1. Introduction

Substance use disorders (SUDs) remain of paramount concern for modern society. The scientific community continues to examine the mechanisms of SUDs in order to develop strategies for improvements in prevention, treatment, and rehabilitation. Psychoactive substance use is becoming more prevalent over time. Over 2% of the world population has had substance dependence, ranging from 1.5% of global disease burden resulting from psychoactive substance use. SUDs are associated with serious comorbidities such as learning and memory impairments, socio-economic burden, and harms to society as a whole [2]. Accumulating data have implicated a role of genetic and epigenetic factors in the development of SUDs [3, 4]. One such candidate is the FMR1 gene (fragile X mental retardation 1). The gene (Xq27.3) has been characterized as a major genetic cause of the Fragile X Syndrome (FXS) [5]. FXS is one of the most common forms of inherited intellectual disability, and is the most common genetic link associated with autism spectrum disorder (ASD) [6, 7]. The encoded RNA-binding protein (FMRP) has a multi-domain structure and though expressed ubiquitously, is most abundant in neurons and testis [8]. This protein regulates synaptic development and plasticity, thus modulating cognitive functions such as learning and memory [9]. FMRP is involved in post-transcriptional RNA metabolism [10], alternative splicing [11], DNA damage response [12], and epigenetic modulation of chromatin function [13].

In FXS, an expansion of more than 200 CGG trinucleotide repeats in the 5′UTR regions of FMR1, named “full mutation”, was found accompanied by abnormal CpG methylation and repressive histone
modifications. These lead to a marked reduction of FMR1 mRNA transcription levels and an FMRP deficiency. This would affect synaptic plasticity and brain function. In less than 1% of FXS patients, mutations are found in the coding region of FMR1 [14]. An expansion of CGG repeats within the 55–200 (a “premutation” state) range is linked to two different clinical conditions: fragile X-associated primary ovarian insufficiency (FXPOI) and fragile X-associated tremor/ataxia syndrome (FXTAS). For unknown reasons, the permutation causes overproduction of FMR1 mRNA but normal or slightly reduced protein levels [15]. A gain-of-function toxic effect of elevated FMR1 mRNA levels has been proposed to explain this phenomenon [16].

The role of FMR1 gene in SUDs has not been well studied. However, a number of recent investigations indicate that SUDs and FMR1-associated disorders might share common underlying mechanisms. Thus, alcohol abuse seems to intensify behavioral problems (such as aggression and impulsivity) in FXS [17], and is associated with increased neurological deterioration in FXTAS [18]. A case of a premutation male adult with FXTAS and a long history of methadone use showed faster disease progression than what is typically observed [19]. Authors hypothesized that the prolonged narcotic use had accelerated symptoms and exacerbated mRNA toxicity of the underlying condition. Similarities in causes of drug addiction and ASD have also been outlined, especially in their neural circuits and molecular signaling pathways [20]. Additionally, studies in mice have identified FMRP as a novel mediator of cocaine-induced behavioral and synaptic plasticity [21], which also mediates dopamine activity in forebrain neurons [22]. In view of these findings, the current

Table 1. Socio-demographic characteristics of participants.

| Gender | Male | Female |
|--------|------|--------|
| Group  | Alcohol dependence | 10 (62.5%) | 6 (37.5%) |
|        | Drug dependence     | 12 (70.6%) | 5 (29.4%) |
|        | Control group       | 5 (45.5%)  | 6 (54.5%) |
| Total  | 27 (61.4%)          | 17 (38.6%) |

| Age range, years old | Group | Alcohol dependence | Drug dependence | Control group | Total |
|----------------------|-------|---------------------|------------------|--------------|-------|
| 21–30                |       | 2 (12.5%)           | 1 (5.9%)         | 1 (9.1%)     | 4 (9.1%) |
| 31–40                |       | 5 (31.3%)           | 11 (64.7%)       | 1 (9.1%)     | 17 (38.6%) |
| 41–50                |       | 3 (17.6%)           | 5 (29.4%)        | 6 (54.5%)    | 15 (34.1%) |
| 51–60                |       | 1 (5.9%)            | 1 (5.9%)         | 3 (27.3%)    | 7 (15.9%) |
| Over 60              |       | 0 (0.0%)            | 0 (0.0%)         | 0 (0.0%)     | 1 (2.3%)  |

| Education            | Group | Alcohol dependence | Drug dependence | Control group | Total |
|----------------------|-------|---------------------|------------------|--------------|-------|
| Primary              |       | 1 (6.3%)            | 0 (0.0%)         | 0 (0.0%)     | 1 (2.3%) |
| Secondary            |       | 8 (50.0%)           | 11 (64.7%)       | 3 (27.3%)    | 22 (50.0%) |
| Bachelor/Master degree |     | 6 (37.5%)           | 6 (35.3%)        | 7 (63.6%)    | 19 (43.2%) |
| Doctoral degree      |       | 0 (0.0%)            | 0 (0.0%)         | 1 (9.1%)     | 1 (2.3%) |
| No degree            |       | 0 (0.0%)            | 0 (0.0%)         | 0 (0.0%)     | 1 (2.3%)  |

| Marital status       | Group | Alcohol dependence | Drug dependence | Control group | Total |
|----------------------|-------|---------------------|------------------|--------------|-------|
| Married              |       | 3 (18.8%)           | 5 (29.4%)        | 7 (63.6%)    | 15 (34.1%) |
| Living with a partner without marriage |       | 2 (12.5%)           | 3 (17.6%)       | 3 (27.3%)    | 8 (18.2%) |
| Divorced             |       | 4 (25.0%)           | 1 (11.8%)        | 7 (63.6%)    | 15 (34.1%) |
| Separated/without divorce |     | 2 (12.5%)           | 0 (0.0%)         | 1 (9.1%)     | 4 (9.1%)  |
| Single               |       | 2 (12.5%)           | 7 (41.2%)        | 1 (9.1%)     | 13 (29.5%) |

| Household             | Group | Alcohol dependence | Drug dependence | Control group | Total |
|----------------------|-------|---------------------|------------------|--------------|-------|
| Living alone         |       | 6 (37.5%)           | 5 (29.4%)        | 0 (0.0%)     | 11 (25.0%) |
| Living with spouse/partner |     | 1 (6.3%)            | 5 (29.4%)        | 5 (45.5%)    | 11 (25.0%) |
| Living with spouse/partner and child/children | | 3 (18.8%) | 3 (17.6%) | 5 (45.5%) | 11 (25.0%) |
| Living alone with child/children |       | 2 (12.5%)           | 0 (0.0%)         | 0 (0.0%)     | 2 (4.5%)  |
| Living with parents  |       | 4 (25.0%)           | 4 (23.5%)        | 0 (0.0%)     | 8 (18.2%) |
| Living with other people/not from the family |       | 0 (0.0%)            | 0 (0.0%)         | 1 (9.1%)     | 1 (2.3%)  |

| Working status       | Group | Alcohol dependence | Drug dependence | Control group | Total |
|----------------------|-------|---------------------|------------------|--------------|-------|
| Full time employed   |       | 5 (31.3%)           | 4 (23.5%)        | 10 (90.9%)   | 19 (43.2%) |
| Part time employed   |       | 6 (37.5%)           | 1 (11.8%)        | 1 (9.1%)     | 14 (31.8%) |
| Student              |       | 0 (0.0%)            | 0 (0.0%)         | 0 (0.0%)     | 1 (2.3%)  |
| Unemployed           |       | 7 (41.2%)           | 1 (5.9%)         | 1 (9.1%)     | 10 (22.7%) |

| Criminal status      | Group | Alcohol dependence | Drug dependence | Control group | Total |
|----------------------|-------|---------------------|------------------|--------------|-------|
| Registered or arrested by the police over the last year |       | 0 (0.0%)            | 0 (0.0%)        | 0 (0.0%)    | 2 (4.5%)  |
| Registered or arrested by the police before the last year |       | 3 (18.8%)           | 7 (41.2%)       | 0 (0.0%)    | 10 (22.7%) |
| Never registered or arrested by the police |       | 13 (81.3%)          | 8 (47.1%)       | 11 (100%)   | 32 (72.7%) |
The study was based on the hypothesis that FMR1 is involved in SUDs development and/or manifestation. To address this hypothesis, we examined relative FMR1 mRNA expression levels and their sex-dependence in leukocytes from patients with alcohol or drug dependence in comparison with healthy controls. In addition, data related to socio-demographic characteristics of the participants and the specifics of their substance use is addressed.

2. Material and methods

2.1. Participants

This study included 44 participants distributed across three groups. The first group consisted of 16 patients with alcohol dependence (AD) (mean age ± SD = 43 ± 9 years, range 27–57, 10 males and 6 females), included in a program for detoxification and psychosocial rehabilitation at the Sofia State psychiatric hospital for treatment of drug and alcohol addiction. The second group consisted of 17 persons with drug dependence (DD; heroin) (mean age ± SD = 41 ± 8 years, range 27–62, 12 males and 5 females), from programs including treatment with opioid agonists (methadone or substitol) at two clinical centres in Sofia. Diagnoses were confirmed by review of the medical records. The ICD-10 diagnostic system was used. The third control group (C) comprised of 11 people, who were selected from the general population based on a declaration that they do not suffer from chronic diseases, do not use any illicit drugs and their use of alcohol is no more frequent than 1–2 times a week (low-alcohol beverage). Controls matched as closely as possible to the first two groups in terms of age and gender (mean age ± SD = 47 ± 9 years, range 31–58, 5 males and 6 females). All participants were adults.

2.2. Procedure

Written informed consent, for participation in the study and data collection, was obtained from each participant prior to the study. All participants donated a 5–6 ml blood sample for molecular-genetic analysis. The collected blood samples were transferred to the laboratory and processed within 2 h to avoid RNA degradation. Participants also completed a questionnaire with data related to their socio-demographic status. The questionnaire filled in by the people from the AD and DD groups included several extra questions related to drug or alcohol use. The study was carried out in accordance with the Declaration of Helsinki and approved and regulated by the Local Research Ethical Committee (1/20.03.2019).

2.3. Obtaining socio-demographic and alcohol/drug use data

A questionnaire was constructed especially for the purposes of the study. It collected the following information from participants in all groups – age; sex; educational, marital, household, working, criminal, and medical status; presence of relatives with problem substance use. The extra questions for AD and DD groups covered the age of onset, years of use of the main substance, presence of problem use of other substances.

Table 2. Substance-use related characteristics of participants.

| Chronic diseases | Absence of chronic disease | Kidney stones | Diabetes | Panic disorder | Heart-related problems | Hypertonia |
|------------------|---------------------------|---------------|----------|----------------|------------------------|-----------|
| Group            | Alcohol dependence        | 10 (62.5%)    | 1 (6.3%) | 1 (6.3%)       | 2 (12.5%)              | 1 (6.3%)  |
|                  | Drug dependence           | 17 (100%)     | 0 (0.0%) | 0 (0.0%)       | 0 (0.0%)               | 0 (0.0%)  |
| Total            | 27 (82.0%)                | 1 (3.0%)      | 1 (3.0%) | 1 (3.0%)       | 2 (6.0%)               | 1 (3.0%)  |

| Previous treatment related to substance use |
|---------------------------------------------|
| Never been in treatment before              | One previous treatment | More than one previous treatment |
| Group                                        |                         |                               |                               |
| Alcohol dependence                          | 2 (12.5%)               | 3 (18.8%)                     | 11 (68.8%)                    |
| Drug dependence                             | 4 (23.5%)               | 2 (11.8%)                     | 11 (64.7)                    |
| Total                                       | 6 (18.2%)               | 5 (15.2%)                     | 22 (66.7%)                   |

| Age of onset, years old                     |
|---------------------------------------------|
| Before 20                                   | 21–30                    | 31–40                        | After 41                     |
| Group                                        |                         |                               |                               |
| Alcohol dependence                          | 11 (68.7%)              | 2 (12.5%)                    | 2 (12.5%)                    | 1 (6.3%) |
| Drug dependence                             | 12 (70.6%)              | 3 (17.6%)                    | 1 (5.9%)                     | 1 (5.9%) |
| Total                                       | 23 (69.7)               | 5 (15.2%)                    | 3 (9.1%)                     | 2 (6.0%) |

| Years of use of the main substance           |
|---------------------------------------------|
| Up to 10 years                              | More than 10 years      |
| Group                                        |                         |                               |
| Alcohol dependence                          | 7 (43.8%)               | 9 (56.2%)                    |
| Drug dependence                             | 7 (41.2%)               | 10 (58.8%)                   |
| Total                                       | 14 (42.4%)              | 19 (57.6%)                   |

| Regular use of other substances              |
|---------------------------------------------|
| Yes                                         | No                        |
| Group                                        |                           |                               |
| Alcohol dependence                          | 6 (37.5%)                | 10 (62.5%)                   |
| Drug dependence                             | 12 (70.6%)               | 5 (29.4%)                    |
| Total                                       | 18 (54.5%)               | 15 (45.5%)                   |

| Relatives with substance use                |
|---------------------------------------------|
| First degree relatives                      | Second degree relatives   | No relatives                 |
| Group                                        |                           |                               |
| Alcohol dependence                          | 8 (50.0%)                | 5 (31.3%)                    | 3 (18.8%)                   |
| Drug dependence                             | 2 (11.8%)                | 0 (0.0%)                     | 15 (88.2%)                  |
| Total                                       | 10 (30.3%)               | 5 (15.2%)                    | 18 (54.5%)                  |
2.4. Isolation of total RNA and cDNA synthesis

Total RNA was isolated using QiAamp RNA Blood mini kit (Qiagen) following the manufacturer's recommendations. An optional step was included to eliminate any potential genomic DNA contamination (RNase-free DNase set, Qiagen). The concentration and quality of all RNA samples was determined spectrophotometrically (BioSpec-nano Spectrophotometer - Shimadzu Biotech) and electrophoretically on 1.5% agarose gel in 1xTAE. Only isolates with absorption ratio of 260/280 nm > 1.8 were subjected to subsequent analyses. An aliquot of 500ng RNA from each sample was used to synthesize complementary DNA (cDNA) by FIREScript RT cDNA Synthesis Mix (Solis Biodyne) according to the manufacturer's protocol. Reactions not containing reverse transcriptase or RNA template were used as negative controls. The synthesized cDNA was immediately frozen and stored at -30 °C.

2.5. qRT-PCR analysis

Quantitative RT-PCR (qRT-PCR) was performed on the synthesized cDNA templates to detect relative mRNA expression levels of \( \text{FMR1} \) gene in the three study groups by 5x HOT FIREPol EvaGreen qPCR Mix Plus (no ROX) (Solis Biodyne). The housekeeping gene \( \text{ATP5b} \) was used as a reference gene to normalize the target gene expression. The primers used are as follows: for \( \text{FMR1} \) F-GCAGATTCCATTTCATGATGTCA, R-ACCACCAACAGCAAGGCTCT, product length 122bp [15] and for \( \text{ATP5b} \) F-TCACCCAGGCTGGTTCAGA and R- AGTGGCCAGGGTAGGCTGAT, product length 80bp [23]. The reaction was performed in a 10 \( \mu \)l volume containing: 1x qPCR Mix, 0.3 \( \mu \)M of each primer set, DNase-RNaseFree water and 1\( \mu \)l of 5-fold diluted cDNA on PikoRealTM Real-Time PCR System (Thermo Fisher Scientific Inc.). The cycling conditions included 15 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 60 °C, 45 s at 72 °C, and final elongation for 5 min at 72 °C. No template control (NTC) was added in each experiment. Data were analyzed using PikoReal Software version 2.1 (Thermo Fisher Scientific Baltics UAB). Standard curves were generated to assess the efficiency of the amplification.

2.6. Data analysis

Socio-demographic characteristics and those related to the substance use are analyzed by descriptive statistics (crosstabs). Relative quantification of \( \text{FMR1} \) gene expression data was performed using the Relative Expression Software Tool (REST) [24]. Statistical evaluation was done by hypothesis test, which is included in the REST software. Values of \( p \) below 0.05 were considered significant.

3. Results

3.1. Analysis of socio-demographic and substance-use related characteristics of participants

Socio-demographic characteristics and substance-use related characteristics of the participants in the study are presented in Table 1 and Table 2, respectively. The participants in the three groups were relatively balanced for sex and age. The comparison between groups showed a presence of higher educational status (predominantly university degree) among people from the control group; whereas in the AD and DD groups, people with a high school degree dominated. The results revealed the marital status of the control group was more stable – more than 90% of them were married or lived with a partner (and children). In comparison, approximately 60% of alcohol and 50% of drug dependent people were either not married or divorced/separated from their partners and...
typically lived either alone or with their parents. The working status of people with alcohol and drug dependence was also much worse than that of the controls. Less than 30% of them had a full time job compared with more than 90% of healthy controls. The difference between groups in terms of their legal status was even larger. Nobody from the control group has ever had problems with the police. In contrast, approximately 1/4 of people with alcohol-and 1/2 of drug-dependent individuals had problems with the police in the past. In terms of health status, an interesting fact was that approximately 1/3 of the people from the AD group had another chronic disease; whereas the other studied groups did not declare serious accompanying health issues.

The characteristics related to substance abuse revealed the following picture: approximately 2/3 of people from both AD and DD groups had experienced more than one previous treatment; about 70% started to use substances before they turned 20 years old; about 1/2 of both SUD groups had used their preferred substance for more than 10 years; approximately 1/3 of participants from the AD group and about 2/3 of those from the DD group regularly used other substances.

Regarding an inheritance pattern, 50% of people with an AD and about 10% of people with a DD declared to have a first-degree relative (a father and/or a mother), who experienced problem use of alcohol or illicit substances. In contrast, none of the controls declared to have a first-degree relative with alcohol or illicit drug use.

3.2. Analysis of FMR1 mRNA levels in people with alcohol or drug dependence, and controls

The transcription levels of FMR1 in the three studied groups were estimated (Figure 1). We found that FMR1 mRNA expression in people with alcohol dependence was down-regulated compared to controls with 25% decrease (AD vs. C, p = 0.005; Figure 2A). No significant changes in the relative expression of FMR1 were observed in the group with drug dependence compared to controls (Figure 2B).

![Figure 3](image-url) Relative expression of FMR1 mRNA in A – men with alcohol dependence (AD-M) vs. men from the control group (C-M); B – females with alcohol dependence (AD-F) vs. female from the control group (C-F), C – men with drug dependence (DD-M) vs. men from the control group (C-M) and D – females with drug dependence (DD-F) vs. females from the control group (C-F).
3.3. Sex-dependent expression of FMR1 mRNA

Further, we tested whether the differences in FMR1 mRNA expression were sex-specific. The results showed that men with alcohol dependence had a significant 40% decrease in FMR1 mRNA expression ($p = 0.001$) when compared to the men from the control group (Figure 3 – A). However, no significant differences were found between females with alcohol dependence and females from the control group (Figure 3 – B). Interestingly, a similar pattern was observed among people with drug dependence. Men from the DD group had lower expression levels of FMR1 mRNA when compared to men from the control group with 24% decrease ($p = 0.015$) (Figure 3 – C). Again, the decrease was sex-specific as no significant difference was found between the women from the DD group compared to female controls (Figure 3 – D).

Contrasting to controls, where the expression levels seemed to be rather heterogeneous, those in these two male groups with alcohol or drug dependence were relatively clustered and shifted downwards.

4. Discussion

The current results indicate a correlation between SUDs and mRNA expression levels of FMR1 gene. However, no causal connection can be concluded. The detected downregulation of FMR1 in alcohol and drug abusers may be a predisposing event or a consequence of psychoactive substance use. SUDs have a multidimensional nature, which is a result of the combined interaction between biological, psychological and social parameters [25]. It is difficult to differentiate the causative role of a single factor. However, their cumulative effect contributes to disease manifestation.

As hypothesized, people with alcohol or drug dependence, included in this study, shared a common profile. In terms of social-demographic characteristics, data from this study indicated that people with SUDs were predominantly male. This trend is typical of these target groups as globally SUDs are twice as common in men than in women [1]. In the current study, we observed that substance dependence individuals had lower levels of education, typically were divorced or separated from their partners, and lived alone or with their parents. Their working status was unstable. Problems with the police were evident in both groups; although they were more present in the DD group. In terms of health status, a stable tendency in the profile of people with alcohol dependence was the presence of a chronic disease contrasting to drug-dependent individuals. In terms of characteristics related to substance use, onset of use was typically before the age of 20, the use of substances lasted for many years; usually the dependent individuals had undergone at least one previous addiction treatment and had close relatives with psychoactive substance use. Though an inheritance pattern could be suggested, we can not conclude whether it is of genetic, epigenetic or psycho-social nature. The observed socioeconomic, psychosocial and health differences between groups could be primary contributing or additive variables to the findings here.

Regarding FMR1 status, statistically significant FMR1 down-regulation was found in the group with alcohol dependence, and in male representatives of both the AD and DD groups. To our knowledge, this is the first data on a possible role for FMR1 in people with alcohol and/or drug dependence. The observed FMR1 down-regulation does not match that normally observed in disease variants. The down-regulation was less than that observed in a fullmutation state as found in FXS, where only residual mRNA is observed. It did not correspond with the permutation state observed where FMR1 mRNA levels were several times elevated, though resultant FMRP protein was normal or slightly reduced [26]. On the basis of these observations, a novel FMR1-associated mechanism mediating alcohol/drug addictions could be proposed.

A sex-specific pattern of expression of FMR1 gene was shown by Singh et al. in male mouse brain where FMR1 was down-regulated in an age-dependent manner [27]. In female mouse brain the same authors reported for a downregulation in adult mice and elevated levels of FMR1 in old mice [28]. The authors argue that the observed effects might be related to impaired neuronal functions leading to cognitive disturbance during aging including learning and memory.

The present observation is that FMR1 down-regulation tends to be sex-specific, which may not be unusual when examining other FMR1-associated disorders. Thus, although FXS affects both males and females, the frequency of the fullmutation allele in the total population is much higher in males [29] and men are in general more severely affected than women [30]. Specificity in sex-distribution is also observed in the premutation-associated disorders, as the incidence of premutation alleles in the total population is almost three times higher in females [29]. While FXPOI is specific to women, FXTAS affects mostly male premutation carriers.

In conclusion, the present study groups with psychoactive substance dependence shared a common profile regarding socio-demographic and substance-use related characteristics. Statistically significant FMR1 down-regulation was found in the group with alcohol dependence, and in male subgroups with alcohol and drug dependence. This observed effect was more pronounced in people with alcohol-than in drug-dependence. As far as we know, this is the first study on FMR1 mRNA relative expression in alcohol and drug dependent persons. The data imply a role of FMR1 gene in SUDs mechanisms, probably in a sex-dependent manner. However, a few limitations of the current study could be pointed. The study includes relatively small number of persons under investigation and is limited only to mRNA levels assessment. The observed FMR1 down-regulation pattern does not easily fit in known disease variant models, such that novel FMR1-associated mechanisms in SUDs require more research. Further more detailed studies using larger cohorts are needed, which includes determination of CGG-repeat number, CpG methylation analysis, and FMRP levels.

Declarations

Author contribution statement

M. Krasteva: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Y. Koycheva: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.
R. Racheva: Analyzed and interpreted the data; Wrote the paper.
T. Taseva, T. Raycheva and S. Simeonova: Performed the experiments.
B. Andreev: Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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