
Review

An "Omic" Overview of Fragile X Syndrome

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Simple Summary: Fragile X syndrome (FXS) is a neurodevelopmental disorder and remains the most frequent inherited cause of intellectual disabilities. Fragile X patients are at great risk to develop behavior problems including autism, anxiety, aggressivity and hyperactivity. FXS results from a mutation leading to the absence of a single protein, the Fragile X Mental Retardation Protein (FMRP). Most of the study aiming to understand the physiopathology of FXS are centered on the effect of its absence on protein synthesis. However, besides protein synthesis regulation, FMRP has several other functions, needing to be more understood, that could play a significant role in FXS. The goal of the present work is to review some of these functions and to put them into perspective in order to get a more comprehensive understanding of FXS physiopathology.

Abstract: Fragile X syndrome (FXS) is a neurodevelopmental disorder associated with a wide range of cognitive, behavioral and medical problems. It arises from the silencing of the fragile X mental retardation 1 (FMR1) gene, and consequently, in the absence of its encoded protein, FMRP (Fragile X Mental Retardation Protein). FMRP is a ubiquitously expressed and multifunctional RNA-binding protein, primarily considered as a translational regulator. Pre-clinical studies of the past two decades have therefore focus on this function to relate FMRP's absence to the molecular mechanisms underlying FXS physiopathology. Based on these data, successful pharmacological strategies were developed to rescue fragile X phenotype in animal models. Unfortunately, these results did not translate into human, as clinical trials using same therapeutic approaches did not reach the expected outcomes. These failures highlight the need to put into perspectives the different functions of FMRP in order to get a more comprehensive understanding of FXS pathophysiology. In this review, FMRP's involvement on noteworthy molecular mechanisms are pointed out; ultimately contributing to various biochemical alterations composing the fragile X phenotype.

Keywords: Fragile X Syndrome; FMRP; RNA-binding protein; Physiopathology; Genomic; Transcriptomic; Proteomic; Metabolomic

1. Introduction

The "Omic" term refers to the wide range of high throughput experimental approaches aiming to get a holistic depiction of the different biomolecules within a living organism. These techniques intent to get a better understanding of systems biology throughout a comprehensive overview of the genes (genomic), RNA (transcriptomic), proteins (proteomic) and metabolites (metabolomic) making up a cell or a tissue. However, when performing such studies, one should avoid the mistake of only getting a simplistic "Omic" portrait of its biological sample (Figure 1A). Indeed, an aware experimenter should take in consideration the large and inherent diversity within each class of biological molecules and the complex nature of the different interactions between them (Figure 1B-C).

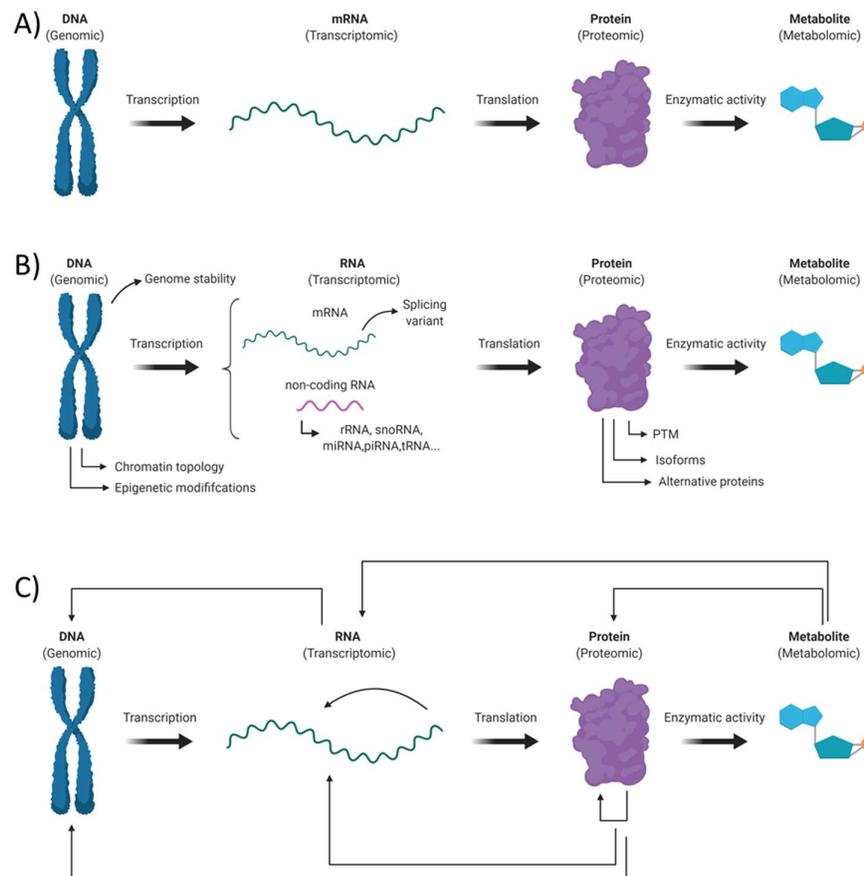


Figure 1. Schematic representation of the different “Omic” categories: **A)** A simplistic “Omic” representation in which a gene encode a single mRNA which is further translated in a unique protein that can, ultimately, exerted its biological activity, which in this example, produced a metabolite. **B)** A large diversity of biomolecules exist within each “Omic” categories. **C)** Schematization of the different interaction between component of each “Omic” categories.

Fragile X syndrome (FXS) is a X-linked neurodevelopmental disorder with an approximative prevalence of 1/4000 in males and 1/8000 in females [1]. FXS is the leading inherited cause of intellectual disabilities (ID) and the most prevalent monogenic cause of autism spectrum disorders (ASD). The severity of ID varies from one individual to another; being usually moderate to severe in men while it is most often mild to borderline in women. It is estimated that between 30 and 50% of patients with FXS will present ASD. Fragile X (FX) is also characterized by a broad spectrum of challenging behaviors (anxiety, aggression, self-injury, attention deficit disorder (ADHD), social withdrawal), physical (facial dysmorphism, macroorchidism, joints hypermobility) and self-limited medical problems (recurrent otitis media, seizures). Furthermore, all FX-related comorbidities are found with variable penetrance, resulting in a large phenotypic heterogeneity across this population [2–5].

Fragile X syndrome typically originates from the dynamic expansion of CCG trinucleotide repeats found in the 5' UTR of the FMR1 gene (Xq27.3) [6]. A fully mutated allele possesses more than 200 repeats, which results in the transcriptional silencing of the gene throughout an epigenetic mechanism, and ultimately, in the loss of expression of the Fragile X Mental Retardation Protein (FMRP) [6,7]. Mosaicism is commonly observed in the fragile X population, with a prevalence ranging from 12-41% in males [8,9]. Repeats-size

and methylation mosaicisms have been described in FXS and are both associated with a detectable FMRP expression, a higher IQ and milder behavioral problems [10–13] (Figure 2). X chromosome inactivation ratio in fragile X females (proportion of cells that have inactivated the fully mutated X chromosome) have also been correlated with a better prognosis [10].

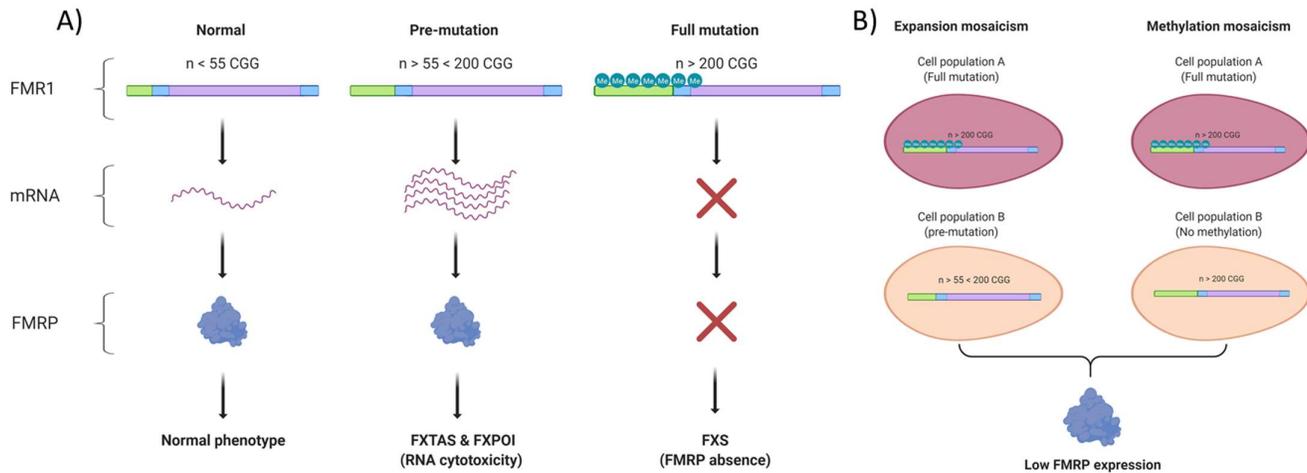


Figure 2. Genetic of Fragile X syndrome: **A)** CGG trinucleotide repeats are found between the promoter and the first exon of the FMR1 gene. A normal allele has less than 55 while a fully mutated one has more than 200. A full mutation leads to the silencing of the FMR1 gene throughout an epigenetic mechanism which is, in part, characterized by the methylation (Me) of the gene. A pre-mutation allele has between 55 and 200 CGG repeats and can lead to the fragile X tremor and ataxia syndrome and the fragile X primary ovarian insufficiency. **B)** Two types of mosaicism are observed in Fragile X. Expansion mosaicism is characterized by a cell population carrying a pre-mutation and another with a fully mutated and methylated allele. On the other hand, methylation mosaicism is characterized by two populations of fully mutated cells, but only one of them carries a methylated allele. Both types of mosaicism result in low FMRP expression.

The fragile X mental retardation protein is expressed ubiquitously across the body, but to a higher degree in the brain and gonads [14,15]. FMRP possesses both nuclear localization and exportation signals, two Argentin domains as well as two hnRNP homology domains (KH) and one arginine-glycine-glycine box (RGG) [16]. The last three domains provide FMRP RNA binding capacity. FMRP is able to recognize both RNA motifs, such as pseudoknots structure, SoSLIP and G-quartets [17–19], as well as RNA sequences [20]. The RNA binding capacity of FMRP is central to its molecular function. Indeed, missense mutations in either KH domains impair FMRP ability to bind RNA and are associated with many typical FX phenotypes, including ID, facial dysmorphism and macroorchidism [21,22]. Despite the fact that FMRP is involved in many stages of RNA metabolism, translational regulation remains its foremost function [23–25].

FMRP functions are essential for normal neurodevelopment, as illustrated by the many neuroanatomical defects harbored by fragile X patients. Indeed, abnormalities in white and grey matter volume of different brain regions have been observed in both young and adult patients [26–28]. Furthermore, post-mortem studies of fragile X brains have revealed an aberrant spine morphology, characterized by longer, thinner and denser dendrites [29,30]. Synaptic plasticity is impaired in absence of FMRP and seems to be related to cortex hyperexcitability observed in FX patients and several animal models [31–33]. This hyperexcitability seems to be the result of an enhanced long-term depression (LTD) and reduced long-term potentiation (LTP); the contribution of both mechanisms being different according to brain regions [34]. In some patients, the absence of FMRP may

also affect other classic neurochemicals pathways, including the dopaminergic, cholinergic and serotonergic-mediated pathways [35,36].

The preclinical research conducted over the past 20 years has mainly focused on the excitatory glutamate transmission mediated by group 1 (Gp1) metabotropic glutamate receptor (mGluR 1 and mGluR5) and the γ -aminobutyric acid (GABA) related inhibition to link the lack of FMRP to the mechanisms underlying FXS physiopathology (Figure 3). The hypothesis based upon those observations are centered around FMRP's ability to regulate the translation of its targeted mRNA. In this conjecture, FMRP would act as a chief regulator of Gp1 mGluR signaling by mediating the translation of proteins involved in its signal transduction and by functionally opposing the pro-translational effects of Gp1 mGluR stimulation [24]. The upregulation of Gp1 mGluR signalling results in aberrant LTD and LTP found in the *Fmr1* KO mouse hippocampus [37,38]. Deficits in GABAergic inhibition are also found in the mouse model of FXS. Indeed, FMRP has been shown to regulate the translation of some GABA receptors subunits and many enzymes involved in GABA metabolism and transport [39–42]. Taken together, those alterations are tough to create an imbalance between excitatory and inhibitory neurotransmission and are believed to be the origin of the cognitive and behavioral phenotypes found in FXS.

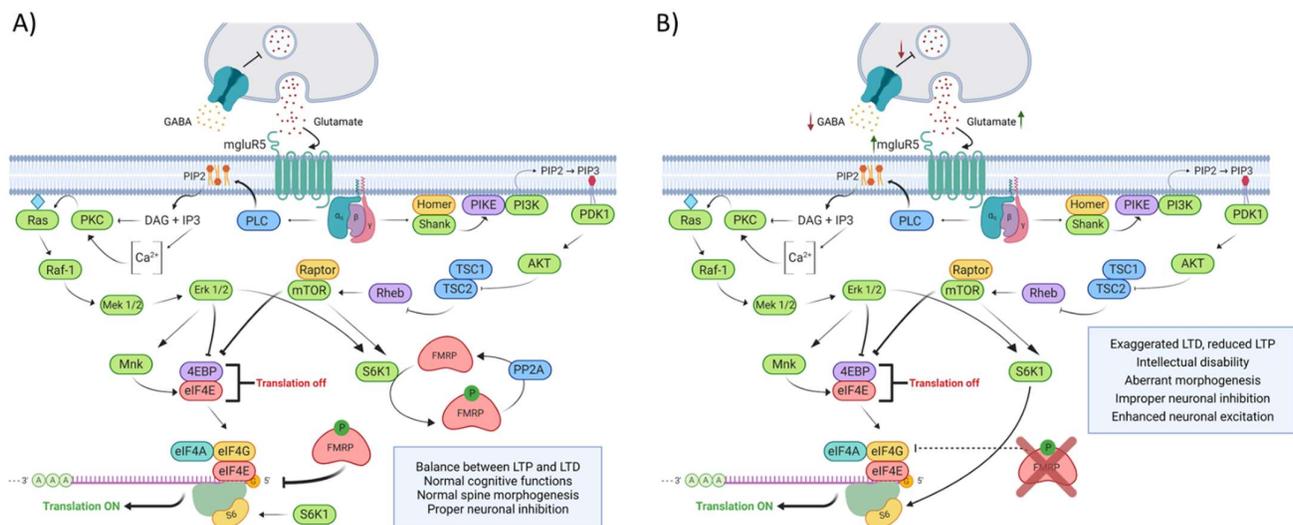


Figure 3. Schematization of the conceptual model of FXS physiopathology, resulting from an imbalance between excitatory (glutamate) and inhibitory (GABA) neurotransmission. **A)** Activation of GABA receptors at the presynaptic neurons inhibits the release of glutamate into the synaptic cleft. Glutamate stimulation of mGluR5 at the postsynaptic neuron lead to the activation of many downstream effectors, mainly the Ras/Raf/Mek/ERK and PI3K/AKT/mTOR signaling cascades. These pathways lead, among other things, to the translation of proteins which promotes LTD and impairs LTP. **B)** GABA output is decreased in the absence of FMRP, which lead to an improper neuronal inhibition. The resulting enhanced glutamate release promotes the activation of mGluR5, which ultimately lead to the an exaggerated LTD and a reduced LTP.

This model of FXS physiopathology has been corroborated over the years by a plethora of animal model-driven studies. Indeed, restoration of GABA and glutamate neurotransmission in *Fmr1* KO mice have repeatedly been associated with the rescue of FX biochemical and behavioral phenotypes [43–50]. Unfortunately, the hype carried out by those preclinical investigations did not translate into human, as clinical trials aimed to inhibit mGluR5 or to enhance the GABAergic system did not completely fulfill the expected outcomes [51–59]. Lessons can be learned from this inability to translate the success generated in the mouse model to the fragile X patient. Firstly, a better understanding of human physiopathology is needed. Given the accumulation of evidence regarding the limitation of the KO mouse to fully replicate human phenotype, studies aiming to fulfill

this task must focus on human subjects and models [60,61]. Secondly, one should "think outside of the ribosome" when searching for new mechanisms underlying FXS pathophysiology. The bulk of the research addressing this topic has solely considered FMRP as a translational regulator. The assumption that FXS is merely a disease of erroneous translation compose an oversimplification of the broad diversity of biochemicals and cellular alterations composing the fragile X phenotype. Finally, one should always keep in mind the large phenotypic heterogeneity found across the FX population when undergoing human-based researches

This work proposes an overview of some of the numerous molecular mechanisms in which FMRP is involved and the multidimensional nature of the biochemicals defects found in fragile X (Figure 4). This will be accomplished by reviewing some of the different alteration observable in FXS in an "Omic-dependant" manner. Furthermore, this review will focus, has much as possible, on researches conducted in human subjects or models.

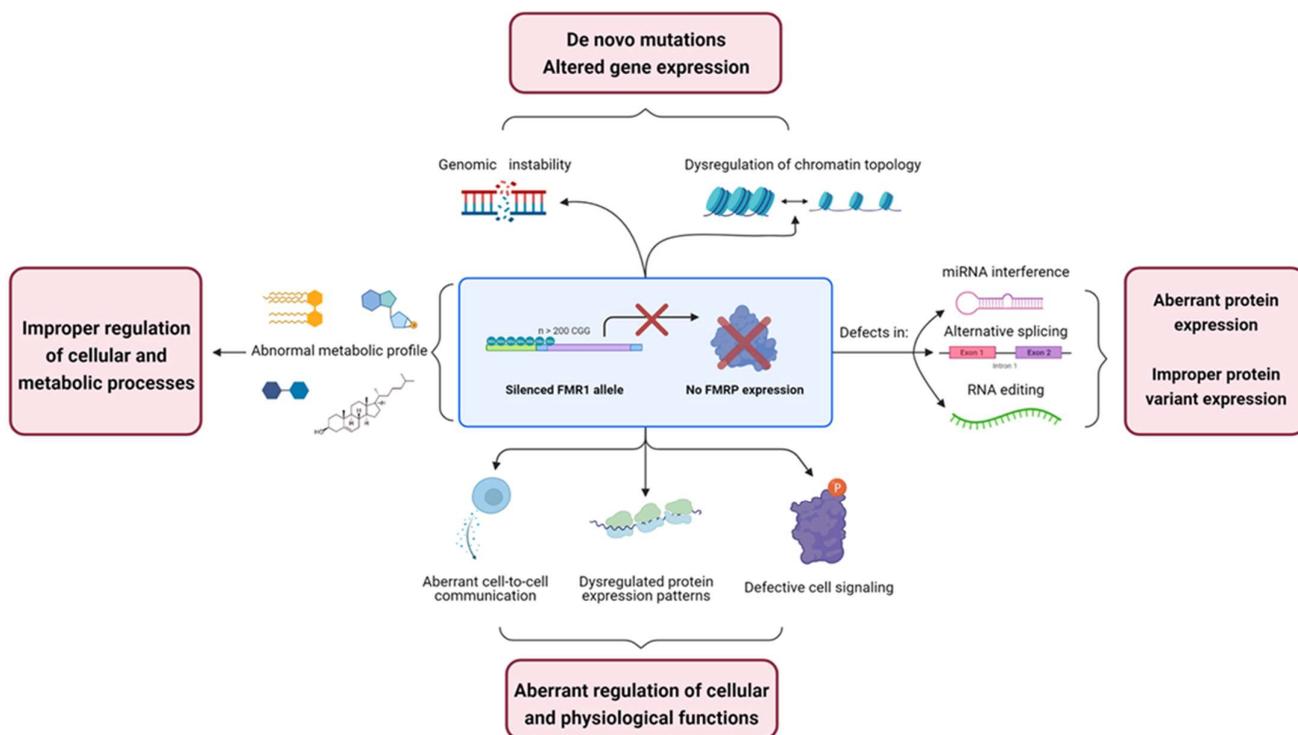


Figure 4. Summary of some of the various biochemicals alterations composing the fragile X phenotype on each "Omic" category.

2. Genomic Alterations in Fragile X Syndrome

Even though the vast majority of FMRP is cytoplasmic, a certain proportion is located in the nucleus. Some isoforms in which the nuclear export signal is spliced out are, indeed, found predominantly in this cellular compartment [62–64]. Those observations initially raised the hypothesis that FMRP may have actual nuclear function.

2.1. FMRP Is Involved in Genomic Stability Maintenance

One of the first reports of FMRP genomic functions came from the study of Alpatov et al., in which they brought strong evidence of FMRP implication in DNA damage response (DDR)[65]. They discovered that FMRP, through its Agenet domains, binds the chromatin via the methylated tails of histones H3. Mouse embryonic fibroblasts (MEF) treated with replication stress agent showed a FMRP-dependant induction of histone 2A variant H2A.X phosphorylation (γ H2A.X), a core component of the DNA repair complex[66]. They also showed that under these conditions, more FMRP is located in the nucleus and

that some of it colocalize with γ H2A.X. FMRP transitory expression can rescue γ H2A.X induction in HeLa and MEF cells, but mutations impairing Agenet domain binding to the chromatin proof to be less efficient in the matter. The precise role of FMRP in DDR remains, nonetheless, to be fully elucidated.

Evidences of the drosophila homolog of FMRP (dFMRP) involvement in the Piwi-interacting RNA (piRNA) pathway have also been reported [67,68]. The piRNA pathway is involved in the transcriptional silencing and enzymatic degradation of transposable elements, which have the ability to replicate and insert into new loci [69]. The piRNA mediated inhibition is crucial for the maintenance of the genome integrity, as transposition events can lead to DNA breaks and improper recombination events.

Taken together, those studies point toward a role for FMRP in the maintenance of genomic stability, which further suggests that fragile X patients may be subject to an abnormal increase of mutations caused by an accumulation of DNA damages and improper transposition events. This phenomenon can have deleterious effect on long-lasting cells, such as neurons, and potentially have repercussion on the wider nervous circuit in which affected neurons are connected.

2.2 Alteration of Chromatin Topology in FXS

Regulation of the genome three-dimensional structure, also referred as chromatin topology, is crucial for the spatiotemporal control of gene expression. This tightly regulated mechanism is mediated, in part, by post-translational modifications (PTM) of histones. These modifications dictate histone's interactions with other proteins and modulate the reorganization of chromatin topology, two mechanisms which in turn, can bilaterally modulate transcription. Indeed, some histone PTM exacerbate a repressive control over gene expression, while others promote it [70].

Many chromatin modifier enzymes are encoded by FMRP mRNA targets [71]. Reports of widespread dysregulation of histones PTM have been described in the mouse model of FXS, thus supporting this observation. Indeed, Korb and al have monitored an increase of several histone modifications associated with an active chromatin topology in cortex neurons of Fmr1 KO mice. Ensuing RNA-seq analysis consequently revealed more upregulated genes among all dysregulated transcripts. Furthermore, they showed that pharmacological inhibition of the transcription factor Brd4 (a protein that binds to acetylated histones and promotes transcription [72]) normalize spine morphology, aberrant behaviour and partially rescue gene expression in the same model [73]. A more recent report has described an overall increase in H3 lysine 36 trimethylation in the hippocampus of the Fmr1 KO mouse [74], a PTM also associated with active gene expression.⁷⁰

Those studies highlighted the fact that the loss of FMRP promotes alteration in chromatin topology which can be further materialized into a dysregulation of gene expression. Mutations in chromatin remodeling genes have also been repeatedly associated with neurodevelopmental disorder such as autism and ID [76,77]. Taken as a whole, those observations suggest that the alteration in histone PTM observed in the FMR1 KO mouse may promote aberrant gene expression, which in turn, can promote key fragile X phenotypes.

3. Transcriptomic Alterations in Fragile X Syndrome

By its capacity to bind both RNA and protein, FMRP act as an adapter to facilitate the interaction between those two classes of biomolecules. This pivotal function allows FMRP to mediate various RNA processing mechanisms other than its traditional ability to regulate translation by ribosome stalling or eIF4E sequestration [20].

3.1. FMRP Mediates Micro RNA-Related Interference

Micro RNAs (miRNA) are 19-24 nucleotides long non-coding RNAs involves in post-transcriptional regulation of approximately 30% of human genes[78]. Upon transcription, newly synthesis primary miRNAs are processed into a partially unpaired stem-loop

precursor (pre-miRNA) by the nuclear ribonuclease Drosha. Pre-miRNAs are then exported through exportin 5 to the cytoplasm, where they are further processed by the ribonuclease Dicer into double-stranded RNA. The functional strand of this RNA duplex makes up the mature miRNA and is loaded into the RISC complex (RNA induced silencing complex) through its association with members of the Argonaute (AGO) protein family. From there, miRNA-containing RISC complex binds to complementary mRNA and either promotes its degradation or inhibits its translation [79,80].

FMRP is a well established effector of the miRNA pathway. Indeed, FMRP is found in RISC and directly interact with different components of those ribonucleoprotein (RNP) complexes, including AGO1, AGO2 and Dicer and binds to both mature and pre-miRNAs [81–85]. FMRP binding to miRNAs is mediated by its KH domains and is believed to favour miRNA annealing to their complementary mRNA [83]. As such, *in vitro* experiments have shown that the presence of FMRP in RISC complex can both promote and inhibit miRNAs-related interference. The function of FMRP in that miRNA-containing RNP complex is modulated by many factors, including the affinity of FMRP to the targeted mRNA and protein-protein interactions [81,83,86].

First evidence of the involvement of the miRNA pathway into fragile X physiopathology was brought by Jin and al. In this study, they showed that a downregulation of AGO1 alters FMRP-related translational control and promotes certain FXS phenotypes [82]. Moreover, this work establishes FXS as the first neurologic disease associated with alteration in the miRNA pathway. Since then, an accumulation of experimental evidence has linked such deficit to the absence of FMRP and, consequently, to mechanisms underlying fragile X physiopathology. For example, the miR-125b mediated translational repression of NR2A (a sub-unit of the glutamate receptor NMDA) is FMRP-dependent and regulate spines morphogenesis [87]. Defects in axon guidance can also be linked to aberrant miRNA regulation in FXS. Indeed, Halevy and al. have used induced pluripotent stem cells derived from fragile X subjects' fibroblasts to associate a downregulation of hsa-miR-382 to this neuronal alteration [88]. FMRP was also shown to modulate the miR-125a mediated interference of PSD-95, a protein involved in post-synaptic AMPA receptors endocytosis and consequently, in synaptic plasticity regulation [89,90]. PSD-95 and miR-125a are respectively found up and downregulated in synaptoneurosomes of *Fmr1* KO mice. Even more interestingly, a recent miRNA profiling performed in two independent cohorts have identified an increase of miR-125a in urine of fragile X boys [91].

The miRNA pathway represents a third mechanism, with ribosome stalling and eIF4E sequestration, from which FMRP can exert its regulatory effect on translation [20]. Correction of an improper micro-RNA interference therefore represents a potent therapeutic approach to rectify FXS biochemical alterations [79,92]. Furthermore, as shown by Putkonen and al. [81], circulatory miRNA may also constitute a great source of peripheral biomarkers that can be used as quantitative and objective tools by clinicians [93].

3.2. *Alternative Splicing and FMRP*

The first insight of FMRP involvement in alternative splicing came from the study of Didiot and al. in which they showed that FMRP affects alternative splicing of its own mRNA. Indeed, they showed that FMRP binds its own mRNA through 2 G-quartets located closely to a known alternative spliced site in exon 15. Consequently, they observed that both overexpression and silencing of FMRP leads to aberrant FMR1 splicing pattern [94]. Further evidence of FMRP implication into a more widespread regulation of alternative splicing comes from studies conducted in *Drosophila*. Indeed, the knock-down of dFMRP leads to the deregulation of more than 100 splicing events [95]. Moreover, only a handful of dFMRP mRNA targets have seen their splicing pattern influence by this genetic manipulation [96]. Those observations were recently transposed into the mouse model of fragile X. Indeed, Shah and al. reported a reduction of approximately 30% of exon skipping event in *Fmr1* KO mice hippocampus. Furthermore, many of the genes found with defective

splicing are linked to neuronal transmission and autism[74]. FMRP is also known to interact with various proteins involved in pre-mRNA alternative splicing, one of which being the splicing factor RBM14[97,98]. Together, FMRP and RBM14 modulate the splicing of Tau and Protrudin, two genes involved in neuronal cells differentiation and dendrites growth. The improper splicing pattern of those two genes observed in the hippocampus of Fmr1 KO mice therefore suggests that FMRP involvement in the regulation of pre-mRNA splicing can contribute to FXS physiopathology [98].

Alternative splicing is one of the abounding mechanisms engaged in the temporal and cell-specific regulation of gene expression and in the promotion of the proteome functional diversity. Maintenance of its homeostasis is thus mandatory to ensure appropriate cell function, especially in neural tissue, where it participates in virtually all facets of neurons activity [99]. Despite the accumulating number of evidences regarding FMRP role toward alternative splicing in animal models, the clear functions of FMRP in this molecular mechanism remain to be fully elucidated. More importantly, no study has yet corroborated those observations in specimens derived from fragile X patients.

3.3. FMRP Absence May Lead to Defect in RNA Editing

The ADAR (adenosine deaminase acting on RNA) family of enzymes catalyzes the post-transcriptional modification of adenosine to inosine in double-strand pre-mRNA. Inosine is interpreted as guanosine by the translational and splicing machinery. As such, A to I edition in mRNA may lead to variable amino acids incorporation into corresponding proteins and contribute to the regulation of its alternative splicing. In other words, RNA editing represents another mechanism by which different protein variants can emanate from one genomic entity. Furthermore, proper ADAR editing is believed to be crucial for normal neuronal functions, as many mRNA edited by those enzymes encoded ion channels, neurotransmitter receptors and protein involved in synaptic transmission [100].

The initial observation of FMRP presence into ADAR-containing RNP complexes raised the hypothesis that FMRP may act as a RNA editing mediator [101]. Co-immunoprecipitation experiments report a direct interaction between FMRP and ADAR2, a partnership that was shown to inhibit ADAR2 RNA editing activity [102,103]. A consequent FMRP-dependent dysregulation of RNA editing was further observed in various models of FXS. Indeed, both FMRP silencing and mutations impairing KH domains RNA binding have proved to increase ADAR activity and to engender erroneous editing for some of the mRNA studied [101–103]. Those transcripts, which include the calcium channel Cav1.3 and glutamate receptor subunits GluK2, GluA2 and GluA4, are involved in synaptic modulation, thereby suggesting that RNA editing impairments could have a significant contribution to FXS physiopathology [102,103]. However, those studies had only considered the editing pattern of a preselected pool of mRNAs. A transcriptome wide characterization of RNA editing defects in fragile X could therefore bring a clearer portrait of FMRP involvement in RNA editing and the contribution of this mechanism to FXS phenotype.

3. Proteomics Alterations in Fragile X Syndrome

Expression patterns of numerous proteins are known to be deregulated in FXS. Those proteins exhibit a wide array of biological and cellular functions, ranging from receptors, enzymes, translation factors and more. The expected pathophysiological consequences of FMRP's absence should therefore be as various as the nature of the proteins found improperly expressed in FXS.

3.1. Rate of protein synthesis alteration: a still misunderstood hallmark of FXS

Alteration in the rate of protein synthesis remains a focal point of fragile X physiopathology since the assent of the general agreement regarding FMRP as a translational regulator. The first reported evidence of FMRP's ability to regulate translation came from studies conducted in rabbit reticulocyte lysate, in which it was shown to inhibit the

general translational rate in a dose-dependent manner[104,105]. Since then, a plethora of studies have utilized a variety of FX animal models, in combination with a profusion of experimental workflow, to firmly establish the relation between the absence of FMRP and an increase in rate of protein synthesis[106–108].

Most of the work addressing this issue has been performed in order to conceptualize the mGLuR theory, and consequently, in hippocampal slices prepared from Fmr1 KO mice. This experimental approach, which allows the combination of electrophysiological and metabolic measurements, had greatly contributed to the advancement of knowledge regarding synaptic and biochemical defects underlying the absence of FMRP and supports the development of various preclinical studies[43,109]. In this sense, rescue of the fragile X phenotype in Fmr1 KO mice following targeted pharmacological treatments has repeatedly been associated with a normalization of the aberrant protein synthesis[44,45,47,110,111]. Such reports strengthen the role of FMRP in the maintenance of translational homeostasis to ensure normal neurologic and behavioural phenotypes and further establish the rate of protein synthesis alteration as one of the most potent monitoring biomarkers for FXS.

Despite being one of the most prominent and studied alterations in animal models, only a limited number of reports have studied protein synthesis defects in human, mainly due to limitations regarding sample nature and accessibility. As such, two studies have used fibroblasts obtained from a skin biopsy of FX patients to monitor an increase in translational rate in fragile X derived cells [112,113]. Another substantial contribution concerning this issue came from two studies of the Beebe Smith group, in which positron emission tomography was used to measure a decrease in the *in vivo* integration of [¹¹C] leucine into nascent brain proteins of 15 FX patients under propofol sedation and a similar, but not significant, trend of perturbation in 9 FX patients under dexmedetomidine anaesthesia[114,115]. Finally, we recently reported that the rate of protein synthesis is also decreased in freshly extracted peripheral blood mononuclear cells (PBMCs) of FX patients (Dionne et al., 2021, under revision). These studies indubitably shown that the perturbation in translational homeostasis found in animal models is replicated in fragile X patients. Furthermore, all those studies made the common observation that some FX patients displayed a rate of protein synthesis within control range, thereby suggesting that any therapeutic intervention aiming to restore translational homeostasis may not be beneficial for all of them. The tissue-specific trend of perturbation and variability observed into the FX population seemingly demonstrates that the protein synthesis defects inherent to the absence of FMRP is more nuanced and complex in human than in mice. Indeed, protein synthesis in KO mice was constantly shown to be solely upregulated in all tissue or cell types tested so far [113,116,117].

These observations clearly demonstrated that defects regarding protein synthesis are still misunderstood in humans, and that several questions remain to be addressed to rectify the situation. The first one would be to establish if the normalization of the aberrant protein synthesis rate can be achieved by a targeted pharmacological treatment, and further confirm the usefulness of the rate of protein synthesis measurement as a monitoring biomarker for FXS. The development of such an objective and quantitative tool would represent a major step forward for any future FX clinical trials [118]. Another substantial advancement would be to identify the proteins that are dysregulated during the timeframe that protein synthesis rate measurement is conducted. The same experimental approach could be used to study how FX cells respond to different stimuli. Such experiments could provide valuable information on molecular mechanism underlying fragile X physiopathology and yield a relevant source of peripheral biomarkers [116].

3.2. Cell Signalling Defects in FXS

Cell signalling is a complex communication network that governs cell fundamental mechanisms and coordinate their activity. Through this process, extracellular signals

activate specific cell surface receptors before being transduced by signalling cascades into a wide variety of intracellular responses. The Ras/Raf/MEK/ERK and the PI3K/AKT/mTOR pathways are two major actors of those cell signalling transduction mechanisms. Indeed, those ubiquitous pathways are pivotal for a broad range of cellular (cell proliferation and differentiation, transcription and translation regulation) and physiological (metabolism, synaptic plasticity, development) processes [119,120].

FMRP, through its ability to regulate the translation of its targeted mRNA, acts as a chief mediator of the two aforementioned pathways. Indeed, analyses conducted in different mouse brain regions and HEK293 cells have shown that FMRP binds the mRNA of various effector and regulator of those signalling cascades [25,71,121]. Consequently, a basal hyperphosphorylation of ERK and AKT (reflecting the hyperactivation of their respective pathways) as well as a delayed and weaker ERK phosphorylation following Gp1 mGLUR stimulation are observable in the hippocampus and cortex of Fmr1 KO mice [122–125]. Those alterations can be directly linked to exaggerated mGLUR5 signalling and contribute to the aberrant synaptic phenotype and dysregulated protein synthesis rate observed in this model. As such, the normalization of ERK and AKT phosphorylation status in KO mice have frequently been associated with the correction of the improper translational rate, LTD and behavioural phenotypes; thereby establishing measurements of ERK and AKT activation status as one of the most potent biomarkers of therapeutic efficiency for FXS [44,45,109,110,126].

The hyperactivation of the Ras/Raf/MEK/ERK and PI3K/AKT/mTOR pathways found in the brain of KO mice is also observable in a variety of human samples. Indeed, an increase in the phosphorylation of MEK, ERK, AKT and mTOR have been described in post-mortem brains of FX patients [127,128]. These molecular alterations are also replicated in more accessible and less invasive tissues, such as fibroblasts, PBMCs and blood platelets [112,127,129]. Most notably, our research group showed that the phosphorylation of ERK and AKT measured in blood platelets is correlated with the IQ of FX patients. Furthermore, a 3 months lovastatin treatment corrected the exaggerated ERK overactivation in platelets and have been associated with a clinical improvement [129,130]. However, no study has yet shown that a reduction of the AKT pathway hyperactivation is achievable following a targeted treatment in human trials.

Taken together, these observations clearly illustrate the relevance of using cell signalling alterations in FXS as biomarkers of therapeutic efficiency in human trials. In addition, it could also be interesting to study whether the signalling defects observed outside of neurons can contribute to the physiopathology of FXS. Indeed, most studies have solely focused on neurons-related defects. Given the multifunctional nature of these signalling pathways and the fact that their hyperactivation is replicated in peripheral cells, it is very likely that their deregulation in non-neuronal tissues could contribute, to some extent, to a number of extra-neuronal manifestations or symptoms.

3.3. *The Matrix metalloproteinase-9 Involvement in FXS Physiopathology*

The matrix metalloproteinase-9 (MMP9) is a zinc-dependent endopeptidase which targets many components of the extracellular matrix (ECM). In neuronal tissue, MMP9 enzymatic activity towards the perineuronal net (a network of loosely organized ECM components that supports several synaptic processes [131,132]) promotes dendritic spines elongation and regulate synaptic plasticity [133–136].

Colocalization studies conducted in mouse neurons has shown that MMP9 mRNA is bound and subjected to FMRP translational control [137]. Consequently, an increase in MMP9 expression has been reported in the frontal cortex and hippocampus of FX patients and Fmr1 KO mice [137]. Furthermore, genetic deletion of MMP9 in Fmr1 KO mice rescue several FX phenotypes, including the aberrant spine morphogenesis and synaptic plasticity [138]. Those observations prompt the uses of minocycline, an MMP9 inhibitor, as a targeted treatment for FXS [139].

Minocycline treatments were shown to successfully reduce MMP9 activity, reverse the improper spine morphogenesis and improve anxiolytic behaviour and locomotor activity in Fmr1 KO mice [140,141]. During clinical trials, minocycline mildly improves FX patient's behaviour, with a more pronounced effect on irritability and anxiety [142,143]. Dziembowska and al., further reported that the increase MMP9 plasmatic activity is reduced in some FX patients following a 3 months minocycline treatment [144]. Even though they did not observe any correlation between MMP9 inhibition and global clinical improvement, their results strongly suggest that the plasmatic measurement of MMP9 can be used as a monitoring biomarker for FXS. A thorough validation within a larger FX cohort is, however, mandatory to confirm that statement.

Those reports clearly demonstrated that MMP9 is a key contributor to FXS pathophysiology. However, the precise mechanism by which MMP9 activity contributes to fragile X phenotype remains to be fully elucidated. Despite this, measurement of MMP9 proteolytic activity remains one of the promising biomarkers of FXS, as shown by the fact that the MMP9 dysregulation monitored in KO mice and post-mortem patient brains is replicated in FX blood.

3.4. Aberrant cytokines profile: A sign of immune dysfunctions in FXS?

Cytokines are crucial mediators of cell signalling events occurring within the immune system and are secreted in response to various stimuli. A wide variety of extra-immune cells, including neurons and microglia, express cytokines and their respective receptors. Cytokines are consequently involved in the regulation of several neuronal functions, in both developing and mature brain, as well as in the nervous system response to infections and injuries. Such processes are important for normal brain operation, since they can modulate cognition and emotional processing [145].

Very little is known about a possible immune dysfunction in FXS since only a handful of exploratory studies have been reported. As such, an *in situ* comparative transcriptomic analysis has shown that dysregulated transcript from FMR1 KO mouse embryos hippocampus and cortex primarily harbour an immunological signature [146]. A further study observed a decreased IL-6 and TNF- α mRNA expression in KO mouse adult hippocampus [147]. Moreover, FX PBMCs shown an increase in the production of pro-inflammatory cytokines IL-6 and IL-12p40 after being conjointly stimulated with lipopolysaccharides (LPS) and DHPG, a Gp1 mGluR agonist. Interestingly, FX leucocytes did not display any alteration in cytokine production when solely stimulated with LPS [148]. However, two studies have shown that circulating levels of several pro-inflammatory cytokines are actually decreased in FX serum and plasma [149,150]. Despite being somewhat contradictory, those reports strongly suggest the existence of immune dysfunctions in FXS. This statement is supported by a recent systemic analysis of the medical diagnosis conduct over 5736 FX patients. Indeed, Yu et al. found a higher prevalence of various infectious diseases and an underrepresentation of autoimmune disorders in the FXS population [151].

An alteration in cytokines secretion or response could contribute to several FX phenotypes, as it might increase the patient's sensitivity to neurological damage induced by environmental, pathological or xenobiotic exposure. However, more detailed studies are needed to better characterize the potential immune dysfunctions found in FX and the mechanism by which the absence of FMRP leads to such impairments.

4. Metabolomic Alterations in Fragile X Syndrome

Many studies have associated an aberrant metabolic profile with FMRP's loss of expression. Indeed, metabolomic screenings conducted in Fmr1 KO mice and FX patients have shown that glucose, lipid, and several neurotransmitter metabolisms are deregulated in FXS [152,153]. The fact that FMRP binds to the mRNA of many key regulators of the metabolism can lay a rationale to those observations [71,152]. But above all, those

results show that the extra-neuronal phenotype caused by FMRP's absence must not be underestimated, and therefore, deserves to be better characterized.

4.1. *The Hypocholesterolemic Phenotype of FXS*

Cholesterol is a ubiquitous lipid and an essential component of eukaryotic membranes. It is known to play an important role in the maintenance of plasmatic membranes structural integrity and in the regulation of its functions [154]. Approximately 25% of all cholesterol within human body is found in the brain. Indeed, a large portion of this cholesterol is located in the myelin sheaths of axons and in dendritic spines membranes [155]. Maintenance of cholesterol homeostasis is therefore crucial for proper neurodevelopment and cognitive functions [156].

A prime example of a neurodevelopmental disease caused by an improper cholesterol metabolism is the Smith-Lemli-Opitz syndrome (SLOS), which exhibit mild to moderate ID and ASD has one of its most prevalent comorbidities [157,158]. SLOS is a recessive condition caused by mutations in the gene encoding the 7-dehydrocholesterol reductase, which catalyze the last step of cholesterol biosynthesis. As such, SLOS patients typically present low levels of circulating cholesterol [158]. A higher prevalence of hypocholesterolemia was also found in an etiologically diverse autistic population [159–161]. Moreover, very low levels of circulating cholesterol (below the 10th centiles) were associated with higher risk of ID, anxiety and depression in men with ASD [161].

These observations promoted the investigation of peripheric levels of cholesterol in FXS. Indeed, two independent reports have observed lower plasmatic levels of LDL (light density lipoprotein), HDL (high-density lipoprotein) and total cholesterol in FX men [162,163]. Several parameters of those cholesterol profiles were also shown to be correlated with the severity of the behavioral impairments found in these patients. Interestingly, no associations were observed between plasmatic level of the proprotein convertase subtilisin/kexin type 9 (PCSK9) and total/LDL cholesterol in FX patients, despite the existence of a clear correlation in CTL individuals [163]. PCSK9 is secreted from hepatocytes and binds to LDL receptors, which promotes their degradation and favorite higher plasmatic concentration of cholesterol. Moreover, a recent report showed that the phosphorylation level of PCSK9, a PTM that enhances its binding affinity for the LDL receptor, is decreased in samples from FX patients [164]. These observations, which suggest a dysregulation of PCSK9 activity in FXS, therefore provides the first clues regarding the molecular mechanism responsible for the hypocholesterolemia found in this population.

Since hypocholesterolemia is a common to FXS and ASD, it would be interesting to investigate if the same biochemical mechanisms underlying this phenotype are shared between both conditions. Such research could provide a better understanding of both diseases physiopathology and potentially pave the way for therapeutic approach that could be beneficial for individuals with FXS and ASD. Furthermore, it will also be interesting to validate if low cholesterol level could be used as prognosis marker of ASD and other comorbidities in FX patients.

4.2. *The Cyclic AMP Metabolism Is Defective in Fragile X*

Cyclic AMP (cAMP) is a second messenger involved in the transduction of a plethora of signalling pathways. The intracellular levels of cAMP are regulated by two classes of enzymes: adenylate cyclase (AC) and phosphodiesterase (PDE). Adenylate cyclase is responsible for cAMP synthesis. The majority (9 out of 10) of AC isoforms are membrane-bound enzymes that are regulated by various G protein-coupled receptors (GPCR)[165]. On the other hand, PDE catalyze cyclic nucleotides (cAMP and cGMP) degradation. Some PDE subfamilies are cAMP (PDE4,7 and 8) or cGMP (PDE5,6 and 9) specific, while the other (PDE1,2,3,10 and 11) possesses specificity for both cyclic nucleotides[166]. Intracellular levels of cAMP promote the activity of various kinase such as PKA and ERK[167], and regulate gene expression through the activation of the transcription factor CREB[165].

As such, cAMP is involved in several cognitive and neuronal processes, including stress, anxiety, memory, synaptic plasticity, synaptic transmission and spines morphogenesis [168–170]

The pioneer work of Berry-Kravis and al. in the 90s provided the first evidence of a defective cyclic AMP metabolism in FXS. Indeed, they reported that blood platelets and lymphoblastoid cell lines from FX patients produced lower levels of cAMP compared to controls and other matched individuals with ID and ASD diagnostics [171–173]. Furthermore, they reported that FMRP overexpression in mouse neurons enhance cAMP production, thereby highlighting the contribution of FMRP in cAMP pathway [174]. These observations were further replicated in animal models of FXS, more precisely the *Fmr1* KO mouse and the *dFMR1* null *Drosophila* [175].

These studies paved the way for the elaboration of the "cAMP theory of fragile X", which lays its foundation in the aberrant expression pattern of proteins involved in the cAMP cascade. Indeed, mRNAs of several AC isoforms and members of PDE subfamilies are targeted by FMRP [71,121]. This theory further states that the downregulation of cAMP signalling, both downstream and independently of Gp1 mGluR activation, contributes to FXS physiopathology [176]. This theory is corroborated by several studies conducted in KO mice, in which the reversal of several FX phenotypes was associated with an upregulation of the cAMP pathway. Indeed, pharmacologic treatments that either stimulate adenylate cyclase activity, both directly or through the activation of AC-coupled GPCR or inhibits specific PDE have proof to be efficient therapeutic approach [177–180].

The accumulation of evidence regarding the defects in the cyclic AMP signaling in FXS and their contribution to the disease physiopathology are undeniable. As such, many effectors of this pathway represent potent therapeutic targets. Furthermore, as shown by the work of Berry-Kravis and al., quantification of cAMP production in peripheral blood cells could serve as objective biomarkers to assay treatment efficacy.

4.3. The Amyloid- β Precursor Protein and Its Secreted Metabolites

The Amyloid precursor protein (APP) is a dendritic transmembrane protein which is involved in several neuronal functions, including axonogenesis, neurite outgrowth and neuronal adhesion[181]. APP can be proteolytically processed by two distinct pathways. When undergoing the amyloidogenic pathway, APP is sequentially cleaved by the β -secretase BACE1 and the γ -secretase complex to produce the neurotoxic secreted β -APP (sAPP β) and the amyloid- β (A β) peptides, which either takes the form of a 40 (A β 40) or 42 (A β 42) amino acids long chains. A β peptides are the major component of the amyloid plaques found in the brain of patients with Alzheimer's disease and are therefore associated with neurodegeneration. Alternatively, APP can be processed by α -secretases to generate the secreted α -APP (sAPP α), which similarly to APP, bears a wide array of neurotrophic functions [182].

FMRP was shown to bind to APP mRNA and inhibits its translation through interactions with the miRNA machinery [183]. As such, levels of APP, sAPP α and A β peptides were shown to be increased in brains of *Fmr1* KO mice and linked to several key alterations composing the FX phenotype [184,185]. In concordance with those observations, APP haploinsufficiency was associated with the rescue of repetitive behaviour, anxiety, mGluR-LTD and spine morphology in *Fmr1* KO mice [186]. Those alterations regarding APP expression and catabolism are also replicated in humans. Indeed, levels of APP, sAPP α and A β peptides were shown to be upregulated in post-mortem brains samples and various peripheral cells from FX patients [184,186]. Plasmatic levels of both forms of secreted APP and A β peptides were also shown to be increased in children with FXS, while only A β 42 seems to be decreased in adult patients. Interestingly, circulating levels of secreted APP and A β peptides were also shown to be deregulated in children with ASD. Indeed, samples collected from those patients showed an increased in sAPP α and a decreased in sAPP β and both form of A β peptides [187–190]. Moreover, levels of sAPP α and

total sAPP were shown to be reduced following a treatment with acamprosate in children with ASD, both with and without FXS [191].

The dysfunctions regarding APP expression and processing monitored in FXS suggest that those defects could contribute significantly to the mechanisms underlying the disease physiopathology. Furthermore, peripheral levels of APP and its secreted metabolites represent promising biomarkers for the monitoring of treatment efficacy and for the prediction of ASD comorbidities in FX patients.

5. Conclusion

FMRP is a multifunctional protein that plays a role in several biochemical processes. The phenotypical consequences of its absence are diverse, and many are still misunderstood. Future researches should focus on the elucidation of those interrogations. Such discovery would promote a better comprehension of the overall mechanism underlying FXS physiopathology and pave the way for new therapeutic approaches and the establishment of objective biomarkers to improve clinical management.

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