



***FMRI* gene mutations cause neurodevelopmental-degenerative disorders: Importance of fragile X testing in Serbia**

Mutacije *FMRI* gena uzrokuju razvojne i degenerativne poremećaje nervnog sistema: značaj testiranja na nestabilni X hromozom u Srbiji

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Scientific advances in biomedicine have enabled translation of preclinical research breakthroughs to clinical trials during the last decades. Resources required for such effort are typically available in developed countries such as the USA. Kennedy Krieger Institute in Baltimore, Maryland is an internationally recognized institution dedicated to improving the lives of individuals with disorders of the brain, spinal cord, and musculoskeletal system (<https://www.kennedykrieger.org>). Clinical Trials Unit (CTU) at the Institute is one of the top-level institutions in the world that conduct the advances in translational medicine. The Unit helps advance treatment, prevention, and possible cures (<https://www.kennedykrieger.org/research-training/centers-labs-cores/clinical-trials-unit>). This and other similar institutions bring together world-leading experts in clinical research in order to provide state-of-the-art treatment of previously untreatable disorders for participants. One of them is fragile X syndrome (FXS), the hallmark of Fragile X-associated disorders (FXD), which is at the forefront of translational efforts to develop such targeted treatments. Specifically, FXS is the most translated among all neurodevelopmental disorders in human clinical trials. Namely, preclinical breakthroughs have generated much interest by the field to translate them into humans with FXS, and possibly autism spectrum disorder (ASD), a major public and economic health burden on society worldwide. Specifically, a recent search of www.clinicaltrials.gov, the National Institute of Health (NIH) sponsored website, and literature search revealed that 22 double-blind, placebo-controlled clinical trials^{1,2} have been registered in humans with FXS, as required by the Food and Drug Administration (FDA) Act of 2007. Gaps in translating the above successes have been identified. At present, no symptomatic or disease modifying treatments for

FXS have received regulatory approval. Then the most respectable medicinal regulatory authorities in the world (e.g. FDA) greatly benefit from efforts of leading clinical and research experts in the USA to address these gaps and advance these clinical trials of humans with FXS that were conducted mostly from 2008 to 2015¹⁻³. To briefly backtrack, collaborative effort among scientific institutions in developed, and some developing countries, is key to the establishment of local specialty fragile X clinics, and even clinical and research consortiums, which is of a vital importance for the successful translation of such treatment advances. Here, we highlight the importance of the Fragile X Clinical & Research Consortium (FXCRC). The Consortium is a collaborative endeavor initiated in 2006 by the National Fragile X Foundation (NFXF) to advance clinical practice and facilitate coordinated, collaborative multi-site research on FXS, which currently consists of 28 clinics in the USA and Canada. The Consortium has formed several committees designed to address common issues with regard to best practices in evaluation and treatment, strategies for supporting and enhancing clinic work, and research priorities, such as Clinical Trials Committees. Next, Fragile X Clinical and Research Consortium Registry and Database (FORWARD) is the Center for the Disease Control (CDC) funded project now 5-year renewed through 2020 (PI: Brown, 1 U01 DD001189-01) in which the Consortium works closely with the CDC and the NFXF. The project helps establish standards of care, facilitate the conduct of multi-institutional clinical research projects, coordinate and organize research across sites, build a reliable, dynamic patient registry and assist member clinics in data collection and analysis, including effective and relevant outreach and surveillance. The Institute is one of the sites for the FORWARD

project (<https://www.kennedykrieger.org/research-training/current-research-projects/clinical-research/fragile-x-clinical-and-research-cooperative>) (PI: Budimirovic). In addition, in 2014, Dr Budimirovic also led the Consortium's Clinical Committee effort on expert Consensus Document on ASD in FXS featured on the NFXF website⁴, which was then followed by a large cohort evidence-based study that characterized ASD in FXS⁵. The Consensus Documents are supported in part by the CDC-funded grant and created and reviewed by a variety of FXCRC clinicians with many clinical backgrounds in order to offer the most up-to-date insight on a variety of subjects related to Fragile X.

An increase in life expectancy and decrease in mortality in developing countries, particularly in low, middle and upper middle-income countries, many of them in transition, has been noted during the past several decades. These positive trends are by and large because of improvements in their quality of life and diet and their health care systems, especially in disease and disorder prevention, early diagnosis, and appropriate treatments. Among other benefits, the progress delays the onset and decreases the severity of chronic diseases including neurodevelopmental [e.g., FXS, ASD, non-FXS intellectual disabilities (ID)], and neurodegenerative [i.e., fragile X-associated tremor ataxia syndrome (FX-TAS)] disorders^{6–8}. For example, challenging behaviors seen in a higher proportion of children with FXS who meet criteria for ASD than those who don't can significantly impact an individual's academic and adaptive functioning, limiting their participation in the community. There are three levels of interventions aimed at decreasing the implications of these disabilities: society (healthy public policy)⁹, health-care community (e.g., through education activity, training, and public campaigns)¹⁰ and individual (e.g., counseling, screening, and availability of information about clinical preventive services)¹¹. In Serbia, the incidence of FXD as the umbrella of FXS, non-FXS ID or FX-TAS is still unknown, which is a common case for these countries in transition. Consequently, socio-economic factors such as poverty and lack of adequate research support (e.g., inadequate laboratory equipment and diagnostic test) interfere with the early diagnosis and treatment of these disorders. Hence, it is often necessary to develop the collaborative programs and network between developed country and upper middle income countries such as Serbia. The effort contribute to building sustainable research capacity in these countries aimed at reaching long-term goals: prevention, early diagnosis of the FXD full mutation and adequate treatment strategies. In Serbia, such strategies don't exist but they need to include genetic, social and economic resources to prevent the above noted negative impact of FXS on brain development and function resulting in functionally interfering behavioral and other problems that negatively affect the quality of life of these individuals and their families. Together, there is a compelling need to develop new networks and an international multidisciplinary study in Serbia in order to describe the feasibility of screening for frequency of FXD among pediatric and adult populations with relevant disabilities, and to start building their Registry, Database and Repository.

FMR1 gene mutations. There has been great progress in the fragile X field since the discovery of the Fragile X Mental Retardation 1 (*FMR1*) gene in 1991 by Drs. Ben Oostra, David Nelson, and Stephen Warren^{12–14}. The gene is located on the Xq27.3 chromosome. There are two types of *FMR1* mutations that expand the number of CGG triplet repeats: normal (30–45) triplets, full-mutation (FM): > 200 triplets, and premutation (PM); 55–200 triplets^{15, 16}. The impact of 45–54 CGG nucleotide repeats in the *FMR1* gene has not been studied enough¹⁷. The mutations of the *FMR1* gene cause both neurodevelopmental and neurodegenerative disorders under the umbrella of FXD. FXS is a global neurodevelopmental disorder caused by the FM mutation in the promotor region of the *FMR1* gene that leads to epigenetic (hypermethylation) silencing and thus, to the absence or reduction of its encoding protein: fragile X mental retardation protein (FMRP)¹⁸. Affecting up to 1 in 2500 boys, FXS is the leading cause of an inherited form of ID and the most known monogenetic cause of ASD that is purely behaviorally defined by Diagnostic and Statistical Manual of Mental Disorders (DSM)-5 [American Psychiatric Association, 2013], in contrast to FXS that is a medical/genetic disorder characterized by ASD in up to 2 out of 3 boys^{6, 7, 19, 20}. As such, FXS is the most studied ASD model 'disorder of synapse' due to their profound clinical and biological overlaps⁴. Specifically, FMRP, an RNA-binding protein that is heavily expressed in the brain, targets approximately 4% of the transcribed mRNAs in the brain, and as many as 842 of these transcribed mRNAs converge on the same pathway as idiopathic ASD^{16, 21–25}. Therefore, the lack of a specific treatment for both FXS and ASD has propelled the need to develop core targeted therapies. Briefly, since FMRP acts as a "brake", the absence of the fragile X gene's encoded protein in FXS causes up-regulation of metabotropic glutamate receptor 5 (mGluR5), mTOR, MMP9, RAS, GSK3-beta, and PI3K signaling, and down-regulation of GABA, and cAMP [and protein kinase (PKA), CAMP response element binding protein (CREB) signaling, which leads to an imbalance in neural excitation/inhibition^{7, 16, 26}. Collectively, these findings constitute 'the mGluR theory' by Bear et al.²⁶, providing the basis for the aforementioned clinical trials¹⁷. In addition, preclinical studies have shown decreased activity of insulin signaling, and that metformin can normalize at least the cAMP [and cAMP-dependent PKA and CREB]^{27, 28}.

Prevalence of PM is in up to 1 : 150 women and 1 : 400 men, which is about 10 times more common than FM¹⁶. Currently, 1.5 million individuals are affected with the PM ("carriers") in the US, and over 20 million worldwide. FX-TAS is a serious adult neurological disorder that results in cognitive, gait, and motor deficits in approximately 40–50% of carrier men and 16% of carrier women, with an average age of onset at 62 years⁸. Importantly, pathophysiological mechanisms underlying PM and FM are different and distinct. In PM, the accumulation of mRNA becomes toxic to the cell. However, in FM, the *FMR1* epigenetic silencing results in the lack of FMRP. Furthermore, PM causes different clinical phenotypes: for example, it can cause anywhere from minimal/moderate to severe cases such as fragile X-

associated primary ovarian insufficiency (FX-POI), early menopause in adult females (not the focus of this article) or FX-TAS in adult males.

FMRI mutations diagnosis. The genetic diagnosis of FXD has much improved over time. The chromosome cytogenetic test was the only available diagnostic tool for FXD during the 1970s and 1980s. Nowadays, molecular diagnosis for FXD is possible using the most advanced method. The “*FMRI* DNA” (Southern blot fragile X testing in the text) is “standard of care” for determining the presence of *FMRI* mutations. Advanced methods assess not only the degree of CGG expansion, but also *FMRI* gene promoter methylation status (mPCR)⁷. Furthermore, the quantification of the spectrum of methylation characteristics in patients with *FMRI* expansions is available by the use of mPCR^{29,30}. Also, in order to improve the diagnosis, prognosis and treatment options for affected individuals, quantification of FMRP (qFMRP) is useful³¹. This is also of relevance for asymptomatic carriers with PM, and rare asymptomatic individuals with FM but *FMRI* is not silenced (these individuals have FMRP levels that are ~20% of normal individuals)^{32,33}. Regardless, new kits have created easy-to-use, accessible, and high performance methods for laboratories. These new kits can measure the methylation fraction for each *FMRI* allele at a higher resolution than the Southern blot. In addition, there is an opportunity to detect mosaic alleles in affected individuals. For example, scientists at Asuragen, Inc from Texas in collaboration with fragile X experts in the USA have developed Amplidex[®] and Xpansion[®] Interpreter *FMRI*, which offer a very successful suite^{29,30}. The advanced test also measures the presence and the number of AGG nucleotide triplets between CGGs (AGG interruptions), called ‘speed bumps’, which are important for the stability of *FMRI* PM allele. For example, a risk of PM expansion into FM in the offsprings increases 3–4 times in absence of AGGs (vs 2 among 60–80 CGGs) in a carrier³⁴. The most frequent clinically relevant situation involves the mother with PM and her son with FM. Fragile X testing detects more than 99% of individuals with FXD, including the carriers¹⁶. There are three general circumstances in which fragile X testing should be considered: (i) clinical symptoms that suggest FXD, including any adult over 50 with features of FX-TAS such as cognitive, gait, and motor deficits, especially in combination with a positive family history of fragile X; (ii) a family history of FXD and intellectual or learning disabilities, or (iii) ASD and family or personal history of a fragile X carrier^{18,35,36}. One survey revealed that almost 38% of parents of children eventually diagnosed with FXS, underwent more than 10 symptom-related visits to their health care professional before the fragile X diagnostic test was ordered³⁷. From this, it is not surprising that the average age of the diagnosis of FXS is 35 to 37 months³⁸. Typically, physicians do not consider it without a family history of ID or other dysmorphic features. However, these features are not present in approximately one third of individuals with FXS³⁹.

Fragile X testing can provide information not only for the diagnosis, but also for the treatment and prevention of FXD^{16,18}. Guidance statements from professional organiza-

tions such as the American College of Medical Genetics, and American Academy of Child & Adolescent Psychiatry, American Academy of Child Neurology emphasize the need for fragile X testing in individuals diagnosed with ASD⁴⁰. Furthermore, the FXCRC has specific guidelines that begin with care by a physician-led team with expertise in FXD. Nevertheless, general clinical practice and available literature reveal that only one third of individuals with ASD are tested for *FMRI* mutations⁴¹. In countries such as Serbia, clinicians, patients and their families are by and large not familiar with the type of inheritance and phenotypes of FXD, or with the availability of precise genetic fragile X testing. Thus, there is a need to enhance the knowledge about FXD and fragile X testing. However, opportunities for medical education are limited, even at the medical school education level. Their faculties have to invest time and resources to research, and they occasionally have to organize such education programs⁴². Medical professionals often have no incentives to attend these lectures. Thus, as the first step, a well-designed educational-informative survey needs to reach a wide range of these medical health professionals. This is probably the best way to access their knowledge about FXD, and to begin an educational aspect that would disseminate this knowledge. This is also important because despite early interventions, only 9% male and 44% female patients with FXS reach a high level of independence as adults. Furthermore, while there is a need to balance between the individual needs, and the distribution of public health resources, the early diagnosis of FXS may also qualify these individuals for clinical trials. The vast majority of the aforementioned clinical trials in FXS targeted excitatory/inhibitory imbalances (14/22, 64%)^{1,2,43,44}. Yet, over the last few years, some of these key trials failed to meet the primary efficacy end points, including the well-powered study by Berry-Kravis et al.⁴⁵ (2016) that studied the mGluR5 antagonist mavoglurant. Nevertheless, such “negative” results in the clinical trials actually provide us with valuable lessons for designing future treatment studies in FXS, ASD, and other neurodevelopmental disorders^{1,45,46}. For example, follow-up analyses of the arbacofen study (GABA-B agonist) showed that one of shortcomings in the design and outcome measures failed to capture areas of positive response to the newly developed therapeutics^{47,48}. Applying these ‘lessons learned’ a trofinetide (NNZ-2566) phase II trial conducted in adolescents and adults with FXS by the Neuren Pharmaceutical applied the Fragile X Syndrome Rating Scale which covers a wide range of behavioral symptoms, including FXS and ASD in FXS^{49,50}. It is a worthwhile effort to continue to validate this new behavior rating scale covering the core FXS behavioral phenotypes and associated symptoms. Close collaboration between the study sponsor (Neuren Pharmaceutical) and the experts in the field has been another necessary progress needed to move the field ahead faster.

In conclusion, the help from the USA in the form of collaboration is much needed for Serbia in the field of FXD, and ASD, which is currently either nonexistent or negligibly developed. Thus, building relationships with the NFXF and

the Consortium and its Clinical Trial Committee in the USA aims toward a long-term purpose of this article: to found and establish the first fragile X clinic in Serbia, and probably in the South East Europe. That also sets a stage for building a fragile X Registry, Database, and Repository in Serbia for which external international funds will be critical. In the meantime, an extensive survey has been designed as a tool for the first FXD KAP (knowledge, attitude, and practices) study in Serbia, called “Applied knowledge to

early detection of genetic disorders caused by mutations of fragile X-chromosome: the most common genetic cause of ASD”. The study has been approved by Ethics Committee (IRB) of School of Medicine, University of Belgrade, Serbia (No 29/IX-6; September 21, 2016; PI: Budimirovic, co-PI: Protic). The study will be conducted among physicians in primary health care in Serbia and the last grade medical students at School of Medicine, University of Belgrade, Serbia and the results will be obtained in the next few months.

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