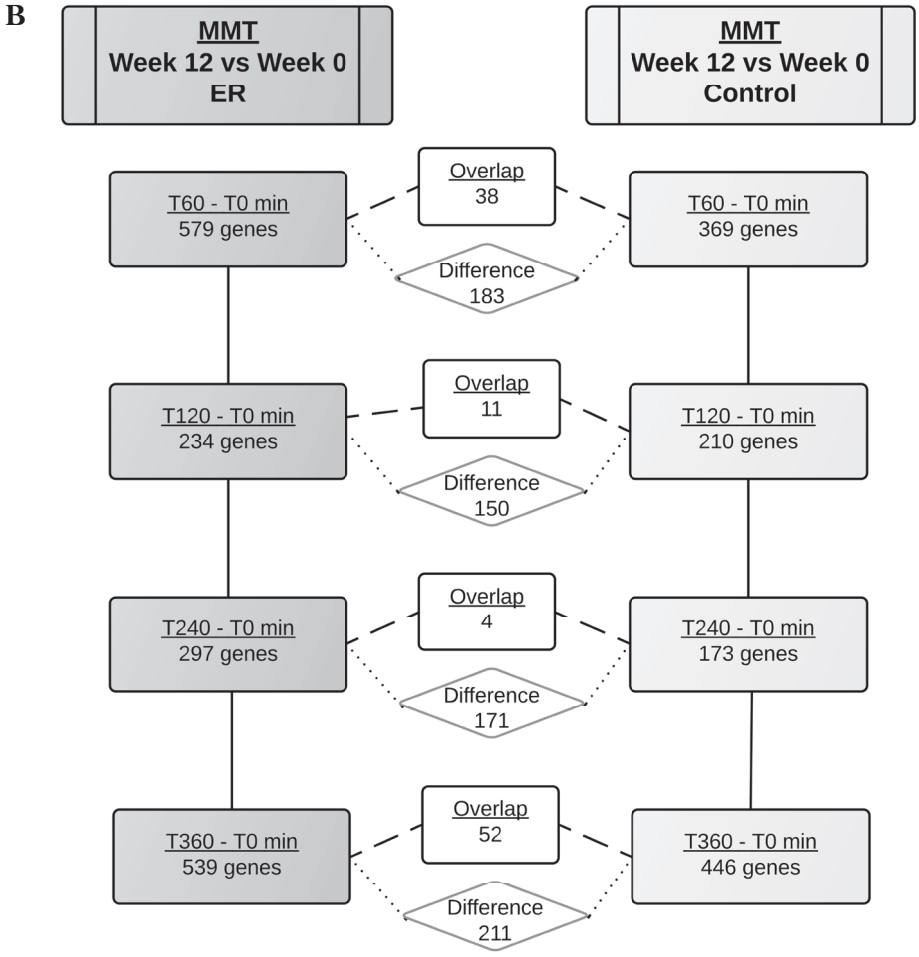


Figure 2. Significant changes in gene expression during challenge tests. Number of genes differentially expressed ($P<0.05$) upon 20% ER (left track) and control (right track) diet during **a.** an OGTT at 30, 60, and 120 minutes, and **b.** an MMT at 60, 120, 240 and 360 min. In the middle the number of genes is depicted that were overlapping between the two diets and genes that showed a significant difference in response between the two diets.

To identify what gene sets were enriched upon the ER diet and upon the control diet during the OGTT (figure 3a) and during the MMT (figure 3b) Gene Set Enrichment Analysis was performed. Figure 3 displays the number of gene sets changed for each time point per challenge comparing ER to control.

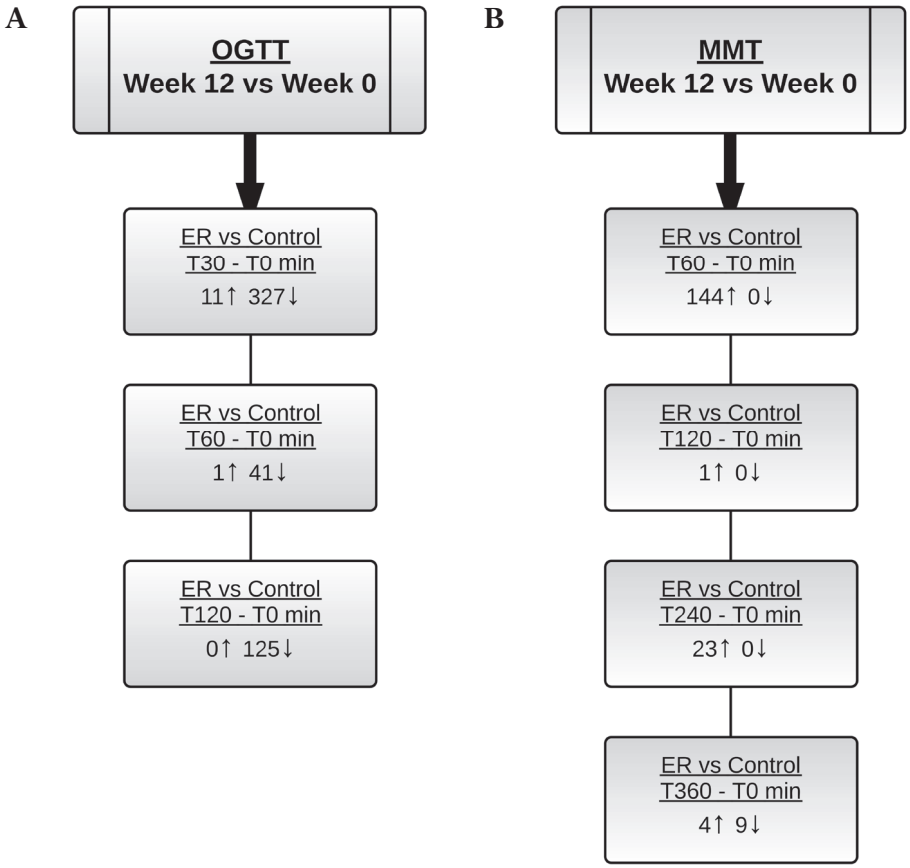


Figure 3. Significantly changed gene sets during the challenge tests between ER and control. Numbers of gene sets differently changed between ER and control diet at **a.** 30, 60, and 120 minutes during the OGTT and **b.** 60, 120, 240, and 360 minutes during the MMT. Each postprandial value was first compared to the its fasting (T0) value (data not shown), then within the diet group (data not shown) and finally between the diet groups (data shown).

To identify a robust change in response gene sets were selected that were significantly changed upon ER for at least two out of three time points during the OGTT which resulted in 44 gene sets. Clusters of these genes sets are visualised in supplementary figure 1. These included OXPHOS, cell adhesion, and DNA replication and were decreased postprandially upon ER. 12 weeks of 20% ER increased OXPHOS (figure 4a), cell adhesion (figure 4b), and DNA replication (figure 4c) related gene sets in the fasted state, and decreased these gene sets during the OGTT. For the MMT four gene sets were significantly changed upon ER for at least two out of the four time points during the MMT (supplementary figure 2).

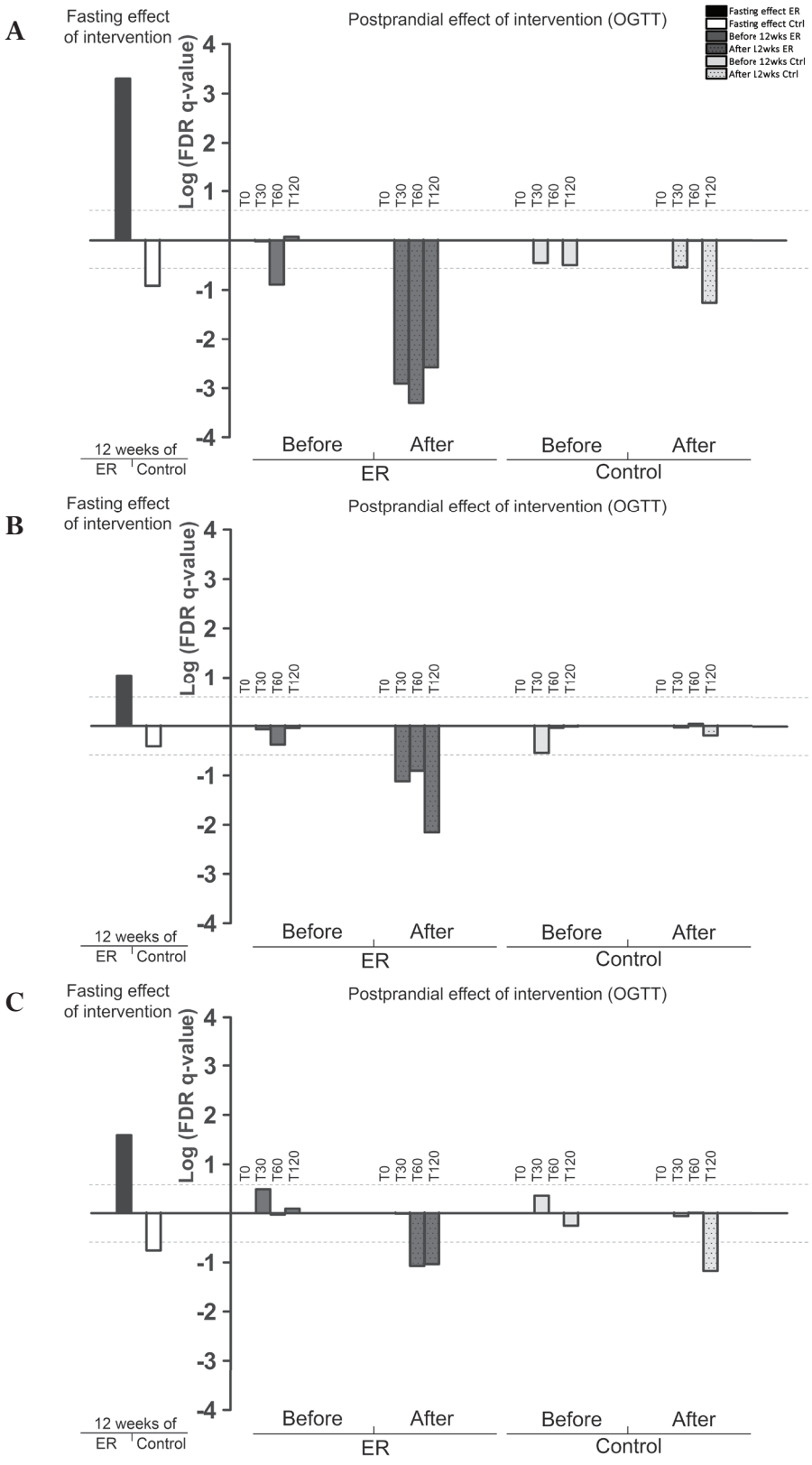


Figure 4. Significant gene set changes in the fasted state and during the OGTT. Comparison between fasting and postprandial time points upon 12 weeks ER and control diet for the OGTT, for gene sets involved in **a.** oxidative phosphorylation, **b.** cell adhesion, and **c.** DNA replication. Left side shows effect in the fasted state upon 12 weeks ER or control. Right side shows postprandial effects at time points 30, 60, and 120 minutes during the OGTT before and after ER or control. Each bar represents the logarithmic FDR q-value. Direction of change, i.e. up or down, is based on the Normalized Enrichment Score from GSEA. Dotted line = significance cut off for FDR q-value of 0.25 (Log value = (-)0.60).

Shift in metabolism

Because the largest changes of ER, during fasting as well as postprandially during an OGTT, were found on OXPHOS gene expression, and OXPHOS is characteristic for a metabolic shift of immune cells, we further analysed this potential metabolic shift by characterisation of responses of other important genes upon ER. We determined whether genes involved in glycolysis were affected and examined lactate dehydrogenases (LDH-A, LDH-B, LDH-D) and pyruvate dehydrogenase kinase isozyme 4 (PDK4). None were significantly different ($P>0.05$) between or within the ER and control groups. As a metabolic shift goes together with monocyte differentiation towards M1 or M2 macrophages, we examined potential priming towards M1 or M2 macrophages by investigating ER-induced gene expression changes for M1 and M2 markers (supplementary figure 3). On the gene expression level a significantly increase upon ER was observed for M2-related gene set Cluster of differentiation 163 (CD163) and Integrin beta 2 (ITGB2/CD18), and M1 markers Macrophage Receptor (MARCO) and C-C chemokine receptor type 2 (CCR2) while M1 markers L-selectin (SELL) and Cluster of differentiation 80 (CD80) decreased significantly. To identify potential correlations between M1 and M2 markers and genes from the OXPHOS pathways that were significantly changed, we created a correlation heatmap for the significantly changed M1/M2 markers (Supplementary figure 4). A positive correlation was observed between OXPHOS genes, and a modest negative correlation was observed between the OXPHOS genes and M1 markers.

Discussion

Within this study we aimed to investigate if the application of challenge tests before and after an ER intervention magnified the effects on health using transcriptomics. We studied the effect of 12 weeks of 20% ER on whole genome gene expression in human PBMCs both in the fasted state, and upon an OGTT and an MMT. In the fasted state, 20% ER induced expression of genes involved in OXPHOS, cell adhesion, energy metabolism, immune system, and cell cycle. During the OGTT, 20% ER consistently reduced expression of genes involved in OXPHOS, cell adhesion, and DNA replication. During the MMT, the results of 20% ER were less consistent at several time points.

We identified two other studies that observed effects of ER on OXPHOS gene expression in blood or PBMCs, but opposite effects compared to our study were found. One study showed that eight weeks of 35% ER led to a decrease in expression of OXPHOS related genes in PBMCs from nine obese men [9], but no control group was present. Another study showed that six weeks of 900 kcal/d (comparable to 40-45% ER) led to a decrease in OXPHOS in whole blood from nine obese participants that lost a high percentage weight, and nine obese participants that lost a low percentage of weight [23]. It must be noted that the size of our study population was much larger with respectively 40 participants and 32 participants in the ER and control group compared to the nine participants per group in above mentioned studies. The length of the intervention and intensity of the intervention might also have affected the outcome, with 12 weeks of relatively mild 20% ER in our intervention versus eight and six weeks strong 35-45% ER in the other studies. Also in other tissues and organisms opposite results are found for ER on oxidative stress as described by Ristow et al. [24]. Therefore theories for both a decrease or increase in OXPHOS upon ER exist. A decrease in OXPHOS gene expression is explained by a decreased amount of nutrients that can be oxidised, resulting in a decrease in reactive oxygen species (ROS), which could be beneficial as it may decrease oxidative damage inside cells. Whereas an increase in OXPHOS is explained by the need to oxidise as many nutrients as possible, as this generates more ATP than using the nutrients for glycolysis. This increase in OXPHOS, results in an increase in ROS, which could be beneficial as it may increases the coping strategy towards oxidative stress and therefore also other stressors [24-26] leading to a greater adaptive capacity and more metabolic flexibility of the cell. This increase in ROS and OXPHOS expression has been shown before in tissues from rodents [27, 28] but not in human studies. An increase in OXPHOS gene expression may also be explained by an increase in biogenesis of mitochondria. We did however not see an upregulation in expression of genes coding for mitochondrial ribosomal proteins which are involved in mitochondrial biogenesis (data not shown), and therefore we do not assume an increase in mitochondria biogenesis due to ER. A newer and more specific explanation for an increase in OXPHOS may be found in a shift in immunometabolism. An increase in gene sets involved in OXPHOS may indicate a shift in favour of oxidative metabolism. On average, 22% of PBMCs are monocytes, which can shift their metabolism from glycolysis to OXPHOS and can be primed to differentiate into different type of macrophages [13]. OXPHOS is characteristic for

M2 macrophages, and glycolysis for M1 macrophages. Although ER upregulated OXPHOS gene sets and two other M2 specific gene sets, i.e. galactose metabolism and regulation of ornithine decarboxylase and M1 and M2 macrophages did show a trend towards M2 macrophages, some M1 macrophage markers and M1 specific gene sets were upregulated as well in our study. Based on this gene expression data no clear conclusion can be drawn concerning a ER-induced shift in immunometabolism, but future research could investigate the effects of ER on metabolism of immune cells using different techniques.

An interesting result within our study is that OXPHOS increased strongly in the fasted state upon ER, but decreased postprandially during the OGTT upon ER. An explanation for this opposite response may be the shift from shortage of energy due to ER to a sudden overload of energy during the OGTT. Upon ER, the postprandial decrease in OXPHOS was already visible after 30 minutes. Despite no energy deficit, glucose also evoked a decrease in OXPHOS in the control group, but only after 120 minutes. This time difference may indicate a faster response, and higher flexibility towards a stressor upon an ER intervention. This is also in line with the above mentioned theory on the increased coping mechanism due to increased OXPHOS in the fasted state upon ER, reflected by a fast reduction in OXPHOS after an stressor such as the oral glucose load. Similar to OXPHOS, gene sets related to DNA replication increased in the fasted state upon ER, and decreased postprandially during the OGTT. DNA replication is a process activated at all times in a cell's life cycle to maintain enough cells to construct a tissue. Although others also showed increases in expression of DNA replication genes upon long- and short term ER in liver from mice [29], it is difficult to explain this increase as a biological function from gene expression data in PBMCs, especially since these cells do not divide. In addition, cell adhesion related gene sets also increased in the fasted state upon ER and decreased during the OGTT upon ER. Within these gene sets mainly genes encoding for different integrins were increased, which may reflect an increased attachment of immune cells to the vascular wall. Which is not in line with the expected improvement in vascular health. The postprandial decrease is also opposite to previous findings on high fat challenges that increased cell adhesion molecules protein expression in healthy, lean participants [30, 31]. It should be noted that we examined gene expression instead of protein expression.

To ensure that the detected effects of 20% ER are not due to subpopulation shifts within PBMCs, we examined lymphocyte/monocyte-ratio, and showed that the changes in gene expression are not due to a change in subpopulation of PBMCs both in the fasted state as well as after both challenges. For the pathway data analyses we used strict selection steps as gene sets needed to be changed between the diet groups, within the ER group, and at least at two time points postprandially, or conclusion are therefore based on consistent postprandial findings. A point of attention is that in this study participants had an OGTT on the first day and an MMT the next day. The effect of the OGTT on the cells might not have been restored the next day when the MMT was executed. This could have affected the fasting values on the day of the MMT and maybe also the postprandial response, which might explain the minimal ER-induced changes of the MMT challenge.

The main question we had was whether a challenge test had added value compared to testing in the fasted state for magnification of health effects of ER. The challenge test did not result in a larger number of genes or gene sets changed upon ER compared to fasting. In this respect one might question the added value of a challenge test. However, the data clearly showed that the use of an OGTT in combination with transcriptomics shows an increased phenotypic flexibility upon ER as reflected by a faster and more pronounced downregulation of OXPHOS, cell adhesion, and DNA replication during the OGTT after ER, assuming a more healthy state in these participants and displaying the significance of dynamic measurement versus measurement in the static fasting state. How these fining can be translated to more functional health outcome measures remains to be evaluated. An interesting finding of the challenges test is the large interindividual variation in gene expression response upon the challenge tests. This finding gives new opportunities to explore the use of the response towards a challenge to find and predict responders and non-responders of an intervention.
In conclusion, 12 weeks of 20% ER increased phenotypic flexibility as reflected by a faster and more pronounced decrease in expression of genes related to OXPHOS, cell adhesion, and DNA replication during an OGTT. The OGTT in combination with transcriptomics can be used to measure phenotypic flexibility in an ER-intervention which could not be determined with a static measurement in the fasted state. The translation of this to finding to ER-related health outcome measures needs further investigation.

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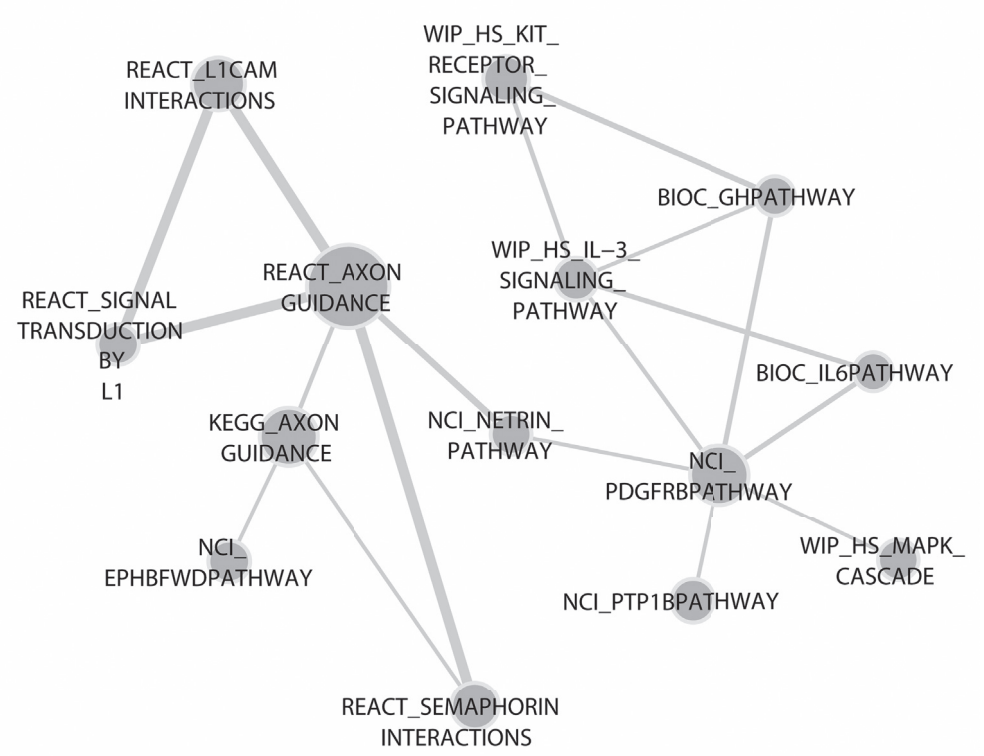
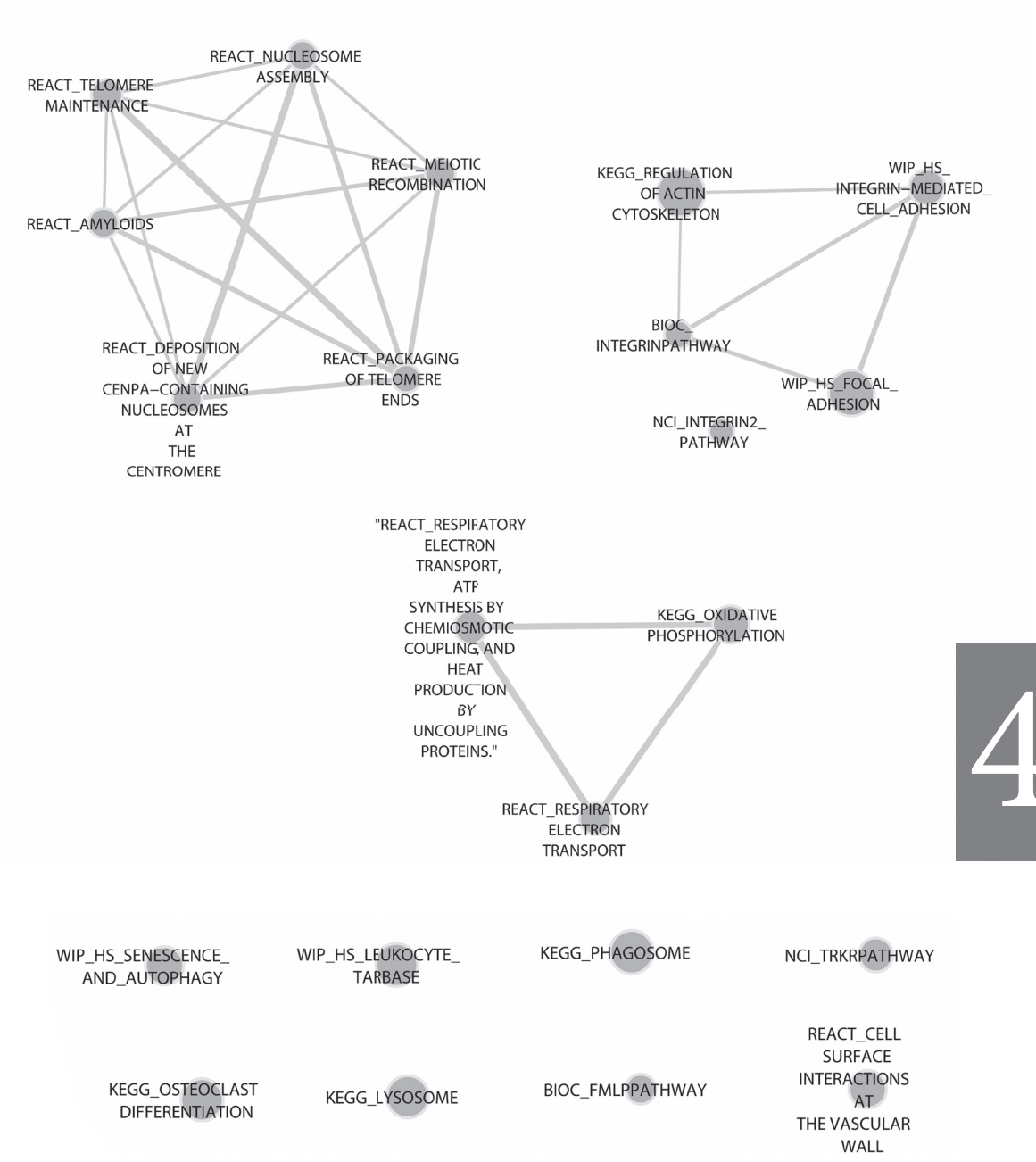
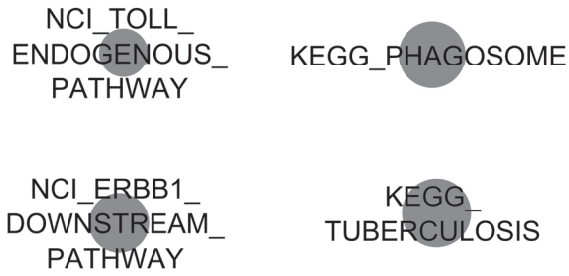
Supplementary information



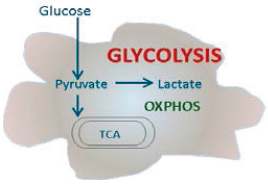


Supplementary figure 1. Visualisation of gene set clusters changed during the OGTT. Depicted are the clusters of gene sets that were changed during the OGTT. Selection of these gene sets was as follows: (1) gene sets had to be significantly (P>0.05) different between the ER and control diet for at least two postprandial time points, and (2) had to be significantly different for at least two postprandial time points within the ER diet group.



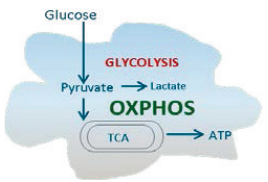
Supplementary figure 2. Visualisation of gene set clusters changed during the MMT. Depicted are the clusters of gene sets that were changed during the MMT. Selection of these gene sets was as follows: (1) gene sets had to be significantly (P>0.05) different between the ER and control diet for at least two postprandial time points, and (2) had to be significantly different for at least two postprandial time points within the ER diet group.

A **M1 macrophage**



Gene	SLR	P-value
MARCO	0.18	0.005
CCR2	0.14	0.018
CXCL9	0.13	0.120
ITGAX	0.10	0.050
IL1RN	0.10	0.178
TLR4	0.05	0.511
TNF	0.04	0.697
CXCL10	0.04	0.761
IDO1	0.03	0.609
IL23A	0.00	0.968
COX20	-0.04	0.215
SELL	-0.06	0.017
SOCS1	-0.07	0.233
ITGA1	-0.08	0.190
IL6	-0.11	0.113
CCL2	-0.12	0.205
CD80	-0.14	0.019
IL1B	-0.25	0.243

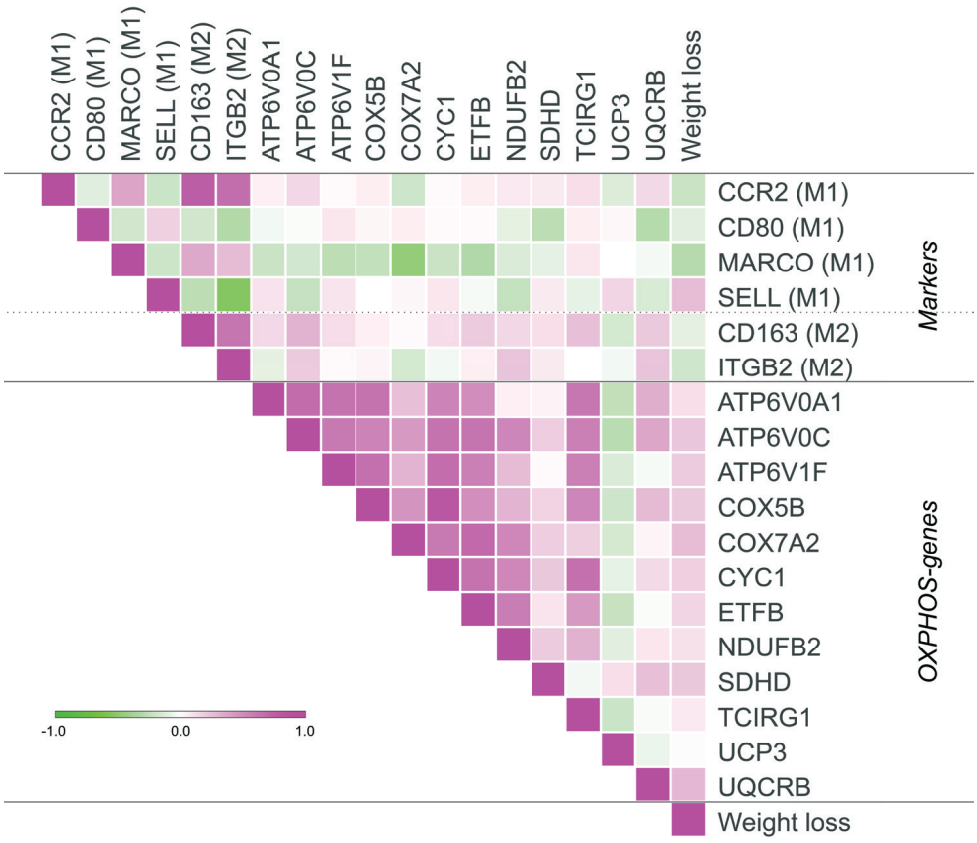
B **M2 macrophage**



Gene	SLR	P-value
CD163	0.18	0.046
ICAM1	0.10	0.085
VEGFA	0.08	0.113
ITGB2	0.07	0.038
TGM2	0.06	0.253
IL10	0.03	0.594
TGFB1	0.02	0.468
CX3CR1	0.02	0.570
MRC1	0.00	0.944
FCER2	-0.06	0.527
ITGB5	-0.07	0.327
NR3C2	-0.08	0.108

-0.15 0.15 P-value <0.05

Supplementary figure 3. Macrophage markers. Depicted are the signal log ratios (SLR) and P-value within the 20% energy restriction group for **a.** M1 markers and **b.** M2 markers.



Supplementary figure 4. Correlation heat map of M1/M2 markers and OXPHOS genes. Depicted is the signal log ratio of the gene expression of M1- and M2-markers and oxidative phosphorylation genes in the fasted state upon ER. Scale: green = correlation score of -1, pink = correlation score of 1

Supplementary table 1. Gene Set Enrichment Analysis. Cut-off value FDR q-value 0.25.
Supplementary table 1a. Gene sets upregulated in PBMCS upon twelve weeks of 20% ER compared to control.

Gene set	NES	FDR q-value
REACT_AMYLOIDS	2.803	0.000
KEGG_SYSTEMIC LUPUS ERYTHEMATOSUS	2.479	0.000
REACT_RESPIRATORY ELECTRON TRANSPORT	2.466	0.000
REACT_MEIOTIC RECOMBINATION	2.416	0.000
REACT_PACKAGING OF TELOMERE ENDS	2.415	0.000
KEGG_OXIDATIVE PHOSPHORYLATION	2.407	0.000
NCI_HDAC_CLASSI_PATHWAY	2.330	0.000
REACT_NUCLEOSOME ASSEMBLY	2.307	0.000
REACT_TELOMERE MAINTENANCE	2.296	0.000
REACT_RESPIRATORY ELECTRON TRANSPORT, ATP SYNTHESIS BY CHEMIOSMOTIC COUPLING, AND HEAT PRODUCTION BY UNCOUPLING PROTEINS.	2.279	0.000
REACT_DEPOSITION OF NEW CENPA-CONTAINING NUCLEOSOMES AT THE CENTROMERE	2.272	0.000
REACT_REGULATION OF ORNITHINE DECARBOXYLASE (ODC)	2.271	0.000
REACT_SWITCHING OF ORIGINS TO A POST-REPLICATIVE STATE	2.231	0.000
REACT_ORC1 REMOVAL FROM CHROMATIN	2.220	0.000
NCI_AURORA_A_PATHWAY	2.211	0.001
WIP_HS_PROTEASOME_DEGRADATION	2.212	0.001
REACT_MITOTIC G1-G1_S PHASES	2.175	0.001
WIP_HS_ELECTRON_TRANSPORT_CHAIN	2.177	0.001
REACT_SYNTHESIS OF DNA	2.166	0.001
KEGG_ALZHEIMER'S DISEASE	2.155	0.002
REACT_CHROMOSOME MAINTENANCE	2.156	0.002
KEGG_HUNTINGTON'S DISEASE	2.142	0.002
REACT_REMOVAL OF LICENSING FACTORS FROM ORIGINS	2.144	0.002
WIP_HS_OXIDATIVE_PHOSPHORYLATION	2.136	0.002
REACT_CDK-MEDIATED PHOSPHORYLATION AND REMOVAL OF CDC6	2.109	0.002
KEGG_SALMONELLA INFECTION	2.104	0.002
REACT_REGULATION OF DNA REPLICATION	2.091	0.003
REACT_ACTIVATION OF CHAPERONES BY IRE1ALPHA	2.084	0.003
KEGG_PROTEASOME	2.078	0.003
REACT_APOPTOSIS	2.076	0.003
KEGG_PARKINSON'S DISEASE	2.062	0.003
REACT_DEGRADATION OF BETA-CATENIN BY THE DESTRUCTION COMPLEX	2.065	0.003
REACT_SIGNALING BY WNT	2.066	0.003
REACT_REGULATION OF ACTIVATED PAK-2P34 BY PROTEASOME MEDIATED DEGRADATION	2.051	0.003
WIP_HS_SENESCENCE_AND_AUTOPHAGY	2.048	0.004
REACT_VIF-MEDIATED DEGRADATION OF APOBEC3G	2.049	0.004
WIP_HS_OXIDATIVE_STRESS	2.035	0.004
NCI_HDAC_CLASSIII_PATHWAY	2.040	0.004
REACT_VPU MEDIATED DEGRADATION OF CD4	2.025	0.004
REACT_CDC20_PHOSPHO-APC_C MEDIATED DEGRADATION OF CYCLIN A	2.025	0.004
REACT_G1_S TRANSITION	2.019	0.004
WIP_HS_FATTY_ACID_BETA_OXIDATION	2.014	0.004
REACT_MEIOTIC SYNAPSIS	2.026	0.004
NCI_LIS1PATHWAY	2.029	0.004
WIP_HS_LEUKOCYTE_TARBASE	2.020	0.004
REACT_DESTABILIZATION OF MRNA BY AUF1 (HNRNP D0)	2.010	0.004
REACT_UBIQUITIN-DEPENDENT DEGRADATION OF CYCLIN D	2.005	0.005
KEGG_LYSOSOME	2.003	0.005
REACT_SCF(SKP2)-MEDIATED DEGRADATION OF P27_P21	2.004	0.005
REACT_ASSEMBLY OF THE PRE-REPLICATIVE COMPLEX	1.999	0.005
REACT_REGULATION OF APOPTOSIS	1.992	0.005
KEGG_PERTUSSIS	1.988	0.005
KEGG_DRUG METABOLISM - OTHER ENZYMES	1.980	0.006

REACT_APC_C_CDH1 MEDIATED DEGRADATION OF CDC20 AND OTHER APC_C_CDH1 TARGETED PROTEINS IN LATE MITOSIS_EARLY G1	1.974	0.006
NCI_AURORA_B_PATHWAY	1.973	0.006
REACT_UBIQUITIN-DEPENDENT DEGRADATION OF CYCLIN D1	1.970	0.006
REACT_G1_S DNA DAMAGE CHECKPOINTS	1.968	0.006
NCI_MYC_REPRESSPATHWAY	1.969	0.006
REACT_REGULATION OF APC_C ACTIVATORS BETWEEN G1_S AND EARLY ANAPHASE	1.968	0.006
WIP_HS_MAPK_CASCADE	1.956	0.006
REACT_ACTIVATION OF APC_C AND APC_C_CDC20 MEDIATED DEGRADATION OF MITOTIC PROTEINS	1.964	0.006
REACT_CYCLIN E ASSOCIATED EVENTS DURING G1_S TRANSITION	1.956	0.006
REACT_P53-DEPENDENT G1 DNA DAMAGE RESPONSE	1.958	0.007
KEGG_PENTOSE PHOSPHATE PATHWAY	1.956	0.007
REACT_SIGNAL TRANSDUCTION BY L1	1.960	0.007
NCI_CD8TCRDOWNSTREAMPATHWAY	1.962	0.007
REACT_UBIQUITIN MEDIATED DEGRADATION OF PHOSPHORYLATED CDC25A	1.958	0.007
REACT_P53-INDEPENDENT G1_S DNA DAMAGE CHECKPOINT	1.951	0.007
NCI_EPHBFWDPATHWAY	1.940	0.007
NCI_INTEGRIN2_PATHWAY	1.938	0.007
REACT_P53-INDEPENDENT DNA DAMAGE RESPONSE	1.931	0.008
REACT_AUTODEGRADATION OF THE E3 UBIQUITIN LIGASE COP1	1.929	0.008
REACT_SCF-BETA-TRCP MEDIATED DEGRADATION OF EMI1	1.925	0.008
REACT_P53-DEPENDENT G1_S DNA DAMAGE CHECKPOINT	1.922	0.008
REACT_S PHASE	1.922	0.008
REACT_APC_C-MEDIATED DEGRADATION OF CELL CYCLE PROTEINS	1.919	0.008
REACT_CYCLIN A_CDK2-ASSOCIATED EVENTS AT S PHASE ENTRY	1.911	0.009
REACT_APC_C_CDC20 MEDIATED DEGRADATION OF SECURIN	1.910	0.009
REACT_REGULATION OF MITOTIC CELL CYCLE	1.904	0.009
REACT_AUTODEGRADATION OF CDH1 BY CDH1_APC_C	1.905	0.009
NRF2_TARGETS	1.905	0.009
REACT_APC_C_CDC20 MEDIATED DEGRADATION OF MITOTIC PROTEINS	1.901	0.009
KEGG_AMINO SUGAR AND NUCLEOTIDE SUGAR METABOLISM	1.883	0.011
REACT_STABILIZATION OF P53	1.881	0.011
REACT_M_G1 TRANSITION	1.869	0.013
REACT_DNA REPLICATION PRE-INITIATION	1.864	0.014
KEGG_PRION DISEASES	1.856	0.014
REACT_DNA REPLICATION	1.856	0.014
NCI_MAPKTRKPATHWAY	1.852	0.015
REACT_SEMAPHORIN INTERACTIONS	1.839	0.017
KEGG_SHIGELLOSIS	1.837	0.017
REACT_METABOLISM OF NUCLEOTIDES	1.826	0.019
BIOC_INTEGRINPATHWAY	1.818	0.020
KEGG_OSTEOCLAST DIFFERENTIATION	1.816	0.020
REACT_FATTY ACID, TRIACYLGLYCEROL, AND KETONE BODY METABOLISM	1.813	0.021
REACT_CDT1 ASSOCIATION WITH THE CDC6_ORC_ORIGIN COMPLEX	1.803	0.023
REACT_CYCLIN D ASSOCIATED EVENTS IN G1	1.794	0.025
REACT_MITOTIC M-M_G1 PHASES	1.795	0.025
REACT_FORMATION OF THE HIV-1 EARLY ELONGATION COMPLEX	1.788	0.026
REACT_G1 PHASE	1.774	0.027
PPARA_TARGETS	1.775	0.027
KEGG_NON-SMALL CELL LUNG CANCER	1.780	0.027
KEGG_ADIPOCYTOKINE SIGNALING PATHWAY	1.776	0.028
WIP_HS_MITOCHONDRIAL_LC-FATTY_ACID_BETA-OXIDATION	1.779	0.028
REACT_DNA STRAND ELONGATION	1.776	0.028
KEGG_PPAR SIGNALING PATHWAY	1.777	0.028
REACT_CLASS I MHC MEDIATED ANTIGEN PROCESSING & PRESENTATION	1.750	0.034
REACT_FORMATION OF THE EARLY ELONGATION COMPLEX	1.749	0.034
WIP_HS_FOCAL_ADHESION	1.751	0.034
WIP_HS_TOLL-LIKE_RECEPTOR_SIGNALING_PATHWAY	1.750	0.034
KEGG_CHRONIC MYELOID LEUKEMIA	1.744	0.035
KEGG_REGULATION OF ACTIN CYTOSKELETON	1.741	0.035
KEGG_FATTY ACID METABOLISM	1.741	0.035

NCI_MET_PATHWAY	1.742	0.035
KEGG_TOLL-LIKE RECEPTOR SIGNALING PATHWAY	1.728	0.039
REACT_CELL CYCLE CHECKPOINTS	1.723	0.040
KEGG_AMYOTROPHIC LATERAL SCLEROSIS (ALS)	1.719	0.041
KEGG_PORPHYRIN AND CHLOROPHYLL METABOLISM	1.715	0.042
REACT_REGULATION OF MRNA STABILITY BY PROTEINS THAT BIND AU-RICH ELEMENTS	1.716	0.042
KEGG_BACTERIAL INVASION OF EPITHELIAL CELLS	1.713	0.042
WIP_HS_CELL_CYCLE	1.693	0.050
REACT_HIV-1 TRANSCRIPTION ELONGATION	1.680	0.053
KEGG_AMOEBIASIS	1.681	0.053
KEGG_TUBERCULOSIS	1.681	0.053
REACT_INTERLEUKIN-1 SIGNALING	1.682	0.054
WIP_HS_CYTOKINES_AND_INFLAMMATORY_RESPONSE	1.683	0.054
REACT_SEMA4D IN SEMAPHORIN SIGNALING	1.665	0.056
REACT_RNA POLYMERASE II TRANSCRIPTION ELONGATION	1.666	0.057
REACT_CELL CYCLE, MITOTIC	1.671	0.057
REACT_TAT-MEDIATED ELONGATION OF THE HIV-1 TRANSCRIPT	1.666	0.057
REACT_FORMATION OF RNA POL II ELONGATION COMPLEX	1.663	0.057
NCI_TRKRPATHWAY	1.670	0.057
KEGG_GALACTOSE METABOLISM	1.666	0.057
REACT_PYRIMIDINE METABOLISM	1.668	0.057
REACT_AXON GUIDANCE	1.666	0.058
REACT_FORMATION OF HIV-1 ELONGATION COMPLEX CONTAINING HIV-1 TAT	1.660	0.058
REACT_FORMATION OF HIV-1 ELONGATION COMPLEX IN THE ABSENCE OF HIV-1 TAT	1.657	0.059
NCI_NETRIN_PATHWAY	1.656	0.059
BIOC_KERATINOCYTEPATHWAY	1.655	0.059
WIP_HS_INTEGRIN-MEDIATED_CELL_ADHESION	1.643	0.064
REACT_ANTIGEN PROCESSING_ UBIQUITINATION & PROTEASOME DEGRADATION	1.641	0.064
KEGG_CHEMOKINE SIGNALING PATHWAY	1.637	0.066
REACT_TRNA AMINOACYLATION	1.634	0.067
REACT_UNFOLDED PROTEIN RESPONSE	1.633	0.067
KEGG_BLADDER CANCER	1.628	0.069
REACT_L1CAM INTERACTIONS	1.623	0.072
NCI_IL12_2PATHWAY	1.624	0.072
NCI_FOXM1PATHWAY	1.619	0.073
BIOC_PPARAPATHWAY	1.616	0.074
REACT_TRANSFERRIN ENDOCYTOSIS AND RECYCLING	1.617	0.074
REACT_METABOLISM OF LIPIDS AND LIPOPROTEINS	1.616	0.074
KEGG_FATTY ACID ELONGATION	1.599	0.083
WIP_HS_G1_TO_S_CELL_CYCLE_CONTROL	1.592	0.086
BIOC_IL1RPATHWAY	1.590	0.087
KEGG_VASOPRESSIN-REGULATED WATER REABSORPTION	1.590	0.087
WIP_HS_ERBB_SIGNALING_PATHWAY	1.586	0.088
NCI_PRLSIGNALINGEVENTSPATHWAY	1.586	0.088
WIP_HS_FLUOROPYRIMIDINE_ACTIVITY	1.587	0.088
NCI_E2F_PATHWAY	1.583	0.089
KEGG_CHAGAS DISEASE (AMERICAN TRYPANOSOMIASIS)	1.574	0.094
REACT_PHASE II CONJUGATION	1.571	0.096
WIP_HS_EPITHELIUM_TARBASE	1.571	0.096
NCI_P38ALPHABETAPATHWAY	1.567	0.097
BIOC_PYK2PATHWAY	1.567	0.097
WIP_HS_REGULATION_OF_TOLL-LIKE_RECEPTOR_SIGNALING_PATHWAY	1.565	0.098
REACT_METABOLISM OF AMINO ACIDS AND DERIVATIVES	1.558	0.102
KEGG_GLUTATHIONE METABOLISM	1.557	0.102
WIP_HS_MAPK_SIGNALING_PATHWAY	1.553	0.105
NCI_ERBB2ERBB3PATHWAY	1.548	0.108
NCI_TCRCALCIUMPATHWAY	1.543	0.110
NCI_ILK_PATHWAY	1.543	0.111
NCI_IFNGPATHWAY	1.539	0.113

BIOC_FMLPPATHWAY	1.536	0.115
WIP_HS_NOD_PATHWAY	1.530	0.117
KEGG_RNA POLYMERASE	1.530	0.117
KEGG_DNA REPLICATION	1.530	0.118
KEGG_ERBB SIGNALING PATHWAY	1.531	0.118
REACT_TRANSCRIPTIONAL REGULATION OF WHITE ADIPOCYTE DIFFERENTIATION	1.524	0.121
NCI_P53DOWNSTREAMPATHWAY	1.520	0.123
KEGG_PYRIMIDINE METABOLISM	1.509	0.132
KEGG_NEUROTROPHIN SIGNALING PATHWAY	1.508	0.132
REACT_APOPTOTIC EXECUTION PHASE	1.509	0.132
KEGG_STARCH AND SUCROSE METABOLISM	1.504	0.134
NCI_MTOR_4PATHWAY	1.500	0.136
KEGG_LEISHMANIASIS	1.499	0.136
BIOC_IL6PATHWAY	1.500	0.137
BIOC_MAPKPATHWAY	1.496	0.137
REACT_METABOLISM OF RNA	1.496	0.138
KEGG_NATURAL KILLER CELL MEDIATED CYTOTOXICITY	1.496	0.138
BIOC_RASPATHWAY	1.492	0.140
REACT_SIGNALLING TO RAS	1.491	0.140
NCI_RET_PATHWAY	1.489	0.141
KEGG_FOCAL ADHESION	1.488	0.141
KEGG_ENDOMETRIAL CANCER	1.483	0.144
REACT_METABOLISM OF WATER-SOLUBLE VITAMINS AND COFACTORS	1.483	0.144
WIP_HS_RANKL-RANK_SIGNALING_PATHWAY	1.477	0.145
NCI_SMAD2_3NUCLEARPATHWAY	1.483	0.145
WIP_HS_REGULATION_OF_ACTIN_CYTOSKELETON	1.480	0.145
NCI_CXCR3PATHWAY	1.480	0.145
KEGG_PROSTATE CANCER	1.477	0.146
BIOC_TNFR1PATHWAY	1.478	0.146
BIOC_INSULINPATHWAY	1.477	0.146
NCI_P75NTRPATHWAY	1.472	0.149
BIOC_IL2PATHWAY	1.472	0.149
WIP_HS_APOPTOSIS	1.470	0.149
KEGG_PROGESTERONE-MEDIATED OOCYTE MATURATION	1.470	0.150
NCI_RB_1PATHWAY	1.468	0.150
NCI_S1P_S1P1_PATHWAY	1.466	0.151
KEGG_EPITHELIAL CELL SIGNALING IN HELICOBACTER PYLORI INFECTION	1.460	0.156
REACT_IRON UPTAKE AND TRANSPORT	1.458	0.156
NCI_CDC42_PATHWAY	1.460	0.156
KEGG_ENDOCYTOSIS	1.457	0.157
REACT_INSULIN RECEPTOR RECYCLING	1.455	0.158
KEGG_NOD-LIKE RECEPTOR SIGNALING PATHWAY	1.453	0.160
REACT_SLC-MEDIATED TRANSMEMBRANE TRANSPORT	1.443	0.167
KEGG_ACUTE MYELOID LEUKEMIA	1.445	0.167
REACT_TRAF6 MEDIATED INDUCTION OF PROINFLAMMATORY CYTOKINES	1.444	0.167
KEGG_AXON GUIDANCE	1.441	0.168
KEGG_T CELL RECEPTOR SIGNALING PATHWAY	1.441	0.168
REACT_GPVI-MEDIATED ACTIVATION CASCADE	1.439	0.169
KEGG_FRUCTOSE AND MANNOSE METABOLISM	1.438	0.170
NCI_HIF1_TFPATHWAY	1.435	0.170
NCI_TOLL_ENDOGENOUS_PATHWAY	1.436	0.171
KEGG_CYTOSOLIC DNA-SENSING PATHWAY	1.435	0.171
REACT_TOLL LIKE RECEPTOR 3 (TLR3) CASCADE	1.432	0.172
REACT_INTRINSIC PATHWAY FOR APOPTOSIS	1.432	0.172
KEGG_BIOSYNTHESIS OF UNSATURATED FATTY ACIDS	1.427	0.172
REACT_SIGNALING BY EGFR	1.428	0.173
NCI_PDGFRBPATHWAY	1.426	0.173
KEGG_CELL CYCLE	1.423	0.173
NCI_PS1PATHWAY	1.428	0.173
KEGG_MAPK SIGNALING PATHWAY	1.424	0.174
WIP_HS_SIGNALING_OF_HEPATOCYTE_GROWTH_FACTOR_RECEPTOR	1.423	0.174
WIP_HS_INSULIN_SIGNALING	1.428	0.174

KEGG_MELANOMA	1.429	0.174
NCI_PLK1_PATHWAY	1.421	0.174
NCI_CERAMIDE_PATHWAY	1.422	0.174
NCI_FOXOPATHWAY	1.419	0.175
NCI_PTP1BPATHWAY	1.418	0.175
NCI_VEGFR1_2_PATHWAY	1.429	0.175
REACT_SIGNALLING TO ERKS	1.419	0.175
REACT_CELL SURFACE INTERACTIONS AT THE VASCULAR WALL	1.420	0.175
REACT_CHOLESTEROL BIOSYNTHESIS	1.416	0.176
KEGG_STAPHYLOCOCCUS AUREUS INFECTION	1.415	0.176
REACT_RHO GTPASE CYCLE	1.413	0.178
NCI_NFAT_TFPATHWAY	1.407	0.182
REACT_M PHASE	1.407	0.183
KEGG_COLLECTING DUCT ACID SECRETION	1.403	0.183
NCI_REG_GR_PATHWAY	1.406	0.183
REACT_REGULATION OF LIPID METABOLISM BY PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA (PPARALPHA)	1.402	0.183
REACT_HIV INFECTION	1.403	0.183
KEGG_PEROXISOME	1.403	0.184
WIP_HS_IL-1_PATHWAY	1.403	0.184
REACT_PYRUVATE METABOLISM AND CITRIC ACID (TCA) CYCLE	1.404	0.185
REACT_TOLL LIKE RECEPTOR 7_8 (TLR7_8) CASCADE	1.396	0.188
REACT_SIGNALING BY RHO GTPASES	1.393	0.191
WIP_HS_SELENIUM_PATHWAY	1.390	0.193
REACT_MRNA PROCESSING	1.390	0.193
REACT_TOLL LIKE RECEPTOR 9 (TLR9) CASCADE	1.390	0.193
NCI_HEDGEHOG_GLIPATHWAY	1.388	0.194
WIP_HS_NUCLEAR_RECEPTORS	1.383	0.199
BIOC_TOLLPATHWAY	1.383	0.199
KEGG_GNRH SIGNALING PATHWAY	1.378	0.202
NCI_IL8CXCR2_PATHWAY	1.378	0.202
REACT_TRAF6 MEDIATED INDUCTION OF NFKB AND MAP KINASES UPON TLR7_8 OR 9 ACTIVATION	1.379	0.203
KEGG_SYNAPTIC VESICLE CYCLE	1.373	0.203
WIP_HS_TGF_BETA_SIGNALING_PATHWAY	1.376	0.204
BIOC_STRESSPATHWAY	1.373	0.204
KEGG_PHAGOSOME	1.374	0.204
REACT_P75 NTR RECEPTOR-MEDIATED SIGNALLING	1.374	0.205
BIOC_ERKPATHWAY	1.374	0.205
NCI_FCER1PATHWAY	1.370	0.206
NCI_HNF3BPATHWAY	1.369	0.207
NCI_ATF2_PATHWAY	1.365	0.210
BIOC_HIVNEFPATHWAY	1.366	0.210
KEGG_COLORECTAL CANCER	1.364	0.210
REACT_MYD88 DEPENDENT CASCADE INITIATED ON ENDOSOME	1.364	0.211
REACT_RNA POL II CTD PHOSPHORYLATION AND INTERACTION WITH CE	1.353	0.216
NCI_PI3KPLCTRKPATHWAY	1.358	0.216
WIP_HS_ANDROGEN_RECEPTOR	1.353	0.216
KEGG_COMPLEMENT AND COAGULATION CASCADES	1.354	0.216
REACT_MITOTIC PROMETAPHASE	1.357	0.217
REACT_METABOLISM OF MRNA	1.355	0.217
NCI_FGF_PATHWAY	1.356	0.217
REACT_MAP KINASE ACTIVATION IN TLR CASCADE	1.354	0.217
KEGG_VIBRIO CHOLERAE INFECTION	1.351	0.217
NCI_IL1PATHWAY	1.354	0.217
REACT_INTEGRIN CELL SURFACE INTERACTIONS	1.351	0.217
BIOC_GHPATHWAY	1.355	0.217
WIP_HS_FOLATE_METABOLISM	1.349	0.218
NCI_FAK_PATHWAY	1.346	0.221
REACT_CHEMOKINE RECEPTORS BIND CHEMOKINES	1.347	0.221
WIP_HS_ANGIOGENESIS_OVERVIEW	1.344	0.222

REACT_RECYCLING PATHWAY OF L1	1.344	0.222
KEGG_PATHOGENIC ESCHERICHIA COLI INFECTION	1.341	0.225
WIP_HS_INTRINSIC_PATHWAY_FOR_APOPTOSIS	1.340	0.225
NCI_HNF3APATHWAY	1.339	0.226
NCI_AVB3_OPN_PATHWAY	1.339	0.226
REACT_SIGNALLING BY NGF	1.337	0.227
NCI_UPA_UPAR_PATHWAY	1.334	0.231
REACT_HOST INTERACTIONS OF HIV FACTORS	1.333	0.231
REACT_PROTEIN FOLDING	1.332	0.231
NCI_SYNDECAN_2_PATHWAY	1.327	0.236
KEGG_MUCIN TYPE O-GLYCAN BIOSYNTHESIS	1.323	0.242
REACT_MRNA CAPPING	1.320	0.245
NCI_NECTIN_PATHWAY	1.318	0.245
WIP_HS_SIGNAL_TRANSDUCTION_OF_S1P_RECEPTOR	1.319	0.245
REACT_METABOLISM OF VITAMINS AND COFACTORS	1.318	0.245
NCI_LYSOPHOSPHOLIPID_PATHWAY	1.317	0.246
REACT_TRANSPORT OF GLUCOSE AND OTHER SUGARS, BILE SALTS AND ORGANIC ACIDS, METAL IONS AND AMINE COMPOUNDS	1.316	0.246
REACT_FORMATION AND MATURATION OF MRNA TRANSCRIPT	1.315	0.247
BIOC_EGFPATHWAY	1.313	0.248

Supplementary table 1b. Gene sets downregulated in PBMCs upon twelve weeks of 20% ER compared to control.

Gene set	**NES**	**FDR q-value**
KEGG_TASTE TRANSDUCTION	-1.786	0.221

Chapter 5

Comparison of postprandial whole genome gene expression response in peripheral blood mononuclear cells between an oral glucose tolerance test and a mixed meal test

5

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In preparation

Abstract

Background: The peripheral blood mononuclear cell (PBMC) transcriptome has been shown to respond to long-term dietary interventions and to acute dietary interventions such as challenge tests. Examples of dietary challenge tests are the oral glucose tolerance test (OGTT), the high fat challenge test, and the mixed meal test (MMT). The difference in changes in gene expression due to differences in macronutrient composition of dietary challenge tests has not yet been fully elucidated.

Objective: We aimed to investigate the effect of the addition of other macronutrients to glucose on the changes in gene expression in PBMCs, by comparing the postprandial response of an OGTT with an MMT on whole genome gene expression of PBMCs.

Methods: 68 healthy, overweight men and women, aged 50-65, completed an OGTT and an MMT, the MMT was made out of the 75 g glucose of the OGTT plus 60 g fat and 20 g protein. PBMCs whole genome gene expression was compared postprandially between the OGTT and the MMT at timepoints 0, 60, and 120 minutes. Changes in insulin, glucose, triglycerides (TAGs), and non-esterified-fatty acid (NEFA) levels, as well as the blood cell subpopulation were compared at timepoints 0, 60, 120 and 240 minutes.

Results & Conclusions: In conclusion, the addition of fat and protein to glucose in a challenge test leads to a different postprandial response for all metabolites measured except for insulin. The postprandial changes in gene expression showed a higher number of overlap than the number of different changes in gene expression between the MMT and the OGTT. The difference in changes in gene expression was present for gene sets involved in cell cycle and platelet activation. Both the MMT as the OGTT downregulated gene sets involved in fatty acid metabolism, TCA cycle, and OXPHOS. Based on the high overlap in gene expression changes and the related metabolic pathways, we hypothesize that glucose in a challenge test is the main denominator of the postprandial changes in gene expression in the first two hours, which might mainly be regulated via insulin.

Introduction

Nutritional challenge tests are gaining more attention in nutrition research as a tool to magnify the differences in health states and to measure phenotypic flexibility [1]. Well-known nutritional challenge tests are the oral glucose tolerance test (OGTT), a high fat challenge, and the mixed meal test (MMT). An OGTT consists of a high load of glucose and measures how well the body is able to clear this glucose from the blood. OGTTs are widely applied in the clinic to diagnose insulin resistance in participants, by measuring the time course of glucose and insulin levels in the blood upon two hours. A high fat challenge consists of a high load of fat and can contain different types of fatty acids such as saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), or polyunsaturated fatty acids (PUFAs). Studies have used the high fat challenge test to magnify differences in health states and showed for example that high fat challenges resulted in more pronounced triglyceride (TAGs) responses in obese and obese diabetic participants compared to lean participants [2]. Also the postprandial triglyceride response has been shown to be superior to fasting levels for the assessment of cardiovascular disease risk as reviewed by Boren et al. [3], and Pirillo et al. [4].

An MMT consists of a combination of macronutrients including carbohydrates, protein and fat, and has a higher energy content. Based on the composition, an MMT reflects a more commonly consumed meal than a high fat challenge or an OGTT [5].

In addition to the postprandial responses of glucose, insulin, and TAGs, omics technologies can be applied to study postprandial responses upon challenge tests. We previously showed that the use of the combination of transcriptomics with a high fat challenge test magnified differences between lean and obese individuals when compared to the fasting state [6], when peripheral blood monocular cells (PBMCs) were studied. PBMCs are relevant cells to study during postprandial responses since they are easy accessible over time as they circulate in the blood and have been shown multiple times to reflect diet-related changes in their gene expression [7, 8].

Some studies have been performed that compared the postprandial response of the transcriptome between different type of dietary challenges, such as different types of fatty acids [2] and a high fat challenge versus an OGTT [9]. The difference in changes in gene expression due to differences in macronutrient composition has not yet been fully elucidated. Especially not for the comparison of a single nutrient load such as glucose to a more complex nutritional composition reflecting a commonly consumed meal. In this study we aimed to investigate the gene expression changes during two challenge tests that had either glucose alone (OGTT) or the same amount of glucose with the addition of fat and protein (MMT), resembling a more commonly consumed meal. We compared the postprandial gene expression changes at different timepoints between an MMT and an OGTT in PBMCs from 68 healthy, overweight participants.

Materials and Methods

Participants

81 healthy, overweight, Caucasian men and women, aged 50-65, with a BMI 25-35kg/m^2 were included in this study. Exclusion criteria included: weight change of ≥ 3kg in the preceding three months, current smokers, substance abuse, excess alcohol intake, pregnancy, diabetes, cardiovascular disease, cancer, gastrointestinal disease e.g. inflammatory bowel disease or irritable bowel syndrome, kidney disease, liver disease, pancreatitis, use of medications likely to interfere with energy metabolism, appetite regulation and hormonal balance (including: anti-inflammatory drugs or steroids, antibiotics, androgens, phenytoin, erythromycin or thyroid hormones), having any metallic or magnetic implants such as pacemakers, and claustrophobia.

Nutritional challenge tests

A total number of 72 participants completed the oral glucose tolerance test (OGTT) and 68 participants completed also a mixed meal test (MMT) the next day. The OGTT consisted of 75 g of glucose in 250 mL of water, the MMT consisted of 75 g of glucose, 60 g of palm oil and 20 g of protein (Protifar, Nutricia) mixed with 320 g of water [5]. Insulin and glucose concentrations were measured in the fasted state and during these challenge tests by the laboratory of Imperial College London, United Kingdom. Triglycerides were measured in the fasted state and during these challenge tests by the laboratory of Medical University Varna (MUV, Varna, Bulgaria). Peripheral blood mononuclear cells (PBMCs) were collected in the fasted state, and postprandially at 30, 60, and 120 minutes during the OGTT and at 60, 120, 240, and 360 minutes during MMT. Each subject provided written informed consent. The study was executed at Imperial College London (London, UK) and approved by the Brent Ethics Committee (REC ref: 12/LO/0139) and was registered at clinicaltrials.gov as NCT01684917. All fasting values were based on the timepoint 0 on the day of the OGTT.

PBMC RNA isolation and microarray processing

PBMCs were isolated from whole blood using BD Vacutainer® Cell Preparation Tubes™ according to the manufacturer's instructions. Total RNA was isolated from PBMC samples using Trizol reagent (Invitrogen, Breda, The Netherlands) and purified using Qiagen RNeasy Micro Kit (Qiagen, Venlo, The Netherlands). RNA integrity was checked with Agilent 2100 bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). From the isolated RNA we used the timepoints 0, 60 and 120 minutes for microarray analysis, these were present during the OGTT and the MMT. 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 Gene 1.1 ST arrays targeting 19 697 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 [10]. Microarray signals were normalized using robust multichip average (RMA) [11]. Genes with normalized signals >20 on at least 100 arrays were defined as expressed and selected for further analysis. Significant different expression of individual genes were tested using the LIMMA R library [12]. Changes were considered significant when P-value was <0.05 in a t-test/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 [13]. Gene sets with a false discovery rate (FDR q-value) <0.25 were defined as significantly regulated. Next to general GSEA, we performed single sample GSEA as described in [14].

Postprandial data analysis on gene expression and gene set level was performed as follows: (1) each postprandial value was compared to its fasting value (T30-T0, T60-T0, etc.); and (2) to determine the difference in postprandial response between the MMT and OGTT these values were compared between the two challenge tests [(MMT(T60-T0)) - (OGTT(T60-T0)), etc.].

Overlap in genes and gene sets

The overlap in gene sets at different time points was visualised using UpSetR [15]. The selection of overlapping gene sets during the postprandial courses of both challenge tests was performed using the following steps (1) exclusion of significantly different changed gene sets [FDR q-value>0.25 for (MMT(T0)) - (OGTT(T0)), FDR q-value>0.25 for (MMT(T60-T0)) - (OGTT(T60-T0))], etc.], and (2) gene sets should be significantly changed (FDR q-value<0.30) at at least two timepoints within the challenge tests [FDR q-value<0.30 for (MMT(T60-T0)) and for (MMT(T120-T0)), or for (MMT(T60-T0)) and for (OGTT(T60-T0)), or (MMT(T120-T0)) and (OGTT(T60-T0)), etc.].
Enrichment Map is a Cytoscape plugin [16] and was used for functional enrichment visualisation and clustering. Array data have been submitted to the Gene Expression Omnibus under accession number GSE88794.

Statistical analysis of biochemical measurements

The statistical package SPSS (IBM Corp. Released 2013. IBM SPSS Statistics for Windows (Version 22.0. Armonk, NY: IBM Corp.) was used for analysis of the biochemical measurements. Statistical comparisons of repeated measures in the challenge tests were performed by linear mixed models for repeated measures (covariance type: compound symmetry). Comparisons of postprandial responses were preformed using delta values (changes from baseline) as dependent variables, and 'Challenge', 'timepoint(mins)', and 'Challenge x timepoint(mins)' as fixed effects. If the fasting values were significantly different upon the intervention, then the delta value from the fasting values from before the intervention and after the intervention, were used as covariate.

Results

Baseline characteristics and fasting blood parameters of the 68 participants of which microarray data of both challenge tests at all were present, are summarised in table 1.

Table 1. Baseline characteristics of 68 participants.

	Participants (n=68)
Gender	32♂ / 36♀
Age (y)	59 [50,65]
Weight (kg)	83.3 ± 12.2
BMI (kg/m^2)	29.0 ± 2.7
Waist circumference	99.0 ± 10.0
Body fat (%)	34.0 ± 7.6
Glucose (mmol/L)	5.17 ± 0.57
Insulin (mmol/L)	15.8 ± 7.08
Triglycerides (mmol/L)	1.29 ± 0.49
NEFAs (mmol/L)	0.37 ± 0.17
Lymphocytes (# giga/L)	1.63 ± 0.44
Monocytes (# giga/L)	0.46 ± 0.15
Lymphocytes/monocytes-ratio	4.00 ± 1.62
Neutrophils (# giga/L)	3.00 ± 1.56

Data represent mean and ±SD, or median and [range].
♂: Men ♀: Women.

Postprandial effect of MMT and OGTT on blood parameters

Significant different postprandial changes between the MMT and the OGTT were observed for plasma levels of glucose, triglycerides, and NEFAs as well as for numbers of lymphocytes, monocytes, neutrophils and the lymphocytes/monocytes-ratio, (table 2, figure 1). The postprandial plasma insulin response was not significantly different between the MMT and the OGTT. Baseline differences were observed for plasma levels of glucose, insulin and NEFAs, and for the blood cell population of monocytes, the lymphocytes/monocytes-ratio and the neutrophils.

Table 2. Effect of a mixed meal test and oral glucose tolerance test on postprandial blood parameters. Depicted are the fasting levels (0 min) followed by the change in response compared to fasting (60 min vs 0 min) for plasma levels of glucose, insulin, triglycerides, non-esterified fatty acids (NEFAs), and blood numbers of lymphocytes, monocytes, neutrophils, and the lymphocytes/monocytes-ratio. Significance is shown for the postprandial response within the challenge test and between both challenge tests as calculated by linear mixed models.

		TIME				P-value		
		0 min	Δ60 min	Δ120 min	Δ240 min	Baseline (0 min)	Timepoint (mins)[1]	Challenge x Timepoint(mins)[2]
Glucose (mmol/L)	MMT	5.07 ± 0.52	1.61 ± 1.48	0.62 ± 1.43	-0.72 ± 0.83	0.02	<0.001	<0.001
	OGTT	5.17 ± 0.57	2.92 ± 2.25	0.98 ± 2.00	-1.32 ± 0.63		<0.001	
Insulin (mmol/L)	MMT	18.33 ± 7.54	91.04 ± 64.30	60.49 ± 42.10	6.43 ± 13.08	<0.001	<0.001	0.785
	OGTT	15.82 ± 7.08	87.37 ± 61.09	55.05 ± 55.14	-2.32 ± 5.53		<0.001	
Triglycerides (mmol/L)	MMT	1.34 ± 0.52	0.21 ± 0.50	0.48 ± 0.67	0.75 ± 0.74	0.36	<0.001	<0.001
	OGTT	1.29 ± 0.49	0.08 ± 0.28	-0.06 ± 0.22	0.06 ± 0.34		<0.001	
NEFAs (mmol/L)	MMT	0.33 ± 0.14	-0.23 ± 0.13	-0.26 ± 0.13	-0.07 ± 0.14	0.008	<0.001	<0.001
	OGTT	0.37 ± 0.17	-0.28 ± 0.17	-0.34 ± 0.17	-0.04 ± 0.17		<0.001	
Lymphocytes (# giga/L)	MMT	1.69 ± 0.46	-0.04 ± 0.22	0.12 ± 0.32	0.41 ± 0.39	0.079	<0.001	<0.001
	OGTT	1.63 ± 0.44	-0.11 ± 0.23	0.08 ± 0.27	0.75 ± 0.70		<0.001	
Monocytes (# giga/L)	MMT	0.43 ± 0.13	-0.05 ± 0.09	0.04 ± 0.11	0.12 ± 0.11	0.042	<0.001	0.062[†]
	OGTT	0.45 ± 0.15	-0.09 ± 0.09	0.01 ± 0.10	0.13 ± 0.14		<0.001	
Lymphocytes/ monocytes-ratio	MMT	4.25 ± 1.54	0.65 ± 1.34	0.07 ± 1.04	-0.09 ± 0.95	0.022	<0.001	0.039
	OGTT	4.00 ± 1.62	0.68 ± 1.27	0.10 ± 1.00	0.43 ± 1.05		<0.001	
Neutrophils (# giga/L)	MMT	2.70 ± 0.87	0.31 ± 0.30	0.55 ± 0.51	0.70 ± 0.61	0.049	<0.001	<0.001[††]
	OGTT	3.00 ± 1.56	0.15 ± 0.28	0.19 ± 0.43	0.35 ± 0.75		<0.001	

Data represent mean and ±SD. MMT: mixed meal test; OGTT: oral glucose tolerance test. [1]Timepoint(mins) = Effect of time within challenge test; [2]Challenge x timepoint(mins) = differences between challenge on the response. [†]This interaction effect was significant when corrected for fasting (P=0.001); [††]This interaction effect was not significant when corrected for fasting (P=0.085).

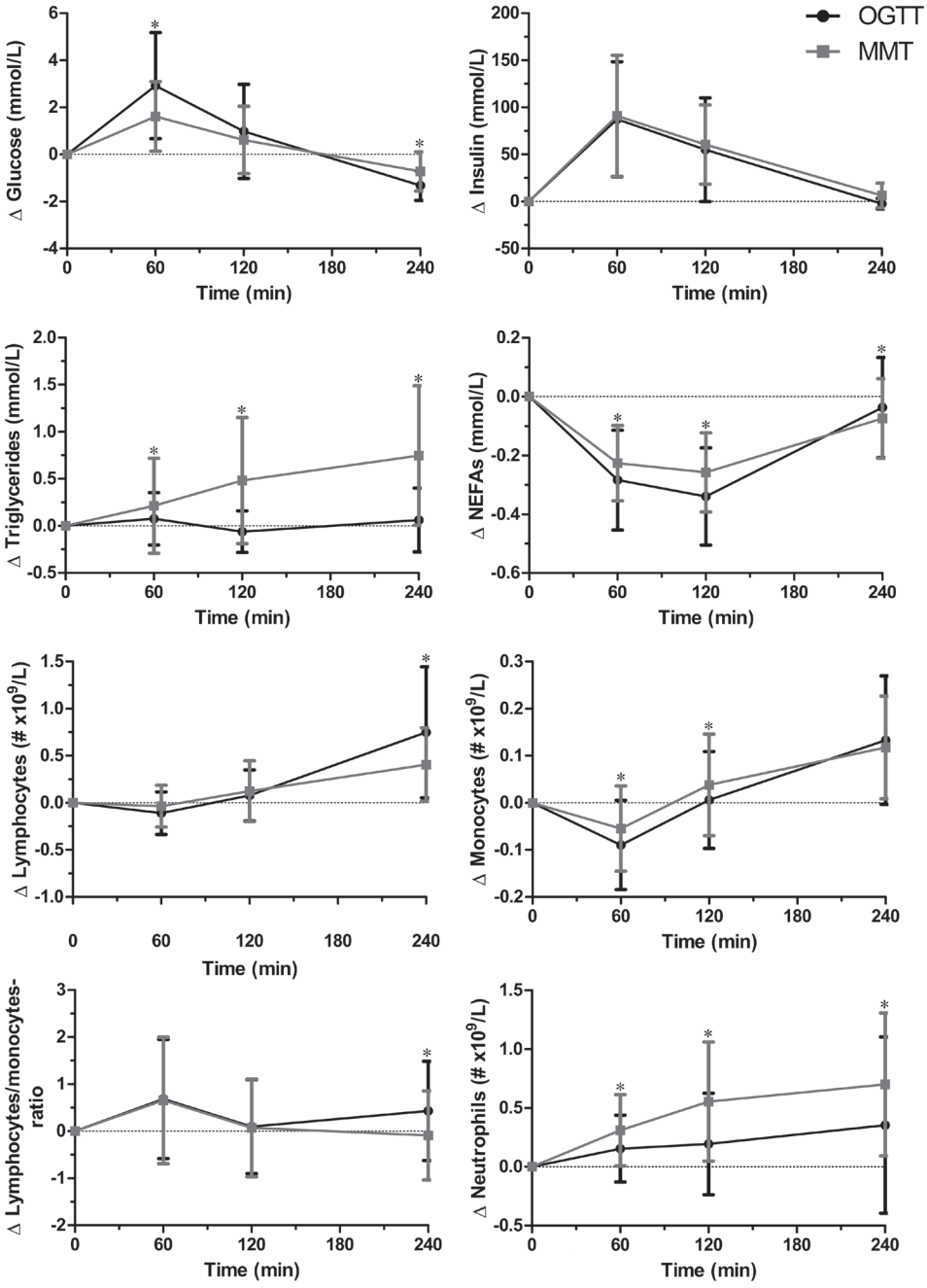

Figure 1. Effect of a mixed meal test and oral glucose tolerance test on postprandial blood parameters. Data represent mean and ±SD. Depicted is the postprandial response, as a change in response compared to fasting (60 min vs 0 min) of the MMT (■) and OGTT (●) for levels of glucose, insulin, triglycerides, non-esterified fatty acids (NEFAs), and numbers of lymphocytes, monocytes, neutrophils, and the lymphocytes/monocytes-ratio. An asterisk (*) indicates that values are statsitically different between the MMT and the OGTT at that timepoint.

Effect of an MMT and an OGTT on postprandial gene expression

To identify the difference and overlap in effect of the MMT and OGTT on whole genome gene expression in PBMCs, gene expression responses during the OGTT and MMT were compared. Figure 2a shows the comparisons in postprandial response between the two challenge tests at 60 and 120 minutes. The difference in postprandial gene expression changes between both challenges ranged from 196 to 372 genes, and 1684 genes were changed in the fasted state. The overlap in postprandial gene expression changes between both challenges ranged from 1304 to 1903 genes. To further elucidate the overlap in gene expression changes between the different timepoints we compared each timepoint between the MMT and OGTT with each other (figure 2b).

To identify the difference and overlap in potential gene sets and signalling routes that changed during the MMT and OGTT, Gene Set Enrichment Analysis (GSEA) was performed. Figure 3a shows the comparisons in postprandial response for gene sets between the two challenge tests at 60 and 120 minutes. The difference in postprandial gene sets between both challenges ranged from 42 to 55 gene sets, and 249 gene sets were changed in the fasted state. The overlap in postprandial gene sets between both challenges ranged from 11 to 90 gene sets. To further elucidate the overlap in gene sets between the different timepoints, we compared each timepoint between the MMT and OGTT with each other (figure 3b). Gene sets significantly changed per timepoint are shown in supplementary table 1 (MMT) and supplementary table 2 (OGTT).

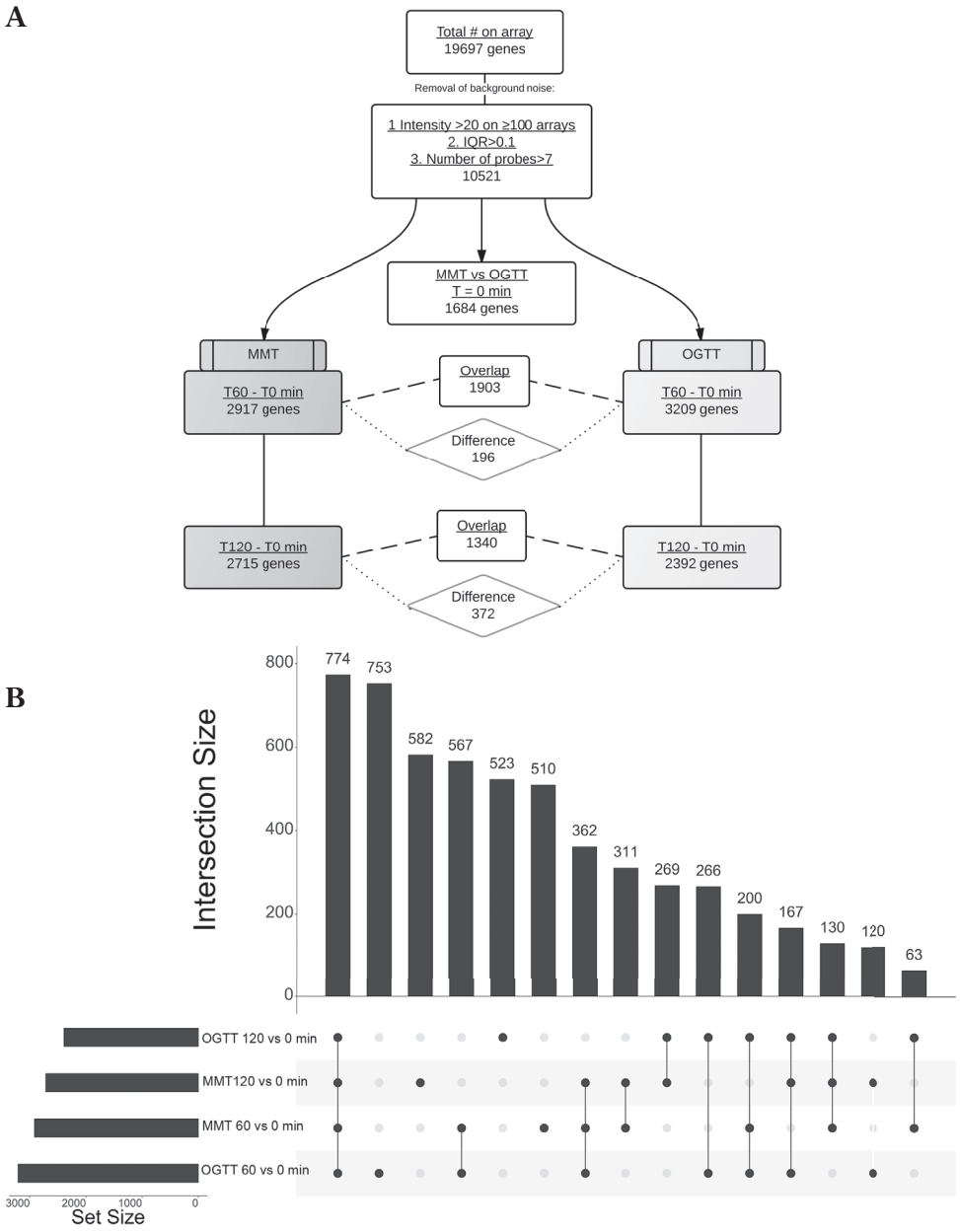

Figure 2. Significant changes in gene expression during the mixed meal test and the oral glucose tolerance test. **a.** Depicted is the stepwise selection of genes in microarray analysis, based on intensity, IQR, and number of probes. The significant ($P<0.05$) changed gene expression between or within the mixed meal test (MMT, left track) and oral glucose tolerance test (OGTT, right track) is shown. **b.** This graph shows the overlap of the number of genes that was significantly changed ($P<0.05$) during the OGTT and MMT at different timepoints. The horizontal bars depict the total number of genes of which the expression was significantly changed for each timepoint; the vertical bars depict the number of overlapping genes for the different timepoints. Overlapping timepoints are indicated by (●).

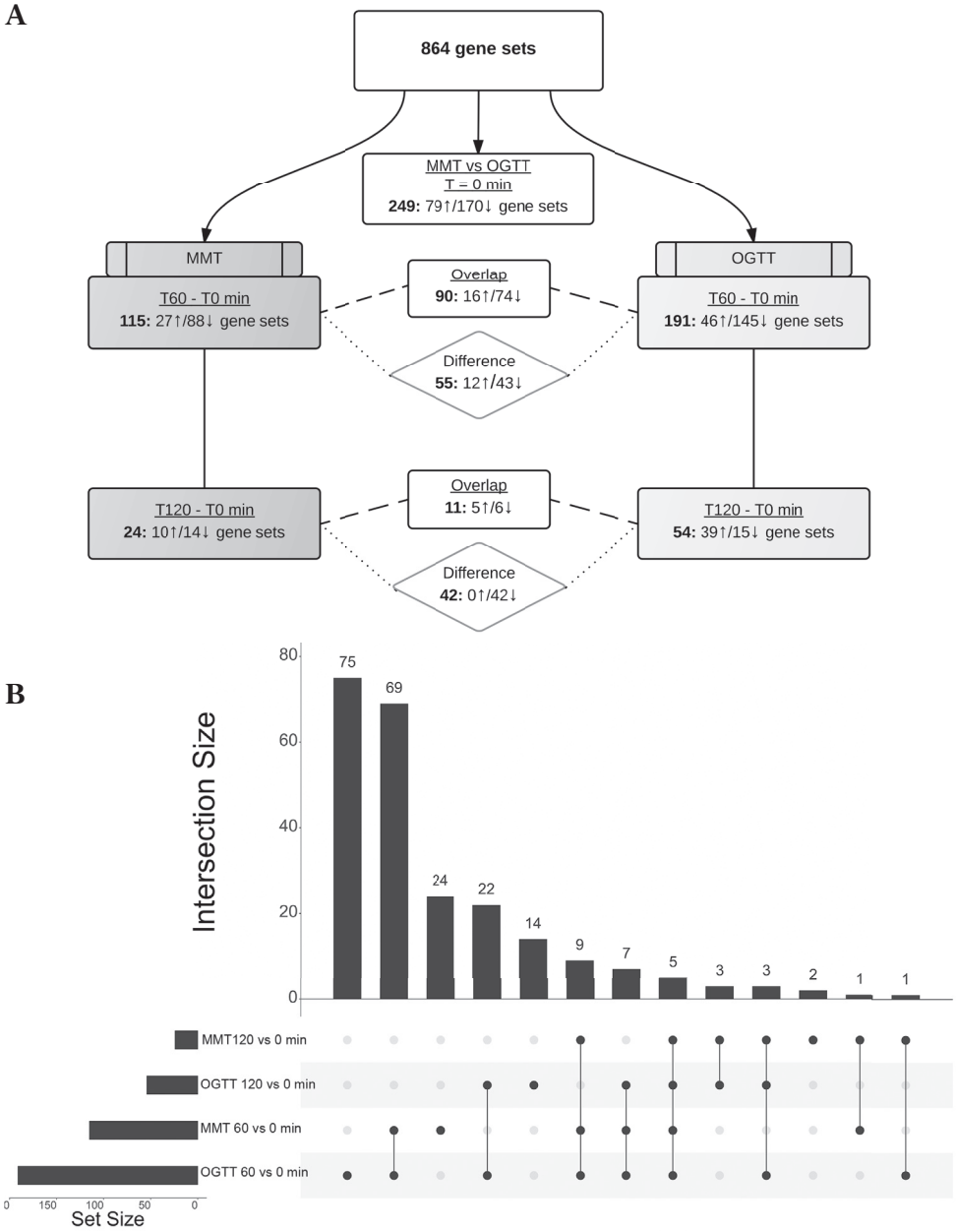

Figure 3. Significant changes in gene sets during the mixed meal test and the oral glucose tolerance test.
a. Depicted is the result of Gene Set Enrichment Analysis, showing the significantly changed (FDR q-value<025) gene sets during the mixed meal test (MMT, left track) and oral glucose tolerance test (OGTT, right track). Total numbers are shown in bold, direction of gene sets is shown as: ↑ up, and ↓ down. **b.** This graph shows the overlap of the number of gene sets that was significantly changed (FDR q-value<0.25) during an oral glucose tolerance test (OGTT) and mixed meal test (MMT) at different timepoints. The horizontal bars depict the total number of gene sets that was significantly changed for each timepoint; the vertical bars depict the number of overlapping gene sets for the different timepoints. Overlapping timepoints are indicated by (●).

Because of the high number in overlap between the MMT and OGTT, we selected the overlapping gene sets between the OGTT and the MMT. The exact selection procedure is described in the methods section of this paper. Briefly, gene sets different in the fasted state (0 minutes) between the challenge test were excluded, and overlap was required at at least two timepoints. This selection resulted in 69 overlapping gene sets, which were clustered based on the genes that were enriched in these gene sets. Gene sets that showed a large overlap between the MMT and OGTT could be identified as downregulated clusters involved in lipid metabolism, TCA cycle, oxidative phosphorylation (OXPHOS), transport, iron transport, tryptophan metabolism, lymphocyte tarbase, HLA-immune, and cell adhesion; and as upregulated clusters involved in immune cell type-specific, and WNT-signalling; and as a group of miscellaneous gene sets (supplementary figure 1). The data of the gene sets involved in energy metabolism are visualized in figure 4. Gene sets involved in lipid metabolism were downregulated at 60 and 120 minutes during both challenge tests, whereas gene sets involved in beta-oxidation related gene sets were downregulated only at 120 minutes during both challenge tests. OXPHOS related gene sets were significantly downregulated at 60 minutes only during both challenge tests. TCA cycle related gene sets were changed at 60 minutes during both challenge tests, and at 120 minutes only during the OGTT.

		Normalised Enrichment Score				FDR q-value			
		MMT		OGTT		MMT		OGTT	
		T60-T0	T120-T0	T60-T0	T120-T0	T60-T0	T120-T0	T60-T0	T120-T0
Fatty acid metabolism	REACT_METABOLISM OF LIPIDS AND LIPOPROTEINS	-1.74	-1.58	-1.93	-1.56	0.14	0.37	0.02	0.41
	REACT_SPHINGOLIPID METABOLISM	-1.53	-1.42	-1.52	-1.51	0.23	0.39	0.17	0.47
	KEGG_PPAR SIGNALING PATHWAY	-1.48	-1.30	-1.52	-1.09	0.27	0.44	0.17	0.78
	REACT_REGULATION OF LIPID METABOLISM BY PPARALPHA	-1.76	-1.68	-1.65	-1.71	0.14	0.28	0.11	0.25
	REACT_FATTY ACID, TRIACYLGLYCEROL, AND KETONE BODY METABOLISM	-1.63	-1.94	-1.81	-1.74	0.15	0.13	0.05	0.21
	PPARA_TARGETS	-1.50	-2.01	-1.77	-2.03	0.26	0.18	0.06	0.15
	WIP_HS_MITOCHONDRIAL_LC-FATTY_ACID_BETA-OXIDATION	-1.30	-1.79	-1.03	-1.74	0.45	0.21	0.66	0.22
	WIP_HS_FATTY_ACID_BETA_OXIDATION	-0.70	-1.74	-1.17	-1.71	0.96	0.23	0.48	0.24
TCA cycle	WIP_HS_TCA_CYCLE	-1.86	-1.69	-1.64	-1.94	0.06	0.26	0.11	0.22
	REACT_PYRUVATE METABOLISM AND CITRIC ACID (TCA) CYCLE	-1.68	-1.50	-1.54	-1.89	0.13	0.36	0.16	0.19
OXPHOS	KEGG_HUNTINGTON'S DISEASE	-1.73	-0.92	-1.56	-1.18	0.14	0.89	0.15	0.74
	KEGG_OXIDATIVE PHOSPHORYLATION	-1.52	-0.48	-1.42	-0.75	0.24	1.00	0.26	0.95
		-1.8 ... -1				FDR q-value <0.25			

Figure 4. Selection of gene sets overlapping between the mixed meal test and oral glucose tolerance test involved in energy metabolism. Depicted are the energy metabolism related gene sets that were significantly changed at at least two timepoints. Numbers reflect the normalized enrichment score (NES) which indicates the magnitude of the change and is illustrated in blue. Furthermore the significance is shown in dark pink as FDR q-value<0.25; gene sets with a FDR q-value between 0.25-0.30 are shown in light pink; PPAR: Peroxisome proliferator-activated receptor; Gene set 'KEGG_HUNTINGTON'S DISEASE' contains genes involved in oxidative phosphorylation.

We also analysed the responses of those gene sets per individual using single sample GSEA (ssGSEA) (figure 5). Large interindividual variation was found at all timepoints. Each individual had an overall consistent response for each cluster of gene sets but does not consistently respond between the OGTT and the MMT.

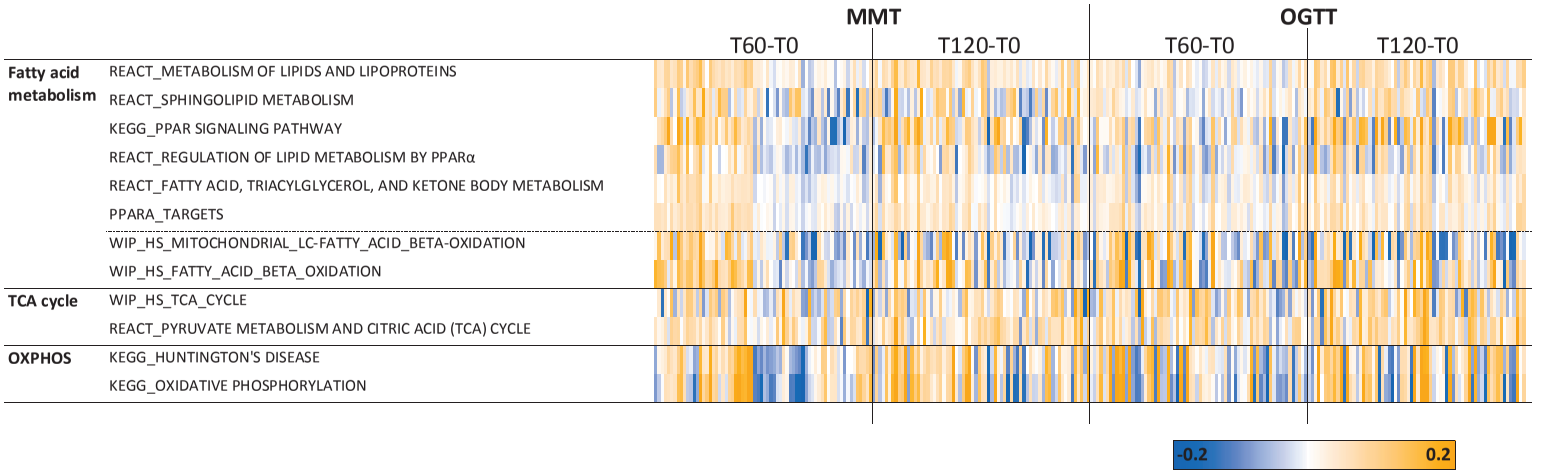

Figure 5. Selection of gene sets overlapping between the mixed meal test and oral glucose tolerance test involved in energy metabolism displayed for each individual. Depicted is the result of single sample Gene Set Enrichment Analysis (ssGSEA), each column representing an individual which is shown for the 68 subjects at 60 and 120 minutes during the mixed meal test (MMT) and at 60 and 120 during the oral glucose tolerance test (OGTT). This graph shows the individual response to the MMT and OGTT for each individual and displays the high variation in response for each gene set.

Discussion

In this study we aimed to compare the postprandial changes in gene expression in PBMCs during an OGTT and during an MMT. The difference between the MMT and the OGTT was much smaller than the overlap during the MMT and the OGTT for both the number genes and the number of gene sets. The difference between OGTT compared to the MMT was reflected by an increase of gene sets involved in cell cycle, and a decrease of gene sets involved in platelet activation. The overlap between the MMT and the OGTT included a downregulation of gene sets involved in fatty acid metabolism, TCA cycle, and OXPHOS.

We found one other study that compared the postprandial changes in gene expression in PBMCs upon two challenge tests composed of different macronutrients. In this study timepoint 60 minutes during an OGTT and timepoint 240 minutes during an oral lipid tolerance test (OLTT) were compared in 23 subjects [9]. But contrary to our findings a large difference in gene expression response was found. This might be explained by the difference in timepoints that they compared with each other [9]. Furthermore, the OLTT contained only 11 grams of unspecified carbohydrates, while the MMT and OGTT used in our study had an overlap of 75 grams of glucose.

Although we had an overlap in our challenge tests of 75 grams of glucose, the 60 grams of fat and 20 grams of protein in the MMT likely explain the finding that all blood parameters were significantly different between the two challenge tests, except for insulin. Insulin might be explanatory for some of the effects that were found to overlap between both challenges. Insulin is known to decrease NEFA levels by decreasing lipolysis [17], which is reflected by the postprandial increase in insulin at 60 minutes and the postprandial decline in NEFAs at 60 and 120 minutes in the blood. This decrease in plasma levels of NEFAs might be explanatory for the downregulation of beta-oxidation related gene sets at 120 minutes. In contrast to insulin, plasma levels of glucose were different during the MMT and the OGTT. Blood glucose response curves at 60 minutes were significantly higher after the OGTT compared to the MMT. In humans, a high protein breakfast was shown to delay the gastric emptying compared to a high carbohydrate breakfast [18]. The observed lower postprandial peak in glucose may be due to a delay in gastric-emptying, initiated by the protein in the MMT. In parallel with this higher plasma glucose level during the OGTT compared to the MMT, TCA cycle showed a prolonged downregulation up to 120 minutes for the OGTT, which was not present at the MMT. The postprandial plasma TAGs were higher during MMT compared to the OGTT, which can be explained by the presence of 60 grams of fat in the MMT. We did however not see any differences in gene expression related to the increased amount of fat in the MMT challenge and the paralleled increase in plasma TAG. Previous studies showed clear transcriptional changes upon high fat challenges [2, 6, 9]. These studies however never examined the response up to two hours. The effects of the fat component in the challenge test may be present at a later time point, for example four to eight hours postprandially. With respect to the differences in blood cell subpopulation, we found that lymphocytes and monocytes decreased more during the OGTT than during the MMT but this did not lead to a significant difference in ratio between these cell types at 60 and 120 minutes postprandially.

Therefore we expect that a change in cell population in the PBMCs is not be responsible for the observed effects on gene expression. At 240 minutes, the lymphocytes/monocytes-ratio was increased during the OGTT due to a more pronounced increase in lymphocytes. Increases in lymphocytes have been shown before during challenge tests but no differences between challenges tests, consisting of fat, glucose or both, or only water, were found [19, 20].
The percentage of neutrophils in the blood increased more after the MMT compared to the OGTT. A postprandial increase of neutrophils has been shown before in healthy participants receiving an OGTT, an OLTT and a combined OGTT + OLTT [19, 20]. But interestingly the response was not different between the challenges and also visible upon consumption of water alone.

At baseline, differences between blood plasma levels of glucose, insulin, NEFAs, and difference in numbers of monocytes, neutrophils, and lymphocytes/monocytes-ratio were found. Due to the original design of the study, the order of challenge tests was not randomized. The OGTT was given on the first day, followed by the MMT the next day. Based on the baseline differences in clinical chemistry, blood cell subpopulations and gene expression measurement, we expect that the effects of the OGTT might still have been present the next morning. This might not only have affected baseline measurements but also the postprandial response. Therefore, during the selection of the overlap in gene sets, gene sets that were significantly different at baseline were excluded. It also points to the importance of a standardized meal the evening before a challenge test. In this study we showed a huge interindividual variation in response to the challenges. Interestingly, also intra-individual variation was present as the same subject seemed not to respond in the same way for both challenges test. Dividing participants based on their changes in gene expression during a challenge test could be of interest to explain variation in blood parameters as well and to show the coping strategy of different individuals with a nutritional stressor.

In conclusion, the addition of fat and protein to glucose in a challenge tests leads to different postprandial responses of plasma markers except for insulin. The overlap in changes in gene expression between the MMT and OGTT was much bigger than the differences for both the number of genes and the number of gene sets. Based on the high overlap in changes in gene expression and the related metabolic pathways, we hypothesize that glucose in a challenge test is the main denominator of the postprandial changes in gene expression in the first two hours, which might mainly be regulated via insulin.

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Supplementary information

		Normalised Enrichment Score				FDR q-value			
		MMT		OGTT		MMT		OGTT	
		T60-T0	T120-T0	T60-T0	T120-T0	T60-T0	T120-T0	T60-T0	T120-T0
Transport	REACT_TRANSMEMBRANE TRANSPORT OF SMALL MOLECULES	-1.62	-1.37	-1.79	-1.43	0.15	0.39	0.05	0.62
	REACT_SLC-MEDIATED TRANSMEMBRANE TRANSPORT	-1.61	-1.36	-1.76	-1.42	0.16	0.39	0.07	0.64
	REACT_METAL ION SLC TRANSPORTERS	-1.61	-1.58	-1.41	-1.32	0.16	0.34	0.26	0.69
	REACT_TRANSPORT OF GLUCOSE AND OTHER SUGARS, BILE SALTS AND ORGANIC ACIDS, METAL IONS AND	-1.57	-1.31	-1.57	-1.32	0.19	0.43	0.14	0.69
Iron transport	REACT_TRANSFERRIN ENDOCYTOSIS AND RECYCLING	-1.71	-1.00	-1.73	-1.07	0.12	0.78	0.07	0.78
	REACT_IRON UPTAKE AND TRANSPORT	-1.96	-1.66	-1.93	-1.49	0.02	0.30	0.02	0.50
Cell types	KEGG_T CELL RECEPTOR SIGNALING PATHWAY	1.77	1.89	1.73	1.61	0.09	0.16	0.09	0.20
	WIP_HS_T_CELL_RECEPTOR_SIGNALING_PATHWAY	1.85	1.69	1.57	1.21	0.09	0.29	0.16	0.65
	NCI_CD8TCRPATHWAY	1.99	1.39	1.61	0.92	0.09	0.67	0.15	0.93
	BIOC_NKCELLSPATHWAY	-1.55	-1.29	-1.49	-0.83	0.22	0.45	0.20	0.90
	BIOC_NKTPATHWAY	0.95	1.80	1.45	1.59	0.83	0.15	0.24	0.22
Tryptophan metabolism	KEGG_TRYPTOPHAN METABOLISM	-1.61	-1.79	-1.67	-1.32	0.16	0.23	0.10	0.68
	WIP_HS_TRYPTOPHAN_METABOLISM	-1.59	-1.66	-1.94	-1.83	0.18	0.28	0.02	0.20
WNT signalling	WIP_HS_WNT_SIGNALING_PATHWAY	1.75	1.48	1.58	1.11	0.10	0.53	0.15	0.74
	WIP_HS_WNT_SIGNALING_PATHWAY_AND_PLURIPOTENCY	1.91	1.50	1.74	1.18	0.07	0.49	0.09	0.69
	KEGG_WNT SIGNALING PATHWAY	1.93	1.87	1.58	1.25	0.07	0.12	0.15	0.59
	KEGG_HEDGEHOG SIGNALING PATHWAY	1.56	1.68	1.75	1.40	0.24	0.28	0.10	0.37
	KEGG_BASAL CELL CARCINOMA	1.79	1.54	1.71	1.64	0.09	0.45	0.09	0.17
Lymphocyte Tarbase	WIP_HS_LYMPHOCYTE_TARBASE	-1.75	-1.30	-1.92	-1.35	0.14	0.45	0.02	0.67
	WIP_HS_MUSCLE_CELL_TARBASE	-1.71	-1.39	-1.85	-1.41	0.12	0.40	0.04	0.63
	WIP_HS_EPITHELIUM_TARBASE	-1.78	-1.19	-1.82	-1.22	0.13	0.59	0.05	0.77
Immune, HLA	KEGG_ANTIGEN PROCESSING AND PRESENTATION	-1.91	-1.66	-2.02	-1.39	0.03	0.27	0.01	0.63
	KEGG_PHAGOSOME	-2.12	-1.74	-2.28	-1.30	0.01	0.22	0.00	0.68
	KEGG_VIRAL MYOCARDITIS	-1.71	-1.40	-1.73	-0.79	0.13	0.40	0.07	0.93
Cell adhesion	REACT_L1CAM INTERACTIONS	-1.50	-1.58	-1.51	-1.59	0.26	0.36	0.18	0.44
	REACT_SIGNAL TRANSDUCTION BY L1	-1.74	-1.47	-1.87	-1.93	0.15	0.38	0.03	0.16
	NCI_NECTIN_PATHWAY	-1.59	-1.44	-1.38	-1.20	0.18	0.39	0.29	0.76
	REACT_TIGHT JUNCTION INTERACTIONS	1.52	1.76	1.96	1.56	0.27	0.20	0.05	0.25
	NCI_NCADHERINPATHWAY	-1.73	-1.66	-1.73	-1.35	0.14	0.26	0.07	0.68

Supplementary figure 1. continued on next page

		Normalised Enrichment Score				FDR q-value			
		MMT		OGTT		MMT		OGTT	
		T60-T0	T120-T0	T60-T0	T120-T0	T60-T0	T120-T0	T60-T0	T120-T0
Other	BIOC_RAC1PATHWAY	-1.78	-1.28	-1.65	-1.06	0.12	0.46	0.11	0.78
	KEGG_BACTERIAL INVASION OF EPITHELIAL CELLS	-1.56	-1.07	-1.60	-1.27	0.21	0.70	0.13	0.74
	KEGG_COMPLEMENT AND COAGULATION CASCADES	-1.43	-1.73	-1.76	-1.31	0.34	0.23	0.07	0.68
	KEGG_DORSO-VENTRAL AXIS FORMATION	-1.47	-1.50	-1.71	-1.42	0.29	0.37	0.08	0.64
	KEGG_DRUG METABOLISM - OTHER ENZYMES	-1.59	-1.01	-1.82	-1.39	0.17	0.77	0.05	0.62
	KEGG_GLYCOSPHINGOLIPID BIOSYNTHESIS - LACTO AND NEOLACTO SERIES	1.44	1.52	1.52	1.88	0.35	0.46	0.19	0.03
	KEGG_LYSOSOME	-2.20	-1.42	-2.38	-1.07	0.01	0.40	0.00	0.78
	NCI_HEDGEHOG_GLIPATHWAY	-1.68	-1.83	-1.80	-1.82	0.13	0.25	0.05	0.19
	KEGG_PROTEIN PROCESSING IN ENDOPLASMIC RETICULUM	-1.92	-1.62	-1.53	-1.67	0.04	0.30	0.17	0.30
	NCI_AJDISS_2PATHWAY	-1.52	-0.86	-1.47	-0.75	0.24	0.93	0.21	0.95
	NCI_ANGIOPOIETINRECEPTOR_PATHWAY	-1.53	-1.39	-1.43	-0.95	0.23	0.40	0.25	0.81
	NCI_EPHBFWDPATHWAY	-1.74	-1.53	-1.92	-1.51	0.14	0.37	0.02	0.48
	NCI_P53REGULATIONPATHWAY	-1.46	-1.41	-1.62	-1.80	0.30	0.39	0.12	0.20
	NCI_RAC1_PATHWAY	-1.52	-0.78	-1.41	-0.80	0.24	0.98	0.26	0.92
	NCI_SYNDECAN_2_PATHWAY	-1.53	-0.92	-1.60	-0.74	0.23	0.88	0.13	0.94
	REACT_KINESINS	1.62	0.87	1.47	0.84	0.18	0.93	0.23	0.96
	REACT_SEMAPHORIN INTERACTIONS	-1.63	-1.41	-1.60	-1.40	0.14	0.40	0.13	0.65
	WIP_HS_DIURNALLY_REGULATED_GENES_WITH_CIRCADIAN_ORTHOLOGS	-1.71	-1.81	-1.97	-1.59	0.12	0.25	0.02	0.44
	WIP_HS_SIDS_SUSCEPTIBILITY_PATHWAYS	1.63	1.26	1.49	1.71	0.18	0.75	0.21	0.11
	REACT_PHASE II CONJUGATION	-1.93	-1.54	-1.66	-1.22	0.03	0.37	0.11	0.78
	REACT_BIOLOGICAL OXIDATIONS	-1.72	-1.09	-1.63	-1.06	0.13	0.68	0.12	0.78
	REACT_ACTIVATION OF CHAPERONES BY IRE1ALPHA	-1.41	-1.07	-1.66	-1.77	0.36	0.70	0.10	0.23
	NCI_IL2_STAT5PATHWAY	1.82	1.66	1.90	1.16	0.09	0.31	0.07	0.70
	NCI_PS1PATHWAY	1.57	1.74	1.07	0.94	0.23	0.23	0.72	0.91
	NCI_IL8CXCR2_PATHWAY	-1.71	-1.47	-1.95	-1.10	0.12	0.38	0.02	0.77
	REACT_INSULIN RECEPTOR RECYCLING	-1.55	-0.85	-1.60	-1.07	0.21	0.94	0.13	0.78
	NCI_PDGFRBPATHWAY	-1.67	-1.04	-1.67	-1.01	0.13	0.71	0.10	0.77
	NCI_NETRIN_PATHWAY	-1.71	-0.88	-1.43	-1.11	0.12	0.92	0.25	0.77
	NCI_EPOPATHWAY	-1.71	-1.75	-1.79	-1.51	0.12	0.24	0.05	0.46
	NCI_GMCSF_PATHWAY	-1.61	0.86	-1.63	-0.82	0.16	0.94	0.12	0.91
	REACT_SIGNALING BY RHO GTPASES	-1.68	-1.51	-1.67	-1.33	0.13	0.38	0.10	0.69
	KEGG_FC GAMMA R-MEDIATED PHAGOCYTOSIS	-1.72	-1.32	-1.73	-1.04	0.13	0.42	0.07	0.77
	REACT_RHO GTPASE CYCLE	-1.72	-1.53	-1.70	-1.34	0.13	0.36	0.08	0.66

-1.8 ... -1

1 ... 1.8

FDR q-value <0.25

Supplementary figure 1. Selection of gene sets overlapping between the mixed meal test and oral glucose tolerance test. This figure shows the energy metabolism related gene sets that were significantly changed (FDR q-value<0.25) at two timepoints. Numbers reflect the normalized enrichment score (NES) which indicates the magnitude of the change. Blue illustrates downregulation, orange illustrates upregulation. Furthermore the significance is shown in dark pink as FDR q-value<0.25; the colours of gene sets with a FDR q<0.30 is shown in light pink.

Supplementary table 1. Gene Set Enrichment Analysis showing the significantly changed gene sets during the mixed meal test (MMT). Cut-off value FDR q-value 0.25.
Supplementary table 1a. Gene sets upregulated in peripheral blood mononuclear cells at 60 minutes during the MMT.

Gene set	NES	FDR q-value
WIP_HS_WNT_SIGNALING_PATHWAY_AND_PLURIPOTENCY	1.907	0.067
NCI_NFAT_TFPATHWAY	1.966	0.068
KEGG_WNT SIGNALING PATHWAY	1.929	0.069
WIP_HS_TCR_SIGNALING	1.870	0.079
WIP_HS_T_CELL_RECEPTOR_SIGNALING_PATHWAY	1.847	0.086
NCI_CD8TCRPATHWAY	1.994	0.086
KEGG_BASAL CELL CARCINOMA	1.793	0.090
NCI_IL2_STAT5PATHWAY	1.815	0.091
BIOC_TCRPATHWAY	1.761	0.093
KEGG_T CELL RECEPTOR SIGNALING PATHWAY	1.768	0.094
WIP_HS_WNT_SIGNALING_PATHWAY	1.749	0.096
KEGG_PRIMARY IMMUNODEFICIENCY	1.772	0.099
REACT_PLATELET AGGREGATION (PLUG FORMATION)	1.793	0.100
NCI_BETACATENIN_NUC_PATHWAY	1.817	0.102
BIOC_IL2RBPATHWAY	1.707	0.128
NCI_TCR_PATHWAY	1.690	0.139
NCI_CD8TCRDOWNSTREAMPATHWAY	1.670	0.147
WIP_HS_SELENIUM_METABOLISM_AND_SELENOPROTEINS	1.676	0.148
WIP_HS_SIDS_SUSCEPTIBILITY_PATHWAYS	1.632	0.177
BIOC_MCALPAINPATHWAY	1.638	0.178
REACT_KINESINS	1.621	0.184
REACT_NCAM SIGNALING FOR NEURITE OUT-GROWTH	1.594	0.214
REACT_VOLTAGE GATED POTASSIUM CHANNELS	1.581	0.224
NCI_PS1PATHWAY	1.570	0.233
REACT_G ALPHA (Z) SIGNALLING EVENTS	1.563	0.236
KEGG_HEDGEHOG SIGNALING PATHWAY	1.556	0.238
KEGG_ECM-RECEPTOR INTERACTION	1.540	0.247

Supplementary table 1b. Gene sets downregulated in peripheral blood mononuclear cells at 60 minutes during the MMT.

Gene set	NES	FDR q-value
WIP_HS_TYPE_II_INTERFERON_SIGNALING_(IFNG)	-2.134	0.005
KEGG_PHAGOSOME	-2.119	0.005
KEGG_INFLUENZA A	-2.099	0.006
REACT_INTERFERON GAMMA SIGNALING	-2.134	0.006
KEGG_STAPHYLOCOCCUS AUREUS INFECTION	-2.081	0.006
REACT_CYTOKINE SIGNALING IN IMMUNE SYSTEM	-2.138	0.007
KEGG_LYSOSOME	-2.203	0.007
KEGG_TUBERCULOSIS	-2.063	0.008
REACT_INTERFERON SIGNALING	-2.149	0.008
WIP_HS_NOD_PATHWAY	-1.980	0.021
REACT_IRON UPTAKE AND TRANSPORT	-1.960	0.024
REACT_PHASE II CONJUGATION	-1.928	0.034
KEGG_ANTIGEN PROCESSING AND PRESENTATION	-1.911	0.035
WIP_HS_SQUAMOUS_CELL_TARBASE	-1.915	0.035
KEGG_PROTEIN PROCESSING IN ENDOPLASMIC RETICULUM	-1.918	0.036
WIP_HS_TCA_CYCLE	-1.864	0.058
NCI_IL8CXCR1_PATHWAY	-1.705	0.115
REACT_TRANSFERRIN ENDOCYTOSIS AND RECYCLING	-1.706	0.117
BIOC_RAC1PATHWAY	-1.780	0.118
NCI_EPOPATHWAY	-1.706	0.119

NCI_IL8CXCR2_PATHWAY	-1.713	0.122
WIP_HS_DIURNALLY_REGULATED_GENES_WITH_CIRCADIAN_ORTHOLOGS	-1.707	0.122
NCI_NETRIN_PATHWAY	-1.709	0.122
KEGG_OSTEOCLAST DIFFERENTIATION	-1.781	0.124
WIP_HS_MUSCLE_CELL_TARBASE	-1.713	0.125
REACT_PYRUVATE METABOLISM AND CITRIC ACID (TCA) CYCLE	-1.681	0.125
REACT_BIOLOGICAL OXIDATIONS	-1.723	0.126
REACT_RHO GTPASE CYCLE	-1.718	0.126
KEGG_VIRAL MYOCARDITIS	-1.713	0.128
REACT_SIGNALING BY RHO GTPASES	-1.681	0.128
NCI_HEDGEHOG_GLIPATHWAY	-1.683	0.129
NCI_IL6_7PATHWAY	-1.723	0.129
KEGG_ASTHMA	-1.667	0.129
KEGG_SYSTEMIC LUPUS ERYTHEMATOSUS	-1.669	0.130
WIP_HS_EPITHELIUM_TARBASE	-1.781	0.131
KEGG_LEISHMANIASIS	-1.683	0.132
KEGG_PERTUSSIS	-1.661	0.132
REACT_INTERFERON ALPHA_BETA SIGNALING	-1.669	0.132
KEGG_FC GAMMA R-MEDIATED PHAGOCYTOSIS	-1.724	0.133
BIOC_CASPASEPATHWAY	-1.685	0.133
NCI_PDGFRBPATHWAY	-1.671	0.133
NCI_NCADHERINPATHWAY	-1.725	0.136
REACT_METABOLISM OF LIPIDS AND LIPOPROTEINS	-1.741	0.136
WIP_HS_LYMPHOCYTE_TARBASE	-1.755	0.137
KEGG_NOD-LIKE RECEPTOR SIGNALING PATHWAY	-1.728	0.137
REACT_SIGNALING BY INTERLEUKINS	-1.731	0.138
NCI_EPHBFWDPATHWAY	-1.742	0.140
NCI_IFNGPATHWAY	-1.651	0.141
KEGG_HUNTINGTON'S DISEASE	-1.733	0.142
REACT_REGULATION OF LIPID METABOLISM BY PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA (PPARALPHA)	-1.756	0.142
REACT_INTERLEUKIN-1 SIGNALING	-1.646	0.144
REACT_SEMAPHORIN INTERACTIONS	-1.631	0.144
BIOC_FMLPPATHWAY	-1.639	0.145
WIP_HS_TOLL-LIKE_RECEPTOR_SIGNALING_PATHWAY	-1.641	0.145
KEGG_MINERAL ABSORPTION	-1.634	0.145
KEGG_HERPES SIMPLEX INFECTION	-1.636	0.145
REACT_FATTY ACID,TRIACYLGLYCEROL, AND KETONE BODY METABOLISM	-1.632	0.146
REACT_SIGNAL TRANSDUCTION BY L1	-1.743	0.146
NCI_BARD1PATHWAY	-1.641	0.147
KEGG_TOLL-LIKE RECEPTOR SIGNALING PATHWAY	-1.623	0.149
REACT_TRANSMEMBRANE TRANSPORT OF SMALL MOLECULES	-1.624	0.151
REACT_SLC-MEDIATED TRANSMEMBRANE TRANSPORT	-1.615	0.156
NCI_GMCSF_PATHWAY	-1.608	0.156
KEGG_TRYPTOPHAN METABOLISM	-1.612	0.157
REACT_METAL ION SLC TRANSPORTERS	-1.609	0.158
NCI_P53DOWNSTREAMPATHWAY	-1.598	0.167
KEGG_DRUG METABOLISM - OTHER ENZYMES	-1.594	0.169
WIP_HS_TRYPTOPHAN_METABOLISM	-1.585	0.176
NCI_NECTIN_PATHWAY	-1.586	0.178
REACT_TRANSPORT OF GLUCOSE AND OTHER SUGARS, BILE SALTS AND ORGANIC ACIDS, METAL IONS AND AMINE COMPOUNDS	-1.571	0.195
KEGG_BACTERIAL INVASION OF EPITHELIAL CELLS	-1.562	0.206
WIP_HS_LEUKOCYTE_TARBASE	-1.557	0.209
NCI_LIS1PATHWAY	-1.552	0.211
REACT_INSULIN RECEPTOR RECYCLING	-1.554	0.211
BIOC_NKCELLSPATHWAY	-1.545	0.217
WIP_HS_REGULATION_OF_TOLL-LIKE_RECEPTOR_SIGNALING_PATHWAY	-1.547	0.217
WIP_HS_IL-3_SIGNALING_PATHWAY	-1.541	0.220
REACT_SPHINGOLIPID METABOLISM	-1.529	0.231
NCI_SYNDECAN_2_PATHWAY	-1.531	0.233
NCI_ANGIOPOIETINRECEPTOR_PATHWAY	-1.529	0.233
NCI_TOLL_ENDOGENOUS_PATHWAY	-1.518	0.235
NCI_RAC1_PATHWAY	-1.525	0.235
KEGG_METABOLISM OF XENOBIOTICS BY CYTOCHROME P450	-1.519	0.236
NCI_AJDISS_2PATHWAY	-1.520	0.238
KEGG_OXIDATIVE PHOSPHORYLATION	-1.521	0.239
REACT_UNFOLDED PROTEIN RESPONSE	-1.511	0.242
WIP_HS_TRANSPORT_OF_GLUCOSE_AND_OTHER_SUGARS,_BILE_		

SALTS_AND_ORGANIC_ACIDS,_METAL_IONS_AND_AMINE_COMPOUNDS	-1.512	0.243
NCI_IL3_PATHWAY	-1.506	0.247

Supplementary table 1c. Gene sets upregulated in peripheral blood mononuclear cells at 120 minutes during the MMT.

Gene set	NES	FDR q-value
NCI_NFAT_TFPATHWAY	2.048	0.064
NCI_CD8TCRDOWNSTREAMPATHWAY	1.909	0.190
KEGG_T CELL RECEPTOR SIGNALING PATHWAY	1.890	0.156
BIOC_TCRPATHWAY	1.886	0.122
KEGG_WNT SIGNALING PATHWAY	1.869	0.119
WIP_HS_GPCRS,_OTHER	1.869	0.099
NCI_TCRCALCIUMPATHWAY	1.799	0.169
BIOC_NKTPATHWAY	1.799	0.149
REACT_TIGHT JUNCTION INTERACTIONS	1.761	0.198
NCI_PS1PATHWAY	1.735	0.229

Supplementary table 1d. Gene sets downregulated in peripheral blood mononuclear cells at 120 minutes during the MMT.

Gene set	NES	FDR q-value
PPARA_TARGETS	-2.014	0.178
KEGG_STAPHYLOCOCCUS AUREUS INFECTION	-1.991	0.118
REACT_FATTY ACID,TRIACYLGLYCEROL, AND KETONE BODY METABOLISM	-1.941	0.133
WIP_HS_SQUAMOUS_CELL_TARBASE	-1.899	0.157
KEGG_MINERAL ABSORPTION	-1.805	0.230
KEGG_TRYPTOPHAN METABOLISM	-1.792	0.230
WIP_HS_MITOCHONDRIAL_LC-FATTY_ACID_BETA-OXIDATION	-1.789	0.209
KEGG_FATTY ACID METABOLISM	-1.759	0.245
NCI_EPOPATHWAY	-1.751	0.238
WIP_HS_FATTY_ACID_BETA_OXIDATION	-1.745	0.231
KEGG_PHAGOSOME	-1.742	0.218
KEGG_COMPLEMENT AND COAGULATION CASCADES	-1.728	0.229
REACT_INTERFERON SIGNALING	-1.725	0.218
REACT_INTERFERON GAMMA SIGNALING	-1.653	0.246

Supplementary table 2. Gene Set Enrichment Analysis showing the significantly changed gene sets during the oral glucose tolerance test (OGTT). Cut-off value FDR q-value 0.25.

Supplementary table 2a. Gene sets upregulated in peripheral blood mononuclear cells at 60 minutes during the OGTT.

Gene set	NES	FDR q-value
NCI_NFAT_TFPATHWAY	2.174	0.008
REACT_REGULATION OF BETA-CELL DEVELOPMENT	2.004	0.041
REACT_TIGHT JUNCTION INTERACTIONS	1.957	0.046
NCI_IL2_STAT5PATHWAY	1.902	0.066
REACT_REGULATION OF GENE EXPRESSION IN BETA CELLS	1.897	0.057
REACT_VIRAL MRNA TRANSLATION	1.829	0.098
BIOC_TCRPATHWAY	1.824	0.087
REACT_PEPTIDE CHAIN ELONGATION	1.815	0.084
WIP_HS_TCR_SIGNALING	1.805	0.082

REACT_EUKARYOTIC TRANSLATION ELONGATION	1.798	0.078
REACT_INFLUENZA VIRAL RNA TRANSCRIPTION AND REPLICATION	1.780	0.084
KEGG_HEDGEHOG SIGNALING PATHWAY	1.750	0.102
REACT_L13A-MEDIATED TRANSLATIONAL SILENCING OF CERULOPLASMIN EXPRESSION	1.745	0.098
WIP_HS_WNT_SIGNALING_PATHWAY_AND_PLURIPOTENCY	1.740	0.094
REACT_3 -UTR-MEDIATED TRANSLATIONAL REGULATION	1.738	0.090
KEGG_T CELL RECEPTOR SIGNALING PATHWAY	1.735	0.087
REACT_FORMATION OF A POOL OF FREE 40S SUBUNITS	1.727	0.088
NCI_CD8TCRDOWNSTREAMPATHWAY	1.714	0.092
KEGG_BASAL CELL CARCINOMA	1.711	0.090
REACT_GTP HYDROLYSIS AND JOINING OF THE 60S RIBOSOMAL SUBUNIT	1.697	0.096
REACT_EUKARYOTIC TRANSLATION INITIATION	1.648	0.136
REACT_RIBOSOMAL SCANNING AND START CODON RECOGNITION	1.647	0.132
REACT_TRANSLATION INITIATION COMPLEX FORMATION	1.637	0.136
KEGG_PRIMARY IMMUNODEFICIENCY	1.627	0.140
NCI_CD8TCRPATHWAY	1.608	0.154
REACT_CAP-DEPENDENT TRANSLATION INITIATION	1.605	0.151
REACT_ACTIVATION OF THE MRNA UPON BINDING OF THE CAP-BINDING COMPLEX AND EIFS, AND SUBSEQUENT BINDING TO 43S	1.605	0.146
KEGG_RIBOSOME	1.602	0.143
REACT_EUKARYOTIC TRANSLATION TERMINATION	1.601	0.139
WIP_HS_WNT_SIGNALING_PATHWAY	1.583	0.154
KEGG_WNT SIGNALING PATHWAY	1.578	0.154
WIP_HS_T_CELL_RECEPTOR_SIGNALING_PATHWAY	1.567	0.161
WIP_HS_CYTOPLASMIC_RIBOSOMAL_PROTEINS	1.563	0.160
REACT_FORMATION OF THE TERNARY COMPLEX, AND SUBSEQUENTLY, THE 43S COMPLEX	1.549	0.172
KEGG_GLYCINE, SERINE AND THREONINE METABOLISM	1.541	0.176
REACT_NONSENSE MEDIATED DECAY INDEPENDENT OF THE EXON JUNCTION COMPLEX	1.531	0.183
KEGG_GLYCOSPHINGOLIPID BIOSYNTHESIS - LACTO AND NEOLACTO SERIES	1.523	0.190
REACT_NETRIN-1 SIGNALING	1.512	0.197
REACT_TRANSLATION	1.495	0.214
BIOC_IL12PATHWAY	1.495	0.208
WIP_HS_SIDS_SUSCEPTIBILITY_PATHWAYS	1.488	0.213
BIOC_CTLA4PATHWAY	1.487	0.210
REACT_KINESINS	1.467	0.233
NCI_TAP63PATHWAY	1.462	0.236
WIP_HS_P38_MAPK_SIGNALING_PATHWAY	1.459	0.235
BIOC_NKTPATHWAY	1.453	0.239

Supplementary table 2b. Gene sets downregulated in peripheral blood mononuclear cells at 60 minutes during the OGTT.

Gene set	**NES**	**FDR q-value**
KEGG_STAPHYLOCOCCUS AUREUS INFECTION	-2.513	0.000
KEGG_LYSOSOME	-2.380	0.000
WIP_HS_SQUAMOUS_CELL_TARBASE	-2.348	0.000
KEGG_PHAGOSOME	-2.279	0.000
REACT_CYTOKINE SIGNALING IN IMMUNE SYSTEM	-2.174	0.002
KEGG_TUBERCULOSIS	-2.125	0.003
REACT_INTERFERON GAMMA SIGNALING	-2.099	0.003
REACT_INTERFERON SIGNALING	-2.072	0.006
KEGG_ANTIGEN PROCESSING AND PRESENTATION	-2.024	0.012
WIP_HS_STATIN_PATHWAY	-1.992	0.014
KEGG_INFLUENZA A	-1.976	0.016
WIP_HS_TYPE_II_INTERFERON_SIGNALING_(IFNG)	-1.982	0.016
WIP_HS_DIURNALLY_REGULATED_GENES_WITH_CIRCADIAN_ORTHOLOGS	-1.969	0.016

NCI_IL8CXCR2_PATHWAY	-1.953	0.019
WIP_HS_LYMPHOCYTE_TARBASE	-1.918	0.019
NCI_EPHBFWDPATHWAY	-1.925	0.020
WIP_HS_NOD_PATHWAY	-1.911	0.020
REACT_SIGNALING BY INTERLEUKINS	-1.919	0.020
WIP_HS_TRYPTOPHAN_METABOLISM	-1.939	0.020
REACT_IRON UPTAKE AND TRANSPORT	-1.925	0.020
NCI_IL8CXCR1_PATHWAY	-1.934	0.021
REACT_METABOLISM OF LIPIDS AND LIPOPROTEINS	-1.926	0.022
KEGG_OSTEOCLAST DIFFERENTIATION	-1.867	0.032
REACT_SIGNAL TRANSDUCTION BY L1	-1.868	0.033
WIP_HS_MUSCLE_CELL_TARBASE	-1.845	0.040
KEGG_ASTHMA	-1.848	0.040
NCI_TOLL_ENDOGENOUS_PATHWAY	-1.822	0.048
WIP_HS_EPITHELIUM_TARBASE	-1.817	0.049
KEGG_DRUG METABOLISM - OTHER ENZYMES	-1.824	0.049
REACT_TRANSMEMBRANE TRANSPORT OF SMALL MOLECULES	-1.792	0.051
WIP_HS_TOLL-LIKE_RECEPTOR_SIGNALING_PATHWAY	-1.807	0.051
REACT_INTERLEUKIN-1 SIGNALING	-1.793	0.052
REACT_FATTY ACID, TRIACYLGLYCEROL, AND KETONE BODY METABOLISM	-1.807	0.053
NCI_HEDGEHOG_GLIPATHWAY	-1.799	0.053
NCI_IL6_7PATHWAY	-1.793	0.053
REACT_INTERFERON ALPHA_BETA SIGNALING	-1.799	0.055
NCI_EPOPATHWAY	-1.794	0.055
PPARA_TARGETS	-1.769	0.063
REACT_SLC-MEDIATED TRANSMEMBRANE TRANSPORT	-1.755	0.065
KEGG_TOLL-LIKE RECEPTOR SIGNALING PATHWAY	-1.759	0.066
NCI_PI3KCIPATHWAY	-1.756	0.067
KEGG_COMPLEMENT AND COAGULATION CASCADES	-1.760	0.067
NCI_NCADHERINPATHWAY	-1.734	0.070
KEGG_VIRAL MYOCARDITIS	-1.729	0.070
REACT_TRANSFERRIN ENDOCYTOSIS AND RECYCLING	-1.732	0.070
KEGG_FC GAMMA R-MEDIATED PHAGOCYTOSIS	-1.727	0.071
KEGG_PERTUSSIS	-1.739	0.071
WIP_HS_LEUKOCYTE_TARBASE	-1.734	0.071
REACT_MYD88 DEPENDENT CASCADE INITIATED ON ENDOSOME	-1.734	0.073
KEGG_LEISHMANIASIS	-1.739	0.073
REACT_TRAF6 MEDIATED INDUCTION OF NFKB AND MAP KINASES UPON TLR7_8 OR 9 ACTIVATION	-1.740	0.074
REACT_TOLL LIKE RECEPTOR 7_8 (TLR7_8) CASCADE	-1.720	0.074
WIP_HS_IL-3_SIGNALING_PATHWAY	-1.714	0.075
NCI_UPA_UPAR_PATHWAY	-1.715	0.076
KEGG_DORSO-VENTRAL AXIS FORMATION	-1.710	0.076
REACT_TOLL LIKE RECEPTOR 9 (TLR9) CASCADE	-1.708	0.077
REACT_RHO GTPASE CYCLE	-1.699	0.082
KEGG_GRAFT-VERSUS-HOST DISEASE	-1.675	0.100
NCI_PDGFRBPATHWAY	-1.670	0.101
WIP_HS_REGULATION_OF_TOLL-LIKE_RECEPTOR_SIGNALING_PATHWAY	-1.662	0.101
KEGG_TRYPTOPHAN METABOLISM	-1.667	0.102
REACT_SIGNALING BY RHO GTPASES	-1.670	0.102
REACT_ACTIVATION OF CHAPERONES BY IRE1ALPHA	-1.662	0.103
WIP_HS_B_CELL_RECEPTOR_SIGNALING_PATHWAY	-1.664	0.103
REACT_PHASE II CONJUGATION	-1.655	0.106
REACT_REGULATION OF LIPID METABOLISM BY PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA (PPARALPHA)	-1.651	0.107
BIOC_RAC1PATHWAY	-1.652	0.107
WIP_HS_TCA_CYCLE	-1.641	0.112
BIOC_CASPASEPATHWAY	-1.642	0.114
NCI_IFNGPATHWAY	-1.635	0.117
NCI_GMCSF_PATHWAY	-1.633	0.117
REACT_BIOLOGICAL OXIDATIONS	-1.629	0.119
NCI_P53REGULATIONPATHWAY	-1.624	0.120

NCI_PTP1BPATHWAY	-1.624	0.121
REACT_PLATELET ACTIVATION	-1.626	0.121
NCI_TXA2PATHWAY	-1.619	0.123
REACT_CELL SURFACE INTERACTIONS AT THE VASCULAR WALL	-1.614	0.123
REACT_SIGNALING BY NOTCH	-1.614	0.124
BIOC_RHOPATHWAY	-1.615	0.126
KEGG_VIBRIO CHOLERAE INFECTION	-1.608	0.128
KEGG_AUTOIMMUNE THYROID DISEASE	-1.602	0.131
KEGG_NOD-LIKE RECEPTOR SIGNALING PATHWAY	-1.598	0.132
NCI_SYNDECAN_2_PATHWAY	-1.599	0.132
REACT_SEMAPHORIN INTERACTIONS	-1.595	0.132
KEGG_BACTERIAL INVASION OF EPITHELIAL CELLS	-1.602	0.132
REACT_INSULIN RECEPTOR RECYCLING	-1.596	0.133
NCI_VEGFR1_2_PATHWAY	-1.585	0.139
REACT_P75 NTR RECEPTOR-MEDIATED SIGNALLING	-1.577	0.140
NCI_ALPHASYNUCLEIN_PATHWAY	-1.576	0.140
KEGG_TOXOPLASMOSIS	-1.578	0.140
KEGG_ALLOGRAFT REJECTION	-1.585	0.141
KEGG_TYPE I DIABETES MELLITUS	-1.582	0.141
REACT_TRANSPORT OF INORGANIC CATIONS_ANIONS AND AMINO ACIDS_OLIGOPEPTIDES	-1.579	0.141
WIP_HS_ALPHA_6_BETA_4_SIGNALING_PATHWAY	-1.579	0.142
REACT_TRANSPORT OF GLUCOSE AND OTHER SUGARS, BILE SALTS AND ORGANIC ACIDS, METAL IONS AND AMINE COMPOUNDS	-1.572	0.143
REACT_CELL DEATH SIGNALLING VIA NRAGE, NRIF AND NADE	-1.567	0.147
NCI_ERBB1_DOWNSTREAM_PATHWAY	-1.565	0.148
WIP_HS_SENESCENCE_AND_AUTOPHAGY	-1.561	0.149
KEGG_HUNTINGTON'S DISEASE	-1.562	0.150
REACT_PLATELET DEGRANULATION	-1.554	0.152
REACT_INTERLEUKIN-3, 5 AND GM-CSF SIGNALING	-1.557	0.152
KEGG_HERPES SIMPLEX INFECTION	-1.555	0.153
REACT_PYRUVATE METABOLISM AND CITRIC ACID (TCA) CYCLE	-1.544	0.159
REACT_SIGNALLING BY NGF	-1.544	0.160
BIOC_METPATHWAY	-1.545	0.160
REACT_FORMATION OF PLATELET PLUG	-1.545	0.162
REACT_NUCLEOTIDE-BINDING DOMAIN, LEUCINE RICH REPEAT CONTAINING RECEPTOR (NLR) SIGNALING PATHWAYS	-1.540	0.162
REACT_G ALPHA (12_13) SIGNALLING EVENTS	-1.538	0.163
KEGG_PROTEIN PROCESSING IN ENDOPLASMIC RETICULUM	-1.533	0.168
NCI_P38ALPHABETAPATHWAY	-1.529	0.170
REACT_TRAF6 MEDIATED INDUCTION OF PROINFLAMMATORY CYTOKINES	-1.530	0.170
NCI_INTEGRIN2_PATHWAY	-1.521	0.172
KEGG_PPAR SIGNALING PATHWAY	-1.522	0.172
KEGG_PORPHYRIN AND CHLOROPHYLL METABOLISM	-1.523	0.172
WIP_HS_SIGNALING_OF_HEPATOCYTE_GROWTH_FACTOR_RECEPTOR	-1.519	0.173
KEGG_ENDOCRINE AND OTHER FACTOR-REGULATED CALCIUM REABSORPTION	-1.524	0.174
NCI_INTEGRIN_A9B1_PATHWAY	-1.517	0.174
REACT_SPHINGOLIPID METABOLISM	-1.524	0.174
KEGG_NEUROTROPHIN SIGNALING PATHWAY	-1.515	0.175
KEGG_INTESTINAL IMMUNE NETWORK FOR IGA PRODUCTION	-1.508	0.177
REACT_NUCLEAR EVENTS (KINASE AND TRANSCRIPTION FACTOR ACTIVATION)	-1.512	0.177
REACT_L1CAM INTERACTIONS	-1.511	0.177
REACT_PURINE METABOLISM	-1.508	0.178
REACT_CLATHRIN DERIVED VESICLE BUDDING	-1.509	0.179
WIP_HS_EGF_RECEPTOR_SIGNALING_PATHWAY	-1.505	0.179
KEGG_NATURAL KILLER CELL MEDIATED CYTOTOXICITY	-1.502	0.181
WIP_HS_OXIDATIVE_PHOSPHORYLATION	-1.498	0.185
WIP_HS_PROSTAGLANDIN_SYNTHESIS_AND_REGULATION	-1.489	0.195
NCI_P53DOWNSTREAMPATHWAY	-1.486	0.196
BIOC_NKCELLSPATHWAY	-1.487	0.196
KEGG_RHEUMATOID ARTHRITIS	-1.480	0.201

KEGG_APOPTOSIS	-1.481	0.201
REACT_RESPONSE TO ELEVATED PLATELET CYTOSOLIC CA2+	-1.477	0.203
REACT_TRANS-GOLGI NETWORK VESICLE BUDDING	-1.476	0.204
REACT_NRAGE SIGNALS DEATH THROUGH JNK	-1.471	0.204
KEGG_AMOEBIASIS	-1.471	0.205
NCI_AJDISS_2PATHWAY	-1.472	0.205
REACT_MAP KINASE ACTIVATION IN TLR CASCADE	-1.473	0.206
KEGG_FC EPSILON RI SIGNALING PATHWAY	-1.465	0.209
WIP_HS_NOTCH_SIGNALING_PATHWAY	-1.466	0.210
REACT_HEMOSTASIS	-1.456	0.216
NCI_CXCR4_PATHWAY	-1.459	0.216
KEGG_GLYCEROPHOSPHOLIPID METABOLISM	-1.457	0.217
KEGG_MINERAL ABSORPTION	-1.458	0.217
REACT_PLATELET ACTIVATION TRIGGERS	-1.439	0.240

Supplementary table 2c. Gene sets upregulated in peripheral blood mononuclear cells at 120 minutes during the OGTT.

Gene set	NES	FDR q-value
REACT_NONSENSE MEDIATED DECAY INDEPENDENT OF THE EXON JUNCTION COMPLEX	2.114	0.018
REACT_EUKARYOTIC TRANSLATION TERMINATION	2.095	0.012
REACT_EUKARYOTIC TRANSLATION ELONGATION	2.090	0.010
REACT_PEPTIDE CHAIN ELONGATION	2.076	0.010
REACT_VIRAL MRNA TRANSLATION	2.043	0.013
REACT_REGULATION OF GENE EXPRESSION IN BETA CELLS	2.030	0.013
REACT_REGULATION OF BETA-CELL DEVELOPMENT	2.009	0.014
REACT_FORMATION OF A POOL OF FREE 40S SUBUNITS	2.009	0.013
REACT_NONSENSE-MEDIATED DECAY	2.006	0.012
REACT_INFLUENZA VIRAL RNA TRANSCRIPTION AND REPLICATION	2.002	0.011
REACT_CHEMOKINE RECEPTORS BIND CHEMOKINES	1.972	0.014
REACT_NONSENSE MEDIATED DECAY ENHANCED BY THE EXON JUNCTION COMPLEX	1.968	0.014
REACT_TRANSLATION	1.942	0.019
REACT_3 -UTR-MEDIATED TRANSLATIONAL REGULATION	1.931	0.020
KEGG_RIBOSOME	1.915	0.023
REACT_L13A-MEDIATED TRANSLATIONAL SILENCING OF CERULOPLASMIN EXPRESSION	1.907	0.024
NCI_NFAT_TFPATHWAY	1.900	0.024
WIP_HS_CYTOPLASMIC_RIBOSOMAL_PROTEINS	1.897	0.024
KEGG_GLYCOSPHINGOLIPID BIOSYNTHESIS - LACTO AND NEOLACTO SERIES	1.884	0.026
REACT_GTP HYDROLYSIS AND JOINING OF THE 60S RIBOSOMAL SUBUNIT	1.871	0.028
REACT_EUKARYOTIC TRANSLATION INITIATION	1.839	0.038
REACT_CAP-DEPENDENT TRANSLATION INITIATION	1.836	0.037
REACT_METABOLISM OF MRNA	1.780	0.063
WIP_HS_CYTOKINES_AND_INFLAMMATORY_RESPONSE	1.723	0.106
REACT_CLASS A_1 (RHODOPSIN-LIKE RECEPTORS)	1.717	0.108
WIP_HS_SIDS_SUSCEPTIBILITY_PATHWAYS	1.707	0.113
WIP_HS_PEPTIDE_GPCRS	1.704	0.113
NCI_P38ALPHABETADOWNSTREAMPATHWAY	1.691	0.123
BIOC_INFLAMPATHWAY	1.687	0.123
REACT_METABOLISM OF RNA	1.662	0.149
KEGG_BASAL CELL CARCINOMA	1.641	0.171
BIOC_P38MAPKPATHWAY	1.629	0.184
REACT_INSULIN SYNTHESIS AND PROCESSING	1.627	0.180
REACT_TRANSLATION INITIATION COMPLEX FORMATION	1.619	0.186
KEGG_T CELL RECEPTOR SIGNALING PATHWAY	1.606	0.199
REACT_RIBOSOMAL SCANNING AND START CODON RECOGNITION	1.588	0.223

BIOC_NKTPATHWAY	1.587	0.218
WIP_HS_GPCRS,_OTHER	1.585	0.216
REACT_TIGHT JUNCTION INTERACTIONS	1.562	0.248

Supplementary table 2d. Gene sets downregulated in peripheral blood mononuclear cells at 120 minutes during the OGTT.

Gene set	**NES**	**FDR q-value**
PPARA_TARGETS	-2.029	0.148
WIP_HS_TCA_CYCLE	-1.939	0.216
REACT_SIGNAL TRANSDUCTION BY L1	-1.931	0.161
REACT_PYRUVATE METABOLISM AND CITRIC ACID (TCA) CYCLE	-1.890	0.192
WIP_HS_SQUAMOUS_CELL_TARBASE	-1.832	0.229
WIP_HS_TRYPTOPHAN_METABOLISM	-1.829	0.202
NCI_HEDGEHOG_GLIPATHWAY	-1.821	0.190
NCI_P53REGULATIONPATHWAY	-1.801	0.204
REACT_LOSS OF NLP FROM MITOTIC CENTROSOMES	-1.789	0.205
REACT_ACTIVATION OF CHAPERONES BY IRE1ALPHA	-1.766	0.229
KEGG_MINERAL ABSORPTION	-1.760	0.222
REACT_LOSS OF PROTEINS REQUIRED FOR INTERPHASE MICROTUBULE ORGANIZATION FROM THE CENTROSOME	-1.749	0.223
WIP_HS_MITOCHONDRIAL_LC-FATTY_ACID_BETA-OXIDATION	-1.741	0.221
REACT_FATTY ACID, TRIACYLGLYCEROL, AND KETONE BODY METABOLISM	-1.739	0.212
WIP_HS_FATTY_ACID_BETA_OXIDATION	-1.706	0.245

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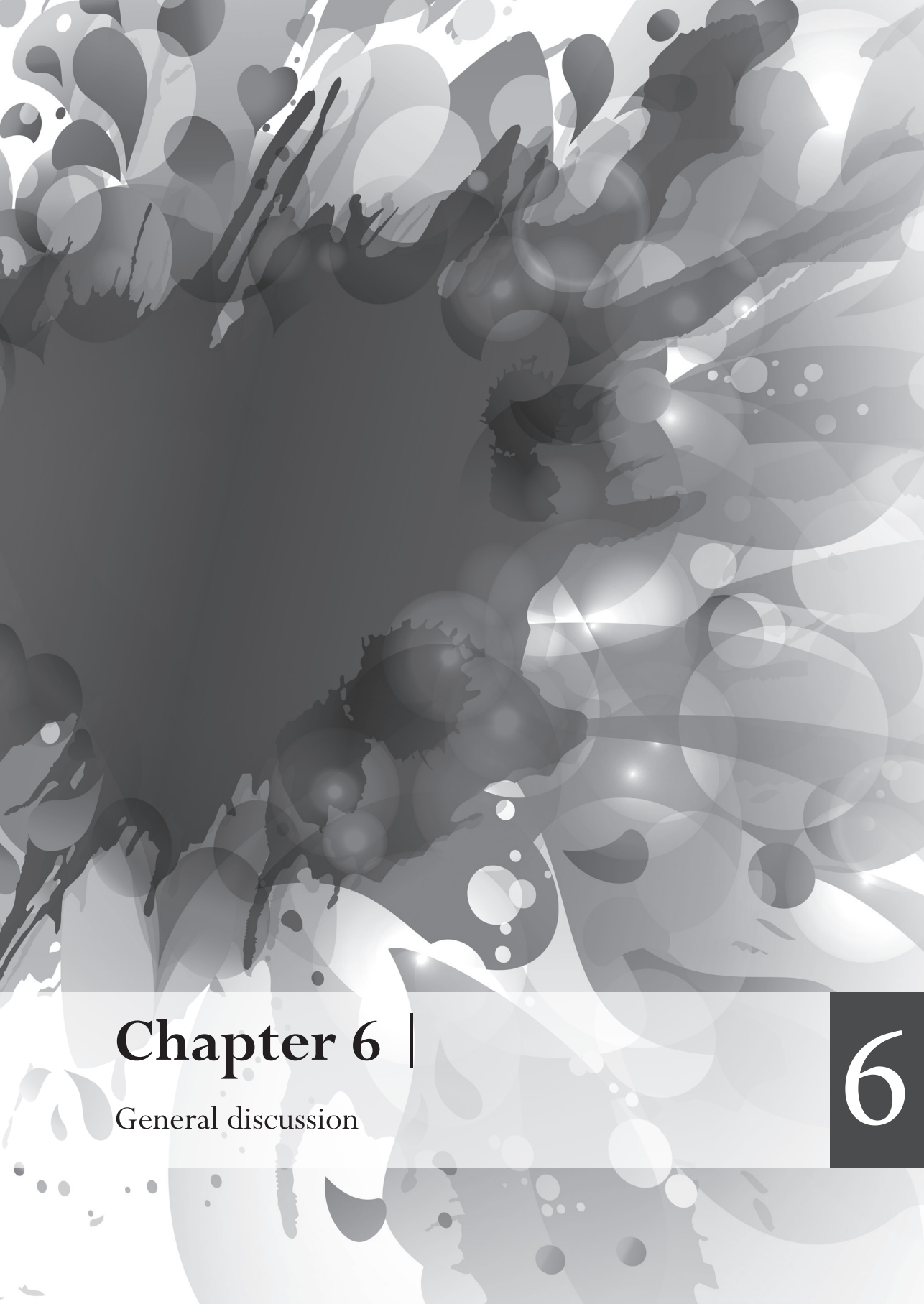

Chapter 6

General discussion

6

The objective of this thesis was to investigate the use of the transcriptome as an early and sensitive marker of diet-related health. In this thesis energy restriction (ER) and caloric restriction (CR) diets were used as models to induce a more healthy state in human participants. The effect of age, as potential marker for health, on the CR-induced effects on the transcriptome was investigated. Furthermore, the effect of the dietary composition during ER, in the form of protein quantity, on the ER-induced effects on the transcriptome was investigated. A potential new tool to magnify the effects of diet on health is the application of challenge tests. These challenge tests were investigated for their potential to magnify diet-related health effects and for the potential to measure changes in phenotypic flexibility. Finally, we compared the changes in gene expression upon two challenge tests differing in nutritional composition.

In this thesis we presented a number of outcomes. First, we showed that upon CR, the transcriptome of PBMCs of healthy young men had a higher responsiveness in immune-response pathways compared to PBMCs of aged men (chapter 2). Also, we showed that the transcriptome of WAT showed a decrease in pathways involved in immune response and inflammasome upon a normal protein ER diet, whereas no such effect was found upon a high protein ER diet. In this study, parameters such as weight loss, glucose, and waist circumference were not different between the two diets (chapter 3). Twelve weeks of ER compared to the control diet was shown to increase phenotypic flexibility as reflected by a faster and more pronounced downregulation of OXPHOS, cell adhesion, and DNA replication during the OGTT (chapter 4). Finally, two challenge tests consisting of either glucose (OGTT) or glucose plus fat and protein (MMT), provoked largely overlapping changes in gene expression in PBMCs (chapter 5). The use of the PBMC transcriptome as a marker of diet-related health, comparison and biological interpretation of the findings, and the future prospects will be discussed in this chapter.

Transcriptome as a marker of diet-related health

To be able to investigate the use of the transcriptome as a marker of diet-related health, we have compared the results presented in these chapters and used the studies as depicted in figure 1. Figure 1 visualises the contribution of each study in the determination of the use of the transcriptome as a marker of diet-related health.

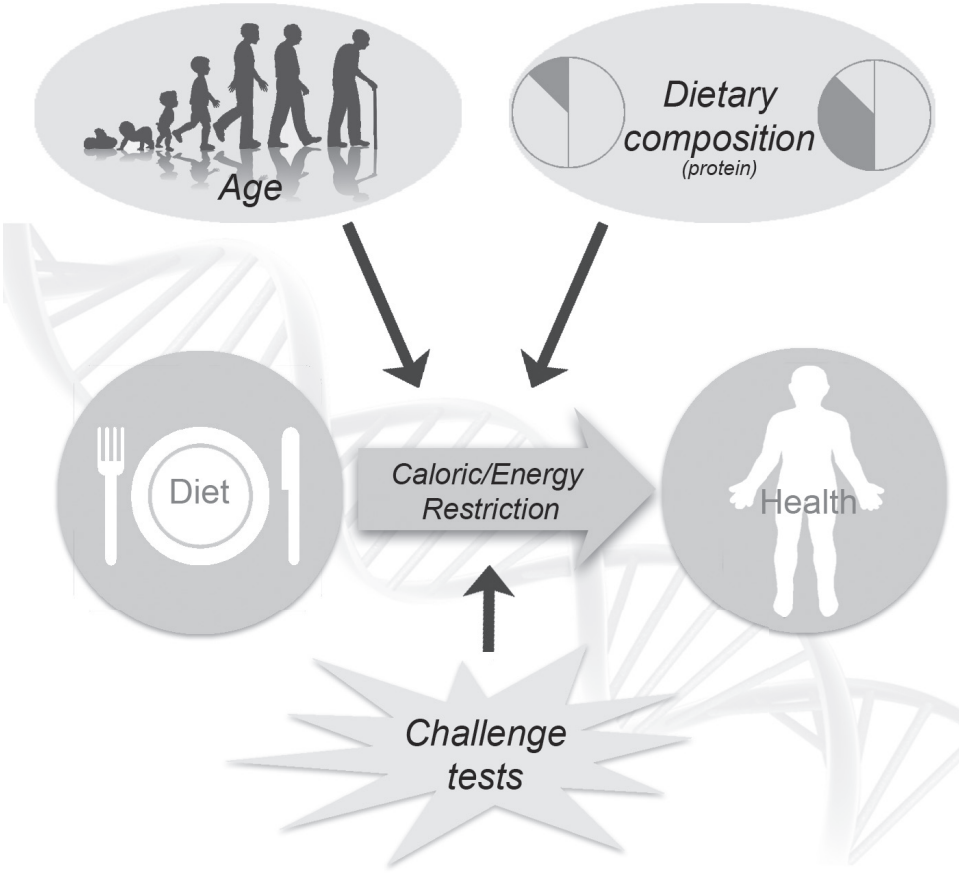

Figure 1. Schematic model used to investigate the use of the transcriptome as a marker of diet-related health.

In chapter 2, we compared the PBMC transcriptome of old and young men upon three weeks of 30% CR. Young men were considered to be more healthy than old men. Interestingly, CR decreased pathways involved in the immune response only in young men and not in old men. This difference in changes in gene expression between individuals in different health states provides evidence that the response of the transcriptome to a dietary intervention in the form of CR can depend on the age and related health state of an individual. This study provided support for the use of the PBMC transcriptome as tool to study diet-related health effects. In chapter 4 we also examined the use of PBMC transcriptome as a tool to study

diet-related health effects. We compared the changes in gene expression upon 12 weeks of 20% ER with the changes in gene expression upon 12 weeks of a control diet. ER leads to weight loss, which brings an individual in a more healthy state. Based upon the finding that ER induced a downregulation of gene sets involved in OXPHOS, cell adhesion, energy metabolism, immune system and cell cycle, but not in the control group, we found additional corroboration for the use of the PBMC transcriptome as a tool to study diet-related health effects. In chapter 3, the effect of a normal protein ER diet on the WAT transcriptome was compared with a high protein ER diet. We showed that a potential beneficial downregulation on inflammation-related pathways was present only in response to the normal protein ER diet and not in response to the high protein ER diet. This finding demonstrates the major impact of dietary composition on the transcriptome and subsequently likely also on health. In this study we found further evidence for the use of the transcriptome as a tool to study diet-related health effects. The response of the PBMC transcriptome to dietary interventions has been shown before for different types of dietary interventions, including fish oil supplementation [1, 2], diets differing in fatty acid composition [3], and low-ER diet [4]. Also, the effect of ER on the WAT transcriptome has been shown [5], although not for different protein quantities. The effect of CR diets, i.e. the study of reduction of energy intake without weight loss, has only been studied in PBMCs from rats [6], and the study presented in this thesis is one of the first studying the effect of CR on the PBMC transcriptome.

Apart from our studies, no other studies have investigated the effect of age or macronutrient composition during CR or ER diets on the transcriptome. In the CR study presented in this thesis, weight loss and glucose levels were similar upon CR in young and old men (chapter 2), but the response of the PBMC transcriptome was different upon CR for young men and old men. Similarly for the macronutrient composition ER study, weight loss did not differ between high protein ER or normal protein ER, while the WAT transcriptome showed less beneficial diet-induced changes on the high protein ER diet as compared to the normal protein ER diet (chapter 3). In conclusion, these studies showed that phenotypic traits such as age and dietary composition, specifically protein quantity, affect the changes in gene expression upon CR and ER independent of the amount of weight loss. This suggests that the transcriptome might be used as an early and sensitive marker of diet-related health. This sensitivity of the transcriptome as a marker was further investigated in chapter 4. In chapter 4, 12 weeks of 20% ER compared to control changed several pathways in the fasted state, while parameters such as glucose and insulin remained similar in the fasted state, providing evidence for the use of the transcriptome as an early and sensitive marker of diet-induced health changes due to weight loss. In conclusion, based on the different outcomes presented in this thesis we provide strong support for the value of the transcriptome of PBMCs as an early marker of diet-related health.

Biological interpretation of energy metabolism-related gene expression changes

Studies in this thesis reveal different changes in gene expression for energy metabolism-related pathways among the various dietary interventions. For example, three weeks of 30% CR (chapter 2) decreased glucose metabolism-related pathways such as the TCA cycle in PBMCs from healthy young men but not in PBMCs from healthy old men. In contrast, 12 weeks of 20% ER (chapter 4) increased glucose metabolism-related pathways such as TCA cycle in PBMCs from overweight middle-aged men and women. Furthermore, these 12 weeks of 20% ER increased lipid metabolism-related pathways such as beta-oxidation and cholesterol biosynthesis. This difference in up- or downregulation of glucose metabolism pathways in PBMCs might point towards a possible mechanism underlying the metabolic adaption of these cells and their coping strategy under different circumstances of restricted energy intake. A period of restricted energy intake for only three weeks might lead to a downregulation of glucose metabolism due to a decreased amount of glucose available to metabolise. When the period of restricted energy is longer, such as 12 weeks, glucose and lipid metabolism might be upregulated, as a possible response to be able to use all available nutrients. Also, beta-oxidation could be increased in concert with increased lipid release from fat storage. Besides the duration of restriction, also age could have influenced the outcomes as young men might have a different coping strategy - being more phenotypically flexible - towards less energy intake compared to older men.

Whereas 12 weeks of ER in PBMCs upregulated pathways involved in beta-oxidation, cholesterol biosynthesis and TCA cycle, 12 weeks of ER in WAT resulted in downregulation of beta-oxidation, cholesterol biosynthesis, TCA cycle, and also triglyceride synthesis. Body fat mass was decreased after 12 weeks ER, explaining a decrease in fatty acid metabolism in WAT due to decreased energy intake. In conclusion, the difference in downregulation between PBMCs and WAT might be explained by the specific function of WAT in lipid metabolism, and more specifically in fat storage and energy release when fasting. When ER is maintained for a period of 12 weeks, influx of fatty acids into WAT and subsequent fatty acid deposition as triglycerides is decreased. In contrast to WAT, PBMCs circulate in the blood and the individual cells are continuously exposed to exocrine and endocrine signals, including the ones secreted by the adipose tissue such as fatty acids, possibly reflecting a more systemic health. The response of PBMCs might not be tissue specific as compared to the response in WAT.

OXPHOS was differentially affected in all studies presented in this thesis, but also previous research is inconclusive on the direction of change of OXPHOS and the meaning of this change [7]. Although extensively discussed in chapter 4, further elaboration on the different response of OXPHOS pathways is warranted. In PBMCs, OXPHOS was downregulated by three weeks of 30% CR in young men and in old men (chapter 2) and upregulated by 12 weeks of 20% ER (chapter 4). A period of restricted energy intake for only three weeks might lead to a downregulation of the OXPHOS machinery possibly due to the decreased

availability of nutrients to metabolise. As discussed in chapter 4, other short-term studies also found a downregulation of OXPHOS [4, 8]. When the duration of restricted energy is extended to 12 weeks, OXPHOS might be upregulated to push the OXPHOS machinery to an active state to use all available nutrients for the generation of ATP via OXPHOS. In contrast to the upregulation of OXPHOS upon 12 weeks of ER in PBMCs, 12 weeks of ER downregulated OXPHOS pathways in WAT. This might be explained by the decrease in body fat mass, leading to a decrease in cellular respiration and the accompanied decrease in expression of OXPHOS-related genes.

In addition to these findings on energy metabolism-related pathways, CR and ER also affected olfactory signalling pathways. Findings on olfactory signalling pathways in human PBMCs and WAT have not yet been described in the literature. Nevertheless, we showed an increase upon 12 weeks of high protein ER (chapter 3) and a decrease upon three weeks of CR in old men only (chapter 2). No effect was seen for olfactory signalling upon 12 weeks of 25% ER (chapter 4). A possible role for olfactory signalling might be found in nutrient sensing which could be diminished in old men and is upregulated due to a high protein quantity in ER. Further research may focus on of the role of olfactory receptors in nutrient sensing and the decrease of these in elderly.

Biological interpretation of immune-related gene expression changes

Also immune-related pathways were affected in all three described intervention studies with ER and CR. Immune response-related pathways were downregulated in PBMCs from young men, and not changed in PBMCs from old men upon three weeks of 30% CR (chapter 2). In contrast, immune response-related pathways were upregulated upon 12 weeks of 20% ER in PBMCs from middle-aged and overweight subjects (chapter 4). Subjects in this last study were aged 50-65 years which could explain a difference in response compared to young men, but also the period of the intervention was four times longer. We hypothesised that young men were more healthy and had a lower inflammation and a decreased need for the immune response, while older men remained in a high inflammation state which could not be reduced by CR. In contrast to the increase in immune response related pathways in PBMCs from subjects aged 50-65 years upon 12 weeks of ER, 12 weeks of ER in WAT decreased immune response related pathways, such as adaptive immunity and immune cell infiltration, in subjects aged 55-70. This difference in immune response might be tissue-specific, as the decrease in fat tissue due to ER might lead to a locally decreased inflammation state, while in PBMCs the immune response might have been affected as a stress response towards less energy, possibly leading to an increased activation state of the immune response.

Gene expression changes in PBMCs during challenge tests

The potential of the transcriptome in combination with challenge tests was investigated to test the hypothesis that challenge tests magnify diet-induced health effects. In chapter 4, we studied the effect of 12 weeks of 20% ER on fasting level, and on the response during challenge tests. Upon

the OGTT, OXPHOS pathways were already significantly downregulated in ER participants at 30 minutes compared to non-ER controls at 240 minutes. The changes in gene expression of PBMCs from ER participants upon an OGTT was faster than of control subjects pointing to an increased phenotypic flexibility after 12 weeks of ER. This faster decrease in expression of the PBMC transcriptome upon a challenge was defined as a higher phenotypic flexibility and reflects theoretically a better health state. Although differences in gene expression changes were present in the fasted state upon ER, using the OGTT made it possible to see a faster and consistent dynamic change in gene expression during this challenge. This dynamic change is not possible to measure in a static fasting sample. In chapter 5 we investigated the effect of different challenge tests on the transcriptome, and showed a downregulation of gene sets involved in fatty acid metabolism, TCA cycle, and OXPHOS for both an OGTT and an MMT in the first two hours after intake. Based on the high overlap in gene expression changes, we hypothesised that the overlap in glucose in these challenge test is the main denominator of the postprandial changes in gene expression, in the first two hours, which might be mainly regulated via insulin.

Strengths and limitations of these human studies on CR and ER

In this thesis we included three different CR and ER intervention studies. First, all studies were randomized dietary intervention studies (chapter 2, 3, 4). Second, the study in young and old men (chapter 2) and the study on the response of WAT (chapter 3) were both completely controlled dietary interventions. During these controlled dietary interventions, 90% of the diet was provided by the University. Due to the fact that the intervention groups in the study on the effect of protein quantity on the WAT transcriptome (chapter 3) were ER diets, this study could also be performed double-blinded (chapter 3). Furthermore, the 12 weeks 20% ER study with nutritional challenge tests (chapter 4, 5) consisted of a large number of participants, 72 in chapter 4 and 68 in chapter 5. The sample size of the other two studies was smaller, 19 in chapter 2 and 22 in chapter 3. Nevertheless, the sample sizes in the presented studies were sufficient to show robust gene expression changes upon CR, ER, and challenge tests. Although, within the study population we did see variations in response.

As studies presented in chapter 2 and 3 were not primarily setup for the exploration of the use of the transcriptome as a marker of diet-related health, some points need to be addressed. Although the sample size showed robust effects, the number of participants was low in the first two studies (chapter 2 and 3). In chapter 2, the WAT biopsy was not used as primary outcome measure of the original study [9] and was on a voluntary basis, leading to a smaller sample size. This makes it more difficult to draw solid conclusions and these studies had a more explorative nature. The period of three weeks of CR in chapter 2, was originally applied to examine possible difference in energy intake after CR [10]. A longer period of CR would have been helpful to compare these results to the results of 12 weeks of ER (chapter 4) and to have more insight in the effect of age on the transcriptome. The study in WAT in chapter 3 on the other hand, had a smaller sample size but also an ER period of 12 weeks and a comparable study population. Finally, the study presented in chapter 3 was designed to investigate the effect of high protein quantity on muscle mass preservation [9]. This was the reason why we studied the effect of a higher than normal protein

quantity. Based on literature, a study designed for actual protein restriction might have reflected the design of the animal studies in which beneficial effects of protein restriction were observed. Most of these studies were used to explore the use of the transcriptome as a marker of diet-related health. Addition of some other measurements would have improved the interpretation of the presented results. Studying a measure for inflammation, such as the measurement of C-reactive protein (CRP), could have been used to determine the inflammation state of the participants. The information on the inflammation state would have been helpful to show whether changes upon the interventions were due to the intervention or due to a coincidental infection such as a common cold. Although the participants were randomized, and this should not have affected the main outcome, maybe it could have been used to explain interindividual differences in response.

The aim of the ER and CR studies in this thesis was to explore the use of the PBMC transcriptome as early and sensitive marker of diet-related health. Within these studies we have used Gene Set Enrichment Analysis (GSEA) as an explorative tool to identify potential gene sets and signalling routes that were changed upon the intervention. GSEA has the advantage that it helps to interpret large amount of genes on functional levels and increases the signal-to-noise ratio [11] leading to the inclusion of genes with non-significant changes that are still important for contribution to changes in pathways and signalling routes. A disadvantage of GSEA is that it is based on the current knowledge of the biological function of genes and gene sets and might be biased towards often-studied genes, pathways, and processes such as those involved in cancer development or HIV. This leads to many gene sets related to cell cycle and viral infections. This might look more important as they might exist with different names leading to a larger cluster of gene sets, than other less studied gene sets. To circumvent this bias we always investigated smaller clusters or individual gene sets and investigated the enrichment of genes present in a gene set and checked in our data set whether these were significantly changed.

Another point to address is the impact of energy output in dietary interventions. Energy expenditure is important for interpretation of CR data as increased energy expenditure might increase the percentage of CR. Due to the decreased energy intake in ER and CR studies, it might be possible that non-exercise activity thermogenesis (NEAT) [12] is decreased as a metabolic adaptation to save energy. Although in all studies the exercise activity was advised to be kept on a habitual level for each participant, the knowledge on energy output due to NEAT-activities was not measured and might have explained differences between individuals. Furthermore as these studies were performed in different seasons, due to 12 weeks of intervention time, this might have influenced the activity of the participants and might also have affected the results.

In this thesis we focused mostly on the use of the PBMC transcriptome as a potential marker of diet-related health. PBMCs are used because of their easy accessibility, in contrast to tissues such as muscle, liver or fat. This value of PBMCs is important and although in all studies pathways involved in energy metabolism were identified, we need to address that PBMCs are immune cells and will probably show more changes towards immune-related pathways. This should be thought of when interpreting the results, but can also be used as an advantage to study more immune-

related genes as potential marker. Also for the potential of a marker of diet-related health, it is impossible to define health solely based on transcriptome data. To circumvent this, we performed ER to increase health, and studied different ages, to be able to study different health states.

Recommendations for future research

To have a clearly defined subset part of the transcriptome that can be used as a marker of diet-related health it is required to show the relation between gene expression and health parameters, such as insulin sensitivity. Another strategy could be the use of the gene expression profile of young participants as a starting point, and compare gene expression profile of other participants with this healthy, young gene expression profile. An example can be found in our study where the response of a subset of genes in old men changed upon CR towards the expression pattern of young subjects (chapter 2, figure 5). Also others have used this approach by comparing the ER-induced changes in gene expression in muscle tissue of middle-aged participants to the gene expression profile of young men [13]. Using this strategy and using young individuals as a starting point, would be helpful in finding a health marker in the PBMC transcriptome.

In this thesis we focused on the use of transcriptomics as a tool to study diet-related health. More omics technologies are gaining attention to be implemented in nutrition research, such as metabolomics, proteomics, genomics, and the microbiomics [14]. If more omics tool can be combined, a more comprehensive phenotype and eventually a more complete marker of diet-related health could arise. Also integration with data from other measurements such as body fat distribution from MRI, that could be different between individuals and could influence their response and health state, can be integrated to have a more comprehensive phenotype. Also based on the outcomes that for example OXPHOS pathways were differently affected by the CR and ER interventions, measurements such as the respiratory capacity of mitochondria might give insight in the actual energy generation of the cells and the possible health state of the cells.

The use of comprehensive phenotyping would also be helpful to study interindividual variations in response. This variation was for example visualised in chapter 3, figure 1, showing that upon 12 weeks ER three participants responded in an opposite manner than the other 19 participants. Also, in chapter 5 we showed through ssGSEA that there was variation in the changes in gene expression upon the challenge tests. A bigger sample size would have made it possible to split the study groups into subgroups with different responses. Using a large sample size would also make it easier to study the role of genetic variants and epigenetics. For example, the influence of genetic variants has been shown for the relationship between apolipoprotein-E carriers and fish oil on gene expression of cholesterol biosynthesis and interferon signalling pathways [15]. Also variants of the adiponectin gene influence the effect of sodium and potassium intake on blood pressure[16]. This kind of genetic information might be of interest in personalised nutrition, such as finding the right amount of sodium or potassium intake for blood pressure. Also challenge tests can help in this more personalised approach for dietary advice as they might be used to cluster individuals in specific responder-groups, as has been done for example based on the response of glucose, insulin, C-peptide concentration, and NEFAs during an OGTT [17].

Although the focus of most dietary intervention studies is on the static, fasting levels, which are usually well-regulated levels, they do not show the ability of an individual to cope with a nutritional stressor. The results of our study showed the added value of a challenge test and future research may therefore focus more on the individual dynamic response to a challenge as this might be used to show an increase in phenotypic flexibility. For example, the use of a challenge test could have been of value in the study of the older population on a CR diet as described in chapter 2. Fasting levels of old men showed a decreased response, mainly for immune-related pathways upon CR, but unknown is whether their phenotypic flexibility was also decreased. Despite the increased immune-related pathways upon baseline, these older men might still be capable of responding to a stressor and might still be phenotypic flexible, especially since they were healthy.

The combination of a challenge test and comprehensive phenotyping in larger study populations undergoing dietary interventions might lead to the understanding of an individual's nutritional need for the prevention of diseases such as diabetes, obesity, and cardiovascular disease. This might lead to a personalised dietary advice for optimal health.

CR mimetics are strategies that result in health benefits of CR without reduction of actual food intake and can be pharmaceutical, nutraceutical, or physiological. Although the adherence to the dietary interventions in our studies was high, in daily life settings subjects do not find it easy to restrict calories. CR mimetics might possible lead to CR benefits without actual CR. Resveratrol has often been studied as a CR mimetic to extend life and health span in model organisms [18]. In human subjects that are obese, have diabetes type II, or have impaired glucose tolerance, resveratrol has been reported to improve metabolic function and improved insulin sensitivity, postprandial glucose levels, and decreased inflammation [19-21]. In contrast, in subjects that were non-obese and had normal glucose tolerance, 12 weeks of resveratrol did not improve plasma lipids, insulin sensitivity, or inflammatory markers [22]. It might however be difficult to find one substitute for CR, since CR affects the complete body and one CR mimetic might not. Finding the most important change due to CR might lead to the identification of a possible CR mimetic, by performing that more mechanistic and function-based studies on the effect of ER and CR. This might be done in for example knock-outs in animal models to investigate the importance of certain genes in the effects of CR and ER. This might eventually help in the discovery of CR mimetics.

Conclusions

Based on the observations in chapter 2, 3, and 4, we conclude that the transcriptome can be used to study diet-related effects on health. We also concluded based on the differential changes in gene expression upon CR at different ages, that the age is an important modulator in the response to CR. The same can be concluded for dietary composition, as a high protein ER diet seemed to reflect less beneficial health effects in changes in gene expression than a normal protein ER diet. Based on the faster and more pronounced changes in gene expression during an OGTT upon 12 weeks of ER, we concluded that the PBMC transcriptome combined with a challenge test can reflect changes in phenotypic flexibility. This makes challenge tests a suitable tool in addition to fasting to study diet-related health effects. Finally, based on the changes in gene expression of the MMT and OGTT, we concluded that glucose in a challenge test is the main denominator of the postprandial changes in gene expression in the first two hours.

Overall, these outcomes lead to the conclusion that the transcriptome, especially in combination with challenge tests, can be used as an early marker of diet-related health. The direct relation to health still needs to be investigated, but the possibility to use the transcriptome as an early marker of diet-related health gives rise to a better understanding of the effects of nutrition on health.

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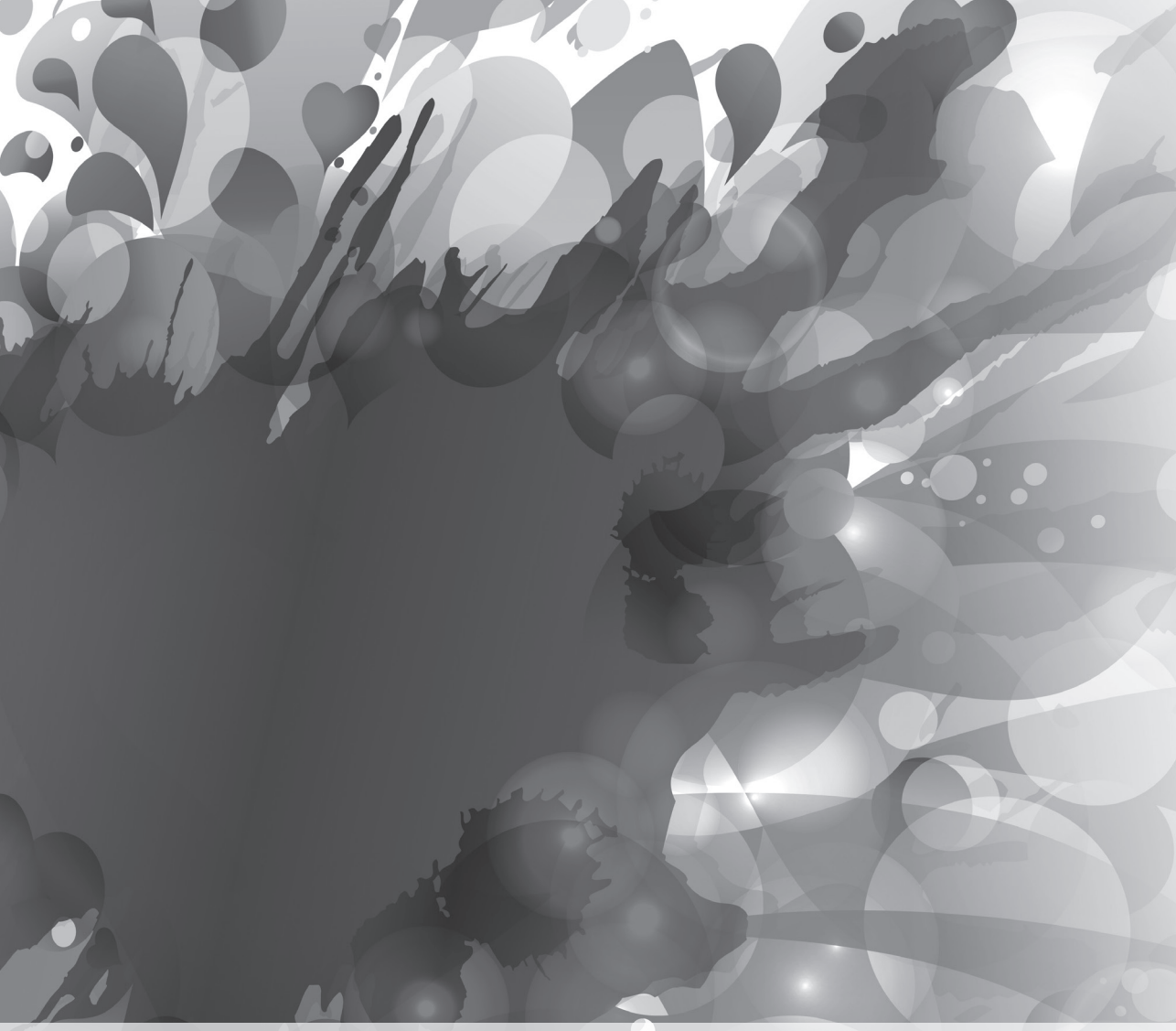


Summary

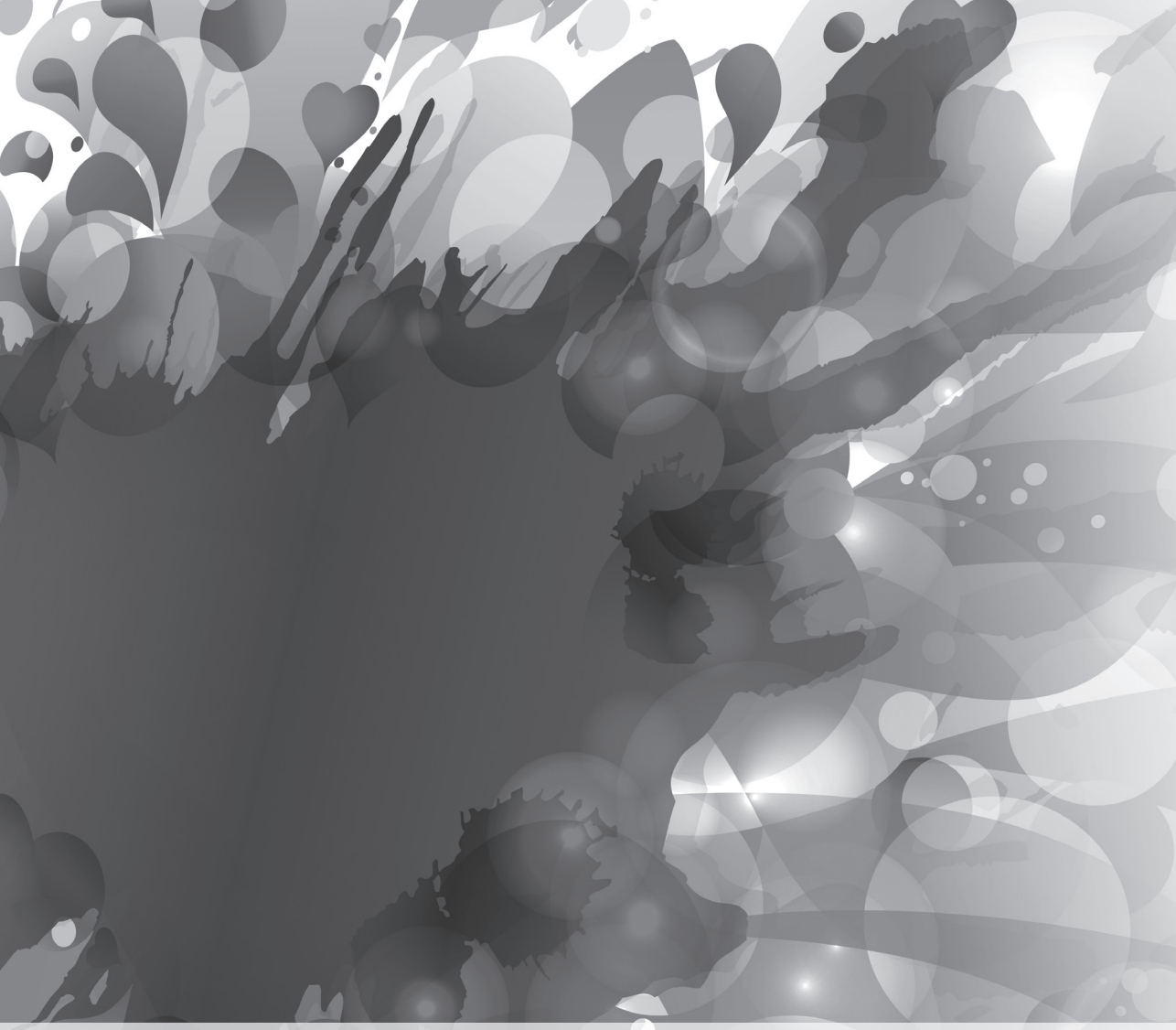
Summary

Background: Nutrition research is facing several challenges with respect to finding diet-related health effects. The effects of nutrition on health are subtle, show high interindividual variations in response, and can take long before they become visual. Recently, the definition of health has been redefined as an organism's ability to adapt to challenges and 'this definition' can be extended to metabolic health. In the metabolic context the ability to adapt has been named 'phenotypic flexibility'. A potential new tool to magnify the effects of diet on health is the application of challenge tests. Combined with a comprehensive tool such as transcriptomics, the study of challenge tests before and after an intervention might be able to test a change in phenotypic flexibility. A dietary intervention well-known to improve health through weight loss is energy restriction (ER). ER can be used as a model to examine the potential of challenge tests in combination with transcriptomics to magnify diet-induced effects on health. As opposed to ER, caloric restriction (CR) is a reduction in energy intake aimed at improving health and life span in non-obese subjects and not directly aimed at weight loss. In this thesis, we aimed to investigate the use of the transcriptome as an early and sensitive marker of diet-related health.

Methods: First we studied the consequences of age on the effects of CR on the peripheral blood mononuclear cells (PBMCs) transcriptome. For that purpose, we compared the changes in gene expression in PBMCs from old men with the changes in gene expression in PBMCs from young men upon three weeks of 30% CR. To study the effect of a change in dietary composition during ER, we compared the changes in gene expression upon a 12 weeks high protein 25% ER diet with the changes in gene expression upon a 12 weeks normal protein 25% ER diet in white adipose tissue (WAT). Next, we investigated the added value of measuring the PBMC transcriptome during challenge tests compared to measuring the PBMC transcriptome in the fasted state to magnify the effects of ER on health. This was investigated by measuring the changes in gene expression upon an oral glucose tolerance test (OGTT) and upon a mixed meal test (MMT), both before and after 12 weeks of 20% ER. Finally, we determined the differences between a challenge test consisting of glucose alone, the OGTT, or consisting of glucose plus other macronutrients, the MMT, on the PBMC transcriptome in diet-related health.

Results: We observed that the transcriptome of PBMCs of healthy young men had a higher responsiveness in immune response pathways compared to the transcriptome of PBMCs of aged men upon CR (chapter 2). Also, we showed that upon a normal protein-ER diet the transcriptome of WAT showed a decrease in pathways involved in immune response and inflammasome, whereas no such effect was found upon a high protein-ER diet. These effect were observed while parameters such as weight loss, glucose, and waist circumference did not change due to the different protein quantities (chapter 3). 12 weeks of 20% ER was shown to increase phenotypic flexibility as reflected by a faster and more pronounced downregulation of OXPHOS, cell adhesion, and DNA replication during the OGTT compared to the control diet (chapter 4). Finally, two challenge tests consisting of either glucose (OGTT) or glucose plus fat and protein (MMT), were shown to result in a larger overlap than difference in the changes in gene expression of PBMCs (chapter 5).

Conclusions: Based on the differential changes in gene expression upon CR at different ages, we concluded that age is an important modulator in the response to CR. As a high protein ER diet induced transcriptional changes seemed to reflect less beneficial health effects than a normal protein ER diet we concluded that the diet composition is important in the health-effect of ER as measured by the transcriptome. Based on the faster PBMCs changes in gene expression during an OGTT upon 12 weeks of 20% ER, we concluded that the PBMC transcriptome combined with a challenge test can reflect changes in phenotypic flexibility. This makes challenge tests a suitable tool to study diet-related health effects. Finally, based on the changes in gene expression of the MMT and OGTT, we conclude that glucose in a challenge test is the main denominator of the postprandial changes in gene expression in the first two hours. Overall, these results lead to the conclusion that the transcriptome, especially in combination with challenges test, can be used as an early marker of diet-related health. The direct relation to health still needs to be investigated, but the possibility to use the transcriptome as an early marker of diet-related health gives rise to a better understanding of the effects of nutrition on health.



Nederlandse samenvatting

Achtergrond: De uitdaging in voedingsonderzoek is het kunnen meten van de effecten van voeding op de gezondheid. Dit komt doordat de gezondheidseffecten van voeding meestal subtiel zijn, veel interindividuele variatie in respons vertonen, en omdat het vaak jaren duurt voordat ze zichtbaar zijn. De definitie van gezondheid is onlangs opnieuw gedefinieerd. De oude definitie van gezondheid was 'een toestand van volledig lichamelijk, geestelijk en maatschappelijk welzijn en niet slechts de afwezigheid van ziekte of andere lichamelijk gebreken'. De nieuwe definitie richt zich meer op het vermogen van een organisme om zich aan te kunnen passen aan veranderingen en uitdagingen. Deze definitie van gezondheid kan worden doorgetrokken naar de stofwisseling, het metabolisme. In deze metabole context wordt het vermogen zich aan te passen 'fenotypische flexibiliteit' genoemd. Een mogelijk hulpmiddel om de meetbaarheid van de gezondheidseffecten van voeding te vergroten is het toepassen van challenge testen. Deze challenge testen zijn bedoeld om te bepalen hoe goed het lichaam in staat is om met een 'challenge', een grote hoeveelheid van een voedingsstof om te gaan. Naast deze challenge testen kunnen we gebruikmaken van transcriptomics: het meten van de mate van expressie van alle genen. De combinatie van een challenge test met transcriptomics kan misschien helpen om een verandering in de fenotypische flexibiliteit door het volgen van een bepaald voedingspatroon te meten. Een voedingspatroon dat bekend staat om zijn positieve effect op gezondheid, vooral voor mensen met overgewicht, is een sterke reductie van de inname van energie, ook wel energie restrictie (ER) genoemd. ER zorgt voor gewichtsverlies, en dit zorgt dan weer voor een betere gezondheid. ER kan daarom gebruikt worden als model voor een verbetering in gezondheid. Hierdoor kan ER ook gebruikt worden als model om aan te tonen of de combinatie van een challenge test en transcriptomics een beter maat is om een effect op de gezondheid te meten dan alleen meten in de nuchtere situatie. De term 'Calorie restrictie (CR)' is ook een verlaging van de hoeveelheid calorieën, maar is, in tegenstelling tot ER, voornamelijk bedoeld ter verbetering van de gezondheid en levensduur van mensen die geén overgewicht hebben. CR is dus niet direct gericht op gewichtsverlies. Het onderzoek in dit proefschrift heeft als doel om te bepalen of het transcriptoom kan dienen als een vroegtijdige en sensitieve marker voor het effect van voeding op de gezondheid.

Methoden: In het eerste onderzoek (hoofdstuk 2) werd de invloed van leeftijd op genexpressie in perifere bloed mononucleaire cellen (PBMC's) bepaald tijdens het volgen van een CR dieet. Na drie weken 30% CR dieet gevolgd te hebben is de genexpressieverandering in PBMC's van oudere mannen vergeleken met de genexpressieverandering in de PBMC's van jongere mannen. In het tweede onderzoek (hoofdstuk 3) werd het effect van de samenstelling van een ER dieet bestudeerd. In dit onderzoek werd het effect van een 12 weken 25% ER dieet met een hoog eiwitgehalte vergeleken met dat van een ER dieet met een standaard eiwitgehalte op genexpressieverandering in vetweefsel. Vervolgens werd in het derde onderzoek (hoofdstuk 4) de toegevoegde waarde van het gebruik van challenge testen voor het vergroten van de meetbaarheid van de gezondheidseffecten van ER onderzocht door de resultaten van challenge testen te vergelijken met de uitkomsten van de nuchtere samples. De resultaten van de veranderingen in genexpressie gedurende een orale glucosetolerantietest (OGTT) en een gemengde maaltijd-test (MMT) werden onderzocht zowel vóor als na een 12 weken-durende

interventie van 20% ER. Tot slot werden in hoofdstuk 5 de verschillen tussen deze twee testen vastgesteld om te zien wat het effect van een test met alleen glucose (OGTT) is ten opzicht van een test met glucose én andere macronutriënten (MMT) op de genexpressie van PBMC's.

Resultaten: We zagen in hoofdstuk 2 dat, na drie weken CR, de genexpressie van de PBMC's van gezonde jonge mannen ten opzichte van de genexpressie van de oudere mannen een grotere response vertoonde in signaalroutes betrokken bij de immuunresponse. Ook toonden we aan, in hoofdstuk 3, dat na een normaal eiwitgehalte-ER dieet, het transcriptoom van vetweefsel een daling vertoonde in signaalroutes betrokken bij de immuunrespons en het inflammasoom, terwijl er geen dergelijk effect werd gevonden na het hoog eiwitgehalte-ER dieet. Dit effect werd waargenomen terwijl parameters zoals gewichtsverlies, glucose, en tailleomtrek niet veranderden als gevolg van de verschillende eiwitgehaltes. In hoofdstuk 4 vonden we dat na het volgen van een 12 weken-durend 20% ER dieet, de fenotypische flexibiliteit van de deelnemers was vergroot. Dit bleek uit een snellere en meer uitgesproken downregulatie van signaalroutes betrokken bij oxidatieve fosforylering, celadhesie, en DNA replicatie tijdens de OGTT. Dit werd niet waargenomen bij de deelnemers die het controle dieet volgden. Tenslotte bleek uit de vergelijking van de challenge testen in hoofdstuk 5, dat een test met enkel glucose (de OGTT) versus een test met glucose plus vet en eiwit (de MMT) er meer overlap dan verschil in de veranderingen in genexpressie in de PBMCs tussen beide testen was.

Conclusies: Op basis van de verschillen in genexpressie na een CR dieet bij deelnemers van verschillende leeftijden, concludeerden we dat het fenotype, en in dit geval dus leeftijd, een belangrijke rol speelt in het effect van CR op genexpressie. De genexpressie veranderingen geïnduceerd door het ER dieet met een hoog eiwitgehalte lijken minder gunstig voor de gezondheid dan een ER dieet met normaal eiwitgehalte. Hieruit concludeerden we dat tijdens ER de voedingssamenstelling een belangrijk rol speelt in het gezondheidseffect, zoals weerspiegeld door de genexpressie, het transcriptoom. Gebaseerd op de snellere en grotere effecten in genexpressieverandering gedurende de OGTT vergeleken met de nuchtere situatie na het ER dieet dan na het controle dieet, concludeerden we dat het meten van het transcriptoom in combinatie met een challenge test, veranderingen in fenotypische flexibiliteit kan weergeven. Dit maakt challenge testen een geschikte tool om de voeding-gerelateerde gezondheidseffecten te bestuderen. Tenslotte, op basis van de genexpressieveranderingen tijdens de MMT en de OGTT, concludeerden we dat in de eerste twee uur postprandiaal de glucose in de challenge testen waarschijnlijk de balangrijkste inducer van de genexpressieveranderingen is. Samenvattend, kunnen we uit deze resultaten concluderen dat het transcriptoom, en voornamelijk in combinatie met een challenge test, kan worden gebruikt als een vroegtijdige marker voor de effecten van voeding. De directe relatie tussen verandering in genexpressie en gezondheid moet nog worden onderzocht, maar de mogelijkheid om genexpressie te gebruiken als marker voor de effecten van voeding, kan bijdragen tot het beter begrijpen van hoe voeding werkt in het lichaam met het uiteindelijke doel de relatie tussen voeding en gezondheid te onderzoeken.

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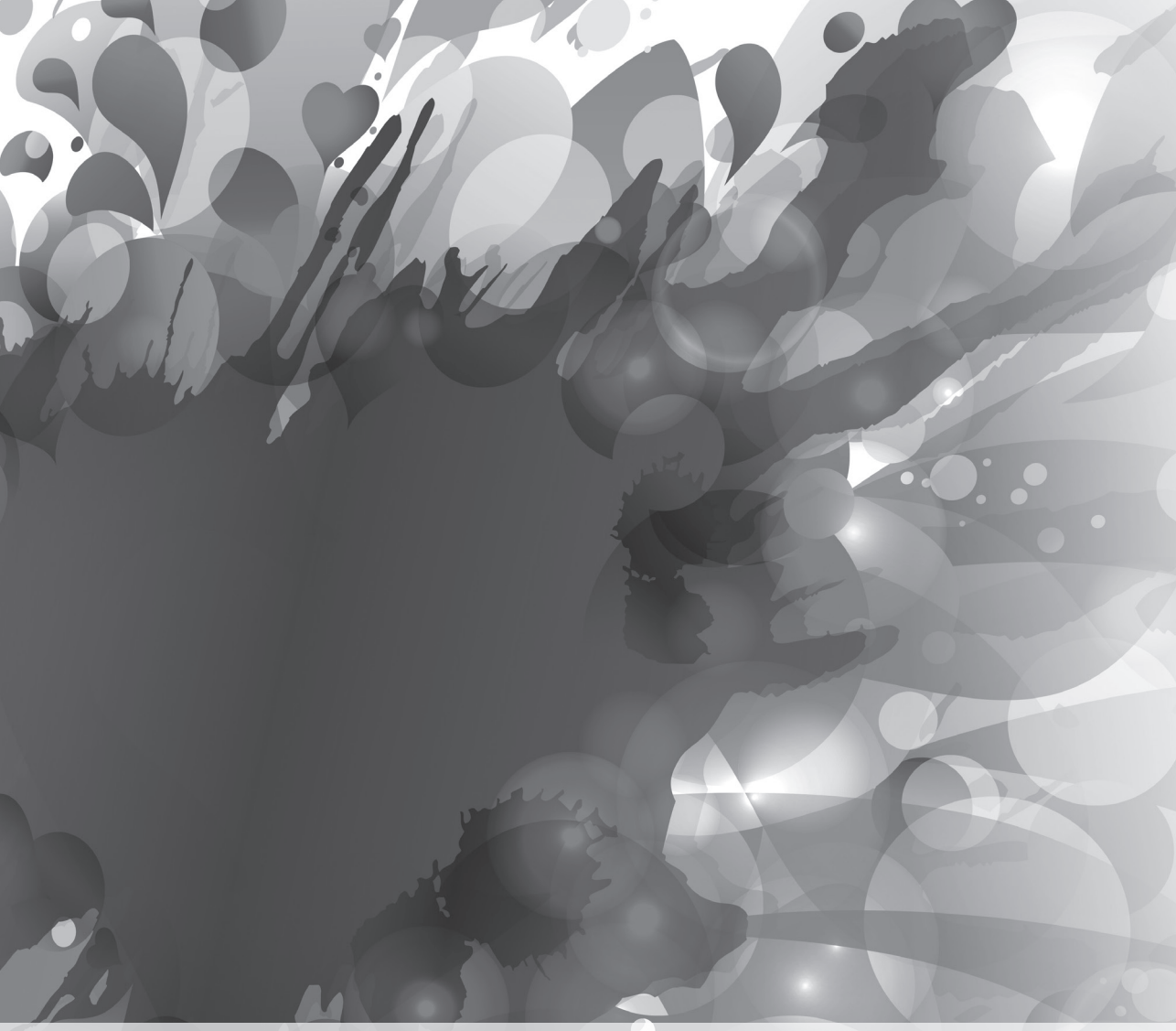
Inge



About the Author

Curriculum Vitae

Inge van Bussel was born on the 7th of September, 1988 in Roermond, the Netherlands. In 2006, she completed secondary school (Gym, *cum laude*) at B.C. Schöndeln in Roermond. In 2007, she started her bachelor Biology at Radboud University Nijmegen. During her bachelors she performed an internship and investigated the 'Role of PACAP in the Ucn1-npEW stress centre' at the Donders Centre for Neuroscience, Institute of Biosciences and Faculty of Science of Radboud University Nijmegen. Upon completion of her bachelor's degree (*cum laude*) in 2009, she continued with a master in Medical Biology at Radboud University Nijmegen and she received her master's degree in 2011 (*bene meritum*). During her master she performed two internships. Her first internship was about 'characterisation of 5-HT2A receptor and DRD3 mutant rats' at Radboud University Nijmegen Medical Centre. For her second internship she went to Cambridge, United Kingdom and performed an internship at the Medical Research Council, Laboratory of Molecular Biology which was about the 'identification of tissue-dependent alternative splicing events within mouse clock genes'.

In March 2012, Inge started her PhD project entitled "blood cell transcriptome as biomarker' under the supervision of Prof. Michael Müller and Dr Lydia Afman, and Prof. Sander Kersten as her promoter, at the Nutrition, Metabolism & Genomics group of the Division of Human Nutrition at Wageningen University. Her PhD project was part of an European Union funded project 'NutriTech'. During her PhD she closely collaborated with members of this international project and presented her work at different symposia, conferences and meetings. In 2013, she was awarded with a poster prize. In addition to her research activities, she organized a study tour to Australia for 25 PhD students of the division of Human Nutrition. Furthermore, she was involved in teaching bachelor students and supervised master students during their master thesis. The results from her PhD project are described in this thesis entitled 'The transcriptome as early marker of diet-related health. Evidence in energy restriction studies in humans.'.

List of publications

Van Bussel, I.P.G., P. Fazelzadeh, G.J.E.J. Hooiveld, and L.A. Afman. "Comparison of postprandial whole genome gene expression response in peripheral blood mononuclear cells between an oral glucose tolerance test and a mixed meal test." *In preparation.*

Van Bussel, I.P.G., P. Fazelzadeh, G. Frost, M. Rundle, and L.A. Afman. "Measuring phenotypic flexibility by using transcriptome time course analyses during challenge tests before and after energy restriction." *In preparation.*

Van Bussel, I.P.G., E.M.P. Backx, C.P.G.M. De Groot, C.P.G.M., M. Tieland, M. Müller, and L.A. Afman *(in press)*. "The impact of protein quantity during energy restriction on genome-wide gene expression analysis in adipose tissue of obese humans." Int J Obes (Lond), *in press.*

Van Bussel, I.P.G., A. Jolink-Stoppelenburg, C. P. De Groot, M. R. Muller, and L. A. Afman (2016). "Differences in genome-wide gene expression response in peripheral blood mononuclear cells between young and old men upon caloric restriction." Genes Nutr 11: 13.

Nonkes, L.J., **I.P.G. van Bussel**, M.M. Verheij, and J.R. Homberg (2011). "The interplay between brain 5-hydroxytryptamine levels and cocaine addiction." Behav Pharmacol 22(8): 723-738.

Overview of completed training activities

Discipline specific activities

Courses	*Organiser*	*Location and year*
NutriScience	VLAG	Wageningen (NL, 2013)
Microarray Analysis using R & Bioconductor	diXa	Maastricht (NL, 2014)
Conferences	*Organiser(s)*	*Location and year*
Nutritional Science days	NWO	Deurne/Heeze (NL,2012, 2013,2015)
Phenotypic flexibility symposium	NutriTech, Bioclaims, PhenFlex, and NuGO	Madrid (ES, 2013)
10th NuGO week	NuGO, TUM	Freising (DE, 2013)
11th NuGO week	NuGO, CRA-NUT	Castellammare di Stabia (IT,2014)
12th NuGO week	NuGO, UB	Barcelona (ES, 2015)
12th European Nutrition Conference FENS	Fase20	Berlin (DE, 2016)
NutriTech Final Symposium	TNO, ILSI	Lisbon (PT, 2016)

General courses

Courses	*Organiser*	*Location and year*
Voice Matters	WGS	Wageningen (NL, 2013)
Competence assessment	WGS	Wageningen (NL, 2013)
Techniques for Writing and Presenting Scientific Papers	WGS	Wageningen (NL, 2013)
Career Assessment	WGS	Wageningen (NL, 2015)
Project and Time Management	WGS	Wageningen (NL, 2015)
Entrepreneurship in and outside Science	WGS	Wageningen (NL, 2015)
Career orientation	WGS	Wageningen (NL, 2016)

Optional activities

Preparation research proposal	WUR	Wageningen (NL, 2012)
PhD tour & organising committee	WUR	Australia & Wageningen (NL, 2013)
NMG-pharma science meetings	WUR	Wageningen (NL, weekly 2012-2016)
Staff seminars	WUR	Wageningen (NL, monthly 2012-2016)

Colophon

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