Original Research

Changes in Plasma Protein Expression Indicative of Early Diet-induced Metabolic Disease in Male Pigs (*Sus scrofa*)

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Recognition of the preclinical stages of metabolic diseases such as diabetes helps to prevent full development of the disease. In our research, we alter the diet composition of pigs to create a model of human metabolic disease. The objective of the current study was to identify plasma proteins and biologic mechanisms that differed in expression between pigs fed a 'cafeteria diet' (considered unhealthy; high in saturated fats) and those fed a 'Mediterranean diet' (considered healthy; high in unsaturated fats). Pigs fed the cafeteria diet showed increased plasma levels of proteins related to LDL ('bad cholesterol'), immune processes, blood clotting, and metal binding. The Mediterranean diet was associated with increased plasma quantities of proteins associated HDL particles ('good cholesterol'), binding of LDL particles, regulation of immune processes, and glycolysis. Pigs fed a cafeteria diet showed molecular signs of diabetes and atherosclerosis—even in the absence of clinical symptoms—which seemed to protect against the development of metabolic disorders. The current results suggest potential biomarkers of the early onset of metabolic syndromes. These biomarkers can help to reveal specific metabolic changes that precede the onset of diabetes, thus enabling the initiation of patient-specific interventions early during pathophysiologic development.

Abbreviations: FT, Fourier transform; MSMS, tandem mass spectrometry.

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Metabolic syndrome is a clinical condition initiated by a deregulated metabolism. Well-known examples of clinical manifestations of metabolic diseases include diabetes, obesity, atherosclerosis, and hypertension; these symptoms are well known to be interrelated, although the criteria that define metabolic syndrome remain under debate.^{8,20,46} The causes underlying the development a metabolic syndrome may be diverse, such as a combination of genetic polymorphisms that lead or predispose to the development of a metabolic defect,^{24,40,44} a lifestyle that negatively interferes with metabolism (including excessive eating, the use of unhealthy food diets, and too little exercise),⁹ or a combination of these factors.^{21,40} Clinical manifestations of metabolic diseases may occur relatively late in life after the initial physiologic deviations remain undetected for many years and too late for interventions to revert to a normal, healthy metabolism by interventions. In the Netherlands, approximately 25% of diabetic people are unaware of their changed metabolic status. Detection of early-onset changes related to metabolic syndromes might help to prevent progression of the disease⁴⁹ or to enable the initiation of interventions at an early stage of disease.

Blood plasma protein and metabolite contents are often useful indicators of the body's metabolic status.^{10–12,45,47,48} Proteomics research in the area of diabetes by using blood plasma, adipose, muscle, and liver protein profiles has identified several changes in plasma protein expression levels in diabetic patients as compared with healthy persons.^{28,29,36} These results showed the breadth of the metabolic deregulation in clinically affected persons. However, the results of such case-control studies are unsuitable for the identification and characterization of metabolic changes related to the preclinical stages of metabolic syndrome. In this context, longitudinal case-control studies are required. However, precisely defining the onset of the preclinical stage in humans remains impossible to predict. Research of early-stage disease is particularly challenging in humans, but such studies are possible by using controlled feeding conditions in model animals.

Pigs and humans share many similarities in metabolism, and pigs are recognized as a model in feeding studies.²² We previously reported that the plasma proteome profiles of control and diabetic pigs differed, especially when the pigs were fed a 'cafeteria diet' high in saturated fat.²¹ In that study, we chemically compared normal and diabetic pigs. We studied the mass spectrometry profiles of blood plasma from both nondiabetic (control) and diabetic pigs fed either the cafeteria diet or a 'Mediterranean diet,' which is high in unsaturated fats. An association study using physiologic data revealed association between peaks and physiologic data related to metabolic diseases such as diabetes and obesity, including body weight, and the size of

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several fat depots, and plasma levels of insulin, glucose, non-esterified fatty acids, triglycerides, cholesterol, VLDL, LDL, and HDL.³⁷ The pathophysiologic deviations of parameters in the diabetic pigs were related mainly to fatty acid and cholesterol transport and metabolism and correlated with specific plasma protein peaks. The differences between diets in corresponding plasma protein expression profiles were larger in diabetic pigs than in controls but related to the same physiologic parameters, suggesting that the changes in control pigs fed the cafeteria diet were the first signs of a diet-induced metabolic syndrome.

From our previous findings,³⁷ we concluded that preclinical detection of the onset of metabolic deregulation related to diet composition might be possible by using plasma proteome profiles and associated physiologic parameters indicating a shift in metabolism toward metabolic syndrome. However, our earlier study did not identify the proteins underlying the potential biomarkers; consequently we were unable to determine the associated biologic mechanisms. The present study revealed the proteins involved and suggested an explanation regarding the metabolic changes underlying metabolic syndrome. The objective of the current research was to identify proteins showing differential expression in blood plasma between pigs fed a Mediterranean diet compared with a cafeteria diet, to discover potential biomarkers that could be used to detect early stages of metabolic syndrome. Furthermore, we analyzed the biologic functions of the identified proteins.

Materials and Methods

Animals. The animal management, experimental protocol, surgical procedures, and animal care involved in this study was approved by the Animal Care and Use Committee of the Department of Animal Sciences (Wageningen UR, Wageningen, The Netherlands), and all efforts were made to minimize pain and distress. The performed research is in strict compliance with the ARRIVE guidelines on animal research.¹⁵ For 10 wk, male YorkshirexLandrace pigs (age, 11 wk; weight, approximately 30 kg) received either a cafeteria diet containing high levels of saturated fat ($n = 5$; Table 1) or a Mediterranean diet consisting of high unsaturated fat ($n = 6$). Physiologic parameters related to body weight gain, body composition, fat metabolism, and diabetes were measured to determine the physiologic effects of the different feeds given to the animals.³⁷ At 9 wk, EDTA blood samples were collected after 16 h of fasting and used for plasma proteome determination. Briefly, blood samples collected in heparinized (150 IU, USP Lithium Heparin, 10 mL Venoject, Terumo, Leuven, Belgium) or EDTA (0.47 mol/L EDTA, 10 mL Venoject, Terumo) tubes were immediately chilled at 0 °C in ice water and then centrifuged at 4 °C for 10 min at 1500 $\times g$. Plasma aliquots were stored at -80 °C for later analyses.³⁷

Proteomics and bioinformatic analysis. Label-free analyses (nanoLC–Fourier transform [FT]–tandem mass spectrometry [MSMS]) were performed^{19,31} to identify the differentially expressed proteins. In short, protein extracts were heated in SDS gel loading buffer. After electrophoresis of 16 μg protein, gel digestions were performed by using trypsin on 10 slices as described.³³ The samples were analyzed by injecting 18 μL sample over a preconcentration column (prepared inhouse; 0.10 $\times$ 32 mm, 5- μm beads, Magic C18 AQ 200 Å, Bruker Nederland, Elisabethhof, Netherlands) at a constant pressure of 270 bar (typically resulting in a flow rate of approximately 7 μL /min). Peptides were eluted from the preconcentration column onto an analytical column (prepared inhouse; 0.10 $\times$ 250 mm; 3- μm beads; Magic AQ 200 Å C18, Bruker Nederland) by using an acetonitrile gradient at a flow rate of 0.5 μL /min (EASY-nLC

1000, Thermo Scientific, Waltham, MA). The gradient consisted of an increase from 8% to 33% acetonitrile in water containing 5 mL/L acetic acid over 50 min, followed by a rapid (3 min) increase in the acetonitrile concentration to 80% (with 20% water and 5 mL/L acetic acid in both acetonitrile and water) as a column-cleaning step.

A microcross assembly (model P777, Upchurch Scientific, IDEX Health and Scientific, Middleboro, MA) was positioned between the preconcentration and analytical columns. An electrospray potential of 3.5 kV was applied directly to the eluent by using a stainless-steel needle fitted into the waste line of the micro cross. Full-scan positive-mode FTMS spectra were measured between mass-to-charge ratios of 380 and 1400 (LTQ-Orbitrap XL, Thermo Electron, San Jose, CA) at high resolution (60,000) as described.¹⁹ Automatic gain control target values were set to 10,000 for the ion trap and 500,000 for the Orbitrap (FT), or maximal ion times of 100 μs (ion trap) and 500,000 ms (FT) were used. Collision-induced fragmented (isolation width, 2 m/z; 30% normalized collision energy; activation Q, 0.25; and activation time 15 ms) tandem MS (MSMS) scans of the 4 most abundant 2+- and 3+-charged peaks in the FTMS scan were recorded in data-dependent mode in the linear trap (MSMS threshold, 5000; 45 s exclusion duration for the selected m/z $\pm$ 25 ppm [MSMS threshold, 5000]).

FTMS runs with all MSMS spectra obtained were analyzed by using MaxQuant 1.3.0.5⁴ at default settings for the Andromeda search engine^{4,5} except that extra variable modifications were set for deamidation of asparagine and glutamine. A *Sus scrofa* database was downloaded from UniProt (<http://www.uniprot.org>) and used with a contaminants database that contains sequences of common contaminants, including BSA (accession no. P02769, bovine serum albumin precursor), trypsin (P00760, bovine; P00761, porcine), keratin K22E (P35908, human), keratin K1C9 (P35527, human), keratin K2C1 (P04264, human), and keratin K1CI (P35527, human). The data from the 10 slides used per sample were combined, whereas the individual samples from different pigs were kept separate.

For protein identification, peptides and proteins with a false discovery rate of less than 1% and proteins with at least 2 identified peptides (at least one of which was unique) were accepted. Filtering and further bioinformatic analysis of the MaxQuant–Andromeda workflow output and the analysis of the protein expression ratios between the 2 diets were performed by using the Perseus 1.3 module (available in the MaxQuant suite). Reversed hits were deleted from the MaxQuant results. Proteins showing up- or downregulation of expression were selected by using *t*-test analysis, with a *P* value of less 0.05 indicating significance. Up- and downregulated proteins were further investigated by using the UniProt (<http://www.uniprot.org/>) and Gene Ontology (<http://www.geneontology.org/>) websites to obtain the biologic functions of the proteins to evaluate their biologic activities.

All proteomic LC–MSMS measurements were done at Biqualys (Wageningen, Netherlands).

Results

We identified 208 proteins in total. Of these, 18 proteins showed increased expression in pigs fed the cafeteria diet, whereas 16 proteins were increased in those on the Mediterranean diet (Tables 2 and 3). The *P* values of these differences ranged from less than 0.0001 to less than 0.05, and the ratios of protein expression between the 2 diets differed by 17% to 3700%. Gene ontology analysis of these proteins by using gene and protein databases revealed that the differentially expressed

Table 1. Composition of the diets containing high unsaturated fat (Mediterranean diet) and high saturated fat (cafeteria diet)

Component	Mediterranean diet			Cafeteria diet		
	Amount	Gross energy (Mj/ kg)	Energy intake (%)	Amount	Gross energy (Mj/ kg)	Energy intake (%)
Soy beans, extracted	51.3	0.37	1.60	51.3	0.37	1.60
Potato protein	50	0.69	2.94	50	0.69	2.94
Wheat gluten meal	118.3	1.82	7.79	118.3	1.82	7.79
Cellulose	100	0.10	0.45	100	0.10	0.45
Sucrose	100	1.89	8.08	100	1.89	8.09
Fructose	150	2.77	11.84	150	2.77	11.84
Native pea starch	150	3.06	13.08	150	3.06	13.09
Animal fat (lard)	0	0	0	250	12.12	51.86
Trisun 80, high oleic sunflower oil	55.9	2.82	12.08	0	0	0
Canola	142.5	7.20	30.78	0	0	0
Corn oil	45	2.27	9.72	0	0	0
Fish oil	6.6	0.32	1.35	0	0	0
Cholesterol (extra)	0	0	0	10	0.48	2.04
Chalk marl	11	0	0	11	0	0
Monocalcium phosphate	9.9	0	0	9.9	0	0
Sodium chloride	4.7	0	0	4.7	0	0
Mineral/vitamin premix	2	0.01	0.05	2	0.01	0.05
L-Lysine hydrochloride	2.8	0.06	0.26	2.8	0.06	0.26
Total (g/kg)	1000			1010		
Gross energy (Mj/kg)	23.4	23.38		23.4	23.37	

From reference 37 (used with permission).

proteins can be grouped according to their molecular function; several proteins have multiple molecular functions.

Transport and metabolism of fatty acids and cholesterol. As shown in Table 2, 4 proteins related to fatty acid and cholesterol transport and metabolism have higher plasma levels in pigs fed the cafeteria diet compared with the Mediterranean diet. Three of these proteins are related to lipid transport through LDL—apolipoprotein B, plasma platelet-activating factor acetyl hydrolase, and F1SCV9 [homologous to apolipoprotein B])—and the remaining protein, lecithin cholesterol acyltransferase, plays a role in lipid metabolism. In addition, 3 other proteins showed increased expression in animals on the Mediterranean diet (Table 3): the apolipoprotein M protein is part of the HDL lipid transport particle; by removing lipid hydroperoxides, glutathione peroxidase reduces the negative effects of oxidizing lipid molecules; and vitronectin functions as a scavenger receptor for binding LDL particles.

Immune functions. *Complement system.* Table 2 indicates that 4 proteins related to the complement system are more highly expressed in the plasma of pigs fed the cafeteria diet compared with the Mediterranean diet. Three of them—complement component 1 subcomponents R and S and complement component 2—are involved in activation of the classic complement cascade, and the SerpinF2 protein is a peptidase inhibitor that is involved in blood coagulation. Furthermore, 2 of the 3 proteins that were increased in the animals given the Mediterranean diet (F1S0J1 [similar to apolipoprotein R] and F1S0J2 [the product of *C4BPA*]) belong to the complement control module, and the 3rd (vitronectin, the product of *VTN*) acts to prevent membrane damage (Table 3).

Immune cell adhesion. Compared with those fed the Mediterranean diet, pigs fed the cafeteria diet had higher plasma levels of 2 proteins related to immune cell adhesion: plasma platelet-activating factor acetyl hydrolase, which is involved in the

activation of monocyte chemotaxis, and lymphocyte cytosolic protein 1 (the product of *LCP1*), which promotes the activation of T cells (Table 2). indicates that. In contrast, 4 proteins showed the opposite expression profile (Table 3). All 4 proteins (including orosomucoid, ORM1) regulate immune processes, and 3 of them (E-cadherin, vitronectin, and the product of *FN1* [fibronectin 1]) contribute to the regulation of cell adhesion.

Peptidases. The group of proteins with peptidase activity is diverse, consisting of 3 proteins from the peptidase S1 family, 4 serine endopeptidases and an endopeptidase inhibitor, a carboxypeptidase, and a peptidase activator. The expression of 7 of the 10 proteins was greater in the plasma of pigs fed the cafeteria diet compared with the Mediterranean diet. These proteins represented a diverse expression profile, which included 2 of the S1 peptidase family proteins (complement components 1R and 2), 3 of the serine endopeptidases and the endopeptidase inhibitor (complement component 1S, SERPINF2, LOC100516921, and CD109 [a platelet-specific antigen]), and carboxypeptidase 2. In contrast, the remaining 3 peptidases (that is, vitronectin, F1S0J1, and F1S0J2) showed increased expression in all groups fed the Mediterranean diet (Table 3).

Metal-binding proteins. The differentially expressed metal-binding proteins could be divided according to whether they bound iron (3 peptides, hemoglobin-related), zinc (carbonic anhydrase 1 and carboxypeptidase 2), or calcium (complement subunits, LCP1, LOC100620511). Except for a single calcium-binding protein, all proteins showed higher expression in pigs fed the cafeteria diet than in those given the Mediterranean diet (Table 2).

Blood clotting. Table 2 indicates that 2 proteins (plasma platelet-activating factor acetyl hydrolase and SerpinF2) involved in the blood clotting cascade were increased in the plasma of pigs fed the cafeteria diet compared with the Mediterranean diet. No

Table 2. Molecular functional analysis of plasma proteins with increased expression in pigs fed for 10 wk on a cafeteria diet (containing high levels of saturated fats) compared with pigs on a Mediterranean diet (containing high levels of unsaturated fats)

Protein	Cafeteria:		Complement	Peptidase	Metal bind- ing	Immune cell adhesion	Blood clotting
	Mediterranean expression ratio	Fatty acid and cholesterol					
Apolipoprotein B	1.59	X					
Lecithin cholesterol acyltransferase	1.39	X					
Plasma platelet-activating factor acetyl- hydrolase	1.94	X				X	X
F1SCV9 (uncharacterized)	1.53	X					
Complement component C1R	1.48		X	X	X		
Complement component C1S	1.33		X	X	X		
Complement component 2	1.50		X	X			
SERPINF2 (α 2-antiplasmin precursor)	1.17		X	X			X
CD109 (a putative macroglobulin)	4.71			X			
F1RK01 (product of <i>CPB2</i>)	1.32			X	X		
F1S133 (LOC100516921; uncharacterized)	1.27			X			
Hemoglobin subunit β	37.07				X		
Hemoglobin α chain	1.53				X		
F1RXC1 (product of <i>CA1</i> ; carbonic anhydrase 1)	2.74				X		
HBB; hemoglobin subunit β	1.50				X		
F1RK02 (product of <i>LCPI</i> ; uncharacter- ized)	1.86				X	X	
F1SRR8; uncharacterized protein	1.38						
F1SCV8; uncharacterized protein	1.60						

proteins whose expression was increased in Mediterranean-fed pigs or decreased in cafeteria-fed animals were obtained.

Glycolysis. Quantities of 2 proteins involved in the glycolytic pathway—L-lactate dehydrogenase β chain and fructose-biphosphate aldolase A—were lower in plasma of pigs on the cafeteria diet compared with animals fed the Mediterranean diet. No other differences in plasma protein expression were found.

Discussion

In this study, we identified potential plasma proteome biomarkers to facilitate preclinical detection of metabolic disease in a male pig model. In addition, we report the biologic mechanisms with which the differentially expressed proteins are associated. In the Netherlands, approximately 25% of diabetics are unaware of their changed metabolic status,¹³ which may threaten their overall health and increase future healthcare costs. Early detection of changes related to metabolic syndromes may help to prevent disease progression⁹ and to promote early intervention. Biomarkers may facilitate detection of early-onset metabolic syndrome. We argued that proteins that show different plasma expression levels between the 2 diets are indicative of subtle metabolic changes that precede phenotypic shifts toward clinical metabolic disease. Furthermore, given the multifactorial nature of metabolic diseases, a useful biomarker will need to represent multiple proteins. In this study, we followed diet-related metabolic changes in pigs, which we used as models for humans in light of the many physiologic similarities between pigs and humans.^{22,34} Therefore, we assumed that metabolic deregulation due to diet composition will also show many similarities between the 2 species. For 10 wk, the pigs were fed either a healthy Mediterranean-style diet high in unsaturated fats or an unhealthy cafeteria-style diet high in saturated fats. None of our pigs developed diet-induced clinical symptoms, such as obesity and insulin resistance, but our previous results

on proteomics clearly indicated that the cafeteria diet induces physiologic changes that are related to the development of the metabolic disease diabetes.³⁷ In the current study, we used nondiabetic pigs to identify the proteins involved and the underlying biologic and possible pathophysiologic mechanisms. Together our previous and current results imply that these same changes in protein expression will occur in diabetic pigs but to a greater extent. In addition, our previous study³⁷ used physiologic parameters measured at 16 h after feeding to reveal associations between changes in protein expression levels and the development of metabolic syndrome. Therefore such parameters may reflect permanent metabolic changes and not just short-term meal-induced differences in the plasma proteome. It should be noted that the pigs did not show clinical signs of the metabolic syndrome. Here we extend our previous study³⁷ by reporting proteomic indicators associated with early stages in the development of metabolic syndrome.

In pigs fed the unhealthy cafeteria diet, the expression levels of proteins active in LDL transport were increased, whereas those on the healthy Mediterranean diet showed increased levels for HDL transport proteins and LDL-removal receptors. Both LDL and HDL transport fatty acids and cholesterol in the blood, but HDL can be removed from the blood much more easily than LDL,^{1,35} leading to LDL-cholesterol accumulation in macrophages and atherosclerotic plaques; consequently HDL-cholesterol is considered ‘good cholesterol’ and LDL-cholesterol as ‘bad cholesterol.’ These results suggest that fats and cholesterol in the healthy diet are transported mainly by HDL transport particles, whereas those in the unhealthy diet primarily are delivered through an LDL-associated mechanism. Therefore, our results suggest a direct connection between the diet and the functionality and metabolism of fat and cholesterol transport particles. A previous review of proteomics studies regarding diabetes reported the differential expression of several apolipoproteins in both serum and plasma.³⁶ Our results are in

Table 3. Molecular functional analysis of plasma proteins with increased expression in pigs fed for 10 wk on a Mediterranean diet (containing high levels of unsaturated fats) compared with pigs on a cafeteria diet (containing high levels of saturated fats)

Protein	Cafeteria: Mediterranean expression ratio	Fatty acid and chole- sterol	Complement	Peptidase	Metal binding	Immune cell adhesion	Glycolysis
Apolipoprotein M	0.59	X					
Glutathione peroxidase	0.51	X					
P48819 (product of <i>VTN</i> ; vitronectin)	0.82	X	X			X	
F1S0J2 (product of <i>C4BPA</i> ; uncharacterized)	0.49		X				
F1S0J1 (product of <i>C4BPA</i> ; uncharacterized)	0.67		X				
HABP2 (hyaluronan binding protein 2)	0.33			X			
Antithrombin III	0.86			X			
F1SS24 (product of <i>FN1</i> ; uncharacterized)	0.68			X		X	
F1SRC8 (LOC100620511; uncharacterized, possible C-type lectin)	0.70				X		
Orosomucoid 1 (product of <i>ORM1</i> ; α 1 acid glycoprotein)	0.59					X	
CDH1 (E-cadherin)	0.54					X	
L-lactate dehydrogenase B chain (LDH heart unit)	0.51						X
Fructose-bisphosphate aldolase A	0.23						X
Transthyretin (prealbumin)	0.79						
Secreted phosphoprotein 24	0.53						
F1STN0 (product of <i>SMPDL3B</i> ; uncharacterized)	0.22						

agreement with these studies and extend them to preclinical conditions. In addition, 2 plasma proteins involved in glycolysis were less expressed in pigs fed the cafeteria diet, perhaps suggesting poor glucose utilization and metabolism in these pigs as compared with those given the healthy diet. A low capacity of the body to use glucose is a characteristic feature of obesity, metabolic syndrome, and diabetes.^{6,7}

In summary, diets rich in saturated fatty acids induce a series of metabolic changes. Accumulation of fat leads to obesity and diabetes through insulin resistance and decreased adiponectin expression leading to lipotoxicity. This effect, among others, changes the expression of glycolysis-related proteins and shifts the transport of cholesterol from the preferred HDL to LDL. This series of events ultimately leads to the altered phenotypes characteristic of metabolic syndromes.

Diet composition differentially regulated several immune-related genes. The cafeteria diet led to higher levels of proteins of the complement system, chemotaxis, and T-cell activation, whereas the Mediterranean diet promoted the expression of regulatory units of the complement system and proteins protective against activation of complement and cell adhesion. Thus these results may suggest that the unhealthy diet activates the proinflammatory immune system, whereas the healthy diet, to maintain immune homeostasis, activates regulatory and protective mechanisms against immune-associated damage. Similar diet-induced activation of the immune system occurs in diabetic mice as well.^{13,36,39} Furthermore, several studies show a relationship between diabetes and differential expression of immune-related proteins, including inflammatory proteins and vitronectin.³⁶ Because our pigs did not yet show overt clinical signs of metabolic syndrome, these results suggest that these proteins might be valuable as biomarkers to indicate early signs of metabolic

and inflammatory deregulation. In addition, inflammation has been observed in adipose tissue of obese persons,⁴³ perhaps due to the increased expression of immune-associated proteins, but our pigs did not yet demonstrate any increase in fat deposition. So, again, these proteins might be regarded as biomarkers of early onset of metabolic disease, before any clinical symptoms can be measured.

The diet-induced changes in these effects on immune processes may be related to the elevated expression in pigs fed the cafeteria diet of 2 proteins involved in blood clotting. The combination of excessive cholesterol, immune activation, and aberrant blood clotting may lead to and accelerate the development of atherosclerosis.³ Indeed, our pigs showed early, subclinical signs of developing atherosclerotic lesions—more often in diabetic pigs than in control pigs and nondiabetic pigs fed the cafeteria diet than the Mediterranean diet.^{16,17,42} The increased expression of these plasma proteins suggests that these processes are already initiated in the preclinical stage.

Several peptidases, proteases, and hydrolases showed greater expression in the pigs fed the Mediterranean diet than the cafeteria diet. This group of proteins is diverse and includes iron-, zinc-, and calcium-binding proteins. The functions of these metals likewise are diverse: iron is important for oxygen and carbon dioxide transport (hemoglobin proteins); zinc is involved in the functioning of many enzymes involved in protein anabolism, tissue renewal, and immune function;^{30,41} and calcium plays a role in many biologic functions, including cellular metabolism, tissue remodeling, blood clotting, and transport.^{18,23,25-27,32} An association between diabetes and hemoglobin expression has been reported.³⁶ Together, these results suggest a greater capacity of pigs fed the Mediterranean diet to maintain and restore whole-body homeostasis with regard to many life functions.

Finally, several plasma peptidases were differentially expressed in relation to both diets, but we were unable to elucidate an underlying biologic mechanism. This difficulty may reflect the diverse composition of this protein group, which includes several proteases with opposing functions. Alternatively, this molecular function may be a nonspecific effect of diet or may point toward unknown physiologic changes. Overall our results indicate that diet composition affects plasma protein composition. Furthermore, the effects of diet on the plasma proteome seem to be indicative of the health aspects of the diet. Therefore, we conclude that these differentially expressed plasma proteins can be used as biomarkers for adjusting diet composition to prevent metabolic diseases long before the onset of clinical symptoms.

Figure 1 summarizes the results and illustrates the complexity of developing a biomarker for a multiphenotypic disease. In particular, the figure shows distribution of various biologic mechanisms between the 2 dietary groups. Furthermore, the Figure clearly shows the multifactorial background of the metabolic disease phenotypes and that all factors should be considered when developing a predictive biomarker.

The increase in the expression of the plasma proteins between the 2 dietary conditions ranged from 17% to 3700%. This result correlates well with previously reported plasma proteome profiles,³⁷ which showed that differences in plasma protein expression between the 2 diets in nondiabetic pigs were much smaller than those between nondiabetic and diabetic pigs, especially when they received the unhealthy cafeteria diet. This pattern may relate to the stage of metabolic syndrome: early during disease development, when the body can still cope with the adverse effects of the cafeteria diet, compared with overt metabolic syndrome with clinical symptoms, when the body has lost its ability to compensate. Our current experimental design included a period of 10 wk during which the pigs daily consumed 2 very different diets—more extreme conditions than the usual situation in humans. Similar effects might occur in humans after prolonged consumption of less extreme diets and should be explored in future human cohort studies.

Detecting the earliest molecular indications of metabolic deregulation is important to prevent full development of metabolic syndrome. Our biomarkers are unlikely to be influenced by the recent intake of a meal, because we measured the physiologic parameters 16 h after the pigs' last feeding. The differences in the diet-associated expression ratio of the biomarker proteins was modest (17%)² to 3700% for a single protein. To detect differences in biologic processes, we recommend using a combination of proteins as biomarkers for early-stage metabolic disease, rather than a single protein. The best biomarker perhaps might be one that contains 1 or 2 proteins from each molecular function group. A broad biomarker might detect the first sign of a developing metabolic syndrome. However, a multiprotein biomarker is often more predictive for a trait compared with a single protein biomarker.³⁸ By using state-of-the-art knowledge of the plasma proteome, multiprotein biomarkers can be developed. This multiprotein biomarker approach may be useful in predicting and designing healthy food compositions. For translational biomedicine, the multiprotein biomarkers need to be validated in human cohorts before they can be implemented in the clinical setting.

Although outside the scope of the present work, it is important to relate our current results to biomarker work in human metabolic disease, especially diabetes. Both human and mouse studies indicate the breadth of metabolic syndrome, including

Figure 1. Schematic representation of the biologic mechanisms that differed between pigs fed the Mediterranean diet compared with the cafeteria diet. The mechanisms are organized according to the metabolic disease phenotype that might develop from these preclinical aberrations.

diabetes, and the related biomarkers identified.^{28,29,36} Several of the biomarkers we found here were reported to occur in diabetic human patients, including the proteins related to fatty acid and cholesterol transport, immune- and inflammatory proteins, and hemoglobin.^{28,29,36} Here we report that these metabolic changes can be measured before any clinical sign of the disease, and we argue that, if correlates can be found in humans, preclinical evaluation of people at risk may prevent the onset of metabolic disease including diabetes.

In summary, we have detected several proteins in the plasma of pigs that are differentially expressed in response to feed composition and potential early, preclinical signs of metabolic syndrome. Furthermore, unlike previously published data, these altered protein expressions might be used as biomarkers to detect early stages of metabolic disease.

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