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REVIEW
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Mass Spectrometry-based Hair Metabolomics for Biomarker Discovery

Yu Ra Lee[1,2], Jongki Hong[2,3], and Bong Chul Chung[1,2]*

[1]*Molecular Recognition Research Center, Korea Institute of Science and Technology, Seoul 02792, Korea*
[2]*KHU-KIST Department of Converging Science and Technology, Kyung Hee University, Seoul 02447, Korea*
[3]*College of Pharmacy, Kyung Hee University, Seoul 02447, Korea*

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Abstract : Metabolomics makes it possible to analyze the interrelationships between various signaling molecules based on the metabolic pathways involved by using high-resolution devices. This approach can also be used to obtain large-scale metabolic information to identify the relevant pathways for disease diagnosis and prognosis and search for potential biomarkers. In the fields of medicine and forensics, hair analysis is used to detect various metabolites in the body. Hair can be harvested readily in a noninvasive manner and is easier to transport and store than blood and urine. Another advantage from a forensic viewpoint is that hair reflects all the components of body fluids. In addition, because of the unique coating structure of hair, it can be used for measurements without changing or destroying its adsorbed components. In this review, the pretreatments for hair analysis, instrumental conditions and clinical applications are discussed. Especially, the clinical use of hair metabolomics in the diagnosis of various diseases and the limitations of the technique are described.

Keywords : hair, metabolomics, biomarker, steroids

Introduction

Interest in metabolomic studies has grown rapidly in the past few years.[1] In addition, the analysis of human hair for genetic (DNA) and drug testing, which started in the 1960s, is a common technique these days, as hair is easy to collect and yields highly accurate results.

However, the hair roots are required for genetic and paternity testing. This is because the relevant biological information is present in the hair mother cells of the hair bulb. However, in the case of alcohol, tobacco, and narcotic consumption, the component of interest remains in the keratin protein layer of hair. Thus, these substances can be detected even in hair without roots.

Hair grows relatively slowly, and both endogenous compounds and those present in the environment are incorporated from the blood into the hair during its growth. This is reflected in the average chemical composition of hair over several months.[2] In particular, the analysis of the slow-growing hair matrix provides a suitable index for quantitatively evaluating the integrated hormone levels over several months.[3] Hair gradually forms a thick wall on its outer layer just before it is exposed to the outside environment and permanently records information related to the state of the minerals present in the human body. Normally, hair grows by 0.03 cm per day and 1 cm per month. Therefore, if one collects 3–4 cm of hair, one can obtain health-related information for the previous 3–4 months. In particular, hair stores 10–50 times more mineral information than blood and urine and can be analyzed more accurately.

Hair that grows 0.3 mm daily contains a wealth of information related to the levels of essential minerals, such as magnesium, potassium, iron, sodium, and calcium, in the body as well as heavy metals such as mercury, lead, and cadmium. The essential minerals found in hair are indispensable elements and play an important role in various physiological functions such cell generation and the stimulation of cell activity. Thus, by determining the contents of these minerals, it is possible to evaluate the metabolic rate, stress, immunity, and adrenal gland and thyroid gland status.

The medical conditions that can be prevented based on the results of hair examinations include chronic fatigue, obesity, diabetes, metabolic syndrome, atopy and skin

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*Reprint requests to Bong Chul Chung
https://orcid.org/0000-0002-4449-0043
E-mail: bcc0319@kist.re.kr

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diseases, osteoporosis, arteriosclerosis, high cholesterol, and hypertension. These illnesses are caused by poor lifestyle and eating habits for long periods and vary from mental illnesses.

Hair analysis has also become increasingly important for detecting the presence of substances of abuse, both in clinical and forensic toxicology investigations.[4] Hair fiber offers several advantages over other biological matrices (blood and urine), including a larger window of detection, ease of collection, and sample stability.[5] Hair samples are extremely valuable for testing for long-term drug use. Moreover, as stated above, a key advantage of hair analysis is that hair samples are easy to obtain and can be acquired noninvasively. In addition, they do not have any additional storage requirements and can be kept at room temperature for long periods.

Hair is a strong matrix that is stable at room temperature, can be handled and transported with ease, cannot be tampered with readily during collection, can be collected noninvasively, and has a high resistance to decay in post-mortem cases.[6,7]

Metabolomics is a comprehensive technique to systematically analyze and quantify the changes in the behavior and secretion of metabolites within cells or tissues and reinterpret the metabolite network by linking the various metabolomic groups with their related physiological and pathological conditions. The main purpose of using metabolomics in current medicine is to determine the mechanism of drug action or disease; discover or measure biomarkers for diagnosis, prognosis, toxicity; and evaluate the therapeutic effects of drugs. Since human diseases and health disorders are caused by changes in the metabolism (metabolic pathways) of the body, metabolomics is increasingly being used for the discovery and identification of diagnostic biomarkers and therapeutic targets.[8,9] Metabolomics allows for a comprehensive analysis of metabolites and their associations with the metabolic processes related to various pathways.[10] Therefore, in the case of unknown metabolites, it is important to study the diseases they may cause as well as their correlations. Research in this direction has already begun recently.

Finally, it has been shown based on multivariate statistical analysis that it is possible to determine the gender of a person through the principal component analysis (PCA) and orthogonal partial least-squares discriminant analysis (OPLS-DA)[11] of hair.

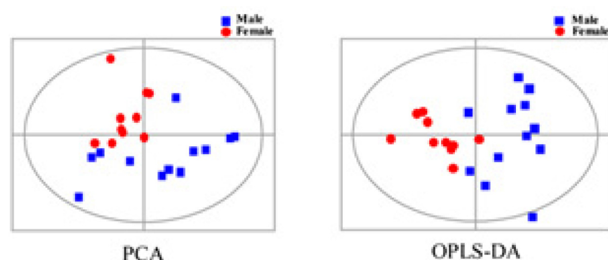

Figure 1. Score plots obtained from PCA and OPLS-DA of hair from healthy controls.

In this review, we briefly introduce the pretreatments performed on hair samples prior to analysis and describe the clinical applications of hair metabolomics.

Sample Preparation

Washing for decontamination

An important step in the development of a decontamination procedure is to produce artificially contaminated specimens that be used for testing the efficiency of the washing process to remove the chemicals[12] and heavy metal ions[13] deposited on the surface of hair. The decontamination of hair is an important step that requires further examination.[14] Prior to analysis, the hair sample to be tested must be decontaminated by washing with a variety of solvents such as methanol, acetone,[15] and dichloromethane[16] to remove any oil or surface contaminants present.[17]

Alkaline hydrolysis

Alkaline hydrolysis, which completely dissolves the hair matrix, allows for the solubilization of all the drugs present in the hair sample.[18] For alkaline hydrolysis, 1 mL of 1 M NaOH is added to the hair sample and allowed to react at 80°C for 1 h. The temperature can be kept at 70-90°C. After the sample has cooled to room temperature, 1 mL of acetate buffer (pH 5.2) is added to the mixture, and its pH is adjusted to 5–6 by adding 0.1 mL of 2 M HCl. Finally, the mixture is extracted twice using 2.5 mL of hexane/ethyl acetate (3:2, v/v) by mechanical shaking for 10 min.[19]

Acidic hydrolysis

For acidic hydrolysis, 1 mL of 0.6 M HCl is added to the hair sample, and the mixture is incubated at 40°C overnight. Alternatively, 1 mL of 1 M HCl is added to the hair sample, and the sample is kept overnight at 50°C.[20] After heating, 0.9 mL of 2 M NaOH is used to adjust the pH of the mixture to 11–12. Then, derivatization[21] or extraction is performed using diethyl ether.[22] Another acidic hydrolysis method used to remove the protein components involves heating the hair sample with 6 M HCl at 110°C for 16 h.[23] Acid hydrolysis could be used to extract basic metabolites due to protonation of the nitrogen atoms present in the molecules.[24]

Ultrasonication

Sonication is used to improve the digestion performance of enzymes. The most important parameters affecting the process are the cavitation frequency, ultrasound intensity, and type of solvent, bubbled gas, and external temperature used.[25] To maximize the extraction efficiency of the hair steroids, the effects of the treatment time (15, 30, 60, and

Table 1. Instrumental conditions for hair analysis using liquid chromatography system-mass spectrometer.

Sample preparation	Instrumental condition	Mobile phase	Analytes	Ref.
soak for 5 min or 5 h in a 1 mg/mL solution of cocaine base or HCl salt	Waters MALDI HDMS SYNAPT	A: methanol B: 10 mM ammonium bicarbonate, pH 10	cocaine	30
extracted with phosphate buffer and liquid-liquid extraction	Ultimate 3000 pump (Thermo Fisher, Les Ulis, France) and Orbitrap mass spectrometer (Q-Exactive)	A: 0.1% formic acid in water (ammonium formate 2 mmol/L) B: 0.1% formic acid in 1% water, ACN/MeOH, 50:50, v/v	untargeted screening	31
extracted with buffer:solvent mixture at 37°C for 18 h	Agilent 6550 QTOF coupled to a 1290 Infinity UHPLC system	A: 0.05% formic acid in 10 mM ammonium formate B: 0.05% formic acid in acetonitrile	7-amino-flunitrazepam, 7-amino-clonazepam, 7-amino-nitrazepam, acetylmorphine, alimemazine, alprazolam, amphetamine, etc.	32
under sonication at 45°C	A Quattro Premier tandem mass spectrometer (Waters)	A: 0.1% formic acid in water B: 0.1% formic acid in methanol	33 basic drugs (amphetamines, cocaine, opiates, opioids and metabolites)	33
added 3 mL NaOH 1N and incubated 95°C for 10 min	Acquity ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA) coupled to a Micromass Quattro Ultima Platinum (Waters)	A: 5 mM formic acid in water B: 5 mM formic acid in acetonitrile	9-tetrahydrocannabinol	34
under sonication at 45°C	Agilent 6460 triple quadrupole mass spectrometer coupled to a 1290 Infinity UHPLC system	A: 5 mM ammonium formate containing 0.1% formic acid B: methanol/acetonitrile 1:1 with 0.1% formic acid	synthetic cannabinoids, synthetic cathinones, ketamine, piperazines and amphetamine	35
incubated with methanol for 24 h on a rolling mixer	UFLC system from Shimadzu coupled QTRAP® 6500 from SCIEX	A: 0.2 mM NH_4F in water/methanol 97:3, v/v B: 0.2 mM NH_4F in water/methanol 3:97, v/v	steroid hormone	36
under sonication at 40°C	Thermo ULTIMATE 3000 HPLC system coupled to a Thermo single-stage Orbitrap (Exactive) MS system	A: water with 5 mM ammonium formate and 0.1% formic acid B: methanol/acetonitrile 1:1 with 0.1% formic acid	drugs of abuse and pharmaceutical drugs	37
hydrolysis with 1 mL of 2.5 M NaOH at 60°C for 25 min	Ultimate 3000 LC system (Thermo Fisher, Les Ulis, France) coupled a Thermo Scientific Q Exactive mass spectrometer	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile	cannabinoids and metabolites	38
extracted with methanol-HCl mixture	Agilent 1100 LC system coupled 3000 triple quadrupole mass spectrometer	A: 0.1% formic acid in water B: formic acid in methanol	methamphetamine, amphetamine, methylenedioxymetamphetamine, methylenedioxyamphetamine, ketamine, norketamine, dehydronorketamine, 6-acetylmorphine, morphine, codeine	39
soak in 300 uL of methanol	MDS Sciex hybrid quadrupole time-of-flight mass spectrometer	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile	cannabinoids	40
incubated with methanol at 37°C for 16 h	Agilent 1200 HPLC system coupled 3200 QTRAP tandem mass spectrometer	A: mixture of methanol-water (20:80, v/v) containing ammonium acetate (2 mM) B: mixture of methanol-water (80:20, v/v) containing ammonium acetate (2 mM)	antiretroviral drugs	41

Table 1. Continued.

Sample preparation	Instrumental condition	Mobile phase	Analytes	Ref.
extracted with DW at 80°C for 30 min	Agilent 1100 LC system coupled 4000 triple quadrupole mass spectrometer	A: 2.5 mM ammonium formate/DW (25:75, v/v) B: 2.5 mM ammonium formate/methanol (25:75, v/v)	nicotine and cotinine	42
incubated with acidic aqueous buffer at 100°C for 1 h	Waters Acquity UHPLC coupled with a triple quadrupole mass spectrometer	A: 0.3% formic acid in acetonitrile B: 5 mM ammonium formate pH 3	hallucinogenic drugs	43
incubated with 3 mL of 0.1 M HCl at 53°C for overnight	Agilent 1100 HPLC system coupled Sciex Triple Quad 5500 mass spectrometer	A: 60% (v/v) methanol in water B: 0.1% formic acid in acetonitrile	methamphetamine	44
incubated with acidic aqueous buffer at 100°C for 1 h	Waters Acquity UHPLC coupled with a triple quadrupole mass spectrometer	A: 0.1% formic acid in acetonitrile B: 5 mM ammonium formate pH 3	antidepressant and anxiolytic drugs	45
incubated with 0.5 mL of a mixture acetic acid/methanol (20:80, v/v) at 38°C for 12 h	Waters Acquity UPLC H-Class LC system coupled triple quadrupole mass spectrometer	A: 0.1% ammonium in water B: 0.1% ammonium in methanol	21 endocrine disrupting chemicals	46
under sonication for 2 h	Shimadzu UFLC 20A LC system coupled IT-TOF mass spectrometer	A: 0.1% formic acid in water B: 0.1% formic acid in acetonitrile	untargeted screening	47
incubated with 2 mL of a mixture acetic acid/methanol (15/85, v/v) at 38°C for overnight	Agilent 1200 LC system coupled to a 6410 Agilent triple quadrupole mass spectrometer	A: methanol B: 5 mM ammonium acetate aqueous solution	endocrine-disrupting compounds	48
Incubated with potassium hydroxide at 54°C for 16 h	Waters UPLC coupled with a QTOF mass spectrometer	A: 0.5% formic acid in water B: acetonitrile	untargeted screening	49

Table 2. Instrumental conditions for hair analysis using gas chromatography system-mass spectrometer.

Sample preparation	Instrumental condition	Used column	Analytes	Ref.
incubated with 1 mL 2M NaOH at 38°C overnight	Agilent GC-MSD instrument	HP-5MS (30 m × 0.25 mm, 0.25 μm)	organic pollutants	50
incubated with 2 mL MeOH at 50 °C for 18 h	Agilent 6850 GC system coupled 5975 MSD	HP-5MS (30 m × 0.25 mm, 0.25 μm)	cocaine and its derivatives	51
incubated with 1 mL 1M NaOH at 70°C for 15 min	Agilent GC-MSD instrument	HP-5MS (30 m × 0.25 mm, 0.25 μm)	phenobarbital	52
incubated with 2 mL 1N NaOH at 50°C for 18 h	Agilent 7890A GC system coupled 7000C triple quadrupole mass spectrometer	HP-5MS (30 m × 0.25 mm, 0.25 μm)	cannabinoids	53
incubated with 1mL 1 M NaOH at 90°C for 15 min	Agilent 7890B GC system coupled 7000B triple quadrupole mass spectrometer	Zebron ZB-5MSi (30 m × 0.25 mm, 0.25 μm)	cannabinoids	54
incubated with Proteinase K enzyme at 37.5°C for 50 min	Agilent 7890A GC system coupled 5975 MSD	BP-X5 SGE Forte Capillary column (30 m × 0.25 mm, 0.25 μm)	cannabinoids	55
incubated with 1 mL 1M NaOH at 90°C for 15 min	Varian CP-3800 GC system coupled Saturn 4000 MS/MS ion trap mass detector	VF-5MS capillary column (30 m × 0.25 mm, 0.25 μm)	cannabinoids	56
extracted with 1mL 0.1M HCl at 37°C for 16 h (overnight)	Agilent 6890 GC system coupled Leco® Pegasus® IV time-of-flight (TOF) mass spectrometer	DB-5MS (30 m × 0.25 mm, 0.25 μm)	drug analysis	57
extracted with methanol in an ultrasonic bath (~5 h)	Varian CP-3800 GC system coupled Saturn 2200 MS/MS ion trap mass detector	Zebron capillary column (30 m × 0.25 mm, 0.25 μm)	external contamination	58
after pulverized, incubated with 1 mL of acetonitrile at 40°C for overnight	Agilent 7890A GC system coupled 7000A triple quadrupole mass spectrometer	HP-5MS (30 m × 0.25 mm, 0.25 μm)	pesticide analysis	59
extracted with methanol or methanol/dichloromethane in an ultrasonic bath at 40°C for 1 h	Agilent 7890 GC system coupled 5975 MSD	Ultra-1 capillary column (25 m × 0.2 mm, 0.33 μm)	steroid hormone	19

120 min at 25°C) and temperature (25, 40, and 60°C for 60 min) during sonication with methanol have been evaluated. The effect of the extraction solvent used during ultrasonication has also been evaluated. For this, hair samples were incubated with methanol (0.5 mL) or methanol/dichloromethane (1:2 or 2:1) for 60 min at 40°C.[19]

Enzymatic hydrolysis

For the enzymatic hydrolysis of hair, added 500 μL of a 1,4-dithiothreitol solution (12 mg/mL in tris(hydroxymethyl) aminomethane-hydrochloric acid buffer solution (0.1 M, pH 7.2)) is added. The mixture is then incubated at 40°C for 12 h.[26]

Instrumental Conditions

Most experimental instrument for hair analysis consists of the use of liquid chromatography-mass spectrometry. Here is one example we used to explain the instrumental conditions for untargeted profiling.[27-29] An ACQUITY™ ultra-performance liquid chromatography system (Waters, Milford, MA, USA) coupled to a Q-Tof Premier™ quadrupole/time-of-flight hybrid mass spectrometer system from Waters (Milford, MA, USA) were used. The gradient elution system consisted of solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) and was controlled as follows: 0–3 min, 5% B; 3–10 min, 5–50% B; 10–11.5 min, 50–95% B; 11.5–12 min, 95–5% B. The gradient was then returned to the initial concentration (5% B) and held for 2 min before running the next sample. Except for previous results, instrument conditions used in other studies were showed in Table 1. also classically used gas chromatography-mass spectrometry method is also summarized in Table 2. It was confirmed that most of the GC-MS methods were analyzed using a column composed of 5% phenyl polysilphenylene-siloxane.

Applications of Hair Metabolomics

Forensic science

Forensic chemistry uses biological samples such as urine and hair to evaluate the authenticity of psychotropic and other drugs in order to determine whether they are suitable for use as well as to measure the contents (purities) of their various components.

Drug confirmation tests performed on hair samples obtained during drug crime investigations are not only used to complement urine analysis but also for diagnosing drug use and checking for chronic drug abuse.

Moreover, the duration of drug use can be determined several months after use based on the detection range. For example, methamphetamine is a highly addictive central nervous system stimulant, and the changes in human hair metabolites after excessive methamphetamine use have been analyzed. Through network analysis, it has been shown that the concentrations of glycosphingolipids, sphingolipids, glycerophospholipids, and ether lipids as well as the metabolism of amino acids (glycine, serine, and threonine; cysteine and methionine) are affected by methamphetamine use.[60]

In addition, from a forensic point of view, a method for simultaneously analyzing the drugs and steroids present in hair samples has been developed.[61]

However, there are a few limitations associated with hair analysis. The differences in the hair growth rate with age, gender, ethnicity, as well as the individual variations between subjects make it difficult to interpret the concentrations of metabolites in hair. In addition, it is also difficult to estimate the time and volume details from hair segment analysis, as the drug integration mechanism of the hair matrix is not yet fully understood.[62] In addition, hair samples can be manipulated through cosmetic treatments, and the drug concentrations can be altered, resulting in false negatives. In particular, the oxidative bleaching of hair samples under alkaline conditions has a significant effect on drug concentration. However, recently, a method to identify the metabolites altered by oxidative beauty therapies was developed based on nontargeted hair metabolomics analysis. [63]

Androgenic alopecia

Androgenic alopecia is a well-known condition that occurs because of increased male hormone secretion.[64] However, androgenic alopecia in females (female-pattern baldness) differs from that in males. The causes of female-pattern baldness are not as clear as those of male-pattern baldness. Therefore, in a previous study, we performed untargeted metabolomics to comprehensively analyze the

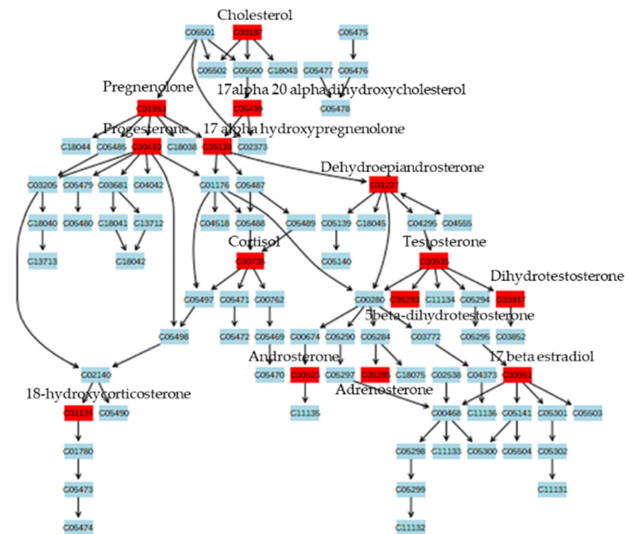

Figure 2. Steroid hormone biosynthesis with significant differences in male groups. Metabolites in red are significantly different for patients and controls.

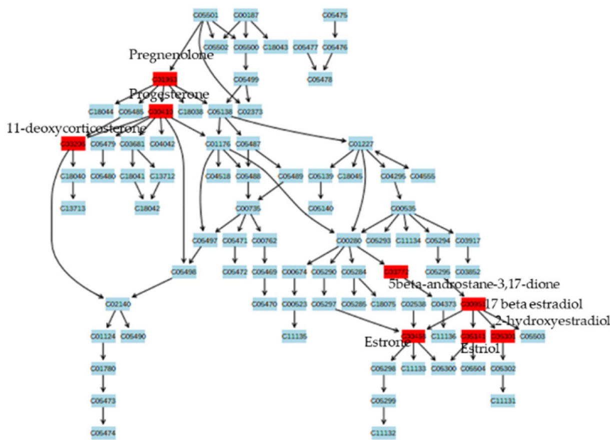

Figure 3. Steroid hormone biosynthesis with significant differences in female groups. Metabolites in red are significantly different for various groups.

metabolites and metabolic pathways of androgenic alopecia using human hair samples.[29] We were able to elucidate the extensive metabolic changes associated with androgenic alopecia based on sex.

In the case of the male groups, steroid hormone biosynthesis and the androgen metabolic pathways were significantly altered.

On the other hand, in the case of the female groups, steroid hormone biosynthesis and the estrogen metabolic pathways were significantly altered.

Hair follicles are among the most highly proliferative tissues. Therefore, we had also analyzed the levels of the polyamines and metabolites involved in cell proliferation in hair samples taken from patients with androgenic alopecia.[21,65]

Neurodegenerative diseases

Neurodegenerative diseases are associated with several types of cognitive impairment, and the cortisol concentration in hair may reflect the level of chronic stress.[66] The metabolic ratio of 7β-hydroxycholesterol (7β-OHC) to cholesterol can be used as a predictable index for evaluating cognitive impairment. The ratios for the abnormal cognition (mild cognitive impairment (MCI) + Alzheimer's disease (AD)) and vasospastic angina (VA) groups were found to be significantly different.[67]

In addition, an analysis of the glucocorticoid levels in hair samples from patients with Parkinson's disease showed that the level of hair cortisone was significantly higher in the patients.[68]

Hair analysis during various trimesters of pregnancy

Hair metabolites are an important source of information in pregnancy research and are used to study the metabolic mechanisms and complications related to pregnancy.[2, 49, 69] The 40-week gestation period is divided into three trimesters. The period from the moment of confirmation of pregnancy to 13 weeks is called the first trimester, that from 14 weeks to 28 weeks is called the second trimester, and that from 28 weeks until delivery is called the third trimester. As pregnancy progresses, the concentrations of various metabolites change. The intermediates of glycolysis and the tricarboxylic acid cycle, such as pyruvic acid,

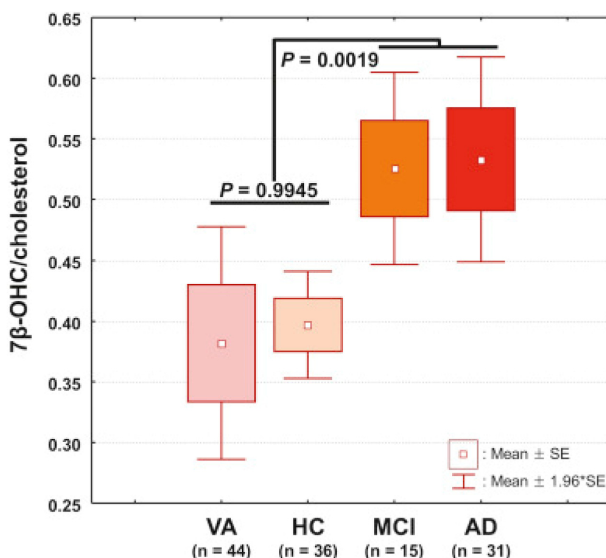

Figure 4. Box plot of hair metabolic ratios of 7β-hydroxycholesterol (7β-OHC) to cholesterol.

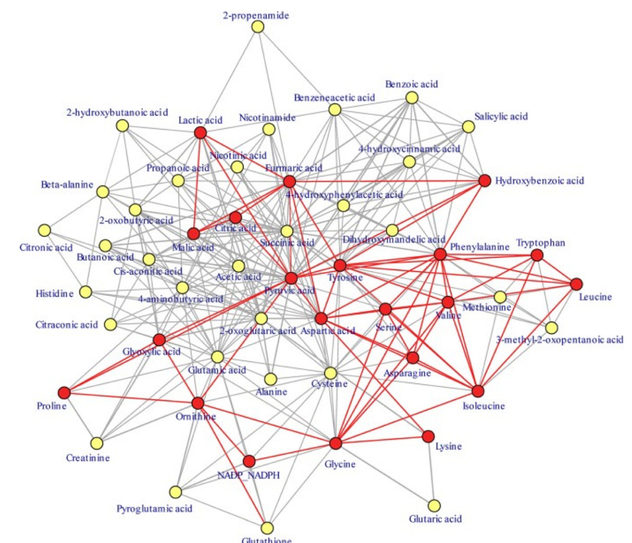

Figure 5. Metabolic network showing relationships between trimester-related metabolic pathways and various metabolites. Red circles represent metabolites that are significantly altered throughout pregnancy while yellow circles represent those that are not.

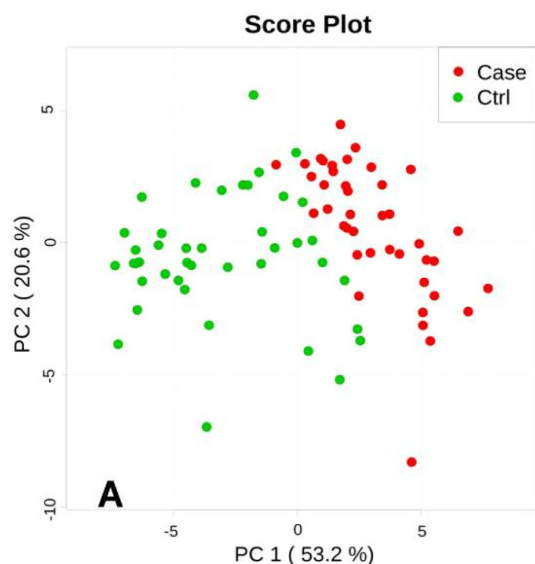

Figure 6. Principal component analysis (PCA) score plot. Red circles represent fetal growth restriction cases while green circles represent normal controls.

fumaric acid, citric acid, and malic acid, link the various metabolic pathways. In particular, carbohydrate metabolism is significantly altered during pregnancy.[49]

In addition, based on an analysis of hair samples, we were able to confirm that there is a difference in the PCA results for the group with fetal growth restriction and those for normal controls. In particular, there were significant differences in the lactate, levulinate, 2-methyloctadecanate, tyrosine, and margarate levels of these groups.[2]

The onset of intrahepatic cholestasis of pregnancy, a maternal liver disease, can lead to sudden consequences, including fetal death and stillbirth. Attempts are being made to predict this disease based on predictive biomarkers using maternal hair samples. However, the results obtained so far have been unsatisfactory, as the hair samples collected at the onset of the disease did not show metabolic changes, suggesting rapid development.[70]

Perspective and limitations

In this review, we discussed hair metabolomics, including the sample preparation techniques used and the clinical applications of the method. We believe this review provides insights for improving the currently used approaches for hair analysis. In the future, hair metabolomics will be used more widely not only in forensic science but also in many other fields.

From a forensic viewpoint, hair analysis should be a complementary approach rather than the primary technique. Since there is a possibility of false positive results owing to the external contamination of the hair sample, an efficient decontamination procedure is required.[71] Hair readily adsorbs contaminants even after short-term exposure. For example, environmental substances and shampoos adhere to its surface. In addition, the hair matrix may contain endogenous metabolites and other substances related to long-term exposure. Therefore, methods are being developed to profile both transiently exposed chemicals and endogenous metabolites in the same hair sample.[72]

A recent study confirmed that there are differences in the levels of metabolites with the hair color, suggesting that it is necessary to consider the hair color and hair segments in subsequent studies on hair metabolites.[73]

Thus, owing to their various advantages, metabolomics methods based on the noninvasive analysis of hair samples have been used in many clinical studies.

Acknowledgments

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