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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.

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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 metho-

dologies 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 M Ω 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 13,000g 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 13,000g for 5 minutes, the collected proteins were washed with 150 µL of 10% TCA, vortexed, and centrifuged at 13,000g for 10 minutes. The precipitated

proteins were rewashed with 250 µL distilled H₂O, vortexed and centrifuged under the same condition three times. The resulting pellets were then resuspended with 50 mM NH₄HCO₃ (pH 8.5), digested with 4 µg of trypsin for 8 hours at 37°C, and dried in a vacuum centrifuge to remove NH₄HCO₃. 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-H₂ and 20 µL of 4% formaldehyde-D₂, 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/H₂O to pH 2.0–3.0 and applied onto the reverse phase C18 column pre-equilibrated with 200 µL of 0.1% TFA/H₂O (pH 2.0–3.0). The column was also washed with 200 µL of 0.1% TFA/H₂O (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

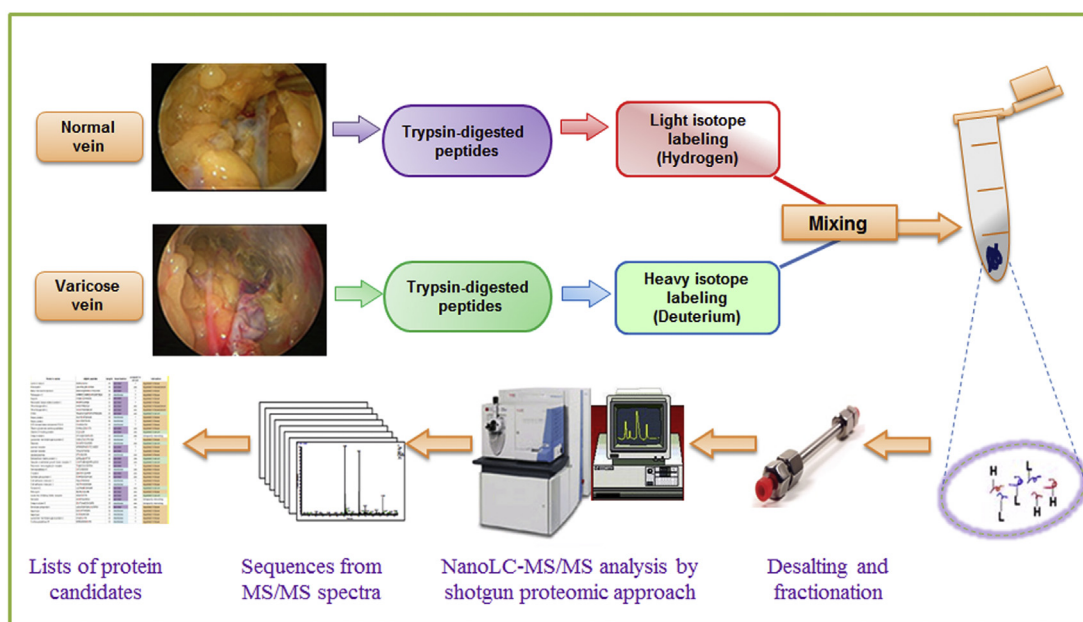


Figure 1. Schematic representation of the procedures used to analyze and compare protein expression. Quantitative shotgun analyses of proteomic changes in clinical samples of varicose vein tissues were performed by using HILIC-C18 for peptide separation and nanoLC-MS/MS coupled with stable isotope dimethyl 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 μ L of 0.1% FA in H₂O 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- μ L sample of collected fractions was loaded onto an Agilent Zorbax XDB C18 precolumn (0.35 mm, 5 μ m) and separated with a C18 column (i.d., 75 μ m \times 15-cm, 3 μ m). 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

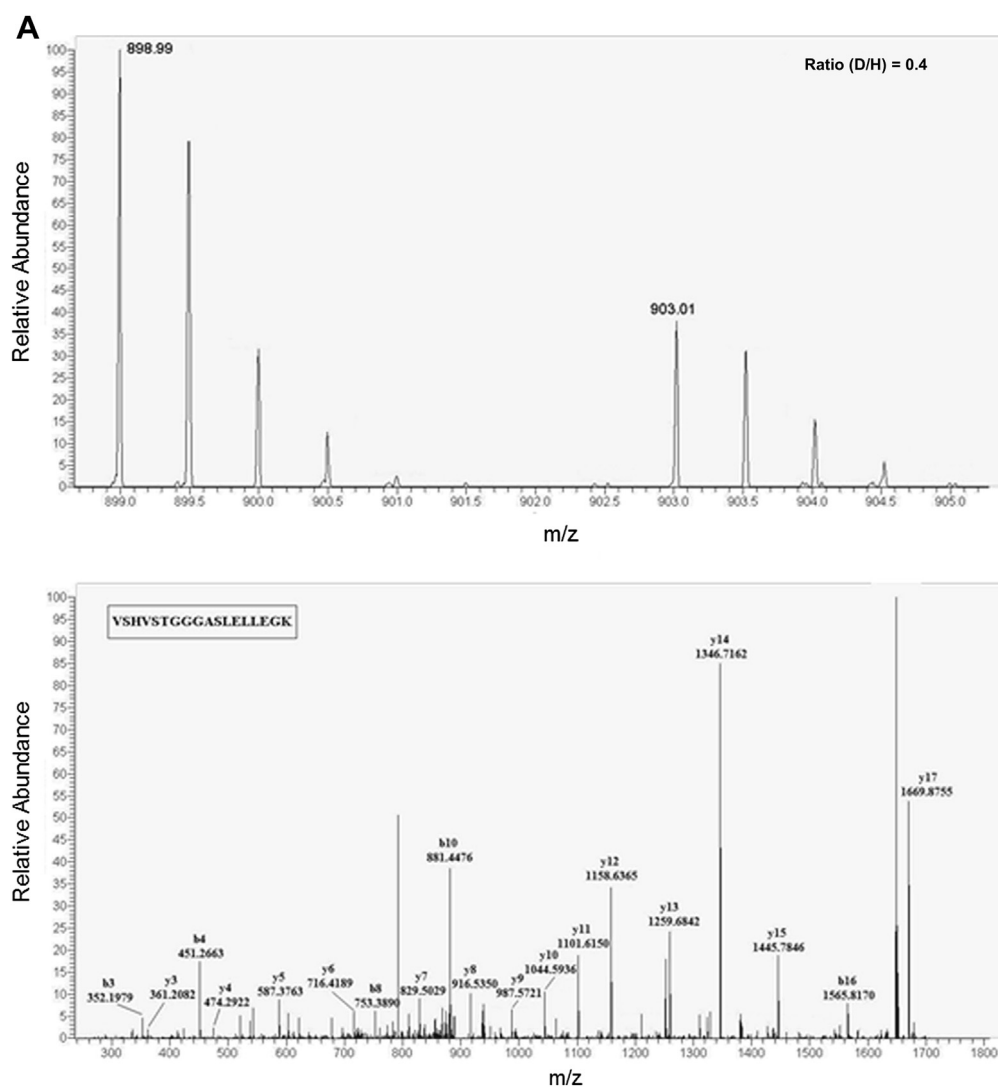


Figure 2. The MS/MS CID spectra and ratios of isotopic pairs (quantification ratios) of the representative peptides (A) VSHVSTGGGASLELEGGK for phosphoglycerate kinase 1 and (B) AEGSDVANAVLDGADCIMLSGETAK for pyruvate kinase isozymes M1/M2. Peptide quantification ratios (D/H) for normal (hydrogen labeling) 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.

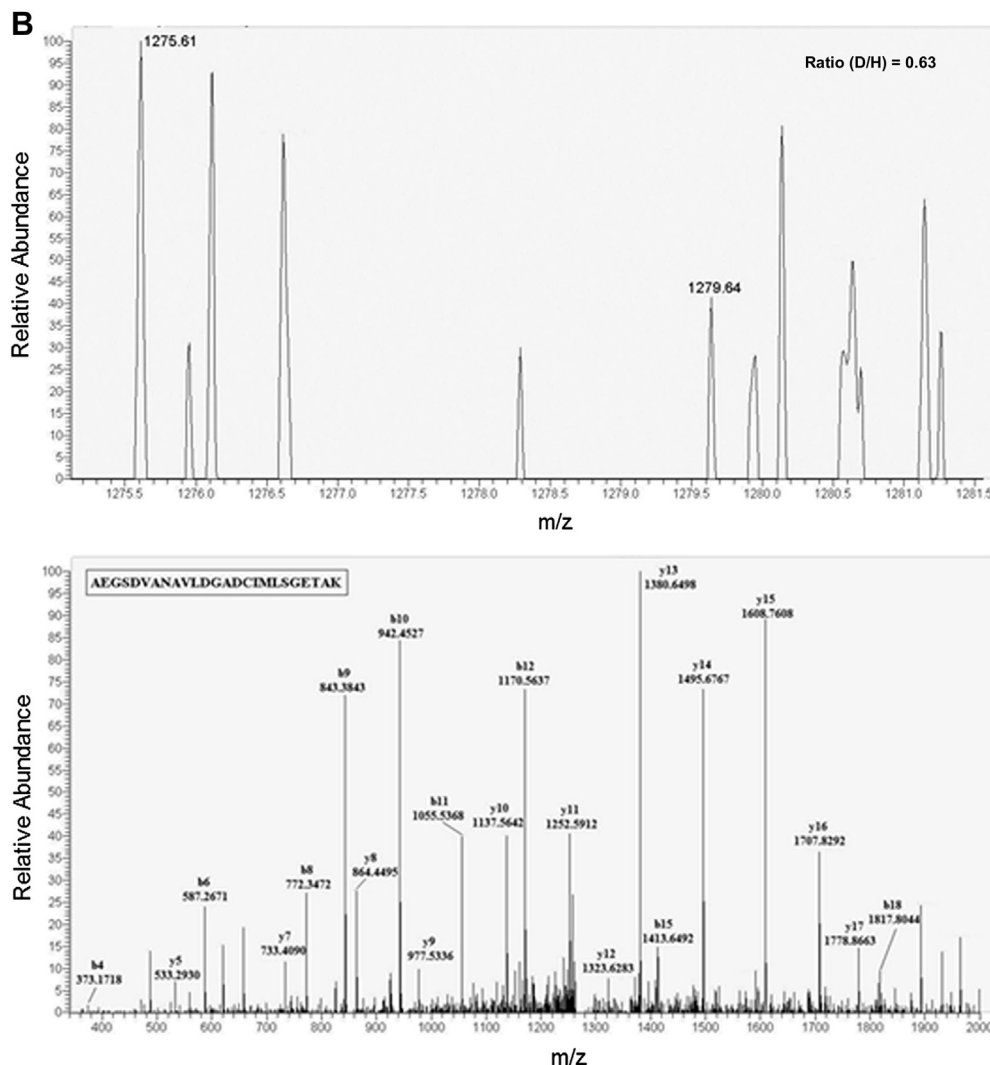


Fig. 2. (continued).

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- μ g 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 D₄ and H₄-labeled peptides (VSHVSTGGGASLELLEGGK) 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 D₄- and H₄-labeled peptides (AEGSDVA-NAVLDGADCIMLSGETAK) 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

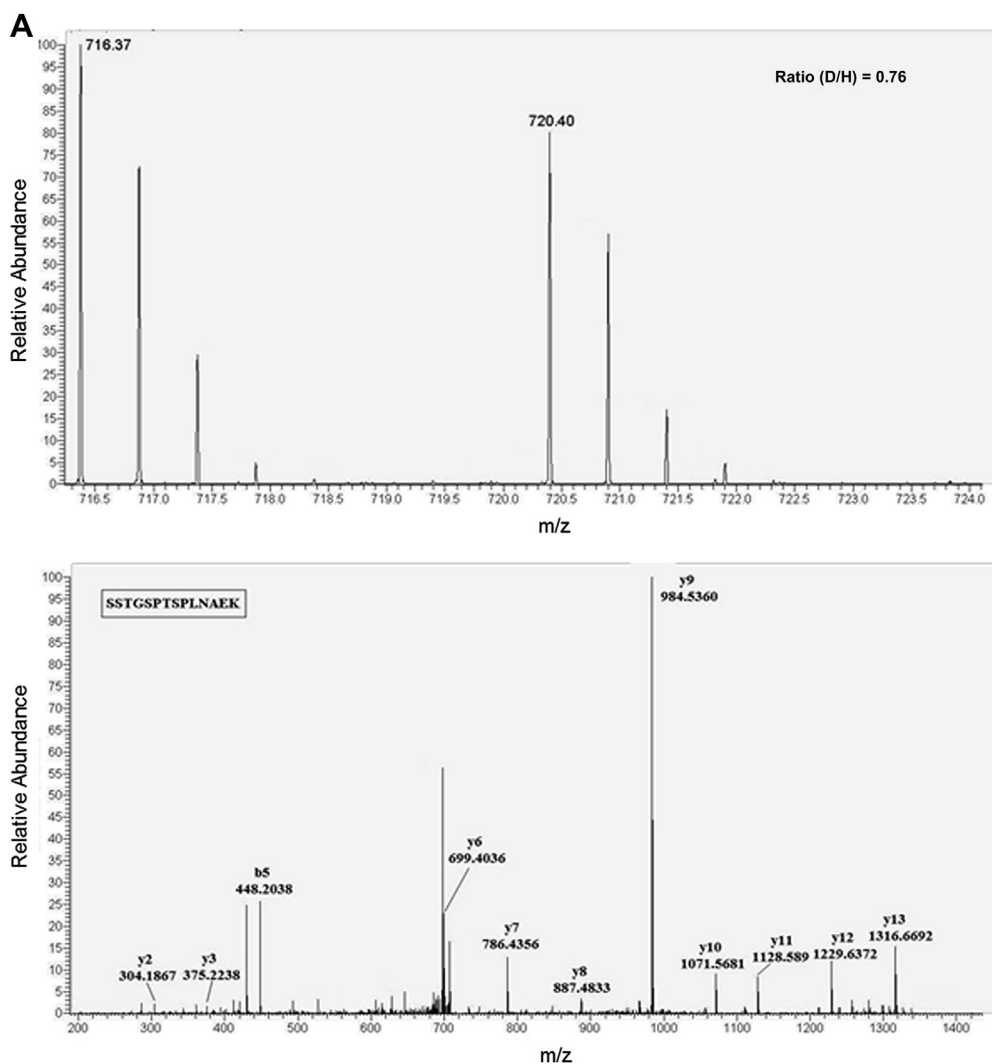


Figure 3. The MS/MS CID spectra and ratios of isotopic pairs (quantification ratios) of the representative (A) peptides SSTGSPTSPLNAEK for myosin light chain kinase and (B) EAFSLFDK for calmodulin. The peptide quantification ratio (D/H) for normal (hydrogen labeling) and varicose veins (deuterium labeling) 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.

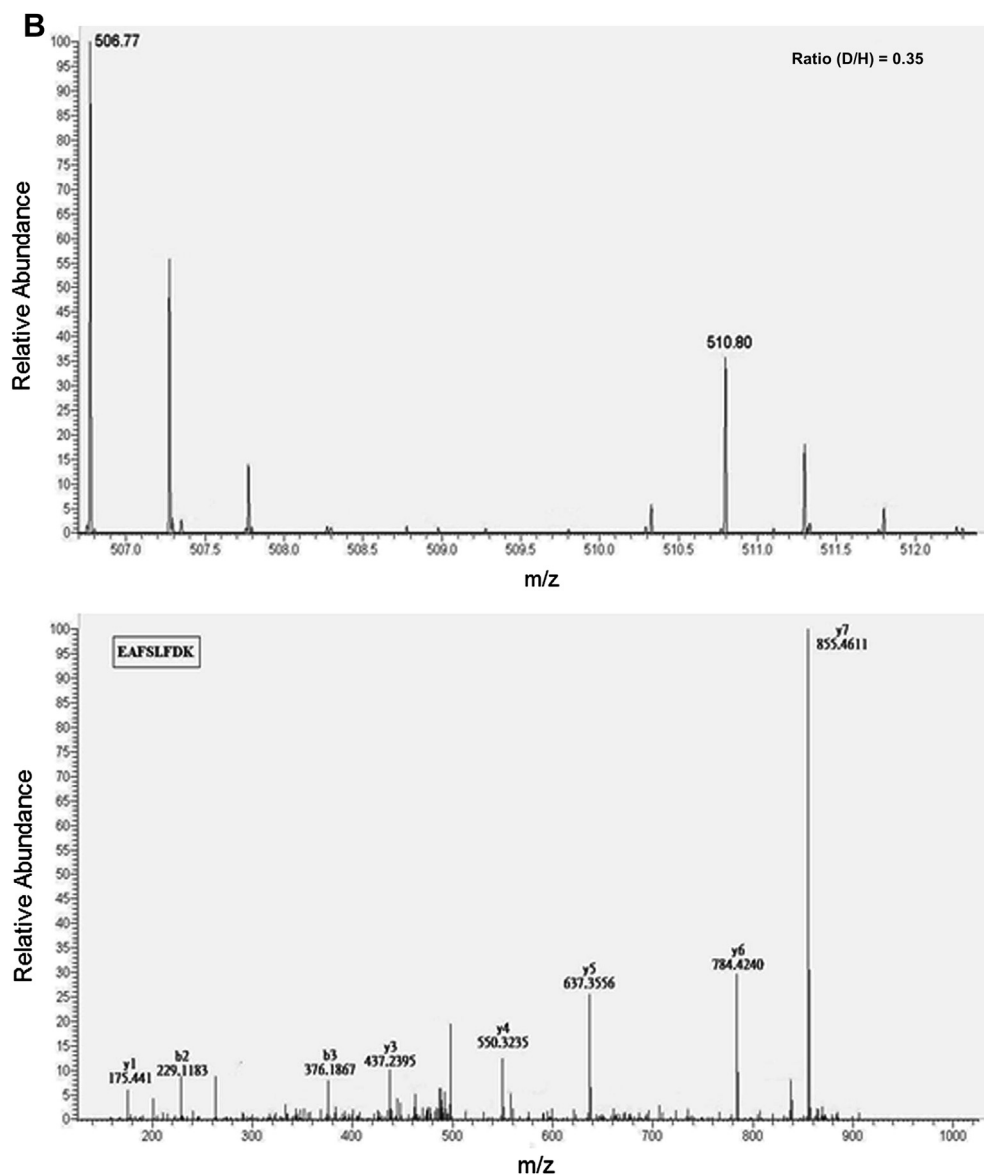


Fig. 3. (continued).

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 (SSTGSPSTPLNAEK) 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 \pm 0.197
Energy generation						
Glycolysis						
2	Glucose-6-phosphate isomerase	P06744	184/8	8.42/63.15	18	0.45 \pm 0.130
3	Phosphoglycerate mutase 1	P18669	624/16	6.67/28.80	25	0.44 \pm 0.068
4	Fructose-bisphosphate aldolase A	P04075	2946/69	8.30/39.42	50	0.52 \pm 0.107
5	Phosphoglycerate kinase 1	P00558	951/33	8.30/44.61	42	0.5 \pm 0.077
6	Pyruvate kinase isozymes M1/M2	P14618	3147/65	7.96/57.94	39	0.55 \pm 0.106
7	Triose phosphate isomerase	P60174	4452/49	5.65/30.79	50	0.61 \pm 0.139
8	α -enolase	P06733	7624/71	7.01/47.17	43	0.61 \pm 0.071
Respiration and fermentation						
9	Mitochondrial ATP synthase β subunit	P06576	107/6	5.26/56.56	15	0.79 \pm 0.154
Anaerobic glycolysis						
10	L-Lactate dehydrogenase B chain	P07195	442/18	5.71/36.64	22	0.64 \pm 0.059
Energy utilization						
11	Calmodulin	P62158	777/13	4.09/16.84	36	0.37 \pm 0.235
12	Myosin light chain kinase	Q15746	1168/23	5.85/210.72	13	0.61 \pm 0.320

Protein expression ratios of D/H shown here are the mean \pm standard deviation of triplicate measurements.

D = deuterium labeling (varicose veins); H = hydrogen labeling (normal veins).

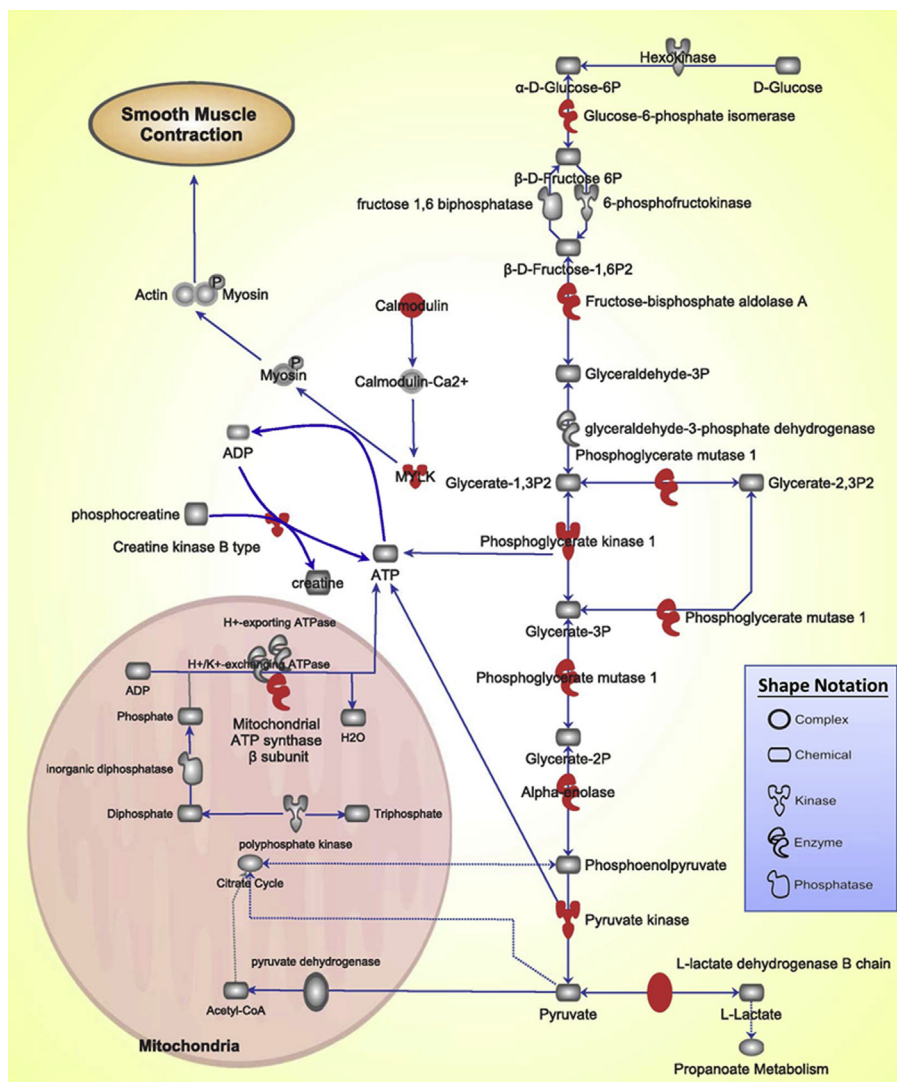


Figure 4. Schematic representation of derived pathways associated with ATP synthesis and utilization. The networks of these identified proteins mapped to canonical pathways from the Ingenuity Pathways Analysis (Ingenuity Systems) library were used to compare significantly downregulated protein molecules 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.

Acknowledgments

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References

- [1] Lin SD, Chang KP, Yang YL, Lee SS, Lin TM, Tsai CC, et al. Gross anatomy of primary varicose veins observed in endoscopic surgery. *Ann Plast Surg* 2002;49:559–66.
- [2] Clark A, Harvey I, Fowkes FG. Epidemiology and risk factors for varicose veins among older people: cross-sectional population study in the UK. *Phlebology* 2010;25:236–40.
- [3] Cesarone MR, Belcaro G, Nicolaidis AN, Geroulakos G, Griffin M, Incandela L, et al. 'Real' epidemiology of varicose veins and chronic venous diseases: the San Valentino Vascular Screening Project. *Angiology* 2002;53:119–30.
- [4] Karathanos C, Sfyroeras G, Drakou A, Roussas N, Exarchou M, Kyriakou D, et al. Superficial vein thrombosis in patients with varicose veins: role of thrombophilia factors, age and body mass. *Eur J Vasc Endovasc Surg* 2012;43:355–8.
- [5] Franks PJ, Wright DD, Moffatt CJ, Stirling J, Fletcher AE, Bulpitt CJ, et al. Prevalence of venous disease: a community study in west London. *Eur J Surg* 1992;158:143–7.
- [6] Bergan JJ, Schmid-Schönbein GW, Smith PD, Nicolaidis AN, Boisseau MR, Eklof B. Chronic venous disease. *N Engl J Med* 2006;355:488–98.
- [7] Callam MJ. Epidemiology of varicose veins. *Br J Surg* 1994;81:167–73.
- [8] Nael R, Rathbun S. Treatment of varicose veins. *Curr Treat Options Cardiovasc Med* 2009;11:91–103.
- [9] Lin SD, Tsai CC, Lin TM, Lee SS, Chang KP, Lai CS. Endoscope-assisted correction of primary varicose veins. *Ann Plast Surg* 2000;44:241–9.
- [10] Lin SD, Chang KP, Lu DK, Lee SS, Lin TM, Tsai CC, et al. Endoscope-assisted management of varicose veins in the

- posterior thigh, popliteal fossa, and calf area. *Ann Plast Surg* 2002;48:10–20.
- [11] Partsch H. Varicose veins and chronic venous insufficiency. *Vasa* 2009;38:293–301.
- [12] Gohel MS, Davies AH. Pharmacological agents in the treatment of venous disease: an update of the available evidence. *Curr Vasc Pharmacol* 2009;7:303–8.
- [13] Shingler S, Robertson L, Boghossian S, Stewart M. Compression stockings for the initial treatment of varicose veins in patients without venous ulceration. *Cochrane Database Syst Rev* 2011;11: CD008819.
- [14] Rose SS, Ahmed A. Some thoughts on the aetiology of varicose veins. *J Cardiovasc Surg (Torino)* 1986;27:534–43.
- [15] Sansilvestri-Morel P, Nonotte I, Fournet-Bourguignon MP, Rupin A, Fabiani JN, Verbeuren TJ, et al. Abnormal deposition of extracellular matrix proteins by cultured smooth muscle cells from human varicose veins. *J Vasc Res* 1998;35: 115–23.
- [16] Raffetto JD, Khalil RA. Mechanisms of varicose vein formation: valve dysfunction and wall dilation. *Phlebology* 2008; 23:85–98.
- [17] Lengyel I, Acsády G. Histomorphological and pathobiochemical changes of varicose veins. A possible explanation of the development of varicosis. *Acta Morphol Hung* 1990;38: 259–67.
- [18] Chiou SH, Wu CY. Clinical proteomics: current status, challenges, and future perspectives. *Kaohsiung J Med Sci* 2011; 27:1–14.
- [19] MacCoss MJ, McDonald WH, Saraf A, Sadygov R, Clark JM, Tasto JJ, et al. Shotgun identification of protein modifications from protein complexes and lens tissue. *Proc Natl Acad Sci U S A* 2002;99:7900–5.
- [20] Ideker T, Thorsson V, Ranish JA, Christmas R, Buhler J, Eng JK, et al. Integrated genomic and proteomic analyses of a systematically perturbed metabolic network. *Science* 2001; 292:929–34.
- [21] Wu CJ, Chen YW, Tai JH, Chen SH. Quantitative phosphoproteomics studies using stable isotope dimethyl labeling coupled with IMAC-HILIC-nanoLC-MS/MS for estrogen-induced transcriptional regulation. *J Proteome Res* 2011; 10:1088–97.
- [22] Santos HM, Kouvonen P, Capelo JL, Corthals GL. Isotopic labelling of peptides in tissues enhances mass spectrometric profiling. *Rapid Commun Mass Spectrom* 2012;26:254–62.
- [23] Boersema PJ, Aye TT, van Veen TA, Heck AJ, Mohammed S. Triplex protein quantification based on stable isotope labeling by peptide dimethylation applied to cell and tissue lysates. *Proteomics* 2008;8:4624–32.
- [24] Yoshida T. Peptide separation in normal phase liquid chromatography. *Anal Chem* 1997;69:3038–43.
- [25] Yoshida T. Calculation of peptide retention coefficients in normal-phase liquid chromatography. *J Chromatogr A* 1998; 808:105–12.
- [26] Alpert AJ. Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. *J Chromatogr* 1990;499:177–96.
- [27] Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 1999; 20:3551–67.
- [28] Hirosawa M, Hoshida M, Ishikawa M, Toya TMASCOT. multiple alignment system for protein sequences based on three-way dynamic programming. *Comput Appl Biosci* 1993;9:161–7.
- [29] Thingholm TE, Jensen ON, Robinson PJ, Larsen MR. SIMAC (sequential elution from IMAC), a phosphoproteomics strategy for the rapid separation of monophosphorylated from multiply phosphorylated peptides. *Mol Cell Proteomics* 2008; 7:661–71.
- [30] Hou W, Ethier M, Smith JC, Sheng Y, Figeys D. Multiplexed proteomic reactor for the processing of proteomic samples. *Anal Chem* 2007;79:39–44.
- [31] Pichler P, Köcher T, Holzmann J, Mazanek M, Taus T, Ammerer G, et al. Peptide labeling with isobaric tags yields higher identification rates using iTRAQ 4-plex compared to TMT 6-plex and iTRAQ 8-plex on LTQ Orbitrap. *Anal Chem* 2010;82:6549–58.
- [32] Webster J, Oxley D. Protein identification by MALDI-TOF mass spectrometry. *Methods Mol Biol* 2012;800:227–40.
- [33] Raffetto JD, Ross RL, Khalil RA. Matrix metalloproteinase 2-induced venous dilation via hyperpolarization and activation of K⁺ channels: relevance to varicose vein formation. *J Vasc Surg* 2007;45:373–80.
- [34] Haviarová Z, Janegová A, Janega P, Durdík S, Kováč P, Stvrtinová V, et al. Expression of constitutive nitric oxide synthase isoforms in varicose vein wall; preliminary results. *Int J Vasc Med* 2011;2011:204723.
- [35] Sansilvestri-Morel P, Rupin A, Jaisson S, Fabiani JN, Verbeuren TJ, Vanhoutte PM. Synthesis of collagen is dysregulated in cultured fibroblasts derived from skin of subjects with varicose veins as it is in venous smooth muscle cells. *Circulation* 2002;106:479–83.
- [36] Woodside KJ, Hu M, Burke A, Murakami M, Pounds LL, Killewich LA, et al. Morphologic characteristics of varicose veins: possible role of metalloproteinases. *J Vasc Surg* 2003; 38:162–9.
- [37] Butler TM, Siegman MJ, Mooers SU, Narayan SR. Myosin-product complex in the resting state and during relaxation of smooth muscle. *Am J Physiol* 1990;258:C1092–9.
- [38] Butler TM, Pacifico DS, Siegman MJ. ADP release from myosin in permeabilized smooth muscle. *Am J Physiol* 1989;256: C59–66.
- [39] Butler TM, Narayan SR, Mooers SU, Siegman MJ. Rapid turnover of myosin light chain phosphate during cross-bridge cycling in smooth muscle. *Am J Physiol* 1994;267:C1160–6.
- [40] Wallimann T, Wyss M, Brdiczka D, Nicolay K, Eppenberger HM. Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. *Biochem J* 1992;281: 21–40.
- [41] Banks RD, Blake CC, Evans PR, Haser R, Rice DW, Hardy GW, et al. Sequence, structure and activity of phosphoglycerate kinase: a possible hinge-bending enzyme. *Nature* 1979;279: 773–7.
- [42] Bernstein BE, Hol WG. Crystal structures of substrates and products bound to the phosphoglycerate kinase active site reveal the catalytic mechanism. *Biochemistry* 1998;37: 4429–36.
- [43] Mazurek S. Pyruvate kinase type M2: a key regulator of the metabolic budget system in tumor cells. *Int J Biochem Cell Biol* 2011;43:969–80.
- [44] Cheung WY. Calmodulin plays a pivotal role in cellular regulation. *Science* 1980;207:19–27.
- [45] Kamm KE, Stull JT. Dedicated myosin light chain kinases with diverse cellular functions. *J Biol Chem* 2001;276:4527–30.
- [46] Kamm KE, Stull JT. The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Ann Rev Pharmacol Toxicol* 1985;25:593–620.
- [47] Stull JT, Lin PJ, Krueger JK, Trewheella J, Zhi G. Myosin light chain kinase: functional domains and structural motifs. *Acta Physiol Scand* 1998;164:471–82.
- [48] Somlyo AP, Somlyo AV. Signal transduction and regulation in smooth muscle. *Nature* 1994;372:231–6.
- [49] Singer HA. Ca²⁺/calmodulin-dependent protein kinase II function in vascular remodelling. *J Physiol* 2012;590: 1349–56.

- [50] Elsharawy MA, Naim MM, Abdelmaguid EM, Al-Mulhim AA. Role of saphenous vein wall in the pathogenesis of primary varicose veins. *Interact Cardiovasc Thorac Surg* 2007;6: 219–24.
- [51] Sansilvestri-Morel P, Rupin A, Badier-Commander C, Kern P, Fabiani JN, Verbeuren TJ, et al. Imbalance in the synthesis of collagen type I and collagen type III in smooth muscle cells derived from human varicose veins. *J Vasc Res* 2001;38:560–8.