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Dietary fibre and protein do not synergistically influence insulin, metabolic or inflammatory biomarkers in young obese Göttingen minipigs

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Abstract

The effects of dietary fibre (DF) and protein on insulin response, lipidaemia and inflammatory biomarkers were studied in a model experiment with juvenile obese Göttingen minipigs. After 20 weeks feeding on a high-fat fructose-rich low-DF diet, forty-three 30-week-old minipigs (31·3 (SEM 4·0) kg body weight) were allocated to low- or high-DF and -protein diets for 8 weeks in a 2 × 2 factorial design. High DF contents decreased ($P = 0·006$) while high protein increased ($P < 0·001$) the daily gain. High protein contents increased fasting plasma concentrations of glucose ($P = 0·008$), NEFA ($P = 0·015$), ghrelin ($P = 0·008$) and non-fasting LDL:HDL ratios ($P = 0·015$). High DF increased ghrelin ($P = 0·036$) and C-peptide levels ($P = 0·011$) in the non-fasting state. High protein increased the gene expression of fructose-bisphosphatase 1 in liver tissue ($P = 0·043$), whereas DF decreased fatty acid synthase expression in adipose tissue ($P = 0·035$). Interactions between DF and protein level were observed in the expression of leptin receptor in adipose tissue ($P = 0·031$) and of PPARγ in muscle ($P = 0·018$) and adipose tissue ($P = 0·004$). In conclusion, high DF intake reduced weight gain and had potential benefit on β-cell secretory function, but without effect on the lipid profile in this young obese model. High dietary protein by supplementing with whey protein did not improve insulin sensitivity or lipidaemia, and combining high DF with high protein did not alleviate the risk of metabolic abnormalities.

Key words: **Wheat bran: Whey protein: Obesity: Metabolic syndrome: Miniature pig model**

Globally, the obesity epidemic is increasing at an alarming rate and especially the increase in childhood obesity is of concern[1]. Although obesity itself is not a disease, the condition often causes endocrine and metabolic changes clustered into the so called the metabolic syndrome (MetS) that typically arises in parallel with obesity[2–4]. The MetS represents a complex pathophysiological cluster characterised by excessive ectopic lipid accumulation causing inflammation, insulin resistance, hypertension and hyperlipidaemia leading to type 2 diabetes (T2D) and CVD[5].

Lifestyle modifications, especially through a healthy diet, have attracted great interest to alleviate MetS development. Increased consumption of dietary fibre (DF) has been proven to ameliorate postprandial dyslipidaemia and insulin responses, which could effectively modulate T2D and CVD[6,7]. DF can influence digestion and absorption processes at all sites of the gastrointestinal tract. Thus, DF, depending on its composition, can delay gastric emptying[8], impede the digestion processes in the small intestine[9], influence the metabolic outcome of the microbiota in the large intestine[10] and influence the release of gastrointestinal satiety hormones[11], which alone or in combination will improve postprandial glycaemia and insulin responses[9]. Although acute intervention studies have indicated soluble DF as most efficient in regulating glycaemia and cholesterol levels[12,13], controlled intervention studies have indicated that diets high in insoluble cereal fibre may improve insulin resistance[14] to a larger extent than what is the case with more soluble DF sources[12,15]. Another dietary constituent that is known to influence the MetS is content and quality of protein[16]. A recent study showed that a diet high in either animal or plant protein reduced liver fat, insulin resistance and hepatic inflammation[17]. It has also been found that milk protein, whey protein in particular, has insulinotropic properties by affecting the release of incretin hormones and insulinotropic amino acids[18]. Moreover, whey protein provided as a pre-meal has been found to delay gastric emptying[19] and reduce glycaemic response after consuming a carbohydrate-rich diet[20,21].

Although DF and protein have been the subject of many investigations, the interactive effects of DF and protein are poorly understood. In the present study, we investigated

Abbreviations: DF, dietary fibre; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; HKG, housekeeping genes; IFN-γ, interferon-γ; MetS, metabolic syndrome; sAT, subcutaneous adipose tissue; T2D, type 2 diabetes.

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the possible ameliorating effects on MetS biomarkers in an obese porcine model for childhood obesity[22] by the use of enzyme-treated wheat bran as DF supplement and hydrolysed whey protein as protein supplement alone or combined. Göttingen minipigs have a small body size and have previously been used for the development of MetS models because of the anatomical and physiological similarities with adolescent and adult humans[23,24]. We hypothesised that DF and whey protein and their combinations will have a positive effect on plasma insulin, lipid biomarkers, inflammatory biomarkers and expression of genes associated with nutrient metabolism in young obese Göttingen minipigs.

Materials and methods

Animals and handling

The care and housing of animals were done according to Danish laws and regulations regarding humane care and use of animals in research (The Danish Ministry of Justice, Act on Animal Experiments no. 474 of 15 May 2014, as stipulated in the executive order no. 12 of 7 January 2016) and performed under license obtained from the Danish Animal Experimentation Inspectorate, Ministry of Food, Agriculture and Fisheries. Animal health was monitored closely by observing behaviour (appetite, activity, interactions with humans and neighbouring pigs) and signs of disease.

A total of forty-three female Göttingen minipigs (Ellegaard Göttingen minipigs), raised at Aarhus University, Department of Animal Science, Foulum, Denmark, were used in the study. The minipigs were received at the facilities at 8 weeks of age in four separate blocks over 8 months. At arrival, the minipigs were housed in pairs and fed a standard minipig chow diet (Special Diet Services, Dietex International, UK) according to breeders' recommendations for 1 week followed by separation and a 1-week gradual transition to a high-fat low-DF diet containing 20 % fructose (LOFLOP) for the following 20 weeks of *ad libitum* feeding to induce obesity. The minipigs were housed in individual pens (1·5 m × 2·4 m for pigs <50 kg, and 3 m × 2·4 m > 50 kg). Water was provided *ad libitum* from drinking nipples, and the pens were provided with wood shavings as bedding material for the first 22 weeks. When entering the intervention of the current experiment, the bedding was removed and replaced with a rubber mattress, and toys were provided to satisfy the minipigs' rooting behaviour.

At an average body weight (BW) of 31·3 (SEM 4·0) kg (30 weeks old), the minipigs were transferred to one of four experimental diets over 3 d to gradually reach 100 % of the experimental diet on day 4 using fixed amounts of feed corresponding to 2·6 % of BW. The four experimental diets were a diet low in DF and protein (LOFLOP), a diet low in DF and high in protein (LOFHIP; 6·8 % whey protein hydrolysate added), a diet high in DF and low in protein (20 % enzyme-treated wheat bran added) and a diet high in DF and protein (HIFHIP; 6·8 % whey protein hydrolysate and 20 % enzyme-treated wheat bran added). Wheat bran was delivered by Lantmännen Cerealia AB and was enzymatically treated with cell wall-degrading enzymes (xylanase, glucanase and cellulase) by DuPont Industrial Biosciences Aps as described by Nielsen *et al.*[10]. Whey protein hydrolysate (Lacprodan® HYDRO.REBUILD) was provided by Arla Foods Ingredients Group P/S. The experimental diets were produced at the feed mill at Aarhus University and stored at –20°C. Ingredients and nutrient composition of the experimental diets are shown in Table 1.

After the transition to the experimental diets, voluntary feed intake was recorded for a total of 7 weeks (first week and then for three times every second week). BW was measured at baseline, at fourth and eighth weeks after starting the intervention to calculate daily weight gain and weight gain per unit of feed intake. Anthropometric measurements including length, chest and abdomen circumference (cm) were taken using measuring tape at the end of the experimental intervention (week 28).

Sample collection

Before (week 20 of *ad libitum* feeding with LOFLOP) and after the intervention (week 28), fasting blood and tissue samples were taken as described in detail by Curtasu[22]. Briefly, after an overnight fasting (feed removed at 15·00), the minipigs were anaesthetised with 0·1 ml/kg BW of Zolitil mixture containing 50 mg/ml tiletamine/zolazepam (Vibrac SA, Carros, France), 2·5 mg/ml butorphanol (Torbugesic® Vet, Scan Vet Animal Health A/S), 12·5 mg/ml ketamine (Ketaminol Vet, Intervet Denmark) and 12·5 mg/ml xylazine (Rompun, Bayer Health Care AG), and blood samples were collected from the jugular vein by veno-puncture. Following which the minipigs were moved into a left recumbent position, and a liver biopsy sample (50 mg) was taken with a biopsy pistol (Pro-MagTM I 2.5, Argon Medical Devices, Inc.) and a 14 G × 10 cm needle (Argon Medical Devices, Inc.) after shaving and disinfection of the skin with 0·5 % chlorhexidine solution in 85 % alcohol (Abena A/S) and subcutaneous injection of a local anaesthetic (Procamidor VET, 20 mg/ml, Richter Pharma, AG). Biopsies were supervised by ultrasound scanning by using a 6–18 MHz linear probe (MyLabTM Five VET, Biosound Esaote, Inc.) and the incision closed with a surgical staple. For sampling of subcutaneous fat and muscle tissue, the right hind leg of the minipig was cleaned, sterilised and anaesthetised locally as described for the liver biopsy. After a 15–20 mm incision of the skin, approximately 100 mg of subcutaneous adipose tissue (sAT) was collected, followed by collection of 50–100 mg muscle tissue from the semitendinosus muscle by use of the biopsy pistol. The sAT were snap frozen in liquid N_2 and stored at –80°C until analysis. Muscle and liver tissues were placed in sterile tubes containing RNAlater (Sigma-Aldrich Co. LLC).

After sampling, a few drops of Streptocillin® vet. (Boehringer Ingelheim Danmark A/S) were administered to the incision site and closed with surgical staples. Urine was collected by attaching an absorbent tampon under the tail (covering the urethra) using Omniplast adhesive fabric tape (Hartmann, Germany) before the minipigs were transferred back to their pens for recovery.

Following the 8-week intervention, the animals were weighed and euthanised in the 9th week over 3 d. At euthanasia at the end of the experiment, the minipigs had not had their feed removed, so samples collected reflect the non-fasting state. For sampling, the minipigs were weighed and anaesthetised with

Table 1. Ingredients (g/kg as-fed), chemical composition (g/kg DM) and energy distribution (percentages) of the experimental diets

Items	LOFLOP	LOFHIP	HIFLOP	HIFHIP
Ingredients (g/kg as-fed basis)				
Wheat starch	233	167	66	0
Whole grain wheat (roller milled)	150	150	150	150
Wheat bran (finely milled)	125	125	125	125
Enzyme-treated wheat bran	–	–	200	200
Wheat gluten	65	65	32	32
Fish meal	20	20	20	20
Whey protein hydrolysate	–	68	–	68
Fructose	200	200	200	200
Animal fat (lard)	150	150	150	150
Vitamins and minerals	57	56	57	56
Chemical composition (g/kg DM)				
DM (g/kg as-fed basis)	913	913	919	919
Ash	62	65	73	76
Crude protein (N × 6·25)	113	179	114	175
HCl fat	174	180	188	187
Available carbohydrates	577	522	456	387
Sugars				
Fructose	225	223	224	221
Glucose	1	1	7	7
Sucrose	7	7	9	8
Starch	344	292	216	150
Dietary fibre*	100	106	191	205
NSP (soluble NSP)	69 (8)	75 (12)	136 (22)	136 (15)
AX (soluble AX)	44 (5)	46 (7)	86 (16)	85 (12)
RS†	2	1	1	1
AXOS	5	3	12	17
Fructans	6	8	11	9
Klason lignin	19	20	30	41
Gross energy (MJ/kg DM)	20·7	21·5	21·3	21·7
Energy FAO/WHO (MJ/kg DM)‡	18·9	19·3	18·0	17·9
Protein (%)	10·2	15·7	10·8	16·6
Fat (%)	34·1	34·4	38·7	38·6
Carbohydrates (%)	51·9	45·9	43·1	36·7
Dietary fibre (%)	3·8	4·0	7·4	8·0

LOFLOP, low-fibre low-protein diet; LOFHIP, low-fibre high-protein diet; HIFLOP, high-fibre low-protein diet; HIFHIP, high-fibre high-protein diet; AX, arabinoxylan; RS, resistant starch; AXOS, arabinoxylan-oligosaccharides.
* Dietary fibre = NSP + fructans + RS + AXOS + Klason lignin.
† Determined by enzymatic resistant starch assay (AOAC method 2002.02).
‡ Calculated nutrient concentration and energy conversion factors (FAO) for protein (17 kJ/g), fat (37 kJ/g), carbohydrates (17 kJ/g) and total dietary fibre (8 kJ/g).

Zolitil mixture as described above. The pigs were then fitted with a catheter in the ear vein for possible supplementary anaesthesia and put in a supine position for sampling from the jugular vein. Following which the abdominal cavity was opened by a midline incision and a blood sample was quickly taken from the portal vein. The minipigs were then euthanised with an overdose of sodium pentobarbital followed by exsanguination. The entire gastrointestinal tract was removed, and the small intestine and colon sections were tied off to keep contents in place while measuring the length. Liver, kidney and heart weights were recorded, and tissue samples from the right medial liver lobe were frozen immediately for fat analysis. Urine samples were collected by removing the urinary bladder and direct puncture to determine pH at room temperature with a pH meter and a sample was stored at –80°C for further analysis. Backfat thickness was measured by ultrasound scanning (MyLabTM Five VET, Biosound Esaote, Inc.) with a 6–18 MHz linear probe. Measurements were taken in the area of the *longissimus dorsi* muscle over the last rib while the animal was in a hanging position. The distance from the skin to the last layer of fat was measured at each recording and exported for calculation.

Blood samples taken at fasting and at slaughter were collected in lithium heparin, K3 ethylene-diamine-tetra acetic acid and K3 ethylene-diamine-tetra acetic acid/Aprotinin inhibitor (10 000 kIU/ml blood, Nordic Pharma Ltd.), centrifuged for 12 min at 3300 rpm (1220 rcf) at 4°C and plasma was aliquoted for analyses and stored at –80°C.

Analytical methods

All chemical analyses of the diets were performed in duplicate on freeze-dried samples and analysed as previously described[10]. Liver fat was determined based on Bligh and Dyer's method[25]. Briefly, liver samples were homogenised in double amount of methanol using an Ultra-Turrax homogeniser (IKA Labortechnik) in an ice bath. Homogenate (600 mg) was mixed with 1·0 ml water, 1·5 ml methanol and 1·0 ml chloroform, the mixture was shaken for 1 min and 1·0 ml water and 2·0 ml chloroform were added and shaken for 1 min. The mixture was centrifuged at 3000 rpm (1220 rcf) for 5 min to get phase separation and 2·00 ml of the lower chloroform phase was taken out, dried and the residue was weighed for determination of fat content.

Concentrations of glucose, fructosamine, lactate, NEFA, HDL, LDL, total cholesterol, TAG, albumin, aspartate transaminase, alanine transaminase and γ-glutamyltransferase in lithium heparin plasma were analysed using the ADVIA 1650 Chemistry system (Siemens Diagnostics) according to the manufacturer's instructions (Siemens Diagnostics Clinical Methods for ADVIA 1650). Urinary glucose, creatinine and total protein analyses were conducted with the same system. Millipore MILLIPLEX MAP Human Metabolic Hormone bead panel kit (HMHEMAG-34K, Merck Millipore, Merck KGaA) was used to determine insulin, glucagon, C-peptide and ghrelin (active), glucose-dependent insulinotropic polypeptide (GIP) and total glucagon-like peptide-1 (GLP-1) in K3 ethylene-diamine-tetra acetic acid/Aprotinin inhibitor plasma. Cytokines (interferon γ (IFN-γ), IL-2, IL-4, IL-10, IL-12 and IL-18) in K3 ethylene-diamine-tetra acetic acid plasma were measured using a Millipore MILLIPLEX MAP Porcine bead panel kit (PCYTMAG-23K, Merck Millipore, Merck KGaA, Darmstadt, Germany). Both kits were run according to the manufacturer's instructions on a Luminex MAGPIX system (Luminex Corporation).

RT-PCR was performed on liver, muscle and sAT to analyse gene expression (online Supplementary Table S1). In liver tissue, total RNA extraction was performed using the NucleoSpin RNA Plus kit (Macherey-Nagel GmbH & Co., KG.) according to the manufacturer's instructions. Total RNA extraction from muscle and sAT was operated using TRI Reagent® Solution (Ambion, Applied Biosystems) based on the manufacturer's guidelines. RNA transcription, cDNA synthesis and RT-PCR quantification were conducted as previously described[26]. Glyceraldehyde 3-phosphate dehydrogenase, β-actin and hypoxanthine phosphoribosyl transferase 1 were tested as potential housekeeping genes (HKG). Gene expression data were obtained as Ct values (cycle number for which logarithmic plots cross a calculated threshold) and used to calculate ΔCt values as the difference between Ct of the target gene and mean Ct of HKG. Liver glyceraldehyde 3-phosphate dehydrogenase exhibited changes with regard to DF and protein interaction, whereas sAT glyceraldehyde 3-phosphate dehydrogenase exhibited changes with regard to protein effect. As a result, mean of β-actin and hypoxanthine phosphoribosyl transferase 1 was used as HKG for liver tissue, whereas mean of β-actin and glyceraldehyde 3-phosphate dehydrogenase was used as HKG for muscle and β-actin were used as HKG for sAT. Relative gene expression was determined using the (1+efficiencies)$^{-\Delta\Delta Ct}$ method where $\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{LOFLOP}$. Results were reported as fold changes. C-reactive protein expression in muscle and sAT as well as muscle leptin receptor expression are not reported due to detection limit.

Calculations

Porcine obesity index was calculated as previously described[27]:

$$POI\,(l/cm) = (\pi \times (1/3) \times BS \times (Abr^2 + Cr^2 + Ab \times Cc))/BS \times 10^3, \quad (1)$$

where BS, Abr, Cr, Ab and Cc are body size (length), abdomen radius, chest radius, abdomen circumference and chest circumference, respectively.

Body surface area was calculated as follows[28]:

$$BSA\,(m^2) = 0{\cdot}121BW^{0{\cdot}575}, \quad (2)$$

Weights of liver, heart and kidney and lengths of small intestine and colon at slaughter were calculated relative to BW (kg) at slaughter.

Values of fasting blood glucose (mM) and insulin (MIU/l) were used to calculate the homeostatic model assessment for insulin resistance (HOMA-IR) and β-cell function (HOMA-β) as previously described[29]:

$$\text{HOMA-IR} = (\text{insulin} \times \text{glucose})/22{\cdot}5, \quad (3)$$

$$\text{HOMA-}\beta = 20 \times \text{insulin}/(\text{glucose} - 3{\cdot}5). \quad (4)$$

Statistical analysis

The pig was regarded as the experimental unit. According to the power calculations for TAG and total cholesterol, 6–8 minipigs completing the study were expected to give sufficient statistical power ($\alpha < 0{\cdot}05$; $\beta = 0{\cdot}80$). All data analyses were accomplished using the MIXED procedure of SAS (SAS Institute, Inc.) based on the normal mixed model:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + t_k + \varepsilon_{ijk}, \quad (5)$$

where Y_{ijk} is the dependent variable; μ is the overall mean; α_i and β_j are the fixed effects of DF level (i = LOF or HIF) and protein level (j = LOP or HIP); $(\alpha\beta)_{ij}$ is the interaction among fixed effects; t_k is the random effect of block ($k = 1, 2, 3$ or 4) and ϵ_{ijk} is the residual error. If an interaction was detected, we conducted a pairwise comparison with groups adjusted by multiple comparisons with Tukey–Kramer *post hoc* test. Values are presented as least square means with standard error of the means (SEM). A log transformation was performed for HOMA-β, ratios of glucose:creatinine and protein:creatinine in urine at the end of experiment, urinary ratios of glucose:creatinine and protein:creatinine at baseline to obtain variance homogeneity and values of back-transformed data are presented with a 95 % CI. Pearson correlation of delta values for glucose in the portal vein and jugular vein against daily starch intake was performed by using GraphPad Prism 8.0 (GraphPad Software Inc.). Effects are reported significant when $P < 0{\cdot}05$, while $P < 0{\cdot}10$ was considered as a tendency. For the statistical analysis of anthropometric measurements block 2 was excluded due to absence of data.

Results

Diets, feed intake, body weight, morphometric and organ indices

The diets contained almost equal amounts of fat and gross energy as shown in Table 1. As planned, the crude protein content was 56 % higher in the two high-protein diets, and the DF content was approximately doubled in the two high-DF diets, it was also reflected in the relative energy contributions from protein and DF. The increase was in the form of NSP, AX, AXOS and Klason lignin due to the inclusion of enzyme-treated wheat bran

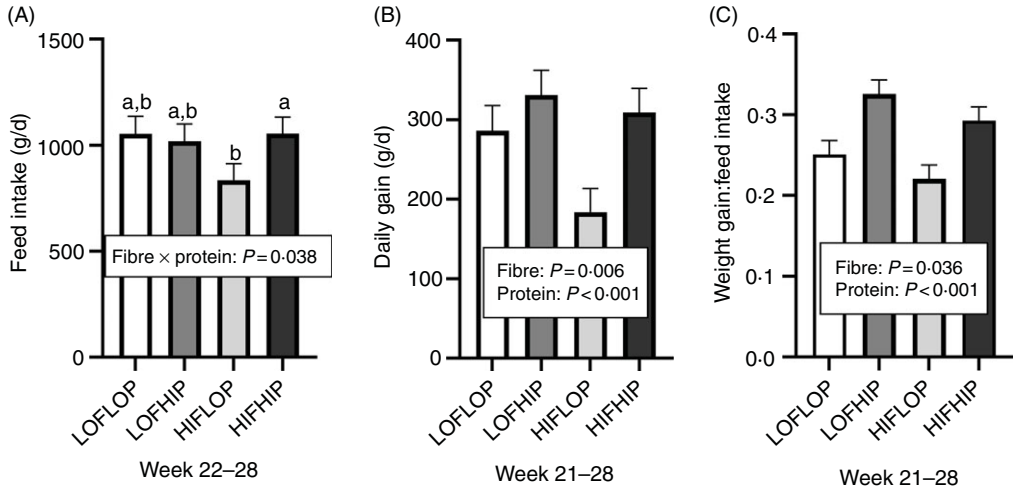

Fig. 1. Feed intake (A), daily weight gain (B) and weight gain per unit of feed intake (C) of Göttingen minipigs fed *ad libitum* during 8 weeks. Values are least-squared means with standard errors represented by vertical bars. Minipigs were regarded as the experimental units, *n* 10 for low-fibre low-protein diet (LOFLOP), *n* 10 for low-fibre high-protein diet (LOFHIP), *n* 12 for high-fibre low-protein diet (HIFLOP) and *n* 11 for high-fibre high-protein diet (HIFHIP). [a,b] Unlike letters are presented in the figure for the significant interaction ($P < 0·05$) after adjustment for multiple comparisons by the Tukey–Kramer *post hoc* test. Only significant *P* values are presented in the figure.

in the high-DF diets. Along with the higher crude protein and DF, the starch content was lower: 150 g/kg DM in the HIFHIP diet compared with 344 g/kg DM in the LOFLOP diet.

The forty-three minipigs that for 20 weeks had been intervened by an energy-dense diet (diet LOFLOP) with baseline characteristics as in Table S2, increased their BW from 31·3 (SEM 4·0) kg before the intervention to 46·8 (SEM 5·4) kg after 8 weeks with no difference between the four diets (data not shown; $P > 0·10$). The daily feed intake recorded in weeks 22–28 showed a significant interaction between DF and protein ($P = 0·038$) with the lowest feed intake observed with high-fibre low-protein diet (Fig. 1). The daily weight gain as well as the gain per unit of feed intake was lower with the high-fibre diets (–20 %, $P = 0·006$; –11 %, $P = 0·036$) compared with low-fibre diets and higher with the high-protein diets (+36 %, $P < 0·001$; +31 %, $P < 0·001$) compared with low-protein diets (Fig. 1). At the end of intervention, there was no significant effect of dietary treatment on any of the morphometric measurements of the pigs either in length, chest circumference, abdomen circumference, porcine obesity index or body surface area and backfat thickness was also not significantly affected by either DF or protein levels (online Supplementary Fig. S1).

The relative weight (% of BW) of liver, heart, kidney and length (m/kg) of small intestine and colon as well as liver fat content at slaughter is shown in online Supplementary Fig. S2. The relative weight of the kidney was higher in the protein groups (0·20 *v.* 0·17 % of BW, $P = 0·010$), whereas none of the other responses was affected by dietary treatment.

Clinical parameters in fasting and non-fasting plasma

Plasma concentrations of clinical parameters in the fasting (jugular vein) and non-fasting state (jugular and portal vein taken at euthanasia) are shown in Table 2. At fasting, there were significantly higher plasma glucose concentrations with the high-protein diets compared with low-protein diets (6·9 *v.* 5·8 mM, $P = 0·008$), and an interaction of DF with protein in fructosamine ($P = 0·022$), but no difference between groups by pairwise comparisons. There were also higher NEFA concentrations (241 *v.* 161 μM, $P = 0·015$), higher albumin (44 *v.* 40 g/l, $P = 0·018$) and aspartate transaminase concentrations (28 *v.* 23 U/l, $P = 0·043$) with the high-protein compared with low-protein diets in fasting plasma. In the non-fasting samples, a borderline significant increase in LDL concentration was observed with high-protein diets in jugular (1·7 *v.* 1·2 mM, $P = 0·054$) and portal venous plasma (1·8 *v.* 1·3 mM, $P = 0·049$). Moreover, the higher protein content also resulted in significantly higher LDL:HDL ratios in plasma from both the jugular vein (0·88 *v.* 0·65, $P = 0·015$) and the portal vein (0·92 *v.* 0·67, $P = 0·014$) in the non-fasting state as compared with pigs fed the low-protein diets. For the other responses, no significant effects of dietary treatment were seen. Non-fasting plasma levels of glucose and lactate were not significantly changed by either DF or protein levels, but, surprisingly, glucose for the HIFHIP diet was higher in the jugular vein than the portal vein. Overall, the difference in glucose concentration between the portal and jugular vein was positively related to the intake of starch (r 0·494, $P > 0·001$, online Supplementary Fig. S3).

Fasting and non-fasting concentrations of plasma hormones and inflammatory cytokines

Fasting and non-fasting concentrations of hormones and inflammatory cytokines in plasma from the jugular vein are shown in Table 3. Fasting ghrelin concentrations were significantly higher with the high-dietary protein diets (28 *v.* 15 pg/ml, $P = 0·008$) but higher with the high-DF diets in the non-fasting state (21 *v.* 15 pg/ml, $P = 0·036$). At fasting, GIP concentrations were significantly lower (37 *v.* 60 pg/ml, $P = 0·008$) by the high-DF diets compared with low-fibre diets, whereas an interaction between DF and protein was observed in the non-fasting state ($P = 0·009$) with significantly higher values of the LOFLOP diet than the other three diets. The high-DF diets induced a borderline significant increase in fasting levels of total GLP-1 ($P = 0·053$), while diets with high protein content tended to increase it in the

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Table 2. Concentrations of clinical parameters in fasting (jugular vein) and non-fasting plasma (jugular and portal vein) in Göttingen minipigs fed diets low or high in dietary fibre and protein
(Mean values with their standard errors)

	Diet*					*P*		
Item	LOFLOP	LOFHIP	HIFLOP	HIFHIP	SEM	Fibre	Protein	F × P†
Fasting, jugular vein								
Glucose (mM)	5·7	6·8	5·8	6·9	0·42	0·771	0·008	0·917
Fructosamine (μM)	261	282	269	261	8·3	0·308	0·325	0·022
NEFA (μM)	166	197	156	284	36	0·275	0·015	0·162
Lactate (mM)	2·0	1·8	1·5	1·6	0·24	0·154	0·779	0·506
TAG (mM)	0·53	0·50	0·62	0·65	0·07	0·072	0·990	0·660
LDL (mM)	1·6	1·9	1·4	1·7	0·25	0·361	0·265	0·888
HDL (mM)	1·7	1·7	1·7	1·6	0·20	0·900	0·359	0·450
LDL:HDL	1·0	1·2	0·84	1·1	0·16	0·320	0·096	0·773
Total cholesterol (mM)	4·2	4·6	4·3	3·9	0·71	0·519	0·925	0·444
Albumin (g/l)	42	45	39	43	1·5	0·097	0·018	0·779
AST (U/l)	30	23	25	23	2·3	0·305	0·043	0·301
ALT (U/l)	25	24	23	23	1·8	0·253	0·737	0·832
GGT (U/l)	61	70	63	64	6·5	0·728	0·357	0·373
Non-fasting, jugular vein								
Glucose (mM)	5·6	6·3	6·3	7·8	0·60	0·062	0·065	0·496
Lactate (mM)	1·9	1·7	1·8	1·7	0·27	0·798	0·567	0·763
TAG (mM)	0·64	0·57	0·62	1·2	0·19	0·098	0·119	0·076
LDL (mM)	1·3	1·9	1·1	1·5	0·28	0·177	0·054	0·608
HDL (mM)	1·9	1·8	1·8	1·7	0·18	0·356	0·246	0·854
LDL:HDL	0·66	0·98	0·63	0·79	0·10	0·255	0·015	0·382
Total cholesterol (mM)	4·1	4·2	3·8	3·7	0·61	0·341	0·932	0·798
Non-fasting, portal vein								
Glucose (mM)	7·7	7·7	6·9	7·5	0·82	0·516	0·689	0·697
Lactate (mM)	4·4	4·0	4·6	4·5	0·90	0·656	0·777	0·872
TAG (mM)	1·0	0·82	1·2	1·5	0·35	0·088	0·729	0·240
LDL (mM)	1·4	2·0	1·2	1·6	0·29	0·241	0·049	0·636
HDL (mM)	2·0	1·8	1·9	1·8	0·18	0·442	0·280	0·724
LDL:HDL	0·68	1·0	0·66	0·82	0·10	0·279	0·014	0·332
Total cholesterol (mM)	4·3	4·6	3·8	3·9	0·68	0·240	0·693	0·779

AST, aspartate transaminase; ALT, alanine transaminase; GGT, γ-glutamyl transferase.
* Minipigs were regarded as the experimental units, *n* 10 for low-fibre low-protein diet (LOFLOP), *n* 10 for low-fibre high-protein diet (LOFHIP), *n* 12 for high-fibre low-protein diet (HIFLOP) and *n* 11 for high-fibre high-protein diet (HIFHIP).
† F × P, interaction between fibre and protein level.

non-fasting state ($P = 0{\cdot}055$). The C-peptide concentrations in non-fasting plasma were higher when the minipigs were fed the high-DF diets (60 *v.* 32 pg/ml, $P = 0{\cdot}011$) and a tendency ($P = 0{\cdot}076$) for higher concentrations with the high dietary levels of protein. There was no effect of diet on insulin concentrations, homeostatic model assessment for insulin resistance and HOMA-β in the fasting state, and only a tendency ($P = 0{\cdot}062$) for a higher non-fasting concentration of insulin with high dietary protein content. High DF content gave rise to higher fasting concentrations of IL-12 (0·75 *v.* 0·59 ng/ml, $P = 0{\cdot}047$) and IFN-γ (5·8 *v.* 4·3 ng/ml, $P = 0{\cdot}011$) compared with low-DF diets, whereas an interaction between DF and protein was seen in fasting concentrations of IL-4 ($P = 0{\cdot}022$) but with no differences between the groups by pairwise comparisons. In the non-fasting state, only IFN-γ levels were significantly higher with a high content of DF in the diet (2·3 *v.* 1·6 ng/ml, $P = 0{\cdot}001$).

Clinical parameters in urine at fasting and slaughter

In the fasting state, there was a significant interaction between DF and protein level in creatinine concentrations of the urine ($P = 0{\cdot}036$) with LOFLOP having a significantly higher concentration than the two high-DF diets (Table 4). There was no effect of diet on urinary glucose and protein concentrations and glucose:creatinine and protein:creatinine ratios ($P > 0{\cdot}10$) in the fasting state. At the non-fasting state, where urine was taken directly from the bladder at euthanasia, a high dietary protein content resulted in a significantly higher pH of the urine (5·6 *v.* 5·1, $P < 0{\cdot}001$) and higher protein concentrations (85 *v.* 46 mM, $P = 0{\cdot}032$) compared with the low dietary protein diets. An interaction of DF and protein was observed in protein:creatinine ratio ($P = 0{\cdot}048$) with higher values of HIFHIP diet than the three other diets.

Relative gene expression of liver, muscle and subcutaneous adipose tissue

Biopsies were taken from the liver, muscle and sAT to study the relative expression of genes involved in carbohydrate and lipid metabolism, inflammation and transcription factors (Table 5). No effects were observed in gene expression in the liver except for an increase in the expression of fructose-bisphosphatase 1 with a high protein content of the diet (1·27 *v.* 0·96, $P = 0{\cdot}043$). In muscle tissue, there were interactions between DF and protein in the expression of acetyl-coenzyme A carboxylase α ($P = 0{\cdot}020$), PPARγ ($P = 0{\cdot}004$) and TNF ($P = 0{\cdot}026$), where the LOFHIP induced significantly higher expression of PPARγ than the other three diets in the *post hoc* analysis. In sAT, high DF content reduced the expression of fatty acid synthase (FASN) (0·70 *v.* 1·20, $P = 0{\cdot}035$), whereas an interaction between DF and protein was

Table 3. Fasting and non-fasting concentrations of circulating hormones and inflammatory cytokines in Göttingen minipigs fed diets low or high in dietary fibre and protein
(Mean values with their standard errors; 95 % confidence intervals)

	Diet*									*P*		
Item	LOFLOP	95 % CI	LOFHIP	95 % CI	HIFLOP	95 % CI	HIFHIP	95 % CI	SEM	Fibre	Protein	F × P†
Fasting												
Ghrelin (pg/ml)	14		18		16		38		5·6	0·055	0·008	0·073
GIP (pg/ml)	72		47		40		33		8·8	0·008	0·083	0·286
GLP-1 (pg/ml)	228		258		308		317		38	0·053	0·600	0·769
C-peptide(pg/ml)	28		33		35		38		5·8	0·307	0·468	0·928
Glucagon (pg/ml)	245		266		260		208		29	0·435	0·511	0·180
Insulin (pM)	45		55		57		55		16	0·688	0·815	0·663
HOMA-IR	1·9		2·6		2·4		2·4		0·84	0·891	0·639	0·654
HOMA-β‡	62	36, 107	50	28, 89	56	33, 95	50	29, 84	–	0·858	0·553	0·878
IL-2 (ng/ml)	0·20		0·30		0·35		0·28		0·07	0·186	0·763	0·128
IL-4 (ng/ml)	0·36		1·43		1·32		0·79		0·35	0·605	0·473	0·028
IL-10 (ng/ml)	0·36		0·50		0·49		0·47		0·08	0·418	0·343	0·229
IL-12 (ng/ml)	0·59		0·60		0·71		0·78		0·11	0·047	0·575	0·691
IL-18 (ng/ml)	0·75		0·96		1·08		0·97		0·15	0·178	0·757	0·231
IFN-γ (ng/ml)	4·4		4·1		5·3		6·2		0·59	0·011	0·552	0·292
Non-fasting												
Ghrelin (pg/ml)	14		17		20		23		3·0	0·036	0·343	0·997
GIP (pg/ml)	589[a]		316[b]		248[b]		295[b]		61	0·003	0·111	0·009
GLP-1 (pg/ml)	258		304		292		354		29·3	0·142	0·055	0·771
C-peptide (pg/ml)	23		41		50		69		11	0·011	0·076	0·945
Glucagon (pg/ml)	234		277		248		264		27	0·977	0·280	0·602
Insulin (pM)	71		76		55		85		9·0	0·668	0·062	0·171
IL-2 (ng/ml)	0·09		0·20		0·22		0·23		0·05	0·131	0·311	0·342
IL-4 (ng/ml)	0·12		0·55		0·58		0·57		0·21	0·248	0·329	0·302
IL-10 (ng/ml)	0·15		0·34		0·31		0·38		0·10	0·264	0·177	0·533
IL-12 (ng/ml)	0·32		0·30		0·35		0·36		0·04	0·278	0·930	0·656
IL-18 (ng/ml)	0·26		0·45		0·46		0·50		0·10	0·209	0·243	0·468
IFN-γ (ng/ml)	1·6		1·6		2·3		2·4		0·21	0·001	0·621	0·732

HOMA-IR, homeostatic model assessment for insulin resistance; HOMA-β, homeostatic model assessment for β-cell function; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like polypeptide; IFN-γ, interferon γ.
[a,b] Unlike superscript letters in a row are presented for the significant interaction ($P < 0·05$) after adjustment for multiple comparisons by the Tukey–Kramer *post hoc* test.
* Minipigs were regarded as the experimental units, *n* 10 for low-fibre low-protein diet (LOFLOP), *n* 10 for low-fibre high-protein diet (LOFHIP), *n* 12 for high-fibre low-protein diet (HIFLOP) and *n* 11 for high-fibre high-protein diet (HIFHIP).
† F × P, interaction between fibre and protein level.
‡ Values are back transformed after log transformation and are expressed as means with lower and upper 95 % confidence intervals.

Table 4. Clinical parameter concentrations of urine collected at fasting and non-fasting state of Göttingen minipigs fed diets low or high in dietary fibre and protein
(Mean values with their standard errors; 95 % confidence intervals)

	Diet*									*P*		
Item	LOFLOP	95 % CI	LOFHIP	95 % CI	HIFLOP	95 % CI	HIFHIP	95 % CI	SEM	Fibre	Protein	F × P†
Fasting												
Creatinine (mM)	17[a]		9·1[a,b]		7·1[b]		8·4[b]		2·4	0·018	0·160	0·036
Glucose (mM)	2·8		21		13		20		8·1	0·530	0·074	0·379
Protein (mM)	360		753		243		435		193	0·239	0·116	0·583
Glucose:creatinine‡	0·13	0·02, 0·81	0·80	0·14, 4·8	0·69	0·13, 3·7	1·0	0·17, 5·9	–	0·163	0·118	0·279
Protein:creatinine‡	14	6·1, 34	35	16, 79	24	12, 49	38	17, 85	–	0·465	0·100	0·597
Non-fasting												
pH	5·0		5·6		5·1		5·6		0·13	0·707	<0·001	0·896
Creatinine (mM)	10		10		10		7·6		1·6	0·387	0·327	0·416
Glucose (mM)	0·72		0·46		0·37		0·54		0·17	0·422	0·852	0·179
Protein (mM)	43		84		49		87		19	0·788	0·032	0·945
Glucose:creatinine‡	0·05	0·03, 0·07	0·04	0·03, 0·07	0·03	0·02, 0·05	0·06	0·04, 0·10	–	0·815	0·258	0·137
Protein:creatinine‡	3·9[b]	2·0, 7·7	7·5[b]	3·8, 15	4·0[b]	2·1, 7·6	24[a]	12, 46	–	0·040	<0·001	0·048

[a,b] Unlike superscript letters in a row are presented for the significant interaction ($P < 0·05$) after adjustment for multiple comparisons by the Tukey–Kramer *post hoc* test.
* Minipigs were regarded as the experimental units, *n* 10 for low-fibre low-protein diet (LOFLOP), *n* 10 for low-fibre high-protein diet (LOFHIP), *n* 12 for high-fibre low-protein diet (HIFLOP) and *n* 11 for high-fibre high-protein diet (HIFHIP).
† F × P, interaction between fibre and protein level.
‡ Values are back transformed after log transformation and are expressed as means (95 % confidence interval).

Table 5. Gene expression of selected genes in liver, skeletal muscle and subcutaneous adipose tissue after dietary intervention in Gottingen minipigs (Mean values and 95 % confidence intervals)

Item		Diet*								*P*		
Gene	Pathway	LOFLOP	95 % CI	LOFHIP	95 % CI	HIFLOP	95 % CI	HIFHIP	95 % CI	Fibre	Protein	F × P†
Liver tissue												
SLC2A5	Carbohydrate metabolism	1·00	0·40, 2·50	1·10	0·44, 2·75	0·94	0·39, 2·26	0·32	0·13, 0·78	0·071	0·126	0·092
SLC2A4	Carbohydrate metabolism	1·00	0·53, 1·88	1·36	0·72, 2·55	0·85	0·47, 1·55	0·80	0·44, 1·47	0·198	0·680	0·476
SLC2A8	Carbohydrate metabolism	1·00	0·65, 1·54	0·89	0·58, 1·38	1·21	0·80, 1·84	0·81	0·53, 1·24	0·752	0·110	0·381
HK1	Carbohydrate metabolism	1·00	0·76, 1·32	1·15	0·87, 1·52	1·08	0·82, 1·41	0·92	0·70, 1·20	0·492	0·891	0·179
FBP1	Carbohydrate metabolism	1·00	0·60, 1·67	1·32	0·79, 2·21	0·91	0·55, 1·50	1·22	0·74, 2·02	0·511	0·043	0·956
ACACA	Fatty acid metabolism	1·00	0·69, 1·45	1·18	0·82, 1·71	1·12	0·80, 1·57	1·04	0·73, 1·47	0·981	0·853	0·502
ACLY	Fatty acid metabolism	1·00	0·74, 1·35	0·80	0·59, 1·08	1·18	0·89, 1·55	0·85	0·64, 1·13	0·454	0·060	0·706
FASN	Fatty acid metabolism	1·00	0·47, 2·11	1·24	0·59, 2·61	1·49	0·76, 2·95	0·89	0·43, 1·80	0·914	0·627	0·324
ADIPOR1	Fatty acid metabolism	1·00	0·70, 1·43	1·03	0·74, 1·48	1·10	0·78, 1·55	1·14	0·80, 1·62	0·412	0·770	0·964
PPARγ	Lipid transcription factors	1·00	0·59, 1·70	1·28	0·75, 2·19	1·25	0·75, 2·09	0·86	0·51, 1·44	0·716	0·738	0·195
CRP	Immune system/inflammation	1·00	0·18, 5·64	0·97	0·17, 5·50	0·72	0·13, 3·89	1·45	0·26, 7·95	0·949	0·487	0·475
IL6	Immune system/inflammation	1·00	0·52, 1·91	1·42	0·74, 2·70	1·44	0·80, 2·59	0·96	0·52, 1·78	0·990	0·872	0·248
TNF	Immune system/inflammation	1·00	0·69, 1·44	1·12	0·78, 1·62	1·23	0·87, 1·75	1·31	0·92, 1·86	0·319	0·633	0·873
CCL5	Immune system/inflammation	1·00	0·59, 1·69	1·51	0·89, 2·56	1·30	0·79, 2·14	1·53	0·92, 2·55	0·481	0·167	0·526
Muscle tissue												
SLC2A4	Carbohydrate metabolism	1·00	0·66, 1·52	1·03	0·68, 1·57	1·12	0·76, 1·66	0·89	0·60, 1·33	0·929	0·554	0·465
PFKM	Carbohydrate metabolism	1·00	0·42, 2·39	0·98	0·41, 2·35	0·83	0·35, 1·97	0·89	0·37, 2·11	0·237	0·818	0·729
ACACA	Fatty acid metabolism	1·00	0·63, 1·58	1·91	1·20, 3·03	1·72	1·11, 2·65	1·35	0·87, 2·12	0·561	0·350	0·020
FASN	Fatty acid metabolism	1·00	0·49, 2·05	3·27	1·59, 6·71	1·52	0·78, 2·94	1·41	0·71, 2·81	0·556	0·133	0·067
ADIPOR1	Fatty acid metabolism	1·00	0·63, 1·59	0·97	0·61, 1·55	0·95	0·60, 1·50	1·05	0·66, 1·66	0·923	0·728	0·559
PPARγ	Lipid transcription factors	1·00^{b}	0·69, 1·44	2·39^{a}	1·66, 3·45	1·70^{a,b}	1·22, 2·38	1·34^{a,b}	0·93, 1·93	0·999	0·117	0·004
IL6	Immune system/inflammation	1·00	0·64, 1·57	1·10	0·70, 1·73	1·55	1·02, 2·35	0·92	0·59, 1·41	0·504	0·234	0·114
TNF	Immune system/inflammation	1·00	0·62, 1·62	1·81	1·12, 2·94	1·83	1·15, 2·91	1·52	0·95, 2·44	0·194	0·300	0·026
CCL5	Immune system/inflammation	1·00	0·61, 1·63	1·54	0·94, 2·51	1·44	0·91, 2·27	1·49	0·93, 2·39	0·428	0·310	0·358
Subcutaneous adipose tissue												
SLC2A4	Carbohydrate metabolism	1·00	0·60, 1·68	1·56	0·93, 2·63	1·27	0·78, 2·07	0·84	0·51, 1·39	0·403	0·965	0·051
FASN	Fatty acid metabolism	1·00	0·50, 2·00	1·39	0·69, 2·79	0·85	0·44, 1·66	0·56	0·28, 1·11	0·035	0·768	0·125
ADIPOR1	Fatty acid metabolism	1·00	0·68, 1·48	1·42	0·96, 2·09	1·30	0·91, 1·86	0·84	0·58, 1·22	0·529	0·718	0·048
LEPR	Fatty acid metabolism	1·00^{b}	0·34, 2·94	0·89^{b}	0·31, 2·60	2·29^{a}	0·81, 6·48	0·60^{b}	0·21, 1·71	0·457	0·007	0·031
LEP	Fatty acid metabolism	1·00	0·47, 2·14	0·90	0·42, 1·93	0·99	0·48, 2·05	0·98	0·47, 2·06	0·887	0·837	0·846
ADIPOQ	Fatty acid metabolism	1·00	0·65, 1·53	1·29	0·84, 1·98	1·44	0·98, 2·14	0·90	0·60, 1·36	0·960	0·532	0·092
CIDEC	Fatty acid metabolism	1·00	0·70, 1·43	1·11	0·78, 1·59	1·20	0·86, 1·66	0·75	0·53, 1·06	0·576	0·266	0·115
PPARγ	Lipid transcription factors	1·00	0·52, 1·93	1·60	0·83, 3·11	1·61	0·85, 3·04	0·94	0·49, 1·80	0·929	0·748	0·018
IL6	Immune system/inflammation	1·00	0·47, 2·13	0·84	0·39, 1·79	0·46	0·22, 0·95	0·58	0·28, 1·19	0·136	0·923	0·594
TNF	Immune system/inflammation	1·00	0·49, 2·03	1·42	0·71, 2·85	1·52	0·78, 2·97	1·21	0·61, 2·40	0·565	0·876	0·198
CCL5	Immune system/inflammation	1·00	0·65, 1·54	1·27	0·82, 1·97	1·27	0·85, 1·90	1·22	0·80, 1·85	0·626	0·653	0·488

SLC2A5, solute carrier family 2 (facilitated glucose/fructose transporter) member 5, Glut5; SLC2A4, solute carrier family 2 (facilitated glucose transporter) member 4, Glut4; SLC2A8, solute carrier family 2 (facilitated glucose transporter) member 8, Glut8; HK1, hexokinase 1; FBP1, fructose-bisphosphatase 1; PFKM, phosphofructokinase, muscle; ACACA, acetyl-coenzyme A carboxylase α; ACLY, ATP-citrate lyase; FASN, fatty acid synthase; CCL5, C-C motif chemokine ligand 5/encodes RANTES; ADIPOR1, adiponectin receptor 1; LEPR, leptin receptor; LEP, leptin; ADIPOQ, adiponectin; CIDEC, cell death inducing DFFA like effector C; CRP, C-reactive protein, pentraxin-related.

[a,b] Unlike superscript letters in a row are presented for the significant interaction ($P < 0{\cdot}05$) after adjustment for multiple comparisons by the Tukey–Kramer *post hoc* test.

* Minipigs were regarded as the experimental units, *n* 10 for low-fibre low-protein diet (LOFLOP), *n* 10 for low-fibre high-protein diet (LOFHIP), *n* 12 for high-fibre low-protein diet (HIFLOP) and *n* 11 for high-fibre high-protein diet (HIFHIP). Data are reported relative to LOFLOP.

† F × P, interaction between fibre and protein level.

seen in the expression of adiponectin receptor 1 (ADIPOR1) ($P = 0{\cdot}048$), leptin receptor ($P = 0{\cdot}031$) and PPARγ ($P = 0{\cdot}018$) without differences between groups after adjustment for multiple comparisons.

Discussion

The current study intended to investigate the possible ameliorating effects of DF and protein on MetS biomarkers in an obese porcine model for childhood obesity[22]. The daily feed intake for 6–9-month-old female minipigs is restricted to 300–400 g in order to prevent obesity[30]. Although the low-protein diets in this study had slightly lower protein content than recommended standard diet (11 *v.* 13 %), the daily protein intake of these growing minipigs was covered with the *at libitum* feeding pattern (104 g with the low-protein diets *v.* 39–52 g with the standard diet). Obesity was induced in the model by feeding a high-fat high-fructose diet (diet LOFILOP), and after 20 weeks, the pigs were on average approximately twice as heavy as Göttingen minipigs fed according to manufacturers' recommendations (31 *v.* 16 kg at 7 months of age)[30]. The heavy fat accumulation created a preclinical stage with increased fasting glucose, signs of insulin deregulation and mild signs of liver inflammation (online Supplementary Table S2). Similar effects have been found in other studies with Göttingen minipigs[22,31,32].

In the current 8-week intervention period with diets varying in DF and protein, the minipigs continued to gain weight although at a different rate depending on the dietary composition. Thus, the pigs fed the diet high in DF and low in protein consumed less feed and, consequently, gained less weight than the pigs on the other diets. The lower feed intake of the high-DF low-protein diet and the contrasting effects of DF and protein on weight gain are most likely related to: first, a lower nutrient digestibility, modified nutrient absorption and influence of DF on satiety[9,10]; and second, positive influence of high protein on growth and modest influence on satiety. In earlier epidemiologically studies[33,34], a positive effect of high protein in particular of protein of animal origin on weight gain[34], has also been observed presumably reflecting that the protein quality plays a crucial role. It should be noted that the low-DF low-protein diet used during development of obesity[22] and continued to be used in one of the dietary treatment (LOFLOP) in the current intervention study was specifically designed to provide a reduced protein content in order to redirect energy from lean tissue accretion to adipose tissue storage in this way diminishing muscle mass for glucose regulation[35]. These aspects have to be taken into consideration when evaluating the effects of the high-protein diets on several biomarkers. For instance, the concentrations of albumin and aspartate transaminase in plasma are most likely a reflection of changed protein catabolism and turnover rates in the liver with a high dietary protein intake[36] rather than a marker for the MetS[37]. To support this view, relative organ weight[30], liver fat[35] and plasma inflammatory cytokines[22] were found to be in the normal range.

While we can expect the digestibility to be lower with the high-DF diets because of the insoluble nature of DF making it resistant to microbial degradation[10,38], there are also indications of satiety induced by gut hormones with DF. GIP was significantly reduced by high DF both at fasting and at non-fasting but with significantly higher levels during non-fasting than fasting. GIP stimulates insulin secretion in a manner related to the absorption of especially glucose and fat[39]. The lower GIP level with high-DF diets and higher GIP level with the low-DF low-protein diet is without doubt a reflection of the influx of glucose being higher for the latter than the former diet. The GLP-1 level also showed borderline increase with the high-DF diets at the fasting stage, indicating an effect of DF on satiety after overnight fasting; the effect most likely being caused by the slower absorption of nutrients[40] and possibly also triggered by fasting levels of SCFA being released from slowly fermented DF[41]. GLP-1 increases rapidly after a meal and plays a key role in the regulation of insulin secretion and sensitivity thereby reducing post-meal glucose concentrations and improves β-cell proliferation[42]. Overall, although no significant change was observed in insulin sensitivity with high DF at the fasting state, GLP-1 secretion induced by a possible slower nutrient absorption could potentially be protective against β-cell exhaustion. In contrast to GIP and GLP-1, the results on ghrelin diverge from the general understanding that DF can suppress ghrelin concentrations and thereby reduce the feeling of hunger[43] as the non-fasting level ghrelin was higher in the high-DF diets without influencing either feed intake or weight gain. A previous study with high-DF diets also demonstrated that satiety feeling may be unrelated to the ghrelin response and did not affect the following food intake[6]. It has also been found that an AX-enriched meal increases serum ghrelin levels in healthy human subjects with normal glucose tolerance[44]. However, given the *ad libitum* feeding pattern and expected delayed nutrient absorption with high DF, increased ghrelin levels could also occur earlier in the low DF groups, and as a result, there was no overall influence on either feed intake or weight gain.

We have previously shown that an AX-rich diet did not change postprandial glucose responses of pigs but induced a lower postprandial peak in insulin in portal vein, hepatic vein and mesentery artery compared with a low-DF Western-style diet[45]. In the present study, we did not see any effect of the high-DF diets on either glucose or insulin, which is in line with a study with human subjects with normal glucose tolerance where an AX-rich diet was found not to influence glucose or insulin responses[44]. Although we did not observe significant changes in insulin concentrations, we found higher non-fasting levels of C-peptide with the high-DF diets, which may be an indicator of improved secretory function of pancreatic β-cells. However, the higher level of C-peptide occurred without neither homeostatic model assessment for insulin resistance nor HOMA-β being influenced by DF. Of note, in our comparisons between species, it needs to be acknowledged that there are indications that C-peptide may have different functions in minipigs and humans[32,46].

During the progression of obesity in this juvenile model, we did not observe any relationship between obesity development and IL-12 and IFN-γ[22]. After 8 weeks of dietary interventions, however, elevated concentrations of IL-12 in the fasting state and IFN-γ in the fasting and non-fasting state were observed

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in pigs fed the high-DF diets. These results are in contrast to our expectations but generally in agreement with a recent study where it was concluded that the diet high in DF from enzyme-treated wheat bran did not affect low-grade inflammation[47]. The reason for lack of effect is most likely that the degree of inflammation is much less at obesity than under a pathogen infection where DF have been found to have immunomodulatory effects by reducing IL-12, IFN-γ and TNF-α production[48]. Moreover, the MetS, especially the systemic inflammation, has been strongly associated with general and central obesity[49], the absence of significant changes in obesity markers such as obesity index, backfat thickness and liver fat in the current study corresponded with the lack of effect on most inflammatory biomarkers except IFN-γ. The rationale of measuring at fasting and non-fasting is that inflammatory cytokines have been reported to change in response to a meal[50].

The two high-protein diets caused higher fasting glucose concentrations, a tendency for higher urinary glucose concentrations and a tendency for higher non-fasting insulin, GLP-1 and C-peptide concentrations but no differences in fasting insulin. The higher fasting glucose level is presumably caused by gluconeogenic amino acids including branched-chain amino acid, as indicated by the higher concentration of glucose in the jugular than the portal vein and higher urine pH at non-fasting. Although high branched-chain amino acid content of whey protein has shown insulionotropic effects in a short-term study[18], the long-term effects are contradictory[51], and a positive association between high circulating branched-chain amino acid and obesity was found in our previous study[22], which may have detrimental effects on glucose and lipid homoeostasis of obese minipigs[52]. The increased expression of liver fructose-bisphosphatase 1, the rate-limiting enzyme in gluconeogenesis, on the high-protein diets is probably also related to a higher gluconeogenesis as also observed in young subjects with newly diagnosed T2D[53]. Other studies have shown that high-protein diets, despite their beneficial effects on satiety, weight loss, and blood lipids, under certain conditions may modulate amino acid metabolic signature and be a factor in insulin resistance and T2D development[51,54]. Moreover, a human intervention study with the same type of diets did not induce a higher GLP-1 response with high-protein diets[55], whereas this was the case when whey protein was provided as a pre-meal in an acute study[19]. The difference in response to protein in the current and the 12-week human intervention study[55] on GLP-1 compared with acute studies[19,56] could indicate that whey protein elicit responses by different mechanisms when provided acutely compared with a chronic intake.

Neither whey protein nor DF influenced total cholesterol in plasma but whey protein increased non-fasting LDL and the LDL:HDL ratio. A higher fasting NEFA concentration was also observed when feeding the high-protein diets. Combined these data indicate an unfavourable progression towards dyslipidaemia when feeding the high-protein diets. In a recent 12-week intervention study with human subjects with abdominal obesity, it was found that whey protein in combination with a low-DF diet reduced total fasting cholesterol, whereas high DF had the opposite effect[16].

Obesity-driven insulin resistance in white adipose tissue, liver and skeletal muscle is the primary cause of T2D and is linked to obesity-associated metabolic abnormalities such as dyslipidaemia and inflammation[57]. Therefore, we explored how DF and protein interventions would affect the expression of key regulatory genes involved in carbohydrate and lipid metabolism, inflammation and transcription factors in liver, muscle and sATs. We observed an increased relative gene expression of PPARγ in muscle tissue in the LOFHIP group, which potentially can be linked to impaired insulin sensitivity. PPARγ plays an important role in regulating insulin action in skeletal muscle[58], and mRNA and protein expression of PPARγ has been found to be higher in muscle tissue of humans with severe insulin resistance[58,59]. These data also corroborate the higher fasting glucose concentrations and tendency for higher non-fasting insulin concentrations in high-protein diets. It appears that DF may attenuate the detrimental effect of protein as increased gene expression of PPARγ was only found in diet with high protein and low DF content.

The high-fibre low-protein diet induced a significantly elevated expression of leptin receptor in the adipose tissue, the signalling pathway through which leptin controls energy balance[60]. This may suggest improved responsiveness to leptin and potentially be associated with the reduced feed intake and weight gain in this particular group. A lower leptin receptor mRNA abundance in the sAT has been associated with morbid obesity when compared with lean human subjects and strongly correlated with insulin sensitivity[61]. A previous study also found that leptin receptor overexpression in adipose tissue of leptin receptor transgenic mice could reduce BW and fat deposition[62]. As a central enzyme involved in lipid biosynthesis, FASN gene expression in adipose tissue has been associated with visceral fat accumulation and impaired insulin sensitivity[63]. In our study, however, the relative expression of FASN was suppressed significantly by the high DF content, which potentially can explain the lower weight gain and improved non-fasting C-peptide concentrations in the high DF groups.

Our study has several strengths but also some weaknesses. First, we performed a randomised long-term *ad libitum* intervention study with well characterised and controlled diets. Second , our sample size based on the power calculation was sufficient to allow a reliable detection of changes in metabolic biomarkers. Third, we present very comprehensive clinical parameters both at non-fasting and fasting. Fourth, we use innovative ingredients including whey protein hydrolysate as protein source and enzyme-related wheat bran as DF source. The study, however, also has limitations that need to be acknowledged. First, we cannot exclude that the growth of animals may have masked the effects of DF and protein; thus, further studies are needed to clarify whether DF and high protein can synergistically affect the MetS in an adult obese model with stable weight. Second, a lean control group of pigs feeding on a regular diet was not included as the primary purpose of this study was to investigate the effects of DF and protein on obesity. Third, since whey protein and enzyme-treated wheat bran were the only protein and fibre source used to increase dietary protein and DF levels, we cannot conclude whether the effects were induced by the specific sources or by the levels of protein and DF, and further

studies on other DF and protein sources are needed. Moreover, in this paper, we have only presented mRNA data and protein expression of the regulatory genes were not analysed. Since mRNA expression is not always correlated with protein expression as well as with the activation or function of corresponding proteins, the gene expression findings need to be interpreted with caution and warrant further studies.

In conclusion, the present study demonstrates that an 8-week dietary intervention with DF and protein did not improve glucose and insulin response directly in an obese minipig model. However, we demonstrated that a diet enriched with DF from enzyme-treated wheat bran reduced weight gain and had a potential beneficial effect on β-cell secretory function but without effects on lipid biomarkers. Diets enriched in whey protein hydrolysate tended to increase post-meal insulin levels and several markers related to lipid and carbohydrate metabolism in an unfavourable way. In contrast to our hypothesis, a combination of high- or low-DF and -protein diets did not show a synergistic effect on insulin sensitivity, postprandial lipaemia, metabolic or inflammatory biomarkers associated with the MetS as it was also found in a recent human intervention study[55] and concluded in a recent review on the impact of DF consumption on insulin resistance and the prevention of T2D[15].

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K. E. B. K., H. N. L. and M. S. H. conceived and designed the research; M. V. C., H. N. L., and M. S. H performed the animal experiments; H. N. L. and Y. X. analysed the data; P. K. T. provided data consultation; Y. X. wrote the paper; all co-authors contributed to draft review.

The authors declare that there are no conflicts of interest.

Supplementary material

For supplementary material referred to in this article, please visit https://doi.org/10.1017/S0007114520003141

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