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1 **Title page**

2 Title: Association of 1-deoxy-sphingolipids with steatosis but not steatohepatitis nor fibrosis
3 in Non-Alcoholic Fatty Liver Disease

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32

33 Disclosures

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41

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45

46 **List of abbreviations**

47	ALP	Alkaline phosphatase
48	ALT	Alanine aminotransferase
49	AST	Aspartate aminotransferase
50	BMI	Body Mass Index
51	C ₁₆ -SA	C ₁₆ -sphinganine
52	C ₁₆ -SO	C ₁₆ -sphingosine
53	C ₁₇ -SO	C ₁₇ -sphingosine
54	C ₁₈ -SA	C ₁₈ -sphinganine
55	C ₁₈ -SO	C ₁₈ -sphingosine
56	C ₁₈ -phyto-SO	C ₁₈ -phyto-sphingosine
57	C ₁₉ -SO	C ₁₉ -sphingosine
58	C ₂₀ -SA	C ₂₀ -sphinganine
59	C ₂₀ -SO	C ₂₀ -sphingosine
60	CD	Confidence Distribution
61	CI	Confidence Interval
62	deoxy-SA	deoxy-sphinganine
63	deoxy-SO	deoxy-sphingosine
64	DSB	1-Deoxy Sphingoid Base
65	dSL	deoxy SphingoLipids
66	FLIP	Fatty Liver Inhibition of Progression
67	γGT	gamma Glutamyl Transpeptidase
68	HbA _{1c}	Haemoglobin A _{1c}
69	HDL-C	High Density Lipoprotein-Cholesterol

70	HOMA	Homeostatic Model Assessment
71	IFG	Impaired Fasting Glucose
72	IGT	Impaired Glucose Tolerance
73	IR	Insulin Resistance
74	LDL-C	Low Density Lipoprotein-Cholesterol
75	MetS	Metabolic Syndrome
76	NAFL	Non-Alcoholic Fatty Liver
77	NAFLD	Non-Alcoholic Fatty Liver Disease
78	NASH	Non-Alcoholic Steatohepatitis
79	NASH \geq F2	Non-Alcoholic Steatohepatitis with significant fibrosis
80	OGTT	Oral Glucose Tolerance Test
81	PPAR	Peroxisome Proliferator-Activated Receptor
82	SA	Sphinganine
83	SA-dienine	Sphingadienine
84	SO	Sphingosine
85	S1P	Sphingosine-1-Phosphate
86	SphK	Sphingosine Kinase
87	SPT	Serine-Palmitoyl Transferase
88	TG	TriGlycerides
89	T2DM	Type 2 Diabetes Mellitus
90	WC	Waist Circumference
91		
92		

93 **Abstract**

94 **Background:** Non-Alcoholic Fatty Liver Disease (NAFLD) is the most important cause of
95 chronic liver disease in the western world. In some individuals liver steatosis can be
96 accompanied by inflammation and cell damage (Non-Alcoholic Steatohepatitis, NASH), and
97 even liver fibrosis. Sphingolipids are a heterogeneous class of lipids and essential
98 components of the plasma membrane and plasma lipoproteins. The atypical class of deoxy-
99 sphingolipids have been implicated in the metabolic syndrome and type 2 diabetes.

100 **Aim:** To determine if circulating (deoxy)sphingolipids are associated with NAFLD and its
101 different entities, steatosis, inflammatory changes (inflammation and ballooning) and
102 fibrosis.

103 **Methods:** Sphingolipids were analysed by LC-MS after hydrolysing the N-acyl and O-linked
104 headgroups in plasma of obese adults who underwent a liver biopsy in suspicion of NAFLD.

105 **Results:** 288 patients were included, liver status was as follows: 17.7% control, 16.3% NAFL
106 (Non-Alcoholic Fatty Liver or isolated steatosis), 51.7% NASH without significant fibrosis and
107 14.3% NASH with significant fibrosis. There was no association between typical sphingolipids
108 and NAFLD and its different entities. There was a statistically significant association between
109 the presence of steatosis and the concentrations of deoxy-sphinganine (exp(B) 11.163 with
110 CI [3.432, 36.306] and $p < 0.001$) and deoxy-sphingosine (exp(B) 8.486 with CI [3.437, 20.949]
111 and $p < 0.001$). There was no association between these deoxy-sphingolipids and activity of
112 the steatohepatitis, nor was there any association with fibrosis. Differences in deoxy-
113 sphingolipids also correlated independently with the presence of the metabolic syndrome,
114 but not diabetes.

115 **Conclusion:** Deoxy-sphingolipids are elevated in patients with steatosis compared to those
116 without fatty liver, but not different between the different NAFLD subtypes, suggesting that
117 deoxy-sphingolipid bases might be involved in steatogenesis, but not in the further
118 progression of NAFLD to NASH nor in fibrogenesis.

119

120 **Introduction**

121 **NAFLD**

122 Non-Alcoholic Fatty Liver Disease (NAFLD) is the most important cause of chronic liver
123 disease in the western world. In some individuals liver steatosis can be accompanied by liver
124 cell damage, inflammation, and even liver fibrosis. The latter can lead to cirrhosis which can
125 further result into end-stage liver disease[1]. The pathogenesis of NAFLD is still not
126 completely elucidated. Presumably, the integrated action of numerous conditions acting in
127 parallel (genetic predisposition, adipose tissue dysfunction, insulin resistance (IR), oxidative
128 stress, lipotoxicity, gut dysbiosis...) results in NASH, giving rise to the multiparallel hit
129 hypothesis[2]. Different studies have shown that fibrosis grade is the strongest predictor for
130 hepatic- and extra-hepatic complications[3, 4], whereas steatohepatitis is considered the
131 driving force of disease progression and adverse outcomes.

132 **Sphingolipids**

133 Sphingolipids are a heterogeneous class of bioactive lipids with a plethora of functions
134 playing important roles in almost all major aspects of cell biology including metabolism,
135 inflammation, autophagy and cell adhesion and migration[5]. Furthermore, they are major
136 components of cell membranes and contribute to plasma lipoprotein formation. The
137 enzyme Serine-Palmitoyl Transferase (SPT) catalyses the first and rate-limiting step in the *de*
138 *novo* synthesis of sphingolipids, which converts L-serine and palmitoyl-CoA into 3-keto-
139 sphinganine. The latter is converted into sphinganine (SA). SA is N-conjugated with another
140 fatty acid to form dihydro-ceramide. When desaturated at C4 it forms ceramide, the
141 building block for the more complex sphingolipids (Fig. 1)[6]. Ceramidase converts ceramide
142 into sphingosine (SO), the most abundant long-chain sphingoid base in mammalian cells.

143 Sphingosine-1-Phosphate (S1P) is formed after phosphorylation of SO by two Sphingosine
144 Kinases (SphK1 and -2)[7]. In plasma, SO is the most common sphingoid base, followed by
145 sphingadienine (SA-dienine) and SA[8]. Ceramide and S1P have been implicated in key steps
146 of the pathophysiology of NAFLD in animal models[7]. Data in humans are, however, scarce.
147 L-serine and palmitoyl-CoA are the preferred substrates for SPT, however, other substrates
148 can be used, including acyl-CoA with carbon chain lengths ranging from C12 to C18.
149 Furthermore, SPT can use other amino acid substrates beside L-serine: L-alanine and to a
150 certain extent L-glycine. The use of these alternative substrates generates a category of
151 atypical sphingolipids: the 1-Deoxy Sphingolipids (dSLs) that lack the C1-OH group of the
152 regular sphingolipids (Fig. 1). dSLs are not metabolised to form complex sphingolipids nor
153 are they degraded by the regular sphingoid catabolism pathway[9]. The function of these
154 dSLs is unknown. dSL levels were shown to be increased in plasma of patients with the
155 Metabolic Syndrome (MetS) and Type 2 Diabetes Mellitus (T2DM)[9]. Furthermore, they
156 could play a role in the pathogenesis of T2DM with the dSLs being cytotoxic for insulin
157 producing cells as they induce senescence and multiple cell death pathways[10]. NAFLD,
158 MetS and T2DM are closely related and originate from the same underlying pathological
159 processes, such as IR, lipotoxicity, dyslipidaemia and chronic inflammation[11]. Since there
160 is a correlation between the MetS, T2DM and (deoxy)sphingolipids, on the one hand, and a
161 close relationship between the MetS, T2DM and NAFLD, on the other hand, we aimed at
162 investigating whether :

- 163 1. circulating (1-deoxy) sphingoid bases are associated with NAFLD and its different
164 components, steatosis, cell damage & inflammatory changes (lobular inflammation
165 and ballooning) and fibrosis;

166 2. associations between deoxy-sphingolipids, on the one hand, and the MetS or T2DM,
167 on the other hand, are driven by the presence of NAFLD and its different
168 components.

169 **Patients & Methods**

170 **Study participants**

171 The protocol of patient selection has previously been described[12]. Briefly, patients visiting
172 the obesity clinic of the Antwerp University Hospital were consecutively recruited. Every
173 patient underwent a metabolic and hepatologic work-up both approved by the Ethics
174 Committee of the Antwerp University Hospital (reference 6/25/125, Belgian registration
175 number B30020071389) in order to screen for NAFLD. Subjects had to be 18 years or older.
176 As T2DM harbours potential confounders, patients with a known history of diabetes were
177 excluded. However, patients were included if diabetes was *newly* diagnosed. Sample
178 collection for sphingolipid analysis was taken before any glucose lowering therapy was
179 started. Patients with significant alcohol consumption were excluded. A liver biopsy was
180 proposed if there was a suspicion of liver disease, these were mainly performed outside the
181 setting of bariatric surgery in order to exclude severe liver abnormalities prior to surgery.
182 Only patients who underwent a liver biopsy were included for this study. Biopsies deemed
183 insufficient for adequate reading by the pathologist were excluded from further analysis.
184 The different pathological features of NAFLD were scored according to both the NASH
185 Clinical Research Network Scoring System and the Fatty Liver Inhibition of Progression
186 (FLIP)-algorithms[13, 14]. The diagnosis of NASH required the combined presence of
187 steatosis, ballooning and lobular inflammation. Based on liver histology, patients were
188 classified in 4 distinct groups reflecting different stages of severity of the disease:

- 189 • *No-NAFLD*: no histological evidence for steatosis, inflammation, ballooning and
190 fibrosis
- 191 • *Isolated steatosis* (or NAFL): histological evidence of steatosis with activity score
192 (ballooning+inflammation) ≤ 1 and fibrosis stage ≤ 1
- 193 • *NASH without significant fibrosis (fibrosis stage (F) < 2)*: histological evidence of
194 steatosis with ballooning score ≥ 1 , inflammation score ≥ 1 and fibrosis stage ≤ 1
- 195 • *NASH with significant fibrosis ($\geq F2$) (so called “fibrotic NASH”)*: histological evidence
196 of steatosis with ballooning score ≥ 1 , inflammation score ≥ 1 and fibrosis stage ≥ 2 .

197 Patients with histological evidence of a liver disease other than NAFLD or patients with
198 NAFLD but who could not be unequivocally categorised in one of the 4 groups were
199 excluded.

200 **Sphingolipid analysis**

201 The protocol for analysing plasma sphingoid bases has previously been described[15].
202 Briefly, sphingoid bases were determined in fasting plasma samples. Sphingolipids in plasma
203 are present in a broad variation of subspecies. They can be saturated and are usually
204 acylated with another fatty acid. Furthermore, most of these sphingolipids are conjugated
205 to an O-linked head group. To analyse the sphingoid backbones, the sphingolipids were
206 subjected to a sequential acid and base hydrolysis. Acid hydrolysis specifically breaks the N-
207 alkyl chain, whereas alkaline conditions lead to a release of the O-linked head group. The
208 sphingoid bases were analysed using Liquid Chromatography/Mass Spectrometry. Analysed
209 bases included C₁₆-sphinganine (C₁₆-SA), C₁₆-sphingosine (C₁₆-SO), C₁₇-sphingosine (C₁₇-SO),
210 C₁₈-sphinganine (C₁₈-SA), C₁₈-sphingosine (C₁₈-SO), C₁₉-sphingosine (C₁₉-SO), C₂₀-sphinganine

211 (C₂₀-SA), C₂₀-sphingosine (C₂₀-SO), sphingadienine (SA-dienine), C₁₈-phyto-sphingosine (C₁₈-
212 phyto-SO), deoxy-sphinganine (deoxy-SA) and deoxy-sphingosine (deoxy-SO).

213

214 **Statistical analysis**

215 *1. The role of sphingolipids in the different entities of NAFLD*

216 Data were analysed with SPSS version 24. Means were compared by independent t testing
217 or non-parametric tests when appropriate. To investigate the association of sphingoid bases
218 with the different entities of NAFLD (steatosis, steatohepatitis and fibrosis), the following
219 groups were compared: 'No-NAFLD' vs. 'NAFL' to investigate the association of the
220 sphingoid bases in steatosis; 'NAFL' vs. 'NASH <F2' to investigate the potential association
221 of the sphingoid bases in steatohepatitis and eventually 'NASH <F2' vs. 'NASH ≥F2' to
222 investigate the potential association of the sphingoid bases in fibrogenesis. Sphingoid bases
223 that showed association with NAFLD (and its stages) in this first crude analysis were further
224 analysed using binary logistic regression. Since NAFLD is closely related to the MetS and
225 diabetes mellitus type 2, interaction with or confounding is possible. Furthermore, certain
226 lipid lowering therapies can have a profound effects on NAFLD. Adjustment was done using
227 metabolic markers (*e.g.* homeostasis model assessment (HOMA)-IR, presence of the MetS,
228 presence of T2DM or Impaired Fasting Glucose (IFG) and the use of lipid lowering therapy)
229 as covariates and/or interaction terms in a binary logistic regression.

230 *2. Deoxy-sphingoid bases and metabolic parameters*

231 To investigate whether the associations between deoxy-sphingolipids, on the one hand,
232 and the MetS or T2DM, on the other hand, are driven by NAFLD, the possible
233 confounding/interaction effect of NAFLD was checked using binary logistic regression
234 analysis. Furthermore, correlations between deoxy-sphingolipids and other metabolic
235 parameters (*e.g.* triglycerides and HOMA-IR) were checked. To investigate these
236 correlations, Pearson's correlation coefficient was used when appropriate. If not, non-

237 parametric testing was performed: Kendall's tau-b was used if there were a great number of
238 tied ranks, in others cases Spearman's rho was used.

239

240

241 RESULTS

242 ***1. The role of sphingolipids in the different entities of NAFLD***

243 288 consecutive patients with a complete dataset were included. Their liver phenotype was
244 as follows: 17.7% No-NAFLD, 16.3% NAFL, 51.7% NASH <F2 and 14.3% NASH ≥F2. This is an
245 obese study population with a mean BMI of 39.6 kg/m² (SD 6.4) (Table 1). Log
246 transformation of the concentration of sphingoid bases was used in statistical analysis since
247 the distribution of these bases was skewed (resulting in extreme exp (B) values).

248

249 *1a. Sphingoid bases in No-NAFLD vs. NAFL*

250 There were 47 patients with isolated liver steatosis and 51 (26.6%) patients with no
251 histological evidence for the presence of NAFLD (No-NAFLD). There was a statistically
252 significant difference in age, waist and HbA_{1c}, but there was no difference in BMI, presence
253 of the Mets and presence of T2DM. There was no use of fibrates and statin use was not
254 significantly different in both groups (Supplementary Table 1). There was a statistically
255 significant association between the presence of steatosis and the concentrations of deoxy-
256 SA (exp(B) 6.708 with CI [1.243, 36.195] and p 0.027) and deoxy-SO (exp(B) 4.851 with CI
257 [1.315, 17.890] and 0.018). There was no interaction effect of metabolic markers in the
258 association of steatosis and the sphingoid bases deoxy-SA and deoxy-SO, and statistical
259 significance was maintained for the DSBs (deoxy-SA and deoxy-SO) even after correction for
260 confounding (*e.g.* by metabolic markers).

261

262 *1b. Sphingoid bases in NAFL vs. NASH <F2*

263 149 patients had NASH, compared to 47 patient with isolated steatosis. There was a
264 statistically significant difference in the presence of the MetS, HOMA-IR and liver enzymes
265 (Supplementary Table 2). There was a statistically significant difference in plasma
266 concentrations of C₁₆-SO, C₁₇-SO, C₁₈-SO and SA-dienine. Further analysis did, however, not
267 show an association between NASH and C₁₆-SO (exp(B) 0.159 with CI [0.016, 1.595] and
268 p=0.118) nor C₁₇-SO (exp(B) 0.106 with CI [0.008, 1.342] and p=0.083). There was a
269 statistically significant association between NASH and C₁₈-SO (exp(B) 0.036 with CI [0.001,
270 0.991] and p 0.049) and SA-dienine (exp(B) 0.037 with [0.002, 0.725] and p 0.030). There
271 was no interaction effect by the metabolic markers. However, after correction for the
272 presence of the MetS there was a loss of the statistically significant association between
273 NASH, on the one hand, and C₁₈-SO (exp(B) 0.043 with CI [0.001, 1.405] and p 0.077) and SA-
274 dienine (exp(B) 0.062 with CI [0.003, 1.414] and p 0.081), on the other hand.

275

276 *1c. Sphingoid bases in NASH <F2 vs. NASH ≥F2*

277 41 patients with NASH had evidence of significant fibrosis (fibrotic NASH), compared to 149
278 (26.6%) NASH patients who had no significant fibrosis (Supplementary Table 3). There was a
279 statistically significant association between fibrotic NASH and the concentrations of C₁₉-SO
280 (exp(B) 0.157 with CI [0.025, 0.986] and p=0.048) and SA-dienine (exp(B) 0.013 with CI
281 [0.001, 0.342] and p=0.009). There was no interaction between metabolic markers and the
282 sphingoid bases C₁₉-SO and SA-dienine. After correcting for HOMA-IR, however, there was
283 loss of statistical significance for the association between fibrotic NASH and C₁₉-SO (exp(B)
284 after correction 0.203 with CI [0.028, 1.495] and p=0.118). Furthermore, there was a loss of

285 statistical significance for the association between fibrotic NASH and SA-dienine after
286 correcting for gender (exp(B) after correction 0.048 with CI [0.001, 1.213] and p=0.065).

287

288 ***2. Deoxy-sphingoid bases and metabolic - parameters***

289 Log transformation of the concentration of sphingoid bases was used in binary regression
290 analysis since the distribution of these bases was skewed (resulting in extreme exp (B)
291 values).

292 *2a. Metabolic syndrome*

293 There was a highly statistically significant association between MetS and the concentrations
294 of deoxy-SA (exp(B) 29.934 with CI [8.457, 105.949] and p <0.001) and deoxy-SO (exp(B)
295 29.024, with CI [9.796, 85.998] and p <0.001). There was no interaction with the metabolic
296 markers (including T2DM) nor with NAFLD, nor was there loss of statistical significance after
297 correction for the metabolic markers (including T2DM and HOMA-IR) nor NAFLD

298 *2b. Type 2 diabetes mellitus*

299 There was a highly statistically significant association between the presence of T2DM and
300 the concentrations of deoxy-SA (exp(B) 14405.280 with CI [13.084, 15860091.190] and p
301 0.007) and deoxy-SO (exp(B) 7.953 with CI [1.832, 34.515] and p 0.006). There was no
302 interaction with metabolic markers (including MetS) nor with NAFLD. After correction for
303 the MetS, however, there was a loss of a statistically significant association between T2DM
304 and deoxy-SA (exp(B) after correction 1.964 with CI [0.275, 14.048] and p=0.501) and deoxy-
305 SO (exp(B) after correction 2.138 with CI [0.400, 11.432] and p=0.374).

306 *2c. Correlation with other metabolic parameters*

307 There was a statistically significant correlation between the DSBs and triglycerides (Fig 2A
308 and 2B; Spearman's rho 0.553 and $p < 0.001$ for deoxy-SA and Spearman's rho 0.581 and p
309 < 0.001 for deoxy-SO) and between deoxy-SA and IR. There was no statistically significant
310 correlation between deoxy-SO and IR (Fig 2C and 2D; Spearman's rho 0.1138 and p 0.021 for
311 deoxy-SA and Spearman's rho 0.104 and p 0.081 for deoxy-SO) (Table 2).

312

313 Discussion

314 Sphingolipids are essential components of the cell membrane and plasma lipoproteins. They
315 play a complex role as signalling molecules in multiple processes and have been suggested
316 to be of pathophysiological relevance in NASH. dSLs, an atypical category of sphingolipids,
317 are generated when SPT uses L-alanine instead of L-serine. dSLs are neither metabolised to
318 complex sphingolipids nor degraded by the sphingolipid catabolism pathways. Their
319 function is not clear, but they seem involved in T2DM and the MetS.

320 In a large, well characterised, prospectively screened cohort with ultimately histological
321 diagnosis of NAFLD we observed a highly significant association between NAFLD and 1-
322 deoxy sphingoid bases (DSBs). We found no statistically significant difference between the
323 different stages of NAFLD, suggesting that dSLs are related to steatosis, but are not
324 implicated in the further progression of NAFLD to NASH and not associated with fibrosis.
325 Furthermore, this study confirms the association of deoxy-sphingolipids with the MetS but
326 not diabetes.

327 In a study with 99 individuals, Bertea et al. found a statistically significant difference in plasma
328 dSLs levels when comparing T2DM patients with control. Although there was a clear
329 difference between these groups regarding gender, BMI and other metabolic markers, there
330 was no correction performed, questioning these findings. Furthermore, they described a
331 possible predictive role of dSLs for the presence of T2DM, although this case-control study
332 did not lean itself to perform prediction analysis [9]. Also Othman et al investigated the role
333 of dSLs in the MetS and T2DM. They found a significant difference between plasma levels of
334 dSLs when comparing healthy volunteers with patients with the MetS or patients with
335 T2DM, but found no difference between plasma levels of dSLs when comparing T2DM with

336 MetS. In this study, there was a statistically significant difference in BMI and waist
337 circumference when comparing patients with T2DM to those with the Mets, and a (near
338 statistically) significant difference when comparing triglycerides, but there was no
339 correction performed [16].

340 Human data regarding the association between sphingolipids, more specifically, dSLs on the
341 one hand, and the presence of NAFLD, on the other, is scarce. Gordon *et al* described a
342 diverse panel of 20 plasma lipids, including the dSL 1-deoxy-ceramide, capable of
343 distinguishing NAFLD from NASH in 88 patients with liver histology categorised as normal,
344 steatotic, NASH, or cirrhotic. Although patients were selected on an “all-comers” approach
345 where liver biopsy was obtained during surgery (gastric bypass, liver transplant, multi organ
346 transplant, hernia repair...), the study population was obese with a mean fasting glucose >
347 100 mg/dL (other metabolic conditions were not mentioned). The possible effects of these
348 conditions on the results was not assessed, meaning that an overestimation of the
349 discriminate power of these lipids is possible [17]. Gai *et al.* reported a correlation between
350 NAFLD and dSLs in 80 individuals, but failed to specify the contribution of these dSLs in the
351 different entities of NAFLD. Furthermore, they did not provide a clear overview of the main
352 (metabolic) characteristics of these patients and failed to check for the confounding or
353 interactive effect of metabolic factors [18]. The role of dSLs in the MetS, T2DM and NAFLD is
354 not fully understood, but they seem to play a role in the control of metabolism. A study by
355 Zuellig *et al.* showed that dSLs compromised insulin secretion and triggered senescence and
356 cell-death in insulin producing cells triggered by multiple pathways, including cytoskeletal
357 remodelling, senescence, necrosis and apoptosis favouring hyperglycaemia. [10] Although
358 we found an increasing presence of *de novo* T2DM with a more severe phenotype of NAFLD,
359 there was no significant interaction between *de novo* T2DM nor IR and dSLs when

360 investigating the association with steatosis, nor was there loss of significance after
361 correcting for T2DM or IR (data for T2DM not shown) suggesting that hyperglycaemia or IR
362 *per se* is not a determinant of dSL formation in the pathophysiology of NAFLD. On the other
363 hand, dSLs do not significantly differ between the 3 NAFLD categories whereas IR and
364 impairment of glycaemic control worsen as disease worsens (despite comparable age and
365 BMI). This observation hence does not support an important role for dSLs in impairment of
366 glucose metabolism in NAFLD. Wei *et al.* also speculated that the formation of dSLs in T2DM
367 is not caused by hyperglycaemia *per se*, but rather associated with metabolic changes in
368 T2DM [19].

369 Another possible explanation in the association between dSLs and NAFLD is a shift in the
370 utilization of alanine instead of serine by SPT. Mardinoglu *et al.* revealed a L-serine
371 deficiency in NAFLD using a genome scale metabolic model of hepatocytes. As seen in
372 literature, there was a significant association between T2DM and DBSs[9] and between the
373 MetS and dSLs[16], which was also the case in our population. However, after correcting for
374 the MetS, there is a loss of statistical significance in the association between dSLs and
375 T2DM. Furthermore, this association between dSLs and MetS is not driven by the presence
376 of NAFLD (since the lack of a significant interaction). Although it is not clear what exact role
377 the DSBs play in steatosis, they seem to be associated with metabolic dysregulation, which
378 might eventually lead to steatosis.

379 Sphingolipid metabolism has been proposed as a target for pharmacological therapy of
380 NASH. Othman *et al* showed that fibrates have a DSBs lowering effect, independent of a
381 triglyceride lowering effect [19]. It is not clear whether this is a peroxisome proliferator-
382 activated receptor (PPAR) α or non-PPAR α mediated effect[20]. Gai et al. described that the

383 activation of Farnesoid X Receptor reduces dSLs plasma levels in a high fat-fed mouse model
384 and in 1-deoxysphinganine-treated mice [18]. Another group found that oral
385 supplementation of serine suppressed the formation of dSLs in an hereditary sensory and
386 autonomic neuropathy type 1 (HSAN1) animal model and in humans[21, 22]. Serine
387 palmitoyltransferase long chain base subunit 1 and 2 mutations in SPT (as seen in HSAN1)
388 induce a permanent shift in the substrate preference from L-serine to L-alanine[6]; dSLs
389 formation in metabolic diseases is, however, not caused by a mutation in SPT but by a
390 dysregulation in carbohydrate and fatty acid metabolism [19]. Our data are linking dSLs
391 more to steatosis than to steatohepatitis and fibrosis (at least in a cross-sectional analysis).
392 Steatosis is not *per se* harmful and can even be protective, questioning the therapeutic
393 potential of modulating dSLs in the treatment of NASH. Nevertheless, reducing steatosis can
394 be a target for NAFLD treatment, given the presumed role of lipotoxicity. The exact potential
395 impact of modulating sphingolipid metabolism on NASH progression is hence to be
396 determined.

397 In this study, patients were not included based on a *a priori* suspicion of liver disease .This
398 selection procedure resulted in a series representing the whole spectrum of NAFLD,
399 whereas retrospective series of biopsy proven NAFLD patients tend to be skewed towards
400 more severe subtypes. On the other hand, analysis was performed cross-sectionally on a
401 population that is predominantly Caucasian. A causal link between deoxy-sphingolipids and
402 steatosis is therefore difficult to determine. Finally, the reported data only handles the
403 sphingoid base concentrations. Although they reflect the total sphingoid base composition
404 for all the individual sphingolipid subclasses, further investigation of these different
405 subclasses is needed to provide a more detailed understanding of the pathological role of

406 the sphingolipidome in NAFLD and its potential role in the development of therapeutic
407 agents.

408 **Conclusion:** dSLs are increased in relation to steatosis independently of their correlation
409 with metabolic markers, but are not further increased when the spectrum of NAFLD
410 worsens, questioning their role as a therapeutic target for NASH.

411

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Tables

Table 1: Patients characteristics according to the histology-based classification

		TOTAL N=288 (100%)	No-NAFL N=51 (17.7%)	NAFL N=47 (16.3%)	NASH <F2 N=149 (51.7%)	NASH ≥F2 N=41 (14.3%)	p-value
General	Age (years)	43.8 (±12.3)	38.1 (±10.6)	43.5 (±12.3)	45.4 (±12.4)	45.0 (±12.4)	0.003*
	Gender: men	96 (33.3%)	8 (15.7%)	10 (21.3%)	53 (35.6%)	25 (61.0%)	<0.001***
Anthropometry	BMI (kg/m ²)	39.6 (±6.4)	38.2 (±4.9)	39.9 (±7.1)	38.9 (±6.0)	43.5 (±7.4)	0.001**
	Waist (cm)	118.8 (±13.2)	111.93 (±10.4)	118.1 (±13.6)	117.9 (±11.3)	132.1 (±13.1)	<0.001**
Metabolic markers	TG (mg/dL)	155.5 (±78.5)	137.2 (±66.6)	150.3 (±106.9)	157.1 (±72.8)	177.9 (±69.4)	0.009**
	LDL-C (mg/dL)	124.7 (±36.8)	121.5 (±43.9)	128.8 (±33.2)	123.4 (±35.7)	128.5 (±35.5)	0.674*
	HDL-C (mg/dL)	48.9 (±13.7)	53.2 (±13.4)	51.3 (±15.9)	48.6 (±13.5)	41.9 (±9.3)	<0.001**
	Statin use (%)	30 (10.4)	2 (3.9)	3 (6.4)	22 (14.8)	3 (7.3)	0.087***
	HbA1c (%)	5.7 (±0.5)	5.4 (0.3)	5.6 (±0.3)	5.6 (±0.4)	6.1 (±1.0)	<0.001**
	Glucose mg/dL	86.2 (±22.4)	80.2 (±7.4)	82.4 (±11.0)	85.1 (±12.4)	102.1 (±49.8)	<0.001**
	HOMA-IR	4.0 (±3.2)	3.1 (±3.2)	2.9 (±1.8)	3.8 (±2.4)	6.8 (±5.1)	<0.001**
	MetS	155 (56.2%)	20 (40.8%)	17 (37.8%)	87 (61.3%)	31 (77.5%)	<0.001***
T2DM	30 (10.5%)	1 (2.0%)	3 (6.5%)	16 (10.9%)	10 (24.4%)	0.004***	
Liver enzymes	AST (U/L)	31.7 (±17.5)	24.4 (±8.0)	25.5 (±8.5)	30.7 (±11.8)	51.5 (±31.5)	<0.001**
	ALT (U/L)	45.6 (±27.3)	31.0 (±11.3)	36.1 (±18.7)	45.8 (±21.6)	74.0 (±43.0)	<0.001**
Sphingoid bases	C ₁₆ -SA	0.469 (±0.221)	0.460 (±0.227)	0.441 (±0.159)	0.491 (±0.249)	0.432 (±0.156)	0.673**
	C ₁₆ -SO	15.491 (±5.313)	15.991 (±5.880)	16.584 (±4.753)	15.379 (±5.568)	14.026 (±3.828)	0.067**
	C ₁₇ -SO	6.233 (±2.007)	6.533 (±2.365)	6.665 (±1.686)	6.180 (±2.053)	5.555 (±1.498)	0.018**
	C ₁₈ -SA	3.659 (±1.401)	3.599 (±1.588)	3.512 (±1.229)	3.726 (±1.459)	3.662 (±1.126)	0.822*
	C ₁₈ -SO	84.161 (±20.682)	86.927 (±24.394)	89.051 (±17.530)	83.308 (±21.246)	78.050 (±14.864)	0.008**
	C ₁₈ -phytoSO	0.110 (±0.038)	0.115 (±0.052)	0.114 (±0.029)	0.110 (±0.037)	0.100 (±0.026)	0.135**
	C ₁₉ -SO	1.815 (±0.794)	1.875 (±0.839)	1.814 (±0.702)	1.871 (±0.858)	1.541 (±0.508)	0.200**
C ₂₀ -SA	0.024 (±0.108)	0.021 (±0.010)	0.024 (±0.012)	0.025 (±0.110)	0.025 (±0.009)	0.024**	

	C ₂₀ -SO	0.195 (±0.068)	0.188 (±0.079)	0.199 (±0.060)	0.198 (±0.071)	0.190 (±0.052)	0.388**
	SA- diene	31.376 (±9.161)	33.168 (±11.686)	33.966 (±7.963)	31.063 (±8.688)	27.315 (7.080)	<0.001**
	Deoxy- SA	0.074 (±0.042)	0.064 (±0.040)	0.077 (±0.042)	0.076 (±0.042)	0.077 (±0.040)	0.099**
	Deoxy- SO	0.110 (±0.075)	0.088 (±0.075)	0.106 (±0.062)	0.117 (±0.077)	0.118 (±0.076)	0.019*

No histological evidence of Non-Alcoholic Fatty Liver Disease (No-NAFLD), Non-Alcoholic Fatty Liver (NAFL), Non-Alcoholic Steatohepatitis with no significant fibrosis (NASH <F2), Non-Alcoholic Steatohepatitis with significant fibrosis (NASH ≥ F2), Body mass index (BMI), Triglycerides (TG), Low density lipoprotein cholesterol (LLD-C), High density lipoprotein cholesterol (HDL-C), Haemoglobin A_{1c} (HbA_{1c}), Homeostatic model assessment insulin resistance (HOMA-IR), Metabolic syndrome (MetS), aspartate aminotransferase (AST), alanine aminotransferase (ALT), C₁₆-Sphinganine (C₁₆-SA), C₁₆-Sphingosine (C₁₆-SO), C₁₇-Sphingosine (C₁₇-SO), C₁₈-Sphinganine (C₁₈-SA), C₁₈-Sphingosine (C₁₈-SO), C₁₉-Sphingosine (C₁₉-SO), C₂₀-Sphinganine (C₂₀-SA), C₂₀-Sphingosine (C₂₀-SO), Sphingadiene (SA-diene), C₁₈-phyto-sphingosine (C₁₈-phyto-SO), deoxy-sphinganine (deoxy-SA), deoxy-sphingosine (deoxy-SO). SD or percentage of the total group is presented between brackets. p-values: *ANOVA, **Kruskal-Wallis, ***Pearson Chi-square.

Table 2: Correlation with metabolic and inflammatory parameters

	Triglycerides		HOMA-IR	
	Correlation coefficient*	p	Correlation coefficient*	p
Deoxy-SA	0.553	<0.001	0.138	0.021
Deoxy-SO	0.581	<0.001	0.104	0.081

* Spearman's rho. Homeostatic model assessment insulin resistance (HOMA-IR), deoxy-sphinganine (deoxy-SA) deoxy-sphingosine (deoxy-SO)

Supplementary materials

Supplementary Table 1: Patient characteristics for the comparison No-NAFLD vs. NAFL

		No-NAFLD N=51	NAFL N=47	p
General	Age (years)	38.1 (±10.6)	43.5 (±12.3)	0.021**
	Gender: men	8 (15.7%)	10 (21.3%)	0.475***
Anthropometry	BMI (kg/m ²)	38.2 (±4.9)	39.9 (±7.1)	0.190**
	Waist (cm)	111.93 (±10.4)	118.1 (±13.6)	0.007**
Metabolic markers	TG (mg/dL)	137.2 (±66.6)	150.3 (±106.9)	0.814*
	LDL-C (mg/dL)	121.5 (±43.9)	128.8 (±33.2)	0.369**
	HDL-C (mg/dL)	53.2 (±13.4)	51.3 (±15.9)	0.415*
	Statin use (%)	2 (3.9)	3 (6.4)	0.580***
	HbA1c (%)	5.4 (0.3)	5.6 (±0.3)	<0.001*
	Glucose (mg/dL)	80.2 (±7.4)	82.4 (±11.0)	0.611*
	HOMA-IR	3.1 (±3.2)	2.9 (±1.8)	0.652*
	MetS	20 (40.8%)	17 (37.8%)	0.763***
	T2DM	1 (2.0%)	3 (6.5%)	0.259***
Liver enzymes	AST (U/L)	24.4 (±8.0)	25.5 (±8.5)	0.254*
	ALT (U/L)	31.0 (±11.3)	36.1 (±18.7)	0.248*
Imaging	Steatosis on US	30 (61.2%)	40 (95.2%)	<0.001***
Pathology	Steatosis 0/1/2/3 (%)	100/0.0/0.0/0.0	0.0/80.9/17.0/2.1	<0.001***
	Ballooning 0/1/2 (%)	100/0.0/0.0	68.1/31.9/0.0	<0.001***
	Inflammation 0/1/2 (%)	100/0.0/0.0	72.3/27.3/0.0	<0.001***
	Fibrosis 0/1/2/3/4 (%)	100/0.0/0.0/0.0/0.0	93.6/6.4/0.0/0.0/0.0	0.067***
Sphingoid bases	C ₁₆ -SA	0.460 (±0.227)	0.441 (±0.159)	0.890*
	C ₁₆ -SO	15.991 (±5.880)	16.584 (±4.753)	0.384*
	C ₁₇ -SO	6.533 (±2.365)	6.665 (±1.686)	0.384*
	C ₁₈ -SA	3.599 (±1.588)	3.512 (±1.229)	0.773*
	C ₁₈ -SO	86.927 (±24.394)	89.051 (±17.530)	0.328*
	C ₁₈ -phytoSO	0.115 (±0.052)	0.114 (±0.029)	0.526**
	C ₁₉ -SO	1.875 (±0.839)	1.814 (±0.702)	0.897**
	C ₂₀ -SA	0.021 (±0.010)	0.024 (±0.012)	0.236*
C ₂₀ -SO	0.188 (±0.079)	0.199 (±0.060)	0.281**	

	SA-diene	33.168 (±11.686)	33.966 (±7.963)	0.387**
	Deoxy-SA	0.064 (±0.040)	0.077 (±0.042)	0.023**
	Deoxy-SO	0.088 (±0.075)	0.106 (±0.062)	0.014**

* Mann-Whitney U, ** independent sample T-test, *** chi-square. Ballooning and Inflammation are graded according to SAF/Flip-algorithm. No histological evidence of Non-Alcoholic Fatty Liver Disease (no-NAFLD), Non-Alcoholic Fatty Liver (NAFL), Significant fibrosis (\geq F2), Body mass index (BMI), Triglycerides (TG), Low density lipoprotein cholesterol (LDL-C), High density lipoprotein cholesterol (HDL-C), Haemoglobin A_{1c} (HbA_{1c}), Homeostatic model assessment insulin resistance (HOMA-IR), Metabolic syndrome (MetS), aspartate aminotransferase (AST), alanine aminotransferase (ALT), C₁₆-Sphinganine (C₁₆-SA), C₁₆-Sphingosine (C₁₆-SO), C₁₇-Sphingosine (C₁₇-SO), C₁₈-Sphinganine (C₁₈-SA), C₁₈-Sphingosine (C₁₈-SO), C₁₉-Sphingosine (C₁₉-SO), C₂₀-Sphinganine (C₂₀-SA), C₂₀-Sphingosine (C₂₀-SO), Sphingadiene (SA-diene), C₁₈-phyto-sphingosine (C₁₈-phyto-SO), deoxy-sphinganine (deoxy-SA), deoxy-sphingosine (deoxy-SO). SD or percentage of the total group is presented between brackets.

Supplementary Table 2: patient characteristics for the comparison NAFL vs. NASH

		NAFL N=47	NASH <F2 N=149	p
General	Age (years)	43.5 (±12.3)	45.4 (±12.4)	0.350**
	Gender: men	10 (21.3%)	53 (35.6%)	0.067***
Anthropometry	BMI (kg/m ²)	39.9 (±7.1)	38.9 (±6.0)	0.486*
	Waist (cm)	118.1 (±13.6)	117.9 (±11.3)	0.952**
Metabolic markers	TG (mg/dL)	150.3 (±106.9)	157.1 (±72.8)	0.113*
	LDL-C (mg/dL)	128.8 (±33.2)	123.4 (±35.7)	0.373**
	HDL-C (mg/dL)	51.3 (±15.9)	48.6 (±13.5)	0.285*
	Statin use (%)	3 (6.4)	22 (14.8)	0.133***
	HbA _{1c} (%)	5.6 (±0.3)	5.6 (±0.4)	0.514*
	Glucose (mg/dL)	82.4 (±11.0)	85.1 (±12.4)	0.151*
	HOMA-IR	2.9 (±1.8)	3.8 (±2.4)	0.026*
	MetS	17 (37.8%)	87 (61.3%)	0.006***
	T2DM	3 (6.5%)	16 (10.9%)	0.386***
Liver enzymes	AST (U/L)	25.5 (±8.5)	30.7 (±11.8)	0.005*
	ALT (U/L)	36.1 (±18.7)	45.8 (±21.6)	0.001*
Imaging	Steatosis on US	40 (95.2%)	139 (96.5%)	0.699***
Pathology	Steatosis 0/1/2/3 (%)	0.0/80.9/17.0/2.1	0.0/36.9/38.3/24.8	<0.001***
	Ballooning 0/1/2 (%)	68.1/31.9/0.0	0.0/50.3/49.7	<0.001***
	Inflammation 0/1/2 (%)	72.3/27.3/0.0	0.0/58.4/41.6	<0.001***

	Fibrosis 0/1/2/3/4 (%)	93.6/6.4/0.0/0.0/0.0	70.5/29.5/0.0/0.0/0.0	0.001^{***}
Sphingoid bases	C ₁₆ -SA	0.434 (±0.141)	0.478 (±0.233)	0.469*
	C₁₆-SO	16.212 (±4.426)	15.087 (±5.262)	0.031*
	C₁₇-SO	6.511 (±1.610)	6.045 (±1.960)	0.023*
	C ₁₈ -SA	3.464 (±1.238)	3.712 (±1.392)	0.689*
	C₁₈-SO	86.492 (±17.300)	82.173 (±20.123)	0.005*
	C ₁₈ -phytoSO	0.111 (±0.029)	0.108 (±0.035)	0.229*
	C ₁₉ -SO	1.760 (±0.667)	1.800 (±0.806)	0.839**
	C ₂₀ -SA	0.023 (±0.011)	0.025 (±0.010)	0.315**
	C ₂₀ -SO	0.194 (±0.060)	0.196 (±0.067)	0.773**
	SA-diene	32.700 (±7.803)	30.254 (±8.491)	0.028**
	Deoxy-SA	0.079 (±0.041)	0.077 (±0.042)	0.867**
	Deoxy-SO	0.115 (±0.075)	0.117 (±0.077)	0.441**

* Mann-Whitney U, ** independent sample T-test, *** chi-square. Ballooning and Inflammation are graded according to SAF/Flip-algorithm. Non-Alcoholic Steatohepatitis with no significant fibrosis (NASH <F2), Non-Alcoholic Steatohepatitis with significant fibrosis (NASH ≥ F2), Body mass index (BMI), Triglycerides (TG), Low density lipoprotein cholesterol (LDL-C), High density lipoprotein cholesterol (HDL-C), Haemoglobin A_{1c} (HbA_{1c}), Homeostatic model assessment insulin resistance (HOMA-IR), Metabolic syndrome (MetS), aspartate aminotransferase (AST), alanine aminotransferase (ALT), C₁₆-Sphinganine (C₁₆-SA), C₁₆-Sphingosine (C₁₆-SO), C₁₇-Sphingosine (C₁₇-SO), C₁₈-Sphinganine (C₁₈-SA), C₁₈-Sphingosine (C₁₈-SO), C₁₉-Sphingosine (C₁₉-SO), C₂₀-Sphinganine (C₂₀-SA), C₂₀-Sphingosine (C₂₀-SO), Sphingadiene (SA-diene), C₁₈-phyto-sphingosine (C₁₈-phyto-SO), deoxy-sphinganine (deoxy-SA), deoxy-sphingosine (deoxy-SO). SD or percentage of the total group is presented between brackets.

Supplementary Table 3: Patient characteristics for the comparison NASH <F2 vs. NASH ≥F2

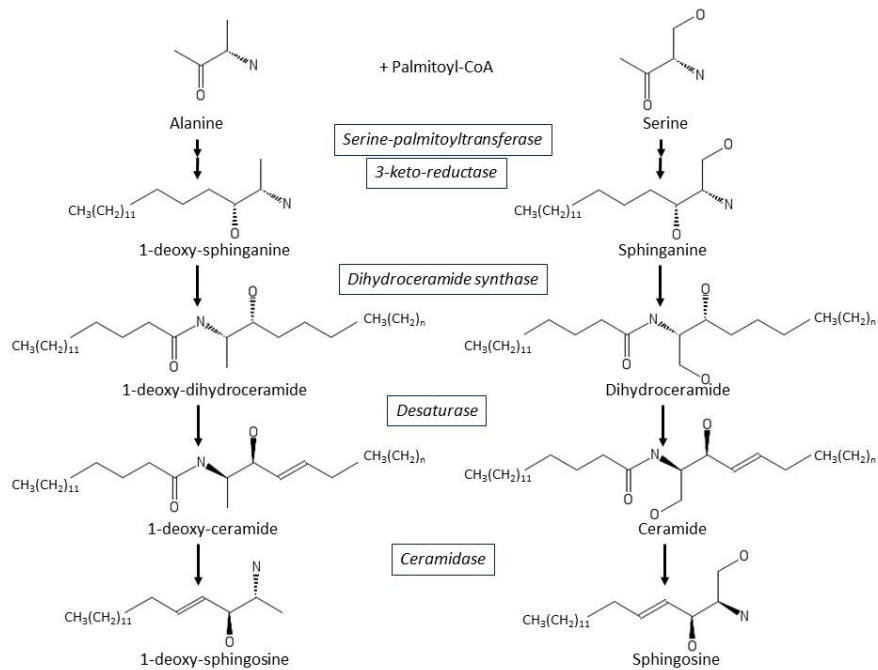
		NASH <F2 N=149	NASH ≥F2 N=41	p
General	Age (years)	45.4 (±12.4)	45.0 (±12.4)	0.855**
	Gender: men	53 (35.6%)	25 (61.0%)	0.003^{***}
Anthropometry	BMI (kg/m²)	38.9 (±6.0)	43.5 (±7.4)	<0.001*
	Waist (cm)	117.9 (±11.3)	132.1 (±13.1)	<0.001**
Metabolic markers	TG (mg/dL)	157.1 (±72.8)	177.9 (±69.4)	0.059*
	LDL-C (mg/dL)	123.4 (±35.7)	128.5 (±35.5)	0.420**
	HDL-C (mg/dL)	48.6 (±13.5)	41.9 (±9.3)	0.002*
	Statin use (%)	22 (14.8)	3 (7.3)	0.212***
	HbA_{1c} (%)	5.6 (±0.4)	6.1 (±1.0)	<0.001*
	Glucose (mg/dL)	85.1 (±12.4)	102.1 (±49.8)	<0.001*
	HOMA-IR	3.8 (±2.4)	6.8 (±5.1)	<0.001*
	MetS	87 (61.3%)	31 (77.5%)	0.058***
Liver enzymes	T2DM	16 (10.9%)	10 (24.4%)	0.027^{***}
	AST (U/L)	30.7 (±11.8)	51.5 (±31.5)	<0.001*

	ALT (U/L)	45.8 (±21.6)	74.0 (±43.0)	<0.001*
Imaging	Steatosis on US	139 (96.5%)	37 (±94.9)	0.632***
Pathology	Steatosis 0/1/2/3 (%)	0.0/36.9/38.3/24.8	0.0/14.6/31.7/53.7	0.001***
	Ballooning 0/1/2 (%)	0.0/50.3/49.7	0.0/51.2/48.8	0.920***
	Inflammation 0/1/2 (%)	0.0/58.4/41.6	0.0/29.3/70.7	<0.001***
	Fibrosis 0/1/2/3/4 (%)	70.5/29.5/0.0/0.0/0.0	0.0/0.0/58.5/39.0/2.5	<0.001***
Sphingoid bases	C ₁₆ -SA	0.491 (±0.249)	0.432 (±0.156)	0.375*
	C ₁₆ -SO	15.379 (±5.568)	14.026 (±3.838)	0.206**
	C ₁₇ -SO	6.180 (±2.053)	5.555 (±1.498)	0.083**
	C ₁₈ -SA	3.726 (±1.459)	3.662 (±1.126)	0.687*
	C ₁₈ -SO	83.308 (±21.246)	78.050 (±14.864)	0.250*
	C ₁₈ -phytoSO	0.110 (±0.037)	0.100 (±0.026)	0.126*
	C₁₉-SO	1.871 (±0.858)	1.541 (±0.508)	0.046**
	C ₂₀ -SA	0.025 (±0.011)	0.026 (±0.009)	0.528**
	C ₂₀ -SO	0.198 (±0.071)	0.190 (±0.052)	0.958*
	SA-diene	31.063 (±8.688)	27.316 (7.078)	0.008**
	Deoxy-SA	0.077 (±0.042)	0.077 (±0.040)	0.832**
	Deoxy-SO	0.117 (±0.078)	0.118 (±0.076)	0.895**

* Mann-Whitney U, ** independent sample T-test, *** chi-square. Ballooning and Inflammation are graded according to SAF/Flip-algorithm. Non-Alcoholic Steatohepatitis with no significant fibrosis (NASH <F2), Non-Alcoholic Steatohepatitis with significant fibrosis (NASH ≥ F2), Body mass index (BMI), Triglycerides (TG), Low density lipoprotein cholesterol (LDL-C), High density lipoprotein cholesterol (HDL-C), Haemoglobin A_{1c} (HbA_{1c}), Homeostatic model assessment insulin resistance (HOMA-IR), Metabolic syndrome (MetS), aspartate aminotransferase (AST), alanine aminotransferase (ALT), C₁₆-Sphinganine (C₁₆-SA), C₁₆-Sphingosine (C₁₆-SO), C₁₇-Sphingosine (C₁₇-SO), C₁₈-Sphinganine (C₁₈-SA), C₁₈-Sphingosine (C₁₈-SO), C₁₉-Sphingosine (C₁₉-SO), C₂₀-Sphinganine (C₂₀-SA), C₂₀-Sphingosine (C₂₀-SO), Sphingadiene (SA-diene), C₁₈-phyto-sphingosine (C₁₈-phyto-SO), deoxy-sphinganine (deoxy-SA), deoxy-sphingosine (deoxy-SO). SD or percentage of the total group is presented between brackets.

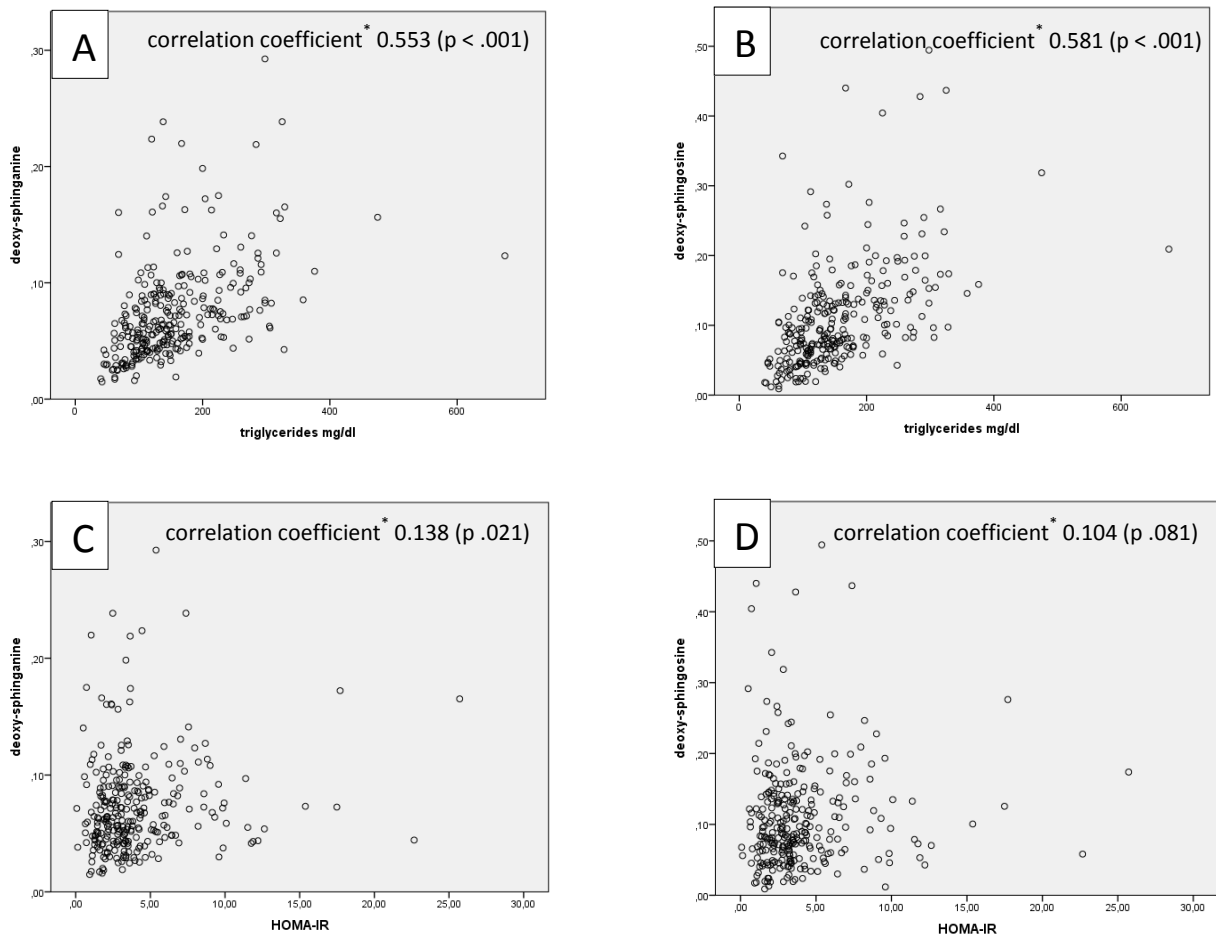
Legends to the Figures

Figure 1: Biosynthesis of sphingolipids and 1-deoxy-sphingolipids.



The enzyme serine-palmitoyltransferase (SPT) catalyses the first and rate-limiting step in the *de novo* synthesis of sphingolipids, which converts L-serine and palmitoyl-CoA into 3-keto-sphinganine. The latter is converted into sphinganine (SA). SA is N-conjugated with another fatty acid to form dihydro-ceramide. When desaturated at C4 it forms ceramide. Ceramidase converts ceramide into sphingosine.

Figure 2: Correlation of fasting plasma sphingoid bases and metabolic markers.



A: Correlation of fasting plasma deoxy- sphinganine and triglyceride levels; B: Correlation of fasting plasma deoxy- sphingosine and triglyceride levels; C: Correlation of fasting plasma deoxy- sphinganine concentrations and HOMA-IR; D: Correlation of fasting plasma deoxy- sphingosine concentrations and HOMA-IR. * Spearman's rho correlation coefficient and associated p-value