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Evaluating CHARGE Syndrome in Congenital Hypogonadotropic Hypogonadism Patients Harboring CHD7 Variants

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Abstract:	Purpose: Congenital hypogonadotropic hypogonadism (CHH) is a rare genetic disease caused by gonadotropin releasing hormone deficiency, and can also be part of complex syndromes (e.g. CHARGE syndrome). CHD7 mutations were reported in 60% of patients with CHARGE syndrome, and in 6% of CHH patients. However, the definition of CHD7 mutations was variable, and the associated CHARGE signs in CHH were not systematically examined. Methods: Rare sequencing variants (RSVs) in CHD7 were identified through exome sequencing in 116 CHH probands, and were interpreted according to ACMG guidelines. Detailed phenotyping was performed in CHH probands who were positive

	for CHD7 RSVs, and genotype-phenotype correlations were evaluated. Results: 16% (18/116) of CHH probands harbor heterozygous CHD7 RSVs, and detailed phenotyping was performed in 17 of them. 80% (4/5) of CHH patients with pathogenic or likely pathogenic CHD7 variants were found to exhibit multiple CHARGE features, and 3 of these patients were reclassified as CHARGE syndrome. In contrast, only 8% (1/12) of CHH patients with non-pathogenic CHD7 variants exhibit multiple CHARGE features (p=0.01). Conclusion: Pathogenic or likely pathogenic CHD7 variants rarely cause isolated CHH. Therefore a detailed clinical investigation is indicated to clarify the diagnosis (CHH vs. CHARGE) and to optimize the clinical management.

Prof. James P. Evans
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Lausanne, October 4th, 2017

Dear Prof. Evans,

We would like to thank you for the acceptance of our manuscript GIM-D-17-00362R1, entitled "Evaluating CHARGE Syndrome in Congenital Hypogonadotropic Hypogonadism Patients Harboring CHD7 Variants" for publication in *Genetics in Medicine*.

As per the acceptance email, we have supplied all of the requested information including higher resolution figures and final version of supplementary data. We attest that we have received and archived written consent for participation/publication from every individual whose data is included. We will not be requesting Open Access for this article.

Please feel free to let us know if you have any additional questions or needs, and again thank you for the opportunity to publish our manuscript in your worthy journal.

Sincerely yours,

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Reviewer Comments:

Reviewer #1: This manuscript describes results of a whole exome sequencing project from 116 individuals with congenital hypogonadotrophic hypogonadism (CHH). The authors report that, among individuals with pathogenic variants in CHD7, several have additional features of CHARGE syndrome that led to reclassification of their diagnosis.

Generally, the manuscript is well written and the data are nicely presented. However, the conclusions drawn about some of the individuals lack evidence that would be required to establish or refute a clinical diagnosis of CHARGE syndrome.

We thank the Reviewers for their diligent review of our manuscript and their insightful suggestions. We have responded each of the points below.

A major concern is that the authors do not use the most recent clinical diagnostic criteria for CHARGE syndrome (published by Hale et al, American Journal of Medical Genetics, 2016), significantly limiting the interpretation of their results. In the Hale paper, minor criteria for CHARGE syndrome are de-emphasized and pathogenic CHD7 variants are included as a major criterion. Use of these new criteria by the authors would lead to reassignment of all 5 individuals in this study with pathogenic or likely pathogenic CHD7 variants for whom adequate clinic information is available (patient 4 has SCC abnormalities and patient 5 has no temporal bone CT or MRI).

The Reviewer brings up an interesting point regarding the use of the Hale criteria for the diagnosis of CHARGE syndrome, recently published in American Journal of Medical Genetics [1].

We acknowledge that the Hale proposal, which includes the presence of a pathogenic *CHD7* variant as one of the major criteria, may provide a unique genetic aspect to the diagnosis of CHARGE syndrome. However, our study aims to analyze the genotype-phenotype correlation of *CHD7* variants in CHH. Thus, we feel that inclusion of genotype information within the phenotype classification (i.e. diagnosis of CHARGE syndrome) could lead to a bias in the results. Therefore, we used the Verloes criteria (the purely clinical diagnostic criteria for CHARGE syndrome) which are still widely in use [2].

In addition, one concern with the Hale proposal lies in the fact that their definition of a pathogenic *CHD7* variant is not clear. The discussion section of the Hale paper states that missense variants are specifically required to be *de novo* and present in another CHARGE patient. This is not consistent with the now widely-accepted guidelines from the American College of Medical Genetics and Genomics (ACMG). The extra level of stringency recommended by Hale et al. can lead to the under-classification of truly pathogenic variants. Furthermore, as most *CHD7* variants in CHH patients are missense rather than the frameshift and nonsense mutations primarily seen in CHARGE syndrome [3-5], this point regarding

interpretation of missense variants becomes especially critical. We have addressed the Hale proposal in the Discussion (Page 18, paragraph 1).

In addition, the conclusions drawn are not novel, as similar observations were previously reported by Bergman et al, albeit on a smaller cohort of individuals (36 patients; Bergman et al, J Clin Endocrinol Metab, 2012).

Multiple studies have addressed the presence of *CHD7* mutations in CHH/KS patients [3, 4, 6-8], however, the results are conflicting. Two studies reported *CHD7* mutations only in KS with additional CHARGE-like features [7, 8]. In contrast, three studies with a larger number of CHH probands found *CHD7* mutations in isolated CHH patients [3, 4, 6]. These divergent findings likely result from the lack of systematic phenotyping of CHARGE features in CHH patients, as well as the use of different genetic definitions of "mutations". Our study addressed these two limitations by applying the ACMG guidelines for variant interpretation and performing detailed phenotyping in CHH patients, with a goal of contributing to a consensus within the field.

Several minor concerns, if addressed, will also strengthen the paper:

We thank the Reviewer for the careful review of our manuscript. We have corrected the following points as the Reviewer suggested.

1. pg 5 last line, "harbor" is misspelled.
Corrected

2. pg 7, first sentence "combining a detailed phenotyping focusing" makes no sense.
Corrected

3. pg 10, para 2, second to last line, add an "s" to "harbor"
Corrected

4. pg. 11, first sentence, change "was utilized" to "were utilized"; line 4 change to "presented (add "ed"); line 11, is "bilateral vestibular" supposed to modify some other word that is missing?; line 18, "bilateral transmission hearing loss" is not a medical term; line 23 (last line), change to "presented with coloboma"
Corrected.

5. pg 12, first line, is the external ear malformation unilateral?; line 2, remove "a" before "mild bilateral"; line 5 should read "Patient (no s) 4"; for Patient 5, add that no temporal bone CT or MRI has been done (this is highly pertinent to the ability to establish a diagnosis (or not)); line 11 change "defect" to "pathogenic variant"; line 16 change to "exhibit (no s)".
Corrected.

6. mental retardation is no longer an acceptable term; the correct term is "intellectual disability".
Corrected.

7. pg 13, line 5 from the bottom, "the sister" is ambiguous; use proband #s.
Corrected.

8. pg 14, line 9, remove "being"
Corrected.

9. pg 15 line 2 add "the" between "in" and "CHH"; line 12, the phrase "question regarding the" is awkward (consider replacing with "question of") 10. pg 16, line 16 remove "a" between "have" and "higher"; line 22 "their study" is ambiguous; line 17 add "the" before "Bergmann"; also, the correct spelling is "Bergman"
Corrected.

11. pg 17, line 3 remove "the" between "all" and "five"
Corrected.

12. the term "mutations" should be replaced with "pathogenic variants" throughout the paper, to be in line with current human genetics nomenclature.

We agree with the Reviewer that the term of 'variant' and 'pathogenic variant' are the current recommended nomenclature, and we used these terms for all the findings from this study. However, when we cite previous reports, we prefer to use the exact term used in those reports (e.g. mutations), in order to avoid a misrepresentation of the original reports.

13. Table 1, "median organ" is a vague term. Do the authors mean "mediastinal organ"? If so, this term should be used, and more clinical detail provided.

We have corrected this term. Clinical details of mediastinal organ malformation were provided in the main text (Page 12, paragraph 3 for Patient 1; Page 13, paragraph 2 for Patient 4, Page 14, paragraph 3 for Patient 8, Table 1 legend for Patient 9).

14. Table 2, the additional M340V in the unaffected father is predicted benign, yet the authors propose that it could serve to modify the phenotype of the affected children. This seems less likely than the variant having no effect, given the broad variability in clinical expression among individuals with CHARGE features.

We agree with the Reviewer's point that variable expressivity is commonly seen in familial cases of CHARGE syndrome. The phenotypic variation observed within members carrying the pathogenic variant (p.A1107V) in Family 4 can be due to variable expressivity, as previously reported in CHARGE syndrome. However, we cannot exclude a modifying or contributing role of the p.M340V in this family. We have edited the text to reflect this (Page 13, paragraph 2).

15. Table S1 would be easier to follow if the variants were mapped onto the patients as in Table 1.

As per the Reviewer's suggestion, we have added a column with Patient number in Table S1 in order to match the Patient number in Table 1.

Reviewer #2: Xu and colleagues describe the results of gene panel testing in a large cohort of patient with congenital HH. They focus on the resulting CHD7 rare variants found. The strength of this study is the good phenotyping of the patients with such a CHD7 RSV.

We thank the Reviewer for their positive comments on our manuscript.

I have a few, mostly minor, comments:
Regarding the conclusion on compound htz CHD7 variant (the benign variant adding to the phenotype), the authors should be more careful, the syndrome is known to be highly variable so there is a high chance of coincidence here.

We agree with the Reviewer's comment. We have added this point in the Result section (Page 13, paragraph 2).

Page 17: The authors discuss the relationship of CHD7 with several other proteins known to be involved in HH / KS like FGFR1. Nothing is said about FGF8. Recently it was found that CHD7 and FGF8 are linked in the early development of the cerebellum. Is a similar relationship possible for the GnRH neuronal development?

We thank the Reviewer for this insightful comment. Indeed, no study has examined the interaction between CHD7 and FGF8 in GnRH neuronal development. We have added this point in the discussion (Page 19, paragraph 1)

The authors should stress another reason for which careful re-evaluation of patients with a CHD7 mutation is extremely important. If the diagnoses CHARGE syndrome is made we know that the clinical variability is huge, even with the same pathogenic variant (e.g. within families and mono-zygotic twins). This has consequences for the off-spring of these patients: the phenotype of a child inheriting the variant cannot be predicted and may be more severe than in the parent. Genetic counseling is thus very important.

We thank the Reviewer for this pertinent suggestion. We have added this point in the Discussion (Page 19, paragraph 2).

Some typo's are present in the manuscript:
key words: hypogonaidsm -> hypogonadism
page 12: patients 4 -> patient 4
page 12: no additional CHARGE feature -> features

page 14: futher -> further
Fig 1A and fig 2 are hardly readable (characters are blurred)

We apologize for the oversight of the typos, and have corrected them. We have also provided figures with higher resolution, although the blurring noted by the Reviewer was most likely introduced during the generation a PDF file (for the review process) from our original figures.

Reference:

1. Hale, C.L., et al., *Atypical phenotypes associated with pathogenic CHD7 variants and a proposal for broadening CHARGE syndrome clinical diagnostic criteria.* Am J Med Genet A, 2016. **170a**(2): p. 344-54.
2. Bergman, J.E., et al., *CHD7 mutations and CHARGE syndrome: the clinical implications of an expanding phenotype.* J Med Genet, 2011. **48**(5): p. 334-42.
3. Marcos, S., et al., *The prevalence of CHD7 missense versus truncating mutations is higher in patients with Kallmann syndrome than in typical CHARGE patients.* J Clin Endocrinol Metab, 2014. **99**(10): p. E2138-43.
4. Balasubramanian, R., et al., *Functionally compromised CHD7 alleles in patients with isolated GnRH deficiency.* Proceedings of the National Academy of Sciences of the United States of America, 2014. **111**(50): p. 17953-8.
5. Janssen, N., et al., *Mutation update on the CHD7 gene involved in CHARGE syndrome.* Human Mutation, 2012. **33**(8): p. 1149-1160.
6. Kim, H.G., et al., *Mutations in CHD7, Encoding a Chromatin-Remodeling Protein, Cause Idiopathic Hypogonadotropic Hypogonadism and Kallmann Syndrome.* American Journal of Human Genetics, 2008. **83**(4): p. 511-519.
7. Jongmans, M.C., et al., *CHD7 mutations in patients initially diagnosed with Kallmann syndrome--the clinical overlap with CHARGE syndrome.* Clin Genet, 2009. **75**(1): p. 65-71.
8. Bergman, J.E., et al., *The results of CHD7 analysis in clinically well-characterized patients with Kallmann syndrome.* J Clin Endocrinol Metab, 2012. **97**(5): p. E858-62.

Evaluating CHARGE Syndrome in Congenital Hypogonadotropic Hypogonadism Patients Harboring *CHD7* Variants

Short running title: *CHD7* in CHH: phenotype-genotype correlation

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Abstract

Purpose: Congenital hypogonadotropic hypogonadism (CHH) is a rare genetic disease caused by gonadotropin releasing hormone deficiency, and can also be part of complex syndromes (e.g. CHARGE syndrome). *CHD7* mutations were reported in 60% of patients with CHARGE syndrome, and in 6% of CHH patients. However, the definition of *CHD7* mutations was variable, and the associated CHARGE signs in CHH were not systematically examined.

Methods: Rare sequencing variants (RSVs) in *CHD7* were identified through exome sequencing in 116 CHH probands, and were interpreted according to ACMG guidelines. Detailed phenotyping was performed in CHH probands who were positive for *CHD7* RSVs, and genotype-phenotype correlations were evaluated.

Results: 16% (18/116) of CHH probands harbor heterozygous *CHD7* RSVs, and detailed phenotyping was performed in 17 of them. 80% (4/5) of CHH patients with pathogenic or likely pathogenic *CHD7* variants were found to exhibit multiple CHARGE features, and 3 of these patients were reclassified as CHARGE syndrome. In contrast, only 8% (1/12) of CHH patients with non-pathogenic *CHD7* variants exhibit multiple CHARGE features (p=0.01).

Conclusion: Pathogenic or likely pathogenic *CHD7* variants rarely cause isolated CHH. Therefore a detailed clinical investigation is indicated to clarify the diagnosis (CHH vs. CHARGE) and to optimize the clinical management.

Key words: Congenital hypogonadotropic hypogonadism; Kallmann syndrome; CHARGE syndrome; chromodomain helicase DNA binding protein 7.

INTRODUCTION

Congenital hypogonadotropic hypogonadism (CHH) is a rare genetic disorder (1 in 4,000 to 10,000)[1] caused by isolated gonadotropin-releasing hormone (GnRH) deficiency, leading to absent or incomplete puberty and infertility. Non-reproductive features such as anosmia, hearing impairment, cleft lip/palate, and scoliosis are often seen in patients with CHH, and are considered CHH-associated phenotypes.[2-4] In particular, anosmia is present in 50% of CHH patients and this co-occurrence is termed Kallmann syndrome (KS). CHH can be part of a complex syndrome such as Bardet-Biedl Syndrome, septo-optic dysplasia, CHARGE syndrome, and others.[5] CHARGE syndrome has a prevalence of 1 in 15,000 to 17,000.[6] The CHARGE acronym represents a non-random cluster of multi-organ malformations including coloboma, heart defects, choanal atresia, retardation of growth and development, genital hypoplasia and ear anomalies.[6] Based on the clinical severity (i.e. the number of major and minor CHARGE signs), a patient can be diagnosed as typical, partial or atypical CHARGE syndrome.[7] Despite the distinct diagnostic criteria, several phenotypes such as hearing impairment and hypogonadotropic hypogonadism overlap between CHH and CHARGE syndrome.

CHARGE syndrome is an autosomal dominant disease. *CHD7*, encoding chromodomain helicase DNA binding protein 7, is the major causative gene for CHARGE syndrome. CHD7 plays an important role in chromatin modeling and transcription regulation, and it regulates genes implicated in neural crest guidance.[8-10] Heterozygous mutations in *CHD7*, mainly nonsense or frameshift, were found in 60% of CHARGE cases (ranging from 33% to 90%).[6] Nearly all cases of CHARGE syndrome are sporadic (>97%), and most CHARGE patients harbor *de novo CHD7* mutations (when tested).[6]

In contrast, CHH is a genetically heterogeneous disease, with mutations observed in > 20 genes exhibiting varying modes of inheritance.[5] Oligogenicity, defined as mutations in more than one causative gene, occurs in 7% of CHH patients.[11] Thirty percent of CHH patients are considered as familial cases, as they have family members with CHH, delayed puberty or anosmia. Variable expressivity is often observed in familial CHH.[5] Interestingly, *CHD7* mutations have been reported in 6% of CHH patients with or without additional CHARGE-like features.[5] Some have proposed that CHH is a mild allelic form of CHARGE syndrome.[12,13] Still, the correlation between CHARGE-like phenotypes and *CHD7* genotypes in CHH is unclear due to the lack of systematic phenotyping which specifically evaluates CHARGE-like features in CHH patients.

Identifying genotype-phenotype correlations are also hindered by a lack of a consistent definition of mutations across studies. With advanced sequencing technologies and the availability of genetic data in large populations (e.g. 135,000 subjects in the Genome Aggregation Database, gnomAD), previous criteria for mutations based on minor allele frequency and/or prediction algorithms do not appear to be sufficient to define a pathogenic variant. Indeed, the American College of Medical Genetics and Genomics (ACMG) recently updated the guidelines to standardize the interpretation of sequencing variants, outlining 28 different criteria which integrate data from population studies, computational and predictive algorithms, functional assays, segregation analysis, and others.[14] To minimize potential bias in data collection and evaluation, a web-based tool (InterVar) has been developed and validated to facilitate the application of ACMG criteria.[15]

In this study, we aimed to refine the role of *CHD7* in CHH. We performed detailed phenotyping focusing on CHARGE-like features in a large CHH cohort who were positive for rare variants in

CHD7. Subsequently, we used the ACMG guidelines to classify these variants and performed phenotype-genotype correlation.

PATIENTS AND METHODS

Subjects and clinical studies

The clinical and genetic studies were approved by the ethics committee of the University of Lausanne and all participants provided written informed consent prior to study participation. The clinical trial registry number is NCT01601171. The CHH cohort includes 116 unselected probands of non-Finnish European origin (NFE, 61 KS and 55 normosmic CHH). The diagnosis of CHH is defined by: i) absent or incomplete puberty by age 16; ii) low/normal gonadotropin levels in the setting of low serum testosterone/estradiol levels; and iii) otherwise normal anterior pituitary function and normal imaging of the hypothalamic-pituitary area. Olfaction was assessed by self-report and/or formal testing.[16] The 2005 Verloes criteria[7] were used to classify CHARGE-like features and diagnose CHARGE syndrome. In addition to a comprehensive medical exam and review of medical/surgical history, CHD7-positive probands underwent MRI or CT to visualize inner ear structures whenever possible. Audiometry/audiogram and cardiac ultrasound/MRI were performed in cases with clinical suspicion. As a single CHARGE-like feature can be associated with CHH independent of mutations in *CHD7,*[4] only the presence of ≥ 2 additional CHARGE-like features was considered as a significant clinical finding in this study. Both affected and unaffected family members were recruited for clinical characterization and genetic studies when available. The control group consists of 405 unrelated NFE participants from a population-based study, “Cohorte Lausannoise” (CoLaus).[17]

Genetic studies

Exome sequencing in CHH and control cohorts was performed using previously described methods.[18] Non-synonymous rare sequencing variants (RSVs) with MAF < 1% in NFE controls from genome Aggregation Database (gnomAD, http://gnomAD.broadinstitute.org/) in *CHD7*

(NM_017780) and in 23 other known CHH genes were included in this study. The included CHH genes are: *ANOS1* (NM_000216.2), *SEMA3A* (NM_006080), *FGF8* (NM_033163.3), *FGF17* (NM_003867.2), *SOX10* (NM_006941), *IL17RD* (NM_017563.3), *AXL* (NM_021913), *FGFR1* (NM_023110.2), *HS6ST1* (NM_004807.2), *PCSK1* (NM_000439), *LEP (*NM_000230), *LEPR* (NM_002303), *FEZF1 (*NM_001024613), *NSMF* (NM_001130969.1), *PROKR2* (NM_144773.2), *WDR11* (NM_018117), *PROK2* (NM_001126128.13), *GNRH1* (NM_000825.3), *GNRHR* (NM_000406.2), *KISS1* (NM_002256.3), *KISS1R* (NM_032551.4), *TAC3* (NM_013251.3), *and TACR3* (NM_001059.2). All *CHD7* variants were confirmed by Sanger sequencing, as well as any RSVs in the remaining 23 CHH genes in these patients. Variants are reported in agreement to HGVS nomenclature.[19]

CHD7 RSVs were interpreted according to the ACMG guidelines.[14] For missense variants, InterVar (http://wintervar.wglab.org/) was used for the automated interpretation, and included information on segregation, phenotype and structural modeling data. Variants were classified into 5 categories: pathogenic, likely pathogenic, uncertain significance, likely benign and benign. Pathogenic or likely pathogenic RSVs in other CHH genes were also reported in the study. In addition, the identified *CHD7* RSVs were also classified by the commonly used criteria based on SIFT and PolyPhen2: a variant is categorized as pathogenic if predicted to be deleterious by either SIFT and/or PolyPhen2. Finally, the Bergman system,[20] a classification system for missense *CHD7* variants in CHARGE syndrome, was also used to categorize identified *CHD7* RSVs.

Statistical analysis

Gene-collapsed rare variant association (RVA) test in CHH vs. controls was performed to

compare rare variant allele frequencies by two-tailed Fisher's exact test. Fisher's exact test was also used to compare the percentage of patients in different groups as appropriate. The significance level was set at $p < 0.05$ (two-sided).

RESULTS

CHD7 RSVs are significantly enriched in CHH versus controls

Seventeen heterozygous *CHD7* RSVs, including 2 protein truncating variants (PTVs) and 15 missense variants, were identified in 18 of the 116 CHH probands (Table S1, Figure 1A). Only 1 missense RSV (p.M340V) was found in the 405 controls from the CoLaus cohort. The allele frequency of *CHD7* RSVs was significantly higher in CHH probands relative to CoLaus controls (7.8%, 18/232, vs. 0.1%, 1/810; p = 1.6E-11).

The PTVs included a splicing variant (c.2613+5G>A) which was previously shown to result in the skipping of exon 9 and found in an unrelated normosmic CHH patient,[12] and a nonsense variant (p.R2428*) previously reported in an unrelated CHARGE syndrome patient (www.chd7.org). Among the 15 missense RSVs, five were located in CHD7 functional domains (Figure 1A-B). Structural modeling predicted that all variants within the functional domains (except p.A1107V) were deleterious (Figure 1B). Segregation analysis was performed in 16/18 (89%) pedigrees. Three RSVs were *de novo*, including the nonsense and 2 missense, and the others RSVs were inherited (Table S1, Figure 2). One affected family member was found to harbor compound heterozygous RSVs (Figure 2, Family 4). Genetic data for family members of the remaining 2 probands were not available.

Further classifying the variants according to the ACMG guidelines, we found that 5 variants were pathogenic or likely pathogenic, 7 variants were of uncertain significance, and 5 were benign or likely benign (Table S1).

Genotype-phenotype correlation

Detailed phenotyping of additional CHARGE signs was performed in 17/18 CHH probands, and the data were utilized for subsequent genotype-phenotype correlation analysis (Table 1). The remaining proband was unavailable for clinical follow-up.

All five of the probands harboring pathogenic or likely pathogenic *CHD7* variants also presented with anosmia (i.e. Kallmann syndrome, KS). Further, four out of five probands also exhibit CHARGE-associated features.

Patient 1 is a female proband previously diagnosed with KS. Although she was also noted to have coloboma and pulmonary artery stenosis, she did not have further evaluation for CHARGE syndrome. Genetic testing revealed that she harbored a *de novo* nonsense *CHD7* variant (p.R2428*). Follow-up MRI showed bilateral hypoplasia of semi-circular canals, confirming the diagnosis of typical CHARGE syndrome (i.e. 2 major signs & 2 minor CHARGE signs).

Patient 2 is a male patient born with bilateral choanal atresia that was subsequently surgically repaired. He had normal growth and development during childhood, without evident CHARGE signs. Further medical evaluation for absent puberty revealed isolated hypogonadotropic hypogoandism and anosmia (Sniffins' Stick 5/16, < 5%tile), leading to the diagnosis of KS. He was found to harbor a *de novo CHD7* RSV (p.Y1412D). Additional detailed phenotyping revealed mild external ear defect, audiogram indicated mild bilateral conductive hearing loss, and MRI showed hypoplasia of olfactory bulbs, semi-circular canal malformation and VII nerve hypoplasia. No heart defects were identified in the cardiac evaluation. The patient exhibited 2 major and 3 minor CHARGE signs, consistent with the diagnosis of typical CHARGE syndrome.

Patient 3 is a male proband who was referred to our clinical service with the diagnosis of KS. He also presented with coloboma and facial palsy. He was found to harbor a *de novo CHD7* missense RSV (p.C989Y). Further detailed phenotyping revealed bilateral external ear malformation, and audiogram indicated mild bilateral sensorineural hearing loss. Cardiac and inner ear MRIs were normal. Base on these findings (i.e. 1 major & 3 minor CHARGE signs), he was re-classified as having atypical CHARGE syndrome.

Patient 4 is a female proband diagnosed with KS with a history of congenital heart malformation (mitral valve prolapse), synkinesia and scoliosis. She was found to harbor *CHD7* p.A1107V, and a subsequent inner ear CT revealed semi-circular canal hypoplasia. She exhibited 1 major and 2 minor CHARGE signs, but did meet the diagnostic criteria for CHARGE syndrome. Expanding the genetic testing in this family, we found that both the proband's mother and sister harbored the same *CHD7* p.A1107V variant, while the proband's sister inherited an additional benign variant (p.M340V) from their father (Figure 2). This affected sister also had KS and all of the CHARGE features present in the proband, as well as the additional features of unilateral sensorineural hearing loss and unilateral renal hypoplasia, resulting in a diagnosis of atypical CHARGE syndrome (Table 2). Their mother, who carried the heterozygous p.A1107V variant, was reproductively normal but exhibited two CHARGE signs (semi-circular canal hypoplasia and hearing impairment), while the father was phenotypically normal without any signs of CHARGE or CHH (Table 2). The phenotypic difference between the two unaffected sisters can be the result of the variable expressivity of *CHD7* pathogenic variants.[21,22] However, it is also possible that the additional *CHD7* p.M340V variant, although predicted to be benign, may contribute to the phenotypic difference observed between the two affected siblings.

Patient 5 is a male proband with isolated KS. Both he and his affected brother were found to harbor the *CHD7* p.F1019C variant. The patient exhibited no additional CHARGE features, however, a radiological evaluation of the inner ear structures was not performed. Interestingly, he was the only patient in this group to harbor an additional pathogenic variant in another CHH gene (*SEMA3E* p.R619C).[18]

Overall, the majority (80%, 4/5) of CHH probands with pathogenic or likely pathogenic *CHD7* RSVs exhibited at least two additional CHARGE features, and three of these patients met the diagnostic criteria for CHARGE syndrome (Table 1).

Non-pathogenic *CHD7* variants (uncertain significance, likely benign, or benign) were found in 12 CHH patients. Notably, almost half (42%, 5/12) of these patients were found to harbor a pathogenic or likely pathogenic variant in other CHH genes (Table 1, Figure 2). Patient 6 was the only patient to exhibit ≥ 2 additional CHARGE features. She had KS with sensorineural hearing loss and intellectual disability, yet did not meet the diagnostic criteria for CHARGE syndrome. She was found to harbor an additional PTV in *FGFR1* (p.R365Kfs*5). Three patients with CHH exhibited a single additional CHARGE feature: sensorineural hearing loss in Patient 7, mild heart defect (mild dilatation of right ventricle, aortic root and ascending aorta) in Patient 8, and intellectual disability in Patient 12. The remaining seven patients did not exhibit any additional CHARGE feature, although some had other CHH-associated phenotypes, such as synkinesia, dental or skeletal defect (Table 1).

In summary, a significantly higher proportion of CHH patients with pathogenic or likely pathogenic *CHD7* RSVs exhibited multiple CHARGE features, compared to those with non-

pathogenic *CHD7* variants (80%, 4/5 vs. 8%, 1/12, p = 0.01). Indeed, three CHH patients with pathogenic or likely pathogenic *CHD7* RSVs were reclassified as having CHARGE syndrome following detailed clinical investigation, while none of the probands with non-pathogenic variants merited diagnostic reclassification (60%, 3/5, vs. 0%, 0/12, p = 0.02).

Genotype-phenotype correlation based on different classification of *CHD7* RSVs

The *CHD7* missense variants found in CHH probands were further classified using SIFT / PolyPhen2 and the Bergman system (Table 3). The Bergman system integrates information from structural modeling, prediction algorithms (Polyphen-2 and Align-GVGD), segregation and population data.[20] This system has been used to evaluate missense *CHD7* variants in CHARGE syndrome, but has not previously been applied in CHH. 73% (11/15) of *CHD7* RSVs were categorized by SIFT and/or PolyPhen2 as damaging. Using the Bergman system, only 2 of the 15 missense RSVs were classified as probably pathogenic, 2 RSVs as uncertain significance, and the remaining variants were probably benign. The Bergman system yielded results similar to the ACMG criteria, yet appeared to be more stringent as it demonstrated 100% sensitivity and 100% specificity in predicting *CHD7* RSVs resulting in CHARGE syndrome (Table 3).

DISCUSSION

In this study, we identified 17 *CHD7* RSVs in 16% (18/116) of CHH probands. Overall, *CHD7* RSVs are significantly enriched in the CHH cohort versus controls (7.8% vs. 0.1%, p = 1.6E-11), supporting the implication of *CHD7* in CHH. Herein, we demonstrated that applying the updated ACMG guidelines[14] revealed an excellent correlation between the pathogenicity of *CHD7* RSVs and the observed clinical severity in CHH (i.e. additional CHARGE-like features). Indeed, 80% of *CHD7* RSVs classified as pathogenic or likely pathogenic cause multiple CHARGE features among CHH probands, resulting in reclassification of 3 patients to CHARGE syndrome according to the Verloes criteria. These clinical findings are in sharp contrast with patients harboring the 12 *CHD7* RSVs classified as non-pathogenic (i.e. uncertain significance, benign or likely benign), as only one patient exhibited ≥ 2 additional CHARGE signs. Notably, this patient also harbors an additional pathogenic variant in *FGFR1*, a known CHH gene with pleiotropic effects. Further, these data raise the question of the role of non-pathogenic variants in CHH. These variants are likely not the major cause of CHH, and indeed pathogenic or likely pathogenic variants in another CHH gene were found in 5/12 (42%) of these patients. However, we cannot exclude the possibility that these seemingly benign *CHD7* variants act as genetic modulators of the overall CHH phenotype as supported by the statistical enrichment of *CHD7* RSVs in CHH.

Our study is the first to systematically evaluate CHARGE features in a large cohort of CHH probands. Radiologic examination of the inner ear to investigate semi-circular canal hypoplasia (a major sign of CHARGE syndrome) is rarely performed in CHH patients, i.e. 0~38% of probands were tested in previous studies.[12,13,23] In this present study, we systematically phenotyped the CHH probands and performed inner ear MRI/CT in 77% (14/18) of them. Our data suggest that the ACMG criteria can identify a subset of CHH patients requiring a complete

clinical evaluation for CHARGE signs (those with pathogenic or likely pathogenic *CHD7* variants) versus those who have low risk of having additional CHARGE signs. We found that 75% (3/4) of probands with pathogenic or likely pathogenic variants in *CHD7* also had pathology of the inner ear compared to 0% (0/10) in probands with non-pathogenic *CHD7* RSVs. As detailed phenotyping demands an enormous clinical effort and medical resources, the capacity to identify patients with a high-risk for additional CHARGE features is extremely important.

Further, we classified the *CHD7* RSVs with commonly used criteria based on SIFT and/or PolyPhen2, as well as the Bergman system - a prediction algorithm developed for evaluating missense variants in CHARGE syndrome. In contrast to ACMG criteria and Bergman system, SIFT and/or PolyPhen2 classified > 70% of RSVs to be deleterious. It is well-known that both SIFT and PolyPhen2 have higher accuracy in predicting loss-of-function variants relative to benign variants, which leads to a higher false positive rate.[24] To date, only one study has attempted to assess the functional impact of CHD7 mutants in a model system. Balasubramanian and colleagues found that 75% (9/12) of CHH-associated and 100% (4/4) of CHARGE-associated CHD7 mutations are loss-of-function in a zebrafish model. However, it is not possible to differentiate the mutations resulting in CHH from those causing CHARGE syndrome. While the functional data from Balasubramanian et al. is compelling, it is important to note that segregation data were only presented for 3 families. The advantage of the ACMG criteria and the Bergman system used in the present study is the ability to integrate a broad spectrum of evidences, including segregation data.

Although similar results were obtained using ACMG criteria and the Bergman system, it is important to note that the Bergman system weights heavily for *de novo CHD7* variants - a feature more commonly found in CHARGE syndrome compared to CHH. This may explain the

minor differences in the results of the two classifications. In our cohort, the Bergman system had 100% sensitivity and specificity for identifying causative variants for CHARGE syndrome (defined by the Verloes criteria). Recently, Hale et al. proposed to broaden CHARGE syndrome clinical diagnostic criteria by including pathogenic *CHD7* variants as major criteria.[25] While these proposed criteria aim to improve the diagnosis in patients with atypical presentation by integrating molecular findings into the diagnosis process, the current study aims to analyze the genotype-phenotype correlation of *CHD7* variants in CHH. Thus, the inclusion of genotype information within the phenotype classification (i.e. diagnosis of CHARGE syndrome) may lead to a bias in the results. Furthermore, the Hale proposal requires that *CHD7* missense variants be '*de novo* and recurrent' in patients to be considered pathogenic. This is not consistent with the now widely-accepted guidelines from ACMG. The extra level of stringency recommended by Hale et al. can lead to the under-classification of truly pathogenic missense variants—an especially critical point for our study as most *CHD7* variants identified in CHH are missense, rather than the predominant nonsense and frameshift variants seen in CHARGE syndrome.[6,13,23]

Interestingly, all five CHH patients found to harbor pathogenic or likely pathogenic *CHD7* RSVs in this study were anosmic (i.e. Kallmann syndrome), consistent with three of five prior studies identifying *CHD7* mutations exclusively in KS patients.[12,13,23,26,27] Further, anosmia and hypogonadotropic hypogonadism were reported to be highly correlated in patients with CHARGE syndrome.[28] Indeed, murine studies showed a developmental role of *Chd7* in both GnRH neurons and the olfactory system: (i) the expression of *Chd7* in the embryonic olfactory placode at E10.5-E11 is temporally and spatially consistent with the genesis of GnRH neurons;[12] (ii) *Chd7*-haploinsufficiency is associated with decreased cellular proliferation in the olfactory placode, along with downregulation of expression of *Fgfr1* and *Bmp4*, two morphogens critical for GnRH early neuron development;[29] and (iii) *Chd7* heterozygous knockout (KO) mice

exhibit reduced olfaction, delayed pubertal onset and reduced GnRH neuron number, mimicking Kallmann syndrome.[29,30] Notably, a recent study showed that Chd7 regulates the embryonic expression and signaling of Fgf8 in mice. Heterozygous defects in *Chd7* and *Fgf8* exhibit a synergistic effect in cerebellar vermis development.[31] As FGF8 is a critical morphogen for both GnRH neuron fate specification[32,33] and olfactory bulb development,[34] further studies are warranted to elucidate the epistatic interaction between *FGF8* and *CHD7* in GnRH neuron biology in both human and animal models.

In the era of Sanger sequencing, CHH probands were often not screened for variants in *CHD7* due in part to its prohibitively large size. With the wide use of next-generation sequencing technologies in both diagnostic and research settings, *CHD7* RSVs are increasingly being found in CHH patients. Therefore, our study has implications for future clinical genetic practice when *CHD7* RSVs are identified in patients with CHH. It is important to study the genetic segregation and apply ACMG guidelines to classify the pathogenicity of the variant. If the variant is predicted to be pathogenic or likely pathogenic, there is a high risk for the patient to exhibit additional CHARGE features, or even undiagnosed CHARGE syndrome. Furthermore, appropriate genetic counseling based on the presence of pathogenic *CHD7* variants in CHH families is critical, as a high degree of variable expressivity is often observed in CHARGE syndrome families.[21,22] Thus even in CHH probands who have pathogenic *CHD7* variants and only minor CHARGE features, the same mutation in future generations may result in a more severe CHARGE phenotype. In conclusion, a comprehensive clinical screening of CHARGE-like features is indispensable in the patients with pathogenic *CHD7* variants in order to clarify the diagnosis (CHH versus CHARGE syndrome) and to provide an optimized clinical follow-up as well as a tailored genetic counseling.

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Figure legends

Figure 1. *CHD7* rare sequencing variants identified in CHH probands. (A) Schematic of the CHD7 protein (1-2997 amino acid) and locations of identified RSVs. Pathogenic or likely pathogenic RSVs according to guidelines from American College of Medical Genetics are noted in red. The functional domains of CHD7 in both (A) and (B) are noted as follows: blue, chromodomain 1; green, chromodomain 2; yellow, helicase N; pink, helicase C; purple, BRK. **(B)** Structural model of CHD7 chromo- and helicase domains. The location of RSVs is indicated by arrows. The ATP-binding domain is depicted in cyan (located in the helicase N-lobe).

Figure 2. Informative pedigrees of CHH probands. CHH, congenital hypogonadotropic hypogonadism; RSV, rare sequencing variant; square, male; circle, female; arrow, proband; +, wild type.

Supplementary material

Table S1. *CHD7* rare sequencing variants identified in CHH probands

Disclosure statement: The authors have nothing to disclose.

Figure 1

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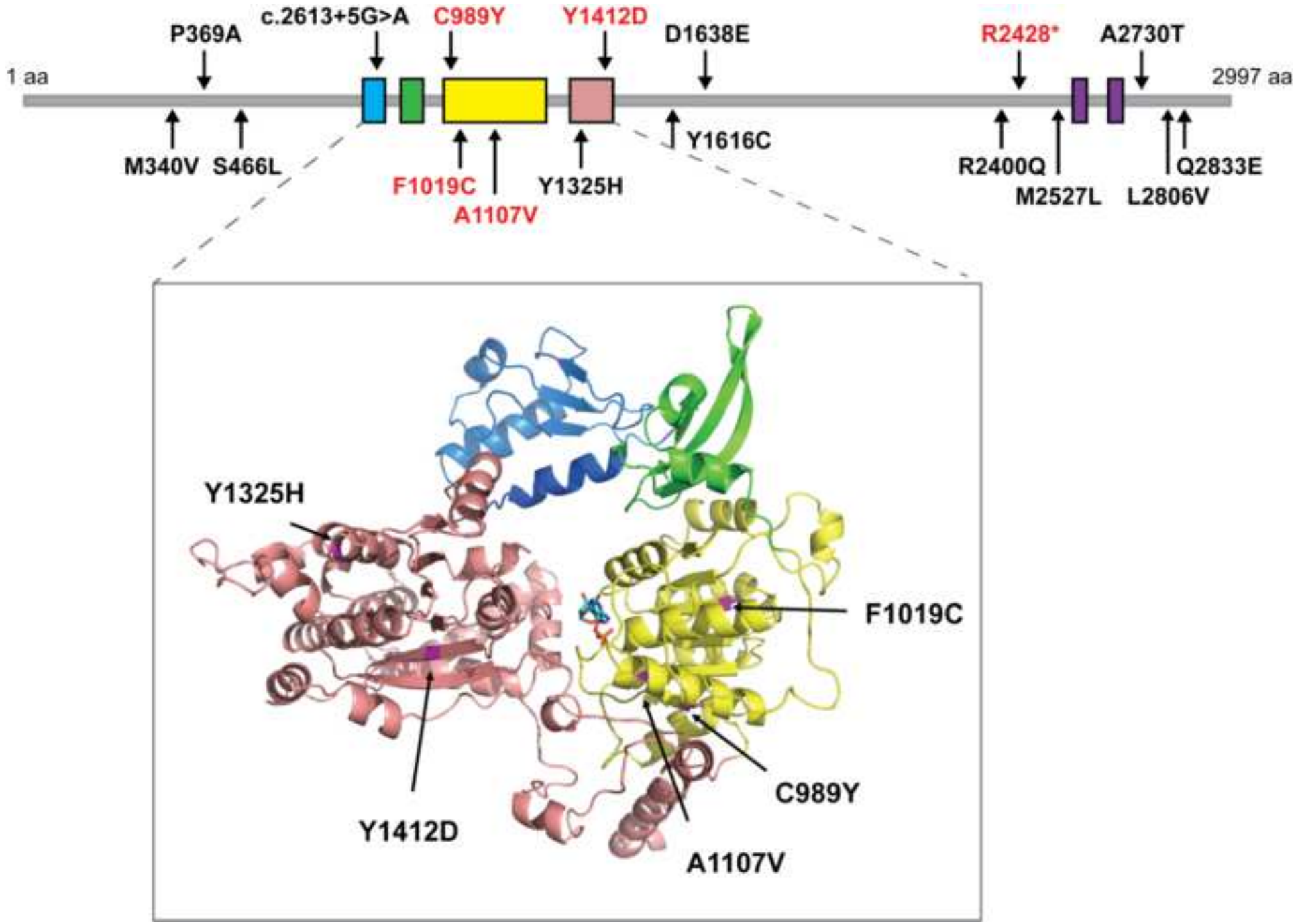

Figure 2

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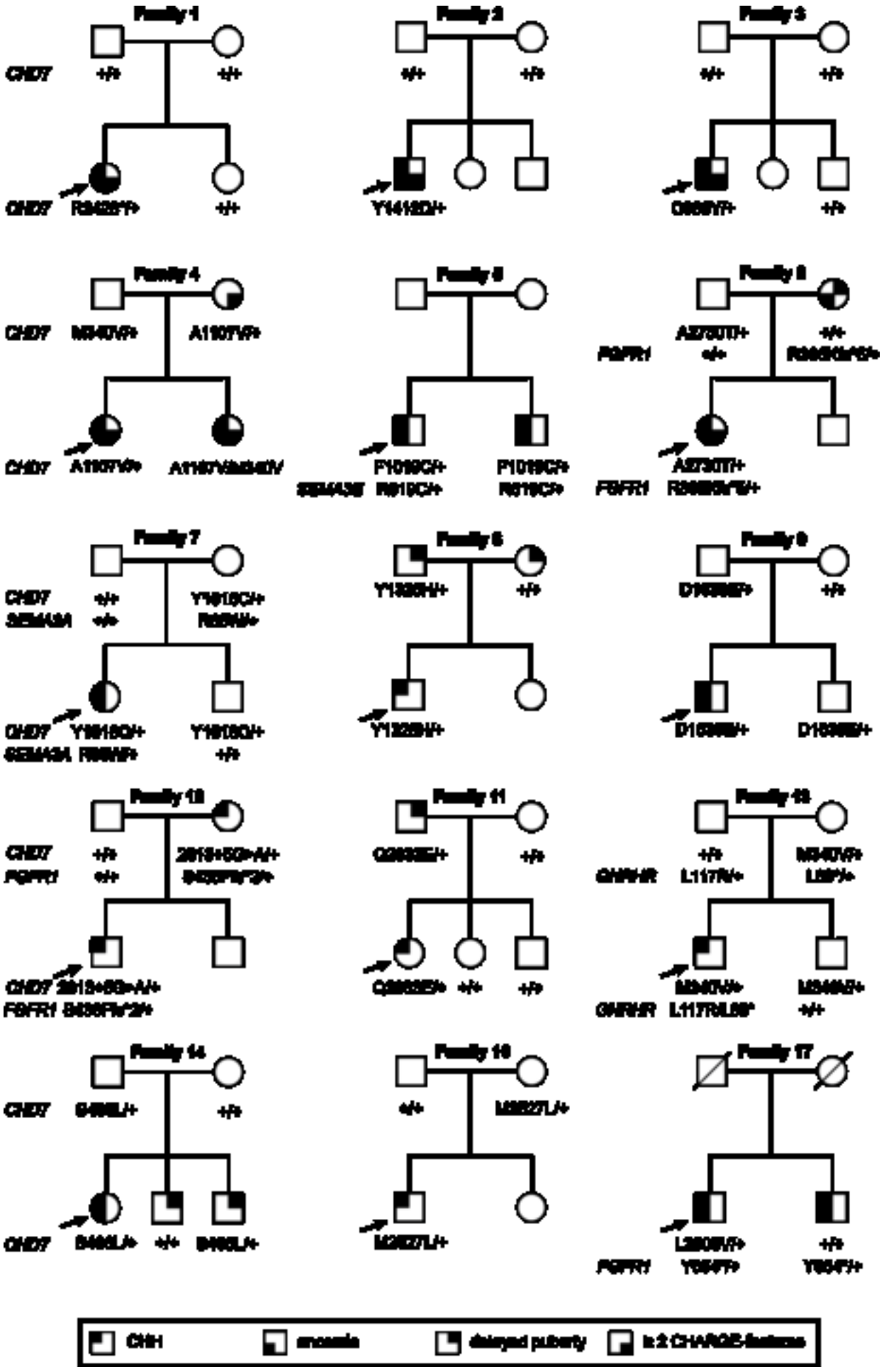

Table 1. Phenotype-genotype correlation in CHH probands with *CHD7* RSVs

Pt	Initial Dx	*CHD7* RSVs	Other pathogenic variants	CHARGE-features								Other phenotypes	Final Dx	ACMG Criteria
				Major			Minor							
				C	A	SCC	RED	E	DI	MO	HPD			
1	KS	p.R2428*	-	+	-	+	-	-	-	+	+	nasal cartilage distortion	CHARGE	P
2	KS	p.Y1412D	-	-	+	+	+	+	-	-	+	ovale palate	CHARGE	LP
3	KS	p.C989Y	-	+	-	-	+	+	-	-	+	atropy of optic chiasma	Atypical CHARGE	LP
4	KS	p.A1107V	-	-	-	+	-	-	-	+	+	see Table 2	KS	LP
5	KS	p.F1019C	*SEMA3E* p.R619C	-	-	U	-	-	-	-	+	dental defects	KS	LP
6	KS	p.A2730T	*FGFR1* p.R365Kfs*5	-	-	-	+	-	+	-	+	optic nerve atropy, CLP, CC agenesis	KS	U
7	KS	p.Y1616C	*SEMA3A* p.R66W	-	-	-	+	-	-	-	+	-	KS	U
8	nCHH	p.Y1325H	-	-	-	-	-	-	-	+	+	-	nCHH	U
9[M]	KS	p.D1638E	-	-	-	-	-	-	-	M	+	synkinesia, dental defect	KS	U
10	nCHH	c.2613+5G>A	*FGFR1* p.S436Ffs*3	-	-	-	-	-	-	-	+	skeletal defect	nCHH	U
11	nCHH	p.Q2833E	-	-	-	U	-	-	-	-	+	-	nCHH	U
12	nCHH	p.M340V	-	-	-	-	-	-	+	-	+	-	nCHH	B
13	nCHH	p.M340V	*GNRHR* p.[L89*];[L117R]	-	-	-	-	-	-	-	+	dental defect, astigmatism	nCHH	B
14	KS	p.S466L	-	-	-	U	-	-	-	-	+	-	KS	B
15	nCHH	p.R2400Q	-	-	-	-	-	-	-	-	+	-	nCHH	LB
16	nCHH	p.M2527L	-	-	-	-	-	-	-	-	+	dental defect, astigmatism	nCHH	LB
17	KS	p.L2806V	*FGFR1* p.Tyr654*	-	-	-	-	-	-	-	+	-	KS	B

CHH, congenital hypogonadotropic hypogonadism; RSV, rare sequencing variant; Pt: patient; Dx: diagnosis; KS: Kallmann syndrome; nCHH, normosmic CHH; CHARGE-features and CHARGE diagnosis are according to Verloes 2005 Criteria;(Verloes 2005) C, coloboma; A, choanal atresia; SCC: semi-circular canals dysplasia; RED, rhemboencephalic dysfunction; E, ear anomalies; ID, intellectual disability; MO, mediastinal organ malformation (heart and esophagus); HPD: hypothalamic-pituitary dysfunction; U, unknown; P: pathogenic, LP: likely pathogenic, U: uncertain significance, B: benign, LB: likely benign. M, Marfan syndrome was confirmed by the identification of *FBN1* p.R1692del and aorta root dilation in this proband; CLP, cleft lip and palate; CC, corpus callosum.

Table 2. Detailed phenotype in the family of Patient 4

	proband	sister	mother	father
***CHD7* RSVs**	**[A1107V]; [WT]**	**[A1107V];[M340V]**	**[A1107V];[WT]**	**[WT];[M340V]**
CHARGE major signs				
coloboma	-	-	-	-
choanal atresia	-	-	-	-
semi-circular canal	+	+	+	-
CHARGE minor signs				-
rhombencephalic dysfunction	-	+	+	-
ear anomalies	-	-	-	-
intellectual disability	-	-	-	-
hypothalamic-pituitary dysfunction	+	+	-	-
malformation mediastinal organs	+	+	-	-
CHH associated phenotypes				-
synkinesia	+	+	-	-
scoliosis	+	+	+	-
renal defect	-	+	-	-
Diagnosis summary	**KS with multiple CHARGE features**	**Atypical CHARGE syndrome**	**normal reproduction with CHARGE features**	**unaffected**

KS, Kallmann syndrome; RSV, rare sequencing variant; WT, wild type.

Table 3. Comparison of different classification systems for *CHD7* RSVs

Pt	*CHD7* RSVs	Other mutations	Final Dx	Multiple CHARGE features	Classification of *CHD7* RSVs		
					ACMG criteria	Bergman	SIFT / PPH2
1	p.R2428*	-	CHARGE	+	P	-	-
2	p.Y1412D	-	CHARGE	+	LP	P	P
3	p.C989Y	-	Atypical CHARGE	+	LP	P	P
4	p.A1107V	-	KS	+	LP	U	P
5	p.F1019C	*SEMA3E* p.R619C	KS	-	LP	U	P
6	p.A2730T	*FGFR1* p.R365Kfs*5	KS	+	U	B	P
7	p.Y1616C	*SEMA3A* p.R66W	KS	-	U	B	P
8	p.Y1325H		nCHH	-	U	U	P
9	p.D1638E	-	KS	-	U	B	P
10	c.2613+5G>A	*FGFR1* p.S436Ffs*3	CHH	-	U	-	-
11	p.Q2833E	-	nCHH	-	U	B	B
12	p.M340V	-	nCHH	-	B	B	B
13	p.M340V	*GNRHR* p.[L89*];[L117R]	nCHH	-	B	B	B
14	p.S466L	-	KS	-	B	B	P
15	p.R2400Q	-	nCHH	-	LB	B	P
16	p.M2527L	-	nCHH	-	LB	B	B
17	p.L2806V	*FGFR1* p.Y654*	KS	-	B	B	P

KS, Kallmann syndrome; nCHH, normosmic CHH; RSV, rare sequencing variants; ACMG criteria;(Richards, Aziz et al. 2015) Bergman system;(Bergman, Janssen et al. 2012) PPH, PolyPhen2. P, pathogenic; B, benign; U, uncertain significance; LP, likely pathogenic; LB, likely benign.

Table S1. *CHD7* rare sequencing variants identified in CHH probands

c.HGVS	p.HGVS	Patient No	MAF		Segregation	Domain	*in silico* prediction				CHD7 database	ACMG criteria
			matched	all			PPH2	SIFT	Align-GVGD	Structural		
Protein truncating RSVs												
c.7282C>T	p.R2428*	1	0	0	***de novo***	-	-	-	-	-	P	P
c.2613+5G>A	-	10	0	1E-04	affected parent	-	-	-	-	-	P	U
Missense RSVs												
c.1018A>G	p.M340V	12,13	0.007 [H]	0.005	parent carrier / NA	-	T	T	T	-	B	B
c.1105C>G	p.P369A	18*	6E-05	3E-05	parent carrier	-	D	T	T	-	B	U
c.1397C>T	p.S466L	14	0.002	0.001	parent carrier	-	T	D	T	-	B	B
c.2966G>A	p.C989Y	3	0	0	***de novo***	Helicase N	D	D	D	D	-	LP
c.3056T>G	p.F1019C	5	0	0	parent carrier	Helicase N	D	D	D	D	-	LP
c.3320C>T	p.A1107V	4	0	0	affected parent	Helicase N	D	D	D	M	-	LP
c.3973T>C	p.Y1325H	8	2E-04	6E-05	affected parent	Helicase C	D	D	D	D	-	U
c.4234T>G	p.Y1412D	2	0	0	***de novo***	Helicase C	D	D	D	D	-	LP
c.4847A>G	p.Y1616C	7	0	0	parent carrier	-	D	D	D	-	-	U
c.4914T>G	p.D1638E	9	3E-05	1E-05	parent carrier	-	D	D	M	-	-	U
c.7199G>A	p.R2400Q	15	0	4E-06	NA	-	D	T	M	-	-	LB
c.7579A>C	p.M2527L	16	0.004 [H]	0.002	parent carrier	-	T	T	T	-	B	LB
c.8188G>A	p.A2730T	6	2E-05	2E-05	parent carrier	-	D	T	T	-	-	U
c.8416C>G	p.L2806V	17	0.004	0.001	discordant between affected siblings	-	D	T	M	-	U	B
c.8497C>G	p.Q2833E	11	0	0	affected parent	-	T	T	T	-	-	U

MAF, minor allele frequency in gnomAD database; matched, ethnically-matched population in gnomAD; all: all populations in gnomAD; H, variants have been detected in homozygous status in gnomAD database. NA: not available; Domain is determined as previously reported;[1] PPH-2, PolyPhen2; D, damaging predicted by Polyphen-2, SIFT, align-GVGD, or structural modeling; M, minor defect by structural modeling or mildly deleterious by align-GVGD; T, benign by Polyphen-2, SIFT or align-GVGD; CHD7 database, www.chd7.org; ACMG criteria;[2] P, pathogenic; B, benign; U, uncertain significance; LP, likely pathogenic; LB, likely benign. *Clinical information unavailable on this patient.

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1. Janssen N, Bergman JEH, Swertz MA, et al. Mutation update on the CHD7 gene involved in CHARGE syndrome. *Human mutation.* 2012;33(8):1149-1160.
2. Richards S, Aziz N, Bale S, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genetics in medicine : official journal of the American College of Medical Genetics.* 2015;17(5):405-424.

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