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Characteristics of a nationwide cohort of patients presenting with Isolated Hypogonadotropic Hypogonadism (IHH)

Marco Bonomi, MD[1,2], Valeria Vezzoli, PhD[2], Csilla Krausz, MD[3], Fabiana Guizzardi, PhD[2], Silvia Vezzani, MD[4], Manuela Simoni, MD[4], Ivan Bassi, BSc[2], Paolo Duminuco, BSc[2], Natascia Di Iorgi, MD[6], Claudia Giavoli, MD[1,7], Alessandro Pizzocaro, MD[8], Gianni Russo, MD[9], Mirella Moro, MD[2], Letizia Fatti, MD[2], Alberto Ferlin, MD[10], Laura Mazzanti, MD[11], Maria Chiara Zatelli, MD[12], Salvo Cannavò, MD[13], Andrea M. Isidori, MD[14], Angela Ida Pincelli, MD[15], Flavia Prodam, MD[16], Antonio Mancini, MD[17], Paolo Limone, MD[18], Maria Laura Tanda, MD[19], Rossella Gaudino, MD[20], Mariacarolina Salerno, MD[21], Pregnolato Francesca[22], MS, Mohamad Maghnie, MD[6], Mario Maggi, MD[3], Luca Persani, MD[1,2] on behalf of the Italian Network on Central Hypogonadism (NICe group)*

[1]Department of Clinical Sciences and Community Health, University of Milan, Milan, Italy.
[2]IRCCS Istituto Auxologico Italiano, Division of Endocrine and Metabolic Diseases & Lab. of Endocrine and Metabolic Research, Milan, Italy.
[3]Department of Biomedical, Experimental and Clinical Sciences "Mario Serio", University of Florence, Florence, Italy.
[4]Unit of Endocrinology, Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Modena, Italy; Department of Medicine, Endocrinology, Metabolism and Geriatrics, Italy; Azienda USL of Modena, Italy.
[5]Center for Genomic Research, University of Modena and Reggio Emilia, Modena, Italy
[6]Department of Pediatrics, IRCCS Giannina Gaslini, University of Genoa, Genoa, Italy
[7] Endocrinology and Metabolic Diseases Unit, Fondazione IRCCS Cà Granda Ospedale Maggiore Policlinico, Milan, Italy
[8] Endocrinology Unit, IRCCS, Humanitas Research Hospital, Rozzano, Milan, Italy
[9] IRCCS San Raffaele Hospital, Department of Pediatrics, Milan , Italy
[10]Unit of Andrology and Reproductive Medicine, Department of Medicine, University of Padova, Padova, Italy
[11] Department of Medical and Surgical Sciences, Alma Mater Studiourm, University of Bologna, Bologna, Italy

[12] Section of Endocrinology and Internal Medicine, Department of Medical Sciences, University of Ferrara, Ferrara, Italy

[13] Department of Clinical and Experimental Medicine, Endocrinology Unit, University of Messina, Messina, Italy.

[14] Department of Experimental Medicine, Sapienza University of Rome, Rome, Italy

[15]Division of Internal Medicine, Endocrinology and Diabetology Unit, San Gerardo Hospital, University of Milan Bicocca, Monza, Italy

[16] Department of Health Sciences, University of Eastern Piedmont, Novara, Italy

[17] Operative Unit of Endocrinology, Catholic University of the Sacred Heart, Rome

[18] Endocrinology, Diabetes and Metabolic Disease Unit, A.O. Ordine Mauriziano di Torino, Torino, Italy

[19] Dipartimento di Medicina Clinica e Sperimentale- Università dell'Insubria sede di Varese

[20] Department of Surgical Sciences, Dentistry, Gynecology and Pediatrics, University of Verona, Verona, Italy

[21] Department of Translational Medical Sciences, University of Naples “Federico II”, Naples, Italy

[22]IRCCS Istituto Auxologico Italiano, Experimental Laboratory of Immunological and Rheumatologic Researches, Milan, Italy

** All members of the NICe group are listed in the acknowledgment.*

Corresponding author:

Marco Bonomi, MD

UO di Medicina Generale ad Indirizzo Endocrino-Metabolico

Piazzale Brescia 20 - 20149 Milano

Phone: +39-02619112390 - Fax: +39-02619112777

email: luca.persani@unimi.it

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Abstract

Objective: IHH is a rare disorder with pubertal delay, normal (normoosmic-IHH, nIHH) or defective sense of smell (Kallmann syndrome, KS). Other reproductive and non-reproductive anomalies might be present altough information on their frequency are scanty, particularly according to the age of presentation.

Design: Observational cohort study carried out between January 2008-June 2016 within a national network of academic or general hospitals

Methods:. We performed a detailed phenotyping of 503 IHH patients with: 1) manifestations of hypogonadism with low sex steroid hormone and low/normal gonadotropins; 2) absence of expansive hypothalamic/pituitary lesions or multiple pituitary hormone defects. Cohort was divided upon IHH onset (PPO, pre-pubertal onset or AO, adult onset) and olfactory function: PPO-nIHH (n=275), KS (n=184), AO-nIHH (n=36) and AO-doIHH (AO-IHH with defective olfaction, n=8).

Results: 90% of patients were classified as PPO and 10% as AO. Typical midline and olfactory defects, bimanual synkinesis and familiarity for pubertal delay were found also among the AO-IHH. Mean age at diagnosis was significantly earlier and more frequently associated with congenital hypogonadism stigmata in KS patients. Synkinesis, renal and male genital tract anomalies were enriched in KS. Overweight/obesity are significantly associated with AO-IHH rather than PPO-IHH.

Conclusions: KS patients are more prone to develop a severe and complex phenotype than nIHH. The presence of typical extra-gonadal defects and familiarity for PPO-IHH among the AO-IHH patients indicates a common predisposition with variable clinical expression. Overall, these findings improve the understanding of IHH and may have a positive impact on the management of patients and their families.

Introduction

Isolated Hypogonadotropic Hypogonadism (IHH) is a rare disorder with a still undetermined prevalence, estimated as 1:4-10,000 boys (1), and approximately 3-5 fold lower in females (2,3,4). IHH is characterized by abnormal pubertal development and/or infertility, low sex steroid and low/inappropriately normal gonadotropin levels (5,6). However, an adult-onset IHH in patients that had previously completed their puberty has been also described (2,7,8,9,10). IHH is termed Kallmann's syndrome (KS) or normosmic IHH (nIHH) when associated with a defective or normal sense of smell (6). In the recent years the previous view of KS and nIHH as two distinct clinical entities has been questioned (11). Although the pathogenesis of IHH is still frequently unexplained, a strong genetic background (12) is known to be largely shared between KS and nIHH. Indeed, these two entities may coexist within unique familial settings, thus suggesting they may constitute variable phenotypic manifestations of shared genetic defects (13,14,15,16). Furthermore, IHH may be associated with other developmental anomalies, such as midline defects, hearing impairment, renal anomalies, bimanual synkinesia in both sexes and micropenis and/or cryptorchidism in male patients (2,6). However, there is no information on the frequency of the phenotypical manifestations of IHH, in particular according to the age of presentation or to the nIHH or KS subclassification.

Aim of the present study is to give the clinical characterization of IHH in the largest cohort ever collected in a nation-wide collaboration involving either academic and general hospitals.

Subjects and Methods

Patient population

The entire cohort consists of 503 subjects (376 males, M and 127 females, F) recruited by the NICe group since 2008. The study, accomplishing the Declaration of Helsinki, was approved by the Ethic Committee of the coordinating institution (GR-2008-1137632), and all patients or their tutors gave a written informed consent. Anonimous patient data, referred to the time of diagnosis, before any therapy, were collected either prospectively or retrospectively and a clinical database was created. Inclusion criteria were: 1) signs/symptoms of hypogonadism associated low sex steroid hormone and inappropriately low/normal gonadotropins; 2) absence of expansive hypothalamic/pituitary lesions or multiple pituitary hormone defects (MPHD). Patients with pubertal defect were selected among those that did not enter or complete spontaneous pubertal development (testes volume <12 mL or primary amenorrhea). Adult males were selected among the patients presenting with a complete pubertal development and the combination of a) loss of libido, b) erectile dysfunction, c) loss of spontaneous nocturnal erections, d) testosterone <8 nmol/L with low/normal gonadotropins. Adult females were selected among the patients presenting with the combination of a) secondary amenorrhea for 6 months <40 years of age, b) low/normal gonadotropins. All basal blood sampling were performed before 9:00 AM, after an adequate fasting period, and the low hormonal levels were always confirmed at least twice. In order to omit the functional hypothalamic defects, exclusion criteria were: 1) body mass index <18.5 kg/m^2 (17); 2) intensive exercise (>5 hours/week); 3) chronic illness. Patients diagnosed with IHH during adolescence were reexamined after therapy withdrawal between 17-20 years, in order to exclude a constitutional delay of puberty.

Protocols

Patients and/or their parents underwent standard interviews on family history, with particular emphasis on the recurrence of delayed/absent puberty, hypogonadism or olfactory defects, and past medical history. Data collected included: 1) pubertal development (Tanner stages); 2) testicular volume (TV) by Prader orchidometer or ultrasonographic (US) assessement; 3) stretched penile length, classified as micropenis according to the available cross-sectional normative data (18,19); 4) presence of gynecomastia; 5) presence of orofacial clefts or tooth agenesis; 6) presence of bimanual synkinesis; 7) hearing or osmic defects. Any signs of puberty in the absence of treatment, such as a TV growth ranging 4-12 mL in males or thelarche in females, was interpreted as a sign of partial spontaneous puberty. The appearance of menarche in females or a testicular volume >12 mL in male was classified as a complete pubertal development. Moreover the following additional investigations were performed at enrolment: 1) abdomen US for the study of potential renal agenesia/hypoplasia; 2) Magnetic Resonance Imaging (MRI) of hypothalamus-pituitary region and rhinencephalon (olfactory bulbs, sulci and tracts); 3) Olfactory test by the Brief Smell Identification Test (BSIT Sensonic, NJ, USA) 4) audiometry test when hearing defect was suspected. Furthermore, patients with olfactory defect were stratified as hyposmic or anosmic, respectively, based on a reduction above or below the 50% of the normal threshold. Based on clinical assessment, the cohort was divided into subgroups: pre-pubertal onset (PPO, in patients with IHH onset before 14 years age of age) or adult-onset (AO, in patients diagnosed in adulthood after an uneventful pubertal development); and depending upon olfactory function. Thus, each subject was classified as belonging to one of the following groups: PPO-nIHH, KS, AO-nIHH and AO-doIHH (AO-IHH with defective olfaction). PPO patients, either KS or nIHH, were further divided into those with totally absent (male: TV<4 mL; female: absent thelarche) or partial pubertal development (male: TV ranging 4-12 mL; female: positive thelarche).

Biochemical assessment: Because we recruited patients with a diagnosis obtained up to 25 years ago, different methods had been used. In the majority of the cases, serum LH, FSH, Estradiol and Testosterone concentrations were measured by electrochemiluminescence immunoassay "ECLIA" from Roche Diagnostic (Roche Diagnostics GmbH, Germany). LH and FSH assays had a lower limit of detection of 0.1 IU/L and a functional sensitivity of 0.2 IU/L. Elecsys® Testosterone II test (Calibrator reference: 05200067 190) had a lower limit of detection of 0.087 nmol/L and a functional sensitivity of 0.4 nmol/L. Elecsys® Estradiol III test (Calibrator reference: 06656048), had a lower limit of detection of 18.4 pmol/L, and a functional sensitivity of 85 pmol/L. The inter- or intra-assay coefficients of variation were <5% in all assays since 2008. Both steroid methods were standardized via isotope dilution-gas chromatography/mass spectrometry.

Statistical methods: statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA). Data were expressed as mean±SE unless otherwise indicated. Continous data from different subsets were compared by means of Mann-Whitney rank-sum test or Kruskal-Wallis with post-hoc Dunn's multiple comparison test as appropriate. Categorical data were tested by Chi-square or Fisher's exact test as appropriate. All *p-values* were two-sided and

$p<0.05$ was considered significant, although in case of multiple comparisons a *p-value* adjusted for the number of comparison was applied. Sex of the patients was consider a factor in the statistical analysis as appeared in the results section and the correlated Figures and Tables. Pairwise comparisons were always performed between KS *vs.* PPO-nIHH and AO-doIHH *vs.* AO-nIHH. Multiple comparison, including also the PPO-nIHH *vs.* AO-nIHH and KS *vs.* AO-doIHH contrasts were only performed for the body mass index and the hormone values variables.

Results

Cohort composition

The cohort composition and clinical information are reported in Table 1. A total of 459 patients (91.2%) were presenting as PPO-IHH (116 F and 343 M), while 44 patients (8.8%), that completed puberty before the reproductive axis failure, were classified as AO-IHH (11 F and 33 M). The 26.5% of the male PPO-group (n=91) presented with a partial spontaneous pubertal development, while 73.5% (n=252) had no signs of spontaneous puberty at >17 years of age. All female PPO patients, instead, were presenting a complete absence of pubertal development. A total of 192 patients were referred to have a smell defect (47 F and 145 M), while 311 patients formed the normosmic groups (80F and 231M).

Age, familiarity and body mass index (BMI) at diagnosis

The mean age at diagnosis was 19.9±0.5 years and 18.3±0.7 years, respectively, for PPO-nIHH and KS, with a significantly earlier diagnosis in KS patients. No differences between males and females were observed in any subgroup except in the AO-nIHH (Table 1).

BMI did not differ among the groups, except for AO-nIHH *vs.* PPO-nIHH (adj. *p-value*=0.0159). Moreover, stratifying the groups in normal-weight (BMI <25 Kg/m^2), overweight (BMI 25-29.99 Kg/m^2) or obese (BMI ≥30 Kg/m^2) subjects it became evident that AO-groups present a higher percentage of overweight/obese then PP-groups. Considering the sex of the patients, we observed statistically significant higher percentages of normal-weight patients in the female than in male PPO-nIHH group (Table 1). The prevalence of obesity among the patients with AO-IHH is higher of the one reported either in the male (15.6 vs 11.5%) or female (20.0 vs 9.3%) adult Italian population by the last survey of the National Institute of Statistics (www.istat.it).

Most cases were sporadic, however a familiarity with IHH was present in 19.0 or 29.6% among the patients with adult or PP onset, respectively, and was more common in the groups with osmic defects (Table 1).

Hormone profiles

Patient hormone profiles were evaluated at diagnosis. LH/FSH levels were evaluated at baseline and after GnRH stimulation in 59.7% and 59.4% of the KS and nIHH subjects, respectively. LH and FSH basal mean values (Fig.1A) were below the lower limit of normal in PPO-groups and low-normal in AO-groups. No significant LH differences between either KS *vs.* PPO-nIHH or AO-doIHH *vs.* AO-nIHH were observed, while basal FSH values were significantly higher in PPO-nIHH compared to KS. Moreover, both LH and FSH basal values were significantly different between KS and AO-doIHH (p=0.001) or PPO- and AO-nIHH (p=0.00001). Acute GnRH stimulation showed a blunted response (Fig.1B) in PPO-groups and a normal response in the AO-groups for both LH and FSH, with significantly different LH peak between PPO- and AO-nIHH (p=0.0001). No sex differences were noted either for basal or stimulated gonadotropin levels in the different groups. In male PPO-groups, gonadotropin levels were also stratified according to the partial or complete absence of pubertal development (Supplementary Fig.1). Mean basal and stimulated LH values were statistically different according to the stage of pubertal development (Supplementary Fig.1), independently of the olfactory status. No differences in mean FSH values were recorded, except basal FSH values between nIHH *vs.* KS absent-puberty groups. Testosterone levels were below the normal range in all male patients (Fig.1C). A significant difference in testosterone levels was only observed in the following comparisons: PPO- *vs.* AO-nIHH (p=0.00001) and KS *vs.* AO-doIHH (p=0.01). Testosterone levels were low in all the male PPO-groups. Estradiol levels were similar and below the normal range in all female groups (Fig.1D).

Developmental anomalies

Information on these defects was recorded in 87-100% of the cases (Table 2). A significantly higher percentage of bimanual synkinesia and renal anomalies was seen in KS compared to PPO-nIHH group. Orofacial clefts or tooth agenesis are significantly more represented in the groups with osmic defect (KS and doIHH) than in normosmic groups (PPO-nIHH and AO-nIHH) (χ^2=4.04; p=0.0445).

No particular differences were noted for all the considered developmental anomalies between sexes, except for the renal anomalies that were exclusively affecting the males.

Analysis of male genital tract anomalies data (Table 3) showed a high percentage of cryptorchidism in PPO-groups associated with a small testicular volume at diagnosis. Interestingly, micropenis or cryptorchidism (either mono- or bilateral) were significantly more represented in KS compared to PPO-nIHH. Nonetheless, cryptorchidism was present in a higher percentage of patients with a complete absent rather than in those with a partial pubertal development. In general, testicular volume at diagnosis was smaller in patients with osmic defect than in normosmic patients with a difference close to be statistically significant only between the two PPO-subgroups (p=0.0437).

Hypothalamus-pituitary MRI imaging

These investigations were performed in all cases. MRI of the hypothalamic-pituitary region was normal in almost all patients irrespective of the groups (Fig.2). However, pituitary hypoplasia, or partial empty sella or non-secreting incidental microlesion were seen in a minority of subjects, with similar percentages among groups or among sexes (Fig.2).

Olfactory evalutation

An accurate olfactory evaluation of the patients was performed by smell test (in 157 KS and 175 nIHH patients) and/or MRI (in 86 KS and 71 nIHH patients). Results of the smell test were compared to percentile norms, based upon 4,000 subjects matched for sex and range of age, reported in the B-SIT Administration Manual. All nIHH patients presented a normal smell identification score. On the other hand, KS and AO-doIHH had a defective sense of smell, with a similar distribution of anosmia or hyposmia (Fig.3A). The MRI evaluation of olfactory structures was normal in the nIHH groups, while it was variably affected in KS patients with pre-pubertal or adult-onset of hypogonadism. A complete aplasia or hypoplasia of the rhinencefalon was seen in the large majority of these subjects (Fig.3B). It appears that the subgroup of KS patients with totally absent puberty are those displaying the more compromised olfactory structure whereas the AO-doIHH (with a normal pubertal development) were the less compromised. No significant differences were noted among sexes.

Discussion

We report the clinical presentation of the largest cohort of IHH patients so far described. The cohort was recruited in various academic and general hospitals across all Italy, thus reflecting the existent clinical practice and included patients experiencing the failure of the reproductive axis before pubertal development (either with partial or complete absent sexual development) or after spontaneous sexual maturation, thus highlighting the wide clinical spectrum of IHH (2,20). The olfaction defects are more frequently associated with a complete GnRH deficiency and with non-reproductive manifestations. Nevertheless, the systematic evaluation of olfactory function surprisingly revealed the existence of morphological/functional defects in olfactory structures also among patients with an adult onset of IHH. Interestingly, familiarity for IHH was detected in variable but significant percentages of patients with pre-pubertal or adult onset of central hypogonadism suggesting a common inheritable predisposition between these two conditions that can be thus considered two extremes of a clinical spectrum of manifestations affecting the GnRH function.

An accurate evaluation of the smell function was performed in a high number of patients previously classified as KS or nIHH, confirming the data reported at diagnosis. After exclusion of 5 nIHH patients with turbinate hypertrophy, none of the patients classified as nIHH presented any defect in the sense of smell at olfactory test or rhinencephalon MRI (Fig.3A,B). Among the KS patients, the percentage of aplastic olfactory structure tended to be associated with a more severe hypogonadal state.

Systematic neuroimaging of the hypothalamic-pituitary region revealed pituitary defects, such as hypoplasia, partial empty-sella or pituitary non-functioning micro-lesions in a minority of cases. These findings were similarly distributed in the different groups and were non associated with specific features.

Interestinlgy, KS patients were diagnosed at an earlier age and have a more frequent familial recurrence than nIHH counterpart. Such differences were statistically significant in the PPO-groups and the trend was conserved also in AO-groups. These data are in agreement with the more severe neonatal phenotype of KS patients, and perhaps with a more frequent involvement of inheritable genetic defects with a higher degree of expressivity and penetrance. Some authors (21,22,23) reported a similar prevalence of cryptorchidism between KS and nIHH groups. In our cohort, congenital micropenis was present only in PPO-groups, as expected (10,24,25), but the prevalence of male genital tract anomalies, including micropenis and cryptorchidism (Table 3), was significantly higher in KS patients, thus indicating a more severe intrauterine androgen deficiency in KS patients. All together, these findings support the idea of an hormonal condition that is generally worse among KS patients. The frequency of micropenis at diagnosis is probably underestimated in our series. This is possibly due to the collection of retrospective data in multiple pediatric and adult centers where other parameters (eg, pre-treatment biochemical values) could be recovered, but the micropenis could not always be confirmed by a physical determination of this parameter.

KS patients also had a higher percentage of associated developmental anomalies (Table 2). Our data regarding the sensorineural hearing loss, which was present only in PPO-groups and with a slightly higher percentage in KS patients, were partially discordant with previous literature, reporting the presence of high percentage of hearing loss in KS patients (24,25) and a total absence in nIHH (24). More importantly, the renal anomalies were only present in PPO-groups and particularly in the male patients with KS. Such association had been reported in the past when renal dysgenesis was considered as a hallmark of the X-linked form of KS (27,28) although such malformations have been described in other KS autosomic dominant/recessive forms. Our findings strongly indicate that kidney anomalies should be suspected only in males with osmic defects.

A limitation of our retrospective study is represented by impossibility to have uniform biochemical analyses. Nevertheless, the majority of the patients had been evaluated with the same methods for gonadotropins and steroids, and no differences in the distribution among the various subgroups were seen when other methods had been used. Altogether, KS and nIHH patients of the present cohort showed overlapping levels of gonadotropins and sex steroids (Fig.1), confirming previous findings (2,6). Reproductive axis activity was indeed more severely affected in PPO- than in AO-groups. In fact, the acute GnRH stimulation lead to a more defective LH response in PPO- than in AO-groups. Moreover, we found significantly lower LH levels in the male patients with a complete defect in comparison with those a partial pubertal development. Although basal FSH levels were significantly higher in nIHH than in KS subgroups with absent pubertal development, GnRH function appears similarly affected among nIHH and KS groups: in particular FSH peak values after GnRH stimulation were comparable among the four groups. Thus, a biochemical marker and/or dynamic test able to differentiate KS/nIHH is still missing (2).

We observed that around 40% of the patients in PPO-groups were overweight/obese (OW/OB). These data are quite similar to what reported in a previous study (10), but the most interesting finding on the association with BMI was detected among the AO-groups. Indeed, the prevalence of obese subjects in the AO-IHH patients was the double than the one reported in a recent survey on the whole Italian population, and the majority of the OW/OB patients in the PPO- and AO-groups were males. It is known that an excess of visceral fat may affect the pulsatile gonadotropin release from the pituitary (7). Moreover, a recent study in a rabbit model demonstrated that a high-fat diet can induce a metabolic syndrome and a central hypogonadism associated with a reduced hypothalamic expression of KISS1 and KISS1R (29). Thus, our data are in accord with the results obtained in animal models supporting the existence of common mechanisms accounting for GnRH function and metabolic regulation (30). In addition, overweight/obesity might represent an acquired cofactor, involved in the onset of IHH among adult subjects that are naturally prone to develop a central failure of the gonadal axis. Thus, in the PPO-group the presence of congenital defects strongly affecting the development, migration and/or activation of GnRH-secreting neurons leads to a severe phenotype with an early onset, independently of the body weight, whereas overweight/obesity may facilitate a delayed IHH onset in carriers of susceptibility alleles with a limited impact on GnRH function. Accordingly, a major interaction between genes and behaviour in IHH has been previously reported in females with functional hypothalamic amenorrhea (31).

In conclusion, we provide the frequency of several IHH clinical characteristics at presentation in the largest cohort ever reported. We demonstrate that IHH clinical spectrum may be even more heterogeneous than previously considered. Despite the existence of a largely common pathogenic background, KS patients are prone to develop a more severe and complex phenotype than nIHH patients. We describe for the first time that AO-IHH is not always a sporadic disease (as previously reported in reff. 9, 10), and together with PPO-IHH they may represent two extremes of a clinical spectrum: they share phenotypical traits (familiarity, olfactory and midline defects, or bimanual synkinesis) and may therefore have a common predisposition. In AO-IHH, the presence of obesity is double than in general Italian population and more frequent than in the pre-pubertal forms, and could thus constitute a relevant factor contributing to the adult onset of the HPG failure. All together, these findings improve the understanding of this disease that may have a positive impact on the future management of IHH patients and their families.

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Table 1. Cohort composition

	PPO-nIHH	KS	*p-value*	AO-nIHH	AO-doIHH	*p-value*	*p-value overall comparison*
Male (n)	**202**	**141**	--	**29**	**4**	--	--
Complete puberty	*0*	*0*	--	*29*	*4*	--	--
Partial puberty	*52*	*39*	--	*0*	*0*	--	--
No puberty	*150*	*102*	--	*0*	*0*	--	--
Female (n)	**73**	**43**	--	**7**	**4**	--	--
Complete puberty	*0*	*0*	--	*7*	*4*	--	--
Partial puberty	*0*	*0*	--	*0*	*0*	--	--
No puberty	*73*	*43*	--	*0*	*0*	--	--
Ratio F:M	*1:2.7*	*1:3.3*	--	*1:4.1*	*1:1*	--	--
Age at diagnosis (yr)	**19.9±0.5**	**18.3±0.7**	*0.0227*	**35.7±2.7**	**33.1±6.3**	*ns*	--
M(yr)	20.6±0.7	18.4±0.8	*0.0253*	37.7±2.4	29.7±5.0	*ns*	--
F(yr)	17.9±0.6	17.8±1.0	*ns*	22.3±2.4	35.7±10.9	*ns*	--
BMI (Kg/m^2)	**24.3±0.3**	**24.8±0.4**	*ns*	**25.6±0.8**	**25.9±1.8**	*ns*	*0.0259*
19≤BMI<25 (%)	*65.9*	*52.4*	*0.0118*	*44.1*	*37.5*	*ns*	*0.0074*
M(%)	*60.5*	*50.4*	--	*42.8*	*25.0*	--	*0.0986*
F(%)	*81.3*	*59.4*	*ns*	*50.0*	*50.0*	*ns*	*0.0367*
25≤BMI<30 (%)	*20.8*	*30.8*	*ns*	*41.2*	*37.5*	*ns*	*0.0189*
M(%)	*23.9*	*31.5*	--	*42.8*	*50.0*	--	*0.0876*
F(%)	*11.9*	*28.1*	--	*33.3*	*25.0*	--	*0.1101*
BMI≥30 (%)	*13.3*	*16.8*	--	*14.7*	*25.0*	--	*0.5515*
M(%)	*15.6*	*18.1*	--	*14.3*	*25.0*	--	*0.7769*
F(%)	*6.8*	*12.5*	--	*16.7*	*25.0*	--	*0.2677*
Familial recurrence (%)	24.2	36.8	*0.0077*	14.7	37.5	*ns*	*0.0081*
M(%)	*22.4*	*35.1*	*0.0190*	*10.7*	*25.0*	*ns*	*0.0147*
F(%)	*29.0*	*42.5*	--	*33.0*	*50.0*	--	*0.6762*

PPO, Pre-pubertal onset; nIHH, normosmic Isolated Hypogonadotropic Hypogonadism; KS, Kallmann syndrome; AO, adult-onset; AO-doIHH, AO-IHH with defective olfaction; M, male; F, female. Comparisons for "age at diagnosis" were carried out using Mann-Whitney test: a *p-value* of 0.025 was considered as critical value after multiple adjustments (0.05/2). The *p-value overall comparison* refers to an initial contrast of the four groups: when statistically significant, pairwise assessments were performed between PPO-nIHH and KS or AO-nIHH and AO-doIHH. Comparisons for BMI (Kg/m^2) were performed using Kruskal-Wallis test with Dunn's *post-hoc* test. All other contrasts among categorical variables were performed using Chi-square or Fisher's exact test as appropriate with a *p-value* of 0.025 as indicated above.

Comparisons of male and female subgroups were made and two were significant at the 5% level: in group AO-nIHH the p-value for "age at diagnosis" was 0.0054; in group PPO-nIHH the p-value for "19≤BMI<25 (%)" was 0.0039.

Table 2. Cohort developmental anomalies*

	PPO-nIHH	**KS**	*p-value*	**AO-nIHH**	**AO-doIHH**	*p-value*	*p-value overall comparison*
Bimanual Synkinesis % (n)	2.5 (6/234)	10.9 (19/174)	*0.0006*	6.1 (2/33)	12.5 (1/8)	*ns*	*0.0031*
Orofacial clefts and/or tooth agenesis % (n)	9.8 (23/235)	14.9 (26/174)	*ns*	0 (0/35)	12.5 (1/8)	*ns*	*0.0296*
Renal anomalies % (n)	0.4 (1/231)	11.5 (20/174)	*<0.0001*	0 (0/35)	0 (0/8)	--	*<0.0001*
Hearing loss % (n)	5.2 (12/231)	7.0 (12/172)	--	0 (0/35)	0 (0/8)	--	*0.4447*

PPO, Pre-pubertal onset; nIHH, normosmic Isolated Hypogonadotropic Hypogonadism; KS, Kallmann syndrome; AO, adult-onset; AO-doIHH, AO-IHH with defective olfaction; *p-value overall comparison* refers to an initial comparison of the four groups: when statistically significant, pairwise comparisons were performed between PPO-nIHH and KS or AO-nIHH and AO-doIHH. In this case, a *p-value* of 0.025 was considered as critical value after multiple adjustments (0.05/2). Comparisons among categorical variables were performed using Chi-square or Fisher's exact test as appropriate.

* The possibility to describe other associated phenotypes, such as daltonism, coloboma, nystagmus, external ear malformations, clinodactyly or vertebral malformations, was given as an open field and they were recorded in a minority of cases (6 KS, 6 PPO-nIHH and 1 AO-nIHH patients) although it is not possible to define the frequency.

Table 3. Male genital tract anomalies

	PPO-nIHH	**KS**	*p-value*	**AO-nIHH**	**AO-doIHH**	*p-value*	*p-value overall comparison*
Micropenis (%)	2.4	7.8	*ns*	0	0	--	--
Partial puberty (%)	0	5.1	*ns*	--	--	--	--
Absent puberty (%)	3.3[b]	8.8[b]	*ns*	--	--	--	--
Male cryptorchidism (%)	21.9	52.7	*<0.0001*	7.4	0	*ns*	*<0.0001*
Monolateral(%)	*4.2*	*24.0*	*<0.0001*	*3.7*	*0*	*ns*	*<0.0001*
Bilateral (%)	*17.7*	*28.7*	*ns*	*3.7*	*0*	*ns*	*0.0078*
Partial puberty (%)	*7.8*	*34.2*	*0.002*	--	--	--	--
Absent puberty (%)	*28.3*[a]	*60.4*[a]	*<0.0001*	--	--	--	--
TV (mL)	4.9±0.2	4.2±0.2	*ns*	18.6±0.7	16.1±0.5	*ns*	*<0.0001*
Partial puberty (mL)	*8.6±0.2*	*8.0±0.3*	*ns*	--	--	--	--
Absent puberty (mL)	*2.5±0.1*	*2.21±0.1*	*0.02*	--	--	--	--

PPO, Pre-pubertal onset; nIHH, normosmic Isolated Hypogonadotropic Hypogonadism; KS, Kallmann syndrome; AO, adult-onset; AO-doIHH, AO-IHH with defective olfaction; TV, testicular volume. *p-value overall comparison* refers to an initial comparison of the four groups: when statistically significant, pairwise comparisons were performed between PPO-nIHH and KS or AO-nIHH and AO-doIHH. Comparisons among all categorical variables were performed using Chi-square or Fisher's exact test as appropriate and a *p-value* of 0.025 was considered as critical value after multiple adjustments (0.05/2). "TV" contrasts were carried out using Kruskal-Wallis test with Dunn's multiple adjustment test.

[a] p<0.01, Absent puberty *vs.* Partial puberty

[b] p=*ns*, Absent puberty *vs.* Partial puberty

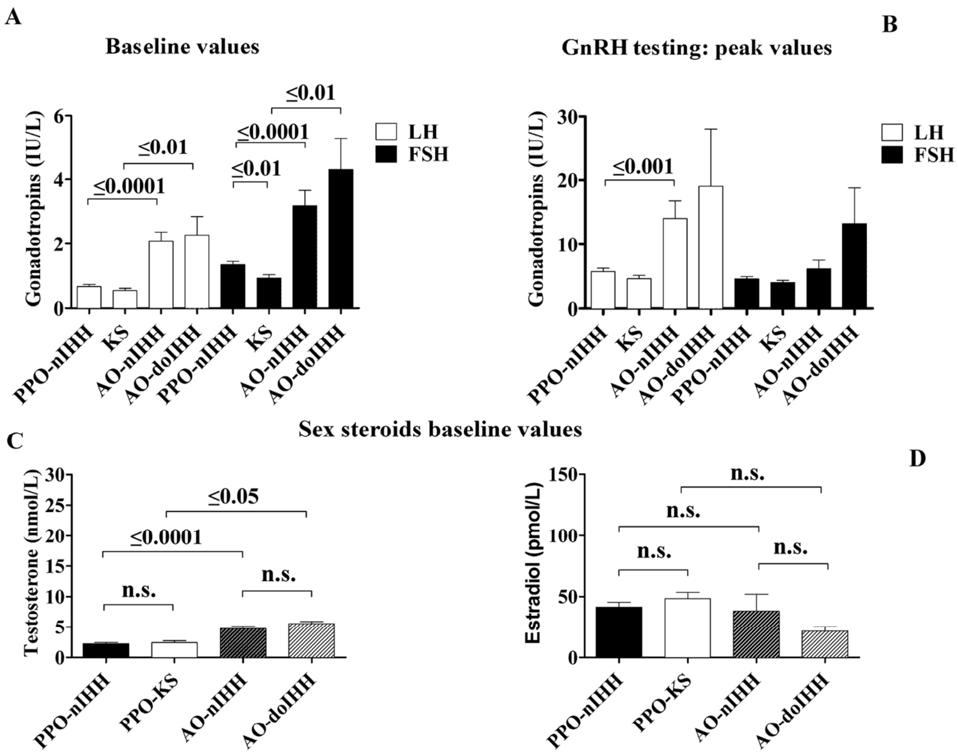

Fig.1 Hormone profile. Serum LH, FSH and sex steroids levels of the whole IHH cohort (n=503; male, n=376; female, n=127). Results represent the baseline (A) and peak stimulated (B) values of the two gonadotropins and the baseline values of male testosterone (C) and female estradiol (D). Gonadotropin stimulation was evaluated following administration of a standard dose of 100 mg GnRH and blood samples for FSH and LH were obtained at the 0' 30', 60', 90' and 120' minutes. Gonadotropin normal basal values are (IU/L): LH >1.7; FSH>1.5. Peri-pubertal peak normal range: LH/FSH=2-3 fold x basal level; adulthood peak normal range: LH=2-5 fold x basal level; FSH:1-2 fold x basal level. Sex steroid normal basal values are: Testosterone 9.9-27.8 nmol/L; Estradiol 36-470 pmol/L. PPO, Pre-pubertal onset; nIHH, normosmic Isolated Hypogonadotropic Hypogonadism; KS, Kallmann syndrome; AO, adult-onset; AO-doIHH, AO-IHH with defective olfaction. Comparisons were carried out using a Kruskal-Wallis with post-hoc Dunn’s multiple comparison test.

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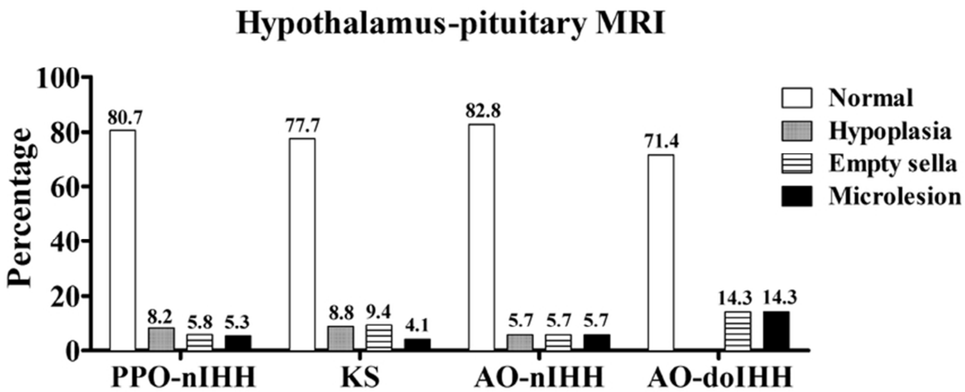

Fig.2: Hypothalamus-pituitary MRI investigations. Results represent the hypothalamus-pituitary MRI investigations in the four groups. PPO, Pre-pubertal onset; nIHH, normosmic Isolated Hypogonadotropic Hypogonadism; KS, Kallmann syndrome; AO, adult-onset; AO-doIHH, AO-IHH with defective olfaction

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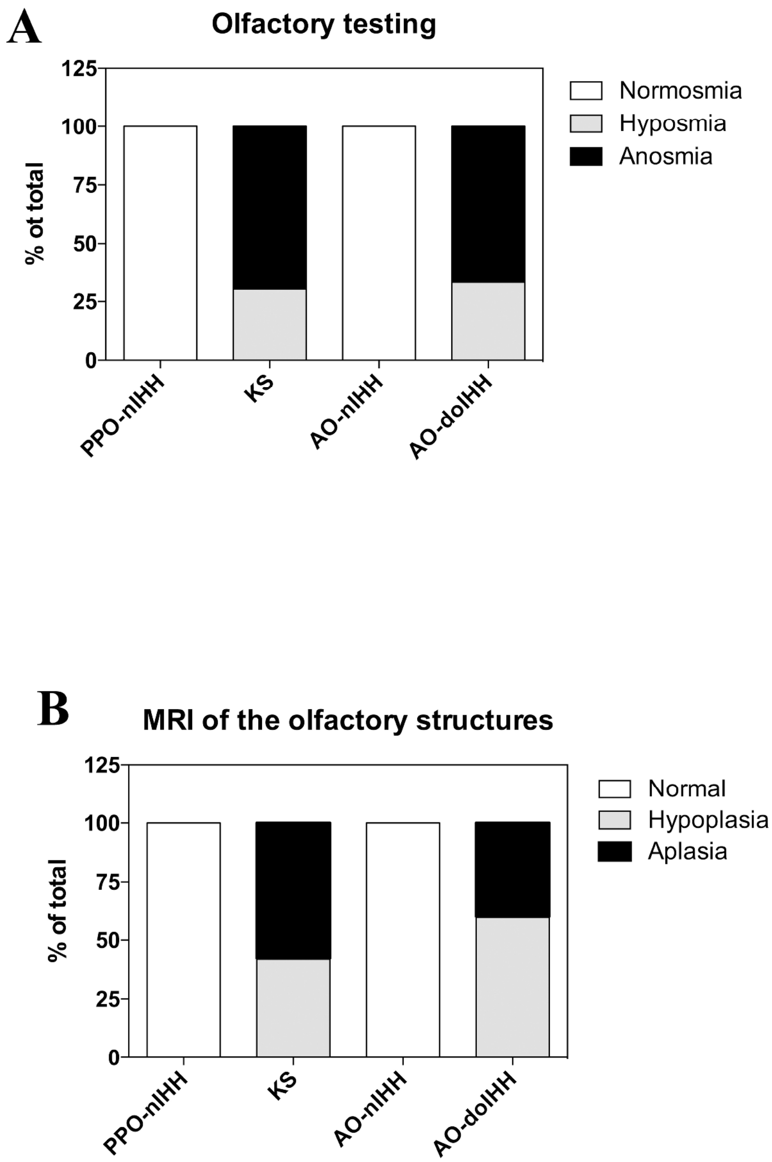

Fig.3: Olfactory testing (A) and MRI investigations of the olfactory structure (B) in the IHH cohort. PPO, Pre-pubertal onset; nIHH, normosmic Isolated Hypogonadotropic Hypogonadism; KS, Kallmann syndrome; AO, adult-onset; AO-doIHH, AO-IHH with defective olfaction; absent, completely absent pubertal development; partial, partial pubertal development; complete, complete pubertal development (=adult onset). Comparisons among categorical variables were performed between KS and AO-doIHH using Chi-square or Fisher's exact test as appropriate and a p-value of 0.025 was considered as critical value after multiple adjustments (0.05/2).

127x192mm (600 x 600 DPI)