Doctorate Program in Molecular Oncology and Endocrinology
Doctorate School in Molecular Medicine

XXVII cycle - 2011–2014
Coordinator: Prof. Massimo Santoro

"Congenital Hypogonadotropic Hypogonadism: novel clinical, genetic and molecular insights"

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“Congenital Hypogonadotropic Hypogonadism: novel clinical, genetic and molecular insights”

I would like to consecrate this work to my family, particularly exhausted by these hard times.

Time often changes and upsets the ancient equilibrium,
and thus creates a vacuum demanding to fill in,
or an overall feeling of fear against things we risk to lose again.
The new challenges to face, instead, are nothing other than signs of the life that's going on.

TABLE OF CONTENTS

LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

1. Maione L, Albarel F, Bouchard P, Gallant M, Flanagan CA, Bobe R, Cohen-Tannoudji J, Pivonello R, Colao A, Brue T, Millar RP, Lombes M, Young J, Guiochon-Mantel A, Bouligand J. R31C GNRH1 Mutation and Congenital Hypogonadotropic Hypogonadism. PLOS One, July 2013, 8 (7), e69616. doi:10.1371/journal.pone.0069616.g004.

2. Maione L., Cantone E., Nettore IC., Cerbone G., De Brasi D., Maione N., Young J., Di Somma C., Sinisi AA., Iengo M., Macchia PE., Pivonello R., Colao A. Flavor impairment: a neglected sensorineural disability in patients with Kallmann Syndrome. *Submitted*.

LIST OF ABBREVIATIONS

B1-5	Breast development, according to Tanner staging
CDS	Coding DNA Sequence
CHH	Congenital Hypogonadotropic Hypogonadism
ERK	Extracellular signal-regulated kinases
FSH	Folliculo-Stimulating Hormone
GFP	Green Fluorescent Protein
GKR	Glycine-Lysine-Arginine peptidic sequence
GnRH	gonadorelin, or gonadoliberin,
GNRH1	gene encoding the GnRH-I peptide
GNRHR	gene encoding the GnRH-I receptor
GPCR	G-protein coupled receptor
HRT	Hormone Replacement Therapy
IP	Inositol Phosphate
KNDy	Kisspeptin, Neurokinin and Dynorphin-secreting neuron
KS	Kallmann Syndrome
LH	Luteinizing Hormone
MAPK	Mitogen Activated Protein Kinase
MTT	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
nIHH	normosmic Isolated Hypogonadotropic Hypogonadism
P1-5	Pubertal development, according to Tanner staging
PCR	Polymerase Chain Reaction
PMNC	Peripheral Blood Mononuclear Cells
SRE	Serum Responsive Element
TMD	Trans-membrane Domain
WB	Western Blot
WT	Wild-type

ABSTRACT

GnRH decapeptide and its receptor are two key-player of the neuroendocrine control of reproduction. Mutations in genes encoding these proteins (*GNRH1* and *GNRHR*) are associated with Congenital Hypogonadotropic Hypogonadism (CHH).

The aim of our work was to screen 450 individuals with CHH for mutations in *GNRH1* and *GNRHR* genes and to determine their deleterious character. Two novel p.W26X and p.R31C mutations in *GNRH1* and two novel p.T269M and p.Y290F mutations in *GNRHR* have been identified in eight CHH individuals from five different families. Molecular and functional characterizations have been performed.

The mutation p.W26X totally disrupts GnRH structure. The occurrence of this variant in two sisters affected by Kallmann Syndrome is not explained by genetics, and offers novel insights to define the pathogenesis in these patients.

The mutation p.R31C introduces a cysteine in position 8 within GnRH. This substitution dramatically alters protein structure and GnRH-dependent signaling, as assessed by impairment of binding, intracellular pathways as MAPK signaling or calcium mobilization, gene expression and biological activity. Moreover, the mutant pre-pro-GnRH is less expressed in various cell lines compared to wild type. Despite the deleterious effects on protein function of p.R31C product, the mode of inheritance suggests a negative dominance with intriguing yet elusive mechanisms.

The mutation p.T269M in *GNRHR* completely abolishes the binding, and produces a complete impairment of GnRH-dependent signals, as evaluated by calcium and MAPK. The homozygous status fully explains disease in two sisters with CHH.

The p.Y290F mutated GnRH receptor is less able to activate the main intracellular GnRH-dependent cascades. The girl with CHH harbors a homozygous mutation from consanguineous parents, consistent with disease.

In conclusion, novel *GNRH1* and *GNRHR* mutations with deleterious properties are reported with their molecular characterization. In two cases, the disease cannot be fully explained by genetic abnormalities, thus opening interesting opportunities for further investigations in this field.

Finally, a section on sensorineural characterization of CHH patients is provided. To this aim, a novel and innovative diagnostic test (Flavor Identification Test) has been developed as part of my Ph.D. thesis. This test has received a National Patent.

INTRODUCTION

Gonadotrope axis physiology

Reproductive function and sexual steroids production are coordinated by the gonadotrope axis, composed by the hypothalamus, the pituitary and the gonads (Fig. 1). The GnRH neurons, substantially settled in little number in the preoptic area and in the arcuate nucleus within hypothalamus, secrete the decapeptide in a pulsatile manner in response to a complex series of endogenous and exogenous *stimuli*. The frequence and the amplitude of GnRH pulses determine the response of gonadotrope cells within the pituitary, which express the GnRH receptor, a G-protein-coupled receptor (GPCR) encoded by the *GNRHR* gene.

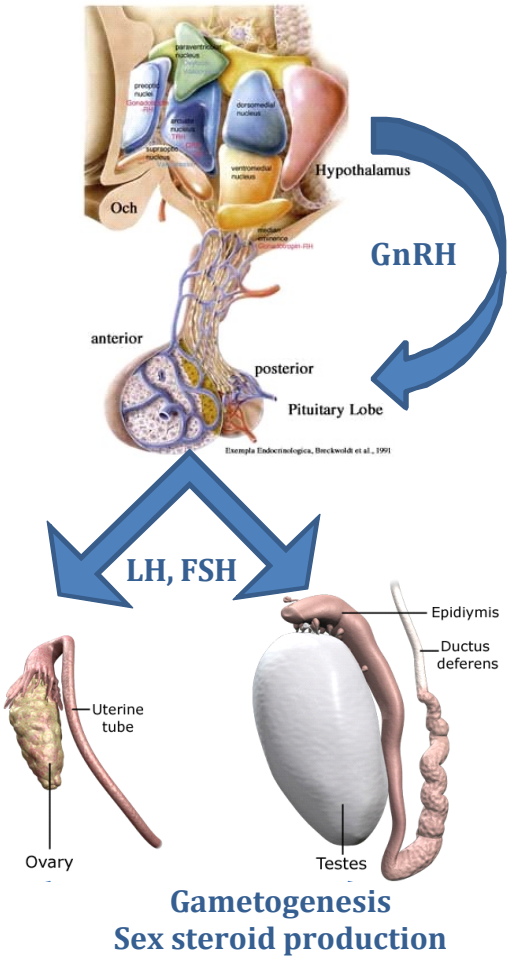


Figure 1- Schematics of gonadotrope axis. GnRH is secreted in the median eminence by axons projecting from preoptic area and arcuate nucleus within hypothalamus. Gonadotrope pituitary cells respond by secreting the gonadotropins LH and FSH that ultimately drive gonadal response in terms of gametogenesis and sex steroids production (adapted from Beckwoldt et al. 1991).

A peculiar and delicate vascular system passing through hypothalamic arteries constitutes the hypothalamo-pituitary portal system, which allows gonadotrope cells to respond to very low ligand concentrations. GnRH-GnRH-R couple triggers an intracellular cascade that ultimately leads to biosynthesis and secretion of the gonadotropins LH and FSH (Fig. 2). These glycoproteins are secreted in the peripheral circulation and stimulate gametogenesis and the hormonal production in target tissues.

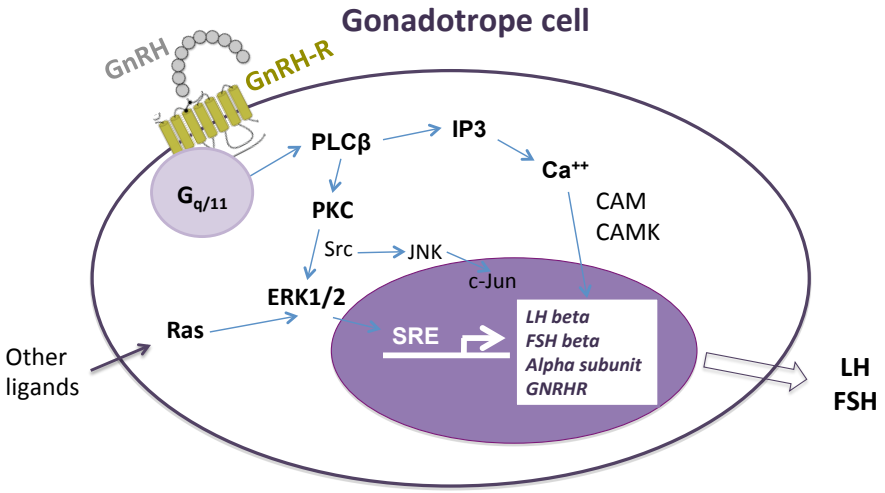
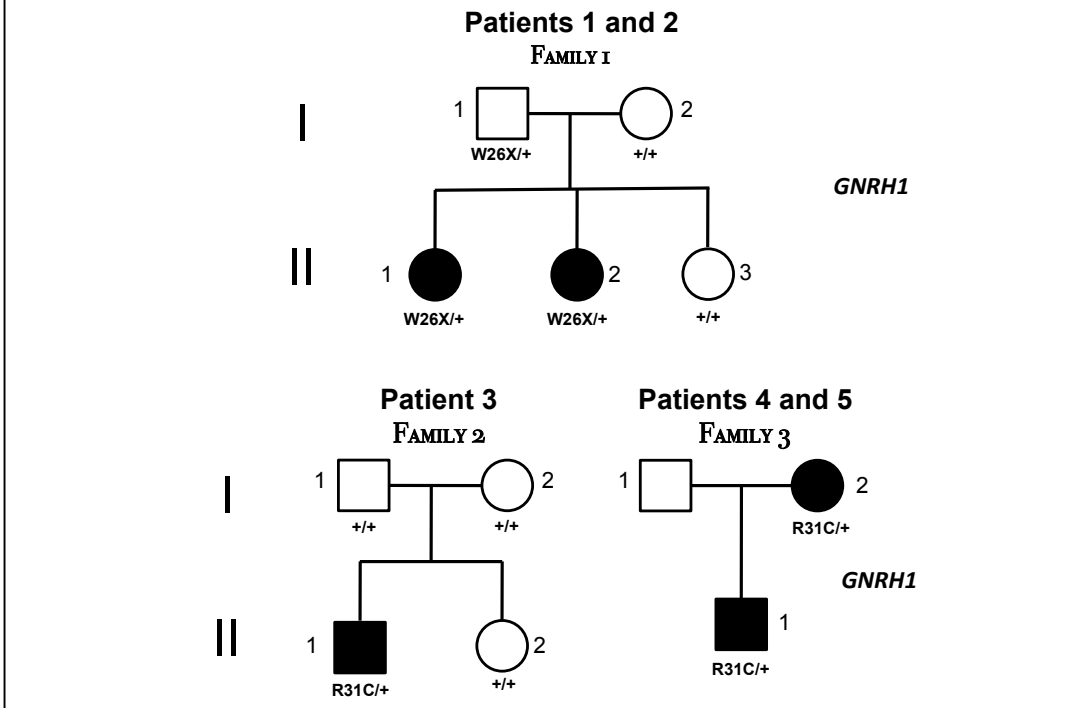


Figure 2 – Schematic of GnRH-dependent signaling.
The gonadotrope cell is exposed to pulsatile stimulation by the GnRH-I. The activation of GnRH receptor signals through different intrcellular effectors, among which mainly by Gq/11 that consequently activates phospholipase C and inositol phosphate / diacyl-glycerole / intracellular calcium mobilization. The activation of the MAPK ERK1/2 seems to pass through Src brokering, or through the activation of Ras by other pathways / co-ligands. These pathways concomitantly lead to increase gonadotropin subunit transcription, and to ultimately induce LH and FSH glycoprotein maturation and secretion.
PLC: phospholipase C; IP3 inositol tri-phosphate; CAM/CAMK: calcium-calmodulin / kinase; PKC: protein kinase C. For other acronyms, please refer to the section "list of abbreviations" within the manuscript.

The GnRH – GnRH-R couple

The GnRH (progonadoliberin-1, UniProtKB P01148) is a peptidic neuro-hormone constituted by 10 amino acids. It is the result of a proteolytic maturation from a 92 amino acids precursor by the pro-hormone convertase-1 that recognizes the GKR amino acidic sequence for cleavage activity (Cheng & Leung, 2005). The decapeptide, encoded by the *GNRH1* gene (ENSG00000147437.8, OMIM 152760) located at 8p11.2, is totally conserved in mammals (Fig. 3).



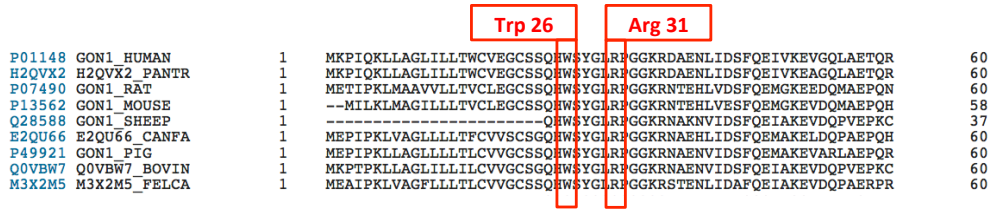


Figure 3 – pedigrees of CHH families with *GNRH1* mutations and interspecies alignment. p.W26X and p.R31C GNRH1 mutations are carried in heterozygosis by all the individuals belonging to family 1 and 2. Sisters from family 1 are affected by Kallmann Syndrome. Filiation in family 2 is confirmed by DNA micro-satellites. All the nucleotide base changes affect highly conserved residues. The interspecies alignment was powered by the UniProt consortium (www.uniprot.org). Clinical and demographic data of all patients are reported in Table 1.

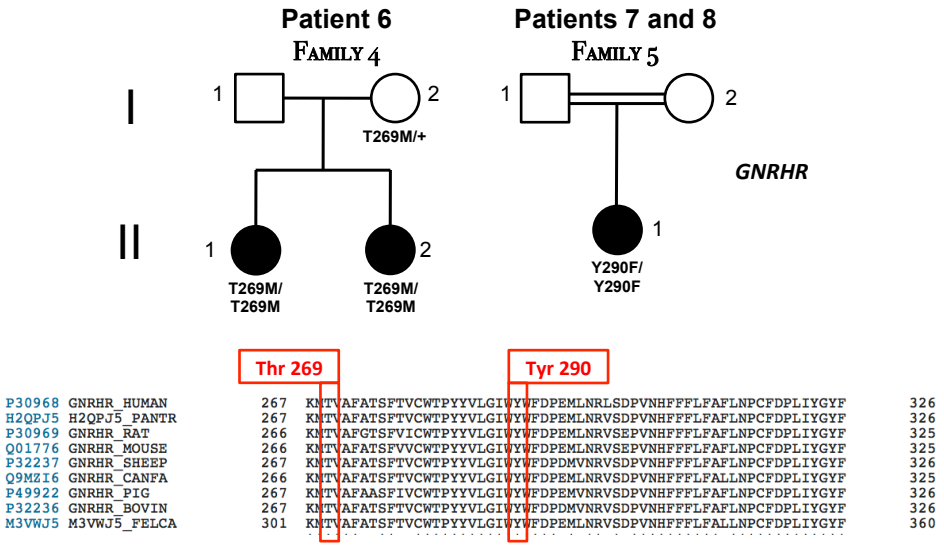


Figure 4 – Pedigrees of CHH families with *GNRHR* mutations and interspecies alignment. All affected patients from family 4 and 5 harbor homozygous *GNRHR* mutations. Parental genetical analysis was only available for the mother of the family 4 (subject I,2). The nucleotide base changes affect highly conserved residues. The interspecies alignment was powered by the UniProt consortium (www.uniprot.org). Clinical and demographic data of all patients are reported in Table 1.

Upstream to the coding sequence, two regions have classically been identified as regulatory regions: a proximal promoter, acting between -548 and -169 base pairs before the transcription start site, and a distal promoter between -1048 and -723 that is believed to act outside the central nervous system (Eraly et al., 1998; Nelson et al., 1998).

The GnRH receptor (GnRH-R, UniProtKB P309683) is encoded by *GNRHR* (ENSG000001091637, OMIM 1388505) located at 4q13.2. The product is a characteristic tail-less GPCR, coupled to a $G_{q/11}$ protein. The absence of a carboxyl-terminal tail in mammalian GnRH receptors is correlated with a lack of rapid desensitization and seems to be crucial for the physiologic response to pulsatile exposition to GnRH-I (Davidson et al., 1994; McArdle et al., 2002a; b). GnRH-R is believed to be expressed at a very low rate on

plasma membranes of gonadotrope pituitary cells (Finch et al., 2009), and is probably gathered mainly in membrane rafts, where it is coupled to different downstream effectors.

The main components of GnRH signaling cascade are represented by $G_{q/11}$ protein, C-beta phospholipase, diacyl-glicerol and tri-phosphate inositol, intracellular calcium mobilization and MAP kinase cascade (Fig. 2). All of these mediators, together with a series of calcium-dependent kinases and transcription factors, are needed to activate LH-beta, FSH-beta and alpha-subunit promoters and to inhibit *GNRHR* transcription.

The frequence and the amplitude of GnRH pulses seem to influence and orientate the transcription of specific gene products. Indeed, low amplitude / low frequence pulses mainly stimulate FSH-beta transcription. Conversely, high-frequence / high-amplitude pulses mainly drive LH-beta transcription (George & Seminara, 2012). The coordinated and dynamic sequence of GnRH stimulation is at the basis of the ovulatory LH surge in women and female mammals and on the other hand is needed to determine the early FSH increase in follicular stage (Gianetti & Seminara, 2008).

Congenital Hypogonadotropic Hypogonadism (CHH)

The CHH represents a well-recognized cause of pubertal failure in men and women (Young, 2012). In the vast majority of patients, the main cause derives from a concomitant deficiency in LH and FSH secretion. This may be reflected by a hypothalamic defect or by a pituitary resistance. Both causes ultimately drive LH and FSH insufficiency, impeding a normal gonadal endocrine and exocrine function. CHH patients, indeed, constantly have sex steroids or gonadal peptides deficiency (Giton et al., 2015; Trabado et al., 2014; Trabado et al., 2011). This impairment is already overt during the early physiologic stages of embryonic gonadotrope activation in mankind (Bouvattier et al., 2012). The prevalence of CHH is around 1:4,000 – 1:10,000 in men, and is believed to be 2-5 fold less frequent in women.

It is about 20 years that the CHH has been extensively studied and classed according to patho-physiological and genetic bases. A global distinction in CHH has to be made between the forms with an ontogenetic abnormality, and those with a deficiency in gonadotrope signaling.

The former CHH is a developmental disease concerning the region comprising and beyond the olfactory placode, from which olfactory nerves as well as GnRH neurons originate and differentiate. The paradigm of this anomaly is Kallmann Syndrome (KS) that is clinically mirrored by the presence of CHH and olfactory impairment (anosmia or hyposmia).

The latter form is resumed solely by the gonadotropin and sex steroids deficiency, without any sign of sensorineural involvement, and is here and thereafter reported as normosmic isolated hypogonadotropic hypogonadism (nIHH). The nIHH seems to be related to an isolated abnormality of neuroendocrine signaling concerning a hypothalamic (GnRH) or a pituitary (GnRH-R or gonadotropins) defect, without overt anatomic or ontogenetic defects. Latest advances demonstrated a delicate neuronal circuitry controlling the correct GnRH homeostasis, passing through kisspeptinergic (Kiss neurons) or neurokininergic (KNDy neurons) systems. These systems represent the pubertal clock in humans and are variably influenced by several endocrine signals, neurotrasmitters and physical cues, integrated in a complex anatomical neuroendocrine system (Pinilla et al., 2012).

Genetics of CHH

Several genes have been associated with CHH. A recently updated list of genes involved in CHH forms and in syndromic associations is reported in Table 1. The list of candidate genes is still expanding, as genetics now explains about 50% of cases. The enrichment of genetic abnormalities in CHH might suggest that more genes will be identified in the future. The products of genes involved in KS are mainly constituted by proteins involved in axon growth and output, in neuron routing and migration, and in mechanisms of cellular attraction or repulsion. These proteins may often interact by constituting a complex framework. Anosmin, the product of *KAL1* gene, is a complex secreted protein able to facilitate neuron migration. Disruption of *KAL1*, located on X-chromosome, as documented in large deletions or point mutations, lead to the severe X-linked form of KS (Franco et al., 1991; Legouis et al., 1991).

FGFR1 is a tyrosin-kinase receptor largely expressed in developing brain that might interact with anosmin. Loss-of-function mutations in *FGFR1* are associated with nIHH, KS and specifically those with midline defects, as labio-palato-schisis and dental agenesis (Dode et al., 2003). *FGF8* and *FGF17* specifically bind *FGFR1* and seem to be responsible of *FGFR1*-like CHH forms (Falardeau et al., 2008). Interactome and networking analyses have allowed the identification of other actors interplaying the FGFs pathways, and have recently been found mutated in CHH patients (Miraoui et al., 2013).

Prokineticin-2 is a secretory protein involved in several physiologic processes, among which neurogenesis. Alterations of this protein, as well as of its cognate receptor prokineticin receptor-2 are associated with KS, nIHH and with multiple pituitary hormone deficiency. These are the only genetic abnormalities in CHH arisen from knocked-out animal models, and not initially from patients (Dode et al., 2006; Matsumoto et al., 2006).

Semaphorin 3A is one of the latest molecules implicated in KS. A large deletion involving *SEMA3A* was associated to a dominant form of familial KS (Young et al., 2012). Other proteins belonging to semaphorin family actually represent good candidates for mutational screening in CHH.

All these reports strongly support the hypothesis that disruption of the complex molecular cues involved in neuronal

migration are fundamental for the understanding of KS pathogenesis.

Table 1 – Genetics of CHH.

	nIHH	KS	Syndromic forms	Associated features
KAL1		+		Renal agenesis, bimanual synkinesis
FGFR1	+	+	+	Midline defects
FGF8	+	+		
FGF17	+	+		
PROK2	+	+		
PROKR2	+	+		
CHD7		+	+	CHARGe sequence[1]
WDR11	+	+		
FEZF1		+		
SEMA3A		+		Renal agenesis
GNRH1	+			
GNRHR	+			
KISS1	+			
KISS1R	+			
TAC3	+			
TACR3	+			
SOX10		+	+	Sensorineural deafness
RNF216			+	Gordon-Holmes sequence[2]
PNPLA6			+	Boucher-Neuhauser sequence[3]
DAX1	+		+	Adrenal insufficiency
PIN1		+		
IL17RD		+		
DUSP6	+	+		
SPRY4	+	+		
LEP			+	Morbid obesity
LEPR			+	Morbid obesity
PSCK1	+		+	Adrenal insufficiency, hair hypopigmentation
BBS1-11			+	Bardet-Biedl sequence[4]
FLRT3		+		

CHH: Congenital Hypogonadotropic Hypogonadism; nIHH: normosmic hypogonadotropic hypogonadism; KS: Kallmann Syndrome.
1: CHARGe syndrome: coloboma of the eye, heart anomalies, choanal atresia, hypogonadotropic hypogonadism, ear abnormalities and/or deafness, facial palsy, cleft palate, and urinary tract dysmorphisms; 2: Gordon-Holmes syndrome: spinocerebellar ataxia, hypogonadotropic hypogonadism, and visual impairment due to chorioretinal dystrophy; 3: Boucher-Neuhauser syndrome: spinocerebellar ataxia, hypogonadotropic hypogonadism and chorioretinal dystrophy; 4: Bardet-Biedl Syndrome: retinitis pigmentosa, obesity, kidney dysfunction, polydactyly, behavioral dysfunction, and hypogonadism.

Genes encoding the actors directly involved in GnRH signaling, like the GnRH ligand (*GNRH1*) and receptor (*GNRHR*) are obvious candidates for nIHH, without impairment in GnRH migration (see after). Genes encoding other important components of the neuroendocrine network have been demonstrated to be altered in nIHH. Indeed the identification of kisspeptin or KNDy neurons offered the opportunity to identify other genes associated to nIHH. Mutations involving the kisspeptin ligand (*KISS1*) have only recently been described (Topaloglu et al., 2012), whereas the alterations in kisspeptin receptor (*KISS1R* or *GPR54*) were already known (de Roux et al., 2003). Neurokinin B (*NKB* or *TAC3*) and its cognate receptor (*NKBR* or *TAC3R*) are two main causes of nIHH (Topaloglu et al., 2009). Isolated disruption of genes encoding gonadotropins also produces nIHH (Weiss et al., 1992). These reports and the clinical pictures associated support the evidence that nIHH may be considered the result of GnRH networking disruption, without any ontogenetic alteration.

Other genes encoding proteins with wider tissue distribution or transcription factors involved in several and shared metabolic intracellular cascades are responsible for syndromic forms that contain CHH. From a clinical point of view, it is important to correctly identify these forms in order to set up the correct clinical approach, and to search for treatable associated conditions. Different syndromes including CHH are reported in Table 1.

The identification of various genes has led scientists to define a pattern of genetic inquiring from a correct "phenotypization" of patients after the first clinical approach, in order to orientate genetic screening (Costa-Barbosa et al., 2013). After exclusion of olfactory disorders, a series of other associated features (like renal or dental agenesis, midline defects, bimanual synkinesis) has to be specifically searched to improve diagnostic power and more appropriately favor genetic counseling. By contrast, it is of note that mutations within the same genes may be associated to different forms and severity of disease. Furthermore, even the same mutations may be accompanied by more or less

severe clinical pictures, indicating a remarkable variable expressivity. Pitteloud et al. first described a family carrying the same FGFR1 mutation in which some individuals presented KS, some other anosmia, nIHH or only a delayed puberty (Pitteloud et al., 2007). As the list of candidate genes lengthens, more cases of oligogenism (i.e. the concurrence of different gene mutations in CHH individuals) have been described. The presence of additional genes in the same individuals may contribute to explain the diversity of disease in probands. One may hypothesize that the participation of various genes creates the backbone and the «fragility» of gonadotrope axis, more or less unveiled by associated factors or *stimuli*, as was already reported in women with hypothalamic amenorrhea (Caronia et al., 2011).

Involvement of GNRH1 and GNRHR in CHH

Despite the obviousness of GnRH receptor implication in CHH, genetic research for *GNRHR* locus has been dampened. Indeed, the previous hypothesis was that, since the administration of GnRH was able to increase FSH and LH in patients with nIHH, a mutation could have unlikely occurred in *GNRHR*. By contrast, numerous cases of nIHH associated with *GNRHR* mutations have been reported since 1997 (de Roux et al., 1997; Kim et al., 2010).

Unlike the GnRH receptor, a natural murine model lacking the peptide GnRH was already described in 1977 (Cattanach et al., 1977). These animals had an isolated hypogonadotropic hypogonadism. In parallel, the gene encoding the GnRH precursor was identified and characterized by Seeburg and colleagues (Adelman et al., 1986). Thereafter, Mason identified a large deletion in this mouse, by establishing a relationship between the GnRH and the hypogonadal mouse (Mason et al., 1986). The transgenic introduction of a 13.5 kb fragment containing the *GNRH1* locus rescued the phenotype and restored fertility in these animals. Despite considerable effort, the involvement of *GNRH1* has been difficult to demonstrate until 2009, when an autosomal recessive form of nIHH was described (Bouligand et al., 2009). The alteration induced a homozygous insertion of an adenine (c.18-19insA) with consequent frame shift and truncation at the level of the signal peptide. Since then only another report has described *GNRH1* mutations associated with nIHH (Chan et al., 2009).

Basic studies and clinical *in vivo* models have contributed to unravel the mechanisms of ligand-receptor coupling and downstream signaling, and the implication of molecular alterations involving these factors in producing human diseases.

AIMS OF THE STUDY

Aim of this work was to screen for mutations of *GNRH1* and *GNRHR loci* in a large series of CHH patients and to find and functionally characterize novel variants.
After a comprehensive clinical evaluation and molecular characterization, a functional study of novel mutants has been performed in order to define the deleterious effect of the mutant proteins and to try to understand the mechanisms of disease in the affected patients.

PATIENTS, MATERIALS AND METHODS

Patients

450 patients with CHH were enrolled in this study. Most patients were recruited from the French Cohort of CHH (Bicetre University Hospital, France). 201 patients out of 449 were affected by KS, whereas 250 suffered from nIHH.

Criteria for KS were:

1) congenital hypogonadotropic hypogonadism, defined by clinical signs or symptoms of hypogonadism and: in men, serum testosterone levels below 1 ng/mL in the presence of low or normal gonadotropins; in women, primary amenorrhea and estradiol levels below 20 pg/ml in the presence of low or normal gonadotropins;

2) complete anosmia, as assessed by a previous olfactory test, or defined by self-report;

3) otherwise normal biochemical tests of anterior pituitary function and ferritin concentrations. nIHH was defined by the same criteria as KS except for olfactory impairment.

In all patients, MRI of pituitary region was performed. In two patients serial blood samplings were drawn in order to assess biochemical and hormonal evolution overtime.

The genetic study was approved by the local Hospital ethics committee and complied with human research guidelines as stated in the Declaration of Helsinki. All the patients gave their written informed consent before genetic analysis and hormone studies.

Genetic analyses

DNA extraction. Genomic DNA was isolated from white blood cells by a commercial kit (FlexiGene® DNA kit, Qiagen, The Netherlands). Briefly, leukocytes nuclei were obtained by centrifugation. Proteins contamination was minimized by a denaturating buffer containing a chaotropic salt and a specific protease. DNA was precipitated in isopropanol, recovered by centrifugation and washed in ethanol. After a proper dryness, DNA was finally resuspended in a proper Tris-HCl pH 8.5 buffer.

RNA extraction. RNA was extracted from PMNC with the Trizol® reagent method (Life Technologies, Carlsbad, CA,

USA). Briefly, whole blood was incubated in EL Buffer (Qiagen, The Netherlands). 6-well plates of cell cultures were washed once in phosphate-buffer saline (PBS) 1x. RNA was extracted by phenol-chloroform method with Trizol® reagent. RNA was finally precipitated in isopropanol, recovered by centrifugation, washed in ethanol and resuspended in RNAse-free water. A spectrophotometer (Nanodrop 1000, Thermo Scienfici, Waltham, MA, USA) was used to measure DNA and RNA concentrations.

Sequencing. Direct genomic sequencing of coding exons and intron-exon junctions of *GNRH1*, *GNRHR* were performed. PCR primers were designed by Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). PCR and sequencing products were purified on a Biomek NXP-96 Laboratory Automation Workstation (Beckman Coulter, Villepinte, France) with Agencourt Ampure XP and Agencourt Cleanseq (Beckman Coulter Genomics, Danvers, MA). Sequencing products were analyzed with an automated capillary sequencer (ABI PRISM 3130xl Genetic Analyzer; Applied Biosystems, Foster City, CA). Electropherogram-derived sequences were compared with NCBI references using SeqScape Software 2.6 (Applied Biosystems, Foster City, CA).
Microsatellites genotyping was performed to confirm filiation (Powerplex 16 System®, Promega, Madison, WI). For GNRH1, apart from coding exons and intron-exon junctions, the up-stream and down-stream promoter encompassing 1100 bp before the transcription start site, and the entire intron 1, whose sequence is usually retained in extra-hypothalamic tissues, were also sequenced.

DNA constructs design and site-directed mutagenesis
The pCMV6 vector containing the wild-type (Tello et al., 2012) *GNRH1* cDNA was obtained by Origene Technologies, Inc. (Rockville, MD, USA, ref no. RG212991) with a modification by a carboxyl-terminal GFP tag. The mutation c.91C>T (p.R31C) has been reproduced with site-directed mutagenesis by QuikChange Stratagene II (Agilent Technologies, CA, USA) according to manufacturer's instructions.
Phosphorylated-5' sense and antisense primers sequences were as follows: 5'-GCACTGGTCCTATGGACTGTCGCCCTG-GAGGAAAGAGAGAT-3'; Antisense: 5'ATCTCTCTTTCCTCCAGG-

GCGACAGTCCATAGGACCAGTGC-3'. WT and mutated sequences have been validated by restriction enzymes and Sangers sequencing thereafter.

The two *GNRHR* missense mutations were prepared using QuikChange Stratagene II from a WT *GNRHR* pcDNA3.1+ vector (Missouri S&T cDNA Resource Center). Phosphorylated-5' sense and antisense primers sequences were as follows: (p.T269M; c.806C>T) Sense: 5'-GCACGGCTGAAGACTCTAAAAATGATGGTTGCATTTGCC-3'; Antisense: 5'-GGCAAATGCAACCATCATTTTTAGAGTCTTCAGCCGTGC-3'; (Y290F; c.869A>T) Sense: 5'-CTACTATGTCCTAGGAATTTGGTTTTGGTTTGATCCTGAAATGTTAA-3'; Antisense: 5'-TTAACATTTCAGGATCAAACCAAAACCAAATTCCTAGGACATAGTAG-3'. Expression construct sequences were confirmed with Sanger sequencing. The WT human *GNRHR* and the two mutants T269M and Y290F were finally modified by CDS cloning into a pCMV-Flag to encode an amino-terminal Flag by a PCR approach. We chose the EcoRI and BglII sites, that are sequentially located in the multilinker region of the receiving pCMV. The artificial construct was obtained by a PCR amplification of the original pcDNA3.1+ *GNRHR* with a former primer 5' to 3' containing: 1) four random nucleotides; 2) a site for the restriction enzyme EcoRI; 3) one base in excess for keeping the "in frame" codonic sequence; 4) 20 nucleotides of the CDS opening deprived of the ATG encoding the starting methionine. The reverse primer was constructed in a similar manner as above, and contained from 5' to 3': 1) four random nucleotides; 2) the site for the restriction enzyme BglII; 3) 20 nucleotides of the CDS closing sequence containing the reverse complement of the stop codon. The customization was therefore: sense 5'-ATTAGAATTCAGCAAACAGTGCCTCTCCTGA-3'; antisense 5'-GCCGAGATCTTCACAGAGAAAAATATCCAT-3'. The product of amplification was then cloned into a pCMV-Flag-Ampi resistant vector pre-digested by EcoRI and BglII by T4 ligase according to ligation procedure. The product of ligation has been finally used for competent XL-Gold transformation and plated in 10 cm-dished containing LB-Agar + ampicillin. Colonies were picked up, cultured in LB-ampicillin and harvested for DNA plasmidic extraction by MIDI-prep commercial kit (Macherey-Nagel, Dueren, Germany). The

correct positioning of the insert was confirmed by digestion with EcoRI/BglII and by Sangers sequencing. All reactions were performed according manufacturers' instructions.

In silico analyses

Inter-species conservation and sequence alignment was performed by means of Homologene on NCBI databases (http://www.ncbi.nlm.nih.gov/homologene). Single nucleotide polymorphisms were detected through NCBI platform (the same as above, /SNP). Primer design was performed using Primer Blast with optimal 60°C-Tm calculations by Schildkraut et Lifson 1967 et Breslauer 1989. Primer sequences where checked by OligoAnalyzer™ software.
Prediction of peptide cleavage was performed by means of Prop v.1.0b ProPeptide Cleavage Site (http://www.cbs.dtu.dk/services/ProP/). Prediction of protein function after selective amino acid substitution was obtained by means of Alamut® (Interactive Biosoftware, Rouen, France), AlignGVD (http://agvgd.iarc.fr/agvgd_input.php/), Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/) and PANTHER Coding SNP Analysis tool (http://www.pantherdb.org/tools/csnpScoreForm.jsp).

Peptide custom and stability

"Wild type" (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2) and "R31C" (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Cys-Pro-Gly-NH2) mutant GnRH were synthesized by a custom peptide manufacturer (Eurogentec®, Liège, Belgium). Molecular weight was confirmed by MALDI-TOF and purity was assessed at ~95% by the manufacturer.
Lyophilized products were suspended in sterile water in order to obtain 0.1 mM aliquots and conserved frozen at -150°C.
LC-MS/MS was performed with Quattro-LCZ triple quadrupole mass spectrometer equipped with the orthogonal electrospray source (Micromass, Manchester, UK) to analyze peptides stability in solution. Peptides solutions (water and cell culture medium) were incubated 24-h at room temperature for these stability studies.

Cell lines
Several cell lines were used for the experiments.

HEK293T (Human Embryonic Kidney 293 cells, ATCC CRL-11268) cells were used for experiments in GnRH-I peptide signaling, as luciferase assays and Western Blot. For this purpose, cells were transiently transfected with pcDNA3.1+ vector of human *GNRHR* (Missouri S&T cDNA Resource Center, Rolla, MO), as they do not express GnRH-R endogenously. They were also used for the studies of pre-pro-hormone GnRH-I (fluorescence studies, MTT assays, western blots) WT and R31C mutant (see after), using GNRH1-GFP tagged pCMV6 (Origene Technologies, Inc., Rockville, MD, USA). Finally, HEK293T cells were used for functional studies of mutant GnRH-Rs (luciferase assay, western blots), after transient transfection of WT or mutant receptors in pcDNA3.1+ or pCMV6-Flag vectors (see after).

COS-7 (CV-1 –simian- in Origin, carrying the SV40 genetic material, ATCC CRL-1651) also do not express GnRH-R. They were used in competition radioligand binding assays in the functional analyses of GnRH-I peptides and of mutant GnRH receptors. They were also used in inositol phosphate (Mason et al., 1986) accumulation in the same studies.

Murine gonadotrope **LbetaT2** cell line was kindly provided by Dr. Mellon laboratory (Turgeon et al., 1996). These cells endogenously express GnRH-R and are able to express gonadotropin subunit transcripts and to secrete mature LH glycoprotein after GnRH treatment. These cells were used for studying mutant GnRH-I peptides by gene expression analyses and by LH assay in supernatants. They were also used for intracellular calcium assays by Fura-2 and for WB.

Att-20 cells (ATCC CRL-11285) are murine corticotrope cells expressing type-1 pro-hormone convertase and are able to cleave pre-pro-hormones containing a GKR sequence (such as pro-opio-melanocortin or pre-pro-GnRH). These cells were used for the studies on pre-pro-GnRH-I, by fluorescence microscopy, WB and MTT assay.

Gn-11 cells originate from a murine embryonic neuroblastoma endogenously expressing GnRH at low rate. They were kindly provided by Radovick group (Zhen et al., 1997). Gn-11 were used for the studies on pre-pro-GnRH-I, by fluorescence microscopy, WB and MTT assay.

A375 melanoma (ATCC CRL-1619) cell line was used for studies on pre-pro-GnRHIs. Cells were transiently transfected with graded doses of WT or mutant *GNRH1*-GFP. The number of GFP-positive cells was computed on a fluorescence microscopy (see after).

H727 neuroendocrine tumor (ATCC CRL-5815) cell line was used as above, especially for studies on pre-pro-GnRH-Is by fluorescence microscopy.

All cell lines were cultured in different cell media in accordance with their specific requirements. The choice of plates and wells was undertaken according to the specific assay and procedure to be performed. All transfection studies were performed by means of Lipofectamin 3000™ reagent (Invitrogen, Cergy Pontoise, France) according to manufacturer's instructions.

Competition radioligand binding assays

The high affinity GnRH analog, [His5,D-Tyr6]-GnRH was radio-iodinated as previously described (Flanagan et al., 1998) and purified using Sephadex chromatography. COS-7 cells were transiently transfected with human GnRH-R DNA construct containing the human WT GnRH-R or the T269M and Y290F *GNRHR* mutants using 6 µg of DNA and 30 µl FuGene HD (Promega Corporation, Madison, WI) per 10 cm dish and seeded into 12-well plates. Two days after transfection cells were washed with HEPES-DMEM containing 0.1 % bovine serum albumin (2 x 1ml, HEPES-DMEM-BSA) and incubated with 125I-[His5,D-Tyr6]-GnRH (100,000 CPM per well). For the study of mutant R31C cys-8 GnRH-I peptide, various concentrations of WT GnRH or R31C GnRH (4 h, 4°C) were used in a total volume of 0.5 ml. For the study of mutant GnRH receptors, various concentrations of sole WT GnRH were administered for 4h at 4°C in a total volume of 0.5 ml. In both studies, cells were washed with phosphate-buffered saline (2 x 1ml) and lysed with NaOH (1 ml, 0.1 M). Cell-bound

radioactivity was counted in a gamma counter and IC-50 values were calculated using GraphPad Prism (GraphPad Software Inc, San Diego).
To determine whether R31C GnRH binds covalently to the GnRH-R, transfected COS-7 cells were incubated with WT GnRH (10-8 M), R31C GnRH (10-5 M) or HEPES-DMEM-BSA alone (2 h, 4° C), washed with HEPES-DMEM-BSA (1ml) and incubated in HEPES-DMEM-BSA (5 ml, 1 h, 4° C) to allow dissociation of non-covalently bound peptide, before the binding assay was performed as above.

Fluorescence microscopy
Cells were seeded in 24-well plates. At about 75% confluence, cells were transfected by either WT GNRH1 pCMV6, R31C, equimolar WT+R31C concentrations, excess of mutant plasmid (WT:R31C 1:4), eGFP-pcDNA3.1 or empty pCMV6 vector using Lipofetacmin3000™ method according to manufacturer's instructions. 24 h after transfection, living cells were visualized under the inverted microscope Olympus IX51 equipped for epifluorescence and phase contrast microscopy (Olympus, Milan, Italy). The images were captured at 40x and acquired with Olimpus Digital Camera F-View II (Olympus, Milan, Italy).
As positive control we used transient transfection with eGFP-pcDNA3.1; empty pCMV6 was always used as negative control. Cell count was calculated by the computer-assisted Cell^F® dedicated software (Olympus Europe Software Informer). The intensity of GFP signal was calculated as average of all visible cells in each microscopic field with compensation method between images and measured after extraction of background. Images were acquired at 1024x1024 pixel resolution and using 0.3 mm Z-stacks.

Measurement of intracellular free calcium concentration ($[Ca2+]_i$)
LbetaT2 cells were kept in serum free medium overnight and then loaded with 2 μM Fura2-AM for 45 min at 37°C. The experiment was conducted in HEPES buffer (in mM; 116 NaCl, 5.6 KCl, 1.2 MgCl2, 5 NaHCO3, 1 NaH2PO4, 20 HEPES ph 7.4) in the presence of extracellular Ca2+ (EGTA 100μM + CaCl2 300μM). Single images of fluorescent emission at 510 nm under excitation at 340 and 380 nm were taken every 5 seconds by a dedicated camera-equipped software. Changes in

$[Ca2+]_i$ in response to GnRHs were monitored using the Fura2 340/380 fluorescence ratio. Basal ratio was arbitrarily considered as 1. Ca2+ responses over basal level were given either as maximal rise of $[Ca2+]_i$ or as area under the curve (for 2 min after agonist addition).

Inositol phosphate accumulation

COS-7 cells were transiently transfected by electroporation with human WT or mutant GnRH-R DNA (10 µg/15 cm dish), seeded into 12-well plates and radiolabeled by overnight incubation with myo-[3H]Inositol (0.5 µCi/well, American Radiolabeled Chemicals, St Louis, Mo). Radiolabeled cells were washed and incubated (30 min, 37° C) in IP medium (HEPES-DMEM-BSA supplemented with 10mM LiCl), then stimulated (60 min, 37° C) with various concentrations of WT GnRH, or WT and R31C GnRH, or equal concentrations of WT GnRH and R31C GnRH. Incubations were stopped by removal of the medium and cells were lysed by addition of formic acid (1 ml, 10 mM). IP were extracted from cell lysates using Dowex 1 X8-200 chromatography and counted using a liquid scintillation counter (Packard). EC-50 and Emax values were calculated using GraphPad Prism (GraphPad Software Inc).

Serum Responsive Element (SRE) luciferase assay

luc2P/SRE/Hygro plasmid (Promega, Madison, WI) was used to test luciferase production in response to MAP kinase activation as a reporter gene system. HEK293T cells ($1.2x\ 10^4$ cells/well) were seeded 72 h before testing in high-glucose Dulbecco's minimal essential medium (DMEM, Invitrogen, Cergy Pontoise, France) containing 2 mM glutamine, 100 IU/mL penicillin, 100 mg/mL streptomycin, and 10% heat-inactivated fetal calf serum at 37°C in 96-well plates. Twenty-four hours before testing, cells were co-transfected in serum free OptiMEM, using Lipofectamine 3000 with the plasmids for human GnRH receptor, luc2P/SRE/Hygro and pMIR-REPORT™ beta-galactosidase vector (Applied Biosystems, Foster City, CA). For the study of mutant GnRHs, WT GnRH, R31C, WT+R31C or vehicle were added at different dilutions (from 10^{-10} to 10^{-6} M). For the study of mutant GnRH receptors, a dose-response curve with serial concentrations of the sole WT GnRH was established. In both studies, after a 5h-incubation cells were harvested and assayed for luciferase activities using a

luminometer (Victor, Perkin Elmer, Waltham, MA, USA). To standardize for transfection efficiency, the relative light units were normalized by the galactosidase activity at optical density. EC-50 and Emax values were calculated using GraphPad Prism (GraphPad Software Inc).

Western blots

For the study on mutant GnRH-I, LbetaT2 cells were starved for 18h in serum-free DMEM before treatments, then exposed to 10^{-8} M of WT, 10^{-8} M of R31C, the combination of 10^{-8} M of each peptide or vehicle. Cells were harvested at 0, 5, 10, 15 and 30 min after treatment.

For the study of mutant GnRH receptors, HEK293T cells were transiently transfected as reported above with either WT or mutant T269M and Y290F *GNRHR*, serum-full medium or vehicle in OptiMem serum-free medium by Lipofectamin 3000. The day after cells were exposed to 10^{-8} M or 10^{-9} M WT GnRH and harvested after 5 or 20 minutes' of exposition to WT GnRH-I.

Western blotting analyses were performed on SDS-page as previously described (Maione et al., 2013a). For protein subcellular fractionation, membrane were extracted by differential centrifugation with dodecyl-beta-maltoside separation (Sigma Aldrich®, St. Louis, MO, USA). Briefly, cells were plated in 6-well plates, transfected by Flag-couples WT or mutant (T269 and Y290F) human *GNRHR* and harvested for protein extraction the following day. After the first centrifugation, pellets were lysated by sonication and resuspended for 1h in 50 uL of a solution containing 4 mg/mL dodecyl-beta-maltoside. Solutions were then centrifuged at 4°C for 1h and supernatants were collected for subsequent blots.

Rabbit anti-phospho-Akt and mouse anti phospho-MEK1/2 (Cell Signaling Technology), mouse anti-total p44/p42 ERK1/2 and anti-phospho-ERK1/2, (Santa Cruz, Dallas, Texas, USA, no. sc-514302, and sc-136521, respectively) were used at various dilutions as appropriate. Murine anti-GnRH-I was used at 1/800 -1/1000 dilution (Santacruz), monoclonal anti alpha-tubulin at 1/2000 (Sigma Aldrich, St. Louis, MO, USA). Mouse anti-Na/K-ATPase (Santacruz) was used at 1/1000 dilution as membrane-specific housekeeping protein. Mouse anti-beta-actin and anti-Flag were used at 1/2500 dilution. For

secondary peroxidase-coupled antibody incubation, membranes were washed and hybridized for 90 minutes at room temperature (anti-mouse or anti-rabbit Fc, 1/5000 or 1/10,000 dilution, as appropriate).
Chemiluminescent detection was performed with ImageQuant LAS 4000 Mini equipment (GE Healthcare, Buckinghamshire, UK). After variable exposition of horse-radish peroxidase substrate (Immobilon®, EMD Millipore, Molsheim, France). Protein quantification was assessed by pixel density quantification with ImageQuant® software.

Gene expression study

LbetaT2 cells were grown at 10^6 cells/well density, in 6-well plates and starved in serum-free DMEM for 18h before tests. After 5 h of incubation with 10^{-8} M WT, R31C, the combination of 10^{-8} M WT + 10^{-8} M R31C peptides or vehicle, cells were washed with 1x PBS and total RNA was isolated using Trizol® (Invitrogen, Germany).
Lhb (murine LH beta subunit) transcript was quantified by real-time RT-PCR, using an ABI Step One Sequence Detector (Applied Biosystem, Foster City, CA, USA) as previously described (Bouligand et al., 2010).

MTT assay

HEK293T, Att-20 and Gn-11 were plated in 96-well plates. Before testing, cells were transfected by using Lipofectamine 3000™ method with the WT human GNRH1 receptor, mutant R31C, combination of WT+R31C plasmids or with empty vector in serum-free OptiMEM. 48h after transfection, GFP signaling was checked and cells were starved in high-glucose DMEM containing 2 mM glutamine, 100 IU/mL penicillin, 100 mg/mL streptomycin, and 10% heat-inactivated fetal calf serum plus the tetrazolium MTT at 37°C for 1 h. After solubilization in isopropanol, the absorbance at 570 nm was measured by VICTOR™ X4 Multilabel Plate Reader. The percentage of viability was normalized upon control (non-transfected) cells (100% viability).

LH assay

LbetaT2 cells were starved overnight in serum-free DMEM. The day of assay cells were exposed to 10^{-8} M WT, R31C, the combination of 10^{-8} M of each peptide or vehicle for 5 h. Cell

culture supernatants were collected and rapidly stored at -80 °C. LH concentration was measured in cell culture media by using a previously described ELISA method (Garrel et al., 2011) with reagents supplied by Dr. Parlow (National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA). The minimum detectable LH concentration was 0.2 ng/ml, and the interassay coefficient of variation was less than 10%.

Statistical analyses

Only nonparametric tests were used. Friedman's test was used to compare three or more matched groups and Kruskall-Wallis test for unmatched groups. These analyses were followed by Dunn's post comparison test. Differences were significant when $p < 0.05$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Statistical analyses were performed using GraphPad Prism version 5.0d (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

A. Genetic Analyses

From the total cohort of CHH patients we identified 10 different variants in *GNRH1* and *GNRHR* coding regions (Fig. 4).

In *GNRH1* we identified the mutations:
- c.18-19insA,
- c.77A>G, p.W26X,
- c.91C>T, p.R31C.

In *GNRHR* we identified:
- c.111T>G, p.I37S,
- c.311A>G, p.Q106R,
- c.710G>A, p.R240Q,
- c.806C>T, p.T269M,
- c.846C>G, p.P282R,
- c.869A>T, p.Y290F,
- c.969A>G, p.Y323C.

Of these mutations, the c.18-19insA in *GNRH1*, and the p.I37S, p.Q106R, p.R240Q, p.P282R and p.Y323C in *GNRHR* were already described and characterized (Beneduzzi et al., 2014; Bouligand et al., 2009; de Roux et al., 1997; Kim et al., 2010; Trarbach et al., 2006). We focused only on mutations never described or functionally characterized, the p.W26X, the p.R31C in *GNRH1*, the p.T269M and the p.Y290F in *GNRHR*. Detailed demographic, clinical, biological and genetic data of individuals carrying these novel variants are reported in Table 2 and Figg. 3 and 4.
The mutation c.77A>G in *GNRH1* consisted in a A>G amino acidic substitution in position 77. Alignment with orthologs revealed a complete conservation of tryptophan in position 3 of GnRH in mammals (Fig. 3).
More importantly, the missense in position 26 of the pre-pro-GnRH generated a stop codon in the third residue of the decapeptide, thus abolishing almost completely the translation product, the ligand sequence and structure. The mutation was carried in heterozygosis in both probands and the father.

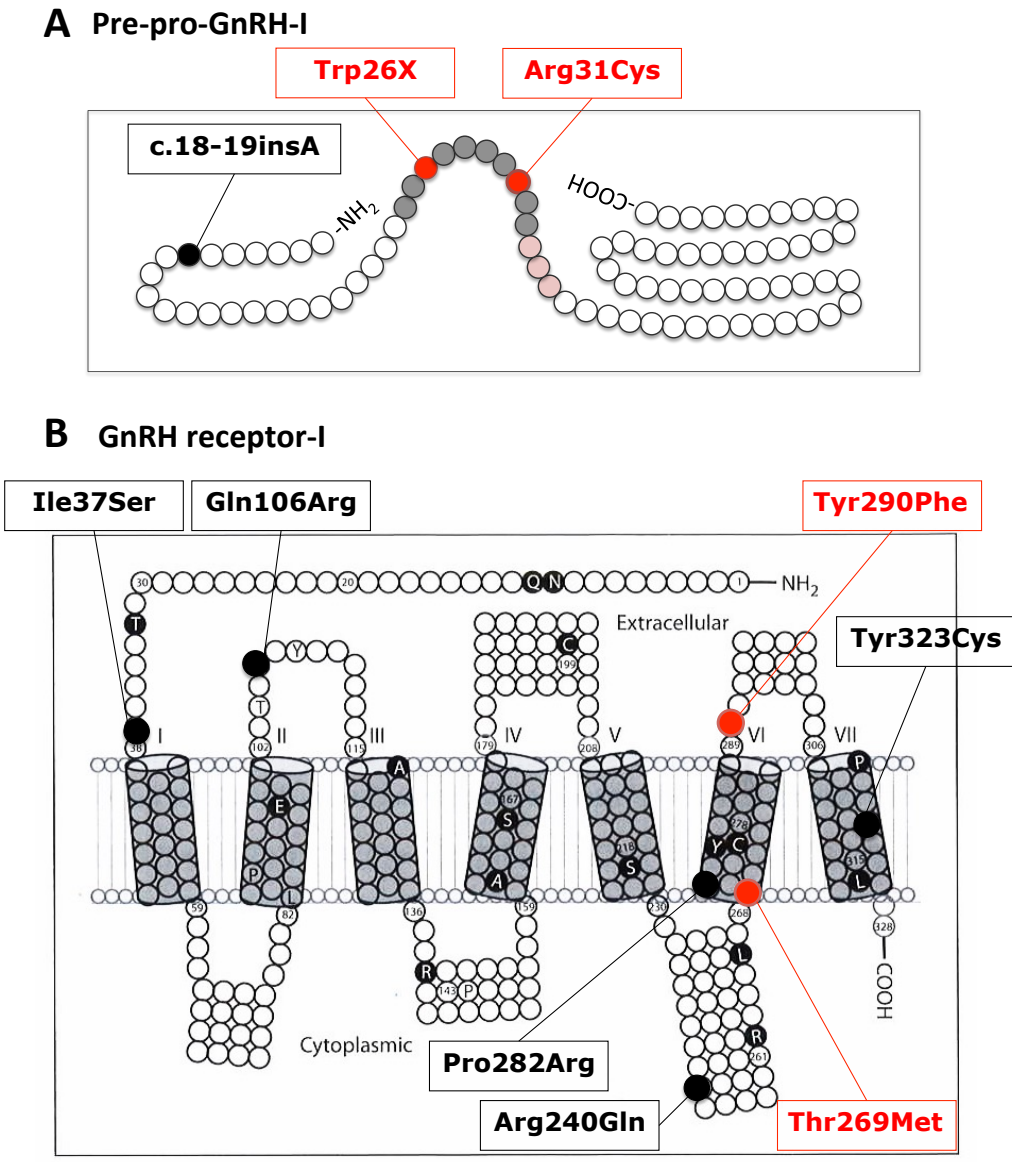


Figure 5 – Schematic two-dimensional representation of the GnRH-I pre-pro-hormone and the GnRH type-I receptor.

Each amino acid is represented by a circle. In both panels, mutations found in the current CHH cohort are reported. Among these, variants already characterized are depicted in black, the novel variants are in red.

A. Pre-pro-GnRH-I. Decapeptidic sequence is shown in grey. The GKR sequence for pro-hormone convertase is shown in pink.
B. The TMDs are depicted as grey box and enumerated from I to VII in Latin numbers. Small black circles denote already described variants associated to CHH.

In order to exclude a contra allelic event in *GNRH1* locus, the complete sequencing of the two regulatory regions of *GNRH1* until -1100 bp before the transcription start site was performed and did not reveal variants. The analysis of the entire sequence of the intron 1, retained in extra hypothalamic tissues, also did not reveal abnormalities. Finally, the GNRH1 sequence from PMNC-cDNA (performed in the patient 1 only) showed the coexistence of WT and mutant form, indicating expression of the WT allele in this patient (Fig. 6A). No mutations were found in other genes involved in CHH, like *GNRHR, FGFR1, PROK2, PROKR2* and *WDR11*.

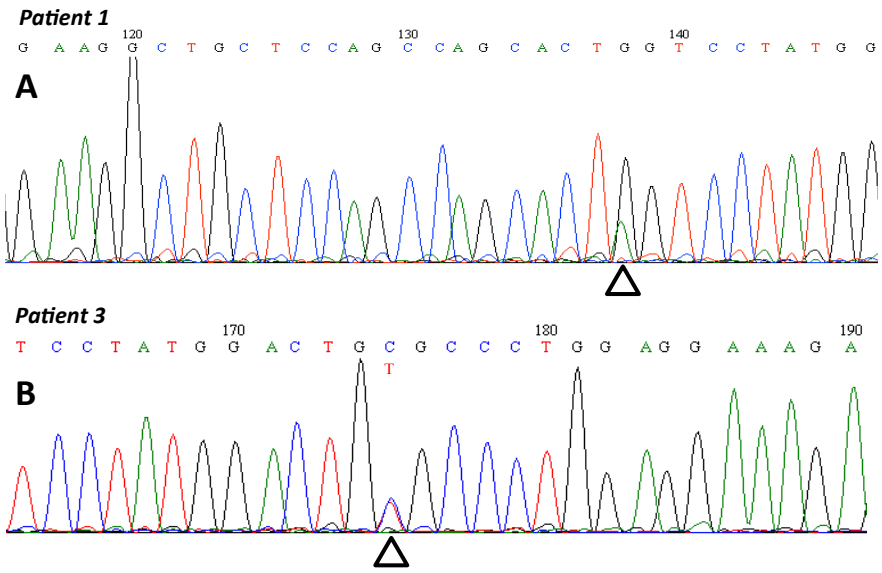


Fig. 6 – GNRH1 sequencing from PMNC-derived cDNA.
RNA was extracted from PMNC of peripheral blood (see Methods). The electropherogram from patient 1 shows the co-existence of WT and the c.77A>G (p.W26X) variant (open arrow, panel A). The electropherogram of patient 3 (B) shows the co-existence of WT and the c.91C>T (p.R31C) variant (open arrow, panel B).

The mutation c.91C>T (p.R31C) in *GNRH1* produced a missense in codon 31, replacing the arginine with a cysteine in the decapeptide position 8 (p.R31C, or cys-8, Fig. 5). In family 2, the boy harbored a heterozygous mutation and his parents are unaffected and do not carry this mutation. Micro-satellites revealed that the mutation is *de novo*. In family 3, the boy carried the heterozygous mutation along with his mother. Both carriers are affected by nIHH. Direct sequencing of genomic regulatory region and the intron 1 did

not reveal abnormalities. Peripheral blood GNRH1 cDNA sequencing revealed the presence of WT and mutant forms, indicating transcriptional production of normal allele (Fig. 6B) and excluding a contra allelic GNRH1 event.

The mutation c.806C>T (p.T269M) in *GNRHR* was carried by two nIHH sisters in homozygosis. Their mother carried the same mutation in heterozygosis, whereas their father was not available for genetic tests (Fig. 4). The missense mutation in *GNRHR* substituted a methionine in place of the threonine in position 269, located in the first residue of the sixth TMD (Fig. 5). The inter species alignment revealed a total conservation of this residue within all mammals (Fig. 4).

The mutation c.869A>T (p.Y290F) in *GNRHR* was carried in homozygosis in the girl affected by nIHH. Familial study was not possible, but clinical inquiry revealed consanguinity in her parents. Missense mutation substituted the tyrosine-290 with a phenylalanine in a critical residue in the third extracellular loop next to the sixth TMD (Fig. 5). The Tyr-290 residue is totally conserved among mammals (Fig. 4).

B. Functional molecular characterization (*in silico* and *in vitro* experiments)

The *GNRH1* mutation c.77A>G (p.W26X) was classified as deleterious by SIFT, PolyPhen-2 and PANTHER prediction tools. Since the stop codon produces a completely truncated peptide, and the decapeptidic structure of GnRH-I is universally shown to be needed for receptor binding, no need for further functional characterization of mutant GnRH has been considered. A comprehensive genetic characterization of contra allelic *GNRH1* locus and of other CHH genes has been assessed (see Section A - Genetic Analyses).

The *GNRH1* mutation c.91C>T (p.R31C) was classified as deleterious by SIFT, PolyPhen-2 and PANTHER. Prop v1.0 predicted no alterations of pro-hormone convertase cleavage site after R31C introduction. R31C and WT decapeptides were stable over 24 h at room temperature in water and culture medium.

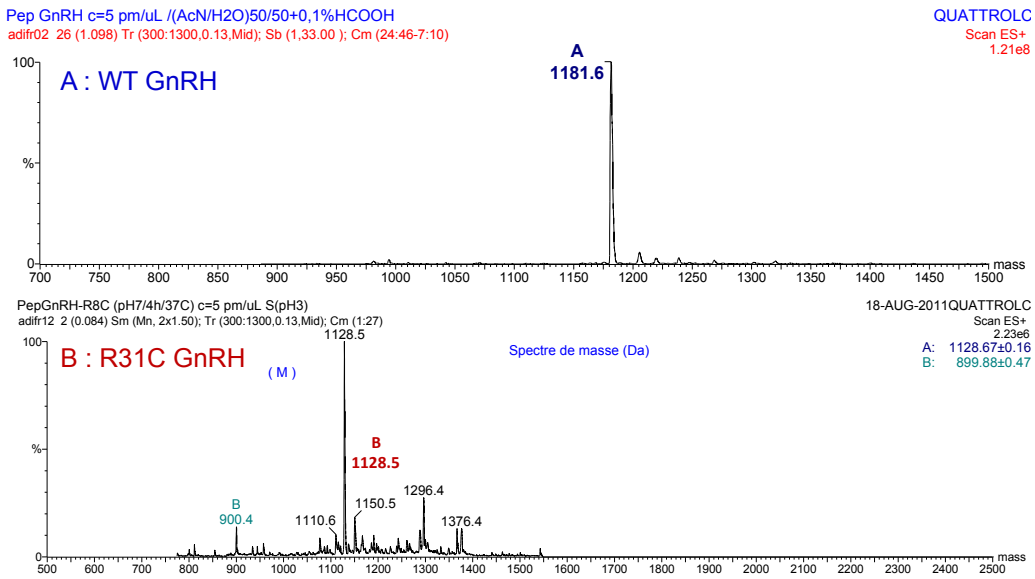


Figure 7 – Mass spectrometry of WT and cys-8 (p.R31C) peptides.
Peptide stability on mass spectrometry-coupled electrospray (Quattro-LC). Peptides were measured in aqueous solution tamponed at pH7 (ammonium acetate) and starved at 37°C overnight. WT and R31C decapeptides are found at the expected molecular weight (1181.6 and 1128.5, respectively). Formation of smaller fragments was absent in WT and negligible in R31C. Formation of R31C dimers was absent.

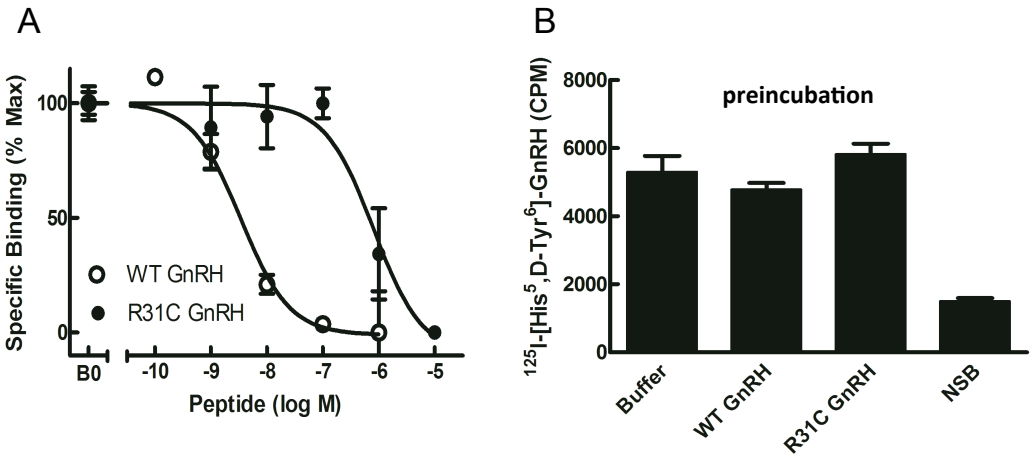


Figure 8 – Binding of WT and R31C GnRH to the GnRH receptor (GnRH-R). (**A**) COS-7 cells were transiently transfected with a human GnRH-R DNA construct and incubated with a ^{125}I-labeled GnRH agonist in the presence of indicated concentrations of WT GnRH or R31C GnRH. IC-50 values are WT GnRH, 2.8 x 10^{-9} M and R31C GnRH, 8.50 x 10^{7} M. Data are means and SEM of a single experiment performed twice in duplicate. (**B**) Transfected COS-7 cells were pre-incubated with buffer alone, WT GnRH (10^{-8} M) or R31C GnRH (10^{-5} M) and then washed to allow dissociation of ligand not covalently attached to the GnRH-R. Pre-incubated cells were then incubated with ^{125}I-labeled GnRH agonist in the absence or presence of saturating concentration of unlabeled WT GnRH (NSB). Data are the means of two experiments performed in triplicate.

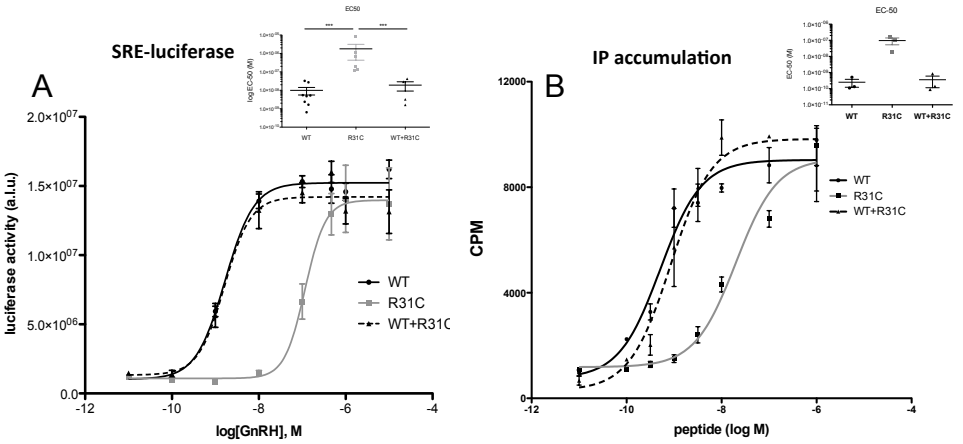


Figure 9 – Dose-response curves.
(**A**) SRE-coupled luciferase assay. HEK293T cells were exposed to graded concentrations of WT GnRH, R31C and equimolar concentrations of WT + R31C during five hours. Luciferase activity arbitrary units by luminometry (a.l.u.) is shown as ratio on beta-galactosidase activity by optical density used as transfection efficiency internal control. In the insert, individual values of EC-50 from n=8 experiences. EC-50 was established at 4 nM for WT and at 313 nM for R31C.
(**B**) IP accumulation. COS-7 cells administered myo-[2-^{3}H]Inositol after transfection. After 16 hours cells were washed and incubated for 1 hour with graded concentrations of WT, R31C and equimolar WT+R31C GnRH. In the insert, individual values of EC-50 from n=3 experiences three times each in triplicate. EC-50 was established at 0.5 nM for GnRH and at 199 nM.

No dimerization of the mutant decapeptide was found at Matrix-Assisted Laser Desorption/Ionization (MALDI) mass spectrometry with time-of-flight (Fig. 7). The cys-8 peptide had lower affinity for GnRH-R than WT GnRH (Fig. 8A). Dose-response curves with SRE-luciferase assay showed an almost 100-fold reduction of R31C agonist versus WT (EC-50 4 and 314 nM, respectively, fig. 9A and insert). Combination of WT and R31C was not able to impair luciferase activity when compared to WT alone (Fig. 9A). The maximal IP generated by both ligands was the same indicating that the R31C GnRH peptide was a full agonist, as was found in the SRE-luciferase assay (fig. 9B and insert). IP dose-response curve with equal concentrations of WT and R31C peptides was indistinguishable from WT GnRH alone (Fig. 9B). ERK1/2 phosphorylation was maximal at 5 min. R31C GnRH showed similar kinetics, but the degree of phosphorylation was reduced (Fig. 10A). Increasing the dose of R31C GnRH from 10^{-8} to 3 x 10^{-7} M in the presence of 10^{-8} M WT GnRH did not reduce ERK1/2 phosphorylation after 5 min of exposure (Fig. 10B). In LbetaT2 cells calcium response by R31C was significantly reduced compared to WT in terms of peak and area under the curve ($p<0.05$). Calcium peak and area under the curve after treatment with the combination of 10^{-8} M GnRH and 10^{-8} M mutant peptide was indistinguishable from WT GnRH alone (Fig. 11).

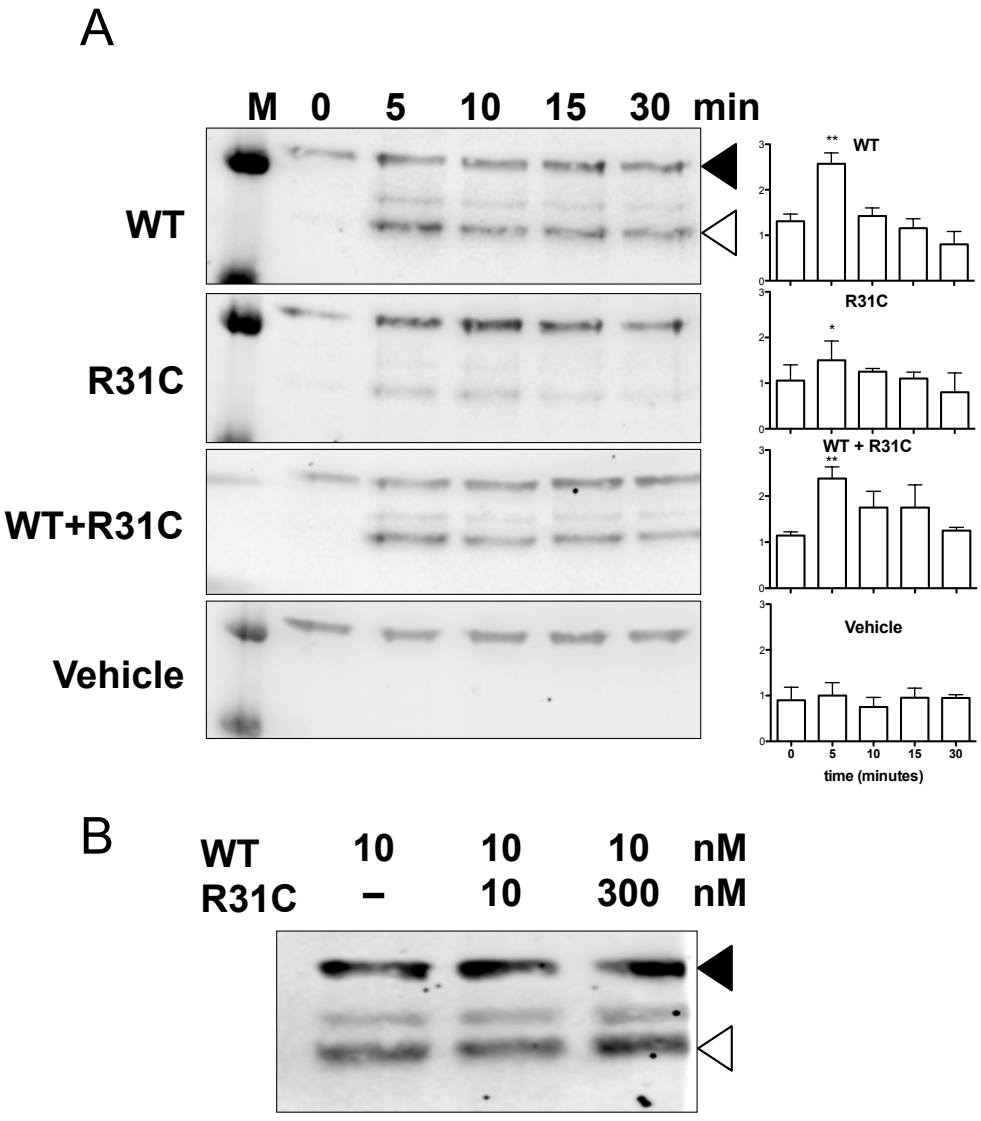


Figure 10 - ERK1/2 phosphorylation by Western blot.
(A) HEK293T cells were starved overnight in serum-free DMEM and then treated with 10 nM WT, 10 nM R31C, 10 nM WT + 10 nM R31C peptides or vehicle for 0, 5, 10 and 30 min. Representative of four experiments. Density quantification is displayed on right-hand, and data are expressed as mean ± SD for four experiments.
(B) LbetaT2 cells were starved overnight in serum-free DMEM and then treated with a constant dose of WT GnRH (10 nM) and increasing (0 – 10 – 300 nM) doses of mutant R31C peptide after 5 min of exposition.
Lysates were analyzed by Western Blotting using phospho-ERK1/2 antibodies (Cell Signaling®, open arrow) and mouse monoclonal anti alpha-tubulin (Sigma Aldrich®, St. Louis, MO, close arrow).

WT GnRH significantly increased *lhb* transcript levels in gonadotropes ($p<0.01$), whereas R31C GnRH did not significantly increase these levels over baseline (not significant, Fig. 12A). WT GnRH strongly stimulated LH secretion over baseline ($p<0.001$). R31C GnRH significantly stimulated LH secretion in LbetaT2 cells supernatants over baseline ($p=0.042$, Fig.12B). However R31C GnRH was unable to influence *lhb* transcription or LH secretion in LbetaT2 cells by WT GnRH (Fig. 12A and B).

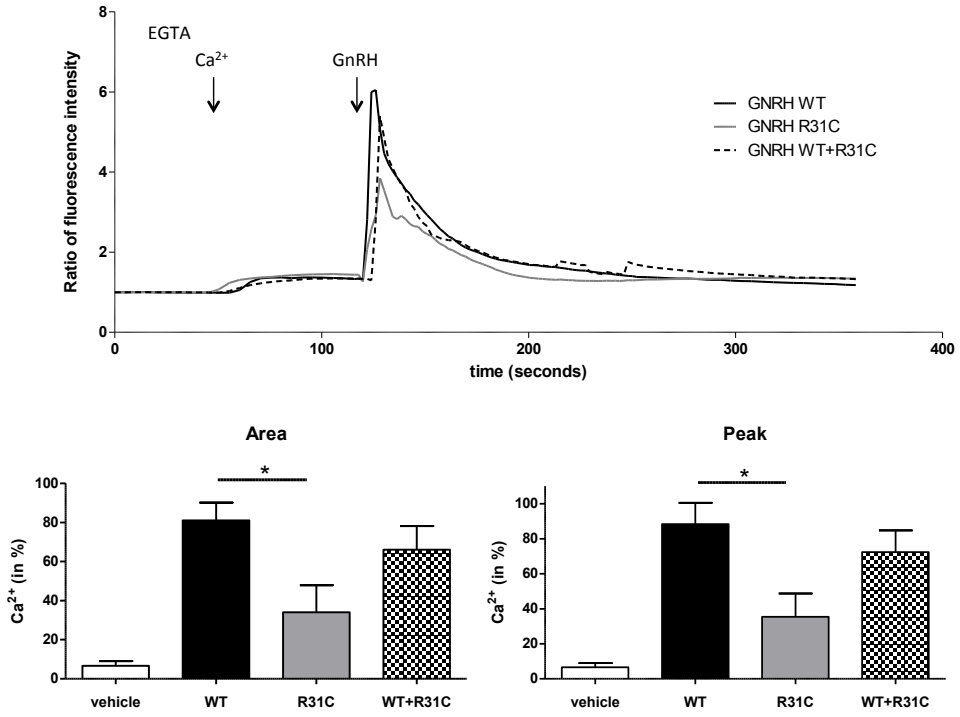


Figure 11 – Effect of treatments on intracellular calcium mobilisation.
Cells were loaded with Fura 2-AM. At the time of the experiment 100μM EGTA was added 30 sec before addition of 300μM of $CaCl_2$ (Ca^{2+}) to the medium as indicated, and cells were then stimulated with 10 nM of wild type (WT), mutant (R31C) or equimolar combination (10 nM WT + 10 nM R31C) or vehicle for 3 min. In the upper panel, representative curves of single cell recording. Each experiment mean was analysed using One-way ANOVA followed by Tukey's Multiple comparison test. In the lower panels, histograms show the means ± SEM (n = 4) of the response to ligands as calcium peak and as area under the curve (AUC). R31C-induced peak and AUC were significantly lower than WT. Combined treatment by WT+R31C did not induce a different calcium peak and AUC compared to WT alone. Representative of at least three independent experiments, *$p<0.05$.

The hypothesis that the cysteine in position 8 of the R31C GnRH might form disulfide bridges with cysteine residues in the receptor was ruled out by the pre-exposition of transfected cells to each of the ligands before extensive washing and performing a competition binding study. No

reduction in radiolabeled GnRH binding was observed, probably indicating that R31C GnRH does not form covalent disulfide bonds with the receptor (Fig. 8B).

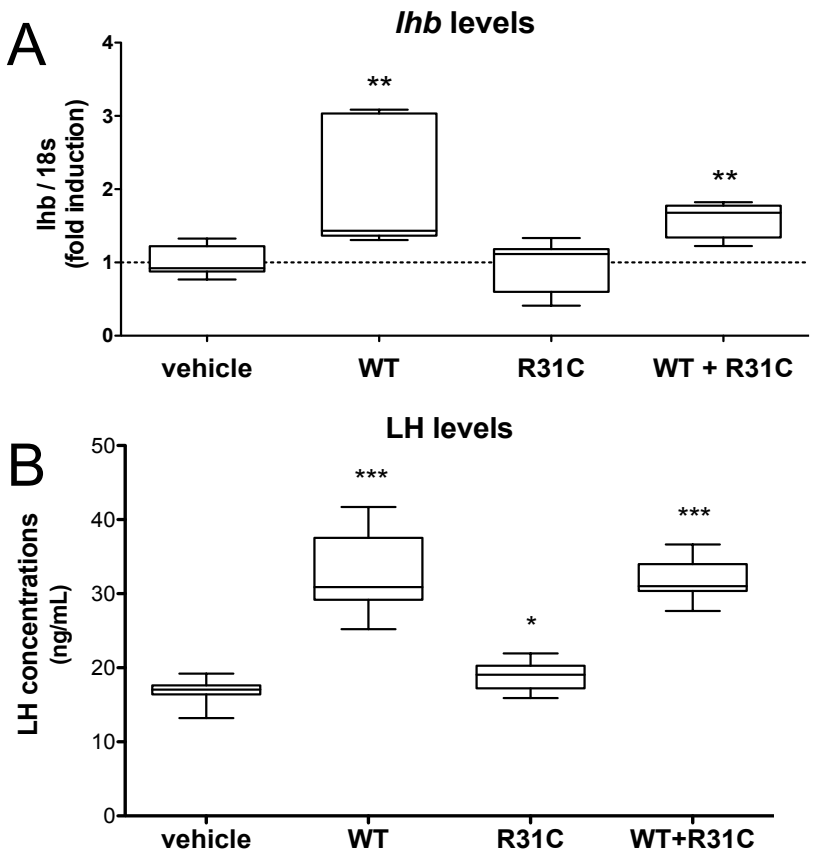


Fig. 12 – Effects of treatments on *Ihb* transcription and LH secretion.
(A) LbetaT2 cells were starved overnight in serum-free DMEM and treated either by 10 nM WT, R31C, 10 nM WT + 10 nM R31C peptides or vehicle. After five hours treatment cells were lysed for RNA extraction (Trizol®). Mouse *Ihb* levels were analyzed by quantitative real time RT-qPCR. Values are expressed as individual ratios on 18S and represented as fold induction on vehicle as means ± SEM, **p<0.01. This experience was conducted n=7 times in duplicates.
(B) LH concentrations from LbetaT2 cells culture *media*. Cells were starved overnight in serum-free DMEM and incubated with 10 nM WT, 10 nM R31C, 10 nM WT + 10 nM R31C, or vehicle for 5 hours. LH levels were measured by a combined rat/mouse RIA. Values are expressed as individual values and as means ± SEM, *p<0.05, ***p<0.001. The experiment was conducted four times and each sample was assayed in duplicates.

After pulsatile administration of WT and cys-8 peptides at 90-min frequency, WT GnRH was more powerful

than R31C GnRH in inducing luciferase activity coupled to SRE reporter gene. Combination of WT and R31C was not able to impair luciferase activity when compared to WT alone (Fig. 13).

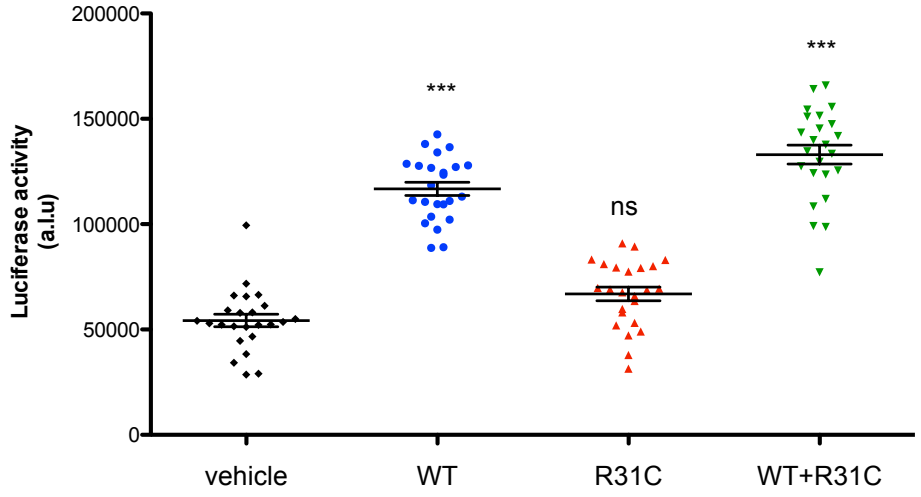


Figure 13 – Luciferase activity after pulsatile treatment by GnRHs
HEK293T cells were transiently transfected with GNRHR and SRE-coupled luciferase reporter gene, and then exposed to four pulses of 10 nM WT, 10 nM R31C and 10+10 nM WT+R31C. After 5 minutes exposure to each ligand, cells were washed, and a subsequent pulse was given 90 minutes after. Five hours after the fourth pulse, cells were harvested for luciferase assay. Luciferase activity arbitrary units by luminometry (a.l.u.) are shown as ratio on beta-galactosidase activity by optical density (used as transfection efficiency internal control). This experience was conducted three times in octaplicates.

Pre-pro-GnRH-GFP sequence and the c91C>T substitution after mutagenesis were verified by Sanger sequencing. Sequencing confirmed also that no CMV promoter, GFP or other sequences were altered. The highest transfection rate was achieved in HEK293T and A375 cells, whereas the lowest rate was found in H727, Gn-11 and Att-20 cells. Expression of murine pro-convertase was exclusively found in Att-20 cells and not in other lineages.
Despite variable transfection rate, in all cell lines tested, the number of GFP-positive cells was lower after R31C than after WT-*GNRH1* (Fig. 14). Co-transfection by WT and different doses of R31C did not impair GFP signal except than in Att-20 cells, in which a slight but not significant reduction in GFP signal was observed after high doses of R31C (Fig. 14).

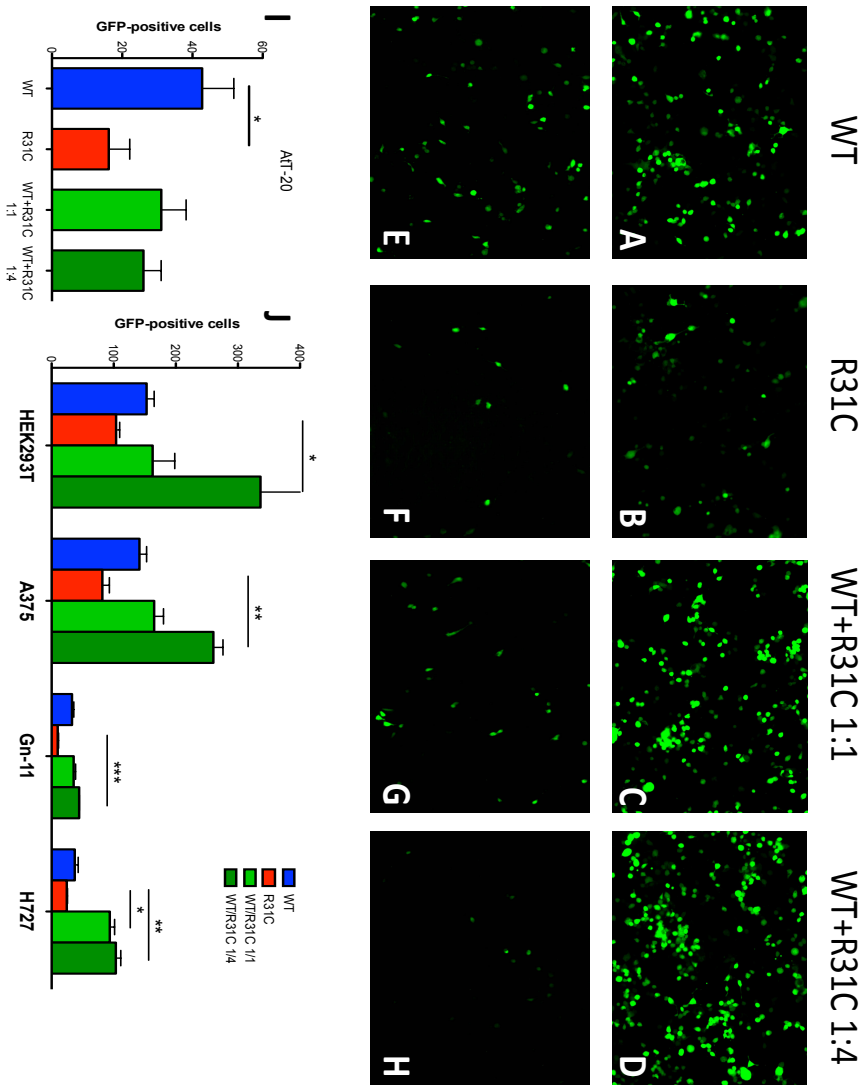


Fig. 14 – Efficiency of *GNRH1* transfection by GFP-positive cell count.
In upper panels, GFP positive cells are depicted by 20x magnification fuorescence microscopy.
Transfection was performed by Lipofectamine3000™ method with either 75 ng WT GNRH1 pCMV6 (A and E), 75 ng R31C (B and F), 75+75 ng WT+R31C (C and G), or 75+300 ng WT+R31C (D and H) in HEK293T (from A to D) or Att-20 (from E to H) cells.
(I) Quantification of GFP-positive cells in Att-20 cells. *p<0.05 by Dunn's test. Experience conducted n=6 times.
(J) Quantification of GFP-positive cells in HEK293T, A375, Gn-11 and H727 cells. *p<0.05, **p<0.01, ***p<0.001 by Dunn's test. Experience conducted n=3 times.

Apart from the number of GFP-positive cells, also the intensity of GFP signal was reduced in HEK293 cells after R31C transfection compared to WT (Fig. 15). No change in HEK293T, Gn-11 and Att-20 cell viability was observed 24, 48 and 72 h after effective transfection by WT and R31C as assessed by MTT assay (see Fig. 16A, B and C).

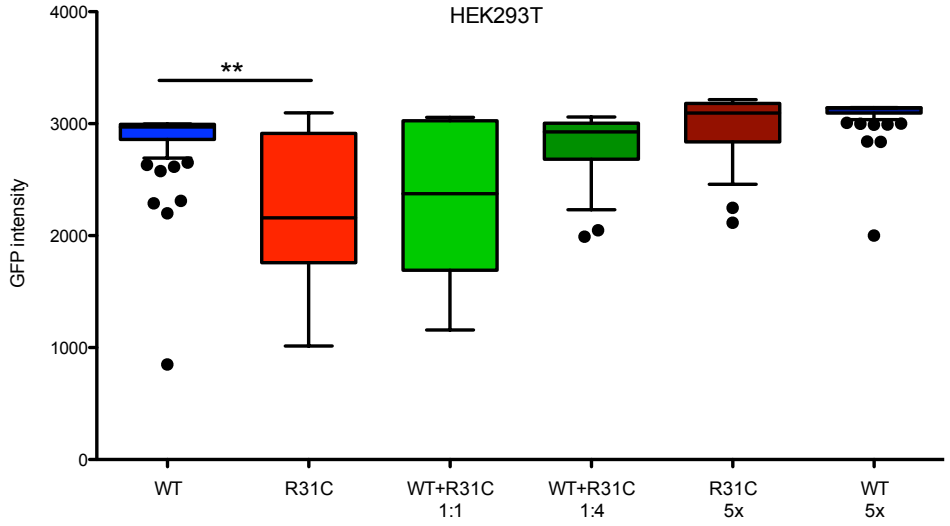


Figure 15 – GFP intensity in HEK293T cells.
Cells were seeded in 6-well plates and transfected by either 75 ng WT GNRH1 pCMV6, 75 ng R31C, 75+75 ng WT+R31C, 75+300 ng WT+R31C, 375 ng R31C or 375 ng WT plasmids with Lipofectamin3000™ method. The day after, living cells were visualized on the Olympus Fluorescent Microscope (see Methods). Pixel quantification was normalized over background. The same exposition duration was used for each acquisition. Pixel brute quantification was drawn in all visible GFP-positive cells in the microscopic field by Cell^F software. Data are expressed as Box & Whiskers (10°-90° centile). **p<0.01.

The *GNRHR* mutation T269M was classified as deleterious by SIFT, PolyPhen-2 and PANTHER. Binding and competition studies showed a complete absence of radioiodinated GnRH signal in COS-7 cells after transient transfection with T269M construct when compared to WT *GNRHR* (Fig. 17).

Similarly, dose-response curve of IP accumulation was completely abolished after treatment with logarithmic doses of GnRH (Fig. 18).

ERK1/2 phosphorylation was very low 5 min after 10^{-8} M GnRH administration in HEK293T cells transfected with the mutant receptor compared to those expressing WT *GNRHR*. No change in pMEK1/2 or pAKT activation was observed after 5 min of GnRH-I exposition (Fig. 19). Subcellular protein extraction revealed the presence of Flag-coupled T269M mutant in membrane fractions (Fig. 20). In transiently-transfected 293 cells no increase of luciferase activity coupled to serum responsive elements was detected after graded doses of GnRH ligand exposition in T269M transfected cells, in comparison to WT *GNRHR* (Fig. 21).

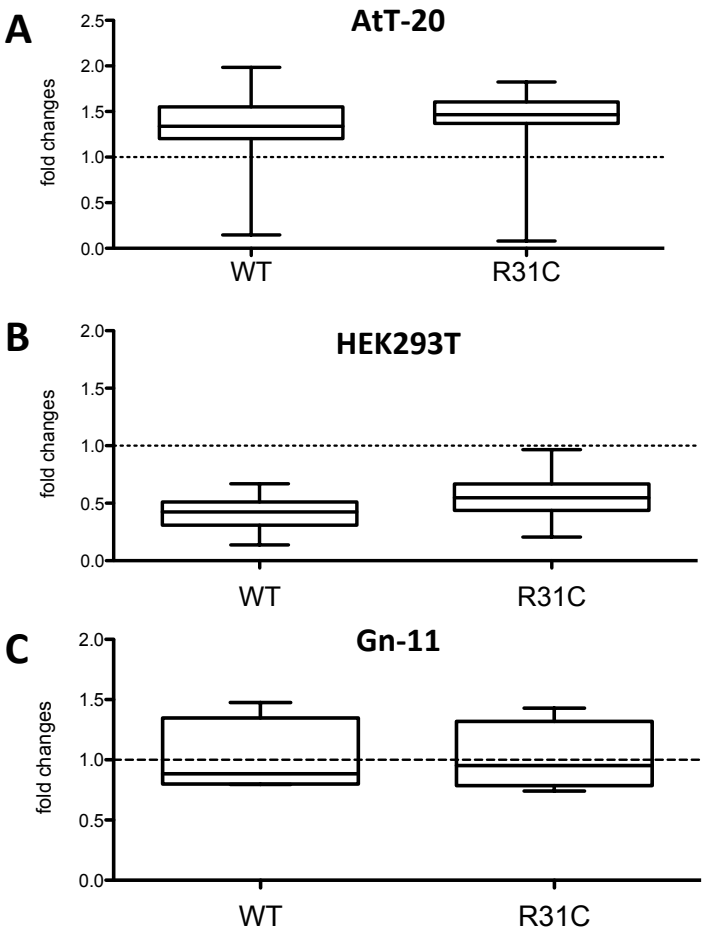


Figure 16 – Cell viability by MTT method in cells transfected with GNRHs.
Att-20 (A), HEK293T (B) and Gn-11 (C) cell lines were transfected with 35 ng/ well wild-type (WT), mutant (R31C) *GNRH1* or empty pCMV vector in a 96-well plate by Lipofectamin3000™. 48h after transfection, cells were exposed to MTT salt + serum, and finally dissolved in isopropanol. Absorbance was measured at 570 nm by Victor (PerkinElmer, see Methods).
Data are expressed as fold changes over controls (dotted line) of median values (Box & Whiskers, min-max). This experiment was conducted n=4 times in 12-plicates.

The *GNRHR* mutation Y290F was predicted to be damaging by SIFT, PolyPhen-2 and PANTHER. Binding studies showed a dramatic reduction of GnRH radioiodinated signal in transiently-transfected COS-7 cells compared to WT *GNRHR* (Fig. 17).

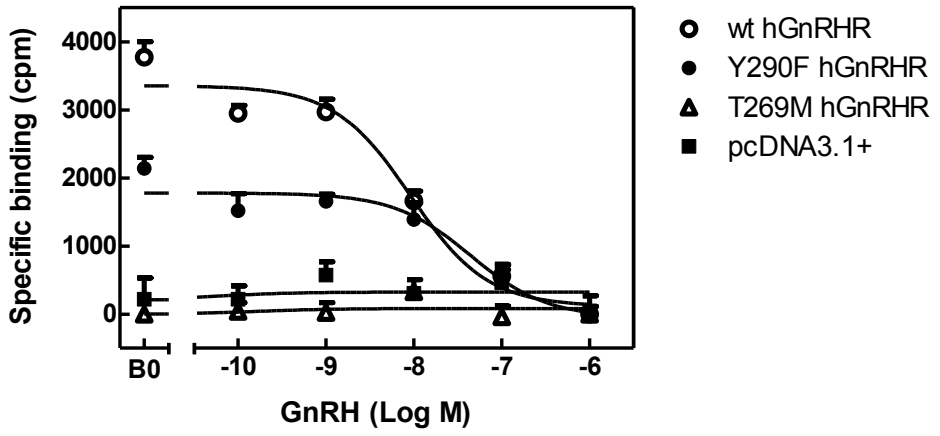


Figure 17 – Binding of GnRH-I to WT and mutant GnRH receptors (hGnRHR). COS-7 cells were transiently transfected with wild-type (wt), Y290F, T269M human GnRH-R DNA constructs or empty pcDNA3.1+ vector and incubated with a ^{125}I-labeled GnRH agonist in the presence of graded concentrations of GnRH-I. Data are means and SEM of a single experiment performed twice in triplicate.

A dose-response curve showed a weaker induction of IP accumulation when compared to WT *GNRHR*-transfected cells. Apart from higher EC-50, also the Emax was consistently lower than that induced by WT-*GNRHR* transfection (FIG. 18). GnRH-induced ERK1/2 phosphorylation was lower in Y290F-transfected 293 cells than in those expressing WT GnRH receptor, with no changes in pMEK1/2 or pAKT (FIG. 19). Fractionated protein extraction revealed the presence of Flag-tagged Y290F in membrane fractions, similarly to WT *GNRHR* (Fig. 20).

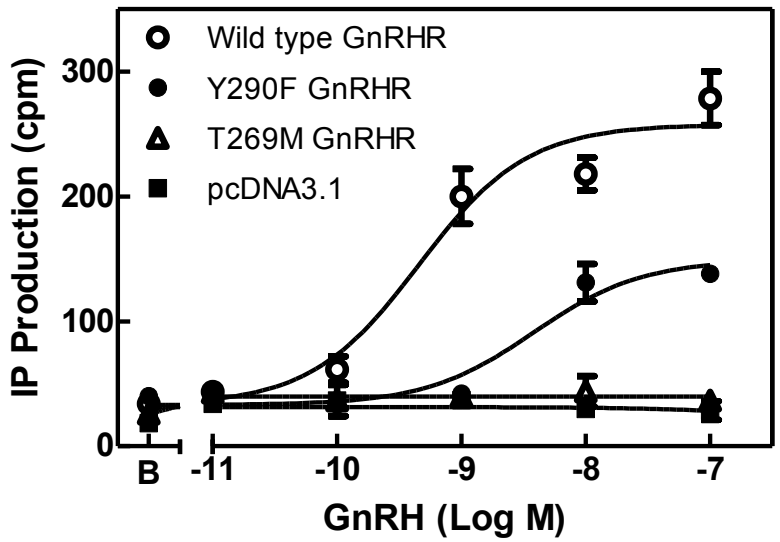


Figure 18 – Intracellular inositol phosphate (IP) accumulation.
COS-7 cells were transiently transfected with Wild-type, Y290F, T269M human GnRH-R or empty vector (pcDNA3.1+) and washed before the addition of myo-[2-^{3}H]Inositol. After 16 hours cells were washed and incubated for 1 hour with graded concentrations of GnRH-I. This experience was conducted three times each in triplicate.

A blunt response in terms of SRE-coupled luciferase activity was noted only at the highest GnRH doses, with a remarkable reduction of EC-50 (18 *vs* 1.9 nM, respectively, $p<0.001$) and especially of Emax ($p<0.0001$) compared to WT-transfected cells (FIG. 21).

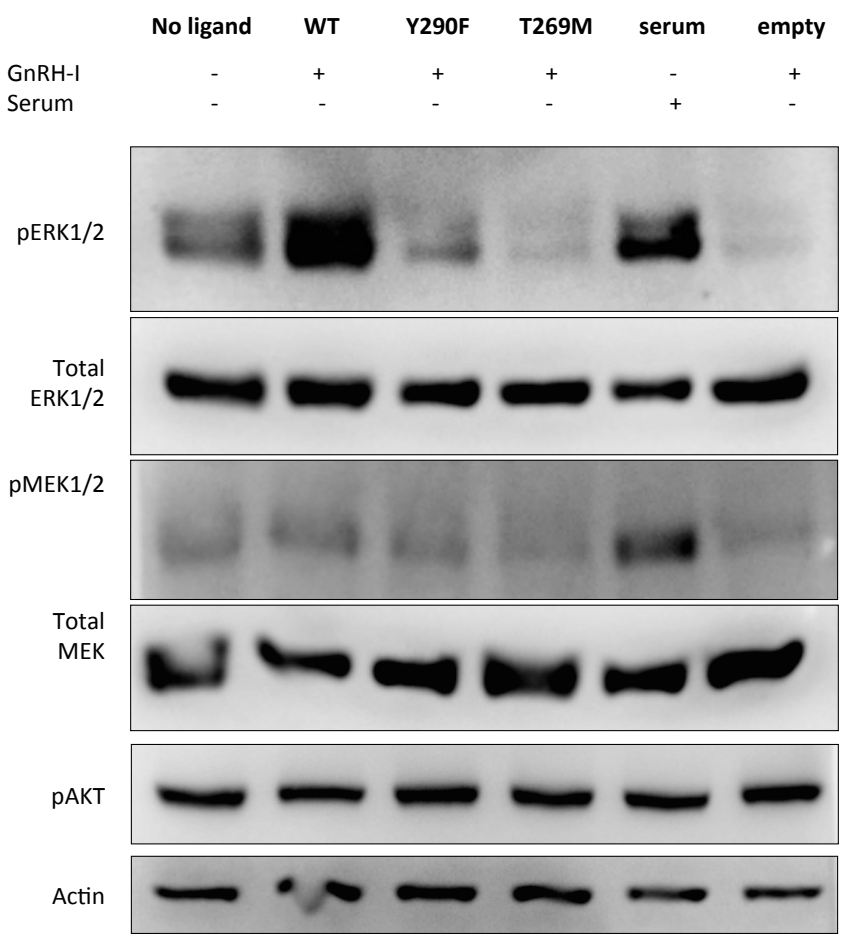


Figure 19 – Signaling of different GnRHRs by Western Blot.
HEK293T cells were transfected by either wild-type human GNRHR-pcDNA3.1+, or same doses of mutant Y290 and T269M GNRHR, or empty vector. GnRH-I was administered at 10 nM for five minutes, then cells were washed and lysated as described in Methods' Section. This experiment was conducted four times.

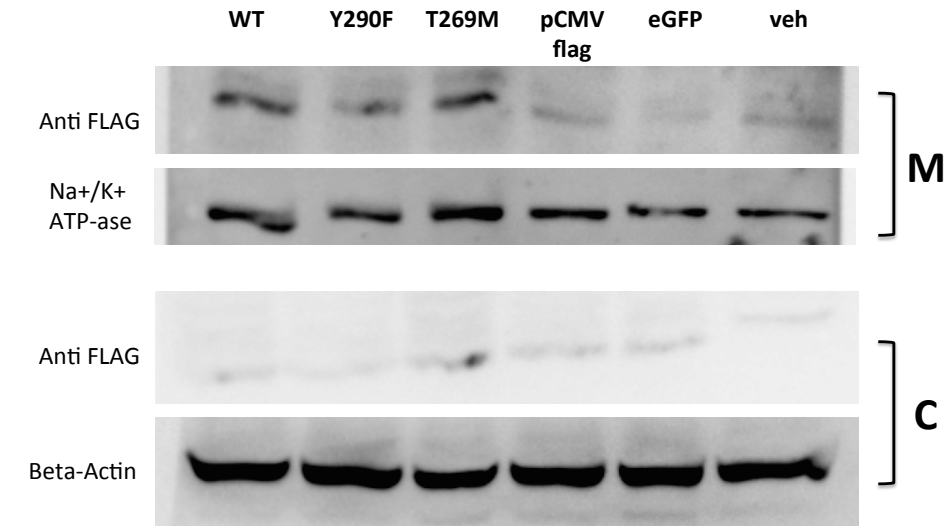


Figure 20 – GnRHR-Flag by Western Blot of subcellular protein extracts
HEK293T were transiently transfected with plasmid containing human WT, Y290F, T269M constructs, pCMV-Flag or eGFP. Proteins from membrane (M) or cytoplasm (C) fractions were differentially extracted through the dodecyl-beta-maltoside and centrifugation method (see Methods). WT: wild type; eGFP: green-fluorescent protein pCMV plasmid; veh: vehicle (non transfected).

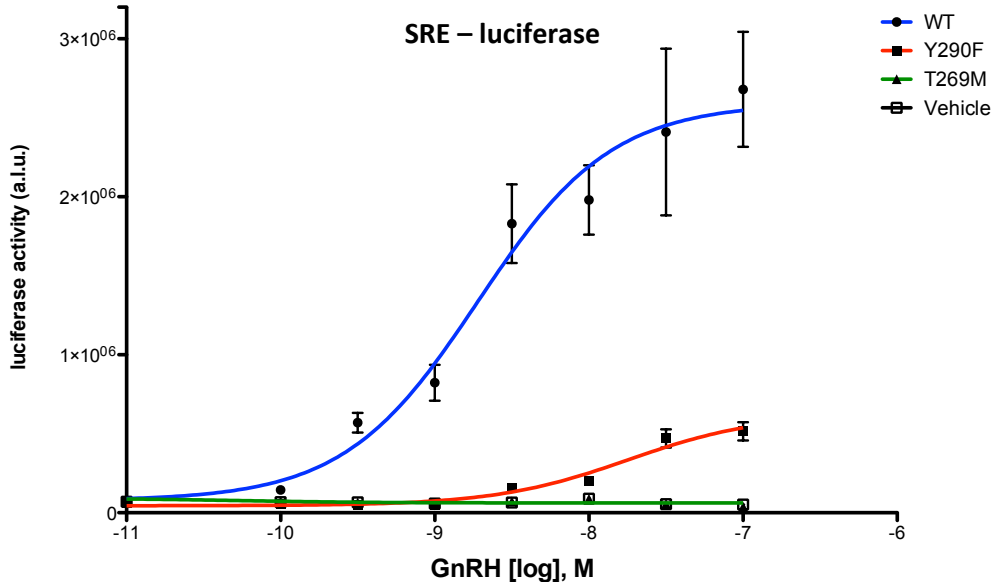


Figure 21 – SRE-coupled luciferase assay activated by different GNRHRs.
HEK293T cells were transfected by wild-type, Y290F, T269M human GnRH-R or empty pcDNA3.1+ vector, and exposed the day after to graded concentrations of wild-type GnRH-I during five hours. Luciferase activity arbitrary units by luminometry (a.l.u.) are shown as ratio on beta-galactosidase activity by optical density used as transfection efficiency internal control. This experience was conducted six times.

C. Clinical characteristics of CHH patients harboring *GNRH1* and *GNRHR* mutations

The *GNRH1* mutation c.77A>G (p.W26X) was present in two sisters (Fig. 3) originating from France. Their mother originated from continental France, whereas their father was born in Martinique (Béké Caucasian group). Clinical inquiry of their parents was unremarkable. At clinical examination, there were no associated signs except for anosmia. The clinical picture, dominated by primary amenorrhea, absent puberty and anosmia, evoked a typical Kallmann Syndrome. MRI supported this diagnosis by showing the classical rhinencephalic abnormalities. No other associations were found at clinical examination. At the time of visit they were 32 and 28 years old (patients 1 and 2, respectively) and regularly received estro-progestins. One of them conceived a healthy daughter after several cycles of GnRH pump.

The R31C *GNRH1* mutation was found in two unrelated French families with nIHH. In both cases mutation was in heterozygosis.

In patient 3, nIHH was sporadic (see Results, Section A). The boy presented at 19 years old with a failure to progress throughout puberty. At physical examination he had a partial pubertal development with 12-mL bilateral testes volume, and no olfactory impairment. Hormone assays revealed hypogonadism in the absence of any secondary cause (see Table 2). In patients 4 and 5 the *GNRH1* mutation segregated with disease. The boy had a partial form of CHH with a 10-12 mL testis volume, low serum testosterone and low gonadotropins, with a P3 pubertal stage. He had no anosmia. His mother had primary amenorrhea and conceived after ovarian stimulation, although diagnosis was established only at 65 years of age, when hormone assays revealed inappropriately low gonadotropins in regard to age.

A clinical reevaluation to assess for nIHH reversal has been tried in patients 3 and 4. After 2 months of HRT withdrawal, testosterone levels remained within the normal range. A longer observation period, however, put in evidence a progressive insufficiency of gonadotrope axis (see Table 3). Both patients were instructed on their condition of oscillatory and relapsing form of nIHH and definitely put under HRT thereafter.

Table 2 – Demographic, clinical and genetic characteristics of CHH individuals reported in this study.

Index cases (patients)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Phenotype	KS	KS	nIHH	nIHH	nIHH	nIHH	nIHH	nIHH
Gender	F	F	M	M	F	F	F	F
Age at diagnosis	17	13	19	21	65	17	15	21
Familial	+	+	-	+	+	+	+	+/-
Genetic analyses								
GNRH1	W26X/+	W26X/+	R31C/+	R31C/+	R31C/+	Normal	Normal	Normal
GNRHR	Normal	Normal	Normal	Normal	Normal	T269M/T269M	T269M/T269M	Y290F/Y290F
Other CHH-associated genes[1]	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Clinical characteristics								
Olfactory status	Anosmia	Anosmia	Normosmia	Normosmia	Normosmia	Normosmia	Normosmia	Normosmia
Bimanual synkinesis	-	-	-	-	-	-	-	-
Midline defects	-	-	-	-	-	-	-	-
Hypoacusia	-	-	-	-	-	-	-	-
Renal abnormalities	-	-	-	-	-	-	-	-
Biochemical data								
LH (mU/mL)	0.1	0.9	0.7	1.2	4.8	0.9	0.5	1.0
FSH (mU/mL)	0.8	1	1.2	1.5	3.2	1.2	0.9	1.1
LH peak (mU/mL)								
FSH peak (mU/mL)								
LH pulsatility	NA	+	-	-	NA	NA	NA	NA
Estradiol (pg/mL)	7	10	<2	7	<2	12	<10	<10
Total testosterone (ng/mL)			0.3	0.8	NA	0.2	0.3	0.3
AMH (mmol/L)			NA	1.4	<0.4	NA	NA	NA
Inhibin B (ng/mL)			NA	101	<15			
Ferritin (mcg/mL))			63	56	103			
Morphological data								
Testicular volume (mL)			12	10	-			
Ovarian volume (mL)					<10			
MRI findings	Normal	Normal	Normal	Rathke cyst	Normal	Normal	Normal	Normal

Patients enumerated in numbers are described in the manuscript (see Methods' section). CHH : Congenital Hypogonadotropic Hypogonadism ; KS : Kallmann Syndrome ; nIHH : normosmic isolated Hypogonadotropic Hypogonadism ; MRI : Magnetic Resonance Imaging ; 1 : other sequenced genes were : *KAL1, FGFR1, PROK2, PROKR2, WDR11, KISS1, KISS1R, TAC3, TACR3*. A detailed list of CHH-associated genes is reported in Table 1.

Table 3 – Clinical characterization of three CHH patients harboring the R31C *GNRH1* mutation.

	Patient 3	Patient 4	Patient 5
Clinical data			
Sex	M	M	F
Cryptorchidism	No	No	-
Spontaneous puberty	Yes	Yes	Yes
Spontaneous menarche	-	-	No
Delayed puberty	No	No	No
Signs of reversal	Yes	Yes	Yes
Signs of relapse	Yes	Yes	Yes
Spermatogenesis	NA	Yes	-
Induced fertility	-	-	Yes
Spontaneous fertility	-	-	Yes

Spontaneous puberty was defined by the presence of more-than 1 stage of pubertal development according to Tanner Stage. Reversal hypogonadism was assessed as reported in Raivio et al. NEJM 2004. Relapse was defined as re-occurrence of clinical and biochemical signs of hypogonadism.

The c.806C>T (p.T269M) *GNRHR* mutation has been found in two sisters issued from a consanguineous West-African family. Both sisters had a classical form of nIHH. Pubertal development was blunted in both sisters. At clinical examination the first sister had a B2-P2 pubertal stage at the age of 17. No spontaneous menarche was registered. The second sister had no spontaneous menses, nor a pubertal development at the age of 14 (B1, P1). Uterine bleeding was induced by graded doses of transdermal estrogens first. Addition of progestins in a cyclical administration was given thereafter. No symptoms of sex steroids deficiency were developed overtime. Olfactometry, MRI, ferritin levels and analysis of all other pituitary axes were strictly normal.

The c.869A>T (p.Y290F) *GNRHR* mutation occurred in a girl with nIHH, issued from first cousin parents. She reported having had primary amenorrhea, with no breast development. At the time of visit she was 22 years old and she was already administered with estro-progestins, so that evaluation of pubertal stage as well as reproductive organs morpho-volumetry resulted uninformative. No olfactory impairment was detected by olfactometry. Diencephalic MRI did not reveal abnormalities in pituitary region.

DISCUSSION

GnRH is universally considered as the key player in the neuroendocrine control of the reproductive axis (Millar et al., 2004). Phylogenetic studies by several species' comparison indicated the GnRH-receptor couple as ancient as 500 million years. This couple represents a paradigm for understanding not only the reproductive functions, but also for the great amount of basic science studies addressed to the notions of ligand-receptor interaction and the models conceiving receptor desensitization and intracellular signaling deciphering.

GnRH was discovered following pioneer studies conducted notably by Geoffrey Harris who showed that pituitary stalk interruption in a female rat caused fading of ovarian cycles (Green & Harris, 1949).

In the early seventies, two competing groups published the primary structure of LHRH, isolated on the basis of its LH releasing activity (Burgus et al., 1971; Matsuo et al., 1971). In 1976 Roger Guillemin and Andrew Schally shared the Nobel Prize for "their discoveries concerning the peptide hormone production of the brain".

The decapeptide GnRH is processed in hypothalamic neurons from a 92 amino acid precursor by enzymatic cleavage and is packaged in storage granules that are transported down axons to the external zone of the median eminence. The peptide is released in synchronized pulses every 60-120 minutes from the nerve endings of about 1000 neurons into the hypophyseal portal system to stimulate the biosynthesis and the secretion of LH and FSH from pituitary gonadotropes.

The amino acidic sequence of the GnRH receptor was first deduced for the mouse receptor cloned from the pituitary αT3 gonadotrope cell line (Tsutsumi et al., 1992). GnRH receptor has the characteristic features of GPCRs. The NH2-terminal domain is followed by seven α-helical TM domains connected by three extra-cellular domains and three intracellular loop domains (Sealfon et al., 1997). The extracellular domains and superficial regions of the TMD are involved in peptide binding, and the TMD are believed to be involved in receptor configuration and conformational change associated with signal propagation. The activation of GnRH receptor thus is thought to propagate into conformational changes in the

intracellular domains involved in G proteins interaction and other proteins for intracellular signal transduction.
A unique feature of the mammalian type-I GnRH receptor is the absence of a carboxyl-terminal tail, that is present in all other GPCRs and in all of the nonmammalian and mammalian type-II GnRH receptors. This is, therefore, a recently evolved feature that presumably serves an important role in the functioning of the mammalian GnRH receptor.

In this work we conducted a comprehensive genetic, clinical and functional study on new variants occurring within the *GNRH1* and *GNRHR* genes in order to unravel the mechanism of disease in the affected patients, and also to better improve knowledge and understanding of ligand-receptor interactions. The genetic screening revealed ten variants within the coding regions of *GNRH1* and *GNRHR* genes from 16 different CHH patients (Table 2, Figures 3 and 4). We focused on eight individuals with novel or yet uncharacterized variants. All these patients but two sisters suffered from nIHH, confirming that mutations in *GNRH1* and *GNRHR* determine an impairment of GnRH-dependent signaling, without any disruption of the migratory route of GnRH neurons, and thus without associated phenotypes, notably smell defects.

Patients 1 and 2 (family 1) were affected by KS. The nucleotide base change provides a stop codon within the decapeptidic sequence of pre-pro-GnRH, completely empeding the achievement of a normal neuropeptide. We considered that a functional analysis of mutant *GNRH1* was not needed to demonstrate detrimental effects. Given the heterozygous state, we went further on searching for another contra allelic event at *GNRH1* locus. This was excluded by an exhaustive analysis of the entire sequence of intron 1 and of the upstream and downstream *GNRH1* promoters. A further confirmation of the mono allelic event was obtained after direct sequencing of PMNC-derived *GNRH1* cDNA. Indeed, we found the coexistence of WT and W26X transcripts in PMNC from peripheral blood. Despite the deleterious character of this substitution, there are at least three arguments arguing against a causative role in determining disease in these individuals. First, the heterozygous state is not in line with a causal role for CHH. *GNRH1* has been classically associated

with an autosomal recessive form of CHH (Bouligand et al., 2009; Chan et al., 2009). Secondly, the same mutation is carried by the unaffected father. Finally, the probands 1 and 2 are affected by KS, that is in contrast with the obvious phenotype of nIHH, as is the case for the *GNRH1* mutations described to date. In the presence of a normal carrier (father) it is unlikely that this mutation might have impaired the normal differentiation of GnRH neurons and ultimately produced a clinical picture resembling KS. The hypothesis most likely complying with the clinical evidence might lie in a second genetic event that could have occurred to cause KS in this family. It is debatable whether this hypothetical event could determine a complete phenotype itself, or might have contributed in synergy with the *GNRH1* event. Sangers sequencing of the main genes associated with KS in women (*FGFR1, FGF8, PROK2, PROKR2* and *WDR11*) did not reveal abnormalities. It is possible that other genes associated with KS, or other not-yet identified genetic events could have acted in these patients to determine disease. Next-generation sequencing techniques, as well as chromosomal micro-rearrangements could also be useful to unravel the causes of KS in these patients.

For the families 2 and 3 (patients 3, 4 and 5) we found the heterozygous R31C *GNRH1* mutation. The implication of this mutation in determining nIHH was first hypothesized by several arguments: first of all, this variant segregates with disease in one family and is *de novo* in the other; in addition, at least two previous reports describing nIHH cohorts reported the R31C mutation in heterozygosis and with an "apparent" dominant inheritance (Chan et al., 2009; Quaynor et al., 2011); third, the R31C mutation has never been identified in controls. More specifically, this nucleotide base change has not been identified in the databases of the Exome Aggregation Consortium, 1000 Genomes Project, dbSNP, or Exome Variant Server. These findings, together with the position of the nucleotide base change within a CpG island and the extreme rarity of *GNRH1* mutations found hitherto, might also suggest for a mutational hot spot. A second contra allelic event was excluded by sequencing the regulatory regions of genomic *GNRH1* and by sequencing the PMNC-derived cDNA coding regions. We also ruled out oligogenism

by sequencing some genes associated with nIHH and Kallmann Syndrome (*GNRHR, KISS1, KISS1R, TAC3, TACR3, KAL1, FGFR1, PROK2, PROKR2*). Other genes known to be associated with CHH (see Table 1) have not been analyzed.
The R31C peptide (cys-8) had very low activity in receptor binding, SRE-luciferase activation, IP accumulation, Ca2+ mobilization, ERK1/2 phosphorylation, lhb gene expression and LH secretion. Nevertheless we were unable to demonstrate any dominant negative effect on WT GnRH activities (further details in (Maione, 2013 #180) appended in the appendix section).

In order to further explain disease mechanism, we addressed a series of experiments to answer to a couple of questions: does the cys-8 mutant have a dominant effect over WT passing through the pulsatile activity of GnRH and the consequent gonadotrope response? Does the pre-pro-GnRH mutated in position 31 have toxic effects on the GnRH neuron itself?
For the first hypothesis, no impairment of WT-induced luciferase activity was observed after four pulsatile GnRH-I administrations given at 90-minute frequency. Variable frequency, amplitude, or longer exposition, not tested in this work, might be needed to produce impairment in WT-induced signaling. In addition, the rate of GnRH-R expression, known to be very low in gonadotrope cells, might influence results and impede the evidence of a fine-tuning dysregulation (Ballesteros et al., 1998; Finch et al., 2009). The *in vitro* approach thus could not properly reflect the pituitary homeostasis and the complex microenvironment studded by various molecular and cellular components. Therefore, the limits of an *in vitro* approach in this context, together with the negative results of pulsatile assays, prompted us to go further and to directly address the second question.
The second hypothesis is based on a putative toxic effect of mutant pre-pro-GnRH, thereby reducing WT GnRH secretion. This question is also theoretically sustained by other forms of dominant diseases related to neuropeptides developing neurotoxic properties after missense mutation, as already demonstrated for pro-dynorphin in ataxia (Bakalkin et al., 2010) or arginine-vasopressin variants in diabetes insipidus (Birk et al., 2009). An appropriate cell model to address this

hypothesis implies the ability of peptide processing that is characteristic of furin-dependent pro-hormone convertase-1 expressing cells. For this purpose we chose murine Att-20 cells, able to process pro-opio-melanocortin into the mature ACTH and MSH using the same convertase as GnRH neurons, and the Gn-11 cells issued from a murine GnRH-neuroblastoma, endogenously expressing low-levels of murine pre-pro-GnRH. Secondly, we had to track protein expression by GFP tagging. Finally we developed a R31C pre-pro-GnRH by site-directed mutagenesis. The number and the intensity of GFP signal was visibly lower after mutant *GNRH1* transfection than after WT alone. In the absence of other nucleotide aberrations after mutagenesis, it is likely that the reduction in transfection rate and in GFP intensity could be attributed to intrinsic characteristics of the mutant peptide.

The question about a negative dominance upon WT would imply a reduction in GFP signal after co-transfection. Expression of WT in combination of R31C at multiple doses did not affect the entity or the amount of GFP-positive cells. In contrast, co-expression of WT+R31C proteins produced a higher GFP signal in all lineages except than in Att-20. It is intriguing the hypothesis that the absence of GFP overexpression, occurring solely in Att-20, could have impaired WT translation in the sole cell type expressing the pro-hormone convertase. However, the link between the reduction in GFP signal and the putative inability to secrete GnRH is not demonstrated, thus remaining elusive. Furthermore, cell viability is not impaired in Att-20 cells transfected with either WT or mutant *GNRH1*. The absence of repercussions in cell viability comes out against toxic effects of R31C mutation, but does not necessarily imply a non-pathogenic effect.

Apart from direct or indirect toxic effects, R31C mutation might have other harmful properties, such as the suppression of a normal protein processing and the secretion of mature peptides. In particular, the cysteine in position 31 of the pre-pro-hormone might favor the formation of disulfide bridges within other precursors or with proteins coupled to the endoplasmic organelles. Covalent bridges by oxidation of cysteines to disulfide bonds may particularly occur in compartments with oxidizing environments, such as the

endoplasmic reticulum in eukaryotic cells (Darby & Creighton, 1995).
In order to investigate this hypothesis, we recently concluded a partnership with the Department of Biochemistry (Federico II University, Naples). Briefly, supernatants from Att-20, Gn-11 and 293T cells transfected with WT, R31C or various doses of WT+R31C *GNRH1* have been collected, passed through a 0.45 um filter and stored at -80°C. These solutions will be partly evaluated by mass spectrometry for quantification of GnRH decapeptides (by comparison to the specter generated by pure peptides). Part of these solutions will be also employed to perform an in-door bioassay. Filtered solutions will be used as conditioned media to treat *GNRHR*-expressing cells in order to elicit the main GnRH-dependent cascades. The effects after treatment with these supernatants will be compared to those observed by pure GnRH peptide administration.

Clinical observations of patients harboring the R31C mutation are also striking, and might sustain translational research to some extent. Indeed, the presence of R31C mutation is always associated with a partial gonadotrope deficiency, and thus a normal activation of gonadotrope axis is somewhat present in these individuals. The two boys with this mutation have normally progressed through the first stages of puberty and spontaneous testis enlargement has always been observed. In parallel, girls harboring the R31C mutation experience menses and may even conceive spontaneously (Chan, 2011). This seems to suggest that, at least in a clinical setting, a chronic or continuous exposure to the mutant allele produces disease overtime. This is of note, since all experimental procedures conducted hitherto have been performed in bolus, or in a short-course pulsatile manner. Apart from *in vitro* assays, and in the absence of *ex vivo* human surrogates, murine or animal transgenic models should be needed to definitely elucidate the pathogenic mechanism.

Patients 6 and 7 from family 4 with nIHH carry a homozygous T269M *GNRHR* mutation. Clinical phenotype completely resumes a typical form of familial nIHH and is thus consistent with genetic findings. The position 269 is completely conserved in mammals, and is located in the 6^{th} TMD, next to the real end of the third intracellular loop.

Changes in position 269 have never been tested from basic scientists, and so a prediction of consequences was never postulate to date. *In silico* prediction tools suggested damaging consequences. The functional characterization of the mutant receptor revealed a complete abolition of GnRH-dependent signaling, with no elevation of the main downstream effectors of signaling cascade. These dramatic effects seem to be particularly due to the absence of GnRH binding, revealed by competition studies. After subcellular protein fractionation, however, the T269M receptor was found in the plasma membrane, as revealed by Flag signal. All of these experiments, therefore, indicate a complete abolition of ligand coupling.

From a mechanistic point of view, this mutation leads to a pituitary disease, with no response to hypothalamic stimulation. In clinical settings, in order to restore fertility, despite the seminal observations about *GNRHR* mutations (de Roux et al., 1997) the GnRH resistance theoretically abolishes the opportunity to use the GnRH pump, that is considered the best tool to produce ovulation and limit the occurrence of the overstimulation syndrome. Additionally, no chances to overcome pathology are envisaged by the utilization of the emerging neuropeptides kisspeptin and neurokinin B (Sonigo et al., 2012; Young et al., 2013).

The patient 8 from family 5 presents a form of nIHH associated with homozygous Y290F mutation in *GNRHR*. The segregation of classical autosomal recessive disorder is strongly evoked by the notion that her parents, unavailable for genetic testing, were first cousins. The clinical phenotype resembles pure nIHH.

Tyr-290 in TMD 6 is highly conserved among GnRH receptors cloned from different species, implying an important role of this residue in receptor folding, ligand binding and/or receptor activation. Tyr-290 is critical for GnRH I and GnRH II binding. Ala substitution of Tyr-290 or changes of residue 5 within GnRH decapeptides both led to 82-332-fold reduction in affinity (Coetsee et al., 2008; Millar et al., 2004). These data are consistent with the direct interaction between the Tyr-290 of the receptor and the Tyr-5 of GnRH. Studies on I-125-cetrorelix competition have shown that Y290F decreased receptor expression on cell surfaces (Hovelmann et al., 2002).

In silico prediction tools indicated damaging effect with high likelihood. The functional characterization of Y290F receptor mutant reveals a strong impairment in PKC/IP and in MAPK signaling, compared to WT. This impairment is partly determined by binding reduction, as revealed by competition studies, thus confirming the crucial role of Tyr-residue to interact with GnRH.
In clinical settings, this mutation produces a classical form of nIHH. The ideal strategy in order to rescue fertility in this girl should be to prevent pituitary resistance, as in the previous case, and to treat with gonadotropins to stimulate ovulation.

In conclusion, our work expands the spectrum of mutations within *GNRH1* and *GNRHR loci* in CHH. Functional analyses have contributed to provide novel insights in clinical settings as well as in the understanding of the GnRH and receptor coupling. The families described throughout this report are of great interest for reproductive sciences. In addition, the pathophysiology of some clinical pictures, like the occurrence of KS in the family 1, or the dominant nIHH in families 2 and 3, is not yet explained by *in vitro* experiments. Genetic variants occurring in key-player actors in the neuroendocrine control of reproduction are contributing to shed more light in this field. The mechanisms by which a molecular alteration could unbalance gonadotrope axis function and produce disease are of great interest, since a wide spectrum of different treatments is now available to overcome the molecular alterations challenging fertility.

Appendix – the development of a sensorineural test applied to CHH.

Patients with CHH usually experience a strong detriment of their quality of life owing to the hard burden generated by pubertal impairment and lack of steroid production, with consequent injury of sexuality in a delicate stage of life. Along with repercussions related to gonadotrope axis insufficiency, KS patients additionally complain anosmia, a serious sensorineural disturbance that further affects choices and activities of daily life. nIHH patients share with KS the sole endocrine alteration without sensorineural impairment. Nevertheless, a subtle reduction of smell function could not be excluded by the sole clinical inquiry. The identification of a slight smell reduction in a not-otherwise classified CHH individual will orientate the diagnostic process more correctly towards a formal diagnosis of KS and will provide important insights in genetic counseling as well as in the search for specific clinical complications. The olfactometry or a morphologic study of brain region by Magnetic Resonance or CT Scan is thus needed to elucidate and improve a correct clinical definition (Maione et al., 2013b).

During my PhD Course, I had the opportunity to work on the characterization of other aspects of sensorineural disabilities in CHH patients. Indeed, patients experiencing olfactory loss complain flavor disturbance and inability in food identification, with serious repercussions on their global quality of life (Schiffman, 1997). By contrast, KS patients generally do not complain flavor dysfunctions (Hasan et al., 2007). The sense of flavor is a complex gustative ability closely related to taste and smell integrity, that ultimately allows food recognition, oral intake identification and the pleasantness and «palatability» of meals. Aim of this study was to explore the sense of flavor in KS and in nIHH patients, compared to individuals with acquired anosmia and normal controls. Since a validated clinical test assessing flavor was not available, an in-house test has been developed in partnership with GIOTTI aromatic manufacturer (Enrico GIOTTI spa, Scandicci, Florence, Italy). Of note, this novel sensorineural test has been the object of a national patent, identified as Flavor Identification Test, application No. FI2014A000229. The details of the test, the development and the assessment of reliability

and reproducibility are part of an already submitted paper, appended to this manuscript.
Briefly, a series of diluted aromatics were blindly administered, and testers were invited to forcedly choose one among several possible answers identifying the flavor. The sum of correct answers generates a highly-reliable score (Flavor Score).
Our results have shown an unequivocal and dramatic injury in flavor perception in KS, as demonstrated by very low scores compared to controls ($p<0.0001$). This disability resumes what observed in acquired anosmics ($p=ns$) and is not observed in nIHH (Fig. 22). Interestingly, KS subjects are not aware of this impairment, and their quality of life is consistently higher than acquired anosmics with respect to smell disorders, probably reflecting the congenital nature of disease. Along with our report, we have shown that KS patients fail to identify oral intakes.
Flavor deficiency has now to be accounted as a specific sensorineural impairment in KS, whose spectrum of associated disabilities is expanding. KS patients should be apprised of their gustative inability in order to prevent nutritional imbalance and the occurrence of life-threatening accidents.

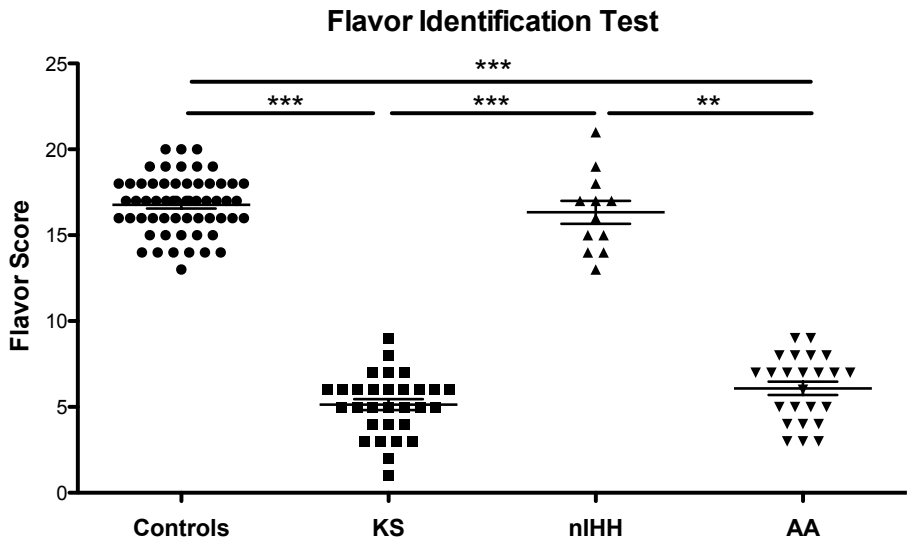


Figure 22 - Flavor test in controls, KS, nIHH and AA patients.
Data are reported as individual values plots. On the y-axis is reported Flavor Score (range 0-21). KS: Kallmann Syndrome; nIHH: normosmic isolated hypogonadotropic hypogonadism; AA: acquired anosmia.
** $p<0.01$; *** $p<0.001$.

ACKNOWLEDGEMENTS

This work, like all things we do with fascination, needs time to be conceived, developed and described. Research activities, in addition, are the ultimate issue of a wider collaborating team, that includes various kinds of experts, teachers, or simply the presence of enthusiastic, friendly people to help, to assist or to share knowledge.

The list of participating people to thank is too ample to be simplistically enumerated in order. This will not give reason and the decent importance to the person/team to whom address these words.

From a cultural and didactic point of view, most thanks are to be given to Jacques (Prof. Young), whose engagement is structurally and institutionally personified in the wide thematic of congenital hypogonadism. Furthermore, the opportunity to study subjects in order to get experience and to follow such delicate patients is a debt with regard to Proff. Sinisi, Bouchard, Pivonello, Macchia, Dr. Albarel, Cerbone, De Brasi, Cavaliere. Prof. Colao, furthermore, has not only trusted in my abilities, but prompted me to apply for a National Patent, and this is to be mentioned.
A remarkable corpus of knowledge in molecular setting is due to Jerome's (Dr. Bouligand) engagement since the time of Master 2. Special thanks in these settings are owed to my direct co-workers in the previous (Drs. Mantel, Lombès, Trabado, Francou, Voican and many others) as well as in the actual (Drs. Macchia, Nettore, Cuomo, De Angelis, Ambrosio, Coppola, Pivonello, Negri, Dentice and many others) laboratory.
The achievement of specific parts of this molecular investigations has been possible thanks to the direct carefulness of scientists able to answer to direct functional questions: Dr. Denoyelle for LH measurements, Dr. Deroussant for his expertise in MS, Dr. Bobe for calcium experiences, Dr. Gallant and Flanagan for binding studies, Prof. Millar for his acute, punctual and at the same time paternalistic tips about the complex ever-evolving field of GnRH.

A kind and thankful think goes to my family, directly or indirectly involved in the progression of my overall activities. Finally, I feel the obligation to thank lots of friends and people who at different titles have interchanged to give their contribution.

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OPEN ACCESS Freely available online

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R31C *GNRH1* Mutation and Congenital Hypogonadotropic Hypogonadism

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Abstract

Normosmic congenital hypogonadotropic hypogonadism (nCHH) is a rare reproductive disease leading to lack of puberty and infertility. Loss-of-function mutations of *GNRH1* gene are a very rare cause of autosomal recessive nCHH. R31C *GNRH1* is the only missense mutation that affects the conserved GnRH decapeptide sequence. This mutation was identified in a CpG islet in nine nCHH subjects from four unrelated families, giving evidence for a putative "hot spot". Interestingly, all the nCHH patients carry this mutation in heterozygosis that strikingly contrasts with the recessive inheritance associated with frame shift and non-sense mutations. Therefore, after exclusion of a second genetic event, a comprehensive functional characterization of the mutant R31C GnRH was undertaken. Using different cellular models, we clearly demonstrate a dramatic reduction of the mutant decapeptide capacity to bind GnRH-receptor, to activate MAPK pathway and to trigger inositol phosphate accumulation and intracellular calcium mobilization. In addition it is less able than wild type to induce *lh-beta* transcription and LH secretion in gonadotrope cells. Finally, the absence of a negative dominance *in vitro* offers a unique opportunity to discuss the complex *in vivo* patho-physiology of this form of nCHH.

Citation: Maione L, Albarel F, Bouchard P, Gallant M, Flanagan CA, et al. (2013) R31C *GNRH1* Mutation and Congenital Hypogonadotropic Hypogonadism. PLoS ONE 8(7): e69616. doi:10.1371/journal.pone.0069616

Editor: Andrew Wolfe, John Hopkins University School of Medicine, United States of America

Received February 11, 2013; **Accepted** June 11, 2013; **Published** July 25, 2013

Funding: This work was supported in part by grants from Paris-Sud 11 University (Bonus Qualité Recherche 2009, Attractivité Univ. Paris Sud 2010), PHRC HYPOPROTEO P081212 and Fondation pour la Recherche Médicale. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The gonadotropin-releasing hormone (GnRH) is essential in mammalian reproduction. This decapeptide, released from hypothalamic GnRH-neurons, triggers an intracellular cascade involving IP3 accumulation, calcium mobilization and MAPK phosphorylation through its cognate receptor GnRHR. The activation of these signaling pathways ultimately stimulates the synthesis and secretion of gonadotropins (LH and FSH) by pituitary gonadotrope cells.

The decapeptide sequence is conserved among most mammals and the amino and carboxyl termini are conserved in mammals and invertebrates [1–4].

Mutations of the human *GNRH1* gene, encoding a 92 amino-acid pre-pro-GnRH, are a very rare cause of normosmic congenital hypogonadotropic hypogonadism (nCHH). A frame shift resulting in a failure to translate the GnRH peptide sequence gives rise to nCHH with autosomal recessive inheritance [5]. Subsequently a p.R31C *GNRH1* mutation in which arginine is substituted by cysteine has been described [6,7]. This is the sole mutation affecting the GnRH decapeptide sequence. The arginine in position 8 of the GnRH decapeptide has been shown to be crucial for biological activity [2–4] and shown to interact with an acidic residue in the mouse [8] and in the human [9] GnRH-Rs. This mutation, though identified in two nCHH families in two independent series [6,7], has not been characterized. In these families, the somehow afflicted individuals were heterozygous. This observation is surprising as the frame shift *GNRH1* mutation only resulted in nCHH in homozygous patients [5].

Here we report on the identification of p.R31C mutation in three individuals in two additional unrelated nCHH families. All the individuals are heterozygous for the mutation. We have undertaken a comprehensive molecular characterization of the

mutation in order to understand the mechanism of nCHH in these individuals.

Results

Genetic analysis

We identified the *GNRH1* c.91C>T (p.R31C) mutation in two unrelated French families with nCHH. Demographic, clinical, biological and genetic data are reported in Table 1. Interestingly, the two pedigrees are very different in terms of presentation (Fig. 1). In family 1, nCHH is sporadic. The boy (II.1) presented at 19 years old with a failure to progress through puberty. At physical examination he had a partial pubertal development with 12-mL bilateral testes volume. He had no olfactory impairment. Hormone assays revealed very low testosterone levels and low gonadotropin levels. No secondary causes were found for central hypogonadism (see Table 1). *GNRH1* heterozygous mutation in this boy was *de novo*, as ascertained by micro-satellites analysis.

In the second family the *GNRH1* mutation was present in heterozygosis and segregated with disease. The boy (II.1) was diagnosed having hypogonadotropic hypogonadism because of small testis volume (10 mL at left, 12 mL at right testis), low serum testosterone and low gonadotropins. His pubertal stage was P3 according to Tanner. He had no anosmia. Common causes of secondary hypogonadism were excluded. His mother (I.2) was affected by primary amenorrhea, and conceived after ovarian stimulation by exogenous gonadotropins, although a formal diagnosis was not established at that time. She was re-evaluated later at the age of 65, and hormone assays revealed low sex steroids accompanied with inappropriately low gonadotropins. She had no other apparent secondary causes (see Table 1). The father (I.1) was not available for genetic analysis.

We did not identify a second genetic event after genomic regulatory region analysis of *GNRH1* locus and *GNRH1* cDNA sequencing in all *propositii*.

Predictive analysis

The *GNRH1* c.91C>T nucleotide substitution did not create any donor or acceptor aberrant splice site according to prediction tools with Alamut® software. This nucleotidic substitution induces a missense at codon 31, replacing Arginine-31 by a Cysteine in the pre-pro-GnRH (*GNRH1* p.R31C).

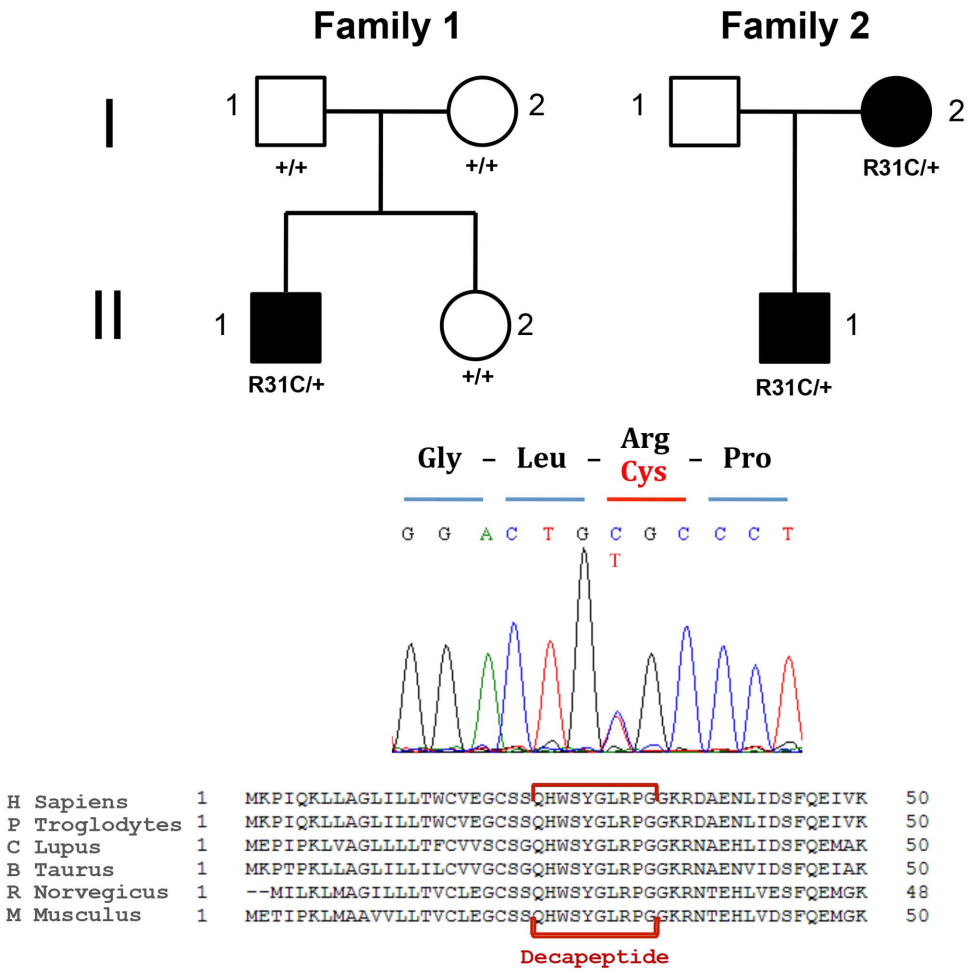

Figure 1. Pedigree of nCHH families carrying c.91C>T (p.R31[R, C]) *GNRH1* mutation. In the family 1, the *propositus* (II.1) has a *de novo* mutation and his filiation has been confirmed by DNA microsatellites. In the family 2 the mutation was transmitted from the mother (I.2) to her son (II.1). She required medical assistance for procreation. Clinical and demographic data of all patients are reported in Table 1. Electropherogram represents the heterozygous mutation in the individual II.1 from family 1. In the panel below, pre-pro-GnRH amino acid sequence conservation. Decapeptide is shown in red.
doi:10.1371/journal.pone.0069616.g001

Table 1. Clinical, biological and genetic characterization of patients.

	II.1 (Family 1)	II.1 (Family 2)	I.2 (Family 2)
Clinical data			
Sex	M	M	F
Age at diagnosis	19	21	65
Total testosterone (ng/mL) *	0.8	0.77	NA
Estradiol (pg/mL) *	NA	7	<12
LH (mU/mL) *	1	1	2.8
FSH (mU/mL) *	0,9	1.4	5.8
LH pulsatility	NA	Absent	NA
AMH (mmol/l) *	NA	1.4	<0.4
Inhibin B (ng/mL) *	NA	101	<15
Ferritin (mcg/mL)	63	56	103
Testicular volume (mL)	12	10	-
Ovarian volume (mL)	-	-	<10
Pituitary and olfactory IRM	Normal	3-mm Rathke's cyst	Normal
Olfactometry**	Normal	Normal	Normal
Associated features	None	None	None
Genetic data			
GNRHR1	normal	normal	normal
KISS1	normal	normal	normal
GPR54	normal	normal	normal
PROK2	normal	normal	normal
PROKR2	normal	normal	normal
FGFR1	normal	normal	normal
FGF8	normal	NA	NA
NELF	NA	normal	normal
TAC3	normal	normal	normal
TACR3	normal	normal	normal
GNRH1 promoter	normal	normal	normal
GNRH1 exon 1	normal	normal	normal
GNRH1 intron 1	normal	normal	normal

NA: not available.

*Reference ranges for hormone levels and method characteristics. Total testosterone: 3.5–8.5 ng/mL for adult men (RIA, Orion Diagnostica device, Spectria®, Espoo, Finland; detection limit: 0.01 ng/mL; intra- and interassay coefficients of variation (CVs): 3.2% and 4.6%); estradiol: 15–35 pg/mL for men, <16 for post-menopausal women (RIA, Orion Diagnostica, Spectria®; detection limit: 2 pg/mL; intra- and the interassay CVs: 2.8% and 5.8%); LH: 1.4–8 mU/mL for men, >30 mU/mL for post-menopausal women; FSH: 1.4–10 mU/mL for men, >30 mU/mL for post-menopausal women (for LH and FSH: Immunotech device, Beckman Coulter, Praha, Czech Republic; detection limits: 0.1 IU/L for both FSH and LH; intra- and interassay CVs: <6.3% for FSH, <6.7% for LH); AMH: 22–38 pmol/L for men, 14–48 for women (enzyme immunometric assay, Immunotech reagents, Beckman Coulter Company, Marseille, France; detection limit: 0.4 pmol/L; intra and interassay CVs: <12.3% and <14.2%,); Inhibin B: 80–327 pg/mL for men, <20 pg/mL for post-menopausal women (enzyme immunometric assay, Oxford Bio-Innovation reagents, Serotec, Oxford, UK; detection limit: 15 pg/mL; intra- and interassay CVs: 4.2% and 10.2%,).

**Subjective olfactometry was performed by a computed-assisted validated test [35].

doi:10.1371/journal.pone.0069616.t001

Prop v.1.0b predicted that this missense did not abolish proconvertase-dependent cleavage site for pre-pro-GnRH maturation into the decapeptide (amino-acids 24 to 33).

Alignment with orthologs revealed conservation of arginine in position 8 of GnRH in mammals (Fig. 1). This amino acid exchange was classified as deleterious by various *in silico* prediction tools (see methods section). More importantly, experiments on the substitution of arginine 8 with a variety of amino acids demonstrate that it is crucial for binding and signaling [10]. Arginine 8 was further shown by mutagenesis studies in the GnRH-R that it interacted with an acidic residue in the extracellular loop three of the mouse (glutamate 301) and the human (aspartate 302) GnRH-Rs [8,9].

In vitro molecular characterization

R31C and wild-type (WT) decapeptides were stable over 24-hours at room temperature in water and culture medium. No dimerization of the mutant decapeptide was found (Fig. S1).

The R31C GnRH peptide bound the GnRH-R with an affinity more than 100-fold lower than that of WT GnRH (Fig. 2A and 2B). Dose-response curves with SRE-luciferase assay showed an almost 100-fold reduction of R31C agonist *versus* WT (Fig. 3A). The EC-50 was 4 nM for WT GnRH and 314 nM for R31C GnRH ($p<0.0001$, Fig. 3B). ERK1/2 phosphorylation was maximal at 5 minutes. R31C GnRH showed similar kinetics, but the degree of phosphorylation was reduced (Fig. 4A).

WT GnRH induced a rapid and transient calcium mobilisation in LbetaT2 cells. Calcium response by R31C was significantly reduced compared to WT in terms of peak and area under the curve ($p<0.05$, see Fig. 5). Pre-treatment with 100 nM GnRH-antagonist cetrorelix abolished responses to both ligands (data not shown). The IP accumulation dose-response curve demonstrated that the R31C GnRH (EC-50, 199 nM) was more than a 100-fold less potent than WT GnRH (0.5 nM), ($p<0.01$, Fig. 6A and B). The maximal IP generated by both ligands was the same indicating that the R31C GnRH peptide was a full agonist, as was found in the SRE-luciferase assay.

WT GnRH significantly increased *lhb* transcript levels in gonadotropes ($p<0.01$), whereas R31C GnRH did not significantly increase these levels over baseline (not significant, Fig. 7A).

WT GnRH strongly stimulated LH secretion over baseline ($p<0.001$). R31C GnRH significantly stimulated LH secretion in LbetaT2 cells supernatants over baseline ($p = 0.042$, Fig. 7B).

The above findings clearly show that in binding, signaling and functional assays, the R31C GnRH is less potent than WT GnRH. Since the heterozygous condition was associated with nCHH in our patients, that could suggest a dominant negative effect, we studied the effects of combining WT GnRH with R31C GnRH.

We hypothesized that the cysteine in position 8 of the R31C GnRH might form disulfide bridges with cysteine residues in the receptor that are not in disulfide bridges. There are three such cysteines: cysteine 218, cysteine 279 and cysteine 317. In order to examine this possibility the effects of R31C GnRH pre-incubation on subsequent binding of a radio-labelled GnRH analog was examined. We determined from the competition binding study that R31C GnRH would occupy 50% of receptors. As a control, we utilized WT GnRH at 10^{-8} M, which also occupies 50% of receptors. After treatment, the cells were extensively washed to remove all non-covalently bound peptide. Subsequent binding of radiolabeled GnRH analog was the same in these treatments as well as in medium-treated cells (Fig. 2B). These findings indicate that both WT GnRH and R31C GnRH can be fully removed from the receptor, indicating that R31C GnRH does not form covalent disulfide bonds with the receptor.

Dose-response curves generated from SRE-coupled luciferase were indistinguishable from WT GnRH administered alone (Fig. 3A).

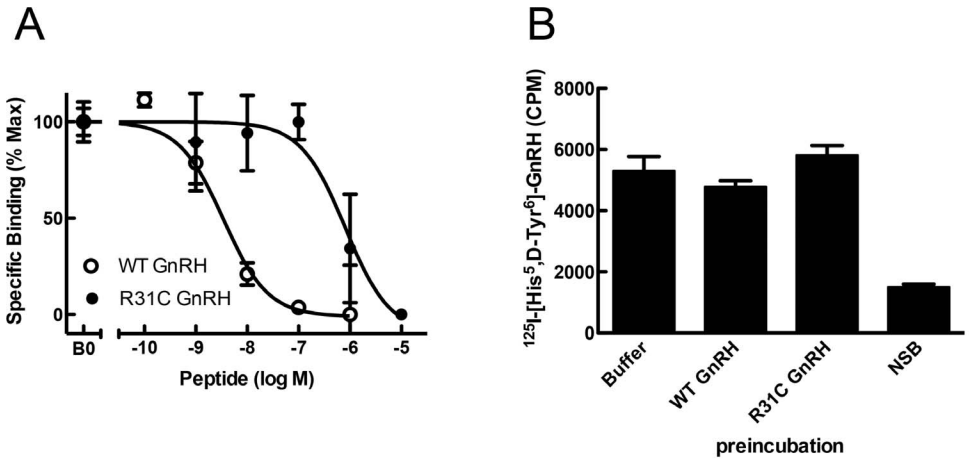

Figure 2. Binding of WT and R31C GnRH to the GnRH receptor (GnRH-R). (**A**) COS-7 cells were transiently transfected with a human GnRH-R DNA construct and incubated with a ^{125}I-labeled GnRH agonist in the presence of indicated concentrations of WT GnRH or R31C GnRH. IC-50 values are WT GnRH, 2.8×10^{-9} M and R31C GnRH, 8.50×10^{-7} M. Data are given as means and SD of two experiments performed in duplicate. (**B**) Transfected COS-7 cells were pre-incubated with buffer alone, WT GnRH (10^{-8} M) or R31C GnRH (10^{-5} M) and then washed to allow dissociation of ligand not covalently attached to the GnRH-R. Pre-incubated cells were then incubated with ^{125}I-labeled GnRH agonist in the absence or presence of saturating concentration of unlabeled WT GnRH (NSB). Data are the means of two experiments performed in triplicate.
doi:10.1371/journal.pone.0069616.g002

After pulsatile administration of each ligand at 90-minute frequency, WT GnRH was more potent than R31C GnRH to induce luciferase activity coupled to SRE reporter gene. Combination of WT and R31C was not able to impair luciferase activity when compared to WT alone (Fig. S2).

Treatment of LbetaT2 and GnRH-R-expressing HEK293T cells with 10 nM WT GnRH in combination with 10 nM R31C GnRH induced similar kinetics of ERK1/2 phosphorylation compared to 10 nM WT alone (Fig. 4A). Increasing the dose of R31C GnRH from 10 nM to 300 nM in the presence of 10 nM WT GnRH did not reduce ERK1/2 phosphorylation at 5 minutes exposure (Fig. 4B). Calcium peak and area under the curve after treatment with the combination of 10 nM GnRH and 10 nM mutant peptide was indistinguishable from WT GnRH alone (Fig. 5). IP dose-response curve with equal concentrations of WT and R31C peptides was indistinguishable from WT GnRH alone

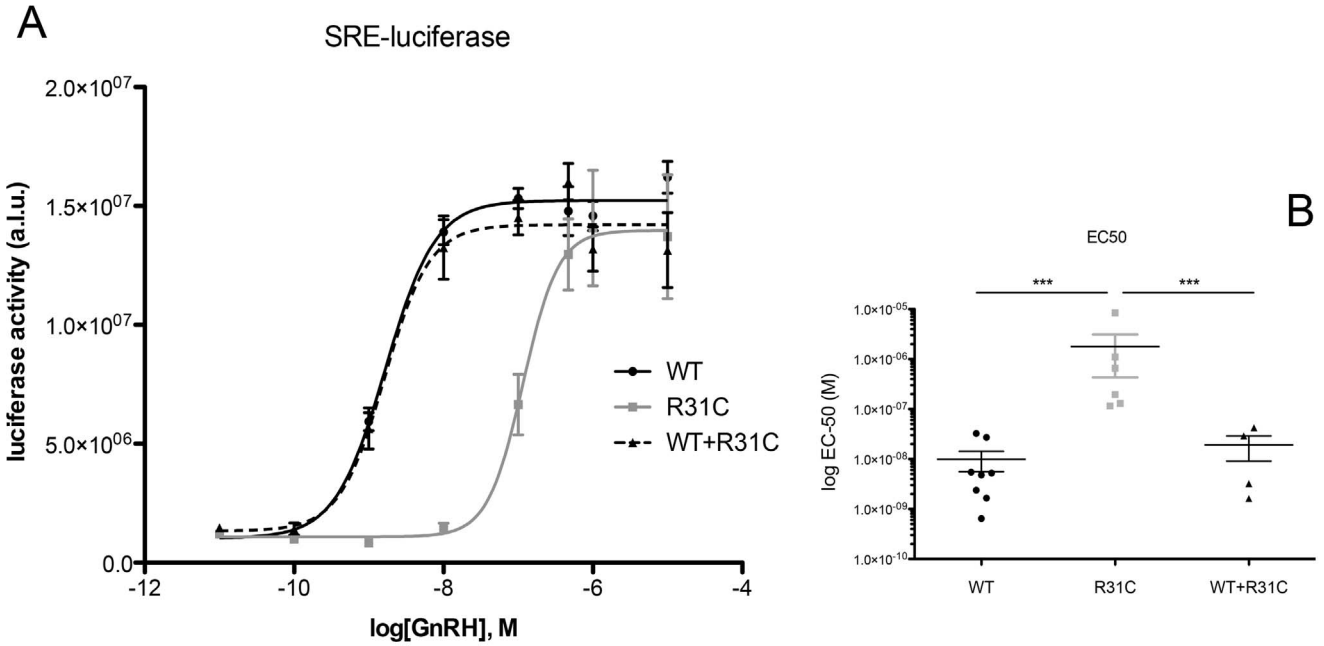

Figure 3. SRE-coupled luciferase assay. (**A**) HEK293T cells were exposed to graded concentrations of wild type (WT) GnRH, R31C and equimolar concentrations of WT + R31C during five hours. Luciferase activity arbitrary units by luminometry (a.u.) have been shown as ratio on beta-galactosidase activity by optical density used as transfection efficiency internal control. This experience was conducted eight times. (**B**) Representation of individual values of EC-50 (calculated with Arcsin "P" root transformation) from n = 8 dose-response experiments. WT GnRH EC-50 was calculated at 4±1.2 nM, R31C EC-50 at 313±130 nM. ***$p<0.0001$.
doi:10.1371/journal.pone.0069616.g003

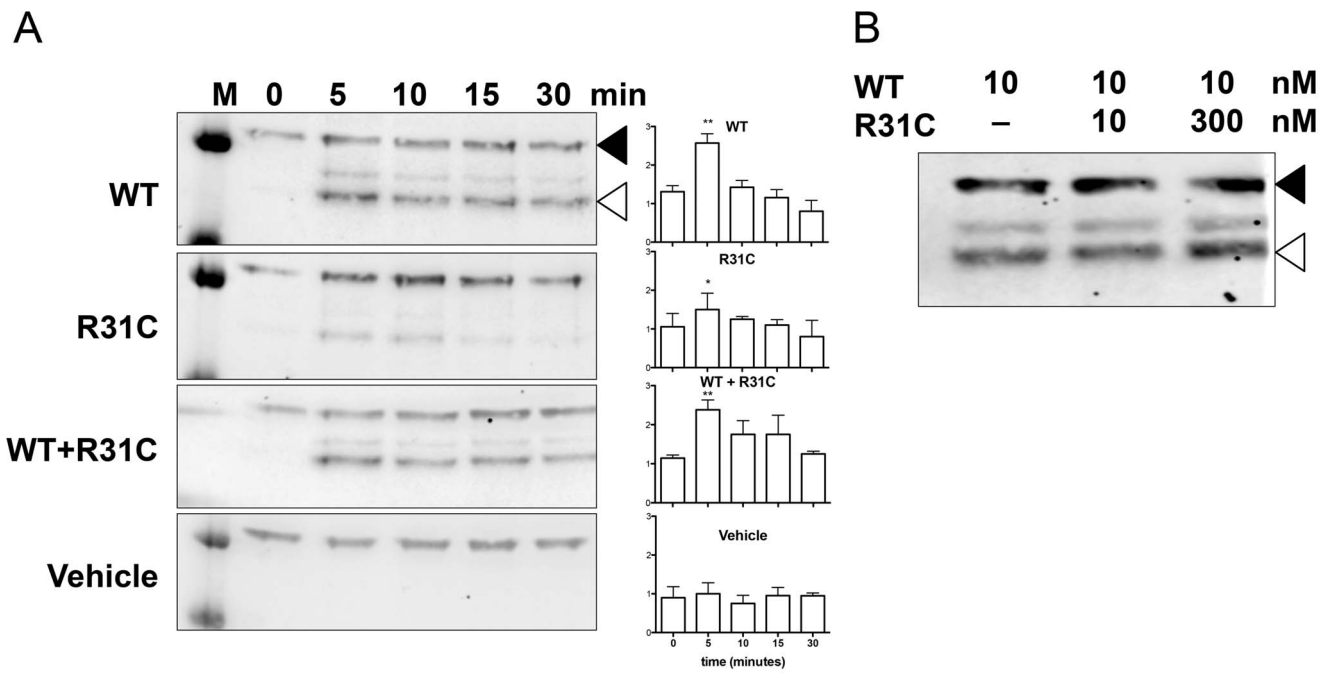

Figure 4. ERK1/2 phosphorylation by Western blot. (**A**) LbetaT2 cells were starved overnight in serum-free DMEM and then treated by 10 nM WT, 10 nM R31C, 10 nM WT +10 nM R31C peptides or vehicle for 0, 5, 10 and 30 minutes. Representative of four experiments. Density quantification is displayed on right-hand, and data are expressed as mean ± SD for four experiments. *p<0.05; **p<0.01 over baseline. (**B**) LbetaT2 cells were starved overnight in serum-free DMEM and then treated by a constant dose of WT GnRH (10 nM) and increasing (0–10–300 nM) doses of mutant R31C peptide after 5 minutes exposition. Lysates were analyzed by Western Blotting using phospho-ERK1/2 antibodies (Cell Signaling®, open arrow) and mouse monoclonal anti alpha-tubulin (Sigma Aldrich®, St. Louis, MO, close arrow). Similar results on ERK intensity and kinetics were obtained using transiently transfected HEK293T cells.
doi:10.1371/journal.pone.0069616.g004

(Fig. 6). This indicates that a full range of doses of R31C GnRH does not influence the ability of WT to bind and activate the GnRH-R. R31C GnRH was also unable to influence the stimulation of *lhb* transcription (Fig. 7A) or LH secretion (Fig. 7B) from LbetaT2 cells by WT GnRH.

Discussion

GNRH1 mutations are a very rare cause of nCHH [5–7,11,12]. The *GNRH1* p.R31C mutation is the only description of an amino acid change in GnRH. Yet this mutation has been described in nine individuals in four separate families. This mutation was first reported in association with nCHH in heterozygosis and a dominant inheritance was postulated [6], contrasting with the previously described recessive transmission of the frame shift mutation, which fails to transcribe the GnRH sequence [5]. Some nCHH individuals with the R31C mutation concomitantly had variations in other nCHH genes perhaps suggesting oligogenism. More recently, the R31C mutation was reported in a girl with no variations in other nCHH and Kallmann Syndrome-related genes [7]. This is of interest since this study analyzed all known genes associated with nCHH and Kallmann Syndrome aiming to establish the prevalence of oligogenism in these syndromes. This mutation was considered not sufficient to explain the phenotype because of the heterozygous inheritance [7]. In the present study, we have found the same mutation in three additional nCHH patients from two unrelated families and unrelated to the previously described families with the R31C mutation. Although sex steroid and gonadotropin levels undoubtedly related to CHH, more-than 4 mL testicular volume in patients at presentation suggested a residual functional activity of hypothalamic-pituitary-gonadal axis, probably reflecting heterozygosity.

The identification of this recurrent mutation in four unrelated families, the finding of a *de novo* event in one family and the location of the nucleotide base change within a CpG islet are three consistent arguments for a mutational «hot spot». As interest, this amino acid is the most variable among vertebrate and invertebrate GnRHs [1–4]. The R31C GnRH mutation has never been identified in controls and segregation with the phenotype has been found in all affected families (see Table 2). Furthermore, this nucleotide base change has not been identified in 5989 individuals from the Exome Sequencing Project (ESP) cohort (NHLBI GO, Seattle, WA; URL: http://evs.gs.washington.edu/EVS; October, 2012). Thus, this implies that this missense mutation is likely responsible for the nCHH phenotype. However, although the R31C GnRH had very low activity in receptor binding, SRE-luciferase, IP, Ca^{2+}, ERK1/2 signaling and in *lhb* gene expression and LH secretion, we were unable to demonstrate any dominant negative effect on WT GnRH activities.

Previous genetic studies established that *GNRH1* loss of function mutations lead to an autosomal recessive nCHH. Thus we first searched for another genetic event at *GNRH1* locus. This was excluded by an exhaustive analysis of the entire sequence of intron 1 present in the hypothalamic primary transcript, the sequence of the upstream and downstream *GNRH1* promoters and the cDNA coding regions [13,14]. We also ruled out oligogenism by sequencing the main genes associated with nCHH and Kallmann Syndrome [15–17], although *WDR11*, *CHD7* and *SEMA3A*, other genes known to be associated with CHH, have not been analyzed. Furthermore, we cannot entirely exclude the contribution of a gene not known to be associated with CHH. Next generation

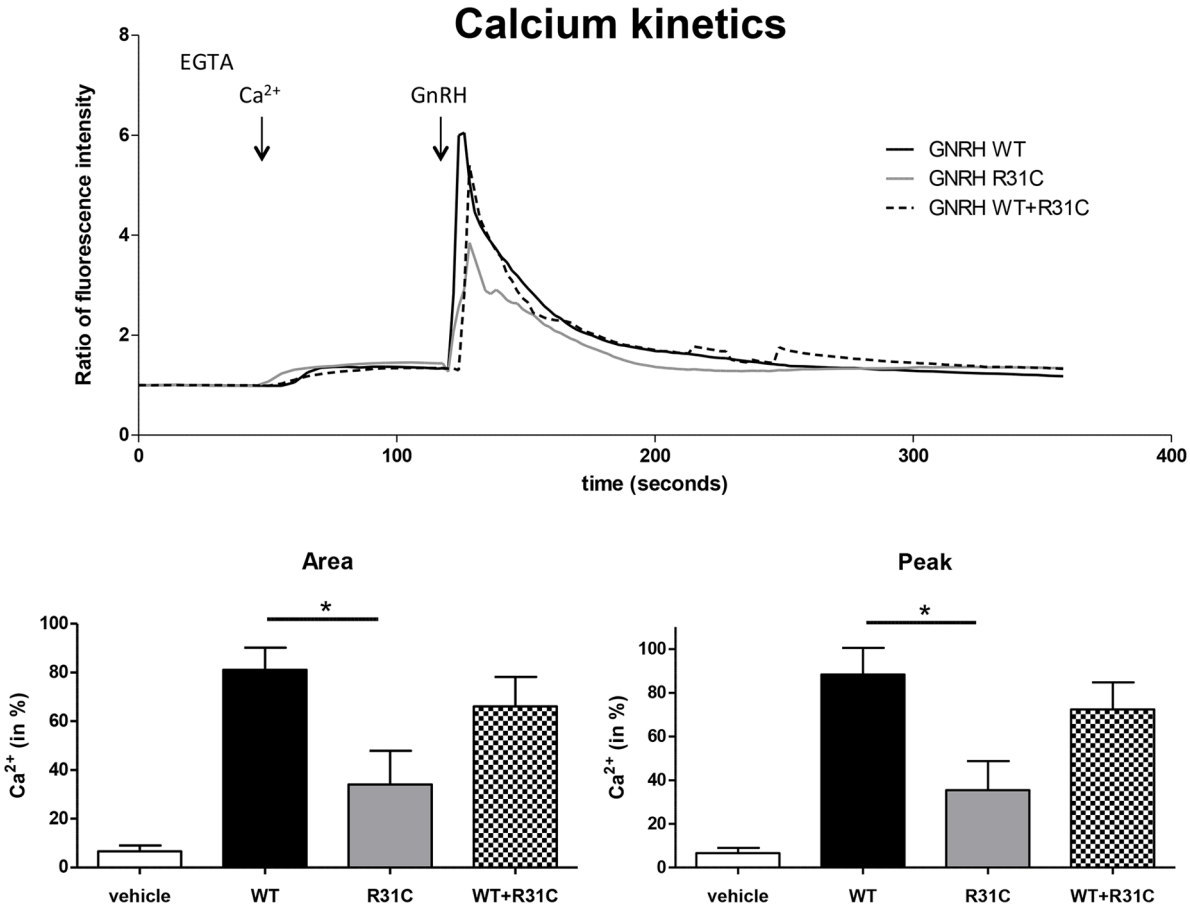

Figure 5. Effect of treatments on intracellular calcium mobilization. Cells were loaded with Fura 2-AM. At the time of the experiment 100 μM EGTA was added 30 sec before addition of 300 μM of $CaCl_2$ (Ca^{2+}) to the medium as indicated, and cells were then stimulated with 10 nM of wild type (WT), mutant (R31C) or equimolar combination (10 nM WT +10 nM R31C) or vehicle for 3 min. In the upper panel, representative curves of single cell recording. Each experiment mean was analysed using One-way ANOVA followed by Tukey's Multiple comparison test. In the lower panels, histograms show the means ± SEM (n = 4) of the response to ligands as calcium peak and as area under the curve (AUC). R31C-induced peak and AUC were significantly lower than WT. Combined treatment by WT+R31C did not induce a different calcium peak and AUC compared to WT alone. Representative of at least three independent experiments, *$p<0.05$.
doi:10.1371/journal.pone.0069616.g005

sequencing methods could be helpful to find a possible second genetic defect.

In order to investigate the possible mechanisms whereby R31C GnRH might influence the actions of WT GnRH, as it is apparently the case in the patients, we set about first examining the activity of the R31C GnRH in receptor binding and variety of signaling pathways, as well as its ability to stimulate LH release. We then examined its ability to affect WT GnRH actions in these systems. R31C GnRH had a binding affinity of more than 100-fold lower than that of WT GnRH. Consistent with this observation, R31C GnRH had similar reductions in potency (increased EC-50 values) in stimulation of SRE-luciferase and IP generation. However, R31C GnRH was able to elicit the same maximal stimulation of SRE-luciferase and IP, clearly demonstrating that it is a full agonist in recruiting these signaling pathways. This indicates that it has no antagonistic activity and it is therefore unlikely to explain the phenotype of the heterozygous patients. Supporting these observations, R31C peptide determined weaker responses in contrast to those of WT GnRH on ERK1/2 phosphorylation, Ca^{2+} mobilization, *lhb* transcription and LH secretion.

Further studies confirmed our interpretation that R31C GnRH does not antagonize WT GnRH actions at the receptor level. When R31C GnRH, at concentrations varying from 10^{-10} M to 10^{-6} M, was added in combination with WT GnRH at 10^{-9} M in various experiments, it failed to have any impact on receptor binding, SRE-luciferase, IP generation and Ca^{2+} signaling or on *lhb* transcription and LH secretion. These findings are in accordance with our understanding of structure-activity relations of GnRH analogs and their interaction with the GnRH-R. Arginine 8 is crucial for the correct conformation of mammalian GnRH and for its binding to the receptor [1–4]. In particular arginine 8 has been demonstrated to be crucial for the interaction with the aspartate 302 of the human GnRH-R and the glutamate 301 of the mouse GnRH-R [8,9,18].

The mode of inheritance, apparently dominant in four families, is thus not explained by a simple negative dominance in the parameters that we have measured. There are other possibilities that were not investigated here. Firstly, the mutant peptide may interact with molecules within the GnRH neuron to impair activity or induce toxicity, thereby reducing WT GnRH secretion. Aberrant transcription products might be retained in the endoplasmic reticulum or could act as neurotoxic agents, as already demonstrated for pro-dynorphin in ataxia [19] or arginine-vasopressin variants in diabetes insipidus [20]. Another

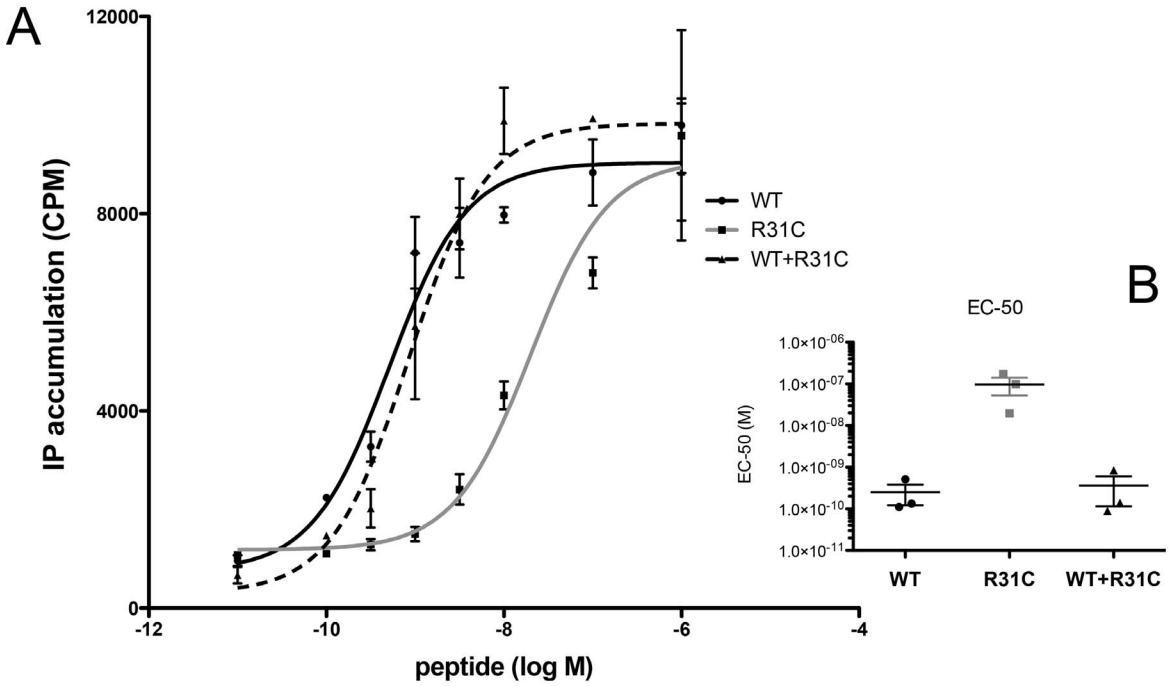

Figure 6. IP accumulation. (**A**) COS-7 cells were transiently transfected with human GnRH-R expression vector and washed before the addition of myo-[2-^{3}H]Inositol. After 16 hours cells were washed and incubated for 1 hour with graded concentrations of WT, R31C and equimolar WT+R31C GnRH. This experience was conducted three times each in triplicate. (**B**) Representation of individual values of EC-50 (calculated with Arcsin "P" root transformation) from n = 3 dose-response experiments. WT GnRH EC-50 was calculated at 0.52±0.22 nM, R31C EC-50 at 198.8±75.9 nM, WT+R31C EC-50 at 0.63±0.42 nM.
doi:10.1371/journal.pone.0069616.g006

possibility is that, in GnRH neurons, the R31C GnRH precursor forms an aberrant intermolecular disulfide bridge with the WT GnRH precursor, which disrupts correct folding of the molecule leading to detection by surveillance proteins and trafficking to lysosomes for degradation of both peptides. In addition, the heterodimer may be resistant to processing.

Although a wide range of cellular models was used, they do not necessarily reflect *in vivo* events. Despite abundant literature, the molecular mechanisms underlying GnRH-R down-regulation and pulse deciphering remain currently poorly understood [21–23]. It is of note that SRE-luciferase activity was not impaired after four pulses at 90-minute frequency (Fig. S2). Nevertheless a slow and progressive loss of pituitary response to mutant GnRH might require a longer pulsatile exposure. In this context, a transgenic mouse model may shed light on the mechanism of the dominant negative effect [24].

In conclusion, together with our report, four nCHH families carrying R31C *GNRH1* heterozygous mutations have been identified. As this is a putative «hot spot» mutation it is likely to be identified in other nCHH families. The families harboring the R31C mutation are of great interest for reproductive sciences since the pathophysiology is not explained by *in vitro* experiments. There is herein an obvious opportunity to study novel aspects of GnRH signaling *in vivo* and/or to identify novel genes modulating GnRH reproductive function.

Materials and Methods

Patients

From a cohort of 410 patients with congenital hypogonadism we screened for a panel of mutations including *GNRH1* (see below). The study was approved by the Paris Sud University Hospital ethics committee and complied with human research guidelines as stated in the Declaration of Helsinki. Patients gave their written informed consent before genetic analysis and hormone studies.

Hormone assays

Serum LH, FSH, inhibin B, plasma testosterone and estradiol concentrations were measured by immunoradiometric, enzyme-linked immunoabsorbent, or radioimmuno-assays [25] as reported in Table 1.

Genetic testing

Genomic DNA was isolated from white blood cells (WBC). Direct genomic sequencing of *GNRH1* was performed by sequencing all exons and exon-introns junctions (NG_016457.1), up-stream and down-stream promoter encompassing 1100 bp before start site of transcription (Fig. S3). Direct genomic sequencing of coding exons and intron-exon junctions of *GNRHR1*, *KISS1*, *GPR54*, *NELF*, *TAC3*, *TACR3*, *FGF8*, *FGFR1*, *PROK2* and *PROKR2* was performed as previously described [26]. PCR primers were designed by Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) [26]. PCR and sequencing products were purified on a Biomek NXP-96 Laboratory Automation Workstation (Beckman Coulter, Villepinte, France) with Agencourt Ampure XP and Agencourt Cleanseq (Beckman Coulter Genomics, Danvers, MA). Sequencing products were analyzed with an automated capillary sequencer (ABI PRISM 3130xl Genetic Analyzer; Applied Biosystems, Foster City, CA). Electropherogram-derived sequences were compared with NCBI references using SeqScape Software 2.6 (Applied Biosystems, Foster City, CA).

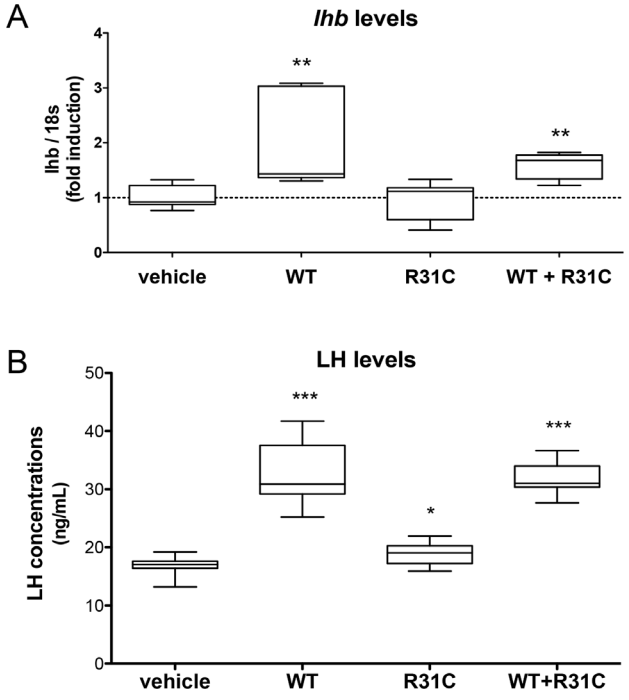

Figure 7. Effects of treatments on *lhb* transcription and LH secretion. (**A**) LbetaT2 cells were starved overnight in serum-free DMEM and treated either by 10 nM WT, R31C, 10 nM WT +10 nM R31C peptides or vehicle. After five hours treatment cells were lysed for RNA extraction (Trizol®). Mouse *lhb* levels were analyzed by quantitative real time RT-qPCR. Values are expressed as individual ratios on 18S and represented as fold induction on vehicle as means ± SEM, **p<0.01. This experience was conducted n = 7 times in duplicates. (**B**) LH concentrations from LbetaT2 cells culture *media*. Cells were starved overnight in serum-free DMEM and incubated with 10 nM WT, 10 nM R31C, 10 nM WT +10 nM R31C, or vehicle for 5 hours. LH levels were measured by a combined rat/mouse RIA. Values are expressed as individual values and as means ± SEM, *p<0.05, ***p<0.001. The experiment was conducted four times and each sample was assayed in duplicates.
doi:10.1371/journal.pone.0069616.g007

Microsatellites genotyping was performed to confirm filiation (Powerplex 16 System®, Promega, Madison, WI).

Total RNA was extracted from cells (WBC or cells in culture) with the Trizol® reagent (InVitrogen, Cergy Pontoise, France). RT-PCR and direct cDNA sequencing was performed as previously reported [26,27]. Complete CDS of *GNRH1* transcripts (NM_0001083111.1 and NM_000825.3) were analyzed with primers for RT-PCR and sequencing in Table S1.

Molecular Characterization of the GnRH R31C mutant

Peptide custom and stability. "Wild type" (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2) and "R31C" (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Cys-Pro-Gly-NH2) mutant GnRH were synthesized by a custom peptide manufacturer (Eurogentec®, Liège, Belgium). Molecular weight was confirmed by MALDI-TOF and purity was assessed at ~95% by the manufacturer.

Lyophilized products were suspended in sterile water in order to obtain 0.1 mM aliquots and conserved frozen at −150°C.

LC-MS/MS was performed with Quattro-LCZ triple quadrupole mass spectrometer equipped with the orthogonal electrospray source (Micromass, Manchester, UK) to analyze peptides stability in solution. Peptides solutions (water and cell culture medium) were incubated 24-h at room temperature for these stability studies.

Table 2. Recurrence of c.91C>T (p.R31C) *GNRH1* base change in subjects from CHH families and in healthy controls.

Authors	Reference	Year	CHH	Healthy individuals
Vagenakis et al.	[11]	2005	0/26	NA
Chan et al.	[6]	2009	4/310	0/192
Topaloglu et al.	[36]	2009	0/50	0/100
Quaynor SD. et al.	[7]	2011	2/48[a]	0/188
Current study		2013	3/410[b]	0/545[b]
Total			**9/844*** **	**0/1025**

NA: not applicable; *** p<0.0001 by Fisher's test between CHH patients and healthy controls. [a]: the mother of the female *propositus* has been represented as a carrier but no clinical data are reported. [b]: 120 CHH patients and 345 healthy subjects have been added to previously published data [5].
doi:10.1371/journal.pone.0069616.t002

In silico prediction of peptide cleavage was performed by means of Prop v.1.0b ProPeptide Cleavage Site (http://www.cbs.dtu.dk/services/ProP).

Prediction of protein function after selective amino acid substitution was obtained by means of Alamut® (Interactive Biosoftware, Rouen, France), AlignGVD (http://agvgd.iarc.fr/agvgd_input.php/), Polyphen2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/) and PANTHER Coding SNP Analysis tool (http://www.pantherdb.org/tools/csnpScoreForm.jsp).

Cell lines

Various cell lines were used for the experiments. HEK293T (ATCC CRL-11268) and COS-7 (ATCC CRL-1651), which do not express GnRH-R, were used after transient transfection with expression vector of human *GNRHR* (Missouri S&T cDNA Resource Center, Rolla, MO). Murine gonadotrope LbetaT2 cells were kindly provided by Dr. Mellon laboratory [28]. These cells endogenously express GnRH-R and are able to express gonadotropin subunit transcripts and to secrete mature LH glycoprotein after GnRH treatment [28].

Competition radioligand binding assays

The high affinity GnRH analog, [His^5, D-Tyr^6]-GnRH was radio-iodinated as previously described [29] and purified using Sephadex chromatography [30]. COS-7 cells were transiently transfected with a human GnRH-R DNA construct containing the human GnRH-RII carboxy-terminal to enhance expression [31] using 6 µg of DNA and 30 µl FuGene HD (Promega Corporation, Madison, WI) per 10 cm dish and seeded into 12-well plates. Two days after transfection cells were washed with HEPES-DMEM containing 0.1% bovine serum albumin (2×1 ml, HEPES-DMEM-BSA) and incubated with ^{125}I-[His^5, D-Tyr^6]-GnRH (100,000 CPM per well) and various concentrations of WT GnRH or R31C GnRH (4 h, 4°C) in a total volume of 0.5 ml. Cells were washed with phosphate-buffered saline (2×1 ml) and lysed with NaOH (1 ml, 0.1 M). Cell-bound radioactivity was counted in a gamma counter and IC-50 values were calculated using Graphpad Prism (GraphPad Software Inc, San Diego).

To determine whether R31C GnRH binds covalently to the GnRH-R, transfected COS-7 cells were incubated with WT GnRH (10^{-8} M), R31C GnRH (10^{-5} M) or HEPES-DMEM-BSA alone (2 h, 4°C), washed with HEPES-DMEM-BSA (1 ml) and incubated in HEPES-DMEM-BSA (5 ml, 1 h, 4°C) to allow

dissociation of non-covalently bound peptide, before the binding assay was performed as above.

Serum Responsive Element (SRE) luciferase assay

luc2P/SRE/Hygro plasmid (Promega, Madison, WI) was used to test luciferase production in response to MAP kinase activation as a reporter gene system. HEK293T cells (1.2×10^4 cells/well) were seeded 72 h before testing in high-glucose Dulbecco's minimal essential medium (DMEM, Invitrogen, Cergy Pontoise, France) containing 2 mM glutamine, 100 IU/mL penicillin, 100 mg/mL streptomycin, and 10% heat-inactivated fetal calf serum at 37°C in 96-well plates. Twenty-four hours before testing, cells were co-transfected in serum free OptiMEM, using Lipofectamine 2000 (Invitrogen, Cergy Pontoise, France) with the plasmids for human GnRH receptor, luc2P/SRE/Hygro and pMIR-REPORT™ beta-galactosidase vector (Applied Biosystems, Foster City, CA). WT GnRH, R31C, WT+R31C or vehicle were added at different dilutions (from 10^{-10} to 10^{-6} M). After 5 h-incubation cells were harvested and assayed for luciferase activities as previously described [32], using a luminometer (Victor, Perkin Elmer, Waltham, MA). To standardize for transfection efficiency, the relative light units were normalized by the galactosidase activity at optical density. EC-50 and Emax values were calculated using Graphpad Prism (GraphPad Software Inc).

Inositol phosphate (IP) accumulation

COS-7 cells were transiently transfected by electroporation with human GnRH-R DNA (10 µg/15 cm dish), seeded into 12-well plates and radiolabelled by overnight incubation with myo-[3H]Inositol (0.5 µCi/well, American Radiolabeled Chemicals, St Louis, Mo). Radiolabelled cells were washed and incubated (30 min, 37°C) in IP medium (HEPES-DMEM-BSA supplemented with 10 mM LiCl), then stimulated (60 min, 37°C) with various concentrations of WT GnRH or R31C GnRH or equal concentrations of WT GnRH and R31C GnRH. Incubations were stopped by removal of the medium and cells were lysed by addition of formic acid (1 ml, 10 mM). IP were extracted from cell lysates using Dowex 1 X8-200 chromatography and counted using a liquid scintillation counter (Pack ard). EC-50 and Emax values were calculated using Graphpad Prism (GraphPad Software Inc) [33].

ERK1/2 Western blot

LbetaT2 cells were starved 18 h in serum-free DMEM before treatments, then exposed to 10 nM of WT, 10 nM of R31C, the combination of 10 nM of each peptide or vehicle. Cells were harvested at 0, 5, 10, 15 and 30 minutes after treatment. Western blotting analyses were performed as previously described [32].

Measurement of intracellular free calcium concentration ($[Ca^{2+}]i$)

LbetaT2 cells were kept in serum free medium overnight and then loaded with 2 µM Fura2-AM for 45 min at 37°C. The experiment was conducted in HEPES buffer (in mM; 116 NaCl, 5.6 KCl, 1.2 $MgCl_2$, 5 $NaHCO_3$, 1 NaH_2PO_4, 20 HEPES ph 7.4) in presence of extracellular Ca^{2+} (EGTA 100 µM + $CaCl_2$ 300 µM). Single images of fluorescent emission at 510 nm under excitation at 340 and 380 nm were taken every 5 Sec. Changes in $[Ca^{2+}]i$ in response to GnRHs were monitored using the Fura2 340/380 fluorescence ratio. Basal ratio was arbitrarily considered as 1. Ca^{2+} responses over basal level were given either as maximal rise of $[Ca^{2+}]i$ or as area under the curve (for 2 min after agonist addition).

Gene expression study

LbetaT2 cells were grown at 10^6/well in 6-well plates and starved in serum-free DMEM 18 h before tests. After 5 hours incubation with 10 nM WT, R31C, the combination of 10 nM WT +10 nM R31C peptides or vehicle, cells were washed with 1× PBS and total RNA isolated using Trizol® (Invitrogen, Germany).

Lhb (murine LH beta subunit) transcript was quantified by real-time RT-PCR, using an ABI Step One Sequence Detector (Applied Biosystem, Foster City, CA) as previously described [27]. Primers are provided in Table S1.

LH assay

LbetaT2 cells were starved overnight in serum-free DMEM. The test day cells were exposed to 10 nM WT, R31C, the combination of 10 nM of each peptide or vehicle during five hours. Cell culture supernatants were collected and rapidly stored at −80°C. In cell culture media, LH concentration was measured using a previously described ELISA method [34] with reagents supplied by Dr. Parlow (National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA). The minimum detectable LH concentration was 0.2 ng/ml, and the interassay coefficient of variation was less than 10%.

Statistical analyses

Only nonparametric tests were used. Friedman's test was used to compare three or more matched groups and Kruskall-Wallis test for unmatched groups. These analyses were followed by Dunn's post comparison test. Differences were significant when $p<0.05$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Statistical analyses were performed using GraphPad Prism version 5.0d (GraphPad Software Inc., San Diego, CA).

Supporting Information

Figure S1 Peptide stability on mass spectrometry-coupled electrospray (Quattro-LC). Peptides were measured in aqueous solution at pH 7 and starved overnight at 37°C. WT and R31C decapeptides are found at the expected molecular weights (1181.6 and 1128.5, respectively). Formation of smaller fragments was absent in WT and negligible in R31C. Formation of R31C dimers was absent.
(PPT)

Figure S2 Luciferase activity after pulsatile exposure. HEK293T cells were transiently transfected with GNRHR and SRE-coupled luciferase reporter gene, and then exposed to four 90 min-spaced pulses of 10 nM WT, 10 nM R31C and 10+10 nM WT+R31C. After 5 minutes exposure to each ligand, cells were washed, and a subsequent pulse was given 90 minutes after. Five hours after the last pulse, cells were harvested for luciferase assay. Luciferase activity arbitrary units obtained by luminometry (a.l.u.) are shown as ratio on beta-galactosidase activity by optical density (used as transfection efficiency internal control). This experience was conducted three times (n = 8 replicates for each experiment).
(PPT)

Figure S3 Genomic localization of human *GNRH1* and related transcription and translation products. Two main regulatory regions are located upstream the trascription start site: the proximal promoter mainly regulates hypothalamic trascript, whereas the distal promoter controls a longer *GNRH1* transcript (retaining entire intron 1 sequence) in the extra-cerebral tissues. Amino acids are represented by letters from the international nomenclature. In the prepropeptide GnRH, functional domains

are represented for the signal peptide (23 amino acids, blue), decapeptide GnRH (purple), and GnRH-associated peptide (GAP) (56 amino acids, green) (adapted from Bouligand et al., NEJM, 2009).
(PPT)

Table S1 Primer sets used for experiments.
(DOC)

Acknowledgments

We are grateful to Dr. Alain Deroussent (Gustave Roussy Institute and CNRS, Mass spectrometry platform IFR54, Villejuif, France) for technical support on MS platform; we also thank Isabelle Boucly and Lenaig Lamour for genetic sequencing technical support and Chantal Denoyelle for helping in LH dosages. We finally thank Dr. Adela Voican for helping to edit the manuscript.

Author Contributions

Conceived and designed the experiments: LM JB JY AGM. Performed the experiments: LM RB RPM CAF MG RP ML JCT. Analyzed the data: LM JB RB. Contributed reagents/materials/analysis tools: FA PB AC ML TB. Wrote the paper: LM JB JY.

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PLOS ONE

Flavor impairment: a neglected sensorineural disability in patients with Kallmann Syndrome

--Manuscript Draft--

Manuscript Number:	PONE-D-15-08121
Article Type:	Research Article
Full Title:	Flavor impairment: a neglected sensorineural disability in patients with Kallmann Syndrome
Short Title:	Flavor impairment in Kallmann Syndrome
Corresponding Author:	Luigi Maione Universita degli Studi di Napoli Federico II Naples, ITALY
Keywords:	Kallmann Syndrome, flavor, taste, hypogonadism, olfaction, fertility
Abstract:	Background. Kallmann Syndrome (KS) associates congenital hypogonadism to olfactory impairment. Our aim was to evaluate flavor function and flavor-related handicap in KS patients. Patients and Methods. 30 patients with KS, 12 with normosmic hypogonadism (nIHH), 24 with acquired anosmia (AA) and 58 healthy controls entered the study. All participants filled questionnaires for dietary habits, olfaction-related quality-of-life, and self-determined smell, flavor and taste abilities prior to undergo standardized olfactometry and gustometry. Each subject underwent flavor test, developed with orally-administered aqueous aromatic solutions, consisting in identifying 21 different compounds by choosing each out of five alternative items. Flavor score (FS) was calculated as the sum of correct answers (range 0-21). Results. Flavor perception by self-assessment was similar between KS, nIHH and controls, and was largely reduced in only AA. FS was similar between KS (5.4±1.4) and AA (6.4±1.9), and lower than nIHH (16.2±2.4,$p<0.001$) and controls (16.8±1.7,$p<0.0001$). FS showed strong reproducibility and correlated with olfactory scores in the overall population. KS and AA patients identified aromatics eliciting trigeminal stimulation better than pure odorants. Olfaction-related quality-of-life was more impaired in AA than in KS. Conclusions. This is the first report showing flavor impairment in KS. This contrasts with what generally evidenced in routinely clinics, since KS patients, contrarily to AA, do not complain flavor inability, perhaps owing to the congenital nature of dysfunction. Flavor injury should be accounted as a specific KS impairment, because of important detrimental effects on physical and mental health and on quality-of-life. KS patients might be apprised of flavor inability also to prevent unexpected and life-threatening accidents.
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Question	**Response**
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Competing Interests You are responsible for recognizing and disclosing on behalf of all authors any competing interest that could be	The authors have declared that no competing interests exist

perceived to bias their work, acknowledging all financial support and any other relevant financial or non-financial competing interests. Do any authors of this manuscript have competing interests (as described in the PLOS Policy on Declaration and Evaluation of Competing Interests)? **If yes**, please provide details about any and all competing interests in the box below. Your response should begin with this statement: *I have read the journal's policy and the authors of this manuscript have the following competing interests:* **If no** authors have any competing interests to declare, please enter this statement in the box: "*The authors have declared that no competing interests exist.*" * typeset	
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Methods section of your manuscript. Please enter the name of the IRB or Ethics Committee that approved this study in the space below. Include the approval number and/or a statement indicating approval of this research. **Animal Research (involved vertebrate animals, embryos or tissues)** All animal work must have been conducted according to relevant national and international guidelines. If your study involved non-human primates, you must provide details regarding animal welfare and steps taken to ameliorate suffering; this is in accordance with the recommendations of the Weatherall report, "The use of non-human primates in research." The relevant guidelines followed and the committee that approved the study should be identified in the ethics statement. If anesthesia, euthanasia or any kind of animal sacrifice is part of the study, please include briefly in your statement which substances and/or methods were applied. Please enter the name of your Institutional Animal Care and Use Committee (IACUC) or other relevant ethics board, and indicate whether they approved this research or granted a formal waiver of ethical approval. Also include an approval number if one was obtained. **Field Permit** Please indicate the name of the institution or the relevant body that granted permission.	
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Additional data availability information:	

Dear Academic Editor
PLOS One

Naples, February, the 20th, 2015

Dear Editor,

Please find enclosed the manuscript entitled "Flavor impairment: a neglected sensorineural disability in patients with Kallmann Syndrome" by Maione et al.
This is the first report focusing on flavor function in patients with Kallmann Syndrome, a rare endocrine disease affecting gonadotrope axis as well as olfaction.

Along with this Research Article, we demonstrate that patients with Kallmann Syndrome, though unaware of flavor disability, fail not only to detect volatile compounds (anosmia) but also to identify orally-administered aromatic solutions (flavor) when compared to patients with normosmic hypogonadism and controls. This disability resembles what is observed in acquired anosmics, who complain a marked inconvenience of flavor and perceive a remarkable handicap in relation to this impairment.

The sense of «flavor» is complex, and involves every aspect of the routinely life, holding a very high impact on the general wellbeing and health. The social and relational aspects of foods and mealtimes, the economic and cultural repercussions related to flavor represent an original and in our opinion interesting investigation. The finding of a newly misdiagnosed sensorineural impairment in these patients might be of crucial importance for scientific community and for physicians who usually take care of these patients.

Finally, the disclosure of this injury has enabled us to develop and standardize a new clinically-relevant chemosensory test with high reliability, whose results are offered for the first time in this manuscript.
We trust in scientific consistence not less than originality of this work and thus we look forward to hearing from your opinion. We sincerely think these contents meet your Journal's scope.

The authors hereby confirm that neither the manuscript nor any part of it has been published or is being considered for publication elsewhere. By signing this letter each of the Authors acknowledges that he or she participated sufficiently in the work to take public responsibility for its content.

The Authors also declare that the work will not be submitted for publication elsewhere until the decision of your editorial board.

We just ask not to be revised by Dr. Stehpanie Seminara and Dr. WF Crowley Jr, belonging to Massachussets General Hospital Reproductive Endocrinological Group, for competing interest.

Best regards,

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Flavor impairment: a neglected sensorineural disability in patients with Kallmann Syndrome

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Abbreviated title: Flavor impairment in Kallmann Syndrome

Keywords: Kallmann Syndrome, flavor, taste, hypogonadism, olfaction, fertility

Word count: 2992.

Number of figures and tables: 3 figures, 1 table.

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ABSTRACT

Background. Kallmann Syndrome (KS) associates congenital hypogonadism to olfactory impairment. Our aim was to evaluate flavor function and flavor-related handicap in KS patients.

Patients and Methods. 30 patients with KS, 12 with normosmic hypogonadism (nIHH), 24 with acquired anosmia (AA) and 58 healthy controls entered the study. All participants filled questionnaires for dietary habits, olfaction-related quality-of-life, and self-determined smell, flavor and taste abilities prior to undergo standardized olfactometry and gustometry. Each subject underwent flavor test, developed with orally-administered aqueous aromatic solutions, consisting in identifying 21 different compounds by choosing each out of five alternative items. Flavor score (FS) was calculated as the sum of correct answers (range 0-21).

Results. Flavor perception by self-assessment was similar between KS, nIHH and controls, and was largely reduced in only AA. FS was similar between KS (5.4±1.4) and AA (6.4±1.9), and lower than nIHH (16.2±2.4,$p<0.001$) and controls (16.8±1.7,$p<0.0001$). FS showed strong reproducibility and correlated with olfactory scores in the overall population. KS and AA patients identified aromatics eliciting trigeminal stimulation better than pure odorants. Olfaction-related quality-of-life was more impaired in AA than in KS.

Conclusions. This is the first report showing flavor impairment in KS. This contrasts with what generally evidenced in routinely clinics, since KS patients, contrarily to AA, do not complain flavor inability, perhaps owing to the congenital nature of dysfunction. Flavor injury should be accounted as a specific KS impairment, because of important detrimental effects on physical and mental health and on quality-of-life. KS patients might be apprised of flavor inability also to prevent unexpected and life-threatening accidents.

BACKGROUND

Kallmann syndrome (KS; MIM 308700, 147950, 244200, 610628, 612370, 612702) is defined by the association between congenital hypogonadotropic hypogonadism (CHH) and olfactory dysfunction (1, 2).

Gustative abilities are poorly evaluated in KS patients. Basic taste evaluated with electrogustometry has been found normal in KS (3). The sense of flavor is a complex gustative ability closely related to taste and smell integrity, that ultimately allows food recognition, oral intake identification and the pleasantness and «palatability» of meals. Patients experiencing olfactory loss, rather than lone olfactory impairment, mainly complain flavor disturbance and inability in food identification, with serious repercussions on their global quality of life (4). In contrast, KS patients generally do not complain gustatory or flavor dysfunctions (3).

Aim of this study was to explore the sense of flavor in KS patients, in comparison to patients with congenital normosmic isolated hypogonadism (nIHH), those with acquired forms of anosmia (AA), and normal healthy controls.

MATERIAL AND METHODS

Patients

The present study was approved by the Local Ethics Committee (Comitato Etico Università Federico II, related reference number 253/13). All the participants were adult and gave their written informed consent, in keeping with Italian Bioethics Law and the Declaration of Helsinki.

30 patients with KS, 12 with nIHH, 24 with AA, and 58 normal subjects entered the study. Criteria for inclusion of KS patients included: 1) congenital hypogonadotropic hypogonadism, defined by clinical signs or symptoms of hypogonadism and: in men, serum testosterone levels below 1 ng/mL in the presence of low or normal gonadotropins; in women, primary amenorrhea and estradiol levels below 20 pg/ml in the presence of low or normal gonadotropins; 2) complete anosmia, as assessed by a previous olfactory test, or defined by self-report; 3) otherwise normal biochemical tests of anterior pituitary function and ferritin concentrations. nIHH was defined by the same criteria as KS except for olfactory impairment. AA was due to trauma (*n*=3), chronic nasal disease such as rhinosinusitis with or without polyps (*n*=7), viral infections (*n*=5), tumors (*n*=2), previous surgery (*n*=5) or unknown causes (*n*=2). Subjects with neurodegenerative disorders were excluded.

Healthy controls had neither smell impairment nor reproductive disease at clinical enquiry, and were enrolled by participating at an educational and medical prevention program campaign (Campus Salute Onlus, Naples, Italy; www.campussalute.it). We excluded all controls having current or chronic sinusopathies, nasal polyps, viral or seasonal rhinitis, and those suffering from either neurodegenerative or ascertained cognitive disorders. We also excluded individuals being administered drugs known to interfere with taste or smell abilities (anti-thyroidal, antibiotics, griseofulvin, lithium, penicillamine, procarbazine, rifampin, anxiolytic, antipsychotic, antiepileptic, antidepressants, amiodarone, digoxin, statins, chemotherapic drugs). Characteristics of the sample population are summarized in Table 1.

Food intake was restricted to drinking water within 3 hours before the tests. Alike, smoking was not allowed within the same time period.

Questionnaires

Prior to the evaluation of chemosensory functions, patients and controls were invited to self-define their ability in sensorineural perception. Each participant assessed olfaction (smell ability), flavor (food and oral intakes identification ability) and taste (basic taste ability) by means of a color-intuitive ten-grade arbitrary scale ranging from 0 (minimum) to 10 (maximum). In order to evaluate dietary habits, patients and controls were submitted to the PREDIMED questionnaire, whose scores range from 0 to 14 (5). High scores mostly comply with Mediterranean diet style and related food preference. All participants filled the questionnaire for olfactory disorders (QOD) that is

115 particularly designed for anosmic populations. High QOD scores denote strong detriment of
116 olfactory related quality of life (6).
117
118 *Smell test*
119 All the subjects were submitted to the Sniffin' Sticks® test (SS-Burghart Medical Technology,
120 Wedel, Germany), in which odorants are presented in felt-tip pen-like sticks. The test consisted of
121 three subtests for the study of odor threshold (T), odor discrimination (D), and odor identification
122 (I), respectively, as well described by Hummel et al. in 2007 (7).
123 Each subtest result was then summed up to a composite score, known as TDI score. Anosmia has
124 been defined by a TDI<16, while normosmia, according to the subjects' age, by a TDI>29 for the
125 subjects between 18 and 53 years, and by a TDI>28 for those older than 53.
126
127 *Taste function*
128 Basic gustatory test has been extensively described by Pingel et al. (8). Four tastants were tested:
129 1) 10 g/L (29 mM) sucrose, for the «sweet»; 2) 324 mg/L (1 mM) quinine hydrochloride, for the
130 «bitter»; 3) 1.0 g/L (0.017 M) sodium chloride, for the «salty»; 4) 0.2 g/L (1 mM) citric acid, for the
131 «sour».
132 These substances were soluted in distilled water and administered regionally. A drop of
133 approximately 20 microliters of each solution was applied on the upper surface of the tongue
134 Before application of each taste solution, the mouth was rinsed twice with distilled water. After
135 presentation of the stimulus each patient or control was invited to pick one of the five descriptors
136 ("sweet", "sour", "salty", "bitter", or "water/no taste"). The Taste Score was calculated as the sum of
137 correctly identified tastants and ranged from 0 to 16. Hypogeusia was defined for a TS <13 (8).
138
139 *Flavor test*
140 The flavor test was developed in aqueous solutions containing aromatic extracts, kindly provided
141 by GIOTTI manufacturer (Enrico Giotti spa, Scandicci, Firenze, Italy). Each compound was
142 previously tested and marketed for alimentary use according to Italian and EU current regulations
143 (European Food Safety Authority Regulation EC n. 178/2002; EC n. 854/2004; Italian G.U. n.
144 139/2004). A series of 20 extracts has been selected in accordance to the routinely dietary use.
145 More details on aromatic bases employed for the current test are reported in Supplemental Table
146 1. Each tastant, originally contained in a 30-mL amber bottle, was diluted in purified water
147 according to manufacturer's instructions, stored at 4°C in aliquots for mono-usage after preparation
148 and maintained at room temperature 20 minutes before administration. An aliquot of approximately
149 1 milliliter of each tastant was administered in oral cavity through a Pasteur pipette and maintained
150 approximately five seconds; mouth was then rinsed twice with purified water before the
151 administration of the following tastant. At each administration, participants were invited to identify

the aromatic by forcedly choosing the correct solution among five proposed items. A total of 21 aromatics (including one blank) were sequentially administered. The flavor score (FS) was calculated as the sum of correctly identified aromatics and ranged from 0 to 21. The flavor test has been deposited for patent license (application patent No. FI2014A000229).

Statistical analyses

Data are reported as mean ± standard deviation (SD) and as individual values in the figures. The non-parametric ANOVA Dunn's test was used to compare quantitative data across the KS, nIHH, AA and control groups. The $\chi 2$ test and Fisher's exact test were used to compare categorical data. Spearman's rank was applied to define correlations, and Bland-Altman plots and linear regression to assess reproducibility. Differences were considered statistically significant at $p<0.05$. Univariate and multivariate analyses using logistic regression models were conducted to identify determinants of flavor function scores. Statistical analyses and graphics were performed using Prism software, version 5.0f (GraphPad Software Inc., La Jolla CA) and the SPSS 16.0 package (SPSS, Inc., Chicago, IL).

RESULTS

Patients with AA were older than KS, nIHH and controls ($p<0.01$ *versus* all) and had higher BMI than controls and KS ($p<0.01$ and $p=0.026$, respectively). No difference in age and BMI was found across KS, nIHH and controls. Population characteristics are given in Table 1.

Sensorineural perception

Odor perception by self-assessment was impaired at similar extent in KS and AA patients. It was consistently lower in anosmics (KS and AA) than in both controls ($p<0.0001$) and nIHH ($p<0.0001$, Fig. 1A). Taste perception by self-assessment was similar across the four groups (Fig. 1B). Flavor perception was not different between KS, nIHH and controls. On the contrary, AA patients reported lower flavor perception than controls, nIHH and KS ($p<0.001$ for all, Fig. 1C).

Chemosensory evaluation

KS and AA patients resumed similar TDI scores on olfactometry (9.4±2.1 vs 9.7±2.7, respectively, $p=0.62$). TDI scores in KS and AA were consistently lower than either nIHH (36.3±5.6, $p<0.001$ for both) or controls (37.2±4.3, $p<0.001$ for both, Fig. 1D). Significance did not change after analysis of each subtest (threshold, discrimination and identification) taken separately.

Taste Score by gustometry was not different across KS (15.1±1.2), nIHH (15.8±0.6), AA (15.1±1.2) and controls (15.2±1.3, see Fig. 1E). Mild hypogeusia was found in 3 controls, 1 KS and 1 AA patient (p ns).

FS was dramatically lower in KS (5.4±1.4) than in nIHH (16.2±2.4, $p<0.001$) and in controls (16.8±1.7, $p<0.0001$). No difference was found between KS and AA (6.4±1.9, p=ns) (Fig. 1F). Differences in FS did not change after adjustment for age and BMI. A strong correlation was found between TDI and FS scores in the entire population ($r=0.89$, $p<0.0001$, Fig. 2A). Nonetheless, correlation was abrogated when considered the anosmic (AA+KS, Fig. 2B) and the normosmic (controls+nIHH, Fig. 2C) populations separately.

Univariate analysis in the overall population showed that FS score correlated with gender ($r=0.4$, $p<0.0001$), odor self-assessment ($r=0.86$, $p<0.0001$), QOD – Quality of Life ($r=0.77$, $p<0.0001$) and TDI ($r=0.94$, $p<0.0001$) scores. Correlations remained significant after adjustment for gender. After adjustment for TDI score, only odor self-assessment score still held significance ($r=0.52$, $p=0.039$).

Univariate analysis in KS population showed that FS score was inversely related to QOD – Quality of Life scores ($r=-0.78$, p 0.013), whereas TDI scores did not correlate with QOD items.

Unlike controls and nIHH, KS and AA patients were more able to identify aromatics with property to elicit a trigeminal response than those with a pure olfactory action ($p<0.0001$ and $p<0.01$, respectively, Fig. 3). Nevertheless, the ability in identifying compounds with trigeminal action in KS and AA was weaker than in controls ($p=0.028$).

Test/re-test analyses revealed strong reproducibility at both normosmic and anosmic ranges (Supplemental Fig. 1).

All populations resumed similar PREDIMED scores (Table 1). When analyzing details for food preference, however, AA seemed to consume more frequently legumes and nuts' servings than controls (Supplemental Table 2).

QOD – Quality of Life score was higher in AA than in controls ($p<0.0001$), in nIHH ($p<0.0001$) and in KS ($p<0.05$). It was also higher in KS than in controls and in nIHH ($p<0.05$ for both).

DISCUSSION

KS is a developmental disorder that associates congenital hypogonadism and anosmia because of the common ontogenesis of GnRH neurons within olfactory placode. Olfactory impairment, though variable, is a well-recognized disability in KS patients (2). In contrast, gustative function has been poorly evaluated in KS. Functional evaluations exploring basic taste were not abnormal in a small series of KS patients (3). In the same work, an integer sense of flavor was reported in KS, which prompted the Authors to hypothesize that congenital anosmics might learn by accentuating other aspects of foods to compensate for their dysfunction (3).

Flavor is a complex sense deriving from the combination between odorant and gustatory inputs, and is variously influenced by aspect, texture and temperature (9, 10). In our series KS patients have reported an intact perception of flavor by self-assessment, in contrast with individuals with AA, who also complain difficulties in oral intakes identification.

Analysis of olfactory and gustative functions through standard chemosensory tests revealed similar anosmic scores for KS and AA by sniffin' sticks, and normogeusic scores for all groups by taste test. This indicates that the degree of olfactory impairment is similar in the anosmic populations, and thus differences in FS might not be due to subtle weakening of smell ability.

It is important to consider that no test is available in routinely clinics to assess flavor. We developed a test to assess this function based on the identification of oral liquid intakes. This test explores the capacity of identifying different aromatics dissolved in aqueous solutions, thus allowing standardization and eliminating putative confounding factors, like consistency, temperature and appearance (color and shape). Flavor test has shown high reproducibility in both normal individuals and anosmic populations [see Supplemental Fig. 1].

We clearly show a dramatic injury of flavor perception in KS, that is totally similar to that observed in AA subjects. Flavor impairment seems not be due to peculiar dietary habits, as all populations tested resumed similar scores and food choices, consistent with medium adherence to Mediterranean Diet regimens. A more detailed evaluation of flavor test shows that the distribution of correct answers is not widely scattered, but some compounds are more frequently perceived than others. In particular, KS and AA are more able to identify compounds with trigeminal action than those actually known as pure odorants. Despite this, aromatics with a trigeminal component are less repeatedly perceived in KS and AA than in normosmics, in line with evidences reporting an overall fainting of trigeminal stimulation in anosmics (11, 12).

AA patients were older and with higher BMI than KS, nIHH and controls. FS, however, does not correlate with age or BMI. On the contrary, gender represents a stronger determinant for FS, in the same way as known for olfactometry, in which better performances are registered in young women

(13). Flavor Score completely correlates with olfactometric scores, thus representing an optimal tool to detect clear-cut anosmic from normosmic individuals. Correlation is abolished when considered anosmic or normosmic populations separately. Although TDI score is not particularly designed to detect better or worse sniffers among normosmics or anosmics, Flavor Score might produce different information from simple olfactory volatile impressions that probably could derive from various combinatory inputs by oral intakes. This deserves to be more appropriately evaluated, also in specific subpopulations, i.e. involved in perfume, cooking or enogastronomic activities. A mild hypogeusia has been found in only few subjects, and was not related to a particular subpopulation. Furthermore, taste scores in these individuals settled just below the lower limits of normality, reflecting a mild impairment likely not affecting results. Taste scores did not correlate with flavor scores. We did not use «umami» sense among tastants because barely recognizable or distinguishable from «salty».

Sex steroid deficiency *per se* seems not to have a role in flavor perception. Flavor score is strictly normal in patients with nIHH, who share the same steroidogenic defect as KS. Similarly, hormonal treatment does not have a role in determining FS. In the overall CHH population, three patients were not or were insufficiently supplemented with hormonal treatments, and resumed similar scores. Treatment by HRT or gonadotropins also did not affect FS results. No difference in FS was registered on the basis of the different mutations in either KS or nIHH genes tested.

In agreement with what observed in routinely clinics and in previous reports, we confirm that KS patients are scantly aware of flavor disabilities. Patients were asked specifically about their ability to perceive flavors, aromatics and to recognize foods and oral intakes in their routinely life. In our experience, only few KS subjects reported a clear conscience of this inadequacy, whereas the vast majority was not alerted about this. In line with this finding, the detriment of quality of life related to the sensorineural inabilities in KS is less critical than in AA, probably reflecting the congenital nature of the disorder in the former.

It is of interest that, in anosmic population, FS negatively correlated with the QOD – Quality of Life statements, whereas TDI did not. This may reflect a major importance of flavor dysfunction in determining quality of life detriment. The importance to use validated questionnaires in this setting is underlined by the difficulties of a reproducible olfactory ability self-evaluation by simply asking the patient about the presence or the degree of hyposmia on a visual analogic scale (6, 14). The scores deriving from the Questionnaire for Olfactory Disorders, particularly designed for anosmic populations, revealed higher impairment in AA than in KS.

AA patients, who resumed the highest QOD – Quality of Life scores, also scored the lowest QOD – Sincerity Statements (data not shown), indicating a tendency to give socially desired answers particularly in this population. This may be due to the older age of AA, since it is known that age may affect QOD results (6). Alternatively, higher QOD scores in AA may more properly reflect a

global detriment in general wellbeing, which is more strongly perceived in the acquired form of this dysfunction. Although other quality-of-life questionnaires have not been specifically tested in this study, QOD items were designed to explore the complain about daily life problems of disosmic patients, and have been shown to well correlate with established tests for quality of life and mood states, as the Beck's Depression Inventory or the Mood Inventory tests (6).

Apart from overt anosmics, it will be interesting to evaluate flavor in hyposmic patients, who may partially have different kinds of odorous impressions. The main question is to explore whether, through this complete qualitative test, these patients may achieve a full recognition of food, whereas intermediate scores are resumed at olfactometry. Apart from the scope of this study, it will be also intriguing to assess flavor function and food identification tasks in patients suffering from dys- or ageusia, whose main complain seems to solely affect the basic taste ability.

Flavor disability is both a social and a medical concern. This function is closely associated with the pleasantness and the sociability of mealtimes, and invariably influences relationships and social behaviors (4, 15-17). From a medical point of view, recognition of nutrients may prevent ingestion of spoilt or contaminated foods. Thus, flavor alteration is not merely an inconvenience but can also impact physical and mental health. Abnormal taste perception in KS might have serious health consequences by elevating the risk of food-borne illness and by altering food choices with potential repercussions on nutritional status. Moreover, flavor and taste dysfunction may lead to an increased risk of allergic reactions in people with food sensitivities and have an impact even on survival (18).

In conclusion, this is the first report showing that, along with smell impairment, KS patients also fail to identify oral intakes. Flavor deficiency has to be accounted as a specific sensorineural impairment in KS, whose spectrum of associated disabilities is expanding. KS patients should be apprised of their gustative inability in order to prevent nutritional imbalance and life-threatening accidents.

ACKNOWLEDGEMENTS

We thank the Campus Salute Onlus for joining this collaborationship with Federico II University, and for allowing the enrolment a proper sample attained from educational and preventive medical programs particularly addressed to general population. We are grateful to Dr. Corinne Eloit for her competences and expertise in the domain of sensorineural abilities, and for her inspiring intuitions. We are indebted with Donato Creti, Francesca Trapani and the whole team of Enrico GIOTTI spa for the neutral commitment and the facility to prepare and distribute the aromatic bases used for this study.

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Legend to Figures

Figure 1 – Sensorineural perception and evaluation in healthy controls, KS, nIHH and AA patients.

Data are reported as individual values in all panels. Details for each sensorineural test are given in Methods' Section. KS: Kallmann Syndrome; nIHH: normosmic isolated hypogonadotropic hypogonadism; AA: acquired anosmia. ** $p<0.01$; *** $p<0.001$.

Panel A. Olfactory perception by self-assessment (0-10 color-intuitive arbitrary scale). Higher values denote better perception.

Panel B. Gustative perception by self-assessment (0-10 color-intuitive arbitrary scale). Patients and controls were instructed to indicate their subjective ability to perceive basic tastes (sweet, salty, sour or bitter).

Panel C. Flavor perception by self-assessment (0-10 color-intuitive arbitrary scale). Patients and controls were particularly instructed to indicate their subjective ability to perceive flavors and identification of different foods by ingestion.

Panel D. Olfactory function evaluated by Sniffin' Sticks® test (SS-Burghart Medical Technology, Wedel, Germany, see Methods). On the y-axis is reported the relative TDI Score (range 0-48).

Panel E. Gustatory sensitivity evaluated by gustometry (see Methods). On the y-axis is reported Taste Score (range 0-16).

Panel F. Flavor function evaluated by flavor test (see Methods). On the y-axis is reported Flavor Score (range 0-21).

Figure 2 – Linear regression analysis between olfactometry (TDI) and Flavor Score.

Panel A. Correlation between flavor score and olfactometry by TDI score in the overall population. Individual plots depict the different populations tested. On the x-axis olfactometry by TDI scores; on the y-axis the flavor scores. The overall population is represented. $R=0.89$, $p<0.0001$. Slope is 0.45 ± 0.03, 95% CI [0.39; 0.49].

Panel B. Correlation between flavor score and TDI score in the anosmic population (KS + AA). $R=0.16$, p 0.27.

Panel C. Correlation between flavor score and TDI score in the normosmic population (controls + nIHH). $R=0.12$, p 0.36.

KS: Kallmann Syndrome; nIHH: normosmic isolated hypogonadotropic hypogonadism; AA: acquired anosmia.

Figure 3 – Flavor perception according to aromatic characteristics.

Histograms represent the rate of correct answers given for compounds with trigeminal or pure odorant properties (see also Ref. 19 and 20). Compounds with putative trigeminal or putative

mixed stimulation were not considered. Aromatics with trigeminal properties (black bars) here reported are peppermint, mustard and garlic. Aromatics with pure odorant properties (white bars) are vanilla, almond, chocolate, tea, honey, banana, coffee, peach and chestnut. Statistical significance by Fishers' exact test is given. A strong difference was also found between controls and nIHH versus KS ($p<0.0001$ for both), and AA ($p<0.0001$ for both, data not shown).
KS: Kallmann Syndrome. AA: acquired anosmia. nIHH: normosmic isolated hypogonadotropic hypogonadism. * $p<0.05$; ** $p<0.01$; **** $p<0.0001$.

Table 1. Characteristics of sample population.

	Controls	KS	nIHH
N.	58	30	12
Sex ratio, males (%)	41 (71)	24 (80)	10 (67)
Age (range, ys)	32 ± 10 (21-63)	29 ± 9 (18-54)	23.2 ± 5.5 (18-31)
BMI (kg/m^2)	23 ± 3	24 ± 4	23.7 ± 3.1
Mutations, N.			
KAL1		9	
FGFR1/FGF8		5	
PROK2/PROKR2		2	1
GNRH1/GNRHR			2
PREDIMED Score	8 ± 2	6.8 ± 1.6	8 ± 1.6
QOD-Quality of Life Score	4.2 ± 1.6	13.1 ± 6.5 [c]	3.8 ± 1.7

Data are reported as mean ± standard deviation, or as number (percentage). KS: patients with Kallmann Syndrome; nIHH: patients with normosmic isolated hypogonadotropic hypogonadism; AA: patients with acquired anosmia. PREDIMED: 14-Item Mediterranean Diet assessment (see Methods); QOD: Questionnaire for Olfactory Disorders (see Methods). a: $p<0.001$ vs Controls, KS and nIHH; b: $p<0.05$ vs Controls and KS; c: $p<0.05$ vs Controls and nIHH; d: $p<0.0001$ vs Controls and nIHH; e: $p<0.05$ vs KS.

Figure 1
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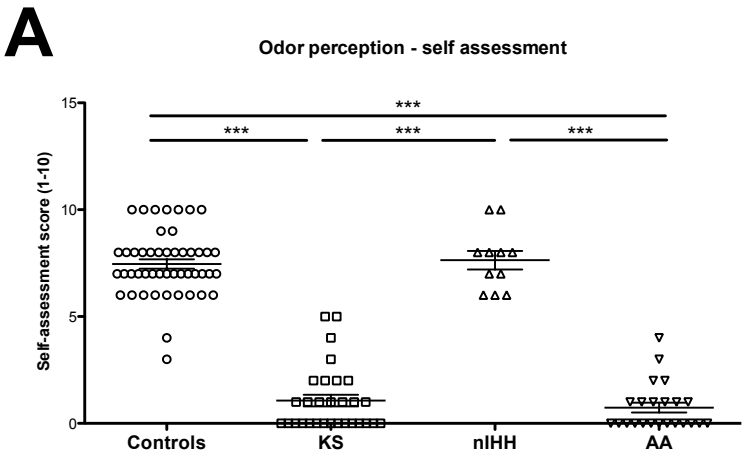
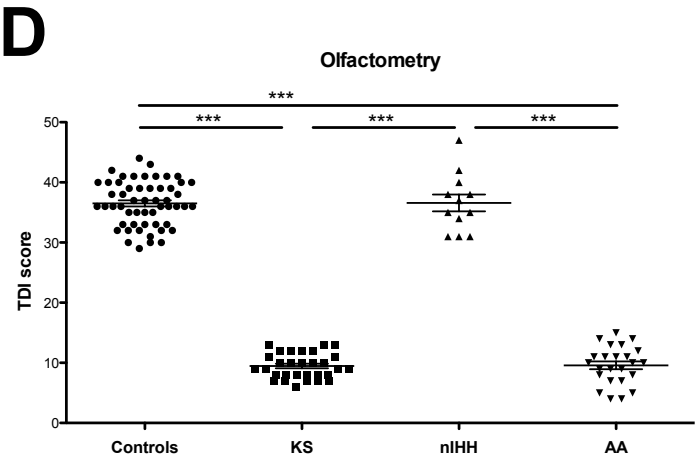
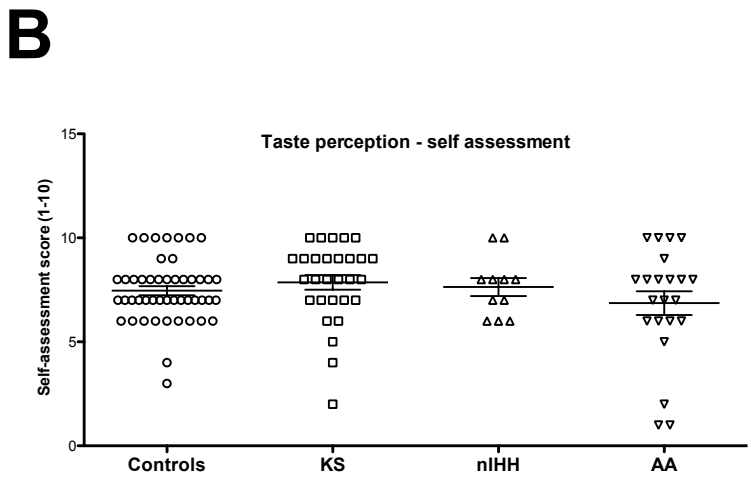
Fig. 1

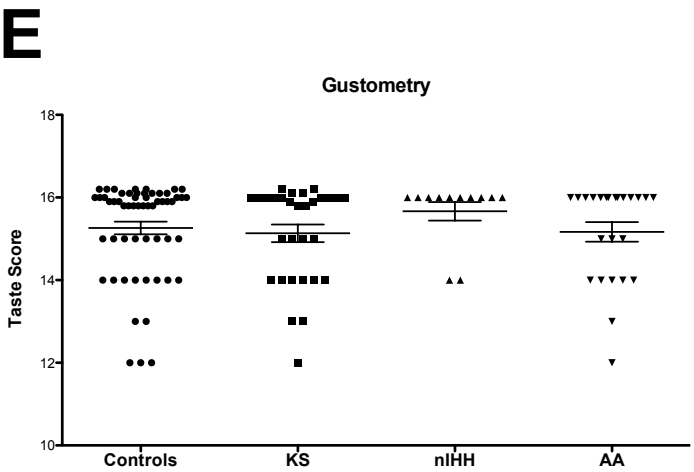
SENSORINEURAL PERCEPTION

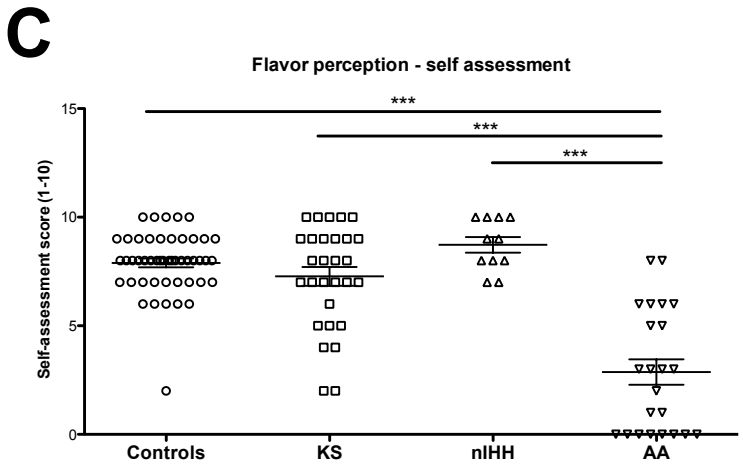
CHEMOSENSORY EVALUATION











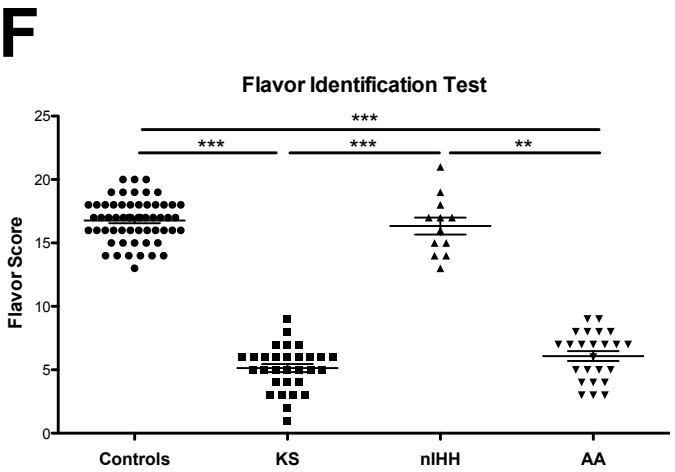


Figure 2
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Fig. 2

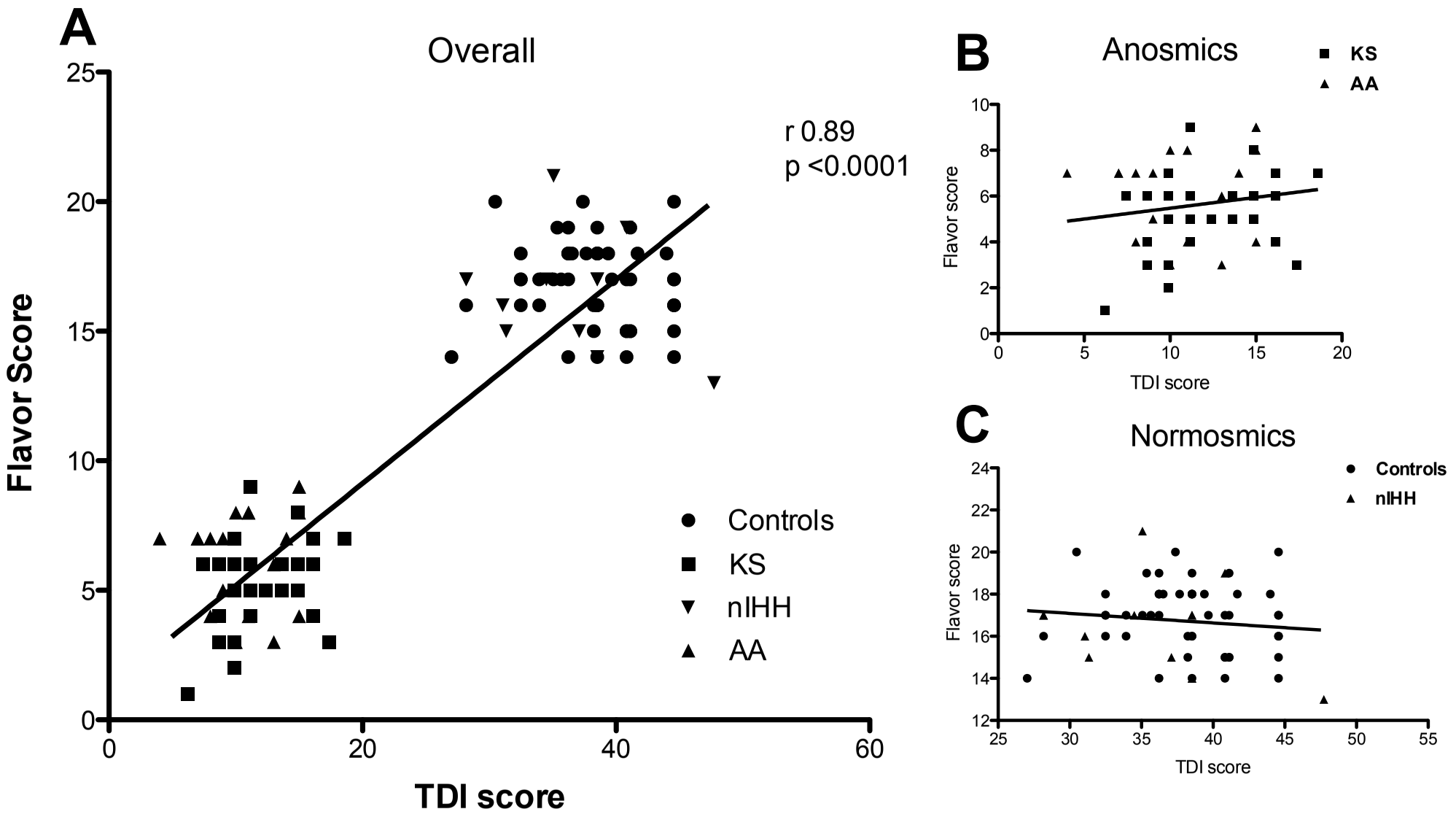

Figure 3
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Fig. 3