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Martijn C.G.J. Brouwers, Jacqueline de Graaf, Marleen M.J. van Greevenbroek, Anna M. Georgieva, Carla J.H. van Der Kallen, et al.. Parabolic relation between plasma triglycerides and LDL-cholesterol in familial combined hyperlipidemia. The multiple-type hyperlipidemia explained?. *Clinical Science*, 2007, 114 (5), pp.393-401. <10.1042/CS20070314>. <hal-00479401>

HAL Id: hal-00479401

<https://hal.science/hal-00479401v1>

Submitted on 30 Apr 2010

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Parabolic relation between plasma triglycerides and LDL-cholesterol in familial combined hyperlipidemia

The multiple-type hyperlipidemia explained?

Short running title: parabolic relation between triglycerides and LDL

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Key words: cardiovascular disease, coronary artery disease, apolipoprotein B, VLDL, epidemiology, diagnosis

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Abstract:

Background: familial combined hyperlipidemia (FCHL) is a highly prevalent genetic lipid disorder that accounts for a substantial number of premature cardiovascular events. To date, FCHL has been complicated by the different lipid phenotypes that are present within one family and one individual patient over time

Objective: in the present study, we hypothesized that a parabolic relation between plasma triglycerides and LDL-cholesterol can explain this so-called 'multiple-type hyperlipidemia' in FCHL.

Methods: our hypothesis was tested in two well-documented FCHL cohorts (Maastricht: n=145; Nijmegen: n=299) that were followed over time with 5-years interval. Three groups were constructed depending on plasma triglycerides: group A (individuals with both measurement below 1.5 mmol/L), group B (one measurement below and one above 1.5 mmol/L) and group C (both measurement above 1.5 mmol/L).

Results: in both male but not female cohorts, a significant, positive relation between plasma triglycerides and LDL-cholesterol was observed in group A (Maastricht: $p=0.02$; Nijmegen: $p=0.001$), a significant, negative relation in group C (Maastricht: $p=0.01$; Nijmegen: $p=0.02$), and a relation intermediate to group A and C in group B. In contrast, both apolipoprotein B levels and the prevalence of cardiovascular disease were related with plasma triglycerides in a more linear fashion.

Conclusions: a parabolic relation between plasma triglycerides and LDL-cholesterol explains the 'multiple-type hyperlipidemia' in FCHL. In addition, the linear relation between triglycerides and both apolipoprotein B levels and prevalence of cardiovascular disease substantiate the use of apolipoprotein B instead of LDL-cholesterol in the diagnosis of FCHL and the prediction of cardiovascular disease.

Introduction

Almost 30 years ago, Goldstein et al. were the first to delineate familial combined hyperlipidemia (FCHL) as a genetic hyperlipidemia [1]. Nowadays it is well established that in Western societies FCHL is a highly prevalent disease (1:100) with an approximately five-fold increased risk of premature cardiovascular complications [1, 2]. It has been estimated that 10% of patients with premature coronary artery disease are affected by FCHL [1]. Many FCHL patients exhibit features of the metabolic syndrome, such as insulin resistance [3], visceral obesity [2, 4], fatty liver [5, 6], low HDL-cholesterol and high blood pressure [7]. With the current epidemic of obesity, it is expected that the prevalence of metabolic syndrome, and hence FCHL, will increase even further with a subsequent increase in the incidence of cardiovascular complications. Despite the progressive insights in the clinical syndrome, several important questions around FCHL remain. One of the most challenging is the puzzle of the different plasma lipid phenotypes observed in relatives in FCHL families, or even more confusing within one FCHL patient [1, 8], i.e. hypercholesterolemia, hypertriglyceridemia or the combination of both. This phenomenon is called the 'multiple-type hyperlipidemia' and it has resulted in the requirement that an elaborate diagnostic procedure in several relatives is needed to robustly establish FCHL in a family and subsequently in one FCHL patient. Furthermore, once a subject is diagnosed as 'affected', he or she might be non-affected at the next visit due to the same phenomenon. Efforts have therefore been made to circumvent this problem by redefining FCHL with parameters that are more specific and more stable over time, such as apolipoprotein B (apo B)[9, 10].

Previous studies of Veerkamp and colleagues have demonstrated that the switch in triglyceride phenotype, i.e. from normotriglyceridemia to hypertriglyceridemia or vice versa, is associated with changes in body mass index and insulin resistance in FCHL [11, 12]. More recently, another metabolic factor, i.e. hepatic fat as reflected by plasma alanine aminotransferase levels, was added to this list in a second Dutch FCHL cohort [13]. As the metabolic pathways contributing to the switch in triglyceride phenotype are being elucidated, no factors have yet been linked to the switch in cholesterol phenotype. In the current study, we anticipated that the switch in cholesterol phenotype depends on changes in plasma triglycerides.

Careful study of kinetic data on synthesis and metabolism of lipoprotein particles, as reviewed by Packard et al.[14], has led us to hypothesize that plasma triglycerides and LDL-cholesterol levels are part of a metabolic continuum that follows a parabolic relation, i.e. at low triglyceride levels (<1.5 mmol/L), LDL- cholesterol increases in parallel with increasing triglycerides levels, whereas a further increase in triglycerides above the triglyceride cut-off of 1.5 mmol/L is associated with a decline in LDL-cholesterol. This complex relation would be due to the secretion of different species of very low-density lipoprotein (VLDL) particles by the liver, i.e. VLDL2 and triglyceride-rich VLDL1. Since VLDL2-particles – which are mainly catabolized into LDL-particles - are secreted when plasma triglycerides are low [14], a positive relation between plasma triglycerides and LDL-cholesterol can be anticipated when plasma triglycerides are less than 1.5 mmol/L. In contrast, when plasma triglycerides increase, VLDL1-particles become abundant, which are not all catabolized into LDL-particles [14]. Furthermore, these LDL-particles are cholesterol-depleted due to CETP-mediated cholesteryl ester exchange with VLDL1-particles [14]. As a consequence, we hypothesized that, at high triglycerides (> 1.5 mmol/L), the relation between plasma triglycerides and LDL-cholesterol is negative (see Figure 1 for detailed explanation).

A parabolic relation between plasma triglycerides and LDL-cholesterol could theoretically account for the switch in cholesterol phenotype in FCHL. Therefore, in the present study we investigated the presence of such a parabola in two previously reported, well-defined FCHL cohorts that have been followed over time, i.e. the Maastricht and Nijmegen FCHL cohorts [11, 13]. In addition, we studied the longitudinal relation between plasma triglycerides and apo B - hallmarks of FCHL - and the prevalence cardiovascular disease - the clinically relevant endpoint of FCHL.

Methods

Study populations

FCHL family members were collected and followed prospectively in two cohorts: the Maastricht and Nijmegen cohort. The Maastricht FCHL pedigrees ($n = 145$ subjects) were originally recruited based on the cut-off values of 6.5 mmol/L and/or 2.3 mmol/L

for plasma cholesterol and triglycerides, respectively. Their baseline (1999) and follow-up (2004) characteristics have recently been described in detail [13]. The Nijmegen FCHL families (n = 299 subjects) were originally recruited based on the 90th percentile diagnostic criteria for hypercholesterolemia and hypertriglyceridemia, which has been described in detail elsewhere, together with their baseline (1994) and follow-up (1999) characteristics [11].

In both cohorts, type 2 diabetes mellitus was an exclusion criterion for the index patient (= proband) [7, 11]. No subjects carried the apo E2/E2 genotype. Furthermore, FCHL patients were withdrawn from lipid lowering medication for at least two weeks prior to both visits. The presence of cardiovascular disease (CVD) was defined by angina pectoris, myocardial infarction, stroke, peripheral vascular disease or vascular surgery. The studies were approved by the ethics committee of the University Medical Center Nijmegen and the University Hospital Maastricht/Maastricht University. All subjects gave written informed consent.

Measurements

Subjects visited the study centers after an overnight fast. Weight and height were measured with participants wearing light clothing only, and the body mass index (BMI) was calculated as weight divided by height squared (kg/m^2).

Fasting plasma triglycerides, total cholesterol, HDL-cholesterol and apo B were determined as described elsewhere [7, 11, 15]. LDL-cholesterol was calculated with the Friedewald formula ($\text{LDL-cholesterol} = \text{total cholesterol} - \text{HDL-cholesterol} - (\text{plasma triglycerides} * 0.45)$) [16]. Since this formula is only valid for plasma triglycerides < 4.5 mmol/L [16], subjects with triglycerides above this value were excluded from analyses (six subjects from the Maastricht cohort and seven subjects from the Nijmegen study population). In the Nijmegen FCHL cohort, LDL-cholesterol was also approximated by subtracting VLDL-cholesterol and HDL-cholesterol from total cholesterol levels. VLDL-cholesterol was measured by ultracentrifugation [9].

Statistical analyses

Baseline characteristics of the cohorts are presented as mean (SD) or median (interquartile ranges). A Student's T-test was used to detect differences in age between FCHL probands (= index patient of one FCHL pedigree) and their relatives, and between Maastricht and Nijmegen probands/relatives. The anthropometric and lipid parameters were compared by linear regression with inclusion of age and sex. To test the possibility of a parabolic relation between plasma concentrations of triglycerides and LDL-cholesterol as an explanation for the multiple-type hyperlipidemia in FCHL, three groups were defined (figure 1): a group with plasma triglycerides <1.5 mmol/L both at baseline and at follow-up (group A), a group with triglycerides >1.5 mmol/L at both time points (group C), and the remaining group in whom plasma triglycerides were <1.5 mmol/L at one time-point and >1.5 mmol/L at the other moment (group B). The cut-off value of 1.5 mmol/L was used since it is commonly accepted that from this value there is substantial cholesteryl ester exchange between VLDL1 and LDL-particles (figure 1) [14]. Since sex hormones are known to influence lipid metabolism [17], all analyses were conducted for male and female subjects separately. A mixed linear model for repeated measurements with random intercept was used accounting for repeated measurement in one individual. A significant relation should therefore be interpreted as the intra-individual relation averaged for all subjects in the particular subgroup. According to our hypothesis, we anticipated a significant, positive relation in group A, a significant, negative relation in group C and a relation intermediate to group A and C in group B (figure 1). In addition, the regression slopes of the different subgroups were compared with each other by using interaction terms. All analyses were conducted with SPSS 13.0 statistical package (SPSS Inc. Illinois, USA).

Results

Baseline characteristics of Maastricht and Nijmegen FCHL study populations

Baseline characteristics of Maastricht and Nijmegen FCHL probands and their relatives are presented in Table 1. Maastricht and Nijmegen FCHL probands were significantly

older when compared to their relatives. The seemingly marked difference in total cholesterol levels between FCHL probands and relatives was lost after correction for age and sex ($p = 0.07$ for Maastricht and $p = 0.32$ for Nijmegen). In contrast, age- and sex-adjusted analyses revealed that FCHL probands still displayed significantly higher plasma triglycerides (Table 1).

Probands and relatives in the Maastricht population were significantly more obese than in the Nijmegen FCHL cohort (Table 1). Furthermore, small, but significant differences were observed for plasma total cholesterol and HDL-cholesterol levels between Maastricht and Nijmegen FCHL relatives.

As previously reported [11, 13], the proportion of subjects who switched lipid phenotype during follow-up, i.e. from normotriglyceridemia to hypertriglyceridemia, from normocholesterolemia to hypercholesterolemia or vice versa, was similar in both cohorts (30% and 28% in Maastricht and Nijmegen, respectively).

Longitudinal parabolic relation between plasma triglycerides and LDL-cholesterol

A possible parabolic relation between plasma triglycerides and LDL-cholesterol, to explain the multiple-type hyperlipidemia in FCHL, was first evaluated in males from both FCHL study populations. As shown in Figure 2 (panel I), mixed linear regression analysis revealed a 5-year positive relation between plasma triglycerides and LDL-cholesterol for Maastricht FCHL men in group A (plasma triglycerides below 1.5 mmol/L at both time points, $p = 0.02$) and group B (plasma triglycerides below and above 1.5 mmol/L at different time points, $p = 0.2$). This relation was significant, but negative for men in group C (plasma triglycerides above 1.5 mmol/L at both time points, $p = 0.01$). According to our hypothesis, the slopes of the regression lines, indicated as β , decreased with increasing plasma triglycerides ($\beta_a = 1.8$, $\beta_b = 0.3$, $\beta_c = -0.7$, figure 2, panel I). Furthermore, these slopes were all significantly different from each other (β_a versus β_b : $p = 0.02$; β_b versus β_c : $p = 0.003$; β_a versus β_c : $p < 0.001$, Hochberg corrected). Comparable results were obtained in the male Nijmegen cohort (Figure 2, panel II). Of additional interest, very similar results were obtained when LDL-cholesterol levels, approximated by subtracting VLDL-cholesterol and HDL-cholesterol from total

cholesterol levels, were taken for analysis (figure 2, panel II, inset). Therefore, the use of the Friedewald formula does not account for the observed parabola.

The results for the female Maastricht and Nijmegen FCHL cohorts were less consistent: despite a trend towards a parabolic relation in the Maastricht cohort ($\beta_a = 0.6$, $p = 0.02$; $\beta_b = 0.5$, $p = 0.2$; $\beta_c = -0.3$, $p = 0.1$), no significant relation was observed in any subgroup of the Nijmegen cohort ($\beta_a = -0.1$, $p = 0.5$; $\beta_b = -0.2$, $p = 0.3$; $\beta_c = 0.01$, $p = 0.9$).

Parabolic relation between plasma triglycerides and LDL-cholesterol within individual male FCHL patients

Further evidence for the above presented longitudinal relation between plasma triglycerides and LDL-cholesterol in the male FCHL population was assessed by investigating whether one individual can pass completely through the parabolic curve. For this, at least three consecutive measurements in one individual were required with plasma triglycerides values below, around and above the parabolic peak value of 1.5 mmol/L. Although such series of data are hard to obtain for each individual, 13 male subjects in the combined study population were measured more than twice and fulfilled each of the following plasma triglycerides criteria: one measurement around 1.5 mmol/L, i.e. between the arbitrary values of 1.4 and 1.6 mmol/L, one measurement below 1.4 mmol/L and one measurement above 1.6 mmol/L. Of the 13 subjects who met these criteria, 11 subjects showed an increase in LDL-cholesterol levels when plasma triglycerides values increased from low to moderate levels, whereas a further increase was accompanied with a decrease in LDL-cholesterol (Figure 3)

Longitudinal relation between plasma triglycerides and apo B and prevalence of cardiovascular disease

Since elevated apo B levels – the equivalent of the amount of atherogenic particles in plasma – are a hallmark of FCHL, we subsequently analyzed the longitudinal relation between plasma triglycerides and apo B in the male Maastricht and Nijmegen FCHL cohorts. Of interest, no parabolic relation was observed. At the lower plasma triglycerides range, both cohorts displayed an increase in apo B with increasing triglycerides, which was significant for the Nijmegen cohort (group A: $\beta_a = 0.2$, $p < 0.001$), but not for the

Maastricht population (group A: $\beta_a = 0.3$, $p = 0.10$) (Figure 4, panels I and II). A further increase in plasma triglycerides was not associated with a decrease in plasma apo B levels in either of the cohorts (Figure 4, panel I and II), in contrast to what has been observed for LDL-cholesterol. As a consequence, the LDL-cholesterol:apo B ratio, which is indicative of LDL-particle size, did decrease with increasing plasma triglycerides ($p < 0.001$ for group C in both Maastricht and Nijmegen cohorts; Figure 4, panel I and II, insets). When both male FCHL cohorts were combined to analyze the prevalence of CVD in the three triglycerides subgroups, the highest prevalence was observed in group C ($p = 0.04$ for group C versus group B, $p = 0.006$ for group C versus group A; logistic regression with correction for age; Figure 4, panel III). Of note, the seriously increased prevalence of CVD in group C was similar in both cohorts (40% and 35% in Maastricht and Nijmegen, respectively).

Discussion

It has been estimated that approximately ten percent of survivors of a premature myocardial infarction is affected by FCHL [1]. FCHL is currently viewed as a genetically complex disease, implying that multiple genes in combination with environmental factors are responsible for the different metabolic pathways that eventually result in the characteristic hyperlipidemic phenotype [18]. On top of this complexity, there is the multiple-type hyperlipidemia, i.e. the presence of different lipid phenotypes within one family and one individual FCHL patient, that complicates its diagnosis, research and treatment. In the present study, we anticipated that a parabolic relation between plasma triglycerides and LDL-cholesterol can account for the switch in plasma cholesterol phenotype in FCHL. This hypothesis was based on the fact that the liver is able to secrete different VLDL-particles with different catabolic fates, depending on the actual plasma triglycerides concentrations [14].

Our hypothesis was verified in two well-documented FCHL cohorts that have been followed over time [11, 13]. We recently demonstrated that the switch in lipid phenotype is very similar in both cohorts [11, 13] and we currently showed that baseline

characteristics of the Maastricht and Nijmegen FCHL cohorts were comparable with regard to plasma lipid levels and prevalence of CVD. This provided us with the unique opportunity to test our hypothesis in two comparable, but independent FCHL cohorts. Both male Maastricht and Nijmegen cohorts clearly demonstrated a longitudinal parabolic relation between plasma triglycerides and LDL-cholesterol. This relation was also present in individual male patients with observations in each of the different triglycerides segments, substantiating the evidence that one individual can indeed pass completely through the parabolic curvature. In addition, this relation was not simply due to the use of the Friedewald formula, since similar results were obtained with an alternative approximation of LDL-cholesterol. The results in the female cohorts were less consistent, which is probably due to the well-known inter- and intra-individual scattering effects of estrogens on lipid levels in a population with different estrogen levels [17].

We believe that the present results, combined with our previously obtained data, account for the multiple-type hyperlipidemia in FCHL, as shown in Figure 5. In previous studies we have shown that the switch in plasma triglycerides is accompanied by changes in obesity, insulin resistance and the amount of hepatic fat [11-13]. As these metabolic factors cause a switch in plasma triglycerides, they automatically induce changes in plasma LDL-cholesterol, and – when substantial – cause a switch in cholesterol phenotype.

A striking observation in the current study is the absence of a parabolic, and presence of an almost linear relation between plasma triglycerides and the amount of atherogenic particles in plasma, as reflected by apo B levels. This observation is pivotal in several respects. First, given the fact that the majority of the plasma apo B is accounted for by LDL-particles [19], the discrepancy between the curves for LDL-cholesterol and apo B is most likely explained by a change in LDL-particle composition, i.e. the development of small-dense LDL as shown by a decrease in the LDL-cholesterol:apo B ratio. This finding is in concordance with our hypothesis. Second, while the parabolic relation for LDL-cholesterol offers an explanation for the multiple-type hyperlipidemia in FCHL, the linear relation for apo B may serve as a solution for the accompanying diagnostic

difficulties, since it is expected that apo B levels switch less frequently from normal to abnormal over time. Indeed, many reports have shown a lower temporal variability for apo B levels and therefore advocated the use of apo B instead of cholesterol levels in diagnosing FCHL [9, 10, 20, 21]. Last and most importantly, the present study suggests that apo B levels are superior to LDL-cholesterol in predicting cardiovascular events, at least in hypertriglyceridemic FCHL patients, since the relation between plasma triglycerides and the prevalence of cardiovascular disease was also linear. Again, it is anticipated that the presence of small-dense LDL particles, which are more atherogenic than buoyant LDL particles [22, 23], is responsible for this discrepancy. These findings corroborate the use of apo B in the ongoing debate of LDL-cholesterol versus apo B levels in predicting cardiovascular events [24].

In summary, the present five-year follow-up study in two well-documented FCHL cohorts has demonstrated that plasma triglycerides and LDL-cholesterol are related in a parabolic fashion in male FCHL family members. These findings account for the 'multiple-type hyperlipidemia' in FCHL and therefore improve our understanding of the natural history of this highly prevalent disease. Furthermore, the more linear relation between plasma triglycerides and both apolipoprotein B levels and the prevalence of cardiovascular disease may provide a solution to this complex phenomenon and further advocates the use of apolipoprotein B instead of LDL-cholesterol levels in the diagnosis of FCHL and the prediction of cardiovascular complications.

Acknowledgements

The authors would like to thank P.P.W.J. Brouwers for graphical support. T.W.A. de Bruin was supported by a grant of the Netherlands Organization for Scientific Research (no. 900-95-297).

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Figure legends

Figure 1: Hypothesized parabolic relation between plasma triglycerides and LDL-cholesterol. In normotriglyceridemic conditions, triglycerides are mainly incorporated in VLDL2-particles, which are mostly degraded into LDL-particles. Therefore, an increase in VLDL2-particles is expected to lead to an increase in plasma triglycerides and LDL-cholesterol, explaining part A of the parabolic curve in panel II. A further increase in plasma triglycerides is accompanied by an increased secretion of triglyceride-rich VLDL1-particles. VLDL1-particles exchange cholesteryl-ester and triglycerides with LDL- (and HDL-) particles, mediated by cholesteryl-ester transfer protein (CETP), resulting in cholesterol depleted LDL-particles. Many cholesteryl-esters transferred to VLDL1-particles leave the plasma cholesterol pool, since it is estimated that a large proportion of the VLDL1-particles is not catabolized to VLDL2 and eventually to LDL, but is directly taken up as VLDL-remnants by, among others, the liver. This would explain the negative correlation between plasma triglycerides and LDL-cholesterol (part C of the parabolic curve in panel II). Part B of the parabolic curve in panel II depicts the transition from VLDL2-secretion to VLDL1-secretion by the liver. This occurs, on average, at plasma triglycerides 1.5 mmol/L. The three dashed lines in panel II indicate the three subgroups that have been constructed to test our hypothesis of a parabolic relation, see materials and methods sections.

Figure 2: 5-year intra-individual relation between plasma triglycerides and LDL-cholesterol in male Maastricht (panel I) and Nijmegen (panel II) FCHL cohorts. (Maastricht: group A: $n = 19$, $\beta_a = 1.8$, $p = 0.02$; group B: $n = 19$, $\beta_b = 0.3$, $p = 0.2$; group C: $n = 20$, $\beta_c = -0.7$, $p = 0.01$. Nijmegen: group A: $n = 46$, $\beta_a = 1.0$, $p = 0.001$; group B: $n = 37$, $\beta_b = 0.2$, $p = 0.3$; group C: $n = 51$, $\beta_c = -0.2$, $p = 0.02$). The inset of panel II shows the results for LDL-cholesterol approximated by subtracting VLDL-cholesterol and HDL-

cholesterol from total cholesterol in the Nijmegen FCHL population. Regression lines are drawn from the minimum to the maximum triglycerides value of each subgroup.

Figure 3: Intra-individual relation between plasma triglycerides and LDL-cholesterol for 13 FCHL family members with each three consecutive measurements below triglycerides 1.4 mmol/L, between 1.4 and 1.6 mmol/L and above 1.6 mmol/L (Panel A to M). Dashed vertical lines indicate plasma triglycerides 1.4 mmol/L and 1.6 mmol/L. Panel N depicts the average relation. Error bars represent SEM. Analyzed with Wilcoxon's non-parametric test for paired samples.

Figure 4: 5-year intra-individual relation between plasma triglycerides and apo B levels in male Maastricht (panel I) and Nijmegen (panel II) FCHL cohorts. The insets show the relation between plasma triglycerides and the LDL-cholesterol:apo B ratio, which is indicative of the LDL-particle size. Panel III depicts the relation of the three triglycerides subgroups with the prevalence of CVD. For this purpose, the male Maastricht and Nijmegen cohorts were combined to increase statistical power (analyzed with logistic regression with correction for age).

Figure 5: Conceptual framework of the parabolic relation between plasma triglycerides and LDL-cholesterol as an explanation for the multiple-type hyperlipidemia in FCHL. The grey area represents the 95th percent confidence interval for the combined male Maastricht and Nijmegen cohorts. The horizontal and vertical lines indicate the cut-off values for hypercholesterolemia en hypertriglyceridemia, respectively, as defined by the NCEP ATPIII criteria. Abbreviations: NL: normolipidemia; HC: hypercholesteromia; HTG: hypertriglyceridemia

Table 1. Baseline characteristics of FCHL probands and relatives in Maastricht and Nijmegen cohort

	Maastricht FCHL cohort		Nijmegen FCHL cohort	
	Relatives	Probands	Relatives	Probands
Male / female	50 / 68	8 / 13	115/147	19 /11
Age, years	41.1 ± 15.8	52.3 ± 11.1*	38.2 ± 15.8	48.9 ± 16.4*
BMI (kg/m ²)	25.8 ± 4.2	28.5 ± 4.3	23.9 ± 3.5†	25.3 ± 4.2‡
Total Cholesterol, mmol/L	5.4 ± 1.2	6.4 ± 1.1	5.6 ± 1.3†	6.3 ± 1.3
Triglycerides, mmol/L	1.2 (0.9 – 1.7)	2.1 (1.3 – 2.4)*	1.4 (0.8 – 1.8)	2.1 (1.6 – 3.0)*
Apolipoprotein B, g/L	1.1 ± 0.3	1.4 ± 0.2*	1.1 ± 0.3	1.3 ± 0.3
HDL-cholesterol, mmol/L	1.0 ± 0.2 [0.8 / 1.0]	0.9 ± 0.3 [0.9 / 0.9]	1.2 ± 0.3 [1.1 / 1.3]†	1.0 ± 0.3 [0.9 / 1.2]
LDL-cholesterol, mmol/L	3.9 ± 1.1	4.6 ± 1.2	3.7 ± 1.2	4.3 ± 1.3
CVD, %	11 [15 / 8]	33 [42 / 27]	11 [16 / 7]	37 [62 /15]

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Data are presented as mean \pm SD or as median (interquartile range); Data between square brackets indicate males and females, respectively; all analyses are Hochberg corrected.

* $p < 0.05$, proband versus relative, Student's T-test

† $p < 0.05$, relative Nijmegen versus Maastricht, age and sex corrected

‡ $p < 0.05$ proband Nijmegen versus Maastricht, age and sex corrected

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