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# Loss-of-function variants in *SEMA3F* and *PLXNA3* encoding semaphorin-3F and its receptor plexin-A3 respectively cause idiopathic hypogonadotropic hypogonadism

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**PURPOSE:** Idiopathic hypogonadotropic hypogonadism (IHH) is characterized by absent puberty and subsequent infertility due to gonadotropin-releasing hormone (GnRH) deficiency. IHH can be accompanied by normal or compromised olfaction (Kallmann syndrome). Several semaphorins are known potent modulators of GnRH, olfactory, and vomeronasal system development. In this study, we investigated the role of Semaphorin-3F signaling in the etiology of IHH.

**METHODS:** We screened 216 IHH patients by exome sequencing. We transiently transfected HEK293T cells with plasmids encoding wild type (WT) or corresponding variants to investigate the functional consequences. We performed fluorescent IHC to assess SEMA3F and PLXNA3 expression both in the nasal region and at the nasal/forebrain junction during the early human fetal development.

**RESULTS:** We identified ten rare missense variants in *SEMA3F* and *PLXNA3* in 15 patients from 11 independent families. Most of these variants were predicted to be deleterious by functional assays. SEMA3F and PLXNA3 are both expressed along the olfactory nerve and intracranial projection of the vomeronasal nerve/terminal nerve. PLXNA1-A3 are expressed in the early migratory GnRH neurons.

**CONCLUSION:** SEMA3F signaling through PLXNA1-A3 is involved in the guidance of GnRH neurons and of olfactory and vomeronasal nerve fibers in humans. Overall, our findings suggest that Semaphorin-3F signaling insufficiency contributes to the pathogenesis of IHH.

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## INTRODUCTION

Idiopathic hypogonadotropic hypogonadism (IHH) is a rare genetic disorder characterized by complete or partial pubertal failure caused by gonadotropin-releasing hormone (GnRH) deficiency. According to olfactory function, IHH is divided into two major forms: normal sense of smell (normosmic IHH, nIHH) and inability to smell, anosmia, defined as Kallmann syndrome (KS). However, this distinction is often blurred, probably reflecting the wealth of pathophysiological mechanisms and their variety of combinations in individual cases. Up to the present, nearly 50 genes have been reported to be associated with IHH;[1] these account for nearly 50% of all cases thus suggesting that other associated genes remain to be discovered. To date, the genes implicated in IHH impact GnRH neuron ontogenesis, GnRH neuron migration and/or axon growth, GnRH secretion, and/or gonadotrope function. GnRH-secreting neurons are unique neuroendocrine cells as they originate in the nasal placode, outside the central nervous system, during embryonic development, and migrate to the hypothalamus along the vomeronasal and terminal nerves (VNN, TN).[2] This process is evolutionarily conserved and follows a similar spatiotemporal pattern in all mammals,[2] including humans.[3,4] Maldevelopment of this neuroendocrine system results in hypothalamic hypogonadotropic hypogonadism. Unraveling new genetic pathways involved in the development or function of GnRH neurons is relevant for understanding the basis of pathogenesis leading to IHH in humans.

Proper navigation of growing axons and neurons during embryonic development depends on the action of guidance cues, which include semaphorins, a large and diverse family of secreted and membrane-associated proteins.[5] Correct targeting of GnRH neurons and olfactory/vomeronasal projections have been shown to depend on the orchestrated action of this family of guidance cues.[6] Variants in members of class 3 semaphorins, *SEMA3A*, *SEMA3E*, and *SEMA3G*, have been associated with IHH.[7–9]

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To exert its functions, SEMA3s bind to Neuropilin coreceptors (NRP1 and NRP2) in heterocomplexes with PlexinA1-4 (PLXNA1-4) receptors to activate plexin signal transduction.[10] Loss of function of Plxna1, Nrp1, and Nrp2 have been linked to defective GnRH neuron development in mice,[9,11–13] and nonsynonymous heterozygous variants in *PLXNA1* and *NRP2* have been identified in KS individuals.[12]

Among SEMA3s, there is semaphorin-3F (SEMA3F), a secreted protein that serves as a guidance cue to repel late-arriving olfactory axons that express neuropilin-2 (Nrp2) receptor to the olfactory bulbs.[14] SEMA3F, PLXNA3, and NRP2 have a spatiotemporal expression consistent with a possible role in regulating the GnRH system, olfactory, and vomeronasal development.[3,13,14]

## MATERIALS AND METHODS

### Patients

A total of 216 IHH patients (nIHH, $n = 181$ and KS, $n = 35$) from 178 independent families recruited in Turkey were included in the study. IHH patients had absent pubertal development by age 13 and 14 in girls and boys respectively and low or normal basal gonadotropin levels in the face of low estradiol/testosterone levels. The KS patients additionally had anosmia/hyposmia. The levels of olfactory function were determined based on self-reporting and physical examination by administering a culturally appropriate ten-item (mint, lemon, soap, etc.) smell test.

### DNA sequencing and rare variant analyses

The genomic DNA samples for exome sequencing (ES) were prepared as an Illumina sequencing library, and in the second step, the sequencing libraries were enriched for the desired target using the Illumina Exome Enrichment protocol. The captured libraries were sequenced using Illumina HiSeq 2000 Sequencer (Macrogen, Seoul, Korea). The reads were mapped against UCSC (https://genome.ucsc.edu/cgi-bin/hgGateway) hg19. The presence of significant variants was verified by Sanger sequencing on an Applied Biosystems PRISM 3130 auto sequencer. The details of DNA sequencing and rare variant analyses including burden testing are found in the Supplementary Information.

### Cell-based functional assays

To test the functionality of the variants identified in KS and nIHH probands, we transiently transfected HEK293T cells with plasmids encoding the human wild-type proteins (SEMA3F and PLXNA3) or the corresponding variants and investigated whether the *SEMA3F* secretory capacity or *PLXNA3* protein maturation and trafficking of transfected cells was affected. Details of the cell-based functional assays are provided in Supplementary Materials and Methods.

Collection and processing of human fetuses and immunohistochemistry for GnRH were performed as previously described[3,15] and detailed in the Supplementary Materials and Methods.

## RESULTS

We identified heterozygous or hemizygous missense variants in *SEMA3F* (HGNC:10728) and *PLXNA3* (HGNC:9101) genes, respectively in 15 patients from 11 unrelated families. Clinical and molecular genetic characteristics of the patients and their alterations are shown in Table 1 and Supplementary Table 1, respectively. The location of the variants on *SEMA3F* and *PLXNA3* gene diagrams and the pedigrees are depicted in Figs. 1 and 2. We found six rare *SEMA3F* variants located in critical regions for the proper protein function or its dimerization (Fig. 1a). We identified one variant (T29M) located close to the signal sequence (SS) of the protein, one (P452T) located within the SEMA domain, one (A652S) in the Ig domain, and three (R699W, P722L, T724M) located in the basic motif of the protein (Fig. 1a). Seven IHH patients carried variants in *PLXNA3* respectively in the SEMA binding domain (R108C), in the Plexin-Semaphorin-Integrin (PSI2) domain (S646P), and in the Ig domain (IPT3 region) (L1086V), and RAS-GAP domain (R1359C), which suggest that these variants are likely to affect ligand and coreceptor interactions (Fig. 2a).

**Table 1.** Clinical characteristics of individuals with *SEMA3F* and *PLXNA3* variants.

| Family/individual number | Gene/variant | Age at diagnosis | Sex | Obesity | Olfaction | Reproductive phenotype |
|---|---|---|---|---|---|---|
| A I-2 | *SEMA3F* p.Pro452Thr | 28 | F | No | Anosmic | No delayed puberty or infertility reported |
| A II-1 | *SEMA3F* p.Pro452Thr | 20 | F | No | Anosmic | Absent puberty, primary amenorrhea |
| A II-2 | *SEMA3F* p.Pro452Thr | 12 | M | Obese | Anosmic | Absent puberty, cryptorchidism |
| B II-1 | *SEMA3F* p.Pro452Thr | 10.5 | M | Overweight | Normosmic | Cryptorchidism |
| C II-1 | *SEMA3F* p.Arg699Trp | 16 | F | No | Normosmic | Absent puberty, primary amenorrhea |
| D II-2 | *SEMA3F* p.Thr29Met | 17 | M | Obese | Hyposmic | Absent puberty, cryptorchidism |
| E II-1 | *SEMA3F* p.Pro722Leu | 14 | M | Obese | Normosmic | Absent puberty |
| F II-1 | *SEMA3F* p.Ala652Ser | 35 | M | No | Hyposmic | Absent puberty, micropenis, infertility |
| G II-2 | *SEMA3F* p.Thr724Met<br>*PLXNA3* p.Ser646Pro | 20 | F | No | Normosmic | Primary amenorrhea, delayed menarche at age 16 |
| G II-6 | *SEMA3F* p.Thr724Met<br>*PLXNA3* p.Ser646Pro | 14 | F | No | Normosmic | Absent puberty |
| H II-1 | *PLXNA3* p.Arg108Cys | 19 | M | No | Normosmic | Absent puberty |
| H II-2 | *PLXNA3* p.Arg108Cys | 17 | M | No | Normosmic | Absent puberty |
| I II-1 | *PLXNA3* p.Arg108Cys | 21 | M | No | Hyposmic | Absent puberty, cryptorchidism |
| J II-1 | *PLXNA3* p.Leu1086Val | 14 | M | Overweight | Normosmic | Absent puberty, micropenis |
| K II-1 | *PLXNA3* p.Arg1359Cys | 18 | F | Obese | Normosmic | Absent puberty, primary amenorrhea |

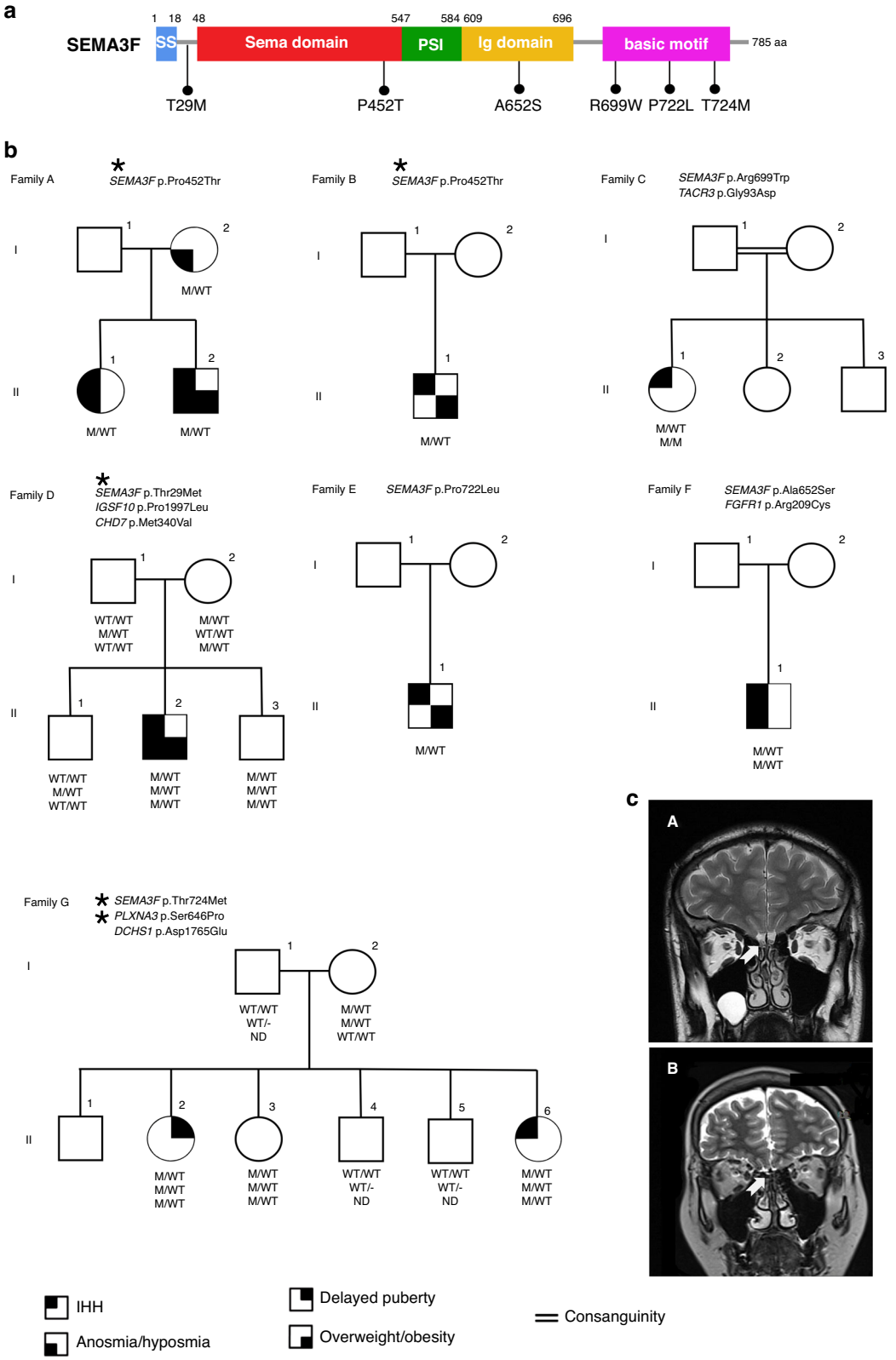


**Fig. 1** ***SEMA3F* variants in the etiology of idiopathic hypogonadotropic hypogonadism.** (**a**) The variants are depicted on the functional gene diagram of *SEMA3F*. (**b**) The pedigrees of seven families with *SEMA3F* variants are shown. Affected males and females are represented by black squares and black circles respectively. White square symbols indicate unaffected male family members, white circle symbols represent unaffected female family members, and the double line indicates consanguinity. Under each symbol are the genotypes in the same order as the gene and variant descriptions, with WT and M denoting wild type and variant, respectively. IHH idiopathic hypogonadotropic hypogonadism, ND not determined, – denotes allele naturally absent in males as *PLXNA3* is on the X chromosome. (**c**) The T2-weighted magnetic resonance image (MRI) from a healthy control (**A**) and from patient D II-2 (**B**). The arrows point to normal (**A**) and aplastic olfactory bulbs (**B**).

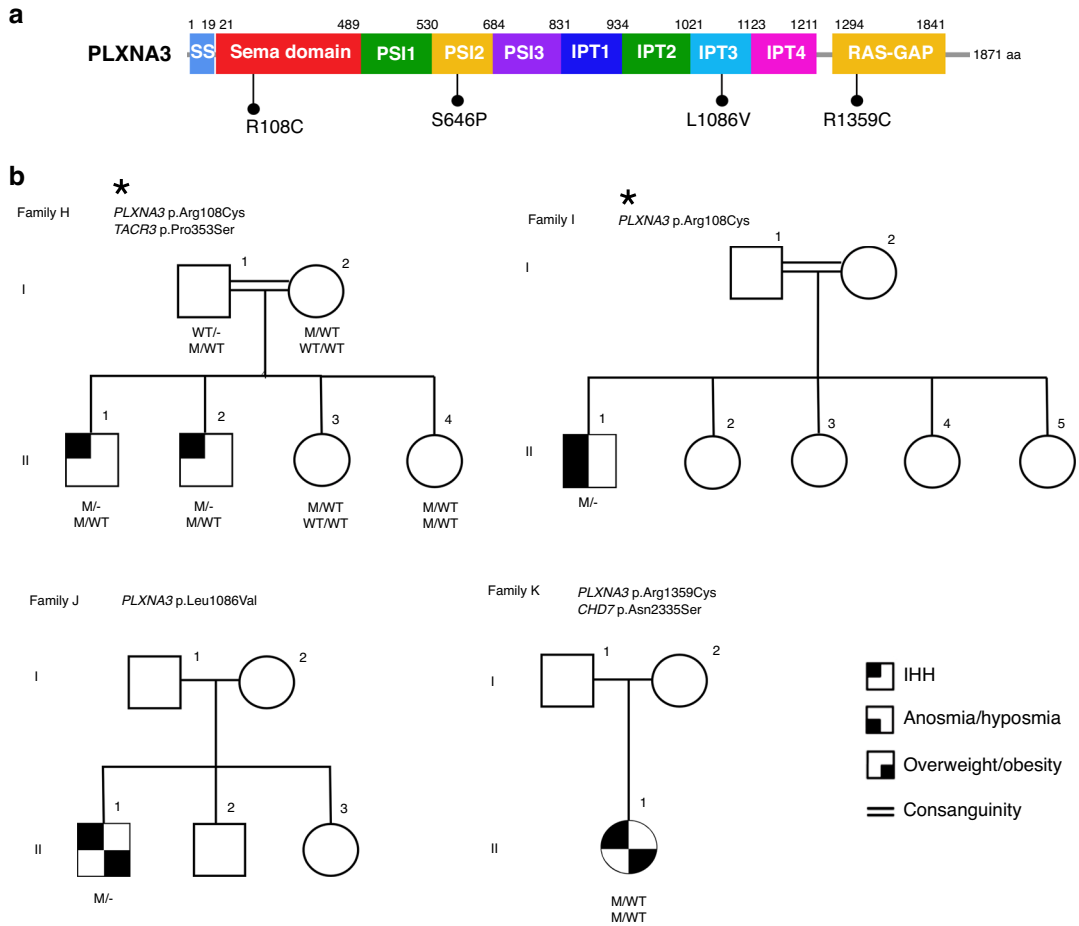


**Fig. 2 *PLXNA3* variants in patients with idiopathic hypogonadotropic hypogonadism.** (**a**) The diagram of *PLXNA3* showing the positions of the missense variants found in patients. (**b**) The pedigrees of the four families with *PLXNA3* variants are shown. Note that patients in family G in Fig. 1 also have *PLXNA3* variants in addition to *SEMA3F* variants. Affected males and females are represented by black squares and black circles, respectively. White square symbols indicate unaffected male family members, white circle symbols represent unaffected female family members, and the double line indicates consanguinity. Under each symbol are the genotypes in the same order as the gene and variant descriptions, with WT and M denoting wild type and variant, respectively. Since *PLXNA3* is on the X chromosome male individuals are expected to have only one allele. IHH idiopathic hypogonadotropic hypogonadism, – denotes allele naturally absent in males as *PLXNA3* is on the X chromosome.

To avoid harmless polymorphisms, in addition to quality and read depth filtering, we filtered the protein altering heterozygous/hemizygous variants by setting a threshold occurrence of 1:10,000. These ten variants were either not seen or extremely rare with a minor allele frequency <0.0001 in the two largest reference population databases, TOPMed and gnomAD. Similarly, these variants were not reported in a much smaller but regional database, the Greater Middle East Variome (GME) (except for the *SEMA3F* R699W variant appearing once in 163 Turkish alleles). Sanger sequencing confirmed the presence of these variants. No variant was seen in ClinVar. These variants were all classified as variants of uncertain significance (VUS) by American College of Medical Genetics and Genomics/Association for Molecular Pathology (ACMG/AMP) classification.[16] Detailed information regarding these variants is given in Supplementary Table 1.

In the etiology of IHH, almost all causative gene alterations show phenotypic variability and segregations in families are often irregular as in some of the pedigrees in this study. Oligogenic etiology[17] and clinical reversibility,[18] among others, are well recognized in this condition to explain these complex pedigrees.

In 6 of the 11 kindreds (54%), there was at least one more gene known to be associated with IHH (oligogenecity).[1,17] This high rate is consistent with our recent observation of the increased detection of oligogenecity in the etiology of IHH,[19] which was earlier reported to be 10–20%.[17] This probably reflects the increased number of recognized genes for IHH as well as recent diagnostic popularity of exome sequencing. Overall, the inheritance pattern of IHH due to the *SEMA3F* variants is consistent with an autosomal dominant inheritance with variable penetrance and expressivity, irrespective of the inheritance whether it is familial or sporadic, which was repeatedly observed in recent IHH gene discoveries.[20] The inheritance of IHH due to the *PLXNA3* variants is consistent with the X-linked recessive pattern. With the exception of one *SEMA3F* (p.Pro452Thr) and one *PLXNA3* (p.Arg108Cys) variant, all other variants were encountered once. Notably, the sisters in family G had variants in both *SEMA3F* and *PLXNA3*, both inherited from their apparently unaffected mother, but their phenotypes were not more severe. In fact, these sisters had variable phenotypes. While the proband (G II-6) suffered from complete IHH, the other affected sister (G II-2) had only delayed menarche. Yet, a third sister (G II-3), even though she has the same genotype as the affected two sisters, did not display any abnormal pubertal or reproductive abnormalities. We cannot readily explain the inconsistencies of the phenotypes among these three sisters who have the same genotype. However, it is increasingly well appreciated in the genetics of IHH that oligogenic contribution with variably reduced penetrance complicates pedigrees.[21] Overall, 6 of 15 (40%) patients (5 of them with *SEMA3F* variants) had

compromised olfaction. In two of them, brain magnetic resonance images (MRIs) were obtained, which showed hypoplastic/aplastic olfactory bulbs. Figure 1c depicts the absent olfactory bulbs in case D II-2 due to p.Thr29Met in *SEMA3F*. These data are consistent with previous rodent studies showing the role of *SEMA3F* in the proper projections and fasciculations of the olfactory nerve and thus in the development of the olfactory bulb.[14] Interestingly, the same *SEMA3F* variant (Pro452Thr) was associated with normosmia or anosmia in two different families (families A and B, respectively). On the other hand, male patients in both families had cryptorchidism, indicating the presence of severe congenital hypogonadism. In congenital IHH, fetal pituitary gonadotropin secretion is low, leading to inadequate testosterone levels in fetal serum. As the testicular descent and growth of phallus are androgen-dependent, consequently, boys with severe IHH present with micropenis, cryptorchidism, and hypospadias at birth.[22] Again, there was a discrepancy in olfactory function in patients carrying the same *PLXNA3* variant (p.Arg108Cys) in two different kindreds (families H and I, Fig. 2a, b). These observations suggest that there is a complex input to the olfactory phenotype. To date, all Semaphorin signaling gene variant discoveries were carried out solely in the KS patient cohorts.[9,12,23] This may have obscured the breadth of the phenotypic spectrum associated with this versatile family of molecules. Our population, on the contrary, is made up of all-inclusive IHH patients regardless of their olfactory functions. Therefore, our study has an unrestricted ability to observe all

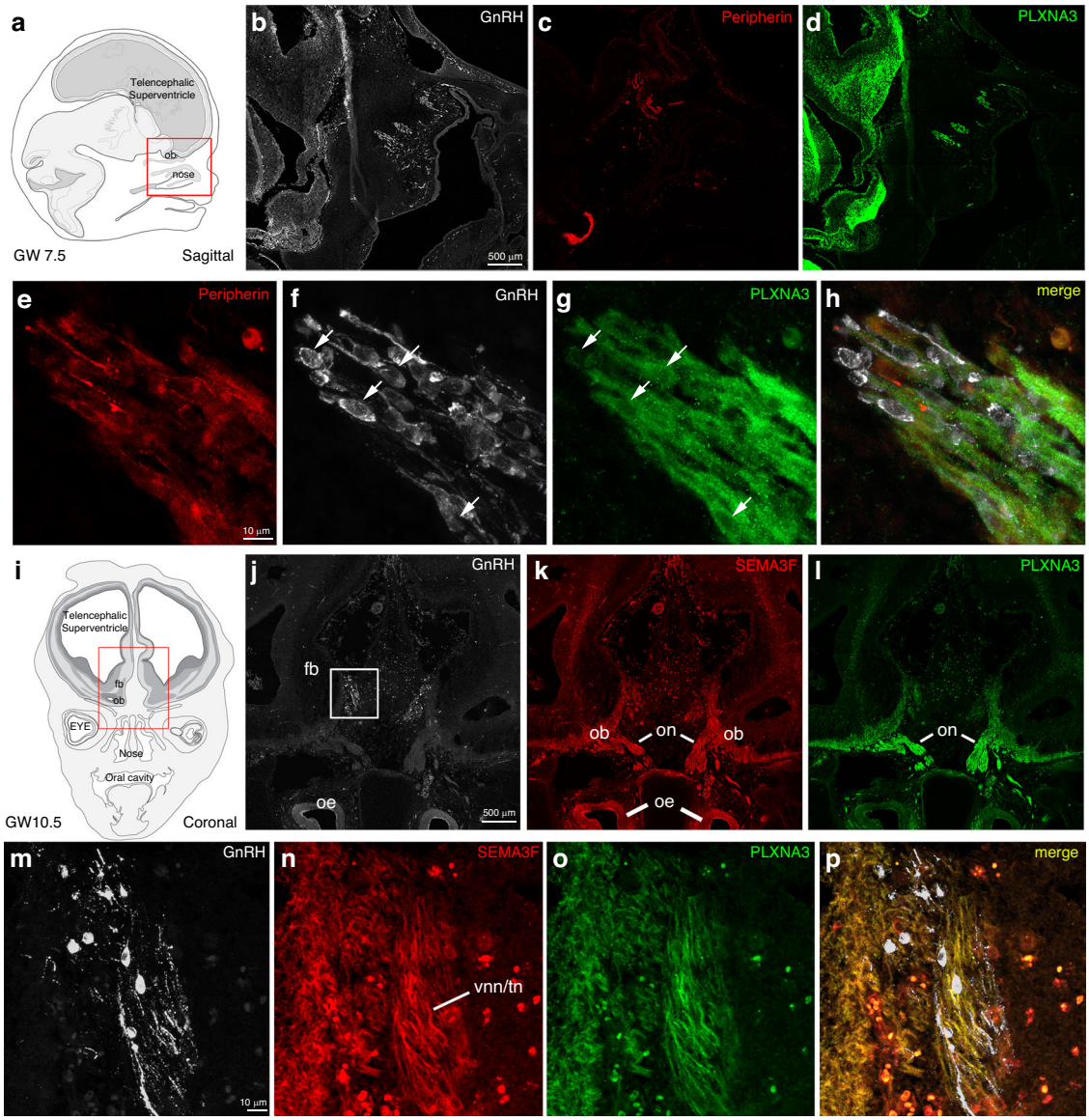


**Fig. 3 Semaphorin-3F and Plexin-A3 are expressed along the migratory route of gonadotropin-releasing hormone (GnRH) neurons in human fetuses.** Fluorescent immunohistochemistry (IHC) was performed to assess SEMA3F and PLXNA3 expression both in the nasal region (**a**) and at the nasal/forebrain junction (**i**) during the early fetal development. (**b**–**d**) Representative immunostaining for GnRH, Peripherin, and Plexin-A3 on sagittal sections of a GW7.5 human fetus. (**e**–**h**) Magnification of the boxed area in (**b**), revealing that PLXNA3 is found in the peripherin-positive olfactory/vomeronasal scaffold, as well as in GnRH neurons (arrows) during their migration through the nasal compartment. (**j**–**l**) Representative pictures of immunolabelings for GnRH, PLXNA3, and SEMA3F on coronal sections of a GW10.5 human fetus. (**m**–**p**) Magnification of the boxed area in (**j**). The olfactory and vomeronasal/terminal nerves still show immunoreactivity for PLXNA3 at the nose/forebrain junction. SEMA3F is strongly expressed in this region and detected along the PLXNA3-positive nerves. GnRH neurons migrating inside the forebrain do not longer express PLXNA3. fb forebrain; ob olfactory bulbs, oe olfactory epithelium, on olfactory nerves, vnn/tn vomeronasal/terminal nerves. Scale bars: (**b**,**j**) = 500 µm; (**e**,**m**) = 10 µm.

possible phenotypes. Case in point, in a previous screening study of *PLXNA1* variant cases in the same cohort as in this study, we found nine cases, among which only one third had compromised olfaction.[19] In contrast, the seminal publication on this gene was from an all anosmic (KS) cohort.[12]

Western blot analysis revealed normal protein content of *SEMA3F* variants in whole cell lysates (Supplementary Fig. 1a, b). By contrast, *SEMA3F*s harboring the P452T, T29M, and T724M missense variants showed impaired SEMA3F secretion, as shown by western blot analysis of the conditioned media (Supplementary Fig. 1a, c). The P722L variant, on the other hand, had secreted levels of protein similar to the wild type. Since the family E proband has *SEMA3F* P722L only, it is curious if this variant is responsible for the IHH phenotype. Even though *SEMA3F* P722L is an extremely rare variant, the functional studies did not support its potential deleterious ramifications. Therefore, it is unlikely that this variant is solely responsible for the IHH phenotype in the family E proband. Interestingly this proband also has a variant in *QRFP* (NM_198180:c.G277A:p.E93K), which is heavily expressed in the hypothalamic arcuate nucleus and is known to regulate food intake.[24] Its product, Pyroglutamylated RFamide peptide 43, has been shown to stimulate the hypothalamic–pituitary–gonadal axis via gonadotropin-releasing hormone in rats.[25] However, no literature exists associating this gene with IHH in humans. Given the extreme obesity and IHH of this patient, it is tempting to think that variants in these two genes together contributed to the IHH phenotype of this patient. Since SEMA3F is a bifunctional guidance molecule that can exert both axon-repulsive and -attractive effects depending on its gradient and on receptors' composition on target cells,[26] future studies will be aimed at understanding how different SEMA3F concentrations and different receptors' complexes may affect GnRH neuronal migration and/or olfactory and vomeronasal axon orientation. We also tested the effect of the *PLXNA3* variants (with the exception of R1359C and L1086V) on receptor synthesis by western blot analyses on transfected HEK293T cells producing *PLXNA3* WT, *PLXNA3* S646P, and *PLXNA3* R108C (Supplementary Fig. 2). The fact that the two functionally untested variants (L1086V and R1359C) are located, respectively, in the Ig domain (IPT3 region) and in the RAS-GAP domain (R1359C) suggests that these variants are likely to affect ligand and coreceptor interactions, based on the structural model of the protein.[27]

Immunoblots on cell lysates from HEK293T cells transfected with *PLXNA3* WT or with the mutant proteins revealed the expression of a band for PLXNA3 at the expected molecular weight (220 kDa), which was absent in mock-transfected cells (Supplementary Fig. 2a). However, cells expressing the mutant *PLXNA3*s presented two isoforms (Supplementary Fig. 2a), which suggests glycosylation modifications at the level of the ER ("immature forms of proteins").

We next immunostained transfected HEK293 cells for PLXNA3 and Calnexin, a marker of the endoplasmic reticulum (ER). WT PLXNA3 was predominantly localized on the plasma membrane (Supplementary Fig. 2B). In contrast, S646P, but not R108C, variant showed PLXNA3 localization exclusively in the ER (Supplementary Fig. 2B, C), indicating that the variant S646P disrupts cell surface localization of PLXNA3. Overall, these data revealed the existence of two PLXNA3 isoforms, which raises two functional hypotheses: (1) these variants could affect the processing and/or maturation of the glycosylation of the receptor with a subsequent accumulation of PLXNA3 in ER, and/or (2) these variants could affect the protein trafficking of the receptor from the ER to the cell surface. The observation that the PLXNA3 S646P variant was predominantly found within the ER, rather than at the cell surface, supports those hypotheses. Interestingly, all probands carrying variants in PLXNA3 were males (Table 1). These data together with the fact that PLXNA3 lies on the X chromosome suggest that these individuals have likely minimal signaling through PLXNA3 and argue in favor of the pathogenic effect of PLXNA3 variants in IHH.

With the exception of NRP2,[3] expression of SEMA3F signaling pathway has never been investigated in the developing human GnRH and olfactory system. Thus, we evaluated the expression pattern of SEMA3F and PLXNA1-A4 receptors in sagittal and coronal sections of human fetal heads (gestational weeks post amenorrhea: GW, GW7.5 and GW10.5), together with the expression of GnRH and peripherin, a marker of the developing

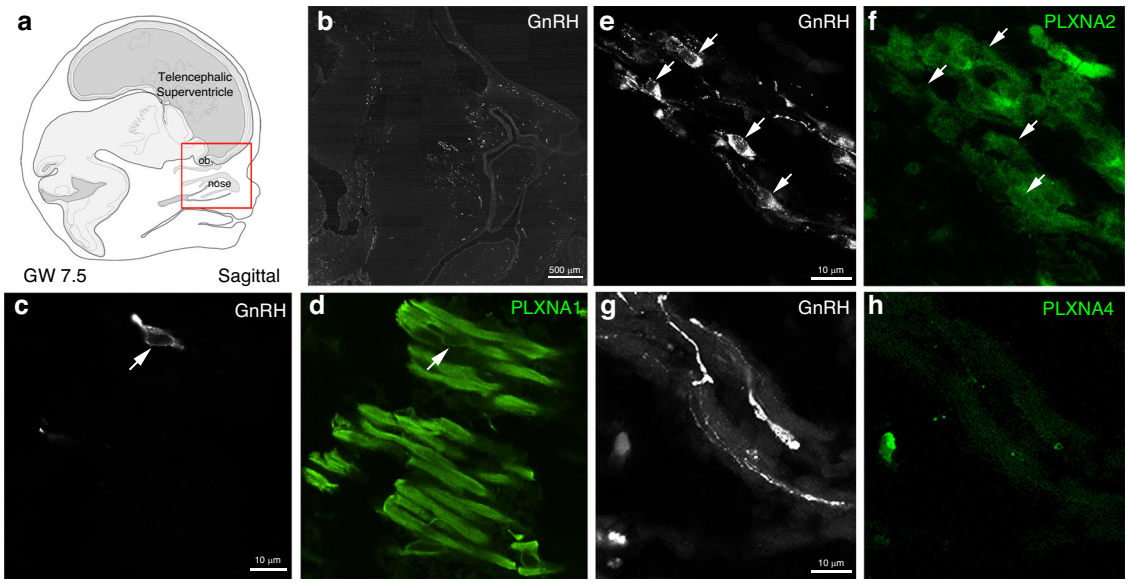


**Fig. 4 PLXNA1 and PLXNA2 could also contribute to SEMA3F signaling in the human fetal nose.** Since SEMA3F can signal not only through PLXNA3, but through the PLXNA1-A4 family, additional stainings were performed to check for PLXNA1-A4 expression in the nasal compartment of a GW7.5 human fetus (**a**,**b**). In addition to PLXNA3, migrating gonadotropin-releasing hormone (GnRH) neurons and the olfactory/vomeronasal scaffold also express PLXNA1 (**c**,**d**) while PLXNA2 was detected in GnRH neurons and other cells of the migratory mass, but not in the olfactory/vomeronasal projections (**e**,**f**). However, PLXNA4 immunoreactivity was only found in scattered cells, distant from the migratory route of GNRH neurons (**g**,**h**). ob olfactory bulbs. Scale bars: (**b**) = 500 µm; (**e**,**c**,**g**) = 10 µm.

olfactory and vomeronasal fibers[3,28] (Figs. 3, 4). At GW7.5, the great majority of GnRH neurons migrate in chains across the nasal septum along vomeronasal/terminal peripherin-positive fibers directed toward the forebrain (Fig. 3b, c, e, h), in agreement with our previous immunohistochemical study.[3] *PLXNA3* expression was found along the VNN and TN (Fig. 3d–h), as well as in migratory GnRH neurons (see arrows in Fig. 3f, g). At GW11, about 80% of GnRH neurons have been reported to enter the brain and migrate toward their final target areas.[3] Our immunofluorescence analysis of a GW10.5 fetus indeed revealed robust GnRH immunoreactivity in migratory cells invading the developing forebrain (Fig. 3i, j, m). Similarly to the mouse olfactory system, where SEMA3F is secreted by early-arriving olfactory axons and deposited at the anterodorsal olfactory bulb (OB),[14] in GW10.5 human fetuses, *SEMA3F* is expressed along the olfactory nerve (on), by the developing olfactory epithelium (oe) and by the VNN and TN entering the brain (Fig. 3k, n). *SEMA3F* expression was absent in human migratory GnRH neurons (Fig. 3m, n). At this fetal stage, like at GW7.5, *PLXNA3* is expressed along the VNN and TN, which also express its ligand (Fig. 3l–p). However, at GW10.5 GnRH neurons are PLXNA3-negative (Fig. 3m, o, p), thus suggesting that PLXNA3 could be downregulated in GnRH neurons that entered the brain compartment.

To assess the expression pattern of the other PLXN receptors through which *SEMA3F* can possibly signal, we next performed double immunofluorescence staining of sagittal sections of GW7.5 fetuses using antibodies raised against GnRH and PLXNA1, PLXNA2, and PLXN4. This analysis revealed that GnRH neurons migrating in the nasal compartment express PLXNA1 and PLXNA2 (Fig. 4a–f; arrows) but not PLXNA4 (Fig. 4g, h). Also, PLXNA1 was found to be expressed by the VNN and TN that form the migratory scaffold for GnRH neurons (Fig. 4d). GnRH neurons migrate together with a heterogeneous coalescence of placode-derived and neural crest-derived migratory cells and olfactory axons, collectively called the migratory mass (MM).[29] At this stage, we observed a mixed mass of GnRH neurons and other cells migrating across the nasal mesenchyme toward the telencephalon expressing PLXNA2 (Fig. 4e, f). Overall, these results show that SEMA3F and PLXNA3 are both expressed along the olfactory nerve (on) and along the intracranial projection of the VNN and TN. Our immunohistochemical data also indicated that the signal-transducing receptors PLXNA1-A3 are expressed in early migratory GnRH neurons, therefore suggesting that SEMA3F signaling through PLXNA1-A3 could be involved in the guidance of GnRH neurons and of olfactory and vomeronasal nerve fibers in humans.

## DISCUSSION

Our findings suggest that SEMA3F signaling is necessary for correct human puberty onset and reproduction. To substantiate this point, we present clinical, molecular, genetic, and in vitro functional data from 15 patients who belonged to 11 independent families. These probands all presented with pubertal failure and were clinically diagnosed with IHH or KS. With the prevalent involvement of oligogenicity and clinical reversal in the pathogenesis of IHH, it is often challenging to determine which variant is causing the disease phenotype. In families C and F, the other variants (a homozygous *TACR3* and a heterozygous *FGFR1*, respectively) were already classified in the ClinVar database as pathogenic and likely pathogenic for IHH, respectively. We believe that these additional variants were the main driving force of the IHH phenotypes. The effects of the SEMA3F variants on the phenotypes were predicted to be minor in these two family probands. Not considering the patients from these two families, our study reveals that variants in *SEMA3F* and *PLXNA3* are present in 6.0% of patients with nIHH/KS.. The results of this study indicate that genes in the SEMA3F signaling pathway are among the most frequently mutated ones in congenital GnRH deficiency, together with *FGFR1* and *CHD7*.[1]

With regard to any previous implication of SEMA3F in human puberty/reproduction, in a large genome-wide association study, a single-nucleotide polymorphism near *SEMA3F* was found to be strongly correlated with age at first birth, which indicates earlier puberty timing and reproductive success,[30] suggesting the relevance of *SEMA3F* to reproductive function in humans.

To support further our message, we provide unequivocal human embryonic data showing the expression of SEMA3F along the developing human GnRH migratory pathway. Moreover, our data provide compelling evidence that all the receptors required for the SEMA3F signaling, including PLXNA3, a key component of the SEMA3F holoreceptor complex,[31] are expressed by the human GnRH and olfactory/vomeronasal systems. Based on the expression of SEMA3F signaling pathway in the nasal compartment of early human fetuses, and on the chemotactic role of this semaphorin reported in other species,[31] our data raise the hypotheses that SEMA3F/PLXNA3 could regulate GnRH neuronal migration and olfactory/vomeronasal axonal elongation in humans. Notably, our data indicated that the signal-transducing receptors PLXNA1-A3 are expressed in early migratory GnRH neurons. This is consistent with a recent investigation[13] highlighting cooperation of Plxna1 and Plxna3 in the formation of the GnRH migratory scaffold in mice, based on which the human orthologue of Plxna3 (i.e., *PLXNA3*), like PLXNA1, was proposed to be a candidate gene for variant screening in patient with KS. Indeed, in that study[13] the authors have shown that, while mice lacking plxna1 or plxna3 present only mild or absent defects in the development of the GnRH neuron and olfactory systems, double-mutant *Plxna1*$^{-/-}$; *Plxna3*$^{-/-}$ animals phenocopied the full spectrum of nasal axon and GnRH neuron defects typical of KS. In line with the oligogenic nature of KS, these data suggest that *Plxna1* synergizes with *Plxna3* during nasal axon guidance required for proper GnRH neuron migration.

The existing literature indicates that the SEMA3F signals through Nrp2/Plxna3 holoreceptor complex, while Sema3a signaling occurs mainly via Nrp1/Plxna1.[6,31] Importantly, Nrp2 and its ligand, Sema3f, are expressed by olfactory sensory neurons in rodents in a complementary manner that is important for establishing olfactory map topography.[14] Despite these evidences, based on typical GnRH migration patterns and expected numbers of GnRH neurons in the Sema3f deficient mice, this guidance molecule was reported to be dispensable for GnRH neuron migration.[32] However, similarly to the recently described cooperation of *Plxna1* with *Plxna3*,[13] it is possible that *Sema3f* may synergize with other Semaphorins to regulate the proper GnRH migratory process and/or olfactory axonal elongation. Moreover, pubertal and adult reproductive phenotypes of *Sema3f* $^{-/-}$ mice, such as the timing of puberty onset, first estrus, fertility index, litter size, etc. have not been investigated. Besides, species-specific differences in Sema3s functions between mice and humans may exist. In fact, significant species differences, even with well-established reproductive genes including *TAC3/TACR3* and *KISS1/KISS1R*, have been previously documented. Specifically, patients bearing variants in *TAC3* and *TACR3*[33] have sexual infantilism and infertility due to GnRH deficiency. In contrast, a recent transgenic Tacr3(-/-) mouse model demonstrated that normal sexual maturation occurs in these mice, albeit some significant reproductive defects are evident in adulthood.[34] Likewise, while humans with *KISS1*[35] and *KISS1R*[36] variants typically suffer from pubertal failure and infertility, a considerable proportion of female Kiss1 and Kiss1r knockout mice exhibit normal estrous cyclicity, and some male Kiss1- and Kiss1r-null mice exhibit spermatogenesis.[37] These species differences underscore the importance of human studies in puberty/reproduction research.

Finally, a recent work provided compelling evidence that SEMA3s-mediated signaling drives the development of

hypothalamic melanocortin circuits and that variants in these pathways cause obesity.[27] Interestingly, the hypothalamic pro-opiomelanocortin (POMC)-expressing neurons have been repeatedly observed to send projections to and be in close contact with kisspeptin/neurokinin B/dynorphin (KNDy) cells.[38,39] A plethora of investigations have recently identified the role of the hypothalamic KNDy neuronal population as the GnRH pulse generator.[1,39] Importantly, in the study of van der Klaauw and colleagues, one of the patients harboring a *PLXNA3* variant (D1710N) had hypogonadotropic hypogonadism in addition to obesity.[27] However, since the majority of the probands in that study were prepubertal age children, the true prevalence of hypogonadotropic hypogonadism among heterozygotes may be underestimated.[27] Although we noted in this study that a significant proportion of the patients (40%) was obese, *SEMA3F/PLXNA3* variants were not statistically more enriched in the subset of obese IHH patients than in our general IHH cohort, where 56 patients (26.0%) featured overweight or obesity ($p = 0.24$). It would be interesting in the future to carefully look at larger and multiple IHH cohorts and determine whether statistical significance in the frequency of obesity or metabolic disorders could emerge when analyzing larger populations and probands carrying variants in SEMA3F signaling pathway.

In summary, we provide clinical, genetic, molecular/cellular, and developmental evidence to implicate variants in SEMA3F signaling in the etiology of IHH. We propose that this phenotype could be exerted via a direct deleterious effect of these variants on the GnRH neuronal migration and potentially a harmful impact of *SEMA3F/PLXNA3* variants on the development of hypothalamic melanocortin circuits involved in the regulation of energy homeostasis, which are also known to influence GnRH secretion and reproduction. Each of these events or a combination could hence negatively affect the development and function of the hypothalamic–pituitary–gonadal axis. Further studies aimed at addressing the contribution of SEMA3F and PLXNA3 in each one of the abovementioned developmental processes may uncover new mechanisms underlying human disorders characterized by central hypothalamic dysfunction and infertility.

## URLS

HGNC, https://www.genenames.org/
GenBank, https://www.ncbi.nlm.nih.gov/genbank/
TOPMed, https://bravo.sph.umich.edu/freeze5/hg38/
gnomAD Browser, https://gnomad.broadinstitute.org/
Greater Middle East Variome Project (GME), http://igm.ucsd.edu/gme/data-browser.php
InterVar, http://wintervar.wglab.org/
ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/
Combined Annotation Dependent Depletion (CADD), https://cadd.gs.washington.edu
MutationTaster, http://www.mutationtaster.org
PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/
SIFT, http://sift.bii.a-star.edu.sg/
LRT, http://www.genetics.wustl.edu/jflab/lrt_query.html

## DATA AVAILABILITY

The functional studies data that support the findings of this study are available on reasonable request from the corresponding author. The significant genetics variant data that support the findings of this study are available in a public database, ClinVar, where the data have been deposited. The following variants with associated clinical information were submitted deposited to ClinVar. ClinVar accession numbers are shown in parentheses after each variant: SEMA3F p.Pro452Thr (SCV001438328), p.Arg699Trp (SCV001438333), p.Thr29Met (SCV001438332), p.Pro722Leu (SCV001438335), p.Ala652Ser (SCV001438336), p.Thr724Met (SCV001438338). PLXNA3 p.Ser646Pro (SCV001438339), p.Arg108Cys (SCV001438342), p.Leu1086Val (SCV001438354), p.Arg1359Cys (SCV001438811). The raw human genetics data are not publicly available due to consent form restrictions.

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Conceptualization: A.K.T., L.D.K., P.G. Data curation: I.T., B.Y., F.G. Formal analysis: D.J., G.D., P.G., L.D.K. Funding acquisition: A.K.T., P.G. Investigation: L.D.K, G.T. Resources: A.D.C, H.C.E., A.D.K., B.O., E.I., E.M., E.D., P.C., A.Y., S.Y.A., S.O.D., O.E., F.D., G.A. Writing—original draft: A.K.T., L.D.K., P.G. Writing—review & editing: A.K.T., P.G.

## ETHICS DECLARATION

All individuals and/or their legal guardians provided written informed consent, and the study was approved by the Ethics Committee of the Cukurova University Faculty of Medicine and by the institutional review board of the University of Mississippi Medical Center.

## COMPETING INTERESTS

The authors declare no competing interests.

## ADDITIONAL INFORMATION

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