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Review

Aetiological coding sequence variants in non-syndromic premature ovarian failure: From genetic linkage analysis to next generation sequencing

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ABSTRACT

Premature ovarian failure (POF) is a frequent pathology affecting 1–1.5% of women under 40 years old. Despite advances in diagnosing and treating human infertility, POF is still classified as being idiopathic in 50–80% of cases, strongly suggesting a genetic origin for the disease. Different types of autosomal and X-linked genetic anomalies can originate the phenotype in syndromic and non-syndromic POF cases. Particular interest has been focused on research into non-syndromic POF causative coding variants during the past two decades. This has been based on the assumption that amino acid substitutions might modify the intrinsic physicochemical properties of functional proteins, thereby inducing pathological phenotypes. In this case, a restricted number of mutations might originate the disease. However, like other complex pathologies, POF might result from synergistic/compensatory effects caused by several low-to-mildly drastic mutations which have frequently been classified as non-functional SNPs. Indeed, reproductive phenotypes can be considered as quantitative traits resulting from the subtle interaction of many genes. Although numerous sequencing projects have involved candidate genes, only a few coding mutations explaining a low percentage of cases have been described. Such apparent failure to identify aetiological coding sequence variations might have been due to the inherent molecular complexity of mammalian reproduction and to the difficulty of simultaneously analysing large genomic regions by Sanger sequencing.

The purpose of this review is to present the molecular and cellular effects caused by non-synonymous mutations which have been formally associated, by functional tests, with the aetiology of hypergonadotropic non-syndromic POF. Considerations have also been included regarding the polygenic nature of reproduction and POF, as well as future approaches for identifying novel aetiological genes based on next generation sequencing (NGS).

Contents

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1. Introduction

Human infertility can be considered a public health concern since it affects ~15% of couples worldwide. Up to 30% of cases are caused by exclusive female factors, such as endometriosis, tubal disease and ovulation dysfunction (Smith et al., 2003). At least 14% of women show signs of hypofertility related to a decrease in ovarian reserve which, in some cases, evolves to early menopause (Santoro, 2011). Premature ovarian failure (POF) affects 1–1.5% of women under 40 years old and ~0.1% under the age of 30 (Conway, 2000; Coulam et al., 1986; Luborsky et al., 2003). In most cases, POF can be considered as the final stage of primary ovarian insufficiency (POI), a heterogeneous disease involving ovarian function impairment and irregular ovulation (Cox and Liu, 2014; Nelson, 2009). Although POI has been proposed recently as conditions describing ovarian dysfunction leading to infertility, the more classic term POF will be used in the present review. From a clinical point of view, POF has been defined as 4–6 months of amenorrhoea before the age of 40 related to high follicle stimulating hormone (FSH) plasma levels (>40UI/l) (Coulam, 1982). Hypergonadotropic hypogonadism in such patients results from an ovarian inability to close (via hormone signalling) a negative feedback loop on the synthesis of pituitary-secreted gonadotropins. POF women suffer primary (PA) or secondary amenorrhoea (SA), depending on the occurrence (SA) or not (PA) of menarche (Timmreck and Reindollar, 2003). In both cases, ovarian dysfunction can be found as an isolated phenotype (non-syndromic) or accompanying concomitant medical conditions (e.g. Turner's, BPES X syndromes). Distinct mechanisms (which might be deregulated at multistep levels) have been proposed for explaining the POF phenotype. This pathology might result from the development of a few follicles during embryogenesis, as well as from their abnormal recruitment. Enhanced follicular atresia could also lead to an early depletion of follicular stock (Goswami and Conway, 2005; Persani et al., 2011).

Aetiologically, POF has been linked to iatrogenic events (especially pelvic surgery and anti-cancer treatment), autoimmune conditions, infectious agents (viral oophoritis), metabolic disorders (galactosaemia) and environmental factors (Dragojević-Dikić et al., 2010; Goswami and Conway, 2005; Laissue et al., 2008; Persani et al., 2010). Unfortunately, despite advances in diagnosing and treating human infertility, POF is still classified as being idiopathic in 50–80% of cases, strongly suggesting a genetic origin for the disease. Differing types of autosomal and X-linked genetic anomalies, such as large chromosomal rearrangements and sequence point mutations, can originate the phenotype in syndromic and non-syndromic POF cases. Ovarian failure in Turner's syndrome (XO monosomy) might be caused by the haploinsufficiency of genes located on critical X chromosome regions which escape inactivation (Elsheikh et al., 2002; Zinn and Ross, 1998). X chromosome deletions and translocations have led to POF loci (POF-1, POF-2 and POF-3) being identified which might contain critical candidate genes (Davison et al., 2000; Lacombe et al., 2006; Marozzi et al., 2000; Powell et al., 1994; Tharapel et al., 1993). *FMR1* premutations displaying an intermediate number (between 55 and 200) of CGG repeats located on the 5′UTR region of the gene as well as *FMR2* microdeletions have been linked to an increased predisposition to POF (Allingham-Hawkins et al., 1999; Murray et al., 1998, 1999; Sherman, 2000). Sequence point mutations of a transcription factor (*FOXL2*) originate the ovarian phenotype in the BPES syndrome (Beysen et al., 2009; Crisponi et al., 2001).

Particular interest has been focused on research into non-syndromic POF causative coding variants during the past two decades. This has been based on the assumption that amino acid substitutions might modify the intrinsic physicochemical properties of functional proteins, thereby inducing pathological phenotypes. In this case, a restricted number of mutations might originate the disease. However, like other complex pathologies, POF might result from synergistic/compensatory effects caused by several low-to-mildly drastic mutations which have frequently been classified as non-functional SNPs (Gibson, 2012; Kryukov et al., 2007). Indeed, reproductive phenotypes can be considered quantitative traits resulting from the subtle interaction of many genes (L'Hôte et al., 2010).

Although numerous sequencing projects have involved candidate genes, only a few coding mutations explaining a low percentage of cases have been described. Mutations in *FSHR*, *LHCGR*, *NR5A1*, *NOBOX*, *FOXL2*, *FIGLA*, *BMP15*, *NANOS3* and *STAG3* have been formally validated as being causative of non-syndromic POF (Aittomäki et al., 1995; Beau et al., 1998; Caburet et al., 2014; Di Pasquale et al., 2004; Doherty et al., 2002; Laissue et al., 2008; Latronico et al., 1996; Lourenço et al., 2009; Qin et al., 2007; Rannikko et al., 2002; Rossetti et al., 2009; Santos et al., 2014; Touraine et al., 1999; Wu et al., 2013; Zhao et al., 2008). Such apparent failure to identify aetiological coding sequence variations might have been due to the inherent molecular complexity of mammalian reproduction and to the difficulty of simultaneously analysing large genomic regions by Sanger sequencing.

The purpose of this review is to present the molecular and cellular effects caused by non-synonymous mutations which have been formally associated by functional tests with the aetiology of hypergonadotropic non-syndromic POF. To present this data, the relevant POF genes have been classified into three distinct groups: genes encoding gonadotropins receptors, transcription factor (TF) genes and other types of gene. Considerations have also been included regarding the polygenic nature of reproduction and POF, as well as future approaches for identifying novel aetiological genes based on next generation sequencing (NGS).

2. Genes encoding gonadotropin receptors: follicle stimulating hormone receptor (*FSHR*) and luteinising hormone/choriogonadotropin receptor (*LHCGR*)

Subtle regulation of the hypothalamic-pituitary-gonadal (HHG) axis is crucial in humans for proper sexual development and gonad function. Gonadotropin-releasing hormone (GnRH) neurons migrate across the cribiform plate into the hypothalamus during embryo development to contact the hypophyseal-portal vascular system (Tobet and Schwarting, 2006). These cells secrete the GnRH peptide (in a pulsatile fashion) which in turn stimulates the pituitary synthesis and secretion of the follicle stimulating (FSH) and luteinising (LH) hormones. FSH and LH bind to specific gonadal transmembrane receptors named FSHR and LHCGR, respectively to regulate particular reproductive functions. The negative feedback loop in the pituitary synthesis of gonadotropins is closed by the secretion of steroid and non-steroid substances (e.g. oestradiol, progesterone, inhibins A and B, gonadotrophin surge-attenuating factor-GnSAF) (Messinis, 2006; Messinis et al., 2014; Plant, 2008). FSHR participates in regulating ovarian physiology in females by stimulating oestrogen synthesis and follicle development while LHCGR-related effects

include ovulation and ovarian steroidogenesis (Ascoli et al., 2002; Dias et al., 2002).

Human *LHCGR* and *FSHR* genes are located on the short arm of chromosome 2. Several transcript sizes and alternatively spliced variants have been reported in distinct species for both *LHCGR* and *FSHR* mRNAs (Menon and Menon, 2012 and references therein). It has been shown that during antral follicle development, there is increasing *FSHR* expression in the ovaries. Concurrently, theca-interstitial cells have low *LHCGR* expression levels which progressively increase, secondary to distinct paracrine factors stimuli (e.g. estradiol and FSH). *LHCGR* expression is transitorily downregulated by the LH preovulatory surge but becomes completely recovered during the luteal phase (LaPolt et al., 1990; Peegel et al., 1994). Both *LHCGR* and *FSHR* genes encode G-protein-coupled transmembrane factors, specifically those belonging to G protein-coupled receptor rhodopsin/β2 adrenergic receptor-like family A. Structurally, they have an extracellular domain, a short intracellular domain and seven signal transduction-related transmembrane α-helix domains (Figs. 1 and 2) (Ascoli and Segaloff, 1989; Ascoli et al., 2002; Dias et al., 2002; Fredriksson et al., 2003; Puett et al., 2007; Vassart et al., 2004). Crystallisation assays have shown that FSH binds to the FSHR concave region and that the FSH-FSHR ectodomain complex can become dimerised in solution (Fan and Hendrickson, 2005, 2008). LHCGR structural features (studied by *in silico* modelling of its ectodomain) have shown that it is similar to that described for FSHR (Menon and Menon, 2012; Puett et al., 2007).

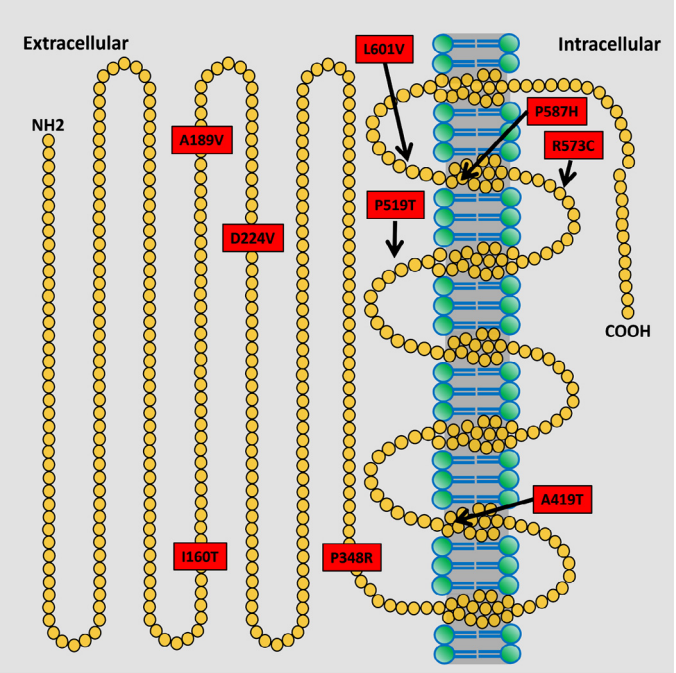

Fig. 1. FSHR mutations causative of non-syndromic POF.

2.1. FSHR

FSHR was the first gene for which open reading frame (ORF) mutations were seen to be related to POF aetiology (Aittomäki et al., 1995). As for other candidate POF genes, the initial mapping of the chromosomal region encompassing *FSHR* was assessed by genetic linkage analysis in affected families. Linkage analysis locates disease-causing loci by identifying genetic markers which are co-inherited and linking them to a phenotype of interest. This technique (which has enabled mapping genetic diseases since 1982) has been based on the observation that genomic regions (and consequently genes) residing physically close in a chromosome remain linked during meiosis (Bird et al., 1982). The logarithm of the odds (LOD) score statistical method has been widely used for assessing whether genetic marker data in families are linked to a particular phenotype (Dawn Teare and Barrett, 2005; Morton, 1955). LOD scores over 3 (meaning a statistically significant 0.001 p value) have classically been considered to be positive evidence of linkage (Chotai, 1984).

The genetic linkage analysis described by Aittomäki et al. (1995) involved 6 POF families from a geographically-isolated Finnish subpopulation which had previously led to the description of a hypergonadotropic ovarian dysgenesis locus (named ODG1) (Aittomäki, 1994; Aittomäki et al., 1995). More precisely, using microsatellite markers across the genome led to locating the *ODG1* locus on chromosome 2p21, a region encompassing the *FSHR* and *LHCGR* candidate genes (Aittomäki et al., 1995). Sequencing analysis identified homozygous c.566C>T (p.Ala189Val) transition in the *FSHR* gene in ~30% of these patients. Functional assays showed that this mutation (located in the protein's extracellular ligand-binding domain) reduced receptor binding ability and impaired cAMP synthesis, secondary to recombinant FSH stimulation (Aittomäki et al., 1995). It should be stressed that the high incidence of the p.Ala189Val mutation in the Finnish population might be attributable to a founder effect as *FSHR* mutations are rare in other ethnic populations (Da Fonte Kohek et al., 1998; De la Chesnaye et al., 2001; Jiang et al., 1998; Layman et al., 1998; Liu et al., 1998; Sundblad et al., 2004; Whitney et al., 1995). Eight additional *FSHR* mutations leading to POF (present at homozygous or compound heterozygous state), for which *in vitro* functional tests have been performed, have been described to date (Fig. 1 and Table 1) (Allen et al., 2003; Beau et al., 1998; Doherty et al., 2002; Kuechler et al., 2010; Meduri et al., 2003; Touraine et al., 1999). Further mutations lacking functional tests potentially related to POF have been described as well as sequence variants associated with male infertility (Desai et al., 2013; Siegel et al., 2013). It is worth noting that most women carrying pathogenic *FSHR* mutations display a severe ovarian phenotype characterised by primary amenorrhoea, high gonadotropin plasma levels and low (or undetectable) estradiol levels. This phenotype is similar to that observed in female *Fshr*$^{-/-}$ knockout (KO) mice which were affected by lack of puberty, high gonadotropin levels, low oestrogen and progesterone plasma levels, incomplete follicular development and sterility (Abel et al., 2000; Dierich et al., 1998).

2.2. LHCGR

Concerning *LHCGR* and POF aetiology, the first causative mutation was described by Latronico et al. (1996). The LHCGR-c.1660T>C (p.Arg554Ter) homozygous mutation was detected in a single Brazilian family which was affected by reproductive phenotypes (three pseudohermaphrodite 46,XY siblings having Leydig-cell hypoplasia and a 46,XX sister having amenorrhoea). This mutation was located in the third cytosolic loop of the protein and was predicted to truncate a significant functional region of the protein (Fig. 2 and Table 1). Although this mutation has not been tested by a functional assay, its presence in males and females belonging to a family affected by specific reproductive signs, as well as inherent genetic and biochemical characteristics (a homozygous nonsense mutation), strongly suggested a deleterious effect (Latronico et al., 1996). Complete ovarian resistance was reported thereafter in a 46XX woman carrying the homozygous *LHCGR* c.1777G>C (p. Ala593Pro) variant. A functional test for this mutation revealed mutant receptor impairment for stimulating adenylyl cyclase in response to hCG (Toledo et al., 1996).

Other female patients have been described from families in which 46XY individuals have displayed sex development dysfunction. A 46XX woman carrying the *LHCGR* c.1822_1827del mutation

Table 1
Mutations causative of non-syndromic POF.

	Mutations causative of non-syndromic POF			
	DNA	Protein	Protein domain	Reference
FSHR	c.479C>T	p.Ile160Thr	Extracellular	Beau et al., 1998
	c.566C>T	p.Ala189Val	Extracellular	Aittomäki et al., 1995
	c.671A>T	p.Asp224Val	Extracellular	Touraine et al., 1999
	c.1043C>G	p.Pro348Arg	Extracellular	Allen et al., 2003
	c.1255G>A	p.Ala419Thr	Transmembrane helix 2	Doherty et al., 2002
	c.1555C>A	p.Pro519Thr	Extracellular loop 2	Meduri et al., 2003
	c.1717C>T	p.Arg573Cys	Intracellular loop 3	Beau et al., 1998
	c.1760C>A	p.Pro587His	Transmembrane helix 6	Kuechler et al., 2010
	c.1801C>G	p.Leu601Val	Extracellular loop 3	Touraine et al., 1999
LHCGR	c.1660T>C	p.Arg554Ter	Intracellular loop 3	Latronico et al., 1996
	c.1777G>C	p.Ala593Pro	Transmembrane helix 6	Toledo et al., 1996
	c.1822_1827del	p.Leu608_Val609del	Transmembrane helix 7	Latronico et al., 1998
	c.1060A>G	p.Glu354Lys	Extracellular	Stavrou et al., 1998
NR5A1	c.390delG	p.Pro131fs	Ligand-binding domain	Lourenço et al., 2009
	c.666delC	p.Asn222fs	Ligand-binding domain	Lourenço et al., 2009
	c.386C>T	p.Pro129Leu	Hinge region	Lourenço et al., 2009
	c.691_699del	p.Leu231_Leu233del	Hinge region	Lourenço et al., 2009
	c.877G>A	p.Asp293Asn	Ligand-binding domain	Lourenço et al., 2009
	c.13T>G	p.Tyr5Asp	N-terminal region	Jiao et al., 2013
	c.704C>T	p.Pro235Leu	Ligand-binding domain	Camats et al., 2012
	c.763C>T	p.Arg255Cys	Ligand-binding domain	Philibert et al., 2013
	c.768delC	p.Asp257fs	Ligand-binding domain	Suwanai et al., 2013
NOBOX	c.1064G>A	p.Arg355His	Homeodomain	Qin et al., 2007
	c.271G>T	p.Gly91Trp	ND	Bouilly et al., 2011
	c.349C>T	p.Arg117Trp	ND	Bouilly et al., 2011
	c.907C>T	p.Arg303Ter	Homeodomain	Bouilly et al., 2011
	c.1025G>C	p.Ser342Tyr	Homeodomain	Bouilly et al., 2011
	c.1048G>T	p.Val350Leu	Homeodomain	Bouilly et al., 2011
	c.331G>A	p.Gly111Arg	ND	Bouilly et al., 2015
	c.1112A>C	p.Lys371Thr	ND	Bouilly et al., 2015
	c.1856C>T	p.Pro619Leu	C-terminus	Bouilly et al., 2015
FIGLA	c.15_36del	p.Gly6fs	N-terminus	Zhao et al., 2008
	c.419_421del	p.Asn140del	ND	Zhao et al., 2008
FOXL2	c.560G>A	p.Gly187Asp	ND	Laissue et al., 2009a
BMP15	c.704A>G	p.Tyr235Cys	Prodomain	Di Pasquale et al., 2004
	c.202C>T	p.Arg68Trp	Prodomain	Rossetti et al., 2009
	c.413G>A	p.Arg138His	Prodomain	Rossetti et al., 2009
	c.443T>C	p.Leu148Pro	Prodomain	Rossetti et al., 2009
	c.631C>T	p.Glu211Ter	Prodomain	Dixit et al., 2006
NANOS3	c.457C>T	p.Arg153Trp	ND	Wu et al., 2013
	c.358C>A	p.Glu120Lys	Zinc finger domain	Santos et al., 2014
STAG3	c.968delC	p.Phe187fs	STAG domain	Caburet et al., 2014

(p.Leu608_Val609del), located within the seventh transmembrane helix, was affected by oligoamenorrhoea and infertility (Latronico et al., 1998). Such mutation was functionally related to the protein's intracellular retention which led to a decrease in the amount of cell surface receptors (Latronico et al., 1998). A further female displaying primary amenorrhoea was a carrier of the homozygous *LHCGR* c. 1060A>G (p.Glu354Lys) sequence variant (Stavrou et al., 1998). At protein level, this mutation is located in the extracellular domain adjacent to the first transmembrane helix. *In vitro* experiments have shown a complete loss of mutant protein function as HEK293 transfected cells lack cAMP synthesis after hCG stimulation (Stavrou et al., 1998). Interestingly, two sisters affected by the homozygous IVS10-G>A splice mutation (which led to the deletion of amino acids Tyr317 to Ser324) were affected by a mild phenotype, as they had regular menses for years and normal LH plasma levels (Bruysters et al., 2008). Although the receptor was expressed and functionally competent to respond to LH stimulus, in this case, it had reduced potency. Furthermore, an 8-year-old girl has been reported carrying *LHCGR* compound heterozygous mutations (p.Ile152Thr and c.537-3C>A splice acceptor mutation leading to exon 7 skipping). These mutations were also present in her brother who was affected by Leydig cell hypoplasia (Qiao et al., 2009). An *in vitro* functional test for p.Ile152Thr mutation revealed significant impairment of hCG binding and signal transduction. Concerning

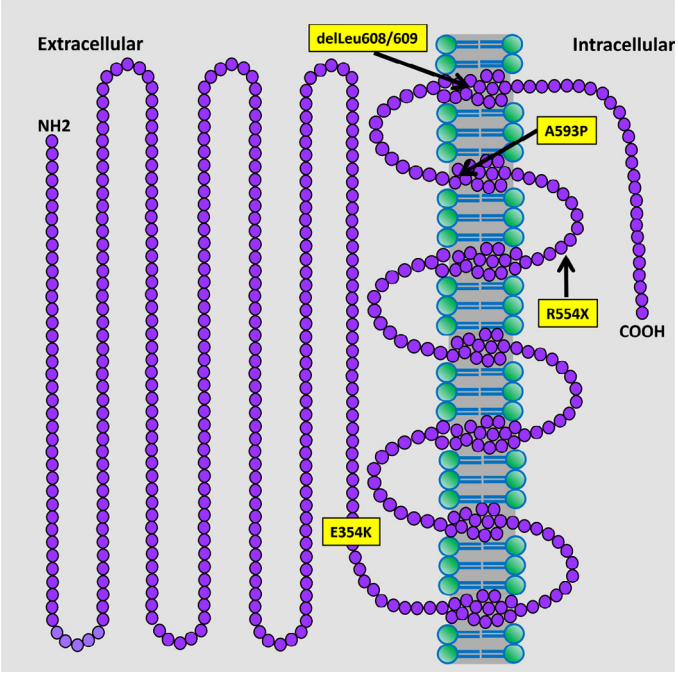

Fig. 2. LHCGR mutations causative of non-syndromic POF.

animal models, two *Lhcgr*-KO models have been produced (Lei et al., 2001; Zhang et al., 2001). These animals had small ovaries, thin uteri, a lack of preovulatory follicles or corpora lutea and a delay regarding the age of vaginal opening. These mice could be considered close phenocopies of the corresponding human mutations.

3. TF-encoding genes

TF are key molecular actors which are responsible for direct gene regulation. These proteins, which seem to be encoded by ~1900 genes in the human genome, recognise 6–12 bp long degenerate DNA sequences for regulating target genes (Lander et al., 2001; Vaquerizas et al., 2009; Venter et al., 2001). A specific gene's regulatory region can be transcriptionally modulated by multiple TF which, in turn, can simultaneously regulate many targets. Distinct mechanisms (motifs) have been described for regulating such complex networks, such as the autoregulatory loop, the feed-forward regulatory circuit and the multi-input motif (MacQuarrie et al., 2011). Embryonic female gametogenesis and adult reproductive physiology are multistep processes which have been subtly modulated in terms of gene expression which partially depends on TF function. Studies, mostly performed on genetically-modified mice, have shown that several TF (e.g. *Blimp1*, *Pou5f1*, *Prdm14*, *Gata4*, *Nr5a1*, *Zglp1*, *Emx2*, *Lhx8*, *Lhx9*, *Nanog*, *Sohl1*, *Sohl2*, *Foxo3a*, *Nobox*, *Figla*, *Tbp2*, *Taf4b* and *Foxl2*) regulate molecular cascades associated with primordial germ cell specification and migration, meiosis, follicle development and ovulation (Jagarlamudi and Rajkovic, 2012; Jagarlamudi et al., 2010). Mutations in these genes could thus lead to analogues to human POF phenotypes. Concerning these, only *FIGLA, NOBOX* and *FOXL2* mutations have been functionally associated with some cases of non-syndromic POF aetiology. This might have been due to Sanger sequencing limitations since this technique does not permit simultaneous parallel sequencing of numerous genes. Indeed, since several POF genes are transcription factors regulating numerous targets in an interactive lattice, Sanger sequencing might be challenging due to the significant length of the genomic candidate regions. NGS approaches should overcome this drawback in the near future for identifying novel molecular actors responsible for POF (see below).

3.1. NR5A1

The nuclear receptor subfamily 5 group A member 1 (NR5A1), also known as steroidogenic factor 1 (SF1), is one of the main proteins implicated in mammalian gonadal differentiation and modulation of steroidogenesis via hypothalamic-hypophysis axis regulation (Lalli et al., 2013; Lin and Achermann, 2008; Młynarczuk and Rękawiecki, 2010; Schimmer and White, 2010). The *NR5A1* gene is located on 9q33.3 and encodes a 461 residue protein from the orphan nuclear receptor family (Oba et al., 1996; Taketo et al., 1995). Structurally, SF1 includes an N-terminal zinc finger DNA-binding domain (DBD), an A box, a ligand binding domain, a proline-rich hinge region and an AF-2 activation domain located at the protein's C-terminal region. *SF1* displays a large spectrum of spatiotemporal expression which is related to distinct tissue's specific functions. *SF1* expression has been detected in particular tissue from adult organs, such as pituitary gland (gonadotroph cells), hypothalamus, hypocampus, ovary (granulosa and theca cells, corpus luteum), testis (Leydig and Sertoli cells), adrenal cortex, and spleen (Schimmer and White, 2010). It has also been detected early in the genital ridge and thereafter during hypophysis, hypothalamus, adrenal gland and gonad development steps. In mice, *Sf1* is expressed during testis determination and differentiation in the formation of the bipotential gonads as well as in Leydig and Sertoli cells to regulate steroid hormones and the anti-Müllerian hormone (AMH) (Hanley et al., 1999; Ikeda et al., 1994). SF1 regulates the transcription of several genes in the ovaries, such as *StAR*, *CYP17A1*, *CYP11A1* (*P450scc*), *AMH*, *CYP19A1* (aromatase), *INHA*, *NR0B1*, *3β-HSD*, *SCP-2*, *HDL-R* and *NP-C1* (Hoivik et al., 2010; Mendelson and Kamat, 2007). The mouse constitutive KO model of *Sf1* was mainly affected by male-to-female sex reversal of the external genitalia and the animals died secondary to adrenal failure (Luo et al., 1994; Sadovsky et al., 1995). A conditional mouse KO model in which *Sf1* was specifically disrupted in granulosa cells led to infertility due to having hypoplastic ovaries, a reduced amount of oocytes and the absence of corpora lutea (Jeyasuria et al., 2004; Pelusi et al., 2008). More recently, a hypomorphic model of *Sf1*, produced by the ablation of *Cited2* in mice, was related to gonadal ectopic cell migration (Combes et al., 2010). *Cited2*$^{-/-}$ females also displayed a transient downregulation of pro-ovarian genes (*Foxl2*, *Rspo1*, and *Wnt4*). This feature led to screening *CITED2* mutations in POF women and proposing new variants which were potentially related to the disease's pathogenesis (Fonseca et al., 2012). *SF1* mutations have been described in humans as being causative of primary adrenal failure, 46, XY and XX gonadal dysgenesis and POF (de Mello et al., 2011; Ferraz-de-Souza et al., 2011). Sequence variants may be associated with hypospadias, bilateral anorchia, male factor infertility, adrenal tumourogenesis, polycystic ovary syndrome and endometriosis.

Lourenço et al., identified the first *NR5A1* mutations causative of non-syndromic POF (Lourenço et al., 2009). Direct sequencing was used for screening individuals from four families lacking adrenal dysfunction but affected by 46XY sex development anomalies and 46XX POF for *NRA5A1* coding mutations. Such mutations were identified in women having primary or secondary amenorrhoea: c.877G>A (p.Asp293Asn, homozygous), c.3G>A (p.Met1Ile heterozygous), c.390delG (heterozygous) and c.666delC (heterozygous) (Fig. 3 and Table 1). The c.390delG (p.Pro131fs) and c.666delC (p.Asn222fs) sequence variants were predicted to create a truncated 295 amino acid long protein lacking the major part of the ligand binding and AF2 domains. *NR5A1* was analysed using an identical approach in 25 women displaying sporadic POF. In the same study, two of these individuals had non-conservative sequence variants. The first patient displayed an in-frame 9-bp (c.691_699del) heterozygous deletion which led to the loss of three residues (p.Leu231_Leu233del) located in the ligand binding domain. The second individual carried the c.368G>C (p.Gly123Ala) and c.386C>T (p.Pro129Leu) mutations in a heterozygous state. All these variants were absent in a significant number of control alleles. *In vitro* functional tests on embryonic kidney (tsa201) and Chinese hamster ovary (CHO) cells showed severe transactivation disturbances of the p.Pro129Leu, p.Leu231_Leu233del and p.Met295Ter mutations on

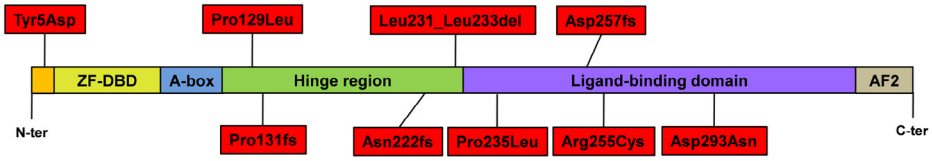

Fig. 3. NRA5A1 mutations causative of non-syndromic POF.

both *CYP11A1* and *CYP19A1* promoters. The p.Asp293Asn mutation had a less drastic functional effect on both *CYP* promoters.

Since the report by Lourenço et al. (2009), several studies have described screening for *NR5A1* mutations in POF women (Camats et al., 2012; Janse et al., 2012; Jiao et al., 2013; Lakhal et al., 2012; Philibert et al., 2013; Suwanai et al., 2013; Voican et al., 2013).

Although various sequence variants which were potentially related to the disease pathogenesis have been described, solely the c.13T>G (p.Tyr5Asp), c.704C>T (p.Pro235Leu), c.763C>T (p.Arg255Cys), and c.768delC (p.Asp257fs) mutations (found in heterozygous state) have been formerly validated as causative of POF by functional tests (Camats et al., 2012; Jiao et al., 2013; Philibert et al., 2013; Suwanai et al., 2013). These mutations (located in the protein's N-terminal region (p.Tyr5Asp) and ligand binding domain (p.Pro235Leu, p.Arg255Cys, p.Asp257fs) were linked to distinct degrees of transactivation disturbance in target gene promoters.

In summary, *NR5A1* ORF mutations are responsible for various phenotypes, including 46XX gonadal dysgenesis and POF. To date, 8 *NR5A1* mutations have been proved to be causative of POF by functional assays. Further mutations deserve functional assays to establish their potential pathogenicity.

3.2. NOBOX

The *newborn ovary homeobox* (*NOBOX*) gene, which encodes a homeodomain transcriptional regulator, is located on chromosomes 6 and 7q35 in mouse and human species, respectively (Suzumori et al., 2002). *NOBOX* is expressed in the gonads in both species, preferentially by metaphase II oocytes from primordial follicles (Huntriss et al., 2006; Rajkovic et al., 2004; Suzumori et al., 2002). It has also been reported that NOBOX is present in oocytes beyond the primary developmental stage and that the latest stages involve downregulation of its expression (Belli et al., 2013). Human NOBOX has a characteristic 60 residue region (the homeodomain) which is highly conserved with that from rodents (>90% identity) (Suzumori et al., 2002). NOBOX regulates transcription via specific binding elements (e.g. the TAA/GTTG/A sequence) located on target genes' promoter regions (Choi and Rajkovic, 2006). It has been postulated that many relevant genes for female reproduction (e.g. *Gdf9*, *Bmp15*, *Pou5f1*, *Zar1*, *c-Mos*, *Oog1*, *Pad6*, *Oosp1*, *Oct4*) are directly or indirectly regulated by NOBOX because KO mice have shown differential expression patterns compared to wild type (WT) animals (Rajkovic et al., 2004). Direct binding to promoter regions has been demonstrated for *Gdf9*, *Pou5f1* and *Pad6* (Choi et al., 2010; Choi and Rajkovic, 2006). Very recently, it has been shown that NOBOX and FOXL2 can form a complex to regulate specific target genes in granulosa cells (Bouilly et al., 2014). Female *Nobox*$^{-/-}$ KO mice have displayed a dramatic postnatal reduction of oocyte number secondary to meiosis dysfunction in oogonia and impaired primordial to primary follicle transition (Rajkovic et al., 2004). Electron microscopy has revealed an increase in adherens junctions between unseparated oocytes within syncytial follicles when assessing KO animals' ovaries, which might have been related to cell adhesion dysfunction (Lechowska et al., 2011). Moreover, these experiments led to proposing that the infertile phenotype of female *Nobox* null mice might be associated with abnormal signalling between germ and somatic cells. All the above findings supported a potential role for NOBOX mutations during POF pathogenesis.

In a first attempt to establish whether encoding mutations in this gene might be a common cause of non-syndromic POF, 30 Japanese patients were screened by direct sequencing for *NOBOX* sequence variants but no positive results were found (Zhao et al., 2005).

Thereafter, Qin et al. described the first *NOBOX* mutation related to POF aetiology (Qin et al., 2007). The complete *NOBOX* encoding sequence was sequenced in a panel of 96 POF women. The c.1064G>A (p.Arg355His) mutation was novel among the sequence variations identified in that study and absent in the control population. This mutation was located in the protein's homeodomain and involved an amino acid (Arg355) which is highly conserved during vertebrate species' evolution (Fig. 4 and Table 1). Functional binding experiments, based on electrophoretic mobility shift assays (EMSA), have revealed a drastic decrease in protein binding to specific DNA-binding elements (Qin et al., 2007). Further assays, using equimolar amounts of WT and mutant protein, revealed that the p.Arg355His mutation might have a dominant negative effect and suggested that the NOBOX homeodomain may function as a dimer.

More recently, direct sequencing of the *NOBOX* open reading frame in 178 Caucasian, Senegalese, and Bantu women revealed 6 non-synonymous sequence variants in heterozygous state: c.271G>T (p.Gly91Trp), c.349C>T (p.Arg117Trp), c.907C>T (p.Arg303Ter), c.1025G>C (p.Ser342Tyr), c.1048G>T (p.Val350Leu), c.1444G>A (p.Gly472Ser) (Bouilly et al., 2011). All of the above (excepting p.Gly472Ser) were not found in a control panel of women from the same ethnic origin having a high evolutionary conservation score, thereby suggesting a pathogenic effect. Moreover, p.Arg303Ter, p.Ser342Tyr and Val350Leu were located in the homeodomain, suggesting potential functional disruption of mutant proteins to regulate relevant genes implicated in ovarian development and physiology. Two functional assays demonstrated initially that the p.Gly91Trp, p.Arg117Trp, p.Arg303Ter, p.Ser342Tyr and p.Val350Leu mutations were causative of POF (Bouilly et al., 2011). An EMSA approach displayed a disruption of mutant proteins directly binding to the NOBOX-specific consensus binding sequence. Luciferase experiments, using human *GDF9* promoter region which included a NOBOX binding element, then revealed a 50% reduction of *GDF9* transcriptional activity. Interestingly, this study reported the highest prevalence (>5%) of causative mutations identified in a POF cohort during a single gene analysis. A more recent study, carried-out on 200 Chinese POF patients, did not lead to finding NOBOX coding variants related to the disease's pathogenesis (Qin et al., 2009). Bouilly et al. reported that two previously described (p.Gly91Trp, p.Arg117Trp) and three novel mutations (p.Gly111Arg, p.Lys371Thr and p.Pro619Leu) have impaired transactivation properties on target promoters (Fig. 4) (Bouilly et al., 2015).

3.3. FIGLA

The *FIGLA* gene which is located on chromosomes 2 and 6 in human and mouse species, respectively, encodes a basic helix-loop-helix (bHLH) transcription factor (Huntriss et al., 2002; Liang et al., 1997). Structurally, the bHLHL domain has ~60 residues which form

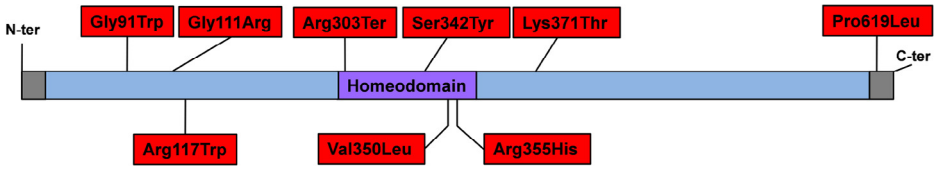

Fig. 4. NOBOX mutations causative of non-syndromic POF.

two helices (separated by a variable loop region) and a DNA-binding domain (Jones, 2004; Massari and Murre, 2000). It has been shown that *Figla* is oocyte specific and regulates critical functions during early folliculogenesis (Liang et al., 1997; Soyal et al., 2000). Figla protein is present in mice from E13.5 onwards and binds to a conserved E-box motif (CANNTG) located on *Zp* (*zona pellucida*) genes' promoter region to modulate their transcription (Liang et al., 1997). Furthermore, the human FIGLA protein binds to the *ZP2* promoter region, which suggests similar functions for human and mouse species (Bayne et al., 2004). Female *Figla* KO mice have become infertile due to accelerated postnatal loss of oocytes from primordial follicles (Soyal et al., 2000). More recent experiments involving *Figla* null mouse lines and expression microarrays have displayed target genes, such as *kit*, *Dppa3*, *Pou5f1*, and some *Nlrp* family members further downstream (Joshi et al., 2007). It has also been shown that *Figla* inhibits male specific genes which have suggested dichotomic molecular functions during germ cell development (Hu et al., 2010). *FIGLA* is also expressed early in humans, showing a significant increase at the time of primordial follicle development (Bayne et al., 2004).

FIGLA non-synonymous mutations were seen to be related to POF pathogenesis in a 2008 study (Zhao et al., 2008) where the gene's complete encoding sequence was directly sequenced in 100 Chinese POF women. Three previously undescribed sequence coding variants were identified: c.11C>A (p.Ala4Glu), c.15_36del (p.Gly6fs) and c.419_421del (p.Asn140del). The p.Ala4Glu variant was present in one individual from the control population (1/304). The p.Gly6fs mutation was not present in the control population and led to the synthesis of a truncated 66 amino acid long product. The mutant protein's inherently pathogenic effect was related to haploinsufficiency. Yeast two-hybrid functional assays for protein/protein interaction testing were performed for FIGLA-p.Ala4Glu and FIGLA-p.Asn140del mutants. The TCF3 (also known as E2A) protein HLH domain, which was previously shown to form heterodimers with FIGLA, was used as prey (Bayne et al., 2004; Liang et al., 1997). It was shown that the p.Asn140del mutation impaired FIGLA/TCF3-HLH interaction (Table 1). By contrast, p.Ala4Glu did not have a functionally pathogenic effect. Since the report by Zhao et al., no further studies have been reported involving sequencing analysis of the *FIGLA* coding region.

3.4. FOXL2

The forkhead box (FOX) family of proteins was originally described during a study of *Drosophila* mutants in the *Fkh* gene (Weigel and Jäckle, 1990; Weigel et al., 1989). FOX factors are widely distributed in animals and fungi, as at least 2000 members have been identified in more than 100 species (Benayoun et al., 2011). Fifty FOX genes have been located in the human genome to date (Jackson et al., 2010). Many biological and molecular processes have been related to FOX proteins, such as immunoregulation, cell cycle control and survival, body axis establishment, glucose homeostasis, cancer onset and development, stem cell biology, chromatin remodelling and organ development (including testes and ovaries) (for review, see Benayoun et al., 2010; Eijkelenboom and Burgering, 2013; Lam et al., 2013; Ro et al., 2013; Sanchez et al., 2014; Uhlenhaut and Treier, 2011).

The human *FOXL2* was mapped in 2001 to 3q23 and described as a 2.7 kb monoexonic gene encoding a 376 amino acid protein displaying a forkhead domain and a 14 residue long polyalanine (poly-Ala) stretch (Crisponi et al., 2001). *FOXL2* is expressed in mammalian ovaries from gonadal determination to adult life, thereby underlining its functional role during development and fertility maintenance (Cocquet et al., 2002; Crisponi et al., 2001; Pannetier et al., 2003). It has been shown that *Foxl2* is expressed during mouse hypophysis where it may have a function during organogenesis (Ellsworth et al., 2006). Interestingly, several studies have shown a key role for *FOXL2* during central regulation of FSHB synthesis by pituitary gonadotropic cells (Fortin et al., 2014; Ghochani et al., 2012; Justice et al., 2011; Lamba et al., 2010; Roybal et al., 2014; Tran et al., 2011, 2013). It might thus be considered that FOXL2 plays an important dual pituitary/ovary role in regulating mammalian reproduction. Constitutive mouse KO models of *Foxl2* have been created which mainly displayed premature massive depletion of follicular stock and craniofacial malformations, similar to those observed in BPES patients (Schmidt et al., 2004; Uda et al., 2004).

Concerning human disease, fine physical mapping and Sanger sequencing techniques have led to defining *FOXL2* mutations as causative of the blepharophimosis–ptosis–epicanthus inversus (BPES) syndrome (Crisponi et al., 2001). This pathology is transmitted in an autosomal dominant fashion and is clinically characterised by complex eyelid malformations and POF (Crisponi et al., 2001; Verdin and De Baere, 2012; Zlotogora et al., 1983). Two types of BPES have been defined which depend on POF presence (BPES type I) or absence (BPES II) (Zlotogora et al., 1983). More than 140 intragenic *FOXL2* mutations have been reported to date and included in a public database (http://medgen.ugent.be) (Beysen et al., 2009; Verdin and De Baere, 2012). Mutations not affecting *FOXL2* ORF and complete gene deletions have also been described (Beysen et al., 2009). The first studies led to proposing a genotype-phenotype correlation: mutations leading to truncated protein synthesis before the poly-Ala tract were related to BPES type I while those leading to poly-Ala stretch expansion were linked to BPES type II. No correlations were established concerning elongated and truncated FOXL2 proteins with a complete forkhead domain as well as those translated from missense mutations (De Baere et al., 2001, 2003; Verdin and De Baere, 2012). Many functional tests have been performed to determine *FOXL2* mutations' potentially pathogenic effects. For instance, the *in vitro* expression of the 24-alanine version displayed subcellular mislocalisation and aggregation of the mutant protein as well as transactivation disturbances of target gene promoters (Caburet et al., 2004; Moumné et al., 2008). Missense mutations located in the forkhead domain were also linked to aggregation and subcellular mislocalisation but, coherently, were linked to more drastic transactivation disturbance of target genes (Beysen et al., 2008). Furthermore, distinct theoretical and experimental models have been used for exploring *FOXL2* missense mutations' molecular and functional effects (Dipietromaria et al., 2009; Todeschini et al., 2011).

Several studies have been undertaken concerning non-syndromic POF to establish whether FOXL2 mutations might be causative of the phenotype but only three sequence variants in heterozygous state have been identified: c. 772T>A (p. Tyr258Asp), c. 661_690del (p.Ala221_Ala230del) and c.560G>A (p.Gly187Asp) (Gersak et al., 2004; Harris et al., 2002; Laissue et al., 2009a; Verdin and De Baere, 2012 and references therein). Functional tests were only performed for the p.Gly187Asp mutation located C-terminal to the forkhead DNA binding domain (Table 1). More precisely, KGN granulosa-like cells were transfected with plasmids encoding WT or mutant (FOXL2-G187D) versions of *FOXL2* in phase with the green fluorescent protein (GFP) sequence. Luciferase reporter assays were carried out using the promoter region of *FOXL2* itself, an artificial specific FOXL2 promoter (named 2xFLRE) or the promoter of *OSR2*, a gene related to periocular mesenchyma development. Although subcellular mislocalisation was not observed, the protein's mutant version displayed a reduction in its transactivation properties regarding *FOXL2* and *2xFLRE* promoters, thereby underlining the p. Gly187Asp mutation's pathogenic effect (Laissue et al., 2009a). No functional effect was identified on the *OSR2* promoter which correlated with the absence of palpebral phenotype in the affected patient. Since then, no further *FOXL2* mutations have been described in non-syndromic POF women. It is worth noting that the recurrent FOXL2 c.402C>G (p.Cys134Trp) somatic mutation has been

related to the pathogenesis of ovarian granulosa cell tumours (Benayoun et al., 2010; Rosario et al., 2014; Shah et al., 2009). Indeed, it has been postulated that FOXL2 might act as a tumour suppressor gene or an oncogene.

4. Other POF causative genes

4.1. BMP15 (GDF9B)

BMP15 (also known as *GDF9B*), which is located on the human Xp11.2 chromosome, encodes a protein from the TGF-β superfamily of growth factors. TGF-β molecules, which include BMP, GDF, inhibins, activins and other proteins, are widely expressed in vertebrate species during many development processes, including female reproduction (Chang et al., 2002; Juengel and McNatty, 2005; Laissue et al., 2008; Massagué, 1998; Otsuka, 2010; Persani et al., 2011; Shimasaki et al., 2004). Particularly, *BMP15* is almost solely expressed by oocytes from early stages of follicular maturation and during all stages (Dube et al., 1998; Erickson and Shimasaki, 2003; Shimasaki et al., 2004).

BMP15, just like other TGF-β proteins, is synthesised as an inactive preproprotein having three domains: a signal peptide located in the N-terminal region, a prodomain (propeptide) and a mature region in the C-terminal region (Chang et al., 2002). The protein's mature region is released to the extracellular compartment after a series of post-translational processing steps (dimerisation and proteolytic cleavage) to bind to target cells' serine/threonine kinase types I and II receptors. Phosphorylation of the type II receptor activates the R-SMAD/SMAD intracellular pathway, resulting (after nuclear signalling translocation) in target gene activation or inhibition (Massagué, 2012; Massagué et al., 2005; Weiss and Attisano, 2013). BMP15 can form homodimers (BMP15:BMP15) or heterodimers (BMP15:GDF9) when it binds to its close paralogue growth differentiation factor 9 (GDF9). The secreted soluble dimer mature domain binds to receptors located on granulosa cell surface to participate in key steps regarding ovarian function, such as granulosa cell proliferation and follicle maturation, ovulation rate modulation, oocyte competence determination and regulating granulosa cell sensitivity to FSH (Fabre et al., 2006; Hashimoto et al., 2005; Moore and Shimasaki, 2005; Persani et al., 2011, 2014). It has been shown that BMP15:GDF9 heterodimers act specifically in mice and humans via a molecular complex which includes BMPR2 (serine/threonine kinase type II) and ALK4/5/7 (serine/threonine kinase type I) receptors as well as the ALK6 co-receptor (Peng et al., 2013). Functional assays have shown that BMP15:GDF9 heterodimers are more bioactive than homodimeric (BMP15:BMP15 or GDF9:GDF9) molecules in both species (Peng et al., 2013). These findings have partly explained these molecules' complex biological behaviour in mammalian species. Indeed, natural and artificial mutant models of *BMP15* and *GDF9* have shown intriguing phenotypes. Female homozygous *Bmp15* KO mice have shown subfertility secondary to reduced ovulation rate while *Gdf9*$^{-/-}$ animals have become completely infertile (Dong et al., 1996; Yan et al., 2001). Double KO mice (*Bmp15*$^{-/-}$ and *Gdf9*$^{+/-}$) have revealed a less severe ovarian phenotype than that observed in single (*Bmp15*$^{-/-}$ or *Gdf9*$^{+/-}$) animals (Yan et al., 2001). *BMP15* and *GDF9* heterozygous missense mutations in sheep have been related to hyperfertility and infertility phenotypes, respectively (Demars et al., 2013; Galloway et al., 2000; Hanrahan et al., 2004; Laissue et al., 2008).

Di Pasquale et al. (2004) described the first *BMP15* encoding mutation related to POF aetiology. The heterozygous *BMP15* c.704A>G (p. Tyr235Cys) mutation was identified by Sanger sequencing in two related Italian patients affected by ovarian dysgenesis and primary amenorrhoea. Tyr to Cys amino acid substitution (located in the protein's pro-region) was related to a deleterious dominant negative effect. The protein's mutant recombinant version had impaired proliferation activity regarding granulosa cells (Di Pasquale et al., 2004). Our group and others have undertaken massive Sanger sequencing projects of the *BMP15* encoding region in panels of POF patients during the last 10 years which have led to identifying more than 15 missense variants (Persani et al., 2014 and references therein). All of them were located in the protein's pro-region, except for c.13A>C, (p.Ser5Arg) and c. 985C>T (p.Arg329Cys) which affected the signal peptide and the mature domain, respectively (Lakhal et al., 2009, 2010; Rossetti et al., 2009; Wang et al., 2010).

Rossetti et al. tested the activity of some missense mutations found in POF individuals in an elegant functional reporter assay (Rossetti et al., 2009). Only the c.202C>T (p.Arg68Trp), c.413G>A (p.Arg138His) and c.443T>C (p.Leu148Pro) heterozygous mutations were clearly validated as causative of the phenotype (Fig. 5 and Table 1). Interestingly, the additional c.631C>T homozygous mutation found in an Indian POF patient could be assumed to be aetiological because it generated a truncated (p.Glu211Ter) protein potentially lacking the mature domain (Dixit et al., 2006).

Intriguingly, although *BMP15* has been shown to play a crucial role during mammalian ovary development and function, only five mutations modifying the protein sequence can be considered aetiological. This scenario led to our group engaging in a recent functional exploration of whether *BMP15* promoter polymorphism (c.-9G>C) might be related to the POF phenotype. Indeed, it has been established that this variant modifies the paired-like homeodomain transcription factor 1 (PITX1) binding site and that the c.-9G allele leads to *BMP15* promoter transactivation disturbances (Fonseca et al., 2014). This might suggest that fine-tuning of *BMP15* expression is critical for normal human ovarian physiology. It is worth noting that *GDF9* missense variants have been described in POF patients and dizygotic twinning but none has been functionally characterised to date (Laissue et al., 2008; Persani et al., 2014).

4.2. NANOS3 *and* STAG3

NANOS3 and *STAG3* are the most recently reported genes for which mutations have been validated (by functional assays) as being causative of non-syndromic POF (Table 1) (Caburet et al., 2014; Santos et al., 2014; Wu et al., 2013).

Three *NANOS* genes (*NANOS* 1 through 3) which encode CCHC zinc finger RNA interacting proteins have been described in mice and humans. The NANOS protein binds to the Pumilio co-factor to repress translation (Sonoda and Wharton, 2001; Wang et al., 2002; White et al., 2001). It has been shown that the *NANOS* genes are

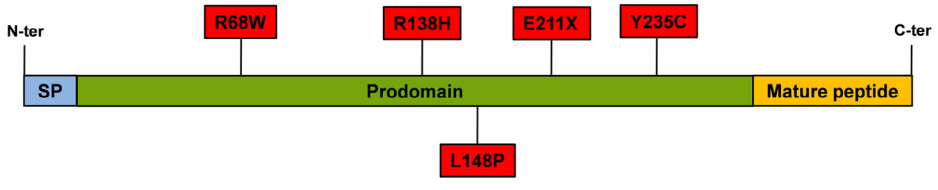

Fig. 5. BMP15 mutations causative of non-syndromic POF.

important for germ cell development in several species (Lai and King, 2013 and references therein). The single *Nanos* gene in *Drosophila*, first reported as a maternal effect gene, has been mainly linked to maintaining germ cell migration, survival and fate as well as germ stem cell self-renewal (Asaoka-Taguchi et al., 1999; Forbes and Lehmann, 1998; Kobayashi et al., 1996; Wang and Lehmann, 1991; Wang and Lin, 2004). The mouse KO model of *Nanos2* revealed specific male infertility while both female and male *Nanos3*$^{-/-}$ animals had reduced gonad size and were infertile because primordial germ cells were not maintained during migration (Suzuki and Saga, 2008; Suzuki et al., 2008; Tsuda et al., 2003). Attenuated *Nanos3* mRNA levels have been related to a significant decrease in germ cell number (Wu et al., 2013). *NANOS1*, *NANOS2* and *NANOS3* screening for mutations by Wu et al. in Chinese POF patients has revealed three missense variants: *NANOS1*-c.413C>T (p. Pro138Leu), *NANOS2*-c.39C>G (p.Leu13Phe) and *NANOS3*-c.457C>T (p.Arg153Trp) (Wu et al., 2013). Only the NANOS3 p.Arg153Trp mutation was novel since it was not present in public SNP databases and/or in control individuals from the same ethnic origin. The arginine residue in position 153 was strictly conserved among mammalian species, which suggested a key functional role. *In vitro* assays have shown that the p.Arg153Trp mutation is related to the synthesis of an unstable protein product (Wu et al., 2013). A subsequent *NANOS3* mutational analysis in 85 Brazilian POF women led to identifying the novel c.358C>A (p.Glu120Lys) homozygous mutation in two sisters having primary amenorrhoea (Santos et al., 2014). This mutation, located in the zinc finger domain, was related to cell death and underlined a NANOS3-mediated protective effect against apoptosis in primordial germ cells (Santos et al., 2014).

Concerning *STAG*, a combination of genome-wide genetic linkage homozygosity mapping was used in a previous study to establish the potential genetic defect underlying POF aetiology in a highly consanguineous Palestinian family (Caburet et al., 2012). Genotyping (using ~450 microsatellite markers and an SNP gene chip) and genetic analysis led to locating genomic candidate regions on chromosome 7 encompassing around 12.5Mb (Caburet et al., 2012). Thereafter, exome and Sanger sequencing led to identifying a 1 bp deletion (c.968delC) in the stromal antigen 3 (*STAG3*) gene in affected women from the same family (Caburet et al., 2014). The *STAG3* gene (also known as *Scc3*/*Irr1* in *S. cerevisiae*) encodes one of the four cohesin multi-protein complex subunits (Mehta et al., 2013 and references therein). It has been shown that cohesin has crucial functions during DNA replication and mitosis, gene expression, DNA repair and meiosis by allowing correct chromosomal pairing and segregation (Ball et al., 2014; Bardhan, 2010; Mehta et al., 2013). It has been predicted that the frameshift *STAG3* c.968delC mutation described by Caburet et al. can produce a truncated protein (p.Phe187fs) affecting the STAG domain which potentially lacks the armadillo (ARM)-type interaction domain (Caburet et al., 2014). Although the protein's precise effect has not been assessed at mRNA or protein level, functional evidence has been presented by studying homozygous mice lacking *Stag3*. Post-natal ovaries from *Stag3*$^{-/-}$ animals have shown severe and early ovarian dysgenesis while foetal oocytes have displayed arrested axial element assembling beyond the leptotene stage of prophase I. It has also been seen that synapsis between homologues became disrupted and that centromeric sister chromatid cohesion became lost (Caburet et al., 2014). Interestingly, more recent findings have proposed *STAG3* as a strong candidate gene for causing human male infertility (Llano et al., 2014).

A similar approach to that presented above for *STAG3* mapping was adopted very recently concerning two sisters affected by non-syndromic POF from a consanguineous Muslim Arab family (De Vries et al., 2014). The homozygous c.613C>T (p.Gln205X) novel mutation was identified in the synaptonemal complex central element protein 1 (*SYCE1*) gene by using homozygosity mapping, whole-exome and Sanger sequencing. The SYCE1 factor is a component of the synaptonemal complex which is necessary for maintaining closely-related paired chromosome homologues during meiosis (Costa and Cooke, 2007; Costa et al., 2005).

Female KO mice which were homozygous for *Syce1* have been seen to be infertile due to arrested meiosis at prophase I, lacking synaptonemal complexes and having a severe reduction of follicles (Bolcun-Filas et al., 2009). No functional tests were performed to assess the potential pathogenic impact of the *SYCE1* c.613C>T sequence variant. However, its intrinsic nature (a novel homozygous nonsense mutation), the experimental approach used to identify it and the evident ovarian phenotype displayed by female *Syce1*$^{-/-}$ mice strongly suggested it as an aetiological factor.

5. The polygenic nature of reproduction and POF

Mammalian species' fertility and successful reproduction depend on many molecular factors' precise function in males and females during distinct phases, such as sex determination, meiosis and gametogenesis, hormone synthesis and action, fecundation, early development, implantation and post-implantation. Correctly accomplishing the above has meant that both coding and regulatory genomic regions have been selected during evolution to guarantee the ability to produce life offspring (fitness). It has been shown that hundreds of genes are implicated in reproduction (having been particularly well-studied in mouse models) (Jagarlamudi et al., 2010; Matzuk and Burns, 2012; Matzuk and Lamb, 2002, 2008; Roy and Matzuk, 2006). Furthermore, this molecular network is finely regulated in terms of gene expression, thereby underlining the complex processes related to physiological and pathological conditions.

For instance, it has been shown that gene dosage is a critical feature for mammalian gender determination. Interestingly, although *NR0B1* (also known as *DAX1*) mutations have been associated with X-linked primary adrenal insufficiency and hypogonadotropic hypogonadism, gene duplications have been related to dosage sensitive male to female sex reversal (Bardoni et al., 1994; McCabe, 2007). More recently, a hypomorphic mouse model of *Sf1* was created by the genetic ablation of CBP/p300-interacting transactivator with ED-rich tail 2 (*Cited2*) (Combes et al., 2010). Gonad development was delayed in *Cited2*$^{-/-}$ males and their testis structure was disrupted. Furthermore, ectopic cell migration and a transient delay in *Foxl2* and *Wnt4* expression were recorded in XX *Cited2*$^{-/-}$ gonads. These findings led our group to hypothesise that *CITED2* encoding mutations in humans might be related to non-syndromic POF aetiology. Indeed, we identified the *CITED2* c.604C>A (p.Pro202Thr) mutation as a strong candidate mutation for POF aetiology (Fonseca et al., 2012). Precise gene expression also seems to be a critical condition during folliculogenesis. For example, growth factor proteins (especially *TGFβ*) have been described as important molecules during cross-talk between intraovarian signalling compartments. Indeed, fine modulation of *BMP2*, *BMP4*, *BMP5*, *BMP6*, *BMP7*, *BMP15*, *GDF9* and *INHA* is necessary for physiological bidirectional communication between oocytes, granulosa cells and theca cells (Chang et al., 2002; Knight and Glister, 2001). As mentioned above, regulatory variants in the *BMP15* promoter region may thus contribute towards POF aetiology (Fonseca et al., 2014).

It is worth noting that reproduction physiology implies complex phenotypes which can be considered quantitative traits. Indeed, fertile individuals vary regarding their reproductive characteristics (e.g. age of menarche, number of follicles at birth, ovarian reserve, testicular weight, sperm count and motility, plasma hormone levels), thereby underlining the fact that phenotypes in mammalian reproduction may be regulated by quantitative trait loci (QTL) (Laissue et al., 2008; L'Hôte et al., 2010). It is worth noting that QTL can be defined as chromosomal regions carrying genes (encoding and regulatory regions) which are responsible for complex, measurable phenotypes. The latter assumption was explored by studying

particular mouse models allowing QTL mapping (Laissue et al., 2009b). The interspecific recombinant congenic strains (IRCS) model has been especially useful for mapping short QTL responsible for male (testis and prostate weight, sperm nucleus shape, sperm survival) and female phenotypes (embryonic lethality and resorption) (Laissue et al., 2009c; L'Hôte et al., 2007; Vatin et al., 2012). Interestingly, some of these findings have led recently to identifying human *ALPP* encoding sequence variants as being related to recurrent spontaneous abortion and *in vitro* fertilisation success (Vatin et al., 2014).

All in all, the scenario presented above underlines physiological and pathological reproductive phenotypes' polygenic nature and that the dysregulation of any of these molecular cascades (due to genetic variants) might contribute towards or cause distinct forms of infertility, including POF. However, it remains to be determined how many genes may be responsible for most non-syndromic POF cases.

Similarly to other complex diseases, it is likely that the common disease–common variant hypothesis may not be related to POF pathogenesis (Botstein and Risch, 2003; Gibson, 2012; Pritchard and Cox, 2002). The foregoing was assumed before the introduction of genome wide association studies (GWAS) which showed that most genetic variance did not arise ("missing heritability" concept) from individual SNPs mapped via this technique (Eichler et al., 2010; Gibson, 2012; Maher, 2008; Manolio et al., 2009). Several other models (e.g. the rare allele model, the infinitesimal model, the broad sense heritability model) have been proposed for explaining heritability and the amount of participating sequence variants in complex diseases affecting humans (Gibson, 2012 and references therein). However, accurately predicting how many genes may be related to non-syndromic POF pathogenesis is difficult because high throughput genomic sequencing has not been performed on large panels of patients.

6. NGS approaches and future directions

After the first description of the Human Genome, genomics has significantly evolved to contribute for a better understanding of the molecular basis of medicine (Guttmacher and Collins, 2002; Lander et al., 2001). Genotyping (e.g. via Genome Wide Association Studies, GWAS) and genome-wide sequencing (e.g. via NGS) studies have accelerated the identification of a relevant number of genes that are causal and risk factors for both rare and common human diseases (Boycott et al., 2013; Green and Guyer, 2011; Kiezun et al., 2012; Visscher et al., 2012; Zimmern and Khoury, 2012). Studying human pathology's genetic architecture (defined as the number, frequency and effect of aetiological alleles) has evolved exponentially since the introduction of NGS technologies involving hybrid capture (Albert et al., 2007; Gnirke et al., 2009; Hodges et al., 2007; Shendure and Ji, 2008). Significant advances have been made in bioinformatics, making the analysis of a large amount of omics data affordable and more accurate (Berger et al., 2013).

NGS, which was presented for the first time in 2007, marked the beginning of a new era concerning the analysis of human genome sequences (Albert et al., 2007; Gnirke et al., 2009; Hodges et al., 2007; Shendure and Ji, 2008). Before the arrival of this technique, Sanger sequencing was widely used for screening variants potentially causing monogenic and complex diseases. However, studies involving numerous genes and/or large genomic regions were particularly challenging due to inherent technical limitations. In fact, Sanger sequencing permits a read length encompassing up to 700 base-pairs (bp) per reaction. On the contrary, the NGS approach allows simultaneously analysing millions of bp in only hours, thereby facilitating large-scale exploration of the human genome. The first NGS studies and many projects nowadays have been focused on researching novel recessive disease-related sequence variants, particularly those caused by homozygous mutations. The molecular origin of >3500 rare diseases has been determined to date, with NGS having significantly contributed towards this (Boycott et al., 2013). Such aetiological variants are relatively easy to identify because most are novel (absent in databases of sequence variants) and/or are exclusively present in several members of the same family. This allows easy screening of candidate variants in public SNP databases and filtering them between affected and non-affected individuals from the same family. It is important to note that familial cases of hypofertility/infertility, including POF, are rare because genetic causative variants are under strong negative selection. Successful screening attempts, via NGS, have been reported for monogenic dominant Mendelian disorders. However, this approach implies more drawbacks because heterozygous mutations (logically) occur more frequently, thereby involving complex filtering to select potentially deleterious mutations.

At present, three NGS main approaches ("formats") are normally used. They mainly depend on the length of the genome region being analysed: whole-genome sequencing (WGS), whole-exome sequencing (WES) and custom target sequencing microarrays (TSM). WGS is principally used for research purposes while WES and TSM are used for both research and diagnosis. We and others have proposed that NGS is a coherent and efficient tool for establishing a molecular diagnosis for monogenic diseases for which clinical and genetic definition is challenging because of overlapping phenotypes (Ortega-Recalde et al., 2013; Patiño et al., 2014; Tan et al., 2014; Woods et al., 2014). Many NGS panels of candidate genes are used today as part of diagnostic pipelines for distinct pathologies (Biesecker and Green, 2014; Rehm, 2013). Several diagnostic tests and prognostic tools for monogenic and complex diseases are used in clinical environments. In addition, human genomics based on microarray assays and potent bioinformatics analysis have permitted to study large amounts of data which have enhanced the discussion on potential genetic models (e.g. the rare allele model, the broad sense heritability model, the infinitesimal model) underlying frequent diseases (Gibson, 2012). To date, NGS has not been widely used for some complex pathologies in which several variants might contribute towards the phenotype, because data analysis highlights remarkable complexity, especially for simultaneous interactive network exploration. However, some reports concerning complex traits (e.g. cancer, triglyceride plasma levels, HDL and LDL proteins, obesity, type 1 diabetes, blood pressure) as well as in non-invasive prenatal screening tests (NIPT) have been proposed (Kiezun et al., 2012; Koboldt et al., 2013; Lange et al., 2014; Ross and Cronin, 2011; Shyr and Liu, 2013).

Concerning POF, as mentioned above, two studies using NGS technologies (exome sequencing) have been performed in family-related cases of POF (Caburet et al., 2014; De Vries et al., 2014). A very recent TSM study of 70 candidate genes has identified potential novel genes (*ADAMTS19* and *BMPR2*) and mutations related to the disease pathogenesis (Fonseca et al., 2015).

It is worth noting that main NGS technology drawbacks imply variable rates of positive and negative results, and difficulties for studying highly repetitive genomic regions. Thus, NGS positive results must be validated by Sanger sequencing in all cases.

However, due to the exponential cost decrease of high-throughput sequencing, it has been proposed that NGS results could be confirmed by a second round of assays using the same technical approach (replicates) (Robasky et al., 2014; Zhang et al., 2014).

Future directions would involve testing a significant number of isolated (non-family-related) non-syndromic POF cases. This approach could be facilitated by designing custom microarrays including coding and regulatory regions for a large number of POF candidate genes. It would also be possible to perform exome sequencing assays in which a subset of genes (the POF candidates) might be specifically analysed. Furthermore, whole-genome

sequencing might reveal further disease pathogenesis-related regions. NGS technology will certainly involve a key tool for mapping genome variations participating in ovarian-related physiological and pathological conditions. It could be used in the near future regarding a variety of ovarian dysfunctions for diagnostic and predictive purposes.

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