Food & Function

Linking the chemistry and physics of food with health and nutrition

www.rsc.org/foodfunction

Volume 3 | Number 10 | October 2012 | Pages 987–1100

ISSN 2042-6496

COVER ARTICLE

Almoosawi *et al.*

Differential effect of polyphenol-rich dark chocolate on biomarkers of glucose metabolism and cardiovascular risk factors in healthy, overweight and obese subjects: a randomized clinical trial

RSC Publishing

2042-6496(2012)3:10;1-Y

Food & Function

Dynamic Article Links

Cite this: *Food Funct.*, 2012, **3**, 1035

www.rsc.org/foodfunction

PAPER

Differential effect of polyphenol-rich dark chocolate on biomarkers of glucose metabolism and cardiovascular risk factors in healthy, overweight and obese subjects: a randomized clinical trial

S. Almoosawi,*[a] C. Tsang,[b] L. M. Ostertag,[c] L. Fyfe[d] and E. A. S. Al-Dujaili[d]

Received 12th March 2012, Accepted 1st July 2012
DOI: 10.1039/c2fo30060e

The association between excess cortisol and various parameters of metabolic syndrome including hypertension, insulin resistance and dyslipidaemia is increasingly recognised. The present single-blind randomised placebo-controlled cross-over study compared the effect of polyphenol-rich dark chocolate (DC) on biomarkers of glucose metabolism, lipid profile, and blood pressure (BP) in females with BMI $\geq$ 25 kg m^{-2} ($n = 21$) and females with BMI < 25 kg m^{-2} ($n = 21$). Volunteers consumed 20 g of DC containing 500 mg polyphenols or a placebo DC with negligible polyphenol-content daily for 4 weeks, separated by a 2-week washout period. Systolic BP and diastolic BP decreased after 4 weeks of polyphenol-rich DC. Placebo raised fasting insulin, homeostasis model assessment of insulin resistance (HOMA-IR) and salivary cortisol, an effect that was significantly different from polyphenol-rich DC which had a negligible effect on fasting insulin, HOMA-IR and salivary cortisol. Females with BMI $\geq$ 25 kg m^{-2} responded less favourably to placebo than lean females and consequently had higher fasting insulin and HOMA-IR, in addition to a lower quantitative sensitivity check index (QUICKI) after ingestion of placebo compared to polyphenol-rich DC. No significant changes in lipid profile were observed. This study provides evidence for the metabolic benefits of consuming polyphenol-rich dark chocolate while demonstrating the possibility of adverse effects occurring with polyphenol-poor chocolate placebo.

Introduction

Obesity is a multifactorial condition linked to numerous cardiometabolic risk factors including insulin resistance, hypertension and dyslipidaemia.[1,2] In recent years, evidence for the role of abnormal cortisol metabolism in mediating the association between obesity and cardiometabolic risk factors, particularly hyperinsulinaemia and insulin resistance, have been described.[2–5] This evidence is based on the central role of cortisol in regulating glucose and blood pressure (BP) homeostasis, and the ability of cortisol to promote oxidative stress and decrease nitric oxide bioavailability.[6,7] Current experimental and epidemiological studies provide evidence for a protective role for polyphenol-rich dark chocolate (DC) and cocoa polyphenols on markers of glucose regulation, blood pressure and lipid profile.[8–18] This protective role has been ascribed to the antioxidant properties of DC and cocoa polyphenols and their ability to modulate nitric oxide bioavailability.[14,17] However, few studies have examined the involvement of the endocrine system in mediating the cardiometabolic health-effects of polyphenols. We have previously reported that the consumption of small portions of DC by overweight and obese individuals improves fasting capillary glucose and blood pressure levels without significantly altering urinary cortisol excretion.[8] To overcome the limitations of our previous study and to elaborate on our findings further, the current study aimed to examine the effect of 4-week consumption of DC containing 500 mg polyphenols on cardiometabolic risk factors, cortisol metabolism and antioxidant status across a range of BMI, the objectives being to establish whether consumption of polyphenol-rich DC elicits a differential response between lean and overweight females.

Methods

Design

The study used a single-blind randomised placebo-controlled cross-over design where each subject acted as their own control. Following a 1-week run-in phase, eligible subjects were randomly assigned to receive 20 g DC containing 500 mg polyphenols or

[a]*MRC Human Nutrition Research, Elsie Widdowson Laboratory, 120 Fulbourn Road, Cambridge CB1 9NL, UK. E-mail: suzana.almoosawi@mrc-hnr.cam.ac.uk; Fax: +44 (0)1223 437 515; Tel: +44 (0)1223 437 623; +44 (0)1223 426 356*
[b]*Health & Food Science School of Contemporary Sciences, University of Abertay, Dundee, UK*
[c]*Rowett Institute of Nutrition and Health, University of Aberdeen, Aberdeen, UK*
[d]*Department of Dietetics, Nutrition and Biological Sciences, Queen Margaret University, Queen Margaret Drive, Edinburgh, UK*

20 g placebo DC. Participants followed each intervention for 4 weeks, after which they were crossed-over to the next intervention separated by a 2-week washout period. The study included healthy, non-smoking lean (BMI = 18.5–24.99 kg m^{-2}) and overweight females (both overweight, BMI $\geq$ 25 kg m^{-2} and obese, BMI $\geq$ 30 kg m^{-2}) with no history of diabetes, hypertension or cardiovascular disease. Individuals taking dietary supplements, BP or cholesterol-lowering drugs, and those with soy and nut allergies were excluded. Smokers were excluded to minimise the confounding effect of nicotine consumption on hypothalamic–pituitary–adrenal axis activity. Participants gave written consent and completed a lifestyle questionnaire before being screened for fasting blood glucose, total cholesterol, BP and BMI to determine their eligibility. The study was conducted according to the guidelines laid down in the Declaration of Helsinki. All experiments were performed in compliance with the relevant laws and institutional guidelines and all procedures were approved by the university ethics committee.

Diet

Table 1 provides a summary of the nutrient composition of the polyphenol-rich DC and the placebo used in this study (data provided by the manufacturer who supplied the chocolate: Barry Callebaut, Lebbeke, Belgium). The polyphenol-rich DC contained 500 mg of polyphenols, as estimated by the Folin-Ciocalteu method, and 65.5% of cocoa solids. The 500 mg dose was selected on the basis of a preliminary study.[8] The placebo was a DC matched for taste, texture, colour and macronutrient composition to the polyphenol-rich DC, but which contained no polyphenols. The participants were asked to distribute their DC dose over the day and to maintain their usual dietary intakes. A list of polyphenol-rich foods and beverages was provided and participants were instructed to refrain from eating these products during the entire study duration.[8] Participants were asked to fill in a 3-day (two weekdays and one weekend) diet and physical activity diary during the run-in phase and at the end of each dietary intervention. Diet diaries were analysed for energy and macronutrient intake using Windiet software (Windiet Research, Univation Ltd, Robert Gordon University, Aberdeen, UK). Compliance with the study's protocol was assessed by direct interviewing, returning of empty chocolate foils and assessment of diet diaries.

Table 1 Nutritional composition of 20 g of placebo or polyphenol-rich dark chocolate

Component	Placebo dark chocolate	Polyphenol-rich dark chocolate
Total polyphenols (mg)[a]	NG[c]	500
Epicatechin and catechin (mg)[b]	NG	18.99
Epicatechin and catechin ratio	—	9 : 1
Theobromine (mg)	130.00	163.40
Caffeine (mg)	14.30	17.54
Energy (kJ)	425.80	425.80
Total fat (g)	7.34	7.34
Saturated fat (g)	4.62	4.62
Monounsaturated fat (g)	2.46	2.46
Polyunsaturated fat (g)	0.26	0.26
Trans fat (g)	0.04	0.04
Protein (g)	1.34	1.34
Carbohydrate (g)	7.44	7.44
Magnesium (mg)	36.00	42.00
Sodium (mg)	1.24	0.38
Potassium (mg)	340.00	150.00

[a] Estimated using Folin-Ciocalteu method. [b] Measured by HPLC. [c] NG, negligible.

Measurements

All tests were conducted at the start and at the end of each treatment period. Participants were asked to consume the same diet the day before their appointment and to avoid heavy physical activity and alcohol 24 h before testing. Blood samples were obtained by venepuncture following a 12 h fast. Fasting glucose was measured using a commercial hexokinase assay (Sentinel, UK). Fasting insulin was measured using a commercial insulin ELISA kit (Mercodia, Sweden). Homeostasis model assessment of insulin resistance (HOMA-IR), homeostasis model assessment of β-cell function (HOMA-β), quantitative sensitivity check index (QUICKI) and revised-QUICKI were calculated based on published equations.[19–21] Assessment of lipid profile and liver enzymes (aspartate aminotransferase, troponin) was undertaken at the Clinical Biochemistry Laboratory, Royal Infirmary of Edinburgh, Scotland, UK, using an automated platform (Olympus, UK). Serum non-esterified fatty acids were quantified using an automated enzymatic colorimetric method (Wako, Germany) at the Rowett Institute of Nutrition and Health, Aberdeen, UK. Urinary polyphenol content and antioxidant status were measured in 24 h urine samples using the Folin-Ciocalteu method for estimation of total polyphenols[22] and urinary oxygen radical absorbance capacity,[23] respectively. A validated automated A&D Medical UA-767 BP monitor (A&D medical, San Jose, CA, USA) was used to measure BP after a rest of 10 min. Three values were taken at 2 min intervals to increase the reliability of the results. Salivary cortisol and cortisone were analysed in duplicates using an enzyme-linked immunosorbent assay (ELISA) according to previously described methods.[24,25] The data was then used to calculate the cortisone/cortisol ratio which serves as an index of 11β-hydroxysteroid dehydrogenase activity.[26] Monitoring the activity of this enzyme helps detect changes in the peripheral metabolism of cortisol.[26]

Statistical analysis

All statistical analyses were performed using SPSS for Windows, version 16.0.0 (SPSS Inc, Chicago, IL, USA). Sample size was calculated based on data from the pilot study[8] to permit the detection of a 0.5 ± 0.6 mmol L^{-1} reduction in fasting glucose with a power of $(1 - \beta) = 0.8$ and $\alpha = 0.05$ (~22 subjects per group). Continuous normally distributed data are expressed as mean $\pm$ SD unless otherwise stated. Differences in baseline characteristics between the two BMI categories (BMI < 25 kg m^{-2} and BMI $\geq$ 25 kg m^{-2}) were examined using an independent sample *t*-test with the BMI category as the grouping variable and age, anthropometrical data, biochemical measurements, blood pressure, salivary free cortisol/cortisone ratio, and urinary antioxidant capacity as the dependent variables.

For multiple comparisons, data were analysed with a three-factor repeated measure analysis of variance with time (pre- and post-) and treatment (DC and placebo) as the two within-subject

factors and BMI category as the between-subject factor. The three-factor analysis of variance (ANOVA) detects differences in response between the two BMI categories, as well as comparing the effect of the two treatment groups (DC and placebo) and the pre and post-treatment values of outcome variables. Bonferroni adjustment was used to account for multiple testing. For statistically significant between-subject effects, data is presented separately for the two BMI categories. Significant within-subject effects were further explored by paired Student's *t*-test.

Changes in physical activity, energy, and macronutrient intake were assessed with a mixed between-within subject analysis of variance with time as the within-subject factor and BMI as the between-subject factor.

Association between changes in biomarkers of glucose metabolism, blood pressure and lipid profile, and BMI were assessed using stepwise regression according to the following equation: $y = (a_1x_1) + (a_2x_2) + (a_3x_3) + a_4$, in which: y is the change in outcome from baseline per person per treatment group, $x_1$is the dummy variable (0 for placebo and 1 for polyphenol-rich DC), x_2 is the BMI expressed as a continuous variable, x_3 is the product term for the dummy variable and BMI, and a_{1-4} are the β coefficients.

Characteristics of participants

The study included 42 volunteers (41 Caucasian and 1 Hispanic), of which 21 had a normal BMI range (mean BMI = 21.64 ± 2.23 kg m^{-2}), 13 were overweight (mean BMI = 26.98 ± 1.25 kg m^{-2}) and 8 were obese (mean BMI = 32.23 ± 1.56 kg m^{-2}). The flow of participants through the study is shown in Fig. 1.

Compared to females with BMI < 25 kg m^{-2}, females with BMI ≥ 25 kg m^{-2} had marginally higher fasting insulin ($P = 0.053$) and significantly greater total cholesterol: high-density lipoprotein ratio ($P = 0.043$), systolic blood pressure (SBP) ($P < 0.001$) and diastolic blood pressure (DBP) ($P < 0.001$). The overweight group also had a significantly higher physical activity level ($P < 0.0001$), waist circumference ($P < 0.0001$), hip circumference ($P < 0.0001$), waist/hip ratio ($P = 0.023$) and percentage body fat ($P < 0.0001$). Females with BMI < 25 kg m^{-2} had higher percentage body water than females with BMI ≥ 25 kg m^{-2} ($P < 0.0001$). No differences in other variables were observed.

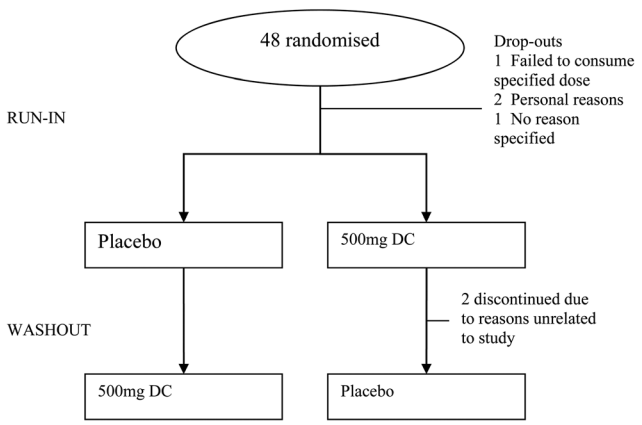

Fig. 1 Flow of participants through the study.

Glucose metabolism and blood pressure

There was a significant effect of treatment on fasting glucose, fasting insulin, HOMA-IR, QUICKI, revised QUICKI, SBP, and DBP. A significant treatment-by-time interaction was also observed for these variables suggesting that the change from baseline across the two treatment groups (polyphenol-rich DC *vs.* placebo) was not similar (Table 2). The response to polyphenol-rich DC and placebo differed by BMI as indicated by the significant between-subject effect. Consumption of polyphenol-rich DC for 4 weeks reduced fasting glucose and HOMA-IR from baseline by 0.58 ± 0.68 mmol l^{-1} ($P < 0.001$ by paired Student's *t*-test) and 1.03 ± 0.76 units ($P = 0.041$), respectively in females with BMI ≥ 25 kg m^{-2}. There were no significant changes in these parameters in females with BMI < 25 kg m^{-2} (fasting glucose: $P = 0.268$; HOMA-IR $P = 0.603$). Polyphenol-rich DC did not reduce fasting insulin levels from the baseline in either the overweight ($P = 0.269$) or lean group ($P = 0.268$). Polyphenol-rich DC decreased SBP in lean and overweight females by 3.41 ± 6.08 ($P = 0.014$) and 4.48 ± 7.82 mmHg ($P = 0.016$), respectively. In contrast, only females with BMI ≥ 25 kg m^{-2} experienced a 3.22 ± 3.75 mmHg reduction in DBP following a 4 week consumption of polyphenol-rich DC ($P < 0.001$).

Females with BMI ≥ 25 kg m^{-2} responded less favourably to placebo DC and consequently had higher fasting insulin (2.73 ± 5.08 mU l^{-1} increase from baseline; $P = 0.023$), HOMA-IR (0.66 ± 1.26 unit increase; $P = 0.03$) and lower QUICKI (0.01 ± 0.02 unit decrease; $P = 0.033$) levels after consumption of the placebo. This effect was not observed in lean females ($P > 0.05$).

Differences between BMI categories and between the effect of polyphenol-rich DC and placebo on glucose metabolism and blood pressure were further reinforced by the stepwise regression analysis (Fig. 2 and 3). As observed, polyphenol-rich DC reduced fasting glucose ($P = 0.002$) and DBP ($P = 0.001$) to a greater extent in subjects with a higher BMI. Insulin, on the other hand, remained relatively stable at the end of the 4 week treatment with polyphenol-rich DC. By contrast, the detrimental effects of the placebo, on insulin ($P = 0.014$), HOMA-IR ($P = 0.001$) and QUICKI ($P = 0.005$) were more pronounced in females with elevated BMI than those with a lower BMI.

Antioxidant status and glucocorticoid levels

There was a significant effect of treatment and treatment-by-time interaction on urinary total polyphenols and antioxidant capacity (Fig. 4). Polyphenol-rich DC increased total polyphenols excretion and improved antioxidant status as indicated by the rise in urinary total polyphenols levels and ORAC, whereas consumption of placebo increased oxidative stress as noted by the fall in ORAC.

There was a significant treatment effect on mean daily salivary cortisol ($P = 0.006$). Placebo DC raised mean daily salivary cortisol from 10.52 ± 65.64 nmol l^{-1} to 26.34 ± 40.08 nmol l^{-1}, while polyphenol-rich DC did not alter any of these parameters (Fig. 5). There were no significant changes in salivary cortisone or the cortisone/cortisol ratio.

No significant between-subject effects were detected by three-factor ANOVA suggesting that changes in urinary antioxidant capacity and salivary glucocorticoids were similar across the two

Table 2 Effect of polyphenol-rich dark chocolate and placebo on biomarkers of glucose metabolism, blood pressure and anthropometry in lean females ($n = 21$) and overweight females ($n = 21$) as shown by a three-factor ANOVA

		Polyphenol-rich dark chocolate					Placebo					Three-factor ANOVA	
		Before		After		Δ[d]	Before		After		Δ	Wilk's lambda	
Variable	BMI	Mean	95% CI	Mean	95% CI		Mean	95% CI	Mean	95% CI		Treatment	Treatment × time
Glucose (mmol l^{-1})	BMI < 25	4.64	(4.23, 5.05)	4.71	(4.21, 5.21)	−0.14	4.50	(4.03, 4.97)	4.98	(4.46, 5.50)	0.27	0.028	0.003
	BMI ≥ 25	5.06	(4.57, 5.55)	4.94[b]	(4.46, 5.42)	−0.58	4.49	(3.93, 5.05)	4.93	(4.45, 5.41)	−0.01		
Insulin (mU L^{-1})	BMI < 25	7.96	(6.61, 9.31)	7.68	(6.29, 9.07)	−0.40	7.56	(6.19, 8.93)	7.67	(6.17, 9.17)	−0.01	0.03	0.007
	BMI ≥ 25	9.53	(7.80, 11.26)	9.48	(8.03, 10.93)	−0.72	8.81	(7.06, 10.56)	12.21[b]	(9.91, 14.51)	2.73		
HOMA-IR	BMI < 25	1.62	(1.31, 1.93)	1.61	(1.35, 1.87)	−0.20	1.42	(1.08, 1.76)	1.68	(1.32, 2.04)	0.07	0.005	0.001
	BMI ≥ 25	2.19	(1.67, 2.71)	2.12	(1.67, 2.57)	−0.36	1.83	(1.28, 2.38)	2.78[b]	(2.07, 3.49)	0.66		
QUICKI	BMI < 25	0.36	(0.35, 0.37)	0.37	(0.36, 0.38)	0.01	0.37	(0.36, 0.38)	0.36	(0.35, 0.37)	0.00	0.04	0.002
	BMI ≥ 25	0.35	(0.34, 0.36)	0.35	(0.34, 0.36)	0.01	0.36	(0.35, 0.37)	0.34	(0.33, 0.35)	−0.01		
Revised-QUICKI	BMI < 25	0.91	(0.82, 1.00)	0.97	(0.83, 1.11)	0.10	1.01	(0.87, 1.15)	0.90	(0.78, 1.02)	−0.06	0.026	0.016
	BMI ≥ 25	0.86	(0.76, 0.96)	0.80	(0.63, 0.97)	0.11	0.98	(0.91, 1.05)	0.76	(0.66, 0.86)	−0.05		
SBP (mmHg)	BMI < 25	106.86	(103.53, 110.19)	102.98[a]	(99.42, 106.54)	−3.87	106.71	(104.02, 109.40)	107.81	(103.61, 112.01)	1.10	0.02	0.007
	BMI ≥ 25	119.29	(114.29, 124.29)	114.81[a]	(110.78, 118.84)	−4.48	120.13	(116.82, 123.44)	118.10	(114.91, 121.29)	−2.03		
DBP (mmHg)	BMI < 25	71.70	(69.77, 73.63)	70.35	(68.25, 72.45)	−1.35	71.86	(69.89, 73.83)	73.68	(71.05, 76.31)	1.83	0.008	0.003
	BMI ≥ 25	79.25	(75.66, 82.84)	76.03[a]	(72.09, 79.97)	−3.22	79.35	(75.80, 82.90)	80.33	(76.78, 83.88)	0.98		
Weight (kg)	BMI < 25	59.85	(55.90, 63.81)	59.86	(56.03, 63.70)	0.01	60.25	(56.52, 63.97)	60.20	(56.21, 64.20)	−0.05	0.003	0.266
	BMI ≥ 25	79.33	(75.59, 83.06)	79.04[c]	(75.38, 82.71)	−0.29	79.38	(75.63, 83.13)	79.66[c]	(75.95, 83.37)	0.28		
WC (cm)	BMI < 25	74.33	(71.66, 77.01)	73.91	(71.49, 76.34)	−0.42	75.81	(71.87, 79.75)	74.31	(71.78, 76.84)	−1.50	0.488	0.294
	BMI ≥ 25	89.69	(85.38, 94.00)	90.14	(85.96, 94.33)	0.45	88.79	(84.83, 92.74)	87.10	(80.51, 93.70)	−1.68		
WHR	BMI < 25	0.76	(0.74, 0.78)	0.76	(0.74, 0.78)	0.00	0.77	(0.75, 0.80)	0.76	(0.74, 0.78)	−0.01	0.699	0.524
	BMI ≥ 25	0.81	(0.78, 0.83)	0.81	(0.78, 0.84)	0.00	0.80	(0.78, 0.83)	0.80	(0.78, 0.83)	0.00		

[a] $P \leq 0.05$ compared to pre-treatment. [b] $P \leq 0.001$ compared to pre-treatment. [c] Significant difference in means between post-polyphenol-rich dark chocolate and placebo. [d] Post-treatment - baseline.

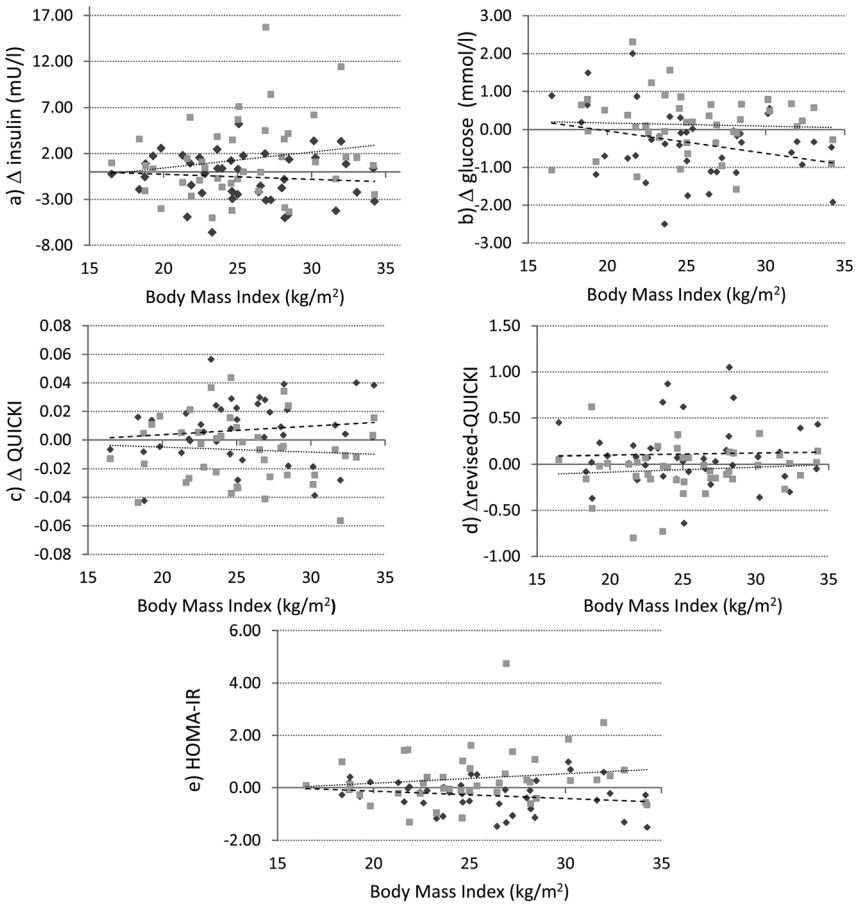

Fig. 2 Body mass index determines the magnitude of the effect of polyphenol-rich dark chocolate (◆) and placebo (■) on biomarkers of glucose metabolism. Regression equations were as follows: (a) placebo $y = 0.174\text{BMI} - 3.6621$ $R^2 = 0.033$; polyphenol-rich DC $y = -0.0542\text{BMI} + 0.8192$ $R^2 = 0.0086$ (b) placebo $y = -0.0084\text{BMI} + 0.3429$ $R^2 = 0.0024$; polyphenol-rich DC $y = -0.0592\text{BMI} + 1.1467$ $R^2 = 0.0903$ (c) placebo $y = -0.0003\text{BMI} + 0.002$ $R^2 = 0.0047$; polyphenol-rich DC $y = 0.0006\text{BMI} - 0.0083$ $R^2 = 0.0157$ (d) placebo $y = 0.0054\text{BMI} - 0.1925$ $R^2 = 0.0094$; polyphenol-rich DC $y = 0.0022\text{BMI} + 0.053$ $R^2 = 0.0009$ (e) placebo $y = 0.0365\text{BMI} - 0.5603$ $R^2 = 0.0235$; polyphenol-rich DC $y = -0.0283\text{BMI} + 0.4364$ $R^2 = 0.0433$. Trend line for polyphenol-rich dark chocolate (----) and placebo (·····).

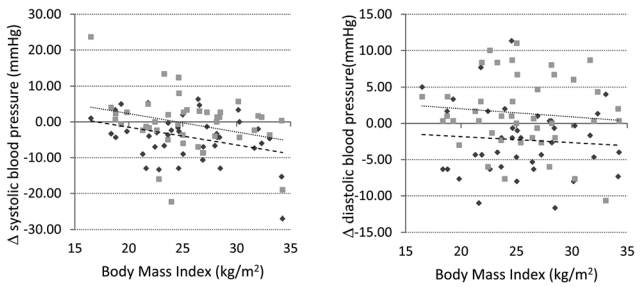

Fig. 3 Body mass index determines the magnitude of effect of polyphenol-rich dark chocolate (◆) and placebo (■) on systolic and diastolic blood pressure. Regression equations were as follows: (a) placebo $y = -0.5134\text{BMI} + 12.584$ $R^2 = 0.0852$; polyphenol-rich DC $y = -0.496\text{BMI} + 8.4338$, $R^2 = 0.1126$) placebo $y = -0.111\text{BMI} + 4.2265$ $R^2 = 0.0096$; polyphenol-rich DC $y = -0.0829\text{BMI} - 0.1179$ $R^2 = 0.0062$. Dots stand for change from baseline. Trend line for polyphenol-rich dark chocolate (----) and placebo (·····).

BMI categories. Consequently, data for urinary antioxidant capacity and salivary glucocorticoids is presented for the combined two BMI categories.

Additional findings

No significant changes in anthropometric data were observed following polyphenol-rich DC consumption or placebo. However, a significant difference in body weight was detected between the placebo and the active polyphenol-rich DC group at the end of the 4 week treatment (Table 2). Additional analyses using a paired Student's *t*-test demonstrated that there was a difference of 0.57 ± 1.08 kg between weight values at the end of the 4 week treatment with placebo compared to post-polyphenol-rich DC in overweight females ($P = 0.011$) but not in lean females (difference in weight between post-placebo and post-DC $= 0.06 \pm 1.78$ kg; $P = 0.088$). The inclusion of the change in body weight as a covariate in three-factor ANOVA or regression did not alter the main effects of treatment (data not shown).

There were no significant changes in energy or macronutrient intake from baseline or between polyphenol-rich DC and placebo (Fig. 6). No significant changes in lipid profile, liver enzymes, or physical activity from baseline or between polyphenol-rich DC and placebo were found (data not shown).

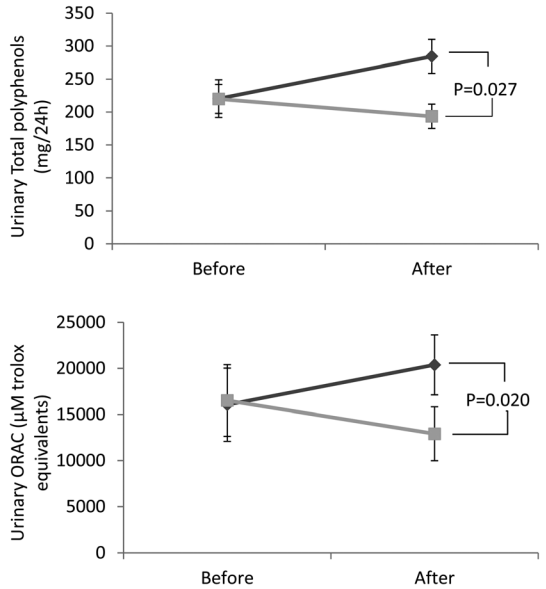

Fig. 4 Changes in total urinary polyphenol content and oxygen radical absorbance capacity (ORAC) after 4 weeks of polyphenol-rich dark chocolate (◆) and placebo (■) ($n = 41$). Data are expressed as means ± SEM.

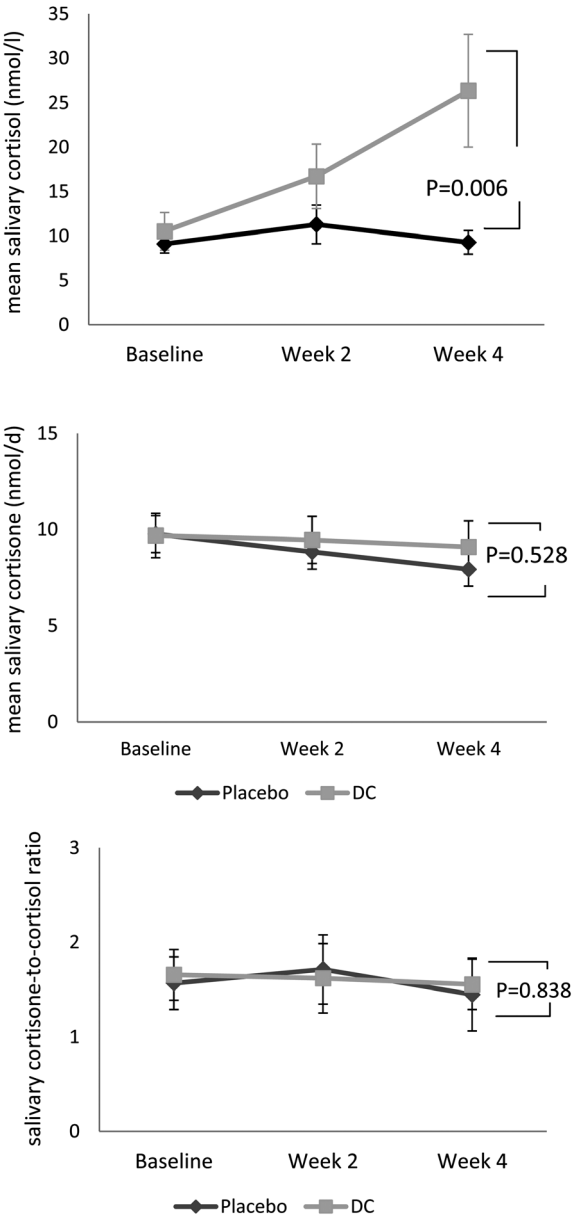

Fig. 5 Effect of polyphenol-rich DC (◆) and placebo (■) on mean daily salivary cortisol, cortisone and cortisone/cortisol ratio ($n = 40$). Results represent mean ± SEM of three salivary collection obtained in morning (9.00–10.00 am), afternoon (12.00–1.00 pm) and evening (4.00–5.00 pm). All samples were analysed in duplicates.

Discussion

The present study demonstrates that a 4 week consumption of DC containing 500 mg polyphenols by lean and overweight females reduces BP and improves glucose regulation as indicated by the reduction in fasting glucose and HOMA-IR. Such findings are consistent with our preliminary data,[8] epidemiological[10] and intervention studies on healthy volunteers,[11] hypertensives[9] and glucose-intolerant hypertensives.[12] The data are also in accordance with the most recent findings of Davison *et al.*[13] who reported beneficial effects of polyphenol-rich cocoa on cardiometabolic markers in subjects with BMI $\geq$ 25 kg m^{-2}. More importantly, the current study demonstrates that overweight and obese subjects respond more effectively to the hypoglycaemic and hypotensive properties of polyphenol-rich DC than their lean counterparts. To our knowledge, this is the first study that allows the direct comparison of the effect of polyphenol-rich DC on glucose, lipid and BP between the two BMI categories. Such differences in response may be attributed to the raised metabolic risk factors seen in individuals with BMI $\geq$ 25 kg m^{-2} which were predicted in the current study by the higher baseline waist circumference, waist/hip ratio, total cholesterol: high-density lipoprotein ratio, and BP compared to lean individuals.

In contrast to previous human studies, no significant changes in fasting insulin were observed in this trial following consumption of polyphenol-rich DC. Some animal studies have failed to report any improvements in insulin in some models of obesity but not in others, which could indicate that the underlying cause of insulin resistance may dictate a subject's responsiveness to polyphenol-rich DC. Alternatively, the use of a more robust study design which included a DC placebo rather than a white chocolate placebo could have eliminated any possible effect of varying magnesium and theobromine content between the active DC and the placebo. This implies that, unlike other studies which did not control for differences in macronutrient and micronutrient content, any changes in the assessed parameters in our study can be directly related to the presence of DC polyphenols rather than other DC components. The latter could be further supported by the significant rise in urinary antioxidant capacity seen following ingestion of polyphenol-rich DC.

In the present intervention, reductions in SBP and DBP of 4.17 and 2.29 mmHg, respectively, were observed. These values are consistent with a recent meta-analysis of 10 randomised controlled trials involving 297 volunteers which demonstrated that, on average, polyphenol-rich DC and cocoa reduce SBP and DBP by 4.5 and 2.5 mmHg, respectively.[14] However, when

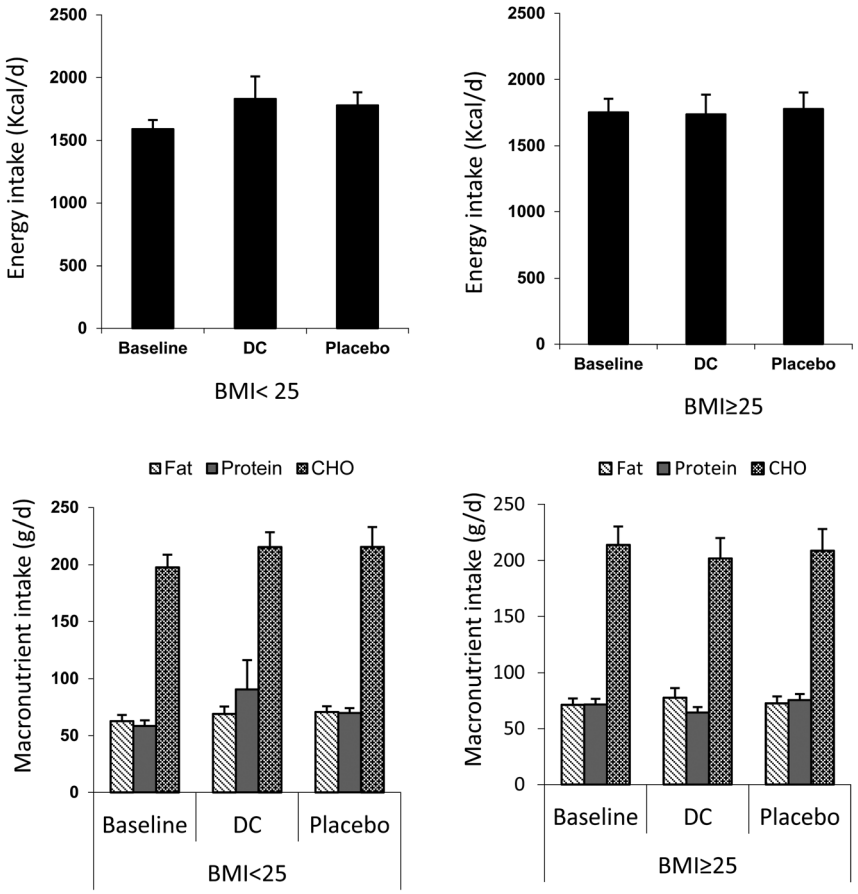

Fig. 6 Energy and macronutrient intake in lean females ($n = 21$) and overweight females ($n = 21$) at baseline and during the polyphenol-rich DC and placebo intervention.

comparing changes in BP in the main study to the preliminary DC study,[8] it can be observed that the magnitude of reduction in SBP and DBP was lower at the end of the 4 week main DC intervention (SBP − 4.17 mmHg from baseline, DBP − 2.29 mmHg from baseline) than at the end of the 2 week preliminary study (SBP − 6.98 mmHg from baseline, DBP − 5.62 mmHg from baseline). This is partially in agreement with the aforementioned meta-analysis wherein Desch *et al.*[14] demonstrated greater reductions in SBP and DBP in short-term 2 week trials as opposed to long-term 4–18 week interventions, suggesting that adaptation might occur to high polyphenol doses over time. Regardless of the latter, small improvements in BP such as observed in our study could have important implications to population prevalence of cardiovascular disease. In the Zutphen Elderly cohort, the highest tertile of cocoa consumption was related to a 3.7 and 2.1 mmHg reduction in SBP and DBP, respectively, compared to the lowest tertile of cocoa consumption. This cocoa-related reduction in BP was linked with a 45 and 50% reduction in long-term risk of cardiovascular disease and all-cause mortality, respectively. More recently, data from the European Prospective Investigation into Cancer and Nutrition, involving 19 357 people, have shown that even smaller reduction of 1.0 mmHg in SBP and 0.9 mmHg in DBP, induced by moderate chocolate consumption, could decrease the long-term risk of myocardial infarction and stroke by 39%.

The absence of an effect of polyphenol-rich DC on lipid profile is in contrast to previous research[9,12,15,16] and meta-analytical data.[17] However, it is consistent with some studies that did not observe any changes in lipid profile after ingestion of polyphenol-rich DC.[11,18] Most previous studies that reported changes in lipid profile included participants who had a high lipid profile and who consumed 100 g DC containing 500 mg polyphenols.[9,12] Thus, the lack of a significant effect in our study could be attributed to the normal lipid levels of our study's population or differences in the composition of the chocolate products used.

The present study adds to current knowledge by demonstrating that overweight and obese individuals could be more adversely affected by the consumption of polyphenol-poor DC than lean individuals, as indicated by the significant rise in fasting insulin, HOMA-IR, and the marginal decline in QUICKI following placebo. In fact, using the linear regression equations, it could be estimated that a 4 week consumption of polyphenol-deficient DC by a woman with a BMI = 30 kg m^{-2} could increase fasting insulin and HOMA-IR by 1.56 mU l^{-1} and 0.53 units, respectively, compared to an estimated 0.17 mU l^{-1} and 0.24 unit increase in insulin and HOMA-IR in a woman with a BMI = 22 kg m^{-2}. Such changes in insulin sensitivity have not been reported by previous studies possibly due to the use of lean individuals, the short duration of the trials, and the use of tailored iso-caloric diets.[9,11,12,18] Nevertheless, our findings are

consistent with studies reporting that polyphenol-poor chocolate and cocoa-flavoured food products have an insulin index 50–60% higher than that predicted by their glycolic index.[27] Consumption of commercially available cocoa has also been shown to enhance postprandial insulin secretion in lean young adults.[28] This hyperinsulinaemic effect has been attributed to the presence of several insulinotropic compounds in cocoa and chocolate including stearic acid, caffeine, theobromine, serotonin, phenylethylamine, cannabinoid-like fatty acids and amino acids like leucine, phenylalanine and arginine.[28] However, to our knowledge, no study has as yet identified the exact component responsible for the hyperinsulinaemic effect of commercially available cocoa or chocolate. Moreover, we did not have any information regarding the chemical composition of the placebo or the process by which it is produced. This would have helped clarify whether the manufacturing process could have altered the chemical matrix of the placebo rendering it more harmful to health. Regardless of this, our findings raise concern considering the widespread consumption and availability of polyphenol-deficient chocolate on the market.

In addition to the hyperinsulinaemic effects, placebo DC increased oxidative stress and salivary cortisol levels in both lean and overweight females. These findings were in contrast to the improved antioxidant status and unaltered cortisol levels seen following treatment with polyphenol-rich DC. Cocoa butter is known to induce oxidative stress both *in vitro* and *in vivo*.[29] High-fat feeding has also been shown to stimulate basal and stress induced hypothalamic activity in animal studies.[30,31] Moreover, in humans, high-fat low-carbohydrate diets,[32] mixed meals,[33] and lipid and insulin infusions[34] have been shown to increase cortisol production. Together, this evidence may imply that in the absence of polyphenols, high-fat products such as chocolate may adversely affect metabolism. Consequently, the importance of polyphenols may lie in their ability to counteract the deleterious effects of high-fat and polyphenol-deficient products as opposed to providing any additional hormonal benefits.

One of the main limitations of the present study is the observed rise in body weight seen at the end of the placebo intervention in the overweight group, which may have accounted for the rise in insulin resistance seen following the consumption of placebo. Although we did not observe any changes in energy intake or physical activity, under-reporting of energy intake cannot be excluded in overweight individuals and may have accounted for the observed rise in body weight. The placebo also contained a high amount of fat and sugar and no polyphenols. Given the known association between excess cortisol and oxidative stress[7] and the ability of cortisol in the presence of hyperinsulinaemia to promote fat accumulation,[35] it could be speculated that the rise in body weight in the overweight group following treatment with polyphenol-deficient DC may have occurred due to the detrimental effect of the placebo on insulin and cortisol metabolism. This hypothesis deserves further research considering the ability of polyphenols to modulate glucocorticoid metabolism[36,37] and the potential implications such findings may have in forwarding our understanding of the association between diet and disease. Recently, several experimental studies in rodents have shown that polyphenol-rich cocoa can promote thermogenesis and inhibit high-fat diet-induced obesity,[38] and that cacao-derived epicatechin can promote the oxidative capacity of muscle.[39] Recent epidemiological evidence has also found that frequent consumption of chocolate is inversely associated with BMI.[40] This evidence, combined with the findings from the current study, suggests that further research is needed to understand the role of polyphenols in modulating metabolism and weight regulation.

Conclusions

In conclusion, the present study demonstrates that overweight females respond more favourably to the glucose- and blood pressure-lowering effects of polyphenol-rich dark chocolate than females with a normal BMI range. Polyphenol-rich dark chocolate did not affect cortisol metabolism. However, an adverse effect of polyphenol-deficient dark chocolate on insulin sensitivity, antioxidant status and cortisol metabolism was observed suggesting that the importance of polyphenols in chocolate may lie in their ability to avoid the adverse effects of polyphenol-deficient dark chocolate. This suggests the need for further research to improve our understanding of the relation between polyphenols and health.

Conflicts of interest

The authors declare no conflicts of interest. SA was responsible for conceptualizing the research question, designing and conducting the trial, analysing and interpreting the data, and writing the manuscript. CT provided essential reagents and assisted with antioxidant analysis. LMO conducted non-esterified fatty acid analysis. LF and EASA supervised the research, assisted with data interpretation, and reviewed the manuscript. EASA assisted with hormonal analysis. All authors commented on the draft of the manuscript and approved the final version.

Abbreviations

BP	Blood pressure
DBP	Diastolic blood pressure
DC	dark chocolate
HOMA-β	homeostasis model assessment of β-cell function
HOMA-IR	homeostasis model assessment of insulin resistance
SBP	systolic blood pressure
QUICKI	quantitative sensitivity check index
ORAC	oxygen radical absorbance capacity

Acknowledgements

We would like to thank all the volunteers who took part in this study as well as Barry Callebaut for supplying the chocolate products.

References

1 S. M. Grundy, Obesity, metabolic syndrome, and cardiovascular disease, *J. Clin. Endocrinol. Metab.*, 2004, **89**(6), 2595–2600.

2 E. Ferrannini, B. Balkau, S. W. Coppack and J. M. Dekker, *et al.*, Insulin resistance, insulin response, and obesity as indicators of metabolic risk, *J. Clin. Endocrinol. Metab.*, 2007, **92**(8), 2885–2892.
3 V. Vicennati and R. Pasquali, Abnormalities of the hypothalamic–pituitary–adrenal axis in nondepressed women with abdominal obesity and relations with insulin resistance: evidence for a central and a peripheral alteration, *J. Clin. Endocrinol. Metab.*, 2000, **85**, 4093–4098.
4 M. Duclos, P. M. Pereira, P. Barat, B. Gatta and P. Roger, Increased cortisol bioavailability, abdominal obesity and the metabolic syndrome in obese women, *Obesity*, 2005, **13**, 1157–1166.
5 S. Kidambi, J. M. Kitchen, C. E. Grim, H. Raff, J. Mao, R. J. Singh and T. A. Kitchen, Association of adrenal steroids with hypertension and the metabolic syndrome in blacks, *Hypertension*, 2007, **49**, 704–711.
6 T. Iuchi, M. Akaike, T. Mitsui and Y. Ohshima, *et al.*, Glucocorticoid excess induces superoxide production in vascular endothelial cells and elicits vascular endothelial dysfunction, *Circ. Res.*, 2003, **92**, 81–87.
7 G. Bjelaković, S. Beninati, D. Pavlović and G. Kocić, *et al.*, Glucocorticoids and oxidative stress, *Journal of Basic and Clinical Physiology and Pharmacology*, 2007, **18**, 115–127.
8 S. Almoosawi, L. Fyfe, C. Ho and E. Al-Dujaili, The effect of polyphenol-rich dark chocolate on fasting capillary whole blood glucose, total cholesterol, blood pressure and glucocorticoids in healthy overweight and obese subjects, *Br. J. Nutr.*, 2010, **103**(6), 842–850.
9 D. Grassi, S. Necozione, C. Lippi and G. Croce, *et al.*, Cocoa reduces blood pressure and Insulin resistance and improves endothelium-dependent vasodilation in hypertensives, *Hypertension*, 2005, **46**, 398–405.
10 B. Buijsse, C. Weikert and D. Drogan, *et al.*, Chocolate consumption in relation to blood pressure and risk of cardiovascular disease in German adults, *Eur. Heart J.*, 2010, **31**, 1616–1623.
11 D. Grassi, C. Lippi, S. Necozione, G. Desideri and C. Ferri, Short-term administration of dark chocolate is followed by a significant increase in insulin sensitivity and a decrease in blood pressure in healthy persons, *Am. J. Clin. Nutr.*, 2005, **81**, 611–614.
12 D. Grassi, G. Desideri, S. Necozione and C. Lippi, *et al.*, Blood pressure is reduced and insulin sensitivity increased in glucose-intolerant, hypertensive subjects after 15 days of consuming high-polyphenol dark chocolate, *J. Nutr.*, 2008, **138**, 1671–1676.
13 K. Davison, A. M. Coates, J. D. Buckley and P. R. Howe, Effect of cocoa flavanols and exercise on cardiometabolic risk factors in overweight and obese subjects, *Int. J. Obes.*, 2008, **32**, 1289–1296.
14 S. Desch, J. Schmidt, D. Kobler and M. Sonnabend, *et al.*, Effect of cocoa products on blood pressure: systematic review and meta-analysis, *Am. J. Hypertens.*, 2010, **23**(1), 97–103.
15 S. Baba, M. Natsume, A. Yasuda and Y. Nakamura, *et al.*, Plasma LDL and HDL cholesterol and oxidized LDL concentrations are altered in normo- and hypercholesterolemic humans after intake of different levels of cocoa powder, *J. Nutr.*, 2007, **137**(6), 1436–1441.
16 S. Baba, N. Osakabe, Y. Kato and M. Natsume, *et al.*, Continuous intake of polyphenolic compounds containing cocoa powder reduces LDL oxidative susceptibility and has beneficial effects on plasma HDL-cholesterol concentrations in humans, *Am. J. Clin. Nutr.*, 2007, **85**(3), 709–717.
17 L. Jia, X. Liu and Y. Y. Bai, *et al.*, Short-term effect of cocoa product consumption on lipid profile: a meta-analysis of randomized controlled trials, *Am. J. Clin. Nutr.*, 2010, **92**, 218–225.
18 D. Taubert, R. Berkels, R. Roesen and W. Klaus, Chocolate and blood pressure in elderly individuals with isolated hypertension, *JAMA, J. Am. Med. Assoc.*, 2003, **290**, 1029–1030.
19 D. R. Matthews, J. P. Hosker, A. S. Rudenski, B. A. Naylor, D. F. Treacher and R. C. Turner, Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man, *Diabetologia*, 1985, **28**, 412–419.
20 T. M. Wallace, J. C. Levy and D. R. Matthews, Use and abuse of HOMA modeling, *Diabetes Care*, 2004, **27**, 1487–1495.
21 G. Perseghin, A. Caumo, M. Caloni, G. Testolin and L. Luzi, Incorporation of the fasting plasma FFA concentration into QUICKI improves its association with insulin sensitivity in nonobese individuals, *J. Clin. Endocrinol. Metab.*, 2001, **86**, 4776–4781.
22 V. L. Singleton and J. A. Rossi, Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents, *Am. J. Enol. Vitic.*, 1965, **16**(3), 144–158.
23 B. Ou, M. Hampsch-Woodill and R. L. Prior, Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe, *J. Agric. Food Chem.*, 2001, **49**(10), 4619–4626.
24 E. A. S. Al-Dujaili and S. Ashmore, Effect of Glycaemic Index of the Diet on Salivary Cortisol and Testosterone Levels in Women, *Endocrine Abstracts*, 2007, vol. 13, 286.
25 H. Baghdadi, E. A. S. Al-Dujaili, S. Almoosawi, F. Howie and J. I. Mason, Application of a Highly Specific and Sensitive ELISA for the Estimation of Cortisone in Biological Fluids, Endocrine Abstract, 2010, vol. 16, p. 152.
26 M. Palermo, C. H. Shackleton, F. Mantero and P. M. Stewart, Urinary free cortisone and the assessment of 11β-hydroxysteroid dehydrogenase activity in man, *Clin. Endocrinol.*, 1996, **45**, 605–611.
27 S. Holt, J. Brand-Miller and P. Petocz, An insulin index of foods: the insulin demand generated by 1000 kJ portions of common foods, *Am. J. Clin. Nutr.*, 1997, **66**(5), 1264–1276.
28 J. Brand-Miller, S. H. A. Holt, V. de Jong and P. Petocz, Cocoa powder increases postprandial insulinemia in lean young adults, *J. Nutr.*, 2003, **133**, 3149–3152.
29 J. A. Vinson, J. Proch, P. Bose and S. Muchler, *et al.*, Chocolate is a powerful *ex vivo* and *in vivo* antioxidant, an antiatherosclerotic agent in an animal model, and a significant contributor to antioxidants in the European and American diets, *J. Agric. Food Chem.*, 2006, **54**(21), 8071–8076.
30 N. M. Morton, L. Ramage and J. R. Seckl, Down-regulation of adipose 11β-hydroxysteroid dehydrogenase type 1 by high-fat feeding in mice: a potential adaptive mechanism counteracting metabolic disease, *Endocrinology*, 2004, **145**, 2707–2712.
31 A. J. Drake, D. E. Livingstone, R. Andrew and J. R. Seckl, *et al.*, Reduced adipose glucocorticoid reactivation and increased hepatic glucocorticoid clearance as an early adaptation to high-fat feeding in Wistar rats, *Endocrinology*, 2005, **146**, 913–919.
32 R. Basu, R. Singh, A. Basu, C. M. Johnson and R. A. Rizza, Effect of nutrient ingestion on total-body and splanchnic cortisol production in humans, *Diabetes*, 2006, **55**, 667–674.
33 R. H. Stimson, A. M. Johnstone, N. Z. Homer and D. J. Wake, *et al.*, Dietary macronutrient content alters cortisol metabolism independently of body weight changes in obese men, *J. Clin. Endocrinol. Metab.*, 2007, **92**(11), 4480–4484.
34 D. J. Wake, N. Z. Homer, R. Andrew and B. R. Walker, Acute *in vivo* regulation of 11β-hydroxysteroid dehydrogenase type 1 activity by insulin and intralipid infusions in humans, *J. Clin. Endocrinol. Metab.*, 2006, **91**, 4682–4688.
35 M. Ottosson, K. Vikman-Adolfsson, S. Enerbäck, G. Olivecrona and P. Björntorp, The effects of cortisol on the regulation of lipoprotein lipase activity in human adipose tissue, *J. Clin. Endocrinol. Metab.*, 1994, **79**(3), 820–825.
36 A. G. Atanasov, A. A. Dzyakanchuk, R. A. Schweizer, L. G. Nashev, E. M. Maurer and A. Odermatt, Coffee inhibits the reactivation of glucocorticoids by 11β-hydroxysteroid dehydrogenase type 1: a glucocorticoid connection in the anti-diabetic action of coffee?, *FEBS Lett.*, 2006, **580**(17), 4081–4085.
37 R. A. Schweizer, A. G. Atanasov, B. M. Frey and A. Odermatt, A rapid screening assay for inhibitors of 11β-hydroxysteroid dehydrogenases (11β-HSD): flavanone selectively inhibits 11β-HSD1 reductase activity, *Mol. Cell. Endocrinol.*, 2003, **212**(1–2), 41–49.
38 N. Matsui, R. Ito, E. Nishimura, M. Yoshikawa, M. Kato and M. Kamei, *et al.*, Ingested cocoa can prevent high-fat diet-induced obesity by regulating the expression of genes for fatty acid metabolism, *Nutrition*, 2005, **21**(5), 594–601.
39 L. Nogueira, I. Ramirez-Sanchez, G. A. Perkins, A. Murphy, P. R. Taub and G. Ceballos, *et al.*, (−)-Epicatechin enhances fatigue resistance and oxidative capacity in mouse muscle, *J. Physiol.*, 2011, **589**(Pt 18), 4615–4631.
40 B. A. Golomb, S. Koperski and H. L. White, Association between more frequent chocolate consumption and lower body mass index, *Arch. Intern. Med.*, 2012, **172**(6), 519–521.