

## Commentary on *NOBOX* Mutations in Premature Ovarian Insufficiency

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### Abstract

*NOBOX* is an ovarian specific transcription factor that plays an important role in follicular growth and survival. Nineteen *NOBOX* variants have been previously associated with premature ovarian insufficiency (POI). Disease severity in patients with heterozygous and homozygous mutations largely overlap however, hampering genotype-phenotype correlations. We recently reported the first case of biallelic truncating mutations (NM\_001080413.3 (NOBOX):c.826C>T, p.(Arg276\*) and NM\_001080413.3(NOBOX):c.1421del, p.(Gly474Alafs\*76)) of *NOBOX* in two Belgian sisters with POI. Both variants were identified by whole exome sequencing (WES) and classified as pathogenic following ACMG guidelines. Our findings suggest that haploinsufficiency of *NOBOX* can be well tolerated. Furthermore, compound heterozygosity for the two *NOBOX* variants was very likely responsible for the severe POI phenotype in the two sisters, who presented with primary amenorrhea (PA), delayed puberty and hypergonadotropic hypogonadism. In addition, we searched for *NOBOX* variants in a cohort of 151 unrelated POI patients and showed a prevalence of *NOBOX* mutations of 1.3%, contrasting with previous reports of *NOBOX* mutation prevalence in POI patients up to 9%. A new *NOBOX* variant was identified in our cohort (NM\_001080413.3 (NOBOX): c.259C>A, p.(Pro87Thr)) and reported for the first time in the present paper.

**Keywords:** *NOBOX* gene, Delayed puberty, Premature ovarian insufficiency, Next generation sequencing, Hypergonadotropic hypogonadism, Amenorrhea

### Main text

We have recently reported two novel, biallelic truncating mutations of the Newborn Ovary homeoBOX-encoding gene (*NOBOX*) (OMIM: 610934) in two Belgian sisters presenting a severe form of non-syndromic premature ovarian insufficiency (POI). Both sisters had an absence of spontaneous breast development and primary amenorrhea with hypergonadotropic hypogonadism. *NOBOX* variants

(NM\_001080413.3 (NOBOX):c.826C>T, p.(Arg276\*) and NM\_001080413.3(NOBOX):c.1421del, p.(Gly474Alafs\*76)), were identified by WES performed in trio in the index case and her two parents. Sanger sequencing confirmed the inheritance of both mutated alleles at a compound heterozygous state by the affected sister, parents were heterozygous carriers of each variant [1].

POI is a female specific syndrome that affects 1% to up to

3.7% of women under their 40s and constitutes an important cause of female infertility [2,3]. POI is characterized by a vast heterogeneity in its clinical presentations and etiologies [4]. A genetic origin is identified in at least 20-25% of POI patients [5]. Recognized genetic origins of POI mainly include chromosomal abnormalities, *FMR1* premutations and single gene mutations [5-11]. More recently, oligogenic inheritance has also been suspected in POI [12,13]. Affected women can present primary amenorrhea with delayed puberty or normal pubertal development, secondary amenorrhea or oligoamenorrhea associated with hypergonadotropic hypogonadism [2]. *NOBOX* is a well-known POI causing gene. It encodes an ovarian specific transcription factor expressed in primordial germ cells, germ cell cysts, primordial follicles and growing oocytes [14,15]. It plays an important role in follicular growth and survival beyond the primordial stage [15]. The absence of *Nobox* accelerates postnatal oocyte loss and abolishes the transition from primordial to growing follicles in mice. Ovarian histology of *Nobox*<sup>-/-</sup> mice showed at day 0 after birth, the presence of oocytes clustered in germ cell cysts and primordial follicles, while at day 7, only few primordial and primary without secondary follicles and at day 14 complete loss of most. All *Nobox*<sup>-/-</sup> mice had atrophic ovaries and no oocytes at 6 weeks of age. *Nobox*<sup>+/-</sup> mice were however fertile up to 9 months of life, supporting that haploinsufficiency of *Nobox* in mice was well tolerated, which contrasts with human data, where dominant inheritance was mostly observed [1,15]. In women, *NOBOX* mutations have been associated with POI both in autosomal recessive and dominant manner [1]. Interestingly, in previously reported POI cases related to *NOBOX* mutations, the severity of the clinical presentation was not associated with the number of affected alleles [1]. In fact, patients with heterozygous mutations can present primary amenorrhea (PA) and delayed puberty; homozygous mutations, although more rarely reported, can be associated with both secondary and primary amenorrhea. The absence of correlation between the number of affected alleles and the severity of the clinical presentation of POI can potentially be related to an oligogenic nature of POI and a complex genetic background in which other susceptibility genes might modulate the patient's phenotype [11-13]. In the past, the potential implication of heterozygous *NOBOX* variants was suggested to be related to *NOBOX* haploinsufficiency. However, our report shows that the haploinsufficiency of *NOBOX* in human could be tolerated as the patient's mother who was heterozygous carrier of c.1421del was menopausal at 52 years [1]. This last mechanism is in accordance with mice models as no defect on follicular growth beyond the primordial stage has been observed in *Nobox*<sup>+/-</sup> mice [15]. Recent functional studies of *NOBOX* variants identified in POI patients supported rather a dominant negative effect of mutated proteins at the heterozygous state. Actually, *NOBOX* mutated proteins can be instable and induce intracellular aggregates, partial sequestration of wild type protein, nuclear localization impairment and cell toxicity [16]. In our report, a dominant negative impact was not suspected

for the c.1421del variant as it was inherited from a non-POI mother. However, we could not exclude this mechanism for the c.826C>T variant inherited from the healthy father, as his family story was less relevant [1]: the father was an only child, his maternal and paternal aunts were unable to conceive without any reported underlying cause of infertility, and no sequencing for *NOBOX* mutations could be performed in his parents and aunts. Although no histologic analysis of ovarian tissue was performed in our patients, the complete absence of breast development and primary amenorrhea demonstrate a total absence of estrogen production by granulosa cells likely related to an absence of follicular development. As we did not perform functional studies, we cannot exclude that this severe POI clinical presentation presented in 2 sisters could be due to the total absence of one or both mutated proteins. In fact, both mutations lead to a premature stop codon, which can induce a nonsense mediated mRNA decay and subsequently an absence of truncated protein synthesis [17]. Nineteen *NOBOX* variants associated to POI have been reported up to date [1,18,19]. We have previously reported details on 17 variants [1]. A recent publication [19] described 4 previously reported *NOBOX* variants, which included the c.131G>T (p.Arg44Leu) variant showing impaired autophagosomal degradation by *in vitro* testing [16,18]. A novel mutation (c.1626del, p.(Phe543SerfsTer7)) has been recently reported by Rossetti et al [13]. According to the ACMG classification [20], this new variant is pathogenic (PSV1, PM2, PP5). The carrier patient presented primary amenorrhea with no other details on pubertal development. We note that this patient was also carrier of 2 other pathogenic and likely pathogenic mutations, respectively in *FIGLA* (*OMIM*: 608697) and *NR5A1* (*OMIM*: 184757) genes which could support an oligogenic nature of POI [12,13].

Different studies reported a prevalence of 5.6% to up to 9% of *NOBOX* mutations in POI cohorts [18,19,21,22]. These cohorts included mainly European and/or North-African and Sub-Saharan African POI patients. We performed genetic screening for *NOBOX* variants by next generation sequencing in a POI cohort of 151 unrelated POI patients, including mainly Caucasian patients (59%), Subsaharian-African and North African patients (30%). We showed the presence of only one other *NOBOX* variant (NM\_001080413.3 (*NOBOX*): c.259C>A, p.(Pro87Thr)) at the heterozygous state in a Caucasian patient from Italian origin [10]. This variant has never been reported previously and is classified as a variant of uncertain significance (VUS) by ACMG guidelines (PM2, BP4). It has been submitted to LOVD database, following URL have been attributed: <https://databases.lovd.nl/shared/variants/0000839554>.

The prevalence of *NOBOX* mutations in our POI cohort is 1.3% (n=2) with respectively 2.2% in Caucasian patients (n=89) and 0% in the other ethnic groups (n=62). Interestingly, only 9 out of the 20 *NOBOX* variants identified up to date (including the new one described in the present report) were classified as pathogenic or likely pathogenic based on the ACMG

**Table 1:** Reported NOBOX genetic variants.

N	NOBOX variants (cDNA, protein)	Zygoty	POI phenotype	F	FS	ACMG classification*	Revised classification	References
1	c.131G>T, p.Arg44Leu	NI	SA	0.00148	Yes	B	LB	16, 18, 19
2	c.259C>A, p.(Pro87Thr)	HZ	SA	0.00000402	NP	VUS	VUS	Present paper
3	c.271G>T, p.Gly91Trp	HZ	PA+ delayed puberty SA (1 patient at 22years †)	0.00214	Yes	B	VUS	1, 19
4	c.331G>A, p.Gly111Arg	HZ	SA	0.0000638	Yes	LB	VUS	1
5	c.349C>T, p.Arg117Trp	HZ Ho	PA+delayed puberty, PA, SA PA, SA	0.0766	Yes	B	B	1, 19
6	c.454G>A, p.Gly152Arg	HZ	Early menopause	0.00331	Yes	B	LB	1, 19
7	c.567delG, p.Thr190HisfsTer13	Ho	PA	0	Yes	LP	LP	1
8	c.826C>T, p.(Arg276Ter)	HZ#	PA, no PD	0	NP	P	P	1
9	c.907C>T, p.Arg303Ter	HZ	SA	0.00000401	Yes	P	P	1
10	c.1025G>C, p.Ser342Thr	HZ	SA	0.00000402	Yes	VUS	VUS	1
11	c.1048G>T, p.Val350Leu	HZ	SA	0	Yes	P	P	1
12	c.1064G>A, p.Arg355His	HZ	SA	0.0011	Yes	VUS	LP	1
13	c.1078C>T, p.(Arg360Ter)	Ho	NI	0.00000403	NP	P	P	1
14	c.1112A>C, p.Lys371Thr	HZ	SA	0.00195	Yes	B	VUS	1
15	c.1345C>T, p.Arg449Ter	HZ	SA	0.0000251	Yes	P	P	1
16	c.1354G>A, p.Asp452Asn	HZ	PA, SA	0.11	Yes	B	LB	1, 19
17	c.1421del, p.(Gly474AlafsTer76)	HZ#	PA, no PD	0	NP	P	P	1
18	c.1489delT, p.(Cys497ValfsTer53)	Ho	PA, incomplete PD	0.0000287	NP	P	P	1
19	c.1626del, p.(Phe543SerfsTer7)	HZ	PA	0.00000657	NP	P	P	13
20	c.1856C>T, p.Pro619Leu	HZ	PA, SA (†)	0.00111	Yes	B	LB	1

B: Benign; FS: Functional Study showing a negative impact of the mutation on the protein function; F: GnomAD allele frequency; HZ: Heterozygous; HO: Homozygous; LB: Likely Benign; LP: Likely Pathogenic; †: Same patient carrying variants (no precision in cis or trans); N: Variant number; NI: No Indication; NP: Not Performed; P: Pathogenic; PD: Pubertal Development; PA: Primary Amenorrhea; SA: Secondary Amenorrhea. #: Variants found in heterozygous compound state; VUS: Variant of Uncertain Significance; \*: ACMG classification according to Varsome (<https://varsome.com/>).

guidelines, according to VARSOME analysis (Table 1). Ten *NOBOX* variants associated with POI have been classified as benign, likely benign or VUS, despite their negative impact on the protein function as shown by *in vitro* testing (Table 1). Such discordance between a pathogenic *in vitro* mutated protein function and a benign or likely benign classification by the ACMG guidelines may be due to other *in vivo* factors such as other specific molecular pathways or specific thresholds of critical protein activity or a more complex genetic background such as an oligogenic spectrum and/or multifactorial factors that cannot be reproduced *in vitro*. These factors could modulate the expression of gene mutations and protein functions so that the clinical expressivity of *NOBOX* mutations could be associated with variable phenotypes [1]. Another potential explication to such discordance between *in vitro* pathogenic effect and a predicted ACMG benign effect can be due to some classification bias in Varsome (<https://varsome.com/>). In fact, some ACMG benign classification criteria, should be used with caution when dealing with POI, especially, BS1 (allele frequency is greater than expected for disorder) and BS2 (observed in a healthy adult individuals for a recessive (homozygous), dominant (heterozygous) or X-Linked (hemizygous) disorder with full penetrance expected at an early stage) [20]. In fact, POI prevalence is estimated between 1 and 3.7% before the age of 40 [3]. Such relatively frequent disorder can consequently be induced by mutations that are more frequent than 1% in the general population. For Varsome interpretation, variants with frequencies exceeding those of already reported gene variants are considered as benign (BS1) even when they are relatively rare, especially for POI (<1%). Furthermore, the ACMG guidelines use population frequency as one of the main criteria to select between a pathogenic or a benign state, however, causal POI genes often display an effect of sexual dimorphism. In fact, POI is a specific female syndrome, which underlying genetic cause, can be transmitted by asymptomatic, healthy and fertile male carrying potentially pathogenic variants. Nevertheless, these men are still included in the population database, which introduces interpretation bias. GnomAD population frequencies of POI gene variants, should focus only on female population frequencies of such variants. Next, there is the fact of incomplete penetrance of some variants, as we have shown for the variant NM\_001080413.3(*NOBOX*):c.1421del, p.(Gly474Alafs\*76), carried by the non-POI mother of both affected sisters [1]. Then, we cannot exclude that similar cases could be included in GnomAD as well. All these cautions in interpretation are not taken into account by Varsome neither for BS1 nor BS2 criteria and thus require manual revision. Furthermore, while using GnomAD as a selection criteria is common practice, it has to be noted that persons who are included in the database have only been selected on the basis of not having had a serious childhood congenital disease. Then, POI women might be included in this population, potentially not being aware that they are affected by POI at the time of sampling for GnomAD, either because they are

pre-symptomatic or misdiagnosed. In Table 1, we have already included PS3 criteria to variants classification when *in vitro* testing showed negative impact of the mutation on protein function. However, when we exclude criticized BS1 and/or BS2, potential source of bias, the classification of some benign, likely benign or VUS variants changes (Revised classification, Table1).

To conclude, *NOBOX* mutation prevalence in our cohort of 151 POI patients was of 1.3%, consistent with the prevalence of many highly penetrant POI genes [5]. With our new report, twenty *NOBOX* mutations were associated to POI at the time of writing this paper. POI phenotypes varied with no systematic correlation between the number of mutated *NOBOX* alleles and the severity of the POI phenotype. This apparent discrepancy can be due to a potential dominant negative effect of some mutations as well as potential oligogenic nature of POI. We have previously reported the first case of compound heterozygous truncating mutations of *NOBOX* in outbred patients, generalizing biallelic *NOBOX* null mutations as a rare cause of severe POI with delayed puberty and primary amenorrhea. Furthermore, our findings suggested that *NOBOX* haploinsufficiency could be well tolerated.

## Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Author Contributions Statement

A.S: conception and design, acquisition, analysis and interpretation of data, drafting the article. J.D: conception and design, critical revision of the manuscript. A.G: analysis, interpretation of data and revision of the manuscript. S.V.D.: substantial contributions to conception and design, revision of the manuscript. X.P : analysis, interpretation of data and revision of the manuscript, M.A: substantial contributions to conception and design, critical revision of the manuscript. A.D: conception and design, acquisition of the data, analysis and interpretation of the data, critical revising for important intellectual content. All authors gave final approval of the version to be published.

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