

¹H-NMR based serum metabolomic signatures imperative in retinal neurodegeneration and development of Diabetic Retinopathy

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Abstract

Aim: To identify serum metabolomic fingerprints using ¹H-NMR in Diabetic Retinopathy (DR). **Methods and Materials:** ¹H-NMR was performed on 32 subjects [11 type 2 diabetic patients each without DR (group A) and with DR (group B) along with 10 control (group C)]. **Results:** The study unraveled ¹H-NMR based serum metabolomic fingerprints of diabetic retinopathy showed significant variations in Ribitol, D-glucose, Fructose-6-phosphate, Uridine Diphosphate-N-acetyl glucosamine and Glycerophosphocholine. The study envisaged that abnormal polyol metabolism and accumulation of ribitol contributory to development of diabetic retinopathy. Presence of Uridine Diphosphate-N-acetylglucosamine in blood has been also confirmed its probable role in progressive neurodegeneration in diabetic retinopathy. NMR spectra also indicated that downregulation of glycerophosphocholine is directly related to retinol metabolism and subsequent unusual chromophore synthesis facilitating retinal degeneration. **Conclusion:** Five metabolomic fingerprints have been identified and found to be unique in Diabetic Retinopathy (DR).

Keywords: Serum metabolomics, ¹H-NMR Spectroscopy, Abnormal polyol metabolism, Retinal neurodegeneration, Diabetic Retinopathy, Retinol metabolism

Introduction

Diabetic retinopathy (DR) is a microvascular [1] and neurodegenerative [2] complication of eye and it is the leading cause of blindness and visual impairment among working-age persons in developed countries. Although stringent control of both blood glucose levels and hypertension are crucial to halt the progression of the disease, the suggested goals are difficult to achieve in many patients, and, consequently, DR develops during the evolution of the disease. It has been predicted that global prevalence of diabetes will increase from 366 million in 2011 to 552 million in 2030 [3]. These data indicate the considerable worldwide public health affliction from diabetic

retinopathy and the importance of identifying risk factors. Metabolomics is the attempt to reckon all small endogenous molecules from cells, tissue or biofluids [4]. Metabolome is an elusive level of organization than the proteome or transcriptome for comprehending an intricate biological system. A major advantage of metabolomics is the ability to uncover novel and potentially pertinent metabolites which can be the basis of therapeutic approaches or diagnostic indicators [5]. Identification of prognostic DR specific markers are indispensable since metabolic dysfunction may persist for several years before manifesting as clinical diabetes [6] and subsequent irreversible development of diabetic retinopathy. So, an imperative impetus exists for novel cost effective diagnostic means for the documentation of metabolomics biomarkers and risk prognosticators for

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comprehensive understanding of the avenues in which metabolic aberrations contribute to diabetic retinopathy pathophysiology.

Metabolomics of eye has been achieved earlier employing animal models having artificially induced diabetic retinopathy. Since, human vitreous and ocular biopsy samples are scarcely available for experimental analysis [7]. The current study is exploiting $^1\text{H-NMR}$ to document metabolomics biomarkers provides scope to document molecular and/or sub-molecular information from serum sample. Raman and NMR spectroscopy of vitreous in DR are reported in literature [8, 9]. However, application of $^1\text{H-NMR}$ in diagnosis of DR using serum has not been reported so far. Despite substantial development in molecular pathology, there is lack of specific biomarkers for the diagnosis and prediction of DR. So, pathological manifestations need to be scrutinized at varied levels that could be the exploration of serum components contributory to relevant molecular pathology.

Materials and methods

The study has been performed with 31 subjects. Consecutive 11 diabetes patients without diabetic retinopathy in either eye (Group A) and 10 diabetes patients with diabetic retinopathy in any or both eyes (group B) attending retina clinic of a tertiary care teaching institute were included in the study. Inclusion criteria were: type 2 diabetes mellitus patients; age above 30 years; without any major co-morbidity. Exclusion criteria were: photocoagulation in the preceding 6 months; previous intravitreal injection/vitreo-retinal surgery history of glaucoma; renal failure, neoplasia, acute bacterial / viral infection, any other endocrine disorder, Patients known to suffer from any autoimmune disease and liver disease were also excluded. Ten age and sex matched healthy volunteers (group C) were included as control. The study was endorsed by the Institutional ethics committee and the study subjects were recruited after obtaining written informed consent. The study followed the tenets of the

Result

Mean duration of diabetes in Group A (Diabetes) and Group B (Diabetic Retinopathy) were 11.2 ± 3.1 years and 15.2 ± 7.1 years respectively ($p=1.47955\text{E-}06$). Mean HbA1C in Group A and Group B were $6.4 \pm 1.4\%$ and $8.5 \pm 2.7\%$ respectively ($p=2.36842\text{E-}05$). Mean HbA1C in group C (Normal) was $4.9 \pm 0.8\%$. Proportion of male subjects in group A, B and C were 54.5%, 63.6% and 50% respectively. NMR spectroscopy of Diabetes, Diabetic retinopathy and control samples revealed that the ribitol has been upregulated (Peak no. 3.98 ppm, 3.68 ppm, 3.897 ppm and 3.709 ppm) in DR compared to diabetic and normal condition. All of its NMR peaks (^1H) peaks are present in both diabetic and DR, but absent in

Declaration of Helsinki. Six ml of peripheral blood samples were collected and preserved in collection tubes (BD Vacutainer Plus Plastic K2 EDTA Tubes) and transported to the laboratory within 5 minutes of collection. The red blood cells were removed by centrifugation at 3000 rpm (revolutions per minute) for 15 minutes to separate erythrocytes from serum. Serum samples were then transferred into cryogenic tube and stored at -80°C . Frozen samples were initially kept at 4°C for 60-90 min before bringing it to room temperature for NMR data acquisition.

NMR Spectroscopy and identification of metabolites: NMR spectra were documented at 298 K using a Bruker 600 MHz (for ^1H) AVANCE AVIII spectrometer. Serum samples were thawed by gradual temperature gradation and homogenized using a vortex mixer. Following centrifugation (8000 rpm, 5 min), 600 μl of each sample was transferred into 5 mm NMR tubes. Samples (200 μl) were diluted in D_2O (400 μl) containing 1 mM sodium salt of 3- (trimethylsilyl) propionic-2, 2, 3, 3, d_4 acid (TSP). Solvent residual peak of D_2O was considered as standard in NMR experiments. Water peak were suppressed for minimizing the noise of NMR spectrum. NMR spectra were acquired on a 400 MHz (9.4 T) magnet interfaced to a spectrometer (Avance 400; Bruker, Rheinstetten, Germany), and kept at 30°C throughout the experiment, as described previously. In the NMR spectra, each peak corresponded to a chemical component that could be identified by its chemical shift. Before comparison, spectra were digitized in 1000 bins of equal length so that all peaks in the spectra were taken into account. Once digitized, the spectra were fed into MestReNova (Ver. 9.0.0.12821, MeastReLab 2013, USA). After that, ppm and intensities have been checked and compared with Human Metabolome database (HMDB) [10], we were able to inquire which NMR peaks (i.e. metabolites) were responsible for the differences. Finally, correlations between metabolites have been revealed with KEGG pathway explorer [11] and their probable roles have been explored in retinal degeneration in progression of diabetic retinopathy.

control-NMR spectra. Consequently, riboflavin, has been found to be downregulated in DR due to its breakdown during disease progression. Glucose levels (Peak no. 3.889 ppm (s), 3.538 ppm (s), 3.457 ppm (m)) in case of both Diabetics and DR have been found to be higher compared to control. The expression of Uridine Diphosphate-N-acetyl glucosamine also found to be upregulated in case of DR compared to Diabetes and diabetic retinopathy. Riboflavin has been found to be significantly down regulated in DR compared to diabetes and normal condition. Chemical shifts of major metabolites have been identified through comparison with HMDB database have been mentioned in Table 1. The intensity of each metabolite has been shown in stacked format in Figure 1 (a, b, c).

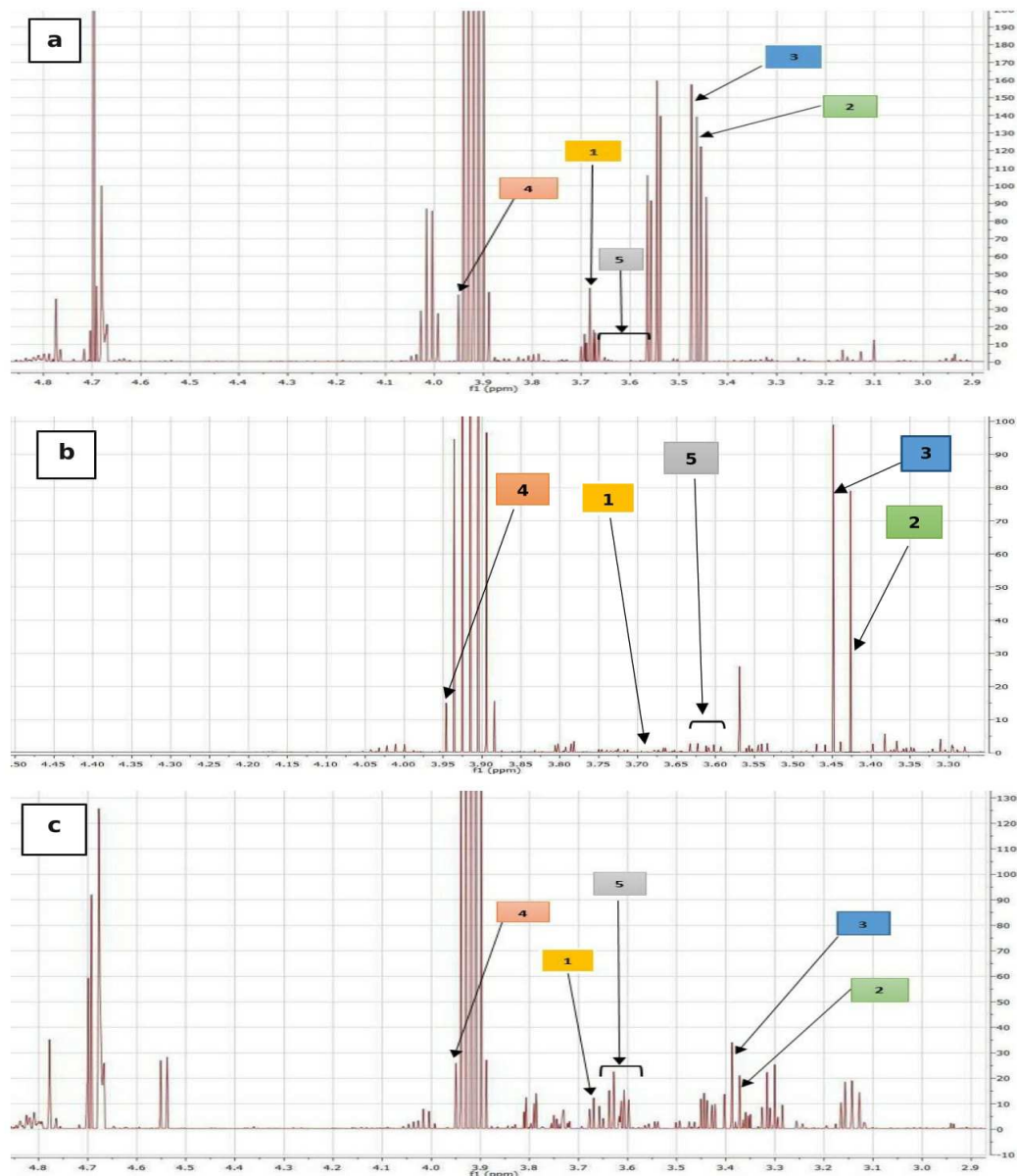


Figure 1: Stacked ^1H NMR spectra of a. Diabetic Retinopathy; b. Diabetes Sample; c. Normal Sample 1. Ribitol; 2. Fructose-6-Phosphate; 3. D-glucose; 4. Uridine Diphosphate-N-acetyl glucosamine; 5 Glycerophosphocholine

Table-1: Chemical shift of major metabolites identified through comparison with HMDB database.

Metabolites	¹ H NMR chemical shifts (ppm)		Altered status in diabetic retinopathy	Spectra type
	Found	HMDB database		
Ribitol	3.98	3.8	Upregulated	Multiplets
	3.68	3.68	Unchanged	Singlet
	3.897	3.83	Upregulated	Multiplets
	3.709	3.70	Unchanged	Singlet
Fructose-6-phospate	3.947	3.94	Upregulated	Singlet
	3.545	3.54	Upregulated	Singlet
D-glucose	3.899	3.897	Upregulated	Singlet
	3.538	3.536	Upregulated	Multiplets
Uridine Diphosphate-N-acetyl glucosamine	3.553	3.553	Unchanged	Singlet
	3.899	3.867	Upregulated	Multiplets
Glycerophosphocholine	3.64	3.645	Downregulated	Singlet
	3.59	3.598	Downregulated	Singlet
	3.58	3.572	Downregulated	Singlet

Discussion

Ribitol (pentitol, sugar alcohol, polyol) is a metabolic end product produced by the reduction of ribose in human fibroblasts and erythrocyte. Export of ribitol across the cell membrane indicates that can be expelled from the body without metabolic transformation. Several inborn defects of metabolism with abnormal polyol concentrations in body fluids are known to date. Most of these defects can be diagnosed by the assessment of urinary concentrations of polyols. Microvascular complications like diabetic retinopathy are associated with cellular accumulation of sugar alcohols since excessive amount of glucose triggers polyol or sugar alcohol metabolism and consequent buildup of polyols in cells [12]. World-wide metabolic screening does not include the assessment of polyols in body fluids and presence of ribitol in serum of DR patients has not been reported so far. Elevated levels of Ribitol and D-Arabitol in all body fluids have been indicative of abnormal polyol metabolism in leukoencephalopathy and peripheral neuropathy of unknown origin [13]. Here in this study first time we reported elevated levels of ribitol in blood associated with abnormal polyol metabolism may be contributory to development of diabetic retinopathy and progressive retinal neurodegeneration. Four peaks confirmed the presence of ribitol in blood of diabetic retinopathy affected individuals (Peaks no. 3.98 ppm, 3.68 ppm, 3.897 ppm, 3.709 ppm).

Presence of Uridine Diphosphate-N-acetylglucosamine was evident from the appearance of identification of three peaks at 3.553 ppm, 3.889 ppm and 3.924 ppm. Uridine Diphosphate-N-acetylglucosamine (uridine 5'-diphosphate-GlcNAc, or UDP-Glc-NAc) is an acetylated aminosugar nucleotide. UDP-GlcNAc is the substrate for alteration of nucleocytoplasmic proteins at serine and threonine residues with N-acetyl glucosamine (O-GlcNAc). Nutrient recognizing in mammals is done through the hexosamine biosynthetic pathway (HSP), which generates uridine 5'-diphospho-N-acetylglucosamine (UDP-Glc-NAc) as its end product. Mammals respond to nutrient excess by activating O-GlcNAcylation (addition of O-linked N-acetylglucosamine). O-GlcNAc addition (and elimination) is important to histone remodeling, transcription, apoptosis, proliferation and proteasomal degradation. This nutrient-responsive signaling cascade also modulates insulin signaling pathway. Alterations in O-GlcNAc metabolism are connected with various human diseases including diabetes mellitus and neurodegeneration [14]. Due to the chemical makeup of UDP-GlcNAc, it is well suited to serve as a glucose sensor in that it is a high-energy metabolic intermediate that requires and/or responds to glucose, fatty acid, amino acid and nucleotide metabolism for their synthesis. Elevated levels of O-GlcNAc have an effect on insulin-stimulated glucose uptake. These

evidences show UDP-Glc-Nac may have a pivotal role in progressive retinal neurodegeneration and development of diabetic retinopathy.

Three peaks (3.909 ppm, 3.88 ppm, 3.91 ppm) showed the presence of Glycerophosphocholine in blood, which is a key intermediate in Retinol metabolism [15]. It has been observed DR patients showed significant down regulation of Glycerophosphocholine since decreased level of β -carotene or vitamin-A results in a state of retinal damage. Impaired retinol metabolism leads to atypical activation of retinol into 11-cis retinal (chromophore of rhodopsin) and consequent buildup of Retinol Binding Protein (RBP) within cell, which ultimately triggers unusual pigment formation in rod and cone cell facilitating progressive retinal degeneration.

D-glucose and Fructose-6-phosphate levels also found to be increased in diabetes and diabetic retinopathy conditions. The elevated levels of D-glucose and Fructose-6-phosphate indicates higher level of glycolysis to breakdown excess amount glucose. Changes in glucose utilization and redox status in diabetic eyes contribute to the progression of retinopathy by affecting osmolarity, promoting oxidative damage, and altering neurotransmission.

In conclusion the current study successfully identified ¹H-NMR based serum metabolomic fingerprints of diabetic retinopathy showing significant alterations in Ribitol, D-glucose, Fructose-6-phosphate, Uridine Diphosphate-N-acetyl glucosamine and Glycerophosphocholine. The study envisaged that abnormal polyol metabolism and accumulation of ribitol were contributory to the development of diabetic retinopathy. Presence of Uridine Diphosphate-N-acetylglucosamine in blood also indicate its probable role in progressive neurodegeneration in diabetic retinopathy. NMR spectra also indicated that downregulation of glycerophosphocholine is directly related to retinol metabolism and subsequent unusual chromophore synthesis facilitating retinal degeneration.

Conclusion

The results not only indicate that the metabolomic signatures have sufficient evidence to be used as candidate prognosticators for future therapeutic approaches but also have the potential to be developed into a diagnostically useful tool for a comprehensive understanding of pathobiology and disease mechanism in Diabetic Retinopathy.

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