Potential biomarker proteins for aspiration pneumonia detected by shotgun proteomics using buccal mucosa samples: A cross-sectional study

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Abstract

Background: Aspiration pneumonia (AP), which is a major cause of death in the elderly, does present with typical symptoms in the early stages of onset, thus it is difficult to detect and treat at an early stage. In this study, we identified biomarkers that are useful for the detection of AP and focused on salivary proteins, which may be collected non-invasively. This is because expectorating saliva is often difficult for elderly people, so we collected salivary proteins from the buccal mucosa.

Methods: We collected samples from the buccal mucosa of six patients with AP and six control patients (no AP) in an acute-care hospital. Following protein precipitation using trichloroacetic acid and washing with acetone, the samples were analyzed by liquid chromatography and tandem mass spectrometry (LC-MS/MS). We also determined the number of cytokines and chemokines in non-precipitated samples from buccal mucosa.

Results: Comparative quantitative analysis of LC-MS/MS spectra revealed 179 and 181 proteins that were significantly higher and lower in abundance, respectively, in the AP group compared with the control group. We identified eight proteins (chloride intracellular channel protein 3, b-defensin 4A, protein S100-A7A, Purkinje cell protein 4-like protein 1, glutamine synthetase, protein S100-A7, cytosolic 5'-nucleotidase 3A, pyridoxal kinase) that were detected in all of the AP samples with high coverage, indicating that these proteins are promising AP biomarker candidates. In addition, the abundance of C-reactive protein (CRP) in oral samples was highly correlated with serum CRP levels, suggesting that oral CRP levels may be used as a surrogate to predict serum CRP in AP patients. A multiplex cytokine/chemokine assay revealed that MCP-1 tended to be low, indicating unresponsiveness of MCP-1 and its downstream immune pathways in AP.

Conclusion: Our findings suggest that oral salivary proteins, which are obtained non-invasively, can be utilized for the detection of AP.

Background

Aspiration pneumonia (AP) is caused by inhaling saliva, food, or vomit, which results in bacterial infection [1–3]. Aspiration, defined as the inhalation of oropharyngeal or gastric contents into the larynx and lower respiratory tract, is often the result of impaired swallowing resulting from dysphagia, head/neck/esophageal cancers, esophageal stricture, chronic obstructive pulmonary disease, or seizures. This allows oral and/or gastric contents to enter the lung, especially in patients with an inefficient cough reflex [1]. In addition to swallowing, impaired consciousness, because of degenerative neurologic disease or cardiac arrest, is also a risk factor for AP[2, 4].

Although the detection of the causative bacteria results in prompt treatment with antibiotics, it is often difficult to distinguish the infectious from non-infectious bacteria in oral microbiota. Oral bacteria are present at various sites within the human oral cavity [5]. In addition to the oral microbiota, recent reports indicate that lung microbiota is involved in pneumonia [6, 7]. Boaden et al. identified 103 different

bacterial phylotypes from oral microbiota in patients suffering from acute stroke [8]. In addition, there are also a variety of causative bacteria. One study identified 67 pathogens detected in 95 institutionalized elderly patients with severe AP [4].

It is unclear whether AP represents a distinct entity from typical pneumonia [1, 9, 10]. Based on a previous report which estimated that AP accounts 5–15% of the cases of community-acquired pneumonia, Mandell et al. proposed that AP should not be considered a distinct entity, but rather part of a continuum that also includes community- and hospital-acquired cases of pneumonia [1]. Recent studies have indicated that the composition of salivary proteins reflects oral and systemic conditions [11]. For example, salivary proteins may apply to the detection of localized oral diseases, such as head and neck cancer [12] and Sjogren's syndrome [13, 14], as well as systemic diseases, such as diabetes mellitus [15–17], and viral infections [18]. Based on reports regarding a relationship between oral proteomics and disease, we suspect that some salivary proteins may be used as biomarkers for the detection of AP at the early stage of onset.

It is not easy for elderly people, particularly bedridden patients with neurologic or cerebrovascular disease, to eject a sufficient volume of saliva. In addition, saliva production is likely decreased because of decreased chewing frequency or drug treatment. In the present study, we collected saliva proteins from the buccal mucosa, where the ostia of Stensen’s ducts are located. By cleaning the buccal mucosa before sample collection, contaminates are readily removed. Using shotgun proteomics for a comparative quantitative analysis between AP and control patients, we identified eight candidate AP biomarkers. We also found a significant correlation between serum and oral C-reactive protein (CRP) levels. In addition, we evaluated a panel of cytokines and chemokines to determine the responsiveness of immune-related proteins.

Methods

Subjects

We collected samples from the buccal mucosa of six AP and six control patients in an acute-care hospital in Ishikawa Prefecture (Japan) from September 2021 to December 2021. The characteristics of the 12 patients are listed in Tables 1 and S1. Five of the six AP patients and one of the six controls had been treated with antimicrobial agents at the time of collection. Five of the AP patients had a history of AP, whereas none of the control ones had been previously diagnosed with AP. Medical information (age, gender, body mass index, underlying diseases, blood test data, and dietary intake method) was obtained from the electronic medical records. Oral conditions were assessed by the Oral Health Assessment Tool (OHAT) [19]. The number of remaining teeth and the presence of intra-oral bleeding were determined and the buccal mucosa was assessed for dryness.

Table 1
Characteristics of the subjects

	AP ($n = 6$)			Ctrl ($n = 6$)			*P*-value[†]
Age (years)	87.2	±	5.5	78.3	±	10.3	0.09
Body Mass Index	15.3	±	0.2	21.6	±	3.6	0.00
Blood data							
CRP (mg/dl)	4.99	±	4.27	1.80	±	2.86	0.16
WBC (10^3/μl)	8.07	±	3.58	8.57	±	4.30	0.83
Alb (g/dl)[*]	2.78	±	0.59	2.80	±	1.62	0.98
Oral states							
Residual teeth	6.8	±	8.5	8.2	±	12.8	0.84
OHAT	3.2	±	2.2	1.2	±	1.2	0.08

[†] *P* values were calculated by Student's unpaired *t*-test.

[*]Albumin concentrations of the AP ($n = 4$) and control ($n = 4$) patients. The other data were obtained from the AP ($n = 6$) and control ($n = 6$) patients.

Sample collection from the buccal mucosa

Before sample collection, a dentist confirmed that at least 2 h had passed since the previous meal. After removing visible food residue, samples were collected from the buccal mucosa using a Hummingood sponge brush (Molten Corporation, Hiroshima, Japan), which had been dipped into 5 mL saline in a 50mL tube and squeezed briefly onto the side of the tube. The samples were collected by placing the brush on the buccal mucosa, rubbing "back and forth" 10 times at a rate of 1 rub/second. The sponge was returned to the saline-containing tube, pressed, and squeezed tightly. After collection, the samples were stored at − 20°C, thawed, and centrifuged at 3,000 × g for 5 min at 4°C. The supernatants were used for further analysis by LC-MS/MS and multiplex assays.

LC-MS/MS analysis

The supernatant was precipitated using trichloroacetic acid (TCA) and washed with acetone. The precipitate was air-dried at room temperature and dissolved in 40 μL of 6 M Urea containing 50 mM triethylammonium bicarbonate. After measuring the protein concentration with a Pierce BCA Protein Assay Kit (ThermoFisher Scientific), 1 μg of protein was alkylated reductively and digested with trypsin. The digested peptides were fractionated, purified, and analyzed by nano-liquid chromatography and tandem mass spectrometry (LC-MS/MS; Thermo Orbitrap QE plus: ThermoFisher Scientific). A comparative analysis of the detected protein and label-free quantitation was performed using Proteome Discoverer software (Thermo Fisher Scientific).

Multiplex cytokine assay

After measuring protein concentration using the BCA Protein Assay Kit, the supernatants (not precipitated by TCA) were applied to a LEGENDplex Human Inflammation Panel 1 (13-plex: IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, IL-33) in a V-bottom Plate (BioLegend, San Diego, USA). The concentrations of the target proteins were standardized to total protein concentration.

Results

Sample collection and patient information

We collected buccal mucosa samples from AP (n = 6, age 79–93 years old) and non-AP (Control; n = 6, age 66–93 years old) patients. Three samples (AP#3, AP#5, and AP#6) were collected from the patients with Parkinson's disease, three (AP#2, AP#4, and Control#1) from those with Alzheimer's disease, and two (Control#2 and Control#6) with Lacunar infarction. The duration from the onset to the collection time varied from 1 to 18 days (Table S1). Body mass index was low in the AP group (Table 1). No significant differences were observed in the blood for CRP concentration, several white blood cells, or serum albumin concentration. While there was no difference in the number of residual teeth, the OHAT score tended to be higher in the AP group, indicating an unhealthy oral state of the AP patients.

Comparative quantitative analysis of oral proteins

After TCA precipitation and acetone wash, protein solutions were obtained with concentrations ranging from 0.65 to 2.49 mg/mL. The LC-MS/MS analysis detected a total of 3,528 proteins including 3,253, 157, and 118 proteins at high-, middle-, and low-confidence levels, respectively, based on the false discovery rate (FDR; Table S2). No significant difference was observed in the distribution of abundance (Figure S1). Among the proteins, the abundance of 179 and 181 proteins was high in the AP group and the control group, respectively (Fig. 1). Principal component analysis (PCoA) revealed that the AP #4 sample was distinct from the other 11 samples, and there was no significant difference in the PCoA profiles between the AP and control groups (Figure S2).

Using the significantly higher abundant proteins from each group, we carried out a gene ontology enrichment analysis [20, 21]. No significant results (P < 0.05 by FDR) were observed for biological process or molecular function for the 179 proteins that were higher in the AP group. In contrast, the molecular functions, nucleosome-binding and small molecule binding, were detected using the 181 proteins that were expressed higher in the control group. Although histones are involved in bacterial killing as a major component of neutrophil extracellular traps [22], the involvement of these proteins in the non-occurrence of AP is unknown.

Serum and Oral C-reactive protein (CRP)

Ouellet-Morin et al. reported a moderate-to-strong association between CRP measured in saliva and serum (r = 0.72) [23]. In the present study, there was no significant difference in CRP concentration either

in the blood (P = 0.16) or oral cavity (P = 0.71) between the AP and control groups. Nonetheless, Spearman's correlation coefficient was 0.86 (P = 0.001) between blood and oral CRP levels, indicating that serum CRP may be predicted non-invasively using oral CRP values, which is consistent with the previous report [23] (Fig. 2). It is unclear why the abundance of oral CRP was relatively high in Control #3.

Detected proteins

Glycoproteins

A variety of glycoproteins play important roles in innate and adaptive immunity [24]. In the AP group, we found a low abundance of α-2-HS-glycoprotein (Fetuin; Abundance Ratio AP/Control = 0.38, P = 0.02) and β-2-glycoprotein 1 (Ratio = 0.39, P = 0.04), both of which are primarily present in blood and exhibit various physiological functions [25, 26]. Alpha-2-HS-glycoprotein, a negative acute-phase reactant [27], is decreased during the early period of sepsis but begins to increase from 72 days post-inflammation [28]. The β-2-glycoprotein 1 possesses the dual capability of up- and down-regulating complement and the coagulation systems, depending upon the external stimulus as well as CRP and thrombomodulin [26]. It is unclear whether its lower abundance is associated with the occurrence of AP.

S100 protein family

The S100 proteins, a family of calcium-binding cytosolic proteins, are known as damage-associated molecular pattern molecules and they exhibit a variety of intracellular and extracellular functions [29]. Protein S100A-7A (Ratio = 6.51, P < 0.01) and S100A-7 (Ratio = 4.33, P = 0.02) were higher in the AP group; however, there was no difference in the levels of other S100 protein family members.

Cytokines and chemokines

The LC-MS/MS detected some interleukins (ILs), however, there was no difference in IL-1α, IL-8, or IL-18 levels between the groups. Isoform 2 of IL-19 and isoform 2 of IL-36β, but not IL-36α, were higher in the AP group (Ratio > 100, P < 0.01), whereas IL-36γ also tended to be high (Ratio = 3.26, P = 0.14). For the chemokines, C-C motif chemokine 28 was low in the AP group (Ratio = 0.39, P < 0.01). The IL-36 cytokines, which include IL-36α, IL-36β, IL-36γ, and IL-36Ra, belong to the IL-1 family and exert pro-inflammatory effects on various target cells, such as keratinocytes, synoviocytes, dendritic cells, and T cells [30]. Ramadas et al. showed that IL-36γ is upregulated in airway epithelial cells and involved in chemokine (neutrophil chemoattractants CXCL1 and CXCL2) production and neutrophil influx in mice challenged with a house dust mite extract [31]. In contrast, the abundance of the IL-36 receptor antagonist protein also tended to be higher in AP samples compared with the control samples (P = 0.089). Our data suggest that various IL-36-related signaling pathways are involved in the onset of AP.

Non-salivary proteins

In the Human Body Fluid Proteome database, 2,871 proteins have been registered as saliva proteins as of May 2022 [32], and 1,973 of the 3,528 proteins detected in the present study were registered as salivary

proteins in the database. Of the 1,555 non-salivary proteins, 130 were in high abundance in the AP group, whereas only six were detected with > 20% coverage. Mago Nashi Homolog 2 (Magoh2) was detected in five of the six AP samples, but not in any of the Control samples. Although Magoh proteins contribute to exon junction complexes [33], it is unclear whether the Magoh2 protein is involved in the onset of AP.

Multiplex cytokine assay

In our shotgun proteomics analysis, we did not detect peaks for IL-1β, IL-6, TNF-α, or MCP-1 (Table S2). In the multiplex cytokine/chemokine assay, the values for IL-6 and TNF-α were under the limit of detection (IL-6, < 6.80 pg/mL; TNF-α, < 0.73 pg/mL) in most of the oral samples. Using the supernatant without TCA precipitation, we also conducted a multiplex cytokine/chemokine assay. The protein concentrations of the supernatant ranged from 0.012 to 0.27 mg/mL, which were likely dependent on the strength of rubbing. Among the 13 cytokine and chemokine proteins, IL-1β, MCP-1, IL-8, and IL-18 were detected in all 12 samples. The concentrations were normalized to the total protein concentration. There were no significant differences between the AP and control groups, although MCP-1 levels tended to be lower (P = 0.065 by Mann–Whitney U test; Fig. 3).

Biomarker candidates

Besides the proteins described above, the LC-MS/MS analysis detected various proteins, some of which the function are unknown. An essential requirement for AP biomarkers is that the amount must be sufficiently high to be detected by common and convenient assays, such as ELISA, western blot analysis, or immunochromatography. Among the 360 proteins with a P-value < 0.05, we selected up to 60 proteins with a coverage of > 20%. We then selected 36 proteins that were detected in all six AP patients (Table 2). Among the 36 proteins selected, eight proteins (chloride intracellular channel protein 3, β-defensin 4A, protein S100-A7A, Purkinje cell protein 4-like protein 1, glutamine synthetase, protein S100-A7, cytosolic 5'-nucleotidase 3A, pyridoxal kinase) were higher in the AP group (Fig. 4). The remaining 28 proteins were lower in the AP group and included properdin, high mobility group nucleosome-binding domain-containing protein 5, beta-2-glycoprotein 1, C-C motif chemokine 28, and alpha-2-HS-glycoprotein. Using the 32 proteins detected in all of the samples, we prepared a cluster heat map (Fig. 5), which separated the AP and control samples into separate clusters.

Table 2
Proteins with higher abundance between the AP and control groups

Accession (Uniprot ID)	Protein name	Coverage [%]	Abundance Ratio (AP) / (Control)	*P*-Value
O95833	Chloride intracellular channel protein 3	83	6.56	0.001
Q86SG5	Protein S100-A7A	69	6.51	0.000
A6NKN8	Purkinje cell protein 4-like protein 1	32	6.28	0.002
P15104	Glutamine synthetase	60	5.55	0.003
O15263	Beta-defensin 4A	36	5.10	0.011
P31151	Protein S100-A7	87	4.33	0.024
Q9H0P0	Cytosolic 5'-nucleotidase 3A	42	4.16	0.041
O00764	Pyridoxal kinase	37	4.13	0.019
Q03591	Complement factor H-related protein 1	60	0.46	0.037
O60911	Cathepsin L2	62	0.44	0.023
P27918	Properdin	27	0.43	0.042
P82970	High mobility group nucleosome-binding domain-containing protein 5	27	0.42	0.048
O60234	glia maturation factor gamma	51	0.41	0.048
Q92522	Histone H1x	28	0.41	0.015
P80723	Brain acid soluble protein 1	80	0.40	0.024
P02751	fibronectin	24	0.39	0.015
P02749	Beta-2-glycoprotein 1	58	0.39	0.041
Q9NRJ3	C-C motif chemokine 28	21	0.39	0.008
P02765	Alpha-2-HS-glycoprotein	47	0.38	0.018
P46527	Cyclin-dependent kinase inhibitor 1B	62	0.37	0.006
P29966	Myristoylated alanine-rich C-kinase substrate	51	0.37	0.029
O76070	Gamma-synuclein	75	0.37	0.028
P58062	Serine protease inhibitor Kazal-type 7	62	0.36	0.007

The proteins were detected in all the six AP samples with > 20% coverage. *P* values were calculated by an ANOVA test and adjusted by the Benjamini-Hochberg method.

Accession (Uniprot ID)	Protein name	Coverage [%]	Abundance Ratio (AP) / (Control)	*P*-Value
A8MWD9	Putative small nuclear ribonucleoprotein G-like protein 15	25	0.36	0.030
P31025	Lipocalin-1	61	0.36	0.025
Q9H1E3	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1	54	0.35	0.021
P20930	Filaggrin	34	0.33	0.012
P02766	Transthyretin	73	0.32	0.009
P07305	Histone H1.0	21	0.30	0.006
P56134	ATP synthase subunit f, mitochondrial	26	0.28	0.005
P07476	Involucrin	73	0.28	0.003
O75396	Vesicle-trafficking protein SEC22b	30	0.28	0.037
Q6P5S2	Protein LEG1 homolog	41	0.24	0.001
O95274	Ly6/PLAUR domain-containing protein 3	45	0.23	0.001
O75556	Mammaglobin-B	49	0.22	0.004
P41439	Folate receptor gamma	53	0.17	0.026

The proteins were detected in all the six AP samples with > 20% coverage. *P* values were calculated by an ANOVA test and adjusted by the Benjamini-Hochberg method.

Discussion

Underlying diseases can affect the composition of oral proteins. Figura et al. reported lower concentrations of S100-A16, ARP2/3, and VPS4B in the saliva of the Parkinson's disease group compared with the healthy control group [34]. Although the three proteins were detected with high confidence in the present study, there was no significant difference between the three Parkinson's disease samples (AP#3, AP#5, and AP#6) and the other nine samples. Concerning Alzheimer's disease, Contini et al. reported higher levels of S100A8, S100A9, α-defensins, and cystatins A and B in patients with Alzheimer's disease compared with healthy volunteers [35]. Although our analysis detected α-defensin, there was no significant difference between the three samples from Alzheimer's disease patients (AP#2, AP#4, and Control#1) and the other nine samples. These data suggest that the changes in the protein concentration disappear in patients suffering from AP.

Intracellular chloride channels are known for their role in cardiovascular, neurophysiology, and tumor biology [36]. Although chloride intracellular channel protein 3 (CLIC3) is shown to be involved in malignancies, Brum et al. reported that CLIC3 is involved in bone formation [37]. Therefore, we need to examine carefully whether CLIC3, which varies in abundance depending on body weight, is a putative biomarker for AP because there was a difference in body weight between the AP and control groups. In addition, there was a significant difference in body mass index between the AP and control groups. Thus, this protein may instead be involved in osteoporosis.

Beta-defensin 4A (hBD-4) is a member of the beta-defensin family of antimicrobial peptides that are expressed in the oral epithelium [38]. The hBD-4 exerts its antimicrobial activity by disrupting the cell membrane of Gram-negative and -positive bacteria and fungi [39]. The hBD-4 expression is upregulated in the bronchial and bronchiolar epithelium of patients with chronic lower respiratory tract infections. Thus, elevated hBD-4 levels in buccal mucosa samples may indicate infection in the oral cavity and/or airways [40].

The S100 protein family consists of approximately 20 members, which are not only involved in cell proliferation, differentiation, migration, and apoptosis but are also thought to be closely related to cancer and neurodegenerative diseases [41]. Of these, S100-A7 is abundant in the saliva of patients with systemic sclerosis [42] and has recently been reported to act as an antimicrobial peptide. The S100-A7 is produced and [43, 44] released by epithelial cells on the tongue and has been shown to exhibit antimicrobial activity against *Escherichia coli* (*E. coli*) [45]. The S100-A7 may be a useful biomarker for AP.

Purkinje cell protein 4-like 1 (PCP4L1) is a member of the calpacitin family [43, 44], however, there are only a few reports describing its function. Morgan et al. reported in 2012 that the isoleucine-glutamine motif of Pcp4l1, but not the full-length PCP4L1, binds to calmodulin and inhibits calmodulin-dependent kinase II [46]. Bulfone et al. showed that PCP4L1 is expressed only in the central nervous system [44]. Concerning cytosolic 5'-nucleotidase 3A (NT5C3A), Fu et al. reported that treating human macrophages with JNJ872, a potent drug used against influenza A viruses, resulted in NT5C3A expression [47]. Miller et al. proposed that NT5C3A is a candidate biomarker in plasma for the diagnosis of sports concussions; however, it is unclear whether Pcp4l1 and NT5C3A are involved in AP.

Cytokines and chemokiness are known markers of inflammation in response to bacterial infection. Although MCP-1 is a chemokine that recruits monocytes to the foci of active inflammation [48, 49], it was detected in all samples by the multiplex assay and the values tended to be lower in the AP group compared with the control group (Fig. 3). McGrath-Morrow et al. showed that in a lower respiratory tract model of E. coli infection, the host defense against the bacterium was mediated by MCP-1 and its receptor, CCR2 [50]. Low MCP-1 levels in AP patients may explain the reduced resistance to infection [51].

Conclusion

In this study, we identified putative biomarkers applicable to the detection of AP at an early stage. We found eight candidate proteins that may be considered biomarkers of AP. This study had several limitations, which included the varied duration from onset to collection (1–18 days post-onset). It remains unclear whether the candidate proteins identified in this study increase or decrease in the early stages of the disease. To address this issue, long-term prospective studies need to be conducted that evaluate samples from pre-onset to the onset of AP.

List of abbreviations

AP, aspiration pneumonia; CRP, C-reactive protein; LC-MS/MS, nano-liquid chromatography and tandem mass spectrometry

Declarations

Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of Kanazawa University and the acute-care hospital and conducted following the Declaration of Helsinki and the Microorganism Safety Management Regulations of Kanazawa University. Written informed consent was obtained from the participants or his/her guardians before inclusion in the study. The participants' participation in this study was voluntary and the procedure for withdrawing consent was explained.

Consent for publication

All authors consent to the publication of this manuscript.

Availability of data and materials

The LC-MS/MS data are described in Table S2.

Competing interests

The authors declare no potential conflicts of interest with respect to the study.

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Author contributions

KoO designed this study, analyzed the data, and wrote the manuscript. ME conducted the experiments, analyzed the data, and drafted a part of the manuscript. TH and HN collected the oral samples. TK conducted the multiplex cytokine/chemokine assay and revised the manuscript for the intellectual

content. TS contributed to the selection of biomarker candidates. KaO contributed to the data analysis. HS launched a research project including this study. SO and JS supervised this study. SO contributed to the ethical approval. All authors contributed to the revision of the manuscript.

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Figures

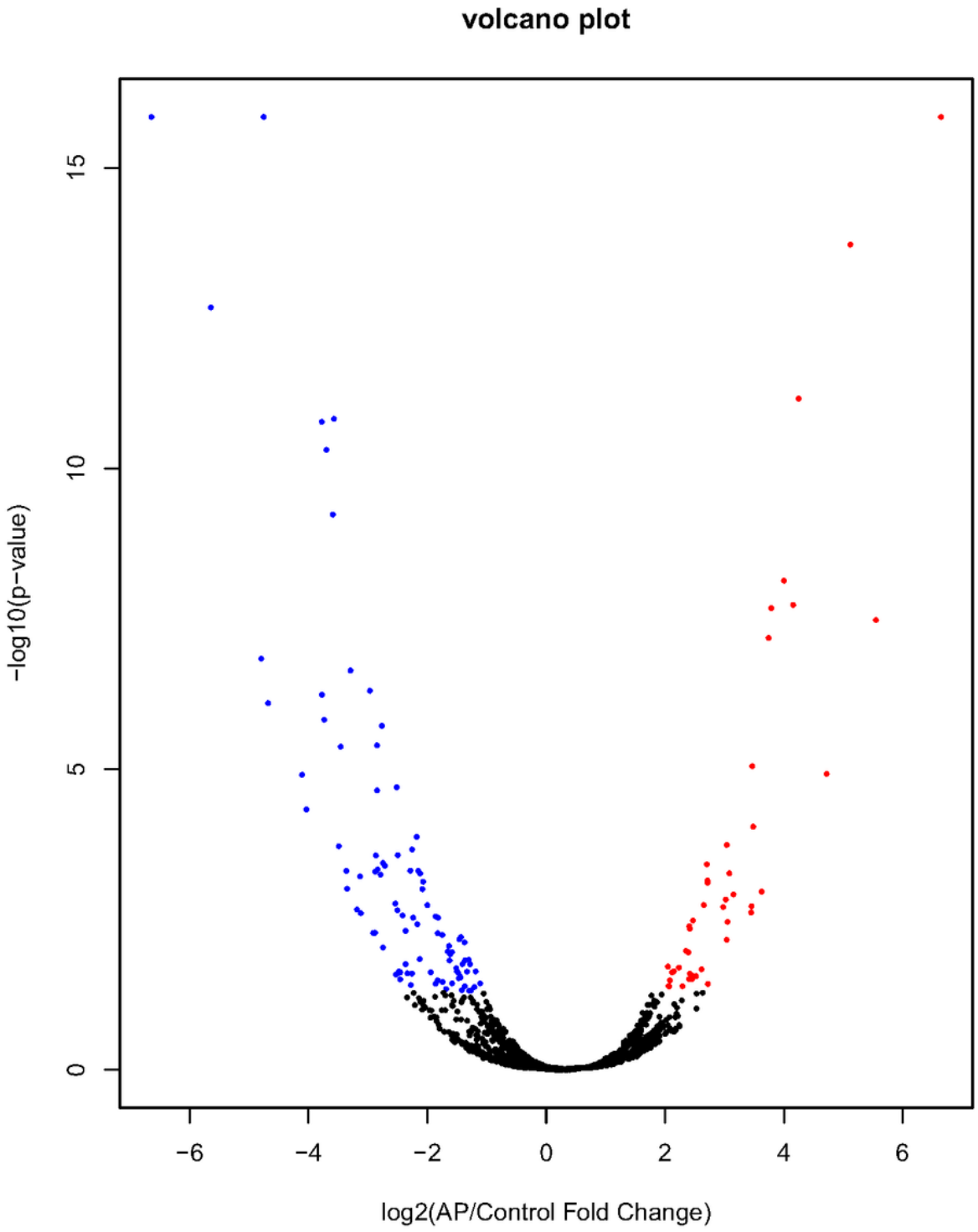

Figure 1

Volcano plots of the proteomic data. The plots were generated using the abundance ratio = Log_2(AP/Control). *P* values were calculated by ANOVA and adjusted by the Benjamini-Hochberg method.

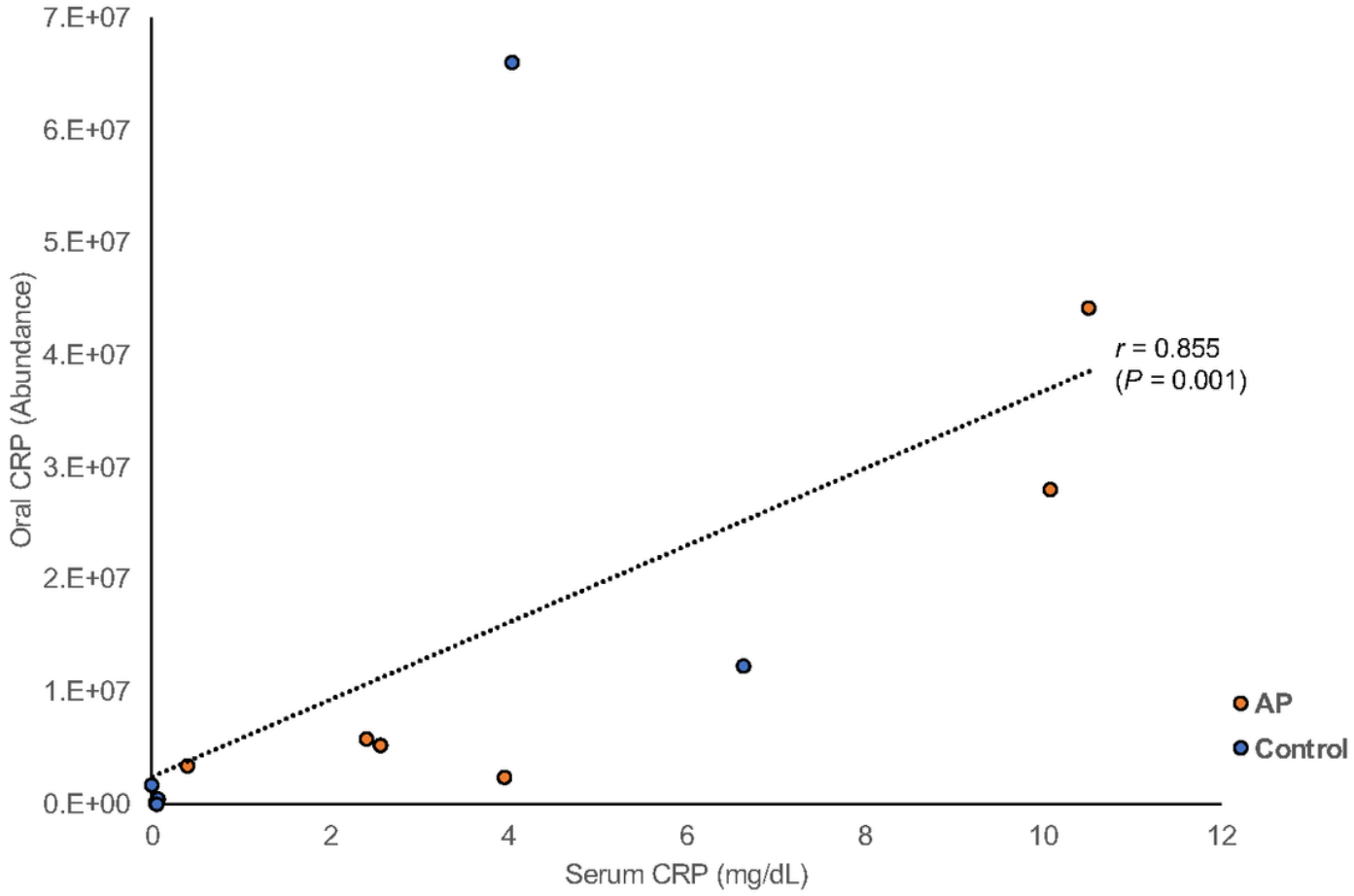

Figure 2

Correlation between serum and oral CRP levels (Spearman's rank correlation coefficient).

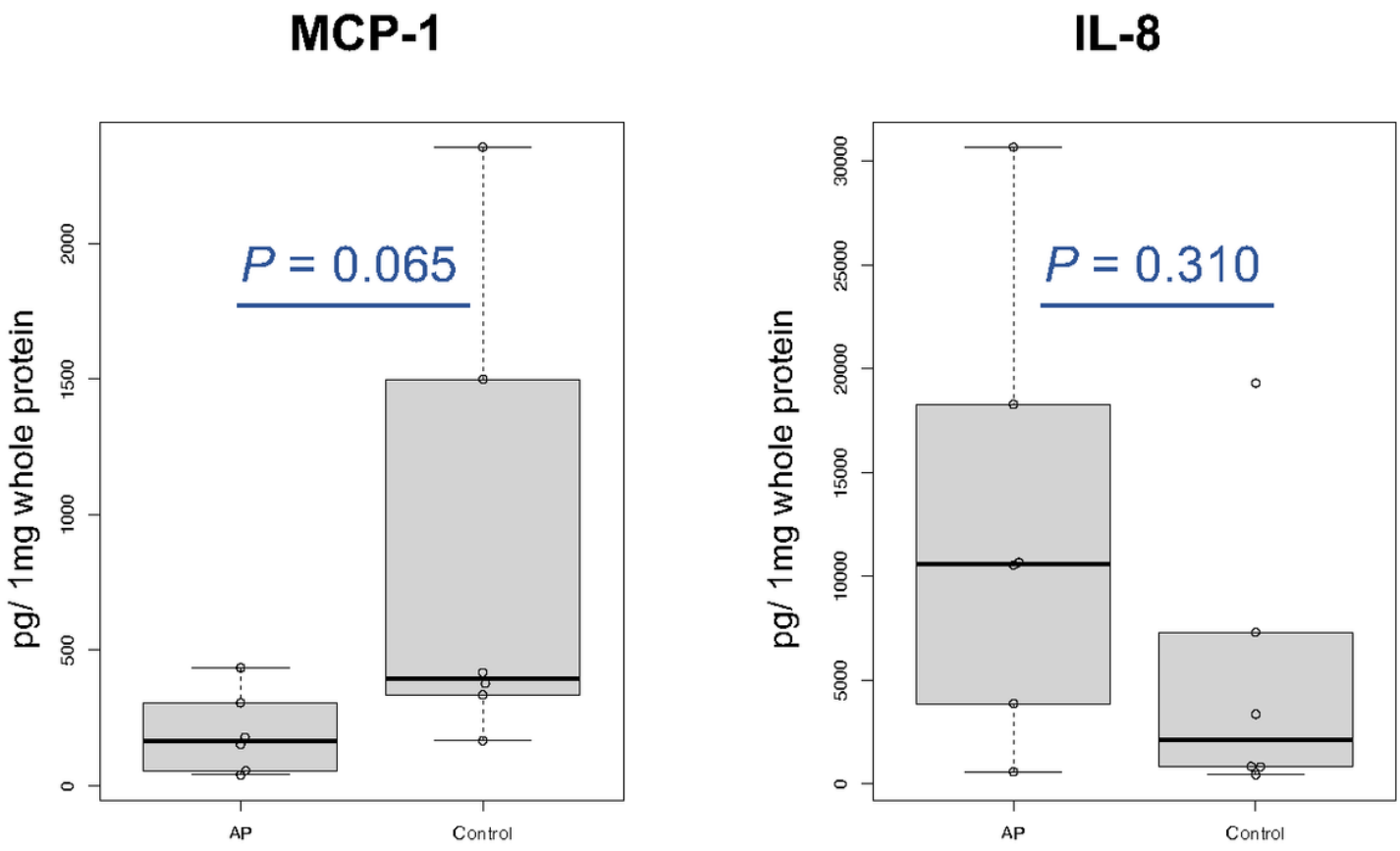

Figure 3

Results of the multiplex cytokines/chemokines assay. *P* values were calculated by the Mann–Whitney U test.

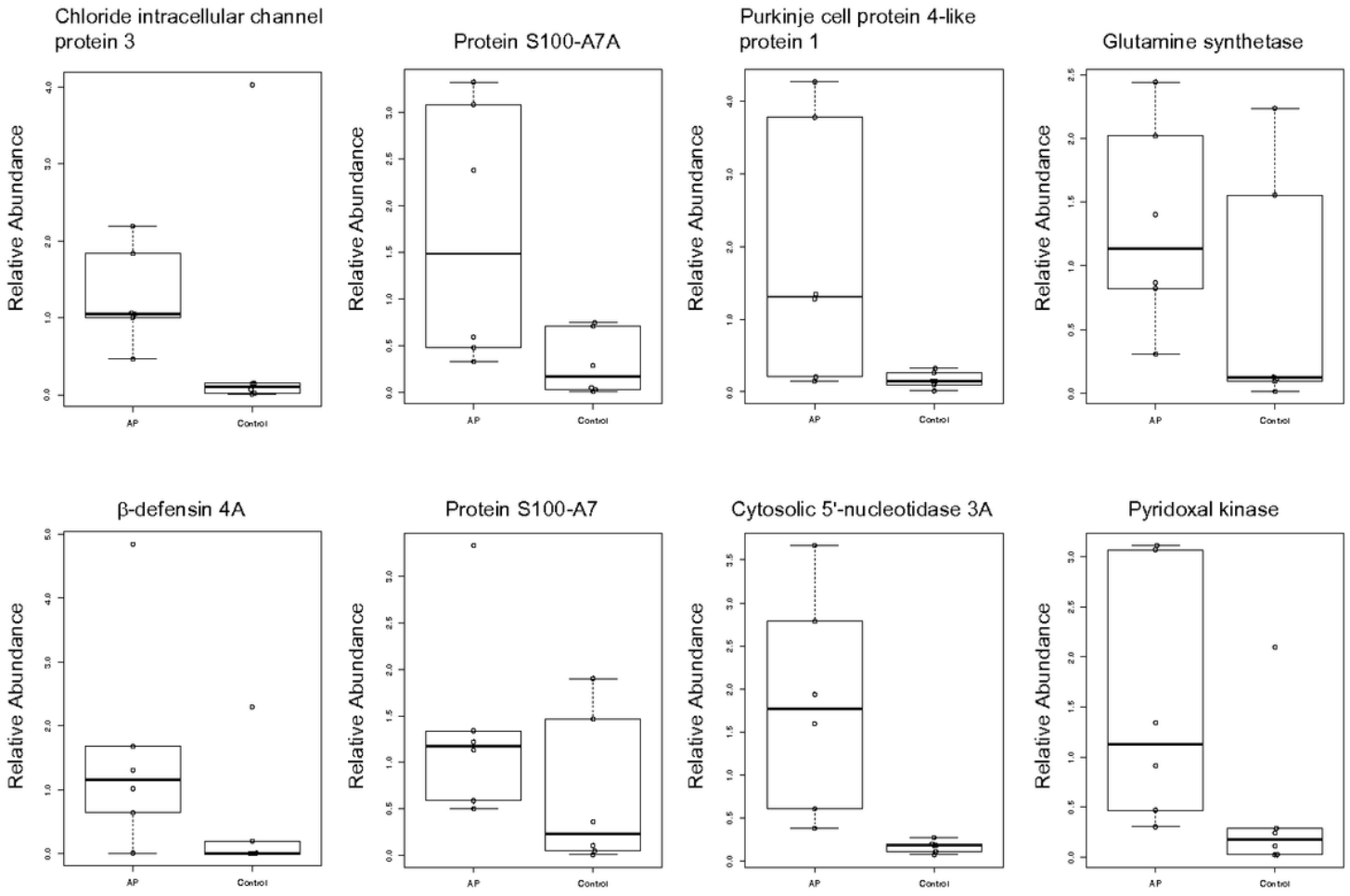

Figure 4

Abundance of the eight proteins that were higher in the AP samples. The proteins were detected in all six AP samples with a coverage of >20%. The *P* values were calculated by ANOVA and adjusted by the Benjamini-Hochberg method and listed in Table 2.

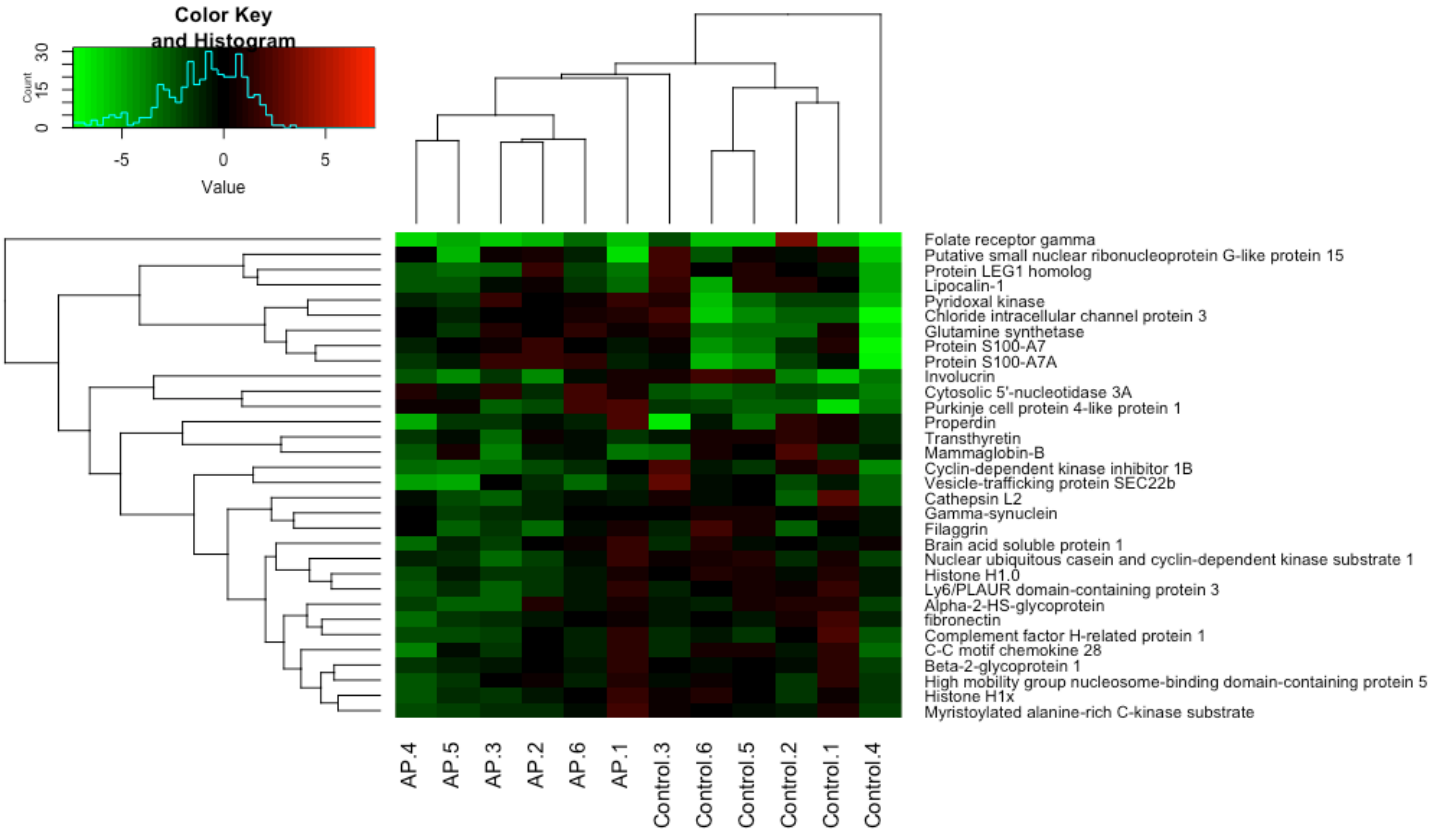

Figure 5

Heatmap using the relative abundance of 32 proteins. The proteins were detected with a coverage of > 20% and a *P*-value < 0.05.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTables.xlsx