DATASET BRIEF

Proteomic analysis of the secretome of human umbilical vein endothelial cells using a combination of free-flow electrophoresis and nanoflow LC-MS/MS

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Human umbilical vein endothelial cells are the most widely used *in vitro* model for endothelial cells. Their secreted proteins, however, have not been comprehensively analysed so far. In this study, we accomplished to map the secretome of human umbilical vein endothelial cells by combining free-flow electrophoresis with nanoflow LC-MS/MS. This comprehensive analysis provides a basis for future comparative studies of protein secretion by endothelial cells in response to cardiovascular risk factors and is available on our website http://www.vascular-proteomics.com.

Keywords:
Atherosclerosis / Cell biology / Extracellular proteins / Protease inhibitors / Proteomic methods / Secreted proteins

Endothelial cells form the inner lining of all vessels and play a central role in the pathogenesis of cardiovascular disease, including atherosclerosis, the major cause of morbidity and mortality in the Western world [1]. To date, proteomic data sets have been published on intracellular proteins and microparticles of human umbilical vein endothelial cells (HUVECs) [2–5]. A proteomic analysis of secreted proteins, however, has not been performed so far, partially due to difficulties in analysing conditioned medium of HUVEC cultures. HUVECs require serum supplements for their survival, which mask secreted proteins in their conditioned medium. In this study, we characterized the secretome of HUVECs by using a combination of free-flow electrophoresis (FFE) and nanoflow LC-MS/MS to overcome the complexity of the serum supplement.

HUVECs were isolated from human umbilical cords as described previously [6]. Immunofluorescence staining confirmed the presence of the endothelial marker VE-cadherin (CD144, Fig. 1). Before sampling, the endothelial monolayer was washed extensively with plain medium to minimize cross-contamination with bovine proteins and incubated in serum-replacement medium overnight. The conditioned medium was then separated by FFE to reduce sample complexity (see online Methods in Supporting Information). Figure 2A shows the corresponding pH of each fraction in the 96-well collection plate. To verify their protein content, fractions were separated by SDS-PAGE and stained with silver. As highlighted in Fig. 2B, fractions 27 to 55 corresponding to the pH range 3–9 contained most of the proteins. Based on their complexity, fractions were pooled, subject to tryptic digestion, and analysed by LC-MS/MS (LTQ Orbitrap XL, ThermoFisher Scientific). FFE separation and LC-MS/MS analysis were both performed on two different sets of conditioned media. Spectra were searched against a combined bovine/human database using the Sequest and X!Tandem algorithm. Peptide and peptide probabilities were computed using the Scaffold software (v2.0, Proteomesoftware). In total, 374 proteins satisfied the filter criteria of 80% peptide probability and 95% protein probability. The putative match of 182 human proteins was further confirmed by visual inspection of the spectra (Supporting Information Table). The entire MS/MS data set

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Abbreviations: FFE, free-flow electrophoresis; **HUVECs,** human umbilical vein endothelial cells

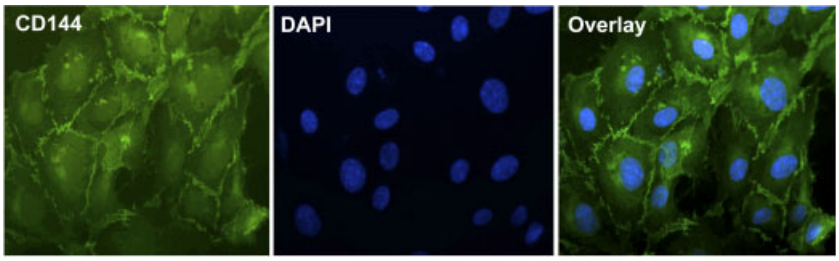

Figure 1. Immunofluorescence analysis. Endothelial cells were stained for CD144 (VE-cadherin). Nuclei were counterstained by DAPI (blue).

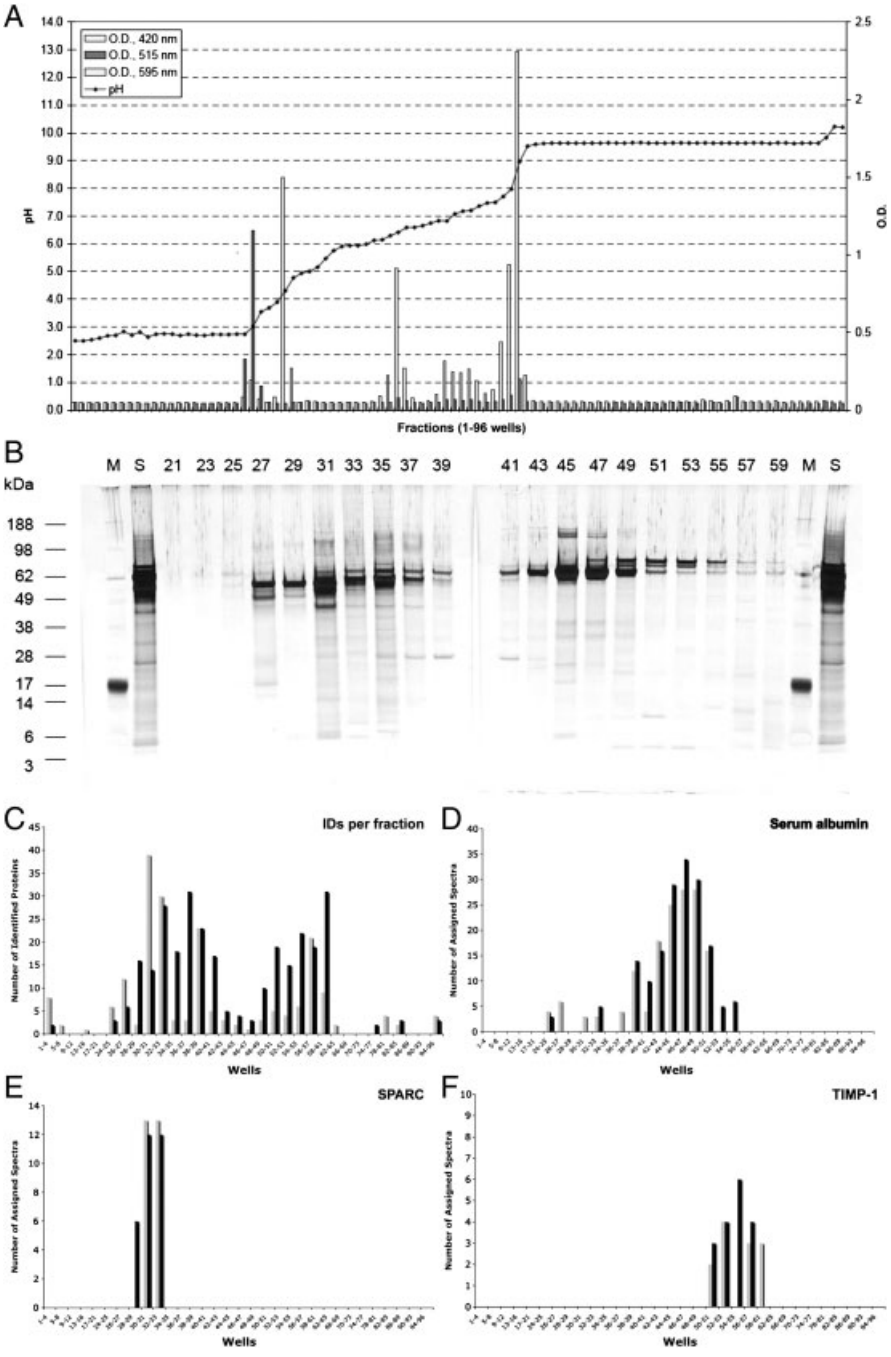

Figure 2. FFE separation. (A) pH gradient and the distribution of p*I* markers in the 96-well collection plate at the time of the p*I* marker test performed immediately before sample application. (B) After FFE, the fractions were separated by SDS-PAGE and stained with silver. Each fraction is labelled with the number of the well. ''S'' denotes ''sample before FFE fractionation'' and ''M'' for ''marker''. (C) Plot depicting the number of identified proteins throughout the 96-well plate. (D) Total number of spectra obtained for albumin. SPARC (E) and TIMP-1 (F) are confined to specific fractions in the 96-well collection plate. The similar number of assigned spectra in the two different biological replicates (grey and black bars) demonstrates the consistent recovery after FFE.

Table 1. Secretome of HUVECs

Accession no.	Entry name	Protein name	MW (kDa)	Coverage (%)	Unique peptides	Unique spectra	Assigned spectra	Location
Proteinases								
P03956	MMP1_HUMAN	Interstitial collagenase[a)]	54.0	41	17	18	81	S
P08253	MMP2_HUMAN	72 kDa type IV collagenase	73.8	7	4	5	16	S
P50281	MMP14_HUMAN	Matrix metalloproteinase-14	65.9	4	2	2	7	M
Q13443	ADAM9_HUMAN	ADAM 9	90.6	7	4	4	11	M/S
O14672	ADA10_HUMAN	ADAM 10	84.1	3	2	2	6	M
Q13444	ADA15_HUMAN	ADAM 15	87.7	6	3	3	4	M
P15144	AMPN_HUMAN	Aminopeptidase N	109.5	20	18	18	59	M
O95084	PRS23_HUMAN	Serine protease 23	43.0	14	4	4	5	S
Proteinase inhibitors								
P01033	TIMP1_HUMAN	Metalloproteinase inhibitor 1[a) b)]	23.2	32	5	5	34	S
P01034	CYTC_HUMAN	Cystatin-C[a) b)]	15.8	50	4	5	24	S
P19823	ITIH2_HUMAN	Inter-α-trypsin inhibitor heavy H2 chain	106.4	7	6	6	20	S
P01023	A2MG_HUMAN	α-2-Macroglobulin	163.3	3	4	4	43	S
Extracellular matrix components								
P09486	SPRC_HUMAN	SPARC[a) b)]	34.6	35	12	17	96	S
P98160	PGBM_HUMAN	Basement membrane-specific heparan sulfate proteoglycan core protein	468.8	6	21	22	51	M
P09382	LEG1_HUMAN	Galectin-1[a)]	14.7	54	7	8	44	S
P17931	LEG3_HUMAN	Galectin-3	26.2	10	2	2	4	M/S
P23142	FBLN1_HUMAN	Fibullin-1	77.3	3	2	2	3	S
Q12805	FBLN3_HUMAN	EGF-containing fibulin-like extracellular matrix protein 1[a)]	54.6	20	9	10	36	S
Q08629	TICN1_HUMAN	Testican-1	49.1	4	2	2	12	S
Q9H8L6	MMRN2_HUMAN	Multimerin 2, Emilin 3	104.4	3	2	2	6	S
P08572	CO4A2_HUMAN	Collagen α-2(IV) chain	167.6	3	3	3	4	S
P05997	CO5A2_HUMAN	Collagen α-2(V) chain	144.9	1	2	2	2	S
P21980	TGM2_HUMAN	Protein-glutamine γ-glutamyltransferase 2	77.3	4	2	2	4	S
Q9Y4K0	LOXL2_HUMAN	Lysyl oxidase homolog 2[a)]	86.7	4	2	2	7	S
Insulin-like growth factor-binding proteins								
P18065	IBP2_HUMAN	IGF-binding protein 2[a)]	35.1	33	8	10	53	S
P22692	IBP4_HUMAN	IGF-binding protein 4[a)]	27.9	16	3	4	15	S
Q16270	IBP7_HUMAN	IGF-binding protein 7[a)]	29.1	20	5	5	31	S
O00622	CYR61_HUMAN	Protein CYR61, IGF-binding protein 10	42.0	12	4	5	6	S
Growth factors and related proteins								
P29279	CTGF_HUMAN	Connective tissue growth factor	38.1	15	4	4	32	S
Q12841	FSTL1_HUMAN	Follistatin-related protein 1[a) b)]	34.9	12	4	4	29	S
Q9UBP4	DKK3_HUMAN	Dickkopf-related protein 3	38.3	10	3	3	9	S
P09341	GROA_HUMAN	Growth-regulated α protein, CXCL1	11.3	6	1	1	2	S
Inflammation-related proteins								
Q01638	ILRL1_HUMAN	Interleukin-1 receptor-like 1	63.4	7	4	5	29	S

Table 1. Continued

Accession no.	Entry name	Protein name	MW (kDa)	Coverage (%)	Unique peptides	Unique spectra	Assigned spectra	Location
P26022	PTX3_HUMAN	Pentraxin-related protein PTX3[a)]	42.0	20	6	6	29	S
Q969H8	CS010_HUMAN	Uncharacterized protein C19orf10 Interleukin 25	18.8	24	3	3	15	S
P05067	A4_HUMAN	Amyloid β A4 protein[a)]	86.9	7	3	3	3	M
P0C0L4	CO4A_HUMAN	Complement C4A, C4B	192.8	2	2	2	2	S
P05156	CFAI_HUMAN	Complement factor I	65.7	4	2	2	6	S
Protein S100 family								
P31151	S10A7_HUMAN	Protein S100-A7	11.5	35	3	3	9	S
P05109	S10A8_HUMAN	Protein S100-A8	10.8	42	4	4	11	S
P06702	S10A9_HUMAN	Protein S100-A9	13.2	39	3	3	6	S
Coagulation and related proteins								
P05121	PAI1_HUMAN	Plasminogen activator inhibitor 1[a) b)]	45.1	11	3	3	9	S
P10646	TFPI1_HUMAN	Tissue factor pathway inhibitor	35.0	9	3	3	9	S
Q13201	MMRN1_HUMAN	Multimerin 1, emilin 4	138.1	6	4	5	9	S
AAB59458	VWF_HUMAN	von Willebrand factor[a)]	309.3	4	9	10	18	S
Annexins and calcium ion-binding proteins								
P07355	ANXA2_HUMAN	Annexin A2[a) b)]	38.6	42	11	12	34	M
P08758	ANXA5_HUMAN	Annexin A5[a) b)]	35.9	9	4	4	13	M
O43852	CALU_HUMAN	Calumenin	37.1	8	2	2	6	C
P27797	CALR_HUMAN	Calreticulin[a)]	48.1	21	7	10	35	C
O94985	CSTN1_HUMAN	Calsyntenin-1	109.8	2	2	2	4	M
Miscellaneous secreted proteins								
P55145	ARMET_HUMAN	Protein ARMET	20.3	23	3	3	5	S
P61769	B2MG_HUMAN	β-2-Microglobulin[a)]	13.7	19	2	2	6	S
Membrane antigens and receptors								
P13987	CD59_HUMAN	CD59 glycoprotein[a)]	14.2	20	3	4	22	M
P13598	ICAM2_HUMAN	Intercellular adhesion molecule 2	30.7	15	3	3	20	M
Q9NPY3	C1QR1_HUMAN	Complement component C1q receptor	68.6	9	3	3	6	M
Q9UNN8	EPCR_HUMAN	Endothelial protein C receptor	26.7	29	6	9	80	M/S
P30530	UFO_HUMAN	Tyrosine-protein kinase receptor UFO	97.4	3	2	2	6	M
P33151	CADH5_HUMAN	Cadherin-5, VE-Cadherin, CD144	87.5	13	8	9	28	M
P43121	MUC18_HUMAN	Cell surface glycoprotein MUC18, CD146	71.6	8	5	5	9	M
P07996	TSP1_HUMAN	Thrombospondin 1	129.4	18	17	18	64	M
Miscellaneous membrane proteins								
P07237	PDIA1_HUMAN	Protein disulfide-isomerase, proly-4-hydroxylase, beta polypeptide[a)]	57.1	34	16	17	33	C
P30101	PDIA3_HUMAN	Protein disulfide-isomerase A3[a)]	56.8	13	6	6	10	C
Q15904	VAS1_HUMAN	Vacuolar ATP synthase subunit S1	52.0	11	4	4	11	M
Q12907	LMAN2_HUMAN	Vesicular integral-membrane protein VIP36	40.2	7	2	2	3	C
Q99536	VAT1_HUMAN	Synaptic vesicle membrane protein VAT 1 homolog, lectin, mannose-binding 2	41.9	8	2	2	3	M

Table 1. Continued

Accession no.	Entry name	Protein name	MW (kDa)	Coverage (%)	Unique peptides	Unique spectra	Assigned spectra	Location
O95810	SDPR_HUMAN	Serum depriviation-response protein	47.1	7	2	2	8	M
Lysosomal proteins								
P11279	LAMP1_HUMAN	Lysosome-associated membrane glycoprotein 1, CD107a	44.7	8	3	3	6	M
P07339	CATD_HUMAN	Cathepsin D	44.5	5	2	2	7	C
P42785	PCP_HUMAN	Lysosomal Pro-X carboxypeptidase, angiotensinase C	55.8	6	3	3	3	M/C
P07602	SAP_HUMAN	Proactivator polypeptide (Saposin-A)[a)]	58.1	10	5	5	14	S

Minimum: 95% protein probability, 80% peptide probability; M, Membrane; S, Secreted and C, Cytoplasm.
a) Previously identified in the secretome of human microvascular endothelial cells [9].
b) Previously identified in the secretome of rat endothelial cells [8].

is deposited in PRIDE (http://www.ebi.ac.uk/pride/, accession numbers: 9291-9353 plus 9751).

Figure 2C shows the number of identified proteins in each fraction. As expected based on the high conductivity of the anodic and cathodic stabilization media [7], fractions 24–61 contained most proteins. Albumin spreads over multiple fractions (Fig. 2D), but the percentage of albumin scans was comparable to the best results reported by others [8] (14.8 and 11.9% of all identified spectra were assigned to albumin in the first and second biological replicate, respectively). Unlike albumin, most proteins were reproducibly confined to few fractions (Figs. 2E and F) and most of the secreted and membrane proteins identified have not been previously reported in proteomic analyses of conditioned medium from endothelial cells [8, 9] (see footnote to Table 1). Representative MS/MS spectra are shown in Supporting Information Fig. 1. Unsupervised pathway analysis (Ingenuity System, Mountain View, CA) built four dominant protein association networks (Supporting Information Fig. 2) and returned ''coagulation system'', ''IGF-1 signalling'', ''complement system'' and ''leukocyte extravasation signalling'' as top canonical pathways.

Factors released by endothelial cells play a key role in vascular homeostasis. In this study, we explored the possibilities of FFE to overcome the complexity of proteins in conditioned medium of cell cultures. Briefly, a carrier ampholite is loaded with the protein sample and continuously streamed [10–12]. The separation takes place due to an electrical field perpendicular to the direction of the flow and proteins are collected separately depending on their p*I*. FFE has been widely used in proteomics, including the separation of cell organelles [13, 14] or plasma samples [7, 11], but to our knowledge, no analysis of cell culture medium has been performed thus far. FFE offers three major advantages for the analysis of conditioned medium: First, because of the high loading capacity, large volumes of conditioned medium (24 mL were used in this study) can be concentrated, desalted and prefractionated in a single run. Second, albumin, the most abundant component in the culture medium, is contained in 14 fractions out of 96 wells. Thus, FFE can separate low-abundant proteins from high-abundant compounds in the conditioned medium. Third, the prefractionation is performed in the liquid-phase minimizing potential sample loss and providing maximum compatibility with subsequent analysis by LC-MS/MS.

Traditional cardiovascular risk factors, such as hypercholesterolemia, hypertension, diabetes and cigarette smoking, induce endothelial dysfunction. Our approach of combining FFE with LC-MS/MS partially overcomes the problems inherent to large-scale analyses of protein secretion [15] and will assist future studies of endothelial protein release in response to cardiovascular risk factors [16].

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The authors have declared no conflict of interest.

References

[1] Mayr, M., Mayr, U., Chung, Y. L., Yin, X. *et al.*, Vascular proteomics: linking proteomic and metabolomic changes. *Proteomics* 2004, *4*, 3751–3761.

[2] Peterson, D. B., Sander, T., Kaul, S., Wakim, B. T. *et al.*, Comparative proteomic analysis of PAI-1 and TNF-alpha-derived endothelial microparticles. *Proteomics* 2008, *8*, 2430–2446.

[3] Banfi, C., Brioschi, M., Wait, R., Begum, S. *et al.*, Proteome of endothelial cell-derived procoagulant microparticles. *Proteomics* 2005, *5*, 4443–4455.

[4] Bruneel, A., Labas, V., Mailloux, A., Sharma, S. *et al.*, Proteomics of human umbilical vein endothelial cells applied to etoposide-induced apoptosis. *Proteomics* 2005, *5*, 3876–3884.

[5] Scheurer, S. B., Rybak, J. N., Rosli, C., Neri, D., Elia, G., Modulation of gene expression by hypoxia in human umbilical cord vein endothelial cells: a transcriptomic and proteomic study. *Proteomics* 2004, *4*, 1737–1760.

[6] Mayr, M., Metzler, B., Kiechl, S., Willeit, J. *et al.*, Endothelial cytotoxicity mediated by serum antibodies to heat shock proteins of Escherichia coli and Chlamydia pneumoniae: immune reactions to heat shock proteins as a possible link between infection and atherosclerosis. *Circulation* 1999, *99*, 1560–1566.

[7] Nissum, M., Kuhfuss, S., Hauptmann, M., Obermaier, C. *et al.*, Two-dimensional separation of human plasma proteins using iterative free-flow electrophoresis. *Proteomics* 2007, *7*, 4218–4227.

[8] Pellitteri-Hahn, M. C., Warren, M. C., Didier, D. N., Winkler, E. L . *et al.*, Improved mass spectrometric proteomic profiling of the secretome of rat vascular endothelial cells. *J. Proteome Res.* 2006, *5*, 2861–2864.

[9] Flora, J. W., Edmiston, J., Secrist, R., Li, G. *et al.*, Identification of *in vitro* differential cell secretions due to cigarette smoke condensate exposure using nanoflow capillary liquid chromatography and high-resolution mass spectrometry. *Anal. Bioanal. Chem.* 2008, *391*, 2845–2856.

[10] Hannig, K., Kowalski, M., Klock, G., Zimmermann, U., Mang, V., Free-flow electrophoresis under microgravity: evidence for enhanced resolution of cell separation. *Electrophoresis* 1990, *11*, 600–604.

[11] Nissum, M., Foucher, A. L., Analysis of human plasma proteins: a focus on sample collection and separation using free-flow electrophoresis. *Exp. Rev. Proteomics* 2008, *5*, 571–587.

[12] Moritz, R. L., Simpson, R. J., Liquid-based free-flow electrophoresis-reversed-phase HPLC: a proteomic tool. *Nat. Methods* 2005, *2*, 863–873.

[13] Sengelov, H., Borregaard, N., Free-flow electrophoresis in subcellular fractionation of human neutrophils. *J. Immunol. Methods* 1999, *232*, 145–152.

[14] Zischka, H., Braun, R. J., Marantidis, E. P., Buringer, D. *et al.*, Differential analysis of *Saccharomyces cerevisiae* mitochondria by free-flow electrophoresis. *Mol Cell Proteomics* 2006, *5*, 2185–2200.

[15] Pula, G., Mayr, U., Evans, C., Prokopi, M. *et al.*, Proteomics identifies thymidine phosphorylase as a key regulator of the angiogenic potential of colony-forming units and endothelial progenitor cell cultures. *Circ. Res.* 2009, *104*, 32–40.

[16] Mayr, M., Zhang, J., Greene, A. S., Gutterman, D. *et al.*, Proteomics-based development of biomarkers in cardiovascular disease: mechanistic, clinical, and therapeutic insights. *Mol. Cell. Proteomics* 2006, *5*, 1853–1864.

