"Identification of Distinctive Metabolic Signatures of autoimmune inflammatory diseases using NMR based Metabolomics Approach"

Summary

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Summary

The sophisticated immune system has developed to protect humans from infectious pathogens as its principal role. The inability to distinguish between self and non-self is commonly referred to as an immunological deficiency, and it is at the origin of autoimmune disorders. There are around 100 unique autoimmune disorders, some of which are organ-specific, such as Takayasu arteritis (TA) and Rheumatic arthritis (RA), and some of which are multi-organ immunological disorders, such as systemic lupus erythematosus (SLE). With the introduction of developing molecular immunology techniques and pioneering clinical laboratory work based on evidence, major advances in diagnosis and ailment diagnoses, as well as improvements in prognosis, have been made over the last decade. Takayasu arteritis is an uncommon, idiopathic chronic inflammatory condition affecting the aorta and its major branches. It contributes to large artery stenosis, occlusion, and aneurysmal degeneration.

Traditionally, autoimmune disorders have been considered uncommon but have now been shown to affect 3-5 percent of the population through extensive epidemiological studies; it has now been shown to affect 3-5 percent of the population, with the most common of these disorders being autoimmune thyroid disease, type I diabetes (T1D) and systemic lupus erythematosus (SLE). More notably, however, there are almost 100 different autoimmune diseases, some of which are organ-specific such as Takayasu arteritis (TA), Rheumatic arthritis (RA), while some of which represent a spectrum of immunological dysfunctions affecting multiple organs such as SLE.

Multiple genes with specific gene mutations, HLA susceptible, non-HLA loci as well as epigenetic mechanisms have been implicated in specific autoimmune diseases. The majority of autoimmune disorders have multiple genetic factors that play a part, rather than being monogenic. While there have been a number of early studies that show correlations in human autoimmune diseases with the major histocompatibility complex (MHC), the findings have also failed to lead to associations that have substantial predictive strength for the clinician.

In the production of autoimmunity, many environmental factors have been implicated. The most popular mechanism which activates autoreactive T and B cells is 'molecular mimicry'. 'Epitope spreading' is a process that results in multiple neo-epitopes being produced. Furthermore, microbiota and diet (e.g., vitamin D, iodine and gluten) may also lead to loss of resistance by modulating innate and adaptive immunity.

However, through a large number of molecular studies exploring not only genetic causes, but also the role of epigenetics, our understanding of human autoimmune disease has and continues

to be established. In addition, advances have been made in the methodology of laboratory research, including serological standardization and the development of modern autoantibody tests**.** In addition, increased knowledge of geoepidemiology has resulted in a much greater understanding of what happens to particular patients during a tolerance violation. For all physicians, autoimmunity is a challenge; however, the prognosis for patients with these diseases has increased significantly over the past decade and we foresee more changes in the future.

Because systemic inflammation has a substantial impact on metabolism, metabolomics was employed to investigate inflammatory models in people and animals. Various investigations indicated that inflammation alters metabolite levels, providing valuable insights into the aetiology of these diseases and exposing multiple possible biomarkers for disease evaluation. Metabolomics has developed as a particularly viable high-throughput technique for the investigation of complex biological samples in recent years. It provides more precise insights into an organism's pathophysiological condition by analyzing the total low molecular weight metabolites (metabolome), which are generated in response to genetic changes and/or environmental stimuli. This analytical method was made possible by NMR spectroscopy's versatility as a powerful tool for detecting and quantifying a wide range of cellular metabolites in biological materials. When combined with pattern recognition techniques, it makes substantial contributions to the classification of an organism's phenotype, opening up many scientific disciplines for metabolomic research.

Metabolomics has newly now become modern technique for the identification of biomarker molecules and biochemical responses caused by a disease or its therapeutic action, using mass spectrometry (MS) and high resolution proton nuclear magnetic resonance (NMR) to access metabolite profiles in biofluids or tissue extracts. Applying recent developments in the study of autoimmune diseases (ADs) using metabolomic methods, including multiple sclerosis (MS), rheumatoid arthritis (RA), inflammatory bowel diseases (IBD), autoimmune diabetes et al. Many experiments have shown that AD patients can be distinguished using metabolic profiling followed by well-established data analysis methods, including main component analysis (PCA) and partial least square analysis (PLS), involving subtypes of certain diseases and stable individuals. Not only do these metabolites influence the synthesis of oxygen, amino acids and lipids, but they also include changes in neurotransmitters, nucleotides, immune responses and anti-inflammatory responses. For the identification, treatment, and detection processes of subsequent diseases, knowledge of specific metabolomic signatures in ADs could be useful.

Metabolomics a new-sprung cousin to genomics and proteomics- is an analytical approach to metabolism that involves a comparative and quantitative analysis of low molecular weight metabolites and their intermediates concentration profiling in affected biological systems (typically urine, blood plasma/serum/ synovial fluid, cell lysates, or tissue extracts). Genomics, transcriptomics and proteomics analysis further complemented with metabolomics information, which allows offers the potential to understand the entire biological system including health or disease processes operating in that system –so-called systems biology approach. With its ability to discover disease-related biomarkers and underlying biochemical processes, nowadays, metabolomics is utilized virtually in all aspects of biomedical research directed to enhance the understanding of the disease and health processes. The complete paradigm is based on the fact that a pathophysiological condition or therapeutic intervention results in a distinct and characteristic change in the biochemical composition profiles of biofluids and metabolomics aims to identify these changes. The biochemical changes -that correlate with a disease (or disease type/grade) and treatment response- then permit the clinical researchers to raise the diagnosis of disease including, early disease detection, monitoring response to treatment and patient stratification for therapy. The molecular biomarkers validated using wide-range of human populations form the basis for new clinical diagnostic assays.

Nowadays, Proton Nuclear Magnetic Resonance (^{1}H NMR), Gas Chromatography-Mass Spectrometry (GC-MS) and Liquid Chromatography-Mass Spectrometry (LC-MS) are well-established powerful analytical methods for generating metabolomics profiles. For the analysis of complex, biological samples like bio-fluids, these techniques have their advantages and disadvantages. For instance, GC-MS requires derivatization, which lengthens the sample preparation time. In general LC-MS and GC-MS need more time-consuming sample preparation. NMR offers several clear advantages compared to other analytical and biochemical analysis methods used for metabolomics studies: (a) applicable to a variety of biological and clinical samples, tissue extracts, and even cell lysates, (b) rapid, quantitative, and offers the potential for high-throughput (i.e. analysis of >100 samples/day is attainable), (c) least-destructive (i.e. the prepared sample can be used in many consecutive NMR experiments or the same sample can be analysed by the other-other analytical techniques after the NMR experiments are completed), (d) unbiased (i.e., all protonated metabolites present in a biological mixture are detectable irrespective of their physical properties) (e) Peaks in the NMR spectra can be consistently assigned to the particular metabolite, based on their chemical shifts and multiplet patterns and (f) virtually it requires no sample preparation and furnishes highly reproducible results. These are the main grounds that NMR has become the method of

choice for studying metabolic changes associated with distinct human pathologies and also gaining tremendous popularity in pharmaco-metabolomics studies as well. Nowadays, the sensitivity of NMR is also not a major issue; even nanogram detection limits are possible with novel pulse methodology and appropriate instrumentation.

The main objective in Metabolomics is to study all the metabolites within a biological sample in an unbiased manner. The metabolomics studies involve a number of specimens together for the analysis. For metabolomics, well-planned experiment design and execution are required along with univariate and multivariate statistical analysis is needed for pattern recognition and interpretation of metabolomics data. Hence, the NMR spectroscopy coupled with multivariate statistical analysis permits the identification of metabolic disturbances and distinct metabolic pathways associated with the disease, but it also allows the recognition of metabolic signatures which have their potential diagnostic and prognostic implications for clinical management of the disease.

The NMR-based metabolomics approach has already been helpful in identification of metabolic markers for inflammatory autoimmune disease such as Systemic lupus erythematosus (SLE), Takayasu arteritis (TA), rheumatoid arthritis (RA), and gout disease etc. The identification of disease biomarkers plays a critical role not only in early disease diagnosis or risk prediction but also in classification and disease progression or assessment prognosis and treatment response. Establishment of these biomarkers in routine clinical use has the potential to provide insight into the pathogenesis of disease states and discover diagnostic markers for therapeutic targets. Thus, the metabolic biomarkers have the power to increase the overall survival and quality of patient life in, addition to saving huge expenses for the society. The primary objective of the research presented in this thesis was to evaluate the use of high-resolution NMR spectroscopy together with multivariate analysis based metabolomics for identifying and characterizing potential biomarkers of TA patient. This thesis consists of original research work in which the applicability of NMR Metabolomics in identifying biomarkers of disease dependent changes has been explored. Metabolomic studies were performed on a broad range of subjects ranging from healthy volunteers to patients with active and inactive stage of the disease. The research objectives undertaken have been discussed briefly.

In this Ph.D. thesis, NMR-based metabolomics was used for metabolic profiling to identify possible unique metabolic profiles and biomarker candidates for the active and inactive stages of Takayasu arteritis, where it is possible to manipulate unpredictable changes in metabolite levels to discover novel mechanistic information in health and disease. This advancement in the area parallels the two papers used in this study, beginning with article I. Here, we present the quantitative evaluation of disease activity, which is critical for the efficient management of Takayasu Arteritis patients (TA). Active inflammation is distinguished by activated glutaminolysis and decreased glycolytic flow. We believe that the circulatory Glutamine/Glucose ratio (QGR) can be used to detect active inflammation in TA.

Using high field (800 MHz) NMR spectroscopy, the quantitative profiles of circulatory metabolites involved in glutaminolysis (Glutamine and Glutamate) and those that estimate glycolytic flux (Glucose and lactate) were examined. When compared to inactive-TA patients, active-TA patients' serum levels of glutamine and lactate were considerably lower, indicating that these individuals had activated glutaminolysis and lowered glycolytic activity. This is reinforced further by considerably lower QGR and lactate to glucose ratio (LGR) levels in active TA patients compared to inactive TA patients. As a result, we feel that circulatory QGR has the potential to be used as a surrogate marker for assessing disease activity in TA patients. However, using this ratio in clinical settings would need further research on large patient cohorts as well as procedure modification to increase accuracy.

In paper II, Using serum samples, Histidine was found to have substantial anti-oxidative and anti-inflammatory effects. It also acts as a precursor for histamine biosynthesis and is important in immune-mediated inflammatory disorders. Based on this hypothesis, we postulated that circulating Histidine might be used to detect active inflammation and hence monitor disease activity in TA. Circulating histidine levels were substantially lower in active TA patients compared to both inactive TA patients and NC, but there was no statistically significant difference between inactive TA and NC. The circulatory histidine/formate ratio might be used as a surrogate marker to improve the diagnostic screening of TA patients, both active and inactive. Furthermore, they may be investigated for their value in assessing disease progression and suggesting treatment in TA patients. However, for therapeutic relevance, further research on large patient cohorts must be conducted in a longitudinal approach.

In the current Ph.D. research, NMR-based metabolomics was shown to be a feasible method for identifying QGR as potential biomarkers of both the active and inactive stages of Takayasu arteritis (chapter II), as well as histidine as a biomarker in chapter III. Although understandable

in certain ways, it is important to remember that the biological evidence acquired from metabolomic investigations, in terms of potential biomarkers, must be verified before making any statements. In particular, a useful biomarker, in particular, must be quantifiable, reproducible, and related to important biochemical results. As a consequence, it would be beneficial to expand the current study to additional autoimmune disorders in order to further examine the validity of the data provided in this thesis. NMR and MS, in particular, have been shown to be complementary and strong analytical techniques for the full assessment of the metabolome. An integrated NMR-MS metabolomic method will enhance overall metabolite identification and provide the opportunity to broaden the scope of metabolomic research. Furthermore, a focused examination of a single route may be required to properly understand certain metabolically caused changes. Finally, the ability to integrate NMR with other analytical platforms may provide additional information on the autoimmune metabolome, allowing for a better understanding of the metabolic consequences caused by self-reactive antibodies.

Recent technological advancements have increased the ability to investigate the metabolite compositions of biological samples linked with a variety of illnesses. This additional layer of knowledge substantially supplements the previously discovered genomic, transcriptomic, and proteomic technologies. In the near future, studies integrating these multiple levels of biological information will give essential knowledge for detecting the biological pathways that work in each illness and providing each patient with a specific molecular profile. Technologies for metabolomics investigation must enhance sensitivity in order to be less time-consuming and costly, and their precision must be increased by the associated analytic algorithms. Furthermore, if large cohort studies are to be undertaken, there is a considerable need for improvement in the throughput of most metabolomics systems. Furthermore, sampling and clinical selection methods must be consistent in order to assure sample accuracy and the reduction of technical and biological confounders. Although metabolomics is an emerging technique, it is quickly expanding, and new findings will undoubtedly improve our understanding of the molecular basis of rheumatic diseases in the coming years, leading to better outcomes for these patients.

We have shown that the serum metabolic profiling of TA patients and identified possible biomarkers for the diagnosis of TA differentiated from its active and inactive states in comparison to HC for the first time use NMR-based metabolomics techniques. The discovery of a relation between potential serum biomarkers in TA patients suggests that NMR might be a useful technique for evaluating specific pathogenic pathways in TA patients' inflamed arteritis.

There is an urgent need to confirm and enlarge this observation in a large cohort of patients, incorporating metabolomic research with cytokine and autoantibody analysis to develop testing that can identify a reaction with no need for interpretative care, bringing the age of specifically clinical practice closer. In the future, we would need to go through the actions of the various medications on the cellular metabolite, unequivocally identifying the metabolite NMR signals accountable for the differentiation between the treatments using advanced multivariate data analysis for biomarker profiling.