Genetics, Lifestyle, and Low-Density Lipoprotein Cholesterol in Young and Apparently Healthy Women

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BACKGROUND: Atherosclerosis starts in childhood but low-density lipoprotein cholesterol (LDL-C), a causal risk factor, is mostly studied and dealt with when clinical events have occurred. Women are usually affected later in life than men and are underdiagnosed, undertreated, and understudied in cardiovascular trials and research. This study aims at a better understanding of lifestyle and genetic factors that affect LDL-C in young women.

METHODS: We randomly selected for every year of age 8 women with LDL-C ≤1st percentile (≤50 mg/dL) and 8 women with LDL-C ≥99th percentile (≥186 mg/dL) from 28 000 female participants aged between 25 to 40 years of a population-based cohort study. The resulting groups include 119 and 121 women, respectively, of an average 33 years of age. A gene-sequencing panel was used to assess established monogenic and polygenic origins of these phenotypes. Information on lifestyle was extracted from questionnaires. A healthy lifestyle score was allocated based on a recently developed algorithm.

RESULTS: Of the women with LDL-C ≤1st percentile, 19 (15.7%) carried mutations that are causing monogenic hypocholesterolemia and 60 (49.6%) were genetically predisposed to low LDL-C on the basis of an extremely low weighted genetic risk score. In comparison with control groups, a healthier lifestyle was not associated with low LDL-C in women without genetic predispositions. Among women with LDL-C ≥99th percentile, 20 women (16.8%) carried mutations that cause familial hypercholesterolemia, whereas 25 (21%) were predisposed to high LDL-C on the basis of a high-weighted genetic risk score. The women in whom no genetic origin for hypercholesterolemia could be identified were found to exhibit a significantly unfavorable lifestyle in comparison with controls.

CONCLUSIONS: This study highlights the need for early assessment of the cardiovascular risk profile in apparently healthy young women to identify those with LDL-C ≥99th percentile for their age: first, because, in this study, 17% of the cases were molecularly diagnosed with familial hypercholesterolemia, which needs further attention; second, because our data indicate that an unfavorable lifestyle is significantly associated with severe hypercholesterolemia in genetically unaffected women, which may also need further attention.

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Clinical Perspective

What Is New?

- The application of a 1-step comprehensive analysis of established monogenic and polygenic origins of hypo- and hypercholesterolemia using a novel next-generation sequencing based gene panel.
- This 1-step analysis includes the detection of copy number variation, thereby excluding the need to run multiplex ligation-dependent probe amplification tests (only available for low-density lipoprotein receptor) to detect large duplications and deletions in all canonical low-density lipoprotein genes.
- Novel use of genetic risk scores for low low-density lipoprotein cholesterol.

What Are the Clinical Implications?

- This study demonstrates the need for early cardiovascular risk assessment in apparently healthy young (25–40 years) women to identify those with low-density lipoprotein cholesterol levels ≥99th percentile for their age.
- In our study, 17% of these women presented with molecularly defined, but undiagnosed and untreated familial hypercholesterolemia that dictates lifelong exposure to high low-density lipoprotein cholesterol levels if left untreated.
- Apart from genetic evaluation, our study shows that, in the majority of cases, an unfavorable lifestyle is associated with severe hypercholesterolemia, which may call for timely lifestyle evaluation and advice to prevent future cardiovascular complications.

Women are known to be protected, in general, from cardiovascular disease (CVD) during childbearing age, but CVD-related morbidity and mortality increases sharply following menopause,[1] which coincides with a steep increase of plasma levels of low-density lipoprotein cholesterol (LDL-C).[2] LDL-C is a well-recognized major treatable risk factor of CVD[3,4] and is therefore the main focus of our study. Although CVD ranks as the leading cause of death in women, preventive strategies including lipid-lowering treatment remain significantly underused.[3,5] Despite an impressive decline of CVD mortality over the past 40 years, this is surprisingly not observed in young women.[6] In line, women report less intensive treatment for dyslipidemia than men, including those with documented CVD.[5,7] These observations are potentially fueled by the assumption that young premenopausal women are protected from CVD.[8,9] This interesting topic has not been widely studied, however.

Plasma LDL-C is affected by both genetics and lifestyle. It is estimated that heritability explains 40% to 50% of plasma LDL-C levels.[10] Thus far, however, only a handful of monogenic disorders of LDL metabolism have been described: for low LDL-C it includes abetalipoproteinemia (OMIM#200100) and primary hypobetalipoproteinemia (HBL; OMIM#615558). Although these disorders are very rare, it is important to note that each of the respective genes, ie, *MTTP*, *APOB*, *PCSK9*, and *ANGPTL3* and their products are effectively targeted with drugs to lower LDL-C in clinical care. On the other side of the LDL spectrum, one can discern familial hypercholesterolemia (FH; OMIM#143890) attributable to mutations in *LDLR, PCSK9*, or *APOB*[11] or autosomal recessive hypercholesterolemia (OMIM#603813) attributable to mutations in *LDLRAP1*.[12] Although the prevalence of HBL is not well documented (estimated between 1 in 1000 and 3000[13]), the prevalence of monogenic hypercholesterolemia is well studied and concerns one of the most frequent genetic disorders known to date, with a prevalence of 1 in 217 and 250 in Northern Europe and the United States, respectively.[14,15] Besides large effects of rare variants in the above-mentioned genes on LDL-C, hypercholesterolemia can also have a polygenic origin with a combination of common genetic variants, with each having a small impact, associated with this lipid trait.[16]

Besides genetic determinants, there is also evidence of an association between lifestyle and plasma lipids, independent of genetic risk.[17] Smoking, obesity, sedentary lifestyle, and an unhealthy diet are all associated with unfavorable plasma lipid levels including LDL-C,[18] but whether these relations are of a causal nature is not clear. These factors can be combined into a healthy lifestyle score that was recently reported to be independently associated with CVD.[17]

In the present study, we aimed to address the main factors driving LDL-C levels in young premenopausal women to ultimately improve their cardiovascular health management. To this purpose, we carefully selected apparently healthy women with the highest and lowest LDL-C for their age from Lifelines, a large Dutch population-based cohort study.[19,20] Targeted next-generation sequencing was subsequently used to assess established mono- and polygenic origins of these phenotypes, whereas a recently described healthy lifestyle score[17] was used to investigate associations between lifestyle and plasma LDL-C levels.

METHODS

Participants

Lifelines is a large population-based prospective cohort study and biobank that includes a total of 167 729 individuals from the north of the Netherlands. This cohort has been described in detail in previous reports.[19,20] The study protocol was approved by the Medical Ethical Committee of the University Medical Center Groningen in the Netherlands and all participants have provided written informed consent. The data and

materials of this study are available on reasonable request following the access procedure of Lifelines.[21]

At baseline, participants filled out questionnaires and underwent a basic physical examination while biomaterials such as blood and 24-hour urine were collected. Fasting blood was drawn from all participants for clinical chemistry measurements including plasma levels of cholesterol, LDL-C (direct measurement), high-density lipoprotein cholesterol (HDL-C), triglycerides, glucose, and Hb1Ac. Participants were included between 2006 and 2013.

Selection of Study Groups

Of the 89 050 female participants of Lifelines, 27 958 (31%) were aged between 25 and 40 years. A total of 1583 women (5.7%) were excluded because of CVD (classified as myocardial infarction, stroke, or coronary surgery), diabetes mellitus, use of lipid-lowering drugs (statin, ezetimibe, or fibrates), or because of secondary causes of dyslipidemia, ie, aberrant thyroid function (thyrotropin <0.4 mU/L, thyrotropin >4.0 mU/L, thyroxine <9 pmol/L, or thyroxine >24 pmol/L), abnormal liver function (alanine aminotransferase ≥40 U/L, or aspartate aminotransferase ≥35 U/L), or kidney dysfunction (estimated using the Modification of Diet in Renal Disease formula; estimated glomerular filtration rate ≤45 mL/min). The remaining women were defined to be apparently healthy.

We and others have shown that LDL-C increases with age.[2] To avoid an age-based selection bias in our study, we therefore randomly selected 8 women per year of age with either extremely low LDL-C (≤1st percentile for age; ≤50 mg/dL) or extremely high LDL-C (≥99th percentile for age; ≥186 mg/dL), respectively. Ages 25 and 26 were grouped because of an overall shortage of women of this age.

As controls for our monogenic analysis, we selected women with normal plasma LDL-C from the Genome of the Netherlands study from which sequencing data were available.[22] This population-based study included 250 trios (parents and 1 child) for which whole-genome sequencing data are available. To ensure a selection of unrelated women, we started with female offspring. After filtering for missing LDL-C measurements, 121 samples were left. We subsequently selected women in the 20- to 45-year age range. Women with LDL-C <50 mg/mL or >190 mg/mL were excluded, which rendered a final set of 94 women. The selected women have an average LDL-C of 105 mg/dL (54–166), and are 32 years of age (20–44). Sequencing data analysis and variant filtering for the control group were performed by using the same analysis pipeline that was used for the groups with either low or high LDL-C.

The healthy lifestyle score of women in whom no genetic component for extremely low or extremely high LDL-C could be identified was compared with 2 control groups from the Lifelines study: (1) Lifelines control group I: 60 apparently healthy women between 25 and 40 years of age with LDL-C levels between 89 and 108 mg/dL (4 women per year of age were randomly selected following the same inclusion criteria as described above for the women with extreme LDL-C levels); and (2) Lifelines control group II: all apparently healthy women with LDL-C levels between 54 and 186 mg/dL and aged between 25 and 40 years of age (n=25 898).

Next-Generation Sequencing

To assess monogenic and polygenic causes of extreme LDL-C levels in the selected individuals, we developed a custom target sequencing kit. We targeted the coding regions of 11 genes involved in monogenic LDL-C disorders (*LDLR, APOB, PCSK9, LDLRAP1, APOE, ABCG5, LIPA, STAP1, MTTP, ANGPTL3,* and *SAR1B*) (Table I in the online-only Data Supplement). To study a possible polygenic cause of hypo- or hypercholesterolemia, this kit also includes probes targeting 12 single-nucleotide polymorphisms that were chosen for their contribution to a polygenic risk score for plasma LDL-C, based on genome-wide association studies on lipid traits (Table II in the online-only Data Supplement).[23] The sequencing platform and workflow analyses used are detailed in the online-only Data Supplement.

Screening for Monogenic Origins of Hypo- and Hypercholesterolemia

Variants were defined as rare if the minor allele frequency was <0.1% in the general population and in a matched population genetic data set (the Genome of the Netherlands study[22]; see the online-only Data Supplement). We set out to assess whether these variants may be causally related to hypo- or hypercholesterolemia. There is, unfortunately, no consensus on the procedure to attribute causality or pathogenicity to variants detected by next-generation sequencing,[24] but 3 classes of genetic variants have recently been proposed to be causal in monogenic LDL-C disorders[25] and we have used this as lead. These include: (1) mutations leading to a premature truncation of the encoded protein (nonsense, indels, or frameshift mutations) or to an alteration of mRNA splicing; (2) missense variants predicted to be deleterious by each of 5 in silico prediction algorithms (online-only Data Supplement); and (3) mutations described in publicly available archive of genetic variations associated with clinical phenotypes: Human Genome Mutation Database,[26] FH mutation database,[27,28] and ClinVar.[29,30] All rare genetic variations identified in our study are listed in Tables III to V in the online-only Data Supplement including variations of uncertain clinical significance.

Detection of copy number variations was performed using a recently published dedicated tool: CoNVaDING (Copy Number Variation Detection In Next-generation sequencing Gene panels).[31] Detected copy number variations were validated using either multiplex ligation-dependent probe amplification, or by long-range or real-time polymerase chain reaction (detailed in the online-only Data Supplement).

Genetic Risk Score Calculation

It is known that extreme cholesterol phenotypes can result from the accumulation of common small-effect LDL-C affecting alleles (phenocopying monogenic LDL-C disorders).[23] The overall effect can be calculated, and this results in a so-called weighted genetic risk score (wGRS: the weighted sum of the estimated per-allele effect in LDL-C changes; Table II in the online-only Data Supplement). To study a possible polygenic cause of hypo- or hypercholesterolemia, we have used a compilation of 12 single-nucleotide polymorphisms with the highest power to discriminate between FH mutation-negative individuals and the general population based on the referred

literature.[16,23,32,33] The calculated wGRS values for the high and low LDL-C groups of women were compared with the wGRS values of participants from the Genome of the Netherlands study (n=498).[22] Selected women with a wGRS <10th percentile (wGRS <0.549) or >90th percentile (wGRS >1.17) of wGRS controls were considered as having an extreme wGRS that was considered to be causally related to the phenotype.[24]

Evaluation of Lifestyle Behavior With a Lifestyle Score

To investigate the association between lifestyle and extreme LDL-C levels in women, we used a recently described healthy lifestyle score.[17] Points were given for the major lifestyle parameters including smoking status (0: current smoking; 1: no current smoking), obesity (0: body mass index ≥30 kg/m^2; 1: body mass index <30 kg/m^2), physical activity (0: sedentary lifestyle; 1: weekly ≥150 minutes moderate physical activity or ≥75 minutes intensive physical activity); and eating habits (at least 4 of the following characteristics equals 1 point: fruits [≥3 servings per day], vegetables [≥3 servings per day], nuts [≥1 serving per week], grains [≥3 servings per day], fish [≥2 servings per week], milk products [2–3 servings per day], processed meats [no more than 1 serving per week], unprocessed red meats [≤3 servings per week], and sugar-sweetened beverages [no more than 1 serving per week]). A maximum of 4 points reflects a very healthy lifestyle.

Statistical Analysis

Statistical analyses were performed with R studio software (v0.99.903; R Project for Statistical Computing) or IBM SPSS Statistics, version 22.0 (IBM Corp). Normally distributed clinical parameters were reported as mean and standard deviation and were statistically tested with the Student *t* test. Not normally distributed variables were presented as median and interquartile range. Mann-Whitney *U* test was used for comparison between groups. Percentages were compared by using χ^2 test. The distribution of the healthy lifestyle score between women without a genetic origin of hypo- or hypercholesterolemia was statistically compared with dedicated control groups using χ^2 3×2. Figures were created with the library ggplot2 R package (Wickham H. ggplot2: Elegant Graphics for Data Analysis. New York, NY: Springer; 2009).

RESULTS

Clinical Parameters

We selected 121 and 119 women with very low or very high LDL-C plasma levels using cutoff values that correspond to age- and gender-specific 1st and 99th percentiles, respectively.[2] The baseline characteristics of the selected groups are presented in Table 1. Because of the age block design used, the 2 groups were of the exact same age (averaging 33 years), whereas LDL-C levels are 5-fold higher in the high LDL-C group than in the low LDL-C group (43 versus 201 mg/dL; *P*<0.001).

In comparison with women with low LDL-C, those with high LDL-C presented with significantly lower HDL-C and significantly higher triglycerides. These marked differences in lipid and lipoprotein levels coincide with significantly higher body mass index and systolic blood pressure (*P*<0.001 for both). Finally, glucose levels tended to be greater in the high LDL-C group (*P*=0.07), whereas an elevation in glycohemoglobin in this group was highly statistically significant (*P*=0.004).

Figure I in the online-only Data Supplement shows that targeted sequencing resulted in a mean coverage depth of 436X per sample for each base, and that 99.8% of the targeted regions were covered at least 30 times.

Table 1. Clinical Parameters of Selected Groups

	Low LDL-C (n=121)	High LDL-C (n=119)	*P* Value
Age, y	33±4.4	33±4.4	NS
Total cholesterol, mg/dL	116±15	275±23	<0.001
LDL-C, mg/dL	43±8	201±19	< 0.001
HDL-C, mg/dL	68±15	52±12	<0.001
Triglycerides, mg/dL	53 (35–71)	133 (89–159)	<0.001
BMI, kg/m^2	24.4±4.6	27.9±5.1	<0.001
SBP, mm Hg	114±10	119±11	<0.001
Physically active, n (%)	29 (24)	13 (11)	0.045
Smoking, n (%)	29 (24)	21 (18)	0.033
Glucose, mmol/L	4.7±0.4	4.8±0.5	0.070
HbA1c, %	5.3 (5.2–5.5)	5.5 (5.3–5.6)	0.004

Baseline characteristics of selected women with LDL-C ≤1st percentile for age or LDL-C ≥99th percentile for age.

BMI indicates body mass index; HbA1c, glycohemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; NS, not significant; and SBP, systolic blood pressure.

Hypocholesterolemia In Young Women Has a Strong Genetic Component

Monogenic

We identified 2 premature stop codons, 2 splice acceptor variants, 7 frameshift mutations, and 1 missense mutation that are causally related to the phenotype. These mutations were identified in *APOB* (15 participants), in *PCSK9* (2 participants), and in *ANGPTL3* (2 participants) (Table III in the online-only Data Supplement). It is interesting to note that 2 novel heterozygous pathogenic copy number variations (1 deletion including exon 1 of *PCSK9* and one deletion including exons 22–24 of *APOB*) were identified and validated (in the online-only Data Supplement). In total, the proportion of individuals with mutations linked to monogenic hypocholesterolemia was 19 of 121 individuals (15.7%) (Figure A). An additional 11 individuals (9%) had heterozygous rare variants in *APOB*, *PCSK9*, or *MTTP*, but these did not meet the strict pathogenic mutation selection criteria that we used and were therefore annotated as "women

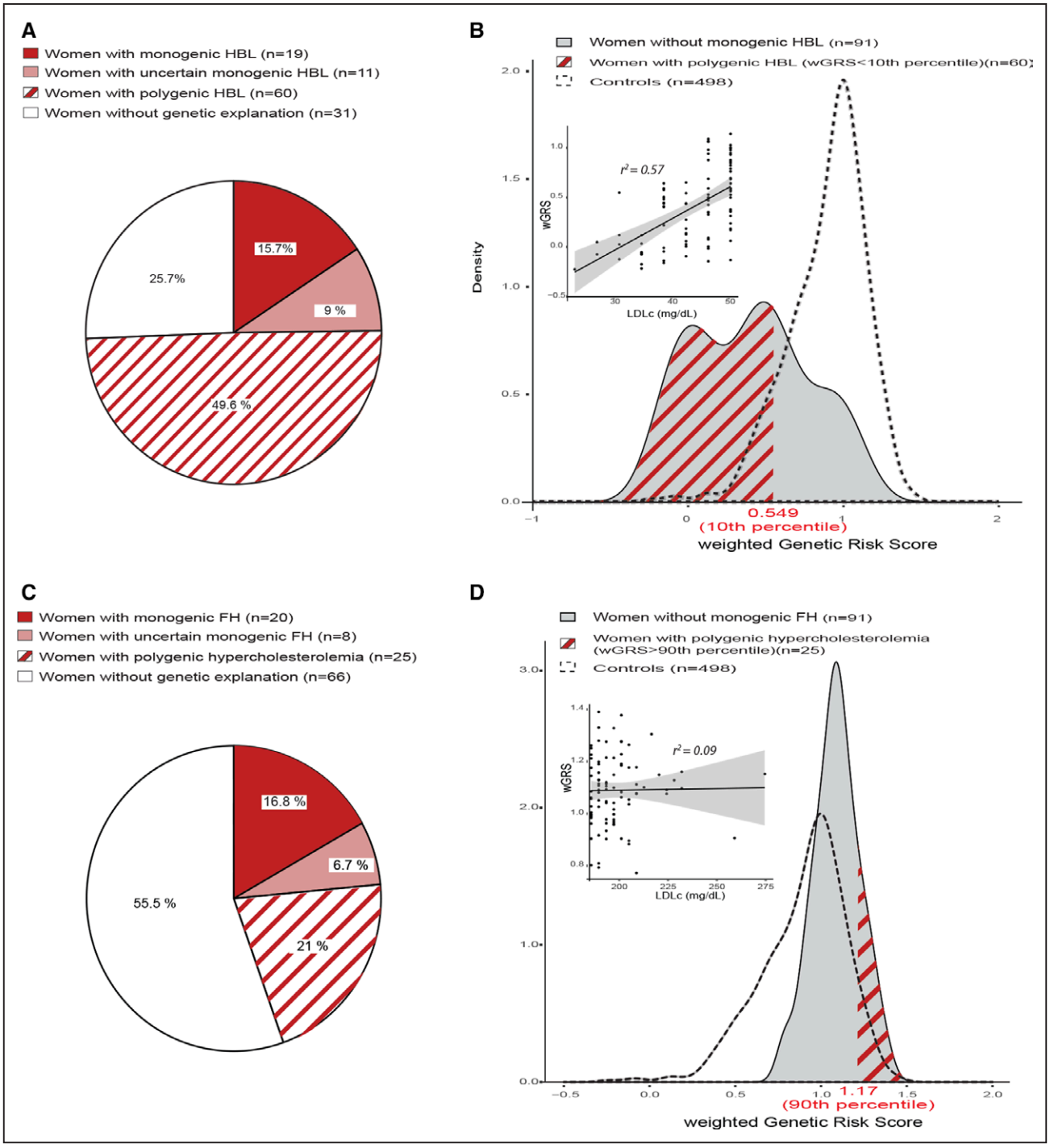

Figure. Monogenic and polygenic components of hypo- and hypercholesterolemia in young and apparently healthy women from LifeLines.

A, Prevalence of genetic determinants of hypobetalipoproteinemia (HBL) in young women with low-density lipoprotein cholesterol (LDL-C) ≤1st percentile. Among 121 women, 19 (15.7%) have a monogenic (pathogenic) HBL mutation (mutations in apolipoprotein B, proprotein convertase subtilisin/kexin type 9, and angiopoietin-like 3), 11 (9%) present a mutation of clinically uncertain significance in HBL genes, 60 (49.6%) present an extremely low-weighted genetic risk score (wGRS <10th percentile), whereas in 31 (25.7%), no genetic origin for a low LDL-C phenotype could be identified. **B,** Distribution of wGRS for women with LDL-C ≤1st percentile in comparison with the control group. The distribution of wGRS in women without a mutation in HBL genes (n=91) (average wGRS=0.40 [–0.225, 1.142]) is significantly shifted to the left in comparison with controls (average wGRS=0.896 [–0.239, 1.43]) (*P*=2.2E-16). Sixty women (49.6%) without a mutation in HBL genes have extremely low wGRS (<10th percentile [wGRS <0.549]). The embedded graph shows the correlation between LDL-C and wGRS in all 91 HBL mutation-negative women. LDL-C plasma levels significantly correlated with wGRS (Pearson correlation r^2=0.57, *P*=5.2E-9). Sequencing data from 498 unrelated individuals from the Genome of the Netherlands (*Continued*)

Figure Continued. project were used to determine the distribution of wGRS in a control population. **C,** Prevalence of genetic determinants of hypercholesterolemia in young women with LDL-C ≥99th percentile. Among 119 women, 20 (16.8%) have a monogenic (pathogenic) familial hypercholesterolemia (FH) mutation (mutations in low-density lipoprotein receptor or apolipoprotein B), 8 (6.7%) present a mutation of uncertain clinical significance in FH related genes, whereas 25 (21%) present an extreme wGRS (>90th percentile). In 66 (55.5%), no genetic origin for hypercholesterolemia could be identified. **D,** Distribution of wGRS for women with LDL-C ≥99th percentile in comparison with the control group. The distribution of wGRS in FH mutation-negative individuals (n=91) (average wGRS=1.25 [–0.225, 1.142]) is significantly shifted to the right in comparison with controls (average wGRS=0.896 [–0.239, 1.43]) (*P*=3.4e-13). Twenty-five women (21%) of FH mutation-negative women have extremely high wGRS (>90th percentile [wGRS>1.17]). The embedded graph shows the correlation between LDL-C and wGRS in FH mutation-negative women (n=91). LDL-C plasma levels do not correlate with wGRS (Pearson correlation r^2=0.09). Sequencing data from 498 unrelated individuals from the Genome of the Netherlands project were used to determine the distribution of wGRS in a control population. wGRS indicates weighted genetic risk score.

with uncertain monogenic hypobetalipoproteinemia (HBL)" (Figure A; Table III in the online-only Data Supplement). It is important to note that none of the pathogenic variants identified, were found in the high LDL-C or control groups (Tables IV and V in the online-only Data Supplement), which validates the extreme genetic approach used. In addition, none of the rare genetic variants identified in the control group was considered pathogenic (Table V in the online-only Data Supplement), which points at the usefulness of prediction algorithms and their ability to assign pathogenicity. In the entire cohort, we did not find homozygosity or compound heterozygotes in any of the genes studied. Furthermore, no mutations were identified in the other genes known to be involved in monogenic LDL-C disorders. Overall, plasma LDL-C levels were significantly lower in women with a monogenic origin of HBL than in the remainder of women in this group (37±8.5 versus 44±6.7 mg/dL; *P*<0.001) (Figure II in the online-only Data Supplement).

Table 2. Involvement of the Lifestyle Habits of Women in Whom No Genetic Origin of HBL Could Be Identified in Comparison With Control Cohorts

	Women Without Genetic HBL (n=31)	Lifelines Control Group I (n=60)	*P* Value	Lifelines Control Group II (n=25 898)	*P* Value
Healthy lifestyle factors, % (n)					
No current smoking	71 (22)	75 (45)	0.332	75 (19 458)	0.583
No obesity	90 (28)	87 (52)	0.612	86 (22 157)	0.450
Regular physical activity	39 (12)	28 (17)	0.314	42 (10 762)	0.745
Healthy diet	29 (9)	20 (12)	0.332	16 (4128)	0.046
Healthy lifestyle score, % (n)			0.204		0.153
Favorable (3/4 factors)	45 (14)	33 (20)		36 (9262)	
Intermediate (2 factors)	26 (8)	45 (27)		43 (11 120)	
Unfavorable (0/1 factors)	29 (9)	22 (13)		21 (5516)	
Demographic, lipid, and clinical characteristics					
Age, y	32±4.0	33±4.4	0.344	33±4.7	0.061
Total cholesterol (mg/dL)	120±15	166±15	<0.001	174±27	<0.001
LDL-C, mg/dL	46±4	97±8	<0.001	104±27	<0.001
HDL-C, mg/dL	62±15	62±12	0.338	58±15	0.145
Triglycerides, mg/dL	53 (39–65)	71 (53–97)	0.006	71 (53–89)	<0.001
SBP, mm Hg	114±9	117±11	0.192	116±11	0.195
BMI, kg/m^2	23.3±4.6	25.4±5.1	0.056	25.2±4.8	0.021
Glucose, mmol/L	4.6±0.4	4.7±0.5	0.110	4.7±0.6	0.204
HbA1c, %	5.2 (5.2–5.4)	5.4 (5.2–5.6)	0.052	5.4 (5.2–5.5)	0.021

Comparison of baseline characteristics and healthy lifestyle factors between women without genetic HBL with: (1) control group I consisting of 60 randomly selected women with normal LDL-C plasma levels between 89 and 108 mg/dL and (2) control group II composed of 25 898 women between 25 and 40 years of age and with LDL-C levels between 54 and 186 mg/dL. No obesity is defined as BMI <30 kg/m^2, physical activity is defined as weekly ≥150 minutes moderate physical activity or ≥ 75 minutes intensive physical activity, and the definition of a healthy diet is described in Methods.

BMI indicates body mass index; HbA1c, glycohemoglobin; HBL, hypobetalipoproteinemia; HDL-c, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; and SBP, systolic blood pressure.

Polygenic

To evaluate the polygenic component of the HBL phenotype, we compared the distribution of wGRS values of the women without monogenic HBL with controls of the Genome of the Netherlands study (n=498).[22] Overall, the distribution of wGRS was significantly shifted to the left for women without monogenic HBL. In fact, we identified a highly significant lower wGRS in these women than in controls (P=2.2E-16) (Figure B). Of the 91 women without monogenic HBL, 60 had an extremely low wGRS (<10th percentile of the controls; wGRS <0.5487) which may explain their very low LDL-C plasma levels (Figure B). This is supported by the notion that, in this group, the wGRS was strongly correlated with LDL-C plasma levels (P=5.2E-9, Pearson r^2=0.57; Figure B, Inset). Combined, these data suggest that very low LDL-C plasma levels in this group are likely of polygenic origin in 50% of the cases (60 of 121 women). Taken together, our genetic analysis highlights a strong genetic component in 66% (16% monogenic+50% polygenic) of the cases of hypocholesterolemia studied.

Lifestyle

To investigate the association between lifestyle and low LDL-C levels, we compared lifestyle factors between women in whom no genetic origin for HBL was identified with 2 control groups of similar ages (see Methods for details). Separately, the lifestyle factors were not different when comparing women without genetic HBL with Lifelines control groups I and II (Table 2). Only a healthier diet in comparison with Lifelines control group II just reached statistical significance (16% versus 29%; P=0.046). An assumedly healthy lifestyle score,[17] which combines the studied lifestyle factors, was not significantly different between hypocholesterolemic women and Lifelines control groups I and II (Table 2).

Extreme High LDL-C Plasma Levels in Young Women are Mainly Associated With Lifestyle

Monogenic

We identified 12 causal mutations in *LDLR* and 1 causal missense mutation in *APOB* (Table IV in the online-only Data Supplement). *LDLR* mutations included 2 splice variants, 9 missense mutations, and 1 large (2 exons) deletion. The latter has been validated by using multiplex ligation-dependent probe amplification as detailed in the online-only Data Supplement. Taken together, 20 of 119 (16.8%) women were molecularly diagnosed with FH (Figure C). Eight individuals (6.7%) carried variants in *LDLR* or *APOB* of uncertain clinical significance. Furthermore, no mutations were identified in the other genes known to be involved in monogenic LDL-C disorder. Again, none of the pathogenic mutations identified were found in the low LDL-C or control groups (Tables III and V in the online-only Data Supplement) and no homozygotes or compound heterozygotes for supposed deleterious mutations were identified. The mean LDL-C level in women with monogenic FH was significantly higher than in women without monogenic FH (221±26.4 versus 199±15.1 mg/dL; P<0.001; Figure III in the online-only Data Supplement).

Polygenic

The comparison of wGRS values of women without monogenic FH with controls of the Genome of the Netherlands study[22] showed that 25 of 91 (21%) had an extremely high wGRS (>90th percentile [wGRS > 1.17]). Figure D shows that the distribution of wGRS is significantly shifted to the right for FH mutation-negative individuals in comparison with controls (P<0.001). In contrast to our findings in the low LDL-C group, the wGRS does not show a direct correlation with LDL-C plasma levels in these individuals (Pearson r^2=0.09; Figure D, Inset). These data suggest that the polygenic component is a moderate driver of high LDL-C plasma in this study group.

Lifestyle

Table 3 shows that women without genetically defined hypercholesterolemia have lower prevalence rates of healthy lifestyle factors in comparison with Lifelines control group II (no current smoking: 59% versus 75%, P=0.002; no obesity: 56% versus 86%, P<0.001). Overall, the healthy lifestyle score appeared to be significantly different between women without genetic hypercholesterolemia versus Lifelines control group II (P<0.001): 2.5-fold more women presented with an unfavorable healthy lifestyle score (52% versus 21%). Conversely, fewer women were classified to exhibit a favorable healthy lifestyle score (21% versus 36%).

CONCLUSIONS

Genetic and lifestyle factors are known to modulate plasma levels of LDL-C but have not been well described previously in young women. This correlates with the notion that women are naturally protected against CVD[9]; however, it is also known that CVD represents the number 1 cause of death in women,[34] and that the underlying pathology, atherosclerosis, starts in early childhood.[35]

Because LDL-C represents a major and modifiable risk factor for CVD, we set out to study this parameter in apparently healthy premenopausal women with the lowest LDL-C (≤1st percentile; 50 mg/dL), or highest LDL-C (≥99th percentile; 186 mg/dL) for their age. In other words, the women studied here represent 2% of the general Dutch population in which we assessed monogenic and polygenic origins of these extreme LDL-C levels and investigated possible associations with

Table 3. Involvement of the Lifestyle Habits of Women in Whom No Genetic Origin of FH Could Be Identified in Comparison With Control Cohorts

	Women Without Genetic FH (n=66)	Lifelines Control Group I (n=60)	*P* Value	Lifelines Control Group II (n=25 898)	*P* Value
Healthy lifestyle factors, % (n)					
No current smoking	59 (39)	75 (45)	0.011	75 (19 458)	0.002
No obesity	56 (37)	87 (52)	<0.001	86 (22 157)	<0.001
Regular physical activity	32 (21)	28 (17)	0.670	42 (10 762)	0.108
Healthy diet	23 (15)	20 (12)	0.474	16 (4128)	0.863
Healthy lifestyle score, % (n)			0.002		<0.001
Favorable (3/4 factors)	21 (14)	33 (20)		36 (9262)	
Intermediate (2 factors)	27 (18)	45 (27)		43 (11 120)	
Unfavorable (0/1 factors)	52 (34)	22 (13)		21 (5516)	
Demographic, lipid, and clinical characteristics					
Age, y	33±4.5	33±4.4	0.891	33±4.7	0.498
Total cholesterol, mg/dL	271±19	166±15	<0.001	174±27	<0.001
LDL-C, mg/dL	197±15	97±8	<0.001	104±27	<0.001
HDL-C, mg/dL	50±12	62±12	<0.001	58±15	<0.001
Triglycerides, mg/dL	133 (106–168)	71 (53–97)	<0.001	71 (53–89)	<0.001
SBP, mm Hg	120±10	117±11	0.164	116±11	0.038
BMI, kg/m^2	29.3±5.5	25.4±5.1	<0.001	25.2±4.8	<0.001
Glucose, mmol/L	4.9±0.5	4.7±0.5	0.151	4.7±0.6	0.047
HbA1c, %	5.5 (5.3–5.7)	5.4 (5.2–5.6)	0.106	5.4 (5.2–5.5)	0.016

Comparison of baseline characteristics and healthy lifestyle factors between women without genetic FH with: (1) control group I consisting of 60 randomly selected women with normal LDL-C plasma levels between 89 and 108 mg/dL and (2) control group II composed of 25 898 women between 25 and 40 years of age and with LDL-C levels between 54 and 186 mg/dL. No obesity is defined as BMI <30 kg/m^2, physical activity is defined as weekly ≥150 minutes moderate physical activity or ≥75 minutes intensive physical activity and the definition of a healthy diet is described in Methods.

BMI indicates body mass index; FH, familial hypercholesterolemia; HbA1c, glycohemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; and SBP, systolic blood pressure.

lifestyle. Of note, we only investigated rare genetic variants (predicted to be pathogenic) that were exclusively associated with either low LDL-C or high LDL-C while none of these variants were found in women with normal LDL-C.

Selecting apparently healthy women from the general population for extreme LDL-C levels unexpectedly rendered 2 groups with opposed overall cardiometabolic phenotypes (Table 1), revealing LDL-C as an interesting biomarker. In the hypocholesterolemic women, we identified a remarkable strong genetic component (accounting for 66% of the cases), although a healthy lifestyle score was not significantly different in these women in comparison with controls. In the hypercholesterolemic women, the genetic component was less strong than in the hypocholesterolemic group, but it is important that 17% of these women were diagnosed with FH because of mutations in canonical genes. Remarkably, we identified a significant unfavorable lifestyle in 52% of the women without genetic hypercholesterolemia (in comparison with 21% in controls).

Genetic Determinants of Severe Hypocholesterolemia

Studies into the genetics of HBL have so far been almost exclusively restricted to patients who were referred to the clinic.[13] Our population-based study identified a monogenic origin for HBL in 16% of the cases. In 50% of the cases, our data suggest a polygenic predisposition for HBL. The latter novel finding could be strengthened with a strong linear, highly statistically significant relation between wGRS and LDL-C. Combined, our study shows, to the best of our knowledge, for the first time, that genetic variation in established LDL genes and loci associated with plasma LDL-C concentration can account for HBL in two-thirds of the women with LDL-C below the first percentile. We realize that, for any of the rare variants of predicted pathogenicity, segregation analysis in families and experimental laboratory studies, as well, are needed to prove functionality. This is in the current study not only true for included heterozygotes for, eg, rare *ANGPTL3* variants, but also for excluded variants in *APOB* and *PCSK9* because these were assigned to be of unknown significance (Tables III

and IV in the online-only Data Supplement). We would like to emphasize further that, in our extreme genetics study, all rare variants investigated were exclusively found in 1 of the 2 outer tails of the LDL-C distribution curve and absent in a control group. Taken together, the estimate that two-thirds of low LDL-C can be accounted for by genetics is, in our opinion, reasonable. This suggests that our current knowledge on the molecular basis of HBL may be quite complete.

Genetic Determinants of Severe Hypercholesterolemia

By using very strict criteria to assign causality to mutations, we here show that 17% of young women with LDL-C ≥99th percentile (ie, ≥186 mg/dL) for their age have molecularly defined FH. Recently, Abul-Husn et al[36] and Khera et al[25] reported much lower percentages of 2.5% and 1.7%, respectively, in individuals with LDL-C levels >190 mg/dL who participated in prospective cohort studies or coronary artery disease case studies. The marked differences may be related to the ethnic origins of the study subjects and the genetic screening methods used, but a more likely explanation for this large discrepancy is related to the fact that we studied young women (mean age 33 years), whereas Abul-Husn et al and Khera et al studied older men and women (mean ages of 53 and 61 years, respectively). When considering that women between 35 and 59 years of age show a 42% increase of LDL-C, it becomes clear that having an LDL-C ≥186 mg/dL as a young woman is an extreme phenomenon, whereas this is a more common finding in older women (for more detail, see cross-sectional Lifelines data[2]). Similarly, Wang et al[24] recently reported a much higher prevalence of causal FH mutations (53.7%) in patients with clinically ascertained FH with LDL-C >262 mg/dL clearly illustrating that stricter selection criteria for FH studies render higher success rates of molecular diagnosis.

As expected, most subjects with FH were heterozygote carriers for mutations in *LDLR* (90%), whereas 10% had mutations in *APOB*. Although Lifelines participants are predominantly inhabitants of the northern provinces of the Netherlands, comparable percentages have previously been reported of 9169 participants carrying FH mutations who underwent family cascade screening for FH in the Netherlands between 2003 and 2010.[37]

Lifestyle and Severe Hypo- and Hypercholesterolemia

To our surprise, the use of LDL-C as the sole selection parameter for this study led to the inclusion of women with an overall beneficial or detrimental cardiovascular/metabolic phenotype (Table 1) at the extreme tails of the LDL-C distribution. As such, LDL-C emerged as an interesting biomarker for cardiovascular health in this cohort. This finding further sparked our interest in the association between lifestyle and plasma LDL-C levels. We therefore studied lifestyle parameters of women without genetically related hypo- and hypercholesterolemia and 2 Lifelines control groups (60 women of similar age but with normal LDL-C, and 25 898 women aged between 25 and 40 years of age with LDL-C levels between 54 and 186 mg/dL). This analysis was conducted using a recently published healthy lifestyle score.[17]

When comparing our LDL-C study groups with controls, lifestyle was not associated with low LDL-C in women without genetic HBL. In contrast, lifestyle was significantly associated with severe hypercholesterolemia in young women without genetically associated hypercholesterolemia. To validate the use of the respective healthy lifestyle parameters to study the relation with LDL-C levels, Table VI in the online-only Data Supplement shows that the prevalence rates of each single healthy lifestyle factor decrease significantly with increasing LDL-C levels ($P<0.001$ for all parameters) in 26 541 Lifelines women between 25 and 40 years of age. In this large control cohort, an unfavorable lifestyle score was also significantly associated with increased LDL-C (Figure IV in the online-only Data Supplement). With this validation, our findings support close monitoring of especially obesity and smoking because these lifestyle parameters are associated with severe hypercholesterolemia in young women.

Genetic Screening and Lifestyle Evaluation Are Key Components for Hypercholesterolemia

The repercussions of genetic and lifestyle components of extreme LDL-C plasma levels described in this study are important in terms of molecular diagnostics, therapeutic care, and possibly future research directions to improve cardiovascular health in women.

The diagnosis of FH is becoming more and more dependent on molecular characterizations, because improvements in lifestyle, diet, and the use of lipid-lowering medication have changed the clinical expression of FH over the past decades.[38] For example, patients less frequently present with physical manifestations like tendon xanthomas and corneal arcus, and also the progression of CVD in index patients and family members seems to have attenuated over the past decades.[38] Finding a causal mutation in an index patient is thus an important step for screening of potentially affected family members: on average, for each novel index patient with FH, 8 family members with FH are identified.[39] In addition, knowing the precise

molecular diagnosis may guide the choice of therapy and secure a better prognosis in FH patients by receiving reimbursement for expensive PCSK9 antibodies.[40] It is important to note that our study in young individuals identified more FH-causing mutations than other studies of older individuals with a comparable LDL-C cutoff.[25,36] This suggests that, for FH screening in the young, the use of age- and sex-based LDL-C values (eg, the 99th percentile) may render better results than using a general fixed LDL-C cutoff. In other words, the current study points at an age- and sex-based LDL-C threshold for initiating molecular diagnostics in suspected female FH patients. Combining the 95th or 99th percentile of LDL-C with the Dutch Lipid Clinic Network Score may further improve the potential yield of finding mutations.

Our next-generation sequencing gene panel combined with a dedicated bioinformatics pipeline allows for a 1-step comprehensive analysis of established monogenic and polygenic factors that affect plasma LDL-C. This also includes the detection of copy number variations, thereby excluding the need of running multiplex ligation-dependent probe amplification tests to detect larger duplications and deletions in *LDLR* for FH diagnostics, while filling the gap of a lack of commercial multiplex ligation-dependent probe amplification kits of *APOB* and *PCSK9*. Platforms like this may enable rapid and comprehensive molecular assessment of individuals with suspected FH, and rare symptomatic hypocholesterolemia, as well. Our data substantiate that polygenic hypercholesterolemia is a common hereditary form of high LDL-C levels that is generally not accounted for in routine genetic testing. Individuals with polygenic hypercholesterolemia may therefore be falsely reassured when monogenetic tests are normal, although these individuals should be informed that they remain predisposed for increased CVD risk.

Our results furthermore support the need of a nationwide lipid-screening program in young women, which is underlined by the notion that our study cohort with high LDL-C represents 1% of the premenopausal female population of which 17% was found to have FH. These women are underdiagnosed and undertreated and are at increased risk of CVD.[41] However, despite the notion that recent reviews suggest that statins are probably not teratogenic and not directly linked with congenital anomalies,[42] these drugs are still avoided in pregnancy, and women with FH have to be intensively informed and medically well monitored during pregnancy.

Finally, this study suggests that an unfavorable lifestyle is significantly associated with severe hypercholesterolemia. Such a relation may be left unnoticed in extreme cases with a mere focus on a genetic origin of this phenotype.

Study Limitations

The state-of-the-art genetic pipeline used in the study makes use of prediction algorithms and clinical databases to assign pathogenicity to variants. However, statements of pathogenicity are not always based on appropriate functional (in vitro and in vivo) evidence, whereas segregation analysis in affected families is mostly lacking. This may have led to an overestimation of the effects of rare variants in our study. However, we have only identified and studied variants that were unique to individuals with LDL-C ≤1st percentile or LDL-C ≥99th percentile while they were not found in controls. This supports the hypothesis that these variants are functionally related to the LDL-C phenotypes. Furthermore, we used the same criteria as recently published to annotate variants as (likely) pathogenic.[25] The pathogenic missense variants identified in our study are described in Results in the online-only Data Supplement.[43]

This study demonstrates the need for early cardiovascular risk assessment in young women to identify those with severely elevated LDL-C levels for their age (≥99th percentile, ie, 1 in 100). First, because 17% of these women presented undiagnosed and untreated FH that dictates lifelong exposure to high LDL-C levels if left untreated. Second, because our study suggests that an unfavorable lifestyle is associated with severe hypercholesterolemia. Although this study cannot account for any causality, comprehensive lifestyle evaluation of patients with severe hypercholesterolemia (mimicking patients with mutations in canonical genes) is indicated; however, replication of our findings is warranted.

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Disclosures

None.

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