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RESEARCH ARTICLE

Suction blister fluid as potential body fluid for biomarker proteins

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Early diagnosis is important for effective disease management. Measurement of biomarkers present at the local level of the skin could be advantageous in facilitating the diagnostic process. The analysis of the proteome of suction blister fluid, representative for the interstitial fluid of the skin, is therefore a desirable first step in the search for potential biomarkers involved in biological pathways of particular diseases. Here, we describe a global analysis of the suction blister fluid proteome as potential body fluid for biomarker proteins. The suction blister fluid proteome was compared with a serum proteome analyzed using identical protocols. By using stringent criteria allowing less than 1% false positive identifications, we were able to detect, using identical experimental conditions and amount of starting material, 401 proteins in suction blister fluid and 240 proteins in serum. As a major result of our analysis we construct a prejudiced list of 34 proteins, relatively highly and uniquely detected in suction blister fluid as compared to serum, with established and putative characteristics as biomarkers. We conclude that suction blister fluid might potentially serve as a good alternative biomarker body fluid for diseases that involve the skin.

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1 Introduction

Early disease diagnosis and disease monitoring are critical factors in the correct physical symptom determination and subsequent therapy. Biomarkers that specifically and sensitively reflect the involvement of biological pathways may facilitate the diagnosis, disease monitoring, and development of treatments. Most often, analysis of plasma and/or serum has been employed for measurement of biomarkers involved in a whole variety of diseases. Currently, analysis of plasma is commonly used for diagnosis of diseases and measurement and confirmation of disease status [1]. Therefore, it is an important body fluid to search for novel biomarkers not only to be used as diagnostic tools, but also to elucidate new molecular pathways involved in diseases and mechanisms explaining altered homeostatic conditions [2]. Novel technologies, such as proteomics approaches, are able to provide rapid access to extensive plasma proteomes, thereby allowing detection of multiple biomarkers [3]. While

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Abbreviations: CRPS1,: complex regional pain syndrome 1; **HMGB 1,** high mobility group box 1; **PM,** potential markers; **RPC,** reversed phase chromatography; **SCX,** strong cation exchange

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plasma is the most important and readily available source for systemic biomarkers, other biological matrices have been explored to screen for biomarkers that are expected to operate at a more local level. Some examples of such alternative biological matrices are tumor tissue [4], cerebrospinal fluid [5], and suction blister fluid [6].

Suction blister fluid is largely derived from the interstitial fluid, which is the place where many important biomarkers are expected to be found. Compared to skin biopsies used to analyze local mediators, suction blister fluid is obtained by means of a less invasive sampling technique. Suction blister fluid can be used as body fluid to detect drugs [7], study mediators of inflammation [8], and other biomarkers present at the local level of the skin. For several diseases, including epidermal necrolysis [9], scleroderma [10], and CRPS1 [6], suction blister fluid has been used as a source to identify/quantify potential biomarkers. In these cases and in other studies [11–14], the variations of protein contents measured in suction blister fluid samples were demonstrated to be sufficient for reliable measurement of biomarkers. Suction blister fluid has not been studied in depth at the proteome level yet and may thus be a potential new body fluid to be used for diagnosis of coetaneous diseases.

Proteomic mapping of suction blister fluid is an initial step in the search for potential biomarkers that may act at the local level of the interstitial fluid or the epidermis. Here we describe the analysis of the suction blister proteome with 2-DE and with a 2-D-HPLC MS/MS based approach in order to demonstrate the usefulness of suction blister fluid as body fluid for diagnostic purposes. When proteomic approaches are used to analyze suction blister fluid, the dynamic range of proteins present in this body fluid is a major bottleneck as in other body fluids such as plasma [1, 15]. Since many biomarkers are low abundant proteins, these biomarkers are consequently masked by the more high abundant proteins [16, 17]. The efficient removal of abundant proteins in plasma proteomics is a well-explored path to reach for the lower abundant proteins. Therefore, also in our work we first depleted suction blister fluid for high abundant proteins using methods already established for plasma proteome analysis. Subsequently, protein profiles of depleted suction blister fluids, nondepleted suction blister fluids and serum were compared with 2-DE. Next, we used nanoflow multidimensional HPLC MS/MS strategies to analyze more in-depth depleted suction blister fluid and depleted serum and compared qualitatively protein abundances between both body fluids. Then, potentially interesting proteins identified in suction blister fluid that were already in use as (serum) biomarkers or that might be used as such are discussed in view of their potential as suction blister fluid derived biomarkers. For this, we focused on skin related diseases and proteins that were present in suction blister fluid in much higher concentrations than in serum.

2 Materials and methods

2.1 Suction blister fluid collection

The seven control individuals who donated suction blister fluid for this study were all female with a mean age of 34.2 ± 10.7 (SD) years. Induced suction blisters from these individuals were collected, pooled, and used for our experiments. Blisters were induced by means of a suction method [18, 19]. A seven-hole (5 mm diameter per hole) skin suction chamber was positioned on the ventral side of the skin of the forearm. A vacuum of 300 mm Hg negative pressure was applied with an Atmoforte 350A aspirator pump (ATMOS Medizintechnik, Lenzkirch, Germany), which was reduced after 15 min to 250 mm Hg and again, 15 min later, reduced to 200 mm Hg. This negative pressure was maintained until blisters containing sufficient fluid had been developed, but not longer than 2.5 h (Fig. 1A). The blisters (typically between 30 and 100 μL in volume) were punctured (Fig. 1B) and the contents of the blisters were pooled into a 1.5 mL Eppendorff conical polypropylene tube and centrifuged for 5 min at 1.600 × *g*. All samples were stored in 1 mL conical polypropylene tubes at −80°C until analysis.

2.2 Collection of serum

Serum was provided by the Department of Clinical Chemistry of the Leiden University Medical Centre, The Netherlands. It was a mixture of serum from 30 healthy individuals and was stored at −80°C for at most 3 wk.

2.3 Removal of abundant proteins

Human serum and suction blister fluid samples were depleted of abundant proteins with one of the following three

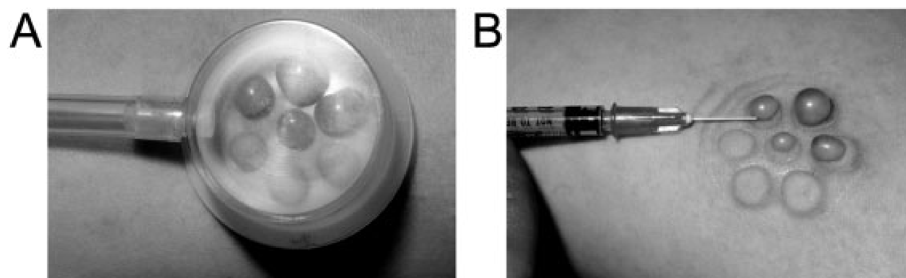

Figure 1. (**A**) Suction chamber during the development of blisters; (**B**) puncture of blisters and collection of suction blister fluid.

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different methodologies: (i) albumin depletion (Vivascience spin columns, Hannover, Germany); (ii) depletion for six high abundant proteins (Agilent, Palo Alto, USA); and (iii) depletion for 12 high abundant proteins (Beckman Coulter, Fullerton, USA). The albumin depletion and depletion for 12 high abundant proteins were spin column based, while the depletion for six high abundant proteins was HPLC-column based. In all cases, depletion was carried out according to the instructions of the manufacturer. Initial examination of the biological variation of blister fluid and serum can be found in the Supporting Information as 1-D and 2-D gel images of individual blister fluid and serum samples. For the 2D gels, PDQuest was used to count the average numbers of spots in the gels. The average protein concentration determined in these body fluids is also given. For suction blister fluid, which has a protein concentration (~16 μg/μL) that is five times lower then serum (~80 μg/μL), five times more sample was used compared to serum, in this case 50 μL (~800 μg). For this, equal volumes of suction blister fluid from seven healthy volunteers were pooled. The depletions of six high abundant proteins yielded ~100 μg of proteins, while the depletions of 12 high abundant proteins yielded ~40 μg of proteins (clean-up efficiencies of 87 and 95%, respectively).

2.4 2-DE

Proteins (approximately 100 μg in case of the albumin depleted samples or the samples depleted for six high abundant proteins and 40 μg in case of the samples depleted for 12 high abundant proteins) in suction blister fluid samples were concentrated with the 2-D cleanup kit from GE Healthcare (Piscataway, USA) and subsequently dissolved in 450 μL rehydratation mix (7 M Urea, 2 M thiourea, 4% CHAPS, 0.3% DTT, 12 μL/mL Destreak reagent from GE Healthcare and 5 μL/mL IPG-buffer pH 3–10 from GE Healthcare). The dissolved samples were then transferred to a 24 cm Immobiline Drystrip pH 3–11 Non-Linearized (GE Healthcare) and allowed to reswell overnight. Separation of the proteins in the first dimension according to their isoelectric point was done with an IPGphor from GE Healthcare (max 50 μA per strip, 20°C, S1 gradient at 1000 V for 3 h, S2 step-N-hold at 1000 V for 1 h, S3 gradient at 8000 V for 2 h, S4 step-N-hold at 8000 V for 6 h and finally S5 step-N-hold at 500 V for 6 h.) The strip was then first submerged for 10 min in 4 mL DTT (10 mg/mL in equilibration buffer) for reduction, then submerged for 10 min in 4 mL IAC (25 mg/mL in equilibration buffer) for alkylation and finally washed for 5 min with equilibration buffer. The equilibration buffer consisted of Tris (50 mM), Urea (6 M), glycerol (30% v/v), SDS (2% v/v) in water. The strip was then transferred onto the second dimension gel and sealed with agarose. The separation into the second dimension was subsequently performed overnight (at 1 W *per* gel) on 12% SDS-PAGE with an Ettan Dalt Twelve system (GE Healthcare) according to the manufacturer's instructions. The running buffer was ten times diluted from a commercially available (Bio-Rad, München, Germany) 10 × SDS electrophoresis buffer (250 mM Tris, 1.92 M Glycine, 1.0% w/v SDS, pH 8). The gels were stained according to Shevchenko *et al.* [20] with slight modifications. In short, the gels were sensitized with sodium thiosulphate (0.04%) after fixing and washing. Then, silver nitrate (0.1%) impregnated the gels at 4°C for 20 min. Sodium carbonate (3%) with formalin (0.05%) was used for developing the gels, while EDTA (1.4%) was used to stop the development process. A GS710 calibrated densitometer (Bio-Rad) was used for scanning of the gels. PDQuest (Bio-Rad, Veenendaal, The Netherlands) was used for spot analysis.

2.5 2-D-HPLC with Fourier Transform ICR MS/MS (2-D-HPLC MS/MS) detection

After protein fractionation with the columns that deplete for 12 high abundant proteins, the unbound fractions were concentrated to 50 μL using 5 kD centrifugal filters (Millipore, Bedford, USA). The concentrates were then diluted with 500 μL ammonium bicarbonate (50 mM) and subsequently concentrated to 50 μL. This was done two times followed by evaporation to dryness with a SpeedVac. To ensure maximal digestion the following procedure according to Dirksen *et al.* [21] was used: samples were redissolved in 50 μL urea (8 M) and 5 μL Lys-C (0.1 μg/μL H_2O). Mixtures were incubated at 37°C for 4 h. Then, reduction and alkylation was carried out by first adding 2 μL DTT (1 mM) at 50°C and incubating for 15 min followed by adding 2 μL iodoacetamide (2 mM) at 20°C and allowing to alkylate for 15 min. After this step the whole sample was diluted with 150 μL ammonium bicarbonate (100 mM) in order to lower the urea concentration below 2 M. Finally, 10 μL trypsin (0.1 μg/μL in 1 mM HCl) was added. The resulting mixture was incubated overnight at 37°C. After digestion, the resulting peptides were desalted using C18 containing pipette-tips (in-house made from 3 M Empore C18 filters and Aqua 5 μm C18 200 Å particles (Phenomenex, Torrance, CA, USA). For this, the peptide tips with sample were first washed with RP chromatography (RPC) mobile phase A. Then, the samples were eluted with RPC mobile phase B, evaporated to dryness (SpeedVac) and finally redissolved in 10 μL strong cation exchange (SCX) mobile phase A. The mobile phases used were as follows: RPC mobile phase A consisted of 0.1 M acetic acid, while RPC mobile phase B contained 80% ACN in 0.1 M acetic acid. SCX mobile phase A consisted of 20% ACN and 0.05% formic acid. SCX mobile phase B consisted of 20% ACN, 0.05% formic acid, and 0.5 M NaCl.

For the first dimension of the 2D-HPLC separation, offline SCX was carried out on two 50 × 0.8 mm Zorbax BioSCX II (3.5 μm) columns coupled in series. Aliquots of 10 μL were injected for every analysis. The total flow rate from the pumps (Shimadzu LC-9A, Duisburg, Germany) was set at 450 μL/min and splitted in a 1:9 ratio resulting in a flow rate of approximately 50 μL/min through the columns. The first 10 min of the analysis were under isocratic conditions using

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100% SCX mobile phase A. From 10 to 40 min the concentration of SCX mobile phase B increased linearly from 0 to 40%. After this, the concentration of SCX mobile phase B increased to 90% within 0.1 min and was kept there until $t = 45$ min. Within 2 min the SCX mobile phase composition was returned to the initial condition to re-equilibrate the column. Detection was carried out using UV at 220 nm (Shimadzu SPD-6A). Fractions (30 in total) of 1.0 min (50 μL) were collected and evaporated to dryness (SpeedVac).

For the second dimension of the 2D-HPLC separation, RPC was done with a similar setup as described by Pinkse *et al.* [22] on an Agilent 1200 system (Agilent Technologies, Waldbronn, Germany) coupled on-line to a 7-Tesla LTQ-FT mass spectrometer (Thermo Electron, Bremen, Germany). The collected and dried fractions from the first dimension were redissolved in 10 μL RPC-mobile phase A prior to injecting 2 μL aliquots from every fraction from the first dimension. Peptides were separated using gradient chromatography, which was performed as follows: during the first 10 min 100% RPC mobile phase A was used (flow rate 5 μL/min). This was done to allow the whole injected sample to be trapped on a precolumn (100 μm internal diameter, 2 cm length, packed in-house with Aqua™ C18 RP), while the effluent from the precolumn was directed to the waste. After 10 min, the precolumn was switched in-line with the analytical column (50 μm internal diameter, 20 cm length, packed in-house with ReproSil-Pur 3.5 μm 120 Å C18-AQ, Dr. Maisch, Ammerbuch, Germany). The elution strength was then increased to 40% RPC mobile phase B in two stages: first a 5 min linear gradient (0–10% RPC mobile phase B), then a 30 min linear gradient (10–40% RPC mobile phase B) was applied. These steps were followed by a 5 min block-elution using 100% RPC mobile phase B. Re-equilibration was then performed using starting conditions. The effluent was directed to the MS (7-Tesla LTQ-FT-ICR-MS; Thermo Electron) 10 min after injection. An ESI interface was used to ionize the peptides. The effluent was sprayed *via* emitter tips (made in-house), butt-connected to the analytical column, directly into the MS, operating in the positive ion-mode. The mass spectrometer was set in data dependent mode, automatically switching between MS and MS/MS acquisition. Full scan MS spectra (from m/z 300–1500) were acquired in the FT-ICR with a resolution of 100 000 at m/z 400 after accumulation to target value of 500 000. The two most intense ions at a threshold above 5000 were selected for collision induced fragmentation in the linear IT at a normalized collision energy of 35% after accumulation to a target value of 15 000.

2.6 Data analysis of 2-D-HPLC MS/MS

All 30 data files resulting from the 30 fractions analyzed by MS were merged and subjected to the MASCOT search engine (Matrix Science, London, UK; version 2.1.02) using the Swiss-Prot database (version UniProt-Swiss-Prot_48.3 database). Carbamidomethyl cysteine was set as fixed modification, oxidized methionine was set as variable modification and possible missed cleavage of trypsin was set as 1. The mass tolerance of the precursor ion was set at 5 ppm and of the fragments at ± 0.8 Da. The results from the searches were exported to, and analyzed with Scaffold (version 01_05_19; false positive rate of <1%) and X!Tandem (Minimal protein ID probability: 99%, number of unique peptides: at least 2, minimal peptide ID probability: 95%). Scaffold was used to visualize and analyze multiple datasets in a comprehensive manner. For this, X!Tandem was used in combination with MASCOT scoring in Scaffold to enhance peptide identification(s). Our converted data to a Scaffold file is available as Supporting Information.

Scaffold files and Excel files with detailed descriptions of the identified proteins are given. Additionally in the Supporting Information general information and gel images describing the initial exploration of the biological variation regarding the proteins in blister fluid and serum are provided.

3 Results and discussion

3.1 Evaluation of different depletion strategies and initial comparison of suction blister fluid and serum proteomes using 2-DE

When proteomics approaches are used to analyze plasma, the dynamic range of proteins present in this body fluid is a major bottleneck [1, 15]. A comparable problem is encountered in the analysis of suction blister fluid. Since disease specific biomarkers are typically present as very low abundant proteins, these biomarkers are consequently masked by the high abundant proteins [16, 17]. This is why efficient removal of abundant proteins is essential prior to biomarker identification. This specific removal of high abundant proteins is a cumbersome operation by itself, in which no ideal methodology is available yet. Usually, other proteins than the designated proteins for depletion are also depleted due to nonspecific binding [23]. However, even after efficient removal of high abundant proteins, still many biomarkers are expected to be present in concentrations that are eight to ten decades lower than the subsequent most abundant proteins [24].

For evaluation purposes, different depletion methodologies were examined for their efficiency, specificity, and reproducibility in depleting suction blister fluid. After collection of suction blister fluid and serum, 2-DE was carried out with albumin depleted samples and samples depleted for 6 or 12 high abundant proteins. Similar albumin percentages (of approximately 40–50%) of the total protein contents were found in suction blister fluid and in serum before albumin depletion (by determining the total protein concentrations before and after albumin depletion with a 2D-Quant kit from GE Healthcare; data not shown) [1]. As expected, suction blister fluid contained total protein concentrations approximately five times less as compared to serum [14]. Compara-

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ble relative 2-D gel profiles were observed for several of the abundant proteins, *e.g.*, the Igs, transferrins, prealbumin, haptoglobulins, and antitrypsins when the albumin depleted suction blister fluid proteome (Fig. 2A) was compared with the albumin depleted serum proteome (shown by Sanchez *et al.* [25]).

With the depletion strategy from Agilent, the following proteins should be removed: albumin, Igs (IgG, IgM, IgA), transferrin, α1-antitrypsin and haptoglobin. The depletion methodology from Beckman also depleted the following proteins: apolipoprotein A-I and A-II, α1-acid glycoprotein, α1-macroglobulin, and fibrinogen. Figure 2 shows representative silver stained 2-D gels. Figure 2A is a reference gel and visualizes the protein composition of suction blister fluid depleted for serum albumin. In 2B a gel of serum, depleted for 6 high abundant proteins is shown, whereas gels of suction blister fluid, depleted for 12 and 6 high abundant proteins are shown in Figs. 2C and D, respectively. A direct comparison of the gels obtained for serum and suction blister fluid (2B and D, respectively), both depleted for six high abundant proteins, reveals the strong similarities. A direct comparison of the depletion efficiencies and specificities in suction blister fluid with the strategies that deplete for 6 and 12 proteins, respectively, can be made from the gels in 2C and D. We encircled in red the positions of several high abundant proteins in blister fluid and serum. Circled in green are areas in gel 2B and D in which significant more proteins spots are visible in the gel obtained for blister fluid when compared to that for serum.

By comparing the 6 and 12 depleted suction blister fluid samples with nondepleted and albumin depleted suction blister fluid samples, we observed that both the 6 and the 12 depletion strategies had high protein depletion specificities and efficiencies (see Fig. 2 and gel images in the Supporting

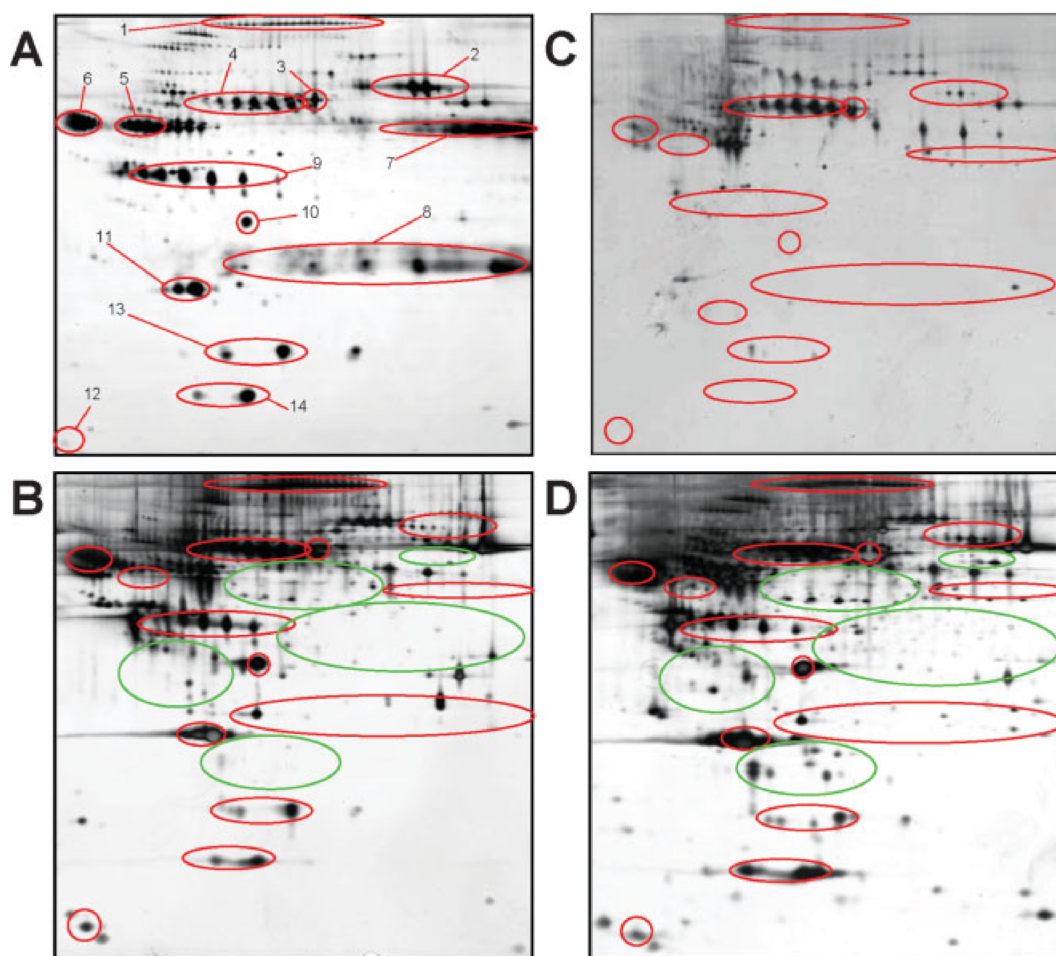

Figure 2. Visualization of depletion strategy and comparison of blister fluid and serum proteome by 2-DE. **(A)** Albumin depleted suction blister fluid; **(B)** serum depleted for six high abundant proteins; **(C)** suction blister fluid depleted for 12 high abundant proteins; **(D)** suction blister fluid depleted for six high abundant proteins. Encircled in red are positions of the following high abundant proteins: (1) α2-macroglobulin, (2) transferrin, (3) albumin, (4) hemopexin, (5) α1-antitrypsin, (6) antichemotrypsin, (7) Igs heavy chain, (8) Igs light chain, (9) haptoglobin, (10) transthryretin, (11) apolipoprotein-A1, (12) apolipoprotein-A2, (13) haptoglobin α2-chain, and (14) prealbumin. Encircled in green are areas in the gels in which significant more protein spots are visible in blister fluid than in serum.

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information). High specificities for both depletion strategies with serum were already described [26, 27]. Because the specificities and reproducibility's obtained for the strategies that deplete for 6 and 12 high abundant proteins were similar and the strategy that depletes for 12 high abundant proteins resulted in the highest enrichment factor, this depletion strategy was used for subsequent 2-D-HPLC MS/MS experiments.

3.2 Comparison of suction blister fluid and serum proteomes using 2-D-HPLC MS/MS and stringent data analysis

Subsequently, in order to increase the coverage of the suction blister fluid and serum proteomes, 2-D-HPLC MS/MS was conducted. After depletion of suction blister fluid and serum for 12 high abundant proteins, the enriched proteins in both body fluids were cleaved in solution by tryptic digestion. The resulting peptides were separated in the first dimension in 30 fractions with SCX chromatography. Each fraction was subsequently analyzed with RPC connected on-line to an LTQ-FT-MS. The data files resulting from the 2-D-HPLC MS/MS measurements were combined and analyzed using MASCOT, Scaffold, and X!Tandem as described in the experimental section. With the stringent settings used, 401 proteins were detected in suction blister fluid. For human serum this number was 240. An overview of all proteins and peptides detected is available as Supporting Information in a Scaffold file. Using parallel searches in a reversed database we were able to derive that less than 1% of these identifications could be false. For this, all spectra were searched against the reversed Swissprot database with the taxonomy set to human. The same algorithm as used for our real protein database search to evaluate this false positive rate of the peptide identifications was used. This reversed database was constructed by using the reversed order of amino acid sequences of every protein in a similar way as described by Peng *et al.*, Kislinger *et al.*, and Moore *et al.* [28–30]. Although, we realize that this analysis if far from comprehensive, we note that comparable numbers of proteins were found in previous 2-D-HPLC MS/MS proteome analyses on human serum, when similarly high database search stringencies were used [26, 31–33]. In these studies, the amounts of serum proteins ranged from 325 to 622. When we submitted our data to the IPI_human database (version 3.19) under similar stringencies as we used for our SwissProt search, we found 628 and 410 proteins for suction blister fluid and serum, respectively (data not shown). Although originating from capillary blood, the number of proteins detectable in suction blister fluid is larger than in human serum when a same total amount of protein sample is used. This finding may result from additional proteins present in suction blister fluid originating from lymph nodes and tissue leakage proteins, or might indicate that the dynamic range problem in depleted suction fluid is

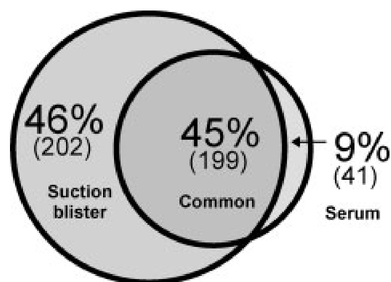

Figure 3. Venn-diagram of overlapping proteins found (percentages and numbers) both in suction blister fluid and serum and proteins uniquely found in one of the two matrices. Data obtained from 2-D-HPLC MS/MS. Numbers of proteins detected are given between parenthesis and as a percentage. (Minimal protein ID probability: 99%, number of uniquely detected peptides: at least 2, minimal peptide ID probability: 95%.)

smaller than in serum. Indicative of the latter is that less than half of the proteins detected in suction blister fluid were also detected in human serum (Fig. 3). All proteins uniquely found in suction blister fluid can easily be accessed *via* a Scaffold file, which can be found in the Supporting Information.

Next, suction blister fluid and serum were correlated in order to get a clearer view of the similarities between these body fluids. For this semi-quantitative analysis, the amount of uniquely detected peptides in suction blister fluid and serum were compared for individual proteins. Therefore, the relative extent to which the proteins are present in suction blister fluid and serum was very roughly estimated by the number of uniquely detected peptides found per protein in both body fluids according to similar principles as used by Scholten *et al.*, Sanders *et al.*, and Ishihama *et al.* [34–36] for rough determination of protein abundances. The ratios between the number of uniquely detected peptides per protein found in suction blister fluid and serum were plotted from high to low ratios using a logarithmic scale (Fig. 4). Using this semi-quantitative analysis we deduct that many proteins are of similar relative abundance in the extracted proteome of both suction blister fluid and serum. Only a few proteins were detected with significant more uniquely detected peptides in serum than in suction blister fluid (right end part of Fig. 4). On the contrary, a large number of proteins were detected with significant more uniquely detected peptides in suction blister fluid when compared to serum. This is shown in the left part of Fig. 4. This plot, together with the 2-DE data and Fig. 3 all indicate the high similarities between suction blister fluid and serum when looking only at the 10–20 most abundant proteins. In the case of the suction blister fluid, zooming into the lower abundance fraction of the proteins reveals a larger number of additional proteins than in the case of serum.

We used the DAVID gene functional classification program (2006 version; medium stringency, http://david.abcc.ncifcrf.gov/) to cluster the proteins found in serum and

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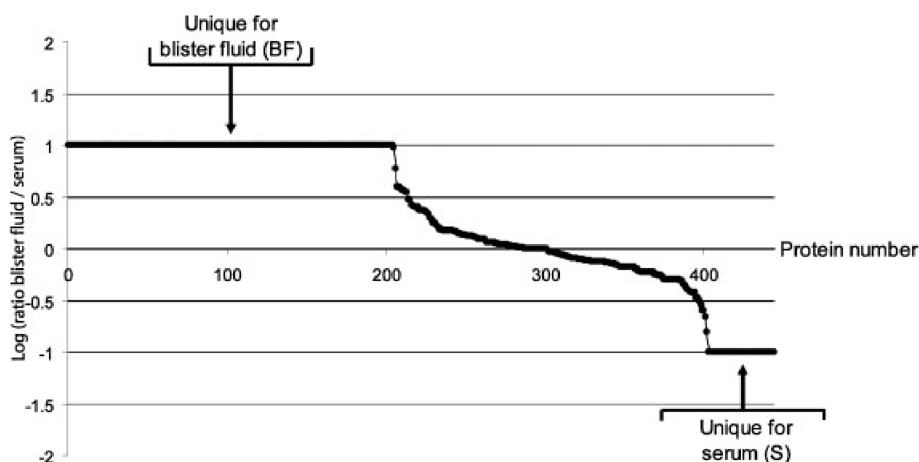

Figure 4. Protein ratios graph representing the logarithm of the ratios between the uniquely detected peptides per protein found in suction blister fluid and serum. Higher ratios than 10 or lower than 0.1 were set at 10 and 0.1, respectively.

suction blister fluid. For an explanation of what the DAVID algorithm is and does, we refer to (http://david.abcc.ncifcrf.gov/helps/functional_annotation.html and http://david.abcc.ncifcrf.gov/content.jsp?file=functional_classification.html#fuzzy). Figure 5A and B shows diagrams of the different protein classes for all the proteins found in both body fluids. The areas in the pie graphs represent percentages of numbers of proteins found per class. Evidently, in these graphs the 12 high abundant proteins, which were depleted from the samples prior to analysis, are not depicted. These graphs therefore represent the approximately 5% of proteins left in both body fluids after depletion. While serum only has one protein class not found in suction blister fluid, DAVID appointed eight protein classes to suction blister fluid, which were not appointed to serum. These protein classes all represent cell-based processes like metabolism, protein synthesis, oxidation state management, and cell shape. Therefore, it is likely to assume that these additional protein (classes) result from cell leakage processes during blister formation in addition to blood related proteins that diffuse *via* extracellular spaces into the blisters. Although this preliminary conclusion requires a more thorough investigation, it hints at the potential of studying protein levels at the location of the disorder.

3.3 Suction blister fluid as potential body fluid for biomarkers

We like to stress that in this study we have not identified any new biomarkers. By analyzing samples under identical conditions we did examine which proteins are easier to detect in blister fluid than in serum. Of these latter proteins, a biased list was constructed that is depicted in Table 1. All these proteins were functionally reviewed to find out whether they have ever been annotated as disease markers (M) or potential markers (PM). In the following section, this literature review is summarized.

3.3.1 Proteins described as biomarkers acting at the local level (light gray rows in Table 1)

As suction blister fluid is expected to be an ideal body fluid for analyzing localized disease mediated effects, we first focus on proteins described previously as markers involved in diseases at the local level of the epidermis [6, 8] (light gray rows in Table 1).

3.3.1.1 Beta-2-microglobulin, C-reactive protein and psoriasin

Psoriasis is a common skin disease, which is characterized as an inflammatory and hyperproliferative skin disorder. Interestingly, upregulated levels of beta-2-microglobulin, C-reactive protein, and psoriasin have been found in psoriatic skin biopsies [37–39]. Additionally, in abnormal differentiation of epithelial cells like skin cancers, upregulated levels of psoriasin have also been demonstrated [39]. A preliminary study on psoriatic skin in which psoriasin was identified as up regulated protein in suction blister fluid demonstrates the usefulness of this body fluid [40]. Beta-2-microglobulin and C-reactive protein are also involved in psoriasis and moreover C-reactive protein is known to be upregulated in skin cancer. For these diseases, it is expected that suction blister fluid would be a good candidate body fluid for analysis of these potential markers.

3.3.1.2 Cystatin A and ezrin

Cystatin A, which is thought to regulate invasion and metastasis of malignant cells, is a potential marker for monitoring the differentiation of malignant cells in skin tumors. Instead of using skin tumor sections, suction blister fluid collection is potentially a less invasive technique for monitoring suspected skin for these cancers. Ezrin is detected in suction blister fluid with 11 uniquely detected peptides in control individuals, while it was not detected in serum. As

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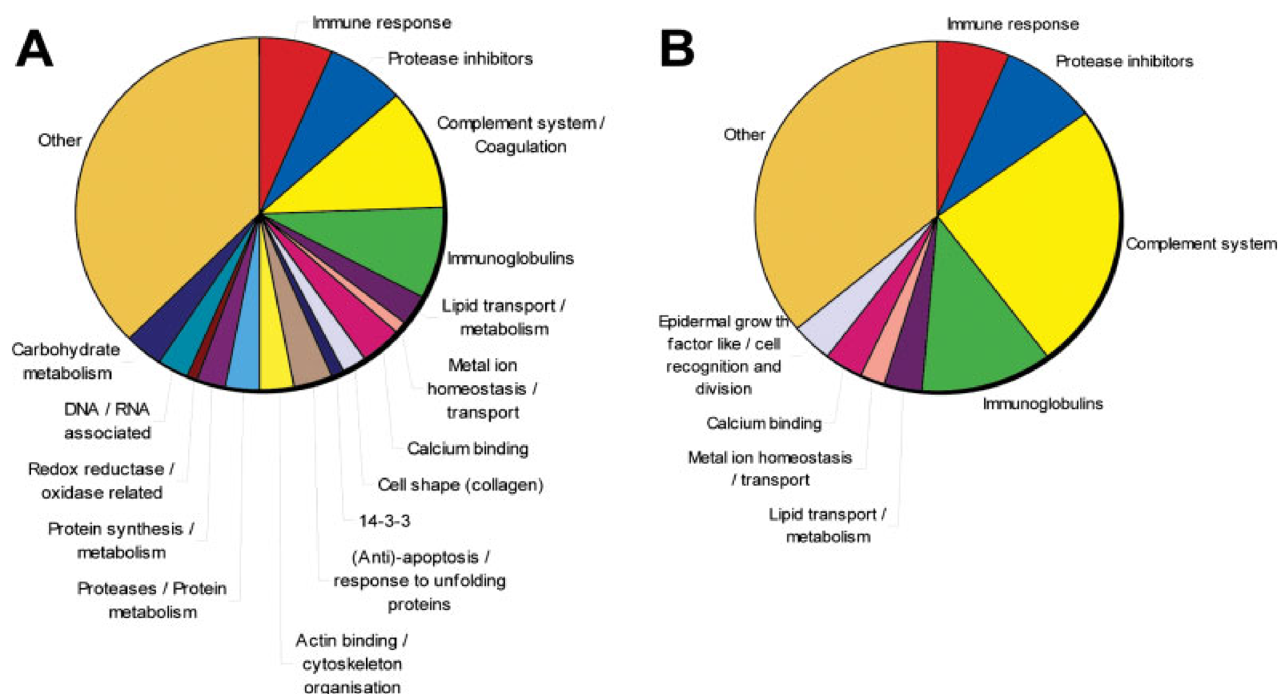

Figure 5. Pie graphs representing proteins uniquely detected in suction blister fluid (A) and serum (B) sorted by DAVID functional classification into different characteristic functions.

ezrin is upregulated in some skin cancers [41], suction blisters taken from the epidermis adjacent to these skin cancers may be valuable sources for biomarker analysis.

3.3.1.3 GST P1

It is well known that GST P1 is overexpressed in some multidrug resistant tumor cells [42, 43]. This might implicate resistance of tumor cells to chemotherapy, but also a cellular stress response. These observations however, still indicate GST P1 inhibitors to be potential coassisting drugs together with cytostatics [31, 44–49]. In addition, GST P1 may be used as a marker protein for monitoring cancer development during treatment or recurrence of malignant cancer cells. For skin cancer, suction blister fluid probably is able to extract this marker protein from the cutaneous cancerous cells locally. The marker is then present in higher concentration than in blood, in which it will be diluted. As we already readily detected GST P1 in suction blister fluid from control individuals, it will also be possible to measure upregulation induced by skin cancer.

3.3.2 Proteins described as biomarkers acting at the systemic and local level

Some proteins in Table 1 were exclusively detected in suction blister fluid or with significant more uniquely detected peptides per protein compared to serum (dark gray rows in Table 1).

3.3.2.1 Cystatin C

This protein is an indicator of renal functioning. In this study, six peptides from cystatin C were uniquely detected in suction blister fluid in contrary to serum in which no cystatin C was detected. Blisters taken from skin adjacent from the kidneys might provide therefore valuable extra information about renal cell leakage and therefore also renal functioning.

3.3.2.2 Interleukin-6 signal transducer (gp130)

Interleukin-6 is involved in the growth and differentiation of numerous cell types [50]. It stimulates the immune response to trauma (especially tissue damage) and is produced by T cells and macrophages. The IL-6 receptor consists of two subunits, namely an interleukin-6 specific subunit and gp130, a signal transducing subunit. After binding of interleukin-6 to the interleukin-6 receptor with low affinity, gp130 binds to the binary complex resulting in a high affinity interaction of the interleukin-6 receptor with interleukin-6, allowing interleukin-6 mediated cell signaling. Scleroderma is an autoimmune disease of the connective tissue which is characterized by the formation of skin and organ sclerosis. Elevated serum levels of gp130 might indicate activation of the interleukin-6 system and the development of sclerotic lesions [51]. Since gp130 is detected in suction blister fluid of control individuals, upregulated gp130 concentrations in sclerotic skin are expected to be detected in suction blister fluid in an earlier stadium than in serum.

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Table 1. List of 34 proteins found in suction blister fluid, which are already used as biomarker or possible biomarker detected in other body fluids (mostly serum)

Nr	Protein	Body fluid/biopts	Biomarker level	Disease(s)	Ratio uniquely detected peptides Suction blister fluid/serum
1	Amyloid P component	S	PM	Beta-2-microglobulin amyliodosis [58], liver associated [59, 60]	12/13
2	Beta-2-microglobulin	S	PM	Psoriasis [37]	7/3
3	C-reactive protein	S	PM, M	Muscle strength loss [61], cancer [62], psoriasis [38], cardiovascular [63]	5/7
4	S100A7 (psoriasin)	Sk	PM	Psoriasis [39], Skin cancer [64]	4/5
5	Cartilage oligomeric matrix protein	S, Sy	M	Arthritis [65-67]	5/2
6	Clusterin	S, S, S	PM	Heart disease/infarction related and diabetes [68], liver associated [69], squamous cell carcinoma [70]	23/20
7	Colony stimulating factor 1	S	PM	Endometrial adenocarcinoma [71]	3/3
8	Complement component C5a	S	PM	Cardiovascular related [72]	60/75
9	Complement component H	U	M	Bladder cancer [73]	41/84
10	Cystatin A	Sk	PM	Malignancy in human epidermal keratinocytes [74]	8/2
11	Cystatin C	S	M	Renal function [75, 76]	6/0
12	Ezrin	T	PM	Skin tumor related [41]	9/0
13	GST Pi	T	PM	Skin tumor related [77]	7/7
14	Haptoglobin related protein	S	M	Malignant lymphoma [78]	2/4
15	Hepatocyte growth factor activator	S	M	Prostate cancer [79]	9/23
16	High-mobility group box 1	S, C	PM	Cerebral and myocardial ischemia [80], cutaneous lupus erythematosus [52]	11/0
17	Histidine-rich glycoprotein	S	PM	Alzheimer's disease [81]	23/21
18	IGF-binding protein 3	S, S	PM	Liver disease [82], tiredness in highly trained sportsmen [83]	7/11
19	Interleukin-18	S, Sk, U	PM, M	Coronary artery disease and diabetes [84], gastric cancer [85], HIV related [86], kidney related [87, 88], ovarian cancer [89], psoriasis [90], graft-*versus*-host disease [91], breast cancer related [92], cardiovascular related [93], lung disease related [94]	4/0
20	Interleukin-1 receptor antagonist protein	S, Sk	PM	Psoriasis [95], gynaecological cancers [96], heart transplant and bypass related [97], psoriatic arthritis related [98]	3/0
21	Interleukin-6 signal transducer (gp130)	S, C	M	Heart failure related [99, 100], HIV infection [101], tumor related [102], scleroderma [51], chronic renal failure [103], fibromyalgia [104], inflammatory disorders of the skin [105]	3/2
22	Insulin-like growth factor binding protein-4	S	PM	Chronic renal failure [55]	3/0
23	Acid-labile subunit insulin-like GF	S	M	Growth disorders [106]	24/21
24	Lipopolysacharide-binding protein	S	M	Bacterial infection in cirrhotic patients [107]	3/8
25	Orosomucoid	U	M	Bladder cancer [108], cardiovascular related [56]	11/14
26	Paraoxonase 1	S	PM, M	Liver function monitoring after transplantation [109], vascular dementia [110]	9/0
27	Periostin	S	PM	Bone metastases from breast cancer [57]	3/0
28	Phosphoglycerate kinase 1	S	PM	Pancreatic ductal adenocarcinoma [111]	15/0
29	Phospholipid transfer protein	S	PM	Accelerated atherosclerosis in type 2 diabetes [112], coronary heart disease [113]	5/0
30	Prostaglandin D2 synthase	S, C	M	Perilymphatic fistula [114], CSF leakage and related [115–117], diabetes [118], hydrocephalus [119]	3/3
31	Protein Z	S	PM	Ischaemic stroke [120]	3/6

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Table 1. Continued

Nr	Protein	Body fluid/biopts	Biomarker level	Disease(s)	Ratio uniquely detected peptides Suction blister fluid/serum
32	Amyloid A	S	PM	Adipose related inflammation [121]	3/5
33	Sex hormone-binding globulin	S	PM, M	Bone fracture risk in men [122], insulin resistance in obese males [123], cardiovascular mortality in elderly men [124]	10/4
34	Thioredoxin	S, Sk	M	Oxidative stress in patients with rheumatoid arthritis [53], skin cancer related [54]	5/0

These proteins are discussed in the text as (examples) of possible biomarkers to be detected in suction blister fluid. Light gray rows: skin related biomarkers and therefore likely candidates for suction blister fluid biomarkers. Dark gray rows: proteins found only in or in much higher concentrations in suction blister fluid. S = serum; Sk = skin; Sy = synovial fluid; T = tumor; U = urine; C = CSF; M = marker; PM = potential marker.

The fact that some other proteins in Table 1 are detected with more uniquely detected peptides in serum than in suction blister fluid does not implicate that they are not interesting markers to be analyzed in suction blister fluid. Proteins found in suction blister fluid that are already in use as marker or identified as potential marker with serum as body fluid, are obviously most interesting as potential suction blister fluid based markers if they are skin disease related.

3.3.2.3 Protein markers in suction blister fluid with possible additional diagnostic value

For the cytokine high mobility group box 1 (HMGB 1), it is unlikely that this potential cerebral and myocardial ischemia marker can confidently be detected in suction blister fluid as cerebrospinal fluid and serum are the obvious methods of choice, respectively, for diagnosis of these diseases. But suction blister fluid might be used in addition in order to get more specific and reliable results due to the involvement of HMGB 1 in other events. As an example, the involvement of HMGB 1 in cutaneous lupus erythematosus [52] might result in elevated concentrations of HMGB 1 in serum giving rise to a false positive diagnosis on myocardial ischemia. Since cutaneous lupus erythematosus is localized to the skin, additional monitoring HMGB 1 in suction blister fluid can potentially rule out a myocardial ischemia if elevated HMGB 1 concentrations are found in the skin.

Other examples in which monitoring biomarkers in suction blister fluid can give additional diagnostic information are suction blister fluid analysis of interleukin-18, interleukin-1 receptor antagonist and thioredoxin. By comparing protein levels between suction blister fluid and serum, increased confidence can be gained in the origin of the process causing the elevated biomarker concentrations. These important biomarkers are namely involved in several diseases, including rheumatoid arthritis [53] and some skin cancers [54] and are therefore not very specific. For this purpose, especially thioredoxin might be an ideal candidate to be monitored in suction blister fluid.

3.3.2.4 Protein markers in suction blister fluid without additional value

Other proteins in Table 1, which were detected with much more uniquely detected peptides compared to serum (Nr. 22, 26–29, 33; Table 1), are probably not suitable to be used as suction blister fluid biomarker for the mentioned diseases, since all these diseases are either systemic or organ related. In these cases it is expected that blood or a body fluid taken adjacent to or at the area of the disease is the obvious choice for sample collection. The fact that these proteins were detected with much more uniquely detected peptides compared to serum probably is caused by normal tissue leakage effects. An important reason to use suction blister fluid as body fluid in some of these cases, would be that it is much less invasive than biopsies. In these cases, the blisters probably have to be applied near the localized effect [55–57].

From Table 1 it becomes clear that various proteins are used to diagnose different diseases and that some proteins can be used to diagnose or monitor multiple diseases. This makes multiple screening of protein markers to diagnose or monitor one disease advantageous in terms of increasing specificity. Therefore, measuring different proteins in suction blister fluid might be a good biomarker screening method. Two examples of possible “combination marker” screening strategies can be named.

Firstly, when looking at skin related cancers, monitoring psoriasin, cystatin A, ezrin, and thioredoxin is an example of obtaining more specific output regarding possible skin cancers. As these biomarkers are found in different skin cancers, monitoring them might indicate the

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specific form of skin cancer. Additional serum monitoring can even strengthen the diagnosis and exclude other diseases.

Secondly, psoriasis might be diagnosed with more certainty and specificity when beta-2-microglobulin, C-reactive protein, psoriasin, interleukin 18, and interleukin-1 receptor antagonist protein levels in suction blister fluid (or a subset of these) are measured. If only one protein is measured as biomarker, upregulation can result from many different diseases (See Table 1).

4 Concluding remarks

As an initial step for the search for biomarkers acting at the local level of the interstitial fluid, the proteome of artificial suction blisters was analyzed using 2-DE and 2-D-HPLC MS/MS. Suction blister fluid is regarded to reflect the protein composition present in the interstitial fluid. It was shown with 2-DE that at the level of the most abundant proteins present in serum and suction blister fluid were quite alike. Also after depletion of six high abundant proteins, the 2-D gel profile of the in this manner depleted proteome was similar for suction blister fluid and serum. 2-D-HPLC MS/MS (SCX-RP) analysis confirmed the similarity of the depleted protein profiles of suction blister fluid and serum with respect to the abundant proteins. Using 2-D-HPLC MS/MS and stringent identification settings 401 proteins were detected in suction blister fluid compared to 240 proteins in serum. Nearly all proteins detected in serum were also detected in suction blister fluid. Of the proteins detected in suction blister fluid several may be related to cell mediated processes and thus resulted probably from cell leakage of epidermal cells during blister formation. The proteins in suction blister fluid were looked at in order to identify proteins that might act as biomarkers, resulting in a list of 34 proteins of interest. In case of the suction blister fluid, zooming into the lower abundance protein fraction reveals a larger number of additional proteins than in the case of serum. Although we realize that the blister formation process (>2 h at 37°C) may lead to artefact proteins, as also sometimes observed when biopsies are taken, we conclude that suction blister fluid may serve as an interesting alternative body fluid for biomarker analysis at localized areas of interest.

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