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Jean-Charles Martin[1], Claire Bal-dit-Sollier[2], Jean-Marie Bard[3], Denis Lairon[1], Michel Bonneau[4], Chantal Kang[2], Murielle Cazaubiel[5], Corinne Marmonier[6], Pascale Leruyet[7], Constance Boyer[6], Hassan Nazih[3], Catherine Tardivel[1], Catherine Defoort[1], Marion Pradeau[1], Imene Bousahba[1,8], Habib Hammou[8], Ljubica Svilar[1], Ludovic Drouet[2]

*Address correspondence* to JCM: C2VN, 27 boulevard Jean-Moulin, Faculté de Médecine La Timone, 13385 Marseille, France; e-mail: jean-charles.martin@univ-amu.fr; phone: +33 7 77 23 00 34.

[1]C2VN, INRAE, INSERM, Aix Marseille Université, Marseille, France: JCM, DL, CT, CD, MP, IB, LS.

[2]LTA-IVS INSERM U689, Hôpital Lariboisière, Paris, France: CBDS, LD

[3]Institut de Cancérologie de l'Ouest, Centre de Recherche en Nutrition Humaine Ouest, EA 2160 - IUML FR3473, CNRS, Université de Nantes, Nantes, France: JMB, HN

[4]UCEA, INRAE, Jouy-en-Josas, France: CK, MB

[5]BIOFORTIS, Saint-Herblain, France: MC

[6]CNIEL, Paris, France: CM, CB

[7]Lactalis R&D, Retiers, France: PL

[8]Université Oran 1, Oran, Algeria: HH

All data reported in this manuscript will be made available upon request, pending application and approval.

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*Running head*: Biomarkers and biological effects of dairy fat.

**Clinical trial registration number** 2008-A01145-50

**Abbreviations used**:

BCAA, branched-chain amino acids; DF, dairy fat; ESI, electrospray ionization; FBM, familial hypercholesterolemia Bretoncelles-Meishan; FID, flame ionization detector; FWHM, full width at half-maximum; HCA, hierarchical cluster analysis; HILIC, hydrophilic interaction liquid chromatography; HMDB, human metabolome database; HRMS, high-resolution mass spectrometry; KEGG, Kyoto encyclopedia for genes and genomes; LC/MS, liquid chromatography/mass spectrometry; MS, mass spectrometry; NCD, normal-energy collision-induced dissociation; NIPALS, non-linear iterative partial least squares; OPLS, orthogonal partial least squares; PLS, partial least squares; PLS-DA, PLS discriminant analysis; QC, quality control; R1, plant-fat diet; R2, summer dairy-fat diet; R3, winter dairy-fat diet; R4, winter dairy-fat diet (R3) with added calcium; RP, reversed-phase; UPLC, ultra-high-performance liquid chromatography; VIP, variable importance in projection; W4M, workflow for metabolomics.

## Abstract

Health effects of dairy fats (DF) are difficult to evaluate, as DF intakes are hard to assess epidemiologically and DF have heterogeneous compositions that influence biological responses. We set out to find biomarkers of DF intake and assess biological response to a summer DF diet (R2), a winter DF diet (R3), and a R3 supplemented with calcium (R4) compared to a plant-fat-based diet (R1) in a randomized clinical trial (n=173) and a 2-year study in mildly metabolically disturbed downsized pigs (n=32). Conventional clinical measures were completed by LC/MS plasma metabolomics/lipidomics. The measured effects were modeled as biological functions to facilitate interpretation.

DF intakes in pigs specifically induced a U-shaped metabolic trajectory, reprogramming metabolism to close to its initial status after a one-year turnaround. Twelve lipid species repeatably predicted DF intakes in both pigs and humans (6.6% errors). More broadly, in pigs, quality of DF modulated the time-related biological response (R2: 30 regulated functions, primarily at 6 months; R3: 26 regulated functions, mostly at 6–12 months; R4: 43 regulated functions, mostly at 18 months). Despite this heterogeneity, 9 functions overlapped under all 3 DF diets in both studies, related to a restricted area of amino acids metabolism, cofactors, nucleotides and xenobiotic pathways and the microbiota. In conclusion, over the long-term, DF reprograms metabolism to close to its initial biological status in metabolically-disrupted pigs. Quality of the DF modulates its metabolic influence, although some effects were common to all DF. A resilient signature of DF consumption found in pigs was validated in humans.


**Keywords:** multiplex biomarkers, interspecies validation**,** metabolic trajectory, biological functions, biological pathways, dairy fat quality, deep phenotyping

# 1 Introduction

Dairy fat (DF) (butter, cheese, and whole milk) is an important component of regular foodways in developed countries, providing ~15% of total daily energy intake [1], and DF intakes are predicted to increase by a further 20% worldwide in the 2018–2027 period [2]. In the past decade, the long-held thinking that DF is bad for health has been challenged [3], and the balance of benefits to harms has swung back in favor of dairy products [4]. Dairy products can beneficially influence many aspects of health, such as cardiovascular disease, obesity, cancer, bone health, aging, and more [4], but the role of the DF moiety is often overlooked. There is therefore a need for epidemiological studies to get a better evaluation of DF intakes than the dietary questionnaires currently used, which are often not accurate enough to connect DF intakes to health outcomes. In fact, efforts to discover DF biomarkers are based either on short-term DF exposure (2 or 3 months) or on estimates of DF intake taken from food frequency questionnaires [5], both of which are exposed to biases. Short-term controlled nutritional interventions in humans also have weaknesses, such as attrition, low compliance [6], logistical hurdles, and insufficient time to induce a stable biological response [7]. Furthermore, DF biomarkers are often limited to circulating DF fatty acids [8] and rarely address the plasma lipidome (which includes all lipid species bearing fatty acids) [9] that can provide more detailed information. Finally, dairy fatty acid composition undergoes significant seasonal and geographical variations [10] that shift unsaturated-to-saturated fatty acids ratio and *trans*-vaccenic and rumenic acids to lower values in winter milk. Such natural changes are significant enough to modify the health properties of DF, but this has so far only been shown in research on certain cardiovascular risk factors in animals [11] and humans [12]. Adding calcium to dairy products is expected to bring further health benefits. It induces calcium soaps of saturated fatty acids, which lowers their absorption and leads to improvement in some cardiovascular risk factors such as total and LDL cholesterol [13].

There are other potential benefits of calcium-enriched DF that also warrant further investigation.

We previously reported an 8-week randomized nutritional trial in humans that found no statistical differences between vegetable fat, summer DF and winter DF and no clear advantage of calcium supplementation of winter DF on LDL cholesterol, which was the primary endpoint of that clinical study [14]. Our data suggested that milk fat could be consumed as part of a normal balanced diet without increasing cardiometabolic risk factors. Here we exploited this previous work to validate, in human volunteers, persistent biomarkers of DF found in a 2-year-long nutritional trial carried out in parallel with the same DF in a downsized colony of pigs selected for natural spontaneous human-like cholesterol levels and responsiveness to lipid food intake [15, 16], known as the 'familial hypercholesterolemia Bretoncelles Meishan' (FBM) pig colony. We also evaluated the overall and specific long-term biological response to each DF in these FBM pigs and compared it to the response found in the short-term clinical intervention. The goal is to overcome the limitations of controlled nutritional intervention studies that tend to be too short-term to obtain a biological steady state after a nutritional challenge with DF.

For this purpose, in addition to conventional clinical biology, we used deep phenotyping based on metabolomics and lipidomics. Both these -omics provide an unbiased assessment of food exposure and serve as a substitute for self-reported food intake [17]. They can also reveal a broad spectrum of DF-induced metabolic effects.

The assessment of cardiovascular outcomes to complement our previous study [14] is the subject of another upcoming report.

# 2 Methods

## 2.1 Clinical study design

This study was performed according to Good Clinical Practices and in compliance with French regulations the tenets the Declaration of Helsinki, and was recorded under number 2008-A01145-50. Informed consent was obtained from all volunteers. The trial started on 16 March 16 2009 (first volunteer in) and ended on 21 May 2010 (last volunteer out). The design was a two-center, randomized, double-blind study with four parallel arms managed and monitored by Biofortis Mérieux Nutrisciences, Nantes. Detailed description of the study design, volunteer selection process, and full diets can be found in [14] and in the supplementary material for the clinical study that had a primary outcome based on LDL cholesterol [14]. On the basis of previous experience with similar inclusion criteria, a mean LDL cholesterol of 1.62 g/L was expected with a standard deviation of 0.2 g/L at inclusion. With an alpha risk of 5%, the number of individuals to include was estimated as 47 in each diet group for a power of 90% and 36 in each group for a power of 85%. An original set of 528 volunteers with serum LDL cholesterol ≥ 1.3 g/L were screened for inclusion and exclusion criteria, and then the final total of 173 volunteers were randomized into 4 dietary groups (supplementary material—clinical study, Figure 2), each bringing 38% energy as lipids: R1 providing plant fat, R2 providing a summer dairy fat, R3 providing a winter dairy fat, and R4 providing the same winter fat but enriched with added calcium (supplemental clinical study design, Table 1 and Table 2). Fatty acid composition is reported in Table 1, and population data are reported in Table 2 and supplemental online material. The raw fats prepared for the human study were also used to prepare the pig diet, and the process was supervised by private-sector companies Lactalis, Entremont Alliance, and BEL (supplemental dairy fat preparation). Like for the clinical study, the fatty acid composition of each type of fat was kept constant throughout the nutritional protocol (Table 1) by adjusting, as and when necessary, with DF varying in fatty acid composition.

## 2.2 Nutritional study in pigs

The downsized colony of pigs known as the FBM pig colony [18] has been selected for natural spontaneous human-like cholesterol levels that are in part explained by a homozygous R84C mutation in the hotspot of the LDL receptor, and for responsiveness to lipid food intake [15, 16]. This study was approved by the Jouy-en-Josas–AgroParisTech institutional animal care and use committee under Agreement 12/048, and complied with the ARRIVE guidelines 2.0, in accordance with EU Directive 2010/63/EU. The study used 32 pigs, i.e. 8 pigs per nutritional group, that were randomly selected at 5 months of age and assigned to each dietary group (R1 to R4), which counted half females and half barrows, and kept for 24 months on the 4 diets. The sample size was chosen based on the diet-induced 25% decrease in measured intra-ventricular coronary artery atherosclerosis [15]. Venous blood was drawn from each pig at the start (T0) and then every 6 months until month 24 (T24). The dietary groups were similar to those of the clinical study, i.e. characterized by the same types of DF (Table 1). The study was synchronized with the human trial, starting from May 2008 for the first pigs in, and ending April 2011 for the last pigs out. Detailed diet composition data and complementary information are reported in the supplemental pig study design.

## 2.3 Blood analyses

For both individual humans and pigs, fasted blood samples were drawn to perform clinical biology tests (see supplemental Tables S1 and S3), total fatty acids analysis [19], and both metabolomics analysis and lipidomics analysis [20]. Blood samples were centrifuged to obtain a plasma that was then aliquoted in Eppendorf tubes and stored at -80°C until analysis.

Metabolomics and lipidomics

In all instances, sample preparation and analyses were performed randomly and within uninterrupted consecutive series. Polar/semi-polar compounds in plasma were extracted by adding 400 µL of ice-cold methanol to 100 µL of plasma and a 100-µL aliquot of each sample

placed in two separate vials, one for polar analysis using HILIC LC/MS and one for semi-polar analysis using RP-C/MS.

For non-polar metabolites (lipidomics analysis), compounds were extracted using the conventional Folch extraction method by adding 800 µL of ice-cold chloroform:methanol (1:1 v/v) to 100 µL of plasma in a glass tube placed in a clean glass insert to be analyzed by RP-LC/MS.

The polar/semi polar and lipid samples were separated using an UltiMate™ 3000 HPLC system (Thermo Scientific, Les Ulis, France) coupled to a Q-Exactive™ Plus quadrupole-orbitrap high-resolution hybrid mass spectrometer (HRMS) (Thermo Scientific, Les Ulis, France) equipped with electrospray ionization source (H-ESI II). The method is fully described in supplementary material LCMS.

Complementary total plasma fatty acid composition analysis was performed using gas chromatography with a flame ionization detection system [19], starting from 250 µL of plasma.

### 2.4 LCMS data preprocessing

Mass spectra data files were converted to mzXML format using the open source ProteoWizard application. Peak detection, alignment and curation were performed using XCMS, and the analytical drift was corrected using the linear correction module provided in the free web tool W4M [21].

#### 2.4.1 Mass feature annotations

Feature annotations for the HILIC and RP streams were performed by matching peaks against in-house libraries of authentic standards (~1300 metabolites) covering the bulk of primary metabolism and run under identical conditions [22]. Lipid LC/MS annotations were performed by matching the XCMS-generated data matrix to lipids identified in samples by

148 $MS^2$ spectral matching using LipidSearch™ software (Thermo Fisher Scientific, Les Ulis,
149 France) with the in-house W4M data tool [22].

150 Each annotated metabolite (Supplemental Tables S1 and S3) was assigned a biological role
151 based on HMDB Metabocards, PubChem descriptions, and KEGG pathways. Complementary
152 information was found in PubMed publications where available. The annotated metabolites
153 were then grouped according to their functional role (Supplemental Tables S2 and S4). Lipid
154 species were grouped using an HCA procedure (Supplemental Tables S2 and S4)[22].
155 Metabolites and lipid clusters were analyzed utilizing a hierarchical PLS procedure as
156 described in [22] in which each functional set combining metabolites or each cluster
157 combining lipids can be translated into a workable composite score for each individual.

158 ## 2.5 Statistical analyses and data display

159 Univariate statistical analysis, HCA, heatmapping, pathway enrichment and visualization,
160 ANOVA-simultaneous component analysis for two-factor analysis, and receiver operating
161 characteristic (ROC) curves were processed using the online tool MetaboAnalyst [23]. Gender
162 imbalance effects in both the pig and human studies was corrected using the batch effect
163 correction tool in MetaboAnalyst. Multivariate statistical analyses (principal component
164 analysis, non-hierarchical and hierarchical PLS–DA) were performed using SIMCA 12
165 software (Umetrics, Umea, Sweden).

166 All data were 'auto-scaled' before multivariate statistical analysis and log2-transformed for
167 univariate analyses when the criteria for normal distribution were not met.

168 The significant threshold for importance-in-projection (VIP) analysis on PLS-DA variables
169 was calculated by utilizing a normal probability plot indicating which metabolites from the
170 corresponding VIP value deviated the most from a normal distribution due to treatment.
171 Hierarchical PLS-DA modelling was performed based on the contribution of separate
172 orthogonal LS-DA calculated from all functional sets of metabolites or lipid clusters, and used

to generate a composite score value for each functional set [22]. The functional metabolic/lipid blocks were 'weighted' to take into account the number of variables per block [24]. For lipid blocking, the lipid species were grouped according to clusters calculated by HCA (Ward method).

Venn plots were constructed using the online tools Venny 2.0.2 (bioinfogp.cnp.csic.es/tools/venny) and Draw Venn diagram (bioinformatics.psb.ugent.be/webtools/Venn/). Word cloud analysis was performed using the freely-available web tool WordItOut (worditout.com).

Biomarker extraction and validation were performed essentially according to the procedure detailed in [22]. The method workflow is summarized in the supplemental biomarker workflow.

## 3 Results

The pig diets only differed in terms of their fat moiety. The summer DF R2 was lower in saturated fatty acids and higher in unsaturated fatty acids than the winter DFs R3 and R4 (Table 1). The plant-based fat R1 provided as much total saturated fatty acids as the summer DF R2, but with much less myristic acid. It was also higher in polyunsaturated fatty acids, especially linoleic acid, than both the summer and winter DF. w6-to-w3 ratio was much closer to the recommended value of 5 in DF than in plant-based fat (Table 1).

The Pig *vs* Human data comparisons included (respectively) 33 *vs* 42 clinical variables, 22 plasma fatty acids in both species, 161 *vs* 147 annotated plasma metabolites, and 292 *vs* 410 lipid species (both featured in 20 different lipid classes). Detailed compositions are listed in Supplemental Tables S1 and S3, respectively.

196 Anthropometric and zoometric outcomes did not differ between individuals in the 4 dietary
197 settings (Table 2 and Table 3). The weight gain rate in pigs was greatest between the 12$^{th}$ and
198 18$^{th}$ month of the nutritional challenge, corresponding to 18 and 24 months of age.

199 ## 3.1 Biomarker investigation in pigs

200 *DF* vs *non-DF comparisons:*

201 Our first aim was to identify biomarkers of DF intake that can be used in epidemiological
202 studies, regardless of the length of exposure to DF. Since we wanted to apply the pig results to
203 the human study, we used the common omics data collected in both species. We thus retained
204 22 common plasma fatty acids, 130 common lipid species, and 74 common metabolites
205 (Supplemental Table S5).

206 For that purpose, we selected the variables that most differentiated the non-DF control pigs
207 from DF-consuming pigs from 6 months onwards (at T6, T12, T18 and T24 months of dietary
208 exposure). At each timepoint, the discriminating variables were selected using either the VIP
209 criteria or the score contribution criteria from the PLS analysis as well as a *t*-test (adjusted *P*
210 value < 0.05). The most common variables in at least 3 timepoints were selected and pooled
211 from each test. Twelve variables were retained, i.e. two fatty acids (C14:0, C20:5n-3), three
212 triglycerides (TG(15:0/16:0/18:2), TG(18:2/20:4/20:4), TG(18:1/18:1/20:3)), one cholesteryl
213 ester (ChE(C22:5)), four phosphatidylinositols (PI(16:0/16:1), PI(16:0/18:1), PI(18:0/18:1),
214 PI(18:0p/20:4)), and two phosphatidylcholines (PC(31:1), PC(33:1)) (Supplemental Figure
215 S1). When used in a PLS-DA model, these 12 variables clearly discriminated the non-dairy *vs*
216 dairy diets at any timepoint post-DF exposure (cross-validation ANOVA *P*-value from $6 \times 10^{-8}$
217 to $3.7 \times 10^{-12}$) but not pre-DF exposure (*P*-value not significant) (Supplemental Figure S2).
218 We also used the pigs prior to the dietary challenges to test our biomarkers using a ROC
219 predictor. The model was trained with the T6 pigs to estimate dairy/non-dairy intake at T0,
220 T12, T18 and T24 (Figure 1). Predictive performance was almost 100% for both DF *vs* R1

plant-fat-fed pigs at each timepoint (Figure 1). In addition, when considered together and irrespective of time, the prediction of purposely-left-out pigs using the ROC estimator, whether DF-fed or plant-fat-fed, had an error rate of only 8.4% (Supplemental Table S6). There were no major differences between the 3 DF diets using the DF intake predictors compared to the R1 plant-fat diet (Figure 2A). However, for DF there was a time effect on predictor scores, with the greatest deviations observed during the first year followed by a tendency to return towards baseline values in the second year (Figure 2B). This observed effect included the conventional cardiovascular risk biomarkers (Supplemental Figure S3). This trajectory was specific to DF diet intake conditions, since the time-course response in non-DF pigs did not follow the same pattern (Figure 2C). Hence the question that arose was whether the biomarkers could distinguish mid-term (6–12 months) *versus* long-term (18–24 months) DF intake, but analysis found that they failed to do so (not shown). The biomarker status of pigs fed only DF (R2, R3, R4) was statistically different from baseline status prior to DF intake (T0) at every timepoint (T6, T12, T18, T24) (Supplemental Figure S4). Conversely, in pigs fed only plant-fat diet, biomarker status showed no change from T0 to T12 months ($P > 0.05$) but then differences emerged in the second year ($P < 0.05$), indicating a significant but lagging time effect on biomarker status in non-DF-fed pigs (Figure 2C and Supplemental Figure S5).

### 3.2 Validation of the pig dairy fat biomarkers in Human

We then set out to validate the selected 'dairy'-variable signatures of DF consumption over a long time-period in pigs in the narrower-period companion clinical trial. For that purpose, we randomly kept out one fifth of the human individuals, performed in 5 iterations. The 'dairy' status of the 1-out-of-5 excluded subjects was then predicted in each iteration using the same ROC modelling procedure as used with pigs while using the remaining 4-out-of-5 subjects. The overall performance indicated that 62.5% of 'non-DF' individuals and 95% of 'DF'

246 consumers were correctly assigned, given a sensitivity of 0.88 and a specificity of 0.806
247 (Supplemental Figure S6). From there, we calculated a dairy score index combining the 12
248 selected variables using the NIPALS algorithm [22] (Figure 3). For each of the 5 ROC-
249 sigmoid curve models, the asymptotic values were determined, and their mean value of
250 0.6344 was chosen as the dairy score index threshold value (Supplementary Figure S6). At
251 this threshold value, the performances of the dairy score was 5% errors and 7.7% undefined
252 for predicting 'non-DF' individuals, and 6.6% errors and 6.6% undefined for predicting 'DF'
253 individuals at both 95% and 99% confidence intervals (Figure 3).

254 ### 3.3 Biological trajectory elicited by dietary treatment in pigs

255 Biological response was investigated by combining all the variables in a PLS-DA model with
256 time as class variable, using either DF-fed pigs or only plant-fat-fed pigs. In the loading plot
257 of the PLS-DA analysis, the time-trajectory of biological response followed a very different
258 course between DF-fed and plant-fat-fed pigs (Figure 4). Beyond a turning point at 12
259 months, the trajectory diverged to follow opposite directions between the dairy and non-dairy
260 diets. From this point on, the trajectory with DF diets tended to return to initial baseline
261 conditions (Figure 4), mirroring the response pattern of the DF intake biomarkers (Figure 2).
262 All 3 DF diets followed this same trajectory (Supplemental Figure S7). Note that DF-fed pigs
263 followed a similar time–course on all diet-sensitive clinical, metabolomics and lipidomics
264 variables (Supplemental Figure S8).
265 When the variables were clustered into functional ontologies (see method [22], and
266 Supplemental Table S2), the metabolic trajectory observed in the DF-fed pigs (between T0–
267 T24 and T6–T12 months, Figure 5B) mainly reflected a rebalancing in amino acids including
268 tryptophan metabolism towards microbiota-related metabolism, vascular regulations and
269 related metabolic disorders, and across a broad section of the captured lipidome (Figure 5).

Not all the measured variables were equally sensitive to the dietary challenge: 10 clinical variables, 21 metabolomics variables and 150 lipidomics variables were found to be highly sensitive to the 4 diets over the time-course of the experiment (Supplemental Table S7). With these most diet-sensitive variables, the difference in biological effect was 4 times lower between the 3 DF diets than between DF diets and the plant-fat diet (R2X[1] *vs* R2[X2], (Figure 4A)). For these variables, the time factor was responsible for 15% of the biological variability, the dietary factor was responsible for 19.5%, and the time × diet interaction was responsible for 10.3% (ASCA analysis, Figure 4).

### 3.4 Specific biological response to dairy fats *vs* non-dairy fat over time in pigs

We then investigated the differential effect of plant-fat *vs* DF at all timepoints, using the variables clustered according to functional ontologies (metabolites and clinical variables) or statistical clusters (lipids; see above). In comparison to plant-fat-fed pigs, 67 'blocks' were highly significant in DF-fed pigs at all timepoints (adjusted *P*-value < 0.001), and 28 blocks out of 67 differentiated the non-DF control diet *vs* all 3 DF diets (Supplemental Table S8). These 28 blocks corresponded to 12 unique clusters, irrespective of time. Among them, 6 lipid clusters (#1, 2, 6, 7, 8, 9) were affected at most (at least three) of the timepoints (Figure 6). In some lipid clusters, DF-fed pigs showed a decline *vs* control pigs in mainly monoenoic fatty acids such as TG (lipid cluster 1) and mainly n-6 fatty acid species in ChE and TG (lipid cluster 2; Figure 7). Conversely, n-6 fatty acids increased with DF intake in lipid cluster 8, which was dominated by TG species. Finally, DF consumption was also characterized by an increase in PI species and 30–35 carbon PC species (esterifying dairy fatty acids; lipid cluster 9) and in ω3 and dairy fatty acids in cholesteryl esters (lipid cluster 7). We also evidenced a differential regulation of metabolites involved in amino acid metabolism and metabolic disorders at later timepoints under DF diets (Figure 6).

## 3.5 Specific biological response across the dairy fat diets in pigs

After comparing DF diets *vs* the plant-fat diet, we turned to focus on the effects differentiating only the 3 DF from 6 months up to 24 months (ANOVA with adjusted *P*-value at 0.05). First, we determined the main effects of each diet by determining the recurrence of the biological effects over time. Second, we identified the time-period that revealed most of the effects (Figure 8). In fact, adding calcium to winter DF substantially changed its biological properties. Compared to winter DF alone (R3), winter DF with added calcium (R4) induced more diverse effects (n=43 *vs* 26 functions, respectively) that manifested at a later time (T18 *vs* T6/T12) and were mostly related to mitochondrial function, oxidative stress control, and various metabolic regulations *vs* hemostasis and energy metabolism (Figure 8). In comparison, the summer DF diet (R2) elicited a different pattern of biological responses that were mostly related to lipid metabolism (lipid cluster 7) but also related to amino acid (including tryptophan) metabolism (Figure 8). The greatest number of the summer DF (R2) effects was observed mainly at earlier timepoints (T6).

We then performed data comparisons between the human and pig studies. However, contrary to the metabolic functions, lipidomics comparison based on lipid-cluster similarity could not be performed as there were too many differences in lipid species between the two sample sets.

## 3.6 Overlap between Humans and Pigs

### 3.6.1 Non-dairy vs dairy fat

In the human trial, 19 biological functions were significantly modified between the DF diets *vs* the plant-fat diet. Of these 19 functions, xenobiotics, microbiota metabolism, antioxidants, metabolic disorders, oxidative stress and kidney functions showed the greatest recurrence in the 2-year pig trial (Figure 9). Tryptophan and tyrosine metabolisms and inflammation were common to both humans and pigs, but only lasted over the short term (6 months) in pigs (Figure 9). Branched-chain amino acid metabolism and gene expression regulation also occurred at an earlier time in pigs (6 months) and then reoccurred later (24 months). The

metabolites forming these biological functions corresponded to fairly diverse regions of primary metabolic pathways (using the web tool 'MetaboAnalyst'; see under Methods), including some amino acid metabolism pathways, nucleotide metabolism, and cofactor and xenobiotics metabolism (Supplemental Figure S9). These regulations common to both humans and pigs would thus represent the most constant hallmark of DF effect.

### 3.6.2 Across dairy fat-diets

Analysis focused on comparisons between DF diets only found that results from the clinical study showed only weak overlap with results from the pig study, on factors such as vascular health for R2 *vs* R3 and R4 diets, and catecholamines and branched-chain amino acid metabolisms for R3 *vs* R2 and R4 diets (Supplemental Figure S10).

# 4 Discussion

This study was designed to reveal biomarkers and effects of DF intakes in both a long-term pig model and a short-term human clinical trial. We used animals from a downsized colony of pigs selected for natural spontaneous human-like cholesterol level and responsiveness to lipid food intake [15, 16, 18], and human volunteers with serum LDL cholesterol ≥ 1.3 g/L [14]. In pigs, most of the CVD risk factors were lower at the starting timepoint (T0) than later in their life (Supplemental Figure S3 and Supplemental Table S2), presumably reflecting a more general metabolic disruption occurring over time. However, in this report, our purpose was to go beyond cardiovascular pathology to reveal the range of metabolic regulations associated with various DF in a background context of mild metabolic disorder.

In the pig study, approximately one third of the variables measured were found to be sensitive to the influence of diet (DF *vs* non-DF), resulting in a significant but limited effect (20% plus 10% in interaction with time, Figure 4). The effect was nonlinear, showing a large shift from initial status until a turning point at one year after which there was a second phase (T12 to T24 months) marked by a reverse trajectory (Figure 4). However, from this turning point, the DF diets led to an opposite pattern of response to that of the plant-fat diet, rebalancing the metabolic regulations towards the initial setting (Figure 4 and Supplemental Figure S8). With a lifespan of 15–20 years, the pigs in our study were challenged from post-weaning to young adulthood (from 5 or 6–30 months of age), and so the observed effect cannot be due to age-related metabolic alterations. However, we cannot rule out other metabolic adjustments occurring from the post-weaning period to adulthood [25]. Nevertheless, in this life period (post-weaning to young adulthood), we expected to see a metabolic shift [25], as observed in the plant-fat-fed pigs (Figure 4C), and not a trajectory of return to the initial setting. Furthermore, the kinetics of the greatest dietary influence (over the first 6 months) was desynchronized with growth rate, which is normally maximal between 12 and 18 months (Table 2). This result shows that over a long-term (one year) period, the DF used (but not the

plant-fat control) were sufficiently biologically active to trigger efficient corrective regulations in our metabolically-disrupted pig model. The main corrective regulations involved metabolism-related alteration, tryptophan metabolism and microbiota metabolites, which are both key factors for sustaining health [26, 27], amino acid metabolism and vascular health-related metabolites, and regulations that shape several plasma lipid groups. These metabolic adjustments were also accompanied by corrections of some CVD risk markers (Supplemental Figure S3), suggesting that the influence of DF on biological status could be considered beneficial to health. We do not know whether selecting another life-period or using non-disrupted metabolic conditions would have reproduced a similar pattern. However, this finding nevertheless challenges whether it is appropriate to use short-term nutritional clinical trials to estimate long-term influences of diets on biological status, as also concluded in other rare very-long-term nutritional interventions [28, 29]. Insufficient length of DF exposure could provide explanations for certain inconclusive results found elsewhere with dairy fats [3].

Despite this time-dependency (Figure 4), we were nevertheless able to find a resilient signature of DF intake in the pig study. The signature came from total plasma fatty acids analysis (C14:0 and C20:5n-3) and from the plasma LC/MS/MS lipidomics analysis (3 TG species, 4 PI species, 1 PC specie and 1 ChE specie). We could not evaluate the performance of our signature on other proposed biomarkers of milk-fat intake such as C15:0 and *trans*-16:1n-7 [5, 30, 31], as these fatty acids were not measured in both pigs and humans. Note that there are concerns around using such fatty acids including heptadecanoate as biomarkers of DF intake, as they might not be only specific to DF [32]. However, one of the lipid species in the signature found here (TG15:0/16:0/18:2) included C15:0 and flagged lipid cluster 8 which mostly comprises dairy fatty acids (Figure 7). This signature of long-term DF exposure in pigs was validated in the short-term 2-month human clinical trial in which the lipid variables were

assembled into a predictive score of DF intake generated by an equation using a PLS algorithm [22, 33-35]. The robustness and generalizability of our DF intake signature were confirmed by the interspecies validation, its high sensitivity (0.883) and specificity (0.81) (Figure 4), and the area under the ROC curve value (0.82) (Figure 1), irrespective of DF quality or duration of intake. To make our findings applicable in epidemiological practice, the variables expressed in relative intensities have to be quantified in absolute values and further tested in a vast trial. Nevertheless, our study goes beyond previous work highlighting biomarkers of DF intake based on food frequency questionnaires [5, 36] or short-term intervention studies, which can prove inaccurate.

A striking finding was that compared to plant-fat diet, the DF diets had a greater overall effect on the plasma lipidome than on the plasma metabolome (Figure 6), deeply changing lipid-species and lipid-class contents over time (Figure 7). This would of course mirror dietary fatty acid composition, but the changes that occurred reached beyond this simple influence as they appeared to constantly affect two lipid clusters, i.e. decreasing omega-6 fatty acids and increasing omega-3 fatty acids in specific lipid classes (cholesterol esters, PC and TG). This could at least partly result from the lower intake of linoleic acid in DF diets (Table 1). DF also influenced sphingolipids. Equally striking was the fact that the DF also increased omega-6 fatty acids in another co-regulated complex lipid cluster (cluster 6). In addition, dairy-origin fatty acids accumulated in specific TG and were not more broadly scattered among the whole set of lipid classes. Our analysis showed that DF intake increased PI species. Similar findings were also reported in human post-prandial plasma after intake of full-fat dairy foods [37]. These observations are relevant to the selection of several PI species in our composite biomarker signature of DF intake. Our in-depth lipidomics approach therefore shows that DF diets deeply influenced the lipids at lipid-species scale. This study thus finds that focusing solely on circulating fatty acids and lipid classes could mislead efforts to conclude on the complex

influence of DF on lipid metabolism. The lipidome should be examined more comprehensively whenever possible, otherwise important information could be missed.

Cross-analysis of the metabolic functions with differential patterns of change between the plant-fat and DF diets in the pig study and the human clinical study identified 9 functions overlapping in both trials. These 9 functions form the foundation of effects of DF *vs* plant-fat diet, which targeted important regulations for health, such as antioxidant function or oxidative stress, kidney function, and metabolic regulations including branched-chain amino acid metabolism. This connects with epidemiological studies showing benefits of DF diets, such as protecting against the most prevalent chronic non-communicable diseases (obesity, type-2 diabetes, CVD, osteoporosis, some cancers) and even mortality, with very few adverse effects reported [4]. Note the impact of the milk fats on circulating microbiota metabolites, which shows that DF affect microbiota activity/composition, as already reported [38], as shown here where DF intake was found to rebalance microbiota metabolism over time (Figure 4). This new potential of milk fat to help maintain health and prevent disease warrants further exploration. Mapping the individual metabolites of the above 9 metabolic functions into biochemical pathways made it possible to identify the molecular regulations associated with DF intake (Supplemental Figure S10). These regulations common to both human and pig studies corresponded to a numerous but narrow set of regions of primary metabolism, including some aspects of amino acid metabolism, nucleotide metabolism, and cofactor and xenobiotic metabolism

The difference in biological response was four times higher between dairy *vs* non-DF diets than across the three DF diets (Figure 4A). The differences across the DF diets concerned metabolites more than lipid regulations (Figure 8). Winter DF (R3) and summer DF (R2) appeared to change a similar number of biological functions, mostly in the early time-period (~30 biological functions affected at around 6 months). However, the summer DF induced more recurrent specific effects, ranging from various lipid metabolisms to amino acid metabolisms

including cysteine and homocysteine metabolism. The seasonal changes in milk fat fatty acid composition are therefore great enough to influence the biological status of individuals beyond plasma fatty acid composition and besides the classical markers of CVD risk already reported [11, 12, 39]. Adding calcium to the 'R3' winter DF (to afford R4) greatly changed its properties, increasing both its biological impact and recurrency (43 *vs* 26 biological functions changed), but still differentiated it from summer-season DF. This differentiation between R2 and R4 included various important functions for metabolic regulation and defense against stress, with a shift in activity period to a later timepoint (6 months with R2 and 18 months with R4). Here we used young pigs with no apparent comorbidities. It would now be instructive to investigate whether the observed differences in molecular 'omics'-based phenotypes translate into health improvement later in life. Nevertheless, our long-term pig study demonstrated that DFs are not all alike and can exert differential biological effects.

This analysis of DF effects in pigs was repeated on data from the 2-month clinical study. However, the results did not match well with the pig study, likely due to the length of dietary exposure that may have been too short and possibly also to the influence of other components of human diet not found in the pig diets, and/or to interspecies differences. However, our analysis of the number of biological functions affected nevertheless showed that winter DF had the strongest—but limited—specific biological effects, whereas the winter DF with added calcium conversely had the lowest metabolic impact.

## 5 Conclusion

Interspecies validation of the combination of biomarkers found here provides a robust and universal biomarker signature for assessing DF intake in various clinical settings. Research is now warranted to evaluate whether this signature can also estimate amounts of intake and be readily extended to epidemiological practice.

In pigs, the effect of the DF diets accounted for ~20% of biological variation and another 10% in interaction with the time factor. The effect is U-shaped, specifically bringing metabolism—including some markers of risk for CVD—back towards the initial biological status after a turning point at one year. On the long-term, milk fats can thus trigger specific compensatory effectors in these metabolically-disrupted pigs in order to bring homeostasis back to the younger healthier status, which means that DF could be considered beneficial in this condition. Whether this would also happen in humans in the context of a far more diversified food intake has yet to be confirmed, but has been suggested elsewhere [40]. Our findings nevertheless challenge the relevance of using short-term nutritional clinical studies to conclude on the effects of long-term nutritional exposure to DF.

Despite the time-related drift in biological response shown in pigs, we also found recurrent DF-specific biological effects (BCAA metabolism, antioxidant/oxidative stress control, kidney function, metabolic regulations) that overlapped with the short-term clinical human trial. These metabolic functions corresponded to molecular regulations associated with a narrow area of primary metabolism, including some amino acid metabolic pathways as well as nucleotide, xenobiotic and cofactor metabolic pathways that may point to benefits of consuming DF. Note too that DF has a potential impact on microbiota metabolic activity, which warrants further research.

The deep phenotyping employed here in the pig study was able to differentiate the biological influence of each DF diet over different time-windows (early for R2, intermediate for R3, later for R4) and to various extents, especially when calcium was added (R4 *vs* R3). Whether these differences at metabolic systems level translate into health improvements later in life warrants further investigation, but it is already clear that quality of the DF matters.

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**Table 1.** Composition (in %) of the main fatty acids in the dietary fats.

| **Fatty acids** | **R1** | **R2** | **R3 and R4** |
|---|---|---|---|
| **Total saturated fatty acids** | 64 | 64 | 74 |
| **C14:0** | 0.9 | 10 | 10 |
| **C16:0** | 40 | 27 | 37 |
| **Total monounsaturated fatty acids** | 29 | 30 | 22 |
| **C18:11*t* (*trans*-vaccenic acid)** | 0.0 | 4.5 | 1.5 |
| **Total polyunsaturated fatty acids** | 20 | 4.0 | 2.0 |
| **C18:2n-6** | 19 | 1.4 | 1.4 |
| **C18:3n-3** | 0.3 | 0.9 | 0.3 |
| **C18:9*c*,11*t* (rumenic acid)** | 0.0 | 0.8 | 0.5 |
| **w6/w3 ratio** | 63.3 | 1.6 | 4.7 |

R1: plant fat, R2: summer dairy fat, R3: winter dairy fat, R4: winter dairy fat with added calcium

**Table 2**. Anthropometric data on the 173 volunteers who completed the nutritional challenge (mean±SD).

| Variable | R1: plant fat | R2: summer dairy fat | R3: winter dairy fat | R4: winter dairy fat + calcium | Adjusted *P*-value between groups |
|---|---|---|---|---|---|
| Males | 16 (39%) | 17 (37%) | 16 (36%) | 14 (34%) | 0.972* |
| Females | 25 (61%) | 29 (63%) | 29 (64%) | 27 (66%) | |
| Age | 51.3±10.7 | 47.8±12.0 | 50.0±12.1 | 50.8±11.7 | 0.430 |
| BMI 8 weeks | 24.5±3.2 | 25.0±2.6 | 24.5±2.9 | 24.9±2.6 | 0.705 |
| Smoker–yes | 4 | 2 | 6 | 5 | 0.486* |
| Smoker–no | 37 | 44 | 39 | 36 | |
| SBP–baseline (mm Hg) | 127±16 | 125±14 | 124±13 | 128±14 | 0.58 |
| SBP–8 weeks (mm Hg) | 124±12 | 126±15 | 126±15 | 127±13 | 0.51 |
| DBP–baseline (mm Hg) | 76±11 | 77±10 | 79±9 | 78±7 | 0.38 |
| DBP–8 weeks (mm Hg) | 75±9 | 76±9 | 76±9 | 75±8 | 0.93 |
| WC–baseline (in cm) | 82±11 | 84±10 | 84±10 | 82±8 | 0.46 |
| WC–8 weeks (in cm) | 82±11 | 84±10 | 83±10 | 82±8 | 0.98 |

*Chi² test

DBP: diastolic blood pressure; SBP: systolic blood pressure; WC: waist circumference

**Table 3**. Zoometric data on the 32 pigs used in the study, including 4 females and 4 castrated males in each diet group (mean±SD).

| Variable | R1 plant fat | R2 summer dairy fat | R3 winter dairy fat | R4 winter dairy fat + calcium | adjusted *P*-value between groups |
|---|---|---|---|---|---|
| Age at start (months) | 5.6±0.6 | 5.8±0.6 | 6.6±0.5 | 4.5±0.7 | 0.59 |
| T0_body weight (kg) | 22.4±2.0 | 22.0±3.6 | 22.3±2.6 | 18.0±4.0 | 0.59 |
| T6_body weight (kg) | 34.8±5.3 | 43.6±7.5 | 40.2±5.2 | 40.7±7.8 | 0.91 |
| T12_body weight (kg) | 65.2±4.7 | 72.1±11.4 | 73.8±8.9 | 74.0±11.7 | 0.59 |
| T18_body weight (kg) | 95.7±3.3 | 112.7±11.4 | 106.4±10.4 | nd | nd |
| T24_body weight (kg) | 121.2±7.2 | 121.5±20.8 | 112.8±14.1 | 124.3±13.1 | 0.99 |
| T24_systolic blood pressure (mm Hg) | 168.6±14.8 | 183.8±20.3 | 167.7±21.1 | 171.4±16.4 | 0.91 |
| T24_diastolic blood pressure (mm Hg) | 109.6±11.0 | 121.5±12.1 | 128.6±13.6 | 104.9±37.1 | 0.91 |
| T24_bladder weight (g) | 223.8±27.6 | 258.8±43.43 | 343.1±66.2 | 390.6±117.9 | 0.91 |
| T24_liver weight (g) | 1446.3±232.5 | 1147.5±123.3 | 1172.5±154.9 | 1293.8±152.6 | 0.91 |
| T24_right kidney weight (g) | 123.1±6.3 | 116.9±8.6 | 133.9±7.2 | 122.5±13.2 | 0.99 |
| T24_left kidney weight (g) | 122.5±9.0 | 115.0±8.2 | 120.6±7.2 | 125.0±12.6 | 0.99 |
| T24_lung weight (g) | 570.0±12.9 | 572.5±85.8 | 767.5±103.7 | 695.0±64.8 | 0.91 |
| T24_bone mineral density (g/cm²) | 1589±71 | 1686±86 | 1575±47 | 1710±66 | 0.91 |
| T24_CMO (g) | 2665±304 | 2694±224 | 2482±189 | 3052±299 | 0.91 |
| T24_body area (cm²) | 1651±110 | 1643±92 | 1568±84 | 1842±101 | 0.88 |
| T24_% fat | 38.8±2.0 | 34.7±2.9 | 41.3±1.9 | 33.0±3.0 | 0.59 |
| T24_fat mass (g) | 45270±2122 | 40964±7386 | 49084±4670 | 40808±6721 | 0.91 |
| T24_lean mass (g) | 73215±6726 | 72911±9983 | 68877±5271 | 69769±10868 | 0.99 |

**Figure legends**

**Figure 1**. Receiver operating characteristic (ROC) curve using the 12 selected biomarkers of dairy fat intake combined with a PLS-DA algorithm to predict dairy fat-fed *vs* non-dairy fat-fed pigs. The model was trained with the T6 pigs (panel A) to estimate dairy/non-dairy intake at T0, T12, T18 and T24 (panel B). Predictions were almost 100% correct for dairy-fed *vs* non-dairy-fed pigs at each timepoint.

**Figure 2**. Panel **A**, Statistical proximity in the multivariate response of the 12 indicators of dairy fat intakes in pigs across dietary groups. Upper panel: loading plot of a PLS-DA analysis with Y as diet factor showing the *c* vector summarizing the overall score for the dairy-fat biomarkers in each dietary group. Lower panel: HCA using the *c* vector and showing the statistical proximity of the dairy-fat biomarker responses in each dietary group. Panel **B**, time–course trajectory of dairy-fat intake using the dairy-fat intake biomarkers, as assessed in the loading plot of a PLS-DA analysis (with Y as time factor) and using the *c* vector (upper panel) or an HCA (lower panel). Panel **C**, time–course trajectory of plant-fat intake using the selected biomarkers, as assessed in the loading plot of a PLS-DA analysis (with Y as time factor) and using the **c** vector (upper panel) or an HCA (lower panel).

**Figure 3**. Prediction rate of dairy-fat intake in the clinical study using the biomarkers of dairy-fat intake by pigs and combined into a score using an equation calculated with the NIPALS algorithm. ND: not determined.

**Figure 4**. Panel **A**, Statistical proximity in the multivariate response of the diet-sensitive variables (10 clinical variables, 21 metabolomics variables and 150 lipidomics variables; Supplemental Table S7, see results) in pigs across dietary groups (R1, plant fat; R2, summer dairy fat, R3, winter dairy fat, R4, winter dairy fat with added calcium). Upper panel: loading plot of a PLS-DA analysis with Y as diet factor showing the *c* vector summarizing the overall

score for the diet-sensitive variables in each dietary group. Lower panel: HCA using the *c* vectors and showing the statistical proximity of responses in the diet-sensitive variables in each dietary group. Panel **B**, time–course trajectory of biological responses in dairy-fat-fed pigs using all the measured variables (clinical, metabolomics, lipidomics), assessed in the loading plot of a PLS-DA analysis (with Y as time factor) and using the *c* score (a proxy for the group barycenter; upper panel) or an HCA (lower panel). Panel **C**, time–course trajectory of biological responses in plant-fat-fed pigs using all the measured variables, as assessed in the loading plot of a PLS-DA analysis (with Y as time factor) and using the *c* score (upper panel) or an HCA (lower panel). Panel **D**, calculation of the influence of either time or diet factors on the selected dairy fat-sensitive variables and time × variable interactions, using ANOVA simultaneous component analysis (ASCA).

**Figure 5.** Variables modeled as clusters (lipids) or functional groups (metabolites) differing in pig plasma between T6–T12 months and T0–T24 months. **Panel A**, normal probability plot showing the shift in normal distribution of the PLS-DA VIP values indicating very significant variables. **Panel B**, Student's *t*-test with *P* values of each variable and expressed in –log10. **Panel C**, HCA (Ward method) and variable-intensity heatmap showing T0 and T24 or T6 and T12 pigs and lipid clusters or metabolic functions. Dark grey: positive values, light gray: negative values, in relative intensity.

**Figure 6**. Venn plot showing the metabolic systems-based biological response to dairy fats compared to the non-dairy-fat diet over time in the pig study. The variables were combined into metabolic functions or lipid clusters (see Methods section) and compared between dairy-fat-fed and non-dairy fat-fed pigs over time.

**Figure 7**. Lipid composition of selected lipid clusters in the plasma of pigs fed the various diets. Percentages of lipid classes are displayed in circles, and the component lipid species are shown as a heatmap where red colors indicate a relative increase and blue colors indicate a

relative decrease. The list of lipid species for each timepoint (T6, T12, T18, T24) is given in the same order as they appear in the heatmaps.

**Figure 8**. Specific biological response to each dairy fat in pigs over time using the metabolic systems approach (see Methods section). For each biological function, character size relates to its recurrence over time. For the time dimension, character size corresponds to the number of biological functions influenced at each timepoint. R2, summer dairy-fat diet; R3, winter dairy-fat diet; R4, the R3 diet with added calcium.

**Figure 9**. Venn plot of common biological responses at metabolic systems scale in both pigs and humans. The most common shared functions are given.

**A**

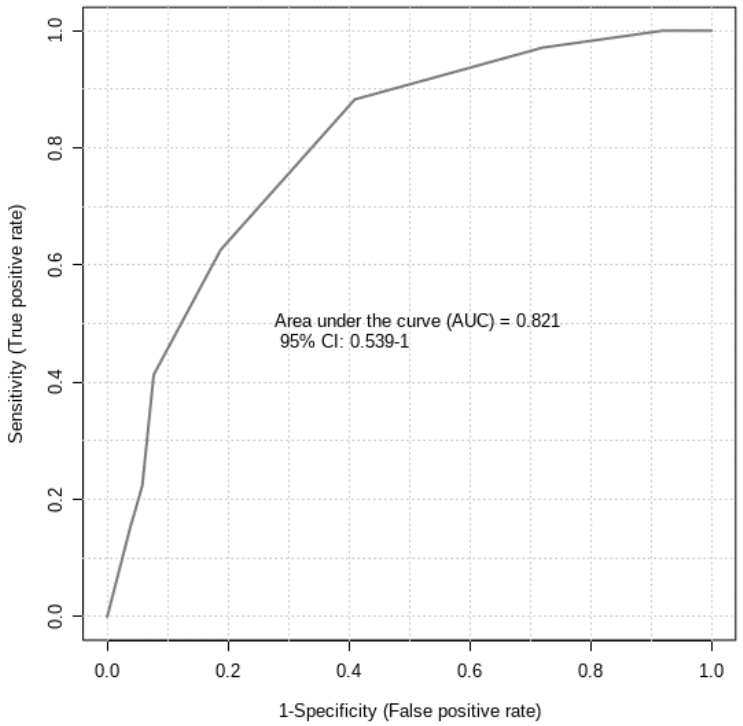



**FIGURE 1**

**B**

| Time | T0 | T12 | T18 | T24 |
|---|---|---|---|---|
| Dairy intake pigs | 0 | 100% | 100% | 92% |
| Non dairy intake pigs | 79% | 100% | 100% | 100% |

Figure 2

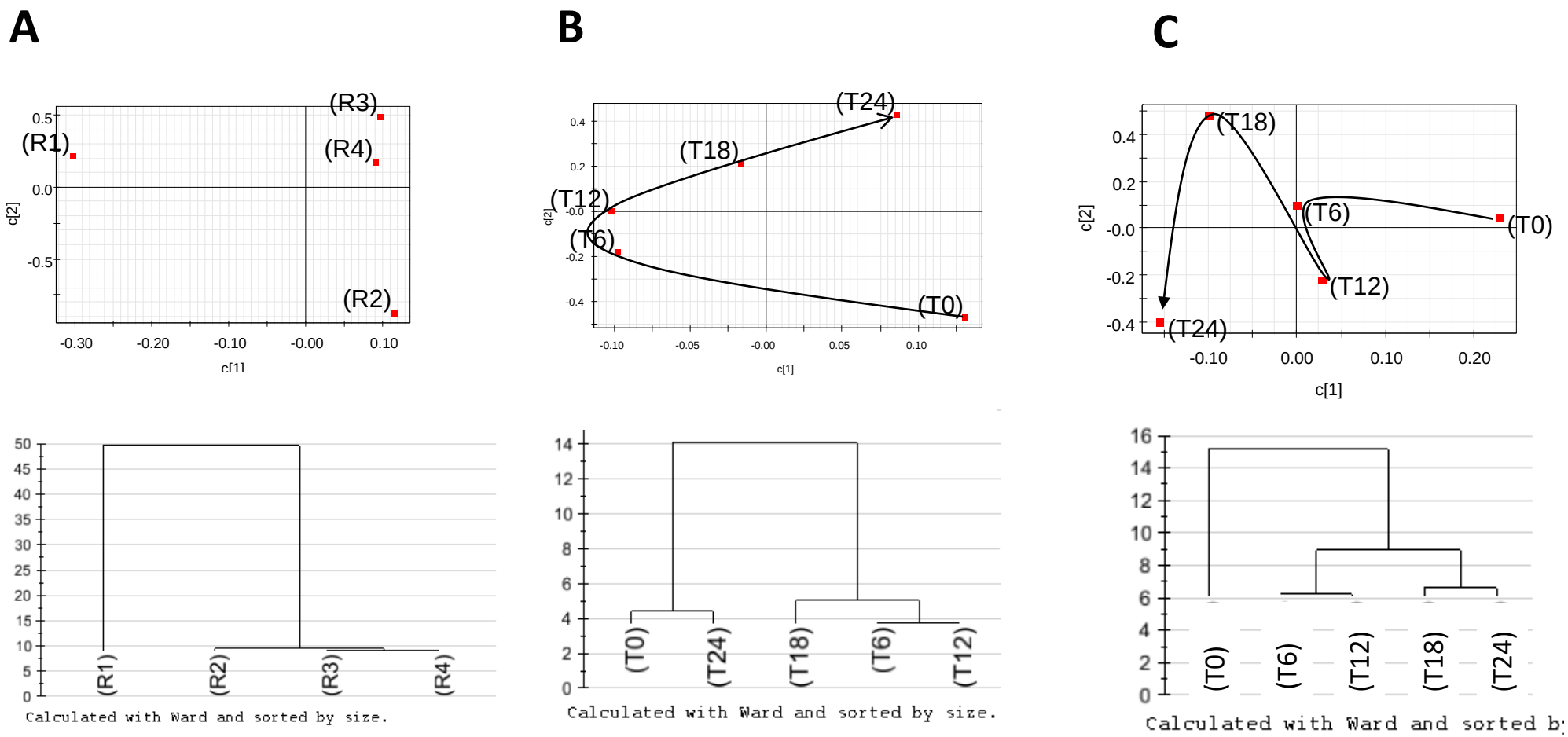
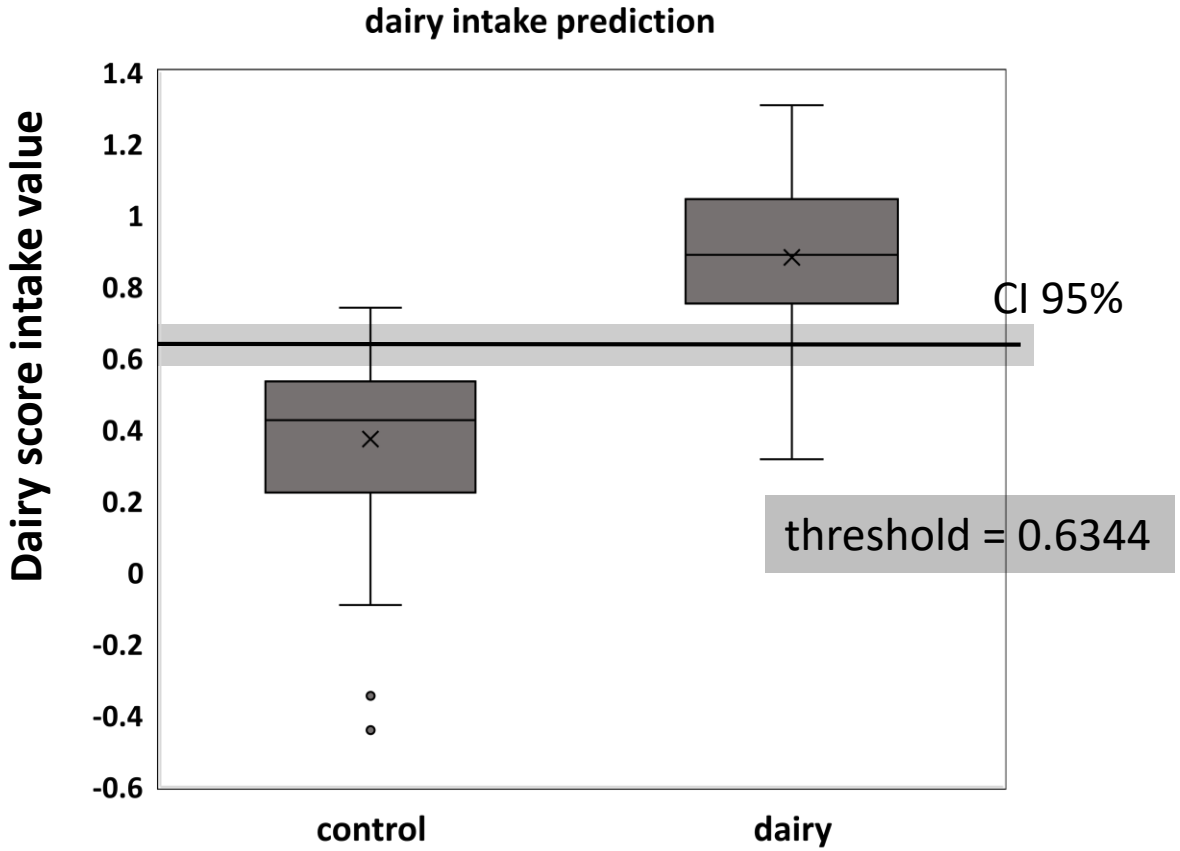

A
B
C
(R1)
(R2)
(R3)
(R4)
(T0)
(T6)
(T12)
(T18)
(T24)
c[1]
c[2]
Calculated with Ward and sorted by size.

**Figure 3**

Human study

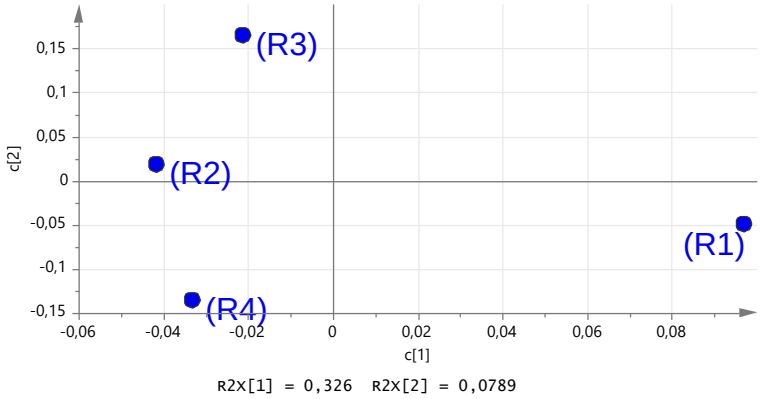
**dairy intake score** =
-0.00101505***(C14:0)** -0.0007791***(C20:5n-3)** -0.1561508***(ChE(22:5))** +0.5204846***(PC(31:1))** +0.02480472***(PC(33:1))** +0.27208***(PI(16:0/16:1))** -0.5702826***(PI(16:0/18:1))** -0.7779556***(PI(18:0/18:1))** +11.724626***(PI(18:0p/20:4))** +0.01611526***(TG(15:0/16:0/18:2))** -0.05135144***(TG(18:1/18:1/20:3))** +0.0499953***(TG(18:2/20:4/20:4))** +0.9796806

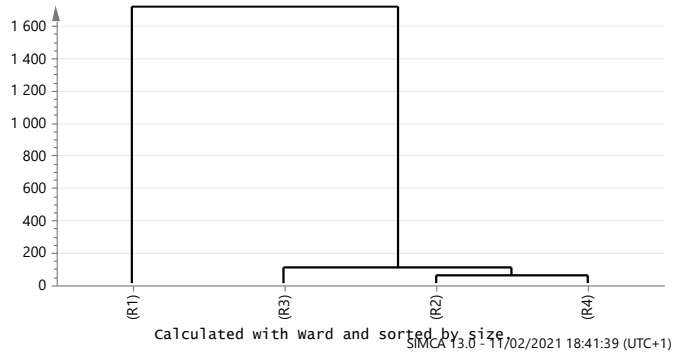



| CI 95% | control | dairy | ND |
|---|---|---|---|
| control | 34 | 2 | 3 |
| dairy | 8 | 105 | 8 |
| | | | |
| CI 99% | control | dairy | ND |
| control | 34 | 2 | 3 |
| dairy | 8 | 102 | 11 |

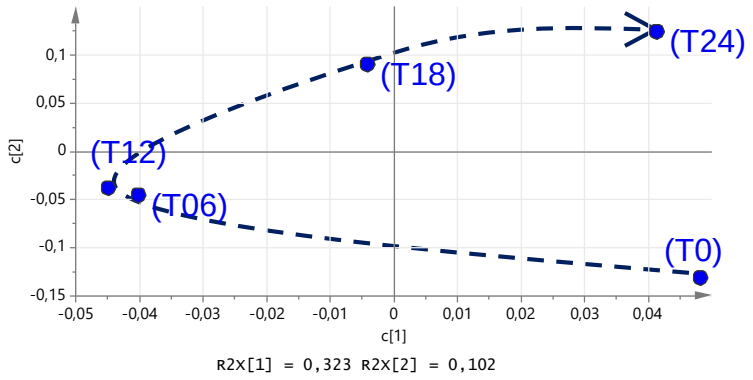
Figure 4

**A**



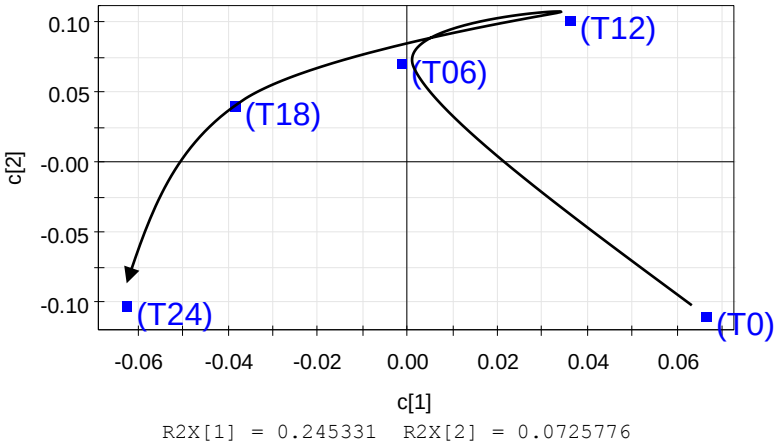



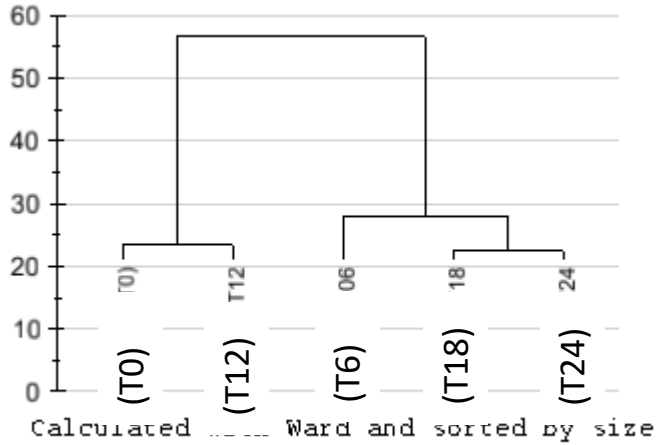

**B**





**C**

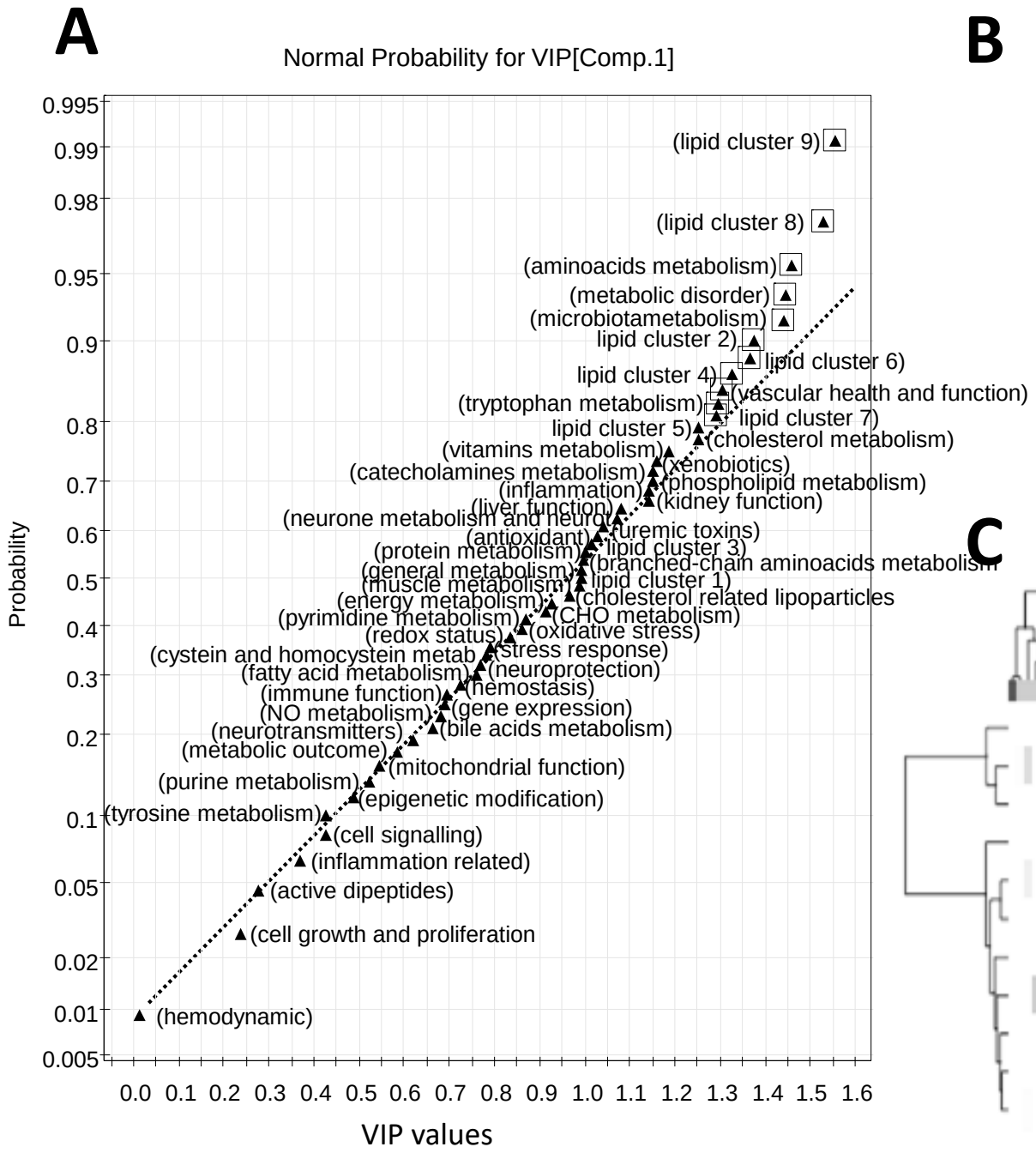


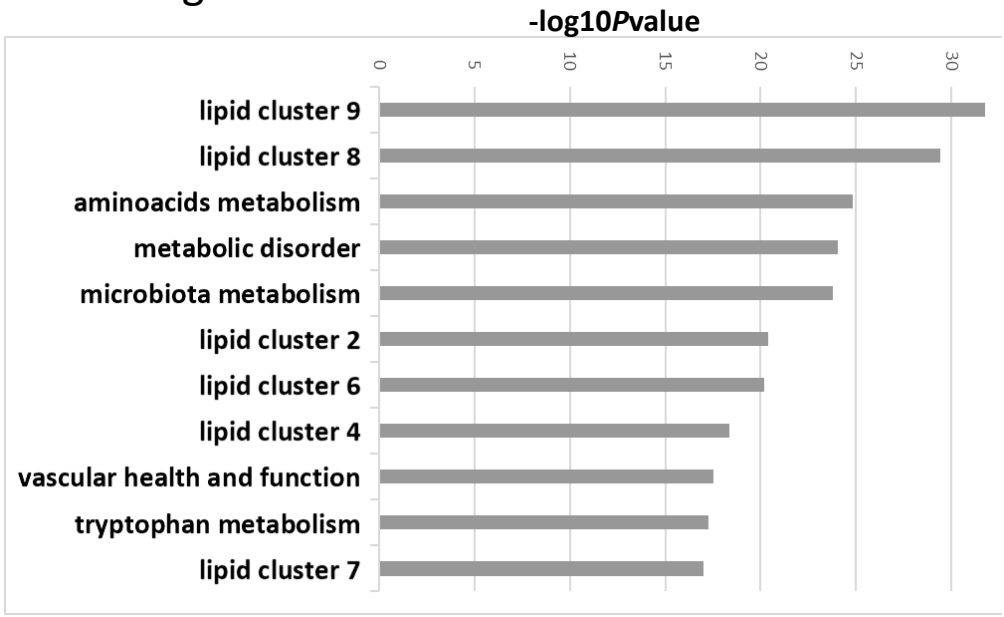




**D**

| | % of explained variance | Permutation p-value |
|---|---|---|
| time | 15.00 | 0.001 |
| diet | 19.42 | 0.001 |
| Interaction | 10.28 | 0.013 |
| Residuals | 55.01 | 0.000 |

Figure 5


A
Normal Probability for VIP[Comp.1]
Probability
VIP values
0.995
0.99
0.98
0.95
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.05
0.02
0.01
0.005
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.1 1.2 1.3 1.4 1.5 1.6
(lipid cluster 9)
(lipid cluster 8)
(aminoacids metabolism)
(metabolic disorder)
(microbiotametabolism)
lipid cluster 2)
lipid cluster 6)
lipid cluster 4)
(vascular health and function)
(tryptophan metabolism)
lipid cluster 7)
lipid cluster 5)
(cholesterol metabolism)
(vitamins metabolism)
(xenobiotics)
(catecholamines metabolism)
(phospholipid metabolism)
(inflammation)
(kidney function)
(liver function)
(neurone metabolism and neurot
(uremic toxins)
(antioxidant)
lipid cluster 3)
(protein metabolism)
(branched-chain aminoacids metabolism
(general metabolism)
lipid cluster 1)
(muscle metabolism)
(cholesterol related lipoparticles
(energy metabolism)
(CHO metabolism)
(pyrimidine metabolism)
(oxidative stress)
(redox status)
(stress response)
(cystein and homocystein metab
(neuroprotection)
(fatty acid metabolism)
(hemostasis)
(immune function)
(gene expression)
(NO metabolism)
(bile acids metabolism)
(neurotransmitters)
(metabolic outcome)
(mitochondrial function)
(purine metabolism)
(epigenetic modification)
(tyrosine metabolism)
(cell signalling)
(inflammation related)
(active dipeptides)
(cell growth and proliferation
(hemodynamic)


B
-log10Pvalue
0
5
10
15
20
25
30
lipid cluster 9
lipid cluster 8
aminoacids metabolism
metabolic disorder
microbiota metabolism
lipid cluster 2
lipid cluster 6
lipid cluster 4
vascular health and function
tryptophan metabolism
lipid cluster 7

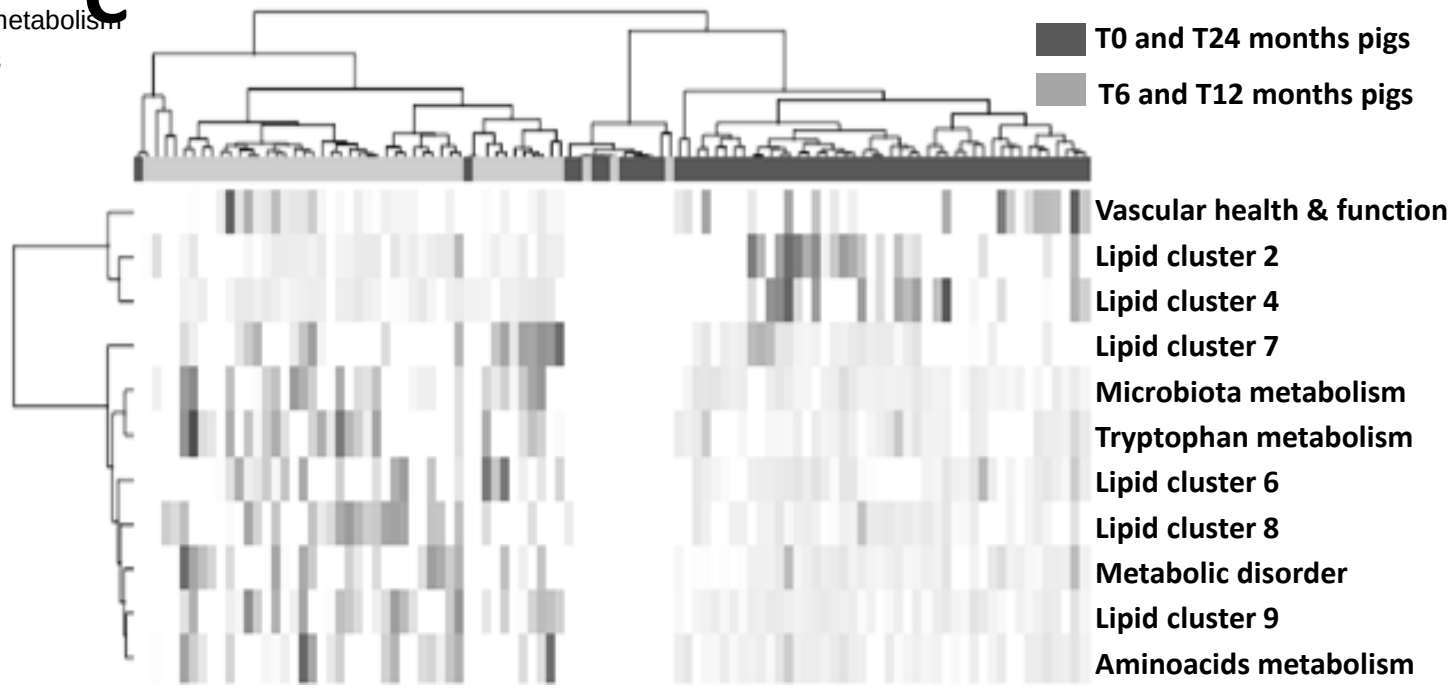
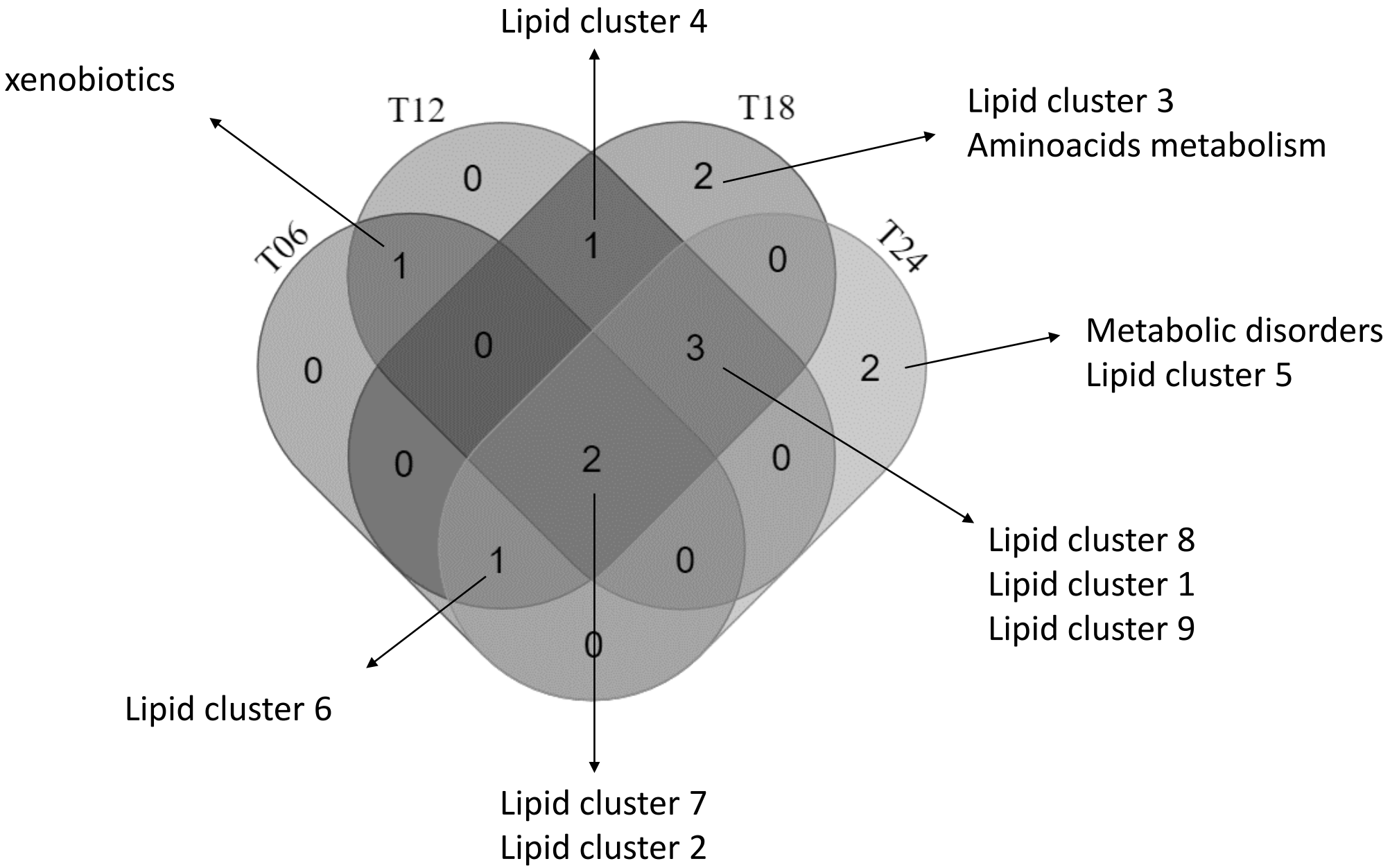
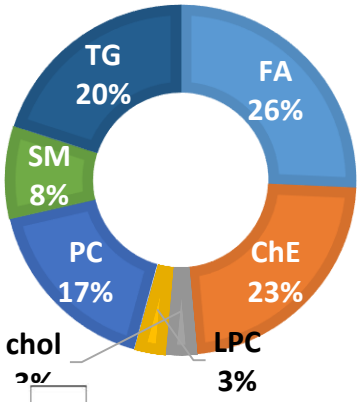
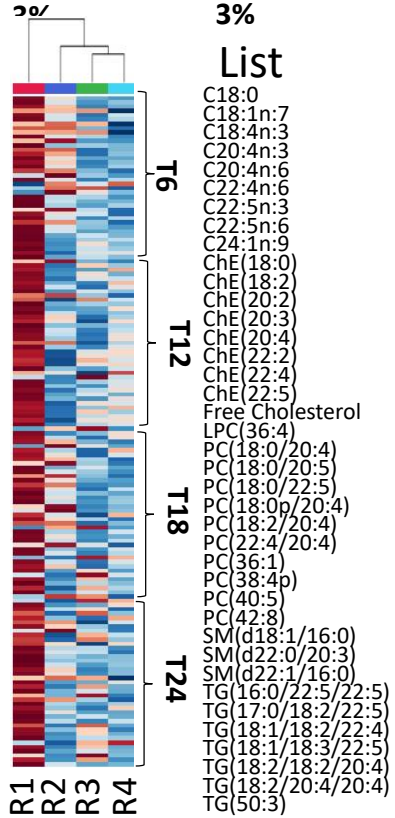

C
T0 and T24 months pigs
T6 and T12 months pigs
Vascular health & function
Lipid cluster 2
Lipid cluster 4
Lipid cluster 7
Microbiota metabolism
Tryptophan metabolism
Lipid cluster 6
Lipid cluster 8
Metabolic disorder
Lipid cluster 9
Aminoacids metabolism

Figure 6


Lipid cluster 4
xenobiotics
T12
T18
Lipid cluster 3
Aminoacids metabolism
T06
T24
0
2
1
1
0
0
0
3
2
0
2
0
1
0
0
Metabolic disorders
Lipid cluster 5
Lipid cluster 8
Lipid cluster 1
Lipid cluster 9
Lipid cluster 6
Lipid cluster 7
Lipid cluster 2

## LIPID CLUSTER 2
(62% w6 lipid species)

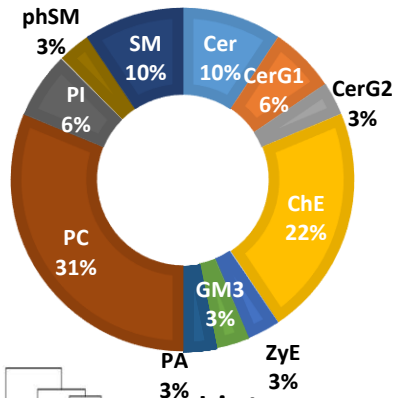


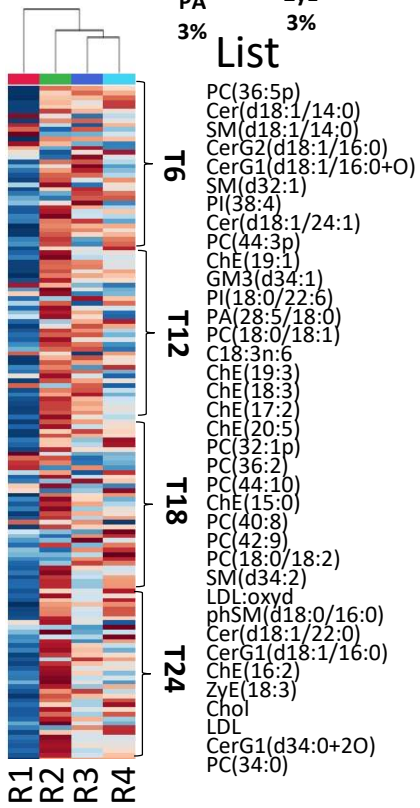




List

C18:0
C18:1n:7
C18:4n:3
C20:4n:3
C20:4n:6
C22:4n:6
C22:5n:3
C22:5n:6
C24:1n:9
ChE(18:0)
ChE(18:2)
ChE(20:2)
ChE(20:3)
ChE(20:4)
ChE(22:2)
ChE(22:4)
ChE(22:5)
Free Cholesterol
LPC(36:4)
PC(18:0/20:4)
PC(18:0/20:5)
PC(18:0/22:5)
PC(18:0p/20:4)
PC(18:2/20:4)
PC(22:4/20:4)
PC(36:1)
PC(38:4p)
PC(40:5)
PC(42:8)
SM(d18:1/16:0)
SM(d22:0/20:3)
SM(d22:1/16:0)
TG(16:0/22:5/22:5)
TG(17:0/18:2/22:5)
TG(18:1/18:2/22:4)
TG(18:1/18:3/22:5)
TG(18:2/18:2/20:4)
TG(18:2/20:4/20:4)
TG(50:3)

## LIPID CLUSTER 7
(chol esters, w3 lipids, sphingolipids)

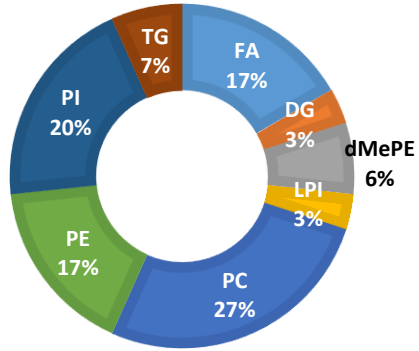


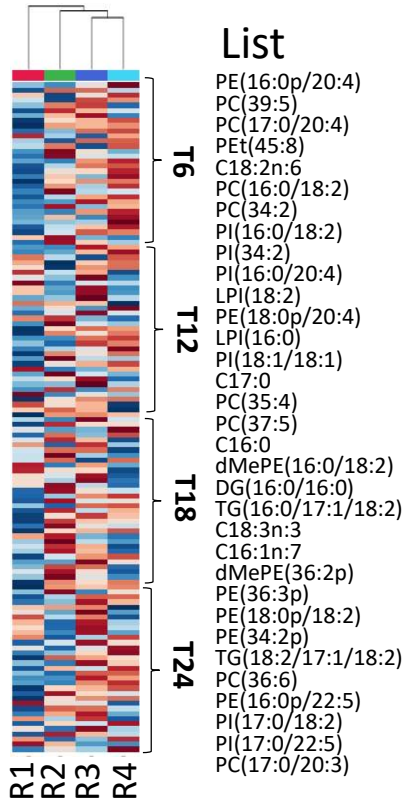




List

PC(36:5p)
Cer(d18:1/14:0)
SM(d18:1/14:0)
CerG2(d18:1/16:0)
CerG1(d18:1/16:0+O)
SM(d32:1)
PI(38:4)
Cer(d18:1/24:1)
PC(44:3p)
ChE(19:1)
GM3(d34:1)
PI(18:0/22:6)
PA(28:5/18:0)
PC(18:0/18:1)
C18:3n:6
ChE(19:3)
ChE(18:3)
ChE(17:2)
ChE(20:5)
PC(32:1p)
PC(36:2)
PC(44:10)
ChE(15:0)
PC(40:8)
PC(42:9)
PC(18:0/18:2)
SM(d34:2)
LDL:oxyd
phSM(d18:0/16:0)
Cer(d18:1/22:0)
CerG1(d18:1/16:0)
ChE(16:2)
ZyE(18:3)
Chol
LDL
CerG1(d34:0+2O)
PC(34:0)

## LIPID CLUSTER 6
(mainly w6 in PI and PE lipids)

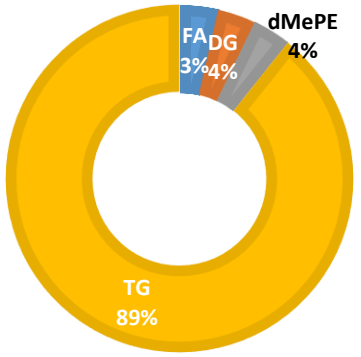


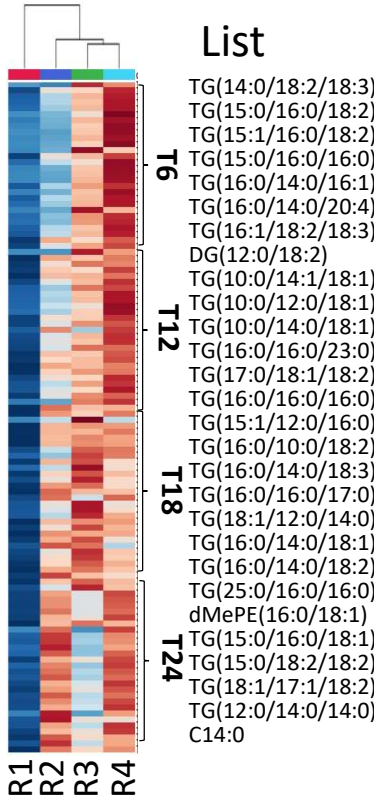




List

PE(16:0p/20:4)
PC(39:5)
PC(17:0/20:4)
PEt(45:8)
C18:2n:6
PC(16:0/18:2)
PC(34:2)
PI(16:0/18:2)
PI(34:2)
PI(16:0/20:4)
LPI(18:2)
PE(18:0p/20:4)
LPI(16:0)
PI(18:1/18:1)
C17:0
PC(35:4)
PC(37:5)
C16:0
dMePE(16:0/18:2)
DG(16:0/16:0)
TG(16:0/17:1/18:2)
C18:3n:3
C16:1n:7
dMePE(36:2p)
PE(36:3p)
PE(18:0p/18:2)
PE(34:2p)
TG(18:2/17:1/18:2)
PC(36:6)
PE(16:0p/22:5)
PI(17:0/18:2)
PI(17:0/22:5)
PC(17:0/20:3)

## LIPID CLUSTER 8
(mainly dairy FA in TG)

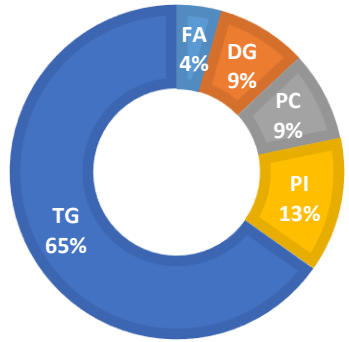


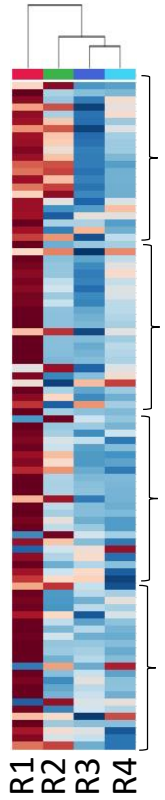




List

TG(14:0/18:2/18:3)
TG(15:0/16:0/18:2)
TG(15:1/16:0/18:2)
TG(15:0/16:0/16:0)
TG(16:0/14:0/16:1)
TG(16:0/14:0/20:4)
TG(16:1/18:2/18:3)
DG(12:0/18:2)
TG(10:0/14:1/18:1)
TG(10:0/12:0/18:1)
TG(10:0/14:0/18:1)
TG(16:0/16:0/23:0)
TG(17:0/18:1/18:2)
TG(16:0/16:0/16:0)
TG(15:1/12:0/16:0)
TG(16:0/10:0/18:2)
TG(16:0/14:0/18:3)
TG(16:0/16:0/17:0)
TG(18:1/12:0/14:0)
TG(16:0/14:0/18:1)
TG(16:0/14:0/18:2)
TG(25:0/16:0/16:0)
dMePE(16:0/18:1)
TG(15:0/16:0/18:1)
TG(15:0/18:2/18:2)
TG(18:1/17:1/18:2)
TG(12:0/14:0/14:0)
C14:0

## LIPID CLUSTER 1
(65% w9 lipids)

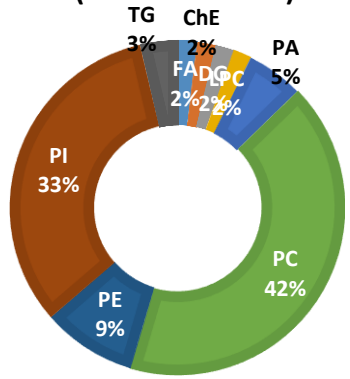


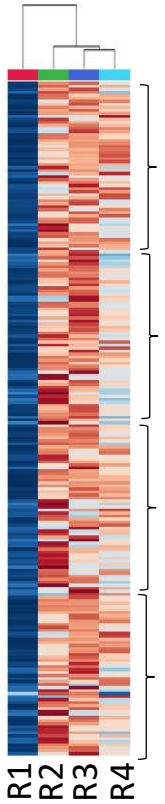




List

C20:1
TG(18:1/18:1/20:3)
TG(18:1/18:2/18:2)
TG(16:0/18:1/18:1)
TG(18:2/18:2/18:2)
TG(18:1/18:1/18:2)
DG(18:1/18:2)
TG(16:0/18:1/18:2)
DG(18:2/18:2)
TG(18:3/18:2/18:2)
TG(18:1/18:2/24:1)
TG(18:1/17:1/20:4)
TG(26:1/18:1/18:2)
TG(26:1/18:2/18:2)
TG(25:1/18:1/18:1)
TG(27:0/18:1/18:2)
TG(18:2/17:1/20:4)
PC(17:4/18:1)
PC(37:4)
PI(19:0/20:4)
TG(18:1p/16:0/18:1)
PI(17:0/20:4)
PI(18:0/20:3)

## LIPID CLUSTER 9
(enriched in PI)



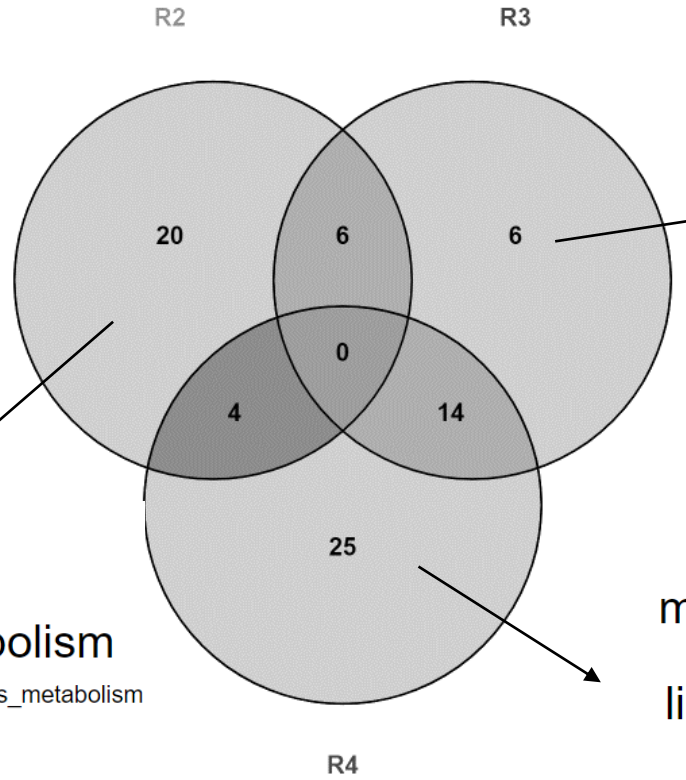




List

PI(16:0e/18:1)
PI(34:3)
PA(22:5/21:1)
PC(15:0/18:2)
PC(33:2)
PC(34:3)
PC(33:1)
PC(35:2)
PI(17:0/18:1)
PA(20:4/23:1)
PC(15:0/18:1)
PC(34:4)
PC(35:3)
PA(27:6/18:0)
PC(17:0/18:2)
PI(16:0/16:0)
PI(18:0/16:0)
PI(18:0/18:1)
PI(36:1)
PI(16:0/18:1)
PI(15:0/18:1)
PI(16:0/16:1)
PC(31:1)
PC(15:0/16:0)
PC(31:0)
PC(29:0)
PC(30:0)
PI(38:4p)
PI(18:0e/18:2)
PI(18:0p/20:4)
PC(15:0/20:4)
HDL
PC(17:0/20:5)
PI(18:0e/18:1)
PC(16:0/16:1)
PC(32:1)
PC(40:2)
PE(18:0p/22:6)
PI(16:0/22:6)
TG(20:0e/14:0/18:1)
TG(20:0e/16:0/16:0)
C20:5n:3
PC(16:0/20:5)
ChE(14:0)
PE(16:0p/18:1)
PI(18:0e/22:5)
PC(18:1/18:2)
DG(20:0/18:1)
PC(36:3)
PI(18:1/18:2)
LPC(18:3)
PC(16:0/18:3)
PE(38:1p)
PE(18:0p/18:1)
PE(36:1p)
PI(16:0/18:3)

Figure 7

Figure 8




**N= 26 biological clusters**

fatty_acid_metabolism
lip_cluster_3
tryptophan_metabolism
lip_cluster_8
kidney_related_function
tyrosine_metabolism
gene_expression
lip_cluster_6
lip_cluster_9
CHO_metabolism
aminoacids_metabolism
hemostasis
vascular_health
antioxidant
microbiota_metabolism
phospholip_metabolism
lip_cluster_2
lip_cluster_1
stress_response
immune_function
lip_cluster_5
energy_metabolism

T24
T18 T6 T12

**N= 30 biological clusters**

tryptophan_metabolism
gene_expression
lip_cluster_6
mitochondrial_function
cystein_and_homocystein_metabolism
cholesterol_related_metabolism
vitamins_metabolism
lip_cluster_3
lip_cluster_8
lip_cluster_2
branched-chain_aminoacids_metabolism
lip_cluster_9
lip_cluster_7
cell_growth_and_proliferation
lipd_cluster_4
oxidative_stress
aminoacids_metabolism
fatty_acid_metabolism
energy_metabolism
vascular_health
active_dipeptide
neurone_metabolism_and_neurotransmitter
phospholip_metabolism

T18 T12
T24 T6

**N= 43 biological clusters**

metabolic_disorder
tryptophan_metabolism
oxidative_stress
lip_cluster_9
xenobiotics
pyrimidine_metabolism
antioxidant
stress_response
lip_cluster_3
general_metabolism
kidney_related_function
branched-chain_aminoacid_metabolism
microbiota_metabolism
mitochondrial_function
hemostasis
catecholamine_metabolism
immune_function
lip_cluster_8
redox_status
fatty_acid_metabolism
lip_cluster_2
lip_cluster_6
cystein_and_homocystein_metabolism
energy_metabolism
tyrosine_metabolism
vascular_health
CHO_metabolism
metabolic_outcome

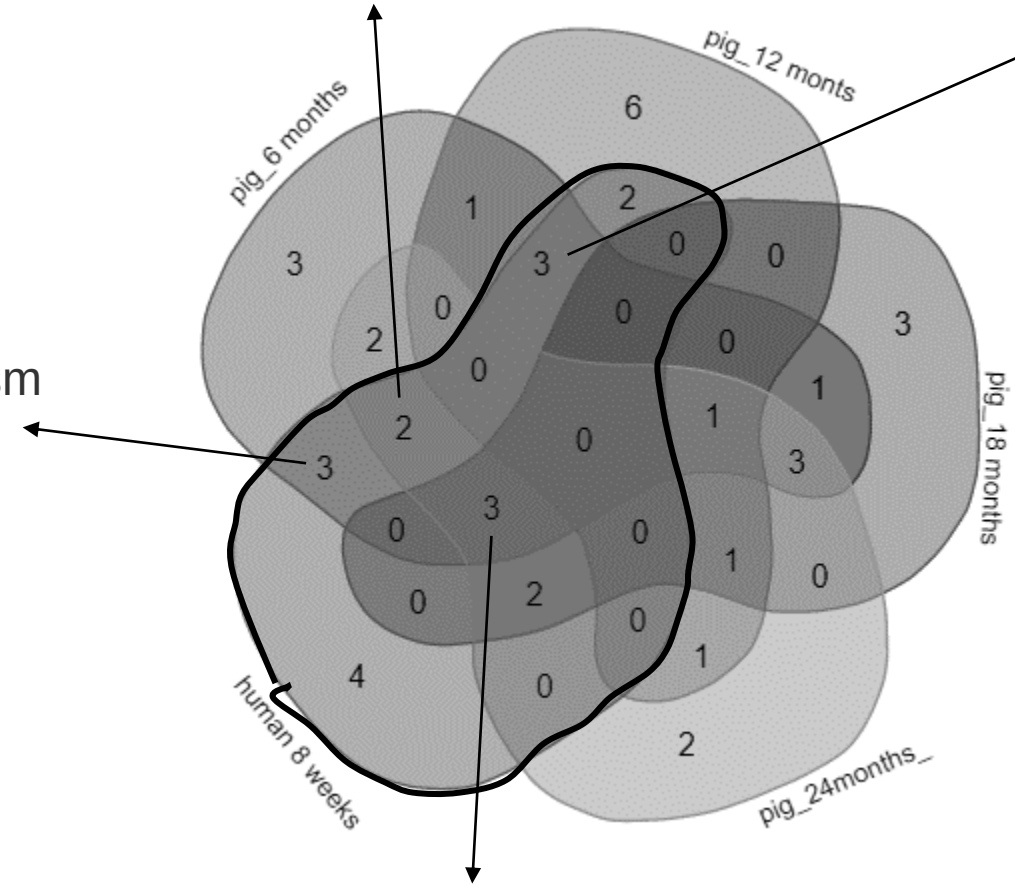
T18
T24
T12 T6

Figure 9

branched-chain aminoacids metabolism
gene expression

xenobiotics
microbiota metabolism
antioxidant

tryptophan metabolism
tyrosine metabolism
inflammation

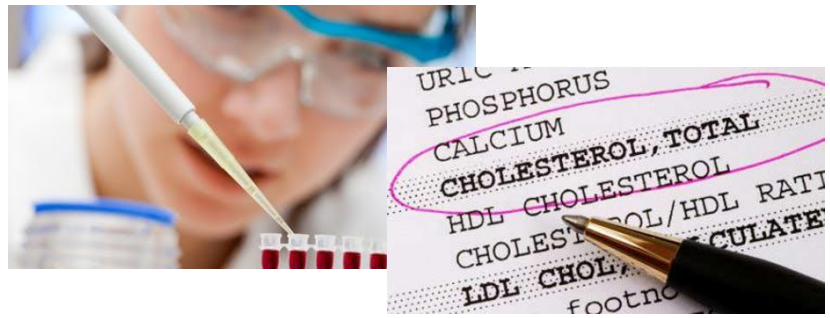



metabolic disorder
oxidative stress
kidney function

173 volunteers (63,5% ♀, 36,5% ♂)
1.6 ≤ LDL-cholesterol < 2.2 g/l

**X**

32 downsized pigs (50% castrated ♂ et ♀)
Mild hypercholesterolemia
(LDL receptor R84C mutation)




Conventional clinical status

« omics » (metabolomics and lipidomics)

- Plant-fat R1
- Summer dairy fat R2
- Winter dairy fat R3
- Winter dairy fat + $Ca^{2+}$ R4

T0 T2months T6months T12months T18months T24months