ANGPTL8 (Betatrophin) is Expressed in Visceral Adipose Tissue and Relates to Human Hepatic Steatosis in Two Independent Clinical Collectives

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ABSTRACT

Angiopoietin-like protein 8 (ANGPTL8)/betatrophin expression in visceral adipose tissue and associations with circulating fatty acid profile have not yet been investigated.

Forty subjects were included in a cross-sectional study, 57 in a dietary weight reduction intervention. Circulating Angiopoietin-like protein 8/betatrophin was measured in all subjects. Liver and adipose tissue were sampled and plasma fatty acids and tissue Angiopoietin-like protein 8/betatrophin expression were evaluated in the cross-sectional study. In the intervention study oral glucose testing and liver magnetic resonance scanning at baseline and after 6 months were performed. Angiopoietin-like protein 8/betatrophin mRNA was increased in visceral compared to subcutaneous adipose tissue ($p < 0.001$). Circulating ANGPTL8/betatrophin correlated with liver steatosis ($r = 0.42$, $p = 0.047$), triacylglycerols ($r = 0.34$, $p = 0.046$), saturated ($r = 0.43$, $p = 0.022$), monounsaturated ($r = 0.51$, $p = 0.007$), and polyunsaturated fatty acids ($r = -0.53$, $p = 0.004$). In the intervention study, baseline Angiopoietin-like protein 8/betatrophin correlated with age ($r = 0.32$, $p = 0.010$) and triacylglycerols ($r = 0.30$, $p = 0.02$) and was increased with hepatic steatosis ($p = 0.033$). Weight loss reduced liver fat by 45 % and circulating Angiopoietin-like protein 8/betatrophin by 11 % (288 ± 17 vs. 258 ± 17 pg/ml; $p = 0.015$). Angiopoietin-like protein 8/betatrophin is related to liver steatosis, while visceral adipose tissue represents an additional site of expression in humans.

Abbreviations

AUC	Area under the curve
AT	Adipose tissue
BMI	Body mass index
ELISA	Enzyme-linked immunosorbent assay
FA	Fatty acids
FAME	Fatty acid methyl esters
HOMA-IR	Homeostasis model-assessment of insulin resistance
mRNA	Messenger ribonucleic acid
NAFLD	Nonalcoholic fatty liver disease
SCAT	Subcutaneous adipose tissue
T2D	Type 2 diabetes mellitus
VAT	Visceral adipose tissue

Introduction

In a recent animal study, Angiopoietin-like protein 8 (ANGPTL8), also known as betatrophin, human C19ORF80, lipasin, or RIFL was reported to promote pancreatic beta-cell mass proliferation in rodents while ameliorating glucose metabolism [1, 2]. However, this finding could not be reproduced by others [3]. In humans, most [4–8] but not all [9, 10] clinical studies in type 2 diabetes mellitus (T2D) observed correlations of circulating ANGPTL8/betatrophin with variables of glucose homeostasis. Moreover, even diabetic nephropathy and retinopathy have been related to a complex network of involved biomarkers including circulating ANGPTL8/betatrophin [11–13]. Otherwise, no relationship of ANGPTL8/betatrophin with C-peptide levels could be established in diabetic patients [14, 15]. The latter is supported by experimental data indicating that associations of ANGPTL8/betatrophin and glucose metabolism cannot largely be explained by enhanced insulin secretion [16, 17]. Therefore, effects on insulin sensitive tissues may contribute to the observed relationship.

Nonalcoholic fatty liver disease (NAFLD) predisposes humans to T2D [18] as NAFLD is closely related to insulin resistance, resulting in hyperinsulinemia, hyperglycemia and hypertriacylglycerolemia [19, 20]. The liver was reported as a major site of ANGPTL8/betatrophin expression in humans [21]. Moreover, ANGPTL8/betatrophin is associated with dyslipidemia in rodents and humans [3, 8, 9], particularly being involved in triacylglycerol metabolism [22]. Accordingly, we hypothesized that liver fat and ANGPTL8/betatrophin production could interact locally and thereby show interrelations with insulin resistance and circulating lipid profiles. Therefore, in a cross-sectional cohort and an independent intervention study, we tested the hypothesis that hepatic steatosis is related to ANGPTL8/betatrophin in humans, and that liver fat reduction due to hypocaloric dieting reduces circulating ANGPTL8/betatrophin. We, moreover, evaluated whether blood lipids and main fatty acid (FA) species in plasma are associated with ANGPTL8/betatrophin. As adipose tissue (AT) represents an alternative site of ANGPTL8/betatrophin mRNA expression [21] we finally determined whether in humans, visceral adipose tissue (VAT) and subcutaneous adipose tissue (SCAT) show comparable expression levels of ANGPTL8/betatrophin and how these relate to circulating levels.

Subjects and Methods

Study groups and ethics

We investigated subgroups from 2 independent studies. The study protocol was approved by the institution's human research committee, respectively. Informed consent was obtained from all patients for being included in the study. Informed consent was obtained after full explanation of the purpose and nature of all procedures used. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008.

We included subjects from the cross-sectional INSIGHT (German Clinical Trials-Register: DRKS00005450) study [23]. This group comprised 24 participants characterized by the presence of histologically proven liver steatosis but absence of T2D, and 16 subjects without hepatic steatosis, insulin resistance or T2D. We further analyzed 57 participants of the B-SMART (Clinical-Trials.gov Identifier: NCT00956566) study. The B-SMART study was designed as a prospective, randomized trial to compare weight loss and changes of metabolic and cardiovascular variables with reduced carbohydrate or reduced fat hypocaloric diets [24].

Basic characteristics, clinical chemistry and assays

Medical history and anthropometry were evaluated. Oral glucose tolerance testing was performed in B-SMART subjects [24]. Insulin resistance was estimated by the homeostasis model-assessment of insulin resistance (HOMA-IR), blood glucose, insulin, lipids, and inflammatory markers were measured by standard methods in all subjects in certified Clinical Chemistry Laboratories [23, 24]. Circulating ANGPTL8/betatrophin was analyzed by enzyme-linked immunosorbent assay (ELISA; Wuhan Eiaab Science, Wuhan, China; E11644h, intra-assay coefficient of variation ≤ 4.8 %, inter-assay coefficient of variation ≤ 7.2 %). Circulating adiponectin (ALPCO Immunoassays, Salem, NH, USA; #47-ADPHU-E01) was measured in B-SMART participants.

Sampling of tissue specimen

In INSIGHT subjects, liver specimen were harvested from a healthy liver segment and histopathology was evaluated according to accepted criteria [23]. Liver surgery was indicated for non-malignant or malignant indication. Samples of SCAT and VAT were taken by knife extraction in the same surgical setting. All samples were immediately flash frozen in liquid nitrogen and stored at −80 °C until analysis.

Lipid analysis

Lipids were extracted from plasma of INSIGHT subjects (n = 27) and analyzed for FA species [25]. FA concentrations are expressed as the percentage of the total area of all FA peaks (% of total fatty acid methyl esters, FAME).

Oral glucose tolerance test

After an overnight fast, blood samples were obtained in B-SMART subjects at baseline and after oral glucose loading (75 g glucose/500 ml) at 15, 30, 45, 60, 75, 90, and 120 min for analysis of glucose and insulin [24].

Magnetic resonance spectroscopy of the liver

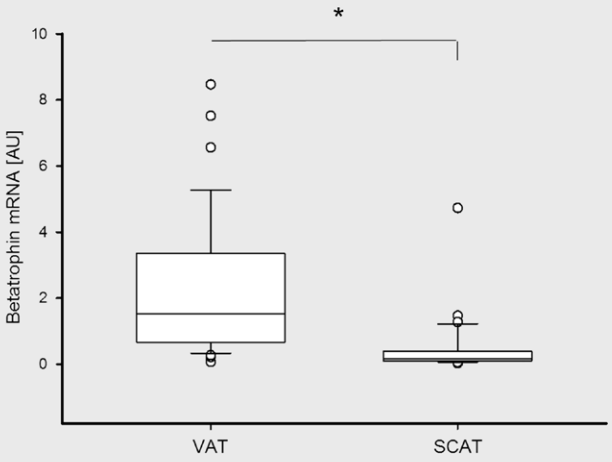
All included B-SMART subjects had completed a magnetic resonance (MR) scan at baseline (n = 57) and after 6 months dietary intervention (n = 52) [24]. Intrahepatic lipid (IHL) content was assessed by ^{1}H MR spectroscopy of the liver [24].

Real time PCR

ANGPTL8/betatrophin messenger mRNA was analyzed in liver tissue, SCAT and VAT of INSIGHT subjects by means of real-time PCR [26]. Primer sequences used for analysis were as follows: 5′-CTGTCCCGTAGCACCTTCTG-3′ and 5′-CAGAAGGTGCTACGGGACAG-3′ for ANGPTL8/betatrophin; 5′-TGACACTGGCAAAACAATGCA-3′ and 5′-GGTCCTTTTCACCAGCAAGCT-3′ for hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1), which was used for normalization.

Statistics

Data were analyzed with IBM SPSS 22.0 (Chicago, IL, USA). Data are given as mean ± SEM if not otherwise stated. Logarithmic transformation of data was performed if required. Depending on data distribution the following procedures were used: Pearson's or Spearman's rank correlation coefficient, Kruskal-Wallis test with Bonferroni post hoc adjustment for multiple testing, Student's t-test or Mann–Whitney U-test. Significance level was defined as 2-sided $p < 0.05$.



▶ **Fig. 1** ANGPTL8/betatrophin mRNA in subcutaneous vs. visceral adipose tissue. ANGPTL8/betatrophin mRNA was detectable in 92 % of VAT samples in INSIGHT study subjects and 75 % SCAT samples. Levels of ANGPTL8/betatrophin mRNA were significantly increased in human VAT compared to SCAT ($p < 0.001$). SCAT: Subcutaneous adipose tissue; VAT: Visceral adipose tissue. Boxes span from 25th–75th percentile, error bars indicate 10th and 90th percentile; * $p < 0.05$.

Results

ANGPTL8/betatrophin mRNA expression in INSIGHT subjects

We detected ANGPTL8/betatrophin mRNA in the liver of 97 % of all INSIGHT subjects, in VAT of 92 % and in SCAT of 75 %. No association of either SCAT ($r = -0.16$, $p = 0.41$), VAT ($r = -0.27$, $p = 0.13$) or liver ($r = -0.13$, $p = 0.47$) ANGPTL8/betatrophin mRNA with circulating levels was found. Otherwise, after adjustment for age circulating ANGPTL8/betatrophin correlated with VAT mRNA ($r = -0.33$, $p = 0.058$) and significantly with combined mRNA levels of liver tissue, VAT and SCAT ($r = -0.36$, $p = 0.029$). Moreover, liver mRNA was related to circulating ANGPTL8/betatrophin after adjustment for HOMA-IR ($r = -0.36$, $p = 0.034$). Remarkably, in AT we observed significantly higher ANGPTL8/betatrophin mRNA levels in VAT as compared to SCAT ($p < 0.001$; ▶ **Fig. 1**), where expression levels were negligible.

Associations of ANGPTL8/betatrophin with NAFLD related variables in INSIGHT

Characteristics of the INSIGHT group are given in ▶ **Table 1**. Circulating ANGPTL8/betatrophin was numerically increased in NAFLD vs. non-NAFLD subjects, but this did not reach statistical significance ($p = 0.80$; ▶ **Table 1**). Circulating levels were positively correlated with hepatic steatosis ($r = 0.42$, $p = 0.047$; ▶ **Fig. 2**), age ($r = 0.44$, $p = 0.008$), serum triacylglycerols ($r = 0.34$, $p = 0.046$), plasma saturated FA ($r = 0.43$, $p = 0.022$; **Suppl. Fig. 1S**) and plasma monounsaturated FA ($r = 0.51$, $p = 0.007$; **Suppl. Fig. 2S**). By contrast, a negative association of circulating ANGPTL8/betatrophin and plasma polyunsaturated FA was observed ($r = -0.53$, $p = 0.004$; **Suppl. Fig. 3S**). After adjustment for age circulating ANGPTL8/betatrophin was negatively related to low density lipoprotein (LDL) ($r = -0.35$, $p = 0.042$) and high density lipoprotein (HDL) ($r = -0.37$, $p = 0.029$).

Hepatic ANGPTL8/betatrophin mRNA correlated with the NAS ($r = 0.36$, $p = 0.023$), hepatic steatosis ($r = 0.52$, $p = 0.009$; **Suppl. Fig. 4S**), hepatocyte ballooning degeneration ($r = 0.46$, $p = 0.003$) and HOMA-IR ($r = 0.35$, $p = 0.030$). Otherwise, no associations were found regarding further variables of glucose metabolism.

Modification of ANGPTL8/betatrophin by long term dietary intervention in B-SMART subjects

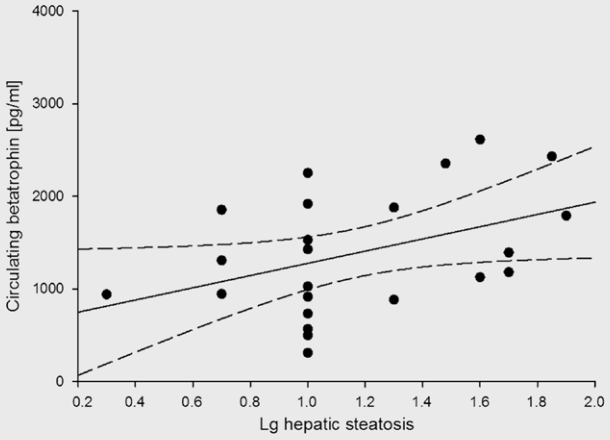
Baseline characteristics of B-SMART participants are given in ▶ **Table 2**. Baseline analysis of circulating ANGPTL8/betatrophin in the B-SMART cohort revealed a correlation with age ($r = 0.32$, $p = 0.010$) and serum triacylglycerols ($r = 0.30$, $p = 0.020$; **Suppl. Fig. 5S**). A close to significant correlation was also observed with area under the curve (AUC) insulin during the oral glucose tolerance test ($r = 0.25$, $p = 0.052$; **Suppl. Fig. 6S**). With adjustment for age, the correlation with insulin AUC became significant ($r = 0.33$, $p = 0.012$), while the correlation with serum triacylglycerols remained unchanged ($r = 0.32$, $p = 0.02$). When stratified according to IHL content, participants with hepatic steatosis had elevated ANGPTL8/betatrophin compared to participants with normal liver fat content ($p = 0.033$; ▶ **Fig. 3**). With dieting, body weight decreased 6.9 kg (−7.4 %) together with reductions in IHL by 45 % and plasma ANGPTL8/betatrophin by 11 % (288 ± 17 vs. 258 ± 17 pg/ml; $p = 0.015$; ▶ **Fig. 4**). Circulating ANGPTL8/betatrophin reductions tended to be more pronounced on the reduced fat compared with the reduced carbohydrate diet (−37 ± 17 vs. −20 ± 17 pg/ml).

▶ **Table 1** Characteristics of INSIGHT participants [23].

Characteristics	Non NAFLD	NAFLD	p-Value
n (% male)	16 (31)	24 (46)	–
Age (years)	54 ± 4	60 ± 3	0.24
Body mass index (kg/m²)	23.8 ± 0.9	27.0 ± 1.4	0.075
Waist circumference (cm)	87.2 ± 3.7	96.3 ± 3.0	0.044
NAFLD activity score (0–8)	0.2 ± 0.1	2.2 ± 0.2	< 0.001
Ballooning degeneration (0–2)	0	0.3 ± 0.1	0.28
Liver inflammation (0–3)	0.2 ± 0.1	0.7 ± 0.2	0.075
Fibrosis (0–4)	1.1 ± 0.3	1.1 ± 0.3	0.90
Liver steatosis (%)	0.4 ± 0.3	20.6 ± 4.0	< 0.001
Alanine-transaminase (U/l)	41 ± 16	47 ± 8	0.16
C-reactive protein (mg/dl)	1.2 ± 0.6	2.3 ± 0.8	0.13
Triacylglycerols (mmol/l)	1.2 ± 0.1	1.3 ± 0.1	0.50
LDL cholesterol (mmo/l)	3.3 ± 0.3	3.2 ± 0.2	0.90
HDL cholesterol (mmol/l)	1.3 ± 0.1	1.2 ± 0.1	0.77
Blood glucose (mmol/l)	4.9 ± 0.2	5.2 ± 0.3	0.27
HOMA-IR	1.8 ± 0.6	2.6 ± 0.6	0.12
HbA1c (mmol/mol)	35.2 ± 1.9	36.4 ± 1.4	0.51
Circulating ANGPTL8/betatrophin (pg/ml)	1 016.5 ± 191.1	1 213.9 ± 203.5	0.80

Data are given as means ± S.E. or absolute numbers, respectively

HDL: High-density lipoprotein; HOMA-IR: Homeostasis model of insulin resistance; LDL: Low-density lipoprotein; NAFLD: Nonalcoholic fatty liver disease; NAS: NAFLD activity score



▶ **Fig. 2** Association of circulating ANGPTL8/betatrophin with hepatic steatosis in INSIGHT subjects. Scatter plots illustrate correlation of circulating ANGPTL8/betatrophin with logarithmized liver steatosis (r = 0.42, p = 0.047). Lines indicate the 95 % confidence intervals.

Discussion

Our study indicates that ANGPTL8/betatrophin mRNA is expressed at higher levels in human visceral compared to subcutaneous adipose tissue and we expand previous findings by showing correlations of ANGPTL8/betatrophin with main fatty acid species in human plasma. We further confirm regulation of ANGPTL8/betatrophin by nutritional interventions, and associations with liver steatosis, age, parameters of insulin resistance and lipid metabolism in 2 independent collectives.

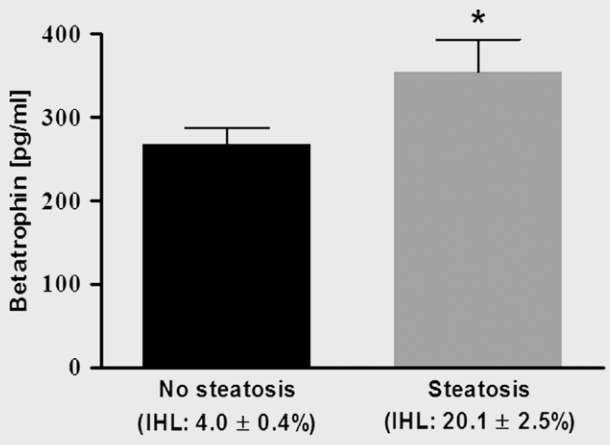
A central, yet not fully elucidated aspect is the tissue specific contribution to circulating ANGPTL8/betatrophin levels. The mRNA expression of ANGPTL8/betatrophin was reported to be very high in the liver compared to other organs and tissues in humans, while in mice AT was identified as an additional expression site [1, 21]. In our study, hepatic ANGPTL8/betatrophin mRNA correlated with circulating levels after adjustment for HOMA-IR, supporting the liver as a main contributor. However, contrasting with earlier findings [1, 21] ANGPTL8/betatrophin mRNA was also abundantly expressed in VAT in our subjects. This observation was supported by a correlation of VAT mRNA with age adjusted circulating ANGPTL8/betatrophin. Otherwise, human expression patterns in fat tissues were previously analyzed in commercially available cDNA arrays and characteristics of the donor population remain therefore unreported [21]. Our specimen were harvested intraoperatively under perfused conditions from characterized patients. Variable results could therefore be at least partially attributed to potential differences regarding the donor groups. We moreover observed ANGPTL8/betatrophin mRNA expression in VAT to be higher com-

▶ **Table 2** Baseline characteristics of B-SMART participants [24].

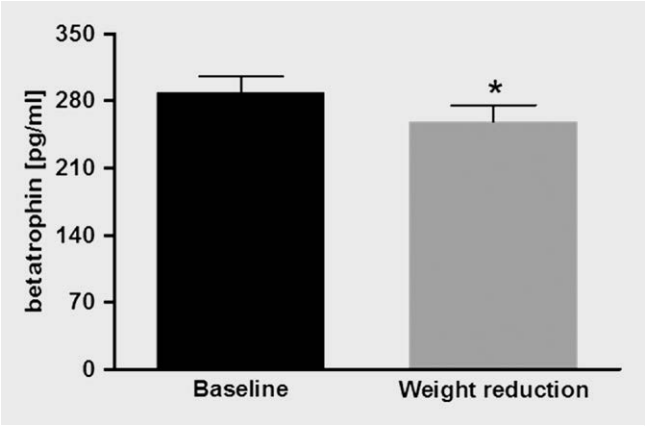
Characteristics	Low carb	Low fat	p-Value
n (% male)	27 (7)	30 (20)	–
Age (years)	44 ± 2	46 ± 2	0.55
Body mass index (kg/m²)	33.0 ± 1.0	33.1 ± 0.7	0.93
Waist circumference (cm)	100 ± 2	102 ± 2	0.47
IHL (%)	6.5 ± 1.4	9.8 ± 1.7	0.16
Alanine aminotransaminase (U/l)	21 ± 2	30 ± 4	0.06
C-reactive protein (mg/dl)	1.2 ± 0.2	1.6 ± 0.3	0.32
Triacylglycerols (mmol/l)	1.2 ± 0.1	1.2 ± 0.1	0.92
LDL cholesterol (mmol/l)	2.9 ± 0.1	3.2 ± 0.1	0.14
HDL cholesterol (mmol/l)	1.3 ± 0.1	1.3 ± 0.1	0.82
Blood glucose (mmol/l)	5.5 ± 0.1	5.4 ± 0.1	0.75
HOMA-IR	1.3 ± 0.2	1.8 ± 0.2	0.11
Circulating ANGPTL8/ betatrophin (pg/ml)	279 ± 23	297 ± 25	0.62

Data are given as means ± S.E. or absolute numbers, respectively

HDL: High-density lipoprotein; HOMA-IR: Homeostasis model of insulin resistance; IHL: Intrahepatic lipids; LDL: Low-density lipoprotein



▶ **Fig. 3** Associations of ANGPTL8/betatrophin with hepatic steatosis in the B-SMART cohort. Stratification according to IHL revealed that subjects with hepatic steatosis (20.1 ± 2.5 % IHL, n = 15) had elevated ANGPTL8/betatrophin compared to nonsteatotic (4.0 ± 0.4 % IHL, n = 42) subjects (p = 0.033). Data are shown as mean ± SEM; IHL, intrahepatic lipids; * p < 0.05.



▶ **Fig. 4** Association of ANGPTL8/betatrophin with weight loss in B-SMART. After a mean weight loss of 6.9 kg (= 7.4 % initial body weight) due to dietary intervention accompanied by a 45 % IHL reduction, ANGPTL8/betatrophin decreased by 11 % (p = 0.015). Data are shown as mean ± SEM; IHL:Intrahepatic lipids; * p < 0.05.

pared to SCAT, while previous studies showing lower ANGPTL8/betatrophin mRNA expression in AT did not separately focus on VAT and SCAT [1, 21]. This further contributes to explain contrasting results. Support for our findings comes from a recent study in humans, where the authors also detected ANGPTL8/betatrophin mRNA expression in human SCAT [27]. In accordance with the latter and earlier findings from others [1, 21, 27, 28] SCAT expression in our study was relatively low. Moreover, hypothesizing that previous studies mainly investigated ANGPTL8/betatrophin mRNA expression in SCAT, our data provide evidence for the first time that VAT represents another contributor to circulating ANGPTL8/betatrophin in humans. Taken together, the liver and AT could variably conribute to circulating ANGPTL8/betatrophin in humans, whereby age, nutrition and insulin levels could represent main regulating factors [22, 27].

Multiple lines of evidence link ANGPTL8/betatrophin to lipid metabolism [22]. Particularly, an important role of ANGPTL8/betatrophin in hepatic very low density lipoproteins (VLDL) production has been proposed [21, 22]. In these studies, Angptl8/betatrophin knockout mice showed reduced plasma triacylglycerol levels after refeeding, which was explained by reduced VLDL secretion in line with enhanced lipoprotein lipase activity [29]. Conversely, forced ANGPTL8/betatrophin expression produced hypertriacylglycerolemia [3]. In support of these experimental data we observed significant associations of ANGPTL8/betatrophin with circulating triacylglycerols in 2 independent human collectives. We further expand previous findings by suggesting a contrasting relationship with polyunsaturated vs. saturated and monounsaturated FA in plasma. This could be of interest since ANGPTL8/betatrophin is known to be nutritionally regulated [4, 21, 22] whereby our intervention supports the impact of dietary manipulation also in the long term. Whether, however, this effect is finally mediated by the observed reduction of liver lipids, a decreased intake in, that is, SFA with a consecutively modified circulating FA profile, a combination of these factors or other yet unknown influences cannot be answered with our current study design. Otherwise, our data evolve the hypothesis that different FA species could have varying impact on the regulation of ANGPTL8/betatrophin. Indeed, treatment with polyunsaturated FA represents an established therapeutic approach in reducing elevated plasma triacylglycerols [30]. A potential role of ANGPTL8/betatrophin in that regard remains, however, to be defined.

Insulin resistance is known to be related to NAFLD [31, 32]. In our study, circulating ANGPTL8/betatrophin correlated with insulin AUC, at least after adjustment for age. This correlation disappeared following 45 % liver lipid reduction with concomitant ANGPTL8/betatrophin decrease. Furthermore both, circulating and hepatic ANGPTL8/betatrophin correlated significantly with histological proven liver steatosis. Moreover, hepatic mRNA expression was related to HOMA-IR, while circulating levels correlated with hepatic mRNA expression after adjustment for HOMA-IR. Therefore, our findings suggest that hepatic lipid content could play a role to explain the previously observed association of ANGPTL8/betatrophin with variables of glucose metabolism [4–8]. In support of the fact that liver fat is accepted as the most important predictor of human insulin resistance [18], significantly elevated circulating ANGPTL8/betatrophin was recently reported in NAFLD patients, while the observed association was abolished after statistical adjustment for insulin resistance [33]. Thereby it is of major significance that dyslipidemia and particularly hypertriacylglycerolemia are acknowledged features of hepatic insulin resistance [20, 31]. Therefore, although we found a significant association of ANGPTL8/betatrophin with hepatic steatosis, we hypothesize that the lack of difference regarding circulating ANGPTL8/betatrophin in in our NAFLD vs. non-NAFLD subjects was rather explained by minor differences between groups in lipid metabolism than by liver steatosis per se.

Our study is limited by lack of mechanistic evidence and cannot prove causality. Nevertheless, our study showed a robust association between ANGPTL8/betatrophin and hepatic steatosis in 2 independent cohorts using in vivo and ex vivo gold standard methods for liver lipid quantification. While we investigated a limited number of human subjects, associations between ANGPTL8/betatrophin and NAFLD were evident in both cohorts.

In our study, circulating ANGPTL8/betatrophin was determined using the N-terminus recognizing ELISA and samples in both of our studies were obtained and stored under comparable standard conditions. Using the N-terminus recognizing ELISA, Fenzl et al. [10] vs. Hu et al. [5] observed about 4-fold higher ANGPTL8/betatrophin levels in healthy controls with comparable BMI. In our study, liver ANGPTL8/betatrophin mRNA correlated with the ELISA determined levels, providing evidence for the validity of the measurements. However, circulating ANGPTL8/betatrophin levels were about 4-fold higher in INSIGHT compared to B-SMART subjects. Otherwise, the detected differences are clearly in line with previous reports showing comparable ranges of variability [4, 5, 8, 10, 33]. However, with our current study design, we cannot fully explain the observed differences.

Our data suggest the liver and visceral adipose tissue as contributors to circulating ANGPTL8/betatrophin in humans. Hepatic lipid accumulation as critical determinant of dyslipidemia and insulin resistance is related to human ANGPTL8/betatrophin. Future studies need to account for hepatic lipid content and circulating fatty acid profile as variables to variation in ANGPTL8/betatrophin.

Study registration: Clinical-Trials.gov Identifier: NCT00956566; German Clinical Trials-Register: DRKS00005450.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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