CHAPTER 2

LITERATURE REVIEW

2.2. Definition and prevalence of the *FMR1* premutation

The *FMR1* gene is located at Xq27.3. It spans 38 kilobase (kb) and encodes a 4.4 kb transcript consisting of 17 exons (23). There are essentially four allelic forms of the gene with respect to the CGG repeat length. They are referred to as common, intermediate, premutation, and full mutation.

The presence of a full mutation in the *FMR1* gene, consisting of over 200 CGG repeat results in silencing of the gene by hypermethylation. Consequently, no mRNA is produced, and the lack of the gene product, The absence of FMRP, product of *FMR1* gene is responsible for FXS (24). FXS is characterized by mental retardation, macroorchidism, and mild facial abnormalities (25, 26). In the general population, approximately 1/4000 males have FXS and by inference, about 1/6000 females (4).

Premutation alleles contain between 55-199 repeat. These alleles are unmethylated and repeat tracks can become unstable when transmitted from mother to their child. Premutation alleles are at risk of expansion into a full mutation in the next generation (27). Premutation in the *FMR1* gene associated with primary ovarian insufficiency (POI) and the Fragile X-associated tremor/ataxia syndrome (FXTAS) (8, 28). Approximately 1/250 females and 1/800 males carry a premutation alleles.

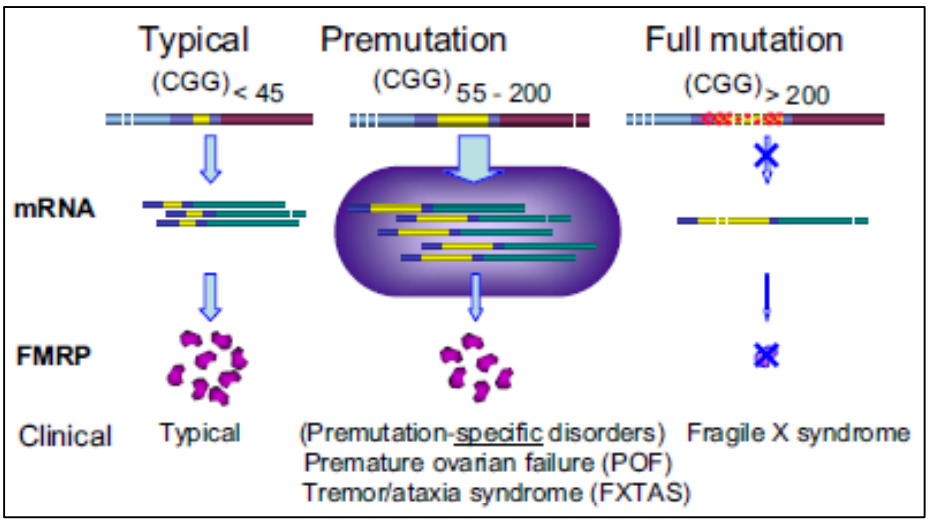

Figure 2.1. Expression of *FMR1* in normal women, premutation carriers, and full mutation carriers. Adapted from (27)**.**

Alleles in the intermediate range (45-54 repeats) are defined as such because of their repeat size and because they have been identified in the general population with no known cases of unstable transmission (25).

Typical alleles contain less than 45 CGG repeat. The individu have these alleles there is no impairment associated to *FMR1* gene.

2.2. Premutation-associated primary ovarian insufficiency

FMR1 is a highly conserved gene that is composed of 17 exons, that span about 40 kilobases (kb) of DNA and encodes an mRNA of 3,9 kb. The *FMR1* premutation is now a well-established cause of POI and the leading known cause of inherited POI. Approximately 20% of female premutation carriers develop POI (2). Overall, the entire distribution of the age at menopause for premutation carriers is shifted to the younger age by about five years compared with noncarriers (13).

POI is defined as amenorrhea before the age of 40 years and the presence of an elevated follicle stimulating hormone (FSH) level in serum (29, 30). It is a heterogeneous disorder affecting 1% of reproductive-age women in the general population.

In most cases, the cause of POI is not identified (11, 12, 31). The known causes include autoimmune, genetic, iatrogenic following surgical, radiotherapeutic or chemotherapeutic intervention as in malignances, and environment (11, 12). Autoimmune disease may cause damage in ovaries, that is associated with autoimmune regulator polyglandular failure type 1 or autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) and polyglandular failure type 2 (11).

Chromosomal abnormalities are frequently seen, especially in young women with POI. The association of chromosomal abnormalities and POI has been identified. Deletion in Forkhead Transcription Factor 2 (*FOXL2)*, a gene on chromosome 3 coding a winged heix, forkhead transcription factor will blockade granulose cells differentiation, early activation of primordial follicles and subsequent oocyte apoptosis (11). Mutations in galactose 1-phosphate uridyl transferase (*GALT*) gene will result in *GALT* enzyme deficiency and therefore build up galactose metabolites including galactose 1-phosphate and galactiol in multiple cell types that cause cell damage. The patients carrying these mutation clinically present mental retardation, liver failure, renal insufficiency and POI in female patients. There are many other autosomal genes involved in mechanism leading to POI, such as Inhibin α Subunit (*INHA)* gene located on chromosome 2,

Ataxia Telangiextasia Mutated (*ATM)* gene on chromosome 11, Polymerase γ (*POLG)* gene on chromosome 15, BecQ Protein like 3-DNA Helicase (*BLM),* Autoimmune Regulator Polyglandular Failure *(AIRE),* Eukaryotic Translation Initiation Factor 2B, Subunit 2 *(EIFB2B-2),* Eukaryotic Translation Initiation Factor 2B, Subunit 4 *(EIFB2B-4),* Eukaryotic Translation Initiation Factor 2B, Subunit 5 (*EIFB2B-5),* Luteinizing Hormone/Choriogonadotrophine receptor *(LHCGR),* Steroidogenic Acute Regulatory Protein (*STAR),* 17-α Hydroxilase/17,20-lyase enzyme (*CYP17A1)* (11).

Table 2.1. Causes of primary ovarian insufficiency Adapted from (11)**.**

Causes	Gene identification	Gene location
Genetic		
X chromosome		
X chromosome monosomy		
45X/46XX mosaic		
X chromosome translocations or partial deletions		
BMP15	Bone morphogenetic protein 15	Xp11·2
DIAPH2	Diaphanous	Xq22
FMR1 premutation	Fragile X mental retardation protein 1	Xq27·3
FMR2	Fragile site, X-linked, E	Xq28
Autosomal genes		
INHA	Inhibin α subunit	2q33-q36
FOXL2	Forkhead transcription factor	3q23
GALT	Galactose-1-phosphate uridyl transferase	9p13
ATM	Ataxia telangectasia mutated	11q22·3
POLG	Polymerase (DNA directed) γ	15q25
BLM	RecQ protein-like-3 DNA helicase	15q26·1
AIRE	Autoimmune regulator polyglandular failure type 1	21q22·3
EIF2B-2	Eukaryotic translation initiation factor 2B, subunit 2	14q24
EIF2B-4	Eukaryotic translation initiation factor 2B, subunit 4	2p23
EIF2B-5	Eukaryotic translation initiation factor 2B, subunit 5	3q27
Autosomal genes affecting follicle function		
FSHR	FSH receptor	2p21-p16
LHCGR	Luteinizing hormone/choriogonadotrophin receptor	2p21
STAR	Steroidogenic acute regulatory protein	8p11·2
CYP17A1	17-α hydroxylase/17,20-lyase enzyme	10q24·3
Follicle destruction		
Autoimmune		
AIRE	Autoimmune regulator polyglandular failure type 1 or APECED (autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy)	21q22·3
HLA association	Polyglandular failure type 2	
Iatrogenic		
Surgery		
Chemotherapy		
Radiation therapy		

The X-chromosome is involved in mechanisms underlying POI as demonstrated in Turner syndrome, which in most cases is the result of the complete loss of the X-chromosome, where the patients experience early ovarian failure. Deletion and/or translocation of genes on the X-chromosome are critical to ovarian function, which also affects the correct alignment of chromosomes during meiosis, eventually leading to follicular atresia. Candidate genes or loci on the X-chromosome that cause familial or sporadic POI have been identified such as Bone Morphogenetic Protein 15 (*BMP15),* Diaphanous 2 *(DIAPH2),* FSH Receptor 1*(FSHPRH1),* X (inactive) Specific Transcript *(XIST),*Angiotensin II Subtype 2 *(AT2)*, and SRY (Sex Determining Region Y) Box 3 (*SOX3)* (14, 32).

Once an increased risk for POI had been established among premutation carriers, surveys were conducted to determine the frequency of premutation carriers among women with idiopathic POI. Using studies that identified women primarily through reproductive endocrinology clinics and clearly distinguished familial from sporadic POI, the estimated frequency of women who are premutation carriers is 11.5% (familial POI) and 3.2% (sporadic POI) (13).

The association between CGG repeat size in *FMR1* gene and female reproductive phenotype was identified, includes a continuum of diminished ovarian reserve, resulting in menstrual cycle alteration (short, irregular and skipped cycles), hormonal fluctuations, and decreased fertility (2, 16). Some of the variation in the estimation of penetrance is probably due, in part, to the increasing probability of primary ovarian insufficiency with increasing number of CGG repeats. Surprisingly, however, this relationship is nonlinear. Indeed, the

risk appears to increase with increasing premutation repeat size between 59 and 99, thereafter the risk of POI become plateaus even decreases for women with repeat size over 100 (13).

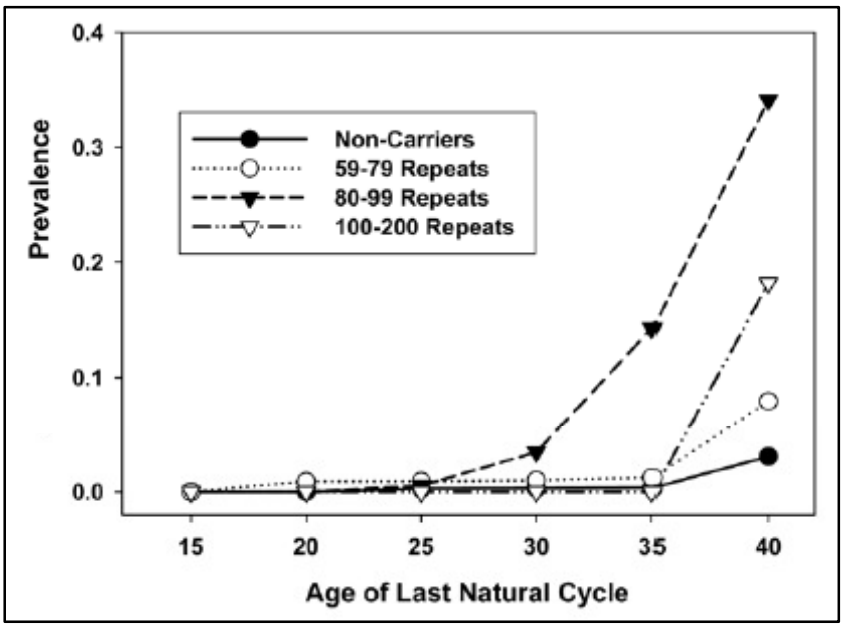

Figure 2.2. Relationship of primary ovarian insufficiency prevalence and CGG repeat size in *FMR1* gene. Based on a data set of 429 *FMR1* premutation carriers and 517 non-carriers. Adapted from (13).

The mechanisms of POI related to the *FMR1* premutation is unclear. Attractive possibilities include decreased number of follicles in the initial pool, an accelerated rate of atresia of follicles, or some other mechanisms impairing follicles function (2). All these possibilities mechanisms are consistent with the hypothesis that a continuum of impaired ovarian function exists in women with the *FMR1* premutation.

There are many hypotheses of underlying mechanisms of POI associated with *FMR1* gene including the potential role of FMRP in the ovary and the *FMR1* mRNA gain-of-function toxicity (33, 34). Conway et al (1998) hypothesize a

toxic effect of premutation alleles and suggested that effect may reduce the follicle pool at birth. Other hypothesis that have been suggested are mislocalization of premutation transcripts (35) or abnormal binding of other proteins to the repeat tract (36).

FMRP is a ubiquitously expressed RNA-binding protein, and several common alternatively spliced isoforms are found in approximately equal abundance (37). FMRP is expressed in several areas that may affect any of developmental steps including follicular development. It is expressed in the brain, in regions that are critical for hormonal regulation, in spermatogonia in the testis, in primordial grem cells in the fetus, and in the oocyte and granulosa cells of developing follicles in adult ovaries (38-40). Bachner et al (1993) demonstrated that in the mature ovary no specific *FMR1* expression signal was found, but enhanced expression were seen in the fetal ovary. At this developmental stage proliferation of oogonia takes place. It is suggested that *FMR1* serves a special function during germ cell proliferation in males and females (41). Studies from Drosophila showed that d*FMR1* null ovaries contain egg chambers with both fewer and supranumerary germ cells. FMRP also controls the levels of *Cbl* mRNA in the ovary and that reducing *Cbl* gene dosage by half rescues the d*FMR1* oogenesis phenotypes. This result suggested that FMRP controls germline proliferation during oogenesis by regulating the expression of cbl in the developing ovary (42). In Drosophila, d*FMR1* is also known to be involved in germ cells and oocyte specification (43). Therefore, mutations that lead to an

altered pattern of ovarian FMRP expression, such as promoter mutations, could lead to POI in premutation carriers (33).

Conway et al. (1995) proposed that a premutation allele is underexpressed in fetal ovaries, leading to a reduction in the number of oocytes at birth. Decreased expression of FMRP from premutation alleles during germ cell proliferation may adversely affect the number of oocytes produced, thus reducing the available pool in adults (44).

Another possibility is that a particular isoform of FMRP, crucial during oogenesis is less efficiently produced from premutation alleles. Murine studies have shown that FMRP is particularly strongly expressed during the mitotic phases of oogenesis and so any changes in expression at this critical time could dramatically reduce the number of oocytes. Against this hypothesis are the results of studies on at least 11 isoforms of *FMR1* showing no differences between tissues of normal compared with premutation alleles. Moreover, analysis of the ovaries from a 16-week-gestation fetus carrying a full mutation allele that did not express FMRP suggested that the number of oocytes was not markedly reduced (33). The risk for ovarian dysfunction is not increased among full mutation carriers; thus the molecular mechanism underlying this premutation-associated disorder is unrelated to the reduction of FMRP (16).

The possible explanation of this phenomenon appears to be no detrimental effect on ovarian function because the inactivation of *FMR1* gene by hypermethylation. Another theory is that although FMRP is absent in the full

mutation, a back up protein provides its function to regulate oocytes production (16).

The increased *FMR1* mRNA is postulated to result in a RNA gain-of-function toxicity and recent work suggests that in addition to the reproductive effects in female premutation carriers, male who carry the fragile X premutation may develop an adult-onset neurological disorder related to the RNA gain-of-function toxicity (45). It has been demonstrated that at the molecular level, individuals with premutation alleles show elevated *FMR1* mRNA levels, 2-10 times higher than normal, in peripheral blood leukocytes, but normal or reduced FMRP levels. The increased levels of *FMR1* mRNA may have some adverse (toxic) effects on germ cells, perhaps destabilizing meiotic arrest or increasing the attrition rate, again causing a more rapid depletion of the germ cell pool. This dominant gain-of-function mechanisms is well supported for the other known premutation-associated disorder, FXTAS (20, 27, 46). *FMR1* expression studies identified transcripts in granulose cells of ovarian follicles (39, 40). Interestingly, transcripts were present in maturing follicles only, not those in the early stages (40).

Previous neurohistological studies on brains of four symptomatic elderly *FMR1* gene premutation carriers demonstrated neuronal degeneration in the cerebellum and the presence of eosinophilic intranuclear inclusion in both neurons and astroglia that show a positive reaction with anti-ubiquitin antibodies, which suggests a link with the proteasome degradation pathway. These findings suggest that the premutation tracts in the *FMR1* mRNA may attract high quantities of

CGG-binding proteins with a consequent cumulative cytotoxic effect that may lead to intranuclear inclusion formation and ultimately neuronal cell death (28).

Hagerman et al. (2004) proposed model to explain how mutations in one gene may lead to different diseases, including a lack of FMRP leading to clinical features of fragile X and the RNA toxic gain-of-function model for FXTAS. In this model, specific protein interactions with the 5 UTR of the *FMR1* mRNA are altered as a consequence of expansion of the CGG repeat. In the premutation range, the expanded CGG repeat would lead to excess binding of one or more proteins, owing to increased mRNA copy number, increased number of CGG repeats, and/or altered secondary/tertiary RNA structure. This excess binding depletes the proteins from the cellular pool, resulting in the loss of their normal functions in other regulatory processes. The sequestration process would also trigger the accumulation or abnormal processing of proteins by the proteasomal degradation pathway, leading to inclusion formation with associated ubiquitinated proteins, proteasomal subunits, and stress-response (HSP) proteins (28).

It is possible that the POI presenting in female carriers may be caused by a toxic RNA effect in the ovary as well (47).

2.3. Premutation mice is an excellent model to study primary ovarian insufficiency

A transgenic knock in mice has been developed and might be an excellent model to study POI because the process of the genital system development and folliculogenesis in mice is similar with that in the human ovaries (17, 18).

In mice, development of the genital system proceeds sequentially, beginning with differentiation of the primordial germ cells (PGCs) and formation of the genital ridge. The PGCs of the mice embryo are first detected at 7-8 days *post coitum* (dpc) as a small population of alkaline phosphatase-expressing cells in the extraembryonic tissue at the root of the allantois (48). The oocytes in the neonatal ovary are the result of migration, and subsequent proliferation of PGCs from the allantois to the gonadal ridge during fetal development of the female mice. PGCs will be continued to proliferate, forming clonal, interconnected clumps, yielding a total of 40,000 PGCs (49, 50).

Several autosomal genes encoding transcription factors mediate early events in the development of the indifferent gonads, PGCs development and survival. Disruption of the genes Lim homeobox 1 (*Lhx1*), Wilm's tumour 1 (*Wt1*), or steroidogenic factor 1 (*Sf1*), results in arrested gonadal development (51, 52). Bone morphogenetic protein 4 gene (*BMP4)* regulates the formation of PGC precursors (53). The others genes that have been identified for regulation of PGCs development and survival are Stem cell factor (*SCF*) (54) (55), leukaemia inhibitory factor (*LIF*) (56), interleukin 4 (57), Neuregulin β (58), transforming growth factor β, and activin (59).

Female germ cells enter meiotic prophase and proceed through the leptotene, zygotene, and pachytene stages of meiosis, arresting in the last stage of meiotic prophase I, the diplotene stage, around the time of birth. No new oocytes or follicles are formed during the remainder of life. Whereas in some of these follicles growth is immediately initiated, most primordial follicles remain dormant until initial recruitment occurs. Thus, the primordial follicles constitute the resting stock of non-growing follicles, the ovarian reserve, which is progressively depleted during reproductive life, resulting in limited fertility in female mice older than 12 months (60).

Ovarian follicles support oocyte growth and maturation and produce steroid hormones critical for the function of the reproductive tract. The earliest stage of follicular development is the primordial stage. Primordial follicles form during the neonatal period as clusters of germ cells are invaded by pre-granulosa cells. Activation of primordial follicles begins the process of follicular development, initiating a cascade of events that will end either in death of the follicles through atresia or in ovulation (60).

The period of follicle growth between the recruitment of the primordial follicle and its rescue by FSH can take at least three weeks. Similar to humans, this process of recruitment and selection is a very wasteful process. Only a few, about ten to twelve in the mice, of the hundreds of antral follicles are selected by FSH, whereas the others are removed through a process called atresia which involves apoptosis (60).

During fertile life, the mice displays the so-called estrous cycle. The estrous cycle is composed of a series endocrine, behavioral and physiologic events that typically occur every 4-6 days throughout the reproductive life span unless interrupted by pregnancy, pseudopragnacy, or unestrus. These cycle can be divided into several stages, that is, pro-estrous, estrous, met-estrous and di-estrous. The process is regulated by hormones secreted by the pituitary gland and ovarian follicles. Most often the stage of the estrous cycle is determined by examining smears of cells from the vagina (60).

The strategy of developing and using mice models with spontaneous or induced mutations to investigate ovarian dysfunction is extremely powerful, and has provided important insights into the mechanistic basis of POI and subsequent infertility (61, 62).

In order to study POI related to the fragile X premutation in an animal system, a transgenic knock-in mice model has been developed in which the CGG(8) allele in the endogenous murine *Fmr1* gene was replaced, via homologous recombination, with a human Nhel-Xhol fragment containing a CGG(99) allele (19).

As in humans, there is moderate repeat instability during transmission in the premutation mice. Another finding with the derived expanded CGG repeat line is that, as in humans, the level of *Fmr1* mRNA in brain homogenates increases by nearly 3-fold compared to controls (63). These premutation mice have allowed us to model the pathophysiology of FXTAS in males carrying the premutation, including deficits in motor function, learning, and memory, the occurrence of

intranuclear inclusions in neurons, and the transgenerational repeat instability occurring in fragile X syndrome (64).

The observations in premutation mice suggest a direct role of the *Fmr1* gene in the pathogenesis of FXTAS, either by CGG expansion per se or by elevated *Fmr1* transcript levels (RNA-gain-of-function). It is important to note that these elevated *Fmr1* mRNA levels in brain tissue were already present by one week of age, which underscores the potential for as yet undocumented developmental consequences of excess message production in both FXTAS and POI in female carriers of the premutation. Thus far, detailed studies focused on ovarian function in homozygous female mice with an expanded CGG-repeat are lacking (37).