Plasma proprotein convertase subtilisin kexin type 9 is a heritable trait of familial combined hyperlipidaemia

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ABSTRACT

The aim of the present study was to investigate the relationship between circulating PCSK9 (proprotein convertase subtilisin kexin type 9) and FCHL (familial combined hyperlipidaemia) and, when positive, to determine the strength of its heritability. Plasma PCSK9 levels were measured in FCHL patients (n = 45), NL (normolipidaemic) relatives (n = 139) and their spouses (n = 72). In addition, 11 FCHL patients were treated with atorvastatin to study the response in PCSK9 levels. PCSK9 levels were higher in FCHL patients compared with NL relatives and spouses: 96.1 compared with 78.7 and 82.0 ng/ml (P = 0.004 and P = 0.002 respectively). PCSK9 was significantly associated with both TAG (triacylglycerol) and apolipoprotein B levels (P < 0.001). The latter relationship was accounted for by LDL (low-density lipoprotein)–apolipoprotein B (r = 0.31, P = 0.02), not by VLDL (very-low-density lipoprotein)–apolipoprotein B (r = 0.09, P = 0.49) in a subgroup of subjects (n = 59). Heritability calculations for PCSK9 using SOLAR and FCOR software yielded estimates of 67–84% respectively (P < 0.0001). PCSK9 increased from 122 to 150 ng/ml in 11 FCHL patients treated with atorvastatin (40 mg) once daily for 8 weeks (P = 0.018). In conclusion, plasma PCSK9 is a heritable trait associated with both FCHL diagnostic hallmarks. These results, combined with the significant rise in PCSK9 levels after statin therapy, warrant further studies in order to unravel the exact role of PCSK9 in the pathogenesis and treatment of this highly prevalent genetic dyslipidaemia.

INTRODUCTION

FCHL (familial combined hyperlipidaemia) is the most prevalent genetic dyslipidaemia in Western society (estimated prevalence 1 in 100) and accounts for a substantial proportion of cases with premature myocardial infarction [1]. The current opinion on its pathogenesis is that the typical dyslipidaemia, i.e. elevated plasma

Key words: cholesterol, dyslipidaemia, proprotein convertase subtilisin kexin type 9 (PCSK9), sterol-regulatory-element-binding protein 2 (SREBP2), statin.

Abbreviations: BMI, body mass index; FCHL, familial combined hyperlipidaemia; HDL, high-density lipoprotein; HOMA-IR, homoeostasis model assessment for insulin resistance; LDL, low-density lipoprotein; NL, normolipidaemic; PCSK9, proprotein convertase subtilisin kexin type 9; SREBP2, sterol-regulatory-element-binding protein 2; TAG, triacylglycerol; VLDL, very-low-density lipoprotein.

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apolipoprotein B and TAG [triacylglycerol (triglyceride)] levels, is the consequence of hepatic VLDL (very-low-density lipoprotein) overproduction, in the background of hepatic steatosis and insulin resistance, combined with an impaired clearance of remnants and LDL (low-density lipoprotein) particles [2]. Several genetic defects have already been implicated in the clearance pathway, such as the apolipoprotein A1/C3/A4/A5 gene cluster and the lipoprotein lipase gene [3–5].

PCSK9 (proprotein convertase subtilisin kexin type 9) is an important, inverse regulator of LDL particle clearance, since it promotes the degradation of the LDL receptor in hepatocytes by two different pathways, either intracellularly or as a systemically secreted protein that binds the LDL receptor, resulting in their combined internalization and subsequent degradation [6]. A high expression of PCSK9 is therefore positively associated with plasma LDL cholesterol levels [6]. Of interest, recent studies have suggested that PCSK9 is also implicated in the regulation of VLDL production [7,8]. PCSK9 is therefore an intriguing candidate to evaluate its involvement in the FCHL phenotype.

The systemic release of PCSK9 enables its measurement in plasma, which can be used to gain more insights into the relationship of PCSK9 with lipoprotein metabolism in large cohort studies. In the present study, we have measured plasma PCSK9 in a cohort of FCHL patients, NL (normolipidaemic) relatives and their spouses to examine its relationship with the FCHL phenotype and, when positive, to determine the strength of its heritability. Furthermore, a subgroup of FCHL patients was treated with standard lipid-lowering therapy to study the response in PCSK9 levels.

MATERIALS AND METHODS

Subjects

FCHL patients, NL relatives and their spouses visited our lipid clinic during the period 2003–2005, after an overnight fast, 3 days abstinance from alcohol and 2 weeks withdrawal from lipid-lowering medication [9]. Diagnosis of the FCHL-affected state was based on plasma apolipoprotein B levels >1.2g/l and plasma TAG levels >1.5 mmol/l [10]. Relatives of FCHL patients who did not fulfil these criteria at the time of their visit were designated as ‘normolipidaemic relatives’. These subjects were included as an additional control group, because previous studies have shown that several metabolic and vascular disturbances, such as fatty liver and arterial stiffness, are not only confined to FCHL patients, but are also present in NL relatives [11,12].

In addition, a subgroup of FCHL patients (n = 11) also visited our clinic before and after 8 weeks treatment with atorvastatin (40 mg) once daily. This substudy was designed as a single-arm, open-label study, without placebo controls. Subjects were asked to stop any lipid-lowering medication 4 weeks prior to initiation of the study (five subjects had been treated with statin monotherapy, three subjects with a combination therapy of a statin and fibrate and the remaining three subjects had not received any lipid-lowering therapy at the start of the study). Atorvastatin (40 mg) at bedtime was prescribed for 8 weeks as monotherapy.

All subjects gave written informed consent. The study was approved by the local medical ethical review board of the Maastricht University.

Measurements

Subjects were weighed in their underwear. BMI (body mass index) was calculated as weight divided by height squared. Waist circumference was measured in the standing position midway between the lower rib and the spina iliaca anterior superior.

Systolic and diastolic blood pressures were measured twice in sitting position after 10 min of rest (Omron 705CP).

Blood was collected, after an overnight fast, in pre-cooled EDTA-treated tubes. After centrifugation at 150g for 15 min at 4 °C, plasma aliquots were stored at −80 °C. Plasma TAGs, apolipoprotein B, total cholesterol, HDL (high-density lipoprotein)-cholesterol, insulin and glucose were measured as described previously [9]. An updated computer model, which was based on formula introduced by Matthews et al. [13] in 1985, was used to estimate HOMA-IR (homoeostasis model assessment for insulin resistance; (http://www.dtu.ox.ac.uk).

Plasma PCSK9 levels were measured in stored samples with the previously described PCSK9 dual monoclonal antibody sandwich ELISA [14]. Samples were shipped on solid CO2 and stored at −70 °C prior to analysis. The freeze–thaw stability was excellent with >90% recovery even after four freeze–thaw cycles. The intra-assay coefficient of variation was 3.9–8.9% [15]. The recently reported diurnal variation in plasma PCSK9 levels has introduced some additional scattering in PCSK9 concentrations [16], since blood was drawn between 09.00 h and 12.00 h, irrespective of the affected state.

In a subset of subjects, apolipoprotein B levels were also determined in the VLDL and LDL subfractions, isolated by ultracentrifugation as described by Redgrave et al. [17]. Apolipoprotein B levels in the VLDL subfraction were measured by gel electrophoresis according to the method of Karpe and Hamsten [18], whereas apolipoprotein B levels in the LDL subfraction were measured using the same method as in total plasma.

Statistical analyses

Results are presented as means ± S.D. or as median (interquartile range) in the case of non-normal
Plasma PCSK9 in FCHL

Table 1  Characteristics of FCHL patients, NL relatives and their spouses

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Spouses</th>
<th>NL relatives</th>
<th>FCHL patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male/female) (n)</td>
<td>39/33</td>
<td>65/74</td>
<td>17/28</td>
</tr>
<tr>
<td>Age (years)</td>
<td>51 ± 12</td>
<td>45 ± 15*</td>
<td>54 ± 13</td>
</tr>
<tr>
<td>Current smoking (%)</td>
<td>25</td>
<td>26</td>
<td>27</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.4 ± 4.1</td>
<td>25.2 ± 4.4</td>
<td>28.0 ± 3.9†</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>91.1 ± 12.7</td>
<td>92.5 ± 12.4†</td>
<td>99.2 ± 10.4†</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>131 ± 19</td>
<td>132 ± 18†</td>
<td>142 ± 17†</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>83 ± 11</td>
<td>84 ± 10</td>
<td>88 ± 10†</td>
</tr>
<tr>
<td>Antihypertensive medication (%)</td>
<td>14</td>
<td>20†</td>
<td>42†</td>
</tr>
<tr>
<td>TAG (mmol/l)</td>
<td>1.1 (0.8–1.7)</td>
<td>1.2 (0.9–1.7)†</td>
<td>2.0 (1.8–3.3)†</td>
</tr>
<tr>
<td>Apolipoprotein B (g/l)</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.3</td>
<td>1.4 ± 0.2†</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>5.4 ± 1.0</td>
<td>5.5 ± 1.8</td>
<td>7.0 ± 1.1†</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.3</td>
<td>0.9 ± 0.3†</td>
</tr>
<tr>
<td>Lipid-lowering medication (%)</td>
<td>8</td>
<td>21†</td>
<td>52†</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.7 (0.3–1.2)</td>
<td>0.9 (0.3–1.3)†</td>
<td>1.3 (0.9–1.8)†</td>
</tr>
</tbody>
</table>

†P < 0.05 compared with spouses; ††P < 0.05 compared with spouses, age- and sex-adjusted.

RESULTS

General characteristics of the study population

Characteristics of FCHL patients (n = 45), NL relatives (n = 139) and their spouses (n = 72) are shown in Table 1. NL relatives were significantly younger and there were more women in the FCHL group (P = 0.08) when compared with their spouses. Both FCHL patients and NL relatives were more abdominally obese and exhibited higher systolic blood pressure, plasma TAG and HOMA-IR levels when compared with their spouses. Plasma apolipoprotein B and diastolic blood pressure were elevated in FCHL patients when compared with their NL relatives and spouses (Table 1).

Plasma PCSK9 levels in FCHL patients, NL relatives and their spouses

Plasma PCSK9 levels (interquartile range) were significantly higher in FCHL patients in comparison with their spouses: 96.1 ng/ml (73.7–132.9) compared with 82.0 ng/ml (65.3–97.9), P = 0.002, and also in comparison with their NL relatives: 96.1 ng/ml (73.7–132.9) compared with 78.7 ng/ml (60.6–100.3), P = 0.004. No differences were observed between NL relatives and spouses (P = 0.51).

Additional adjustment for history of use of lipid-lowering and antihypertensive medication, BMI or waist circumference, HOMA-IR, current smoking and systolic/diastolic blood pressure did not affect the outcomes substantially (results not shown). Furthermore, re-analysis with only FCHL patients naïve to any lipid-lowering medication (n = 21) also demonstrated significantly higher PCSK9 levels when compared with spouses (P = 0.04), indicating that statin therapy did not

Heritability calculations

Heritability calculations were performed to estimate the extent to which the variation in plasma PCSK9 levels could be accounted for by genetic factors. Heritability calculations were performed exactly as described previously [9]. In short, the FCOR subprogram of the SAGE software package [19] was used to estimate the intraclass correlations for sibling pairs. The maximum heritability estimate ($h^2$) can be derived from the intraclass correlation ($r$): $h^2 = 2r$. In addition, the variance component model implemented in SOLAR [20], which includes all family relationships, was also used to estimate the familiality of PCSK9. Heritability estimates were also calculated for the apolipoprotein B and normalized TAG traits, both used as a reference. The transformation to normality was made prior to the heritability calculations.

Intraclass correlations for siblings were calculated as $r = 0.30$ for plasma PCSK9, $r = 0.28$ for plasma TAG, $r = 0.32$ for apolipoprotein B and $r = 0.33$ for TAG levels. These intraclass correlations were used to estimate the heritability ($h^2$) of plasma PCSK9 levels, which was calculated as $h^2 = 2r = 0.60$ for plasma PCSK9. Heritability estimates were also calculated for the apolipoprotein B and normalized TAG traits, both used as a reference. The transformation to normality was made prior to the heritability calculations.

distribution. Non-normally distributed parameters (TAG, HOMA-IR and PCSK9) were log-transformed prior to analyses. First, differences in PCSK9 concentrations between FCHL patients, NL relatives and their spouses were calculated with linear regression with adjustment for age and sex. Next, the associations between PCSK9 (main determinant) and apolipoprotein B and TAG (main outcomes) were determined. These analyses were adjusted for age, sex and family state, i.e. FCHL patient (yes or no) or NL relative (yes or no), included as dummy variables.

The change in plasma PCSK9 levels after treatment with atorvastatin was analysed with a paired-samples Student’s t test.

All statistical analyses were carried out with the use of SPSS (Statistical Package of Social Sciences) version 16.0 for Windows.
account for the observed differences in PCSK9 level between the groups of interest.

**Relationship between plasma PCSK9 levels and diagnostic hallmarks of FCHL**

To gain more insights into the relationship between plasma PCSK9 levels and diagnostic hallmarks of FCHL, we subsequently constructed multivariate regression models with plasma apolipoprotein B and TAG levels as dependent variables. Age- and sex-adjusted regression slopes for PCSK9 were significant for both variables in the overall population ($\beta = 0.002$, $P < 0.001$ for apolipoprotein B; $\beta = 0.002$, $P < 0.001$ for TAG; Table 2, model 1). Subsequent additional adjustment for family state, i.e. spouse, NL relative or FCHL patient, did not substantially affect these associations (Table 2, model 2).

However, the overall model goodness of fit increased significantly for both apolipoprotein B and TAG when family state was added to the model ($P < 0.001$ for the $R^2$ change for both variables, Table 2). This was explained by the variable FCHL patient (yes/no) in the case of apolipoprotein B ($P < 0.001$) and by the variables FCHL patient (yes/no) and NL relative (yes/no) in the case of TAG ($P < 0.001$ and $P = 0.04$ respectively). These results indicate that other factors in addition to PCSK9 also contribute to the FCHL phenotype.

Of interest, additional adjustment for HOMA-IR did not affect the model for apolipoprotein B (Table 2, model 3), whereas the overall model goodness of fit for plasma TAG changed from 0.50 to 0.63 ($P < 0.001$; Table 2, model 3). PCSK9 remained a significant determinant of both FCHL diagnostic hallmarks.

**Associations of plasma PCSK9 levels with apolipoprotein B in lipid subfractions**

Since plasma PCSK9 levels were significantly associated with both plasma TAGs (which are predominantly present in VLDL particles) and apolipoprotein B levels (which represent, in particular, LDL particle number and also IDL (intermediate-density lipoprotein) and VLDL particle number), we subsequently measured apolipoprotein B concentrations in VLDL and LDL subfractions in a sample of FCHL patients and NL relatives ($n=59$), as reported previously [21]. Of note, this subset of subjects displayed similar age, sex distribution and apolipoprotein B levels when compared with the FCHL patients and NL relatives presented in Table 1 (results not shown). Figure 1 shows that plasma PCSK9 levels were not significantly related to VLDL apolipoprotein B levels ($r = 0.09$, $P = 0.49$; Figure 1A), whereas a significant relationship was observed for LDL apolipoprotein B levels ($r = 0.31$, $P = 0.02$, Figure 1B).

**Heritability of plasma PCSK9 levels**

Since plasma PCSK9 levels were significantly associated with the FCHL phenotype, we subsequently estimated the extent to which plasma PCSK9 levels could be accounted for by genetic factors. Heritability calculations were performed in all FCHL relatives, i.e. the NL relatives and FCHL patients combined, resulting in 188 sibling pairs in total. The intraclass correlation ($r$) estimated with SAGE FCOR [19] in these 188 sibling pairs was 0.42 ± 0.05 ($P < 0.0001$), corresponding to a maximum heritability of 0.84 ($2r = h^2$). Sex-specified analyses also yielded significant outcomes: brother–brother (59 sibling pairs): $r = 0.22 ± 0.11$, $P = 0.05$; sister–brother (84 sibling pairs): 0.33 ± 0.04, $P < 0.0001$; sister–sister (45 sibling pairs): 0.44 ± 0.08, $P < 0.0001$. These maximum heritability estimates were high when compared with the estimates for apolipoprotein B and TAG: $r = 0.05 ± 0.05$, $P = 0.30$ and $r = 0.16 ± 0.05$, $P = 0.0006$ respectively.

Heritability estimates for PCSK9 levels when all family relationships were included, as estimated with SOLAR [20], were maximally 0.67 ± 0.19 ($P < 0.0001$). The heritability of apolipoprotein B and TAG was 0.25 ± 0.21 ($P = 0.10$) and 0.67 ± 0.22 ($P < 0.0001$) respectively.

**Plasma PCSK9 levels before and after treatment with atorvastatin**

Previous studies have demonstrated that plasma PCSK9 levels rise with statin therapy [22,23]. To study whether a similar effect can be observed in a population characterized by elevated plasma PCSK9 levels, we measured PCSK9 levels in 11 FCHL patients treated with statin therapy.
with atorvastatin (40 mg) once daily for a period of 8 weeks. LDL apolipoprotein B levels (interquartile range) decreased from 1.2 (0.9–1.3) g/l to 0.7 (0.6–0.8) g/l \( (P < 0.001) \). Median plasma PCSK9 increased significantly from 122.0 (81.9–188.0) ng/ml to 150.0 (113.0–228.0) ng/ml after 8 weeks of treatment \( (P = 0.018) \), Figure 2). The change in LDL apolipoprotein B levels was not associated with the change in PCSK9 levels in this small cohort \( (r = -0.18, P = 0.6) \).

**DISCUSSION**

The present study demonstrated that elevated circulating PCSK9 levels are a consistent feature of FCHL. Heritability calculations showed that a substantial proportion of the variation in plasma PCSK9 levels is accounted for by genetic factors. Our results therefore suggest that plasma PCSK9 levels are involved in the complex genetic background of FCHL.

Further analyses revealed that plasma PCSK9 levels are related to both diagnostic hallmarks of FCHL, i.e. plasma TAG and apolipoprotein B. Nevertheless, FCHL status remained associated with both hallmarks independent of PCSK9, implying that PCSK9 is not solely responsible for the FCHL phenotype. Indeed, previous reports and also the present study have assigned an important role to insulin resistance, as reflected by HOMA-IR, in the development of the hypertriglyceridaemic phenotype [24]. Furthermore, several other genetic defects have already been implicated in FCHL, such as upstream transcription factor 1 and the apolipoprotein A1/C3/A4/A5 gene cluster [3,4].

The relationship between plasma PCSK9 and apolipoprotein B levels, reflecting the total number of atherogenic particles in plasma, was largely explained by LDL particle number, as apolipoprotein B levels measured in the LDL, but not in the VLDL, subfraction were also related to PCSK9. These results are in agreement with the recently published association between LDL particle clearance and plasma PCSK9 levels; that is, PCSK9 is negatively associated with LDL particle clearance and thus positively related to LDL particle number in plasma [25]. The explanation for the relationship between circulating PCSK9 and plasma TAG levels in FCHL is, however, less clear. Although recent studies have linked PCSK9 to VLDL production [7,8], we did not observe a relationship between VLDL apolipoprotein B and PCSK9. However, the present study was not able to distinguish between VLDL production and clearance, which together determine VLDL apolipoprotein B levels.

Several explanations could be put forward for the high circulating PCSK9 levels in FCHL. A genetic cause is very likely, given the high heritability estimates that were observed for plasma PCSK9 (ranging from 67 to 84%). This novel finding implies that a maximum of 67–84% of the variability in plasma PCSK9 levels is accounted for by genetic factors. It should, however, be noted that other factors, such as a shared environment, can also contribute to these heritability estimates. Nevertheless, these values were higher than the estimates for the FCHL diagnostic hallmarks (10–25% for apolipoprotein B and 32–67% for TAG), which are in line with our previous report [9]. These outcomes advocate the use of the PCSK9 trait in the genetic delineation of complex lipid disorders, such as FCHL.

A direct, genetic explanation for the high PCSK9 levels in FCHL could be mutations in the PCSK9 gene itself. Some of these mutations have been associated with altered plasma PCSK9 levels [26]. Of interest, the PCSK9 gene is located on chromosome 1p32, which is near a locus that has repeatedly been associated with plasma apolipoprotein B levels in quantitative trait locus linkage
analyses [27–29]. Furthermore, Abifadel et al. [30] have demonstrated that mutations in the PCSK9 gene may be responsible for hyperlipidaemia in some but not all FCHL pedigrees [30].

An alternative, indirect explanation for the elevated plasma PCSK9 levels could be found in the pathways that determine the expression of PCSK9, either genetic or environmental in origin. PCSK9 is under the transcriptional control of at least two proteins, i.e. SREBP2 (sterol-regulatory-element-binding protein 2) and hepatocyte nuclear factor 1 and hepatocyte nuclear factor 1 transcriptional control of at least two proteins, i.e. or environmental in origin. PCSK9 is under the transcriptional control of at least two proteins, i.e. SREBP2 (sterol-regulatory-element-binding protein 2) and hepatocyte nuclear factor 1 [31–33]. Of interest, SREBP2 has also other downstream effects, such as increased LDL receptor expression and increased cholesterol synthesis (6,34). In this context, it is of interest that van Himbergen et al. [35] recently reported that plasma lathosterol levels, a plasma marker of cholesterol synthesis, are consistently elevated in FCHL patients [35]. Elevated plasma PCSK9 and lathosterol levels in FCHL patients could therefore point to a common causative pathway, i.e. SREBP2 activation, a possibility that deserves further investigation. Of note, a positive relationship between plasma lathosterol and PCSK9 levels has recently been demonstrated in cohorts of subjects after prolonged fasting [36,37].

SREBP2 activation also accounts for the statin-induced expression of the LDL receptor and the subsequent reduction in LDL cholesterol levels [6]. Previous reports have demonstrated that statin therapy is accompanied by a rise in plasma PCSK9 levels [22,23], which leads to the degradation of the LDL receptor and is thought to blunt the cholesterol-lowering effects of statin therapy [6]. It has recently been speculated that the physiological SREBP2-mediated suppression of PCSK9, as observed during prolonged fasting, aims to preserve plasma LDL cholesterol levels [16]. Apparently, non-physiological impairment of HMGCoA (3-hydroxy-3-methylglutaryl-CoA) reductase, and subsequent stimulation of PCSK9 and the LDL receptor, results in a net decrease in LDL cholesterol levels. In this respect, the observed increase in PCSK9 levels after statin therapy in FCHL patients is not unique, although previous reports have not confined their findings specifically to this entity [22,23]. It is nevertheless of clinical relevance to report this observation, since it suggests that particular FCHL patients who are characterized by already elevated PCSK9 levels could benefit from PCSK9-antagonizing therapy to augment the effects of statin therapy. These antagonizing drugs are currently under development [38].

In conclusion, the present study has demonstrated that plasma PCSK9 is a highly heritable trait that is associated with both diagnostic hallmarks of FCHL. Our results warrant further genetic studies with plasma PCSK9 as a trait (potentially combined with lathosterol levels) to gain more insights into the development of the FCHL phenotype. Furthermore, the further rise in plasma PCSK9 levels after initiation of statin therapy suggests that FCHL patients in particular could benefit from PCSK9-antagonizing therapy.

**AUTHOR CONTRIBUTION**

Martijn Brouwers and Marleen van Greevenbroek designed the study protocol. Data collection was performed by Martijn Brouwers. Laboratory analyses were performed by Jason Trount, Angela Bonner Freeman and Robert Konrad. Statistical analyses were performed by Martijn Brouwers and Ake Lu. Martijn Brouwers, Marleen van Greevenbroek, Jason Trount, Niolaas Schaper, Robert Konrad and Coen Stehouwer interpreted the data and wrote the paper.

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