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Impact of diets rich in olive oil, palm oil or lard on myokine expression in rats†

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It has recently emerged that myokines may be an important skeletal muscle adaptive response to obesogenic diets in sedentary subjects (who do not exercise). This study aimed to assess the influence of various high fat (HF) diets rich in either crude palm oil (cPO), refined palm oil (rPO), olive oil (OO) or lard on the modulation of myokine gene expression in the gastrocnemius. Five groups of 8 rats were each fed HF or control diet for 12 weeks. Systemic parameters concerning glucose, insulin, inflammation, cholesterol, triglycerides (TG) and transaminases were assessed by routine methods or ELISA. Akt and ACC phosphorylation were analyzed by WB in the soleus. Mitochondrial density, inflammation, and the gene expression of 17 myokines and the apelin receptor (Apj) were assessed by qPCR in the gastrocnemius. We found that HF diet-fed rats were insulin resistant and Akt phosphorylation decreased in the soleus muscle, but without any change in *Glut4* gene expression. Systemic (IL-6) and muscle inflammation (NFκB and IκB) were not affected by the HF diets as well as TBARS, and ASAT level was enhanced with OO diet. Soleus pACC phosphorylation and gastrocnemius mitochondrial density were not significantly altered. The gene expression of some myokines was respectively increased (*myostatin* and *Il-15*) and decreased (*Fndc5* and *apelin*) with the HF diets, whatever the type of fat used. The gene expression of two myokines with anti-inflammatory properties, *Il-10* and *myonectin*, was dependent on the type of fat used and was most increased respectively with cPO or both rPO and OO diets. In conclusion, high-fat diets can differentially modulate the expression of some myokines, either in a dependent manner or independently of their composition.

Introduction

A diet that is too rich in fat ultimately leads to a range of chronic diseases (obesity, diabetes, cardiovascular diseases, cancers, etc.) that constitute public health problems whose importance will increase significantly in the coming years.^{1–5} Such diets can not only have deleterious effects on the liver or heart, but recently it has also been shown that the skeletal muscle could also be affected. High fat diets can lead to the following: a change in muscle strength or resistance⁶ which differs according to the type of fiber (slow or fast),⁷ a change

in fiber composition, muscle atrophy,⁸ a modification of the biogenesis and enzymatic content of the mitochondria,⁹ and an accumulation of intramuscular lipids which can ultimately lead to insulin resistance.¹⁰

Depending on countries and circumstances, diets may contain fats that have very different compositions. For example, the Mediterranean diet is based on olive oil, while palm oil is widely used in some African countries or in Malaysia. On the other hand, some people have a diet rich in animal meat. These different fats have a complex chemical composition that can vary not only according to the nature and degree of saturation of their triglycerides, but also according to the content of minor compounds represented mainly by polyphenols, phytosterols, carotenoids, vitamins, etc.

In past decades, it has been shown that the skeletal muscle is also an endocrine organ through the secretion of multiple myokines.¹¹ These myokines have extremely varied effects and can affect several organs including the bone, liver, adipose tissue and heart. They can have paracrine or endocrine effects depending on the case, and be involved in multiple pathologies including alterations in carbohydrate metabolism and

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even diabetes.^{12–14} For a certain number of these myokines, physical exercise is the trigger for the increase in their expression by the muscle.¹⁵ As a result, some myokines may even eventually be released into the bloodstream. On the other hand, there may be a close reciprocal relationship between the level of certain myokines and the metabolic status of a subject, for example during obesity or type II diabetes.^{16–18} In these pathologies, the subjects are generally sedentary and in that case, their metabolic status may become the determining factor in the variation of myokine expression in relation to their basal rate in the absence of physical exercise.¹⁹

A high level of saturated fatty acids in the blood has been shown to have a deleterious effect on the skeletal muscle by inhibiting both muscle fiber regeneration²⁰ and muscle protein synthesis,²¹ and also by increasing the level of ROS which leads to proteasome-mediated protein degradation.²² For some of these effects, the chemical structure of fatty acids seems to be an important parameter in the responses obtained.²³ A direct effect or consequence of the above-mentioned effects could be a change in the expression of the myokines produced by the muscle, which could lead to distant effects on other organs such as the heart, adipose tissue or bone.^{24–26}

Some studies on cellular models, like C₂C₁₂ myoblasts, have already shown that fatty acid treatments of skeletal muscle cells were able to modulate the expression of some myokines.^{27–31}

These results obtained on cellular models led us to compare the influence of different high-fat diets on animal models because high-fat diets constitute an important factor in the development of some chronic non-communicable diseases.³² Adipose tissue is strongly impacted by lipid-rich diets. Interestingly, it has been shown that there is a cross-talk between the muscle and adipose tissue that is mediated by adipokines and myokines.²⁶ Thus, the effects of diets could have a direct impact on the muscle or an indirect one through the action of adipokines secreted in response to these diets.

The aim of this study was to measure the impact of high-fat diets with different types of fats on myokine expression. We favoured an animal model that does not exercise, but which is not hindered in its movements, in order to be close to the living conditions of a sedentary subject. These animals were fed a high-fat diet enriched either with fats from plant origin (crude PO (cPO), refined PO (rPO), olive oil (OO)) or with those from animal origin (Lard). This is the first study that compares the effects of different types of edible fats on myokine expression in relation to insulin signalling and inflammation in a sedentary rat model.

Materials and methods

Animals and diets

Forty young male Wistar rats (Charles River, L'Arbresle, France) aged 6 weeks were housed under conditions of constant temperature (20–22 °C) and humidity (45–50%). A stan-

dard dark cycle (20.00–08.00 hours) was imposed. Rats were randomized into five groups and were fed for 12 weeks one of the five following semi-purified diets: (a) control diet containing 5% lipid as soybean oil (11% energy from fat) (Control), (b) HF diets (55% energy from fat) rich in cPO (with 2.5% soybean oil and 30% cPO), (c) HF diets rich in rPO (with 2.5% soybean oil and 30% rPO), (d) HF diets rich in OO with 2.5% soybean oil and 30% OO, and (e) HF diets rich in lard (with 2.5% soybean oil and 30% lard). cPO and rPO were supplied by SANIA company (Côte d'Ivoire), and OO (virgin olive oil) was bought in a supermarket and chosen for their large current consumption. Each group of rats was termed by the type of fat corresponding to its diet. Rats were given free access to water and food during the whole experiment and their body growth was determined weekly. During the first three months, the average amount of food ingested per animal was recorded and was around 28 g day⁻¹. These values do not vary significantly between groups. For the entire duration of the diet, we did not detect any animals whose feeding behaviour appeared to be different from that of other animals. High-fat diets usually reported in the literature contain 30–85% energy from fat in high-fat obesity-induced studies. The two most common fat levels for inducing obesity in rodents are 45 and 60% energy from fat. In our study, we chose an intermediate level of dietary fat between these two levels of about 55% energy from fat. A detailed composition of both experimental diets and oils is given in the ESI Tables 1 and 2,[†] respectively. All HF diets were supplemented with 2.5% soybean oil to maintain an adequate intake of linoleic acid and to avoid growth alterations resulting from linoleic acid deficiency. Two animals per cage were kept free to move around in cages of surface size 940 cm². These animals were not subjected to physical exercise. All experimental procedures were approved by the local ethical committee in Montpellier, France (Reference CEEA-LR-12002).

Intraperitoneal glucose tolerance test

Intraperitoneal glucose tolerance test (IPGTT) was performed seven days before rat sacrifice as previously described.³³ Blood glucose was measured immediately prior to the injection and 20, 30, 40, 60, 90, and 120 min afterward by using glucose strips and a commercial glucometer (AccuChek Active, Roche Diagnostics, USA). The area under the curve (AUC) was calculated by the trapezium method. The AUC values are expressed as g glucose per L per 120 min.

Rat sacrifice and sampling

One week after IPGTT, 16-hour fasted rats were anaesthetised with pentobarbital (Ceva Santé Animale, Libourne, France). Blood was collected and centrifuged at 1000 g for 10 min at 4 °C and plasma was collected and stored at –80 °C until analysis. Rat gastrocnemius was quickly removed; one piece was immediately frozen in liquid nitrogen and then kept at –80 °C until analysis. The left soleus and right soleus were immersed respectively in a 0.7 μM insulin solution or PBS without

insulin at 37 °C for 15 minutes. The muscles were then rinsed and frozen in liquid nitrogen.

Routine biochemical analyses

The blood contents of glucose, total cholesterol, HDL-cholesterol and triglycerides, ASAT and ALAT, were determined using standard methods on a COBAS automated analyser (Roche Diagnostics, France). TBARS was measured in both plasma and liver homogenates, according to the method of Sunderman *et al.*³⁴

Plasma insulin (Mercodia, France) and interleukin-6 (IL-6) (Fisher Scientific, France) levels were quantified with ELISA kits. Insulin resistance (IR) was evaluated by the homeostasis model of IR (HOMA-IR) using the following formula: [fasting plasma glucose level (mmol L⁻¹) × fasting plasma insulin level (mIU L⁻¹)]/22.5.³⁵

Reverse transcription and qRT-PCR analysis

Total RNA from the gastrocnemius was extracted with Trizol reagent (Invitrogen Life Technologies, Cergy Pontoise, France) by using a FastPrep-24 homogenizer (MP biomedical, France). Reverse transcription reactions were performed on 500 ng total RNA by using a reverse transcription Takara Kit (Takara Bio Europe, France) and RT-qPCR done using the LightCycler® 480 SYBR Green I Master (Roche Applied Science, France). The results were normalized to the *Rplp0* gene. The used primer sequences are given in Table 1.

Relative mitochondrial DNA quantification

Approximately 25 mg of the gastrocnemius muscle was finely cut and incubated with proteinase K overnight at 56 °C. After incubation with RNase A, DNA was extracted by using the NucleoSpin tissue kit (Macherey-Nagel, France). After appropriate dilution, qPCR was performed and the ratio of *Cox-1* (mitochondrial gene) to *18S* (nuclear gene) was then calculated.

Protein isolation and western blot analysis

Whole-cell protein lysates from the gastrocnemius were prepared in the following lysis buffer: 20 mM Tris pH 8, 50 mM DL-dithiothreitol, 2 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X100, 1 mM PMSF, 1 mM orthovanadate and 1% (v/v) of antiprotease cocktail (Sigma-Aldrich, Saint-Quentin-Fallavier, France). Proteins were resolved by SDS-PAGE and then transferred to nitrocellulose membranes using refrigerated Tris-glycine transfer buffer at 20 V overnight. The membranes were blocked in 5% nonfat milk in PBS (without Tween) for 1 h at room temperature. The membranes were then incubated overnight with primary antibody in blocking buffer. After washes in PBS/Tween under gentle agitation, the membranes were incubated for 45 min in the dark with the fluorescent-labeled secondary antibodies and finally quantified using the Odyssey infrared imaging system (LI-COR, Lincoln, USA). Primary antibodies: acetyl-CoA carboxylase (ACC), protein kinase B (Akt), phospho-protein kinase B (p-Akt, Ser 473), nuclear factor kappa B (NF-κB p65), inhibitor of nuclear factor kappa B

Table 1 Primer sequences used for real-time RT-PCR

Gene	Forward 5'–3'	Reverse 5'–3'
Pparα	CGGTGTGTATGAAGCCATCTT	GATCAGCATCCCGTCTTTTGT
Ppar-γ	CCCAATGTTGCTGATTACA	GGACGCAGGCTCTACTTTGA
RplpO	CACTGGCTGAAAAGGTCAAGG	GACTTGGTGTGAGGGGCTTA
Myostatin	ATCTGAGAGCCGTCAAGACTCC	CAGTCAAGCCCCAAAGTCTCTCC
Fndc5	GAGGTGCTGATCATCGTCGT	GAGCAAGCACTGAAAGGGTTT
Il-6	AGAGGAGACTTCACAGAGGATACC	AATCAGAATTGCCATTGCACAA
Lif	AAGTACCATGTGGGCCATGT	GGACCACCGCACTAATGACT
Fstl1	AGCGAGTGTGCCATGAAG	GAGTGGAAGAGATAGGGAAGC
Il-13	ATCGAGGAGCTGAGCAACAT	ATATCCTCTGGGTCTGTGG
Il-8	ACCGAAGTCATAGCCACACT	CGCCATCGGTGCAATCTATC
Igf-1	TGGTGGACGCTCTTCAGTTC	GCTTCAGCGGAGCACAGTAC
Decorin	CACTCCAGGAGCTTCGACTCCAC	AGTGGGTTGCCGCCAGTTC
Il-10	AGAGAAGCTGAAGACCCTCTG	AAAATCATCTTTCACCTGTCC
Il-15	ACTACCTGTGTTTCCTTCTCAAC	TTGGCCTCTGTTTTAGGG
Apelin	GGCTAGAAGAAGGCCAAC	CTGTGGAGTAAAGAGACTGG
Apj	GGTGCTTGCTGAGTTGCT	TAGATGGCAGGGATGAGA
Musclin	AAGGCATTCTCCGTGGACTT	CCAGCCGAGAGTCTGTCAA
Gdf-15	GTGTTGCTGGTGCTCTCGTG	CGGTGTTTCAATCTTCCCAG
Tgfb	GACCGCAACAACGCAATCT	GACAGCCACTCAGGCGTATC
Gdf-11	TTCCGCCAGCCACAAAGCAAC	ATGATCCAGTCCCAGCCAAAAG
Myonectin	TGTTGTTGAAAAGGTGCGGTA	TCAGCTTTAAATCCGCTTCA
Tfam	AGCTAAACACCCAGATGCAAA	GCCTTGAAGGGTTATCTTGG
Pgc1α	TGTGGAAGTCTCTGGAAGTGC	CGGCCGTAAGTGAGATGAAT
Cox-1	GGAGCAGTATTCGCCATCAT	CGAACCTCCGACTTTCGTTC
18S	GAGGTGAAATCTTGGACCGG	

Abbreviations: *Apj*: apelin receptor; *Cox-1*: cytochrome c oxidase 1; *Fndc5*: fibronectin type III domain-containing protein 5; *Fstl1*: follistatin-like protein 1; *Gdf-11,15*: growth differentiation factor 11,15; *Igf-1*: insulin like growth factor 1; *Il-6,8,10,13,15*: interleukin-6,8,10,13,15; *Lif*: leukemia inhibitory factor; *Pgc-1α*: (Ppargc-1a) peroxisome proliferator-activated receptor gamma coactivator-1a; *Ppar-α*: peroxisome proliferator-activated receptor alpha; *Ppar-γ*: peroxisome proliferator-activated receptor gamma; *RplpO*: large ribosomal protein; *Tfam*: mitochondrial transcription factor A; *Tgfb*: transforming growth factor β; 18S: ribosomal RNA18S.

(I κ B- α) (Cell Signaling, Leiden, The Netherlands); phosphoacetyl CoA carboxylase (pACC, Ser 79) (Merck Millipore, Guyancourt, France); glucose transporter Glut 4 (GLUT 4) (Abcam, Cambridge, UK); tubulin (Sigma-Aldrich, Saint-Quentin-Fallavier, France).

Statistical analysis

The results were expressed as means $\pm$ SD, $n = 7$ –8 animals per group. The sample size was calculated from the expected difference in the AUC of the IPGTT between the control and HF diet groups. We expected an AUC about 1.20 times higher in the HF groups than in the control group. A standard deviation of 15% of the higher value could be allowed. For a type 1 risk α of 0.05 and power (1- β) of 80%, the sample size required was eight per group. Statistical analyses were based on nonparametric tests: a Mann–Whitney U test was used to compare the control group to all high fat groups together (named in tables and figures as p1(HF/Ctrl)). A Kruskal Wallis test followed by a Dunn *post hoc* test was used to compare each high fat group to each other (named in tables and figures as p2(Fat/Fat)). The limit of statistical significance was set at $p < 0.05$. Statistical analyses were performed using StatView program (SAS Institute, Cary, NC, USA) and R program (R Foundation for Statistical Computing).

Results

Effectiveness of diets

The diet enriched with OO resulted in an increase in the percentage of liver monounsaturated fatty acids, while diets enriched with PO or lard resulted in an increase in the percentage level of liver saturated fatty acids (Fig. 1A). As expected, the weights of rats fed the high-fat diet were significantly higher than those fed the control diet. However, no significant differences were observed between the high-fat (HF) diets themselves (Fig. 1B). Together, these results validate the effectiveness of the diets.

All HF diets alter insulin signalling and glucose response

The plasma insulin level, HOMA-IR, and muscle expression of the glucose transporter GLUT4 were not affected by the diets (Tables 2 and 3).

As the muscle plays an important role in glucose metabolism through insulin signalling, this pathway was evaluated in the soleus by the level of Akt phosphorylation, which ultimately leads to the increase of membrane localization of the glucose transporter GLUT4. When compared to the control group, all HF diets led to a very significant reduction in the double pAkt/Akt ratio in the soleus in the presence (+I) or absence (–I) of preincubation with insulin; however, no differences between the HF diets themselves were found (Fig. 2A and B). In our study, we showed that a 12-week HF diet induces insulin resistance regardless of whether the fat is rather saturated or monounsaturated. As a result, we observed that the AUC of the IPGTT was elevated overall by HF regimes

with no significant differences among the HF diets themselves (Fig. 2C).

Blood and muscle lipid parameters

No significant changes in blood TG, total cholesterol, and HDL cholesterol were found for all groups (Table 2). TG storage in visceral adipose tissue was significantly higher in animals fed the high-fat diets, but TG storage in the liver was significantly higher only with olive oil diet (Table 2). No significant change of the oxidative stress parameter TBARS was found. In addition, no changes in the expression of acetyl-CoA carboxylase (ACC) phosphorylation (Fig. 3A and B) were found, suggesting that muscle lipid synthesis was not significantly affected by the diets. The gene expression of both transcription factors *Ppara* and *Ppar γ* was not significantly altered with the diets; however, a slight non-significant decrease for *Ppara* with the HF diet was observed (Table 3).

ASAT transaminase was significantly increased by the OO diet (ALAT being significantly increased by OO only compared to rPO and cPO), which could be a consequence of an effect on the liver.^{33,36,37}

No increase of muscle inflammation by any HF diet

Plasma IL-6 and gastrocnemius NF κ -B or I κ β protein levels did not change (Table 3), indicating that systemic or muscle inflammation was not enhanced by the HF diets.

Effects of diets on muscle mitochondria

Mitochondrial DNA (mtDNA) density was assessed by qPCR on the gastrocnemius by calculating the ratio of the *Cox-1* mitochondrial gene to the *18S* nuclear gene. No significant variation in mtDNA density could be found, while the gene expression of the transcription factor Tfam shows only a very weak upward almost significant change ($p = 0.058$) (Table 3). In our study, the level of expression of *Pgc1 α* , which corresponds to an activator of mitochondriogenesis and an inducer of the expression of irisin,³⁸ did not change.

Myokine expression in the gastrocnemius

The gene expression of 17 myokines and the apelin receptor (Apj) is presented in Table 4. *Myostatin*, *Il-15*, *fndc5/irisin* and *apelin* gene expression was significantly affected by fat level regardless of their composition, whereas only a nonsignificant change with $p < 0.1$ was observed for *Fstl-1*, *Il-8*, and *Igf-1* gene expression. The gene expression of other myokines was not influenced by fat. In the latter case, it was possible that the number of animals used was insufficient (the lack of test power) to conclude to a diet effect.

The gene expression of both myokines, *apelin* and *fndc5/irisin*, was significantly decreased between the control group and the HF diet groups altogether, while that of *myostatin* and *Il-15* was significantly increased (Table 4). However, for these four myokines, the fatty acid composition of the fats did not influence their expression.

Interestingly, the type of diet fat significantly regulated the gene expression of Il10 and myonectin, two myokines with

Fig. 1 Diets have an effect on rat weights and liver lipids. Rats were fed their respective diets for 12 weeks and liver fatty acids were quantified by gas chromatography from liver tissue (A), $p1(HF/Ctrl)$ = p -value of a Mann–Whitney statistical test when comparing all HF groups together with the control group (Ctrl), and $p2(Fat/Fat)$ = p -value of a Kruskal–Wallis statistical test when comparing each high fat group to each other and NS = non-significant ($p > 0.05$). Histograms corresponding to significantly different averages are topped with different letters. Rats were weighed every week (B), and the stars indicate a significant difference between the control and all high fat diet groups together. Results were expressed as mean values $\pm$ SD, $n = 7$ – 8 animals per group. Ctrl control diet, cPO crude palm oil diet, rPO refined palm oil diet, OO olive oil diet, NS nonsignificant, HF high fat.

anti-inflammatory properties (Fig. 4). The gene expression of *Il10* was not significantly changed when the control group was compared to all animals fed the HF diets. However, a significant increase in *Il10* gene expression level was observed with the cPO diet compared to both rPO and OO diets.

The gene expression of *myonectin* was dependent on both the level and the type of fat (Fig. 4). When compared to the control diet, the HF diet groups as a whole induced a significant increase of *myonectin* gene expression. When comparing

only fat diets to each other, a significant increase of *myonectin* gene expression level was observed with the rPO and OO diets compared to cPO diet. The level of *Il10* or *myonectin* gene expression with the lard-rich diet was intermediate between that of cPO and rPO or OO.

The gene expression of interleukins 6, 8, 13 and 15 was also analyzed (Table 4). *Il-15* gene expression was significantly increased in animals fed the HF diet, while that of *Il-8* only showed a decreasing trend in the same animals. Finally, no variation in *Il-6* and *Il-13* gene expression was observed.

Table 2 Effect of diets on systemic parameters from plasmas

	Control	cPO	rPO	OO	Lard	p1(HF/Ctrl)	p2(Fat/Fat)
Visceral AT (g)	28.6 ± 8.2a	44.7 ± 9.1b	46.4 ± 8.3b	42.7 ± 10.5b	45.9 ± 11.4b	0.0002*	NS
Blood TG, mM	1.18 ± 0.55	0.99 ± 0.30	1.21 ± 0.60	1.01 ± 0.31	1.01 ± 0.42	NS	NS
Liver TG, mg g ⁻¹	31.54 ± 9.89	29.11 ± 16.84a	29.38 ± 16.87a	61.95 ± 27.26b	38.92 ± 11.79a	NS	0.0275*
Total cholesterol, mM	2.00 ± 0.41	1.75 ± 0.42	1.89 ± 0.46	1.72 ± 0.28	1.55 ± 0.26	0.078◆	NS
HDL-Cholesterol, mM	1.49 ± 0.30	1.34 ± 0.24	1.48 ± 0.36	1.33 ± 0.20	1.06 ± 0.33	NS	NS
Blood TBARS, mM	5.12 ± 0.86	5.13 ± 0.60	5.26 ± 1.04	4.79 ± 0.88	4.90 ± 0.60	NS	NS
IL6 (pg mL ⁻¹)	14.7 ± 7.7	14.6 ± 8.1	13.1 ± 6.9	14.5 ± 11.5	14.3 ± 9.5	NS	NS
HOMA-IR	26.4 ± 27.5	39.9 ± 24.6	55.4 ± 30.2	31.6 ± 29.2	30.3 ± 23.5	NS	NS
Insulin (µg L ⁻¹)	3.05 ± 2.81	4.33 ± 2.59	5.90 ± 3.12	3.37 ± 2.85	3.73 ± 2.66	NS	NS
ALAT (UI L ⁻¹)	17.13 ± 10.86	19.25 ± 9.15a	22.75 ± 17.02a	49.88 ± 23.73b	26.38 ± 9.18ab	0.084◆	0.0080*
ASAT (UI L ⁻¹)	74.13 ± 8.61	73.88 ± 5.11a	64.29 ± 7.74a	101.38 ± 34.40b	74.13 ± 8.37a	NS	0.0034*

Rats were fed their respective diets for 12 weeks. The plasmas were processed as described in Materials and methods. The results were expressed as means ± SD, $n = 7-8$ animals per group. p1(HF/Ctrl) = p -value of a Mann-Whitney statistical test when comparing all HF groups together with the control group (Ctrl), and p2(Fat/Fat) = p -value of a Kruskal-Wallis statistical test when comparing each high fat group to each other. ◆ = indicates a change with $p < 0.1$, * indicates significance at $p < 0.05$, and NS indicates $p > 0.05$. Ctrl control diet, cPO crude palm oil diet, rPO refined palm oil diet, OO olive oil diet, TBARS thiobarbituric acid reactive substances, TG triglycerides.

Table 3 Muscle parameters

	Control	cPO	rPO	OO	Lard	p1(HF/Ctrl)	p2(Fat/Fat)
mtDNA density (qPCR)	1.00 ± 0.20	0.93 ± 0.21	0.98 ± 0.31	1.37 ± 0.59	0.86 ± 0.35	NS	NS
Tfam (qPCR)	1.00 ± 0.12	1.11 ± 0.15	1.18 ± 0.64	1.22 ± 0.21	1.10 ± 0.27	0.058◆	NS
Pgc1α (qPCR)	1.00 ± 0.20	1.23 ± 0.47	0.88 ± 0.16	0.87 ± 0.38	0.98 ± 0.23	NS	NS
Ppara (qPCR)	1.00 ± 0.28	0.89 ± 0.63	0.72 ± 0.23	0.68 ± 0.21	0.88 ± 0.39	0.073◆	NS
Pparγ (qPCR)	1.00 ± 0.26	0.88 ± 0.10	0.92 ± 0.25	1.00 ± 0.15	1.19 ± 0.39	NS	NS
NF-κB (WB)	1.00 ± 0.49	1.08 ± 0.7	1.23 ± 0.85	1.17 ± 0.88	1.39 ± 1.18	NS	NS
Iκβ (WB)	1.00 ± 0.70	0.80 ± 0.18	0.87 ± 0.14	0.86 ± 0.20	0.77 ± 0.32	NS	NS
Murf1 (qPCR)	1.00 ± 0.30	0.83 ± 0.27	0.82 ± 0.26	0.91 ± 0.22	0.90 ± 0.36	NS	NS
Atrogin (qPCR)	1.00 ± 0.28	1.20 ± 0.64	0.77 ± 0.24	1.08 ± 0.39	1.18 ± 0.78	NS	NS
GLUT4 (WB)	1.00 ± 0.53	0.82 ± 0.18	1.01 ± 0.21	1.00 ± 0.31	1.18 ± 0.41	NS	NS

Rat gastrocnemius was processed as described in Materials and methods for western blot or qPCR analysis. The control groups were set at 1. The statistical rules are identical to those described in Table 2. The abbreviations used for genes are the same as in Table 1. NFκB nuclear factor kappa B, Iκβ inhibitor of nuclear factor kappa B, Ctrl control diet, cPO crude palm oil diet, rPO refined palm oil diet, OO olive oil diet.

Discussion

The aim of this work was to evaluate the influence of 4 high-fat diets on some myokine gene expression in a rat model that did not exercise. Analyses performed on a number of systemic and muscle parameters allowed us to characterize the effects of these diets on this animal model. However, each fat used was a complex mixture of fatty acids with different levels of unsaturation, and also of minor compounds (polyphenols, phytosterols, carotenoids, vitamins, etc.). Recently, some studies have focused on the impact of the degree and nature of fatty acid unsaturation on muscle physiology. It was found by us and others that MUFA and PUFA may enhance muscle anabolism, insulin sensitivity, and mitochondrial beta oxidation and diminish inflammation, all these actions counteracting in some way the effects of obesity.³⁹⁻⁴¹

The palm oil refining process is well described in Mba *et al.*⁴² Schematically, the crude (red) palm oil rich in carotenoids is subjected to a chemical treatment to remove gums, then to a clay treatment to absorb pigments, then to a deodorization process and finally to a fractionation process that separates the palm solid stearin fraction from the liquid

palm olein. cPO and rPO have close fatty acid compositions of about 50% SFA, 40% MUFA, and 10% PUFA,^{42,43} that of lard is about 41% SFA, 47% MUFA, and 12% PUFA,²⁶ and that of OO is about 14% SFA, 77% MUFA, 9% PUFA.^{44,45} Olive oil has less saturated and more monounsaturated fatty acids than palm oil or lard. In addition to glycerides, oils but not lard contain several classes of minor components (polyphenols, carotenoids, vitamin E and others). cPO contains a higher quantity of carotenoids (500–700 ppm for cPO and 2.2 to 16.7 ppm for OO), but most of them are removed by the refining process in rPO.^{46,47} With regard to the polyphenols, OO (virgin) is the richest oil in polyphenols (around 100 ppm for OO and 50 ppm for cPO), and the refining process of cPO eliminates about 50% of those initially contained in cPO.^{48,49} These proportions were also confirmed in our laboratory on oils used in our diets (data not shown). As a consequence, the antioxidant capacity of rPO measured by the scavenging DDPH radical is also lowered by 50%.⁴⁸ In the case of vitamin E, due to the refining process, the content of alpha-tocopherol, alpha-, gamma- and delta-tocotrienols is rather higher in rPO than in cPO.⁴⁶ On the other hand, the content of alpha tocopherol in OO is comparable to that of palm oil, whereas its tocotrienol

Fig. 2 High fat diets alter glucose metabolism. The soleus muscles were incubated for 15 min either in 0.7 μ M insulin or in PBS alone before processing for western blot experiments. Representative western blot of either total Akt or phospho-Akt (Ser 473) with tubulin as a normalizing gene (A). The histograms of blot quantification (B). For each diet, IPGTT after a 16 h fasting was performed 1 week before sacrifice. The area under curve (AUC) of blood glucose level evolution during IPGTT was reported (C). The statistical rules ($p1$, $p2$) are identical to those described in Fig. 1A. The results are expressed as mean values $\pm$ SD, $n = 7-8$ animals per group. AUC area under curve, IPTG intraperitoneal glucose tolerance test, Akt protein kinase B, Ctrl control diet, cPO crude palm oil diet, rPO refined palm oil diet, OO olive oil diet, NS nonsignificant, HF high fat.

content is much lower.^{42,50} Other compounds such as phytosterols could also contribute to the biological effects of these fats, with olive oil being the richest, followed by lard and palm oil.⁵¹

In our study, it may seem surprising that blood TG levels do not increase significantly in animals fed a high-fat diet, but other studies corroborate this fact.^{39,52} Instead, the storage of TG is rather carried out in adipose tissue and in the liver, depending on the diet. As far as the muscle is concerned, a previous study showed that high-fat diets containing either 5% or 30% fat did not significantly increase the amount of fat in the muscle (whether the fat was saturated or not).³⁹

Tested HF diets did not induce inflammation and TBARS variation or significant changes at the mitochondrial level. With regard to inflammation, the situation was found different from that of the liver, where olive oil has been shown to promote an inflammation process.³³ In the study of Feillet-Coudray *et al.*,³⁹ a HF diet with either 5% or 30% of lipid also

led to a nonsignificant change of PGC1 α protein level in the muscle.

Myokines are represented by numerous peptides or proteins that are produced by the skeletal muscle and released in the circulation. They can act in an autocrine or paracrine fashion on the muscle, or by exerting an endocrine effect on distant tissues.^{12,14,16} A number of studies have examined the effect of exercise on myokine expression. Here, we instead focused on studying the effect of different high-fat diets on basal myokine expression, which generally corresponds to the situation experienced by obese sedentary subjects.

Gene expression of myokines *apelin*, *fndc5/irisin*, *myostatin* and *Il-15* depends on the level of fat but not on their fatty acid composition

Our results showed that the fatty acid composition of the fats did not influence the expression of all four myokines, *apelin*, *fndc5/irisin*, *myostatin* and *Il-15*. With regard to *apelin* expression, one can observe some discrepancy in the literature.

Fig. 3 Effect of high fat diets on muscle lipid synthesis. The soleus muscle incubated in insulin or in PBS as in Fig. 2. Representative western blot of either total ACC or phospho-ACC (Ser 79) in the soleus muscle from rats, with tubulin as a normalizing gene (A). Blot quantification from all animals (B). The statistical rules are identical to those described in Fig. 1A. The results are expressed as mean values $\pm$ SD, $n = 7-8$ animals per group. ACC acetyl coenzyme A carboxylase Ctrl control diet, cPO crude palm oil diet, rPO refined palm oil diet, OO olive oil diet, NS nonsignificant, HF high fat.

Indeed, in two sedentary mice studies, a decrease in the muscle mRNA expression of *apelin* was reported after 8 weeks of HF diet,⁵³ while no significant variation was observed after 22–28 weeks of HF diet.⁵⁴ In a rat study, a significant increase of *apelin* expression was observed in the gastrocnemius muscle but not in the soleus.⁵⁵ With regard to *Apj* expression, in our study, HF diet led only to an increasing trend of *Apj* expression, while in the Ji *et al.* study,⁵³ it led to a significant decrease of mRNA and protein expression. Apelin has been shown to be produced primarily by muscle and fat tissue with a probable paracrine/autocrine action.¹³ In mice fed a high-fat diet, it has been shown that treatment with apelin can improve insulin sensitivity and increase the oxidation capacity of fatty acids.⁵⁶

With regard to FNDC5/irisin, data related to its relationship with obesity are quite variable according to the authors. In the study of Mazur-Bialy *et al.*,⁵⁷ a 12-week HF diet led to a decrease of blood irisin in sedentary mice. Since most of the blood irisin comes from the proteolytic cut of the membrane

FNDC5 precursor which is abundantly expressed in the muscle,⁵⁸ it is likely that the muscle expression of *Fndc5* gene is also reduced. In the work of Seo *et al.*,⁵⁹ after a 10-week HF diet (with a concomitant garlic supplementation during 4 weeks), FNDC5 protein level remained unchanged in the rat gastrocnemius, whereas that of circulating irisin was higher. In the work of Rocha-Rodrigues *et al.*,⁶⁰ after a 17-week HF diet, a nonsignificant decrease of FNDC5 or circulating irisin was observed. In a work on obese or lean Zucker rats, irisin level was slightly but significantly increased in the diaphragm muscle in obese rats.⁶¹ Irisin is known to induce a better use of glucose by the muscle and to increase lipid oxidation through the transformation of white into brown adipose tissue.^{62,63} Also, in T2M patients, the level of irisin is found to be lower.⁶⁴ Therefore, these pieces of evidence suggest that the decrease in *apelin* or *Fndc5* gene expression would be associated with a deleterious effect induced by all HF diets regardless of their composition in fatty acids.

With regard to myostatin expression, Dong *et al.* found a significant increase in mouse gastrocnemius myostatin mRNA after a 12-week HF diet.⁶⁵ In addition, a higher level of myostatin expression and secretion by myotubes from obese women was also reported.⁶⁶ An interesting aspect of Dong's study is that the inhibition of myostatin level by the peptibody technique led to an increase in lipid oxidation and Akt phosphorylation and also an increase in the expression of the glucose transporter Glut-4 in muscle. In our study, the increase in myostatin that we observed was accompanied by a decrease in Akt phosphorylation. These results suggest that myostatin level could significantly influence lipid and glucose metabolism as well as insulin resistance.⁶⁷

Furthermore, in Dong's study, it was also shown that myostatin negatively regulates the expression of irisin. This is consistent with our results showing a negative correlation between *myostatin* and *irisin* gene expression (Fig. 5). It should be noted that in our study, a full analysis of the correlation matrix was performed, and only those that were highly significant ($p < 0.001$) were showed. It is worth noting that these correlations are independent of the diet since the points for each regime are regularly arranged along the lines. Interestingly, we also found a negative correlation between myostatin and apelin, and a positive one between myostatin and musclin (Fig. 5), showing a complex interplay between these myokines. Since irisin and apelin have concordant effects on lipid oxidation and glucose uptake, it seems logical that the correlations with myostatin are similar. Musclin is known to significantly increase muscle endurance by increasing mitochondrial biogenesis.⁶⁸ It may be possible that the increase in musclin that we observed would be a reaction aimed to counteract the deleterious effects of increased myostatin.

Another aspect of the action of myostatin is the regulation of muscle mass. While the inhibitory effect of myostatin on muscle growth is now well established,⁶⁹ conversely, treatment with apelin has been shown to increase muscle growth, an effect that may counteract the adverse effects of aging.⁷⁰ Thus, the increase in myostatin expression and the decrease in

Table 4 Myokine and Apj expression

	Control	cPO	rPO	OO	Lard	p1(HF/Ctrl)	p2(Fat/Fat)
<i>Myostatin</i> , qPCR	1.00 ± 0.58a	1.75 ± 1.02b	1.64 ± 0.83b	1.80 ± 0.54b	1.53 ± 0.77b	0.033*	NS
<i>Il-15</i> , qPCR	1.00 ± 0.32a	1.37 ± 0.49b	1.36 ± 0.22b	1.46 ± 0.48b	1.29 ± 0.61b	0.036*	NS
<i>Apelin</i> , qPCR	1.00 ± 0.31a	1.01 ± 0.71b	0.83 ± 0.40b	0.52 ± 0.27b	0.56 ± 0.37b	0.012*	NS
<i>Fndc5</i> qPCR	1.00 ± 0.30a	0.75 ± 0.49b	0.75 ± 0.32b	0.71 ± 0.26b	0.68 ± 0.32b	0.024*	NS
<i>Fstl-1</i> , qPCR	1.00 ± 0.33	1.48 ± 0.54	1.32 ± 0.56	1.21 ± 0.30	1.35 ± 0.36	0.073♦	NS
<i>Igf-1</i> , qPCR	1.00 ± 0.34	0.77 ± 0.22	0.80 ± 0.20	0.82 ± 0.20	0.79 ± 0.32	0.090♦	NS
<i>Il-8</i> , qPCR	1.00 ± 0.34	0.82 ± 0.24	0.75 ± 0.28	0.75 ± 0.49	0.70 ± 0.33	0.091♦	NS
<i>Il-6</i> , qPCR	1.00 ± 0.55	0.91 ± 0.48	0.73 ± 0.40	0.79 ± 0.32	0.73 ± 0.37	NS	NS
<i>Il-13</i> , qPCR	1.00 ± 0.94	2.56 ± 1.81	1.41 ± 0.73	1.45 ± 0.78	1.97 ± 1.32	NS	NS
<i>Lif</i> , qPCR	1.00 ± 0.46	1.81 ± 1.95	1.37 ± 0.76	0.86 ± 0.31	1.13 ± 0.53	NS	NS
<i>Decorin</i> , qPCR	1.00 ± 0.30	0.82 ± 0.12	0.92 ± 0.18	0.99 ± 0.34	0.79 ± 0.25	NS	NS
<i>Musclin</i> , qPCR	1.00 ± 0.80	1.87 ± 1.14	1.50 ± 0.96	1.38 ± 1.05	1.27 ± 0.82	NS	NS
<i>Gdf-15</i> , qPCR	1.00 ± 0.36	1.32 ± 1.41	0.92 ± 0.30	0.75 ± 0.34	0.95 ± 0.60	NS	NS
<i>Tgfb</i> , qPCR	1.00 ± 0.16	0.78 ± 0.18	1.02 ± 0.24	1.01 ± 0.23	0.83 ± 0.28	NS	NS
<i>Gdf-11</i> , qPCR	1.00 ± 0.33	1.19 ± 0.39	0.99 ± 0.35	1.12 ± 0.42	0.92 ± 0.26	NS	NS
<i>Apj</i> , qPCR	1.00 ± 0.32	1.29 ± 0.56	1.38 ± 0.65	1.45 ± 0.65	1.33 ± 0.39	0.085♦	NS

Rat gastrocnemius was processed as described in Materials and methods for qPCR analysis. The control groups were set at 1. The statistical rules are identical to those described in Table 2. The abbreviations used for genes are the same as in Table 1. *Ctrl* control diet, *cPO* crude palm oil diet, *rPO* refined palm oil diet, *OO* olive oil diet, *NS* nonsignificant, *HF* high fat.

Fig. 4 Gene expression of both myokines, *Il10* and *myonectin*, in the gastrocnemius muscle depends on the type of fat used in the diet. At the end of the 12-week diet, RNA was extracted from the gastrocnemius and retrotranscribed. Real time qPCRs were then performed on *Il10* (A) or on *myonectin* (B) myokines. The statistical rules (p1, p2) are identical to those described in Fig. 1A. The results are expressed as mean values ± SD, *n* = 7–8 animals per group. *IL-10* interleukine 10, *Ctrl* control diet, *cPO* crude palm oil diet, *rPO* refined palm oil diet, *OO* olive oil diet, *NS* nonsignificant, *HF* high fat.

apelin expression that we observed should logically lead to a decrease in muscle proteins. In our study, we observed a significant decrease of the relative gastrocnemius weight for all high fat diets in comparison with the control diet but not of the absolute weight (data not shown). The increase of myostatin gene expression that we observed was not accompanied by the activation of an atrophic process, since neither the expression of *Murf 1* nor *Atrogin-1* E3-ubiquitin-ligases was altered (Table 3), suggesting that the effect of myostatin could

be solely a metabolic effect. However, it is worth noting that, whereas muscle atrophy is generally associated with obesity,⁷¹ it is still difficult to relate such regulating effects to muscle mass. Indeed, during high-fat diets as well as for obese patients, the possible accumulation of lipids or water in the muscle makes it difficult to interpret the gross weight of these tissues.⁷²

The increase of *Il-15* gene expression that we observed may be rather beneficial since this myokine was shown to lower

Fig. 5 Myokine expression/correlation in the gastrocnemius according to diet fat. The correlation analysis of myokines was performed using Statview software. A complete correlation matrix was analyzed, but only those myokine pairs that are highly correlated ($p < 0.001$) are shown in this figure. Ctrl control diet, cPO crude palm oil diet, rPO refined palm oil diet, OO olive oil diet, NS nonsignificant, HF high fat.

white fat and to increase both mitochondrial activity and glucose uptake.^{73,74} However, in the case of *Il-15* and *Il-6* gene expression, our results are not in agreement with those of Shing.⁷⁵

Finally, the results reported in Table 4 indicate that 11 of the 17 myokines analyzed showed no variation in their gene expression whatever the level or the composition of fat. However, for some of them, a higher number of animals in the

groups could reveal significant differences by increasing the power of the statistical tests. This could be the case for *Igf1*, *fstl1* and *Il8* gene expression for which only a change associated to $p = 0.091$ was observed. It should be noted that the downward trend we observed for *Igf-1* gene expression in high-fat diets would deserve to be confirmed in a more in-depth study since this myokine has an important action on muscle growth.⁷⁶

Gene expression of anti-inflammatory myokines IL-10 and myonectin depends on fat diet composition

Such an effect of a cPO diet may be due to oil compounds other than fatty acids since there is little difference in the fatty acid composition between cPO and rPO. On the other hand, we noted that OO, which is nevertheless rich in polyphenols and vitamin E, did not induce an increase in *Il-10* gene expression. Since the content of carotenoids is much higher in cPO than in OO or rPO, one hypothesis could be that beta-carotene could be partly responsible for the effects of crude (red) palm oil. However, a recent study has shown that the treatment of rats with beta-carotene did not lead to an increase of liver protein IL-10.⁷⁷ Therefore, the gene expression of *Il-10* in the muscle after treatment with beta carotene would need to be studied afterwards. On the other hand, since the content of tocotrienol is even higher in rPO than in cPO and OO, a hypothesis based on vitamin E seems difficult to support. The same may be true for polyphenolic compounds whose content is higher in OO than in cPO or rPO. It is now well established that IL-10 has interesting anti-inflammatory properties.⁷⁸ Moreover, the hydrodynamic injection of the *Il-10* gene in mice prevented both obesity and glucose intolerance induced by a HF diet.⁷⁹ In a mice model with a muscle overexpression of IL-10, a 3-week or 16-week HF diet induced obesity but improved insulin sensitivity and reduced inflammation in the skeletal muscle.^{80,81} Dagdeviren *et al.* have also shown that muscle IL-10 overexpression protects mice against aging-associated muscle insulin resistance and inflammation.⁸² Therefore, the effect of a cPO diet on *Il-10* gene expression would in principle be beneficial for reducing muscle inflammation and insulin resistance induced by HF diets. In our study, we did not observe any significant reduction of the systemic level of IL-6 protein or muscle *Il-6* gene expression. One hypothesis could be that the induction of muscle IL-10 was not sufficient to result in significant effects on these parameters. Indeed, literature data indicate that the level of *Il-10* gene expression in the muscle, both at basal level and during exercise, is generally low, whereas a 80-fold increase in the circulating level of IL-10 can be induced by endurance exercise.⁸³

Myonectin, also known as CTRP15, was found recently as a nutrient responsive myokine secreted by the skeletal muscle in the circulation and involved in the metabolism of fatty acids in response to exercise.⁸⁴ We found that *myonectin* gene expression was significantly higher in rats fed the HF diet than those in the control group. This is consistent with the work of Peterson *et al.* (cited above) in which *myonectin* gene expression in the diaphragm was higher in obese than in lean

Zucker rats,⁶¹ but not in the soleus and plantaris from high fat diet-fed mice,⁸⁴ suggesting that sensitivity to this type of diet may be species-dependent.

Interestingly, when comparing fats, a significant increase in *myonectin* expression level was observed for the rPO and OO diets in comparison with cPO diet. It is worth noting that a similar effect of rPO and OO with respect to cPO can be observed for the gene expression of both *Il-10* and *myonectin*, with lard having an intermediate effect. As a consequence, the tocotrienol or polyphenol content of oils could not explain such difference in *myonectin* expression. Myonectin has been shown to share some properties with irisin. In particular, it promotes glucose and fatty acid uptake and oxidation in both liver and adipose tissue.^{84,85} In addition, myonectin has been shown to have a protective effect on the heart,⁸⁶ and recently myonectin has been suggested to be a potential marker of both prediabetes and type 2 diabetes.^{87,88}

The aim of our study was to analyze the impact of different diets on a fairly large number of myokines, in order to guide and target future research aimed at elucidating the mechanisms that could explain the most important effects observed, which are probably different for each myokine. Understanding the action of diets on myokine expression involves understanding the action of each compound contained in fats on skeletal muscle cells. With regard to fats, the triglycerides provided by chylomicrons after digestion, or those provided by VLDL are hydrolyzed either by lipoprotein lipase on the surface of capillaries or by triacylglycerol lipases in adipose tissue, thus releasing fatty acids into the circulation that will serve as energy fuel for the muscle cell which uses them preferentially. Therefore it would be relevant to study the action of individual fatty acids on the cells. Two types of effects will then be possible: those that are mainly due to the greater or lesser quantity of fatty acids and those that are due to the structure of the fatty acid. A small number of studies carried out on cultured muscle cell models have already begun to show interesting effects of fatty acids on both the expression of myokines and secondly on more global responses such as atrophy, proliferation and insulin resistance mechanisms. To begin with, it has been shown that the structure of the fatty acid is an important parameter since for example DHA and EPA have been shown to be protective against palmitate-induced atrophy of C₂C₁₂ mouse myoblasts.^{21,27} Interestingly, in the study by Granados *et al.*,²⁸ oleic acid did not affect the expression of IL15 contrary to its trans isomer elaidic acid, indicating that subtle differences in the structure of these fatty acids can have an impact on responses. In principle, several mechanisms could be imagined that could differentiate the effect of fatty acids according to their structure, like differences in the CD36 entry of the fatty acid,⁸⁹ or intracellular mode of action like reticulum endoplasmic stress.⁹⁰ Unfortunately, the mechanisms that could explain the impact of the fatty acid structure on cellular responses are still very poorly understood, and for the moment, studies are limited to the simple observation of induced effects. Another study by Sánchez *et al.*³⁰ investigated the effects of treating C₂C₁₂ cells with an equimolar mixture of

oleic acid and linoleic acid. This mixture induced an increase in IL6 mRNA and protein in the medium, but only IL15 mRNA and not protein. Such treatment showed no effect on irisin expression and production. The differences observed between these *in vitro* studies and ours indicate that studies in cell models and those in animal models are necessary and complementary. Indeed, a high-fat diet is a complex mixture of nutrients that induces a superposition of effects. On the other hand, the cross-talk between organs such as muscle and fat tissue, as mentioned in the Introduction, can also make the interpretation of responses even more complex.

Thus, the limitation of our study is that it does not presently make it possible to attribute to which compound contained in the fats tested the observed effects are due. In a future work, it would be very interesting to test the action of the individual components of oils.

Conclusion

Our study reveals that high-fat diets can differentially modulate the expression of myokines, either in a dependent manner or independently of their composition. Interestingly, among the myokines studied, two of them with anti-inflammatory properties were found to have a fat-dependent expression. These results highlight the interesting link between the lipid composition of diets and both the expression and effects of myokines on muscle function. However, since diet-induced variations in the expression levels of such myokines are rather small compared to the effects generally obtained with exercising subjects in other studies,⁹¹ added to the fact that different myokines may have antagonistic effects, one must be cautious in drawing conclusions about the actual metabolic consequences of these variations. Furthermore, given the close and reciprocal links between the action of myokines and adipokines, it would be very interesting in future work to assess the expression of adipokines as well.

Author contributions

Badia E, Cristol JP, Absalome M, and Camara-Cisse M supervised the entire study. Badia E, Cristol JP, Raynaud F and Gauze-Gnagne C analyzed and interpreted the data. Badia E, Gauze-Gnagne C, Morena M, and Koffi G were involved in the writing of the article. Coudray C and C did the diet design. Gauze-Gnagne C and Lauret C performed qPCR experiments, Raynaud F and Djohan F performed western blots experiments, and Djohan F managed the feeding of animals. Feillet-Coudray C and C handled animal sacrifice and organ harvesting.

Conflicts of interest

The authors have no conflict of interest to declare.

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