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LONG-TERM CONSEQUENCES OF PATERNAL CANNABIS USE ON AUTISM CANDIDATE GENES. A CROSS-SECTIONAL STUDY PROPOSAL

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Abstract

The few existing studies concerning the effect of cannabis consumption on the male germ cells, generated sufficient evidence to indicate that paternal cannabis consumption might be involved in faulty sperm DNA methylation, at genes related to neurodevelopmental disorders, including ASD. Limitations of the exiting literature revolve around small sample sizes, insufficient reported data, unaccounted confounding variables and contradictory findings.

The objective of this research proposal is to address current literature gaps and to bring clarification regarding previous findings, by using a larger sample size, and further explore the potential dose-effect relationship between cannabis exposure and the three ASD-related genes: *DLGAP2, HCN1,* and *NR4A2.* Our hypotheses assume that participants that have consumed cannabis for longer periods of time and in larger quantities will have higher levels of hypomethylation at these genes, comparing with the participants that have consumed less cannabis for shorter periods of times, or that haven't consumed at all. The results will push this emerging field further, and by extension, will generate more research questions, on topics like safety consumption thresholds, the permanence of epigenetic change and, if weather or not these changes can be inherited. Cumulated, the answers could possibly reshape preconception counselling advice offered to future fathers and influence the policy makers in prioritizing prevention efforts regarding hazardous cannabis consumption.

Keywords: ***cannabis consumption; male germ cells; DNA methylation; neurodevelopmental disorders; ASD.***

Introduction

Autism Spectrum Disorder (ASD) is a complex, multifactorial neurodevelopmental condition (Salari et al., 2022). While it is widely understood that the environment can influence the integrity and maintenance of the epigenome in both parents, paternal factors on how the environment impacts the epigenetic mechanisms involved in ASD have been understudied (Soubry, 2018). Recent research shows that paternal factors like age, obesity, and smoking can increase the risk of ASD (Sandin et al., 2016; Surén et al., 2014). With cannabis consumption and potency constantly rising, epidemiological studies reveal strong associations between cannabis legalization and recent spikes in ASD incidence in The United States (US) (Reece & Hulse, 2019). Maternal cannabis exposure has already been linked to neurodevelopmental delay and autistic deficits in infants (Corsi et al., 2020). However, the research concerning the effect of cannabis consumption on male germ cells and its ASD-associated risk is limited. Men in their peak reproductive years comprise the largest group of cannabis consumers (Mauro et al., 2018). More research is needed to understand how paternal cannabis use affects the epigenetic mechanisms involved in ASD. The answers could reshape preconception counseling advice offered to future fathers and influence policymakers to prioritize prevention efforts.

Human studies

Murphy et al. (2018) conducted the first study investigating the differences in sperm DNA methylation profiles between cannabis consumers (n = 12) and non-consumers (n = 12). The authors used Reduced Representation Bisulfite Sequencing (RRBS) to generate 5 million CpG sites per participant. Following a two-tailed independent t-test, 6640 CpG sites were found significantly differentially methylated at ≥10% methylation difference, 78.3% of sites being hypomethylated in the consumer group. Disks large-associated protein 2 (*DLGAP2*) gene was among the regions identified as being significantly differentially methylated. *DLGAP2* plays a crucial role in synapse organization and neuronal signalling; its dysregulation is associated with ASD and schizophrenia (Rasmussen et al., 2017). While the study is directionally promising, it is far from being generalizable due to a limited evaluation of a dose-effect relationship between cannabis exposure and changes in methylation, no accounting for possible confounders (e.g., nutrition, alcohol use, etc.), and the cross-sectional study design restricted potential inferences regarding the reversibility and heritability of these epigenetic changes.

However, linear regressions on the data set found significant associations between Δ9-trans-tetrahydrocannabinol **(**THC) levels and the methylation level of 177 genes, including *DLGAP2* (results not reported).

To further investigate the effect of cannabis exposure on the methylation profile of the *DLGAP2* gene, Schrott et al. (2020) conducted a follow-up study from Murphy et al. (2018). The authors examined the original sperm samples, using Quantitative Bisulphite Pyrosequencing (QBP). A significantly hypomethylated CpG site was found, in addition to the nine previously identified. The strong correlations between methods (p = .02), and the same direction of change, provide increased validity to both studies. However, using the original small sample impacts the generalizability of the findings, and potential confounders are still unaccounted for. The authors also looked at the relationship between the gene's methylation status and its mRNA expression using 28 conceptal brains. They found that as methylation increased, the mRNA expression significantly decreased (r = - .38, p < .05). Nardone et al. (2014) had previously reported mRNA disruptions in the brains of individuals with autism. Thus, understanding how cannabis consumption can impact sperm DNA methylation, and further influence gene expression its crucial, given the critical role of *DLGAP2* in neuronal signalling. However, the authors analyzed whole brains instead of specific areas where *DLGAP2* is most highly expressed (hippocampus, striatum), potentially diminishing the results.

In a subsequent study, Schrott et al. (2021) scrutinized the reversibility of the cannabis-associated epigenetic changes, in 18 cannabis users and 24 non-users. Sperm samples collected before and after 11 weeks of cannabis abstinence were compared using Whole-Genome Bisulfite Sequencing (WGBS). Significant differential methylation was found for 163 CpG sites before, and 127 sites after abstinence. The stark difference in the number of differentially methylated CpG sites found here compared to Murphy et al. (2018) findings (6640 sites), could be due to differences between the THC levels of the users in the two studies. This could also relate to the *DLGAP2* gene, here found hypomethylated in the "after" samples, but not before abstinence, contradicting previous findings (Schrott et al., 2020). The small sample size, although the largest used so far in current research, could have also impacted the results. Consequently, we emphasize the need for more research regarding a dose-effect relationship between cannabis exposure and methylation changes in ASD-candidate genes. Using Ingenuity Pathway Analysis, the authors assessed the functional roles of all differentially methylated genes. Neurodevelopmental disorders, cognitive impairment and learning were among the top

10 strongest 'disease and function associations' (p = .0008), supporting the existing evidence regarding cannabis's effects on early-life development.

The current human research shows significant associations between cannabis exposure and faulty sperm DNA methylation. Some impacted genes are associated with neurodevelopmental disorders, including an ASD-associated gene, *DLGAP2*. Moreover, a dose-effect relationship between exposure and methylation status appears to exist.

Animal studies

Testing for a causal relationship and looking to replicate the human-based findings, Murphy et al. (2018) exposed nine sexually-mature male rats to 2 mg/kg THC (mimicking human moderate intake), for 12 days, comparing them to controls (n = 8). Following similar analyses in their human study (RRBS), the authors found 627 altered genes associated with THC exposure. However, the administered solutions contained ethanol, and the effect of its interaction with THC on methylation was not accounted for.

Levin et al. (2019), following identical replication of the Murphy et al. (2018) study procedure, bred THC-exposed and unexposed rats to drug-naive females. The first offspring (n_{THC} = 14, $n_{control}$ = 12) were studied for neurobehavioral outcomes. The authors found a significant attention impairment and an increase in habituation of locomotor activity, in the offspring of the THC-exposed male rats. Both behaviours are common in individuals with ASD (Ogino et al., 2018). Although the effects became evident only in adulthood, they were significant despite the father's limited exposure to, and modest (2 mg/kg) administration of, THC. This further raises questions concerning a dose-effect relationship and safe consumption thresholds. Nevertheless, the results should be carefully interpreted due to the unaccounted interactional effect between ethanol and THC.

As an extension of their exploratory human study regarding the *DLGAP2* gene, Schrott et al. (2020) investigated the possibility of intergenerational effects of paternal cannabis use in rats. First, the authors found the *Dlgap2* gene significantly differentially methylated in the THC-exposed rats from Murphy et al. (2018) study. Subsequently, they dosed two randomized groups of rats with either 4 mg/kg THC or vehicle control, for 28 days. QBP was used to analyse the sperm. However, only half of the CPG sites were identified this time. Rats were then bred with drug-naive females, and offspring's brains were collected in adulthood (n_{THC} = 6, $n_{control}$ = 8). Analyses between the father's sperm epigenome and the offspring's hippocampi and nucleus accumbens were performed. A weak positive relationship (p = .02) was found only in the nucleus accumbens, supporting current beliefs regarding inheritance of negative health

outcomes through sperm epigenome (Tang et al., 2015). It is important to note that this study also found an insignificant positive inverse relationship between methylation status and gene expression. This finding, and the lack of methylated CpG sites in the hippocampi, may be due to interspecies variation, variations in THC dosage and route of administration, or possibly reflects the small sample size of the tests.

The evidence coming from animal data complements the human research findings. The *Dlgap2* gene was found significantly hypomethylated in offspring of THC-exposed male rats, supporting the possibility of intergenerational effects.

In vitro studies

Research with induced pluripotent stem cells (iPSC) also supports a link between paternal cannabis consumption and increased ASD risk in offspring. Schrott et al. (2022), investigated the impact of cannabis exposure on the DNA methylation of two groups of genes essential for early-life development. Spermatogonial stem-cells (SSC) (n = 3), derived from differentiating cells exposed to 50nM of cannabis extract, were compared to control (n = 3), using bisulfite pyrosequencing. Significant DNA methylation differences were found for two ASD-related genes: hyperpolarization-activated cyclic nucleotide-gated channels (*HCN1*) and nuclear receptor subfamily 4 group A member 2 (*NR4A2*). *HCN1* is involved in neurotransmission and synaptic plasticity, mutations being associated with impaired motor learning and memory (Seo et al., 2015). *NR4A2* is implicated in the differentiation and maintenance of dopaminergic neurons during neurodevelopment, with deletions being associated with intellectual disability, language impairment, and autism (Lévy et al., 2018). Addressing the epigenetic inheritance issue, the authors verified if the SSC-derived cells would potentially further carry the modifications. Results were non-significant, although they almost reached significance for *NR4A2*. A larger sample size is needed to clarify this.

This study proposal addresses the limitations and contradictions found in the studies cited above by using a larger sample of participants, investigating the potential dose-effect relationship between cannabis exposure and methylation profiles in ASD-related genes, and accounting for possible confounders. The study's results will complement existing and future research regarding the heritability of these epigenetic changes. If this relationship exists, the immediate relevant question should be about safe consumption thresholds. Thus, we define our research questions as follows: 1. Is paternal cannabis use associated with faulty methylation profiles in ASD-related genes? 2. How is cannabis consumption associated with changes in sperm DNA methylation, at ASD-related genes? We propose the following directional

hypotheses to answer these questions: 1. There will be a significant difference in hypomethylation between cannabis consumers and non-consumers, for the *DLGAP2*, *HCN1*, and *NR4A2* genes. 2.a. There will be a positive relationship between the frequency of cannabis consumption and hypomethylation levels of *DLGAP2*, *HCN1*, and *NR4A2* genes: as consumption frequency increases, the hypomethylation levels will also increase. 2.b Higher creatinine-adjusted THC levels will be associated with higher hypomethylation levels for *DLGAP2*, *HCN1*, and *NR4A2* genes.

Study Design

This research proposes a between-subjects cross-sectional design. Experimental manipulation involving dosing participants with cannabis goes against the ethical principle of minimizing harm. Moreover, Murphy et al. (2018) and Schrott et al. (2021) employed cross-sectional designs, so we consider our choice justified. This correlational and predictive design aims to test hypotheses and explore relationships. Henceforth, we will use "cannabis" to refer to marijuana and its derived products containing substantial amounts of THC (National Institute on Drug Abuse, 2019).

To test the first hypothesis, the predictor variable will be *cannabis consumption*, with two levels, consumption and non-consumption, reflecting two groups of participants, similar to previous research. Cannabis consumption will be biologically verified in urine samples, as we need to measure consumption for the second hypothesis as well. We excluded blood analyses for this, due to being an invasive method. While hair testing is more time-sensitive than urine samples (Musshoff & Madea, 2006), the latter are the fastest and cheapest option, enabling accurate comparisons to previous samples. Using hair samples would make sense only if their sensitivity would extend beyond 90 days post use. Based on Schrott et al. (2020, 2022) findings, the three outcome variables will be *hypomethylation profiles* for *DLGAP2*, *HCN1* and *NR4A2* genes. The hypomethylation levels will be expressed as the percentage of unmethylated cytosines at specific CpG sites (Peinado, 2011). Like previous authors, we will consider the genes differentially hypomethylated if the difference is $\geq 10\%$. The data will be generated using Bisulfite Pyrosequencing on semen samples collected from participants, as Schrott et al. (2020) did in their *DLGAP2*-focused study. Although previous research used pyrosequencing methods with higher resolution and accuracy, such as RRBS and WGBS (Murphy et al., 2018; Schrott et al., 2021), we consider Bisulfite Pyrosequencing a better option. Besides being the most cost-effective, it can quantify DNA methylation levels faster than the other methods. We overcame its limited coverage by defining the targeted genes (Seiler Vellame et al., 2021).

The second hypothesis (2.a) assumes a relationship between long-term consumption and a higher degree of hypomethylation. It also assumes a dose-effect relationship between cannabis and the degree of epigenetic change (2.b) (Murphy et al., 2018). The existing literature does not provide a clear solution regarding the best methodology for measuring cannabis use. For example, The Marijuana Screening Inventory (Alexander & Leung, 2004), evaluates frequency in terms of number of smoked joints/day rather than evaluating consumption over months/years. We consider The Cannabis Use Problems Identification Test (CUPIT) (Bashford et al., 2010), developed to evaluate the frequency in conjunction with problematic use, a better choice for measuring our predictor of interest: *frequency of cannabis use*. The variable will have four levels: a. no consumption: a CUPIT-score of zero, b. occasional consumption: a score < 12, c. moderate consumption: a score of 12-20, and d. heavy consumption: a score > 20. The latter reflects long-term consumption, above 90 days. For Hypothesis 2.b., the continuous predictor variable *creatinine-adjusted THC levels,* will be measured in urine samples. Creatinine-adjusted levels are more accurate than unadjusted THC levels for measuring drug use over time. The former account for variations in urine concentration, which can affect the latter interpretation (Huestis & Smith, 2007). The outcome variables for both Hypothesis 2.a and 2.b, are identical to the first hypothesis: *methylation profile* of each specified gene, expressed in percentages.

Based on Murphy et al. (2018), we will match the participants in terms of age, race, ethnicity, height, weight, education level, employment status, and marriage, to minimize unsystematic variation (Field, 2013). Similarly, due to their potential epigenetic impact (Soubry, 2018), the following confounding variables will be evaluated or biologically measured: other *recreational/illicit drugs use* in urine samples; *hazardous drinking* with The Alcohol Use Disorders Identification Test-Concise (AUDIT-C) (Bush et al., 1998), *breath alcohol levels* (breathalyzer); and *mental health diagnosis*, with a focus on ASD, psychotic disorders and intellectual disability (self-reporting).

Participants

Despite the small number of participants, previous research yielded significant results. Schrott et al. (2021) had 42 participants in their study, aged 18-40 years old. We will also recruit a similar number (40 participants), but per category, enrolling in total 160 English-speaking adult male participants. We will apply the same age limits to reflect the age-based consumption trend-analyses (Mauro et al., 2018). Volunteers will be recruited in centers and institutes focused on treating cannabis addiction, listed on the National Health Service (NHS)

official website. Ideally, the participants with a CUPIT score > 20 will have a clinically established diagnosis (Cannabis Use Disorder), providing more power to the study design and results. We will also recruit community participants by advertising the research in online forums and social media (e.g., GrassCity Forum) and college campuses. All participants will be recruited from the Greater London area, United Kingdom (UK). Participants will be excluded if they: use recreational/illicit drugs (except cannabis), have a diagnostic or a family history of ASD/ psychotic disorders/intellectual disability (except cannabis-induced psychotic disorder), have an AUDIT-C score > 4, or register a breath alcohol level > 0.0. These inclusion/exclusion criteria are replicated from previous research (Murphy et al., 2018; Schrott et al., 2021) and ensure the possibility of data comparison across studies.

Materials

The Cannabis Use Problems Identification Test (CUPIT) (Bashford et al., 2010), is a brief self-reporting instrument designed to detect problematic cannabis use and discriminate diagnostic subgroups along a severity continuum (non-problematic, risky, problematic use). The questionnaire contains 16 items, divided into two subscales: 'impaired control' (10 items; five measuring consumption frequency and intensity, and five measuring impaired control), and 'problems' (six items evaluating cannabis-induced problems) (see Appendix). All items have a Likert scale type of response, ranging from one to nine. Reversed scoring applies only to item 9. An overall score is obtained by adding the raw scores of all items, ranging from 0 (no use at all) to 82 (daily/multiple-daily use, severely problematic, in the past 12 months). Important cut-off points are 12 (risk of developing cannabis use disorder in the following year) and 20 (current cannabis use disorder). The authors report excellent internal consistencies for each subscale, with Cronbach's alpha values of $\alpha = 0.92$, respectively 0.83.

The Alcohol Use Disorders Identification Test-Concise (AUDIT-C) (World Health Organization, 1998), is a brief alcohol screening reliably identifying active alcohol use disorders. AUDIT-C has three items, with five answer choices from 0 - 4 (see Appendix). The total score ranges between 0 - 12, obtained by adding the raw scores. In men, a score ≥ 4 is considered positive for active alcohol use disorders. The reported internal consistency is $\alpha = .98$. The question regarding past or current *mental health diagnoses* will be "Do you or anyone in your family has or had an ASD, a psychotic disorder or intellectual disability?" (Xie et al., 2020). A dichotomic ("Yes/No") type of answer is expected.

Identically with Murphy et al. (2018) and (Schrott et al., 2020, 2021), DNA will be extracted from semen according to Qiagen's Puregene DNA Purification Protocol (Qiagen,

2023). Genomic DNA (gDNA) will be quantified with NanoDrop 2000 spectrophotometer. The Bisulfite Pyrosequencing and PCR amplification will be performed using the PyroMark CpG Assay Design Software (Qiagen). We cannot use the same instrument that previous authors used (Pyromark Q96 MD Pyrosequencing Instrument-Qiagen), as it is discontinued and reagents will not be available during analyses. Instead, we will use an upgraded version: PyroMark Q48 Autoprep System. Primers and PCR conditions can be found in Table 1 (Appendix). Gas chromatography–mass spectrometry (GC–MS) will determine the creatinine-adjusted THC levels. GC–MS will also verify other recreational/illicit drug use.

Procedure

We will enroll participants between September 1st, 2023 - March 31st, 2024. After reviewing the Participant Information Sheet and Informed Consent Form, with the right to withdraw and anonymity clearly stated, they will be invited to the initial screening visit following written consent. During this, demographic data will be collected, and two clinical psychologists will evaluate the participants (instruments detailed in the Materials section), ensuring that ethical research protocols are followed. *Breath alcohol levels* will be measured. Subsequently, an appointment to a collaborating laboratory will be made at the time-convenience of each participant to provide the biological samples. During the appointment, the participants will receive a General Data Protection Regulation form containing relevant information about the protection of the privacy and confidentiality of their data, as well as the purpose of its use. All participants will receive independent sterile containers and instructions on collecting the semen and urine samples. After completing the data collection phase, every participant will be invited to a debriefing session. Urine samples will be analyzed within 13 hours from collection to ensure the capture of low-range THC levels (Kale, 2019). Genomic analyses will be run following liquefaction at room temperature, within 60 minutes of collection. Like Schrott et al. (2020), DNA will be extracted from semen according to Qiagen's (2023) protocol: following cell lysis and Proteinase K digestion, RNase A solution will be applied to remove contaminating RNA from the samples. After precipitation and centrifugation, DNA will be isolated in 30 µl of nuclease-free water (Schrott et al., 2020). Alternatively, gDNA will be stored at −80◦C until use. Similarly to Schrott et al. (2020), gDNA will be treated with sodium bisulfite to convert unmethylated cytosines to uracils, methylated cytosines remaining cytosines. The resulted bisulfite-modified DNA (10-20 ng) will then be used for PCR amplification and bisulfite pyrosequencing. To ensure the accuracy and reproducibility of the results, assay validation will be performed in triplicate, as previous

authors did: mixtures of fully methylated/unmethylated DNA will be compared to a gradient of methylated-control DNA.

Ethics

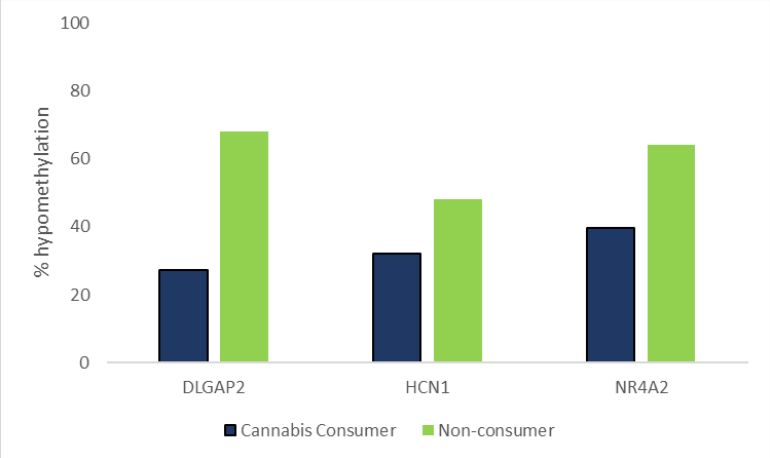
This research proposal involves enrolling human participants from NHS-affiliated clinics, collecting biological samples and applying psychometric tools. Hence, we will apply for ethical approval at King's College London Research Ethics Committee and NHS Research Ethics Committee. We will apply for a license from Human Tissue Authority, because urine is considered "relevant material" under the Human Tissue Act (2004). In compliance with the BPS Code of Human Research Ethics (Oates et al., 2021), participation in this study is voluntary. Participants can withdraw at any time without consequences or benefit loss. This will clearly be stated in the informed consent form, along with the research purpose, procedures, possible foreseeable risks/discomforts, benefits, the study's scientific value and contact details for additional questions and participants' rights. In compliance with UK's Data Protection Act (2018), participants' privacy and data confidentiality will be ensured and any direct identifiers (e.g., names, addresses) will be removed. Because this research involves participants that consume cannabis, a mind-altering product, some of the participant's capacity to consent may be compromised. To manage this, we will ensure a longer response time (seven days) and provide a detailed participant information sheet that can be reviewed when they are not under the influence. If providing the samples or being psychologically evaluated goes against the patients' moral, religious, or personal ethical code, we will fully respect that, withdrawal being possible at any time. During data collection, several risks to participants may occur. Providing semen samples can cause distress, as this can be an incredibly intimate process. If participants feel physical or emotional distress during screening day, the clinical psychologists on site will attend to them, providing professional advice and comfort. Some participants may feel anxiety or discomfort during the biological sample collection. The medical staff present on site will attend to any related problems. During the debriefing session, participants will receive a written sheet containing a summary of the study's purpose and procedures, contact details for further questions, information about support services and resources, and a participation thank you message. All participants will be compensated with 50 GPB, and local transportation fees supported by participants in connection to this study will be reimbursed. This study will not employ deception techniques. Thus, no debriefing will be required regarding this aspect.

Analysis and Reporting

Data analysis will be performed in SPSS v28.01.01.4. Initial analyses will generate descriptive statistics and facilitate data cleaning. Means, standard deviations, and percentages will be reported for all variables. Frequency distributions (histograms) will be generated to characterize the sample and data distribution patterns relevant to our inferential analyses. Missing data will be omitted. Outliers will be converted to the group mean (± two standard deviations) to avoid biased results and to limit their impact (Field, 2013). As in Murphy et al. (2018), participants will be matched based on demographic variables (see Study Design) by a stratified random sampling. The *cannabis consumption* variable will define the two groups: consumers vs. non-consumers.

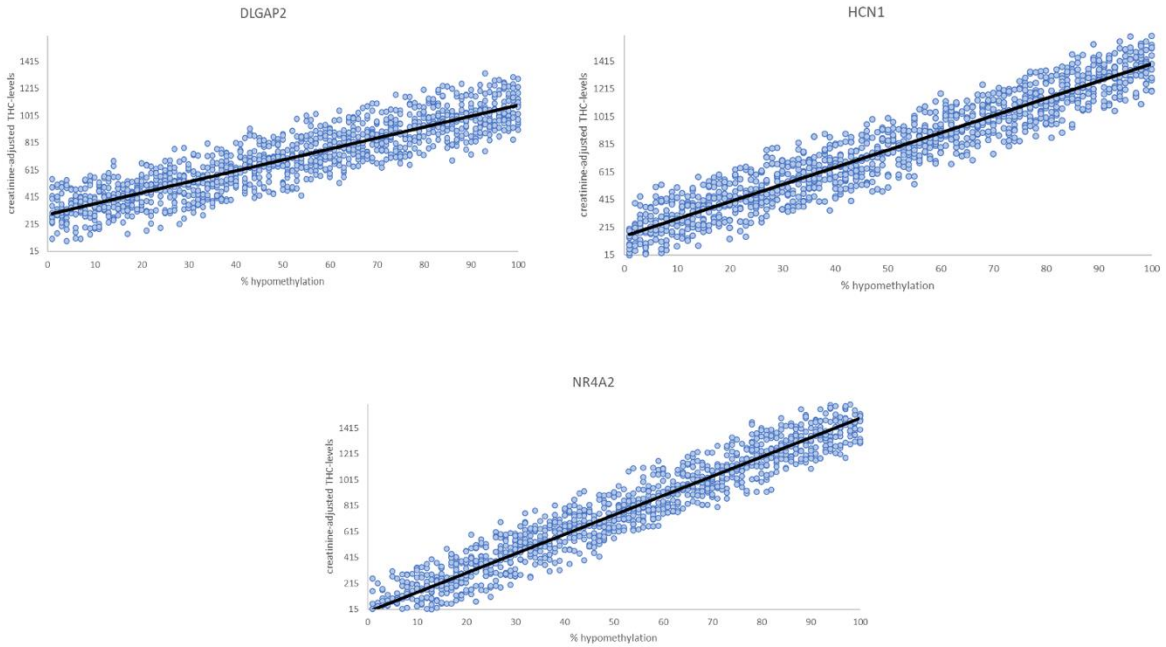
Like Schrott et al. (2020), we will remove CpG sites with missing data or < 5X coverage after pyrosequencing, reporting the total numbers before and after. Assuming that normality and homogeneity of variances are met, a one-tailed independent *t*-test will compare the mean hypomethylation levels of *DLGAP2*, *HCN1*, and *NR4A2* between cannabis users and controls, testing the first hypothesis. A Bonferroni correction will be applied to adjust for multiple comparisons, controlling the family-wise error rate (Type I error) (Field, 2013). The Bonferroni-corrected alpha level will be $\alpha = .05/3 = .0167$, with *p*-values falling beneath this threshold being considered statistically significant. Mean methylation levels, standard deviations, *t*-values, degrees of freedom, and *p*-values will be reported for each gene, as well as an interpretation of the results, including the significance status. A chart similar to Figure 1 will reflect the results. Further, we will calculate the percentage of hypomethylation difference for each gene between the two groups and compare it with the >10% methylation difference previously obtained (Schrott et al., 2020, 2022b). If the assumptions for the *t*-test are violated, Welch's *t*-test will be employed instead.

Figure 1. **Example of bar chart (hypothetical data)**



To test Hypothesis 2.a and determine the direction of the relationship between the frequency of cannabis use and the hypomethylation level of the three ASD-genes, we will run a multivariate multiple regression (MMR). *Frequency of cannabis use* is a categorical predictor variable with four levels a. no consumption, b. occasional consumption, c. moderate consumption, and d. heavy consumption. 'No consumption' will represent the baseline, and three dummy variables will be created to represent the other three consumption levels. Given that all three genes are ASD-related, we suspect that there is a degree of correlation between them. To control for this, we chose the MMR instead of three independent multiple linear regressions (Field, 2013). We presume that the linearity, independence of errors and homoscedasticity assumptions will be met. We will use either Wilks' Lambda or Pillai's Trace tests (depending on whether or not the multivariate normality assumption is met) to assess the overall significance of the relationship between consumption frequency and the three ASD-genes. We will interpret the outputs' *t*-values, *p*-values and β-coefficients to evaluate this relationship for each independent gene. A large statistically significant *t*-value (absolute value) will indicate a large effect of the afferent consumption level on the hypomethylation level of a gene relative to its standard error, a small *t*-value indicating a small effect (Field, 2013). A statistically significant positive β-coefficient, as well as higher β-values for "heavy consumption" compared to lower consumption levels, will suggests a positive relationship between consumption frequency and hypomethylation levels of a gene.

Figure 2. **Example of scatterplots (hypothetical data)**



Presuming that the linearity, normality, and homoscedasticity assumptions are met, a Pearson correlation analysis will test Hypothesis 2.b, determining if higher creatinine-adjusted THC-levels are associated with higher hypomethylation levels in the given genes. A Bonferroni-correction will account for multiple comparisons. The Bonferroni-adjusted alpha will be $\alpha = .05/3 = .0167$, with *p*-values falling beneath this threshold being considered statistically significant. A statistically significant positive correlation coefficient *r* would support the hypothesis, with values closer to 1 indicating a higher relationship strength (Cohen, 1992). The correlation coefficients and associated *p*-values will be reported and interpreted. Scatter plots will be created to visualize the relationship, similar to Figure 2. A Spearman's rank correlation will be employed if one or more assumptions are violated.

Conclusion

The few existing studies concerning the effect of cannabis consumption on male germ cells generated sufficient evidence to indicate that paternal cannabis consumption might be involved in faulty sperm DNA methylation at genes related to neurodevelopmental disorders, including ASD. Two human studies showed significant hypomethylation of the *DLGAP2* gene in cannabis consumers compared to non-consumers. The results were replicated in rodent studies. *DLGAP2* has a critical role in synapse organization and neuronal signaling. Furthermore, studies *in vitro* showed significant hypomethylation of *HCN1* and *NR4A2* genes, in spermatogonial stem-cells exposed to THC extract, compared to unexposed cells. *HCN1* is involved in neurotransmission and synaptic plasticity, and *NR4A2* is implicated in the differentiation and maintenance of dopaminergic neurons during neurodevelopment. Dysregulations in these three genes are associated with ASD or ASD-related phenotype. Although insufficient, the evidence also suggests that there might be a dose-effect relationship between cannabis exposure and the methylation status of *DLGAP2*. Limitations of the existing literature revolve around small sample sizes, insufficient reported data, unaccounted confounding variables, and contradictory findings. Considering this, we formulated this study proposal to address current literature gaps: clarifying previous findings by using a larger sample size, and further exploring the potential dose-effect relationship between cannabis exposure and the three specified ASD-related genes.

Special ethical considerations were accounted for. Not only that this study proposal involves human participants, but it also involves sample collection: semen and urine. While providing a urine sample might be common practice, providing a semen one is not. Aside from the fundamental rights of data protection, anonymity, and withdrawal from the study,

participants' moral, religious, and personal ethical standards must be respected. Physical or emotional discomfort can arise in multiple ways, as providing a semen sample is a very intimate act. Moreover, the question regarding a mental health diagnosis could also cause discomfort, as participants might feel self-doubt and affect the ones prone to negative views of themselves. All these ethical aspects, as well as others, need to be carefully considered and attended to.

Our hypotheses assume that participants that have consumed cannabis for longer periods and in larger quantities will have higher levels of hypomethylation at the discussed genes compared with the participants that have consumed less cannabis for shorter periods or that have not consumed at all. The results will push this emerging field further, and, by extension, will generate more research questions, on topics like safety consumption thresholds, the permanence of epigenetic change and, if whether these changes can be inherited. Cumulated, the answers could possibly reshape preconception counseling advice offered to future fathers and influence policymakers to prioritize prevention efforts regarding hazardous cannabis consumption. Further research should also focus on protective factors, as not every cannabis consumer will likely have faulty DNA methylation profiles. If these protective factors (e.g., exercise) could be influenced, they could be promoted during counseling sessions.

Several limitations can be inputted to this study proposal. For example, excluding participants that consume other types of drugs impacts the generalizability of the results, as a portion of cannabis consumers are likely to consume other recreational drugs as well. Moreover, we have not discussed the epigenetic interactional effect between other drugs and cannabis on the methylation status of ASD-related genes. Future research should consider this, given the teratogenic effect of drugs in general. Another limitation is related to the self-reporting measures, such as the CUPIT or AUDIT-C questionnaires. Self-reporting measures have a higher degree of bias than objective measures, as recollection can be affected by time passing, leading to over- or underestimation of the reported answers. That being said, the proposed study design is apt to answer the research questions and provides ecological validity, as we want to enroll participants with a large age range and various demographic backgrounds. Furthermore, similar to previous research, using biological samples will give more power to the results, allowing deeper inferences, provide research value, and advancing this emerging field.

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APPENDIX

Table 1. Primers and PCR conditions (Murphy et al., 2018; Schrott et al., 2022)

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')	Sequencing Primer (5'→3')	PCR Cycling Conditions*
DLGAP2	GGGAATGGTAA ATTTTGATAGGA	BTN-CACAATA ACCCCAAAAATATTICC	GTTTGTTTTTA TAATTATAT	94°C - 66°C/64°C - 72°C × 5 at each annealing temp, then 94°C - 62°C - 72°C x 55
HCN1	GAGGTTTTTGGG GTTTAGAAGAAGATTG	CCAAATACAAAT TACCCTCTTTTAGG	TTTTGGGGAGTGTGT	94°C - 68°C/66°C - 72°C x 5 at each annealing temp, then 94°C - 64°C - 72°C x 55
NR4A2	GGGTTTAGGGG AAAGTGAAGT	ACTAACCCTAA CCCCCAATATACCTTTAT	GTTAGGTAGGA AATATATTAAAG	94°C - 64°C/62°C/60°C - 72°C x 5 at each annealing temp, then 94°C - 58°C - 72°C x 55

*All reactions will be performed with a heated lid at 99 degrees. Reactions will begin at 95 degrees for 15 minutes. Following 30 seconds at each cycling temperature, samples will be heated at 72 degrees for 10 minutes and then cooled at 4 degrees.

Figure 2. **Example of Demographic Data reporting. The image was captured from Murphy et. al. (2018).**

Table 1. Participant characteristics.

	Cannabis Users	Non-Users	*p*
Demographics/Physical/Cognitive			
Age in years (SD)	21.8 (3.8)	25.8 (6.7)	NS
Race (% Caucasian)	58.3	75	NS
Height in inches (SD)	69.1 (2.4)	69.7 (2.5)	NS
Weight in pounds (SD)	164.7 (30.2)	176.1 (39.7)	NS
IQ Est (SD)	116.3 (14.6)	115.5 (11.5)	NS
THC concentration – unadjusted ng/mL (SD)	260.8 (228.9)	0.0 (0.0)	$p < 0.01$
THC concentration – Creatinine adjusted ng/mL (SD)	329.8 (460.9)	0.0 (0.0)	$p < 0.05$
Semen Analysis			
Duration of abstinence before sample in days (SD)	3.8 (1.2)	4.3 (1.3)	NS
Interval between ejaculation and analysis in minutes (SD)	19.6 (9.9)	25.0 (10.0)	NS
Volume in mL (SD)	3.2 (1.4)	3.4 (1.2)	NS
Semen pH (SD)	8.4 (0.2)	8.4 (0.1)	NS
Sperm Concentration (SD)	58.1 (26.5)	96.3 (49.7)	$p < 0.05$
% Motile (SD)	63.3 (8.5)	63.8 (16.0)	NS
% Normal Morphology (SD)	3.3 (3.3)	3.5 (2.0)	NS