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1	Title page
2	Title: Association of 1-deoxy-sphingolipids with steatosis but not steatohepatitis nor fibrosis
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### 33 Disclosures

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# 46 List of abbreviations

47	ALP	Alkaline phosphatase
48	ALT	Alanine aminotransferase
49	AST	Aspartate aminotransferase
50	BMI	Body Mass Index
51	C <sub>16</sub> -SA	C <sub>16</sub> -sphinganine
52	C <sub>16</sub> -SO	C <sub>16</sub> -sphingosine
53	C <sub>17</sub> -SO	C <sub>17</sub> -sphingosine
54	C <sub>18</sub> -SA	C <sub>18</sub> -sphinganine
55	C <sub>18</sub> -SO	C <sub>18</sub> -sphingosine
56	C <sub>18</sub> -phyto-SO	C <sub>18</sub> -phyto-sphingosine
57	C <sub>19</sub> -SO	C <sub>19</sub> -sphingosine
58	C <sub>20</sub> -SA	C <sub>20</sub> -sphinganine
59	C <sub>20</sub> -SO	C <sub>20</sub> -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 <sub>1c</sub>	Haemoglobin A <sub>1c</sub>
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 ≥ 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

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

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

Methods: Sphingolipids were analysed by LC-MS after hydrolysing the N-acyl and O-linked
 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 CI [3.432, 36.306] and p<0.001) and deoxy-sphingosine (exp(B) 8.486 with CI [3.437, 20.949] 110 and p<0.001). There was no association between these deoxy-sphingolipids and activity of 111 112 the steatohepatitis, nor was there any association with fibrosis. Differences in deoxysphingolipids also correlated independently with the presence of the metabolic syndrome, 113 but not diabetes. 114

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.

#### 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 cell damage, inflammation, and even liver fibrosis. The latter can lead to cirrhosis which can 124 further result into end-stage liver disease[1]. The pathogenesis of NAFLD is still not 125 126 completely elucidated. Presumably, the integrated action of numerous conditions acting in parallel (genetic predisposition, adipose tissue dysfunction, insulin resistance (IR), oxidative 127 128 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

Sphingolipids are a heterogeneous class of bioactive lipids with a plethora of functions 133 134 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-ketosphinganine. The latter is converted into sphinganine (SA). SA is N-conjugated with another 139 fatty acid to form dihydro-ceramide. When desaturated at C4 it forms ceramide, the 140 141 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.

Sphingosine-1-Phosphate (S1P) is formed after phosphorylation of SO by two Sphingosine 143 144 Kinases (SphK1 and -2)[7]. In plasma, SO is the most common sphingoid base, followed by sphingadienine (SA-dienine) and SA[8]. Ceramide and S1P have been implicated in key steps 145 146 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 can be used, including acyl-CoA with carbon chain lengths ranging from C12 to C18. 148 149 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 atypical sphingolipids: the 1-Deoxy Sphingolipids (dSLs) that lack the C1-OH group of the 151 152 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 dSLs is unknown. dSL levels were shown to be increased in plasma of patients with the 154 155 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 processes, such as IR, lipotoxicity, dyslipidaemia and chronic inflammation[11]. Since there 159 is a correlation between the MetS, T2DM and (deoxy)sphingolipids, on the one hand, and a 160 161 close relationship between the MetS, T2DM and NAFLD, on the other hand, we aimed at investigating whether : 162

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

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

169 Patients & Methods

### 170 Study participants

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

No-NAFLD: no histological evidence for steatosis, inflammation, ballooning and
 fibrosis

- Isolated steatosis (or NAFL): histological evidence of steatosis with activity score
   (ballooning+inflammation) ≤1 and fibrosis stage ≤1
- NASH without significant fibrosis (fibrosis stage (F)<2): histological evidence of</li>
   steatosis with ballooning score ≥1, inflammation score ≥1 and fibrosis stage ≤1
- NASH with significant fibrosis (≥F2) (so called "fibrotic NASH"): histological evidence
   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]. Briefly, sphingoid bases were determined in fasting plasma samples. Sphingolipids in plasma 202 203 are present in a broad variation of subspecies. They can be saturated and are usually acylated with another fatty acid. Furthermore, most of these sphingolipids are conjugated 204 205 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 Nalkyl chain, whereas alkaline conditions lead to a release of the O-linked head group. The 207 208 sphingoid bases were analysed using Liquid Chromatography/Mass Spectrometry. Analysed 209 bases included  $C_{16}$ -sphinganine ( $C_{16}$ -SA),  $C_{16}$ -sphingosine ( $C_{16}$ -SO),  $C_{17}$ -sphingosine ( $C_{17}$ -SO), C<sub>18</sub>-sphinganine (C<sub>18</sub>-SA), C<sub>18</sub>-sphingosine (C<sub>18</sub>-SO), C<sub>19</sub>-sphingosine (C<sub>19</sub>-SO), C<sub>20</sub>-sphinganine 210

- 211 (C<sub>20</sub>-SA), C<sub>20</sub>-sphingosine (C<sub>20</sub>-SO), sphingadienine (SA-dienine), C<sub>18</sub>-phyto-sphingosine (C<sub>18</sub>-
- 212 phyto-SO), deoxy-sphinganine (deoxy-SA) and deoxy-sphingosine (deoxy-SO).

#### 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 with the different entities of NAFLD (steatosis, steatohepatitis and fibrosis), the following 218 groups were compared: 'No-NAFLD' vs. 'NAFL' to investigate the association of the 219 sphinghoid bases in steatosis; 'NAFL' vs. 'NASH <F2' to investigate the potential association 220 221 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 analysed using binary logistic regression. Since NAFLD is closely related to the MetS and 224 225 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) as covariates and/or interaction terms in a binary logistic regression. 229

230 2. Deoxy-sphingoid bases and metabolic parameters

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

#### 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  $\geq$ F2. This is an 245 obese study population with a mean BMI of 39.6 kg/m<sup>2</sup> (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

There were 47 patients with isolated liver steatosis and 51 (26.6%) patients with no 250 251 histological evidence for the presence of NAFLD (No-NAFLD). There was a statistically 252 significant difference in age, waist and HbA<sub>1c</sub>, but there was no difference in BMI, presence of the Mets and presence of T2DM. There was no use of fibrates and statin use was not 253 254 significantly different in both groups (Supplementary Table 1). There was a statistically significant association between the presence of steatosis and the concentrations of deoxy-255 SA (exp(B) 6.708 with CI [1.243, 36.195] and p 0.027) and deoxy-SO (exp(B) 4.851 with CI 256 257 [1.315, 17.890] and 0.018). There was no interaction effect of metabolic markers in the association of steatosis and the sphingoid bases deoxy-SA and deoxy-SO, and statistical 258 significance was maintained for the DSBs (deoxy-SA and deoxy-SO) even after correction for 259 260 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 statistically significant difference in the presence of the MetS, HOMA-IR and liver enzymes 264 (Supplementary Table 2). There was a statistically significant difference in plasma 265 concentrations of C<sub>16</sub>-SO, C<sub>17</sub>-SO, C<sub>18</sub>-SO and SA-dienine. Further analysis did, however, not 266 show an association between NASH and C<sub>16</sub>-SO (exp(B) 0.159 with CI [0.016, 1.595] and 267 p=0.118) nor C<sub>17</sub>-SO (exp(B) 0.106 with CI [0.008, 1.342] and p=0.083). There was a 268 269 statistically significant association between NASH and C<sub>18</sub>-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 was no interaction effect by the metabolic markers. However, after correction for the 271 272 presence of the MetS there was a loss of the statistically significant association between NASH, on the one hand, and  $C_{18}$ -SO (exp(B) 0.043 with CI [0.001, 1.405] and p 0.077) and SA-273 dienine (exp(B) 0.062 with CI [0.003, 1.414] and p 0.081), on the other hand. 274

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 statistically significant association between fibrotic NASH and the concentrations of C<sub>19</sub>-SO 279 (exp(B) 0.157 with CI [0.025, 0.986] and p=0.048) and SA-dienine (exp(B) 0.013 with CI 280 [0.001, 0.342] and p=0.009). There was no interaction between metabolic markers and the 281 282 sphingoid bases C<sub>19</sub>-SO and SA-dienine. After correcting for HOMA-IR, however, there was loss of statistical significance for the association between fibrotic NASH and  $C_{19}$ -SO (exp(B) 283 after correction 0.203 with CI [0.028, 1.495] and p=0.118). Furthermore, there was a loss of 284

statistical significance for the association between fibrotic NASH and SA-dienine after
 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

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

292 2a. Metabolic syndrome

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

298 2b. Type 2 diabetes mellitus

There was a highly statistically significant association between the presence of T2DM and the concentrations of deoxy-SA (exp(B) 14405.280 with CI [13.084, 15860091.190] and p 0.007) and deoxy-S0 (exp(B) 7.953 with CI [1.832, 34.515] and p 0.006). There was no interaction with metabolic markers (including MetS) nor with NAFLD. After correction for the MetS, however, there was a loss of a statistically significant association between T2DM and deoxy-SA (exp(B) after correction 1.964 with CI [0.275, 14.048] and p=0.501) and deoxy-SO (exp(B) after correction 2.138 with CI [0.400, 11.432] and p=0.374).

306 *2c. Correlation with other metabolic parameters* 

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

#### 313 Discussion

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

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

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

MetS. In this study, there was a statistically significant difference in BMI and waist circumference when comparing patients with T2DM to those with the Mets, and a (near statistically) significant difference when comparing triglycerides, but there was no 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. Gorden et al described a diverse panel of 20 plasma lipids, including the dSL 1-deoxy-ceramide, capable of 342 343 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 where liver biopsy was obtained during surgery (gastric bypass, liver transplant, multi organ 345 346 transplant, hernia repair...), the study population was obese with a mean fasting glucose > 100 mg/dL (other metabolic conditions were not mentioned). The possible effects of these 347 conditions on the results was not assessed, meaning that an overestimation of the 348 349 discriminate power of these lipids is possible [17]. Gai et al. reported a correlation between NAFLD and dSLs in 80 individuals, but failed to specify the contribution of these dSLs in the 350 different entities of NAFLD. Furthermore, they did not provide a clear overview of the main 351 352 (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 not fully understood, but they seem to play a role in the control of metabolism. A study by 354 355 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 remodelling, senescence, necrosis and apoptosis favouring hyperglycaemia. [10] Although 357 358 we found an increasing presence of *de novo* T2DM with a more severe phenotype of NAFLD, there was no significant interaction between de novo T2DM nor IR and dSLs when 359

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

Another possible explanation in the association between dSLs and NAFLD is a shift in the 369 utilization of alanine instead of serine by SPT. Mardinoglu et al. revealed a L-serine 370 371 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 the MetS, there is a loss of statistical significance in the association between dSLs and 374 T2DM. Furthermore, this association between dSLs and MetS is not driven by the presence 375 376 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)  $\alpha$  or non-PPAR $\alpha$  mediated effect[20]. Gai et al. described that the

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

In this study, patients were not included based on a *a priori* suspicion of liver disease .This 397 selection procedure resulted in a series representing the whole spectrum of NAFLD, 398 399 whereas retrospective series of biopsy proven NAFLD patients tend to be skewed towards more severe subtypes. On the other hand, analysis was performed cross-sectionally on a 400 population that is predominantly Caucasian. A causal link between deoxy-sphingolipids and 401 402 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 for all the individual sphingolipid subclasses, further investigation of these different 404 405 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 therapeutic407 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.

### 412 **REFERENCES**

- Caldwell, S. and C. Argo, *The natural history of non-alcoholic fatty liver disease*. Dig Dis, 2010.
   **28**(1): p. 162-8.
- 415 2. Caligiuri, A., A. Gentilini, and F. Marra, *Molecular Pathogenesis of NASH*. Int J Mol Sci, 2016.
  416 **17**(9).
- Angulo, P., et al., *Liver Fibrosis, but No Other Histologic Features, Is Associated With Long- term Outcomes of Patients With Nonalcoholic Fatty Liver Disease.* Gastroenterology, 2015. **149**(2): p. 389-97.e10.
- 4. Ekstedt, M., et al., *Fibrosis stage is the strongest predictor for disease-specific mortality in*421 *NAFLD after up to 33 years of follow-up.* Hepatology, 2015. **61**(5): p. 1547-54.
- Hannun, Y.A. and L.M. Obeid, *Sphingolipids and their metabolism in physiology and disease.*Nat Rev Mol Cell Biol, 2018. **19**(3): p. 175-191.
- 424 6. Bode, H., et al., *HSAN1 mutations in serine palmitoyltransferase reveal a close structure-*425 *function-phenotype relationship.* Hum Mol Genet, 2016. **25**(5): p. 853-65.
- 426 7. Nikolova-Karakashian, M., Sphingolipids at the Crossroads of NAFLD and Senescence. Adv
  427 Cancer Res, 2018. 140: p. 155-190.
- 428 8. Quehenberger, O., et al., *Lipidomics reveals a remarkable diversity of lipids in human plasma*.
  429 J Lipid Res, 2010. **51**(11): p. 3299-305.
- 430 9. Bertea, M., et al., *Deoxysphingoid bases as plasma markers in diabetes mellitus*. Lipids
  431 Health Dis, 2010. 9: p. 84.
- 432 10. Zuellig, R.A., et al., *Deoxysphingolipids, novel biomarkers for type 2 diabetes, are cytotoxic*433 *for insulin-producing cells.* Diabetes, 2014. 63(4): p. 1326-39.
- 434 11. Mikolasevic, I., et al., *Nonalcoholic fatty liver disease A multisystem disease*? World J
  435 Gastroenterol, 2016. 22(43): p. 9488-9505.
- 436 12. Verrijken, A., et al., *Prothrombotic factors in histologically proven nonalcoholic fatty liver*437 *disease and nonalcoholic steatohepatitis.* Hepatology, 2014. **59**(1): p. 121-9.
- 438 13. Kleiner, D.E., et al., *Design and validation of a histological scoring system for nonalcoholic*439 *fatty liver disease.* Hepatology, 2005. **41**(6): p. 1313-21.
- 44014.Bedossa, P., Utility and appropriateness of the fatty liver inhibition of progression (FLIP)441algorithm and steatosis, activity, and fibrosis (SAF) score in the evaluation of biopsies of442nonalcoholic fatty liver disease. Hepatology, 2014. **60**(2): p. 565-75.
- Penno, A., et al., *Hereditary sensory neuropathy type 1 is caused by the accumulation of two neurotoxic sphingolipids.* J Biol Chem, 2010. **285**(15): p. 11178-87.
- 445 16. Othman, A., et al., *Plasma deoxysphingolipids: a novel class of biomarkers for the metabolic syndrome?* Diabetologia, 2012. 55(2): p. 421-31.
- 447 17. Gorden, D.L., et al., *Biomarkers of NAFLD progression: a lipidomics approach to an epidemic.*448 J Lipid Res, 2015. 56(3): p. 722-36.
- 44918.Gai, Z., et al., Farnesoid X receptor activation induces the degradation of hepatotoxic 1-<br/>deoxysphingolipids in non-alcoholic fatty liver disease. Liver Int, 2020. 40(4): p. 844-859.
- 451 19. Othman, A., et al., *Plasma 1-deoxysphingolipids are predictive biomarkers for type 2 diabetes*452 *mellitus.* BMJ Open Diabetes Res Care, 2015. **3**(1): p. e000073.
- 45320.Othman, A., et al., Fenofibrate lowers atypical sphingolipids in plasma of dyslipidemic454patients: A novel approach for treating diabetic neuropathy? J Clin Lipidol, 2015. 9(4): p. 568-45575.
- 456 21. Othman, A., et al., Lowering plasma 1-deoxysphingolipids improves neuropathy in diabetic
   457 rats. Diabetes, 2015. 64(3): p. 1035-45.
- 458 22. Garofalo, K., et al., Oral L-serine supplementation reduces production of neurotoxic
  459 deoxysphingolipids in mice and humans with hereditary sensory autonomic neuropathy type
  460 1. J Clin Invest, 2011. 121(12): p. 4735-45.

# Tables

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

		TOTAL			NASH <f2< th=""><th>NASH ≥F2</th><th></th></f2<>	NASH ≥F2	
		N=288			N=149	N=41	p-value
		(100%)	N=51 (17.7%)	N=47 (16.3%)	(51.7%)	(14.3%)	
	Age	43.8	38.1	43.5	45.4	45.0	0.000*
Conoral	(years)	(±12.3)	(±10.6)	(±12.3)	(±12.4)	(±12.4)	0.003
General	Gender:	96	8	10	53	25	<0.001 <sup>***</sup>
	men	(33.3%)	(15.7%)	(21.3%)	(35.6%)	(61.0%)	<0.001
	BMI	39.6	38.2	39.9	38.9	43.5	0.001**
Anthropometr	(kg/m²)	(±6.4)	(±4.9)	(±7.1)	(±6.0)	(±7.4)	0.001
у	Waist	118.8	111.93	118.1	117.9	132.1	<0.001**
	(cm)	(±13.2)	(±10.4)	(±13.6)	(±11.3)	(±13.1)	<0.001
	TG	155.5	137.2	150.3	157.1	177.9	0.009**
	(mg/dL)	(±78.5)	(±66.6)	(±106.9)	(±72.8)	(±69.4)	0.009
	LDL-C	124.7	121.5	128.8	123.4	128.5	0.674*
	(mg/dL)	(±36.8)	(±43.9)	(±33.2)	(±35.7)	(±35.5)	0.074
	HDL-C	48.9	53.2	51.3	48.6	41.9	<0.001**
	(mg/dL)	(±13.7)	(±13.4)	(±15.9)	(±13.5)	(±9.3)	<0.001
	Statin	30	2	3	22	3	0.097***
	use (%)	(10.4)	(3.9)	(6.4)	(14.8)	(7.3)	0.087
Metabolic	HbA1c	5.7	5.4	5.6	5.6	6.1	<0.001**
markers	(%)	(±0.5)	(0.3)	(±0.3)	(±0.4)	(±1.0)	<0.001
	Glucose	86.2	80.2	82.4	85.1	102.1	<0.001**
	mg/dL	(±22.4)	(±7.4)	(±11.0)	(±12.4)	(±49.8)	<0.001
	HOMA-	4.0	3.1	2.9	3.8	6.8	<0.001**
	IR	(±3.2)	(±3.2)	(±1.8)	(±2.4)	(±5.1)	<0.001
	MetS	155	20	17	87	31	<0.001****
		(56.2%)	(40.8%)	(37.8%)	(61.3%)	(77.5%)	
	T2DM	30	1	3	16	10	0.004***
		(10.5%)	(2.0%)	(6.5%)	(10.9%)	(24.4%)	
	AST	31.7	24.4	25.5	30.7	51.5	<0.001**
Liver on types	(U/L)	(±17.5)	(±8.0)	(±8.5)	(±11.8)	(±31.5)	<0.001
Liver enzymes	ALT	45.6	31.0	36.1	45.8	74.0	<0.001**
	(U/L)	(±27.3)	(±11.3)	(±18.7)	(±21.6)	(±43.0)	<0.001
	C 5A	0.469	0.460	0.441	0.491	0.432	0.672**
	C <sub>16</sub> -3A	(±0.221)	(±0.227)	(±0.159)	(±0.249)	(±0.156)	0.075
	050	15.491	15.991	16.584	15.379	14.026	0.067**
	C <sub>16</sub> -30	(±5.313)	(±5.880)	(±4.753)	(±5.568)	(±3.828)	0.007
	0-50	6.233	6.533	6.665	6.180	5.555	0.018**
	C <sub>17</sub> -30	(±2.007)	(±2.365)	(±1.686)	(±2.053)	(±1.498)	0.018
	C	3.659	3.599	3.512	3.726	3.662	0 822*
Sphingoid	C <sub>18</sub> -3A	(±1.401)	(±1.588)	(±1.229)	(±1.459)	(±1.126)	0.822
hases	C	84.161	86.927	89.051	83.308	78.050	0.008**
50303	C <sub>18</sub> -30	(±20.682)	(±24.394)	(±17.530)	(±21.246)	(±14.864)	0.008
	C <sub>18</sub> -	0 1 1 0	0 115	0 11/	0 1 1 0	0 100	**
	phytoS	(+0.038)	(+0.052)	(+0.029)	(+0.037)	(+0.026)	0.135
	0	(±0.050)	(±0.032)	(±0.023)	(±0.037)	(±0.020)	
	C10-50	1.815	1.875	1.814	1.871	1.541	0.200**
	C19-50	(±0.794)	(±0.839)	(±0.702)	(±0.858)	(±0.508)	0.200
	Car-SA	0.024	0.021	0.024	0.025	0.025	0.024**
	C20-3A	(±0.108)	(±0.010)	(±0.012)	(±0.110)	(±0.009)	0.027

C <sub>20</sub> -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  $\geq$  F2), Body mass index (BMI), Triglycerides (TG), Low density lipoprotein cholesterol (LLD-C), High density lipoprotein cholesterol (HDL-C), Haemoglobin A<sub>1c</sub> (HbA<sub>1c</sub>), Homeostatic model assessment insulin resistance (HOMA-IR), Metabolic syndrome (MetS), aspartate aminotransferase (AST), alanine aminotransferase (ALT), C<sub>16</sub>-Sphinganine (C<sub>16</sub>-SA), C<sub>16</sub>-Sphingosine (C<sub>16</sub>-SO), C<sub>17</sub>-Sphingosine (C<sub>17</sub>-SO), C<sub>18</sub>-Sphinganine (C<sub>18</sub>-SA), C<sub>18</sub>-Sphingosine (C<sub>18</sub>-SO), C<sub>19</sub>-Sphingosine (C<sub>19</sub>-SO), C<sub>20</sub>-Sphinganine (C<sub>20</sub>-SA), C<sub>20</sub>-Sphingosine (C<sub>20</sub>-SO), Sphingadiene (SA-diene), C<sub>18</sub>-phyto-sphingosine (C<sub>18</sub>-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*	р	Correlation coefficient*	р	
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

		No-NAFLD	NAFL	
		N=51	N=47	р
Conorol	Age (years)	38.1 (±10.6)	43.5 (±12.3)	0.021**
General	Gender: men	8 (15.7%)	10 (21.3%)	0.475***
A	BMI (kg/m <sup>2</sup> )	38.2 (±4.9)	39.9 (±7.1)	0.190 <sup>**</sup>
Anthropometry	Waist (cm)	111.93 (±10.4)	118.1 (±13.6)	0.007**
	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 <sup>*</sup>
Metabolic	Statin use (%)	2 (3.9)	3 (6.4)	0.580***
markers	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 <sup>*</sup>
	ALT (U/L)	31.0 (±11.3)	36.1 (±18.7)	0.248 <sup>*</sup>
Imaging	Steatosis on US	30 (61.2%)	40 (95.2%)	<0.001***
	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***
Pathology	Inflammatio n 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***
	C <sub>16</sub> -SA	0.460 (±0.227)	0.441 (±0.159)	0.890*
	C <sub>16</sub> -SO	15.991 (±5.880)	16.584 (±4.753)	0.384*
	C <sub>17</sub> -SO	6.533 (±2.365)	6.665 (±1.686)	0.384*
Sphingoid	C <sub>18</sub> -SA	3.599 (±1.588)	3.512 (±1.229)	0.773 <sup>*</sup>
bases	C <sub>18</sub> -SO	86.927 (±24.394)	89.051 (±17.530)	0.328
	C <sub>18</sub> -phytoSO	0.115 (±0.052)	0.114 (±0.029)	0.526
	C <sub>19</sub> -SO	1.875 (±0.839)	1.814 (±0.702)	0.897
	C <sub>20</sub> -SA	0.021 (±0.010)	0.024 (±0.012)	0.236
	C <sub>20</sub> -SO	0.188 (±0.079)	0.199 (±0.060)	0.281

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

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 (LLD-C), High density lipoprotein cholesterol (HDL-C), Haemoglobin A<sub>1c</sub> (HbA<sub>1c</sub>), Homeostatic model assessment insulin resistance (HOMA-IR), Metabolic syndrome (MetS), aspartate aminotransferase (AST), alanine aminotransferase (ALT), C<sub>16</sub>-Sphinganine (C<sub>16</sub>-SA), C<sub>16</sub>-Sphingosine (C<sub>16</sub>-SO), C<sub>17</sub>-Sphingosine (C<sub>17</sub>-SO), C<sub>18</sub>-Sphinganine (C<sub>18</sub>-SA), C<sub>18</sub>-Sphingosine (C<sub>18</sub>-SO), C<sub>20</sub>-Sphingosine (C<sub>20</sub>-SA), C<sub>20</sub>-Sphingosine (C<sub>20</sub>-SA), deoxy-sphingosine (C<sub>20</sub>-SO). SD or percentage of the total group is presented between brackets.

		NAFL N=47	NASH <f2 N=149</f2 	p
Conoral	Age (years)	43.5 (±12.3)	45.4 (±12.4)	0.350**
General	Gender: men	10 (21.3%)	53 (35.6%)	0.067***
Anthronomotry	BMI (kg/m <sup>2</sup> )	39.9 (±7.1)	38.9 (±6.0)	0.486*
Anthropometry	Waist (cm)	118.1 (±13.6)	117.9 (±11.3)	0.952**
	TG (mg/dL)	150.3 (±106.9)	157.1 (±72.8)	0.113 <sup>*</sup>
	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 <sup>*</sup>
Motabolic	Statin use (%)	3 (6.4)	22 (14.8)	0.133***
markers	HbA1c (%)	5.6 (±0.3)	5.6 (±0.4)	0.514 <sup>*</sup>
	Glucose (mg/dL)	82.4 (±11.0)	85.1 (±12.4)	0.151 <sup>*</sup>
	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***
	AST (U/L)	25.5 (±8.5)	30.7 (±11.8)	0.005*
Liver enzymes	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	0.0/80.9/17.0/2.1	0.0/36.9/38.3/24.8	<0.001****
	0/1/2/3 (%) 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****

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

	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	C <sub>16</sub> -SA	0.434 (±0.141)	0.478 (±0.233)	0.469 <sup>*</sup>
bases	C <sub>16</sub> -SO	16.212 (±4.426)	15.087 (±5.262)	0.031 <sup>*</sup>
	C <sub>17</sub> -SO	6.511 (±1.610)	6.045 (±1.960)	0.023*
	C <sub>18</sub> -SA	3.464 (±1.238)	3.712 (±1.392)	0.689 <sup>*</sup>
	C <sub>18</sub> -SO	86.492 (±17.300)	82.173 (±20.123)	0.005 <sup>*</sup>
	C <sub>18</sub> -phytoSO	0.111 (±0.029)	0.108 (±0.035)	0.229 <sup>*</sup>
	C <sub>19</sub> -SO	1.760 (±0.667)	1.800 (±0.806)	0.839**
	C <sub>20</sub> -SA	0.023 (±0.011)	0.025 (±0.010)	0.315**
	C <sub>20</sub> -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  $\geq$  F2), Body mass index (BMI), Triglycerides (TG), Low density lipoprotein cholesterol (LLD-C), High density lipoprotein cholesterol (HDL-C), Haemoglobin A<sub>1c</sub> (HbA<sub>1c</sub>), Homeostatic model assessment insulin resistance (HOMA-IR), Metabolic syndrome (MetS), aspartate aminotransferase (AST), alanine aminotransferase (ALT), C<sub>16</sub>-Sphinganine (C<sub>16</sub>-SA), C<sub>16</sub>-Sphingosine (C<sub>19</sub>-SO), C<sub>17</sub>-Sphingosine (C<sub>17</sub>-SO), C<sub>18</sub>-Sphinganine (C<sub>18</sub>-SA), C<sub>18</sub>-Sphingosine (C<sub>19</sub>-SO), C<sub>20</sub>-Sphinganine (C<sub>20</sub>-SA), C<sub>20</sub>-Sphingosine (C<sub>20</sub>-SA), deoxy-sphingosine (C<sub>20</sub>-SO). SD or percentage of the total group is presented between brackets.

		NASH <f2< th=""><th>NASH ≥F2</th><th>2</th></f2<>	NASH ≥F2	2
		N=149	N=41	μ
Conoral	Age (years)	45.4 (±12.4)	45.0 (±12.4)	0.855**
General	Gender: men	53 (35.6%)	25 (61.0%)	0.003****
A athreas are star.	BMI (kg/m <sup>2</sup> )	38.9 (±6.0)	43.5 (±7.4)	<0.001 <sup>*</sup>
Anthropometry	Waist (cm)	117.9 (±11.3)	132.1 (±13.1)	<0.001**
	TG (mg/dL)	157.1 (±72.8)	177.9 (±69.4)	0.059 <sup>*</sup>
	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***
Metabolic markers	HbA1c (%)	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 <sup>***</sup>
	T2DM	16 (10.9%)	10 (24.4%)	0.027***
Liver enzymes	AST (U/L)	30.7 (±11.8)	51.5 (±31.5)	<0.001*

Supplementary Table 3: Patient characteristics for the comparison NASH <F2 vs. NASH  $\geq$ F2

	ALT (U/L)	45.8 (±21.6)	74.0 (±43.0)	<0.001 <sup>*</sup>
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 <sup>***</sup>
	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 <sub>16</sub> -SA	0.491 (±0.249)	0.432 (±0.156)	0.375 <sup>*</sup>
	C <sub>16</sub> -SO	15.379 (±5.568)	14.026 (±3.838)	0.206 <sup>**</sup>
	C <sub>17</sub> -SO	6.180 (±2.053)	5.555 (±1.498)	0.083**
	C <sub>18</sub> -SA	3.726 (±1.459)	3.662 (±1.126)	0.687 <sup>*</sup>
	C <sub>18</sub> -SO	83.308 (±21.246)	78.050 (±14.864)	0.250 <sup>*</sup>
	C <sub>18</sub> -phytoSO	0.110 (±0.037)	0.100 (±0.026)	0.126 <sup>*</sup>
	C <sub>19</sub> -SO	1.871 (±0.858)	1.541 (±0.508)	0.046**
	C <sub>20</sub> -SA	0.025 (±0.011)	0.026 (±0.009)	0.528 <sup>**</sup>
	C <sub>20</sub> -SO	0.198 (±0.071)	0.190 (±0.052)	0.958 <sup>*</sup>
	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  $\ge$  F2), Body mass index (BMI), Triglycerides (TG), Low density lipoprotein cholesterol (LLD-C), High density lipoprotein cholesterol (HDL-C), Haemoglobin A<sub>1c</sub> (HbA<sub>1c</sub>), Homeostatic model assessment insulin resistance (HOMA-IR), Metabolic syndrome (MetS), aspartate aminotransferase (AST), alanine aminotransferase (ALT), C<sub>16</sub>-Sphinganine (C<sub>16</sub>-SA), C<sub>16</sub>-Sphingosine (C<sub>16</sub>-SO), C<sub>17</sub>-Sphingosine (C<sub>17</sub>-SO), C<sub>18</sub>-Sphinganine (C<sub>18</sub>-SA), C<sub>18</sub>-Sphingosine (C<sub>18</sub>-SO), C<sub>20</sub>-Sphinganine (C<sub>20</sub>-SA), C<sub>20</sub>-Sphingosine (C<sub>20</sub>-SO), Sphingadiene (SA-diene), C<sub>18</sub>-phyto-sphingosine (C<sub>18</sub>-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