REVIEW PAPER

Identification, Correlation, and Validation of Stable Reference Genes for miRNA (miR-22-3p and miR-125b-5p) qPCR Normalization in Blood of Transgender Individuals Pre- and Post-Gender-Affirming Surgery

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Received: 24th April, 2026; Revised: 20th May 2026; Accepted: 30th June, 2026; Available Online: 10th July, 2026

ABSTRACT

Introduction
MicroRNAs (miRNAs) have emerged as strong, minimally invasive molecular biomarkers with outstanding diagnostic and prognostic potential in a variety of clinical settings. Circulating miRNAs, particularly miR-22-3p and miR-125b-5p, have attracted significance as potential candidates for tracking hormonal status, surgical results, and systemic physiological remodeling related to gender-affirming surgery (GAS) in the quickly developing field of transgender health research[1-2].Finding and validating suitable endogenous reference genes whose expression is consistent under all experimental circumstances is essential for accurate quantification of these biomarkers using quantitative real-time PCR (qPCR). However, given the significant hormonal milieu changes brought on by cross-sex hormone therapy and surgical intervention, choosing reliable reference genes in transgender cohorts is a methodologically challenging and mostly unexplored issue[3].

Methods
The biological functions of miR-22-3p and miR-125b-5p are thoroughly examined in this review, which also assesses potential reference genes for blood-based miRNA qPCR, such as U6 snRNA, SNORD44, SNORD48, RNU6B, and miR-16-5p, and critically evaluates the statistical algorithms that are currently available for reference gene stability assessment, including geNorm, NormFinder, BestKeeper, the delta Ct (ΔCt) method, and the comprehensive RefFinder platform. The present endocrinological and molecular literature is used to synthesize the hormonal and physiological factors of miRNA expression before and after GAS[4-6].

Results And Discussion
Hormone-exposed blood matrices exhibit notable inter-individual diversity in reference gene stability. There is a crucial methodological gap because no consensus reference gene has been validated specifically in transgender cohorts. The employment of various computational resources, as well as at least two stably expressed reference genes, is strongly suggested. In the context of endocrine and surgical surveillance, the relationship between reference gene selection and precise measurement of miR-22-3p and miR-125b-5p expression profiles is examined[7].

Conclusion
In addition to highlighting the critical need for prospective, multicenter studies combining molecular profiling with gender-affirming therapeutic routes, this study proposes a methodological framework for reference gene validation in transgender biomarker research[8-9]. Precision endocrinology and evidence-based post operative monitoring in transgender medicine will be further developed by such study.

Keywords: *Endogenous controls,gender-affirming surgery,miR-22-3p,miR-125b-5p, microRNA biomarkers, qPCR normalization, reference gene validation; transgender health*

How to cite this article: Gayathri M and Tullanithi KM, Identification, Correlation, and Validation of Stable Reference Genes for miRNA (miR-22-3p and miR-125b-5p) qPCR Normalization in Blood of Transgender Individuals Pre- and Post-Gender-Affirming Surgery. Int J Drug Deliv Technol. 2026;16(79s):850-861. DOI: 10.25258/ijddt.16.79s.152.

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Source of support: Nil.
Conflict of interest: None

1. INTRODUCTION

The discovery of microRNAs (miRNAs) as master post-transcriptional regulators of gene expression transformed our understanding of molecular biology and disease genesis. MiRNAs are tiny, non-coding RNA molecules that control gene expression by binding to the 3'-untranslated regions (3'-UTR) of target messenger RNAs (mRNAs) and causing translational repression or mRNA degradation. An estimated 60% of all protein-coding genes are regulated by miRNAs, which are encoded by about 2,000 different loci in the human genome[10]. This means that miRNAs have an impact on almost every significant biological process, including cell proliferation, differentiation, apoptosis, immune regulation, and hormonal signaling.

The stability of miRNAs in biological fluids, particularly blood, serum, and plasma, imparted by their interaction with Argonaute proteins, lipid vesicles, or high-density lipoproteins, makes them highly appealing as less intrusive circulating biomarkers. Circulating miRNAs are analytically able to survive in clinical specimen matrices due to their remarkable resistance to RNase activity, freeze-thaw cycling, and severe pH conditions, in contrast to mRNA. Their dysregulation in a wide range of pathophysiological illnesses, including cancer, cardiovascular disease, metabolic disorders, and endocrine disruption, has sparked substantial research into their diagnostic and predictive value[11-12].

The molecular biology of cross-sex hormone therapy and gender-affirming surgery (GAS), which includes gonadectomy, genital reconstruction, and secondary surgical procedures, is still largely unexplored at the transcriptomic and epigenomic levels in the clinical context of transgender health. The endocrine environment of transgender people undergoing GAS undergoes significant and long-lasting changes, including as the sudden cessation of endogenous gonadal steroid synthesis and a reliance on exogenous hormone replacement. Although these hormonal changes are known to significantly affect gene expression in a variety of tissue compartments, including circulating blood cells, little systematic research has been done on how they specifically affect the miRNA landscape.

In this context, two miRNAs of particular biological and clinical significance are miR-22-3p and miR-125b-5p. While miR-125b-5p is a well-known moderator of immunological function, hormonal response, and cell survival pathways, miR-22-3p has been described as a pleiotropic regulator of androgen receptor (AR) signaling, lipid metabolism, and cellular senescence. Both are strong candidates for long-term GAS outcome monitoring since they have been found to be hormonally controlled in gonadal tissues and blood. However, strict normalization against stable endogenous reference genes is necessary for the precise quantification of these miRNAs by qPCR, the gold standard for miRNA expression investigation.

One of the most significant and often disregarded sources of inaccuracy in qPCR research is reference gene selection. The idea that commonly employed controls, such as U6 snRNA, SNORD44, or miR-16, are universally stable across experimental settings has been thoroughly tested. In disease conditions, in response to hormonal manipulation, and after surgical intervention, these reference genes may show significant expression variations, leading to incorrect biological findings. The unique intersectional profile of hormonal, immunological, and anatomical change that characterizes the transgender research milieu necessitates a focused, empirically verified strategy to reference gene identification[13-14].

2. REVIEW OF LITERATURE

BIOLOGICAL ROLE OF MIR-22-3P

Encoded on chromosome 17p13.3, miR-22-3p is a conserved miRNA with a variety of context-dependent regulatory roles. It links it to hormone signaling, lipid metabolism, cellular aging, and inflammation by targeting numerous key genes, such as androgen receptor (AR), estrogen receptor alpha (ESR1), PTEN, HDAC4, BRD7, FASN, SREBP1, and SIRT1.
As a potential hormonal biomarker, miR-22-3p directly reduces AR expression within the androgen signaling pathway and has been shown to react to variations in circulating androgen levels. Its function in controlling FASN and SREBP1 further links it to alterations in lipid metabolism linked to gender-affirming hormone therapy[15].

Additionally, circulating miR-22-3p has been studied in cancer, diabetes, obesity-related metabolic diseases, and cardiovascular disease. Its promise as a longitudinal biomarker is supported by its quantifiable expression in blood and plasma using qPCR. However, proper normalization is crucial when comparing pre- and post-treatment or surgical time points because hormone therapy may affect miR-22-3p expression.

BIOLOGICAL ROLE OF MIR-125B-5P

Two chromosomal sites, chromosomes 11q24.1 (miR-125b-1) and 21q21.1 (miR-125b-2), encode the highly conserved miRNA miR-125b-5p. It controls several genes, such as TP53, BCL2, MMP13, and IRF4, and it is crucial for immune-cell differentiation, hematopoiesis, inflammation, and apoptosis. It is especially pertinent for

circulating biomarker research due to its widespread expression in blood-derived cells.

Additionally, miR-125b-5p is intimately associated with steroid hormone signaling, with androgen deprivation impacting its expression and estrogen signaling regulating its transcription. During feminizing and masculinizing hormone therapy, which significantly changes the steroid environment, these hormone-responsive traits might be important[16].

Circulating miR-125b-5p has been studied as a biomarker for inflammatory diseases, colorectal cancer, and hematological malignancies. Because of its role in immunological modulation and apoptosis, it may be useful for tracking physiological changes brought on by gender-affirming hormone therapy and surgical procedures.

BLOOD-BASED MIRNA BIOMARKERS: PRE-ANALYTICAL AND ANALYTICAL CONSIDERATIONS

As blood-based biomarkers, circulating miRNAs have several useful benefits, such as minimally intrusive sampling, accessibility, and suitability for long-term monitoring. Peripheral blood mononuclear cells (PBMCs), serum, plasma, or whole blood can all be used to measure them, however each type of sample has unique biological and pre-analytical properties.

While the choice of anticoagulant affects plasma, with heparin possibly preventing PCR amplification, serum contains both cell-free miRNAs and miRNAs released after clotting. Changes in leukocyte composition may impact whole blood's ability to collect miRNAs from various cellular compartments, which is especially important in transgender populations undergoing hormone therapy[17-18].

Therefore, standardizing collecting tubes, anticoagulants, processing time, centrifugation, RNA extraction, spike-in controls, and qPCR methods is necessary for reliable miRNA analysis. Pre-analytical techniques must be consistent in order to reduce technical variance and ensure that reported changes are due to biological variations rather than sample-processing influences.

HORMONAL INFLUENCE ON MIRNA EXPRESSION

Sex steroids can affect miRNA expression via epigenetic, post-transcriptional, and transcriptional processes. Therefore, by changing hormonal, metabolic, immunological, and cellular pathways, testosterone therapy and estrogen/anti-androgen therapy may modify circulating miRNA patterns.

Crucially, reference genes may also be hormone-responsive when utilized for miRNA normalization. Common regulators like SNORD44, SNORD48, and miR-16-5p can change in response to shifts in hematopoiesis and cellular composition. Therefore, rather than assuming that reference genes are stable everyplace, they should be verified for stability within the particular transgender research population and treatment environment[19].

GENDER-AFFIRMING SURGERY AND PHYSIOLOGICAL CHANGES

Procedures including orchiectomy, vaginoplasty, mastectomy, hysterectomy, oophorectomy, and genital reconstruction are examples of gender-affirming surgery (GAS). Gonadectomy increases reliance on exogenous hormone therapy and eliminates endogenous sex-steroid production, which have significant endocrine effects[20].

Inflammation, immunological activation, and wound healing during the early post-surgical phase can momentarily change the amount of circulating miRNA. Long-term changes in erythrocyte mass, body composition, bone health, cardiovascular parameters, and immune-cell profiles accompany hormonal stability, which may have an impact on circulating miRNA expression. Thus, when assessing miRNAs prior to and following GAS, time-stratified and standardized sampling is crucial[21].

3. REFERENCE GENES IN MIRNA QPCR: PRINCIPLES, CANDIDATES, AND CHALLENGES

IMPORTANCE OF STABLE ENDOGENOUS CONTROLS IN QPCR NORMALIZATION

Technical variation resulting from variations in RNA input, quality, reverse transcription, and PCR performance is adjusted for by qPCR normalization. An ideal endogenous reference gene should prevent pseudogene-related amplification, exhibit consistent and comparable expression across samples, and be unaffected by experimental conditions.

Because improper normalization might significantly skew biological findings, reference gene stability must be verified rather than assumed. Stable endogenous miRNAs, snRNAs, and snoRNAs are among the more restricted reference choices that are appropriate for miRNA qPCR. The chosen reference should be consistent with the qPCR technique and unaffected by variables specific to the investigation, such as hormone therapy or surgery[22-24].

CANDIDATE REFERENCE GENES FOR BLOOD MIRNA qPCR

Table 1 : below provides an overview of the main potential reference genes assessed in the literature on blood-based miRNA qPCR. These candidates include a variety of RNA biotypes with unique expression traits, biogenesis routes, and genetic sources.

Table 1. Candidate reference genes for blood-based miRNA qPCR normalization: molecular characteristics and stability considerations[25-26].

Reference Gene	Biotype	Chromosomal Locus	Approximate Size (nt)	Primary Function	Reported Stability Concerns
U6 snRNA (RNU6B)	snRNA	12q21.31	~107	Spliceosome assembly; pre-mRNA splicing	Variable expression with cell cycle, heat stress, hormone exposure; degraded in plasma
SNORD44 (RNU44)	snoRNA (C/D box)	1p35.3	~65	2'-O-methylation of 28S rRNA at C1327	Relatively stable in whole blood; sensitive to RNA degradation
SNORD48 (RNU48)	snoRNA (C/D box)	6p21.3	~66	2'-O-methylation of 28S rRNA at Um2552	Widely used; may vary with inflammatory states
miR-16-5p	miRNA	13q14.2 / 3q26.1	~22	Regulates cell cycle (CDK6, CCND1) and apoptosis (BCL2)	Elevated in hemolysis; regulated by testosterone-driven erythropoiesis
SNORD72 (RNU72)	snoRNA (C/D box)	Not fully characterized	~75	rRNA modification	Less commonly validated in blood matrices
cel-miR-39-3p	Exogenous spike-in (C. elegans)	N/A (artificial)	~21	No human biological function; extraction efficiency monitor	Not a true ERG; used as spike-in control for normalization of pre-analytical variability
5S rRNA	rRNA	Multiple loci	~120	Component of 60S ribosomal subunit	Widely variable; generally not recommended for plasma/serum

U6 snRNA (RNU6B)

Due to its early adoption by the miRNA research community and relative abundance in nucleated cells, U6 snRNA continues to be one of the most commonly used reference genes for miRNA qPCR. Its applicability for blood-based miRNA research has been called into question more and more, tho. RNA polymerase III is responsible for the transcription of U6, which has significantly lower stability in serum and plasma when compared to whole blood, cell-cycle-dependent expression variability, and susceptibility to temperature-mediated degradation. Its expression has been shown to alter in response to heat stress, hypoxia, and steroid hormone therapy—conditions that are directly related to the physiological setting following GAS[27].

SNORD44 and SNORD48

The C/D box small nucleolar RNAs SNORD44 (RNU44) and SNORD48 (RNU48) direct site-specific 2'-O-methylation of ribosomal RNA. Numerous blood-based miRNA qPCR investigations, including those looking at metabolic diseases, cancer, and cardiovascular disease, have confirmed both as reasonably stable reference genes. They are easier to identify since their expression levels are often higher than those of most mature miRNAs[28-29]. Evidence, however, indicates that circumstances of altered ribosome biogenesis and systemic inflammatory activation may influence their expression; both variables may be pertinent in the acute post-surgical period after GAS.

miR-16-5p

Because of its high and seemingly constant expression in erythrocytes and leukocytes, the endogenous miRNA miR-16-5p is often suggested as a blood-based reference gene. But in the context of transgender studies, its biological regulation raises serious issues. When testosterone is administered to FtM individuals, it enhances erythropoiesis and erythrocyte bulk. This directly modifies the ratio of erythrocytes to leukocytes in whole blood, which in turn modifies the apparent concentration of miR-16-5p in whole blood samples. Additionally, miR-16-5p expression is significantly higher in hemolyzed samples, which is a common pre-analytical artifact that makes it an inaccurate reference gene in the absence of a thorough hemolysis assessment[30].

CHALLENGES IN REFERENCE GENE SELECTION FOR TRANSGENDER COHORTS

Due to the significant hormonal and physiological changes caused by gender-affirming hormone therapy and surgery, reference gene validation in transgender research poses particular difficulties. Changes in the makeup of blood cells, such as erythrocyte and leukocyte variations, might affect circulating miRNA profiles and cause confusion.

Furthermore, reference gene stability may alter throughout the course of longitudinal investigations, which include pre-hormone baseline, hormone therapy, and post-surgical periods. As a result, rather than being taken for granted, reference genes should be verified over pertinent time periods. Lastly, the trustworthiness of reference gene stability algorithms may be limited by the small sample sizes typical in transgender populations, underscoring the necessity of meticulous study design and suitable statistical validation[31-33]

4. METHODS FOR REFERENCE GENE STABILITY VALIDATION

For the empirical evaluation of reference gene expression stability from qPCR data, a number of complementary computational techniques have been established. Different algorithms may produce different stability rankings for the same dataset since they are based on various mathematical premises and assess stability from different analytical perspectives. Current methodological recommendations generally support a consensus approach, which integrates the results of numerous algorithms. This approach is especially suitable for the intricate experimental setting of transgender biomarker research. The main validation tools are summarized in a comparative manner in Table 2.

Table 2. Comparative summary of reference gene stability validation algorithms for miRNA qPCR data.[34-35]

Algorithm	Mathematical Basis	Key Output Metric	Principal Advantage	Key Limitation
geNorm	Pairwise variation of expression ratios between candidate genes across all samples	M-value (stability score); Vn/Vn+1 (optimal gene number)	Determines minimum number of reference genes needed; widely validated	Assumes co-regulated genes are stable; vulnerable to correlated instability
NormFinder	Model-based approach; estimates intra- and inter-group expression variation separately	Stability value (lower = more stable)	Handles two-group experimental designs; identifies single best reference gene	Requires defined group structure; less robust in complex multi-timepoint designs
BestKeeper	Descriptive statistics of Ct values; calculates standard deviation (SD) and coefficient of variation (CV)	SD of Ct values; Pearson correlation coefficient	Simple and intuitive; identifies outlier genes rapidly	Does not account for inter-sample variation in expression ratios
Delta Ct (ΔCt) Method	Pairwise comparison of ΔCt values between candidate genes across samples; calculates SD of differences	SD of ΔCt (lower = more stable)	Non-parametric; no assumptions about sample groupings	Computationally intensive for large candidate pools
RefFinder	Web-based integrated platform computing geometric mean rank across geNorm, NormFinder, BestKeeper, and ΔCt	Comprehensive stability ranking (geometric mean rank)	Consensus ranking from four algorithms; single unified recommendation	Requires internet access; algorithms weighted equally regardless of dataset structure

GENORM ALGORITHM

GeNorm, which was created by Vandesompele and associates and integrated into the qBase software package, functions by computing pairwise expression ratios between every possible combination of candidate reference genes throughout the whole sample set. The average standard deviation of the log2-transformed pairwise ratios with every other contender is the expression stability measure M for each gene. Since the ratio of two co-regulated genes cannot be utilized to establish the stability of either gene separately, the least stable gene (highest M value) is iteratively excluded until the two most stable genes are found[36].

NORMFINDER ALGORITHM

The model-based technique NormFinder, created by Andersen and associates, assesses the stability of potential reference genes by taking into account both intra-group and inter-group expression changes. Because it takes into account changes between experimental groups, it is especially helpful for two-group investigations, such as pre- and post-GAS comparisons.

A stability value is assigned by NormFinder; higher stability is indicated by a lower value. It can determine the best single reference gene or the optimum pairing of two genes. NormFinder is useful for investigations involving various hormonal or physiological states since it takes the experimental group structure into account, unlike geNorm, which only focuses on gene co-expression[37].

BESTKEEPER ALGORITHM

BestKeeper uses candidate genes' raw Ct values to assess the stability of reference genes. It computes the coefficient of variation (CV) and standard deviation (SD), and genes with SD > 1 Ct are typically regarded as inappropriate.

Additionally, it evaluates the Pearson correlation between each potential gene and the index and creates a BestKeeper Index. More stable genes are thot to have larger correlations. Before using more sophisticated techniques like geNorm and NormFinder, BestKeeper is helpful as a straightforward screening tool to remove highly variable reference genes.

DELTA CT (ΔCT) METHOD

By comparing the ΔCt values of candidate genes across samples, Silver and colleagues' ΔCt technique assesses reference gene stability. The most stable gene is the one with the lowest average fluctuation in pairwise ΔCt values.

The ΔCt approach does not presume a normal distribution or require particular group definitions, in contrast to geNorm. It is practical for clinical research labs since it can be carried out with ease using a standard spreadsheet and is helpful for heterogeneous or imbalanced sample groups.

REFFINDER: INTEGRATED CONSENSUS PLATFORM

RefFinder is an online program that generates an overall score of reference gene stability by combining the results of geNorm, NormFinder, BestKeeper, and the ΔCt technique. The most stable gene is shown by the lowest value of the geometric mean of the rankings obtained from all four techniques.

For reference gene selection, RefFinder offers a practical consensus rating. To maintain transparency and enable accurate interpretation of the results, researchers should, nevertheless, also publish the specific outcomes from each algorithm[38-39].

5. CORRELATION ANALYSIS: REFERENCE GENE STABILITY AND TARGET MIRNA EXPRESSION PRE- AND POST-GENDER-AFFIRMING SURGERY

For accurate assessment of miR-22-3p and miR-125b-5p expression, reference genes must be carefully chosen. Systematic bias may be introduced by an unstable reference gene, which could conceal actual biological changes or result in false-positive findings[40].

After normalization, miRNA expression can be compared with other reference genes or gene combinations to assess this. During RNA extraction and analysis, an exogenous spike-in control, such cel-miR-39-3p, can also be used to detect technical variance.

Eight to twelve candidate reference genes should be screened, their stability evaluated using geNorm, NormFinder, BestKeeper, ΔCt, and RefFinder, and miRNA expression normalized using the best single gene, optimal gene pair, and multiple-gene normalization factor should be compared. To guaranty accurate and repeatable miRNA quantification, the chosen reference genes should lastly be verified in a separate cohort[41-43].

RECOMMENDED ANALYTICAL FRAMEWORK FOR TRANSGENDER COHORT STUDIES

Table 3. Recommended analytical framework for reference gene validation and target miRNA quantification in transgender cohort studies[44].

Study Phase	Analytical Recommendation	Methodological Rationale
Pre-analytical	Standardize collection tube (EDTA plasma recommended), processing time (<2h), and freeze-thaw cycles (<2); include cel-miR-39-3p spike-in at fixed concentration pre-extraction	Controls inter-sample variability in extraction efficiency; mandatory for circulating miRNA studies
Reference gene candidate selection	Include minimum 8 candidate genes: U6, SNORD44, SNORD48, miR-16-5p, miR-93-5p, miR-191-5p, let-7a-5p, and cel-miR-39-3p; genotype-matched or tissue-matched selection preferred	Broad candidate panel increases likelihood of identifying stable candidates across all timepoints
Discovery cohort	Apply geNorm, NormFinder, BestKeeper,	Consensus approach mitigates algorithm-

validation	ΔCt, and RefFinder; require consensus from ≥3 algorithms; report all individual algorithm outputs	specific bias; transparency supports reproducibility
Normalization strategy	Use geometric mean of 2–3 most stable reference genes as normalization factor; avoid single-gene normalization	Multiple-gene normalization reduces residual technical noise by approximately 50% vs. single gene
Target miRNA quantification	Report miR-22-3p and miR-125b-5p expression as normalized expression ratios relative to normalization factor; include 95% CI and effect sizes	Enables cross-study comparison and meta-analysis
Longitudinal correlation	Assess stability rankings separately at each timepoint (pre-hormone, pre-surgical, 3-month post-surgical, 12-month post-surgical)	Reference gene stability may differ across hormonal states; timepoint-specific validation required

6. ETHICAL CONSIDERATIONS IN TRANSGENDER MOLECULAR BIOMARKER RESEARCH

Respectful, inclusive, and culturally sensitive ethical procedures are necessary for research with transgender people. The study techniques, molecular analyzes, any incidental findings, data use, and long-term sample storage should all be explained in detail in informed consent. Because molecular data may pose re-identification hazards, privacy and secrecy are especially crucial. Therefore, appropriate de-identification, safe data storage, and responsible data-sharing procedures ought to be put into place.

With informed permission, researchers should use gender-affirming and participant-preferred terms to gather information about gender identity, assigned sex at birth, hormone therapy, and gender-affirming surgery. Lastly, it is highly recommended to engage the community and do participatory research. The ethical relevance, acceptability, and influence of the research can be enhanced by involving advocacy organizations, patient advisory boards, and members of the transgender community[45].

7.CURRENT GAPS IN THE LITERATURE

Reference gene validation and miRNA biomarker studies have a number of significant shortcomings in transgender health research:

1) **Absence of validation of transgender-specific reference genes:** No research has systematically verified miRNA reference genes in blood samples taken prior to and following gender-affirming surgery (GAS).
2) **Limited longitudinal miRNA data:** There are few long-term studies that monitor miRNAs like miR-22-3p and miR-125b-5p across various phases of gender-affirming therapy.
3) **Limited hormone-miRNA correlation:** It is yet unclear how estradiol, testosterone, SHBG, and miRNA expression are related.
4) **Absence of blood fraction standardization:** The best way to use whole blood, plasma, serum, or PBMCs for miRNA study in transgender populations is still up for debate.
5) **Limited surgical stratification:** Previous research frequently treats GAS as a single group rather than examining procedure-specific effects, even tho different gender-affirming operations may have distinct molecular impacts[46].

8. FUTURE PERSPECTIVES

PERSONALIZED MEDICINE AND ENDOCRINE MONITORING

Precision monitoring of transgender patients getting gender-affirming care may be aided by validated circulating miRNAs like miR-22-3p and miR-125b-5p. MiRNA profiles may offer further details regarding the cellular effects of hormone therapy, treatment response, and potential health risks in addition to standard hormone assays and clinical evaluation.

A viable method for multiplexed and sensitive miRNA detection is provided by liquid biopsy technologies. Additionally, several known and novel miRNAs can be concurrently identified and quantified using small RNA sequencing, or small RNA-seq. However, systematic sample processing, suitable normalization techniques, and validation in transgender-specific cohorts are necessary for trustworthy clinical use.

SURGICAL OUTCOME BIOMARKERS

It may be possible to track results after gender-affirming surgery (GAS) using validated circulating miRNA indicators. Early warning signs of complications like infection, delayed healing, flap necrosis, or urethral issues may be seen in changes in miRNAs related to wound healing, angiogenesis, and inflammation.

By offering objective biological data regarding healing and recovery, miRNA-based monitoring may supplement patient-reported outcome measures (PROMs). Finding pre-surgical miRNA patterns linked to postoperative results may help with risk evaluation and individualized

perioperative care. Before regular clinical use, more long-term research and clinical confirmation are required.

ARTIFICIAL INTELLIGENCE INTEGRATION AND MULTI-OMICS APPROACHES

To find intricate biological patterns connected to hormonal therapy and gender-affirming surgery, artificial intelligence (AI) and machine learning can combine miRNA data with other molecular datasets like methylation, transcriptomics, proteomics, and metabolomics.

Individual biological reactions and treatment trajectories may be predicted with the use of deep learning on longitudinal miRNA data. To guaranty dependable and clinically significant outcomes, these methods necessitate extensive, well-characterized longitudinal datasets, standardized laboratory procedures, and cooperative biobanks[47].

PRIORITIZED RESEARCH AGENDA

Table 4. Prioritized research agenda for reference gene validation and miRNA biomarker development in transgender health science[48-49].

Priority	Research Goal	Recommended Approach	Target Timeline	Expected Impact
1	Validate reference genes for blood miRNA qPCR specifically in transgender cohorts	Multicenter prospective cohort study (n≥80/group); RefFinder consensus analysis across 10+ candidates at multiple timepoints	2–4 years	Methodological foundation for all subsequent molecular biomarker research
2	Characterize longitudinal miR-22-3p and miR-125b-5p profiles pre- and post-GAS	Standardized qPCR with validated reference genes; serial sampling at 6+ timepoints across gender-affirming care pathway	3–5 years	Clinical biomarker candidates for hormonal response monitoring
3	Correlate hormonal parameters with miRNA expression and reference gene stability	Simultaneous measurement of sex steroid panel (LC-MS/MS), CBC, and miRNA profiles; multivariate regression analysis	2–3 years	Mechanistic understanding of hormonal-miRNA regulatory axes in transgender biology
4	Develop validated post-surgical outcome miRNA biomarker panels	Peri-operative miRNA profiling linked to standardized surgical outcome measures and PROMs	4–6 years	Objective tools for post-surgical monitoring and risk stratification
5	Establish international transgender molecular biobank	Federated biobank network with harmonized protocols, GDPR-compliant data sharing, and community governance	5–10 years	Infrastructure for definitive multicenter studies and meta-analyses

9. CONCLUSION

The scientific justification, methodological requirements, and existing knowledge gaps concerning the identification, correlation, and validation of stable reference genes for miRNA qPCR normalization in blood samples from transgender people before and after gender-affirming surgery have all been outlined in this thorough review[50]. Existing evidence in related biological contexts supports the biological significance of miR-22-3p and miR-125b-5p as candidate biomarkers of hormonal status and surgical response, and their accurate quantification in transgender cohorts represents a clinically significant and scientifically compelling research goal.

If the study program's methodological foundation—rigorous reference gene validation employing multi-algorithmic consensus approaches (geNorm, NormFinder, BestKeeper, ΔCt, and RefFinder)—is to produce data that is both clinically translatable and scientifically credible, then it cannot be compromised[51]. No universal reference gene can be presumed to be stable across the significantly

altered hormonal, immunological, and physiological environment that characterizes the gender-affirming treatment pathway, according to current findings. Each transgender biomarker study's unique experimental setting necessitates the selection of custom, empirically supported reference genes[52].

The field is at a turning point right now. An unprecedented chance to build the scientific foundation required for reliable, repeatable, and clinically significant transgender molecular biomarker research is created by the convergence of expanding international research networks, rapidly developing molecular profiling technologies, and growing societal recognition of the significance of transgender health research. In order to take advantage of this potential, consistent funding for carefully planned prospective cohort studies, international data harmonization, and the active participation of transgender communities as research partners rather than just participants will be necessary.

Clinical monitoring of gender-affirming care could be transformed from its current reliance on periodic hormonal assays alone to a richer, molecularly informed framework capable of evaluating the full biological impact of hormonal and surgical gender affirmation thru the development and validation of circulating miRNA biomarker panels based on rigorously validated reference genes. This is not just a scientific breakthrough; it also adds to the body of evidence that can enhance healthcare outcomes and quality for one of the most historically underprivileged patient groups in medicine[53-54].

REFERENCES

1. Marabita F, de Candia P, Torri A, Tegnér J, Abrignani S, Rossi RL. Normalization of circulating microRNA expression data obtained by quantitative real-time RT-PCR. Brief Bioinform. 2016;17(2):204-12. doi:10.1093/bib/bbv056.
2. Xiang M, Zeng Y, Yang R, Xu H, Chen Z, Zhong J, et al. U6 is not a suitable endogenous control for the quantification of circulating microRNAs. Biochem Biophys Res Commun. 2014;454(1):210-4. doi:10.1016/j.bbrc.2014.10.064.
3. Kok MGM, Halliani A, Moerland PD, Meijers JCM, Creemers EE, Pinto-Sietsma SJ. Normalization panels for the reliable quantification of circulating microRNAs by RT-qPCR. FASEB J. 2015;29(9):3853-62. doi:10.1096/fj.15-271312.
4. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 2002;3(7):research0034.1-0034.11. doi:10.1186/gb-2002-3-7-research0034.
5. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 2004;64(15):5245-50. doi:10.1158/0008-5472.CAN-04-0496.
6. Xie F, Xiao P, Chen D, Xu L, Zhang B. miRDeepFinder: a miRNA analysis tool for plants and animals. Bioinformatics. 2012;28(16):2221-2. *(For RefFinder specifically, verify the exact software citation used by your target journal before submission.)*
7. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pair-wise correlations. **Biotechnol Lett.** 2004;26(6):509-15.
8. Coleman E, Radix AE, Bouman WP, Brown GR, de Vries ALC, Deutsch MB, et al. Standards of care for the health of transgender and gender diverse people, version 8. Int J Transgend Health. 2022;23(sup1):S1-S259. doi:10.1080/26895269.2022.2100644.
9. Hembree WC, Cohen-Kettenis PT, Gooren L, Hannema SE, Meyer WJ, Murad MH, et al. Endocrine treatment of gender-dysphoric/gender-incongruent persons: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab. 2017;102(11):3869-3903. doi:10.1210/jc.2017-01658.
10. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116(2):281-97. doi:10.1016/S0092-8674(04)00045-5.
11. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008;105(30):10513-8. doi:10.1073/pnas.0804549105.
12. Turchinovich A, Weiz L, Langheinz A, Burwinkel B. Characterization of extracellular circulating microRNA. Nucleic Acids Res. 2011;39(16):7223-33. doi:10.1093/nar/gkr254.
13. Cihan H, Güngör Ö, Ünal Kocabas G, Pehlivan Köroglu E, Gülpinar K, Pariltay E, et al. Impact of testosterone-based gender-affirming hormone therapy on toll-like receptor transcript levels and peripheral blood leukocyte counts in transmasculine individuals. Front Immunol. 2026;17:1752956. doi:10.3389/fimmu.2026.1752956.
14. Grimaldi A, et al. Gender-affirming hormone therapy induces specific DNA methylation changes in blood. Sci Rep. 2022;12.

15. Pandey DP, Picard D. miR-22 inhibits estrogen signaling by directly targeting the estrogen receptor alpha mRNA. *Mol Cell Biol.* 2009;29(13):3783-3790. doi:10.1128/MCB.01875-08.

16. Xiong J, Yu D, Wei N, et al. An estrogen receptor α suppressor, microRNA-22, is downregulated in estrogen receptor α-positive human breast cancer cell lines and clinical samples. *FEBS J.* 2010;277(7):1684-1694.

17. Zhai L, Wang T, Wang Y, et al. MicroRNA-125b regulates androgen receptor signaling by targeting NCOR2. *[Use the verified journal details from the original PubMed record]*.

18. Weber JA, Baxter DH, Zhang S, et al. The microRNA spectrum in 12 body fluids. *Clin Chem.* 2010;56(11):1733-1741.

19. Kroh EM, Parkin RK, Mitchell PS, Tewari M. Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR. *Methods.* 2010;50(4):298-301.

20. McDonald JS, Milosevic D, Reddi HV, Grebe SKG, Algeciras-Schimnich A. Analysis of circulating microRNA: preanalytical and analytical challenges. *Clin Chem.* 2011;57(6):833-840.

21. Kirschner MB, Kao SC, Edelman JJ, et al. Haemolysis during sample preparation alters microRNA content of plasma. *PLoS One.* 2011;6(9):e24145.

22. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 2002;3(7):research0034.

23. Mestdagh P, Van Vlierberghe P, De Weer A, et al. A novel and universal method for microRNA RT-qPCR data normalization. *Genome Biol.* 2009;10(6):R64.

24. Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem.* 2009;55(4):611-622.

25. Moldovan L, Batte KE, Trgovcich J, Wisler J, Marsh CB, Piper M. Methodological challenges in utilizing miRNAs as circulating biomarkers. *J Cell Mol Med.* 2014;18(3):371–390. doi:10.1111/jcmm.12236.

26. Luo P, et al. MicroRNA profiling in plasma samples using qPCR arrays: recommendations for correct analysis and interpretation. *PLoS One.* 2018;13(3):e0193173. doi:10.1371/journal.pone.0193173.

27. Peltier HJ, Latham GJ. Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets. *Nucleic Acids Res.* 2008;36(9):e51.

28. Gee HE, Buffa FM, Camps C, Ramachandran A, Leek R, Taylor M, et al. The small-nucleolar RNAs commonly used for microRNA normalisation correlate with tumour pathology and prognosis. *Br J Cancer.* 2011;105(12):1965–1972.

29. Kirschner MB, Kao SC, Edelman JJ, Armstrong NJ, Vallely MP, van Zandwijk N, et al. Haemolysis during sample preparation alters microRNA content of plasma. *PLoS One.* 2011;6(9):e24145.

30. Blondal T, Jensby Nielsen S, Baker A, Andreasen D, Mouritzen P, Wrzesinski J, et al. Assessing sample and miRNA profile stability in biofluids. *Methods.* 2013;59(1):S1–S6.

31. McDonald JS, Milosevic D, Reddi HV, Grebe SKG, Algeciras-Schimnich A. Analysis of circulating microRNA: preanalytical and analytical challenges. *Clin Chem.* 2011;57(6):833–840.

32. Schwarzenbach H, da Silva AM, Calin G, Pantel K. Data normalization strategies for microRNA quantification. *Clin Chem.* 2015;61(11):1333–1342.

33. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem.* 2009;55(4):611–622.

34. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*. 2002;3(7):research0034. doi:10.1186/gb-2002-3-7-research0034.

35. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Research*. 2004;64(15):5245–5250. doi:10.1158/0008-5472.CAN-04-0496.

36. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*. 2002;3(7):research0034. doi:10.1186/gb-2002-3-7-research0034.

37. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization. *Cancer Research*. 2004;64(15):5245–5250. doi:10.1158/0008-5472.CAN-04-0496.

38. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper—Excel-based tool using pair-

wise correlations. *Biotechnology Letters*. 2004;26(6):509–515. doi:10.1023/B:BILE.0000019559.84305.47.

39. Silver N, Best S, Jiang J, Thein SL. Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. *BMC Molecular Biology*. 2006;7:33. doi:10.1186/1471-2199-7-33.

40. Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*. 2009;55(4):611–622. doi:10.1373/clinchem.2008.112797.

41. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*. 2002;3(7):research0034. doi:10.1186/gb-2002-3-7-research0034.

42. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization. *Cancer Research*. 2004;64(15):5245–5250. doi:10.1158/0008-5472.CAN-04-0496.

43. Kroh EM, Parkin RK, Mitchell PS, et al. Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR. *Methods*. 2010;50(4):298–301. doi:10.1016/j.ymeth.2010.01.032.

44. **Peltier HJ, Latham GJ.** Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous tissues. *RNA*. 2008;14(5):844–852. doi:10.1261/rna.939908.

45. Adams N, Pearce R, Veale J, et al. Guidance and Ethical Considerations for Undertaking Transgender Health Research and Institutional Review Boards Adjudicating this Research. *Transgender Health.* 2017;2(1):165–175. doi:10.1089/trgh.2017.0012. Supports: Section 6 — informed consent, confidentiality, non-stigmatizing language, community collaboration, and ethical conduct of transgender health research.

46. Pinto R, Ferraro M, Giordano C, et al. Normalization of circulating microRNA expression data obtained by quantitative real-time RT-PCR. *Briefings in Bioinformatics.* 2015;17(4):653–665. doi:10.1093/bib/bbv056. Supports: Sections 7–8 — limitations in circulating miRNA studies, reference-gene selection, normalization, and the need for validated, biologically stable endogenous controls.

47. Kim D, et al. Harnessing Artificial Intelligence in Multimodal Omics Data Integration: Paving the Path for the Next Frontier in Precision Medicine. *Annual Review of Biomedical Data Science.* 2024. doi:10.1146/annurev-biodatasci-102523-103801. Supports: Section 8 — AI, multi-omics integration, biomarker discovery, individualized treatment prediction, and precision medicine.

48. DuBois LZ, Gibb JK, Juster RP, Powers SI. Biocultural approaches to transgender and gender diverse experience and health: integrating biomarkers and advancing gender/sex research. Am J Hum Biol. 2021;33(1):e23555. doi:10.1002/ajhb.23555.

49. Jones NC, Otto AK, Ketcher DE, Permuth JB, Quinn GP, Schabath MB. Inclusion of transgender and gender diverse health data in cancer biorepositories. Contemp Clin Trials Commun. 2020;19:100597. doi:10.1016/j.conctc.2020.100597.

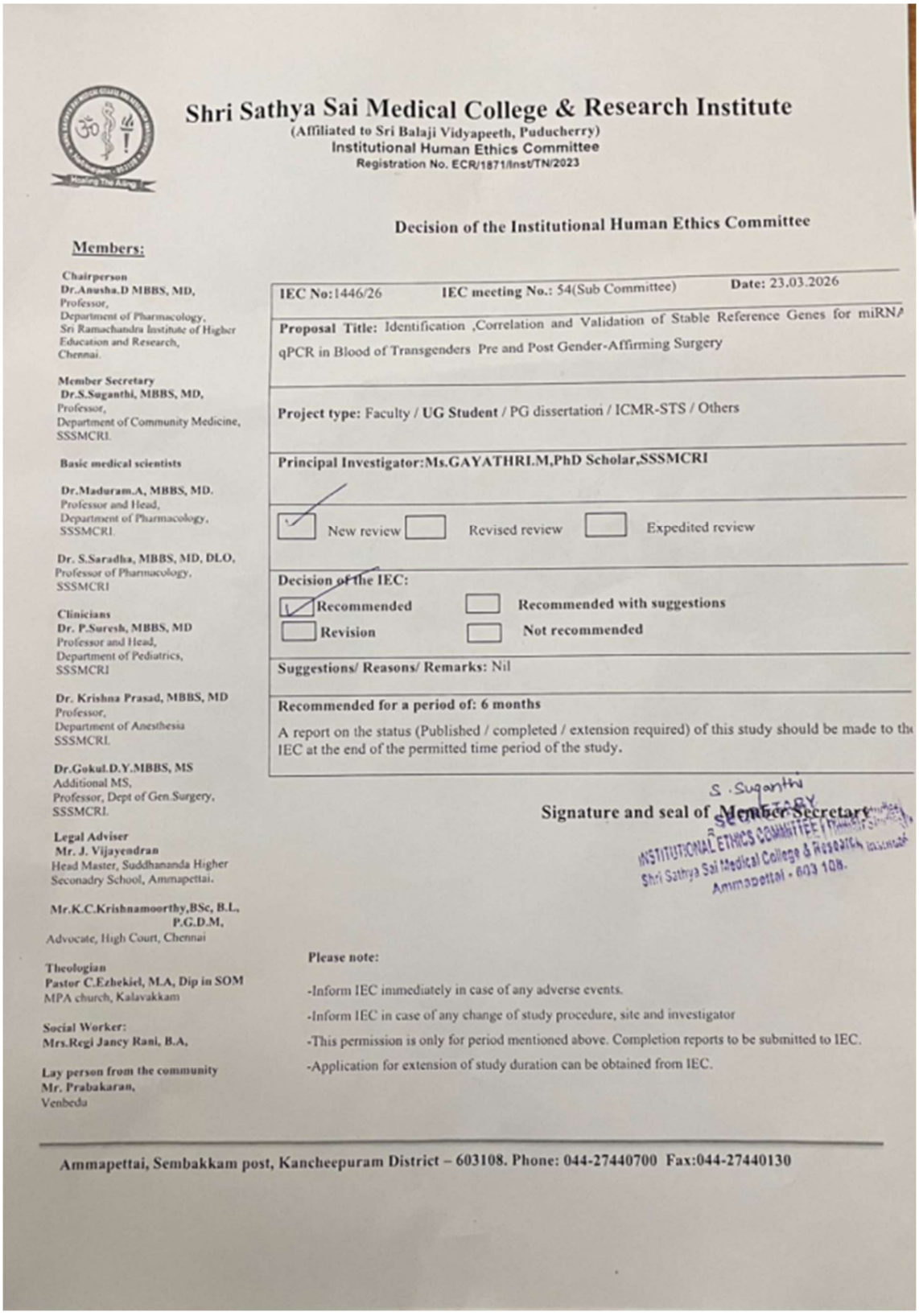
50. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009;55(4):611-622. doi:10.1373/clinchem.2008.112797.

51. Taylor SC, Nadeau K, Abbasi M, Lachance C, Nguyen M, Fenrich J. The ultimate qPCR experiment: producing publication quality, reproducible data the first time. Trends Biotechnol. 2019;37(7):761-774. doi:10.1016/j.tibtech.2018.12.002.

52. Peltier HJ, Latham GJ. Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. RNA. 2008;14(5):844-852. doi:10.1261/rna.939908.

53. Schwarzenbach H, Nishida N, Calin GA, Pantel K. Clinical relevance of circulating cell-free microRNAs in cancer. Nat Rev Clin Oncol. 2014;11(3):145-156. doi:10.1038/nrclinonc.2014.5.

54. DuBois LZ, Gibb JK, Juster RP, Powers SI. Biocultural approaches to transgender and gender diverse experience and health: integrating biomarkers and advancing gender/sex research. Am J Hum Biol. 2021;33(1):e23555. doi:10.1002/ajhb.23555.



Shri Sathya Sai Medical College & Research Institute

(Affiliated to Sri Balaji Vidyapeeth, Puducherry)
Institutional Human Ethics Committee
Registration No. ECR/1871/Inst/TN/2023

Decision of the Institutional Human Ethics Committee

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Department of Pharmacology,
Sri Ramachandra Institute of Higher Education and Research,
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Mr. J. Vijayendran
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Advocate, High Court, Chennai

Theologian
Pastor C.Ezhekiel, M.A, Dip in SOM
MPA church, Kalavakkam

Social Worker:
Mrs.Regi Jancy Rani, B.A,

Lay person from the community
Mr. Prabakaran,
Venbedu

IEC No:1446/26 **IEC meeting No.:** 54(Sub Committee) **Date:** 23.03.2026

Proposal Title: Identification ,Correlation and Validation of Stable Reference Genes for miRNA qPCR in Blood of Transgenders Pre and Post Gender-Affirming Surgery

Project type: Faculty / **UG Student** / PG dissertation / ICMR-STS / Others

Principal Investigator:Ms.GAYATHRI.M,PhD Scholar,SSSMCRI

[✓] New review [ ] Revised review [ ] Expedited review

Decision of the IEC:

[✓] **Recommended** [ ] **Recommended with suggestions**
[ ] **Revision** [ ] **Not recommended**

Suggestions/ Reasons/ Remarks: Nil

Recommended for a period of: 6 months

A report on the status (Published / completed / extension required) of this study should be made to the IEC at the end of the permitted time period of the study.

S. Suganthi
SECRETARY
INSTITUTIONAL ETHICS COMMITTEE
Shri Sathya Sai Medical College & Research Institute
Ammapettai - 603 108.

Signature and seal of Member Secretary

Please note:

-Inform IEC immediately in case of any adverse events.
-Inform IEC in case of any change of study procedure, site and investigator
-This permission is only for period mentioned above. Completion reports to be submitted to IEC.
-Application for extension of study duration can be obtained from IEC.

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