Review Article

Evaluation of Metabolomics in Chemical Pathology Research in Pakistan: A Less Explored Path and New Frontier

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Abstract

Metabolomics involves the study of unique chemical fingerprints left behind by specific cellular processes. Metabolomics encompasses the analysis of both "endogenous" compounds, such as amino acids, lipids, cofactors, nucleotides, carbohydrates, hormones, etc., and "exogenous" metabolites including drugs, toxins, environmental contaminants, pesticides, herbicides, etc. Initially, metabolic analyses heavily relied on nuclear magnetic resonance (NMR), but recent advancements in mass spectrometry (MS) and Tandem MS have expanded the horizons of research, service, and education in this field. In this overview, we delve into the domain of mass spectrometry from the perspective of Chemical Pathologists. To establish a baseline, we conducted a search using the PakMediNet search engine. Our literature review yielded a total of 1167 articles, of which 1155 were excluded, and 12 were included. Our findings indicated that while MS is utilized for method development and biomarker evaluation in the fields of basic biological sciences and the pharmaceutical industry, there are limited collaborative efforts with Chemical Pathologists for clinical applications. It is imperative to harness the advancements in MS for research and development, as this technology is a driving force behind progress in service and education. Looking ahead, areas such as newborn screening, diagnostics for inherited metabolic disorders, heavy metals analysis, and toxicology hold great potential for research in collaboration with Chemical Pathologists. To achieve these goals, the formation of working groups, establishing partnerships with institutes possessing relevant expertise, and providing funding opportunities are essential steps forward.

Introduction

The term "metabolomics" was first introduced in the early 2000s, representing a comprehensive effort to analyse the full spectrum of micro molecules, or metabolites with a molecular weight (MW) of up to 1500 Da, found within biological systems [1]. Metabolomics is a rapidly evolving field of research like genomics, transcriptomics, and proteomics. Its primary objective is to uncover the dynamic biochemical processes occurring within cells, tissues, and organisms, while also exploring exogenous" metabolites such as drugs, toxins, environmental contaminants, pesticides, herbicides, and more. Metabolites form a diverse collection of small molecules with relatively low molecular weights, encompassing lipids, amino acids, peptides, nucleic acids, organic acids, vitamins, thiols, and carbohydrates [2]. The analysis of metabolic profiles found in common biofluids like saliva, blood, urine, and faeces provides a valuable technique for assessing and predicting pathological conditions, dietary patterns, and medication toxicity. Unlike other "omics" disciplines, metabolites and their concentrations precisely reflect the underlying metabolic activities and the state of cells and tissues. This makes metabolomics an exceptionally effective approach, offering the most accurate representation of the molecular phenotype [2-4].

Metabolome profiling employs two primary approaches: targeted and untargeted methods. Targeted and semi-targeted metabolomics studies are aimed to precisely identify and quantify a specific subset of metabolites present in biological samples [5]. The goal is to provide accurate measurements of known metabolites. In contrast, untargeted studies focus on identifying and comparing as many signals as possible within a given sample set. The primary objective is to identify and categorize these signals as metabolites by utilising metabolomics databases. Untargeted studies are particularly valuable for uncovering previously unidentified metabolites, especially when these unknown compounds may serve as biomarkers in specific research areas [5]. This distinction between targeted and untargeted approaches allows researchers to choose the most suitable method based on their research objectives and the depth of metabolomic analysis required.

Metabolite exploration in biological fluids and tissues is a critical process in metabolomics research, achievable through various technological platforms such as nuclear magnetic resonance spectroscopy (NMR), Gas Chromatography-Mass Spectrometry (GC-MS), and Liquid Chromatography-Mass Spectrometry (LC-MS) [6]. Among the most common spectroscopic analytical techniques, NMR stands out for its ability to detect and analyse a wide spectrum of organic compounds in the micro-molar range without the need for derivatization. New NMR techniques (e.g., 1.2 GHz spectrometers), advances in cryoprobe, microprobe or sub-microprobe technologies, along with novel pulse sequence designs, significantly improve the sensitivity of NMR experiments and significantly decrease the lower limits of metabolite detection and quantification. In particular, the lower limit of detection for ultra-high-field NMR instruments (high nanomolar concentrations) is not too different than that of triple quadrupole MS instruments [8,9]. Mass spectrometry (MS) is gaining popularity in high-throughput metabolomics due to its greater sensitivity and is often combined with other methods such as chromatography-MS [6,7]. Due to the diversity of the metabolome, regardless of whether you choose untargeted or targeted metabolomics techniques, both approaches have their challenges. For instance, volatile organic compounds are best analysed using the GC-MS platform, while semi-polar molecules can be examined using LC-MS which can identify and quantify metabolites with high sensitivity and specificity without requiring chemical derivatization. However, it is important to note that some metabolite loss can occur during sample processing and this loss can be mitigated by combining multiple technological platforms.

Metabolomic research is still relatively new and emerging in many countries including Pakistan. Although some research has been conducted in this area, it remains in the early stages of development, requiring further investment and advancement in the field. There are significant challenges hindering the progress of metabolomic research, primarily stemming from a lack of funding and resources. One of the major impediments to the growth of metabolomic research in many countries including Pakistan is the inadequate funding available for research projects. This shortage of financial support limits the capacity of researchers to procure essential equipment and reagents necessary for conducting metabolomic analyses effectively. As a result, the potential for in-depth and comprehensive research in this field is stifled.

Another critical issue is the shortage of qualified personnel with expertise in metabolomics, which makes it difficult to conduct high-quality research and analyses in this field. Bridging this expertise gap is essential for advancing metabolomic studies in the country. The objective of this review is to identify prospective, cross sectional, experimental analytics studies carried out using Mass spectrometry techniques undertaken by Chemical Pathologists. By scrutinizing existing research, this review aims to pinpoint research gaps within the field of metabolomics. Subsequently, it will provide valuable approaches and suggestions to address these gaps. Efforts to address these challenges should include advocating for increased funding for metabolomic research, promoting collaboration with international experts and institutions, and establishing training programs to cultivate a skilled workforce in metabolomics. By addressing these issues and fostering a conducive environment for metabolomic research, scientists in the developing countries can make significant strides in advancing this emerging field and contributing to the global body of knowledge in metabolomics.

Material and Methods

In this overview, the research domain of Mass spectrometry was explored with Chemical Pathologists perspective from Pakistan. Pakmedinet was used as search engine using following keywords "Metabolomics", "Mass spectrometry", "Pakistan". The criteria

for inclusion in the shortlisting were limited to articles written by Pakistani authors, with no restrictions on the date or time of publication. Studies comprising reviews, letters to the editor, surveys, abstracts only, opinion papers, hypothesis, viewpoints, animal studies, basic sciences/nonclinical studies, article full text in language other than English and articles without any chemical pathologists listed as authors were omitted.

The two reviewers autonomously compiled the data using a predesigned pro forma enlisting the region of study publication, number of study participants, time period of recruitment, and findings of study.

Results

The databases searched revealed a total of 1167 articles. Based on the stringent inclusion criteria as depicted in Flowchart 1, 12 articles were included in the final analysis based on autonomous evaluation by two investigators with an excellent agreement of κ statistic = 0.90. Review 1articles n=157, abstracts only n=67, case reports n=116, case series n=72, frequency reports n=87, no Chemical Pathologists in authors n=656 were excluded using the predetermined criteria. Table 1 presents a comprehensive overview of the articles included in this systematic review published from 2016-2022.

Flowchart 1: Flowchart Illustrating the Author's Search Strategy for Metabolomics in Chemical Pathology – A detailed, step-by-step depiction of the process used to identify and analyse relevant studies, databases, and metabolic pathways relevant to chemical pathology research.

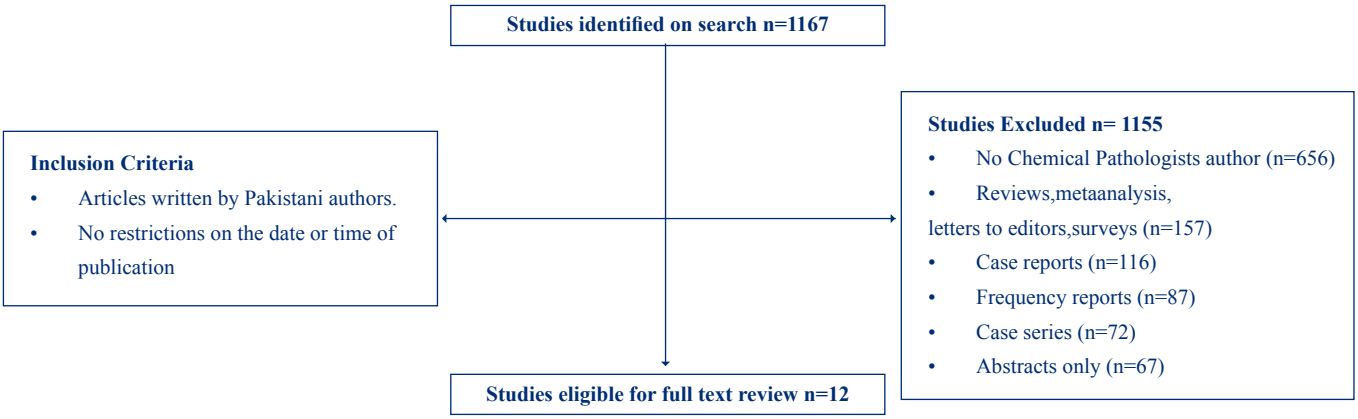

Table 1: Summary of Studies Related to the Evaluation of Metabolomics in Chemical Pathology – A concise overview of key studies, highlighting their contributions to the field.

S. No	Author(s)	Citation #	Region of Pakistan	Year of publication	Institute	Analyte measured	Technique used	Sample size (n)	Brief recommendation
1	Naz S et al.	[12]	Rawalpindi, Punjab	2021	Armed Forces Institute of Pathology, Rawalpindi, Pakistan	Vitamin D	Liquid chromatography- Tandem mass spectrometry (LC-MS)	120	LC-MS is highly sensitive, specific, cheaper method for vitamin D detection useful for guiding management of patients with malabsorption syndrome on D2 therapy
2	Naz S et al.	[13]	Rawalpindi, Punjab	2020	Armed Forces Institute of Pathology, Rawalpindi, Pakistan	Vitamin D	Liquid chromatography- Tandem mass spectrometry (LC-MS)	120	LC-MS is highly sensitive, specific method for VIT D detection and quantitation

3	Hafeez A et al.	[14]	Rawalpindi, Punjab	2018	Armed Forces Institute of Pathology, Rawalpindi, Pakistan	Organic acid	Gas chromatography-Mass spectrometry	110	Urine organic acid must be interpreted in. Context of complete clinical, nutritional, and biochemical findings for diagnosis of inherited metabolic diseases. Analytical protocols must be designed for better interpretation of results
4	Aamir M et al.	[15]	Rawalpindi, Punjab	2016	Armed Forces Institute of Pathology, Rawalpindi, Pakistan	Cannabinoid detection in hairs of addicts	Liquid chromatography- Tandem mass spectrometry (LC-MS	60	LC-MS can simultaneously detect 2 important cannabis metabolites I hair of cannabis users and hence can be used as an effective monitoring tool. While it is less time consuming and laborious, low it is plagued with low sensitivity
5	Musharraf SG et al.	[16]	Karachi, Sindh	2016	Dr Panjwani centre for molecular medicine and drug research, international centre for chemical and biological sciences, University of Karachi, Pakistan	Serum metabolites in acute leukaemia	Gas chromatography-Tandem Quadrupole Mass spectrometry	72	Serum metabolites can serve as early diagnostic biomarker for acute leukaemia detection.
6	Khan AH et al.	[17]	Karachi, Sindh	2018	Department of Pathology and laboratory medicine, AKU, Karachi, Pakistan	Kidney Stone Analysis	Fourier transform infrared spectroscopy (FTIR)	449	FT-IR has several advantages over chemical analysis including its use in smaller samples study of all stone layers, faster detection of stone crystals
7	Khan A et al.	[18]	Mardan, KPK	2018	Department of Environmental sciences, Abdul Wali khan University, Mardan, Pakistan	4 NSAIDS (Paracetamol,diclofenac,ibuprofen, codeine)and BDZ(Diazepam,Lorazepwm,Bromazepam,Temazepam) In untreated sewage	Liquid chromatography-Tandem quadrupole Mass spectrometry		Toxicity levels of mentioned chemicals must be identified and its effect in marine ecosystem, soil and groundwater should we study
8	Younas A et al.	[19]	Rawalpindi, Punjab	2020	Armed Forces Institute of Pathology, Rawalpindi, Pakistan	Cannabinoid detection in hairs of addicts	Liquid chromatography-Tandem mass spectrometry (LC-MS)	151	Hair is a better diagnostic indicator for chronic cannabis detection than urine

9	Jafri, L et al.	[20]	Karachi, Sindh	2022	Department of Pathology and laboratory medicine, AKU, Karachi, Pakistan	Gallstone constitution	Fourier transform infrared spectroscopy (FTIR)	117	Gallstone library created using FTIR allows for more synergy and less intensive gallstone analysis between different clinical laboratory
10	Dawood S et al.	[21]	Kharian, Punjab	2021	CMH kharian medical college, / National university of medical sciences, Pakistan	Drug intoxication in travel related crimes	Immunoassay (Index test), Triple Quadrupole Liquid chromatography-Mass spectrometry	77	Urine drug screening using immunoassays is a rapid, effective, and convenient way of drug intoxication in emergency however the use is limited by an array of false negative and positive results. Hence factors influencing false results must be informed to clinicians for better treatment outcome measures.
11	Aamir M et al.	[22]	Rawalpindi, Punjab	2021	Department of Clinical Pathology and Endocrinology, Armed Forces Institute of Pathology, Rawalpindi, Pakistan	2,4,6 Trinitrotoluene (TNT) and 4-ADNT,2 ADNT,2,4 ADNT and 2,6 ADNT screening in ordnance factory workers	Liquid chromatography-Tandem mass spectrometry (LC-MS)		LC MS can be used for simultaneous quantification of TNT and its metabolites allowing good accuracy for TNT explosion screening in industry workers of Pakistan
12	Khan ZUN et al.	[23]	Karachi, Sindh	2022	Department of Pathology and laboratory medicine, AKU, Karachi, Pakistan	Urine Organic Acids	Gas chromatography-Mass spectrometry (GCMS)	85	Five Organic acids namely, isovaleric acid, homovanillic acid, suberic acid, adipic acid, indole acetic acid serve as important discriminatory factor between children with autism spectrum disorder and developing children

Liquid chromatography- Tandem mass spectrometry (LC-MS), Fourier transform infrared spectroscopy (FTIR), Gas Chromatography-Mass spectrometry (GCMS), Trinitrotoluene (TNT).

Discussion

Metabolomics research holds the potential to revolutionize various scientific and medical domains, spanning from drug development to personalized medicine. As the amount of data produced by metabolomics studies continues to grow, there is a need for enhanced data analysis techniques. A validation study conducted by Naz S et al. [12] using LC-MS coupled with a quadrupole- tandem mass spectrometer and Electron spray Ionization to analyse serum 25 hydroxy vitamin D (D2 and D3 metabolites) in an adult population with an N=120 sample size. This study yielded impressive results, with a recovery rate of 98% and 97.5% for D3 and while the recovery rate was 97% and 98% for D2. The percentage relative standard deviation (RSD) was found to be 0.8% and 1.3% respectively, with minimal cross-reactivity with 24,25 hydroxy vit D and 25,26 di-hydroxy vitamin D metabolite than the routinely used immunoassays [12]. The utilization of this highly sensitive, specific, and minimally cross-reactive tandem MS method can offer cost-effective, and standardized results for vitamin D assessment, especially in tertiary care settings that handle substantial workloads [12]. Similar findings were replicated in a study done by Naz S et al.where they compared vitamin D testing methods and elucidated the limitations of immunoassays, highlighting the advantages of the novel LCMS/MS technique [13]. In the realm

of vitamin D analysis, metabolomic technology has emerged as a superior alternative to immunoassays. However, it's worth noting that this transition has yet to take place in Pakistan. The persistence of immunoassay usage in our country can be attributed to significant gaps that still exist in this regard. These gaps primarily stem from the limited availability of resources and the inadequate training of chemical pathologists in Pakistan. Hafeez A et al a retrospective study focusing on the diagnostic and analytical challenges involved in interpreting urine organic acid profiles in a cohort of 110 individuals over a two year-period, using gas chromatography mass spectrometry as their analytical technique [14]. Notably, within this dataset, 25% of the samples were subject to rechecking or recall by the authors due to preanalytical factors such as delayed sample arrival at the laboratory or the collection of samples randomly. The study concluded that pre-analytical control is an obligatory prerequisite for urine organic acid testing, and the profile must be interpreted in context of complete clinical, nutritional, and biochemical findings for an accurate diagnosis of inherited metabolic disorders. It is also imperative that analytical protocols be carefully designed to facilitate a more precise interpretation of the results [14]. These findings emphasize the complexity and sensitivity of urine organic acid profiling and underscore the significance of a holistic approach to ensure accurate and clinically meaningful results in the realm of metabolic disorder diagnosis.

In a study conducted by Aamir M et al, they developed and validated an LCMS technique for the detection of cannabis metabolites in the hair from chronic cannabis users. This LCMS method allowed for the simultaneous measurement of both cannabis metabolites, including THC and THC-COOH. It presented a distinct advantage over GCMS, primarily because it required fewer derivatization steps before analysis, resulting in a comparatively shorter analysis time [15]. Musharraf SG et al. utilized magnetic resonance spectroscopy (NMR) in their metabolomics research involving acute lymphoblastic leukemia (ALL) patients [16]. Using NMR technology, this case-control study compared patients with ALL to healthy controls and people with aplastic myeloid leukemia (AML). The findings revealed significant metabonomic variations between ALL and acute myeloid leukemia AML patients when compared to the control groups. These variations encompassed abnormal glycolysis, disruptions in the tricarboxylic acid (TCA) cycle, alterations in lipoprotein profiles, changes in choline metabolism, and fluctuations in fatty acid metabolism [16]. In the field of metabolomics, NMR is progressively gaining popularity as a reliable method. NMR offers precise insights into metabolite profiles, making it particularly well-suited for metabolomic studies involving biofluids. Its capability to provide a comprehensive overview of metabolites makes NMR-based metabolomics a valuable tool in this field. These studies highlight the importance of advanced analytical techniques like LCMS and NMR in elucidating metabolite profiles and their applications in diverse areas of research, from forensic analysis to disease diagnosis.

Khan AH et al.conducted a comprehensive analysis of kidney stones, employing two distinct methods: Fourier Transform Infrared (FT-IR) spectroscopy and chemical analysis. Their study encompassed a substantial sample size of 449 patients, and the comparison between these two methods yielded a reasonably good level of agreement, as reflected by a kappa statistic of 0.57 (with a 95% confidence interval ranging from 0.5 to 0.64). It's worth nothing that there were some disparities in the examination of 77 of the stones [17]. FT-IR spectroscopy emerged as a valuable method in this context. It demonstrated robustness in its ability to analyse kidney stones and was advantageous for utilizing only a small proportion of the sample. It generally allowed for the positive identification of the majority of components present in kidney stones [17]. This research underscores the effectiveness of FT-IR spectroscopy as a powerful tool for the analysis of kidney stones. Its ability to provide accurate and reliable results while conserving sample material makes it a valuable asset in the field of stone analysis and diagnosis.

Khan A et al. conducted a study focusing on the presence of pharmaceuticals, including non-steroidal anti-inflammatory drugs (NSAIDs) and four benzodiazepines/anti-depressants (ADs), in municipal wastewater in Northwest Pakistan. They utilized the Liquid Chromatography with a Triple Quadrupole Tandem Mass Spectrometry (LC-MS/MS) technique to analyze these substances [18]. The study's findings revealed significantly elevated concentrations of NSAIDs in both sewage and surface water. Specifically, substances like paracetamol and ibuprofen exhibited the highest levels. Given these findings, a comprehensive investigation into the potential toxicities of these specific chemicals is imperative. Furthermore, the study emphasizes the critical need to comprehend their potential impact on vital environmental components, including the marine ecosystem, soil quality, and groundwater systems [18]. This research highlights the importance of monitoring and addressing the presence of pharmaceuticals in wastewater, as their persistence in the environment can have far-reaching consequences. Understanding the potential risks to both human health and the environment is crucial for effective management and mitigation strategies.

Younas A et al . carried out a study aiming to assess the precision of Cannabinoids testing using LC-MS/MS in human hair and comparing it with urine samples from civil heavy vehicle drivers (total n=151) in Punjab [19]. The results of the study revealed that the diagnostic accuracy of Cannabinoids detection in hair reached 94%, whereas in urine samples, it was 83%. The Receiver Operating Characteristic (ROC) curve analysis demonstrated an area under the curve of 0.79 for urine samples and an impressive 0.96 for hair samples. These findings highlight the feasibility of hair as an alternative matrix for testing due to its non-invasive collection method, enhanced diagnostic effectiveness, and a more extended window of detection in comparison to urine [19]. This research underscores the advantages of employing hair

as a matrix for Cannabinoids testing, particularly in scenarios involving heavy vehicle drivers, where accurate and non-invasive testing methods are of paramount importance. The higher accuracy and extended detection window in hair samples make it a valuable option for such assessments.

A study conducted by Aamir M et al focused on the development and validation of an LC-MS/MS technique with the aim of simultaneously measuring Trinitrotoluene (TNT) and its metabolites in urine samples collected from workers in an ordnance factory [22]. The results of the study revealed that the utilization of LC-MS/MS with an APCI (Atmospheric Pressure Chemical Ionization) source yielded offered significant advantages in terms of speed, sensitivity, and specificity. Consequently, this approach shows promise as an effective means of screening for TNT exposure among individuals working in ordnance factory settings [22]. This research highlights the potential of LC-MS/MS with APCI as a valuable tool for monitoring and assessing TNT exposure, particularly in occupational settings where such exposure may occur. Its advantages in terms of accuracy and efficiency make it a promising method for ensuring the safety of workers in such environments. Furthermore, the application of metabolomics to analyse TNT is a novel and innovative aspect of this study, demonstrating the utility of mass spectrometry in testing and assessing exposure levels in occupational settings. In a case-control study conducted by Khan ZUN et al., the aim was to compare the urine organic acid profiles of children diagnosed with autism spectrum disorder (ASD) and typically developing (TD) children and identify biomarkers that could aid in diagnosing ASD, using GCMS [23]. The study yielded important findings, with several organic acids, including 3-hydroxyisovaleric acid, homovanillic acid, adipic acid, suberic acid, and indole acetic acid exhibiting significant differences between the two groups. These differences held promise as potential biomarkers for aiding in the diagnosis of ASD. With accuracy rate of 88.2% subjects were categorized correctly as ASD or TD group based on their urine organic acid profiles. These identified biomarkers are valuable for future research focused on understanding of the etiology of ASD in children [23]. This research highlights the potential utility of urine organic acid profiles as a diagnostic tool for ASD and underscores the significance of identifying biomarkers to aid in early diagnosis and intervention for children with this condition.

The concentration of research studies in Punjab underscores its role as the central hub of academic and research endeavours in Pakistan. Given its status as the most populous province, Punjab possesses a greater abundance of resources, educational institutions, and researchers, consequently leading to a higher volume of research initiatives. Sindh and Khyber Pakhtunkhwa (KPK) also feature prominently in the academic research landscape. Conversely, the absence of studies in Baluchistan highlights a crucial aspect of the nation's research and academic landscape, spotlighting the disparities in research focus across different regions and the potential implications for the development and comprehension of Pakistan's diverse locales. Efforts to promote and support research in regions with lower research activity, such as Baluchistan, can contribute to a more balanced and holistic approach to research and academic development throughout the country. Addressing these disparities can lead to a more equitable distribution of resources and research opportunities, ultimately benefiting the entire nation.

Even the expertise is quite different; the result interpretation of vitamins, steroid hormones, or immunosuppressive drugs requires a given training; for metabolomics, the training is much more complex and involves the acquisition of knowledge for the subsequent statistical approach and chemometrics. Indeed, without the latter, any metabolomic result cannot be interpreted. Hence, this underscores the dire necessity for comprehensive metabolomics training, both in Pakistan and on a global scale [24].

Conclusion

Metabolomic is an emerging field and promising avenue for chemical pathologists and researchers. Its recognition as a vital tool in medical research and diagnostics has opened exciting prospects for chemical pathologists. One of the significant advantages is its potential to revolutionize disease diagnosis and personalized medicine. By analysing the complex metabolic profiles of individuals, chemical pathologists can gain deeper insights into the underlying biochemical mechanisms of diseases. This information provides physicians with the means to make more precise diagnoses, personalized medication regimens, and an improved understanding of disease progression. Furthermore, metabolomics could provide understanding on the complex interactions between genetics, environment, and lifestyle, revealing how these factors impact health and susceptibility to illness. Chemical pathologists have a vital role for advancing medical research and improving healthcare outcomes for the diverse and dynamic population as metabolomics continues to advance in their country.

The review highlights the necessity of making a coordinated effort to close the gap between scattered MS applications in basic sciences and pharmaceuticals and their clinical integration, in cooperation with Chemical Pathologists. Interdisciplinary research and knowledge exchange can be stepped up through the creation of cooperative working groups and strategic alliances with established institutes. Additionally, creating specific funding possibilities will enable professionals and researchers to effectively progress MS-focused scholarly studies.

In conclusion, metabolomics has the potential to bring about transformative changes in healthcare and research. By fostering collaboration, supporting interdisciplinary efforts, and providing the necessary resources, the developing countries can harness the full potential of metabolomics for the benefit of their populations.

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