Gender-Affirming Medical Care: Longitudinal Effects on DNA Methylation and Psychological Well-Being in Transgender and Gender Diverse Youth

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Executive Summary

Transgender and gender diverse (TGD) youth exhibit higher levels of anxiety, depression, and stress than their cisgender peers. Psychological survey and questionnaire responses have shown that these adverse mental health outcomes are significantly ameliorated by the receipt of gender-affirming medical care, which includes puberty suppression, gender-affirming hormones (GAH), and surgery. However, few studies have investigated the biological effects of gender-affirming care on TGD individuals, and even fewer have explored this question specifically regarding TGD youth. The present study begins to address this gap by analyzing parallel changes in mental health and DNA methylation from blood samples of TGD youth as they receive GAH. Our results reproduce previous findings of improvements in anxiety and stress after a period of time on GAH. Furthermore, this study highlights the limitations of epigenetic aging as a biological indicator of mental health and well-being in this cohort but suggests a role for average M-value (a measure of overall DNA methylation) in predicting self-reported anxiety and stress. Lastly, the present study identifies six differentially methylated CpG sites after 12 months of GAH in transmasculine youth and reproduces the loss of methylation observed at four sites previously reported in transmasculine adults. Not only does this study provide additional psychologically based support for the benefits of GAH, but it also offers new insights into the impact of GAH on the methylome of TGD youth in terms of epigenetic aging and differential methylation and their associations with psychological well-being.

This thesis is part of a larger clinical study that is investigating the effects of GAH on TGD youth's psychological and biological health through analyses of survey responses, DNA methylation (DNAm), telomerase activity, and telomere length, the latter three of which are biological markers that have been associated with stress in humans. These measures are obtained from three timepoints over the first year of an individual's treatment in order to assess longitudinal changes. Our eventual analyses of telomerase activity and telomere length will provide new insights into the effects of GAH on youth’s biological health since reduced telomerase activity/increased telomere attrition confers a greater risk of bone marrow failure, pulmonary fibrosis, oncogenic transformation, and degenerative diseases (Lin & Epel, 2022; Mangaonkar & Patnaik, 2018; Shammas, 2011). Our present DNAm analyses demonstrate a novel application of epigenetic aging algorithms in a cohort of TGD youth and more broadly investigate GAH-induced differential methylation. These findings help elucidate the effects of GAH on the epigenome of TGD youth, which can be used to inform future studies that will evaluate the clinical significance of such epigenetic alterations.
This project has approval from the Princeton Institutional Review Board and the recruitment process is currently underway, with TGD youth between the ages of 11 and 25 being recruited to participate. All enrolled individuals currently are or will be receiving puberty suppression or GAH from physicians at the Rutgers—Robert Wood Johnson Medical School. Professor Kristina Olson (Princeton University Department of Psychology), an expert in transgender youth psychology, has generously provided the questionnaires that are used to assess psychological well-being. Physician-scientist Professor Daniel Notterman has also been closely involved in the development of this cohort: his laboratory explores epigenetic biomarkers of stress in adolescents, and his expertise in this area is directly relevant to the present study. In accordance with study protocol, peripheral blood mononuclear cells (PBMCs) are collected from each participant before they receive gonadotropin-releasing hormone analogues to suppress puberty or sex hormones to promote the development of secondary sex characteristics. Once a participant begins an intervention, two additional samples are collected after 3-5 months and 12 months. A total of 40 subjects are currently enrolled, and this number is expected to increase over the coming months. Preliminary qualitative findings have shown increased telomerase activity induced by estrogen or testosterone in human foreskin fibroblasts, although further validation is required (Babu, 2022). A preliminary demographic analysis of the currently enrolled subjects has shown that the present cohort resembles other cohorts published in the literature in terms of gender identity, race/ethnicity, and age, with the majority identifying as White/European, not Hispanic/Latinx, and transmasculine.

Both this thesis and the larger clinical study will extend current understandings of general methylation changes during gender-affirming medical care, provide biologically based analysis for the mental health benefits of gender-affirming care through analyses of epigenetic aging, differential methylation, and telomere attrition, and link these measures to changes in psychological well-being.

**Background**

Despite gaining greater visibility in the U.S. due to sustained activism efforts, increased media representation, and legislation and policy changes (Gillig et al., 2018; Michelson et al., 2018), TGD individuals continue to face workplace discrimination, unequal access to healthcare, and threats to their personal safety (Bockting et al., 2020). The effects of this gender identity-based discrimination contribute to TGD individuals’ higher risk for suicide and serious mental illness (SMI) than their cisgender peers; other contributing factors include (but are not limited to) distress about one’s physical body and a lack of support from family and peers (Bockting et al., 2013; Clements-Nolle et al., 2006; Olson et al., 2015; Terada et al., 2011; Witcomb et al., 2018). At particular risk for these adverse mental health effects are TGD youth, who often experience school victimization based on their gender identity and limited interactions with gender-affirming healthcare (Almeida et al., 2009; Clark et al., 2018; Grossman & D’Augelli, 2007; Hoffman et al., 2009; MacApagal et al., 2016; Russell et al., 2011). Given the
high rate of SMI, suicidal ideation, and suicide attempts in TGD youth, it is crucial to develop practices and attitudes that better support them in their experiences.

These adverse mental health outcomes can be partly attributed to gender dysphoria, which the *DSM-V* defines as “the distress that may accompany the incongruence between one’s experienced or expressed gender and one’s assigned gender” (American Psychiatric Association, 2013). The gold standard for the management of gender dysphoria is commonly referred to as the “Dutch approach” for its origins in the Netherlands (Cohen-Kettenis et al., 2011). The protocol involves a multi-step process to ensure that gender affirming care is appropriately delivered to those who need it, while gradually informing patients of the risks and outcomes associated with the different options. It begins with a diagnostic phase, after which a team consultation determines the patient’s eligibility and the most appropriate form of care to pursue, which can include psychological, medical, or surgical interventions. Widely accepted medical interventions for the reduction of gender dysphoria include gonadotropin-releasing hormone analogues (GnRHas) to suppress puberty and GAH to induce the development of secondary sex characteristics that better align with an individual’s embodiment goals; these interventions are the focus of the present study.

Per the Dutch protocol, patients with a diagnosis of gender dysphoria and at least 12 years of age are eligible to receive GnRHas as a means of puberty suppression, a largely reversible cessation of puberty that is often accompanied by drastic reductions in patients’ distress about their physical maturation (Cohen-Kettenis et al., 2011). A study of 70 transgender adolescents in the Netherlands also found significant decreases in depressive symptoms and behavioral problems after the initiation of GnRHas (De Vries et al., 2011). Transgender youth aged 16 or older who have been meeting regularly with a psychiatrist are then eligible to receive GAH, which involves the administration of 17β-estradiol or testosterone esters to induce “feminizing” or “masculinizing” puberty, respectively. This process promotes the development of desired secondary sex characteristics. Lastly, at 18 years of age, individuals become eligible for gender-affirming surgical procedures. A given patient can undergo any or all of these options; regardless of the path upon which a patient decides, the dramatic reductions in patient stress reported by Cohen-Kettenis et al. (2011) suggest that the Dutch approach to gender-affirming care—in the form of therapy, puberty suppression, GAH, or surgery—is beneficial for the mental well-being of transgender children and adolescents (Cohen-Kettenis et al., 2011). This is the approach utilized in the present study.

Growing evidence for the positive psychological effects of gender-affirming medical care has primarily relied upon studies of self-reported surveys (Cohen-Kettenis et al., 2011; Nguyen et al., 2018). For example, a prospective six-year follow-up study in the Netherlands found that puberty suppression followed by GAH and gender-affirming surgery resolved both gender dysphoria and issues of psychological functioning such that the transgender individuals’ well-being resembled (or even surpassed) that of age-matched controls (De Vries et al., 2014). Similarly, a recently published follow-up study including 315 transgender and nonbinary youth across the U.S. found increases in positive affect, appearance congruence, and life satisfaction, as
well as decreases in anxiety and depression, after two years on GAH (Chen et al., 2023). Although true outcomes data in this field are limited in number, these preliminary findings are promising in regard to the psychological benefits of gender-affirming care and demonstrate that gender dysphoria can be reduced in TGD populations.

While these questionnaire-based approaches are widely used, there is increasing interest in supplementing this methodology with biological markers that can assess psychological state and inform intervention efficacy; one such possibility is the field of epigenetics, which holds promise for biological assessments of stress, aging, and even mental illness. The term “epigenetics” refers to the collection of largely reversible biological processes that can alter gene expression without changing the genes themselves (L. Zhang et al., 2020). DNA methylation (DNAm) is one of the best-studied epigenetic mechanisms and involves the reversible addition of a small molecule to a cytosine residue in a DNA strand (Moore et al., 2012). Most DNAm occurs at cytosines that immediately precede a guanine residue, and these sites are referred to as CpG sites or loci. In the late 20th century, researchers discovered that DNAm serves as a mechanism for the regulation of gene expression (Compere & Palmiter, 1981), with most—but not all—DNAm being associated with reduced gene expression (Moore et al., 2012). This finding opened new possibilities for investigations into developmental processes and potential biomarkers of several diseases and conditions, from human cancer to biological aging and stress.

DNAm studies have led to the development of “epigenetic clocks,” which are machine learning algorithms that were originally developed to accurately predict chronological and biological age using the methylation status of a subset of CpGs (Horvath, 2013; Horvath & Raj, 2018). When considered in isolation, each selected CpG’s methylation status contributes very little predictive information to chronological age. However, when the CpG sites are considered together within the parameters used by the algorithm, their collective methylation status holds significant predictive power—an individual’s methylation data can provide a “methylation age” that closely corresponds to their chronological age. Clocks that were designed to accurately predict chronological age are referred to as “first-generation” clocks; the first-generation clocks utilized in this study are the Horvath and Hannum clocks, named after their creators (Hannum et al., 2013; Horvath, 2013).

The methylation ages generated by epigenetic clocks can be indicative of a variety of age-related conditions. An epigenetic age that is higher than one’s chronological age (i.e., positive epigenetic age acceleration) is associated with cognitive decline, cellular senescence, and reductions in predicted remaining lifespan (Horvath & Raj, 2018). Clocks that account for physiological features of aging and that were specifically designed to predict remaining lifespan are known as “second-generation” clocks and comprise the PhenoAge and GrimAge clocks, which are also included in the present study (Levine et al., 2018; Lu et al., 2019). Second-generation clocks have been shown to be strongly associated with social determinants of health, with individuals subject to adversity more likely to experience positive epigenetic age acceleration (Raffington & Belsky, 2022).
In addition to its role in the development of epigenetic clocks, differential DNAm has also been implicated as a potential biomarker of SMI. A variety of genomic regions display differing levels of DNAm between unaffected individuals and people with anxiety or depression (Alladi et al., 2018; Li et al., 2019). Moreover, these DNAm changes in blood samples have also been observed in brain tissue, suggesting a functional link between blood DNAm and symptoms of anxiety and depression (Boström et al., 2017; Carlberg et al., 2014; Emeny et al., 2017). However, no studies have yet evaluated these broader methylation patterns in a cohort of TGD youth receiving gender-affirming medical care; the study at hand is poised to address this gap through the following aims and methods.

Aims & Methods

Aim 1: Psychological Well-Being

Investigate the effects of gender-affirming medical interventions (puberty blockers or GAH) on the psychological well-being of TGD youth through analyses of psychological evaluations, epigenetic age acceleration, and average DNAm.

There already exists support for the mental health benefits of gender-affirming care as assessed through self-reported measures (Chen et al., 2023; De Vries et al., 2011, 2014; Green et al., 2022; Turban, King, et al., 2020). The present study seeks to validate these findings using psychological evaluations administered to TGD youth in this cohort. These questionnaire-based findings are extended by evaluating psychological well-being through the biologically based method of epigenetic aging. Since accelerated epigenetic aging is associated with increased stress, assessing baseline levels and longitudinal changes in epigenetic aging over the course of gender-affirming care has the potential to provide biological support for the benefits of such care on the psychological well-being of TGD youth (as well as to more fundamentally support the use of epigenetic age acceleration as a possible indicator of psychological well-being). Epigenetic age calculations from TGD youth in the present cohort are also compared to similar calculations from adolescents participating in the Future of Families and Child Wellbeing Study (FFCWS; Future of Families and Child Wellbeing Study, n.d.). Lastly, since higher levels of global DNAm have been associated with anxiety (Bortoluzzi et al., 2018; Murphy et al., 2015) and stress (Harkess et al., 2016; Rusiecki et al., 2012), I evaluate putative associations between average DNAm and self-reported anxiety and stress in the present cohort.

Aim 2: Differential Methylation

Identify differentially methylated probes (DMPs) associated with 3-5 months and 12 months of GAH and evaluate the reproducibility of GAH-associated DMPs from transmasculine adults in a cohort of transmasculine youth.

DNAm changes associated with puberty are well-documented in cisgender adolescents, and recent work has investigated similar changes in transgender adults (Shepherd et al., 2022).
However, DNA methylation changes have not yet been evaluated in transgender adolescents experiencing GAH-induced puberty. The present study addresses this gap in the literature by identifying individual probes whose methylation status differs significantly after the first 3-5 months and 12 months of GAH. This study also extends previous findings of GAH-associated DMPs in transmasculine adults (Shepherd et al., 2022) by evaluating changes in their methylation status in a cohort of transmasculine youth.

Key Findings

Psychological Well-Being

After 3-5 months of GAH, TGD youth’s self-reported anxiety and stress improved significantly. When analyzed separately, transmasculine youth also reported significantly improved anxiety levels after 3-5 months of GAH. These improvements in anxiety and stress recapitulate similar findings in other cohorts of TGD youth after GAH (Chen et al., 2023; Green et al., 2022).

Before starting GAH, TGD youth (and transmasculine youth specifically) reported levels of anxiety and depression that were significantly higher than those reported by the age-matched general population. This finding reiterates the literature that identifies disproportionately high levels of anxiety and depression in young TGD populations (Nunes-Moreno et al., 2022; Pattison et al., 2021; Reisner et al., 2015; Witcomb et al., 2018). Although still significantly elevated in TGD youth, self-reported anxiety and depression approached levels of the general population after 3-5 months of GAH. In fact, anxiety levels reported by transmasculine youth were no longer significantly elevated from the general population after 3-5 months, indicating that in as little as several months, GAH may alleviate the disproportionately high anxiety that can be experienced by transmasculine youth.

In addition to significant effects on mental health and well-being, GAH also affected the evaluations of physical appearance reported by this cohort. TGD youth (and transmasculine youth in particular) reported significant decreases in depersonalization after 3-5 months of GAH. TGD youth also reported significant improvements in Transgender Congruence Scale scores—as well as the Appearance Congruence Subscale—after 3-5 months of GAH; this finding was true for transmasculine youth as a subgroup as well. Improved TCS scores suggest higher feelings of congruence between one’s gender identity and physical body, while higher Appearance Congruence scores indicate higher levels of comfort with physical appearance specifically. This finding is expected given that GAH promotes the development of desired secondary sex characteristics, thus aligning one’s body more closely with one’s embodiment goals and improving appearance comfort.

Both TGD youth in general and transmasculine youth specifically reported significant decreases in dissatisfaction with their primary sex characteristics after 3-5 months of GAH, with unchanging dissatisfaction with their secondary and neutral characteristics. In a similar vein, TGD youth’s desire to change primary, secondary, and neutral characteristics
decreased significantly after 3-5 months of GAH, suggesting that GAH induces physical changes that better align with TGD youth’s embodiment goals.

While there were significant changes in TGD youth’s self-reported mental health and physical appearance evaluation after 3-5 months of GAH, there were no corresponding changes in epigenetic age acceleration that reached statistical significance. However, epigenetic age acceleration appeared to trend upward in all four clocks after one year of GAH, with a statistically significant increase in PhenoAge epigenetic age acceleration after one year of GAH.

Self-reported levels of anxiety and stress were positively correlated with average M-values at baseline and after 3-5 months of GAH. Since positive M-values are indicative of a higher methylated than unmethylated signal (Du et al., 2010), increased average DNAm may be an indicator of anxiety and stress in TGD youth. This relationship between average DNAm and anxiety/stress in TGD youth is consistent with studies that found global hypermethylation in anxious vs. nonanxious adolescents (Bortoluzzi et al., 2018) and adults (Murphy et al., 2015), as well as a study that found a positive correlation between perceived stress and global DNAm in adults (Harkess et al., 2016). Global levels of DNAm have also been reported to vary by sex and race/ethnicity (F. F. Zhang et al., 2011) and are being investigated as possible biomarkers for various types of cancer (Hoffmann & Schulz, 2005; Hsiung et al., 2007; Pufulete et al., 2003). Although further validation with larger cohorts is required, results from the present study suggest a role for average DNAm in predicting TGD youth’s self-reported anxiety and stress levels.

Differential Methylation

Although the differential methylation analyses identified no significant CpGs after 3-5 months of GAH in TGD youth or transmasculine youth specifically, six CpGs reached suggestive significance after 12 months of testosterone in transmasculine youth; these CpGs are: cg0616709, cg21895450, cg16655805, cg05126421, cg20386316, and cg14583750.

Lastly, four CpG sites identified as differentially methylated in transmasculine adults throughout one year of GAH (Shepherd et al., 2022) were also identified in the present cohort of transmasculine youth. In both cohorts (transmasculine adults and youth), the β-values of these four sites decreased over the first year of GAH, with the most significant decrease occurring at cg23256579. According to Shepherd et al. (2022), the decrease in β-values in transmasculine individuals approached methylation levels observed in cisgender men. Since a similar trend was observed in transmasculine youth, it appears that masculinizing GAH induces progressive DNAm changes at these four CpG sites in a manner that approaches the methylation profile of cisgender individuals with the same gender identity.
Conclusions & Policy Recommendations

This study reproduces previous findings of improvements in self-reported anxiety and stress after GAH initiation; it also signifies the first investigation of GAH-induced changes to DNAm in a cohort of TGD youth. Larger sample sizes and longer follow-up durations are necessary to confirm this study’s preliminary findings that epigenetic age acceleration of TGD youth is largely unaltered after 3-5 months of GAH but may increase after 12 months of GAH. More robust validation is also required for the present study’s differential methylation findings. Despite the preliminary nature of these results, this study provides novel insights into the impact of GAH on the methylome of TGD youth and proposes average DNAm as an indicator of anxiety and perceived stress in this population. Continuing this research will not only offer new understandings of DNAm changes in puberty but will also lead to a more complete understanding of the biological mechanisms underpinning GAH; deepening this understanding is crucial to allow for physicians, policymakers, families, and most importantly, TGD youth themselves to make better informed decisions about their care.

In spite of growing evidence demonstrating the psychological benefits of the Dutch approach, gender-affirming medical care has become increasingly controversial. Limited knowledge about the biological effects of gender-affirming medical interventions have led jurisdictions across the U.S. to alter health legislation and restrict access to this care. Over 100 pieces of such legislation have been introduced across the U.S. in 2023 alone, severely limiting access to gender-affirming care for minors (Map: Attacks on Gender Affirming Care by State - Human Rights Campaign, n.d.). Many of these proposals cite the lack of data on gender-affirming interventions as reason enough to impose full-scale bans, emphasizing the imminent policy need for further psychological and biological research in this field. The present study offers new insights into the association between psychological and biological effects of gender-affirming medical interventions (specifically, GAH). Findings from this study can directly inform the current debate on the approaches needed to support TGD youth, with the goal of developing the necessary information to promote sound, fact-based policy-making about this important aspect of healthcare.
References


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