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## **Deep phenotyping and biomarkers of various dairy fat intakes in an 8-week randomized clinical trial and 2-year swine study**

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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.

## 1 **Abstract**

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

10 DF intakes in pigs specifically induced a U-shaped metabolic trajectory, reprogramming  
11 metabolism to close to its initial status after a one-year turnaround. Twelve lipid species  
12 repeatably predicted DF intakes in both pigs and humans (6.6% errors). More broadly, in pigs,  
13 quality of DF modulated the time-related biological response (R2: 30 regulated functions,  
14 primarily at 6 months; R3: 26 regulated functions, mostly at 6–12 months; R4: 43 regulated  
15 functions, mostly at 18 months). Despite this heterogeneity, 9 functions overlapped under all  
16 3 DF diets in both studies, related to a restricted area of amino acids metabolism, cofactors,  
17 nucleotides and xenobiotic pathways and the microbiota. In conclusion, over the long-term,  
18 DF reprograms metabolism to close to its initial biological status in metabolically-disrupted  
19 pigs. Quality of the DF modulates its metabolic influence, although some effects were  
20 common to all DF. A resilient signature of DF consumption found in pigs was validated in  
21 humans.

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

## 24 1 Introduction

25 Dairy fat (DF) (butter, cheese, and whole milk) is an important component of regular  
26 foodways in developed countries, providing ~15% of total daily energy intake [1], and DF  
27 intakes are predicted to increase by a further 20% worldwide in the 2018–2027 period [2]. In  
28 the past decade, the long-held thinking that DF is bad for health has been challenged [3], and  
29 the balance of benefits to harms has swung back in favor of dairy products [4]. Dairy products  
30 can beneficially influence many aspects of health, such as cardiovascular disease, obesity,  
31 cancer, bone health, aging, and more [4], but the role of the DF moiety is often overlooked.  
32 There is therefore a need for epidemiological studies to get a better evaluation of DF intakes  
33 than the dietary questionnaires currently used, which are often not accurate enough to connect  
34 DF intakes to health outcomes. In fact, efforts to discover DF biomarkers are based either on  
35 short-term DF exposure (2 or 3 months) or on estimates of DF intake taken from food  
36 frequency questionnaires [5], both of which are exposed to biases. Short-term controlled  
37 nutritional interventions in humans also have weaknesses, such as attrition, low compliance  
38 [6], logistical hurdles, and insufficient time to induce a stable biological response [7].  
39 Furthermore, DF biomarkers are often limited to circulating DF fatty acids [8] and rarely  
40 address the plasma lipidome (which includes all lipid species bearing fatty acids) [9] that can  
41 provide more detailed information. Finally, dairy fatty acid composition undergoes significant  
42 seasonal and geographical variations [10] that shift unsaturated-to-saturated fatty acids ratio  
43 and *trans*-vaccenic and rumenic acids to lower values in winter milk. Such natural changes  
44 are significant enough to modify the health properties of DF, but this has so far only been  
45 shown in research on certain cardiovascular risk factors in animals [11] and humans [12].  
46 Adding calcium to dairy products is expected to bring further health benefits. It induces  
47 calcium soaps of saturated fatty acids, which lowers their absorption and leads to  
48 improvement in some cardiovascular risk factors such as total and LDL cholesterol [13].

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

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

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

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

71

## 72 2 Methods

### 73 2.1 Clinical study design

74 This study was performed according to Good Clinical Practices and in compliance with  
75 French regulations the tenets the Declaration of Helsinki, and was recorded under number  
76 2008-A01145-50. Informed consent was obtained from all volunteers. The trial started on 16  
77 March 16 2009 (first volunteer in) and ended on 21 May 2010 (last volunteer out). The design  
78 was a two-center, randomized, double-blind study with four parallel arms managed and  
79 monitored by Biofortis Mérieux Nutrisciences, Nantes. Detailed description of the study  
80 design, volunteer selection process, and full diets can be found in [14] and in the  
81 supplementary material for the clinical study that had a primary outcome based on LDL  
82 cholesterol [14]. On the basis of previous experience with similar inclusion criteria, a mean  
83 LDL cholesterol of 1.62 g/L was expected with a standard deviation of 0.2 g/L at inclusion.  
84 With an alpha risk of 5%, the number of individuals to include was estimated as 47 in each  
85 diet group for a power of 90% and 36 in each group for a power of 85%. An original set of  
86 528 volunteers with serum LDL cholesterol  $\geq 1.3$  g/L were screened for inclusion and  
87 exclusion criteria, and then the final total of 173 volunteers were randomized into 4 dietary  
88 groups (supplementary material—clinical study, Figure 2), each bringing 38% energy as  
89 lipids: R1 providing plant fat, R2 providing a summer dairy fat, R3 providing a winter dairy  
90 fat, and R4 providing the same winter fat but enriched with added calcium (supplemental  
91 clinical study design, Table 1 and Table 2). Fatty acid composition is reported in Table 1, and  
92 population data are reported in Table 2 and supplemental online material. The raw fats  
93 prepared for the human study were also used to prepare the pig diet, and the process was  
94 supervised by private-sector companies Lactalis, Entremont Alliance, and BEL (supplemental  
95 dairy fat preparation). Like for the clinical study, the fatty acid composition of each type of fat  
96 was kept constant throughout the nutritional protocol (Table 1) by adjusting, as and when  
97 necessary, with DF varying in fatty acid composition.

## 98 2.2 Nutritional study in pigs

99 The downsized colony of pigs known as the FBM pig colony [18] has been selected for  
100 natural spontaneous human-like cholesterol levels that are in part explained by a homozygous  
101 R84C mutation in the hotspot of the LDL receptor, and for responsiveness to lipid food intake  
102 [15, 16]. This study was approved by the Jouy-en-Josas–AgroParisTech institutional animal  
103 care and use committee under Agreement 12/048, and complied with the ARRIVE guidelines  
104 2.0, in accordance with EU Directive 2010/63/EU. The study used 32 pigs, i.e. 8 pigs per  
105 nutritional group, that were randomly selected at 5 months of age and assigned to each dietary  
106 group (R1 to R4), which counted half females and half barrows, and kept for 24 months on  
107 the 4 diets. The sample size was chosen based on the diet-induced 25% decrease in measured  
108 intra-ventricular coronary artery atherosclerosis [15]. Venous blood was drawn from each pig  
109 at the start (T0) and then every 6 months until month 24 (T24). The dietary groups were  
110 similar to those of the clinical study, i.e. characterized by the same types of DF (Table 1). The  
111 study was synchronized with the human trial, starting from May 2008 for the first pigs in, and  
112 ending April 2011 for the last pigs out. Detailed diet composition data and complementary  
113 information are reported in the supplemental pig study design.

## 114 2.3 Blood analyses

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

### 119 Metabolomics and lipidomics

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



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

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

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

134 Complementary total plasma fatty acid composition analysis was performed using gas  
135 chromatography with a flame ionization detection system [19], starting from 250  $\mu$ L of  
136 plasma.

137

## 138 [2.4 LCMS data preprocessing](#)

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

### 143 [2.4.1 Mass feature annotations](#)

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

148 MS<sup>2</sup> spectral matching using LipidSearch<sup>TM</sup> 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 log<sub>2</sub>-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

173 to generate a composite score value for each functional set [22]. The functional  
174 metabolic/lipid blocks were ‘weighted’ to take into account the number of variables per block  
175 [24]. For lipid blocking, the lipid species were grouped according to clusters calculated by  
176 HCA (Ward method).

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

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

184

### 185 3 Results

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

192 The Pig vs Human data comparisons included (respectively) 33 vs 42 clinical variables, 22  
193 plasma fatty acids in both species, 161 vs 147 annotated plasma metabolites, and 292 vs 410  
194 lipid species (both featured in 20 different lipid classes). Detailed compositions are listed in  
195 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<sup>th</sup> and  
198 18<sup>th</sup> 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

221 plant-fat-fed pigs at each timepoint (Figure 1). In addition, when considered together and  
222 irrespective of time, the prediction of purposely-left-out pigs using the ROC estimator,  
223 whether DF-fed or plant-fat-fed, had an error rate of only 8.4% (Supplemental Table S6).  
224 There were no major differences between the 3 DF diets using the DF intake predictors  
225 compared to the R1 plant-fat diet (Figure 2A). However, for DF there was a time effect on  
226 predictor scores, with the greatest deviations observed during the first year followed by a  
227 tendency to return towards baseline values in the second year (Figure 2B). This observed  
228 effect included the conventional cardiovascular risk biomarkers (Supplemental Figure S3).  
229 This trajectory was specific to DF diet intake conditions, since the time-course response in  
230 non-DF pigs did not follow the same pattern (Figure 2C). Hence the question that arose was  
231 whether the biomarkers could distinguish mid-term (6–12 months) *versus* long-term (18–24  
232 months) DF intake, but analysis found that they failed to do so (not shown). The biomarker  
233 status of pigs fed only DF (R2, R3, R4) was statistically different from baseline status prior to  
234 DF intake (T0) at every timepoint (T6, T12, T18, T24) (Supplemental Figure S4). Conversely,  
235 in pigs fed only plant-fat diet, biomarker status showed no change from T0 to T12 months ( $P$   
236  $> 0.05$ ) but then differences emerged in the second year ( $P < 0.05$ ), indicating a significant  
237 but lagging time effect on biomarker status in non-DF-fed pigs (Figure 2C and Supplemental  
238 Figure S5).

### 239 3.2 Validation of the pig dairy fat biomarkers in Human

240 We then set out to validate the selected ‘dairy’-variable signatures of DF consumption over a  
241 long time-period in pigs in the narrower-period companion clinical trial. For that purpose, we  
242 randomly kept out one fifth of the human individuals, performed in 5 iterations. The ‘dairy’  
243 status of the 1-out-of-5 excluded subjects was then predicted in each iteration using the same  
244 ROC modelling procedure as used with pigs while using the remaining 4-out-of-5 subjects.  
245 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).

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

### 278 3.4 Specific biological response to dairy fats vs non-dairy fat over time in pigs

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

### 294 3.5 Specific biological response across the dairy fat diets in pigs

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

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

### 311 3.6 Overlap between Humans and Pigs

#### 312 3.6.1 Non-dairy vs dairy fat

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



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

### 325 3.6.2 Across dairy fat-diets

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

330

331

## 332 4 Discussion

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

342 In the pig study, approximately one third of the variables measured were found to be sensitive  
343 to the influence of diet (DF vs non-DF), resulting in a significant but limited effect (20% plus  
344 10% in interaction with time, Figure 4). The effect was nonlinear, showing a large shift from  
345 initial status until a turning point at one year after which there was a second phase (T12 to  
346 T24 months) marked by a reverse trajectory (Figure 4). However, from this turning point, the  
347 DF diets led to an opposite pattern of response to that of the plant-fat diet, rebalancing the  
348 metabolic regulations towards the initial setting (Figure 4 and Supplemental Figure S8). With  
349 a lifespan of 15–20 years, the pigs in our study were challenged from post-weaning to young  
350 adulthood (from 5 or 6–30 months of age), and so the observed effect cannot be due to age-  
351 related metabolic alterations. However, we cannot rule out other metabolic adjustments  
352 occurring from the post-weaning period to adulthood [25]. Nevertheless, in this life period  
353 (post-weaning to young adulthood), we expected to see a metabolic shift [25], as observed in  
354 the plant-fat-fed pigs (Figure 4C), and not a trajectory of return to the initial setting.

355 Furthermore, the kinetics of the greatest dietary influence (over the first 6 months) was  
356 desynchronized with growth rate, which is normally maximal between 12 and 18 months  
357 (Table 2). This result shows that over a long-term (one year) period, the DF used (but not the

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

372 Despite this time-dependency (Figure 4), we were nevertheless able to find a resilient  
373 signature of DF intake in the pig study. The signature came from total plasma fatty acids  
374 analysis (C14:0 and C20:5n-3) and from the plasma LC/MS/MS lipidomics analysis (3 TG  
375 species, 4 PI species, 1 PC specie and 1 ChE specie). We could not evaluate the performance  
376 of our signature on other proposed biomarkers of milk-fat intake such as C15:0 and *trans*-  
377 16:1n-7 [5, 30, 31], as these fatty acids were not measured in both pigs and humans. Note that  
378 there are concerns around using such fatty acids including heptadecanoate as biomarkers of  
379 DF intake, as they might not be only specific to DF [32]. However, one of the lipid species in  
380 the signature found here (TG15:0/16:0/18:2) included C15:0 and flagged lipid cluster 8 which  
381 mostly comprises dairy fatty acids (Figure 7). This signature of long-term DF exposure in pigs  
382 was validated in the short-term 2-month human clinical trial in which the lipid variables were

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

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

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

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

427 The difference in biological response was four times higher between dairy *vs* non-DF diets than  
428 across the three DF diets (Figure 4A). The differences across the DF diets concerned  
429 metabolites more than lipid regulations (Figure 8). Winter DF (R3) and summer DF (R2)  
430 appeared to change a similar number of biological functions, mostly in the early time-period  
431 (~30 biological functions affected at around 6 months). However, the summer DF induced more  
432 recurrent specific effects, ranging from various lipid metabolisms to amino acid metabolisms

433 including cysteine and homocysteine metabolism. The seasonal changes in milk fat fatty acid  
434 composition are therefore great enough to influence the biological status of individuals beyond  
435 plasma fatty acid composition and besides the classical markers of CVD risk already reported  
436 [11, 12, 39]. Adding calcium to the 'R3' winter DF (to afford R4) greatly changed its properties,  
437 increasing both its biological impact and recurrency (43 vs 26 biological functions changed),  
438 but still differentiated it from summer-season DF. This differentiation between R2 and R4  
439 included various important functions for metabolic regulation and defense against stress, with  
440 a shift in activity period to a later timepoint (6 months with R2 and 18 months with R4). Here  
441 we used young pigs with no apparent comorbidities. It would now be instructive to investigate  
442 whether the observed differences in molecular 'omics'-based phenotypes translate into health  
443 improvement later in life. Nevertheless, our long-term pig study demonstrated that DFs are not  
444 all alike and can exert differential biological effects.

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

## 452 5 Conclusion

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

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

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

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

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495



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

<b>Fatty acids</b>	<b>R1</b>	<b>R2</b>	<b>R3 and R4</b>
<b>Total saturated fatty acids</b>	64	64	74
<b>C14:0</b>	0.9	10	10
<b>C16:0</b>	40	27	37
<b>Total monounsaturated fatty acids</b>	29	30	22
<b>C18:1<i>t</i> (<i>trans</i>-vaccenic acid)</b>	0.0	4.5	1.5
<b>Total polyunsaturated fatty acids</b>	20	4.0	2.0
<b>C18:2n-6</b>	19	1.4	1.4
<b>C18:3n-3</b>	0.3	0.9	0.3
<b>C18:9<i>c</i>,11<i>t</i> (rumenic acid)</b>	0.0	0.8	0.5
<b>w6/w3 ratio</b>	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 <i>P</i> -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

\*Chi<sup>2</sup> 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 <i>P</i> -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/cm <sup>2</sup> )	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 (cm <sup>2</sup> )	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  $\times$  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  $-\log_{10}$ . **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.

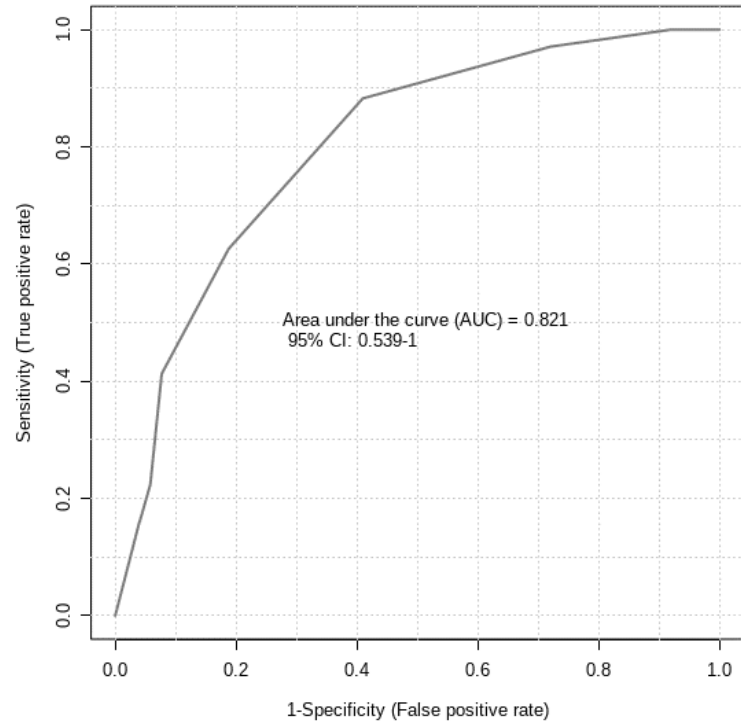
**Figure 7.** Lipid composition of selected lipid clusters in the plasma of pigs fed the various diets. Percentages of lipid classes are displayed in circles, and the component lipid species are shown as a heatmap where red colors indicate a relative increase and blue colors indicate a



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.

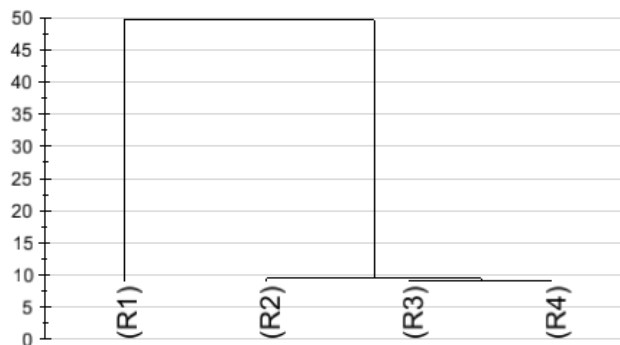
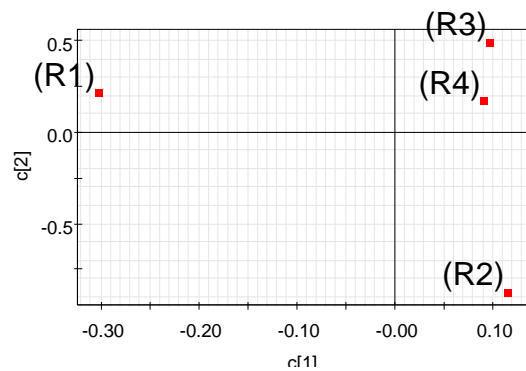
**Figure 9.** Venn plot of common biological responses at metabolic systems scale in both pigs and humans. The most common shared functions are given.

**A****FIGURE 1****B**

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

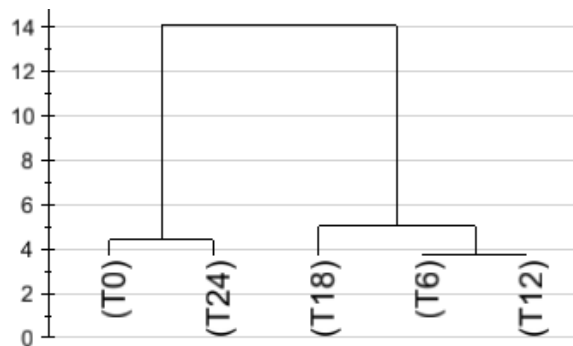
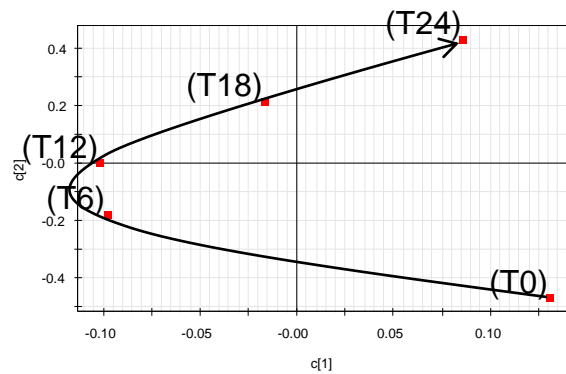
Figure 2

**A**



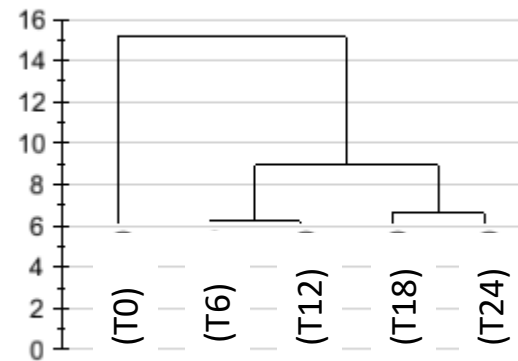
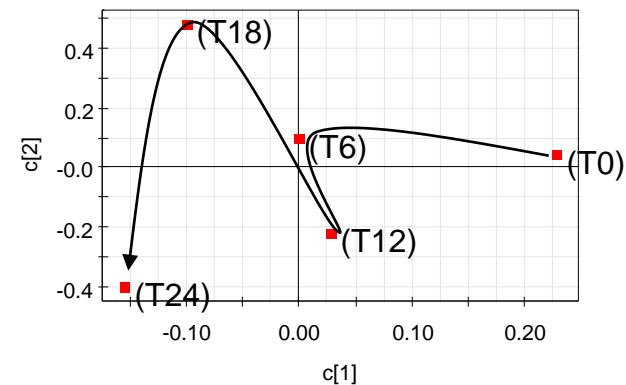
Calculated with Ward and sorted by size.

**B**



Calculated with Ward and sorted by size.

**C**



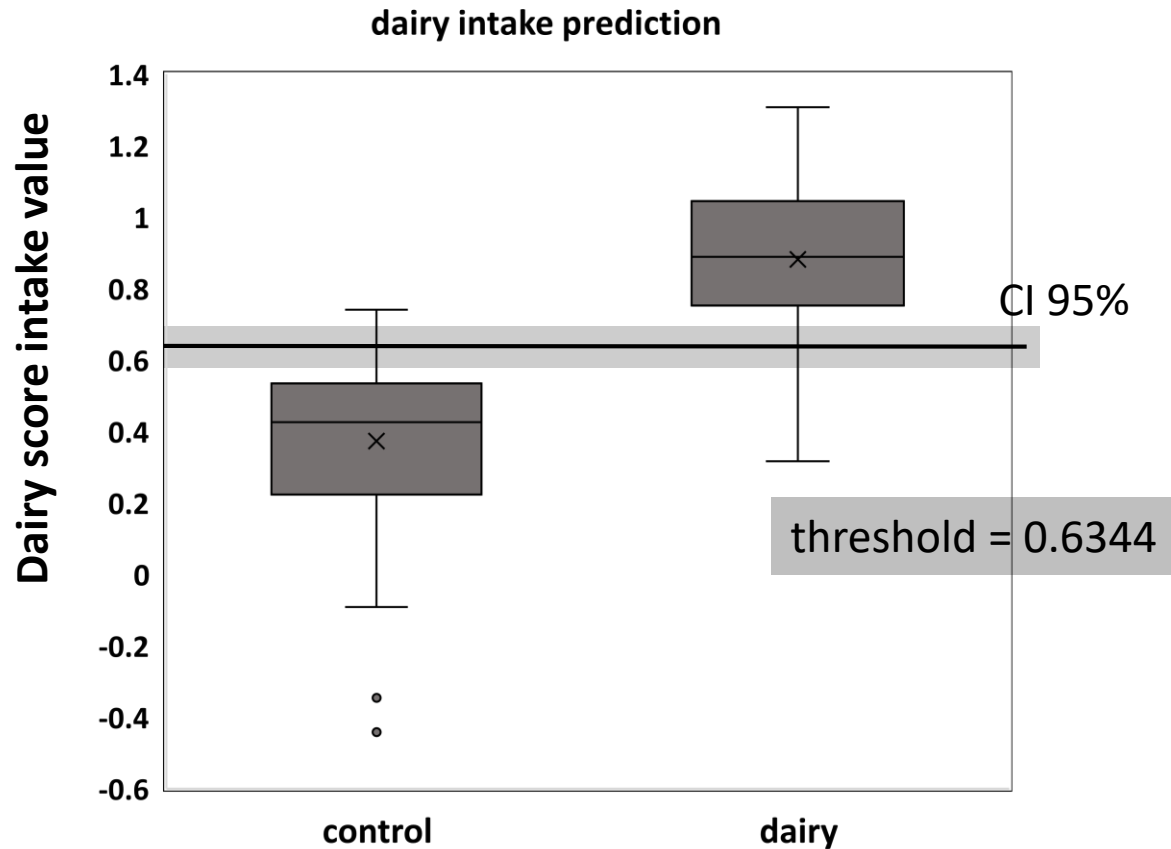
Calculated with Ward and sorted b:

Figure 3

Human study

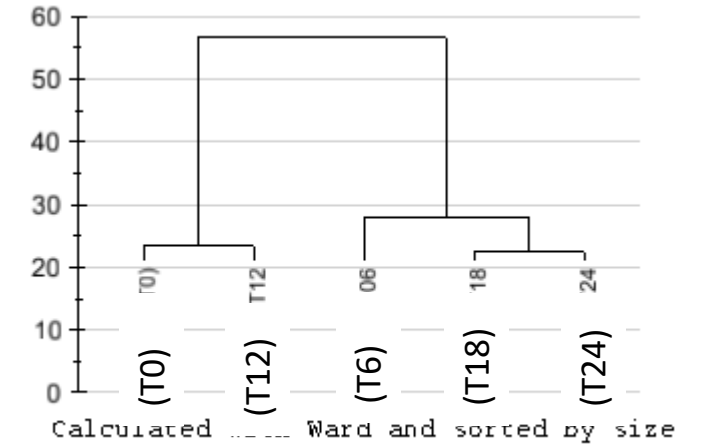
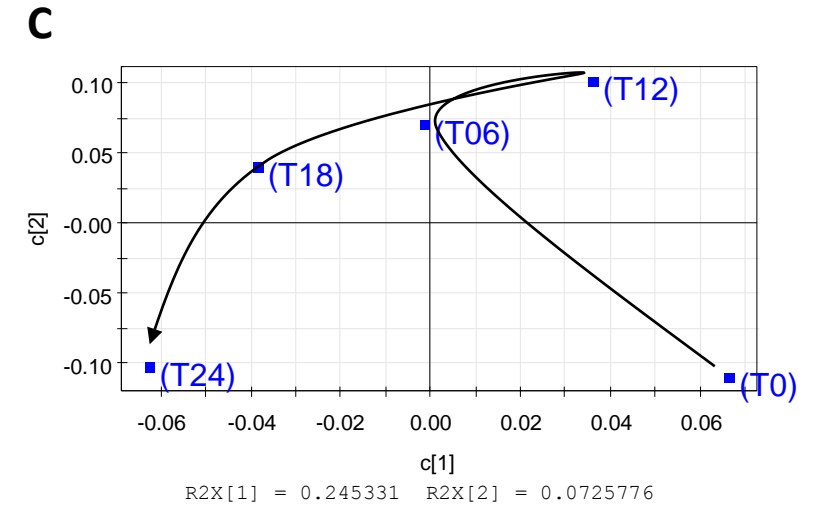
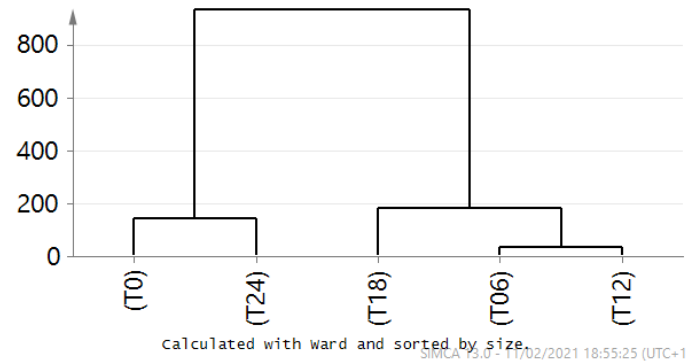
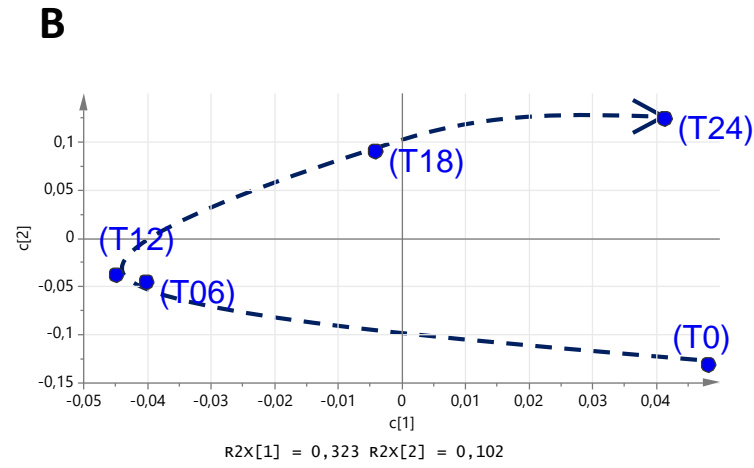
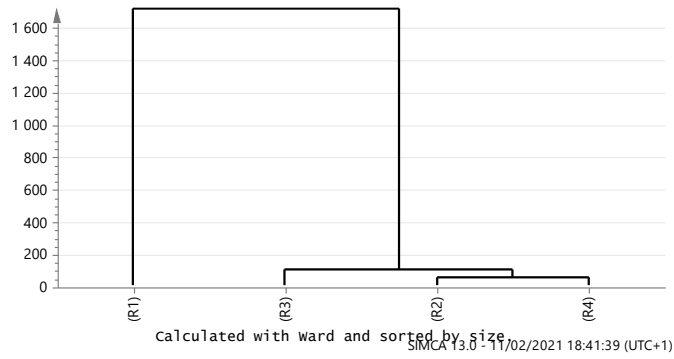
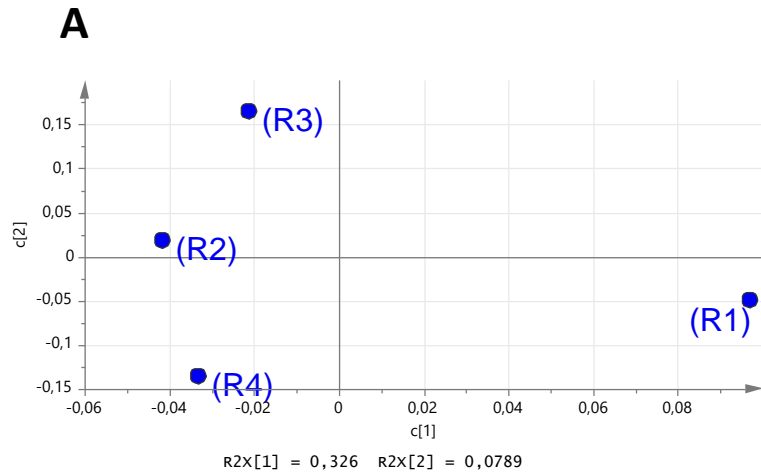
**dairy intake score =**

$$\begin{aligned}
 & -0.00101505 * (\mathbf{C14:0}) - 0.0007791 * (\mathbf{C20:5n-3}) - 0.1561508 * (\mathbf{ChE(22:5)}) + 0.5204846 * (\mathbf{PC(31:1)}) + 0.02480472 * \\
 & (\mathbf{PC(33:1)}) + 0.27208 * (\mathbf{PI(16:0/16:1)}) - 0.5702826 * (\mathbf{PI(16:0/18:1)}) - 0.7779556 * (\mathbf{PI(18:0/18:1)}) \\
 & + 11.724626 * (\mathbf{PI(18:0p/20:4)}) + 0.01611526 * (\mathbf{TG(15:0/16:0/18:2)}) - 0.05135144 * (\mathbf{TG(18:1/18:1/20:3)}) \\
 & + 0.0499953 * (\mathbf{TG(18:2/20:4/20:4)}) + 0.9796806
 \end{aligned}$$



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



**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

**A**

Normal Probability for VIP[Comp.1]

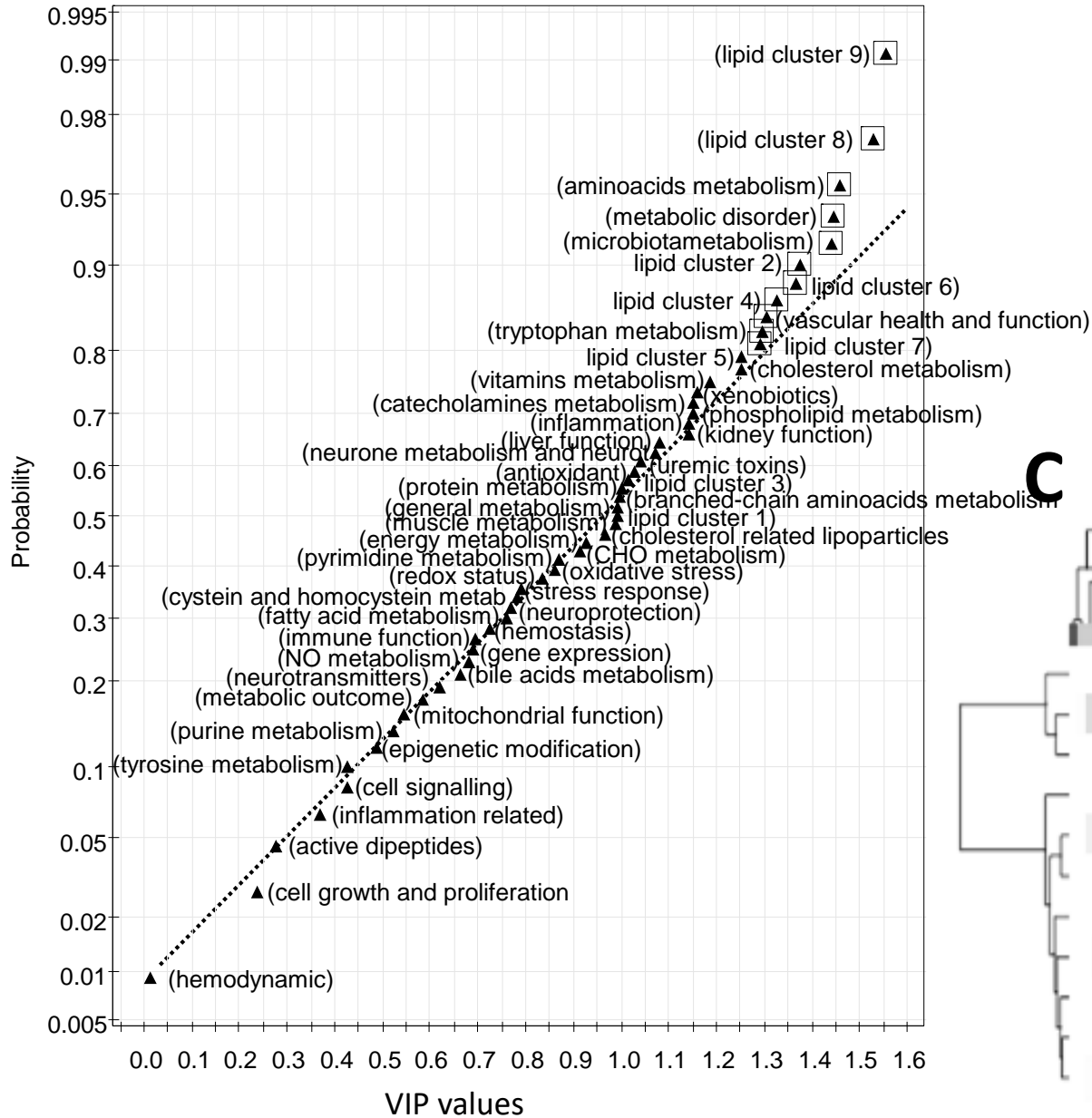


Figure 5

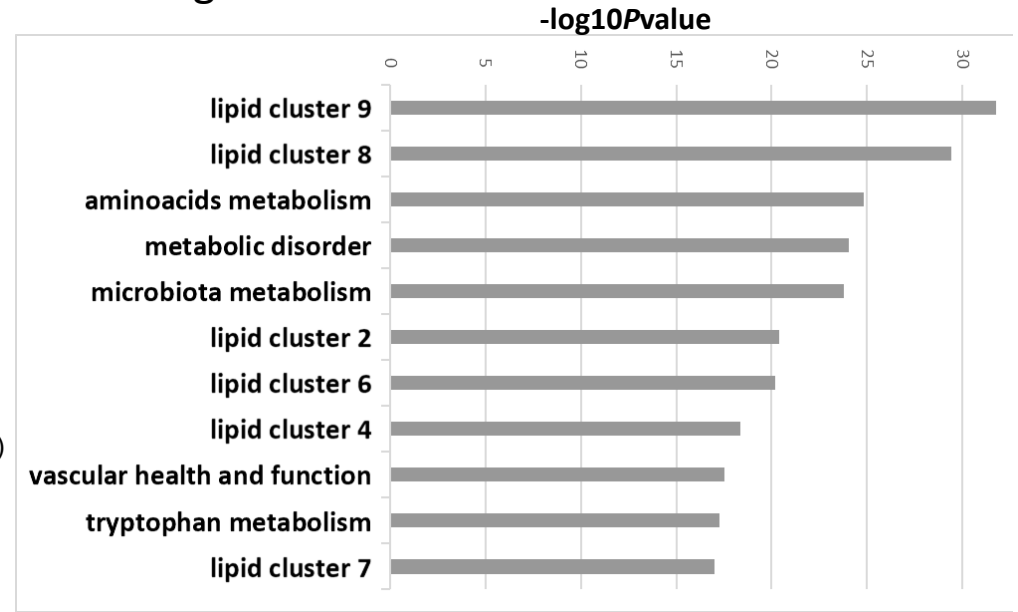
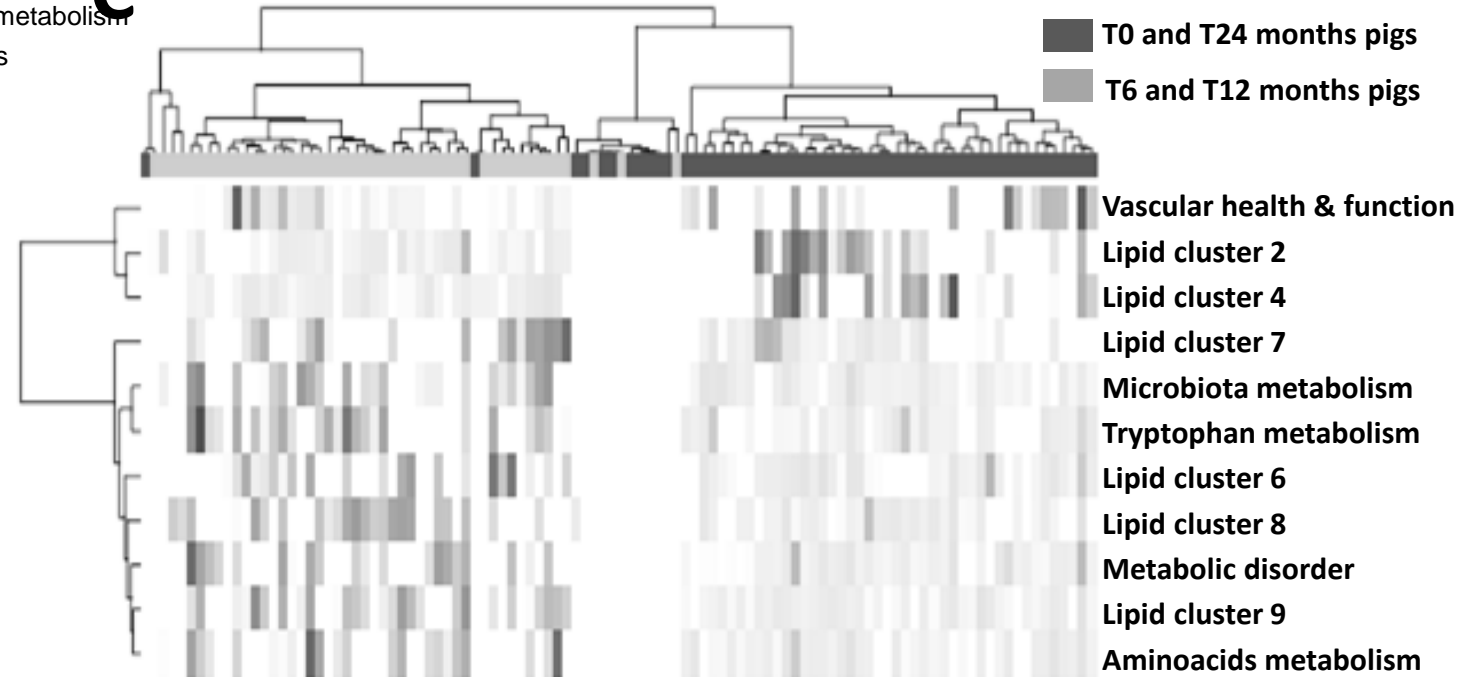
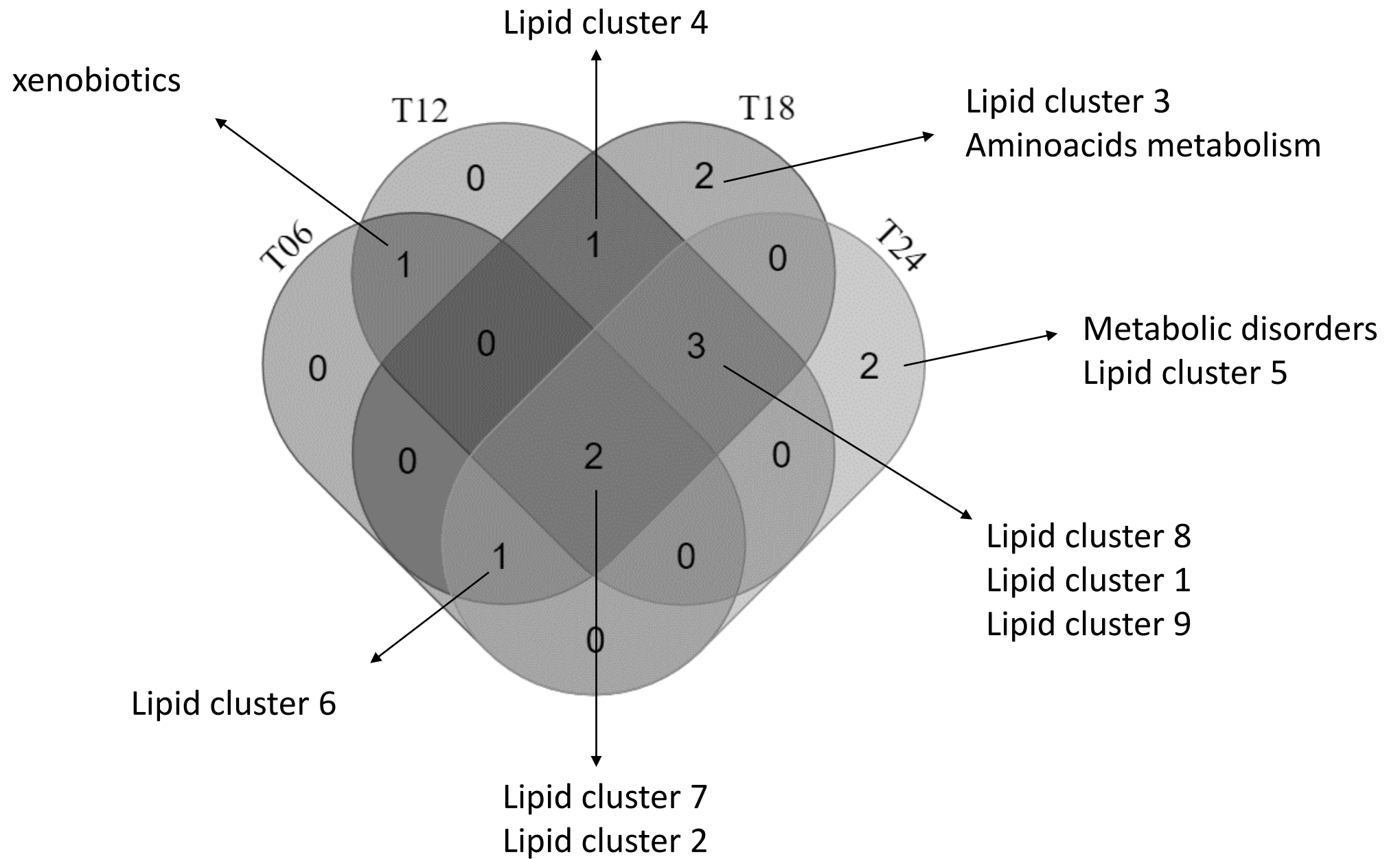
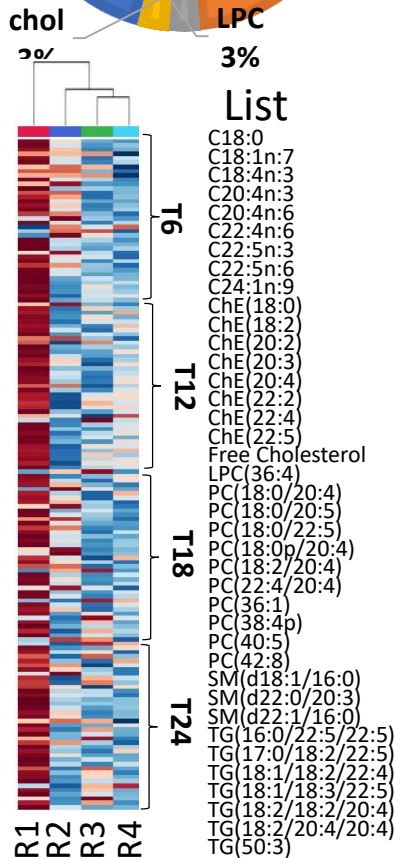
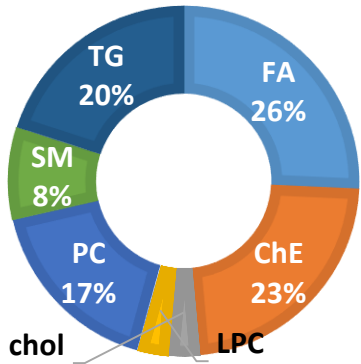
**B****C**

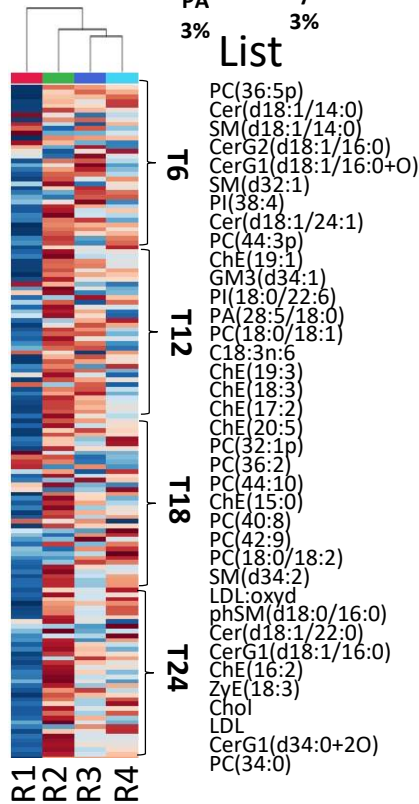
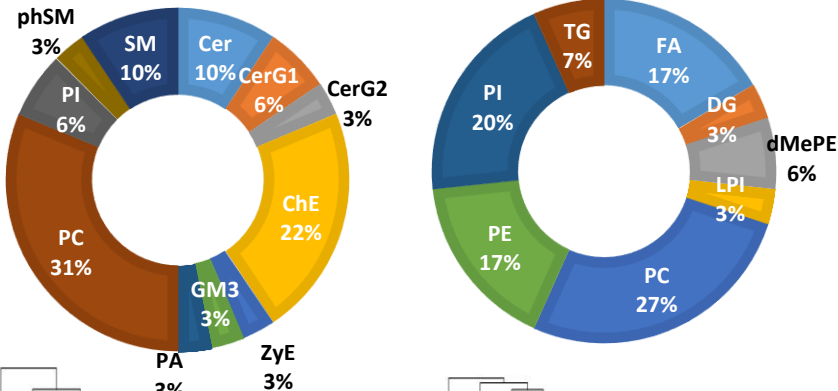
Figure 6



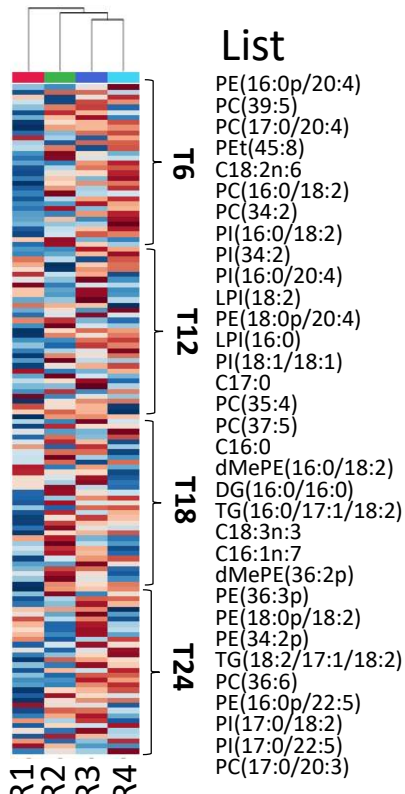
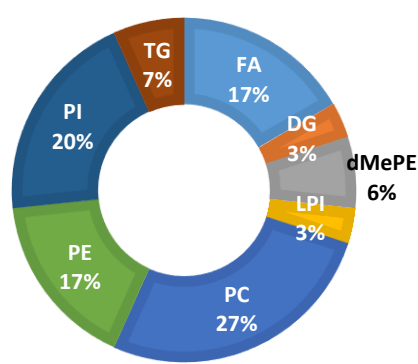
**LIPID CLUSTER 2**  
(62% w6 lipid species)



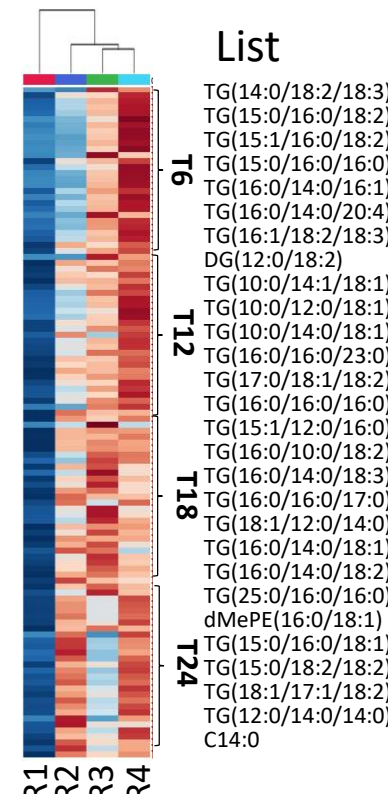
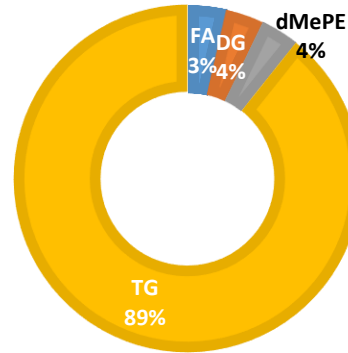
**LIPID CLUSTER 7**  
(chol esters, w3 lipids, sphingolipids)



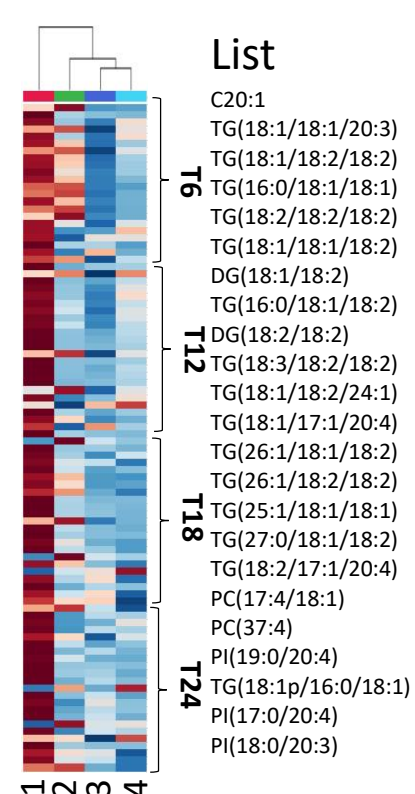
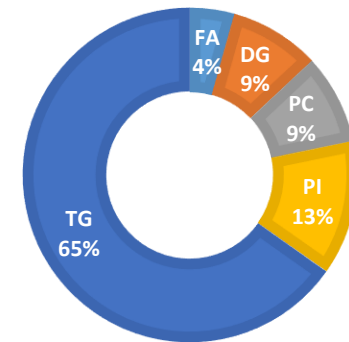
**LIPID CLUSTER 6**  
(mainly w6 in PI and PE lipids)



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



**LIPID CLUSTER 1**  
(65% w9 lipids)



**LIPID CLUSTER 9**  
(enriched in PI)

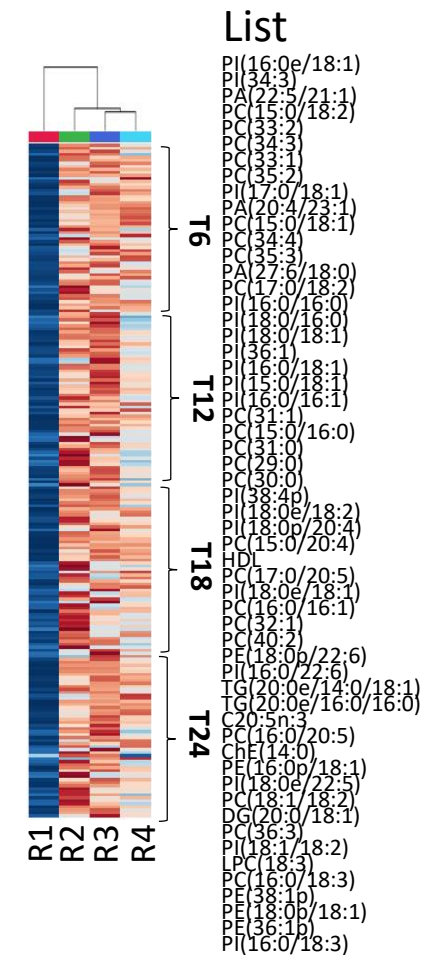
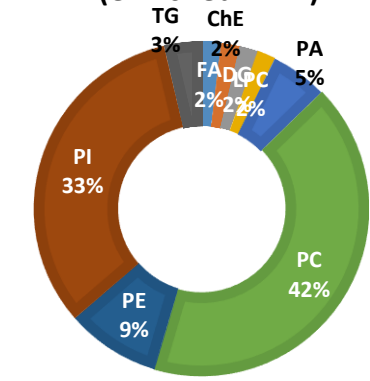


Figure 7



Figure 8

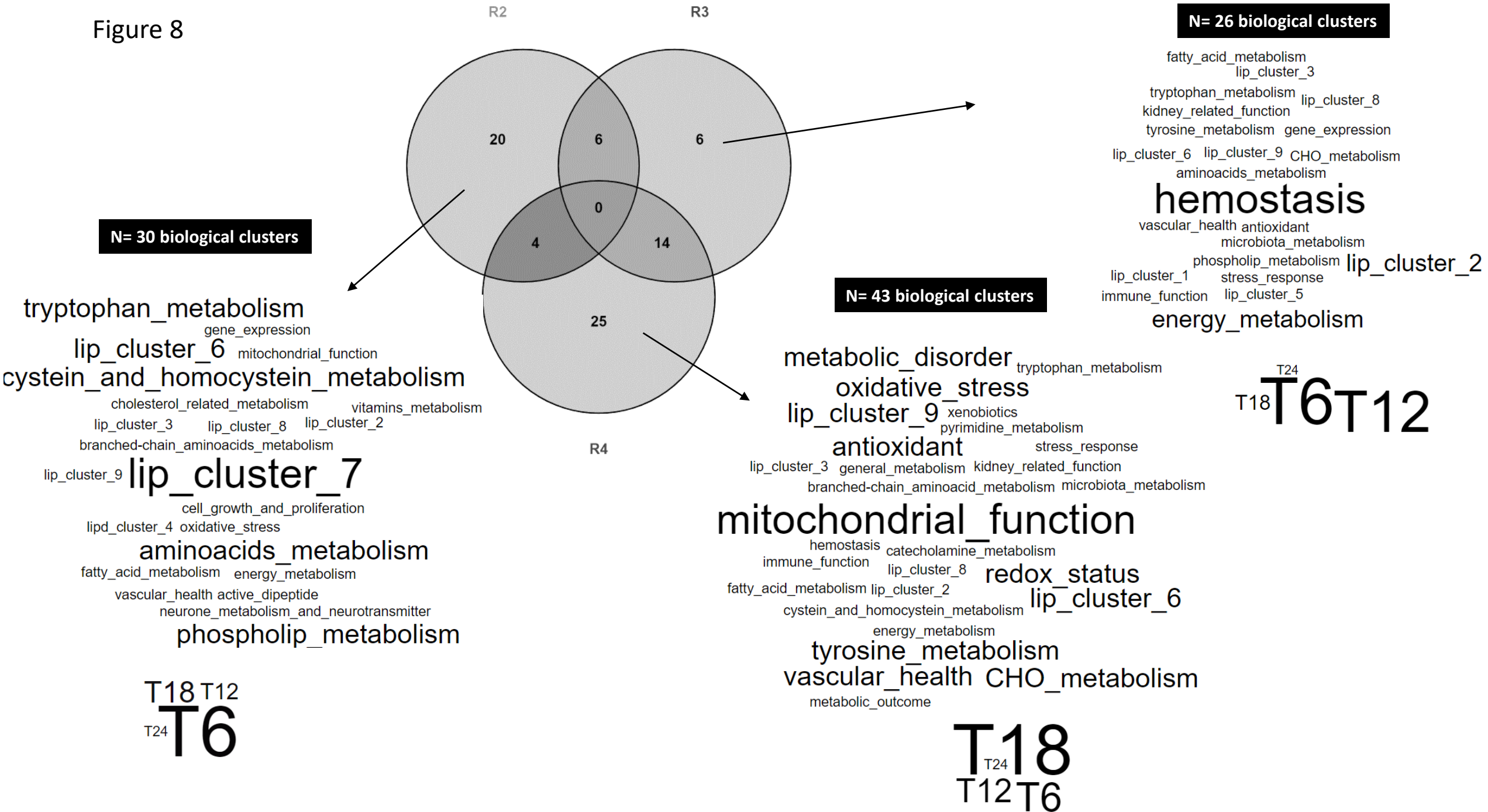
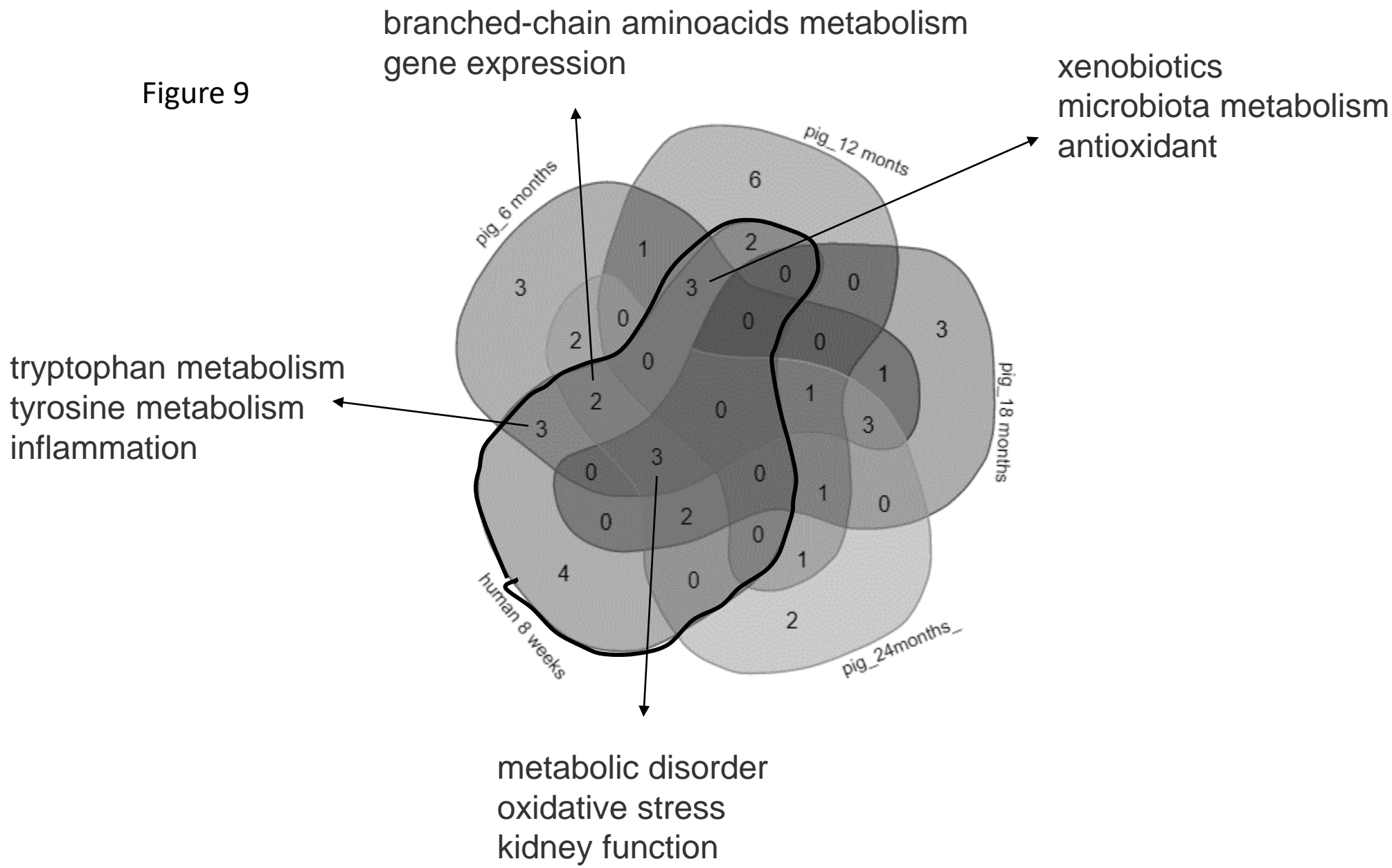




Figure 9



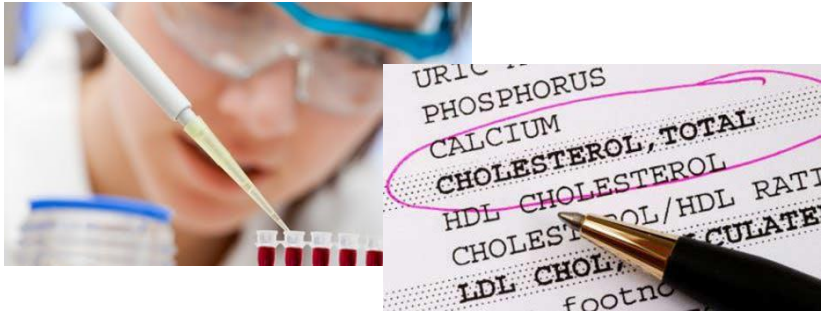


173 volunteers (63,5% ♀, 36,5% ♂)  
 $1.6 \leq \text{LDL-cholesterol} < 2.2 \text{ g/l}$

**X**



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



Conventional clinical status

Plant-fat  
R1

Summer  
dairy fat  
R2

Winter  
dairy fat  
R3

Winter dairy fat  
+ Ca<sup>2+</sup>  
R4



« omics » (metabolomics and lipidomics)

