Peptidomic Analysis of Cartilage and Subchondral bone in OA patients

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Abstract

Background:

The objective of this study was to develop a method for directly analyzing osteochondral samples straight out of the operating room without cell culturing, thereby enabling identification of potential peptide biomarkers to better understand the mechanisms involved in the development of osteoarthritis and pain.

Material and Methods:

Osteochondral plugs from wounded and macroscopically non-wounded zones of the femur condyle were collected from 6 patients with manifest osteoarthritis (OA) undergoing total knee arthroplasty (TKA). The samples were demineralized and supernatant was collected and isotopically marked with Tandem Mass Tag (TMT) labeling and analyzed using liquid chromatography coupled with tandem mass spectrometry LC-MS/MS.

Results:

Using peptidomics, 6292 endogenous peptides were identified. Five hundred sixty six peptides (8 identified endogenous peptides) differed significantly (p-value 0.10) from wounded zones compared to non-wounded zones.

Conclusion:

This pilot study shows promising results for enabling peptidomic analysis of cartilage and bone straight out of the operating room. With further refinement, peptidomics can potentially become a diagnostic tool for OA, and improve the knowledge of disease progression and genesis of pain.

Keywords: Cartilage/ Mass spectrometry/ Neuropeptides/ Osteoarthritis/ Pain
Introduction

In osteoarthritis (OA), pain is the main symptom and the major cause for seeking medical care [1-3]. Yet, the field of pain mechanisms in OA is poorly understood and largely unexplored. Studies suggest pain is affected both by mechanisms involving neuropeptide signaling [4, 5], and peripheral and central sensitization [6, 7], but also by psychological factors altering the pain perception [8]. With such complexity the profession calls for more effective guidelines in the determination of which patient category can be helped by surgical intervention.

The defined clinical and radiological criteria used today to diagnose OA have generally poor sensitivity and neither visualize the onset and early signs of disease nor predict disease progression [9-11]. Many patients are, in early stages of OA, asymptomatic and when diagnosed they already have extensive cartilage deterioration. Moreover, in OA the cartilage pathology is widely discussed but there is an indication that subchondral bone changes play an equally important role in disease progression [12, 13].

Given the low sensitivity of current diagnostic methods there has been an increasing interest in findings biomarkers to detect pathological developments in the osteoarthritic joint, predominantly by analyzing synovial fluid, serum and plasma [14-17]. To date, due to the high mineral composition of bone and the dominance of collagen in the extracellular matrix of cartilage, traditional extractions protocols cannot be applied. Solid bone and cartilage tissue have therefore been difficult to study using protein and peptide analysis methods.

Peptidomics, by which endogenous protein fragments are characterized and quantified by mass spectrometry, has already been shown to play an increasingly important role in finding biomarkers in fields such as cancer and neurodegenerative diseases [18, 19].

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Peptides play a key role in many regulatory processes and there are both hormones and signalling molecules that are active as endogenous peptides, the largest group being neuropeptides.

In this pilot study we explore the possibility to perform peptidomic analysis directly on knee tissue samples to identify peptides that may be involved in pain signaling and also serve as biomarkers of OA. We developed a method for peptide extraction and analysis of endogenous peptides from osteochondral biopsies taken from wounded and macroscopically non-wounded cartilage areas in osteoarthritic knee tissue samples for subsequent analysis by liquid chromatography mass spectrometry (LC-MS) using the Tandem Mass Tag (TMT) technique for quantification. The method introduces the possibility of broadening the search and identification of potential biomarkers as well as extending our knowledge of pain and pathological mechanism involved in OA.

**Materials and methods**

**Selection of samples**

The samples used in the present study were de-identified left-over tissues from 6 patients with OA who underwent Total Knee Replacement (TKR) surgery at the Department of Orthopaedics at Sahlgrenska University Hospital, Gothenburg, Sweden. Tissue sampling was approved by patients and followed a procedure approved by the Ethics Committee at University of Gothenburg.
Peptide extraction

Tissue samples were collected during surgery and immediately taken to the laboratory for biopsy taking. An osteochondral plug (about 10 mm length, 2 mm diameter) was drilled out manually from femur condyle samples using a T-Lok Bone Marrow Biopsy needle (Argon Medical Devices, USA).

Samples were evaluated by an orthopedic surgeon with large knowledge of osteochondral damages. Three samples were taken from a macroscopically healthy area of the lateral femur condyle with unwounded cartilage (UOA) and three samples were taken from an area with severe osteoarthritis and wounded cartilage (WOA). Samples were weigh adjusted and stored at -80°C in Eppendorf Low bind tubes (Eppendorf, Germany).

Peptide extracts were prepared by demineralizing the samples using 1.2 M HCl and 20% Acetonitrile (v/v) over night at 4°C on a rolling mixer. The supernatant was collected after centrifugation at 14,000 x g for 10 min at 4°C. Five microliters were used for Bradford Protein Assay (BIO-RAD Bradford Quick Start Protein Assay System, Bio-Rad Laboratories Inc. USA). The samples were volume adjusted to the same protein concentration. Sample volumes corresponding to 10 µg protein was transferred to 1.5 ml Eppendorf LoBind tubes and lyophilized. Aliquots of 1 M tri-ethyl ammonium bicarbonate (TEAB, 17 µl), 8 M Guanidium hydrochloride (Gua-HCl, 50 µl), and water (100 µl) were added and the samples and vortexed. TCEP (200 mM, 4 µl) was added and the samples were incubated at 55°C while shaking. After letting samples cool to room temperature, 400 mM iodoacetamide (4 µl) was added and the samples were incubated for 30 min at room temperature in the dark. TMT reagents source (0.8 mg) were dissolved in acetonitrile (41 µl). TMT reagent solution (15.3 µl) (Thermo Fisher Scientific, USA) was added to each sample followed by incubation for 1 h at room temperature while shaking. The labeling reaction was quenched by addition of
5% hydroxylamine (v/v, 9.5 µl) and incubation for 20 min at room temperature. The samples were combined into TMT 6-plex sets, each set consisting of three samples of tissue from unwounded areas and three samples from osteoarthritis tissue. MWCO-30 kDa ultrafiltration devices (Vivacon 2, 30 MWCO HY, VNO2H21) were washed by loading a solution of 100 mM TEAB and 3 M Gua-HCl and centrifuging at 2,500 x g for 60 min at room temperature. Samples were spun through the filter devices and the flow-through, containing the peptide fraction, was collected. An aliquot of 50 mM ammonium bicarbonate was spun through and combined with the peptide extract to improve yield. Water (1.2 ml) and 10% trifluoroacetic acid (TFA; v/v, 200 µl) were added to the peptide extracts to acidify the samples to pH<3. The peptide extracts were desalted by SPE (SEP-PAK C\textsubscript{18}, Waters) operated using a vacuum chamber. The SPE cartridges were washed with 80% acetonitrile, 0.1% TFA (Buffer B) (v/v, 1 ml) and equilibrated with 0.1% TFA (Buffer A) (2 x 1 ml). After loading the samples, the cartridges were washed with Buffer A (2 x 1 ml) and subsequently eluted with Buffer B (1 ml). The elutes were lyophilized by vacuum centrifugation and stored at -80°C pending LC-MS analysis.
Figure 1.

Sample demineralization and protein/peptide extraction

Reduction and alkylation

TMT 6-plex labeling

Combine TMT multiplex

MWCO-30 kDa Ultrafiltration

LC-MS (Q-Exactive)

Peptide identification (PEAKS)

Bradford Protein Assay
LC-MS/MS Analysis

The samples were dissolved in 2% ACN, 0.1% TFA (v/v, 7 µl). Aliquots of 5 µl were loaded on a Dionex nano-LC instrument (Ultimate 3000 RSLC, Thermo Fisher Scientific, USA) fitted with a 75 µm x 2 cm trap column (PepMap Acclaim C\textsubscript{18}, Thermo Fisher Scientific, USA) and a 75 µm x 50 cm separation column (PepMap Acclaim C\textsubscript{18}, Thermo Fisher Scientific, USA), coupled to a Q-Exactive mass spectrometry instrument (Thermo Fisher Scientific, USA). Peptide separation was performed using a 160 min gradient running from 3 to 45 % of mobile phase B (84% ACN, 0.1% formic acid). The mass spectrometer was operated in the positive ion mode. The instrument settings for the MS scans were: resolution 70,000; \textit{m/z} range 400-1,600; max injection time 250 ms; AGC target 1e6. Data-dependent acquisition was used to record up to 10 consecutive MS\textsuperscript{2} spectra per full scan spectrum, selecting precursor ions in decreasing order of intensity, and using 20 s dynamic exclusion, and charge state exclusion to exclude signals with unassigned charge, charge 1 and $>$5. The isolation window was set to 1.2 \textit{m/z}. The instrument settings for the MS\textsuperscript{2} scans were: resolution 35,000 for endogenous peptides and 17,500 for tryptic peptides; fixed first mass \textit{m/z} 100; max injection time 120 ms for endogenous peptides and 60 ms for tryptic peptides; AGC target 1e5.

Data Analysis

Automatic \textit{de novo} sequencing and peptide identification by sequence database searching was performed using the software PEAKS Studio (Bioinformatics Solutions Inc, Canada). The following settings were used: database: UniProt/SwissProt; taxonomy: homo sapiens; parent mass error tolerance: 20 ppm; enzyme: none; fixed modifications: carbamidomethylation, TMT6-plex; variable modifications: oxidation of methionine. Peptide identifications were validated by the commonly used method target-decoy approach, using a target false discovery

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rate (FDR) of 5% [20]. TMT reporter ion intensities were determined using the software Proteome Discoverer 2 (Thermo Fisher Scientific, USA). Spectral clustering was performed using MSCluster to match spectra representing the same peptide in different TMT sets and the cluster list was annotated using the peptide identification results from PEAKS [21]. TMT ion intensity ratios were normalized to the average ratio within the TMT set and log-transformed.

**Protocol for efficient peptide extraction from osteochondral plug**

A key to a successful analysis is efficient peptide extraction [22]. The inorganic matrix of bone with high abundance of hydroxyapatite complicates the extraction process. Previous reports have shown that protein extraction from bone tissue is significantly improved by removing the inorganic matrix before the extraction process [23, 24]. Bone samples were therefore initially demineralized by incubation in 1.2 M HCl.

The mean protein concentration obtained from the 6 patients in UOA and WOA zones of the OA samples, as determined by the Bradford protein assay were per biopsy sample, 28.3 µg (SD 17.7) from macroscopic healthy tissue (UOA), and 46.7 µg (SD 20.7) from osteoarthritic tissue (WOA).

**Statistics**

Data was adjusted to be normally distributed by log transformation. Data was plotted to confirm the distribution. The difference between mean values for the peptide abundance from unwounded (UOA) and wounded (WOA) zones from the 6 patients investigated was tested for statistical significance (90% significance level p ≤0.1) using two tailed paired t-test. Due
to the high dimension of data and multiple hypothesis testing multiple testing adjustment analysis with Holm’s and Hochberg’s method was made.

**Results**

**Endogenous peptides identification from wounded and unwounded zones from OA samples**

Extracts of endogenous peptides, prepared by molecular weight cut-off (MWCO) ultrafiltration, were analyzed by LC-MS in the data-dependent mode, and peptide identification was performed by database searching. The term endogenous peptides refer to peptides that are not obtained by tryptic digestion of the sample proteins, as is the most common types of peptides analyzed in proteomics.

In total, 6292 endogenous peptides were identified, derived from 915 proteins (889 protein groups). Out of these, 601 peptides (derived from 156 proteins) carried TMT label. 462 of the identified peptide chains were able to match with the database used for identification. A complete list of identified and matched peptides and their corresponding proteins is provided in Additional File 1.
Figure 2.

A.

Osteocalcin

B.

Complement C3

x5

C.

Collagen alpha chain
In the six patients included in the study, a total of 566 endogenous peptides were found significantly differing with a p-value≤0.1 in unwounded zones compared to wounded osteoarthritic zones. Out of the significant differing findings, only 8 proteins and endogenous peptides were identified in the database search. A complete list of identified endogenous peptides and proteins can be found in Additional File 1. After multiple testing adjustments there was no significant difference in peptides expressed in wounded and unwounded zones.

**Table 1 presents proteins identified found in 3 or more patients with a p-value≤0.1.**

**Discussion**

This is to our knowledge the first reported peptidomic study of cartilage and subchondral bone carried out in human samples. Previous studies have investigated protein expression in synovial fluid and the synovial membrane. Although we did experience difficulties with labeling and quantification of peptides we feel that the results are important to report as peptidomics on solid tissue can potentially bring many answers to the field of OA research.

We did find a group of peptides varying significantly between the groups tested but none of these differed significantly when we did multiple testing adjustments.

In our study we identified multiple peptides related to proteins involved in neuronal signaling. None of the neuro related proteins were found to differ significantly between unwounded and wounded zones but are interesting in their presence in subchondral bone and cartilage for the deepening of our knowledge on the pain mechanisms involved in OA.
Previously reported OA associated proteins

One of the interesting findings is the expression of Complement C3. Recent findings indicate that complement cascade activation may be crucial for the development of OA [25]. Our findings indicate a difference in complement C3 in macroscopically healthy compared to wounded zones of OA tissue. Complement C3 was decreased in wounded zones compared to unwounded zones and a reason for this is probably that even though the healthy tissue was taken from unwounded zones, the biopsies came from patient with manifest OA. Complement C3 activations can be seen both in early and advanced stages of OA but is predominantly thought to play an important role in early stages of osteoarthritis development [26]. Knockout mice for C3 have however not shown to be protected against the development of OA and the reason for this is that compensatory mechanisms with coagulations factors activate C5 leading to a complement cascade activation even though C3 is not present [27].

Changes to the subchondral bone may be of equal importance to the pathogenesis of OA. Recent studies suggest that a decrease in the subchondral bone density may be related to OA [28]. Moreover, knockout mice models with defect type I collagen in bone show progressive cartilage destruction [29]. In our biopsies both cartilage and subchondral bone was present. We found a decrease in collagen I in the wounded zones correlating with previous findings that changes in the subchondral bone may be related to disease progression in OA.

With a progressive OA there is also evidence of increased bone turnover and metabolic activity in the subchondral bone [30-32]. Osteocalcin is the most abundant noncollagenous protein found in bone and plays a major role in bone formation. Previous studies have suggested an initial decrease in Osteocalcin in early OA followed by an increase as the disease progresses.
In early stages of rheumatoid arthritis, plasma components such as fibrinogen aggregate in the joint and possibly initiate the inflammatory cascade. Excessive fibrin deposits in the synovial membrane are thought to play a role in pannus formation and the disease progression [33, 34]. Activation of the inflammation cascade can also be seen in OA and fibrinogen may play an equally important role in early stages of the disease. In our data we saw decreased levels of fibrinogen in the wounded tissue compared to the unwounded zones present in 5 out of 6 patients.

**Neuropeptide signaling pathways in OA**

Multiple endogenous peptides involved in neurotransmitter signaling were identified although none of them differed significantly between the unwounded and wounded areas. The findings were however interesting in our quest for understanding the joint pain mechanisms better.

An endogenous peptide sequence derived from VPS10 domain-containing receptor SorCS2-receptor was present in all 6 patients investigated. Seen in murine models, SorCS2 predominantly plays an important role in the development of the central nervous system [35]. SorCS2 has recently been associated with crucial ligament rupture and post-traumatic osteoarthritis in animal models [36, 37] and is linked to activation of neuropeptide pathways [38].

Vesicular acetylcholine transporter (SLC18A3) is responsible for the acetylcholine transportation into synaptic vesicles and subsequently crucial for acetylcholine secretion [39]. Peptide sequences from SLC18A3 were present in all 6 patients. Previous studies have shown that various choline and acetylcholine transporters are expressed in synovial tissue and cartilage from patients with rheumatoid arthritis and OA however SLC18A3 was absent [40].

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SH3 and multiple ankyrin repeat domains protein 2 (SHANK2) is involved in synaptic plasticity through its regulation of NMDA receptors as well as regulating the spine [41]. SHANK2 endogenous peptide sequences were present in all 6 patients. Animal models indicate SHANK2 may play an important role in chronic pain mechanism, knockout mice have shown decreased sensitivity to chronic pain [42].

4.3 Analytical considerations and limitations of the study

We had only a small amount of material, investigating osteochondral plugs from 6 patients undergoing total knee replacement. To be able to identify clinical relevant significant differences, with so many peptides identified, a larger group of patients is needed. Moreover, a vast majority of the significant peptides were not identified in the database search. With increasing peptidomic research we hope the database is expanding which in the future can lead to a better peptide identification process.

The TMT labeling was found to be incomplete, which resulted in that quantitative data could only be obtained for a fraction of all identified peptides. We did following up series with other control tissue that showed that the TMT markers were very thermal sensitive and that they needed to be adjusted to room temperature a couple of hours for a better labelling. While it may distort the quantitative results, we still feel these results are worth reporting.

The present pilot study shows that the TMT multiplex LC-MS/MS method is useful for identification of endogenous peptides in cartilage and subchondral bone from OA patients. With this new method it is possible to analyze endogenous peptides in tissue directly taken from surgical procedures without any cell culturing needed. The findings open up for further
studies potentially deepening our understanding of the development of pain and the pathogenesis of OA.

**Contribution,**

HZ, KB and MB designed the study. JG and BG performed the experimental work and with TS analyzed the data. JG performed the MS analysis. HZ, MB, KB and JG critically evaluated the study. BG drafted the manuscript, JG and MB finalized the article. All authors discussed on the work and gave final approval.

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**Ethics approval**

Tissue sampling was approved by patients and followed a procedure approved by the Ethics Committee at University of Gothenburg. Since tissue samples were de-identified the need for an ethical approval was waived.

**Conflict of Interest statement**

The authors have declared no conflict of interest.
Funding

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References

Legends

Figure 1: Schematic workflow of the steps in analysis of cartilage and subchondral biopsy from OA patients using peptidomics. Samples from 6 patients were included in the study. 3 biopsies were taken from wounded (WOA) and unwounded (UOA) zones. Peptides were extracted and iTRAQ labeled. Labeled samples were then fractioned using ultrafiltration and analysed on a LC-MS (Q-Exactive).

Figure 2. Identification and relative quantification of endogenous peptides. Fragment ion mass spectrum of identified endogenous peptides (a) Osteocalcin 89-100, (b) Complement C3 742-747, and (c) Collagen alpha chain 1212-1216. The spectra are annotated with matching b- and y-ions. The six TMT reporter ions of m/z 127-131 in the lower mass region give the relative abundance of the peptides in osteoarthritis (WOA) tissue and tissue from unwounded zones (UOA) from a single patient.
### Table 1

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<tr>
<th>Gene</th>
<th>Identification Uniprot</th>
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<th>Sample mean relative difference (WOA-UOA)</th>
<th>p-value</th>
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| 139 | BGN  | Biglycan | DTSGVLDPP | 0.635 |
| 140 | HBA1 | Hemoglobin subunit alpha | GAHAEGYGAEL | 0.637 |
| 141 | FGA  | Fibrinogen alpha chain | LAEGGGVR | 0.641 |
| 142 | FAM160A2 | FTS and Hook-interacting protein | HASWARGP | 0.643 |
| 143 | PLIN4 | Perilipin-4 | EERAGVLRSVCGLL | 0.643 |
| 144 | CASZ1 | Zinc finger protein castor homolog 1 | AAGAGARTPAL | 0.648 |
| 145 | COL4A3 | Collagen alpha-3(IV) chain | GSPGLPGSPGPP | 0.652 |
| 146 | VIM  | Vimentin | RSSAVRLR | 0.655 |
| 147 | VIM  | Vimentin | GPTASRPSRSSVYTT | 0.656 |
| 148 | VIM  | Vimentin | VTTSTRTY | 0.658 |
| 149 | VIM  | Vimentin | TNLDSPLVLDTH | 0.664 |
| 150 | FGB  | Fibrinogen beta chain | RREEAPLRPAPPSISGGGYR | 0.666 |
| 151 | KIF14 | Kinesin-like protein KIF14 | GIDSGK | 0.674 |
| 152 | COL1A1 | Collagen alpha-1(I) chain | ISVPGMGP | 0.681 |
| 153 | ALB  | Serum albumin | VAAQAALG | 0.685 |
| 154 | COL5A1 | Collagen alpha-1(VIII) chain | DRRMGGVPGALG | 0.685 |
| 155 | VIM  | Vimentin | LNLRETNL | 0.687 |
| 156 | VIM  | Vimentin | ALRPTSTR | 0.689 |
| 157 | VIM  | Vimentin | PSTRSRLY | 0.689 |
| 158 | CRNKL1 | Crooked neck-like protein 1 | EEEVKANPHN | 0.690 |
| 159 | COL2A1 | Collagen alpha-1(II) chain | PIGPPGERG | 0.691 |
| 160 | COL2A4 | Collagen alpha-1(XXIV) chain | MGYPGPPGV | 0.691 |
| 161 | VIM  | Vimentin | ALRPTSTRSLYA | 0.694 |
| 162 | COL1A1 | Collagen alpha-1(I) chain | DGGRYYRA | 0.697 |
| 163 | VIM  | Vimentin | VPGVR | 0.705 |
| 164 | COL1A2 | Collagen alpha-2(1) chain | GGYDPFGYDFDFYR | 0.705 |
| 165 | COL1A2 | Collagen alpha-2(1) chain | QGAPSVPGAPRGPGAPGISPGAG | 0.707 |
| 166 | COL1A1 | Collagen alpha-1(I) chain | PAGPRPGPP | 0.707 |
| 167 | COL2A1 | Collagen alpha-1(II) chain | PAGAPQPGAPGPA | 0.708 |
| 168 | VIM  | Vimentin | PGGYVYATRSSA | 0.709 |
| 169 | HBA1 | Hemoglobin subunit alpha | FFHD1L | 0.712 |
| 170 | PLA2G2A | Phospholipase A2, membrane associated | KEAAL | 0.712 |
| 171 | VIM  | Vimentin | TRYSTLG | 0.714 |
| 172 | BGLAP | Osteocalcin | LYQWLG | 0.715 |
| 173 | BAIA3 | BA1-associated protein 3 | PDPAAQGQLGT | 0.717 |
| 174 | VIM  | Vimentin | LADAIN | 0.717 |
| 175 | COL11A2 | Collagen alpha-2(XI) chain | PSSPGPGAPSGP | 0.717 |
| 176 | WWC1 | Protein KIBRA | LDLLEDLQAT | 0.719 |
| 177 | COL1A2 | Collagen alpha-2(I) chain | DGDFFYRA | 0.719 |
| 178 | BGLAP | Osteocalcin | ELADHIGFQEAYRFYGPV | 0.719 |
| 179 | COL1A2 | Collagen alpha-2(1) chain | DFGYDGDFYRA | 0.722 |
| 180 | COL3A1 | Collagen alpha-1(III) chain | GMRMGPGSGPGP | 0.723 |
| 181 | ITH4  | Inter-alpha-trypsin inhibitor heavy chain | AIHRFK | 0.723 |
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| 183 | COL1A2 | Collagen alpha-2(I) chain | YDFGYDGDFYRA | 0.724 |
| 184 | ANKRD13C | Ankyrin repeat domain-containing protein 13C | PGDEEEAAALGQTFT | 0.725 |
| 185 | VIM  | Vimentin | GPTASRPSRSSRYVTSTRTY | 0.727 |
| 186 | SMARCA1 | Probable global transcription activator | DYCMIWRGYE | 0.728 |
| 187 | PLA2G2A | Phospholipase A2, membrane associated | YKFSN | 0.733 |
| 188 | IGF1N | Immunoglobulin-like and fibronectin type III domain-containing protein 1 | PVAGLSDSG | 0.734 |
| 189 | HECA | Headcase protein homolog | YGRSPGPSGPQSPPTG | 0.734 |
| 190 | VIM  | Vimentin | GPGTAASRPSSS | 0.734 |
| 191 | COL1A2 | Collagen alpha-2(V) chain | PPGAGPGGSPGPSGPQ | 0.734 |
| 192 | AATK | Serine/threonine-protein kinase LMTK1 | SRRTVSPAPT | 0.735 |
| 193 | VIM  | Vimentin | VYTTSTRTY | 0.737 |
| 194 | FGA  | Fibrinogen alpha chain | SGEIDFLAEGGGVR | 0.741 |
| 195 | COL1A2 | Collagen alpha-2(I) chain | YGDIFYR | 0.744 |
| 196 | FGA  | Fibrinogen alpha chain | VPGNF | 0.745 |
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| 201 | WIZ  | Protein Wiz | QDAGLHLDPQ | 0.749 |
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| 204 | BGLAP | Osteocalcin | QEAYRFYGPV | 0.755 |
| 205 | SLC18A3 | Vesicular acetylcholine transporter | ISFGSLVA | 0.758 |
| 206 | NCOA1 | Nuclear receptor coactivator 1 | QSDNDSATCSADED | 0.758 |
| 207 | DIAPH3 | Protein diaphanous homolog 3 | DLHDKFVT | 0.760 |</p>
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