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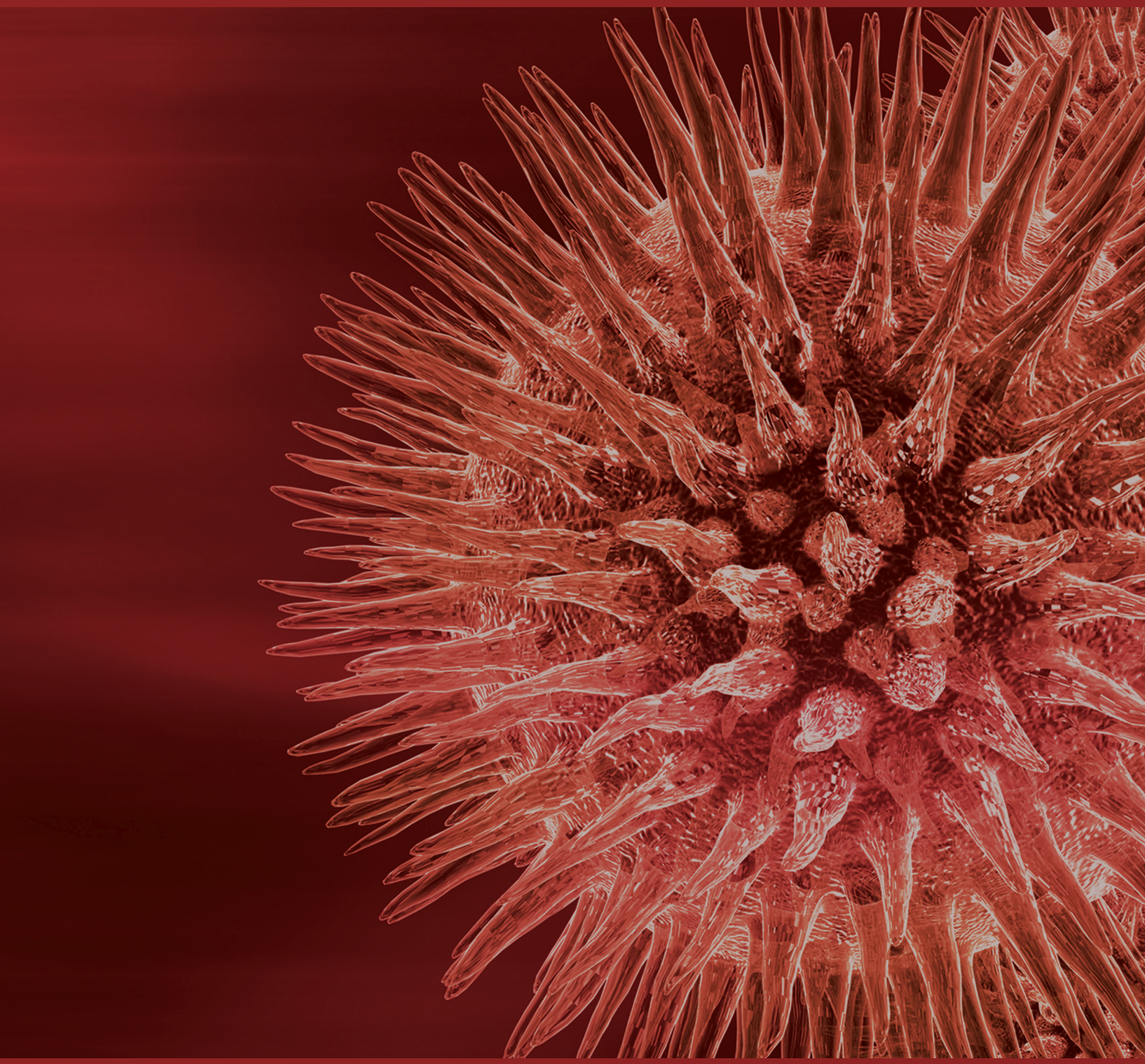
Human Body Fluid

Guest Editors: Shih-Bin Su, Terence Chuen Wai Poon, and Visith Thongboonkerd



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Editorial

Human Body Fluid

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Human body fluids are considered as attractive sources for clinical markers. As for disease diagnosis and prognosis, advantages of body fluid testing include low invasiveness, low cost, and rapid sample collection and processing. Besides, altered protein expression profiles in body fluids reflect the change of physiological states and cellular networks of the diseased tissue/organ. Thus, analysis of human body fluid has become one of the most promising approaches to discover biomarkers or reveal pathophysiological mechanisms for human diseases. Human body fluid analysis is inherently challenging due to their unique characteristics such as protein complexity and the wide dynamic range of protein abundances. With the remarkable advances in the methods for sample preparation, proteomics technology, and quantitation, it is now possible to analyze body fluids with higher sensitivity and robust experimental design. As the recognition of the importance of "Translational Medicine" which is the process of turning appropriate biological discoveries into drugs and medical devices that can be used in the diagnosis and/or treatment of patients, a great quantity of researches were focusing on the human sample including human body fluids. Thus, it is conceivable that the new insight unveiled by human body fluid analysis will attract a wide audience abroad including researchers from the field of basic sciences, bioinformatics, analytical chemistry, molecular biology, and hospital.

In the study of H. Y. Wu et al., authors performed the first differential proteomic profiling between peritoneal dialysates from chronic glomerulonephritis (CGN) patients at the early and middle stage of continuous ambulatory peritoneal dialysis (CAPD) treatment. The changed proteins provide clues to the PD-induced loss of proteins from the peritoneum and assist the identification of potential biomarkers for noninvasive monitoring of peritoneal damage.

M. H. Yang et al. also characterized the peritoneal dialysate proteins from diabetes mellitus (DM) by proteomic tools. CGN peritoneal dialysate was used as control. Differentially expressed proteins in DM samples may indicate a situation for possible drug treatment and predictors of peritonitis for a validation study in the future.

P. Badiee reviewed the evaluation of different human body fluids, pleural Effusion, bronchoalveolar lavage fluid, peritoneal fluid, urine, pericardial effusion, blood, cerebrospinal fluid (CSF), synovial fluid, and saliva, for diagnosing fungal infections. Routine laboratory tests for the diagnosis of FI include urinalysis and blood analysis while analyses of both CSF and serum can improve the accuracy of the diagnosis.

K. Bořecká et al. studied the use of Coefficient of Energy Balance (CEB) values in a large CSF samples ($n = 8183$) to demonstrate that CEB enables more exact assessment of actual energy state in the CSF compartment than glucose and lactate alone. This study suggested that CEB combined with CSF cytology has a great importance for diagnosis, differential diagnosis, and early therapy of CNS diseases.

H. C. Yen et al. discussed potential interferences on the analysis of F_2-IsoPs and F_4-NPs in CSF by GC/NICI-MS by present analytical methods. Proper TLC purification for obtaining reliable chromatograms for F_2-IsoPs quantification in CSF is suggested as well as the necessity of adding

additional holding of the column at 280°C for a period of time following data acquisition during F_2-IsoPs and F_4-NPs analysis to avoid potential interferences on subsequent F_4-NPs quantification in CSF.

Acknowledgments

The guest editors of this special issue thank the reviewers and all authors for providing important contributions in this issue. The contributions in this issue discuss novel insights, advanced assessment as well as diagnosis. Of course, the selected topics and papers are not a comprehensive representation of the area of this special issue. Nonetheless, they represent the rich knowledge that we have the pleasure of sharing with the readers.

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Terence Chuen Wai Poon
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Review Article

Evaluation of Human Body Fluids for the Diagnosis of Fungal Infections

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Invasive fungal infections are a major cause of morbidity and mortality in immunocompromised patients. Because the etiologic agents of these infections are abundant in nature, their isolation from biopsy material or sterile body fluids is needed to document infection. This review evaluates and discusses different human body fluids used to diagnose fungal infections.

1. Introduction

Invasive fungal infections (IFI) are increasing in immunocompromised patients due to the improved management of high-risk patients with novel treatment methods. The risk of IFI has been increasing over the last decades mostly because the medical treatments used in specific setting of patients are correlated with severe and prolonged immunosuppression. Although some types of IFI are rare, they are associated with significant morbidity and mortality [1].

The main etiologic agents include yeasts, filamentous fungi, and dimorphic fungi. Common yeasts are predominantly *Candida* spp. including *Candida albicans*, *Candida glabrata*, *Candida kefyr*, *Candida parapsilosis, Candida tropicalis* [2], and *Cryptococcus* spp. Other organisms such as *Trichosporon* spp. can be involved in serious conditions. The filamentous fungi are classified as having either septate (e.g., *Aspergillus* spp.) or aseptate hyphae (Mucorales) [3]. Less common pigmented molds (dematiaceous, that is, darkly pigmented) including *Pseudallescheria* can infect some human organs, especially in the central nervous system (CNS). Finally, the dimorphic fungi are filamentous at 25°C and room temperature and yeast-like in host tissues or when incubated at 35 to 37°C. These fungi, which are true pathogens, include *Blastomyces*, *Histoplasma*, *Coccidioides*, *Paracoccidioides,* and *Penicillium marneffei* and are endemic in specific geographic regions [4–6].

Analysis of all body fluids is essential for the diagnosis of IFI and may be more informative than serum analysis [7]. Invasive diagnostic procedures such as bronchoscopy, thoracoscopy, percutaneous catheter drainage, or open surgery are usually necessary to confirm the diagnosis but are not possible in some critical patients. For pyogenic abscesses, percutaneous catheter drainage is an established technique with a success rate of 80%–90% [8, 9], although in deep systemic mycosis of poorly localized nature, the success rate is reported to be low [10].

To date no typical clinical picture has emerged, so the management of infections is critical and the isolation and identification of the fungal pathogen are important for prompt, appropriate therapy. To identify deep-seated IFI and provide appropriate care according to international criteria, isolation of fungi from biopsy specimens [11] or drainage from any focal fluid sample is necessary [8, 9, 12].

In high-risk patients, the isolation of fungi during followup may appear to contradict the patient's physiological and clinical condition, which may not reflect the severity of the infection. Need for early antifungal therapy is still controversial, especially when *Candida* is isolated from peritoneal fluid, urine, or pulmonary specimens [13–16]. The isolation of fungi from more than one specimen of urine, peritoneal

fluid, or blood culture is a reliable criterion for systemic IFI [1].

The methods available for the diagnosis of fungal elements in human body fluids include routine mycological methods such as direct microscopic examination and culture in specific fungal media, serological methods such as the latex antigen test, galactomannan, and mannan antigen or antibody detection by ELISA, and molecular methods such as nested PCR, real-time PCR, and PCR ELISA. Determining the susceptibility of the isolated fungi to antifungal agents can help improve the clinical management of systemic mycosis [3, 17]. Early diagnosis with prompt antifungal therapy or even surgery might be warranted to save the patient's life [18]. This review evaluates and discusses different human body fluids that can be used in the diagnosis and management of IFI.

2. Pleural Effusion

Pulmonary fungal infections are difficult to confirm. The isolation of fungi (especially yeasts) as the pathogenic agent is controversial, so determining the prevalence and management of these infections has been difficult. A previous report demonstrated that *Candida* colonization could be found in respiratory samples obtained by bronchopulmonary lavage, endotracheal aspirate, or protected specimen brushing in critically ill patients [19]. One of the criteria for the diagnosis of pulmonary FI is an obvious lung lesion on chest X-ray and isolation of fungi from the pleural effusion or blood. The pleural fluid obtained in many cases of fungal pleuritis [20] is a reliable specimen for the diagnosis of pulmonary infections. Pulmonary cryptococcal infection is identified by microscopic examination or positive cryptococcal antigen in percutaneous needle aspiration fluid [18]. For the diagnosis of *Aspergillus* fungus ball, surgical specimens from the pleural cavity have been used, but the value of pleural fluid is not known [21].

3. Bronchoalveolar Lavage Fluid

Pulmonary fungal infection, especially pulmonary aspergillosisis is routinely diagnosed by examination of bronchoalveolar lavage fluid (BAL). There are series of recommendations for performing BAL fluid, according to European Respiratory Society [22, 23].

Conventional mycological techniques like culture and histological examination of BAL fluid are the most commonly used ones for the diagnosis of these infections and the detection of the fungal cell wall antigen can be performed by galactomannan (GM) test [24] and PCR [25] in BAL samples. The sensitivity and specificity of galactomannan Ag test can vary in serum and BAL fluid and they are usually lower in BAL than in serum [24]. According to Musher et al., the sensitivity and specificity of the GM EIA in BAL fluid with positive culture result for *Aspergillus* were 61% and 98% with an index cutoff 1.0 and 76% and 94% with an index cutoff of 0.5, respectively [26]. The sensitivity and specificity of qPCR assay in BAL fluid were 67% and 100%, respectively [26].

As oxygenation is likely to deteriorate during the BAL collection, there are difficulties in performing BAL fluid collection in some critical patients like hematology patients with complication rates approaching 15% [27] and patients with borderline oxygenation who require elective preprocedure intubation and ventilation.

4. Peritoneal Fluid

For some patients a permanent vascular access cannot be used, and peritoneal dialysis is performed instead. In such patients, fungal peritonitis [28] is one of the most serious complications. This infection is a rare but potentially fatal complication. Fungal peritonitis accounted for 3.6% [29] and from 5% to 22% [30, 31] of all peritonitis episodes. The mortality rate was from 20% to 30% in one study [32] and 60% to 70% in another [33]. However, in some areas the rates can be much higher, and peritonitis is associated with high rates of morbidity and mortality.

Direct microscopic examination of the peritoneal fluid was useful for confirming suspected IFI in 60% of patients in one study [29]. In patients in the intensive care unit with peritonitis, grade C scores (at least three risk factors) predicted yeast isolation from peritoneal fluid with 84% sensitivity, 50% specificity, 67% positive predictive value, 72% negative predictive value, and an overall accuracy of 71% [34]. About 43.4% of the patients with perforated peptic ulcer [35] had positive peritoneal fluid fungal culture for yeast, including *Candida* spp. The agents responsible for peritonitis according to Martos et al. were *Candida parapsilosis* (4), *C. albicans* (2), *C. tropicalis* (1), *C. glabrata* (1), *C. famata* (1) and *Fusarium oxysporum* (1) [29].

5. Urine

Candiduria is clearly not a disease and is common in hospitalized patients. Clinical findings vary and can include asymptomatic candiduria (previously healthy patients, predisposed outpatients, or predisposed inpatients), symptomatic candiduria (cystitis, pyelonephritis, prostatitis, epididymo-orchitis, or urinary tract fungus balls), and clinically unstable candiduria [36]. Most patients are asymptomatic. *C. albicans* is the yeast most commonly isolated from urine, accounting for 50% to 70% of the isolates in various studies [37–39].

Samples should be collected with the clean-voided urine culture method. Candiduria may occur due to contamination of urine during sampling with perineal flora, especially in older women or when vaginal discharge is present. In these cases, sampling must be repeated and it is often necessary to obtain the urine specimen by sterile bladder catheterization. If the second specimen culture yields no yeasts, contamination can be assumed and no further diagnostic procedures are needed.

Once contamination is ruled out, colonization of the bladder, perineum, or indwelling urinary catheter must be considered. To verify infection from colonization, the number of organisms in the urine must be quantified. The first studies done in 1970s used renal biopsies to establish renal

involvement [31, 40]. In patients without a catheter or with a short indwelling catheter, proven renal infection was found with as few as 10 000 to 15 000 yeasts/mL and as many as 40 000 yeasts/mL in the urine [41]. In addition, criteria such as pyuria, the presence of pseudohyphae [42], and the finding of casts [43, 44] in the urine were considered to distinguish between colonization and urinary tract infection, but these features are of limited practical use in clinical terms. Species such as *Candida glabrata* naturally cannot produce pseudohyphae, and *C. albicans* can be induced to form pseudohyphae by varying pH and nutrient conditions. It was also thought that antibody-coated yeasts in the urine could be used as a marker for infection [45, 46].

Some special disorders such as acquired or congenital disturbances of urine flow, occult diabetes mellitus, genitourinary structural abnormalities, bacterial infections, diminished renal function, structural abnormalities of the kidney, and metabolic abnormalities merit particular attention because they are the predisposing factors for candiduria [47, 48]. Candiduria may be due to hematogenous seeding of the kidney cortex in the course of disseminated candidiasis [37]. Experimental studies of hematogenous renal candidiasis in animal models indicated that any concentration of *Candida* spp. in the urine was significant for renal involvement [49]. Even if infection of the urinary tract by *Candida* spp. can be confirmed, surprisingly, physicians do not always follow the patients and antifungal therapy is not always warranted [37–39]. This issue needs to be addressed in further studies of the patients with candiduria.

6. Pericardial Effusion

Fungal pericarditis occurs mainly in immunocompromised patients [50] due to endemic fungi such as *Histoplasma* and *Coccidioides,* opportunistic fungi (*Candida*, *Aspergillus*), and semifungi including *Nocardia* and *Actinomyces* [51–53]. The clinical picture of fungal myocarditis comprises of the full spectrum of pericardial diseases [54]. Fungal pericarditis is diagnosed mainly by staining and culturing pericardial effusion or tissue samples. The samples must be analyzed promptly and should undergo Gram, acid-fast, and fungal staining, followed by cultures [55]. Molecular assays can serve as a reliable method for the diagnosis of fungal endocarditis [56].

7. Blood

Diagnostic methods for IFI in the blood vary in sensitivity and specificity. Blood culture for the diagnosis of filamentous fungi is not specific and for *Candida* spp., with a sensitivity about 50% [57, 58]. Candidemia is diagnosed when *Candida* spp. are isolated from at least one blood culture [59]. The mortality rates for candidemia range from 5% in intensive care units in the USA to 71% in liver transplant recipients [60].

Serological methods such as the beta glucan assay in serum may be useful for identifying IFI by all etiologic agents except *Cryptococcus* and *Zygomycetes*, with sensitivity rates of 64% to 77%, although specificity may be decreased in patients with certain concurrent bacterial infections [61, 62]. The serum galactomannan assay was found to have 95% sensitivity and specificity for invasive *Aspergillus* infections [63], and the serum mannan assay had a 67% specificity and 90% sensitivity for the diagnosis of systemic candidiasis in patients with fungemia [64, 65].

Molecular methods have recently been used to detect systemic IFI in serum and whole blood samples. The pan-fungal PCR assay [61] detects the small-subunit rRNA gene sequence of major fungal organism groups, with a sensitivity of 80% and a specificity of 95.6% [66]. Other molecular methods include PCR ELISA, nested and real-time PCR, which are used to detect fungal DNA in blood samples. These methods can improve the monitoring of the patients after antifungal therapy [67, 68]. In patients with IFI who respond to antifungal therapy, PCR ELISA assays became negative after 14 days of treatment and remained positive in patients who did not respond [69]. The other advantage of molecular methods for blood samples, especially in serial sampling, is the earlier appearance of positive PCR findings compared to other diagnostic methods based on radiological symptoms. Culture methods can take 8–10 days, whereas PCR can yield results in 4-5 hours. In patients who were followed weekly, the PCR findings became positive before clinical manifestations and radiological findings appeared. In other words, the PCR results became positive during the incubation period of the infection [66, 70, 71].

8. Cerebrospinal Fluid

Many fungi can cause CNS infection in high-risk patients. Infection in immunocompetent patients has also been reported [72]. Among patients with hematologic malignancies, CNS disease accounts for 9.4% of filamentous IFI [73]. The diagnosis is based on the analysis of cerebrospinal fluid (CSF). In adult normal CSF, the white blood cell (WBC) count may be as high as 5/mL with a predominance of lymphocytes. In normal CSF the glucose concentration is from 50% to 60% of serum values [74].

In patients with fungal meningitis infection, the WBC count is variable and lymphocytic pleocytosis (*Cryptococcus*, *Candida*) is present together with a predominance of neutrophils (*Aspergillus*, *Blastomycosis*) or eosinophilia (*Coccidioides*) [75].

On direct microscopic examination, hyphae or pseudohyphae can occasionally be seen in *Candida* or other infections. Indian ink should be used for the diagnosis of *Cryptococcus neoformans*. Fungal cultures are mostly positive in *C. neoformans* and candidal meningitis and are less frequently positive in other IFI [76]. Larger volumes of CSF obtained with repeated lumbar puncture can increase the chances of positive culture results.

Glucose and protein concentrations in CSF are the sensitive indicators of CNS pathology. The physician should know the normal reference range in each region because the measurements are technique dependent and the normal range varies depending on laboratory procedures. In the normal population, CSF glucose is about two thirds of the

Table 1: Use of body fluid for the detection of fungal infections.

Site of infection	Sample	Most Etiologic fungal agents
Systemic infection	Blood	*Candida* spp., *Aspergillus* spp., *Cryptococcus* spp.
Urinary tract and systemic infection	Urine	*Candida* spp. especially *C. albicans*, *Cryptococcus* spp.
Lung	Pleural effusion, Bronchoalveolar lavage fluid	*Candida* spp., *Aspergillus* spp., *Cryptococcus* spp., Zycomycetes fungi family
Peritoneum	Peritoneal	*Candida* spp., and rare filamentous fungi like *Fusarium oxysporum or Aspergillus* spp.
Joint	Synovial fluid	*Candida* spp., *Aspergillus fumigatus*
Heart	Pericardial effusion	Endemic fungi such as *Histoplasma* and *Coccidioides* or opportunistic fungi like *Candida*, *Aspergillus* and semi-fungi including *Nocardia* and *Actinomyces*
Central Nervous Infection	Cerebrospinal fluid	*Cryptococcus neoformans*, *Candida* spp., *Aspergillus* spp., *Blastomyces dermatitis*, *Pseudallescheria* and *Histoplasma capsolatum*
Oral	Saliva	*Candida* spp.

Table 2: Diagnostic methods for the detection of opportunistic fungal infections in body fluids.

Etiologic agents	Diagnostic methods
Aspergillosis	*Pleural effusion or Bronchoalveolar lavage fluid* (i) Direct examination, cultures, histopathologic demonstration (ii) Galactomannan antigen detection, Beta-D-glucan assay*, Polymerase chain reaction *Serum* (i) *Aspergillus* antibody test (precipitins) for chronic pulmonary aspergillosis (ii) *Aspergillus* IgG antibodies, *Aspergillus* IgE test (precipitins) (iii) Galactomannan and beta-D-glucan levels double sandwich enzyme-linked immunosorbent assay (ELISA) (iv) 1,3-Beta-D-glucan
Candidiasis	*Blood, CSF, Urine, or other body fluid* (i) Isolating a *Candida* species from multiple or repeat cultures (ii) Beta-D-glucan and other antigen and metabolite assays (iii) Polymerase chain reaction
Cryptococosis	*Cerebrospinal fluid, Blood or Serum, BAL, Urine* (i) India ink smear (ii) Culture (iii) latex agglutination test or enzyme linked immunosorbent for Cryptococcal antigen testing
Mucormycosis	*Sterile body fluid from site of infection* (i) Histopathopathologic examination (ii) Culture
Other fungal infection	*Sterile body fluid from site of infection* (i) Histopathopathologic examination (ii) Culture and isolation of fungal species in cultures of involved biologic materials

* 1,3-Beta-D-glucan, a cell wall composition of many fungi, may be positive in patients with a variety of invasive fungal infections, it is typically negative in patients with mucormycosis or cryptococcosis.

serum glucose concentration and does not exceed 300 mg/dL regardless of serum levels. The adult range of CSF protein concentration reaches 18 to 58 mg/dL between 6 and 12 months of age [77]. In fungal brain infections, CSF glucose is generally low and protein is generally high, with exceptionally high levels in cryptococcal infections [74].

The sensitivity and specificity rates for CSF antigen testing are above 90% for *Cryptococcus* and *Histoplasma* [74, 75] and

the galactomannan CSF assay for the diagnosis of invasive aspergillosis has been investigated [63]. In addition, PCR analyses can indicate the presence of DNA from different fungal species in CSF [78].

9. Synovial Fluid

Fungal joint infection due to yeast and filamentous fungi is rare and its diagnosis and treatment can be challenging even for skilled doctors. The etiologic agents reported most frequently are *Candida* and *Aspergillus* spp. In invasive *Aspergillus* infections, dark fluid is aspirated from the joint. In joints with *Aspergillus fumigatus* infection, synovial fluid analysis showed cell count between 7300 and 128 000 cell/mL, predominantly neutrophils [79].

In recent years the incidence of periprosthetic joint infection (total knee replacement) has increased. In one study, most fungal knee infections were caused by *Candida* species (80%), of which *C. parapsilosis* was the most common (50%) [80]. The definitive diagnosis and identification of the etiologic agent were yielded by aspiration of synovial fluid in most cases, but in some cases intraoperative and tissue biopsy culture were needed [81].

10. Saliva

Saliva is the first defense against the many pathogenic organisms in oral cavity. With regard to IFI, the most important role of saliva is defense against pathogenic fungi with special enzymes. *Candida albicans* is the most important fungal species in the oral cavity. The primary mechanism of salivary defense is secretory immunoglobulin, which inactivates fungi via binding and/or agglutination of the microorganisms [82]. Other defense mechanisms have also been hypothesized, such as lactoferrin, lysozyme, and histamine, particularly histatin-5, which can bind to the surface of heat shock protein homologues of *C. albicans* and lead to internalization and death of this organism [83]. The clinical signs of oral candidiasis have been investigated by counting *Candida* organisms in saliva [84], and a sensitive PCR detection system has been used to diagnose *Pneumocystis carinii* pneumonia [85].

Tables 1 and 2 presented the use of body fluid and diagnostic methods for the detection of opportunistic fungal infections in body fluids.

11. Conclusions

The examination of human body fluids is necessary to diagnose IFI, even though invasive methods may be needed to obtain samples for analysis. Routine laboratory tests for the diagnosis of FI include urinalysis and blood analysis. The second line for the diagnosis of systemic mycosis is the CSF. Analyses of both CSF and serum can improve the accuracy of the diagnosis. Other human body fluids should be obtained and analyzed for IFI according to specific conditions.

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Research Article

Analytical Variables Affecting Analysis of F_2-Isoprostanes and F_4-Neuroprostanes in Human Cerebrospinal Fluid by Gas Chromatography/Mass Spectrometry

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F_2-isoprostanes (F_2-IsoPs) are a gold marker of lipid peroxidation *in vivo*, whereas F_4-neuroprostanes (F_4-NPs) measured in cerebrospinal fluid (CSF) or brain tissue selectively indicate neuronal oxidative damage. Gas chromatography/negative-ion chemical-ionization mass spectrometry (GC/NICI-MS) is the most sensitive and robust method for quantifying these compounds, which is essential for CSF samples because abundance of these compounds in CSF is very low. The present study revealed potential interferences on the analysis of F_2-IsoPs and F_4-NPs in CSF by GC/NICI-MS due to the use of improper analytical methods that have been employed in the literature. First, simultaneous quantification of F_2-IsoPs and F_4-NPs in CSF samples processed for F_4-NPs analysis could cause poor chromatographic separation and falsely higher F_2-IsoPs values for CSF samples with high levels of F_2-IsoPs and F_4-NPs. Second, retention of unknown substances in GC columns from CSF samples during F_4-NPs analysis and from plasma samples during F_2-IsoPs analysis might interfere with F_4-NPs analysis of subsequent runs, which could be solved by holding columns at a high temperature for a period of time after data acquisition. Therefore, these special issues should be taken into consideration when performing analysis of F_2-IsoPs and F_4-NPs in CSF to avoid misleading results.

1. Introduction

Reliable assessment of oxidative stress *in vivo* has been important for investigating the roles of oxidative stress in the pathogenesis or progression of diseases [1]. F_2-isoprostanes (F_2-IsoPs) are prostaglandin (PG)-like compounds derived from lipid peroxidation of arachidonic acid (AA, C20:4 ω-6), which is abundant in all kinds of cells, initiated by free radicals independent of the cyclooxygenase pathway. They are initially formed as esterified form on phospholipids and can be released into surrounding body fluids to become free form via the action of enzymes with the phospholipase-like activities [2–5]. There are four regioisomers of F_2-IsoPs, but the 5-series and 15-series regioisomers are the major regioisomers formed *in vivo* [6]. F_2-IsoPs have been well recognized as the most reliable and specific marker of lipid peroxidation *in vivo* and is a widely used marker of oxidative damage due to several favorable characteristics [7]. On the other hand, F_4-neuroprostanes (F_4-NPs) are generated from the lipid peroxidation of docosahexaenoic acid (DHA, C22:6 ω-3) via similar mechanisms, but eight regioisomers are produced [8]. As shown by Yin et al., 4-series and 20-series regioisomers are the most abundant regioisomers of F_4-NPs generated *in vitro* and *in vivo* [9]. Measurement of F_4-NPs in CSF or brain tissue has been considered as a more selective marker for neuronal oxidative damage because DHA is enriched in neurons [10].

Gas chromatography/negative-ion chemical-ionization mass spectrometry (GC/NICI-MS) is the most sensitive and robust method for routine quantification of F_2-IsoPs and F_4-NPs in biological samples [11, 12], which is required for body fluids with low levels of free F_2-IsoPs and F_4-NPs or limited availability, such as cerebrospinal fluid (CSF). Different assay platforms and further modifications for GC/NICI-MS analysis of F_2-IsoPs and F_4-NPs were present in the literature as discussed in our previous paper [13], but most

of the them were modified from procedures from the groups of Roberts or Morrow [8, 14, 15]. As previously reviewed by us, methods involving liquid chromatography/mass spectrometry (LC/MS) were superior than the GC/NICI-MS method primarily in the aspect of identification of different regioisomers, but it could only be used for quantification of highly abundant free F_2-IsoPs in urine or total levels of F_2-IsoPs in plasma, which consisted of abundant esterified F_2-IsoPs from lipoproteins, due to its lower sensitivity [16]. Moreover, the unique lipid chromatography/atmospheric pressure chemical ionization mass spectrometry (LC/APCI-MS) method developed by Yin et al. was only used to identify different regioisomers of F_4-NPs in liver tissue and DHA oxidized *in vitro* [9], whereas detection of F_2-IsoPs and F_4-NP in CSF in the literature so far, including our studies, was only performed by the GC/NICI-MS method [8, 13, 17–21].

Although the GC/NICI-MS method has been recognized as the reference method to quantify F_2-IsoPs and F_4-NP levels in body fluids, several variants of analytical settings exist in the literature for GC/MS analysis. For example, the area on silica recovered from thin-layer chromatography (TLC) plates should be smaller for F_2-IsoPs analysis than that for F_4-NPs analysis to obtain better chromatographic separation [13, 17], but Corcoran et al. simultaneously analyze total (free plus esterified) levels of F_2-IsoPs, F_4-NPs, and isofurans, another product of lipid peroxidation from AA, in human CSF without showing any chromatogram when they employed a very different method without the step of TLC purification [18]. Because the amount of CSF needed for F_4-NPs is much higher for F_2-IsoPs analysis, it is also a tempting idea to perform simultaneous analysis of the same sample when the amount of CSF samples available is very little. On the other hand, during our previous investigation on F_4-NPs of human CSF, we noticed obvious retention of unknown compounds from the previous samples on GC columns at *m/z* 593.5, which was used to detect F_4-NPs, by using the temperature ramp commonly indicated to analyze F_2-IsoPs or F_4-NPs [15, 22]. We speculated that when this problem was not noticed, the results of F_4-NPs quantification in CSF might not be reliable. Accordingly, we also wondered whether analysis of F_4-NPs in CSF could also be affected by the retention effect following analysis of F_2-IsoPs of CSF or other body fluids since it was very common to analyze different samples for either F_2-IsoPs or F_4-NPs interchangeably during routine operation.

In this report, we first investigated whether simultaneous analysis of F_2-IsoPs and F_4-NPs for CSF samples processed for F_4-NPs analysis is feasible by comparing chromatograms and results of F_2-IsoPs quantification from CSF samples processed for F_2-IsoPs and F_4-NPs separately. Furthermore, we systematically examined whether significant amount of substances was retained in GC columns from previously injected samples by simultaneously recording chromatograms at *m/z* 569.4, *m/z* 593.5, and *m/z* 573.4, which were masses employed to detect F_2-IsoPs, F_4-NPs, and $[^2H_4]$-15-F_{2t}-IsoP (internal standard), respectively, after analyzing F_2-IsoPs and F_4-NPs in CSF or analyzing F_2-IsoPs in urine and plasma. We also evaluated whether the peaks from the retained substances could overlap with the peaks for quantification of F_2-IsoPs, F_4-NPs, or $[^2H_4]$-15-F_{2t}-IsoP (internal standard) at their corresponding masses. Finally, the beneficial effect of additional holding of the column at a high temperature after regular data acquisition in the method setting on removing retained peaks was investigated.

2. Materials and Methods

2.1. Test Samples. For the testing in this study, we used three pooled human CSF samples with different levels of F_2-IsoPs and F_4-NPs, which were designated as L-CSF, M-CSF, and H-CSF for low levels, medium levels, and high levels of these compounds, respectively. L-CSF sample was from patients with normal pressure hydrocephalus, whereas M-CSF and H-CSF samples were from patients with aneurysmal subarachnoid hemorrhage (aSAH). These CSF samples were pooled from specimen collected during our previous study on aSAH that have been published [13, 17], in which we showed that CSF samples from aSAH had much higher levels of F_4-NPs than that from non-aSAH controls including those with normal pressure hydrocephalus [13]. Moreover, one test plasma sample and one test urine sample were also pooled samples from normal subjects used in the previous studies [23].

2.2. Sample Processing and GC/MS Detection for Analysis of Free F_2-IsoPs in CSF, Plasma, and Urine. The methods of analyzing free F_2-IsoPs in human body fluids was modified from the procedures described by the Morrow's group [11, 14]. Some of major modifications on solid-phase extraction (SPE) and GC/MS settings have been indicated in our previous publications [13, 17]. In brief, an appropriate volume of body fluids was added into 3 mL of ultrapure water containing the internal standards and the pH was adjusted to pH 3, which was followed by two runs of SPE purification with C_{18} columns and then silica columns. The internal standard used for CSF and plasma was $[^2H_4]$-15-F_{2t}-IsoP [14], whereas that used for urine was $[^2H_4]$-8-F_{2t}-IsoP to avoid interferences peaks from endogenous substances in urine samples [24]. $[^2H_4]$-15-F_{2t}-IsoP and $[^2H_4]$-8-F_{2t}-IsoP were purchased from Cayman Chemical, which were designated as 8-iso prostaglandin $F_{2\alpha}$-d_4 (catalog number 316350) and iP$F_{2\alpha}$-IV-d_4 (catalog number 316230), respectively, in the catalog of Cayman Chemical. These organic solvents in vials containing the two internal standards purchased from Cayman Chemical were evaporated and resuspended in a fixed amount of ethanol followed by the calibration of true concentration of the internal standard in the ethanol solution by the method of Milne et al. [11]. In this study, 0.6 mL of CSF, 0.5 mL of plasma, and 0.05 mL of urine samples were mixed with 100 pg, 250 pg, and 330 pg of the internal standards, respectively, for each sample preparation. Because the concentrations of M-CSF and H-CSF samples were known to be much higher than L-CSF, only 0.4 mL and 0.24 mL of M-CSF and H-CSF samples, respectively, were used along with 0.6 mL of L-CSF for the test described in this study.

The elution and washing steps for SPE purification were the same as the method of Milne et al. [14], but 6-mL disposable columns with 500 mg sorbent (J. T. Baker) was used and SPE was operated on a negative-pressure vacuum manifold column processor (J. T. Baker), which was connected to a vacuum pump capable of controlling the speed of SPE flow, to simultaneously process multiple samples [17]. The eluate from C_{18} columns was dried very briefly with 2 g anhydrous sodium sulfate in glass vials before being processed by silica columns. The analyte in final eluate from silica columns was dried, converted to PFB esters by using PFBB and DIPE, purified by thin-layer chromatography (TLC), and extracted by ethyl acetate according to the methods of Milne et al. [14]. The scarping range on TLC plates was 1 cm above and 1 cm below the TLC standard, methyl ester of $PGF_{2\alpha}$. After the second derivatization by *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and dimethylformamide (DMF), which was stored over CaH_2, and further dryness by nitrogen gas, the analyte was dissolved in an appropriate amount of undecane, which was also stored on CaH_2, for further injection into the GC/MS [14].

F_2-IsoPs in the samples and the internal standards added into the samples were detected at *m/z* 569.4 and 573.4, respectively, by the mode of selected ion monitoring. The ion detected by GC/NICI-MS was the trimethylsilyl ether derivative of the carboxylate anion of F_2-IsoPs or the internal standard, which has been well illustrated by us [13] or Milne et al. [22]. Amount of F_2-IsoPs in the samples was quantified by multiplying amount of $[^2H_4]$-15-F_{2t}-IsoP or $[^2H_4]$-8-F_{2t}-IsoP added into the samples with the ratio of the peak height of F_2-IsoPs to that of the corresponding internal standard. Settings and instrumentation of GC/NICI-MS used was the same as what we have previously described [13], in which the 6890 GC/5975 MS and DB-1701 capillary column from Agilent were used, except the inclusion of 2-min holding at 300°C at the end of temperature ramp (190°C to 300°C at 18°C/min) for the acquisition of chromatograms followed by additional holding of the GC column at 280°C with the detector off. The second holding was omitted for some examinations in this study; otherwise, this additional holding time was set as 20 min for routine analysis of CSF and plasma samples and 10 min for urine samples based on the testing results described in this study.

2.3. Sample Processing and GC/MS Detection for Analysis of Free F_4-NPs in CSF. The procedures for analysis of free F_4-NPs in human CSF have been described previously in our publication [13], which were modified from the method of Arneson and Roberts II [15], except that 250 pg of the internal standard $[^2H_4]$-15-F_{2t}-IsoP was used with 1.5 mL of CSF. For M-CSF and H-CSF, 1 mL and 0.6 mL of samples, respectively, were used and diluted to 1.5 mL with water before adding into 3 mL of water. The whole process and principle are similar to those for analysis of free F_2-IsoPs in body fluids. One difference was that the washing solvent for silica SPE columns in the second SPE was ethyl acetate [14] and ethyl acetate/heptane (75 : 25) [15] for F_2-IsoPs analysis and F_4-NPs, respectively. Another difference was that the scraping area of TLC plates for extraction of F_4-NPs was 1 cm below and 3 cm above the TLC standard [8, 13] instead of the 2 cm range performed for F_2-IsoPs analysis described in Section 2.2. Different from other methods described in the literature, we previously established the method by overlaying the chromatograms of CSF samples with that of products from *in vitro* oxidation of DHA at *m/z* 593.5 to identify the range of peaks for quantification of F_4-NFs [13, 16]. In this study, as for the F_2-IsoPs analysis, additional 2-min run at 300°C was included for the acquisition of chromatograms. The setting of additional 30-min holding at 280°C without turning the detector on was included in the method for our routine analysis, which was based on the observation from this study, was purposely omitted for some tests in this report. Amount of F_4-NPs in samples was quantified by multiplying amount of $[^2H_4]$-15-F_{2t}-IsoP added into samples with the ratio of the peak area of F_4-NPs at *m/z* 593.5, which was defined by the range of peaks of oxidized DHA, to that of $[^2H_4]$-15-F_{2t}-IsoP.

2.4. Statistical Analysis. The significance of the difference for the data between the 2 groups was evaluated using a two-tailed independent *t*-test with SPSS software (SPSS Inc.). The Levene test was also conducted to determine whether *p* values should be obtained under the assumption of equal variance or unequal variance. Statistical significance was considered when *p* values were smaller than 0.05.

3. Results

3.1. Results of F_2-IsoPs Analysis in CSF for Samples Processed for F_2-IsoPs versus Samples Processed for F_4-NPs. To address the question that whether simultaneous analysis of F_2-IsoPs and F_4-NPs for the same CSF sample processed for F_4-NPs analysis was appropriate, we first analyzed F_2-IsoPs and F_4-NPs simultaneously for L-CSF, M-CSF, and H-CSF samples processed for F_4-NPs analysis from three independent replicates of each sample. The results of F_2-IsoPs quantification were then compared with those for the corresponding three CSF samples processed for F_2-IsoPs analysis in a separate experiment. As shown by Table 1, we found that differences in mean values of F_2-IsoPs from samples processed in two different ways relative to the values from samples processed for F_2-IsoPs were small for L-CSF (5.2%) and M-CSF (1.7%) but were very high for H-CSF (66%). Despite of having the small difference for values of F_2-IsoPs quantified from two different methods (5.2%), there was statistical significance between values of F_2-IsoPs for L-CSF processed for F_2-IsoPs analysis versus for F_4-NPs analysis. It could be a systematic difference due to amount of CSF samples or internal standards used in two different operations. On the other hand, due to wider TLC scraping ranges for F_4-NPs analysis, the abundance of the peaks adjacent to the peak of F_2-IsoPs became much higher for all samples tested. The chromatograms at *m/z* 569.4 of H-CSF processed in two different ways are shown to illustrate such alteration (Figure 1). The results showed that the peak resolution for samples processed for F_4-NPs was worse due to significant overlap with the adjacent peaks and

the baseline was higher for the peak of F_2-IsoPs compared with that processed for F_2-IsoPs.

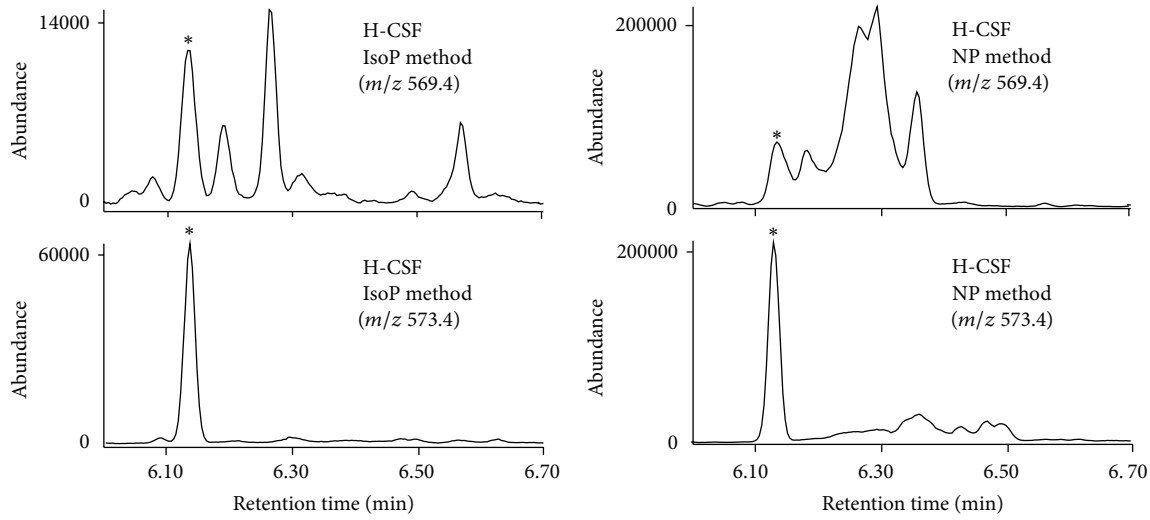
3.2. Evaluation on the Extent of Sample Retention on GC Columns without Additional Holding of the Column at the High Temperature after Analysis of F_4-NPs for the M-CSF Sample. For GC/MS settings, all methods that originated from the groups of Morrow and Roberts in the literature for F_2-IsoPs and F_4-NPs indicated the temperature ramp of 190 to 290 or 300°C without additional holding [11, 12, 14, 15]. However, during the analysis of F_4-NPs in human CSF for our previous study on aSAH [13], we noticed that some residual substances on the GC column after each analysis tended to be retained and eluted out together with the next samples, which could be detected at *m/z* 593.5, although the degree of this effect varied from sample to sample. To more systematically examine such effect at different masses, in this study, we first examined chromatograms at *m/z* 569.4, *m/z* 573.4, and *m/z* 593.5 for 4 injections of undecane, indicated as "Wash," following analysis of F_4-NPs in CSF. Although *m/z* 569.4 was not used for detection of F_4-NPs, it was important to monitor all masses used because analysis of F_2-IsoPs may be performed right after analysis of F_4-NPs during routine operation.

All the sources and processing of samples were the same for different figures for this test. Only M-CSF was used for this purpose. To simulate the method frequently applied, second holding of the GC column at 280°C with the detector off was not conducted although we included 2-min holding at 300°C at the end of temperature ramp to make the acquisition of chromatograms more complete. As shown by Figure 2, chromatograms in the range of 3.6 min to 8.6 min (the end of data acquisition) of the 4 undecane washes after the injection of M-CSF sample indicated that there were obvious unknown peaks retained in the column from the previous CSF sample at *m/z* 573.4 and *m/z* 593.5 but not *m/z* 569.4. Some peaks of washes were at the retention time for the quantification of target compounds, such as one peak of Wash-2 at *m/z* 573.4 and three peaks of Wash-1 at *m/z* 593.5, although the abundance of the peaks from these washes was much less than that from the CSF sample. Moreover, the peak *a* of Wash-1 at *m/z* 573.4 and the peak *b* of Wash-2 at *m/z* 593.5 were very large although they did not overlap with the retention time of F_4-NPs peaks.

Furthermore, we wondered how the chromatograms would look like and the degree of retention would be when 5 CSF samples were analyzed consecutively without additional holding. To examine this issue, multiple processed M-CSF samples dissolved in undecane were pooled together and multiple injections from the same pooled sample were performed. We found that the large peaks, peak *a* and peak *b*, at *m/z* 573.4 and *m/z* 593.5, respectively, showed up behind the target peaks during the injections of real samples, which were similar to the pattern found in Figure 2 (middle and right panels of Figure 3). Moreover, small but obvious peaks at the retention time for the quantification of target peaks also appeared in Wash-2 at *m/z* 573.4 and 4 Washes at *m/z* 593.5 (middle and right panels of Figure 3). On the contrary, there was no obvious obscure peak beyond background signals in the chromatograms at *m/z* 569.4 (left panel of Figure 3).

3.3. Evaluation on the Extent of Sample Retention on GC Columns without Additional Holding of the Column at the High Temperature after Analysis of F_2-IsoPs for the M-CSF, Plasma, and Urine Samples. The same pattern of examination described in Section 3.2 was also applied to M-CSF, plasma, and urine samples processed for F_2-IsoPs analysis. The chromatograms of 5 consecutive sample injections from the same pooled sample dissolved in undecane and 4 undecane washes were monitored. Although *m/z* 593.5 was not used for analysis of F_2-IsoPs, any retained compounds detectable at *m/z* 593.5 might interfere with the analysis of F_4-NPs of the next samples. The amount of M-CSF, plasma, and urine samples indicated in Section 2 was used and processed as for our routine analysis of F_2-IsoPs. The chromatograms at *m/z* 569.4 in a short range of retention time showed that we had proper analysis of F_2-IsoPs for those samples by our routine analysis (Figure 4(a)). The comparison on chromatograms in the range of 4.4 min to 8.6 min (the end of data acquisition) at *m/z* 593.5 for this test on CSF, plasma, and urine is shown by Figure 4(b), in which that for Wash-4 is not shown because of low abundance. Because the amount of the CSF sample processed for F_2-IsoPs analysis was less than that for F_4-NPs and the scarping area for TLC purification was also smaller, the abundance of peaks at *m/z* 593.5 was much smaller, but there was also a relatively large peak (peak *c*) at the end of chromatograms of Sample-3 to Sample-5 and Wash-1 to Wash-2 (left panel of Figure 4(b)) similar to those observed during F_4-NPs analysis (Figure 3). Interestingly, a group of the retained peaks (*d* peaks) in the range of retention time for F_4-NPs quantification or the large peak (peak *e*) at the end of chromatograms rises markedly for Sample-3 to Sample-5 and remained in high abundance for Wash-1 and Wash-2 (middle panel of Figure 4(b)). On the contrary, there was no apparent peak from retained substances in the chromatograms of the samples and there were only minimal signals in the chromatograms of Wash-1 and Wash-2 for urine samples (right panel of Figure 4(b)). Moreover, chromatograms of all samples and washes at *m/z* 569.4 had no obvious retention effect (see Supplemental Figure 1 of the Supplementary Material available online at http://dx.doi.org/10.1155/2013/810915), while only some peaks with low abundance showed up in Wash-1 and Wash-2 after injections of plasma samples at *m/z* 573.4 (Supplemental Figure 2). Because the amount of internal standard monitored at *m/z* 573.4 was very large, such low-abundant residual peaks should not affect the quantification of the internal standard.

3.4. Chromatograms Monitored during the Second Holding of the Column after Normal Acquisition of Data. Next, we monitored possible presence of peaks in the chromatograms at all three masses representing residual substances from the first injection of samples during additional holding of the column at 280°C for 30 min after normal acquisition of data. When the same processed CSF sample used in Figure 2 was analyzed for F_4-NPs, there was no peak with appreciable



(a) IsoP method (b) NP method

Figure 1: Comparison on the patterns of chromatograms at *m/z* 569.4 for the H-CSF sample processed for F_2-IsoPs (IsoP method) and for F_4-NPs (NP method). Because the amount of samples and internal standard used were different for different methods, the abundance of peaks for the internal standard at *m/z* 573.4 was also shown. The peaks of F_2-IsoPs and $[^2H_4]$-15-F_{2t}-IsoP at *m/z* 569.4 and *m/z* 573.4, respectively, are indicated by the asterisks.

amount of abundance at *m/z* 569.4 during this second holding time (upper panel of Figure 5(a)), but significant amount of residual substances could be detected at *m/z* 573.4 and 593.5 (middle and lower panels of Figure 5(a)). Moreover, based on the patterns of the surrounding peaks, peak *a* and peak *b* indicated in Figures 2 or 3 might be present during this second holding and then moved to the retention time in the range of data acquisition during next runs.

The chromatograms during the second holding were also monitored for the same processed CSF, plasma, and urine samples used in Figure 4. The results showed that many peaks in substantial amount could be detected for plasma samples, whereas small but obvious peaks from the CSF and urine samples were also detectable during the additional 30-min holding at 280°C (Figure 5(b)). Peaks that were likely to be peak *c*, *d* peaks, and peak *e* were also found on the chromatograms. Moreover, there was no obvious signal above background signals at *m/z* 569.4 for CSF, plasma, and urine samples (Supplemental Figure 3(a)). The abundance of the retained peaks in the chromatograms at *m/z* 573.4 was also very low (Supplemental Figure 3(b)).

3.5. Demonstration on the Elimination of Retention Effect by Additional Holding of GC Columns at a High Temperature at the End of Each Sample Analysis. To avoid potential interferences of the retention effect on the subsequent analysis of F_4-NPs in CSF at *m/z* 593.5, we previously needed to conduct multiple injections of undecane to monitor the conditions of GC columns, which was a troublesome process. To eliminate this problem during sample analysis in a more efficient way, we have incorporated additional holding of the column at 280°C, the highest temperature tolerated by the column recommended by the manufacturer, into the method for each injection without the detector on, which could reduce contamination of the ion source. The holding time was determined by the degree of improvement on removing retained peaks, which was confirmed by the absence of obvious peaks in the subsequent washes and disappearance of retained peaks during the second holding as what monitored in Figure 5 and in this section.

To demonstrate the effectiveness of such modification on the method setting, chromatograms for the F_4-NPs quantification of the 5 consecutive CSF samples without and with the additional 30-min holding were compared (Figure 6). The left panel of Figure 6 is the same as the right panel of Figure 3 except showing a narrower range of retention time, which focuses on peaks for F_4-NPs quantification. The results indicated that the patterns of F_4-NPs peaks were gradually altered and the baseline of the peaks gradually elevated from CSF-1 through CSF-5 when there was no additional holding after acquisition of chromatograms (left panel of Figure 6). However, such interference was not found when the additional holding was conducted (right panel of Figure 6). Moreover, the large peak *b* at the end of chromatograms (right panel of Figure 3) and other small interfering peaks in the range of the retention time (left panel of Figure 6) for F_4-NPs quantification in the chromatograms at *m/z* 593.5 of the subsequent 4 undecane washes could be observed after the CSF analysis when additional holding was not performed. That disappeared after additional 30-min holding was included in the method (right panel of Figure 6 and right panel of Supplemental Figure 4). Although there were some visible peaks in the chromatogram of Wash-2 even with additional holding, the abundance was too low to affect the quantification. On the other hand, the peak *a* and other small

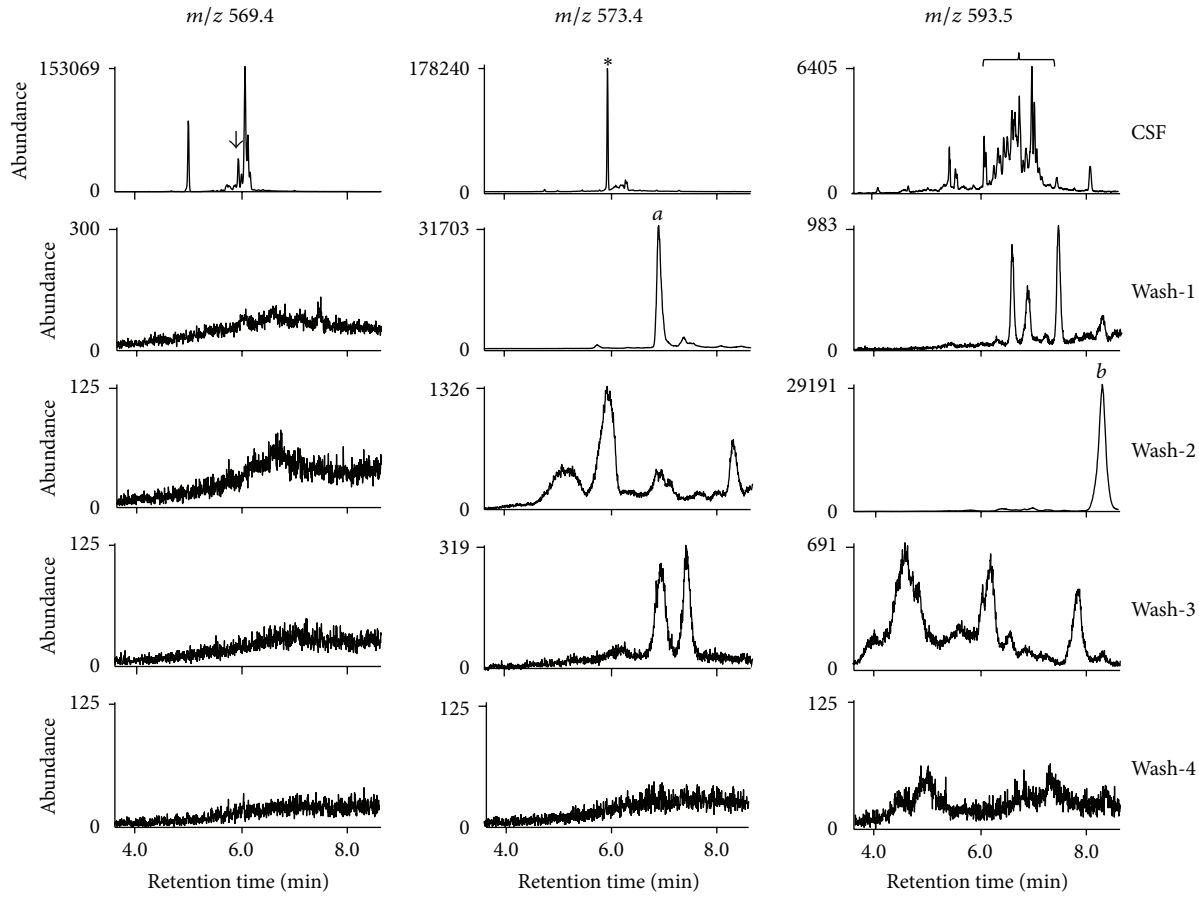


Figure 2: Chromatograms at *m/z* 569.4, *m/z* 573.4, and *m/z* 593.5 for the M-CSF sample processed for F_4-NPs analysis and the 4 subsequent injections of undecane alone (Wash-1 to Wash-4). The chromatograms in the range of 3.6 min to 8.6 min (the end of data acquisition) were compared. The peak of F_2-IsoPs is indicated by the arrow. The peak of $[^2H_4]$-15-F_{2t}-IsoP is indicated by the asterisk. Peak *a* and peak *b* indicated in the text were peaks of the retained substances from the sample with very high abundance. The range of retention time for quantification of F_4-NPs peaks at *m/z* 593.5, which is defined by that for the peaks of oxidized DHA, is indicated by the brace.

Table 1: Results of quantification of F_2-IsoPs and F_4-NPs in three test CSF samples that have been processed for F_2-IsoPs (IsoP method) or F_4-NPs (NP method) analysis.

	NP method		IsoP method	% of difference for F_2-IsoPs[c]
	F_4-NPs (pg/mL)	F_2-IsoPs (pg/mL)	F_2-IsoPs (pg/mL)	
L-CSF	57.7 ± 5.1 (8.8%)	19.5 ± 0.1 (0.5%)	20.6 ± 0.4 (1.7%)[a]	5.2%
M-CSF	109.0 ± 9.9 (9.1%)	56.9 ± 1.7 (3.0%)	55.9 ± 0.9 (1.7%)	1.7%
H-CSF	1023.4 ± 116.7 (11.4%)	141.0 ± 4.0 (2.8%)	85.0 ± 0.8 (0.9%)[b]	66.0%

There were three replicates for each CSF sample. Nine CSF samples were processed and analyzed together. Data were presented as mean ± standard deviation (coefficient of variation). Coefficient of variation was the ratio of standard deviation to mean presented as percentage and was used to represent within-run imprecision. [a]$p < 0.05$ ($p = 0.007$ under assumption of equal variance versus values of F_2-IsoPs for L-CSF processed by the NP method). [b]$p < 0.05$ ($p = 0.001$ under assumption of unequal variance versus values of F_2-IsoPs for H-CSF processed by the NP method). [c]The difference between the mean values of two methods divided by the mean values from IsoP method is calculated and represented as % of difference.

peaks during runs of CSF samples or washes at *m/z* 573.4 (middle panel of Figure 3) also disappeared (left panel of Supplemental Figure 4).

The quantification results of CSF-1 to CSF-5 in Figure 6 are listed in Table 2. The trend of increase in the latter injections of samples without additional holding was clear. The quantification results were significantly greater and the within-run imprecision, presented as coefficient of variation, was higher when samples were analyzed without additional holding than that with additional holding (Table 2). In contrast, the method without the additional holding did not result in significantly higher values of F_2-IsoPs in CSF when the same test was performed on the CSF sample processed for F_2-IsoPs analysis (Table 3).

Furthermore, the comparison on the chromatograms in the absence and presence of second holding of the column

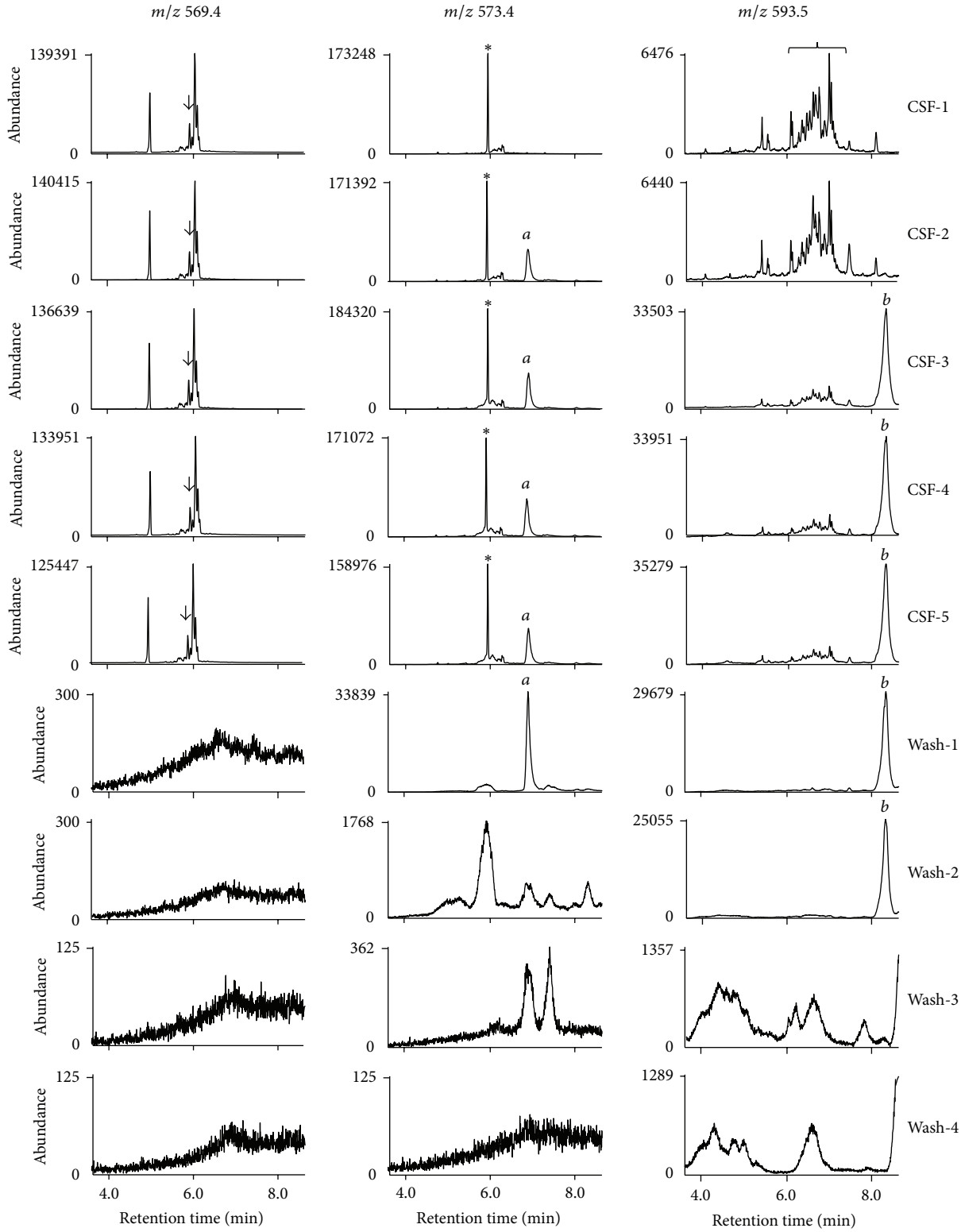


FIGURE 3: Chromatograms at *m/z* 569.4, *m/z* 573.4, and *m/z* 593.5 for the 5 consecutive injections of the same pooled M-CSF sample processed for F_4-NPs analysis (CSF-1 to CSF-5) and the 4 subsequent injections of undecane alone (Wash-1 to Wash-4). The chromatograms in the range of 3.6 min to 8.6 min (the end of data acquisition) were compared. The peaks of F_2-IsoPs at *m/z* 569.4 are indicated by the arrows. The peaks of $[^2H_4]$-15-F_{2t}-IsoP at *m/z* 573.4 are indicated by the asterisks. The range of retention time for quantification of F_4-NPs peaks at *m/z* 593.5, which is defined by that for the peaks of oxidized DHA, is indicated by the brace.

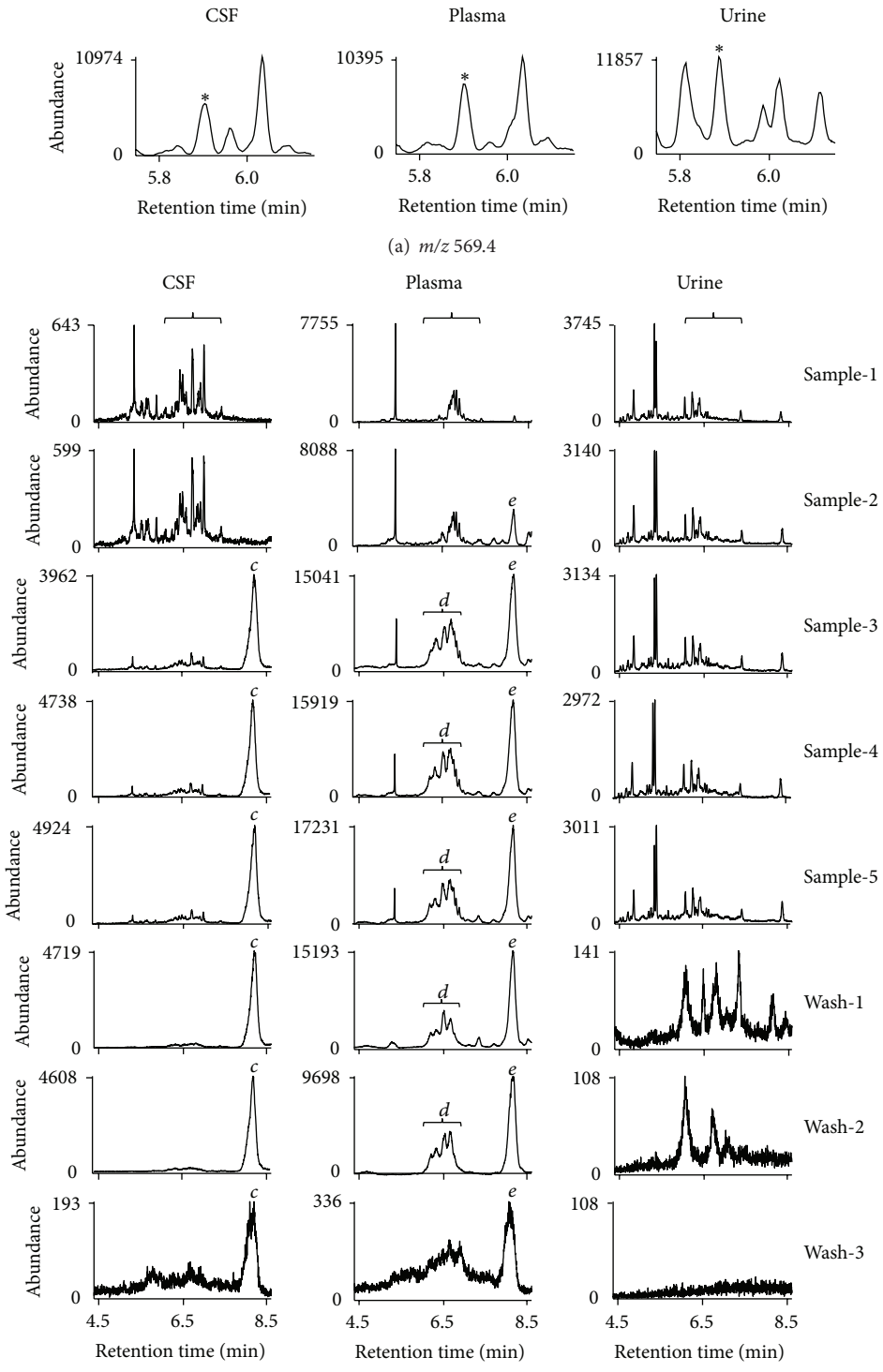
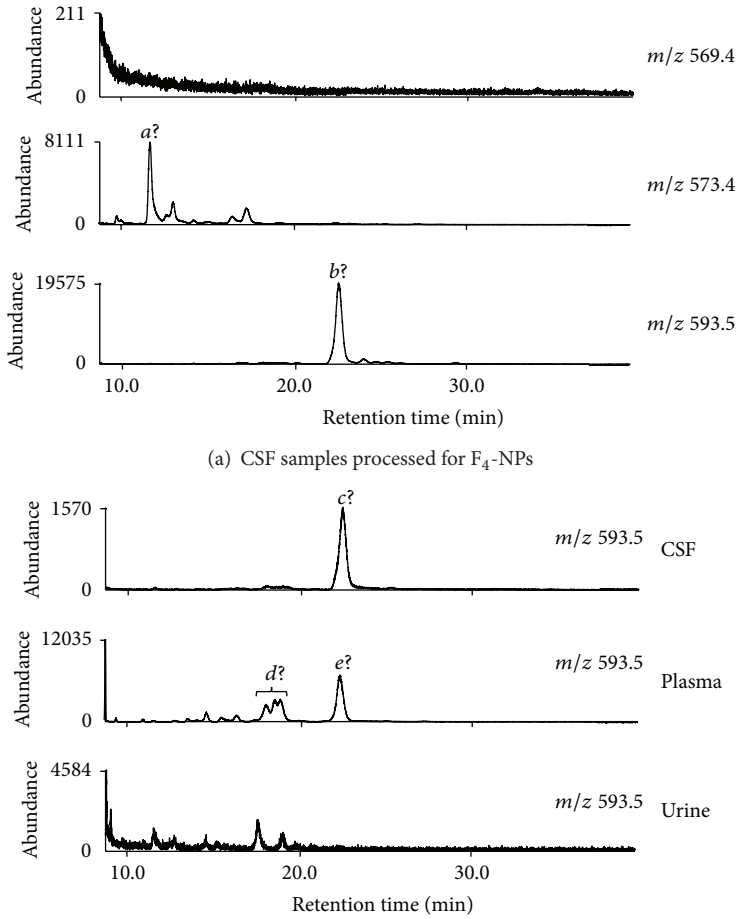


FIGURE 4: (a) Chromatograms at *m/z* 569.4 in a narrower range of retention time for the same pooled M-CSF sample, the plasma sample, and the urine sample processed for F_2-IsoPs analysis. The peaks of F_2-IsoPs are indicated by asterisks. The concentrations of F_2-IsoPs quantified by the routine operation described in Section 2 were 54.9 pg/mL, 13.5 pg/mL, and 851 pg/mL for the CSF sample, the plasma sample, and the urine sample, respectively. (b) Chromatograms for the 5 consecutive injections of the same M-CSF sample (left panel), the same plasma sample (middle panel), and the same urine sample (right panel) processed for F_2-IsoPs analysis (Sample-1 to Sample-5) and the 3 subsequent injections of undecane alone (Wash-1 to Wash-3) at *m/z* 593.5. The chromatograms in the range of 4.4 min to 8.6 min (the end of data acquisition) were compared. The range of retention time corresponding to that for F_4-NPs quantification is indicated by the braces although these samples were not used for F_4-NPs quantification.



(a) CSF samples processed for F_4-NPs

(b) Samples processed for F_2-IsoPs

Figure 5: The chromatograms monitored for the additional 30-min holding (8.6 min to 38.6 min) of the column at 280°C after normal acquisition of data. (a) The chromatograms at *m/z* 569.4, *m/z* 573.4, and *m/z* 593.5 for the same M-CSF sample described in Figure 2. (b) The chromatograms at *m/z* 593.5 for the same CSF, plasma, and urine samples described in Figure 4.

Table 2: Results of quantification of F_4-NPs from 5 consecutive injections of the same pooled sample.

	F_4-NPs levels (pg/mL)	
	No additional holding	30-min holding
CSF-1	119.9	113.8
CSF-2	130.2	114.0
CSF-3	144.4	117.7
CSF-4	145.5	119.3
CSF-5	147.5	121.7
Mean ± SD	137.5 ± 12.0	117.3 ± 3.4*
(CV%)	(8.7%)	(2.9%)

The sample number was designated according to the sequence of injection. The quantification data for samples analyzed without additional holding corresponds to chromatograms shown by the left panel of Figure 6, whereas that with 30-min holding corresponds to chromatograms shown by the right panel of Figure 6. CV is coefficient of variation that is the ratio of standard deviation (SD) to mean. * $p < 0.05$ ($p = 0.017$ under assumption of equal variance) between two sets of data obtained by two different methods.

Table 3: Results of quantification of F_2-IsoPs from 5 consecutive injections of the same pooled sample.

	F_2-IsoPs levels (pg/mL)	
	No additional holding	30-min holding
CSF-1	56.1	54.8
CSF-2	55.5	55.3
CSF-3	54.8	55.2
CSF-4	54.1	56.4
CSF-5	53.8	55.5
Mean ± SD	54.9 ± 1.0	55.4 ± 0.6
(CV%)	(1.8%)	(1.1%)

The sample number was designated according to the sequence of injection. There was no statistical significance between two sets of data obtained by two different methods ($p = 0.281$ under assumption of equal variance).

for the same samples described for Figure 4, in which samples were processed for F_2-IsoPs analysis, was conducted.

Because chromatograms of urine samples did not exhibit any appreciable amount of peaks from retained substances that may further affect subsequences analysis of next samples at all three masses detected (Figure 4, Supplemental Figures 1,

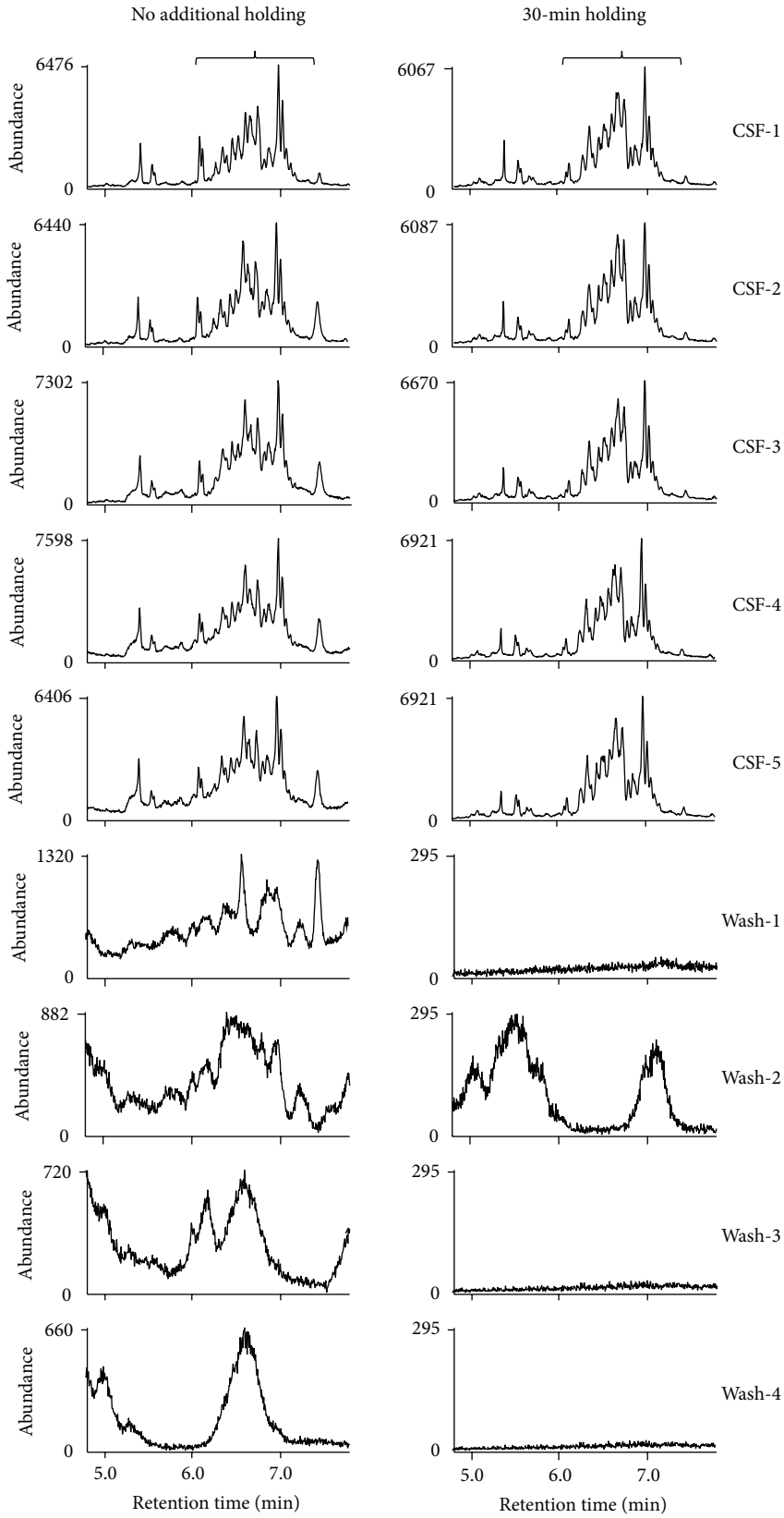
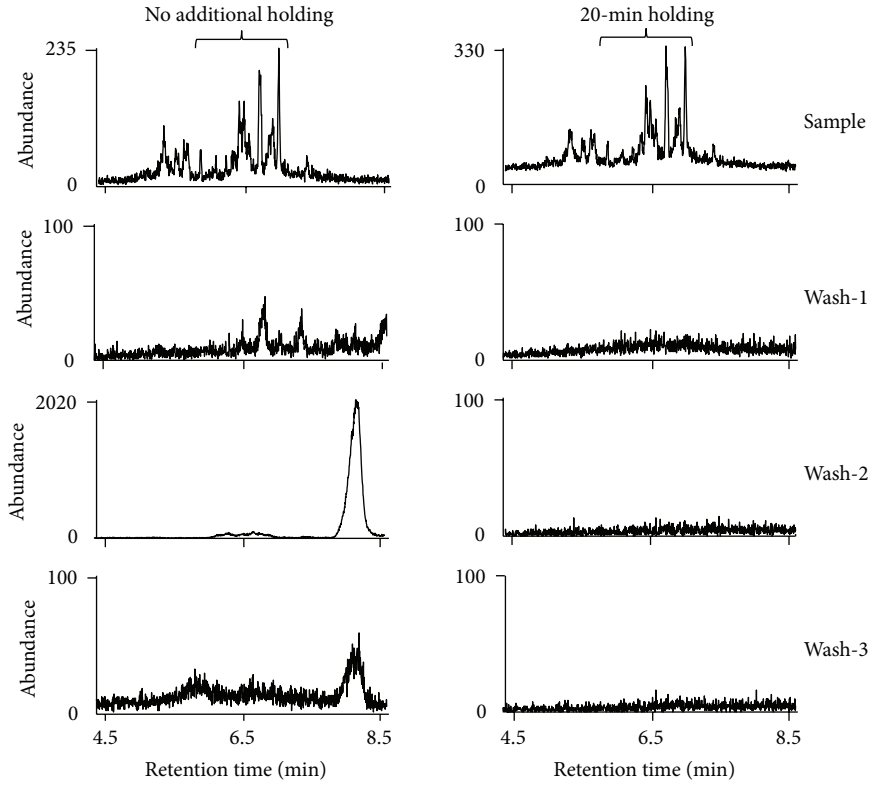
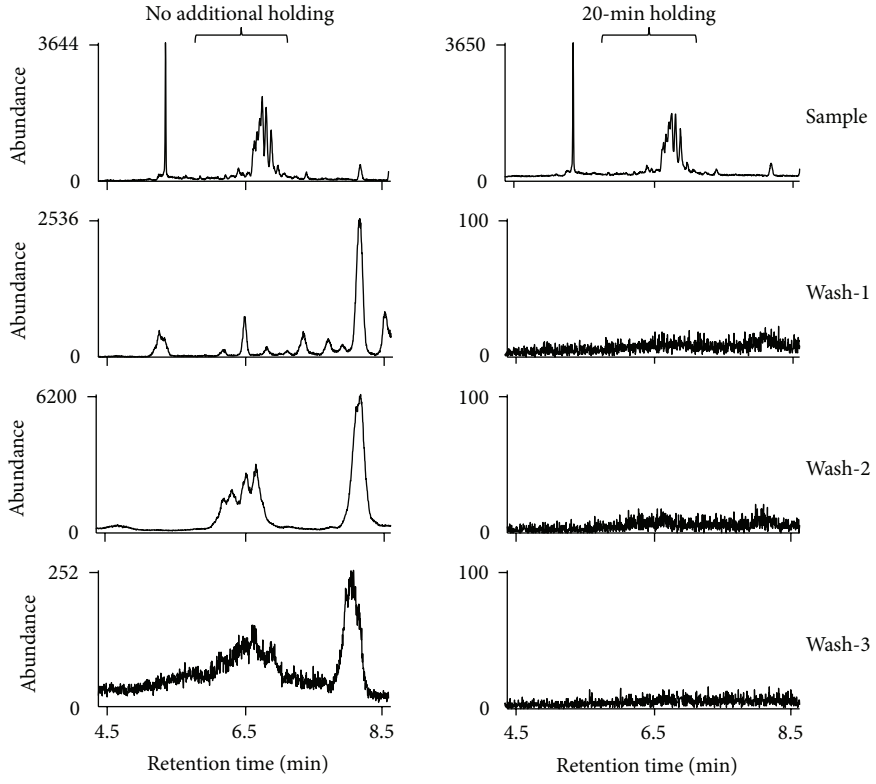


Figure 6: Comparison on the chromatograms for the 5 consecutive injections of the same pooled M-CSF sample processed for F_4-NPs analysis (CSF-1 to CSF-5) at *m/z* 593.5 without (left panel) and with (right panel) additional 30-min holding of the column at 280°C at the end of data acquisition. For better viewing of the peaks of F_4-NPs and the adjacent peaks, the chromatograms in the range of 4.8 min to 7.8 min were compared. The peaks for F_4-NPs quantification are indicated by the braces.



(a) CSF



(b) Plasma

Figure 7: Comparison on the chromatograms at m/z 593.5 for one CSF sample (a) or one plasma sample (b) processed for F_2-IsoPs analysis that has been used in Figure 4 without (left panel) and with (right panel) additional 30-min holding of the column at 280°C at the end of data acquisition. The range of retention time corresponding to that for F_4-NPs quantification is indicated by the braces although these samples were not used for F_4-NPs quantification.

2), this test was not conducted for urine samples. The results indicated that peaks from retained substances at *m/z* 593.5 in the chromatograms without the holding disappeared when the second holding was added in the method for both the CSF sample (Figure 7(a)) and the plasma sample (Figure 7(b)).

4. Discussion

Detection of F_2-IsoPs has been widely applied in a large number of clinical studies, showing great utilities of this marker [16, 25]. Although quite a few of studies have detected F_2-IsoPs in human CSF, such as our study on aSAH [17] and several publications on Alzheimer's disease (AD) [19, 21, 26], so far only three groups have measured F_4-NPs in human CSF, which included the original report of Roberts et al. on AD [8], our studies on aSAH [13] and traumatic brain injury (TBI) [27], and the studies of Corcoran et al. on aSAH and TBI [18]. However, variations in analytical protocols and further modifications for the GC/NICI-MS analysis of these compounds without description of methods in detail or the demonstration on chromatographic data with acceptable resolution of peaks may lead to questionable results.

In this study, we demonstrated that simultaneous analysis of F_2-IsoPs and F_4-NPs for the human CSF samples processed for F_4-NPs analysis caused much more overlap of the peak for F_2-IsoPs and the adjacent peaks at *m/z* 569.4 compared with the sample that was processed for F_2-IsoPs analysis. Consequently, the quantification results of F_2-IsoPs became inaccurate and unreliable if CSF samples had very high levels of F_2-IsoPs and F_4-NPs. This indicates that proper TLC purification is critical to avoid the interferences on quantification of F_2-IsoPs from substances with retention time that overlapped with the retention time of F_2-IsoPs, such as other related lipid peroxidation products, PGs, and their metabolites. These compounds may not be all removed by SPE. In the literature, few studies simply quantified F_2-IsoPs and F_4-NPs simultaneously for brain tissues processed by the same TLC purification when using the methods of Milatovic et al. [28], Milne et al. [29], or Zhang et al. [30]. This practice may be avoided because brain tissues contain much higher amount of these compounds than CSF samples, but it might be followed for CSF samples because the availability of CSF samples is usually very limited. Great care therefore should be taken. Furthermore, although we did not perform methods described by Nourooz-Zadeh et al. [31] or Corcoran et al. [18] that analyzed F_2-IsoPs and F_4-NPs simultaneously for human brain tissue [31] or human CSF [18] using different methods without the step of TLC purification, the same concern raised by us should also be applied to these two platforms.

The results from this study also revealed for the first time that significant amount of unknown compounds with low volatility were retained in the GC column from human CSF and plasma sample processed for either F_2-IsoPs or F_4-NPs analysis by using the regular GC elution method that originated from the methods established by the group of Roberts or Morrow [8, 11, 12, 14, 15]. Urine samples did not cause much retention effect based on the observation from the current examination possibly because we routinely started with a much less amount of urine than what was indicated in the literature. However, we cannot exclude the possibility that this phenomenon could also be enhanced by greater loading amount of samples into the GC columns even with urine samples. Although the residual substances at *m/z* 573.4 theoretically would not affect quantification of the internal standard because of the presence of relatively large amount of the internal standard, it was important to note that unknown substances at *m/z* 573.4 were also retained in the GC column substantially from CSF samples during F_4-NPs analysis. Furthermore, many of major peaks detected during the additional holding time shown in Figure 5 appeared to show up as those peaks of the undecane washes shown in Figures 2 and 3 at the end of chromatograms or in the range of retention time for F_4-NPs quantification. These peaks from retained substances therefore tended to move to the earlier retention time in the subsequent injections.

The quantification of F_4-NPs at *m/z* 593.5 for human CSF was more prone to be interfered by the retention effect from residual substances in the previous samples. When a proper method was not applied to avoid such effect, continuous injections of samples could enhance this problem by the accumulation of those substances in the GC column and enhancement of background signals. As demonstrated by our examinations, although the abundance of the interfering peaks eluted out by the undecane washes following one injection of the sample from human CSF was less than that of F_4-NPs peaks at *m/z* 593.5 and was not likely to have a major effect on quantification of F_4-NPs for the next sample (Figure 2), consecutive 5 injections of the same sample for F_4-NPs quantification in the CSF sample enhanced the baseline and caused falsely higher values of F_4-NPs (Figure 6 and Table 2), which could be prevented by the additional holding time at 280°C after normal acquisition of chromatographic data. In other words, the interfering effect from the retained substances could not be predicted by simply observing the chromatograms of undecane injections after a single sample injection. Although we did not test the potential interference on F_4-NPs quantification in CSF following 5 consecutive analyses of plasma samples processed for F_2-IsoPs analysis, we suspected that a group of unknown peaks (*d* peaks) in high abundance at *m/z* 593.5 from the retention of the previous plasma sample with the retention time in the range of that for F_4-NPs peaks (Figure 4), which was much greater for those peaks from retention of the previous CSF sample processed for F_4-NPs analysis in the same range (Figure 2), were highly likely to interfere with subsequent quantification of F_4-NPs in CSF as well. Because this retention effect did not interfere with F_2-IsoPs analysis at *m/z* 569.4 for CSF, plasma, or urine samples, it would not have been noticed by most researchers who only measured F_2-IsoPs levels in human body fluids. However, this problem would be a major problem when both F_2-IsoPs analysis and F_4-NPs analysis were carried out interchangeably on the same GC/MS equipment without performing the additional holding at 280°C.

The time needed for the second holding of the column at the high temperature varied for different analysis and should be evaluated by observing the traces of chromatograms during the long holding after the regular acquisition of

data, by comparing signals in undecane washes after sample injections with and without additional holding, and by comparing results of quantification with and without holding after multiple sample injections. The holding time therefore should also be different for different labs with different GC/MS settings or different format in sample processing, but our current study has demonstrated the rationale and necessity of such evaluation.

Taken together, this work has indicated the importance of proper TLC purification for obtaining reliable chromatograms for F_2-IsoPs quantification in CSF and revealed the necessity of adding additional holding of the column at 280°C for a period of time following data acquisition during F_2-IsoPs and F_4-NPs analysis to avoid potential interferences on subsequent F_4-NPs quantification in CSF. Although GC/NICI-MS is not a commonly used technique and is hard to manage, the GC/NICI-MS method remains to be the most sensitive and robust method for detecting F_2-IsoPs or F_4-NPs in body fluids. Many seemingly minor variations in analytical methods that may have a major impact on the reliability of results should therefore be carefully evaluated.

Acknowledgment

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Research Article

Coefficient of Energy Balance: Effective Tool for Early Differential Diagnosis of CNS Diseases

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Urgent examination of cerebrospinal fluid (CSF) provides immediate important information about the character of central nervous system (CNS) impairment. Although this examination includes energy parameters such as glucose and lactate concentrations, it does not commonly use Coefficient of Energy Balance (CEB). In this study, we focused on CEB because it enables more exact assessment of actual energy state in the CSF compartment than glucose and lactate alone. CEB informs about the actual functioning condition of present cells, and it does not require any other analysis or costs. Using Kruskal-Wallis ANOVA, we examined a large CSF sample ($n = 8183$) and we compared CEB values among groups with different cytological syndromes. We found a statistically significant difference of CEB between the group with granulocyte pleocytosis and the control group. These results indicate a high degree of anaerobic metabolism caused by the oxidative burst of neutrophils. Similarly, we found a statistically significant difference of CEB between the control group and groups with tumorous oligocytosis plus pleocytosis and monocyte pleocytosis. This difference can be attributed to the oxidative burst of macrophages. Our findings suggest that CEB combined with CSF cytology has a great importance for diagnosis, differential diagnosis, and early therapy of CNS diseases.

1. Introduction

Unlike blood analysis, an examination of CSF allows the following of immune and metabolic processes in CNS without disturbing other organ systems as the CSF compartment is considerably autonomous. The urgent (fundamental) examination of CSF should be accessible everywhere 24 hours a day to provide important information in the decision-making diagnostic and therapeutic algorithms [1, 2]. The urgent CSF examination includes an analysis of energy balance, determination of blood-CSF barrier permeability, spectrophotometric analysis, total cell number and cytological investigation, and possibly a measurement of "quick" destruction markers. The examination should be complemented by an analysis of CRP (C-reactive protein) concentration in blood to give a picture of the system inflammation state [1–7].

Biochemical parameters of energy metabolism in the CSF compartment typically comprise a concentration of the energetic substrate, that is, glucose in CSF and blood, glucose quotient Q_{glu} ($Q_{glu} = \lfloor \text{glucose}_{CSF} \rfloor / [\text{glucose}_{blood}]$), and concentration of the product of anaerobic metabolism, that is, lactate in CSF [2, 4]. Glucose is actively transported across blood-CSF barrier; therefore, its CSF concentration is dependent on concentration in serum [8]. An importance of lactate for distinction of bacterial meningitis from aseptic/viral meningitis is well known [9–11], as well as a prognostic usefulness of lactate in many CNS diseases [12]. Because the concentration of lactate is substantially influenced by an amount of glucose that comes in the metabolic processes, it is not the absolute indicator of an anaerobic metabolism extent. The Coefficient of Energy Balance (CEB) links consumption of glucose, production of lactate, and production of ATP to

one mutual mathematical formula [6]; therefore, the inverse relationship of lactate and glucose concentrations multiplies the sensitivity of CEB. CEB accurately and sensitively evaluates the actual anaerobic metabolism extent in a territory adjacent to the CSF compartment [5–7, 13–16]. Unlike glucose or lactate, CEB can stratify a metabolic turnover to more layers corresponding with different diseases. CEB has been described in local studies [5–7, 13–16], but it has not been widely used and well known yet. According to recent studies the Coefficient of Energy Balance correlates better ($\rho = -0.770$) with CSF cellularity than lactate or glucose concentrations in purulent meningitis [15], and CEB value below 10.00 has 100.0% sensitivity and 92.1% specificity for diagnosis of purulent meningitis [13]. An evaluation of metabolic turnover in the CSF compartment by CEB in combination with number of cells and cytology enables the determination of the features and intensity of inflammation. Thus CEB has a crucial importance for differentiating the cause of CNS disability [15].

To calculate CEB, it is important to understand the processes of energetic metabolism of glucose. Glucose as an energy substrate is changed by glycolysis to pyruvate in CNS. If a sufficient amount of oxygen is dissolved in CSF compartment, the glycolysis takes place primarily in an *aerobic* way. In aerobic glycolysis pyruvate is further metabolized by the Krebs cycle and the respiratory chain to CO_2 and H_2O producing 38 molecules of adenosine triphosphate (ATP) from 1 molecule of glucose. If the amount of oxygen in the CSF compartment is low, the extent of *anaerobic* glycolysis increases. In anaerobic glycolysis pyruvate is converted to lactate producing only 2 ATP molecules from 1 molecule of glucose [4, 13]. Obviously, this energy state is disadvantageous, especially for CNS tissue. The Coefficient of Energy Balance [6] is a mathematical expression of the described biochemical processes:

$$\mathrm{CEB} = 38 - 18 * \frac{\lfloor \mathrm{lactate}_{\mathrm{CSF}} \rfloor}{[\mathrm{glucose}_{\mathrm{CSF}}]}. \quad (1)$$

CEB determines the average number of ATP molecules produced from 1 molecule of glucose in current circumstances in the CSF compartment and thus expresses the proportion of anaerobic glucose metabolism [5–7, 13–16].

Under the physiological conditions in CSF, the considerable amount of oxygen is dissolved there and a natural consumption of glucose is continuously supplied from blood. As a result, concentration of glucose is quite high (Q_{glu} more than 0.55), concentration of lactate is low, and value of CEB within aerobic metabolism is in the reference range, that is, 28.00–38.00 [3, 5, 13, 14].

Serous inflammations can be either of infectious (e.g., viruses, *Treponema pallidum,* and *Borrelia* sp.) or noninfectious etiology (autoimmune diseases, paraneoplastic impairment, after cerebral bleeding, during reparations, and regeneration of damaged CNS tissue). When serous CNS inflammation occurs, a permeability of blood-CSF barrier increases, humoral immune components and cells penetrate CSF, and activated immune system raises energy demands.

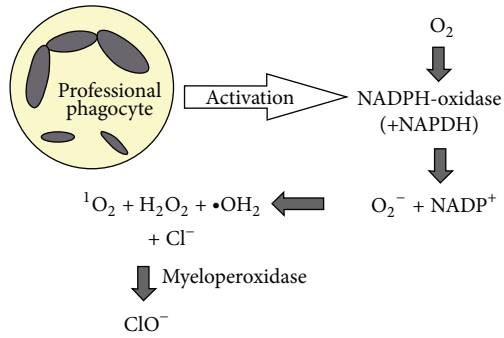

Figure 1: The oxidative burst of professional phagocytes (neutrophils, macrophages): the oxidative burst is induced by activation of NADPH-oxidase producing reactive oxygen species, by which phagocytes destroy devoured pathogens. Then myeloperoxidase catalyzes oxidation of chlorides to high bactericidal hypochlorites with participation of hydrogen peroxide [5, 22–28].

Limited tissue reserves of glucose [11] may cause an advanced extent of anaerobic metabolism in cerebrospinal fluid. In consequence, concentration of glucose slightly decreases (Q_{glu} under 0.55), concentration of lactate slightly increases, and value of CEB ranges from 10.00 to 28.00 [5–7, 13–16].

Diametrical difference in energy state is caused by the so-called "oxidative (respiratory) burst of professional phagocytes" when purulent CNS inflammation or another kind of pathologies occurs. See Figure 1.

During this process a consumption of oxygen is substantial, so an intensive anaerobic metabolism of glucose is developed, and as a result, concentration of glucose in CSF significantly decreases (or Q_{glu}), concentration of lactate significantly increases, and value of CEB distinctly falls, ranging between 10.00 and highly negative values [5–7, 13–16]. Professional phagocytes may be neutrophils in case of purulent inflammation (of infectious etiology—usually extracellular bacteria—or noninfectious etiology—after a reperfusion of ischemic parts incurred as a consequence of vasospasms after subarachnoid hemorrhage) [5–7, 13–16, 29–33]. Professional phagocytes may be also macrophages, in case of an elimination of intracellular bacteria and yeasts or molds (*Mycobacterium tuberculosis, Listeria monocytogenes,* rarely *Borrelia* sp., *Treponema pallidum, Cryptococcus neoformans,* and *Candida albicans*) or at the malignant meningeal infiltration, MMI [5–7, 13–16]. This means that CEB values from 10.00 to highly negative values are usually accompanied by granulocyte pleocytosis (the oxidative burst of neutrophils) and minor tumorous pleocytosis or oligocytosis, monocyte pleocytosis or oligocytosis, or rarely lymphocyte pleocytosis or oligocytosis (the oxidative burst of macrophages). An adherence of macrophages to tissue—an epicenter of pathological process—may sometimes cause a minority representation of monocyte-macrophage cells in CSF, while lymphocytic cells predominate in cerebrospinal fluid. A high scope of anaerobic metabolism in the cerebrospinal fluid compartment should evoke a suspicion of an infection caused by the above-mentioned microbes or CNS cancer.

In this study we focused on CEB because it has a greater information potential than glucose or lactate itself. CEB enables one to accurately express the range of anaerobic glucose metabolism, and it can stratify a metabolic turnover. It is commonly and early available within a fundamental (urgent) CSF examination, without the necessity of any other analysis or costs. We examined a large CSF sample from patients with variant neurological symptoms and compared CEB values among eight cytological groups to match the findings with the above-mentioned studies. We hypothesized that values of CEB would be significantly different in groups accompanied by the oxidative burst of professional phagocytes. This means that significantly different CEB would be in the group with granulocyte pleocytosis or tumorous pleocytosis/oligocytosis, possibly monocyte pleocytosis. Our results confirm this hypothesis. They suggest that CEB, commonly assessed with CSF cytology, can differentiate between variant pathologies in CNS specify and accelerate differential diagnosis and early targeted therapy of CNS diseases.

2. Materials and Methods

We analyzed 8183 CSF samples in the Laboratory for CSF and Neuroimmunology (the Expert laboratory in CSF analysis in Czech Republic, http://www.likvor.cz). In all cases, physicians requested this examination because of a suspicion of some neurological disease (there were signs of meningeal irritation, focal neurological findings, febrile disorders, disturbances of consciousness, complications after head injury or neurosurgical operations, etc.). Firstly we examined a total cell number in the Fuchs-Rosenthal chambre, basic biochemistry, secondly we made a qualitative cytological examination, and thirdly we assumed levels of IgG, IgA, IgM, and their CSF/S quotients, and β2-m in CSF and serum and its CSF/S quotient. In the cytological examination we used standard (MGG = May-Grünwald + Giemsa-Romanovski) and special staining techniques in an effort to detect malignant elements (toluidine blue stain, Papanicolaou) and the presence of CNS tissue destruction (Oil Red 0) by detecting of lipophagic macrophages. Cytological samples were obtained from CSF using a gentle sedimentation technique. With immunoglobulins, their intrathecal oligoclonal synthesis, if existent, was determined numerically (Reiber).

According to the findings in cytology we divided the sample into 8 groups: control group (n = 235), granulocyte oligocytosis (GO, n = 64), granulocyte pleocytosis (GP, n = 766), monocyte oligocytosis (MO, n = 2699), monocyte pleocytosis (MP, n = 1457), lymphocyte oligocytosis (LO, n = 1200), lymphocyte pleocytosis (LP, n = 1610), and tumorous oligocytosis plus pleocytosis (TO + TP, n = 152). We included TO in the same group as TP because of small sample size and the same etiology. *Pleocytosis* is determined as the number of leukocytes in the Fuchs-Rosenthal chamber greater than 4/1 μL; *oligocytosis* as the number of leukocytes in the Fuchs-Rosenthal chamber in reference range (to 4/1 μL), but with a pathological cell composition—the attributive adjective according to the predominant cells (except TP/TO, where the adjective tumorous is in case of any presence of malignant cells in CSF).

Table 1: Reference ranges of selected parameters of basic CSF examination (determined at our laboratory) [3, 4, 17–21].

Parameter	Age	Range	Unit
Energy metabolism			
Q_{glu}		0.55–0.65	—
$Lactate_{CSF}$		0.7–2.1	mmol/L
Cytology			
Number of leukocytes		0–4	/1 μL
Cytological composition		Lymphocyte : monocyte = 7 : 3	
Permeability of blood-CSF barrier			
Total $protein_{CSF}$ (TP_{CSF})	0–2 weeks	450.0–1090.0	mg/L
	2–4 weeks	510.0–1010.0	
	1–3 months	240.0–650.0	
	3–6 months	230.0–370.0	
	6–12 months	170.0–350.0	
	1–10 years	160.0–310.0	
	11–18 years	160.0–400.0	
	19–40 years	240.0–490.0	
	41–50 years	270.0–600.0	
	51–60 years	290.0–670.0	
	61–70 years	260.0–790.0	
Albumin quotient (Q_{alb}) Q_{alb} = [$albumin_{CSF}$]/[$albumin_{blood}$]	0–2 weeks	5.6–23.2	$\times 10^{E-3}$
	2–4 weeks	7.6–16.4	
	1–3 months	2.3–10.6	
	3–6 months	2.0–4.8	
	6–12 months	1.4–4.5	
	1–10 years	1.0–4.5	
	11–18 years	1.0–5.0	
	19–30 years	1.7–5.7	
	31–40 years	1.8–6.2	
	41–50 years	2.0–7.2	
	51–60 years	2.1–8.9	
	61–70 years	2.2–9.9	
Inflammatory activity			
Beta-2-$microglobulin_{CSF}$		0.2–2.0	mg/L
Intrathecal synthesis-IgG (Reiber)		0	%
Intrathecal synthesis-IgA (Reiber)		0	%
Intrathecal synthesis-IgM (Reiber)		0	%

We defined the control group as a group with results of biochemical and cytological CSF analysis in the reference range, including beta-2-microglobulin concentration in CSF and zero intrathecal synthesis in classes IgG, IgA, and IgM according to Reiber's calculation—see decision limits in Table 1. Although these criteria may not be considered to be fully convincing, there is virtually no other way of further differentiation (we do not perform lumbar puncture

Table 2: CEB with defined cytological syndromes.

Cytology	N	CEB						
		Median	Minimum	Maximum	1st quartile	3rd quartile	2.5th percentile	97.5th percentile
Controls	235	31.31	27.45	34.30	30.36	32.05	28.46	33.41
GO	64	29.55	7.00	35.03	26.24	31.12	10.06	33.26
GP	766	4.68	−2 996.50	33.55	−40.99	20.81	−1 158.70	31.75
LO	1 200	31.12	−1 249.00	35.11	30.14	31.97	24.06	33.35
LP	1 610	29.77	−3 980.50	37.36	26.39	31.24	−3.29	33.26
MO	2 699	30.45	−457.69	37.95	28.70	31.68	11.44	33.42
MP	1 457	25.96	−1 360.00	36.50	16.82	29.56	−52.05	32.66
TO + TP	152	21.80	−835.00	33.40	−1.66	29.52	−558.70	32.49

Descriptive statistics of CEB in 8 groups according to cytology; GO: granulocyte oligocytosis, GP: granulocyte pleocytosis, LO: lymphocyte oligocytosis, LP: lymphocyte pleocytosis, MO: monocyte oligocytosis, MP: monocyte pleocytosis, TO + TP: tumorous oligocytosis + pleocytosis.

Table 3: Results of post hoc multiple comparisons.

Compared groups	Level of significance
GO-GP	$P < 0.01$
GO-MO	No difference
GO-MP	$P < 0.01$
GO-LO	$P < 0.01$
GO-LP	No difference
GO-TO + TP	$P < 0.01$
GO-controls	$P < 0.01$
GP-MO	$P < 0.001$
GP-MP	$P < 0.001$
GP-LO	$P < 0.001$
GP-LP	$P < 0.001$
GP-TO + TP	$P < 0.01$
GP-controls	$P < 0.001$
MO-MP	$P < 0.001$
MO-LO	$P < 0.01$
MO-LP	$P < 0.01$
MO-TO + TP	$P < 0.001$
MO-controls	$P < 0.01$
MP-LO	$P < 0.001$
MP-LP	$P < 0.001$
MP-TO + TP	No difference
MP-controls	$P < 0.001$
LO-LP	$P < 0.001$
LO-TO + TP	$P < 0.001$
LO-controls	No difference
LP-TO + TP	$P < 0.01$
LP-controls	$P < 0.001$
TO + TP-controls	$P < 0.001$

The comparison of groups with each other; GO: granulocyte oligocytosis, GP: granulocyte pleocytosis, LO: lymphocyte oligocytosis, LP: lymphocyte pleocytosis, MO: monocyte oligocytosis, MP: monocyte pleocytosis, TO + TP: tumorous oligocytosis + pleocytosis.

in healthy people). Samples with parameters not meeting the above criteria were automatically regarded as samples from patients with pathological CSF findings.

Concentrations of glucose in CSF and serum, total protein in CSF, and lactate in CSF were analyzed photometrically (Synchron Lx by Beckman; Viva by Siemens); concentration of beta-2-microglobulin$_{CSF}$ was analyzed by nephelometry (Array by Beckman, BN II by Siemens); Q_{glu} and CEB were calculated.

Descriptive methods, Kolmogorov-Smirnov test, box-and-whiskers plots, and Kruskal-Wallis ANOVA with pairwise posthoc multiple comparisons were used for statistical analysis.

3. Results and Discussion

Descriptive statistics of CEB with individual cytological syndromes in cerebrospinal fluid and results of testing of concordance among 8 groups are summarized in Tables 2 and 3.

Because the distribution of CEB in our data set is not normal and CEB values are often strongly negative, we performed the transformation to $CEB_{transf}(CEB_{transf} = \log(40 - CEB))$ for creation of box-and-whiskers plots—see Figures 2 and 3.

As can be seen from Tables 2 and 3 and Figure 2, we found a statistically significant difference of CEB between the group with granulocyte pleocytosis and the control group ($P < 0.001$), respectively, significantly decreased values of CEB, as well as in comparison with all other cytological groups, that is, LO, LP, MO, MP, TO + TP and GO ($P < 0.001$ or $P < 0.01$). Similarly, the group with TO + TP presents the statistically significant difference of CEB from controls ($P < 0.001$) and other cytological groups ($P < 0.001$ or $P < 0.01$), except for MP (between TO + TP and MP is no statistical difference). Consequently, our results support the above-mentioned theory of the oxidative burst of professional phagocytes and probability of simultaneous occurrence of anaerobic scope of CEB and certain cytological patterns.

Also the groups with MP and LP show a significant difference in comparison with controls ($P < 0.001$). But the same difference is between MP and GP, LP and GP ($P < 0.001$): this means that the values of CEB in GP are even significantly lower than in MP and LP (see Tables 2 and 3, Figures 2 and 4 again). MP can accompany cases of infection by intracellular bacteria, yeasts or molds, or malignant meningeal infiltration.

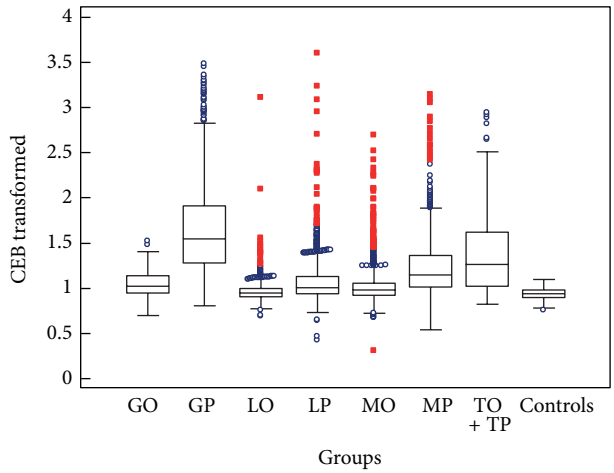

Figure 2: CEB with defined cytological syndromes in CSF (box-and-whiskers plots; GO: granulocyte oligocytosis, GP: granulocyte pleocytosis, LO: lymphocyte oligocytosis, LP: lymphocyte pleocytosis, MO: monocyte oligocytosis, MP: monocyte pleocytosis, TO + TP: tumorous oligocytosis + pleocytosis).

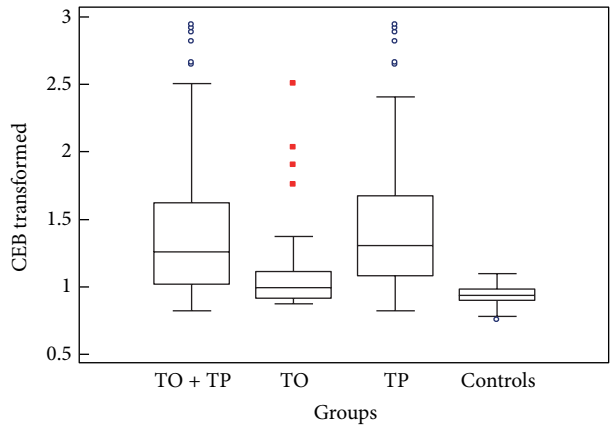

Figure 3: CEB with tumorous pleocytosis and tumorous oligocytosis (box-and-whiskers plots, CEB in the common TO + TP group versus separated TO and TP groups; TO: tumorous oligocytosis, TP: tumorous pleocytosis).

Thus a decline of CEB to an anaerobic ratio can be expected in these cases, but they represent only a small proportion of the large group of MP. CEB values ≤10.00 are in 16.3% (237/1457) of MP and 5.8% (93/1610) of LP (see Figure 4). Furthermore, inflammatory process with the oxidative burst of macrophages is conditioned by the activation of specific immune components; therefore, it can fully develop after a certain interval. In case of severe immunodeficiency, it may not happen at all [13, 34, 35].

The interquartile range of CEB in the LP group is from 26.39 to 31.24, in the LO group from 30.14 to 31.97; these ranges correspond to serous inflammatory processes in CNS, which LP and LO usually accompany. Although the oxidative burst of phagocytes in terms of infection by intracellular bacteria in LP, LO can be sometimes expected, these cases are even less frequent than in MP (see Figure 4). Characteristic cytological picture of serous neuroinfections is LP, but we can also find GP, MP, and MO according to the stage. In all these cases the CEB is slightly decreased. Although the group of lymphocyte oligocytosis is not by any means identical with the control group, we found no difference of CEB values between LO and the control group. Lymphocyte oligocytosis is a cytological picture of autoimmune processes (such as multiple sclerosis) or chronic, nonpurulent, CNS inflammations (e.g., spirochaetal, neuroborreliosis, neurosyphilis). In these affections the oxidative burst of professional phagocytes is outstanding, which is supported by our findings.

The variance of CEB in TP in comparison with other groups still raises so far as this group is divided into two subgroups TO and TP, despite the small number of measurements (small number of measurements in the group with TO and TP is caused by low prevalence of these severe disorders in population). It can be seen in Table 4 and Figure 3.

In contrast to these results, the group with GO shows less significant difference of CEB in comparison with controls ($P < 0.01$) and no difference between GO and MO, LP. It may be caused by the fact that finding of GO is not usually caused by purulent neuroinfection. This cytological pattern appears in the initial stages of nonpurulent inflammations and in early stage of cerebral ischemia [36].

It should also be remembered that granulocyte pleocytosis and oligocytosis may rarely occur after subarachnoid hemorrhage, where the pseudopurulent noninfectious inflammation is induced by chemotactic and stimulatory effects of cytokines, the complement component C5a, and adhesion molecules; that is, both cytology and CEB correspond to the oxidative burst [29–33].

When we divide all values of CEB into three subgroups according to the following cut offs [5–7, 13–16]: 10.00 (cut off for the oxidative burst) and 28.00 (lower reference limit), we can construct Figure 4.

As can be seen in Table 2, Figures 2 and 4, the huge extent of CEB values is evident in the GP group: there are strongly negative values but also only slightly reduced values and values in reference ranges. CEB values ≤10.00 are in 57.8% (443/766) of GP, and CEB values within 10.00–28.00 are in 31.4% of GP. A part of CEB values above 10.00 probably corresponds to the early stages of serous neuroinfections, where nonspecific immune cells (neutrophils) may preponderate within a short time. Another part of these values may be caused by the so-called *preventive neuroprotection* when a systemic infection by neurotropic pathogens (e.g., typically the sepsis by *Neisseria meningitis*) is present. An activation of brain capillaries endothelium occurs, and then the permeability of blood-brain barrier increases—an influx of humoral immune components and immunocompetent cells to CSF raises to prepare this initially immune-privileged space for prospective invasion of pathogen and fully fledged inflammation process [3, 5, 7, 16, 36]. In this case, patterns imitating neuroinfection can be seen (GP or LP, hyperproteinorrhachia with an important share of immunoglobulins, acute phase proteins, complement, etc.), but there are no oxidative burst phagocytes yet. Just CEB enables distinguishing whether

Table 4: CEB with tumorous pleocytosis and tumorous oligocytosis.

Cytology	N	CEB						
		Median	Minimum	Maximum	1st quartile	3rd quartile	2.5th percentile	97.5th percentile
TO	31	31.13	−280.40	32.50	27.28	31.72	−223.50	32.49
TP	121	19.72	−835.00	33.40	−6.73	27.94	−671.73	32.49
TO + TP	152	21.80	−835.00	33.40	−1.66	29.52	−558.70	32.49
Controls	235	31.31	27.45	34.30	30.36	32.05	28.46	33.41

Descriptive statistics of CEB in the common TO + TP group versus separated TO and TP groups; TO: tumorous oligocytosis, TP: tumorous pleocytosis.

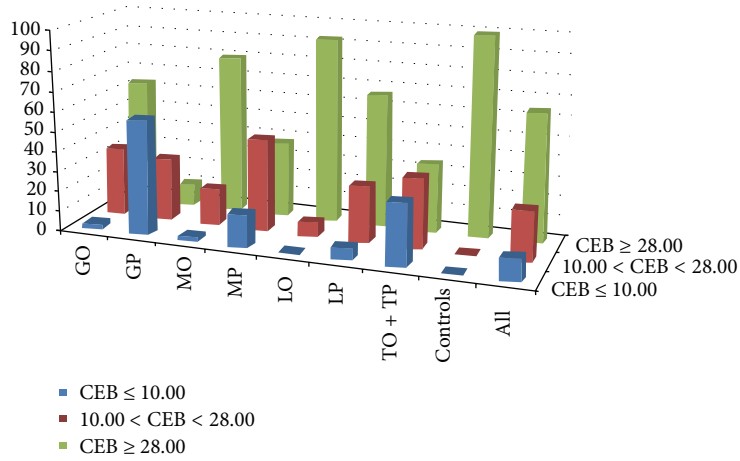

Figure 4: Distribution of relative frequency of CEB subgroups: percentage of individual cytological groups in strata of extent of anaerobic metabolism according to CEB (CEB ≥28.00 is in the reference range; CEB between 10.00 and 28.00 corresponds with advanced extent of anaerobic metabolism in serous CNS inflammations; CEB ≤10.00 corresponds with the oxidative burst of professional phagocytes).

there is indeed an ongoing inflammation or whether it is absent (only the preventive neuroprotection) [5, 6, 14]. Another nonnegligible part of CEB values above 10.00 in GP is caused by an artificial blood admixture; 33.8% (259/766) of GP with CEB above 10.00 has erythrocyte count >100/1 μL.

Inclusion of data from patients with *secondary infection* after trauma or neurosurgical performance may bring a slight modification of results to our data set. In these cases the input of pathogens to the immune unprepared space causes slow acceleration of inflammation, and in addition there are often many other complications like production of glucocorticoids due to stress, a primary disease of CNS itself, a noninfectious inflammation after neurosurgical performance, a contamination of CNS by nonpathogenic microbes, usually a protective antibiotic therapy, polymorbidity, and so forth. These patients do not often develop an adequate inflammatory response, including cellularity of cerebrospinal fluid. A detection of metabolic manifestation of the oxidative burst by CEB significantly contributes to the solution of this difficult situation [5, 6, 37, 38].

Figure 4 shows that the highest percentage of CEB values less than 10.00 is in the GP group, the second largest in the group TO + TP; groups with LO, GO, and MO have very low share of these values.

To sum up, the "anaerobic shift" of CEB towards the values below the lower reference limit always reliably reveals the presence of pathology in CNS.

4. Conclusion

Advances in laboratory technologies affect an investigation of cerebrospinal fluid; processes occurring in CNS can be more accurately characterized by still new biomarkers measured at specialized centers. But we target the most fundamental energy parameters in CSF because they provide the first information about pathological processes in CNS to the physician, and they are generally available, low-cost, and irreplaceable. The Coefficient of Energy Balance enables more exact assessment of actual energy state in the CSF compartment than glucose and lactate alone, and not only does it specify and accelerate diagnostic and therapeutic decision, but it also determines the direction of further examinations.

Using a large data set (n = 8183) we found statistically significant difference of CEB values in cytological groups accompanied by the oxidative burst of professional phagocytes. Our results confirm the ability of CEB to distinguish between variant pathologies in CNS, and thus support the benefit of CEB as an instrument for early differential diagnosis and early targeted therapy of CNS diseases. We believe that the combination of CEB and cytology in CSF examinations is crucial because cytology describes the presence of concrete cells, and CEB adds information about the functioning condition of these cells. Of course, it is also necessary to build diagnosis on all clinical and laboratory findings comprehensively, not only on one or several laboratory findings.

CEB can reveal the presence of a serious disease of the central nervous system already at the level of the basic (urgent) analysis of cerebrospinal fluid. We are convinced that a calculation of CEB should be included into an urgent CSF analysis and automatically set in a Laboratory Information System. Further studies of CEB in relation to specific clinical diagnosis are needed to clarify sensitivity and specificity of CEB. Results from CSF open the possibility of using CEB for examinations of other body fluids.

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Research Article

Proteomic Profiling for Peritoneal Dialysate: Differential Protein Expression in Diabetes Mellitus

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Peritoneal dialysis (PD) is an increasingly accepted modality of renal replacement therapy. It provides the advantages of having a flexible lifestyle, stable hemodynamics, and better preservation of residual renal function. To enhance our understanding of the peritoneal dialysate of diabetes mellitus (DM), peritoneal dialysate proteins were identified by two-dimensional gel electrophoresis (2DE) combined with reverse-phase nano-ultra performance liquid chromatography electrospray ionization tandem mass spectrometry (RP-nano-UPLC-ESI-MS/MS) followed by peptide fragmentation patterning. To validate the differential proteins, ELISA and Western blotting analyses were applied to detect candidate proteins that may be related to DM. We performed 2DE on the peritoneal dialysate samples, with detection of more than 300 spots. From this, 13 spots were excised, in-gel digested, and identified by RP-nano-UPLC-ESI-MS/MS. Ten of these showed significant differential expression between the DM and chronic glomerulonephritis (CGN) peritoneal dialysate samples. In this study, we conducted a comparative proteomic study on these two groups of dialysate that may provide evidence for understanding the different peritoneal protein changes. These proteins may not be new biomarkers; however, they may indicate a situation for possible drug treatment and can be the predictors of peritonitis for a validation study in the future.

1. Introduction

Peritoneal dialysis (PD) is a treatment for patients with severe chronic kidney disease such as renal failure or renal insufficiency. The process uses the patient's peritoneum in the abdomen or coelom as a membrane across which fluids and dissolved substances (electrolytes, urea, glucose, proteins, and other small molecules) are exchanged between the blood and the peritoneal fluid (to which a high-glucose solution has been infused) [1]. PD is today an increasingly accepted modality of renal replacement therapy. The problem with this therapy is that, upon prolonged treatment, peritoneal fibrosis with subsequent loss of ultrafiltration (UF) ability and fluid overload could occur, which will result in the necessity of discontinuation of PD. An additional problem would be also the onset of peritonitis, which might occur upon prolonged

treatment [2]. Recurrent peritonitis and bioincompatibility of the peritoneal dialysate (e.g., high blood glucose, high osmolality, acidic pH, and elevated glucose degradation products) are the leading mechanisms attributed to peritoneal fibrosis [3]. Increasing levels of the dialysate/plasma creatinine ratio (D/P Cr) on the peritoneal equilibrium test (PET) indirectly reflect possible peritoneal fibrosis-related UF failure [4]. So far, invasive peritoneal biopsy is the only method in current daily practice which can provide both definite evidence and prediction of peritoneal fibrosis. Therefore, a noninvasive diagnostic method is urgently needed. Several studies have been focused on the analysis of spent dialysate composition, including biochemistry profiles [5], cytokines [6], or proteomics [7, 8]. Among these, proteins are the final biological factors for performing biochemical reactions and physiological responses. Several studies have reported the unique proteomic profiling of pleural effusion [9, 10] or urine [11, 12]. Analyzing proteomic presentation of the dialysate may provide clues for both understanding the mechanisms of peritoneal damages and noninvasively predicting the changes of peritoneal transport characters. Brauner et al. reported that the increased amount of novel protein (daintain/allograft inflammatory factor-1, AIF-1) is detected during the early stage of PD initiation and peritonitis [13]. Another study also reported that the elevation of vitamin D-binding protein, haptoglobin, and α-2 microglobulin was noted in the diabetes mellitus (DM) dialysate [14]. Furthermore, the target proteomic changes may function as novel biomarkers for disease monitoring or detection in vitro.

Finding biomarkers in the complex biological matrix of proteins in the peritoneal dialysate for DM is a challenge. Although there are numerous proteins, lipids, and metabolic products in dialysate samples, disease biomarkers often appear at low concentrations. High-abundance proteins such as albumin, IgG, and fibrinogen are dominant and may repress the signals of the low-abundance proteins. In recent years, several maturing analytical tools have been applied in proteomic research, such as two-dimensional gel electrophoresis (2DE) and mass spectrometry analysis, which have provided much information on body fluid analysis [14, 15].

PD is one modality of the renal replacement therapy and is adopted increasingly all over the world due to more stable hemodynamic and biochemistry level and less cost than hemodialysis. End-stage renal disease due to DM is not contraindicated for peritoneal dialysis. DM and chronic glomerulonephritis (CGN) are the most common etiologies of end-stage renal disease (ESRD) receiving maintenance peritoneal dialysis [1]. In this proteomic study, we assembled a list of proteins observed in peritoneal dialysates from DM and CGN patients. To enhance our understanding of the DM proteome, the peritoneal dialysate proteins were identified by 2DE and nano-ultra performance liquid chromatography electrospray ionization tandem mass spectrometry (nano-UPLC-ESI-MS/MS) followed by peptide fragmentation patterning. This study is designed to establish an optimal technique for a proteomic map of DM proteins. The database provides not only analysis of PD proteins from DM patients but also suggests potential diagnostic protein biomarkers for further investigation.

2. Materials and Methods

2.1. Peritoneal Dialysate Collection. Enrolled patients received regular peritoneal dialysis for more than three months, without recent major comorbidity, surgery, or hospital admission within 1 month before the study. Human peritoneal dialysate specimens were collected after standard PET from 12 DM and 12 CGN patients. Two groups of patients were matched by age and gender. The Human Experiment and Ethics Committee of the Chi-Mei Medical Center approved the study protocol, and all subjects signed informed consents to the study performed. This study was approved by the Institutional Research Board and executed according to the Helsinki Declaration Principles. Written informed consents were received from all participating patients. Each peritoneal dialysate specimen (100 mL) was centrifuged at 1000 ×g at 4°C for 10 min. The supernatant was stored in polypropylene tubes at −80°C. The protein concentrations of the peritoneal dialysate samples were measured using a fluorescence-based protein quantification detection kit (Quant-iT Fluorometer, Qubit Protein Assay Kit, Q33212, Invitrogen).

2.2. Two-Dimensional Gel Electrophoresis (2DE). The protein in the peritoneal dialysates (containing 120 μg protein) was precipitated with 10% w/v trichloroacetic acid (TCA) in acetone. The precipitates were washed with acetone three times and dissolved in an isoelectric focusing (IEF) rehydration buffer for 2DE analysis. IEF strips (pH 3–10, IPGphor, Amersham Biosciences, Uppsala, Sweden) were developed through a stepwise incremental voltage program: 30 V for 16 hr, 500 V for 1 hr, 1000 V for 1 hr, and 8000 V for 4 hr, with a total power of 34 kV hr. Then the strips were subjected to a two-step equilibration in buffers: the first step containing 6 M urea, 30% glycerol, 2% SDS, and 50 mM Tris-HCl (pH 8.8) with 1% w/v dithiothreitol (DTT, Affymetrix/USB, 15395) and for the second step, with 2.5% w/v iodoacetamide (IAA, Amersham Biosciences, RPN6302V). The strips were then transferred onto the second-dimensional SDS-PAGE equipment and developed on 1.0 mm thick gradient (8–16%) polyacrylamide gels at 10°C.

2.3. Silver Staining. The gels were fixed in 40% v/v ethanol and 10% v/v acetic acid in water overnight and then incubated in a buffer solution containing 30% v/v ethanol, 6.8% w/v sodium acetate, and 0.312% w/v sodium thiosulfate for 30 min. After washing three times in water for five minutes each, the gels were stained for 30 minutes in 0.25% w/v silver nitrate solution containing 0.02% w/v formaldehyde. Development was performed for 10 min in a solution consisting of 2.5% w/v sodium carbonate and 0.01% w/v formaldehyde. Acetic acid solution (5% v/v) was used to stop the development; then the stained gels were rinsed three times in water for five minutes each.

2.4. Image Acquisition and Analysis. The stained gels were scanned using an ImageScanner and LabScan 3.00 software

(Amersham Biosciences). Image analysis was carried out using the ImageMaster 2D, version 2002.1 (Amersham Biosciences). In this study, we performed 2DE on three repeats of each sample, respectively. To prevent variation between different 2DE gels, all volumes of protein spots in the 2DE map were normalized by using the ratio of each spot over the sum.

2.5. Statistics and Tryptic In-Gel Digestion. Protein spots were selected by using the criteria: protein expressions are a twofold difference between DM and CGN patients (greater than twofold or less then 50%). Statistical significance was determined by using the two-tailed Fisher exact test at $P < 0.05$. The protein spots were excised manually, reduced, alkylated, and then digested using sequence grade trypsin (V511A, Promega) by standard protocols [16].

2.6. Protein Identification. The tryptic digests of the proteins were separated and identified using a flow rate of 400 nL/min with an RP-nano-UPLC system (nanoACQUITY UPLC, Waters, Milford, MA, USA) coupled to an ion trap mass spectrometer (LTQ Orbitrap Discovery Hybrid FTMS, Thermo, San Jose, CA, USA) equipped with an electrospray ionization source. For RP-nano-UPLC-ESI-MS/MS, a sample (2 μL) of the desired peptide digest was loaded into the reverse phase column (Symmetry C18, 5 μm, 180 μm × 20 mm) by an autosampler. The RP separation was performed using a linear acetonitrile gradient from 99% buffer A (100% D.I. water/0.1% formic acid) to 85% buffer B (100% acetonitrile/0.1% formic acid) in 45 min using the micropump. The separation was performed on a C18 microcapillary column (BEH C18, 1.7 μm, 75 μm × 100 mm) using the nanoseparation system. As peptides eluted from the microcapillary column, they were electrosprayed into the ESI-MS/MS with the application of a distal 2.1 kV spraying voltage with heated capillary temperature of 200°C. Each cycle of one full-scan mass spectrum (m/z 400–2000) was followed by four data-dependent tandem mass spectra with collision energy set at 35%.

2.7. Database Search. For protein identification, Mascot software (Version 2.2.1, Matrix Science, London, UK) was used to search the Swiss-Prot human protein sequence database. For proteolytic cleavages, only tryptic cleavage was allowed, and the number of maximal internal (missed) cleavage sites was set to two. Variable modifications of cysteine with carboxyamidomethylation, methionine with oxidation, serine/threonine/tyrosine with phosphorylation, and asparagine/glutamine with deamidation were allowed. Mass tolerances of the precursor peptide ion and fragment ion were set to 20 ppm and 1 Da, respectively. Positive protein identifications were defined when the Mowse scores greater than 100 were considered significant ($P < 0.05$). Proteins were initially annotated by similar searches using UniProtKB/Swiss-Prot databases.

2.8. Western Blotting. Four proteins, apolipoprotein A-I (APOA1), apolipoprotein A-IV (APOA4), eukaryotic translation initiation factor 4A isoform 1 (EIF4G1), and Zn-alpha2-glycoprotein (AZGP1), were analyzed by Western blotting. Each peritoneal dialysate sample (1 μg/μL, 10 μL) was electrophoresed through a precast gel (NuPAGE Novex 4–12% Bis-Tris Gel, 1.5 mm, 10 wells, Invitrogen, Carlsbad, CA, USA). Proteins were transferred from the gel to a polyvinyl difluoride (PVDF) membrane (Millipore, Bedford, CA, USA) by means of the semidry technique using the Criterion Blotter (Bio-Rad) at 100 V for 60 min and blocked with 5% milk in PBS (adjusted to pH 7.4) containing 0.05% Tween-20.

The membranes were then incubated overnight with rabbit anti-APOA1 (2840-1, Epitomics), rabbit anti-APOA4 (3109-1, Epitomics), anti-EIF4G1 (E8949-54, Labome), and rabbit anti-AZGP1 (ab117275, Abcam) diluted to 1 μg/mL. After washing, the membrane was incubated with alkaline peroxidase-conjugated affinipure goat anti-rabbit IgG (111-035-003, Immuno Research) diluted 1/10000 for 1 hr. The proteins were detected with an enhanced chemiluminescence (ECL) system. The quantitative analysis of Western blotting was carried out using the ImageQuant-TL-7.0 software, version 2010 (Amersham Biosciences) [17].

2.9. ELISA Assay. Each peritoneal dialysate sample was analyzed for the concentrations of APOA1 (E-80AP1, Immunology Consultants Laboratory), alpha-1-microglobulin/bikunin preproprotein (AMBP) (E90217Hu, Uscn Life Science Inc.), mutant retinol-binding protein (RBP4) (E90929Hu, Uscn Life Science Inc.), and haptoglobin (HP) (E-80HPT, Immunology Consultants Laboratory) in duplicate, using commercially available enzyme-linked immunosorbent assay (ELISA) kits. The protein concentrations were tested by standard protocols, according to the suggestion of the manufacturers. The model of ELISA reader was Multiskan EX, which was made by Thermo Scientific (Vantaa, Finland). Statistical significance was determined by using the two-tailed Fisher exact test at $P < 0.05$ and ROC analysis.

3. Results and Discussion

Peritoneal dialysis is one of the therapeutic options for ESRD patients. The treatment involves introduction of a high glucose concentration of fluid to the patient peritoneum, which causes waste products in the blood to diffuse across the peritoneal membrane. DM is one of the major diseases treated by PD, in which the high glucose concentration may accelerate damage to the peritoneal membrane by increasing the permeability and decreasing the ultrafiltration efficiency. It will cause peritoneal membrane dysfunction and the patient may have fluid overload-related complications or eventually require hemodialysis. Long-term high blood glucose level will result in multiple organs damage due to either microangiopathy or macroangiopathy. Diabetes is the most common etiology of new uremic patients receiving dialysis therapy. Meanwhile, chronic glomerulonephritis is also one of the leading causes of end-stage renal disease. It presented with long-term proteinuria and/or hematuria and slowly

decreasing renal function, predominantly involving kidneys. Comparison of proteomic differentiation in peritoneal dialysis fluid from DM and CGN patients provides opportunity to noninvasively searching evidence or predicting biomarkers of glucotoxicity-related damage of peritoneum. It is impossible to obtain "healthy" control peritoneal dialysate. Thus, we utilized the peritoneal dialysate from CGN patients as control samples and compared them with the DM peritoneal dialysate. In this study, we described the observation of significant differential expression of several proteins in DM peritoneal dialysate.

3.1. 2DE Map of Peritoneal Dialysate. We performed 2DE on the peritoneal dialysate from DM and CGN patients, and more than 300 spots were detected using the image analysis software (Figure 1(a)). The protein identifications in the 2DE analysis of the peritoneal dialysate were compared with the 2DE images of normal human plasma (downloadable from the Swiss-Prot/TrEMBL website: http://world-2dpage.expasy.org/swiss-2dpage/docs/publi/inside1995.html). The most abundant proteins in the peritoneal dialysates were matched with the plasma 2DE map (according to the MW, pI, and pattern of spots) such as albumin, microglobulin, haptoglobin, glycoprotein, fibrinogen, and immunoglobulin.

The criteria of proteins selected were protein expressions of more than twofold difference between the DM and CGN peritoneal dialysate (greater than twofold or less then 50%). Statistical significance was determined by using the two-tailed Fisher exact test at $P < 0.05$. In this study, 13 of the protein spots showed significant differential expression between the DM and CGN peritoneal dialysate samples (Figure 1(b)). However, three of those spots were not identified (D1, C5, and C6 in Figure 1(b)). Ten identified proteins and their functions are listed in Tables 1 and 2. These proteins can be classified into two major functional groups as binding/transport and acute-phase/immune response.

3.2. Protein Identification by RP-Nano-UPLC-ESI-MS/MS. The fragmentation spectra from the RP-nano-UPLC-ESI-MS/MS analysis were searched against a nonredundant protein database by MASCOT. When a protein was identified by three or more unique peptides possessing MASCOT scores, no visual assessment of spectra was conducted and the protein was considered present in the sample. All Mascot results were manually confirmed by visual assessment of the MS/MS spectra for overall quality. In addition, the criterion for manual validation reported by Jaffe et al. was used [18]. It requires a readily observable series of at least 4 y ions.

For the ten identified proteins, 4 upregulated and 6 downregulated proteins were found in DM peritoneal dialysate. The ten proteins were positively identified as apolipoprotein A-IV (APOA4), Zn-alpha2-glycoprotein (AZGP1), eukaryotic translation initiation factor 4A isoform 1 (EIF4G1), human class I histocompatibility antigen (HLA-A) (upregulation), albumin (ALB), alpha-1-microglobulin/bikunin preproprotein (AMBP), apolipoprotein A-I (APOA1), immunoglobulin G1 Fc fragment (IGHG1), mutant retinol-binding protein (RBP4), haptoglobin alpha2 (HP) (downregulation), which may serve as potential protein indicators of DM.

3.3. DM-Related Proteins. To validate the differential proteins identified by nano-UPLC-ESI-MS/MS, ELISA and Western blotting analyses were applied to detect candidate proteins that may be related to DM. In this study, 6 proteins were selected and confirmed by ELISA or Western blotting.

Figure 2 shows the representation of the ELISA analysis results of peritoneal dialysate samples. The individual values and the means with standard errors for each protein were also showed in the Figure 2. In the peritoneal dialysate samples, AMBP, APOA1, RBP4, and HP were detected to be strongly downregulated in the DM patients ($^{*}P < 0.05$, $n = 12$). The receiver operating characteristic (ROC) curve analyses showed that the differential proteomic features were potential protein biomarkers for DM (Figure 3). The areas of the ROC curve in the 95% confidence interval of AMBP, APOA1, RBP4, and HP were 0.7847, 0.8958, 0.9931, and 0.9097, respectively.

The identified candidate proteins that may be related to DM are confirmed differential protein expression with Western blotting. Each peritoneal dialysate sample, containing 10 μg of protein, was applied into a precast gel and transferred to a PVDF membrane. Figure 4 shows the representation of the Western blotting analysis results of APOA1, APOA4, EIF4G1, and AZGP1 in the peritoneal dialysate samples. Compared with Western blotting of the β-actin standard, the concentrations of candidate proteins in peritoneal dialysate samples were detected and analyzed by using the quantitative analysis software ImageQuant-TL-7.0. The P values between CGN and DM were less than 0.05 (Figure 5, $^{*}P < 0.05, n = 12$). The protein-protein interaction pathways were performed by String 9.0 Web software. Ten proteins identified in this study were marked by red arrows (Figure 6).

Several identified proteins in this study were reported as peritoneal dialysate proteins, such as APOA1, HP, ALB, IGHG1, and AZGP1 [15, 19]. In our study, some proteins were also indicated that may relate to DM.

HP is synthesized in the liver as a proprotein, processed by proteolytic cleavage into α and β subunits, and released into the bloodstream as a tetrameric protein. The expression of the HP subunits has caught the attention of investigators because of their exceptionally high degree of stimulation by cytokines and dexamethasone [20]. In this study, the expression of HP was downregulated in DM patients. HP is a defensive response protein, which may control the equilibrium between tolerance and immunity to nonself antigens. In addition, high blood glucose may cause chronic systemic inflammation. Thus, DM patients need to take medication to control blood glucose and inflammation, which may due to the downregulation of HP.

APOA4, a 46 kDa plasma apolipoprotein, is synthesized predominantly in the small intestine and is an antiatherogenic and antioxidative plasma glycoprotein involved in reverse cholesterol transport. The increased expression of APOA4 was found in chronic kidney disease (CKD) patients [21]. In previous studies, plasma APOA4 levels were increased in DM patients, especially in noninsulin-dependent diabetes mellitus (NIDDM) patients [22, 23]. The increase in expression of APOA4 may be related to hypertriglyceridemia (hTG), hyperglycemia, hormonal dysregulation,

Table 1: The 10 peritoneal dialysate proteins identified with higher confidence levels (at least three unique peptide sequences matched) in this study.

Accession no.	Protein name	Expression	MW (Da)	*PI* Value	Mascot score	Match queries	Sequence coverage	Peptide
P02768	Albumin (ALB)	Downregulation	69367	8.2	66	4	17%	K.FQNALLVR.Y K.AVMDDFAAFVEK.C + oxidation (M) K.KVPQVSTPTLVEVSR.N
P02760	Alpha-1-microglobulin/bikunin preproprotein (AMBP)	Downregulation	38999	5.95	349	7	21%	R.ETLLQDFR.V K.WYNLAIGSTCPWLK.K + carbamidomethyl (C); R.GECVPGEQEPEPILIPR.V + carbamidomethyl (C) R.EYCGVPGDGDEELLRFSN.- R.VVAQGVGIPEDSIFTMADR.G R.VVAQGVGIPEDSIFTMADR.G + oxidation (M) R.ETLLQDFRVVAQGVGIPEDSIFTMADR.G + deamidated (NQ)
P02647	Apolipoprotein A-I (APOA1)	Downregulation	30778	5.39	244	12	44%	K.VQPYLDDFQK.K K.WQEEMELYR.Q + oxidation (M) R.THLAPYSDELR.Q K.VQPYLDDFQKK.W R.DYVSQFEGSALGK.Q R.VKDLATVYVDVLK.D K.VEPLRAELQEGAR.Q + deamidated (NQ) K.VEPLRAELQEGAR.Q K.LLDNWDSVTSTFSK.L R.QKVEPLRAELQEGAR.Q + Gln->pyro-Glu (N-term Q) R.QKVEPLRAELQEGAR.Q K.DSGRDYVSQFEGSALGK.Q
P01857	Immunoglobulin G1 Fc fragment (IGHG1)	Downregulation	36106	6.95	154	5	36%	K.NQVSLTCLVK.G + carbamidomethyl (C) K.FNWYVDGVEVHNAK.T R.EPQVYTLPPSRDELTK.N + deamidated (NQ) R.TPEVTCVVVDVSHEDPEVK.F + carbamidomethyl (C) R.WQQGNVFSCSVMHEALHNHYTQK.S + carbamidomethyl (C); deamidated (NQ)

Table 1: Continued.

Accession no.	Protein name	Expression	MW (Da)	*PI* Value	Mascot score	Match queries	Sequence coverage	Peptide
P02753	Mutant retinol binding protein (RBP4)	Downregulation	23010	6.29	235	4	84%	-.FSGTWYAMAK.K -.FSGTWYAMAK.K + oxidation (M) K.KDPEGLFLQDNNVAEFSVDETGQMSATAK.G K.KDPEGLFLQDNNVAEFSVDETGQMSATAK.G + oxidation (M)
P00738	Haptoglobin alpha2 (HP)	Downregulation	45205	6.46	77	4	21%	K.LPECEADDGCPKPPEIAHGYVEHSVR.Y + 2 carbamidomethyl (C) K.AVGDKLPECEADDGCPKPPEIAHGYVEHSVR.Y + 2 carbamidomethyl (C) K.LPECEADDGQPPPKCIAHGYVEHSVR.Y + 2 carbamidomethyl (C); deamidated (NQ) K.AVGDKLPECEADDGQPPPKCIAHGYVEHSVR.Y + 2 carbamidomethyl (C); deamidated (NQ)
P06727	Apolipoprotein A-IV (APOA4)	Upregulation	45399	5.23	1109	19	43%	K.VNSFFSTFK.E R.LTPYADEFK.V R.LEPYADQLR.T K.ALVQQMEQLR.Q R.LTPYADEFKVK.I K.LVPFATELHER.L R.DKVNSFFSTFK.E K.LGEVNTYAGDLQK.K K.KLVPFATELHER.L K.VKIDQTVEELRR.S K.LNHQLEGLTFQMK.K K.LKEEIGKELEELR.A K.SELTQQLNALFQDK.L K.LGPHAGDVEGHLSFLEK.D K.SLAELGGHLDQQVEEFR.R R.ENADSLQASLRPHADELK.A R.QKLGPHAGDVEGHLSFLEK.D R.ENADSLQASLRPHADELKAK.I

Table 1: Continued.

Accession no.	Protein name	Expression	MW (Da)	*PI* Value	Mascot score	Match queries	Sequence coverage	Peptide
P25311	Zn-alpha2-glycoprotein (AZGP1)	Upregulation	34259	5.71	739	24	48%	K.SQPMGLWR.Q K.CLAYDFYPGK.I + carbamidomethyl (C) K.WEAEPVYVQR.A K.AREDIFMETLK.D K.AYLEEECPATLR.K + carbamidomethyl (C) K.QKWEAEPVYVQR.A + Gln->pyro-Glu (N-term Q) K.QKWEAEPVYVQR.A K.QKWEAEPVYVQR.A + deamidated (NQ) K.AYLEEECPATLRK.Y + carbamidomethyl (C) K.YYYDGKDYIEFNK.E R.QDPPSVVVTSHQAPGEK.K + Gln->pyro-Glu (N-term Q) R.AKAYLEEECPATLRK.Y + carbamidomethyl (C) K.EIPAWVPFDPAAQITK.Q R.QDPPSVVVTSHQAPGEKK.K + Gln->pyro-Glu (N-term Q) R.QVEGMEDWKQDSQLQK.A + Gln->pyro-Glu (N-term Q) R.QVEGMEDWKQDSQLQK.A + deamidated (NQ); Gln->pyro-Glu (N-term Q); oxidation R.QVEGMEDWKQDSQLQK.A R.QVEGMEDWKQDSQLQK.A + deamidated (NQ) R.QVEGMEDWKQDSQLQK.A + oxidation (M) R.QVEGMEDWKQDSQLQK.A + deamidated (NQ); oxidation (M) K.NILDRQDPPSVVVTSHQAPGEK.K K.NILDRQDPPSVVVTSHQAPGEK.K + deamidated (NQ) K.HVEDVPAFQALGSLNDLQFFR.Y K.NILDRQDPPSVVVTSHQAPGEKK.K
Q04637	eukaryotic translation initiation factor 4A isoform 1 (EIF4G1)	Upregulation	175491	5.32	469	8	28%	R.QFYINVER.E K.GYDVIAQAQSGTGK.T K.GVAINMVTEEDKR.T K.MFVLDEADEMLSR.G R.DFTVSAMHGDMDQK.E K.LQMEAPHIIVGTPGR.V R.GIYAYGFEKPSAIQQR.A K.LNSNTQVVLLSATMPSDVLEVTK.K

Table 1: Continued.

Accession no.	Protein name	Expression	MW (Da)	*PI* Value	Mascot score	Match queries	Sequence coverage	Peptide
P01892	Human class I histocompatibility antigen (HLA-A)	Upregulation	40922	6.46	129	3	36%	R.VNHVTLSQPKIVK.W K.SNFLNCYVSGFHPSDIEVDLLK.N + carbamidomethyl (C) K.SNFLNCYVSGFHPSDIEVDLLK.N + carbamidomethyl (C); deamidated (NQ)

Table 2: Subcellular location and protein function of 10 proteins with higher confidence levels identified in peritoneal dialysate of CGN and DM.

Accession no.	Protein name	Subcellular location	Biological process	Molecular function	Protein function
P02768	Albumin (ALB)	Secreted	Cellular response to starvation Hemolysis by symbiont of host erythrocytes Maintenance of mitochondrion location	Binding protein	Serum albumin, the main protein of plasma, has a good binding capacity for water, Ca2+, Na+, K+, fatty acids, hormones, bilirubin, and drugs. Its main function is the regulation of the colloidal osmotic pressure of blood. Major zinc transporter in plasma, typically binds about 80% of all plasma zinc.
P02760	Alpha-1-microglobulin/bikunin preproprotein (AMBP)	Secreted	Cell adhesion Heme catabolic process Negative regulation of immune response	Serine-type endopeptidase inhibitor activity Calcium oxalate binding Small molecule binding Calcium channel inhibitor activity	Inter-alpha-trypsin inhibitor inhibits trypsin, plasmin, and lysosomal granulocytic elastase. Inhibits calcium oxalate crystallization
P02647	Apolipoprotein A-I (APOA1)	Secreted	Cholesterol metabolism Lipid metabolism Lipid transport Steroid metabolism Transport	Binding protein	Participates in the reverse transport of cholesterol from tissues to the liver for excretion by promoting cholesterol efflux from tissues and by acting as a cofactor for the lecithin cholesterol acyltransferase (LCAT). As part of the SPAP complex, activates spermatozoa motility.
P01857	Immunoglobulin G1 Fc fragment (IGHG1)	Secreted, membrane	Complement activation, classical pathway Innate immune response	Antigen binding	G1m marker
P02753	Mutant retinol binding protein (RBP4)	Secreted	Glucose homeostasis Gluconeogenesis response to insulin stimulus	Retinol binding Retinol transporter activity	Delivers retinol from the liver stores to the peripheral tissues. In plasma, the RBP-retinol complex interacts with transthyretin; this prevents its loss by filtration through the kidney glomeruli.
P00738	Haptoglobin alpha2 (HP)	Secreted	Defense response Metabolic process	Catalytic activity	Haptoglobin combines with free plasma hemoglobin, preventing loss of iron through the kidneys and protecting the kidneys from damage by hemoglobin, while making the hemoglobin accessible to degradative enzymes. Uncleaved haptoglobin, also known as zonulin, plays a role in intestinal permeability, allowing intercellular tight junction disassembly, and controlling the equilibrium between tolerance and immunity to nonself antigens.

Table 2: Continued.

Accession no.	Protein name	Subcellular location	Biological process	Molecular function	Protein function
P06727	Apolipoprotein A-IV (APOA4)	Secreted	Cholesterol efflux Cholesterol homeostasis Cholesterol metabolic process Chylomicron assembly Chylomicron remodeling High-density lipoprotein particle remodeling Hydrogen peroxide catabolic process Innate immune response in mucosa Leukocyte adhesion Lipoprotein metabolic process Multicellular organismal lipid catabolic process	Antioxidant activity Cholesterol transporter activity Copper ion binding Phosphatidylcholine binding Phosphatidylcholine-sterol O-acyltransferase activator activity Protein homodimerization activity	May have a role in chylomicrons and VLDL secretion and catabolism. Required for efficient activation of lipoprotein lipase by ApoC-II; potent activator of LCAT. Apoa-IV is a major component of HDL and chylomicrons.
P25311	Zn-alpha2-glycoprotein (AZGP1)	Secreted	Antigen processing and presentation Cell adhesion Immune response Lipid catabolic process Negative regulation of cell proliferation	Fatty acid binding Protein transmembrane transporter activity Ribonuclease activity	Stimulates lipid degradation in adipocytes and causes the extensive fat losses associated with some advanced cancers. May bind polyunsaturated fatty acids.
Q04637	Eukaryotic translation initiation factor 4A isoform 1 (EIF4G1)	Cytosol	Cell death Insulin receptor signaling pathway	DNA binding Translation initiation factor activity	Component of the protein complex eIF4F, which is involved in the recognition of the mRNA cap, ATP-dependent unwinding of 5′-terminal secondary structure and recruitment of mRNA to the ribosome.
P01892	Human class I histocompatibility antigen (HLA-A)	Golgi membrane Plasma membrane	Type I interferon-mediated signaling pathway Regulation of immune response Interspecies interaction between organisms	Binding protein	Involved in the presentation of foreign antigens to the immune system.

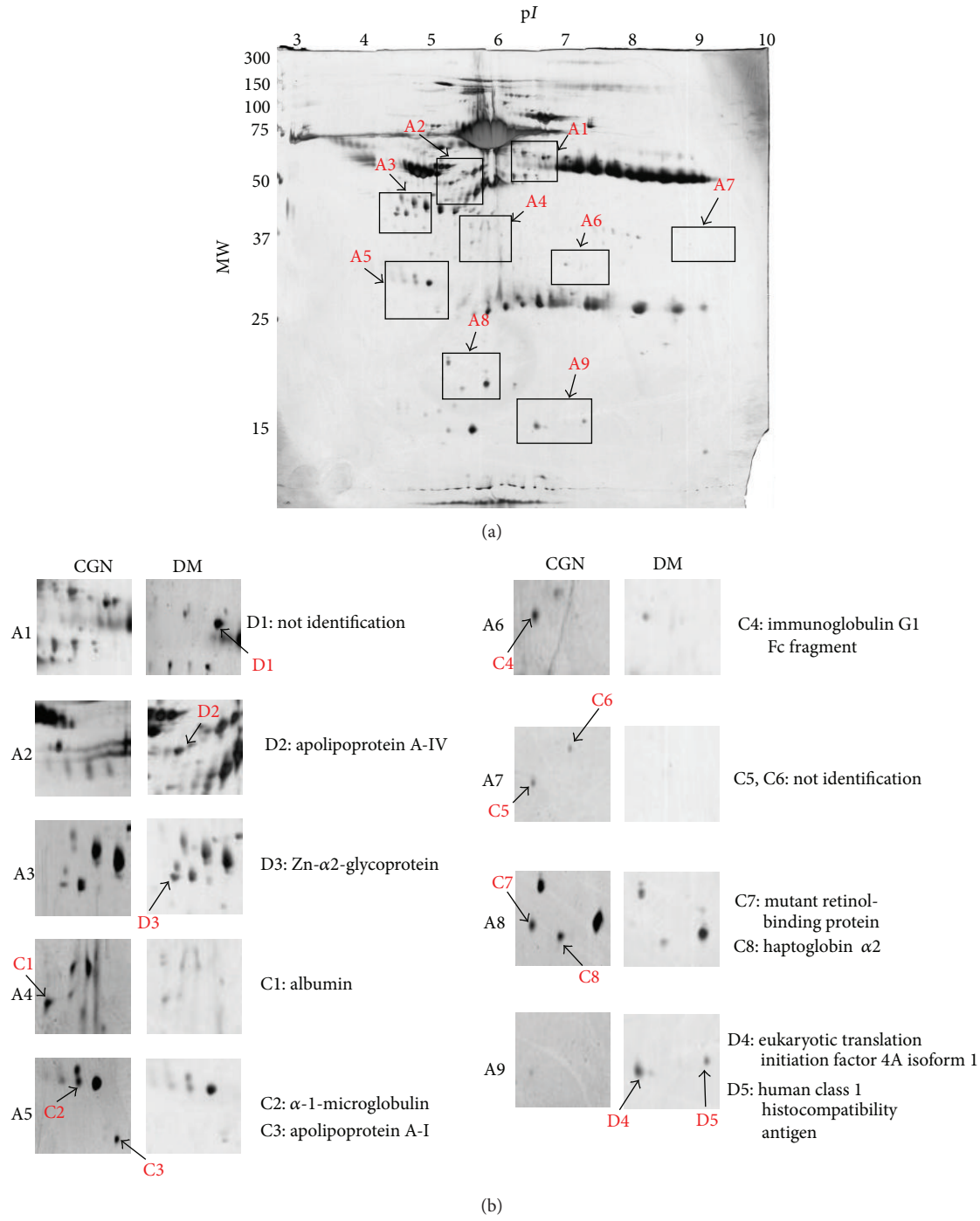

Figure 1: Representative 2DE maps of peritoneal dialysate samples (a) CGN and (b) compared with DM. Peritoneal dialysate samples were separated for 2DE analysis (pH 3–10), and 120 μg protein of each sample was loaded into each gel. The analysis of each sample was repeated in three gels.

inflammation, and to a lesser extent to HDL cholesterol level [24]. However, the metabolism pathway and relationship between APOA4 and DM were unclear.

APOA1 is a component of the high-density lipoprotein responsible for the cholesterol transport into liver. APOA1 was associated with DM showing downregulation in DM patients [25]. Our 2DE, Western blotting, and ELISA results were consistent with those of previous studies [26–29].

RBP4 is a novel adipocytokine (adipocyte-secreted hormone), which shows upregulation in insulin-resistant animal models. In the clinical study, it is reported that the RBP4 level is higher in type 2 DM, gestational DM, and metabolic syndrome patients [30, 31]. However, in a study reported by Krill et al., it was found the RBP concentrations measured in DM serum samples were significantly lower than those in the healthy group [32–34]. Also, in another report, the mean

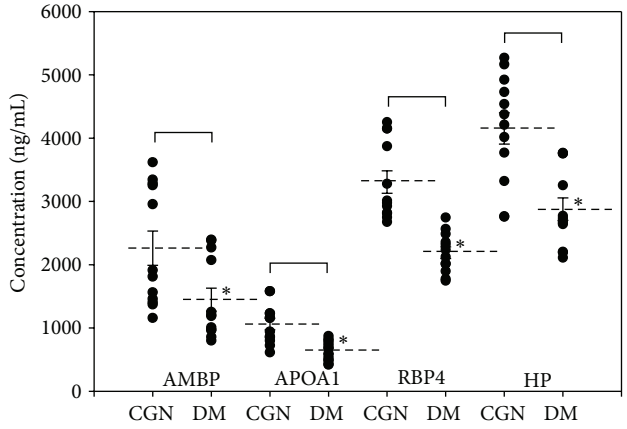

Figure 2: Confirmation of AMBP, APOA1, RBP4, and HP expressions by ELISA analysis ($n = 12$, 3 repeats, $^*P < 0.05$). Scatter plots of the individual values for each protein are shown.

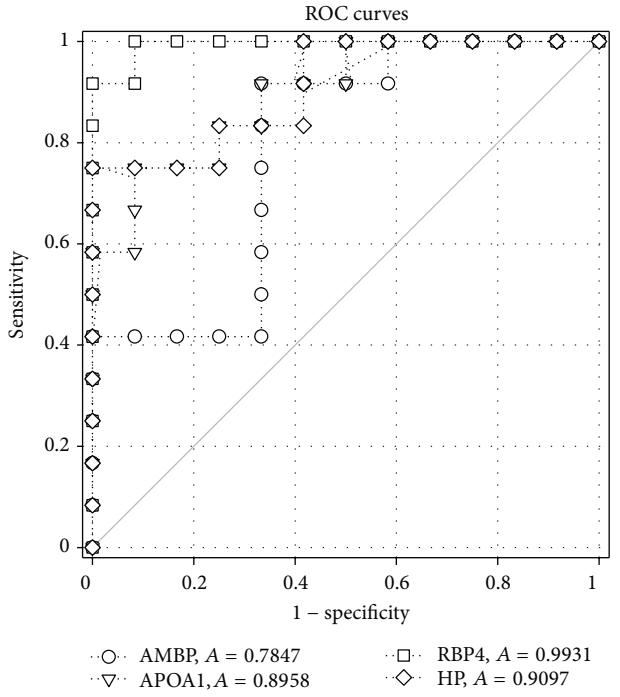

Figure 3: ROC curve analysis for AMBP, APOA1, RBP4, and HP in the peritoneal dialysate samples of DM patients. The estimated areas under the curves are 0.7847, 0.8958, 0.9931, and 0.9097, respectively.

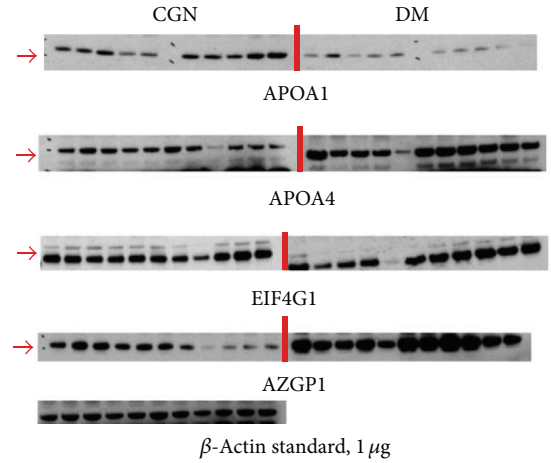

Figure 4: Confirmation of APOA1, APOA4, EIF4G1, and AZGP1 expressions by Western blot analysis. All peritoneal dialysate samples of CGN and DM patients were confirmed by Western blot analysis and the representatives are shown in this figure.

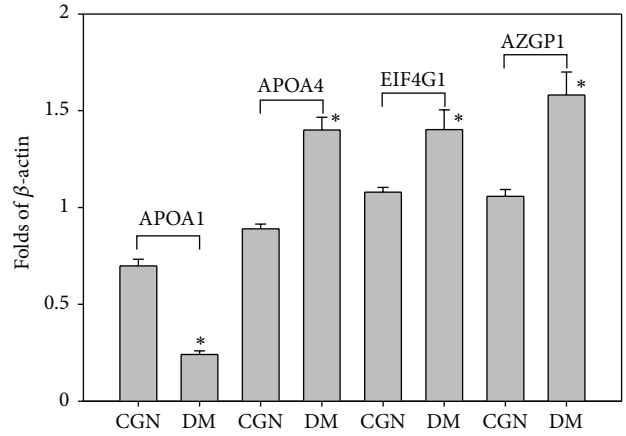

Figure 5: Protein expression in peritoneal dialysate samples of CGN and DM patients. The quantitative analysis of Western blotting was carried out using the ImageQuant-TL-7.0 software.

serum vitamin A and RBP levels were significantly lower in the insulin-dependent diabetes mellitus (IDDM) patients. The linear regression between vitamin A and RBP levels was statistically significant [35]. In our study, the RBP4 level was downregulated in DM group. However, the role of RBP4 in DM is still challenged.

There are not many reports about the role of AZGP1 in DM patients. AZGP1 is a candidate gene for age-dependent changes in the genetic control of obesity. In the study reported by Gohda et al., AZGP1 mRNA level was upregulated in the type 2 DM mice [36]. This is consistent with our experimental results.

AMBP is a glycoprotein also known as protein HC, present in body fluids. In the study reported by Shore et al., the level of AMBP in urine was significantly elevated in the NIDDM patients, which may be related to high blood sugar level and poor glycaemic control [37]. Although this is inconsistent with our experimental result, there are other reports which supported our data [38, 39].

The expression of EIF4G1 was upregulated in the DM patients in our study. Unfortunately, the literature mentioning the relationship between EIF4G1 and DM was unavailable. It may be worthy to reexplore this biomarker in a future study.

Previous studies have indicated that HP, AMBP, and AZGP1 were the candidate biomarkers for DM [40–42]. We also observed the changed levels of RBP4 and EIF4G1 in DM. In conclusion, those results support the hypothesis that

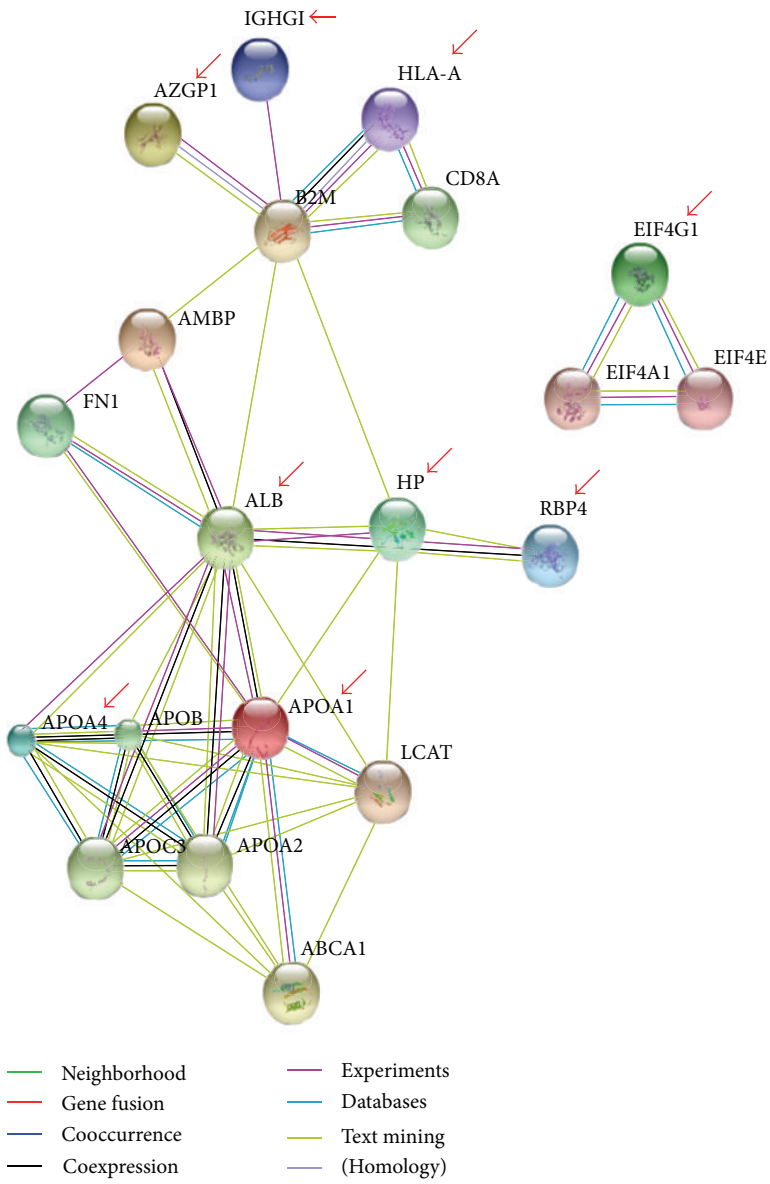

Figure 6: The protein-protein interaction pathways were performed. Proteins identified in this study were marked by red arrows.

those proteins may associate in the metabolism of DM. These proteins may not be sensitive diagnosis biomarkers for DM; however, they may be indicators of alteration in glycemic control.

4. Conclusions

Many investigations have focused on the development of biomarkers as a diagnostic tool. Proteomic approaches are powerful tools for analyzing proteins in complex mixtures. 2DE and MS analysis are useful for the analysis of human samples in a clinical research environment; they can be utilized when identifying the origins of samples of body fluids, analyzing protein phenotypes, monitoring disease processes, and searching for new disease markers.

There are only a few reports concerning proteomic analysis for the investigation of clinical peritoneal dialysate. This may be due to difficulties with obtaining "healthy" control specimens of peritoneal dialysate. Thus, in this study, we utilized the peritoneal dialysate from CGN patients as control samples and compared them with the DM peritoneal dialysate. Experimental results showed that ten proteins were differentially expressed in DM peritoneal dialysate samples. Although the sample number was not large, most of the proteins with differential expression in peritoneal dialysate samples were also found in plasma or serum. These proteins may not be new biomarkers; however, they may indicate a situation for possible drug treatment or glycemic control. In this paper, we may not be able to provide a final decision for the function or presence of these proteins, but these data support the need to continue and expand proteomic

analysis of total proteins in DM peritoneal dialysate. The results of these studies still need to be verified by larger clinical studies. In conclusion, these proteins are valuable for the identification of differentially expressed proteins involved in the proteomics database and screening biomarkers for further study of DM.

Conflict of Interests

No competing financial interests exist.

Authors' Contribution

Ming-Hui Yang and Shih-Bin Su contributed equally to this work as first authors.

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Research Article

Comparative Proteomic Analysis of Peritoneal Dialysate from Chronic Glomerulonephritis Patients

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Peritoneal dialysis (PD) frequently contributes to peritoneal damage which cannot be easily identified without invasive techniques, implying the urgent need for biomarkers and revealing mechanisms. Chronic glomerulonephritis (CGN) is one of the leading causes of receiving dialysis treatment. Here, we attempted to analyze the peritoneal dialysate collected from CGN patients when they receive continuous ambulatory peritoneal dialysis (CAPD) treatment for the first time and after a year to reveal the protein changes that resulted from PD. Proteins were displayed by two-dimensional gel electrophoresis (2DE). Altered gel spots were digested followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis for protein identification. Eight proteins were found to have differential expression levels between two groups. Their differential expressions were validated by Western blots in other sets of peritoneal dialysates. Proteins identified with higher levels in the first-time dialysate suggested their dominant appearance in CGN patients, while those that showed higher levels in peritoneal dialysate collected after one year may result from initial peritoneal inflammation or changes in the permeability of the peritoneum to middle-sized proteins. All the identified proteins may provide a perceptiveness of peritoneal changes caused by PD and may function as potential biomarkers or drug targets.

1. Introduction

Peritoneal dialysis (PD) is a widely used method of renal replacement therapy which also can lead to pathologic damage of the peritoneum and patient fatality. One potential mechanism is glucotoxicity to the peritoneal membrane [1]. However, it is not possible to identify peritoneal change without invasive techniques up to now; therefore, biomarkers that can be used for recognizing initial damages caused by PD are of urgent need. Nevertheless, insufficient information about proteins that are changed during peritoneal dialysis or between different kinds of diseases makes pathogenic

mechanism unclear. The studying of fluid from dialysis may reveal potential markers or shed light on the mechanisms of peritonitis.

In recent decades, analyses of peritoneal dialysate by proteomic approaches have opened up progresses in this field. A descriptive study was performed on the dialysate of nine paediatric PD children patients by SDS-PAGE [2]. A total of 189 proteins, involved in acute phase proteins, complement factors, hormones, coagulation factors, and apolipoproteins, were identified. Evidence shows that daintain/allograft inflammatory factor-1 (AIF-1) increases in the first spent dialysate from patients newly receiving continuous ambulatory peritoneal dialysis (CAPD), suggestion a potential marker of CAPD peritonitis [3]. Lin et al. have compared the protein profile of peritoneal dialysate from 16 patients with and without peritonitis [4] by two-dimensional gel electrophoresis (2DE), which identified β2-microglobulin as potential biomarker for CAPD peritonitis. Tyan et al. also analyzed 12 peritoneal dialysate collected from patients before and after peritonitis by 2DE and identified 10 proteins that were significantly differentially expressed between two groups [5]. A proteomic analysis was conducted on peritoneal dialysate from CAPD patients whose peritoneal membranes are of high (H), high average (HA), low average (LA), and low (L) transport rates [6]. Five proteins, identified as serum albumin in a complex with myristic acid and triiodobenzoic acid, α1-antitrypsin, complement component C4A, immunoglobulin κ light chain, and apolipoprotein A-I, were found to show differed levels among groups. Another laboratory works on analyzing the protein composition of peritoneal dialysate from patients receiving peritoneal dialysis with different concentration of glucose [7]. Four proteins, alpha-1-antitrypsin, fibrinogen beta chain, transthyretin, and apolipoprotein A-IV, were found to be underexpressed in the highest osmolar solution. The result provides potential targets for future therapeutic implementation in preventing inflammatory processes induced by the exposure to dialysis solutions. Besides, Wang et al. have compared the protein profile of diabetic peritoneal dialysate versus normal peritoneal fluid [8]. According to the western confirmation results, vitamin D-binding protein, haptoglobin, and α-2-microglobulin showed higher levels in the DM samples, while complement C4-A and IGK@ protein were of lower levels compared to the control samples.

Chronic glomerulonephritis (CGN), nephroangiosclerosis, and diabetes were identified as the most common causes of chronic renal failure [9, 10]. CGN is characterized by slowly inflammatory changes in glomerular capillaries generally leading to irreversible renal failure. Here, we intend to analyze the peritoneal dialysate collected from CGN patients when they receive CAPD treatment for the first time and after a year to point out the protein changes due to CGN-induced accumulation of uremic toxins or the inflammation caused by the oxidative stress. The altered protein targets may shed light to the mechanism of peritoneal change and function as clues to initial damage of peritoneal membrane.

2. Material and Methods

2.1. Patient Sample Preparation. Ten CGN patients were recruited in this study. Their peritoneal dialysates were collected first time at their early stage of receiving maintenance dialysis treatment (E1–10). After one year of CAPD treatment, their peritoneal dialysates were collected again to present the middle stage of treatment (M1–10). The clinical data of these patients were provided in Supplementary Table A (see Supplementary Material available online at http://dx.doi.org/10.1155/2013/863860). This study was approved by the Institutional Research Board and carried out according to the Helsinki Declaration Principles. All participating subjects have provided written informed consents. Each sample was centrifuged at 1000 ×g at 4°C for 10 min, and the supernatants were stored at −20°C until further use. An aliquot of each sample was used to measure protein concentration.

2.2. Protein Precipitation. Peritoneal dialysates from seven of the ten patients were used for protein profiling by two-dimensional gel electrophoresis (2DE) analysis, while thoes from the other three patients were used for further validation of the altered proteins by Western blotting analysis. Peritoneal dialysates collected from two stages, E1–E7 and M1–M7 (each containing 140 μg of protein), were run individually, and triplicate analyses were performed for each sample. Trichloroacetic acid in cold acetone (1 : 9) containing 0.1% dithiothreitol was added to each sample and then was placed at −20°C overnight to facilitate protein precipitation. Samples were next centrifuged at 18000 g for 30 min to collect the pellets. After three times of washing with acetone (−20°C) containing 0.1% dithiothreitol, the pellets were left air dried. Each pellet was dissolved in a 250 μL rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 2% ampholytes, and 120 mM dithiothreitol) and carefully sonicated for 60 min.

2.3. Two-Dimensional Gel Electrophoresis (2DE) and Image Analysis. Protein samples were loaded onto immobilized pH gradient (IPG) gel strips for the first-dimensional gel electrophoresis. To minimize evaporation and urea crystallization, IPG cover oil (0.8 mL) was applied on top of each gel strip. Isoelectric focusing (IEF) was run following a stepwise incremental voltage program on an IPGphor Isoelectric Focusing System (GE Healthcare Life Sciences, Taipei, Taiwan). Samples were run under the following settings: 30 V for 16 h, 500 V for 1 h, 500 V for 1 h, and 8000 V for 4 h. After IEF, the strips were first placed in equilibration buffer with 1% dithiothreitol w/v for 15 min followed by in 2.5% w/v iodoacetamide for another 15 min. The IPG strip was placed on a 1.0 mm thick 12% polyacrylamide gel and was run at 300 V for 4-5 h for the second-dimensional gel electrophoresis. The visualization of the gels was achieved by using silver staining. The stained gels were scanned at a resolution of 300 dpi using an ImageScanner operated by LabScan 3.00 software (GE Healthcare Life Sciences). Image spots were detected and matched by using ImageMaster 2D software (GE Healthcare). The spot detection was manually

checked. Each spot intensity was normalized by the total intensity volume of all spots detected on the gel. To identify the differentially expressed protein spots during the CAPD treatment, spot intensities of the sample from middle-stage groups were compared to those from the early-stage groups. Ratios were statistically analyzed using Student's t-test, and a P value <0.05 was considered significant. Protein spots with altered expression were excised from the gels and subjected to protein identification.

2.4. In-Gel Digestion. The protein spots were washed with 25 mM ammonium bicarbonate and 50% acetonitrile/25 mM ammonium bicarbonate. After being washed three more times with 25 mM ammonium bicarbonate and 50% acetonitrile/25 mM ammonium bicarbonate, the gel fragments were placed at 56°C for 1 h in a solution containing 10 mM dithiothreitol, 55 mM iodoacetamide, and 25 mM ammonium bicarbonate for protein reduction and alkylation. Gel fragments were added with 10 μL of a 0.1-μg/μL modified trypsin digestion buffer in 25 mM ammonium bicarbonate and were incubated at 37°C overnight. After tryptic digestion, the supernatants were transferred to Eppendorf tubes, and the remaining peptides were further extracted from the gel pieces by incubation with a solution of 50% acetonitrile and 5% formic acid.

2.5. Protein Identification Using Liquid Chromatography-Mass Spectrometry and Database Searching. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used for protein identification. Tryptic peptides were fractionated in a C18 microcapillary column (75 μm i.d. × 15 cm) at a flow rate of 200 nL/min with a nano-HPLC system (LC Packings Nederlands B.V., Amsterdam, The Netherlands). A 40 min gradient from 0 to 60% buffer B (80% acetonitrile, 0.1% formic acid) was used to elute the peptides. The eluate was electrosprayed into an ion trap mass spectrometer (LCQ DECA XP Plus; Thermo Electron Corp., San Jose, CA, USA) using a distal 1.3 kV spraying voltage. Each cycle of one full scan mass spectrum (m/z 150–2000) was followed by three data-dependent tandem mass spectra on the three most intense peaks. For mass spectrometry data analysis, Xcalibur BioWorks 3.3 software (Thermo Fisher Scientific, Waltham, MA, USA) was used to generate peak lists. The resulting DTA files were merged and searched against Swiss-Prot human protein sequence database by using Mascot software (Version 2.2.1, Matrix Science, London, UK). The search parameters were set as follows. A maximum of two missed cleavage sites were allowed. Variable modifications were set on cysteines (carboxyamidomethylation), methionine (oxidation), and asparagine and glutamine (deamidation). The mass tolerance of both the precursor peptide ions and the fragment ion was set to 0.5 Da. For peptide identification, the peptides identified with expected values <0.05 were acceptable. Proteins identified with a Mascot score above the significant hit threshold ($P < 0.05$) and more than two rank 1 peptides were considered significant.

2.6. Western Blotting. Samples, each contained 25 μg of proteins, were loaded in 1.0 mm × 10-well gradient gels (NuPAGE Bis-Tris, 4% to 12%) purchased from Invitrogen (Carlsbad, CA, USA). Proteins were resolved in the gel and transferred onto nitrocellulose membranes. The membranes were blocked in 5% nonfat milk in Tris-buffered saline with Tween 20 (TBST, containing 3.0 g/L Tris, 14.4 g/L glycine, and 0.5% Tween 20 with pH adjusted to 8.3) at room temperature for 1 h. The membranes were then washed three times with TBST and probed with antibodies at 4°C overnight. The primary antibodies and corresponding concentrations used were as follows: anti-Ig mu chain C region (1 : 2000) (MyBioSource, Vancouver, BC, Canada); antifibrinogen gamma chain (1 : 1000) (Abcam, Cambridge, UK); anti-C-reactive protein (1 : 500) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); anti-Ig delta chain C region (1 : 500) (Santa Cruz Biotechnology, Inc.); anti-alpha-1-antitrypsin (1 : 1000) (Abcam); anti-histidine-rich glycoprotein (1 : 1000) (R&D Systems, Minneapolis, MN, USA); antiapolipoprotein A-I (1 : 5000) (Epitomics, Burlingame, CA, USA); and antiserum amyloid P (1 : 5000) (Abcam). After being washed three times with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies (1 : 2000–1 : 5000) at room temperature for 1 h, membranes were then washed with TBST for three more times and then developed with enhanced chemiluminescence detection. The volume densitometry of each band was determined using ImageMaster 2D software.

3. Results

3.1. Analysis of Individual Peritoneal Dialysate Samples Using 2DE. To reveal the protein profile change due to CAPD treatment, 2DE was used in this study. Peritoneal dialysates were collected from seven CGN patients at the beginning of receiving CAPD treatment, which was used to present the early stage of peritoneal dialysate sample (E1–E7). These patients were treated with CAPD, and the middle-stage peritoneal dialysate samples were collected after a year. Each sample was analyzed individually and repeated three times.

To evaluate the reproducibility of the 2DE gels, twenty-one gels were subjected to image analysis. We found that approximately 95% of the spots can be detected within each triplicate experiment. Since the discrepancy of the gel images was determined by the reproducibility of the presence of each protein spots, here we counted the number of gels in which each spot appeared to evaluate the variance within each sample. Owing to the high similarity between the triplicate gels, a submaster gel can be generated from each patient sample at two different states. By combining all submaster gels (14 images), a master gel containing 368 spots was obtained. Among them, 163 (44.3%), 101 (27.4%), and 63 (17.1%) spots can be detected in 7, 6, and 5 gels of the early-stage gels (E1–E7), respectively, consisting of 88.8% of all the datable spots (Figure 1(a)). Besides, the remaining 14 (3.8%), 7 (1.9%), 11 (2.9%), 5 (1.4%), and 4 (1.1%) spots were detected in 4, 3, 2, 1, and 0 of the E1–E7 gels, respectively. As for the M1–M7 groups, as depicted in Figure 1(b), 9.5% of the 368 spots, 189 (51.4%), 96 (26.1%), and 44 (12.0%) spots, were found in 7, 6, and 5 gels, respectively. The other 11 (3.0%), 10 (2.7%), 6 (1.6%), and 5 (0.8%) spots were detected in 4, 3, 2, and 1

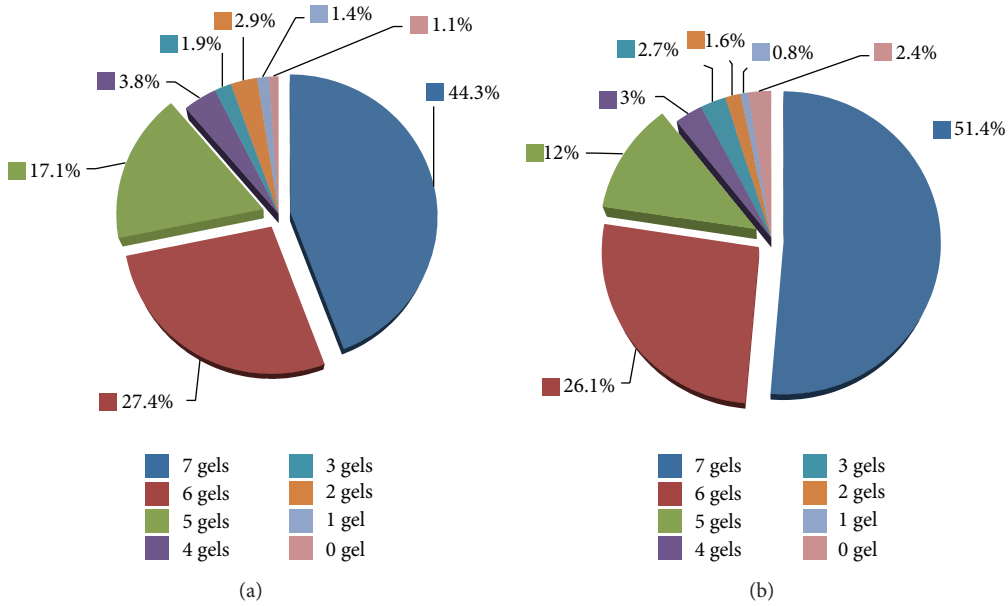

Figure 1: Evaluation of the individual difference within each of the 7 peritoneal dialysates collected from (a) early stage and (b) middle stage. A total of 386 spots were detected in the master gel of those 14 gels. Among the E1–E7 gels, 163 (44.3%), 101 (27.4%), 63 (17.1%), 14 (3.8%), 7 (1.9%), 11 (2.9%), 5 (1.4%), and 4 (1.1%) spots were detected in 7, 6, 5, 4, 3, 2, 1, and 0 of the early-stage gels, respectively. Among the M1–M7 gels, 189 (51.4%), 96 (26.1%), 44 (12.0%), 11 (3.0%), 10 (2.7%), 6 (1.6%), 5 (0.8%), and 9 (2.4%) spots were detected in 7, 6, 5, 4, 3, 2, 1, and 0 of the middle-stage gels, respectively.

of the middle-stage gels, respectively, while 9 (2.4%) spots were absent in all gels. The absence of spots in one group suggests that differences between early- and middle-stage peritoneal dialysate exist. Although several proteins showed differential expression, no consistent, statistically valid ($P < 0.05$) differences between the E1–E7 and M1–M7 groups were detected.

After evaluation of the similarity between samples, E1–E7 and M1–M7 (each with triplication) consisting of 42 gels were comparatively analyzed to identify CAPD induced protein change. Gel spots were analyzed by image analysis software for spot detection, spot matching, intensity normalization, and result output. Figure 2 was of two representative gels of early-stage (Figure 2(a)) and middle-stage peritoneal dialysate samples. The differential change of each spot was estimated by Student's t-test. Spots with expression levels that varied between E1–E7 and M1–M7 samples with a P value <0.05 were considered significant. A total of 4 spots (Figure 2(a)) had significantly higher protein expression levels in the early-stage peritoneal dialysate than that in the middle stage. Six spots (Figure 2(b)) showed significantly higher protein expression levels in the peritoneal dialysate after one year of CAPD treatment.

3.2. Protein Identification by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Analysis. To identify these 10 altered protein spots, P01–P10 gel spots were excised, enzymatically digested, and subjected to LC-MS/MS analysis. Each spot was identified by searching the raw data against human protein database. Detailed information about each spot was listed in Table 1. All the proteins were identified with at least three peptides, suggesting that protein identification is reasonably accurate. The 10 spots were found to represent 8 proteins. Among them, 4 protein spots, showing higher levels in the early-stage sample, P01, P02, and P03/P04, were identified as Ig mu chain C region, fibrinogen gamma chain, and C-reactive protein, respectively. Other 6 spots, Ig delta chain C region (P05), alpha-1-antitrypsin (P06), histidine-rich glycoprotein (P07/P08), apolipoprotein A-I (P09), and serum amyloid P-component (P10), were identified with higher level in middle-stage samples. As listed in Table 1, for most proteins, the experimental molecular weight (MW) measured by 2D PAGE corresponded to the theoretical MW annotated in protein sequence database. For Ig mu chain C region (P01) and Ig delta chain C region (P05), their observed MW was higher than the theoretical value, which may have resulted from the posttranslational modification (e.g., glycosylation). The experimental isoelectric point (pI) of most proteins matched the theoretical value found in the database. It has been known that protein modifications also can alter pI. Therefore, we hypothesized that inconsistencies between observed and predicted pI (e.g., P06 and P07/P08) may indicate the presence of protein modification or processing. The subcellular location and protein functions were analyzed through gene ontology and summarized in Table 2.

3.3. Protein-Protein Interaction Network Analysis. Proteins rarely act alone. Most of the time, they team up to carry out biological functions. Mapping of protein-protein interaction can help unravel the complicated molecular relationships in living systems. Here, in order to elucidate the main molecular interactions and biological connections, a protein-protein

Table 1: Identification of 10 altered spots, of which 4 showed higher levels in the early-stage PD samples, while 6 had higher levels in the middle stage.

Spot number[a]	Protein name	Swiss-Prot accession no.	Mr (E/T)[b] (kDa)	pI (E/T)[c]	No. of identified peptides	Sequence coverage (%)	Mascot score	Fold change ± SD[d]	Alteration (M/E)
P01	Ig mu chain C region	P01871	68/49	6.5/6.4	5	10.2	215	0.34 ± 0.20	Down
P02	Fibrinogen gamma chain	P02679	45/51	5.7/5.4	6	11.8	258	0.09 ± 0.02	Down
P03	C-reactive protein	P02741	25/25	5.5/5.5	4	16.2	145	0.13 ± 0.06	Down
P04	C-reactive protein	P02741	25/25	5.4/5.5	5	20.1	156	0.21 ± 0.09	Down
P05	Ig delta chain C region	P01880	70/47	6.2/6.8	3	6.3	124	2.91 ± 0.65	Up
P06	Alpha-1-antitrypsin	P01009	46/47	4.7/5.4	6	12.8	301	55.51 ± 13.71	Up
P07	Histidine-rich glycoprotein	P04196	53/59	5.6/7.0	4	6.7	186	8.13 ± 1.20	Up
P08	Histidine-rich glycoprotein	P04196	53/59	5.7/7.0	5	8.4	211	13.11 ± 3.05	Up
P09	Apolipoprotein A-I	P02647	25/31	5.3/5.6	4	12.9	201	23.67 ± 2.89	Up
P10	Serum amyloid P-component	P02743	27/25	5.7/6.1	6	24.5	298	5.78 ± 1.09	Up

[a]The spot numbers are designated in Figures 2(a) and 2(b).

[b]Mr (E/T): experimental observation of apparent molecular weight in 2-DE/theoretical molecular weight calculated from protein sequence database.

[c]pI (E/T): experimental observation of pI in 2-DE/theoretical pI calculated from protein sequence database.

[d]Fold change and standard deviation calculated from the protein spot intensity in middle-stage samples (M) versus that in early-stage samples (E).

Table 2: Gene ontology analysis of 8 differentially expressed proteins.

Spot number[a]	Protein name	Subcellular location	Biological process	Molecular function
P01	Ig mu chain C region	Plasma membrane	Immune response	Antigen binding
P02	Fibrinogen gamma chain	Extracellular space	Platelet activation, platelet degranulation, protein polymerization, response to calcium ion, and signal transduction	Cell surface binding and metal ion binding
P03/P04	C-reactive protein	Extracellular space	Acute-phase response, complement activation, regulation of lipid storage, regulation of macrophage, opsonization, protein polymerization, response to hypoxia, and response to lead ion	Cell surface binding, cholesterol binding, choline binding, low-density lipoprotein particle binding, and metal ion binding
P05	Ig delta chain C region	Extracellular space	Immune response	Antigen binding
P06	Alpha-1-antitrypsin	Extracellular space	Acute-phase response, platelet activation, platelet degranulation, and regulation of proteolysis	Serine-type endopeptidase inhibitor activity
P07/P08	Histidine-rich glycoprotein	Extracellular space	Angiogenesis, chemotaxis, regulation of angiogenesis, regulation of cell adhesion, regulation of cell growth and proliferation, regulation of fibrinolysis, platelet activation and degranulation, regulation of immune, response to tumor cell, and regulation of gene expression	Cell surface binding, cysteine-type endopeptidase inhibitor activity, heme binding, heparan sulfate proteoglycan binding, heparin binding, immunoglobulin binding, metal ion binding, serine-type endopeptidase inhibitor activity, and zinc ion binding
P09	Apolipoprotein A-I	Extracellular space	Cholesterol metabolism, lipid metabolism, lipid transport, steroid metabolism, sterol metabolism, and transport	Cell surface binding, heme binding, heparin binding, immunoglobulin binding, metal ion binding, and zinc ion binding
P10	Serum amyloid P-component	Extracellular space	Acute-phase response and protein folding	Metal ion binding and unfolded protein binding

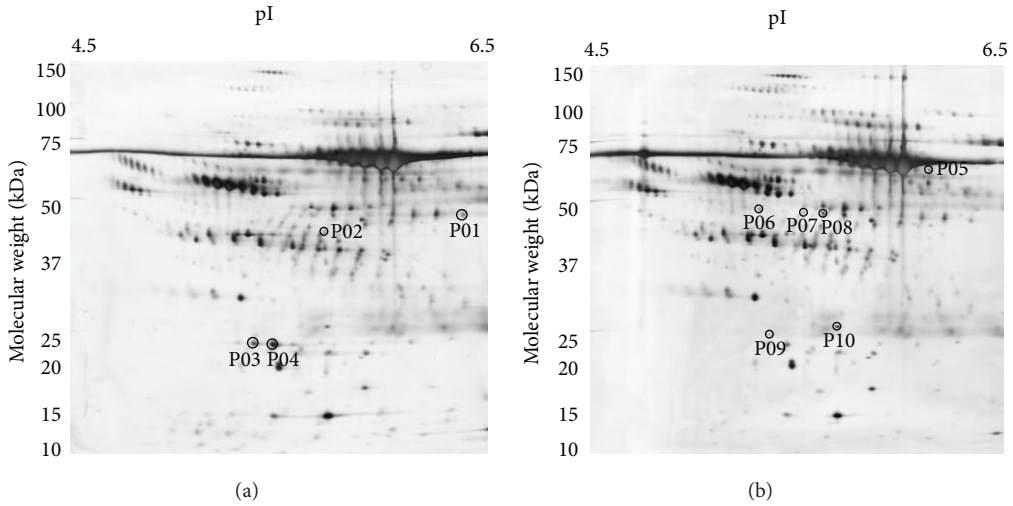

Figure 2: Representative 2DE gels of the (a) early-stage and (b) middle-stage samples. Each sample containing 140 μg was analyzed separately by 2DE (pH 4–7). The analysis of each sample was repeated three times. About 300 protein spots were detected on each gel, and a total of 386 protein spots were contained in the final master gel. Comparing between the two groups, 10 proteins were found to have differential levels, of which, 4 spots (P01–P04) had higher levels in the early-stage samples (pointed out by arrows in panel (a)), while 6 spots (P05–P10) showed higher levels in the middle-stage ones (indicated by arrows in panel (b)).

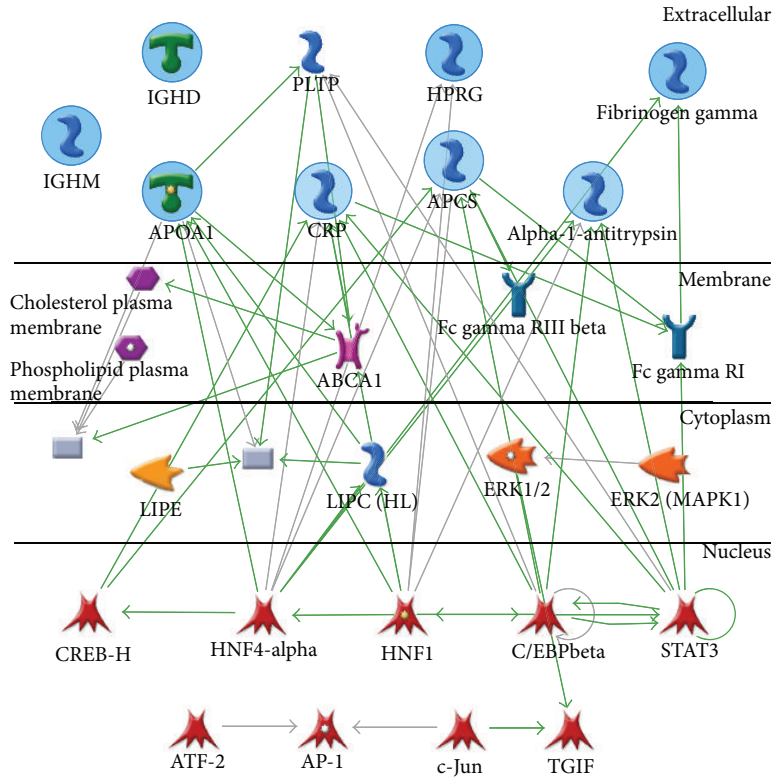

Figure 3: Interaction network analysis of 8 proteins that showed altered levels between early- and middle-stage groups. Proteins identified in this study, alpha-1-antitrypsin, serum amyloid P-component (APCS), C-reactive protein (CRP), fibrinogen gamma, apolipoprotein A-I (APOA1), histidine-rich glycoprotein (HPRG), Ig delta chain C region (IGHD), and Ig mu chain C region (IGHM), were marked with a circle. Full protein names for the abbreviation and legend for molecules used in MetaCore networks are provided in Supplementary Table B and Supplementary Figure A.

interaction network was constructed based on the 8 altered proteins by MetaCore (GeneGo, Inc., St Joseph, MI, USA). (illustrated in Figure 3). The full name of the abbreviation was provided in Supplementary Table B. The indication of the shape and color of the molecule was depicted in Supplementary Figure A. The protein molecules in the interaction network were laid out according to their cellular localizations. The network consisted of 25 proteins, among which, 6 proteins, alpha-1-antitrypsin, serum amyloid P-component (APCS), C-reactive protein (CRP), fibrinogen gamma chain, apolipoprotein A-I (HDL), histidine-rich glycoprotein (HPRG) were identified in our study (indicated with circles). These 6 proteins were involved in a network that contained receptor ligand (e.g., HDL), binding protein (e.g., phospholipid transfer protein, PLTR), receptor (e.g., high affinity immunoglobulin gamma Fc receptor I, Fc gamma RI), ion channel (e.g., ATP-binding cassette subfamily A member 1, ABCA1), kinase/phosphatase (e.g., ERK1/2), phospholipase (e.g., endothelial lipase, LIPE), and various transcription factors (e.g., c-Jun).

3.4. Validation of Protein Expression by Western Blotting. To further confirm the results of 2DE analysis, Western blotting was used to evaluate the expression levels of altered proteins in peritoneal dialysates from three other CGN patients (E8–E10 versus M8–M10). The fold change in the expression of each identified protein was obtained by dividing the mean band density of middle-stage sample by that of the early-stage samples (M/E). The expression levels of the three proteins, which were considered with higher levels in early-stage samples by 2DE, were confirmed by using Western blotting in three separate samples. As shown in Figure 4(a), Ig mu chain C region (P01, fold change = 0.15), fibrinogen gamma chain (P02, fold change = 0.21), and C-reactive protein (P03/P04, fold change = 0.08) were detected. As for those proteins that were found to have higher levels in middle-stage samples, the values of fold change evaluated by Western blotting were 4.6, 3.3, 5.4, 3.9, and 7.5 for Ig delta chain C region (P05), alpha-1-antitrypsin (P06), histidine-rich glycoprotein (P07/P08), apolipoprotein A-I (P09), and serum amyloid P-component (P10), respectively (Figure 4(b)). These results are consistent with that from 2DE analysis.

4. Discussion

In order to comprehensively realize the protein group involved, a protein network analysis of the 8 altered proteins was done to deduce their interacting proteins. Six of the 8 identified proteins can be linked through protein-protein interaction, suggesting their close association in the system.

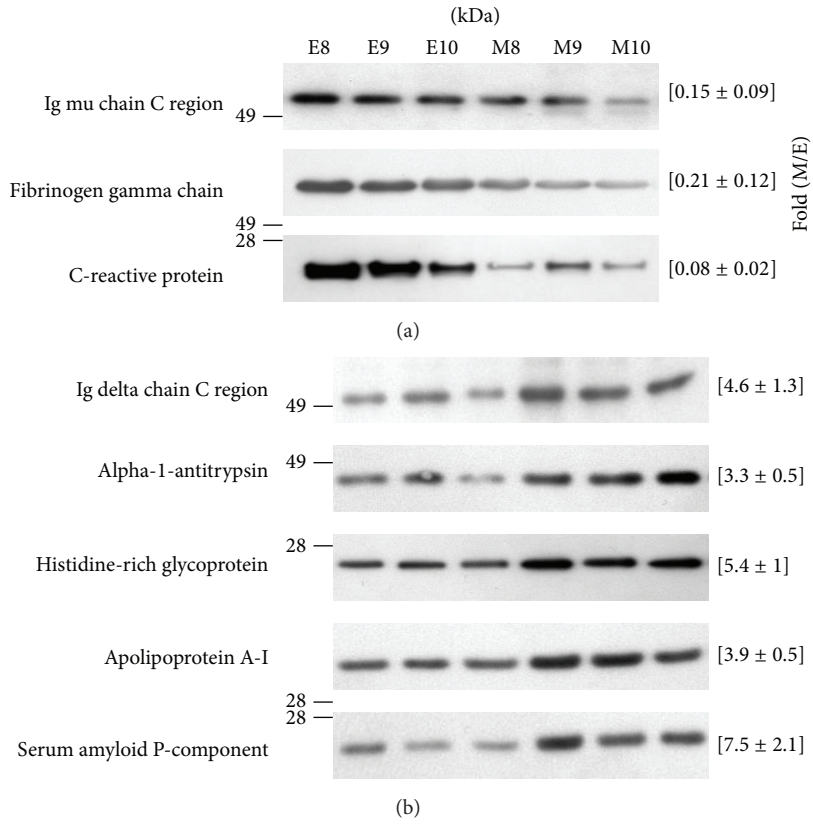

Figure 4: Western blotting of the identified proteins in peritoneal dialysates collected from other three CGN patients at their first time (E08–E10) and after a year (M08–M10) of receiving CAPD treatment to further validate these protein candidates. The mean band density of E08–E10 and M08–M10 was calculated and used to obtain the fold change between the two groups (M/E). The fold is indicated in the bracket to the right of each set of bands. Western blotting confirmed that (a) Ig mu chain C region, fibrinogen gamma, and C-reactive protein show higher expression levels in the early-stage samples than in the middle-stage samples, while (b) Ig delta chain C region, alpha-1-antitrypsin, histidine-rich glycoprotein, apolipoprotein A-I, and serum amyloid P-component were observed to have higher levels in the dialysate after one year of CAPD treatment.

Some interesting proteins were also recruited in the network and were found to be related to the process of inflammation or immune response. ABCA1 deficiency can lead to impaired cholesterol efflux to HDL or apolipoprotein A-I (APOA1), decreased apoE secretion, and increased secretion of inflammatory cytokines and chemokines [11]. In the deletion of Fc gamma RI, antibody responses were elevated, implying its role as a control point in the development of immune responses [12]. The copy number variation of the FCGR3B gene (Fc gamma RIII beta) is found to be associated with immune complex clearance [13]. The downstream moles in cytoplasm and nucleus, such as ERK1/2, c-Jun, AP-1, ATF-2, and STAT3, have been known to participate in inflammatory response [14–17]. Those interacting proteins indicate that these differentially expressed proteins are involved in peritoneal inflammation.

We speculated that proteins identified with higher levels in the first-time dialysate suggested their prevailing expression in CGN patients. Ig mu chain C region (P01) was identified with higher levels in the initial peritoneal dialysate. According to a previous work, patients with primary glomerulonephritis showed IgM as dominant molecules in glomerular by immunofluorescence [18]. A recent work found that mouse with Ig mu chain C region gene knockout was devoid from the glomerular damage, implying the requirement of Ig mu chain C region in the development of glomerulonephritis. Several lines of evidence supporting the correlation between fibrinogen and glomerulonephritis have been obtained [19]. Our finding of the appearance of fibrinogen gamma chain (P02) in the peritoneal dialysate from CGN patients is consistent with those previous works. The expression of human C-reactive protein (P03/P04) in the lupus-prone NZB/NZW F1 mice has delayed the initiation of glomerulonephritis and death [20], suggesting that high level of C-reactive protein may present a self-protective process against glomerulonephritis.

Proteins that have higher levels in peritoneal dialysate after one year of CAPD treatment may shed light on the mechanism or potential marker for initial peritoneal damage. The main role of Ig delta chain C region (P05) in immune system has been believed to be B-cell receptor. The expression of Ig delta chain C region is required in mature B cells and a key modulator of the humoral immune response (reviewed in [21]). It has been known that alpha-1-antitrypsin

(P06) is detectable in peritoneal dialysate of peritonitis-free patients. In peritonitis-free patients, alpha-1-antitrypsin prevents the proteolytic action and cell activation, leading to platelet activating factor synthesis. However, the inactivation of its function by oxidants resulted in proteolytic injury and unrestrained synthesis of inflammatory mediators during peritonitis [22]. Histidine-rich glycoprotein (P07/P08) has been shown to bind heparin [23], plasminogen [24], and fibrinogen [25] and to regulate aspects of the immune system [26, 27]. Histidine-rich glycoprotein is an important regulator of immune complexes uptake by monocytes [28]. Levels of plasma histidine-rich glycoprotein have been found to decrease during acute states of disease such as advanced liver cirrhosis [29], AIDS, renal disease, asthma, and pulmonary disease [30]. Histidine-rich glycoprotein can interact with different cell surface receptors and bind a wide range of ligands, and it has been involved in the regulation of numerous biological functions, particularly angiogenesis, immune complex clearance, and coagulation (reviewed in [31]). Apolipoprotein A-I (P09) was higher in middle-stage than in early-stage samples, which is consistent with previous studies in which patients with high transport rates in peritoneum also had higher apolipoprotein A-I in dialysate when compared to those with low transport rates [6]. Apolipoprotein A-I is the major component of HDL and functions as an acceptor for the sequential transfer of phospholipids and free cholesterol from peripheral tissue. It also facilitates the transport of cholesterol to the liver and other tissue for excretion and steroidogenesis. [32]. The loss of apolipoprotein A-I in peritoneal dialysate effluents led to the decrease of apolipoprotein A-I and HDL in plasma [33], which implied the reason why patients with high solute transport rates are prone to develop atherosclerosis [34]. Serum amyloid P component (P10) is an acute phase protein, structurally related to C-reactive protein, which is synthesized in response to the proinflammatory cytokines early in the inflammatory. Serum amyloid P component interacts with inflammatory and complement factors, acting as an opsonin that reacts with nuclear autoantigens in systemic autoimmunity [35]. Mice with targeted deletion of serum amyloid P component gene lead to the development of immune complex glomerulonephritis. In adult female mouse, homozygous mutant mouse with serum amyloid P component gene knockout increases glomerulonephritis [36]. Both studies designated that this protein is crucial for the development of glomerulonephritis. The observation of those proteins showing differential levels at two stages in peritoneal dialysate may provide novel aspects for the study initial peritoneal change related to peritoneal dialysis. These proteins may help elucidate the mechanism of inflammation, peritoneal structural change, kidney damage, atherosclerosis, angiogenesis, and fibrosis related to dialysis.

This study has its limitations. It is very difficult to identify peritoneal damage unless invasive methods are used. Therefore, we cannot define the condition of peritoneal change, but we can assume that the peritoneum has slightly changed after the long-term CAPD treatment. This is a major obstacle in our experimental research. In addition, the small number of patients may contribute to the limited biological and clinical relevance of our findings. To address this issue, the differentially expressed proteins were further validated in another set of 3 CGN patients. This work has provided potential targets and mechanisms of the change due to peritoneal dialysis processes, which have been confirmed by mass spectrometry data and Western blotting. Before being applied clinically, further experiments are considered necessary to evaluate those targets in a sufficient number of participants.

In conclusion, to our knowledge, this is the first study to show differential proteomic profiling between peritoneal dialysates from CGN patients at the early and middle stage of CAPD treatment. The identified proteins may provide clues to the PD-induced loss of proteins from the peritoneum and assist the identification of potential biomarkers for noninvasive monitoring of peritoneal damage. Further studies of these potential proteins are needed for accessing their roles in both basic research and clinical monitoring.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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