How to measure health improvement?

Assessment of subtle shifts in metabolic phenotype

Parastoo Fazelzadeh

THESIS COMMITTEE

Promotors

Prof. Dr A.H. Kersten

Professor of Nutrition, Metabolism and Genomics

Wageningen University & Research

Prof. Dr J.P.M. van Duynhoven

Special Professor Magnetic Resonance in relation to Food

Wageningen University & Research

Co-promotor

Dr M.V. Boekschoten

Researcher, Division of Human Nutrition

Wageningen University & Research

Other members

Prof. Dr W.H.M. Saris, Maastricht University, The Netherlands

Prof. Dr E.J.M. Feskens,Wageningen University & Research, The Netherlands

Dr J.A. Westerhuis, University of Amsterdam, The Netherlands

Dr J. Bouwman, TNO, Zeist, The Netherlands

This research was conducted under the auspices of the Graduate School VLAG
(Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

How to measure health improvement?

Assessment of subtle shifts in metabolic phenotype

Parastoo Fazelzadeh

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Thursday 11 May 2017
at 11 a.m. in the Aula.

Parastoo Fazelzadeh
How to measure health improvement?
Assessment of subtle shifts in metabolic phenotype
188 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2017)
With references, with summary in English

ISBN: 978-94-6343-073-9
DOI: 10.18174/403573

CONTENTS

To my beloved parents...

1

Introduction

Human health is impacted by a complex network of interactions between biological pathways, mechanisms, processes, and organs. To maintain internal homeostasis, many of these processes need to act in a continuously changing environment in response to stimuli such as exercise, diet, infections, stress, and temperature (Figure 1). The ability to appropriately respond to changes in environment is referred to as 'phenotypic flexibility'. Diseases develop when and where these adaptive processes fail [1].

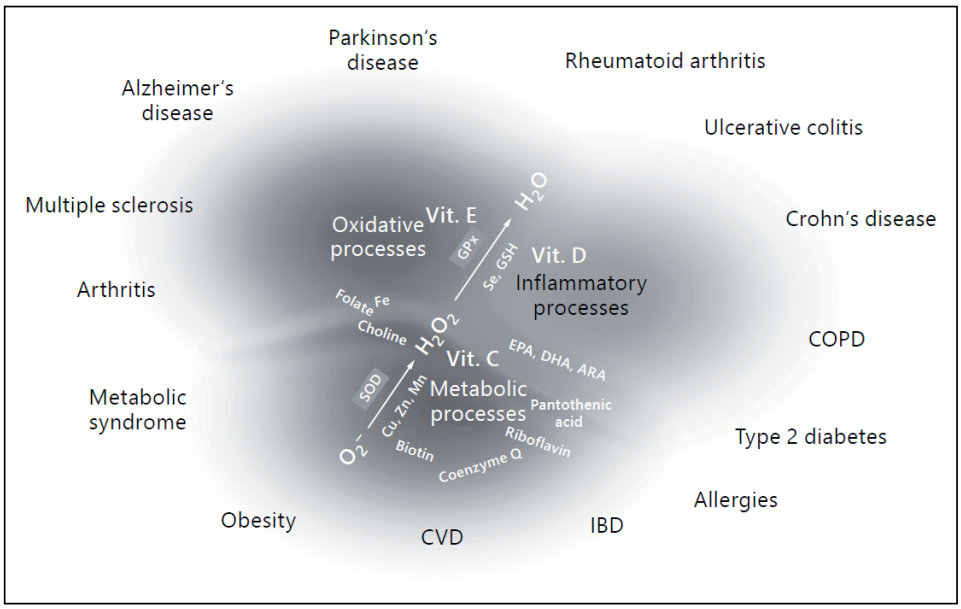


Figure 1. Many disease onsets (outer circle) are related to lifestyle; however, the mechanisms of disease progression and the processes that maintain optimal health are different. This figure shows three crucial processes including the capacity to maintain flexibility in metabolic, oxidative, and inflammatory stress. Here, flexibility is considered as the capacity to maintain optimal homeostasis. CVD = Cardiovascular disease; IBD = inflammatory bowel disease; IR = insulin resistance; SOD = superoxide dismutase. (Reproduced from van Ommen B. and Wopereis, S) [1].

The metabolic phenotype provides a readout of the metabolic state of an individual and is a product of interactions between several factors such as genetics, diet, lifestyle, environment and gut microbiota [2, 3]. To maintain health, understanding these relationships will be one of a major challenges for the next decades. To address this challenge, system biology powered by genomics, proteomics, bioinformatics, and metabolomics is providing a novel methodological framework to unravel the connections between health states [1, 4, 5]. This concept is illustrated in Figure 2, which shows the application of high-throughput 'omics' techniques and elabo-

rate statistical analyses tools to help resolve the complex relationship between (bio) chemicals in human systems and their interaction with other variables including environment /lifestyle to produce the measured phenotype [6].

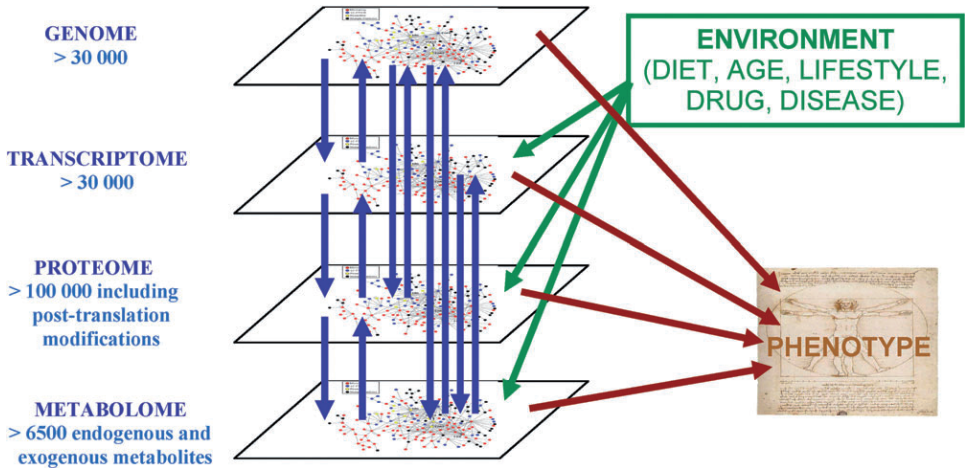


Figure 2. A schematic overview of the complex interactions of functional levels including metabolome, proteome, transcriptome and genome in biological systems. In general, the phenotype is considered as a product of the complex interaction of components from all of the functional levels and environmental effects. (Reproduced from Dunn et al.) [7].

The question now is how to measure a subtle shift towards a healthy phenotype. As lifestyle and diet induced effects are rather small, sensitive methods are needed to capture these slight changes.

Currently, the biomarkers used in health care research are not based on optimal health maintenance but on quantifying disease [1]. Since optimal health is not simply the absence of disease, and since the underlying biology of health and disease may be fundamentally different, a new generation of biomarkers is needed to quantify all aspects of systems flexibility, with the aim of bringing more insight into the lifestyle factor that promote health optimization [1].

In this thesis I describe two ways to monitor phenotype shifts; either by looking directly into the baseline metabolome of tissues or by looking at the ability to restore metabolic homeostasis in blood upon a dietary challenge. Both metabolomics and transcriptomics will be deployed to get a comprehensive view on the metabolic phenotype.

Phenotyping via snapshot measurements in organs

Most studies that aim to assess a metabolic phenotype rely on circulating biofluids such as blood and urine [8, 9]. Such body fluids can be obtained in a straightforward manner from volunteers and are considered to mirror the metabolic events that occur in organs. It should be noted, however, that tissues and organs might be more sensitive to report on the metabolic phenotype as each organ has strikingly different metabolic patterns [10].

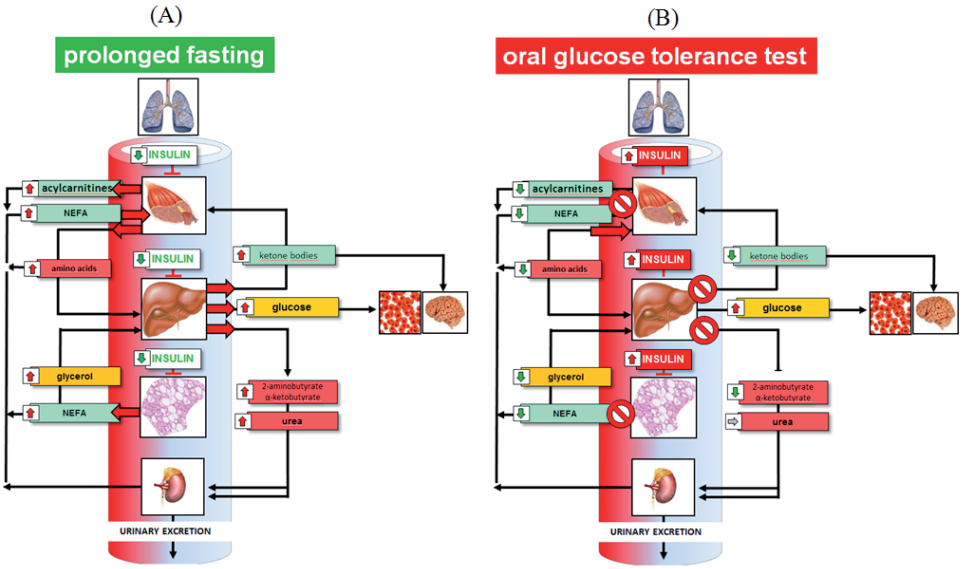


Figure 3. Schematic depiction of the metabolic control mechanisms that define (A) the catabolic (fasting) state and (B) the anabolic (postprandial) state. Metabolically active tissues have been indicated that predominantly define the metabolic phenotype (muscle, liver, adipose) (Reproduced from van Ommen *et al*. 2014)[11].

Although metabolism for a major part takes place in liver, muscle and adipose tissue (Figure 3), only few human studies have considered these tissues for defining metabolic phenotypes [12-15]. Skeletal muscle is an interesting organ to study phenotypic shifts as the phenotype can be altered through exercise and nutrition. Current insights are mostly based on animal studies [16, 17]. The few metabolic studies that involve human muscle biopsies are mostly related to a few metabolites or to specific metabolite classes [14, 15]. Therefore, a comprehensive view of the effects on various stimuli on the muscle metabolome is still lacking.

Phenotyping via challenge responses

Phenotypic flexibility is a key factor in the relationship between human health and nutri-

tion and points to the concept of the human body as an orchestrated machinery that adapts to external stimuli [11]. Considering 'health' as the ability to cope with daily challenges [18], current approaches focusing on disease risk biomarkers are not sufficiently sensitive to detect the effects of lifestyle factors (including exercise training and nutritional interventions), which in general aim to improve and sustain health. Instead, one would like to design new biomarkers that tell something about the resilience capacity of an organism, which may be achieved by studying the response to specific perturbing stimuli.

In recent years, the application of meal challenges to detect subtle difference in metabolic resilience has gained much interest in the nutrition field [19-21]. The best known example is the Oral Glucose Tolerance Test (OGTT), which specifically probes the resilience of glucose metabolism [22]. In addition, a so called mixed meal challenge—involving a standardized meal comprising protein, lipids and glucose— has been employed to more broadly target phenotypic flexibility in multiple organs [23]. Consistent with this notion, it was shown that the application of a mixed meal challenge in combination with plasma metabolomics and proteomics profiling revealed additional metabolic changes beyond what was observed under non-perturbed conditions [24].

Similarly, by applying a highly controlled 4 day challenge protocol—which included 36 h fasting, Oral Glucose and Lipid Tests (OGTTs & OLTTs), liquid test meals, physical exercise, and cold stress—to young healthy male subjects, inter-individual variation was increased even in phenotypically similar subjects. Specifically, the challenges enabled the identification of specific metabotypes that were not observable in baseline metabolite profiles [25]. These findings have provided detailed information on the complex metabolic changes induced by OGTTs/OLTTs and have given novel insights into the regulation of glucose and lipid metabolism. In this study, we examined whether a response to a mixed-meal challenge could provide readout for a shift in phenotypic flexibility upon diet-induced weight loss in abdominally obese male subjects.

Metabolomics

Metabolomics is the 'systematic study of the unique chemical fingerprints which specific cellular processes leave behind', this pertains in particular to the scientific study of their small-molecule metabolite profiles [26]. As the end products of cellular processes, the metabolome represents the collection of all metabolites in a biological cell, tissue, organ or organism [27].In recent years, several strategies to identify and quantify multiple metabolites using sophisticated analytical technologies have emerged. As a direct functional readout of the physiological state of an organism, 'metabolomics', is considered to 'act as spoken language,

broadcasting signals from the genetic architecture and the environment' [28, 29].

A range of analytical technologies including mass spectrometry, liquid or gas chromatography and NMR has been employed to analyse metabolites in different organisms, tissues, and fluids. Complementary approaches have to be established to extract, detect, quantify, and identify as many metabolites as possible [30].

Metabolite levels are closely linked to the phenotype of an organism. Accordingly, metabolomics can be considered as bridging the gap between genotype and phenotype [31], providing a more complete view of how cells function, as well as identifying novel or striking changes in specific metabolites. In particular, studying tissue-derived metabolite profiles is probably the closest way to get to the phenotype, yet it is not a well-developed technology. A major bottleneck to achieve extended coverage is the limited amount of tissue that can be obtained from human studies. The recent development of a comprehensive targeted metabolic profiling platform which was optimized and validated for small muscle biopsies paved the way to unravel the observed phenotypical differences at a more comprehensive level [32]. In this thesis we covered several metabolite classes including amines, acylcarnitines, organic acids, oxylipins and nucleotides. Our hypothesis was to study phenotypic shifts at not only the single metabolite level but also at the level of related pathway (Table 1).

Table 1. Metabolites classes measured in this thesis

Metabolite classes	Platform	Relevant pathways per platform
Amino acids & Biogenic amines	Accq-Tag UPLC-MS/MS (33) & NMR (34)	Stimulate protein synthesis, Muscle recovery from exercise
Acylcarnitines	Accq-Tag UPLC-MS/MS (33)	Mitochondrial transport of fatty and amino acids
Organic acids	GCMS (35)	TCA cycle, Carbohydrate metabolism and Fatty acid oxidation
Oxylipins	LCMS (36)	Changes in inflammatory lipid metabolism
Nucleotides	Enzymatic assays (32)	Energy metabolism

Transcriptomics

Transcriptomics uses high-throughput genomic methods, such as microarray analysis or RNA sequencing, to assess the expression of thousands of genes. Knowing the pattern of gene expression at a given time allows the identification of gene sets that are differentially expressed in response to challenge/intervention. In this way, it can be used to get more insights into the genome-wide effects of a specific stimulus, which in turn can help us to discover the effects of regulatory pathways involved in transcriptional regulation.

In relation to human health, transcriptomics has already proven to be extremely valuable to distinguish not only a pathological state from a non-pathological state, but it can also show more detailed patterns that reflect different stages of a specific disease progression [37, 38]. Hence, it can be also useful to study shifts in phenotype following specific challenges/interventions. By integrating the knowledge derived from transcriptomics, genetics and other available tools, our understanding of the phenotype flexibility is expected to greatly improve.

Gene expression studies can be performed both by directly looking into organ-specific gene expression or by looking at peripheral blood mononuclear cells (PBMC's). The suitability of PBMCs for gene profiling is related to their active metabolism [39] and their accessibility by simple venipuncture [40, 41], thereby allowing for a repeated time-series analysis of changes in gene expression in response to dietary interventions. Still, it should be considered that while transcriptomic profiling of organs allows a direct view of gene expression in the tissue of interest, PBMC's are used as proxies for systemic effects. Importantly, a study by Rudkowska *et al.* supports the use of PBMCs as a surrogate model for skeletal muscle gene expression in nutrigenomics studies, as shown by the strong correlation (r=0.84, $P<0.0001$) between transcript expression levels of PBMCs and skeletal muscle tissue after n-3 PUFA supplementation [42].

Model populations for observing phenotype shifts

Frail and healthy elderly subjects: effect of prolonged training

Populations around the world are aging rapidly. This trend is likely to increase further over the next few decades [43]. Due to the association of senescence with a wide range of diseases, there is an increase in demand for care at this stage of life. One of the major age-related functional declines is characterized by loss of skeletal muscle mass and function, often resulting in frailty. Physical activity is one of the most effective strategies to improve muscle mass and strength in adults [44, 45]. Different claims exist about the effectiveness of resistance-type exercise to overcome muscle loss and weakness in older adults, including its ability to improve quality of life [46], and even reversing aging in skeletal muscle [47]. In order to elucidate the complex processes that occur in ageing skeletal muscle, we assessed the effect of prolonged resistance-type exercise training in skeletal muscle of older subjects to examine the possible shift from an older to younger phenotype. Looking directly into muscle gene / metabolite profiles, we expect to gain more insight into muscle health-related factors as well as into the potential underlying molecular mechanisms.

Obese subjects: effect of weight loss

The importance of maintaining metabolic flexibility as a key factor to maintain optimal health calls for new research on the relationship between nutrition and health. An important aspect of phenotypic flexibility is the capacity to switch from carbohydrate to fat oxidation and vice-versa in response to certain external stimuli, which is considered crucial for optimal metabolic homeostasis. This capacity is in particular compromised in the obese state. As is shown in Figure 4, there is an association between the obese, insulin resistant, and T2D phenotype, as shown by an impaired fat oxidation during fasting and an impaired switch from fat oxidation to the glucose oxidation after a meal [48] or after insulin stimulation [49]. Based on these considerations, we expect an improvement in the metabolic response in obese subjects after weight loss (following 4 weeks of low calorie diet). Therefore, we examined whether the response to a mixed meal challenge could serve as a readout for a shift in phenotypic flexibility upon weight loss in obese subjects.

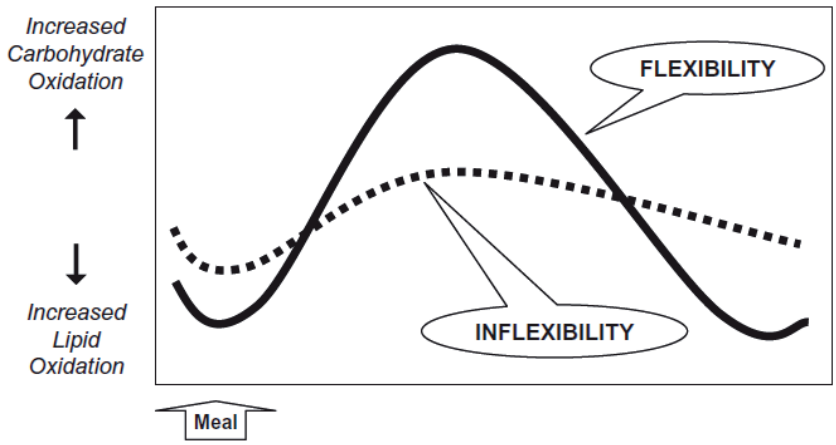


Figure 4. An overview of the post absorptive and postprandial adaptations in skeletal muscle substrate oxidation. A low respiratory quotient (RQ) indicates a relatively high fat oxidation and vice versa. The closed line represents a normal, healthy individual; the dotted line represents metabolic inflexibility (Reproduced from Corpeleijn et al. [50]).

Objective and outline of this thesis

In this thesis, I aim to track shifts in metabolic phenotype through two different approaches. First, I investigated whether prolonged resistance-type exercise training could shift the elderly phenotype toward a young phenotype, representing a more healthy status (Chapter 2 and 3). To that end, I used an organ specific approach focused on skeletal muscle tissue. The major aim was to better understand the molecular characteristics of the frailty phenotype (as

an advanced stage of ageing) by evaluating the gene expression and metabolite profile in skeletal muscle of frail older, healthy older, and young subjects. Moreover, I aimed to specify the effect of resistance-type exercise training on the skeletal muscle transcriptome/metabolome in older people.

In Chapter 4 and 5 of this thesis I examined whether a mixed meal challenge response could provide an appropriate readout for a shift in phenotypical flexibility upon weight loss in obese male subjects. We combined metabolic profiling and whole genome gene expression in PBMCs to comprehensively capture the metabolic mechanisms that underlie phenotypic flexibility. We were particularly interested in finding out whether the study of the effect of weight loss on the postprandial response would provide a more sensitive readout than the study of the effect of weight loss under fasting homeostatic conditions.

REFERENCES

1. van Ommen B, Wopereis S: **Next-Generation Biomarkers of Health.** *Nestle Nutr Inst Workshop Ser* 2016, **84:**25-33.
2. Dumas ME, Maibaum EC, Teague C, Ueshima H, Zhou B, Lindon JC, Nicholson JK, Stamler J, Elliott P, Chan Q, Holmes E: **Assessment of analytical reproducibility of 1H NMR spectroscopy based metabonomics for large-scale epidemiological research: the INTERMAP Study.** *Anal Chem* 2006, **78:**2199-2208.
3. Nicholson JK, Holmes E, Wilson ID: **Gut microorganisms, mammalian metabolism and personalized health care.** *Nat Rev Microbiol* 2005, **3:**431-438.
4. Loscalzo J, Kohane I, Barabasi AL: **Human disease classification in the postgenomic era: a complex systems approach to human pathobiology.** *Mol Syst Biol* 2007, **3:**124.
5. Fabre AC, Cornette R, Slater G, Argot C, Peigne S, Goswami A, Pouydebat E: **Getting a grip on the evolution of grasping in musteloid carnivorans: a three-dimensional analysis of forelimb shape.** *J Evol Biol* 2013, **26:**1521-1535.
6. Harris RC, Tallon MJ, Dunnett M, Boobis L, Coakley J, Kim HJ, Fallowfield JL, Hill CA, Sale C, Wise JA: **The absorption of orally supplied beta-alanine and its effect on muscle carnosine synthesis in human vastus lateralis.** *Amino Acids* 2006, **30:**279-289.
7. Dunn WB, Broadhurst DI, Atherton HJ, Goodacre R, Griffin JL: **Systems level studies of mammalian metabolomes: the roles of mass spectrometry and nuclear magnetic resonance spectroscopy.** *Chem Soc Rev* 2011, **40:**387-426.
8. van der Greef J, van Wietmarschen H, van Ommen B, Verheij E: **Looking back into the future: 30 years of metabolomics at TNO.** *Mass Spectrom Rev* 2013, **32:**399-415.
9. Shaham O, Wei R, Wang TJ, Ricciardi C, Lewis GD, Vasan RS, Carr SA, Thadhani R, Gerszten RE, Mootha VK: **Metabolic profiling of the human response to a glucose challenge reveals distinct axes of insulin sensitivity.** *Molecular Systems Biology* 2008, **4**.
10. Berg JM TJ, Stryer L.: **Biochemistry. Each Organ Has a Unique Metabolic Profile. .** New York: W H Freeman; 2002. **:**Section 30.32,.
11. van Ommen B, van der Greef J, Ordovas JM, Daniel H: **Phenotypic flexibility as key factor in the human nutrition and health relationship.** *Genes Nutr* 2014, **9:**423.
12. Hanzu FA, Vinaixa M, Papageorgiou A, Parrizas M, Correig X, Delgado S, Carmona F, Samino S, Vidal J, Gomis R: **Obesity rather than regional fat depots marks the metabolomic pattern of adipose tissue: an untargeted metabolomic approach.** *Obesity (Silver Spring)* 2014, **22:**698-704.
13. Moreno-Navarrete JM, Jove M, Ortega F, Xifra G, Ricart W, Obis E, Pamplona R, Portero-Otin M, Fernandez-Real JM: **Metabolomics uncovers the role of adipose tissue**

PDXK in adipogenesis and systemic insulin sensitivity. *Diabetologia* 2016, **59:**822-832.

14. Gueugneau M, Coudy-Gandilhon C, Théron L, Meunier B, Barboiron C, Combaret L, Taillandier D, Polge C, Attaix D, Picard B, et al: **Skeletal Muscle Lipid Content and Oxidative Activity in Relation to Muscle Fiber Type in Aging and Metabolic Syndrome.** *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 2014.
15. Hargreaves M, McKenna MJ, Jenkins DG, Warmington SA, Li JL, Snow RJ, Febbraio MA: **Muscle metabolites and performance during high-intensity, intermittent exercise.** *J Appl Physiol (1985)* 1998, **84:**1687-1691.
16. Garvey S, Dugle J, Kennedy A, McDunn J, Kline W, Guo L, Guttridge D, Pereira S, Edens N: **Metabolomic profiling reveals severe skeletal muscle group-specific perturbations of metabolism in aged FBN rats.** 2014, **15:**217-232.
17. Houtkooper RH, Argmann C, Houten SM, Canto C, Jeninga EH, Andreux PA, Thomas C, Doenlen R, Schoonjans K, Auwerx J: **The metabolic footprint of aging in mice.** *Sci Rep* 2011, **1:**134.
18. Huber M, Knottnerus JA, Green L, Horst Hvd, Jadad AR, Kromhout D, Leonard B, Lorig K, Loureiro MI, Meer JWMvd, et al: **How should we define health?** *BMJ* 2011, **343**.
19. Berthiaume N, Zinker BA: **Metabolic responses in a model of insulin resistance: comparison between oral glucose and meal tolerance tests.** *Metabolism* 2002, **51:**595-598.
20. Lin S, Yang Z, Liu H, Tang L, Cai Z: **Beyond glucose: metabolic shifts in responses to the effects of the oral glucose tolerance test and the high-fructose diet in rats.** *Mol Biosyst* 2011, **7:**1537-1548.
21. Zhao X, Peter A, Fritsche J, Elcnerova M, Fritsche A, Haring HU, Schleicher ED, Xu G, Lehmann R: **Changes of the plasma metabolome during an oral glucose tolerance test: is there more than glucose to look at?** *Am J Physiol Endocrinol Metab* 2009, **296:**E384-393.
22. Ho JE, Larson MG, Vasan RS, Ghorbani A, Cheng S, Rhee EP, Florez JC, Clish CB, Gerszten RE, Wang TJ: **Metabolite profiles during oral glucose challenge.** *Diabetes* 2013, **62:**2689-2698.
23. Stroeve JH, van Wietmarschen H, Kremer BH, van Ommen B, Wopereis S: **Phenotypic flexibility as a measure of health: the optimal nutritional stress response test.** *Genes Nutr* 2015, **10:**459.
24. Pellis L, van Erk MJ, van Ommen B, Bakker GC, Hendriks HF, Cnubben NH, Kleemann R, van Someren EP, Bobeldijk I, Rubingh CM, Wopereis S: **Plasma metabolomics and proteomics profiling after a postprandial challenge reveal subtle diet effects on human metabolic status.** *Metabolomics* 2012, **8:**347-359.
25. Krug S, Kastenmuller G, Stuckler F, Rist MJ, Skurk T, Sailer M, Raffler J, Romisch-Margl

W, Adamski J, Prehn C, et al: **The dynamic range of the human metabolome revealed by challenges.** *Faseb j* 2012, **26:**2607-2619.

26. Daviss B: **Growing pains for metabolomics.** In *TheScientist* | April 25, 2005.
27. Jordan KW, Nordenstam J, Lauwers GY, Rothenberger DA, Alavi K, Garwood M, Cheng LL: **Metabolomic characterization of human rectal adenocarcinoma with intact tissue magnetic resonance spectroscopy.** *Dis Colon Rectum* 2009, **52:**520-525.
28. Jewett MC, Hofmann G, Nielsen J: **Fungal metabolite analysis in genomics and phenomics.** *Curr Opin Biotechnol* 2006, **17:**191-197.
29. Gieger C, Geistlinger L, Altmaier E, Hrabe de Angelis M, Kronenberg F, Meitinger T, Mewes HW, Wichmann HE, Weinberger KM, Adamski J, et al: **Genetics meets metabolomics: a genome-wide association study of metabolite profiles in human serum.** *PLoS Genet* 2008, **4:**e1000282.
30. Villas-Bôas SG, Roessner U, Michael A. E. Hansen, Smedsgaard J, Nielsen J: *Metabolome Analysis: An Introduction.*2006.
31. Fiehn O: **Metabolomics--the link between genotypes and phenotypes.** *Plant Mol Biol* 2002, **48:**155-171.
32. Alves RAM, Dane A, Harms A, Strassburg K, Seifar R, Verdijk L, Kersten S, Berger R, Hankemeier T, Vreeken R: **Global profiling of the muscle metabolome: method optimization, validation and application to determine exercise-induced metabolic effects.** *Metabolomics* 2015, **11:**271-285.
33. Noga MJ, Dane A, Shi S, Attali A, van Aken H, Suidgeest E, Tuinstra T, Muilwijk B, Coulier L, Luider T, et al: **Metabolomics of cerebrospinal fluid reveals changes in the central nervous system metabolism in a rat model of multiple sclerosis.** *Metabolomics* 2012, **8:**253-263.
34. Mihaleva VV, Korhonen SP, van Duynhoven J, Niemitz M, Vervoort J, Jacobs DM: **Automated quantum mechanical total line shape fitting model for quantitative NMR-based profiling of human serum metabolites.** *Anal Bioanal Chem* 2014, **406:**3091-3102.
35. Koek MM, Jellema RH, van der Greef J, Tas AC, Hankemeier T: **Quantitative metabolomics based on gas chromatography mass spectrometry: status and perspectives.** *Metabolomics* 2011, **7:**307-328.
36. Strassburg K, Huijbrechts AM, Kortekaas KA, Lindeman JH, Pedersen TL, Dane A, Berger R, Brenkman A, Hankemeier T, van Duynhoven J, et al: **Quantitative profiling of oxylipins through comprehensive LC-MS/MS analysis: application in cardiac surgery.** *Anal Bioanal Chem* 2012, **404:**1413-1426.

37. Bomprezzi R, Ringner M, Kim S, Bittner ML, Khan J, Chen Y, Elkahloun A, Yu A, Bielekova B, Meltzer PS, et al: **Gene expression profile in multiple sclerosis patients and healthy controls: identifying pathways relevant to disease.** *Hum Mol Genet* 2003, **12:**2191-2199.
38. Maas K, Chan S, Parker J, Slater A, Moore J, Olsen N, Aune TM: **Cutting edge: molecular portrait of human autoimmune disease.** *J Immunol* 2002, **169:**5-9.
39. Kussmann M, Raymond F, Affolter M: **OMICS-driven biomarker discovery in nutrition and health.** *J Biotechnol* 2006, **124:**758-787.
40. Liew CC, Ma J, Tang HC, Zheng R, Dempsey AA: **The peripheral blood transcriptome dynamically reflects system wide biology: a potential diagnostic tool.** *J Lab Clin Med* 2006, **147:**126-132.
41. Marriage BJ, Clandinin MT, MacDonald IM, Glerum DM: **The use of lymphocytes to screen for oxidative phosphorylation disorders.** *Anal Biochem* 2003, **313:**137-144.
42. Rudkowska I, Raymond C, Ponton A, Jacques H, Lavigne C, Holub BJ, Marette A, Vohl MC: **Validation of the use of peripheral blood mononuclear cells as surrogate model for skeletal muscle tissue in nutrigenomic studies.** *Omics* 2011, **15:**1-7.
43. Lutz W, Sanderson W, Scherbov S: **The coming acceleration of global population ageing.** *Nature* 2008, **451:**716-719.
44. Snijders T, Verdijk LB, van Loon LJ: **The impact of sarcopenia and exercise training on skeletal muscle satellite cells.** *Ageing Res Rev* 2009, **8:**328-338.
45. Morley JE: **Editorial: Sarcopenia Revisited.** *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 2003, **58:**M909-M910.
46. Puetz TW, O'Connor PJ, Dishman RK: **Effects of chronic exercise on feelings of energy and fatigue: a quantitative synthesis.** *Psychol Bull* 2006, **132:**866-876.
47. Melov S, Tarnopolsky MA, Beckman K, Felkey K, Hubbard A: **Resistance exercise reverses aging in human skeletal muscle.** *PLoS One* 2007, **2:**e465.
48. Corpeleijn E, Mensink M, Kooi ME, Roekaerts PM, Saris WH, Blaak EE: **Impaired skeletal muscle substrate oxidation in glucose-intolerant men improves after weight loss.** *Obesity (Silver Spring)* 2008, **16:**1025-1032.
49. Kelley DE, Goodpaster B, Wing RR, Simoneau JA: **Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss.** *Am J Physiol* 1999, **277:**E1130-1141.
50. Corpeleijn E, Saris WH, Blaak EE: **Metabolic flexibility in the development of insulin resistance and type 2 diabetes: effects of lifestyle.** *Obes Rev* 2009, **10:**178-193.

2

Expression of protocadherin gamma in skeletal muscle tissue is associated with age and muscle weakness

Roland Hangelbroek*
Parastoo Fazelzadeh*
Michael Tieland
Mark Boekschoten
Guido Hooiveld
John van Duynhoven
James Timmons
Lex Verdijk
Lisette de Groot
Luc van Loon
Michael Müller

*Both authors contributed equally

Published in Journal of Cachexia, Sarcopenia and Muscle (2015).

ABSTRACT

The skeletal muscle system plays an important role in the independence of older adults. In this study we examine differences in the skeletal muscle transcriptome between healthy young and older subjects and (pre-) frail older adults. Additionally, we examine the effect of resistance-type exercise training on the muscle transcriptome in healthy older subjects and (pre-) frail older adults.

Baseline transcriptome profiles were measured in muscle biopsies collected from 53 young, 73 healthy older subjects and 61 frail older subjects. Follow-up samples from these frail older subjects (31 samples) and healthy older subjects (41 samples) were collected after 6 months of progressive resistance-type exercise training. Frail older subjects trained twice per week and the healthy older subjects trained three times per week.

At baseline genes related to mitochondrial function and energy metabolism were differentially expressed between older and young subjects, as well as between healthy and frail older subjects. 307 genes were differentially expressed after training in both groups. Training affected expression levels of genes related to extracellular matrix, glucose metabolism and vascularization. Expression of genes that were modulated by exercise training were indicative of muscle strength at baseline. Genes that strongly correlated with strength belonged to the protocadherin gamma gene cluster (r=-0.73).

Our data suggest significant remaining plasticity of ageing skeletal muscle to adapt to resistance-type exercise training. Some age-related changes in skeletal muscle gene expression appear to be partially reversed by prolonged resistance-type exercise training. The protocadherin gamma gene cluster may be related to muscle denervation and re-innervation in ageing muscle.

KEYWORDS: ageing, frailty, skeletal muscle, transcriptomics

INTRODUCTION

The number of people aged above 65 has increased rapidly over the past few decades, and is likely to increase progressively[1]. Because senescence is associated with a wide range of afflictions, including physical disability, cancer, heart disease and diabetes, the demand for care for older people will further increase. The loss of skeletal muscle mass and function with aging leads to frailty and results in the loss of independence of older adults.

Frailty, and related sarcopenia, are very complex and many factors contribute to their aetiology. This includes physical inactivity, malnutrition, hormonal changes and changes within the muscle [2-4]. Mitochondrial function decreases with age [5], fast-twitch muscle fibres demonstrate a smaller cross-sectional area[6], protein synthesis capacity is reduced [7], anabolic signals are less effective [4, 8], and there are fewer satellite cells to regulate adaptive responses to stimuli [9]. In older adults muscle cells can also undergo continuous cycles of denervation and reinnervation, which can lead to both weakness and loss of muscle mass [10, 11].

One of the most effective strategies to improve muscle mass and strength in adults is physical exercise [9, 12]. Resistance-type exercise is particularly suitable to curtail muscle loss and muscle weakness in older people. In accordance, quality of life is improved after participating in resistance-type exercise training [13]. Some even claim that resistance-type exercise training reverses ageing in skeletal muscle [14].

To elucidate some of these complex processes that occur in skeletal muscle during ageing, we examined the effects of prolonged resistance-type exercise training in frail and healthy older subjects on the skeletal muscle transcriptome. By comparing genome-level gene expression in frail and pre-frail older subjects, healthy older subjects and young subjects we aim to better understand the molecular causes of frailty. Secondly, we aimed to determine the effect of resistance-type exercise training on the skeletal muscle transcriptome in both frail and healthy older people.

METHODS

Experimental Design

We collected a total of 259 muscle biopsy samples from pre-frail and frail older subjects (61 subjects, 92 samples), healthy older subjects (73 subjects, 114 samples) and young males (53 subjects, 53 samples). Some of these samples were follow-up samples taken after 24 weeks of resistance-type exercise training (31 samples from the frail older subjects, 41 samples from the healthy older subjects). Training for both groups was similar and consisted of progressive full-body resistance-type exercise training. However, the frail older group had training sessions twice per week, whereas the healthy older group trained three times per week. In addition, subjects took a protein or control drink for the duration of the study. The healthy older group received a 15 gram portion of milk protein or control supplement at breakfast. The frail older group received a similar drink containing 15 gram supplement drink (milk protein or control) at breakfast and lunch. More details can be found in the respective papers [15, 16]. Table 1 shows the characteristics of our study population at baseline. Table 2 shows the effect of the training intervention on the older subjects that were included in this study and where follow-up data is available.

Subjects

Biopsies from frail and pre-frail older subjects were collected from participants of two studies performed by Tieland *et al.* [15, 17]. For these studies frail and pre-frail older subjects were selected based on the Fried criteria for frailty [2]. These subjects will hereafter be referred to as frail older subjects. These characteristics are unintentional weight loss, weakness, self-reported exhaustion, slow walking speed, and low physical activity. Subjects in the healthy older group were not considered frail by any of these criteria at the start of the intervention study[16]. Several additional baseline samples from healthy older subjects were collected from several studies from our group [18, 19]. These samples were taken before any intervention was undertaken and serve as additional reference samples. Baseline samples from young subjects were from healthy male subjects [20]. These were also taken before any intervention took place and serve as reference samples. All studies were approved by the medical ethical committee of either Wageningen University or Maastricht University and comply with the Declaration of Helsinki.

Muscle biopsy

Muscle samples were obtained with a 5 mm Bergstrom muscle biopsy needle from the

Musculus vastus lateralis, after local anaesthesia of the skin and fascia. Samples were freed from any visible blood and non-muscle tissue and immediately frozen in liquid nitrogen and then stored at -80° C. All samples were obtained in the morning, in an overnight fasted state, with at least 3 days of no heavy physical activity.

Sample preparation and microarray analysis

Total RNA was isolated from the skeletal muscle tissue by using Trizol reagent (Invitrogen, Breda, Netherlands). Thereafter RNA was purified using the Qiagen RNeasy Micro kit (Qiagen, Venlo, Netherlands) and RNA quality was checked using an Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, Netherlands). Total RNA (100 ng) was labelled using an Ambion WT expression kit (Life Technologies, Bleiswijk, Netherlands) and hybridized to human whole genome Genechip Human Gene 1.1 ST arrays coding 19.732 genes, (Affymetrix, Santa Clara, CA). Sample labelling, hybridization to chips and image scanning was performed according manufacturer's instructions.

Data analysis

Microarray signals were normalized using robust multichip average (RMA). Data was filtered using Universal exPression Codes filtering (UPC) with a 50% cut-off, corresponding to a 50% likelihood that a gene is expressed [21]. Significant differences of individual genes were tested using the limma R library [22]. Baseline differences were tested between the three groups (frail older, healthy older or young). Our model included gender, supplementation and group. For the effect of exercise we included subject, gender, time and supplementation in the model. The training effect for frail older and healthy older subjects was analysed separately due to differences in training frequency. P-values were adjusted using false discovery rate (FDR) [23]. A q-value below 0.05 was considered significant. Pathway analyses were performed using Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity) on the filtered dataset with the UPC filtered genes used as the background. A sparse partial least squares (sPLS) model for leg extension 1RM was made using the caret R library [24]. The dataset was split into a training set (75%) and a testing set (25%) before fitting the model using cross-validation. This model was validated using 10 times repeated 10-fold cross-validation. Final number of components for the sPLS model selected by grid search was 3. Principal Component Analysis (PCA) was performed using the FactoMineR R library [25]. Plots were made using the R libraries ggplot2 and gplots[26, 27].

RESULTS

Baseline differences in transcriptome

Large differences in gene expression profiles between young and older adults (healthy and frail) were found, with the expression of 5228 genes significantly different between young subjects and both groups of older subjects. However, fold changes of the majority of these genes were relatively subtle, which suggested small but consistent differences between these groups. Only 825 genes out of these 5228 genes showed fold changes higher than 1.2. Venn-diagrams can be found in supplementary figures 1A and 1B. The top 20 genes that were significantly different at baseline between the three groups are presented in table 3. Top canonical pathways reported by IPA include oxidative phosphorylation, TCA cycle and glucose metabolism (supplementary figure S2).

Table 4 shows the top 20 genes that were different between healthy and frail older subjects at baseline. Top genes included METTL21C, FRZB and non-coding RNA. Pathways that were significantly different between the frail and healthy older subjects were related to glucose metabolism and RNA processing (supplementary figure S2). In general, expression of genes related to glucose metabolism were lower in both frail older and healthy older subjects compared to young, with frail older subjects showing the lowest expression of the groups. Principal Component Analysis (PCA) summarizes this observation, where the healthy older subjects seemed to be between the frail older subjects and the young subjects on the first two components (supplementary figure S3 and S4). While pathways related to mitochondrial function were some of the most significantly affected pathways, the fold changes of the individual mitochondrial genes were relatively small but consistent. Fold changes for these mitochondrial genes were in the range of 1.1 and 1.2.

Effect of prolonged resistance-type exercise training

Prolonged resistance-type exercise training showed a significant effect on the gene expression profiles in both frail and healthy older people (431 and 1395 significantly changed genes, respectively). 307 genes were changed in both groups after resistance-type exercise training. Changes in expression of all these overlapping genes were in the same direction. A table with the top 20 genes changed by training is presented in table 5. Training resulted in the differential expression levels of many genes that are related to the connective tissue and the extracellular matrix, including collagen genes and laminin genes, suggesting significant tissue remodelling due to the training. Upstream analysis using IPA showed that TGF-β signalling-related genes were significantly activated in both groups, primarily due to the increased

expression of collagen and laminin genes (supplementary figure S5). Other significant genes include myofibrillar proteins such as myosin heavy chain isoforms and troponin isoforms.

Genes related to glucose metabolism shifted away from the expression levels of the older subjects at baseline towards the levels of the younger phenotype. This trend is reflected in many other genes, where the majority of genes significantly changed by exercise training shifted towards 'younger' expression levels (325 genes out of 431 in the frail older subjects, 1106 out of 1395 in the healthy older subjects). Figure 3 shows a heat map of 184 genes that are significantly changed by training in both groups and are significant when comparing young with either frail or healthy older subjects. Most of these genes shift towards younger levels.

To further analyse the relationship between the 307 genes that are robustly after training in both groups changed (q-value < 0.05) we performed sPLS regression to calculate leg extension 1RM based on gene expression in the baseline samples. The aim was to evaluate whether differences in expression of the genes that were changed by training represent the overall strength of the muscle at baseline. The samples obtained after training were excluded for this analysis. A plot of the predicted leg extension 1RM strength against the measured leg extension 1RM strength is presented in figure 4. Gene importance for the final model is presented in table 6. Cross-validation mean R2 of the model was 0.73, the mean RMSE was 17.7. The RMSE for the withheld testing set was 19.1. The top genes contributing to the model include genes from the protocadherin gamma gene cluster, CTNNBIP1, C20orf26 (CFAP61), C12orf75 and USP54. We calculated the eigengene for all protocadherin gamma genes and correlated this eigengene with leg extension 1RM. The protocadherin gamma eigengene showed a strong direct negative correlation (Pearson r = -0.73) with 1RM leg extension strength. A plot of this negative correlation is presented in supplementary figure S7.

DISCUSSION

In this study we compared the transcriptomes of skeletal muscle of healthy young, healthy older, and frail older subjects to better understand the skeletal muscle related part of the frail phenotype. A schematic overview of our findings is presented in figure 5. To our knowledge this is the first study investigating the effect of age on the muscle transcriptome to include frail and pre-frail older subjects. We observed clear and pronounced differences at baseline between young and older subjects. In our data frailty seems to present itself in the muscle transcriptome primarily as a more advanced stage of ageing (see also supplementary figures S3 and S4). This may, at least partly, be due to the higher average age of the frail group. There

is, however, still significant overlap in age due to the high variation in age in both groups (79.8 ± 8.9, 74.1 ± 4.5 mean age and standard deviation for frail and healthy older subjects respectively, table 1 and 2).

Baseline differences between young and older subjects

There were significant differences in genes related to mitochondrial function and oxidative phosphorylation (supplementary figure S2). It is well known that mitochondrial function is impaired in older adults [5] which can be a responsive feature to muscle inactivity [28] and mitochondrial protein carbonylation [29]. In this case the average expression of mitochondrial genes is lowest in the frail older subjects at baseline. These expression differences may also represent a lower abundance of mitochondria [6].

There are two genes among the top differentially expressed genes between the three baseline groups that are as yet unknown, C20orf26 (CFAP61) and C12orf75. Both these genes have a higher expression levels in the older subjects and training appears to attenuate expression of these genes. Not much is known about the function of CFAP61 except that it is highly expressed in skeletal muscle tissue and may be related to calcium signalling and/or energy conversion [30]. C12orf75 may be related to cell proliferation and stem cell signalling [31].

In our data frail subjects showed significantly higher expression levels of METTL21C when compared to both the young and healthy older subjects, with a greater fold change difference between healthy and frail subjects than between the young and the older subjects (table 5). Training decreases the mean expression of METTL21C in both groups, but this does not reach significance using our significance cut-off. However, in the frail group it does reach a q-value of 0.08 after training, showing a fold change of -1.58. METTL21C encodes for a protein-lysine methyltransferase belonging to a group of proteins that are involved in methylation of chaperone proteins, where METTL21C appears to methylate HSP70 and HSP90 [32] and has recently been found to be associated with skeletal muscle development [33]. In vitro inhibition of METTL21C expression in myoblasts showed impaired myotube differentiation and calcium signalling, suggesting that METTL21C plays an important role in the function of muscle cells and possibly also the overall quality of the muscle.

Effect of prolonged resistance-type exercise training

The majority of genes that significantly changed following prolonged resistance-type exercise training showed a shift in the expression levels towards levels observed in the younger group (figure 3). A previous study has shown a similar effect[14]. Indeed, Melov *et al.* state that training reverses the effect of ageing. While there is a shift towards younger expression levels,

this does not necessarily mean that there is reversal of ageing. A more likely explanation is that the skeletal muscle in these older subjects have been 'detrained' due to more sedentary lifestyle when compared with healthy younger controls. Physical inactivity is a major contributor to age-related muscle loss and weakness and is one of the criteria of frailty [2]. In this way participation in prolonged resistance-type exercise training is likely to shift gene expression to younger levels. Furthermore, in our data the young subjects had higher muscle strength (table 1). Training leads to subtle but consistent changes in the muscle transcriptome [34]. Thus, a shift towards younger expression levels would be consistent with the increased strength after more prolonged resistance-type exercise training.

The genes that shift towards younger expression levels include genes related to the extracellular matrix, vascularisation, glucose metabolism and muscle contraction (supplementary figures S2, S5 and S6). The muscle biopsies were taken at least three days after the last training session. Thus, we are not observing acute effects of a single bout of resistance-type exercise, but rather longer term consistent changes in gene expression. Notably absent among the changes induced by prolonged resistance-type exercise training, however, are the primary differences observed when we compare young and older subjects: mitochondrial function. Possible explanations are that these changes are too subtle to pick up after 24 weeks of resistance-type exercise training or that prolonged resistance-type exercise training does not significantly affect these genes. Timing of the muscle biopsies relative to the last training session may also be a factor. It may be that expression of these mitochondrial genes only change acutely after resistance-type exercise rather than chronically.

Prolonged resistance-type exercise training showed fewer significantly affected genes in the frail group. Part of this can be explained by the differences in treatment. The healthy older subjects had training sessions three times per week whereas the frail older subjects received two sessions per week. The load of the training was also lower in for the frail subjects. However, it may also be that the frail are less capable of adapting to the additional stress of prolonged resistance-type exercise training. Fortunately, the frail subjects still showed a significant response to the training stimulus despite their less adaptive phenotype [2]. Others have already reported that older adults in general have a decreased response to resistance-type exercise on a transcriptome level [35], and this may also play a role in the smaller response in the frail older subjects compared to the healthy older subjects.

Gene expression and muscle strength

Prolonged resistance-type exercise training led to strength increases in all individuals to the point that training increased strength levels in the frail older subjects close to the levels observed

in the healthy older subjects at baseline (Figure 1). However, it did not necessarily lead to increases in lean body mass in all individuals (figure 2). This suggests that we primarily observed an increase in muscle quality, cross-bridge cycling efficiency, calcium handling and/or neuromuscular adaptation rather than an increase in muscle cross-sectional area. Our data provide evidence suggesting disturbances in axon guidance and muscle innervation in the older subjects.

We performed sPLS regression analysis to calculate leg strength based on expression levels of the genes that are robustly changed after prolonged exercise training in both groups in the baseline samples. Our rationale was that since these genes are changed in both groups after training, where the leg extension 1RM is significantly higher, that expression of these genes could also reflect muscle strength at baseline without training. We were able to build a reasonably accurate regression model to calculate leg extension 1RM at baseline based on gene expression (mean cross-validation R2 of 0.73 and RMSE of 17.7, figure 4). Thus, expression of the genes robustly changed by exercise also seems to be indicative of muscle strength, not only after training but also prior to prolonged exercise training. This suggests that expression of these genes may be used as a biomarker to training status prior to study entry.

Several of the most important variables in our sPLS model for muscle strength belonged to the protocadherin gene cluster. Genes of the protocadherin gamma gene cluster were significantly different between frail older subjects, healthy older subjects and young subjects at baseline. Expression of these genes also went down after training in both groups. Older subjects had higher expression of this gene than young subjects and expression was highest in the frail older subjects. There are good indications that this gene cluster is relevant for neuromuscular performance. Many of the genes from this cluster are also significantly changed after training in both groups. Protocadherin gamma genes ranked very highly in the variable importance for our correlative model for leg extension 1RM (table 6).

Protocadherin gamma genes are primarily expressed in neural tissues such as the brain and the spinal cord and appears to be involved in axonal guidance [36]. Protocadherin proteins show homeophilic binding to other protocadherin proteins and in this way these proteins provide recognition sites for axonal binding. By expressing different protocadherin gamma genes from the gene cluster axons can be guided to different locations [37]. In knockout mice these genes appear to be indirectly related to muscle function: knock-out mice show severe muscle weakness and tremors, although this is attributed to loss of spinal motor neurons [38, 39]. It may also be that it is expressed at the muscle side of the synapse to facilitate axon guidance towards muscle, and increased expression in this case is due to the denervation-reinnervation cycles seen in ageing muscle. Therefore, we hypothesise that as muscle loses innervation it increases expression of the protocadherin gamma cluster to facilitate axon binding from other nerves.

Protocadherin gamma is not the only significant group of genes related to neuromuscular function that we found in our data. There are several other genes differentially expressed between frail and healthy older subjects that are related to the innervation of muscle, including acetylcholine esterase (AChE) and kyphoscoliosis peptidase (KY). Both play important roles in the function of the neuromuscular junction [40, 41]. The top differentially expressed gene at baseline, unc-13 homolog C (UNC13C), is involved in neurotransmitter release [42, 43]. Both MYLK4 and IGFN1 are also among the top significantly different genes between the three groups at baseline. Little is known about MYLK4 and IGFN1, but both have been indirectly associated with neuromuscular function. MYLK4 has been shown to be significantly down-regulated in AChE knockout mice, together with KY, suggesting that it is somehow involved in the signal transduction [44]. Like KY, IGFN1 has been associated with both muscle structure and neurological function [45, 46]. This gene also binds EEF1A2, which is the gene associated with the wasted (Wst) mouse phenotype [46, 47]. This phenotype shows significant immunological and neuromuscular defects [48]. IGFN1 has many splicing variants, which suggests that it plays a pleiotropic role in the muscle. Another indication of denervation is the increased expression of the perinatal myosin heavy chain isoform MYH8 in older subjects. Previous studies have found that expression of this gene is increased in tissue where the muscle fibres have lost innervation [49, 50].

CONCLUSIONS

Our data suggests a significant remaining plasticity of ageing skeletal muscle to adapt to regular resistance type exercise. Many age-related changes in skeletal muscle gene expression are partially reversed by prolonged resistance-type exercise training. Expression of the genes robustly changed following prolonged resistance-type exercise training in frail and healthy older subjects did not only reflect the effect of training itself, but also reflected muscle strength at baseline. Expression of the protocadherin gamma gene cluster is negatively correlated with muscle strength in our data and may be related to muscle denervation and re-innervation.

Clinical relevance

We have identified a gene cluster that may be related to denervation and re-innervation cycles in the muscle. Loss of motor neurons has been suggested to play an important role in age-related muscle weakness and sarcopenia, but is unfortunately not yet fully understood. Prolonged resistance-type exercise training was able to modulate the expression of proto-

cadherin gamma. Hence, studying the expression of this gene may provide novel insights on whether or not denervation and re-innervation is modulated by interventions or lifestyle factors such as nutrition and physical activity. Furthermore, in this paper we show that steady-state gene expression provides information on the strength of the muscle itself. This suggests that steady-state gene expression could potentially be used as a tool to provide insight into muscle strength of a subject, but theoretically also other muscle health-related factors.

Limitations

While we have a large sample size for such a study, we also have a very heterogeneous study population. The ratio of male to female among both groups of older adults is not entirely equal. We have adjusted for this in our statistical analyses where possible. Another limitation is that the muscle biopsies from the frail older adults were obtained from a study performed at Wageningen University, whereas the muscle biopsies from the healthy older adults and the young adults were obtained from studies performed at Maastricht University. Protocols for muscle biopsy collection and preparation in Wageningen are based on those from Maastricht and thus are very similar, but there may still be some bias that we cannot account for statistically. The microarray analyses were performed within the same lab at the same time by the same technician, which means that batch effects should be minimal. The protocols for the prolonged resistance-type exercise training were slightly different for the frail older adults and the healthy older adults. The primary difference being that the healthy older adults trained three times per week and the frail older adults trained twice per week. As a consequence, the training stimulus for the frail older adults was somewhat lower in these individuals and this could partially explain the decreased response among the frail older subjects. This difference in treatment also prevented us to compare the training responses in both groups directly.

ACKNOWLEDGEMENTS

This project is funded by Top Institute Food and Nutrition, a public-private partnership on precompetitive research in food and nutrition. The public partners are responsible for the study design, data collection and analysis, decision to publish, and preparation of the manuscript. The private partners have contributed to the project through regular discussion. JA Timmons is supported by an MRC grant on Human Ageing (G1100015).

REFERENCES

1. Lutz W, Sanderson W, Scherbov S: **The coming acceleration of global population ageing.** *Nature* 2008, **451:**716-719.
2. Fried LP, Tangen CM, Walston J, Newman AB, Hirsch C, Gottdiener J, Seeman T, Tracy R, Kop WJ, Burke G, McBurnie MA: **Frailty in Older Adults: Evidence for a Phenotype.** *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 2001, **56:**M146-M157.
3. Clark BC, Manini TM: **Sarcopenia≠ dynapenia.** *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 2008, **63:**829-834.
4. Brook MS, Wilkinson DJ, Phillips BE, Perez-Schindler J, Philp A, Smith K, Atherton PJ: **Skeletal muscle homeostasis and plasticity in youth and ageing: impact of nutrition and exercise.** *Acta Physiologica* 2015**:**n/a-n/a.
5. Short KR, Bigelow ML, Kahl J, Singh R, Coenen-Schimke J, Raghavakaimal S, Nair KS: **Decline in skeletal muscle mitochondrial function with aging in humans.** *Proceedings of the National Academy of Sciences of the United States of America* 2005, **102:**5618-5623.
6. Evans WJ, Lexell J: **Human aging, muscle mass, and fiber type composition.** *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 1995, **50:**11-16.
7. Breen L, Phillips SM: **Skeletal muscle protein metabolism in the elderly: interventions to counteract the'anabolic resistance'of ageing.** *Nutrition & metabolism* 2011, **8:**68.
8. Cuthbertson D, Smith K, Babraj J, Leese G, Waddell T, Atherton P, Wackerhage H, Taylor PM, Rennie MJ: **Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle.** *The FASEB Journal* 2005, **19:**422-424.
9. Snijders T, Verdijk LB, van Loon LJ: **The impact of sarcopenia and exercise training on skeletal muscle satellite cells.** *Ageing research reviews* 2009, **8:**328-338.
10. Lexell J: **Evidence for nervous system degeneration with advancing age.** *The Journal of nutrition* 1997, **127:**1011S-1013S.
11. Brooks SV, Faulkner JA: **Skeletal muscle weakness in old age: underlying mechanisms.** *Medicine and science in sports and exercise* 1994, **26:**432-439.
12. Morley JE: **Editorial: sarcopenia revisited.** *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 2003, **58:**M909-M910.
13. Arent S, Landers D, Etnier J: **The Effects of Exercise on Mood in Older Adults: A Meta-Analytic.** *Journal of Ageing and Physical Activity* 2000, **8:**407-430.
14. Melov S, Tarnopolsky MA, Beckman K, Felkey K, Hubbard A: **Resistance exercise reverses aging in human skeletal muscle.** *PLoS One* 2007, **2:**e465.

15. Tieland M, Dirks ML, van der Zwaluw N, Verdijk LB, van de Rest O, de Groot LCPGM, van Loon LJC: **Protein Supplementation Increases Muscle Mass Gain During Prolonged Resistance-Type Exercise Training in Frail Elderly People: A Randomized, Double-Blind, Placebo-Controlled Trial.** *Journal of the American Medical Directors Association* 2012, **13:**713-719.
16. Leenders M, Verdijk LB, Van der Hoeven L, Van Kranenburg J, Nilwik R, Wodzig WKWH, Senden JMG, Keizer HA, Van Loon LJC: **Protein supplementation during resistance-type exercise training in the elderly.** *Medicine and science in sports and exercise* 2013, **45:**542-552.
17. Tieland M, van de Rest O, Dirks ML, van der Zwaluw N, Mensink M, van Loon LJC, de Groot LCPGM: **Protein Supplementation Improves Physical Performance in Frail Elderly People: A Randomized, Double-Blind, Placebo-Controlled Trial.** *Journal of the American Medical Directors Association* 2012, **13:**720-726.
18. Wall BT, Hamer HM, de Lange A, Kiskini A, Groen BB, Senden JM, Gijsen AP, Verdijk LB, van Loon LJ: **Leucine co-ingestion improves post-prandial muscle protein accretion in elderly men.** *Clinical nutrition* 2013, **32:**412-419.
19. Leenders M, Verdijk LB, van der Hoeven L, van Kranenburg J, Nilwik R, van Loon LJ: **Elderly men and women benefit equally from prolonged resistance-type exercise training.** *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 2013, **68:**769-779.
20. Gorissen SH, Burd NA, Hamer HM, Gijsen AP, Groen BB, van Loon LJ: **Carbohydrate coingestion delays dietary protein digestion and absorption but does not modulate postprandial muscle protein accretion.** *The Journal of Clinical Endocrinology & Metabolism* 2014, **99:**2250-2258.
21. Piccolo SR, Sun Y, Campbell JD, Lenburg ME, Bild AH, Johnson WE: **A single-sample microarray normalization method to facilitate personalized-medicine workflows.** *Genomics* 2012, **100:**337-344.
22. Smyth GK: **Limma: linear models for microarray data.** In *Bioinformatics and computational biology solutions using R and Bioconductor*. Springer; 2005: 397-420
23. Benjamini Y, Hochberg Y: **Controlling the false discovery rate: a practical and powerful approach to multiple testing.** *Journal of the Royal Statistical Society Series B (Methodological)* 1995**:**289-300.
24. Kuhn M: **Building predictive models in R using the caret package.** *Journal of Statistical Software* 2008, **28:**1-26.
25. Husson F, Josse J, Le S, Mazet J: **FactoMineR: multivariate exploratory data analysis and data mining with R.** *R package version* 2013, **1:**102-123.

26. Wickham H: *ggplot2: elegant graphics for data analysis.* Springer; 2009.
27. Warnes GR, Bolker B, Bonebakker L, Gentleman R, Huber W, Liaw A, Lumley T, Maechler M, Magnusson A, Moeller S: **gplots: Various R programming tools for plotting data.** *R package version* 2009, **2**.
28. Steinbacher P, Feichtinger RG, Kedenko L, Kedenko I, Reinhardt S, Schönauer A-L, Leitner I, Sänger AM, Stoiber W, Kofler B: **The Single Nucleotide Polymorphism Gly482Ser in the PGC-1α Gene Impairs Exercise-Induced Slow-Twitch Muscle Fibre Transformation in Humans.** 2015.
29. Valls MRB, Wilkinson DJ, Narici MV, Smith K, Phillips BE, Caporossi D, Atherton PJ: **Protein carbonylation and heat shock proteins in human skeletal muscle: relationships to age and sarcopenia.** *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 2014**:**glu007.
30. Dymek EE, Smith EF: **A conserved CaM- and radial spoke–associated complex mediates regulation of flagellar dynein activity.** *The Journal of Cell Biology* 2007, **179:**515-526.
31. Kikuchi K, Fukuda M, Ito T, Inoue M, Yokoi T, Chiku S, Mitsuyama T, Asai K, Hirose T, Aizawa Y: **Transcripts of unknown function in multiple-signaling pathways involved in human stem cell differentiation.** *Nucleic Acids Research* 2009, **37:**4987-5000.
32. Cloutier P, Lavallée-Adam M, Faubert D, Blanchette M, Coulombe B: **A Newly Uncovered Group of Distantly Related Lysine Methyltransferases Preferentially Interact with Molecular Chaperones to Regulate Their Activity.** *PLoS Genet* 2013, **9:**e1003210.
33. Huang J, Hsu Y-H, Mo C, Abreu E, Kiel DP, Bonewald LF, Brotto M, Karasik D: **METTL21C Is a Potential Pleiotropic Gene for Osteoporosis and Sarcopenia Acting Through the Modulation of the NF-κB Signaling Pathway.** *Journal of Bone and Mineral Research* 2014, **29:**1531-1540.
34. Egan B, Zierath Juleen R: **Exercise Metabolism and the Molecular Regulation of Skeletal Muscle Adaptation.** *Cell Metabolism* 2013, **17:**162-184.
35. Raue U, Trappe TA, Estrem ST, Qian H-R, Helvering LM, Smith RC, Trappe S: *Transcriptome signature of resistance exercise adaptations: mixed muscle and fiber type specific profiles in young and old adults.*2012.
36. Chen WV, Alvarez FJ, Lefebvre JL, Friedman B, Nwakeze C, Geiman E, Smith C, Thu CA, Tapia JC, Tasic B: **Functional significance of isoform diversification in the protocadherin gamma gene cluster.** *Neuron* 2012, **75:**402-409.
37. Chen WV, Maniatis T: **Clustered protocadherins.** *Development* 2013, **140:**3297-3302.
38. Wang X, Weiner JA, Levi S, Craig AM, Bradley A, Sanes JR: **Gamma protocadherins are required for survival of spinal interneurons.** *Neuron* 2002, **36:**843-854.

39. Weiner JA, Wang X, Tapia JC, Sanes JR: **Gamma protocadherins are required for synaptic development in the spinal cord.** *Proceedings of the National Academy of Sciences of the United States of America* 2005, **102:**8-14.
40. Fischbach GD, Frank E, Jessell TM, Rubin LL, Schuetze SM: **Accumulation of acetylcholine receptors and acetylcholinesterase at newly formed nerve-muscle synapses.** *Pharmacological Reviews* 1978, **30:**411-428.
41. Blanco G, Coulton GR, Biggin A, Grainge C, Moss J, Barrett M, Berquin A, Maréchal G, Skynner M, van Mier P: **The kyphoscoliosis (ky) mouse is deficient in hypertrophic responses and is caused by a mutation in a novel muscle-specific protein.** *Human molecular genetics* 2001, **10:**9-16.
42. Basu J, Betz A, Brose N, Rosenmund C: **Munc13-1 C1 Domain Activation Lowers the Energy Barrier for Synaptic Vesicle Fusion.** *The Journal of Neuroscience* 2007, **27:**1200-1210.
43. Brose N, Rosenmund C, Rettig J: **Regulation of transmitter release by Unc-13 and its homologues.** *Current Opinion in Neurobiology* 2000, **10:**303-311.
44. Lin H-Q, Choi R, Chan K-L, Ip D, Tsim KW-k, Wan DC-c: **Differential gene expression profiling on the muscle of acetylcholinesterase knockout mice: A preliminary analysis.** *Chemico-Biological Interactions* 2010, **187:**120-123.
45. Baker J, Riley G, Romero MR, Haynes AR, Hilton H, Simon M, Hancock J, Tateossian H, Ripoll VM, Blanco G: **Identification of a Z-band associated protein complex involving KY, FLNC and IGFN1.** *Experimental Cell Research* 2010, **316:**1856-1870.
46. Mansilla F, Dominguez CAG, Yeadon JE, Corydon TJ, Burden SJ, Knudsen CR: **Translation elongation factor eEF1A binds to a novel myosin binding protein-C-like protein.** *Journal of Cellular Biochemistry* 2008, **105:**847-858.
47. Chambers DM, Peters J, Abbott CM: **The lethal mutation of the mouse wasted (wst) is a deletion that abolishes expression of a tissue-specific isoform of translation elongation factor 1α, encoded by the Eef1a2 gene.** *Proceedings of the National Academy of Sciences* 1998, **95:**4463-4468.
48. Newbery HJ, Gillingwater TH, Dharmasaroja P, Peters J, Wharton SB, Thomson D, Ribchester RR, Abbott CM: **Progressive loss of motor neuron function in wasted mice: effects of a spontaneous null mutation in the gene for the eEF1A2 translation factor.** *Journal of Neuropathology & Experimental Neurology* 2005, **64:**295-303.
49. Schiaffino S, Gorza L, Pitton G, Saggin L, Ausoni S, Sartore S, Lømo T: **Embryonic and neonatal myosin heavy chain in denervated and paralyzed rat skeletal muscle.** *Developmental biology* 1988, **127:**1-11.
50. Sun H, Li M, Gong L, Liu M, Ding F, Gu X: **iTRAQ-coupled 2D LC–MS/MS analysis on differentially expressed proteins in denervated tibialis anterior muscle of Rattus norvegicus.** *Molecular and Cellular Biochemistry* 2012, **364:**193-207.

Table1. Subject characteristics of the baseline only subjects

	FE	HE	YO
N (male / female)	24 / 6	27 / 5	53 / 0
Age (years)	79.8 ± 8.9	74.1 ± 4.5	21.3 ± 2.4
Height (m)	1.71 ± 0.09	1.73 ± 0.08	1.84 ± 0.06
Weight (kg)	80 ± 12.4	75.9 ± 12.9	76.5 ± 10.3
BMI (kg / m^2)	27.3 ± 4.2	25.2 ± 3.2	22.6 ± 3
Body Fat (%)	28.8 ± 7.2	23.4 ± 5.5	15.4 ± 4.6
Lean Mass (kg)	52.1 ± 6.3	55.5 ± 8.6	61.9 ± 6
Leg Extension 1RM (kg)	65 ± 20	68 ± 17	124 ± 20
Leg Press 1RM (kg)	127 ± 31	155 ± 41	203 ± 36

Mean±SD, FE=frail older subjects, HE=healthy older subjects, YO=young male subjects.

Table 2. Subject characteristics of the subjects with before and after samples.

	FE pre	FE post	HE pre	HE post
N (male / female)	11 / 20		26 / 15	
Age (years)	76.5 ± 7.0		69.9 ± 5.0	
Height (m)	1.66 ± 0.09		1.71 ± 0.09	
Weight (kg)	78.5 ± 13.6	79.6 ± 14.1*	76.7 ± 13.2	77.1 ± 13.2
BMI (kg / m^2)	28.5 ± 4.1	29 ± 4.3 *	26.1 ± 2.8	26.2 ± 2.8
Body Fat (%)	36.7 ± 8.5	36.6 ± 8.8	25.9 ± 5.9	24.4 ± 5.9 *
Lean Mass (kg)	46.1 ± 10.0	46.9 ± 9.9 *	54.6 ± 11.1	56.0 ± 11.3 *
Leg Extension 1RM (kg)	59 ± 18	81 ± 24 *	81 ± 17	114 ± 23 *
Leg Press 1RM (kg)	130 ± 35	178 ± 49 *	179 ± 40	230 ± 50 *

FE = frail older subjects, HE = healthy older subjects, YO = young male subjects. * indicates a significant effect of resistance-type exercise training ($p < 0.05$).

Table 3. Top 20 genes significantly different between young and old.

Gene	FC FE vs. HE	FC FE vs. YO	FC HE vs. YO	q-value FE vs. HE	q-value FE vs. YO	q-value HE vs. YO
IGFN1	-1.12	-6.43	-5.71	0.64	0.00	0.00
UNC13C	1.07	6.20	5.78	0.60	0.00	0.00
MYLK4	-1.35	-5.13	-3.81	0.07	0.00	0.00
C12orf75	1.45	4.54	3.13	0.01	0.00	0.00
SLC38A1	-1.11	-3.50	-3.14	0.55	0.00	0.00
HCN1	1.16	3.36	2.90	0.30	0.00	0.00
MYH8	1.16	3.28	2.83	0.50	0.00	0.00
C20orf26	1.36	3.39	2.49	0.00	0.00	0.00
NR4A3	-1.86	-3.70	-2.00	0.02	0.00	0.00
FAM83B	1.11	2.91	2.63	0.39	0.00	0.00
DAAM2	-1.06	-2.51	-2.36	0.42	0.00	0.00
NNMT	1.33	2.71	2.04	0.03	0.00	0.00
ZNF382	1.21	-2.09	-2.54	0.05	0.00	0.00
TPPP3	1.33	2.62	1.97	0.01	0.00	0.00
COL28A1	1.07	2.36	2.20	0.42	0.00	0.00
METTL21EP	1.05	-2.19	-2.29	0.78	0.00	0.00
HIST1H3E	1.11	-2.08	-2.30	0.30	0.00	0.00
SNORD115-32	1.02	2.17	2.14	0.91	0.00	0.00
SERPINA5	1.04	-2.11	-2.19	0.89	0.00	0.00
METTL21C	1.98	2.84	1.44	0.00	0.00	0.03

FE = frail older subjects, HE = healthy older subjects, YO = young men.

Table 4. Top 20 genes significantly different between the frail elderly and the healthy elderly at baseline.

Gene	FC FE vs. HE	q-value FE vs. HE
METTL21C	1.98	0.00
NR4A3	-1.86	0.02
VTRNA1-1	-1.71	0.00
MIR206	1.58	0.00
SNORA38B	-1.58	0.00
S100A8	1.51	0.04
FRZB	-1.48	0.03
HES1	-1.47	0.00
P2RY13	1.46	0.00
MIR133B	1.45	0.01
C12orf75	1.45	0.01
SNORA60	-1.44	0.00
SNORD60	-1.43	0.00
LYZ	1.43	0.03
SNORD80	-1.42	0.00
SNORD82	-1.42	0.00
SNORD29	-1.41	0.00
EVI2B	1.40	0.01
UPK3A	-1.40	0.00
ID1	-1.40	0.00

FE = frail older subjects, HE = healthy older subjects.

Table 5. Top 20 genes significantly different in both frail elderly and healthy elderly subjects after training.

Gene	FC FE training	FC HE training	q-value FE training	q-value HE training
FRZB	1.97	1.55	0.00	0.00
IGFN1	1.58	1.80	0.04	0.00
MYLK4	1.45	1.69	0.01	0.00
COL3A1	1.45	1.68	0.01	0.00
ANKRD2	-1.44	-1.61	0.01	0.00
THBS4	1.34	1.66	0.05	0.00
PFKFB3	1.61	1.38	0.01	0.01
COL4A1	1.35	1.46	0.00	0.00
CAPN6	1.37	1.45	0.03	0.00
COL1A2	1.35	1.45	0.03	0.00
EDNRB	1.24	1.56	0.01	0.00
GCNT2	-1.51	-1.28	0.00	0.00
C20orf26	-1.24	-1.54	0.03	0.00
C12orf75	-1.42	-1.33	0.01	0.00
CCDC80	1.34	1.40	0.03	0.00
OLFML2B	1.38	1.34	0.00	0.00
SPARC	1.28	1.44	0.00	0.00
COL4A2	1.32	1.37	0.00	0.00
LGI1	-1.31	-1.33	0.02	0.00
ACOT11	-1.30	-1.34	0.04	0.00

FE = frail older subjects, HE = healthy older subjects.

Table 6. Variable importance and coefficients of the top 20 variables for the sPLS model.

Gene	Variable Importance	Coefficient
C20orf26	100	-1.93835
PCDHGA10	99.3099	-0.828
PCDHGB5	97.59634	-1.17955
PCDHGB1	95.06749	-1.02454
CTNNBIP1	91.85769	1.12848
USP54	90.58105	-0.85727
PCDHGA8	83.51363	-0.91253
PCDHGB7	82.41083	-0.80005
MYOZ2	81.3163	-0.61206
PCDHGA11	78.54377	-0.6435
C12orf75	78.18419	-0.82036
PCDHGA7	77.82971	-0.85683
PCDHGA2	76.42625	-0.91682
HEXIM2	75.94954	0.241251
GRSF1	75.24609	0.38796
GCNT2	75.11434	-1.0698
FBP2	72.2918	0.262855
PLEKHO1	68.54881	0.238599
CRY2	68.13805	-1.59754
PABPC4	67.4792	0.848579

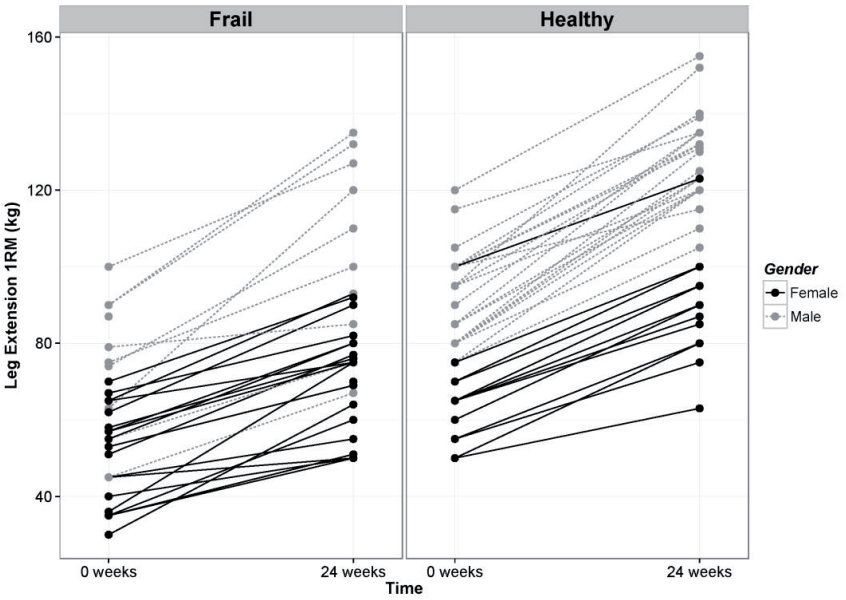


Figure 1. A and B – Change of leg extension 1RM after prolonged exercise training for each individual. Left are frail elderly subjects, right are healthy elderly subjects.

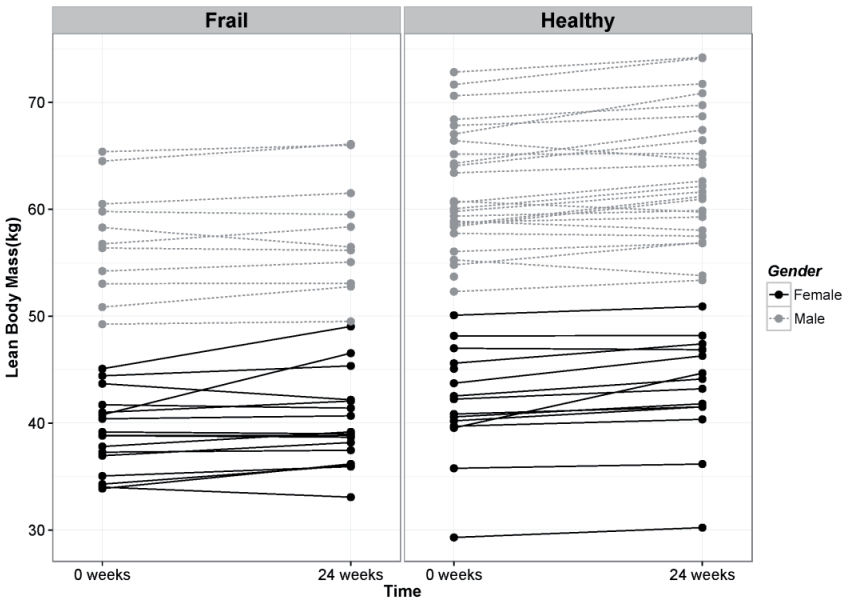


Figure 2. A and B. Change of lean body mass after prolonged exercise training for each individual. Left are frail older subjects, right are healthy older subjects.

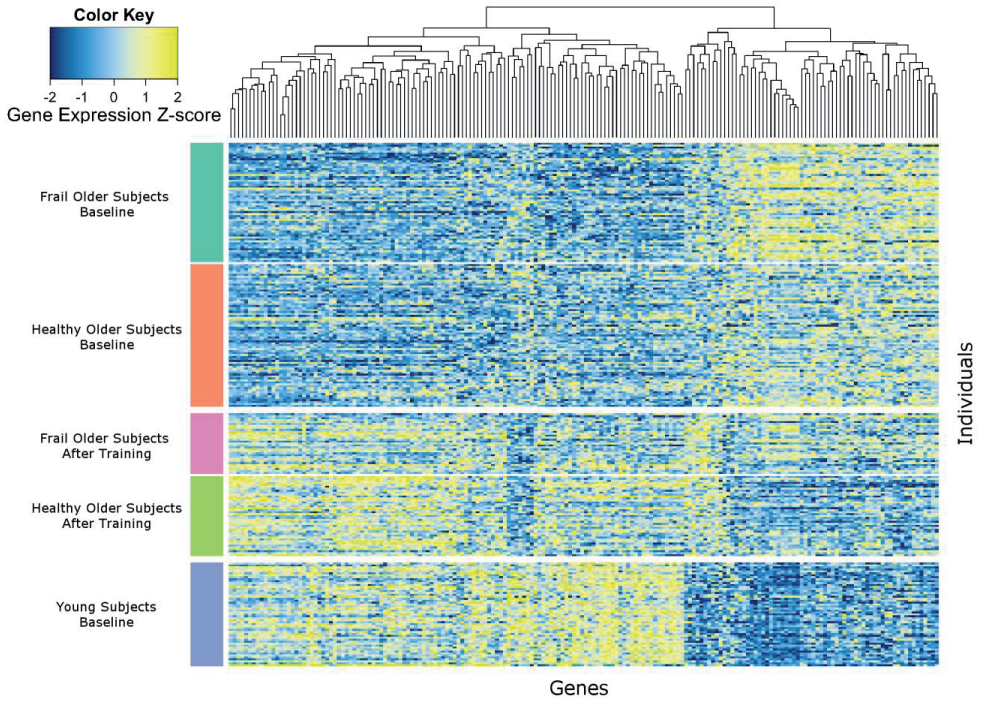


Figure 3. Heatmap of 184 genes that are significantly different between young and older subjects, and are significantly changed by prolonged resistance-type exercise training in both groups.

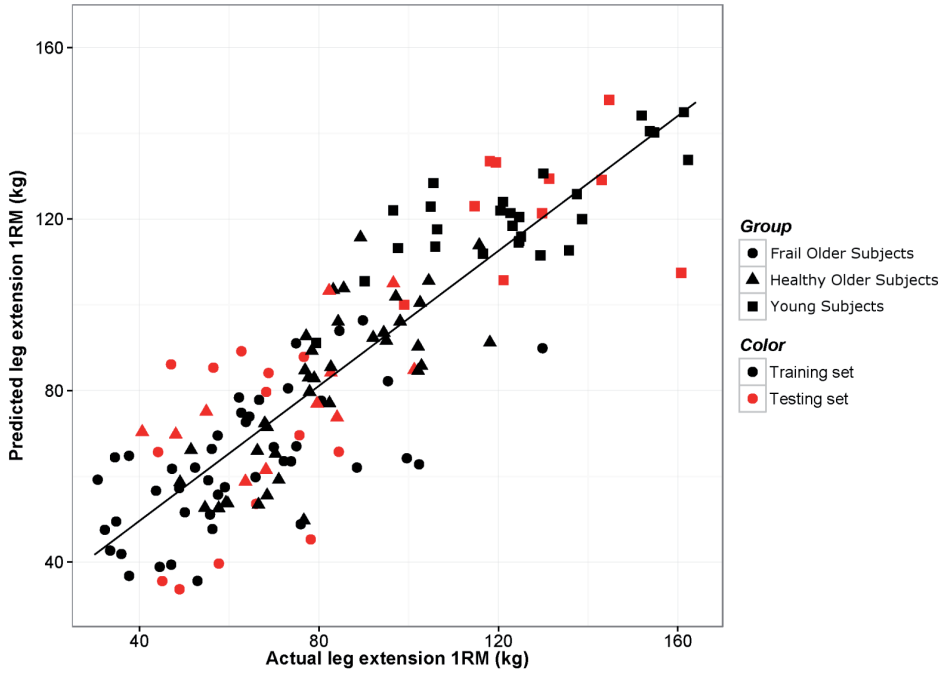


Figure 4. Scatter plot of predicted leg extension 1RM of the baseline samples using sPLS and the actual measured 1RM. Red dots indicate samples that were part of the testing data set (25% of the total data set).

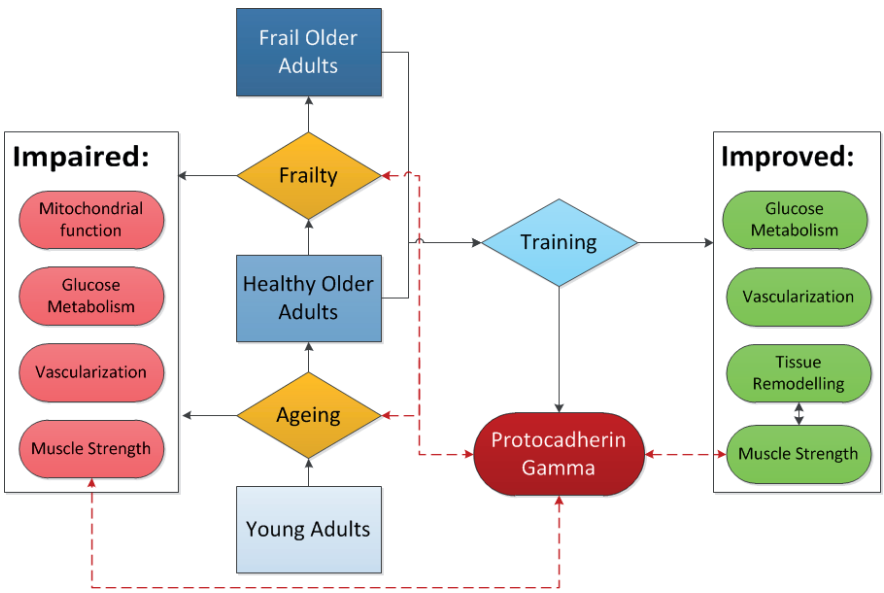


Figure 5. Schematic summary of our findings.

SUPPLEMENTARY MATERIAL

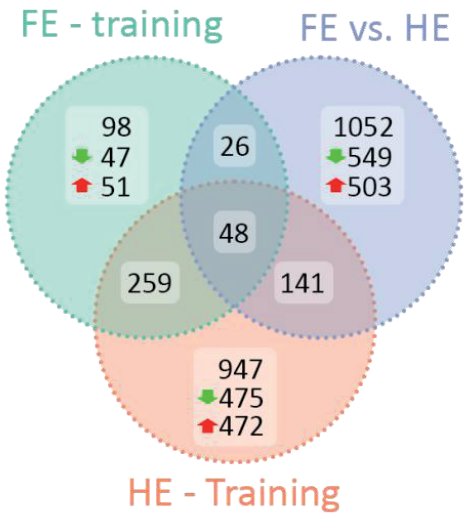


Figure S1A. Venn diagram of significant genes (q-value < 0.05) changed after prolonged exercise training in frail (FE – training, green) and healthy older subjects (HE – training, red). FE vs HE are genes significantly different at baseline between frail and healthy older subjects (blue).

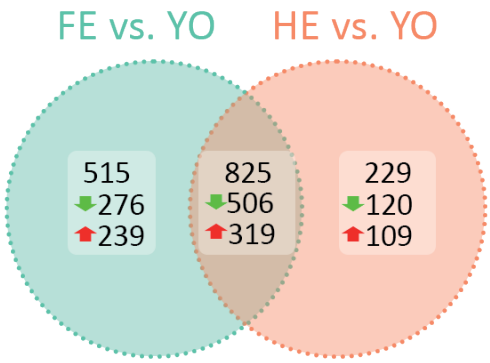


Figure S1B. Venn diagram of significant genes (q-value < 0.05) at baseline between the young (YO) group and both frail (FE) and healthy older subjects (HE). Due to the high number of significant genes with relatively small differences an additional fold-change cut-off was used for these data (fold change > 1.2).

Figure S2. Top 40 (by p-value) significantly changed canonical pathways from Ingenuity Pathway Analysis. First two columns represent the prolonged exercise training effect in frail (FE) and healthy (HE) older subjects. Last three columns represent baseline differences between frail older subjects (FE), healthy older subjects (HE), and young subjects (YO). Colours indicate significance.

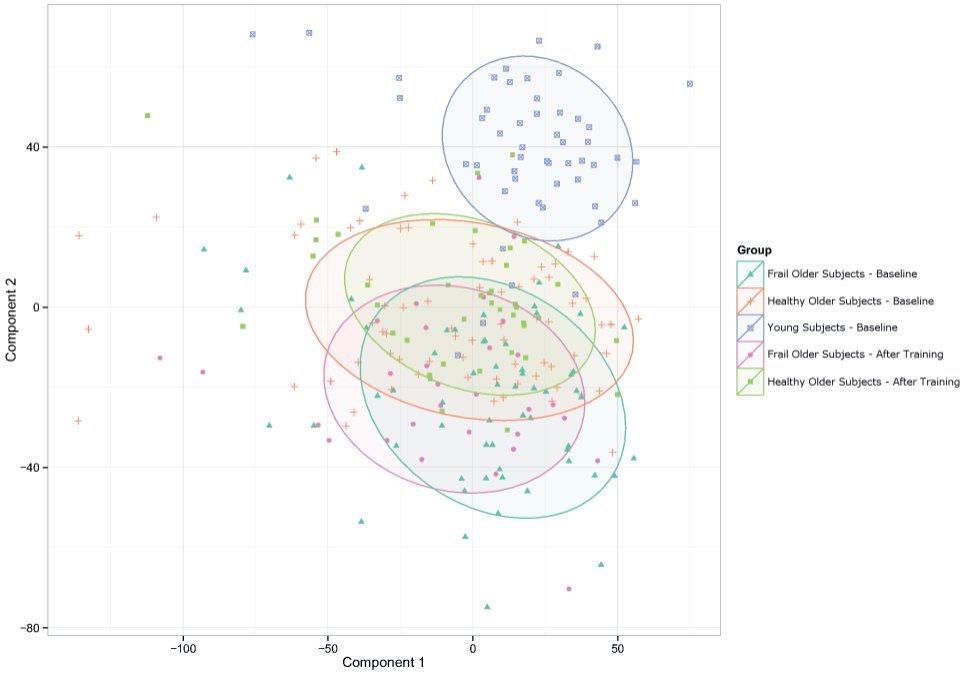


Figure S3. PCA of all UPC filtered genes for all groups, before and after prolonged exercise training. Ellipses indicate 95% confidence interval.

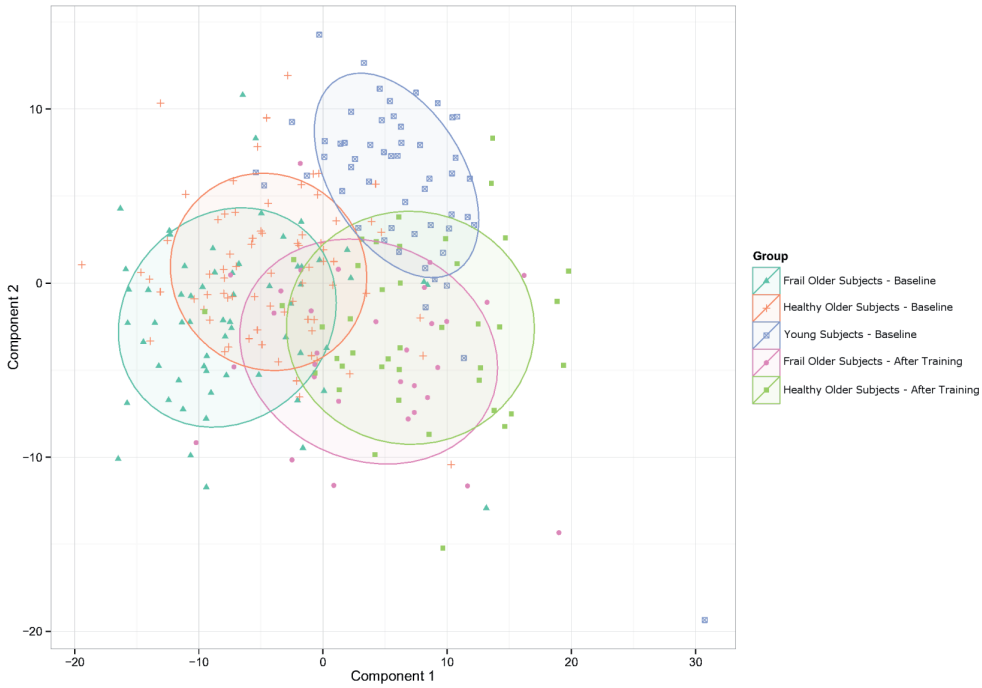


Figure S4.PCA of 307 exercise responsive genes for all groups, before and after prolonged exercise training. Ellipses indicate 95% confidence interval.

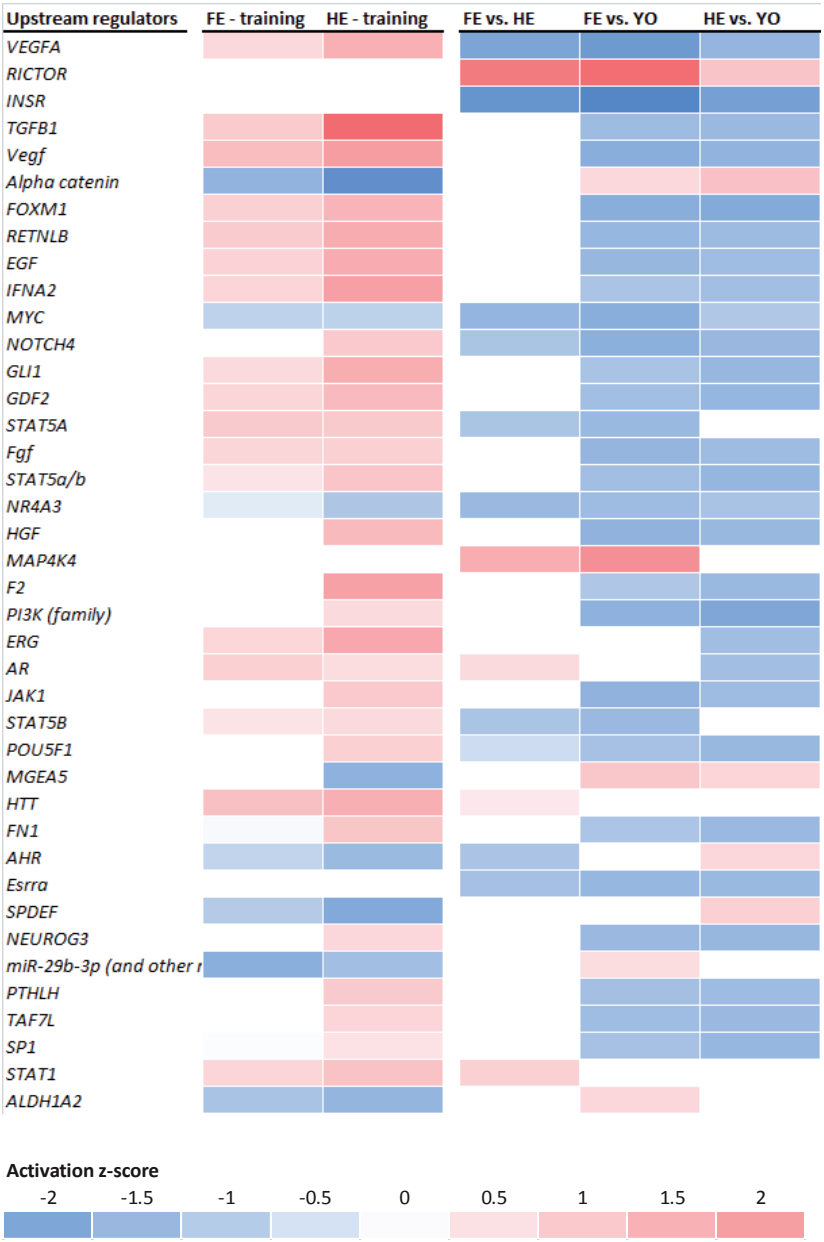


Figure S5. Top 40 (by score) significant upstream regulators from Ingenuity Pathway Analysis. First two columns represent the prolonged exercise training effect in frail (FE) and healthy (HE) older subjects. Last three columns represent baseline differences between frail older subjects (FE), healthy older subjects (HE), and young subjects (YO). Colours indicate predicted activation.

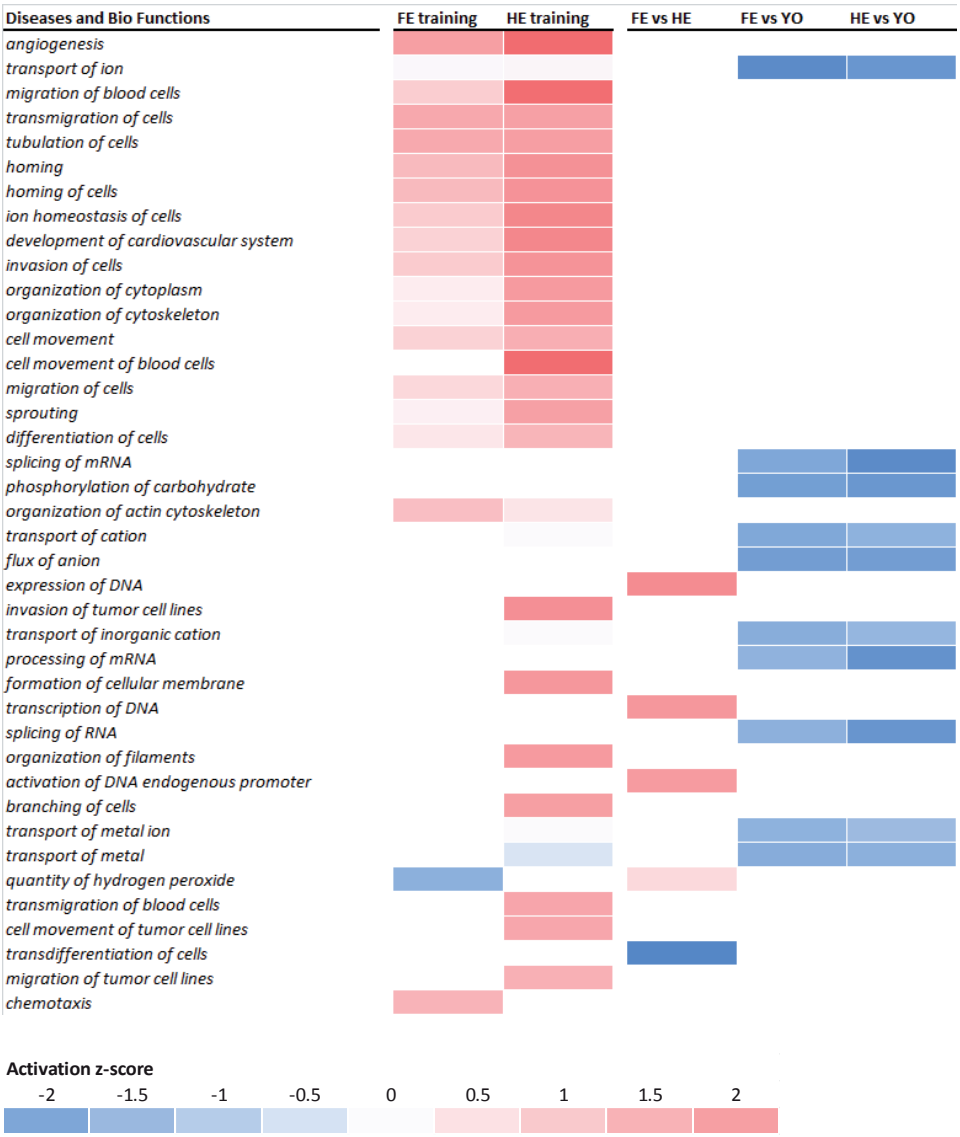


Figure S6. Top 40 (by score) significantly changed diseases and functions from Ingenuity Pathway Analysis. First two columns represent the prolonged exercise training effect in frail (FE) and healthy (HE) older subjects. Last three columns represent baseline differences between frail older subjects (FE), healthy older subjects (HE), and young subjects (YO). Colours indicate predicted activation.

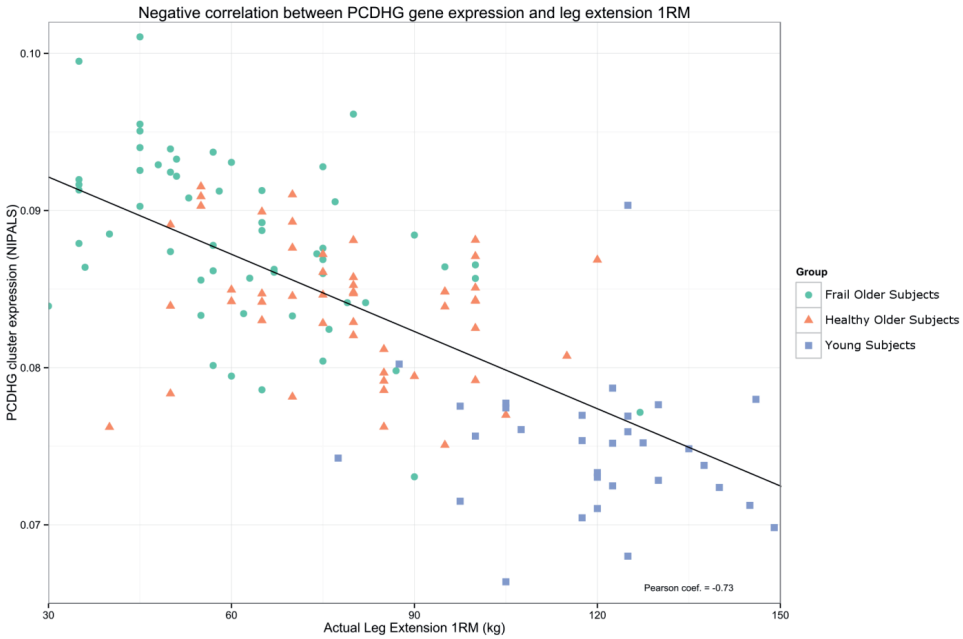


Figure S7. Negative Correlation between the PCDHG gene cluster (eigengene as calculated using the NIPALS algorithm) and leg extension 1RM.

3

The muscle metabolome differs between healthy and frail subjects of older age

Parastoo Fazelzadeh*
Roland Hangelbroek*
Michael Tieland
Lisette de Groot
Lex Verdijk
Luc van Loon
Age Smilde
Rodrigo Alves
Jacques Vervoort
Michael Müller
John van Duynhoven
Mark Boekschoten

*Both authors contributed equally

Published in The Journal of proteome research (2016).

ABSTRACT

Populations around the world are aging rapidly and concomitant loss of physiological functions negatively affects quality of life at older age. A major contributor to the frailty syndrome of ageing is loss of skeletal muscle. In this study we assessed the skeletal muscle biopsy metabolome of healthy young, healthy older and frail older subjects to determine the effect of age and frailty on the metabolic signature of skeletal muscle tissue. Moreover, effects of prolonged whole-body resistance type exercise training on the muscle biopsy metabolome of older subjects were examined. The baseline metabolome was measured in muscle biopsies collected from 30 young, 66 healthy older subjects and 43 frail older subjects. Follow-up samples from frail older (24 samples) and healthy older subjects (38 samples) were collected after 6 months of prolonged resistance-type exercise training. Young subjects were included in order to have a reference for expected shifts of the older towards a younger metabolic phenotype. Primary differences in skeletal muscle metabolite levels between young and healthy older subjects were related to mitochondrial function, fiber type, and tissue turnover. Similar differences were observed when comparing frail with healthy older subjects. Prolonged resistance-type exercise training revealed a correlative adaptive response of amino acids, especially branched chain amino acids, and genes related to tissue remodeling. The impact of exercise on branched chain amino acids derived acylcarnitines in older subjects points to a downward shift in branched chain amino acid catabolism upon training. We observed modest correlations between muscle metabolite and plasma levels. This prohibits the use of the latter as read-outs of muscle metabolism and stresses the need for direct assessment in muscle tissue biopsies.

KEYWORDS: muscle biopsy, frailty, aging, tissue remodeling

INTRODUCTION

Populations around the world are aging rapidly and concomitant loss of physiological functions compromises independence at older age. It has been estimated that people older than 60 y will make up 22% of the world population and people older than 80 y will account for 4.4% of the world population in 2050 [1]. A major contributor to the frailty syndrome of ageing is skeletal muscle loss, which can lead to increased disability in the older population. In most people muscle mass and strength start to decline around the age of 35 y with more progressive muscle loss observed after the age of 65 y [2-6].

From a recent study [7] we learned that prolonged resistance-type exercise training partially shifts the skeletal muscle transcriptome of older subjects toward an expression pattern observed in muscle tissue of young subjects, with changes in gene expression related to vascularisation, tissue remodelling and glucose metabolism. We hypothesize a similar shift towards the young phenotype in the muscle metabolome after resistance-type exercise training in older subjects. The transcriptome analysis also revealed substantial differences between healthy young men, healthy older subjects and frail older subjects before any intervention was undertaken. Particularly genes related to mitochondrial function were downregulated in older subjects compared to young.

Although the effects at gene expression level are expected to be reflected in metabolic regulation, our insights are limited to only few studies, mostly in animals. Recent work showed that ageing affects glucose and fatty acid metabolism in muscle of mice [8]. A study on aged rats suggests a muscle group-specific perturbation of lipid and glucose metabolism consistent with mitochondrial dysfunction [9]. A recent study in humans showed that lipid content and oxidative activity in skeletal muscle are related to muscle fiber type in ageing and metabolic syndrome [10]. Another human study showed that upon training mitochondrial function and intermediary metabolism were reprogrammed in insulininsensitive obese subjects [11]. Within these human studies the deployed metabolic profiling platforms had limited coverage and focused on specific sub-metabolomes.

A major bottleneck in achieving extended coverage was the limited amount of muscle tissue material that can be obtained from human studies. The recent development of a comprehensive targeted metabolic profiling platform [12] optimized and validated for small muscle biopsies paved the way to understand the observed phenotypical differences at a more comprehensive level. Here we establish the effect of aging and frailty on the skeletal muscle metabolome. We further examine the impact of prolonged resistance-type exercise training on the metabolome of frail and healthy older subjects. We included a population of young subjects in our study in order to have a reference for expected shifts of the older towards a younger metabolic phenotype.

MATERIALS AND METHODS

Experimental design

Muscle biopsies (*Vastus lateralis*) and circulating metabolites samples were collected from pre-frail, frail older [13, 14], healthy older [15, 16] and young subjects [17]. Medical history of all subjects was evaluated by medical questionnaires which were analyzed by a physician. Subjects who were unable to participate in the training due to pain, were excluded prior to starting the intervention. Included subjects who showed severe discomfort during the training sessions were excluded from further participation in the study. Baseline metabolite levels were measured in skeletal muscle tissue of 30 young, 66 healthy and 43 frail older subjects (Table 1). We also measured metabolites in plasma and serum for 50 young, 76 healthy and 62 frail older subjects (Supplementary Table S1). The transcriptome of our muscle biopsy set was measured in an earlier study[7].

Samples from healthy young male subjects were derived from several studies performed within our group, in which exactly the same technique and processing was used for sample collection. We used young male subjects as a reference group and samples were only taken at baseline. Samples from the frail and healthy older subjects were obtained from two study centers. More details of the studies can be found in the respective papers [13, 16].

In addition, muscle biopsies were obtained after 6 months of resistance-type exercise training for 38 healthy and 24 frail older subjects. All muscle biopsies and circulating metabolites samples were obtained in the morning, in an overnight fasted state, after standardized meal the evening before, and no strenuous physical activity for 3 days prior to muscle biopsy collection.

Fried criteria [18] were used to assess the frailty in older subjects, in which frailty is viewed in terms of the unintentional weight loss, weakness, self-reported exhaustion, slow walking speed, and low physical activity. Based on the above mentioned criteria, the healthy older subjects were not considered frail or pre-frail at the start of the intervention study [19].

In our study population, all older subjects regardless of their health status (frail or healthy) improved in muscle performance following 6 months resistance-type exercise training (Supplementary Figure S1 Table S2) as illustrated by significantly increased leg extension and leg press strength after training (P-value < 0.01) [7, 13, 19]. Both healthy older and frail older subjects followed similar progressive full-body resistance type exercise training. In brief, the training consisted of a 5 minute warm-up on a cycle ergometer, followed by 4 sets on the leg-press and leg-extension machines. In addition, 3 sets on chest press, lat pulldown, ped-dec and vertical row machines were performed (Technogym, Rotterdam, The Netherlands).However, the healthy older subjects trained 3 times per week and frail subjects trained 2 times per week.

Moreover, subjects received a protein or control supplement during the study. Healthy older subjects took 15 gram milk protein or control drink at breakfast and frail older subjects took a similar 15 gram drink (milk protein or control) at breakfast and lunch every day throughout the entire 6 months intervention. Full details can be found in the earlier papers on the phenotypical impact of training on our study population [13, 19].All studies were approved by The Medical Ethics Committee of either Wageningen University or Maastricht University and comply with the Declaration of Helsinki.

Metabolomics analysis of circulating metabolites

Amino acids and biogenic amines were derivatized (Acc-Tag) in 5 µL aliquots of plasma. Samples were analyzed using an ACQUITY UPLC system with autosampler (Waters, Etten-Leur, The Netherlands) coupled with a Xevo Tandem quadrupole mass spectrometer (Waters) operated using QuanLynx data acquisition software (version 4.1; Waters). An Accq-Tag Ultra column (Waters) was used. The Xevo TQ was used in the positive-ion electrospray mode and all analytes were monitored in Multiple Reaction Monitoring (MRM) using nominal mass resolution. Acquired data were evaluated using TargetLynx software (Waters), by integration of assigned MRM peaks and normalization using proper internal standards[20].

Acylcarnitines, trimethylamine-N-oxide, choline, betaine, deoxycarnitine and carnitine were analyzed in 5 µL plasma, spiked with an internal standard, using a UPLC-MS/MS. Also here an Accq-Tag Ultra column was used. The Xevo TQ was used in the positive-ion electrospray mode and all analytes were monitored in Multiple Reaction Monitoring (MRM) using nominal mass resolution. In-house developed algorithms [21] were applied using the pooled QC samples to compensate for shifts in the sensitivity of the mass spectrometer over the batch.

Organic acids were measured by GC-MS using 50 µL of plasma sample prepared using a two-step derivatization procedure with subsequent oximation using methoxyamine hydrochloride (MeOX) and silylation using N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA. Samples were measured on an Agilent GC (7890A) coupled to Agilent Quadrupole-MS with EI source (Agilent MSD 5975C). Separation was performed using a HP-5MS column (30 m x 0.25 m x 0.25 µm; Agilent). The raw data were pre-processed using Agilent MassHunter Quantitative Analysis software for GC-MS (Agilent, Version B.04.00), and quantitation of metabolite response was calculated as the peak area ratios of the target analyte to the respective internal standard. In-house developed algorithms were applied using the pooled QC samples to compensate for shifts in the sensitivity of the mass spectrometer over the batch. Serum metabolite concentrations determined by NMR were measured as described by Mihaleva *et al.* [22]. In short, serum samples were ultrafiltrated and automated quantum mechanical line shape fitting of ^{1}H NMR spectra was performed using PERCH.

In tissue metabolome analysis

Metabolites were extracted from 10 mg of wet muscle tissue. This tissue was further lyophilized and weighted to determine the dry tissue mass. After pulverizing the tissue, metabolites were extracted using methanol/chloroform/water (MCW). The extraction method used in this study has extensively been described and characterized elsewhere [12].

Amines, acylcarnitines and oxylipins were measured using the platforms also used for measurement of these metabolites in plasma. The validation of these methods for human tissue biopsies is described in detail elsewhere [21]. To compensate for shifts in the sensitivity of the mass spectrometer over multiple batches of measurements, in-house developed algorithms were applied [21]. The metabolite response was determined by the peak area ratio of the target analyte to the appropriate internal standard. These response ratios were used in the subsequent data analysis. ATP, ADP, creatine and phosphocreatine were determined spectrophotometrically using established enzymatic assays. For the first 3 metabolites, commercially available fluorimetric assay kits were used (BioVision cat.# K354-100, K355-100 and K635-100) following the manufacturer's instructions. Phosphocreatine was measured according to a colorimetric assay kit protocol described by Szas *et al.*[23]. In total 96 metabolites including amine, acylcarnitines, organic acids, oxylipins and a number of nucleotides were measured.

Statistical analysis

Statistical analysis was performed on log-transformed data. We used analysis of variance (ANOVA) for between group comparisons at baseline. *P*-value<0.05 was considered significant. We used linear mixed models for assessment of the training effect. Our model included exercise training, subject, sex, protein supplementation and within subject correlation. Analyses of the training effect was performed separately for frail and healthy older subjects due to differences in training frequency. To summarize acylcarnitines into one single metabolite (eigen metabolite), the Non-linear Iterative partial least squares (NIPALS) algorithm [24] of the mixOmics R library was used to calculate the Singular Value Decomposition (SVD) of acylcarnitines. The mixOmics R library was used to perform multilevel sparse partial least squares (sPLS) [25, 26]for integration of metabolomics and transcriptomics. A canonical correlation cut-off of 0.80 was used for building the network. FactoMineR was used to perform principal component analysis (PCA) [27]. All analyses were done using R (version 3.02).

ASCA (ANOVA Simultaneous Component Analysis) was performed to determine global differences on metabolites. ASCA is a multivariate method that partitions variation in the data and enables to interpret these partitions by simultaneous component analysis [28]. Analysis was performed separately for the frail and healthy older subjects, using delta values of each metabolite for each individual (value after training - value before training) with supplement, sex and their interaction in the model as factors. Analysis was done under Matlab (version R2012a).

RESULTS

Baseline comparisons between healthy older, frail older and young subjects

Comparison of the circulating metabolites profiles of young and healthy older subjects revealed a range of metabolites that differed significantly between both groups. Analysis of variance (ANOVA) showed that amino acids and acylcarnitines were responsible for the main differences in circulating metabolites between older and young subjects. These differences were in line with previous observations of age-related effects on the circulating metabolome [8, 29, 30].

Figure 1 shows a principal component analysis (PCA) of the biogenic amine profiles of the muscle biopsies obtained from young, healthy older and frail older subjects, before and after exercise. We observed clear age-related differences in the biogenic amine profiles of skeletal muscle, as well as a difference between healthy and frail older subjects. The PCA plot also revealed an effect of prolonged resistance-type exercise training on amine concentrations in muscle tissue. PCA revealed a similar effect for organic acids in muscle (Supplementary Figure S2).

ANOVA yielded a series of skeletal muscle metabolites that significantly differ between healthy older subjects and young subjects. Many of these metabolites are amino acids and organic acids (Table 2). The outcome of ANOVA modeling of muscle metabolites in healthy vs. frail older subjects is presented in Table 3.

Effect of prolonged resistance-type exercise training in frail and healthy older subjects

In the PCA plot in Figure 1 we can observe that prolonged resistance-type exercise training has an effect on the biogenic amine profile of skeletal muscle tissue of frail and healthy older subjects. The trends in the PCA plot suggest that upon training both healthy and frail older subjects shift towards a younger phenotype (see also the plot for the average value of isoleucine in Supplementary Figure S3,a). The variation in this PCA plot may not only be due to phenotype (young, healthy and frail old) and exercise, but also to sex and protein supplementation. Hence we performed ANOVA Simultaneous Component Analysis (ASCA) to account for these different sources of biological variation. We observed that prolonged resistance-type exercise training had a weak effect on muscle tissue metabolite levels. No significant interaction of protein supplementation with training in both frail and healthy older subjects could be observed. Interaction of sex with training was not significant in healthy older subjects, but was significant in frail older subjects (*P*-value=0.03).

Next we used linear mixed models on univariate metabolite levels to account for phe-

notype, exercise, protein supplementation and sex as sources of biological variation. We observed that prolonged resistance-type exercise training affected muscle levels of acylcarnitines in both the healthy older and frail older subjects (Table 4 and 5, respectively). These effects pertain to propionyl (C3), methylmalonyl (C4-DC), and isovaleryl (C5) acylcarnitines as products of the oxidation of amino acids, branched chain amino acids (BCAA), and C6-C20 acylcarnitines as products of fatty acid oxidation [31]. For several acylcarnitines the exercise effects were nearly significant (*P*-value >0.05), hence we used the singular value decomposition to summarize the levels of fatty acid derived and amino acid derived acylcarnitines (denoted as FAAC and AAAC, respectively in Figure 4 and 5). Subsequently, we also performed linear mixed models to determine the effect of training on the fatty acid derived and amino acid derived acylcarnitines. The amino acid derived acylcarnitines levels were significantly decreased after training for both healthy and frail older subjects (see example of the effects for propionylcarnitine in Supplementary Figure S3, b).

We used multilevel sPLS to integrate muscle changes in the transcriptome and metabolome after training. Here the goal was to investigate the interaction of two matched data sets and the selection of subsets of either positively or negatively correlated variables across all subjects. This multivariate approach highlighted the training effects within subjects separately from the biological variation between subjects. We applied canonical mode, which highlights the strongest correlations between the two data sets. Subsequently, we selected significantly changed genes based on training (FDR<0.05) in both frail and healthy older subjects. We observed that amino acids, particularly branched chain amino acids, correlate with genes related to connective tissue/extracellular matrix such as collagen, laminin and SPARC (Figure 2).

Correlation between muscle and plasma metabolites levels

Within this study we simultaneously collected fasting plasma and muscle biopsies. We investigated to what extent baseline plasma metabolite levels reflect muscle metabolite levels. We first constructed separate correlation heatmaps for muscle and plasma metabolites within each compartment. In Figure 3a-b one can observe that metabolites that are in the same group of metabolites (e.g. amino acids, acylcarnitines) are correlated to each other within both plasma and muscle. However, the correlation heatmap of muscle and plasma metabolites (Figure 3c) showed only minor to moderate correlations between muscle and plasma metabolites (Pearson correlation between 0.3 and 0.5). The correlation networks (Figure 3d) show that strongest correlations pertain to 3-hydroxybutyric acid, 4-hydroxyproline, proline, branched chain amino acids and several acylcarnitines. Correlation of serum and muscle metabolites are presented in Supplementary Figure S4. Results are generally in line with plasma-muscle metabolite correlations.

DISCUSSION

Comprehensive metabolic profiling of muscle biopsies: age and resistance-type exercise training effects in a heterogeneous study population

Comprehensive metabolic profiling of muscle biopsies revealed baseline differences between the muscle biopsy metabolomes of healthy young, healthy older and frail older subjects. Furthermore, distinct effects of prolonged resistance-type exercise training in the latter two groups could be observed. Critical was the deployment of five robust profiling platforms using a single and thoroughly validated muscle-biopsy extraction procedure [12]. The analytical variation in these profiles, was around 20-30%, which was smaller than the biological variation in the muscle biopsies. In order to separate the sources of biological variation in our heterogeneous study population we relied on univariate linear mixed models. The metabolic effects of sex and supplementation however turned out to be relatively minor compared to phenotype and response to prolonged resistance-type exercise training. A multivariate approach (ASCA) did not show any significant effects of training, probably because not all sources of biological variation could adequately be accounted for [32]. In the following discussion we limit ourselves to univariate approach since it more strictly accounts for heterogeneity of our study population.

Baseline comparisons

TCA cycle metabolites (succinic acid, fumaric acid, 2-ketoglutaric acid) were lower in the healthy older subjects compared to young subjects. These differences were accompanied by lower levels of ATP, ADP, branched chain amino acids and acylcarnitines in the healthy older subjects. This indicates impaired mitochondrial function or a lower number of mitochondria in the muscle of the older subjects. It may also be a reflection of the lower habitual physical activity of the older subjects. These observations are in line with data on the transcriptomics profile of these subjects, which show that genes related to mitochondrial function and oxidative phosphorylation have decreased expression in the older subjects compared to young subjects, with the lowest expression occurring in the frail older subjects [7]. This is also in agreement with other studies [8, 33-37], where the mitochondrial electron-transport chain is also among the significantly different pathways in muscle metabolism when comparing older and young subjects.

Worth mentioning are the lower levels of 4-hydroxyproline and proline in the healthy older subjects compared to the young subjects. Both of these amino acids have been associated with collagen turnover [38, 39]. Two precursors of proline, ornithine and arginine, have higher muscle levels in the older subjects. Higher levels of ornithine and arginine, together with lower levels of 4-hydroxyproline and proline, may be due to dysfunction of the mitochondrial ornithine aminotransferase [40], leading to accumulation of arginine and ornithine. This is in

line with slightly lower expression of genes related to tissue remodeling, such as collagen in the older subjects compared to the young subjects [7].

In healthy older subjects we observed higher muscle levels of β-isoamino butyric acid (BAIBA) than in young subjects. Hypothetically, this metabolite is produced upon exercise by expression of PGC-1α and has been proposed as a myokine stimulating browning of white adipose tissue and hepatic β-oxidation [41]. In our study population we observe gene transcription downregulation of PGC-1α target genes in healthy older subjects with respect to young and in frail older subjects with respect to healthy older subjects [7]. At posttranslational level activity of PGC-1α is modulated by NAD^+ dependent deacetylation by SIRT1 (Figure 4-b) [42]. Since NAD^+ levels are decreasing with age [43], we expect also here a downregulation of PGC-1α [44]. The expected decrease of β-isoamino butyric acid (BAIBA) levels is however only observed when comparing frail to healthy older subjects, whereas we observe a significant increase in BAIBA in healthy older subjects compared to young subjects. Thus, the observed changes in baseline levels of BAIBA levels in muscle in our study population do not appear to be consistent with age-related down regulation of PGC-1α. Hence we cannot confirm the recently stated hypothesis that BAIBA acts as a PGC-1α induced myokine. An explanation could be that in our study differences in PGC-1α expression are caused by age and frailty dependent processes, whereas previously described PGC-1α -mediated effects on BAIBA were caused by acute exercise.

Two polyamines, spermine and spermidine, were found to be significantly different in the frail compared to the healthy older subjects. Polyamines are involved in tissue regeneration and cell proliferation and differences are associated with both exercise and muscle pathology [45-47]. The genes directly involved in the polyamine pathway are however not differentially expressed between frail and healthy older subjects. Hence the observed differences in polyamine levels between the frail and healthy older subjects are more likely to be attributed to effects at the level of enzyme activity or metabolite transport. Previous studies have shown that perturbations in polyamine metabolism are associated with neuromuscular disorders [48, 49]. On the transcriptome level we indeed found indications of neuromuscular perturbations in the frail older subjects [7]. In addition, increased spermine levels were recently linked to skeletal muscle atrophy [50]. However, our data suggests that at baseline this process is in the opposite direction when comparing frail and healthy older subjects, even though frail older subjects generally have less skeletal muscle and are likely to exhibit more extensive muscle atrophy. Ost *et al.* recently reported that spermidine is increased in the skeletal muscle of mice overexpressing uncoupling protein 1. The authors proposed that this might be an adaptive response to cope with the additional oxidative stress [51].

The level of carnosine is decreased in healthy older subjects relative to young subjects, and in frail relative to healthy older subjects. Carnosine is an abundant metabolite in muscle where it plays an important role in intracellular pH buffering [52]. Carnosine has also been associated

with chelation of metal ions and antioxidant activity [53]. Carnosine levels are higher in type II muscle fibers compared to type I. A likely explanation for the significantly lower levels of carnosine in healthy and frail older subjects is therefore the decrease of muscle fiber II/I ratio with respectively age and lack of exercise[54].

Several oxylipins derived from linoleic acid (LA) and α-linoleic acid (ALA) occur at higher levels in the muscle of the healthy older subjects compared to young subjects. On the other hand, metabolites derived from the Δ-6 desaturase product dihomo-γ-linoleic acid (DGLA) are reduced in healthy older subjects. We postulate that due to reduced Δ-6 desaturase activity linoleic acid and α-linoleic acid accumulate in the muscle of the healthy older subjects, whereas downstream Δ-6 desaturase product dihomo-γ-linoleic acid products are depleted [55].

Effect of prolonged resistance-type exercise training

We compared the effect of prolonged resistance-type exercise training in both healthy and frail older subjects with all metabolites using multilevel sPLS. There was a profound correlation between the adaptive response to training between the transcriptome and amino acids in the muscle metabolome (canonical correlations between 0.7 and 0.8). There were particularly high correlations between expression changes of extracellular matrix genes and amino acids. Although it is unlikely that there is a direct link between expression of these genes and levels of these metabolites, it does imply that these changes in amino acid levels are part of the adaptive response to resistance-type exercise training.

At the metabolite level, the most striking effects of resistance-type exercise training in frail and healthy older subjects were observed for the C3 (propionyl) and C5 (isovaleryl) muscle acylcarnitines derived from branched chain amino acids. After training, the amino acid derived acylcarnitines showed a significant decrease both in the healthy and frail older subjects, accompanied by an increase of branched chain amino acids. A likely explanation is that the flux-determining mitochondrial branched chain α-keto acid hydrogenase (BCKDH) complex [56] has a compromised response to prolonged resistance-type exercise training. As is schematically depicted in Figure 4-a, the BCKDH complex can respond to exercise via different mechanisms. PGC-1 is a known activator of BCKDH, but training did not have an effect on its gene expression in our study. Exercise is known to increase NAD^+ levels [44] and could thus activate PGC-1α in a post-translational manner via SIRT1. Apparently also this mechanism is not activated by training in the older subjects. These effects are specific for branched chain amino acids oxidation and no significant effects on fatty acid derived acylcarnitines were found. A decrease in branched chain amino acids oxidation may stimulate mTOR related pathways activation and protein synthesis [57, 58], which is beneficial for older subjects. As this mechanism occurs at enzymatic level, the available metabolomics and transcriptomics data can however not confirm this hypothesis and in future studies proteomics would be called for.

Correlation of plasma and muscle metabolome

The weak correlations between plasma and muscle metabolite levels indicate that plasma levels only partially reflect muscle metabolism, even though muscle is one of the largest metabolically active tissues in the human body. This seems to suggest that these metabolites are also produced by other metabolic compartments. In a recent study, lack of correlation between acylcarnitine levels in plasma and tissues in mice was attributed to differences in turnover in plasma and muscle compartments, and contribution of other compartments than muscle to plasma acylcarnitine levels [59]. The same rationale very likely also applies to amino acids involved in collagen metabolism (proline, 4-hydroxyproline), which can also be formed in bone. Adipose tissue is also a metabolically active compartment for branched chain amino acids besides muscle [60], and this will likely weaken plasma-muscle level correlations. As a consequence, correlations between plasma metabolites and muscle metabolites are too modest to support their use as direct read-outs of muscle metabolism [61, 62]

CONCLUSION

The major differences in muscle metabolome of healthy older and young subjects relate to mitochondrial function, fiber-type composition, and tissue turnover. Similar differences were observed when comparing frail older subjects with healthy older subjects. Prolonged resistance-type exercise training showed a correlative adaptive response of amino acids and genes responsible for tissue remodeling. The effect of exercise on amino acid derived acylcarnitines in healthy and frail older subjects points towards decreased branched chain amino acids catabolism likely due to attenuated activation of the flux-determining mitochondrial branched chain α-keto acid hydrogenase complex in older subjects. Only modest correlations between muscle metabolite and plasma levels were found, which prohibits the use of the latter as read-outs of muscle metabolism.

ACKNOWLEDGEMENTS

This research was financially supported by Top Institute Food and Nutrition, Wageningen, The Netherlands, was part of the research program of the Netherland Metabolomics Centre (NMC), part of the Netherlands Genomics Initiative (NGI). TI Food and Nutrition is a public-private partnership on precompetitive research in food and nutrition. The public partners are responsible for the study design, data collection and analysis, decision to publish, and preparation of the manuscript. The private partners have contributed to the project through regular discussion. The authors gratefully acknowledge the staff of the NMC Demonstration and Competency Centre for performing the expert metabolic profiling work on plasma and muscle biopsies. We also thank Dr. Velitchka V. Mihaleva for expert deconvolution of1H NMR spectra of serum.

REFERENCES

1. PD UN: **Department of Economic and Social Affairs.Population Ageing and Development .New York.** September 2012.
2. Frontera WR, Hughes VA, Fielding RA, Fiatarone MA, Evans WJ, Roubenoff R: *Aging of skeletal muscle: a 12-yr longitudinal study.*2000.
3. Goodpaster BH, Park SW, Harris TB, Kritchevsky SB, Nevitt M, Schwartz AV, Simonsick EM, Tylavsky FA, Visser M, Newman AB: **The loss of skeletal muscle strength, mass, and quality in older adults: the health, aging and body composition study.** *J Gerontol A Biol Sci Med Sci* 2006, **61:**1059-1064.
4. Sayer AA, Robinson SM, Patel HP, Shavlakadze T, Cooper C, Grounds MD: **New horizons in the pathogenesis, diagnosis and management of sarcopenia.** *Age Ageing* 2013, **42:**145-150.
5. Sayer AA, Syddall H, Martin H, Patel H, Baylis D, Cooper C: **The developmental origins of sarcopenia.** *J Nutr Health Aging* 2008, **12:**427-432.
6. Sayer AA, Syddall HE, Martin HJ, Dennison EM, Anderson FH, Cooper C: **Falls, sarcopenia, and growth in early life: findings from the Hertfordshire cohort study.** *Am J Epidemiol* 2006, **164:**665-671.
7. Hangelbroek RWJ, Fazelzadeh P, Tieland M, Boekschoten MV, Hooiveld GJEJ, van Duynhoven JPM, Timmons JA, Verdijk LB, van Loon LJC, de Groot LCPGM, Müller M: **Expression of protocadherin gamma in skeletal muscle is associated with muscle weakness and age.** *In press* 2015.
8. Houtkooper RH, Argmann C, Houten SM, Canto C, Jeninga EH, Andreux PA, Thomas C, Doenlen R, Schoonjans K, Auwerx J: **The metabolic footprint of aging in mice.** *Sci Rep* 2011, **1:**134.
9. Garvey SM, Dugle JE, Kennedy AD, McDunn JE, Kline W, Guo L, Guttridge DC, Pereira SL, Edens NK: **Metabolomic profiling reveals severe skeletal muscle group-specific perturbations of metabolism in aged FBN rats.** *Biogerontology* 2014, **15:**217-232.
10. Gueugneau M, Coudy-Gandilhon C, Théron L, Meunier B, Barboiron C, Combaret L, Taillandier D, Polge C, Attaix D, Picard B, et al: **Skeletal Muscle Lipid Content and Oxidative Activity in Relation to Muscle Fiber Type in Aging and Metabolic Syndrome.** *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 2014.
11. Huffman KM, Koves TR, Hubal MJ, Abouassi H, Beri N, Bateman LA, Stevens RD, Ilkayeva OR, Hoffman EP, Muoio DM, Kraus WE: **Metabolite signatures of exercise training in human skeletal muscle relate to mitochondrial remodelling and cardiometabolic fitness.** *Diabetologia* 2014, **57:**2282-2295.

12. Alves RAM, Dane A, Harms A, Strassburg K, Seifar R, Verdijk L, Kersten S, Berger R, Hankemeier T, Vreeken R: **Global profiling of the muscle metabolome: method optimization, validation and application to determine exercise-induced metabolic effects.** *Metabolomics* 2015, **11:**271-285.
13. Tieland M, Dirks ML, van der Zwaluw N, Verdijk LB, van de Rest O, de Groot LC, van Loon LJ: **Protein supplementation increases muscle mass gain during prolonged resistance-type exercise training in frail elderly people: a randomized, double-blind, placebo-controlled trial.** *J Am Med Dir Assoc* 2012, **13:**713-719.
14. Tieland M, van de Rest O, Dirks ML, van der Zwaluw N, Mensink M, van Loon LJ, de Groot LC: **Protein supplementation improves physical performance in frail elderly people: a randomized, double-blind, placebo-controlled trial.** *J Am Med Dir Assoc* 2012, **13:**720-726.
15. Wall BT, Hamer HM, de Lange A, Kiskini A, Groen BB, Senden JM, Gijsen AP, Verdijk LB, van Loon LJ: **Leucine co-ingestion improves post-prandial muscle protein accretion in elderly men.** *Clin Nutr* 2013, **32:**412-419.
16. Leenders M, Verdijk LB, van der Hoeven L, van Kranenburg J, Nilwik R, van Loon LJ: **Elderly men and women benefit equally from prolonged resistance-type exercise training.** *J Gerontol A Biol Sci Med Sci* 2013, **68:**769-779.
17. Gorissen SH, Burd NA, Hamer HM, Gijsen AP, Groen BB, van Loon LJ: **Carbohydrate coingestion delays dietary protein digestion and absorption but does not modulate postprandial muscle protein accretion.** *J Clin Endocrinol Metab* 2014, **99:**2250-2258.
18. Fried LP, Tangen CM, Walston J, Newman AB, Hirsch C, Gottdiener J, Seeman T, Tracy R, Kop WJ, Burke G, McBurnie MA: **Frailty in Older Adults: Evidence for a Phenotype.** *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* 2001, **56:**M146-M157.
19. Leenders M, Verdijk LB, Van der Hoeven L, Van Kranenburg J, Nilwik R, Wodzig WK, Senden JM, Keizer HA, Van Loon LJ: **Protein supplementation during resistance-type exercise training in the elderly.** *Med Sci Sports Exerc* 2013, **45:**542-552.
20. Noga MJ, Dane A, Shi S, Attali A, van Aken H, Suidgeest E, Tuinstra T, Muilwijk B, Coulier L, Luider T, et al: **Metabolomics of cerebrospinal fluid reveals changes in the central nervous system metabolism in a rat model of multiple sclerosis.** *Metabolomics* 2012, **8:**253-263.
21. van der Kloet FM, Bobeldijk I, Verheij ER, Jellema RH: **Analytical Error Reduction Using Single Point Calibration for Accurate and Precise Metabolomic Phenotyping.** *Journal of Proteome Research* 2009, **8:**5132-5141.
22. Mihaleva VV, Korhonen SP, van Duynhoven J, Niemitz M, Vervoort J, Jacobs DM: **Auto-**

mated quantum mechanical total line shape fitting model for quantitative NMR-based profiling of human serum metabolites. *Anal Bioanal Chem* 2014, **406:**3091-3102.

23. Szasz G: **Reaction-rate method for gamma-glutamyltransferase activity in serum.** *Clin Chem* 1976, **22:**2051-2055.
24. Wold H: *Path models with latent variables: The NIPALS approach.* Acad. Press; 1975.
25. Le Cao KA, Martin PG, Robert-Granie C, Besse P: **Sparse canonical methods for biological data integration: application to a cross-platform study.** *BMC Bioinformatics* 2009, **10:**34.
26. Lê Cao K-A, González I, Déjean S: **integrOmics: an R package to unravel relationships between two omics datasets.** *Bioinformatics* 2009, **25:**2855-2856.
27. Sébastien Lê JJ, François Husson: **FactoMineR: An R Package for Multivariate Analysis.** *Journal of Statistical Software* 2008.
28. Smilde AK, Jansen JJ, Hoefsloot HCJ, Lamers R-JAN, van der Greef J, Timmerman ME: **ANOVA-simultaneous component analysis (ASCA): a new tool for analyzing designed metabolomics data.** *Bioinformatics* 2005, **21:**3043-3048.
29. Berger A, Milgram E, Mitchell M, Lawton K, Hanson R, Kalhan S, Milburn M: **The metabolomics of aging.** *Faseb Journal* 2007, **21:**A1040-A1040.
30. Lawton KA, Berger A, Mitchell M, Milgram KE, Evans AM, Guo L, Hanson RW, Kalhan SC, Ryals JA, Milburn MV: **Analysis of the adult human plasma metabolome.** *Pharmacogenomics* 2008, **9:**383-397.
31. Laferrere B, Reilly D, Arias S, Swerdlow N, Gorroochurn P, Bawa B, Bose M, Teixeira J, Stevens RD, Wenner BR, et al: **Differential metabolic impact of gastric bypass surgery versus dietary intervention in obese diabetic subjects despite identical weight loss.** *Sci Transl Med* 2011, **3:**80re82.
32. Saccenti E, Hoefsloot HJ, Smilde A, Westerhuis J, Hendriks MWB: **Reflections on univariate and multivariate analysis of metabolomics data.** *Metabolomics* 2014, **10:**361-374.
33. Balagopal P, Rooyackers OE, Adey DB, Ades PA, Nair KS: **Effects of aging on in vivo synthesis of skeletal muscle myosin heavy-chain and sarcoplasmic protein in humans.** *Am J Physiol* 1997, **273:**E790-800.
34. Short KR, Vittone JL, Bigelow ML, Proctor DN, Nair KS: **Age and aerobic exercise training effects on whole body and muscle protein metabolism.** *Am J Physiol Endocrinol Metab* 2004, **286:**E92-101.
35. Yarasheski KE, Welle S, Nair KS: **Muscle protein synthesis in younger and older men.** *JAMA* 2002, **287:**317-318.

36. Yarasheski KE, Zachwieja JJ, Bier DM: **Acute effects of resistance exercise on muscle protein synthesis rate in young and elderly men and women.** *Am J Physiol* 1993, **265:**E210-214.
37. Rooyackers OE, Adey DB, Ades PA, Nair KS: **Effect of age on in vivo rates of mitochondrial protein synthesis in human skeletal muscle.** *Proc Natl Acad Sci U S A* 1996, **93:**15364-15369.
38. Kjaer M: **Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading.** *Physiol Rev* 2004, **84:**649-698.
39. Gosselin LE: **Skeletal Muscle Collagen: Age, Injury and Disease.** In *Sarcopenia–Age-Related Muscle Wasting and Weakness.* Springer; 2011: 159-172
40. Morris SM, Jr.: **Arginine metabolism: boundaries of our knowledge.** *J Nutr* 2007, **137:**1602s-1609s.
41. Roberts LD, Bostrom P, O'Sullivan JF, Schinzel RT, Lewis GD, Dejam A, Lee YK, Palma MJ, Calhoun S, Georgiadi A, et al: **beta-Aminoisobutyric acid induces browning of white fat and hepatic beta-oxidation and is inversely correlated with cardiometabolic risk factors.** *Cell Metab* 2014, **19:**96-108.
42. Gomes AP, Price NL, Ling AJ, Moslehi JJ, Montgomery MK, Rajman L, White JP, Teodoro JS, Wrann CD, Hubbard BP, et al: **Declining NAD(+) induces a pseudohypoxic state disrupting nuclear-mitochondrial communication during aging.** *Cell* 2013, **155:**1624-1638.
43. Imai S, Guarente L: **NAD+ and sirtuins in aging and disease.** *Trends Cell Biol* 2014, **24:**464-471.
44. Overmyer KA, Evans CR, Qi NR, Minogue CE, Carson JJ, Chermside-Scabbo CJ, Koch LG, Britton SL, Pagliarini DJ, Coon JJ, Burant CF: **Maximal oxidative capacity during exercise is associated with skeletal muscle fuel selection and dynamic changes in mitochondrial protein acetylation.** *Cell Metab* 2015, **21:**468-478.
45. Pegg AE, Mccann PP: **Polyamine Metabolism and Function.** *American Journal of Physiology* 1982, **243:**C212-C221.
46. Turchanowa L, Rogozkin VA, Milovic V, Feldkoren BI, Caspary WF, Stein J: **Influence of physical exercise on polyamine synthesis in the rat skeletal muscle.** *European Journal of Clinical Investigation* 2000, **30:**72-78.
47. Lee NKL, Maclean HE: **Polyamines, Androgens, and Skeletal Muscle Hypertrophy.** *Journal of Cellular Physiology* 2011, **226:**1453-1460.
48. Kaminska AM, Stern LZ, Russell DH: **Altered Muscle Polyamine Levels in Human Neuromuscular Diseases.** *Annals of Neurology* 1981, **9:**605-607.

49. Kaminska AM, Stern LZ, Russell DH: **Polyamine Accumulation in Normal and Denervated Neonatal Muscle.** *Experimental Neurology* 1981, **72:**612-618.
50. Bongers KS, Fox DK, Kunkel SD, Stebounova LV, Murry DJ, Pufall MA, Ebert SM, Dyle MC, Bullard SA, Dierdorff JM, Adams CM: **Spermine oxidase maintains basal skeletal muscle gene expression and fiber size and is strongly repressed by conditions that cause skeletal muscle atrophy.** *Am J Physiol Endocrinol Metab* 2015, **308:**E144-158.
51. Ost M, Keipert S, van Schothorst EM, Donner V, van der Stelt I, Kipp AP, Petzke KJ, Jove M, Pamplona R, Portero-Otin M, et al: **Muscle mitohormesis promotes cellular survival via serine/glycine pathway flux.** *Faseb j* 2015, **29:**1314-1328.
52. Sale C, Artioli GG, Gualano B, Saunders B, Hobson RM, Harris RC: **Carnosine: from exercise performance to health.** *Amino Acids* 2013, **44:**1477-1491.
53. Boldyrev AA, Aldini G, Derave W: **Physiology and pathophysiology of carnosine.** *Physiol Rev* 2013, **93:**1803-1845.
54. Harris RC, Wise JA, Price KA, Kim HJ, Kim CK, Sale C: **Determinants of muscle carnosine content.** *Amino Acids* 2012, **43:**5-12.
55. Kalish BT, Fallon EM, Puder M: **A tutorial on fatty acid biology.** *JPEN J Parenter Enteral Nutr* 2012, **36:**380-388.
56. Shimomura Y, Murakami T, Nakai N, Nagasaki M, Harris RA: **Exercise promotes BCAA catabolism: effects of BCAA supplementation on skeletal muscle during exercise.** *J Nutr* 2004, **134:**1583s-1587s.
57. Um SH, D'Alessio D, Thomas G: **Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1.** *Cell Metab* 2006, **3:**393-402.
58. Newgard CB: **Interplay between lipids and branched-chain amino acids in development of insulin resistance.** *Cell Metab* 2012, **15:**606-614.
59. Schooneman MG, Achterkamp N, Argmann CA, Soeters MR, Houten SM: **Plasma acylcarnitines inadequately reflect tissue acylcarnitine metabolism.** *Biochim Biophys Acta* 2014, **1841:**987-994.
60. Herman MA, She P, Peroni OD, Lynch CJ, Kahn BB: **Adipose tissue branched chain amino acid (BCAA) metabolism modulates circulating BCAA levels.** *J Biol Chem* 2010, **285:**11348-11356.
61. Brosnan JT: **Glutamate, at the Interface between Amino Acid and Carbohydrate Metabolism.** *The Journal of Nutrition* 2000, **130:**988.
62. van de Poll MC, Siroen MP, van Leeuwen PA, Soeters PB, Melis GC, Boelens PG, Deutz NE, Dejong CH: **Interorgan amino acid exchange in humans: consequences for arginine and citrulline metabolism.** *The American Journal of Clinical Nutrition* 2007, **85:**167-172.

Table 1. Characteristics of subjects of which skeletal muscle tissue biopsies were studied

	Young	Healthy older	Frail older
N (male / female)	30/0	47/19	25/18
Age (years)	21.7 ± 2.5	71.7 ± 5.2	77.5 ± 8.0
Height (m)	1.83 ± 0.06	1.72 ± 0.08	1.67 ± 0.09
Weight (kg)	76.7 ± 11.8	75.9 ± 13.3	77.5 ± 11.1
BMI[a] (kg / m^2)	22.6 ± 2.7	25.5 ± 3.0	27.5 ± 3.7
Body Fat (%)	14.9 ± 4.9	24.5 ± 5.6	32.1 ± 8.8

Data was presented as mean ±SD. a: body mass index.

Table 2. Muscle metabolites that are significantly different between healthy older and young males [a]

Metabolite	*P*-value	FC[b] (Older/Young)
TCA Cycle		
Succinic acid	0.02	0.76
2-ketoglutaric acid	0.03	0.76
Fumaric acid	0.04	0.82
Lactic acid	0.05	0.69
Energy		
ATP	<0.01	0.75
ADP	0.01	0.88
Branched chain amino acids		
Valine	<0.01	0.81
Leucine	<0.01	0.81
Isoleucine	0.03	0.84
Acylcarnitines		
Acetylcarnitine (C2)	<0.01	0.49
Malonylcarnitine (C3-DC)	<0.01	0.46
Intracellular buffering		
Carnosine	<0.01	0.7
Arginine, Proline Pathway		
Ornithine	<0.01	1.55
Arginine	<0.01	1.34
4-hydroxy-proline	0.01	0.69
Proline	0.02	0.84
Glycylglycine	0.05	0.87
Methionine	<0.01	0.8

Table 2 continued

Other amino acids		
Lysine	<0.01	1.44
Aspartic acid	<0.01	1.45
Oxylipins		
LA (LOX)		
9-HODE	<0.01	1.4
13-HODE	0.01	1.37
13-KODE	0.03	1.3
LA(CYP450)		
9,10-EpOME	0.04	1.39
ALA (LOX)		
9-HOTrE	0.01	1.71
DGLA (LOX)		
15S-HETrE	0.02	0.83
8-HETrE	0.03	0.85
Aminobutyric acids		
β amino isobutyric acid	<0.01	1.61
α-aminobutyric acid	0.01	0.82

a: Out of a comprehensive muscle biopsy metabolic profile those metabolites are presented that are significantly (*P*-value =<0.05) different between young and old subjects according to univariate ANOVA models. b: Fold Change

Table 3. Muscle metabolites that are significantly different between frail and healthy older subjects.[a]

Metabolite	Group	Sex	Interaction	FC[b](Frail/Healthy)
TCA Cycle				
Citric acid	<0.01	NS [c]	NS	0.54
Acylcarnitines				
Isovalerylcarnitine (C5)	<0.01	NS	NS	0.42
Octenoylcarnitine (C8)	0.03	0.03	NS	0.77
Malonylcarnitine(C3-DC)	0.02	NS	NS	0.77
Carnitine (C0)	0.01	NS	NS	0.75
Intracellular buffering				
Carnosine	0.01	NS	NS	0.8
Oxylipins				
LA (CYP450)				
12,13DiHOME	0.04	NS	0.03	1.18
DGLA (LOX)				
8HETrE	0.03	NS	NS	0.81
15SHETrE	<0.01	NS	NS	0.77
Polyamine metabolism				
Spermidine	0.01	0.02	NS	1.24
Spermine	0.04	NS	NS	0.9
Other amino acids				
Histidine	<0.01	NS	NS	0.79
Asparagine	0.01	NS	NS	0.81
Taurine	0.01	NS	NS	0.79
Serine	0.01	NS	NS	0.86
Glycine	0.02	NS	NS	0.81
oacetylserine	0.02	NS	NS	0.9
Homoserine	0.02	NS	NS	0.85
Tyrosine	0.02	NS	NS	0.83
Tryptophan	0.02	0.04	NS	0.83
Methionine	0.02	NS	NS	0.83
Glutamine	0.03	NS	NS	0.82
Pyroglutamic acid	0.03	NS	NS	0.83
Glutamic acid	0.04	NS	NS	0.82
Glycylglycine	<0.01	NS	NS	0.77
Aminobutyric acids				
β amino isobutyric acid	0.05	NS	NS	0.79

a: Out of a comprehensive muscle biopsy metabolic profile those metabolites are presented that are significantly (*P*-value =<0.05) different between frail and healthy older subjects according to a univariate ANOVA models that accounted for Group effects (Frail vs. Healthy), Sex effect, and Group and Sex interaction; Significance of Group (Frail vs. Healthy older subjects) and Group & Sex interactions has been indicated; b:fold change; c: not significant

Table 4. Training effect on muscle metabolites in healthy older subjects[a]

Metabolite	Training	FC(post/pre training)[b]
Pipecolic acid	0.002	1.64
Isovalerylcarnitine (C5)	0.005	0.56
Linoleylcarnitine (C18:2)	0.01	0.61
Oleylcarnitine (C18:1)	0.01	0.7
Propionylcarnitine (C3)	0.01	0.73
Palmitoylcarnitine (C16)	0.02	0.75
11.12.EpETrE	0.03	1.26
Tetradecenoylcarnitine (C14:1	0.03	0.47
AAAC[c]	0.02	0.77
FAAC[d]	NS	0.96

a: Metabolites are presented that significantly (*P*-value =<0.05) differ pre- and Post-training in healthy older subjects according to univariate linear mixed models. We note that we constructed also linear mixed models that account for other factors and their interactions, but these were not found to be significant. b: fold change, c,d: Single Value Decomposition (SVD) were calculated for amino and fatty acid acylcarnitines, denoted as AAAC and FAAC, respectively.

Table 5. Training effect on muscle metabolites in frail older subjects [a]

Metabolite	Training	Supplement	Sex	Interaction	FC (post/pre training)[b]
Propionylcarnitine (C3)	<0.01	NS[c]	NS	NS	0.75
Glucose	<0.01	NS	<0.01	NS	1.35
Lactic acid	0.01	NS	<0.01	NS	1.55
Tetradecenoylcarnitine (C14:1)	0.03	NS	NS	NS	2.00
Methionine	0.04	<0.01	NS	NS	1.22
Tryptophan	0.04	NS	NS	NS	1.21
β Alanine	0.05	NS	NS	0.01	0.92
Isoleucine	0.05	<0.01	0.02	NS	1.21
Myristoylcarnitine (C14)	0.05	NS	NS	NS	1.73
AAAC[d]	0.01	NS	NS	NS	0.76
FAAC[e]	NS	NS	NS	NS	1.95

a: Metabolites are presented that significantly (*P*-value =<0.05) differ pre- and post-training in frail older subjects according to univariate linear mixed models that account for Supplement, Sex and (Training and Supplement) Interaction. We note that we constructed also linear mixed models that account for other interactions, but these were not found to be significant. b: fold change, c: Not Significant, d & e: Single Value Decomposition (SVD) were calculated for amino acid and fatty acid acylcarnitines (AAAC and FAAC, respectively).

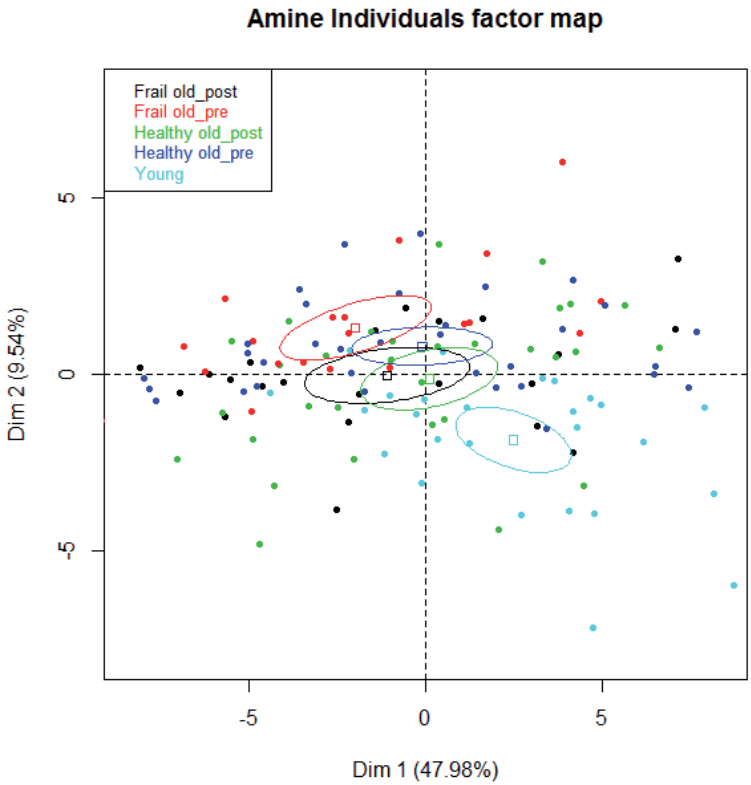


Figure 1. Principal component analysis (PCA) plot of biogenic amines detected in muscle biopsies. To visualize whether groups are significantly different from each other, confidence ellipses (95% Confidence Interval) were drawn around them.

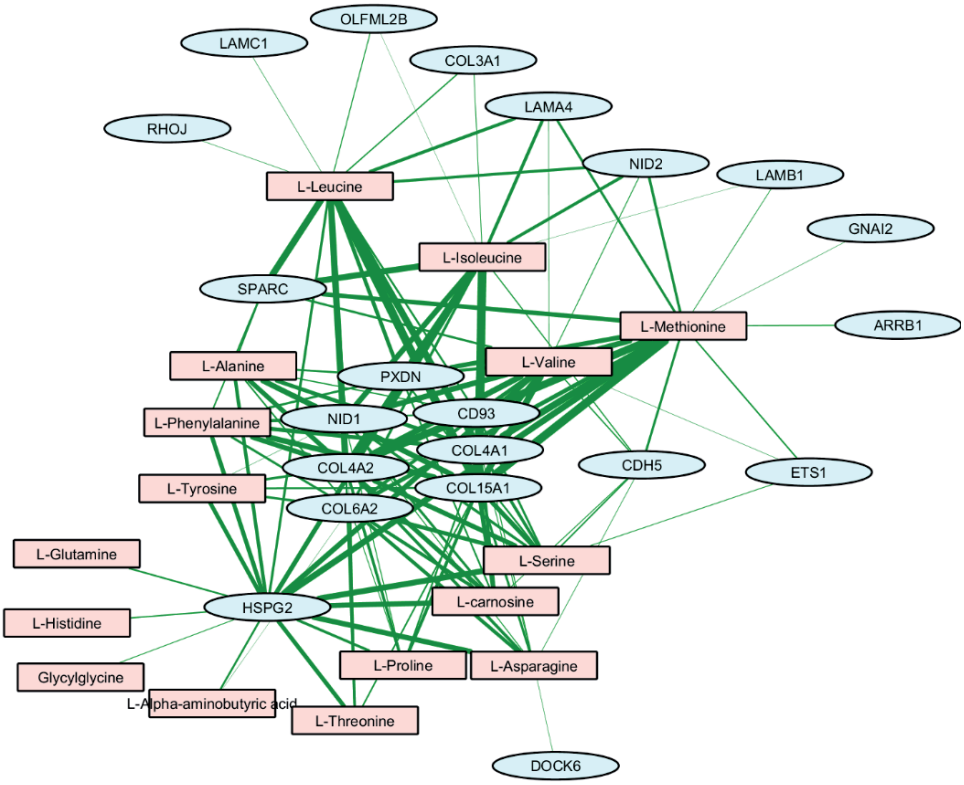
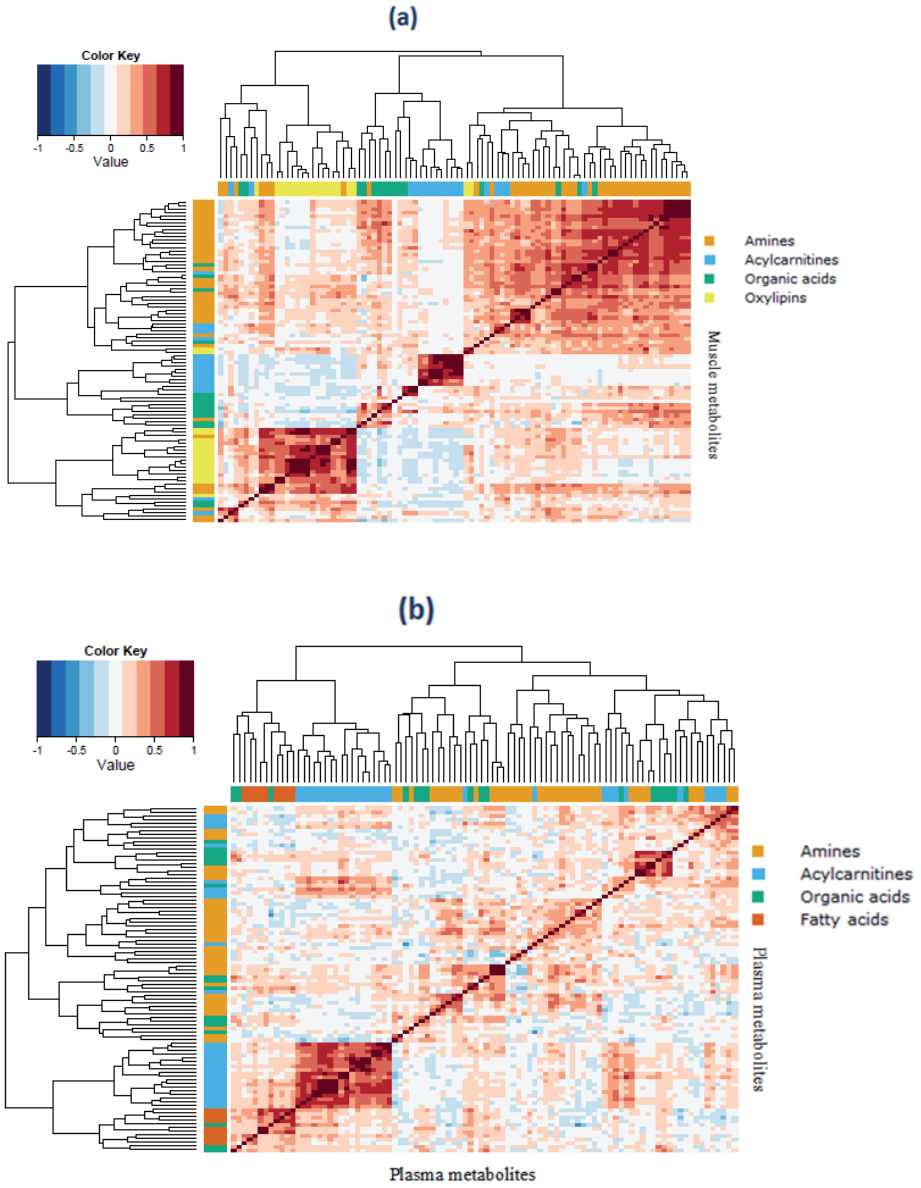


Figure 2. Correlation network of muscle metabolites and genes. Only significantly changed genes were selected (FDR<0.05). Metabolite canonical correlation cutoff <= 0.80. Circle: gene, rectangle: metabolite. Green: positive correlation.


(a)
Color Key
-1
-0.5
0
0.5
1
Value
Amines
Acylcarnitines
Organic acids
Oxylipins
Muscle metabolites
(b)
Color Key
-1
-0.5
0
0.5
1
Value
Amines
Acylcarnitines
Organic acids
Fatty acids
Plasma metabolites
Plasma metabolites

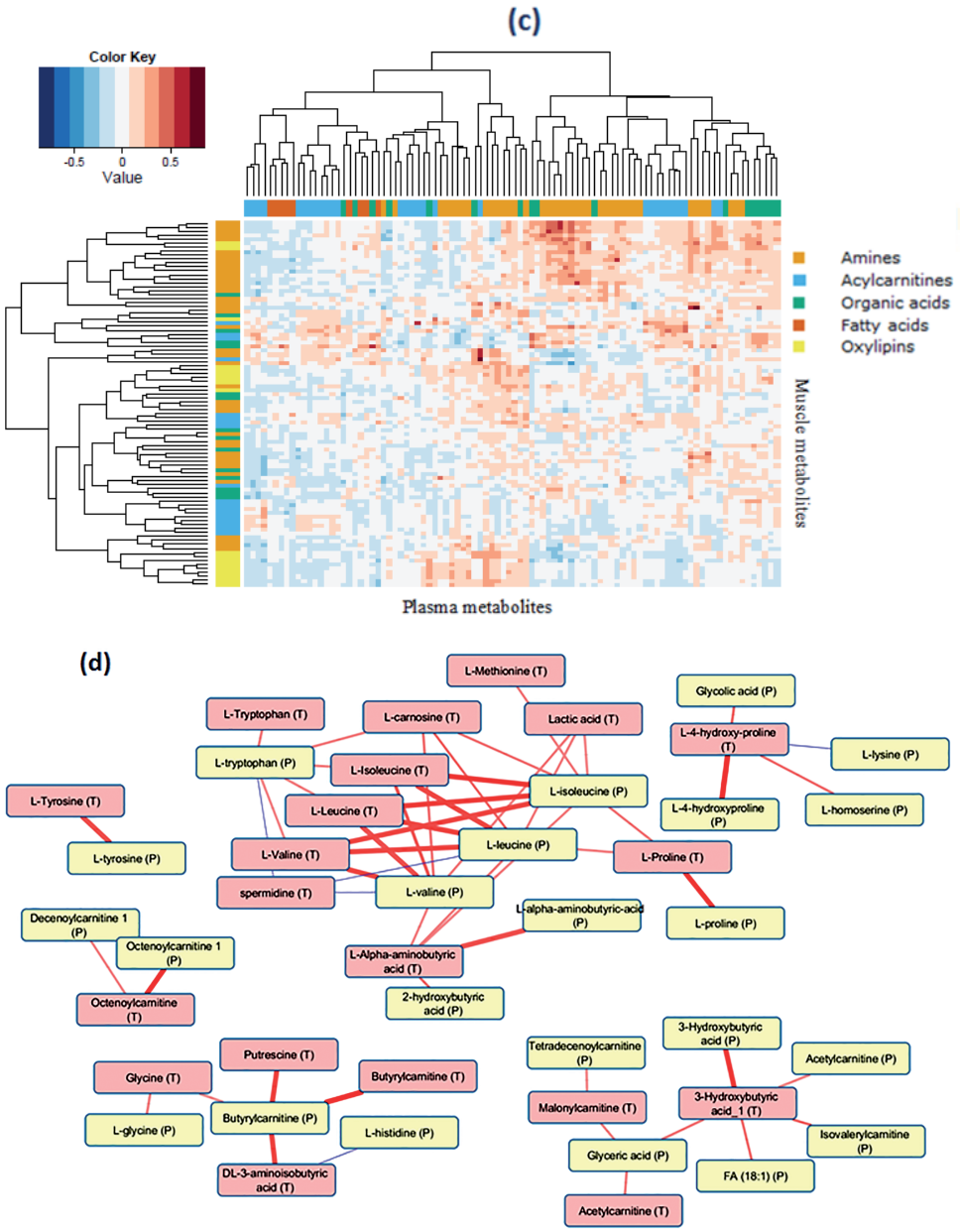


Figure 3. Correlation heatmap of muscle to muscle metabolites (a), plasma to plasma metabolites (b), muscle to plasma metabolites (c). Pearson correlation were used. (d) Correlation network of the most strongly correlated muscle and plasma metabolites (Pearson correlation). Red and blue indicate positive and negative correlations, respectively. Thick lines: correlation ~0.5, thin lines: 0.3 <correlation <0.5. Pink nodes: muscle tissue (T) metabolites and yellow nodes: plasma (P) metabolites.

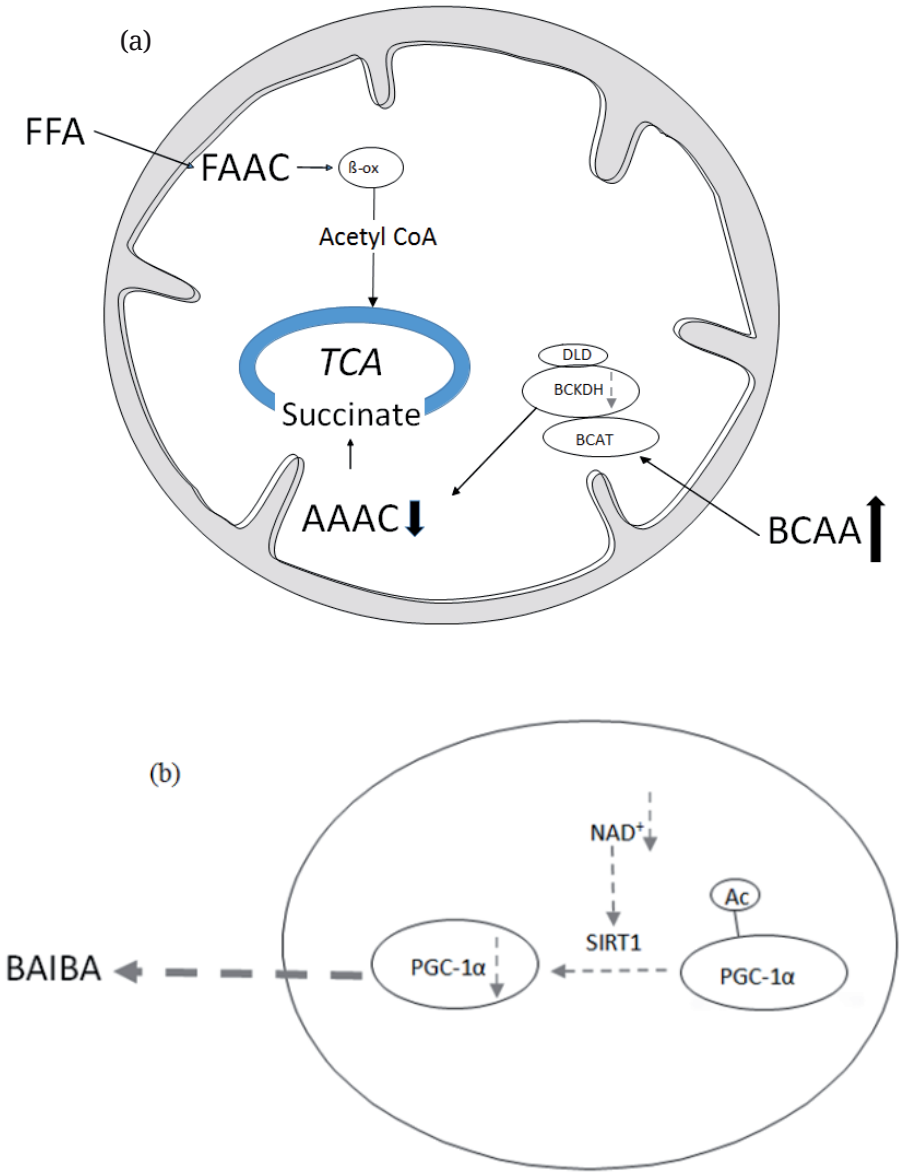


Figure 4. (a) Schematic representation of mitochondrial oxidation of free fatty acids (FFA) and branched chain amino acids (BCAA). Arrows indicate effect of prolonged resistance-type exercise training on older subjects (healthy and frail) on BCAA (increase) and acylcarnitines (decrease) as well as the proposed (dashed-arrows) downregulation of the branched chain α-keto acid hydrogenase (BCKDH) complex. (b) Schematic representation of age-related NAD+ dependent acetylation of PGC1α. Dashed arrows indicate NAD+ and SIRT1 dependent downregulation of PGC-1α and β-isoamino butyric acid (BAIBA).

SUPPLEMENTARY MATERIAL

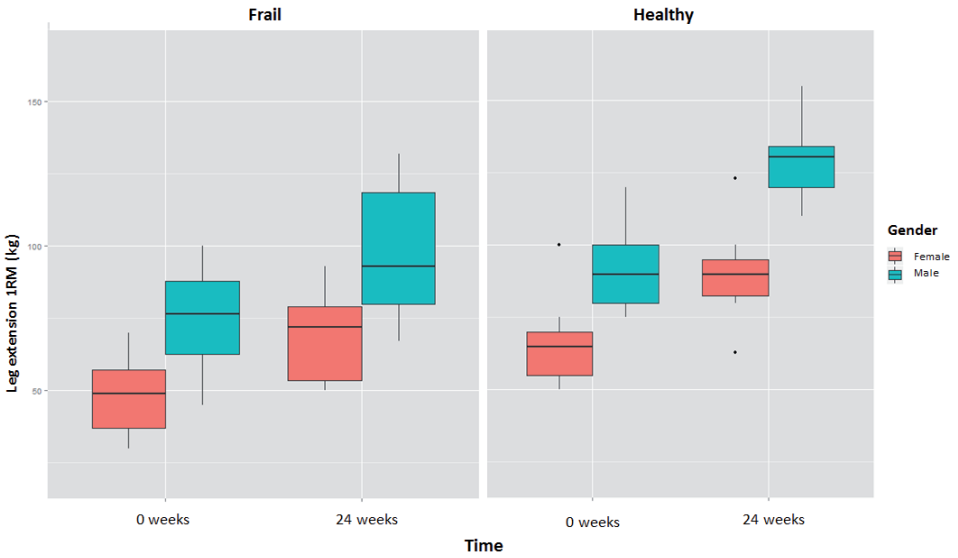


Figure S1. Change of leg extension 1RM after resistance type exercise training. Left are Frail older subjects, right are Healthy older subjects.

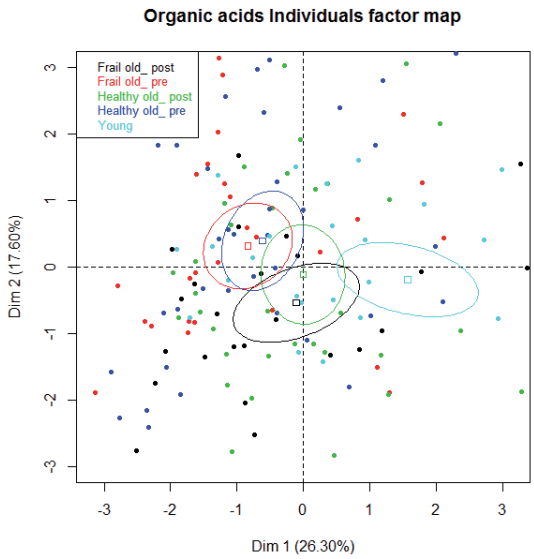


Figure S2. PCA plot of metabolites detected by the organic acid platform in muscle tissue. To visualize whether groups are significantly different from each other, confidence ellipses (95% confidence interval) were drawn around them. Post: after training, pre: before training.

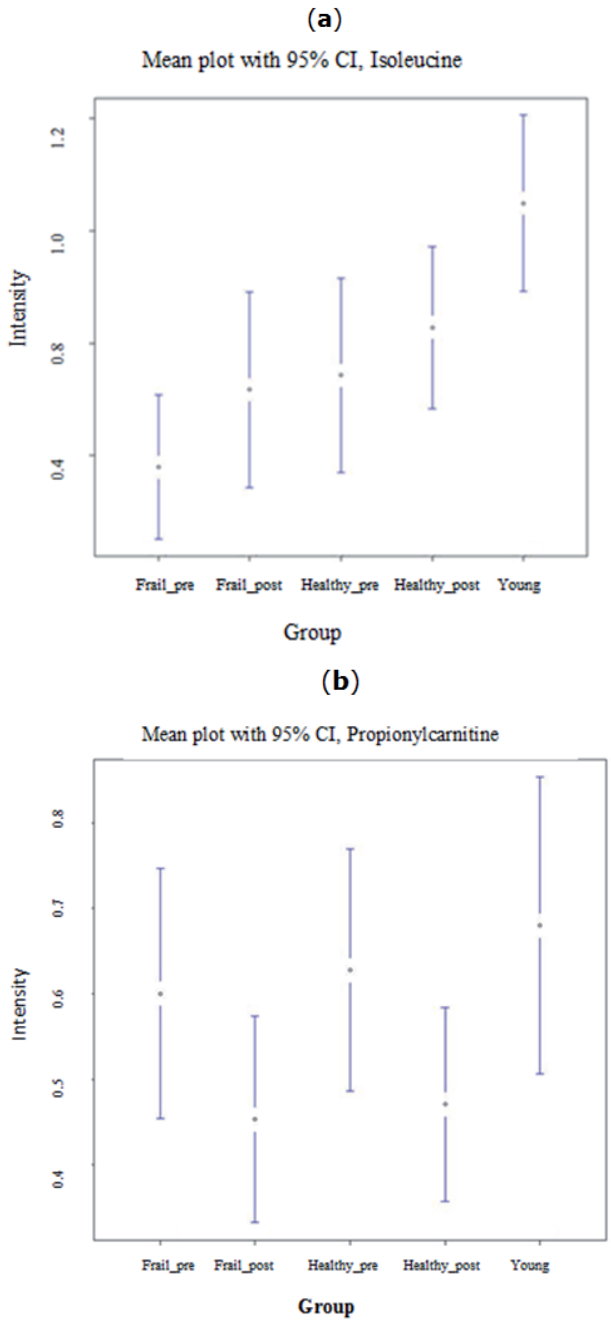


Figure S3. Group means with 95 percent confidence intervals for (a) isoleucine and (b) propionylcarnitine. Standard deviations were relatively constant across groups. Frail_pre: frail older subjects before training, Frail_post: frail older subjects after training, Healthy_pre: healthy older subjects before training, Healthy_post: healthy older subjects after training.

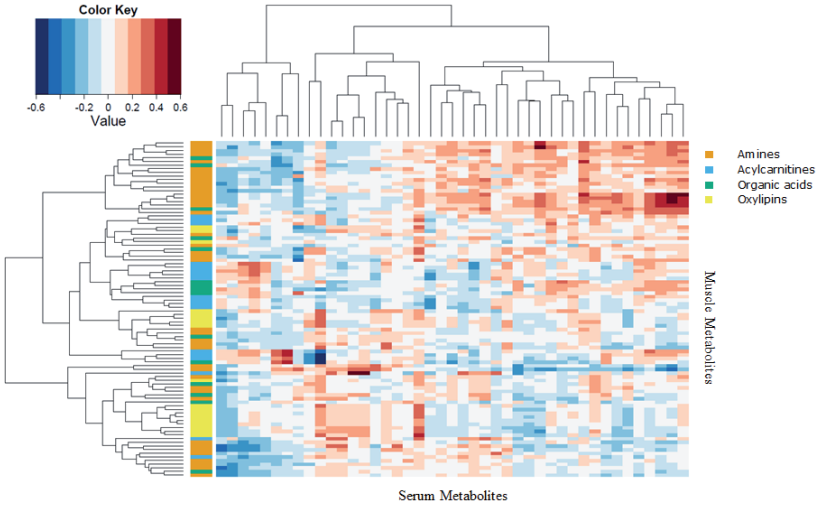


Figure S4. Correlation heatmap muscle and serum metabolite. Pearson correlation were used. Red indicates positive correlation and blue indicates negative correlation.

Table S1. Characteristics of subjects of which plasma samples were studied

	Young	Healthy older	Frail older
N (male / female)	50/0	51/25	35/27
Age (years)	21.7 ± 2.3	71.5 ± 5.1	78.1 ± 8.1
Height (m)	1.83 ± 0.05	1.71 ± 0.08	1.69 ± 0.09
Weight (kg)	76.4 ± 10.3	76.3 ± 12.9	79.2 ± 12.9
BMI[a] (kg / m^2)	23.0 ± 2.9	25.5 ± 3.0	27.8 ± 4.1
Body Fat (%)	15.4 ± 4.5	24.7 ± 5.7	32.66 ± 8.7

a:body mass index.

Table S2. Characteristics of the older subjects with before and after training samples.

	Frail older pre[a]	Frail older post [b]	Healthy older pre	Healthy older post
N (male / female)	10/14		22/16	
Age (years)	76.0 ± 7.0		69.3 ± 4.0	
Height (m)	1.66 ± 0.09		1.70 ± 0.09	
Weight (kg)	77.7 ± 13.6	78.5 ± 14.0	75.9 ± 13.4	76.2 ± 13.6
BMI[c] (kg / m2)	27.9 ± 3.8	28.2 ± 3.9	26.0 ± 2.8	26.0 ± 2.8
Body Fat (%)	35.4 ± 8.3	35.2 ± 8.8	26.4 ± 5.4	24.7 ± 5.0*
Lean Mass (kg)	46.9 ± 9.9	47.4 ± 10.1*	53.6 ± 11.2	55.1 ± 11.5
Leg Extension 1RM (kg)	60.1 ± 18.9	82.7 ± 23.3*	79.8 ± 18.2	112.6 ± 22.5*
Leg Press 1RM (kg)	130.1± 33	176 ± 43.0*	175.9 ± 40.4	227.0 ± 49.9*

a:pre indicates before training. b: post indicates after training. c: Body Mass Index. * indicates a significant effect of resistance-type exercise training (*P*-value < 0.05).

4

Weight loss moderately affects the mixed meal challenge response of the plasma metabolome and transcriptome of peripheral blood mononuclear cells in obese subjects

Parastoo Fazelzadeh
Roland Hangelbroek
Peter Joris
Casper Schalkwijk
Diederik Esser
Lydia Afman
Thomas Hankemeier
Doris Jacobs
Velitchka Mihaleva
Sander Kersten
John van Duynhoven
Mark Boekschoten

Submitted

ABSTRACT

The response to dietary challenges has been proposed as a more accurate measure of metabolic health than static measurements performed in the fasted state. We examined whether the response to a mixed-meal challenge could provide a readout for a weight lossinduced phenotype shift in abdominally obese male subjects. Fifteen lean subjects (BMI= 23.0±2.0kg/m^2) were compared to 29 abdominally obese subjects (BMI= 30.3±2.4 kg/m^2) in order to have a benchmark for weight loss-induced phenotypic shifts. Levels of several plasma metabolites were significantly different between lean and abdominally obese at baseline as well as during postprandial metabolic responses. Genes related to oxidative phosphorylation in peripheral blood mononuclear cells (PBMCs) were expressed at higher levels in abdominally obese subjects as compared to lean subjects at fasting, which was partially reverted after weight loss. The impact of weight loss on the postprandial response was modest, both at the metabolic and gene expression level in PBMCs. We conclude that mixed-meal challenges are not necessarily superior to measurements in the fasted state to assess metabolic health. Furthermore, the mechanisms accounting for the observed differences between lean and abdominally obese in the fasted state are different from those underlying the differences observed during the postprandial response.

KEYWORDS. Mixed-meal challenge, Metabolic health, Phenotype shift

INTRODUCTION

Healthy individuals are able to respond to external stimuli by keeping biological parameters within narrow homeostatic bandwidths. As a consequence, current approaches focusing on disease risk biomarkers mostly lack the sensitivity to detect the effects of lifestyle and dietary interventions aimed at improving or sustaining health. Hence, there is a growing awareness that 'health' should not just be defined as 'the absence of disease', but is more accurately described as 'resilience of homeostatic control', i.e. the ability to cope with daily challenges [1] without drifting out of the regulated homeostatic/allostatic zone [2]. The concept of the human body as an orchestrated machinery that continuously adapts to a changing environment has been embraced by the nutritional field and coined as 'phenotypic flexibility'[3]. The earlier introduced concept of 'metabolic flexibility' departs from a more narrow definition of phenotypic flexibility and specifically refers to the ability of organs to change fuel use depending on availability [4]. The capacity to switch from carbohydrate to fat oxidation and vice versa depending on their supply and demand is crucial for optimal metabolic homeostasis, and thus an important aspect of phenotypic flexibility. In both concepts, the resilience capacity can be tested by the assessment of the stress response to short-term perturbations.

Specific dietary challenge tests have been described, each probing the resilience of different metabolic regulatory processes. The best known challenge test is the oral glucose tolerance test (OGTT), which specifically probes the resilience of glucose metabolism [5]. The oral lipid and protein tolerance tests (OLTT and OPTT, respectively) probe the resilience of other, and partially overlapping metabolic regulatory processes. The use of a mixed-meal challenge, comprising protein, glucose and lipids, has been proposed to target multiple organs and more broadly encompass phenotypic flexibility [6]. By challenging metabolic regulatory processes by means of a dietary challenge, dynamic changes in nutrient metabolism might be uncovered, allowing better exploration of the individual capacity to cope with metabolic stressors [7].

Next to metabolite profiling, which has already been shown to be useful in revealing the complex changes upon dietary challenges, gene expression of peripheral blood mononuclear cells (PBMCs) has been brought forward as readout of biological processes such as inflammation, metabolism [8], oxidative stress and inflammatory status [9-11]. PBMCs can be easily collected in adequate quantities for transcriptomics studies, and it has been shown that nutritional components can modulate pro- and anti-inflammatory mechanisms [8, 12]. A previous study has indicated that long-term consumption of a Mediterranean diet (high content of unsaturated fats and polyphenols) reduces metabolic stress and oxidative phosphorylation activity in PBMCs obtained from overweight men and women [13]. Furthermore, it has also been shown that diet induced weight loss modulates immune-inflammatory and antioxidant

responses and mRNA expression in PBMCs [14].

We examined whether a mixed-meal challenge response could provide a sensitive readout for a shift in phenotypical flexibility upon weight loss in abdominally obese male subjects. By combining metabolite profiling in plasma and whole genome gene expression in PBMCs, we aimed at comprehensively describing the changes in the metabolome and gene expression underlying phenotypic flexibility. We were in particular interested whether the effect of weight loss on the postprandial response would provide a more sensitive readout than the observation in the fasting state. In order to have a benchmark for this comparison, we first compared fasting baseline and postprandial response between lean and abdominally obese subjects. Subsequently, we assessed the effect of dietary weight loss on the fasting baseline and postprandial response in abdominally obese men.

MATERIALS AND METHODS

Subject characteristics

Fifteen lean men with a waist circumference below 94 cm (BMI= 23.0 ± 2.0kg/m^2) and 29 abdominally obese men with a waist circumference between 102-110 cm participated in the study (BMI= 30.3 ± 2.4 kg/m^2). Baseline characteristics of subjects of which microarrays and metabolomics were performed are displayed in Table1. All volunteers were apparently healthy and did not receive proton pump inhibitors, anti-hypertensive medication or drugs known to affect lipid or glucose metabolism.

Study design

Full details of the study have been published before [15]. In brief, abdominally obese men received a mixed-meal challenge prior to and after an 8-week weight loss or no-weight loss control intervention. Lean subjects were included as a reference group and only received the mixed meal challenge at baseline (Figure 1). Prior to the intervention (D1), all subjects underwent a mixed-meal challenge. The standardized mixed meal consisted of two muffins and 300 ml 0% fat milk, which provided 4.6 MJ (4598 KJ or 1100 kcal): 56.6 g fat, 26.5 g protein and 121 g carbohydrate. Blood samples were collected at fasting and immediately after ingestion of the challenge over 4 hours at regular intervals (0, 30, 60, 120, 180, 240 minutes). Subjects assigned to diet-induced weight-loss (WL, Figure 1) program consumed a commercially available very low energy diet (Modifast; Nutrition et Santé, Benelux, Breda, The Netherlands) for 4-5 weeks providing 2.1 MJ/day. Once the waist circumference was below 102 cm (the NCEP ATP II cut-off value) within this period, subjects consumed a mixed-solid energy-restricted diet up to 4.2 MJ/day with a recommended composition for the following 1-2 weeks. Then, subjects

consumed a diet matching their energy requirements to maintain their newly achieved body weights (weight-stable conditions) for at least 2 weeks. Subjects underwent the same mixed-meal challenge again after the weight loss intervention (D2). Blood samples were collected at the same time intervals as on D1. Subjects assigned to the control (CTRL, Figure 1) intervention underwent the same tests, but maintained their habitual diet, physical activity levels and alcohol consumption during 8 weeks. The period between the first and second measurements was the same for the weight loss intervention (WL) and control (CTRL) groups. More details on the composition of low energy diet and mixed meal challenge can be found elsewhere [15].

Sample Collection

On the day before blood sampling, subjects were asked not to perform any strenuous physical exercise or to consume alcohol. On the morning of blood sampling - after a 12 hour overnight fast (from 8 PM) subjects were only allowed to drink a glass of water in the morning. Subjects were also asked not to consume high-fat foods on the day prior to the test days and to come to the test centre by public transport or car to standardize measurements as much as possible.

An intravenous cannula was inserted and blood samples were taken at fasting and after mixed meal consumption both before and after the weight loss intervention at 6 time points (fasting (T0) and 30, 60,120,180 and 240 minutes in the postprandial state). Metabolic profiling was performed on all time points and transcriptomics analysis of PBMCs only at fasting (T0) and after 4 hours in the postprandial state (T4).

PBMC and RNA isolation

PBMCs were isolated before and 4 h after mixed meal challenge by using BD Vacutainer Cell Preparation Tubes. RNA was isolated (RNeasy Micro kit, Qiagen, Venlo, the Netherlands), quantified (Nanodrop ND 1000, Nanodrop technologies, Wilmington, DE, USA) and integrity was checked by an Agilent 2100 Bioanalyser with RNA 6000 microchips (Agilent Technologies, South Queensferry, UK). Samples were included for microarray analysis if RNA integrity number (RIN) was > 7.

Microarray processing

PBMC samples from 15 lean and 29 abdominally obese subjects yielded enough RNA of sufficient quality at all collection points to perform microarray analysis. Microarray analysis was performed for each individual at fasting (T0) and 4 h (T4) in the postprandial state. Total RNA was labelled using a one-cycle cDNA labelling kit (MessageAmp™ II-Biotin Enhanced Kit; Ambion, Inc., Nieuwerkerk a/d IJssel, Netherlands) and hybridized to GeneChip® Human

Gene 1.1 ST Array targeting 19 738 unique gene identifiers (Affymetrix, Inc. Santa Clara, CA, USA). Sample labelling, hybridization to chips, and image scanning were performed according to the manufacturers' instructions.

Microarray analysis

Microarray signals were normalized using robust multichip average (RMA). Data was filtered using Universal expression Codes filtering (UPC) with a 50% cut-off, corresponding to a 50% likelihood that a gene is expressed [16]. Significant differences of individual genes were tested using the limma R library [17]. At fasting, the expression of genes between groups was defined as different when P was < 0.05 in a t-test with empirical Bayes correction. Gene expression was defined as postprandial changed between T0 and T4 h when the P was< 0.05 in a paired t-test with empirical Bayesian correction. Data were further analysed with gene set enrichment analysis (GSEA) using pre-ranked lists based on the t-statistic [18]. Gene sets with a false discovery rate (FDR Q<0.2) were defined as significantly regulated. Plots were made using the R libraries ggplot2 and gplots [19, 20].

Plasma metabolic profiling

Amino acids and biogenic amines were derivatized (Acc-Tag) in 5 µL aliquots of plasma and analyzed using an ACQUITY UPLC system equipped with autosampler (Waters, Etten-Leur, The Netherlands) and coupled to a Xevo Tandem quadrupole mass spectrometer (Waters) operated using QuanLynx data acquisition software (version 4.1; Waters). An Accq-Tag Ultra column (Waters) was used. The Xevo TQ was used in the positive-ion electrospray mode and all analytes were monitored in Multiple Reaction Monitoring (MRM) using nominal mass resolution. Acquired data were evaluated using TargetLynx software (Waters), by integration of assigned MRM peaks and normalization using proper internal standards [21]. Acylcarnitines, trimethylamine-N-oxide, choline, betaine, deoxycarnitine and carnitine were analyzed in 5 µL plasma, spiked with an internal standard, using a UPLC-MS/MS. Also here an Accq-Tag Ultra column was used. The Xevo TQ was used in the positive-ion electrospray mode and all analytes were monitored in Multiple Reaction Monitoring (MRM) using nominal mass resolution. In-house developed algorithms[22] were applied using the pooled QC samples to compensate for shifts in the sensitivity of the mass spectrometer over the batch. Organic acids were measured by GC-MS using 50 µL of plasma sample prepared using a two-step derivatization procedure with subsequent oximation using methoxyamine hydrochloride (MeOX) and silylation using N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA. Samples were measured on an Agilent GC (7890A) coupled to Agilent Quadrupole-MS with EI source (Agilent MSD 5975C). Separation was performed using a HP-5MS column (30 m x 0.25 m x 0.25 µm; Agilent). The raw

data were pre-processed using Agilent MassHunter Quantitative Analysis software for GC-MS (Agilent, Version B.04.00), and quantitation of metabolite response was calculated as the peak area ratios of the target analyte to the respective internal standard. Oxylipins were analyzed as previously described [23]. In short, compound extraction was performed with SPE using a hydrophilic-lipophilic balance and samples were analyzed by LC using a C18 column coupled to ESI on a triple quadrupole mass spectrometer, where oxylipins were detected in negative ion mode using dynamic SRM. while chromatographic separation was achieved with a C18 column. Peak areas of target metabolites were corrected by appropriate internal standards and calculated response ratios were used throughout the analysis. In-house developed algorithms were applied using the pooled QC samples to compensate for shifts in the sensitivity of the mass spectrometer over the batch. Serum metabolite concentrations determined by NMR were measured as described by Mihaleva *et al.* [24]. Briefly, high resolution ^{1}H NMR spectra were acquired on ultrafiltrated serum samples at 300K using a Bruker Avance III 600 MHz spectrometer. Automated quantum mechanical line shape fitting of the^{1}H NMR spectra was performed using PERCH NMR software to obtain absolute concentrations of 44 metabolites.

Metabolomics Statistical analysis

We used Spearman rank correlations to correlate plasma metabolite concentrations and phenotypical parameters (LBM, HOMA). These correlations were also determined for nadir and Δ-nadir acylcarnitine values [25]. Nadir acylcarnitine values were defined as the lowest value achieved during the 4 hours after the meal. Δ-nadir was calculated as difference between nadir and T0 values. The postprandial response was considered as the incremental area under the curve (iAUC) [26]. For the iAUC calculation, the values at fasting (T0) were subtracted from the total AUC. Comparison between abdominally obese and lean group was performed using analysis of variance (ANOVA). To evaluate the intervention effect on fasting metabolites (T0) and the intervention effect on postprandial response (iAUC) in abdominally obese subjects, statistical analysis was performed using linear mixed models. A $P<0.05$ was considered to be statistically significant.

Local false discovery rate (lFDR) was used to correct for multiple testing [27-29]. All calculations were performed under R (version 3.2.1).

RESULTS

Metabolic differences between lean and abdominally obese subjects

In order to have a benchmark for the comparison of abdominally obese subjects before and after weight loss, we first considered the difference between lean and abdominally obese subjects (Figure 1). In the fasted state, 19 plasma metabolites were significantly different between abdominally obese subjects and lean subjects (Table 2), including various amino acids, a fatty acid derived acylcarnitine (FAAC), and metabolites related to the TCA cycle. For a number of oxylipins we observed significantly lower plasma levels in the abdominally obese subjects compared to the lean subjects. Upon the mixed meal challenge, four types of postprandial metabolic responses could be recognized, i.e. metabolite changes related to glucose metabolism, amino acid metabolism, lipid metabolism, and ketogenesis [30]. Figure 2 presents typical postprandial curves for these response types. Both lean and abdominally obese subjects followed similar responses over time. Metabolites for which the longitudinal response, expressed as iAUC, significantly ($P<0.05$) differed between abdominally obese and lean subjects are presented in Table 2. The 8 metabolites that were different between lean and abdominally obese included a number of amino acids, but not branched chain amino acids. Interestingly, 2-hydroxyisovalerate, a catabolite of branched chain amino acids was also significantly different.

In the fasted state, 1862 genes were differently expressed ($P< 0.05$) in PBMCs between lean and abdominally obese subjects. The mixed meal challenge changed the expression of 1305 genes in abdominally obese and 1707 genes in lean subjects. The response to the meal challenge of 1537 genes differed between lean and abdominally obese (Δ abdominally obese T4-T0 vs. Δ lean T4-T0). A total of 359 genes were both differentially expressed between abdominally obese and lean after fasting and differed between abdominally obese and lean subjects in response to the mixed meal challenge (Figure S1). In the fasting samples, GSEA identified gene sets that were enriched in abdominally obese compared to lean subjects (FDR <0.2), which mainly belonged to oxidative phosphorylation and the electron transport chain. Gene sets involved in immune regulation were enriched in lean compared to abdominally obese subjects (Supplementary Table S1). In the postprandial state, genes related to immune pathways and glucose metabolism were enriched in abdominally obese as compared to lean (Δ abdominally obese T4-T0 vs. Δ lean T4-T0, Supplementary Table S2). In total, in the fasting state 9 gene sets were positively enriched and 9 gene sets were negatively enriched in abdominally obese compared to lean subjects (FDR<0.2). In the postprandial state, 114 gene sets were upregulated in abdominally obese subjects compared to lean subjects, while 15 gene sets were downregulated.

Impact of weight loss on fasting metabolism in abdominally obese subjects

All subjects who received the low-calorie diet significantly lost weight ($P<0.05$) (Table 1). To establish whether

weight loss has any effect on the fasting levels of metabolites in abdominally obese subjects, Δ values were calculated per metabolite per subject (T0 after –T0 before). Table 3 shows the three metabolites that were different before and after the weight loss intervention in abdominally obese subjects in the fasted state. We also assessed the connection between weight loss and ratios between acetylcarnitine (C_2) and longer chain (C_n) acylcarnitines. These (C_2/C_n) acylcarnitine ratios have previously been suggested as read-outs for lipid β-oxidation and indeed significantly correlated with HOMA (Supplementary Material, Table S3). Although these correlations suggest that acylcarnitine ratios partly reflect insulin resistance, we could not find any significant effect of weight loss on these acylcarnitine ratios, whereas we did observe an improvement in the HOMA index [15].

We next determined the effect of weight loss on gene expression in PBMCs in the fasted state. Weight loss changed the expression of 835 genes in the abdominally obese subjects (T0 after weight loss vs. T0 before weight loss, Figure S2). GSEA identified a number of differently enriched (FDR <0.04) pathways, including respiratory electron transport, and oxidative phosphorylation, which were negatively enriched after weight loss in abdominally obese subjects. Gene sets related to immune regulation and insulin signalling were positively enriched after weight loss (Supplementary Table S4). After weight loss, genes in oxidative phosphorylation and to a lesser extent in carbohydrate metabolism showed expression levels that were closer to expression levels in lean subjects (Figure 3).

Impact of weight loss on postprandial metabolic response in abdominally obese subjects

The weight loss intervention had a subtle effect on the postprandial metabolic response in plasma, of which several examples are presented in Figure 2. For 11 metabolites the postprandial response as expressed as incremental area under the curve (iAUC) was significantly different. For a major part these metabolites consisted of oxylipins derived from enzymatic oxidation of arachidonic acid (Table 3). We also observed shifts in the response to the mixed-meal challenge after the control intervention (Supplementary Table S5).

To identify differences in PBMC gene expression in response to the mixed-meal challenge before and after weight loss, differences were calculated as Δ values in both groups (T4-T0, D2 vs. T4-T0, D1). We identified 384 genes that showed a different postprandial response after the weight-loss intervention as compared to the response before weight loss. Furthermore, we identified 226 genes that showed a different postprandial response after the control inter-

vention (Figure S2). GSEA showed that oxidative phosphorylation and the electron transport chain were the main affected pathways. Before the intervention oxidative phosphorylation and the electron transport chain were downregulated upon the mixed-meal challenge, whereas after the weight loss intervention oxidative phosphorylation and the electron transport chain were upregulated (Figure 3). In contrast, gene sets related to the immune system and carbohydrate metabolism showed the opposite pattern with down-regulation upon a mixed-meal challenge before the intervention and a dampened response or even up-regulation upon a mixed-meal challenge after the intervention (Figure 3, Supplementary Table S6).

In short, GSEA showed that at fasting, 161 gene sets were upregulated and 120 gene sets were down regulated (FDR< 0.2) in the WL abdominally obese group (Supplementary Table S4), while in the abdominally obese control group 219 gene sets were upregulated and 3 gene sets were downregulated (data not shown). GSEA showed that upon mixed meal challenge, 6 gene sets were upregulated and95 gene sets were down regulated (FDR< 0.2) in WL abdominally obese group (Supplementary Table S6), while in CTRL abdominally obese group only 45 gene sets were downregulated (data not shown).

DISCUSSION

In this study, we examined whether a standardized mixed-meal challenge could reveal a weight loss-induced shift from the abdominally obese phenotype towards a lean one. We were particularly interested in finding out whether measurement of the response to a dietary challenge would reveal additional changes in metabolic phenotype compared to measurements in the fasting state.

Metabolic differences between lean and abdominally obese

The observed differences in the fasting levels of BCAAs reflect differences in metabolic homeostasis between lean and abdominally obese and are in line with previous observations [31]. The shift in fasting levels of these metabolites can be explained by their positive correlation with insulin resistance, which was significantly different for the lean and abdominally obese subjects [31, 32]. We could however not confirm previously observed differences in metabolites related to the TCA cycle between the abdominally obese and lean subjects [31]. For a range of oxylipins significantly lower plasma levels were observed in abdominally obese subjects relative to the lean group, which we attribute to a decrease in enzymatic lipid oxidation.

Recently, ratios between C_2/C_n acylcarnitines were brought forward as putative read-outs for β-oxidation rate [7]. Although these ratios correlated with HOMA (Supplementary Table

S3), we could not find a difference of C_2/C_n acylcarnitine ratios between the lean and abdominally obese groups, even though insulin sensitivity differed between the two groups. This suggests that while these ratios are correlated with insulin sensitivity, they do not provide an increased sensitivity compared to classical markers.

The lean and abdominally obese subjects also differed in their postprandial mixed meal response. The individual variation, however, was rather large, as shown in Figure 2. An elaborate interpretation of the differences in response between lean and abdominally obese is given in the Supplementary Material. Briefly, we could reproduce previously observed differences in the postprandial responses of amino acids between lean and abdominally obese [33]. We could, however, not reproduce earlier observations showing differences in the postprandial responses of free fatty acids between lean and abdominally obese. We attributed this to dietary intake of fatty acids during the postprandial phase. We also did not observe differences in postprandial response of fatty acid derived acylcarnitines and hence could not confirm earlier observations that lean and abdominally obese differ in β-oxidation of fatty acids [33]. Also no differences in postprandial responses of BCAAs between lean and abdominally obese could be discerned, very likely also due to direct dietary intake of amino acids. We did however observe a striking difference for a BCAA transamination product, i.e. 2-hydroxyisovalerate ($P< 0.05$). This metabolite require the branched chain amino transferase (BCAT) and keto acid dehydrogenase (BCKDH) complex for further catabolism to propionic acid. The function of the BCKDH complex is known to be compromised in insulin resistant subjects, leading to increased fasting levels of BCAA [33]. Compromised BCKDH function should also increase postprandial accumulation of other upstream metabolites such as 2-hydroxyisovalerate. This is indeed what was observed in the abdominally obese relative to lean subjects [34]. We also observed difference for a range of oxylipins after a mixed meal challenge in lean and abdominally obese subjects. A recent study did however not show differences in postprandial response of oxylipins between lean and abdominally obese subjects upon high fat challenges [35]. This indicates that the postprandial response of oxylipins is not only determined by the lean-abdominally obese phenotype but also depends on the composition of the meal challenge.

Although the lean and abdominally obese showed several distinct differences in the postprandial metabolic response, these differences were less pronounced when compared to a previous study using an OGTT challenge [36]. That study showed blunted responses of BCAAs and FFAs, in contrast to our study. We attribute this to dietary intake of these metabolites since our mixed meal challenge was quite rich in protein and fatty acids. This likely shrouds observation of differences in postprandial response due to phenotypical differences.

Gene sets related to oxidative phosphorylation were higher expressed in abdominally obese in comparison to lean subjects at baseline in the fasting state. This is consistent with

a previous study that found that dietary background can affect PBMC gene expression. Consumption of monounsaturated fatty acids and Mediterranean diet compared with the saturated fatty acid diet decreased the expression of genes involved in oxidative phosphorylation in abdominally overweight males and females [13]. Differences were also found in gene sets related to the immune system. These gene sets were lower expressed in abdominally obese when compared to lean subjects. However, these effects were relatively small, yet consistent with a previous study that also showed rather small differences in a number of immune related gene sets that showed lower expression in abdominally obese subjects when compared to lean subjects [12].

In contrast to the fasting state, we observed that in the postprandial state expression of genes related to immune pathways was increased in abdominally obese relative to lean subjects. In addition, we observed larger changes in expression of gene sets involved in carbohydrate metabolism in the abdominally obese, which could be due to differences in insulin sensitivity and the activity of energy metabolism pathways. The observed differences in PBMC gene expression between abdominally obese and lean subjects at baseline and after the mixed meal challenge point towards a difference in haemostasis and immune function between the two groups.

Based on the number of genes differently expressed between lean and abdominally obese we can conclude that our mixed meal challenge was not able to magnify differences in transcriptional response in PBMC between lean and abdominally obese when compared to the fasting state (fasting: 1862 genes and postprandial: 1537 genes). This is in contrast to a study by Esser *et al.* demonstrating that an OLTT challenge with 95g of fat high in SFA or MUFA increased the number of genes significantly differentially expressed between lean and abdominally obese subjects. This study also showed a higher number of changed genes in abdominally obese relative to lean subjects when comparing the MUFA and the SFA challenges. The authors hypothesised that a MUFA challenge is more potent in inducing transcriptional differences between lean and abdominally obese in PBMCs than a SFA challenge. Our mixed meal challenge was higher in carbohydrate and protein and lower in fat content. These fats were also predominantly saturated, which may explain the weaker gene expression response to our mixed meal challenge. This weaker response can also be explained by large variety in response induced by the absorption a complex mixture of nutrients.

Effect of weight loss on fasting metabolism in abdominally obese subjects

The effect of weight loss on fasting metabolism of abdominally obese subjects only involved two amino acids and creatinine. This stands in contrast to the previously observed changes in baseline levels of BCAAs, AAACs, carnitine and metabolites of the TCA cycle [37, 38]. It is also in contrast to the observed improvement of insulin sensitivity upon weight loss

in our study population, indicating that the abdominally obese phenotype partially reversed towards a lean one [31, 32]. Furthermore, we did not observe an effect of weight loss on C_2/C_n acylcarnitine ratios.

In our study, weight loss affected the expression of 835 genes in the fasting state. Weight loss resulted in lower expression of genes involved in oxidative phosphorylation PBMCs at baseline. These genes also show lower expression levels in lean compared to abdominally obese individuals (Figure 3). Furthermore, baseline expression of genes involved in carbohydrate metabolism is different between lean and abdominally obese subject. However, the expression levels of these genes do not seem to change towards the expression levels observed in the lean upon weight loss. Moreover, gene sets involved in immune regulation were higher expressed after weight loss, while these gene sets were enriched in lean subjects compared to abdominally obese subjects before the intervention. A possible explanation for the observed differences in expressions of immune-related genes is a shift in cell population occurring during the study. Unfortunately, differentiated blood cell counts were not performed in the current study so we cannot confirm this. Therefore, we cannot conclude that the observed differences are due to a difference in immune status between lean and abdominally obese.

We also observed significant differences in the control group, which was not expected since our control group matched in BMI and waist circumference at the start of the intervention and the control group did not lose any weight. These differences suggest a seasonal effect; however, due to the design of the study this explanation is unlikely. Our remaining explanation is that the participants in the control group changed their behaviour during the course of the study.

Effect of weight loss on postprandial metabolic response in abdominally obese subjects

The predominant effect of the weight loss intervention on the postprandial response in abdominally obese subjects involved 3 amino acids, 5 oxylipins, choline, creatine and glucose. Several differences in the postprandial response that were observed between lean and abdominally obese subjects, however, were not observed after weight loss in the abdominally obese. Lower glucose levels after weight loss suggests that the mixed meal challenge stimulates a more prolonged insulin response, which is in line with previous observations [6]. In our study the weight loss intervention induced a small number of shifts in fasting metabolite level; the impact of the mixed meal on the postprandial metabolic response involved more metabolites but was still subtle. This is in line with Geidenstam et al. who also found only a modest number of differences in the postprandial metabolic response upon OGTT, mostly branched and aromatic acids, after weight loss and a maintenance period in abdominally

obese subjects. The authors attribute this to the heterogeneity in the postprandial response of abdominally obese to an OGTT challenge. The heterogeneity in postprandial response to a mixed meal most likely also explains why also in our study only modest postprandial effects of weight loss were observed. In contrast, a recent study by Kardinaal *et al.* [39], suggested that a change in challenge response is a more sensitive biomarker of metabolic resilience than changes in fasting metabolism. This discrepancy might be due to either their particular study population, which consisted of a homogeneous group of males with metabolic syndrome, whereas within our study the subjects were a more heterogeneous group of healthy abdominally obese. The study from Kardinaal *et al.* also used a high fat challenge, which may provoke a more targeted postprandial response as the mixed meal used in our study. In our study the strong and rapid direct uptake of amino acids and fatty acids from the mixed meal may have shrouded observation of postprandial metabolic differences. Therefore the recent recommendation of a mixed meal as the optimal challenge for demonstrating subtle improvements in metabolic flexibility has been expounded with regard to the composition of such a challenge [6].

Genes related to carbohydrate metabolism were downregulated in abdominally obese subjects upon a mixed meal challenge after weight loss compared to before the weight loss intervention. Figure 3 shows that the reduced response of genes in the carbohydrate metabolism to the challenge results in expression levels that are more comparable to expression levels observed in lean subjects. As insulin sensitivity is also improved after weight loss the attenuation of the expression of genes in carbohydrate metabolism seems consistent with this improvement in insulin sensitivity. Similarly, genes related to inflammatory pathways were also showed a dampened response in the abdominally obese after weight loss, which is also more in line with the response observed in lean subjects. Overall, we conclude that the post-prandial responses to weight loss seem to shift towards the lean response after weight loss with regard to carbohydrate metabolism and the inflammatory response. Although the observed shift suggest that subjects after weight loss have a more lean phenotype, they do not reach the level of lean subjects, which is in line with the actual weight loss. Furthermore, at the end of the study the subjects were maintaining weight. This may result in a less pronounced response when comparing to studies in which subjects were still losing weight at the time of measurement.

CONCLUSION

Our results are in line with recent observations that the metabolic phenotype of abdominally obese and lean subjects is different with respect to both the plasma metabolome and PBMC gene expression in the fasting state. The difference in phenotypic flexibility between

lean and abdominally obese was also reflected in the mixed meal postprandial response of the plasma metabolome and PBMC gene expression. The difference in response of a downstream metabolite of a branched chain amino acid suggests compromised function of the BCKDH complex in the abdominally obese. The metabolites and genes that differ at baseline show minor overlap with those that are differ in their postprandial response. This indicates that the mechanisms accounting for the observed differences between lean and abdominally obese in the fasted state are different from the mechanisms underlying the differences during the postprandial mixed meal response.

Compared to the difference in lean-abdominally obese metabolic phenotype, weight loss had a small effect on the fasting plasma metabolome. Also the effect of weight loss on baseline gene expression in PBMCs was smaller; the main effect was a shift of genes related to oxidative phosphorylation towards the lean phenotype. The impact of weight loss on the mixed meal postprandial response of plasma metabolites and PBMC gene expression was modest and mainly point to altered enzymatic lipid oxidation and carbohydrate metabolism upon weight loss. The weight loss induced shifts of baseline and postprandial response of plasma metabolome and PBMC gene expression showed little overlap with the differences between lean-abdominally obese metabolic phenotype.

The modest number of significant differences in postprandial metabolic responses between lean-abdominally obese and abdominally obese before and after weight loss may be explained by the complex composition of the mixed meal. Hence, the composition of meal challenges should be considered carefully in order to provoke a distinct postprandial metabolic response.

ACKNOWLEDGEMENT

This research was financially supported by Top Institute Food and Nutrition, Wageningen, The Netherlands, was part of the research program of the Netherland Metabolomics Centre (NMC), part of the Netherlands Genomics Initiative (NGI). TI Food and Nutrition is a public-private partnership on precompetitive research in food and nutrition. We thank Amy Harms (Demonstration and Competency Laboratory, NMC Leiden) for providing metabolomics profiles of high quality and Jenny Jansen (Nutrition, Metabolism and Genomics group, Wageningen University) for performing microarray analyses.

REFERENCES

1. Huber M, Knottnerus JA, Green L, Horst Hvd, Jadad AR, Kromhout D, Leonard B, Lorig K, Loureiro MI, Meer JWMvd, et al: **How should we define health?** *BMJ* 2011, **343**.
2. van Ommen B, Keijer J, Heil SG, Kaput J: **Challenging homeostasis to define biomarkers for nutrition related health.** *Mol Nutr Food Res* 2009, **53:**795-804.
3. van Ommen B, van der Greef J, Ordovas JM, Daniel H: **Phenotypic flexibility as key factor in the human nutrition and health relationship.** *Genes Nutr* 2014, **9:**423.
4. Corpeleijn E, Saris WH, Blaak EE: **Metabolic flexibility in the development of insulin resistance and type 2 diabetes: effects of lifestyle.** *Obes Rev* 2009, **10:**178-193.
5. Ho JE, Larson MG, Vasan RS, Ghorbani A, Cheng S, Rhee EP, Florez JC, Clish CB, Gerszten RE, Wang TJ: **Metabolite profiles during oral glucose challenge.** *Diabetes* 2013, **62:**2689-2698.
6. Stroeve JH, van Wietmarschen H, Kremer BH, van Ommen B, Wopereis S: **Phenotypic flexibility as a measure of health: the optimal nutritional stress response test.** *Genes Nutr* 2015, **10:**459.
7. Krug S, Kastenmuller G, Stuckler F, Rist MJ, Skurk T, Sailer M, Raffler J, Romisch-Margl W, Adamski J, Prehn C, et al: **The dynamic range of the human metabolome revealed by challenges.** *Faseb j* 2012, **26:**2607-2619.
8. Afman L, Milenkovic D, Roche HM: **Nutritional aspects of metabolic inflammation in relation to health--insights from transcriptomic biomarkers in PBMC of fatty acids and polyphenols.** *Mol Nutr Food Res* 2014, **58:**1708-1720.
9. Crujeiras AB, Parra D, Goyenechea E, Martinez JA: **Sirtuin gene expression in human mononuclear cells is modulated by caloric restriction.** *Eur J Clin Invest* 2008, **38:**672-678.
10. Crujeiras AB, Parra D, Milagro FI, Goyenechea E, Larrarte E, Margareto J, Martinez JA: **Differential expression of oxidative stress and inflammation related genes in peripheral blood mononuclear cells in response to a low-calorie diet: a nutrigenomics study.** *Omics* 2008, **12:**251-261.
11. Sheu WH, Chang TM, Lee WJ, Ou HC, Wu CM, Tseng LN, Lang HF, Wu CS, Wan CJ, Lee IT: **Effect of weight loss on proinflammatory state of mononuclear cells in obese women.** *Obesity (Silver Spring)* 2008, **16:**1033-1038.
12. Esser D, van Dijk SJ, Oosterink E, Lopez S, Muller M, Afman LA: **High fat challenges with different fatty acids affect distinct atherogenic gene expression pathways in immune cells from lean and obese subjects.** *Mol Nutr Food Res* 2015, **59:**1563-1572.

13. van Dijk SJ, Feskens EJ, Bos MB, de Groot LC, de Vries JH, Muller M, Afman LA: **Consumption of a high monounsaturated fat diet reduces oxidative phosphorylation gene expression in peripheral blood mononuclear cells of overweight men and women.** *J Nutr* 2012, **142:**1219-1225.
14. de Mello VD, Kolehmanien M, Schwab U, Pulkkinen L, Uusitupa M: **Gene expression of peripheral blood mononuclear cells as a tool in dietary intervention studies: What do we know so far?** *Mol Nutr Food Res* 2012, **56:**1160-1172.
15. Joris PJ, Plat J, Kusters YH, Houben AJ, Stehouwer CD, Schalkwijk CG, Mensink RP: **Diet-induced weight loss improves not only cardiometabolic risk markers but also markers of vascular function: a randomized controlled trial in abdominally obese men.** *Am J Clin Nutr* 2016.
16. Piccolo SR, Sun Y, Campbell JD, Lenburg ME, Bild AH, Johnson WE: **A single-sample microarray normalization method to facilitate personalized-medicine workflows.** *Genomics* 2012, **100:**337-344.
17. Smyth GK: **Limma: linear models for microarray data.** In *Bioinformatics and computational biology solutions using R and Bioconductor*. Springer; 2005: 397-420
18. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP: **Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles.** *Proc Natl Acad Sci U S A* 2005, **102:**15545-15550.
19. Wickham H: *ggplot2: elegant graphics for data analysis.* Springer; 2009.
20. Warnes GR, Bolker B, Bonebakker L, Gentleman R, Huber W, Liaw A, Lumley T, Maechler M, Magnusson A, Moeller S: **gplots: Various R programming tools for plotting data.** *R package version* 2009, **2**.
21. Noga MJ, Dane A, Shi S, Attali A, van Aken H, Suidgeest E, Tuinstra T, Muilwijk B, Coulier L, Luider T, et al: **Metabolomics of cerebrospinal fluid reveals changes in the central nervous system metabolism in a rat model of multiple sclerosis.** *Metabolomics* 2012, **8:**253-263.
22. van der Kloet FM, Bobeldijk I, Verheij ER, Jellema RH: **Analytical error reduction using single point calibration for accurate and precise metabolomic phenotyping.** *J Proteome Res* 2009, **8:**5132-5141.
23. Strassburg K, Huijbrechts AM, Kortekaas KA, Lindeman JH, Pedersen TL, Dane A, Berger R, Brenkman A, Hankemeier T, van Duynhoven J, et al: **Quantitative profiling of oxylipins through comprehensive LC-MS/MS analysis: application in cardiac surgery.** *Anal Bioanal Chem* 2012, **404:**1413-1426.

24. Mihaleva VV, Korhonen SP, van Duynhoven J, Niemitz M, Vervoort J, Jacobs DM: **Automated quantum mechanical total line shape fitting model for quantitative NMR-based profiling of human serum metabolites.** *Anal Bioanal Chem* 2014, **406:**3091-3102.
25. Ramos-Roman MA, Sweetman L, Valdez MJ, Parks EJ: **Postprandial changes in plasma acylcarnitine concentrations as markers of fatty acid flux in overweight and obesity.** *Metabolism* 2012, **61:**202-212.
26. Carstensen M, Thomsen C, Hermansen K: **Incremental area under response curve more accurately describes the triglyceride response to an oral fat load in both healthy and type 2 diabetic subjects.** *Metabolism* 2003, **52:**1034-1037.
27. Strimmer K: **fdrtool: a versatile R package for estimating local and tail area-based false discovery rates.** *Bioinformatics* 2008, **24:**1461-1462.
28. Strimmer K: **A unified approach to false discovery rate estimation.** *BMC Bioinformatics* 2008, **9:**303.
29. Box GEP, Jenkins, G.M., and Reinsel G.C. : **Time Series Analysis: Forecasting and Control.** 1994.
30. Shaham O, Wei R, Wang TJ, Ricciardi C, Lewis GD, Vasan RS, Carr SA, Thadhani R, Gerszten RE, Mootha VK: **Metabolic profiling of the human response to a glucose challenge reveals distinct axes of insulin sensitivity.** *Mol Syst Biol* 2008, **4:**214.
31. Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF, Haqq AM, Shah SH, Arlotto M, Slentz CA, et al: **A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance.** *Cell Metab* 2009, **9:**311-326.
32. Newgard CB: **Interplay between lipids and branched-chain amino acids in development of insulin resistance.** *Cell Metab* 2012, **15:**606-614.
33. Badoud F, Lam KP, Perreault M, Zulyniak MA, Britz-McKibbin P, Mutch DM: **Metabolomics Reveals Metabolically Healthy and Unhealthy obese Individuals Differ in their Response to a Caloric Challenge.** *PLoS One* 2015, **10:**e0134613.
34. Podebrad F, Heil M, Reichert S, Mosandl A, Sewell AC, Bohles H: **4,5-dimethyl-3-hydroxy-2[5H]-furanone (sotolone)--the odour of maple syrup urine disease.** *J Inherit Metab Dis* 1999, **22:**107-114.
35. Strassburg K, Esser D, Vreeken RJ, Hankemeier T, Müller M, van Duynhoven J, van Golde J, van Dijk SJ, Afman LA, Jacobs DM: **Postprandial fatty acid specific changes in circulating oxylipins in lean and aobese men after high-fat challenge tests.** *Molecular Nutrition & Food Research* 2014, **58:**591-600.

36. Geidenstam N, Spegel P, Mulder H, Filipsson K, Ridderstrale M, Danielsson AP: **Metabolite profile deviations in an oral glucose tolerance test-a comparison between lean and obese individuals.** *Obesity (Silver Spring)* 2014, **22:**2388-2395.
37. Shah SH, Crosslin DR, Haynes CS, Nelson S, Turer CB, Stevens RD, Muehlbauer MJ, Wenner BR, Bain JR, Laferrere B, et al: **Branched-chain amino acid levels are associated with improvement in insulin resistance with weight loss.** *Diabetologia* 2012, **55:**321-330.
38. Wahl S, Vogt S, Stuckler F, Krumsiek J, Bartel J, Kacprowski T, Schramm K, Carstensen M, Rathmann W, Roden M, et al: **Multi-omic signature of body weight change: results from a population-based cohort study.** *BMC Med* 2015, **13:**48.
39. Kardinaal AF, van Erk MJ, Dutman AE, Stroeve JH, van de Steeg E, Bijlsma S, Kooistra T, van Ommen B, Wopereis S: **Quantifying phenotypic flexibility as the response to a high-fat challenge test in different states of metabolic health.** *Faseb j* 2015, **29:**4600-4613.
40. Wang TJ, Ngo D, Psychogios N, Dejam A, Larson MG, Vasan RS, Ghorbani A, O'Sullivan J, Cheng S, Rhee EP, et al: **2-Aminoadipic acid is a biomarker for diabetes risk.** *J Clin Invest* 2013, **123:**4309-4317.
41. Pellis L, van Erk MJ, van Ommen B, Bakker GC, Hendriks HF, Cnubben NH, Kleemann R, van Someren EP, Bobeldijk I, Rubingh CM, Wopereis S: **Plasma metabolomics and proteomics profiling after a postprandial challenge reveal subtle diet effects on human metabolic status.** *Metabolomics* 2012, **8:**347-359.
42. Saccenti E, Hoefsloot HJ, Smilde A, Westerhuis J, Hendriks MWB: **Reflections on univariate and multivariate analysis of metabolomics data.** *Metabolomics* 2014, **10:**361-374.
43. Shrestha A, Mullner E, Poutanen K, Mykkanen H, Moazzami AA: **Metabolic changes in serum metabolome in response to a meal.** *Eur J Nutr* 2015.
44. Matthews DE, Marano MA, Campbell RG: **Splanchnic bed utilization of glutamine and glutamic acid in humans.** *Am J Physiol* 1993, **264:**E848-854.
45. Adeva MM, Calvino J, Souto G, Donapetry C: **Insulin resistance and the metabolism of branched-chain amino acids in humans.** *Amino Acids* 2012, **43:**171-181.
46. Elia M, Livesey G: **Effects of ingested steak and infused leucine on forelimb metabolism in man and the fate of the carbon skeletons and amino groups of branched-chain amino acids.** *Clin Sci (Lond)* 1983, **64:**517-526.

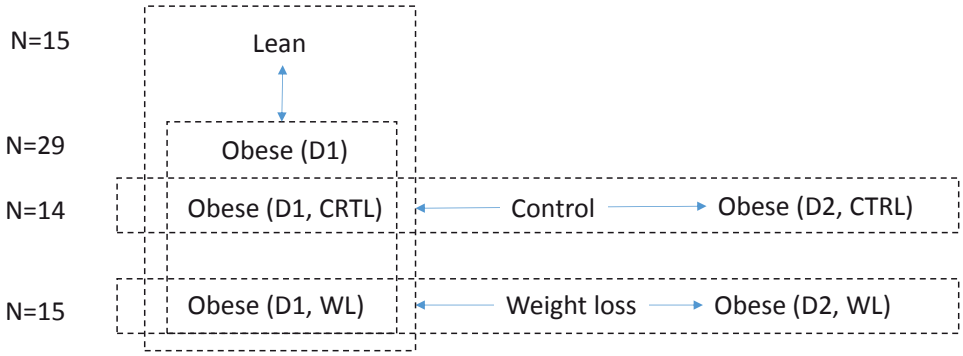


Figure 1. Schematic overview of the study design. D1, D2: before and after intervention, respectively. WL: weight loss intervention group, CTRL: control group. The arrows indicate the phenotype comparisons made in this study (lean vs abdominally obese, before and after interventions.

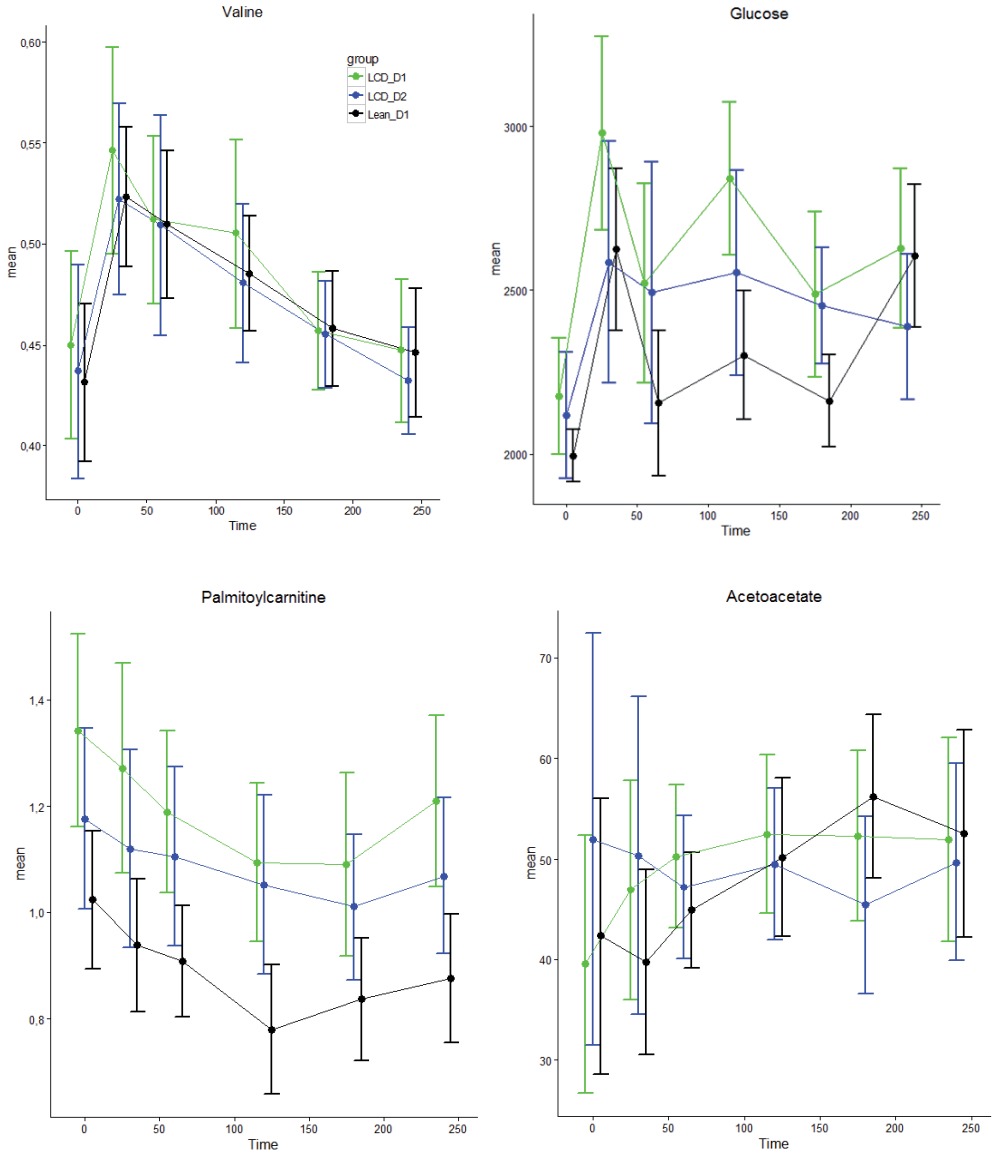


Figure 2. Postprandial mixed meal response curves of, valine, glucose, palmitoylcarnitine and acetoacetate, as representative metabolites from glycolysis, amino acid metabolism, lipolysis and ketogenesis, respectively. Mean curves are presented for lean (black) and abdominally obese subjects are presented , the latter before (green) and after (blue) weight loss intervention (see Figure 1). The bars represent variation within these groups.

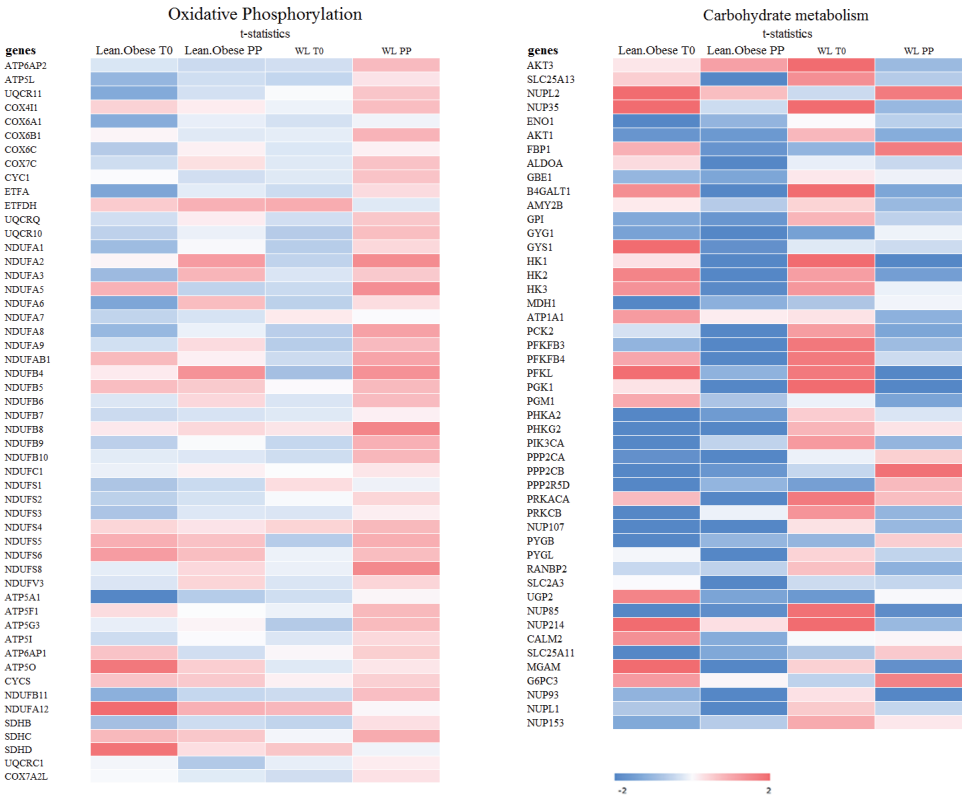


Figure 3. Overview of lean-abdominally obese differences in expression of genes related to (Left) oxidative phosphorylation and (Right) carbohydrate metabolism in PBMCs at fasting (T0) and during postprandial response (ΔLean vs. Δ Abdominally obese postprandial) to a mixed meal challenge. Also shown are effects of weight loss on gene expression in PBMCs of abdominally obese subjects at baseline (abdominally obese after weight loss vs. abdominally obese before weight loss) and during postprandial phase (Δ abdominally obese after weight loss vs. Δ abdominally obese before weight loss). The heatmap is based on moderates t-statistics for each comparison which has the same interpretation as an ordinary t-statistic except that the standard errors have been moderated across genes using a simple Bayesian model.

Table 1. Characteristics of lean subjects and abdominally obese subjects before and after weight loss or control interventions.

	Lean	WL(D1)	WL(D2)	CTRL(D1)	CTRL(D2)
Number	15	14	14	15	15
Age (y)	47.4 ±17	44 ± 15		44.8 ± 14.0	
Weight (kg)	73 ± 7.5	96.6 ± 8.6	87.3 ± 8.6*	98.9 ± 9.9	98.1 ± 9.5
Height (m)	1.8 ± 0.1	1.8 ± 0.1		1.8 ± 0.1	
BMI (kg/m^2)	23.0 ± 2.0	30.0 ± 1.8	26.9 ± 1.6*	30.7 ± 2.9	30.4 ± 2.8
Waist circumference (cm)	85.4 ± 6.7	106.8 ± 3.6	95.7 ± 4.5*	106.8 ± 3.9	106.2 ± 3.8
Hip circumference (cm)	95.9 ± 4.1	108.4 ± 4.9	102.7 ± 4.5*	109.5 ± 7.0	109.3 ± 7.8
LBM (kg)	54.2 ± 6.7	67.1 ± 6.9	61.5 ± 8.9	69.2 ± 10.4	69.8 ± 10.7
Fat mass (%)	19.0 ± 6.5	29.5 ± 4.4	25.8 ± 5.0	29.8 ± 4.3	28.3 ± 4.1
Glucose (mmol/L)	5.1 ± 2.9	5.3 ± 0.47	5.0 ± 0.33	5.3 ± 0.48	5.3 ± 0.37
Insulin (uU/mL)	7.0 ± 1.7	12.5 ± 5.5	7.8 ± 3.4*	12.0 ± 6.5	12.1 ± 5.2

Data are presented as mean ± SD. *: A significant effect of weight loss (P<0.05). WL: weight loss intervention, CTRL: control group, D1, D2: before and after intervention (see also Figure 1), BMI: Body mass index, LBM: lean body mass.

Table 2. Significant (P<0.05, lFDR< 0.2) differences between fasting metabolite concentrations (FC) and postprandial mixed meal response (iAUC) in plasma between lean and abdominally obese subjects.

	***P*(Fasting)**	**FC (Abdominally obese T0/Lean T0)**	***P*(iAUC)**
Acylcarnitines			
Palmitoylcarnitine	<0.01	1.25	
Amino acids and related metabolites			
Serine	<0.01	0.86	
Beta alanine	0.02	1.07	
Asparagine	0.02	0.9	
Creatinine	<0.01	1.21	
Isoleucine	<0.01	1.24	
Alanine	<0.01	1.18	<0.01
Tyrosine	<0.01	1.2	
Valine	0.01	1.13	
Keto-Leucine	0.01	1.13	
Carnitine	0.02	1.07	
Leucine	0.03	1.11	
Proline			<0.01
Methylmalonic acid			<0.01
Threonine			0.01
Histidine			0.01
Methionine			0.01
Phosphocholine			0.01
2.hydroxyisovalerate			0.01
Oxylipins			
5-HETE	<0.01	0.61	
12-13-EpOME	0.01	0.65	
9-HODE	0.02	0.71	
9-HOTrE	0.02	0.68	
TCA cycle and related metabolites			
Glyceric acid	<0.01	0.95	
Beta glucose	0.02	1.09	
Alpha glucose	0.02	1.08	

FC: Fold change. Differences in response were calculated on the basis of iAUC. iAUC: incremental area under the curve.

Table 3. Effect of weight loss (P<0.05, lFDR<0.2) on fasted state (T0, FC) metabolite concentrations and postprandial mixed meal challenge response (iAUC) in plasma of abdominally obese subjects.

Metabolites	*P*	FC(WL)	*P*(iAUC)
Amino acids and related metabolites			
Glycine	<0.01	1.18	
Creatinine	<0.01	0.88	
Glutamine			<0.01
Histidine			<0.01
Creatine			<0.01
Pyroglutamic acid			<0.01
Glutamic acid	<0.01	0.66	
Oxylipins			
TXB2			<0.01
PGE2			<0.01
12S.HHTrE			<0.01
5.HETE			<0.01
11.HETE			0.02
TCA cycle and related metabolites			
Glucose			0.01
Acylcarnitines			
Choline			0.01

FC: Fold change (T0, after weight loss/T0, before weight loss). Differences in postprandial response were determined on the basis of iAUC. iAUC: incremental area under the curve significant at *P*<0.05.

SUPPLEMENTARY MATERIAL

Postprandial metabolic response in lean and abdominally obese subjects

Despite the significant inter-individual variation between the time-responses, yet differentiating features can be recognized according to four dominant postprandial metabolic events [30, 41] : glycolysis, lipolysis, ketogenesis, proteolysis and amino acid oxidation

Glycolysis In lean volunteers the mixed meal provoked a prolonged insulin response, in line with previous observations [6]. As a consequence postprandial effects on glycolysis, lipolysis and ketogenesis can be expected [30]. Indeed, the initial postprandial increase of glucose was rapidly followed by a strong insulin-induced decrease. This pattern was also observed for lactate [30] and downstream TCA metabolites [41]. The postprandial response of glucose did not differ significantly between the lean and abdominally obese group (lFDR> 0.2), which was also the case for most downstream TCA metabolites, except for citrate.

Lipolysis The essential fatty acids linoleic acid (LA) and alpha linolenic acid (ALA) follow the characteristic insulin-induced non esterified fatty acid response (NEFA), which is a strong decrease followed by an increase after 1 hour (Supplementary Figure S3-a). Hence in our study mixed meal challenge did not induce the lag phase response for longer chain ($C_{>16}$) fatty acids [41] that was previously observed for another mixed meal challenge. Apparently, for mixed meal challenge in our study, insulin-induced depletion of FFAs is dominant over intake of FFAs. Also the FFA response did not differ significantly between the lean and abdominally obese group.

Within this study we assessed oxylipins since they have been associated with a range of inflammatory mechanisms. The enzymatic oxidation pathways of the oxylipins are known, but our attempt to model their postprandial response with a recently introduced first order kinetic model [42] was not successful. Only modest correlations between oxylipins and their precursors were found (Supplementary Table S7) which we attribute this to complexity of having both an insulin effect combined with dietary intake of fatty acids in the mixed meal challenge. The postprandial response of several oxylipins was however different between the lean an abdominally obese group, which suggests differences in lipid oxidation between the abdominally obese and lean groups.

Once the free fatty acids (FFAs) are destined as fuel they enter the mitochondria as acylcarnitines in order to undergo stepwise β-oxidation to acetylcarnitine which enters the TCA cycle for energy production. Plasma levels of fatty acid derived acylcarnitines (FAAC, Supplementary Table S8) reflect increased FFA availability and/or a shift in β-oxidation flux. In the lean subjects, the mixed meal challenge induced a monotonous postprandial decrease of both fatty acid derived acylcarnitines and acetylcarnitine, in line with reduced availability of

FFAs and/or reduced β-oxidation upon switching from b-oxidation to glycolysis [43]. The ratio between C_2 (acetyl) carnitine and fatty acid derived acylcarnitines has been proposed as direct readouts for intracellular β-oxidation [7]. During the postprandial phase we observe that for the C_2/FAAC ratios interindividual difference is more pronounced at fasting rather than response (Supplementary Figure S3-b). We could however not observe a difference between the abdominally obese and lean groups for these C_2/FAAC rations.

Nadir acylcarnitine levels have been proposed as a single parameter summary of their postprandial response [25]. We found correlations with phenotypical parameters such as HOMA and lean body mass (Supplementary Table S9). Although this suggests that postprandial acylcarnitine responses are related to fatty acid oxidation flux, we could not observe statistically significant differences between the lean and abdominally obese group.

Ketogenesis In lean volunteers we observe a monotonous postprandial decrease of the ketone bodies β-hydroxybutyric acid, and acetone (Supplementary Figure S3-c) which is in line with previous observations upon administration of mixed meal challenge[41]. The decrease can be explained by direct inhibition of ketogenesis by insulin and indirectly by decrease of FFA due to inhibition of lipolysis. Acetoacetate however, goes up due to enhanced postprandial amino acid flux (Figure 1). In the abdominally obese none of the ketone bodies showed a postprandial response.

Proteolysis and amino acid oxidation Upon administration of the mixed meal challenge insulin-induced inhibition of proteolysis is expected. An indication for an impact on muscle metabolism is the weak postprandial decrease in creatinine for lean subjects (Supplementary S5-d), which is in contrast to a previous observation [41]. The postprandial response in the abdominally obese did however not differ significantly from[33] the lean group.

Unfortunately, observation at the level of amino acids is obscured by direct dietary uptake upon the mixed meal challenge [33]. Nevertheless, still several amino acids differ in their postprandial response in lean vs abdominally obese, in line with a recent study [33]. In this study these effects were attributed to impairment of the TCA cycle in abdominally obese, this was confirmed by differences in postprandial response of citrate and succinate.

The mixed meal challenge induces a strong postprandial increase of BCAA which can be explained by direct uptake and transamination of other dietary amino acids in the splanchnic bed [44]. After the steep increase of BCAAs, their levels drop rapidly due to insulin-induced uptake in muscle or other tissues[45]. Within the muscle, BCAAs (leucine in particular) are rapidly transaminated to branched chain α-keto acids (BCKAs) and excess nitrogen is subsequently released as glutamine. Indeed we observed a second peak in the glutamine response curve which we attribute to BCAA's transferring their amine group (Supplementary Figure S3-e) [46]. The BCKAs are oxidized to $C_{4\text{-}5}$-CoA derivatives of CoA to finally produce acetoace-

tate, C_2(acetyl)-CoA and C_3 (proprionyl)-CoA, all of which can enter the TCA cycle [45]. Excess levels of the C_{3-5} fatty acids appear in plasma as amino acid derived acylcarnitines (AAAC, Supplementary Table S3) which can be considered as proxies of BCAA oxidation. The kinetic profile of the AAACs generally follows the BCAA profile (only propionyl and isovaleryl with a bit delay compare to BCAAs. During the postprandial phase we observe that for the BCAA/AAAC ratios inter-individual differences are more pronounced at fasting rather than for their postprandial response (Supplementary Figure S3-f). The postprandial response of the BCAAs and AAACs does not differ between lean and abdominally obese, but interestingly we observe significant differences in the response of two branched chain keto acids (α-ketoisovaleric acid, 2-hydroxyisovalerate) and methylmalonic acid (Supplementary Figure S3-g). This indicates that in the abdominally obese group oxidation of mitochondrial BCAA by the BCKDH complex might be impaired, causing temporary postprandial accumulation of these downstream BCAAs catabolites[34].

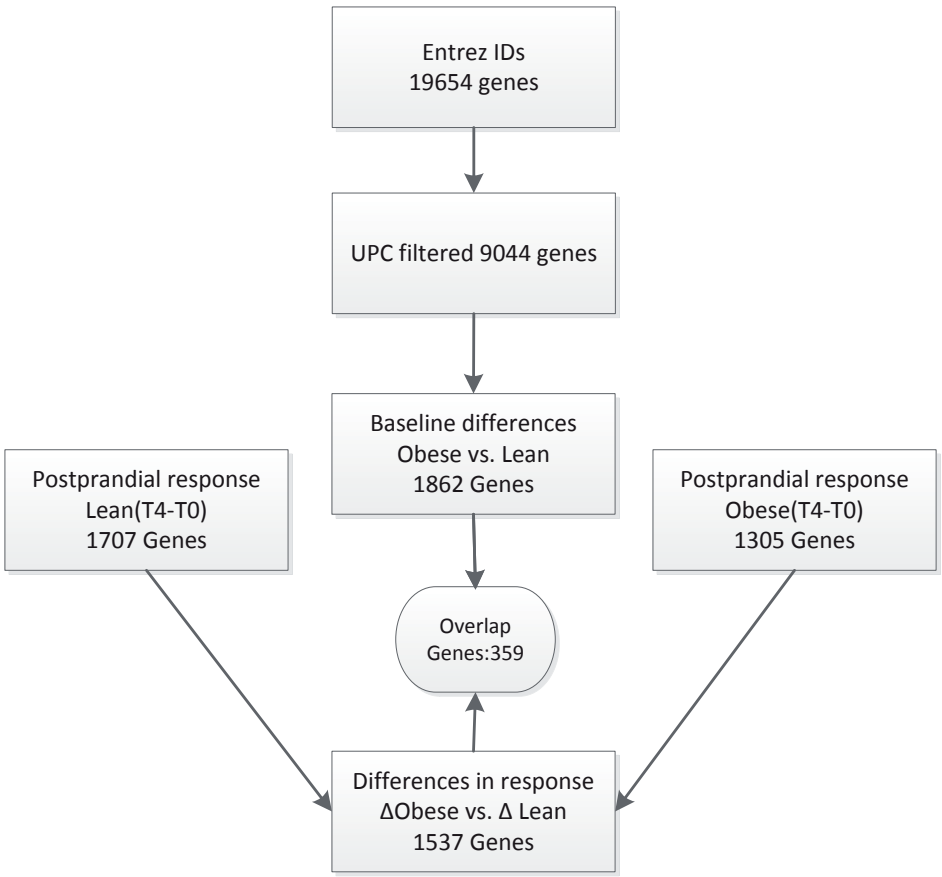


Figure S1. Flow diagram showing the number of genes of which the expression was significantly different between lean (n=15) and abdominally obese (n=29) subjects at fasting, the number of genes that changed in expression after a mixed meal challenge and the number of genes that changed differently in expression in abdominally obese relative to the lean subjects after a mixed meal challenge. A change was significant if $P < 0.05$.

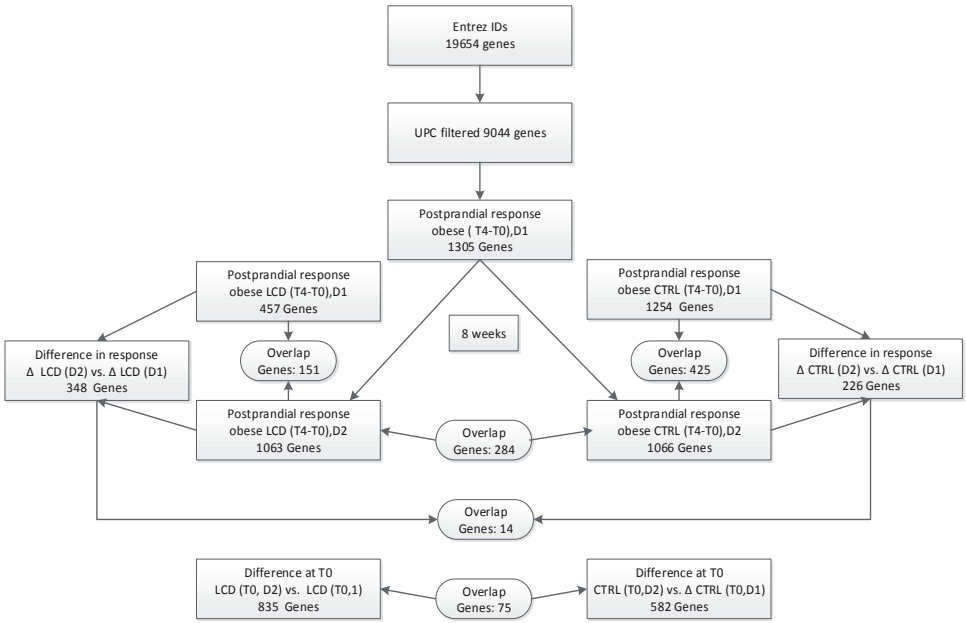
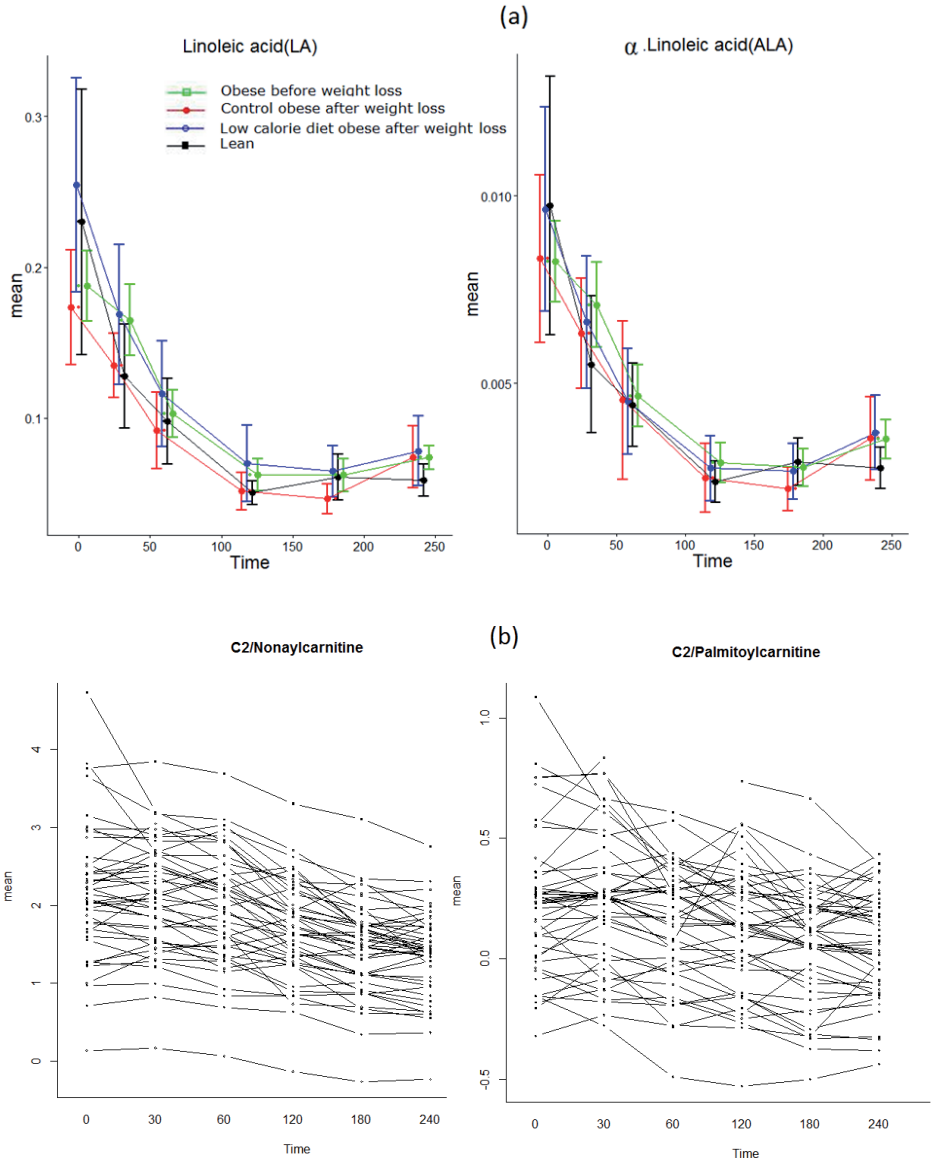
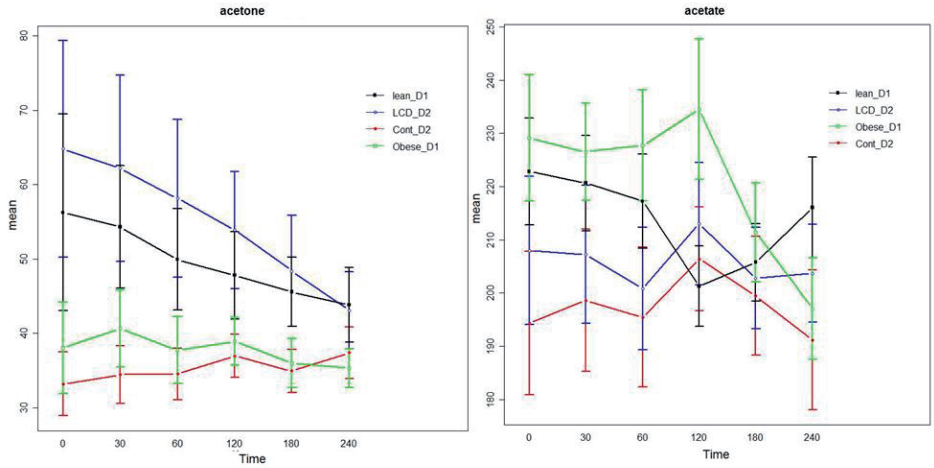
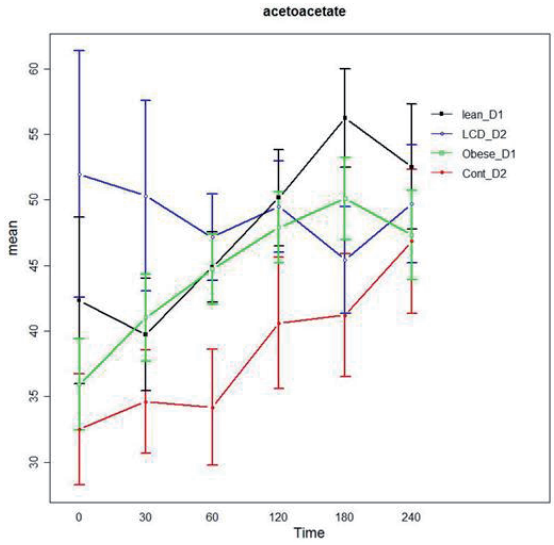


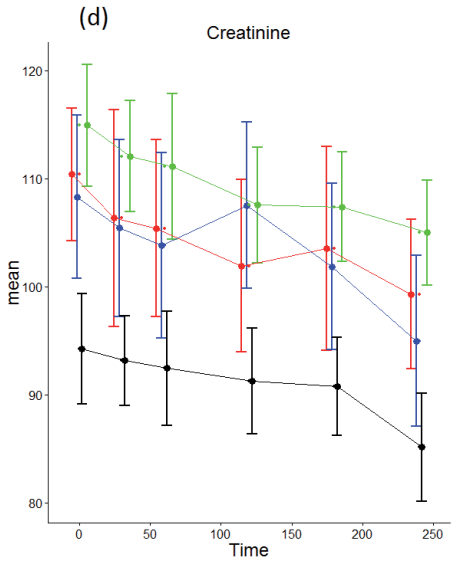
Figure S2. Flow diagram showing the number of genes of which the expression was significantly different in abdominally obese (n=29) before weight loss (D1) in response to mixed meal challenge, the number of genes that changed significantly in expression before (D1) and after (D2) intervention (WL, CRTL) in response to mixed meal challenge, the number of genes that changed significantly at T0 comparing subjects in WL or CTRL before and after intervention. A change was significant if $P < 0.05$.

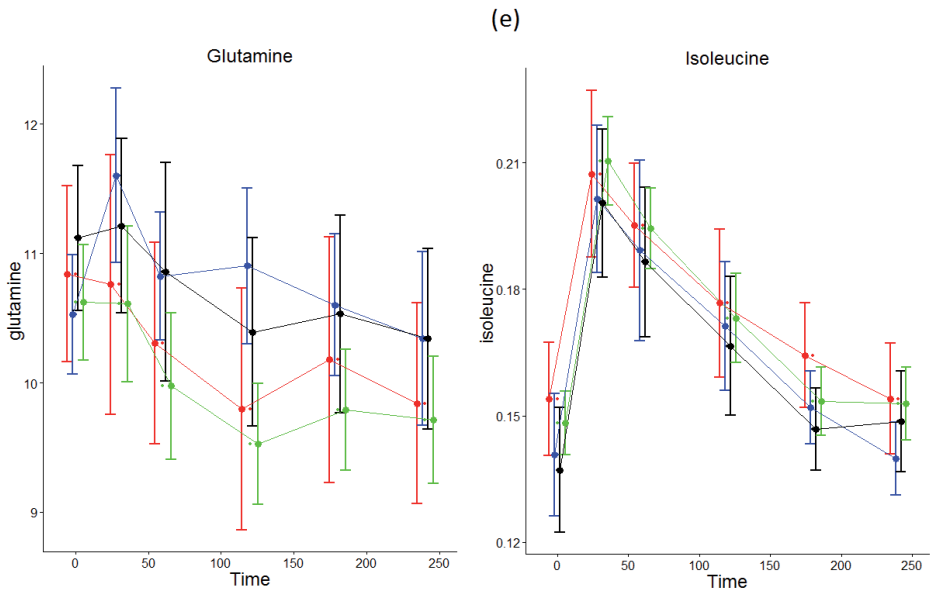

(a)
Linoleic acid(LA)
α .Linoleic acid(ALA)
Obese before weight loss
Control obese after weight loss
Low calorie diet obese after weight loss
Lean
mean
Time
(b)
C2/Nonaylcarnitine
C2/Palmitoylcarnitine
mean
Time

(c)


acetone
mean
Time
lean_D1
LCD_D2
Cont_D2
Obese_D1
acetate
mean
Time
lean_D1
LCD_D2
Obese_D1
Cont_D2


acetoacetate
mean
Time
lean_D1
LCD_D2
Obese_D1
Cont_D2


(d)
Creatinine
mean
Time
120
110
100
90
80
0
50
100
150
200
250


(e)
Glutamine
glutamine
Time
12
11
10
9
0
50
100
150
200
250
Isoleucine
isoleucine
Time
0.21
0.18
0.15
0.12
0
50
100
150
200
250

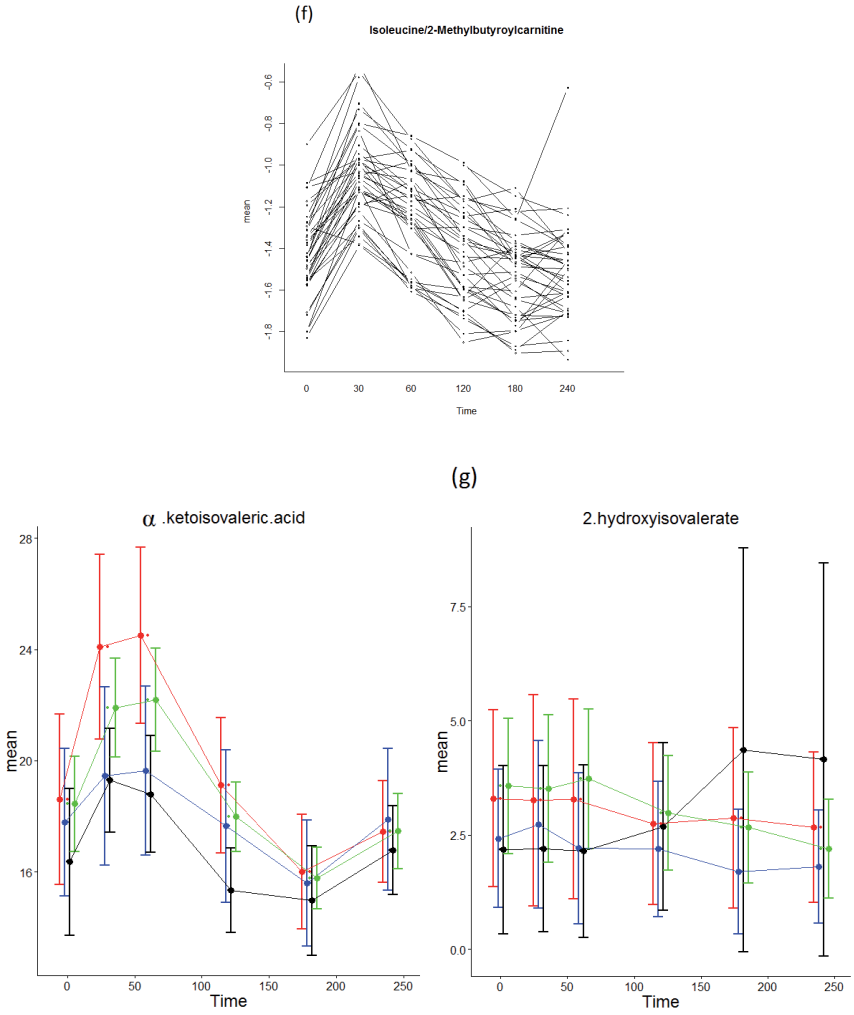


Figure S3. Mean postprandial response curves for linoleic acid and α-linolenic acid (a). Mean postprandial curve (lean subjects only) of ratio between acetylcarnitine (c2) and fatty acid derived acylcarnitines; each color indicates one individual (b). Mean postprandial curve of ketone bodies (c), carnitine (d), glutamine and isoleucine (e). Individual plot (lean subjects only) of ratio between Isoleucine and 2-ethylbutyroylcarnitine; each color indicate one individual (f); Mean postprandial curve for alpha ketoisovaleric acid and 2-hydroxyisovalerate (g).

Table S1. Overview of significantly (FDR<0.2) positively /negatively enriched gene sets between abdominally obese vs. lean subjects at fasting (T0).

Positively enriched gene sets

NAME	SIZE	NES	FDR q-val
REACT_RESPIRATORY ELECTRON TRANSPORT	56	2.08	0.01
WIP_HS_OXIDATIVE_PHOSPHORYLATION	46	2.03	0.02
KEGG_OXIDATIVE PHOSPHORYLATION	92	2.02	0.01
REACT_RESPIRATORY ELECTRON TRANSPORT	69	2.00	0.01
WIP_HS_ELECTRON_TRANSPORT_CHAIN	76	1.92	0.04
KEGG_PARKINSON'S DISEASE	86	1.91	0.04
REACT_DESTABILIZATION OF MRNA BY KSRP	15	1.84	0.08
REACT_DEADENYLATION-DEPENDENT MRNA DECAY	39	1.84	0.07
KEGG_RNA DEGRADATION	60	1.80	0.11

Negatively enriched gene sets

NAME	SIZE	NES	FDR q-val
NCI_IL23PATHWAY	18	-2.08	0.08
WIP_HS_NOTCH_SIGNALING_PATHWAY	24	-2.00	0.09
BIOC_IL1RPATHWAY	22	-1.89	0.20
NCI_ATF2_PATHWAY	27	-1.86	0.18
BIOC_KERATINOCYTEPATHWAY	35	-1.84	0.18
BIOC_NTHIPATHWAY	20	-1.81	0.20
WIP_HS_OXIDATIVE_STRESS	18	-1.79	0.19
KEGG_OSTEOCLAST DIFFERENTIATION	94	-1.77	0.20
NCI_AP1_PATHWAY	36	-1.77	0.18

Size indicates the number of total genes which are involve in the corresponding pathway. (NES): normalised enrichment scores and (FDR): false discovery rate.

Table S2. Overview of significantly (FDR<0.2) up- and down-regulated gene sets during postprandial mixed meal challenge response between abdominally obese and lean subjects.

Upregulated gene sets

NAME	SIZE	NES	FDR q-val	Direction	
				obese	Lean
NCI_IL6_7PATHWAY	35	2.13	0.01	up	down
NCI_IFNGPATHWAY	35	2.01	0.04	up	down
KEGG_LYSOSOME	97	2.01	0.03	up	down
KEGG_TUBERCULOSIS	114	2.00	0.02	up	down
WIP_HS_TYPE_II_INTERFERON_SIGNALING_(IFNG)	27	1.99	0.02	up	down
KEGG_STAPHYLOCOCCUS AUREUS INFECTION	28	1.99	0.02	up	down
KEGG_PORPHYRIN AND CHLOROPHYLL METABOLISM	18	1.98	0.02	up	down
KEGG_INFLUENZA A	121	1.97	0.02	up	down
KEGG_LEISHMANIASIS	51	1.96	0.02	up	down
KEGG_PERTUSSIS	43	1.94	0.02	up	down
KEGG_STARCH AND SUCROSE METABOLISM	20	1.89	0.04	up	down
WIP_HS_EPITHELIUM_TARBASE	200	1.89	0.04	up	down
NCI_ENDOTHELINPATHWAY	36	1.89	0.03	up	down
REACT_TOLL LIKE RECEPTOR 7_8 (TLR7_8) CASCADE	60	1.89	0.03	up	down
REACT_MYD88 DEPENDENT CASCADE INITIATED ON ENDOSOME	60	1.89	0.03	up	down
NCI_UPA_UPAR_PATHWAY	19	1.89	0.03	up	down
REACT_TOLL LIKE RECEPTOR 9 (TLR9) CASCADE	62	1.87	0.03	up	down
KEGG_SALMONELLA INFECTION	52	1.86	0.04	up	down
WIP_HS_PROSTAGLANDIN_SYNTHESIS_AND_REGULATION	16	1.86	0.04	up	down
REACT_METABOLISM OF CARBOHYDRATES	88	1.84	0.04	up	down
KEGG_PHAGOSOME	94	1.83	0.05	up	down
REACT_TRAF6 MEDIATED INDUCTION OF NFKB AND MAP KINASES UPON TLR7_8 OR 9 ACTIVATION	59	1.82	0.05	up	down
NCI_ANTHRAXPATHWAY	16	1.82	0.05	up	down
NCI_ATF2_PATHWAY	27	1.81	0.05	up	down
REACT_GOLGI ASSOCIATED VESICLE BIOGENESIS	42	1.81	0.05	up	down
REACT_CLATHRIN DERIVED VESICLE BUDDING	49	1.81	0.05	up	down
REACT_TRANS-GOLGI NETWORK VESICLE BUDDING	49	1.80	0.05	up	down
KEGG_OSTEOCLAST DIFFERENTIATION	94	1.78	0.06	up	down
BIOC_KERATINOCYTEPATHWAY	35	1.78	0.06	down	down
WIP_HS_IL-6_SIGNALING_PATHWAY	35	1.78	0.06	up	down
KEGG_NEUROACTIVE LIGAND-RECEPTOR INTERACTION	38	1.77	0.06	up	down
WIP_HS_LEUKOCYTE_TARBASE	107	1.76	0.07	up	down

Table S2. Upregulated gene sets continued

REACT_MEMBRANE TRAFFICKING	97	1.75	0.07	up	down
BIOC_TIDPATHWAY	15	1.75	0.07	up	down
NCI_TOLL_ENDOGENOUS_PATHWAY	19	1.75	0.07	up	down
WIP_HS_OXIDATIVE_STRESS	18	1.74	0.08	up	down
KEGG_COMPLEMENT AND COAGULATION CASCADES	15	1.74	0.08	up	down
NCI_HES_HEYPATHWAY	28	1.74	0.07	up	down
BIOC_IL1RPATHWAY	22	1.73	0.08	up	down
REACT_P75 NTR RECEPTOR-MEDIATED SIGNALLING	62	1.73	0.07	up	down
REACT_MAP KINASE ACTIVATION IN TLR CASCADE	42	1.73	0.08	up	down
WIP_HS_REGULATION_OF_TOLL-LIKE_RECEPTOR_SIGNALING_PATHWAY	96	1.72	0.08	up	down
KEGG_MALARIA	23	1.72	0.07	up	down
NCI_LYSOPHOSPHOLIPID_PATHWAY	38	1.72	0.07	up	down
KEGG_FRUCTOSE AND MANNOSE METABOLISM	22	1.71	0.08	up	down
WIP_HS_SENESCENCE_AND_AUTOPHAGY	61	1.69	0.10	up	down
WIP_HS_SQUAMOUS_CELL_TARBASE	94	1.69	0.10	up	down
REACT_CYTOKINE SIGNALING IN IMMUNE SYSTEM	154	1.69	0.10	up	down
REACT_GLUCOSE METABOLISM	44	1.68	0.10	up	down
NCI_HNF3APATHWAY	18	1.68	0.10	up	down
REACT_ASSOCIATION OF TRIC_CCT WITH TARGET PROTEINS DURING BIOSYNTHESIS	23	1.67	0.11	up	down
KEGG_FATTY ACID METABOLISM	26	1.67	0.10	down	down
WIP_HS_NOD_PATHWAY	28	1.67	0.10	up	down
REACT_INTERLEUKIN-1 SIGNALING	34	1.67	0.11	up	down
REACT_ERK_MAPK TARGETS	17	1.66	0.11	up	down
REACT_MAPK TARGETS_ NUCLEAR EVENTS MEDIATED BY MAP KINASES	25	1.66	0.11	up	down
WIP_HS_PHYSIOLOGICAL_AND_PATHOLOGICAL_HYPERTROPHY_OF_THE_HEART	17	1.65	0.11	down	down
KEGG_PENTOSE PHOSPHATE PATHWAY	19	1.65	0.12	up	down
REACT_TRAF6 MEDIATED INDUCTION OF PROINFLAMMATORY CYTOKINES	51	1.65	0.12	up	down
KEGG_GALACTOSE METABOLISM	17	1.64	0.12	up	down
WIP_HS_IL-3_SIGNALING_PATHWAY	37	1.63	0.12	up	down
REACT_SIGNALING BY INTERLEUKINS	85	1.63	0.13	up	down
WIP_HS_GPCR_LIGAND_BINDING	24	1.63	0.13	up	down
REACT_INTERFERON SIGNALING	75	1.63	0.12	up	down
REACT_NRAGE SIGNALS DEATH THROUGH JNK	30	1.62	0.13	up	down
REACT_HEXOSE TRANSPORT	33	1.62	0.13	up	down
NCI_P38ALPHABETADOWNSTREAMPATHWAY	29	1.62	0.13	up	down
KEGG_SPHINGOLIPID METABOLISM	23	1.62	0.13	up	down
NCI_GMCSF_PATHWAY	31	1.62	0.13	up	down
WIP_HS_KIT_RECEPTOR_SIGNALING_PATHWAY	39	1.62	0.13	up	down

Table S2. Upregulated gene sets continued

KEGG_TOLL-LIKE RECEPTOR SIGNALING PATHWAY	69	1.62	0.13	up	down
NCI_MAPKTRKPATHWAY	27	1.61	0.13	up	down
REACT_GLUCOSE TRANSPORT	33	1.61	0.13	up	down
KEGG_FC GAMMA R-MEDIATED PHAGOCYTOSIS	73	1.61	0.13	up	down
NCI_RB_1PATHWAY	42	1.61	0.13	up	down
KEGG_RHEUMATOID ARTHRITIS	48	1.60	0.14	up	down
BIOC_NTHIPATHWAY	20	1.59	0.14	up	down
WIP_HS_IL-5_SIGNALING_PATHWAY	29	1.59	0.14	up	down
WIP_HS_TOLL-LIKE_RECEPTOR_SIGNALING_PATHWAY	69	1.59	0.15	up	down
REACT_TOLL LIKE RECEPTOR 3 (TLR3) CASCADE	56	1.59	0.15	up	down
REACT_INFLAMMASOMES	16	1.58	0.15	up	down
KEGG_PPAR SIGNALING PATHWAY	22	1.58	0.15	up	down
NCI_ARF_3PATHWAY	19	1.58	0.15	up	down
REACT_INTERFERON GAMMA SIGNALING	54	1.57	0.15	up	down
NCI_RAC1_PATHWAY	44	1.57	0.16	up	down
REACT_INTERFERON ALPHA_BETA SIGNALING	43	1.57	0.16	up	down
KEGG_ADIPOCYTOKINE SIGNALING PATHWAY	40	1.57	0.16	up	down
KEGG_NEUROTROPHIN SIGNALING PATHWAY	88	1.56	0.17	up	down
WIP_HS_MRNA_PROCESSING	114	1.55	0.17	up	down
WIP_HS_FOLATE_METABOLISM	29	1.55	0.17	up	down
WIP_HS_LYMPHOCYTE_TARBASE	305	1.55	0.17	up	down
REACT_RECYCLING PATHWAY OF L1	26	1.55	0.17	up	down
BIOC_HIVNEFPATHWAY	45	1.55	0.17	up	down
KEGG_NOD-LIKE RECEPTOR SIGNALING PATHWAY	43	1.55	0.17	up	down
REACT_NUCLEAR EVENTS (KINASE AND TRANSCRIPTION FACTOR ACTIVATION)	20	1.54	0.17	up	down
WIP_HS_APOPTOTIC_EXECUTION_PHASE	22	1.54	0.18	up	down
KEGG_GLYCOLYSIS _ GLUCONEOGENESIS	33	1.54	0.18	up	down
WIP_HS_INTRINSIC_PATHWAY_FOR_APOPTOSIS	16	1.54	0.17	up	down
NCI_IL8CXCR1_PATHWAY	22	1.54	0.18	up	down
REACT_G ALPHA (12_13) SIGNALLING EVENTS	47	1.54	0.18	up	down
NCI_CDC42_PATHWAY	52	1.54	0.18	up	down
REACT_CHAPERONIN-MEDIATED PROTEIN FOLDING	33	1.53	0.19	up	down
WIP_HS_TNF-ALPHA-NF-KB_SIGNALING_PATHWAY	161	1.52	0.19	up	down
NCI_TXA2PATHWAY	40	1.52	0.19	up	down
NCI_CERAMIDE_PATHWAY	37	1.52	0.19	up	down
REACT_L1CAM INTERACTIONS	50	1.52	0.19	up	down
KEGG_AMOEBIASIS	40	1.52	0.19	up	down
NCI_HIF1_TFPATHWAY	44	1.52	0.19	up	down
KEGG_BLADDER CANCER	21	1.51	0.19	up	down
NCI_RHOA_REG_PATHWAY	25	1.51	0.19	up	down
NCI_KITPATHWAY	37	1.51	0.19	up	down

Table S2. Upregulated gene sets continued

NCI_RAC1_REG_PATHWAY	28	1.51	0.19	up	down
NCI_S1P_S1P3_PATHWAY	20	1.51	0.19	up	down

Downregulated gene sets

NAME	SIZE	NES	FDR q-val	Direction obese	Lean
NCI_CD8TCRPATHWAY	47	-2.12	0.02	down	up
REACT_GENERATION OF SECOND MESSENGER MOLECULES	29	-1.91	0.13	down	up
REACT_BRANCHED-CHAIN AMINO ACID CATABOLISM	15	-1.87	0.12	down	up
REACT_BIOSYNTHESIS OF THE N-GLYCAN PRECURSOR	24	-1.83	0.14	down	up
WIP_HS_GENERIC_TRANSCRIPTION_PATHWAY	15	-1.81	0.12	down	up
NCI_TCR_PATHWAY	58	-1.70	0.26	down	up
KEGG_T CELL RECEPTOR SIGNALING PATHWAY	81	-1.69	0.24	down	up
NCI_CD8TCRDOWNSTREAMPATHWAY	46	-1.69	0.22	down	up
WIP_HS_INFLAMMATORY_RESPONSE_PATHWAY	15	-1.68	0.20	down	up
BIOC_AMIPATHWAY	17	-1.67	0.20	up	up
KEGG_ABC TRANSPORTERS	19	-1.67	0.18	up	up
REACT_TCR SIGNALING	57	-1.66	0.18	up	up
REACT_GENERIC TRANSCRIPTION PATHWAY	134	-1.65	0.18	down	up
BIOC_CSKPATHWAY	17	-1.62	0.19	down	up
NCI_IL2_STAT5PATHWAY	25	-1.61	0.20	up	up

Size indicates the number of total genes which are involve in the corresponding pathway. (NES): normalised enrichment scores and (FDR): false discovery rate.

Table S3. Correlation of the fasting plasma metabolome at baseline with HOMA

A	**Acylcarnitine**	**$C_2/C_{n\ vs.}$ HOMA**		**$C_{2vs.}$ HOMA**		**$C_{n\ vs.}$ HOMA**	
	C_n	*P*	ρ	*P*	ρ	*P*	ρ
	AAAC						
	C_4 (butyryl)	<0.001	-0.44	0.70	-0.04	<0.001	0.41
	C_3 (propionyl)	0.01	-0.30			0.003	0.38
	C_5(2-methylbutyroyl)	0.09	-0.21			0.02	0.28
	C_0 (carnitine)	0.1	-0.20			0.07	0.22
	FAAC						
	C_6 (hexanoyl)	<0.001	-0.55			0.01	0.31
	C_{18} (stearoyl)	0.4	-0.10			0.9	0.01
	C_{16} (palmitoyl)	0.07	-0.22			0.1	0.20
	C_{14} (myristol)	0.1	-0.20			0.3	0.10
B						***AA vs. HOMA***	
	BCAA					*P*	ρ
	Isoleucine					0.06	0.34
	Leucine					0.02	0.30
	Valine					0.02	0.30
	Amino acids					*P*	ρ
	Tyrosine					<0.001	0.57
	Phenyl alanine					0.02	0.30
	2aminoadipic acid					0.06	0.24

AAAC: amino acid derived acylcarnitine, FAAC: fatty acid derived acylcarnitines. AA: amino acid.
ρ: Spearman's rank correlation coefficient. Fasting levels (T0) of all subjects (lean and abdominally ob ese) before
and after interventions were included.

Table S4. Overview of significantly (FDR<0.05) upregulated and downregulated gene sets in WL abdominally obese subjects at fasting (T0) before and after weight loss intervention (D2 vs. D1)

Upregulated gene sets

NAME	SIZE	NES	FDR q-val
NCI_IL12_2PATHWAY	45	2.27	0
KEGG_T CELL RECEPTOR SIGNALING PATHWAY	81	2.14	0.01
KEGG_T CELL RECEPTOR SIGNALING PATHWAY	81	2.14	0.01
BIOC_NTHIPATHWAY	20	2.05	0.02
BIOC_PPARAPATHWAY	35	2.03	0.02
NCI_FGF_PATHWAY	26	1.99	0.03
NCI_FCER1PATHWAY	51	1.97	0.03
REACT_NRAGE SIGNALS DEATH THROUGH JNK	30	1.95	0.03
REACT_SIGNALING BY RHO GTPASES	77	1.94	0.03
KEGG_SALMONELLA INFECTION	52	1.94	0.03
REACT_RHO GTPASE CYCLE	77	1.94	0.03
BIOC_IL1RPATHWAY	22	1.92	0.03
NCI_TCRCALCIUMPATHWAY	18	1.91	0.03
WIP_HS_NOD_PATHWAY	28	1.9	0.03
KEGG_NEUROTROPHIN SIGNALING PATHWAY	88	1.9	0.03
NCI_AP1_PATHWAY	36	1.89	0.03
NCI_IL23PATHWAY	18	1.88	0.03
REACT_SIGNALING BY GPCR	152	1.88	0.03
REACT_INTERACTIONS OF THE IMMUNOGLOBULIN SUPERFAMILY (IGSF)	31	1.88	0.03
KEGG_LEISHMANIASIS	51	1.88	0.03
KEGG_CARBOHYDRATE DIGESTION AND ABSORPTION	19	1.87	0.03
NCI_ERBB4_PATHWAY	20	1.86	0.03
NCI_HIF1_TFPATHWAY	44	1.86	0.03
KEGG_TOLL-LIKE RECEPTOR SIGNALING PATHWAY	69	1.86	0.03
KEGG_NOD-LIKE RECEPTOR SIGNALING PATHWAY	43	1.84	0.04
WIP_HS_RANKL-RANK_SIGNALING_PATHWAY	41	1.84	0.04
REACT_G ALPHA (12_13) SIGNALLING EVENTS	47	1.84	0.04
WIP_HS_TOLL-LIKE_RECEPTOR_SIGNALING_PATHWAY	69	1.83	0.04
NCI_EPHBFWDPATHWAY	21	1.83	0.03
NCI_ATF2_PATHWAY	27	1.83	0.03
WIP_HS_INSULIN_SIGNALING	113	1.82	0.04
NCI_IL1PATHWAY	26	1.82	0.04
KEGG_OSTEOCLAST DIFFERENTIATION	94	1.81	0.04
REACT_GPCR DOWNSTREAM SIGNALING	143	1.81	0.04
NCI_CD8TCRDOWNSTREAMPATHWAY	46	1.8	0.04
BIOC_KERATINOCYTEPATHWAY	35	1.8	0.04
NCI_CXCR4_PATHWAY	78	1.8	0.04
REACT_ION CHANNEL TRANSPORT	18	1.79	0.04

Table S4. Upregulated gene sets continued			
KEGG_MTOR SIGNALING PATHWAY	36	1.79	0.04
BIOC_TOLLPATHWAY	28	1.79	0.04

Downregulated gene sets

NAME	SIZE	NES	FDR q-val
KEGG_PARKINSON'S DISEASE	86	-2.5	0
REACT_RESPIRATORY ELECTRON TRANSPORT	56	-2.45	0
KEGG_OXIDATIVE PHOSPHORYLATION	92	-2.42	0
WIP_HS_OXIDATIVE_PHOSPHORYLATION	46	-2.36	0
WIP_HS_ELECTRON_TRANSPORT_CHAIN	76	-2.24	0
REACT_FORMATION OF THE TERNARY COMPLEX, AND SUBSEQUENTLY, THE 43S COMPLEX	39	-2.21	0
REACT_PLATELET DEGRANULATION	46	-2.19	0
REACT_CDK-MEDIATED PHOSPHORYLATION AND REMOVAL OF CDC6	45	-2.17	0
REACT_UBIQUITIN-DEPENDENT DEGRADATION OF CYCLIN D1	45	-2.15	0
REACT_UBIQUITIN-DEPENDENT DEGRADATION OF CYCLIN D	45	-2.13	0
KEGG_PROTEASOME	40	-2.13	0
REACT_DESTABILIZATION OF MRNA BY AUF1 (HNRNP D0)	50	-2.12	0
REACT_SCF(SKP2)-MEDIATED DEGRADATION OF P27_P21	49	-2.11	0
REACT_REGULATION OF APOPTOSIS	51	-2.09	0
REACT_REGULATION OF ACTIVATED PAK-2P34 BY PROTEASOME MEDIATED DEGRADATION	45	-2.09	0
REACT_RESPONSE TO ELEVATED PLATELET CYTOSOLIC CA2+	50	-2.07	0
WIP_HS_PROTEASOME_DEGRADATION	56	-2.07	0
REACT_P53-INDEPENDENT DNA DAMAGE RESPONSE	45	-2.04	0
REACT_REGULATION OF ORNITHINE DECARBOXYLASE (ODC)	44	-2.03	0.01
REACT_AUTODEGRADATION OF CDH1 BY CDH1_APC_C	55	-2.02	0
REACT_VIF-MEDIATED DEGRADATION OF APOBEC3G	49	-2.02	0
REACT_UBIQUITIN MEDIATED DEGRADATION OF PHOSPHORYLATED CDC25A	45	-2.02	0
REACT_APC_C_CDC20 MEDIATED DEGRADATION OF SECURIN	55	-2.01	0.01
REACT_P53-INDEPENDENT G1_S DNA DAMAGE CHECKPOINT	45	-1.99	0.01
REACT_CDT1 ASSOCIATION WITH THE CDC6_ORC_ORIGIN COMPLEX	49	-1.98	0.01
REACT_DNA REPLICATION PRE-INITIATION	60	-1.97	0.01
REACT_M_G1 TRANSITION	60	-1.97	0.01
REACT_ACTIVATION OF APC_C AND APC_C_CDC20 MEDIATED DEGRADATION OF MITOTIC PROTEINS	56	-1.97	0.01
REACT_APC_C_CDC20 MEDIATED DEGRADATION OF MITOTIC PROTEINS	56	-1.97	0.01
REACT_CYCLIN E ASSOCIATED EVENTS DURING G1_S TRANSITION	54	-1.97	0.01
REACT_SCF-BETA-TRCP MEDIATED DEGRADATION OF EMI1	48	-1.96	0.01
REACT_CDC20_PHOSPHO-APC_C MEDIATED DEGRADATION OF CYCLIN A	56	-1.95	0.01
REACT_MITOTIC G1-G1_S PHASES	76	-1.93	0.01
REACT_SYNTHESIS OF DNA	73	-1.92	0.01
REACT_CYCLIN A_CDK2-ASSOCIATED EVENTS AT S PHASE ENTRY	56	-1.91	0.01
REACT_REGULATION OF APC_C ACTIVATORS BETWEEN G1_S AND EARLY ANAPHASE	61	-1.91	0.01

Table S4. Downregulated gene sets continued			
KEGG_RIBOSOME	66	-1.91	0.01
KEGG_HUNTINGTON'S DISEASE	126	-1.89	0.01
REACT_FORMATION OF A POOL OF FREE 40S SUBUNITS	74	-1.88	0.01
REACT_ASSEMBLY OF THE PRE-REPLICATIVE COMPLEX	53	-1.88	0.01
REACT_REGULATION OF DNA REPLICATION	58	-1.87	0.01
REACT_G1_S TRANSITION	72	-1.87	0.01
REACT_AUTODEGRADATION OF THE E3 UBIQUITIN LIGASE COP1	47	-1.86	0.01
REACT_VIRAL MRNA TRANSLATION	63	-1.86	0.01
REACT_PREFOLDIN MEDIATED TRANSFER OF SUBSTRATE TO CCT_TRIC	18	-1.86	0.01
REACT_COOPERATION OF PREFOLDIN AND TRIC_CCT IN ACTIN AND TUBULIN FOLDING	18	-1.85	0.01
REACT_INFLUENZA VIRAL RNA TRANSCRIPTION AND REPLICATION	63	-1.85	0.01
REACT_SWITCHING OF ORIGINS TO A POST-REPLICATIVE STATE	58	-1.85	0.01
BIOC_PROTEASOMEPATHWAY	20	-1.84	0.01
REACT_ORC1 REMOVAL FROM CHROMATIN	58	-1.84	0.01
REACT_VPU MEDIATED DEGRADATION OF CD4	47	-1.84	0.01
KEGG_ALZHEIMER'S DISEASE	112	-1.83	0.01
REACT_RIBOSOMAL SCANNING AND START CODON RECOGNITION	44	-1.83	0.01
REACT_EUKARYOTIC TRANSLATION TERMINATION	65	-1.82	0.02
REACT_REMOVAL OF LICENSING FACTORS FROM ORIGINS	58	-1.81	0.02
REACT_P53-DEPENDENT G1 DNA DAMAGE RESPONSE	51	-1.81	0.02
REACT_S PHASE	82	-1.8	0.02
REACT_P53-DEPENDENT G1_S DNA DAMAGE CHECKPOINT	51	-1.8	0.02
REACT_APC_C-MEDIATED DEGRADATION OF CELL CYCLE PROTEINS	63	-1.79	0.02
REACT_TRANSLATION INITIATION COMPLEX FORMATION	44	-1.78	0.02
REACT_REGULATION OF MITOTIC CELL CYCLE	63	-1.77	0.02
REACT_STABILIZATION OF P53	48	-1.77	0.02
REACT_NONSENSE MEDIATED DECAY INDEPENDENT OF THE EXON JUNCTION COMPLEX	70	-1.77	0.02
REACT_G1_S DNA DAMAGE CHECKPOINTS	51	-1.76	0.02
REACT_SIGNALING BY WNT	60	-1.75	0.02
REACT_EUKARYOTIC TRANSLATION ELONGATION	67	-1.75	0.02
REACT_DEGRADATION OF BETA-CATENIN BY THE DESTRUCTION COMPLEX	60	-1.74	0.02
REACT_REGULATION OF BETA-CELL DEVELOPMENT	67	-1.73	0.03
REACT_INSULIN SYNTHESIS AND PROCESSING	101	-1.72	0.03
KEGG_SPLICEOSOME	111	-1.71	0.03
REACT_REGULATION OF GENE EXPRESSION IN BETA CELLS	67	-1.71	0.03
REACT_POST-ELONGATION PROCESSING OF INTRONLESS PRE-MRNA	17	-1.7	0.03
REACT_CHROMOSOME MAINTENANCE	47	-1.69	0.03
REACT_NUCLEOSOME ASSEMBLY	25	-1.69	0.03
PPARA_TARGETS	81	-1.68	0.04
REACT_PROCESSING OF CAPPED INTRONLESS PRE-MRNA	17	-1.68	0.04

Size indicates the number of total genes which are involve in the corresponding pathway. (NES): normalised enrichment scores and (FDR): false discovery rate

Table S5. Fasting (T0) and postprandial effects of abdominally obese that underwent the control intervention.

Metabolites	FC(T0)	iAUC
		P
Acylcarnitines		
2.Methylbutyroylcarnitine	1.13	
Propionylcarnitine	1.17	
Isobutyrylcarnitine	1.22	0.04
Stearoylcarnitine		0.01
Octanoylcarnitine		0.04
Decanoylcarnitine		0.04
Amino acids and related metabolites		
Choline	1.02	
Methionine	1.17	
Phosphocholine	1.15	
Dimethylglycine	1.21*	
Leucine	1.06	
Isoleucine	1.07	
Arginine	1.15*	
Tyrosine	1.07	0.02
Valine	1.1*	<0.01 *
N6.N6.N6.trimethyl.L.lysine	1.08*	
Ornithine	1.06	
Phenylalanine	1.1	
2.aminoadipic.acid	1.12	0.01
Sarcosine		0.01
Dimethylglycine		0.03
Dimethylamine		0.04
TCA cycle and related metabolites		
Aspartic acid	1.18	
Fumaric acid		<0.01*
Oxylipins		
12.13.DiHOME		<0.01

FC: Fold change (T0, T2, before and after control intervention, respectively), iAUC: Incremental area under the curve, *: Significant at $P<0.05$,lFDR<0.2.

Table S6. Overview of significantly (FDR<0.2) upregulated and downregulated gene sets in abdominally obese subjects in response to mixed meal challenge before and after weight loss intervention (Δ T4-T0, D2, vs. Δ T4-T0, D1).

Upregulated gene sets

NAME	SIZE	NES	FDR q-val	Direction	
				D2	D1
WIP_HS_OXIDATIVE_PHOSPHORYLATION	46	2.09	0.03	up	down
KEGG_OXIDATIVE PHOSPHORYLATION	92	1.94	0.09	up	down
WIP_HS_ELECTRON_TRANSPORT_CHAIN	76	1.87	0.14	up	down
REACT_RESPIRATORY ELECTRON TRANSPORT	56	1.84	0.14	up	down
REACT_RESPIRATORY ELECTRON TRANSPORT	69	1.83	0.12	up	down
KEGG_PARKINSON'S DISEASE	86	1.77	0.18	up	down

Downregulated gene sets

NAME	SIZE	NES	FDR q-val	Direction	
				D2	D1
KEGG_GALACTOSE METABOLISM	17	-2.26	0.00	down	up
NCI_HIF1_TFPATHWAY	44	-2.07	0.03	down	up
WIP_HS_ESTROGEN_SIGNALING_PATHWAY	17	-2.01	0.05	down	up
KEGG_CARBOHYDRATE DIGESTION AND ABSORPTION	19	-1.92	0.06	down	up
BIOC_KERATINOCYTEPATHWAY	35	-1.93	0.07	down	up
REACT_NRAGE SIGNALS DEATH THROUGH JNK	30	-1.93	0.08	down	up
WIP_HS_PHYSIOLOGICAL_AND_PATHOLOGICAL_HYPERTROPHY_OF_THE_HEART	17	-1.95	0.08	down	up
NCI_IL6_7PATHWAY	35	-1.82	0.09	down	up
REACT_ION TRANSPORT BY P-TYPE ATPASES	17	-1.82	0.09	down	up
NCI_EPOPATHWAY	27	-1.80	0.09	down	up
WIP_HS_IL-3_SIGNALING_PATHWAY	37	-1.88	0.09	down	up
BIOC_NTHIPATHWAY	20	-1.83	0.09	down	up
WIP_HS_SREBP_SIGNALLING	22	-1.85	0.09	down	up
NCI_ERBB2ERBB3PATHWAY	31	-1.80	0.09	down	up
KEGG_PHOSPHATIDYLINOSITOL SIGNALING SYSTEM	55	-1.84	0.09	down	up
WIP_HS_WNT_SIGNALING_PATHWAY	28	-1.83	0.10	down	up
REACT_ION CHANNEL TRANSPORT	18	-1.86	0.10	down	up
NCI_IL2_1PATHWAY	46	-1.81	0.10	down	up
WIP_HS_RANKL-RANK_SIGNALING_PATHWAY	41	-1.86	0.10	down	up
WIP_HS_KIT_RECEPTOR_SIGNALING_PATHWAY	39	-1.77	0.12	down	up
REACT_GLUCOSE TRANSPORT	33	-1.75	0.12	down	up
REACT_HEXOSE TRANSPORT	33	-1.73	0.12	down	up
NCI_MAPKTRKPATHWAY	27	-1.74	0.13	down	up

Table S6. Downregulated gene sets continued				
KEGG_ADIPOCYTOKINE SIGNALING PATHWAY	40	-1.75	0.13	down up
WIP_HS_MICRORNAS_IN_CARDIOMYOCYTE_HYPERTROPHY	53	-1.76	0.13	down up
NCI_ATF2_PATHWAY	27	-1.75	0.13	down up
KEGG_STARCH AND SUCROSE METABOLISM	20	-1.72	0.13	down up
NCI_AVB3_OPN_PATHWAY	26	-1.73	0.13	down up
NCI_IL1PATHWAY	26	-1.74	0.13	down up
NCI_NECTIN_PATHWAY	19	-1.71	0.13	down up
NCI_AMB2_NEUTROPHILS_PATHWAY	25	-1.71	0.13	down up
NCI_AR_TF_PATHWAY	39	-1.71	0.13	down up
KEGG_NEUROTROPHIN SIGNALING PATHWAY	88	-1.68	0.14	down up
WIP_HS_IL-1_PATHWAY	42	-1.68	0.14	down up
BIOC_IL1RPATHWAY	22	-1.69	0.14	down up
KEGG_FOCAL ADHESION	93	-1.68	0.15	down up
REACT_SIGNALLING TO ERKS	25	-1.69	0.15	down up
NCI_IL23PATHWAY	18	-1.65	0.15	down up
REACT_RHO GTPASE CYCLE	77	-1.65	0.15	down up
REACT_P75 NTR RECEPTOR-MEDIATED SIGNALLING	62	-1.65	0.15	down up
REACT_SIGNALING BY RHO GTPASES	77	-1.65	0.15	down up
NCI_ANGIOPOIETINRECEPTOR_PATHWAY	34	-1.67	0.15	down up
KEGG_ECM-RECEPTOR INTERACTION	15	-1.65	0.16	down up
NCI_PI3KCIPATHWAY	41	-1.66	0.16	down up
BIOC_IL7PATHWAY	15	-1.64	0.16	down up
REACT_INTEGRIN CELL SURFACE INTERACTIONS	37	-1.64	0.16	down up
BIOC_GLEEVECPATHWAY	21	-1.63	0.16	down up
WIP_HS_IL-7_SIGNALING_PATHWAY	22	-1.65	0.16	down up
NCI_VEGFR1_2_PATHWAY	56	-1.63	0.16	down up
REACT_MITOCHONDRIAL TRNA AMINOACYLATION	15	-1.66	0.16	down up
WIP_HS_FOCAL_ADHESION	91	-1.63	0.16	down up
REACT_SIGNALLING BY NGF	160	-1.66	0.16	down up
BIOC_PPARAPATHWAY	35	-1.62	0.17	down up
REACT_INTERACTIONS OF THE IMMUNOGLOBULIN SUPERFAMILY (IGSF) MEMBER PROTEINS	31	-1.58	0.17	down up
KEGG_MISMATCH REPAIR	18	-1.59	0.17	down up
REACT_NCAM SIGNALING FOR NEURITE OUT-GROWTH	21	-1.59	0.17	down up
WIP_HS_INTEGRIN-MEDIATED_CELL_ADHESION	59	-1.62	0.17	down up
REACT_NETRIN-1 SIGNALING	19	-1.57	0.17	down up
WIP_HS_EGF_RECEPTOR_SIGNALING_PATHWAY	116	-1.58	0.17	down up
KEGG_FC EPSILON RI SIGNALING PATHWAY	51	-1.59	0.17	down up
WIP_HS_IL-4_SIGNALING_PATHWAY	36	-1.59	0.17	down up
NCI_GMCSF_PATHWAY	31	-1.59	0.17	down up
KEGG_ALDOSTERONE-REGULATED SODIUM REABSORPTION	17	-1.58	0.17	down up
REACT_REGULATION OF GLUCOKINASE BY GLUCOKINASE REGULATORY PROTEIN	27	-1.58	0.17	down up
NCI_NETRIN_PATHWAY	19	-1.58	0.17	down up
NCI_NETRIN_PATHWAY	19	-1.58	0.17	down up
NCI_ATM_PATHWAY	27	-1.59	0.17	down up
BIOC_EDG1PATHWAY	15	-1.58	0.17	down up

Table S6. Downregulated gene sets continued

REACT_GTP HYDROLYSIS AND JOINING OF THE 60S RIBOSOMAL SUBUNIT	83	-1.57	0.17	down	up
REACT_SIGNALING BY INTERLEUKINS	85	-1.57	0.17	down	up
BIOC_TNFR2PATHWAY	16	-1.61	0.17	down	up
KEGG_RIBOSOME BIOGENESIS IN EUKARYOTES	57	-1.60	0.17	down	up
REACT_EFFECTS OF PIP2 HYDROLYSIS	15	-1.61	0.17	down	up
NCI_FAK_PATHWAY	47	-1.60	0.17	down	up
NCI_IFNGPATHWAY	35	-1.60	0.18	down	up
BIOC_INTEGRINPATHWAY	26	-1.56	0.18	down	up
WIP_HS_EBV_LMP1_SIGNALING	16	-1.56	0.18	down	up
NCI_MTOR_4PATHWAY	58	-1.60	0.18	down	up
REACT_SIGNALLING TO RAS	18	-1.60	0.18	down	up
NCI_CXCR4_PATHWAY	78	-1.55	0.18	down	up
KEGG_TIGHT JUNCTION	61	-1.54	0.18	down	up
NCI_CD40_PATHWAY	23	-1.55	0.18	down	up
REACT_GLOBAL GENOMIC NER (GG-NER)	27	-1.55	0.18	down	up
KEGG_INOSITOL PHOSPHATE METABOLISM	42	-1.55	0.19	down	up
KEGG_HOMOLOGOUS RECOMBINATION	17	-1.54	0.19	down	up
BIOC_IL2RBPATHWAY	28	-1.54	0.19	down	up
NCI_REG_GR_PATHWAY	54	-1.53	0.19	down	up
KEGG_VEGF SIGNALING PATHWAY	45	-1.53	0.19	down	up
REACT_NUCLEAR IMPORT OF REV PROTEIN	30	-1.53	0.19	down	up
WIP_HS_INSULIN_SIGNALING	113	-1.53	0.19	down	up
REACT_APOPTOTIC EXECUTION PHASE	36	-1.50	0.19	down	up
NCI_ERBB4_PATHWAY	20	-1.50	0.20	down	up

Size indicates the number of total genes which are involve in the corresponding pathway.(NES): normalised enrichment scores and (FDR): false discovery rate

Table S7. Overview of downstream oxylipins produced from AA, LA, ALA, DGLA, EPA, and DHA by the COX, LOX and CYP oxidizing enzymes. Spearman correlations between oxylipins and their precursors (AA and LA) have been presented (P<0.05)

Precursor	Downstream oxylipin	Enzyme	*P*	ρ
AA	5.HETE	5-LOX	<0.001	0.34
	11.HETE	COX2	<0.001	0.37
	PGE2	COX	<0.001	0.24
	TXB2	COX	<0.001	0.22
	12S.HHTrE	COX0	<0.001	0.17
LA	12.13.EpOME	CYP450	NS	
	9.HODE	5-LOX	NS	
	13.HODE	15-LOX	<0.001	0.30
	12.13.DiHOME	CYP450	0.003	-0.14
	9.12.13.TriHOME	CYP450	<0.001	-0.42
ALA	9.HOTrE	LOX	<0.001	-0.20
DGLA	PGF1a	COX		
EPA	17.18.DiHETE	CYP450		
DHA	19.20.DiHDPA	CYP450		
	10.HDoHE	Auto-oxidation		

ρ: Spearman rank correlation coefficient, NS: Not significant.

Table S8. Amino acid (AAAC) and fatty acid (FAAC) derived acylcarnitines covered in this study.

	-Carnitine	Abbreviation	Origin
AAAC (C_{2-5})	Acetyl-	C_2	Leu, Ile
	Proprionyl-	C_3	Ile, Val
	Isobutyryl-	C_4	Val
	2-Methylbutyryl-	C_5	Ile
	Isovaleryl-	C_5	Leu
FAAC (C_{6-18})	Hexanoyl-	$C_{6:0}$	Caproic, hexanoic acid
	Octanoyl-	$C_{8:0}$	Octanoic acid
	Octenoyl-	$C_{8:1}$	Octenoic acid
	Decanoyl-	$C_{10:0}$	Decanoiccapric acid
	Myristoyl-	$C_{12:0}$	Myristic acid
	Tetradecanoyl	$C_{14:0}$	
	Palmitoyl-	$C_{16:0}$	Palmitic acid
	Stearoyl-	$C_{18:0}$	Stearic acid
	Linoleyl-	$C_{18:2}$	

Table S9. Correlation of ΔNadir and Nadir of plasma acylcarnitines levels with phenotypic parameters.

	ΔNadir vs. HOMA		ΔNadir vs. LBM		Nadir vs. HOMA		Nadir vs. LBM	
Acylcarnitines	***P***	**ρ**	***P***	**ρ**	***P***	**ρ**	***P***	**ρ**
Acetylcarnitine (C_2)	0.01	0.30			0.03	0.26		
2Methylbutyrilcarnitine (C_4)	0.04	-0.25						
Deoxycarnitine (C_0)	0.03	-0.26						
Tetradecanoylcarnitine (C_{14})			0.005	0.34				
Linoleylcarnitine ($C_{18:2}$)			0.02	0.37				
Octanoylcarnitine (C_8)			0.02	0.30				
Tetradecenoylcarnitine (C_{14})			0.02	0.27				
Hexanoylcarnitine (C_6)					0.001	0.39		
Butyrylcarnitine (C_4)					0.0003	0.44		
Propionylcarnitine (C_3)					0.001	0.39		
Carnitine (C_0)							0.01	0.30
Isobutyrylcarnitine (C_4)							0.01	-0.31

LBM: Lean body mass, ρ: Spearman's rank correlation coefficient. Only correlations with $p<0.05$ have been indicated.

5

Global testing of shifts in metabolic phenotype

Parastoo Fazelzadeh
Huub Hoefsloot
Thomas Hankemeier
Jasper Most
Sander Kersten
Ellen Blaak
Mark Boekschoten
John van Duynhoven

In preparation

ABSTRACT

It has been proposed that the ability of a person to respond to a metabolic stressor reflects the capacity to adapt to a new situation and that this can provide a better indication of health and disease risk than a measurement at fasting.For individual metabolites such correlations can be established, but this provides a fragmented view on the involved pathways. This can be overcome by Goeman's global testing approach, which explicitly considers that metabolites are connected in pathways. The application of Goeman's global test to two intervention studies indicates that it can provide a direct view on involvement of *a priori* defined pathways in phenotype shifts. In one study obese subjects received a mixed meal challenge before and after weight loss and in a second study obese subjects received a high fat mixed meal challenge before and after a polyphenol intervention. The effect of weight loss intervention on *a priori* defined metabolic pathways observed in the fasting and postprandial plasma metabolome was consistent with previous studies. In both studies Goeman's global test showed that interventions affected more metabolic pathways at fasting than during the postprandial response. For the population of the weight loss intervention study, Goeman's global test revealed that HOMA correlated with the fasting levels of the TCA cycle, BCAA catabolism, the lactate, Arginine-Proline and Phenylalanine-Tyrosine pathways. For the population of the polyphenol intervention study, HOMA correlated with fasting metabolite levels of TCA cycle, fatty acid oxidation and Phenylalanine-tyrosine pathways. Also these correlations were more pronounced for metabolic pathways in the fasting state, than during the postprandial response. Hence we cannot confirm that the postprandial metabolic response to a mixed meal challenge provides a more sensitive read-out for a shift in phenotypic flexibility than fasting metabolism.

Keywords. Goeman's global test,phenotypic flexibility

INTRODUCTION

Health is maintained by well-orchestrated interactions between physiological processes. These processes have to function in a changing environment and thus they collectively strive to maintain homeostasis by continuous adaptations. The ability to adapt to stressors such as diet and exercise has been coined as phenotypical flexibility (PF) and has been proposed as a measure for health [1, 2]. PF has been brought forward as a broad concept [1], but comprises the well-established concept of metabolic flexibility (MF), which is the efficiency of the postprandial switch between fasting lipid catabolism to postprandial carbohydrate anabolism [3]. Several studies have claimed that the ability of a person to respond to a metabolic stressor reflects the capacity to adapt to the new situation and this provides a better indication of health and disease risk comparing to fasting measure [4, 5]. Challenge tests have been put forward to measure the phenotypic flexibility of a biological system, meaning how well the system is able to undo the perturbation of a challenge and bring the system back to steady state.

In earlier studies univariate statistics was used to find differences between metabolic phenotype, at the level of baseline metabolism or in the response to a challenge. This approach is compromised by the multiple testing problem, and also cannot unambiguously establish whether a phenotype shift is better reflected in a shift in baseline metabolism or in an altered response to a dietary challenge.

In this work we will explore an approach where we exploit prior knowledge on involvement of metabolic pathways in shifts in baseline metabolism or altered postprandial responses. In order to exploit this prior knowledge we will use Goeman's global test, which is a robust test whether metabolites that are connected in a pathway collectively respond to a change in conditions [6].

We will explore this approach in two studies, where mixed-meal challenges were carried out to assess the efficacy of two types of dietary interventions. The first study examined whether a mixed meal challenge response could provide a readout for a shift in phenotypical flexibility upon weight loss in obese male subjects. This study showed a significant effect of weight loss on improved insulin sensitivity [7] and thus provides a relevant case for testing our approach. In the second study, the effect of long term polyphenol consumption on phenotypical flexibility was also assessed by means of a mixed meal challenge. The polyphenol intervention did not have an effect on insulin sensitivity and MF but increased baseline and postprandial fat oxidation as compared to placebo [8]. In both studies the responses of amino acids and acylcarnitines were measured, as well as metabolites related to the TCA cycle. This set of metabolites was selected since previous studies most convincingly related them with the

ability to effectively switch from lipid to carbohydrate metabolism in the postprandial phase, i.e. metabolic flexibility. We explore Goeman's global testing approach to assess the effect of the weight loss and polyphenol intervention on both baseline and mixed meal challenge response. Furthermore, we will use this approach to establish correlations between phenotypical parameters related to insulin sensitivity and metabolic flexibility.

MATERIALS AND METHODS

Subject characteristics

Weight loss intervention study. 29 abdominally overweight / obese men (BMI= 30.3 ± 2.4kg/m^2) participated in the study. None of the subjects were diagnosed with clinical diseases. Subjects characteristics can be found in Supplementary Table S1-a.

Polyphenol intervention study. 38overweight and obese subjects (BMI= 29.7 ± 0.5 kg/m^2) participated in this study, 28 subjects were randomly selected for metabolomics analysis. Characteristics of the subjects who completed the study are summarized in Supplementary Table S1-b.

Study design

Weight loss intervention study. Lean subjects were only studied cross-sectional, and obese/overweight subjects before and after random assignment to a weight-loss intervention of 8 weeks. Before the weight loss intervention (D1), all subjects underwent a mixed meal challenge test and subsequently blood samples were collected during 4 hours. Subjects assigned to either a weight-loss (WL) or control (CTRL) programme for 8 wk. After this period (D2), subjects again underwent a mixed meal challenge and blood samples were collected at the same time intervals as on D1. HOMA was measured at both D1 and D2. Details on the design can be found in an earlier study [7] .

Polyphenol intervention study. In this randomized, double-blind, placebo-controlled, parallel-intervention trial, subjects received either a polyphenol supplement (epigallocatechin gallate and resveratrol; 282 and 80 mg/d, respectively) or a placebo (partly hydrolyzed microcrystalline cellulose-filled capsules) for a period of 12 wk to assess effects of polyphenol supplementation on tissue-specific insulin sensitivity (primary outcome) and metabolic profile, skeletal muscle oxidative capacity, fat oxidation, and lipolysis (secondary outcomes). The supplementation period started the day after the last baseline measurement in week 0 and was continued throughout measurements in week 12. In total, subjects were asked to visit the university 10 times for medical screening, 3 clinical investigation days (CIDs) before the

start of supplementation (within 7 d), 3 control visits during the supplementation period, and 3 CIDs in the last week of supplementation (within 7 d). In this study we use the data from CID2 where high-fat mixed meal (HFMM) challenges was performed. At CID2, besides HOMA also energy expenditure (EE), respiratory quotient (RQ), fat and carbohydrate oxidation were measured by indirect calorimetry by using the open-circuit ventilated hood system (Omnical; Maastricht University) and were calculated according to the formulas of Weir and Frayn, respectively. Also a hyperinsulinemic-euglemic clamp with an isotope labelled glucose infusion tracer was performed to assess rate of disappearance (Rd, as measured for peripheral insulin sensitivity) and endogenous glucose production (% EGPm a measure for hepatic insulin resistance). Full details of the study can be found elsewhere [8].

Sample Collection

Weight loss intervention study. Subjects were asked not to perform any strenuous physical exercise or to consume alcohol and high-fat foods on the day before blood sampling. Blood samples were taken at fasting and after mixed meal consumption both before and after the weight loss intervention at 6 time points (fasting (T0) and 30, 60,120,180 and 240 minutes in the postprandial state). The standardized mixed meal consisted of two muffins and 300 ml 0% fat milk, which provided 1100 kcal: 56.6 g fat, 26.5 g protein and 121 g carbohydrate. Metabolic profiling was performed on all time points.

Polyphenol intervention study. After inserting a cannula into the antecubital vein, substrate oxidation was measured for 30 min under fasting conditions (T0) and for 4 h after the ingestion of a liquid high-fat mixed meal (HFMM) (625 kcal, 61% of energy from fat, 33% of energy from carbohydrate, 6% of energy from protein), which was consumed within 5 min at t = 0. Blood samples were taken under fasting (0 min) and postprandial (t = 30, 60, 90, 120, 150, 180, 210, and 240 min) conditions. Full details of the study can be found elsewhere [8].

Plasma metabolic profiling

Amino acids and biogenic amines, organic acids and acylcarnitines were measured for both the weight loss and polyphenol intervention studies, with a total 170 metabolites. Amino acids and biogenic amines in plasma were derivatized (Acc-TAG) and measured by a UPLC system which was interfaced to quadrupole mass spectrometer. Acylcarnitines in plasma were also measured by UPLC-MS, but without derivatisation. Organic acids in plasma were measured by GC-MS, after oximation and silation derivatization. Oxylipins were only analyzed for the weight loss study. First an SPE extraction was performed and subsequently aLC separation coupled to ESI on a triple quadrupole mass spectrometer. Oxylipins were detected in negative ion mode using dynamic SRM. Full details of these platforms have been described in earlier

studies [9-12]. Serum metabolites were measured by NMR in a quantitative manner, full experimental details can be found in earlier work [11]. In short, serum samples were ultrafiltrated and automated quantum mechanical line shape fitting of ^{1}H NMR spectra was performed using PERCH in order to obtain absolute metabolite concentrations.

Statistical analysis

Analysis of variance (ANOVA) was used for between group comparisons at baseline. Linear mixed model was used to assess the fasting comparison and difference of response effect between groups. $P<0.05$ was considered to be statistically significant. To account for multiple testing, local false discovery rates (lFDR) were calculated for each metabolite [13, 14]. The postprandial response was considered as incremental area under the curve (iAUC), taking the first measurement (t0) as a reference. The iAUC was calculated in two ways. First, by considering that the AUC comprises both negative AUC (AUC−) and positive AUC (AUC+) contributions, we refer to this value as iAUC. Secondly, we also calculated the positive iAUC where the absolute values contributing to the curve were summed up, we refer to these values as piAUC [15, 16]. For both iAUC and piAUC we use the trapezoidal calculation method [17].

For pathway analysis, Goeman's global test for metabolomics was applied to test groups of covariates (or features) for association with a response variable using the global test R library [6, 18]. In this approach *a priori* biological information, such as knowledge of pathways is used as cellular processes arise as the result of many reactions between metabolic intermediates [19]. In the metabolomics field, predefined groups of pathways or functional modules can be used [19-21]. More detail about this approach can be find elsewhere [6, 18]. Nadir acylcarnitine values were defined as the lowest value achieved during the 4 hours after the meal [22]. Δ nadir was calculated as difference between nadir and T0 values. All analyses were done using R (version 3.1.2).

RESULTS

Univariate assessment of intervention effects and correlations with phenotype parameters

Weight loss intervention study. Figure 1A and B shows the *P*-value distribution of values for the weight loss effect on respectively fasting metabolite levels and their postprandial response as expressed by iAUC. As it is shown in the figure there is a more pronounced effect at fasting as compared to the postprandial response. Comparing obese subjects that underwent the weight loss and control interventions at fasting and with respect to challenge response, we found that

a range of metabolites are significantly different between two groups in either comparison. Metabolites that are significantly different between groups at fasting upon weight loss (Supplementary Table S2A) are not changed upon a mixed meal challenge (Supplementary Table S2B). Branched chain amino acids (BCAA) and amino acid derived acylcarnitines (AAAC) were among the most significantly different metabolites between groups at baseline.

Polyphenol intervention study. The P-value distribution of the polyphenol intervention effect on metabolite baseline and response (iAUC) values are presented in Figure 1C and D, respectively. The P-value distribution shows that only a small number of metabolites are significantly different between two groups either at fasting or in their postprandial response. This is also in line with the small number of metabolites on which the polyphenol intervention had a significant effect according to univariate testing (Supplementary Table S3A and 3B).

Correlation with phenotype parameters. For the weight loss study population a number of univariate correlations between metabolite levels and HOMA can be established [12]. The correlations between HOMA and branched chain amino acids, phenylalanine and tyrosine are in line with previous studies [23, 24]. In addition we could also find correlations between HOMA and C_2/C_n acylcarnitine ratios, which were recently brought forward as putative readouts for β-oxidation rate [25]. In an earlier study, we also found correlation between HOMA and Δ nadir and nadir acylcarnitine levels, in particular for amino acid derived ones [12]. The Δ nadir and nadir acylcarnitine values can be considered as a single parameter summary of their postprandial response [22] and the correlations with HOMA suggest they are related to fatty acid oxidation flux.

For the polyphenol intervention study, besides HOMA also ΔRQ and fat oxidation were measured via indirect calorimetry. We can observe a number of significant correlations between these phenotypical parameters with metabolites at fasting (Supplementary Table S4). These correlations however provide a scattered view on involvement of metabolic pathways. We also assessed whether fasting plasma C_2/C_n acylcarnitine ratios correlated with HOMA, ΔRQ, and fat oxidation. The only significant effect was correlation of C_2/2-methylbutyroylcanitine with HOMA (P= 0.02, ρ=-0.31). For the population of the polyphenol intervention study, correlation of Δ nadir and nadir acylcarnitine levels with phenotypic parameters (HOMA, ΔRQ, fat oxidation) did not show any significant effects.

Goeman's global test for assessment of intervention effects on plasma metabolome

Metabolites from the TCA cycle and the lactate pathway were grouped according to the KEGG database. For amino acid and fatty acid derived acylcarnitines no pathway information has been entered in KEGG. Hence we grouped metabolites according to branched amino

acid catabolism, comprising branched chain amino acids and derived acylcarnitines. Fatty acid derived carnitines (C8-C18) were also grouped in a pathway. We also grouped phenylalanine and tyrosine, since both these amino acids have consistently been associated with insulin resistance [24]. Oxylipins derived from arachidonic acid were also grouped in a pathway. An overview of metabolites collected in pathways is given in Table 1. The result of Goeman's global testing for assessment of lean vs obese differences and effect of weight loss on obese is presented in Table **2**. Three pathways, including the TCA cycle and BCAA catabolism are different between obese and lean subjects at fasting. The combination of phenylalanine and tyrosine was also significantly different between lean and obese. The last two pathways were also different between obese subjects before and after a weight loss intervention. Regarding the postprandial metabolic response, the difference between obese and lean subjects was limited to lactate and the Arg-Pro pathway. We note that here we present effects for iAUC values, which account for positive and negative contributions. The effects are similar when piAUC values are considered (Supplementary Table S5). The Goeman's global test could however not reveal significant (P<0.05) weight loss induced differences in postprandial response in obese subjects. The effect of weight loss on the enzymatic oxidation pathway of arachidonic acid did not reach statistical significance (P<0.09). The Goeman's global testing approach was also deployed to reveal pathways that were affected by the polyphenol intervention. No significant effect could however be observed for neither baseline metabolite levels nor the metabolic postprandial responses, irrespective of whether iAUC or piAUC values were used.

Goeman's global test for assessment of correlations of plasma metabolome with phenotypical variables

The populations of the weight loss and polyphenol intervention studies were well characterized with respect to insulin sensitivity (HOMA), metabolic flexibility (RQ) and baseline fat oxidation (Table 1a and 1b). HOMA was determined in both the weight loss and polyphenol intervention studies, whereas RQ and baseline fat oxidation were only determined in the polyphenol study. We explored whether the Goeman's global testing approach would provide a means to establish correlations between phenotype parameters and metabolic pathways. Goeman's global test revealed that for the population of the weight loss intervention study HOMA correlated with the fasting levels of metabolites from the TCA cycle, BCAA catabolism, lactate-glucose, Arginine-Proline and Phenylalanine-tyrosine pathways (Table 3). For this population the correlation between pathways and HOMA were more pronounced at fasting than for the postprandial response as expressed by iAUC (Table 3) or piAUC (Supplementary Table S5).

For the polyphenol study Goeman's global test revealed that HOMA is correlated with TCA cycle (P=0.02) and fatty acid oxidation pathways (P=0.06) as observed in fasting levels (Ta-

ble 3). For ΔRQ, fat oxidation, hepatic insulin resistance and peripheral insulin sensitivity no correlations could be established for any of the pathways when fasting levels were considered. Goeman's test also found no correlations between any of the phenotypical parameters (HOMA, ΔRQ, fat oxidation, hepatic insulin resistance, peripheral insulin sensitivity) and postprandial responses (iAUC and piAUC) when these were grouped in pathways.

DISCUSSION

Global testing of intervention effects on metabolic pathways. We applied Goeman's global test to determine whether sets of metabolites that are connected within a pathway collectively respond to an intervention. Our aim was to examine whether a phenotypic flexibility can be defined as a shift in baseline metabolism or by the postprandial metabolic response. A study by Hendrickx et al. revealed that Goeman's global test can be used to determine if the behaviour of a group of metabolites within the same pathway, is related to a specific outcome of interest [6]. We applied the Goeman's global test on two studies where volunteers respectively underwent a weight loss and polyphenol intervention. The weight loss intervention caused a more pronounced shift in baseline levels rather than in postprandial response as is shown in *P* distribution plot in Figure 1. For the weight loss intervention Goeman's global tests indeed showed significant effects at pathway level at fasting (Table 2). Metabolites involved in the TCA cycle have significantly different pattern between obese and lean subjects at fasting, which is in line with other studies [24]. Moreover, we observed an effect of weight loss on BCAA catabolism, and the combination of Phe-Tyr, which is in line with previous observations of changes in metabolic profiles accompanying an improvement in insulin resistance [23, 24]. In Chapter 4 we observed that the main metabolites for which the postprandial response was different before/after weight loss were oxylipins derived from arachidonic acid by enzymatic oxidation. Although the individual *P* values were significant for arachidonic acid derived oxylipins, when we test them collectively in Goeman's test the effect of weight loss on the postprandial response of this pathway was only $P = 0.09$.

In the dietary polyphenol intervention study, the *P* distribution plot indicated that only a small number of metabolites were affected at baseline or in their postprandial response. This was also reflected in the small number of metabolites that showed a significant effect according to univariate testing (Supplementary Table S3). Goeman's global test indeed did not reveal pathways that were significantly different before and after the polyphenol intervention. This is in line with the relative small effect of the polyphenol intervention on phenotypical parameters. Although the prolonged polyphenol supplementation stimulated fat oxidation and

increased mitochondrial capacity comparing to placebo, no significant effect on tissue-specific insulin sensitivity and metabolic flexibility in obese subjects was observed [8].

Goeman's global testing of correlations between pathways and phenotype parameters. The observed correlations of HOMA with metabolites involved in TCA cycle, BCAA catabolism and lactate pathway can be explained by their positive correlation with insulin resistance [23, 24]. However, Goeman's global test showed that the changes in postprandial response are smaller than the change at T0 after the intervention (Table 3). In the population of the weight loss study, more pathways were significant than for the polyphenol intervention study, in particular at fasting. As the HOMA range of obese subjects of two studies is comparable (Supplementary Table S1a, S1b), the lack of pronounced effects in polyphenol study might be due to lack of power and/or confounding with other phenotype parameters.

CONCLUSION

The application of Goeman's global test to two intervention studies indicates that it can provide a direct view on involvement of *a priori* defined pathways in phenotype shifts. The effect of weight loss intervention on *a priori* defined metabolic pathways was consistent with previous studies, as well as correlation of pathways with insulin sensitivity as described by HOMA. Goeman's global test, indicated that metabolic flexibility is more strongly reflected in pathways observed at baseline, than in their postprandial response. Hence we cannot confirm that the postprandial metabolic response to a mixed meal challenge provides a more sensitive read-out for a shift in phenotypic flexibility than fasting metabolism.

ACKNOWLEDGEMENT

This research was financially supported by Top Institute Food and Nutrition, Wageningen, The Netherlands, was part of the research program of the Netherland Metabolomics Centre (NMC), part of the Netherlands Genomics Initiative (NGI). TI Food and Nutrition is a public-private partnership on precompetitive research in food and nutrition. We thank Amy Harms from the Demonstration and Competency Laboratory at the Netherlands Metabolomics Centre (Leiden University) for expert training and delivery of high-quality metabolic profiles.

REFERENCES

1. van Ommen B, van der Greef J, Ordovas JM, Daniel H: **Phenotypic flexibility as key factor in the human nutrition and health relationship.** *Genes Nutr* 2014, **9:**423.
2. Huber M, Knottnerus JA, Green L, Horst Hvd, Jadad AR, Kromhout D, Leonard B, Lorig K, Loureiro MI, Meer JWMvd, et al: **How should we define health?** *BMJ* 2011, **343**.
3. Corpeleijn E, Saris WH, Blaak EE: **Metabolic flexibility in the development of insulin resistance and type 2 diabetes: effects of lifestyle.** *Obes Rev* 2009, **10:**178-193.
4. Elliott R, Pico C, Dommels Y, Wybranska I, Hesketh J, Keijer J: **Nutrigenomic approaches for benefit-risk analysis of foods and food components: defining markers of health.** *Br J Nutr* 2007, **98:**1095-1100.
5. Bakker GC, van Erk MJ, Pellis L, Wopereis S, Rubingh CM, Cnubben NH, Kooistra T, van Ommen B, Hendriks HF: **An antiinflammatory dietary mix modulates inflammation and oxidative and metabolic stress in overweight men: a nutrigenomics approach.** *Am J Clin Nutr* 2010, **91:**1044-1059.
6. Hendrickx DM, Hoefsloot HC, Hendriks MM, Canelas AB, Smilde AK: **Global test for metabolic pathway differences between conditions.** *Anal Chim Acta* 2012, **719:**8-15.
7. Joris PJ, Plat J, Kusters YH, Houben AJ, Stehouwer CD, Schalkwijk CG, Mensink RP: **Diet-induced weight loss improves not only cardiometabolic risk markers but also markers of vascular function: a randomized controlled trial in abdominally obese men.** *Am J Clin Nutr* 2016.
8. Most J, Timmers S, Warnke I, Jocken JW, van Boekschoten M, de Groot P, Bendik I, Schrauwen P, Goossens GH, Blaak EE: **Combined epigallocatechin-3-gallate and resveratrol supplementation for 12 wk increases mitochondrial capacity and fat oxidation, but not insulin sensitivity, in obese humans: a randomized controlled trial.** *Am J Clin Nutr* 2016, **104:**215-227.
9. Noga MJ, Dane A, Shi S, Attali A, van Aken H, Suidgeest E, Tuinstra T, Muilwijk B, Coulier L, Luider T, et al: **Metabolomics of cerebrospinal fluid reveals changes in the central nervous system metabolism in a rat model of multiple sclerosis.** *Metabolomics* 2012, **8:**253-263.
10. van der Kloet FM, Bobeldijk I, Verheij ER, Jellema RH: **Analytical Error Reduction Using Single Point Calibration for Accurate and Precise Metabolomic Phenotyping.** *Journal of Proteome Research* 2009, **8:**5132-5141.
11. Mihaleva VV, Korhonen SP, van Duynhoven J, Niemitz M, Vervoort J, Jacobs DM: **Automated quantum mechanical total line shape fitting model for quantitative NMR-based profiling of human serum metabolites.** *Anal Bioanal Chem* 2014, **406:**3091-3102.

12. Fazelzadeh P, Hangelbroek RWJ, Joris P, Mensink RP, Esser D, Afman L, Hankemeier T, Jacobs D, Mihaleva VV, Kersten S, et al: **Moderate effect of weight loss in obese volunteers on metabolic flexibility** *In progress* 2017.
13. Strimmer K: **fdrtool: a versatile R package for estimating local and tail area-based false discovery rates.** *Bioinformatics* 2008, **24:**1461-1462.
14. Strimmer K: **A unified approach to false discovery rate estimation.** *BMC Bioinformatics* 2008, **9:**303.
15. Carstensen M, Thomsen C, Hermansen K: **Incremental area under response curve more accurately describes the triglyceride response to an oral fat load in both healthy and type 2 diabetic subjects.** *Metabolism* 2003, **52:**1034-1037.
16. Pellis L, van Erk MJ, van Ommen B, Bakker GC, Hendriks HF, Cnubben NH, Kleemann R, van Someren EP, Bobeldijk I, Rubingh CM, Wopereis S: **Plasma metabolomics and proteomics profiling after a postprandial challenge reveal subtle diet effects on human metabolic status.** *Metabolomics* 2012, **8:**347-359.
17. Senn S: **Analysis of serial measurements in medical research.** *Bmj* 1990, **300:**680.
18. Goeman JJ, van de Geer SA, de Kort F, van Houwelingen HC: **A global test for groups of genes: testing association with a clinical outcome.** *Bioinformatics* 2004, **20:**93-99.
19. Kanehisa M, Goto S: **KEGG: kyoto encyclopedia of genes and genomes.** *Nucleic Acids Res* 2000, **28:**27-30.
20. Kanehisa M, Goto S, Furumichi M, Tanabe M, Hirakawa M: **KEGG for representation and analysis of molecular networks involving diseases and drugs.** *Nucleic Acids Res* 2010, **38:**D355-360.
21. Kanehisa M, Goto S, Hattori M, Aoki-Kinoshita KF, Itoh M, Kawashima S, Katayama T, Araki M, Hirakawa M: **From genomics to chemical genomics: new developments in KEGG.** *Nucleic Acids Res* 2006, **34:**D354-357.
22. Ramos-Roman MA, Sweetman L, Valdez MJ, Parks EJ: **Postprandial changes in plasma acylcarnitine concentrations as markers of fatty acid flux in overweight and obesity.** *Metabolism* 2012, **61:**202-212.
23. Newgard CB: **Interplay between lipids and branched-chain amino acids in development of insulin resistance.** *Cell Metab* 2012, **15:**606-614.
24. Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF, Haqq AM, Shah SH, Arlotto M, Slentz CA, et al: **A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance.** *Cell Metab* 2009, **9:**311-326.
25. Krug S, Kastenmuller G, Stuckler F, Rist MJ, Skurk T, Sailer M, Raffler J, Romisch-Margl W, Adamski J, Prehn C, et al: **The dynamic range of the human metabolome revealed by challenges.** *Faseb j* 2012, **26:**2607-2619.

Table 1. Collection of metabolites in pathways for Goeman's global testing

Pathway	*Number of metabolites in the group*	*Metabolites*
TCA cycle	8	Citric acid, malic acid,2-ketoglutaric acid, succinic acid, fumaric acid, pyroglutamic acid, cis-aconitic acid, pyrovate
BCAA catabolism	8	C3, C4, C4DC, C5, C5, Val, Leu, Ile
Fatty acid oxidation	17	C8, C10, C12, C14, C16, C18
Lactate pathway	2	Lactate, Pyruvate, Glucose
Arg, Pro	6	Methionine, Proline, Ornithine,Citrulline,4-hydroxyproline, Arginine
Phe, Tyr	2	Phenylalanine, Tyrosine
Enzymatic oxidation of arachidonic acid	5	TXB2, PGE2, 12S.HHTrE, 5.HETE, 11.HETE

Table 2. Goeman's global testing of differences in metabolic pathways (Table 1) between lean and obese volunteers and the effect of a weight loss (WL) intervention (compared to a control (CRT) intervention). Differences were tested for baseline values (T0) and postprandial response (iAUC).

Pathways and number of metabolites		Fasting		Response	
		T0 Obese vs lean	Δ T0 WL vs CRTL	iAUC Obese vs lean	ΔiAUC WL vs CRTL
TCA cycle	8	0.01	0.2	0.4	0.3
BCAA catabolism	7	0.02	0.02	0.5	0.5
Fatty acid oxidation	17	0.07	0.4	0.7	0.5
Lactate pathway	3	0.06	0.5	0.03	0.3
Arg, Pro	6	0.5	0.1	0.02	0.6
Phe, Tyr	2	0.01	0.03	0.3	0.3
Enzymatic oxidation of Arachidonic acid	5	0.6	0.5	0.3	0.09

Table 3. Goeman's global test results for correlations between baseline values, postprandial response (expressed as iAUC) and HOMA. The Goeman's global tests were performed separately for volunteers from the weight loss and polyphenol intervention studies. No significant effects were observed of effect of polyphenol intervention on postprandial response. Note that no arachidonic acid (AA) metabolites (oxylipins) were measured for the polyphenol intervention study.

Pathways and number of metabolites		Fasting		Postprandial response
		Weight loss intervention group	Polyphenol intervention group	Weight loss intervention group
TCA cycle	8	0.01	0.02	0.4
BCAA catabolism	7	0.04	0.4	0.6
Fatty acid oxidation	17	0.7	0.06	0.4
Lactate pathway	3	0.05	0.9	0.02
Arg, Pro	6	0.04	0.6	0.7
Phe, Tyr	2	0.02	0.04	0.5
Enzymatic oxidation of arachidonic acid	5	0.7	NA	0.4

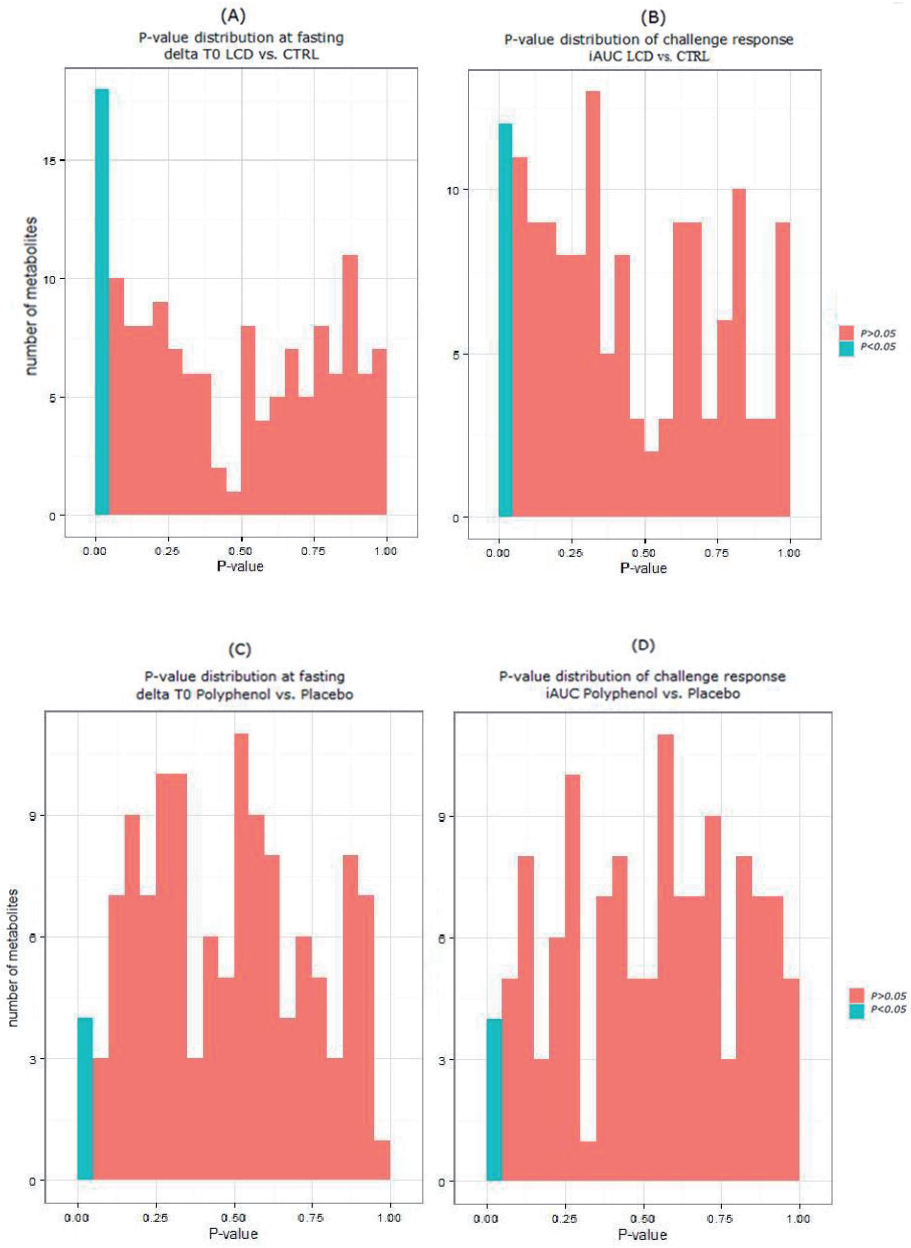


Figure 1. *P*- value distribution of effect of weight loss on baseline values in obese subjects (ΔWL T0 vs. Δ CTRL T0) (A), effect of weight loss on challenge response values in obese subjects (iAUC WL vs. iAUC CTRL) (B), effect of polyphenol intervention on baseline values in obese subjects (Δ PolyphenolT0 vs. Δ Placebo T0) (C), effect of polyphenol intervention on challenge response values in obese subjects (iAUC Polyphenol vs. iAUC Placebo) (D). Metabolites with $P<0.05$ were coloured green and otherwise red.

SUPPLEMENTARY MATERIAL

Table S1-a. Characteristics of lean subjects and obese subjects before and after weight loss (WL) or control (CRTL) interventions.

	Lean	WL(D1)	WL(D2)	CTRL(D1)	CTRL(D2)
Number	15	14	14	15	15
Age(y)	47.4 ± 4.5	44 ± 3.7		44.8 ± 3.4	
BMI(kg/m^2)	23.0 ± 0.6	30.0 ± 0.5	26.9 ± 0.5*	30.7 ± 0.7	30.4 ± 0.7
Glucose (mol/L)	5.1 ± 0.07	5.3 ± 0.12	5.0 ± 0.09	5.3 ± 0.09	5.3 ± 0.37
HOMA(IR)	1.62±0.1	3.00±0.4	1.75±0.2*	2.89±0.4	2.92±0.3

Data are presented as mean ± SEMs. *: A significant effect of weight loss (P<0.05). WL: weight loss, CTRL: control, D1, D2: before and after intervention (see also Figure 1), BMI: Body mass index.

Table S1-b. Characteristics of the subjects of the polyphenol study before and after intervention.

	Placebo		Polyphenol	
	Week 0	Week 12	Week 0	Week 12
Number	14		13	
Age (y)	41.4 ± 2.5		35 ± 3.1	
BMI (kg/m^2)	28.5 ± 0.8		29.6 ± 0.8	
Glucose(mol/L)	5.1 ± 0.1	5.1 ± 0.1	5.1 ± 0.07	5.1 ± 0.1
HOMA(IR)	2.2± 0.4	2.3 ± 0.3	1.9 ± 0.2	1.7 ± 0.1
RQ	0.78 ± 0.01	0.82 ± 0.02	0.80 ± 0.01	0.79 ± 0.01
ΔRQ	0.08 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.08 ± 0.01
Fat oxidation	0.08 ± 0.006	0.06 ± 0.01	0.07 ± 0.005	0.07 ± 0.008

Data are means ± SEMs. BMI: Body mass index

Table S2A. Univariate comparisons of effect of weight loss intervention on baseline values in obese subjects (ΔWLT0 vs. Δ CTRL T0)

Metabolite	P	lFDR
Amino acids & related metabolites		
Creatinine	<0.01	NS
Glycine	<0.01	NS
2 aminoadipic acid	0.01	NS
Isoleucine	0.01	NS
Leucine	0.01	NS
Creatine	0.02	NS
Carnitine	0.02	NS
Betaine	0.02	NS
4 hydroxyproline	0.03	NS
Tyrosine	0.05	NS
Valine	0.05	NS
Acylcarnitines		
Propionylcarnitine (C3)	<0.01	NS
2-Methylbutyroylcarnitine (C5)	<0.01	NS
TCA cycle & related metabolites		
Succinic acid	0.03	NS
Glutamic acid	0.04	NS

P<0.05 was considered significant. lFDR: local false discovery rate
NS:not significant

Table S2B. Comparison of effect of weight loss intervention on challenge response values in obese subjects (iAUC WL vs. iAUC CTRL)

Metabolites	*Day*Condition*	lFDR
Amino acids & related metabolites		
SDMA	0.02	NS
Dimethylglycine	0.03	NS
Methionine	0.04	NS
Asparagine	0.04	NS
Succinate	0.04	NS
Arginine	0.05	NS
Acylcarnitines		
Myristoyl (C12)	0.05	NS
Choline	0.05	NS
Oxylipins		
12.13.DiHOME	<0.01	NS
12S.HHTrE	0.04	NS
9.10.DiHOME	0.04	NS

P<0.05 was considered significant subjects according to univariate linear mixed models.lFDR: local false discovery rate
NS: not significant

Table S3A. Univariate comparisons of effect of polyphenol intervention on baseline values in obese subjects (Δ Polyphenol T0 vs. Δ Placebo T0)

Metabolite	P	lFDR
Amino acids & related metabolites		
Histidine	0.03	NS
Dopamine	0.05	NS
Acylcarnitines		
Octanoyl (C8)	0.01	NS
Decanoyl (C10)	0.03	NS

P<0.05 was considered significant.lFDR: local false discovery rate
NS:not significant

Table S3B. Comparison of effect of the polyphenol intervention on challenge response values in obese subjects (iAUC Polyphenol vs. iAUC Placebo)

Metabolite	Day*Group	lFDR
Amino acids & related metabolites		
Cystathionine	0.01	NS
Acylcarnitines		
Myristoyl (C12)	0.01	NS
Deoxy (C0)	0.01	NS
Isovaleryl (C5)	0.05	NS
Organic acids		
3 Hydroxybutyric acid	0.04	NS

P<0.05 was considered significant. lFDR: local false discovery rate
NS:not significant

Table S4. Univariate correlation of the fasting plasma metabolome of volunteers in polyphenol intervention study with HOMA, fat oxidation and ΔRQ.

	ΔRQ		HOMA		FAT Oxidation	
Metabolites	P	ρ	P	ρ	P	ρ
Proline	<0.01	-0.42				
Tiglylcarnitine	<0.01	-0.39				
Isovalerylcarnitine	<0.01	-0.38				
Glutathione	0.01	0.34	0.03	-0.30		
Betaine	0.01	-0.33				
Glycylglycine	0.02	0.32				
Methionine.sulfone	0.03	0.30				
Saccharopine	0.03	-0.30				
Pivaloylcarnitine	0.04	-0.30				
DL.3.aminoisobutyric acid	0.04	-0.28				
Propionylcarnitine	0.05	-0.28				
3.Methoxytyramine			<0.01	0.48		
Citricacid			<0.01	-0.46		
Beta.Alanine			<0.01	0.41		
Acetylcarnitine			<0.01	-0.37		
Oleylcarnitine			0.01	-0.36		
Asparagine			0.02	-0.33		
4.hydroxy.proline			0.02	0.32		
Norepinephrine			0.03	-0.32		
Tyrosine			0.03	0.31		
Cis.aconitic acid			0.03	-0.30		
Homocysteine					<0.01	0.40
S.Methylcysteine					<0.01	0.40
Kynurenine					0.01	0.36
Succinic acid					0.01	-0.34
Cysteine					0.02	0.32
Cystathionine					0.04	0.28

ρ: Pearson correlation coefficient.

Table S5. Goeman's global testing of differences in metabolic pathways observed during the postprandial response, expressed as piAUC. Goeman's global tests were performed separately for volunteers from the weight loss and polyphenol intervention studies. No significant effects were observed for effect of weight loss or polyphenol intervention on postprandial response, expressed as piAUC (data not shown).

Pathways and number of metabolites		Difference obese vs lean	Correlation with HOMA for weight loss intervention group
TCA cycle	8	NS	NS
BCAA catabolism	7	NS	NS
Fatty acid oxidation	17	NS	NS
Lactate pathway	3	0.06	0.05
Arg, Pro	6	0.1	NS
Phe, Tyr	2	NS	NS
Enzymatic oxidation of arachidonic acid	5	NS	NS

NS:not significant

6

General discussion

Recapturing aim

The metabolic phenotype is the product of interactions between several factors such as genetics, diet, lifestyle, and environment. The aim of this thesis was to measure health improvements by assessing subtle shifts in metabolic phenotype. Two approaches were applied to identify a phenotype shift. First, by looking at the effect of prolonged resistance-type exercise training on skeletal muscle tissue in older subjects and examining the possible shift toward younger subjects as a reference for a healthier phenotype. Second, by observing the response to a dietary challenge in obese subjects and examining the possible shift toward lean subjects as a reference for a healthier phenotype.

Main findings of this thesis

Chapter 2 and 3 of this thesis showed how the significant remaining plasticity of ageing skeletal muscle can adapt to resistance-type exercise training. It was shown that frail and healthy older subjects are two distinct phenotypes according to the skeletal muscle tissue metabolite profiles and that exercise training shifts aged muscle towards a more younger phenotype. It was also shown that the effect of exercise on amino acid derived acylcarnitines (AAAC's) in older subjects points towards decreased branched chain amino acid catabolism, possibly due to compromised activation of the branched chain α-keto acid hydrogenase (BCKDH) complex. The protocadherin gamma gene cluster was identified as a possible contributor to aged-muscle denervation and re-innervation. Moreover, it was found that plasma is not a direct indicator of muscle metabolism, emphasizing the need for direct assessment of metabolism in muscle tissue.

Chapter 4 and 5 of this thesis addresses whether a mixed meal challenge response could provide a readout for a shift in phenotype upon weight loss in obese male subjects. It was concluded that the impact of weight loss on the mixed meal postprandial response of plasma metabolites and PBMC gene expression was modest. Furthermore, assessment of metabolic health at the fasted state and during a post-prandial test yield distinct types of information.

Training induced shifts of older phenotypes toward young

In this thesis, healthy and frail older subjects were subjected to supervised resistance-type exercise training, a widely accepted strategy for tackling muscle wasting and weakness. Our study suggests a significant remaining plasticity of ageing skeletal muscle to adapt to regular resistance-type exercise and also a shift in the expression of a subset of genes towards levels observed in the younger group. At the gene expression level, the most striking finding in our study was the association of protocadherin gamma gene cluster (related to denervation and re-innervation) in skeletal muscle with age and muscle weakness, which might provide novel

insights into whether or not denervation and re-innervation is modulated by interventions or lifestyle factors such as nutrition and physical activity. Nevertheless, there is a lack of the knowledge at the pathway level to be able to target the expression of protocadherin gamma to improve age-related muscle weakness and functional decline (Chapter 2).

It has been shown that resistance-type exercise combined with amino acid ingestion elicits the greatest anabolic response and may help older subjects in producing a 'youthful' muscle protein synthetic response [1]. However, it is still debated whether older subjects respond in a similar manner to that of younger adults [2]. In this thesis, it was found that skeletal muscles of older subjects move toward a younger phenotype after resistance-type exercise training. Accordingly, resistance-type exercise in the elderly causes skeletal muscle to adopt features of more youthful skeletal muscle.

The primary differences in skeletal muscle metabolite levels between healthy older and young subjects were related to mitochondrial function, fibre type, and tissue turnover (Chapter 3). At the metabolite level, the most remarkable effects of resistance-type exercise training in older subjects were a significant decrease in the level of amino acid derived acylcarnitines (AAAC's) accompanied by an increase in branched chain amino acids level after training. This suggests a compromised response of a flux-determining mitochondrial branched chain α-keto acid hydrogenase (BCKDH) to prolonged resistance-type exercise training[3]. A decrease in branched chain amino acids oxidation may stimulate mTOR (a protein complex influencing protein synthesis and breakdown) related pathways activation [4, 5] and consequently protein synthesis, which is likely beneficial for older subjects. However, this mechanism occurs at the level of enzymatic activity, which prevents us from reaching strong conclusions based on the present data. Dickinson et al. demonstrated that mTOR signalling is necessary to stimulate the rate of muscle protein synthesis after resistance exercise [6]. A meta-analysis by Markofski et al. showed that there is no difference in fasting protein synthesis rates between young and old skeletal muscle, but both total and phosphorylated mTOR were elevated in muscle from fasted elderly individuals [7]. This may be due to either a compensatory response reflecting increased relative muscle loading in daily life or may be due to a dysregulation of the mTOR complex, influencing muscle mass in a complex and non-linear manner [8].

The gains in muscle strength and function in response to resistance-type exercise reflect improvements in multiple physiological factors, including coordination, muscle mass, and neuromuscular function. These improvements are observed in most healthy subjects that take up a resistance exercise program. Whether this also holds true for older subjects with pre-existing skeletal problems or disease such as diabetes is not fully clear [2]. A study by Churchward et al. demonstrated that there are no non-responders to the benefits of resistance-type exercise training (regarding lean body mass, fiber size, strength or function) in

older subjects. This implies that resistance-type exercise should be promoted without restriction to support healthy aging in the older population [9]. In our study population, all older subjects regardless of their health status (frail or healthy)and gender improved in muscle performance following 6 months of resistance-type exercise training, as illustrated by a significantly increase in leg extension and leg press strength post training [10-12]. However, lean body mass did not necessarily increase in all subjects, indicating that the strength increase must be primarily due to an increase in muscle quality, calcium handling, cross-bridge cycling, and neuromuscular adaptation instead of an increase in muscle cross-sectional area [10]. A review by Timmons et al., however, concluded that in human studies the demonstration of the efficacy of resistance-type training in older subjects is compromised by 'population stratification' [13]. Many human studies report spurious observations based on small sample size and high inter-subject variation. Therefore, not all researchers in the field agree on the scale of heterogeneous outcomes from exercise training. Using larger-scale prolonged studies and improving diagnostics of an individual's potential to respond to standardised physical training will help to perform studies with sufficient statistical power to allow for population stratification [13]. The key question is whether these kinds of studies are actually feasible? In this context, the standardisation of protocols (such as exercise load, meal composition, etc.) will be of prime importance to enable integration of independent studies. For body fluids the use of such standardised protocols has now become common practice in large scale metabolomics studies [14-16].

Weight loss induced shift of obese phenotypes

This thesis examined the phenotypic flexibility after applying a mixed meal challenge test with a combination of carbohydrates, fat and protein, i.e., an oral protein–glucose–lipid tolerance test. The assumption that underlies the use of mixed meal challenges is that it triggers all aspects of phenotypic flexibility [17].The observed plasma glucose responses in response to the mixed meal challenge were similar as compared to an OGTT, while the insulin response was prolonged in comparison with the glucose response [18]. The postprandial metabolic response was found to be different between obese and lean subjects, yet the difference was not as pronounced as for the OGTT challenge [19], which may be explained by the complexity of our mixed meal, affecting multiple metabolic pathways. A study by Esser at al. revealed that a MUFA challenge is more potent in eliciting differences between lean and obese subjects as compared to a SFA challenge [20]. Notably, the lipid composition of the mixed meal in our study is similar to SFA. It is possible that the usage of one single nutrient might be more effective at evoking differences in phenotypic responses between subjects. In addition, it allows for better understanding of the underlying biological mechanisms.

A mixed meal challenge is expected to provoke a more prolonged insulin response [17], possibly allowing for better differentiation between individuals. However, differentiation based on postprandial mixed meal responses was modest. Although a clear improvement in insulin sensitivity could be observed, the weight loss intervention had only a subtle effect on fasting and especially postprandial metabolites[21, 22] [4]. A recent study by Kardinaal *et al.* found that the changes observed in response to a challenge are a more sensitive biomarker of metabolic resilience than changes in fasting concentrations [23]. Compared to our study, the discrepancy might be due to the population under study, as the study by Kardinaal *et al.* involved subjects with the metabolic syndrome, whereas in our study the subjects were healthy overweight/obese. An alternative explanation may be differences in the type of challenge used. Indeed, Kardinaal *et al.* performed a high fat challenge, whereas in our weight loss study a mixed meal challenge was applied.

Therefore, based on our data we conclude that there is a moderate effect of weight loss on the mixed meal challenge response of the plasma metabolome and transcriptome of peripheral blood mononuclear cells of obese subjects. A recent study also found a modest number of differences in postprandial response to an OGTT between obese and lean subjects [19]. These differences were discussed qualitatively, without attempt to discuss statistical significance. The authors noticed a much stronger heterogeneity in the OGTT postprandial response of obese compared to lean. It is very likely that the heterogeneity in postprandial response to a mixed meal also explains why in our study only modest effects of weight loss were observed. Moreover, it is possible that the differences between lean and obese in the postprandial metabolic switch may be concealed by the direct influx of dietary amino acids and fatty acids.

FUTURE PERSPECTIVES

How to follow up with the challenge concept?

Chapter 4 and 5 showed, that the impact of weight loss on the mixed meal postprandial response of plasma metabolites and PBMC gene expression was modest. Based on these observation, the concept of using dietary challenge test to amplify differences in phenotypic flexibility between individuals may itself be challenged.

Firstly, the design of the challenge test should be carefully considered. Currently, there is no consensus on the composition of challenge tests for quantifying phenotype flexibility. Measuring a person's phenotypic flexibility can be performed by perturbing the homeostasis of that person, and quantifying the response of single or multiple markers during the postprandial phase. In Figure 1 the main physiological processes relevant to phenotypical flexibil-

ity are shown [17]. The overview in this figure illustrates the multitude of processes that can be perturbed by dietary challenges. Even a single dietary challenge like OGTT will impact on multiple organs, either directly or indirectly. In the postprandial phase also these organs will mutually interact as they are networked in our body. Unravelling the direct and indirect postprandial responses induced by single challenges is challenging but feasible [24]. It has been claimed that a multiple challenge such as a mixed meal is preferable since it more closely resembles metabolic perturbations encountered in daily life [17]. The outcome of the analysis in Chapter 4 however indicate that the metabolic response to a mixed meal challenge is difficult to be interpreted, even when using a range of profiling platforms to widely cover the plasma metabolome.

Secondly, those metabolic markers should be identified that can provide information on specific organ-dependent processes involved in phenotypical flexibility. Such processes can be measured by identifying the dynamics of the metabolic stress response in blood [17]. Upon a mixed meal challenge, the system is however flooded with dietary amino acids, fatty acids, carbohydrates, which may conceal the detection of the effects of the intervention on pathways involved in phenotypical flexibility. This can be resolved by observing the postprandial dynamics of acylcarnitines as acylcarnitines are only produced in the body and can be considered as proxies of amino acid and fatty acid metabolism. Both in muscle metabolomics (Chapter 3) and in the challenge responses (Chapter 4), clear effects of phenotype shifts were observed in the levels of acylcarnitines. A study by Ramos-Roman suggested the use of postprandial changes in plasma acylcarnitine concentrations as markers of fatty acid flux in overweight subjects [25]. Plasma acylcarnitines also have been proposed as biomarkers of insulin resistance and metabolic inflexibility in adults [26, 27], although this notion was recently contested [28]. It has been suggested that increased acylcarnitine levels in obese subjects may not only be due to an impairment in metabolism, but may also represent a natural response to an excess supply of lipid. Indeed, the increased production of acylcarnitine could result from excess fatty acid flux originating from lipid stored either intracellularly or peripherally [25]. These findings underscore the need for a better understanding of the postprandial response of acylcarnitines during the transition from fasting state to fed states in determining of how substrate overload might contribute to postprandial metabolic dysfunction.

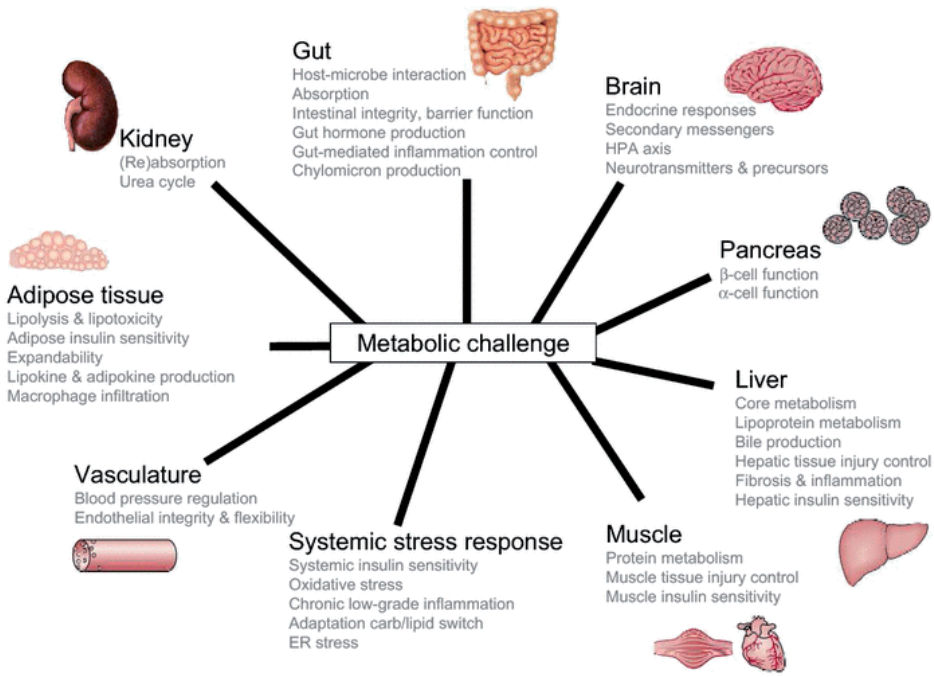


Figure 1. Overview of the physiological processes involved in phenotypic flexibility. By applying an optimal nutritional challenge, health effects related to nutrition can be quantified. (Figure reproduced from Stroeve *et al.* [17]).

Integration of metabolome with transcriptome.

Data integration is now a very commonly used practice in the life sciences and refers to the situation where, for a given system, multiple sources of data are available that are studied integratively to improve knowledge discovery [29]. It is becoming increasingly accepted that the integration of 'omics' data, such as transcriptomics, proteomics, and metabolomics will provide a better understanding of biological systems [30]. One of the challenges in data integration is the generation of interpretable results, which should help answer questions such as: which variables from multi-omics data sets are correlated with each other and which relevant variables provide the most insight into the biology underlying the experimental hypotheses?

This challenge can be addressed by performing variable selection, while combining the two types of variables in the modelled integration process. Gonzalez *et al.* developed and implemented a useful approach: a variant of partial least squares (PLS) regression called sparse PLS [30, 31], which simultaneously integrates and selects variables using lasso penalization [32]. In Chapter 2 sPLS regression analysis was carried out to calculate leg strength based on the expression levels of genes that are robustly changed after prolonged exercise training in

both groups in the baseline samples. It was shown using sPLS that there is a link between muscle strength and protocadherin gamma gene cluster. In Chapter 3 sPLS was used to integrate muscle changes in transcriptome and metabolome after training. We observed that amino acids, particularly branched chain amino acids, correlate with genes related to connective tissue/extracellular matrix. A direct link between the expression of these genes and the levels of these metabolites is unlikely, yet it does imply that these changes in amino acid levels are part of the adaptive response to resistance-type exercise training. In Chapter 4 it was shown that the effect of a stressor (mixed meal) on PBMC gene expression is rather weak. Accordingly, using PBMC gene expression to measure phenotypic flexibility might not be an appropriate approach. Moreover, integrated interpretation of the PBMC transcriptome with the plasma metabolome is difficult since they both represent systemic effects, meaning that they reflect the response of multiple organs to a phenotype shift. Besides that, many regulatory mechanisms occurs at the enzymatic level, requiring a proteomics type of approach. Furthermore, attempts to integrate the plasma metabolome, proteome and gene expression in PBMCs may be hampered due to different time-scales in regulation of metabolism, protein turnover and gene expression. Integration of different omics levels (metabolomics, proteomics, transcriptomics) applied to body fluids sampled from the systemic circulation will therefore not likely be successful. An organ-focussed approaches that integrate multiple omics levels using system biology approaches is expected to be more feasible [33]. In such approaches, the metabolome, proteome and transcriptome can be sampled from a single compartment, and time-dependent relationships can be modelled in a direct manner.

Metabolomics profiling of tissues: how to move on?

Chapter 3 showed that the correlation between skeletal muscle and plasma metabolites is rather weak. This finding indicates that plasma metabolites provide a poor reflection of muscle metabolism, suggesting that they cannot be used as a substitute biomarker. Current procedures for obtaining tissue biopsies from human volunteers are labour intensive, can cause discomfort, pain and in some cases risk, thereby raising the bar for obtaining ethical approval. Examples are liver biopsy or repeated muscle biopsies. As a consequence, most human studies focusing on organ metabolomics and/or transcriptomics are carried out on a minimal number of volunteers and are poorly powered. To allow for larger scale organ specific studies, we need innovative tissue sampling methods that can be used with high-throughput and without discomfort and risk for human volunteers. Availability of less invasive/painful sampling techniques would in particular enable larger scale studies on adipose tissue and skeletal muscle.

The Bergstrom needle muscle biopsy technique has long been considered as the gold stan-

dard in muscle tissue sampling [34] and has provided a wealth of information for researchers. However, its major drawback is that it is moderately invasive, requiring an incision through the skin and fascia ranging from 5 to 10 mm [34]. Consequently, this technique may not be suitable for research designs that require repeated sampling. As a minimally invasive technique 'microbiopsy' (or fine needle aspiration) has been implemented using a small gauge needle for tissue sampling without the need for an incision [35, 36], allowing for less invasive collection of skeletal muscle samples. However, the small sample size obtained is often deemed insufficient for certain analyses. Using a recently developed robust extraction procedure, metabolites could be recovered at high yields and in a reproducible manner [37, 38]. For dietary studies, Beynen and Katan [39] adapted the original needle-biopsy method of Hirsch *et al.* [40] by using an 18-gauge needle, and reported collecting a 'small amount of visible material'. This mini-biopsy technique collects essentially small globules of fat sufficient for dietary studies. Another study compared two sampling methods of subcutaneous adipose tissue using either a 14 gauge needle with incision versus a 16 gauge needle without incision to examine biomarkers of cancer risk [41]. Having less invasive sampling techniques and using small amounts of tissue would enable larger scale human studies to more accurately define phenotypical shifts due to diet or lifestyle interventions.

REFERENCES

1. Breen L, Phillips SM: **Skeletal muscle protein metabolism in the elderly: Interventions to counteract the 'anabolic resistance' of ageing.** *Nutr Metab (Lond)* 2011, **8:**68.
2. Greig CA, Gray C, Rankin D, Young A, Mann V, Noble B, Atherton PJ: **Blunting of adaptive responses to resistance exercise training in women over 75y.** *Exp Gerontol* 2011, **46:**884-890.
3. Shimomura Y, Murakami T, Nakai N, Nagasaki M, Harris RA: **Exercise promotes BCAA catabolism: effects of BCAA supplementation on skeletal muscle during exercise.** *J Nutr* 2004, **134:**1583s-1587s.
4. Newgard CB: **Interplay between lipids and branched-chain amino acids in development of insulin resistance.** *Cell Metab* 2012, **15:**606-614.
5. Um SH, D'Alessio D, Thomas G: **Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1.** *Cell Metab* 2006, **3:**393-402.
6. Dickinson JM, Fry CS, Drummond MJ, Gundermann DM, Walker DK, Glynn EL, Timmerman KL, Dhanani S, Volpi E, Rasmussen BB: **Mammalian target of rapamycin complex 1 activation is required for the stimulation of human skeletal muscle protein synthesis by essential amino acids.** *J Nutr* 2011, **141:**856-862.
7. Markofski MM, Dickinson JM, Drummond MJ, Fry CS, Fujita S, Gundermann DM, Glynn EL, Jennings K, Paddon-Jones D, Reidy PT, et al: **Effect of age on basal muscle protein synthesis and mTORC1 signaling in a large cohort of young and older men and women.** *Exp Gerontol* 2015, **65:**1-7.
8. Phillips BE, Williams JP, Gustafsson T, Bouchard C, Rankinen T, Knudsen S, Smith K, Timmons JA, Atherton PJ: **Molecular networks of human muscle adaptation to exercise and age.** *PLoS Genet* 2013, **9:**e1003389.
9. Churchward-Venne TA, Tieland M, Verdijk LB, Leenders M, Dirks ML, de Groot LC, van Loon LJ: **There Are No Nonresponders to Resistance-Type Exercise Training in Older Men and Women.** *J Am Med Dir Assoc* 2015, **16:**400-411.
10. Hangelbroek RWJ, Fazelzadeh P, Tieland M, Boekschoten MV, Hooiveld GJEJ, van Duynhoven JPM, Timmons JA, Verdijk LB, de Groot LCPGM, van Loon LJC, Müller M: **Expression of protocadherin gamma in skeletal muscle tissue is associated with age and muscle weakness.** *Journal of Cachexia, Sarcopenia and Muscle* 2015.
11. Tieland M, Dirks ML, van der Zwaluw N, Verdijk LB, van de Rest O, de Groot LCPGM, van Loon LJC: **Protein Supplementation Increases Muscle Mass Gain During Prolonged Resistance-Type Exercise Training in Frail Elderly People: A Randomized, Double-Blind, Placebo-Controlled Trial.** *Journal of the American Medical Directors Association* 2012, **13:**713-719.

12. Leenders M, Verdijk LB, Van der Hoeven L, Van Kranenburg J, Nilwik R, Wodzig WKWH, Senden JMG, Keizer HA, Van Loon LJC: **Protein supplementation during resistance-type exercise training in the elderly.** *Medicine and science in sports and exercise* 2013, **45:**542-552.
13. Timmons JA, Gallagher IJ: **Molecular studies of exercise, skeletal muscle, and ageing.** *F1000Res* 2016, **5**.
14. Dona AC, Jimenez B, Schafer H, Humpfer E, Spraul M, Lewis MR, Pearce JT, Holmes E, Lindon JC, Nicholson JK: **Precision high-throughput proton NMR spectroscopy of human urine, serum, and plasma for large-scale metabolic phenotyping.** *Anal Chem* 2014, **86:**9887-9894.
15. Beckonert O, Keun HC, Ebbels TM, Bundy J, Holmes E, Lindon JC, Nicholson JK: **Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts.** *Nat Protoc* 2007, **2:**2692-2703.
16. Emwas AH, Luchinat C, Turano P, Tenori L, Roy R, Salek RM, Ryan D, Merzaban JS, Kaddurah-Daouk R, Zeri AC, et al: **Standardizing the experimental conditions for using urine in NMR-based metabolomic studies with a particular focus on diagnostic studies: a review.** *Metabolomics* 2015, **11:**872-894.
17. Stroeve JH, van Wietmarschen H, Kremer BH, van Ommen B, Wopereis S: **Phenotypic flexibility as a measure of health: the optimal nutritional stress response test.** *Genes Nutr* 2015, **10:**459.
18. Casas-Agustench P, Lopez-Uriarte P, Bullo M, Ros E, Gomez-Flores A, Salas-Salvado J: **Acute effects of three high-fat meals with different fat saturations on energy expenditure, substrate oxidation and satiety.** *Clin Nutr* 2009, **28:**39-45.
19. Geidenstam N, Spegel P, Mulder H, Filipsson K, Ridderstrale M, Danielsson AP: **Metabolite profile deviations in an oral glucose tolerance test-a comparison between lean and obese individuals.** *Obesity (Silver Spring)* 2014, **22:**2388-2395.
20. Esser D, van Dijk SJ, Oosterink E, Lopez S, Muller M, Afman LA: **High fat challenges with different fatty acids affect distinct atherogenic gene expression pathways in immune cells from lean and obese subjects.** *Mol Nutr Food Res* 2015, **59:**1563-1572.
21. Shah SH, Crosslin DR, Haynes CS, Nelson S, Turer CB, Stevens RD, Muehlbauer MJ, Wenner BR, Bain JR, Laferrere B, et al: **Branched-chain amino acid levels are associated with improvement in insulin resistance with weight loss.** *Diabetologia* 2012, **55:**321-330.
22. Wahl S, Vogt S, Stuckler F, Krumsiek J, Bartel J, Kacprowski T, Schramm K, Carstensen M, Rathmann W, Roden M, et al: **Multi-omic signature of body weight change: results from a population-based cohort study.** *BMC Med* 2015, **13:**48.

23. Kardinaal AF, van Erk MJ, Dutman AE, Stroeve JH, van de Steeg E, Bijlsma S, Kooistra T, van Ommen B, Wopereis S: **Quantifying phenotypic flexibility as the response to a high-fat challenge test in different states of metabolic health.** *Faseb j* 2015, **29:**4600-4613.
24. Wopereis S, Wolvers D, van Erk M, Gribnau M, Kremer B, van Dorsten FA, Boelsma E, Garczarek U, Cnubben N, Frenken L, et al: **Assessment of inflammatory resilience in healthy subjects using dietary lipid and glucose challenges.** *BMC Med Genomics* 2013, **6:**44.
25. Ramos-Roman MA, Sweetman L, Valdez MJ, Parks EJ: **Postprandial changes in plasma acylcarnitine concentrations as markers of fatty acid flux in overweight and obesity.** *Metabolism* 2012, **61:**202-212.
26. Adams SH, Hoppel CL, Lok KH, Zhao L, Wong SW, Minkler PE, Hwang DH, Newman JW, Garvey WT: **Plasma acylcarnitine profiles suggest incomplete long-chain fatty acid beta-oxidation and altered tricarboxylic acid cycle activity in type 2 diabetic African-American women.** *J Nutr* 2009, **139:**1073-1081.
27. Mihalik SJ, Goodpaster BH, Kelley DE, Chace DH, Vockley J, Toledo FG, DeLany JP: **Increased levels of plasma acylcarnitines in obesity and type 2 diabetes and identification of a marker of glucolipotoxicity.** *Obesity (Silver Spring)* 2010, **18:**1695-1700.
28. Schooneman MG, Napolitano A, Houten SM, Ambler GK, Murgatroyd PR, Miller SR, Hollak CE, Tan CY, Virtue S, Vidal-Puig A, et al: **Assessment of plasma acylcarnitines before and after weight loss in obese subjects.** *Arch Biochem Biophys* 2016, **606:**73-80.
29. Gomez-Cabrero D, Abugessaisa I, Maier D, Teschendorff A, Merkenschlager M, Gisel A, Ballestar E, Bongcam-Rudloff E, Conesa A, Tegnér J: **Data integration in the era of omics: current and future challenges.** *BMC Systems Biology* 2014, **8:**1-10.
30. Lê Cao K-A, González I, Déjean S: **integrOmics: an R package to unravel relationships between two omics datasets.** *Bioinformatics* 2009, **25:**2855-2856.
31. Le Cao KA, Martin PG, Robert-Granie C, Besse P: **Sparse canonical methods for biological data integration: application to a cross-platform study.** *BMC Bioinformatics* 2009, **10:**34.
32. Tibshirani R: **Regression shrinkage and selection via the lasso: a retrospective.** *Journal of the Royal Statistical Society: Series B (Statistical Methodology)* 2011, **73:**273-282.
33. Zewail AH: **Physical Biology: From Atoms to Medicine.** 2008:567
34. Bergstrom J: **Percutaneous needle biopsy of skeletal muscle in physiological and clinical research.** *Scand J Clin Lab Invest* 1975, **35:**609-616.
35. Friedmann-Bette B, Schwartz FR, Eckhardt H, Billeter R, Bonaterra G, Kinscherf R: **Similar changes of gene expression in human skeletal muscle after resistance exercise and multiple fine needle biopsies.** *J Appl Physiol (1985)* 2012, **112:**289-295.

36. Pietrangelo T, D'Amelio L, Doria C, Mancinelli R, Fulle S, Fano G: **Tiny percutaneous needle biopsy: An efficient method for studying cellular and molecular aspects of skeletal muscle in humans.** *Int J Mol Med* 2011, **27:**361-367.
37. Alves RAM, Dane A, Harms A, Strassburg K, Seifar R, Verdijk L, Kersten S, Berger R, Hankemeier T, Vreeken R: **Global profiling of the muscle metabolome: method optimization, validation and application to determine exercise-induced metabolic effects.** *Metabolomics* 2015, **11:**271-285.
38. Chen S, Hoene M, Li J, Li Y, Zhao X, Haring HU, Schleicher ED, Weigert C, Xu G, Lehmann R: **Simultaneous extraction of metabolome and lipidome with methyl tert-butyl ether from a single small tissue sample for ultra-high performance liquid chromatography/mass spectrometry.** *J Chromatogr A* 2013, **1298:**9-16.
39. Beynen AC, Katan MB: **Rapid sampling and long-term storage of subcutaneous adipose-tissue biopsies for determination of fatty acid composition.** *Am J Clin Nutr* 1985, **42:**317-322.
40. Hirsch J, Farquhar JW, Ahrens EH, Jr., Peterson ML, Stoffel W: **Studies of adipose tissue in man. A microtechnic for sampling and analysis.** *Am J Clin Nutr* 1960, **8:**499-511.
41. Campbell KL, Makar KW, Kratz M, Foster-Schubert KE, McTiernan A, Ulrich CM: **A pilot study of sampling subcutaneous adipose tissue to examine biomarkers of cancer risk.** *Cancer prevention research (Philadelphia, Pa)* 2009, **2:**37-42.

Summary

Human health is impacted by a complex network of interactions between biological pathways, mechanisms, processes, and organs, which need to be able to adapt to a continuously changing environment to maintain health. This adaptive ability is called 'phenotypic flexibility'. It is thought that health is compromised and diseases develop when these adaptive processes fail. As the product of interactions between several factors such as genetic makeup, diet, lifestyle, environment and the gut microbiome, the 'metabolic phenotype' provides a readout of the metabolic state of an individual. Understanding these relationships will be one of a major challenges in nutrition and health research in the next decades. To address this challenge, the development of high-throughput omics tools combined with the application of elaborate statistical analyses will help characterize the complex relationship of (bio) chemicals in human systems and their interaction with other variables including environment and lifestyle to produce the measured phenotype.

An important aim of this thesis was to identify phenotype shifts by looking at effect of prolonged resistance-type exercise training on skeletal muscle tissue in older subjects and the possible shift toward the features of younger subjects as a reference for a healthier phenotype. A second aim was to identify phenotype shifts by looking at the response to a challenge in obese subjects and the possible shift toward lean subjects as a reference for a healthier phenotype.

Chapter 2 and 3 of this thesis show how the significant remaining plasticity of ageing skeletal muscle can adapt to resistance-type exercise training. The data indicate that frail and healthy older subjects have two distinct phenotypes according to the skeletal muscle tissue metabolite profiles and that exercise training shifts aged muscle towards a younger phenotype. We showed that the effect of exercise on amino acid derived acylcarnitines (AAAC's) in older subjects points towards decreased branched chain amino acid catabolism, likely due to compromised activation of the branched chain α-keto acid hydrogenase (BCKDH) complex. Furthermore, we found that the protocadherin gamma gene cluster might be involved in aged-muscle denervation and re-innervation. Finally, plasma was found to be a poor indicator of muscle metabolism, emphasizing the need for direct assessment of metabolites in muscle tissue.

Chapter 4 of this thesis examines whether a mixed meal challenge response provides a readout for a shift in phenotype upon weight loss in obese male subjects. We concluded that weight loss moderately affects the mixed meal challenge response of both plasma metabolome and transcriptome of peripheral blood mononuclear cells in obese subjects. Measurements at the fasted and postprandial state also provide us with a different type of information. In Chapter 5 it is demonstrated that the global testing of pathways could provide a concise summary of the multiple univariate testing approach used in Chapter 4.

In Chapter 6 it is discussed how the findings of this thesis increase our understanding of how to measure phenotypic flexibility as a proxy of health. In this thesis it is shown that the correlations between tissue and plasma metabolites are rather weak, emphasising the need to perform organ-specific studies. Availability of less invasive/painful sampling techniques and the use of small amounts of tissue would enable larger scale human studies on adipose tissue and skeletal muscle to more accurately define phenotypical shifts due to diet or lifestyle interventions. With respect to the assessment of phenotypical flexibility by omics approaches, significant complications can be expected in trying to relate plasma metabolism to PBMC gene expression. Organ-focussed approaches that integrate multiple omics levels using system biology approaches are considered to be a lot more promising.

Acknowledgement

It is really hard to explain how much I appreciate the educational and official systems and supports in the Netherlands that made my life easier to stay in the small city of Wageningen, “The City of Life Sciences”, and to gather knowledge. Thanks to WUR and all the people working here to keep its rank high in the world. Indeed, I would like to mention some specific persons with whom I was involved directly.

At first I would like to thank my first supervisor **Prof. Michael Müller**. Prof. Michael, you are a great man with vast knowledge about Nutrigenomics. I was so lucky that I had the opportunity to be one of your PhD students. We had nice and useful discussions every Friday. The first slide of your all presentations is unforgettable to me: “We are what we eat”.

My supervisor **Prof. Sander Kersten**, I am really pleased to be one of your PhD students. You are a great man with brilliant thoughts and ideas. Your inspiration helped me to look at various issues from different angles to increase the quality of my work. Thank you for all the support and help.

My supervisor **Prof. John van Duynhoven**, we had non-stopping discussions over the last four years. You were always positive, full of ideas which helped me to progress continuously. Without your efforts and guidance it should not have been possible to finish my thesis on time. You always kept things up to date in research which is appreciable, I admire your vast knowledge in many areas that inspired me really. You never minded disturbing you; even during weekends you sent me new feedbacks over my work. Your appreciation and inspiration brought me in this position today. “Hope” is a word that is highly “correlated” with your personality and I am happy that I also feel the same internally. I’ll never forget your efforts during my PhD period.

My supervisor **Dr. Mark Boekschoten**, I call you “Sure man” because every time that I asked something the answer was “Sure” with a kind smile. You are a very kind person who likes to support by all means. I do appreciate all your attempts to make life flexible for me. The first months of my PhD your lovely daughter Alice was born, now she is 4.5 years old, she can talk, she can play, she can walk; This shows that how much time it takes to get a PhD and how much time a supervisor should spend for one PhD candidate to make her/his research done.

I would like to thank my paranymphs **Charlotte** and **Roland** for accepting my request on the big day in my educational life.

Dr. Guido Hooiveld, how I can explain you. I borrow a phrase from Frits’ Thesis; ”Elke lab nodig een Guido”. En ik wil graag zeggen: “Natuurlijk”. When I started analysing array data, everything looked like a puzzle to me. You took my hand through each step and put all the pieces of a puzzle in a right position and those together made my thesis. We emailed a lot over R problems. Now, when I look back to all emails I can’t stop laughing at myself regarding the subjects of my emails to you. Here are some: “ Question” , “Vraagen”, ”I got an error” ,

"one more question", last question", "sorry for disturbing you again". Never ever forget our discussion over "R".

Dear **Prof. Jacques Vervoort**, we had nice discussion through different topics from science to culture, and history. Your knowledge about different topics is adorable. I had very nice time working with you for my master thesis. I still wonder how you can survive eating two apples for lunch! I never forget your favourite story about difference between males and females which states that years ago males used to chase animals for hunting and females used to stay at home with children eating all the time and that makes the difference!

Dear **Prof. Wim Saris**, I would like to thank you for involving me in the Diogenes and Muscle Health projects. You were always so calm with a beautiful warm smile.

My best colleague **Roland Hangelbroek**, I really enjoyed four years of my life working with you. You are smart, creative, supportive, calm person. As a muscle builder, you really improved our muscle projects from confusing points to exploring the Protocadherin gamma gene cluster. This is really awesome. We have some common phrase such as "Don't worry about it", with pure American accent or "we need to wrap it up now". Whenever I look at a bird I also remember your childhood as a very silent good boy: "binnen zitten en vogel kijken"!! Beside science we also progress in lekker broed maken met Katinka konijn! Many, many, many thanks for a good time.

Dear **Phillip de Groot**, you are a good bioinformatician. You helped me a lot with analysing microarray data. I always remember you on 23rd February as we both celebrate this special day as our birthday.

Age, Huub, Johan, you are all good biostatisticians. I liked it when we discussed statistical topics, and I learned a lot from that.

Shohreh, I am really proud of you, my compatriot. You are a strong person. I learned from you how to overcome difficult moments of my life. Even at very tough moments, your face was covered with a beautiful big smile. My especial thanks to you and Jenny for all the work you did to generate the wet lab and microarray data.

Juri, you know some Persian sentences like "Kafsh khoob ast", "Kafsh ghashang nist" and I learned even more Dutch like "lekker binnen zitten, lekker drinken, lekker eten, goed filmpje kijken". Thank you so much for nice moments.

Mechteld, Jenny, Mieke, Karin, Carolien, I would like to thank your kindness and support. Thanks for the pleasant talks we had at the coffee corner. We chat about Dutch culture and language together. Those discussions helped me to pass the Inburgering exam. Thank you so much.

Lydia you have vast knowledge about PBMC gene expression and helped me alot to improve my work. **Diederik** you are a very good colleague. You brought piece and calm to last

moments of my PhD with your nice book. You know that I am the only who have 2 copies, one on my desk and one at home. It helped me to organise my own book. Your contribution on the forth chapter was very significant. Thanks you both for your help.

I would like to thank Pink Ladies Community **(Inge,Nikkie,Katja,Jvalini** and of course **the fish!)**. I would like to also thank **Jocelijn, Charlotte, Lily, Aafke, Wieneke, Micheal, Wilma, Sophie, Rogier, Merel, Marco, Klaske, Rinke** and **Philip,** all of you are cordial and helpful. **Ya, Neeraj, Montserrat** we used to have one thing in common, "Missing sun" in the Nederlands. We had lots of discussions about it. Still, when we had a sunny week in July, again we complained about no air-condition at home! So, you see humans can be so strange! When you get something that you wish, you expect something else! I wish you all the best and success with your PhD and of course with your life!

I would like to thank **Didi, Jasmijn, Gea, Cornelia, Adrienne, Yvonne, Vesna** and **Ilkay** for their support in many official tasks; without your support the completion of my PhD thesis would not have been possible. You are the key to smooth organizational and financial things.

Jesse van dam, I never forget your support all during my Master and PhD time. Bioinformatics was a completely new field for me and without your help I could not stand at where I am now. We were in the same group for most of our projects from 2010 to 2012. We really had great time, especially evening walking and cooking after long day work. You are one of the unforgettable people in my mind. I wish you all happiness through your life.

Dear **Jan Niels,** you are a good man, so kind, so supportive. I do appreciate all your attempts to help me with different challenges that I faced here. Every time I called you, you were at my door in no time. I never forget all your kindness to me. Also we practiced Dutch language sometimes. We laughed so many times over this wrong phrase of mine, "Dat is a klopt". I like this Dutch phrase also "Logees en vis zijn drie dagen fris."

I would like to thank the thesis committee members for their time to evaluate my thesis and joining in my PhD defence day.

I have to commend the persistent patience, perseverance and support of my parents, my beautiful sisters, my lovely brother and family members during my PhD period. My kind, supportive father, you left us so early; I know today your dreams come true and you are so happy for your daughter's success. You are always in my heart. "God bless you", my hero. My special thanks goes to my mother, a symbol of patience and strength. Thank you very much.

I may have missed some people to mention their name here, THANK YOU ALL.

May 2017, Wageningen

About the author

CURRICULUM VITAE

Parastoo Fazelzadeh was born on February 23, 1977 in Amol, Iran. In 1995, she completed high school in Amol. Thereafter she started her bachelor in biology at Avicenna University of Hamedan. She worked in Pars hospital in Tehran and for years she supervised a clinical and pathological laboratory. She started her master in Bioinformatics at Wageningen University in 2010. The study was made possible through obtaining a competitive NUFFIC fellowship. She did her master thesis at the department of Biochemistry of Wageningen University, where she investigated the metabolic control of obesity with large scale datasets from a human cohort (> 700 individuals) who were involved in a weight loss/weight maintenance study.

She performed her second master thesis at the National Genotype Center in Genopole, Evry, France, where she investigated the relationship of genetic information as obtained from large scale SNP studies to metabolic and anthropometric data of a European study related to obesity. One of the results obtained from this study is that there is a clear relationship between the rate limiting steps in the urea cycle, CPS1, to weight maintenance.

Immediately after getting her MSc Degree in Bioinformatics, Parastoo started her PhD project “How to measure health improvements? Assessment of a subtle shift in metabolic phenotype”, at the Nutrition, Metabolism and Genomics group of Wageningen university under the supervision of Prof.dr. Sander Kersten, Prof.dr. John van Duynhoven and Dr. Mark Boekschoten. During her PhD project, she performed challenge studies in humans with a focus on phenotypic flexibility and health. During her PhD project, Parastoo was also involved in teaching and supervising students during their master thesis.

LIST OF PUBLICATIONS

Network Analysis of Metabolite GWAS Hits: Implication of CPS1 and the Urea Cycle in Weight Maintenance.
Matone A, Scott-Boyer MP, Carayol J, **Fazelzadeh P,** Lefebvre G, Valsesia A,Charon C, Vervoort J, Astrup A, Saris WH, Morine M, Hager J.
PLoS One. 2016 Mar 3;11(3):e0150495

The Muscle Metabolome Differs between Healthy and Frail Older Adults.
Fazelzadeh P, Hangelbroek RW, Tieland M, de Groot LC, Verdijk LB,van LoonLJ, Smilde AK, Alves RD, Vervoort J, Müller M, van Duynhoven JP, Boekschoten MV. J Proteome Res. 2016 Feb 5;15(2):499-509. doi: 0.1021/acs.jproteome.5b00840. Epub 2016 Jan 22.

Expression of protocadherin gamma in skeletal muscle tissue is associated with age and muscle weakness.
Hangelbroek RW, **Fazelzadeh P**, Tieland M, Boekschoten MV, Hooiveld GJ,van Duynhoven JP, Timmons JA, Verdijk LB, de Groot LC, van Loon LJ, Müller M.
J Cachexia Sarcopenia Muscle. 2016 Feb 2. doi: 10.1002/jcsm.12099.

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

Weight loss moderately affects the mixed meal challenge response of the plasma metabolome and transcriptome of peripheral blood mononuclear cells in obese subjects.
Fazelzadeh P , Hangelbroek R, Joris P, Schalkwijk C, Esser D, Afman L, Hankemeier T, Jacobs D, Mihaleva V, Kersten S, van Duynhoven J, Boekschoten M.
(Submitted).

Global testing of shifts in metabolic phenotype.
Fazelzadeh P,Hoefsloot H, Hankemeier T, Most J, Kersten S, Blaak E, Boekschoten M and van Duynhoven J.
(Manuscript in preparation).

Weight loss-induced cellular stress in subcutaneous adipose tissue and the risk for weight regain in overweight and obese adults.
Roumans NJ, Vink RG, Bouwman FG, **Fazelzadeh P**, van Baak MA, Mariman EC.
Int J Obes (Lond). 2016 Dec 5. doi: 10.1038/ijo.2016.221

Diet-induced weight loss decreases adipose tissue oxygen tension with parallel changes in adipose tissue phenotype and insulin sensitivity in overweight humans.
Vink RG, Roumans NJ, *Čajlaković M*, Cleutjens JP, Boekschoten MV, **Fazelzadeh P**, Vogel MA, Blaak EE, Mariman EC, van Baak MA, Goossens GH.
Int J Obes (Lond). 2017 Feb 9. doi: 10.1038/ijo.2017.38.

Adipose tissue gene expression is differentially regulated with different rates of weight loss in overweight and obese humans.
Vink RG, Roumans NJ, **Fazelzadeh P**, Tareen SH, Boekschoten MV, van Baak MA, Mariman EC.
Int J Obes (Lond). 2017.

Adipose tissue meal-derived fatty acid uptake is not enhanced after diet-induced weight loss in overweight and obese adults.
Vink RG, Roumans NJ, van der Kolk B, **Fazelzadeh P**, Boekschoten MV, Mariman EC and van Baak MA
(Submitted to American Journal of Clinical Nutrition)

A role for adipose tissue inflammation and ECM remodelling in the risk of weight regain after weight loss.
Roumans NJ, Vink RG, **Fazelzadeh P**, van Baak MA and Mariman EC.
(Accepted for publication in The American Journal of Clinical Nutrition).

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

OVERVIEW OF COMPLETED EDUCATIONAL ACTIVITIES

Discipline specific activities

Introduction to nutritional metabolomics (Copenhagen, Denmark, 2014)
Signal proteins related to diet and exercise (Oslo, Norway, 2014)
Nutrigenomics of food, NUGO week (Castellammare di Stabia, Napoli, Italy, 2014)
Advanced visualisation, integration and biological interpretation of -omics data (Wageningen, 2014)
Dutch Nutritional Science Days (Heeze, Nederland, 2015)
12th Annual conference of the Metabolomics Society (Dublin, Ireland, 2016)

General activities

VLAG PhD week (Venlo, Nederlands, 2012)
Longitudinal data analysis, Statistics workshop (Wageningen, 2012)
Techniques for Writing and Presenting a Scientific Paper (Wageningen, 2013)
Patenting workshop (Wageningen, 2013)
Biosystem data analysis course (Amsterdam, 2013)
Reviewing a Scientific Paper (Wageningen, 2014)

Optionals

Preparation of research proposal (Wageningen, 2012)
Nutritional Genomics and Genetics (Wageningen, 2013)
NMG scientific meetings (2012-2016)
PhD tour (USA, 2015)

The studies presented in this thesis were performed within the framework of TI Food and Nutrition and the Netherlands Metabolomics Centre.

Financial support from Wageningen University , TI Food and Nutrition for printing this thesis is gratefully acknowledged.

Cover design : ahmaddboostani@yahoo.com
Layout : briyan.2209@gmail.com

Printed by Digiforce – Proefschriftmaken.nl