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Randomized Control Trials

Increasing plant protein in the diet induces changes in the plasma metabolome that may be beneficial for metabolic health. A randomized crossover study in males

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SUMMARY

Background & aim: Dietary shifts replacing animal protein (AP) with plant protein (PP) sources have been associated with lowering cardiometabolic risk (CMR), but underlying mechanisms are poorly characterized. This nutritional intervention aims to characterize the metabolic changes induced by diets containing different proportions of AP and PP sources in males at CMR.
Design: This study is a 4-week, crossover, randomized, controlled-feeding trial in which 19 males with CMR followed two diets providing either 36 % for the control diet (CON-D) or 64 % for the flexitarian diet (FLEX-D) of total protein intake from PP sources. Plasma nontargeted metabolomes (LC-MS method) were measured in the fasted state and after a high-fat challenge meal at the end of each intervention arm. Lipogenesis and protein synthesis fluxes, flow-mediated dilatation (FMD) and gluco-lipidic responses were assessed after the challenge meal. Data were analyzed with mixed models, and univariate and multivariate models for metabolomics data.
Results: In both arms CMR improved with time, with decreased body weight (−0.9 %), insulin resistant (−34 %, HOMA-IR, Homeostatic Model Assessment for Insulin Resistance) and low-density lipoproteins (LDL)-cholesterol (−11 %). Diet had no effect on FMD or metabolic fluxes, but a trend ($0.05 < p \leq 0.1$) was observed for a stronger decrease in HOMA-IR and lower postprandial glucose after FLEX-D *vs* CON-D. The abundance of 21 and 37 metabolites differed between diets at fasted and fed states, respectively, including food intake biomarkers of AP (methylhistidine, eicosapentaenoic acid, hydroxyprolines) and PP sources (trigonelline, N-acetyl-ornithine). In fasted or fed states, indole acrylic acid and indole propionic acid, both products of tryptophan catabolism, were higher after FLEX-D *vs* CON-D, while the indispensable amino acids-related metabolites alpha-aminoadipic acid, hydroxymethylbutyric acids and propionylcarnitine were lower. In the postprandial state only, the ω-oxidation products dodecanedioic, tetradecanedioic and hexadecanedioic acids were higher after FLEX-D *vs* CON-D.

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Conclusions: Despite little changes in risk factors after 4 wk, this study evidenced subtle metabolic adaptations in amino acids and lipid metabolism and gut microbiota activity occurring after higher PP source intake that may be beneficial to CMR.
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Abbreviations list

AP	animal protein
ASR	absolute synthesis rates
AUC	area under the curve
CM	cardiometabolic
CON-D	control diet
CRP	C-reactive protein
DNL	*de novo* lipogenesis
FLD	flowmetry by faser doppler
FLEX-D	flexitarian diet;
FMD	flow mediated dilation
FSR	fractional synthesis rate
LiMM-PCA	linear mixed model-principal component analysis
NEFA	non-esterified fatty acids
PP	plant protein
PS	protein synthesis
TG	triglycerides
total-C	total cholesterol
TMAO	trimethylamine-oxide
WC	waist circumference

1. Introduction

The transition toward more sustainable diets is advocated to promote both planetary and human health and involves favoring plant protein (PP) sources, including whole grains, legumes, nuts and seeds, over animal protein (AP) sources, especially red and processed meat [1–4]. Epidemiological studies have shown beneficial effects of PP-rich diets on cardiovascular and metabolic disease risks [5–9], some of the leading causes of morbi-mortality largely preventable by diet [10]. Nevertheless, there is a lack of highly controlled intervention studies designed to specifically investigate the impact of a protein shift towards more PP and less AP on cardiometabolic (CM) risk factors and overall metabolism, especially in real-life settings where diverse AP and PP sources are supplied within whole diets [11,12]. This explains the currently limited knowledge regarding the metabolic adaptations and impacts on disease-related processes of such a dietary shift, and warrants research to reduce the gap between mechanistic explanations and epidemiological data. Among the hypotheses available in the literature, the induced metabolic changes and beneficial health effects could be partially mediated by the differences in amino acid (AA) profile between AP and PP [12]. In this regards, in a preclinical study, we did show that PP protect against high-fat-induced metabolic disorders by increasing AA reshuffling through transamination and routing of carbohydrates and lipids towards dispensable AA synthesis [13]. On the other hand, a more integrative analytical approach like metabolomics could provide more detail on the mechanisms and potential metabolic reorientations related to this dietary shift [11]. Two cross-sectional studies examining the specific association between protein sources and the plasma metabolome reported that many AA and derivatives, acylcarnitines, organic acids and lipids were differentially associated with AP and PP intakes [14,15]. The effect of a shift from AP to PP on the plasma metabolome was also investigated in two interventions, respectively showing differences in AA and AA derivatives after a soy-based diet [16] and a better lipid profile after a vegetarian diet [17,18], compared to AP-rich diets with similar total energy intake and macronutrient distribution.

These studies focused on post-absorptive conditions and did not assess the postprandial response or metabolic shifts occurring during this critical period. Analyzing changes in the plasma metabolome during the postprandial response to challenge meals is a way to characterize phenotypic flexibility [19], and to evidence subtle metabolic dysregulations between healthy and unhealthy individuals that provide valuable insights into the development of CM diseases [20]. While the composition of challenge meals used throughout the literature is highly variable, mixed meal containing all macronutrients with a high lipid fraction have been reported to be particularly effective in challenging phenotypic flexibility [20] and evaluating vascular function due to their proinflammatory and pro-atherogenic effects [21].

Here, we aimed to characterize and compare the metabolic changes induced by a dietary shift from two-thirds AP, as currently observed in most countries with Western-type diets [22,23], to a flexitarian diet with one-third AP of the total protein intake. This reduction, although substantial, allows to keep most of categories of animal products in the diet, thereby requiring less drastic dietary changes while promoting dietary diversity.

To do so, we enrolled 19 males at higher CM risk in an intervention study with a high level of dietary control, and assessed the changes in the plasma metabolome in both the post-absorptive state and the postprandial (in response to a high-fat challenge meal) state, as the primary outcome. To further characterize the diet effect on CM health and overall metabolism, we assessed vascular function and estimated metabolic fluxes, such as protein synthesis (PS) and *de novo* lipogenesis (DNL), and assayed circulating markers of glucose and lipid homeostasis, inflammation and endothelial function (adhesion molecules, nitrites, and nitrates) as secondary outcomes.

2. Subjects and methods

2.1. Participants

All recruited participants were males aged 25 to 55 y, overweight or obese (BMI 25–35 kg/m^2), weighing >73 kg, with an enlarged waist circumference (WC) (≥94 cm) and who fulfilled at least one of the following conditions: high fasting plasma triglyceride (>1.49 g/L); high fasting glycemia (≥5.6 mmol/L); low fasting HDL-cholesterol (<1.03 mmol/L); high systolic (≥130 mmHg) or diastolic (≥85 mmHg) blood pressure. Exclusion criteria, including diabetes, hypertension, cardiovascular and gastrointestinal

diseases, are detailed in Supplemental Text S1. Participant eligibility was assessed during the baseline visit by a physician and a trained dietician. Procedures for this study were in accordance with the ethical standards of the Helsinki Declaration. Participants were recruited from the free-living population in the metropolitan area of Clermont-Ferrand (France) through various communication channels, including social networks, mailing lists, newspapers, and flyers. The ethical committee (the Committee for the Protection of Individuals Sud-Méditerranée I, France) approved the main study protocol on 13th December 2019 (RBHP 2019 PICKERING 2). Minor modifications were further approved on 26th November 2020 and 13th July 2021, mostly to broaden inclusion criteria due to recruitment difficulties (see Supplemental Text S1 and Supplemental Table S1). The study was registered at the French National Agency for the Safety of Medicines and Healthcare Products (2019-A02447-50) and clinicaltrials.gov (NCT04236518). All study participants provided written informed consent. This study took place at Clinical Research Centre/Clinical Pharmacology Centre, University Hospital, Clermont-Ferrand, France from August 2020 to August 2022.

2.2. Study design

The PROVEGOMICS study was based on a two-period randomized crossover, open, monocentric, controlled-feeding design. Participants followed a control diet with predominantly AP sources (CON-D) and a flexitarian diet with predominantly PP sources (FLEX-D) in a randomized order (Fig. 1). The primary outcome was change in the plasma metabolome. The two intervention periods lasting 4 weeks each were separated by a 2-week wash-out period, which could be adjusted (1–5 weeks) if scheduling conflicts arose. Visits to the center were on days 1, 14, 28, and 29 of each intervention period. Participants were instructed to avoid strenuous physical activity the day before a visit, to fast overnight and not to smoke on the morning of the visit scheduled at ~8.00 am. Within each intervention period, we measured body weight and WC on days 1, 14 and 28, and body composition (assessed by impedancemetry, QuadScan, BodyStat) on days 1 and 28. Post-absorptive (fasted) blood samples were also collected on days 1, 14 and 28. The visit at day 28 included a postprandial (fed) test with a 6-h follow-up after a high-fat challenge meal (900 kcal; 80 E% lipids, 13 E% carbohydrates, 7 E% proteins) identical for all participants and intervention periods, as detailed in Supplemental Text 1. Blood samples were collected ~90 min before (t-90) and immediately before (t0) the consumption of the challenge meal and at 30, 60, 120, 240 and 360 min after. PS and DNL were assessed by isotopic labeling using ^2H_2O tracing [13]. Participants consumed a priming dose of ^2H_2O at 99.8 % (DLM-2259-250-0, Cambridge Isotope Laboratories) of 5 mL/L of body water (estimated by Chumlea equation

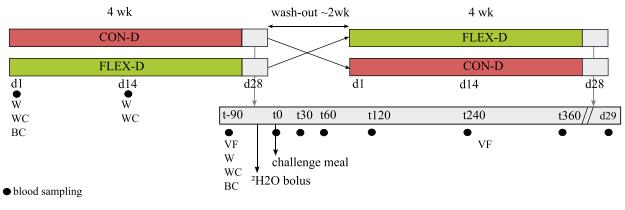

Fig. 1. Design of the two-period, randomized, crossover, four-week controlled-feeding intervention with a control (CON-D) or a flexitarian diet (FLEX-D). At the end of each intervention period, participants underwent a postprandial exploration following a high-fat challenge meal (900 kcal, 80 % lipids). Time points t-90, t0, t30, t60, t120, t240, t360 are minutes relative to challenge meal intake. BC, body composition; VF, vascular function; W, weight; WC, waist circumference.

[24]) in 4 oral boluses over a 1-h period immediately before the meal ingestion, followed by free access to 0.5 % ^2H drinking water until the end of the visit at ~5.00 pm to maintain the ^2H enrichment of body water. Endothelial function was measured in the post-absorptive state 90 min before meal ingestion and in the postprandial state 255 min after meal ingestion. Participants had an afternoon snack at the end of the follow-up before leaving the center and consumed a standardized dinner at home. The next morning (d29), participants came back to the center to give a post-absorptive blood sample before beginning the wash-out period. The visit sequence was repeated in the second period of the intervention with the other diet. All blood samples were immediately centrifuged after collection (10 min, 3000 *g*, 4 °C), plasma was frozen in liquid nitrogen, and stored at −80 °C until further analysis.

2.3. Study diets and compliance monitoring

During the intervention, three times a week dietary-controlled lunches and dinners freshly prepared from a central kitchen were delivered to participants. Diets, based on a weekly rotation, were designed to provide either 35 % (CON-D) or 65 % (FLEX-D) of total protein intake from PP sources. Diets included diverse protein sources such as red meat, poultry, dairy products, fish, eggs, grains and legumes (Supplemental Table S2), without excluding any of the major protein sources. Dietary advice on snacks and breakfast was provided to limit intake of protein-rich foods and prevent any major changes in dietary habits between intervention periods (Supplemental Text S1). To assess dietary compliance, participants reported the eaten proportion of the food provided, any deviation from a standardized breakfast, and all other food and beverages consumed. A three-day weighted food record was conducted between the baseline visit and the beginning of the first intervention period to estimate actual food consumption. The daily dietary data were analyzed using Nutrilog Online (Nutrilog) and the French food composition database CIQUAL.

2.4. Analyses

2.4.1. Plasma biochemistry

Plasma concentrations of insulin, glucose, triglycerides (TG) and non-esterified fatty acids (NEFA) were measured in all post-absorptive blood samples (d1, d14 and d28 t0) and postprandial plasma samples (d28 t30, t60, t120, t240, and t360 min). Lactate, urea, uric acid, creatinine, total cholesterol (total-C), HDL-C and LDL-C were also measured in all the post-absorptive blood samples. Insulin concentration was assessed using a commercial ELISA kit (10-1113-01, Mercodia) and the NEFA concentration using a commercial kit (NEFA-HR (2), Fujifilm Wako), while the remaining measurements were conducted using appropriate kits on an automated analyzer (ABX Pentra C400, Horiba Medical). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as previously defined [25]. In post-absorptive states at the beginning and the end of each intervention period (d1 and d28 t0), concentrations of plasma cytokines (CCL2, IL-1β, IL-10, IL-6, TNF-α) and adhesion molecules (P-selectin, E-selectin, VCAM-1, ICAM-1) were assayed by multiplexed immuno-assays (LXSAHM-09, Bio-techne R&D Systems). High sensitivity C-reactive protein (CRP) concentration was measured by ELISA (QK1707, Bio-techne R&D Systems), and nitrite, nitrate and malondialdehyde were measured using mass spectrometry-based methods as previously described [26,27]. Inter and intra-assay CV values obtained for the various biochemistry parameters are presented in Supplemental Table S3.

2.4.2. Measurements of vascular function

Vascular endothelial function was assessed by the non-invasive flow mediated dilation (FMD) technique at the end of each intervention period (d28) in both post-absorptive and postprandial states. Measurements were made on the left brachial artery above the antecubital fossa by following a procedure fully compliant with the reference method [28], using a high-resolution ultrasound system with a 7–12 MHz linear array 190 transducer (Vivid S5, GE Healthcare, France), a mechanical arm device (Vascular Imaging, Amsterdam, Netherlands) for precise three-dimensional movements, and automated edge-detection software (Hemodyn 3M apparatus, Dinap SRL, Argentina) for image analysis. FMD was calculated as follows:

$$FMD(\%) = (D_{max} - D_{basal}) / D_{basal} \quad (1)$$

where D_{basal} is the baseline diameter measured over a 1-min period before occlusion, and D_{max} is the maximal diameter post-occlusion measured over a 3-min period at peak flow.

Palm microcirculation was assessed at the same time as FMD using the non-invasive flowmetry by laser Doppler (FLD) technique, which enables continuous measurement of endothelial-dependent reactivity in local microcirculation, using the laser Doppler system PeriFlux 5010 (Perimed). Details of the specific endpoint values assessed are available in Supplemental Fig. S1.

2.4.3. Isotopic measurements for study compliance and tracer-based flux assessments

Animal and plant products differ in their natural abundances of stable N and C isotopes, leading to distinct $\delta^{15}N$ and $\delta^{13}C$ fingerprints in the body that can be used to estimate the relative dietary intake more objectively than using self-reported data [29]. Thus, to monitor diet compliance throughout each diet intervention period, $\delta^{15}N$ and $\delta^{13}C$ were measured in plasma proteins collected at d1, d14 and d28 t0 by elemental analyzer-isotope ratio mass spectrometry (EA-IRMS, Isoprime; VG Instruments).

At the end of each intervention period (d28), PS and DNL fluxes were quantified using the precursor-product ^2H labeling method. PS and DNL were calculated from the ^2H enrichments measured by EA-IRMS (TCEA-IRMS Delta V advantage; Thermo Fisher) in plasma water and the corresponding product pool, i.e., in plasma protein for PS and in TG from chylomicron-free plasma (mostly very low density lipoprotein (VLDL)-TG) for DNL using the following formulas, as previously reported in full [13]:

$$\text{FSR}\ (\%/\text{d}) = \frac{100}{\text{t}\ (\text{d})} \times \ln\left[\frac{\text{n*E}_{\text{plasma}}\ (\%) - \text{E}_{\text{P0}}(\%)}{\text{n*E}_{\text{plasma}}\ (\%) - \text{E}_{\text{P}}(\%)}\right] \quad (2)$$

$$\text{ASR}\ (\text{g}/\text{d}) = \frac{\text{FSR}\ (\%/\text{d})}{100} \times \text{plasma product pool size}\ (\text{g}) \quad (3)$$

where t (d) is the period of ^2H labeling before plasma sampling (for PS, we used plasma sampled at d29 to assess the mean daily FSR value, while for DNL we used plasma from d28 t360 to assess the postprandial FSR value); $n*E_{plasma}$ is the maximum ^2H enrichment achievable in the product with E_{plasma} (%) being the ^2H enrichment of plasma water (remaining stable during the labeling period) and n the number of ^2H incorporation sites in the product (for PS, we used $n = 2.2$ as the mean of the n values for each amino acid according to literature values [30] and for DNL we used $n = 0.48$ as previously described [13]); E_P and E_{P0} (%) are the ^2H enrichments of product (plasma protein for PS and VLDL-TG for DNL) at time t and before ^2H_2O exposure (d28 t-90), respectively. The product pool sizes for PS and DNL were estimated using plasma albumin and TG in chylomicron-free plasma, respectively. Plasma albumin and TG concentrations were determined using an automated analyzer and commercial kits, as described previously. FSR (%/d) and ASR (g/d) are the fractional and absolute synthesis rates, respectively. Since the FSR and ASR measurements were performed in plasma over a period short enough to limit tracer recycling, the results essentially reflect the hepatic values.

2.4.4. Plasma metabolomics

Detailed methods of metabolomics analyses by liquid chromatography coupled to high-resolution mass spectrometry are provided in Supplemental Text S2. Briefly, plasma metabolites were extracted by methanol-assisted protein precipitation [31] and analyzed using a U3000 liquid chromatography system coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific, France) fitted with an electrospray source operated in the positive and negative ion modes. To cover a wider range of metabolites, analyses were duplicated with both HILIC and C18 columns associated with negative and positive ionization modes, respectively. All samples were analyzed within the same batch, and quality control samples were used to check the consistency of the analytical results in terms of signal and retention time stability during the experiments. Raw data inspection, processing and filtering were done using Xcalibur software (Thermo Fisher Scientific, France) and the Workflow4metabolomics interface [32]. Features were annotated with the in-house database according to accurately measured masses and chromatographic retention times [31].

2.5. Statistical analyses

2.5.1. Clinical and phenotypic data

Data are reported as means ± SD. Statistical analyses were performed with SAS Studio version 3.81 (SAS Institute). Post-absorptive and postprandial kinetics data were analyzed separately using mixed models (PROC MIXED) for repeated measurements, with diet (CON-D or FLEX-D), time (d1, d14 and d28 for post-absorptive data; t0, t30, t60, t120, t250 and t360 min for postprandial data; week 1, 2, 3 and 4 for dietary data), period (first or second), diet*time and diet*period as fixed effects, participant as a random effect and diet and time as repeated factors. For postprandial biochemical data (glucose, insulin, TG, and NEFA), the value before the dietary interventions (calculated as the mean of post-absorptive values at d1 of each intervention period) was introduced in the model as a covariate. The differences between CON-D and FLEX-D were tested as preplanned comparisons at each time point, using *ad hoc* contrasts under the mixed model with Bonferroni multiple testing correction. The data were transformed by logarithmic or Box-Cox transformation to ensure the normality of the residuals, when needed. Data measured only once per intervention period (DNL and PS) were analyzed using similar mixed models but without the time effect and diet*time interaction.

2.5.2. Metabolomics data

Metabolomics data were analyzed using the linear mixed model-principal component analysis (LiMM-PCA) method [33,34] (Supplemental Text S3) using the limpca package [35] running on R (version 4.2.0), which involved five steps: 1) dimension reduction using PCA; 2) fitting a linear mixed model that describes the experimental factors (diet, time, period, diet*time, diet*period as fixed effects and participant as a random effect) for each retained PCA component; 3) computation of an effect matrix; 4) for each experimental factor, estimation of the contribution to the total variance of

the dataset and statistical significance; 5) graphical representation of the effect matrix using PCA. Independent LiMM-PCA analyses were conducted for HILIC and C18 data, as well as for post-absorptive (d1, d14, d28 t0) and postprandial datasets (d28 t0, t30, t60, t120, t240, t360), with prior pareto-scaling of the raw MS data and outlier identification by PCA. Briefly, the statistical results indicate the contribution of each experimental factor to the overall variance and its significance, and for each significant factor, loading scores associated with individual metabolomics features represent their contribution to sample discrimination based on that specific experimental factor. LiMM-PCA loading for the diet effect (l_D) at post-absorptive and postprandial states are referred as $l_{D\text{-postab}}$ and $l_{D\text{-postpr}}$ respectively, while the LiMM-PCA loading for the interaction diet*time effect in the post-absorptive state is referred to as ($l_{I\text{-postab}}$).

Features with $|\, l_D \,| > 0.01$ or $|\, l_{I\text{-postab}} \,| > 0.01$ and a confirmed annotation were further tested using univariate mixed model statistical analysis to assess the diet or diet*time effect at the individual metabolite level. The Workflow4Metabolomics Galaxy interface [32] was used, with diet as a fixed effect, time as a repeated factor, and participant as a random effect (Mixedmodel module, based on lmerTest R package model [36]). Raw data were log-transformed prior to analysis, and *p*-values were corrected for multiple testing using the false discovery rate method. To characterize the direction of the diet effect on each selected metabolite (i.e., increased with CON-D or FLEX-D) in the post-absorptive state, we calculated the ratio of the mean metabolite intensity at d28-t0 after FLEX-D over the corresponding value after CON-D. For the postprandial state, we calculated the ratio of the mean postprandial total area under the curve (AUC) of metabolite intensity after FLEX-D over the corresponding value after CON-D. Total AUC (time points day 28-t0, t30, t60, t120, t240, t360) was calculated using the trapezoidal method in SAS Studio. Spearman correlations were calculated (PROC CORR, SAS) between annotated metabolites contributing to diet discrimination in the post-absorptive state as assessed by LiMM-PCA ($|\, l_{D\text{-postab}} \,| > 0.01$) and post-absorptive plasma levels of CM risk markers including glucose, insulin, HOMA-IR, triglycerides, total-C, LDL-C and HDL-C levels at d0, d14 and d28-t0 during CON-D and FLEX-D, with *p*-value adjustment for multiple testing using the false discovery rate method.

For all statistical analyses, the significance thresholds were set at $p \leq 0.05$ and we refer to a trend when $0.05 < p \leq 0.1$.

3. Results

Nineteen participants completed the study, and their data were included in the final post-absorptive analyses (Supplemental Fig. S2). Participant baseline characteristics are shown in Table 1. The postprandial data obtained after the challenge meal at the end of each dietary intervention were collected from 18 participants for FLEX-D (one participant was excluded due to poor tolerance of the ^2H_2O intake) and 14 participants for CON-D (five participants were excluded due to vomiting (n = 2) or incomplete meal ingestion (n = 3)). Detailed harms are presented in Supplemental Table S4.

3.1. Dietary data and protocol compliance

Daily food intake self-reports (Table 2) confirmed that participants were overall compliant with the experimental design and achieved the targeted nutritional manipulation. FLEX-D contained a higher proportion of PP compared to CON-D (63.9 % *vs* 35.5 %, *p*-diet <0.001) but total protein intake was lower (13.3 E% *vs* 16.3 E%, *p*-diet <0.001) and carbohydrate intake was higher (45.7 E% *vs* 43.2 E %, *p*-diet <0.01). During the FLEX-D intervention, participants also had a higher fiber intake, 8.5 g/d more than during the CON-D intervention (*p*-diet <0.001), because of the higher fiber content in the PP sources. Vitamin E (+42 %), folate (+20 %), vitamin K1 (+15 %), Ca (+30 %), Fe (+8 %) and Mn (+11 %) intakes were significantly higher in FLEX-D than CON-D, while vitamin A (−25 %), vitamin D (−61 %), thiamin (−25 %), riboflavin (−13 %), niacin (−38 %), pantothenic acid (−10 %), vitamin B12 (−55 %), Na (−24 %), Se (−14 %), Zn (−12 %), EPA (−62 %) and DHA (−78 %) intakes were lower (*p*-diet <0.05) (Supplemental Table S5).

The CON-D was designed to meet the protein source ratio currently observed in the French adult population. In line with this objective, during the CON-D arm (compliance measures) the protein intake and PP proportion were similar to those reported at the baseline (3-day food record) (total protein: 16.3E% *vs* 16.4E%; PP: 35.5 % *vs* 36.0 %, respectively). Results of tracing $\delta^{15}N$ and $\delta^{13}C$ were

Table 1
Baseline characteristics and dietary habits of study participants.[a]

	Total N = 19	Sequence FLEX-D − CON-D N = 10	Sequence CON-D − FLEX-D N = 9
Anthropometric and metabolic characteristics			
Age, y	41.7 (7.1)	40.2 (7.8)	43.4 (6.2)
Weight, kg	88.5 (7.3)	86.5 (4.7)	90.7 (9.2)
BMI, kg/m^2	28.4 (2.4)	28.1 (1.8)	28.8 (3.0)
Waist circumference, cm	102.7 (5.9)	103.1 (6.6)	102.3 (5.3)
Plasma triglycerides, g/L	2.1 (0.7)	2.3 (0.6)	2.0 (0.8)
Plasma glucose, mmol/L	5.2 (0.3)	5.4 (0.3)	5.1 (0.3)
Plasma HDL-C, mmol/L	1.1 (0.2)	1.1 (0.3)	1.1 (0.2)
Systolic blood pressure, mmHg	125.7 (8.9)	124.3 (9.8)	127.3 (8.0)
Diastolic blood pressure, mmHg	77.8 (6.9)	75.6 (8.0)	80.3 (4.7)
Nutrient intake			
Energy, kcal/d	2212.6 (417.0)	2208.0 (539.8)	2217.7 (251.9)
Protein, E%	16.4 (2.3)	17.2 (2.2)	15.5 (2.0)
Plant protein, % total protein intake	36.0 (8.0)	36.6 (10.1)	35.4 (5.5)
Carbohydrates, E%	41.5 (5.5)	43.1 (6.0)	39.7 (4.5)
Lipids, E%	37.8 (5.1)	36.0 (5.2)	39.8 (4.3)
SFA, E%	16.7 (3.5)	15.4 (3.3)	18.1 (3.2)
MUFA, E%	12.3 (2.2)	11.4 (2.6)	13.2 (1.1)
PUFA, E%	4.3 (1.2)	3.9 (0.9)	4.7 (1.3)
Alcohol, E%	1.7 (2.1)	1.2 (1.9)	2.4 (2.3)
Fiber, g/d	21.4 (5.6)	21.8 (5.9)	21.0 (5.6)

[a] Data are means (SD). BMI, body mass index; HDL-C, high density lipoprotein-cholesterol; MUFA, mono-unsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

Table 2
Dietary intake during the 4-week nutritional intervention with a control (CON-D) or a flexitarian diet (FLEX-D)[a].

	CON-D (n = 19)	FLEX-D (n = 19)	*p*-diet	*p*-time	*p*-diet*time
Energy, kcal/d	2236.0 (377)	2275.2 (402.5)	>0.1	>0.1	>0.1
Protein, E%	16.3 (1.0)	13.3 (0.8)	<0.001	>0.1	0.023
Plant protein, %total protein intake	35.5 (5.0)	63.9 (4.5)	<0.001	0.060	<0.001
Carbohydrates, E%	43.2 (3.4)	45.7 (3.2)	0.027	>0.1	>0.1
Lipids, E%	34.7 (2.8)	34.4 (2.5)	>0.1	>0.1	>0.1
SFA, E%	13.4 (1.5)	13.9 (1.4)	>0.1	>0.1	>0.1
MUFA, E%	11.8 (1.2)	11.6 (1.4)	>0.1	>0.1	0.072
PUFA, E%	4.7 (0.5)	4.3 (0.5)	0.005	0.056	>0.1
Alcohol[b] E%	2.1 (1.4)	1.6 (1.6)	0.039	>0.1	>0.1
Fiber, g/d	28.0 (5.5)	36.5 (6.2)	<0.001	>0.1	>0.1

[a] Data are means (SD). The main effects of diet, time, period and the interactions diet*time and diet*period were analyzed using a mixed model for repeated measurements, but only the effects of diet, time and diet*time are presented in the table. The effects of period and diet*period were not significant (*p*-value >0.05) for all presented variables.
[b] Analysis was conducted on log-transformed data.

consistent with good overall compliance as the isotopic fingerprints resulting from the changes in protein sources targeted in each dietary intervention were as expected from the recorded dietary data (Supplemental Fig. S3).

3.2. Body composition, plasma markers related to cardiometabolic status, and metabolic fluxes

Participants experienced a slight weight loss during the 4-week dietary interventions (−0.7 kg, i.e., −0.9 %, *p*-time <0.05) that was not significantly different between diets (Fig. 2), accompanied by a small reduction in WC (−0.9 %, *p*-time = 0.080). While lean mass did not significantly change after either diet intervention, fat mass decreased (−6%) with the FLEX-D but not with the CON-D (*p*-diet*time <0.05).

During the dietary intervention, and irrespective of the diet, there was an overall improvement in metabolic health, with reduced post-absorptive levels of total-C (−9%), LDL-C (−11 %), insulin (−29 %), glucose (−7%), creatinine (−5%) and lactate (−10 %), lower HOMA-IR (−34 %) and lower total-C-to-HDL-C ratio (−4%) (*p*-time <0.05) (Fig. 2 and Supplemental Figs. S4 and S5). There was a trend for larger HOMA-IR decrease with FLEX-D than CON-D (−38 % vs −30 %, *p*-diet*time = 0.080). Plasma urea also decreased more with FLEX-D than CON-D (−15 % vs −8%, *p*-diet*time <0.01). Post-absorptive TG, NEFA and uric acid concentrations remained unchanged during the intervention. Postprandial TG, NEFA, and insulin concentrations after the challenge meal were not significantly affected by the diet, whereas there was a trend for lower postprandial glycemia after FLEX-D than CON-D (*p*-diet = 0.082) (Fig. 3).

Several inflammation markers were assayed (Supplemental Table S6). There was a trend for a decrease in CRP during the dietary intervention regardless of the diet (−38 %, *p*-time = 0.092). On the contrary, there was a trend for increased IL-10, an anti-

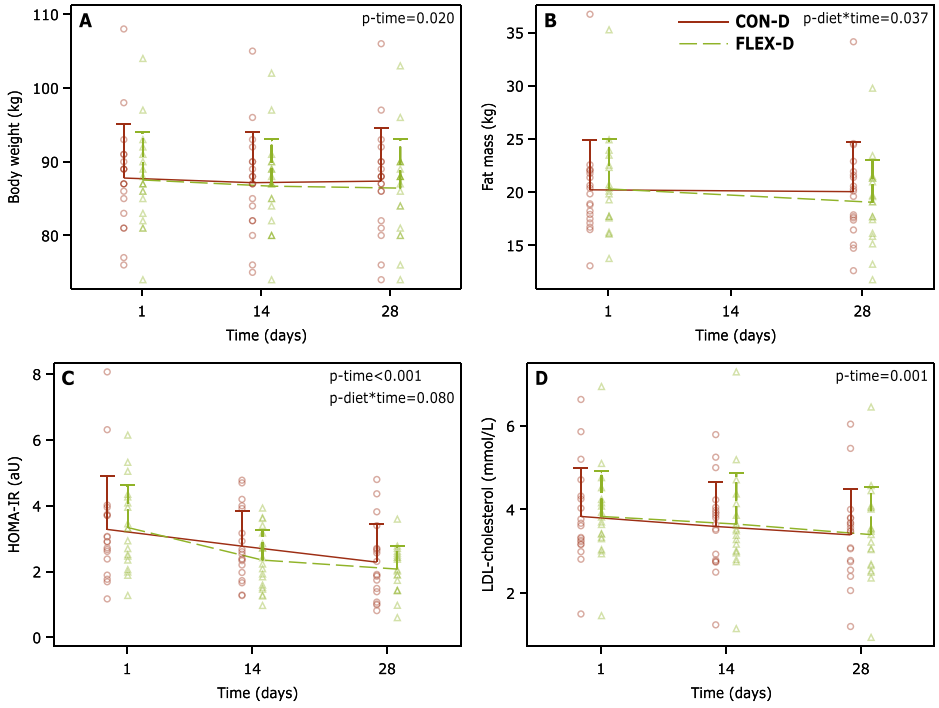

Fig. 2. Changes in body weight (A), fat mass (B), insulin resistance (HOMA-IR) (C) and low-density lipoprotein (LDL)-cholesterol post-absorptive concentration (D) during a 4-week intervention with a control (CON-D, n = 19) or a flexitarian diet (FLEX-D, n = 19). Data are means + SD. The effects of diet, time, period and the interactions diet*time and diet*period were analyzed using a mixed model for repeated measurements. Fat mass and HOMA-IR data were log-transformed prior to statistical analysis. aU, arbitrary units. *p*-values >0.1 are not shown.

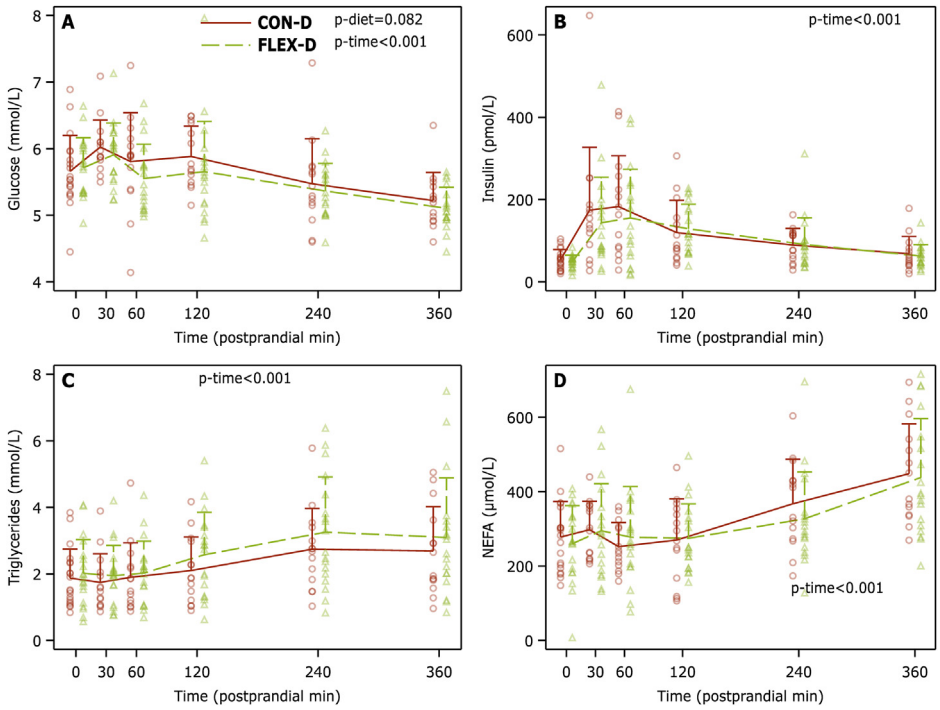

Fig. 3. Postprandial changes in plasma glucose (A), insulin (B), triglycerides (C) and non-esterified fatty acids (NEFA) (D) concentrations following a high-fat meal challenge after a 4-week intervention with a control (CON-D, n = 14) or a flexitarian diet (FLEX-D, n = 18). Data are means +SD. The effects of diet, time, period, baseline value and the interactions diet*time and diet*period were analyzed using a mixed model for repeated measurements. Effects of time, diet and time*diet are presented in the figure, with *p*-values >0.1 not shown. The effect of the baseline value was significant (*p*-baseline <0.05) for glucose, insulin, triglycerides and NEFA. The effect of period was not significant for all presented variables (*p*-period >0.05). The interaction diet*period was significant for NEFA only (p-diet*time = 0.032). Insulin, glucose, and triglyceride data were log-transformed before statistical analysis.

inflammatory cytokine, with the FLEX-D only (FLEX-D +8 %; CON-D −0.8 %, *p*-diet*time = 0.053). Pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and chemoattractant CCL2 did not change with time or diet, nor did plasma malondialdehyde, an oxidative stress marker.

The FSR and ASR values of DNL and PS fluxes estimated at the end of each intervention period were similar between CON-D and FLEX-D (Table 3).

3.3. Vascular and endothelial functions

There was a trend for a decreased FMD in response to the challenge meal (*p*-time = 0.075), but there was no significant effect of the diet (CON-D or FLEX-D) on FMD (Fig. 4). The basal brachial artery diameter (mean 4.27 mm) remained unchanged during the intervention. FLD parameters (Supplemental Table S7) and post-absorptive plasma nitrite and adhesion molecules (ICAM, VCAM, E-selectin, P-selectin) (Supplemental Table S8) were not significantly modified by the diet and only a few FLD parameters were modified after the challenge meal. Plasma nitrate increased over time during FLEX-D but remained stable during CON-D (*p*-diet*time <0.05).

Table 3
Fractional synthesis rate (FSR) and absolute synthesis rate (ASR) of plasma protein synthesis and plasma *de novo* lipogenesis after a 4-week intervention with a control (CON-D) or a flexitarian diet (FLEX-D)[a].

	CON-D (n = 14)	FLEX-D (n = 17)
Protein synthesis		
FSR, %/d	4.37 (1.42)	4.52 (1.16)
ASR, g/d	6.11 (2.27)	6.37 (1.87)
***de novo* lipogenesis**		
FSR, %/d	13.16 (5.76)	13.70 (5.10)
ASR, g/d	0.48 (0.29)	0.56 (0.36)

[a] Data are means (SD). The effects of diet, period and the diet*period interaction were analyzed using a mixed model and were not significant for any of the variables presented (*p*-values >0.1).

3.4. Post-absorptive and postprandial metabolome signatures

The post-absorptive and postprandial plasma metabolomes were analyzed and quantified by LC-MS. A total of 3120 and 4114

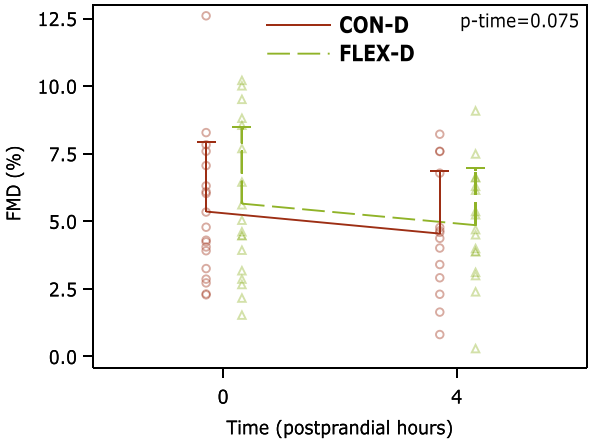

Fig. 4. Brachial artery flow-mediated dilatation (FMD) in response to post-occlusion hyperemia in the post-absorptive state and 4h after a high-fat challenge meal after a 4-week intervention with a control (CON-D) or a flexitarian diet (FLEX-D). Data are means +SD. The effects of diet, time, period and the interactions diet*time and diet*period were analyzed using a mixed model for repeated measurements. $n_{AP\text{-}diet\ t0} = 19$, $n_{AP\text{-}diet\ t4} = 14$, $n_{PP\text{-}diet\ t0} = 18$, $n_{PP\text{-}diet\ t4} = 17$. *p*-values >0.1 are not shown.

features of the metabolome were included in processing the C18 and HILIC column data, respectively. Outliers were identified by PCA and removed from further analyses (see Supplemental Text S4 for a detailed description). The contribution of the diet to the overall variance ranged from 0.80 % to 1.06 % (p-diet <0.01) according to the four LiMM-PCA models fitted, depending on post-absorptive/postprandial status and the column (Supplemental Table S9). The diet*time interaction was significant for the C18 post-absorptive model (p-diet*time <0.05). The results from the HILIC and C18 columns were merged for subsequent analyses. Among the 1309 features contributing to the diet effect ($|l_D| > 0.01$) in the post-absorptive and postprandial states, a total of 111 and 118, respectively, were annotated and thus identified as metabolites, 82 of them being common to the two conditions (Supplemental Table S10). These diet-discriminating metabolites included amino acids and derivatives, aromatic compounds (indoles, imidazopyrimidines or phenolic compounds), lipid related metabolites (acylcarnitines, dicarboxylic acids, bile acids), organic acids, aliphatic compounds, carbohydrate compounds (sugar acids or saccharides) and nucleosides and derivatives. A description of all identified metabolites is presented in Supplemental Table S11.

The diet effect was further tested for the identified metabolites using univariate mixed models. The diet effect was significant (p-diet <0.05) for 21 metabolites in post-absorptive plasma and 37 metabolites in postprandial plasma, respectively, with 11 of them being in common (Fig. 5). Six of the common metabolites were lower after FLEX-D than CON-D, namely lysine, alpha-amino adipic acid, methylhistidine, eicosapentaenoic acid, benzyl alcohol, and aminosalicylic acid. Four of the common metabolites were higher after FLEX-D than CON-D, namely 2,6-dihydroxybenzoic acid, N-acetyl-ornithine, indole acrylic acid, and alpha-hydroxyhippuric acid. Overall, the direction of the diet effect was consistent between the post-absorptive and post-prandial states, except for indole propionic acid, which was more abundant at the post-absorptive state at d28, but its postprandial AUC was lower after FLEX-D than after CON-D (Supplemental Fig. S6). Among the 652 features contributing to the diet*time effect in the post-absorptive state ($|l_{\text{I-postab}}| > 0.01$), 50 were identified as metabolites and 4 had a significant diet*time effect as assessed by univariate analysis, namely phenoxypropionic acids, phenoxybutyric acid, pyridoxic acid and N-acetyl-ornithine.

When considering the contribution of each metabolite to the overall diet discrimination (l_D value), together with magnitude of the differences between each diets in the postabsorptive (Fig. 6) or postprandial (Fig. 7) states, few metabolites scored highly on both dimensions. In the post-absorptive state, the five metabolites that showed the highest FLEX-D-to-CON-D fold change (the ratio of the mean metabolite intensity at d28-t0 after FLEX-D over the corresponding value after CON-D) were alpha-hydroxyhippuric acid, o-hydroxyhippuric acid, indole propionic acid, indoleacrylic acid and trigonelline, while the five metabolites with the lowest values were acetaminophen glucuronide, acetaminophen, adenosine, methylhistidine and benzyl alcohol. In the postprandial state, the five metabolites with the highest FLEX-D-to-CON-D fold change (the ratio of the mean postprandial total AUC of metabolite intensity after FLEX-D over the corresponding value after CON-D) were alpha-hydroxyhippuric acid, o-hydroxyhippuric acid, indoleacrylic acid, taurodeoxycholic acid and N-acetyl-ornithine, while the five metabolites with the lowest values were acetaminophen glucuronide, acetaminophen, indole propionic acid, methylhistidine and phenoxypropionic acids.

Post-absorptive and postprandial changes in metabolite abundance with marked differences between diets and/or of particular biological interest are presented in Supplemental Fig. S6 (indoleacrylic acid, indole propionic acid and trimethylamine oxide), Supplemental Fig. S7 (N-acetyl-ornithine and alpha-aminoadipic acid), and Supplemental Fig. S8 (dodecanedioic acid, tetradecanedioic acid and hexadecanedioic acid).

Spearman correlation analyses between plasma metabolites contributing to the overall diet discrimination at the post-absorptive state ($|l_{\text{D-postab}}| > 0.01$, n = 111) and plasma markers of glucose and lipid homeostasis (n = 7) are presented in

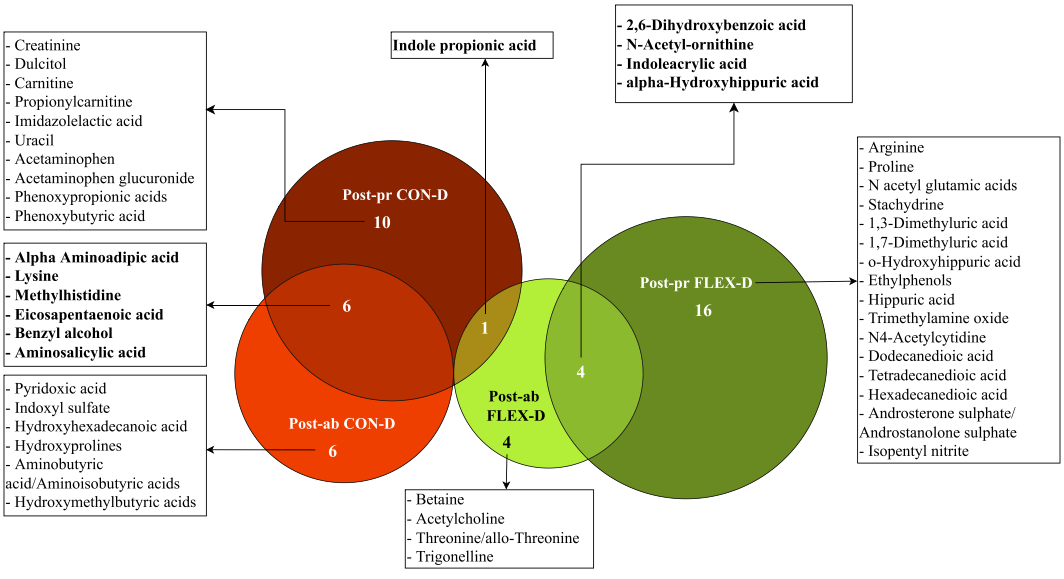

Fig. 5. Plasma metabolites that differed during a dietary intervention with a control (CON-D) or a flexitarian diet (FLEX-D). Metabolites were assessed in the post-absorptive state during a 4-week diet adaptation period (post-ab) or at the end of each intervention period during a 6h follow-up after a high-fat challenge meal (post-pr). The metabolites listed significantly contributed to the overall diet effect ($|l_D| > 0.01$) and had a significant diet effect when tested individually (univariate mixed model analysis; p-diet <0.05). Metabolites in bold differed between diets in both postprandial and post-absorptive states, as assessed by the univariate analysis. Differences in metabolite abundance were based on the ratio of the mean metabolite intensities at d28-t0 of FLEX-D over CON-D for the post-absorptive state and the ratio of mean total AUC of metabolite intensities of FLEX-D over CON-D for the postprandial state.

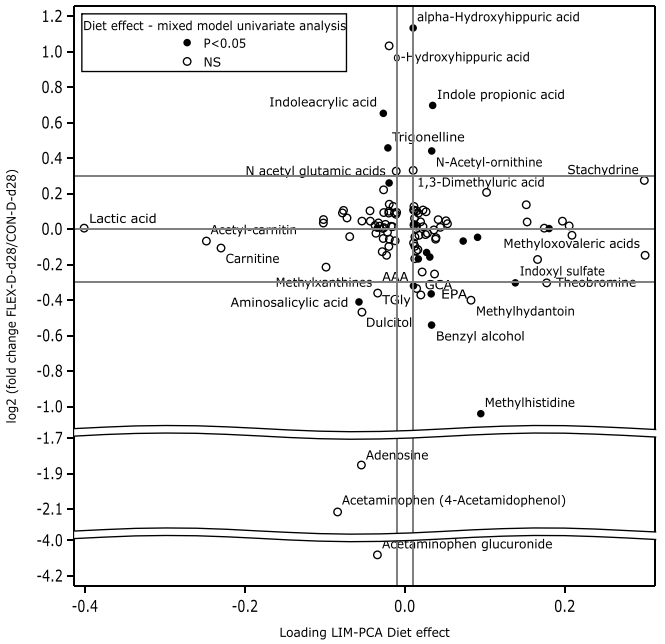

Fig. 6. Log2-transformed fold change values of post-absorptive plasma metabolites after a 4-week intervention with a control (CON-D) or a flexitarian diet (FLEX-D) according to their contribution to the overall diet effect as assessed by the LiMM-PCA model ($| l_{D\text{-}postab} | > 0.01$). Metabolites shown by filled circles were also significantly modified by the diet as assessed by univariate analysis (p-diet <0.05) while those with empty circles were not (not significant (NS), p-diet >0.05). AAA, alpha amino adipic acid; EPA, eicosapentaenoic acid; GCA, glycocholic acid; TGly, N-Tiglylglycine.

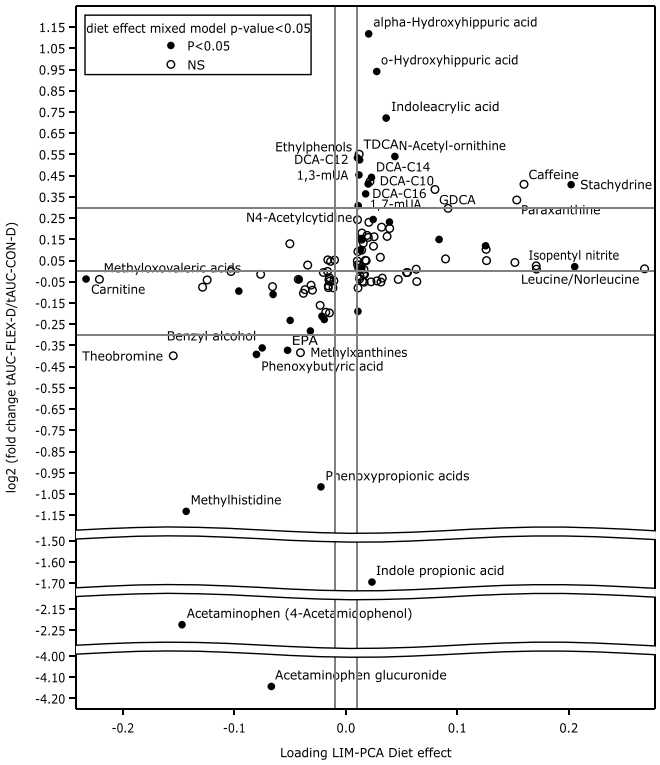

Fig. 7. Log2-transformed fold change values in metabolite postprandial total area under the curve (tAUC) measured after a high-fat challenge meal after a 4-week intervention with a control (CON-D) or a flexitarian diet (FLEX-D) according to their contribution to the overall diet effect as assessed by the LiMM-PCA model ($| l_{D\text{-}postpr} | > 0.01$). Metabolites shown by filled circles were also significantly modified by the diet as assessed by univariate analysis (p-diet <0.05) while those with empty circles were not (not significant (NS), p-diet >0.05). 1,3-mUA, 1,3-dimethyluric acid; 1,7-mUA, 1,7-dimethyluric acid; DCA-C10, sebacic acid; DCA-C12, dodecanedioic acid; DCA-C14, tetradecanedioic acid; DCA-C16, hexadecanedioic acid; EPA, eicosapentaenoic acid; GDCA, glycodeoxycholate; TDCA, taurodeoxycholic acid.

Supplemental Fig. S9. Seventeen metabolites were significantly correlated with at least one clinical marker. Among the metabolites with a significant diet effect when tested individually (univariate mixed model analysis; p-diet <0.05), lysine was negatively correlated with TG ($\rho = -0.42$), indoxyl sulfate was positively correlated with insulin ($\rho = 0.41$), and hydroxyhexadecanoic acid was positively correlated with LDL-C ($\rho = 0.40$). Interestingly, short-chain acylcarnitines (acetyl-carnitine, propionylcarnitine, butyrylcarnitine) and branched-chain amino acid product hydroxymethylglutaric acid were positively correlated to plasma glucose or insulin.

4. Discussion

In this randomized crossover, controlled feeding trial, while most CM risk factors were slightly improved regardless of the diet, the short-term flexitarian transition induced changes in the metabolic profile and the plasma metabolome that suggested a positive shift, with changes in AA and lipid metabolism and gut microbiota activity reported in the literature as negatively associated with CM risk.

4.1. Clinical endpoints and CM risk factors

The improvements in CM risk factors (weight, post-absorptive total cholesterol, LDL-cholesterol and HOMA-IR) after both interventions may have been driven by the higher quality of the experimental diets compared to prevailing dietary habits, or the adoption of a healthier behavior during the trial.

Diet had no effect on many endpoints of CM risk, such as circulating lipids, inflammation cytokines, adhesion molecules or the measures of endothelial function (FMD and FLD). The small sample size, the short duration of the study, and the strong effect of the intervention, irrespective of the diet, may have contribute to the absence of between-diet effects. In line with our results, some clinical trials have reported no beneficial effects of PP-rich diets on inflammation [37–40], while others showed improvements in lipid profiles after vegetarian compared to omnivore diets [17,41,42]. Regarding endothelial function, there is a lack of studies exploring the specific effects of flexitarian diets, with most reports relating to the Mediterranean diet [43]. However, we found that fat mass decreased with the flexitarian diet amid trends of decreases in HOMA-IR and postprandial glycemia and increase in anti-inflammatory cytokine IL-10, which is in line with reports of beneficial or null effects on glucose homeostasis of substituting AP with PP sources [44]. Together, these results suggest a 4-week flexitarian diet has modest beneficial effects on the CM profile.

4.2. Food intake biomarkers in plasma metabolome

A higher number of diet-discriminating metabolites were identified in the postprandial state than in the post-absorptive state, even though the same challenge meal was used for both diets. Postprandial tests may therefore highlight metabolic effects that would be missed otherwise [20].

Many of the diet-discriminating metabolites identified in the post-absorptive or postprandial states are validated or candidate biomarkers of intake of animal (methylhistidine, creatinine, carnitine, hydroxyprolines, eicosepentaenoic acid, dulcitol) or plant

foods (N-acetyl-ornithine, stachydrine, trigonelline, 1,7-dimethyluric acid, 1,3-dimethyluric acid, 2,6-dihydroxybenzoic acid, hippuric acid) and the changes in abundance observed in this trial are mostly consistent with previous findings (see Supplemental Text S5 [45–48]). However, the distinction between food intake biomarkers and effect biomarkers is not straightforward. For instance, trigonelline in urine has been positively associated with legume intake as well as improved glucose homeostasis [49], through exploratory mechanisms involving insulin secretion and oxidative stress [50].

4.3. Protein and AA and metabolism

The flexitarian and control diets resulted in similar synthesis of plasma proteins, in line with a report using a similar methodology showing equivalent skeletal muscle protein synthesis following vegan or omnivore diets [51]. This is also consistent with our previous findings in rats showing that, compared to AP, PP do not alter protein synthesis but remodel AA metabolism due to their less optimal AA profile for direct incorporation into proteins, leading to increased metabolic reshuffling of AA that is potentially beneficial for CM health because of the additional energy expended and the effect on intermediary and lipid metabolism [13]. In line with this, we evidenced changes in diet-discriminating metabolites suggesting subtle shifts in AA metabolism. The flexitarian diet resulted in higher postprandial levels of metabolites related to non-indispensable AA metabolism, such as arginine, proline and N-acetylglutamic acids. In contrast, post-absorptive and/or postprandial levels of metabolites related to indispensable AA were lower after the flexitarian diet: lysine and its oxidation product alpha-aminoadipic acid; imidazolelactic acid, a catabolic product of histidine; branched-chain amino acids (BCAA) catabolic products such as hydroxymethylbutyric acids (possibly β-hydroxy-β-methylbutyrate, a well-known metabolite of leucine [52]); and propionyl-carnitine. In addition, there was a trend for lower hydroxymethylglutaric acid, a leucine catabolic product, in the postprandial state after the flexitarian diet. BCAA and propionyl-carnitine have been associated with AP-rich diets [11] and meat intake [53,54], while alpha-aminoadipic acid remained stable during a one-year Mediterranean diet intervention [55] and has been positively associated with total protein intake [14]. Furthermore, there are well-established associations between BCAA and propionyl-carnitine and insulin resistance and diabetes [56,57]. A similar association was recently reported for alpha-aminoadipic acid [58–60], with preliminary evidence suggesting that alpha-aminoadipic acid could inhibit insulin signaling and promote gluconeogenesis [58]. In the present study, propionyl-carnitine and hydroxymethylglutaric acid were positively correlated with HOMA-IR.

4.4. Lipid and microbiota-related metabolisms

While postprandial plasma DNL, mostly reflecting hepatic activity, was not modified by diets, several changes in lipid-related metabolites were observed with the flexitarian diet, some of which only occurred during the postprandial phase, such as higher levels of several dicarboxylic acids (dodecanedioic, tetradecanedioic and hexadecanedioic acids). We also observed a trend for higher postprandial C10-acylcarnitine, a β-oxidation-related metabolite, with the flexitarian diet. Dicarboxylic acids are products of ω-oxidation, a minor endoplasmic reticulum pathway mostly active in the liver and kidney, which provides an alternative way to reduce the accumulation of toxic fatty acids in conditions with increased lipid flux, such as diabetes or high-fat diets [61]. Our results suggest that the flexitarian diet may have activated compensatory mechanisms to cope with lipid overload and saturated β-oxidation in the postprandial state, although more specific studies are needed to further investigate this hypothesis.

The flexitarian diet led to higher post-absorptive levels of metabolites resulting from tryptophan catabolism by gut microbiota, such as indole propionic acid (IPA) and indoleacrylic acid, but lower post-absorptive levels of indoxyl sulfate (the hepatic metabolite of indole). Indole derivatives are thought to improve the intestinal immune response, regulation of the intestinal barrier and gastrointestinal motility, and stimulate glucagon-like peptide 1 secretion [62]. More specifically, IPA has been negatively associated with diabetes risk [63,64], cardiovascular disease risk and obesity [65]. While IPA has been positively associated with PP intake [14] and vegetarian diets [17], indoxyl sulfate has been negatively associated with PP intake [14]. Interestingly, a positive association has been reported between tryptophan intake and IPA plasma levels only when fiber intake was high [64], which is a situation similar to the high-fiber flexitarian diet provided here (Table 2). While indole contributes to some of the above-mentioned beneficial effects of indole derivatives, indoxyl sulfate is also a uremic toxin that has deleterious effects on the vascular system [66]. Diets with contrasted protein sources have been reported to induce changes in gut microbiota activity and/or composition [17,67], and the shifts in indole metabolites observed in the present study may indicate such changes in gut microbiota. Regarding the postprandial response, the flexitarian diet increased trimethylamine-oxide (TMAO) levels. While plasma TMAO has been associated with cardiovascular risk [68], it is also dependent on the dietary background in a way that remains to be fully elucidated. Thus, TMAO levels have been reported to be lower after plant-based diets compared to animal-based diets [69] but also higher after wholegrain rich diets [70], this last finding being in line with the higher fiber content of the flexitarian diet. Furthermore, we did not quantify plasma TMAO concentrations, which may have been within physiological range after both experimental diets and deserve further quantitative investigation.

5. Conclusion

Our study has several strengths. The effect of the diet was characterized using multiple complementary approaches, including exploratory metabolomics, quantitative assessment of metabolic fluxes, and functional measurements such as FMD. Furthermore, we characterized the metabolic response in the post-absorptive state and after a high-fat challenge meal, allowing for the assessment of phenotypic flexibility. The trial also benefited from important advantages to identify specific metabolic changes related to diet, including a high level of dietary control but also AP/PP ratios either close to those reported in Western diets [71] or reasonable to be adopted by the adult population while minimizing long-term health risks and environmental impacts [72].

There are several limitations, including a relatively small size of the study population, a short duration of the intervention, and a brief wash-out period, with carryover effects that cannot be fully excluded. As expected, the interpretation of the metabolic changes associated with the shift in dietary protein is not straightforward, as the experimental diets differed by several dietary factors (e.g., total protein intake, fiber, micronutrients), that could all have contributed to the observed effects.

To conclude, despite small differences in CM changes between diets at the end of this chronic intervention, the realistic increase in PP sources resulted in several metabolic adaptations. In particular, changes related to AA and lipid metabolism or changes that could be ascribed to gut microbiota activity are in line with decreased CM risk. Overall, these results provide new mechanistic insights for

understanding the metabolic effects of a transition towards diets richer in PP. The changes in tryptophan metabolites call for further investigations of gut microbiota activity and composition. Furthermore, our characterization of changes in the plasma metabolome specifically induced by AP-rich or PP-rich diets warrants replication in larger trials.

Statement of authors' contributions to manuscript

HF, J-FH, FM, SP, DR designed research; AA, CC, FC, MC, JD, PJ, GL, VM, SP, GP, M-AV conducted the experiment and generated the data; HF, GL, MTF, SP analyzed data or performed statistical analysis; GL wrote the first draft with major inputs of HF and SP and critical comments from J-FH, CL-M, FM, CP, LG-D, DR; HF and SP had primary responsibility for final content; All authors have read and approved the final manuscript.

Data sharing

Data described in the manuscript, code book, and analytic code will be made available upon request.

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Conflict of interest

Caroline Perreau, Catherine Lefranc-Millot and Laetitia Guérin-Deremaux are employees of Roquette Freres. Gaïa Lépine PhD grant was funded by INRAE and Roquette, under the scientific supervision of Sergio Polakof and Hélène Fouillet.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2024.10.009.

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