Journal of Proteomics xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Original Article

# Systems characterization of differential plasma metabolome perturbations following thrombotic and non-thrombotic myocardial infarction

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ARTICLE INFO

*Article history:*
Received 20 January 2017
Received in revised form 3 March 2017
Accepted 18 March 2017
Available online xxxx

*Keywords:*
Metabolomics
Myocardial infarction
Coronary artery disease
Systems biology
Network analysis

ABSTRACT

Myocardial infarction (MI) is an acute event characterized by myocardial necrosis. Thrombotic MI is caused by spontaneous atherosclerotic plaque disruption that results in a coronary thrombus; non-thrombotic MI occurs secondary to oxygen supply-demand mismatch. We sought to characterize the differential metabolic perturbations associated with these subtypes utilizing a systems approach. Subjects presenting with thrombotic MI, non-thrombotic MI and stable coronary artery disease (CAD) were included. Whole blood was collected at two acute time-points and at a time-point representing the quiescent stable disease state. Plasma metabolites were analyzed by untargeted UPLC-MS/MS and GC–MS. A weighted network was constructed, and modules were determined from the resulting topology. To determine perturbed modules, an enrichment analysis for metabolites that demonstrated between-group differences in temporal change across the disease state transition was then conducted.

*Biological significance:* We report evidence of metabolic perturbations of acute MI and determine perturbations specific to thrombotic MI. Specifically, a module characterized by elevated glucocorticoid steroid metabolites following acute MI showed greatest perturbation following thrombotic MI. Modules characterized by elevated pregnenolone metabolites, monoacylglycerols, and acylcarnitines were perturbed following acute MI. A module characterized by a decrease in plasma amino acids following thrombotic MI was differentially perturbed between MI subtypes.



## 1. Introduction

Heart disease is the most prevalent cause of global mortality [1]. While heart disease is a ubiquitous disease with respect to prevalence, there is significant heterogeneity in outcomes [2–4] and in the incidence and presentation of acute events such as acute myocardial infarction (AMI) [5]. Given this heterogeneity, elucidating the metabolic perturbations associated with the transition from a stable disease state such as stable coronary artery disease (sCAD), to an acute event such as AMI is of critical importance. While myocardial necrosis is a pathological characteristic common to all acute myocardial infarctions, there are multiple proximate causes of AMI [6]. A classification system based on etiology has been developed and includes 6 types [6]. Of interest is differentiating AMI caused by spontaneous atherosclerotic plaque disruption that results in a coronary thrombus (thrombotic MI), versus AMI caused by a deficit in oxygen supply secondary to other non-thrombotic causes such as vasospasm or stress cardiomyopathy. This distinction is important as the course of treatment differs between types and misclassification may result in negative outcomes such as iatrogenic bleeding [7].

The pathophysiology of thrombotic MI can be conceptualized as a "perfect storm" in which a vulnerable atherosclerotic plaque ruptures or is disrupted in the presence of thrombogenic blood [8]. While plaque rupture or disruption is a prerequisite for coronary thrombosis, it is not sufficient. The insufficiency has been demonstrated by autopsy study of cases of sudden cardiac death in which evidence of a healing thrombus (as opposed to pathological) was found in 69% of cases [9]. This

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http://dx.doi.org/10.1016/j.jprot.2017.03.014
1874-3919/

characterization of thrombotic MI suggests that a biological processes or metabolic response distinct from a plaque rupture or disruption may provide sufficient amplification for pathological thrombosis. Elucidation of such amplifying factors in thrombotic MI includes identifying metabolites that are differentially abundant at the time of thrombotic MI compared to a quiescent stable disease state. To demonstrate specificity for thrombotic MI, these factors must not be differentially abundant secondary to downstream ischemia/necrosis or medical and pharmacological interventions that individuals undergoing MI receive. Furthermore, to the authors knowledge, an examination of the metabolic perturbations following AMI differentiated by etiological subtype (thrombotic versus non-thrombotic) has not yet been conducted. We have thus executed such a study evaluating the plasma metabolome of subjects experiencing AMI differentiated by subtype at the acute event state and the quiescent stable disease state.

Metabolic phenotyping is well suited for studying disease state transitions as changes in metabolite concentrations are dependent on genetic factors, environmental influences, and gene-environment interactions [10]. Since blood plasma functions as a liquid carrier, plasma contains enzymes, lipoproteins, hormones, nutrients, metabolic waste products, and many other small molecules dissolved or in suspension [11] it has the advantage of being a repository of metabolic changes from all tissues and therefore reflective of the state of the entire organism at the time of sampling. However, attributing the root cause of changes in metabolite concentration in plasma or serum across state transitions is not straightforward. Developing a mechanistic reconstruction of metabolic pathway impacts is complicated by the following: (1) plasma contains intermediates and products of multiple metabolic pathways, (2) the tissue source of metabolites in plasma may not be known, and (3) curated metabolic pathway models may not generalize to plasma when a preponderance of the model is localized to unobserved cellular compartments such as mitochondria. Consequently, a data-dependent network reconstruction of the plasma metabolome for determining related sets of metabolites was conducted. This is consistent with the function of metabolites as substrates, products, and regulatory factors within discrete biological processes.

In this study, we recruited three patient groups: (1) subjects presenting with acute thrombotic MI, (2) subjects presenting with acute non-thrombotic MI, and (3) subjects presenting with stable CAD undergoing cardiac catheterization. Plasma metabolites were extracted from whole blood collected from each subject during both the acute/procedural phase and at a follow-up time-point (approximately 3 months later) regarded as a subject's quiescent stable disease phase. We used a data-dependent strategy to identify “modules” of related metabolites based on network topology. The methodology used for discovering metabolite modules belongs to a class of techniques known as “weighted network analysis” [12] and was originally developed as a framework for analyzing gene co-expression networks [13]. After module discovery, we then sought to evaluate whether any of the modules were significantly associated with the transition from the stable disease state to the acute disease state and demonstrated phenotype specificity. We report multiple modules enriched for related metabolites that distinguish acute MI from stable CAD, a module enriched for metabolites differing between thrombotic MI and non-thrombotic MI, and a module enriched for differences across all three groups.

## 2. Methods

A graphical overview of the analytical approach utilized in this study is presented in the graphical abstract (Fig. 1).

### 2.1. Study cohort

A patient cohort was recruited to allow for determining the unique plasma metabolomic signature of AMI differentiated by proximate cause [14]. A novel criterion was developed for discriminating between thrombotic and non-thrombotic MI patients as widely accepted guidelines for such discrimination were unavailable. This criterion was sufficiently stringent to minimize misclassification through the elimination of borderline/non-definitive cases. In addition to enrolling AMI patients, subjects with stable coronary artery disease presenting for an elective procedure requiring cardiac catheterization were enrolled. As thrombotic MI is defined with respect to a present characteristic (thrombosis) while non-thrombotic MI is defined by an absence of a characteristic, our cohort was suitable for demonstrating specificity for MI secondary to thrombosis with proper control. Specifically, the non-thrombotic MI group was utilized to control for the presence of metabolic changes associated with ischemia and myocardial necrosis. The stable CAD group was used to control for the presence of underlying constitutive atherosclerotic disease factors that would be present in thrombotic MI subjects but may not be present in non-thrombotic MI subjects. Both groups were utilized to control for metabolic changes associated with cardiac catheterization. Participants were recruited from two hospitals following approval by the University of Louisville Institutional Review Board. Participants were provided with written informed consent and the study was conducted in accordance with the ethical standards defined in the 1964 Helsinki declaration. A total of 11 thrombotic MI, 12 non-thrombotic MI, and 15 stable CAD subjects were eligible and enrolled in the study. Further details on the human subject cohort and enrollment criterion are provided in the Supplement (Supplementary Table 1).

### 2.2. Plasma metabolomics

Whole blood was collected from study subjects immediately prior to cardiac catheterization (denoted T0) and 6-hour post catheterization (denoted T6). These samples represented acute phase time-points for MI subjects. To represent a subject's quiescent phase, whole blood was again collected at a follow-up time-point approximately 3 months after the cardiac catheterization procedure. Plasma samples were then prepared for identification and quantification of metabolite relative abundances by Metabolon, Inc. (Research Triangle Park, NC). The metabolite extraction process was conducted using the Microlab STAR® system, an automated liquid handling workstation (Hamilton Company, Reno, NV). After adding a recovery standard, methanol was added to precipitate proteins and vigorous shaking was applied using a GenoGrinder 2000 (Glen Mills, Metuchen, NJ). The resulting extract containing metabolites was divided into five aliquots—one each for positive and negative ion mode ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) with electrospray ionization, one for polar metabolite quantification (UPLC-MS/MS with negative ion mode electrospray ionization), and one for gas chromatography–mass spectrometry (GC–MS) analysis. UPLC-MS/MS analysis was conducted using a Waters ACQUITY UPLC (Milford, MA) and a Thermo Scientific (Waltham, MA) Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated with a scanning range from 80 to 1000 $m/z$ and 35,000 mass resolution. Separate Waters UPLC BEH C18 (2.1 × 100 mm, 1.7 μm) columns were used for positive and negative ion optimized conditions using water and methanol containing 0.1% formic acid (positive ion optimization) or water and acetonitrile with 10 mM ammonium formate (negative ion optimization). The aliquots for GC–MS analysis were dried under vacuum for a minimum of 18 h and derivatized under dried nitrogen using bistrimethylsilyltrifluoroacetamide. Each aliquot was then separated on a 5% diphenyl/95% dimethyl polysiloxane fused silica column (20 m × 0.18 mm ID; 0.18 μm film thickness) with helium as the carrier gas and a temperature ramp from 60° to 340 °C in a 17.5 min period. GC–MS analysis was then conducted utilizing a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer with electron impact ionization. The scan range was from 50 to 750 $m/z$ and had unit mass resolving power. Molecular identification was performed by

Please cite this article as: P.J. Trainor, et al., Systems characterization of differential plasma metabolome perturbations following thrombotic and non-thrombotic myocardial infarc..., J Prot (2017), http://dx.doi.org/10.1016/j.jprot.2017.03.014