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Tocolysis and Neurodevelopment of Children Born Very Preterm

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The singular French PCSK9-p.Ser127Arg gain-of-function variant: A significant player in cholesterol levels from a 775-year-old common ancestor

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ABSTRACT

Background and aims: PCSK9 is a key regulator of LDL-cholesterol levels. PCSK9 gain of function variants (GOFVs) cause autosomal dominant hypercholesterolemia (ADH). The first described PCSK9-GOFV, p.Ser127Arg, almost exclusively reported in France, represents 67 % of the PCSK9 French GOFVs due to a founder effect. Few other carriers are reported in South Africa and Norway. This study aims to estimate when the common ancestor lived and to describe a cohort of p.Ser127Arg carriers.

Methods: Eight families and 14 p.Ser127Arg carriers were genotyped and phenotyped. Haplotypes were constructed using 11 microsatellites around PCSK9 and 6 intragenic single nucleotide polymorphisms (SNPs). To add to the biological analysis, eight additional p.Ser127Arg carriers, 12 carriers of other PCSK9-GOFVs, 93 LDLR loss of function variant (LOFV) carriers and 49 non-carriers subjects were phenotyped.

Results: The most common ancestor of p.Ser127Arg was estimated to have lived 775 years ago [95 % CI: 575-1075]. French Protestants exiled after the revocation of the Edict of Nantes in 1685 AD likely brought the variant to South Africa and Norway. As expected for ADH subjects, carriers of LDLR-LOFV, the p.Ser127Arg, or

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other PCSK9-GOFVs showed significantly higher LDL-C levels than that of the non-carriers. Interestingly, LDL-C levels are higher for LDLR-LOFVs and for the reduced secreted p.Ser127Arg than for secreted PCSK9-GOFVs, suggesting a greater effect of the p.Ser127Arg. Conversely, HDL-C was significantly lower for LDLR-LOFV and p.Ser127Arg carriers.

Conclusions: This first report from a large cohort of PCSK9-p.Ser127Arg carriers provides observations suggesting a stronger hypercholesterolemic potential of the mutated pro-PCSK9 compared with the secreted mature protein. This work also provides additional data to support the association between PCSK9 and HDL metabolism, and molecular evidence that this variant appeared in France around 1248 AD (Graphical Abstract = Fig. 1).

1. Introduction

Autosomal dominant hypercholesterolemia (ADH) is one of the most common monogenic diseases, with a prevalence of heterozygotes of 1:310 [1,2]. ADH is characterized by high plasma levels of low-density lipoproteins cholesterol (LDL-C) due to an alteration in the LDL catabolic pathway. Lifelong exposure to elevated levels of LDL-C promotes early atherosclerotic plaque development and premature cardiovascular disease (CVD) [3].

ADH may result from variants in four genes encoding the low-density lipoprotein receptor (LDLR, *LDLR* gene) [4], apolipoprotein B (ApoB, *APOB* gene) [5], proprotein convertase subtilisin/kexin type 9 serine protease (PCSK9, *PCSK9* gene) [6], and apolipoprotein E (ApoE, *APOE* gene) [7]. The respective contribution of these four genes to ADH slightly varies from one country to another. In French ADH patients the *LDLR* is implicated in 73.9 % of the cases, while variants in *APOB* and *PCSK9* are responsible for 6.6 % and 0.7 % of the cases respectively [8], and in *APOE* for less than 1 % [9]. In Norwegian ADH patients with a positive molecular diagnosis result, the *LDLR* is implicated in 94 % of the cases, *APOB* and *PCSK9* in 5.4 % and 0.6 % of the cases respectively [10].

The first ADH causative gene identified was *LDLR* encoding the LDLR [4]. Loss of function variants (LOFVs) in *LDLR* cause Familial Hypercholesterolemia (FH) (MIM# 606945) with an initially estimated prevalence of heterozygous cases of 1:500. More than 3500 pathogenic/likely pathogenic variants have been reported in *LDLR* [a], the vast majority being private variants. Few *LDLR*-LOFVs have been identified with a higher prevalence in genetically homogeneous and isolated populations, due to founder effects. Four variants in geographically diverse areas account for >80 % of French Canadian ADH patients [11], three others account for 90 % of Afrikaner ADH patients [12], two *LDLR*-LOFVs account for >65 % of ADH cases in Finland [13], one other is present in 35 % of Jewish-Ashkenazi ADH families in Israel [14], one *LDLR*-LOFV accounts for >80 % of ADH cases in Lebanon [15], and two *LDLR*-LOFVs account for 30 % of all ADH cases in Norway [10].

ApoB is the structural apolipoprotein of LDL, and the major ligand for the LDLR. While truncating variants in *APOB* cause hypobetalipoproteinemia, missense variants can cause hypercholesterolemia by encoding a ligand-defective ApoB protein, familial defective apolipoprotein B-100 (FDB) [5] (MIM# 144010). The p.Arg3527Gln FDB-causing variant was first reported in the late 1980s and is the most common FDB variant [5]. This variant was found in 97 % of FDB patients in a cohort from 8 European countries [16]. A few other variants leading to hypercholesterolemia have subsequently been described in the following years and all are located in the region of the *APOB* encoding the receptor binding domain [17].

PCSK9 undergoes autocatalytic cleavage of its precursor form (74-KDa) to produce its mature and active form (60-KDa) which is secreted in the circulation [18]. PCSK9 binds to the LDLR at the cell surface through the combined interactions between the PCSK9 catalytic domain and the LDL receptor epidermal growth factor domain A, and the PCSK9 pro-domain and the LDLR β -propeller [19]. Independently of its catalytic activity, PCSK9 binds the LDLR at the cell surface and drives it to the lysosome where the complex is degraded and the LDLR thus fails to

recirculate to the cell surface [18]. PCSK9 also has an heparan sulfate-binding site in its prodomain, which interacts with cell-surface heparin-like molecules to facilitate the LDLR interaction [20]. In the cell, PCSK9 interacts with the ER-resident GRP94 (glucose-regulated protein of 94 kDa) chaperone and this binding mitigates PCSK9-mediated LDLR degradation [21], increases the protein abundance of GRP94 and prevents ER stress [22]. Thus demonstrating co-chaperone functionality and consistent with the notion that PCSK9 can act as a chaperone for pre-mature ER-resident LDLR and promote its trafficking to the cell surface [23]. PCSK9 impacts the metabolism of triglyceride-rich lipoprotein metabolism modestly [24]. The first described PCSK9 gain of function variant (GOFV), p.Ser127Arg [6], affects the serine at position 127 which is highly conserved between species and plays an important role in the recognition of the autocatalytic cleavage site [18]. Compared to wild type PCSK9 and most of the others PCSK9-GOFVs, the p.Ser127Arg variant appeared to have a 66 % reduction in autocatalytic cleavage activity, leading to higher levels of unprocessed pro-PCSK9 relative to the mature PCSK9 [18]. The p.Ser127Arg has also been shown to have a higher binding affinity for the LDLR, through the enhanced interaction between the PCSK9 pro-domain and the LDLR β -propeller [19]. And, although considerably reduced compared to wild type PCSK9, the secreted PCSK9-p.Ser127Arg have a higher binding affinity for heparan sulfate proteoglycans which facilitates the cell surface LDLR interaction [25], and is defective for LDL binding which facilitates its binding to the LDLR and the PCSK9/LDLR degradation [26].

This variant was also associated with an overproduction of ApoB-containing lipoproteins (VLDL, IDL and LDL particles), based on kinetic studies in human patients [27]. This is consistent with the ~2-fold higher ApoB-100 production rate reported for homozygous FH patients [28]. The mechanism by which secreted PCSK9-GOFVs affect LDL catabolism is either through an increase in the variant half-life or an increased affinity for the LDLR, all resulting in an enhanced receptor degradation [18]. Although the mechanism remains unclear, p.Ser127Arg and PCSK9-p.Asp129Gly [29] appears to be the only PCSK9-GOFVs to present higher levels of unprocessed pro-PCSK9. At higher levels within the cell, proPCSK9-p.Ser127Arg has been reported to bind the LDLR and probably increases its degradation [30]. HDL particles from carriers of a PCSK9-GOFV are less effective in inhibiting oxidative damage, platelet aggregation, inflammatory alterations and apoptosis relative to FH patients and normolipidemic controls [31]. PCSK9-GOFVs may therefore affect HDL metabolism in addition to their profound effects on LDL metabolism. Further, a potential difference in effect of secreted PCSK9-GOFVs and the proPCSK9-p.Ser127Arg is plausible.

The PCSK9-p.Ser127Arg GOFV was initially identified in two ADH families from Pays de la Loire and Normandy regions in western France. In the two families, the p.Ser127Arg is carried on the same haplotype suggesting that they share a common ancestor [6]. In addition to the two first French families, one South African ADH family with three affected relatives, all carriers of the p.Ser127Arg, was reported within eleven carriers of a PCSK9 ADH-causing variant [29,32]. Among 10 Norwegian ADH families with a PCSK9 variant, three carried the p.Ser127Arg [10]. No p.Ser127Arg carriers have been reported from other countries so far [33–36]. In the phenotype-genotype reporting database ClinVar [b], there are 6 entries for this variant, one from a French team (AC)

reporting 11 carriers, and the five others from teams in Portugal, the Netherlands and three in the USA (San Francisco, Baltimore, Seattle) who each report one carrier [c]. Thus, the p.Ser127Arg is not more common than other PCSK9 ADH-causing variants in the general population aside from the French population where it accounts for 67 % of the PCSK9 ADH-causing variants to a founder effect [8].

This study describes the largest cohort of carriers of the PCSK9-p.Ser127Arg GOFV with the aims to estimate when the most recent common ancestor carrying the variant lived and to compare this variant's biological effects with those of LDLR-LOFVs and secreted PCSK9-GOFVs.

2. Patients and methods

2.1. Patients and families

The 22 probands (19 French patients were recruited between 2012 and 2020 through the French National Research Network for Primary Dyslipidemia from all over France; 1 from South Africa (family SAfr) [29] and 2 from Norway (probands #13 and #14)) were included in the genotyping analysis following the identification of the heterozygous PCSK9-c.381T > A variant (NM_174936.4), encoding the PCSK9-p.Ser127Arg variant, in routine genetic diagnostic analyses. Families from 8 of the 22 probands were enlarged, the HC2, HC92, HC806, and SAfr families have been previously reported [6,29,37,38]. The HC748, HC2062, HCL1, HCL2 and SAfr families are presented in Fig. 2. Thus, a total of 57 carriers of the p.Ser127Arg variant were genotyped: the 22 probands and 35 relatives from the 8 families (Table S1) [5 in HC2 [37], 17 in HC92 [6], 4 in HC748, 3 in HC806 [38], 2 in HC2062, 1 in HCL1, in HCL2 and 2 in SAfr [29] and (Supplementary Table S1)].

For the lipid values analysis, 8 unrelated carriers of a heterozygous PCSK9-GOFV leading to a secreted protein were also included: p.Asp35Tyr, p.Leu108Arg, p.Arg215Cys, p.Phe216Leu [6], p.Arg218Ser,

p.Arg357His, p.Arg357Cys and p.Arg469Trp. And, 8 other unrelated carriers of the p.Ser127Arg, 3 carriers of the p.Arg496Trp and 1 carrier of the p.Asp374His variant in PCSK9 from the national registry REFERCHOL (Registre Français de l'hyperCHOLestérolémie Familiale) [3] were added in the cohorts. The 93 carriers of a heterozygous LDLR-LOFV (nonsense, frameshift, splice site, major rearrangements) were collected in a previous study [39]. The 49 subjects non-carriers, of a LDLR-LOFV or a PCSK9-GOFV, are 15 unrelated from the families included in the genotyping analysis (5 in HC2 [37], 8 in HC92 [6], 1 in HC806 [38] and 1 in HC2062) and 34 unrelated subjects from the LDLR-LOFV carriers families.

The study was conducted according to the French, Norwegian and South African bioethics regulations, and all subjects gave informed consent.

2.2. Genotyping

All relatives from the 8 families (83 relatives) and 14 probands were genotyped for 11 microsatellites spanning 19.6 Mb around PCSK9 (NC_000001.11, NM_174936.4, NP_777596.2, GRCH38p14) and 6 Single Nucleotide Variants (SNVs) located in exon 1: p.Leu23dup, intron 2: c.400-201 A > G, intron 3: c.524-90 C > G and c.524-68 C > G, intron 4: c.657 + 82 G > A, and exon 9: p.Val474Ile (Fig. 3). The microsatellites PCR products (fluorescent-labeled specific primers) were analyzed in three multiplex runs on a Genetic Analyzer (Applied Biosystems 3500 Series). GeneMapper® software was used to define each individual genotype. SNPs in exons 1 and 4 were genotyped by Sanger sequencing and analyzed using CodonCode Aligner® Software. SNPs in exon 3 and exon 9 were genotyped using TaqMan® SNP Genotyping Assays. In the 8 families, haplotypes were reconstructed manually. Primers sequences are available on request to the corresponding author.

2.3. Estimation of tMRCA

The age of the most recent common ancestor (tMRCA) of p.Ser127Arg carriers was estimated from the length of the haplotypes shared by the carriers using the *EstiAge* software [41]. For each p.Ser127Arg carrier, haplotypes were reconstructed using either familial information when available or using a maximum parsimony principle as recommended when using *EstiAge*. Only microsatellite variants were considered. A variant rate of 0.001 per meiosis was assumed and a stepwise variant model was considered. Allele frequencies were estimated on 15 non-carrier individuals (unrelated from families: 5 in HC2, 8 in HC92, 1 in HC806 and 1 in HC2062 (Supplementary Table S1) who were genotyped at the same 18 variants as the carriers.

2.4. Multiple of median for total cholesterol, LDL-C, HDL-C, and TG levels

The multiple of median (MoM) for the total cholesterol (TC-MoM), LDL-C (LDL-MoM), HDL-C (HDL-MoM), and triglyceride (TG-MoM) values measure the deviation of an individual test result from the median of a reference population. It allows the comparison of lipid levels adjusted for age and sex using data from a French population of children [42] and a Dutch population of adults [43]. The MoMs are determined as the ratio of the lipid profile of the patient to the lipid profile of the 50th percentile of a reference population with same sex and age (i.e. TC-MoM = TC patient/TC 50th percentile ref. pop.). For patients on regular treatment for whom pre-treatment values were not available, the untreated LDL-C value (indicated in brackets in Fig. 1) was estimated using the correction factors for statins ± ezetimibe medication given by a meta-analysis of 71 reports [40].

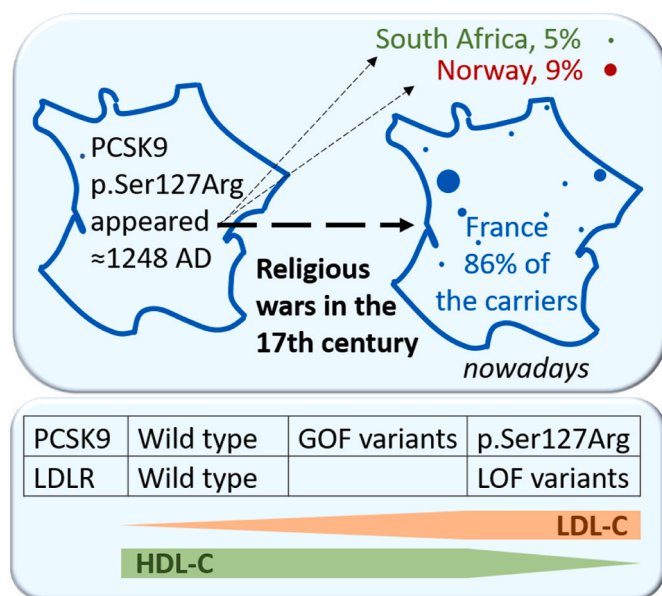


Fig. 1. Up: The PCSK9-p.Ser127Arg gain of function variant (GOFV) appeared in France around 1248 AD and French Protestants exiled religious wars in the 17th century likely brought it to South Africa and Norway. Nowadays the variant is almost exclusively reported in France with 86 % of the carriers.

Down: Carriers of LDLR loss of function variants (LOFVs), the p.Ser127Arg, or other PCSK9-GOFVs show significantly higher LDL-C levels than that of the non-carriers. And, LDL-C levels are higher for LDLR-LOFVs and p.Ser127Arg carriers than for other PCSK9-GOFVs carriers. Conversely, HDL-C levels are significantly lower for LDLR-LOFVs and p.Ser127Arg carriers than for other PCSK9-GOFVs carriers and non-carriers.

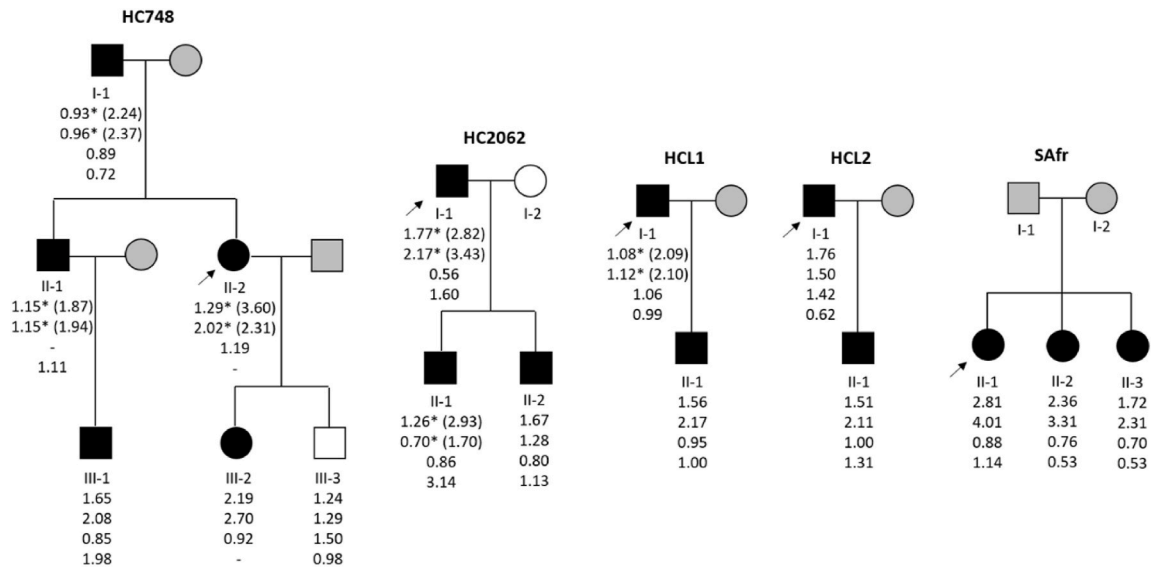


Fig. 2. Pedigree of five ADH families carrying the PCSK9-p.Ser127Arg variant.

Heterozygous variant carriers are indicated by the black symbols, white symbols indicate non-carriers, and grey symbols indicate individuals that have not been analyzed. The characteristics of each individual are represented as follows: individual number, MoM-total cholesterol, MoM-LDL-Cholesterol, MoM-HDL-Cholesterol and MoM-TG. *Under cholesterol-lowering therapy. For patients on regular treatment for whom pre-treatment values were not available, the untreated LDL-C value (indicated in brackets) was estimated using the correction factors for statins ± ezetimibe medication given by a meta-analysis of 71 reports [40].

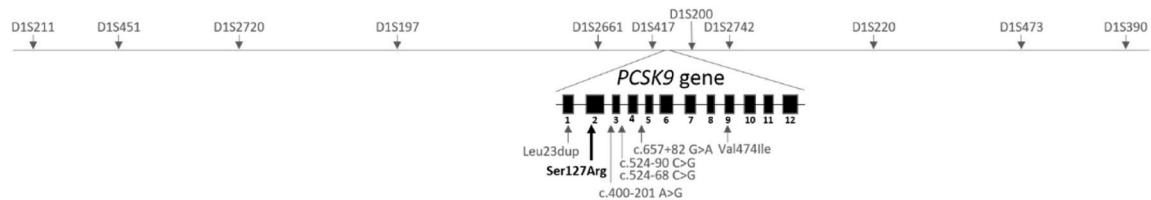


Fig. 3. Diagram of the 19.6 Mb region at chromosome 1p.32 flanking the PCSK9 gene.

The 12 PCSK9 exons are represented by the black rectangles. The p.Ser127Arg variant is shown in black. The 11 microsatellites are above the gene and the 6 intragenic SNPs are below it.

Table 1
Haplotypes of the 19.6 Mb region including the PCSK9 gene (Fig. 3) in the 8 families and 14 index cases.

Family	Nb carriers / Nb Meiosis	Reference	HC2	HC92	HC748	HC806	HC2062	HCL1	HCL2	SAfr	Probands													
			7/6	21/20	5/4	4/2	3/2	2/1	2/1	3/0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Variant	Position **	ancestral	Haplotypes																					
D1S211	43765684-43765876	163	163	181/183	183	169	165	175/177	163	165/175	163	163	165	183	163	183	185	183	165	205	183	169	181	
D1S451	45291668-45291843	175	175	175	175	175	175	175/177	175	175	175	175	177	177	175	179	175	175	179	177	177	175	167	177
D1S2720	47442211-47442451	237	237	235	237	241	237	237/241	237	237	237	237	237	237	237	237	237	237	237	237	237	240	235	237
D1S197	50284845-50284966	136	136	134	138	140	136	132	142	138	136	132	136	134	134	138	134	142	136	132	142	138	-	138
D1S2661	53869043-53869230	186	186	182	182	-	-	186	186	182	186	190	188	186	186	182	188	188	188	186	188	188	186	186
D1S417	54848120-54848302	188	188	188	188	188	188	188	188	188	188	188	188	188	188	188	188	188	188	188	188	188	188	188
p.Leu23dup	55039904_55039906	L9	L9	L9	L9	L9	L9	L9	L9	L9	L9	L9	L9	L9	L9	L9	L9	L9	L9	L9	L9	L9	L9	L9
p.Ser127Arg	55044016	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
c.400-201A>G	55046322	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
c.524-90C>G	55052188	G	G	G	G	G	G	G	G	C	G	G	G	G	G	G	G	G	G	G	G	G	G	C
c.524-68C>G	55052210	G	G	G	G	G	G	G	G	C	G	G	G	G	G	G	G	G	G	G	G	G	G	G
c.657+82G>A	55052493	A	A	A	A	A	A	A	A	G	A	A	A	A	A	A	A	A	A	A	A	A	-	A
p.Val474Ile	55058564	A	A	A	A	A	A	A	A	A/G	A	A	A	A	A	A	A	A	A	A	A	A	A	A
D1S200	55544489-55544660	169	169	169	169	169	169	169	169	167	169	169	173	169	169	169	169	169	169	163	169	169	169	169
D1S2742	56227761-56228005	248	248	248	260	248	248	246	246	256/258	248	248	244	246	248	248	248	248	246	246	246	258	244	244
D1S220	58820634-58820876	235	235	223	235	235	235	221/225	223/233	237	233	235	223	235	237	233	223	235	235	235	233	235	235	237
D1S473	61441151-61441388	240	236	242	240	240	240	252	236	242	234	236	242	242	244	242	244	244	244	234	244	240	244	236
D1S390	63334381-63334586	208	196	200/204	208	208	208	200/204	212	200	212	208	204	208	204	208	196	208	212	196	204	204	204	204

Microsatellites' number refers to the number of tandem CA repetitions, or AGAA repetitions for D1S390. Recombinant alleles are highlighted in dark grey. The most likely ancestral haplotype is highlighted in light grey. * Fig. 2. ** GRCh38p14.

3. Results

3.1. The p.Ser127Arg variant frequency within the French PCSK9-GOFV remains high

The p.Ser127Arg variant in *PCSK9* accounts for 67 % of the French PCSK9-GOFV due to a founder effect [8]. Nowadays, the frequency of the p.Ser127Arg variant within the French PCSK9-GOFV in our diagnostic academic laboratories remains high and is still in favor of a founder effect. Indeed, the p.Ser127Arg variant accounts for 67 % in Lyon, 72 % in Paris, 69 % in Boulogne-Billancourt, and 70 % in the French registry for familial hypercholesterolemia. While, the p.Ser127Arg is not more common than the other PCSK9-GOFV in Norway (30 %, MPB personal data) and South Africa (8 %, DB personal data).

3.2. The p.Ser127Arg common haplotype is 204 kb long

The genotyping of the 97 individuals (57 carriers and 40 non-carriers of the p.Ser127Arg variant in *PCSK9*) for, at first, the 6 intragenic SNPs and 3 flanking microsatellites (D1S417, D1S200, D1S2742) revealed that all the variant carriers share the same intragenic haplotype, L9-A (Table 1). This common haplotype, carrying the p.Ser127Arg variant, is 204 kb long and flanked by variants D1S417 and c.524-90C > G. Interestingly, the longer haplotype previously identified in the HC2 and HC92 French families [6], L9-A-G-G-A-A, is shared by all the French carriers from this study plus one of the two Norwegian probands (#13) (Table 1).

3.3. The first carrier of the p.Ser127Arg lived between 948 and 1563 AD in western France

To estimate the age of the most recent common ancestor (tMRCA), additional microsatellites variants (D1S211, D1S451, D1S2720, D1S197, D1S2661, D1S220, D1S473, D1S390) were genotyped (Tables 1 and Supplementary Table S1). The most likely ancestral haplotype carrying the PCSK9-p.Ser127Arg variant is 163-175-237-136-186-188-L9-A-G-G-A-A-169-248-235-240-208 (Table 1). And the most recent common ancestor was estimated to have lived about 31 generations ago with a 95 % CI of 23–43. Assuming that one generation equals 25 years, the variant would therefore be about 775 years old, with a 95 % CI between 575 and 1075 years. The variant would therefore have appeared around 1248 AD, between 948 and 1448 AD. Altogether, these data estimate that the most recent common ancestor with the p.Ser127Arg variant lived between 948 and 1448 AD.

The geographical location of the 22 probands carrying the PCSK9-p.Ser127Arg variant shows that the majority of them live in France (19/22, 86 %) and that half of them live in the Pays de la Loire region in western France (Fig. 4). This observation suggests that the most recent common ancestor with the p.Ser127Arg lived in western France, were French Huguenots lived in the end of the 17th century (Fig. 5), and his or her descendants later migrated to eastern France, Norway and South Africa.

3.4. Increased TC, LDL-C, TG and decreased HDL-C levels for p.Ser127Arg carriers

We compared the p.Ser127Arg biological effects with those of LDLR-LOFVs and secreted PCSK9-GOFVs. The secreted PCSK9-GOFV are mainly located in the catalytic domain and characterized with an increased LDLR affinity (Fig. 6).

As expected for ADH subjects, and as observed for LDLR-LOFV carriers, PCSK9-GOFV carriers (either the p.Ser127Arg or a PCSK9-GOFV) showed significantly higher total and LDL-cholesterol (LDL-C) levels, adjusted for age and sex (TC-MoM and LDL-MoM), than non-carriers (Table 2, Supplementary Table S2). Interestingly, LDL-C levels are similar for LDLR-LOFV carriers and p.Ser127Arg carriers and both are

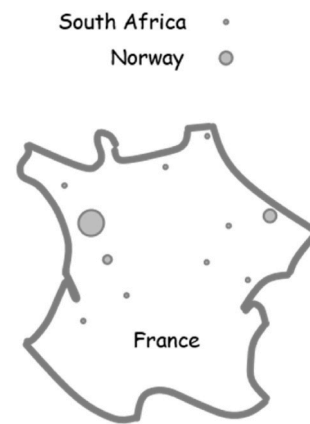


Fig. 4. Geographic location of the 22 probands carrying the PCSK9-p.Ser127Arg variant. The size of the circles represents the number of p.Ser127Arg heterozygous carriers, from $n = 6$ for the largest to $n = 1$ for the smallest.



Fig. 5. Emigration of French protestants to countries of refuge at the end of the 17th century. Based on a map from Protestantism virtual museum [d]. Black dots represent cities of refuge (host cities) and regions in grey represent the major emigration zones.

higher than the levels for secreted PCSK9-GOFV carriers.

Conversely, HDL-C levels were significantly lower for the LDLR-LOFV and p.Ser127Arg carriers than for the non-carriers (Table 2, Supplementary Table S2), which contrasts with previous reports showing no effect of PCSK9-GOFVs on HDL-C levels [32,37,44]. However, a non-significant reduction in HDL-C was previously observed in 11 carriers of a PCSK9-GOFV, including the p.Ser127Arg variant, compared to 11 non carriers [31]. The reduced HDL-C levels observed here is significant, likely because of the larger size of the sample compared to the study with 11 subjects in each group [31], and/or likely due to the type of PCSK9-GOFV. Indeed, the 11 carriers from the previous study did not all carry the p.Ser127Arg variant [31] while carriers from this cohort all harbor the p.Ser127Arg variant. This might suggest a specific effect of the p.Ser127Arg variant on HDL-C levels. However, even if not significant, HDL-C levels were lower for secreted PCSK9-GOFVs than for non-carriers and were not different from those of p.Ser127Arg carriers (Table 2, Supplementary Table S2).

Triglyceride levels were also significantly higher for PCSK9-GOFV carriers than for the non-carriers (Table 2, Supplementary Table S2),

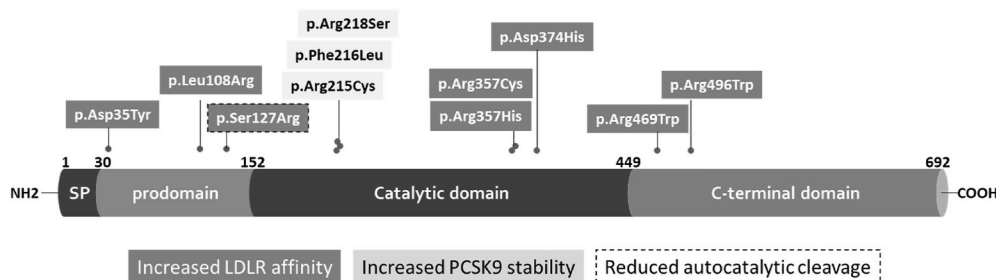


Fig. 6. PCSK9-GOFVs distribution within the protein domains.

Table 2

Comparison of lipid values between carriers of a LDLR-LOFV and a PCSK9-GOFV.

	Age	Sex	TC-MoM	LDL-MoM	HDL-MoM	TG-MoM
non-carriers N = 49	42.0 (24.5–50.0)	4W-11M	1.09 (0.95–1.19)	1.0 (0.92–1.25)	1.1 (1.02–1.25)	0.98 (0.57–1.17)
LDLR-LOFV carriers N = 93	34.0 (21.0–44.0)	53W-40M	1.9 (1.63–2.12)	2.45 (2.10–3.00)	0.90 (0.75–1.04)	1.0 (0.79–1.35)
<i>p</i> value ^a	0.0413	0.0524	<0.0001	<0.0001	<0.0001	0.2481
p.Ser127Arg carriers N = 30	40.0 (26.8–53.0)	9W-13M	2.09 (1.78–2.28)	2.55 (2.12–3.16)	0.96 (0.75–1.11)	1.18 (0.98–1.89)
<i>p</i> value ^a	0.9678	0.3931	<0.0001	<0.0001	0.0003	0.0055
<i>p</i> value ^b	0.1662	0.3931	0.0488	0.6747	0.7539	0.0066
secreted PCSK9-GOFV carriers N = 12	42.5 (25.3–57.3)	5W-3M	1.70 (1.34–1.93)	1.88 (1.44–2.45)	1.05 (0.85–1.31)	0.89 (0.73–1.24)
<i>p</i> value ^a	0.6305	0.3931	<0.0001	<0.0001	0.2681	0.8206
<i>p</i> value ^b	0.1427	0.3931	0.0788	0.0188	0.0745	0.5693
<i>p</i> value ^c	0.4949	0.9243	0.0173	0.0497	0.1522	0.0236
PCSK9-GOFV carriers N = 42	41.5 (26.8–54.3)	14W-16M	2.0 (1.67–2.26)	2.48 (1.83–3.14)	0.98 (0.80–1.16)	1.09 (0.87–1.71)
<i>p</i> value ^a	0.8609	0.3931	<0.0001	<0.0001	0.0009	0.0384
<i>p</i> value ^b	0.0701	0.3931	0.4484	0.4210	0.2621	0.0568
<i>p</i> value ^c	0.7794	0.9243	0.3285	0.4071	0.5453	0.3460
<i>p</i> value ^d	0.6100	0.4109	0.0751	0.1485	0.2918	0.0930

Continuous variables are expressed as Median with its interquartile ranges (1st quartile–3rd quartile). Lipid values are in MoM. The Chi-square test (with Yates correction) and Mann–Whitney test were used to compare qualitative and quantitative values, respectively.

^a Two-tailed *p* value for comparison to non-carriers.

^b Two-tailed *p* value for comparison to LDLR-LOFV carriers.

^c Two-tailed *p* value for comparison to p.Ser127Arg carriers.

^d Two-tailed *p* value for comparison to secreted PCSK9-GOFV carriers.

in agreement with the overproduction of ApoB-containing lipoproteins (VLDL, IDL and LDL) observed in carriers of the p.Ser127Arg PCSK9-GOFV [27].

4. Discussion

According to the worldwide ADH prevalence estimated at 1 in 310 [1,2], around 217,340 subjects in France may have heterozygous ADH which led to the creation of the French Registry of Familial Hypercholesterolemia [3,8]. Among the investigated probands included in the French Registry (N = 2054 patients), 1150 (56 %) carry an ADH-variant including 1.8 % (N = 21 patients) in the PCSK9 gene. The most frequent variant is the p.Ser127Arg detected in 14 patients (67 %) [8]. Currently it is still detected in 73 % of the carriers of a PCSK9-GOFV. This low genetic heterogeneity in the French population supports the founder origin of the p.Ser127Arg variant. In a multinational study, to evaluate the PCSK9 inhibitor alirocumab response, among the 17 p.Ser127Arg carriers 12 are from France, three from Norway, and two from South Africa, suggesting a French founder effect [36]. PCSK9 variants are distributed in localized geographical regions and a limited number of persons. The absence of variants overlapping between Dutch and Japanese populations indicates geographically isolated variants of recent origin [36]. A founder effect is also reported for loss of function variants in PCSK9 leading to hypocholesterolemia. The two common PCSK9-LOFVs, p.Tyr142* and p.Cys679*, in African Americans are rare in European Americans and most carriers of either variant share the same haplotype, suggesting that each is inherited from a single founder [45].

From this large cohort of PCSK9-p.Ser127Arg carriers we estimated the age of the most recent common ancestor carrying the variant, using the Estiage software. We estimated that the variant would have appeared between 948 and 1448 AD in western France.

Today, the majority (86 %) of probands carrying the PCSK9-p.Ser127Arg are in France and half of them are in western France. Identification of p.Ser127Arg carriers in Norway and South Africa likely comes from the exile of French Protestants which took place after the revocation of the Edict of Nantes in 1685 and the resultant migration of the Huguenots.

The French king Louis XIV revoked the religious aspect of the Edict of Nantes in 1685 AD by signing the Edict of Fontainebleau imposing a ban on Protestantism on French territory [d]. Subsequently the number of Protestants living in France experienced a very strong decline, through massacre, assassination, exile or gradual conversion to Catholicism. About 1200 of the 200,000 Protestants (1/4 of the French Protestants, the Huguenots) who left France from 1685 to 1715 AD chose to leave for Africa (Fig. 5) [e]. From 1688 to 1691, 178 families made the journey from Rotterdam, Netherlands, to the Dutch settlement at the Cape in South Africa [f]. Nowadays, descendants of Huguenots represent 20 % of the Afrikaner population and most have a French surname [g]. Other Huguenots, a very small minority, settled further afield at the end of the 17th century: in the Protestant states of northern Europe (Denmark, Norway, Sweden) as far as Saint Petersburg and overseas in or in the British colonies from North America [g]. A second wave of Protestant immigration to the Kingdom of Denmark and Norway occurred in the 18th century and was driven mainly by economic reasons. As in other countries of Northern Europe, the settlement of Huguenots in the

Kingdom of Denmark and Norway took place under good reception conditions which encouraged descendants of Huguenots, already settled in Switzerland and the Netherlands, to come and join the Huguenots already present [g]. In the 18th century, the French Consul in Bergen, Norway, was a Huguenot refugee who was joined by part of his family from Vendée in the Pays de la Loire region, France [h]. A more recent migration towards South Africa and/or Norway cannot be excluded.

We have assumed the generation time of 25 years, as frequently used in human population-genetics studies. As suggested by different studies, we could have used a generation time of 20 [d], 30 [d] or 26.9 [46] years. The PCSK9-p.Ser127Arg would therefore be about 620 [95 % CI: 460–860], 930 [95 % CI: 690–1290] or 834 [95 % CI: 619–1157] years old. The p.Ser127Arg would therefore have appeared between 733 and 1563 AD, still before the revocation of the Edict of Nantes in 1685 AD.

The ApoB-p.Arg3527Gln variant responsible for the FDB phenotype is reported in many ethnicities, most notably Europeans. Worldwide p.Arg3527Gln carriers have the same haplotype (XbaI, MspI, EcoRI), suggesting that all carriers share the same ancestor [47]. In addition, the prevalence of this disorder in Switzerland is 1/209-230⁴⁸. This extremely high prevalence in Switzerland, and the prevalence gradient that persists in the neighboring regions (high prevalence in eastern France and southern Germany, low prevalence in Italy), suggest that the ApoB-p.Arg3527Gln may have developed in the common ancestor of the population living in this region today [48]. Based on a molecular and genetic approach the age of the ApoB-p.Arg3527Gln was estimated at 6750 years ago [47]. The PCSK9-p.Ser127Arg appears to be much more recent which could explain why it is less distributed worldwide.

The LDLR-p.Tyr400_Phe402del responsible for the FH phenotype is only reported in Gran Canaria and accounts for 68 % of FH [49]. This LDLR variant likely appeared 387 years ago [50], even more recently than the PCSK9-p.Ser127Arg. This could explain, beyond the island geographic isolation, the exclusivity of this variant to the Gran Canaria.

In this study, heterozygous carriers of the PCSK9-p.Ser127Arg variant showed increased total cholesterol, LDL-C and triglycerides levels as expected from previous reports. Interestingly, LDL-C levels are similar for LDLR-LOFV carriers and p.Ser127Arg carriers and both are higher than the levels for secreted PCSK9-GOFV carriers. This could suggest a greater effect of the p.Ser127Arg variant compared to secreted PCSK9-GOFVs, possibly due to a stronger potential of the mutated proPCSK9 compared to the secreted mature protein, to the additive effect of the proPCSK9-p.Ser127Arg intracellularly and the few secreted PCSK9-p.Ser127Arg, and or to the ER stress probably induced by the ER-retained proPCSK9-p.Ser127Arg. However, this observation should be treated with caution due to the small number of PCSK9-GOFV carriers (N = 12) and the various variants with different mechanisms and levels of severity in terms of elevated LDL-C. Indeed, it has been shown that the p.Asp374Tyr and p.Arg496Trp variants were each equal in severity to p.Ser127Arg whereas p.Asp35Tyr and p.Phe216Leu were not as severe [36].

In contrast, HDL-C levels were significantly lower for the LDLR-LOFV and p.Ser127Arg carriers compared to non-carriers. This finding is consistent with previous reports for LDLR-LOFVs carriers [31]. And, although it contrasts with previous reports showing no effect of PCSK9-GOFVs on HDL-C levels [32,37,44], this finding is consistent with the non-significant reduction in HDL-C levels observed in carriers of a PCSK9-GOFV, including the p.Ser127Arg [31]. Concordantly, an increase in HDL-C levels in FH Lebanese patients carrying the same LDLR-LOFV is observed with the PCSK9-p.Leu23dup variant which is known to be associated with lower LDL-C levels [15]. Furthermore, no differences in HDL-C levels were reported between carriers of a PCSK9-LOFV and non-carriers [45,51] while PCSK9-inhibitors treatment increased the HDL-C level by 7.0 % (+0.1 mmol/L [95 % CI, 0.09; 0.11], $p < 0.0001$) and apolipoprotein A1 (ApoA1) by 3.7 % (+0.1 g/L [95 % CI, 0.001; 0.1], $p = 0.0003$) [52]. Nevertheless, this increase in HDL-C is marginal compared to the drastic drop in total cholesterol, LDL-C and non-HDL-C. Finally, even if not significant, HDL-C levels were

lower for secreted PCSK9-GOFVs than for non-carriers and were not different from those of p.Ser127Arg carriers. Which is not in favor of a specific effect of the p.Ser127Arg on HDL-C levels but requires confirmation in larger samples. However, it should be noted that this reduction in HDL-C levels in ADH subjects (*0.9 in MoM and *0.8 mg/dL) is marginal compared with the massive increase in LDL-C levels (*2.3 in MoM and mg/dL) or total cholesterol levels (*1.8 in MoM and mg/dL).

5. Conclusion

This is the first report of a large cohort of PCSK9-p.Ser127Arg carriers and the first study to date a PCSK9-GOFV. We provide molecular evidence that the p.Ser127Arg originates from France. We cannot, however, rule out the likelihood that a founder from somewhere else brought the variant to western France between 948 and 1563 AD. All together, these observations suggest a stronger potential of the mutated pro-PCSK9 compared to the secreted mature protein and add data to support similar effects of LDLR-LOFVs and PCSK9-GOFVs on HDL-C levels while HDL functions are differentially affected [31]. The underlying mechanisms at the origin of this reduction in HDL-C levels for each ADH-variant remains to be determined. Are they similar or specific to each type of ADH-variant? Is it a direct effect of the mutated proteins or a consequence of the high LDL concentrations?

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Ethical approval

The study was performed in accordance with French bio-ethics regulations with adherence to the Declaration of Helsinki principles. Informed consent was obtained from all subjects involved in the study. The research project received IRB approval (research project trial #05-07-06 approved by the French Consultative Committee for the Protection of Person in Biomedical Research, Paris, DC-2008-512) and the approval N°920434 by “Commission Nationale de l’Informatique et des Libertés” for samples from Lyon (GENELIP/ASAP study; clinical trial registration number: NCT03939039, <https://clinicaltrials.gov/ct2/show/NCT03939039>). DNA samples from South Africa were imported with an MTA between INSERM U1148 and the University of Cape Town (n°IE-2022-2335) and received conservation authorization in France (CODECOH DC-2022-5287). Genotyping data from Norway were imported with a DTA (Approval PVO: 23/14566; Approval REK: 618217) between INSERM U1148 and Unit for Cardiac and Cardiovascular Genetics, Department of Medical Genetics, Oslo University Hospital.

CRedit authorship contribution statement

Yara Azar: Investigation, Data curation, Visualization, Writing – original draft, Writing – review & editing. **Thomas E. Ludwig:** Formal analysis. **Hugo Le Bon:** Investigation. **Thea Bismo Strøm:** Resources, Investigation, Writing – original draft, Writing – review & editing. **Olivier Bluteau:** Resources. **Mathilde Di-Filippo:** Resources, Writing – original draft, Writing – review & editing. **Alain Carrié:** Resources. **Hedi Chtioui:** Resources. **Sophie Béliard:** Resources. **Oriane Marmontel:** Resources. **Annie Fonteille:** Resources. **Maite Gebhart:** Resources. **Noël Peretti:** Resources. **Philippe Moulin:** Resources, Writing – original draft, Writing – review & editing. **Jean Ferrières:** Resources. **Alain Pradignac:** Resources. **Michel Farnier:** Resources. **Antonio Gallo:** Resources. **Cécile Yelnik:** Resources. **Dirk Blom:** Resources,

Writing – original draft, Writing – review & editing. **Emmanuelle Génin**: Formal analysis. **Martin Prøven Bogsrud**: Resources. **Trond P. Leren**: Resources. **Catherine Boileau**: Funding acquisition. **Marianne Abifadel**: Funding acquisition. **Jean-Pierre Rabès**: Resources. **Mathilde Varret**: Supervision, Funding acquisition, Data curation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.atherosclerosis.2024.118596>.

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