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**Five-year follow-up of waist circumference, insulin and ALT levels in familial combined hyperlipidemia**

Short title: ALT and lipid phenotype in FCHL

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**Keywords:** hypertriglyceridemia, lipids, lipoproteins, liver, steatosis

## **Abstract**

**Introduction:** Familial combined hyperlipidemia (FCHL), an entity with many features of the metabolic syndrome, is characterized by changes in cholesterol and triglyceride phenotype over time. This study was conducted to investigate the relation of alanine aminotransferase (ALT) levels, used as a surrogate for the amount of hepatic fat, with the switch in triglyceride phenotype and the increased susceptibility to develop hypertriglyceridemia in FCHL.

**Methods:** Body mass index, waist circumference, plasma triglycerides, insulin and ALT levels were measured in 145 FCHL family members and 54 spouses at baseline and after a five-year follow-up.

**Results:** A switch from normotriglyceridemia to hypertriglyceridemia or vice versa, as observed in 22 of 145 FCHL family members, was associated with changes in plasma ALT levels ( $p=0.001$ ), but not with insulin levels or waist circumference. In five-year follow-up, an intra-individual relation was observed between waist circumference and both plasma triglycerides, insulin and ALT levels. For each waist circumference FCHL patients, but not their normolipidemic relatives, exhibited higher triglyceride and insulin levels than spouses ( $p<0.001$ ). Remarkably, both FCHL patients and the normolipidemic relatives showed higher ALT levels for each waist circumference as compared to spouses ( $p<0.001$  for FCHL patients,  $p=0.035$  for normolipidemic relatives).

**Conclusion:** The present study shows that the longitudinal relation abdominal obesity-ALT is more specific for all FCHL family members, i.e. patients and their normolipidemic relatives, than the relation abdominal obesity-triglycerides. Additionally, the association of ALT with the switch in triglyceride phenotype suggests a central role of the liver in the pathogenesis of FCHL.

## Introduction

Familial Combined Hyperlipidemia (FCHL) is a common primary hyperlipidemia characterized by premature cardiovascular disease [1] and features of the metabolic syndrome, such as insulin resistance [2], abdominal obesity [3] and hypertension [4]. FCHL is, by definition, a 'multiple-type hyperlipidemia', in which different Frederickson's subtypes of hyperlipidemia are present within one family (IIa, IIb and IV, corresponding with isolated hypercholesterolemia, combined hyperlipidemia and isolated hypertriglyceridemia, respectively) [1]. Shortly after the first description of FCHL, it was suggested that the 'multiple-type hyperlipidemia' could occur within one particular FCHL patient as well, and that expression of a hyperlipidemic phenotype at a certain point in time may be due to the combination of genetic and environmental factors [5]. The 'multiple-type hyperlipidemia' within one family makes the clinical diagnosis of FCHL demanding, given the necessity to screen for hyperlipidemia in at least two family members. Furthermore, the 'multiple-type hyperlipidemia' within one individual may lead to misclassification of the affected state, since subjects may be hyperlipidemic at one time-point, but normolipidemic at another measurement. Efforts have therefore been made to simplify and standardize the diagnosis of the FCHL phenotype, among others by including markers that are less variable over time, such as plasma apolipoprotein B levels [6, 7].

The hypertriglyceridemic phenotype in FCHL is the consequence of both an excessive production of VLDL-particles and an impaired clearance of remnants [8-10]. The former has been associated with both hepatic insulin resistance and hepatic fat accumulation [11, 12], which makes them good candidates for explaining the multiple-type hyperlipidemia in FCHL. Indeed, insulin resistance has been associated with the switch in triglyceride phenotype, i.e. from normotriglyceridemia to hypertriglyceridemia or vice versa, in FCHL [13]. To date, the relation between hepatic fat accumulation and the multiple-type hyperlipidemia in FCHL has been unknown. This relation is conceivable given the high prevalence of fatty liver in particularly hypertriglyceridemic FCHL patients [14, 15]. Therefore, the present study was conducted to investigate whether varying amounts of hepatic fat are responsible for the 'multiple-type hyperlipidemia' in FCHL, in particular

the switch in triglyceride phenotype. Since an appreciable proportion of hepatic fat is derived from free fatty acids released from adipose tissue [16], we additionally questioned how the degree of (abdominal) obesity is related to fatty liver, insulin resistance and plasma triglycerides in individual FCHL patients, their normolipidemic relatives and spouses.

For this alanine aminotransferase (ALT) levels were determined with a five-year interval and evaluated in relation to lipid, insulin and obesity parameters in 21 FCHL pedigrees and their spouses. This liver specific enzyme is correlated with liver fat in normal subjects [17] and also in FCHL families [18], and has often been used in longitudinal studies as a marker of nonalcoholic fatty liver disease [19-21], a histological spectrum that comprises hepatic steatosis [22].

## Materials and methods

### *Study population*

FCHL families were ascertained as described previously [23]. Briefly, the diagnosis was established when at least two family members exhibited different types of hyperlipidemia, i.e. hypertriglyceridemia (triglycerides > 2.3 mmol/L), hypercholesterolemia (cholesterol > 6.5 mmol/L) or both, the so-called multiple-type hyperlipidemia. Furthermore, at least one family member had to have a history of premature cardiovascular disease. Exclusion criteria were other genetic hyperlipidemias, such as familial hypercholesterolemia and familial dysbetalipoproteinemia (E2/E2 genotype). FCHL probands, i.e. the index patients from each family, were non-obese (body mass index < 30 kg/m<sup>2</sup>), non-diabetic and had a normal thyroid function. Recently, it was proposed to redefine FCHL according to factors that are more specific for FCHL and constant over time, i.e. apolipoprotein B > 1.2 g/L and triglycerides > 1.5 mmol/L [6]. In the current study, all analyses are conducted with both the traditional and newly proposed criteria of FCHL to assess the impact of these different criteria on the outcomes.

FCHL patients (i.e. all hyperlipidemic members of the established FCHL families), their normolipidemic (NL) relatives (i.e. all normolipidemic members of the established FCHL

families) and their spouses visited the lipid research clinic after an overnight fast and complete abstinence from alcohol for the last three days. Lipid-lowering medication was withdrawn two weeks prior to their visit. Additionally, none of the subjects reported rapid weight loss, took any medication associated with the development of fatty liver [24] or consumed more than two units of alcohol daily. One subject was found to be seropositive for hepatitis C and was therefore excluded from this study. All subjects filled in a questionnaire regarding smoking and dietary habits.

In 2004, subjects were re-recruited in the same order as in 1999, thereby preventing a difference in follow-up period for different subgroups. In total, 21 families, consisting of 199 subjects (FCHL family members and spouses), were studied in both 1999 (baseline) and 2004. Nine of 63 (14%) spouses who were invited for the second visit were lost to follow-up. Three subjects deceased in the follow-up period, the remaining six rejected further participation. They were significantly older than the 54 spouses with a follow-up measurement (75 versus 47 years,  $p < 0.001$ ). There were no differences in age adjusted baseline cholesterol, triglycerides and body mass index (BMI) values. Of all 195 FCHL family members who were invited, 50 subjects (26%) were lost in follow-up because of death ( $n = 8$ ) or reluctance to participate ( $n = 42$ ). Their baseline age, sex, cholesterol, triglyceride and BMI values were not different from the FCHL family members with a follow-up measurement.

All subjects gave written informed consent. The research protocol was approved by the medical ethical review committee at the Academic Hospital Maastricht and Maastricht University.

#### *Anthropometric measurements*

Height was measured with a stadiometer. Weight was determined in underwear. Body mass index (BMI) was calculated as weight divided by height squared ( $\text{kg/m}^2$ ). Waist circumference was measured in standing position at the level of the umbilicus.

#### *Laboratory measurements*

Plasma cholesterol, triglycerides, insulin, apolipoprotein B, glucose and ALT determinations were done as described previously [4, 18]. HOMA-IR (homeostasis

model assessment insulin resistance) was calculated as a measure of insulin resistance [25].

### *Statistical methods*

Data are expressed as mean  $\pm$  standard deviation (SD), or as median with interquartile range between brackets in case of non-normal distribution. An one-way ANOVA was used to detect age differences between FCHL patients, their NL relatives and spouses. Other variables were tested in linear regression models with inclusion of age and gender. Prevalences of hyperlipidemia and changing phenotype between FCHL families and spouses were compared with a chi-square test. Waist circumference, plasma insulin and ALT levels in 1999 and 2004 were compared with the non-parametric Wilcoxon test for related samples. A mixed linear model for repeated measurements with random intercept was constructed to test the five-year longitudinal relations between waist circumference, ALT, insulin and triglyceride levels. Since the repeated measurements within one individual were included in the model as a random factor, the outcome of the model should be interpreted as the intra-individual relation averaged for all subjects. Obviously, such an intra-individual relation is superior to the inter-individual relation that is normally obtained in cross-sectional study designs. Dummy variables were entered in these models to study differences in these longitudinal relations between spouses and FCHL patients (spouse = 0 and FCHL patient = 1), and between spouses and normolipidemic relatives (spouse = 0 and NL relative = 1). All analyses were conducted with SPSS 13.0 statistical package (SPSS Inc. Illinois, USA).

## **Results**

### *Baseline characteristics*

Of all 199 subjects who were studied, 54 were spouses and 145 were FCHL family members derived from 21 FCHL families. At baseline (in 1999), 56 of 145 (39%) subjects fulfilled the traditional FCHL criteria of hyperlipidemia, 29 (52%) were hypercholesterolemic, 15 (27%) were hypertriglyceridemic and 12 (21%) were combined hyperlipidemic. The descriptives of these hyperlipidemic patients, their NL relatives and

their spouses are listed in Table 1. FCHL patients were older than their NL relatives, but not in comparison with their spouses ( $p < 0.001$ , one-way ANOVA). After correction for age and gender, FCHL patients were significantly more (abdominally) obese, and had higher insulin and ALT levels than their NL relatives and spouses (Table 1).

#### *Changes in lipoprotein phenotype expression in 5-year follow-up*

At the second visit in 2004, again 56 of 145 FCHL family members fulfilled the FCHL criteria with an exactly similar distribution among the different phenotypes, but most of these were not the same individuals as at baseline: 43 of 145 (30%) subjects switched lipid phenotype in the follow-up period (Table 2). Twenty-six subjects switched phenotype according to the cholesterol level and 22 subjects switched phenotype according to the triglyceride level (Table 2). In total, 32 of 145 subjects (22%) switched from normolipidemia to FCHL or vice versa (Table 2). When FCHL was defined according to recently proposed criteria (apolipoprotein B  $> 1.2$  g/L and triglycerides  $> 1.5$  mmol/L) [6], the switch from affected to non-affected (or vice versa) was somewhat lower, i.e. 28 of 145 subjects (19%). Only one of these subjects reported a beneficial change in dietary habits, which was accompanied by a switch from hyperlipidemia to normolipidemia. Eight of these subjects changed their smoking habits over time, which did not appear to be associated with the expression of the affected state.

At baseline, 7 of 54 (13%) spouses exhibited the hyperlipidemic phenotype. As expected, this was significantly lower than the 56 of 145 (39%) observed at baseline in the FCHL families ( $p = 0.001$ , chi-square). At the second visit, 12 of 54 (22%) spouses had switched lipid phenotype. However, this proportion was not significantly different from FCHL family members (30% in FCHL versus 22% in spouses,  $p = 0.30$ , chi-square).

#### *Changes in triglyceride phenotype expression in relation with waist circumference, insulin and ALT levels*

The switch in triglyceride phenotype, as observed in 22 FCHL patients, was not associated with changes in waist circumference or plasma insulin levels (Figure 1, panel A and B). Similar results were obtained when BMI or HOMA levels were taken for analysis (data not shown). In contrast, ALT levels were significantly related with the

switch in triglyceride phenotype between 1999 and 2004 ( $p = 0.001$ , Figure 1, panel C). This relation remained significant after correction for waist circumference and plasma insulin levels ( $p = 0.001$ ). Of note, similar results were obtained when these variables were related to the change from normolipidemia to FCHL (or vice versa), which was defined according to the recently proposed consensus, i.e. apolipoprotein B  $> 1.2$  g/L and triglycerides  $> 1.5$  mmol/L ( $p = 0.4$  for waist circumference,  $p = 0.6$  for plasma insulin and  $p = 0.04$  for ALT levels).

*Relation of abdominal obesity with plasma triglycerides, insulin and ALT levels in FCHL patients, NL relatives and spouses*

Since the hyperlipidemic phenotype develops against a background of (abdominal) obesity [26], we subsequently investigated the intra-individual relation between abdominal obesity and plasma triglycerides, insulin and ALT levels in FCHL patients, normolipidemic relatives and spouses.

Despite the absent relation between waist circumference and the switch in triglyceride phenotype, an intra-individual relation was observed between plasma triglycerides and waist circumference in both spouses and FCHL family members (FCHL patients and NL relatives combined). Of interest, the FCHL family members were more susceptible to higher plasma triglycerides than spouses ( $p = 0.004$ ), which was explained by the FCHL patients, i.e. subjects who were hyperlipidemic in 1999 and/or 2004, as shown in Figure 2, panel A. For every waist circumference, FCHL patients had higher triglyceride levels than their spouses ( $p < 0.001$ ). No differences were observed between spouses and the normolipidemic relatives, i.e. subjects who were normolipidemic in 1999 and 2004 ( $p = 0.90$ , Figure 2, panel A). Similar results were obtained for the intra-individual relation between abdominal obesity and plasma insulin levels (Figure 2, panel B). To investigate the role of the liver in this increased susceptibility, the relation between changes in waist circumference and changes in plasma ALT levels was subsequently examined. A five-year intra-individual relation was observed between waist circumference and ALT levels, which was again different between FCHL family members and spouses ( $p < 0.001$ ). However, this time the difference was not only explained by FCHL patients, but also by the NL relatives, as shown in Figure 2, panel C. For every waist circumference, both NL

relatives and FCHL patients exhibited significantly higher ALT levels than their spouses ( $p = 0.035$  and  $p < 0.001$ , respectively). These outcomes were not affected by redefining FCHL according to the newly proposed criteria ( $p = 0.048$  and  $p < 0.001$ , respectively). Replacing waist circumference by BMI and insulin by HOMA-IR yielded very similar results (data not shown).

## Discussion

The present study is the third to present long-term follow-up results in familial combined hyperlipidemia, and the first to incorporate data to evaluate the role of the liver in the pathogenesis of this highly prevalent genetic hyperlipidemia that is associated with premature cardiovascular complications. Longitudinal study designs are essential to study the changes in lipid phenotype over time, the so-called 'multiple-type hyperlipidemia' within one individual, a phenomenon that complicates the diagnosis and elucidation of FCHL.

In the current study, we observed that 43 of the 145 (30%) FCHL family members had a different lipoprotein profile in 2004 in comparison with their initial measurement in 1999. This is in line with the 28 and 34% reported in the two previous long-term follow-up studies in FCHL [27, 28]. However, this proportion was not significantly different from the 22% of spouses who changed lipid phenotype as well. These data suggest that the 'multiple-type hyperlipidemia' within one individual can occur in other conditions besides FCHL as well. Although this conclusion warrants some caution because of the small sample of controls that was used in the present study, it is of interest that Delawi et al. came to similar conclusions in a short-term follow-up study in FCHL patients and controls [29].

The current study focused on factors that are associated with the switch in lipid phenotype in FCHL. We anticipated that a switch in triglyceride phenotype is associated with changes in hepatic fat accumulation. This hypothesis was driven by a recent study reporting that the production of triglyceride-rich VLDL particles is positively associated with the amount of fat in the liver in both normal subjects and patients with type 2 diabetes mellitus [12]. Since liver biopsies were not ethically acceptable in this relatively

healthy population, and magnetic resonance spectroscopy (MRS) was not widely in use at the initiation of this study, we determined plasma ALT levels as a marker of nonalcoholic fatty liver disease to test our hypothesis. ALT levels are correlated with liver fat quantified with ultrasound and MRS in normal subjects and FCHL families [17, 18], and have often been implemented as a surrogate for nonalcoholic fatty liver disease in longitudinal studies [19-21]. We found that ALT levels are indeed associated with the switch in triglyceride phenotype, suggestive of changes in hepatic fat as the driving force behind the change in triglyceride phenotype in FCHL. Of note, Westerbacka and colleagues have demonstrated that hepatic fat accumulation can indeed be a very reversible phenomenon [30]. Other factors that have previously been associated with the switch in triglyceride phenotype, i.e. obesity and insulin resistance [13, 27], were not associated in the current study. This discrepancy may be explained by a lack of power. The currently observed significant intra-individual relation between (abdominal) obesity and plasma triglycerides in all FCHL patients seems to corroborate that suggestion. Alternatively, since plasma insulin levels reflect insulin resistance of liver, muscle as well as adipose tissue, they may not be a very specific marker for hepatic insulin resistance, which is another cause of VLDL-overproduction [11]. Furthermore, the effects of (abdominal) obesity on the hypertriglyceridemic phenotype are probably mediated through the induction of hepatic steatosis [12, 31]. However, obesity is not a prerequisite for the development of hepatic fat accumulation [30]. The present study therefore shows that of the three markers under investigation, plasma ALT levels are most closely related to the expression of the hypertriglyceridemic phenotype in FCHL.

The potential role of the liver has also been implicated in the pathogenesis of other entities associated with the metabolic syndrome. Several groups have shown that ALT levels, even within the normal range, predict new-onset type 2 diabetes mellitus, and it has been proposed that ALT measurements, an easily obtainable biomarker, could be included in future diabetes prediction algorithms [20, 32]. The present study suggests that the same could be true for the prediction of hypertriglyceridemia in FCHL.

The current results are in line with recent data published by Ter Avest et al. [33]. These authors concluded that elevated apolipoprotein B levels are the consequence of both a primary defect, possibly located in the liver, and of a secondary defect, which could be

due to an increased hepatic free fatty acid flux resulting from expansion of adipose tissue [33]. The present study showed that hepatic fat accumulation, which drives the production of apolipoprotein B containing particles [12, 15], is also dependent on increments in abdominal obesity. In addition, with similar increments in abdominal obesity, not only FCHL patients, but also normolipidemic relatives exhibited significantly higher ALT levels, which is suggestive of an increased genetic susceptibility to develop fatty liver. Indeed, a genetic component was recently demonstrated in the same cohort [18].

An increased (genetic) susceptibility to develop fatty liver could have its origin in different processes, among others by an increased flux of free fatty acids towards the liver [16]. Of interest, Karjalainen et al. previously concluded that an excess hepatic supply of fatty acids, due to an increased release by adipose tissue in both FCHL patients and normolipidemic relatives, could precede the hyperlipidemia in FCHL [34]. These results are very much in concordance with our current data.

Unfortunately, in the present study we were not able to differentiate between different abdominal fat depots with distinctive features, i.e. visceral and subcutaneous obesity. This, together with the use of ALT as a surrogate of hepatic fat, should be regarded as a limitation of this study.

In summary, the present 5-year longitudinal study has underlined the role of the liver in the pathogenesis of FCHL by showing that the switch in triglyceride phenotype is associated with changes in ALT levels and that both FCHL patients and normolipidemic relatives exhibit higher ALT levels with similar increments in visceral obesity. Therefore, genes and metabolic pathways that contribute to the increased susceptibility to develop fatty liver in FCHL deserve our further attention. This will eventually lead to a better understanding of this highly prevalent disease characterized by different types of hyperlipidemia.

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**Figure legends:**

Figure 1: Waist circumference, plasma insulin and alanine aminotransferase (ALT) levels in relation with the triglyceride phenotype switch (NTG: normotriglyceridemia, HTG: hypertriglyceridemia). Calculated with a Wilcoxon non-parametric test for related samples. Data are presented on a log scale.

Figure 2: 5-year intra-individual relation between waist circumference and plasma triglycerides (panel A), insulin (panel B) and ALT levels (panel C) averaged for all spouses, NL relatives and FCHL patients. Regression lines, drawn from the minimum to the maximum waist value of each group, are the resultant of all individual five-year relations. Calculated with mixed linear regression for repeated measurements, age and gender corrected. Triglycerides, insulin and ALT are presented on a log scale.

**Table 1. Baseline characteristics of FCHL subjects, their normolipidemic relatives and spouses**

	Spouses	NL relatives	FCHL
Male / Female	27 / 27	33 / 56	27 / 29
Age, years	47 ± 10	39 ± 16*	50 ± 13
BMI, kg/m <sup>2</sup>	25.5 ± 3.7	25.2 ± 4.1	28.1 ± 3.9†‡
Waist circumference, cm	90.2 ± 10.5	88.8 ± 12.8	96.5 ± 10.5†‡
Cholesterol, mmol/L	5.2 ± 0.8	5.0 ± 0.9	6.7 ± 1.2†‡
Triglycerides, mmol/L	1.1 (0.7 – 1.6)	1.1 (0.9 – 1.4)	2.0 (1.4 – 2.6)†‡
Apolipoprotein B, g/L	1.0 ± 0.2	1.0 ± 0.2	1.4 ± 0.3†‡
Insulin, mU/L	4.9 (2.0 – 9.1)	5.4 (2.0 – 8.9)	8.2 (5.2 – 13.5)†‡
ALT, U/L	14.8 (11.9 – 19.4)	15.9 (11.5 – 20.9)	24.4 (18.3 – 31.1)†‡

Values are expressed as mean ± SD, or median (interquartile range); NL, normolipidemic. The affected state is defined according the traditional criteria.

\* p < 0.001 versus spouses and FCHL; student's T-test

† p < 0.05 versus spouses after correction for age and gender; linear regression,

‡ p < 0.05 versus normolipidemic relatives after correction for age and gender; linear regression

All analyses were Hochberg corrected

**Table 2. Lipid phenotype expression in FCHL in 1999 and 2004**

FCHL		2004				
		NL	HC	HC + HTG	HTG	Total
<b>1999</b>	NL	73	9	2	5	89
	HC	8	17	3	1	29
	HC + HTG	2	3	5	2	12
	HTG	6	0	2	7	15
	Total	89	29	12	15	145

Values represent absolute numbers of subjects



