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Contents

Emerging Biomarkers in Diabetic Kidney Disease: The Necessity for Future Validation Studies, Mechanistic Investigation, and Integration into Risk Models

Diabetes mellitus (DM), a diverse group of diseases, affecting millions of people worldwide and remains to be one of the most difficult health problems.[1] The International Diabetes Federation revealed that the current 537 million individuals with diabetes will increase significantly by the year 2045.[2] Cardiovascular disease, diabetic nephropathy (DN), retinopathy, and neuropathy are the long-term consequences of the condition among others. It is essential to identify patients at risk for these consequences at the earliest. The traditional markers such as blood pressure, lipid profiles, microalbumin, fasting plasma glucose, and HbA1c are not the perfect predictors to recognize the early subclinical organ involvement.[3] This compels the need for novel biomarkers with high sensitivity and specificity to predict the progression of diabetic complications. In addition to offering practical instruments for early renal risk assessment, biomarkers may also provide insight into the etiology of DN or diabetic kidney disease (DKD), which will lead to the development of novel treatment approaches to stop the progression of the disease.

DKD is associated with an increased excretion of albumin, decreased renal function, or both. It affects approximately 30% and 40% of individuals with type 1 and type 2 DM (T2DM), respectively.[4] DN is usually regarded as the primary cause of morbidity and mortality in patients with diabetes. Classical DN and other kidney injury phenotypes associated with diabetes, such as tubulointerstitial damage and vascular nephropathy, are included in DKD. The classic clinical kidney lesions associated with long-term diabetes, particularly type 1 include mesangial enlargement, glomerular basement membrane thickening, Kimmelstiel-Wilson lesions (nodular glomerulosclerosis), and progressive proteinuria.[5] DN frequently causes 30%–50% of end-stage renal disease (ESRD).[6] According to previous reports, between 20% and 40% of people eventually develop ESRD after developing nephropathy.[7,8]

Hyperglycemia in DM sets off a series of pathogenic processes when it is accompanied by hypertension and hyperlipidemia. Endothelin I, angiotensin II, intraglomerular hemodynamic alterations, advanced glycated end products, oxidative stress, and metabolic parameters such as polyols, and diacylglycerols, were found to be associated with DN.[9] Renal cells, including endothelial cells, podocytes mesangial cells, and smooth muscle cells, are affected by hyperglycemia in addition to a variety of inflammatory cells.[10] Tubular injury, inflammation, and fibrosis are the various pathophysiological mechanisms leading to risk for DKD. Various interactions between metabolic abnormalities, renal hemodynamic alterations, immunological dysregulation, and associated inflammatory responses are observed in the pathogenesis.[11] One of the major proinflammatory cytokine, tumor necrosis-alpha (TNF-α) which is released from the activated native kidney cells, such as endothelial cells, glomerular mesangial cells, tubular epithelial cells, and activated monocytes and macrophages is involved in the signaling mechanism. Tumor necrosis factor-alpha receptor (TNFR)-1 and -2 are the circulatory receptors for TNF-α.[12] The signaling finally stimulates the release of monocyte chemoattractant protein-1/C-C chemokine ligand 2 (MCP-1/CCL2), interleukin-1 β, and transforming growth factor-beta (TGF-β)-1 and adversely affect the glomerular function.[13] Either resident or infiltrating macrophages found secreting the TGF-β which later favor the differentiation of fibroblast to myofibroblast in the kidney interstitium resulting in the secretion of extracellular matrix (ECM) proteins.[14] This will promote interstitial fibrosis. The produced ECM proteins in the renal glomerular mesangial cells include laminin, fibronectin, and collagen (I, III, and IV).[15] The activation of protein kinase C has been shown to reduce glomerular blood flow and filtration in DN.[15] Mutation in genes encoding collagen type IV and angiotensin-converting enzymes is also involved in the pathogenesis of DN, in addition to environmental factors.[16] Hence, molecules associated with these processes are expected to be reliable markers for DKD.

Clinical detection of DKD is limited. Therefore, sensitive and specific noninvasive biomarkers are needed for the early detection of DKD. Urinary albumin levels are the conventional method for identifying and categorizing DKD. However, evidences revealed that due to the low sensitivity and specificity, microalbumin cannot be a reliable early biomarker for the diagnosis of DKD.[17] Previous study demonstrated that despite an active therapy, approximately 40% of people with microalbuminuria eventually developed large albuminuria and progressed to ESRD while approximately 30% of patients were stable or recovered to normal.[18] Therefore, albuminuria may not always manifest in DN but may manifest as significant kidney damage. Similarly, unreliability was observed for estimated glomerular filtration rate (eGFR), which remained an indicator of kidney function as well as a measurement of disease progression. Porrini *et al*. reported that eGFR differed from measured glomerular filtration rate (GFR) by ≥30% which is incorrectly staged 30%–60% of chronic kidney disease (CKD) patients.[19] Furthermore, patients with DKD

may present with nonproteinuric or nonalbuminuric status, or may show albuminuria followed by a progressive decline in GFR. This heterogeneity emphasizes the necessity for new biomarkers that capture the underlying mechanisms of tissue damage, inflammation, and oxidative stress associated with diabetic complications.

In addition to making clinical trial design and execution easier, biomarkers linked to kidney disease progression in the early stages of CKD would allow for improved treatment customization for individuals at risk of progressive renal function loss. Although kidney biopsies are the gold standard for diagnosis, they are risky and invasive procedures that provide only moderate prognostic value for glomerular and tubulointerstitial histology.[20] An increased risk of CKD development and clinical outcomes has been linked to a normoalbuminuric range. According to the recent research, nonalbuminuric CKD is becoming more common.[21]

Markers that have been researched extensively include alpha-1-Microglobulin (α1-MG), neutrophil gelatinase-associated lipocalin (NGAL), TNFR1, TNFR-2, kidney injury molecule 1 (KIM-1), and YKL-40. α1-MG, a low-molecular-weight glycoprotein formed in the liver and released into the plasma, can freely filter through the glomeruli and be reabsorbed from the proximal renal tubules. A recent study demonstrated a strong correlation between high blood α1-MG levels and the likelihood of four major complications, such as diabetic retinopathy, peripheral neuropathy, left ventricular hypertrophy, and DN.[22] These reflect various forms of organ damage, and a single biomarker associated with such a wide range of problems will be beneficial for diabetes management. Longitudinal investigations are required to ascertain whether elevated α1-MG levels precede clinical issues or whether serial assessments enhance predictions. NGAL is associated with acute stress and tubular injury, including the loop of Henle and distal tubule. Since it is rapidly responsive to tubular injury, NGAL can provide modest predictive value for the progression of DKD and found elevated in urine before albuminuria.[23] The sensitivity of NGAL was found higher than that of conventional markers, making it a potent tool for diagnosis, staging, and monitoring the disease.[24] However, its level is influenced by inflammation and is less specific for chronic progression, in addition to a lack of a universal threshold value.

Inflammation and tubular damage could predict the progression of DN years before eGFR decline. TNFR-1 and TNFR-2 are associated with kidney disease progression in patients with micro-or macroalbuminuria. Even in patients with normoalbuminuria, circulating soluble (s) TNFR1/TNFR2 and KIM-1 consistently predict DKD outcomes better than albuminuria. Furthermore, in patients with T2 DM with normoalbuminuria, an elevated plasma level of TNFR-1 and TNFR-2 are linked to an increased risk of kidney disease development.[25] However, sTNFR1/sTNFR2 (serum/plasma) is clinically available in some regions, limiting its generalizability.

The other markers whose plasma concentrations were linked to the advancement of DKD are soluble urokinase-type plasminogen activator receptor, monocyte chemotactic protein-1 (CCL2), YKL-40 (a glycoprotein binds to heparin and chitin of ECM) and MCP-1.[26] A podocyte-specific marker, Nephrin (which is crucial for preserving the integrity of the glomerular filtration barrier) is associated to the development of proteinuria and its presence in urine in DN patients with normoalbuminuria detected prior to microalbuminuria and thus can be considered as an early marker of DN.[27] Similar observation on a panel of biomarkers, including stromal cell-derived factor-1 (a CXC chemokine family member), P-selectin, and mtDNA, were found in subclinical DKD patients with normoalbuminuria.[28] Urinary proteomics (e.g., CKD273 classifier) and urinary exosomal miRNAs (e.g., miR-136-5p) are promising markers that require further validation, mainly for their role in the progression of DKD.[29]

The performance of biomarkers may differ across ethnicities and regions owing to genetic, environmental, and lifestyle factors. This emphasizes the need for external validation in diverse cohorts. In addition, the incremental predictive power of novel markers beyond established biomarkers, such as microalbuminuria, must be established. Therefore, more longitudinal studies on heterogeneous populations are warranted. The variability in assay techniques and reference ranges mainly limits generalizability, which specifies the requirement of universal diagnostic thresholds for clinical adoption. Superior prediction scores can be obtained by combining novel biomarkers with imaging and clinical indicators and integrating them into risk models. Multi-analyte panels/machine learning scores: The KidneyIntelX.dkd test (TNFR1, TNFR2, KIM-1, and clinical variables) has updated validation and is being applied for early-stage DKD prognostication in T2DM.[30] However, external validation remains the main limitation of this study. Recently, a chip-based detection system developed by Wayen Biotechnologies (Shanghai, China) using markers such as retinol-binding protein 4, Vitamin D-binding protein, TNFR-2, and KIM-1 in midstream urine collected early morning showed a good discrimination power (area under the curve [AUC] 0.812) with 74% sensitivity and 76% specificity in detecting DKD.[31] Artificial intelligence technologies can combine novel biomarkers with clinical findings to provide a potent strategy for early detection and personalized treatment. Researchers have developed a more accurate risk prediction framework and increased the predictive resilience by combining machine learning algorithms with conventional statistical analysis. Using multimodal data, machine-learning techniques have shown promise in predicting diabetic complications.[32-34] Recently, XGBoost model integrating 15 features of patients including microalbuminuria has demonstrated as a superior model in predicting the accuracy of DKD in T2DM patients.[35] Expanding evidences suggest that targeted metabolite signatures can flag early DKD and

track its progression beyond urine albumin-creatinine ratio (UACR)/eGFR. The application of novel markers will be determined by future validation studies, mechanistic investigations, and incorporation into risk models to determine whether it becomes a standard therapeutic practice in relation to cost, especially in low- and middle-income settings with a high prevalence of diabetes.

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TAA: Study conception, data collection, analysis, interpretation, draft writing, and editing.

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Review Article

A Systematic Review of Sterile Intrauterine Inflammation, Immune-Metabolic Cues, and Epigenetic Programming: The Hidden Path to Preterm Birth

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Abstract

Preterm birth often emerges from inflammatory pathways that unfold in the absence of detectable infection, reflecting a multifaceted interplay between immune activation, metabolic imbalance, environmental stressors, and epigenetic regulation. To clarify how these processes converge, we conducted a systematic review aligned with PRISMA 2020, examining evidence from human observational and interventional studies, mechanistic experiments, and prior systematic reviews across major biomedical databases through March 2025. Forty-eight eligible studies were identified from an initial pool of 2,643 records. Across diverse methodologies, a coherent pattern of four mechanistic themes emerged. Alarmin- and inflammasome-driven signaling—marked by molecules such as IL-1β and S100A12—appeared central to sterile inflammatory activation. A second theme involved metabolic disturbance, particularly obesity-associated impairment in lipid-mediated resolution pathways. Environmental exposures, including fine particulate matter, PFAS, metals, and newly described microplastic contaminants, formed a third mechanistic axis through oxidative stress and epigenetic disruption. A fourth contributor involved alterations in gut or reproductive microbiota and short-chain fatty acid availability, which together influence immune priming.Across these domains, a growing set of translational biomarkers—including amniotic fluid IL-6, extracellular-vesicle microRNAs, maternal cell-free RNA, cfDNA methylation and fragmentomic patterns, and placental T2* MRI (a noninvasive imaging measure of placental oxygenation and perfusion)—show potential for early risk stratification. Interventional signals were noted for omega-3 supplementation, IL-1 pathway modulation, microbiome-targeted strategies, and exposure reduction. Collectively, current evidence supports a model in which sterile inflammation links immune triggers with metabolic, environmental, and epigenetic programming to shape susceptibility to preterm birth. This integrated perspective highlights opportunities for multi-omic screening, biomarker-guided trials, and policy actions aimed at reducing upstream inflammatory drivers.

Keywords: Epigenetic programming, immune-metabolic pathways, multi-omic biomarkers, preterm birth, *sterile intrauterine inflammation*

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Introduction

Preterm birth (PTB) remains the leading cause of neonatal morbidity and mortality worldwide, affecting approximately one in ten live births and contributing to long-term

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neurodevelopmental, cardiometabolic, and respiratory sequelae.[1] Despite substantial advances in obstetric care, the biological pathways that precipitate spontaneous PTB remain incompletely defined, and genuinely effective preventive strategies are still limited.[2] Historically, intrauterine infection was viewed as the principal trigger of inflammation-driven parturition. Increasing evidence, however, demonstrates that a considerable proportion of PTB arises from sterile intrauterine inflammation, a condition of maternal–fetal immune activation that occurs without identifiable pathogens.[3,4] This sterile inflammatory state may originate from metabolic disturbances, nutritional imbalance, environmental exposures, oxidative stress, or epigenetic reprogramming.[5,6] Clarifying these pathways has become a central priority in modern perinatal research.

The concept of fetal origins of adult disease emphasizes how early-life exposures shape lifelong physiological and health trajectories.[7] Epigenetic programming within the placenta and fetus provides a mechanistic link between maternal conditions – including obesity, psychosocial stress, and pollutant exposure – and adverse pregnancy outcomes such as PTB and later cardiometabolic disease.[8,9] Earlier reviews have addressed specific components of these pathways, including microbiome disruption,[10] interleukin-1 (IL-1) signaling,[11] and omega-3 fatty acid-dependent inflammatory resolution.[12,13] Yet no synthesis has bridged immune, metabolic, environmental, and epigenetic processes within the unified framework of sterile intrauterine inflammation. Technological advances in lipidomics,[14] DNA methylation profiling,[15] and environmental epigenetics[16] further highlight the need for an integrated, multiomic interpretation.

To address this gap, we conducted a systematic review of 48 studies encompassing human cohorts, mechanistic animal and *ex vivo* models, and interventional trials. Study selection procedures are depicted in Figure 1, while methodological characteristics are summarized in Tables 1-3. Integrated evidence from these sources reveals four recurring mechanistic endotypes. The first involves alarmin and inflammasome activation, in which molecules such as S100A12 and IL-1β trigger inflammation-mediated preterm labor in the absence of infection.[25,33] The second reflects metabolic and obesity-associated impairment of inflammatory resolution pathways, including reduced production of specialized pro-resolving mediators (SPMs) derived from omega-3 fatty acids.[21,28] The third captures pollutant-associated epigenetic perturbations, where fine particulate matter ≤2.5 μm in diameter (PM2.5), per- and polyfluoroalkyl substances (PFAS), and microplastics alter placental DNA methylation and related regulatory networks.[27,44] The fourth concerns microbiome and short-chain fatty acid (SCFA) dysregulation, characterized by diminished butyrate-producing taxa and altered metabolite availability, which influence immune priming and inflammatory tone.[34,36]

These mechanistic endotypes help explain how robust inflammatory signaling can arise in the absence of infection. Clinical studies show that women presenting with preterm labor and intact membranes often exhibit elevated IL-6 despite negative cultures, demonstrating a sterile intra-amniotic inflammatory phenotype.[20,42] Mechanistic investigations provide further support: activation of the IL-1/nucleotide-binding domain, leucine-rich repeat, and pyrin-domain containing protein 3 (NLRP3) inflammasome accelerates parturition in animal models,[45] and early clinical work suggests that IL-1 pathway blockade may offer therapeutic benefit.[41] Maternal obesity amplifies systemic inflammation and disrupts resolution signaling,[35] whereas supplementation with omega-3 fatty acids has reduced early PTB risk in several clinical contexts.[19,30,31] Environmental exposures, particularly PM2.5 and persistent chemical pollutants, contribute to oxidative stress, placental hypoxia, and disrupted epigenetic regulation.[37,43,46] Recent detection of micro- and nanoplastics in preterm human placentae widens the spectrum of sterile inflammatory triggers.[17,38]

The placenta acts both as a target and mediator of these processes. Altered methylation landscapes,[47] dysregulated

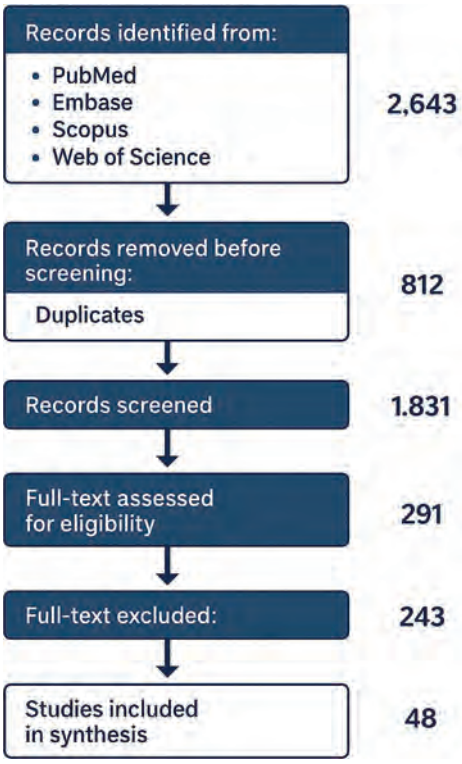
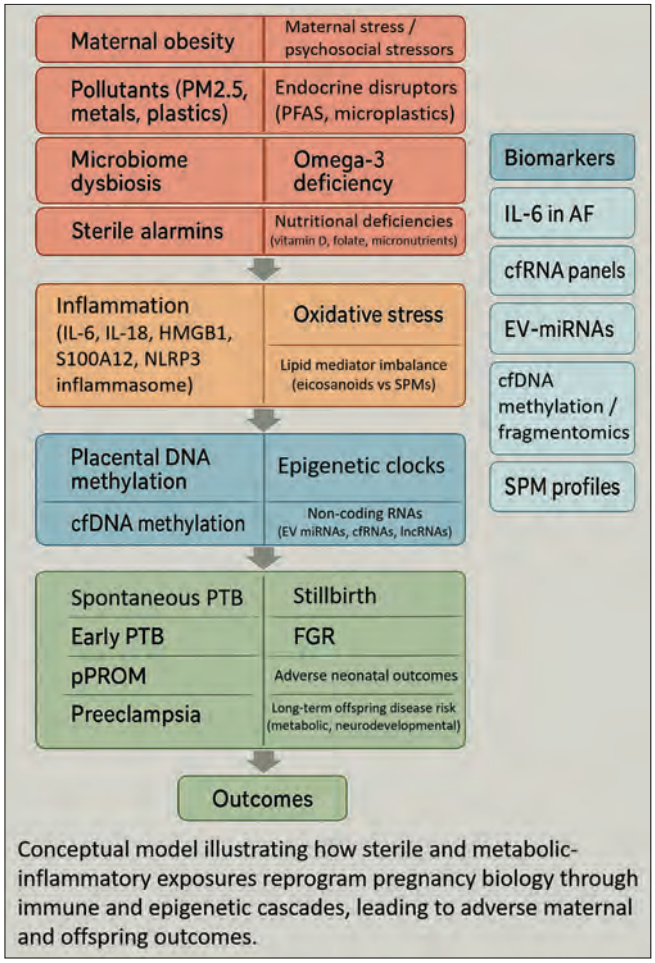


Figure 1: Preferred Reporting Items for Systematic Reviews and Meta-Analyses 2020 flow diagram of study selection. Flow of information through the systematic review process. A total of 2643 records were identified from PubMed, Embase, Scopus, and Web of Science. After removal of 812 duplicates, 1831 records were screened at the title and abstract level. Of these, 291 full-text articles were assessed for eligibility, and 243 were excluded for not meeting the inclusion criteria. Finally, 48 studies were included in the qualitative synthesis of sterile intrauterine inflammation, immune-metabolic cues, and epigenetic programming in preterm birth

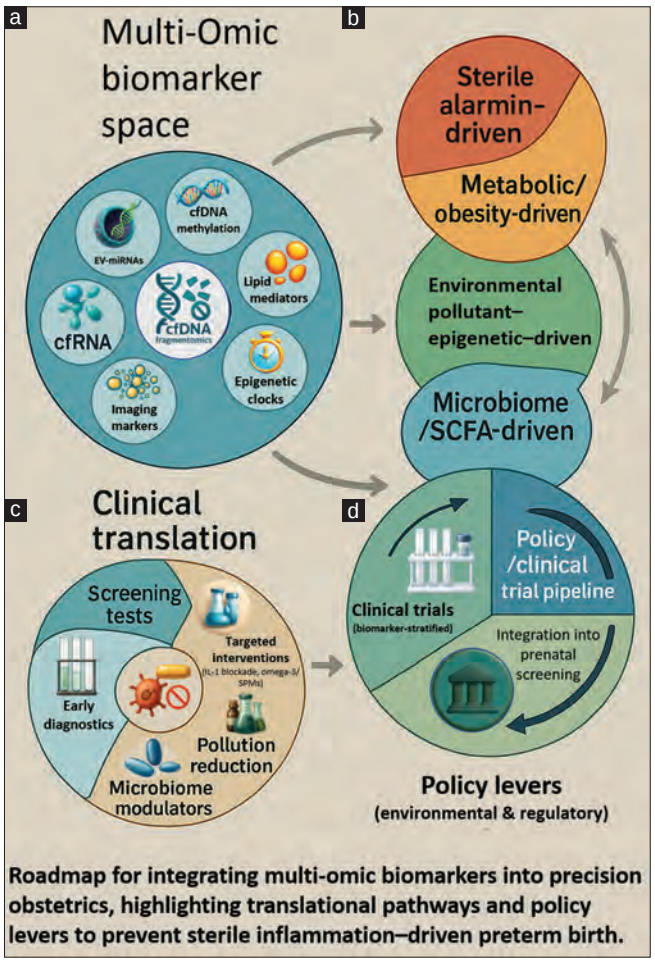
non-coding RNA expression,[18,40] and changes in cell-free DNA (cfDNA) fragmentation patterns[23,39] demonstrate how maternal exposures influence fetal programming. Multiomic biomarkers, including amniotic fluid IL-6 and high-mobility group box-1 (HMGB1),[22,29] extracellular vesicle–derived microRNAs,[24] cell-free RNA (cfRNA) signatures,[48] cfDNA methylation and fragmentomic profiles,[32] and placental oxygenation assessed by T2* magnetic resonance imaging (MRI),[26] now provide non-invasive avenues for monitoring sterile inflammation. These data coalesce into mechanistic clusters illustrated in Figure 2 and support a translational framework outlined in Figure 3 for biomarker-guided screening and intervention. Table 4 extends this framework by summarizing mechanistic animal and *ex vivo* models alongside their translational relevance.

This systematic review synthesizes human and mechanistic evidence linking sterile intrauterine inflammation with metabolic, environmental, microbiome-derived, and epigenetic pathways contributing to PTB. Through this integrative analysis, we aim to provide an updated, comprehensive account



Conceptual model illustrating how sterile and metabolic-inflammatory exposures reprogram pregnancy biology through immune and epigenetic cascades, leading to adverse maternal and offspring outcomes.

Figure 2: Pathophysiological cascade from exposures to adverse pregnancy outcomes. Conceptual model integrating evidence from 48 studies. Maternal, environmental, nutritional, microbiome-related, and sterile alarmin exposures initiate inflammatory, oxidative, and lipid mediator pathways. These converge on epigenetic programming – including placental and cell-free DNA (cfDNA) methylation, non-coding RNAs, and epigenetic clocks – shaping pregnancy biology. Alongside measurable biomarkers (interleukin 6 in amniotic fluid, cfRNA panels, EV-miRNAs, cfDNA methylation/fragmentomics, specialized pro-resolving mediator profiles), these cascades culminate in adverse outcomes such as spontaneous and early preterm birth, preterm premature rupture of membranes, preeclampsia, stillbirth, fetal growth restriction, neonatal morbidity, and long-term offspring disease. cfRNA: Cell-free RNA, CfDNA: Cell-free DNA, IL: Interleukin, pPROM: Preterm premature rupture of membranes, HMGB1: High-mobility group box-1



Roadmap for integrating multi-omic biomarkers into precision obstetrics, highlighting translational pathways and policy levers to prevent sterile inflammation–driven preterm birth.

Figure 3: Multi-omic endotyping and translational roadmap in preterm birth. Integrated roadmap linking biomarker discovery to clinical translation. (a) Multi-omic biomarker space (cell-free DNA [cfRNA] methylation, fragmentomics, cfRNA, EV-miRNAs, lipid mediators, epigenetic clocks, imaging markers). (b) Mechanistic endotypes (sterile alarmin-driven, metabolic/obesity-driven, environmental pollutant–epigenetic–driven, microbiome/short-chain fatty acid-driven). (c) Clinical translation (screening and early diagnostics, targeted interventions such as interleukin 1 blockade and omega-3/specialized pro-resolving mediators, microbiome modulators, pollution reduction). (d) Policy and clinical trial pipeline (biomarker-stratified clinical trials, integration into prenatal screening, and environmental/regulatory levers). This roadmap illustrates how multi-omic biomarkers can enable precision obstetrics, stratify maternal risk, and inform both therapeutic and policy interventions to prevent sterile inflammation–driven preterm birth. cfRNA: Cell-free RNA, CfDNA: Cell-free DNA

Table 1: Core evidence set on sterile intrauterine inflammation, immune-metabolic cues, and epigenetic programming leading to preterm birth: study features and risk of bias assessment

Author	Study design	Setting and sample	Pregnancy window	Exposure/construct	Tissue/sample and assay
Romero *et al*. (2014)[17]	Prospective cohort	Tertiary obstetric center; women with PTL and intact membranes	Mid–late gestation	Sterile intra-amniotic inflammation (culture/PCR-negative) versus no inflammation	Amniotic fluid; IL-6, HMGB1 assays
Stranik *et al*. (2022)[18]	Observational comparative	Women with sPTL and intact membranes	Presentation at sPTL	Sterile versus infectious intra-amniotic states	Cervical fluid; IL-6
Motomura *et al*. (2021)[19]	Mechanistic: *Ex vivo* human membranes + mouse model	Human chorioamniotic membranes; pregnant mice	Late gestation (model)	Alarmin S100A12 exposure (sterile trigger)	*Ex vivo* membrane stimulation; in vivo exposure
Lopez *et al*. (2024)[20]	Mechanistic mouse	Murine parturition models	Late gestation	IL-1β/inflammasome activation	Tissue cytokines; pathway assays
Jiao *et al*. (2024)[21]	Observational (multi-region)	Population-based datasets; spontaneous PTB	All trimesters; windowed analyses	Ambient PM2.5 and constituents	Monitoring/model assignments
Wang *et al*. (2023)[22]	Systematic review/meta-analysis	Multi-study synthesis (China)	All trimesters	Outdoor air pollutants (PM, NO2, etc.)	Literature synthesis; meta-analysis
Thaichana *et al*. (2025)[23]	Retrospective cohort (registry)	Northern Thailand; 2016–2022	All trimesters	PM2.5 exposure	Station/modeled PM2.5 assignment
Guo and Jiang *et al*. (2025)[15]	Meta-analysis	Chinese cohorts/case–controls	Various	PM exposure	Literature synthesis; meta-analysis
Everson *et al*. (2025)[11]	Cohort (placental omics)	Mother–placenta dyads	Delivery	Placental PFAS concentrations	Placenta; epigenome-wide/targeted DNAm
Xie *et al*. (2024)[24]	Prospective cohort	Mother–infant pairs	Delivery	Maternal PFAS exposure (prospective)	Placenta; DNAm profiling
Huff *et al*. (2025)[25]	Cohort (ELGAN subset)	Extremely preterm infants; USA	Delivery	Prenatal metals exposure	Placenta; epigenetic gestational age
Zurub *et al*. (2023)[26]	Systematic review (narrative/structured)	Reproduction and plastics literature	All	Microplastics exposure	Literature synthesis
Jochum *et al*. (2025)[27]	Observational (preprint)	Preterm versus term placentae	Delivery	Placental micro-/nanoplastic burden	Placenta; particle analytics (spectroscopy/microscopy)
Jin *et al*. (2023)[28]	Nested case–control + transcriptomics	Maternal plasma + placenta	Early gestation (plasma); delivery (placenta)	Cell-free RNA biomarkers for PTB	cfRNA (plasma); placental RNA-seq
Gál *et al*. (2024)[12]	Case–control (first trimester)	Maternal plasma	First trimester	Extracellular vesicle small RNAs	EV miRNA sequencing
Ghosh *et al*. (2024)[13]	Prospective nested case–control	Maternal plasma	Early gestation	EV miRNA signatures	EV miRNA assay panels
van Vliet *et al*. (2025)[29]	Prospective cohort	Rotterdam Periconception Cohort	Across gestation (serial)	Placental DNA methylation signals in maternal cfDNA	Maternal plasma cfDNA; methylation profiling
Guo *et al*. (2025)[16]	Case–control (fragmentomics)	Maternal plasma	Mid gestation (typical)	cfDNA genome-wide nucleosome footprints	Plasma cfDNA fragmentomics
Nichols *et al*. (2024)[30]	Observational imaging	Mother–fetus dyads	Mid–late gestation	Placental T2* MRI (oxygenation/perfusion)	MRI T2* mapping; fetal brain metrics
Cetin *et al*. (2024)[7]	Evidence synthesis/guidance (RCT-backed)	Multi-trial context	Prevention window (mid–late gestation)	Omega-3/SPM strategies	Trial evidence; metabololipidomics context

Author	Effect signal (qualitative)*	Risk-of-bias tool and rating	Key strengths	Limitations	Notes for synthesis/endotyping
Romero *et al*. (2014)[17]	Sterile inflammation associated with shorter latency and higher sPTB versus no inflammation	NOS: Low – clear sterile phenotype, prospective sampling	Clinical anchor for sterile construct	Single-center; lab protocol variability	Defines sterile-endotype comparator for synthesis
Stranik *et al*. (2022)[18]	Elevated IL-6 in both sterile and infectious states; supports triage utility	NOS: Moderate – timing/assay heterogeneity	Non-invasive biomarker sampling	Cross-sectional; limited longitudinal follow-up	IL-6 as triage signal to flag sterile versus infectious

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Table 1: Contd...

Author	Effect signal (qualitative)	Risk-of-bias tool and rating	Key strengths	Limitations	Notes for synthesis/ endotyping
Motomura *et al*. (2021)[19]	S100A12 induces sterile inflammation and PTB in mice; mechanistic causality	N/A (preclinical)	Causality for alarmin pathway	Species/translation gap	Inflammasome/alarmin node for endotype mapping
Lopez *et al*. (2024)[20]	IL-1/NLRP3 pivotal during late parturition cascade	N/A (preclinical)	Clear targetable pathway	Species differences	Therapeutic IL-1/ NLRP3 endotype target
Jiao *et al*. (2024)[21]	Positive association overall; constituent-specific risks	NOS: Moderate – exposure misclassification addressed	Large, diverse populations	Residual confounding possible	Exposure endotyping (air toxics mixtures)
Wang *et al*. (2023)[22]	Pooled increased risk with air pollutants	AMSTAR-2: Moderate – heterogeneity explored	Broad evidence overview	Between-study heterogeneity	Prior weights for pollutant domain in meta-regression
Thaichana *et al*. (2025)[23]	Higher PM2.5 linked to higher PTB/LBW	NOS: Moderate–Low – clear cohort with robust confounder control	Large real-world dataset in LMIC setting	Exposure measurement error; spatial misalignment	Regional thresholds/ policy relevance
Guo and Jiang *et al*. (2025)[15]	Positive pooled association; effect modifiers considered	AMSTAR-2: Moderate – explores modifiers/ publication bias	Country-specific synthesis	Publication bias risk	Inputs for moderator analysis in this review
Everson *et al*. (2025)[11]	PFAS associated with broad DNAm perturbations	NOS: Moderate – rigorous assays; observational limits	Tissue-specific DNAm maps	Causality not inferable	PFAS→epigenetic programming path
Xie *et al*. (2024)[24]	PFAS linked with locus-specific DNAm shifts	NOS: Moderate – exposure timing well-defined	Prospective exposure capture	Replication/ platform differences	Cross-cohort confirmatory evidence
Huff *et al*. (2025)[25]	Metals associated with decelerated epigenetic GA (sex-dependent)	NOS: Moderate – targeted high-risk cohort	Sex-stratified analysis	Generalizability beyond ELGAN uncertain	Metals→clock programming signal
Zurub *et al*. (2023)[26]	Signals of risk; mechanistic plausibility for inflammation/ oxidative stress	ROBIS: Moderate – broad scope with emerging field	Comprehensive emerging pollutant framing	Primary human data sparse	Rationale to include plastics in sterile model
Jochum *et al*. (2025)[27]	Higher micro/nanoplastics in preterm placentae	NOS: Preliminary – methods strong; preprint status	First human quantification of placental plastics	Peer review pending; confounding	Novel sterile trigger candidate
Jin *et al*. (2023)[28]	cfRNAs predict PTB; integrated maternal–placental profiles	NOS: Moderate – rigorous profiling; case–control design	Longitudinal sampling window	External validation needed	Early risk stratification biomarker class
Gál *et al*. (2024)[12]	Distinct EV-miRNA signatures in early pregnancy	NOS: Moderate – clear case definition; early window	Earliest window of signal	Specificity to PTB not direct	Platform/analytics applicable to PTB endotyping
Ghosh *et al*. (2024)[13]	Early EV miRNAs associate with later outcomes	NOS: Moderate – prospective sampling	Strong wet-lab methods	Outcome specificity	Supports EV-miRNA workflow for obstetric risk
van Vliet *et al*. (2025)[29]	Feasible serial profiling; gestational dynamics observed	NOS: Low–Moderate – repeated measures; robust QC	Non-invasive placental methylome access	Clinical endpoint linkage pending	Foundation for cfDNA methylation biomarkers
Guo *et al*. (2025)[16]	Genome-wide footprints classify PTB risk	NOS: Moderate – clear analytic pipeline; validation needed	Orthogonal genome-wide signal	Need external validation and calibration	Fragmentomics added to multi-omic panel
Nichols *et al*. (2024)[30]	Placental T2* correlates with fetal brain maturation	NOS: Moderate – imaging confounders addressed	Systems-level physiology biomarker	Access, standardization across scanners	Imaging readout for endotype severity
Cetin *et al*. (2024)[7]	Omega-3 lowers early PTB in defined subsets	AMSTAR-2/ ROBIS: Moderate – heterogeneous RCTs synthesized	Translational and pragmatic guidance	Heterogeneity across trials/doses	Basis for resolution-therapy arm and biomarker monitoring

Effect signal is summarized qualitatively at the protocol stage; no quantitative effect sizes are imputed. Risk-of-bias tools used: NOS (observational studies), RoB-2 (RCTs), AMSTAR-2/ROBIS (systematic reviews/meta-analyses). Mechanistic animal/*ex vivo* studies are noted as N/A but included for pathway-mapping relevance to sterile inflammation endotypes. sPTB: Spontaneous preterm birth, N/A: Not available, RCTs: Randomized controlled trials, PFAS: Polyfluoroalkyl substances, SPMs: Specialized pro-resolving mediators, RoB: Risk of bias, AMSTAR-2: A Measurement Tool to Assess Systematic Reviews, version 2, NOS: Newcastle-Ottawa Scale, PTB: Preterm birth, MRI: Magnetic resonance imaging, cfRNA: Cell-free RNA, CfDNA: Cell-free DNA, miRNAs: Micro RNAs, IL: Interleukin, NLRP3: NLR family pyrin domain-containing 3, HMGB1: High-mobility group box-1, PTL: Preterm Labor, sPTL: Spontaneous PTL, PM: Particulate matter, LBW: Low birth weight, PFAS: Polyfluoroalkyl substances

Table 2: Longitudinal biomarker and assay landscape for sterile intrauterine inflammation and preterm birth

Author	Biomarker class	Specific biomarker (s)/ signatures	Biological source/ sample	Gestational timing	Platform/assay method
Romero *et al*. (2014)[17]	Immune mediators	IL-6; HMGB1 (alarmin) levels in AF	Amniotic fluid	Mid–late gestation; at presentation with PTL	ELISA/clinical immunoassays
Stranik *et al*. (2022)[18]	Immune mediators	Cervical fluid IL-6	Cervicovaginal/ cervical fluid	At sPTL presentation (T2–T3)	Immunoassay
Motomura *et al*. (2021)[19]	Alarmins/DAMPs	S100A12-induced inflammatory signature	*Ex vivo* membranes; maternal/fetal compartments (model)	Late gestation (model)	Cytokine panels; histology; functional readouts
Lopez *et al*. (2024)[20]	Cytokine pathway signaling	IL-1β/NLRP3 inflammasome activation	Placental/uterine tissues (model)	Late gestation (model)	qPCR/protein readouts; pathway inhibition
Colas *et al*. (2014)[8]	Lipid mediators/ SPMs	SPM clusters (RvD, RvE, MaR, PD), eicosanoid profile	Human tissues/fluids (platform paper)	Detectable across gestation (feasibility)	LC–MS/MS targeted metabololipidomics
Nordgren *et al*. (2019)[31]	Lipid mediators/ SPMs	Maternal–infant SPM levels; omega-3 status	Maternal plasma; cord blood	T2–delivery; postpartum pairs	LC–MS/MS metabololipidomics
Cetin *et al*. (2024)[7]	Lipid mediators/ clinical	Omega-3 intake with SPM-informed rationale	Maternal plasma (trials); dietary intake	Prevention windows (T2–T3)	Trial biomarker panels; LC–MS/MS where available
Jin *et al*. (2023)[28]	cfRNA signatures	Maternal plasma cfRNAs + placental RNA profiles	Maternal plasma; placenta	Early gestation (T1–early T2) for plasma	RNA-seq; targeted panels
Gál *et al*. (2024)[12]	EV miRNAs	EV small RNA panels (first trimester)	Maternal plasma	T1 (very early)	EV isolation + small RNA-seq
Ghosh *et al*. (2024)[13]	EV miRNAs	Circulating EV miRNA signatures	Maternal plasma	Early gestation (T1)	EV isolation + targeted miRNA assays
van Vliet *et al*. (2025)[29]	cfDNA methylation (placental)	Placental methylation signals in maternal cfDNA	Maternal plasma cfDNA	Across gestation; serial (preconception–T3)	cfDNA methylation profiling (array/ targeted)
Guo *et al*. (2025)[16]	cfDNA fragmentomics	Genome-wide nucleosome footprint signatures	Maternal plasma cfDNA	Mid gestation (screening window)	Fragmentomics (coverage/periodicity patterns)
Yuen *et al*. (2024)[32]	cfDNA (placental content)/review	Placental fraction; methylation-informed assays	Maternal plasma cfDNA	Across gestation	Review of technologies and analytical considerations
Nichols *et al*. (2024)[30]	Imaging biomarkers	Placental T2* (oxygenation/perfusion)	MRI (placenta + fetal brain)	Mid–late gestation	T2* mapping; quantitative MRI
Hall *et al*. (2024)[33]	Imaging biomarkers	Multimodal placental MRI prior to sPTB <32 weeks	MRI (placenta)	Mid–late gestation; pre-sPTB	Multimodal MRI (incl. T2*)
Apicella *et al*. (2019)[2]	Epigenetic marks	Placental DNAm/histone pathways (context)	Placenta (tissue-level)	Throughout gestation (developmental context)	Review; epigenetic assays landscape
Deng *et al*. (2024)[9]	Epigenetic marks	Placental DNA methylation changes	Placenta	Delivery (cross-sectional)	DNAm arrays/targeted sequencing
Basak *et al*. (2024)[3]	Microbiome-derived metabolites	SCFAs (butyrate) → immune/epigenetic effects	Maternal gut; placenta (mechanistic links)	Across gestation (diet-dependent)	Review of SCFA biology
Kopczyńska and Kowalczyk (2024)[34]	Microbiome-derived metabolites	Short-chain fatty acids; HDAC/GPCR signaling	Maternal circulation; tissues	Across gestation (exposure-dependent)	Review; mechanistic

Author	Associated exposure/trigger context	Clinical outcome (s)	Effect direction/ predictive utility	Longitudinal reproducibility	Integration potential in endotyping	Limitations
Romero *et al*. (2014)[17]	Sterile intra-amniotic inflammation (culture/ PCR-negative)	Shorter amniocentesis-to-delivery; sPTB	↑ IL-6/HMGB1 in sterile cases; prognostic for latency	Repeated AF sampling uncommon	High – anchors sterile endotype	Invasive sampling; center-specific assays
Stranik *et al*. (2022)[18]	Sterile versus infectious IA inflammation triage	sPTB	Discriminatory for inflammation; adjunct triage marker	Cross-sectional; feasible serial in clinics	Moderate – noninvasive triage signal	Specificity to sterile versus infectious limited; timing effects

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Table 2: Contd...

Author	Associated exposure/trigger context	Clinical outcome (s)	Effect direction/ predictive utility	Longitudinal reproducibility	Integration potential in endotyping	Limitations
Motomura *et al*. (2021)[19]	Sterile trigger via alarmin exposure	PTB; neonatal outcomes (model)	Causal link: alarmin → sterile inflammation → PTB	Preclinical serial sampling feasible	High (mechanistic node)	Translation to humans; quantify S100A12 clinically
Lopez *et al*. (2024)[20]	Sterile/parturition cascade activation	Timing of parturition; inflammatory readouts	Pathway pivotal; candidate therapeutic target	Preclinical consistency across experiments	High (therapeutic endotype target)	Species differences; safety in pregnancy
Colas *et al*. (2014)[8]	Resolution biology; diet omega-3 status	Links to inflammatory tone; pregnancy translational studies use platform	Discriminatory lipid mediator signatures	Validated platform; serial capable	High – pairs with omega-3 interventions	Specialized labs; preanalytical handling critical
Nordgren *et al*. (2019)[31]	Dietary omega-3; inflammatory status	Maternal–infant outcomes; inflammation	SPM levels track nutrition/ inflammation; potential protective signal	Some longitudinal pairing; moderate N	Moderate– High – nutritional lever; resolution readouts	Assay availability; heterogeneity in diets
Cetin *et al*. (2024)[7]	Low omega-3; heightened inflammation	Early PTB	Benefit in subsets; monitor with SPM panels	Multi-trial evidence; feasibility for serial draws	High – intervention monitoring biomarker	Trial heterogeneity; dose/timing optimization
Jin *et al*. (2023)[28]	Sterile inflammation; placental stress	PTB	Predictive cfRNA signatures; integrative models	Discovery + internal validation; external needed	High – early noninvasive classifier	Platform harmonization; cohort diversity
Gál *et al*. (2024)[12]	Systemic inflammatory endotype (PE proxy)	Early PE (proxy for inflammatory pregnancy risk)	Early discriminatory signatures	Case–control; needs PTB-focused replication	Moderate – technology path for PTB	Specificity to PTB versus PE; standardization
Ghosh *et al*. (2024)[13]	Inflammation/stress axis	Later PE features	Prognostic early signatures	Prospective nested case–control; reproducible assays	Moderate – transferable to PTB endotyping	Outcome generalization; PTB validation needed
van Vliet *et al*. (2025)[29]	Placental development/aging; sterile programming	Feasibility; trajectory mapping	Feasible serial tracking of placental signals	Serial cohort; strong QC	High – backbone for noninvasive endotyping	Clinical endpoint linkage pending
Guo *et al*. (2025)[16]	Placental stress; sterile inflammation context	PTB	Predictive classifier performance	Discovery + internal validation; external needed	High – orthogonal to methylation/ miRNA	Standardization; prospective validation
Yuen *et al*. (2024)[32]	Noninvasive placental biology	Platform guidance; PTB biomarker potential	Framework for assay selection	N/A (review)	High – informs analytic pipelines	Method heterogeneity; need consensus
Nichols *et al*. (2024)[30]	Placental hypoxia/ sterile inflammation link	Fetal cortical/ subcortical maturation	Correlated placental–fetal maturation indices	Observational serial feasibility varies	High – systems-level readout	Scanner harmonization; access/logistics
Hall *et al*. (2024)[33]	Placental hypoxia/ inflammation	sPTB<32 weeks	Associations with adverse outcomes	Observational; single-timepoint in many	High – complements molecular panels	Motion/artifact; resource intensive
Apicella *et al*. (2019)[2]	Programming and PE; relevant to sterile pathways	PE; placental function	Mechanistic framework for epigenetic readouts	N/A (review)	Moderate – informs loci/ pathways	Not PTB-specific; extrapolation needed
Deng *et al*. (2024)[9]	Inflammatory hypertension axis	PIH/PE outcomes	Candidate loci/pathways relevant to sterile programming	Cross-sectional; replication varies	Moderate – pathway/locus candidates	Condition-specific; PTB linkage indirect
Basak *et al*. (2024)[3]	Dysbiosis; nutrient status	Fetal growth; PTB plausibility	Mechanistic plausibility; therapeutic leads	N/A (review)	Moderate – adjunct to sterile threshold	Human PTB validation needed

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Table 2: Contd...

Author	Associated exposure/trigger context	Clinical outcome (s)	Effect direction/ predictive utility	Longitudinal reproducibility	Integration potential in endotyping	Limitations
Kopczyńska and Kowalczyk (2024)[34]	Obesity/IR; low-fiber diet	Low-grade inflammation; metabolic risk	Mechanistic support for SCFAs as modulators	N/A (review)	Moderate – integrates diet–immune–epi	Translational assays not standardized

Biomarkers are organized by class and mapped to source, timing, platform, and clinical utility. Effect direction is qualitative; prospective validation status is indicated under longitudinal reproducibility. Integration potential reflects expected contribution to multi-omic endotyping of sterile inflammation in preterm birth. T1/T2/T3: First/second/third trimester, RvD/RvE: Resolvins D/E, MaR: Maresins, PD: Protectins, N/A: Not available, IL: Interleukin, NLRP3: NLR family pyrin domain-containing 3, PTB: Preterm birth, sPTB: Spontaneous preterm birth, SCFA: Short-chain fatty acid, SPMs: Specialized pro-resolving mediators, LC: Liquid chromatography, MS: Mass spectrometry, LBW: Low birth weight, HOMA-IR: Homeostatic model assessment of insulin resistance, DAMPs: Damage-associated molecular patterns, NF-κB: Nuclear factor-κB, GPCR: G-protein–coupled receptor, HDAC: Histone deacetylase, IL: Interleukin, MRI: Magnetic resonance imaging, sPTB: Spontaneous preterm birth, EV: Extracellular vesicle, HMGB1: High-mobility group box-1, cfRNA: Cell-free RNA, CfDNA: Cell-free DNA, miRNAs: Micro RNAs, PE: Preeclampsia, PTL: Preterm Labor, sPTL: Spontaneous PTL, qPCR: Quantitative polymerase chain reaction, PCR: Polymerase chain reaction, PIH: Pregnancy-induced hypertension, ELISA: Enzyme-linked immunosorbent assay

of sterile intrauterine inflammation and to outline translational opportunities that may inform precision obstetric strategies and guide future intervention research.

Materials and Methods

This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 statement, ensuring transparent documentation of the search, screening, extraction, and synthesis processes. Although the protocol was developed before the initiation of the review, it was not registered in PROSPERO because the scope integrates clinical, mechanistic, environmental, immunologic, and epigenetic evidence that extends beyond the parameters typically required for registry-based systematic reviews. The methodological procedures were designed to preserve rigor while maintaining flexibility to incorporate translational and experimental studies relevant to sterile intrauterine inflammation.

Eligibility criteria

Studies were considered eligible if they examined sterile intrauterine inflammation, immune–metabolic dysregulation, environmental exposures, or epigenetic programming in relation to PTB or associated maternal–fetal outcomes. Eligible designs included observational cohort and case–control studies, randomized controlled trials, and previously published systematic reviews or meta-analyses. Mechanistic animal and *ex vivo* models were included when they demonstrated translational relevance to human disease pathways. Only peer-reviewed full-text articles published in English between 2000 and 2025 were included. Excluded sources comprised conference abstracts, commentaries, case reports, narrative reviews, editorials, and papers lacking relevance to sterile inflammatory or epigenetic mechanisms.

Information sources and search strategy

Four databases – PubMed, Embase, Scopus, and Web of Science – were searched from inception through March 2025 using controlled vocabulary and free-text terms related to PTB, sterile intrauterine inflammation, immune signaling, lipid mediators, epigenetic programming, microbiome dysregulation, and environmental exposure. No restrictions were applied to population characteristics or study design to ensure maximal sensitivity. Reference lists of included studies and relevant reviews identified through the search were screened manually for additional literature. The search yielded 2643 records; after the removal of 812 duplicates, 1831 unique titles and abstracts were screened. A total of 291 full-text articles were assessed, and 48 studies met all eligibility criteria. The complete selection process is summarized in the PRISMA flow diagram [Figure 1].

Selection process

All titles, abstracts, and full-text articles were independently reviewed by two authors following prespecified inclusion criteria. Discrepancies were resolved by discussion, with arbitration by a third reviewer when needed. Reasons for exclusion at the full-text stage were documented to maintain methodological transparency in accordance with PRISMA recommendations.

Data collection process

A standardized extraction matrix was piloted and refined before use. Extracted data included study design, population and sample characteristics, gestational timing, exposure categories, biospecimens collected, assay platforms, biomarker classes, and clinical or mechanistic outcomes. Additional methodological characteristics were captured, including confounder adjustment, quality of laboratory assays, and length of follow-up. Extraction was performed independently by two reviewers, and inconsistencies were resolved through consensus. No study authors were contacted for additional information.

Data items

Extracted data were organized to map the interplay among exposures, immune pathways, metabolic mediators, epigenetic mechanisms, and pregnancy outcomes within a sterile-inflammation framework. Study characteristics and methodological quality assessments are presented in Table 1. Biomarker domains, analytical platforms, and biological

Table 3: Exposure → Mechanism → Epigenetic/Immune Programming → Outcomes lever map

Exposure domain	Representative exposure (s)/ trigger examples	Primary mechanistic pathway (s)	Epigenetic/molecular programming features	Intermediate biomarkers	Clinical outcomes linked	Intervention levers	Level of evidence	Research gaps/future needs
$PM_{2.5}$ air pollution and constituents[15,21-23]	$PM_{2.5}$ mass; black carbon; metals-bound PM; secondary inorganic aerosols	Oxidative stress; NF-κB activation; placental hypoxia; endothelial dysfunction	Placental DNA methylation shifts; altered miRNA networks; epigenetic age perturbation	Maternal serum IL-6; oxidative stress markers; placenta MRI T2* hypoxia signatures	sPTB; early PTB; LBW; PROM-associated pathways	Ambient air quality improvement; prenatal exposure mitigation; indoor filtration; antioxidant/ resolution-supportive nutrition	High (multi-cohort/ meta-analyses; region-specific consistency)	Source apportionment by constituent; trimester-specific windows; standardize exposure assessment; integrate with molecular endpoints
PFAS exposure[11,24]	PFOA, PFOS, PFHxS mixes measured in placenta/maternal blood	Endocrine disruption; immune modulation; mitochondrial stress	Placental DNAm perturbations at immune/metabolic loci; potential epigenetic clock shifts	cfDNA methylation signals; targeted DNAm panels; inflammatory cytokines	PTB risk plausibility; placental dysfunction; downstream neonatal effects	Policy/regulation; contaminated water remediation; exposure counseling; nutrition to support one-carbon/ antioxidant pathways	Moderate (multiple human cohorts with omic endpoints)	Dose–response; mixtures modeling; longitudinal reversibility; link to sterile biomarker endotypes
Prenatal metals[25]	Cadmium, lead, arsenic mixtures	Oxidative stress; inflammatory signaling; angiogenic imbalance	Placental epigenetic gestational age deceleration (sex-dependent); DNAm alterations	Placental epigenetic clocks; maternal/cord biomarkers	Extremely preterm complications; PTB plausibility	Environmental remediation; nutritional chelation support; regulatory limits	Moderate (targeted high-risk cohorts with robust epigenetics)	Generalizability beyond ELGAN; mechanistic mediation analysis; intervention trials
Micro-/ nanoplastics[26,27]	Polyethylene, polypropylene particles; plasticizers adhered to particles	Particle-induced inflammation; ROS generation; immune activation	Putative placental DNAm/miRNA changes; translocation potential	Placental particle burden analytics; IL-6; oxidative markers	Association with preterm placentae; fertility and pregnancy risks	Plastic exposure reduction; policy on microplastics; biomonitoring pipelines	Emerging (first human quantifications; mechanistic plausibility)	Standardized detection methods; prospective human cohorts; causal mediation to PTB
Maternal obesity/insulin resistance[10,35,36]	High BMI; HOMA-IR; dyslipidemia; low-grade inflammation	Placental macrophage activation; inflammasome priming; impaired resolution	DNAm in metabolic/ inflammatory pathways; miRNA shifts	Adipokines; cytokines; lipid mediator imbalance (SPM deficits)	sPTB risk modulation; PE; fetal programming	Weight/nutrition optimization; physical activity; anti-inflammatory dietary patterns; SPM-supportive omega-3	Moderate (multiple reviews/cohorts; mechanistic coherence)	Endotype-specific thresholds; interaction with pollutants; interventional biomarker endpoints
Omega-3 deficiency/ impaired resolution[7,8,31,37]	Low DHA/EPA intake; reduced SPM biosynthesis (RvD, RvE, MaR, PD)	Resolution deficit; persistent neutrophilic signaling; eicosanoid imbalance	Epigenetic tuning of inflammatory genes; lipid-omic signatures	SPM panels (LC–MS/MS); eicosanoid ratios; hsCRP/cytokines	Early PTB reduction in subsets with supplementation	Dietary DHA/EPA; SPM analogues; precision dosing by baseline status	Moderate–High (RCTs/meta-analyses support benefit; mechanistic readouts available)	Responder profiling; dose/timing; combine with sterile endotype biomarkers
Microbiome dysbiosis/SCFA depletion[3,4,34,38,39]	Reduced butyrate producers; altered vaginal/gut communities	Toll-like receptor tone; GPCR/HDAC signaling; immune tolerance loss	DNAm/histone acetylation modulation; miRNA changes; metabolome shifts	SCFA levels; microbiome profiling; EV miRNAs; cfRNA	PTB plausibility; adverse offspring programming	Dietary fiber; pre/ probiotics; targeted microbiome therapeutics	Moderate (mechanistic + emerging human data)	Causality in human PTB; standardized SCFA assays; microbiome–exposome interactions
Sterile alarmins[19]	S100A12; HMGB1 (alarmins/DAMPs)	Pattern recognition receptor activation; inflammasome/ NLRP3	Downstream transcriptional/ epigenetic inflammatory programs	AF IL-6/HMGB1; cervical IL-6; tissue cytokine panels	Induction of PTB in models; shorter latency clinically (with AF markers)	Target alarmins; block downstream IL-1; resolution-enhancing strategies	High mechanistic plausibility; clinical signals via AF cytokines	Clinical quantification standards; safe modulation strategies in pregnancy

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Table 3: Contd...

Exposure domain	Representative exposure (s)/ trigger examples	Primary mechanistic pathway (s)	Epigenetic/molecular programming features	Intermediate biomarkers	Clinical outcomes linked	Intervention levers	Level of evidence	Research gaps/future needs
Inflammasome–IL-1 axis[5,20,40]	NLRP3 activation; IL-1β signaling	Caspase-1 activation; cytokine cascade; parturition timing control	Transcriptional priming; potential epigenetic feedback	Maternal plasma IL-1 pathway readouts; EV/cfRNA of inflammasome genes	PTB risk (mechanistic); fetal neuroinflammation (model)	IL-1 blockade (safety data); careful peripartum targeting; biomarker-guided	Moderate–High (preclinical + human safety data)	Pregnancy-specific safety windows; selection of sterile endotype candidates
p38 MAPK and parturition clocks[41,42]	Stress kinase activation; redundancy/synergy in parturition signals	MAPK–NF-κB crosstalk; senescence-like signaling	Epigenetic transcription factor networks; chromatin state changes	Phospho-MAPK panels; cytokines; cfRNA modules	Timing of labor; susceptibility to earlier activation	MAPK pathway modulators (experimental); combined resolution strategies	Moderate (theoretical + translational suggestions)	Clinical-grade inhibitors and safety; specificity to sterile PTB
cfDNA placental methylation signatures[29,32,43]	Placental fraction in maternal plasma; targeted/array methylation	Epigenetic memory of exposures and inflammation	Differential methylation; epigenetic clocks; nucleosome positioning interplay	cfDNA methylation panels; placental fraction estimates	PTB risk stratification; growth trajectories	Noninvasive serial profiling; integrate with clinical risk	Moderate (feasibility + early associations)	Assay standardization; multicenter validation; clinical utility trials
cfDNA fragmentomics[16]	Genome-wide nucleosome footprints; fragment size periodicity	Chromatin accessibility/turnover changes in placenta	Footprint patterns reflective of tissue-of-origin activity	Fragmentomics classifiers from plasma	PTB prediction	Combine with methylation and EV/cfRNA for robust models	Emerging–Moderate (discovery + internal validation)	External validation; calibration; prospective screening studies
EV miRNAs and cfRNA panels[12,13,28]	EV miRNA signatures; maternal plasma cfRNAs	Immune activation; stress response; placental communication	Regulatory miRNA networks; transcriptomic shifts	EV isolation + miRNA panels; RNA-seq cfRNA	PE (proxy), PTB risk (direct for cfRNA)	Early screening; track response to interventions	Moderate (multiple early studies with strong lab pipelines)	Cross-cohort harmonization; specificity PTB versus other syndromes
Placental hypoxia imaging (MRI T2*)[30,33]	T2* mapping; multimodal MRI metrics	Oxygenation/perfusion deficits; inflammation-hypoxia interplay	Potential links to epigenetic hypoxia signatures	Placenta T2*; fetal brain maturation indices	sPTB <32 weeks; neurodevelopmental trajectories	Noninvasive monitoring; pair with molecular biomarkers	Moderate–High (two high-quality observational studies)	Scanner harmonization; cost; motion artifacts

Exposure domains are mapped to mechanistic pathways, molecular programming, measurable biomarkers, and clinical outcomes, with actionable intervention levers. Levels of evidence are qualitative, reflecting consistency across cohorts, mechanistic support, and trial data where available. LBW: Low birth weight, HOMA-IR: Homeostatic model assessment of insulin resistance, NF-κB: Nuclear factor-κB, GPCR: G-protein–coupled receptor, HDAC: Histone deacetylase, IL: Interleukin, NLRP3: NLR family pyrin domain-containing 3, PTB: Preterm birth, PM: Particulate matter, MRI: Magnetic resonance imaging, PROM: Premature rupture of membranes, sPTB: Spontaneous preterm birth, PFAS: Polyfluoroalkyl substances, ROS: Reactive oxygen species, EV: Extracellular Vesicle, MAPK: Mitogen activated protein kinase, HMGB1: High-mobility group box-1, cfRNA: Cell-free RNA, CfDNA: Cell-free DNA, miRNAs: Micro RNAs, SCFA: Short-chain fatty acid, SPMs: Specialized pro-resolving mediators, LC: Liquid chromatography, MS: Mass spectrometry, hsCRP: High-sensitivity C-reactive protein, DHA: Docosahexaenoic acid, EPA: Eicosapentaenoic acid, BMI: Body mass index, RCT: Randomized controlled trials, PE: Preeclampsia, RvD/RvE: Resolvins D/E, MaR: Maresins, PD: Protectins, DAMPs: Damage-associated molecular patterns

Table 4: Mechanistic animal and *ex vivo* models elucidating sterile intrauterine inflammation pathways and their translational relevance

Model/System	Exposure/Pathway	Key mechanistic findings	Translational relevance
Ex vivo human chorioamniotic membranes; pregnant mouse[19]	Alarmin S100A12	Sterile cytokine activation in membranes; induced PTB and neonatal mortality in mice	Demonstrates alarmin-driven sterile inflammation; supports alarmins/IL-1 axis targeting
Murine model of parturition[20]	IL-1β/NLRP3 inflammasome activation	IL-1/NLRP3 pathway shown central in triggering PTB processes	Provides mechanistic basis for IL-1/NLRP3 modulation in sterile PTB endotypes
Preterm fetal sheep model[40]	IL-1 receptor blockade	IL-1R antagonism reduced fetal neuroinflammation	Supports biologic plausibility of IL-1 pathway inhibition

Summary of experimental animal and *ex vivo* models demonstrating mechanistic pathways of sterile intrauterine inflammation relevant to preterm birth.
IL: Interleukin, NLRP3: NLR family pyrin domain-containing 3, PTB: Preterm birth

matrices are summarized in Table 2. Table 3 depicts integrated mechanistic pathways linking exposures, immune activation, metabolic events, and epigenetic modifications. Mechanistic models, including *ex vivo* systems and animal studies, are consolidated in Table 4 to support biological plausibility and translational relevance. Outcomes of interest included spontaneous and early PTB, preterm premature rupture of membranes, hypertensive disorders of pregnancy, fetal growth restriction, and neonatal complications. Biomarker categories encompassed cytokines, alarmins, lipid mediators, extracellular-vesicle microRNAs, cfRNA, cfDNA methylation and fragmentomic signatures, SCFA profiles, and imaging-based assessments such as placental T2* MRI.

Risk of bias in individual studies

Risk of bias was evaluated using validated tools appropriate to each study design. Observational studies were appraised using the Newcastle–Ottawa Scale, randomized trials using the RoB-2 framework, and systematic reviews using AMSTAR-2 or ROBIS. Ratings were assigned independently by two reviewers, with disagreements resolved by consensus. Mechanistic animal and *ex vivo* studies were retained for contextual value but were not formally scored; instead, they were annotated descriptively to highlight biological relevance. Risk-of-bias classifications with concise justifications are presented in Table 1.

Synthesis methods

Given the heterogeneity of analytic platforms, study populations, exposure definitions, and biomarker measurements, quantitative meta-analysis was not feasible. Instead, a structured narrative synthesis was conducted. Studies were grouped into four mechanistic domains that emerged consistently across the literature: alarmin and inflammasome activation, metabolic dysregulation and impaired resolution pathways, pollutant-associated epigenetic disruption, and microbiome-derived inflammatory signaling. Within each domain, convergent findings across human cohorts, randomized trials, systematic reviews, and mechanistic models were examined to assess robustness and coherence. Biomarker patterns across gestation are synthesized in Table 2, while integrated mechanistic and translational pathways are illustrated in Table 3 and Figures 2 and 3. Heterogeneity was explored qualitatively by comparing findings across geographical regions, gestational ages, assay platforms, and exposure intensities.

Assessment of reporting bias and certainty of evidence

Reporting bias was assessed qualitatively because of the diversity of study designs and outcomes. For systematic reviews and meta-analyses included in this review, the authors' evaluations of publication bias and heterogeneity were taken into account and are summarized in Table 1. Among primary observational studies, selective reporting was considered likely when sample sizes were small or biomarker measurement panels were limited. Evidence pertaining to emerging topics such as microplastic contamination and cfDNA fragmentomics was judged to be uncertain because of limited replication and variable methodological rigor. Certainty of evidence was assessed using a triangulation approach that integrated consistency across independent studies, plausibility supported by experimental models, and reproducibility across assay platforms. Evidence was considered strongest when multiple well-designed cohorts and randomized trials demonstrated concordant associations, such as in the domains of omega-3 fatty acid supplementation and particulate-matter exposure. These graded assessments informed the overall interpretation and the translational model presented in Figure 3.

Results

Literature selection

The database search yielded 2643 records across PubMed, Embase, Scopus, and Web of Science. After the removal of 812 duplicates, 1831 unique citations were screened via title and abstract. A total of 291 full-text articles were assessed for eligibility, of which 243 were excluded because they focused on infectious etiologies rather than sterile inflammatory processes, did not report pregnancy outcomes, or lacked sufficient biomarker or epigenetic data. Forty-eight studies satisfied all inclusion criteria and were incorporated into the qualitative synthesis. The progression of records through the screening stages is illustrated in the PRISMA flow diagram presented in Figure 1. The final dataset comprised 27 observational cohort or case–control studies, six randomized or quasi-experimental trials, six systematic reviews or meta-analyses, and nine

mechanistic or translational models, forming the analytic corpus for this review.

Characteristics of included studies

Study characteristics are summarized in Table 1, detailing designs, populations, gestational windows, biospecimens, analytical platforms, exposures, outcomes, and risk-of-bias profiles. The included investigations contributed to several interconnected domains related to sterile intrauterine inflammation. Clinical cohorts documented intra-amniotic inflammatory states marked by elevated IL-6 and HMGB1 in the absence of detectable pathogens, findings that were associated with shorter latency to delivery and higher risk of spontaneous PTB.[17,18] Mechanistic studies identified alarmins such as S100A12 and activation of IL-1β and the NLR family pyrin domain-containing 3 (NLRP3) inflammasome as triggers capable of precipitating labor in sterile contexts.[19,20] Environmental epidemiologic studies linked exposure to particulate matter, metals, per-and PFAS, and microplastics to adverse gestational outcomes, often accompanied by altered placental epigenetic landscapes.[11,15,21-27] Investigations focused on maternal metabolic and nutritional conditions revealed that obesity, insulin resistance, and insufficient omega-3 fatty acids potentiate immune activation, impair inflammatory resolution, and induce epigenetic reprogramming within uteroplacental tissues.[7,10,35-37,47] Emerging biomarker studies introduced extracellular-vesicle microRNAs, circulating cfRNA, cfDNA methylation, fragmentomic signatures, and placental T2* MRI as translational indicators of sterile inflammatory processes.[12,13,16,28-30,32,33]

Risk of bias within studies

Overall study quality was moderate across the included evidence. Prospective cohort studies achieved low-to-moderate scores on the Newcastle–Ottawa Scale, supported by structured sampling frameworks and adjustment for maternal covariates, although exposure misclassification and interlaboratory variability remained notable limitations. Randomized trials evaluating omega-3 supplementation and lipid-mediator interventions provided stronger causal inference but varied in dosage, timing, and baseline nutritional profiles.[7,31,37] Systematic reviews and meta-analyses received moderate ratings on AMSTAR-2 or ROBIS due to heterogeneity and potential publication bias.[15,22,26,47] Mechanistic animal and *ex vivo* studies strengthened biological plausibility by demonstrating that sterile alarmin and inflammasome activation can initiate parturition, though extrapolation to human pregnancy requires caution.[19,20] Collectively, heterogeneity in populations, exposures, and biomarkers precluded quantitative meta-analysis, supporting the use of a qualitative synthesis.

Biomarker landscape and longitudinal signals

A comprehensive overview of biomarkers studied across the included literature is provided in Table 2. Amniotic fluid IL-6 and HMGB1 concentrations were consistently increased in women with culture-negative intra-amniotic inflammation and predicted shortened latency and heightened risk of early PTB.[17] Cervical IL-6 further distinguished sterile from infectious inflammatory states.[18] Mechanistic experiments showed that S100A12 and IL-1 β activation of the NLRP3 inflammasome accelerates labor in sterile conditions, reinforcing causal plausibility.[19,20] Lipid-mediator profiling demonstrated gestational variation in SPMs, with higher omega-3 intake associated with more favorable maternal–infant outcomes.[7,8,31,37] Nucleic-acid-based biomarkers yielded early diagnostic potential. cfRNA signatures measured during early pregnancy reflected coordinated maternal–placental transcriptomic activity and predicted PTB risk.[28] Extracellular-vesicle microRNAs, particularly those derived from placental tissues, were linked to susceptibility to preeclampsia (PE) and potentially broader sterile inflammatory phenotypes.[12,13] Maternal plasma cfDNA methylation and fragmentomic patterns correlated with placental health and PTB risk, though additional replication in larger and diverse populations is required.[16,29,32] Imaging studies using placental T2* MRI captured alterations in oxygenation and perfusion that were associated with fetal brain development and spontaneous PTB before 32 weeks.[17,30]

Exposures, mechanisms, and endotype mapping

Table 3 integrates exposure categories with immune, metabolic, and epigenetic mechanisms and corresponding pregnancy outcomes. Across multiple settings, exposure to fine particulate matter ($PM_{2.5}$) demonstrated a consistent association with increased risk of PTB, supported by meta-analytic evidence and biologically plausible epigenetic alterations.[15,21-23] PFAS exposure modified placental DNA-methylation profiles, while prenatal metal exposure influenced epigenetic gestational age in a sex-specific manner.[11,24,25] Micro- and nanoplastic particles recently detected in preterm placental tissues emerged as a novel source of sterile inflammatory activation.[21,27,47] Maternal metabolic stress amplified placental inflammasome activity and disrupted lipid-mediator resolution pathways.[10,17,47] Inadequate omega-3 fatty acid intake reduced synthesis of SPMs, prolonging inflammatory signaling and elevating preterm-birth risk, whereas supplementation mitigated early delivery risk in selected populations.[7,27,37,47] These exposures collectively converged on oxidative stress, mitochondrial dysfunction, and immune activation, producing downstream epigenetic changes such as altered DNA-methylation patterns, microRNA dysregulation, and cfRNA and cfDNA fragmentation. Figure 2 illustrates this mechanistic continuum from environmental or metabolic exposure to immune activation, epigenetic remodeling, and measurable clinical outcomes.

Synthesis across studies

Integration of the evidence revealed a coherent and biologically plausible model of sterile inflammation contributing to PTB. Environmental pollutants, maternal metabolic dysregulation, and nutritional insufficiencies acted as upstream triggers that initiated inflammatory and oxidative cascades. These cascades activated alarmins and inflammasome pathways, which intersected with disruptions in lipid-mediator balance and impaired inflammatory resolution.

Epigenetic programming – including DNA-methylation shifts, microRNA and cfRNA alterations, and cfDNA fragmentomic changes – translated these stressors into sustained biological effects within the placenta. The resulting multi-omic biomarker profiles, encompassing cytokines, lipid mediators, nucleic-acid signatures, and imaging markers, delineated distinct sterile-inflammation trajectories. Clinically, these trajectories were associated with spontaneous and early PTB, preterm premature rupture of membranes, hypertensive disorders, fetal growth restriction, and neonatal morbidity.

Translational roadmap

Figure 3 presents a multi-omic translational framework derived from the synthesized evidence. The figure outlines four primary endotypes – alarmin-driven, metabolic-resolution-deficient, pollutant-epigenetic, and microbiome-derived short-chain-fatty-acid dysregulation – each supported by mechanistic and clinical data. The roadmap situates biomarker discovery, mechanistic validation, and translational application within a unified continuum. Laboratory measures such as cfDNA methylation, fragmentomics, cfRNA, extracellular-vesicle microRNAs, and lipid mediators converge with imaging modalities such as placental T2* MRI to create a suite of potential diagnostic tools. These advances align with targeted interventions, including omega-3 supplementation, IL-1 pathway modulation, and microbiome restoration strategies. The downstream implications extend to the design of biomarker-stratified clinical trials, implementation of precision-obstetric screening programs, and development of policy initiatives targeting air quality and plastic exposure. Together, the findings delineate a multi-tiered framework positioning sterile inflammation as a central driver of PTB and highlight opportunities for early detection and prevention.

Discussion

The synthesis of 48 studies reveals a multidimensional framework, in which sterile intrauterine inflammation, metabolic imbalance, environmental exposures, and epigenetic remodeling converge to shape susceptibility to spontaneous PTB. As detailed in Table 1 and visualized in Figures 2 and 3, the boundaries traditionally drawn between infectious and noninfectious causes of preterm labor do not adequately capture the biological patterns reported across the recent literature. Instead, the evidence supports a model, in which sterile inflammatory signaling operates alongside metabolic and pollutant-induced stress, imprinting durable molecular changes on the placenta and fetal environment. This integrated perspective positions sterile inflammation not as an alternative explanation but as a central axis connecting immune activation, metabolic state, environmental exposures, and the epigenome.

Sterile intrauterine inflammation as a central driver

Foundational clinical work by Romero established that women presenting with preterm labor may demonstrate elevated amniotic IL-6 and HMGB1 even when standard cultures are negative, a pattern now widely recognized as sterile intra-amniotic inflammation.[17,18] Mechanistic studies subsequently clarified how this process unfolds. Gomez-Lopez demonstrated that inflammasome-mediated IL-1β activation amplifies cytokine cascades central to labor initiation, while complementary work showed that alarmins such as S100A12 can precipitate preterm delivery in experimental systems through sterile activation of chorioamniotic immune pathways.[19,20] Together, these clinical and mechanistic findings – summarized across Tables 1 and 2 – support sterile inflammation as a reproducible biological pathway that can initiate parturition independently of infection, with implications for therapeutic targeting of the IL-1 axis and related inflammatory checkpoints.

Environmental pollutants and epigenetic perturbations

A second major theme emerging from this synthesis concerns the role of environmental pollutants as upstream drivers of sterile inflammation. Meta-analytic evidence consistently associates maternal exposure to fine particulate matter ($PM_{2.5}$) with elevated risk of spontaneous PTB, and fetal growth restriction across diverse geographic contexts.[15,21-23] Chemical exposures exert parallel effects. Lee and Zhang independently reported that per- and PFAS reshape DNA-methylation networks governing immune and metabolic regulation in the placenta, effects that align with broader epigenetic vulnerability observed in environmentally stressed pregnancies.[11,24] Everson's work contributed evidence that prenatal metal exposure can shift epigenetic gestational age in a sex-specific manner, reinforcing the connection between environmental stressors and developmental timing.[25] Ragusa provided striking histological confirmation of micro- and nanoplastic particles embedded within human placentae,[27] a finding later supported by mechanistic experiments documenting oxidative stress and inflammasome priming in response to nanoparticle exposure.[26] These convergent streams of evidence, reflected in Table 3, highlight how pollutant exposures activate sterile inflammatory cascades and simultaneously imprint epigenetic signatures with potential implications across gestation and into postnatal life.

Maternal metabolic state and nutritional modulation

Metabolic stress emerged as a key amplifier of sterile immune activation. Observational cohorts demonstrated that maternal obesity and insulin resistance heighten background inflammatory tone through macrophage activation, oxidative stress, and impaired resolution of inflammatory signaling.[10,35,36] Nutritional status further modulated these pathways. Dunstan and Olsen observed that inadequate omega-3 fatty acid intake leads to diminished production of SPMs, prolonging the activity of proinflammatory eicosanoids.[7,8,31,37,47] Carlson and Makrides subsequently confirmed in randomized trials that omega-3 supplementation can meaningfully reduce early PTB in selected populations, though variability in dose, baseline status, and timing contributes to heterogeneous effects across trials.[6,7,37,47] The evidence synthesized in Table 3 suggests that metabolic conditions and nutritional insufficiency constitute modifiable

levers influencing sterile inflammatory biology, with potential relevance for individualized prevention strategies aiming to restore resolution pathways.

Epigenetic programming as a biological memory of exposure

Across all domains – environmental, metabolic, and immunologic – epigenetic modifications appear to function as a biological archive encoding maternal exposures. Multiple studies demonstrated alterations in placental DNA-methylation profiles in pregnancies affected by environmental stress, hypertensive disorders, or metabolic dysfunction, suggesting a convergence of diverse stressors on common epigenetic nodes.[2,9,11,24,25,43,45] Investigations of maternal plasma cfDNA methylation and fragmentomic signatures further revealed their potential to reflect placental stress and predict preterm-birth risk, findings described by groups examining cfDNA architecture across gestation.[16,29,32] Parallel studies by Mouillet, Whitehead, and others identified cfRNA and extracellular-vesicle microRNA patterns that distinguish early-stage placental dysfunction and anticipate PE or spontaneous PTB.[12,13,28] These molecular features, synthesized in Table 2 and integrated in Figure 3, underscore the idea that sterile inflammation and environmental or metabolic stressors leave durable biological marks within the placenta that may influence both immediate obstetric outcomes and longer-term developmental trajectories.

Integration of biomarkers into multi-omic endotypes (with citations)

Although individual biomarkers such as IL-6, HMGB1, cfRNA, EV-miRNAs, and SPMs each provide mechanistic insight, their interpretive strength increases when integrated across molecular layers. Elevated IL-6 and HMGB1 in culture-negative intra-amniotic inflammation have been documented in key clinical studies,[17,18] while inflammasome-related alarmin activation through S100A12 and IL-1 β has been demonstrated in mechanistic models.[19,20] Lipid-mediator and omega-3–derived SPM trajectories associated with preterm-birth risk are described in nutritional and metabolic studies.[7,8,31,37,47] Nucleic-acid biomarkers – including cfRNA panels, EV-miRNAs, and cfDNA methylation or fragmentomic signatures – have shown predictive value for placental dysfunction and spontaneous PTB.[12,13,16,28,29,32] Placental T2* MRI studies further contribute imaging-based readouts of oxygenation and perfusion relevant to early risk detection.[30,33]

Studies summarized in Tables 2 and 4 demonstrate that cytokine responses, lipid-mediator dynamics, nucleic-acid signatures, and imaging metrics converge on shared pathways involving oxidative stress, inflammasome activation, mitochondrial dysfunction, and impaired resolution biology.[10,22,23,26,35,36] When viewed collectively, these signals delineate several mechanistic endotypes – avallarmin-driven, metabolic-resolution-deficient, pollutant-epigenetic, and microbiome-derived short-chain-fatty-acid pathways.[27,34,38,39] Figure 3 illustrates how these endotypes can be conceptualized within a translational roadmap that links molecular discovery to diagnostic development and targeted intervention. This integrative approach positions multi-omic biomarkers as foundational tools for precision obstetrics, enabling early risk stratification, pathway-specific therapy, and alignment of clinical strategies with environmental and nutritional policy efforts.

Convergence of evidence across study types

The coherence of this sterile-inflammation model is strengthened by the convergence of evidence across methodological traditions. Observational epidemiology provided population-level associations across continents and exposure gradients.[15,21,23] Randomized and quasi-experimental trials, supported by meta-analytic syntheses, clarified causal pathways linking nutritional modulation to reductions in early PTB.[7,31,37,47] Mechanistic studies led by Romero, Gomez-Lopez, and others illuminated fundamental inflammatory circuits capable of inducing labor in the absence of infection.[19,20] Omics-driven investigations deepened this mechanistic foundation by demonstrating how pollutants, metabolic stress, and nutrient insufficiency reshape epigenetic architecture and circulating biomarker profiles.[11,16,29] Complementary narrative syntheses further expanded mechanistic plausibility for emerging domains, including microplastics, microbiome metabolites, and complex environmental mixtures.[3,4,26,34,38,39] When these findings are integrated using Tables 1-4 and Figures 2 and 3, a unified biological model emerges in which sterile intrauterine inflammation operates as the integrative nexus connecting immune activation, epigenetic remodeling, metabolic signaling, and environmental exposure within the continuum of PTB.

Strengths, limitations, and future directions

This review brings together a wide span of evidence to clarify how sterile intrauterine inflammation intersects with metabolic imbalance, environmental exposures, and epigenetic remodeling in the pathway to PTB. Its primary strength lies in the integration of mechanistic, clinical, and multi-omic perspectives, allowing concepts that are often studied in isolation to be considered within a single biological framework. By drawing connections between inflammatory signaling, maternal nutritional status, pollutant-driven stress, and molecular changes in placental tissues, the synthesis offers a more coherent understanding of how diverse maternal influences can converge on a common pathway. This breadth also enhances the clinical relevance of the review, as it outlines potential diagnostic and preventive opportunities that span early risk detection, metabolic support, and environmental health.

Several limitations must be acknowledged. The available literature varies considerably in study design, participant characteristics, biospecimen types, and analytic platforms, which complicates direct comparison across investigations. Many studies rely on small or single-center samples, and although they provide valuable mechanistic insight, they

may not fully reflect the complexity of human pregnancy or environmental exposures across populations. Laboratory assays differ in sensitivity and specificity, and variations in sampling timing introduce added uncertainty. The decision not to conduct a meta-analysis reflects the marked heterogeneity of exposures, biomarkers, and outcomes, but it also limits the generation of summary effect estimates. In addition, the protocol was not registered in advance, which constrains the extent to which selection or reporting bias can be excluded despite adherence to established reporting standards.

Future work will need to build on this foundation with studies capable of resolving the remaining uncertainties. Large, multicenter cohorts using harmonized protocols would help evaluate reproducibility and improve the generalizability of biomarker findings. Interventional trials guided by molecular endotypes, rather than treating all individuals with preterm-birth risk as a uniform group, may create opportunities for preventive strategies tailored to specific biological pathways. Advances in genomics, epigenomics, metabolomics, and imaging – combined with increasingly sophisticated computational methods – have the potential to enable early detection of risk with far greater precision than is currently possible. Translating these discoveries into practice will also require attention to environmental and social determinants of maternal health. Strengthened pollution control, targeted nutritional support, and equitable models of maternity care are essential to ensuring that biological insights can lead to meaningful reductions in preterm birth rates on a population level. Sustained collaboration between basic scientists, clinicians, and policymakers will be critical to turn the growing understanding of sterile inflammation into tangible improvements in maternal and neonatal outcomes.

Conclusion

Sterile intrauterine inflammation has become increasingly recognized as a central contributor to the pathway leading to PTB, drawing together immune activation, metabolic imbalance, environmental stressors, and epigenetic remodeling within a single biological continuum. Viewed through an integrative, multi-omic lens, this process reflects more than inflammation occurring without infection. It represents a dynamic network in which maternal physiology and external exposures interact to alter placental function and accelerate the timing of parturition. The collective evidence reviewed here demonstrates that PTB arises not from a uniform mechanism but from overlapping endotypes shaped by variations in maternal health, environmental context, and molecular responses. This synthesis highlights how cytokine activity, lipid-mediator imbalance, nucleic-acid signatures, and advanced imaging can serve as early signals of dysregulated immune–metabolic pathways. These biomarkers, used individually or in combination, offer realistic potential for early detection and more refined risk assessment. Translational opportunities also emerge from this framework. Nutritional interventions that restore resolution biology, immune-modulatory therapies that temper inflammasome activation, strategies that support a healthy maternal microbiome, and public health efforts to reduce pollutant exposure all represent feasible routes toward prevention. Integrating these approaches into maternal-care pathways, particularly through trials that align interventions with underlying biological endotypes, may help shift practice toward more personalized and effective models of obstetric care. Recognizing sterile intrauterine inflammation as a unifying mechanism invites a meaningful reframing of preterm-birth research and clinical management. As multi-omic tools advance and mechanistic insights deepen, the prospect of a precision-based approach to predicting and preventing early birth becomes increasingly tangible. The challenge ahead lies in translating this knowledge into equitable and scalable strategies capable of improving maternal and infant health across diverse populations.

Author contributions

WA, AS, and WP conceptualized and supervised the review. MAB contributed to the literature collection and data extraction. JD and MBAP participated in data analysis and critical content review. INHS, ED, MIIA, and DA was involved in reviewing data evidence. WEKA and MS provided methodological and clinical guidance. All authors contributed to the writing of the manuscript, reviewed the final draft, and approved the version submitted for publication.

Declaration of authorship

The manuscript was entirely conceptualized, written, andedited by the authors without the use of generative AI tools. Standard editorial grammar checks (e.g. Microsoft Wordspell-check) were used solely for language consistency.

Data sharing agreement

No new datasets were generated or analyzed in this study. All data supporting the findings of this systematic review are derived from previously published research cited in the reference list. Therefore, data sharing is not applicable.

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Conflicts of interest

There are no conflicts of interest.

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Review Article

Fatty Liver Promotes Stress-resistant Mitochondria-mediated Hepatic Cellular Proliferation: Implication for the Development of Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the sixth most frequent primary liver cancer with poor prognosis and the primary cause of cancer-related mortality globally. Viral hepatitis, obesity, diabetes mellitus, and excessive alcohol intake are strong predictive factors that induce fatty liver (steatosis) disease and its progression. Chronic inflammatory and oxidative stresses, subsequent to steatosis and associated lipotoxicity, are common etiology toward fatty liver disease progression to fibrosis, cirrhosis, and later to HCC. Cirrhosis is the underlying liver disease in 80% HCC. Cirrhotic livers with HCC have multilesions, including benign, preneoplastic, and neoplastic. Thus, there is reason to think that transformed stress-resistant liver cells in a cirrhotic liver may be progressing into HCC. The deterioration of fats due to oxidative insults and associated carbonyl stress in the cellular milieu is pro-inflammatory and has suspected carcinogenic potentials. Fatty livers are prone to this stress, which further induces antioxidants, phase II detoxification enzymes to cope up with the situation. Within the cells, mitochondrial integrity determines cellular fate. Therefore, intensifying the mitochondrial antioxidant machinery can push the cell to survival under oxidative or inflammatory stress. A series of cell populations transformed themselves with increased metabolic rate and antioxidant status similar to that of cancer stem cells are thus generated. Preclinical exploration of the mechanistic events in these adaptive responses may contribute to a better understanding of the pathophysiology of HCC and will have a promising translational value.

Keywords: Carbonyl stress, fatty liver, hepatocellular carcinoma, inflammation, liver cirrhosis, oxidative stress

Introduction

Hepatocellular carcinoma (HCC) stands as the sixth most common cancer globally and is the fourth leading cause of cancer-related mortality.[1] It is among the most difficult tumors to manage.[2] In addition to viral infections such as hepatitis B and C, risk factors include alcoholic and nonalcoholic fatty liver disease (recently renamed metabolic dysfunction-associated steatotic liver disease (MASLD). Diabetes, obesity, and certain genetic conditions such as hemochromatosis contribute to NAFLD/MASLD.[3,4] Cirrhotic livers are characterized by advanced fibrosis, scar tissues, and formation of multifocal hepatocellular lesions. Clear histological distinctions of hepatocellular lesions into regenerative (benign) dysplastic (premalignant) and neoplastic stages have been described. However, nearly 15%–30% HCC have noncirrhotic origin with the occurrence of a single large neoplastic lesion. In all these cases, chronic oxidative and inflammatory stress, subsequent to steatosis causing lipotoxicity, is common and is associated with the progressive changes of fatty liver condition to fibrosis, cirrhosis, and later to HCC.

The exact pathophysiology that links NAFLD (MASLD) and HCC is not completely known. Obesity, type-2-diabetes mellitus and alcoholic liver diseases (ALD) are independent determinants of MASLD and also HCC.[5] A synergism of

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alcoholism and obesity/diabetes thus may have exacerbated effect. Since fatty liver is the common early manifestation in all these conditions, further complications such as steatohepatitis, fibrosis, and cirrhosis are supposed to be based on fats and their oxidation products. It is generally agreed that oxidative insults to unsaturated lipids produce peroxides, which subsequently converted to epoxides and later to stable aldehydes. These carbonyl molecules (peroxides aldehydes, etc.) can produce carbonyl stress in the cellular milieu by binding with proteins and DNA. 4-Hydroxynonenal, crotonaldehyde, and malondialdehyde are somewhat stable lipid carbonyl molecules with carcinogenic potential.[6] General mechanism involved in the progression of fatty liver disease to HCC have been depicted in Figure 1.[7]

Inflammatory and Oxidative Stress Environment in Fatty Liver Diseases

As insulin resistance increases in obesity and type 2 diabetes, adipocytes release more free fatty acids (FAs) and pro-inflammatory cytokines such as leptin, interleukin-6 (IL-6), and tumor necrosis factor alpha, while adipokine and adiponectin release decreases.[8] These factors combine to produce a microenvironment that raises hepatic inflammation. The nuclear factor kappa B (NF-κB) family of transcription factors, which are known to be crucial in the development and progression of numerous cancer types, is also activated under these conditions.[9] In the case of chronic alcoholism, the excess alcohol will be converted into acetaldehyde and acetate, which disrupts the FA metabolism, leading to increased accumulation of FAs in the liver and thus increasing the production of reactive oxygen species (ROS). Alcohol metabolites, ROS, free FAs, and other epigenetic changes can aggravate inflammatory conditions by activating several inflammatory pathways.[10] Alcohol also increases ROS production via mitochondrial electron transport chain and alcohol metabolism mediated by CYP2E1.[10]

FAs and their oxidized products (eicosanoids, oxidized phospholipids) are endogenous agonists of peroxisome proliferator-activated receptors (PPARs), which are transcription factors that play crucial roles in regulating various cellular functions.[11] PPARα, the primary PPAR isotype found in liver tissue, stimulates the expression of many genes related to FA absorption, acyl-CoA activation, and transport to the mitochondria or peroxisomes, followed by lipoprotein trafficking, β-or ω-oxidation, and ketogenesis.[11]

A mild, age- and sex-dependent, lipid accumulation in the liver has been shown in PPARα deleted mice.[12] In PPARα$^{-/-}$ mice, researchers have noted severe hypoglycemia, low ketone levels, elevated plasma free FAs, and disruptions in β-oxidation and ketogenesis.[13] When compared to wild-type mice, those lacking PPARα and fed a methionine choline diet developed Nonalcoholic steatohepatitis (now termed as metabolic dysfunction-associated steatohepatitis [MASH]).[14] PPAR-α hep$^{-/-}$ mice, similar to whole-body PPARα$^{-/-}$ mice, exhibited steatosis and hypercholesterolemia as the animals aged, without become obese or hyperglycemic, underscoring PPARα's role in liver steatosis progression.[11] This observation has been confirmed in clinical studies. Analyzing mRNA from paired liver biopsies of 85 patients revealed a significant link between reduced PPARα expression and the histological severity of MASH, while no such correlation was found for PPARβ/δ or PPARγ expression.[15] Recent findings indicate that PPARα$^{-/-}$ mice are more prone to diethylnitrosamine (DEN)-induced HCC, with PPARα's anticancer effects being mediated through NF-κB inhibition.[16]

On the other hand, PPARγ expression status is controversial. A couple of preclinical studies have reported that liver-specific disruption of PPARγ in leptin-deficient mice could improve fatty liver conditions, suggesting the role of PPARγ expression in the development of hepatic lipid accumulation. In the HCC condition, there is no expressional variation observed with respect to PPARγ among cirrhotic and noncirrhotic liver, while constant overexpression of PPARγ protein has been found in HCC tissues compared to surrounding normal healthy hepatocytes.[17] Also, a recent study reported a statistically significant decrease in PPARγ expression in HCC tissues, compared to the adjacent hepatocytes.[18] PPARγ agonists have been shown in numerous model systems to

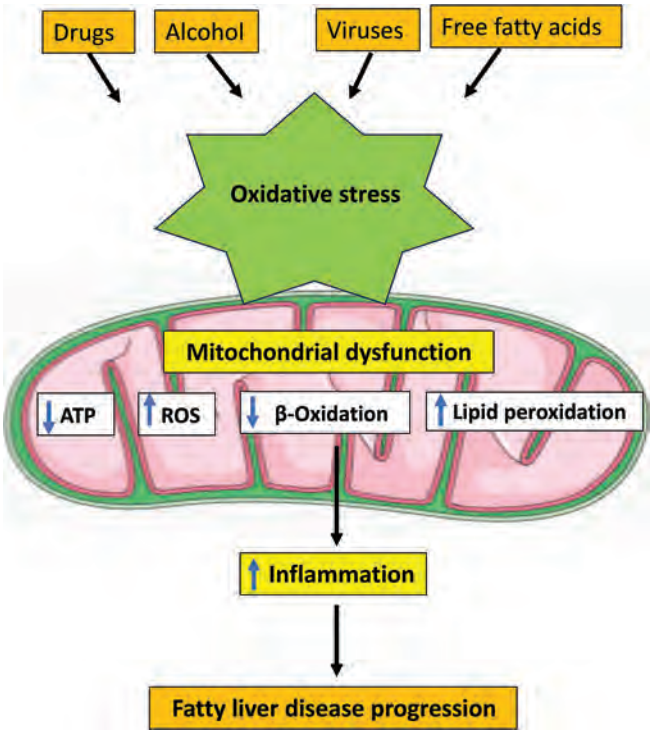

Figure 1: Induction of fatty liver disease progression by mitochondrial dysfunction. Through an array of stimuli, including drugs, alcohol, viruses, free fatty acids (FFAs), and oxidative stress, mitochondrial functionalities can be impaired. Consequently, ATP is depleted, β-FFA oxidation is decreased, and reactive oxygen species accumulation is increased, further inducing lipid peroxidation. This then stimulates inflammatory signalling pathways, thus leading to fatty liver disease progression to cancer development

enhance mitochondrial oxidative phosphorylation, promote mitochondrial biogenesis, and strengthen antioxidant defense, collectively protecting cells from various stress conditions.[19] In addition to preserving mitochondrial function, PPARγ activation also exerts strong antioxidant and anti-apoptotic effects. Studies have shown that mice lacking PPARβ/δ are susceptible to liver damage caused by inflammation. Consistent with this, administering PPARβ/δ has been associated with improvements in hepatic steatosis, as well as reductions in insulin resistance and liver inflammation.[20,21] Furthermore, PPARβ/δ levels were notably lower in a small group of HCC cases compared to the adjacent noncancerous tissue, and the PPARβ/δ agonist GW501516 was observed to inhibit cyclinD1 expression and cell proliferation in hepa1-6 cells.[22] Nonetheless, the role of PPARβ/δ agonists in tumor suppression remains a topic of debate.[23]

Reactive Oxygen Species Adaptation in Tumor

Cell's early response to oxidative stress is to elevate their antioxidant defense and decrease ROS levels, resulting in autophagy to mitigate oxidative injury to biomolecules and organelles.[24,25] The same oncogene signals that enhance ROS signaling also encourage antioxidant adaptive strategies to handle ongoing stress and reduce oxidative damage. This is accomplished by increasing the transcription of NF erythroid 2-related factor 2 (Nrf2) and elevating the baseline Nrf2 antioxidant program.[26] The activation of the Nrf2 pathway enables cells to adapt and survive by controlling the expression of antioxidants, anti-inflammatory agents, and phase II detoxification enzymes. Nrf2 is observed to be overexpressed in various human cancers,[27] clearly indicating that cancer cells can exploit the cytoprotective features of the Nrf2 pathway to enhance their survival.

How cancer cells progressively acquire the expression of Nrf2 and the associated increase in the cellular antioxidant defense are interesting question to be answered. Since fatty liver disease promotes an environment of oxidative and carbonyl stress, evaluating the expression of these transcription factors under such conditions may answer these questions. Nrf2 activity is regulated within the cells by cytosolic inhibitor Kelch-like ECH-associated protein 1, which is involved in its ubiquitination and proteasomal degradation.[28] Inhibition of Nrf2 activity has been found to reduce tumor development and could improve the chemotherapy drugs effectiveness. In a lung cancer-induced mouse model of K-Ras-G12D, Nrf2 pathway has shown to enhance the antitumor activity of cisplatin.[29] Blocking Nrf2 gene expression temporarily with Nrf2 inhibitors is crucial for elevating patient's response toward radiotherapy and chemotherapy. Activation of the Nrf2 pathway can also provide therapeutic advantages in disorders such as multiple sclerosis and various neurodegenerative diseases by lowering oxidative and electrophilic stress.[30] In cancer cells,Nrf2 acts as a double-edged sword : while transient Nrf2 activation contributes to chemoprevention by reducing DNA-damaging reactive oxygen species, sustained or constitutive Nrf2 activation in tumor cells can promote rapid cell proliferation, facilitate evasion of apoptosis or senescence, leading to chemotherapy and radiotherapy resistance. Consequently, both induction and inhibition of Nrf2 activity can be beneficial, although the advantages differ among various patient groups. The mechanism responsible for the stable expression of Nrf2 even after the transformation of resident cells into its carcinogenic form, thereby increasing the cellular antioxidant and detoxification systems, requires further investigation.

Mitochondrial Dysfunction in Metabolic Dysfunction-associated Steatotic Liver Disease

Mitochondrion is the organelle that determines cell fate. The integrity of mitochondrion as a whole depends on the outer and inner mitochondrial membrane stability. Phosphatidylcholine is the predominant lipid of the outer mitochondrial membrane, whereas cardiolipin and phosphatidylethanolamine are the major lipids of the inner mitochondrial membrane, contributing to the stability and organization of the cristae. The electron chain proteins are anchored based on these lipids and function accordingly. Cardiolipin contains four molecules of linoleic acid, and other phospholipids have oleic acids, which are polyunsaturated fats. Therefore, pro-oxidant insults from within or outside this organelle affect its integrity, energy yield, and ultimately induce apoptotic signaling mechanisms entirely regulated by B-Cell lymphoma proteins and caspases. Manganese superoxide dismutase (MnSOD), glutathione hydroperoxidase, thioredoxin (TRX), and aldehyde dehydrogenases are the antioxidant enzymes that protect mitochondria, thus showing anti-apoptotic potential.[31] Peripheral tumor cells of glioma exhibit higher respiratory rate and less antioxidant system, while the neoplastic cells within the tumor mass have increased antioxidant system and develop enhanced chemo resistance.[32] It is agreed that limited access to both molecular oxygen and fuel sources disturb metabolic state of mitochondria, generating ROS. Under such conditions (as in oncogenic and carcinogenic signaling), the cell specifically mitochondria improve their antioxidant system as a protective mechanism that allows survival and maintenance of cell function [Figure 1].

In people with fatty liver disease, liver tissue shows signs of stress, structural and functional defects with mitochondria.[33] These defects include changes in shape, less activity in the respiratory chain, lower Adenosine triphosphate (ATP) levels, more permeable membranes, mtDNA deletions due to stress, and issues with mitochondrial β-oxidation. There is also damage to the mitochondria's structure.[34] Recent studies suggest that mitochondrial dysfunction is a key factor in the start and worsening of MASLD.[35] Cirrhosis of the liver tissue can be explained under this condition; however, it is not clear how exactly this dysfunction contributes to HCC. However, MASLD is linked to less FA oxidation, more free FAs in the liver, and increased liver FA production.[36]

In the early stages of MASLD, the liver uses "mitochondrial flexibility" to adjust mitochondrial activity and size in response to insulin resistance and free FA buildup.[37,38]

Flexibility of which has been lost as the MASH develops. Both MASLD and MASH patients have been found to have enhanced liver mitochondria.[39] But whether this increase is due to more mitochondria being synthesized or improper elimination of damaged mitochondria remains unknown.[40,41] Both mice and humans with MASH showed signs of lower ketogenesis, reduced respiration, mitochondrial uncoupling, and an overactive Krebs cycle.[42] Heightened mitochondrial activity can lead to heightened ROS, which further damages mitochondrial DNA, causing stress in the endoplasmic reticulum, inflammation, and cell death. These conditions may lead to HCC development.[43]

Adaptation of Mitochondria

Mitochondrial signaling pathway dysfunction can significantly affect the development and progression of MASLD/MASH. Patients with early stage of MASLD have been observed with elevated levels of antioxidant enzymes: reduced glutathione (GSH), SOD, and glutathione peroxidase in their blood,[44] which may be an adaptive response to oxidative stress. Activation of the SIRT1-peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α) pathway, can increase the mitochondrial energy metabolism and biogenesis, triggered by activated protein kinase activation, AMP/ATP ratio, or fluctuations in NAD^+ levels.[45] Studies conducted in mice have shown that overexpression of SIRT1 can cause reduction in liver fat and inflammation caused by a high-fat diet by inhibiting the CD36 expression and NF-κB signaling pathways.[46] Mitochondrial dysfunctions are reported in both MASLD and ALD. Early studies have reported about the mitochondrial structural changes leading to megamitochondria in the liver of ALD patients, also alcohol-related liver disease has been observed to have mitochondrial DNA damage.[47,48]

But how these changes affect mitochondrial function in human ALD is not clear. Noninvasive breath tests can show if mitochondrial function is affected.[49,50] More research is needed to understand how mitochondria work in ALD patients, as this might change with the disease stage and drinking habits. Alcohol affects mitochondrial respiration differently in different species. In rats, alcohol reduced mitochondrial respiration in liver mitochondria. Chronic alcohol use also affects the production of proteins needed for respiration, like ATP synthase and cytochrome complexes. However, Recent research in mice, however, revealed elevated mitochondrial respiration and greater concentrations of complex I components in the liver mitochondria.[51,52] Intra-gastric alcohol increased state III respiration and was linked to higher levels of respiratory chain complexes. This method of alcohol delivery also increases liver damage.[53] PGC-1α protein expression was found to be elevated after intragastric alcohol ingestion, but not Mitochondrial transcription factor A, due to the rise in mitochondrial complex components. Furthermore, while alcohol consumption has been demonstrated to largely injure in a zonal-dependent manner, new research has examined whether the alterations in mitochondrial function that occur after prolonged alcohol consumption exhibit an acinar distribution.

Peroxisome Proliferator-activated Receptor Gamma Coactivator 1 Alpha Mediated Mitochondrial Dysfunction in Fatty Liver Disease Progression

Furthermore, it serves as a co-activator for peroxisome proliferator-activated receptor α and γ (PPARα and PPARγ). PGC-1α can induce mitochondrial β-oxidation genes to promote FA oxidation.[54] PGC1α also plays a crucial role in modulating mitochondrial antioxidant defenses within cells. It can elevate manganese superoxide dismutase (MnSOD/SOD2), catalase, peroxiredoxin 5, UCP-TRX reductase (TRXR) 2, and TRX 2, accordingly protecting the cells from dysfunction of mitochondria.[55,56] PGC1-αassociated increase in antioxidant defense is important for preventing mitochondrial dysfunction-associated cell death.[55] In cancer cells of various types, the elevation of PGC-1α helps in cell survival by protecting them from heightened mitochondrial ROS production.[56,57] PGC-1α also plays an important role in regulating mitochondrial antioxidant defense, serving as an adaptive mechanism to meet metabolic demands while preventing the detrimental accumulation of ROS.[58] PGC1α has long been known to play a beneficial role in controlling energy metabolism and homeostasis; its malfunction has been connected to a number of metabolic disorders, including steatohepatitis, Duchenne muscular dystrophy, cardiomyopathy, and other tissue degeneration.[59-62] According to a prior study that used adenovirus transduction, overexpressing PGC1-α can improve lipid metabolism by reducing triacylglycerol buildup and secretion, increasing hepatic oxidation of FAs, and more.[63] Nevertheless, it is still unclear how PGC1-α helps with pathological disorders, including nutrient-related hepatic damage such non-alcoholic fatty liver disease. According to another study, decreased hepatic PGC1-α expression increases oxidative damage and inflammation, which may lead to the development of MASLD.[64] In one study, overexpressed PGC1α mice given a high-fat diet showed improved mitochondrial function and FA oxidation.

The study also discovered that PGC1α is essential for liver steatosis and insulin resistance through an inflammatory response mediated by IL-10.[65] In addition, the same scientists noted a disruption in the mitochondrial process of biogenesis in steatotic livers in a different investigation. However, neither the mitochondrial biogenesis pathway nor PGC1a activity increased mitochondrial mass, despite the increase in PGC1a mRNA expression, which was probably an attempt by hepatocytes to make up for the decreased number of

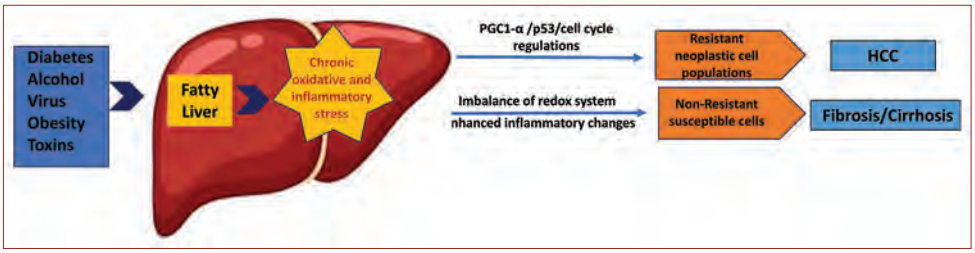

Figure 2: Schematic representation showing hepatocyte adaptation toward hepatocellular carcinoma/cirrhosis various risk factors induced fatty liver associated chronic oxidative and inflammatory stress promote stress susceptile as well as stress resistant cells population in hepatic tissue. Stress resistance is an adaptive mechanism by mitochondria through peroxisome proliferator-activated receptor gamma coactivator 1 alpha/p53 interaction that in turn regulates cell cycle progression, leading to neoplasm and later to hepatocellular carcinoma

mitochondria. Therefore, chronic disruption of the biogenesis of the mitochondrial process may raise oxidative stress and change cellular function in general, which may help MASLD proceed to MASH.[66]

Given the pivotal role of PGC-1α in regulating mitochondrial metabolism, antioxidant defense mechanisms, and adaptation, numerous investigations have explored its involvement in tumor progression, including HCC. Its impact on tumorigenesis remains debated, with some findings suggesting it either supports or hinders cancer development. In human HCC, a decrease in hepatic PGC-1α expression is linked to heightened glycolysis and lower blood glucose levels.[67] Additionally, reduced PGC-1α is linked with the de-differentiation of human hepatoma cell lines due to the disruption of transcription factor, hepatocyte NF 4 alpha, essential for the development of the liver.[68] Supporting the notion of its tumor-suppressive role, Lee *et al*. demonstrated that PGC-1α overexpression enhances the epithelial marker E-cadherin and diminishes HepG2 cell motility, potentially through PPARγ.[69] Furthermore, PGC-1α can interact with p53 and boost the transcription of genes such as TP53-inducible glycolysis and apoptosis regulator, growth arrest and DNA damage-inducible 45, SCO cytochrome C oxidase assembly Protein 2, cyclin-dependent kinase inhibitor 1 (p21), and sestrin2 in human hepatoma cells.[70] In contrast, Bhalla *et al*. discovered that coactivator overexpression increased tumor growth in a xenograft model by boosting lipogenesis; the lack of PGC-1α in a knockout mice reduced DEN-induced liver cancer.[71] Surprisingly, cells that overexpressed the related coactivator PGC-1 β in their livers also showed greater tumor anabolism and ROS scavenging, which resulted in a higher incidence of genetically-induced ($Abcb4^{-/-}$) and chemically-induced liver cancer.[72] Interestingly, the authors found that the liver tumors of these mice had lower levels of PGC-1α and vice versa showed a compensatory increase in PGC-1 β with liver-specific PGC-1α ablation.

While PGC-1α and PGC-1 β are often considered having functional similarity, research indicates that they may regulate different gene activities associated with liver nutrient metabolism. Specifically, PGC-1 β is thought to primarily boost lipogenesis.[72,73] This suggests that PGC-1 β could promote tumor growth, whereas PGC-1α might offer resistance.

In view of all these, it is likely that in progressive stages of fatty liver condition. Hepatic cells can be either susceptible to or resistant to chronic oxidative and inflammatory stress. Susceptible population may die and contribute to Cirrhotic pathology, while resistance cells adapt themselves to survive. The concept of cancer stem cell assumes lower ROS levels, possibly due to higher cellular antioxidant status contribute to self-renewal and differentiation. Enhanced mitochondrial dynamics, including biogenesis, fission, and fusion, allow stem cells to escape apoptosis and maintain stemness. Morphology function of mitochondria also changes in such cancer stem cells. Considering this concept of cancer stem cells, it is likely that he surviving cell in progressively stressed hepatic tissue is the result of mitochondrial adaptation, where enhanced antioxidant system, mitogenesis, and enhanced mitochondrial function could be possible through PGC1-α/p53 mediated signaling [Figure 2].

Conclusion

Preclinical studies have demonstrated that chronic oxidative and inflammatory stress subsequent to hepatic lipid accumulation and exacerbated lipid peroxidation contribute significantly to the progression of fatty liver disease toward cirrhosis and HCC. The adaptation of hepatocyte populations to these stresses, with enhanced metabolic functioning through mitochondrial mechanisms and acquiring resistance to oxidative damage, can promote neoplastic transformation. Specifically, under diabetic or alcoholic fatty liver conditions, elevated FAs and their oxidized derivatives intensify hepatic mitochondrial stress responses, potentially upregulating antioxidant gene expression as an adaptive mechanism. During these adaptive responses,cell cycle checkpoints and tumor suppressors may be regulated towards the survival and proliferation of pre-neoplastic cells.. While transcriptional coactivators of the PGC-1 family are known to modulate mitochondrial biogenesis and oxidative metabolism, and their interaction with tumor suppressor protein p53 can promote neoplastic signaling. Further investigation into the PGC-1α/p53 axis may uncover critical pathways linking metabolic adaptation to hepatocarcinogenesis.

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Conflicts of interest

There are no conflicts of interest.

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Original Article

Relationship between Postload Glucose in Oral Glucose Tolerance Test and Serum Fructosamine Levels in Women with Gestational Diabetes Mellitus in the Second Trimester

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Abstract

Background: Gestational diabetes mellitus (GDM) is a common metabolic condition during pregnancy. While fructosamine (FA) acts as a marker for short-term glycemic management by reflecting average glucose levels over the previous 2–3 weeks, the oral glucose tolerance test (OGTT) is the primary diagnostic tool for evaluating anomalies in glucose metabolism. This study aimed to evaluate the correlation between serum FA and OGTT results to determine their relative effectiveness in monitoring GDM during the second trimester of pregnancy. **Materials and Methods:** A cross-sectional study was conducted at a tertiary care hospital in Chennai during January 2022–December 2022. Seventy-five pregnant women (aged 18–45 years and singleton pregnancy) with confirmed GDM during the 19–24 gestational weeks were included. Plasma glucose and serum FA levels were estimated using standard methods. FA levels were corrected with respect to albumin levels. P <0.05 was regarded as statistically significant. **Results:** Postload plasma glucose was positively correlated with fasting plasma glucose ($P < 0.001$). The study found no significant correlation between OGTT and serum FA levels in pregnant women. Furthermore, during the second trimester of pregnancy, there was no discernible difference ($P > 0.05$) in the FA-albumin-corrected values. **Conclusion:** Serum FA levels in the second trimester did not show any significant correlation with OGTT; hence, they cannot replace the OGTT in diagnosing GDM. It is necessary to conduct additional research based on different trimesters in various clinical settings, as well as on geography and ethnicity.

Keywords: Albumin, fructosamine, gestational diabetes mellitus, oral glucose tolerance test, pregnancy

Introduction

Gestational diabetes mellitus (GDM) is a significant pregnancy complication characterized by the onset of hyperglycemia, typically occurring in the second or third trimester. It affects 2%–5% of pregnancies worldwide, and its prevalence is rising as type 2 diabetes and obesity rates rise.[1,2] Regarding estimates of GD prevalence in India, the literature currently in publication varies; the majority of research claim a prevalence that is slightly greater than what we present here, ranging from 4% to 14%.[3-5] GDM results from impaired glucose tolerance, primarily caused by pancreatic β-cell dysfunction and chronic insulin resistance.[6] Serious hazards associated with this illness include preeclampsia, macrosomia, and a higher chance of type 2 diabetes in later life for both the mother and the child.[7]

The oral glucose tolerance test (OGTT) is widely recognized as the gold standard for diagnosing GDM, as recommended by the World Health Organization (WHO).[8] However, despite its accuracy, OGTT is often criticized for being time-consuming, costly, and uncomfortable for patients.[9] Alternative biomarkers, such as fructosamine (FA), which represents the average blood glucose levels over the preceding 2–3 weeks, have therefore been studied for their possible application in the diagnosis of GDM. In recent years, FA has become more significant as a novel biomarker for hyperglycemia. Through the Maillard reaction, serum proteins are nonenzymatically glycated at the

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amino terminals of arginine and lysine residues to form serum FA.[10] A Schiff base, an aldimine intermediate, is first created by this process. It then goes through an Amadori rearrangement to form a ketoamine linkage or the FA molecule.[10]

Due to the high concentration of albumin in plasma, FA predominantly consists of glycated albumin. FA has advantages over OGTT because it is less expensive and does not require fasting. The potential of FA as a GDM screening test in several studies is debatable. Furthermore, the relationship between FA and OGTT results has been the subject of conflicting research; some studies have found no significant association, while others have found a strong positive correlation.[11,12] This highlights the significance of additional population-based research. This study aimed to evaluate the correlation between FA and OGTT results to determine their relative effectiveness in monitoring GDM during the second trimester of pregnancy.

Materials and Methods

Study design and population

A cross-sectional study was designed for patients with GDM during the second trimester of pregnancy at the Department of OBG and Clinical Biochemistry Lab of a tertiary care center in Chennai, South India. The study was done during the period of January 2022–December 2022. Pregnant women (aged between 18 and 45 years and singleton pregnancy) with confirmed GDM detected during the 19–24 gestational weeks were included. The International Association of Diabetes and Pregnancy Study Groups' criteria were used to diagnose GDM.[13] The study excluded pregnant women who were unwilling to participate in the study. Informed consent was obtained from the patients. The study was conducted according to the standards described in the Declaration of Helsinki, 1975, which was further amended in 2013. This study was approved by the Institutional Ethics Committee (CSP/24/JAN/142/18).

Study procedure

Seventy-five grams of anhydrous dextrose was administered orally in 200 mL of water to the study subjects (during the 19–24 gestational weeks), irrespective of the fasting state.[13] One hour and 2 h postload venous blood (5 mL) was collected in plain red-topped and sodium fluoride tubes. Samples were processed in the clinical biochemistry section in the central laboratory of the hospital. After allowing the blood to clot, the samples were centrifuged at 2000–3000 rpm for 15 min. Plasma was used for the glucose estimation in the Beckman Coulter AU5800 and AU680 analyzers. Subjects with 2 h postload plasma glucose level equal to or above 140 mg/dL were considered as GDM.[13] Serum was separated from the plain blood samples and was stored at − 80°C with designated sample IDs for FA analysis. FA samples were estimated by the nitroblue tetrazolium method using BTS-350.[14] Serum FA-albumin corrected values were calculated based on a given formula of Kunika *et al*.[15]

$$\text{Fructosaine} - \text{Albumin corrected value} = \frac{[\text{Fructosamine Conc}] \times 4}{[\text{Albumin Conc}]}$$

Statistical analysis

Statistical Package for the Social Sciences version 29.0 (IBM, Chicago, Illinois, United States) was used for data analysis, and the mean ± standard deviation (SD) was used to express the results. The Kruskal–Wallis test was employed to compare the means of OGTT and FA levels, whereas Pearson's correlation analysis was used to establish correlations between FA levels and OGTT results. Statistical significance was set at $P < 0.05$.

Results

The total sample size obtained from the study was 75. The average age of the subjects was 27.73 years. The study group consisted of 75 pregnant women with positive GDM confirmed pregnant women of 19–24 gestational weeks. Table 1 depicts the distribution of mean and SD among different age groups and variables – OGTT (fasting, 1 h, and 2 h) and FA. The 2-h postload glucose plasma level was 166.27 ± 20.23 mg/dL, whereas the fasting level was 91.61 ± 12.30 mg/dL. Since only 60 (80%) patients underwent liver function tests, the serum-corrected FA was calculated for those subjects. The mean serum FA and FA-albumin-corrected levels were 445.83 and 470.09 µmol/L, respectively. The correlations between fasting plasma glucose (FPG), 1 h, 2 h, and serum FA levels are shown in Table 2. Postload plasma glucose levels were significantly ($P < 0.001$) correlated with FPG levels. The correlation coefficient between 2 h postload glucose and fasting glucose was higher than the 1 h postload glucose level ($r = 0.589$; 95% confidence interval, 0.418–0.720). However, serum FA or corrected FA did not show any correlation with fasting or postload glucose levels. A negative insignificant correlation was observed between FPG levels during the OGTT and FA or corrected FA levels.

Discussion

The results of the study revealed that the 1 h and 2 h postload plasma glucose levels were significantly correlated with the FPG levels, whereas the serum FA or corrected FA did not

Table 1: Distribution of mean, standard deviation among different age group, and variables – oral glucose tolerance test (fasting, 1 h, and 2 h) and serum fructosamine

Parameters	Mean±SD
Gestational week	19–24
Age (years)	27.73±3.87
Fasting plasma glucose (mg/dL)	91.61±12.30
1-h plasma glucose (mg/dL)	183.00±21.41
2-h plasma glucose (mg/dL)	166.27±20.23
Serum fructosamine (µmol/L)	445.83±92.22
Serum fructosamine-albumin corrected value (µmol/L)[#]	470.09±74.49

[#]Only 60. Values are mean±SD, (n=75). SD: Standard deviation

Table 2: Correlation between oral glucose tolerance test (fasting, 1 h, and 2 h) and serum fructosamine and corrected fructosamine values

Test	Fasting plasma glucose (mg/dL)	1-h plasma glucose (mg/dL)	2-h plasma glucose (mg/dL)
Fasting plasma glucose (mg/dL)			
Correlation coefficient	1	0.528** (95% CI, 0.342–0.675)	0.364** (95% CI, 0.149–0.545)
P	-	<0.001	0.001
1-h plasma glucose (mg/dL)			
Correlation coefficient	0.528** (95% CI, 0.342–0.675)	1	0.589** (95% CI, 0.418–0.720)
P	<0.001	-	<0.001
2-h plasma glucose (mg/dL)			
Correlation coefficient	0.364** (95% CI, 0.149–0.545)	0.589** (95% CI, 0.418–0.720)	1
P	<0.001	<0.001	-
Serum fructosamine (µmol/L)			
Correlation coefficient	−0.049	0.078	0.073
P	0.677	0.506	0.535
Serum fructosamine–albumin corrected value (µmol/L)			
Correlation coefficient	−0.051	0.054	0.158
P	0.698	0.683	0.227

**Correlation is significant at the 0.01 level (two-tailed). CI: Confidence interval

show any correlation with the fasting or postload glucose levels. A negative insignificant correlation was observed between FPG level in OGTT and FA or corrected FA levels. Previous study reported that serum FA and second-trimester glucose challenge/OGTT results were only weakly correlated in a number of mid-pregnancy investigations; FA was a poor predictor of impaired glucose tolerance on the OGTT.[11] FA is more consistent with measurements of average/fasting glucose or short-term blood glucose than with isolated 1- or 2-h OGTT peaks because it represents average glycation over approximately 2–3 weeks, whereas glycated hemoglobin (HbA1c) and FA levels were favorably connected during the second trimester of pregnancy.[16]

The WHO defined GDM as "carbohydrate intolerance of variable severity with the onset or first recognition during pregnancy."[17] Hormonal anomalies that affect insulin sensitivity and pancreatic β-cell dysfunction have been linked to GDM, while the precise cause is uncertain.[18] As the incidence rates of GDM are variable and may possess risks to both maternal and fetal health, it is ideal to screen the GDM in the early stages.[19] Previous studies have demonstrated that FA was somewhat more useful than HbA1C as a rule-in test for the diagnosis of GDM.[20] Thus, FA, reflecting recent blood glucose levels, offers a potential alternative to the OGTT for GDM diagnosis. Women who were screened for GDM had a high risk of developing maturity-onset diabetes; therefore, avoiding obesity in middle age may help these women avoid developing diabetes.[21]

Regarding the identification of GDM, a single assay of serum FA demonstrated a sensitivity of 87.5% and a specificity of 94.5% when compared to the 75 g glucose tolerance test.[22] However, research has shown that it cannot predict the beginning of GDM in the early stages of pregnancy.[12] Furthermore, a previous study found that the sensitivity of FA and HbA1c as indicators of gestational glucose intolerance is extremely low.[23] The Pearson correlation of the OGTT and FA in the current investigation revealed no significant relationship between the patients' serum FA levels and any of the three OGTT parameters (FPG, 1-h, and 2-h). Furthermore, a negative correlation was observed between OGTT and FA levels, a finding that diverges from the established literature, which predominantly reports a positive correlation between these biomarkers. Baker *et al*. reported a substantial ($P < 0.001$) correlation between the concentrations of FA and FPG level ($r = 0.76$).[24] Further study also reported no relationship between the FA level and OGTT outcome.[12] The finding of Gingras *et al*. demonstrated a positive association between OGTT and serum FA.[25] Serum FA levels demonstrated inadequate predictive ability for detecting abnormal gestational glucose tolerance. Since the patients were majorly from the 2nd trimesters, the correlation between serum FA levels and OGTT was insignificant. In contrast to a study by Li and Yang which demonstrated that the FA levels showed a positive association with GCT results during the 28–34 weeks of gestation.[12] But did not correlate with other factors. This suggests that FA levels could potentially be used to screen for GDM during this period. However, the specific cutoff value for this screening should be further determined to assess its clinical implications in diagnosing GDM. Studies vary according to demographic characteristics, glucose load regimens, assay methodologies, timing (which trimester), and whether FA levels are adjusted for blood protein/albumin. Inconsistent results can be partially explained by this heterogeneity; several recent meta-analyses and systematic reviews continue to find evidence insufficient to replace OGTT.[26]

FA and albumin-corrected FA levels vary throughout pregnancy and are influenced by factors such as inflammation, insulin resistance, and abdominal adiposity. A study by Bernier *et al*. demonstrated that there were no significant differences found

in both uncorrected and albumin-corrected FA levels between participants with and without GDM.[26] However, this result should be interpreted cautiously because the statistical power to detect differences was limited, as only 11 of the individuals analyzed in their studies developed GDM during follow-up.

In addition, few significant associations were observed between FA levels and glycemic markers. This suggests that in a population with generally adequate glycemic control, serum FA does not appear to be associated with the development of GDM. Serum FA alone may not be an ideal marker for monitoring blood glucose levels, as its formation and concentration are influenced by both blood glucose and protein concentrations. Therefore, disorders that affect protein metabolism, including nephrotic syndrome, protein-losing enteropathy, pregnancy, malnutrition, and decreased protein synthesis (serum albumin is <3.0 g/dL), as observed in liver cirrhosis, can affect FA levels. Thus, FA levels must therefore be carefully examined in disorders with elevated total protein levels, such as multiple myeloma (caused by elevated immunoglobulins) and polyclonal gammopathies.[27] None of the patients included in this study had these disorders. Instead of being used for an initial diagnosis based on a single OGTT, FA may be helpful for short-term monitoring of glycemic control (e.g. tracking response to medication in GDM over weeks). In addition, an extremely elevated maternal serum FA-Albumin corrected level before delivery in GDM should raise awareness of hypoglycemia in newborns.[28]

Limitations

Due to practical and time constraints, the study was limited to a small sample size and did not account for variations in gestational age among participants. Furthermore, the possibility of selection bias was introduced by recruiting from particular clinical settings, which could have affected the generalizability of the results.

Conclusion

This study found no significant correlation between serum FA levels during the second trimester of pregnancy and OGTT parameters for diagnosing GDM. To confirm its clinical utility in GDM diagnosis, further research in larger, more diverse populations across different gestational weeks is essential.

Author contributions

SPMC conception of the work, interpretation of data, data analysis and draft writing, MAJ design the study, interpretation of data and revision of the manuscript. PMS and BSA contributed to the conception of work, interpretation and draft writing. All authors have approved to submit the manuscript.

Data statement

Data used for this study were obtained from the corresponding author on request.

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Conflicts of interest

There are no conflicts of interest.

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Original Article

Feto Maternal Outcomes Among Overweight And Obese Pregnant Women in a Medical College Hospital, Kerala: An Initial Assessment

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Abstract

Background: Nurses play a crucial role in early risk screening, health education, and preventive interventions to enhance maternal and fetal health outcomes. This cross-sectional study examined the relationship between selected maternal factors and maternal–fetal outcomes among 167 pregnant women. **Materials and Methods:** Data were collected through structured questionnaires and clinical records, focusing on maternal age, body mass index, employment status, type of work, gestational score, and type of delivery. Descriptive statistics described participant characteristics, while Chi-square and Fisher's exact tests were used to assess associations. **Results:** The majority of participants were aged 26–30 years (46.1%), unemployed (58.1%), and involved in moderate work (76%). Most women were multigravida (61.7%) and had a full-term normal delivery (45.5%). The most frequent maternal outcome was abortion (19.2%), followed by gestational diabetes mellitus at 13.8%. Among fetal outcomes, malpresentation (8.4%), intrauterine growth restriction (7.8%), and neonatal hyperbilirubinemia (7.8%) were most common. Statistical analysis revealed a significant association between abortion and employment status ($P = 0.003$) as well as gestational score ($P < 0.0001$). In addition, perineal tear was significantly associated with the type of delivery ($P = 0.004$). No statistically significant associations were found for other outcomes, although some notable trends were observed. **Conclusion:** Employment status, gravidity, and mode of delivery are important predictors of certain maternal and fetal outcomes. These findings underscore the importance of targeted antenatal care, especially for unemployed and multigravida women.

Keywords: Fetal outcomes, maternal outcomes, obesity, overweight, pregnant mothers

Introduction

Although the exact reasons are unknown, maternal obesity and being overweight increase the risk of problems during pregnancy and delivery, as well as newborn death. Gaining too much weight during pregnancy increases the risk of morbidity and death in the mother and unborn child by 1.32 times.[1] A negative outcome for the mother and the child is linked to weight increase that is either greater or less than what is recommended.[2] Gestational diabetes mellitus (GDM) and preeclampsia carry risks for the mother and fetus, respectively, including stillbirth and congenital abnormalities.[3] According to the most alarming health survey in the nation, 15% of rural women and 31.3% of urban women, or nearly one-third, are overweight or obese. The overwhelming and unambiguous evidence attests to the detrimental effects of obesity on their health.[4] Pregnant women will be significantly impacted by the 26% overweight and 29% obese nonpregnant women in the 20–39 age range.[5] Growing data suggests a link between maternal obesity and overweight and poor outcomes for both the mother and the fetus.[6-8] Increased body weight in mothers might have negative effects on the fetus as well as the mother. GDM and preeclampsia carry risks for the mother and fetus, respectively, including stillbirth and congenital abnormalities.

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According to the Institute of Medicine's recommendations for weight gain during pregnancy, pregnancy outcomes are known to be correlated with both prepregnancy body mass index (BMI) and gestational weight gain.[9-11] The Institute of Medicine defines an overweight woman during pregnancy as someone with a prepregnancy BMI between 25 and 29.9 kg/m^2 and an obese woman in pregnancy as prepregnancy BMI of 30 kg/m^2 or greater.[12] It is found to be accurate to let the people know how it affects the outcome of pregnancy. The majority of the literature reviews clearly stated that obesity and overweight issues root pregnancy and delivery complications too. Therefore, the current study aimed to know the weight gain status of antenatal mothers and their outcomes along with any association with selected demographic variables in the selected setting. The investigator also wanted to prevent this unique threat to their life with a tiny step.

Materials and Methods

Study design and setting

A quantitative descriptive study was done among antenatal mothers between the second and third trimesters at the department of obstetrics and gynecology in a tertiary care hospital, Central Kerala, South India, for 2 months (September 2024–October 2024). Pregnant women aged 18–35 years, overweight or obese before pregnancy, willing to participate, and able to read and understand the language (English or Malayalam) were included in the study. Pregnant women categorized as having a high-risk pregnancy by the obstetrician, except for overweight and obesity, were excluded. Formal approval was obtained from the Institutional Ethical Committee (Ref No: 23/EC/24/AIMS-06). Individual informed consent was obtained from each participant before data collection. Confidentiality was maintained throughout the study.

Sample size: Since no previous study was done in this population, the total number of pregnant women who attended the hospital during the period of the study (167 numbers) was selected for the study.

Study procedure

A semistructured checklist was used to assess the fetomaternal outcomes. The checklist for fetomaternal outcomes was validated by three experts, and the internal consistency of 0.8 (Cronbach's alpha) was found feasible for data collection. The investigator met the woman individually and interacted to get marked the responses. The whole data collection took approximately 15 min for each participant. Fetomaternal outcomes are the outcome of pregnancy in terms of antenatal, intranatal, and postnatal complications, type of delivery, fetal outcomes, including birth weight, neonatal intensive care unit admission, and other related complications assessed using a semistructured checklist.

Statistical analysis

The statistics software used in the study was SPSS Version 16, (IBM Corporation, Illinois, US). The descriptive statistics of gestational age and neonatal birth weight were used frequency–percentage distribution, whereas the association found in various variables in the further tables used Chi-square and Fisher's Exact tests. $P < 0.05$ was considered statistically significant.

Results

A total of 167 overweight and obese pregnant mothers were included in the study. The summary of participant characteristics revealed that the study participants had a mean age of 27.74 years (standard deviation [SD] ±3.79). Their average weight was 71.78 kg (SD ± 11.29), and the mean height was 4.41 m (SD ± 21.13). The calculated mean BMI was 28.84 kg/m² (SD ± 5.87), indicating participants were generally overweight, and their frequency distribution is given in Table 1. Regarding the frequency of maternal outcomes, abortion was the most commonly reported outcome (19.2%), followed by GDM (13.8%). Perineal tear and premature rupture of membranes were noted in a smaller proportion (7.8% and 7.2%, respectively). Less common complications included postpartum hemorrhage, oligo/polyhydramnios, and per vaginal discharge. Hypertensive disorders such as PIH and preeclampsia, along with placenta previa, were rare (≤1.2%). These data highlight the need for early screening and antenatal care to manage and reduce the incidence of maternal complications, thereby improving pregnancy outcomes.

In terms of fetal outcomes, malpresentation (8.4%) was the most frequent issue, followed by intrauterine growth

Table 1: Frequency distribution of participant characteristics (n=167)

	Frequency (%)
Age (years)	
≤25	53 (31.7)
26–30	77 (46.1)
≥31	37 (22.2)
Employment	
Employed	70 (41.9)
Unemployed	97 (58.1)
Sedentary work	
Yes	40 (24.0)
No	127 (76.0)
Moderate work	
Yes	127 (76.0)
No	40 (24.0)
Gestational score	
Multi	103 (61.7)
Primi	64 (38.3)
Type of delivery	
Elective lower-segment cesarean section	43 (25.7)
Emergency lower-segment cesarean section	45 (26.9)
Full-term normal delivery	76 (45.5)
Preterm delivery	1 (0.6)
Vacuum delivery	2 (1.2)

restriction (IUGR) and neonatal hyperbilirubinemia (NNH), each at 7.8%. Low birth weight affected 5.4% of the neonates, while preterm birth and fetal distress were reported in 3.6% and 3% respectively. Hypoglycemia, shoulder dystocia, lymphangioma, and macrosomia were rare, occurring in <3% of cases. These findings underscore the importance of continuous fetal monitoring, antenatal checkups, and early interventions to prevent and manage complications, ultimately enhancing neonatal health outcomes and reducing perinatal risks. The participants had a mean gestational age of 37.59 weeks (SD ± 1.69), indicating that most pregnancies reached term. The average birth weight of the babies was 3.13 kg (SD ± 0.42), which falls within the normal range. These findings suggest favorable maternal and neonatal outcomes in the study population.

Employment status and gestational score (P <.0001) showed statistically significant associations with abortion [Table 2]. Abortion was more prevalent among unemployed women and multigravida participants, suggesting possible links to socioeconomic and physiological factors. These findings highlight the need for targeted antenatal support and counseling, particularly for unemployed and multigravida women, to reduce the risk of abortion and improve maternal health outcomes through early identification and care interventions. No statistically significant associations were found between GDM and maternal factors [Table 3]. Type of delivery (P < 0.004) was significantly associated [Table 4]. Other factors were significantly associated with the selected variables.

The association between various maternal characteristics and malpresentation during delivery was analyzed using Chi-square tests [Table 5]. Among age groups, the highest incidence (13%) was observed in women aged 26–30 years, although it was not statistically significant (P = 0.106). BMI categories showed no significant association (P = 0.742), with overweight women having a 6.8% malpresentation rate. Employment status and work type (sedentary or moderate) were also not statistically correlated. Primiparous and multigravida mothers showed similar rates of malpresentation. Elective lower-segment cesarean section (LSCS) had a higher rate (16.3%) than full-term normal delivery (FTND) (3.9%) and other delivery types, but again with no statistical significance (P = 0.221).

The association between maternal factors and IUGR was studied using Chi-square tests [Table 6]. Although none of the associations were statistically significant, trends are observable. Younger mothers (≤25 years) had a higher IUGR rate (13.2%) than older age groups. Underweight women showed no cases of IUGR, while normal BMI had the highest rate (15.8%). Unemployed women had fewer IUGR cases (5.2%) than employed women (11.4%). No notable differences were found between sedentary and moderate work. Primiparous mothers showed a higher IUGR incidence (10.9%) than multiparous. Emergency LSCS had the highest IUGR rate (17.8%) among delivery types.

Although none of the associations were statistically significant in the relationship between maternal variables and NNH, certain patterns emerged. Mothers aged ≥ 31 years showed a higher incidence of NNH (13.5%) than younger groups. Underweight mothers had the highest NNH rate (33.3%), although the sample size was very small. Unemployed women had a slightly higher incidence (9.3%) than employed women (5.7%). Sedentary and moderate work types showed similar results. Primiparous mothers had a slightly lower NNH rate (6.3%) than multigravida. Elective LSCS showed a higher rate of NNH (18.6%) than emergency LSCS and FTND.

Table 2: Association between abortion and maternal factors (n=167)

	Abortion		Total, n (%)	P (Chi-square test)
	No, n (%)	Yes, n (%)		
Age (years)				
≤25	43 (81.1)	10 (18.9)	43 (81.1)	0.907
26–30	63 (81.8)	14 (18.2)	63 (81.8)	
≥31	29 (78.4)	8 (21.6)	29 (78.4)	
BMI (kg/m^2)				
Under weight	3 (100)	0	3 (100)	0.670[@]
Normal	16 (84.2)	3 (15.8)	16 (84.2)	
Over weight	71 (80.7)	17 (19.3)	71 (80.7)	
Obese	45 (78.9)	12 (21.1)	45 (78.9)	
Employment				
Employed	64 (91.4)	6 (8.6)	64 (91.4)	0.003
Unemployed	71 (73.2)	26 (26.8)	71 (73.2)	
Sedentary work				
No	100 (78.7)	27 (21.3)	100 (78.7)	0.220
Yes	35 (87.5)	5 (12.5)	35 (87.5)	
Moderate work				
No	35 (87.5)	5 (12.5)	35 (87.5)	0.220
Yes	100 (78.7)	27 (21.3)	100 (78.7)	
Gestational score				
Multi	74 (71.8)	29 (28.2)	74 (71.8)	0.0001
Primi	61 (95.3)	3 (4.7)	61 (95.3)	
Type of delivery				
Elective lower-segment cesarean section	30 (69.8)	13 (30.2)	30 (69.8)	0.202[@]
Emergency lower-segment cesarean section	38 (84.4)	7 (15.6)	38 (84.4)	
Full-term normal delivery	65 (85.5)	11 (14.5)	65 (85.5)	
Preterm vaginal delivery	1 (100)	0	1 (100)	
Vacuum delivery	1 (50)	1 (50)	1 (50)	

[@]Fisher's exact test. BMI: Body mass index

Discussion

The present study investigated the association between selected maternal factors and fetomaternal outcomes among 167 pregnant women. A notable association was observed between abortion and employment level (P = 0.003), and gestational score (P < 0.0001). This lines up with findings

Table 3: Association between gestational diabetes mellitus and maternal factors (*n*=167)

	GDM		Total, *n* (%)	*P* (Chi-square test)
	No, *n* (%)	Yes, *n* (%)		
Age (years)				
≤25	13 (24.5)	40 (75.5)	13 (24.5)	0.158
26–30	9 (11.7)	68 (88.3)	9 (11.7)	
≥31	7 (18.9)	30 (81.1)	7 (18.9)	
BMI (kg/m^2)				
Under weight	0	3 (100)	0	0.474[@]
Normal	2 (10.5)	17 (89.5)	2 (10.5)	
Over weight	18 (20.5)	70 (79.5)	18 (20.5)	
Obese	9 (15.8)	48 (84.2)	9 (15.8)	
Employment				
Employed	9 (12.9)	61 (87.1)	9 (12.9)	0.191
Unemployed	20 (20.6)	77 (79.4)	20 (20.6)	
Sedentary work				
No	25 (19.7)	102 (80.3)	25 (19.7)	0.159
Yes	4 (10)	36 (90)	4 (10)	
Moderate work				
No	4 (10)	36 (90)	4 (10)	0.159
Yes	25 (19.7)	102 (80.3)	25 (19.7)	
Gestational score				
Multi	15 (14.6)	88 (85.4)	15 (14.6)	0.225
Primi	14 (21.9)	50 (78.1)	14 (21.9)	
Type of delivery				
Elective lower-segment cesarean section	5 (11.6)	38 (88.4)	5 (11.6)	0.603[@]
Emergency lower-segment cesarean section	9 (20)	36 (80)	9 (20)	
Full-term normal delivery	15 (19.7)	61 (80.3)	15 (19.7)	
Preterm vaginal delivery	0	1 (100)	0	
Vaccum delivery	0	2 (100)	0	

[@]Fisher's exact test. GDM: Gestational diabetes mellitus, BMI: Body mass index

Table 4: Association between perineal tear and maternal factors (*n*=167)

	Perineal tear		Total, *n* (%)	*P* (Chi-square test)
	No, *n* (%)	Yes, *n* (%)		
Age (years)				
≤25	50 (94.3)	3 (5.7)	50 (94.3)	0.012[@]
26–30	67 (87)	10 (13)	67 (87)	
≥31	37 (100)	0	37 (100)	
BMI (kg/m^2)				
Under weight	3 (100)	0	3 (100)	0.861[@]
Normal	18 (94.7)	1 (5.3)	18 (94.7)	
Over weight	81 (92)	7 (8)	81 (92)	
Obese	52 (91.2)	5 (8.8)	52 (91.2)	
Employment				
Employed	64 (91.4)	6 (8.6)	64 (91.4)	0.747
Unemployed	90 (92.8)	7 (7.2)	90 (92.8)	
Sedentary work				
No	117 (92.1)	10 (7.9)	117 (92.1)	0.938[@]
Yes	37 (92.5)	3 (7.5)	37 (92.5)	
Moderate work				
No	36 (90)	4 (10)	36 (90)	0.559[@]
Yes	118 (92.9)	9 (7.1)	118 (92.9)	
Gestational score				
Multi	94 (91.3)	9 (8.7)	94 (91.3)	0.554[@]
Primi	60 (93.8)	4 (6.3)	60 (93.8)	
Type of delivery				
Elective lower-segment cesarean section	43 (100)	0	43 (100)	0.004[@]
Emergency lower-segment cesarean section	44 (97.8)	1 (2.2)	44 (97.8)	
Full-term normal delivery	64 (84.2)	12 (15.8)	64 (84.2)	
Preterm vaginal delivery	1 (100)	0	1 (100)	
Vacuum delivery	2 (100)	0	2 (100)	

[@]Fisher's exact test. BMI: Body mass index

by Singh *et al*. who mentioned that socioeconomic stress and lack of pregnancy care access among nonemployed women remarkably contributed to untoward pregnancy outcomes, including abortion.[13] The higher prevalence of abortion among women delivered multiple times may suggest cumulative reproductive stress or deficient spacing between pregnancies, as propped up by findings of Kassa *et al*. who emphasized the significance of birth spacing in preventing maternal complications.[14]

Perineal injuries were importantly associated with the mode of delivery (P = 0.004), with higher occurrences in females undergoing FTND. This decision is consistent with a study by Sharma and Rajalakshmi, which concluded that voluntary normal vaginal deliveries, especially in primiparous women, pose a higher risk of perineal injury than LSCS.[15] Variables such as GDM, IUGR, NNH, and malpresentation did not reveal statistically notable associations with maternal factors, but observable trends stipulated that older age, higher BMI, and elective LSCS were routinely connected with these issues. These trends line up with global inferences by the World Health Organization, which emphasized that maternal obesity and rising age are independent predictors of inauspicious perinatal outcomes, including neonatal problems and delivery interventions.[16]

The data ruled the importance of amalgamating maternal and fetal outcome predictors, such as BMI, type of job, gestational age, and delivery mode into schedule. Empowering nurses on early pinpointing of high-risk pregnancies can raise clinical preparedness. For research in nursing**,** the observed trends – even if precisely non-significant – highlight areas that need in-depth investigation, such as the impact of working status on miscarriage rates and elective LSCS on NNH. This can pilot future longitudinal and intervention researches. The findings call for enhancing antenatal screening policies, prioritizing personal care for nonworking multigravida women, and enhancing resource allocation for maternal–fetal health

Table 5: Association between malpresentation and selected variables (n=167)

	Malpresentation		Total, n (%)	P (Chi-square test)
	No, n (%)	Yes, n (%)		
Age (years)				
≤25	50 (94.3)	3 (5.7)	50 (94.3)	0.106[@]
26–30	67 (87)	10 (13)	67 (87)	
≥31	36 (97.3)	1 (2.7)	36 (97.3)	
BMI (kg/m^2)				
Under weight	3 (100)	0	3 (100)	0.742[@]
Normal	17 (89.5)	2 (10.5)	17 (89.5)	
Over weight	82 (93.2)	6 (6.8)	82 (93.2)	
Obese	51 (89.5)	6 (10.5)	51 (89.5)	
Employment				
Employed	64 (91.4)	6 (8.6)	64 (91.4)	0.941
Unemployed	89 (91.8)	8 (8.2)	89 (91.8)	
Sedentary work				
No	117 (92.1)	10 (7.9)	117 (92.1)	0.678[@]
Yes	36 (90)	4 (10)	36 (90)	
Moderate work				
No	36 (90)	4 (10)	36 (90)	0.678[@]
Yes	117 (92.1)	10 (7.9)	117 (92.1)	
Gestational score				
Multi	94 (91.3)	9 (8.7)	94 (91.3)	0.834
Primi	59 (92.2)	5 (7.8)	59 (92.2)	
Type of delivery				
Elective lower-segment cesarean section	36 (83.7)	7 (16.3)	36 (83.7)	0.221[@]
Emergency lower-segment cesarean section	41 (91.1)	4 (8.9)	41 (91.1)	
Full-term normal delivery	73 (96.1)	3 (3.9)	73 (96.1)	
Preterm vaginal delivery	1 (100)	0	1 (100)	
Vaccum delivery	2 (100)	0	2 (100)	

[@]Fisher's exact test. BMI: Body mass index

Table 6: Association between intrauterine growth restriction and selected variables (n=167)

	IUGR		Total, n (%)	P (Chi-square test)
	No, n (%)	Yes, n (%)		
Age (years)				
≤25	46 (86.8)	7 (13.2)	46 (86.8)	0.228[@]
26–30	73 (94.8)	4 (5.2)	73 (94.8)	
≥31	35 (94.6)	2 (5.4)	35 (94.6)	
BMI (kg/m^2)				
Under weight	3 (100)	0	3 (100)	0.281[@]
Normal	16 (84.2)	3 (15.8)	16 (84.2)	
Over weight	80 (90.9)	8 (9.1)	80 (90.9)	
Obese	55 (96.5)	2 (3.5)	55 (96.5)	
Employment				
Employed	62 (88.6)	8 (11.4)	62 (88.6)	0.135
Unemployed	92 (94.8)	5 (5.2)	92 (94.8)	
Sedentary work				
No	117 (92.1)	10 (7.9)	117 (92.1)	0.938[@]
Yes	37 (92.5)	3 (7.5)	37 (92.5)	
Moderate work				
No	37 (92.5)	3 (7.5)	37 (92.5)	0.938[@]
Yes	117 (92.1)	10 (7.9)	117 (92.1)	
Gestational score				
Multi	97 (94.2)	6 (5.8)	97 (94.2)	0.238[@]
Primi	57 (89.1)	7 (10.9)	57 (89.1)	
Type of delivery				
Elective lower-segment cesarean section	41 (95.3)	2 (4.7)	41 (95.3)	0.100[@]
Emergency lower-segment cesarean section	37 (82.2)	8 (17.8)	37 (82.2)	
Full-term normal delivery	73 (96.1)	3 (3.9)	73 (96.1)	
Preterm vaginal delivery	1 (100)	0	1 (100)	
Vacuum delivery	2 (100)	0	2 (100)	

[@]Fisher's exact test. IUGR: Intrauterine growth restriction, BMI: Body mass index

assessment. Nurses take part a pivotal role in risk assessment, revamping awareness, and prompt referral. Stress should be placed on surveilling women with higher BMI, advanced maternal age, or elective LSCS for fetal issues. Eventually, these indications enhance nurses' role as frontline validates in ensuring safe maternal and newborn outcomes through preemptive, informed, and compassionate care.

Limitations

Total enumerate sampling was used in the study. The sample size could not be calculated as there were no studies available in the specified population. Furthermore, generalization was not possible due to less sample size and single-center study.

Conclusion

This study provides a comprehensive overview of maternal and fetal outcomes concerning selected demographic and clinical factors among pregnant women. While most associations between variables such as age, BMI, employment status, and type of delivery with maternal and fetal complications such as abortion, GDM, IUGR, and NNH were not statistically significant, certain trends were observed. Unemployed and multigravida women showed a higher incidence of abortion, while elective LSCS was more frequently associated with complications, such as NNH and malpresentation. Strengthening maternal health services, particularly for vulnerable groups, can significantly improve outcomes. Future research with larger sample sizes and longitudinal data is recommended to explore causal relationships and develop targeted maternal–fetal health interventions.

Authors' contribution

MJ: Study conception and design, data collection, analysis, and interpretation of results, and draft manuscript. LV: Study conception and design, data collection, analysis, and interpretation of results, draft manuscript, and revision of the manuscript. All authors approved the final version of the manuscript to submit to the journal.

Data sharing agreement

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

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Conflicts of interest

There are no conflicts of interest.

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Original Article

Preclinical Evaluation of Acute and Subacute Toxicity of an Ayurvedic Medication "*Madhusnuhi Rasayana*"

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Abstract

Background: *Madhusnuhi Rasayana* (MR) is an ayurvedic medication known for the improvement of immunity and general health. Despite the medicinal property, the heavy metal toxicity of polyherbal ethnic drugs is a major concern and determination of the safest dose for pharmacological study is important. **Materials and Methods:** The phytochemical screening, organoleptic characteristics, heavy metals content, and microbiological load were analyzed in three different batches of in-house prepared MR that accomplished the prescribed specifications of Ayurvedic Pharmacopoeia. A single acute oral dose (2000 mg/kg body weight) of MR was administered followed by 14day observation and a 28day repeated oral dosing (200, 300, and 400 mg/kg body weight) of MR was administered followed by physiological and structural integrity evaluation conducted in accordance with OECD guidelines to ensure the safety and pharmacological feasible dose in rodent model (Swiss albino). **Results:** The Swiss albino mice had a favorable safety profile following a single oral dose, indicating higher LD_{50} values. The repeated oral administration of MR for 28 days in mice did not alter its biochemical, hematological, and histological parameters, ensuring the unaltered physiological functions and structural integrity of major organs, including testes and ovary. **Conclusions:** For investigating the pharmacological characteristics of MR, a dose of up to 400 mg/kg body weight is advised as safe in mouse models.

Keywords: Acute toxicity, Ayurveda, polyherbal formulations, rasayana, toxicology

Introduction

Ayurveda, an age-old Indian medical tradition, is becoming more and more popular in the world of medicine. The safety and efficacy of ethnic formulations are important considerations in the debate over the full adoption of ayurvedic medicine.[1] The Ayurvedic Pharmacopoeia guidelines are being properly implemented in modern days in the preparation of this formulation. However, due to growing concerns about heavy metal toxicity leading to systemic toxicity, most ayurvedic polyherbal medications, especially those preparations containing metals, are currently under scrutiny. As a result, appropriate steps must be taken, such as choosing plant materials carefully, purifying metals used in preparations, and obtaining scientific confirmation that polyherbal medications meet contemporary safety and effectiveness criteria. Rasayanas are a class of polyherbal preparations, and *Madhusnuhi Rasayana* (MR) is a well-known preparation in Ayurveda used for skin diseases, rheumatoid arthritis, goiter, gout, and nonhealing wounds.[2] There is not much systematic study or scientific literature available using MR.

There are two types of MR: "cheriya" and "valiya" MRs. The polyherbal formulation of MR consists of *Emblica officinalis, Zingiber officinale, Piper longum, Piper nigrum, Terminalia bellirica, Terminalia chebula, Elettaria cardamomum, Cinnamomum zeylanicum, Cuminum cyminum, Cinnamomum tamala, Foeniculum vulgare, Nigella sativa, Piper cubeba, Embelia ribes, Saussurea costus, Operculina turpethum, Piper*

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sylvaticum, Withania somnifera, Clerodendrum serratum, Celastrus paniculatus, Mesua ferrea, Commiphora wightii, Smilax china, clarified butter, sugar, and purified sulfur.[1,2] The use of purified sulfur in MR is a major concern, and so far, no safety assessment of this rasayana has been reported. Therefore, the current study was undertaken to characterize the physicochemical, phytochemical, heavy metal content, and microbial analysis to verify if it is at par with Ayurvedic Pharmacopoeia. An *in vivo* toxicological profiling was also conducted to determine the nontoxic doses for any further preclinical studies.

Materials and Methods

Preparation of "*Madhusnuhi Rasayana*"

An in-house preparation of MR was carried out according to the Sahasrayogam traditional method, comprising the purification of *S. china* (Madhusnuhi), *C. wightii,* and sulfur with the oversight of traditional Ayurveda practitioner K. S. Narayanan Vaidyar (R.M.P. Regd. No. 5551). The polyherbs were dried and powdered, except for *C. wightii* and *S. china,* which were purified before drying and powdered separately. *C. wightii* was purified by mixing with *Curcuma zedoaria* and *Azadirachta indica,* and clarified using boiling butter until it became semisolid. *S. china* was purified by boiling in cow's milk. The MR preparation process was strictly followed by the methodology from Sahasrayoga.[3] The MR was prepared in three batches to test its consistency.

Organoleptic characteristics

According to the Ayurvedic Pharmacopoeia of India: Part II (Formulations), Volume III (1),[4] the organoleptic properties, including loss on drying, total ash, acid-insoluble ash, alcohol soluble extract, water soluble extract, and pH (10% aqueous solution), were evaluated in crude MR.

Heavy metal analysis

Individual batches of MR were weighed (0.1 g) and digested using concentrated HNO_3 and H_2O_2 in a microwave digester. Heavy metals such as lead (Pb) and cadmium (Cd) were analyzed by Inductively coupled plasma atomic emission spectroscopy (ICPAES) (Perkin Elmer-Avio 200).

Microbiology analysis

A serial dilution (10^{-1}–10^{-6}) of MR was prepared from 1 g of MR in 49 mL sterile distilled water. The microbiological analysis included the total aerobic and total yeast count. The total aerobic and yeast counts were determined by the spread plating technique using nutrient agar and Sabouraud dextrose agar as the respective media. The dilution with a countable number of colonies was obtained in 10^{-3} dilution and was used to determine the colony-forming unit (cfu).

Cfu/mL = (Number of colonies in a selected plate × Dilution factor)/Volume added

Fractionation of *Madhusnuhi Rasayana*

About 1 g/100 mL of MR in distilled water was subjected to cold centrifugation (Remi cooling centrifuge-24 BL 9001:2000) at 4500 rpm for 10 min. The residues after removing the supernatant were collected in a beaker, dried out, and dissolved in diethyl ether. The aqueous supernatant and diethyl portion were mixed together in a separating funnel and allowed to separate the two layers. The denser water fractions were collected prior to the nonpolar fractions into respective preweighed beakers. The solvents were evaporated off, using a vacuum concentrator (Eppendorf Concentrator plus), weighed, and dissolved in the respective solvents to get the concentrations.

Preliminary phytochemical analysis

The polar and nonpolar extracts of MR examined for the detection of flavonoid (sodium hydroxide test, ferric chloride test, lead acetate test, and Con. sulfuric acid test), steroid (Salkowski test), tannin (lead acetate test and ferric chloride test), terpenoid (Salkowski test and Con. sulfuric acid), coumarin (sodium hydroxide test), saponin (foam test and froth test), phenol (lead acetate test and ferric chloride test), carbohydrate (Benedict's test), and alkaloid (Mayer's test and Dragendorff's test) following the protocol described by Sofowara,[5] Trease and Evans,[6] Harborne,[7] and Smitha *et al*.[8]

Experimental animals

Both male and female Swiss albino mice were purchased from the College of Veterinary and Animal Sciences, Small Animal Breeding Station, Thrissur, India. They were maintained in ventilated cages in a 12-h dark–light cycle with temperature (22°C–28°C) and 60%–70% relative humidity. Standard rodent meal and unlimited access to filtered water were given to the animals. Institutional Animal Ethics Committee approved the experiment (ACRC/IAEC/20(1)-P2).

Oral acute toxicity analysis

The acute toxicity analysis of MR was carried out following Organisation for economic co-operation and development (OECD) guideline 423.[9] Accordingly, 2000 mg/kg body weight of MR dissolved in 1% gum acacia (250 µL) was given orally as a single dose to three female mice of average body weight along with the same volume of cow milk (Group 1 animals). Animals in Group 2 (vehicle group) were orally administered with 250 µL 1% gum acacia. All the treated animals were initially evaluated for any incidents of death or drastic behavioral change for 4 h and then at 24 h intervals for 14 days. Every week, body weight was tracked, and every 3 days, food and drink intake were noted. Behavioral and neurological abnormalities were also monitored during the course of the study. All of the animals were fasted overnight at the conclusion of the experiment, and a necropsy was conducted the following day.

Repeated administration of *Madhusnuhi Rasayana*

The LD_{50} value (above 2 g/kg body weight) from the acute toxicity research served as the basis for the doses determined for MR in the subacute toxicity evaluation.[10] As a result, three distinct dosages between 200 and 400 mg/kg body weight were chosen and given to the mice orally through gavage over the course of 28 days. Briefly, both sexes of Swiss albino mice (20–25 g) were divided into five groups with five animals

each. Group 1 animals were kept as normal. Group 2 animals administered 1% gum acacia served as vehicle control (VC), and Groups 3–5 animals were given low dose (LD; 200 mg/kg body weight and medium dose (MD; 300 mg/kg body weight) and high dose (HD: 400 mg/kg body weight) of MR, followed by 100 µL milk for all the animals. The animals were monitored for adverse reactions to the drug, such as behavioral changes and mortality during the drug administration period. The body weight and food and water consumption were monitored periodically. All animals were euthanized at the conclusion of the experiment, and a heart puncture was used to obtain blood. All the important organs of male and female mice were excised out to perform histological analysis.

Analysis of blood and other tissues

Hemogram parameter analysis

The blood collected in K3EDTA tubes coated with anticoagulants was used for hematological analysis.

Biochemical analysis

The serum was separated by centrifugation for biochemical analysis. The biochemical parameters analyzed include lipid profile, electrolytes, renal function markers, liver function markers, bilirubin, and total protein. In accordance with the manufacturer's instructions, all of these parameters were examined using commercially available kits (Agappe Diagnostics, Kothamangalam, Kerala, India).

Organosomatic index

Organs were collected and weighed, and the organosomatic index (OSI) was determined using the formula:

OSI = (Organ weight/Body weight) × 100

Heavy metal analysis

About 0.2 g of liver and kidney samples of experimental animals of both the sexes were weighed and digested using 70% HNO_3. The heavy metal lead (Pb) was determined quantitatively by ICP-AES.

Histological analysis

A portion of the vital organs was stored in 10% formaldehyde. Histological sections of these organs were assessed for structural defects at the cellular to organ level following repetitive drug administration, and representative images were captured using a trinocular microscope.

Statistical analysis

Data were expressed as mean ± standard deviation using Tukey's analysis and two-way analysis of variance. The statistical significance was examined using GraphPad Prism (10.31) (GraphPad Software, LLC d.b.a "Dotmatics", 225 Franklin Street.FI.26, Boston, MA 02110). $P < 0.05$ was considered statistically significant.

Results

Characteristics and consistency of *Madhusnuhi Rasayana* in different batches

All the characteristics of the three batches of MR are listed in Table 1. It is noteworthy that all the three batches of MR met the Ayurvedic Pharmacopoeia of India (API) requirements for all the organoleptic qualities. There were minor variations noted in the pH, acidinsoluble ash, and total ash readings, though these changes remained subtle. The cadmium content was below the detection level, and a permissible limit was found for lead content. The total aerobic count as well as the total yeast and mold count of all three batches of MR analyzed were marginally above the limited specifications of the Ayurvedic Pharmacopoeia of India. This slight deviation from acceptable microbial standards suggests the suboptimal storage condition and the lack of preservatives. There was consistency in the yield (%) across three batches of MR. In general, the

Table 1: Characterization of *Madhusnuhi Rasayana* for batch consistency

Parameters	MR1	MR2	MR3	API specification
The organoleptic characteristics (%)				
Loss on drying	3.63	4.03	4.24	-
Total ash	1.86	1.66	1.66	NMT 1.4
Acid-insoluble ash	0.70	0.55	0.65	NMT 0.23
ASF	47.56	68.48	73.51	NLT 40
WSF	50.78		52.79	67.6
pH (10% aqueous solution)	4.94	4.61	4.99	4.01–4.17
Heavy metal analysis (mg/kg)				
Lead	8.866	1.559	1.508	NMT 10.0
Cadmium	BDL	BDL	BDL	NMT 0.3
Microbial analysis (CFU/mL)				
TAC	4.1×10^5	4.5×10^5	4.3×10^5	1×10^5
TYM	5.0×10^3	2.0×10^3	4.0×10^3	1×10^3
Yield of aqueous versus organic solvent fractions				
Nonpolar (diethyl ether)	10.35	10.73	12.6	-
Polar (aqueous)	29.32	28.23	29.07	-

MR1, MR2, and MR3 are three batches of MR prepared independently. ASF: Alcohol soluble fraction, WSF: Water soluble fraction, NMT: Not more than, NLT: Not less than, API: Ayurvedic Pharmacopoeia of India, BDL: Below detection limit, TAC: Total aerobic count, TYM: Total yeast and molds

polar extracts had an increased yield in comparison to the nonpolar MR extracts.

Preliminary phytochemical analysis of *Madhusnuhi Rasayana*

The qualitative phytochemical screening of polar and nonpolar extracts of MR1, MR2, and MR3 detected flavonoid, steroid, carbohydrate, and alkaloid, as shown in Table 2.

Acute toxicity of *Madhusnuhi Rasayana* in mice

During the 14-day monitoring period post single dose MR/vehicle administration, the animals were observed to be active without abnormal changes such as drowsiness, sedation, diarrhea, and the general physique as well as eye color remained unchanged. No abnormal changes observed in the skin or hair. A slight alteration in water and food consumption was observed during the period of experiment, as depicted in Supplementary Figure 1, but no significant body weight loss was observed in either group of animals [Figure 1]. Moreover, no death was observed, and no adverse effects on organs were observed after necropsy of animals [Supplementary Figure 2].

Subacute toxicity of *Madhusnuhi Rasayana* in mice

The body weight as well as the feed and water consumption of male and female Swiss albino mice during the experimental period was recorded and was observed to be without any statistically significant among the groups [Figure 2 and Supplementary Figures 3 and 4].

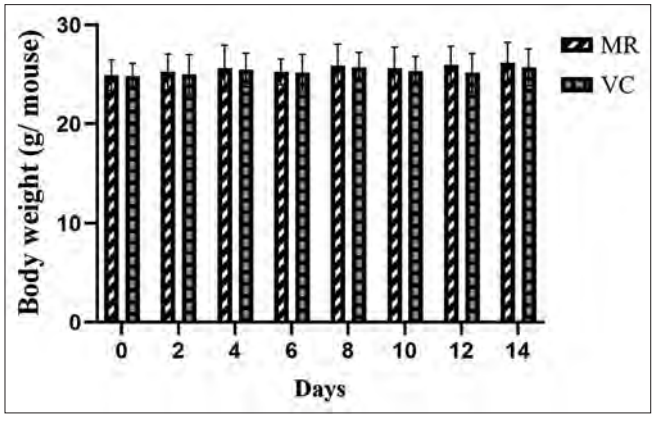

Figure 1: The body weight of mice after the single acute dose administration of *Madhusnuhi Rasayana*. MR: *Madhusnuhi Rasayana*, VC: Vehicle control

Hemogram parameters

Mostly all the hematological parameters, such as differential count (DC), platelets, red blood cells, packed cell volume, hemoglobin (HB), mean corpuscular hemoglobin (MCH), and MCH concentration, remain unchanged in the mice treated with MR when compared with the normal untreated mice as represented in Table 3. A statistically significant ($P < 0.05$) reduction in mean corpuscular volume (MCV) (MD: 400 mg/kg body weight) and total white blood cell count was observed in MR-administered (LD: 200 mg/kg body weight and MD: 400 mg/kg body weight) male mice with respect to the control group but were in the normal range of Swiss albino mice.

Biochemical parameters

Different serum biochemical parameters of male and female mice are represented in Tables 4 and 5, respectively. In both the sexes, renal markers as well as bilirubin and serum protein contents

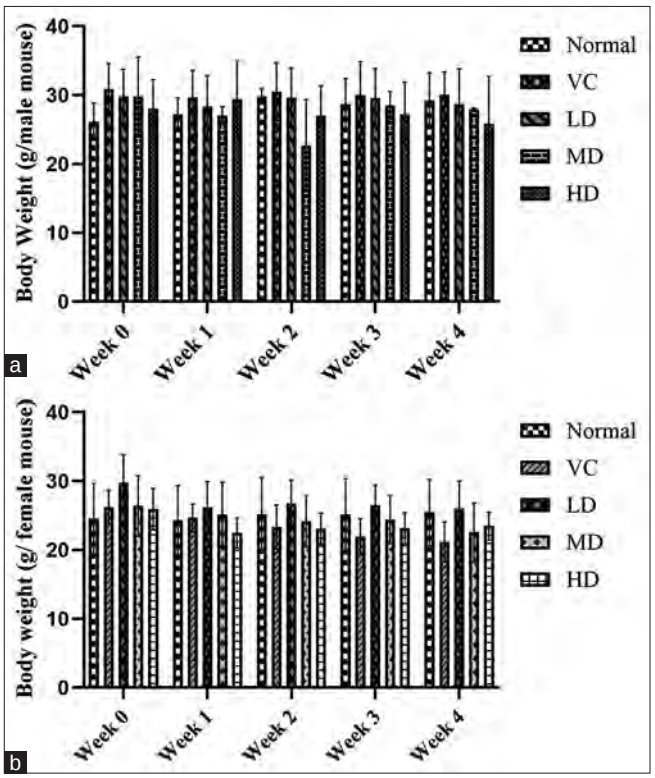

Figure 2: Body weight of male and female Swiss albino mice following repeated oral administration of MR for 28 days. (a) Body weight of male mice, (b) Body weight of female Swiss albino mice. VC: Vehicle control, LD: Low dose, MD: Medium dose, HD: High dose

Table 2: Qualitative screening of phytochemicals in organic versus polar solvent extracts of *Madhusnuhi Rasayana*

Phytochemical MR3	Qualitative test	Nonpolar fraction			Polar fraction		
		MR1	MR2	MR3	MR1	MR2	MR3
Flavonoid	Concentration H_2SO_4 test	++	++	++	++	++	++
Steroid	Salkowski test	++	++	++	–	–	–
Carbohydrate	Benedict's test	+	+	+	++	++	++
Alkaloid	Dragendorff's test	–	–	–	+	+	+

+Level of presence and –absence. MR: *Madhusnuhi Rasayana*

Table 3: Hematological parameters of Swiss albino mice followed by repeated oral administration of *Madhusnuhi Rasayana* at 24-h intervals for 28 days

Parameters	Normal	VC	LD	MD	HD
Male mice					
HB (g/dL)	13.5±0.1	13.9±0.6	13.95±1.2	13.45±0.6	13.5±0.1
RBC (10^6/µL)	7.6±0.6	8.25±0.1	8.05±0.1	8.5±0.4	7.7±0.2
MCV (fL)	63±3	59±1	54±6	51±1.4*	59±1.4
MCH (pg)	18±1	16.5±0.7	17±1	16±0.7	17±0.7
MCHC (g/dL)	28±0.4	28±0.5	31.5±0.7	31±0.7	28±0.7
PCV (%)	48±1	49±1	43.5±4.9	44±0.7	47±0.7
PLT (10^5/µL)	9.95±3.61	7.9±0.1	8.8±0.6	11.2±0.3	10.3±0.6
TC (cell/µL)	13,500±141	10,300±566	6800±1273*	6350±70*	8950±71
Neutrophil (%)	9.5±0.7	9±0.4	10.5±0.7	9.5±0.6	10±0.4
Lymphocytes (%)	86±0.4	87.5±0.7	86.5±0.7	88±0.4	86±0.4
Eosinophils (%)	4.5±0.7	3.5±0.7	3±0.4	4±0.4	4±0.4
Female mice					
HB (g/dL)	13.75±0.1	12±2	13.6±0.1	13.55±0.35	12.65±0.5
RBC (10^6/µL)	7.85±0.1	7.2±1	8.4±0.1	8.35±0.07	8.15±0.6
MCV (fL)	59±1	54±4	56±4	53.5±0.7	58.5±3.5
MCH (pg)	17±0.1	16.5±0.7	16±0.01	16.5±0.7	15.5±2.1
MCHC (g/dL)	29.5±0.7	31±1	28.5±2.1	34±4.9	28.5±2.1
PCV (%)	46.5±1	39±8	47±2.8	44.5±0.7	43.5±0.7
PLT (10^5/µL)	11.3±0.01	8.15±0.35	11.1±2	9.5±1.8	10.55±2.2
TC (cells/µL)	11,200±919	10,750±778	7950±354	7100±283	5750±5233
Neutrophil (%)	9±1	9±1	9.5±0.7	9 0.5±0.7	8±0.01
Lymphocytes (%)	87.5±0.7	87.5±0.7	87.5±0.7	87±1	88.5±0.7
Eosinophils (%)	3.5±0.7	3.5±0.7	4±0.1	3.5±0.7	3.5±0.7

*Level of significance (P<0.05) when these experimental groups were compared to the normal. All other comparisons among groups were nonsignificant. Values are expressed as mean±SD of five animals per group. The values were analyzed using two-way ANOVA (Tukey's multiple comparison test). RBC: Red blood cell, PCV: Packed cell volume, MCV: Mean corpuscular volume, PLT: Platelet count, TC: Total count, SD: Standard deviation, VC: Vehicle control, LD: Low dose, MD: Medium dose, HD: High dose, HB: Hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, SD: Standard deviation

remain unchanged upon MR treatment. The lipid profiling revealed that MR treatment did not affect the serum lipid profile except a slight increase in the triglyceride level in both males and females of high-dose treated animals. The liver enzymes such as serum glutamate–oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) levels showed statistically significant variations, but the values were within the normal range. There was a slight change in the sodium and potassium levels of MR-treated male mice, but no significant change was observed in female mice.

Organosomatic index

The weight of the vital organs after was also assessed to determine the OSI. The OSI of the organs such as the brain, heart, lungs, liver, kidney, stomach, testis, and ovary did not show any statistically significant variation with respect to that of the normal group animals [Supplementary Table 1].

Heavy metal analysis

As traces of lead (pb) content within the permissible limit were noticed in the drug, the heavy metal lead (Pb) was analyzed in the liver and kidney tissue of male and female Swiss albino mice after repeated drug administration for 28 days. The results were found to be below the detection level, implying no lead accumulation in tissues occurred, and this shows the importance of purification of drug.

Histology

Tissue architecture of all vital and reproductive organs of both the sexes was assessed [Supplementary Figure 5]. The tissue sections of the brain, heart, lung, liver, kidney, stomach, and reproductive organs examined were normal in the control (untreated) group of animals. The VC group of animals observed with slight changes in tissue histology such as brain with focal infiltration, lungs with emphysematous changes, and liver and kidney sections with inflammatory cell infiltration while the MR administered animals observed with normal tissue histology.

Discussion

Rasayana in Ayurveda is a stable and potential form of medicine for the prevention and curation of various diseases. As per the Ayurvedic Pharmacopoeia, the stability and shelf life of a drug can be determined by its moisture content. The loss on drying of MR was very low, which implies its maximum stability and prominent shelf life. Alcohol and water solubility of MR within the specification range suggest the better bioavailability of the drug, whereas total ash and acid-insoluble ash content of MR are slightly more than the specification limit, but can be negligible. The main ingredient in MR, *S. china*, is known to accumulate lead (Pb).[11] Lead is a transition heavy metal which can cause

Table 4: Biochemical parameters from experimental male mice on repeated administration (24-h interval) of *Madhusnuhi Rasayana* over 28 days

Parameters	Normal	VC	LD	MD	HD
Lipid profile (mg/dL)					
CHO	99±2.8	110±11	101.5±10.6	99±0.2	125±0.7
TGL	99±24	102±51	81.5±6.4	72±4.0	172±7†
HDL	37.5±0.7	37.5±3.5	41.5±3.5	39±0.5	43±0.4
LDL	42±2.8	52.5±4.9	44±8.5	46±0.7	55±1.4
VLDL	19.5±4.9	20±9.9	16±1.4	14.5±1	26±0.7
Electrolytes (mEq/L)					
Na	144.5±0.7	141±1	145±0.4	146±0.4	140±0.4†
K	8.6±0.1	7.5±0.4*	8.15±0.4	9.1±0.1$^{\#}$	8±0.1†
Cl	101.5±0.7	102.5±2.1	105.5±0.71	105±0.6	104±0.6
HCO_3	25±1	25±1	26±1.0	25.5±0.7	24±1.0
Renal function (mg/dL)					
Urea	42±0.1	40.5±2	35±1	48±1.4	50±0.7
Creatinine	0.45±0.01	0.46±0.06	0.44±0.03	0.49±0.01	0.49±0.01
Liver function (IU/L)					
SGOT	205±32	240±3.0	152±10	184±7	137±7$^{\#}$
SGPT	62.5±1.7	93±35.0	74.5±16.3	63.4±5.0	59±3.1
ALP	110.5±204.1	170.5±14.7	103.5±4.0	90±2.4	142±4.1††
Bilirubin (g/dL)					
Total	0.35±0.07	0.35±0.07	0.3±0.01	0.3±0.03	0.3±0.01
Direct	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.01
Indirect	0.25±0.07	0.3±0.07	0.2±0.03	0.2±0.03	0.2±0.03
Protein (g/dL)					
Total protein	6.8±0.7	6.25±0.49	6.55±0.35	7.4±0.07	6.4±0.07
Albumin	3.05±0.07	3.2±0.01	3.1±0.01	3.2±0.01	3.1±0.01
Globulin	3.75±0.64	3.05±0.49	3.45±0.35	4.2±0.1	3.3±0.1
A/G ratio	0.82±0.12	1.06±0.17	0.90±0.09	0.73±0.04	0.92±0.02

*Level of significance (P<0.05) when this experimental groups were compared to the normal, $^{\#}$Indicates level of significance (P<0.05) when this experimental groups were compared to VC, †Level of significance (P<0.05) when compared with MD group, ††Level of significance (P<0.01) when compared with MD group. Values expressed as mean±SD of 5 animals per group. The values were analyzed using two-way ANOVA (Tukey's multiple comparison test). All other comparisons among groups were nonsignificant. SD: Standard deviation, SGOT: Serum glutamate–oxaloacetate transaminase, SGPT: Serum glutamate pyruvate transaminase, ALP: Alkaline phosphatase, LDL: Low-density lipoprotein, VLDL: Very LDL, HDL: High-density lipoprotein, TGL: Triglyceride, CHO: Cholesterol, VC: Vehicle control, LD: Low dose, MD: Medium dose, HD: High dose

detrimental effects on humans and other organisms.[12] The heavy metals in MR detected with traces of lead (Pb) which was below the specification range while the cadmium (Cd) analyzed in MR was below the detection level, indicating the absence of heavy metal toxicity. A prominence of flavonoids, steroids, carbohydrates, and alkaloids in MR is anticipated, and their presence is expected to exhibit antioxidant, anti-inflammatory, anticancer, immunomodulatory, antimicrobial, and antitumor properties.[12,13] Carbohydrates have a significant role in rasayana as the various polysaccharides can activate macrophages, imposing immunomodulatory property. *P. longum*, *P. nigrum*, and *N. sativa* used in the preparation of MR are known to contain carbohydrates with the macrophage cytotoxicity activation property against tumor cells, hepatoprotective property by providing adjuvant effect, preventing tumor, and inhibiting hemagglutination.[13]

The LD_{50} of MR observed in the *in vivo* safety analysis of MR on Swiss albino mice is higher than 2000 mg/kg body weight based on the observations for a period of 14 days. The observation assessed in acute toxicity analysis had no abnormality in behavioral characteristics, body weight, and feed and water consumption. Moreover, the necropsy of the experimental mice was normal. As per the OECD guideline (407 and 421), the dosage of 2000 mg/kg body weight of MR was considered the safest dose as it did not cause mortality or morbidity of animals. The repeated administration of MR for 28 days was a continuation study focusing on the adverse effects of MR after the acute toxicity analysis to investigate the safe pharmacological dose of MR on animals. Administration of MR in small doses (low dose: 200 mg/kg body weight, MD: 300 mg/kg body weight, and HD: 400 mg/kg body weight) over a long term provides assistance to predict its safety by evaluating hematological, biochemical, and histopathological parameters.

The variations in blood biochemical parameters indicate drug toxicity.[14,15] Moreover, the repeated administration of MR for 28 days resulted in no significant changes in the hematological parameters. However, there were statistically significant differences in males for MCV and total cell count. A low MCV with a normal MCH may suggest that a microcytic response has occurred in that group due to malabsorption or the stress of drug

Table 5: Biochemical parameters from experimental female mice on repeated administration (24-h interval) of *Madhusnuhi Rasayana* over 28 days

Parameters	Normal	VC	LD	MD	HD
Lipid profile (mg/dL)					
CHO	115.5±9.2	88±31	108.5±10.6	113±7	107.5±3.5
TGL	99±44	54±35	143.5±33.2	96.5±6.4	127.5±16.3
HDL	39.5±3.5	37.5±2.1	38.5±0.7	43.5±0.7	42±2.8
LDL	56.5±14.8	40.5±21.9	44±8.5	50.5±7.8	40.5±3.5
VLDL	19.5±9.2	10±7.1	26±2.8	19.0±1.0	25±2.8
Electrolytes (mEq/L)					
Na	142.5±0.7	145.5±4	143.5±0.7	143.5±0.7	140.5±2.1
K	8.3±0.1	8.6±0.7	7.7±0.3	8.2±0.1	7.95±0.4
Cl	103±1.4	103±0.1	102.5±2.12	103.5±0.7	103.5±2.1
HCO_3	26±1	25±1.0	24.5±0.7	24.5±0.7	26±1.4
Renal function (mg/dL)					
Urea	41.5±9.2	34±4	34.5±13	31.5±2.1	39.5±10.6
Creatinine	0.5±0.01	0.44±0.07	0.48±0.01	0.51±0.01	0.48±0.01
Liver function (IU/L)					
SGOT	193.5±26	230.5±74	160.5±8	298±26.8**	173±18††
SGPT	49.5±5.4	93.5±23*	90.5±5.4	109.5±26**	59.5±9†
ALP	110.5±7.2	120±10.1	150.5±19.7*	127±26.4	117±6.4
Bilirubin (g/dL)					
Total	0.4±0.01	0.3±0.01	0.3±0.01	0.3±0.01	0.3±0.01
Direct	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.01
Indirect	0.3±0.01	0.2±0.01	0.2±0.01	0.2±0.01	0.2±0.01
Protein					
Total protein	6.9±0.1	6.2±1.27	6.2±0.28	6.75±0.2	6.4±0.8
Albumin	3.3±0.14	3.1±0.14	3.2±0.1	3.2±0.14	3.25±0.07
Globulin	3.6±0.01	3.1±1.13	3±0.14	3.55±0.07	3.55±0.92
A/G ratio	0.92±0.04	1.06±0.34	1.07±0.01	0.90±0.02	0.95±0.27

*Level of significance (P<0.05) when these experimental groups were compared to the Normal, **Level of significance (P<0.01) when these experimental groups were compared to the normal, †Level of significance (P<0.05) when compared with MD group, ††Level of significance (P<0.01) when compared with MD group. Values expressed as mean±SD of five animals per group. The values were analyzed using two-way ANOVA (Tukey's multiple comparison test). All other comparisons among groups were nonsignificant. SGOT: Serum glutamate–oxaloacetate transaminase, SGPT: Serum glutamate pyruvate transaminase, ALP: Alkaline phosphatase, LDL: Low-density lipoprotein, VLDL: Very LDL, HDL: High-density lipoprotein, TGL: Triglycerides, CHO: Cholesterol, VC: Vehicle control, LD: Low dose, MD: Medium dose, HD: High dose

administration.[16,17] The serum lipid panel revealed statistically nonsignificant disparities in total cholesterol, high-density lipoprotein, low-density lipoprotein, and very low-density lipoprotein levels between the experimental and control groups for both genders of animals. A slight alteration in triglyceride levels in MR-administered groups in comparison to the control groups was observed but was within the normal range of mice. The serum electrolytes in blood and other body fluids are the minerals which have a crucial role in fluid balance, pH balance, nerve signaling, and muscle function.[18,19] All the electrolytes showed no statistically significant distinctions in the female group of mice. In male groups, sodium and potassium displayed significant variations but were within the normal range of Swiss albino mice. Creatinine found in plasma, serum, and urine serves as a renal function marker. The excretion of creatinine is through glomerular filtration at a constant rate; thus, an increase in serum creatinine level indicates a decline in glomerular filtration efficiency.[20,21]

The renal function is assessed by measuring the concentration of serum urea since creatinine is secreted, whereas urea is reabsorbed. However, the renal function markers of the experimental groups in comparison to the control group displayed no alterations, implying the nontoxic nature of drug.[18,19] Creatinine found in plasma, serum, and urine serves as a renal function marker. The excretion of creatinine is through glomerular filtration at a constant rate; thus, an increase in serum creatinine level indicates a decline in glomerular filtration efficiency. The renal function is assessed by measuring the concentration of serum urea since creatinine is secreted, whereas urea is reabsorbed. However, the renal function markers of the experimental groups in comparison to the control group displayed no alterations, implying the nontoxic nature of drug.[20,21]

SGOT, SGPT, and alkaline phosphatase (ALP) are the liver function markers,[22] and an increase in SGOT and SGPT is the result of leakage of these enzymes from the hepatic cells into the bloodstream due to liver cell damage.[23] The epithelial surface of bile duct possesses ALP and its elevation along with hyperbilirubinemia can be caused due to druginduced liver injury.[24] Although there is a significant change in these enzyme levels upon MR treatment, they were well within the normal

range of Swiss albino mice. The HB catabolism product bilirubin from blood enters into the liver and undergoes chemical changes to convert indirect bilirubin to direct bilirubin which will excrete through bile.[24] The repeated administration of MR for 28 days did not alter bilirubin signifying the safety of MR as a drug.

Investigation of heavy metal presence in the vital organs of animals after MR administration showed no evidence of its presence. The metabolism and excretion functioning respective organs liver and kidney of the experimental mice were analyzed for lead accumulation. There were reports regarding lead poisoning in animals with concentration of lead at 0.35 ppm and 10 ppm in the blood, liver, and kidney, respectively.[25] In the current experiment, liver and kidney of MR administered groups of mice along with VC and normal groups were devoid of the heavy metal lead (Pb) indicates MR is safe for consumption without heavy metal toxicity.

Conclusions

The MR was prepared in batches to ensure consistency in its physicochemical properties. The single dosage of MR at 2000 mg/kg body weight was found to be fairly nontoxic to mice. A subacute toxicity study found that mice do not experience significant side effects from 200 mg/kg body weight, 300 mg/kg body weight, or 400 mg/kg body weight of MR based on histological, biochemical, and hematological criteria. Thus, a dose below 400 mg/kg body weight can be taken as a safe dose for any further pharmacological preclinical studies.

Author contributions

Author RKR: Study conception, data collection, analysis, interpretation, draft writing, and editing. Author AKV: data analysis, interpretation, draft writing, and editing. Author ACR: data collection, analysis, interpretation, and draft editing; JCO: Study conception, interpretation, draft writing, and editing. Author LV: Study conception, design, analysis, interpretation, draft writing, and final editing. All authors approved the final version of the manuscript.

Data sharing policy

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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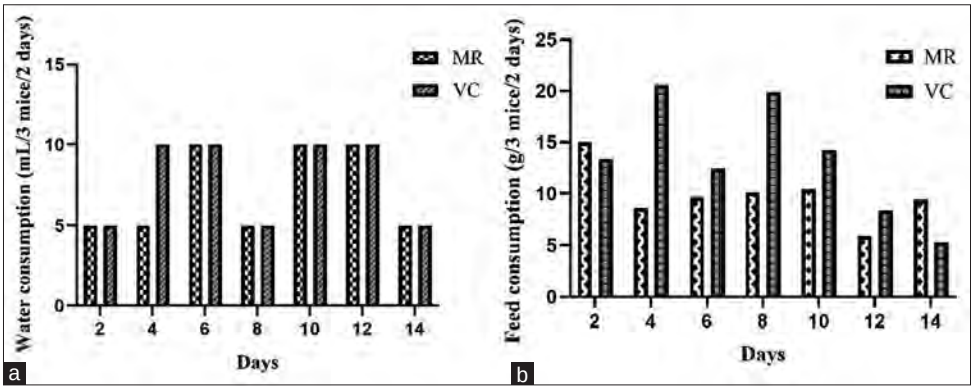
Supplementary Materials

Supplementary file containing Supplementary Table 1 and Supplementary Figures 1-5 linked to the online version of the paper.

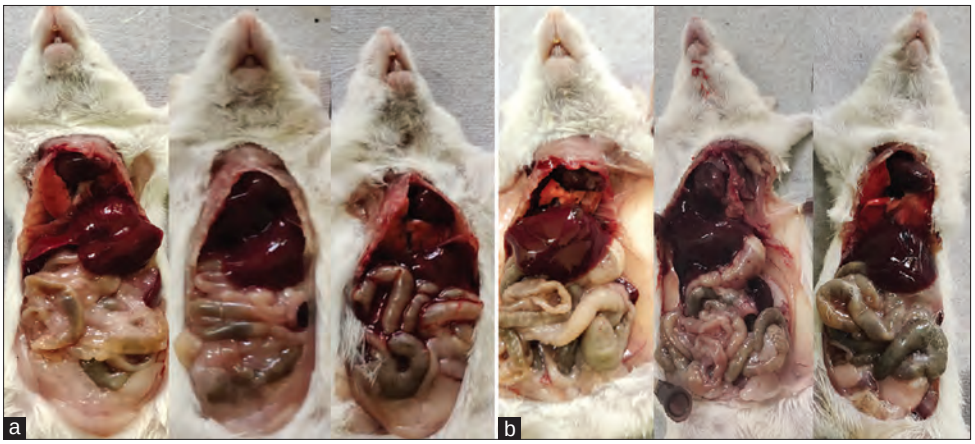
Supplementary Table 1: Organosomatic index of Swiss albino mice following oral subacute administration of *Madhusnuhi Rasayana*

Organs	Normal	VC	LD	MD	HD
Male mice					
Brain	1.10±0.18	1.19±0.11	1.07±0.17	1.29±0.02	1.43±0.35
Heart	0.48±0.05	0.49±0.19	0.47±0.11	0.54±0.05	0.45±0.01
Lungs	0.69±0.17	0.71±0.28	0.62±0.12	0.80±0.18	0.86±0.46
Liver	4.12±0.97	4.25±0.76	4.00±0.35	4.67±0.87	3.77±0.49
Kidney	1.49±0.17	1.49±0.35	1.49±0.22	1.53±0.11	1.45±0.03
Stomach	0.71±0.18	0.65±0.21	0.77±0.21	0.81±0.25	0.98±0.36
Testis	0.65±0.16	0.60±0.13	0.60±0.19	0.60±0.13	0.78±0.32
Female mice					
Brain	1.07±0.17	1.66±0.30	1.44±0.13	1.59±0.22	1.56±0.26
Heart	0.44±0.05	0.52±0.06	0.44±0.08	0.52±0.10	0.45±0.05
Lungs	0.78±0.14	0.85±0.10	0.71±0.15	1.03±0.52	1.09±0.60
Liver	3.16±1.64	4.46±0.38	4.31±0.36	4.52±0.41	4.50±0.20
Kidney	1.11±0.07	1.34±0.20	1.07±0.12	1.18±0.23	1.22±0.04
Stomach	1.02±0.25	0.90±0.52	0.90±0.22	0.97±0.16	0.10±0.01
Ovary	0.09±0.01	0.03±0.02	0.02±0.03	0.02±0.01	0.07±0.01

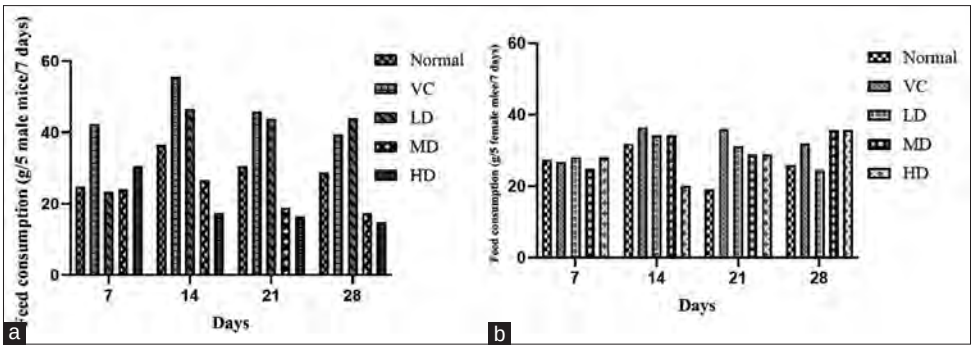
Values expressed as mean±SD of five animals per group. The values were analyzed using two-way ANOVA (Tukey's multiple comparison test). Comparison among the groups was nonsignificant. SD: Standard deviation, VC: Vehicle control, LD: Low dose, MD: Medium dose, HD: High dose



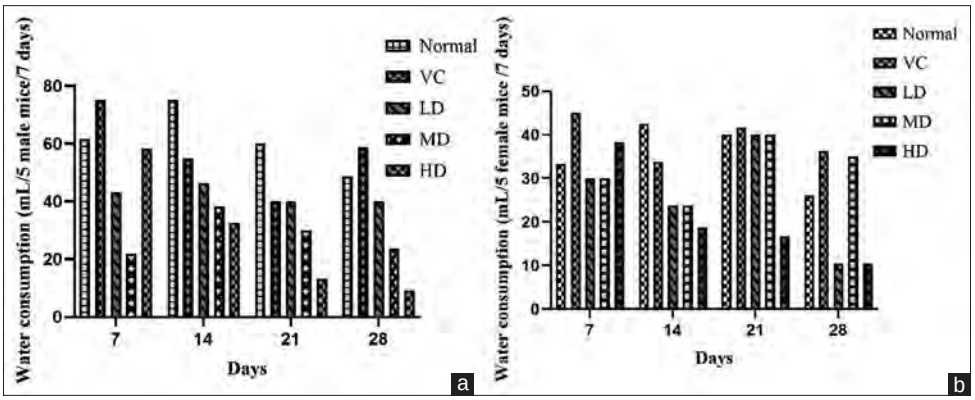
Supplementary Figure 1: The feed and water consumption of Swiss albino mice after the single acute dose administration of MR. (a) The water consumption of mice, (b) The feed consumption of mice. MR: *Madhusnuhi Rasayana*, VC: Vehicle control



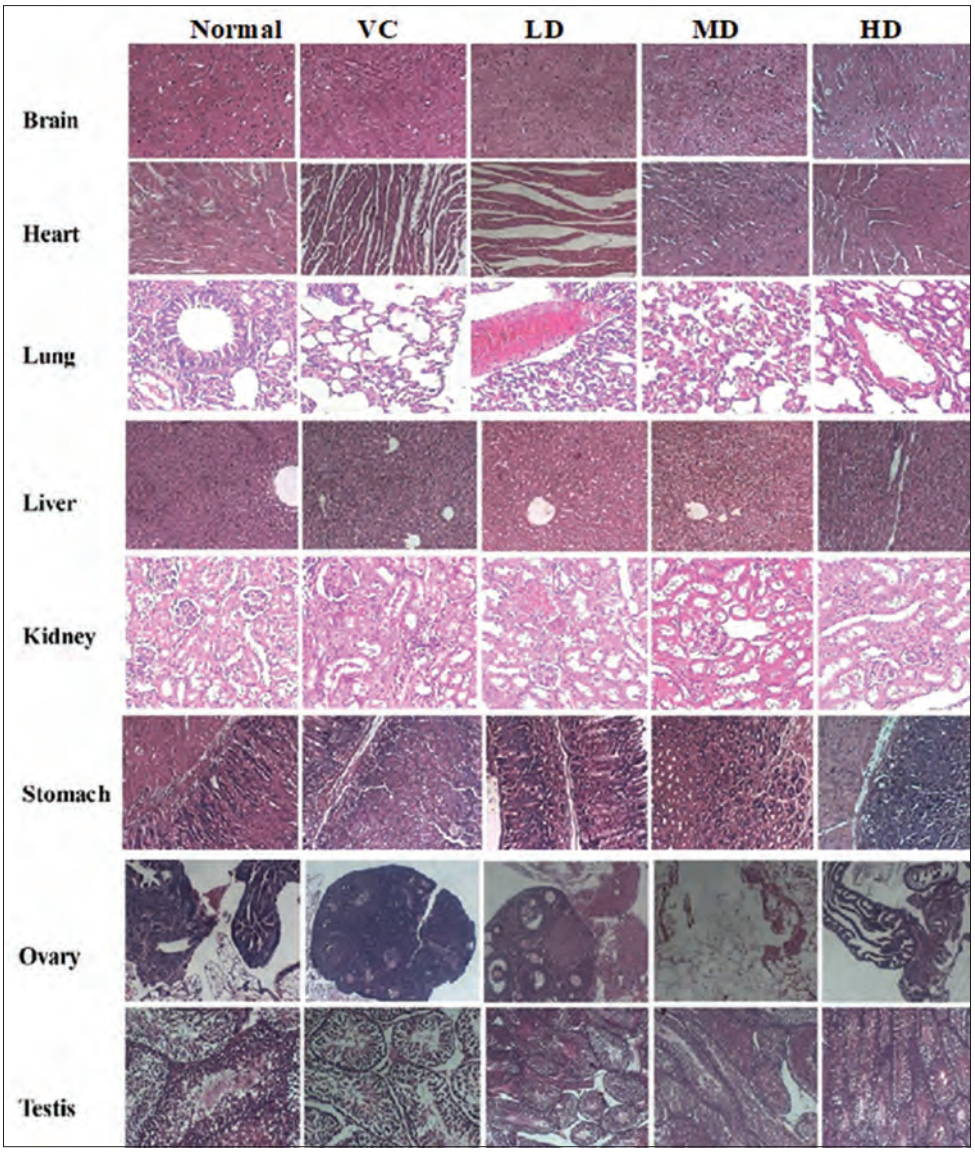
Supplementary Figure 2: The necropsy image of (a) *Madhusnuhi Rasayana* (MR) administered and (b) vehicle control (VC). (a) MR-treated mice, (b) VC-administered mice



Supplementary Figure 3: Feed consumption of (a) male and (b) female Swiss albino mice following repeated administration (24-h interval) of *Madhusnuhi Rasayana* over 28 days. (a) Feed consumption of male mice, (b) Feed consumption of female mice. VC: Vehicle control, LD: Low dose, MD: Medium dose, HD: High dose



Supplementary Figure 4: Water consumption of (a) male and (b) female Swiss albino mice following repeated administration (24-h interval) of *Madhusnuhi Rasayana* over 28 days. (a) Water consumption of male mice, (b) Water consumption of female mice. VC: Vehicle control, LD: Low dose, MD: Medium dose, HD: High dose



Supplementary Figure 5: Histopathological analysis of organs of Swiss albino mice on repeated administration (24-h interval) of *Madhusnuhi Rasayana*. VC: Vehicle control, LD: Low dose, MD: Medium dose, HD: High dose

Case Report

OHVIRA Syndrome in a Preteen: Importance of Wide Septal Resection

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Abstract

Herlyn–Werner–Wunderlich syndrome, another name for obstructed hemivagina and ipsilateral renal anomaly (OHVIRA), is a rare Müllerian anomaly of unclear etiology. This case report describes a case of a 12-year-old girl diagnosed with diadelphic uterus with vaginal septum obstructing one cervix, leading to hematocolpos, hematometra, and hematosalpinx. The patient underwent hysteroscopic septal resection, resulting in unification of the vaginal cavities and resolution of symptoms. This case underscores the importance of early diagnosis and surgical intervention in managing obstructive Müllerian anomalies.

Keywords: Diadelphic uterus, hemivagina, Herlyn–Werner–Wunderlich syndrome, Müllerian anomaly

Introduction

Herlyn–Werner–Wunderlich syndrome, another name for Obstructed Hemivagina and Ipsilateral Renal Anomaly (OHVIRA) syndrome, is a rare congenital disorder that affects the female reproductive and urinary systems. A double uterus, hemivagina, and the lack of a kidney on the same side as the blockage are the three characteristics that define the syndrome. The cause is said to be related to the abnormal development of the Müllerian and Wolffian ducts during fetal development. The syndrome occurs in fewer than 1 in 1,000,000 females.[1] Diagnosis is usually made after menarche. Dysmenorrhea, lower abdomen pain, recurrent infections, pelvic pain with accompanying hematocolpos, and occasionally hematometra are the most frequent clinical manifestations. When a patient has radiological tests such as magnetic resonance imaging (MRI), incidental detection may also take place.

Accurate identification depends on diagnostic imaging. MRI is regarded as the gold standard, even though a pelvic ultrasound (USG) is typically used to scan for abnormalities such as a pelvic tumor or nonexistent kidney. The uterine duplication, the obstructing septum, and the related renal abnormalities may all be clearly seen thanks to the comprehensive imaging of the structures that MRI gives.[2,3] The primary treatment is the surgical resection of the obstructing vaginal septum. This procedure is performed to relieve the obstruction, allow for normal menstrual flow, and preserve the patient's reproductive potential. Delayed intervention may lead to adhesions, chronic pelvic pain, or reduced reproductive potential.[4] This case report describes the details of an adolescent female who initially presented with acute pelvic pain and was finally diagnosed with OHVIRA syndrome. This case highlights the importance of considering Müllerian anomalies in adolescent girls with abdominal symptoms and renal anomalies.

This case report features a patient who was managed with minimally invasive hysteroscopic septal resection, and successfully leading to the complete resolution of symptoms. This report helps to add to the existing literature by directing attention to the importance of early diagnosis, the role of MRI in detecting Müllerian anomalies, and the effectiveness of conservative surgical management in OHVIRA syndrome, thereby helping in preserving fertility.

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Case Report

A 12-year-old girl was presented to the emergency department with complaints of severe abdominal pain. History showed no symptoms of urinary or bowel complaints. She had attained menarche around 8 months ago and had regular cycles since then. She had a similar history in 2 days prior to the presentation, for which she consulted the local hospital and was treated with analgesics. She had no history of any comorbidities or surgeries in the past. General physical examination was unremarkable. The external genitalia were normal. Further examination was performed under anesthesia. USG of the abdomen and pelvis showed an elongated, dilated structure in the midline posterior to the bladder filled with echogenic fluid, and its upper end continuous with the uterus, likely hematocolpos [Figure 1a]. Mild hematometra and hematosalpinx were noted. MRI showed two uterine cornua, two cervices, two vaginal cavities with right-sided hematosalpinx and hematocolpos with lower limit of vaginal obstruction noted approximately 15–20 mm above introitus. The right kidney was not visualized [Figure 1b]. She was posted for hysteroscopic examination and septal resection after obtaining consent and fitness. On examination under anesthesia, a bulge in the anterolateral wall of the right vagina was noted [Figure 2a]. A normal cervix could not be visualized in a per speculum examination. On hysteroscopic examination, the noted bulge was extending high up, and the cervix was visualized at the left side at the apex [Figure 2b]. Proceeded with complete hysteroscopic resection of vaginal septum and evacuation of the accumulated blood. The right cervix was identified, and the two vaginal cavities were successfully unified [Figure 3].[5] She came for a follow-up after 1 month; she had regular, normal cycles. Repeat USG showed no abnormal collection or connections. Hence, no additional interventions were undertaken.

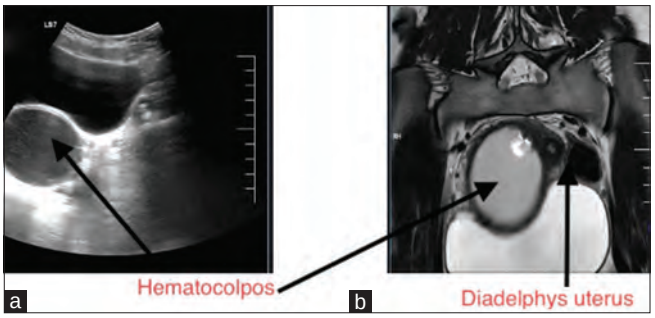

Figure 1: (a) Ultrasound showing hematocolpos and (b) magnetic resonance imaging showing obstruction in lower limit of the vagina

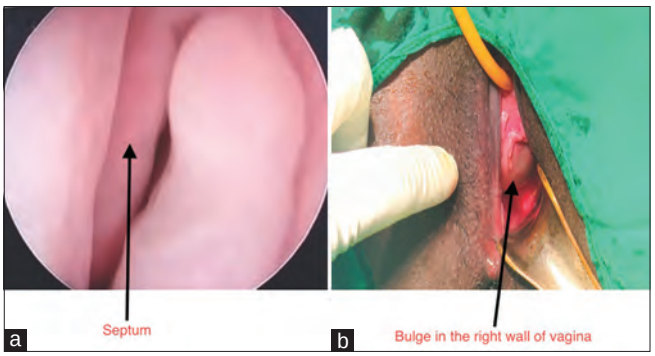

Figure 2: (a) Hysteroscopic view of the visualized bulge in the vagina and (b) per speculum bulge in anterolateral wall of the right vagina

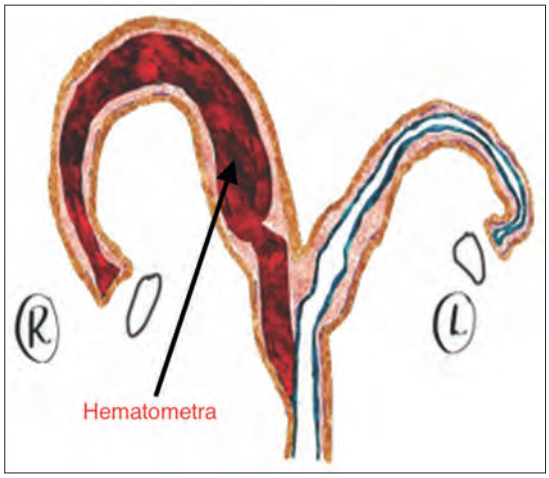

Figure 3: Schematic representation of the findings: two uterine cornua, two cervices, two vaginal cavities with right sides hematosalpinx and hematocolpos

Discussion

OHVIRA syndrome is an uncommon congenital disorder due to anomalies in the development of the Müllerian and Wolffian ducts. It is characterized by a triad of uterine didelphys, a blind hemivagina, and agenesis of the kidney, usually on the same side. This syndrome is commonly seen with pelvic pain, dysmenorrhea, or a palpable mass due to menstrual blood accumulation in the obstructed hemivagina, typically presenting in adolescence.[6,7]

The embryologic origin of OHVIRA is due to changes during the differentiation of the paramesonephric (Müllerian) and mesonephric (Wolffian) ducts. Since renal and reproductive structures originate in close proximity during development, defects in one system may coincide with abnormalities in the other.[8]

Here, a 12-year-old girl presented with uterus didelphys with an obstructing vaginal septum, leading to hematocolpos, hematometra, and hematosalpinx, which is similar to the common presentations described by Hamidi and Haidary[6] and Han *et al*.[7] Many cases are diagnosed soon after menarche due to obstructive symptoms, but few remain undetected until complications arise, like infection, endometriosis, or, rarely, acute abdomen due to rupture or torsion.[9,10]If diagnosis is delayed, it can lead to chronic pelvic pain, impairment of fertility, and also psychological stress. In the case reported by Zivković *et al*.,[4] the obstructed hemivagina remained unrecognized until intrapartum complications developed, pointing to the potential risks of missed or delayed diagnosis.

Imaging is essential for diagnosis. While pelvic USG is a useful first-line tool, particularly in emergency settings,

it may not be enough in detecting complex uterine anomalies. The uterine arrangement and the structure of the vaginal septum can be seen more clearly because to MRI's more precise anatomy.[2,7] Furthermore, if kidney abnormalities, especially unilateral agenesis, are detected by ultrasonography in asymptomatic girls, the clinician should assess the reproductive tract.[11] The vaginal septum is surgically removed to ensure that it doesn't obstruct anything and that future reproductive function can proceed. Early detection and treatment can improve the prognosis, but inadequate or nonexistent therapy might result in long-term issues, including endometriosis or infertility. As a result, early intervention may be regarded as both preventative and therapeutic. This example emphasises to the need of clinical suspicion in adolescent females with nonspecific pelvic problems and documented renal abnormalities.

Conclusion

This case report concludes the necessity of wide septal resection, unlike hymenotomy performed in an imperforate hymen, as inadequate excision may result in re-fusion of the septum and thereby recurrence. A multidisciplinary approach–involving gynecology, urology, radiology, and pediatrics–can facilitate timely diagnosis and reduce complications.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the legal guardian has given her consent for images and other clinical information to be reported in the journal. The guardian understands that names and initials will not be published and due efforts will be made to conceal identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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