**AP2S1** and **GNA11** mutations – not a common cause of familial hypocalciuric hypercalcemia

Silje Hovden¹, Lars Rejnmark², Søren A Ladefoged¹ and Peter H Nissen¹

¹Departments of Clinical Biochemistry and ²Endocrinology and Internal Medicine, Aarhus University Hospital, Aarhus N, Denmark

**Abstract**

**Objective:** Familial hypocalciuric hypercalcemia (FHH) type 1 is caused by mutations in the gene encoding the calcium-sensing receptor (**CASR**). Recently, mutations affecting codon 15 in the gene **AP2S1** have been shown to cause FHH type 3 in up to 26% of **CASR**-negative FHH patients. Similarly, mutations in the gene **GNA11** have been shown to cause FHH type 2. We hypothesized that mutations in **AP2S1** and **GNA11** are causative in Danish patients with suspected FHH and that these mutations are not found in patients with primary hyperparathyroidism (PHPT), which is the main differential diagnostic disorder.

**Design:** Cross-sectional study.

**Methods:** We identified patients with unexplained hyperparathyroid hypercalcemia and a control group of verified PHPT patients through review of 421 patients tested for **CASR** mutations in the period 2006–2014. DNA sequencing of all amino acid coding exons including intron–exon boundaries in **AP2S1** and **GNA11** was performed.

**Results:** In 33 **CASR**-negative patients with suspected FHH, we found two (~6%) with a mutation in **AP2S1** (p.Arg15Leu and p.Arg15His). Family screening confirmed the genotype–phenotype correlations. We did not identify any pathogenic mutations in **GNA11**. No pathogenic mutations were found in the PHPT control group.

**Conclusions:** We suggest that the best diagnostic approach to hyperparathyroid hypercalcemic patients suspected to have FHH is to screen the **CASR** and **AP2S1** codon 15 for mutations. If the results are negative and there is still suspicion of an inherited condition (i.e. family history), then **GNA11** should be examined.

**Introduction**

Familial hypocalciuric hypercalcemia (FHH) is an autosomal dominant inherited disorder characterized by an elevated level of plasma calcium, relatively high level of parathyroid hormone (PTH), low urinary calcium excretion and generally absence of symptoms (1, 2).

Since the 1990s, FHH has been known to be a locus heterogenic disorder associated with at least three different loci designated FHH type 1, 2 and 3 (3, 4, 5, 6). FHH1 is caused by heterozygous inactivating mutations in the gene encoding the calcium-sensing receptor (**CASR**) located on chromosome 3q13.3–q21 (7). The calcium-sensing receptor is a guanine nucleoside-binding protein (G-protein)-coupled receptor (GPCR), which is abundant in the parathyroid glands and in the kidneys, where it is pivotal in the regulation of PTH secretion and renal excretion of calcium. Multiple **CASR** mutations are associated with FHH (8, 9, 10) and consequently genetic analysis of **CASR** is considered the state-of-the-art investigation in FHH (11). However, it has been hypothesized that **CASR** mutations only explain about 65% of the suspected cases with FHH (12). The genes responsible for FHH2 and FHH3 have remained unknown until recently, when Nesbit and coworkers identified that mutations in the genes **GNA11** and **AP2S1**, two genes not previously associated to calcium metabolic phenotypes, are responsible for FHH2 and FHH3 respectively (13, 14).
GNA11 on chromosome 19q3 encodes subunit $\alpha_{11}$ in the trimeric G-protein and mediates the signaling of GPCRs. Inactivating mutations causes FHH2 and have been found in 0–10% of patients without mutations in CASR and AP2S1 (13, 15, 16).

AP2S1 on chromosome 19p13.3 encodes the adaptor protein-2 $\sigma$ subunit, which plays a role in the internalization of membrane proteins, including GPCRs (17). Only mutations affecting codon 15 seem to cause FHH3 and have been identified in 13–26% of patients with suspected FHH without CASR mutations (14, 15, 18, 19).

The phenotype of FHH2 resembles that of FHH1 (3), whereas recent studies indicate that FHH3 is characterized by a higher level of both calcium and magnesium and a lower calcium creatinine clearance ratio (CCCR) than FHH1. Furthermore, a relatively high proportion of FHH3 patients report hypercalcemic symptoms, have a lower bone mineral density (BMD) and may suffer from learning disabilities (15, 19). A recent review indicates that symptomatic hypercalcemic FHH3 patients might benefit from treatment with calcimimetics (20).

Due to a similar clinical presentation and overlapping biochemical measures, FHH is difficult to distinguish from mild primary hyperparathyroidism (PHPT). PHPT often presents with a higher plasma level of PTH and a higher CCCR, whereas FHH patients

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**Figure 1**
Flowchart over the review process of patients tested for CASR mutations and the patient inclusion.

**Figure 2**
Scatterplot showing the distribution of ionized calcium and parathyroid hormone (PTH) in the familial hypocalciuric hypercalcaemia candidate group (FHHc) and in the group of primary hyperparathyroidism before (PHPTbefore) and after (PHPTafter) surgery. The lines indicate the reference intervals (ionized calcium 1.18–1.32 mmol/L and PTH 1.6–6.9 pmol/L).
have milder PTH elevations and a low CCCR (1, 21). International guidelines recommend screening for FHH among patients suspected to have PHPT and considers a CCCR <0.01 as indicative for FHH (22). However, a cut-off value of CCCR <0.02 for referral to genetic testing gives a higher diagnostic accuracy (11, 23). As prognosis and treatment differs between the two disorders, it is important to establish the right diagnosis. PHTP can result in severe side effects such as osteoporosis, kidney stones, cardiovascular disease and depression, whereas the hypercalcemia in FHH caused by CASR mutations, seems not to affect bones, muscles, quality of life or cardiovascular health negatively (2, 24, 25, 26). However, a few cases of chondrocalcinosis and pancreatitis have been reported (1, 21, 27), and a possible association to developing co-occurring PHPT has been proposed (28). Parathyroidectomy is often curative of PHPT, whereas it does not benefit patients with FHH. We have previously shown that non-curative parathyroidectomies are due to FHH in 23% of all cases (9), illustrating the potential consequences of misdiagnosing these two patient groups. Due to the risk of adverse side effects (vocal cord paresis and hypoparathyroidism), parathyroidectomy should be avoided in FHH patients.

To improve the diagnostic approach to FHH patients, we hypothesized that Danish hyperparathyroid hypercalcemic patients suspected of FHH, without mutations in CASR, would have mutations in either AP2S1 or GNA11 explaining the phenotype. We sequenced the two genes in FHH suspected individuals and to rule out a possible genetic overlap between the two disorders, we analyzed the genes in a control group of PHPT patients. Our results indicate that expanding the genetic screening could be valuable in the diagnostic work-up of selected hypercalcemic patients.

Subjects and methods

Patient inclusion

We identified patients (>18 years of age) through review of results from CASR genetic testing performed in our lab on patients with hyperparathyroid hypercalcemia in whom FHH was suspected. The patients were referred from the Department of Endocrinology and Internal Medicine (MEA), Aarhus University Hospital (AUH) from January 2006 to October 2014. The main inclusion criteria for possible FHH 2 or 3 patients were a persistent hypercalcemia (mean ionized calcium level of >1.32 mmol/L) and a concomitant relatively high PTH level (in the upper third of the reference interval or higher equal to plasma PTH >5.0 pmol/L) with no pathogenic variation in CASR. Additionally, a low calcium creatinine clearance ratio (CCCR <0.02), a recurring hypercalcemia after parathyroidectomy and a family history of hypercalcemia were considered. Patients gave written informed consent before participation in the study.

During the review of CASR-tested patients, we identified a control group of PHPT patients. Inclusion depended upon a negative CASR test and successful parathyroidectomy, defined as normocalcemia at least 6 months after surgery. Exclusion criteria for both groups were chronic kidney disease (including previous kidney transplantation) defined as plasma creatinine >140 μmol/L or treatment with lithium (29, 30).

Furthermore, family members of index patients with a presumed pathogenic mutation were invited to participate, and positive responders were screened for the family mutation.

The study was approved by The Health Research Ethics Committee in the Central Denmark Region (no. 1-10-72-174-14) and by the Danish Data Protection Agency (no. 1-16-02-466-14).

Table 1  Biochemical characteristics of the FHH candidate group and the PHPT group. Mean (s.d.)

<table>
<thead>
<tr>
<th></th>
<th>Reference values</th>
<th>FHH candidates (n=33)</th>
<th>PHPT (n=77)</th>
<th>P-Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium ionized (mmol/L)</td>
<td>1.18–1.32</td>
<td>1.40 (0.08)</td>
<td>1.44 (0.07)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PTH (pmol/L)</td>
<td>1.6–6.9</td>
<td>8.33 (2.52)</td>
<td>12.6 (4.5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CCCR</td>
<td>0.011 (0.005)A</td>
<td>0.014 (0.008)B</td>
<td>0.86 (0.08)B</td>
<td>NS</td>
</tr>
<tr>
<td>Magnesium (mmol/L)</td>
<td>0.70–1.10</td>
<td>0.86 (0.05)</td>
<td>0.85 (0.13)B</td>
<td>0.0096</td>
</tr>
<tr>
<td>Phosphate (mmol/L)</td>
<td>0.71–1.53</td>
<td>0.91 (0.12)</td>
<td>0.85 (0.13)B</td>
<td>NS</td>
</tr>
<tr>
<td>25-OHD (nmol/L)</td>
<td>50–160</td>
<td>79 (19)</td>
<td>66 (21)B</td>
<td>0.006</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>35–105</td>
<td>75 (22)</td>
<td>89 (29)C</td>
<td>0.0043</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>60–105</td>
<td>66 (13)</td>
<td>70 (15)C</td>
<td>NS</td>
</tr>
</tbody>
</table>

P values are calculated with Welch's t-test for the difference between the FHH candidates and the PHPT group before surgery.

AData missing from one. BIncomplete data (n=68–74).

25-OHD, 25-hydroxyvitamin D; CCCR, calcium creatinine clearance ratio; FHH, familial hypocalciuric hypercalcemia; PHPT, primary hyperparathyroidism; PTH, parathyroid hormone; NS, not significant.
Biochemical analyses

Biochemical analyses of blood and urine were performed in the clinical setting (diagnosis, treatment and/or follow-up) and measurements of ionized calcium, PTH, CCCR, phosphate, magnesium, alkaline phosphatase, 25-hydroxyvitamin D (25OHD) and creatinine were evaluated. We obtained data from 2006 to 2015 from the University Hospital’s laboratory information system.

Genetic analysis

Genomic DNA was isolated from whole blood as described previously (9).

We performed polymerase chain reaction (PCR) of 12 protein-coding exons (five in AP2S1 and seven in GNA11) including the intron–exon boundaries, using the primer sequences from Nesbit et al. (14) for AP2S1 exon 1, 4 and 5. All other primers were designed using Primer3 (31).

The PCR products were purified using the GFX 96 PCR Purification Kit (Illustra, GE Healthcare) and subjected to bidirectional DNA sequencing as described previously (9). One exception was made with GNA11 exon 1, where the sequencing reaction was modified by addition of 5% DMSO and an initial heat denaturation step (98°C for 5 min) prior to the addition of sequencing reagents (32, 33). Primer sequences, and PCR and sequencing conditions are available on request.

We aligned sequence traces to the reference sequences NM_004069 for AP2S1 and NM_002067 for GNA11 using SeqScape (version 2.7, Life Technologies). All variations from the reference sequences were aligned to the Ensembl (http://www.ensembl.org/Homo_sapiens/Info/Index) (34) and the NCBI SNP databases (http://www.ncbi.nlm.nih.gov/snp/) (35).

Statistical analysis

The statistical analysis was performed with GraphPad Prism version 6. Data are presented as mean and s.d. Welch’s t test was used as the statistical test to determine the differences and a P value <0.05 was considered statistically significant.

Results

We reviewed 421 patients tested for mutations in the CASR (Fig. 1), of whom 25 (5.9%) had FHH1. We excluded 27 patients (6.4%) according to the exclusion criteria and excluded 145 (34.4%) because they were ineligible for...
of the patients had undergone successful parathyroidectomy, and 77 patients from this group were randomly selected to the PHPT control group. The remaining 67 patients (15.9%) were allocated to the FHH 2 and 3 candidate group.

Of the 67 FHH candidates, 12 patients refused participation for reasons unknown and 22 patients did not respond to the invitation. Consequently, 33 patients with suspected FHH aged 31–88 were included. Of the included patients, 39.4% had experienced one or more unsuccessful neck explorations. The rest were considered to have FHH due to low CCCR, absence of hypercalcemic symptoms and/or family history. All patients were followed in the out-patient clinic.

Figure 2 and Table 1 show the biochemical data from the FHH candidate group and the PHPT group. Patients with verified PHPT had pre-operatively statistically significantly higher levels of ionized calcium, PTH and alkaline phosphatase, and statistically significantly lower levels of phosphate and 25-OHD than the FHH 2 and 3 candidates.

Of the 33 FHH suspected patients, we found a pathogenic mutation in AP2S1 in two unrelated patients: a c.43C>T substitution resulting in p.Arg15Leu and a c.44G>A substitution resulting in p.Arg15His. No mutations were identified in GNA11 in the FHH suspected group. Figure 3 and Table 2 show the biochemical values of the two FHH3 patients and the rest of the FHH candidate group. There is a tendency of the FHH3 patients having a lower CCCR and a higher level of ionized calcium and magnesium than the rest of the group.

Sequencing of the AP2S1 and GNA11 genes in the control group revealed that the two patient groups had a similar distribution of polymorphisms in GNA11, whereas the control group had a higher allele frequency of normal variants in AP2S1 than the FHH group (data not shown). No FHH-associated mutations were identified in the PHPT group.

### Patients with mutation in AP2S1

The index patient with p.Arg15Leu had a known family history of hypercalcemia (Fig. 4) as her now deceased mother underwent unsuccessful parathyroidectomy resulting in the screening of the family for FHH. Because of the apparent autosomal dominant inheritance, none of the other family members with hypercalcemia were referred to surgery. Two hypercalcemic and two normocalcemic family members were screened for the p.Arg15Leu mutation, confirming the co-segregation of the c.43C>T genotype.
with hypercalcemia (Table 3). None of the family members had complaints regarding hypercalcemic symptoms. The index patient had a normal BMD (T-score of −0.4 at the lumbar spine, +0.5 at the hip and +0.8 at the forearm). The hypercalcemic son of the index patient suffered from type 1 diabetes, and both he and the sister of the index patient had psychiatric diagnoses/learning disabilities.

As we have previously reported (36), the index patient with the p.Arg15His mutation also had a family history of hypercalcemia believed to be a genetically unverified multiple endocrine neoplasia syndrome (MEN1), but went through multiple parathyroidectomies before the diagnosis of FHH3 was reached (36). Two hypercalcemic and five normocalcemic family members were screened for the p.Arg15His mutation, which resulted in the confirmation of the co-segregation of the c.44G>A genotype with hypercalcemia. Both the index patient and his brother had complaints that could be attributable to hypercalcemia, but had normal BMD except a hip T-score of −1.1 and no psychiatric diagnoses (36).

### Discussion

In our cohort of Danish patients suspected of FHH with a normal CASR gene, we found that two patients (6.1%) had a heterozygous mutation in AP2S1, whereas no mutations were identified in GNA11. Both mutations have previously been reported to be associated with FHH3 (14, 15, 18, 19) and support functional studies indicating that cells with mutated AP2S1 were less sensitive to extracellular calcium (14). We also found that the mutations were absent in 77 verified PHPT patients. Some polymorphisms found to be normal variants in AP2S1 were observed with higher allele frequencies in the PHPT group than those in the FHH candidate group. Whether this has any clinical significance needs further investigation.

Our findings are different from the earlier findings, where 13–26% of CASR-negative patients had AP2S1 mutations and 10% of CASR- and AP2S1-negative patients had mutations in GNA11 (13, 14, 18, 19). We have no evidence that the prevalence of FHH in Denmark differs from other Caucasian populations and would thus expect similar findings. Stratta et al., on the other hand, did not identify mutations in either AP2S1 or GNA11 in a small Italian population (37). One possible explanation for the lower rate of mutation-positive patients found in our study (6.1%) is that the majority of our studied population had a relatively high CCCR between 0.01 and 0.02 (19 of 33 patients). New data suggest that a very low urinary calcium excretion is a characteristic of FHH3 (19), which is supported by the fact that both our genetically verified FHH3 patients had a CCCR <0.01 (mean 0.004). If we only look at the group of patients with a CCCR <0.01 in our data, we find that 14.3% had a mutation in AP2S1, thereby resembling the findings in previous studies.

With regard to our negative findings in GNA11, only two studies describe an association of GNA11 and FHH2, reporting three different mutations (13, 16). Vargas-Poussou et al. found no GNA11 mutations in 220 families investigated (15). The sparse published data on FHH2 and our negative findings in 33 investigated hypercalcemic patients, suggest that mutations in GNA11 are a very rare cause of FHH. Gorvin et al. who report a GNA11 mutation estimate that 65% of FHH patients will be FHH1, 5% FHH3 and <1% FHH2, but do not explain how they reach these estimates (16).

Previously, the FHH3 phenotype was reported to be characterized by increasing PTH and decreasing phosphate with age causing hypophosphatemic osteomalacia in a large kindred (14, 38). This is however not found in other studies (15, 19, 39). It was recently reported that there was no difference between FHH1 and FHH3 in five unrelated cases.

### Table 3 Biochemical characteristics of mutation positive and negative individuals in family F6.

<table>
<thead>
<tr>
<th></th>
<th>Reference values</th>
<th>Mutation positive (n = 3)</th>
<th>Mutation negative (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium ionized (mmol/L)</td>
<td>1.18–1.32</td>
<td>1.53</td>
<td>1.29</td>
</tr>
<tr>
<td>PTH (pmol/L)</td>
<td>1.6–6.9</td>
<td>9.2</td>
<td>4.3</td>
</tr>
<tr>
<td>CCCR</td>
<td></td>
<td>0.003&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Magnesium (mmol/L)</td>
<td>0.70–1.10</td>
<td>0.92</td>
<td>0.77</td>
</tr>
<tr>
<td>Phosphate (mmol/L)</td>
<td>0.71–1.53</td>
<td>0.88</td>
<td>1.1</td>
</tr>
<tr>
<td>25-OHD (nmol/L)</td>
<td>50–160</td>
<td>52</td>
<td>59</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>35–105</td>
<td>95</td>
<td>80</td>
</tr>
<tr>
<td>Creatinine (μmol/L)</td>
<td>60–105</td>
<td>65</td>
<td>69</td>
</tr>
</tbody>
</table>

Means are shown. Mutation positive family members have a p.Arg15Leu mutation in AP2S1.

<sup>a</sup>Data missing from two.

25-OHD, 25-hydroxyvitamin D; CCCR, calcium-creatinine-clearance-ratio; PTH, parathyroid hormone.
(18); however, new data based on a total of 70 FHH3 patients including family members of previously described families indicate that the phenotype of FHH3 differs markedly from FHH1 by higher levels of calcium and magnesium and a lower CCCR (15, 19). Furthermore, Hannan et al. found that the phenotype differed according to the causative mutation in AP2S1, the p.Arg15Leu mutation causes the highest level of calcium and the lowest CCCR, whereas the p.Arg15His mutation causes the lowest level of calcium and a slightly higher CCCR. This tendency is also seen in our FHH3 families, where the p.Arg15Leu mutation results in a mean ionized calcium level of 1.53 mmol/L and a CCCR of 0.003, whereas the p.Arg15His mutation results in a mean ionized calcium level of 1.44 mmol/L and a CCCR of 0.005 (Table 3 and (36)). However, this specific mutation–phenotype correlation is not supported by findings of Vargas-Poussou et al. (15).

Symptoms of hypercalcemia and several other co-morbidities were recently found in FHH3 patients, including low BMD (15), learning disabilities and psychiatric disease (18, 19). Our findings do not allow for such conclusions due to the small number of patients, although our findings of possible hypercalcemic symptoms in one of the identified families do support that FHH3 may be associated with more symptoms than FHH1. Furthermore, we found a p.Arg15His mutation in AP2S1 in a patient clinically suspected of having MEN1, but concluded that he was probably an example of a phenocopy (36). However, further studies of co-morbidities are warranted to establish whether these reported diseases are chance findings or actual complications associated with FHH3.

Hannan et al. introduced a calcium–magnesium–CCCR (CMCR) index (albumin adjusted sCa × sMg/ (100 × CCCR)) to differentiate FHH1 from FHH3 in patients with suspected FHH. They proposed a diagnostic approach, where the CMCR index is applied to all patients with a CCCR <0.01. Patients with a CMCR >5.0 should be referred to screening of AP2S1 first, and if negative, CASR and GNA11 should be analyzed, whereas patients with a CMCR <5.0 should have all three genes tested. However, they also reported a 30% overlap between FHH1 and FHH3 when using the CMCR (19). We applied the CMCR post-analytically on our two verified FHH3 index patients and found that both have a CMCR >5.0. In contrast, one family member had a CMCR <5.0 (4.6), which is in concordance with the 30% overlap.

One of the strengths of this study is that we have included a control group of 77 patients with confirmed PHPT, which ascertains that the reported mutations are not found in PHPT patients. We are also the first to investigate a Danish population of patients with suspected FHH for mutations in AP2S1 and GNA11, and thus, expand the genetic characterization of a thoroughly investigated clinical cohort.

One limitation to the study is the relatively high proportion of decliners and non-responders that may result in selection bias. The group of non-responders had a higher proportion of men and was slightly younger, but was otherwise not different from the included group (data not shown). Another limitation was the difficulty to reach family members in the FHH3 families who did not have regular contact with the probands, which resulted in a too small group of verified FHH3 patients to make any evaluations of a specific FHH3 phenotype robust.

In conclusion, our study of FHH suspected individuals indicate that GNA11 and AP2S1 are not common causes of hypercalcemia in Denmark. In the light of recent findings, it also indicates that patients with FHH3 caused by an AP2S1 mutation affecting codon 15 have a significant FHH phenotype that may be associated with complications. We propose a diagnostic approach to hypercalcemic patients suspected to have FHH, including sequencing the entire coding region of CASR and screening of the mutation hot-spot in AP2S1. In cases with strong suspicion of FHH despite negative CASR and AP2S1 analyses, genetic analysis of GNA11 should be considered.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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