**Abstract**

**Objective:** Normosmic congenital hypogonadotropic hypogonadism (nCHH) is a rare disorder characterised by lack of pubertal development and infertility, due to deficient production, secretion or action of gonadotropin-releasing hormone (GnRH) and, unlike Kallmann syndrome, is associated with a normal sense of smell. Mutations in the \textit{GNRHR} gene cause autosomal recessive nCHH. The aim of this study was to determine the prevalence of \textit{GNRHR} mutations in a group of 40 patients with nCHH.

**Design:** Cross-sectional study of 40 unrelated patients with nCHH.

**Methods:** Patients were screened for mutations in the \textit{GNRHR} gene by DNA sequencing.

**Results:** \textit{GNRHR} mutations were identified in five of 40 patients studied. Four patients had biallelic mutations (including a novel frameshift deletion p.Phe313Metfs*3, in two families) in agreement with autosomal recessive inheritance. One patient had a heterozygous \textit{GNRHR} mutation associated with a heterozygous \textit{PROKR2} mutation, thus suggesting a possible role of synergistic heterozygosity in the pathogenesis of the disorder.

**Conclusions:** This study further expands the spectrum of known genetic defects associated with nCHH. Although \textit{GNRHR} mutations are usually biallelic and inherited in an autosomal recessive manner, the presence of a monoallelic mutation in a patient should raise the possibility of a digenic/oligogenic cause of nCHH.

**Introduction**

Congenital hypogonadotropic hypogonadism (CHH) is characterised by partial or complete lack of pubertal development, secondary to deficient gonadotropin-releasing hormone (GnRH)-induced gonadotropin secretion (1). Diagnosis is based on the existence of low levels of sex hormones associated with low or inappropriate luteinising hormone (LH) and follicle-stimulating hormone (FSH) levels, with no anatomical lesion in the hypothalamic–pituitary tract, and no other pituitary hormone deficiencies. CHH may occur associated with anosmia, a condition referred as Kallmann syndrome, or may occur without associated olfactory abnormalities, referred to as normosmic CHH (nCHH) (1). Genetic studies of patients with CHH have identified monogenic
and oligogenic defects in several genes that regulate the embryonic development or migration of GnRH neurons, or the synthesis, secretion or action of GnRH (2).

The gonadotropin-releasing hormone receptor (GNRHR) gene was one of the first genes to be implicated in nCHH (3, 4). The GNRHR gene is located on chromosome 4q13.2-3 comprises three coding exons and encodes the GnRH receptor (5). The GnRH receptor is expressed mostly at the level of the gonadotrope cells of the pituitary gland, and its activation induces LH and FSH secretion (5). GNRHR mutations explain about 3.5–16% of sporadic cases and up to 40% of familial cases of nCHH (6). Inheritance is autosomal recessive and patients usually have homozygous or compound heterozygous mutations (6).

The aim of this study was to identify and determine the prevalence of GNRHR mutations in a cohort of patients with nCHH.

Subjects and methods
Subjects
The study comprised 40 unrelated Portuguese patients with nCHH (33 men and 7 women), recruited by Portuguese clinical endocrine centres. Inclusion criteria were male and female patients with low serum FSH, LH and sex steroid levels, and failure to enter spontaneous puberty by the age of 18 years or with medically induced puberty below this age and normal sense of smell. Olfactory function was assessed either by olfaction testing or by self-reporting by the patients, depending on the clinical centre. Patients with a history of an acquired cause of hypopituitarism or with abnormal radiological imaging of the hypothalamic–pituitary region were excluded from the study. In mutation-positive patients, additional family members were also studied. The control population consisted of 200 Portuguese unrelated volunteers who were recruited among blood donors. Written informed consent was obtained from all subjects, and the study was approved by the local research ethics committee (Faculty of Health Sciences, University of Beira Interior, Ref: CE-FCS-2012-012).

Genetic studies
Genomic deoxyribonucleic acid (DNA) was extracted from peripheral blood leucocytes using previously described methods (7). Patients were screened for mutations in GNRHR by polymerase chain reaction (PCR) amplification of the three coding exons and exon–intron boundaries and bi-directional sequencing using CEQ DTCS sequencing kit (Beckman Coulter, Fullerton, CA, USA) and an automated capillary DNA sequencer (GenomeLab TM GeXP, Genetic Analysis System, Beckman Coulter). Primer sequences were previously described by Antelli and coworkers (8). The heterozygous frameshift mutation was confirmed by cloning of the PCR products using pGEM-T Easy Vector Systems (Promega Corporation), followed by DNA sequencing of each allele. Genomic sequence variants identified in patients were searched in population variant databases (Exome Aggregation Consortium (ExAC) database) (9) to assess their frequency in the general population. Computational functional prediction analysis (10) was performed to evaluate the impact of the sequence variants on protein function. Unreported variants were excluded in a panel of 200 healthy Portuguese volunteers (400 alleles). Mutation nomenclature followed standard guidelines (11) and was based on the cDNA reference sequence for the GNRHR gene (GenBank accession NM_000406.2). In the patient with a monoallelic mutation in exon 1 of GNRHR, a ~4.5 kb PCR fragment encompassing exons 2 and 3 was analysed to exclude large deletions of either exon. In addition, this patient was screened for digenic/oligogenic mutations by sequencing additional genes related to the hypothalamic–pituitary–gonadal axis (KAL1, FGFR1, GNRH1, GFR8, PROK2, PROK2, KISS1R, CHD7, TAC3 and TACR3) (all primer sequences and PCR conditions are available upon request).

Results
Sequence analysis of the entire coding region of GNRHR, including exon–intron boundary regions, revealed mutations in five (12.5%) patients (Figs 1 and 2). These consisted of a compound heterozygous missense mutation (c.[401T>G];[415C>T]) (p.[Val134Gly];[Arg139Cys]), a compound heterozygous missense/frameshift mutation (c.[317A>G];[847T>C]) (p.[Tyr283His];[Tyr283His]), a compound heterozygous missense/frameshift mutation (c.[785G>A];[937_947del]) (p.[Arg262Gln];[Phe313Metfs*3]), a compound heterozygous missense/frameshift mutation (c.[785G>A];[937_947del]) (p.[Arg262Gln];[Phe313Metfs*3]), a homozygous missense mutation (c.[847T>C];[847T>C]) (p.[Tyr283His];[Tyr283His]) and a heterozygous missense mutation (c.[401T>G];[6A]) (p.Val134Gly). Three of these patients were sporadic cases and two had additional affected siblings that were also tested and shown to share the same mutations.
The clinical characteristics of patients with identified GNRHR mutations are summarised in Table 1.

In the patient with the monoallelic mutation in exon 1 (p.Val134Gly), additional studies were carried out to search for further genetic defects. A PCR amplicon containing GNRHR exons 2 and 3 was partially sequenced and revealed heterozygosity for an intron 2 polymorphism (rs373270328), thereby indicating the presence of two copies of each exon and excluding the possibility of exon deletion as the second mutation in this patient. The screening of other genes related to the hypothalamic–pituitary–gonadal axis, in this patient, revealed an additional heterozygous missense mutation (c.[238C>T];[=]) (p.Arg80Cys) in the PROKR2 gene.

The GNRHR frameshift mutation was identified in two different families and has not been reported before. It consists of an 11 base-pair deletion (c.937_947delTTTTTAAACC), and if translated, would be expected to result in a truncated protein due to a premature termination codon (p.Phe313Metfs*3). This deletion was not found in any of the population variant databases and was excluded in a panel of 200 normal Portuguese controls (400 alleles), on the basis of the different size of the PCR fragments. The remaining missense variants have all been identified as mutations in previous studies and were predicted to have a damaging effect on the encoded protein, by SIFT and PolyPhen-2 analyses (10). Furthermore, these missense mutations were either unreported in the ExAC

Figure 1
Mutations identified in patients with nCHH. (A) Pedigrees of affected individuals. Arrows represent index cases, filled symbols represent affected individuals, open symbols represent unaffected individuals, squares denote men, circles denote women, oblique lines through symbols represent deceased individuals and double line represents consanguineous marriage. Genotypes for tested family members, when available, are presented below each individual. WT, wild-type allele. (B) DNA sequence analysis of normal individuals (above) and patients (below). The positions of the mutations are indicated by asterisks. In the case of the deletion (p.Phe313Metfs*3) (family 2 and 3), only the cloned mutated allele is represented (below) with the deleted sequence (above).
Functional studies have demonstrated that conditions (GNRHR/PROKR2) heterozygous mutation supports this possibility and indicates the need to consider a digenic/oligogenic cause of CHH in patients with GNRHR monoallelic mutations. The GNRHR missense mutations identified in this study have been previously reported in other nCHH patients, namely p.Gln106Arg (3), p.Val134Gly (12), p.Arg139Cys (13), p.Arg262Gln (3) and p.Tyr283His (14). In vitro functional studies have demonstrated that these mutated receptors result in either reduced ligand affinity, reduced signal transduction or abolished plasma membrane expression (3, 12, 13). The 11 base-pair deletion (c.937_947delTTTTTAAACCC) has never been reported before and was identified in two different families from the same geographical region, suggesting a possible inheritance from a common ancestor. This deletion is likely to be highly deleterious due to a frameshift effect and to the formation of a premature stop codon that may lead to the production of a truncated protein (p.Phe313Metfs*3) or to nonsense-mediated messenger ribonucleic acid (mRNA) decay (15), although the latter is less likely due to the location of the mutation in the last exon.

Four patients had biallelic mutations (either homozygous or compound heterozygous), which is in agreement with the typical autosomal recessive inheritance of GNRHR. However, one patient had a GNRHR mutation on one of the alleles (p.Val134Gly), but no mutation on the other allele. Other monoallelic mutations of GNRHR have been reported in patients with CHH and have challenged the traditional view of GNRHR as a recessive gene (16). It has been suggested that such patients with monoallelic mutations may have additional mutations in other genes that act synergistically to produce the phenotype (16). To explore this possibility, we screened this patient for mutations in other commonly implicated genes in CHH and identified an additional heterozygous missense mutation (p.Arg80Cys) in the PROKR2 gene. Interestingly, the same heterozygous PROKR2 mutation has been identified in a Brazilian patient with Kallmann syndrome (17) and has been shown to exert a dominant-negative effect under certain in vitro conditions (18). However, this mutation was also found in two asymptomatic first-degree relatives, leading the authors to conclude that this mutation, by itself, was insufficient to cause the disorder, but that it might contribute to the disease phenotype in association with other factors, such as unidentified mutations in additional genes or epigenetic and environmental effects (17). Indeed, our finding of a digenic (GNRHR/PROKR2) heterozygous mutation supports this possibility and indicates the need to consider a digenic/oligogenic cause of CHH in patients with GNRHR monoallelic mutations.

Digenic and oligogenic inheritance has been identified in several cases of CHH. A study by Sykiotis et al. (19) found that 2.5% of patients with CHH harboured mutations in two or more genes. However, so far, there have been only four reported CHH patients with digenic mutations involving a single heterozygous GNRHR mutation and a mutation in another gene, namely in FGFR1 (16, 19), WDR11 (20) and PROKR2 (p.Val331Met) (16).

Discussion

The overall prevalence of GNRHR mutations in this cohort was 12.5% (five out of 40 patients with nCHH), which is consistent with results presented in other studies (6). Four patients had biallelic mutations (including two patients with a novel frameshift deletion) and one patient had a digenic (GNRHR/PROKR2) heterozygous mutation.

The GNRHR missense mutations identified in this study have been previously reported in other nCHH patients, namely p.Gln106Arg (3), p.Val134Gly (12), p.Arg139Cys (13), p.Arg262Gln (3) and p.Tyr283His (14). In vitro functional studies have demonstrated that these mutated receptors result in either reduced ligand affinity, reduced signal transduction or abolished plasma membrane expression (3, 12, 13). The 11 base-pair deletion (c.937_947delTTTTTAAACCC) has never been reported before and was identified in two different families from the same geographical region, suggesting a possible inheritance from a common ancestor. This deletion is likely to be highly deleterious due to a frameshift effect and to the formation of a premature stop codon that may lead to the production of a truncated protein (p.Phe313Metfs*3) or to nonsense-mediated messenger ribonucleic acid (mRNA) decay (15), although the latter is less likely due to the location of the mutation in the last exon.

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**Table 1** Clinical characteristics of patients with GNRHR mutations.

| Family | Member (a) | Sex | Age of diagnosis (years) | Clinical presentation | Olfaction (b) | Associated features | Basal hormone levels (laboratory normal reference range) | Mutations |
|--------|------------|-----|--------------------------|-----------------------|---------------|---------------------|--------------------------------------------------------|-----------|
| 1      | II-3       | M   | 18.5                     | Delayed puberty. Micropenis. Tanner stage 3. Testicular volume 3 mL | Normal        | Surgery for right-sided cryptorchidism at age 15 years | FSH 1.2 IU/mL (1.0–8.0); LH 0.5 IU/mL (0.7–7.2); total testosterone 0.20 ng/mL (2.60–10.00) | GNRHR p.[Val134Gly];[Arg139Cys] (compound heterozygous) |
| II-1   | M          | 22  | Normal                   |                       |               |                     | FSH 2.4 IU/mL (2.5–10.2); LH <0.1 IU/mL (1.9–2.5); total testosterone 0.33 ng/mL (2.60–10.00) | GNRHR p.[Gln106Arg];[Phe313Metfs*3] (compound heterozygous) |
| II-2   | F          | 18  | Primary amenorrhea       | Normal                |               |                     | FSH 2.2 IU/mL (2.5–10.2); LH 0.9 IU/mL (1.9–2.5); estradiol 15 pg/mL (11–69) | GNRHR p.[Gln106Arg];[Phe313Metfs*3] (compound heterozygous) |
| II-3   | M          | 51  | Delayed puberty. Tanner stage 1. Testicular volume 5 mL | Normal                |               | Low bone mineral density (lumbar spine T-score –2.3; femoral neck T-score –2.8) | FSH 1.1 IU/mL (1.0–8.0); LH 0.4 IU/mL (0.7–7.2); total testosterone 0.40 ng/mL (2.70–11.00) | GNRHR p.[Arg262Gln];[Phe313Metfs*3] (compound heterozygous) |
| II-4   | F          | n/a | Primary amenorrhea       | Normal                |               |                     | FSH 0.4 IU/mL (1.5–33.4); LH <0.07 IU/mL (1.7–15.0); estradiol <11.8 pg/mL (19.5–356.7) | GNRHR p.[Tyr283His];[Tyr283His] (homozygous) |
| II-5   | F          | 39  | Primary amenorrhea       | Normal                |               | Low bone mineral density (lumbar spine T-score –3.2; femoral neck T-score –1.0); Hypercholesterolemia. Secondary sex characteristics had developed due to oral contraceptive use in adolescence | FSH 0.7 IU/mL (4.0–13.0); LH 0.1 IU/mL (1.7–15.0); estradiol <10 pg/mL (20–150) | GNRHR p.[Tyr283His];[Tyr283His] (homozygous) |
| 4      | II-2       | M   | n/a                      | n/a                   | n/a           |                     | FSH 0.5 IU/mL (1.4–18.0); LH 0.7 IU/mL (1.5–9.3); total testosterone 0.09 ng/mL (2.20–8.00) | GNRHR p.Val134Gly (heterozygous); PROKR2 p.Arg80Cys (heterozygous) |

Hormone levels were measured by chemiluminescence immunoassays; bone mineral densities were measured by dual-energy X-ray absorptiometry. (a) As indicated in Figs 1 and 2; (b) self-reported. F, female; FSH, follicle-stimulating hormone; LH, luteinising hormone; M, male; n/a, not available.
Therefore, our patient represents a rare example of digenism involving GNRHR and supports the hypothesis that heterozygous mutations, in genes that normally cause autosomal recessive disease, may affect the same regulatory pathways and result in disease by synergistic heterozygosity. A similar mechanism of synergistic heterozygosity has been proposed for other disorders such as inborn errors of metabolism (21). The involvement of digenism as a cause of CHH suggests that there are multiple layers of redundancy in the hypothalamic–pituitary–gonadal (HPG) control of reproduction and that, at least in the case of less severe mutations, multiple hits in the HPG axis may be required to result in CHH (22).

Our findings of a monoallelic mutation in GNRHR should be viewed with caution as we did not exclude the possibility of a second mutation in non-coding sequences, such as deep intronic or regulatory regions. However, such mutations have not yet been reported in any patient. Although we searched for oligogenicity by sequencing the most commonly affected CHH genes, other rarer loci were not analysed, and it remains to be determined if a more comprehensive genetic analysis (e.g. through whole-exome sequencing) would uncover additional contributing variants. Finally, family members of affected individuals were not always available for analysis and would have been useful for the interpretation of the genetic results.

In conclusion, our study identified a previously unreported mutation of the GNRHR gene, thereby expanding the spectrum of mutations associated with nCHH. In addition, we identified a case of a digenic (GNRHR/PROKR2) heterozygous mutation, suggesting that synergistic heterozygosity within the same regulatory pathway may also play a role in the pathogenesis of the disorder.

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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Author contribution statement
C I G and M C L conceived and designed the study. C I G performed the genetic studies of the patients. J M A, M B, L B, N V and D C collected samples and acquired clinical data of the patients with mutations. C I G and M C L drafted the article and all authors revised it critically for important intellectual content and approved the final manuscript.

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