Peer Review File

Article information: https://dx.doi.org/10.21037/gs-21-577

Reviewer A

This is a case series of 14 FHH patients who were referred for PHPT. This study alerts the novel CaSR mutation can impede the differential diagnosis between PHPT and FHH.

Comment 1: Title, page 1, line 1. The title is "Novel mutations of the calcium-sensing receptor...". So, the authors must provide which cases had novel mutations that have never been reported before in the Result section ant Table 1 or 3.

Reply 1: After conducting a thorough literature research, it seems that all but one (c.554G>A) CaSR gene mutations mentioned in our manuscript are novel. Notably, 9/10 mutations have not yet been reported in the literature or in the CaSR database (www.lovd.nl, www.casrdb.mcgill.ca). We have modified the corresponding paragraph (3.1 CaSR genotyping) in the result section as advised (see Page 11, line 229-232).

Changes in the text: Notably, 9/10 CASR gene mutations have not yet been reported in the literature or in the CASR database (www.lovd.nl, www.casrdb.mcgill.ca). Classification of detected CaSR variants and a schematic representation of CaSR gene structure are shown in Table 2 and Figure 2.

Comment 2: Methods. The years of the study must be provided.

Reply 2: Data were collected retrospectively from patients during 2008 and 2019 (recruitment and follow-up). Data were analyzed and manuscript prepared in 2020 and 2021. The text was modified accordingly (Page 7, line 131-132).

Changes in the text: All patients gave their written informed consent. The data were prospectively documented between 2008 and 2019 and retrospectively analyzed.

Comment 3: Methods, page 6, line 100 and 101; "the majority of patients was assumed for FHH". This should be stated in the Result section and needs to be addressed as well. It would be useful to include information on the total number of patients referred as PHPT during the study period, how many patients underwent CCCR and CE tests, how many patients were assumed for FHH after the biochemical re-checkup, and how many of them received genetic analysis. Likewise, I would suggest providing the number of postoperative patients with elevation of Ca level and how many of them received genetic analysis in the Result section. The authors can show these data as a flow diagram.

Reply 3: All patients were initially referred for PHPT by the endocrinologist or the GP. CCCR and CE was assessed for every patient in this study (Table 1). FHH1 was suspected initially in 8/14 (57.1%) cases, confirmed by the following genetic analysis. PHPT was assumed in 6/14 (42.9%) patients (elevated serum Ca and intact PTH and/or CCCR >0.001 and/or increased 24hU CE), who underwent surgery for PHPT. However, the persistent postoperative elevated serum Ca levels and symptoms lead to further genetic analysis, which subsequently revealed FHH1. We added a new flow diagram (Figure 1) showing the data as advised (Page 10, line 196-205).

Changes in the text: A total of 14 patients (female: n=9 (64.3%), male: n=5 (35.7%), median

age: 50; range 2 to 77 years), initially referred for PHPT by the endocrinologist or the GP to our institution (Medical University of Vienna) for further evaluation and potential treatment, were included in the study. FHH1 was suspected initially in 8/14 (57.1%) cases (elevated serum Ca and normal/slightly increased intact PTH and/or CCCR <0.01 and/or low 24hU CE), confirmed by the following genetic analysis. PHPT was assumed in 6/14 (42.9%) patients (elevated serum Ca and intact PTH and/or CCCR >0.001 and/or increased 24hU CE), who underwent surgery for PHPT. However, the persistent postoperative elevated serum Ca levels and symptoms lead to further genetic analysis, which subsequently revealed FHH1 (Figure 1).

Comment 4: Method, page 7, line 154 and 155. I cannot find on which data the authors conducted the correlation analysis and Fisher's exact test. Moreover, t-tests were performed on the data of the blood tests that generally show non-normal distribution (Table 2). It would be difficult to judge distribution patterns within this small data (14 cases). Instead, distribution patterns of each blood test as it is generally known can be checked. Some blood tests will require log transformation for t-tests.

Reply 4: Thank you for this important indication. Data of correlation analysis and Fisher's exact test were initially included in the manuscript, but removed in further revisions for better comprehension. We have modified the corresponding paragraph (2.6 statistical analyses) in the method section (Page 9, line 191-192).

Changes in the text: T-test was performed using SPSS Statistics 24.0 software (SPSS Inc., Chicago, IL, USA). The results were considered statistically significant if $p<0.05$.

Comment 5: Results, page 8, line 159, "A total of 14 patients, treated at the Medical University of Vienna, were included in the study.". This sentence is not sufficient to explain the target patients. What kind of treatment did they undergo?

Reply 5: We totally agree, that the wording in the first sentence is confusing. All of our study patients were referred for PHPT by the endocrinologist or the GP to our institution for further evaluation and potential treatment. Only patients subsequently diagnosed with FHH1 were included in this study. The text in the paragraph was modified accordingly (Page 10, line 196-199).

Changes in the text: A total of 14 patients (female: n=9 (64.3%), male: n=5 (35.7%), median age: 50; range 2 to 77 years), initially referred for PHPT by the endocrinologist or the GP to our institution (Medical University of Vienna) for further evaluation and potential treatment, were included in the study.

Comment 6: Results, page 8, line 182 to 185. There were only 14 cases. Thus, those mutation data would be more reader-friendly if the localization of each mutation was indicated using a schematic representation of CaSR gene structure with exon numbers.

Reply 6: This is an excellent suggestion. We have added a new figure illustrating the CaSR gene structure showing the localization of each mutation. A schematic representation of CaSR structure has been added (Figure 2; page 11, line 231-232).

Changes in the text: Classification of detected CaSR variants and a schematic representation of CaSR gene structure are shown in Table 2 and Figure 2.

Comment 7: Discussion, page 11-12, line 253-256. It would be better to omit those sentences.
Reply 7: Thank you for this hint. We agree that both statements are rather unsuitable within the general context and therefore deleted from the paragraph. We have deleted the two sentences as advised (Page 15, line 317-324).
Changes in the text: PHPT and FHH have similar biochemical features – elevated serum Ca and PTH levels – which makes the distinction very difficult. Furthermore, the differentiation is impeded by an overlap of diagnostic criteria: Patients carrying inactivating mutations in the CaSR gene, which is indicative of FHH1, can also have PHPT with autonomously hypersecreting parathyroid adenomas or hyperplasia. In 1998, Farnebo et al. reported on the relationship between CaSR downregulation and parathyroid hyperplasia, although without fully understanding the precise nature of this association. The authors also described decreased CaSR expression in parathyroid adenomas (21).

Comment 8: Discussion, page 12, line 274. Please use "in 2011" instead of "recently".
Reply 8: We agree that writing the year is more precise. The sentence has been changed accordingly (Page 16-17, line 349-351).
Changes in the text: In 2011, Frank-Raue et al. analyzed the biochemical parameters and CaSR gene alterations of 139 patients presenting with hypercalcemia and suspected of suffering from PHPT.

Comment 9: Conclusion. It seems inadequate to conclude that the genetic analysis should be performed in every patient. I think sentences of conclusions in the abstract are better.
Reply 9: Thank you for this indication. We agree that the second sentence in conclusion is inadequate. The conclusion paragraph has been adopted accordingly (Page 18, line 390-394).
Changes in the text: We discovered novel CaSR gene mutations, which have not yet been reported in the literature and which considerably impede differential diagnosis of PHPT and FHH1. Our results show that even though the coincidence of FHH and pathohistological proven PHPT seems to exist, parathyroid surgery does not seem to benefit patients later on.

Reviewer B
In this original manuscript by Bhangu et al., authors report their findings from 14 patients carrying mutation of the CASR gene who underwent a screening performed by their surgery team. They report some confounding factors that may mislead physicians between familial hypocalciuric hypercalcemia (FHH) and primary hyperparathyroidism (PHPT). Even if this is of particular interest, several flaws have to be addressed.

MAJOR COMMENTS
Comment 1: introduction, line 64: CCCR is not 'popular', it's un international recommendation (see Eastell et al. JCEM 2014) to discriminate FHH patients from PHPT ones. CCCR>0.01 does NOT indicate PHPT (CCCR>0.02 does). In the so-called grey zone, physicians are encouraged to performed genetic testing (same ref.). When did the use of CCCR start in the authors' team? Can the authors be more specific on the timelines of recruitment?
Reply 1: We apologize for the inappropriate wording and will reformulate the sentence accordingly. The reviewer is absolutely right that PHPT is more likely when CCCR>0.02.

Therefore, we indicated the cut-off points according to the international recommendation in the result section and added the appropriate citation (Eastell et al., JCEM, 2014). Patient recruitment and follow-up data were collected between 2008 and 2019 and we started the use of CCCR approximately at the beginning of the past decade. The sentence has been modified and the proposed citation has been added (Page 5, line 83-85). Data collection period is now specified in the method section (Page 7, line 131-132).

Changes in the text: Twenty-four-hour urinary (24hU) CE and the calculated relation of urinary Ca clearance to creatinine clearance (CCCR) are internationally recommended and thus widely used diagnostic tools to discriminate FHH from PHPT (1,2).

All patients gave their written informed consent. The data were prospectively documented between 2008 and 2019 and retrospectively analyzed.

Comment 2: Methods, line 100: were the patients fasting? was there any screening for medication, chronic kidney disease, etc...? Many other confounding factors can interfere with CCCR and 24h-CE measures.

Reply 2: Thank you for this crucial note as it is very important to take the comorbidities in consideration when assessing CCCR and 24h-CE. None of the patients in our study was fasting at the time of analysis. Importantly, we conducted a specific screening for medication (thiacides). Patients stopped taking the medication at least three weeks prior to CCCR and 24h-CE analysis. Concerning other confounding factors, we reported the related symptoms in Table 1. We modified the corresponding paragraph in the method (Page 8, line 159-160) and result section as advised (Page 10-11, line 217-219).

Changes in the text: Furthermore, interfering factors, such as comorbidities, fasting period and medication (thiazide diuretics), were taken in consideration when assessing CCCR and 24h-CE.

None of the patients presented any relevant comorbidity or was fasting at the time of analysis. Patients stopped taking thiazide diuretics at least three weeks prior to the assessment of CCCR and 24hU CE.

Comment 3: Methods, line 102: what were the indications for surgery? I hope not elevated Ca and/or PTH. What is the usual practice in the team?

Reply 3: According to the current European and German speaking guidelines, surgery is indicated in every patient with PHPT. However, in patients with only moderate hypercalcemia and without symptoms, an observation with close follow-up is also an option, which is offered to the patients. The vast majority of patients, however, decide to undergo surgery in the daily practice. The first paragraph in the result section describes the screening process, as well as the indication for surgery. Furthermore, we added a new flow diagram illustrating this data (Figure 1) (Page 10, line 201-205).

Changes in the text: PHPT was assumed in 6/14 (42.9%) patients (elevated serum Ca and intact PTH and/or CCCR >0.001 and/or increased 24hU CE), who underwent surgery for PHPT. However, the persistent postoperative elevated serum Ca levels and symptoms lead to further genetic analysis, which subsequently revealed FHH1 (Figure 1).

Comment 4: Methods, line 104: My major concern is about the genetic testing. Authors should

apply the ACMG recommendation. IVS should not be considered as pathogenic, unless familial segregation has been performed. Can the authors provide us with more details as how the mutants were characterized as per the ACMG recommendations?

Reply 4: We completely agree with the reviewer that variants of unknown significance are not to be considered pathogenic. As suggested by the reviewer ACMG nomenclature was applied (please compare new table 2 in the revised manuscript) and in addition information available from SIFT analysis and ClinVar are included in table 2 as well. The respective passages in the text (p.....) were changed accordingly. The specific paragraphs in Methods (Page 9, line 185-188), Results (Page 11, line 231-232) and Discussion (Page 16, line 332-344) have been modified. Additionally, a separate table has been added classifying the CaSR variants (Table 2).

Changes in the text: Detected variants were analyzed using Alamut Visual Plus (SophiaGenetics) and classified according to the American College of Medical Genetics and Genomics (ACMG) and ClinVar (14, 15).

Classification of detected CaSR variants and a schematic representation of CaSR gene structure are shown in Table 2 and Figure 2.

In the present study, and alongside other common benign variants, nine different (heterozygous) variants were detected which included nucleotide substitutions that would result in a frameshift, stop codons (e.g., p.(Ile427LeufsX35); p.(Leu71*); p.(Gly366*)) or amino acid substitutions and could thus be causative of FHH1. It is well known that most patients are carriers of so called “private mutations” meaning that those variants are usually not described in the literature and functional assays concerning CaSR-receptor function are not available. This circumstance results in classification of most of the variants as C3 (uncertain significance, according to ACMG), although the observed types of coding effects (frameshift, stop codon) as well as SIFT (sorting intolerant from tolerant) analysis would indicate a pathogenic nature for most of the variants. For p.(Arg544Gln), however, reports are conflicting as to whether this variant is pathogenic or likely benign also resulting in classification of the variant as C3.

Comment 5: Authors state they collected 24h-creatinine in urine: they should provide such information. Especially this is of particular interest to test whether the urine collection was complete or not. This should be expressed as a ratio of body weight. Kidney function should be presented as well.

Reply 5: We completely agree with the reviewer. We kindly apologize for not providing the information (ratio urine collection/body weight), as this is not assessed routinely in our clinic. However, both other parameters (kidney function and 24hU creatinine) have been added to patient characteristics (Table 1).

Changes in the text: Table 1 modified.

Comment 6: Finally, the major finding is that CCCR in this series is as accurate as splitting a coin to discriminate FHH from PHPT patients. Authors should elaborate on more precise tools, already published (for instance Bertocchio et al. JCEM 2018). How could surgeons improve their screening practice? Maybe they should not take any surgical decision based on CCCR if 25(OH)vitamin D is not normal (cf. Table 2).

Reply 6: This is a very important indication. We are grateful to the reviewer for mentioning this interesting diagnostic tool. We have added a new paragraph in the discussion section

elaborating on this newly topic (Page 15, line 303-316).
Changes in the text: In 2018, Bertocchio et al presented an unsupervised risk equation (Pro-FHH) indicating PHPT. The authors concluded that Pro-FHH (100% specificity and 100% positive predictive value) is more effective than 24h-CCCR (96.8% specificity and 90% positive predictive value) in predicting whether a patient had PHPT. Importantly, Pro-FHH is easily calculated as it requires only a measure of calcium, creatinine (in blood and urine), PTH and serum osteocalcin concentrations. However, as stated by the authors, Pro-FHH's weakness is its development based on a retrospective design. Furthermore, Bertocchio et al. included only patients with normal PTH level in their study. In our study, 5/6 patients showed elevated (>73pg/mL) and 1/6 patients showed high-normal (62pg/mL) PTH concentrations prior to surgery. The effectiveness of Pro-FHH needs to be investigated in patients with elevated PTH concentration levels. Therefore, a prospective trial is necessary. However, till new data and findings are available, perhaps a combination of diagnostic tools, such as CCCR, 24hU CE and Pro-FHH, would likely support the screening process.

MINOR COMMENTS
Comment 7: abstract line 38: 'CE' has not been introduced before
Reply 7: Thank you for the note. We have modified the text as advised (Page 3, line 52-55).
Changes in the text: CaSR-genotyping was conducted, various biochemical parameters including twenty-four-hour urinary Ca excretion (24hU CE) and the calculated relation of urinary Ca clearance to creatinine clearance (CCCR), type of surgery and 1-year follow-up data of fourteen patients with proven FHH1 were evaluated.

Comment 8: all over the manuscript: CaSR should refer to the protein CASR to the gene.
Reply 8: Thank you for the note. The term was changed accordingly throughout the manuscript.
Changes in the text:

Comment 9: introduction, line 60: we no longer use the term 'benign' for FHH; patients can have pancreatitis which is not benign.
Reply 9: We agree. The first sentence has been modified as advised (Page 5, line 78-80).
Changes in the text: Familial hypocalciuric hypercalcemia (FHH) is a rare condition, characterized by elevated serum calcium (Ca) and inappropriately normal or slightly elevated parathyroid hormone (PTH) levels.

Comment 10: introduction, line 60: authors should read Dershem R et al. Am J Hum Gen 2020; they'll see how not rare FHH is, especially as compared to PHPT in males... so, FHH is not 'extremely' rare.
Reply 10: Thank you for the hint. The first sentence has been modified as advised (Page 5, line 78-80).
Changes in the text: Familial hypocalciuric hypercalcemia (FHH) is a rare condition, characterized by elevated serum calcium (Ca) and inappropriately normal or slightly elevated parathyroid hormone (PTH) levels.

Comment 11: introduction, line 64: CE is not 'reduced' in FHH, mean CE is just lower in FHH

patients as compared to the one found in PHPT patients. FHH patients can have very high values of CE.

Reply 11: We apologize for the poor wording. Several sentences have been modified accordingly (Page 5, line 81-83; Page 10, line 211-213; Page 14, line 298-299).

Changes in the text: FHH is usually diagnosed with family history and laboratory findings, especially lower urinary excretion of Ca (CE).

In 6/14 (85.7% □3/6 wrongly interpreted for PHPT□) patients lower levels (<2.5 mmol/24h) of 24hU CE were found, indicating FHH.

Lower 24hU CE (<2.5mmol/24h) most likely indicates FHH disorder.

Comment 12: Methods, line 111: authors should read Pekar JD et al. Clin Chem Lab Med 2019

Reply 12: Thank you for the hint. It is an interesting study on agreement rate between ionized calcium (iCa) measurement and total calcium (TCa) or albumin-adjusted calcium measurements. Although we did not indicate iCa but TCa in our manuscript, we nevertheless assessed iCa in every patient. Data can surely be provided if required.

Changes in the text:

Comment 13: Symptoms, linde 135: what is the meaning of discriminating patients 'minimally symptomatic' to others?

Reply 13: This a legitimate question. We intended to differ between patients with no, mild and severe symptoms for a precise classification. However, we understand that this is more confusing than clarifying. Therefore, we will reduce to two groups, asymptomatic and symptomatic. The text in the paragraph was modified accordingly (Page 8, line 164-168; Page 10, line 216-217).

Changes in the text: The disorder is interpreted as symptomatic if patients show a classical course with impairment of the kidney, bone or gastrointestinal tract, kidney stones, osteopenia or osteoporosis, chronic gastroenteritis, symptoms of cardiovascular disease (i.e. arterial hypertension) or hypercalcemic syndrome (i.e. fatigue).

Eight of 14 (57.1%) patients were clinically asymptomatic and 6/14 (42.9%) patients showed classical symptoms of PHPT.

Comment 14: Statistical analysis, line 153: T-tests are not appropriate for such low figures, especially if normality has not been assessed.

Reply 14: Thank you for this note. We provided the p-values as additional information and detailed report of data. We can remove it if requested by the reviewer or editor.

Changes in the text: -

Comment 15: Why did the authors collect blood calcitriol concentration?

Reply 15: The assessment of 25(OH)D and 1,25(OH)D is performed routinely for patients with endocrine disorders at our institution.

Changes in the text: -