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To cite this version:
Sophie Valleix, Guglielmo Verona, Noemie Jourde-Chiche, Brigitte Nédelec, Patrizia Mangione, et al. D25V apolipoprotein C-III variant causes dominant hereditary systemic amyloidosis and confers cardiovascular protective lipoprotein profile. Nature Communications, 2016, pp.10353. 10.1038/ncomms10353. hal-01467259

HAL Id: hal-01467259
https://amu.hal.science/hal-01467259
Submitted on 9 Jun 2021

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D25V apolipoprotein C-III variant causes dominant hereditary systemic amyloidosis and confers cardiovascular protective lipoprotein profile

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Apolipoprotein C-III deficiency provides cardiovascular protection, but apolipoprotein C-III is not known to be associated with human amyloidosis. Here we report a form of amyloidosis characterized by renal insufficiency caused by a new apolipoprotein C-III variant, D25V. Despite their uremic state, the D25V-carriers exhibit low triglyceride (TG) and apolipoprotein C-III levels, and low very-low-density lipoprotein (VLDL)/high-density lipoprotein (HDL) profile. Amyloid fibrils comprise the D25V-variant only, showing that wild-type apolipoprotein C-III does not contribute to amyloid deposition in vivo. The mutation profoundly impacts helical structure stability of D25V-variant, which is remarkably fibrillogenic under physiological conditions in vitro producing typical amyloid fibrils in its lipid-free form. D25V apolipoprotein C-III is a new human amyloidogenic protein and the first conferring cardioprotection even in the unfavourable context of renal failure, extending the evidence for an important cardiovascular protective role of apolipoprotein C-III deficiency. Thus, fibrate therapy, which reduces hepatic APOC3 transcription, may delay amyloid deposition in affected patients.

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There is growing evidence that apolipoprotein C-III (apoC-III), a small exchangeable apolipoprotein carried in the circulation by VLDL and HDL-1, is a master regulator of plasma TG homeostasis and downregulation of its expression has a cardiovascular-protective effect. ApoC-III inhibits the activity of lipoprotein lipase (LPL), the enzyme catalysing the first and rate-limiting step of TG-rich lipoprotein catabolism, and also impairs uptake of TG-rich lipoprotein remnants by the low-density lipoprotein (LDL)-receptor (LDL-R), increasing remnant residence time in the circulation. ApoC-III is a glycosylated apolipoprotein composed of 79 amino acids, synthesized mainly in the liver, and existing in three isoforms, apoC-III1, apoC-III2 and apoC-III4, with, respectively, 0, 1 and 2 residues of sialic acid per molecule, but little is known about sialylation of apoC-III in disease states. Heterozygosity for the rare null allele of APOC3, R19X, has been associated with high levels of plasma HDL cholesterol (HDL-C), low levels of plasma TG and reduced incidence of ischaemic cardiovascular disease (CVD) in the Amish population. Subsequently, genome-wide association studies also identified the Arg19Stop variant in other members of the apoC-III family, including the APOC1, APOC2, APOC4, APOA5, and APOE genes. The unique variant identified was a single-base substitution (c.134A>G) in exon 3 of APOC3, encoding replacement of the negatively charged aspartate residue at position 25 of the mature apoC-III by a valine residue (D25V; Fig. 1). Both of them subsequently developed hypertension and stage II CVD. The proband’s son (IV.1) suffered from sicca syndrome aged 30 years and showed amyloid deposition on salivary gland biopsy (Supplementary Fig. 1), but he had no hypertension and his renal function was normal with a serum creatinine level of 7.3 mg dl\(^{-1}\) and no proteinuria. The proband’s daughter (IV.2) was clinically asymptomatic, and had no amyloid deposits on salivary gland biopsy.

### Amyloid and hypotriglyceridemia in D25V apoC-III carriers.

An initial genetic screening for mutations in all genes recognized until now to be linked to hereditary amyloidosis was negative (see Methods for details). Based on the fact that affected patients with amyloidosis also displayed hypotriglyceridemia and increased plasma levels of HDL-C as compared with non-carriers in the family (Table 1), we next screened several candidate genes including the APOC3, APOC1, APOC2, APOC4, APOA4, APOA5 and APOE genes. The unique variant identified was a single-base substitution (c.134A>G) in exon 3 of APOC3, encoding replacement of the negatively charged aspartate residue at position 25 of the mature apoC-III by a valine residue (D25V; Fig. 1d). All D25V-carriers displayed a 30–50% decrease in plasma apoC-III concentration (Table 1), and there was no correlation between APOC3 promoter polymorphism genotypes and plasma TG levels among family members (Fig. 1a). Therefore, D25V apoC-III variant was uniquely found in family members affected with amyloidosis, hypotriglyceridemia and reduced apoC-III levels, and not in healthy normotriglyceremic family members, demonstrating the co-inheritance of this variant with both the lipidemic and amyloidogenic phenotypic traits.

### Histology and LMD/MS-proteomic analysis of amyloid deposits.

To establish that apoC-III D25V variant is the causative amyloidogenic protein, we analysed amyloid deposits by immunohistochemical staining, immunogold technology and proteomic methodologies. Amyloid deposits were discovered in a total of 17 diverse disease tissues from 6 different affected family members through three generations (kidney, salivary glands, heart, bowel, fat, and bronchia from III.3; kidney, salivary glands, skin, heart, liver from III.5; salivary glands, kidney, skin, kidney, bowel from II.3; salivary glands from IV.1, IV.3 and IV.4).
All patients with sicca syndrome, the first clinical symptom, were carriers of D25V (III.3, III.5, IV.1, IV.3 and IV.4) and had amyloid deposits in salivary gland biopsies. Conversely, clinically non-affected individuals and non-carriers of D25V (III.1 and IV.2) demonstrated no amyloid deposits on salivary gland biopsies. In the proband III.3, all amyloid deposits observed in salivary gland, digestive tract and kidney were Congo red positive and immunoreactive with the anti-apoC-III antibody (Fig. 1e–g). Moreover, extensive amyloid deposits were found around salivary acini, in the interstitial matrix and in the walls of blood vessels, and were associated with areas of fibrosis and massive acinar atrophy (Fig. 1e). Similar pathological findings in salivary glands from younger affected family D25V-carriers (individuals IV.1, IV.3 and IV.4) were found and are shown in Supplementary Fig. 1. Therefore, this extensive amyloid accumulation associated with gland atrophy could explain the sicca symptoms of mouth reported by the patients. Renal histology from affected proband III.3 at an advanced stage of renal insufficiency showed diffuse amyloid deposition in the renal cortex that mostly affected the vascular compartment with abundant amyloid deposits in the walls of renal arterioles leading to lumen obliteration (Fig. 1g,h). Deposits were also observed in the glomeruli with prominent mesangial distribution, within the renal interstitium, and in the peritubular basement membranes with tubular atrophy. Amyloid...
mass spectrometry (LMD/MS)-based proteomics on three diagnosis of apoC-III amyloidosis. Moreover, the fact that anti-apoC-III antibody directly bound to Congo red-staining amyloid areas, whereas there was no binding of antibodies against the other known amyloid fibril proteins. In all 11 affected tissues from 6 D25V-carriers over three generations, the anti-apoC-III antibody consistently and specifically bound to all Congo red-staining amyloid areas, whereas there was no binding of antibodies against the other known amyloid fibril proteins. Moreover, the fact that anti-apoC-III antibody directly bound to the fibrilar aggregates confirms at the ultrastructural level the diagnosis of apoC-III amyloidosis.

In addition to immunofluorescence and immunogold staining, amyloid subtyping was also confirmed by laser microdissection/mass spectrometry (LMD/MS)-based proteomics on three different tissues: digestive tract and heart from proband III.3, and salivary glands from individual IV.3, with several amyloid deposits microdissected per tissue (Fig. 2a,b and Supplementary Table 1). In all proteomes, only the variant apoC-III containing the valine at position 45 (corresponding to position 25 of the mature protein) and not the corresponding apoC-III wild-type peptide with the aspartate identified from the amyloid plaques from the heart and digestive tract, respectively. All amyloid plaques contained only the mutated apoC-III peptide with the wild-type apoC-III sequence with the valine at position 45 (corresponding to position 25 of the mature protein) and not the corresponding apoC-III wild-type peptide with the aspartate.

P component co-localized with the above-mentioned deposits, and vascular, glomerular, tubular and interstitial amyloid deposits were positively and specifically immunoreactive with apoC-III antibody (Fig. 1g). Electron micrographs of the kidney specimen from III.3 showed numerous non-branched and randomly arranged deposits with a fibrillar appearance, measuring 7–10 nm in diameter, typical of amyloid (Fig. 1h). Immunoelectron microscopic analysis subsequently showed that these renal fibrillar aggregates were specifically labelled by gold-conjugated anti-apoC-III antibody (Fig. 1g). Electron micrographs of the kidney specimen from III.3 showed numerous non-branched and randomly arranged deposits with a fibrillar appearance, measuring 7–10 nm in diameter, typical of amyloid (Fig. 1h). Immunoelectron microscopic analysis subsequently showed that these renal fibrillar aggregates were specifically labelled by gold-conjugated anti-apoC-III antibody (Fig. 1g). Electron micrographs of the kidney specimen from III.3 showed numerous non-branched and randomly arranged deposits with a fibrillar appearance, measuring 7–10 nm in diameter, typical of amyloid (Fig. 1h). Immunoelectron microscopic analysis subsequently showed that these renal fibrillar aggregates were specifically labelled by gold-conjugated anti-apoC-III antibody (Fig. 1g). Electron micrographs of the kidney specimen from III.3 showed numerous non-branched and randomly arranged deposits with a fibrillar appearance, measuring 7–10 nm in diameter, typical of amyloid (Fig. 1h). Immunoelectron microscopic analysis subsequently showed that these renal fibrillar aggregates were specifically labelled by gold-conjugated anti-apoC-III antibody (Fig. 1g). Electron micrographs of the kidney specimen from III.3 showed numerous non-branched and randomly arranged deposits with a fibrillar appearance, measuring 7–10 nm in diameter, typical of amyloid (Fig. 1h). Immunoelectron microscopic analysis subsequently showed that these renal fibrillar aggregates were specifically labelled by gold-conjugated anti-apoC-III antibody (Fig. 1g). Electron micrographs of the kidney specimen from III.3 showed numerous non-branched and randomly arranged deposits with a fibrillar appearance, measuring 7–10 nm in diameter, typical of amyloid (Fig. 1h). Immunoelectron microscopic analysis subsequently showed that these renal fibrillar aggregates were specifically labelled by gold-conjugated anti-apoC-III antibody (Fig. 1g).
and histologic and proteomic analyses were consistent with the genetic findings.

MS analysis of apoC-III from D25V-HDLs. To determine the relative amounts of wild-type and mutant apoC-III as well as the proportions of apoC-III isoforms in HDL2 and HDL3, MS was carried out in these lipoproteins from hypotriglyceridemic D25V carriers III.3, IV.3 and IV.4 and normotriglyceridemic non-D25V-carrier III.4. The resulting MS profiles showed that D25V-carriers exhibited a double peak for apoC-III1 and apoC-III2 isoforms (only the profile of individual IV.3 is presented), whereas the non-carrier of D25V had a normal profile (Fig. 3a). The measured mass difference of 15.5 Da between the two signals is in agreement with the difference between the apoC-III wild type (ApoC-III0) and the D25V variant (ApoC-III1) (Table 1).

Table 1: Peptide sequence and mass spectrometric identification of apoC-III in HDL2/3 using LC-MS/MS sequencing.

| Apolipoprotein C-III | Protein identity | Peptide sequence | D25V carrier | Non-carrier | Alignment |
|----------------------|------------------|------------------|--------------|-------------|-----------|
| m/z 2,021.0565       | 61-71            | 61-71            | m/z 2,005.0979 | 61-71       | m/z 2,005.0979 |
| m/z 2,515.2668       | 21-37            | 21-37            | m/z 1,064.6672 | 21-37       | m/z 1,064.6672 |
| m/z 1,506.8412       | 72-78            | 72-78            | m/z 2,153.0186 | 72-78       | m/z 2,153.0186 |
| m/z 1,805.0054       | 81-99            | 81-99            | m/z 1,084.6869 | 81-99       | m/z 1,084.6869 |

Figure 3 | MS characterization of wild-type and D25V apoC-III isoforms in HDL2 and HDL3. (a) MS analysis of apoC-III isoforms from HDL2/HDL3. Representative graphs of apoC-III isoforms from D25V carrier IV.3 and non-carrier III.4 are shown. Three apoC-III isoforms are commonly referred to as apoC-III1, apoC-III2, and apoC-III0, where the index represents the number of sialic acid residues in each isoform. The subscript lower case letter indicates the absence of glycan (apoC-III0) or the presence of a GalNAc-Gal disaccharide (apoC-III0c), respectively. The arrow points to the peak corresponding to the D25V variant of apoC-III1 isoforms with a reduced mass of 15,500 Da (apoC-III1: 9,421.61; apoC-III2: 9,714.40) in comparison to the wild-type apoC-III isoforms (apoC-III1: 9,406.11; apoC-III2: 9,708.90) in comparison to the wild-type apoC-III isoforms (apoC-III1: 9,412.61; apoC-III2: 9,714.40). The variant apoC-III1 and apoC-III2 isoforms are detected only in D25V-carriers. (b) Higher magnification of apoC-III1 from the D25V-carrier IV.3 and a non-carrier III.4. (c) Mass spectrometric identification of apoC-III in HDL2/3 using LC-MS/MS sequencing. The amino-acid sequences obtained using MS/MS are in bold. Specific wild-type or mutated peptides are in red. The observed m/z of peptides (including variable modifications) are listed in the table with their position in the apoC-III protein sequence.
HDL particles were compared on the basis of their total mass. Lipoprotein oxidation was induced by the azo-initiator 2,2'-azo-bis-(2-amidinopropane) hydrochloride (final concentration 1 mM) to inhibit LDL oxidation was assayed at a physiological HDL to LDL ratio.

Specific cholesterol efflux was calculated as $(\text{medium cpm})/(\text{medium cpm} + \text{cell cpm}) \times 100\%$. Specific cholesterol efflux was determined by subtracting nonspecific cholesterol efflux occurring in the absence of cholesterol acceptors (HDL). Relative quantification of each apoC-III isoform in three D25V-carriers (III.3, IV.3 and IV.4) was performed in the three D25V-carriers (III.3, IV.3 and IV.4) and one normolipidemic control subject. In each case 200 µl of serum was analyzed using two GE Healthcare 10/300 GL Superose 6 columns coupled in series, equilibrated and eluted with a buffer containing 0.15 M NaCl, 3 mM EDTA, 0.04% sodium azide, pH 7.4 at 0.4 ml min$^{-1}$. Triglyceride (TG) and total cholesterol (TC) were determined in each fraction. The profile of several lipid classes in the proband’s plasma (Fig. 4a). A detailed analysis of all lipoprotein constituents shows a fivefold increase in HDL2 (Table 3) but a lower apoC-III content in the proband’s HDL2/HDL3 fractions (Supplementary Fig. 2). In addition, LDL particles from III.3 were enriched in esterified and free cholesterol, and in phospholipid, but depleted in TG and protein, a composition typical of hyperalphalipoproteinemic HDL (Table 4). The profile of several lipid classes in the proband’s HDL was altered (Table 5). Although the proband had CKD, his HDL maintained an elevated cholesterol efflux capacity compared with HDL of the normolipidemic control (Fig. 4b), whereas the antioxidative activities of total HDL and small, dense HDL3c were similar between the proband and the normolipidemic control subject (Fig. 4c).

Figure 4 | Lipoprotein studies. (a) Fast protein liquid chromatography (FPLC) size exclusion chromatogram of lipoproteins of III.3 in comparison to a normolipidemic control subject. In each case 200 µl of serum was analyzed using two GE Healthcare 10/300 GL Superose 6 columns coupled in series, equilibrated and eluted with a buffer containing 0.15 M NaCl, 3 mM EDTA, 0.04% sodium azide, pH 7.4 at 0.4 ml min$^{-1}$. Triglyceride (TG) and total cholesterol (TC) were determined in each fraction. (b) Cholesterol efflux capacity of HDL. THP-1 macrophages were cholesterol-loaded by incubation with [3H]cholesterol-labelled acetylated LDL (1 µCi ml$^{-1}$) for 24 h as detailed in the Methods. Cellular cholesterol efflux to HDL3c and total HDL from III.3 and one normolipidemic control subject (15 µg ml$^{-1}$ LDL-PL per well) was measured in serum-free medium for a 4 h chase period. The percentage of cholesterol efflux was calculated as $(\text{medium cpm})/(\text{medium cpm} + \text{cell cpm}) \times 100\%$. Specific cholesterol efflux was determined by subtracting nonspecific cholesterol efflux occurring in the absence of cholesterol acceptors (HDL). (c) Antioxidative activity of HDL. The antioxidative capacity of HDL3c and total HDL from III.3 and one normolipidemic control subject to inhibit LDL oxidation was assayed at a physiological HDL to LDL ratio. HDL particles were compared on the basis of their total mass. Lipoprotein oxidation was induced by the azo-initiator 2,2'-azo-bis-(2-amidinopropane) hydrochloride (final concentration 1 mM). Oxidation was expressed as the concentration of lipid hydroperoxides with conjugated diene structure in LDL.

Table 2 | Relative quantification of apoC-III isoforms in D25V-carriers.

| ApoC-III isoform | HDL2 | HDL3 |
|------------------|------|------|
|                   |  III.4 |  III.3 |  IV.3 |  IV.4 |  III.4 |  III.3 |  IV.3 |  IV.4 |
| D25V +/−         | 8.2   | 11.8  | 3.8   | 5.4   | 11.2  | 17.8  | 5.9   | 5.9   |
| D25V −/−         | 60.3  | 44.1  | 46.4  | 51.4  | 62.7  | 45.9  | 53.7  | 53.7  |
| Ratio WT/D25V Apo-CIII | —     | 2.4   | 2.1   | 2.4   | 2.1   | 2.0   | 2.0   |
| D25V +/−         | 31.5  | 44.1  | 49.8  | 45.2  | 26.1  | 36.3  | 40.4  | 40.4  |
| D25V −/−         | 1.9   | 1.6   | 1.7   | 1.7   | —     | 1.6   | 2.0   | 1.8   |

apoC-III, apolipoprotein C-III; WT, wild type. Relative quantification of each apoC-III isoform in three D25V-carriers (III.3, IV.3 and IV.4) in comparison with a healthy non-D25V-carrier (III.4). The ratio of apoC-III isoforms is modified in the D25V-carriers and less D25V variant than wild-type apoC-III is present in HDL2/HDL3.
hyperalphalipoproteinemia of apoC-III-D25V carriers confer an atheroprotective profile. This was corroborated by clinical data (detailed imaging of coronary angiography, pulse arterial Doppler ultrasound of the lower limbs and supra-aortic trunks, computed tomography (CT) angiography of the aorta and iliac vessels) establishing the absence of atheroma or vascular wall calcification, whereas carotid intima-media thickness was normal in the proband and his sister (III.5). Similarly, there was no record of ischaemic CVD in D25V-patients I.2 or II.1, who died aged 74 and 82 years, respectively.

Analysis of structure, function and aggregation propensity. Predictive analysis of the secondary structure of the apoC-III polypeptide in its lipid-free state revealed that the D25V mutation enhances both its beta-sheet content and aggregation propensity (Fig. 5a,b). Although wild-type and D25V apoC-III initially showed similar circular dichroic spectra, the mutation appears to cause a transition towards prominent beta-sheet content associated with rapid formation of aggregates (Fig. 6a), which were shown to increase the thioflavin T fluorescence, the typical signature of amyloid fibrils (Fig. 6b). Transmission electron microscopy (Fig. 6c) and atomic force microscopy (Fig. 6d) confirmed the presence of fibrillar structures in the D25V apoC-III aggregated material.

The effect of the mutation on the lipid binding was also carried out using dimyristoylphosphatidylcholine (DMPC) multilamellar vesicles. Analysis of the binding curves based on the decrease in turbidity at 325 nm showed that the time required to reduce by 50% the initial turbidity of the vesicles was 168.7 ± 8.1 s for the variant and 114.7 ± 4.2 s for the wild-type, respectively (Fig. 7a), suggesting that the mutation may cause a less efficient lipid binding similar to other apoC-III variants.

### Table 3 | Lipoprotein composition.

| Protein | TG (mg dl⁻¹) | (%) | TC (mg dl⁻¹) | (%) | PL (mg dl⁻¹) | (%) | Total (mg dl⁻¹) |
|---------|--------------|-----|-------------|-----|--------------|-----|----------------|
| Control |              |     |             |     |              |     |                |
| VLDL    | 12.55        | (9.3) | 87.80       | (65.0) | 8.55         | (6.3) | 26.26          | (19.4) | 135.16        |
| IDL     | 4.56         | (20.6) | 8.45        | (38.1) | 2.99         | (13.5) | 6.16           | (27.8) | 22.16         |
| LDL     | 82.90        | (35.2) | 13.72       | (7.2)  | 39.13        | (20.4) | 71.47          | (37.3) | 207.22        |
| HDL2    | 22.79        | (47.4) | 2.86        | (5.9)  | 8.19         | (17.0) | 14.26          | (29.6) | 48.09         |
| HDL3    | 93.15        | (63.1) | 4.63        | (3.1)  | 13.23        | (9.0)  | 36.70          | (24.9) | 147.71        |
| Proband (III.3) | |     |             |     |              |     |                |
| VLDL    | 3.55         | (11.4) | 20.39       | (65.7) | 1.60         | (5.1)  | 5.51           | (17.8) | 31.04         |
| IDL     | 0.42         | (4.9)  | 3.87        | (45.9) | 1.32         | (15.7) | 2.82           | (33.4) | 8.43          |
| LDL     | 45.80        | (31.7) | 11.36       | (7.9)  | 30.98        | (21.4) | 56.53          | (39.1) | 144.67        |
| HDL2    | 117.22       | (47.5) | 5.02        | (2.0)  | 40.19        | (16.3) | 84.63          | (34.3) | 247.06        |
| HDL3    | 62.97        | (56.6) | 2.79        | (2.5)  | 12.72        | (11.4) | 32.79          | (29.5) | 111.27        |

Table 4 | Chemical composition (WT%) of HDL subpopulations.

| Subjects | HDL2b | HDL2a | HDL3a | HDL3b | HDL3c |
|----------|-------|-------|-------|-------|-------|
| CE (WT%) | Proband | 29.8 | 21.1 | 22.2 | 27.0 | 20.1 |
| Control  | 26.0 | 21.6 | 13.1 | 6.2 | 4.2 |
| FC (WT%) | Proband | 7.5 | 3.1 | 3.8 | 6.2 | 4.2 |
| Control  | 6.5 | 4.1 | 3.3 | 4.5 | 1.7 |
| PL (WT%) | Proband | 5.02 | 2.0 | 1.4 | 1.9 | 2.0 |
| Control  | 3.1 | 2.5 | 1.9 | 2.0 | 1.0 |
| TG (WT%) | Proband | 2.2 | 1.4 | 1.4 | 1.9 | 2.1 |
| Control  | 3.1 | 2.5 | 1.9 | 2.0 | 1.0 |
| Total protein (WT%) | Proband | 28.6 | 14.9 | 46.0 | 32.9 | 47.1 |
| Control  | 32.4 | 44.4 | 47.6 | 55.4 | 67.9 |

### Table 5 | Phospholipid and sphingolipid composition (WT%) of total HDL.

| PC | LPC | SM | Cer | PE | PI | PS | PG | PA |
|----|-----|----|-----|----|----|----|----|----|
| Proband | 78.6 | 0.49 | 18.1 | 0.211 | 1.2 | 1.1 | 0.030 | 0.008 | 0.013 |
| Control  | 83.0 | 0.44 | 12.9 | 0.160 | 1.8 | 1.7 | 0.055 | 0.006 | 0.006 |

CE, cholesteryl ester; FC, free cholesterol; HDL, high-density lipoprotein; PL, phospholipid; TG, triglyceride. Data are shown as WT% of total HDL mass. The proband corresponds to individual III.3, and the control to one healthy normolipidemic subject.

Data are shown as WT% of total HDL mass. The proband corresponds to individual III.3, and the control to one healthy normolipidemic subject.
However, the variant was integrated in the vesicles as shown in the native electrophoresis (inset, Fig. 7a). The native electrophoresis revealed the strong propensity of the variant to self-aggregate in its free form when it aggregated and precipitated at the site of deposition (inset, Fig. 7a). Solubility of D25V apoC-III was, however, rescued when the variant was integrated within the vesicles. We have also investigated the effect of the mutation on the well-known inhibition of LPL activity by apoC-III (refs 3,18). The D25V apoC-III variant exhibited the same inhibitory activity when compared with the wild-type (Fig. 7b). It is worth noting that another mutation in position 25 (D25N, designed in silico), with the change in charge but unmodified hydrophobicity, was as efficient as the wild-type in inhibiting LPL activity.19

We finally compared the 3D NMR structure of D25V apoC-III with that of the wild-type counterpart already characterized in the presence of SDS.20 Two-dimensional 1H,15N NMR spectra (Fig. 8a) showed small (<0.2 p.p.m., excluding residue 25) amide chemical shift changes in the variant (Fig. 8b,c), which were clustered within approximately one helical turn (3.6 residues) of the mutation site. No chemical shift perturbations were observed elsewhere in the sequence. The hydrodynamic radii of wild-type and D25V apoC-III micellar complexes, recorded by TRACT relaxation interference experiments, were virtually indistinguishable (Fig. 8d). Moreover, there was no difference in the helical conformation of residues 20–30 (Fig. 8e).

**Discussion**

Here we report a previously unappreciated, naturally occurring APOC3 mutation6–8, which causes low levels of plasma TG and apoC-III and a favourable lipoprotein profile in a French kindred affected with amyloidosis. Concordance between the APOC3 mutation and the presence of amyloid among members of this kindred, coupled with identification of D25V apoC-III within amyloid fibrils from numerous affected tissues and in vitro demonstration of its amyloidogenicity, establishes that the D25V variant also causes amyloidosis.

The phenotype of this new form of amyloidosis is mainly characterized by onset with sicca syndrome as early as age 20 years, secondary to diffuse salivary gland deposits and progressive renal insufficiency. We showed that these clinical symptoms are caused by amyloid deposition in the respective tissues with progressive and widespread amyloid accumulation within different organs. In this novel form of renal amyloidosis, amyloid deposition is characterized by prominent vascular involvement in all compartments of the kidney and ischaemic glomerular lesions that probably account for low-grade proteinuria and severe hypertension. This clinical renal presentation contrasts those described for Leu75Pro apoA1 amyloidosis20 and A-alpha chain amyloidosis31, which typically manifests as a chronic tubulo-interstitial nephritis with amyloid deposits restricted to the inner medulla, or as proteinuria/nephrotic syndrome because of massive glomerular amyloid deposition, respectively, ApoC-III
Lipid binding of ApoC-III was monitored by decrease in turbidity at 325 nm of DMPC vesicles mixed with recombinant wild-type or D25V ApoC-III at a lipid/protein ratio of 2:1 (w/w) over 900 s at 30 °C. The binding curves shown are representative of three independent experiments and expressed as percentage of the initial absorbance of the vesicles at 325 nm. The time required to reduce by 50% the initial signal at 325 nm (t50%) is given as mean ± s.d. of three replicates. Inset, native gel electrophoresis shows the shift in mobility of both proteins after formation of the vesicles with DMPC. Lanes: wild-type apoC-III alone (1) and with DMPC (2); marker (3); D25V apoC-III alone (4) and with DMPC (5). Marker for electrophoretic mobility is a fivefold diluted plasma (see Methods for details) in which the bands corresponding to the major plasma protein components are highlighted: albumin (I), α-1 antitrypsin (II), haptoglobin (III), transferrin (IV), fibrinogen (V) and γ-globulin (VI). The arrow indicates the site of deposition of the samples where clearly the apoC-III variant precipitated. Negative and positive gel polarities indicate the direction of the electrophoretic run.

(b) Inhibition of LPL activity was determined keeping constant the incubation time and measuring the free fatty acid release at different concentrations of the two apoC-III isoforms. Data plotted as mean ± s.d. of three independent experiments.

Figure 7 | Functional properties of wild-type and D25V apoC-III in vitro.

(a) Lipid binding of ApoC-III was monitored by decrease in turbidity at 325 nm of DMPC vesicles mixed with recombinant wild-type or D25V ApoC-III at a lipid/protein ratio of 2:1 (w/w) over 900 s at 30 °C. The binding curves shown are representative of three independent experiments and expressed as percentage of the initial absorbance of the vesicles at 325 nm. The time required to reduce by 50% the initial signal at 325 nm (t50%) is given as mean ± s.d. of three replicates. Inset, native gel electrophoresis shows the shift in mobility of both proteins after formation of the vesicles with DMPC. Lanes: wild-type apoC-III alone (1) and with DMPC (2); marker (3); D25V apoC-III alone (4) and with DMPC (5). Marker for electrophoretic mobility is a fivefold diluted plasma (see Methods for details) in which the bands corresponding to the major plasma protein components are highlighted: albumin (I), α-1 antitrypsin (II), haptoglobin (III), transferrin (IV), fibrinogen (V) and γ-globulin (VI). The arrow indicates the site of deposition of the samples where clearly the apoC-III variant precipitated. Negative and positive gel polarities indicate the direction of the electrophoretic run.

(b) Inhibition of LPL activity was determined keeping constant the incubation time and measuring the free fatty acid release at different concentrations of the two apoC-III isoforms. Data plotted as mean ± s.d. of three independent experiments.

should be added to the list of protein variants associated with hereditary renal amyloidosis, along with apoA-I (ref. 10, apoAII (ref. 11), lysosome23, fibrinogen A24, chain25 and gelsolin26. The diagnosis of apoC-III amyloidosis may be suggested by hypotriglycerideremia, present in all amyloidotic patients from this kindred, a biological marker that is usually absent in patients with other forms of hereditary renal amyloidosis.

Our in vitro studies clearly show that, despite the remarkable differences in folding dynamics and kinetics of aggregation between wild-type and D25V apoC-III in their lipid-free state, their conformation and colloidal stability are similar in the lipido-bound state. Wild-type apoC-III was recently reported to self-aggregate into polymeric ordered structures with a peculiar triangular geometry and a Möbius strip conformation after 3 days of shaking in physiological buffer. Although these structures do not resemble the genuine amyloid fibrils that we obtained under physiological conditions with the D25V variant, it is worth noting that aggregation of lipid-free wild-type apoC-III was preceded by a gradual structural transition that resembles, in circular dichroism (CD) spectroscopy, the conformation rapidly adopted by the D25V variant. There is no evidence suggesting in vivo amyloidogenicity of wild-type apoC-III and demonstration of aggregation in vitro does not necessarily imply amyloidogenicity in vivo. Importantly, the absence of wild-type apoC-III within the ex vivo amyloid fibrils from three patients in this kindred suggests that the wild-type protein does not contribute to amyloid deposition even in the presence of abundant D25V fibrillar seeds, analogous to what we have recently observed in patients with familial β2-microglobulin amyloidosis.

We hypothesize that the preferential incorporation of D25V apoC-III into amyloid fibrils is likely the main cause of the imbalance in the ratio of wild-type/D25V apoC-III observed within HDL particles. It will be worth investigating further in appropriate cellular and/or animal models whether the secretion of the variant may be partially impaired by the intracellular quality control, similar to what we previously described for some of the amyloidogenic variants of apoA-I (ref. 25), in which we found an imbalance in the wild type/variant ratio.

D25V-carriers displayed a reduction in plasma and HDL apoCIII concentration, hypotriglycerideremia associated with a dramatic decrease in the number of VLDL particles, and a concomitant massive increase in the larger HDL2 fraction. We propose that these are indirect effects of the D25V variant because of its very low concentration in plasma (and HDL) fractions because of its preferential association with amyloid fibrils. The lower plasma apoC-III concentration in D25V-carriers probably results in lack of inhibition of LPL and LDL-R. As a consequence, VLDL-TG hydrolysis proceeds at high rate and is followed by rapid uptake of VLDL-remnants (or intermediate density lipoprotein (IDL) in Fig. 4b), provoking the drastic decreases in VLDL and IDL of D25V-carriers (Fig. 4a,b). It has been well established that surface lipids of VLDL, which are liberated after LPL-mediated VLDL-TG hydrolysis, are transferred to HDL by phospholipid transfer protein, resulting in an increase in HDL size and particle number, as confirmed in mice deficient either in LPL27 or in phospholipid transfer protein22 and which lack HDL. Importantly, subjects heterozygous for other loss-of-function APOC3 mutations (K58E28 and R19X29) also present with hypotriglycerideremia and increased plasma HDL.

The favourable lipid/lipoprotein profile of D25V-carriers is remarkable in the context of CKD and/or ESRD, pathological conditions predisposing to premature atherosclerosis and major ischaemic CVD events and commonly associated with an unfavourable lipid profile, an elevated plasma apoC-III concentration and dysfunctional HDL carrying increased amounts of apoCIII, serum amyloid A1 and lipoprotein-associated phospholipase A2 (refs 29–31). Hypertriglycerideremia, often due to accumulation of VLDL remants/IDL in the circulation, is an independent CVD risk factor, and low plasma HDL is a second independent CVD risk factor. D25V-carriers displaying hypotriglycerideremia and very high HDL2 are obviously lacking in CVD risk factors. Moreover, their HDL is functional, as evidenced by their normal antioxidant activity and cholesterol efflux capacity possibly related to their enrichment in sphingomyelin (SM). Furthermore, the lower apoC-III content of HDL may contribute to atheroprotection as HDL particles without apoC-III reduce monocyte adhesion to vascular endothelial cells, whereas apoC-III-rich HDL do not, suggesting that apoC-III may have additional proatherogenic effects on vascular endothelial cells. Thus, the pathologic amyloidogenic properties of D25V apoC-III unexpectedly confer a favourable cardioprotective lipoprotein profile indirectly, most probably through lowering of plasma apoC-III concentration, extending the evidence for an important antiatherogenic role of APOC3 deficiency in ESRD patients.

As reducing the amyloid fibril precursor protein concentration is known to slow amyloid formation and improve prognosis among patients with a variety of systemic amyloidoses, and as PPARα agonists (fibrates) significantly reduce hepatic APOC3 transcription, we postulate that fibrate therapy may have therapeutic potential for treatment of this new form of amyloidosis. Because the safety and side-effect profiles of fibrates are well known, it might be reasonable to prescribe fibrates to the affected individuals from this kindred in an attempt to slow down amyloid formation. If successful, this therapy could represent an alternative to the gene silencing approach currently under evaluation in other systemic amyloidoses.
Methods

Patients. The study was carried out in accordance with the Declaration of Helsinki, and was approved by the University of Marseille Institutional Review Board. All authors vouch for the completeness and accuracy of the analyses and results. Oral and written informed consents were obtained from all participants, including the transfer of biopsy material outside of France.

Figure 8 | NMR spectroscopy. (a) $^1$H,$^15$N-Heteronuclear single quantum coherence (HSQC) spectra of wild-type (WT, blue) and D25V (red) apoC-III. Residue assignments of the variant were based on the Gangabadage assignment of the wild-type protein, using a nuclear overhauser effect spectroscopy (NOESY)-HSQC spectrum (120 ms mixing time) to reassign perturbed residues around the mutation site. Large chemical shift changes from the WT are highlighted with arrows. (b) Amide chemical shift differences between WT and D25V apoC-III, calculated as the weighted combination $\Delta \delta_H = (\Delta \delta_N + (\Delta \delta_D/5)^2)^{1/2}$. An asterisk (*) highlights the position of residue 25. (c) Amide chemical shift differences projected onto the WT NMR structure (pdb2jq3) according to the indicated colour scale. Grey areas indicate unassigned residues or proline. (d) TRACT relaxation interference measurements of WT and D25V apoC-III, determined by integration of the amide envelope and fitting to single exponential decays. Fitted $^15$N relaxation rates $\pm$ s.e. were 7.2 $\pm$ 0.4 s$^{-1}$ (WT) and 7.2 $\pm$ 0.2 s$^{-1}$ (D25V) in the $\alpha$ spin state, and 19.3 $\pm$ 1.0 s$^{-1}$ (WT) and 18.2 $\pm$ 0.4 s$^{-1}$ (D25V) in the $\beta$ spin state, corresponding to hydrodynamic radii values $\pm$ s.e. of 20.3 $\pm$ 0.6 Å (WT) and 19.6 $\pm$ 0.2 Å (D25V). (e) $^1$H strips from the NOESY-HSQC spectrum of D25V apoC-III. Dashed arrows highlight sequential NOEs from the $H_{a}$ atom of residue $i$ to the $H_N$ atom of residue $i+1/i+3$, which are diagnostic of $\alpha$-helical structure.
Genetic analysis. Genomic DNAs were extracted from peripheral blood samples, using the QIAamp DNA mini kit (Qiagen). Coding and consensus splice site regions of APOA1, APOA2, TTR, RAN, FGA, LYZ, GSS, SAA, APOE, APOC3, APOE, APOA4 and APOE genes were amplified by PCR (AmpliTaq Gold; ABI, or Platinum Taq; Invitrogen), according to the manufacturer’s instructions, in a DNA thermal cycler 9700 (Perkin-Elmer). The primer sequences for APOC3 amplification are listed in Supplementary Table 2. The resulting PCR products were purified by QIAquick PCR Purification kit (Qiagen), and sequenced using the Big Dye terminator cycle sequencing kit (DNA sequencing kit; Applied Biosystems) on an automatic genetic analyser (ABI PRISM 3100 genetic analyzer; Applied Biosystems). APOC3 polymorphisms at positions $-455$ (rs285413), $-382$ (rs285412), and $-22$ (rs285411) in the promoter region were genotyped by direct sequencing. Mutation nomenclature was based on the APOC3 transcript reference (NCBI RefSeqDNA accession number NM_000040.1), and nucleotides were numbered according to the cDNA with +1 corresponding to the A of ATG translation initiation codon according to the Human Genome Variation Society guidelines, http://www.hgvs.org/mutnomen/. Therefore, the apoC-III variant is described as D25V.

Histology and immunohistochemistry. Amyloid was detected by Congo red staining of formalin-fixed wax-embedded biopsy sections $^{14}$ (kidney, salivary glands, heart, bowel and bronchia from III.3; kidney, salivary glands, skin, heart, liver from III.5; salivary glands, kidney, skin, bowel from III; salivary glands from IV.1, IV.3, and IV.4). Amyloid typing was performed by indirect immunofluorescence and by immunohistochemistry on formalin-fixed paraffin-embedded samples. Amyloid was stained with 0.25% Congo red in PBS. For the immunofluorescence and by immunoelectron microscopy$^{14}$. For indirect immunofluorescence and by immunoelectron microscopy$^{14}$. For indirect immunofluorescence staining, sections were washed six times with PBS, incubated for 1 h with rabbit polyclonal anti-human IgG antibody (Abnova, 100 ng ml$^{-1}$ in PBS); and for 1 h with gold-labelled strepatavidin (Zymed Laboratories, 1 in PBS). Immunolabelled sections were contrasted using uranyl acetate (1 in PBS) and 1 h with gold-labelled strepatavidin (Zymed Laboratories, 1 in PBS). For the immunogold labelling procedure, thin sections (80 nm) were cut from the routine (glutaraldehyde-fixed, osmicated, Araldite embedded) electron microscope blocks, collected on nickel grids. Grids were floated on one drop of 5% sodium metaperiodate (Sigma Chemical Co) in distilled water for 30 min. Sections were washed with distilled water three times and then saturated for 1 h with 3% BSA in PBS in order to minimize nonspecific labeling. The grids were then incubated overnight at 4°C with primary goat anti-human ApoC3 polyclonal antibody (Abnova, 100 ng ml$^{-1}$ in PBS).

Lipid and tandem MS-based proteomics. Congo red-stained sections from salivary glands (IV.3), heart and digestive tract (III.3) were examined for the apoC-III variant antibody (Abnova). Protein identifications below the 90% confidence level and those identified with peptide sequences that were detected in negative-ion mode. Sample (4 ml) was injected onto a Symmetry Shield RP8 3.5 mmx50 mm reverse phase column (Waters) and separated on the basis of their PL concentrations in an OPTIMA XL 100 K Beckman ultracentrifuge$^{38}$. After centrifugation, each gradient was fractionated in predefined volumes from the meniscus downwards with an Eppendorf 40,000 r.p.m. 44 h in a Beckman XL70 ultracentrifuge at 15°C by a slight modification of the method of Chapman et al.$^{39,40}$. After centrifugation, each gradient was fractionated in predefined volumes from the meniscus downwards with an Eppendorf 40,000 r.p.m. 44 h in a Beckman XL70 ultracentrifuge at 15°C by a slight modification of the method of Chapman et al.$^{39,40}$. After centrifugation, each gradient was fractionated in predefined volumes from the meniscus downwards with an Eppendorf 40,000 r.p.m. 44 h in a Beckman XL70 ultracentrifuge at 15°C by a slight modification of the method of Chapman et al.$^{39,40}$. After centrifugation, each gradient was fractionated in predefined volumes from the meniscus downwards with an Eppendorf 40,000 r.p.m. 44 h in a Beckman XL70 ultracentrifuge at 15°C by a slight modification of the method of Chapman et al.$^{39,40}$.

Fractionation of HDL subpopulations. HDL subpopulations were isolated from plasma (3 ml) by single step, isopycnic non-denaturing density gradient ultracentrifugation in a Beckman SW41 Ti rotor at 40,000 r.p.m. 44 h in a Beckman XL70 ultracentrifuge at 15°C by a slight modification of the method of Chapman et al.$^{39,40}$. After centrifugation, each gradient was fractionated in predefined volumes from the meniscus downwards with an Eppendorf 40,000 r.p.m. 44 h in a Beckman XL70 ultracentrifuge at 15°C by a slight modification of the method of Chapman et al.$^{39,40}$. After centrifugation, each gradient was fractionated in predefined volumes from the meniscus downwards with an Eppendorf 40,000 r.p.m. 44 h in a Beckman XL70 ultracentrifuge at 15°C by a slight modification of the method of Chapman et al.$^{39,40}$. After centrifugation, each gradient was fractionated in predefined volumes from the meniscus downwards with an Eppendorf 40,000 r.p.m. 44 h in a Beckman XL70 ultracentrifuge at 15°C by a slight modification of the method of Chapman et al.$^{39,40}$. After centrifugation, each gradient was fractionated in predefined volumes from the meniscus downwards with an Eppendorf 40,000 r.p.m. 44 h in a Beckman XL70 ultracentrifuge at 15°C by a slight modification of the method of Chapman et al.$^{39,40}$.

Phosphosphingolipidome analysis. For the phosphosphingolipidome analysis, HDL subpopulations (30 mg phospholipid mass determined using a commercially available assay) were added to 4 ml of cold CHCl$_3$/acetic acid (1:2 v/v) containing seven internal lipid standards (4 g of phosphatidylcholine D$_32$ 320, 100 g of phosphatidylinositol (PI) 250, 80 g of phosphatidylethanolamine, 250, 80 g of phosphatidic acid 25:0, 40 g of phosphatidylserine 25:0, 20 g of phosphatidyglycerol 25:0 and 20 g of ceramide 17:0. Avanti Polar Lipids)$.^{41}$ A control sample and a control sample in the absence of all lipids were also collected in the dark and used to ensure control for quality. KEDTA (200 mM) solution was added (1:5 (v/v)) and the mixture was vortexed and centrifuged. The organic phase was dried under nitrogen and lipids were reconstituted into isopropanol/hexane/water (10:5:2 (v/v)). Transferred into LC/MS vials, dried under nitrogen and resuspended in isopropanol/hexane/water (10:5:2 (v/v)), and lipid concentrations were determined by metabolic profiling. Seven principal phospholipid (PL) subclasses (phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylserine and phosphatidic acid) and two principal sphingolipid (SL) subclasses, SM and ceramide, which together comprise 160 individual molecular lipid species and account for >95% of total plasma PL, and SM$^{33,44}$ were assayed by LC/MS/MS. Lipids were quantified using a QTrap 4000 mass spectrometer (AB Sciex), an LC20AD HPLC system, and the Analyst 1.5 data acquisition system (AB Sciex). Quantification of PLs and SLs was performed in positive-ion mode, except for PI species that were detected in negative-ion mode. Sample (4 ml) was injected onto a Symmetry Shield RP8 3.5 mmx50 mm reverse phase column (Waters) and separated on the basis of their PL concentrations in an OPTIMA XL 100 K Beckman ultracentrifuge$^{38}$. After centrifugation, each gradient was fractionated in predefined volumes from the meniscus downwards with an Eppendorf 40,000 r.p.m. 44 h in a Beckman XL70 ultracentrifuge at 15°C by a slight modification of the method of Chapman et al.$^{39,40}$. After centrifugation, each gradient was fractionated in predefined volumes from the meniscus downwards with an Eppendorf 40,000 r.p.m. 44 h in a Beckman XL70 ultracentrifuge at 15°C by a slight modification of the method of Chapman et al.$^{39,40}$. After centrifugation, each gradient was fractionated in predefined volumes from the meniscus downwards with an Eppendorf 40,000 r.p.m. 44 h in a Beckman XL70 ultracentrifuge at 15°C by a slight modification of the method of Chapman et al.$^{39,40}$.
paraoxonase activity, which is inhibited by EDTA. Thereby, this assay employs 12NATURE COMMUNICATIONS | DOI: 10.1038/ncomms10353 | www.nature.com/naturecommunications

TC) isolated from one healthy normolipidemic control subject42,47,48. In this assay, the efflux capacity of HDL46. Briefly, THP-1 monocytes were cultured on 24-well tissue because PL was shown to represent the key component determining cholesterol ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms10353

Antioxidative activity of HDL. Antioxidative activity of HDL (final concentration of each, 10 mg total mass per dl) was assessed at a physiological HDL to LDL ratio towards reference LDL (d = 1.019–1.063 g ml−1; final concentration, 10 mg d−1 TC) isolated from one healthy normolipidemic control subject42,47,48. In this assay, HDL particles were compared on the basis of their total mass concentrations because both protein and lipid components were shown to contribute to the capacity of HDL to inhibit LDL oxidation43,45,50. HDL subfractions were added to LDL directly before oxidation. Lipoprotein oxidation was induced by an azo-initiator, 2,2’-azo-bis-(2-amidinopropane) hydrochloride (final concentration 1 mmol l−1) as model of mild oxidation induced by free radicals in the arterial intravascular42,47,48. Serum was used as a source of LDL for this assay to ensure intact paraoxonase activity, which is inhibited by EDTA. Thereby, this assay employs mild oxidation conditions and integrates the antioxidative activities of several HDL components, that is, apoA-I, antioxidative enzymes and lipophilic low-density- weight antioxidants3,49,50. Accumulation of conjugated dienes was measured as the increment in absorbance at 234 nm. Absorbance kinetics values were corrected for the absorbance of 2,2’-azo-bis-(2-amidinopropane) hydrochloride itself run in parallel as a blank. Three consecutive phases were identified, the lag, the propagation and the end of the propagation phase. The curve was fitted for each of the propagation rate, oxidation rate in the propagation phase and amount of dienes formed at the end of the propagation phase (maximal concentration of dienes) were calculated as markers of antioxidative activity of HDL42,47,48.

MS analysis of apoC-III isoforms from HDL. Twenty micrograms of HDL fraction was dissolved and acidified in 20 μl of 0.1% tris(2-carboxyethyl) phosphine for 15 min with agitation. The sample was desalted using the tips C18 (Millipore) following the manufacturer’s instructions and eluted with 20 μl of saturated matrix solution of sinapinic acid in 50% acetonitrile and 0.5% (v/v) tris(2-carboxyethyl) phosphine. The mass spectrometric measurements were performed using a MALDI TOF/TOF 4800 Proteomics Analyzer mass spectrometer51. The MS spectra from m/z 6,000 to 10,000 were acquired in linear reflector and linear positive ion modes using 1,000 laser shots. For mass spectrometric identification of ApoC-III in HDL/2,3 using LC-MS/MS, 25 μg of HDL fraction was dissolved into 500 mM triethylammonium bicarbonate, 1% SDS. Proteins were reduced with 10 mM dithiothreitol, alkylated with 20 mM iodoacetamide and trypsin digested overnight. The resulting peptides were cleaned up using OMIX C18 100 μl pipette tips (Agilent), and lyophilized before being reconstituted for the LC-MS/MS analysis. The peptides were separated using an Eksigent Ultra nano-LC HPLC system coupled with an AB Sciex Triple TOF 5600 mass spectrometer. The LC separations were performed using a Discovery Bio Wide Pore HPLC column (C18, 3 μm, 100 × 2 mm) at a flow rate of 100 μl min−1. The MS/MS spectra obtained were then searched using the BioWorks data analysis software. The results were analyzed by a multiple peptide approach using the ProFound search system52.

Radionlabelled SAP scintigraphy. Patients II.3 and IV.3 underwent123I-labelled SAP scintigraphy53 (http://www.amyloidosis.org.uk/diagnosis/sap-scintigraphy-3). A mixture in SAP was used for the radiolabeling procedure54. Each patient received about 100 μCi SAP bearing 200 MBq of 123I, corresponding to an effective dose equivalent of ~3 mSv. Thyroid uptake was blocked by oral ingestion of potassium iodide for 2 days before and following isotope injection. Anterior and posterior whole body scintigraphy was performed 24 h after isotope administration using a GE Healthcare-Discovery gamma-camera.

Expression and purification of recombinant ApoC-III. ApoC-III was expressed from a PET23b vector containing the full-length cDNA for human ApoC-III, including the sequence encoding a C-terminal His6-tag preceded by Leu and Glu additional residues56, PET23b/D25V was generated by using a QuickChange site-directed mutagenesis kit (Stratagen). In a 50 ml reaction, 50 ng of plasmid DNA PET- apoC-III was used as template and two synthetic oligonucleotide primers, the forward primer (5’-CCAAGACGCCAGGTTGACCTGCCAGCA GCG-3’) and the reverse primer (5’-CGCTTCTCATGCTAcatCCTGCGGCTG TTG-3’) with the A-to-T base change complementary to opposite strands of the vector, were extended by T7 Turbo DNA polymerase (Stratagen). For PCR, there was an initial cycle of 95 °C for 1 min and 55 °C for 1 min and 68 °C for 2 min. After Den1 primer digestion of the parental deoxynucleosine methylethylated template, the synthesized mutated DNA was transformed into E. coli XL1Blue competent cells. Candidates were screened by sequencing the ApoC3 insert on an ABI 377 prism DNA sequencer.

Prediction of beta aggregation propensity. The Garnier-Osguthorpe-Robson (GOR) method version IV (ref. 53) was used to predict the beta propensity at the most polymorphic sites. The aggregation propensity of wild-type and D25V ApoC-III were predicted using the Zyggregator method54.

Fibrillogenesis. Fibrillogenesis experiments were performed in standard quartz cells stirred at 1,500 r.p.m. (IKA magnetic stirrer) at 37 °C using 100 μM ApoC-III isoforms in PBS, pH 7.4. Aggregation was carried out without seeds of preformed fibrils and the increase in turbidity at 350 nm was monitored. Thioflavin T fluorescence emission57 was measured at the end-point aggregation. Transmission electron microscopy analysis was performed using propane samples stained with 2% (w/v) uranyl acetate using a CM120 microscope at 80 keV. Atomic force microscopy analysis was carried out on 10 μl of fibrillar sample incubated on a freshly cleaved mica substrate for 5 min, then rinsed and dried. Compressed conditions during aggregation of apoC-III were monitored by far-UV CD.

Lipid-binding properties of apoC-III. Association of recombinant wild-type and D25V apoC-III to DMPC multimellar vesicles55 at a lipid-to-protein ratio of 2:1 (w/w), was followed by monitoring the decrease in turbidity of DMPC liposomes at 325 nm as a function of time at 30 °C (ref. 17) in 0.01 M Tris-HCl buffer pH 8 containing 150 mM NaCl, 8.5% KBr, 0.01% Na2SO4 and 0.01% EDTA using a 1 cm path length quartz cell in a Jasco V-650 spectrophotometer equipped with a Peltier temperature controller. The lipid-to-protein molar ratio was measured as the percentage of the decrease in turbidity of DMPC vesicles in the absence of apo-C-III; the time required to reduce the initial absorbance at 325 nm by 50% was determined from the curves to compare the lipid-binding properties of the two apo-C-III isoforms. Electrophoretic mobility of wild-type and D25V apoC-III with and without DMPC was analysed using 0.3% agarose gel electroendosmosis agarose gel in 70 mM barbiturate-sodium barbiturate buffer, pH 8.6 containing 2 m molar calcium lactate. Samples (3 μl) were applied to the gel including fivefold diluted human plasma in 2% bromophenol blue solution used as marker for protein electrophoretic mobility. Electrophoresis was carried out in a 10 °C water thermostated chamber at 20 V cm1. Gel was then fixed for 15 min in a 15% acetic acid solution containing 1% picric acid, dried and stained with Coomassie Blue.

LPL inhibition. Bovine LPL catalytic activity, in the presence of apo-C-III was measured using an emulsion with the same composition as Intralipid 10% (Fresenius-Kabi, Sweden) containing [3H]triolein substrate56. The incubation medium contained 2% (v/v) of the emulsion, 0.1 M NaCl, 60 mg ml−1 BSA, 0.15 M Tris-HCl, pH 8.5, and 16.7 μM lipaseheparin. The medium was pre-incubated with or without each apo-C-III sample for at least 10 min to reach equilibrium. Then 100 μl of lipase (0.25 μg ml−1) was added in a final 200 μl mixture. After 30 min incubation under agitation at 25 °C, the reaction was stopped by adding 2 ml of isopropanol, heptane and 1 M H2SO4 (40:83:1) and 0.5 ml of water. Free fatty acids were extracted using sequential centrifugations in glass tubes for 3 min at 2,580g to separate each mixture in two phases. After the first centrifugation, 800 μl of the upper phase was transferred into the other vials containing alkaline ethanol and then added with 3 ml of heptane before mixing and centrifuging again. After removal of the upper phase, 3 ml of heptane were mixed to the lower phase, which was then centrifuged at above. Supernatant was removed and 800 μl of the lower phase transferred to a vial containing 2 ml of scintillation liquid to count radioactivity and quantify the [3H] free fatty acids (nmol ml−1). The LPL activity was then expressed as percentage of the enzyme activity in the absence of apo-C-III.
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Acknowledgements

Supported by grants from l’Association Française contre l’Amylose, the Institut National de la Santé et de la Recherche Médicale (INSERM) and the French National Reference Center for AL amyloidosis, the UK NHS Research and Development funds, the University College London Amyloidosis Research Fund and grants from the UK Medical Research Council (MR/K001878/1), the Rosetrees Trust/Royal Free Charity PhD programme (M427), the British Heart Foundation (PG080088), the Wellcome Trust Investigator Award (097806/Z/11/Z), the Cariplo Foundation Projects (2014–0700 and 2013–0964), the Telethon Grant GG14127, the INBB (National Institute of Biosostructures and Biosystems), the Italian Ministry of Health and the Italian Ministry of University and Research (Projects FIRB RBFR109E08), Core support for the Centre for Amyloidosis and Acute Phase Proteins is provided by the UK National Institute for Health Research Biomedical Research Centre and unit Funding Scheme. We thank Professors Habib and Laurent Daniel for cardiac and salivary gland biopsies, Drs Gayet and Lebrun for their help in the clinical follow-up of proband III.3 with Dr Noemie Jourde, Ms Lacombe for help with immunohistology analyses, Professor Touchard for helpful discussions, Dr Julie A. Vrana and Jason D. Theis for assistance in proteomics analysis with Ahmet Dogan at the Mayo Clinics, Dr Magali Colombat and François Guillonneau and the Plateforme protéomique de l’Université Paris Descartes (IP5), Sorbonne Paris Cité, 75015 Paris, France, for their proteomic analysis of salivary gland from subject IV.3. We thank all the family members for their participation in this study. We thank Alejandra Carbajal and the Division of Medicine Electron Microscopy Unit, Royal Free Campus, University College London, London for imaging of amyloid fibrils in vitro. We acknowledge the UCL Biological NMR Facility and the Medical Research Council for access to the Bio-medical NMR Centre at the Crick Institute, London, UK, and the staff for their support.

Author contributions

S.V. initiated, conceived and supervised all the clinical, genetic and lipidic studies. B.N. performed all the genetic analysis, A.D.K., M.C., A.K., M.L. and C.D. performed and analysed the lipoprotein studies. N.J.-C. provided blood samples of the patients and coordinated the follow-up of patients; J.-M.G. performed histological analysis on amyloid sample tissues, and F.B. contributed to the experimental design and data analysis of amyloid deposit analysis. A.D. performed the proteomics analysis and interpreted the data. A.M. performed the MS analysis of apoC-III. M.D. and G.G. contributed to the discussion. All the in vitro experimental study was conceived, designed and supervised by V.B. G.V., P.P.M., R.P., C.A.W., A.R. and O.K. performed the research. P.J.T., G.O., M.S., J.C., J.D.G. and P.N.H. contributed to experimental design and discussion. All the authors analysed and interpreted the data. The paper was written by S.V., A.D.K., J.D.G., V.B. and reviewed and approved by all co-authors.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Valleix, S. et al. D25V apolipoprotein C-III variant causes dominant hereditary systemic amyloidosis and confers cardiovascular protective lipoprotein profile. *Nat. Commun.* **7**:10353 doi: 10.1038/ncomms10353 (2016).

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