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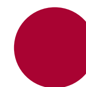

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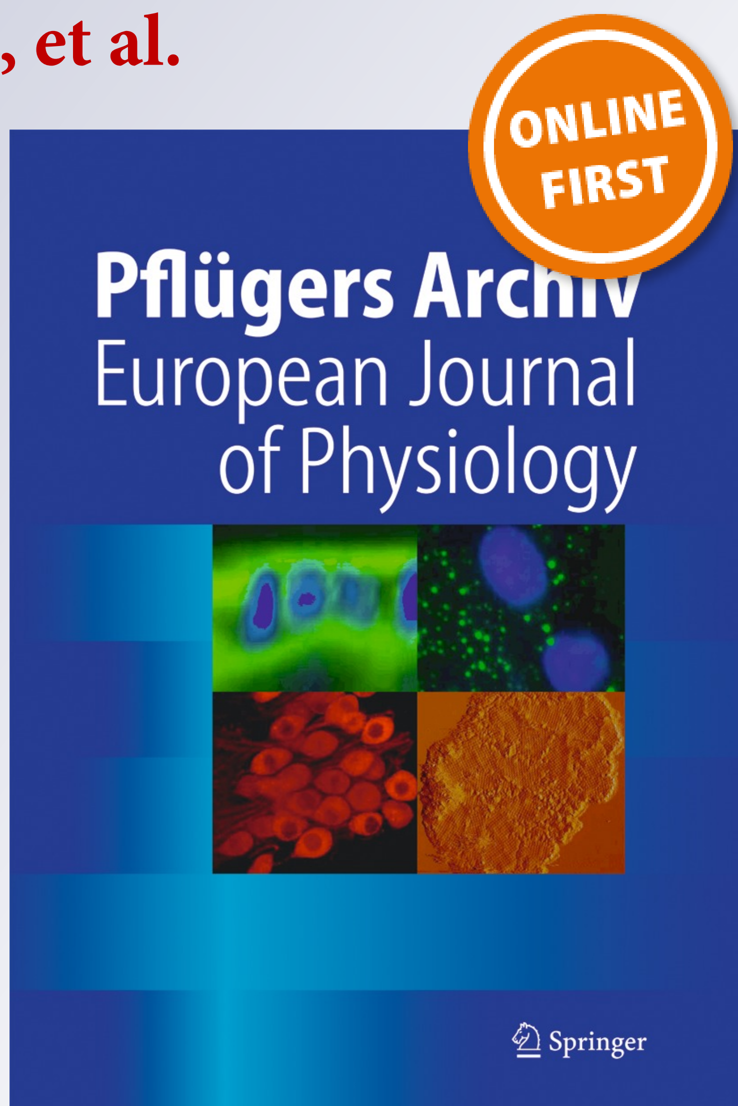

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INTEGRATIVE PHYSIOLOGY

Functional network analysis of obese and lean Göttingen minipigs elucidates changes in oxidative and inflammatory networks in obese pigs

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Abstract The Göttingen minipig model of obesity is used in pre-clinical research to predict clinical outcome of new treatments for metabolic diseases. However, treatment effects often remain unnoticed when using single parameter statistical comparisons due to the small numbers of animals giving rise to large variation and insufficient statistical power. The purpose of this study was to perform a correlation matrix analysis of multiple multi-scale parameters describing co-segregation of traits in order to identify differences between lean and obese minipigs. More than 40 parameters, ranging from physical, cardiovascular, inflammatory and metabolic markers were measured in lean and obese animals. Correlation matrix analysis was performed using permutation test and bootstrapping at different levels of significance. Single parameter comparisons yielded significant differences between lean and obese animals mainly for known physical traits. On the other hand, functional network analysis revealed new co-segregations, particularly in the domain of inflammatory and oxidative stress markers in the obese animals that were not present in the lean. Functional networks of lean or obese minipigs could be utilised to assess drug effects and predict changes in parameters with a certain degree of precision, on the basis of the networks confidence intervals. Comparison of functional networks in minipigs with those of human clinical data may be used to identify common parameters or co-segregations related to obesity between animal models and man.

Keywords Functional network analysis · animal models · obesity · correlation matrix · Göttingen minipig

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Introduction

Translation of the effect of novel therapeutics from animal models of obesity to the corresponding human condition is a major challenge in drug development. A number of animal models are available that mimic various aspects of human obesity. These animal models display varying face, construct and predictive validity and will therefore typically reveal only some of the molecular targets involved in human disease development [5, 18]. They may partially mimic clinical signs and aetiology of the human condition [9], and they may display gene expression, metabolomics or proteomics that only to some extent are comparable to those found in humans. Among others, obese Göttingen minipigs constitute an

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excellent large animal model as they share many characteristics of obesity with the clinical human situation. For example, they can obtain an overweight of more than 100 % and a body fat percentage of around 40–50 %, which is more than what is seen in diet induced rodent models and comparable to what is seen in obese humans [8, 19]. Furthermore, studies with Göttingen minipigs fed with a high-fat diet indicate that they develop early pre-diabetic changes similar to those found in humans [2, 12]. However, qualified selection of parameters to measure and their individual power in predicting clinical efficacy or safety remain a major challenge in pre-clinical drug development. Also, in view of the relatively small number of non-rodents typically used in research for ethical, economical or housing reasons, proper validation of efficacy markers used for novel drug candidates is extremely important.

Pharmacotherapy is aimed at normalising deviations in clinical biomarkers and risk factors that have been demonstrated to relate to obesity and its comorbidities. Therefore, to assess efficacy of novel therapeutics in animal models of disease, such clinical parameters should preferentially behave in a similar manner as in humans in order to increase the predictive power of results obtained in animal experiments to the clinical situation. It is becoming more and more evident that conventional single parameter comparisons between groups do not provide sufficient predictive validity of a model, and that multi-parameter comparisons may improve the understanding of the disease process and therapeutic potential of drug candidates [4].

The purpose of this study was to evaluate co-segregation of multiple phenotypical traits using correlation matrix analysis in order to create patterns of traits characterising the lean vs. the obese state, respectively. Network analysis is commonly used in systems biology in which interactions or relations are investigated on a molecular basis, between proteins, in pathway analysis or gene expression patterns. A comparable approach, but on a higher level of organisation, i.e. network analysis of functional physiological or phenotypical traits was described previously, showing promising results [17, 23]. Multiple parameters or traits were measured ranging from anatomical and physical, cardiovascular, metabolic and inflammatory markers, which are all considered to reflect key processes in the aetiology of obesity and associated comorbidities. This study showed that single parameter comparisons yielded few significant differences between lean and obese animals. However, when subjected to correlation matrix analysis, several important differences were revealed between lean and obese animals, notably within oxidative stress and inflammatory processes. This method may provide improved insight in translational aspects of disease processes between animal models and humans.

Material and methods

Göttingen minipigs

Animals were treated in accordance with the Animal Experimentation Act of Denmark, which is in accordance with the Council of Europe Convention ETS 123. The study was approved by the Danish National Animal Experimentation Board.

Seven ovariectomized (OVX) diet-induced obese and seven lean OVX Göttingen minipigs were used (age 3–4 years, Ellegaard Göttingen Minipigs A/S, Dalmose, Denmark). The minipigs were housed at the Copenhagen University animal research facilities (Taastrup, Denmark) and were fed standard minipig chow (Minipig Maintenance, Altromin, Christian Petersen A/S, Gentofte, Denmark). The lean group was fed restrictively 150 g twice a day whilst the obese group was fed *ad libitum*. The obese pigs had previously been used in pharmacological studies with therapeutic peptides, but had been subjected to wash-out periods exceeding multiple half-lives of these drugs prior to this study.

Phenotypic characterization of the pigs

Body weight (BW) was obtained by weighing the pigs on an ordinary large animal scale on the day of euthanasia. Body composition was determined in connection with flow mediated dilatation (FMD) measurements by use of dual energy X-ray absorptiometry scanning (DEXA) (Hologic Explorer, Santax Medico, Aarhus, Denmark). The DEXA scanning was performed on anaesthetized pigs (intramuscular injection of midazolam 1 mg/kg and ketamine 5 mg/kg) in ventral recumbency with the front and hind limbs stretched backwards. The analysis of body composition was performed using the scanner software package, which estimates the mass of the scanned individual (MASS) and calculates the absolute mass of each tissue (bone; BMC, lean tissue; NONFAT and fat tissue; FAT) in relation to this estimate.

Blood sampling Blood was sampled by venepuncture of the jugular vein in appropriate containers (citrate, EGTA, etc.) from fasted animals (12–18 h) under light sedation with appropriate doses of Zoletil (Tiletamine/zolazepam, Virbac Danmark A/S, Denmark) for later analysis of oxidative stress, inflammatory and metabolic markers. Serum and plasma was collected immediately after centrifugation and stored at −80 or −20 °C until further analysis. Details of each analysis are described below.

Flow mediated vasodilation (FMD) image acquisition, analysis and calculation Pigs were fasted 12–18 h prior to the FMD examination within 4 weeks before euthanasia. Pigs were anaesthetized and a protocol similar to that described

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previously was followed [15]. An ultrasound unit with a 13 MHz linear array transducer was used to acquire 2-D ultrasonographic images of the right brachial artery. Continuous 2-D image recording of all phases of the cardiac cycle for off-line automated edge detection and luminal diameter measurement were performed at defined time periods with constant settings so that a constant number of frames were recorded for all pigs. Pulsed-wave spectral Doppler ultrasonography was used to record blood flow in the brachial artery. The mean angle correction used was 69°±3.5°. For each pig, 2-D longitudinal ultrasonographic images of the artery were obtained during a 30 s period, followed by a 10 s recording of arterial blood flow velocity waveforms obtained via pulsed-wave Doppler ultrasonography before cuff inflation. For induction of reactive hyperaemia, the brachial artery was occluded for 5 min by inflating a blood pressure cuff above 250 mm Hg. An additional 10 s of spectral Doppler ultrasonographic blood flow velocity waveforms was recorded immediately following cuff deflation, and no later than 15 s following cuff deflation, 2-D longitudinal ultrasonographic images of the artery were obtained sequentially during the following 2-minute period. Approximately 8–10 min following cuff deflation, a new 2-D longitudinal baseline recording was obtained during a 30 s period. Nitroglycerine (0.4 mg/dose) was then applied sublingually to evaluate endothelium-independent vasodilatation to exogenous nitric oxide (NO) and immediately hereafter, a 2-D longitudinal recording was performed during the following 2 min. Data were analysed off-line using commercially available software and FMD and flow velocity integral (FVI delta) were calculated as described previously [15].

Biomarkers of oxidative stress and acute phase inflammatory response Plasma biopterins (BH2, BH4) were analyzed by HPLC with coulometric and fluorescence detection as described previously [16]. Total vitamin C (VitC) and Plasma Ascorbate (Asc) were analyzed by HPLC with coulometric detection following reduction of endogenous dehydroascorbic acid (DHA) using tris[2-carboxyethyl]phosphine hydrochloride as described elsewhere [14]. DHA was expressed as a percentage of total VitC. Serum concentrations of Haptoglobin (Hp), C-reactive protein (CRP), serum amyloid A (SAA) and Pig α_1-acid glycoprotein (PAGP) were measured as previously described [10, 11, 20]. After blood sampling, the animals were euthanized by an intravenous overdose of pentobarbital (150 mg/kg) (Pentobarbital 200 mg/ml, Glostrup Pharmacy, Denmark) and necropsy was performed.

Coronary artery function and structure The ventral side of the left ventricle of the heart was quickly excised shortly after euthanasia. The tissue was positioned using pins in a petri dish with a Sylgard® bottom, continuously bathed with freshly oxygenated Krebs's solution (95 % O_2/5 % CO_2/25 mM HCO_3^-), and viewed under a stereo microscope (Leica M80, Leica Microsystems, Heerbrugg, Switzerland) using cold light illumination (Leica CLS 150 XE). The proximal 3–4 cm of the main left coronary artery (LCA) from the opening into the aorta was removed quickly using blunt dissection and transferred to fixative for histological examination. The proximal part (3–4 cm) of the left anterior descending artery (LAD) was carefully removed by blunt dissection and transferred to cold oxygenated Krebs's solution and used for isometric wire myograph experiments. Using a pair of sharpened tweezers and ophthalmic scissors, 2nd and 3rd order side branches of the remaining distal LAD were gently dissected free from surrounding ventricular muscle and transferred to cold oxygenated Krebs's solution for vessel dimension analyses.

Dimension analysis Second and third order LAD segments were transferred to the bath of a pressure myograph (120CP, DMT A/S, Aarhus, Denmark), mounted between two glass pipettes and slowly heated to 37 ° C while the intraluminal pressure was gently raised in stepwise increments to 100 mm Hg. The myograph was placed on the stage of an inverted microscope (Olympus IX71), and the pressurized arteries were viewed at low magnification (4×) through the glass bottom of the bath. A CCD camera connected to Myoview software (both DMT A/S) was used for simultaneous measurement of lumen and external diameters of a 1-2 mm segment of the pressurized artery. Before recording of the diameters, arteries were superfused for 30 min with freshly oxygenated Ca^{2+}-free Krebs's solution (including 2 mM EGTA) to which the L-type Ca^{2+} channel blocker nifedipine (5 μM) was added. This ensured complete relaxation of the arteries in order to obtain the passive structural dimensions of each artery (vessel lumen diameter, wall thickness (Wall Th), and wall/lumen ratio (W/L)).

Coronary artery functional analysis Proximal LAD specimens were cut into segments of about 2–4 mm in length and mounted on two stainless steel pins in a wire myograph (610 M, DMT A/S, Aarhus, Denmark). Optimal lumen diameters (D_o) and maximal potassium stimulated contractions (K^+ max) were identified following a normalization procedure, as previously described [21]. Sensitivities for nifedipine (pD_2 NIF), substance P (pD_2 SP) and bradykinin (pD_2, BRAD) (all obtained from Sigma-Aldrich) were assessed by logistic regression of cumulative concentration response curves constructed on top of 1 μM U46619-induced pre-contractions (U46619 max) using Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

Histopathology of the left coronary artery (LCA) After 48 hours in 4 % formalin, the proximal LCA samples were processed conventionally and embedded in paraffin. Samples were cut into 2–4 μm serial cross-sections for routine

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histology (Hematoxylin and Eosin staining (HE)). A semi-quantitative scoring system was used to evaluate intimal thickening (0=none; 1=mild; 2=moderate; 3=severe) on HE stained sections. Evaluation of samples was done in a blinded fashion.

Metabolic parameters For glucose measurements, 10 μL of plasma was transferred into 500 μL EBIO solution and measured on a Biosen auto analyzer (BIOSEN S_Line, EKF Diagnostics, Cardiff, UK) according to the manufacturer's instructions. Pig insulin content in plasma was measured using luminescence oxygen channeling immunoassay (LOCI), which is a homogenous bead-based assay, as previously described [3].

Fructosamine (FRUC), triglycerides (TG), total cholesterol (CHOL), high density lipoprotein cholesterol (HDL) and low density lipoprotein cholesterol (LDL) were measured on a Cobas® 6000 (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

Correlation matrix of traits and statistical analysis A correlation matrix for all measured traits was calculated for both the lean and obese groups followed by permutation test or bootstrapping in order to identify the probability of having a true correlation at four different levels of significance ($p<0.001$, $p<0.005$, $p<0.01$ and $p<0.05$) using a customized Matlab script (Mathworks, Natick, MA, USA) based on a script kindly provided by G. A. Churchill [17]. A normality test was performed for each trait. Functional network visualisation was done using Gephi software (The Gephi Consortium, Paris, France). Single trait comparisons between groups were performed using Student's unpaired *t* test (two-tailed) or by non-parametric comparison (Mann–Whitney) depending on normality of parameter distribution.

Results

Comparison of all measured traits between lean and obese animals was performed in two different ways. Firstly, in a conventional manner by finding statistically significant differences of parameters between groups using two-sided *t* test analyses, in which differences between groups were considered significant for $p<0.05$. As the purpose of this study was to compare single comparisons to functional network analysis, the *t* test was used to obtain as many potential significant outcomes as possible although the risk of type 1 error is evident. The results of these comparisons are summarized in Table 1. Non-parametric test (Mann–Whitney) gave the same results as *t* test with regard to significance of differences between traits. Secondly, by constructing a correlation matrix of all traits, followed by a permutation test or bootstrapping in order to find statistically significant co-segregations of traits.

Phenotypic characterization of the animals At the time of euthanasia, the obese minipigs weighed about twice as much as the lean and their fat and lean mass were significantly increased or decreased, respectively, both in absolute and relative terms (Table 1).

Apart from obvious differences in physical characteristics between lean and obese animals, there were remarkably few statistical differences in metabolic parameters, i.e. lipid profile and plasma glucose were not different between lean and obese. Surprisingly, plasma insulin was lower and hence, QUICKI index was significantly higher in the obese (Table 1). When comparing biomarkers of oxidative stress and inflammation on an individual basis, no differences were observed between obese and lean animals (Table 1). Main LAD diameter was significantly increased in the obese, in line with an increased heart weight in these animals. However, artery diameter in the obese did not increase proportionally with HW, since absolute diameter per gram heart tissue was significantly lower in the obese as compared to the lean (6.81 ± 0.27 μm/g ($n=15$) vs. 6.04 ± 0.16 μm/g ($n=18$) in lean and obese, respectively). Likewise, an apparent cardiac hypertrophy was observed in the obese, yet, when corrected for BW, HW per kg BW was lower in the obese as compared to lean animals (3.06 ± 0.10 g/kg vs. 2.44 ± 0.12 g/kg in lean and obese, respectively). Contractility, as evidenced by maximum response to high K^+ and U46619, was slightly decreased in obese left coronary arteries. Sensitivity to the dihydropyridine L-type calcium channel blocker nifedipine was higher, whereas that to substance P was lower, in coronary arteries of the obese, compared to those of lean minipigs (Table 1). Dimensions (Lumen, WTh, W/L ratio) of second order coronary arteries were not different between lean and obese. There was no clear evidence of impaired in vivo endothelial function. Absolute responses (FVI delta, FMD and FMD nitro) in obese were significantly higher than those in lean animals, but not when expressed in relative terms (Table 1).

Histopathology of the LCA One pig from the obese group was excluded due to problems during tissue preparation. No differences were found in the degree of intimal thickening between the lean and the obese pigs ($p>0.05$). Mild to moderate intimal thickening with foamy macrophages was present in both groups (5 of 7 lean animals and in all obese animals) while severe intimal thickening was seen in two pigs from the lean group.

Correlation Matrix analysis The strength of correlations between traits was initially evaluated using permutation test (10,000 permutations of every pair of traits). A summary of co-segregation of traits for different levels of significance following permutation is given in Table 2. However, due to missing values for some traits, the total number of observations per trait was in some cases only 5 or 6, instead of 7, as

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Table 1 A summary of averaged results (mean values±standard error of the mean) for all traits in lean and obese Göttingen minipigs

Traits	Lean	Obese
• Physical traits		
Body weight (BW, kg)	50.3±1.6	92.6±5.2 [a]
Mass (MASS, kg)	49.5±1.5	86.7±4.9 [a]
Non-fat mass (NONFAT, kg)	35.3±0.7	49.2±2.7 [a]
Fat mass (FAT, kg)	12.9±1.0	36.4±2.7 [a]
Bone mineral content (BMC, kg))	1.22±0.05	1.08±0.04[a]
Non-fat mass fraction (NONFAT %)	74.0±1.4	58.1±1.6 [a]
Fat mass fraction (FAT %)	26.0±1.4	41.9±1.6 [a]
Heart weight (HW, g)	155.2±5.0	219.3±14.0 [a]
• Metabolic factors		
Fasting plasma glucose (GLUC, mM)	3.80±0.09	3.74±0.36
Fasting plasma insulin (INS, pM)	141.2±32.4	39.16±9.89 [a]
QUICKI	0.38±0.01	0.50±0.03 [a]
C-Peptide (pM)	152.5±21.0	72.75±18.09 (6) [a]
Plasma total cholesterol (CHOL, mM)	1.77±0.11 (6)	1.98±0.10
Plasma triglyceride (TG, mM)	0.28±0.04 (6)	0.46±0.07
Plasma low density lipoprotein (LDL, mM)	0.67±0.05 (6)	0.85±0.10
Plasma high density lipoprotein (HDL, mM)	1.11±0.06 (6)	1.05±0.05
Plasma fructosamine (FRUC, μM)	262.0±7.9 (6)	236.1±9.8
• Biomarkers of oxidative stress and inflammation		
Plasma dihydrobiopterin (BH2, nM)	3.0±0.1	3.4±0.4
Plasma tetrahydrobiopterin (BH4, nM)	19.6±1.8	18.7±1.7
BH2/BH4	0.16±0.02	0.20±0.05
Plasma total ascorbic acid (VitC, μM)	29.5±2.7	33.0±2.0
Plasma ascorbate (Asc, μM)	25.2±2.9	30.3±2.6
Percentage dehydroascorbic acid (%DHA of total VitC)	15.2±2.6	8.8±2.9
(PAGP, μg/mL)	242.6±35.3	229.2±34.0
Haptoglobin (Hp, μg/mL)	2594±484	3115±625
C-reactive protein (CRP, μg/mL)	0.63±0.15	0.51±0.16
Serum amyloid A protein (SAA, μg/mL)	11.0±2.2	18.5±6.9
• Coronary artery traits		
2nd order coronary artery lumen diameter (lumen, μm)	420.2±61.3 (6)	409±51.9 (5)
2nd order coronary artery wall thickness (WTh, μm)	53.5±5.6 (6)	62.0±8.8 (5)
Wall/lumen ratio (W/L)	0.15±0.04 (6)	0.17±0.04 (5)
Left coronary artery optimal diameter (D_o, μM)	1034±83 (6)	1314±70 (6) [a]
Left coronary artery K^+ max response (Kmax, N/m)	21.2±3.2 (6)	19.6±1.5 (6)
U46619 max response (U64619, N/m)	15.9±2.6 (6)	12.8±2.4 (6)
Nifedipine sensitivity (PD2 NIF, $-\text{Log}(IC_{50})$)	8.44±0.23 (6)	9.06±0.09 (6) [a]
Substance P sensitivity (PD2 SP, $-\text{Log}(IC_{50})$)	11.02±0.21 (5)	9.96±0.09 (6) [a]
Bradykinin sensitivity (PD2 BRAD, $-\text{Log}(IC_{50})$)	7.41±0.35 (5)	7.47±0.19 (5)
• In vivo peripheral vascular function		
FVI delta (m/s)	105.4±58.3 (5)	343.5±36.6 (6) [a]
FVI, Δ (%)	113.5±68.6 (5)	556.3±192.6 (6)
Absolute change in luminal diameter (FMD, mm)	0.18±0.05 (6)	0.36±0.05 (6) [a]
FMD, %	9.1±3.1 (6)	12.9±1.8 (6)
Change in luminal diameter post nitro (FMD nitro, mm)	0.13±0.04 (5)	0.29±0.04 (6) [a]
Vascular dilatation post nitro (FMD nitro %)	6.7±2.1 (5)	11.2±1.4 (6)

All numbers are based on seven observations, except when indicated otherwise by the numbers between brackets

[a] denotes statistical significance of differences ($p < 0.05$) between groups as determined by Student *t* test

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Table 2 Co-segregation of traits in lean and obese minipigs according to the level of significance following permutation test

Lean minipigs			Obese minipigs		
Co-segregation of traits at $p<0.001$					
MASS	*BW*	*0.983*	*MASS*	*BW*	*0.997*
FAT	NONFAT %	−0.965	*NONFAT %*	*FAT %*	*−1.000*
FAT	FAT %	0.965	*Asc*	*VitC*	*0.968*
NONFAT %	*FAT %*	*−1.000*			
INS	*QUICKI*	*−0.937*			
Co-segregation of traits at $p<0.005$					
LDL	TG	0.985	*LDL*	*CHOL*	*0.928*
CHOL	WTh	−0.973	FMD %	W/L	0.997
Asc	*VitC*	*0.953*	BH2/4	% DHA	0.801
Co-segregation of traits at $p<0.01$					
FAT	*MASS*	*0.916*	*FAT*	*MASS*	*0.898*
HW	FVI delta	0.963	*NONFAT*	*MASS*	*0.904*
FMD	*FMD %*	*0.991*	*FAT*	*BW*	*0.886*
BW	GLUC	0.894	*NONFAT*	*BW*	*0.910*
FMD	BH2	0.893	INS	C-Pep	0.944
FMD %	BH2	0.869	pD_2 SP	QUICKI	0.956
FMD %	PAGP	0.930	*INS*	*QUICKI*	*−0.901*
BH4	PAGP	0.902	FVI delta	% DHA	−0.886
Co-segregation of traits at $p<0.05$					
NONFAT	*MASS*	*0.850*	NONFAT	FRUC	0.818
FAT	*BW*	*0.874*	MASS	FRUC	0.780
NONFAT	*BW*	*0.865*	BMC	pD_2 NIF	0.899
MASS	NONFAT%	−0.782	D_o	HW	0.916
MASS	FAT%	0.782	TG	FVI delta	−0.745
NONFAT	CHOL	0.915	pD_2 NIF	FVI delta	−0.855
MASS	CHOL	0.877	D_o	FMD	0.905
BW	CHOL	0.852	*FMD*	*FMD %*	*0.958*
LDL	*CHOL*	*0.850*	LDL	FMD nitro	−0.784
D_o	K^+ max	0.894	CHOL	FMD nitro	−0.854
K^+ max	U46619	0.882	LDL	FMD nitro %	−0.793
NONFAT	pD_2 NIF	−0.831	CHOL	FMD nitro %	−0.900
MASS	pD_2 NIF	−0.893	K^+ max	FMD nitro %	0.959
BW	pD_2 NIF	−0.891	pD_2 SP	INS	−0.890
CHOL	pD_2 NIF	−0.917	FMD nitro %	WTh	0.962
U46619	HW	0.902	QUICKI	WTh	−0.871
pD_2 BRAD	FMD	0.990	K^+ max	W/L	0.946
pD_2 BRAD	FMD %	0.982	TG	BH2	0.744
FAT	GLUC	0.775	FVI delta	BH2	−0.926
NONFAT	GLUC	0.774	FVI delta	BH2/4	−0.971
MASS	GLUC	0.880	C-Pep	BH2/4	0.762
pD_2 NIF	GLUC	−0.958	BH2	BH2/4	0.913
D_o	INS	0.799	BH4	BH2/4	−0.792
K^+ max	vessel D	−0.918	TG	Asc	−0.819
INS	vessel D	−0.941	BH2/4	Asc	−0.670
QUICKI	vessel D	0.859	TG	% DHA	0.851
INS	Lumen	−0.910	C-Pep	% DHA	0.867
vessel D	Lumen	0.983	BH2	% DHA	0.816
MASS	WTh	−0.859	Asc	% DHA	−0.880
BW	WTh	−0.909	pD_2 SP	PAGP	0.876
LDL	WTh	−0.919	pD_2 NIF	Hp	−0.871
pD_2 NIF	WTh	0.887	GLUC	Hp	0.858
GLUC	WTh	−0.799	K^+ max	CRP	0.952
pD_2 NIF	W/L	0.909	FMD nitro %	CRP	0.974
Vessel D	W/L	−0.894	Hp	CRP	0.856
Lumen	W/L	−0.951	LDL	SAA	0.774
pD_2 BRAD	BH2	0.884	FVI %	SAA	0.837
C-Pep	BH2	0.768			
pD_2 BRAD	BH4	0.896			
FMD	BH4	0.808			
FMD %	BH4	0.827			
FVI %	Asc	0.964			
D_o	VitC	0.844			
HW	VitC	0.837			
FVI %	VitC	0.950			
FAT	PAGP	−0.798			
NONFAT %	PAGP	0.842			
FAT %	PAGP	−0.842			
pD_2 BRAD	PAGP	0.933			
FMD	PAGP	0.936			
BH4	CRP	−0.913			
BH2/4	CRP	0.868			
BH2/4	SAA	0.871			
CRP	SAA	0.891			

Shown are pairs of traits and their respective correlation coefficient. Italics indicate co-segregations occurring both in lean and obese animals

indicated in Table 1. With this number of observations per trait, respectively 120, 720, or 5040 unique permutations are possible. Therefore, the level of significance of the original correlation may have been overestimated. For this reason, bootstrapping was performed on all traits in order to increase the number of unique combinations beyond 10,000 in order to improve the estimation of the level of significance of correlations between traits.

Table 3 illustrates co-segregation of traits for lean and obese minipigs following bootstrapping. Functional co-segregation networks of traits for lean and obese minipigs are visualised in Fig. 1. The parameters with the most significant level of co-segregation following permutation test are, amongst others, those that are dependent or derived from each other, for example NONFAT % and FAT %, INS and QUICKI, Asc and VitC or similar parameters measured using different methods, e.g. MASS and BW (Table 2). These results can be

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Table 3 Co-segregation of traits in lean and obese minipigs according to the level of significance following bootstrapping

Lean minipigs			Obese minipigs		
Cosegregation of traits at $p<0.001$					
NONFAT %	*FAT %*	*−1.000*	*MASS*	*BW*	*0.997*
			NONFAT %	*FAT %*	*−1.000*
Cosegregation of traits at $p<0.005$					
MASS	*BW*	*0.983*			
FAT	NONFAT %	−0.965			
FAT	FAT %	0.965			
Asc	*VitC*	*0.953*			
Cosegregation of traits at $p<0.01$					
FAT	*MASS*	*0.916*	*FAT*	*MASS*	*0.898*
BW	GLUC	0.894	*NONFAT*	*MASS*	*0.904*
INS	*QUICKI*	*−0.937*	*FAT*	*BW*	*0.886*
BH4	PAGP	0.902	*NONFAT*	*BW*	*0.910*
			LDL	CHOL	0.928
			INS	*QUICKI*	*−0.901*
			Asc	*VitC*	*0.968*
Cosegregation of traits at $p<0.05$					
NONFAT	*MASS*	*0.850*	NONFAT	FRUC	0.818
FAT	*BW*	*0.874*	MASS	FRUC	0.780
NONFAT	*BW*	*0.865*	BMC	pD_2 NIF	0.899
MASS	NONFAT %	−0.782	D_o	HW	0.916
MASS	FAT %	0.782	CHOL	FMD nitro %	−0.900
LDL	TG	0.985	pD_2 SP	INS	−0.890
NONFAT	CHOL	0.915	INS	C-Pep	0.944
MASS	CHOL	0.877	pD_2 SP	QUICKI	0.956
BW	CHOL	0.852	TG	BH2	0.744
MASS	pD_2 NIF	−0.893	FVI	BH2	−0.926
BW	pD_2 NIF	−0.891	FVI	BH2/4	−0.971
U46619	HW	0.902	BH2	BH2/4	0.913
FMD	FMD %	0.991	*BH4*	*BH2/4*	*−0.792*
FAT	GLUC	0.775	TG	Asc	−0.819
NONFAT	GLUC	0.774	TG	% DHA	0.851
MASS	GLUC	0.880	FVI	% DHA	−0.886
pD_2 NIF	GLUC	−0.958	C-Pep	% DHA	0.867
INS	vessel D	−0.941	BH2	% DHA	0.816
INS	Lumen	−0.910	BH2/4	% DHA	0.801
MASS	WTh	−0.859	Asc	% DHA	−0.880
BW	WTh	−0.909	pD_2 SP	PAGP	0.876
CHOL	WTh	−0.973	pD_2 NIF	Hp	−0.871
INS	W/L	0.865	GLUC	Hp	0.858
FMD	BH2	0.893	Hp	CRP	0.856
FMD %	BH2	0.869			
C-Pep	BH2	0.768			
BH4	*BH2/4*	*−0.782*			
Do	VitC	0.844			
HW	VitC	0.837			
FAT	PAGP	−0.798			
NONFAT %	PAGP	0.842			
FAT %	PAGP	−0.842			
FMD	PAGP	0.936			
FMD %	PAGP	0.930			
BH4	CRP	−0.913			
BH2/4	CRP	0.868			

Shown are pairs of traits and their respective correlation coefficient. Italics indicate co-segregation occurring both in lean and obese animals

seen as a performance validation of the analysis method. Although some co-segregation between traits are present in lean as well as obese animals, many co-segregations that can be identified in the lean are not present in the obese or vice versa. Bootstrapping resulted in loss of co-segregations that were present following permutation test alone, as expected (Tables 2 and 3, Fig. 1). Although HW, D_o and BW are significantly different between groups on an average basis (Table 1), HW and D_o appear to co-segregate only in the obese (Table 3 and Fig. 1).

Discussion

Well characterized animal models of disease are a prerequisite for unravelling of disease mechanisms, in particular for predictive validity of findings obtained in animals, for example efficacy of novel therapeutics and their translatability into clinically meaningful biomarkers or endpoints. It is becoming more and more evident that measurement of few biomarkers or endpoints in an animal model is far from sufficient to reliably predict disease progression or efficacy and safety of pharmacotherapy in humans. The clinical presentation of obesity is not merely restricted to body mass index, but to a certain pattern of changed phenotypical traits associated with the condition, such as impaired glucose tolerance, dyslipidaemia, inflammation, altered glucose and insulin levels or an apparent state of redox imbalance [7]. Individually, these traits do not necessarily differ from a normal range but in concert, they may show a deviating pattern as compared to a 'normal' healthy human phenotype. This means that the state of a disease, be it obesity, can usually not be defined as a crisp normal or diseased, but rather as a continuum of changes in various phenotypical traits, with a certain degree of deviation from the normal distribution.

The consequence of a solely hypothesis-driven approach is that, in order to be able to observe significant effects of any novel therapeutic towards normalising disease markers, or at least as compared to untreated controls, one needs an animal model showing statistically significant differences in disease-dependent key phenotypical traits. In other words, a

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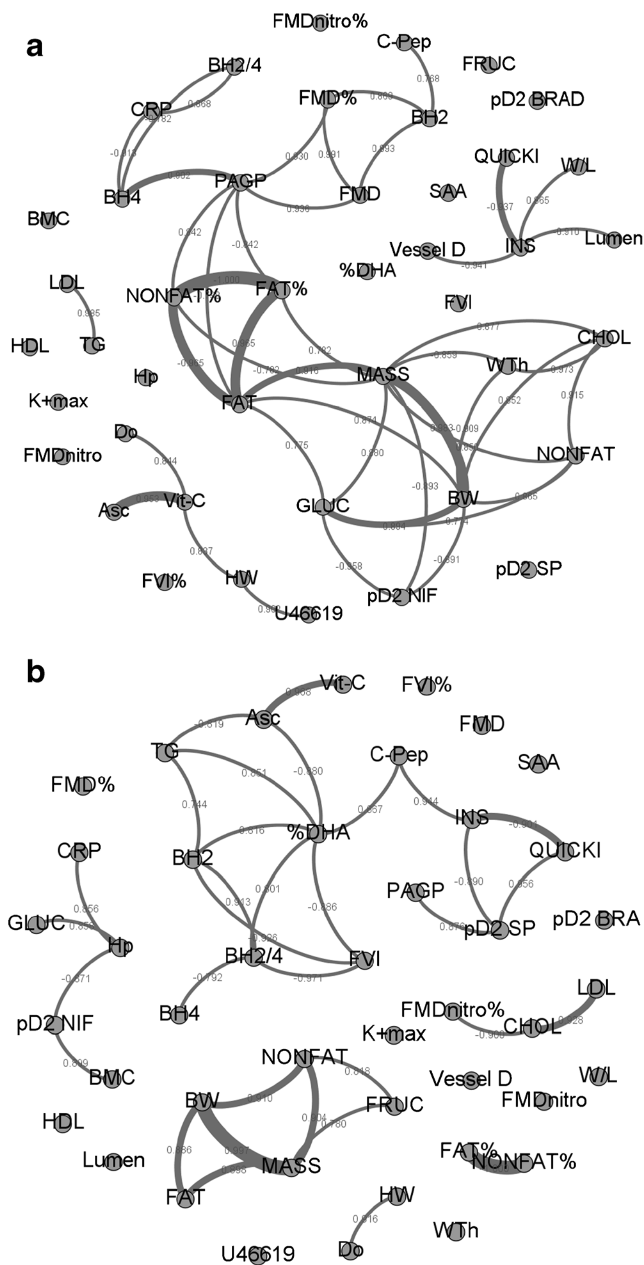

Fig. 1 Functional networks of traits for lean (**a**) or obese (**b**) minipigs following bootstrapping. All traits are represented as *nodes*, whereas significant co-segregations are indicated by *connections between nodes*, also indicating correlation coefficient. The thickness of the edges is proportional to the significance of the correlation, i.e. the *thickest lines* indicate significance of co-segregation with $p<0.001$, whereas the *thinnest lines* indicate significance at $p<0.05$. Trait abbreviations are explained in the text and Table 1. Placement of traits is not identical between lean and obese animals, but is based on shortest distance between nodes with edges according to Gephi Force Atlas

hypothesis-driven approach requires an animal model of disease, in which the parameters of interest are often beyond the usual auto-regulatory homeostatic capacity of the biological system, in order for them to become sufficiently different from healthy subjects. This implies that subjects with borderline changes of disease-dependent phenotypical traits are avoided in animal studies evaluating efficacy of novel therapeutics, whereas extreme changes in traits are preferred in order to increase the probability of observing significant treatment effects. Evidently, this does not translate well to the general overweight population, but perhaps to the obese or morbidly obese population, which account for only a minority of the total population with increased BW.

To illustrate this dilemma, we have shown that in obese minipigs, although they weighed about twice as much as their lean controls and had a significantly higher body fat percentage, their lipid profile, reflected by CHOL, TG and LDL, only tended to be increased, without reaching statistical significance, when compared on an individual parameter basis. Insulin sensitivity, reflected by QUICKI index, was apparently higher in the obese than in the lean, and none of the markers for oxidative stress or inflammation were significantly different between lean and obese animals. Also, the most pronounced intimal thickening was found in the lean group and no significant difference was found between the groups. Peripheral vascular function showed a relative increased reactive hyperaemic response following cuff occlusion in obese compared to lean animals, but also in response to nitroglycerin, whereas coronary artery sensitivity for the dilating effect of substance P was lower, and for the calcium channel blocker nifedipine was significantly higher in obese as compared to lean. This apparent contradiction between vascular and endothelial function in different vascular beds and between lean and obese subjects has previously been shown in animal and human studies and maybe indicative of compensatory mechanisms in response to altered endothelial function or an altered physiological reserve in the obese [6, 1]. Thus, in general, a number of parameters usually associated with obesity were not significantly different between obese and lean animals. This may be due to insufficient statistical power and furthermore, obvious differences in weight and size could have affected FMD and some of the metabolic parameters differently. In addition, the lack of any major metabolic differences between the two groups could be related to the fact that both groups were fed a normal low-fat pig chow albeit at different levels, whereas in humans, obesity is often related to the intake of high fat, high sugar dietary items, which may by themselves affect the metabolic parameters independent of obesity.

Nevertheless, the usefulness of many of the phenotypical traits used in disease state assessment, or effect of pharmacological intervention, may be questionable because of the apparent lack of difference between the obese and the lean Göttingen minipigs.

To address this problem, we utilised a correlation matrix of phenotypical traits and assessed co-segregations between all traits using either permutation with or without bootstrapping. These were used in order to assess the strength of co-segregation between traits, which is of particular importance given the low number of observations in both groups of

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animals. Additionally, no prior assumptions were made as to whether co-segregation of traits was causatively related or not. Dependency of traits, in fact, served as a validation of the method, in that dependent variables would be expected to co-segregate. This was also shown to be the case, since BW and mass, mass versus fat and non-fat mass, total VitC and Asc, plasma insulin and QUICKI, CHOL and LDL and BH4 and BH2/4 ratio correlated with a high level of significance in both lean and obese animals. On the other hand, significant co-segregations were revealed in the lean network that originally may not have been expected, such as plasma insulin and coronary artery diameter, C-peptide and BH2, cholesterol and coronary artery wall thickness, or of Pig α_1-acid glycoprotein (PAGP) versus BH4, fat, fat percentage, non-fat percentage, glucose or peripheral reactive hyperaemia (FMD), respectively. Interestingly, a recent report on the serum concentration of PAGP in obese minipigs showed that in some pig breeds (Ossabaw pigs) but not in Göttingen minipigs, PAGP serum concentrations were indeed significantly increased in obese as opposed to lean pigs [20]. Contrary to this, correlations that may have been expected appeared to be non-significant following bootstrapping. For example, HDL, SAA, FMD and FVI did not appear to co-segregate with any other trait, in either the lean or the obese network, which might suggest that these phenotypical traits are not likely to be predictive for any change in the system under the obese condition in the present experimental setup.

Overall, nine co-segregations of traits were common between lean and obese animals (indicated by italics in Table 3), although their level of significance may, on occasions, be different between lean and obese animals. These co-segregations comprised the dependent relations mentioned above. However, much co-segregation present in the lean network had disappeared in the obese network, whereas others, not present in the lean network, appeared in the obese. From a drug development point of view, such relations may be of considerable interest, since they distinguish the obese from the lean state, and may be used in an exploratory way to assess whether pharmacotherapy can reverse the obesity dependent co-segregations towards the lean-dependent co-segregations. Most notably, for example, is the absence of any relation of %DHA, haptoglobin or FVI with any other trait in the lean, and the appearance of six co-segregations of %DHA, and three relations between haptoglobin and FVI with other traits in the obese, respectively. This would indicate the presence of an oxidative and inflammatory environment in the obese that is not evident in the lean, since the acute phase protein haptoglobin co-segregates with the acute phase protein CRP, blood glucose levels and nifedipine sensitivity and FVI associates with markers of oxidative stress (BH2 and BH2/4) in the obese but not in the lean; both inflammation and oxidative stress have been described as part of the obesity response as previously suggested [22, 13]. Indeed, only in obese animals %DHA co-segregates positively with BH2/BH4, BH2 and TG, and negatively with FVI, indicative of an oxidative environment in these animals. This agrees well with the work of Furukawa and coworkers, who found that increased oxidation in accumulated fat is an important pathogenic mechanism of obesity-associated metabolic syndrome through systemic oxidative stress and inflammation [7]. In principle, by measuring but a few values for one or few traits, the networks could be used to estimate values of other traits, using the correlation coefficients and respective confidence intervals between traits, hereby predicting changes in traits, for example following treatment or other interventions to the system. Furthermore, the present minipig networks should be compared to a similar functional network obtained in other animal models of obesity, or most importantly, with a functional network of similar traits obtained in healthy or obese patients. This is expected to strengthen the translation of the animal model towards the clinical situation dramatically. After all, high-level phenotypical data are common in a clinical setting. Currently, integration of these data is done by experts, but this process could be aided by construction of functional networks as illustrated in the present study.

However, the validity of a network is crucial for the power of any predictions based on that network. In the present case, the number of animals and observations is low, and although bootstrapping or permutation might solve this problem, it cannot be excluded that adding data for a single animal could influence the network topology significantly. Additionally, a high precision and accuracy of the measurements of traits is very important. To illustrate this, many values for serum amyloid A protein (SAA) were below the lowest level of quantification (LOQ) of the assay, implying that the distribution of values for the group were skewed towards the LOQ, and thus not normally distributed. In addition to assay specific issues, biological system-specific issues could result in skewed data. For example, traits may behave linearly within a physiological range, until the system is stressed and traits reach their physiological auto-regulatory limits. Furthermore, other system corrective or compensatory mechanisms modify, mask or overrule the trait of interest, resulting in non-linear, unexpected behaviour. Another issue that might be of importance for the validity of the network is the conditions under which samples or measurements of traits were taken. This, however, is not a network-specific issue, but is of crucial importance for any scientific approach or comparison. Finally, significant correlations between traits could be non-linear. These events could obviously affect the validity of the network, and caution should therefore be taken.

In conclusion, the present results show that in a phenotypically obese group of minipigs, many obesity related parameters were not statistically different from lean animals on an individual comparative basis. However, when visualised as a

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functional network of co-segregations, marked differences between lean and obese animals appeared that could be utilised to better assess the effect of anti-obesity treatment or to evaluate disease progression. Functional network analysis may therefore offer a better understanding of changes in physiological system processes and could lead to a better prediction of clinical outcome of novel therapeutics using relatively small numbers of individuals. Furthermore, the functional network can perhaps form the basis of a translational tool that connects obese minipigs patho-physiological processes to those of human obese patients.

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