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# Differences in lipid deposition and adipose membrane biophysical properties from lean and obese pigs under dietary protein restriction

Ana P. Martins [a,1], Paula A. Lopes [b,1], Marta S. Madeira [b], Susana V. Martins [b], Nuno C. Santos [c], Teresa F. Moura [a], José A.M. Prates [b], Graça Soveral [a,d,*]

[a] REQUIMTE, Dep. Química, FCT-UNL, 2829-516 Caparica, Portugal
[b] CIISA, Faculdade de Medicina Veterinária, Universidade Técnica de Lisboa, 1300-477 Lisboa, Portugal
[c] Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, 1649-028 Lisboa, Portugal
[d] Dep. Bioquímica e Biologia Humana, Faculdade de Farmácia, Universidade de Lisboa, 1649-003 Lisboa, Portugal

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ABSTRACT

Obesity consists in fat accumulation leading to increase in adipose cells number and size. Adipocyte membrane biophysical properties are critical to maintain cellular viability in metabolically healthy obesity. This study investigated the effect of the genetic background and dietary protein restriction on fat tissue lipid composition, adipocyte membrane fluidity and water permeability using the pig as experimental model.

Twenty-four male pigs from distinct genotypes, lean and obese, were fed on normal and reduced protein diets within a 2 × 2 factorial arrangement (two genotypes and two diets). Backfat thickness was two-fold higher in obese than in lean pigs but unrelated to dietary protein level. In contrast, total fatty acids in the subcutaneous adipose tissue were dependent on both breed and diet, with increased lipid content promoted by the fatty genotype and by the restriction of dietary protein. Adipose membranes isolated from obese pig's subcutaneous fat tissue showed higher permeability to water, in line with an increased fluidity. Moreover, the reduced content of dietary protein influenced positively the fluidity of adipose membranes. Neither genotype nor diet affected total cholesterol concentration in the adipose membranes. Membrane-saturated fatty acids' content was influenced by genotype, while membrane-polyunsaturated fatty acids, particularly from the *n*-6 family, was influenced by diet. The ratio of oleic (18:1*c*9)/linoleic (18:2*n*-6) acids was positively correlated with membrane fluidity. All together, these findings reinforce the genetic background as a determinant player on adipose membrane biophysical properties and point to the dietary protein level as an important factor for subcutaneous lipid deposition as well as for regulation of membrane function, factors that may have impact on human obesity and metabolic syndrome.



## 1. Introduction

Obesity is characterized by increased accumulation of triacylglycerols in the adipose tissue from subcutaneous and visceral depots, but also in surrounding organs as kidney, epicardium, skeletal muscle, and blood vessels as the result of hypertrophy and hyperplasia of adipocytes [1]. The adipose tissue is a highly dynamic endocrino-metabolic organ capable of efficient storage and mobilization of lipids to fulfill bioenergetic demands [2]. Lipids are involved in a wide array of processes, not only providing efficient energy storage and specific substrates for cell signaling, but also building membranes within the cell, thus providing compartmentalization.

Cell membranes are primarily composed of lipids and different lipids can alter membrane physical properties such as fluidity and passive permeability. Depending on their composition, cell membranes play an important role in regulating many cellular functions, such as signal transduction, receptor mobility, lipid trafficking, channels and transporters and cell adhesion. Moreover, membrane fluidity and lipid composition have been shown to affect the specific binding of a variety of hormones to their receptors [3].

It is well known that dietary fats can affect the types of lipids present in the organism. In the body, fatty acids derived from the diet and from *de novo* lipogenesis can be further elongated and unsaturated via elongases and desaturases thus generating a variety of lipid species that can then maintain a specific lipid composition [4]. This adaptation, enabling to preserve membrane function as the adipose

* Corresponding author at: REQUIMTE, Dep. Química, FCT-UNL, 2829-516 Caparica, Portugal. Fax: +351 212948550.
*E-mail address:* gsoveral@ff.ul.pt (G. Soveral).
[1] These authors have contributed equally to this article.

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cells expand, is eventually disrupted in morbidly obese individuals, with an outcome favoring secondary diseases such as the metabolic syndrome [5]. How the membrane biophysical properties are regulated to match the specific needs of the membrane expansion is still obscure.

Another aspect related to tissue lipid redistribution was reported using pigs as an experimental model. It has been shown that plasma cholesterol increased dramatically in lean pigs fed low protein diets [6] and that reduced dietary energy and protein in growing pigs significantly increased intramuscular fat [7], while having a minor effect on the amount of subcutaneous adipose tissue [8,9]. Reducing the proportion of protein relative to energy in the diet consistently increases fat deposition. One possible explanation for this effect is that the low protein content restricted muscle growth, resulting in excess energy being converted into intramuscular lipids [8]. Another possibility is that restricted growth from the reduced protein diet (RPD) led to fat redistribution, resulting in an increase of intramuscular fat [7,10].

Considering problems in collecting tissue samples and the multifactorial etiology of obesity in human patients, suitable animal models are essential for a better understanding of the metabolic onset of obesity. Pigs display several anatomical-physiological and metabolic similarities to humans of special interest for the study of genetic or dietary induced obesity [11].

In the present study, pigs with distinct genetic backgrounds, Crossbred (commercial pigs resulting from Large White, Landrace and Pietrain inbreeding) and Alentejana purebred, were selected because they mimic genetically lean and obese animal models respectively, thus providing a valuable comparative model for human obesity. The effect of genotype (lean *versus* obese) as well as the restriction of dietary protein (control or reduced protein diets) on subcutaneous fat deposition was investigated. Additionally, the impact of genotype and RPD at the cell membrane level was determined by assessing adipose membrane lipid composition and biophysical properties such as fluidity and permeability.

## 2. Materials and methods

### 2.1. Experimental design: Animals and diets

This trial was conducted at the facilities of L-INIA (INRB, Vale de Santarem, Portugal) under the guidelines for the care and use of experimental animals, following EU directive 86/609/EEC.

Twenty-four Crossbred (50% Large White, 25% Landrace and 25% Pietrain) and Alentejano purebred entire male pigs with an initial average weight of 60 ± 2 kg were selected. Animals were fed a commercial concentrate diet from weaning until the beginning of the experiment. Thereafter, six animals from each genotype were randomly assigned to one of two diets in a 2 × 2 factorial arrangement (two genotypes and two diets). Pigs were fed individually twice a day whilst water was freely available. Throughout the experiment, pigs were weighted weekly just before feeding. Diets were isoenergetically formulated (16 MJ ME/kg) and differed in crude protein and lysine contents: the control diet contained 17.5% of crude protein and 0.7% of lysine; and the RPD contained 13.1% of crude protein and 0.4% of lysine. The proximate and fatty acid composition of the diets is presented in Table 1.

**Table 1**
Proximate and fatty acid composition of control and reduced protein diets (RPD).

| | Control | RPD |
|---|---|---|
| *Chemical composition (% diet)* | | |
| Dry matter | 89.1 | 89.0 |
| Ash | 4.4 | 4.0 |
| Crude fiber | 4.9 | 4.0 |
| Crude fat | 3.1 | 2.9 |
| Crude protein | 17.5 | 13.1 |
| Calcium | 0.82 | 0.84 |
| Phosphorus | 0.37 | 0.37 |
| Lysine | 0.7 | 0.4 |
| *Fatty acid composition (% total fatty acids)* | | |
| 14:0 | 0.1 | 0.2 |
| 16:0 | 17.3 | 19.6 |
| 16:1c9 | 0.8 | 0.2 |
| 18:0 | 2.6 | 2.6 |
| 18:1c9 | 19.0 | 19.3 |
| 18:1c11 | 1.5 | 1.6 |
| 18:2n-6 | 52.5 | 50.2 |
| 18:3n-3 | 4.9 | 4.3 |
| 20:0 | 0.3 | 0.3 |
| 20:1c11 | 0.4 | 0.5 |

### 2.2. Pigs' tissue sampling

Feed was removed 17–19 h before slaughter. Pigs were slaughtered at a live weight of 93 ± 2 kg (L-INIA experimental abattoir). After electrical stunning and exsanguination, samples from subcutaneous adipose tissue were collected from the right carcass side directly above *longissimus lumborum* muscle. At 24 h postmortem, the backfat thickness was measured in the left carcass side at the last rib position (P2).

### 2.3. Total fatty acids in the subcutaneous adipose tissue

Subcutaneous adipose tissue samples were lyophilized (−60 °C and 2.0 hPa) to constant weight, kept dry at −20 °C, and analyzed within two weeks. Total fatty acids were extracted from the lyophilized samples (ca. 250 mg) [12] using dichloromethane:methanol 2:1 (v/v), converted to methyl esters (FAME) [13] and were determined using a gas chromatograph HP6890A (Hewlett–Packard, PA, USA), equipped with a flame ionization detector and a CP-Sil 88 capillary column (100 m; 0.25 mm i.d.; 0.20 m film thickness; Chrompack, Varian Inc., Walnut Creek, CA, USA) with the chromatographic conditions described [14]. The quantification of total FAME was done using nonadecanoic acid (19:0) from Supelco (Bellefonte, PA, USA), as internal standard.

### 2.4. Isolation of membranes from subcutaneous adipose tissue

Membrane vesicles were prepared from subcutaneous adipose tissue by differential centrifugation with buffer without detergents, using a previously described method [15,16] optimized for pig fat tissue. Crude membranes re-suspended in mannitol–Hepes buffer (100 mM mannitol, 10 mM Tris–Hepes, pH 7.4) were immediately used for experiments. Protein content was determined by the Bradford technique [17].

Vesicle size of all membrane preparations was determined by Quasi-Elastic Light Scattering (Brookhaven Instruments) [18], revealing homogenous populations with monomodal distributions, with a mean hydrodynamic diameter of 342 ± 46 nm.

### 2.5. Fatty acid composition and cholesterol concentration of adipose membranes

Membranes were lyophilized and fatty acids were converted to FAME [19]. The resulting FAME were analyzed by gas chromatography as described in detail [15].

Total cholesterol was extracted from lyophilized membranes as described [15] and was calculated, in triplicate, based on the external standard technique, from a standard curve for peak area versus cholesterol concentration.

### 2.6. Assessment of membrane fluidity

Membrane fluidity was evaluated by measuring the fluorescence anisotropy of two probes incorporated in the membrane: 1,6-diphenyl-1,3,5-hexatriene (DPH), or 1-(4-(trimethylamino)-phenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), from Molecular Probes (Eugene, OR, USA). DPH is incorporated inside the membrane at the fatty acyl group's level, while TMA-DPH is anchored by its cationic moiety at the membrane/water interface probing the membrane region closer to the phospholipids head groups [20]. Measurements of fluorescence anisotropy ($r$) were conducted as previously described [15] and calculated using the equation $r = (I_{VV} - GI_{VH})/(I_{VV} + 2\,GI_{VH})$, where $I_{VV}$ and $I_{VH}$ are the fluorescence intensities; the subscripts indicate the vertical ($V$) or horizontal ($H$) orientations of the excitation and emission polarizers, and $G = I_{HV}/I_{HH}$ is the instrumental factor. The fluorescence intensity data points used for calculations were the average of three identical aliquots (after blank subtraction) measured on a Varian Cary Eclipse fluorescence spectrophotometer (Mulgrave, Australia).

### 2.7. Evaluation of membrane water permeability

Water permeability of membrane vesicles was assessed using the stopped-flow technique (HI-TECH Scientific PQ/SF-53) [21]. Experiments were done at 23 °C for single measurements and from 12 to 37 °C for evaluation of the activation energy, as described [15]. The kinetics of vesicle shrinkage after a hyperosmotic shock (120 mOsM mannitol gradient) was monitored from the time course of scattered light intensity at 400 nm until a stable light scatter signal was attained (Fig. 3A). The osmotic water permeability coefficient ($Pf$) was estimated by fitting the light scatter signal to a single exponential curve and using the equation $Pf = k\ (V_o/A)(1/V_w(\mathrm{osm}_{out})_\infty)$, where $k$ is the exponential time constant, $V_w$ is the molar volume of water, $V_0/A$ is the initial volume to area ratio of the vesicle preparation, and $(osm_{out})_\infty$ is the final medium osmolarity after the applied osmotic gradient. All solution osmolarities were determined from freezing point depression on a semi-micro osmometer (Knauer GmbH, Germany). The activation energy of water transport was obtained from the slope of an Arrhenius plot ($\ln P_f$ as a function of $1/T$) multiplied by the gas constant $R$.

### 2.8. Statistical analysis

Statistical analysis was performed using the Statistical Analysis System (SAS) software package, version 9.1 (SAS Institute, USA). All data were presented as mean and standard error (SE). Data were analyzed using PROC MIXED with variance heterogeneity to determine the significance of the main effects, genotype (lean or obese) and diet (control or RPD), and their respective interaction (genotype × diet). If significant effects were obtained ($P < 0.05$), least squares means were determined using the LSMEANS option and compared using the probability difference procedure (PDIFF option). Pearson's correlation coefficients were calculated with the CORR procedure to establish linear relationships among lipid composition, membrane fluidity and permeability.

## 3. Results

### 3.1. Backfat thickness and fatty acids are increased in obese pigs

Fig. 1 displays the P2 backfat thickness and total fatty acids in the subcutaneous adipose tissue from lean and obese pigs fed on control and RPDs. A clear genotype effect was observed for both parameters. Bakfat thickness at the P2 position was twofold higher in obese than in lean pigs (Panel A; $P < 0.001$) but unrelated to dietary protein level ($P > 0.05$). In a similar manner, obese pigs had a higher amount of total fatty acids in the subcutaneous fat (Panel B; $P < 0.01$). Moreover, a diet effect was also observed with pigs fed on reduced protein showing higher content of total fatty acids in the same tissue ($P < 0.05$). Following these results, we next isolated adipose membranes from pig's subcutaneous adipose tissue in

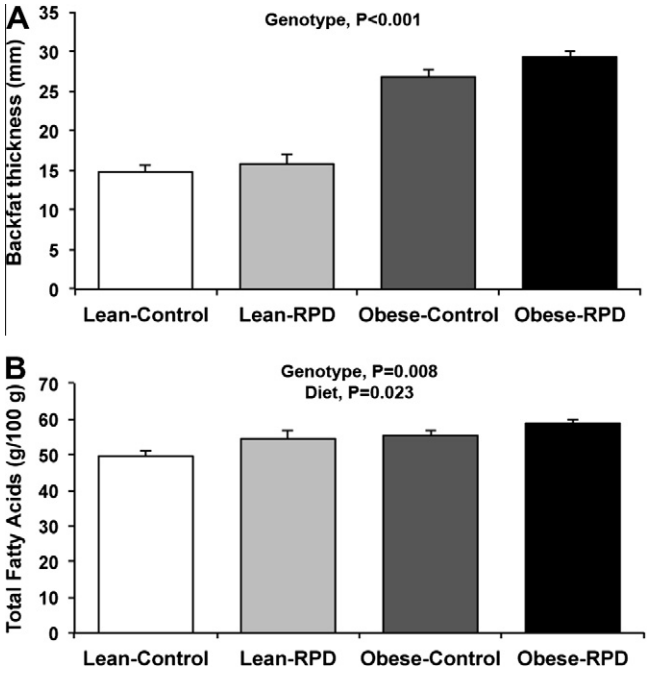


**Fig. 1.** Analysis of subcutaneous adipose tissue. (A) Backfat thickness measured at P2 level, and (B) total fatty acids of lean and obese pigs fed on control and reduced protein (RPD) diets. Statistical significances are shown for the effects of genotype (lean *versus* obese pigs) and diet (control *versus* reduced protein diets) at $P < 0.05$.

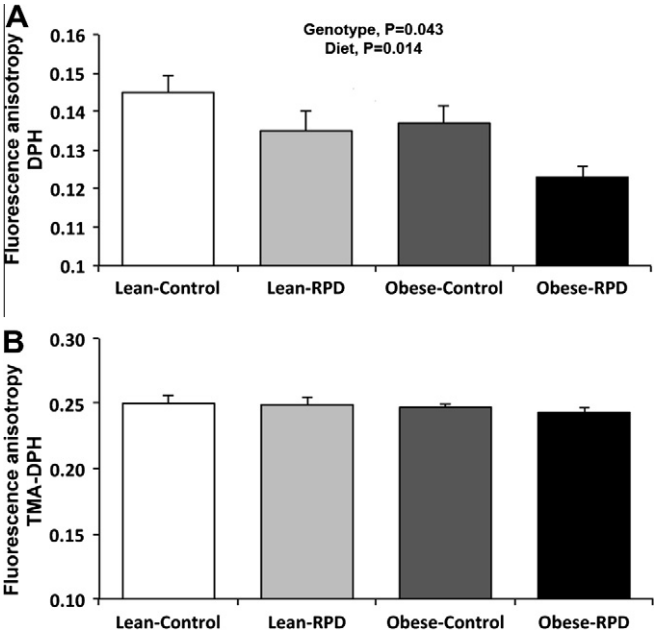


**Fig. 2.** Membrane fluidity measured by fluorescence anisotropy. (A) DPH and (B) TMA-DPH, in adipose membrane vesicles from lean and obese pigs fed on control and reduced protein (RPD) diets. Statistical significances are shown for the effects of genotype (lean *versus* obese pigs) and diet (control *versus* reduced protein diets) at $P < 0.05$.

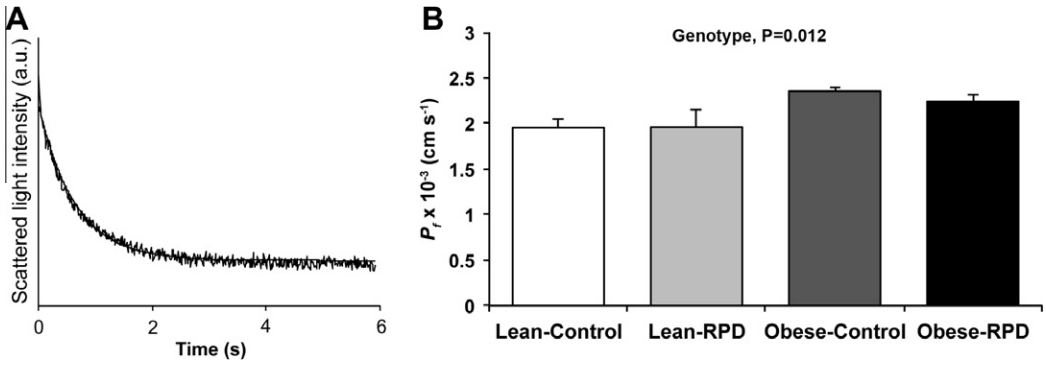


**Fig. 3.** Water permeability of adipose tissue membranes. (A) Typical stopped flow light scatter signal showing membrane vesicles shrinkage due to water outflow after a hyperosmotic shock (gradient 120 mOsM). (B) Water permeability (*Pf*) at 23 °C from lean and obese pigs fed on control and reduced protein (RPD) diets. Statistical significances are shown for the effect of genotype (lean *versus* obese pigs) at $P < 0.05$.

**Table 2**
Cholesterol concentration (mg/g membrane) and fatty acid (% of total FAME) profile of adipose membranes from lean and obese pigs fed on control and reduced protein (RPD) diets.

| | Lean | | Obese | | Significance level | | |
|---|---|---|---|---|---|---|---|
| | **Control** | **RPD** | **Control** | **RPD** | **Genotype** | **Diet** | **Genotype × Diet** |
| Cholesterol | 0.019 ± 0.001 | 0.021 ± 0.004 | 0.018 ± 0.0010 | 0.020 ± 0.004 | 0.688 | 0.453 | 0.863 |
| *Fatty acid profile* | | | | | | | |
| 14:0 | 0.693 ± 0.129 | 0.756 ± 0.108 | 0.780 ± 0.117 | 0.808 ± 0.082 | 0.536 | 0.688 | 0.873 |
| 16:0 | 16.8 ± 1.29 | 17.4 ± 1.44 | 18.1 ± 0.295 | 19.9 ± 0.660 | 0.129 | 0.343 | 0.628 |
| 16:1c7 | 0.278 ± 0.039 | 0.262 ± 0.024 | 0.224 ± 0.008 | 0.204 ± 0.024 | 0.052 | 0.500 | 0.931 |
| 16:1c9 | 1.22 ± 0.225 | 1.06 ± 0.150 | 1.07 ± 0.128 | 1.26 ± 0.095 | 0.894 | 0.917 | 0.289 |
| 17:0 | 0.508 ± 0.044 | 0.402 ± 0.048 | 0.433 ± 0.024 | 0.374 ± 0.045 | 0.231 | 0.063 | 0.577 |
| 17:1c9 | 0.376 ± 0.034 | 0.343 ± 0.034 | 0.288 ± 0.009 | 0.269 ± 0.025 | 0.011 | 0.358 | 0.808 |
| 18:0 | 19.0 ± 0.686 | 18.6 ± 1.01 | 20.9 ± 1.25 | 20.6 ± 0.814 | 0.058 | 0.740 | 0.973 |
| 18:1c9 | 29.9 ± 0.380 | 30.7 ± 1.49 | 31.4 ± 1.03 | 32.7 ± 1.32 | 0.140 | 0.369 | 0.827 |
| 18:1c11 | 2.40 ± 0.158 | 2.15 ± 0.128 | 2.18 ± 0.072 | 2.29 ± 0.056 | 0.725 | 0.540 | 0.132 |
| 18:2*n*-6 | 14.5 ± 1.29 | 11.5 ± 1.32 | 13.8 ± 0.940 | 11.8 ± 0.484 | 0.816 | 0.031 | 0.632 |
| 18:3*n*-3 | 0.526 ± 0.079 | 0.371 ± 0.077 | 0.463 ± 0.072 | 0.356 ± 0.034 | 0.571 | 0.071 | 0.729 |
| 20:0 | 0.244 ± 0.046 | 0.261 ± 0.031 | 0.225 ± 0.026 | 0.301 ± 0.030 | 0.757 | 0.190 | 0.404 |
| 20:1c11 | 0.818 ± 0.102 | 0.905 ± 0.105 | 0.844 ± 0.060 | 0.969 ± 0.098 | 0.636 | 0.271 | 0.841 |
| 20:2*n*-6 | 0.750 ± 0.037 | 0.612 ± 0.045 | 0.511 ± 0.037 | 0.445 ± 0.043 | <0.001 | 0.021 | 0.384 |
| 20:3*n*-3 | 0.346 ± 0.028 | 0.206 ± 0.024 | 0.369 ± 0.041 | 0.318 ± 0.025 | 0.043 | 0.007 | 0.162 |
| 20:4*n*-6 | 2.46 ± 0.383 | 1.20 ± 0.283 | 2.45 ± 0.371 | 1.58 ± 0.112 | 0.558 | 0.004 | 0.546 |
| 20:5*n*-3 | 0.110 ± 0.024 | 0.130 ± 0.033 | 0.102 ± 0.016 | 0.055 ± 0.021 | 0.113 | 0.588 | 0.192 |
| 22:5*n*-3 | 0.044 ± 0.044 | 0.117 ± 0.039 | 0.175 ± 0.043 | 0.117 ± 0.030 | 0.113 | 0.851 | 0.113 |
| *Partial sums* | | | | | | | |
| ΣSFA | 37.2 ± 1.04 | 37.5 ± 1.90 | 40.5 ± 1.44 | 42.0 ± 1.12 | 0.014 | 0.538 | 0.659 |
| ΣMUFA | 35.0 ± 0.549 | 35.4 ± 1.75 | 36.1 ± 1.20 | 37.7 ± 1.45 | 0.221 | 0.440 | 0.649 |
| ΣPUFA | 18.8 ± 1.71 | 14.7 ± 1.82 | 17.8 ± 1.41 | 15.4 ± 0.239 | 0.924 | 0.041 | 0.588 |
| Σ*n*-6 | 17.7 ± 1.62 | 13.8 ± 1.75 | 16.7 ± 1.31 | 14.5 ± 0.578 | 0.925 | 0.039 | 0.526 |
| Σ*n*-3 | 1.03 ± 0.123 | 0.971 ± 0.153 | 1.11 ± 0.111 | 0.846 ± 0.074 | 0.848 | 0.193 | 0.404 |
| Unidentified | 9.02 ± 2.79 | 12.4 ± 4.20 | 5.63 ± 2.10 | 5.47 ± 1.43 | 0.092 | 0.583 | 0.546 |
| *Ratios* | | | | | | | |
| PUFA/SFA | 0.501 ± 0.037 | 0.392 ± 0.039 | 0.443 ± 0.039 | 0.353 ± 0.017 | 0.178 | 0.009 | 0.774 |
| *n*-6/*n*-3 | 18.1 ± 1.69 | 15.4 ± 1.95 | 15.3 ± 0.689 | 17.1 ± 0.738 | 0.750 | 0.807 | 0.176 |
| 18:1c9/18:2*n*-6 | 2.15 ± 0.220 | 2.82 ± 0.301 | 2.34 ± 0.188 | 2.82 ± 0.216 | 0.697 | 0.025 | 0.698 |

FAME = fatty acid methyl esters; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.
Σ SFA = sum of 14:0, 16:0, 17:0, 18:0 and 20:0; Σ MUFA = sum of 16:1*c*7, 16:1*c*9, 17:1*c*9, 18:1*c*9, 18:1*c*11 and 20:1*c*11; Σ PUFA = sum of 18:2*n*-6, 18:3*n*-3, 20:2*n*-6, 20:3*n*-3, 20:4*n*-6, 20:5*n*-3 and 22:5*n*-3; Σ*n*-6 = sum of 18:2*n*-6, 20:2*n*-6 and 20:4*n*-6; Σ *n*-3 = sum of 18:3*n*-3, 20:3*n*-3, 20:5*n*-3 and 22:5*n*-3.

order to study their lipid profile and further correlate those findings with membrane biophysical properties such as fluidity and permeability.

### 3.2. Membrane-saturated fatty acids are influenced by genotype

The lipid composition of adipose membranes from lean and obese genotypes fed on control and reduced protein diets is depicted in Table 2.

Total cholesterol concentration was unaffected by genotype or by diet ($P > 0.05$). The distribution pattern of the main fatty acid classes across groups showed a highest occurrence of saturated fatty acids (SFA) from 37% to 42%, closely followed by monounsaturated fatty acids (MUFA) from 35% to 38% and lastly, polyunsaturated fatty acids (PUFA) from 14% to 19%. The genotype affected the sum of SFA ($P < 0.05$), being the values higher in obese pigs. As for MUFA, only the 17:1*c*9 fatty acid was affected by genotype ($P < 0.05$) with decreased content in obese pigs. Whereas PUFA sum was not affected by the genotype, the 20:2*n*-6 fatty acid was reduced ($P < 0.001$) and the 20:3*n*-3 was increased ($P < 0.05$) in the obese genotype.

### 3.3. Membrane-polyunsaturated fatty acids are influenced by diet

The most important factor affecting the fatty acid profile of adipose membranes was the diet. In fact, the diet influenced the sum

of PUFA, in particular from the *n*-6 family, most at the expenses of 18:2*n*-6 fatty acid ($P < 0.05$) and in a smaller magnitude of 20:2*n*-6 ($P < 0.05$) and 20:4*n*-6 ($P < 0.01$) fatty acids (Table 2), the former in lower amounts in pigs fed a reduced protein diet. In addition, and following the same trend, the 20:3*n*-3 fatty acid was greatly affected by diet ($P < 0.01$). Regarding the fatty acids indexes, diet affected the ratios of PUFA/SFA and 18:1*c*9/18:2*n*-6 in opposite directions. While PUFA/SFA was diminished in reduced protein diets ($P < 0.01$), the 18:1*c*9/18:2*n*-6 was increased ($P < 0.05$), regardless of the genotype.

### 3.4. Fluidity of adipose membranes is dependent on genotype and affected by diet

The relative changes in fluorescence anisotropy of DPH and TMA-DPH in adipose membranes from each group are shown in Fig. 2. With the DPH probe, a significant decrease in fluorescence anisotropy was observed in obese pigs relatively to lean (panel A; $P < 0.05$). Moreover, reduced anisotropy was observed for pigs with reduced protein diets facing their controls (Panel A; $P < 0.05$). Being fluorescence anisotropy inversely related to membrane fluidity, adipose membranes from obese pigs are therefore more fluid. As for TMA-DPH probe, the values observed were similar across groups (Panel B; $P > 0.05$) indicating that the fluidity is mostly changed due to adjustments at the fatty acyl groups' level rather than at the membrane surface.

### 3.5. Permeability of adipose membranes is dependent on genotype

For permeability experiments, membrane vesicles in isotonic buffer were subjected to a hyperosmotic gradient with mannitol and the time course of vesicle volume change was used to calculate membrane water permeability (*Pf*) (Fig. 3A). As shown in Fig. 3B, a significant genotype effect was detected, obese pigs' membranes showing higher permeability values ($P < 0.05$). To investigate whether membrane water channels were involved in this permeability, we assessed the activation energy for water transport (*Ea*) by measuring *Pf* at different temperatures. The *Ea* obtained were similar among groups, ranging from 13.1 ± 0.6 to 14.7 ± 0.8 kcal/mol (54.8 ± 2.4 to 61.5 ± 3.2 kJ/mol) ($P > 0.05$). These relative high *Ea* values suggest that water permeation occurs mainly by diffusion through the lipid bilayer with no contribution of specific protein channels for transport and may therefore correlate with fluidity [22].

### 3.6. Correlation between lipid composition and fluidity

Some interesting Pearson's correlation coefficients were found between membrane's fatty acid composition and fluidity. The correlation of the fluorescence anisotropy obtained by the fluorescent probes among themselves was not significant ($r = -0.082$, $P = 0.731$), pointing to independent membrane biophysical measurements. In contrast, the fluorescence anisotropy from the DPH probe was positively correlated with 18:2*n*-6 fatty acid ($r = 0.442$, $P = 0.035$), PUFA/SFA ratio ($r = 0.483$, $P = 0.020$) and negatively with 18:1*c*9/18:2*n*-6 ratio (r = -0.461, P = 0.027). PUFA/SFA and 18:1*c*9/18:2*n*-6 were found strongly but inversely correlated ($r = -0.840$, $P < 0.001$).

## 4. Discussion

In this study, feeding pigs on a RPD increased the total fatty acid content of the subcutaneous adipose tissue, which is consistent with previous results [9,23]. In contrast, the P2 backfat thickness was not affected by the RPD, which is in agreement with numerous reports [6,8,24,25]. In addition, P2 fat thickness revealed clearly genetic effects on the pattern of fat deposition [6]. As expected, backfat was thicker in the genetically obese than in lean pigs, in accordance with the fact that traditional pig breeds, growing much slower than modern breeds, display higher backfat thickness [8].

Our results evidenced a difference in dietary protein requirement between the two genetic backgrounds. Protein and energy metabolism of growing pigs are markedly dependent on pig's genotype [24,26]. The nutritional requirements of the Alentejano pig, a slow growing, obese porcine breed, seem to differ from those of conventional or high-performing pigs with lower dietary protein requirements [6]. It has been suggested that fast-growing genotypes, such as the lean Crossbred, having a high capacity for muscle growth can use high protein and energy diets without accumulating fat [8]. This is in accordance with the herein results.

Contrary to the reported results on genetically obese mice and rats [3,27], it was recently proposed that the membrane lipid composition of obese healthy humans is different from lean but the fluidity remains unaltered [5]. These notions led us to investigate how adipose membranes from genetically obese pigs could be compared to a lean genotype regarding lipid composition, permeability and membrane fluidity. Furthermore, to our best knowledge, the effect of RPD on membrane lipid composition and biophysical properties has never been investigated.

Overall, all adipose membranes were found to be richer in SFA independently of breed or diet. The distribution pattern of the main fatty acid classes follows the trend SFA > MUFA > PUFA, which is similar to our previous results on fat depots from obese Zucker rats [15] and bovines [16].

An increase in membrane fluidity was observed in the adipose membranes from obese pigs, in accordance with the reported for a wide variety of cell membranes from obese mice and rats [3,27], which was explained by a lower total cholesterol concentration. Yet, in this study, the total cholesterol was unchanged by any factor under study. In addition, membrane incorporation of eicosapentaenoic acid (EPA, 20:5*n*-3) and docosahexaenoic acid (DHA, 22:6*n*-3) that have been demonstrated to significantly alter many basic properties of membranes, including acyl chain order [28], were detected in residual amounts thus not explaining the increased membrane fluidity and permeability. However, this variation may eventually be explained based on the observation that obese pigs had a significantly higher amount of total fatty acids (Fig. 1B). Therefore, even considering that there are no alterations in the total amount of cholesterol, a higher (phospho)lipids to cholesterol ratio may occur on the adipocytes membranes, inducing a decrease on the cholesterol-induced membrane ordering effect and leading to the increased membrane fluidity at the membrane core (lower DPH fluorescence anisotropy) observed for the obese pigs.

Regarding the adipose membrane lipid composition of pigs fed with different protein diets, as previously reported [25], no major differences were observed for the contents of 16:0, 18:0, 18:1*c*9 fatty acids, nor there were differences in the subsequent sums of SFA or MUFA. However, lower contents of linoleic (18:2*n*-6) and arachidonic acids (20:4*n*-6) were detected in pigs fed on RPDs, resulting in a lower PUFA sum, in particular from the *n*-6 family. Since dietary linoleic acid is the precursor for the synthesis of arachidonic acid, it was predictable that both fatty acids vary proportionally and in the same direction.

One consistent change reported in adipose membranes of genetically obese mice was an increase in the ratio oleic (18:1*c*9)/linoleic (18:2*n*-6) acids, a result probably due to an increased desaturation of linoleic acid [3]. This explanation fits well with the increased membrane fluidity observed in pigs fed on RPDs, for which this ratio reached the highest value. In addition, and re-enforcing this finding, membrane fluidity was correlated with 18:1*c*9/18:2*n*-6 ratio,

pointing to a clear compensatory mechanism to maintain membrane biophysical properties. Pigs fed on RPDs also shown a significantly higher content of total fatty acids (Fig. 1B), without changes on the total cholesterol levels. As previously discussed on the context of the comparison between lean and obese animals, this variation profile can also be the main reason for the increased membrane fluidity observed for animals fed on RPD when compared with their controls.

In conclusion, this study demonstrates that the genetic background clearly influences the backfat thickness in pigs, and that the restriction of dietary protein independently increases the total fatty acid content of the subcutaneous adipose tissue. Moreover, the genetic background in obese pigs plays a determinant role on the fluidity of adipose membranes by decreasing membrane rigidity at the core of the bilayer but not at a shallow position, closer to the water/lipid interface. Interestingly, the same effect was observed for pigs fed a RPD. Since relatively small changes in the concentration of membrane lipids, in the order of 10%, are significant enough to alter biophysical properties of the membrane [29], one can speculate that minor concentrations of some fatty acids, due to their geometric structure, may affect lipid packing within cellular membranes thus contributing to the experimentally observed increase in membrane fluidity and permeability. These findings likely reflect membrane adaptation to preserve optimal cellular function.

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