Early changes in tissue amino acid metabolism and nutrient routing in rats fed a high-fat diet: evidence from natural isotope abundances of nitrogen and carbon in tissue proteins

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Abstract

Little is known about how diet-induced obesity and insulin resistance affect protein and amino acid (AA) metabolism in tissues. The natural relative abundances of the heavy stable isotopes of C (δ¹³C) and N (δ¹⁵N) in tissue proteins offer novel and promising biomarkers of AA metabolism. They, respectively, reflect the use of dietary macronutrients for tissue AA synthesis and the relative metabolic use of tissue AA for oxidation or protein synthesis. In this study, δ¹³C and δ¹⁵N were measured in the proteins of various tissues in young adult rats exposed perinatally and/or fed after weaning with a normal- or a high-fat (HF) diet, the aim being to characterise HF-induced tissue-specific changes in AA metabolism. HF feeding was shown to increase the routing of dietary fat to all tissue proteins via non-indispensable AA synthesis, but did not affect AA allocation between catabolic and anabolic processes in most tissues. However, the proportion of AA directed towards oxidation rather than protein synthesis was increased in the small intestine and decreased in the tibialis anterior muscle and adipose tissue. In adipose tissue, the AA reallocation was observed in the case of perinatal or post-weaning exposure to HF, whereas in the small intestine and tibialis anterior muscle the AA reallocation was only observed after HF exposure that covered both the perinatal and post-weaning periods. In conclusion, HF exposure induced an early reorganisation of AA metabolism involving tissue-specific effects, and in particular a decrease in the relative allocation of AA to oxidation in several peripheral tissues.

Keywords: High-fat diets: ¹³C and ¹⁵N natural isotope abundance; Dietary nutrient routing; Protein and amino acid metabolism; Obesity; Insulin resistance

The consumption of high-energy diets rich in fat and sugars has increased worldwide, parallelly the worldwide rise in obesity and metabolic conditions such as type 2 diabetes mellitus and CVD(1,2). Despite high inter-individual variability in sensitivity to gains in weight and adiposity under high-fat (HF) diets in both humans(3–5) and rodents(6,7), HF over-feeding is known to favour obesity and insulin resistance(8) and rodents(6,7), HF over-feeding is known to favour obesity and insulin resistance(8) and metabolic disorders(9) depending on the tissue(18,23). The other reason is the unresolved debate about the possible alterations of the tissue kinetics of protein synthesis and degradation, with only few and conflicting findings about how these fluxes are affected by obesity and insulin resistance(15,16). For instance, proteolysis

Abbreviations: AA, amino acid; CHO, carbohydrate; HF, high fat; NF, normal fat.

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fluxes have mostly been measured at the whole-body level but seldom at the muscle level, with contradictory results, which generally reported no effects of obesity or insulin resistance, or in some cases a proteinolysis increase at the whole-body level\(^{(24-26)}\) or a proteinolysis decrease at the muscle level\(^{(27)}\). Such discrepancies probably reflect the limitations of classic tracer methods when investigating protein kinetics and their alterations\(^{(28)}\). In this context, new and alternative approaches are required in order to gain a more integrated insight into the partitioning and potential reallocation of AA between different anabolic and catabolic fluxes in tissues, so as to ultimately decipher the effects of obesity and insulin resistance on AA and protein metabolism.

To this end, the natural abundances of stable N isotopes in tissue proteins constitute new, relevant and promising biomarkers of AA metabolism in tissues. Dietary protein N is the sole source of body protein N, but body proteins are naturally enriched in the heavier stable N isotope (\(^{15}\)N) over dietary protein. This \(^{15}\)N bioaccumulation (\(\Delta^{15}\)N, also known as the trophic step or discrimination factor) is globally due to discrimination against this heavy isotope during N waste formation and particularly during urea production\(^{(29-33)}\). More precisely, \(\Delta^{15}\)N values vary among tissues as a result of differences in AA composition\(^{(32)}\), but also and mostly as a result of the particular AA metabolism in the tissue, because of the existence of several isotope effects associated with different metabolic pathways such as AA oxidation, as well as protein turnover in tissues\(^{(31,33)}\). Consequently, variations in different body fluxes of the catabolic or anabolic use of AA can result in \(\Delta^{15}\)N variations, and tissue \(\Delta^{15}\)N measurements are integrative biomarkers for the tissue-specific metabolic impact of nutritional and/or physiopathological conditions. For instance, in humans, variations in hair \(^{15}\)N natural abundance values have been shown to reflect changes in N metabolism during pregnancy\(^{(34)}\), nutritional stress\(^{(35)}\), anorexia\(^{(36-37)}\) and cirrhosis\(^{(38)}\), and \(\Delta^{15}\)N values in some animal tissues have been shown to reflect dietary protein quality and anabolic use efficiency\(^{(19,30-33,39-43)}\). More precisely, we have previously shown, by analysing simultaneous multi-tissue \(\Delta^{15}\)N measurements using a multi-compartment model, that tissue protein \(\Delta^{15}\)N values constitute integrated and interpretable biomarkers of tissue AA partitioning between protein synthesis and oxidation\(^{(31)}\).

In addition, the natural abundances of stable C isotopes in tissue proteins are also informative about the dietary origin of tissue AA. In contrast with N, the heavier, stable C isotope (\(^{13}\)C) only modestly bioaccumulates\(^{(32,42)}\), and if the C found in tissue proteins preferentially originates from dietary proteins it can also derive from dietary lipids and carbohydrates (CHO) that are used for non-indispensable AA synthesis\(^{(43-46)}\). Because dietary macronutrients generally differ in their natural \(^{13}\)C abundances, those in tissue proteins may reflect macronutrient use for AA synthesis and consequent routing to tissue proteins, which can vary in line with nutritional and physiopathological conditions\(^{(35,43-45,46-49)}\).

In this study, we used these new C and N isotopic biomarkers in order to better characterise the tissue-specific dysregulations of AA metabolism induced by exposure to an HF diet. C isotopic values were used as indicators of the relative utilisation of dietary macronutrients for tissue AA synthesis, and N isotopic values as indicators for the preferential metabolic use of tissue AA for anabolic \(v\). catabolic purposes. The study was carried out in rats exposed to an HF diet during the perinatal and/or post-weaning periods, as a model of early exposure to HF that is known to increase the risk for obesity and insulin resistance.

### Methods

#### Animals and diets

The animal protocol formed part of a larger study designed to explore the combined effects of diet and exposure to contaminants, whose initial results have already been presented at the 9th Copenhagen Workshop on Endocrine Disrupters – COW2017\(^{(50)}\). In this ancillary study, specific tissues were sampled for isotopic analyses of a subset of animals. Sprague–Dawley dams were pair-fed either a normal-fat (NF) or HF diet during gestation and lactation, from gestational day 8 onwards, while maintaining an equal N intake. After weaning, from postnatal day 21 onwards, male pups were pair-fed with either the NF or HF diet to include all combinations of perinatal and post-weaning dietary exposures (NF-NF, \(n\) 9; NF-HF, \(n\) 7; HF-NF, \(n\) 8; HF-HF, \(n\) 8). Dietary macronutrient compositions were adjusted daily according to the recommendations of Reeves \textit{et al.}\(^{(51)}\) in order to meet the protein and lipid requirements of gestation, lactation and early postnatal growth.

The macronutrient composition of the diets following the stabilisation of food intake (from postnatal day 35) is detailed in Table 1. Compared with the NF diet, the HF diet had the same protein content (16.7 wt%), a higher lipid content (35.3 wt%), and a higher carbohydrate content (48.3 wt%) owing to CHO substitution and a different CHO profile (67 \% maltodextrin and 33 \% sucrose \(v\). 100 \% maltodextrin) for its potential effect on obesity and insulin resistance. Food intake was measured daily throughout the study. On postnatal day 115, the body composition of the animals was measured using NMR-MRI-based technology (EchoMRI\textsuperscript{TM} 700) and oral glucose tolerance tests (OGTT) were performed in order to determine insulin sensitivity. In brief, a glucose bolus (1 g/kg) was

<table>
<thead>
<tr>
<th>Nutrients (wt%)</th>
<th>Normal fat</th>
<th>High fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>16.7</td>
<td>16.7</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>67.7\textsuperscript{†}</td>
<td>38.7†</td>
</tr>
<tr>
<td>Lipids</td>
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</tr>
<tr>
<td>Minerals</td>
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<td>3.5</td>
</tr>
<tr>
<td>Vitamins</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Isotopes (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\delta^{15})N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>–22.2</td>
<td>–22.2</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>–11.3\textsuperscript{‡}</td>
<td>–16.3‡</td>
</tr>
<tr>
<td>Lipids</td>
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<td>–30.4</td>
</tr>
<tr>
<td>Energy$</td>
<td>–13.8</td>
<td>–23.0</td>
</tr>
</tbody>
</table>

\textsuperscript{†} Weighted average of lipids and carbohydrates according to their C content and wt% (Equation (2)).

\textsuperscript{‡} 67 \% maltodextrin, 33 \% sucrose.

\$ Weighted average of lipids and carbohydrates according to their C content and wt% (Equation (2)).
administered orally after an overnight fast, and blood samples were taken from the tail vein before and 15, 30, 60 and 120 min after glucose administration. The blood glucose (OneTouch Ultra Easy glucometer; LifeScan) area under the concentration-time curve (AUC) was calculated using the trapezoid method. The rats were euthanised under pentobarbital anaesthesia on postnatal day 120 after an overnight fast. After an abdominal incision, blood was drawn from the abdominal aorta using a citrate-coated syringe. After centrifugation (12000 g, 2 min), plasma and erythrocytes were rapidly aliquoted, frozen in liquid N2 and stored at −80°C. Plasma insulin levels were measured by ELISA (Millipore). Different tissues were also rapidly collected and stored under the same conditions. The animals were handled according to the recommendations of the Regional Ethics Committee (accreditation number issued by the Ministry for Research: 00837-01).

Feed efficiency and liver protein anabolism efficiency

Feed efficiency was defined as the ratio of body weight at postnatal day 120 relative to cumulative food intake. Liver samples were homogenised and centrifuged (20000 g, 10 min) and the protein content was measured using a bicinchoninic acid assay kit (Bio-Rad). Liver protein anabolism efficiency (%) was defined as the ratio of liver protein content at the time of killing relative to cumulative protein intake over the post-weaning period.

Tissue protein extraction

Frozen tissues were cooled with liquid N2 and pulverised with a pestle and mortar. Tissue proteins were precipitated from 0.1 ml/mg of tissue). Twice, after centrifugation (10000 g, 20 min), the supernatant was removed and the protein pellets were washed with 5-sulfosalicylic acid (10%) and freeze-dried. Subcutaneous adipose tissue (1 g) weighed, freeze-clamped in liquid N2, and stored at −80°C until further analyses. Fat cells were also collected and stored under the same conditions. The animals were handled according to the recommendations of the Regional Ethics Committee (accreditation number issued by the Ministry for Research: 00837-01).

Isotopic analysis

The natural abundances of N and C stable isotopes in samples were expressed relative to standards (atmospheric N2 for 15N/14N and Vienna Pee Dee Belemnitite for 13C/12C, respectively) using the delta notation:

$$\delta^13C = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000$$

where δ is the parts-per-thousand difference in ratio of heavy to light isotopes (13C/12C and 15N/14N) in the samples (Rsample) and standards (Rstandard). We measured 15N and 13C in the proteins in the different tissues sampled, in the faeces, in lipids from adipose tissue and in dietary proteins, CHO and lipids. Isotopic measurements were performed with an isotope-ratio mass spectrometer (Isoprime; VG Instruments) coupled to an elemental analyser (EA Vario Micro Cube; Elementar). Tyrosine was used for calibration and drift correction. δ15N and δ13C values for tissue proteins and faeces were expressed relative to the δ value of dietary proteins using the capital delta notation ($$\Delta_{\text{protein}}$$):

$$\Delta_{\text{protein}} = \delta_{\text{protein}} - \delta_{\text{dietary}}$$

The δ13C of adipose tissue lipids was similarly expressed but relative to dietary lipid δ13C. For each diet, δ13C of non-protein energy sources was calculated as the average δ13C of lipids and CHO weighted by their C content and dietary occurrence as follows:

$$\Delta_{\text{protein}} = \delta_{\text{protein}} - \delta_{\text{dietary}}$$

where δ13C, δ13C and δ13C are, respectively, the δ13C values of dietary lipids, CHO and non-protein energy sources, %C and %L are dietary mass fractions of CHO and lipids and 0 and 0.768 are the reference C mass fractions of CHO and lipids, respectively.

Assessment of the contribution of dietary macronutrients to amino acid synthesis

The C in tissue proteins has various origins. Indeed, even though dietary proteins are preferentially routed to tissue proteins as AA, dietary lipids and CHO are used for the biosynthesis of non-indispensable AA and thus routed to protein synthesis, the degree of which varies according to the dietary macronutrient content(35,44,46). Therefore, by hypothesising an isotopic steady state owing to the entire renewal of tissue proteins since weaning, the δ13C of tissue proteins represents the sum of dietary macronutrient δ13C values weighted by their respective contributions to C in tissue proteins (routing coefficients), in addition to the difference in δ13C between diet and tissue proteins because of isotopic fractionation along metabolic pathways (the trophic step). In our study, the three dietary macronutrients differed in terms of their δ13C and the two diets in terms of their δ13C, because of the different CHO and lipid contents (Table 1). These δ13C signatures of macronutrients and diets allowed us to estimate the routing coefficients for each macronutrient and diet.
In a first step, the contributions of dietary proteins were derived from the following equation:

$$\delta^{13}C_{\text{T}} = p_d \times (\delta^{13}C_p + TSP) + (1 - p_d) \times (\delta^{13}C_{\text{E,NF}} + TSE_d)$$

$$= p_d \times \delta^{13}C_p + (1 - p_d) \times \delta^{13}C_{\text{E,NF}} + TSD,$$  \hspace{1cm} (3)

where $\delta^{13}C_{\text{T}}$, $\delta^{13}C_p$, and $\delta^{13}C_{\text{E,NF}}$ are, respectively, the $\delta^{13}$C values of proteins in a tissue, of dietary proteins and of dietary non-protein energy sources in the post-weaning diet d (d = NF or HF). $p_d$ and $1 - p_d$ are the proportions of protein and non-protein dietary macronutrients routed to C in tissue proteins (the protein and energy routing coefficients, respectively). TSP and TSE$_d$ are the specific trophic steps on dietary protein and energy, and TSD is the global trophic step (TSD = $p_d \times TSP + (1 - p_d) \times TSE_d$). To solve Equation (3), $p$ and TS were assumed to be equal across our diets having an identical and adequate protein content, as $p$ and TS have previously been shown to be extremely consistent over a spectrum of dietary compositions, except for deficient or excessive protein contents$^{(43)}$. By solving Equation (3) simultaneously for each post-weaning diet (NF and HF), we obtained an identifiable system of two linear equations and two unknowns, $p$ and TS, whose solution was as follows:

$$p = \frac{(\delta^{13}C_{\text{E,NF}} - \delta^{13}C_{\text{E,HF}} + \delta^{13}C_{\text{T,NF}} - \delta^{13}C_{\text{T,HF}})}{(\delta^{13}C_{\text{E,NF}} - \delta^{13}C_{\text{E,HF}})}.$$  \hspace{1cm} (4)

$$TS = \frac{(\delta^{13}C_{\text{T,NF}} - \delta^{13}C_{\text{E,NF}}) - (\delta^{13}C_{\text{E,HF}} - \delta^{13}C_{\text{T,HF}})}{\delta^{13}C_{\text{E,NF}} - \delta^{13}C_{\text{E,HF}}}.\hspace{1cm} (5)$$

The contributions of dietary CHO and lipids to C in tissue proteins were then estimated using the following equation:

$$\delta^{13}C_{\text{T}} = p \times \delta^{13}C_p + c_d \times \delta^{13}C_{\text{E}} + (1 - p - c_d) \times \delta^{13}C_4 + TS.$$  \hspace{1cm} (6)

Here, $c_d$ and $1 - p - c_d$ are the proportions of CHO and lipids routed, respectively, to C in tissue proteins (the CHO and lipid routing coefficients) and $\delta^{13}C_{\text{E}}$ and $\delta^{13}C_4$ are their respective $\delta^{13}$C values in diet d. Equation (6) was solved for $c_d$ as follows:

$$c_d = \frac{[\delta^{13}C_{\text{E,NF}} - \delta^{13}C_4 - TS + p \times (\delta^{13}C_4 - \delta^{13}C_p)]}{(\delta^{13}C_{\text{E,NF}} - \delta^{13}C_4)}.$$  \hspace{1cm} (7)

$$c_h = \frac{[\delta^{13}C_{\text{E,NF}} - \delta^{13}C_4 - TS + p \times (\delta^{13}C_4 - \delta^{13}C_p)]}{(\delta^{13}C_{\text{E,HF}} - \delta^{13}C_4)}.$$  \hspace{1cm} (8)

Assessment of tissue amino acid allocation between anabolic and catabolic pathways

We had previously investigated the causes and significance of $\Delta^{15}$N variations across tissues and diets using a multi-compartmental analysis of multi-tissue $\Delta^{15}$N measurements in rats, and showed that the $\Delta^{15}$N of a tissue protein varies as a function of $p_{\text{ox}}$, the proportion of tissue AA that is directed towards oxidation rather than used for protein synthesis$^{(31)}$. In particular, according to model predictions, a rise in $\Delta^{15}$N levels indicates an increase in $p_{\text{ox}}$ in all tissues except muscle, where in contrast it is indicative of a reduction in $p_{\text{ox}}$ because of its opposite sign of isotopic fractionation associated with AA catabolism. Indeed, in most tissues, AA losses for catabolic purposes favour $^{15}$N elimination and hence $^{15}$N retention owing to the isotope effects associated with transamination and liver deamination, whereas under catabolic conditions muscle releases some of the most $^{15}$N-enriched AA (such as Ala and Gln) into the bloodstream, which may favour $^{15}$N elimination from the muscle as previously discussed$^{(31)}$. Our model prediction for the $\Delta^{15}$N and $p_{\text{ox}}$ relationships in splanchnic tissues was further validated experimentally by combining AA tracers and arteriovenous balance methods in ruminants, where we showed that the $\Delta^{15}$N of plasma proteins, which are synthesised in the splanchnic bed and mainly in liver, linearly increases with the splanchnic AA metabolism and oxidation and the liver urea production$^{(29)}$. In the present study, the observed diet-induced $\Delta^{15}$N variations were interpreted in the light of these previous modelling$^{(30)}$ and experimental$^{(29)}$ results: a higher $\Delta^{15}$N was interpreted as indicative of a higher $p_{\text{ox}}$ in all tissues except the muscle, where it was interpreted as indicative of a lower $p_{\text{ox}}$.

Statistical analyses

Because this was an ancillary study during which thirty-two animals received the four dietary treatments studied here, the sample size was fixed. However, we reasoned that the study would be powerful enough with eight rats in each group, because this sample size allowed minimally detectable effect sizes of 0.3‰ using a t test on group means for both $\Delta^{15}$N and $\Delta^{15}$C values, assuming a level of significance of 0.05, a power level of 0.80 and a standard deviation of 0.2‰$^{(52)}$. Such a 0.3‰ effect size was small enough to enable the detection of diet-induced changes in (i) tissue AA metabolism using $\Delta^{15}$N values, according to our previous model predictions$^{(31)}$, and (ii) nutrient routing to tissue protein using $\Delta^{15}$C values, because 0.3‰ is <2% of the difference between dietary CHO and lipid $\Delta^{15}$C values. ANOVA using Tukey’s post hoc test was run under SAS (version 21) in order to determine differences between the groups in terms of body composition, metabolic parameters and $\Delta^{15}$N and $\Delta^{15}$C values ($\alpha = 0.05$). Pearson’s correlation coefficients between $\Delta^{15}$N and metabolic and body composition parameters of interest were evaluated for their significance using the Benjamini–Hochberg false discovery rate (FDR)$^{(53)}$. The FDR correction was used to control the rate of false discoveries among significant correlations, and correlations with an FDR ≤15% were considered as significant. The data are expressed as means and standard deviations.

Results

Body composition, insulin sensitivity and feed efficiency

Post-weaning nutrition was the only factor that affected the body composition and metabolic parameters of the animals. Compared with the NF post-weaning diet, the HF post-weaning diet (i.e. NF-HF and HF-HF v. NF-NF and HF-NF) induced a higher body weight (+28%, 529 (SD 50) g v. 412 (SD 27) g, $P < 0.0001$), with a higher fat mass (+178%, 121 (SD 38) g v. 44 (SD 16) g, $P < 0.0001$) and a higher lean mass (+10%, 354 (SD 35) g v. 322 (SD 36) g, $P < 0.05$). The following metabolic parameters were also

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[References and further details are omitted for brevity. The full text is a detailed study on the assessment of tissue amino acid allocation and its implications on dietary protein and energy routes, statistical analyses, and results related to body composition, insulin sensitivity, and feed efficiency.]
increased under the HF post-weaning diet: plasma insulin levels (+100%, 74 ± 8 (sd 46-88) v. 37 ± 5 (sd 12-3) pm, P < 0.01), glucose OGTT AUC (+19%, 824 ± 105 (sd 54-120min, P < 0.01), feed efficiency (+27%, 66-4 ± 4 (sd 4-8) v. 52 ± 2 (sd 3-4), P < 0.001) and liver protein anabolism efficiency (+18%, 1-98 ± 0.05 (sd 0-05) v. 1-69 (sd 0-10)-%, P < 0.05).

Natural abundances of stable carbon isotopes (Δ13C) in tissue proteins and dietary macronutrient use for amino acid synthesis

Only post-weaning nutrition affected Δ13C values in tissue proteins, faeces and adipose tissue lipids, as presented in Table 2. Δ13C values after HF post-weaning feeding were lower in all the pools sampled, regardless of perinatal nutrition (NF-HF and HF-HF were equal across the diets, whereas the CHO and lipid routings differed in rats fed the normal-fat or high-fat diet. The protein, CHO and lipid routings are the proportions of dietary protein, CHO and lipid C used for amino acid synthesis and consequently routed to C in tissue proteins, and the trophic step is the difference in δ13C between dietary and tissue proteins owing to isotopic fractionation along metabolic pathways (i.e. the coefficients p, c, 1 – p – c and TS of Equation (6), see the ‘Methods’ section). The protein routing and trophic step were assumed to be equal across the diets, whereas the CHO and lipid routings differed in rats fed the normal-fat or high-fat diet.

Table 2. Natural abundances of carbon stable isotopes in tissue proteins, faeces and adipose tissue lipids (Δ13C)*

<table>
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<tr>
<th></th>
<th>NF-NF</th>
<th></th>
<th>HF-NF</th>
<th></th>
<th>NF-HF</th>
<th></th>
<th>HF-HF</th>
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<th></th>
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<th>Post</th>
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<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>n</td>
<td>Mean ± SD</td>
<td>n</td>
<td>Mean ± SD</td>
<td>n</td>
<td>Mean ± SD</td>
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<td>Mean ± SD</td>
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<tr>
<td>Small intestine</td>
<td>2-8 ± 0-1</td>
<td>9</td>
<td>2-9 ± 0-1</td>
<td>8</td>
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<td>0-5 ± 0-1</td>
<td>6</td>
<td>1-0 ± 0-1</td>
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<td>Liver</td>
<td>3-2 ± 0-2</td>
<td>7</td>
<td>3-2 ± 0-1</td>
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<td>6</td>
<td>0-8 ± 0-2</td>
<td>5</td>
<td>1-2 ± 0-2</td>
<td>&lt;0.0001</td>
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<tr>
<td>AT</td>
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<td>6</td>
<td>3-0 ± 0-2</td>
<td>5</td>
<td>0-7 ± 0-1</td>
<td>5</td>
<td>0-7 ± 0-1</td>
<td>4</td>
<td>1-3 ± 0-2</td>
<td>&lt;0.0001</td>
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<tr>
<td>TA muscle</td>
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<td>2-0 ± 0-2</td>
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<td>1-3 ± 0-2</td>
<td>&lt;0.0001</td>
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<tr>
<td>G muscle</td>
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<td>5</td>
<td>0-4 ± 0-2</td>
<td>4</td>
<td>1-3 ± 0-2</td>
<td>&lt;0.0001</td>
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<tr>
<td>S muscle</td>
<td>2-9 ± 0-2</td>
<td>7</td>
<td>2-0 ± 0-2</td>
<td>6</td>
<td>0-3 ± 0-2</td>
<td>5</td>
<td>0-3 ± 0-2</td>
<td>4</td>
<td>1-3 ± 0-2</td>
<td>&lt;0.0001</td>
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<tr>
<td>Heart</td>
<td>2-9 ± 0-2</td>
<td>7</td>
<td>2-0 ± 0-2</td>
<td>6</td>
<td>0-3 ± 0-2</td>
<td>5</td>
<td>0-3 ± 0-2</td>
<td>4</td>
<td>1-3 ± 0-2</td>
<td>&lt;0.0001</td>
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<tr>
<td>Plasma</td>
<td>3-3 ± 0-2</td>
<td>8</td>
<td>3-0 ± 0-2</td>
<td>7</td>
<td>0-7 ± 0-2</td>
<td>5</td>
<td>0-7 ± 0-2</td>
<td>4</td>
<td>1-3 ± 0-2</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>1-8 ± 0-1</td>
<td>8</td>
<td>1-0 ± 0-2</td>
<td>7</td>
<td>0-0 ± 0-2</td>
<td>5</td>
<td>0-0 ± 0-2</td>
<td>4</td>
<td>1-3 ± 0-2</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Faeces</td>
<td>Δ13C (%)</td>
<td>5-9 ± 0-6</td>
<td>8</td>
<td>0-7 ± 0-3</td>
<td>7</td>
<td>0-5 ± 0-2</td>
<td>6</td>
<td>0-5 ± 0-2</td>
<td>5</td>
<td>1-3 ± 0-2</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>AT lipid Δ13C (%)</td>
<td>8-5 ± 0-9</td>
<td>7</td>
<td>8-6 ± 0-7</td>
<td>6</td>
<td>1-5 ± 0-2</td>
<td>5</td>
<td>1-5 ± 0-2</td>
<td>4</td>
<td>1-3 ± 0-2</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NF-NF, rats exposed to the normal-fat (NF) diet during the perinatal and post-weaning periods; NF-HF, rats exposed to the NF diet during the perinatal period and the HF diet during the post-weaning period; NF-HF-HF, rats exposed to the NF diet during the perinatal period and the NF diet during the post-weaning period; HF-HF-HF, rats exposed to the HF diet during both the perinatal and post-weaning periods; peri, perinatal diet; post, post-weaning diet; AT, adipose tissue; TA, tibialis anterior; G, gastronemius; EDL, extensor digitorum longus; S, soleus.

*a, b Mean values with unlike superscript letters were significantly different between groups (P < 0.001).

Table 3. Routing coefficients of dietary macronutrients to tissue proteins in rats fed a normal- or high-fat diet

<table>
<thead>
<tr>
<th></th>
<th>Normal fat</th>
<th></th>
<th>High fat</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Min–max</td>
<td>Mean ± SD</td>
<td>Min–max</td>
</tr>
<tr>
<td>Trophic step (%)</td>
<td>0.63 ± 0.25</td>
<td>(min–max 0.12 – 0.99)</td>
<td>74.2 ± 2.4</td>
<td>(min–max 71.8 – 78.9)</td>
</tr>
<tr>
<td>Protein routing (%)</td>
<td>17.6 – 24.5</td>
<td>13.7</td>
<td>1.3</td>
<td>10.8 – 15.0</td>
</tr>
<tr>
<td>CHO routing (%)</td>
<td>22.4</td>
<td>2.1</td>
<td>12.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Lipid routing (%)</td>
<td>3.4</td>
<td>0.3</td>
<td>2.7 – 3.8</td>
<td>9.5 – 13.3</td>
</tr>
</tbody>
</table>

CHO, carbohydrates.

The protein, CHO and lipid routings are the proportions of dietary protein, CHO and lipid C used for amino acid synthesis and consequently routed to C in tissue proteins, and the trophic step is the difference in δ13C between dietary and tissue proteins owing to isotopic fractionation along metabolic pathways (i.e. the coefficients p, c, 1 – p – c and TS of Equation (6), see the ‘Methods’ section). The protein routing and trophic step were assumed to be equal across the diets, whereas the CHO and lipid routings differed in rats fed the normal-fat or high-fat diet.
routing was 22.4 (sd 2.1)% and 13.7 (sd 1.5)% respectively (Table 3). Therefore, in rats fed the NF and HF diets, where dietary lipids contributed to 13 and 61% of C in non-protein sources (CHO+lipids), dietary lipids contributed to 13% and only 47% of non-protein C routed to tissue proteins (energy routing, see the ‘Methods’ section). It should be noted that, among the tissues studied, erythrocytes were outliers for all nutrient routing, with the highest value for protein routing and the lowest values for CHO and lipid routing (online Supplementary Table S2).

Fig. 1 shows $\Delta^{15}N$ values in faeces and proteins of the small intestine, liver, tibialis anterior and gastrocnemius muscles, and adipose tissue. In adipose tissue proteins, $\Delta^{15}N$ values were up to 0.5‰ higher in rats fed the HF diet after weaning than in those never exposed to the HF diet (NF-HF and HF-HF vs. NF-NF; $P < 0.001$ and $P < 0.05$, respectively), and also tended to
be 0.2% higher following perinatal exposure alone (HF-NF v. NF-NF; P<0.08). In faeces and proteins of the small intestine and tibialis anterior muscle, combined HF exposure during the perinatal and post-weaning periods (HF-HF) resulted in 0.7, 0.4 and 0.3% higher Δ¹⁵N values, respectively, compared with all the other conditions (all P<0.05). These diet-induced Δ¹⁵N variations indicated diet-induced differences in pox, the AA allocation between catabolic and anabolic pathways, in the corresponding tissues. By contrast, Δ²δ¹³C did not differ between the diet groups in proteins of the liver (3.7 (sd 0.2)%e), heart (4.9 (sd 0.3)%e), plasma (4.6 (sd 0.2)%e), and liver proteins were negatively associated with liver protein synthesis and subsequent routing to proteins, the extent of which is determined by the contribution of each dietary macronutrient to AA renewal in the different tissues (i.e. nutrient routing), whereas the use of Δ¹⁵N enabled us to characterise the partitioning of AA between oxidation and protein synthesis pathways in the different tissues (i.e. pox, the proportion of tissue AA that are directed towards oxidation rather than used for protein synthesis). More specifically, our tissue protein δ¹³C measurements were analysed with a new routing model, which allowed quantitative estimation of nutrient routing for each studied diet, whereas our tissue protein δ¹⁵N measurements were interpreted in the light of previous modelling and experimental results, which allowed qualitative estimation of the diet-induced differences in pox. A necessary condition to validate our interpretation of these δ¹³C and δ¹⁵N data was that sufficient time had elapsed since the last diet-shift – that is, δ¹³C and δ¹⁵N were no longer evolving but had reached their final equilibrium values with the post-weaning diet. In our study, nearly all body proteins had very likely reached their isotopic equilibrium at the end of the 100-d post-weaning period. Indeed, according to findings available in the literature, the times necessary to reach δ¹³C and δ¹⁵N equilibrium in rat tissues range from less than or about 50 d in small intestine, liver and plasma to about 100 d in muscles. The only exception was erythrocytes, for which a longer period (130-180 d) has been reported to be necessary to achieve isotopic equilibrium.

### Discussion

During this animal study performed in a carefully controlled dietary environment, we were able to reveal some of the tissue-specific changes that affect protein and AA metabolism in response to HF feeding depending on the exposure window (perinatal and/or post-weaning). This was made possible by the use of a new isotopic approach that consisted in measuring the natural abundances of δ¹³C and δ¹⁵N (δ¹³C and δ¹⁵N) in proteins from a large set of tissues. Using δ¹³C, we were able to determine the contribution of each dietary macronutrient to AA renewal in the different tissues (i.e. nutrient routing), whereas the use of δ¹⁵N enabled us to characterise the partitioning of AA between oxidation and protein synthesis pathways in the different tissues (i.e. pox, the proportion of tissue AA that

### Table 4. Pearson’s correlation coefficients of significant correlations between natural abundances of stable nitrogen isotopes in tissue proteins (Δ¹⁵N) and body composition and metabolic parameters†

<table>
<thead>
<tr>
<th>Body composition</th>
<th>Metabolic parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body weight</td>
</tr>
<tr>
<td>Faeces Δ¹⁵N</td>
<td>0.42*</td>
</tr>
<tr>
<td>Protein Δ¹⁵N</td>
<td></td>
</tr>
<tr>
<td>Small intestine</td>
<td>0.46**</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>0.40*</td>
</tr>
<tr>
<td>G muscle</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.45**</td>
</tr>
</tbody>
</table>

OGTT AUC, area under the concentration–time curve during oral glucose tolerance test; G, gastrocnemius; PAE, protein anabolism efficiency.

† All correlations were significant at a false discovery rate of 15%.

### Dietary macronutrient use for amino acid synthesis

Because the three dietary macronutrients generally differ in terms of their δ¹³C values and can all be used for AA synthesis, δ¹³C in tissue proteins reflects macronutrient use for AA synthesis and subsequent routing to proteins, the extent of which may vary in line with the macronutrient composition of the diet. Compiled data from studies involving a wide variety of dietary conditions suggest a contribution of about 75% of dietary protein to C in tissue proteins with, for instance, protein routing of 74% to bone collagen under a broad range of dietary compositions except for diets markedly deficient or excessive in protein. Here, protein routing was estimated to reach 74-2 (sd 2.4)% across all tissues, which is consistent with the mean values and minor inter-tissue variations recorded by the studies in the literature. Furthermore, taking advantage of the different δ¹³C signatures of dietary CHO and lipid, we were able to distinguish their respective routings for the first time. In the same way as for protein routing, we found few inter-tissue variations regarding the routing of CHO and lipid under each diet, although erythrocyte was an outlier with the lowest values for both CHO and lipid routing. This limited inter-tissue variability probably
reflects the fact that almost all the tissues studied are reliant on a mix of energy fuels during the postprandial period and more on lipids during the post-absorptive period. The only exception is erythrocytes, which are strictly dependent on glucose to fuel their energy metabolism because they lack mitochondria. However, as erythrocytes do not synthesise protein, the lipid and CHO routing calculated in erythrocytes will tend to reflect the mixed utilisation of lipid and CHO in bone marrow. Notwithstanding this, and as discussed above, our routing estimates for erythrocytes may have been partially biased because erythrocytes was the tissue most likely to be influenced by incomplete isotopic turnover.

Interestingly, we found that during NF feeding lipid contributed to the routing of non-protein nutrients in proportion with their dietary occurrence in non-protein nutrients (13%), whereas during HF feeding lipid contributed more to the routing of non-protein nutrients (47%) but less than in proportion with their dietary occurrence in non-protein nutrients (67%). One likely explanation is that dietary lipids cannot enter all the pathways of non-protein C routing to AA: some AA (Ala, Ser, Cys and to a lesser extent Gly) are synthesised from 3C precursors, which are mostly produced during glycolysis, whereas others (Asx, Glx, Pro and Arg) are derived from 4 and 5C precursors produced during the tricarboxylic acid cycle that is fuelled by both glucose and fatty acids via acetyl-CoA. Therefore, an increase in the proportion of lipid in the diet may lead to a proportional rise in lipid-derived C in Asx, Glx Pro and Arg, but not in Ala, Ser, Cys and Gly. In this context, HF-diet-induced changes to the routing of dietary CHO and lipid merely reflected differences in their dietary availability and possible access to the metabolic pathways of AA synthesis, rather than being the consequence of marked changes to the enzymatic activities that occur during adaptation to a new dietary environment.

In our young adult rats, the routing of dietary lipids to tissue proteins thus increased in line with recent, post-weaning, HF exposure, whereas we found no significant effect of earlier, perinatal, HF exposure. This suggests that perinatal HF exposure has no lasting effect on dietary nutrient routing to tissue proteins in later life. It does not preclude the possibility that HF exposure might have affected nutrient routing during the perinatal period in the same way as it did during the post-weaning period, but this could not be traced in tissue protein δ13C at the end of the 100-d post-weaning period. After such a time delay, routing effects limited to the perinatal period are no longer detectable. As discussed above, 100 d were indeed sufficient to reach isotopic equilibrium with the post-weaning diet in nearly all tissues by complete isotopic turnover since the perinatal period(45.54–57), so that the isotopic footprints of earlier effects on nutrient routing had been cleared.

**Tissue amino acid allocation between anabolic and catabolic pathways**

Although all dietary macronutrients contain C, only dietary proteins contain N and are the almost exclusive sources of the N found in tissue proteins. Because bioaccumulation of the heavy isotope 15N is known to occur, our tissue protein δ15N measurements were expressed as Δ15N in order to represent 15N enrichment over dietary protein. Our tissue protein Δ15N measurements ranged from 2 to 5‰; these values were in line with the few findings available in the literature on rodents(30,31,45.54–56.58). Furthermore, the diet-induced Δ15N differences that we observed in some tissue proteins point to diet-induced differences in pox, the relative proportion of AA entering the tissue that is directed toward catabolism. Indeed, according to our previous experimental and modelling results(29.31), a higher δ15N difference between tissue and dietary proteins (i.e. a higher Δ15N) indicates a higher pox in all tissues except the muscle, where it indicates a lower pox. Although we found that HF feeding systematically favoured AA synthesis from dietary fat in all tissues, we saw no associated changes in Δ15N, and thus in pox, in most of the tissues studied (seven out of eleven), namely three skeletal muscles (gastrocnemius, soleus and extensor digitorum longus), the heart, plasma, erythrocytes and liver. In contrast, the present results suggest that HF nutrition affected pox in three other tissues, namely the small intestine (and consequently faeces), tibialis anterior muscle and adipose tissue. In the protein of these three tissues, we observed an HF-induced increase in Δ15N, but according to different patterns: prolonged HF exposure covering both the perinatal and post-weaning periods was required for the small intestine and tibialis anterior muscle, whereas for adipose tissue post-weaning exposure alone was sufficient, and perinatal exposure alone tended to be sufficient, to attain a significant effect. According to our previous works(29,31), the HF-induced increase in protein Δ15N indicated with confidence a more preferential catabolic use of AA (i.e. a higher pox) in the small intestine and the opposite (i.e. a lower pox) in the tibialis anterior. In contrast, as adipose tissue had not been considered in our previous works, a metabolic interpretation of its increase in Δ15N levels was not straightforward. Because a compelling body of evidence supports the reduced expression and activity of enzymes involved in branched-chain AA oxidation (i.e. branched-chain aminotransferase and α-ketoacid dehydrogenase) in adipose tissue in the context of both genetic and diet-induced obesity and insulin resistance(18,39,60), higher Δ15N levels likely indicate less preferential catabolic orientation of AA metabolism in adipose tissue (i.e. a lower pox), as is the case in muscle.

Regarding the tibialis anterior muscle, the rise in its Δ15N level after prolonged HF nutrition – during both the perinatal and post-weaning periods – indicated a lower pox and thus a stronger preferential anabolic orientation of its AA metabolism. Higher protein synthesis in some muscles was likely in the specific context of our study, as it encompassed the early phase of the development of obesity where the gain in fat mass is accompanied by a concomitant gain in lean and muscle masses, as observed here and reported elsewhere(61). During this early phase, the gain in muscle mass has been shown to be associated with an increased fractional protein synthesis rate in the tibialis anterior muscle but not in the soleus muscle(61), which is exactly in line with our observation of a Δ15N increase in the former but not in the latter. Furthermore, limited evidence has suggested that branched-chain α-ketoacid dehydrogenase activity, the rate-limiting step in branched-chain AA oxidation, is reduced in the muscle of obese rodents(62). The rise in Δ15N levels in the protein of the tibialis anterior muscle after prolonged HF exposure could therefore result from an increased...
anabolic use and/or a decreased catabolic use of AA in this tissue. Such a reduction in the relative allocation of AA to oxidation in specific muscles may contribute to the elevation of circulating levels of branched-chain AA and some other AA during obesity and insulin resistance.

Regarding the small intestine, its increased Δ15N levels after prolonged HF nutrition – during both the perinatal and post-weaning periods – indicated a higher pox and thus a weaker preferential anabolic orientation of its AA metabolism. There is a paucity of data regarding the effect of HF feeding on intestinal protein metabolism. In a mouse model of obesity, some cellular changes have recently been observed in the small intestine epithelium that might impair the synthesis of proteins, and particularly those involved in the gut barrier function(63). Interestingly, the similarity between the Δ15N profiles of proteins in the small intestine and faeces reflected the important contribution of enterocyte shedding and intestinal mucins to the N flux entering the colon, and suggests that faeces might be considered as a more accessible proxy for the small intestine when seeking to determine isotopic biomarkers of diet-induced metabolic orientations.

Apart from the above-mentioned effects of the dietary group on Δ15N values in some tissues, we found significant correlations between the Δ15N values in other tissues and certain indicators of their protein anabolism efficiency, which depends on their efficiency of AA anabolic use for protein synthesis ψ. In particular, Δ15N levels in liver protein were negatively correlated with liver protein anabolism efficiency, and Δ15N in gastrocnemius muscle protein was positively correlated with lean mass, which is known to increase in line with muscle mass and muscle protein anabolism efficiency. Such inverse associations between protein Δ15N and efficiency of AA anabolic (ψ) and catabolic (v) use in liver and muscle were in line with our previous model predictions, and resulted from the opposite signs of isotopic fractionation associated with AA catabolism in liver and muscle(51,53). The negative association between liver protein Δ15N and anabolism efficiency had previously been confirmed experimentally in a metabolic tracer study in ruminants, where we have shown that the Δ15N of liver-synthesised plasma proteins increases linearly with liver AA oxidation and urea production(29), but the present study is the first to have demonstrated a positive association between muscle protein Δ15N levels and lean mass. Our results therefore indicate that in different splanchic and peripheral tissues Δ15N could be used as a valuable biomarker of protein anabolism in the considered tissue.

Strengths and limitations of the present study

By using a new isotopic approach consisting in analysing the natural abundances of 13C and 15N in tissue proteins, we were able to unveil the effects of HF feeding on the nutrient routing to tissue AA and on the relative metabolic use of tissue AA for oxidation v and protein synthesis p, respectively. The change in lipid content between diets was confounded with a change in CHO content and profile, so that the HF diet was enriched in fat but also in sucrose compared with the NF diet, a classic dietary pattern for mimicking the contrasted profiles of energy intake in humans while being able to induce obesity and insulin resistance with the fat- and sucrose-enriched diet(64,65). During our nutrient routing estimation by 13C data analysis, for identifiability reasons, the protein routing and trophic steps were assumed to be equal across the diets despite their different compositions. An identical protein routing between diets is very likely given their identical protein content, inasmuch as protein routing has been found notably stable – about 75% – under a broad range of dietary compositions, and our present estimate for protein routing was indeed strictly in line with these previous estimates(48,49,56). In contrast, an identical trophic step between diets of different CHO and lipid contents and profiles is a more questionable simplifying assumption that could have biased our estimates for the CHO and lipid routings, but probably only marginally. Indeed, numerically, our estimate for trophic step 15Ni was marginally affected and calculated in Equations (7) and (8) (e.g. a 25% variation in the trophic step value used in these equations resulted in an absolute difference of <1% in the calculated CHO and lipid routing values). Our routing estimates for erythrocytes may have also been biased because of incomplete isotopic turnover in this tissue, but only marginally as the erythrocytes isotopic values should have reached 80–90% of their final equilibrium values at 100 d, when considering that 130–180 d are required for complete isotopic turnover in this tissue(51,53). A last limitation relates to the interpretation of the diet-induced Δ15N differences in terms of pox, which was based on previous modelling and experimental findings rather than by a devoted modelling analysis of the present data set, because the data set at hand was insufficient for being analysed by such a complex multi-compartmental model as the one we previously developed(51).

In conclusion, our results show that HF feeding increases both dietary fat deposition in adipose tissues and dietary fat routing to the protein of all tissues via AA synthesis from tri-carboxylic acid cycle intermediates. In addition, our Δ15N data also support HF-induced changes to the relative orientation of AA metabolism towards oxidation v protein synthesis in three tissues, with more oxidation in the small intestine but less in the tibialis anterior muscle and adipose tissue.

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The authors’ contributions are as follows: H. F. and J.-F. H. formulated the research question in this ancillary study derived from a larger experiment designed by S. P. and D. Z. S. P. conducted the animal protocol and tissue sampling, O. L. M. conducted the tissue preparation and isotopic analysis and H. F. and N. P. conducted the isotopic data modelling and analysis in collaboration with J.-F. H. and F. M. H. F., J.-F. H., O. L. M. and F. M. analysed and interpreted the data. All authors were involved in the preparation of the manuscript and approved the final manuscript. H. F. had primary responsibility for the final content.

The authors declare that there are no conflicts of interest.
Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114518000326

References