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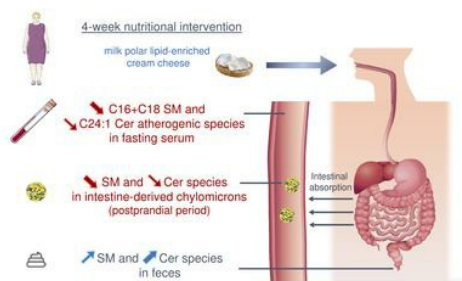
Milk polar lipids favorably alter circulating and intestinal ceramide and sphingomyelin species in postmenopausal women

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1 **Milk polar lipids favorably alter circulating and intestinal**
2 **ceramide and sphingomyelin species in postmenopausal women**

3
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54 oils and dairy companies. M-CM is a member of the scientific advisory board of ITERG, the Industrial
55 Technical Centre for the oils and fats business sector. These activities had no link with the present
56 study. FJ was, and KB is, an employee of ITERG. PG is an employee of ACTALIA Produits Laitiers,
57 an Agri-Food Technical Institute, with a strong specialization in dairy research and development, and
58 food safety. MLa had research collaborations with Mondelez and Bridor without link with the present
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60 Other authors declared no conflict of interest.

61 **ABSTRACT**

62 **BACKGROUND.** High circulating levels of ceramides (Cer) and sphingomyelins (SM) have been
63 associated with cardiometabolic diseases. The consumption of whole-fat dairy products, which
64 naturally contain such polar lipids (PL), is associated with health benefits, but the impact on
65 sphingolipidome remains unknown. We investigated how milk PL supplementation impacts
66 circulating and intestinal SM and Cer composition in association with improvement of cardiovascular
67 markers.

68 **METHODS.** In a 4 week-randomized double-blind controlled study, 58 postmenopausal women
69 consumed daily a cream cheese containing 0, 3 or 5 g of milk PL. Postprandial metabolic explorations
70 were performed before and after the supplementation. SM and Cer species were analyzed in serum,
71 intestine-derived chylomicrons and feces. The ileal content of 4 ileostomy patients was also explored
72 after milk PL intake in a crossover double-blind study.

73 **RESULTS.** Milk PL consumption decreased serum atherogenic C24:1 Cer ($P_{\text{group}} = 0.033$), C16:1
74 ($P_{\text{group}} = 0.007$) and C18:1 ($P_{\text{group}} = 0.003$) SM species. Changes in serum C16+18 SM species were
75 positively correlated with the reduction of total cholesterol ($r = 0.706$, $P < 0.001$), LDL-C ($r = 0.666$,
76 $P < 0.001$) and ApoB ($r = 0.705$, $P < 0.001$). Milk PL decreased the concentration in chylomicrons of
77 total SM ($P_{\text{group}} < 0.0001$) and of C24:1 Cer ($P_{\text{group}} = 0.001$). Saturated SM and Cer species, which are
78 also the major species found in milk PL-enriched cheeses, increased in ileal efflux and feces. There
79 was a marked increase in total fecal Cer after milk PL supplementation ($P_{\text{group}} = 0.0002$). Milk PL also
80 modulated the abundance of some specific SM and Cer species in ileal efflux and feces, suggesting
81 differential absorption and metabolization processes in the gut.

82 **CONCLUSION.** These data demonstrate that milk PL supplementation decreases atherogenic SM
83 and Cer species associated with an improvement of cardiovascular risk markers. Our findings bring
84 new insights on sphingolipid metabolism in the gastrointestinal tract, especially Cer as such signaling

85 molecules potentially participating in the beneficial effect of milk PL. [ClinicalTrials.gov](https://clinicaltrials.gov),
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90 **INTRODUCTION**

91 Sphingolipids (SP) represent a large class of bioactive polar lipids (PL) that play a pivotal role in
92 structural and metabolic functions in the regulation of cardiometabolic homeostasis, intestinal health
93 and inflammatory signaling pathways (1–3). SP metabolism represents a vast and complex network
94 (4), including a plethora of metabolically relevant species regulated by several key enzymes and
95 metabolite fluxes in mammals (5). Dysregulated SP metabolism is related to negative health outcomes
96 (6–8), and an increase in circulating ceramide (Cer) species (particularly C24:1 Cer), which are key
97 precursors of the biosynthesis of several other SP molecules, correlates with markers of
98 cardiometabolic complications (9–11). Sphingomyelins (SM) represent one of the most abundant SP
99 families. High serum SM concentrations correlate with coronary heart diseases in obese patients (13)
100 and high-fat diet increases specifically C16+C18 SM species (i.e., C16:0, C16:1, C18:0 and C18:1
101 species) in rodents (14).

102 There is growing evidence showing that several factors may affect circulating SM and Cer
103 concentrations such as drugs and lifestyle modifications, including exercise and dietary changes (15,
104 16). SP being found in plant and animal cell membranes, the daily SP intake represents approximately
105 0.3-0.4 g / day in humans (17), but the impact of such consumption on the endogenous
106 sphingolipidome remains largely unknown. Cow milk has recently attracted more attention because it
107 naturally contains SM and Cer (~25% of PL in the milk fat globule membrane, MFGM), including
108 C20+22 species (i.e., C20:0, C20:1, C22:0 and C22:1 species) that are found in higher amounts in milk
109 fat than in human blood. Recent meta-analysis and research papers highlighted the beneficial
110 cardiometabolic effects of the consumption of whole fat dairy product, which contain sizeable amounts
111 of milk PL (18, 19). Given that milk PL also contain C16+18 SM and C24:1 Cer species with potential
112 deleterious effects, understanding the impact of milk SP on circulating levels and endogenous SP
113 metabolism is of uttermost importance.

114 Preclinical studies revealed that milk SM supplementation prevents hyperlipemia,
115 hypercholesterolemia, low-grade inflammation, and improves intestinal health related to gut
116 microbiota modulations (2, 20–26). Buttermilk, a by-product of butter industry, represents a natural
117 source of SM-rich MFGM (1-10 g of milk PL / L) (27, 28). In the VALOBAB-C trial, we demonstrated
118 recently that the 4-week consumption of milk PL-enriched test cheese decreases circulating total
119 cholesterol (C) (primary outcome), triacylglycerols (TAG), LDL-C and ApoB, at both fasting and
120 postprandial states, (Supplemental Figure 1) in postmenopausal women at risk of cardiovascular
121 disease (29). However, there are still open questions regarding the potential involvement of the
122 endogenous metabolism and intestinal fate of milk SP species in these benefits. We thus explored the
123 prespecified secondary outcomes of the VALOBAB clinical trial by analyzing SM and Cer molecular
124 species of particular interest in several biological compartments. We aimed to determine how the 4-
125 week supplementation with milk PL impacts the circulating and fecal SM and Cer species at fasting
126 state, and their amount in intestine-derived chylomicrons during the postprandial period, in
127 postmenopausal women at cardiovascular risk. We then verified whether this contributes to the
128 beneficial effects of milk PL on lipid cardiovascular markers. In a complementary study conducted in
129 ileostomy patients, we further aimed to identify the digestive fate of milk SP in the upper
130 gastrointestinal tract after acute consumption of milk PL-rich meals.

131 **RESULTS**

132 **Sphingolipidome of serum SM and Cer molecular species is modified by milk PL**

133 The dietary intervention with milk PL significantly modified the amount of several molecular SP
134 species in fasting serum. In the 5g-PL group, the serum concentrations of the following species
135 decreased between the first (V1) and second (V2) exploration visit: C16:1 SM ($\Delta V2-V1$ CTL: $+0.44$
136 ± 0.97 μM ; 3g-PL: -0.64 ± 0.84 μM ; 5g-PL: -3.35 ± 0.50 μM ; $P_{\text{group}} = 0.007$; *post hoc* analysis: $P_{\text{CTL-5gPL}} = 0.006$), C18:1 SM ($\Delta V2-V1$ CTL: $+1.02 \pm 0.87$ μM ; 3g-PL: -2.21 ± 0.69 μM ; 5g-PL: $-2.99 \pm$
137 0.79 μM ; $P_{\text{group}} = 0.003$; $P_{\text{CTL-5gPL}} = 0.003$) and C20:1 SM ($\Delta V2-V1$ CTL: $+0.62 \pm 0.61$ μM ; 3g-PL: $-$
138 0.71 ± 0.50 μM ; 5g-PL: -1.57 ± 0.48 μM ; $P_{\text{group}} = 0.025$; $P_{\text{CTL-5gPL}} = 0.019$) (Table 1). A decrease in
139 serum C24:1 Cer species was also observed in milk PL groups regardless of dose ($\Delta V2-V1$ CTL: $+0.11$
140 ± 0.08 μM ; 3g-PL: -0.19 ± 0.08 μM ; 5g-PL: -0.37 ± 0.18 μM ; $P_{\text{PL}} = 0.016$), without any effect on the
141 other identified Cer species. No difference between groups was observed in the circulating fasting
142 concentrations of total SM and Cer and of phospholipids (Table 1). Parallel to their amount, changes
143 in the relative abundance of fasting SM and Cer species after intervention, i.e., the proportion of each
144 SM or Cer species in total serum SM or Cer respectively, revealed decreased proportions of C18:1 SM
145 species ($P_{\text{group}} = 0.002$) and C24:1 Cer species ($\Delta V2-V1$ CTL: $+0.65 \pm 0.76\%$; 3g-PL: $-2.67 \pm 0.96\%$;
146 5g-PL: $-2.65 \pm 0.71\%$; $P_{\text{group}} = 0.010$, $P_{\text{PL}} = 0.002$; $P_{\text{CTL-5g}} = 0.021$, $P_{\text{CTL-3g}} = 0.020$; Supplemental Table
147 2). These beneficial effects were associated with the increase of the relative proportions of specific
148 SM and Cer species usually poorly represented in human blood: C20:0 SM ($\Delta V2-V1$ CTL: $+0.14 \pm$
149 0.14% ; 3g-PL: $+0.62 \pm 0.08\%$; 5g-PL: $+0.95 \pm 0.09\%$; $P_{\text{group}} = 0.00005$; $P_{\text{CTL-5g}} = 0.00003$, $P_{\text{CTL-3g}} =$
150 0.010), C22:1 SM ($\Delta V2-V1$ CTL: $-0.02 \pm 0.10\%$; 3g-PL: $+0.65 \pm 0.27\%$; 5g-PL: $+0.56 \pm 0.23\%$; P_{group}
151 $= 0.07$; $P_{\text{PL}} = 0.021$) and C20:0 Cer species ($\Delta V2-V1$ CTL: $-1.44 \pm 0.65\%$; 3g-PL: $+0.38 \pm 0.56\%$; 5g-
152 PL: $+0.47 \pm 0.60\%$; $P_{\text{group}} = 0.057$, $P_{\text{PL}} = 0.016$) (Supplemental Table 2).

154

155 **Milk PL-induced modulations of serum SM and Cer profiles are correlated with the decrease of**
156 **CVD risk markers**

157 Results demonstrated a significant correlation between change in serum SM (particularly C16+18 SM
158 species) and Δ LDL-C, Δ total C and Δ ApoB (Figure 2A, $P < 0.001$). These correlations were mainly
159 mediated by the dietary intervention regardless of milk PL dose (Figure 2B; *versus* no correlation in
160 control group, Figure 2C), as also illustrated in Figure 2D-F by the specific correlations between
161 Δ C16+18 SM species and Δ LDL-C ($r = 0.666$, $P < 0.0001$), Δ total C ($r = 0.706$, $P < 0.0001$), and
162 Δ ApoB ($r = 0.705$, $P < 0.0001$). Fewer correlations were observed between changes in Cer
163 concentrations and those of blood lipids. Because results revealed changes in the SP species
164 proportions (Supplemental Table 2), we analyzed potential correlations with blood lipid concentrations
165 (Figure 2G). Changes in C24+26 Cer species proportions positively correlated with Δ LDL-C ($r =$
166 0.418 , $P = 0.022$), Δ total C ($r = 0.585$, $P < 0.001$) (Figure 2, G and J-K) and Δ ApoB ($r = 0.492$, $P =$
167 0.006). Conversely, variations in C20+22 Cer species proportions negatively correlated with Δ LDL-C
168 ($r = -0.424$, $P = 0.020$) and Δ total C ($r = -0.476$, $P = 0.008$) (Figure 2, H-I). In parallel, we determined
169 the magnitude effect of Δ C16+18 SM, $\Delta\%$ C20+22 and $\Delta\%$ C24+26 Cer species on Total C, LDL-C
170 and ApoB by estimating the regression coefficient associated to each variable in a general linear mixed
171 model. This shows that each variation of 1 μ M of Δ C16+18 SM species would result in a variation of
172 0.0074 mM of LDL-C ($P = 0.0065$), 0.0088 mM of total C ($P = 0.0042$), 0.0017 g / L of ApoB ($P =$
173 0.017). The magnitude effect of $\Delta\%$ C20+22 species on cardiovascular lipid markers was not
174 significant, while each variation of 1% of Δ C24+26 Cer species proportions would result in a variation
175 of 0.039 mM of LDL-C ($P = 0.040$), 0.054 mM of total C ($P = 0.010$) and 0.010 g / L of ApoB ($P =$
176 0.041).

177

178 **Milk PL decrease SM content in intestine-derived chylomicrons and impact their SM and Cer**
179 **molecular profiles**

180 The variations of plasma concentrations of CMRF-bound SM (CMRF-SM) decreased in the 5g-PL
181 group during all the postprandial period ($P_{\text{group}} = 0.015$; $P_{\text{CTL-5g}} = 0.013$), and the variation of plasma
182 CMRF-Cer concentration also tended to decrease ($P_{\text{group}} = 0.053$, $P_{\text{PL}} = 0.051$) (Supplemental Table
183 3). To focus on potential modifications of chylomicron lipid composition regardless of their circulating
184 concentration, we also determined their enrichment in SP by analyzing the SM/TAG and Cer/TAG
185 ratios in CMRF particles. Milk PL reduced significantly CMRF-SM/TAG ratio (Figure 3A, $P_{\text{group}} =$
186 0.00095 , $P_{\text{PL}} = 0.001$; $P_{\text{CTL-3g}} = 0.001$; $P_{\text{CTL-5g}} = 0.009$), notably after lunch that contained the test cream
187 cheese (240-480 min). CMRF-Cer/TAG ratio also significantly decreased in milk PL-treated groups,
188 regardless of dose ($P_{\text{group}} = 0.071$, $P_{\text{PL}} = 0.024$) (Figure 3B). Milk PL effects on SM molecular
189 composition in intestine-derived chylomicrons were mainly mediated by a significant decrease in
190 several CMRF-SM species content relative to CMRF-TAG including C16:0, C16:1, C18:0, C18:1,
191 C20:1, C24:0 and C24:1 SM species (Figure 3, C-E; Supplemental Figure 2). Changes in CMRF-Cer
192 molecular composition was mainly driven by a decrease of C22:0 and C24:1 Cer species content
193 relative to CMRF-TAG (Figure 3, F-H; Supplemental Figure 2).

194

195 **Ileostomy model reveals an important increase of saturated SM and Cer species in ileal efflux**

196 We performed a complementary mechanistic study in ileostomy patients to determine whether the
197 digestive fate of milk PL in the upper gastrointestinal tract may contribute to the above results, notably
198 before absorption and enterocyte metabolism (29). Each milk PL-enriched meal resulted in higher 8h-
199 cumulative ileal efflux of total SM and Cer (CTL: $4.4 \pm 1.3 \mu\text{mol}$; 3g-PL: $143.2 \pm 51.4 \mu\text{mol}$; 5g-PL:
200 $250.2 \pm 117.3 \mu\text{mol}$, $P_{\text{meal}} = 0.04$; CTL: $3.5 \pm 1.0 \mu\text{mol}$; 3g-PL: $67.9 \pm 21.5 \mu\text{mol}$; 5g-PL: 109.1 ± 15.0

201 μmol , $P_{\text{meal}} = 0.005$ respectively, Figure 4A). Detailed molecular composition analysis showed a
202 significant increase in C16:0 SM (CTL: $1.6 \pm 0.4 \mu\text{mol}$; 3g-PL: $32.2 \pm 12.7 \mu\text{mol}$; 5g-PL: 55.7 ± 29.9
203 μmol , $P_{\text{meal}} = 0.04$), C20:0 SM (CTL: $0.3 \pm 0.1 \mu\text{mol}$; 3g-PL: $15.2 \pm 4.8 \mu\text{mol}$; 5g-PL: 26.7 ± 11.6
204 μmol , $P_{\text{meal}} = 0.04$), C22:0 SM (CTL: $0.7 \pm 0.2 \mu\text{mol}$; 3g-PL: $46.9 \pm 15.8 \mu\text{mol}$; 5g-PL: 83.3 ± 36.3
205 μmol , $P_{\text{meal}} = 0.04$) and C16:0 Cer (CTL: $1.4 \pm 0.4 \mu\text{mol}$; 3g-PL: $20.0 \pm 4.4 \mu\text{mol}$; 5g-PL: 31.1 ± 3.1
206 μmol , $P_{\text{meal}} = 0.01$), C20:0 Cer (CTL: $0.1 \pm 0.0 \mu\text{mol}$; 3g-PL: $1.0 \pm 0.3 \mu\text{mol}$; 5g-PL: $1.5 \pm 0.3 \mu\text{mol}$,
207 $P_{\text{meal}} = 0.009$), C22:0 Cer species (CTL: $0.4 \pm 0.1 \mu\text{mol}$; 3g-PL: $17.7 \pm 7.2 \mu\text{mol}$; 5g-PL: 26.4 ± 6.3
208 μmol , $P_{\text{meal}} = 0.02$) (Figure 4, B and C). However, the analysis of SP species relative abundance
209 revealed a reduction in the proportions of atherogenic C16:0, C18:0 SM and C24:1 Cer species (P_{meal}
210 $= 0.02$, $P_{\text{meal}} = 0.04$ and $P_{\text{meal}} = 0.04$ respectively), and an increase in the proportions of C22:0 and
211 C24:0 SM species ($P_{\text{meal}} = 0.005$ and $P_{\text{meal}} < 0.001$) and Cer species ($P_{\text{meal}} = 0.02$ and $P_{\text{meal}} = 0.04$,
212 respectively; Supplemental Figure 3, A and B). Considering that such lipids cannot be absorbed
213 directly as such by enterocytes and that SM digestion is incomplete in the gastrointestinal tract (30),
214 we explored the molecular composition of SM and Cer species in fecal samples collected by the
215 postmenopausal women included in the VALOBAB-C trial.

216 217 **Fecal sphingolipidome is largely enriched in Cer after milk PL supplementation**

218 The 4-week nutritional intervention significantly increased total fecal SM and Cer in milk PL
219 supplemented groups compared to control (Figure 5, A and B) (ΔSM : CTL $-0.09 \pm 0.04 \mu\text{mol}$; 3g-PL
220 $+1.46 \pm 0.95 \mu\text{mol}$; 5g-PL $+1.76 \pm 0.83 \mu\text{mol}$ / g of dry feces, $P_{\text{group}} = 0.006$, $P_{\text{PL}} = 0.001$; ΔCer : CTL
221 $-0.15 \pm 0.08 \mu\text{mol}$; 3g-PL $+4.09 \pm 1.38 \mu\text{mol}$; 5g-PL $+7.69 \pm 2.95 \mu\text{mol}$ / g of dry feces, $P_{\text{group}} = 0.0002$,
222 $P_{\text{PL}} = 0.00006$). Altogether, this increase of total fecal Cer was higher than that of SM ($P = 0.015$, ΔCer
223 *versus* ΔSM in milk PL groups). The detailed molecular analysis revealed a major impact of

224 intervention on saturated SP species, notably an increase of C22:0 SM ($P_{\text{group}} = 0.009$, $P_{\text{PL}} = 0.003$),
225 C24:0 SM ($P_{\text{group}} = 0.011$, $P_{\text{PL}} = 0.002$), C16:0 Cer ($P_{\text{group}} = 0.0005$, $P_{\text{PL}} = 0.0001$), C22:0 Cer ($P_{\text{group}} =$
226 0.00001 , $P_{\text{PL}} = 0.00001$) and C24:0 Cer species ($P_{\text{group}} = 0.00002$, $P_{\text{PL}} = 0.006$) (Figure 5, C-E and I-
227 K, Supplemental Table 4). To a lower extent, the milk PL supplementation also increased the fecal
228 amount of some unsaturated SP species (Figure 5, F-H and L-N, Supplemental Table 4).

229 **DISCUSSION**

230

231 This study is the first to report how the daily consumption of a significant amount of dietary SP present
232 in milk PL impacts the endogenous sphingolipidome in the bloodstream and along the gastrointestinal
233 tract in humans. Firstly, we reveal that the increased intake of milk SM and Cer did not increase their
234 total amount in serum, but the molecular composition of SM and Cer species was markedly improved
235 by the 4-week intervention with milk PL. Notably, the atherogenic C16+18 SM and C24:1 Cer species
236 decreased significantly despite their increased intake from the provided supplementation. These
237 variations even correlated with the beneficial impacts of milk PL on lipid cardiovascular markers
238 reported previously (29). In addition, the Mayo Clinic published the reference values for circulating
239 level of C24:1 Cer species (i.e., 0.65-1.65 μ M, <https://www.mayocliniclabs.com>). Here, we found that
240 serum C24:1 Cer species concentration returned within the normal range after the intervention in the
241 5g-PL group only (V1: 1.96 \pm 0.17 μ M, V2: 1.59 \pm 0.16 μ M, $P_{\text{group}} = 0.033$). Our results demonstrate
242 that milk PL supplementation positively impacts the endogenous sphingolipidome, with the specific
243 decrease of serum SM and Cer species known for being associated with inflammation and metabolic
244 disorders (31, 32). Previous studies reported that high concentrations of serum C18:0, C20:0, and
245 C24:1 Cer species are associated with type 2 diabetes, while high serum levels of C16:0 Cer and C18:0
246 SM species correlate with insulin resistance (33). Regardless of milk PL dose, the analysis of the
247 relative abundance of each SM and Cer species in the bloodstream revealed a significant increase in
248 the proportions of C20:0 SM, C22:1 SM and C20:0 Cer species that are normally poorly detected in
249 human blood but found in non-negligible amount in MFGM. We also estimated to what extent the
250 changes in major SM or Cer species could explain the relationships between milk PL consumption and
251 enhanced lipid cardiometabolic risk factors. According to the estimated regression coefficients,
252 assuming a mean variation of C16+18 SM species of about -22 μ M as observed in the 5g-PL group,

253 its mean effect is expected to be (i) -0.16 mM on LDL-C (with a global effect of -0.34 mM observed
254 in this group) (29), and (ii) -0.19 mM on total C (with an observed effect of -0.4 mM). Moreover,
255 assuming a mean variation of the relative proportions of C24+26 Cer of about -0.83% as observed in
256 the 5g-PL group, its mean effect is expected to be (i) -0.032 mM on LDL-C and (ii) -0.045 mM on
257 total C. These results show that changes in serum total C and LDL-C are significantly associated with
258 changes in serum C16+18 SM species, and to a lower extent with the modulation of the relative
259 proportions of C24+26 Cer species (here mainly driven by the variation of C24:1 Cer species).

260

261 To investigate underlying mechanisms involved in the effects of milk PL consumption on circulating
262 SP species, we first estimated the contribution of intestine-derived chylomicrons, which are the dietary
263 lipid carriers secreted by the small intestine during the postprandial phase. Chylomicrons represent a
264 major source of circulating SM, although the mechanisms by which SM is inserted into these
265 lipoproteins have not been established (34). Dietary SM and Cer are not absorbed as such; their
266 lipolysis products released in the small intestine can be absorbed and a small proportion of their
267 sphingoid bases contribute to the newly formed SP ultimately found in chylomicrons (34). Herein, the
268 4-week milk PL supplementation decreased chylomicron total SM and Cer, especially during the
269 second part of the postprandial period (after test cheese consumption), without change in particle size
270 (i.e., no change in the surface-to-TAG core ratio) (29). These modifications were also observed at
271 species level for almost all SM and Cer species, including those whose concentrations in total serum
272 decreased, namely C16:1 SM, C20:1 SM and C24:1 Cer. Because SP are located at the surface of
273 lipoproteins, this reveals a lower SP amount in the chylomicron composition. Whether this is due to
274 decreased SM synthesis in enterocytes after intervention with dietary SP remains to be elucidated.
275 Milk PL also modified SM molecular profile in chylomicrons with an increase in the proportions of
276 C20 SM species and a decrease in the proportions of C24:1 SM species, possible precursor of C24:1

277 Cer *via* acid SMase (35). These results suggest that these modifications may originate from the gut or
278 from enterocyte metabolism during the intestinal digestion and absorption processes.

279

280 To determine the contribution of SM and Cer in the intestine, we analyzed their molecular profiles in
281 the gut lumen of ileostomy patients after the acute intake of milk PL, as well as in feces of the
282 postmenopausal women after 4-week milk PL supplementation. These analyses revealed an increase
283 of both total SM and Cer in gut contents in milk PL supplemented groups. At a molecular level, the
284 amounts of most detected SP species of interest were significantly increased by milk PL consumption,
285 especially C16:0, C22:0, C24:0 SM and Cer, and also C20:0 SM species. Altogether, SM and Cer
286 species whose amounts increased in gut contents reflect species that are present in milk PL-enriched
287 cheeses. These results are consistent with the fact that SM digestion is incomplete, as only 75-80% of
288 milk SM was reported to be digested and absorbed in humans (36). It has been previously reported that
289 ileal efflux of C16:0 SM was only ~10% of ingested dose *versus* ~20% for C24:0 SM after intake of
290 lower doses of SM, i.e., 50-200 mg (36), suggesting that longer-chain saturated species of SM and Cer
291 are less efficiently digested and absorbed. In addition, the digestion of SM being slow and incomplete,
292 it may induce an important increase of non-digested SM and non-absorbed Cer in the lumen content
293 (3, 30), which may explain the present results. Moreover, fecal metabolites, including the various lipid
294 species normally found in feces, may originate directly from food, but also from host cells, bacterial
295 cell components or indirectly from the molecular conversion of SP by gut microorganisms or host
296 enzymes (37). In a recent study performed in healthy patients, plasma and fecal lipidomic analyses
297 demonstrated that the lipid fraction of fecal samples contains significant amounts of Cer species with
298 only two SM species detected, while plasma samples commonly contain significant amounts of several
299 SM species and lower quantities of Cer (37, 38). Herein, we chose to determine the concentration of
300 12 SM and Cer species of interest in serum and we were also able to quantify all these species in feces.

301 In ileostomy patients, we report higher total SM amount in ileal content compared to total Cer, while
302 total Cer was largely more abundant in the fecal samples compared to total SM. The latter could be
303 the result of several metabolic pathways such as the conversion of dietary SM species in Cer species
304 by host enzymes present in the lumen and in enterocytes. At a molecular level, monounsaturated SM
305 and Cer species increased in both ileal efflux and fecal samples, despite being found in minority in test
306 cheeses compared to saturated species. A potential differential absorption process between
307 monounsaturated and saturated species would thus deserve to be investigated. Interestingly, the major
308 changes reported in the serum after the dietary intervention with milk PL mainly concern
309 monounsaturated SM and Cer species. It may suggest that some modifications of serum and
310 chylomicron SP profiles occur in response to changes in the SP fate in the small intestine.

311 The increased amount of total Cer reported in the fecal samples of milk PL supplemented volunteers
312 could also be the result of gut bacteria metabolism, because several bacteria, including those belonging
313 to the *Bacteroides* genus, were reported to be able to produce SP (39, 40). Very recently, Lee *et al.*
314 demonstrated in female mice that sphinganine, which is the main sphingoid base of SM and Cer present
315 in MFGM, is assimilated by gut bacteria (41). In this study, 99% of gavaged fluorescent sphinganine
316 was assimilated by *Bacteroides* spp.; the remaining 1% by *Prevotella* spp., *Lactobacillus* spp. and
317 *Bifidobacterium* genus (41). Also, *Bifidobacterium* spp., which are known to be increased after milk
318 SM consumption in rodents (42, 43), can release free milk Cer by hydrolyzing milk gangliosides (44).
319 In this context, the contribution of the gut microbiota SP metabolism in the effects of milk PL
320 consumption on the intestinal and circulating SM and Cer profiles cannot be ruled out.

321

322 To further explore potential endogenous mechanisms, we analyzed whole blood cells gene expression
323 of some key enzymes involved in SM synthesis (SM synthase 1 and 2, SGMS1 and SGMS2) and
324 hydrolysis (an acid sphingomyelinase – SMase, also called SM phosphodiesterase 1, SMPD1). We

325 found only slight effects on *SGMS1* and *SGMS2* expression (Supplemental Figure 4), while *SMPD1*
326 expression decreased in milk PL-treated groups compared to control ($P_{\text{group}} = 0.052$, $P_{\text{PL}} = 0.030$). As
327 previously described, acid SMase activity in plasma is increased in acute coronary syndromes (45).
328 However, these results were not likely to explain the changes observed in the circulating
329 sphingolipidome. It would be also relevant to consider the possible contribution of intestinal enzymes
330 given that the small intestine is rich in enzymes known to contribute to SP metabolism, such as alkaline
331 SMase that converts SM in Cer (35). Unfortunately, we could not collect intestinal biopsies from the
332 healthy postmenopausal women in the present study. However, in an 8-week milk PL supplementation
333 performed (0.9 wt%) in high-fat diet fed mice (22), we observed a significant increase of jejunal
334 expression of *Enpp7*, coding for the alkaline SMase, compared to the high-fat control group (1.7-fold
335 change). Interestingly, previous preclinical studies reported opposite impacts of Cer production
336 depending on SMase activity: Cer generated from neutral or acid intestinal SMases are more prompted
337 to exert pro-inflammatory effects while Cer generated from alkaline SMase promote anti-inflammatory
338 pathways (46, 47). The conversion of exogenous SM in Cer by the alkaline SMase could also play a
339 role in the inhibition of cholesterol absorption (47, 48), which is concordant with the present findings
340 and supports the role of SM metabolism in cholesterol absorption. Previous preclinical studies
341 demonstrated that dietary SM are able to play a beneficial role on cholesterol levels and more largely
342 in the prevention of cardiometabolic disorders (25, 26, 49, 50). In mice fed high-fat diets,
343 supplementation with egg SM lowered intestinal absorption of cholesterol and lipids with a reduction
344 of hepatic cholesterol (51). *In vitro*, both SM and Cer inhibit cholesterol absorption in Caco-2 intestinal
345 epithelial cells (47). However, it has been suggested that small SM catabolites, such as Cer and
346 sphingosine, might be the effectors of the beneficial impact of milk SM (2). The present findings
347 consolidate our previous clinical results given that the observed reductions in circulating total
348 cholesterol, LDL-C and ApoB (29) significantly correlate with the reduction of serum pro-

349 inflammatory C16+18 SM in the milk PL-treated groups. In accordance with above mentioned studies,
350 our findings also bring new information and insights on Cer in the gastrointestinal tract as signaling
351 molecules potentially participating in the beneficial effect of milk PL consumption on cholesterol
352 metabolism.

353

354 The present study has several strengths but also some limitations that need to be outlined. The clinical
355 trials were performed in real-life in a targeted population (i.e., overweight postmenopausal women)
356 known to present an important risk of cardiovascular diseases, but results cannot be extrapolated to
357 other populations. We took care to include 4-day dietary records before and after the nutritional
358 intervention to show that volunteers of the three groups did not differentially modify their energy and
359 dietary intakes (Supplemental Table 5). Many parameters of the present study were measured in a
360 limited number of subjects. Nevertheless, we performed for the first time a broad sphingolipidomic
361 analysis, including a large scale of measurements of SM and Cer species at both fasting and
362 postprandial states in various biological compartments: serum, chylomicron fractions, and feces, but
363 also in 8 h-cumulative ileal efflux from ileostomy patients. The sphingolipidome is a complex and
364 dynamic system that encompasses several important SP families, including dihydroceramides,
365 gangliosides or cerebroside (17). Considering the variations in the sphingoid bases, FA and
366 headgroups of SP molecules, the number of species exceeds thousands. SP are localized in cellular
367 membranes (lipid rafts) and are carried by albumin, lipoprotein particles, blood cells and platelets in
368 the bloodstream (52, 53). Based on the present findings, future studies should thus explore the
369 sphingolipidome in other blood compartments and potentially epithelial cells to better understand the
370 fate of milk SM and Cer species. Furthermore, we cannot exclude the potential contribution of other
371 components of the PL fraction/MFGM from buttermilk concentrate and/or the lower milk TAG content
372 in the PL-enriched cheeses in the reported metabolic effects in both trials. Putting aside these

373 limitations, this is to the best of our knowledge the first time that such a wide sphingolipidomic analysis
374 is performed in humans in response to a controlled dietary intervention in the context of
375 cardiometabolic disorders. The present study clearly responds to the need to identify relevant dietary
376 strategies to improve the endogenous SP metabolism, which was highlighted in recent reviews (2, 54).

377

378 The present findings uncover that milk PL supplementation providing particular SP species markedly
379 improved the endogenous sphingolipidome by reducing serum atherogenic C16+18 SM and C24:1 Cer
380 species in overweight postmenopausal women at risk of cardiovascular disease. These reductions in
381 SP were (i) correlated with, and (ii) significantly involved in the decrease of lipid cardiovascular risk
382 markers induced by milk PL intervention. We further demonstrate that despite a significant ingestion
383 of SP provided by milk PL, SM and Cer concentrations decreased in intestine-derived chylomicrons
384 while their concentration increased in gut contents. The related differences in SM and Cer profiles
385 between gut contents and circulating compartments suggest that small intestinal mechanisms occurred
386 during digestion and absorption processes of milk SM and Cer, and that a contribution of the gut
387 microbiota may be possible. Considering that milk PL are naturally found in large amounts in
388 buttermilk, which is still poorly valued in human food, such bioactive lipids could be envisioned as
389 promising ingredients for the development of new functional foods providing health effects in the
390 frame of chronic diseases.

391 **METHODS**

392

393 **VALOBAB-C trial.** Details of the VALOBAB-C study have been published previously (29). Briefly,
394 the multicenter study used a double-blind randomized placebo-controlled parallel design and was
395 conducted in 58 overweight postmenopausal women, without metabolic syndrome but at risk of CVD.
396 The eligibility criteria and sample size calculation have been described previously (29). Volunteers
397 were randomly divided into 3 groups. Randomization was performed electronically using random
398 number generator and supervised by the biostatistician (29). Both volunteers and investigators were
399 kept blind regarding group allocation. Volunteers were subjected to the daily consumption of either
400 control or milk PL-enriched cream cheese (100 g of cream cheese containing 13 g of total fat including
401 0 (control), 3 or 5g-milk PL during 28 days (n = 19; 19 and 20, respectively). The strategic approach
402 was to formulate cheeses with identical total lipid content with partial substitution of TAG by milk PL
403 to avoid increased energy intake. The 3g- and 5g-PL cream cheeses were based on a butterserum
404 concentrate rich in milk PL prepared according to Gassi *et al.* (55) representing a 3- to 5-fold increased
405 daily consumption of milk SM and Cer compared to an estimated intake of dairy SP in Western
406 countries) (Supplemental Table 1) (17). After the “run-in” period, volunteers were subjected to a first
407 exploratory visit (V1), followed by 28 days of intervention and ended by a second exploratory visit
408 (V2). During each visit, overnight fasted-participants received a breakfast meal rich in fat and
409 carbohydrates and 4 h later, they consumed a standardized lunch containing the corresponding test
410 cream cheese, thus dividing the exploratory visit in two specific postprandial periods (0-240 min and
411 240-480 min, as detailed previously) (29). Volunteers were asked to continue their usual diet and
412 physical activity all along the study. Participants were told to avoid the consumption of cheeses other
413 than the test cream cheese, and listed foods that may influence the gut microbiota composition.
414 Particular attention was drawn to standardize the meal consumed the evening before each postprandial

415 exploratory visit. Subjects recorded their food consumption for 4 days before and after the nutritional
416 intervention. No difference in changes in energy and macronutrient intakes, fibers, alcohol, cholesterol
417 and FA intakes was observed between groups (29). The primary outcome was the impact of the 4-
418 week milk PL consumption on fasting serum concentration of total C (29). The predefined secondary
419 outcomes tested in the present study were related to the impact of the dietary intervention on serum,
420 chylomicron and fecal SM and Cer profiles. Considering available samples and practical/technical
421 aspects, some analyses were performed on a subgroup of individuals only (Figure 1).

422

423 **VALOBAB-D trial.** The double-blind RCT was performed in 4 ileostomy patients following a
424 crossover design, as previously described (29) (Supplemental Figure 5). An ileostomy is a surgical
425 opening in the abdomen in which a piece of the ileum is brought outside the abdominal wall to create
426 a stoma through which digestive contents leave the body and are collected in a pouch (ileal efflux).
427 Selected patients according to eligibility criteria were invited to participate to 3 distinct exploratory
428 visits separated by a 4 to 6-week washout period (29). During each visit, overnight fasted patients
429 consumed one of the test cream cheese containing 0, 3- or 5g-milk PL and their ileal efflux was
430 collected over 8 h. Sequences of meal allocation were based on random number generator (29). Both
431 patients and investigators were kept blind regarding meal allocation.

432

433 **Isolation of chylomicron-rich fractions (CMRF).** Isolation of intestine-derived CMRF was
434 performed by ultracentrifugation from plasma collected at different time points as previously described
435 (29, 56).

436

437 **Analysis of serum phospholipids.** Total lipids were extracted from 300 μ L of serum with
438 chloroform:methanol (2:1, v/v) according to the method of Folch (57). After drying under nitrogen,

439 total lipids were determined gravimetrically and were dissolved precisely with 1 mL of
440 chloroform:methanol (2:1, v/v). This stock solution of total lipids was stored at -20 °C. Phospholipid
441 classes were then separated by high-performance liquid-chromatography coupled to an evaporative
442 light-scattering detector (SEDEX LT-ELSD SOLT, HPLC DDL SEDERE, ThermoFisher) (58, 59),
443 using a silica normal-phase column (Lichrospher Si 60, 3 µm, 100 x 4.6 mm, Waters). The
444 chromatographic separation was carried out using a linear binary gradient according to the following
445 scheme: t0 min: 90%A, 10%B 0%C, t20 min 42%A 52%B 6%C, t30 min 32%A 52%B 16%C, t55
446 min 30%A 70%B 0%C, t60 min 90%A 10%B 0%C. Total chromatographic run time was 75 min per
447 sample, which consisted of a 60 min analysis and 15 min to restore initial conditions and re-
448 equilibration. Eluent A consisted of hexane:tetrahydrofuran (99:1, v/v), eluent B of
449 isopropanol:chloroform (80:20, v/v/v) and eluent C of isopropanol:water (50:50, v/v/v). The flow rate
450 of the eluent was 1mL/min. Identification of phospholipids and lysophospholipids was carried out by
451 comparison with the retention time of pure standards (Avanti polar Lipids, USA). Calibration curves
452 for each compound were calculated from the area values of stock solution of pure standards between
453 0.1 to 1 mg / mL. Results were analyzed using Chromeleon software (Thermofisher) and expressed as
454 µg / 100 µL of serum.

455

456 **Analysis of SM and Cer molecular profiles.** Concentrations of SM and Cer molecular species of
457 interest were determined in serum, CMRF, ileal efflux, fecal samples and also in test cream cheese
458 according to the method by Kyrklund (60) which was optimized as previously described (27). Ileal
459 content from patients with ileostomy and fecal samples obtained from VALOBAB-C trial's volunteers
460 were freeze-dried and approximately 15-40 mg of lyophilized matter, accurately weighted, were
461 dissolved in 1mL of apyrogen water prior to lipid extraction. Briefly, for each sample, total lipids were
462 extracted using 2.5 mL of chloroform:methanol (1:2 v/v) in the presence of two deuterium-labelled

463 internal standards (N-heptadecanoyl-D-erythro-sphingosine (C17:0-Ceramide); N-palmitoyl(d31)-D-
464 erythro-sphingosylphosphorylcholine (C16:0D31SM) from Avanti Polar Lipids, Alabama, USA).
465 After 2 h of shaking and centrifugation (10 min, 1900 g), samples were evaporated with liquid nitrogen.
466 The dry samples were dissolved in 1.5mL of chloroform:methanol (1:2 v/v) and sonicated 30 sec on
467 ice. SP were then isolated by saponification with potassium hydroxide during 2 h at 37 °C and then
468 fractionated and desalted using reverse-phase Bond Elut C18 columns. The final elutions were done
469 with 2x1mL of chloroform:methanol (12:1 v/v) and 2x1mL of chloroform:methanol (1:2 v/v) prior to
470 the evaporation of samples with liquid nitrogen. The dry extracts were kept at -20 °C until tandem
471 mass spectrometry analysis (MS/MS). Samples were homogenized in 1mL of chloroform:methanol
472 (1:2 v/v) and analyzed by direct flow injection on a triple-quadrupole mass spectrometer (API 4500
473 QTRAP MS/MS; Sciex Applied Biosystems, Toronto, Canada) in the positive ionization mode using
474 the multiple reaction monitoring (MRM) method. Cer and SM species were measured separately, with
475 two different methods with a flow rate of 200 µl / min (analysis time of 3 min). We quantified 12 SM
476 and Cer species of particular interest regarding cardiovascular risk, being the most abundant in human
477 and also found in bovine milk in different proportions (Table 1). The concentration of each molecular
478 species was calculated from the ratio of its signal to that of the corresponding internal standard. Total
479 Cer and SM concentrations were the sum of the concentrations of the various species. Results are
480 presented based on the assumption of sphingosine d18:1 as the major sphingoid base for determined
481 SM and Cer species. These analyses were performed on a MS/MS platform accredited following EN
482 NF ISO 15189 requirements. The coefficient of variation (CV) for total SM and Cer was 4.4% and
483 5.4% respectively. For the most abundant isoforms (C16:0, C22:0, C24:0, C24:1 SM/Cer), the average
484 CV was $7 \pm 4\%$. The CV for the less abundant isoforms is slightly higher: $17 \pm 5\%$ for Cer and $9 \pm 6\%$
485 for SM species. These elements are in agreement with the Methods and Protocols section of
486 LIPIDMAPS for the analysis of SP (61).

487

488 **Gene expression analysis in whole blood cells.** The PAXgene™ Fresh Whole Blood RNA samples
489 were processed using the PAXgene™ Blood RNA Kit based on column purification of nucleic acids
490 (PreAnalytiX, QIAGEN) as previously described (29). After reverse transcription, real-time PCR
491 assays of *SGMS1* (F-CCTGGTATGCATTTCAACTG; R-TGGCCGCTGTACAGATAGTC), *SGMS2*
492 (F-CAATAGTGGGACGCAGATTC and R-GGACAATCCACCACCAGAAA) *SMPD1* (F-
493 CATCCTGCCAGGTTACATCG; R-CACACCTCCACCATGTCATC) were assessed using a Rotor-
494 Gene 6000 (QIAGEN) and obtained values were normalized to the expression of the housekeeping
495 gene *PGKI* (phosphoglycerate kinase 1, F-CCATGGTAGGAGTCAATCTG; R-
496 AGCTGGATCTTGTCTGCAAC).

497

498 **Statistics.** *VALOBAB-C*: Continuous variables are described as mean \pm SEM. The 4-week intervention
499 impact was determined by comparing the variation of each variable between exploratory visits (i.e.,
500 $\Delta V2-V1$) between groups (P_{group}) (i.e., control *versus* 3g-PL *versus* 5g-PL group; Figure 1). Single
501 time point parameters were analyzed through a general linear model and a subsequent Tukey's *post*
502 *hoc* test. P_{posthoc} corresponds altogether to $P_{\text{CTL vs 3g-PL}}$; $P_{\text{CTL vs 5g-PL}}$ and $P_{\text{3g vs 5g-PL}}$ as mentioned in the
503 text and figures. For parameters analyzed along the postprandial period, a mixed linear modelling
504 (MIXED procedure) was performed to account for within-subject repeated measures, seeking for main
505 effects, at least “group” or time effect and interaction. *Post hoc* analyses were performed following
506 Tukey-Kramer's test to both detail main effects and control for familywise type I error. In case of
507 residual distribution departing from normality, the analyses were performed on ranks. Global “milk
508 PL” effect was also considered as binary factor, and statistical analysis was performed by lumping
509 together milk PL doses in one group *versus* control. Spearman's correlation analyses were also
510 performed between blood lipid markers of cardiovascular risk and serum SM and Cer species grouped

511 in 3 subclasses (i.e., C16+C18; C20+C22 and C24+C26 SM or Cer species). In order to check for any
512 confounding effect, these analyses were also carried-out adjusting for center, age and waist
513 circumference quartiles. Analyses were performed on SAS v9.4 (SAS Institute Inc. Cary, NC, USA)
514 with a two-sided type I error set at 0.05. In order to determine to what extent the changes in SM and
515 Cer species could explain their relationship with lipid markers of cardiometabolic risk, we performed
516 additional analyses. We aimed to adjust the analysis of Δ LDL-C, Δ total C and Δ ApoB variables with
517 Δ C16+18 SM, $\Delta\%$ C20+22 and $\Delta\%$ C24+26 Cer species variables. We first transformed each covariate
518 as a 4 classes ordinal variable, and then checked for a linear relationship between each covariate and
519 each response variable, seeking for almost constant effect from an ordinal class to its neighbor. Since
520 we found merely monotonic relationship, it allowed us to include these covariates in their original
521 continuous form, associated with a unique and relevant regression coefficient, thereby simplifying
522 interpretation. We then reported the magnitude of the effect of Δ C16+18 SM, $\Delta\%$ C20+22 and
523 $\Delta\%$ C24+26 Cer species on Δ total C, Δ LDL-C and Δ ApoB by estimating the coefficient of regression
524 associated to each variable in the mixed linear general model. *VALOBAB-D*: Data are presented as
525 mean \pm SEM and were analyzed with GraphPad Prism 8.3. For normally distributed data (Shapiro-
526 Wilk's test), repeated measures one-way ANOVA were performed followed by Tukey's *post hoc* test.
527 For non-normally distributed data, a Friedman's test was performed followed by Dunn's *post hoc* test.
528 The variation between groups was reported using P_{meal} values and *post hoc* analyses were added
529 directly on corresponding figures using a, b letters. *Graphs*: All graphs and heat maps were created
530 using GraphPad Prism 8.3 (San Diego, CA, USA).

531

532 **Study approval.** Both clinical trials were approved by the Scientific Ethics Committee of Lyon Sud-
533 Est-IV and ANSM (French Agency for the Safety of Health Products) and registered at Clinical Trials

534 (NCT02099032, NCT02146339). The clinical trials were conducted at the Human Nutrition Research
535 Centre Rhône-Alpes (CRNH-RA; Lyon, France) and at the Human Nutrition Research Centre
536 Auvergne (CRNH-A; Clermont-Ferrand, France) according to the Second Declaration of Helsinki and
537 the French Huriet-Serusclet law. All data reported in the current article were obtained from samples
538 stored in the biobank during the clinical studies, for which participants gave a written consent in order
539 prior to inclusion in the study to use the samples for further metabolic analyses. All authors had access
540 to the study data and reviewed and approved the final manuscript.

541

542 **Author Contributions**

543 MLB: conceptualization, validation, formal analysis, investigation, data curation, writing - original
544 draft, visualization; CV: conceptualization, methodology, validation, formal analysis, investigation,
545 data curation, writing - original draft, visualization; ECom and LJ-C: methodology, formal analysis,
546 investigation, data curation; MLe: conceptualization, validation, formal analysis, investigation, data
547 curation; FJ: methodology, validation, formal analysis, investigation; MT, SP, EL, A-EB, KB, JD, AD
548 and CC: investigation; EM: methodology, validation, investigation; CR: formal analysis, data curation;
549 PG, NL and ECot: provided essential resources; AB-D: validation, formal analysis, investigation;
550 MLa: conceptualization; SL-P: conceptualization, methodology, validation, investigation; LO:
551 methodology, formal analysis, data curation; HV: contributed to results interpretation and revised
552 manuscript; CM-B: conceptualization, methodology; DC: conceptualization, methodology, validation,
553 formal analysis, investigation, writing - original draft, data visualization; M-CM: conceptualization,
554 methodology, writing - original draft, data visualization, project administration, supervision and
555 primary responsibility for final article content. All authors read, revised and approved the final
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557

558

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586

587 **Data sharing statement.** According to French law on the publication of biomedical research/clinical
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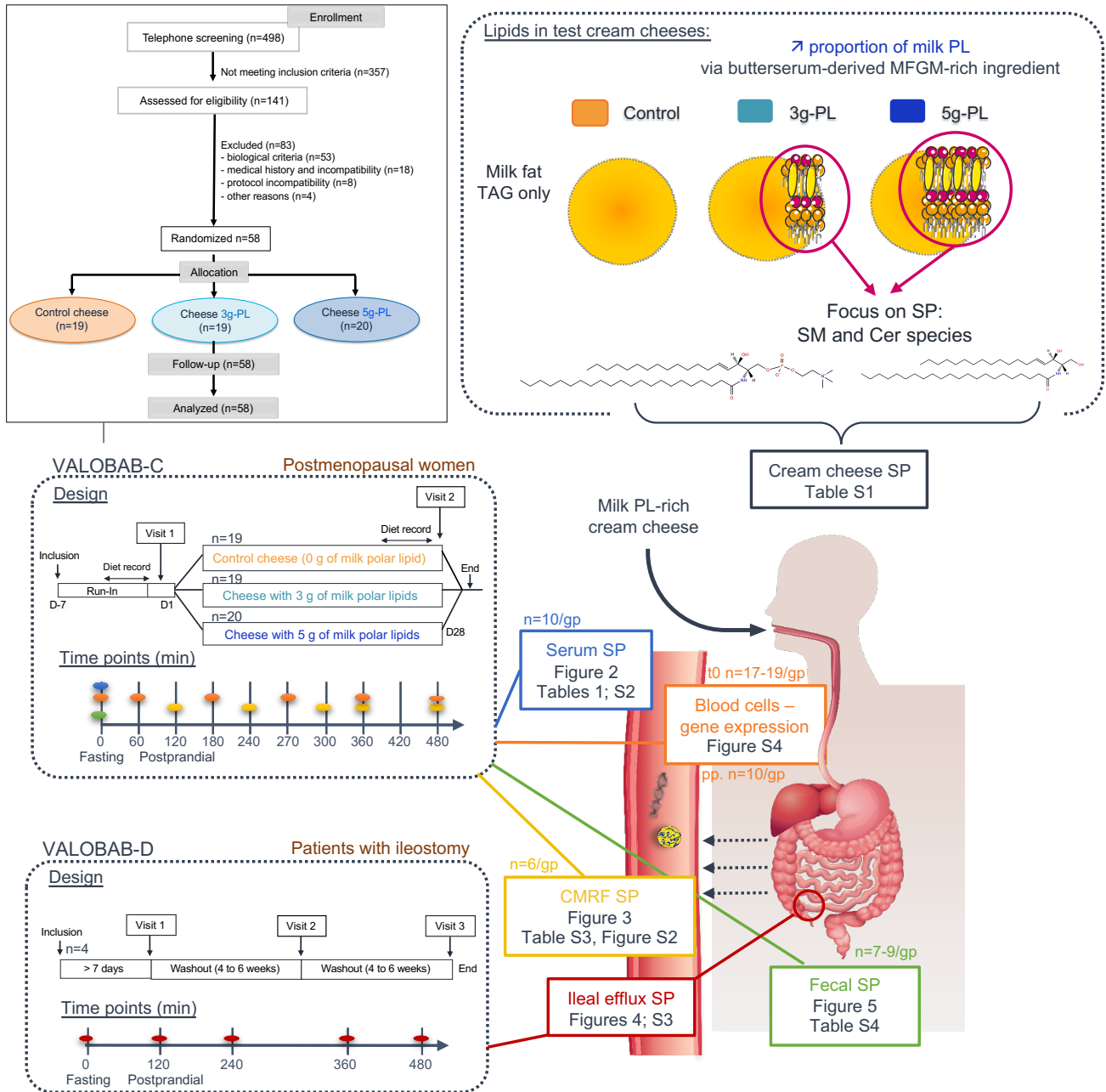
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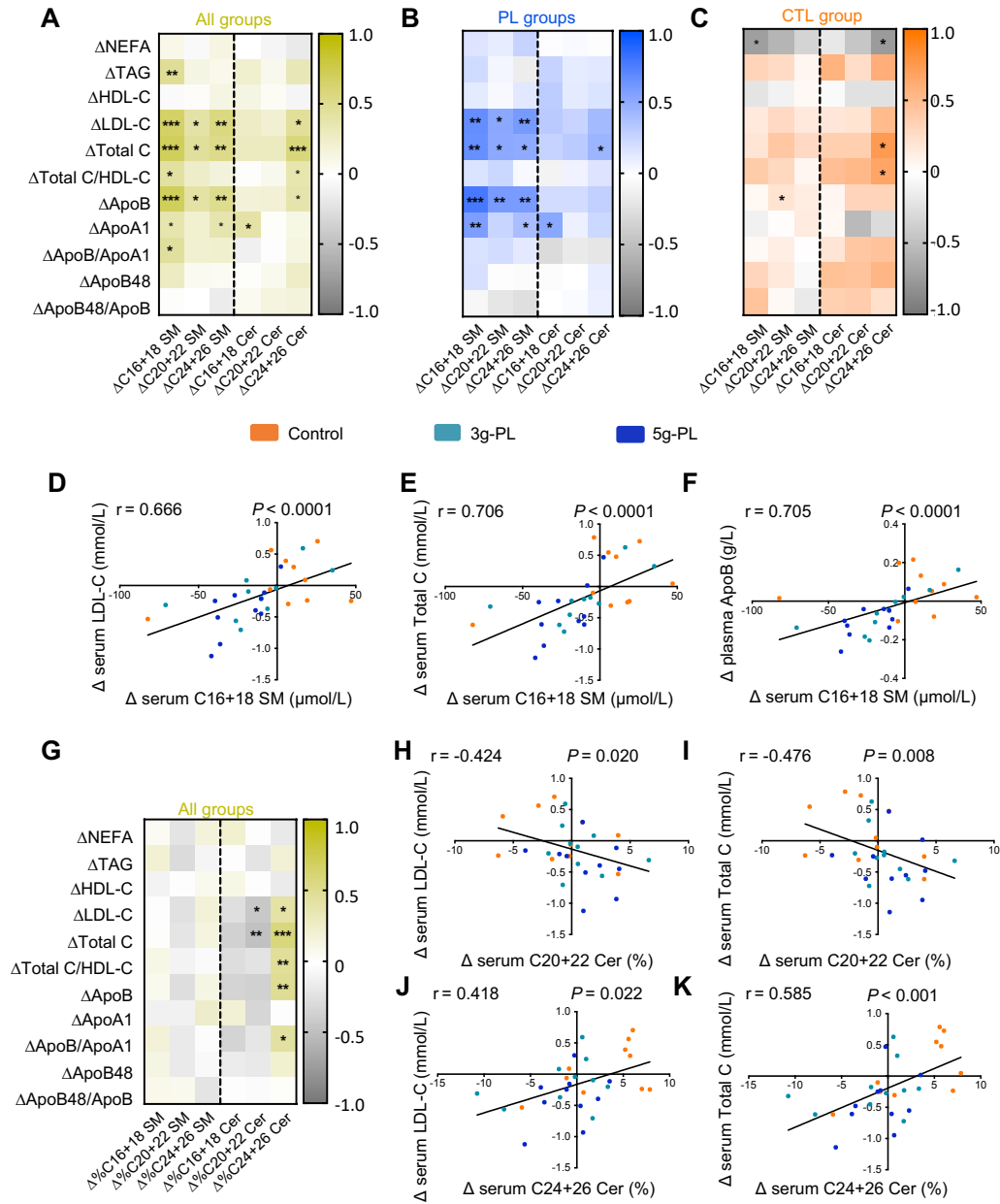
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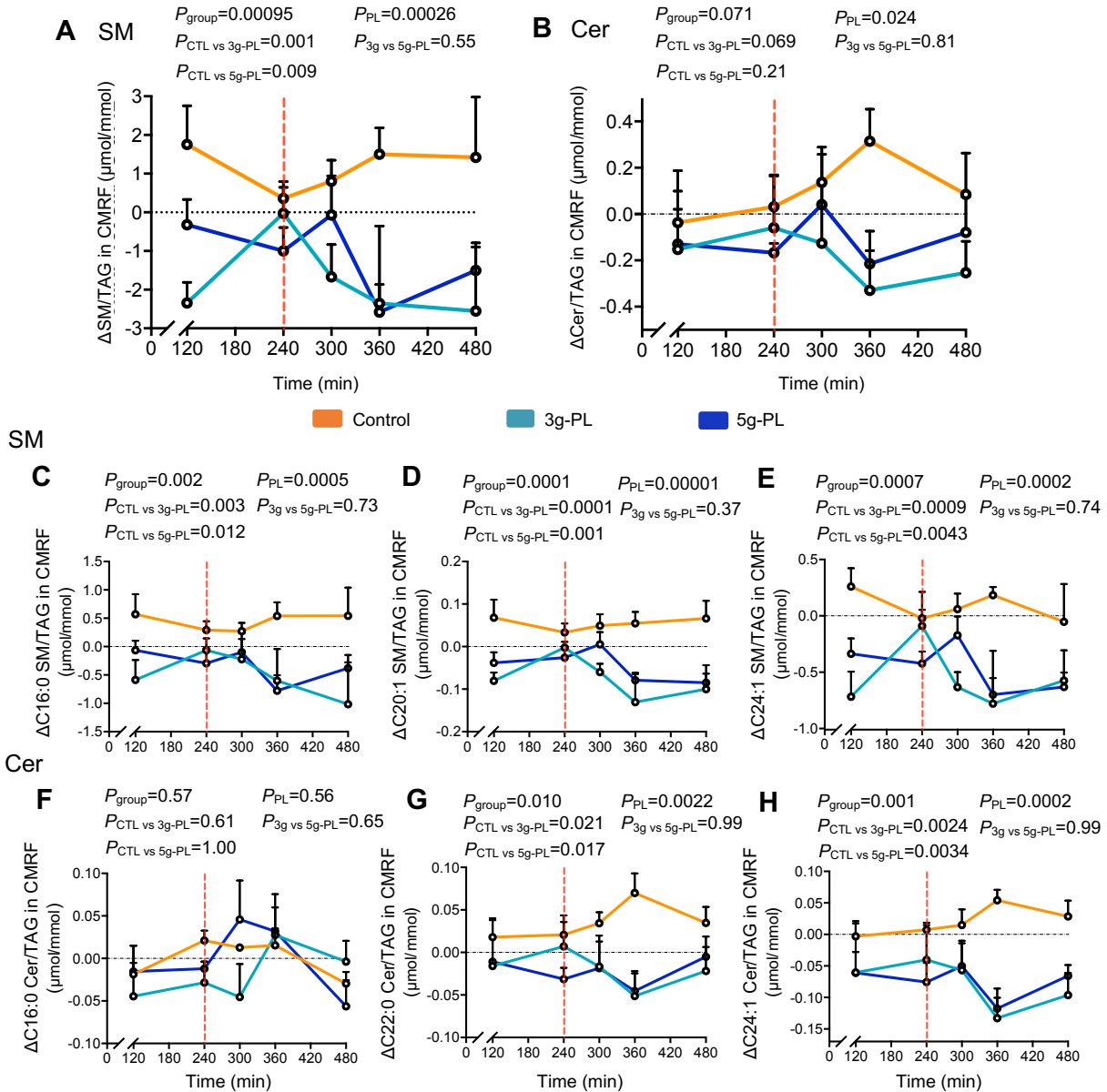
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732 **Figure 1: Design of VALOBAB-C and VALOBAB-D clinical trials and graphical summary of**
 733 **analyses performed on predefined secondary outcomes.** In the VALOBAB-C clinical trial, 58
 734 postmenopausal women were supplemented with test cream cheese containing either 0, 3 or 5 g of
 735 milk PL during 4 weeks. In the VALOBAB-D trial, 4 ileostomy patients were subjected to the acute
 736 consumption of the 3 test cheeses following a cross-over study design. In both trials, during the
 737 exploratory visit, overnight fasted volunteers received a standardized breakfast rich in fat and sugars
 738 at time 0 and a meal containing the test cream cheese at time 240 min of the postprandial period. Tables
 739 and Figures reporting specific results are listed. Cer: ceramides; CMRF: chylomicron-rich fraction;
 740 MFGM: milk fat globule membrane; PL: polar lipids; SP: sphingolipids; SM: sphingomyelins; TAG:
 741 triacylglycerols. Molecular structures were drawn using the LIPIDMAPS® tool.



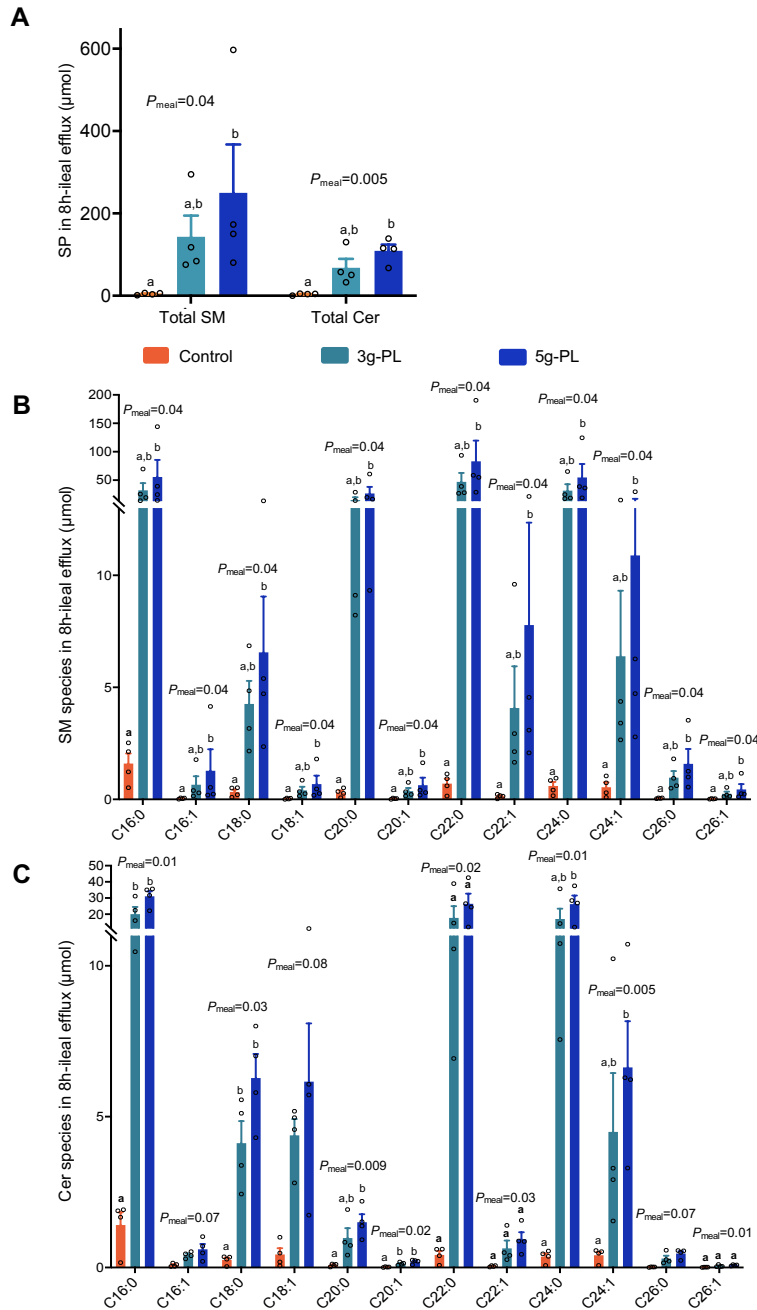
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743 **Figure 2: Major correlations between the impacts of milk PL supplementation on blood lipids**
 744 **and on serum SM and Cer (VALOBAB-C).** (A-C) and (G): Spearman's correlations between blood
 745 lipids and serum SM and Cer species. All data are expressed as Δ V2-V1 at fasting (yellow: all groups
 746 were considered for the analysis (A and G) (n = 30); blue: the 2 groups supplemented with either 3 or
 747 5g-milk PL only (n = 20); orange: the control group only (n = 10). For panels (A-C) and (G), asterisks
 748 in bold represent correlations that remain significant after adjustment for clinical center, quartiles of
 749 volunteer age and waist circumference. Graphs illustrating specific Spearman's correlations between
 750 the intervention impact on C16+18 SM species and on LDL-C (D), Total C (E) and ApoB48 (F);
 751 between C20+22 Cer species proportions (%) and LDL-C (H), and Total C (I); between C24+26 Cer
 752 species proportions (%) and LDL-C (J), and Total C (K). Apo: apolipoprotein; C: cholesterol; Cer:
 753 ceramides; CTL: control; HDL: high density lipoprotein; LDL: low density lipoprotein; NEFA: non-
 754 esterified fatty acids; PL: polar lipids; SM: sphingomyelin; TAG: triacylglycerols.



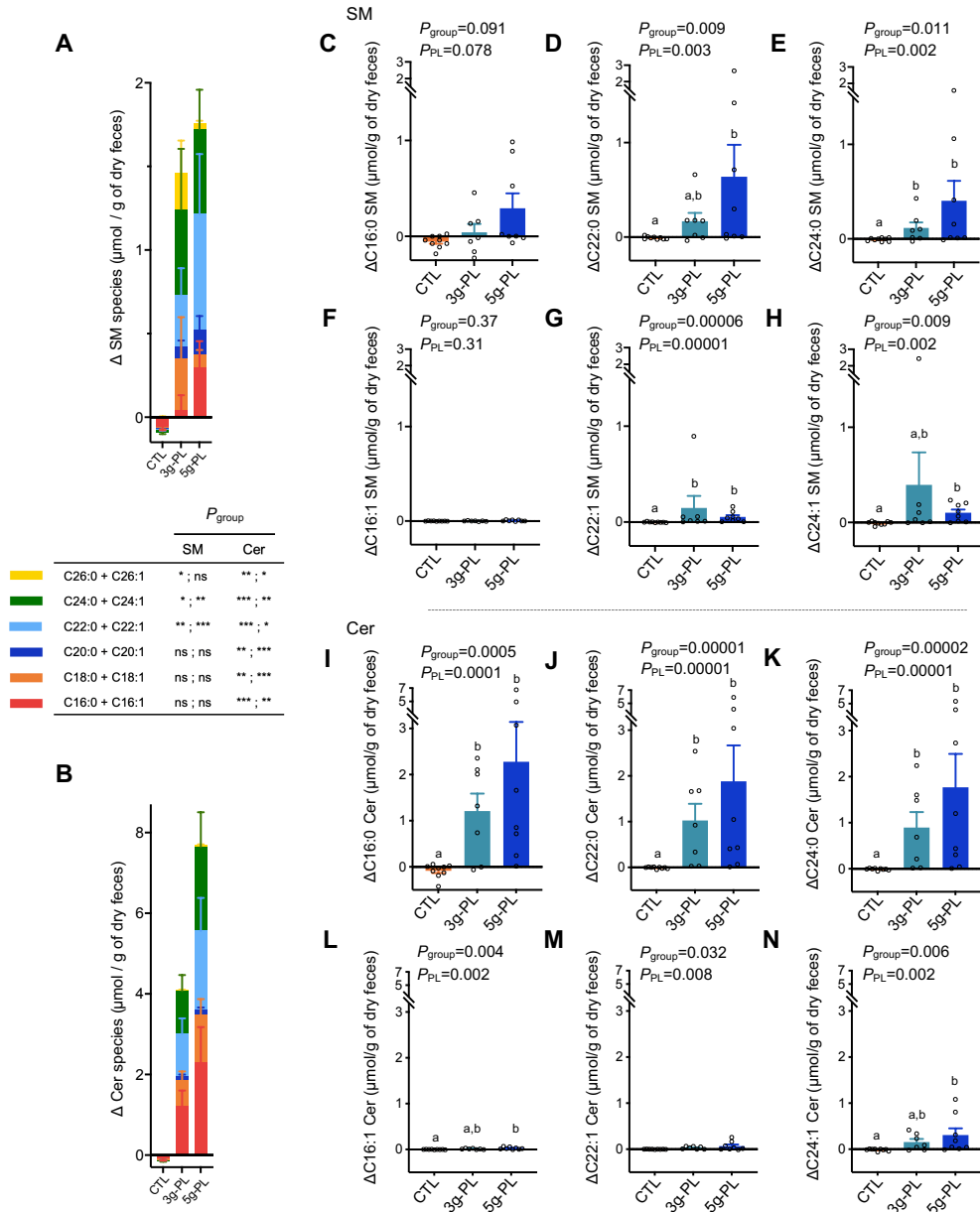
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756 **Figure 3: Milk PL supplementation during 4 weeks modulate SM and Cer molecular**
 757 **composition of plasma CMRF (see complements in Supplemental Table 3 and Supplemental**
 758 **Figure 2, VALOBAB-C trial).** Kinetics of $\Delta V2-V1$ CMRF SM and Cer normalized by CMRF TAG
 759 content (A and B, respectively). Molecular composition analysis of specific SM and Cer species in
 760 CMRF after normalization by CMRF TAG content: C16:1 SM (C), C20:1 SM (D), C24:1 SM (E),
 761 C16:1 Cer (F), C22:0 Cer (G), C24:1 Cer (H). Data are presented as mean \pm SEM (n = 6 / group). The
 762 vertical dotted line represents the intake of the meal including the control or milk PL-rich dairy
 763 (according to group). The P_{group} and P_{posthoc} are shown for the postprandial period from 120 to 480 min.
 764 Statistical analysis was done using a linear mixed model followed by Tukey-Kramer's *post hoc* test.
 765 P_{posthoc} corresponds altogether to $P_{\text{CTL versus 3g-PL}}$; $P_{\text{CTL versus 5g-PL}}$ and $P_{\text{3g versus 5g-PL}}$. Results are presented
 766 based on the assumption of sphingosine d18:1 as the major sphingoid base for determined SM and Cer
 767 species. Cer: ceramides; CMRF: chylomicron-rich fraction; CTL: control; PL: polar lipids; SM:
 768 sphingomyelins; TAG: triacylglycerols.



769

770 **Figure 4: Milk PL ingestion modulate SM and Cer species in ileal efflux in ileostomy patients**
 771 **(see complements in Supplemental Figure 3, VALOBAB-D trial).** Cumulated enrichment over 0-
 772 480 min of total SM and Cer (A). Molecular composition of ileal content efflux after 8 h of
 773 accumulation in SM (B) and Cer (C) species. Data are expressed in µmol and presented as mean ±
 774 SEM (n=4/group) and empty circles represent individual values. Statistical analysis was done using
 775 one-way ANOVA followed by Tukey's *post hoc* test (normal data) or Friedman's test followed by
 776 Dunn's *post hoc* test (non-normal data). Letters "a" and "b" indicate statistically different intervention
 777 effects between groups as calculated by *post hoc* analysis. Results are presented based on the
 778 assumption of sphingosine d18:1 as the major sphingoid base for determined SM and Cer species. Cer:
 779 ceramides; CTL: control; PL: polar lipids; SP: sphingolipids; SM: sphingomyelins.



780

781 **Figure 5: Effect of milk PL supplementation during 4 weeks on SM and Cer species excreted in**
 782 **feces (see complements in Supplemental Table 4, VALOBAB-C trial).** Molecular composition of
 783 SM (A) and Cer (B) in fecal samples (Δ V2-V1). Data are presented as mean \pm SEM (control, n = 9;
 784 3g-PL, n = 7; 5g-PL, n = 8) and expressed in $\mu\text{mol/g}$ of lyophilized feces. Empty circles represent
 785 individual values. Variations of specific SP species present in fecal samples were also determined and
 786 expressed as percentage of total SM and Cer, respectively: C16:0 SM (C), C22:0 SM (D), C24:0 SM
 787 (E), C16:1 SM (F), C22:1 SM (G), C24:1 SM (H), C16:0 Cer (I), C22:0 Cer (J), C24:0 Cer (K), C16:1
 788 Cer (L), C22:1 Cer (M) and C24:1 Cer species (N) (Δ V2-V1). Statistical analysis was done using non-
 789 parametric analysis (non-normal data). Letters “a” and “b” indicate statistically different intervention
 790 effects between groups as calculated by *post hoc* analysis. Results are presented based on the
 791 assumption of sphingosine d18:1 as the major sphingoid base for determined SM and Cer species. Cer:
 792 ceramides; CTL: control; PL: polar lipids; SM: sphingomyelins.

793 **Table 1: Impact of 4-week supplementation with milk PL on serum phospholipids and molecular**
 794 **composition of serum SM and Cer species (see complements in Supplemental Table 2).**

795

	CTL		3g-PL		5g-PL		P_{group}	P_{PL}
	V1	$\Delta\text{V2-V1}$	V1	$\Delta\text{V2-V1}$	V1	$\Delta\text{V2-V1}$		
Serum phospholipids ($\mu\text{g} / 100 \mu\text{L}$)								
Total phospholipids	227.56 \pm 8.68	-7.02 \pm 5.31	220.47 \pm 5.31	-6.69 \pm 5.04	230.34 \pm 5.89	-18.37 \pm 3.74	0.97	0.34
PC	150.28 \pm 5.33	-2.63 \pm 3.58	143.76 \pm 3.87	-3.51 \pm 3.61	154.49 \pm 3.89	-12.51 \pm 2.91	0.078	0.2
PE	4.94 \pm 0.36	-0.14 \pm 0.16	5.15 \pm 0.31	-0.36 \pm 0.28	5.43 \pm 0.28	-0.28 \pm 0.19	0.76	0.49
PI	19.17 \pm 1.92	-1.03 \pm 1.20	19.54 \pm 1.60	-0.84 \pm 1.32	18.83 \pm 1.61	-2.07 \pm 1.56	0.12	0.79
LysoPC	9.57 \pm 0.63	-0.32 \pm 0.38	8.99 \pm 0.47	-0.08 \pm 0.33	9.38 \pm 0.48	-0.75 \pm 0.34	0.38	0.81
SM	43.61 \pm 1.74	-2.9 \pm 1.16	43.03 \pm 1.55	-1.90 \pm 1.20	42.2 \pm 1.87	-2.76 \pm 0.90	0.79	0.68
SM/PC	0.29 \pm 0.01	-0.02 \pm 0.01	0.30 \pm 0.01	-0.01 \pm 0.01	0.27 \pm 0.01	0.00 \pm 0.01	0.098	0.069
Molecular composition of serum SM ($\mu\text{mol} / \text{L}$)								
Total SM	368.11 \pm 29.41	4.35 \pm 24.82	347.52 \pm 20.81	-25.19 \pm 22.15	405.18 \pm 24.22	-36.37 \pm 13.31	0.37	0.17
C16:0 SM	121.11 \pm 14.14	1.95 \pm 7.74	114.84 \pm 10.01	-7.05 \pm 6.31	131.49 \pm 14.10	-12.44 \pm 3.10	0.25	0.12
C16:1 SM	17.82 \pm 2.04	0.44 \pm 0.97 ^a	17.29 \pm 1.49	-0.64 \pm 0.84 ^{a,b}	20.03 \pm 2.12	-3.35 \pm 0.50 ^b	0.007[†]	0.029
C18:0 SM	28.48 \pm 2.78	0.81 \pm 1.40	27.62 \pm 2.20	-2.44 \pm 1.40	31.67 \pm 2.88	-3.40 \pm 1.66	0.13	0.048
C18:1 SM	13.94 \pm 1.69	1.02 \pm 0.87 ^a	13.78 \pm 1.01	-2.21 \pm 0.69 ^{a,b}	14.78 \pm 1.44	-2.99 \pm 0.79 ^b	0.003	0.0007[†]
C20:0 SM	17.83 \pm 1.37	0.79 \pm 1.64	17.72 \pm 1.39	0.73 \pm 1.40	21.12 \pm 1.06	1.29 \pm 0.78	0.95	0.89
C20:1 SM	7.79 \pm 0.59	0.62 \pm 0.61 ^a	7.62 \pm 0.42	-0.71 \pm 0.5 ^{a,b}	8.87 \pm 0.59	-1.57 \pm 0.48 ^b	0.025	0.013
C22:0 SM	30.94 \pm 2.77	1.00 \pm 2.85	28.10 \pm 1.86	0.43 \pm 2.52	35.59 \pm 1.31	0.36 \pm 1.81	0.98	0.8
C22:1 SM	28.30 \pm 1.80	0.69 \pm 2.03	27.31 \pm 1.75	-0.27 \pm 2.42	33.16 \pm 1.37	-1.58 \pm 1.31	0.72	0.87
C24:0 SM	26.71 \pm 2.49	-0.84 \pm 2.63	23.04 \pm 1.6	-2.12 \pm 2.1	28.14 \pm 1.69	-1.89 \pm 1.92	0.91	0.67
C24:1 SM	74.11 \pm 6.12	-2.01 \pm 5.50	69.28 \pm 4.03	-10.75 \pm 5.26	79.27 \pm 4.95	-10.69 \pm 4.28	0.38	0.16
C26:0 SM	0.39 \pm 0.04	-0.03 \pm 0.05	0.33 \pm 0.03	-0.07 \pm 0.05	0.34 \pm 0.02	0.00 \pm 0.04	0.63	0.89
C26:1 SM	0.69 \pm 0.09	-0.08 \pm 0.07	0.59 \pm 0.05	-0.10 \pm 0.05	0.73 \pm 0.08	-0.10 \pm 0.10	0.98	0.85
Molecular composition of serum Cer ($\mu\text{mol} / \text{L}$)								
Total Cer	8.26 \pm 0.54	0.34 \pm 0.59	8.34 \pm 0.66	0.49 \pm 0.79	10.3 \pm 0.77	-0.64 \pm 0.73	0.49	0.64

16:0 Cer	0.76±0.07	-0.01±0.06	0.71±0.07	0.09±0.13	0.86±0.08	-0.03±0.12	0.68	0.77
16:1 Cer	0.48±0.05	-0.05±0.06	0.41±0.04	0.02±0.03	0.45±0.04	-0.03±0.02	0.44	0.33
18:0 Cer	0.55±0.02	-0.02±0.02	0.58±0.03	0.07±0.08	0.65±0.07	-0.10±0.07	0.16	0.92
18:1 Cer	0.1±0.01	-0.01±0.01	0.09±0.01	0.01±0.02	0.11±0.01	-0.01±0.01	0.55	0.61
20:0 Cer	0.89±0.1	-0.07±0.04	0.90±0.10	0.06±0.08	1.00±0.10	-0.03±0.07	0.41	0.30
20:1 Cer	0.19±0.08	0.01±0.03	0.19±0.08	0.00±0.01	0.25±0.09	0.00±0.03	0.94	0.75
22:0 Cer	0.89±0.08	0.09±0.08	0.93±0.09	0.11±0.10	1.25±0.14	0.00±0.13	0.74	0.77
22:1 Cer	0.05±0.01	-0.01±0.01	0.04±0.00	0.00±0.00	0.05±0.00	0.00±0.01	0.76	0.57
24:0 Cer	2.71±0.32	0.30±0.35	2.72±0.26	0.21±0.26	3.54±0.4	-0.06±0.28	0.69	0.55
24:1 Cer	1.48±0.13	0.11±0.08 ^a	1.58±0.19	-0.19±0.08 ^{a,b}	1.96±0.17	-0.37±0.18 ^b	0.033	0.016
26:0 Cer	0.11±0.02	0.01±0.02	0.11±0.01	0.12±0.12	0.14±0.02	0.00±0.02	0.43	0.57
26:1 Cer	0.05±0.01	-0.01±0.01	0.05±0.01	-0.01±0.01	0.05±0.00	-0.01±0.01	0.92	0.81

796

797 Data are presented as mean ± SEM, n = 10 / group. Results are presented based on the assumption of sphingosine d18:1 as
 798 the major sphingoid base for determined SM and Cer species. *P* values presented in bold highlight significant intervention
 799 effect. *P*_{group} represents *P* value associated with group effect as calculated by generalized linear model, while *p*_{PL} represents
 800 *P* value associated with binary effect of milk PL compared to control. † *P* value remains significant (< 0.05) after adjustment
 801 for clinical center, quartiles of volunteer age and waist circumference. ^{a,b} Different superscript letters indicate statistically
 802 different intervention effects between groups as calculated by *post hoc* analysis.