## MINI REVIEW
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# The quest for fragile X biomarkers

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## Abstract

**Background:** Fragile X is the most common form of inherited intellectual disability and the leading known genetic cause of autism. There is currently no cure or approved medication for fragile X although various drugs target specific disease symptoms and a large number of therapeutics are in various stages of clinical development. Multiple recent clinical trials have failed on their primary endpoints indicating that there is a compelling need for validated biomarkers and outcome measures in fragile X.

**Findings:** There are currently no validated blood-based biomarkers to assess disease severity or to monitor drug efficacy in fragile X syndrome. Herein, we review candidate blood protein biomarkers including extracellular-regulated kinase, phosphoinositide 3-kinase, matrix metalloproteinase 9, amyloid-beta and amyloid-beta protein precursor.

**Conclusions:** Bench-to-bedside plans for fragile X syndrome are severely limited by the lack of validated outcome measures. The reviewed candidate biomarkers are at early stages of validation and deserve further investigation.

**Keywords:** Fragile X syndrome; Biomarker; Extracellular-regulated kinase; Phosphoinositide 3-kinase; Matrix metalloproteinase 9; Amyloid beta protein precursor; Amyloid-beta

## Introduction

A biomarker is a measurable and quantifiable biological characteristic that can serve as an indicator of healthy or pathological processes. For example, HDL and LDL are biomarkers for cardiovascular health and autoantibodies are biomarkers for autoimmune disease. Biomarkers are extremely useful in evaluating the clinical benefit of pharmaceutical interventions. A good biomarker assay will be sensitive, specific, rapid, simple to perform, inexpensive and applicable to easily obtained sample material. There is an urgent need to develop such biomarkers for fragile X syndrome (FXS).

## Fragile X syndrome

FXS is the most common form of inherited mental retardation with a frequency of 1 in 2,500 births [1]. FXS results from a mutation in the fragile X mental retardation-1 (*FMR1*) gene, which was discovered by Drs. Ben Oostra, David Nelson and Stephen Warren in 1991. The *FMR1* gene codes for fragile X mental retardation protein (FMRP), an RNA binding protein that plays a critical role in dendrite development. Thus, the absence of FMRP in FXS has a profound effect on synaptic plasticity. The clinical symptoms of FXS include intellectual disability, attention deficit and hyperactivity, anxiety, autistic behaviors, sensory integration problems, speech delay, seizures, hyperextensible joints, hypotonia, postpubescent macroorchidism, flat feet and vertical maxillary excess with protruding ears [2]. There is currently no cure or approved medication for FXS although various drugs target specific disease symptoms and a large number of therapeutics are in various stages of clinical development.

## The compelling need for validated FXS biomarkers

Dr. Mark Bear and colleagues proposed the "mGluR Theory of FXS" in 2004 in which they provided a framework through which overactive signaling through group 1 metabotropic glutamate receptors (mGluRs) (mGluR$_1$ and mGluR$_5$) could contribute to the psychiatric and neurological symptoms of FXS [3]. Mechanistic research the past decade has validated the role of mGluR$_5$ in FXS as well as identified additional membrane receptors, signaling molecules and proteins involved in long-term depression (LTD) that are potential therapeutic targets and disease biomarkers. Compounds targeting these molecules have moved into clinical trials in individuals with

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FXS, which has provided valuable information regarding potential drug efficacy; however, bench-to-bedside plans are severely limited by the lack of validated outcome measures [4]. To facilitate the identification and development of outcome measures for FXS, the NIH organized a panel of experts who evaluated the potential utility of cognitive, behavioral and emotional measures; blood and tissue biomarkers; electrophysiological measures; eye tracking and pupillometry; and neuroimaging tests for FXS [4]. They concluded that there is currently no single or set of endpoints that can serve as an optimal biomarker for FXS clinical trials. The Aberrant Behavior Checklist (ABC) is commonly adopted as the primary outcome measure in FXS clinical trials [4,5]. A recent phase 2 trial conducted by Seaside Therapeutics, Inc. to evaluate the safety and tolerability of the GABA agonist STX209 (*R*-baclofen) in patients with FXS failed on the primary endpoint, which was the ABC-Irritability subscale, although benefit was observed in other measures mostly by posthoc analysis [6]. Novartis has also terminated development of their lead compound for FXS, the mGluR$_5$ inhibitor Mavoglurant (AFQ056), for not meeting the primary endpoint of improved abnormal behaviors compared to placebo. Thus, validated outcome measures and biomarkers could greatly accelerate drug development for FXS. Although recent publications indicate that auditory processing, expressive language sampling, eye tracking and pupillometry, eyeblink conditioning, the

6-factor structured ABC, markerless motion analysis, and the Pediatric Anxiety Rating Scale–Revised (PARS-R) may be viable outcome measures for FXS clinical trials, these tests require considerably more time and money to perform than a simple blood-based biomarker assay. Below, we discuss candidate blood-based biomarkers for FXS including extracellular-regulated kinase (ERK), phosphoinositide 3-kinase (PI3K), matrix metalloproteinase-9 (MMP-9), and amyloid-beta protein precursor (APP) and catabolites (Figure 1).

## Extracellular-regulated kinase

ERK is a component of the mitogen-activated protein kinase (MAPK) signal transduction pathway. ERK signaling can be activated by either protein tyrosine-linked receptors or by G protein-coupled receptors with signaling propagated through a series of phosphorylation reactions. Although there are contradictory results regarding basal phospho-ERK levels and mGluR-induced phosphorylation of ERK in FXS models, a specific inhibitor of the upstream mitogen-activated protein kinase kinases 1 and 2 (MEK1/2) eliminated audiogenic seizure activity in *Fmr1*$^{KO}$ mice [7]. Findings from the Greenough laboratory suggest that the early phase kinetics of ERK activation in lymphocytes is delayed in FXS subjects and could serve as a disease biomarker [8]. ERK activation rates normalize in response to lithium and riluzole treatment [9,10].
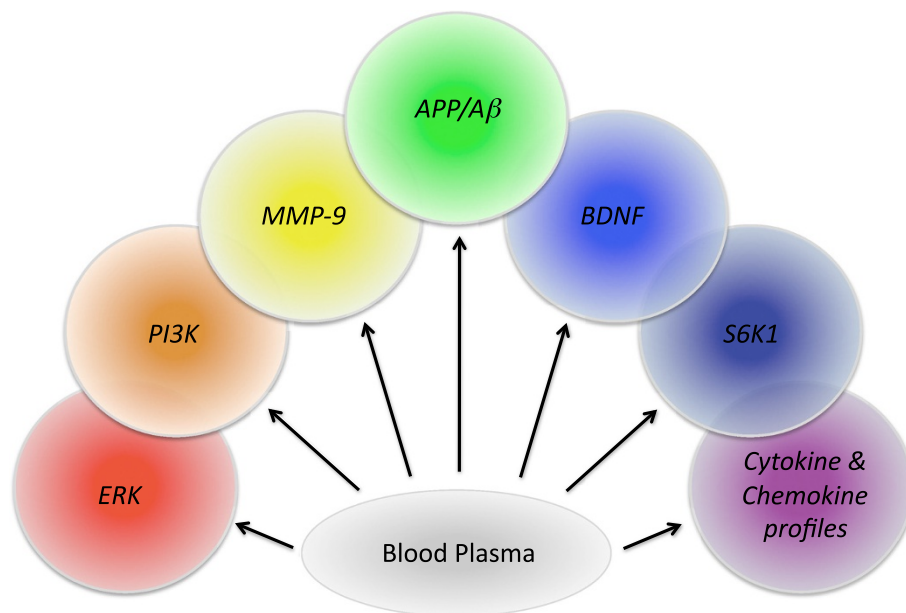


**Figure 1 Potential blood-based biomarkers for FXS.** Candidate blood-based biomarkers for FXS include: extracellular-regulated kinase (ERK), phosphoinositide 3-kinase (PI3K), matrix metalloproteinase-9 (MMP-9), amyloid-beta protein precursor (APP) and catabolites, brain-derived neurotrophic factor (BDNF), p70 ribosomal subunit 6 kinase 1 (S6K1), and cytokine and chemokine profiles.

## Phosphoinositide 3-kinase

PI3Ks are a family of enzymes involved in cell growth, proliferation, differentiation, motility, survival and intracellular trafficking. The catalytic p110β subunit of class I PI3Ks activates protein kinase B (PKB, aka Akt), which is part of the mammalian target of rapamycin (mTOR) signaling pathway. The Zukin laboratory has shown that p110β as well as mTOR phosphorylation and activity are elevated in juvenile $Fmr1^{KO}$ mice [11]. Gross and Bassell demonstrated that FMRP regulates the synthesis of p110β and that peripheral lymphocytes from FXS patients exhibit excessive PI3K activity as well as protein synthesis levels [12]. They utilized an ELISA-based colorimetric assay to detect PI3K activity and a fluorescent metabolic labeling assay to detect protein synthesis in FXS lymphocytes. Both methods could be adapted for clinical evaluation of PI3K activity and protein synthesis levels in FXS lymphocytes in response to drug treatment.

## Matrix metalloproteinase 9

MMPs are involved in the breakdown of the extracellular matrix during processes such as embryonic development, wound healing and learning and memory. MMP-9 is involved in activity-dependent reorganization of dendritic spine architecture. *MMP-9* mRNA is part of the FMRP complex, and translation of MMP-9 is increased at $Fmr1^{KO}$ synapses [13]. The Ethell laboratory found that the antibiotic minocycline decreases MMP-9 in the hippocampus of $Fmr1^{KO}$ mice while promoting dendritic spine maturation and improving anxiety and strategic exploratory behavior [14]. Minocycline also prevents all neuroanatomical defects in FXS flies and improves language and social communication skills, anxiety, attention, irritability, stereotypy, hyperactivity and inappropriate speech in humans [15-17]. The pro- and active-forms of plasma MMP-9 are substantially elevated in FXS individuals compared to typically developing age-matched controls although a significant overall correlation between reduced MMP-9 activity and observed improvement in the Clinical Global Impression-Improvement (CGI-I) was not observed [18]. The preliminary analysis consisted of plasma samples from ten subjects who received minocycline for 3 months with blood samples collected at baseline and after treatment. Six of the ten subjects exhibited some decrease in MMP-9 activity after minocycline treatment and the remaining four showed no change. Five of six patients with decreased MMP-9 activity exhibited improvement in the CGI-I. Thus, a larger study is required to determine if MMP-9 is a viable blood-based biomarker to monitor minocycline efficacy in FXS.

## Amyloid-beta protein precursor and Amyloid-beta

APP and metabolites including amyloid-beta (Aβ) are potential FXS biomarkers. We found that FMRP binds to and regulates the translation of *App* mRNA [19]. In the absence of FMRP ($Fmr1^{KO}$ mice), APP and Aβ are overexpressed. Genetic knockout of one *App* allele in the $Fmr1^{KO}$ mice rescues many disease phenotypes including seizures, anxiety, the ratio of mature versus immature dendritic spines and mGluR-LTD [20]. In human blood plasma, the level of $A\beta_{1-42}$ was significantly reduced in full-mutation FXS adult males compared to age-matched controls while APP and $A\beta_{1-40}$ levels were not altered. These data suggest that $A\beta_{1-42}$ may be a plausible blood-based biomarker for FXS. APP and Aβ are currently under evaluation as blood-based biomarkers in a prospective open-label trial of acamprosate in FXS youth and preliminary results indicate that APP levels are normalized in response to drug treatment [4]. Accumulating evidence from the Lahiri laboratory shows that $A\beta_{1-40}$, $A\beta_{1-42}$ and sAPPβ levels are decreased in plasma of youth with severe autism compared to controls whereas sAPPα levels are elevated [21]. In total, these data suggest that there may be age-dependent differences in APP expression and processing and that these proteins may be valuable biomarkers for both FXS and autism. An important caveat in developing Aβ as a blood-based biomarker is that anticoagulants appear to alter APP processing; thus, blood collection procedures need to be standardized [22]. The advantage of APP and Aβ as blood-based biomarkers for FXS, as opposed to measuring the activity of the aforementioned signaling molecules, is the stability of the proteins in serum.

## Other potential biomarkers

In addition to the biomarkers discussed above, other potential candidates include brain-derived neurotrophic factor (BDNF), p70 ribosomal subunit 6 kinase 1 (S6K1), and cytokine and chemokine profiles. Erickson and colleagues observed increased BDNF levels in a pilot FXS trial testing acamprosate [23]. Hoeffer and colleagues found increased phosphorylation of S6K1 in FXS lymphocytes [24]. Ashwood and colleagues found altered plasma cytokine and chemokine levels in FXS subjects; specifically, they observed elevated interleukin-1 alpha (IL-1α), regulated on activation normal T-cell expressed and secreted protein (RANTES) and 10 kDa interferon gamma-induced protein (IP-10) compared to typically developing controls [25].

## Summary and conclusions

In summary, there are numerous drugs exhibiting promise in preclinical testing for FXS but no validated blood-based biomarkers or behavioral outcome measures for drug efficacy testing. This mini-review highlights research findings demonstrating that ERK activation rates, PI3K and MMP-9 activity levels, and APP and metabolite levels

are potential blood-based biomarkers for FXS. These candidate biomarkers are at early stages of validation and deserve further investigation.

## Abbreviations

Aβ: Amyloid-beta; ABC: Aberrant behavior checklist; APP: Amyloid-beta protein precursor; BDNF: Brain-derived neurotrophic factor; CGI-I: Clinical global impression-improvement; ERK: Extracellular-regulated kinase; *FMR1*: Fragile X mental retardation-1 gene; FMRP: Fragile X mental retardation protein; FXS: Fragile X syndrome; IL-1α: Interleukin-1 alpha; IP-10: 10 kDa interferon gamma-induced protein; LTD: Long-term depression; MAPK: Mitogen-activated protein kinase; MEK: Mitogen-activated protein kinase kinase; mGluR: Metabotropic glutamate receptor; MMP-9: Matrix metalloproteinase 9; mTOR: Mammalian target of rapamycin; PARS-R: Pediatric anxiety rating scale-revised; PI3K: Phosphoinositide 3-kinase; PKB: Protein kinase B; RANTES: Regulated on activation normal T-cell expressed and secreted protein; S6K1: p70 ribosomal subunit 6 kinase 1.

## Competing interests

The author declares that she has no competing interest.

## Authors' contributions

CW prepared the manuscript. The author read and approved the final manuscript.

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