The Effect of Gut Microbiota and Serum Metabolites on Glycemic Variability and Prognosis in Patients With Diabetic Nephropathy: a Prospective Cohort Study

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declaration of secrecy

Research Plan Confirmation and Signature Page

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Title: A Prospective Cohort Study on the Effects of Gut Microbiota and Serum Metabolites on Blood Glucose Variability and Prognosis in Patients with Diabetic Nephropathy

I will conscientiously fulfill my duties as a researcher in accordance with the **China GCP** regulations, personally participating in or directly guiding this clinical study. I have carefully read this protocol and agree to fulfill the relevant responsibilities in accordance with Chinese law, the Declaration of Helsinki, **China GCP**, and this study protocol, and will only implement it after obtaining the consent of the ethics committee, unless measures must be taken to protect the safety, rights, and interests of the study subjects.

Clinical research unit: The 8th Affiliated Hospital of Sun Yat-sen University (Shenzhen Futian)

Principal Investigator: Shen Yunfeng

date： **YTD**

I. Summary of the Research Plan

Diabetic kidney disease (DKD), one of the most significant microvascular complications of diabetes, has become the leading cause of end-stage renal disease. Glycemic variability (GV) is considered closely associated with disease progression in these patients. Recent studies indicate that gut microbiota and their serum metabolites play crucial roles in regulating glycemic variability and disease prognosis. However, the mechanisms by which gut microbiota and their metabolites influence glycemic variability and subsequently affect disease outcomes in DKD patients remain unclear. This study aims to address three key questions: ① Characterizing gut microbiota and serum metabolites in DKD patients with varying disease stages and progression levels, while precisely evaluating the prognostic value of gut microbial markers for adverse outcomes. ② Investigating the relationship between gut microbiota, serum metabolites, and glycemic variability in DKD patients. ③ Determining whether gut microbiota and serum metabolites interfere with glycemic variability to influence disease prognosis. To address these questions, this observational prospective cohort study will enroll 270 patients with diabetic kidney disease. By integrating continuous glucose monitoring (CGM), 16S rRNA high-throughput sequencing, and serum non-targeted liquid chromatography-mass spectrometry (LC-MS), this study evaluated glycemic variability, analyzed gut microbiota and serum metabolites, and constructed and validated predictive models for poor prognosis in diabetic kidney disease (DKD) patients. The findings revealed correlations between gut microbiota, serum metabolites, and glycemic variability, elucidated their prognostic roles in DKD, and provided early identification tools for high-risk DKD patients. We aim to offer novel perspectives and strategies for DKD prognosis management, thereby improving clinical outcomes and quality of life for patients.

2. Preface

2.1 Research Background and Project Justification

2.1.1 Epidemiology and Characteristics of Diabetic Nephropathy

The latest Global Diabetes Atlas, released by the International Diabetes Federation (IDF) in its 10th edition, reveals that approximately 537 million adults worldwide suffered from diabetes mellitus(DM) in 2021. Projections indicate this number will reach 643 million by 2030 and rise to 783 million by 2045 [1]. Diabetic Kidney Disease(DKD), one of the most significant microvascular complications of diabetes, involves chronic kidney damage caused by hyperglycemia. The condition affects the glomeruli, renal tubules, interstitial tissue, and blood vessels, clinically manifesting as persistent albuminuria and/or progressive decline in estimated glomerular filtration rate(eGFR), which may eventually progress to end-stage renal disease(ESRD) [2]. Globally, DKD has become the leading cause of ESRD [3]. The pathophysiological mechanisms of DKD remain complex, with current understanding still requiring further investigation.

The occurrence and development of the disease are related to metabolic disorders, hemodynamic changes and their interaction.

In recent years, an increasing number of studies have shown that DKD is also an inflammatory reactive disease, in which inflammatory pathways play an important role and may be a key factor leading to the progressive damage of organs. In a hyperglycemic environment, inflammatory cell signaling pathways should be activated, mainly including P38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), etc., which in turn activate the NF-κB-related signaling pathway. After activation, NF-κB stimulates the transcription of chemokines, adhesion factors, and pro-inflammatory cytokines, leading to inflammatory cell infiltration in renal tissue and promoting the occurrence and development of DKD [4]. In China, DKD has become the primary cause of end-stage renal disease in middle-aged and elderly patients [5]. Therefore, how to prioritize the prevention, identification, and management of DKD is particularly important. Although renal biopsy is the gold standard for diagnosing DKD, it has not been widely adopted due to its invasive and traumatic nature. Current diagnostic criteria for diabetic kidney disease(DKD) primarily rely on clinical manifestations, including the urine protein-to-creatinine ratio, estimated glomerular filtration rate(eGFR), serum creatinine levels, renal ultrasound findings, and retinopathy presence [6]. However, these indicators demonstrate limited diagnostic sensitivity and specificity for DKD, and fail to effectively assess the severity of renal impairment [7]. **Identifying novel biomarkers to identify high-risk populations with progressive DKD for early intervention could improve patient outcomes, establish dynamic and precise disease progression prediction models, and ultimately enable personalized treatment strategies**.

2.1.2 Variability of Blood Glucose and Diabetic Nephropathy

Glucose variability(GV) typically refers to fluctuations in blood glucose levels or parameters related to glucose homeostasis measured over specific time intervals, including both long-term and short-term glucose fluctuations [8]. Compared to persistent hyperglycemia, high GV can lead to more severe oxidative stress [9]. While chronic hyperglycemia has traditionally been considered the primary risk factor for diabetic complications, studies have also suggested that frequent or wide-ranging glucose fluctuations may serve as independent triggers for such complications. In addition to glycated hemoglobin(HbA1c), GV could be another independent risk factor for diabetic complications [10].

HbA1c has served as the gold standard for blood glucose control for nearly three decades. However, both clinical practice and scientific research have gradually revealed certain limitations of HbA1c. Studies indicate that approximately 20% of diabetic patients' HbA1c values may not accurately reflect their average blood glucose levels [11]. Additionally, as a long-term glucose control indicator, HbA1c fails to capture short-term fluctuations in blood sugar. Since HbA1c is directly related to red blood cell lifespan, any factors affecting cell renewal can impact the accuracy of HbA1 c measurement, which is unrelated to blood glucose levels [12]. In diabetic kidney disease (DKD), as

The decline in eGFR, shortened red blood cell lifespan, and potential decrease in glycated hemoglobin(HbA1c) levels occur independently of blood glucose fluctuations [13]. Ford et al. [14] demonstrated through Deming regression analysis that the correlation between HbA1c and fasting glucose intensifies with renal dysfunction. Hypoglycemia is prevalent among dialysis patients with diabetic kidney disease(DKD) [15], affecting 23.8% to 47.6% of cases, primarily due to reduced gluconeogenesis in residual renal tissue, impaired insulin clearance, and glucose diffusion into red blood cells during hemodialysis [16]. Conversely, hemodialysis and its complications induce glucose metabolism disorders, alter insulin secretion and degradation patterns, and modify drug metabolism, collectively contributing to dialysis-associated hyperglycemia [17].

Reducing the incidence of hypoglycemia and minimizing blood glucose fluctuations are crucial for improving the prognosis of diabetic kidney disease (DKD) patients. Timely and accurate blood glucose monitoring is essential for maintaining good glycemic control. However, self-monitoring of blood glucose (SMBG) faces limitations such as poor adherence and constraints from environmental and time factors. Continuous glucose monitoring systems (CGMS) provide diabetic patients with multiple reliable glucose assessment metrics. These include time in range (TIR) and its derivatives time above range (TAR) and time below range (TBR) for evaluating target-range compliance, daily glucose fluctuations measured by standard deviation (SD), coefficient of variability (CV), and mean amplitude of glycemic excursions (MAGE), as well as daily mean absolute differences (MODD) to monitor intra-day variability. Additionally, the system assesses hyperglycemic index (HBGI) and hypoglycemic index (LBGI) to identify risks of hyperglycemia and hypoglycemia [18]. In 2019, the Advanced Technology and Therapy for Diabetes Committee (ATTD) emphasized in the "International Expert Consensus on Clinical Application of Continuous Glucose Monitoring" that the core monitoring indicator TIR combined with different blood glucose monitoring indicators can serve as a baseline for evaluating glycemic control. In the 2020 China guidelines for the prevention and treatment of type 2 diabetes, TIR was also included in the control targets [19]. Beck et al. [20] used SMBG data from patients in the Diabetes Control and Complications Trial (DCCT) to calculate TIR and found that TIR was independently associated with the progression of diabetic retinopathy (DR) and proteinuria, suggesting that TIR could serve as an effective end-point indicator in clinical trials. Lu et al. evaluated the relationship between HbA 1C estimated from CGM mean glucose and TIR in 2,893 patients with type 2 diabetes, observing a good linear relationship between the two($r=-0.91$, $P<0.001$). This parameter is more reliable than HbA1c data and can be applied to patients with advanced chronic kidney disease (CKD), including those undergoing dialysis. **Currently, few studies have analyzed the relationship between CGM-derived indicators and the risk of adverse renal outcomes in patients with diabetic kidney disease (DKD).**

2.1.3 Metabolism of Intestinal Bacteria and Diabetic Nephropathy

In recent years, there has been growing attention to the role of gut microbiota composition and function in regulating human metabolic processes. The intestinal microbial community participates in key metabolic processes such as food digestion, energy absorption, and glucose metabolism. Disruption of gut microbiota is associated with the occurrence and progression of various common metabolic diseases, including type 1 diabetes mellitus (T1DM) [22], type 2 diabetes mellitus (T2DM) [23], diabetic kidney disease (DKD) [24], and chronic kidney disease (CKD)/end-stage renal disease (ESKD) [25]. In patients with DKD, the gut microbiota typically shows increased pathogenic bacteria and reduced beneficial bacteria [26]. Compared with healthy individuals and diabetic patients without kidney disease (DM), patients with diabetic kidney disease (DKD) exhibit reduced Firmicutes and Prevotella_9, along with increased Proteobacteria and Escherichia-Shigella. Notably, Prevotella_9 and Escherichia-Shigella serve as effective biomarkers to distinguish diabetic nephropathy (DN) from DM [27]. A meta-analysis incorporating 15 studies involving 1,640 participants from DKD, DM, non-DKD, and healthy populations revealed characteristic gut microbiota alterations in DKD patients, including increased Hungatella and Escherichia species alongside decreased butyrate-producing bacteria [28]. These findings demonstrate that DKD induces distinctive changes in the gut microbiota profile.

The imbalance of gut microbiota in diabetic kidney disease (DKD) is significantly associated with clinical indicators such as lipid metabolism, glucose metabolism, and renal function. Gut microbiota may serve as a predictive factor for the onset and progression of DKD, potentially playing a crucial role in its pathogenesis. This includes abnormal gut microbiota metabolites, damage to the intestinal mucosal barrier, increased inflammation, activation of the immune system, and activation of the renin-angiotensin system (RAS) [29]. Gut microbiota-derived uremic toxins can induce the secretion of cytokines and chemokines, increase white blood cell recruitment, promote oxidative stress and inflammation, leading to glomerular and tubular damage and fibrosis, thereby accelerating the development and progression of DKD [30]. Short-chain fatty acids (SCFAs), the final metabolites produced by gut microbiota fermentation of dietary polysaccharides, also play significant roles in multiple aspects of renal physiology. These include regulating hormone secretion and metabolism, lowering blood pressure, participating in immune modulation, reducing inflammation and fibrosis, protecting the intestinal epithelial barrier, and inhibiting endotoxemia [31]. Bile acids are synthesized in the liver and metabolized by gut microbiota into secondary bile acids. These secondary bile acids antagonize Farnesoid X receptors (FXR) and Takeda G protein-coupled receptors 5 (TGR5) in the gut, which are involved in diabetic kidney disease (DKD) [32]. Regarding the intestinal mucosal barrier and inflammation, dysbiosis of gut microbiota leads to an increase in Gram-negative (G-) bacteria. Lipopolysaccharide (LPS), the primary structural component of G-bacteria's outer membrane, can disrupt the intestinal mucosal barrier and leak into the bloodstream. This results in elevated serum LPS levels, promotes secretion of inflammatory cytokines, and causes persistent low-level chronic inflammation along with metabolic endotoxemia. Inflammation plays a critical role in the progression of DKD. **Epidemiological and**

Mechanistic studies have provided strong evidence linking gut microbiota alterations to the development of diabetic kidney disease (DKD). However, the specific gut microbiota and metabolite profiles in patients with DKD at different stages and disease progression levels remain to be elucidated. Notably, the gut microbiota and its metabolites hold significant diagnostic potential for DKD, offering novel perspectives for early disease identification through biomarker applications. Therefore, utilizing these microbial components as biomarkers not only aids in early disease detection but also provides a theoretical foundation for developing innovative therapeutic strategies and intervention approaches.

2.1.4 Hypothesis and Significance of the Research

To date, no studies have explored the impact of gut microbiota on the prognosis of diabetic kidney disease (DKD) patients with varying degrees of glycemic variability. Based on existing literature, we propose the following hypotheses: 1) Glycemic variability and specific gut microbiota serum metabolites serve as predictors of adverse cardiovascular events in DKD patients; 2) The gut microbiota and serum metabolites of DKD patients are associated with glycemic variability (GV), which may influence patient outcomes through gut microbiota metabolism. Our proposed methodology includes: 1) Assessing glycemic variability in DKD patients via continuous glucose monitoring (CGM) to investigate its relationship with prognosis and determine optimal glycemic variability control targets. 2) Utilizing 16S rRNA high-throughput sequencing and serum non-targeted liquid chromatography-mass spectrometry (LC-MS) to analyze the relationship between gut microbiota, metabolites, inflammatory factors, and DKD patient prognosis. This study aims to optimize clinical management strategies, establish risk prediction models, promote interdisciplinary research, and improve patient quality of life. The findings will provide new scientific evidence and tools for precision treatment and prognosis management in DKD patients, demonstrating significant scientific and clinical value.

2.2 Benefit / Risk Assessment of the Study

2.2.1. Benefits received

① **Optimization of glycemic management**: By evaluating glycemic variability with continuous glucose monitoring (CGM), subjects can obtain more accurate glycemic control data, which helps optimize glycemic management, reduce glycemic fluctuations, and reduce the risk of diabetes complications.

② **Personalized treatment:** The findings may reveal the relationship between specific gut bacterial metabolites and prognosis, and provide personalized treatment advice for subjects, such as diet adjustment, probiotics or prebiotics.

③ **Early warning:** If studies find that specific gut bacterial metabolic biomarkers are associated with poor prognosis, subjects could be intervened earlier to prevent cardiovascular events and progression of nephropathy.

2.2.2 Risk profile

2.2.2.1 Known Adverse Effects:

① **Wear continuous glucose monitoring (CGM):** Wearing a CGM device may cause mild stinging or itching of the skin.

② **Blood sample collection:** There may be mild pain during blood collection, and there may be mild bruising or swelling at the site of blood collection.

2.2.2.2 Potential risks:

① **Infection risk:** Any invasive procedure (CGM wearing and blood collection) has a minimal risk of infection. The research team will strictly follow aseptic procedures to minimize this risk.

② **Privacy risk:** The study requires the provision of personal health information, and there is a risk of privacy leakage. The research team will take strict confidentiality measures to ensure the security of the personal information of the subjects.

2.2.3 comprehensive evaluation

The benefits outweigh the risks: While there may be some minor discomfort and potential risks involved, participants will receive more precise blood sugar management advice and personalized treatment plans through their research participation, while contributing to public health. The research team will take all necessary measures to ensure participant safety and privacy, guaranteeing that the benefits outweigh the risks.

III. RESEARCH OBJECTIVES

3.1 Main objective: To explore the relationship between glycemic variability, gut microbiota metabolites and prognosis of patients with DKD.

（1） **Study subjects:** patients with diabetic nephropathy.

（2） **Exposed factors:** glycemic variability (assessed by continuous glucose monitoring CGM); gut microbiota, serum metabolites (assessed by 16S rRNA high-throughput sequencing technology and serum non-targeted liquid chromatography-mass spectrometry LC-MS) and inflammatory factors.

（3） Study endpoint:

① **Primary end point:** progression of DKD. Progression of DKD was defined as renal failure (requiring transplantation or dialysis, or persistent eGFR <15 ml/min/1.73 m2) or a persistent decline of 30% in eGFR from baseline.

② **Secondary End Points:** Ⅰ. Cardiogenic Death: Death caused by cardiac disease or related complications. Ⅱ. Major Adverse Cardiovascular Events (MACE): Including myocardial infarction, stroke, unstable angina pectoris, and other significant cardiovascular events. Ⅲ. Kidney Transplantation: Successful cases of kidney transplantation surgery. Ⅳ. Hypoglycemic Events: Documentation of the frequency and severity of hypoglycemia occurrences.

（4） **Specific objectives:** I. Evaluate the relationship between GV and prognosis in patients with DKD and determine the optimal GV control target. II. Screen for biomarkers of gut microbiota, metabolites, and inflammatory factors associated with DKD prognosis.

3.2 Secondary Purpose

3.2.1 Construct a Predictive Model to Predict the Risk of Adverse Prognosis in Patients With DKD.

（1） Study subjects: patients with diabetic nephropathy.

（2） Exposure factors: glycemic variability index, clinical indicators, intestinal flora, serum metabolites, inflammatory factors.

（3） study OUTCOMES:

① Primary end point: death from any cause, including death from all causes.

② Secondary end point: Unintentional major cardiovascular events (MACE): major cardiovascular events including myocardial infarction, stroke, and unstable angina pectoris.

（4） Specific objectives: Construct a prediction model by incorporating the above indicators to predict the prognosis of DKD patients.

3.2.2 Characteristics of Gut Microbiota and Serum Metabolites in Different GV Groups and Their Correlation With Prognosis

（1） Study subjects: patients with diabetic nephropathy

（2） Exposure factors: different glycemic variability (GV) groups.

（3） Study outcome: characteristics of intestinal microbiota and serum metabolites.

（4） Specific objectives: Through 16S rRNA high-throughput sequencing technology and serum non-target liquid chromatography -Mass Spectrometry (LC-MS) was used to investigate the characteristics of intestinal microflora and serum metabolites in patients with different glycemic variability (GV) groups. The relationship between the bacterial co-abundance groups (CAG) and the functional modules of serum metabolites was further established to explore their correlations with glycemic variability and poor prognosis.

4. Research Content and Innovation

4.1. Research Content

This prospective observational cohort study was designed to investigate the relationship between gut microbiota, serum metabolites, and glycemic variability (GV) and their effect on the prognosis of patients with diabetic nephropathy (DKD).

4.1.1 Assessing the impact of glycemic variability on prognosis in patients with diabetic kidney disease

（1） in prospective cohort studies, patients with DKD were enrolled, and their blood glucose was monitored with a continuous glucose monitor for 14 days, followed by longitudinal follow @-@ up to record adverse prognostic events.

（2） The relationship between glycemic variability and prognosis was analyzed, and the optimal TIR control target for DKD patients was discussed.

4.1.2 Characteristics of gut microbiota and serum metabolites in patients with DKD and their correlation with prognosis

（1） The fecal and blood samples were collected and analyzed by 16S rRNA sequencing, non-targeted metabolites and inflammatory factors.

（2） The biomarkers associated with poor prognosis were screened.

4.1.3. Gut Microbiota and Serum Metabolites in DKD Patients with Different Blood Glucose Variability Groups and Their Correlation with Prognosis

（1） Patients with DKD were divided into high GV group and low GV group according to GV status, and the intestinal microbiota characteristics and serum metabolites characteristics of patients in different GV groups were analyzed.

（2） Based on the association between gut microbiota, serum metabolic biomarkers and prognosis, the relationship between the bacterial co-abundance group (CAG) and the functional modules of serum metabolites was further established to explore their correlation with glycemic variability and poor prognosis.

4.1.4 Construction and validation of adverse event prediction model for DKD patients

（1） Based on the screening of the predictive factors related to adverse events in DKD prognosis, including glycemic variability index, related clinical indicators, intestinal flora, serum metabolites, inflammatory factors, etc., the predictive model was established by using machine learning algorithm, and the performance and stability of the model were evaluated.

（2） independent cohorts of patients with DKD were added as validation cohorts to evaluate the performance and stability of the predictive model.

4.2 Innovativeness

（1） Multidimensional assessment: On one hand, continuous glucose monitoring (CGM) technology is employed to dynamically track glycemic variability in diabetic kidney disease (DKD) patients, capturing detailed fluctuations in blood glucose levels. On the other hand, integrated analysis combines 16S rRNA high-throughput sequencing with serum non-targeted LC-MS to comprehensively evaluate gut microbiota composition and serum metabolite profiles, thereby elucidating their correlations with glycemic variability and disease prognosis.

（2） DESIGN OF PROSPECTIVE COHORT STUDY: A prospective cohort study was designed to enroll patients with different clinical stages of DKD and follow up for 3 years. Adverse prognostic events during follow-up were recorded in detail to ensure the continuity and integrity of the collected data, which provided a strong guarantee for the accuracy and reliability of the study results.

（3） multilevel data integration: the multilevel data, including glycemic variability, clinical indicators, gut microbiota, serum metabolites and inflammatory factors, were integrated to construct a predictive model for DKD prediction.

The risk of adverse prognostic events provides a more comprehensive and accurate tool for clinical risk assessment.

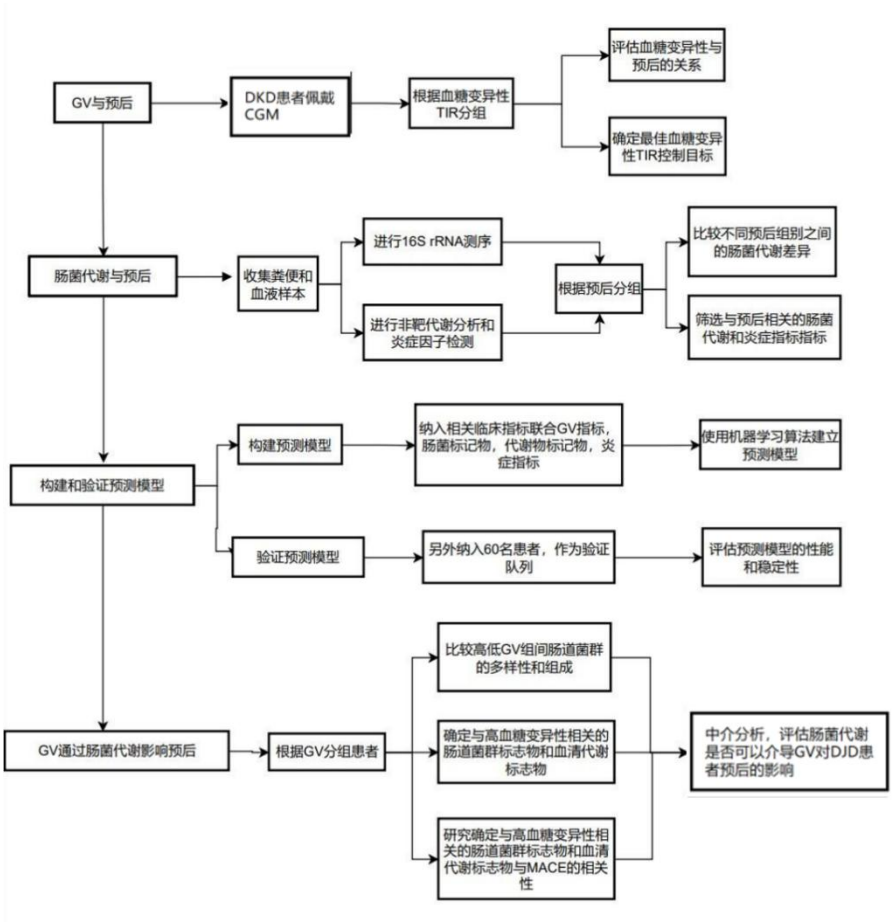
5. Research Design and Technical Route

5.1 General Research Design

this study was a single @-@ center, observational study of patients with type 2 diabetes mellitus and chronic kidney disease, aged 18 to 56 years.

sex, prospective cohort study.

5.2 Technical Roadmap



Note: DKD: Diabetic Kidney Disease; CGM: Continuous Glucose Monitoring; TIR: Time in Range; GV: Glucose Variability.

5.3 Study Site and Study Population

5.3.1 Study site: From July 1, 2025 to December 31,2025, the subjects were recruited in the inpatient department of endocrinology department of the Eighth Affiliated Hospital of Sun Yat-sen University according to the inclusion and exclusion criteria.

5.3.2 Study population: patients with type 2 diabetes and chronic kidney disease (DKD) (n=270)

1) Inclusion Criteria:

① Age 18-65 years;

② The diagnosis of DKD meets the relevant criteria in the "China Diabetes Nephropathy Prevention and Treatment Guidelines (2021 Edition)";

③ Type 2 diabetes mellitus, treated with insulin analogues or oral antidiabetic drugs, diagnosed according to the 1999 WHO criteria;

④ no changes in the antidiabetic regimen were made for at least 3 months before enrollment.

⑤ informed consent was obtained voluntarily.

2) Exclusion criteria:

① The patients with diabetes mellitus complicated with ketoacidosis, primary glomerulonephritis, nephrotic syndrome, lupus nephritis and other primary or secondary kidney diseases.

② The body was in a state of stress such as infection and trauma.

③ women who are pregnant, breastfeeding, or planning to become pregnant next month;

④ severe skin lesions, such as extensive eczema, extensive scarring, extensive tattoos, papular dermatitis, severe edema and psoriasis;

⑤ Sensory surface of the sensor is scarred due to severe skin burns, scalds, sunburns, trauma, ulcers, surgery, etc.

⑥ coagulation dysfunction, anemia or abnormal hematocrit;

⑦ Patients with chronic gastrointestinal diseases or a history of biliary or gastrointestinal surgery were excluded from the study.

⑧ consumed probiotics, prebiotics, or received antibiotic treatment for more than 3 days in the past 3 months; consumed yogurt or yogurt-containing foods or beverages in the past week.

3) Exit criteria:

researcher decided withdrawal

① those who had adverse reactions or serious adverse events or other complications and special physiological changes, and who should be stopped from the study and taken emergency treatment measures such as medication, hospitalization or surgery according to the judgment of the investigator;

② Women became pregnant during the study;

③ Serious violation of the study protocol, such as enrollment in the study despite not meeting the inclusion criteria. Participants may withdraw voluntarily.

① provisional withdrawal of informed consent;

② Participants cannot tolerate certain tests;

③ unforeseen events occurred during the study that made it impossible for the participant to continue the study.

5.4 Methods for Determining the Required Sample Size for Research

This exploratory study investigates the impact of gut microbiota and related metabolites on prognosis in diabetic kidney disease (DKD) patients. To achieve this, patients will be divided into two groups based on adverse prognosis outcomes, with subsequent comparisons of gut microbiota and metabolite differences between groups. To minimize microbial variations caused by individual differences, each group requires at least 30 participants, meaning there must be at least 30 patients with adverse prognosis events. Drawing from existing research, a 2023 study published in *Diabetes Care* [Diabetes Care.2023;46(4):868-873.] included 975 DKD patients with an average follow-up of 4.7 years, reporting 156 major outcomes of DKD progression. Consequently, the total sample size was calculated as 975/156*30=187.5. Considering potential dropouts and incomplete responses, we typically increase the sample size by 10%, ultimately enrolling 210 patients as the modeling cohort. Additionally, 60 DKD patients were included as a validation cohort. During actual recruitment, sample size adjustments may be made based on factors such as recruitment speed and data quality. Regular assessments of sample adequacy should also be conducted to ensure statistical power.

5.5 Investigation Content (CRF Form)

（1） Collect Baseline Information

1） The data collected included demographic information such as age, sex, and education level; personal history such as smoking and drinking; medical history such as the presence of other diseases such as hypertension; medication history such as antidiabetic and antihypertensive drugs; and gastrointestinal status and dietary habits.

2） Physical examination: General condition, height, weight, blood pressure (BP) including systolic (SBP) and diastolic (DBP), waist circumference (WC), hip circumference (HC), waist-to-hip ratio (WHR), etc.

3） The test items included complete blood count, liver and kidney function, HbA1c, TC, TG, HDL-c, LDL-c, FPG, fasting insulin (FIns), and serum uric acid (SUA).

（2） Wear a Continuous Glucose Monitor and Collect Samples

All enrolled subjects were required to wear a continuous glucose monitor (CGM) for 14 days during their first 24-hour hospital stay. Endocrinologists provided personalized dietary guidance based on blood glucose levels, with standardized glucose-lowering regimens. Significant deviations from baseline glucose levels warranted necessary dosage adjustments. Blood glucose variability metrics were collected after 14 days. Additionally, stool and blood samples from all participants were collected and preserved for subsequent analysis.

（3） Follow-up in the Queue:

1） follow-up time: 3 years after discharge.

2） Follow-up methods: outpatient follow-up, telephone follow-up and review of relevant medical records.

3） Outcome measures:

① **Primary end point:** progression of DKD. Progression of DKD was defined as renal failure (requiring transplantation or dialysis, or persistent eGFR <15 ml/min/1.73 m2) or a persistent decline of 30% in eGFR from baseline.

② **Secondary End Points:** I. Cardiogenic Death: Death caused by cardiac disease or related complications. II. Major Adverse Cardiovascular Events (MACE): Including major cardiovascular events such as myocardial infarction, stroke, and unstable angina. III. Kidney Transplantation: Successful cases of kidney transplantation surgery. IV. Hypoglycemic Events: Record the frequency and severity of hypoglycemia occurrences.

（4） 16S RNA Sequencing Analysis and Non-Target Metabolic Analysis

We performed 16S rRNA sequencing, non-target metabolic analysis, and inflammatory factor detection on collected fecal and blood samples to compare differences in gut microbiota, metabolites, and inflammatory factors across different prognostic groups, and to identify prognostic markers. We also compared the differences in gut microbiota, metabolites, and inflammatory factors among patients with high glycemic variability, intermediate glycemic variability, and low glycemic variability. Furthermore, we identified gut microbiota markers and serum metabolic markers associated with high glycemic variability.

（5） Construction and Evaluation of Forecasting Model

First, feature screening was performed using LASSO regression to select variables from five aspects: demographic characteristics, baseline scales, gut microbiota and non-target metabolites, and peripheral blood inflammatory biomarkers. Nine machine learning algorithms were employed to construct classification models, with performance evaluated through resampling training/verification mechanisms. The optimal model was selected via 10-fold cross-validation and performance metrics. Model performance and applicability were assessed using AUROC curves, calibration curves, Brier scores, and decision analysis plots. During training, validation, and testing phases of the optimal model, five-fold cross-validation and learning curves were used to evaluate model fit and stability. Finally, SHAP package was utilized to generate SHAP explanation plots to determine the importance and contribution of individual features.

5.6 Experimental Scheme

Step	Period	*Filter period*	*Period of wearing CGM*			*Follow-up period*		
	Study follow-up	Visit 0	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6
	Study date (days)	1 day before enrollment	1 day after enrollment	7 days after enrollment	14 days after enrollment	Within 1 year after enrollment	Within 2 years after enrollment	Within 3 years after enrollment

1	Selection and exclusion criteria	✓						
2	Sign the informed consent form.	✓						
3	Adorn CGM		✓					
4 clinical data	Basic information of the subjects		✓					
	Clinical diagnosis		✓					
	Personal history		✓					
	History of past illness		✓					
	Check-up		✓					
5 clinical examination Inspection	Routine blood test			✓				
	Liver function			✓				
	Renal function			✓				
	HbA1c			✓				
	Blood fat			✓				
	Fasting insulin (FIns)			✓				
	Blood sugar			✓				
	Thyroid function			✓				
	Coagulation function			✓				
6	Medication during CGM monitoring				✓			
7	Fecal collection		✓					
8	Blood collection		✓					
9	Medications taken after discharge				✓			

10	Download the CGM report				✓			
11	Fecal microbiota sequencing					✓		
12	Metabolite assay in serum					✓		
13	Progression events of DKD during follow-up					✓	✓	✓
14	Assessment of MACE events during follow-up					✓	✓	✓
15	Death events during follow-up		✓	✓	✓	✓	✓	✓
16	Adverse event monitoring		✓	✓	✓			
17	Deviation from plan record		✓	✓	✓	✓	✓	✓
18	Fill in CRF		✓	✓	✓	✓	✓	✓

5.7 Collection, Storage, Use, and Destruction of Research Data and / or Biological Samples

5.7.1 STUDY DATA

Data Collection: This study will collect participants' age, gender, education level, medical history, medication history, and physical measurements including height, weight, blood pressure (BP) (systolic [SBP] and diastolic [DBP]), waist circumference (WC), hip circumference (HC), and waist-to-hip ratio (WHR) through face-to-face interviews, physical examinations, and continuous glucose monitoring (CGM) tests. The CGM will also collect real-time blood glucose data.

Storage: The above information is stored in the medical information platform of our hospital.

Use: All data collected in this study are used for clinical research data analysis and will not be used for commercial purposes.

Destruction: The study will be destroyed in accordance with the regulations 5 years after the study is completed.

5.7.2 Biological Specimen

DATA COLLECTION: Blood and stool samples were collected from the participants and analyzed for complete blood count, liver function, kidney function, glycated hemoglobin, blood lipids, fasting insulin, thyroid function, coagulation function, and gut microbiota.

Storage: All the specimens were stored in the biological sample bank of our hospital.

Use: All data collected in this study are used for clinical research data analysis and will not be used for commercial purposes

road 。

Destruction: The study will be destroyed in accordance with the regulations 5 years after the study is completed.

5.8 Data Management and Statistical Analysis Plan:

5.8.1 Data Management

5.8.1.1 Data Collection Methods and Procedures:

（1） Data collection tools: Electronic Case Report Form (CRF)

（2） The baseline data were collected during the hospitalization of the patients, and the follow-up data were collected at 1, 2 and 3 years.

（3） Collection: Researcher

（4） Data quality control: Training before data collection, focusing on instructions and quality monitoring

5.8.1.2 Data Reception Method and Process:

（1） Data reception method: paper or spreadsheet

（2） Reception time and frequency: Send in real time to the general manager for summary

（3） Recipient: Researchers

（4） Data review: Each CRF must be reviewed and approved by the responsible person before consolidation

5.8.1.3 Data Entry Method and Process:

（1） Input tool: Professional data entry software

（2） Entry person: Researcher

（3） Entry time and frequency: Enter immediately after completing each CRF

（4） Data quality check: Quality control is performed after data entry to ensure accuracy and reasonableness

5.8.1.4 Data Management Process:

（1） data collection: researchers collected clinical data, stool, and serum samples according to standard protocols

（2） Data reception: After each CRF form is reviewed and approved, it is stored in the main file repository.

（3） Data entry: Researchers enter data according to the standard

（4） Data cleaning: After data entry, perform quality control and review to correct and remove non-compliant or duplicate data

（5） Data storage: Data is stored in password-protected electronic storage devices and managed with specialized database software

（6） Data backup: Back up data regularly

（7） Data access: Set permission levels to control data access and ensure security

（8） Security: Storing data in public areas is prohibited to ensure data confidentiality

5.8.2 Statistical Analysis

Descriptive statistical analysis was performed on the collected data. Continuous variables were presented as mean ± standard deviation (or median and interquartile range), while categorical variables were expressed as frequency and percentage. For intergroup comparisons of continuous variables, independent samples t-test or Mann-Whitney U-test was selected based on data normality. In cases involving multiple group comparisons, one-way ANOVA or Kruskal-Wallis test was employed. Categorical variable comparisons utilized chi-square test or Fisher's exact test to assess significant differences between groups. To evaluate relationships among multiple variables, linear regression was applied to continuous variables, logistic regression to categorical variables, and Cox proportional hazards regression to time-to-event data. These analyses adjusted for potential confounders such as age, sex, disease duration, and baseline glucose levels to ensure result accuracy and reliability. Confounding factors were included in regression analyses to minimize their impact. Additionally, propensity score matching was used to balance baseline characteristics between groups, ensuring comparability and enhancing the internal validity of the study results.

After adjusting for various covariates, we examined the association between adverse prognosis, hyperglycemic variability, and the gut microbiota α-diversity index. The relationship between hyperglycemic variability, adverse prognosis, and β-diversity differences was tested using genus-level Bray-Curtis distance-based permutation ANOVA (PERMANOVA). Using MaAsLin, we identified gut microbiota communities potentially associated with adverse prognosis and hyperglycemic variability by comparing hyperglycemic variability groups with hypoglycemic variability groups, and prognostic favorable groups with unfavorable groups. OPLS-DA was employed to identify target metabolites potentially related to hyperglycemic variability and adverse prognosis, with adjustments made through linear regression. Mediation analysis was conducted to evaluate whether gut microbiota metabolism might mediate the association between hyperglycemic variability and adverse prognosis. Sensitivity analysis was performed to test the robustness of the mediation effect.

Based on baseline data, gut microbiota, serum metabolites, and inflammatory factors, predictive models will be constructed using machine learning algorithms (e.g., random forests, support vector machines, neural networks). Feature selection methods (e.g., LASSO regression, recursive feature elimination) will be employed to identify the most prognostic features, thereby enhancing model accuracy and interpretability. Internal stability of the models will be assessed through 10-fold cross-validation, while their generalization ability will be validated using independent validation cohorts.

VI. Ethical Review and Informed Consent

6.1 Ethical Committee Review

This protocol, the written informed consent form, and all materials directly pertaining to the participants must be submitted to the ethics committee.

The study may commence only after obtaining written approval from the ethics committee. Investigators must submit an annual research report to the ethics committee at least once per year. When the study is suspended or completed, investigators must notify the ethics committee in writing. All changes during the study (including revisions to the protocol and/or informed consent forms) must be promptly reported to the ethics committee. No such changes may be implemented without prior ethics committee approval, except for those aimed at eliminating obvious and direct risks to participants. In such cases, the ethics committee will be notified.

6.2 Patient Information and Informed Consent

Researchers must provide participants or their legal representatives with an easily understandable, ethics committee-approved informed consent form, and allow sufficient time for participants or their legal representatives to consider the study. Participants shall not be enrolled until they have obtained a signed written informed consent. Throughout the study period, all updated versions of the informed consent form and written information will be provided to participants. The informed consent form must be retained as an important document for clinical research review.

7. Quality Management Plan

7.1 Measures to Improve the Consistency of Observations

(1) The participating clinical trial institutions are accredited medical device clinical trial facilities, with facilities and conditions meeting the requirements for safe and effective clinical trials. The research department's staffing and equipment meet the trial requirements, and the participating researchers possess the professional knowledge and experience required for the trial medical devices.

(2) The variable factors should be strictly controlled in outpatient cases.

(3) Through the training before clinical trial, the researchers have a full understanding of the clinical trial program and the specific connotation of each index, so as to improve the internal observation consistency and observer consistency of the clinical research data collector, and ensure the reliability of the clinical research conclusion.

(4) The description of subjective symptoms should be objective and not suggestive or inducing; the objective indicators should be checked at the specified time and by the specified method.

(5) Data that deviate significantly from the acceptable range must be verified and the researcher must provide a necessary explanation.

(6) The evaluation of the questionnaire should be made by at least one attending physician and one physician independently. The person who filled in the clinical case report form should have the title of physician or above.

（7） Each clinical research unit should designate a person to check the progress of the clinical trial regularly and verify the data and records carefully.

7.2 Measures to ensure subject compliance should strictly control the variable factors of the subject from the following aspects:

（1） Doctors participating in clinical trials are fixed;

（2） The researcher should explain the process of the study and the obligations of the participants to the participants patiently, so that the participants can cooperate with the study.

（3） The reasons for not meeting the diagnostic criteria should be recorded in detail.

7.3 Researchers Should Follow the Requirements for Case Report Form Completion

All observations and findings in the clinical trial should be verified to ensure the reliability of the data and that the conclusions are derived from the original data. Appropriate data management measures should be implemented during both the clinical trial and data processing phases.

8. Confidentiality Measures

We ensure the confidentiality of participants 'identities and have established strict information security protocols to protect their privacy. (1) During data collection, personal identification information will be processed to safeguard participant privacy: Each participant's name will be assigned a unique identification number. Personal data and case records will be entered and stored by designated sample management personnel. Users will only see the identification number, with no access to personal names or other identifying details. (2) Participant information will be strictly confidential unless required by law. Sample data will be electronically stored in secure systems with password protection, accessible only to authorized personnel. Medical records will be kept at the hospital for researchers' review only. Government agencies, hospital ethics committees, and other relevant researchers may access participant data as required. (3) Research outcomes: Results may be published in medical journals in statistical analysis format, excluding any identifiable participant information and without commercial implications.

9. Expected Progress and Completion Date of the Research Project

Total duration of the project implementation	4 years

Annual Plan Arrangements	
Schedule	**Key contents and outcomes of the stage objectives**
July 2025-May 2026	**Main content of the stage goal:** ① **Patient recruitment and enrollment (2025.7-2025.12)**: Eligible subjects were recruited according to the inclusion and exclusion criteria in the protocol and enrolled. ② **Glucose monitoring and sample collection (2025.7-2025.12):** CGM monitoring was performed for 14 days in enrolled patients, and stool and blood samples were collected. Ensure that the collection, preservation, and transportation of samples met relevant standards and requirements to provide high-quality samples for subsequent analysis. ③ **Preliminary data analysis (2026.1-2026.5):** The collected CGM data were preliminarily analyzed, patients were divided into different groups according to blood glucose variability, and preliminary data analysis report was completed. ④ **Follow-up Initiation (2026.1-2026.5):** A follow-up protocol and team were established to conduct a 3-year observation period for participants. Regular collection of clinical data—including diabetic kidney disease (DKD) progression, major adverse cardiac events (MACE), quality of life assessments, and complication occurrences—provided essential follow-up data for subsequent predictive model development. The follow-up program was successfully initiated and implemented. ⑤ **Additional patient recruitment (2026.1-2026.5):** Start additional recruitment of patients in a validation cohort for validation of the prediction model. **Achievement** ① The recruitment of patients was completed, and the enrollment rate reached the target. ② The collection rate of samples was 100%. ③ The preliminary data analysis report was completed. ④ The follow-up program was initiated and proceeded smoothly. ⑤ additional patients were recruited and the enrollment rate was as planned.
June 2026-December 2027	**Main content of the stage goal:** ① **Sequencing and Metabolic Analysis:** 16S rRNA high-throughput sequencing was performed on collected fecal samples, while serum non-targeted LC-MS analysis was conducted on blood samples to obtain data on gut microbiota and serum metabolites. Bioinformatics methods were employed to analyze sequencing data, identifying the composition and functional characteristics of gut microbiota. Statistical analysis of metabolite data was performed to screen differential metabolites associated with glycemic variability. The final reports were compiled based on both sequencing and metabolic analysis results. ② **Follow-up data collection:** Continue to conduct patient follow-up to ensure the completeness and accuracy of the data. Organize and clean the collected follow-up data to establish a database, providing high-quality data support for subsequent predictive model construction.

	The data integrity rate was over 95%. ③ **Preliminary data analysis:** The collected CGM data were preliminarily analyzed, patients were divided into different groups according to blood glucose variability, and preliminary data analysis report was completed. ④ **Additional patient follow-up**: The additional recruited patients were followed up for 3 years, and the clinical data of patients, including disease progression, occurrence of MACE, quality of life assessment, and occurrence of complications, were collected to provide data support for the validation of the prediction model. The complete rate of follow-up data was more than 95%. **Achievement** ① The 16S rRNA sequencing and non-target metabolic analysis were completed. ② The complete rate of follow-up data was over 95%. ③ The preliminary data analysis report was completed. ④ The complete rate of follow-up data in the verification queue was over 95%.
January 2028-December 2028	**Main content of the stage goal:** ① **Follow-up Data Organization and Analysis:** Conduct comprehensive final organization and analysis of collected follow-up data to ensure data integrity and accuracy. Perform statistical analysis to evaluate patient outcomes, including disease progression, major adverse cardiac events (MACE), quality of life assessment, and complication occurrence. Complete the follow-up data analysis report. ② **Association Analysis:** By integrating glycemic variability grouping results, this study investigates the relationship between gut microbiota characteristics and serum metabolite profiles with clinical outcomes, including disease progression, major adverse cardiovascular events (MACE), quality of life, short-term complications, and overall prognosis. Through correlation analysis and multiple regression analysis, we aim to uncover underlying biological mechanisms and predictive biomarkers, ultimately producing a comprehensive association analysis report. ③ **Prediction Model Development:** By integrating multi-dimensional data including baseline information, gut microbiota profiles, serum metabolite profiles, and inflammatory factors, we constructed predictive models using machine learning algorithms such as random forests, support vector machines, and neural networks. Through cross-validation and other optimization methods, we enhanced the model's predictive performance and stability, completing the initial development of the prediction model. ④ **Model evaluation and validation**: Appropriate evaluation metrics were used to assess the constructed prediction model, verifying its generalization capability and predictive performance across different datasets. Based on the evaluation results, the model was further optimized and adjusted to ensure reliability and practicality, and the model evaluation report was completed. **Achievement：** ① The follow @-@ up data analysis report was completed. ② Complete the association analysis report. ③ The preliminary construction of the prediction model was completed. ④ The model evaluation report was completed.

January 2029-July 2029	**Main content of the stage goal:** ① **Clinical Validation of the Model**: Validate the predictive model using additional recruited patients to evaluate its practical value and feasibility in real-world clinical settings. Based on validation results, make necessary adjustments and improvements to the model. This will facilitate its clinical application and promote its adoption, providing scientific evidence for prognostic assessment and personalized treatment of diabetic kidney disease (DKD) patients. The process will culminate in the completion of a clinical validation report. ② **Complete the project:** write the paper and complete the project report. **Results:** ① The clinical validation report was completed. ② The final report was completed, and the relevant academic papers were written and published.

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