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Research Article

INVESTIGATING DISPARITIES IN METABOLITE CONCENTRATIONS USING GAS CHROMATOGRAPHY MASS SPECTROSCOPY AMONG PATIENTS WITH GESTATIONAL DIABETES MELLITUS AND IN HEALTHY CONTROL PARTICIPANTS

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ABSTRACT

Objective: Metabolomics has been a promising strategy in recent years for understanding the intricate metabolic changes linked to diseases like gestational diabetes mellitus (GDM). Gas chromatography-mass spectrometry (GC-MS) has been used as analytical method in this study to explore the function of metabolomics in comprehending the underlying biochemical alterations in GDM.

Methods: One hundred subjects were enrolled and divided into two groups, Group A with healthy control participants (HCPs) and Group B with GDM patients. Both groups' plasma samples were taken, and concentrations of amino acid metabolites were evaluated using GC-MS analysis.

Results: Leucine and Tyrosine levels were low in HCP, that is, (1.79±0.15 μM and 0.25±0.06 μM), whereas levels were high in GDM patients, that is, (23.58±1.73 μM and 0.466±0.015 μM), respectively. Similarly, tryptophan and histidine levels were low in HCP, that is, (0.41±0.10 μM and 0.271±0.072 μM), and levels were high in GDM patients, that is, (0.871±0.105 μM and 1.916±0.340 μM), respectively. Methionine and Phenylalanine levels were high in HCP (1.063±0.161 and 0.642±0.035), and levels were low in GDM patients (0.765±0.103 and 0.459±0.056), respectively.

Conclusion: The findings of the current study suggested that GDM is associated with an increase in tyrosine, tryptophan, leucine, and histidine levels and a decrease in methionine and phenylalanine levels. Therefore, these amino acids could serve as a diagnostic biomarker and supplementation tool (methionine and phenylalanine) as a management strategy in GDM.

Keywords: Gestational diabetes mellitus, Metabolomics, Healthy control participants, Gas chromatography-mass spectrometry, Amino acid, Biomarker.

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INTRODUCTION

Gestational diabetes mellitus (GDM) is a prevalent condition affecting thousands to millions of pregnant women across the India and globe claiming many lives. Term GDM was coined by O'Sullivan and Mahan in 1964, where GDM was characterized by elevated blood sugar levels detected during pregnancy. It can be marked as a distinctive pregnancy-related condition [1-4]. Its incidence is on the rise, and whereas prevalence of GDM varies between 2% and 38% of pregnancies, contingent on diagnostic criteria and the studied population. In 2017, GDM afflicted approximately 204 million women across the world. It has been forecasted around 308 million women going to get impacted by 2045, predominantly in developing nations [5]. The risk factors associated with GDM comprise being overweight or obese, having an advanced maternal age, and having a family history of diabetes of any type [6]. The increasing prevalence of GDM, much like the rising rates of obesity and Type 2 diabetes worldwide, underscores the need for heightened awareness and action. During pregnancy, β-pancreatic cells adapt through hypertrophy or hyperplasia to meet elevated metabolic demands for fetus organ formation. However, reduced insulin sensitivity condition may be developed, leading to elevated glucose levels. This condition may normally be reversed during post-pregnancy. In contrast to this, in GDM β-cells struggle to adapt to these demands, leading to hyperglycemia, which is further exacerbated by a rapid decline in insulin-receptor-substrate, phosphoinositide 3-kinase, and glucose transporter Type 4 expression, and this damage to organs, including pancreas may lead to irreversible diabetes condition accompany to risk of obesity, sustained insulin resistance, and dyslipidemia. Nevertheless, after childbirth, β-cell function, blood glucose levels, and insulin sensitivity may normalize if properly managed and diagnosed in early stages [6]. These metabolites are small molecules that are the intermediates and end-products of metabolic processes within a living being. These metabolites support growth and are involved the maintenance of cellular functions. Any kind of disturbance in the cellular composition may lead to alteration of metabolomic profile within the biological system, and hence can be utilized further as a predictive and diagnostic biomarker for disease or a medical complication [7]. Metabolomics should be performed with methods that are sensitive, reliable, repeatable, and require the least amount of time to analyze samples. Therefore, a method that is appropriate for that class and specific to that group of metabolites must be used to identify a particular group of metabolites. Because samples can be prepared and analyzed quickly, and because metabolomics uses fewer methods and chemicals than genomics, transcriptomic, and proteomics, it is an easy and affordable way to understand the system [5,8]. There are several different analytical methods that can be used in metabolomics, each with its own strengths and weaknesses. Gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) are the most prevalent platforms utilized in research related to GDM. During metabolomics investigations, these two sophisticated analytical methods are frequently employed to locate and measure tiny molecules in biological materials.

It was found that the levels of different metabolites such as lipids, amino acids, organic acids, and other metabolite levels remarkably differ in clinically diagnosis of potential GDM Subjects [9]. Various factors were

responsible for the altered metabolomic profile in a biological system including environmental, genetic imbalance and its regulation, changes in gut microflora, changes in the concentration of different enzymes, and variation in metabolic pathways leading to GDM-related congenital outcomes. Therefore, identifying and mitigating these metabolomic alterations impact with the help of pathophysiology and phenotype in the early stages of GDM in biological system with helps to alter the fatal outcome.

METHODS

Subjects

Subjects aged between 25 and 35 years of age were selected with GDM (G1 and G2) and non-GDM from a tertiary care hospital, India after approval by Institutional Human Ethical Committee. The study was conducted over period of 1 year. GDM was diagnosed using the International Association of Diabetes and Pregnancy Study Group criteria, which required one or more plasma glucose readings to be equal to or higher than the following: 5.1 mmol/L while fasting, 1 h, 10.0 mmol/L, and 8.5 mmol/L during 2 h. Subject with comorbidities such as polycystic ovarian disease, Hypothyroidism/hyperthyroidism, Osteoporosis, hepatitis, cardiovascular disease, tuberculosis, and human immunodeficiency virus, and on substance abuse were excluded from the study. Before including a participant in the research, an informed consent forms were collected from each patient once they had been made aware of the study. The research adhered to the principles outlined in the Declaration of Helsinki and followed by International Council for Harmonisation (ICH) E6 Guideline for Good Clinical Practice (GCP) standards, with the necessary modifications.

Biochemical assessment

Pre-prandial venous blood (5 mL) was drawn in the morning and was placed in a 10 mL heparin sodium tube. The tube was then centrifuged at 3000 rpm for 10 min. Before analysis, plasma samples were kept at −80°C for storage. For the purpose of removing proteins, 400 µL of each plasma sample was received 800 µL of acetonitrile. All samples were then centrifuged at 30 min at 4°C at 13,000 rpm. Before derivatization, 900 µL of each supernatant was vacuum-evaporated to dryness. Dried samples were derivatized by first adding 50 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide at 40°C for 90 min., followed by 30 µL of methoxylamine hydrochloride (15 mg/mL in pyridine) at 70°C for 60 min. Tetracosane (0.1 mg/mL) was added to 500 µL of heptane as an internal standard, and the mixture was vortexed for 2 min for GC analysis. The efficiency of derivatization was monitored by assessing peak symmetry and absence of underivatized analyte peaks in chromatograms.

The chemical constituents of the sample were analyzed using a GC-MS system comprising an Agilent 8890 GC coupled with a 5977B Mass Selective Detector (Agilent Technologies). Separation was performed using an HP-5MS capillary column (30 m×0.25 mm ID×0.25 µm film thickness). Helium was used as the carrier gas at a constant flow rate of 1.0 mL/min with a split ratio of 1:100. The injection volume was 1.0 µL, and the injection temperature was maintained at 250°C. The head column pressure was set at 9.3 psi. The oven temperature was initially set at 80°C and held for 1 min, then increased to 140°C at a rate of 7°C/min and held for 4 min. Subsequently, the temperature was raised to 180°C at a rate of 5°C/min and held for 6 min, followed by a final increase to 280°C at 5°C/min with a 2 min hold. Mass spectrometric detection was conducted in electron impact mode at 70 eV, with a scan range of m/z 40–550.

Statistical analysis

A statistical analysis was performed to identify significant differences in metabolite concentration between sample groups. Statistical analysis was performed in the Statistical Package for the Social Sciences Version 21.0 and Graphpad Prism version 9. Univariate and multivariate statistical tests, including t-tests, analysis of variance, and principal component analysis, were applied for clinical and metabolic markers assessment, and finally Pearson coefficient correlation was utilized to compare and correlate group-specific metabolic alterations.

RESULTS

Clinical characteristics

In the present study, the amino acid profiling to identify the alteration of plasma amino acid concentration in patients with GDM in comparison to healthy individuals has been performed using GC-MS analysis. The study has been performed on 50 patients with GDM and 50 healthy controls. All the selected patients were female and had a median age of mean age of 26.53±3.19 years for healthy control and 25.08±3.0 years for disease patients (range 20–35 years). The patients had mild-to-severe GDM and were on treatment with metformin and glyburide for at least past 6 months. The demographic and clinical characteristics are summarized in Tables 1 and 2.

Correlation analysis

The correlation between serum Nesfatin level and different clinical parameters, that is, (glycated CD59 [CDC 59], glucose tolerance test (GTT), hemoglobin A1C (HbA1C), fasting blood sugar [FBS], random blood sugar [RBS]) and lipid profile (low density lipoprotein [LDL], total cholesterol [TC]) was determined through Pearson correlation analysis among GDM patients. From the observations in Table 3, the correlation coefficients (r) values indicate a high and statistically significant positive correlation between the serum Nesfatin and the various biomarkers under investigation. The correlation coefficients were 0.8334 for CDC 59, 0.8825 for GTT, 0.7501 for HbA1C, 0.8490 for FBS, and 0.8769 for RBS, with a $p<0.0001$, representing highly significant correlations. These findings suggest that high serum Nesfatin levels are strongly correlated with glucose dysregulation in GDM, as indicated

Table 1: Demographic of GDM and healthy participants

Variables	Categories	n=100 (%)
Age	≤30	35 (35)
	31–33	41 (41)
	34–35	24 (24)
Education	Middle school or below	43 (43)
	High school/Technical school	28 (28)
	Junior college/college	10 (10)
	Master's degree or higher	19 (19)
Occupational category	Government administrator or leader of an enterprise or public institution	15 (15)
	Professional (teacher, doctor, engineering technician, writer, etc.)	10 (10)
	Clerk or relevant personnel	32 (32)
	Commercial and service industry personnel	8 (8)
	Agricultural, forestry, animal husbandry, fishery, and water conservancy production personnel	13 (13)
	Production and transportation equipment operators and related personnel	3 (3)
	Other	19 (19)
Gravidity	1	43 (43)
	2	34 (34)
	3	21 (21)
	≥4	2 (2)
Parity	0	56 (56)
	1	37 (37)
	≥2	7 (7)
Method of pregnancy	Natural conception	64 (64)
	Assisted reproduction	36 (36)
Family history of diabetes?	Yes	43 (43)
	No	57 (57)

Values are presented as n (%)

Table 2: Demographic of and clinical characteristics of GDM and healthy participants

Clinical variables	Healthy patients	Diseased patients	p-value
Age (year)	26.53±3.19	25.08±3.0	0.094
Weight (kg)	66±1.52	70.55±2.55***	<0.001
Height (m)	1.52±0.18	1.46±0.22	0.199
Body mass index (kg/m^2)	21.15±0.81	30.3±2.6***	<0.001
Hb (mg/dL)	12.25±1.2	10.55±2.11***	<0.001
Fasting blood glucose (mg/dL)	80.14±3.02	143.25±5.83***	<0.001
Random blood glucose (mg/dL)	100.18±19.11	150.32±21.30***	<0.001
GTT (mmol/L)	5.56±0.76	11.46±2.37***	<0.001
HbA1C (%)	4.84±0.33	6.67±0.92***	<0.001
LDL (mg/dL)	90.34 4.18	141.68±27.86***	<0.001
TC (mg/dL)	160.3±4.56	224.5±33.81***	<0.001
HDL (mg/dL)	56.7±3.9	42.64±2.577***	<0.001
Nesfatin (ng/mL)	6.44±0.61	5.6±0.42***	<0.001
CDC 59 (SPU)	1.43±0.45	2.07±0.37***	<0.001

Values are presented as Mean±S.D. ***p=<0.001. CDC 59: Glycated CD59, GTT: Glucose tolerance test, HbA1C: Hemoglobin A1C, LDL: Low density lipoprotein, HDL: High density lipoprotein, TC: Total cholesterol, SPU: standard peptide units, GDM: Gestational diabetes mellitus

Table 3: Pearson correlation matrix of various clinical variables with Nesfatin in diseases population

Variable	r	p-value
CDC 59	0.8334	<0.0001
GTT	0.8825	<0.0001
HbA1C	0.7501	<0.0001
FBS	0.8490	<0.0001
RBS	0.8769	<0.0001
LDL	0.8675	<0.0001
TC	0.8034	<0.0001

*r=correlation coefficient, *P*<0.05 was considered as statistically significant. CDC 59: Glycated CD59, GTT: Glucose tolerance test, HbA1C: Hemoglobin A1C, FBS: Fasting blood sugar, RBS: Random blood sugar, LDL: Low density lipoprotein, TC: Total cholesterol

by both short-term (FBS, RBS) and long-term (HbA1C, GTT) glycemic markers, with CDC 59 having the potential to serve as a new marker in the context of the pathophysiology of GDM.

Moreover, the analysis revealed a significant positive correlation of the serum Nesfatin biomarker with lipid variables, evident from the correlation coefficients of 0.8675 for LDL and 0.8034 for TC, both of which were significant at p<0.0001. These results reveal a high correlation between the biomarker and dyslipidemia, which is of utmost significance in GDM due to its relevance to cardiovascular disease in the mother and child. The high correlation coefficients, particularly for GTT (r=0.8825) and LDL (r=0.8675), reflect the strength of the relationship of the biomarker with metabolic dysregulation in GDM. The consistently low p-values (<0.0001) for all variables further support the statistical suitability of these correlations. The correlation analysis of the serum biomarker with these clinical variables in GDM patients is presented in Fig. 1, which provides a clear visual representation of the interrelationships.

Retention time (RT) value analysis

Position of different amino acids was shown by RT values of both the healthy individuals and disease patients, respectively (Table 4).

GC-MS spectra

GC-MS is a precise and sensitive analytical technique that allows for the simultaneous quantification of a wide range of amino acids in

Table 4: RT values of amino acid in healthy and disease patients

S. No.	RT value in healthy subjects	RT value in disease patients	Amino acid
1.	11.07	11.50	Methionine
2.	10.02	10.1	Leucine
3.	16	16.81	Histidine
4.	15.53	15.7	Tyrosine
5.	18.0	18.2	Tryptophan
6.	14.69	14.69	Phenylalanine

RT: Retention time

Table 5: Results of various metabolomics amino acid in healthy control and GDM patients

Amino acid	Healthy control (n=50) (μM)	Patient with GDM (n=50) (μM)	t-value	Df	p-value
Methionine	1.06±0.16	0.77±0.10**	10.70	99	<0.005
Leucine	1.79±0.15	23.59±1.73***	−43.41	99	<0.001
Histidine	0.27±0.07	1.91±0.34**	−40.08	99	<0.001
Tyrosine	0.25±0.06	0.46±0.01*	−18.81	99	<0.01
Tryptophan	0.42±0.10	0.87±0.10***	−22.23	99	<0.001
Phenylalanine	0.64±0.03	0.46±0.05*	10.93	99	<0.05

*p=<0.01, **p=<0.005, ***p=<0.001. GDM: Gestational diabetes mellitus

biological samples. A large amount of variations has been observed in concentrations of amino acids being identified from Table 5 of healthy individuals and disease patients, respectively. It is evident that six amino acids of pharmacological significance, that is, glycine, phenylalanine, tryptophan, tyrosine, methionine, and aspartate showed differences in their concentration in patients and healthy individuals.

Metabolomics profile in spontaneous GDM and healthy control

The representative GC-MS graphs of disease patients and HCs are given in Figs. 2 and 3, respectively. Significant variation can be observed in concentration of amino acid. It is evident that 6 amino acids of pharmacological significance, that is, leucine, methionine, tryptophan, histidine, tyrosine, and phenylalanine, showed differences in their concentration in patients and HCs. Fig. 1 and Table 4 show the peaks at specific RT for each amino acid. For statistical analysis, a t-test was performed using Microsoft Excel and GraphPad Prism. All the tests were two-tailed, and p<0.05 was considered to be statistically significant.

DISCUSSION

In recent years, metabolomics has gained increasing attention in the field of diabetes and, therefore, has a relation in GDM also. By analyzing the small molecules present in blood or cerebrospinal fluid samples, metabolomics can identify changes in metabolic pathways that may be associated with the development or progression of GDM [6].

Among the parameters in the case of GDM, the glycemic parameters (CDC 59, GTT, HbA1C, FBS, RBS) are most critical in the surveillance of pregnancy glucose homeostasis, with GTT being a gold standard for GDM diagnosis and CDC 59 being a potential new biomarker for risk stratification. The lipid parameters (LDL, TC) reflect alterations in lipid metabolism, which in GDM are generally exacerbated and can be an etiological factor for pregnancy complications. The strong correlations obtained indicate that the serum biomarker can prove to be an excellent tool for the evaluation of glycemic as well as lipid profiles in GDM and can prove useful in the identification of patients with increased risk of complications. The data of the Pearson correlation analysis are presented in Table 1, and graphical representations of these correlations are presented in Fig. 1.

There are several different analytical methods that can be used in

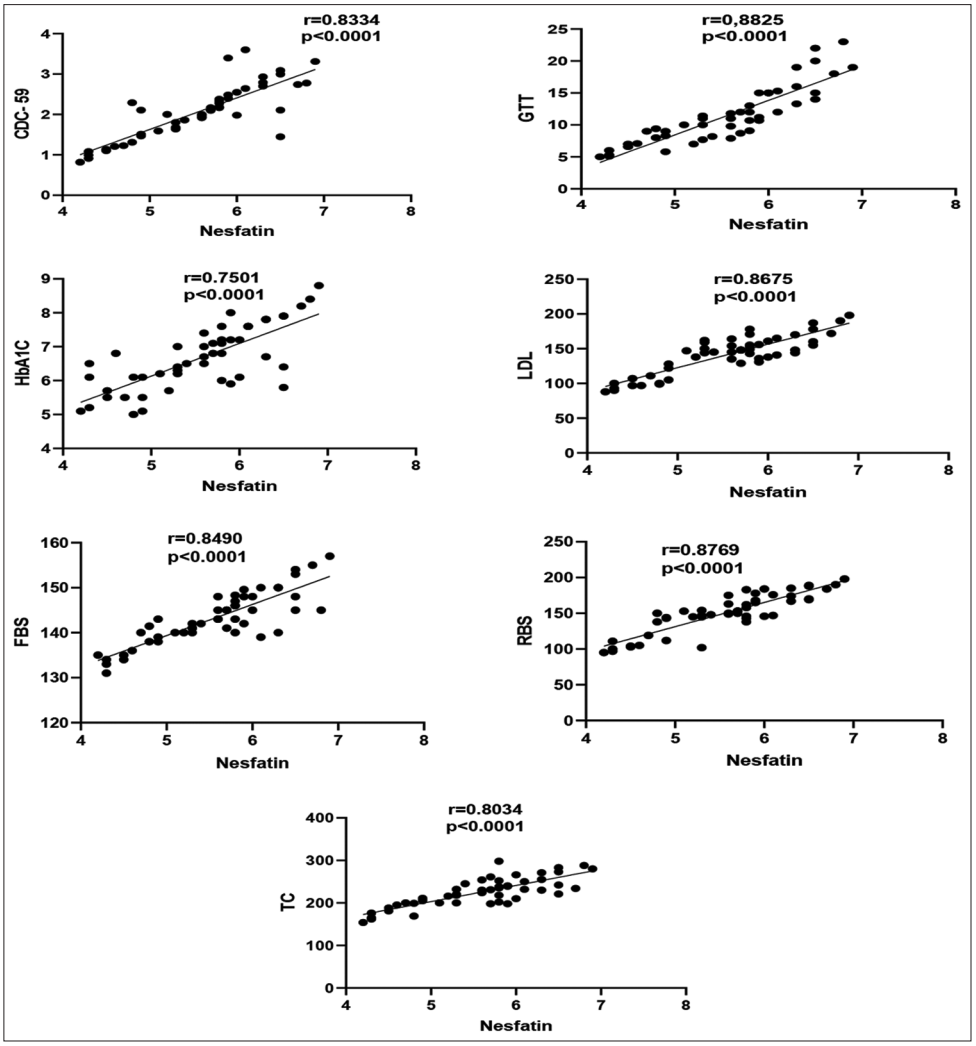

Fig. 1: Pearson correlation analysis of serum Nesfatin (ng/mL) levels with glycated CD59 levels, With diabetic biomarkers (fasting blood sugar, random blood sugar, hemoglobin A1C, glucose tolerance test) and lipid biomarkers (low density lipoprotein, total cholesterol)

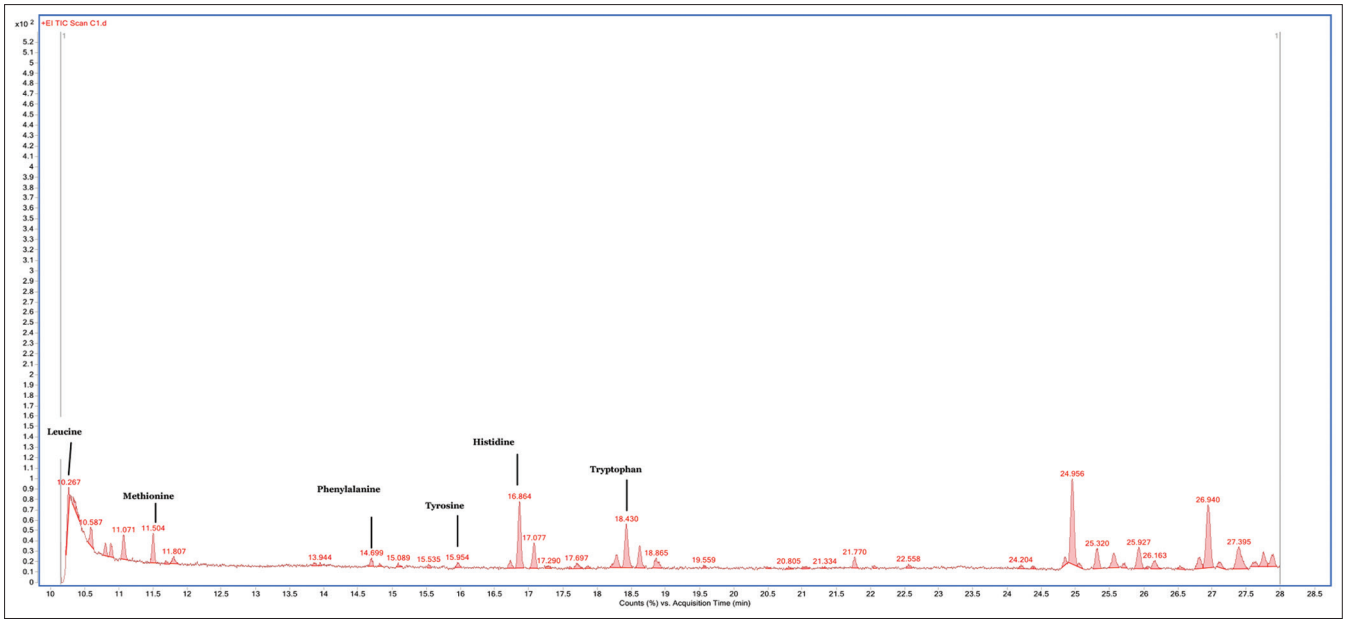

Fig. 2: Representative chromatogram of amino acid by gas chromatography-mass spectrometry for healthy controls

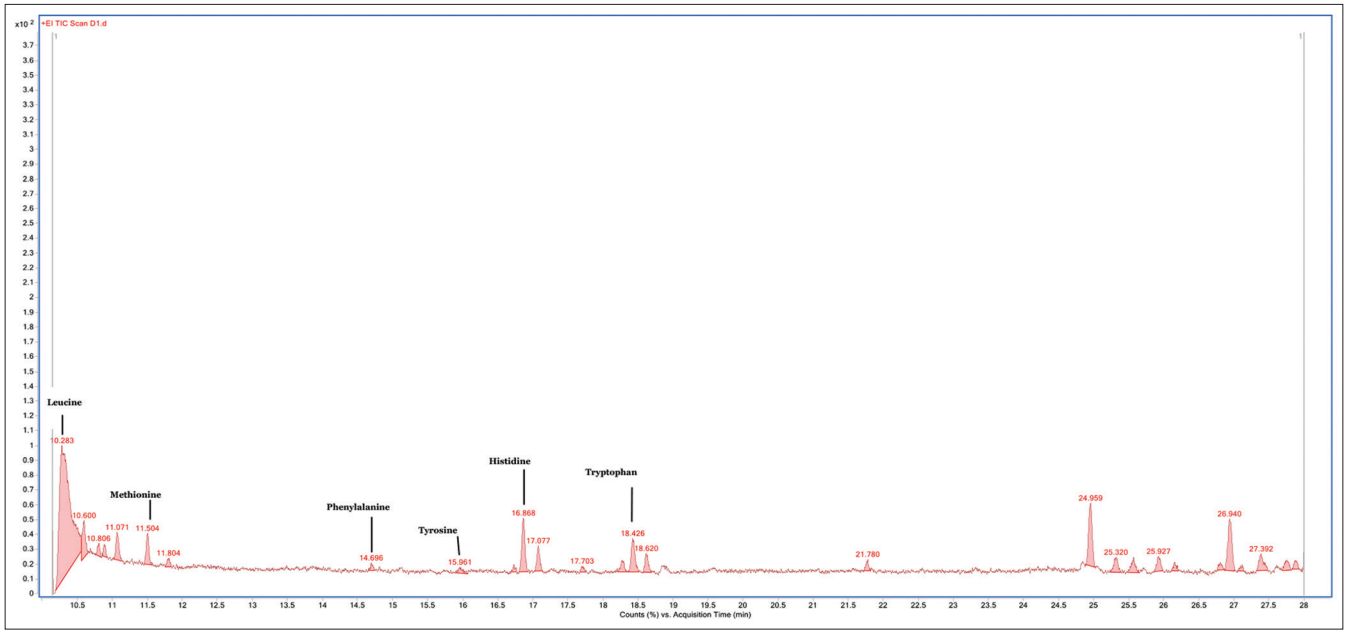

Fig. 3: Representative chromatogram of amino acid by chromatography-mass spectrometry for gestational diabetes mellitus

metabolomics, each with its own strengths and weaknesses. GC-MS and LC-MS are the most prevalent platforms utilized in GDM research. Various amino acids are used in diagnosing the GDM such as Arginine, Alanine, Aspartate, Glutamate, Glycine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Proline, Serine, Threonine, Histidine, Tryptophan, and Tyrosine [6]. The levels of tryptophan, tyrosine, phenylalanine, methionine, histidine, and leucine were measured in GDM patients within this current investigation. We saw the potential for variations in the above amino acids concentration in our study; these changes are discussed below.

Indoleamine 2,3-dioxygenase is frequently more active in GDM, which results in a greater conversion of tryptophan to kynurenine. Oxidative damage, inflammation, and impaired insulin signaling can all be caused by elevated kynurenine levels [10]. There is evidence that tryptophan and its metabolites, such as serotonin and kynurenine, have an impact on insulin sensitivity and β-cell activity. Dysregulation may aggravate glucose homeostasis in GDM. Similar outcomes were shown in our investigation, where a noteworthy rise in tryptophan levels was noted in comparison to a healthy control. Insulin often encourages the body's cells to absorb amino acids like tryptophan. Insulin resistance affects this mechanism in GDM, resulting in increased amounts of tryptophan in the blood. In addition, pregnancy changes the quantities of hormones like cortisol and estrogen, which can affect how tryptophan is metabolized. GDM may amplify these effects, resulting in elevated levels of tryptophan.

Research indicates that high tyrosine plasma levels are linked to insulin resistance and a higher chance of developing GDM. Tyrosine metabolism can alter insulin signaling pathways, and women with GDM have been reported to have abnormal amino acid profiles, specifically increased branched-chain and aromatic amino acids such as tyrosine. In addition, tyrosine levels that are too high might affect insulin production and sensitivity, worsening glucose intolerance during pregnancy [11]. Similar findings were reported in our investigation, which showed a considerable rise in tyrosine levels in GDM patients. An aromatic amino acid called tyrosine is frequently increased in insulin resistance, which is also a symptom of Type 2 diabetes. Because insulin resistance alters the metabolism of amino acids, muscles and other tissues absorb less tyrosine, which raises blood levels. The metabolism of amino acids is mostly controlled by the liver. Subtle liver dysfunctions, such as moderate steatosis or increased hepatic fat, might hinder proper tyrosine breakdown in GDM, leading to plasma buildup.

Numerous studies indicate that in conditions of insulin-resistance and diabetes, the level of circulating methionine and its catabolic derivative cysteine is increased [12,13]. In a study by Burzynska-Pedziwiat*et al.*, GDM women, methionine concentration decreased [14]. A similar correlation was also confirmed by Pappa *et al.* who analyzed amino acid and found that methionine levels were significantly higher in normal pregnant women compared to those with GDM [15]. Similar results were obtained in our study as methionine levels decreased in GDM patients. This fall probably results from increased oxidative stress in GDM, diverting methionine into the transsulfuration pathway for glutathione production to offset reactive oxygen species. Increased fetal demand and methylation activity through S-adenosylmethionine might also reduce maternal methionine levels.

Leucine and histidine are two examples of branched-chain amino acids (BCAAs), which are vital amino acids that are crucial for immunity, nutrition metabolism, and energy balance [16-18]. There is mounting evidence linking BCAAs to an increased risk of type 2 diabetes. Research has shown that GDM patients had higher first-trimester blood levels of leucine and histidine than do healthy pregnant women, which may be a predictor of GDM [14,15]. According to some earlier research, the second trimester's BCAA levels are predictive of GDM. According to a meta-analysis of 432 participants, the GDM group had greater plasma concentrations of BCAAs than the control group [19]. In our investigation, leucine and histidine levels were shown to be higher in GDM patients than in healthy subjects, indicating a substantially comparable reaction. Normally, insulin encourages muscle to absorb and break down BCAAs like leucine. In insulin-resistant conditions (such as GDM), muscle cells are less efficient in absorbing and metabolizing leucine, resulting in its accumulation in the blood. Moreover, the primary location for BCAA catabolism is muscle. Muscle insulin resistance in GDM implies that muscles do not utilize glucose efficiently and digest amino acids like leucine and histidine inefficiently. As a result, there are more BCAAs in the blood.

A study by Spanou *et al.*, shows the levels of phenylalanine significantly reduced in GDM women compared with controls. It is reported that in normal pregnancy phenylalanine decreases during pregnancy probably due to its consumption for nitrogen demands and tissue synthesis [20]. Comparatively similar results were found in our study as phenylalanine levels were low in GDM patients. Greater phenylalanine hydroxylase activity, stimulated by hormonal fluctuations in GDM, hydroxylates more phenylalanine to tyrosine, decreasing its levels. Greater fetal need and competition with BCAAs for transport further decrease maternal phenylalanine levels.

Collectively, our research findings also have the tendency to support the recently developed techniques for accurately diagnosing and managing GDM.

CONCLUSION

Research on the metabolism of amino acids in GDM provides valuable understanding of the complex metabolic changes linked to the condition and might help create more specialized and efficient treatment plans. Several amino acids and associated compounds were discovered to be increased in GDM patients. Tyrosine, tryptophan, leucine, and histidine levels were consistently elevated in the afflicted group, indicating their possible role in the pathogenesis of GDM, and methionine and phenylalanine levels were low in GDM subjects. As these amino acids help in diagnosing the GDM, different findings can be made such as methionine and phenylalanine could be used as a supplementation therapy in GDM patients to upregulate the levels in patients with GDM. To clarify the precise function of these amino acids in the onset and course of illness, more research is required. Different therapeutic and diagnostic techniques, such as modifying methionine or leucine levels to enhance metabolic outcomes, should be investigated because these changed amino acid profiles may aid in the diagnosis of GDM. Furthermore, increased histidine and tryptophan levels may be useful indicators for GDM monitoring and early identification. Therefore, the quantitative evaluation of these amino acids offers encouraging opportunities for improved comprehension, diagnosis, and treatment of GDM through more individualized approaches.

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AUTHOR'S CONTRIBUTIONS

S.B. performed the study. C.P. and P.S. analyzed the data and wrote the manuscript. P.K. and S.B. conducted the literature survey. R.S. conceptualized the study. C.P., P.S, and R.S. performed statistical analysis. R.S., R.K. reviewed the final manuscript draft. All authors have read and agreed to the published version of the manuscript.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

FUNDING

Nil.

INSTITUTIONAL REVIEW BOARD STATEMENT

The research adhered to the principles outlined in the Declaration of Helsinki and followed by ICH E6 GCP standards, with the necessary modifications and approval no. EC/NEW/INST/2024/531/262, by Institutional Human Ethical Committee from Chitkara University, Punjab, India, and Written informed consent for participation in the study was taken from each patient or their relative.

INFORMED CONSENT STATEMENT

Written informed consent for participation in the study was taken from each patient or their relative.

DATA AVAILABILITY STATEMENT

The data and materials are available from the corresponding author on request.

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