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Human Disease Metabolism Research Using Nuclear Magnetic Resonance Metabolomics

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ABSTRACT

A new use of nuclear magnetic resonance (NMR) has emerged in the field of biological samples: NMR-based metabolomics. This technique expands the traditional use of NMR for molecular structure elucidation. But NMR is just as useful in other fields of small molecule biology. Some examples of these methods include quantitative nuclear magnetic resonance (qNMR) for metabolite quantification, stable isotope tracers for drug or nutrient metabolic fate determination, metabolic pathway unraveling and flux analysis, and metabolite-protein interactions for pharmacological effect and regulation understanding. Computational resources and technologies for automating biochemical information extraction from spectra have evolved in parallel, adding depth to our knowledge of systems biochemistry. Saliva, urine, and perspiration have been utilized for medical diagnosis since ancient times. Many conventional medical procedures still rely on the volume, color, and odor of bodily fluids to assess health and diagnose disease. Biomarkers for many diseases have been found thanks to analytical methods that allow for the thorough examination of bodily fluids. A recent interdisciplinary effort has integrated multivariate statistical methods, nuclear magnetic resonance spectroscopy (NMR), and mass spectrometry (MS) to profile alterations in small molecules linked to the development and advancement of human diseases. By analyzing the current and future directions of NMR spectroscopy, this study emphasizes the role of NMR in metabolic studies and small molecule biochemistry more generally.

Key words: Metabolism, NMR-Based Metabolomics, Human Disease

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Introduction:

The spectroscopic method known as Nuclear Magnetic Resonance (NMR) makes use of the energetic transition of nuclear spins when a powerful magnetic field is applied. In the decades following the publication of the first nuclear magnetic resonance (NMR) spectrum in the 1940s, NMR has expanded into a wide variety of analytical chemical applications[1, 2]. The use of nuclear magnetic resonance (NMR) has become increasingly important in the life sciences, particularly in the areas of metabolomics, macromolecule dynamics (including proteins and nucleic acids), and organic molecular identification and structure elucidation (particularly metabolites) [3, 4].

Nuclear magnetic resonance (NMR) spectroscopy provides selected chemical information on molecules in their physiological environment due to its sensitivity to chemical environments. Metabolic research has a long tradition of using NMR. Nucleotide and sugar phosphates, as well as redox species, are phosphorus-containing metabolites that were initially studied in cells and tissues using 31 P NMR [5]. By the end of the 1970s, scientists were hopeful about the prospect of using nuclear magnetic resonance (NMR) to learn more about metabolites in living organisms than they had been able to learn about isolated biomolecules in solution in terms of their structure, mobility, reaction rates, and binding sites [6, 7]. Researchers are still delving into several of these areas with NMR techniques.

Medical, nutritional, toxicological, ecological, and pharmacological disciplines were among the many that made extensive use of radioactive tracers as the de facto method for investigating the metabolic fate of compounds in living systems. With the introduction of labelled supply and improved detection procedures, radioactive isotopes have been gradually substituted by safe stable isotope tracers [8]. The utilization of stable isotope resolved metabolomics (SIRM) has allowed for the determination of absolute metabolic fluxes and the activation states of several metabolic processes across numerous metabolic pathways [9]. When analyzing labeling studies, mass spectrometry (MS) and nuclear magnetic resonance (NMR) are the methods of choice (Lane et al., 2019). One obvious benefit of nuclear magnetic resonance (NMR) in metabolite data deposition is the positional labelling information it can provide.

Technological advancements have ushered in a new age of biological mixture analyses, known as metabolomics. The measurement of metabolites is now widely believed to be accurate. A renewed focus on metabolic research has emerged in the last decade, spurred driven by both technical developments and the critical public need for knowledge of metabolic illnesses. In the end, metabolism is involved in every part of biology [10]. Although mass spectrometry (MS) has been the method of choice for metabolic and metabolomics research because to its sensitivity and extensive coverage, nuclear magnetic resonance (NMR) is still employed by only a minority of researchers. Several benefits are gathered via NMR [10]. The reliability of nuclear magnetic resonance (NMR) measurements is excellent; for example, results may be reliably reproduced between labs (Ward et al., 2010), and, with proper sample storage, instrumental response stability can last for months or even years. There is no need to clean the instrument between samples in standard NMR experiments because the samples are in tubes and no chromatographic procedures are utilized.

Users with different applications can freely share NMR spectrometers without worrying about contamination or carry-over. The quantitative nature of NMR allows for the determination of both relative and absolute amounts of metabolites [11]. Nuclear magnetic resonance (NMR) is a vital technique for structure elucidation since it produces unique spectra for most isomers.

Metabolic Profiling and NMR Metabolomics

Foods, natural extracts, and biological samples have all been characterized by the use of nuclear magnetic resonance (NMR) analysis of complex mixtures, as is done in metabolomics. It is usual practise to use 1H NMR spectra from instruments like NOESY-1D (1D Nuclear Overhauser Effect Spectroscopy) to compile profiles of a great number of metabolites from a wide range of biological samples, including extracts of microbes from the gut microbiome, cells from mammals and plants, and clinical tissues and biofluids like plasma, urine, CSF, or faecal water. Using a 600 MHz NMR spectrometer, around 60 metabolites can be detected in untargeted 1 H NMR spectra of materials (such human urine) with minimal sample preparation required [12, 13]. 1H NMR examination of serum and other blood matrices, as well as tiny molecules (metabolites), can detect different types of lipoproteins. For instance, as shown in Figure 2B, metabolites such as sugars, amino acids, organic acids, and other compounds can be identified through the examination of a human cell

system. These compounds are mostly involved in central carbon metabolism and related pathways. Although most metabolomics studies include dissolved biological samples, high resolution (HR) magic angle spinning (MAS)-NMR can be used to analyze intact tissues as well [14, 15]. Nevertheless, due to the minimal sample preparation, the fact that each molecule frequently causes several signals in the spectrum, and the fact that numerous metabolites can be detected, all 1 H NMR spectra acquired in metabolomics experience significant signal overlap.

Interactions between Metabolite and Protein

Enzymatic and allosteric events, which define and regulate metabolism, require interactions between metabolites and proteins. Although numerous approaches have been devised to investigate macromolecule interactions (such as protein-protein interactions), there is a lack of tools for conducting systematic evaluations of protein-metabolite interactions, and these tools are frequently restricted to hydrophobic metabolites [16]. Protein dynamics in vitro, such as conformational changes in response to ligand binding [17], can be studied using nuclear magnetic resonance (NMR) by tracking amino acid residues in the protein backbone.

The NMR technique has shown to be an exceptional analytical tool for identifying and quantifying chemicals in complicated mixtures. Since NMR-active nuclei are present in all known mixtures, qNMR has found widespread application as a routine analytical method. Very few quantification procedures do not rely on standards, and qNMR is among them. Without the need for chemically identical standards, it can quantitatively assess mixtures of numerous compounds. For the purpose of quantitatively analyzing mixtures of many compounds, this review will cover both the theoretical and practical aspects of qNMR data gathering, spectrum processing, and signal deconvolution/integration. Also covered will be the steps involved in preparing the sample and how various sample circumstances impact the concentration assessment. Although 1H 1D qNMR is still the gold standard for quantitative study of complex mixtures of compounds, heteronuclear 1D and 2D qNMR methods are gaining popularity for application in this type of investigation. Subsequently, a few commonly employed quantitative NMR techniques are reviewed. Subsequently, qNMR is demonstrated in the contexts of metabolomics, natural products, pharmacological research, TCM, and food analysis. We conclude by looking ahead to the potential uses and advancements of Qnmr in the future.

Understanding Protein-Metabolite Interactions Unveils Chemical Communication Principles

A key component of cellular homeostasis is the regulation of numerous cellular processes through metabolicate-protein interactions. Even though metabolites make up the vast majority of cellular components, our comprehension of the interactome between metabolites and proteins is still in its infancy compared to that of protein-protein and protein-DNA interactomes. To systematically identify metabolite-protein interactions in their original context, we offer a chemoproteomic approach [18, 19]. We showed that several of the newly discovered contacts had functional value, and the method succeeded in identifying a network of binding sites and interactions in Escherichia coli, both known and unknown. Thanks to our findings, new enzyme-substrate interactions and instances of metabolite-induced protein complex remodeling have been identified. There are 7,345 potential binding sites and 1,678 interactions in our metabolite-protein interactome. Our findings provide insight into the frequency and processes of enzyme promiscuity, demonstrate the structural and functional principles of chemical communication, and pave the way for the extraction of proteome-wide quantitative parameters of metabolite binding.

Protein structures can be altered either locally or globally as a result of interactions between metabolite and protein [20]. We hypothesized that a universal readout of protein-small molecule interactions might be achieved by detecting ligand-induced structural modifications on a proteome-wide scale. Our approach, which we name LiP-small molecule mapping (LiP-SMap), is based on earlier work and allows us to systematically find proteins that are differentially protease cleavage vulnerable when small molecules bound to proteome extracts.

The LiP-SMap process involves exposing extracts to a small molecule of interest after proteomes have been extracted in a way that maintains the native protein structures. The broad-specificity protease proteinase K is used to perform restricted proteolysis on samples in order to produce fragments of proteins that are specific to their structures [21]. In order to create peptide mixtures suitable for bottom-up proteomic investigation, fragments are then digested using the sequence-specific protease trypsin. We use a label-free quantitative MS

method to compare the LiP patterns of proteomes processed with and without the small molecule, and then we use liquid-chromatography-coupled tandem MS to examine the peptides (Figure 1). A data-independent acquisition-based validation phase and a quantitatively unbiased shotgun proteomic step are components of our pipeline.

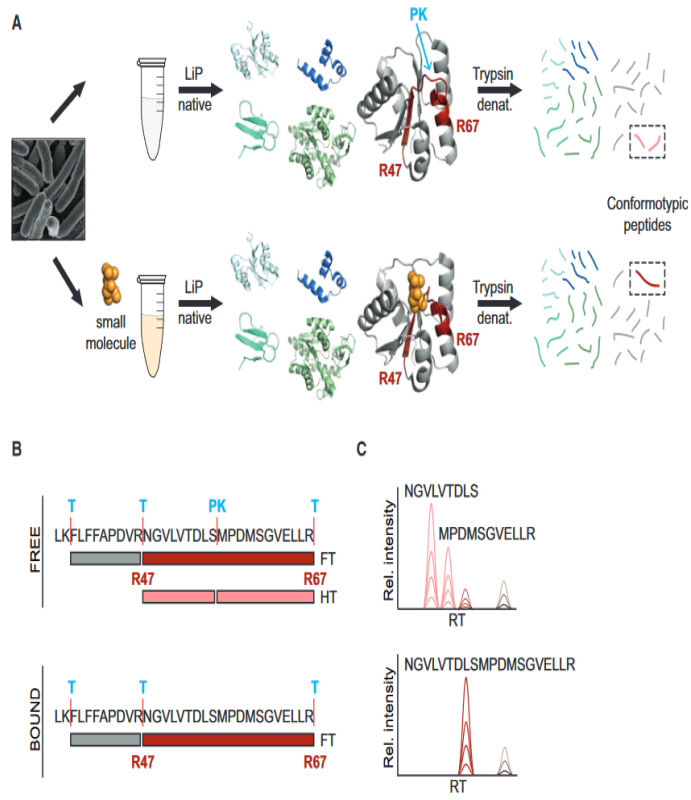

Figure 1: LiP-SMap Approach Workflow: (A) Metabolites are added to or removed from whole-cell lysates that were isolated using native lysis conditions. Changes in local proteolytic sensitivity are brought about by metabolites binding to proteins. Proteinase K (PK) is used for a limited proteolysis step in native circumstances, followed by trypsin for complete digestion in denaturing conditions. This process produces peptides that may be measured by MS. Both the ligand-free version of the protein FixJ (PDB: 1D5W) and its bound form to aspartyl phosphate (PDB: 1DBW) are structurally informative peptides in the example. The peptides that are represented in red are conformotypic peptides, which are specific for either the bound or unbound conformation. A schematic showing FixJ peptides produced with and without aspartyl phosphate is shown in (B). Two peptides with non-tryptic ends (HT) vanish and the concentration of the accompanying fully tryptic peptide (FT) increases because binding precludes PK cleavage. (C) MS reveals the presence of the three peptides.

Ligand-Detected Nuclear Magnetic Resonance Spectroscopy of Protein-Metabolite Interactions in Complex Metabolite Mixtures

Many biological functions are controlled by protein-metabolite interactions. Therefore, comprehending cellular regulation requires first finding such relationships. Unfortunately, there are currently no reliable ways to systematically map protein-metabolite interactions due to the noncovalent nature of the binding [22]. Only few groups of metabolites, including lipids, have been addressed by the few existing methods, most of which rely on mass spectrometry. Our study delves into this matter and demonstrates how ligand-detected nuclear magnetic resonance (NMR) spectroscopy, a tool commonly employed in drug development, can be utilized to methodically detect interactions between proteins and metabolites. To demonstrate the principle, we chose four proteins from different animals and bacteria (AroG, Eno, PfkA, and bovine serum albumin) and found metabolite binders in mixtures including up to 33 different metabolites. Over the whole range of physiologically relevant Kd values, from low micromolar to low millimolar, ligand-detected NMR recorded all of the protein-metabolite interactions that have been reported thus far [23]. The promiscuous binding of citrate, AMP, and ATP—negatively charged metabolites—and the binding of aromatic amino acids to AroG protein were among the many new interactions that we discovered. We evaluated the practical importance of these new interactions with AroG using in vitro enzyme activity tests and found that l-tryptophan, l-tyrosine, and l-histidine are new inhibitors of AroG activity. Therefore, we infer that ligand-detected NMR works well for the methodical discovery of protein-metabolite interactions that are important for biological function.

Escherichia coli's core metabolism and the protein-metabolite connections

The majority of what we know about the connections between metabolism and proteins comes from empirical in vitro investigations, yet these interactions govern practically every biological process. The initial comprehensive investigation into the interplay between polar metabolites and water-soluble proteins in a complete biological subnetwork is presented here [23]. We decided to examine the central metabolism of the well-studied bacterium Escherichia coli in order to gauge the extent of our existing knowledge. We tested 29 enzymes for binding events with 55 intracellular

metabolites using ligand-detected NMR. We identified 98 interactions, with purine nucleotides making up one third of them, when we concentrated on high-confidence interactions with a false-positive rate of 5%. Half of the metabolites we tested did not interact with any enzyme. However, several enzymes interacted with as many as eleven metabolites, and only five of them showed no evidence of metabolite binding at all. Due to their limited chemical resemblance to the reactants of their target, approximately 40% of the interacting metabolites were projected to be allosteric effectors. In vitro tests validated new regulatory roles for five out of eight interactions, including the regulation of the first pentose phosphate pathway enzyme by ATP and GTP.

But instead of watching the protein, a series of ligand-observed NMR tests can be employed to track the binding event via the ligand. Examples of nuclear magnetic resonance (NMR) techniques used to observe ligand binding to non-isotopically labelled proteins include saturation transfer difference (STD) [24], water-ligand observation with gradient spectroscopy (WaterLOGSY), time constant of spin-lattice relaxation in rotating frame (T1rho), and CPMG. The main application of ligand-observed NMR in drug discovery has been high throughput fragment screening.

There are certain requirements for ligand-observed NMR techniques. The ligand is usually given to a large protein (>30 kDa) in excess (10-20 times the protein quantity), and the interactions are usually weak, with dissociation constants (KD) ranging from 1 μM to 10 mM. Proton signal widening or disappearance [25] from the spectrum (Figure 5) indicates that the signal undergoes a substantial relaxation upon binding, which occurs during the quick exchange between the ligand and protein. Gaining binding information from ligand signals is possible through NOEs (STD and water-mediated NOEs, WaterLOSGY) by analyzing bound and unbound states.

The ligand interaction can be epitope mapped and KD can be calculated. Further evaluation of ligand competition in ligand combinations is possible as well [26]. E. coli's core carbon metabolism proteins are one example of an endogenous metabolite-protein interaction that has been systematically identified using ligand-observed nuclear magnetic resonance (NMR).

We tested 29 isolated metabolic enzymes with solutions containing up to 55 different metabolites. With this method, 76 new interactions involving endogenous metabolites and enzymes involved in central metabolism were discovered [27] The availability of pure proteins (or enriched protein cell suspensions) with a predetermined number of metabolites is crucial to this type of technique, even though it is fast to set up from an NMR acquisition standpoint. The interaction of small molecules with macromolecules in biological settings is still an area that is being studied. To illustrate the point, the binding mechanism of ligands to intracellular proteins in living bacteria and cancer cells has been ascertained using whole-cell STD measurements.

Chemometrics as a tool for detecting biomarkers

An essential part of analyzing NMR metabolomics data is multivariate statistical approaches.[28] in When it comes to diseases, environmental stresses, poor diet, or pollutants, multivariate statistics make it easy to see how the metabolome changes on a global scale. We capture the full impact on the system from the external stimuli, not just a few metabolites or certain metabolic pathways. The chemical shifts and intensities that make up a 1D 1H NMR spectrum—a complicated multivariable data set—can reach 32K. The NMR data set is made more comprehensible and easier to understand by employing multivariate statistical methods. Usually, a scoring plot is used to compress the NMR spectrum to a single data point. Multivariate statistical methods have been used to examine both 1D and 2D nuclear magnetic resonance spectra.[29] Supervised and unsupervised multivariate statistical approaches exist. In supervised learning, example classes are introduced, but in unsupervised learning, intrinsic data variances are the only basis. When it comes to NMR metabolomics data, one popular unsupervised method is principal component analysis (PCA). The supervised method known as Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) has lately gained traction as a means to decipher NMR spectra in order to identify biomarkers.the 113th The NMR metabolomics procedure cannot proceed without first pre-processing the data. Data reduction (binning), scaling, and the elimination of solvent areas and noise are all part of the pre-processing of the NMR spectrum. After the 1D 1H NMR spectrum has been baseline corrected and phase corrected to create pure absorption line shapes, the spectrum is either intelligently bucketed (ACD Labs, Toronto, Canada) that uses variable bin sizes to prevent peaks from splitting between bins or uniformly binned with a chemical shift range of 0.01 to 0.04 ppm [30, 31].The number 115 Each bin's numerical value represents the integral of its corresponding spectral region. The unavoidable small and random variations in chemical shifts, phasing, peak form, baseline, etc., in

replicate biological samples are reduced to a minimum by binning NMR spectra. Similarly, NMR spectra acquired from a diverse range of biological substances, including biofluids, exhibit substantial and intrinsic heterogeneity in terms of signal-to-noise ratio and overall quality. It is both unrealistic and unattainable to anticipate consistency in biofluid samples collected from various animals, tissues, or patients. Consequently, in order to remove or reduce signal-to-noise variability caused by overall volume or concentration changes between samples, the NMR spectra must be normalized [33].

To find metabolite biomarkers, we need to isolate the latent variables that differentiate between classes (healthy vs. illness, for example). While principal component analysis (PCA) can reveal the most significant changes in the NMR data, the underlying latent variables (the relationship between classes) might not always lie along the most significant changes. When looking for biomarkers for metabolites [34, 35], OPLS-DA is the way to go. One regression model that takes into account the relationship between dependent variables and class information in multivariate data is OPLS-DA. With OPLS-DA, one component serves as the class predictor, while the remaining components represent variations perpendicular to it. To rephrase, the orthogonal components explain the separation within classes, whereas the predictive component explains the split between classes. OPLS-DA lessens distinction inside classes and places an emphasis on separations between them. Consequently, compared to PCA, OPLS-DA scores plots will show more distinct clusters. Transforming PCA data into OPLS-DA is as easy as adding a class discriminating value as a Y direction vector. One set of data is usually called the control (Y = 0) and the other sets are called the treated (Y = 1) in a two-class system. Two metrics used to evaluate model quality in PCA and OPLS-DA are the quality evaluation score (Q2) and the measure of goodness of fit (R2). Similar in concept to simple linear regressions, a successful model has R2 values ≥ 0.5 (ranging from 0 to 1). While a value of ≥ 0.4 is normal for biological models, an optimal value for Q2 is one. Since over-fitting the data is a typical worry, it is necessary to validate the model before using OPLS-DA, a supervised technique [36]. When validating OPLS-DA models, the leaveone-out strategy is frequently employed. This method involves omitting a portion of the NMR spectra in order to compute a model that can subsequently forecast the data that was omitted. When comparing the original data with the anticipated data.

Analysis of NMR data and processing

In order to process, calibrate, and compute the difference spectra, spectra were run through TopSpin 3.2 (Bruker) with the use of a custom-built Python program (github.com/systemsnmr/metabolite-interactions). Minimizing subtraction artifacts in the final difference spectra required spectra to be calibrated to the DSS reference signal. Each sample's two short-delay T1rho duplicate spectra were compared to serve as an indicator of experimental reproducibility and a quality filter for metabolite stability [37, 38]. Metabolite signals that displayed a discordance of greater than 5% between these two spectra were deemed unstable when exposed to the specified protein and were subsequently omitted from the comprehensive analysis. Also, we didn't include metabolite peaks that seemed to get stronger when the protein was there. The peaks that displayed a significantly negative intensity (less than −0.05) after removing the intensities of the combined short-delay spectra of the protein-metabolite mixtures (T1rho10ms_PM) and free protein (T1rho10ms_P) from the free metabolite reference spectra (T1rho10ms_M) were named as follows: [T1rho10ms_M − (T1rho10ms_PM − T1rho10ms_P) < −0.05].

Recent applications of NMR for biomarker detection. In the United States, the 5-year survival rate for lung cancer is a dismal 15%, while in Europe it drops to 10%. Unfortunately, present diagnostic procedures are insufficient, even though early diagnosis is crucial to improve patient survival [39, 40]. To achieve a consistent pH of 7.00 ± 0.02 in a 540 µL urine sample, 60 µL of 1.5 M phosphate buffer (KH2PO4) containing 0.1% TMSP-d4 was added to create a 600 µL NMR sample. There was a clear metabolic difference between healthy people and lung cancer patients, according to the 1D 1H NMR spectra that were generated (Figure 7). Multivariate statistical analysis using principal component analysis (PCA), partial least squares (PLS), and orthogonal principal component analysis (OPLS-DA) further separated the two groups (Figure 8). The only method that produced a distinct distinction was OPLS-DA. The effects of gender, age, and smoking on the clustering pattern of 2D scores were also investigated [41-44]. The investigation did not find any substantial impact from any of these parameters, which is interesting. Metabolites that were responsible for class separation were identified using the relevant OPLS-DA

loading plot. Lung cancer patients and healthy individuals differed significantly ($P < 0.01$) in hippurate, trigonelline, β-hydroxyisovalerate, α-hydroxyisobutyrate, N-acetylglutamine, and creatinine.The number 130 Investigations into system-wide processes like transformation, progression, proliferation, and metastasis have also made substantial use of NMR metabolomics in cancer cell line pathology studies.[45] The discovery of novel biomarkers and treatments might be the result of this endeavor. An androgen-dependent prostate cancer cell line (LnCAP) was analyzed metabolomically utilizing nuclear magnetic resonance and mass spectrometry, as described by MacKinnon et al. (2012) Methyltrienolone, an androgen receptor agonist, induced a metabolic signature typical of aggressive prostate cancer in LnCAP cells. In particular, certain changes were noted in the levels of myo-inositol, glutathione, amino acids, methionine, phosphocholine, and the ratio of phosphocholine to glycophosphocholine. Clinical treatments could be guided by these possible biomarkers to prevent the unlucky induction of an aggressive and incurable form of prostate cancer by prematurely terminating androgen ablation therapy.

In industrialized nations like the United States, cardiovascular diseases (CVD) rank high among the top killers. One key part of a preventative medical plan is predicting the risk of cardiovascular illnesses. In a research of healthy adults, Bernini et al. (2011) used nuclear magnetic resonance (NMR) metabolomics to find biomarkers linked to cardiovascular disease risk. Analyzing 864 plasma samples thoroughly yielded a CVD risk estimate that was comparable to those of conventional clinical practices. In essence, NMR-determined metabolome changes were associated with conventional CVD risk markers such elevated cholesterol, triglycerides, LDL, and HDL. Along with these well-known risk factors for CVD, other metabolite markers such 3-hydroxybutyrate, αketoglutarate, threonine, and dimethylglycine were also discovered. To produce the NMR samples, 300 µL of sodium phosphate buffer and 300 µL of plasma sample were mixed. All of the plasma samples were analyzed using standard 1D 1H NMR spectra, CPMG diffusion edited spectra, and 1D-NOESY spectra. Obtaining an accurate analysis of known CVD risk factor metabolites and establishing a good association between routine clinical analysis and the NMR data were the key goals of this broad series of NMR investigations. Additionally, a limited group of metabolites that are extremely dependable (P-value < 0.0001) in predicting the risk of cardiovascular disease was the primary focus of the statistical analysis [46]. Interconnected are the high risk and low risk pathways, where a low risk individual's metabolome is pushed toward HDL, α-ketoglutarate, dimethylglycine, and 3-hydroxybutyrate. The opposite is true for high-risk individuals, whose metabolic pathways are altered to prioritize LDL, threonine, and acetoacetate.

Conclusion

Biomarkers that can help in the diagnosis and treatment of a wide range of human disorders have been greatly advanced by NMR metabolomics. Biomarkers for a wide range of diseases, including cancer, neurological disorders, genetic disorders, and infectious diseases, have been found with the help of nuclear magnetic resonance (NMR). Thanks to its robustness, versatility, and ease of application, NMR metabolomics is attractive, and its simplicity in sample preparation is a big part of it. Regular one-dimensional 1H NMR investigations can rapidly provide global metabolomic alterations. Combining two-dimensional nuclear magnetic resonance experiments with chemometrics methods, such as S plots and loading plots, might identify specific metabolites that are associated with a disease and may serve as biomarkers. The biological interpretation of NMR metabolomics data is further supported by statistical analysis, which includes tree diagrams, bootstrap numbers, and T2 hoteling.

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