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Article

Augmented GWAS of Dizygotic Twinning in a New Sample of Mothers of DZ Twins From Australia and New Zealand Highlights *CLYBL* as a New Gene Influencing DZT

Scott D. Gordon[1], Jessica M. Adsett[1], Susan E. List-Armitage[1], Erika de Guzman[2], Grant W. Montgomery[3,4], Emma Vlasiuk[5], Martin A. Kennedy[5], Nikki Hubers[6,7] and Nicholas G. Martin[1]

[1]Department of Genetics and Computational Biology, QIMR Berghofer Medical Research Institute, Australia; [2]Australian Translational Genomics Centre, Queensland University of Technology, Australia; [3]Institute for Molecular Bioscience, The University of Queensland - St Lucia Campus, Australia; [4]Department of Health Science and Technology, Aalborg Universitet, Denmark; [5]Department of Pathology and Molecular Medicine, University of Otago, New Zealand; [6]National Centre for Register-based Research, Aarhus Universitet, Denmark and [7]SMARTBiomed Pioneer Centre, Denmark

Abstract

Dizygotic twinning (DZT) is an important marker of female fertility. The Twinning Genetics Consortium (TGC) recently reported the largest genomewide association meta-analysis (GWAMA) of DZT, identifying six genomewide significant loci and a seventh supported by functional evidence, in 8265 mothers of spontaneous DZ twins (MoDZTs) and 264,567 European-ancestry controls. To identify additional loci, we continued recruitment of MoDZTs in Australia and New Zealand (ANZ). Here, we report a GWAS of a new ANZ2026 sample (1598 MoDZTs and 5317 controls) and a GWAMA combining this sample with the previously reported ANZ2023 dataset. A new locus near *CLYBL* on chromosome 13 was tentatively identified. Because *CLYBL* is associated with vitamin B12 levels linked to fertility, it represents a plausible candidate. However, despite a 51% increase in ANZ cases, only one additional locus emerged, supporting the hypothesis that DZT is an oligogenic rather than highly polygenic trait. Meta-analysis with the TGC2024, the largest existing GWAS of DZT, strengthened signals at several loci, including HLA-G, while weakening others, such as *ZFPM1*.

Keywords: Dizygotic twinning; Fertility; GWAS

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Dizygotic twinning (DZT) is an intrinsically interesting trait and an important marker of female fertility. The Twinning Genetics Consortium (TGC) has recently published the largest genomewide association study (GWAS) meta-analysis of DZT to date, with 8265 mothers of spontaneous dizygotic (DZ) twins (MoDZTs) and 264,567 controls, supplemented by 26,252 DZ twins (independent of the MoDZTs and each twin contributing only half the information of an MoDZT) and 417,433 controls (hereafter TGC2024; Mbarek et al., 2024). All subjects were of European origin. Six significant loci (*GNRH1, FSHR, ZFPM1, IPO8, FSHB* and *SMAD3*) were identified in TGC2024, with two additional loci (17p13.1/SHBG and 6p22.1/HLA-G) strongly implicated by functional data.

Spontaneous DZT varies with maternal age; compared to mothers aged 18 years, the DZ twinning rate in mothers aged 35 is approximately four times higher, but it declines rapidly thereafter (Bulmer, 1970). These age-specific changes likely reflect declining ovarian follicle reserve (Beemsterboer et al., 2006). On the other hand, twinning is associated with increased risks to maternal and infant health (Monden & Smits, 2017; Santana et al., 2016; Santana et al., 2018). Thus, it is important to understand genetic mechanisms regulating DZ twinning and their relationship to fertility and reproductive aging.

In the first successful GWAS of DZ twinning, based on a GWAMA of 1980 MoDZT cases and 12,953 controls (Mbarek et al., 2016), we reported two significant loci associated with DZT, viz. *FSHB*, the structural locus for the follicle-stimulating hormone (*FSH*) beta subunit, and *SMAD3*, which regulates the ovarian response to *FSH*. We replicated these findings in gene-based testing of 'being a DZ twin' in the UK Biobank (Mbarek et al., 2019). Later, we expanded the Australian and New Zealand (ANZ) component of that study to 3273 MoDZT plus 24,009 controls of European ancestry (hereafter, ANZ2023; Gordon et al., 2023), which associated *ZFPM1* (Zinc Finger Protein, FOG Family Member 1) on chromosome 16 and *ADRB2* (β2-adrenergic receptor) on chromosome 5, in addition to replicating the previously reported associations with *FSHB* and *SMAD3* (Mbarek et al., 2016).

From 2021–2024, a new program to recruit additional MoDZT in ANZ has resulted in 1665 additional cases (post-QC) with an online survey and DNA collection for genotyping, while simultaneously QSkin2 became available, an extension of QSkin (Olsen et al., 2012) from which most of the Australian controls for TGC2024 had been drawn. This allowed us to add an additional set of 5317 population-matched controls, genetically unrelated

Corresponding author: Nicholas G Martin; Email: nick.martin@qimrberghofer.edu.au
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(or only distantly related) to the existing set that we had used previously. Both were genotyped on Illumina GSA v3 genotyping chips (collectively, ANZ2026).

The purpose of this article is to describe the collection of a new sample of MoDZTs in Australia and New Zealand (hereafter, ANZ2026) and to evaluate through meta-analysis its augmentation of power to detect DZT genes above that of ANZ2023 and TGC2024.

Methods

The analysis consists of two case/control GWAS where the cases were MoDZT and 'controls' were not known to be cases. Also, cases were surveyed and excluded if the twin pregnancy had involved assisted reproductive technology (ART), and controls were excluded based on close identity-by-descent (IBD) ($\hat{\pi} > 0.1$) to any cases. No phenotype information was used apart from zygosity of the twins, and ART status.

Australia New Zealand Study

Cases. Recruitment of MoDZTs was by public appeal in both countries, including radio and TV publicity following publication of the TGC2024 paper (Mbarek et al., 2024), supplemented by social media and appeals via mother-of-twins clubs. Participants were directed to a country-dependent public website controlled by the respective research group (Australia: https://www.qimrb.edu.au/studies/genetics-of-non-identical-twinninghttps://www.qimrb.edu.au/studies/genetics-of-non-identical-twinning; New Zealand: https://www.otago.ac.nz/gene-structure/research/are-you-the-mother-of-non-identical-dizygotic-twinshttps://www.otago.ac.nz/gene-structure/research/are-you-the-mother-of-non-identical-dizygotic-twins) with a summary of the study and link for interested volunteers, which proceeded to more detailed information, a consent form, collection of personal and contact details, and a link to the survey itself, as approved during institutional ethics review. The survey consisted of two corresponding questionnaires hosted by the commercial service Qualtrics (one per country) with a unique per-participant link and containing questions about the twins' zygosity; use or otherwise of ART (and the type and reason for use); current maternal age; maternal age at each confinement (including the twin and other pregnancies), and the history of other twins in the family. Volunteers whose twins satisfied our criteria for dizygosity (opposite sex, or if same-sex then discordance in physical features plus never mistaken for each other by teachers, grandparents) and who had not been conceived using ART, were invited to donate a saliva sample via a kit (Isohelix or ThermoFisher) mailed to them (with buffer solution; printed instructions; and reply-paid envelope) and returned to the lab in their country.

In Australia there was a total of 2464 respondents of whom we judged 2350 to be MoDZT (based on being opposite sex, having had genetic testing, and/or degree of physical similarity). Of the remainder, 358 (15%) of DZ twin pregnancies involved ART and were excluded. In New Zealand we recruited 836 MoDZTs of whom 115 (14%) had used ART and were excluded. DNA was obtained from 533 cases of whom 487 were genotyped successfully (46 fails), and 426 after phenotypic screening, as above.

DNA for all ANZ cases who donated saliva was extracted at the QIMR Berghofer (by SL-A) and samples genotyped at the Australian Translational Genomics Centre in Brisbane (ATGC, by EdeG) using the Illumina Global Screening Array (GSAv3, Illumina model GSAMD-24v3-0-EA_20034606). DNA samples were obtained from 1527 MoDZTs (1502 passing pre-genotyping QC) of whom 1480 were successfully genotyped (22 fails), reducing to 1425 after again applying the ART and zygosity filters described above. Fifteen cases identified from survey data for the QSkin2 control set (see below) were added (after corresponding filtering). After exclusions for non-European ancestry ($n = 65$) and relatedness (see below) to ANZ2023 ($n = 120$), 1287 Australian and 393 NZ cases were left for analysis, making a total of 1680 cases for ANZ2026. The relatedness exclusions are still available for future updated datasets.

Further QC. Unexpectedly weak associations were seen in initial versions of the ANZ2026 GWAS. Past experience from the ANZ2023 analysis showed that phenotyping error for even a small fraction of the case group could have a noticeable effect on GWAS power, similar to what we observed in ANZ2026, so we decided that it was prudent to identify and remove any such errors as much as practically possible. To this end we recontacted a substantial fraction of case study participants using email, SMS and/or telephone, to confirm zygosity and ART status, independently of the online questionnaire. Of particular interest were mothers reporting same-sex twins, as their zygosity is not infrequently wrongly reported. This included asking the mothers to describe in their own words how they decided the twins' zygosity, and to supply photographs of their twins, when they were willing to do so, for review by project staff. The GWAS presented here was run after removing ineligible individuals identified in this way. At the cutoff date, for the Australian sample (the smaller NZ sample had a broadly similar outcome): out of 1196 mothers recontacted, there were 1018 confirmed DZ and no ART use (so still eligible), 53 with probable MZ twins (excluded), 5 used ART (excluded), 37 incomplete responses (DZ but no response regarding ART); and 81 non-responses even after follow-up. Cases who did not respond to recontact, or for whom there was not a clear result, were retained, as the overall error rate was low, and removing these would have substantially reduced sample size without greatly improving purity of the phenotype. We established in ANZ2023 (Gordon et al., 2023) that even an imperfect phenotype is better for power than a perfect phenotype with disproportionately smaller sample size; for example, we expect <1 unidentified ART case among the 81 nonresponders based on the incidence above. Overall, this process across Australia and NZ removed 82 (~5%) out of an initial 1680 cases at the analysis stage, leaving a final number of 1598. However, the practical effect on the GWAS peaks was quite minor and inconsistent and is barely visible in Manhattan plots. In summary, we see neither overly weak association nor strong false association in ANZ2026 due to poor phenotyping of cases.

Controls. As controls for combined ANZ cases, we used participants in the existing QSkin2 study, a second phase of the QSkin1 study (Olsen et al., 2012) from which we had drawn controls for ANZ2023. QSkin2 is a randomly selected population-based study of skin health from a later cohort of the same Australian population as QSkin1 and MoDZT cases (Jayasinghe et al., 2025). The existing health survey asks whether participants are twins themselves or have other twins in the family, and about zygosity, and use of ART. This enabled us to identify 15 eligible cases (analogous definition to the main case cohort), which we combined with the ascertained cases, and 5317 controls (no DZ twinning history reported) after exclusions due to ancestry and relatedness to cases and ANZ2023 (same rules as cases). QSkin2 control samples were genotyped on the same GSA v3 model, and at the same facility (ATGC), as the case samples.

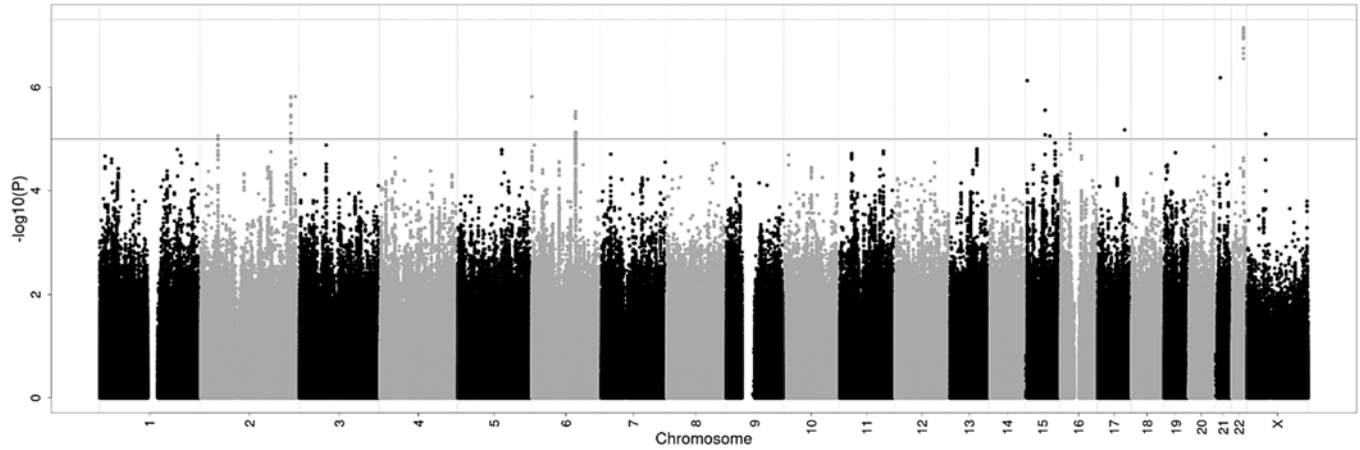


Figure 1. Manhattan plot for ANZ2026 (1598 MoDZT cases, 5317 controls) shows association *p* values calculated by SAIGE, as –log10 (p), after removing markers with minor allele frequency (MAF) < 1% or Rsq < .3. Horizontal axis is genomic position in base pairs, increasing toward the right (hg19). The lower and upper horizontal links indicate suggestive ($p = 10^{-5}$) and genomewide significant ($p = 5 \times 10^{-8}$) levels respectively.
Note: ANZ 2026, Australian and New Zealand 2026; MoDZT, mothers of spontaneous DZ twins.

Data Cleaning and Imputation

ANZ batches of genotyping were called and QC'd as per standard protocols (within and post GenomeStudio, including dropping individuals with <97% call rate and single nucleotide polymorphisms [SNPs] with GenTrain score < 0.6, minor allele frequency (MAF) < 1%, *p* (HWE) < 10^{-6} and call rate < 95%).

A principal components analysis (PCA) for each population was run using *smartpca* (in EigenSoft 7.2.1; original version described in Price et al., 2006) with samples from 1000 Genomes Phase 3 populations used to define the PC axes; all of our genotyping was projected onto those axes. For the ANZ sample, individuals with PC1 and/or PC2 >6sd from the mean of European reference populations were excluded as ancestry outliers (~3% for cases, ~1% for QSkin2 controls).

Observed relatedness was dealt with, depending on the detailed information in registration records, either by (1) dropping duplicates; (2) using SAIGE to accommodate the IBD; or (3) IBD exclusions applied between cases and controls, or between the TGC2024 and ANZ2026 analyses.

In all association analyses, PC1-PC5 were added as covariates, although based on previous experience, there is minimal population stratification after the stated exclusions.

Imputation was run on the Michigan Imputation Server using Eagle (phasing; Loh et al., 2016), minimac4 (imputation; Das et al., 2016) and the HRCr1.1 reference panel, for observed markers passing QC in all relevant batches ($N = 474{,}524$ for ANZ). Filtering on MAF ≥ 0.01 and $r^2 \geq .3$ left a total of $N = 7{,}970{,}886$ potential analysis SNPs for ANZ2026.

Association Analysis (GWAS)

Although ANZ controls closely related to cases ($\hat{\pi} \geq 0.1$) were removed to improve the quality of controls, substantial relatedness was still observed within the case and control groups, which was not unexpected since recruitment for ANZ2026 cases partly relied on recontacting participants in previous family-based studies. Reducing the analysis to an 'unrelateds only' analysis would have considerably reduced the sample size and power of the analysis. Hence, the association tests were run using the R package SAIGE (v 0.45) (Zhou et al., 2018), which uses a Generalised Linear Mixed Model (GLMM) to model relatedness as a part of the initial stage of its analysis and also has a high tolerance to unbalanced case/control ratios — as applies here. SAIGE 'Step 1' (the null GLMM fit) was run with, as input, the phenotype and covariates plus a genomewide set of observed genotypes for markers available in all batches of genotyping (thinned to 118,630 for ANZ2026). The phenotype was the case/control variable, coded 1 = case ($N = 1598$), 0 = control ($N = 5317$). Covariates were the first five genetic principal components (PCs) from the *smartpca* ancestry PCA run (PC1-PC5). SAIGE 'Step 2' was then run in parallel (for speed) across blocks of ~100,000 markers with as input the Variance Ratio and Generalized Linear Mixed Model Association Tests (GMMAT) model estimate files from 'Step 1' plus imputed genotypes (against the HRC r1.1 reference panel, females-only, for the X chromosome) for that block of markers. Step 2 used a MAF cutoff of 0.001 and minimum minor allele count (MAC) of 5. The results for these blocks were then concatenated and filtered based on imputation metadata. For the purposes of results presented here only markers with MAF ≥ 0.01 (1%) and $r^2 \geq .3$ were retained. mBAT-combo (Li et al., 2022) was used to perform gene-based association tests using the supplied 1000 European reference genome for linkage disequilibrium (LD) information.

Results

The results of the SAIGE SNP-based GWAS analysis of our new sample (hereafter referred to as ANZ2026) comprising 1598 MoDZT cases and 5317 QSkin2 controls (Figure 1) evince little apparent support for the hits from our ANZ2023 study (Gordon et al., 2023) or the TGC2024 study that incorporates ANZ2023 (Mbarek et al., 2024). The $\lambda = 0.95$ for the Q-Q plot being less than 1.0 reflects the sparse association signals (Figure 2). This was despite extensive checking of phenotypic QC steps in both cases and controls. As a further check, we interchanged the control samples between ANZ2023 and ANZ2026 and reran the GWAS, with no obvious changes save those due to relative sample sizes. To further assess whether there was any gain in signal from ANZ2026 for DZT association we undertook a series of meta-analyses.

GWAS meta-analysis. GWAMA was performed, after appropriate QC steps, using weighting via the standard error method in Metal (Willer et al., 2010). Both SNP-based and gene-based results are presented. Meta-analyses were performed in various combinations to assess different aspects of results: (1) ANZ2026 + ANZ2023,

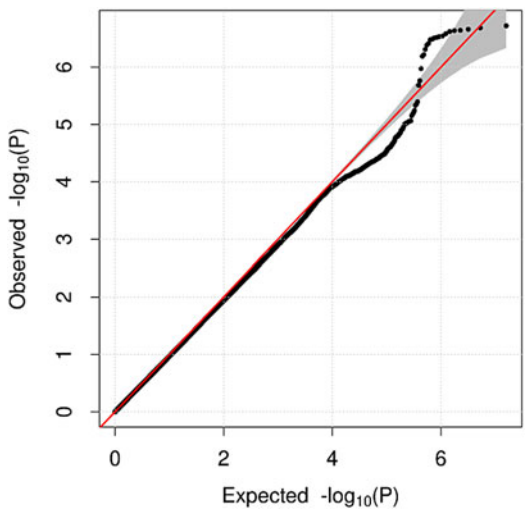


Figure 2. Quantile-Quantile (Q-Q) plots for the ANZ2026 case/control GWAS. Plotted after removing single nucleotide polymorphisms (SNPs) with Rsq <.3 or minor allele frequency (MAF) <1%, as per the Manhattan plots. Shows the observed *p* value (on the vertical axis) as a function of the expected *p* value (horizontal axis) at that position in the *p*-value ranking under the null hypothesis (i.e., case/control status is not associated with any marker; the red line). Lambda = 0.9523 (ANZ, 7,970,886 SNPs).
Note: ANZ 2026, Australian and New Zealand 2026.

to assess the increment in ANZ information (Figures 3 and 4); (2) ANZ2026 + TGC2024 (which already includes ANZ2023) to assess the increment to the global DZT genetics effort from our new collection (Figures 5 and 6). Results for the SNP-based tests are summarized in Table 1 and results for the gene-based tests, in Table 2. Adding ANZ2026 to ANZ2023 produces small increases in significance for six of nine top regions, including those we have most trust in (*FSHB, SMAD3, FSHR, GNRH1*). Adding ANZ2026 to TGC2024 produces similar improvements in significance of those four genes but the hit in *ZFPM1* slightly decreases in significance, while the hit in *IPO8* increases in significance. Another two regions (*HLA-G* and *CLYBL*), which have hovered around the significance threshold in our previous work, each slightly increases in significance, with *CLYBL* now exceeding the genomewide significance threshold. A regional association plot of this chr 13 locus is shown in Figure 7. Gene-based tests in mBAT-combo (Li et al., 2022) showed somewhat different results after adding ANZ2026 to ANZ2023, but highly similar results after adding ANZ2026 to TGC2024 (Table 2). At a genomewide significance threshold of $0.05/N_{\text{tests}} = 2.6 \times 10^{-6}$ conventionally used for gene-based tests, improvements in significance (i.e., decreased *p* value) were seen after adding ANZ2026 to TGC2024, for *ARL14EP* and adjacent *FSHB*, and *SMAD3*. On balance, we are satisfied that ANZ2026 does increase signal for DZT association, though less than we had anticipated.

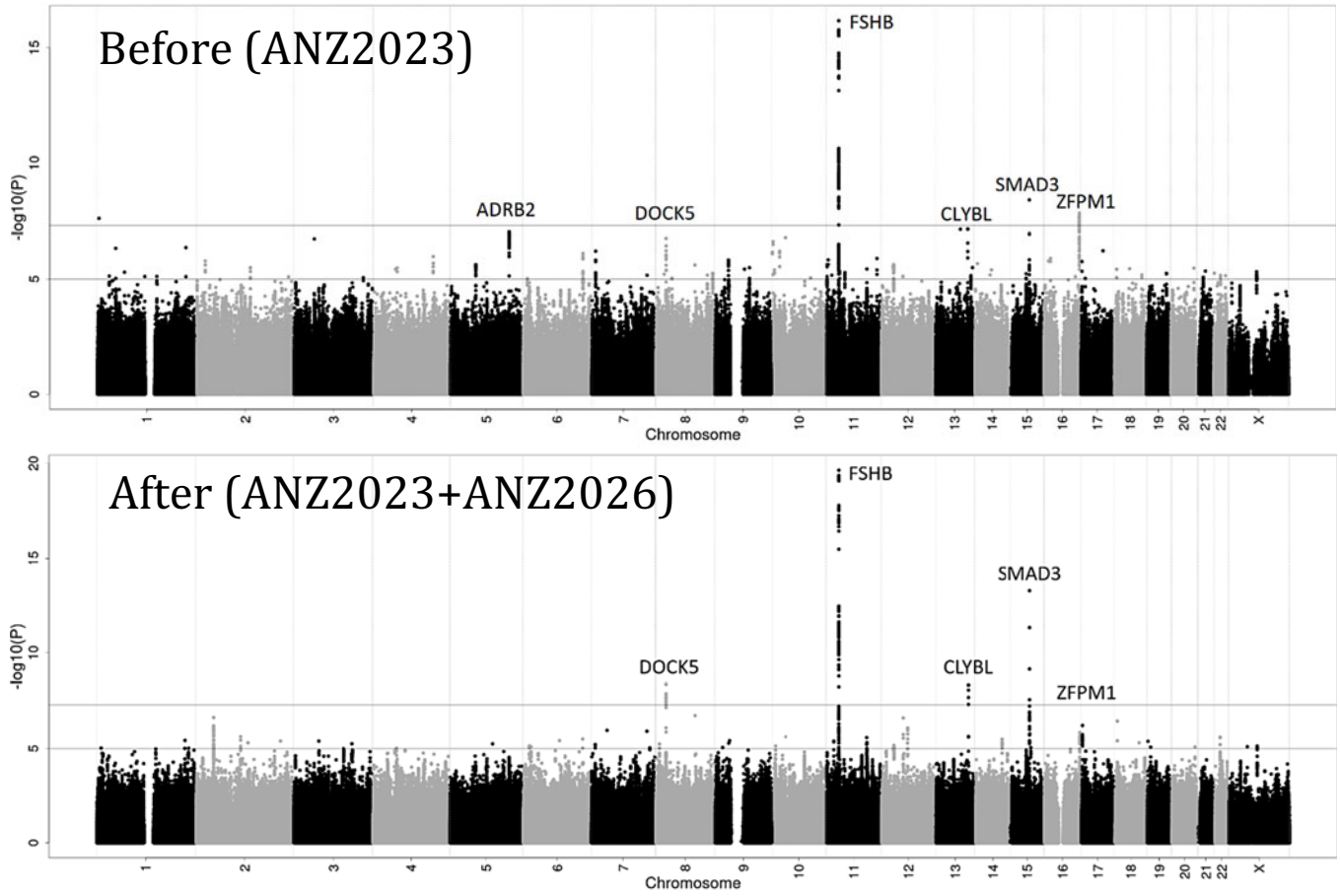


Figure 3. Manhattan plots for additive model (MoDZT vs control) for ANZ2023 alone (top panel) and ANZ2023 + ANZ2026 meta-analysis (bottom). Shows association *p* values calculated by SAIGE (as $-\log_{10}(p)$) after removing markers with minor allele frequency (MAF) < 1% or Rsq <.3. The lower and upper horizontal links indicate suggestive ($p = 5 \times 10^{-5}$) and genomewide significant ($p = 5 \times 10^{-8}$) levels respectively. Labels match the genes quoted for the adjacent peak in Table 1. Top panel shows the results for ANZ2023 (Gordon et al., 2023) *before* meta-analysis with new sample ANZ2026. Bottom panel shows results *after* that meta-analysis (ANZ2023 + ANZ2026).
Note: TGC2024, Twinning Genetics Consortium 2024; ANZ 2023, Australian and New Zealand [component] 2023; ANZ2026, Australian and New Zealand 2026.

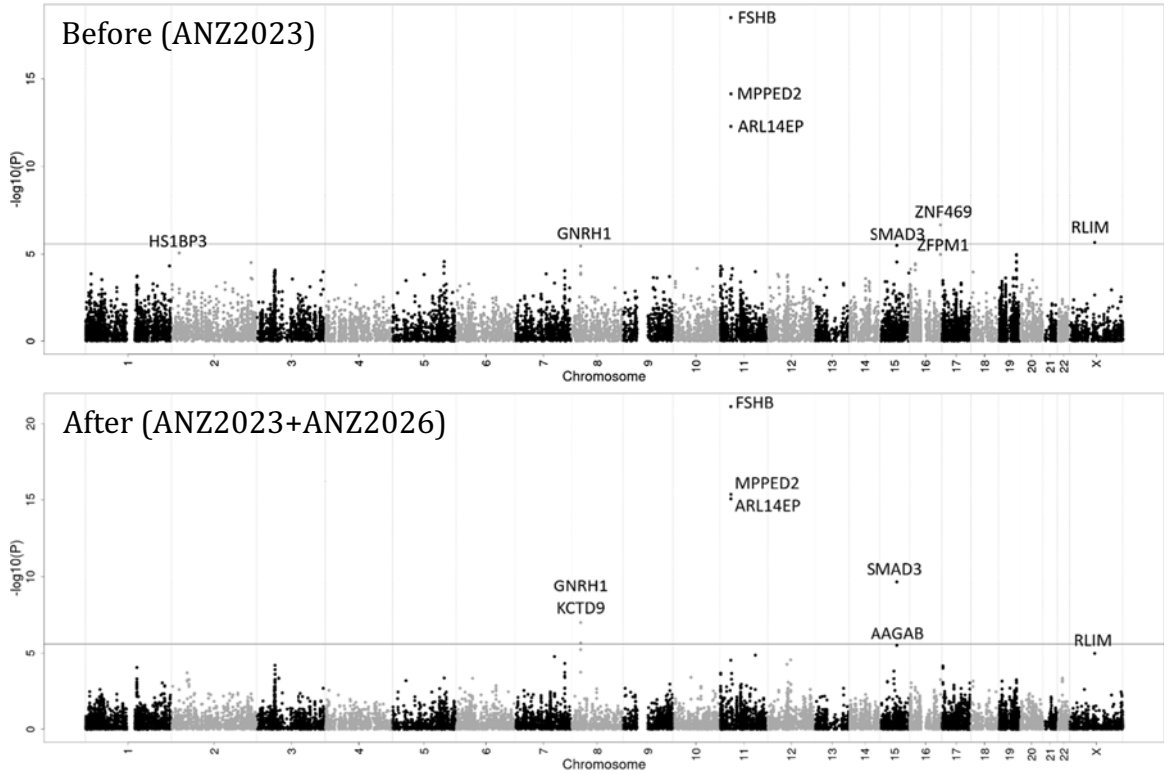


Figure 4. Manhattan Plots for gene-based tests in mBAT-combo (chr1-22, Li et al., 2022) or MAGMA (chr X) (de Leeuw et al., 2015) for ANZ2023 (Gordon et al., 2023, top panel) and ANZ2023 + ANZ2026 meta-analysis (bottom panel); showing composite gene-based *p* value (as $-\log_{10}$ (p)). The horizontal line is at $p = 2.56\text{x}10^{-6}$; $2.53 \text{ x } 10^{-6} = 0.05/N$ = number of tested genes = 19,559; 19,627 (respectively). Labels show the most-associated significant or near-significant gene(s) adjoining the label.
Note: ANZ 2023, Australian and New Zealand [component] 2023; ANZ2026, Australian and New Zealand 2026.

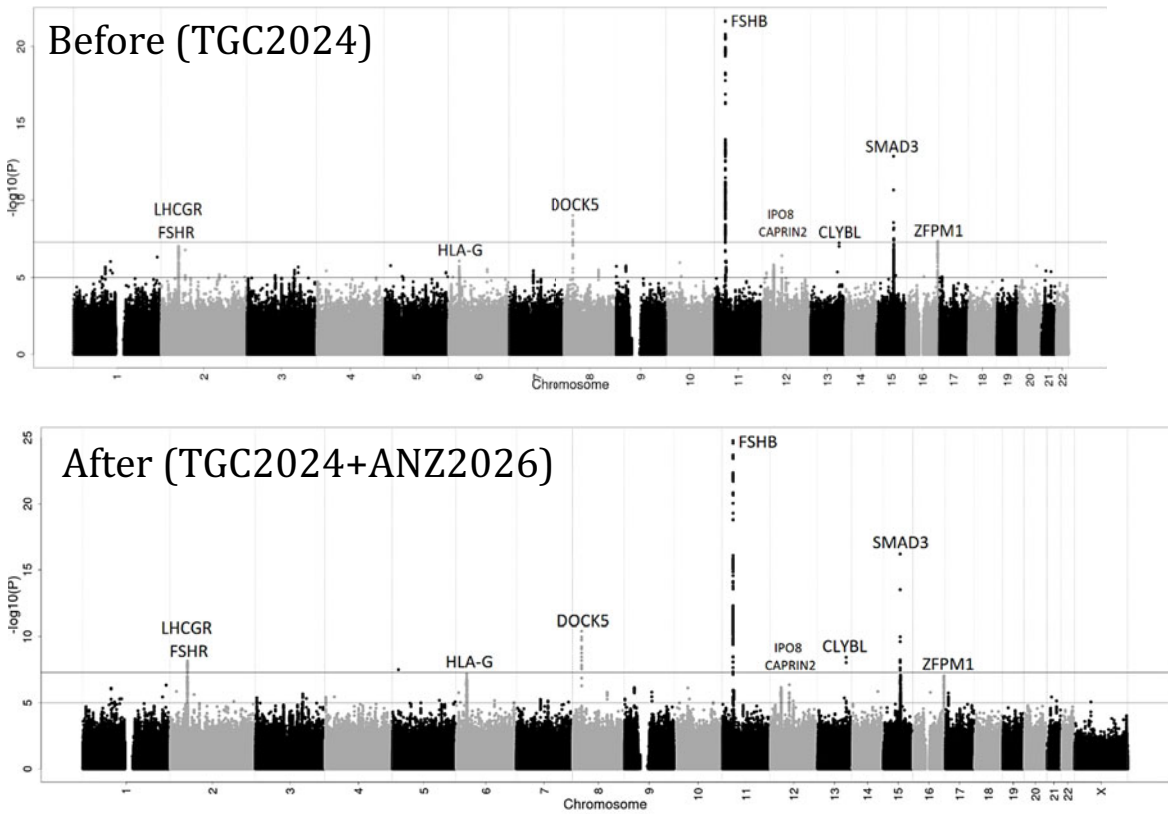


Figure 5. Manhattan plots for additive model (MoDZT vs. control) for TGC2024 (Mbarek et al., 2024; top panel) and TGC2024 + ANZ2026 meta-analyzed (bottom panel). Shows association *p* values calculated by SAIGE ($-\log_{10}$ transformed) after removing markers with minor allele frequency (MAF) < 1% or Rsq < 0.3. Genomewide significance threshold $p = 5 \text{ x } 10^{-8}$ is the upper line. Suggestive threshold 10^{-5} is the lower line. Horizontal axis is genomic position, measured in base pairs, increasing toward the right (hg19). Labels match the genes quoted for the adjacent peak in Table 1. Note that the X-chromosome was not analyzed for TGC2024, so that chromosome is purely ANZ2026.
Note: MoDZT, mothers of spontaneous DZ twins; TGC2024, Twinning Genetics Consortium 2024; ANZ2026, Australian and New Zealand 2026.

Table 1. Comparison of top hits — the most associated single nucleotide polymorphism (SNP) in the TGC2024 results except where not associated (*ADRB2*) in which case the most associated SNP in ANZ2023. Shown for ANZ2023 by itself and TGC2024 (which incorporates ANZ2023) both before and after adding ANZ2026 by meta-analysis

Likely gene (+alternate)	Chrom osome	Position bp (hg19)	P ANZ2023	P, ANZ2023 + ANZ2026	P TGC2024	P, TGC2024 + ANZ2026
ARL14EP (+FSHB)	11	30,226,356	1.68×10^{-16}	2.65×10^{-19}	2.17×10^{-22}	2.62×10^{-25}
SMAD3	15	67,437,863	3.97×10^{-9}	7.70×10^{-14}	1.40×10^{-13}	6.30×10^{-17}
FSHR (+LHCGR)	2	49,449,364	4.57×10^{-3}	8.77×10^{-4}	9.08×10^{-8}	1.80×10^{-8}
ADRB2	5	148,249,230	9.46×10^{-8}	3.00×10^{-5}	0.0859	0.114
HLA-G	6	29,817,617	0.286	0.0209	8.32×10^{-7}	7.11×10^{-8}
GNRH1 (+DOCK5)	8	25,267,103	9.08×10^{-7}	2.88×10^{-8}	9.07×10^{-10}	3.83×10^{-11}
CAPRIN2 (+IPO8)	12	30,919,517	5.30×10^{-4}	1.50×10^{-3}	1.48×10^{-7}	1.43×10^{-6}
CLYBL	13	100,559,123	7.16×10^{-8}	9.37×10^{-9}	5.51×10^{-8}	3.67×10^{-9}
ZFPM1	16	88,528,125	2.09×10^{-8}	3.12×10^{-6}	4.40×10^{-8}	9.13×10^{-8}

Note: TGC2024, Twinning Genetics Consortium 2024; ANZ 2023, Australian and New Zealand [component] 2023; ANZ2026, Australian and New Zealand 2026; RED, stronger signal (with ANZ2026 added); BLUE, weaker signal.

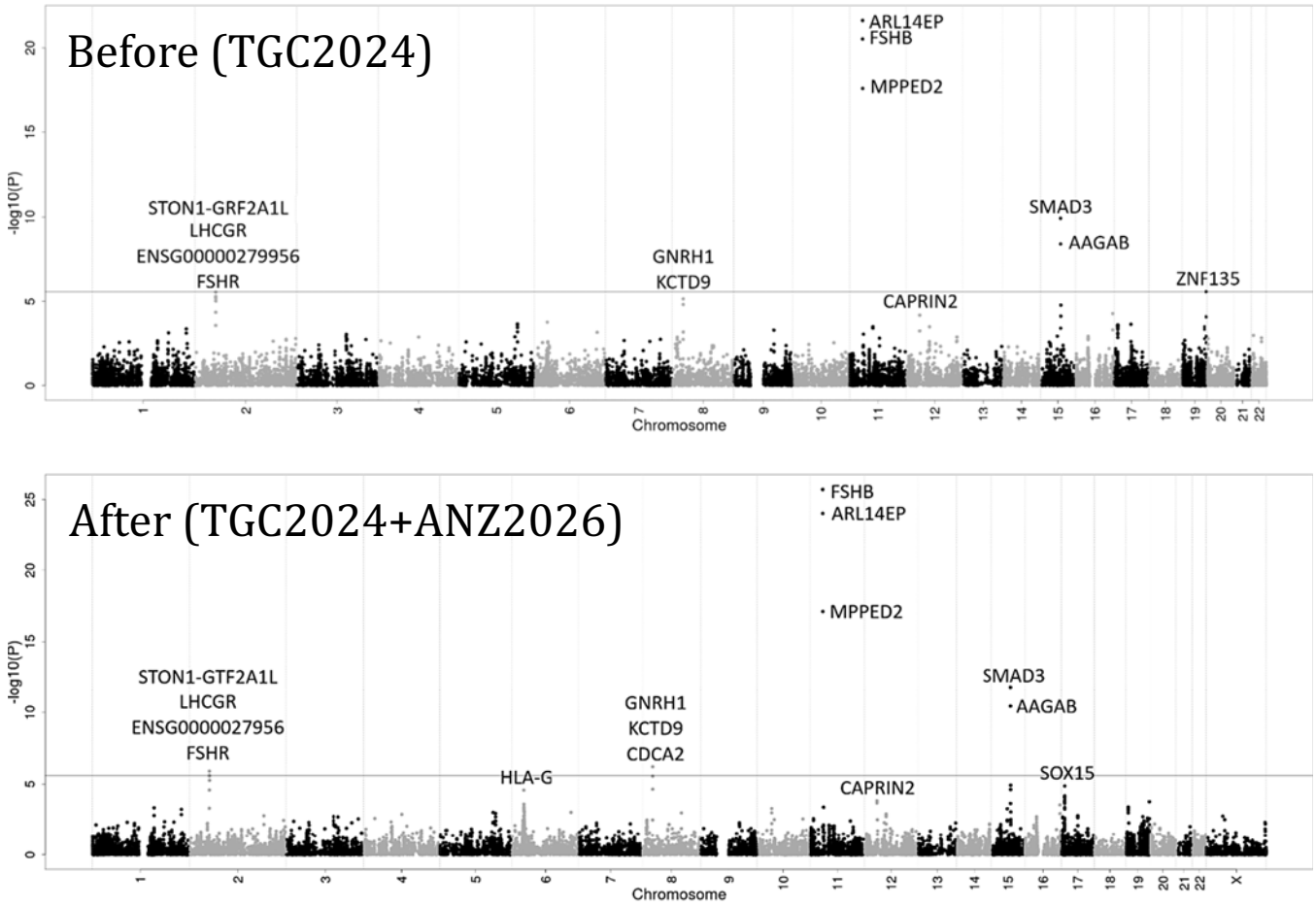


Figure 6. Manhattan plots for gene-based tests in mBAT-combo (chr1-22) or MAGMA (chr X) for TGC2024 and TGC2024 + ANZ2026. The horizontal line is at $0.05/N \approx 2.66 \times 10^{-5}$ and 2.54×10^{-5} respectively (N = number of tested genes = 19,651; 18,831). Labels show the most-associated significant or near-significant gene(s) adjoining the label. Top panel shows the results for TGC2024 (Mbarek et al., 2024) before meta-analysis with new sample ANZ2026. Bottom panel shows results after that meta-analysis (TGC2024 + ANZ2026). Note that the X-chromosome is not available in TGC2024 results, so chr X is just from ANZ2026.
Note: TGC2024, Twinning Genetics Consortium 2024; ANZ2026, Australian and New Zealand 2026.

Table 2. Comparison of mBAT-combo gene-based *p* values for 'top genes' in the genomewide association study (GWAS) results (the gene-based equivalent of Table 1) plus *FSHB, LHCGR, DOCK5, IPO8* (brackets), which sometimes score more highly than the nominal gene, and *ADRB2* (not highly associated in TGC2024). Showing ANZ2023 by itself; TGC2024 (which incorporates ANZ2023); and also after adding ANZ2026 by meta-analysis, as indicated

Likely gene(s)	Chromosome	Position (hg19) (Mbp)	P ANZ2023	P, ANZ2023 + ANZ2026	P TGC2024	P, TGC2024 + ANZ2026
ARL14EP (+*FSHB*)	11	30.23	5.11×10^{-13} (3.03×10^{-19})	8.67×10^{-16} (7.85×10^{-22})	2.47×10^{-22} (3.06×10^{-21})	9.46×10^{-25} (2.00×10^{-26})
SMAD3	15	67.44	3.29×10^{-6}	2.14×10^{-10}	1.17×10^{-10}	1.54×10^{-12}
FSHR (+*LHCGR*)	2	49.45	0.137 (6.35×10^{-3})	0.188 (5.26×10^{-4})	9.49×10^{-6} (5.14×10^{-6})	5.56×10^{-6} (2.48×10^{-6})
ADRB2	5	148.25	2.75×10^{-5}	4.14×10^{-4}	0.0769	0.0747
HLA-G	6	29.82	0.564	0.0321	1.66×10^{-4}	2.71×10^{-5}
GNRH1 (+*DOCK5*)	8	25.27	3.57×10^{-6} (1.48×10^{-4})	1.04×10^{-7} (1.75×10^{-4})	7.12×10^{-6} (1.71×10^{-3})	6.25×10^{-7} (3.31×10^{-3})
CAPRIN2 (+*IPO8*)	12	~30.9	8.32×10^{-4} (1.93×10^{-4})	3.41×10^{-3} (2.58×10^{-3})	6.45×10^{-5} (5.43×10^{-4})	1.49×10^{-4} (2.21×10^{-4})
CLYBL	13	100.6	4.66×10^{-4}	1.95×10^{-3}	9.01×10^{-3}	0.0102
ZFPM1	16	88.53	1.058×10^{-5}	4.91×10^{-3}	4.72×10^{-4}	1.18×10^{-3}

Note: TGC2024, Twinning Genetics Consortium 2024; ANZ 2023, Australian and New Zealand [component] 2023; ANZ2026, Australian and New Zealand 2026; RED, stronger signal (with ANZ2026 added); BLUE, weaker signal.

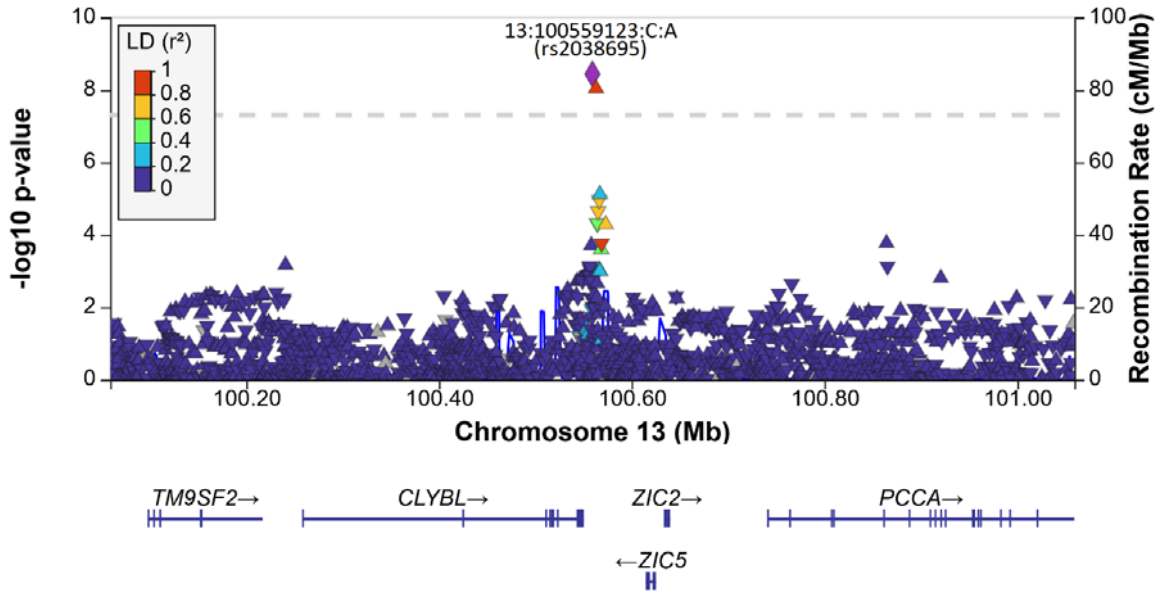


Figure 7. Regional association plot and gene annotation for *CLYBL* region on chromosome 13 (from TGC2024 + ANZ2026 meta-analysis). Plot prepared using LocusZoom.js (Boughton et al., 2021) with LD r^2 estimates derived from 1000 Genomes r3 European genotypes. Grey indicates lack of LD information versus the peak marker.
Note: TGC2024, Twinning Genetics Consortium 2024; ANZ2026, Australian and New Zealand 2026; LD, linkage disequilibrium.

An underlying assumption of polygenic inheritance is that liability is additive across risk loci, whereas it is possible that there are nonlinear effects such as epigenetic interactions. To probe this, we performed conditional GWAS analyses conditioning on the top two risk variants in *FSHB* and *SMAD3*, both singly and together. We hypothesized that there might be subgroups of MoDZT who are wild-type for these loci and whose risk arises from larger effects of the secondary loci such as *FSHR* or *GNRH1*. However, we found no obvious perturbations in significance or effect sizes of other loci in the conditional analyses (data not shown). From this we conclude that the additive model is not challenged, at least with the limited power available to us.

On this basis we proceeded to estimate DZT polygenic risk scores (PRS) in the ANZ2026 sample using weights from the TGC2024 GWAMA, which are entirely independent, and SBayesRC (Zheng et al., 2024), to optimize their combination. Distributions of these PRS are shown in Figure 8. Mean PRS for ANZ2026 MoDZT cases ($n = 1598$) was 0.0895 with *SD* 0.0688 and for controls ($n = 5317$) it was 0.0705 with *SD* 0.0701 (difference in means 0.0189; $t = 9585$, $p = 2.04 \times 10^{-21}$, one-sided).

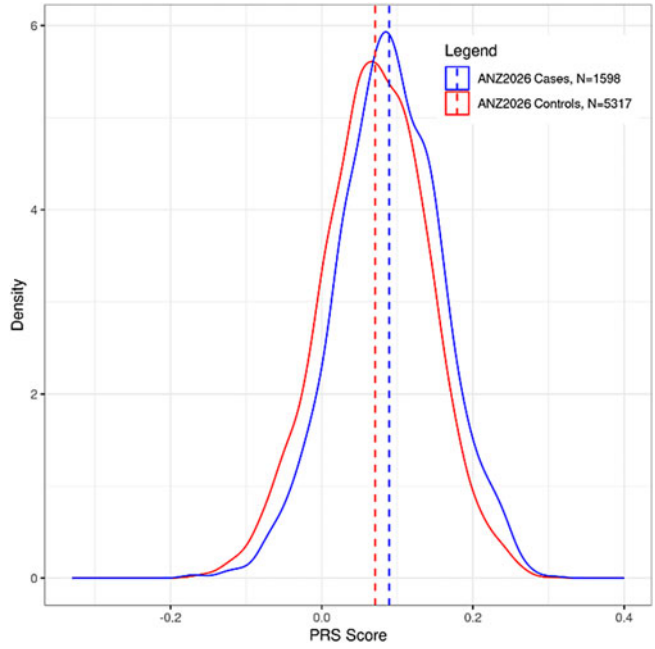


Figure 8. Predicting dizygotic twinning (DZT) status in ANZ2026 from a polygenic risk score (PRS) based on results of TGC2024, with optimization using SBayesRC (using a European UK Biobank LD reference, as TGC2024 is exclusively European origin). For chromosomes 1–22 only. Scores are unstandardized, shown as blue = cases, red = controls. PRSs for ANZ2026 MoDZT cases ($N = 1598$) have mean 0.0895, *SD* 0.0688 and controls ($N = 5317$) have mean 0.0706, *SD* 0.0701 ($t = 9.585$, $p = 1.02 \times 10^{-21}$, one-sided); a difference in means of 0.0189.
Note: TGC2024, Twinning Genetics Consortium 2024; ANZ2026, Australian and New Zealand 2026; MoDZT, mothers of spontaneous DZ twins; LD, linkage disequilibrium.

Discussion

The most interesting finding of our meta-analysis of ANZ2023 and ANZ2026 is our identification of a novel region, *CLYBL* on chromosome 13, (genomewide significant SNP *p* value 3.67×10^{-9}) and confirmation of previously reported key regions associated with DZ twinning — *GNRH1, FSHB, FSHR,* and *SMAD3* — which are involved in the hypothalamic-pituitary-ovarian axis. All of these regions had reached, or were close to, genomewide significance in our previous work.

Despite careful QC of the ANZ2026 sample, ensuring that the mother did not use ART and confirming the DZ zygosity, the increment in GWAS signal at previously established loci was very modest, and in fact some decreased. In particular, *ZFPM1* on chr 16, a novel region first identified in ANZ2023 (Gordon et al., 2023), fell to a suggestive level of significance in the meta-analysis, although genomewide significance was replicated in TGC2024. The lead SNP on chromosome 16 near *ZFPM1* (rs4584807), located in a regulatory region within the first intron of *ZFPM1*, has been shown to be in high LD with SNPs in regions associated with age at menopause (Mbarek et al., 2024), sex hormone-binding globulin (Ruth et al., 2020), and FSH concentrations (Chen et al., 2013). We are unable to explain why the signal was weaker than expected given the increased sample size and can only attribute this to chance or to an undetected error in QC.

One novel locus, *CLYBL* (Citramalyl-CoA Lyase) on chromosome 13, exceeded the genomewide significance threshold in the ANZ2026 meta-analysis. We note the following about this gene: it is a mitochondrial enzyme and is strongly co-expressed with and co-evolved specifically with other components of the mitochondrial B12 pathway (Strittmatter et al., 2014). Approximately 5% of Europeans harbor a premature stop variant in *CLYBL* that has been associated with reduced levels of circulating vitamin B12 (rs41281112) (Strittmatter et al., 2014). However, this SNP is 41 kb away from our top TGC2024 SNP (rs2038695) and in low LD with it, which may imply distinct functional effects. GWAS shows a link between the gene and circulating vitamin B12 levels (Lin et al., 2012). A Scandinavian exome sequencing study confirmed *CLYBL* variants are associated with circulating vitamin B12 levels (Grarup et al., 2013). Nearby SNPs have also been associated with age at menarche, age at menopause, and reproductive aging in large-scale GWAS (Ruth et al., 2021).

Preconception vitamin B12 levels have been linked to favorable reproductive outcomes in both natural pregnancies and those achieved through ART (Gaskins, 2015). For women, it impacts ovulation, egg development, and implantation and deficiencies can contribute to infertility and pregnancy complications (Gaskins & Chavarro, 2018). Prolonged vitamin B12 deficiency has also been suggested to impair fertility by altering ovulation (Bennett, 2001). Taken together, these findings indicate that *CLYBL* may influence DZ twinning through regulation of vitamin B12 metabolism in females and is likely to be successfully replicated in future GWAS. The TGC2024 top SNP (rs2038695 or 13:100559123, hg19) has itself been found associated with Serum Total, Free and/or Bioavailable Testosterone levels in white British males (but not in females-only GWAS) in the UK Biobank ($p \sim 4.7 \times 10^{-14}$ and 4.3×10^{-16}, Tables S1 and S7 in Leinonen et al., 2023; also Tables S5, S6, S8, S9 in Ruth et al., 2020), which is of obvious reproductive relevance, although the interpretation here for a female-specific trait is unclear given the lack of strong association in females.

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Competing interests. None

Ethical standard. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. All studies were approved by the QIMR Berghofer Human Research Ethics Committee (approval P3693, as well as P2034 for QSkin Genetics and P3434 for QSkin2), and additionally (New Zealand study) the Central Health and Disability Ethics Committee, NZ Ministry of Health (approval 2022EXP12825).

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