RESEARCH ARTICLE

Biochemical and metabolomic phenotyping in the identification of a vitamin D responsive metabotype for markers of the metabolic syndrome

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Scope: Metabolic phenotyping promises to be a useful tool in human intervention studies. This study examined whether metabolic phenotyping could identify responders to vitamin D supplementation in terms of the metabolic syndrome.

Methods and results: In a double-blind, randomised placebo-controlled dietary intervention subjects were assigned to receive 15 μg vitamin D_3 or placebo daily. Serum 25-hydroxyvitamin D (25(OH)D) and biochemical markers of the metabolic syndrome were measured at baseline and following the 4-wk intervention. *k*-means clustering and ^{1}H-NMR metabolomic analysis were used to explore responsive phenotypes. Vitamin D supplementation significantly increased serum 25(OH)D to an endpoint concentration of 78.1 ± 20.0 nmol/L ($p < 0.001$). There was no effect of supplementation on the measured markers of the metabolic syndrome. *k*-means cluster analysis based on 13 biochemical markers of the metabolic syndrome and 25(OH)D concentrations revealed five discrete biomarker clusters. One of these clusters, characterised by lower serum 25(OH)D and higher levels of adipokines, showed significant responses in insulin (15% decrease), homestatic model assessment scores (19% decrease) and c-reactive protein (54% decrease). Metabolomic analysis revealed further changes and the extent of change in serum vitamin D correlated negatively with changes in glucose.

Conclusion: Overall, metabolic phenotyping revealed a phenotype that was responsive to vitamin D supplementation.

Keywords:
Metabolic phenotype / Metabolic Syndrome / Metabolomics / Metabotype / Vitamin D

1 Introduction

Vitamin D deficiency, reflected by circulating 25-hydroxyvitamin D (25(OH)D) concentrations < 50 nmol/L [1–5], is prevalent in as much as half of European and US populations [6–8]. There is accumulating evidence to suggest that vitamin D status may play a role in the development of diseases related to the metabolic syndrome. This possible role of vitamin D status is based on the results of case–control studies that report lower serum 25(OH)D concentrations in individuals diagnosed with diabetes [9, 10] and the metabolic syndrome [11, 12] compared with controls. Cross-sectional studies support these findings showing that low serum 25(OH)D concentrations are associated with markers of disturbed glucose metabolism [13–15] and cardiovascular disease [16–18]. Furthermore, a recent meta-analysis concludes that high levels of 25(OH)D among middle-age and elderly populations are associated with a decrease in cardiovascular disease, type 2 diabetes and metabolic syndrome [19]. Intervention studies

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Abbreviations: 25(OH)D, 25 hydroxyvitamin D; **ANOVA,** analysis of variance; **CRP,** c-reactive protein; **GLM,** general linear model; **HOMA,** homestatic model assessment; **NEFA,** non-esterfied fatty acids; **PCA,** principal component analysis; **PLS-DA,** partial least-squares discriminant analysis; **TAG,** triglyceride; **TNF-α,** tumour necrosis factor α; **TSP,** sodium trimethylsilyl [2,2,3,3-$^2H_4$] proprionate; **VIP,** variable importance in the projection

investigating the effects of vitamin D supplementation on glucose metabolism and markers of the metabolic syndrome have yielded mixed results. Administration of supplemental vitamin D metabolites, such as 25(OH)D or 1,25 dihydroxy-vitamin D, to diabetic patients with low baseline vitamin D status resulted in improved insulin secretion and improved glucose concentrations [20, 21]. However, the effect of supplementation in individuals with impaired fasting glucose or insulin resistance is less clear. Some studies have reported that supplementation resulted in significant improvements in insulin sensitivity [22], whereas others report no improvement in markers of glucose tolerance [23] or incidence of type 2 diabetes [24]. Drawing conclusions from these studies is confounded by factors such as the underlying metabolic disease of the patients and the length and the dose of supplementation. Additionally, many studies do not report evidence of achieving levels of 25(OH)D considered beneficial (>75 nmol/L).

Metabolomics is the study of small molecules or metabolites and commonly involves the combination of NMR spectroscopy and multivariate data analysis. The field of metabolomics has contributed greatly to our understanding of the metabolic actions of pharmaceutical agents [25] and in the diagnosis of disease states [26, 27]. The concept of metabolic phenotype has been introduced in metabolomics literature and describes a characteristic metabolic profile reflecting the biochemistry, the physiological status, and the environmental exposures of individuals and populations [28, 29]. There is an expectation that applying this technology in nutrition research will yield valuable information [30]; assigning individuals to a particular metabolic phenotype (metabotypes or nutritypes) will help in understanding the metabolic mechanisms linking diet to a number of chronic diseases [29, 31]. The present study sets out to explore the joint use of targeted and non-targeted metabolic profiling in an intervention study examining the effect of vitamin D supplementation on markers of the metabolic syndrome. To avoid the confounding issue of a metabolic disease, the present study was limited to healthy volunteers. *k*-means cluster analysis was used on targeted plasma biomarkers to create unique clusters or metabolic phenotypes and ^{1}H NMR metabolomic analysis to uncover non-targeted metabolites, which might be involved in any putative link of vitamin D to the metabolic syndrome. Data for the present study were obtained from a dual intervention study of markers of the metabolic syndrome in healthy subjects involving probiotic and vitamin D_3 supplementation.

2 Materials and methods

2.1 Study design

The project was approved by the Research Ethics Committees in University College Dublin and University College Cork (HREC-39-06). All procedures were conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from all subjects. This research is part of a randomised, double-blind placebo-controlled, two centre dietary intervention study. Free-living Caucasians aged 18–65 were recruited by the study researchers at University College Dublin and University College Cork. All volunteers attended a screening session at which their height and weight were measured and a fingerpick blood sample was taken to measure haemoglobin. All women were premenopausal. Exclusion criteria included a BMI <18.5 or >30.0 (kg/m^2), iron deficiency anaemia (haemoglobin <12 g/dL for males and <11 g/dL for females), any chronic or infectious disease and any prescribed medication for such (contraceptive pills were permitted) pregnant or lactating females and persons using hormone replacement therapy.

Upon successful completion of screening participants, 160 subjects, were randomly assigned to one of four treatment groups by an independent researcher using computer-generated random numbers. The four treatment groups were defined as follows: treatment group 1 received daily vitamin D_3 (15 μg) and probiotic (*Lactobacillus salivarius* 10^9 cfu/5 g sachets suspended in maltodextrin); group 2, daily vitamin D_3 and placebo probiotic (maltodextrin); group 3, daily vitamin D_3 placebo and probiotic; and group 4, daily vitamin D placebo and probiotic placebo. The vitamin D_3 and matching placebo were food grade and consumed in capsule form and were identical in appearance and taste, whereas the probiotic and probiotic/placebo (in powder form) were mixed with milk for consumption. The vitamin D_3 capsules and matching placebo capsules were produced by Banner Pharmacaps (Tilburg, The Netherlands). The vitamin D_3 content of the capsules was independently confirmed by laboratory analysis (Consultus, Glanmire, Cork, Ireland). Fasting urine and blood samples were collected before and after a 4-wk dietary intervention. The intervention was carried out in two phases, April 2007 (spring) and November 2007 (autumn). Including the recruitment period, the total duration of the study was January 2007 to December 2007. Compliance was assessed based on serum 25(OH)D concentrations. A reduction in serum 25(OH)D in an individual randomised to vitamin D treatment indicated non-compliance.

2.2 Anthropometric measurements and dietary assessment

Weight and height were determined and BMI (BMI = weight (kg)/height (m^2)) was calculated. Body weight was measured in duplicate to the nearest 0.1 kg with the subject dressed in light clothing, using a Seca Alpha 770 digital scale (CMS Weighing Equipment, London, UK), calibrated in 100 g intervals. Height was measured using a Leicester height measure to the nearest 0.1 cm with the head

positioned in the Frankfort Plane [32] (CMS Weighing Equipment). Dietary intake was assessed by the use of 3-day estimated food record. Food diaries were coded and data were entered into the Weighed Intake Software Program (Tinuviel Software, Anglesey, UK) for analysis. Weighed Intake Software Program uses data from the McCance & Widdowson's ''The Composition of Foods'' plus supplemental volumes to generate nutrient intake data.

2.3 Biofluid collection

On the morning of sample collection following a 12-h overnight fast volunteers collected a first void urine sample, which was immediately placed on ice and brought to the test centre. Urine samples were centrifuged and five 1 mL aliquots were stored at −80°C for analysis. Blood for measurement of leptin, resistin, adiponectin, IL-6 (IL-6), c-reactive protein (CRP), tumour necrosis factor α (TNF-α), insulin, C-peptide and 25(OH)D concentrations was collected in two serum tubes containing clot activator coating, one with gel barrier (Becton Dickinson, Oxford, UK). Blood for cholesterol, triglyceride (TAG) and non-esterfied fatty acids (NEFA) was collected in EDTA-coated evacuated tubes (Becton Dickinson). Samples for glucose analysis were collected in tubes containing fluoride oxalate, and blood for NMR analysis was collected in tubes containing lithium heparin (Becton Dickinson). All samples were inverted eight times. Serum samples were allowed to clot for 30 min before being placed on ice. Plasma samples were placed directly on ice and all samples were processed within 1 h. Samples were centrifuged at 1800 × *g* for 10 min at 4°C and 500 μL aliquots were stored at −80°C until subsequent analysis.

2.4 Biochemical analyses

Biochemical markers were measured using standard commercial kits according to manufacturer's instructions. All samples were run in duplicate. Enzymatic colourimetric assays were used to measure plasma glucose (GLUCOSE Liquicolor Kit, HUMAN, Wiesbaden, Germany), cholesterol (CHOL Enzymatic Endpoint Method, Randox, Crumlin, Antrim, UK), TAG (TRIGS Liquid Reagent GPO-PAP Method, Randox, Crumlin) and NEFA (NEFA-HR(2), Wako Chemicals, Neuss, Germany). Ultrasensitive ELISAs were used for the analysis of serum insulin (Ultrasensitive Insulin ELISA Kit, Mercodia AB, Uppsala, Sweden), serum C-peptide (Ultrasensitive C-peptide ELISA Kit, Mercodia AB), serum IL-6 (Hu IL-6 US Kit, BioSource Europe, Nivelles, Belgium) and serum CRP (hsCRP ELISA Kit, Biocheck, Fosters City, CA, USA). 25(OH)D concentrations were measured using an ELISA (OCTEIA® 25-hydroxy vitamin D, Immuno Diagnostic Systems, Boldon, UK). The quality and accuracy of serum 25(OH)D analysis in the UCC laboratory are assured on an ongoing basis by participation in the Vitamin D External Quality Assessment Scheme (Charing Cross Hospital, London, UK). TNF-α, leptin, adiponectin and resistin were measured in serum using DuoSet ELISA development kits (R&D Systems Europe, Abington, UK).

2.5 Phenotyping by means of biomarker clusters

Cluster analysis was performed using the *k*-means cluster algorithm in SPSS. Before clustering, all biochemical markers were transformed into standardised *z*-scores. The *k*-means method assumes a certain number of clusters, *k*, fixed *a priori* and produces a separation of the objects into non-overlapping groups coming from Euclidean distances. Cluster membership is exclusive and dependent on minimising the Euclidean distance within each cluster and maximising differences between clusters at each step of an iterative procedure. Thus, a cluster represents a group of individuals with similar biochemical characteristics. We examined a split according to five clusters and a maximum of ten iterations were used. In an attempt to validate the cluster analysis, a five-fold cross-validation was carried out. The population was divided randomly into five equal parts and cluster analysis was run five times, each time omitting one of the five parts. In each case, the clusters that emerged were similar to that observed in the original analysis with all subjects included.

2.6 NMR spectroscopy

Urine samples were prepared by the addition of 200 μL phosphate buffer (0.2 mol KH_2PO_4/L, 0.8 mol K_2HPO_4/L) to 500 μL urine. Following centrifugation at 8000 × *g* for 5 min, 10 μL sodium trimethylsilyl [2,2,3,3-$^2H_4$] proprionate (TSP) and 50 μL deuterium were added to 550 μL of the supernatant. Spectra were acquired on a 600-MHz Varian NMR spectrometer using the first increment of a NOESY pulse sequence at 25°C. Spectra were acquired with 16K data points and 128 scans over a spectral width of 9 kHz. Water suppression was achieved during the relaxation delay (1 s) and the mixing time (200 ms). All ^{1}H NMR urine spectra were referenced to TSP at 0.0 ppm and processed manually with Chenomx (version 6) using a line broadening of 0.2 Hz. The spectra were integrated into bins consisting of spectral regions of 0.04 ppm, using Chenomx (version 6). The water region (4.0–6 ppm) was excluded and the data were normalised to the sum of the spectral integral.

Plasma samples were defrosted for 1 h at room temperature and centrifuged at 1000 × *g* for 2 min. Samples were prepared by the addition of 250 μL deuterium and 10 μL TSP to 300 μL plasma. Spectra of samples were acquired by using a Carr–Purcell–Meiboom–Gill pulse sequence with 32K data points and 64 scans. ^{1}H NMR plasma spectra were processed manually with Chenomx

software and were phase and baseline corrected. The spectra were integrated into bins consisting of spectral regions of 0.04 ppm. The water region (4.0–6.0 ppm) was excluded and the data were normalised to the sum of the spectral integral.

2.7 Statistical analysis

All data measurements were performed in a blinded fashion and treatment codes were broken only after all data analysis was completed. Statistical analysis was performed using SPSS® version 15.0 for Windows (SPSS®, Chicago, IL, USA). Independent *t*-tests were used to compare height, weight, BMI and age between males and females at baseline and between vitamin D and placebo groups. Chi-square statistics were used to explore the distribution of males and females between groups. Initial analysis revealed that there was no significant interaction between the probiotic and vitamin D treatment ($p > 0.05$). Therefore, the data were collapsed into two groups: vitamin D supplementation group and placebo group. Differences in the mean change in biochemical measurements following the dietary intervention between vitamin D and placebo groups were compared using general linear models (GLM) analysis of variance (ANOVA) with adjustments for gender and phase. Linear regression analysis was used to investigate the relationship between baseline concentrations of serum 25(OH)D and change in 25(OH)D following the 4-wk intervention.

Biomarker clusters were determined using *k*-means cluster analysis as described previously. Differences in the mean biochemical measurements across clusters were evaluated using ANOVA. Age, BMI, dietary data and plasma fatty acid concentrations were compared between biomarker clusters using GLM analysis with adjustments for gender. For both ANOVA and GLM analyses, where statistically different effects were identified ($p < 0.05$), comparisons were made between clusters using the Bonferroni *post hoc* multiple comparison test. Linear regression analysis was used to investigate the relationships between the change in 25(OH)D concentrations following the intervention and changes in biochemical measurements. Where relationships were identified using regression analysis, ANOVA was used to compare differences in the changes in biochemical markers between tertiles of the change in 25(OH)D concentrations.

Multivariate data analysis was performed using Simca-P+ software (version 11.0; Umetrics, Umeå, Sweden). Data sets were scaled using Pareto scaling for plasma data and unit variance scaling for urine. Principal component analysis (PCA) was applied to explore any trends or outliers in the data. The difference between biomarker clusters was further explored using partial least-squares discriminant analysis (PLS-DA). The variable importance in the projection (VIP) value of each variable in the model was calculated to indicate the contribution to the classification of samples. The quality of all models was judged by the goodness-of-fit parameter (R^2) and the predictive ability parameter (Q^2), which is calculated by an internal cross-validation of the data and the predictability calculated on a leave-out basis. Additionally, the predictability of the models was assessed by creating a training set with 66% of the data and a test set with 34% of the data.

3 Results

In total, 75 males and 85 females, aged between 18 and 63 years, were recruited in the study. Initial cluster analysis performed on baseline data identified two distinct outlying groups of subjects; three subjects with very high IL-6 concentrations and two subjects with very high TNF-α concentrations. After exclusion of the five outlying subjects and individuals with incomplete biochemical profiles, the study sample consisted of 135 participants. The demographic characteristics of the subjects included in the analysis are shown in Table 1. There was no significant difference in the age of males and females; however, BMI was higher for males compared with females.

3.1 Vitamin D intervention and markers of the metabolic syndrome

Of the 135 participants included in the cluster analysis, 9 were identified as having not complied with the intervention protocol and so were excluded from intervention analysis. No subjects reported adverse effects with the supplementation. There was no significant difference in gender distribution, age, BMI (Table 2) or any markers of the metabolic syndrome between the vitamin D and placebo groups at baseline. As expected baseline serum 25(OH)D concentrations were dependant on the intervention phase. Subjects recruited during the spring phase had significantly lower serum 25(OH)D concentrations than those recruited in autumn (51.2 ± 18.0 *vs.* 62.0 ± 24.5 nmol/L, $p = 0.004$). For this reason, GLM analysis of intervention outcomes included adjustments for both gender and phase.

Table 1. Subject demographics

	Males ($n = 60$)	Females ($n = 75$)
Height (m)	1.78 ± 0.07	1.67 ± 0.06
Weight (kg)	79.64 ± 10.54	65.12 ± 9.11
BMI (kg/m^2)	25.05 ± 2.91	23.42 ± 2.71*
Age (years)	34 ± 12	36 ± 12

All values are mean ± SD.
*Significantly different to males at $p < 0.001$ based on independent *t*-test.

Table 2. Subject characteristics, 25(OH)D and biomarkers of the metabolic syndrome at baseline and post-intervention for vitamin D_3 (15 μg/day) and placebo groups

	Placebo ($n = 64$)		Vitamin D_3 ($n = 62$)		*p*-Value
Characteristics					
Gender	25 M	39 F	27 M	35 F	0.609
Age (years)	34 ± 12		36 ± 13		0.404
BMI (kg/m^2)	24.02 ± 2.98		23.83 ± 2.63		0.703
Biomarkers	Baseline	Post	Baseline	Post	*p*-Value
25(OH)D (nmol/L)	53.5 ± 20.9	52.6 ± 16.9	59.7 ± 23.0	78.1 ± 20.0	0.000
Glucose (mmol/L)	5.10 ± 0.58	5.12 ± 0.52	5.02 ± 0.64	5.04 ± 0.68	0.970
Insulin (mU/L)	6.36 ± 3.24	6.59 ± 3.30	5.76 ± 2.99	5.84 ± 3.17	0.790
HOMA	1.48 ± 0.91	1.52 ± 0.83	1.33 ± 0.86	1.34 ± 0.82	0.784
C-peptide (ng/mL)	1.79 ± 0.86	2.16 ± 0.97	1.51 ± 0.91	1.90 ± 0.97	0.787
Cholesterol (mmol/L)	4.64 ± 0.90	4.75 ± 0.93	4.75 ± 1.10	4.89 ± 1.09	0.574
TAG (mmol/L)	1.27 ± 0.54	1.25 ± 0.59	1.22 ± 0.64	1.20 ± 0.63	0.686
NEFA (μmol/L)	501.25 ± 233.18	498.24 ± 219.42	452.42 ± 167.95	478.20 ± 181.42	0.356
IL-6 (pg/mL)	0.10 ± 0.11	0.09 ± 0.10	0.10 ± 0.14	0.12 ± 0.19	0.765
TNF-α (pg/mL)	106.4 ± 253.2	122.8 ± 255.4	125.6 ± 332.7	173.0 ± 423.9	0.326
CRP (mg/L)	1.22 ± 1.42	0.96 ± 1.13	1.69 ± 1.63	1.48 ± 1.70	0.774
Leptin (ng/mL)	5.90 ± 4.22	6.06 ± 4.35	5.19 ± 4.00	5.30 ± 4.07	0.605
Adiponectin (mg/L)	1.57 ± 0.70	1.37 ± 0.63	1.53 ± 0.83	1.22 ± 0.66	0.295
Resistin (ng/mL)	26.02 ± 10.61	26.58 ± 10.17	23.78 ± 13.32	27.60 ± 15.57	0.168

All values are mean ± SD, *p*-values are based on GLM (ANOVA) of between group comparisons of the change in biomarkers, adjusted for gender and intervention phase. M, males; F, females.

Baseline and post-intervention concentrations of serum 25(OH)D and biomarkers of the metabolic syndrome for vitamin D treated and placebo groups are presented in Table 2. Statistical analysis of the change in serum 25(OH)D concentrations indicated a significant increase with vitamin D supplementation compared with the placebo. Multivariate regression analysis indicated a significant inverse relationship between baseline serum 25(OH)D concentrations and the change in serum 25(OH)D with vitamin D supplementation ($\beta = -0.45$, $p = 0.000$). Vitamin D supplementation did not significantly alter any biomarkers of the metabolic syndrome.

Subjects were grouped according to their vitamin D status; 44% were classified as vitamin D deficient (25(OH)D < 50 nmol/L, $n = 56$), 38% as vitamin D insufficient (25(OH)D = 50–75 nmol/L, $n = 48$) and 18% as vitamin D sufficient (25(OH)D > 75 nmol/L, $n = 22$). In order to establish whether the effects of vitamin D repletion are more apparent in vitamin D deficient individuals, the effect of the intervention in this group was examined. Serum 25(OH)D concentrations increased by 23.8 ± 11.0 nmol/L ($p = 0.000$) with vitamin D supplementation in this group, but there was no significant effect on any biomarkers of the metabolic syndrome (data not shown).

3.2 Identification of biomarker phenotypes

Using *k*-means cluster analysis of the 14 biomarkers, five distinct groups were identified. Demographic characteristics, dietary intake data and mean biomarker concentrations for each cluster are presented in Table 3. The clusters were significantly defined by the biochemical parameters and each of the clusters had a distinct biochemical profile. Cluster 1 ($n = 38$) had significantly higher CRP (mg/L) concentrations compared with all other clusters. Cluster 2 had higher glucose (mmol/L) and insulin (mU/L) concentrations and a higher mean homestatic model assessment (HOMA) score compared with all other clusters, whereas C-peptide was significantly higher compared with clusters 3, 4 and 5. Leptin (ng/mL) was also significantly higher in cluster 2 compared with clusters 3 and 4. Cluster 3 had the highest serum 25(OH)D (nmol/L) and NEFA (μmol/L) concentrations. Cluster 4 was characterised by significantly higher TAG (mmol/L). Finally, cluster 5 had low serum 25(OH)D concentrations and significantly higher adiponectin (mg/L) and resistin (ng/mL) concentrations compared with all other clusters. Analysis of the fatty acid concentrations of total plasma lipids highlighted significant differences in plasma fatty acid profiles across the five biomarker clusters; these results are presented in Supporting Information Table S1 and Supporting Information text.

3.3 Biomarker phenotypes and metabolomic profiles

Initial PCA of the ^{1}H NMR plasma data showed three outlying samples. The NMR spectra of these outlying samples were inspected. Two of the spectra were of poor quality, whereas the third had poor water suppression. All three samples were removed from subsequent analysis. A

Table 3. Subject demographics and mean baseline concentration of biomarkers of the metabolic syndrome across biomarker clusters

	Cluster 1 ($n=38$)	Cluster 2 ($n=10$)	Cluster 3 ($n=38$)	Cluster 4 ($n=18$)	Cluster 5 ($n=31$)	p-Value
Characteristics						
Gender	12 M 26 F	5 M 5 F	22 M 16 F	14 M 4 F	7 M 24 F	0.000
Age (years)	35±13	40±15	39±12[5]	33±12	29±10	0.008
BMI (kg/m^2)	25.52±2.93[3,5]	26.65±3.11[3,5]	23.18±2.77	24.65±2.20[5]	22.52±1.84	0.000
Dietary data						
Energy (MJ)	7.69±2.04	9.67±2.38	10.38±3.08[1]	10.33±2.69[1]	8.96±2.59	0.001
TE protein (%)	16.38±3.64	16.50±2.31	16.39±2.97	14.21±1.99	15.91±3.10	0.254
TE carbohydrate (%)	46.21±6.65	49.74±9.03	47.72±6.42	47.90±7.48	49.33±8.13	0.267
TE fat (%)	36.70±6.34	31.58±6.57	34.87±5.12	37.13±7.82	33.61±6.83	0.072
Biomarkers						
25(OH)D (nmol/L)	57.5±26.6	66.4±22.3	**70.4±28.8**[5]	53.1±21.0	46.6±12.3	**0.001**
Glucose (mmol/L)	5.24±0.51[3,5]	**6.26±0.59**[1,3,4,5]	4.82±0.55	4.93±0.40	4.84±0.34	**0.000**
Insulin (mU/L)	7.52±2.07[3,4,5]	**13.11±3.28**[1,3,4,5]	3.65±1.09	5.17±1.90	5.78±2.11[3]	**0.000**
HOMA	1.75±0.52[3,4,5]	**3.66±1.05**[1,3,4,5]	0.79±0.28	1.12±0.38	1.24±0.46[3]	**0.000**
C-peptide (ng/mL)	2.05±0.82[3]	**2.84±0.99**[3,4,5]	1.22±0.79	1.65±0.71	1.60±0.87	**0.000**
Cholesterol (mmol/L)	4.85±0.94	4.93±1.37	**4.95±0.91**	4.23±0.99	4.36±0.90	**0.020**
TAG (mmol/L)	1.25±0.46	1.56±0.82[3,5]	1.02±0.43	**2.07±0.69**[1,4,5]	1.03±0.32	**0.000**
NEFA (μmol/L)	456.58±181.89	422.00±141.41	**543.68±226.66**[4]	344.44±11.63	482.90±211.14	**0.009**
IL-6 (pg/mL)	0.08±0.09	**0.19±0.19**[3]	0.06±0.07	0.13±0.17	0.13±0.12	**0.007**
TNF-α (pg/mL)	127.5±266.4	26.6±55.0	101.4±260.2	**237.4±505.0**	79.4±213.5	**0.315**
CRP (mg/L)	**2.96±1.88**[2,3,4,5]	0.69±0.61	0.90±0.77	1.27±1.20	0.69±0.64	**0.000**
Leptin (ng/mL)	8.29±3.80[3,4,5]	**8.35±4.92**[3,4]	2.66±2.44	3.78±2.96	5.39±3.25[3]	**0.000**
Adiponectin (mg/L)	1.42±0.65	1.24±0.49	1.58±0.52[4]	0.89±0.63	**2.15±0.79**[1,2,3,4]	**0.000**
Resistin (ng/mL)	26.93±13.65[3]	25.70±8.47	18.10±6.90	18.85±8.24	**34.93±11.41**[1,3,4]	**0.000**

All values are mean±SD, p-values for characteristics and dietary data are based on GLM (ANOVA) adjusted for gender, p-values for biomarkers are based on simple ANOVA. The highest mean concentration for each biomarker across clusters is highlighted in boldface, the lowest concentration is underlined. Superscript numbers adjacent to values denote a significant difference between indicated clusters. The superscript number identifies the cluster with the lower concentration. M, males; F, females; TE protein %, protein as percentage of total energy intake; TE carbohydrate %, carbohydrate as percentage of total energy intake; TE fat %, fat as percentage of total energy intake.

Table 4. R^2 and Q^2 values for PLS-DA models based on ^{1}H NMR data of plasma samples, comparing different biomarker clusters

Biomarker cluster	2		3		4		5	
	R^2X	Q^2	R^2X	Q^2	R^2X	Q^2	R^2X	Q^2
1	NM	NM	0.30*	0.07	0.44	0.24	0.36	0.09
2			0.27*	0.08	0.41*	0.15	0.36	0.21
3					0.38*	0.39	NM	NM
4							0.45	0.48

NM, no model created.
*One-component model.

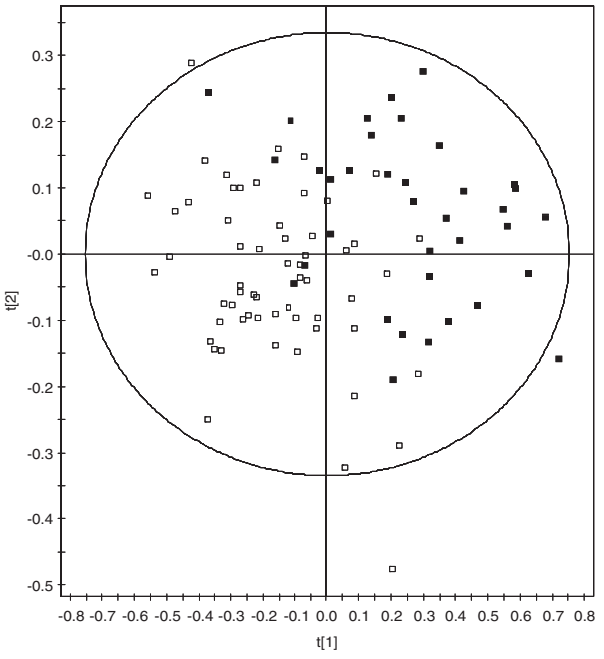

Figure 1. Partial least-squares discriminant analysis (PLS-DA) of ^{1}H NMR plasma data for biomarker cluster 4 *versus* biomarker cluster 5 ($R^2 = 0.45$, $Q^2 = 0.48$). Closed squares (■) represent biomarker cluster 4; open squares (□) represent biomarker cluster 5.

PCA scores plot of all ^{1}H NMR plasma data was created, and the first two components accounted for 59% of variation in the data. Separation of the biomarker clusters was evident. To probe further, the metabolic differences between the different clusters individual pairwise comparisons were performed using PLS-DA. Table 4 presents the R^2 (goodness-of-fit parameter) and Q^2 (predictive ability) values for the PLS-DA models created.

Several robust models were built when comparing plasma profiles of different biomarker clusters. For example, comparison of clusters 4 and 5 resulted in a two-component model (Fig. 1: $R^2X = 0.45$, $Q^2 = 0.48$). Validation of the model, as described in Section 2, indicated that $82.2 \pm 7.4\%$ of the samples were classified correctly. Analysis of the VIP values revealed the metabolites responsible for the separation of the clusters (Table 5). The most discriminating metabolites included lactate, VLDL, LDL and choline. Concentrations of lactate, VLDL and LDL were significantly higher in cluster 4, whereas choline was significantly higher in cluster 5 (Table 5). A one-component PLS-DA model was detected for cluster 3 *versus* cluster 4. Validation indicated that $82.2 \pm 1.5\%$ of the samples were classified correctly. These results are presented in Supporting Information Fig. 1 and Supporting Information Table S2. Examination of the urine metabolomic profiles revealed no significant separation according to biomarker clusters.

3.4 Biomarker phenotypes and response to vitamin D intervention

After the identification and characterisation of biomarker phenotypes, the next step was to ascertain if different biomarker clusters respond differently to dietary intervention with vitamin D. GLM analysis of the biochemical markers for each of the five clusters revealed that individuals in cluster 5 responded positively to the intervention with vitamin D supplementation. No other biomarker clusters displayed significant changes in any of the biochemical markers. For this reason, any further analysis was focused on data collected from biomarker cluster 5 participants.

For cluster 5, baseline characteristics of the vitamin D ($n = 9$) and placebo ($n = 21$)-treated groups were compared using chi-square statistics and independent samples *t*-tests. There was no significant difference in gender distribution, age or BMI (Table 6).

Serum 25(OH)D concentrations increased by 25.8 ± 17.1 nmol/L ($p = 0.009$) in the vitamin D supplemented group. Seven of the nine subjects in this group achieved serum 25(OH)D concentrations at or above 75 nmol/L. Following supplementation with vitamin D, there was a significant reduction in fasting insulin concentrations ($p = 0.011$), HOMA score ($p = 0.006$) and CRP concentrations ($p = 0.011$). Glucose, C-peptide, cholesterol, TAG, NEFA, IL-6, TNF-α, leptin, adiponectin and resistin were not significantly altered.

Regression analysis on data from all subjects in biomarker cluster 5 ($n = 30$) highlighted a significant inverse

Table 5. Relative intensities and VIP values of the most discriminating metabolites for comparison of biomarker clusters 4 and 5

Metabolite	Chemical shift (ppm)	VIP	Cluster 4	Cluster 5	*p*-Value
Lactate[a)]	1.32	6.51	9.82±0.30	6.76±0.21	<0.001
VLDL+LDL	1.28	5.89	10.14±0.39	7.27±0.22	<0.001
Choline	3.24	4.54	4.13±0.15	5.64±0.12	<0.001
Glucose[a)]	3.76	2.40	2.59±0.07	3.11±0.06	<0.001
Acetoacetate	2.24	1.83	0.75±0.03	0.48±0.02	<0.001
Lipid CH_2CH_2CO	1.6	1.84	0.63±0.03	0.36±0.02	<0.001

Clusters 4 and 5 were derived from *k*-means cluster analysis of 14 different biomarkers. All values are expressed as relative intensities and are mean±SD, *p*-values are based on independent samples *t*-test. VIP, variable importance in the projection.
a) More than one chemical shift region had significant VIP values.

Table 6. Biomarker cluster 5; changes in biochemical markers in the vitamin D_3 (15 μg/day) and placebo groups following the 4-wk intervention

	Placebo ($n=21$)		Vitamin D_3 ($n=9$)		*p*-Value
Characteristics					
Gender	4 M	12 F	2 M	7 F	0.842
Age (years)	29±11		30±9		0.922
BMI (kg/m^2)	22.17±1.00		22.55±2.07		0.498
Biomarkers	Baseline	Post	Baseline	Post	*p*-Value
25(OH)D (nmol/L)	43.1±8.9	48.3±14.9	54.1±16.4	79.9±16.2	0.009
Glucose (mmol/L)	4.84±0.32	4.91±0.51	4.89±0.38	4.60±0.60	0.209
Insulin (mU/L)	5.88±2.31	6.99±3.79	5.55±1.80	4.70±2.52	0.011
HOMA	1.26±0.50	1.55±0.96	1.21±0.39	0.98±0.58	0.006
C-peptide (ng/mL)	1.57±0.84	1.90±0.66	1.46±0.83	1.44±0.37	0.243
Cholesterol (mmol/L)	4.35±0.85	4.38±0.99	4.38±1.09	4.36±0.83	0.724
TAG (mmol/L)	1.05±0.30	1.12±0.44	1.00±0.38	0.82±0.19	0.669
NEFA (μmol/L)	499.05±247.18	549.50±241.13	447.78±110.88	482.22±95.89	0.907
IL-6 (pg/mL)	0.14±0.13	0.10±0.08	0.10±0.13	0.11±0.19	0.079
TNF-α (pg/mL)	93.6±256.1	79.7±257.7	50.5±72.7	57.1±73.7	0.769
CRP (mg/L)	0.63±0.53	0.71±0.78	0.93±0.83	0.43±0.42	0.011
Leptin (ng/mL)	5.60±3.39	6.07±3.25	5.30±3.01	5.04±3.18	0.242
Adiponectin (mg/L)	1.92±0.70	1.66±0.62	2.70±0.81	1.98±0.57	0.371
Resistin (ng/mL)	32.82±9.46	30.07±10.22	41.28±13.68	35.40±20.61	0.090

Cluster 5 derived from *k*-means cluster analysis of 14 different biomarkers. All values are mean±SD, *p*-values are based on GLM (ANOVA) of between group comparisons, adjusted for gender and intervention phase. M, males; F, females.

relationship between the change in serum 25(OH)D and the change in glucose concentrations following the intervention ($\beta = -0.502$, $p = 0.006$). Comparing the change in glucose concentrations across tertiles of the change in vitamin D concentrations confirms this association (Fig. 2, $p = 0.002$). An inverse relationship was identified between the change in serum 25(OH)D and the change in adiponectin concentrations ($\beta = -0.464$, $p = 0.011$); however, this relationship did not extend to ANOVA across tertiles of the change in vitamin D ($p = 0.134$). There was no significant relationship between the change in vitamin D and any other markers of the metabolic syndrome measured.

The changes in the metabolomic profiles of biomarker cluster 5 subjects following the intervention with vitamin D_3 were also investigated. Comparison of pre- and post-plasma profiles for subjects receiving supplemental vitamin D_3 resulted in a two-component PLS-DA model (Fig. 3: $R^2X = 0.55$, $Q^2 = 0.37$). Validation of the model, as described in Section 2, indicated that 76.7±8.8% of the samples were classified correctly. Analysis of the VIP values revealed the metabolites responsible for the separation of pre- and post-samples (Table 7). The most significant discriminating metabolites included VLDL+LDL, glucose and glutamine. Similar comparisons in urine profiles revealed no significant differences between pre- and post-samples.

4 Discussion

The metabolic syndrome is characterised by a cluster of metabolic abnormalities including central obesity, insulin resistance, dyslipidaemia, hypertension and a systemic pro-

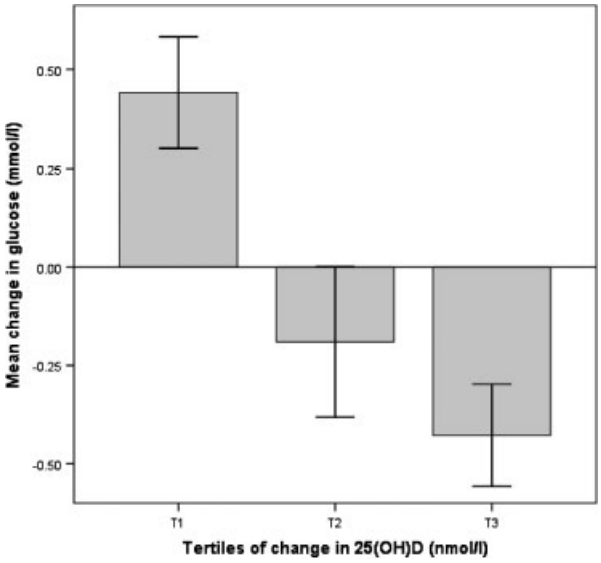

Figure 2. Change in glucose concentration (mmol/L) in subjects in cluster 5 according to tertile of change in serum 25(OH)D (nmol/L). Values are mean ± SEM. The change in 25(OH)D for tertile 1 (T1) = −9.1 ± 1.7, tertile 2 (T2) = 8.1 ± 0.5 and tertile 3 (T3) = 26.6 ± 1.3 nmol/L. ANOVA indicated a significant difference in mean glucose concentration between tertiles of the change in 25(OH)D levels ($p = 0.002$).

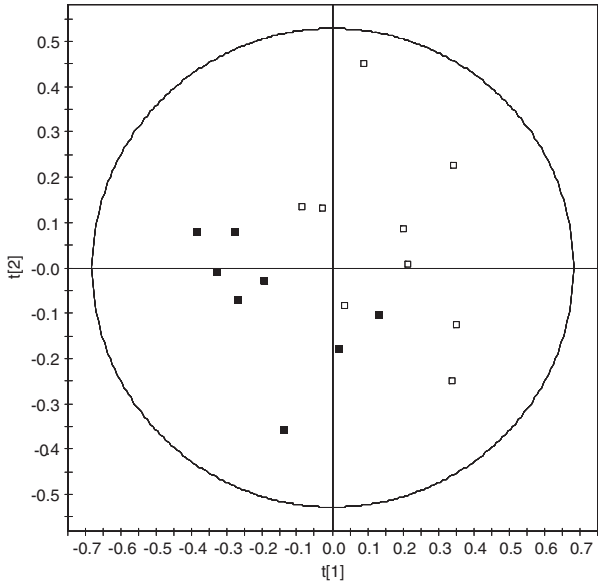

Figure 3. Partial least-squares discriminant analysis (PLS-DA) of ^{1}H NMR plasma data for vitamin D supplemented group in biomarker cluster 5 ($R^2 = 0.56$, $Q^2 = 0.37$). Open squares (□) represent baseline samples; closed squares (■) represent post-samples.

inflammatory state [33]. Recent research suggests that adipokines such as resistin and adiponectin may play a role in pathologies associated with obesity, insulin resistance and the metabolic syndrome [34, 35]. Consequently, the International Diabetes Federation consensus group have recommended consideration of adipokines and other inflammatory markers as possible additions in future definitions of the metabolic syndrome [33]. Several epidemiological studies have suggested that low serum 25(OH)D is associated with an increased risk of having metabolic syndrome [11, 12, 36] and individual components of the metabolic syndrome [13–18, 37]. To date, a limited number of intervention studies have investigated the effects of vitamin D supplementation on markers of the metabolic syndrome. Overall, it appears that vitamin D supplementation may increase insulin secretion and improve glucose tolerance in diabetic patients with low baseline vitamin D status [20, 21], but not in vitamin D sufficient patients [38]. Furthermore, conflicting findings are reported from studies in subjects with impaired glucose tolerance [22, 23] and even less is known as to the effects in healthy populations [24, 39].

The initial findings from the present study showed that daily supplementation with vitamin D_3 for 4 wk in a healthy cohort had no effect on markers of the metabolic syndrome which is in agreement with other such studies [23, 38, 39]. Even when vitamin D deficient individuals were considered alone, no significant changes in any markers of the metabolic syndrome were evident following vitamin D repletion. The next step of the research was to determine whether different metabolic phenotypes could be identified in a group of apparently healthy subjects and whether any of these may be more responsive to vitamin D supplementation in terms of markers of metabolic syndrome. Cluster analysis was performed on a number of targeted markers of the metabolic syndrome resulting in five different metabolic phenotypes. Interestingly, when the effects of the intervention were examined in these biomarker clusters individually, a vitamin D responsive phenotype was identified. Although this vitamin D responsive phenotype consisted of relatively small numbers, the numbers are in line with many other recent vitamin D intervention studies [20, 21, 40]. This responsive phenotype (biomarker cluster 5) was characterised by deficient vitamin D status at baseline (< 50 nmol/L) and higher plasma concentrations of resistin and adiponectin compared with the other clusters. It is noteworthy that when the vitamin D deficient subgroup was studied, their baseline serum 25(OH)D and the change in their serum 25(OH)D following supplementation were very similar to that observed in the vitamin D responsive cluster. Thus, low values of serum 25(OH)D or response to vitamin D supplementation alone are insufficient to elicit a response in terms of the markers of the metabolic syndrome. The results indicated that altered adipokine profiles are needed in addition to low concentrations of vitamin D. It should also be noted that the vitamin D responsive cluster showed normal ranges for fasting glucose, insulin and HOMA levels at baseline. Although elucidating the mechanism of action of vitamin D in terms of the metabolic syndrome is beyond the scope of the current study, it is possible to hypothesise why cluster 5 responded to vitamin D repletion.

Table 7. Relative intensities and VIP values of the most discriminating metabolites for comparison of pre *versus* post-samples from vitamin D treated subjects in biomarker cluster 5

Metabolite	Chemical shift (ppm)	VIP	Pre	Post	*p*-Value
Glycerol-phosphocholine	3.20	3.61	2.44±0.92	1.60±0.29	0.026
lactate	1.32	3.54	7.23±1.08	6.10±1.74	0.125
VLDL+LDL CH_3	0.84	3.38	3.74±0.37	3.10±0.48	0.008
VLDL+LDL CH_2	1.24	3.31	3.52±0.33	2.93±0.43	0.006
Glucose	3.40	3.08	3.46±0.78	2.80±0.29	0.040
Taurine/glucose	3.28	2.62	1.15±0.28	1.53±0.20	0.007
Glutamine	2.48	2.28	0.37±0.11	0.61±0.11	<0.001

Clusters 5 was derived from *k*-means cluster analysis of 14 different biomarkers. All values are expressed as relative intensities and are mean±SD; *p*-values are based on independent samples *t*-test. VIP, variable importance in the projection.

Reports in the literature suggest that vitamin D exerts its action on glucose homeostasis through its effects on insulin sensitivity and insulin secretion. It is possible that this cluster responded to the vitamin D intervention as a result of enhanced pancreatic β-cell function promoted by the higher level of adiponectin and a low level of CRP [41]. Declining β-cell function has been linked to changes in circulating adiponectin and CRP, and *in vitro* studies support a positive role of adiponectin on β-cell function [42].

Metabolic phenotyping in the present study resulted in the identification of five clusters each with their own distinguishing characteristics. For example, biomarker cluster 2 represents an impaired fasting glucose phenotype [43], whereas biomarker cluster 3 could be considered a 'healthy' cluster according to their biochemical profile. To our knowledge, this is the first time that unsupervised biochemical phenotyping was used on an extensive range of targeted biochemical markers. Most studies to date which have sought to explore aspects of baseline phenotype have relied on one marker of phenotype or ratios of baseline phenotypic values.

To develop the concept of metabolic phenotyping, the relationship between biomarker clusters and metabolomic profiles was examined. Overall, the results indicate that biochemical phenotypes are reflected in plasma ^{1}H NMR metabolomic spectra. Taking the example depicted in Section 3; lactate, VLDL, LDL and choline were identified as the key metabolites driving the separation in metabolomic profiles of biomarker clusters 4 and 5. Elevated lactate in the plasma metabolomic profiles of individuals in cluster 4 could reflect an increased energy demand or decreased oxidative capacity [44–47]. With regard to VLDL, LDL and choline concentrations, lipoprotein metabolism would suggest that increased VLDL and LDL concentrations coupled with decreased choline concentrations as seen in biomarker cluster 4 are likely to reflect increased VLDL synthesis in the liver.

To further explore the changes induced following the vitamin D intervention, an untargeted metabolomic approach was applied to cluster 5 and revealed a significant reduction in VLDL+LDL signals, glucose and glycerol phosphocholine. The change in lipid metabolites is important considering the reported associations between circulating 25(OH)D levels and cardiovascular diseases, diabetes and metabolic syndrome. In addition, it would support the hypothesis that the effects of vitamin D are mediated in part though modulation of lipid metabolism [48].

In conclusion, the present study has shown how a targeted approach to metabolic phenotyping using an unsupervised data mining technique can identify discrete clusters with distinct metabolite profiles. This in its own right marks a significant advance in the concept of metabotypes. The finding that this approach identified a vitamin D responsive phenotype is a major milestone for the potential use of such metabotypes in nutritional research. However, further work is necessary to establish the true merit of this approach in understanding the responsiveness to specific dietary interventions, and the incorporation of other phenotypic and genetic factors will be an important focus in the future. In addition to research that can identify metabotypes, work should focus on the biological implications of such metabotypes.

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5 References

[1] Bischoff-Ferrari, H. A., Giovannucci, E., Willett, W. C., Dietrich, T., Dawson-Hughes, B., Estimation of optimal serum concentrations of 25-hydroxyvitamin D for multiple health outcomes. *Am. J. Clin. Nutr.* 2006, *84*, 18–28.

[2] Holick, M. F., Vitamin D deficiency. *N. Engl. J. Med.* 2007, *357*, 266–281.

[3] Lee, J. H., O'Keefe, J. H., Bell, D., Hensrud, D. D., Holick, M. F., Vitamin D deficiency: an important, common, and

easily treatable cardiovascular risk factor? *J. Am. Coll. Cardiol.* 2008, *52*, 1949–1956.

[4] Johnson, D. D., Wagner, C. L., Hulsey, T. C., McNeil, R. B. *et al.*, Vitamin D deficiency and insufficiency is common during pregnancy. *Am. J. Perinatol.* 2010. DOI: 10.1055/s-0030-1262505.

[5] Dong, Y., Pollock, N., Stallmann-Jorgensen, I. S., Gutin, B. *et al.*, Low 25-hydroxyvitamin D levels in adolescents: race, season, adiposity, physical activity, and fitness. *Pediatrics* 2010, *125*, 1104–1111.

[6] O'Sullivan, M., Nic Suibhne, T., Cox, G., Healy, M., O'Morain, C., High prevalence of vitamin D insufficiency in healthy Irish adults. *Ir. J. Med. Sci.* 2008, *177*, 131–134.

[7] Holick, M. F., Vitamin D status: measurement, interpretation, and clinical application. *Ann. Epidemiol.* 2009, *19*, 73–78.

[8] Andersen, R., Molgaard, C., Skovgaard, L. T., Brot, C. *et al.*, Teenage girls and elderly women living in northern Europe have low winter vitamin D status. *Eur. J. Clin. Nutr.* 2005, *59*, 533–541.

[9] Scragg, R., Holdaway, I., Singh, V., Metcalf, P. *et al.*, Serum 25-hydroxyvitamin D3 levels decreased in impaired glucose tolerance and diabetes mellitus. *Diabetes Res. Clin. Pract.* 1995, *27*, 181–188.

[10] Cigolini, M., Iagulli, M. P., Miconi, V., Galiotto, M. *et al.*, Serum 25-hydroxyvitamin D3 concentrations and prevalence of cardiovascular disease among type 2 diabetic patients. *Diabetes Care* 2006, *29*, 722–724.

[11] Ford, E. S., Ajani, U. A., McGuire, L. C., Liu, S., Concentrations of serum vitamin D and the metabolic syndrome among U.S. adults. *Diabetes Care* 2005, *28*, 1228–1230.

[12] Hypponen, E., Boucher, B. J., Berry, D. J., Power, C., 25-Hydroxyvitamin D, IGF-1, and metabolic syndrome at 45 years of age. *Diabetes* 2008, *57*, 298–305.

[13] Chiu, K. C., Chu, A., Go, V. L., Saad, M. F., Hypovitaminosis D is associated with insulin resistance and beta cell dysfunction. *Am. J. Clin. Nutr.* 2004, *79*, 820–825.

[14] Forouhi, N. G., Luan, J., Cooper, A., Boucher, B. J., Wareham, N. J., Baseline serum 25-hydroxy vitamin D is predictive of future glycemic status and insulin resistance. *Diabetes* 2008, *57*, 2619–2625.

[15] Liu, E., McKeown, N. M., Newby, P. K., Meigs, J. B. *et al.*, Cross-sectional association of dietary patterns with insulin-resistant phenotypes among adults without diabetes in the Framingham Offspring Study. *Br. J. Nutr.* 2009, *102*, 576–583.

[16] Auwerx, J., Bouillon, R., Kesteloot, H., Relation between 25-hydroxyvitamin D3, apolipoprotein A-I, and high density lipoprotein cholesterol. *Arterioscler. Thromb. Vasc. Biol.* 1992, *12*, 671–674.

[17] John, W. G., Noonan, K., Mannan, N., Boucher, B. J., Hypovitaminosis D is associated with reductions in serum apolipoprotein A-I but not with fasting lipids in British Bangladeshis. *Am. J. Clin. Nutr.* 2005, *82*, 517–522.

[18] Judd, S. E., Nanes, M. S., Ziegler, T. R., Wilson, P. W. F., Tangpricha, V., Optimal vitamin D status attenuates the age-associated increase in systolic blood pressure in white Americans: results from the third National Health and Nutrition Examination Survey. *Am. J. Clin. Nutr.* 2008, *87*, 136–141.

[19] Parker, J., Hashmi, O., Dutton, D., Mavrodaris, A. *et al.*, Levels of vitamin D and cardiometabolic disorders: systematic review and meta-analysis. *Maturitas* 2010, *65*, 225–236.

[20] Borissova, A. M., Tankova, T., Kirilov, G., Dakovska, L., Kovacheva, R., The effect of vitamin D3 on insulin secretion and peripheral insulin sensitivity in type 2 diabetic patients. *Int. J. Clin. Pract.* 2003, *57*, 258–261.

[21] Rudnicki, P. M., Molsted-Pedersen, L., Effect of 1,25-dihydroxycholecalciferol on glucose metabolism in gestational diabetes mellitus. *Diabetologia* 1997, *40*, 40–44.

[22] von Hurst, P. R., Stonehouse, W., Coad, J., Vitamin D supplementation reduces insulin resistance in South Asian women living in New Zealand who are insulin resistant and vitamin D deficient – a randomised, placebo-controlled trial. *Br. J. Nutr.* 2010, *103*, 549–555.

[23] Tai, K., Need, A. G., Horowitz, M., Chapman, I. M., Glucose tolerance and vitamin D: effects of treating vitamin D deficiency. *Nutrition* 2008, *24*, 950–956.

[24] de Boer, I. H., Tinker, L. F., Connelly, S., Curb, J. D. *et al.*, Calcium plus vitamin D supplementation and the risk of incident diabetes in the Women's Health Initiative. *Diabetes Care* 2008, *31*, 701–707.

[25] Clayton, T. A., Baker, D., Lindon, J. C., Everett, J. R., Nicholson, J. K., Pharmacometabonomic identification of a significant host–microbiome metabolic interaction affecting human drug metabolism. *Proc. Natl. Acad. Sci. USA* 2009, *106*, 14728–14733.

[26] Brindle, J. T., Antti, H., Holmes, E., Tranter, G. *et al.*, Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using 1H-NMR-based metabonomics. *Nat. Med.* 2002, *8*, 1439–1444.

[27] Sreekumar, A., Poisson, L. M., Rajendiran, T. M., Khan, A. P. *et al.*, Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. *Nature* 2009, *457*, 910–914.

[28] Rezzi, S., Ramadan, Z., Fay, L. B., Kochhar, S., Nutritional metabonomics: applications and perspectives. *J. Proteome Res.* 2007, *6*, 513–525.

[29] Holmes, E., Wilson, I. D., Nicholson, J. K., Metabolic phenotyping in health and disease. *Cell* 2008, *134*, 714–717.

[30] Gibney, M. J., Walsh, M., Brennan, L., Roche, H. M. *et al.*, Metabolomics in human nutrition: opportunities and challenges. *Am. J. Clin. Nutr.* 2005, *82*, 497–503.

[31] van Ommen, B., Keijer, J., Kleemann, R., Elliott, R. *et al.*, The challenges for molecular nutrition research 2: quantification of the nutritional phenotype. *Genes Nutr.* 2008, *3*, 51–59.

[32] Cameron, N., *The Measurement of Human Growth. Instrumentation, Surface Landmarks, and Anthropometric Measurements*. Croom Helm, Kent 1984.

[33] Zimmet, P., Magliano, D., Matsuzawa, Y., Alberti, G., Shaw, J., The metabolic syndrome: a global public health problem and a new definition. *J. Atheroscler. Thromb.* 2005, *12*, 295–300.

[34] Matsuzawa, Y., The metabolic syndrome and adipocytokines. *Fed. Eur. Biochem. Soc. Lett.* 2006, *580*, 2917–2921.

[35] Maury, E., Brichard, S. M., Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. *Mol. Cell. Endocrinol.* 2009, *314*, 1–16.

[36] Reis, J. P., von Mühlen, D., Miller, E. R., Relation of 25-hydroxyvitamin D and parathyroid hormone levels with metabolic syndrome among US adults. *Eur. J. Endocrinol.* 2008, *159*, 41–48.

[37] Liu, E., Meigs, J. B., Pittas, A. G., McKeown, N. M. *et al.*, Plasma 25-hydroxyvitamin D is associated with markers of the insulin resistant phenotype in nondiabetic adults. *J. Nutr.* 2009, *139*, 329–334.

[38] Jorde, R., Figenschau, Y., Supplementation with cholecalciferol does not improve glycaemic control in diabetic subjects with normal serum 25-hydroxyvitamin D levels. *Eur. J. Nutr.* 2009, *48*, 349–354.

[39] Fliser, D., Stefanski, A., Franek, E., Fode, P. *et al.*, No effect of calcitriol on insulin-mediated glucose uptake in healthy subjects. *Eur. J. Clin. Invest.* 1997, *27*, 629–633.

[40] Biancuzzo, R. M., Young, A., Bibuld, D., Cai, M. H. *et al.*, Fortification of orange juice with vitamin D(2) or vitamin D(3) is as effective as an oral supplement in maintaining vitamin D status in adults. *Am. J. Clin. Nutr.* 2010, *91*, 1621–1626.

[41] Xiang, A. H., Kawakubo, M., Trigo, E., Kjos, S. L., Buchanan, T. A., Declining beta-cell compensation for insulin resistance in hispanic women with recent gestational diabetes mellitus. *Diabetes Care* 2010, *33*, 396–401.

[42] Rakatzi, I., Mueller, H., Ritzeler, O., Tennagels, N., Eckel, J., Adiponectin counteracts cytokine- and fatty acid-induced apoptosis in the pancreatic beta-cell line INS-1. *Diabetologia* 2004, *47*, 249–258.

[43] World Health Organisation. *Definition and Diagnosis of Diabetes Mellitus and Intermediate Hyperglycemia: Report of a WHO/IDF Consultation*. World Health Organisation, Geneva 2006.

[44] Keun, H. C., Sidhu, J., Pchejetski, D., Lewis, J. S. *et al.*, Serum molecular signatures of weight change during early breast cancer chemotherapy. *Clin. Cancer Res.* 2009, *15*, 6716–6723.

[45] Kim, S.-H., Yang, S.-O., Kim, H.-S., Kim, Y. *et al.*, ^{1}H-nuclear magnetic resonance spectroscopy-based metabolic assessment in a rat model of obesity induced by a high-fat diet. *Anal. Bioanal. Chem.* 2009, *395*, 1117–1124.

[46] Jansson, P. A., Larsson, A., Smith, U., Lönnroth, P., Lactate release from the subcutaneous tissue in lean and obese men. *J. Clin. Invest.* 1994, *93*, 240–246.

[47] DiGirolamo, M., Newby, F. D., Lovejoy, J., Lactate production in adipose tissue: a regulated function with extra-adipose implications. *Fed. Am. Soc. Exp. Biol. J.* 1992, *6*, 2405–2412.

[48] Major, G. C., Alarie, F., Dore, J., Phouttama, S., Tremblay, A., Supplementation with calcium+vitamin D enhances the beneficial effect of weight loss on plasma lipid and lipoprotein concentrations. *Am. J. Clin. Nutr.* 2007, *85*, 54–59.

