ARTICLE

The role of genome size and heterochromatin on maize flowering time

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Abstract: *Maize exhibits extensive variability in vegetative cycle length (VCL), a trait essential for environmental adaptation. This study investigates relationships among genome size, knob heterochromatin content, and VCL in three inbred lines with contrasting flowering times and their F1 hybrids. The latest-flowering line (Floury-2) exhibited the highest DNA and knob heterochromatin contents, whereas the earliest-flowering line (Gaspé) showed the lowest values. Hybrids displayed intermediate values, consistent with an additive inheritance pattern. Significant positive correlations among DNA content, knob content, and VCL were detected across parental lines and hybrids. Within the limited scope of this study, the results suggest that variation in genome size and knob heterochromatin content may influence cell cycle duration and, consequently, flowering time. By integrating cytogenetic and hybrid analyses, this study reveals a cytogenetic basis for flowering time variation and highlights heterochromatin content as an accessible tool for breeding maize adapted to diverse environments.*

Keywords: *DAPI banding, DNA content, F_1 hybrids, knob heterochromatin, vegetative cycle length*

INTRODUCTION

Maize (*Zea mays* subsp. *mays*) is one of the most essential crops for humanity, recognized for its high nutritional value and extensive adaptation to diverse ecogeographical regions. Over 300 maize landraces have been identified worldwide (Castañeda 1990), with 260 found in the Americas, predominantly across environments ranging from lowlands to the Andean highlands (McClintock et al. 1981, Kato et al. 2009, Cámara Hernández et al. 2011). In maize landraces and inbred lines, flowering time is a crucial agronomic trait that influences the growth cycle, yield potential, and adaptability to varied environments. The vegetative cycle length (VCL), i.e. the time between sowing and flowering, varies widely from 35 to 120 days in maize (Aulicino et al. 1992, Melchiorre et al. 2006, 2017, 2020, Colasanti and Muszynski 2009, Realini et al. 2021, Realini et al. 2023).

Among maize inbred lines and landraces cultivated across the Americas, a significant variation in genome size has been reported, with DNA content ranging up to twofold (Poggio et al. 1998, Díez et al. 2013, Realini et al. 2016, Fourastié et al. 2017). Such differences are mainly attributed to the abundance of interspersed retrotransposons, variation in heterochromatic knob regions, and polymorphisms in the number of B chromosomes (Bs) (Poggio et al. 1998,

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SanMiguel and Bennetzen 1998, Meyers et al. 2001, Realini et al. 2016, Fourastié et al. 2017, González and Poggio 2021, González et al. 2022). Knobs are conspicuous heterochromatic regions located at different sub-terminal positions on maize chromosomes (Kato 1976) and can be detected using specific staining techniques such as DAPI banding, which highlights AT-rich repeats (Schweizer 1983, Sumner 1990, González et al. 2013). The variation in knob number, size, and chromosomal distribution among karyotypes has been widely used to identify racial, geographical, and genetic diversity in maize landraces (McClintock et al. 1981, Fourastié et al. 2017, Realini et al. 2018). Besides, knob heterochromatin content has shown a positive correlation with genome size in Argentinean and Bolivian highland landraces (Poggio et al. 1998, Fourastié et al. 2017, González and Poggio 2021). Heterochromatin is characterized by dense packing and low transcriptional activity and replicates later than euchromatin during the synthesis phase (S-phase) (Pryor et al. 1980, Bass et al. 2015, Wear et al. 2017). In maize, an increased heterochromatin content, resulting from a greater number and/or larger size of knobs and associated with a prolonged S-phase, may lead to longer cell cycles and extended VCL (Francis et al. 2008, Greilhuber and Leitch 2013, Realini et al. 2021). Moreover, previous studies in several inbred lines have shown a positive correlation between genome size and flowering time (Rayburn et al. 1994, Comertpay 2019). Indeed, high-altitude landrace populations with reduced genome sizes appear adapted to shorter growing seasons, possibly as a result of greater cold and freeze tolerance (Fourastié et al. 2017, Bilinski et al. 2018, González and Poggio 2021).

It is generally expected that the DNA content of hybrids falls within an intermediate range between that of their parental lines, as observed in interspecific F1 hybrids of *Glandularia* (Ferrari et al. 2019), *Lolium* (Hutchinson et al. 1979) and *Zea* (Palacios 1982), as well as among maize inbred lines (Michaelson et al. 1991, Comertpay 2019). However, Rayburn et al. (1993) reported no significant differences in genome sizes of the maize hybrids tested and their parental lines, whereas Carvalho et al. (2022) found hybrids with larger genome sizes than their parents. These contrasting findings underscore the complex relationship between genome size, heterochromatin content, and VCL in maize hybrids, likely modulated by both natural and artificial selection (Rayburn et al. 1985, González and Poggio 2021, Realini et al. 2021).

This study aims to explore the relationship between genome size, heterochromatin content, and VCL in maize inbred lines with contrasting flowering times and their F1 hybrids. In particular, focusing on the role of knob heterochromatin in genome size variation and its influence on VCL, proposing its use as a cytogenetic tool to support breeding programs aimed at improving maize adaptation to diverse ecogeographical conditions.

MATERIAL AND METHODS

Plant materials

Three maize inbred lines were cultivated under greenhouse conditions at the Instituto Fitotécnico Santa Catalina (IFSC), Facultad de Agronomía, Universidad Nacional de La Plata (lat 34° 46′ S, long 58° 27′ W). The lines analyzed included the late-flowering Floury-2 and the medium-cycle O2M (both developed at IFSC), and the early-flowering Gaspé (originally provided to the IFSC by the University of Illinois, USA).

Artificial hybridizations were performed using a controlled-pollination procedure. Female inflorescences were covered with silicone bags prior to silk emergence to prevent undesired pollination. Hand pollinations were carried out using mature pollen collected from the selected male parents, and the ears were immediately re-covered to ensure isolation. The resulting crosses were O2M × Gaspé and O2M × Floury-2, with O2M used as the common female parent. Mature F1 ears were harvested, and their seeds were sown under the same controlled greenhouse conditions as the parental lines, ensuring reliable comparisons among genotypes. Flowering time, expressed as vegetative cycle length (VCL), was defined as the number of days from sowing to anthesis and was recorded for a minimum of ten individuals per genotype (i.e., parental lines and hybrids). Seed materials were deposited at the Banco de Semillas del Laboratorio "N. I. Vavilov" de la Facultad de Agronomía, Universidad de Buenos Aires.

Estimation of DNA content

Genome sizes were estimated using Feulgen densitometry (Swift 1950); this technique has been chosen for the availability of equipment as well as for its sensitivity in detecting small differences in DNA content. In addition, it provides reliable estimates which are comparable to those obtained by flow cytometry (Doležel et al. 1998). Seeds from the parental lines and F1 hybrids were surface-sterilized and germinated at 25 °C in sterile Petri dishes containing moistened

filter paper. Primary roots were fixed in a 3:1 (v/v) solution of absolute ethanol and acetic acid and stored at 4 °C. As described by Poggio et al. (1998), root tips were excised and hydrolyzed in 5 N HCl at 20 °C for 30 minutes, rinsed three times in distilled water, and stained with Schiff reagent (pH 2.2) for 120 minutes. The material was then washed in SO_2 water (three 10-minute rinses) and subsequently in distilled water for 15 minutes. For mitotic preparations, root tips were squashed on slides with a drop of 45% acetic acid. Coverslips were removed after freezing, and the slides were dehydrated in absolute ethanol and mounted in Euparal.

The DNA content of a somatic cell, the 2C-value, was determined using *Allium cepa* var. *Ailsa Craig* as a reference standard (2C = 33.55 pg; Bennett and Smith 1976) and were expressed in picograms (pg) and megabase pairs (Mbp) assuming 1 pg = 978 Mbp (Doležel et al. 2003). Around 40–50 telophase nuclei per individual were measured, with at least ten individuals analyzed for each parental inbred line and F_1 hybrid.

DAPI banding

Primary root tips were excised from germinated seeds, treated with 0.002 M 8-hydroxyquinoline at room temperature for 3 hours, fixed in a 3:1 ethanol–acetic acid solution, and stored at 4 °C. Fixed root tips were washed in 0.01 M citric acid–sodium citrate buffer (pH 4.6), digested with 2% cellulase (Onozuka R10, Merck) and 20% pectinase (Sigma) at 37 °C for 45 minutes, and squashed onto slides with a drop of 45% acetic acid. Coverslips were then removed by freezing, and the preparations were air-dried.

DAPI (4',6-diamidino-2-phenylindole) banding was performed as described by Sumner (1990), with minor modifications. Mitotic preparations were incubated in methanol in Coplin jars for 2 hours, then washed with McIlvaine citrate buffer. A drop of 2 µg mL DAPI solution was applied over the preparations, covered with plastic coverslips, and incubated at room temperature in a humid, dark chamber for 1 hour. The preparations were then washed alternately with distilled water and McIlvaine buffer and air-dried. Finally, slides were mounted with antifade medium (VectaShield, Vector Labs) and incubated at 37 °C in the dark for two days. Observations were made using a Leica DMLB epifluorescence microscope. Images were captured with a Leica DC 250 digital camera and analyzed using Leica IM 1000 software (version 4.0).

Heterochromatin content was estimated from mitotic metaphase chromosome images by analyzing at least three cells from a minimum of five individuals per inbred line and hybrid. The heterochromatin contents were expressed as the percentage of knob heterochromatin relative to the total chromosomal length, estimated using MicroMeasure software version 3.3 (Reeves and Tear 2000).

Data analysis

Statistical analyses were performed using Microsoft Excel with the Data Analysis ToolPak and SPC add-in. Statistical significance was set at $p < 0.05$, a threshold considered acceptable for verifying the significance of genetic effects (Resende and Alves 2022). DNA content and VCL were compared between parental lines pairs and between parental lines and their F_1 hybrids using Tukey's test ($\alpha = 0.05$; pairwise differences were considered significant at $p < 0.05$, 95% CI), as follows: (1) O2M line, Floury-2 line, and O2M × Floury-2 F1 hybrids; and (2) O2M line, Gaspé line, and O2M × Gaspé F1 hybrids. In addition, Pearson's correlation coefficient was calculated to assess the relationships between mean 2C-value and VCL, 2C-value and heterochromatin content, and VCL and heterochromatin content across parental lines and hybrids.

RESULTS AND DISCUSSION

Maize F_1 hybrids were successfully obtained for crossing of line O2M with Floury-2and with Gaspé. Average VCL values of the parental lines and their hybrids are shown in Table 1. Floury-2 showed the highest mean value (97.6 days), followed by the F1 hybrid O2M × Floury-2 (68.1 days) and O2M (65.0 days); Floury-2 exhibits a significantly higher VCL than that of O2M × Floury-2 ($p = 0.001$) and O2M ($p < 0.001$), and the VCL of the latter is significantly different from the hybrid ($p < 0.001$) (Figure 1a). Similarly, the line O2M shows a higher mean VCL (65.0 days) than O2M × Gaspé (57.7 days) and Gaspé (45.1 days); significant differences were detected between O2M and Gaspé ($p < 0.001$) and between the hybrid and both parentals ($p < 0.001$) (Figure 1b). In this survey, the VCL of hybrid O2M × Floury-2 appears similar to that of O2M, while a less pronounced trend is observed between this parental and O2M × Gaspé (Figures 1a, b). These patterns suggest that the maternal line O2M may contribute more to VCL variation that the paternal lines. Further

Table 1. Mean values of vegetative cycle length (VCL), nuclear DNA content (2C-value), number of knobs, and heterochromatin content for parental inbred lines and their F_1 hybrids. Standard deviation indicated in parenthesis

	Floury-2 line	O2M × Floury-2 (F_1)	O2M line	O2M × Gaspé (F_1)	Gaspé line
VCL (days)	97.6 (2.05)	68.1 (2.44)	65 (1.33)	57.7 (2.58)	45.1 (1.16)
2C-value (pg)	7.04 (0.29)	6.68 (0.1)	6.44 (0.16)	6.15 (0.23)	5.88 (0.41)
2C-value (Mpb)	6885	6533	6298	6015	5750
Number of knobs	10	8	6	5	4
Heterochromatin content (%)	12.12 (0.18)	9.89 (0.11)	7.12 (0.23)	5.74 (0.21)	4.91 (0.28)

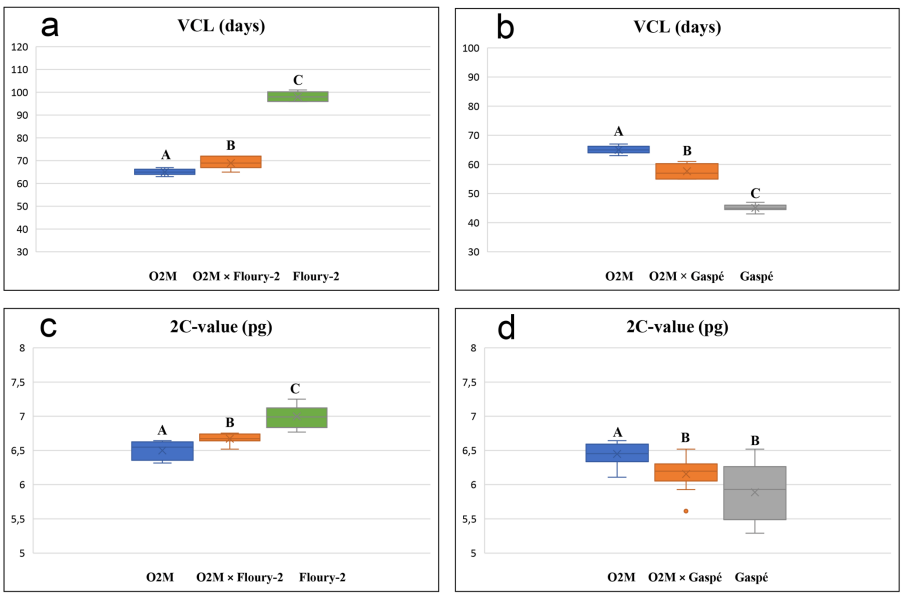

Figure 1. Box-plot graphs. **a:** Comparison of vegetative cycle length (VCL) among the O2M line, Floury-2 line, and their F1 hybrids. **b:** Comparison of VCL among the Gaspé line, O2M line, and their F1 hybrids. **c:** Comparison of 2C-value among the O2M line, Floury-2 line, and their F_1 hybrids. **d:** Comparison of 2C-value among the O2M line, Gaspé line, and their F1 hybrids. Groups labeled with different letters differ significantly (Tukey's test, $p < 0.05$). Whiskers represent standard deviation. pg: picograms.

studies are needed to elucidate the genetic basis of this pattern. The intermediate VCL values observed in these hybrids suggests that flowering time reflects the combined genetic contributions of both parental lines, consistent with the additive inheritance pattern described by Buckler et al. (2009). Recent genomic analyses in maize have revealed a complex architecture largely driven by additive effects (Fan et al. 2024), the plasticity of flowering time across environments (Michel et al. 2022), and candidate genes and SNPs associated with flowering time (Ran et al. 2024). These findings highlight the multifactorial regulation of flowering time in maize. Similar effects have been reported in other species: in rice, flowering time tends to be earlier in hybrids remaining largely governed by additive genetic effects (Tomé et al. 2025) and, in *Eucalyptus* hybrids, additive effects also play a predominant role with practical implications for breeding (Ramalho et al. 2023).

Significant differences in average DNA content (2C-value) were found between Floury-2 (7.04 pg) and O2M (6.44 pg; $p < 0.001$), as well as between Floury-2 and the hybrid (6.68 pg; $p = 0.0013$); whereas O2M and O2M × Floury-2 yielded barely significant differences ($p = 0.0478$) (Table 1; Figure 1c). While O2M and Gaspé (5.88 pg) differed significantly ($p = 0.001$), their hybrid showed an intermediate value (6.15 pg) that differed significantly from O2M ($p = 0.013$) but did not differ significantly from Gaspé ($p = 0.318$) (Figure 1d). The intermediate genome size observed in F_1 hybrids also suggests an additive genetic contribution from both parents, in consistence with previous findings in both natural and artificial hybrids of various plant species (Marques et al. 2012, Pellicer et al. 2013, Ferrari et al. 2019, Zagorski et al. 2020, Matiello and Prochno 2025).

In addition, the correlation between VCL and DNA content average values, was positive and significant (r = 0.891; 95% CI: 0.04–0.99; p = 0.042). This result is consistent with previous reports in diverse maize landraces (Realini et al. 2016, 2021, Bilinski et al. 2018), suggesting a mechanistic link between flowering time and DNA content where larger genomes are associated with slower growth and delayed flowering (Greilhuber and Leitch 2013). Although present findings suggest a potential role of genome size in determining VCL in maize, they should be interpreted within the restricted genetic scope of this study.

DAPI banding is a commonly used technique for chromosomal studies at various taxonomic levels; for instance, Santos et al. (2025) recently analyzed mitotic metaphases to characterize interspecific *Passiflora* hybrids. In the present study, this technique allowed detecting variation in both number and size of heterochromatic knobs (evidenced as DAPI+ bands) among the parental lines and both hybrids (Table 1, Figure 2). While, qualitatively, O2M displayed two large and four medium-sized knobs (Figure 2a, d), O2M × Floury-2 presented five large and three medium-sized knobs (Figure 2b), and Floury-2 exhibited eight large and two medium-sized DAPI+ bands (Figure 2c). The hybrid O2M × Gaspé showed three medium-sized plus two small-sized knobs (Figure 2e) and Gaspé showed four small-sized knobs (Figure 2f). When the heterochromatin content was measured, Floury-2 showed the highest percentage (12.12%), followed by O2M (7.12%) and Gaspé (4.91%); both hybrids exhibited intermediate contents (Table 1). This result is consistent with the variation observed in DAPI banding patterns. Briefly, present results indicate that the late flowering line (Floury-2) presents the highest content of knobs heterochromatin, in contrast fewer and smaller knobs were encountered in the early flowering Gaspé. The survey of Carvalho et al. (2022) reported no association between the number of knobs and the flowering time in hybrids derived from commercial maize lines. These different results may stem from the use of maize lines with distinct genetic backgrounds and from the estimation of heterochromatin content which integrates both the number and size of knobs. This approach has proved useful in describing cytogenetic variability in maize landraces (Fourastié et al. 2017, Realini et al. 2018).

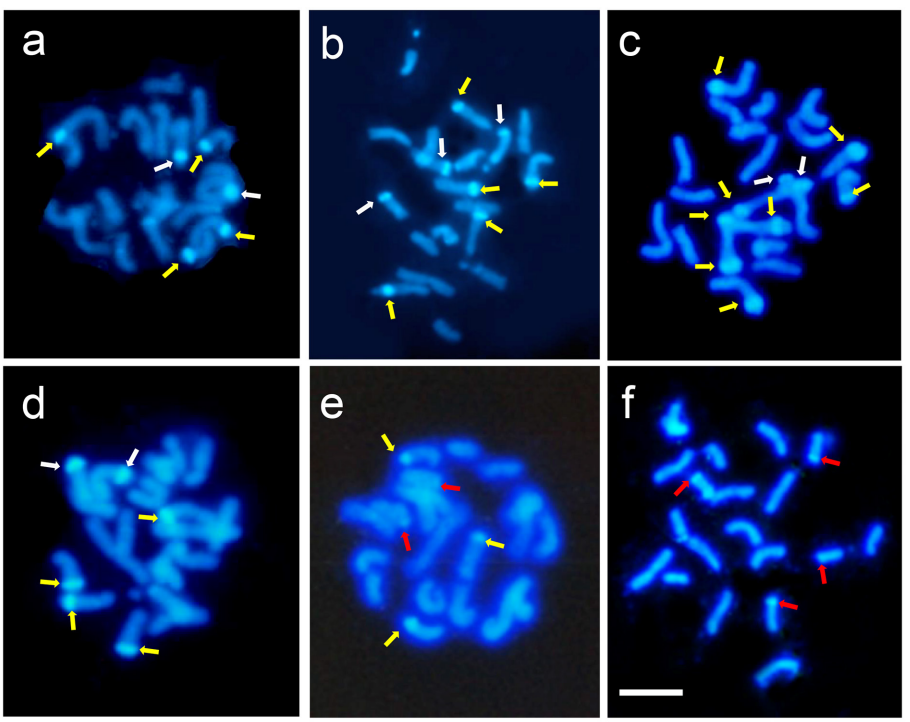

Figure 2. DAPI banding: Knobs as DAPI+ bands in mitotic metaphase chromosomes of maize. **a, d:** O2M line, showing two large and four medium-sized DAPI+ bands. **b:** O2M × Floury-2 F_1 hybrid, with five large and tree medium-sized DAPI+ bands. **c:** Floury-2 line, displaying eight large and two medium-sized DAPI+ bands. **e:** O2M × Gaspé F_1 hybrid, with tree medium-sized and two small-sized DAPI+ bands. **f:** Gaspé line, exhibiting four small-sized DAPI+ bands. White arrows point to large knobs, yellow arrows indicate medium-sized knobs, and red arrows show small knobs. Bar: 10 µm.

In maize landraces, this variation in knob heterochromatin content has been associated with differences in genome size (Poggio et al. 1998, Fourastié et al. 2017). Likewise, Jian et al. (2017) reported significant genome size differences between tropical and temperate inbred lines, with a moderate positive correlation between genome size and the abundance of knob repeats. In the present study, a significant positive correlation was found between the mean knob heterochromatin content and the average 2C-values throughout parental lines and hybrids ($r = 0.889$; 95% CI: 0.04-0.99; $p = 0.043$). In addition, variation in the abundance of transposable elements is thought to play a major role in DNA content variation, since long terminal repeats (LTR) retrotransposons, which represent more than 70% of the nuclear genome, differ in both copy number and chromosomal distribution in maize (SanMiguel and Bennetzen 1998, Meyers et al. 2001, Lamb et al. 2007, Silva et al. 2020). As well, numerical polymorphism of Bs represents another important source of genome size variation (McClintock et al. 1981, Poggio et al. 1998, Fourastié et al. 2017, González et al. 2022). The negative association between Bs and knob heterochromatin, reported in the literature has been interpreted as an interaction contributing to the maintenance of an optimal nucleotype-sensu (Bennett 1972, González and Poggio 2021). This knob–Bs relationship may reflect intragenomic conflicts between repetitive and selfish elements, which could also affect cell cycle duration, the VCL, and adaptive responses across environmental gradients (González and Poggio 2021). Recently, Ruas et al. (2025) reported a trend towards delayed male flowering as increasing Bs´ dosage in several maize lines and hybrids.

Present results also revealed a highly significant and positive correlation between the average heterochromatin content and VCL throughout parental lines and hybrids ($r = 0.944$; 95% CI: 0.37-0.99; $p = 0.016$). The adaptive significance of genome size and heterochromatin variation has been documented in Andean maize landraces, where both cytological features decrease with cultivation altitude (Fourastié et al. 2017). Likewise, in high-altitude Bolivian landraces, genome downsizing was associated with rapid vegetative growth and early flowering, suggesting an adaptation to shorter growing seasons (González and Poggio 2021). Such a relationship has been attributed to two mechanisms: a physical effect influencing cell volume and DNA replication duration, and a regulatory effect in which repetitive elements modulate gene expression (Meagher and Vassiliadis 2005, Comertpay et al. 2019). Maize flowering time is also influenced by major regulatory genes (Buckler et al. 2009, Meng et al. 2011, Navarro et al. 2017, Liang et al. 2019) and by variation in LTR retrotransposons (Lai et al. 2017). Therefore, all of the above physical and regulatory processes, contribute to phenological diversity. In this context, present results suggest that, in maize, germplasms with lower knob heterochromatin content could be useful inputs for breeding programs aiming at obtaining earlier-flowering genotypes.

According to Bilinski et al. (2018), natural selection on DNA content may indirectly act through its influence on the rate of cell production. The duration of the S-phase is known to be affected by nucleotypic effects associated with the amount and volume of nuclear chromatin, as well as by heterochromatin content, which modulates the accessibility of replication origins (Gregory 2001, Francis et al. 2008, Wear et al. 2017). In maize, it has been reported that the densely packed knob heterochromatin is among the last to replicate during S-phase (Pryor et al. 1980, Bass et al. 2015). Within this conceptual framework, the positive correlations observed among VCL, DNA and knob heterochromatin contents, jointly suggest that knob abundance could influence cell cycle duration and flowering time, contributing to maize adaptation. Although based on restricted germplasms samples, current results also suggest that heterochromatin content may serve as an indirect cytogenetic indicator of flowering time. In addition, the present contribution underscores the value of integrating cytogenetic data quantification with hybrid analyses as an accessible strategy for breeding maize suited to diverse environments.

DATA AVAILABILITY

The datasets generated and/or analyzed during the current research are available from the corresponding author upon reasonable request.

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