Ljubljana, 13.3.2023

INFLUENCES ON AND PREVENTION OF SELF-HARM BEHAVIOUR AMONG THE MOST AT-RISK ADOLESCENTS

RESEARCH PROTOCOL

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Slovenian Research Agency Grant No: J3-4534

NCT05765864

1. Scientific background, problem identification and objective of the proposed research

Adolescence is a period of growing up, i.e. the transition from child to adult. The characteristics of this period (e.g. visible physical changes, altered cognitive functioning, etc.) are not only the result of hormonal changes, but also of brain development. The latter has been better studied in recent decades, is influenced by genetically determined developmental pathways and, to a large extent, also by the environment, through epigenetic changes. Between the onset of puberty and around the age of 25, the brain, which has already reached its final size at the age of six, undergoes accelerated change. There is first a loss and then a new formation of a large number of synapses in different areas of the cerebral grey matter, with a delayed and gradual myelination. This occurs in a dorsal-frontal direction, i.e. from the evolutionarily older brain regions responsible for the most basic human functioning (breathing, eating, etc.) towards the evolutionarily younger, frontal brain regions responsible for more complex human functioning (planning, reasoning, anticipating, etc.)[1,2]. As adolescents' cognitive abilities evolve from the concrete to the increasingly abstract, in the process of identity development most adolescents also deal with questions of their own existence (not only "Who am I?" and "What am I like?", but also "Why do I exist?")[3] and, consequently, more often than at other developmental stages, with questions of transience, dying and death[4].
Self-harm behaviour is common in adolescence, with a quarter of adolescents self-harming at some point in their lives withou an intention of dying, and a tenth of Slovenian secondary school pupils having attempted suicide at some point in their lives[5]. In Slovenia, a country with a traditionally high suicide rate, suicide is still the second leading cause of death in the 10-19 age group, with an average of 10 deaths per year over the last 20 years[6,7]. Self-harm behaviours are divided into those in which the person has an intention to die (*suicidal behaviour*) and those in which there are other intentions (*non-suicidal self-injury; NSSI*)[8-10]. There are many reasons for the development and persistence of self-injurious behaviour, divided into risk factors and protective factors, both of which can be roughly individual or external. The distribution, presence and absence of these factors influence the development, persistence and cessation of these behaviours in individuals in complex and not yet fully understood ways[6]. Recently, a growing body of research has focused on demonstrating the influence of the environment on an individual's vulnerability through changes in gene expression, epigenetic modifications. Research, some of which is already being recommended for use in clinical practice, also shows that certain genetic predispositions influence the response of individuals with mental disorders to treatment, both medication and other treatments.
In addition, there is currently no rapid and effective method for ongoing monitoring of adolescents' risk of self-injurious behaviour that would be useful for assessment and monitoring of adolescents after hospital admission.

In the proposed study, we aim to pursue three objectives:
1. To develop a method to identify more effectively the acute and long-term risk of adolescents with the most threatening self-harm behaviours (psychiatric inpatients).
2. To identify the factors that influence the risk of self-harm behaviours and the success of treatment of these behaviours in the most at-risk adolescents (changes in these factors).
3. Develop guidelines for more effective treatment of the most at-risk adolescents.

This will enable us to identify young people at particular risk of severe self-harm behaviour (both death and non-fatal physical harm) more reliably, to target them for more intensive and effective treatment and thus improve their safety, quality of life and prognosis in the short and long term (adulthood).

These objectives will be achieved through the work packages envisaged in the study (Table 2) and described in the following sections.

2. State-of-the-art in the proposed field of research and survey of the relevant literature

The term self-harm covers all forms of behaviour in which a child, young person or adult deliberately harms him/herself[8]. The English guidelines (NICE; National Institute for Health and Care Excellence) define deliberate self-harm (DSH) as "self-harm or self-injury that is independent of the intention of the act"[9]. In clinical and research work with adolescents with self-harm behaviour, it is important to distinguish suicidal behaviour from another common form - non-suicidal self-injury (NSSI). The latter is defined as "the deliberate infliction of injury to one's own body in a way that is not socially acceptable and does not have suicidal intent"[8,10]. These behaviours are most common in adolescents and young adults and include cutting or stabbing the skin, causing burns to the skin (e.g. with cigarette butts), hitting a hard object or body part, scratching until it bleeds, removing scabs from the skin, hitting the head on a hard object and biting the body[8,9,11]. Adolescents who have access to other methods use other methods for NSSI, such as medication for the treatment of their chronic illness[5]. Most patients (by some estimates as high as 90%) use NSSI for self-soothing, to release internal tension and to take control of intolerable emotional or cognitive states, the opposite of suicidal intent (including to prevent a suicide attempt). In this way, the behaviour is maintained in the long term according to the reinforcement principle[11,12]. According to the medical model, NSSI is divided into impulsive, compulsive, stereotyped and severe forms. In clinical work, *the impulsive form* is the most frequent, beginning most often between the ages of 12 and 14 years, and in more than 80% of cases it resolving during adolescence[8]. The behaviour becomes problematic after 5-10 repetitions, when it gains the characteristics of addiction. *The compulsive form* is characterised by repetitive forms of self-injurious behaviour (e.g. nail biting, cuticle scratching, trichotillomania), often with an obsessive component. *The stereotyped form* occurs mainly in the context of intellectual disability or pervasive developmental disorder. The individual repeats monotonous self-injurious behaviours in public without feelings of shame or inhibition (banging his head against the wall, slapping his face, punching himself in the stomach). The most dramatic, but fortunately rare, is *the severe form* where the individual permanently damages a part of the body. This occurs most often in the context of a psychotic experience[12].

2.1. Epidemiology

Suicides are extremely rare in the under-10 age group, with no official statistics recorded in Slovenia in the last 20 years. According to the National Institute of Public Health of the Republic of Slovenia, the number of suicides in the 10-19 age group is around ten per year, and in the 20-24 age group around 15 per year. Similarly to the situation abroad and in adults, Slovenian statistics show a gradual decline in the number of suicides among adolescents over the last 20 years, but suicide remains the second cause of death in this age group[6,7].

The lifetime prevalence of attempted suicide among adolescents in most countries is between 1-4% for boys and 2-10% for girls[6]. Tomori et al. reported in 1996 that among 4706 Slovenian adolescents surveyed between the ages of 14 and 19 years, 10% had ever attempted suicide (of whom 14% were girls and 7% boys) and 44% of adolescents (55% of girls and 31% of boys) had ever thought of killing themselves[13]. In a similar study conducted thirteen years later, in a smaller sample of secondary school students, the prevalence of attempted suicide and suicidal ideation remained approximately the same[5]. In a study conducted in a clinical psychiatric sample of Slovenian adolescents in 2016, 59% reported suicidal ideation and 21% a history of attempted suicide[14]. In addition, in the last year, we have seen a marked increase in visits to emergency child and adolescent psychiatric services for suicidal ideation and attempted suicide, most likely related to the pandemic[15].

The lifetime prevalence of NSSI in adults is reported at 6% (ranging from 2% to 17% according to different studies), while in adolescents it averages around 19%[16,17]. In a sample of Slovenian secondary school students aged 14-19 years, 24% of adolescent females and 12% of adolescent males were found to have ever self-harmed without the intention of dying[5]. Even higher prevalences of self-injurious behaviour are expectedly reported in studies of clinical samples of adolescents, with some reporting annual prevalence rates of up to 50%[18,19]. Lifetime prevalence of NSSI has been reported by 44% of adolescents hospitalised at the Unit for Adolescent Psychiatry of the University Psychiatric Clinic in Ljubljana[14]. Longitudinal NSSI surveys of large samples of young people from the general

population report that most of these behaviours are outgrown by adulthood, despite being problematic[20,21].

2.2. Aetiology

The aetiology of self-injurious behaviour in adolescents involves the interaction of different factors that increase or decrease the risk of suicidal behaviour: risk factors and protective factors (Table 1). Most research to date has been conducted in the area of suicidality, and few researchers have examined factors influencing NSSI during the developmental period, but because of the interconnectedness of the two forms of behaviour, the same factors are often cited for all forms of self-injurious behaviour (with and without the intent to die)[22,23]. They also point out that more attention should be paid, both in research and in general, to resilience, which is mostly presented as the absence of risk factors (e.g. the absence of dysfunctional family relationships, etc.)[23].

Table 1. Risk factors for self-harm (suicide and NSSI) and protective factors specific to adolescents[5,24-74].

Risk factors	**Protective factors**
Individual factors Gender, age (boys, increasing with age) Mental illness (depression, eating disorders) Previous suicide attempts, suicidal ideation or NSSI, hospitalisation Impulsive aggression Alcohol or drug abuse (especially in combination with depression and conduct disorder) Conduct disorder, delinquent behaviour, problems with authorities Neuroticism, pessimism, perfectionism, low self-esteem, poor coping mechanisms Non-heterosexual sexual orientation, non-cis gender identity (LGBTQ) Biological factors*	*Individual factors* Cognitive abilities (intelligence, attention, executive skills) Self-perception of competence, self-worth (self-efficacy, self-image) Temperament and personality (adaptability, sociability, problem-solving ability) Self-regulation (impulse control, arousal and emotion) Positive world view (belief that life has meaning, hope, faith, desire to live, seeking help) Biological factors*
Family, relationships and environment Conflict and domestic violence and all forms of abuse Family history of suicide or attempted suicide, genetic factors Stress factors and traumatic events, school failure Peer violence, online violence, poor peer relationships Loss of romantic relationship, relocation Use of online forums Cluster suicide	*Relationships* Quality of parenting (warmth, structure and control, expectations) Close relationships with competent adults (parents, relatives, mentors) Connection with prosocial, rule-following peers (among older adolescents) *Opportunities and possibilities in the community* Good schools Links with pro-social organisations (sports clubs, music groups, religious organisations) Neighbourhood quality (neighbourhood safety, surveillance, recreation centres, libraries) Quality of social and health care

**Biological factors (due to their relevance to the proposed research, these are presented in more detail)*

While the neurobiology of suicide in adults is well researched, research on suicide in young people is considerably scarcer. Genetic research plays an important role in understanding adolescent suicide. Suicidal behaviour is strongly familial and genetically determined. Research in both twins and adopted children has shown that suicide and attempted suicide are part of a clinical phenotype that runs in families. They found that attempted suicides are more common in families where suicide has occurred and suicide is more common in family members of suicide attempters. They excluded the influence of mental disorders[35]. Furthermore, Zalsman et al. showed an association between a polymorphism in the promoter region of the serotonin transporter gene and a polymorphism in the tryptophan hydroxylase gene (A218C) with aggressive behaviour in suicidal adolescents, but not with attempted suicide[36,37]. One possibility for familial transmission of suicidal tendencies to offspring independently of mental

disorders is thus suggested to be through impulsive aggression[38]. The biological basis for the transmission of this trait to offspring is thought to be related to serotonin metabolism and the low levels of 5-hydroxy indoleacetic acid measured in adolescents' liquor after attempted suicide[39].
At present, even less is known about the impact on NSSI and the scarce research on this topic shows mixed results.
In a twin study, genetic influences explained only thoughts about NSSI, but not this behaviour[40]. In contrast, in a large sample of adult twins, genetic factors were shown to explain around 37% of the variance in NSSI in men and 59% in women[41]. Few studies have looked for the role of specific genes in the expression of NSSI, in particular genes important in the nervous systems for the expression of emotions. Most have focused on the serotonin transporter promoter region polymorphism (5-HTTLPR). No significant effects of 5-HTTLPR on the occurrence of NSSI were found in a sample of male prisoners[42] or among groups of hospitalised adults with a history of attempted suicide or NSSI[43] . Steiger et al. found no association between 5-HTTLPR variants and self-injurious behaviour (NSSI or suicidality) in samples of women with eating disorders. However, in the same study, they showed an association between highly functional variants of the monoamine oxidase A (MAOA) gene and the risk of self-injurious behaviour[44]. As regards polymorphisms of the catechol-O-methyl transferase (COMT) gene, no significant differences were found in a group of adult hospitalised patients with a history of attempted suicide or NSSI[43]. At the same time, a study by Bernegger et al. showed that a haplotype of COMT polymorphisms (consisting of rs737865, rs6269 and rs4633) is associated with NSSI in adults with emotional disorders[45]. The effect of the gene encoding for G protein β3 (GNβ3) on NSSI in people of different ages with depression has also been reported[46].
Hankin et al. reported from studies in two independent population samples of children and adolescents that young people with at least one short variant of the 5-HTTLPR allele expressed a higher frequency of NSSI when experiencing severe peer-related stress[47]. In addition, an association between an adverse emotional environment in childhood and the development of NSSI has also been shown in carriers of the Val polymorphism of the brain derived neurotrophic factor (BDNF) gene compared to carriers of the Met allele[47]. In a sample of Chinese adolescents, associations were found between the experiences of childhood abuse, the MAOA and COMT variants and the occurrence of NSSI. Only in the group of MAOA T allele and COMT Met allele carriers was there no significant association between childhood abuse experiences and the development of NSSI[48].
Important limitations of most of the studies shown are that the sample sizes are too small to detect the effects of single gene variants and as such they are subject to sampling errors. In the only GWAS (genome-wide association study) that included a large sample of the adult population (more than 100000), none of the polymorphisms was found to be significantly associated with self-injurious behaviour, but a few loci that might have an impact on suicidality were shown[49].
In relation to suicide and suicidality, most research has focused on three forms of epigenetic alterations: DNA methylation, microRNA (miRNA) interference and histone modifications[50]. The latter two forms have so far been studied in brain tissue samples from suicide victims, while different areas of DNA methylation have also been studied in samples from other tissues, such as blood. Thus, different potentially important areas of differential methylation are indicated by epigenome-wide methylation studies, methylation studies of regions regulating genes important for the hypothalamic-pituitary-suprarenal axis (HPA axis), the neurotropic system and the serotonin system[50]. Most research supports the link between adverse early experiences, methylation of the NR3C1 glucocorticoid receptor gene and suicidality[50]. However, other HPA-related genes (SKA2, CRH, CRHBP, CRHR1 and CRHR2) have also been described, in particular their methylation changes associated with adverse early experiences[50]. In addition, there is increasing evidence of an association between suicidality and hypermethylation of the BDNF gene in blood samples from both depressed and non-depressed subjects. The association is thought to be independent of the Val66Met polymorphism of this gene[51-54].
The possible influence of epigenetic modifications of the BDNF receptor tyrosine kinase 2 (NTRK2) gene on suicidality is also being investigated, but so far only in post-mortem brain tissue samples[50].
Methylation of several genes in the serotonin system has also been a focus of research into the aetiology of suicidality. A combination of short allele of the serotonin receptor gene (HTR2A) with a history of adverse early life experiences has shown an association with attempted suicide, but subsequent methylation studies have had mixed results[50]. Methylation studies of the tryptophan hydroxylase 2 (TPH2) gene have shown more associations[55].

There is even less research on the epigenetics of NSSI. In a sample of adults with borderline personality disorder, the methylation status of the promoter region of the glucocorticoid receptor gene (NR3C1, exon 1 F) was found to be positively associated with both a history of childhood abuse and the level of self-injurious behaviour[56]. When comparing 15 depressed adolescents of both sexes with NSSI and healthy controls, it was shown that the patients had a higher level of methylation of the promoter region of the SIRT1 gene (at the cytosine-guanine dinucleotide 5 (CpG5) site)[57]. Similarly, abnormal methylation of the promoter region of the proopiomelanocortin (POMC) gene (CpG1 methylation) was shown in depressed adolescents with NSSI[58]. This last study was also performed in a total of 30 adolescents (15 patients and 15 controls). According to our knowledge, no studies evaluating other epigenetic markers of self-injurious behaviour or studies conducted on larger samples have been published so far.

Interplay of protective and risk factors

Several researchers have proposed models according to which suicidal behaviour is the result of an interaction between risk and protective factors. In a longitudinal study of over 1000 young people followed for 29 years, Fergusson and colleagues identified a series of factors that amplified or attenuated the risk of suicidal behaviour in young people with depression. These were family history of suicidal behaviour, childhood sexual abuse, neuroticism, desire to explore and try new things, self-esteem and connectedness with peers. Thus, a positive configuration of these factors (e.g. absence of childhood sexual abuse and family history of suicidal behaviour, low levels of neuroticism, desire to explore and try new things, good self-esteem and absence of deviant peer group) reduced the risk of attempting suicide, while a negative configuration of these factors increased the risk of suicidal behaviour[72]. One model of suicidal behaviour in adolescents is similarly described by Bridge et al (Figure 1)[6]. According to this model, the risk of suicidal behaviour is increased if the adolescent has a mood disorder and impulsivity/aggression. The risk factors or predictors of a higher risk of mood disorder and impulsive behaviour in later puberty may already be present in the pre-pubertal period. The onset of depression in an adolescent poses a risk of suicidal behaviour, which, with additional triggers and stressors and in the absence of protective factors, intensifies and escalates to suicide (Figure 1)[6].

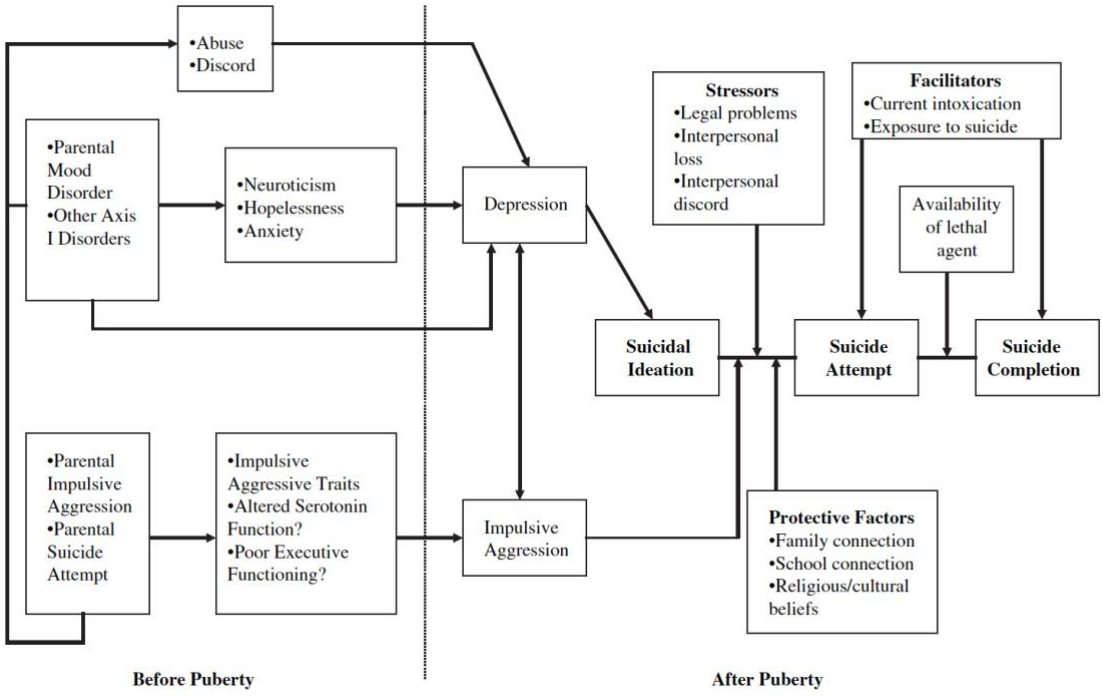

Figure 1: Developmental-transactional model of suicidal behaviour in young people (from Bridge et al. 2006)[6].

3. Detailed description of the work programme

3.1. Content and work programme
The study will include a sample of approximately 200 young people who will be hospitalised for suicide risk (the most at risk in Slovenia) and an approximately equal number of healthy adolescents. At enrolment, we will assess the presence of several factors by reviewing demographic data, clinical diagnosis, self-assessment questionnaires, social assessment, clinical psychological tests, rating scales and blood sampling for genetic analyses. We will longitudinally track autoaggressive events and heteroaggressive events in the patients during hospitalisation and keep a running record of them. We will compare the risk and protective factors of the most at-risk adolescents with a control group of healthy adolescents. We will also reassess the same factors in the most at-risk adolescents after 6 and 18 months of treatment. A detailed description of the study design is given in Figure 2 and Table 2.

3.2. Subjects: Subjects with inclusion and exclusion criteria are described in Table 2 (WP2).

3.3. Methods (Detailed description):

3.3.1. Questionnaires and psychodiagnostic tools:
a. *Enrollment questionnaire, General questionnaire1, General questionnaire2, General questionnaire3*: The questionnaires will cover general and demographic data, information on previous treatments, medication received, mental disorder at discharge (ICD), psychosocial situations (ICD), physical illnesses (ICD), COVID status (vaccinated, recovered, tested), use of psychoactive substances, history of self-harm, suicide attempts, school performance, experience with peers, sexual orientation and identity, residence, family composition, family history of mental disorders, self-harm and suicide attempts.
b. *Columbia Suicide Severity Rating Scale (CSSRS):* The questionnaire is scientifically supported, has the most evidence of utility and efficacy, and is internationally accepted. It has been translated into more than 100 different languages, including Slovene. It is easy to use, suitable for all age groups and adapted for successful use outside the hospital setting, for example in schools, colleges, police, military and elsewhere. It contains 2 screening questions on suicidality and 4 more specific questions, 6 items in total[75].
c. *The Brief Non-Suicidal Self-Injury Assessment Tool (B-NSSI-AT):* used for research purposes to assess the core characteristics of NSSI (form, frequency, function) as well as the secondary characteristics of NSSI (habituation, context of NSSI, perceived impact on life and treatment)[76].
d. *Personality Assessment Inventory-Adolescent (PAI-A)* (https://www.center-pds.si/Katalogtestov/Kliničnitesti/Vprašalnikzaocenoosebnosti-oblikazamladostnike-PAI-A.aspx): an objective-type self-assessment questionnaire for assessing personality in adolescents. It has 264 items to be answered on a 4-point scale. 22 independent scales are obtained (Inconsistency, Rarity, Negative impression, Positive impression, Physical complaints, Anxiety, Anxiety-related disorders, Depressiveness, Mania, Paranoid, Schizophrenia, Borderline traits, Antisocial traits, Alcohol problems, Drug problems, Aggressiveness, Suicidal ideation, Stress, Lack of support, Refusal to deal, Dominance, Warmth).
e. *Trauma Symptom Checklist for Children (TSCC)* (https://www.center-pds.si/Katalogtestov/Kliničnitesti/Vprašalnikotravmatiziranostiotrokinmladostnik. aspx): The questionnaire helps to assess children's/adolescents' experiences of various traumatic experiences, such as physical or sexual violence, peer violence, loss, witnessing violent acts, natural disasters, etc. The questionnaire consists of 6 clinical scales (Anxiety, Depression, Anger, Post-traumatic Stress Symptom, Dissociativeness (two subscales), Sexual Concerns) and 2 validity scales. 6 clinical scales (Anxiety, Depressiveness, Anger, Post-traumatic Stress Symptom, Dissociativeness (two subscales), and 2 validity scales) are included.
f. *Experiences in Close Relationships-Relationship Structures (ECR-RS)*[77,78]: This questionnaire assesses the pattern of attachment to attachment figures (friend, romantic partner, mother, father) in adults and adolescents. The questionnaire has been officially translated into Slovene and used with several samples of Slovene adults and adolescents.

g. *Dynamic Appraisal of Situational Aggression-Youth Version (DASA-YV):* provides a daily assessment of the risk of heteroaggression. The evaluation is efficient and takes less than five minutes. The nurse in charge of each patient completes the DASA-YV once a day[79].
h. *Adolescent Self-Harm Risk Scale (ASHRS):* this is the scale we developed for the present study. It will be used to assess and predict the short-term risk of self-harm behaviour during hospitalisation (assessment at admission) and the long-term risk after discharge (assessment at discharge).
i. *The Level of Personality Functioning Scale-Brief Form 2.0; LPFSBF 2.0:* is a brief, user-friendly instrument that provides a quick impression of the expression of personality pathology. It consists of 12 items classified into two higher-order domains: self-functioning and interpersonal functioning. Participants are asked to rate the 12 items on a four-point Likert scale ranging from 1 (completely false) to 4 (completely true). A total score (sum of all items), a self-functioning score (sum of items 1-6) and an interpersonal functioning score (sum of items 7-12) can be calculated. Satisfactory internal consistency and promising construct validity have been demonstrated. Sensitivity to change after three months of treatment was high[80]. The questionnaire has already been translated for use in the Slovenian population.
j. *Lifetime Incidence of Traumatic Events questionnaire (LITE):* Is a short checklist for screening and assessing the exposure to trauma in children and adolescents. It covers a broad range of potentially upsetting situations that can cause trauma to children and adolescents, such as a car accident, fire, death of a family member, exposure to threats, sexual assault, or witnessing violence. The questionnaire had been validated on a Slovene population of children and adolesscents[81, 82].

k. *Borderline Personality Features Scale (BPFSC-11):* The BPFS-C-11 includes BPD indicators such as affective instability, identity problems and negative attitudes. Responses to the items are on a 5-point Likert scale ranging from 'not at all true' to 'always true'. Studies have shown construct validity of interpretations of BPFS-C-11 scores through positive associations with other measures of BPD and positive associations with measures of BPD correlates, including emotional dysregulation. In a recent sample, the Cronbach's α over 4 years of follow-up was 0.86, 0.85, 0.86 and 0.90, respectively. The scale has been officially translated into Slovenian and will be used with the permission of the authors. It is intended for use by children and adolescents aged 9-11 years and consists of 11 items[83].

l. *Inventory Of Statements About Self-Injury (ISAS):* A self-assessment questionnaire used for research purposes to assess the basic characteristics of NSSI (form, frequency, function, time to event). It also assesses the desire to stop. The questionnaire has been previously translated into Slovene and used in a population of adolescents with self-injurious behaviour[84].

3.3.2. Genetic methods:
DNA isolation: The genomic DNA is going to be isolated according to the established laboratory protocol using FlexGene DNA kit (Qiagen Germany) and 5 mL EDTA-blood sample. Isolated DNA will be stored at 4°C.
Sequencing: Sequencing will be performed on available in-house long-read sequencing technology nanopore PromethION (Oxford Nanopore Technologies). The ability to detect DNA modifications via differences in the electric current intensity produced from a nanopore read of an unmodified base and that of a modified base, enables the detection of DNA methylation in different CpGs from human natural DNA at epigenome-wide level.
Libraries will be prepared using ligation sequencing kit (SQK-LSK 109, Oxford Nanopore Technologies) in following stages: 1. NEBNext Ultra II End repair / dA-tailing, 2. ligation of the native barcodes, 3. ligation of sequencing adapters, 4. flow cell priming and DNA library loading (Flow Cell Priming Kit, Oxford Nanopore Technologies).

The analysis will be conducted using barcoded participants' DNA, enabling the simultaneous analysis of multiple participant's, by pooling the equimolar amount of DNA into a single pool, based on the

observation groups. The sample pooling will reduce the effect of individual epigenome variability and enrich the cohort-specific epigenetic signals, potentially associated with the clinical phenotype.

Bioinformatics
Base Calling: From the raw sequencing signal, the nucleotide sequences will be determined using the software tool Guppy[85], which is based on a predictive neural network model. We will store the identified reads and their reliability in FASTQ files. For subsequent alignments of long reads, we will use the human genome (GRCh38) as a reference.
Variant Calling: Megalodon software (Oxford Nanopore Technologies) will be utilized for determining nucleotide sequences from the raw signal, alignment of readings on human reference genome and variant calling. The reliability of the findings will be additionally confirmed using the software tool Medaka (Oxford Nanopore Technologies). The tool uses a neural network model to identify genetic variants, based on constructed consensus sequences. Variant data will be stored in VCF files.
Quantification and evaluation of short tandem repetitions: Tandem-genotypes[86] and STRique[87] will be used to detect changes in length of tandem repeats, from aligned long DNA reads. In addition to the aligned reads input, the software tool STRique accepts the raw sequencing signal, based on which a hidden Markov model quantifies and evaluates the likelihood of a change. The validated changes in length of tandem repeats will be stored in TSV files.
Methylation sites detection: Different methylation-calling tools will be utilized using the METEORE software tool[88]. The reliability of the methylated CpG islands found in the results will be demonstrated by the prediction models (random forest and multiple linear regression). Additionally, we will compare the detected methylated sites with the results of the Remora software (Oxford Nanopore Technologies). The locations of reliable methylation sites will be stored in BED and TSV files.

3.4. Conduct of the research »Influences on and prevention of self-harm behaviour among the most at-risk adolescents«

The detailed survey process is described in Figure 2 and Table 2.

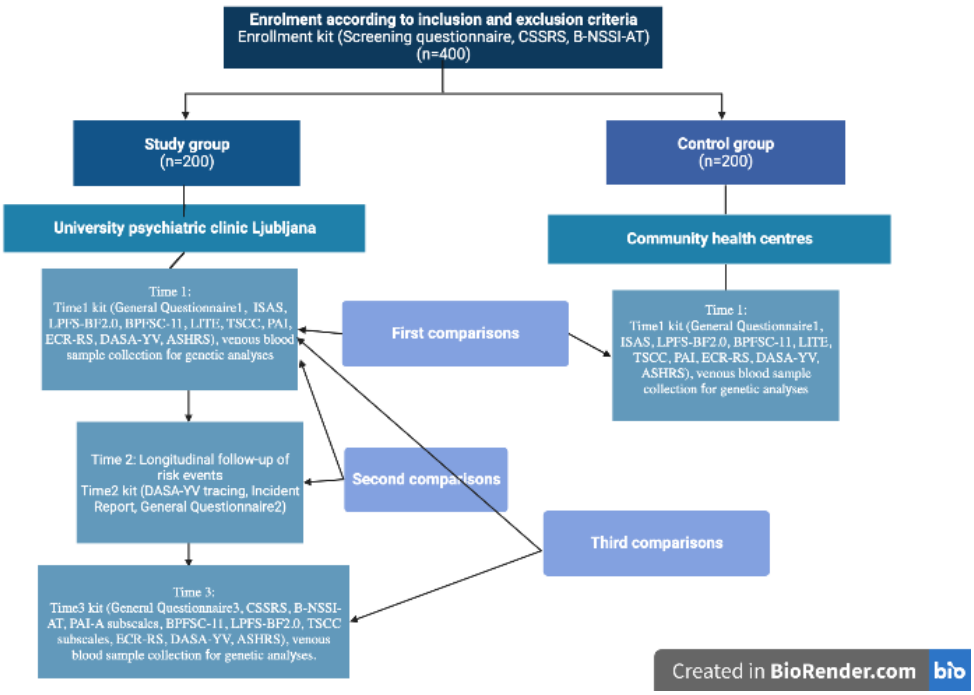

Figure 2. Research flow diagram

Table 2. Detailed definition of the work packages of the research "Influences on and prevention of self-harm behaviour among the most at-risk adolescents".

WP 1: Establishment of a working research module on "Influences on and prevention of self-harm behaviour among the most at-risk adolescents". **Lead organisation:** University of Ljubljana, Faculty of Medicine and Centre for Mental Health, University Psychiatric Clinic Ljubljana (UPKL) **Participating organisations:** the Clinical Institute of Special Laboratory Diagnostics of the Paediatric Clinic of the UKC Ljubljana (KISLD) and the Faculty of Electrical Engineering, Computer Science and Informatics of the University of Maribor (FERI)
Objectives: The first objective of this work package is to prepare instruments for the assessment of the initial condition of hospitalised patients and adolescents from the control group (translation of some questionnaires, purchase of questionnaires), to prepare a detailed protocol for the inclusion of subjects and for the acquisition, storage and analysis of the data (UPKL in collaboration with FERI and KISLD). The second objective is to prepare a GDPR-compliant data entry and storage system in which all the data obtained will be stored securely, anonymised and on an ongoing basis (FERI in collaboration with UPKL and KISLD). The third objective is to train a working group that will include the subjects and capture the data (UPKL in cooperation with FERI and KISLD).
Tasks: **T1: Preparation of work kits.** The PAI and TSCC questionnaires are routinely used in clinical practice in UPKL, the evaluation kits for the study will be purchased from the Centre for Diagnostic Resources, the ECR-RS, CSSRS, ISAS and LITE are already officially translated into Slovenian and freely available, as well as their evaluation method. All questionnaires have been used several times in Slovenian populations and published. The B-NSSI-AT, LPFSBF 2.0 and BPFSC-11 were officially translated and approved by the authors as well as their use in the study (UPKL). The DASA-YV scale has already been officially translated into Slovene and has been in regular use for two years at UPKL. The ASHRS has been prepared and will be validated for the first time during the course of the study. Systematic monitoring of commited and prevented incidents during hospitalisation is a part of the regular process of inpatient follow-up. A General Questionnaire1 was be developed to capture all remaining data at admission (illnesses, medications, diagnoses, psychosocial situations, COVID status, ...), a General Questionnaire2 to capture relevant data at discharge (medications, discharge diagnoses, psychosocial situations, interventions used, etc.) and a General Questionnaire3 to capture relevant data 6 and 18 months after discharge. At this stage, all questionnaires were prepared in sets with the corresponding survey numbers. a. *Enrollment kit* (Screening questionnaire, CSSRS, B-NSSI-AT) b. *Time1 kit* (General Questionnaire1, PAI-A, TSCC, ECR-RS, DASA-YV, ASHRS, LPFSBF 2.0, LITE, BPFSC-11, ISAS), venous blood sample collection for genetic analyses c. *Time2 kit* (DASA-YV tracing, Incident Report (commited and prevented), General Questionnaire2) d. *Time3 kit* (General Questionnaire3, CSSRS, B-NSSI-AT, PAI-A subscales, TSCC subscales, ECR-RS, DASA-YV, ASHRS, LPFSBF 2.0, LITE, BPFSC-11), venous blood sample collection for genetic analyses. **T2: Development of a protocol for subject enrolment and data acquisition, storage and analysis.** This work included the preparation of information materials for subjects and parents/guardians, informed consents/assents and a detailed protocol that will be accessible to the investigators throughout the study. **T3: Preparation of the data entry and storage system:** The data entry and storage information system will be prepared so that it will enable data entry via pre-prepared templates for initial and intermediate reporting of measurements. The system will ensure data privacy, as access will be restricted to anonymized data and allowed only for the purposes of analysis (in line with the General Data Protection Regulation (EU) 2016/679 (GDPR)). The system will ensure that data is safely deleted after the project is completed (according to the GDPR). The information system will be physically located in the Republic of Slovenia, and physical access to the hardware will be limited only to administrative activities by qualified personnel. **T4: Education and training of the working group:** This part will consist of several educational sessions for the different members of the working group: a. Patient involvement, involvement of pupils and students of the control group (assessment of the fulfilment of inclusion/exclusion criteria, information about the study, obtaining consent from adolescents and relatives), b. Collection of questionnaire responses, c. Collection of biological samples, d. Data collection during hospitalisation, e. Follow-up. Initial training sessions will be followed by more frequent follow-up of the study processess, possible complications, necessary adjustments of the protocol according to the initial experience (project manager Maja Drobnič Radobuljac) and re-training if necessary.
Results: **R1: Work kits prepared and multiplied.** **R2: Detailed protocol prepared.** **R3: Data entry and storage system in place.**

R4: Working group prepared.
R5: Report on preparation of materials and working group.

WP 2: Study and control group recruitment, data collection, patient follow-up.
Lead organisation: the Centre for Mental Health, University Psychiatric Clinic Ljubljana (UPKL)
Participating organisations: KISLD and FERI

Objectives:

In this work package we will focus on the involvement of patients and healthy adolescents. The work package includes capturing all patient data at Time 1 and tracking patients during hospitalization until discharge (Time 2) and recapturing data at Time 3 (Figure 2). On the control group side, we will only capture data at Time 1 (Figure 2). This work package has three main objectives: enrollment of hospitalized and healthy adolescents, data collection and follow-up.

Tasks:

T1: Enrollment of hospitalised adolescents. All patients hospitalized in the Intensive Child and Adolescent Psychiatry Unit or the Unit for Adolescent Psychiatry of the Centre for Mental Health UPKL, aged between 13 and 19 years, who will be assessed on admission as suicidal (CSSRS scale) or self-harm with no intention to die (B-NSSI-AT) will be invited to participate in the study. In the case of consent, parents will also be asked for consent for those under 15 years of age. The *exclusion criteria* are confirmed acute psychotic disorder or intellectual disability, severe physical illness (such as cardiovascular or renal disease) or disease of the central nervous system (such as encephalitis, brain injury or haemorrhage, epilepsy), acute poisoning (including with psychoactive substances). The Enrollment Kit and the Time1 Kit will be administered (see Figure 2), and the Time2 Kit will be administered during hospitalisation and at discharge.

T2: Enrollment of healthy adolescents (control group). The control group will include 8th grade students in primary school and 1st year students in secondary school and 1st year students in college, aged 13-19 years, who will attend a regular systematic review. These scheduled regular systematic examinations are accompanied by venous blood sampling. The exclusion criteria will be suicidality (CSSRS scale) or self-injurious behaviour (B-NSSI-AT), a known mental disorder (e.g. depression, bipolar disorder, schizophrenia, mental retardation), severe physical illness (e.g. cardiovascular or renal disease) or disease of the central nervous system (e.g. encephalitis, brain injury or haemorrhage, epilepsy), acute intoxication (including psychoactive substances), and mental disorder, history of suicidality or self-injurious behaviour in a first-degree relative (a parent or a sibling). The Enrollment Kit and the Time Kit1 will be administered (see Figure 2).

T3: Data collection. Data from hospitalised patients will be collected by administering the Enrollment, Time1, Time2 kits (see Figure 2). Adolescents in the control group will have the Enrollment and Time1 kits administered immediately on enrollment, no other data will be collected from these adolescents (see Figure 2). In the event that any of the adolescents approached appeared to be in need of help for self-injurious behaviour or other overt mental disorders, they will be referred for appropriate treatment.

T4: Tracking. Six and 18 months after the end of the hospitalization, we will contact the patients again and administer the Time3 kit (see Figure 2). If any of the adolescents we approach appear to need help for self-injurious behaviour or other overt mental disorders, we will refer them for appropriate treatment.

Results:

R1: Recruited group of subjects.
R2: Recruited control group.
R3: Data collected.
R4: Collected data of the follow-up phase after 6 months.
R5: Report on the characteristics of the treatment and control groups.

WP 3: Analysis of extracted materials, data entry and analysis
Lead organisation: the Centre for Mental Health, University Psychiatric Clinic Ljubljana (UPKL), KISLD and FERI
Participating organisations: UPKL, KISLD and FERI

Objectives:

The first objective is to identify the factors influencing the occurrence of the most threatening forms of self-injurious behaviour in adolescents. A large number of studies have identified both protective and risk factors and their impact, but these are more common in adults, there are fewer studies that distinguish between suicidal behaviour and NSSI, and very few that include newer epigenetic methods. No study has included such a wide range of factors and epigenetic influence.

The second aim is to assess the factors that influence change in risk of self-harm behaviour in the most at-risk adolescents.

The third objective is to develop an instrument (scale) for rapid assessment of short- and medium-term risk of self-harm in adolescents (ASHRS). There are instruments that predict the risk of heteroaggressive behaviour in secure psychiatric wards for adolescents quite reliably, and one of them is used clinically on a daily basis for this purpose (DASA-YV). However, the DASA-YV scale appears to have poor predictive value for self-injurious behaviour in inpatient adolescents (Pintar Babič et al, in press). In order to provide greater safety to hospitalised patients during their care, we aim to develop a scale that is quick and practical for clinical use and that sufficiently assesses the risk of self-harm during hospitalisation. This assessment could be used for adapting the level of protection of the hospitalized adolescent during treatment, thus preventing short and long term health consequences.

Tasks:

T1: **Evaluation of questionnaires.** The questionnaires will be evaluated on an ongoing basis by a trained research associate within UKC according to the manufacturers' instructions.

T2: **Analysis of peripheral blood samples.** Peripheral blood will be drawn from the cubital vein from patients and control group subjects at scheduled routine blood draws (patients at admission, and control group at routine systematic review Time 1), and patients with consents at the 6 and 18-month follow-up (Time 3). The methods of DNA isolation and genetic analysis are presented in Section 3.3.2.

T3: **Data entry into the database.** Data will be entered on an ongoing basis by trained researchers at UPKL and KISLD.

T4: **Data analysis.** The data will be analysed in two phases - a review of the data after 1 year of the study on an estimated 100 subjects and 100 controls, and an analysis of the data from all enrolled subjects. The data will be analyzed as follows.

(1) Analysis with linear regression models, where all measurements after the first period will be taken into account as independent variables (demographic data, genetic data, and results of all solved questionnaires); the dependent variables will be related to the recorded and prevented self-harm behavior and to the results for a rapid assessment of the short- and medium-term risk of self-harm in adolescents (ASHRS). Several models will be created in the analysis, where several combinations of interactions and transformations of independent variables will be considered. The results of this analysis will give us the answer to which factors impact self-injurious behavior.

(2) Survival analysis, which will examine the impact of independent variables (same as in (1)) on the survival rate of the subjects. In this analysis, the fail-to-survive will be considered as any self-injurious behavior. The results of the analysis will give us answers to which factors lead to earlier / later fail-to-survive events and the percentage of self-injurious behavior in connection with the factors.

(3) Cluster analysis will be examined if there are typical groups of subjects with the same behavior and other factors (according to the previously listed independent variables). The analyzes from (1) and (2) will be repeated separately on the found clusters to answer the question of whether the samples from the regression models (1) and the survival analysis models (2) differ according to the cluster of subjects.

Results:

R1: Questionnaires evaluated.

R2: Genetic and biochemical analyses performed.

R3: Data entered into data entry and storage system.

R4: Results of data analyses.

R5: Report on the work carried out.

WP 4: Project coordination, dissemination of findings/results, preparation of treatment guidelines.

Lead organisation: the Centre for Mental Health, University Psychiatric Clinic Ljubljana (UPKL),

Participating organisations: KISLD and FERI

Objectives:

The objectives of this WP are to manage and supervise the project, to disseminate new findings in relation to the set objectives, which will be derived from the project results and, based on these, to prepare guidelines/recommendations for the treatment of adolescents who are most at risk of self-harm.

Tasks:

T1: Project coordination. The tasks of the project coordinator with a detailed implementation plan and timetable are described under point 23.5.

T2: Monitoring the progress of the research and the appropriateness of the protocol and methodology. The progress of the research will be monitored through regular monthly meetings and reviews of the participants and their data collected. The monitoring and reviews will be carried out by the study director (Prof. Dr. Maja Drobnič Radobuljac, MD, PhD, Child and Adolescent Psychiatrist) and the study collaborators (Anja Tomaševič Kramer, MD, Child and Adolescent Psychiatrist, PhD student; Barbara Plemeniti Tololeski, MD, Child and Adolescent Psychiatrist, and Lana Podnar Sernec, resident in Child and Adolescent Psychiatry, PhD student), and by the study collaborators. In case there are problems with the inclusion of adolescents or the application of any of the kits, we will adjust the methodology and inform the researchers involved.

T3: Dissemination of findings/results. The results will be presented at several scientific and professional congresses of child and adolescent psychiatry, psychiatry and suicidology abroad (ESCAP, IACAPAP, DEAPS, ECNP,), at national meetings (ZOMP, ZP SZD, ...), published in open access scientific journals in these fields, indexed in SCI, SSCI.

T4: Preparation of guidelines and recommendations. Based on the findings, reliability assessment of the newly developed scale, we will prepare new recommendations for the inpatient management of adolescents hospitalized in secure psychiatric wards and adolescents after the hospitalization.

Results

R1: Project coordinated, ongoing and final report.

R2: Smooth running of the study, all complications anticipated or resolved immediately on occurrence, report on any protocol changes prepared.

R3: Presentation of results in scientific papers and at conferences.

R4: Guidelines/recommendations prepared.

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