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Deep Vein Thrombosis Exhibits Characteristic Serum and Vein Wall Metabolic Phenotypes in the Inferior Vena Cava Ligation Mouse Model

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WHAT THIS PAPER ADDS

The diagnosis of deep vein thrombosis (DVT) remains challenging as there is no specific and accurate diagnostic biomarker. In this study, metabolomic profiling was applied to identify metabolites as potential biomarkers of DVT. Serum and vein wall extracts of the inferior vena cava ligation DVT mouse model were subjected to liquid chromatography coupled mass spectrometry and NMR spectroscopy. Applying multivariate and univariate statistical analysis allowed for identification of perturbations of carnitine metabolism, sphingolipid metabolism, lipid metabolism, and adenosine metabolism. The findings could direct future studies identifying possible DVT biomarkers in humans and improve our understanding of the underlying metabolic alterations of DVT.

Objectives: Deep vein thrombosis (DVT) is a major health problem, responsible for significant morbidity and mortality. The identification of a simple and effective diagnostic biomarker of DVT remains a challenge. Metabolomics have recently emerged as a new powerful scientific tool to characterise metabolic phenotypes of complex diseases and investigate small molecules in biofluids. The aim of the study was to identify the blood and vein wall metabolomic signature of DVT in a murine experimental model.
Methods: An established inferior vena cava ligation mouse model of DVT (n=10) was used and compared with sham surgery controls (n=10). Comprehensive untargeted metabolic profiling of serum and vein wall extracts was undertaken using liquid chromatography coupled mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy.
Results: Multivariate and univariate statistical analysis demonstrated a differential metabolic profile when comparing DVT mice and control animals. Serum from DVT mice was characterised by differential concentrations of adenosine (decreased in DVT mice 9.6 fold), adenine (decreased 10.6 fold), and tricyclic acid cycle (TCA) intermediates, including citrate, succinate, and fumarate (1.5, 2.3, and 2.8 fold decreases, respectively). L-carnitine was found to be of greater abundance in the serum of DVT animals (67.0 fold change). A number of lipid moiety classes, including sphingomyelins, phosphatidylcholines, and triglycerides, were differentially abundant. Several metabolites were found in vein wall, including acetylcarnitine (increased in DVT mice 1.9 fold), adenosine (increased 2.2 fold), and ceramide (increased 2.7 fold). Correlation analysis illustrated the biochemical relationships between assigned metabolites, with the discriminatory molecules being highly correlated with each other, in both serum and vein wall.
Conclusions: The present findings demonstrate that metabolic dysregulations in DVT centre on energy metabolism, sphingolipid, and adenosine metabolism, representing a DVT specific metabolite signature in a murine experimental model.
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INTRODUCTION

Deep vein thrombosis (DVT) is a major health problem, responsible for significant morbidity and mortality, and imposes a heavy economic burden on healthcare systems.[1] Although most events resolve without complication through spontaneous lysis and recanalisation, DVT can be complicated with life threatening pulmonary embolism,[2] while

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approximately one third of DVT patients develop post-thrombotic syndrome with swelling, pain, skin changes, and/or venous ulceration.[3] Treatment by anticoagulation prevents further thrombus extension, protects from pulmonary embolism, and reduces the risk of chronic lower limb complications. Importantly, unnecessary treatment can result in bleeding. Therefore, accurate and reliable DVT diagnosis is essential. Currently, diagnosis relies on subjective clinical examination and ultrasound imaging.[4] A number of biological markers have been investigated with variable results. D-dimer, the most widely used biomarker, is sensitive but lacks specificity.[5,6] Ongoing research efforts target the utility of alternative blood diagnostic biomarkers able to accurately diagnose DVT, guide length and type of treatment, and potentially identify patients who may benefit from more aggressive therapies than standard anticoagulation.

New molecular technologies and methods have entered the scientific arena, offering the opportunity to revisit this important clinical need. Metabolic profiling has emerged as a new approach to investigate complex metabolic disease and enable precision medicine. Metabolomics is the comprehensive and systematic identification of the small molecules present in differential abundance in biofluids and is affected by various factors such as diet, lifestyle, genetics, disease, environmental factors, and medications. Metabolic profiling approaches to characterising the metabolome can be either targeted or untargeted. In targeted approaches specific metabolites, representative of suspected biological pathways, are analysed in each sample. Non-targeted analysis simultaneously screens multiple small molecules for alterations in their levels within biofluids. This latter approach can identify novel, unrecognised metabolites and pathways, elucidating the pathophysiology of the disease, and highlighting potential biomarkers, biomarker signatures, and/or therapeutic targets. Omics studies, specifically the non-targeted approaches, have allowed us to go from hypothesis led to hypothesis generating studies.

Metabolomics employs high throughput analytical platforms (both nuclear magnetic resonance [NMR] spectroscopy and ultra-performance liquid chromatography mass spectrometry [UPLC-MS]) coupled with statistical modelling to identify metabolites, which are differentially present in the context of health and disease. This provides a platform to screen for candidate biomarkers for DVT diagnosis.[7] NMR is a non-destructive, robust, and quantitative method that provides information on molecular structure, but lacks sensitivity. MS is more sensitive, but requires pre-separation by a chromatographic method and ionisation of the sample. The combination of NMR and MS provides complementary and accurate information on metabolites. Metabolomics has been shown to have utility in vascular disease and metabolic changes have previously been identified, at tissue level, between varicose and non-varicose veins, carotid and femoral atherosclerosis, and stable and unstable carotid plaques.[8,9] Recently, NMR spectrometry was used to identify metabolic changes of DVT in animals of different ages.[10]

This study aimed to elucidate the DVT specific metabolite profile in a murine experimental model.

MATERIALS AND METHODS

Experimental murine model of DVT

The well established inferior vena cava (IVC) ligation mouse model of DVT was developed within the Conrad Jobst Vascular Research Laboratories, University of Michigan, in compliance with United States Department of Agriculture and Animal Agriculture Liaison Committee policies.[11–13] A total of twenty 8–10 week old male Balb/C mice (mean weight 23.7 ± 0.9g) were used. In 10 mice, the inferior vena cava (IVC) below the renal veins and side branches were ligated while posterior branches were cauterised to generate thrombosis as described previously.[12–14] In a further 10 mice, sham laparotomy produced non-DVT control animals. All mice were euthanised at day 2 after IVC ligation or sham surgery. At the time of euthanasia, blood and vein wall were collected. Serum and IVC wall tissue were stored at -80 °C.

Sample preparation and UPLC-MS analysis

Reversed phase (RP) and hydrophilic interaction chromatography (HILIC) UPLC-MS profiling of serum were applied. For profiling of vein wall, organic and aqueous metabolites were extracted serially using a modification of the protocol described by Anwar et al.[15] The vein wall tissue was homogenised using 1mm diameter zirconium beads.

Lipid profiling of serum and vein wall organic metabolite extracts was performed using RP-UPLC-MS as described by Isaac et al.[16] HILIC-UPLC-MS metabolic profiling of serum and aqueous metabolites of vein wall was conducted using a modification of the protocol as described by Spagou et al.[17] Separated samples were analysed with electrospray ionisation (ESI)+ and ESI- modes (supplementary material).

Serum preparation and NMR analysis

For NMR analysis of serum, buffer solution of 0.075 M $Na_2HPO_4x7H_2O$, 4% sodium azide (NaN_3) in H_2O, 20% deuterium oxide (D_2O), and 0.08% 3-(trimethyl-silyl)propionic acid-d4 (TSP) was added to serum samples. ^{1}H-NMR data acquisition was performed as previously described[18] (supplementary material).

Data processing and statistical analysis

The raw MS data were collected in centroid mode (serum and vein wall organic metabolite extracts), pre-processed with XCMS package in R programming software, and the resulting data normalised using a total area normalisation method. Vein wall data were corrected for weight prior to normalisation. The raw data collected in continuum mode (vein wall aqueous extract) were pre-processed using Progenesis QI software (Nonlinear Dynamics, USA), corrected and normalised as above.

For the NMR dataset, NMR spectral data were imported into MATLAB R2014a software (MathWorks, USA) for pre-

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