Evaluation of dynamic changes in interstitial fluid proteome following microdialysis probe insertion trauma in trapezius muscle of healthy women

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Microdialysis (MD) has been shown to be a promising technique for sampling of biomarkers. Implantation of MD probe causes an acute tissue trauma and provokes innate response cascades. In order to normalize tissue a two hours equilibration period for analysis of small molecules has been reported previously. However, how the proteome profile changes due to this acute trauma has yet to be fully understood. To characterize the early proteome events induced by this trauma we compared proteome in muscle dialysate collected during the equilibration period with two hours later in “post-trauma”. Samples were collected from healthy females using a 100 kDa MW cut off membrane and analyzed by high sensitive liquid chromatography tandem mass spectrometry. Proteins involved in stress response, immune system processes, inflammatory responses and nociception from extracellular and intracellular fluid spaces were identified. Sixteen proteins were found to be differentially abundant in samples collected during first two hours in comparison to “post-trauma”. Our data suggests that microdialysis in combination with mass spectrometry may provide potentially new insights into the interstitial proteome of trapezius muscle, yet should be further adjusted for biomarker discovery and diagnostics. Moreover, MD proteome alterations in response to catheter injury may reflect individual innate reactivity.

Microdialysis (MD) is an established technique for the in vivo sampling of various substances from the extracellular space of various tissues giving the possibility to monitor localized molecular events in tissues before changes occurs on the blood level. MD of muscle tissues has been used by our group to study nociceptive and metabolic mechanisms in different chronic musculoskeletal pain states. The exact nociceptive/inflammatory and neuropathic pain mechanisms underlying chronic muscle pain are very complex and not fully clarified and identification of myalgia “signatures” requires a detection method that allows sensitive, specific and reproducible identification, and quantification of potential biomarkers within a high dynamic range. During MD, a catheter with a porous membrane is implanted into the muscle, which is aimed to mimic the functions of a capillary blood vessel, where molecules from extracellular space are diffusing to the physiological saline (perfusion) solution along the concentration gradient and can be collected for following analyses. MD has been initially developed for sampling of rather small molecules (metabolites, amino acids, energy substrates, drugs and neurotransmitters). During the last decade the high molecular weight cut-off MD has demonstrated to be a promising technique for the sampling of protein biomarkers; however this application is rather complex in comparison to small molecule MD. To turn this technique into the robust and reliable daily clinical routine method for in situ monitoring of protein biomarkers, and to explore new clinically relevant biomarkers a deeper method understanding and optimization is needed.

MD causes minimal discomfort to patients and due to the small size of the probe is called a minimally-invasive procedure. Nevertheless, the insertion of MD catheter induces mechanical damage of the surrounding tissue and
blood vessels3–5, which results in local blood flow interruption and bleeding5–7, followed by cascades of fast signaling molecular events6. MD probe implantation itself can lead to inflammatory responses, both acute and chronic6, leading to a variety of substances being released from the damaged tissue, including inflammatory mediators, such as chemokines and cytokines. MD injury can also led to activation of sensory receptors including nociceptors if trauma is localized in the vicinity to them, generating sensation of pain8.

Therefore, there are reasonable concerns about inaccurate interpretation of rather complex stress, wound, wound repair, and disease response events: the disease-related and catheter injury-associated events can involve the same proteins/molecules and thus the sum of events, which can, potentially, compromise study outcomes6.

In order to minimize the influence of needle trauma on experimental outcomes, the "recovery and equilibration" period is often introduced to allow the vascular reaction to return to the normal (or stabilized) state9. Thus an equilibration period of two hours after probe injection is typically used in small molecular MD sampling protocols, and initial fractions under 2 hours are discarded or not analyzed. The same two hours equilibration period is often used even in case of protein biomarkers sampling10–12, thus discarding potentially useful information about the individual innate response, which might be different depending on individual subject condition3.

In this respect a better understanding of processes that occur in tissues during microdialysis is needed. The present study aimed to evaluate changes in the proteome over time following catheter insertion in the trapezius muscle of healthy subjects. We performed proteomic analyses of MD fraction collected immediately after insertion of MD probe (trauma protein fraction (T)) and compared it to the fractions collected 2 hours later (post trauma fraction (PT)).

**Results and Discussion**

In present study we used advantages of high-resolution mass spectrometry for the analysis of peptides obtained from two different muscle dialysate fractions of individual subjects: equilibration period "trauma" fraction (T), collected immediately after catheter insertion and a "post-trauma" fraction (PT) collected 2 hours later. Proteins from dialysate fractions obtained from trapezius muscle interstitium of six healthy female subjects were subjected to minimal sample preparation: desalted, concentrated, and digested with trypsin. Equal amounts of peptides were analyzed in duplicate by LC-MS/MS. The obtained raw files were analyzed using Sequest HT algorithm in Proteome Discoverer (Thermo). The parsimony principle was applied and proteins with similar sequences sharing the same identified peptides were reported as one group.

This straightforward approach allowed the identification of 2516 proteins, in total, merged in to 579 protein groups. A substantial variance between individual samples was observed. Only about 40% of all proteins were identical between all individual subjects from both T and PT periods. Datasets for T and PT samples for proteins detected with top PSMs and identified with the largest number of peptides, were generally the same for all samples (Fig. 1). Proteins identified with top scores were generally the same for T and PT samples (Tables 1 and 2) and includes structural proteins and proteins abundant in plasma/serum. Actin and myosin isoforms are major proteins in muscle cells known to be released into blood in case of muscle damage13. Ten proteins (serum albumin, hemoglobin, carbonic anhydrase-1, immunoglobulins, α1-antitrypsin, serotransferrin, fibrinogen, complement C3, hemoplexin, and prothrombin) were identified with top scores in all fractions are well known as the most abundant blood/serum/plasma proteins (Fig. 1).

Figure 1. Scatter diagram of the protein changes. Scatter plot of T versus PT protein scores (sum of the scores of individual peptides) of all confidently identified proteins. Each dot in the figure represents one of the proteins.
Data files obtained in Proteome Discoverer were exported and further validated in Scaffold software with 90% peptide identification probability, 99% protein identification probability parameters and with minimum 2 peptides required for protein identification, resulting in a 0.0% peptide decoy false discovery rate. Proteins were merged into 212 clusters according to Scaffold's protein cluster analysis algorithm. (Supplementary data S1).

Scaffold software was used to perform a comparative differential analysis of T and PT samples, employing Student’s t-test using normalized spectral abundance factor method (NSAF) to normalize run-to-run variations19. Sixteen proteins were found to have statistically significant differential abundance in T and PT samples (Table 3).

Most of these 16 proteins are known to leak out into the blood in case of tissue damage and represent markers of cellular damage. Four proteins related to muscle energy metabolism: isoform 2 of triosephosphate isomerase; phosphoglycerate kinase; creatine kinase and beta-enolase M-type were significantly higher at initial T and decreased in PT fractions.

Creatine kinase plays a central role in energy transduction especially in skeletal muscle tissues, where rapid energy consumption is needed, and creatine kinase levels/activity in plasma are known to vary between healthy subjects depending on age, gender, race, and physical activity20. In conditions that damage skeletal muscles or brain (heart attacks, myositis, strenuous exercises, muscular dystrophy, cerebral diseases, any mechanical muscle damage), creatine kinase is released from muscle into the blood. The routine test for elevated levels of creatine kinase in blood is traditionally used to detect inflammation of muscles or serious muscle damage21. Phosphoglycerate kinase, triosephosphate isomerase, and muscle specific glycolytic enzyme beta-enolase are essential for efficient energy production. Beta- enolase is also known as a serum marker for muscle damage22.

The levels of 12 proteins (myozenin-1, troponins, vimentin, profilin, RhoGDI2, alpha-crystallin B, histone H1.5, S100-A8 and S100-A9) were increased in PT samples in comparison to initial T samples. Four of them were muscle contraction and calcium signal transduction-related proteins: myozenin-1 and troponins. In skeletal muscle, calcium plays a pivotal role in signal transduction and is essential for cellular processes such as excitation-contraction coupling23. Troponins (Troponin T, fast skeletal muscle, Troponin I, slow skeletal muscle, Isoform 2 of Troponin T, slow skeletal muscle) are regulatory proteins in skeletal and cardiac muscle. Raised

| Protein ID | Protein name                                      | Sum Xcorr | Sum Coverage, % | Mw, kDa |
|------------|--------------------------------------------------|-----------|-----------------|--------|
| 1          | ALBU_HUMAN Serum albumin                         | 6008.93   | 90.31           | 69.3   |
| 2          | HBA_HUMAN Hemoglobin subunit alpha              | 3885.41   | 87.32           | 15.2   |
| 3          | MYG_HUMAN Myoglobin OS                           | 5458.04   | 80.52           | 17.2   |
| 4          | HBB_HUMAN Hemoglobin subunit beta               | 2965.12   | 95.92           | 16.0   |
| 5          | CAH3_HUMAN Carbonic anhydrase 3                 | 1818.56   | 88.85           | 29.5   |
| 6          | TRF_HUMAN Serotransferrin                        | 1807.00   | 66.91           | 77.0   |
| 7          | FIBA_HUMAN Fibrinogen alpha chain                | 1611.09   | 48.38           | 94.9   |
| 8          | HBD_HUMAN Hemoglobin subunit delta              | 1515.40   | 93.20           | 16.0   |
| 9          | MYH7_HUMAN Myosin-7                             | 1373.51   | 28.63           | 223.0  |
| 10         | MYH2_HUMAN Myosin-2                             | 1319.36   | 27.41           | 222.9  |
| 11         | MYH1_HUMAN Myosin-1                             | 1074.00   | 23.36           | 223.0  |
| 12         | CAH1_HUMAN Carbonic anhydrase 1                 | 962.34    | 68.20           | 28.9   |
| 13         | A1AT_HUMAN Alpha-1-antitrypsin                   | 785.71    | 52.63           | 46.7   |
| 14         | FHL1_HUMAN Isoform 5 of Four and a half LIM domains protein | 698.88 | 71.96 | 33.6 |
| 15         | F8WCP0_HUMAN Nebulin                             | 697.60    | 17.01           | 986.1  |
| 16         | HEMO_HUMAN Hemopexin                             | 653.26    | 55.84           | 51.6   |
| 17         | TPIS_HUMAN Isoform 2 of Triosephosphate isomerase | 638.12   | 81.93           | 26.7   |
| 18         | PEBP1_HUMAN Phosphatidylethanolamine-binding protein 1 | 589.24 | 75.40 | 21.0 |
| 19         | MYH3_HUMAN Myosin-3                             | 512.92    | 11.55           | 223.8  |
| 20         | EARP_HUMAN Fatty acid-binding protein, heart     | 475.80    | 63.91           | 14.8   |
| 21         | IGHQ1_HUMAN Ig gamma-1 chain C region            | 457.88    | 50.91           | 36.1   |
| 22         | THR1_HUMAN Prothrombin                           | 425.90    | 32.96           | 70.0   |
| 23         | HBQ1_HUMAN Hemoglobin subunit gamma-1            | 421.21    | 45.58           | 16.1   |
| 24         | D6R35_HUMAN Vitamin D-binding protein            | 420.17    | 50.84           | 53.0   |
| 25         | IGK0_HUMAN Ig kappa chain C region               | 403.16    | 82.08           | 11.6   |
| 26         | BLVRB_HUMAN Flavin reductase (NADPH)              | 395.55    | 75.73           | 22.1   |
| 27         | B7Z9_HUMAN Phosphoglycerate kinase               | 379.70    | 57.33           | 41.4   |
| 28         | K2C1_HUMAN Keratin, type II cytoskeletal 1       | 366.51    | 39.44           | 66.0   |
| 29         | A1AG1_HUMAN Alpha-1-acid glycoprotein 1          | 363.39    | 48.76           | 23.5   |
| 30         | KCRM_HUMAN Creatine kinase M-type                | 363.20    | 49.87           | 43.1   |

Table 1. Top 30 most abundant proteins during the equilibration period. The ranking is based on Peptide Spectrum match (PSM) from high to low.
| Protein ID   | Protein name                     | Mw, kDa | Fold change          |
|-------------|----------------------------------|---------|----------------------|
| S10A9_HUMAN | S10A9_HUMAN                      | 13 kDa  | post-trauma high, trauma low |
| S10A8_HUMAN | S10A8_HUMAN                      | 11 kDa  |                       |
| PROF1_HUMAN | PROF1_HUMAN                      | 15 kDa  |                       |
| LYSC_HUMAN  | LYSC_HUMAN                       | 17 kDa  |                       |
| GD1R2_HUMAN | GD1R2_HUMAN                      | 23 kDa  |                       |
| H15_HUMAN   | H15_HUMAN                        | 23 kDa  |                       |
| TNNT3_HUMAN | TNNT3_HUMAN                      | 30 kDa  | 4.7                   |
| TNNT1_HUMAN | TNNT1_HUMAN                      | 30 kDa  | 7.4                   |
| TNNI1_HUMAN | TNNI1_HUMAN                      | 22 kDa  | 3.1                   |
| MYOZI_HUMAN | MYOZI_HUMAN                      | 32 kDa  |                       |
| VIME_HUMAN  | VIME_HUMAN                       | 54 kDa  | 8.7                   |
| CRYAB_HUMAN | CRYAB_HUMAN                      | 20 kDa  | 17                    |
| TPIS_HUMAN  | TPIS_HUMAN                       | 27 kDa  | 0.3                   |
| PGK1_HUMAN  | PGK1_HUMAN                       | 41 kDa  | 0.2                   |
| KCRM_HUMAN  | KCRM_HUMAN                       | 43 kDa  | 0.06                  |
| ENOB_HUMAN  | ENOB_HUMAN                       | 47 kDa  | 0.1                   |

Table 3. Proteins significantly (p < 0.05) altered between T and PT samples.
troponin level is used as plasma marker of skeletal muscle damage and particularly as an indication of cardiac muscle cell death.

Proteins involved in actin cytoskeleton regulation (vimentin and profilin): Profilin-1 is one of the most important regulators of F-actin dynamics, regulating many intracellular functions implicated to play a role in many pathological conditions25. Vimentin is known to be implicated in the regulation of cell migration and proliferation during the wound healing process26-28; desmin and vimentin are markers of regeneration in muscle disease29-31, and up-regulated vimentin level is a marker of skeletal muscle injury32.

Among proteins which were up-regulated in all PT samples we identified a two proteins with chaperon activities: Rho GDP-dissociation inhibitor 2 (RhoGDI2) and alpha-crystallin B. RhoGDI2 (a member of a small family of chaperone proteins, which controls Rho GTPases33) is linked to apoptosis-induced cytoskeletal reorganization and is potentially associated with oxidative stress, apoptosis, and wound healing34. Alpha-crystallin B (Heat shock protein beta-5, HspB5) is part of the small heat shock protein family and is able to interact with misfolded proteins to prevent protein aggregation and a wide range of cell stress conditions, as well as inhibit apoptosis and contribute to intracellular architecture35. During cell stress, induction and secretion of HSPs leads to pro-inflammatory cytokine and chemokine release activating immune responses36. Alpha-crystallin B was found to be upregulated in trapezius muscle of chronic widespread pain subjects in comparison to healthy control group in our previous MD-study12.

The level of Histone H1.5 was also increased in PT samples. Beside the classical gene-regulating role, extracellular histones bind to receptors and trigger activation of multiple signaling pathways. Histone levels are known to be significantly elevated in response to injury and involved in the regulation of inflammation37. Furthermore, the linker Histone H1.5 was suggested to play a role in the development of muscle38, and may stimulate the proliferative function of 46 of these proteins was identified as “response to stress” (Fig. 4, red color).

Some low-mass proteins, most of them (as much as 74) belonging to extracellular region proteins. Interestingly, the low-molecular weight proteins (< 25 kDa). According to Scaffold analysis, 106 protein groups identified with at least one peptide were corresponding to proteins with molecular weight of less or equal 25 kDa (Supplementary S2). The list of the identified protein was subjected to STRING analysis to reveal functional interactions between the low mass proteins, most of them (as much as 74) belonging to extracellular region proteins. Interestingly, the function of 46 of these proteins was identified as “response to stress” (Fig. 4, red color).

In our previous study we employed the MD technique in combination with 2-D electrophoresis and in-gel digestion to characterize changes in trapezius muscle interstitial proteome in women with chronic myalgia12. However, proteins with low-molecular weight are often underrepresented in in-gel digestion based proteomic studies or their coverage and, by that; validity of their identification is small. The limited number tryptic cleavage sites and by that limited number of generated tryptic peptides in the small proteins should be taken into consideration as well.

From the collected fractions we were able to identify 13 proteins involved in nociception (Table 5) with rather low levels and low reproducibility with some exceptions: high molecular weight kininogen, neutrophyl cytosolic factor 2 and calmodulin 5 were identified with 3 or more peptides. Consistent with previous publications3,15 no significant levels of interleukin were detected in these sampling conditions.

Conclusions
Proteins involved virtually in stress responses, immune system processes, inflammatory responses and nociception were identified in the interstitial fluid MD proteome in healthy pain free subjects. Moreover, the comparison proteins in “trauma” dialysate collected immediately after catheter insertion with proteins from “post-trauma” MD-fraction revealed 16 differentially abundant proteins (p < 0.05). Our results demonstrate that MD-LC-MS/MS is a promising approach to provide new insights into the interstitial proteome of muscle, and potentially can be further adjusted for biomarker discovery and diagnostics. The presence of highly abundant serum and muscle structural proteins, sample complexity, multiple protein isoforms, protein modifications and low abundance of many proteins of interest are the key challenges. Moreover, careful validation of biological relevance of biomarkers is of great importance for personalized medicine.

The damage caused by the MD probe should be taken into consideration when analyzing disease biomarkers using MD.

Materials and Methods
Subjects. Six healthy women (age: 35.1 ± 8.3, BMI: 23.8 ± 2.3) were included in this study. Subjects were instructed not to drink any beverages with caffeine on the day of the study, not to smoke and to avoid
NSAID-medication the week before the study. The participants arrived at the clinic in the morning after having eaten breakfast. A brief interview was then made by one of the physicians checking that the instructions had been followed. All subjects reported that they had followed the instructions. During the study, they were not allowed to

**Figure 2.** Pie charts classifying the identified proteins according to their biological processes (A), cellular components (B) and molecular functions (C). The identified proteins were grouped according to GO annotations.
| Identified Proteins and Clusters | Gene name | Protein ID |
|---------------------------------|-----------|------------|
| Myoglobin                        | MB        | MYG_HUMAN  |
| Phosphatidylethanolamine-binding protein 1 | FERP1 | FERP1_HUMAN |
| Protein S100-A9                  | S100A9    | S10A9_HUMAN|
| Isoform 2 of Fibrinogen alpha chain | FGA | FIBA_HUMAN |
| Protein S100-A8                  | S100A8    | S10A8_HUMAN|
| Superoxide dismutase [Cu-Zn]     | SOD1      | SODC_HUMAN |
| Keratin, type II cytoskeletal 1  | KRT1      | K2C1_HUMAN |
| Neutrophil defensin 1            | DEFA1     | DEFI_HUMAN |
| Bbox GDP-dissociation inhibitor 2 | ARHGDIB | GDIR2_HUMAN |
| Keratin, type I cytoskeletal 10  | KRT10     | K1C10_HUMAN|
| Peptidoglycan recognition protein 1 | PGLYRP1 | PGRP1_HUMAN |
| High mobility group protein B2    | HMGB2     | HMGB2_HUMAN|
| Keratin, type II cytoskeletal 2 epidermal | KRT2 | K2JE_HUMAN |
| Peptidyl-prolyl cis-trans isomerase A | PPIA | PPIA_HUMAN |
| Coflin-1 OS = Homo sapiens       | CFL1      | COF1_HUMAN |
| Fibrinogen beta chain            | FGB       | FIBB_HUMAN |
| Prothrombin                      | F2        | THRB_HUMAN |
| Ubiquitin-40S ribosomal protein S27a | RPS27A   | RS27A_HUMAN|
| Actin, alpha cardiac muscle 1    | ACTC1     | ACTC_HUMAN |
| Complement C3                    | C3        | CO3_HUMAN |
| Myosin-9                         | MYH9      | MYH9_HUMAN |
| Myosin-7                         | MYH7      | MYH7_HUMAN |
| Ig lambda-2 chain C regions      | IGLC2     | LAC2_HUMAN |
| Complement component C4B (Chido blood group | C4B-1 | A2BHY4_HUMAN |
| Metallothionein-1G               | MT1G      | MT1G_HUMAN |
| Ig gamma-1 chain C region        | IGHG1     | IGHG1_HUMAN|
| Alpha-actinin-2                  | ACTN2     | ACTN2_HUMAN|
| Haptoglobin                      | HP        | HPT_HUMAN |
| Ig alpha-1 chain C region        | IGHA1     | IGHA1_HUMAN|
| Ig heavy chain V-III region BUT  | N/A       | HY306_HUMAN|
| Complement factor B              | CFB       | B4E1Z4_HUMAN|
| Ig kappa chain C region          | IGKC      | IGKC_HUMAN |
| Zinc-alpha-2-glycoprotein        | AZGP1     | ZAQG_HUMAN |
| Ig gamma-2 chain C region        | IGHG2     | IGHG2_HUMAN|
| Beta-2-microglobulin             | B2M       | B2MG_HUMAN |
| Ig gamma-4 chain C region        | IGHG4     | IGHG4_HUMAN|
| Thioredoxin                      | TXN       | THIO_HUMAN |
| Isoform 2 of Semenogelin-1       | SEMG1     | SEMG1_HUMAN|
| Isoform 2 of Clusterin           | CLU       | CLUS_HUMAN |
| Apolipoprotein A-IV              | APOA4     | APOA4_HUMAN|
| Angiogenin                       | ANG       | ANGI_HUMAN |
| Cathelicidin antimicrobial peptide | CAMP   | CAMP_HUMAN |
| Cathepsin G                      | CTSG      | CATG_HUMAN |
| Complement C5                    | C5        | CO5_HUMAN |
| Complement factor D              | CFD       | CFAD_HUMAN |
| Hematopoietic lineage cell-specific protein SV = 3 | HCLS1 | HCLS1_HUMAN |
| High mobility group protein B1   | HMGB1     | HMGB1_HUMAN|
| Hornerin                         | HRNR      | HORN_HUMAN |
| Isoform 11 of Fibronectin        | FN1       | FINC_HUMAN |
| Lactoylglutathione lyase          | GLO1      | LGUL_HUMAN |
| Peroxiredoxin-2                  | PRDX2     | PRDX2_HUMAN|
| Platelet basic protein            | PPBP      | CXCL7_HUMAN|
| Protein S100-A7                   | S100A7    | S10A7_HUMAN|
| Ras-related protein Rab-7a       | RAB7A     | RAB7A_HUMAN|

Table 4. Proteins involved in immune system processes.
eat, but they could drink water. All participants gave their informed written consent before the start of the study. The study was approved by the Ethical Committee of Linköping University (Dnr: M10-08) and the methods were carried out in accordance with the approved guidelines.

**Microdialysis.** Microdialysis was performed generally as described in ref. 12. Samples were collected during 20–120 minutes after the catheter insertion (trauma period samples, T) and during 140–200 minutes after the catheter insertion (post-trauma samples, PT). All vials were weighed before the experiment started and after each 20 minutes interval in order to confirm that sampling and fluid recovery (FR) was working according to the perfusion rate set. Vials with visible sign of hemolysis were discarded. The samples were stored on ice to prevent protease activation. The samples were then stored as aliquots in −70°C until analysis.

**Protein extraction and digestion.** Samples were subjected to 3kDa Amicon spin-filter (Merck Millipore) to desalt and concentrate the protein contents. The desalted proteins were dried by speed vacuum concentrator, redissolved in 6 M urea in 25 mM ammonium bicarbonate and incubated at room temperature for at least 30 minutes. The proteins were reduced by incubating in 25 mM DTT for 15 minutes and alkylated with 75 mM iodoacetamide for an additional 15 minutes. The samples were diluted 8 times with 25 mM ammonium bicarbonate and filtered by 3kDa Amicon spin-filter before digestion with trypsin (1:25, w/w trypsin/protein). The digested peptides were dried in speed vacuum concentrator, reconstituted in 0.1% of formic acid in MilliQ water and approximately 0.25 μg was subjected to LC-MS/MS analysis.

**Figure 3.** STRING Bioinformatic analysis of the proteins involved in immune system response shown in Table 4. Colored network nodes represent query proteins. Edges represent protein-protein interactions and include different type of actions depicted by the colored lines. For known interactions: pink, experimentally determined; turquoise, from curated databases. For predicted interactions: green, gene neighborhood; blue, gene co-occurrence. For others interactions: olive green, textmining; black, co-expression; purple, protein homology. Extracellular proteins are marked with red color.
LC-MS/MS analysis. Peptides were separated by reverse phase chromatography on a 20 mm × 100 μm C18 pre column followed by a 100 mm × 75 μm C18 column with particle size 5 μm (NanoSeparatoons, Nieuwkoop, Netherlands) at a flow rate 300 nL/min. EASY-nLC II (Thermo Scientific) by linear gradient of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) (0–100% B in 90 min). Automated online analyses were performed with a LTQ Orbitrap Velos Pro hybrid mass spectrometer (Thermo Scientific) with a nano-electrospray source.

Database searches. Raw files were searched using Sequest HT in Proteome Discoverer (Thermo Fisher Scientific, San Jose, CA, USA; version 1.4.0.288) against a Uniprot Human database (available at UniProtKB website: http://www.uniprot.org/taxonomy/9606) with the following parameters: semi trypsin was used as digestion enzyme; maximum number of missed cleavages 2; fragment ion mass tolerance 0.60 Da; parent ion mass tolerance 10.0 ppm; fixed modification- carbamidomethylation of cysteine; variable modifications - N-terminal acetylation. Data were filtered at 1% false discovery rate, high peptide confidence; rank 1 peptides in top scored proteins.

Data evaluation. Identified proteins were filtered using SCAFFOLD (version 1.4.0.288; Proteome Software Inc., Portland, OR, USA). Identifications were based on a minimum of 2 unique peptides, 90% peptide identification probability (using the Scaffold Local FDR algorithm), and 99% protein identification probability (using the Protein Prophet algorithm), resulting in a 0.0% decoy FDR. Proteins that contained similar peptides and which...
could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. The label-free quantitative analysis of peptides was performed by spectral counting analysis, using normalized spectral abundance factor (NSAF), calculated for each protein to normalize run-to-run variations\textsuperscript{19}, and quantitatively differentiated were statistically analyzed by a t-test. Differences with p values lower than 0.05 were considered statistically significant. Identified proteins were categorized according to gene ontology terms.

String (Search Tool for the Retrieval of Interacting Genes/Proteins, version 10) and Pathway Studio (Elsevier) were used for bioinformatics analysis.

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| Gene name | Protein name | No of peptides sequenced | Mw |
|-----------|--------------|--------------------------|----|
| KNG1      | High molecular weight kininogen | 16 | 71.9 |
| NCF2      | Neutrophil cytosolic factor 2   | 4 | 50.3 |
| CALM5     | Calmodulin 5               | 3 | 15.9 |
| PKIA      | protein kinase inhibitor alpha | 1 | 8.0 |
| RIM5I     | Regulating synaptic membrane exocytosis protein 1 | 1 | 91.5 |
| PRKX      | cAMP-dependent protein kinase catalytic subunit PRKX | 1 | 40.9 |
| PRKDI     | Serine/threonine-protein kinase D1 | 1 | 101.6 |
| IRS1      | Insulin receptor substrate 1 | 1 | 131.5 |
| GFRA1     | GDNF family receptor alpha-1 | 1 | 37.7 |
| NTF3      | Neurotrophin-3             | 1 |  |
| PRKDI     | Protein kinase D1           | 1 | 29.3 |
| KCNC1     | Potassium voltage-gated channel subfamily C member 1 | 1 | 69.5 |
| CCL14     | Chemokine (C-C motif) ligand 14 | 1 | 10.7 |
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Author Contributions
Conceived and designed the experiments: B.Gh., M.V.T., N.G. & B.G. Performed the experiments: B.Gh., M.V.T. Analyzed the data: B.Gh., M.V.T. Contributed reagents/materials/analysis tools: B.Gh., M.V.T. Wrote the paper: B.Gh., M.V.T. B.G., N.G. Reviewed manuscript: B.Gh., M.V.T., B.G., N.G.

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