

A higher glycemic response to oral glucose is associated with higher plasma apolipoprotein C3 independently of BMI in healthy twins

Lutgarda Bozzetto ^{a,*}, Bram J. Berntzen ^b, Jaakko Kaprio ^{c,d}, Aila Rissanen ^b,
Marja-Riitta Taskinen ^b, Kirsi H. Pietiläinen ^{b,e}

^a Department of Clinical Medicine and Surgery, Federico II University Naples, Italy

^b Obesity Research Unit, Research Program for Clinical and Molecular Metabolism, Faculty of Medicine, University of Helsinki, Helsinki, Finland

^c Department of Public Health, Finnish Twin Cohort Study, University of Helsinki, Helsinki, Finland

^d Institute for Molecular Medicine Finland, FIMM, University of Helsinki, Helsinki, Finland

^e Obesity Center, Endocrinology, Abdominal Center, Helsinki University Hospital and University of Helsinki, Helsinki, Finland

Received 16 May 2019; received in revised form 3 October 2019; accepted 4 October 2019

Handling Editor: L. Laviola

Available online ■ ■ ■

KEYWORDS

Apolipoprotein C3;
Glucose metabolism;
Obesity;
Plasma triglyceride;
Twins

Abstract *Background and aims:* Plasma apolipoprotein C3 (ApoC3) is associated with higher plasma triglyceride and type 2 diabetes incidence. We evaluated whether body mass index (BMI) or glucose metabolism were associated with ApoC3 in healthy monozygotic (MZ) twins. *Methods and Results:* Forty-seven MZ twin-pairs (20 men, 27 women), aged 23–42 years, were divided in subgroups according to discordance or concordance for (a) BMI (within-pair difference (Δ) in BMI ≥ 3.0 or < 3.0 kg/m²), or (b) 2-h glucose iAUC, during oral glucose tolerance test (Δ Glucose iAUC ≥ 97.5 or < 97.5 mmol \times 120 minutes). Within these discordant or concordant subgroups, we tested (Wilcoxon signed-rank test) co-twin differences in ApoC3, adiposity measures, insulin-resistance and beta-cell function indices, and plasma and lipoprotein lipids.

In BMI-Discordant ($p = 0.92$) or BMI-Concordant ($p = 0.99$) subgroups, ApoC3 did not differ between leaner and heavier co-twins. In the Glucose-Discordant subgroup, ApoC3 was significantly higher in twins with higher Glucose iAUC than in their co-twins with the lower Glucose iAUC (10.03 ± 0.78 vs. 8.48 ± 0.52 mg/dl; $M \pm SE$; $p = 0.032$). Co-twins with higher Glucose iAUC also had higher waist circumference, body fat percentage, liver fat content, worse insulin-sensitivity and beta-cell function and higher cholesterol and triglyceride in plasma VLDL, IDL, and LDL. In Glucose-Concordant twin-pairs, no significant differences were observed in the explored variables. In all twin-pairs, Δ ApoC3 correlated with Δ in lipids and glucose metabolism variables, the closest relationship being between Δ ApoC3 and Δ VLDL triglyceride ($r = 0.74$, $p < 0.0001$).

Conclusions: While ApoC3 was not related to acquired differences in BMI, it associated with early dysregulation of glucose metabolism independently of obesity and genetic background.

© 2019 Published by Elsevier B.V. on behalf of The Italian Society of Diabetology, the Italian Society for the Study of Atherosclerosis, the Italian Society of Human Nutrition, and the Department of Clinical Medicine and Surgery, Federico II University.

Abbreviations: ApoA1, Apolipoprotein A1; ApoB, Apolipoprotein B; ApoC3, Apolipoprotein C3; BCF, Beta Cell Function; BMI, Body Mass Index; ChREBP, Carbohydrate Response Element–Binding Protein; CVD, Cardiovascular Disease; FOXO1, Forkhead Box O1; HDL, High Density Lipoproteins; HIRI, Hepatic Insulin Resistance Index; HNF4a, Hepatic Nuclear Factor-4a; HOMA-IR, HOMEostatic Model Assessment; iAUC, Incremental Area Under the Curve; ICCs, Intraclass Correlation Coefficients; IDL, Intermediate Density Lipoproteins; ISIC, Insulin Sensitivity Index from C-peptide; ISIIIns, Insulin Sensitivity Index from Insulin; LDL, Low Density Lipoproteins; MZ, Monozygotic; ODI, Oral Disposition Index; OGTT, Oral Glucose Tolerance Test; T2D, Type 2 Diabetes; TRLs, Triglyceride Rich Lipoproteins; VLDL, Very Low Density Lipoproteins; TE, Total Energy.

* Corresponding author. Department of Clinical Medicine and Surgery, Federico II University, 80131, Naples, Italy.

E-mail address: lutgarda.bozzetto@unina.it (L. Bozzetto).

<https://doi.org/10.1016/j.numecd.2019.10.005>

0939-4753/© 2019 Published by Elsevier B.V. on behalf of The Italian Society of Diabetology, the Italian Society for the Study of Atherosclerosis, the Italian Society of Human Nutrition, and the Department of Clinical Medicine and Surgery, Federico II University.

Please cite this article as: Bozzetto L et al., A higher glycemic response to oral glucose is associated with higher plasma apolipoprotein C3 independently of BMI in healthy twins, Nutrition, Metabolism & Cardiovascular Diseases, <https://doi.org/10.1016/j.numecd.2019.10.005>

Introduction

Apolipoprotein C3 (ApoC3) is a key modulator of plasma triglyceride concentrations and a risk factor for cardiovascular disease (CVD) [1]. It is well established that ApoC3 is a main regulator of triglyceride rich lipoproteins (TRL) lipolysis. ApoC3 is an inhibitor of lipoprotein lipase activity [2] and it may impair hepatic TRLs remnant removal [3]. In a recent trial [4], the inhibition of ApoC3 synthesis by a nonsense oligonucleotide dramatically reduced plasma triglycerides in patients with type 2 diabetes (T2D). This study confirmed the pivotal role of ApoC3 in triglyceride metabolism, but, unexpectedly, also showed that ApoC3 inhibition improved insulin resistance and other markers of glycemic control.

In recent longitudinal studies, increased ApoC3 plasma levels were associated with a higher incidence of T2D independently of plasma triglyceride levels and other confounding factors [5–7].

These data would suggest that the regulation of ApoC3 expression may be a link in the crosstalk between glucose and lipid metabolism. Actually, *in vitro* and animal studies showed that ApoC3 stimulates pancreatic β -cell apoptosis [8], and induces islet insulin resistance [9]. Moreover, in rat and human hepatocytes, elevated glucose levels induce expression of ApoC3 via activation of carbohydrate response element-binding protein (ChREBP) and hepatic nuclear factor-4a (HNF4a) [10]. On the other hand, insulin downregulates ApoC3 expression in rat hepatocyte by promoting phosphorylation of the nuclear transcription factor Forkhead box O1 (FOXO1) [11]. Thus, glucose and insulin seem to have opposite actions on ApoC3 expression. However, the balance between glucose and insulin actions on ApoC3 may be more complex in insulin resistance and further studies in humans are needed.

Obesity is associated with disturbances of glucose and lipid metabolism related to hyperinsulinemia. It should be recognized that obesity, as well as its associated metabolic derangements, are highly heritable [12–16]. Monozygotic twins (MZ) represent a unique experimental setting to dissect possible factors influencing lipid metabolism independently of genetic background. Phenotypically discordant MZ twin pairs are of special interest as the two persons with extremes of the phenotype do not differ in their genomic sequence. Therefore, although the possibility remains of somatic mutations, hypervariable gene regions or differences in copy-number variations, the variability in the phenotype is essentially accounted for by environmental factors or gene–environment interaction.

The aim of our study was to evaluate the role of acquired obesity and associated glucose derangement in lipid metabolism with a specific focus on ApoC3 expression. We utilized a MZ twin pair design, excluding those with diabetes, as a method to exclude the impact of genetic background.

Methods

Subjects

This study consisted of 47 MZ twin pairs (20 male 27 female), aged 23–42 years, recruited from population-based FinnTwin16 and FinnTwin12 cohorts ($n = 5147$) [17]. All pairs were Europeans of Finnish ancestry. All subjects were healthy and did not take any regular medications. The study protocols were approved by the ethical committees of the Hospital District of Helsinki and Uusimaa, Finland. Written informed consent was obtained for all participants.

BMI discordant and concordant pairs

Within-pair differences (Δ) in BMI were calculated by subtracting the leaner cotwin's value from the heavier cotwin's value. Thirty twin pairs were defined as discordant for BMI as Δ BMI was ≥ 3 kg/m², while the remaining 17 pairs were concordant (Δ BMI < 3 kg/m²), as previously described [18].

Glucose iAUC discordant and concordant pairs

Δ Glucose iAUC was calculated by subtracting the value of twin with the lower Glucose iAUC from the value of the twin with the higher Glucose iAUC (see below the measurement of Glucose iAUC). Based on the median Δ Glucose iAUC cut-off, 24 twin pairs were defined as discordant (Δ Glucose iAUC ≥ 97.5 mmol \times 120 min) and 23 pairs were defined as concordant (Δ Glucose iAUC < 97.5 mmol \times 120 min) for 2-h glucose iAUC during OGTT.

Dietary habits

Habitual dietary intake was assessed from 3-day food records and analyzed by the Diet32 program (Aivo), based on a national Finnish database for food composition (Fineli, www.fineli.fi, National Institute for Health and Welfare, Nutrition Unit, Helsinki, Finland). Food records were available for 30 twin pairs.

Body composition

Body weight, height, whole-body fat (dual-energy X-ray absorptiometry), abdominal, subcutaneous, and intra-abdominal fat (magnetic resonance imaging) and liver fat (magnetic resonance spectroscopy) were measured as described previously [19]. The magnetic resonance imaging/magnetic resonance spectroscopy measurements were performed in a subsample of 34 twin pairs (24 BMI-Discordant and 10 BMI-Concordant pairs; 17 Glucose iAUC-Discordant and 17 Glucose iAUC-Concordant pairs).

Glucose and insulin during the OGTT

The 75-g OGTT was performed after a 12-h overnight fast with measurements of plasma glucose, serum insulin, and

serum C-peptide at 0, 30, 60, and 120 min. Plasma glucose, serum insulin and serum C-peptide were measured as previously described [20].

Indices of insulin resistance were: HOMA-IR [21]; Matsuda index [22]; Hepatic Insulin Resistance Index (HIRI) [23]; fasting beta-cell function: Beta Cell Function (BCF) [24]; early phase dynamic beta-cell function: Insulin Sensitivity Index from C-peptide (ISIC); Insulin Sensitivity Index from insulin (ISIIns); and insulin secretion adjusted for prevailing insulin resistance: the Oral Disposition Index (ODI) [25] was calculated (ESM) using C-peptide, glucose and insulin measurements from the OGTT measurements. The iAUC after the glucose load (Glucose iAUC) was calculated by the trapezoidal method.

Lipids and apolipoproteins

For lipid measurements, venous blood samples were drawn after a 12-h overnight fast. Serum and lipoproteins concentrations of cholesterol and triglycerides were determined using an automated Konelab 60i analyzer (Thermo Fisher Scientific Oy) by enzymatic methods (Refs. 981812 and 981301). Serum concentrations of apolipoprotein A1 (ApoA1) and apolipoprotein B (ApoB) were measured by immunoturbidometric methods (for ApoA1, Wako Chemicals GmbH and for ApoB, Orion Diagnostica, Espoo, Finland). Serum ApoC3 concentration was measured immunoturbidometrically (Kamiya Biomedical company, Seattle, WA).

Fasting serum lipoproteins (very low-density lipoproteins [VLDL], intermediate-density lipoproteins [IDL] and low density lipoproteins [LDL]) for measurement of detailed lipoprotein particle compositions were separated from fresh blood by sequential flotation ultracentrifugation using a modification of the method of Havel et al. [26].

High density lipoproteins (HDL) was separated and isolated by ultracentrifugation from 0.5 ml serum [27]. Mean HDL particle size was calculated by multiplying the mean size of each HDL subclass by its relative area under the densitometric scan [28].

Statistical analyses

Statistical analyses were performed with Stata/MP statistical software (release 15.0; Stata Corp., College Station, TX, USA). Results are expressed as mean \pm SE unless otherwise specified. Intraclass correlation coefficients (ICCs) were computed as a measure of familial influence on the trait. Comparisons between the co-twins in the BMI or Glucose iAUC groups were made by matched-pairs Wilcoxon signed-rank tests. Sex distributions between the groups were tested by χ^2 test. Pearson's correlations were calculated to examine the relation between Δ ApoC3 and Δ metabolic variables. In these analyses, the Δ was generated by subtracting the value of the twin with the lower Glucose iAUC from the value of the twin with the higher Glucose iAUC for each of the parameters.

Results

Intra-pair similarity in ApoC3, adiposity, and glucose and lipid metabolism and dietary habits in the whole cohort

Clinical characteristics and habitual dietary intake of the individuals in the whole cohort are shown in Table 1.

Intra-pair similarity was first determined by ICC in the whole cohort of all twins to assess familial and probably genetic control over ApoC3, adiposity measures and other metabolic variables (Table 2). ApoC3 levels had a moderate resemblance within the pairs (ICC = 0.46, 95% confidence interval (CI) 0.21 to 0.66, $p < 0.001$). ICC ranged from 0.49 to 0.63 (all $p < 0.001$) for BMI, waist circumference, subcutaneous fat and percent body fat, while there was no within-pair similarity in liver fat and intra-abdominal fat. For glucose metabolism, ICC analysis revealed moderate within-pair similarity in Glucose iAUC (0.52, 95% CI 0.28 to 0.70, $p < 0.001$), Insulin iAUC (0.42, 95% CI 0.15 to 0.64, $p = 0.008$), HOMA (0.36, $p = 0.005$), HIRI (0.34, $p = 0.009$) and BCF (0.57, $p < 0.001$), but not for Matsuda index (0.21, 95% CI -0.08 to 0.47) and ISIC (0.14, 95% CI -0.18 to 0.43). ICC ranged from 0.49 to 0.60 for plasma, LDL and HDL cholesterol (all $p < 0.001$). VLDL triglyceride and plasma triglyceride showed, respectively, very low or no similarity.

As for dietary intake, carbohydrate (percent dietary intake of total energy (%TE)) (0.68, CI 0.15 to 0.32 $p = 0.002$), protein (%TE) (0.52, CI 0.20 to 0.77 $p = 0.03$), total fat (%TE) (0.54, CI 0.07 to 0.78 $p = 0.02$), mono-unsaturated fat (%TE) (0.59, CI 0.14 to 0.81 $p = 0.01$), polyunsaturated n-3 fat (%TE) (0.69, CI 0.34 to 0.85 $p = 0.001$, and fibre (g/die) (0.69, CI 0.34 to 0.85 $p = 0.001$) showed moderate within-pair similarity, while no similarity was shown for total energy intake (kcal), total polyunsaturated fat and n-6 polyunsaturated fat (Table 1).

Intra-pair similarity for ApoC3 and other metabolic variables was similar in Glucose-Discordant or Glucose-Concordant pairs (Table 3). The only exceptions were indices of insulin sensitivity and beta-cell function showing a significant intra-pair similarity in Glucose-Discordant but not in Glucose-Concordant pairs (Table 3).

Intra-pair similarity for carbohydrate, total, saturated, monounsaturated, and polyunsaturated n-3 fat was significant in Glucose-Concordant but not in Glucose-Discordant pairs (ESM Table 2).

Intra-pair similarity for protein and n-3 polyunsaturated fat was significant in Glucose-Concordant but not in Glucose-Discordant pairs (ESM Table 2).

Effects of acquired obesity on glucose and lipid metabolism

Next, we analysed the effects of BMI on glucose and lipid metabolism in the BMI-Discordant group. In the BMI-Discordant pairs (Table 1), the heavier co-twins expectedly differed greatly from the leaner for body fat and its distribution. They weighed on average 18 kg more and had 28% more total fat, 130% more intra-abdominal fat, and 400% more liver

Table 1 Clinical characteristics and dietary habits of the individuals in the whole cohort of monozygotic twins and in BMI-Discordant pairs.

	Whole cohort (n = 94 individuals)	BMI-Discordant (Δ BMI ≥ 3 kg/m ²) (n = 30 pairs)	
		Leaner	Heavier
BMI (kg/m ²)	28.7 \pm 0.6 (19.7–44.7)	25.8 \pm 0.9	32.3 \pm 1.1***
Waist circumference (cm)	93.4 \pm 1.6 (65.2–137.3)	86.1 \pm 2.6	102 \pm 2.9***
Body fat (%)	35.1 \pm 1.0 (10.3–54.3)	31.9 \pm 1.8	41.0 \pm 1.4***
Subcutaneous fat (dm ³)	4.5 \pm 3.3 (1.1–1.5)	3.7 \pm 0.5	6.3 \pm 0.6***
Intra-abdominal fat (dm ³)	1.0 \pm 0.9 (0.1–3.1)	0.60 \pm 0.08	1.4 \pm 0.2***
Liver fat (%)	2.4 \pm 0.5 (0.1–22.4)	0.89 \pm 0.19	4.8 \pm 1.1**
ApoC3 (mg/dl)	9.1 \pm 0.3 (2.7–17.4)	8.9 \pm 0.5	9.4 \pm 0.6
ApoA1 (mg/dl)	138 \pm 2.8 (87–221)	143 \pm 4.9	134 \pm 4.5*
ApoB (mg/dl)	81 \pm 22 (33–171)	74 \pm 2.8	86 \pm 4.6*
Plasma Triglyceride (mmol/l)	1.3 \pm 0.1 (0.42–3.22)	0.94 \pm 0.06	1.2 \pm 1.0*
Plasma Cholesterol (mmol/l)	4.8 \pm 0.1 (2.79–7.27)	4.6 \pm 0.1	4.9 \pm 0.2*
VLDL-Cholesterol (mmol/l)	0.25 \pm 0.02 (0.01–1.24)	0.21 \pm 0.02	0.32 \pm 0.04*
LDL-Cholesterol (mmol/l)	3.0 \pm 0.1 (1.2–5.3)	2.8 \pm 0.1	3.1 \pm 0.1*
HDL-Cholesterol (mmol/l)	1.4 \pm 0.04 (0.6–3.1)	1.5 \pm 0.08	1.3 \pm 0.07***
VLDL-Triglyceride (mmol/l)	0.6 \pm 0.04 (0.1–2.6)	0.5 \pm 0.05	0.8 \pm 0.09*
HbA1c (%)	5.3 \pm 0.03 (4.6–6.2)	5.2 \pm 0.06	5.3 \pm 0.06**
HIRI	30.2 \pm 0.1 (12–59)	28.5 \pm 1.3	32.4 \pm 1.7*
Matsuda	7.9 \pm 0.5 (0.73–22)	9.0 \pm 0.9	6.1 \pm 0.7*
HOMA-IR	1.6 \pm 0.1 (0.3–6.3)	1.2 \pm 0.2	1.9 \pm 0.2**
Energy (kcal/day) ^a	2115 \pm 72 (963–3380)	2044 \pm 114	2206 \pm 130
Carbohydrate (%TE) ^a	42.5 \pm 1.0 (29–68)	43.4 \pm 0.7	41.7 \pm 0.6
Protein (%TE) ^a	17.5 \pm 0.6 (7–35)	16.5 \pm 1.5	16.6 \pm 1.5
Fat (%TE) ^a	34.6 \pm 0.9 (20–53)	34.9 \pm 0.7	35.9 \pm 0.6
Saturated (%TE) ^a	13.4 \pm 0.4 (6–22)	14.4 \pm 0.6	13.2 \pm 0.6
Monounsaturated (%TE) ^a	10.8 \pm 0.3 (6–17)	10.5 \pm 0.3	11.4 \pm 0.3
Polyunsaturated (%TE) ^a	4.7 \pm 0.2 (1.7–8)	4.5 \pm 0.1	5.2 \pm 0.1*
n-3 (%TE) ^a	1.0 \pm 0.07 (0.26–2.7)	1.0 \pm 0.5	1.1 \pm 0.6
n-6 (%TE) ^a	3.8 \pm 0.2 (1.5–6.3)	3.6 \pm 0.2	4.1 \pm 0.2
Fibre (g/die) ^a	18.0 \pm 1.2 (2.4–58)	18.1 \pm 2.1	17.6 \pm 1.4

Data are mean \pm SE (range) *P < 0.05, **P < 0.01, ***P < 0.001 vs. Leaner by matched-pairs Wilcoxon rank sum tests; Δ = heavier *minus* leaner BMI; VLDL: Very Low Density Lipoprotein; LDL: Low Density Lipoprotein; HDL: High Density Lipoprotein; HOMA-IR Homeostatic model assessment Insulin Resistance; HIRI: Hepatic Insulin Resistance Index; TE Total Energy.

^a Data available for 30 twin pairs including 21 BMI-Discordant pairs.

fat than their leaner counterparts. In addition, heavier twins were more insulin resistant, as indicated by higher values of HOMA-IR and lower Matsuda indices, had slightly higher levels of HbA1c and glucose and insulin responses during OGTT than leaner twins (ESM Fig. 1). As for lipid metabolism, heavier twins had significantly higher VLDL and LDL cholesterol, plasma and VLDL triglycerides and ApoB and lower HDL concentrations than their leaner co-twins. Notably, ApoC3 concentrations were comparable between leaner and heavier co-twins, despite robust differences in the body composition and triglyceride levels (Table 1).

Co-twins in BMI-Concordant pairs did not differ for any of the metabolic (glucose- or lipid-related) variables (data not shown).

Relationship between dysregulation of glucose metabolism and features of lipid metabolism

Next, we analyzed the effects of acquired differences in 2 h glucose responses on lipid metabolism in the Glucose-Discordant and Concordant groups (Table 4). Twin pairs discordant or concordant for Glucose iAUC were similar for age (32.9 \pm 1.2 vs. 32.5 \pm 1.2 years; p = 0.84) and sex distribution (11M/13F vs. 9M/14 F; p = 0.64).

Among Glucose-Discordant pairs 8 were BMI-Concordant and 16 were BMI-Discordant.

In the Glucose-Discordant pairs, twins with higher glucose response did not have higher BMI, intra-abdominal or subcutaneous fat. However, they had higher liver fat content and waist circumference. Twins with higher glucose response also had significantly worse whole body insulin sensitivity than their co-twins as indicated by higher HOMA and lower Matsuda index values, as well as worse hepatic insulin sensitivity, as indicated by higher values of HIRI and BCF. Twins with higher glucose response showed signs of impaired beta-cell function as indicated by lower values of ISIC (Table 4) and higher blood glucose levels 30 and 60 min after glucose load (ESM Fig. 2).

Interestingly, co-twins with higher glucose response had significantly higher ApoC3 and ApoB levels (Table 5). They also had significantly higher triglyceride and cholesterol concentrations in plasma, VLDL, IDL and LDL lipoproteins (Table 5).

Dietary pattern did not differ between co-twins in the Glucose-Discordant group (ESM Table 3).

In the Glucose-Concordant pairs, co-twins with higher or lower Glucose iAUC did not differ for any adiposity, glucose

Table 2 Intraclass correlation (ICC) and 95% interval confidence (IC) in whole cohort of 47 monozygotic twin pairs for ApoC3, main measures of glucose and lipid metabolism, and body fat distribution.

	ICC	95% IC	p
ApoC3 (mg/dl)	0.46	0.21–0.66	<0.001
Triglyceride (mmol/l)	0.11	–0.18–0.38	0.233
VLDL-TG (mmol/l)	0.25	–0.03–0.50	0.040
LDL-C (mmol/l)	0.58	0.35–0.74	<0.001
HDL-C (mmol/l)	0.57	0.35–0.74	<0.001
BMI (kg/m ²)	0.51	0.27–0.70	<0.001
Waist circumference (cm)	0.51	0.26–0.70	<0.001
Body fat (%)	0.63	0.43–0.78	<0.001
Subcutaneous fat (dm ³)	0.45	0.18–0.72	<0.001
Intra-abdominal fat (dm ³)	0.20	–0.16–0.51	0.135
Glucose iAUC (mmol/lx120min)	0.52	0.27–0.70	<0.001
Insulin iAUC (μU/mlx120min)	0.42	0.15–0.64	0.008
Liver fat (%)	–0.67	–0.39–0.28	0.648
HOMA-IR	0.36	0.08–0.58	<0.001
Matsuda	0.21	–0.08–0.47	0.077
BCF	0.58	0.32–0.75	<0.001
ISlc	0.14	–0.18–0.43	0.228
HIRI	0.34	0.06–0.58	0.009

VLDL: Very Low Density Lipoprotein; LDL: Low Density Lipoprotein; HDL: High Density Lipoprotein; HOMA-IR Homeostatic model assessment Insulin Resistance; BCF: Beta Cell Function; ISlc: InSulinogenic Index, C-peptide; HIRI: Hepatic Insulin Resistance Index.

and lipid metabolism and dietary variables (Table 5, ESM Table3).

Correlations between ApoC3 and other parameters of glucose and lipid metabolism

To examine the relationship between ApoC3 and glucose and lipid metabolism, we focused on correlations between

the Δs of ApoC3 and glucose and lipid metabolism across the whole cohort (n = 47 pairs) (Fig. 1).

Interestingly, within-pair ΔApoC3 concentrations were positively correlated with within-pair Δglucose (r = 0.30, p = 0.038), and Δinsulin (r = 0.41, p = 0.005) iAUC, and ΔHIRI (r = 0.34, p = 0.020), while they were inversely associated with within-pair differences in Matsuda index (r = –0.40, p = 0.001). There was a borderline relationship also between within-pair difference in ApoC3 and liver fat content (r = 0.34, p = 0.05). As expected, ΔApoC3 was strongly and positively related to ΔVLDL (r = 0.74, p < 0.001) and ΔLDL triglyceride (r = 0.48, p = 0.004).

Discussion

We report here that in a cohort of young and healthy monozygotic twins, within-pair discordance of obesity parameters or glucose response is associated with impaired insulin resistance and robust atherogenic changes in lipid parameters. Interestingly, plasma lipids were associated with higher ApoC3 levels in face of no substantial differences in BMI and subcutaneous/intra-abdominal fat depots. However, percent body fat, waist circumference and liver fat were higher in twins with higher OGTT glucose response. Remarkably, the higher levels of ApoC3 were apparent already in these non-diabetic “healthy twins” at a very early phase of glycaemic derangement.

Our data also showed that ApoC3 levels are moderately similar in MZ twins, even in pairs selected for discordance in BMI. This suggests that familial, probably genetic background is a major determinant of circulating ApoC3. Therefore, the observed within-pair differences in ApoC3

Table 3 Intraclass correlation (ICC) and 95% Interval Confidence (IC) for ApoC3, main measures of glucose and lipid metabolism and body fat distribution by concordance and discordance for glucose iAUC during OGTT.

	Glucose–Discordant Pairs (Δ Glucose iAUC ≥97.5 mmol/lx120min) (n = 24)			Glucose–Concordant Pairs (Δ Glucose iAUC <97.5 mmol/lx120min) (n = 23)		
	ICC	95% IC	p	ICC	95% IC	p
ApoC3 (mg/dl)	0.49	0.11–0.74	0.007	0.48	0.09–0.74	0.009
Triglyceride (mmol/l)	0.15	–0.26–0.55	0.234	0.16	–0.26–0.53	0.227
VLDL-TG (mmol/l)	0.35	–0.05–0.66	0.043	0.17	–0.25–0.54	0.208
LDL-C (mmol/l)	0.63	0.31–0.82	<0.001	0.55	0.19–0.78	0.003
HDL-C (mmol/l)	0.55	0.20–0.78	0.002	0.56	0.20–0.78	0.002
BMI (kg/m ²)	0.57	0.22–0.79	0.002	0.51	0.13–0.76	0.005
Waist circumference (cm)	0.53	0.17–0.76	0.003	0.56	0.19–0.79	0.003
Body fat (%)	0.73	0.46–0.87	<0.001	0.57	0.21–0.79	0.002
Subcutaneous fat (dm ³)	0.51	0.02–0.80	0.021	0.45	–0.04–0.77	0.034
Intra-abdominal fat (dm ³)	0.10	–0.42–0.57	0.357	0.34	–0.17–0.71	0.089
Liver fat (%)	0.03	–0.47– 0.47	0.495	–0.39	–0.51– 0.45	0.559
Glucose iAUC (mmol/lx120min)	0.89	0.77–0.95	<0.001	0.97	0.93–0.99	<0.001
Insulin iAUC (μU/mlx120min)	0.42	0.02–0.70	0.021	0.55	0.17–0.79	0.004
HOMA-IR	0.52	0.16–0.76	0.004	0.19	–0.24–0.56	0.195
Matsuda	0.50	0.12–0.75	0.006	0.06	–0.36– 0.46	0.398
BCF	0.65	0.31–0.85	0.001	0.07	–0.37–0.50	0.377
ISlc	0.16	–0.31–0.56	0.254	0.20	–0.26 – 0.58	0.194
HIRI	0.33	–0.08–0.65	0.056	0.53	0.15–0.78	0.005

VLDL: Very Low Density Lipoprotein; LDL: Low Density Lipoprotein; HDL: High Density Lipoprotein; HOMA-IR Homeostatic model assessment Insulin Resistance; BCF: Beta Cell Function; ISlc: InSulinogenic Index, C-peptide; HIRI: Hepatic Insulin Resistance Index.

Table 4 Glucose metabolism and adiposity measures of monozygotic twins pairs by concordance and discordance for glucose iAUC during OGTT.

	Glucose–Discordant Pairs (Δ Glucose iAUC \geq 97.5 mmol/lx120min) (n = 24)		Glucose–Concordant Pairs (Δ Glucose iAUC <97.5 mmol/lx120min) (n = 23)	
	Twin with Lower Glucose iAUC	Twin with Higher Glucose iAUC	Twin with Lower Glucose iAUC	Twin with Higher Glucose iAUC
Glucose iAUC (mmol/lx120min)	122 \pm 23	302 \pm 24***	153 \pm 21	199 \pm 21***
BMI (kg/m ²)	28.6 \pm 1.1	30.0 \pm 1.1	29.2 \pm 1.2	28.0 \pm 1.0
Waist Circumference (cm)	92.1 \pm 3.2	98.1 \pm 3.3*	92.7 \pm 2.9	90.1 \pm 2.7
Body fat (%)	33.2 \pm 2.1	36.5 \pm 2.0*	35.3 \pm 2.1	35.3 \pm 1.9
Subcutaneous fat (dm ³)	4.3 \pm 0.9	4.6 \pm 0.6	4.6 \pm 0.7	4.4 \pm 0.5
Intra-abdominal fat (dm ³)	0.09 \pm 0.02	1.2 \pm 0.2	0.9 \pm 0.01	1.1 \pm 0.2
Liver fat (%)	1.3 \pm 0.4	4.7 \pm 1.5*	1.6 \pm 0.5	2.1 \pm 0.7
HbA1c (%)	5.2 \pm 0.05	5.3 \pm 0.05	5.26 \pm 0.07	5.23 \pm 0.08
HOMA-IR	1.4 \pm 0.2	2.1 \pm 0.3**	1.5 \pm 0.2	1.40 \pm 0.2
Matsuda	9.4 \pm 1.1	5.9 \pm 0.8***	8.1 \pm 0.9	8.3 \pm 0.9
BCF	0.10 \pm 0.01	0.14 \pm 0.01***	0.13 \pm 0.02	0.10 \pm 0.01
ISIC	1.6 \pm 0.7	0.55 \pm 0.08**	0.73 \pm 0.09	0.70 \pm 0.11
ISIIIns	262 \pm 89	124 \pm 16	172 \pm 26	126 \pm 16
ODI	275 \pm 108	89 \pm 27***	121 \pm 13	131 \pm 26
HIRI	29 \pm 1.7	34 \pm 2.3*	30 \pm 1.9	28 \pm 1.4

Data are mean \pm SE *P < 0.05, **P < 0.01, ***P < 0.001 vs. Twin with Lower Glucose_iAUC; Δ = Twin with Higher Glucose_iAUC minus Twin with Lower Glucose_iAUC. HOMA-IR Homeostatic model assessment Insulin Resistance; BCF: Beta Cell Function; ISIC: InSulinogenic Index, C-peptide; ISIIIns: InSulinogenic Index, Insulin; ODI: Oral Disposition Index; HIRI: Hepatic Insulin Resistance Index.

Table 5 Lipid metabolism in monozygotic twins pairs by concordance and discordance for glucose iAUC during OGTT.

	Glucose–Discordant Pairs (Δ Glucose iAUC \geq 97.5 mmol/lx120min) (n = 24)		Glucose–Concordant Pairs (Δ Glucose iAUC <97.5 mmol/lx120min) (n = 23)	
	Twin with Lower Glucose iAUC	Twin with Higher Glucose iAUC	Twin with Lower Glucose iAUC	Twin with Higher Glucose iAUC
Triglyceride (mmol/l)	0.94 \pm 0.08	1.3 \pm 0.1**	0.98 \pm 0.06	1.0 \pm 0.09
Total Cholesterol (mmol/l)	4.5 \pm 0.1	4.9 \pm 0.2**	4.8 \pm 0.1	4.9 \pm 0.2
VLDL-Cholesterol (mmol/l)	0.24 \pm 0.03	0.35 \pm 0.06*	0.23 \pm 0.02	0.24 \pm 0.03
LDL-Cholesterol (mmol/l)	2.8 \pm 0.1	3.2 \pm 0.1**	3.0 \pm 0.1	3.1 \pm 0.2
IDL-Cholesterol (mmol/l)	0.10 \pm 0.01	0.15 \pm 0.02***	0.11 \pm 0.008	0.13 \pm 0.013
HDL-Cholesterol (mmol/l)	1.3 \pm 0.06	1.3 \pm 0.06	1.5 \pm 0.1	1.4 \pm 0.1
VLDL-Triglyceride (mmol/l)	0.56 \pm 0.07	0.85 \pm 0.13*	0.57 \pm 0.05	0.58 \pm 0.08
LDL-Triglyceride (mmol/l)	0.18 \pm 0.01	0.22 \pm 0.01***	0.20 \pm 0.01	0.21 \pm 0.02
IDL-Triglyceride (mmol/l)	0.077 \pm 0.004	0.092 \pm 0.006**	0.08 \pm 0.005	0.09 \pm 0.007
HDL-Triglyceride (mmol/l)	0.12 \pm 0.01	0.13 \pm 0.01	0.13 \pm 0.01	0.14 \pm 0.02
HDL size (nm)	9.3 \pm 0.09	9.4 \pm 0.07	9.4 \pm 0.1	9.5 \pm 0.08
ApoA1 (mg/dl)	134 \pm 3.6	136 \pm 4.2	141 \pm 6.8	142 \pm 6.7
ApoB (mg/dl)	79 \pm 3.2	88 \pm 5.1*	77 \pm 3	83 \pm 5
ApoC3 (mg/dl)	8.5 \pm 0.5	10.0 \pm 0.8*	9.5 \pm 0.7	9.1 \pm 0.7

Data are mean \pm SE *P < 0.05, **P < 0.01, ***P < 0.001. Δ = Twin with Higher Glucose_iAUC minus Twin with Lower Glucose_iAUC; VLDL: Very Low Density Lipoprotein; IDL: Intermediate Density Lipoprotein, LDL: Low Density Lipoprotein; HDL: High Density Lipoprotein; ApoA1: Apolipoprotein A1; ApoB: Apolipoprotein B; ApoC3: Apolipoprotein C3.

are probably associated with acquired factors that, independently of obesity, genetic background and shared environmental factors, also interfere with glucose homeostasis. Our report is in line with previous studies in Japanese MZ twins showing an ICC for ApoC3 of 0.597 in men and 0.477 in women [29,30]. In addition, APOC3 polymorphisms have been associated with plasma ApoC3, triglyceride metabolism and related cardiovascular risk [31–33].

The higher ApoC3 plasma levels that we observed in co-twins with higher glucose response to OGTT may be

related to a direct effect of hyperglycemia on ApoC3 secretion. *In vitro* evidence shows that glucose induces ApoC3 transcription in the hepatocytes through the activation of Hepatocyte Nuclear Factor 4 Alpha and Carbohydrate-response element-binding protein (ChREBP) [10]. Interestingly, through the same ChREBP pathway, plasma glucose activates *de novo* lipogenesis (DNL) and promotes hepatic triglyceride synthesis [34]. This suggests that ApoC3 transcription is activated in parallel with lipogenic pathways induced by glucose overload. Increased DNL may shift the balance of lipid metabolism favoring

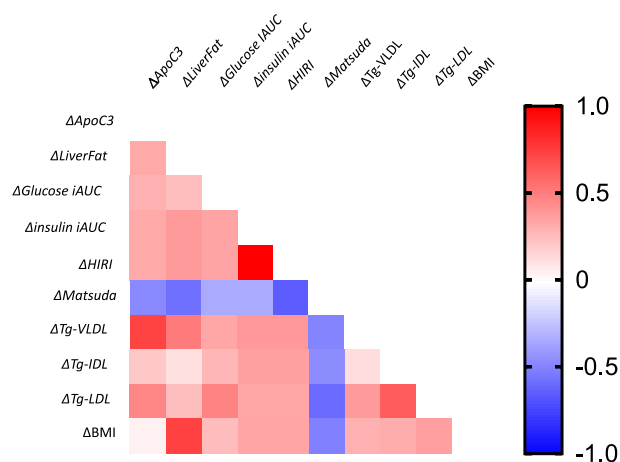


Figure 1 Correlations between within-pair differences (Δ) in serum ApoC3 and plasma glucose and lipid metabolism variables in the whole cohort ($n = 47$ pairs) according to glucose tolerance status. Δ was generated by subtracting the value of the twin with the higher Glucose iAUC from the value of the twin with the lower Glucose iAUC for each of the parameters. Color of the cell denotes the strength and direction of correlations (blue, negative; red, positive) and asterisks mark their significance (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$).

storage of lipids in hepatocytes. If ApoC3 regulates the flux of remnants of triglyceride rich lipoprotein by inhibiting their hepatic reuptake this would aggravate the elevation of circulating triglyceride rich lipoproteins [1,3]. This is in line with our finding that within-pair difference in ApoC3 is strongly correlated with corresponding difference in triglyceride concentrations in VLDL.

Another possibility is that ApoC3 may influence β -cell function, as suggested by emerging evidence from animal and some human studies. In rats, an increase in islet ApoC3 concentrations promoted a local inflammatory milieu, with consequent beta-cell failure [9]. In humans, ApoC3 has been related to a higher susceptibility to type1 diabetes [35]. *In vitro* studies also show that insulin inhibits ApoC3 hepatic transcription [11]. However, so far no evidence shows that low insulin concentrations associate with low ApoC3 levels. It should be recognized that glucose and insulin levels usually increase in parallel in presence of insulin resistance. In our study, co-twins with higher plasma ApoC3 levels also had a significantly increased insulin response to oral glucose as a consequence of lower insulin sensitivity. If insulin-mediated suppression of ApoC3 is impaired and coupled with concomitant stimulation by glycemia, this will enhance ApoC3 expression leading to elevation of triglyceride levels.

In our study, co-twins with increased glycemic response to OGTT also showed higher HOMA-IR and lower Matsuda indices, indicating whole body insulin resistance, as well as higher indexes of hepatic insulin resistance and beta cell function, indicating hepatic and islet insulin resistance. This suggests that, in our glucose “normo-tolerant” young people, both insulin resistance and impaired insulin secretion may contribute to glucose response during OGTT. The differences in peripheral insulin resistance could be related to the observed differences in body fat composition and

distribution. These, in turn, could induce a higher free fatty acids flux to the liver and stimulate triglyceride synthesis and excess fat deposition in hepatocytes. In our study, the higher hepatic insulin resistance observed in twins with high ApoC3 levels was correlated with more hepatic fat content and VLDL production.

In summary, we speculate that based on our analysis of MZ discordant pairs, environmental factors contribute to hepatic insulin resistance and/or liver fat accumulation and visceral obesity deteriorating glycemic homeostasis. Our data suggest that even minor increases of glucose concentrations are associated with increases of ApoC3 plasma levels. Importantly, this action seems to be independent of obesity and genetic and shared environmental background.

Funding

LB has been supported by Federico II University, BB by the Yrjö Jahnsson Foundation, Juho Vainio Foundation and Orion Research Foundation, JK by the Academy of Finland (grants 308248 and 312073), MRT by the Sigrid Jusélius Foundation, KHP by the Academy of Finland Centre of Excellence in Research on Mitochondria, Metabolism and Disease (grant number: 272376), Academy of Finland research grants (314383, 266286), Finnish Medical Foundation, Finnish Diabetes Research Foundation, Gyllenberg Foundation, Finnish Foundation for Cardiovascular Research, Novo Nordisk Foundation, University of Helsinki, Helsinki University Hospital, Government Research Funds, and Sigrid Jusélius Foundation.

Contribution statement

LB conceived and designed the study, wrote the manuscript, collected and analyzed data. BB analyzed data. MRT and KP conceived and designed the study, wrote the manuscript. JK is responsible for the base cohorts from which the pairs were invited to participate. All authors contributed important intellectual content through data interpretation, article drafting, and/or critical revision of the manuscript and all approved its final version for publication. KP is the guarantor of this work and as such, had full access to all data in the study and takes responsibility for the integrity of the data and accuracy of the data analysis.

Declaration of Competing Interest

The authors declare that they have no conflict of interest associated with this manuscript.

Acknowledgements

The authors are grateful to Helina Perttunen and Hannele Hilden, Virve Näätti for technical laboratory assistance, Nina Lundbom, Jesper Lundbom, Antti Hakkarainen for MRI imaging.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.numecd.2019.10.005>.

References

- [1] Taskinen MR, Borén J. Why is apolipoprotein CIII emerging as a novel therapeutic target to reduce the burden of cardiovascular disease? *Curr Atheroscler Rep* 2016;18:59.
- [2] Ebara T, Ramakrishnan R, Steiner G, Shachter NS. Chylomicronemia due to apolipoprotein CIII overexpression in apolipoprotein E-null mice. Apolipoprotein CIII-induced hypertriglyceridemia is not mediated by effects on apolipoprotein E. *J Clin Invest* 1997;99:2672–81.
- [3] Borén J, Watts GF, Adiels M, Söderlund S, Chan DC, Hakkarainen A, et al. Kinetic and related determinants of plasma triglyceride concentration in abdominal obesity: multicenter tracer kinetic study. *Arterioscler Thromb Vasc Biol* 2015;35:2218–24.
- [4] Digenio A, Dunbar RL, Alexander VJ, Hompesch M, Morrow L, Lee RG, et al. Antisense-mediated lowering of plasma apolipoprotein C-III by volanesorsen improves dyslipidemia and insulin sensitivity in type 2 diabetes. *Diabetes Care* 2016;39:1408–15.
- [5] Brahimaj A, Ligthart S, Ikkram MA, Hofman A, Franco OH, Sijbrands EJ, et al. Serum levels of apolipoproteins and incident type 2 diabetes: a prospective cohort study. *Diabetes Care* 2017;40:346–51.
- [6] van Hoek M, van Herpt TW, Dehghan A, Hofman A, Lievever AG, van Duijn CM, et al. Association of an APOC3 promoter variant with type 2 diabetes risk and need for insulin treatment in lean persons. *Diabetologia* 2011;54:1360–7.
- [7] Aroner SA, Yang M, Li J, Furtado JD, Sacks FM, Tjønneland A, et al. C-III and high-density lipoprotein subspecies defined by apolipoprotein C-III in relation to diabetes risk. *Am J Epidemiol* 2017;186:736–44.
- [8] Sol EM, Sundsten T, Bergsten P. Role of MAPK in apolipoprotein CIII-induced apoptosis in INS-1E cells. *Lipids Health Dis* 2009;8:3.
- [9] Ávall K, Ali Y, Leibiger IB, Leibiger B, Moede T, Paschen M, et al. Apolipoprotein CIII links islet insulin resistance to β -cell failure in diabetes. *Proc Natl Acad Sci U S A* 2015;112:E2611–9.
- [10] Caron S, Verrijken A, Mertens I, Samanez CH, Mautino G, Haas JT, et al. Transcriptional activation of apolipoprotein CIII expression by glucose may contribute to diabetic dyslipidemia. *Arterioscler Thromb Vasc Biol* 2011;31:513–51.
- [11] Altomonte J, Cong L, Harbaran S, Richter A, Xu J, Meseck M, et al. Foxo1 mediates insulin action on apoC-III and triglyceride metabolism. *J Clin Invest* 2004;114:1493–503.
- [12] Silventoinen K, Jelenkovic A, Sund R, Yokoyama Y, Hur YM, Cozen W, et al. Differences in genetic and environmental variation in adult BMI by sex, age, time period, and region: an individual-based pooled analysis of 40 twin cohorts. *Am J Clin Nutr* 2017;106:457–66.
- [13] Silventoinen K, Jelenkovic A, Sund R, Hur YM, Yokoyama Y, Honda C, et al. Genetic and environmental effects on body mass index from infancy to the onset of adulthood: an individual-based pooled analysis of 45 twin cohorts participating in the Collaborative project of Development of Anthropometrical measures in Twins (CODATwins) study. *Am J Clin Nutr* 2016;104:371–9.
- [14] Locke AE, Kahali B, Berndt SI, Justice AE, Pers TH, Day FR, et al. Genetic studies of body mass index yield new insights for obesity biology. *Nature* 2015;518:197–206.
- [15] Teslovich TM, Musunuru K, Smith AV, Edmondson AC, Stylianou IM, Koseki M, et al. Biological, clinical and population relevance of 95 loci for blood lipids. *Nature* 2010;466:707–13.
- [16] Mahajan A, Wessel J, Willems SM, Zhao W, Robertson NR, Chu AY, et al. Refining the accuracy of validated target identification through coding variant fine-mapping in type 2 diabetes. *Nat Genet* 2018 Apr;50(4):559–71.
- [17] Kaprio J. The Finnish twin cohort study: an update. *Twin Res Hum Genet* 2013;16:157–62.
- [18] Naukkarinen J, Rissanen A, Kaprio J, Pietiläinen KH. Causes and consequences of obesity: the contribution of recent twin studies. *Int J Obes (Lond)* 2012;36:1017–24.
- [19] Lundbom J, Hakkarainen A, Söderlund S, Westerbacka J, Lundbom N, Taskinen MR. Long-TE ¹H MRS suggests that liver fat is more saturated than subcutaneous and visceral fat. *NMR Biomed* 2010;24:238–45.
- [20] Granér M, Seppälä-Lindroos A, Rissanen A, Hakkarainen A, Lundbom N, Kaprio J. Epicardial fat, cardiac dimensions, and low-grade inflammation in young adult monozygotic twins discordant for obesity. *Am J Cardiol* 2012;109:1295–302.
- [21] Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985;28:412–9.
- [22] Matsuda M, DeFronzo RA. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. *Diabetes Care* 1999;22:1462–70.
- [23] Abdul-Ghani MA, Matsuda M, Balas B, DeFronzo RA. Muscle and liver insulin resistance indexes derived from the oral glucose tolerance test. *Diabetes Care* 2007;30:89–94.
- [24] Van Cauter E, Mestrez F, Sturis J, Polonsky KS. Estimation of insulin secretion rates from C-peptide levels. Comparison of individual and standard kinetic parameters for C-peptide clearance. *Diabetes* 1992;41:368–77.
- [25] Retnakaran R, Qi Y, Goran MI, Hamilton JK. Evaluation of proposed oral disposition index measures in relation to the actual disposition index. *Diabet Med* 2009;26:1198–203.
- [26] Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest* 1955;34:1345–53.
- [27] Blanche PJ, Gong EL, Forte TM, Nichols AV. Characterization of human high-density lipoproteins by gradient gel electrophoresis. *Biochim Biophys Acta* 1981;665:408–19.
- [28] Pérusse M, Pascot A, Després JP, Couillard C, Lamarche B. A new method for HDL particle sizing by polyacrylamide gradient gel electrophoresis using whole plasma. *J Lipid Res* 2001;42:1331–4.
- [29] Cai YP, Hayakawa K, Nishihara R, Kato K. Heritability of serum apolipoprotein concentrations in middle-aged Japanese twins. *J Epidemiol* 2009;19:260–5.
- [30] Hayakawa K, Shimizu T, Ohba Y, Tomioka S. Lifestyle factors affecting intrapair differences of serum apoproteins and cholesterol concentrations in adult identical twins. *Atherosclerosis* 1987;66:1–9.
- [31] Khetarpal SA, Zeng X, Millar JS, Vitali C, Somasundara AVH, Zanoni P, et al. A human APOC3 missense variant and monoclonal antibody accelerate apoC-III clearance and lower triglyceride-rich lipoprotein levels. *Nat Med* 2017;23:1086–94.
- [32] Jørgensen AB, Frikke-Schmidt R, Nordestgaard BG, Tybjaerg-Hansen A. Loss-of-function mutations in APOC3 and risk of ischemic vascular disease. *N Engl J Med* 2014;371:32–41.
- [33] TG and HDL Working Group of the Exome Sequencing Project, National Heart, Lung, and Blood Institute, Crosby J, Peloso GM, Auer PL, Crosslin DR, et al. Loss-of-function mutations in APOC3, triglycerides, and coronary disease. *N Engl J Med* 2014 Jul 3;371(1):22–31.
- [34] Yao Z. Human apolipoprotein C-III - a new intrahepatic protein factor promoting assembly and secretion of very low density lipoproteins. *Cardiovasc Haematol Disord - Drug Targets* 2012;12:133–40.
- [35] Hokanson JE, Kinney GL, Cheng S, Erlich HA, Kretowski A, Rewers M. Susceptibility to type 1 diabetes is associated with ApoCIII gene haplotypes. *Diabetes* 2006;55:834–8.