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ORIGINAL ARTICLE

# Quantitative proteomics analysis of varicose veins: Identification of a set of differentially expressed proteins related to ATP generation and utilization

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**Abstract** Although morphological and anatomical studies indicate that varicose veins are characterized by venous wall weakening and subendothelial fibrosis, the exact underlying biochemical mechanism of their development remains unknown. Additionally, no quantitative proteomic study of venous proteins leading to decreased contractility of varicose veins has been reported to date. Therefore, to elucidate the molecular mechanism of altered vascular contractility, this study performed shotgun proteomic analysis to obtain protein expression profiles in patients with varicose veins. Stable isotope dimethyl labeling coupled with nanoLC-MS/MS revealed downregulation in 12 polypeptides, including myosin light chain kinase, creatine kinase B-type, ATP synthase, phosphoglycerate kinase, and pyruvate kinase. However, analyses of protein species associated with cytoskeletal assembly or with cellular morphology showed no clear up- or down-regulation. These results indicate that defects in ATP generation and utilization may account for the dysfunction

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of vascular smooth muscle following formation of varicose veins. Collectively, the severity of varicose veins depends on the regulatory roles of various protein factors in the metabolic coordination of physiological functions. This pilot study improves understanding of the pathogenesis of varicose veins and lays the foundation for further validation and clinical translation of biomarkers for targeted therapies in treating this disease.


## Introduction

The most common sites of varicose veins are the lower limbs throughout the long saphenous vein and its tributaries [1]. The exact causes of varicose veins are unknown, but factors known to affect the prevalence and incidence of varicose veins include genetic factors, gender, and diet. The disease has also been associated with insufficient physical activity, obesity, occupational activity involving long periods of standing, and advanced age [2—7]. Varicose veins, which are the most common manifestation of chronic venous diseases, affect 25% of women and 15% of men. Up to 80% of the general population has spider veins [8]. An endoscopic-assisted technique for managing primary varicose veins developed by Lin et al. has proven effective for visualizing and clearly identifying characteristic features of varicosities, including supernumerary tributaries, saccular or lateral bulging deformities of vein walls, poor contractility, dilation and tortuous changes in varicose veins, and varicosity of the main channel, its tributaries, and the incompetent perforating veins [1,9,10].

Conservative approaches to reducing the pain and edema associated with varicose veins include compression stockings and oral venotonic medication. Endovenous therapies, including endovenous laser therapy, radiofrequency ablation, and endovenous foam sclerotherapy, have shown low complication rates [8,11—13]. Another hypothesized contributor to the development of varicose veins is cellular aberrations caused by disturbance of the muscle cell/collagen/elastic fiber balance with an unknown genetic defect that causes loss of contractility [14]. Histological studies also show that structural remodeling characterized by accumulations of collagen, proteoglycans, and elastic fibers in smooth muscle cells may decrease venal elasticity while concomitantly increasing inflammation and thrombus [15—17]. Therefore, in smooth muscle cells, dysfunction caused by the cytoskeleton, extracellular matrices or protein factors may be at least partly to explain the partial or total loss of contractile force observed in varicose veins. Although the morphological and pathogenic mechanism(s) underlying the above abnormalities associated with varicose veins have been studied intensively, no quantitative study has analyzed proteomic profiles of venous proteins to elucidate the biochemical development of varicose veins.

Emerging technologies for proteomic analysis have recently made analyzing and identifying complex protein mixtures in biological tissues less tedious and more amenable to routine analysis [18,19]. Although biological research tends to emphasize comprehensive global analysis of cellular systems, reliable and high-throughput analyses of proteome-wide analysis of proteins were not possible until the advent of modern proteomics instrumentation. Proteomic methodologies for molecular and cellular studies of proteins are rapidly evolving and now provide a firm basis for understanding the complex proteome profiles of total protein mixtures from whole tissues or cells from various sources [20].

To elucidate the pathogenesis of altered vascular contractility, this study performed gel-free shotgun proteomic analysis [18,19] coupled with stable isotope dimethyl labeling [18,19,21] and nanoLC-MS/MS [21—23] to characterize and compare the proteins expressed in varicose veins and in nearby portions of normal tissues in the same veins. The quantitative proteomic analysis of differential protein expression levels of normal versus varicose veins revealed at least 455 protein species with high confidence levels. Of these, 12 proteins, which included ATP-generating, ATP-utilizing, and glycolytic enzymes, revealed significant downregulation between normal and diseased tissues. We hypothesized that, as the development of varicose veins progresses, systematic decreases in these enzymes may cause vascular smooth muscle dysfunctions leading to morphological aberrations.

Collectively, varicose veins involve various protein factors with regulatory roles in the metabolic coordination of physiological functions. To our knowledge, this study is the first to describe the pathogenesis of varicose vein formation at the translational level of expressed protein molecules. This pilot study provides data needed for further validation and clinical application of identified biomarkers in targeted therapies for treating varicose veins or arteries.

## Materials and methods

### Chemicals and reagents

Quantitative reagent for protein contents was purchased from Bio-Rad (Hercules, CA, USA). Trichloroacetic acid (TCA), trifluoroacetic acid (TFA), dithiothreitol, iodoacetamide (IAM), ethylenediaminetetraacetic acid (EDTA), sodium deoxycholate, sodium fluoride, formaldehyde-$H_2$, formaldehyde-$D_2$, ammonium bicarbonate ($NH_4HCO_3$), and Triton X-100 were purchased from Sigma Aldrich (St Louis, MO, USA). Acetonitrile (ACN) and sodium phosphate were obtained from Merck (Darmstadt, Germany). Formic acid (FA), sodium acetate, sodium cyanoborohydride and sodium chloride (NaCl) were purchased from Riedel-de Haven (Seelze, Germany). Protease inhibitors (Complete Mini) were purchased from Roche (Mannheim, Germany). Sodium dodecyl sulfate (SDS) and urea were purchased from Amresco (Solon, OH). Modified sequencing-grade trypsin for in-gel digestion was purchased from Promega (Madison, WI, USA). Water was deionized to 18 MΩ by a Milli-Q system (Millipore, Bedford, MA, USA).

## Sample collection

All procedures used in this study were approved by the clinical research ethics committee at Kaohsiung Medical University Hospital. Varicose vein tissue and segmental parts of normal vein samples adjacent to varicose veins as control were obtained from four limbs of four patients who underwent the endoscopic-assisted removal of the varicose veins.

Normal or varicose vein tissue specimens (1 g) were homogenized with a Polytron homogenizer in 1.5 mL extraction buffer containing 10 mM Tris-HCl pH 7.4, 10 mM sodium phosphate, 150 mM NaCl, 0.1% SDS, 2 mM EDTA, 1% sodium deoxycholate, 100 mM sodium fluoride, 1% Triton X-100 and protease-inhibitor cocktail. The homogenates were transferred to 1.5 mL Eppendorf tubes and centrifuged at 13,000*g* for 20 minutes at 4°C to remove debris and insoluble material. Aliquots of the supernatants were assayed with Coomassie protein assay reagent to determine total protein concentration and then stored at −80°C until further analysis.

## Dimethyl labeling and peptide preparation

Volumes of lysates containing 100 μg of total proteins from normal or varicose veins were adjusted to 60 μL and treated with 0.7 μL of 1 M DTT and 9.3 μL of 7.5% SDS at 95°C for 5 minutes before reduction. After the reaction, lysates were further treated with 8 μL of 50 mM IAM at room temperature for 30 minutes alkylation in the dark; the proteins were precipitated by adding 52 μL of 50% TCA followed by incubation on ice for 15 minutes. After removing the supernatant by centrifugation at 13,000*g* for 5 minutes, the collected proteins were washed with 150 μL of 10% TCA, vortexed, and centrifuged at 13,000*g* for 10 minutes. The precipitated proteins were rewashed with 250 μL distilled $H_2O$, vortexed and centrifuged under the same condition three times. The resulting pellets were then resuspended with 50 mM $NH_4HCO_3$ (pH 8.5), digested with 4 μg of trypsin for 8 hours at 37°C, and dried in a vacuum centrifuge to remove $NH_4HCO_3$. Lyophilized peptides for normal and varicose vein samples re-dissolved in 180 μL of 100 mM sodium acetate at pH 5.5 were then treated with 20 μL of 4% formaldehyde-$H_2$ and 20 μL of 4% formaldehyde-$D_2$, respectively [21,23], and mixed thoroughly. Immediately after vortexing for 5 minutes, 20 μL of 3 M sodium cyanoborohydride were added vortexed for 1 hour at room temperature. The resultant liquids were acidified by 10% TFA/$H_2O$ to pH 2.0—3.0 and applied onto the reverse phase C18 column pre-equilibrated with 200 μL of 0.1% TFA/$H_2O$ (pH 2.0—3.0). The column was also washed with 200 μL of 0.1% TFA/$H_2O$ (pH 3.0) and then eluted with a stepwise ACN gradient from 50% to 100% in 0.1% TFA at room temperature.

## Hydrophilic interaction chromatography for peptide separation

Hydrophilic interaction chromatography (HILIC) was performed on an L-7100 pump system with quaternary gradient capability (Hitachi, Tokyo, Japan) using a TSK gel Amide-80 HILIC column (2.0 mm × 150 mm, 3 μm; Tosoh Biosciences, Tokyo, Japan) [24—26] with a flow rate of 200 μL/minute. Two buffers were used for gradient elution: solvent (A), 0.1% TFA in 100% ACN, and solvent (B), 0.1% TFA in water. Each fraction eluted from the reverse-phase C18 column was dissolved in 50 μL of solution containing 85% ACN and 0.1% TFA and then injected into the 100 μL sample loop. The gradient was processed as follows: 0—2% (B) for 4 minutes; 2—8% (B) for 4 minutes; 8—12% (B) for 4 minutes; 12—35% (B) for 54 minutes; 35—100% (B) for 12 minutes; and

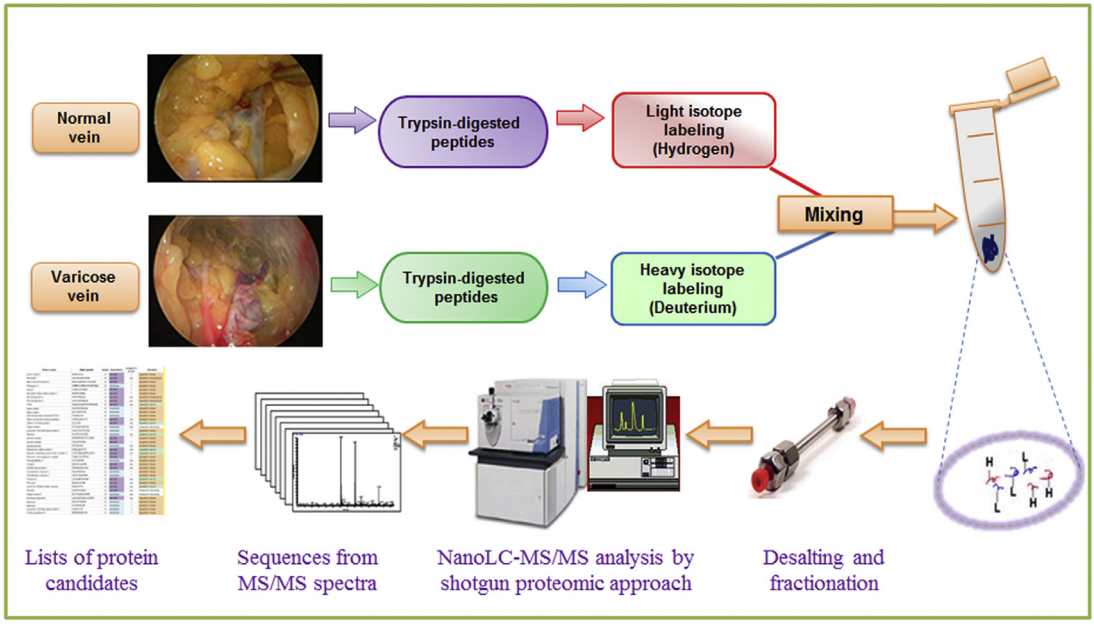


**Figure 1.** Schematic representation of the procedures used to analyze and compare protein expression. Quantitative shotgun analyses of proteomic changes in clinical samples of varicose vein tissues were performed by using HILIC-C18 for peptide separation and nanoLC-MS/MS coupled with stable isotope dimethyl labeling.

100% (B) for 2 minutes. Ten 1-mL fractions were collected and dried in a vacuum centrifuge.

## NanoLC-MS/MS analysis

The lyophilized powders were reconstituted in 10 μL of 0.1% FA in $H_2O$ and analyzed by LTQ Orbitrap XL (Thermo Fisher Scientific, San Jose, CA, USA). Reverse phase nano LC separation was performed on an Agilent 1200 series nanoflow system (Agilent Technologies, Santa Clara, CA, USA). A 10-μL sample of collected fractions was loaded onto an Agilent Zorbax XDB C18 precolumn (0.35 mm, 5 μm) and separated with a C18 column (i.d., 75 μm × 15-cm, 3 μm). The mobile phases used were: (A) 0.1% FA and (B) 0.1% FA in 100% ACN. A linear gradient from 5% to 95% of (B) was applied over a 70-minute period at a flow rate of 300 nL/minute. The peptides were analyzed in positive ion mode by applying a voltage of 1.8 kV to the injection needle. The MS was operated in a data-dependent mode, in which one full scan in the Orbitrap was performed at *m/z* 400—1600 and at a scan rate of 30 ms/scan. Fragmentation was performed in CID mode with a normalized collision energy of 35 V. A repeat duration of 30 seconds was applied to exclude the same *m/z* ions from the reselection for fragmentation. An in-house software system (Xcalibur, version 2.0.7, Thermo-Finnigan Inc., San Jose, CA, USA) was used to manage instrument control, data acquisition, and data processing.

## Protein database search and characterization

Peptides were identified by peak lists converted from the nanoLC-MS/MS spectra by bioinformatic searching against *Homo sapiens* taxonomy in the Swiss-Prot databases for exact matches using the Mascot search program (http://www.matrixscience.com) [27,28]. Parameters were set as follows: mass tolerance, 10 parts/million for precursor ions

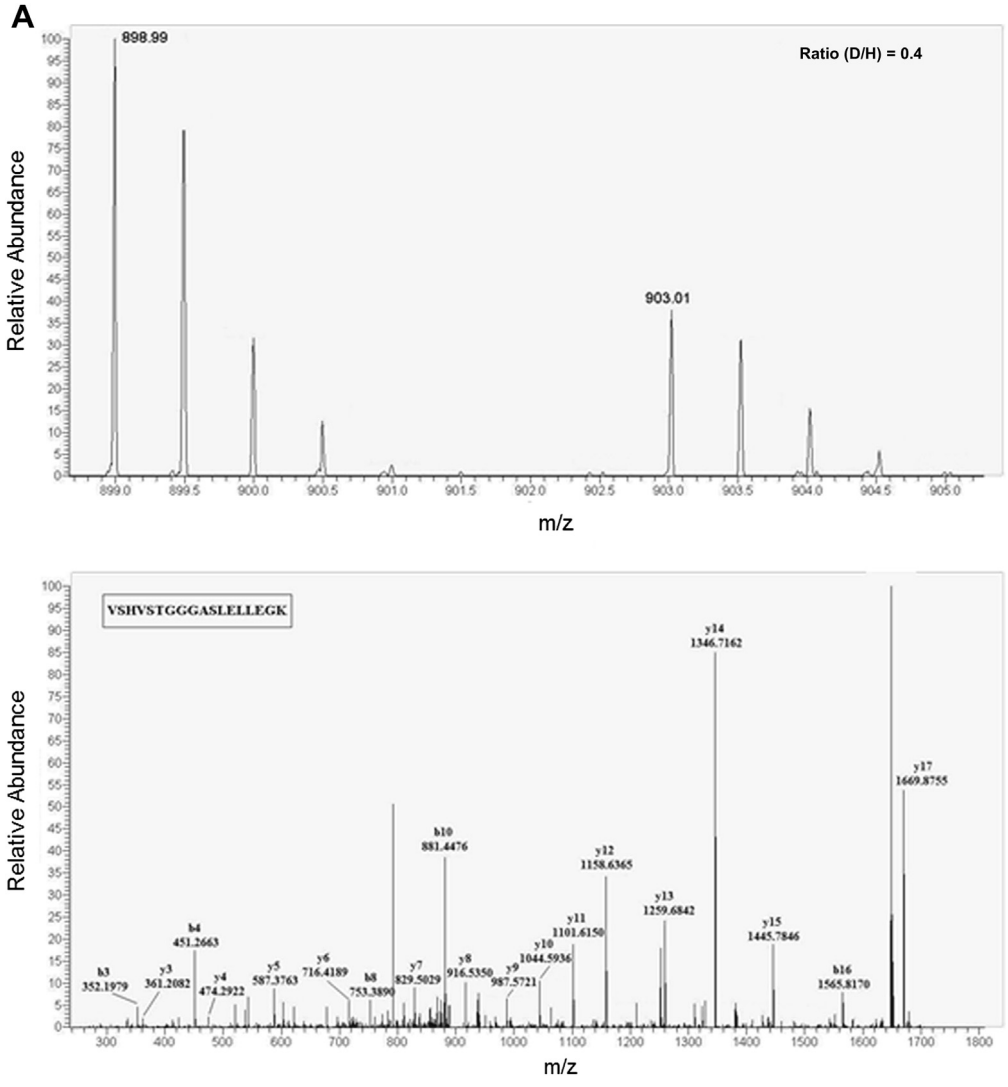


**Figure 2.** The MS/MS CID spectra and ratios of isotopic pairs (quantification ratios) of the representative peptides (A) VSHVSTGGGASLELLEGK for phosphoglycerate kinase 1 and (B) AEGSDVANAVLDGADCIMLSGETAK for pyruvate kinase isozymes M1/M2. Peptide quantification ratios (D/H) for normal (hydrogen labeling) and varicose veins (deuterium labeling) were calculated by Mascot Distiller program using the average area of the first three isotopic peaks across the elution profile. The program merged the Mascot search data and quantification results from each fraction, and peptide ratios for the same sequences obtained from different fractions or at different retention times and charge states were combined.

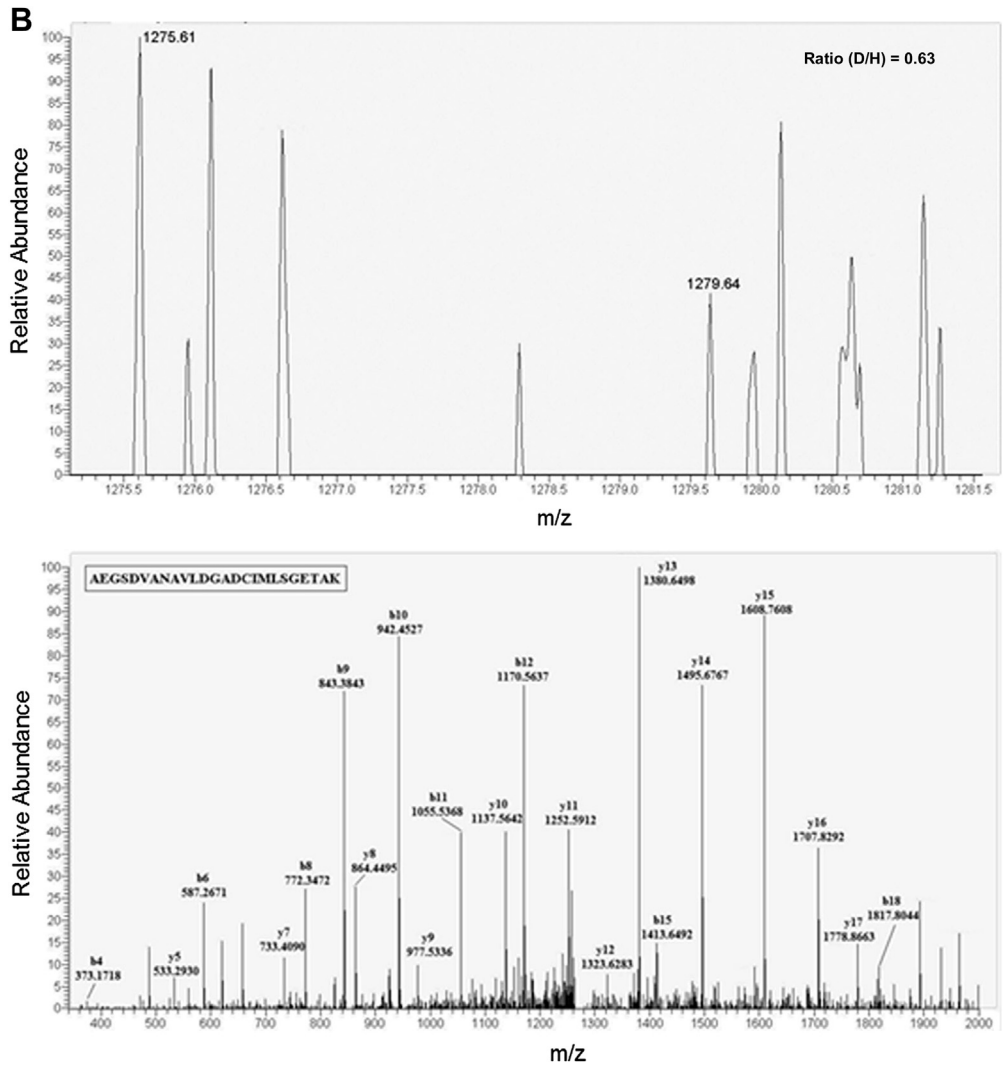


**Fig. 2.** (*continued*).

and 0.8 Da for fragment ions; no missed cleavage sites for trypsin; fixed modification, carbamidomethyl cysteine; quantification, dimethylation; optional modification, oxidized methionine and amidated asparagine/glutamine. A peptide with a Mascot individual ion score higher than 20 was considered positively identified ($p < 0.05$).

The peptide quantification ratios (D/H) for normal (hydrogen labeling) and varicose veins (deuterium labeling) were then calculated using Mascot Distiller program (version 2.3; Matrix Science Ltd., London, UK) for the average area of the first three isotopic peaks across the elution profile. For each fraction, the program merged the Mascot search data and the quantification results by combining peptide ratios with the same sequences even if their fractions, retention times, and charge states differed [21].

### Construction of signaling pathways and network analysis of protein interaction

Pathways and networks of protein interaction and the involved prospective mechanism of ATP synthesis and utilization were derived using software (www.ingenuity.com) developed by Ingenuity Pathways Analysis (IPA; Ingenuity Systems, Redwood City, CA, USA). Protein factors characterized by proteomic analysis were analyzed for associations with mapping related to canonical pathways deposited in the IPA library.

## Results

### NanoLC-MS/MS analysis of protein expression levels

The comparative proteomics analysis of varicose veins in this study was performed by shotgun proteomic approach. Fig. 1 shows the scheme used for sample processing and separation and the subsequent workflow for trypsin digestion, dimethyl labeling and shotgun analysis. Normal and varicose veins were first homogenized and lysed for protein extraction. Under identical conditions, 100-μg samples of total lysates were subjected to trypsin digestion and dimethyl labeling. Respective tryptic peptide samples were mixed at a 1:1 (w/w) ratio and then enriched by the reverse-phase C18 column. Since the enriched peptide population was too complex for full detection and characterization in a single LC-MS/MS run, the enriched peptides were fractionated by HILIC based on polarity difference and harvested into 10 fractions.

Each fraction was analyzed by LC-LTQ-Orbitrap, and the search parameter used to identify the peptides was set to allow for no missed cleavage. Most peptides were separated from one or two adjacent HILIC fractions, and peptides identified by the in-house software were accepted if their individual ion scores were higher than 20, which is the cutoff point used for lower-quality MS/MS spectra [29—31]. The differential protein expression levels of normal versus varicose veins were profiled in the experiments and shown as 455 protein species with a confident identification from a single LC-MS/MS run.

## Identification and quantification of differentially expressed proteins

After the differentially expressed proteins had been confidently identified by dimethyl labeling, enzyme digestion and peptide mass fingerprinting, the peptide quantification ratio (D/H) was obtained by Mascot Distiller program using the average area of the first 3 isotopic peaks across each elution profile [21,22,32]. Figs. 2 and 3 show the peaks for representative peptides, including phosphoglycerate kinase 1, pyruvate kinase isozymes M1/M2, myosin light chain kinase (MYLK) and calmodulin. Notably, Fig. 2A shows the isotopic pairs (quantification ratios) of nanoLC and MS/MS CID spectra, which confirms that the $D_4$ and $H_4$-labeled peptides (VSHVSTGGGASLELLEGK) derived from phosphoglycerate kinase 1, which had *m/z* values of 903.01 (+2) and 898.99 (+2), respectively, and a D/H ratio of 0.4, were eluted simultaneously (~47 minutes) in HILIC fraction 2. The $D_4$- and $H_4$-labeled peptides (AEGSDVANAVLDGADCIMLSGETAK) from pyruvate kinase isozymes M1/M2, which had *m/z* values of 1275.61 (+2) and 1279.64 (+2), respectively, and a D/H ratio of 0.63, were eluted

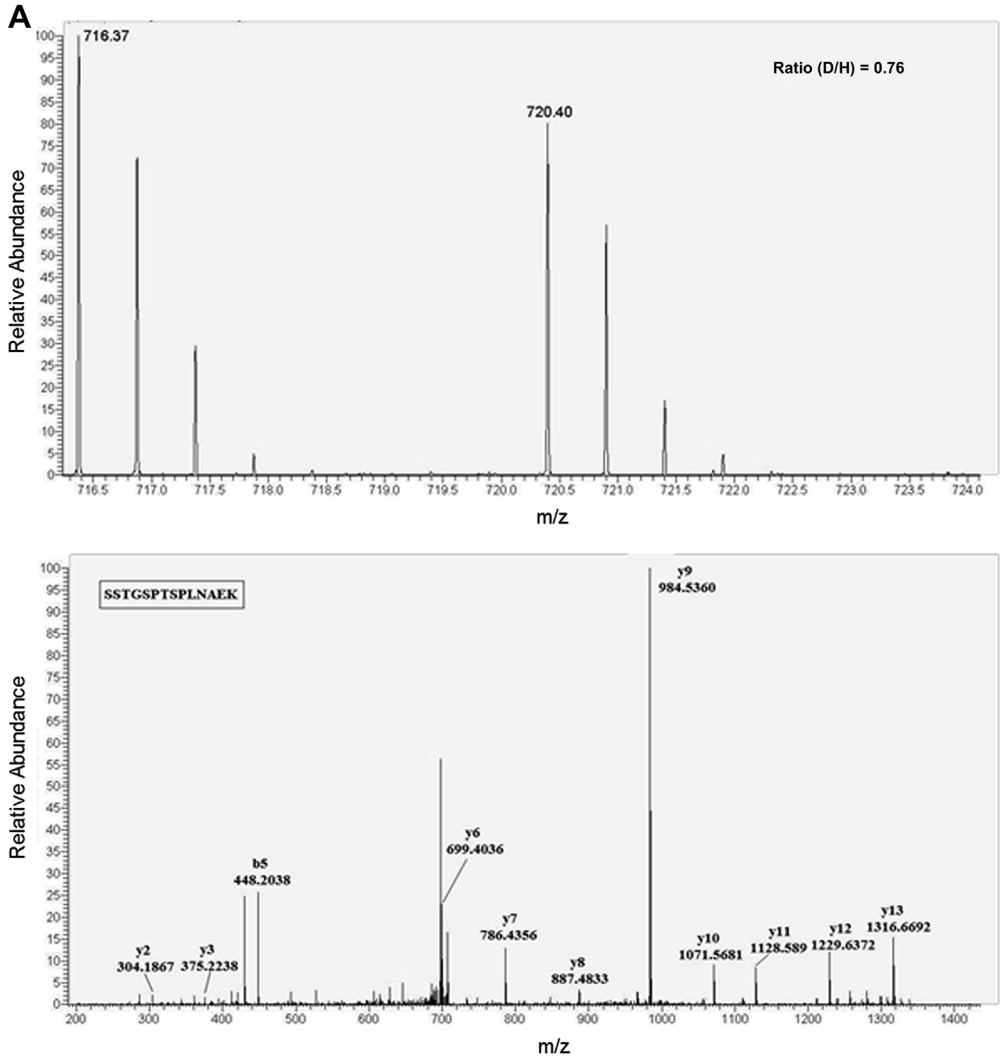


**Figure 3.** The MS/MS CID spectra and ratios of isotopic pairs (quantification ratios) of the representative (A) peptides SSTGSPTSPLNAEK for myosin light chain kinase and (B) EAFSLFDK for calmodulin. The peptide quantification ratio (D/H) for normal (hydrogen labeling) and varicose veins (deuterium labeling) was calculated by Mascot Distiller program using the average area of the first three isotopic peaks across the elution profile. The program merged the Mascot search data and quantification results from each fraction, and peptide ratios for the same sequences obtained from different fractions or at different retention times and charge states were combined.

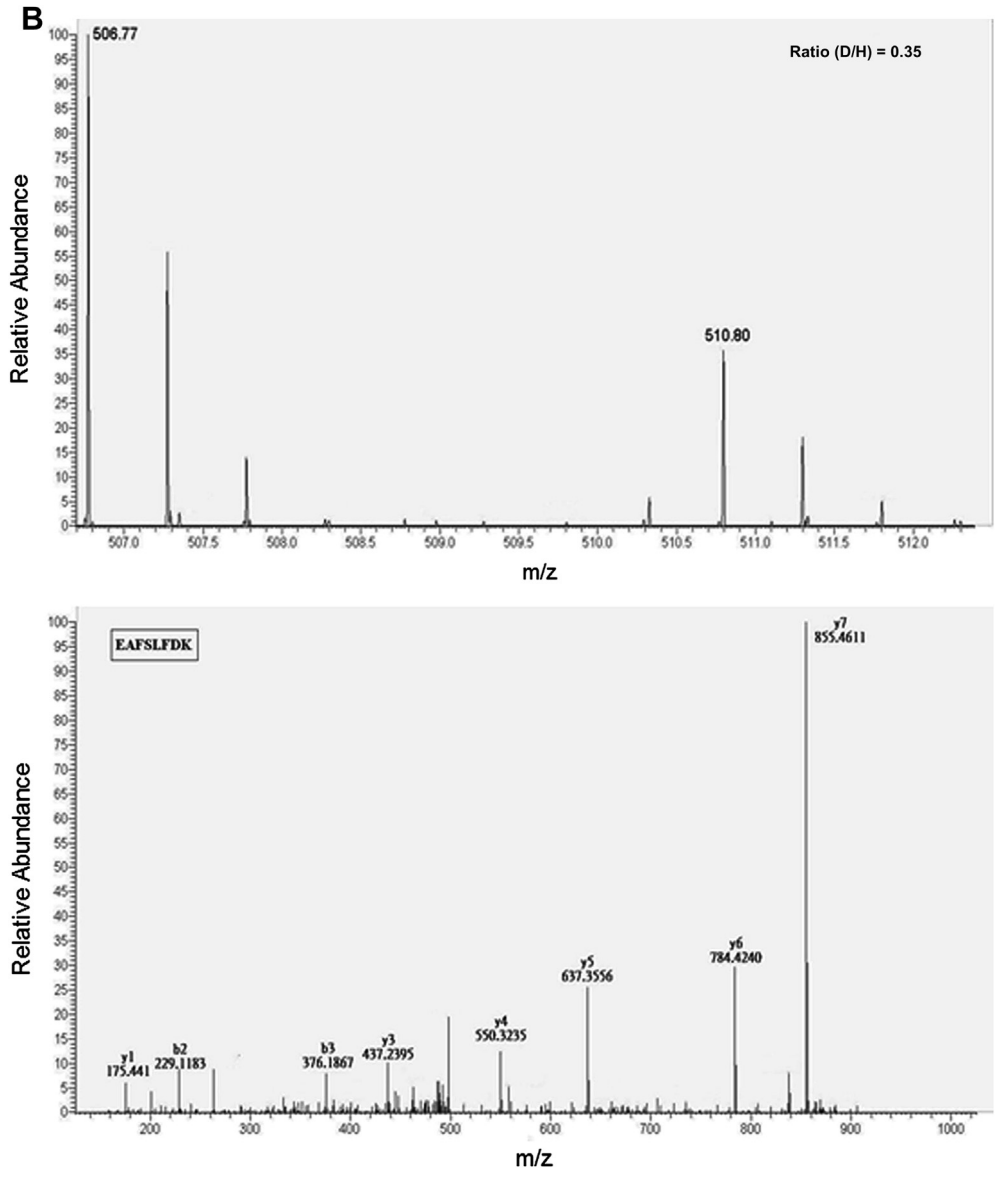


**Fig. 3.** (*continued*).

simultaneously (~68 minutes) in the HILIC fraction 3. Fig. 2B shows the isotopic pairs (quantification ratios) of nanoLC and MS/MS CID spectra. The quantitative ratios showed that these two key enzymes, which generate ATP in the glycolysis pathway, were downregulated in the patients.

In contrast, the isotopic pairs of nano LC and MS/MS CID spectra shown in Fig. 3A revealed that $D_4$- and $H_4$-labeled peptides (SSTGSPTSPLNAEK) derived from MYLK had *m*/*z* values of 716.37 (+2) and 720.40 (+2), respectively, and a D/H ratio of 0.76, were eluted simultaneously (~37 minutes) in the HILIC fraction 4. Fig. 3B showed that the isotopic pairs (quantification ratios) of nano LC and MS/MS CID spectra of $D_4$ - and $H_4$-labeled peptides (EAFSLFDK) from calmodulin, which had *m*/*z* values of 506.77 (+2) and 510.80 (+2), respectively, and a D/H ratio of 0.35, were eluted simultaneously (~56 minutes) in HILIC fraction 3. That is, the patients showed downregulation of two proteins that mediate ATP utilization in smooth muscle contraction. The above results further confirmed the absence of an isotopic effect in the two-dimensional HILIC-C18 separation and excellent efficiency in separating dimethylated peptides fractionated by HILIC column. It was hypothesized that the improved efficiency resulted mainly from the high orthogonality of HILIC and the reverse-phase C18 column.

By using the Mascot Distiller program, data from the Mascot search engine and the quantification results for each fraction were merged into one file in which peptides were combined if more than one peptide had matching sequences (i.e., if they had been harvested from different fractions, at different retention times, or in different charge states). Twelve proteins showed highly consistent downregulation of protein expression, which

signifies that this downregulatory effect may be universal in these patients. All 12 identified proteins were directly or indirectly involved in energy generation and utilization. Eight were glycolysis-related proteins: glucose-6-phosphate isomerase, phosphoglycerate mutase 1, fructose-bisphosphate aldolase A, phosphoglycerate kinase 1, pyruvate kinase isozymes M1/M2, triosephosphate isomerase, α-enolase, and anaerobic glycolysis enzyme identified as lactate dehydrogenase B chain. Two proteins, calmodulin and MYLK, showed associations with ATP utilization and regulation. The remaining two proteins, mitochondrial ATP synthase β subunit and creatine kinase B type, are known to participate in ATP generation and function as energy reservoirs. Table 1 lists their sequence coverage and match scores. The amino acid sequence coverage of the identified proteins varied from 13% to 50%. For example, phosphoglycerate kinase 1 had 42% sequence coverage based on 33 matched peptides; pyruvate kinase isozymes M1/M2 showed 65% sequence coverage based on 39 matched peptides, and fructose-bisphosphate aldolase A showed 50% sequence coverage based on 69 matched peptides.

Although the shotgun proteomic analysis revealed differential expression in 455 proteins, few of the up- or down-regulated protein species that showed associations with cytoskeletal assembly, extracellular matrix formation, enzyme catalysis or inflammation in this quantitative analysis showed clear up- or down-regulation among the patients. Therefore, protein factors involved in ATP utilization and regulation were selected for further analysis.

### Construction of signaling pathways and network analysis of protein interaction

Fig. 4 shows the results obtained by using IPA software for further analysis of the metabolic pathways and key enzymes or proteins that showed associations with ATP utilization and generation. Calcium from the sarcoplasmic reticulum and extracellular spaces is a major regulator of venous smooth muscle function. As it enters the cytosol, it associates with calmodulin to form a complex. The complex then activates MYLK, which uses ATP to promote myosin phosphorylation. By interacting with actin, phosphorylated myosin then upregulates contractile activity in vascular smooth muscle. The results of the above experiment suggest that varicose vein formation may result from attenuation of this contractile force by downregulation of key enzymes that mediate ATP synthesis and utilization.

## Discussion

Biochemical and histological studies show that varicose veins result from incoordination and dysfunction in metabolic pathways and from dysfunctional regulation of expression in many proteins [4,15,33—36]. Morphological evidence generally agree with the fact that structural remodeling characterized by accumulation of collagen, proteoglycans, and elastic fibers in smooth muscle cells decreases elasticity of the veins while concomitantly causing inflammation and thrombus [16,17]. However, the conventional phenotype characterization is insufficient for

**Table 1** Proteins in patients with varicose veins showing downregulation identified by nano liquid chromatography and mass spectrometry.

| Number | Protein ID | Swiss-Prot accession number | Score/match | pI/mass, kDa | Sequence coverage % | Protein expression ratio (D/H) |
|---|---|---|---|---|---|---|
| Energy reservoir | | | | | | |
| 1 | Creatine kinase B-type | P12277 | 3683/53 | 5.34/42.64 | 48 | 0.32 ± 0.197 |
| Energy generation | | | | | | |
| Glycolysis | | | | | | |
| 2 | Glucose-6-phosphate isomerase | P06744 | 184/8 | 8.42/63.15 | 18 | 0.45 ± 0.130 |
| 3 | Phosphoglycerate mutase 1 | P18669 | 624/16 | 6.67/28.80 | 25 | 0.44 ± 0.068 |
| 4 | Fructose-bisphosphate aldolase A | P04075 | 2946/69 | 8.30/39.42 | 50 | 0.52 ± 0.107 |
| 5 | Phosphoglycerate kinase 1 | P00558 | 951/33 | 8.30/44.61 | 42 | 0.5 ± 0.077 |
| 6 | Pyruvate kinase isozymes M1/M2 | P14618 | 3147/65 | 7.96/57.94 | 39 | 0.55 ± 0.106 |
| 7 | Triose phosphate isomerase | P60174 | 4452/49 | 5.65/30.79 | 50 | 0.61 ± 0.139 |
| 8 | α-enolase | P06733 | 7624/71 | 7.01/47.17 | 43 | 0.61 ± 0.071 |
| Respiration and fermentation | | | | | | |
| 9 | Mitochondrial ATP synthase β subunit | P06576 | 107/6 | 5.26/56.56 | 15 | 0.79 ± 0.154 |
| Anaerobic glycolysis | | | | | | |
| 10 | L-Lactate dehydrogenase B chain | P07195 | 442/18 | 5.71/36.64 | 22 | 0.64 ± 0.059 |
| Energy utilization | | | | | | |
| 11 | Calmodulin | P62158 | 777/13 | 4.09/16.84 | 36 | 0.37 ± 0.235 |
| 12 | Myosin light chain kinase | Q15746 | 1168/23 | 5.85/210.72 | 13 | 0.61 ± 0.320 |

Protein expression ratios of D/H shown here are the mean ± standard deviation of triplicate measurements.
D = deuterium labeling (varicose veins); H = hydrogen labeling (normal veins).

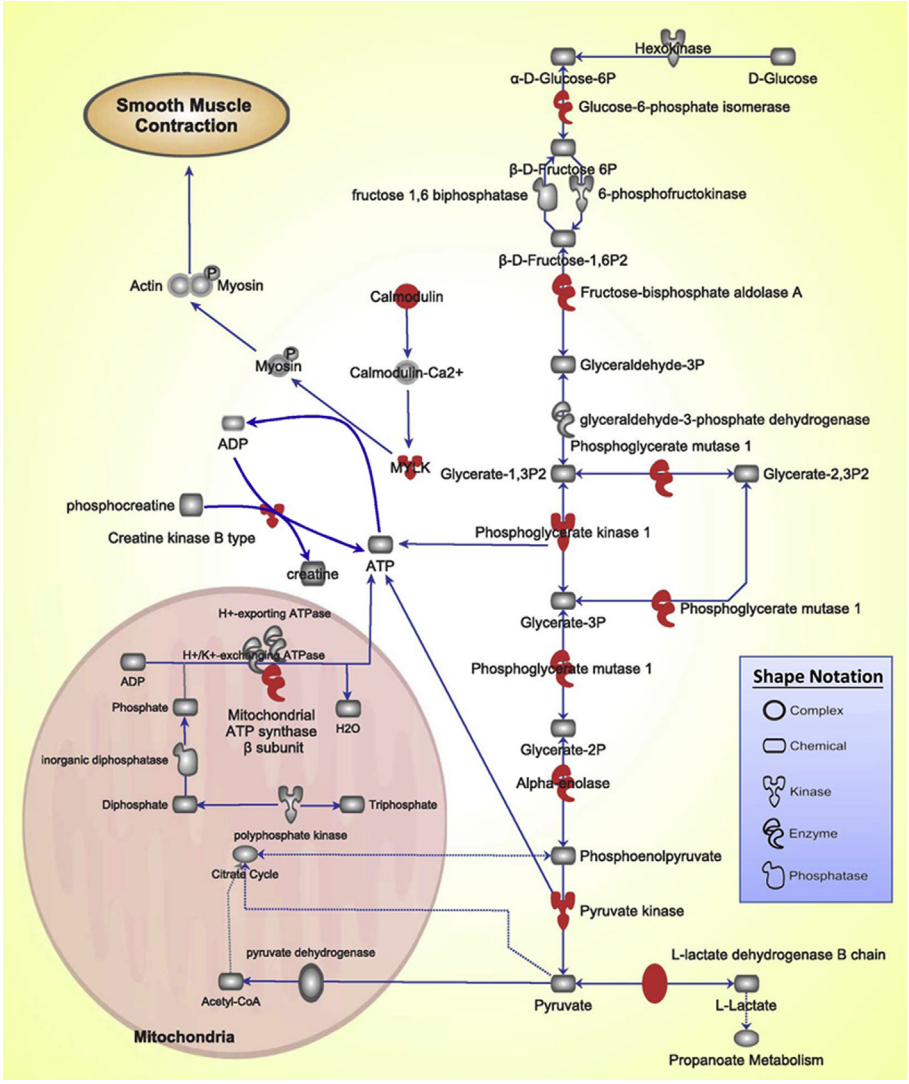


**Figure 4.** Schematic representation of derived pathways associated with ATP synthesis and utilization. The networks of these identified proteins mapped to canonical pathways from the Ingenuity Pathways Analysis (Ingenuity Systems) library were used to compare significantly downregulated protein molecules between normal and diseased tissues. Identified proteins are displayed in red and have different shapes to indicate different functions associated with these molecules, including ATP-generating, ATP-utilizing, and glycolytic enzymatic functions. The blue arrows indicate the biological interrelationships between molecules. All arrows in the figure were supported by at least one reference from the literature, textbooks, or canonical information stored in the Ingenuity Knowledge Base. As the development of varicose veins progresses, systematic decreases in these enzymes may cause dysfunctions in vascular smooth muscle followed by morphological aberrations.

a functional study of factors related to contractile or relaxing machinery. Venous contraction and relaxation have a well-known dependency on the functional interaction of actin, myosin, and other coordinating components in vascular smooth muscle [37—39]. Thus, abnormal vasodilation may result from the reduction or loss of contractile force in smooth muscle cells.

Since the causes of abnormal vasodilation in veins have not been adequately studied and are poorly understood, the aim of this study was to perform a quantitative proteomic analysis to elucidate the protein expression profile and the related pathogenesis of altered vascular contractility in varicose veins. The analysis showed that patients diagnosed with varicose veins had dramatically lower levels of ATP yield, ATP utilization, and glycolytic enzymes compared to normal controls based on the obtained D/H quantification ratios ranging from 0.32 (creatine kinase B-type) to 0.79 (ATP synthase β subunit; Table 1). The metabolic disorder resulting from decreased enzyme expression may be highly unfavorable to the mediating and regulatory functions of vascular smooth muscle in vasodilation or vasoconstriction.

As in all other cells, smooth muscle cells regenerate ATP through three metabolic pathways: oxidative phosphorylation, creatine kinase catalysis, and glycolysis. Among the involved enzymes, a marked decrease in the expression of B-type creatine kinase, which is known to convert phosphate groups from phosphocreatine to ADP, was followed by

formation of ATP and the nitrogenous substance creatine. This showed that, by serving as an intermediate storage pool, phosphocreatine enables instantaneous availability of almost any high-energy phosphate [40]. In the mitochondrial environment, the β subunit of ATP synthase from which ATP is actually generated also revealed down-regulation, which suggests that impairment of its enzymatic function by the proton gradient generated by oxidative phosphorylation may reduce ATP levels in venous tissue.

Phosphoglycerate kinase 1 and pyruvate kinase isozymes M1/M2 are key enzymes for energy release because their catalytic activities initiate phosphate-group transfer reactions. By transferring a phosphate group from 1,3-bisphosphoglycerate to ADP, phosphoglycerate kinase 1 yields ATP and 3-phosphoglycerate [41,42] whereas pyruvate kinase isozymes M1/M2 can catalyze the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP, which generates one pyruvate molecule and one ATP molecule [43]. Phosphoglycerate kinase 1 and pyruvate kinase isozymes M1/M2 had D/H quantification ratios of 0.4 and 0.63, respectively (Fig. 2), which signifies a decreased ATP yield in the varicose vein tissues.

In addition to defects in ATP synthesis and supply, impeded mobilization of cellular components involved in the transformation of chemical energy from ATP to mechanical energy impeded actin—myosin interaction in smooth muscle. As in the skeletal muscle, contraction of smooth muscle in the vein vessel requires calcium ions and ATP. Calcium transported into the cytosol associates with calmodulin, a protein functionally similar to troponin in skeletal muscle [44]. The calmodulin-calcium complex can then activate MYLK, which then further catalyzes the binding of a high-energy phosphate to myosin. Myosin interacts with actin only after MYLK phosphorylation causes muscle contraction [45—49]. The observed D/H quantification ratios of 0.61 for MYLK (Fig. 3A) and 0.37 for calmodulin (Fig. 3B), suggest that ATP utilization of veins remained attenuated.

A continuous supply of large quantities of ATP from metabolic processes of muscle is needed to maintain contraction machinery, energy storage and calcium transport. Inhibition of the glycolysis pathway and down-regulation of ATP utilization not only attenuate various physiological processes, but also attenuate the energy supply needed for physical activity. This study focused on 12 protein factors related to ATP generation and utilization since other protein species known to be associated with cytoskeletal assembly or cellular morphology showed no clear up- or down-regulation among these patients. The availability of low levels of both ATP-generating and ATP-utilizing enzymes may thus partly explain dysfunction of the smooth muscle lining of the vein vessel followed by progressively worsening morphological aberrations in varicose veins (Fig. 4).

This study used stable isotope dimethyl labeling coupled with nanoLC-MS/MS for quantitative shotgun analysis of proteomic changes in clinical samples of varicose vein tissues by LTQ Orbitrap mass spectrometer. In terms of sensitivity and efficiency of proteomic analysis of complex biological tissues, this approach is superior to presample protein fractionation based on 2D-gel electrophoresis followed by LC-tandem MS analysis.

## Conclusion

This report, which is the first characterization and description of energy-oriented enzyme synthesis in varicose veins, corroborates the supposition that primary weakness of the vein wall may have a major role in the pathogenesis of varicosity of venous vessels [14,50]. This study provides novel insights into the biochemical mechanisms of this disease and provides a basis for further studies needed to validate, evaluate, and clinically translate biomarkers into targeted therapies for this disease. Collectively, the severity of varicose veins involves various protein factors that have regulatory roles in metabolic coordination of physiological functions. Further studies are needed to explore other factors such as collagens [51] associated with maintenance of vascular morphology and contractility for improved understanding of the mechanisms underlying vein remodeling and the corresponding formation of varicose veins under specific physiological conditions.

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