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MOLECULAR MECHANISMS OF POSTTRAUMATIC STRESS DISORDER (PTSD) AS A BASIS FOR INDIVIDUALIZED AND PERSONALIZED THERAPY: RATIONALE, DESIGN AND METHODS OF THE SOUTH EASTERN EUROPE (SEE)-PTSD STUDY

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SUMMARY

Posttraumatic Stress Disorder (PTSD) is a major health problem in South Eastern Europe (SEE). Available treatment options are not efficient enough and the course is often chronic. Little is known about molecular mediators and moderators of pathogenesis and therapy. Genetic and epigenetic variation may be one central molecular mechanism.

We therefore established a consortium combining clinical expertise on PTSD from SEE countries Bosnia-Herzegovina (Sarajevo, Tuzla and Mostar), Kosovo (Prishtina) and Croatia (Zagreb) with genetic and epigenetic competence from Germany (Würzburg) in 2011 within the framework of the DAAD (Deutscher Akademischer Austauschdienst)-funded Stability Pact for South Eastern Europe.

After obtaining ethical votes and performing rater trainings as well as training in DNA extraction from EDTA blood between 2011 and 2013, we recruited 747 individuals who had experienced war-related trauma in the SEE conflicts between 1991 and 1999. 236 participants had current PTSD, 161 lifetime PTSD and 350 did not have and never had PTSD.

Demographic and clinical data are currently merged together with genetic and epigenetic data in a single database to allow for a comprehensive analysis of the role of genetic and epigenetic variation in the pathogenesis and therapy of PTSD. Analyses will be done to a great degree by PhD students from participating SEE centers who in addition to participation in the project had an opportunity to take part in spring and summer schools of the DFG (Deutsche Forschungsgemeinschaft) funded Research Training Group (RTG) 1253 and thus meet PhD students from Germany and other countries

We are confident that our project will not only contribute to a better understanding of genetic and epigenetic mechanisms of PTSD as a basis for future individualized and personalized therapies, but also to the academic development of South Eastern Europe.

Key words: *PTSD - South Eastern Europe – genetics - epigenetics - molecular mechanisms - individualized therapy - personalized therapy*

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INTRODUCTION

Background

Posttraumatic stress disorder (PTSD) is a stress-related disorder characterized by symptoms of re-experiencing the trauma such as flash-backs, intrusions or nightmares, avoidance behavior and hyperarousal persisting for more than 1 month post experiencing or witnessing extreme traumatic events involving actual or perceived threat of death or serious injury or threat to one's physical integrity (American Psychiatric Association 2000).

While the lifetime prevalence of PTSD has been estimated at 7% in the US general population (Kessler et al. 2005), it is estimated to be as high as 35% in

people who experienced the war in Bosnia and Herzegovina and 25 % in people who experienced the war in Kosovo (Priebe et al. 2010, Lopes Cardozo et al. 2003). The longitudinal course of PTSD is variable. Approximately 50% of persons diagnosed with PTSD will recover, and the remaining 50% will develop a chronic form of this disorder. Forty percent of these patients will remain symptomatic after 10 years and ten percent after 30 years (Kearns et al. 2012, Koenen et al. 2008, Kessler et al. 2005). In addition to the psychological distress, severe somatic disorders may be a long-term consequence (Dzubur-Kulenovic et al. 2008).

Thus, there is a definite need to develop new therapies for the treatment of PTSD. One approach is research on the pathogeneses of PTSD to improve our understanding of pathways leading to the disorder as a basis for the development of novel therapeutic approaches (Jakovljevic et al. 2012a). Elucidation of these pathophysiological mechanisms can help us in tackling the everlasting question why some individuals develop and some do not develop PTSD following exposure to potentially psychotraumatic events? In other words, which intrapsychic and neurobiological phenomena serve as vulnerability, and perhaps resilience and personal growth factors, following traumatic experiences? (Jakovljevic et al. 2012b).

The etiology of posttraumatic stress disorder (PTSD) is considered to be multifactorial with an interaction of - obviously - traumatic environmental factors as well as genetic factors (Agani et al. 2010, Breslau & Kessler 2001, Kessler et al. 1995). Our knowledge of the clinical and molecular genetic underpinnings of PTSD, including gene-environment interactions and temporally dynamic epigenetic mechanisms as potential mechanistic correlates of environmental influences has recently been reviewed in this and other journals (Zannas et al. 2015, DiGangi et al. 2013, Domschke 2012 Mehta & Binder 2012, Cornelis et al. 2010, Koenen et al. 2009). Given recent methodological advances in particular with regard to epigenetic approaches, research in this field offers itself as a promising venue. The study of epigenetic mechanisms will not only allow to study individuals as defined by their individual genetic variation, but persons defined as individuals with their specific life history documented by their epigenetic variation. An increase in knowledge on genetic and in particular epigenetic mechanisms mediating the effects of trauma may thus provide starting points for the development of individually and even personally tailored therapies.

During a meeting funded by the DAAD Stability Pact for South Eastern Europe in Zagreb in 2011, A. Dzubur-Kulenovic, M. Jakovljevic, K. Domschke and J. Deckert decided to make an effort to design a study on molecular mechanisms of PTSD and apply for funds to the DAAD Stability Pact for South Eastern Europe.

Aims

The study was intended to achieve two aims:

First, given a beneficial effect of MAO-A inhibitors in the pharmacological treatment of PTSD, our previous studies showing association of the more active MAO-A VNTR alleles as well as *MAO-A* gene hypomethylation with panic disorder (Deckert et al. 1999, Domschke et al. 2012) and evidence that not only *MAO-A* gene variation (Reif et al. 2014, Domschke et al. 2008), but even more so in women *MAO-A* methylation status mediates therapeutic success (Ziegler et al. 2016, Domschke et al. 2015), the present project for the first time aimed at an integrative investigation of the role of *MAO-A* related (epi-)genetic variation in the pathogenesis of PTSD and/ or the biological mediation of traumatic events. Furthermore, several other candidate genes of anxiety and depression will be investigated for their genetic/epigenetic impact on PTSD. These include among others the *SLC6A4* gene (Domschke et al. 2014, Koenen et al. 2011, Xie et al. 2011, Wang et al. 2011, Bryant et al. 2010, Kolassa et al. 2010a, Mellmann et al. 2009, Grabe et al. 2009, Koenen et al. 2009, Thakur et al. 2009, Kilpatrick et al. 2007, Lee et al. 2005) and the *FKBP5* gene (Mehta et al. 2011, Xie et al. 2010, Binder et al. 2008).

This comprehensive molecular genetic program is made possible by using synergies with the DFG-funded Collaborative Research Centre CRC-TRR58, projects C02 and Z02.

Second, the present project aims to strengthen the research capacities in participating centers by providing an opportunity for young scientists to obtain methodological and laboratory skills that will improve their further work. This is being achieved through mentorship and study stays in Würzburg in association with the DFG-funded Research Training Group 1253 and educational meetings at the centers from South Eastern Europe as well as through encouraging mutual cooperation between the South Eastern Europe research centers.

SUBJECTS AND METHODS

Ethical votes

Ethical votes at the participating clinical centers were obtained between 2011 and 2013 on the basis of local translations of an information and consent form designed by the Würzburg center.

Participants thus were informed and gave written informed consent according to the principles of the declaration of Helsinki (WMA 2013).

In- and exclusion criteria and psychometric instruments

Inhabitants of South Eastern Europe in research centers in Sarajevo, Prishtina, Tuzla, Zagreb and Mostar

were recruited for the study. Most of them have experienced traumatic events related to war and ethnic cleansing in the wars between 1991 and 1999. Some of them developed PTSD and some did not, and some of those who developed the disorder have later recovered. Also, some of the subjects in the control group were not exposed to trauma. Three groups were defined on the basis of presence or absence of current or lifetime posttraumatic stress disorder (Table 1). Originally, 4 groups with 2 control groups with or without trauma were to be assessed. However, it soon turned out that there were very few individuals in the region who did not experience trauma (n=41 from 350). It was therefore decided to combine the two groups and to perform post-hoc subgroup analyses instead. Assessment scales such as CAPS to determine the presence or absence of current or lifetime PTSD used in the study have been designed based on the diagnostic criteria for PTSD in ICD-10 and DSM IV. DSM 5 criteria for PTSD were adopted by APA in 2013, after the recruitment for our study had already started and adapted scales were not available at that time (American Psychiatric Association 2013). Assessment scales used such as CAPS, the Life Stressor List and the Hofman-Lazarus Coping Scale will allow not only the categorical diagnosis of PTSD, but will also provide information on the severity of PTSD, the severity of trauma and coping style allowing for gene-environment analyses.

Table 1. Three groups of participants were defined depending on the presence of PTSD (according to ICD-10 and DSM IV diagnostic criteria)

Group 1	Group 2	Group 3
Trauma +	Trauma +	Trauma + or -
Current PTSD	Lifetime PTSD	No current or past PTSD („controls“)

At the time of trauma participants were at least 16 years of age and at time of recruitment not older than 65 years of age. The exclusion of subjects who survived trauma in childhood years was based on the frequently described difference in both clinical presentation and course of PTSD and other psychiatric disorders occurring after trauma exposure at young age (Nemeroff 2016). Additional inclusion and exclusion criteria were defined on the basis of clinical criteria and medication (Table 2).

Demographics, clinical history, medication, psychopathology, life events and coping styles were evaluated using standard psychometric instruments in local languages, PTSD-diagnosis being made in accordance with DSM-IV (Table 3). Interviews were performed by medical personnel (psychiatrists, psychologists or psychiatric residents) after rater trainings of the principal investigators by the Sarajevo center at two international coordination meetings and local interviewers by the principal investigators at local meetings.

Table 2. Inclusion and exclusion criteria

Inclusion criteria

- DSM-IV current or life-time PTSD or no PTSD
- ICD-10 diagnosis of enduring personality change after catastrophic experience (F62.0) will be documented as a potentially confounding factor;
- PTSD and traumatization go back to the war period in the respective countries, i.e. in Croatia 1991-1992, Bosnia-Herzegovina 1992-1995 and Kosovo 1998-1999;
- Patients and probands must be at least 16 years of age at time of traumatization.

Exclusion criteria

- Mental retardation (MMSE<25);
- Organic and brain trauma related disorders;
- Epilepsy;
- Psychotic disorders;
- Addiction disorders except smoking;
- Oncological disorders;
- Medication known to affect methylation status, e.g, valproic acid;
- 1st and 2nd degree relation to an already recruited person;
- Patients and probands older than 65 years of age at time of study.

Table 3. Clinical and psychometric evaluation

- Socio-demographic questionnaire incl. smoking status (yes/no current smoker, number of cigarettes/day, duration of smoking);
- General medical history;
- Client Service Receipt Inventory (CSRI – medication, current, dose, duration);
- M.I.N.I. – Screen (Mini International Neuropsychiatric Interview);
- Appropriate section of M.I.N.I 5.0.0;
- MMSE (Mini Mental State Examination);
- Life stressor list, List of traumatic events including frequency and severity of traumatic events;
- CAPS-PTSD (Clinician Administered PTSD Scale);
- BSI (Brief Symptom Inventory);
- Hoffman Lazarus Coping Scale.

Versions of the respective instruments in the local language were used (Dzubur-Kulenovic et al. 2008. Priebe et al. 2010)

EDTA-blood collection and DNA extraction

From all the participants EDTA blood was drawn for genetic and epigenetic analyses and stored at -80°C. DNA extraction was performed at Zagreb (Zagreb samples), Sarajevo (Sarajevo, Tuzla and Mostar samples) and Würzburg (Prishtina samples).

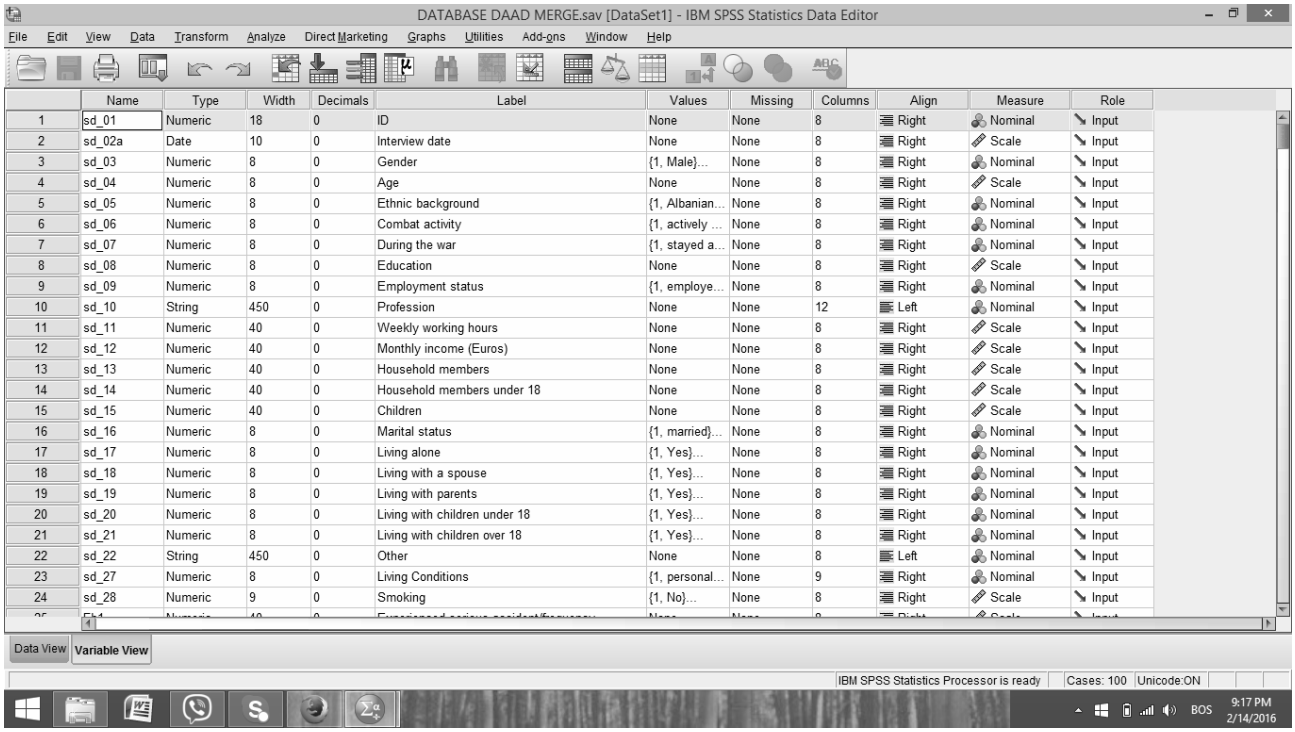

Figure 1. Exemplary screenshot of an excerpt of the SPSS database. 419 demographic and clinical parameters were collected for each participant. They included categorical diagnosis, PTSD severity, trauma severity and coping style

DNA was isolated from human whole blood using FlexiGene DNA Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. In brief, lysis buffer was added to the samples and cell nuclei and mitochondria were pelleted by centrifugation. The pellet was resuspended and incubated in denaturation buffer, containing a chaotropic salt and protease. DNA was precipitated by addition of isopropanol and washed in 70% ethanol. The dried pellet was resuspended in 25mM Tris HCL hydration buffer, pH 7.8. DNA concentration was determined by measuring at 260 and 280 nm (GENios Pro, Tecan, Crailsheim, Germany).

Time of blood drawing, time to storage at -80^{0}C and time of transferal on dry ice to the respective extraction center were documented.

EDTA blood and DNA transport was done on dry ice. The DNA is stored at the Laboratory of Functional Genomics at Würzburg at -80^{0}C.

Database

A SPSS database with the most important clinical variables was designed by the Sarajevo center and disseminated to the other centers (Figure 1).

Participants were pseudonymized and obtained codes 1-200 (Zagreb), 201-400 (Sarajevo), 401-600 (Prishtina), 601-800 (Tuzla) and 801-1000 (Mostar).

The codes are stored at the local centers. Double coding was performed where required by ethical vote.

Demographic and clinical variables were entered at each center by two researchers with random double entry, and sub-databases were merged at Sarajevo center later on. Data freeze was in February 2016.

Genetic and epigenetic data is scheduled to be determined and added by the Würzburg center until June 2016.

A first series of analyses is scheduled to be completed by October 2016. Individual gene-based publications by the PhD students are planned for 2017.

CONSORTIUM AND RESULTS

Consortium

Six psychiatric centers from four countries with principal investigators, collaborators and PhD students committed to take part in the study (Table 4).

The five psychiatric centers involved in the recruitment are located in the countries whose population had experienced severe war-related trauma between 1991 and 1999: Zagreb in Croatia (1991-1992), Sarajevo, Tuzla and Mostar in Bosnia-Herzegovina (1992-1995) and Prishtina in the Republic of Kosovo (1999-1999).

Rater trainings were successfully performed at two international coordination meetings for the principal investigators by the Sarajevo group and at local meetings for collaborators and PhD students by the principal investigators.

Table 4. Members of the SEE-PTSD Study Consortium

Germany	**Bosnia and Herzegovina**
WUERZBURG Principal Investigator: *Jürgen Deckert* Collaborators: *Katharina Domschke* *Bodo Warrings* PhD Student: *Christiane Ziegler*	SARAJEVO Principal Investigator: *Alma Dzubur Kulenovic* Collaborators: *Abdulah Kucukalic* *Alma Bravo Mehmedbasic* *Damir Marjanovic* PhD Students: *Sabina Kucukalic* *Emina Sabic Dzananovic* *Elma Feric Bojic*
Croatia	
ZAGREB Principal Investigator: *Miro Jakovljevic* Collaborators: *Branka Aukst-Margetic* *Dusko Rudan* *Nada Bozina* PhD Students: *Ana Cima Franc* *Nenad Jaksic*	TUZLA Principal Investigator: *Esmina Avdibegovic* Collaborator: *Osman Sinanovic* PhD Students: *Nermina Kravic* *Mirnesa Muminovic*
Kosovo	MOSTAR Principal Investigator: *Dragan Babic* Collaborator: *Marko Pavlovic* PhD Student: *Romana Babic*
PRISHTINA Principal Investigator: *Ferid Agani* Collaborator: *Aferdita Goci Uka* PhD Students: *Shpend Haxhibeqiri* *Valdete Haxhibeqiri* *Blerina Hoxha*	

Eleven SEE PhD students participate in the study and will be able to analyze data for their PhD theses and publications. They took part in annual meetings with German PhD students of the RTG 1253 at Würzburg and at local educational meetings in South East Europe. They received training in DNA extraction from EDTA blood in the Laboratory of Functional Genomics of the Würzburg psychiatric department.

Cohort

Recruitment of patients and probands began in 2013 and was completed by 2015.

The study involved a total of 800 subjects from five sites in three countries (Figure 2).

Out of the total sample, 539 (67%) participants were male, and 261 (33%) female.

A total of 747 participants could be finally included in the study. The studied groups comprise 236 participants (173 males and 63 females) with current PTSD, 161 participants with lifetime PTSD (107 males and 54 females), and 350 participants with no present or past PTSD ("controls", 229 males and 121 females). The number of participants with and without PTSD thus was equal or comparable in the combined sample as well as in most subsamples. The contribution by individual centers was as follows:

Sarajevo: current PTSD n=52 (29 males and 23 females), lifetime PTSD n=50 (28 males and 22 females), controls n=87 (45 males and 42 females).

Prishtina: current PTSD n=38 (18 males and 20 females), lifetime PTSD n=45 (27 males and 18 females), controls n=83 (45 males and 38 females).

Tuzla: current PTSD n=63 (49 males and 14 females), lifetime PTSD n=14 (8 males and 6 females), controls n=76 (53 males and 23 females).

Zagreb: current PTSD n=53 (50 males and 3 females), lifetime PTSD n=22 (19 males and 3 females), controls n=44 (37 males and 7 females).

Mostar: current PTSD n=30 (27 males and 3 females), lifetime PTSD n=30 (25 males and 5 females), controls n=60 (49 males and 11 females).

There were no significant differences with regard to age and gender between groups in the combined sample. Between subsamples there were gender differences due to different populations who had suffered trauma during the war; however care was taken that the control samples were gender-matched.

Experimental program

Variation in several candidate genes including *MAO-A* (Reif et al. 2014, Domschke et al. 2008, Deckert et al. 1999), *SLC6A4* (Domschke et al. 2014, Koenen et al. 2011, Xie et al. 2011, Wang et al. 2011, Bryant et al. 2010, Kolassa et al. 2010a, Mellmann et al. 2009, Grabe et al. 2009, Koenen et al. 2009, Thakur et al. 2009, Kilpatrick et al. 2007, Lee et al. 2005), *TPH2* (Goenjian et al. 2012), *COMT* (Kolassa et al. 2010b, Amstadter et al. 2009), *DAT* (Bailey et al. 2010, Drury et al. 2009, Segmann et al. 2002), *DRD2* (Voisey et al. 2009, Lawford et al. 2006, Lawford et al. 2003), *GAD1* (Weber et al. 2012), *CRHR1* (Amstadter et al. 2011), *FKBP5* (Mehta et al. 2011, Xie et al. 2010, Binder et al. 2008), *OXTR* (Notzon et al. 2016, Feldman et al. 2014), *NPSR1* (Domschke et al. 2011), *BDNF* (Valente et al. 2011), *CNR1* (Lu et al. 2008), *MBP* (Schraut et al. 2014), *RORA* (Logue et al. 2013) and *NPY* (Domschke et al. 2008), as well as methylation status in the *MAO-A* gene (Ziegler et al. 2016, Domschke et al. 2015, Domschke et al. 2012) and other genes such as *FKB5* (Klengel er al. 2013) or *NR3C1* (Vukojevic et al. 2014, Yehuda et al. 2014) will be determined in the Laboratory of Functional Genomics of the Würzburg psychiatric department.

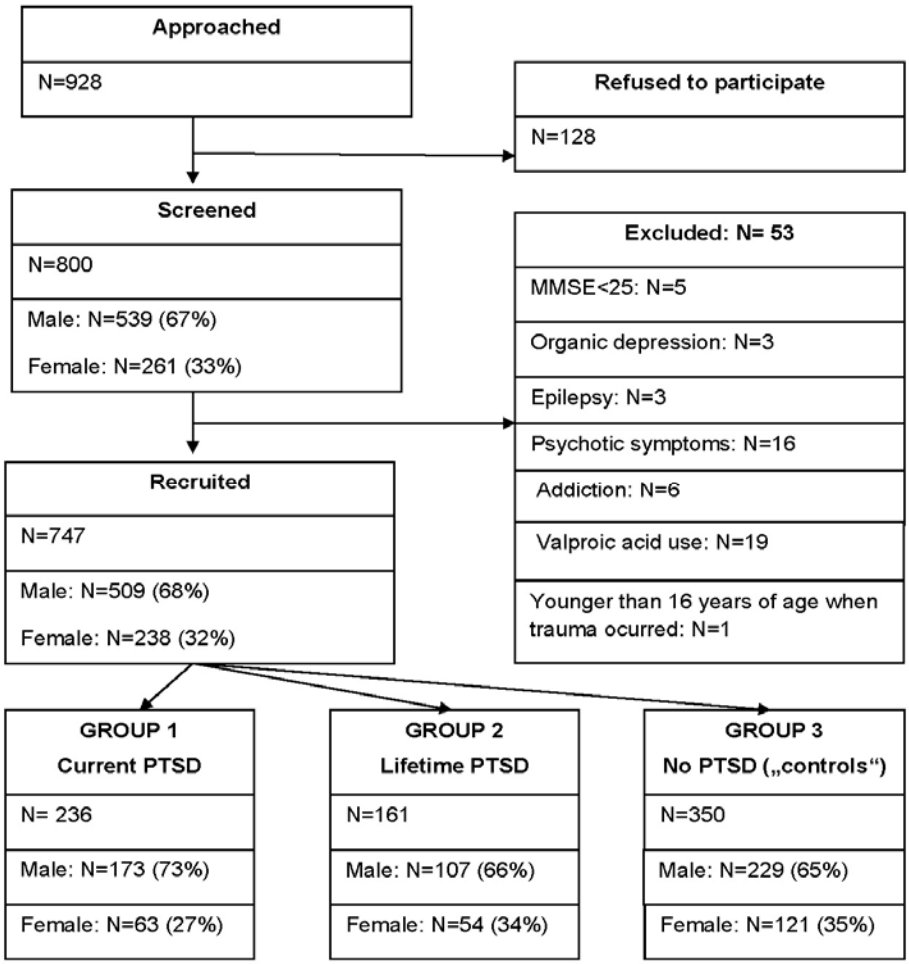

Figure 2. Consort chart of the SEE-PTSD study. Of 928 participants from Bosnia-Herzegowina, Croatia and Kosovo who were approached, 800 could be screened and 747 could finally be included in the study, 350 without PTSD and 397 with either current or lifetime PTSD

The genetic and epigenetic data will be provided to the PhD students and young researchers from South Eastern Europe (3 from Sarajevo, 2 from Tuzla, 1 from Mostar, 2 from Zagreb and 3 from Prishtina) for analysis together with the demographic and clinical data for their PhD thesis or Postdoc Research.

The following analyses are planned:

- A categorical comparison between participants with and without PTSD
- A dimensional comparison of PTSD severity between genotype groups
- Interactions between genotypes and trauma severity on PTSD severity
- Interactions between genotypes and coping style on PTSD severity.

Follow up

Depending on the results, therapeutic intervention studies either using methylation results as a biomarker for psychotherapy need or success or applying a methyl group donor such as S-adenosyl-methionine (SAM) as an adjunct to psychotherapy may be considered.

DISCUSSION

The consequences of war and aggression in the SEE region that took place in the last decade of the 20th century did not only result in heavy loss of human lives and migration but also in the tearing apart of the communal fabric and trust both locally and regionally. This understandably also involves the research community that is suffering from brain-drain and under-resourcing, making it difficult if not impossible for young researchers to embark in serious research as a result of shortages in mentoring and/or funding for translational studies.

This DAAD-funded Stability Pact for South Eastern Europe aimed to overcome this.

In the present project, the following strategies were employed to facilitate scientific collaboration:

With the University of Würzburg an external catalyst gave the impetus to the collaborative project. The design of the study and the methods applied, however, were decided on in a cooperative way during coordination meetings of the consortium with input from every participating center. PhD students from the SEE countries were brought together and met with German and other foreign PhD students during educational meetings and workshops either in the context of the RTG schools or at local meetings. Joint training was provided at several levels for principal investigators as well as PhD students and thus technology was transferred into the SEE region. PhD theses will be supervised by local mentors and mentors from Germany. Data are combined in a joint database which will finally be distributed for data analyses to all centers. A transparent publication policy was developed in a consensual way allowing for publications by every participating center and in particular by the PhD students.

Due to these measures as well as the enthusiasm and idealism in particular of the PhD students and the young researchers over time a highly functional team was built.

Thus, the largest PTSD cohort to date from the region with 397 participants with either current or lifetime PTSD and 350 participants without PTSD, in total 747 participants could be recruited for analyses of molecular mechanism of PTSD.

Still, a lot remains to be done.

The genetic and epigenetic data have to be obtained and integrated into the demographic and clinical database. The complex data have to be analyzed with regard to associations between genetic and epigenetic variation with PTSD as a diagnosis, individual symptoms of PTSD, severity of PTSD, the type and severity of trauma and last not least, the coping styles.

It will be seen if and how different gender distributions, difference in time to trauma and type of trauma due to historical circumstances will affect results across the three countries from South Eastern Europe. Due to the heterogeneity of the control group with regard to trauma, post-hoc analyses will be necessary to compensate this limitation.

With the comprehensive set of psychometric instruments and the genetic and epigenetic data, however, results are expected that will integrate genetic, gene-environment and epigenetic approaches to contribute to a better understanding of the molecular mechanisms of PTSD. This might entail the possibility to further sharpen risk profiles of PTSD-prone individuals and to develop an individually tailored therapy of PTSD based on a genetic and environmental risk factor constellation. Due to the inclusion of epigenetic data mirroring life history such a therapy may justly be called not only individually, but even personally tailored, with the term "person" comprising the genetically defined individuum with its specific life history. Thus, we hope to be one small step closer to answering the everlasting question why some individuals seem to be particularly vulnerable (i.e., and some particularly resilient) in developing PTSD following exposure to potentially psychotraumatic events?

In addition, we are confident that the collaborative SEE-PTSD study will contribute to strengthening research capacities and collaboration in SEE by training and mentoring young PhD students and researchers from the region and bringing them together with PhD students and researchers from Germany and other countries.

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Conflict of interest: None to declare.

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