65781-**Responses to the Reviewers and Editor**

First, we would like to sincerely thank the editor and the reviewers for their time invested to improve the quality of our paper titled **BDNF methylation and mRNA expression in brain and blood of completed suicides in Slovenia**. We addressed all the concerns raised by the reviewers and tried to follow all their suggestions as much as possible.

Detailed responses to comments are given below. Our revision now also includes the revised manuscript in which all the modifications are marked (track changes).

Answers to the Reviewers

Reviewer #1:

1. In the case-control study entitled "BDNF methylation and mRNA expression in brain and blood of completed suicides in Slovenia", the authors explored mRNA levels of different BDNF transcript isoforms and the methylation status of discrete areas of the BDNF gene in an effort to compare two brain areas and blood of suicidal participants with a group of participants who died from cardiac problems. While the value of the analysis is appreciated and the justification by BDNF associations is set by the literature of previous studies, it should be noted that the choice of the control group may determine the findings. What where the control groups used in the cited, previous literature?

   Our response: Thank you for your comment. The choice of control group should be such to not influence the results. Numerous studies (e. g. Ikegame et al., 2013, Kim et al., 2014, Roy et al., 2017) have defined links between epigenetic processes and mental disorders, including for nonfatal suicidal behavior, with DNA methylation established as the most studied mammalian epigenetic mechanism. These studies have shown that *BDNF* methylation in the blood of subjects with mental disorders is usually higher than for that of control subjects. However, studies on *BDNF* methylation specifically in completed suicides are rare. Targeted and whole-genome methylation analyses were used in two studies that showed higher *BDNF* methylation in the brain of suicide victims, compared to controls (Keller et al., 2010, Schneider et al., 2015).

   In the study of Keller et al. (2010) the controls died of two main reasons 'sudden natural death (this is a very broad definition of death with only one limitation – that is death within one hour from the beginning of the symptoms; most common cause of death in this case is due to vascular complications)' and 'transport accident', less common was death due to 'external reasons' and some complication associated with bleeding/tromboemboly. Controls were without psychiatric diagnosis, but two were positive on toxicological analysis for medication on imipramine. In the study of Schneider et al. (2015) the controls died of sudden death (no further explanation was available).

   Fuchikami et al. (2011), Ikegame et al. (2013) included living controls designated as 'healthy controls participated, free of any current or past psychiatric or physical diagnoses and any first-degree relatives with major depression'. In other studies, like Kim et al. (2014), Kang et al. (2013), Roy et al. (2017) they used healthy controls. However, let's take the case of the later study. When the control group was divided according to demographic, e.g. gender and ethnical background the groups became very small, even having only 3 subjects.

In other studies, the approach was longitudinal, and therefore there was no need for controls (Kim et al., 2015, Kang et al., 2018).
Currently, the selection of the control group varies largely among different studies, and therefore one has to take this into consideration when interpreting the results.

2. In addition, and despite the author initial intention, the conclusion reached: "this is the first study that has explored BDNF locus methylation, and the expression of four BDNF transcripts in brain and peripheral blood in the same cohorts" is not considered right. This study could not explore the expression of BDNF transcripts in blood.
   Our response: Thank you for your comment. We changed it into clearer sentence: "This is the first study that aimed to explore BDNF locus methylation, and the expression of four BDNF transcripts in brain and peripheral blood in the same cohorts." Later on in the Conclusions paragraph we stated that "due to the extensively degraded blood RNA we were not able to confirm these effects on mRNA expression".

3. A justification of the areas of the brain to be studied is missing.
   Our response: Thank you for your comment. Our study included two brain areas, Brodmann area 9 and hippocampus. Brodmann area 9 is located in the frontal cortex, more precisely in dorsolateral prefrontal cortex. The dorsolateral prefrontal cortex is an evolutionarily very young acquisition and is involved in maintaining attention, working memory, planning and decision-making, and maintaining self-control.
   Hippocampus is an important part of the limbic system, which is strongly involved in emotion, behavior, motivation and memory. The limbic system is connected by its action to the prefrontal part of the cortex, which contains the previously mentioned Brodmann area 9. The hippocampus is located in the medial temporal lobe of the brain. It is heavily involved in the formation, organization and storage of long-term memory. In addition, it is also important for spatial navigation. It obtains information through various types of neurons, including serotonergic, noradrenergic, GABAergic, and dopaminergic neurons. Both brain areas could therefore be importantly involved in suicidal behavior development.

4. In the discussion the authors mention previous studies evaluated other areas of the brain. Perhaps the inclusion of a summary table, or two, of previous studies exploring BDNF methylation and transcripts in the brain indicating the areas explored and control group types evaluated, and blood, could help with a more reasoned interpretation of their findings.
   Our response: Thank you for your comment and suggestion. However, our article is original research article, and we sincerely feel that proposed additional tables would be more suitable for review type of publication. Therefore, we did not include such tables.

5. Did all blood studies obtain total RNA from whole blood? WBC (white blood cells)? PBMCs (peripheral blood mononuclear cells)?
   Our response: Thank you for your question. Firstly, we would like to add that in many studies of DNA methylation the validation of the results through gene expression (mRNA) is missing (e.g. on post mortem samples Schenider at al. 2015; on living subjects Fuchikami et al. 2011, Ikegame at al. 2013, etc.).
   Actually, among our cited literature only Roy et al. (2017) performed the analysis on both, the RNA and DNA, and they were both extracted from PBMC (Roy et al. 2017). In other studies, (Dwivedi et al., 2003, Kozicz et al. (2008), Pandey et al. (2008)),

where RNA was analyzed, the RNA was extracted from different brain regions and not from blood.
Because the source tissue for DNA methylation is also important, we summed up just for illustration how different are the DNA isolation blood-based tissue samples: Kange et al (2018) and Kim et al. (2014) used the leukocytes, as already mentioned Roy et al. (2017) used PBMCs, while many others used venous/whole blood (Ikegame et al., 2013, Fuchikami et al., 2011, Kang et al., 2013, Kim et al. 2015, or Xu et al., 2016).

6. Table 1, only one p-value is shown, but foot legend indicates 2.
Our response: Thank you for your comment. Only one value remained significant after correction for multiple comparisons. Corrected value (p_{corr} = 0.01) is specified in Results-BDNF methylation, second paragraph.

7. Table 2, indicate meaning of "t" column.
Our response: Thank you for your comment. "t" stands for Student t-test, we removed the column from the table. t value with degrees of freedom in parentheses for nominal statistically significant difference between the study groups is given in Results- BDNF mRNA expression, second paragraph: t(30) = 2.130.

8. Figure 2, it is not clear what numbers 1-14 refer to. Please indicate nucleotide positions in the reference genome.
Our response: Thank you for your comment. Numbers 1-14 refer to the consecutive number of CpG dinucleotides within the studied BDNF region I2. Nucleotide sequences of all BDNF regions that we studied are presented in Supplementary figure 2, including the start and end coordinate of each region (e.g. for BDNF region I2: I2 >chr11:27743690-27744027 (+)) individual CpGs within sequences are marked with consecutive superscripted numbers after (e.g. aagcCG11agctctCG12ag). Here we provide a table with indicated nucleotide positions of the BDNF I2 region in reference genome version GRCh37/Hg19 (February, 2009)):

BDNF region I2	
CpG#[a]	Chr11[b]
1	277743729
2	277743771
3	277743780
4	277743818
5	277743853
6	277743856
7	277743861
8	277743863
9	277743913
10	277743928
11	277743963
12	277743971
13	277743975
14	277744002

Legend: [a]CpG#, consecutive numbers of the CpG dinucleotide; [b]coordinate of cytosine on (+) strand of chromosome 11.

9. The labeling in Supplementary Figure S1. For I2, 5´of Exon I should be explained. The authors mention that I2 is upstream of exon 1, which do not seem coherent if I2 is the labeling for intron2. Please explain clearly the meaning of this labeling.
Our response: Thank you for your comment. The studied regions are labelled with roman numerals (I, II, IV and VI) according to the vicinity of the exons 1, 2, 4 or 6. The region preceding the first exon (I), is divided into two parts, I1 and I2 due to technical reason of maximum amplicon length recommendation for 454 GS Junior sequencing system (400 bp/ region, including 454 GS Junior sequencing primers).

10. Table S1 and other parts of the paper refer to regions I1, I2, IV, VI etc. The meaning of which should be clearly presented in Tables and Figures (they should be auto-explicative), in addition to its first appearance in text.
Our response: Thank you for your comment. The studied regions are labelled with roman numerals (I, II, IV and VI) according to the vicinity of the exons 1, 2, 4 or 6. The region preceding the first exon (I), is divided into two parts, I1 and I2 due to technical reason of maximum amplicon length recommendation for 454 GS Junior sequencing system (400 bp/ region, including 454 GS Junior sequencing primers). We included this information into legend of Figure 1, and into legend of Table 1. We also included this information into legends of Supplementary Figures S1, S2, S3 and S4, and into legends of Supplementary Tables S1, S6, S8, S9 and S10.

11. Review of the English grammar is needed. Remove article "the" before terms like blood in sentences like: "In the blood… "Data obtained from the blood… In addition, the text should be reviewed to avoid repetitions like: "continuum.
Our response: Thank you for your comment. We removed article "the" before term blood. We also removed the repeated "continuum" in the Introduction section. Before article submission English was reviewed and edited by dr. Christopher Berrie (a professional Scientific English Editor and native speaker from UK, we also submitted the declaration). Together with your suggestions we hope the language is now suitable for publication.

Reviewer #2:

12. I suggest the exclusion of the patient with schizophrenia in the control group. Those in the study group are well, but maybe data from that with PD sounds inadequate as is not a main topic in the content, neither it has been analyzed by statistics tools (very difficult being just one case).
Our response: Thank you for your suggestion. We went through other papers, and we determined that also in other papers some controls had for instance medication (e.g. in Keller et al. 2010). After sequencing we also performed the analysis without the patient with schizophrenia, but it did not affect our results, and therefore we decided to keep the initial formation of the groups and not to interfere with the unbiased selection of participants. Furthermore, in the paper of Schenieder et al. (2015), they stated 'Because previous studies [Labonte et al., 2012, 2013] suggested that suicide-associated DNA methylation patterns are independent of the underlying psychiatric disorders and because of the small sample size, we did no further subgrouping of the suicide completers, as to the presence of comorbidities, medication, early-life adversity, or other possible confounding factors.' It is true that they are talking about suicides ('1 individual in the suicide group (S2 had suffered from depression)', but based on these results we decide to leave the one schizophrenia control in the study group and not to

further divide the sample. We believe it is of the highest importance that the person did not die due to suicide, which was the main subject of the investigation.

13. In methods, check if the mix of samples could be adequate to the analysis. Or argue if this method limit the exclusion of the mentioned patient (with schizophrenia in control group).
Our response: Thank you for your comment. You probably meant, if we would use a pool of samples for a suicide victims group and a pool of all control samples to represent the control group. This kind of approach is sometimes used as validation of an 'omic' approach analysis. For instance: a pool sample used for RNA-seq analysis is applied after whole genome DNA methylation analysis of individual samples, since the pooled samples approach could still be informative in case of initial whole genome approach, and it also significantly reduces the costs that would be high for RNA-seq. In our case this was not a method of choice, since we used targeted approach, and therefore it was much more elegant end effective to use two targeted approaches, for DNA methylation and for gene expression.

14. Several works about BDNF methylation could to help enrich the discussion content. **Our response:** Thank you for your suggestions. Our answers are under every suggested article:

- Perroud, N., Salzmann, A., Prada, P., Nicastro, R., Hoeppli, M. E., Furrer, S., & Malafosse, A. (2013). Response to psychotherapy in borderline personality disorder and methylation status of the BDNF gene. Translational psychiatry, 3(1), e207-e207.

The article primarily addresses borderline personality disorder (BPD) but not suicide/suicidal behavior. It is true that under the heading "Patients and methods" the authors state that patients who displayed suicidal/parasuicidal behavior, severe impulsivity control disorders, and anger management problems were referred to psychotherapy. From Table 1 (characteristics of study subjects) we understand that only women participated in the study? Nowhere in Table 1 is it elaborated on how many subjects showed suicidal behavior, impaired impulsivity control, and anger problems. Hence we did not include the suggested reference into our paper.

- González-Castro, T. B., Salas-Magaña, M., Juárez-Rojop, I. E., López-Narváez, M. L., Tovilla-Zárate, C. A., & Hernández-Díaz, Y. (2017). Exploring the association between BDNF Val66Met polymorphism and suicidal behavior: meta-analysis and systematic review. Journal of psychiatric research, 94, 208-217.

We replaced our Pregelj et al (2011) with the suggested Gonzales-Castro et al (2017) systematic review in our Introduction section. The review covers many studies (including our Pregelj et al, 2011) performed on samples from Caucasian and Asian population. Studies explored the most relevant BDNF SNP rs6265.

- Sonal, A., & Raghavan, V. (2018). Brain derived neurotrophic factor (BDNF) and suicidal behavior: A review of studies from Asian countries. Asian journal of psychiatry, 33, 128-132.

The article is interesting. However, the article discusses research done on different ethnic population.

- Ferrer, A., Labad, J., Salvat-Pujol, N., Barrachina, M., Costas, J., Urretavizcaya, M., & Soria, V. (2019). BDNF genetic variants and methylation: effects on cognition in major depressive disorder. Translational psychiatry, 9(1), 1-10. d)

We are aware of the existence of comorbid disorders in suicide completers. The article is interesting but our research focus was suicide, and we did not address other disorders in our study.

15. After Conclusions, but in that section, it should include more data about tendencies observed for remaining suggestion "BDNF is linked to suicidality".
    Our response: Thank you for your suggestion. However, we did not decide to add anything, as all the relevant literature has already been cited in the manuscript, and adding something after conclusion would only duplicate the text and references. We also believe that the conclusion has to be concise and relevant to the topic, and we did our best to achieve this. We hope we fulfilled your expectations.

16. Check if the repetition of references are required or inadequate (those in supplementary material are in the main references).
    Our response: Thank you for your comment. Supplementary data are presented in separate file. By introducing repeating references to supplementary file as well (more precisely to supplementary figure S2), we want to provide the readers information on which regions of the BDNF gene have already been studied for association with mental disorders, and which particular studies did the research.