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A COMPARATIVE STUDY OF METABOLITES IN SERUM AND VENOUS WALL OF VARICOSE VEIN PATIENTS WITH NON VARICOSE VEIN PATIENTS USING HR MAS NMR SPECTROSCOPY.

(Dr Ankur Verma[1], Dr Jitendra Kushwaha[2], Dr Raja Roy[3], Dr Sanjeev Kumar Shukla[4],

1.Mch Resident, Institute of Medical Sciences, Banaras University, Lucknow, UP.((Corresponding Author)

2. Professor. Department of General Surgery, King George's Medical University, Lucknow

3. Former Professor, Centre for Bio Magnetic Research, Sanjai Gandhi Post graduate Medical Sciences, Lucknow

4. Senior Scientist, Sophisticated Analytical Instrument Faculty Division, Central Drug Research Institute, Lucknow

Abstract

Introduction:.Various diagnostic modalities for varicose vein are Doppler USG. Pethysmography, venography. By HR MAS NMR spectroscopy metabolic environment of varicose veins can be studied and findings can be utilised in identification of new markers in pathogenesis, diagnosis and treatment..This research work is to study the metabolic environment of varicose veins by HR MAS NMR Spectroscopy and identification of a diagnostic marker and pathway of disease pathogenesis.

Methods: Serum (n=37) and vein tissue (n=30) taken from varicose vein patient and analysed with HR MAS NMR Spectroscopy and compare with serum (n=79) and vein tissue (n=14) of non-varicose patient.

Results: Higher concentrations of lipoproteins found in serum and of metabolites of lipids and proteins (glutamate, alanine, creatine and myoinositol) in venous wall samples of varicose vein patients as compared to non varicose patients.

Conclusion: Higher concentrations of lipoproteins in serum may be suggestive of role of lipoproteins in diagnosis and of lipid lowering agents in treatment of varicose vein. Higher concentrations of metabolites of lipids and proteins in vein tissue indicates an ongoing inflammatory process thus anti-inflammatory drugs may have a role in treatment of disease.

Introduction

Varicose vein disease is a prevalent disease with significant morbidity and is amongst one of the chronic venous disease presentation. Around 15% to 20% of population in India is suffering from chronic venous disease and in northern India approximately half of the women and a third of men are affected[1]. Duplex ultrasound scanning which is a non-invasive, safe and cost-effective

investigation preferred for assessment of obstruction, turbulence and abnormal reflux but there is individual bias that exists according to its technique or experience[2]. For diagnosis of incompetent venous system in obese patients or patients having lower limb filariasis or pelvic congestion syndrome it is less sensitive and in these cases Magnetic Resonance venography or plathysmography is sometimes needed[3]. Recent studies have identified familial clustering of disease with age, occupation, pregnancy, obesity as key risk factors. The morphological characteristics of the disease have been well known, documenting histological changes in the dilated vein including intimal hyperplasia, disruption of smooth muscle cells and extracellular matrix[4]. Varicose veins also exhibit expression of various transcripts and proteins linked to specific genes regulating cell growth and apoptosis, extracellular matrix metabolism and inflammatory processes. Evidences strongly suggest that primary vein wall pathology is a major contributor in the development of varicose veins[5,6].

Identifying new cellular pathways using metabolomics approaches may address the undisclosed biological events, which have not been picked up by other systems biology approaches, including transcriptomics and proteomics. In previous studies concerning pathophysiology of varicose vein it was found that there is over expression of Matrix Metalloproteinases (MMP) and HIF-1α and HIF-2α, increased levels of PGI-2 and TXA-2, role of integrins with metabolic environment of venous wall showing increased levels of lipid and protein metabolites showing cellular inflammation, proliferation and destruction.

This study aims towards exploring the pathogenesis of varicose vein disease or identifying a metabolite that may be used as a marker for early diagnosis or diagnosis of venous insufficiency in patients having thick skin like filariasis, having lymphovenousedema or having incompetent ileofemoral veins where duplex ultrasound usually fails to diagnose venous incompetency.

Methods

During year 2016-2018, all patients admitted in the Department of General Surgery, King George's Medical University, Lucknow for chronic venous disease were assessed with Duplex Doppler scan. The individuals included in the study were C2-C6 disease as per Clinical-Etiological-Anatomical-Pathophysiological(CEAP) classification excluding population of patients having C1 disease, patients having a metabolic disorder like diabetes or uremia, congenital varicose vein or deranged coagulation profile. The study was conducted with collaboration of Department of Centre for Biomagnetic Resonance (CBMR), Sanjay Gandhi PostGraduate Institute of Medical Research (SGPGI) Lucknow and Sophisticated Analytical Instrument Facility(SAIF) Division, CDRI Lucknow. Ethical clearance(65/Ethics/R.Cell-18) was obtained from Regional ethical committee of King Georges Medical University(KGMU), Lucknow.

Vein tissue samples from varicose vein patients were obtained by stripping and ligation of Great saphenous vein or from hook phlebectomy for treatment of varicosities. Similarly, vein samples were taken for controls during amputation of gangrenous limb or during venous cut down procedures. 2ml of serum were collected from both groups in appendobe vial, all samples were stored at-80^0 centigrade.

On the basis of study design, the groups were classified into Varicose Vein(VV) group (n=37) which included patients having varicose vein and Non-varicose vein(NVV) group (n=79) which included healthy individuals and patients not having varicose vein disease. The demographic pattern is shown below.

	Non-Varicose Group	Varicose Group
CEAP Clinical Score	NA	C2=24 C3=11 C4=01 C5=01 C6=00
Sex	Male=60 Female=19	Male=32 Female=05
Age In Years	Mean=40.6year	Mean=34.2year
Peripheral Arterial Disease	08	00
Hypertension	06	00
Diabetes Mellitus	00	00

NMR experimental setup for serum samples

The NMR spectroscopic study was performed, to observe the variations if any, in the metabolic profile of the varicose vein patients and control group. The data set composed of NVV group(n=79) and VV group(n=37). The samples were subjected to NMR acquisition in native form using Trimethylsilylpropanoic acid (TSP) as an internal standard and as a coaxial insert. The NMR experiments were performed using a BrukerBiospinAvanceIII 800 MHz NMR (BrukerGmBH) spectrometer equipped with a 5mm triple resonance inverse (TCI) 1H/13C/15N cryoprobe with a Z shielded gradient and standard vertical bore, operating at a proton frequency of 800.21 MHz (18.8 T). 1H NMR spectra of serum samples were acquired with water pre-saturation at 300 K with 128 scans and 4 dummy scans. The spectra were acquired using 1D single pulse and Carr-purcell-meiboom-gill (CPMG) pulse sequence with the following experimental parameters: spectral width of 12,820.5 Hz, time domain data points of 64 K, a total relaxation delay of 6.55 s, with a total recording time of approximately 17min. CPMG pulse sequence (PRESET–90-(d - 180 - d) n–Aq) with an echo time of 100ms for samples were performed. All the spectra were processed by applying a line broadening of 0.3 Hz to the FID prior to Fourier Transformation. The recorded spectral data of the samples were pre-processed through manual phase correction followed by absolute baseline correction. Further, they were then characterized as reported in literature and compared with standard NMR spectra of metabolites available in the biological magnetic resonance bank (BMRB, www.bmrb.wisc. edu), Human metabolome data

base (HMDB, www.hmdb.ca) and through NMR suite 8.1 (Chenomx) software.

Statistical analysis

In order to observe the perturbation and significance of the metabolites, for differentiating the groups, a quantification was performed using Quantas (Quantification by Artificial Signal) which is a software based protocol for concentration measurement by externally generating an artificial signal at−1 ppm chemical shift in the 1H CPMG NMR spectra. Well-resolved metabolite signals were integrated manually, and the concentration of the metabolites was calculated in mmol/l, with respect to known concentration of TSP from their respective integral values. Further, in order to classify the groups among cases, the 1H CPMG NMR spectral regions were reduced to discrete binned regions between 0.66 and 8.5 (excluding the region responsible for water i.e. 4.5–5.15 ppm and 5.4-6.75 ppm) of 0.01 ppm bucket size using Bruker AMIX software(ver 3.8.7, BrukerBiospin). The data normalization was performed using sum of absolute intensities in integration mode scaled to total intensity. These binned regions underwent orthogonal signal correction (OSC) using two components and followed by OPLS-DA with full cross validation using 'The Unscrambler X' Software package (ver 10.0.1, Camo ASA).

NMR Experimental Setup for vein tissue samples

At the time of the NMR experiment, the frozen veins sample was thawed at room temperature, samples were washed with D_2O and 30-35 mg wet weight of sample placed into a 4 mm zirconia rotor with a 50 µl capacity. 20 µl of D_2O containing 0.25% TSP was added to the rotor with the veins sample for locking of the spectrometer frequency and TSP served as an internal standard. All NMR experiments were carried out on a BrukerAvance-II 400 MHz spectrometer, operating at a 1 H frequency of 400.13 MHz, equipped with a 5 mm HR-MAS 13C–1 H Z gradient probe with a magic angle gradient, at a room temperature and a sample spinning rate of 4000±1 Hz. Data acquisition and processing were done with Bruker Topspin 2.1.

One dimensional NMR analysis

1 H NMR spectra were acquired using the standard 1D (noesypr 1D) pulse program, a 2.0 s relaxation delay (d1), a 1.99 s acquisition time (AQ), 32K FID data points, a spectral width of 8223.685 Hz and a number of scans of 128. For experiments using presaturation, the transmitter offset was manually set at 4.689 ppm in order to achieve optimal suppression of the residual water signal. The pulse width P1 (15.47 µs) and power level PL9 (55.30 dB) were calculated using command pulsecal for each sample. The calculated P1 and PL9 were almost the same for each sample. 1D T2 relaxation edited 1 H NMR spectra were acquired using the CPMG pulse sequence [RD-90°-{τ -180- τ}n acquisition] and simple presaturation of the water peak was used as a T2 filter to suppress broad signals arising from the macromolecules. All 1D spectra were acquired with 128 transients, 32k complex data points, a spectral width (SW) of 8223.685 Hz and a 2 s relaxation delay, with approximately 10 minutes of acquisition time in each CPMG experiment. The acquired FIDs were Fourier transformed with LB 1Hz, Manual phase correction and automatic baseline correction was performed on each sample. The right side signal of Alanine doublet was

calibrated at 1.47 ppm for all CPMGPR1D spectra.

Multivariate analysis

The CPMG PR1D 1 H NMR spectral range of 0.6 ppm to 5.6 ppm was taken for binning the data to produce a series of sequentially integrated regions of 0.01 ppm in width, which gave 521 equal segments of a spectrum in the sum of intensities integration mode, and spectral intensities were scaled to the total intensity using Bruker AMIX software (version 3.5.5. BrukerBiospin, Germany). The resulting data binning matrices or bucket files having normalized integral values were exported into Microsoft Office Excel 2010. These were further imported to 'The Unscrambler X' software package (version 10.0.1, Camo USA, Norway) for multivariate principal component analysis (PCA).

Results

The 1H NMR characteristic spectra from serum of VV group and NVV group shown in figure 1. There are clearly identified peaks of lipoproteins in varicose vein group. The difference is significant with p value <0.05

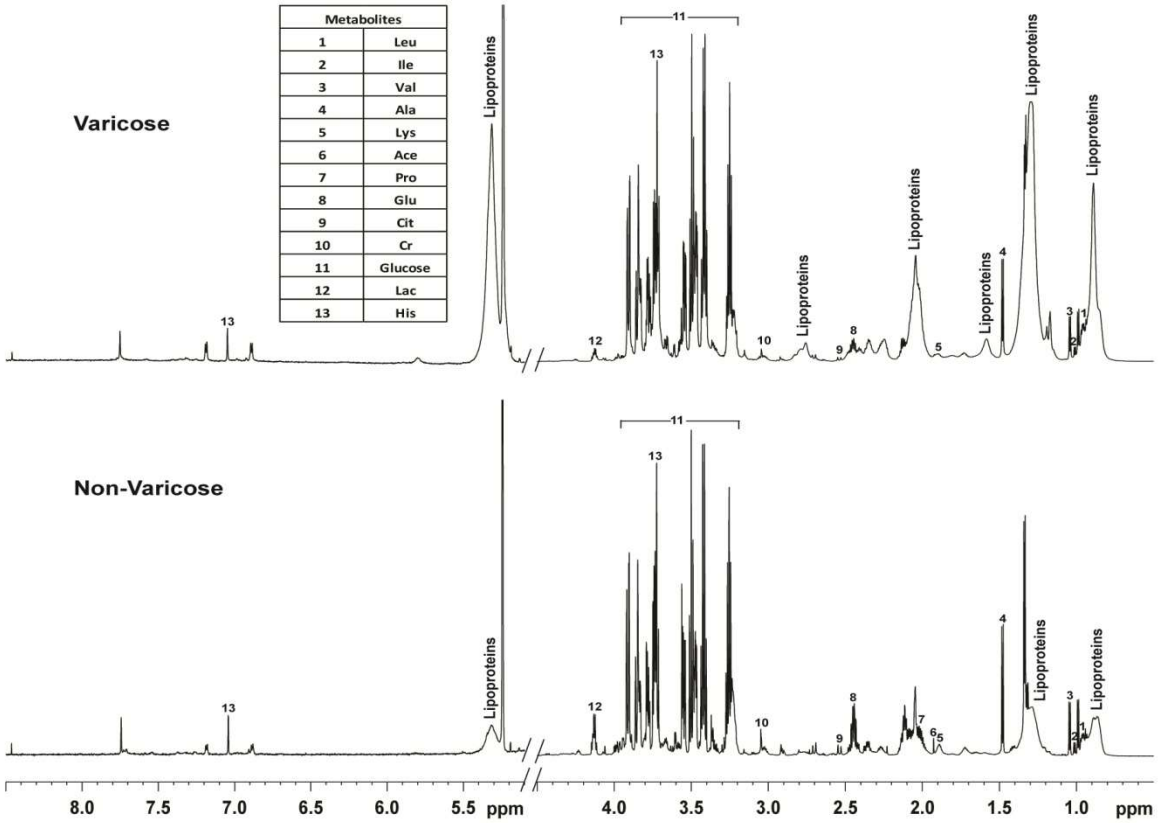

Fig. 1: Carr-Purcell-Meiboom-Gill(CPMG) spectra of varicose and non-varicose serum with associated metabolites

The orthogonal partial least square (OPLS) analysis separating the two classes (varicose and non-varicose) based on their metabolic profiles in serum samples detected using solution state NMR spectroscopy. Probabilistic quotient normalization and unit variance settings were applied for data analysis with $R^2$0.91 and Q^2 was 0.85 with 2-D scatter plot of 1H NMR spectra as shown in figure2. Here it is clearly visible that both the groups are separated enough.

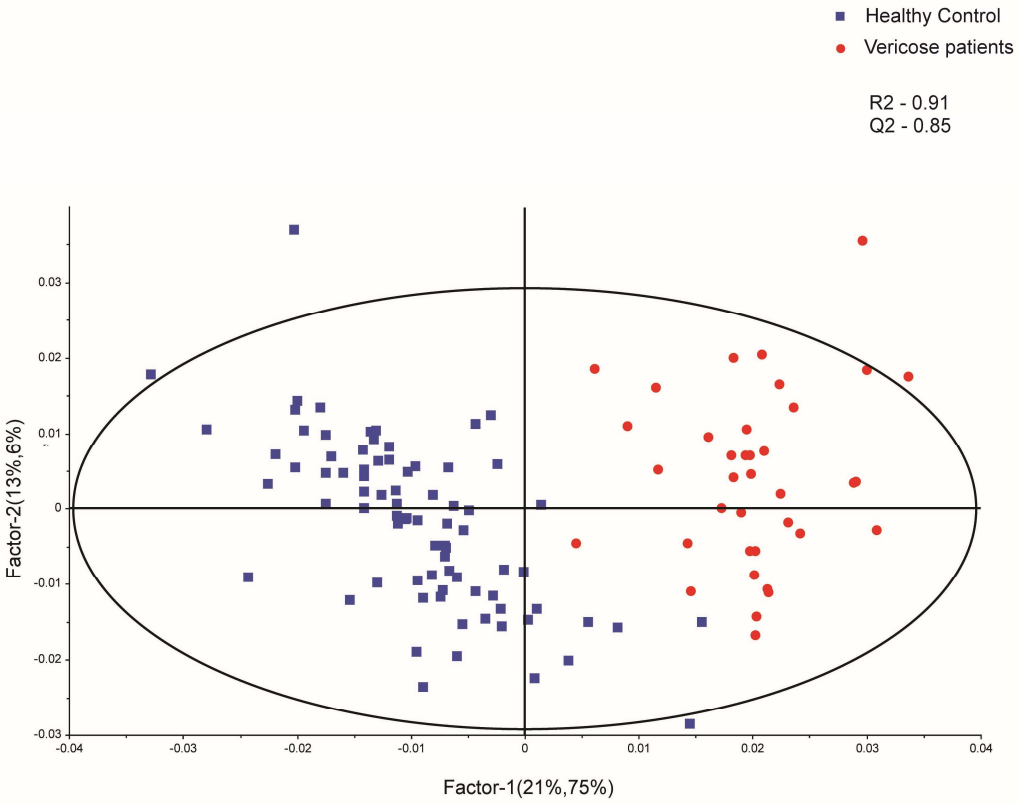

Fig 2: 2D OPLS-DA Scatter plot of 1H NMR spectra of Varicose versus Non-Varicose Serum

Figure3 showing 1D OPLS-DA X-Loading plot of serum metabolites with positive deflection of metabolites ($P<0.05$) in VV group and negative deflection of NVV supporting that VV group serum have higher levels of lipoproteins, glutamate, n-methyl histidine than non-varicose vein patients. Whereas, NVV group have higher levels of lactate, alanine and leucine.

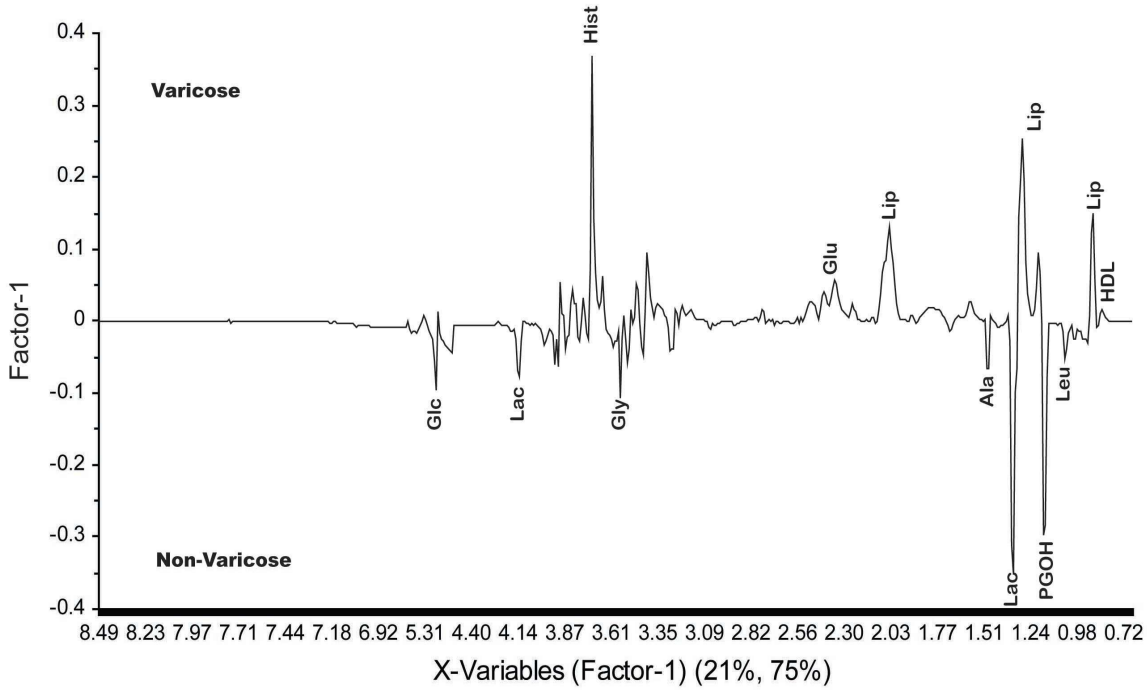

Fig3: 1D OPLS-DA X-Loading Plot of Varicose versus Non-Varicose Serum Spectra

1H NMR characteristic spectra of venous tissue from VV group(n=30) and NVV group(n=14) spectra are shown in Fig. 4. Spectral similarities within each group and differences between the two groups of veins were evident. The large peaks of lipids (unsaturated and saturated) were found in abundance in the NVV group. Creatine, myo-inositol, lactate and glutamate were found in relatively higher levels in VV group.

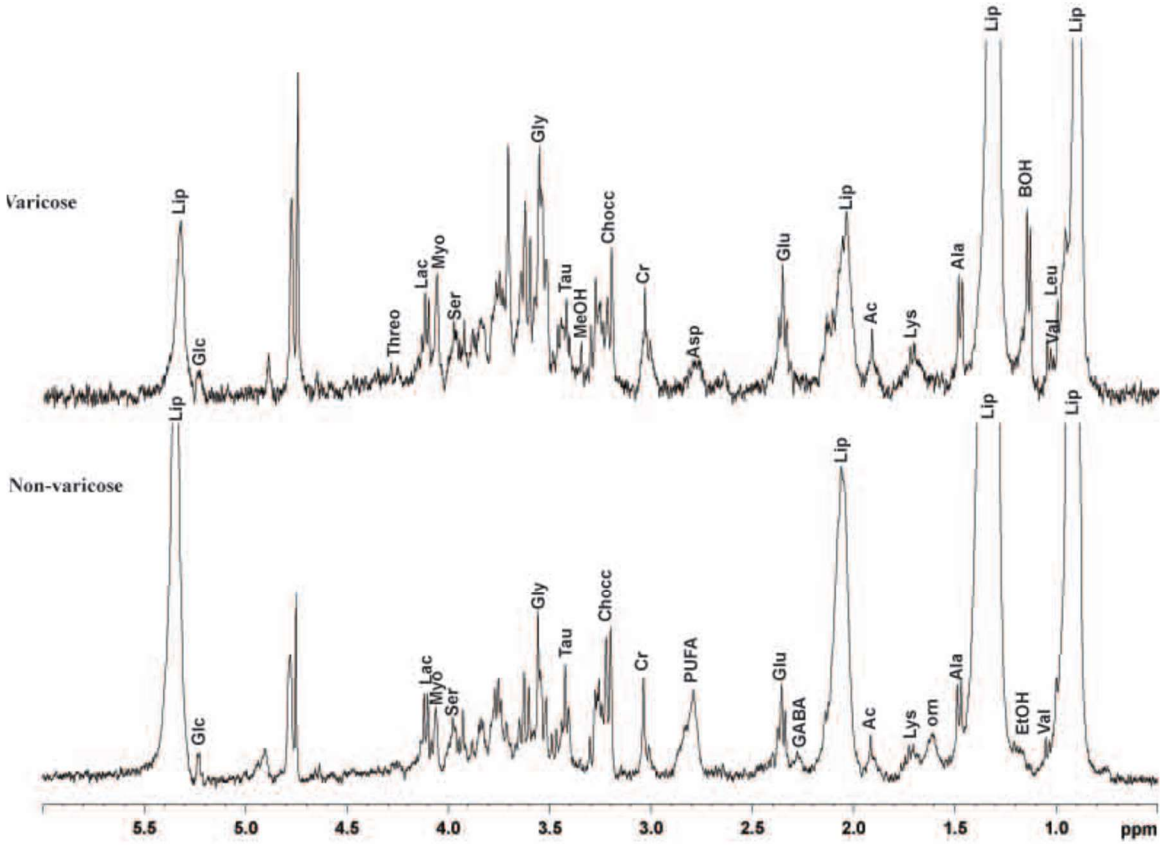

Fig 4: Representative 1H HRMAS NMR spectra of Non-varicose vein and Varicose vein tissue samples

Analysis of the PCA 3D Scatter plot of CPMG spectra of venous tissue of VV group and NVV group as shown in fig. 5 showing clear differentiation of the two groups with PC1 86% and PC2 6%.

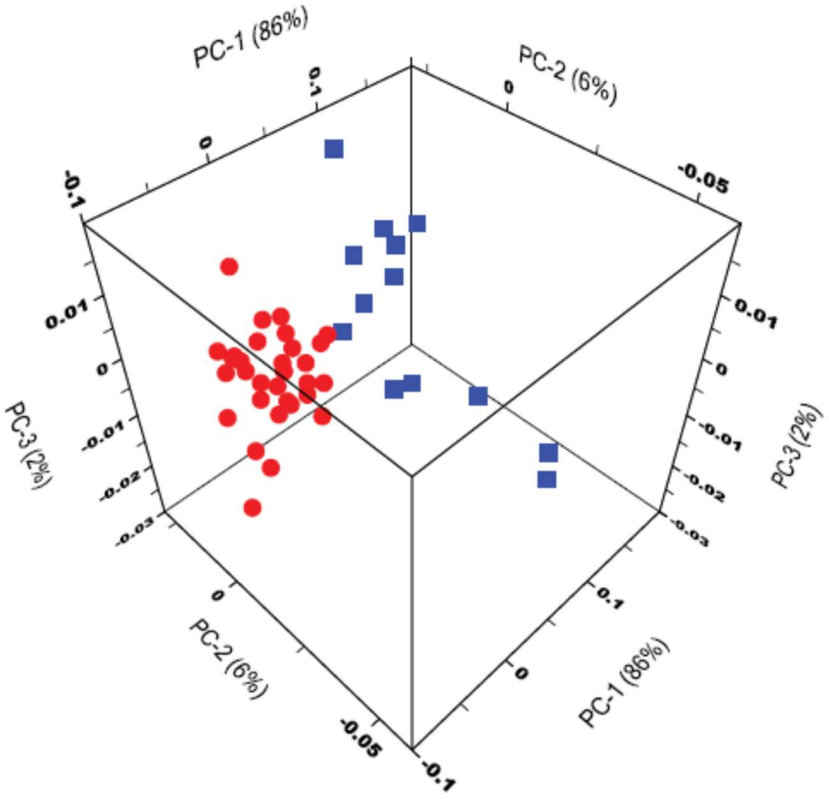

Fig 5: PCA 3D scatter plot of 1H HRMAS NMR spectra of Non-varicose vein versus Varicose vein tissue samples.

Figure 6 showing 1D PCA X-Loading plot of venous tissue samples of VV group and NVV group having positive deflection indicative of metabolites in NVV group and negative deflection showing metabolites in VV group. Lipids, both saturated and unsaturated were higher in NVV group and lysine,taurine, glutamate, creatine, lactate, and myoinositol were higher in VV group tissue samples.

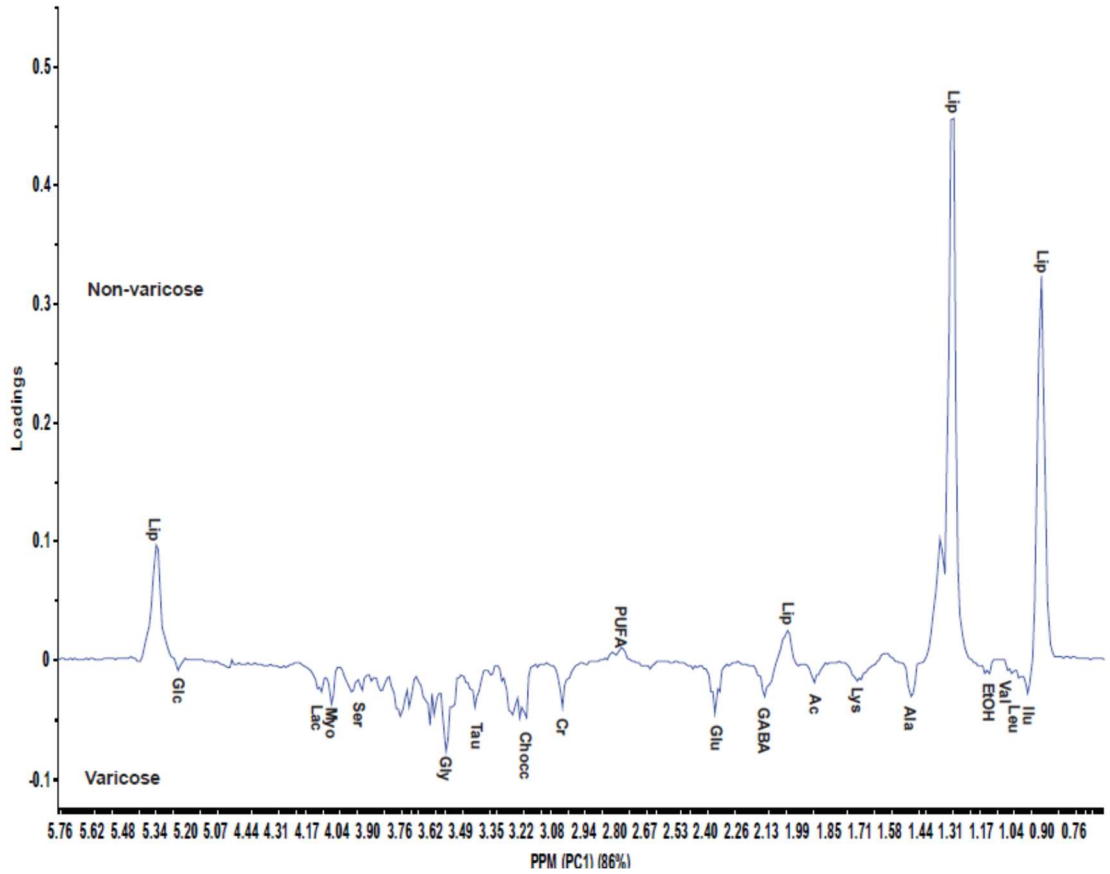

Fig 6 :1D PCA X-Loading plot of Non-varicose vein versus Varicose vein tissue samples

Discussion

In this study we found higher levels of lipoproteins, glutamate and n-methyl histidine in serum of patients having varicose vein. Lipopoproteins are complex molecules with high molecular weight which may be increased due to stasis of blood in varicosities.Lactate and alanine were found to be elevated in the serum samples of non-varicose group. Among vein tissue samples of varicose vein patients having lipid metabolites like myo-inositol and metabolites of amino acids like glutamate, creatine, lactate, leucine, taurine in higher concentration suggestive of increased breakdown of protein and lipids in wall of varicose vein, whereas lipids are intact in vein of non-varicose patients. Pathway analysis provided a potential network of molecular interactions between these metabolites; this network paves the way for further investigation of disease etiology.

Lipoprotein is a biochemical assembly whose primary purpose is to transport hydrophobic lipid molecules .Many enzymes, transporter molecules, antigens, structural proteins, toxins are lipoproteins. It has been demonstrated that lipoproteins, especially HDL and LDL, have important roles in the inflammatory process[7]. Increased levels of these lipoproteins may be associated with increased cardiovascular risk among these patients in future which is not yet established. There

may be role of lipid lowering drugs in these patients similar to established role of flavonoids.

Glutamate one of the key metabolite in many aspects of cellular metabolism, and plays a central role in hepatic amino acid metabolism, glycolysis, gluconeogenesis and the tricarboxylic acid cycle[8]. In addition glutamate is also involved in the production of gamma-aminobutyric acid (GABA) and glutathione[9]. Glutathione, being one of the primary conjugative molecules of phase two detoxification, is an important factor in the cellular defense mechanism and also helps regulate cell proliferation and apoptosis[10]. However there were no discernable changes in glutathione levels detected in the samples.

Taurine, a ubiquitous organic osmolyte, is one of the most abundant amino acids within the body, although it is not involved in protein synthesis. Metabolically derived from cysteine, taurine is involved in the formation of bile salts, regulation of cell volume and modulation of intracellular calcium concentration, essential for the function of signal transduction pathways[11]. Administration of taurine has also been shown to protect from stress-induced vascular endothelial cell apoptosis[12,13], and high levels of taurine is thought to promote cell survival.

Excess **lactate** in serum of samples of control group represents increased anaerobic metabolism of pyruvate by the lactate dehydrogenase (LDH) enzyme,which occurs in a number of states including hypoxia as some patients are having peripheral arterial disease[14].

Myo-inositol, one of the more abundant inositol isomers, is a carbocyclic polyol and is also a structural component of a number of secondary cellular messengers such as inositol phosphates, phosphatidylinositol and phosphatidylinositolphosphate lipids. Myo-inositol, like taurine, predominately operates as an intercellular osmolyte, and is also involved in the Akt-signalling pathway, membrane trafficking and regulating apoptosis. Cytosolic myo-inositol is essential for cell survival and promotes growth and proliferation[15].

Creatine is a nitrogenous organic acid originating from the diet,
and also synthesised in the liver. It is stored in skeletal muscle
where it acts as a cellular energy source[16].Intracellular adenosine triphosphate (ATP) transfers its phosphate group to creatine to form phosphocreatine.25-Phosphocreatine is found in a concentration ranging between 20 and 35 mM in skeletal muscle cells and 5-10 mM in other excitable tissue including brain and smooth muscle[17].

Studies that have already been done for recognizing the metabolic environment in varicose vein patients have shown that higher concentrations of glutamate, taurine, myoinositol, creatine, inosine, phosphatidylcholine, phosphatidylethanolamine and sphingomyelin were present in the Varicose Vein group and triglycerides in non-Varicose Vein group along with differentially expressed miRNAs in VV group i.e., hsa-miR-642a-3p, hsa-miR-4459 and hsa-miR-135a-3p correlated with expression of inosine while miR-216a-5p was correlated with phosphatidylcholine and phosphatidylethanolamine[18,19]. This study has presented a metabolic characterization of serum and vein wall of patients having the VV disease, highlighting the molecular processes underlying VV pathogenesis and progression.

The identified metabolites suggest that the processes of cell proliferation and survival should be further investigated with a view to identify potential therapeutic drug targets. Development of drug therapy in venous disease may help in halting the disease progression as well as a preventative measure for individuals with a genetic propensity for the development of the disease. Factors such as age, medications and past medical history may influence the metabolic profile of vein tissue. However, to our knowledge, there has been no demonstration of the effect of age on metabolic profile of vein tissue so far. The control group included great saphenous vein samples from patients undergoing venous cut down or amputations, which are subject to different biomechanical stresses. These patients specially who underwent amputation are on cardiovascular medications which have a short half-life (less than 24 hours) and neither parent drugs nor their metabolites were detected in our NMR , it is possible that the above factors confounded the presented results by pharmacological effect[20]. It is, nonetheless, important to stress that despite these issues the varicose vein samples were consistently different from the control groups in the statistical modeling, suggesting that any variability within the non-varicose veins groups was far outweighed by the differences between the two groups. Another consideration for the future is CEAP classification, the scheme by which the disease is classified into different grades based on severity and type of presentation, and how CEAP grading relates to metabolic phenotypes[21]. Future studies should potentially explore any differences of metabolites in tissue, serum and urine amongst patients of different disease grades. This could aid clinicians in following disease progression and help tailor therapy accordingly.

Varicose vein disease is a multifactorial disease influenced by both environmental factors such as occupation, obesity, pregnancy and also has a strong familial predisposition. Environmental stimuli in the patient exposes the vein wall to high pressure and hypoxia, which in combination trigger molecular changes in the vein wall leading to vein wall relaxation and dilatation. This work has shown that the metabolic profiles of human varicose veins are systematically different from non-varicose veins tissues, also exemplifying the utility of the metabonomic approach in understanding the molecular changes at cellular level of vascular disease.

Conclusion

This preliminary study demonstrates differential metabolic expression in varicose vein patients as compared with non-varicose vein patients. There is evidence of increased metabolites of proteins and lipids and these differences are not affected by age or sex. Lipids and lipoproteins are relatively higher in serum of varicose vein patients. In future, study of a larger sample size may provide effective treatment for chronic venous disorder and serum analysis may be utilized as an alternative method or as an indicator for response to this treatment. Elucidating the metabolic signature underlying varicose vein disease has the potential to further improve our understanding of biological mechanisms of disease initiation and progression, identify putative biomarkers and targets for prevention and treatment and merits further explanation.

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