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ORIGINAL ARTICLE / ÖZGÜN MAKALE

DETERMINATION OF ALPHA-2-MACROGLUBULIN IN SERUM SAMPLES

SERUM ÖRNEKLERİNDE ALFA-2-MAKROGLUBULİN TAYİNİ

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ABSTRACT

Objective: *Proteomics is one of the fastest growing omics that has been extensively used in clinical studies. Proteomics involves qualitative and quantitative protein analysis in a wide range of samples starting from a single cell to complex biological samples. Protein-based biomarker studies have been applied to many diseases including metabolic diseases, cancer and neuropsychiatric diseases for both diagnostic and prognostic purposes. Alpha-2-macroglubulin (A2MG) is a clinically relevant secreted protein involving in various biological processes including blood coagulation, protein binding and protease inhibition. Current methods for A2MG analysis are limited, as they focus on either immune-specific binding through a certain protein unit or a unique peptide. As a single protein could be in different forms (complexes, modifications, etc) and the biological activity is structure specific, an extensive analysis is necessary. Here a new Mass-Spectrometry (MS) based method was developed for comprehensive A2MG analysis.*

Material and Method: *A reference human serum and A2MG protein standard were used for method development. Proteolytic protein digestion was performed using trypsin and Circular-Dichroism (CD) spectroscopy was used to ensure protein unfolding and denaturation prior to digestion. Targeted MS method was developed to monitor 12 unique peptides for A2MG in serum.*

Result and Discussion: *Monitoring multiple peptides for a single protein enabled to observe biological differences offer a robust and reliable A2MG analysis in serum. The method can also easily be implemented to other proteins. The concept of targeted-MS provides an ideal quantification and validation platform which then can be easily transferred to clinical laboratories.*

Keywords: *Alpha-2-macroglobulin, serum, targeted proteomics*

ÖZ

Amaç: *Proteomik, klinik çalışmalarda yaygın olarak kullanılan en hızlı büyüyen omiklerden biridir. Proteomik, tek bir hücreden başlayarak karmaşık biyolojik örneklere kadar geniş bir örnek yelpazesinde kalitatif ve kantitatif protein analizini içerir. Protein bazlı biyobelirteç çalışmaları, metabolik hastalıklar, kanser ve nöropsikiyatrik hastalıklar dahil olmak üzere birçok hastalığa hem tanısal hem de prognostik*

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amaçlarla uygulanmıştır. Alfa-2-makroglubulin (A2MG), kan pıhtılaşması, protein bağlanması ve proteaz inhibisyonu dahil olmak üzere çeşitli biyolojik süreçlerde yer alan, klinik önemi olan ve salgılanan bir proteindir. A2MG analizi için mevcut yöntemler, belirli bir protein birimi veya benzersiz bir peptit yoluyla immün spesifik bağlanmaya odaklandıklarından sınırlıdır. Tek bir protein farklı formlarda (kompleksler, modifikasyonlar, vb.) olabileceğinden ve biyolojik aktivite yapıya özel olduğundan, kapsamlı bir analiz gereklidir. Bu çalışmada kapsamlı A2MG analizi için yeni bir Kütle Spektrometresi (MS) tabanlı yöntem geliştirildi.

Gereç ve Yöntem: *Bu çalışmada, kapsamlı A2MG analizi için yeni bir Kütle Spektrometresi (MS) tabanlı yöntem geliştirilmiştir. Analitik yöntem geliştirme referans insan serumu ve A2MG protein standardı ile yapılmıştır. Proteolitik protein sindirimi için tripsin kullanılmış ve sindirimden önce ve sonra proteinin denatürasyonu Dairesel-Dikroizm (CD) spektroskopisi kullanılarak test edilmiştir. Hedefli MS yöntemi, serumda A2MG için 12 benzersiz peptidi izlemek için geliştirilmiştir.*

Sonuç ve Tartışma: *Bu çalışmada, biyolojik farklılıkları gözlemlemek için geliştirilen tek bir protein için çoklu peptitlerin ölçülmesi ile sağlam ve güvenilir serumda A2MG analizi geliştirilmiştir. Yöntem, diğer proteinlere de kolayca uygulanabilir. Hedeflenen MS konsepti, daha sonra klinik laboratuvarlara kolayca aktarılabilen ideal bir niceleme ve doğrulama platformu sağlayacaktır.*

Anahtar Kelimeler: *Alpha-2-macroglobulin, hedefli proteomik, serum*

INTRODUCTION

Proteins are the main targets for biomarkers as they are involved in biosynthesis, cell, tissue and organ signaling, cell and tissue structural stability in organisms. Rapidly evolving mass spectrometry (MS)- based technologies in proteomics enable to study proteins in a wide range of samples starting from a single cell[1] to complex biological samples such as blood [2] and tissue. Protein-based biomarker studies have been applied to various diseases including cancer [3] , inflammatory diseases [2, 3], cardiovascular diseases [6], diabetes [7] and even psychiatric disorders [6, 9].

Alpha-2-macroglobulin (A2MG, Uniprot ID: P01023) is a tetrameric glycoprotein with a molecular weight of 725 kDa. It is involved in various biological processes including blood coagulation, protein binding and protease inhibition [10]. It is synthesized by liver and secreted in various biological fluids including serum, saliva, cerebral spinal fluid, ocular fluid and tissues. The average A2MG level in human blood is 1.5-4.0 mg/ml [11].

A2MG is an acute phase protein that can be used to monitor immunological response for both inflammation and infection [12]. Thus, it is also utilized to track the progression of pathological changes in animal models to unravel the inflammatory and infectious diseases [12]. Recent studies have also shown that A2MG has increased significantly in patients who showed severe COVID-19 symptoms. Consequently, the protein is involved in critical biological processes and a valuable biomarker for clinical studies. Hence, a reliable and reproducible quantification of A2MG in serum is vital.

Current methods commonly used in proteomics are two- dimensional gel electrophoresis (2DE) [13], enzyme-linked immunosorbent assays (ELISAs) [14], protein arrays and Mass Spectrometry (MS) based technologies [18,19,20]. Although immunoassays are considered as the gold standard in clinical laboratories, they suffer from several drawbacks such as non-specific binding, batch to batch variation

and cross reactivity [15]. MS based methods have several advantages over immunoassays as they require less sample, provide better specificity due to tandem capacity, low cost and reproducibility [19 -21].

MS based protein quantification is peptide centric and only unique peptide(s) is/are used for both protein identification and quantification. However, a single protein often has more than one representative peptide but only limited number of peptides are used in common practice. In order to reflect the actual biological changes, methods should be validated and the clinical utility must be assessed for all target analytes.

Here, a new targeted MS method was developed for the simultaneous determination of A2MG unique peptides in human serum. This study is the most comprehensive method up to now covering multiple unique peptides for A2MG. The selective measurement in complex biological samples such as serum and plasma provide a reliable quantification and validation platform. Thus, it can be easily transferred to clinical laboratories and enable large scale screening.

MATERIAL AND METHOD

Materials

A reference human serum and A2MG protein standard were obtained from Sigma-Aldrich (St. Louis, MO USA). HPLC grade water, acetonitrile, and formic acid were purchased from Merck (Darmstadt, Germany). Dithiothreitol (DTT), iodoacetamide (IAA), and ammonium bicarbonate (ABC) were purchased from Sigma- Aldrich (St. Louis, MO USA). Sequencing grade trypsin was obtained from Promega (Madison, WI USA).

Protein Digestion

Proteolytic digestion was performed according to the previous study [21]. Five µL of serum sample and 5 µL of A2MG protein standard (0.1 µg/µL) were added to an Eppendorf tube containing 105 µL of 50 mM ammonium bicarbonate. Prior to enzymatic degradation, disulfide bonds were reduced with 10 µL of 45 mM dithiothreitol (DTT) and proteins were denaturated at 65 ◦C for 45 minutes. Cysteine residues were then alkylated using 10 µL of 100mM Iodoacetamide (IAA) for 30 minutes at room temperature. Enzymatic digestion was performed by trypsin at 1:20 (enzyme: protein) molar ratio for 16 hours. Samples were stored at -80 ◦C prior to MS analysis.

Circular-dichroism (CD) Spectroscopy

A 0.2 mg/mL pure A2MG dissolved in 10 mM phosphate buffer (potassium dihydrogen phosphate/disodium hydrogen phosphate) at pH 7.8 was used for CD experiments. CD spectra were recorded with an Jasco J-1500 Circular Dichroism Spectrometer between 180 and 600 nm at 1 point/sec

scan speed. Measurements were carried out at 20 ◦C, using 1 nm band-width and 0.5 nm step size, in a quartz cuvette with 10 mm optical path length. CD spectra were normalized by subtracting the signal of the blanks (10 mM phosphate buffer).

Targeted MS Analysis

Quantitation of twelve A2MG unique peptides was performed by an Agilent 1260 Infinity II HPLC system coupled to an Agilent 6470A triple-quadrupole (QQQ) system (Santa Clara, CA). Peptide separation was performed by Infinity Lab Poroshell 120 EC C18 (3.0 x 150 mm, 2.7 microns) column at 50 ºC over a gradient elution. Flow rate was 0.3 ml/min, mobile phase A was 0.1% formic acid in LC-MS grade water, and mobile phase B was 0.1% formic acid in LC-MS grade acetonitrile. The mass spectrometer was operated in positive mode. The dynamic Multiple Reaction Monitoring (MRM) acquisition was used, the cycle time was 500 ms and the dwell time was at least 20 ms. The voltage of the fragmentor was set to 135 V. The MS method was optimized using Skyline software package (version 21.1.0). The predominant charge state and interference free transitions were screened for all unique peptides. An Agilent MassHunter Quantitative Analysis software was used for data acquisition and processing.

Data Analysis

Raw MS data was processed using the Skyline software package (version 21.1.0) [22]. The peaks were integrated and the peak areas were calculated. Two-stage normalization were applied. In the first stage, the peak areas were normalized using total peak intensities. Then log 2 normalization was applied to normalize the changes that may occur during the analysis.

RESULT AND DISCUSSION

The A2MG protein was first investigated to identify unique peptides and their locations on the crystal structure. The structure has been obtained from Protein Data Bank (PDB; 4ACQ). The peptide uniqueness was confirmed via Uniprot human proteome database. The sequence yields 15 unique tryptic peptides. The position of the peptides on the protein structure is shown in Figure 1. Two peptides, VGFYESDVMGR and LVHVEEPHTETVR are unstructured in the PDB, thus they are excluded from the figure. The tryptic peptide PLLVEPEGLEK is represented as DTVIKPLLVEPEGLEK containing one mis-cleavage. The trypsin is less sufficient when the cleavage site is close to a Proline (P) residue.

The efficiency of the protein denaturation was investigated using CD spectroscopy. CD Spectroscopy was used for checking the unfoldedness of the protein upon addition of a reducing agent (DTT), and heating. It was also used for confirming the complete digestion. For this purpose, CD spectra of the protein in the buffer, after DTT and heat treatment, and after digestion were measured (Figure 2).

A2MG in the phosphate buffer before any treatment shows a CD spectrum, suggesting a secondary structure comprising alpha helices and beta sheets, as seen in its three-dimensional structure. After adding DTT, and heating of the protein, the CD spectrum changed to have features of polyproline II conformation which is a signature for the secondary structure of unfolded state of proteins. After trypsin digestion, A2MG lost all secondary structure elements, suggesting the complete degradation.

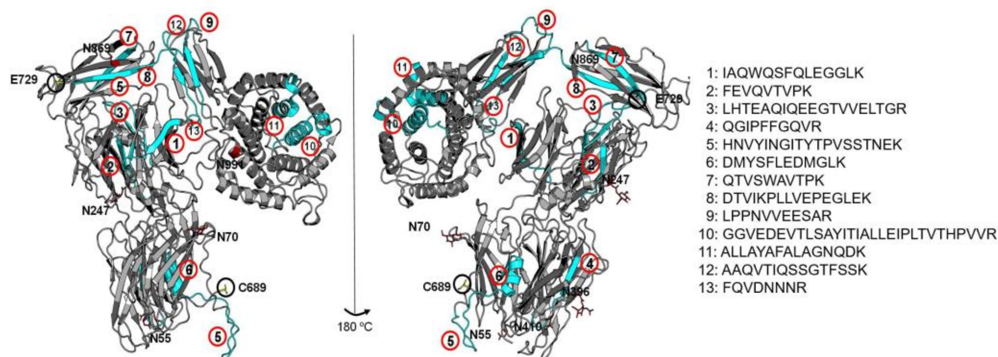

Figure 1. The A2MG protein structure and the position of unique peptides (shown in turquoise) in the protein sequence

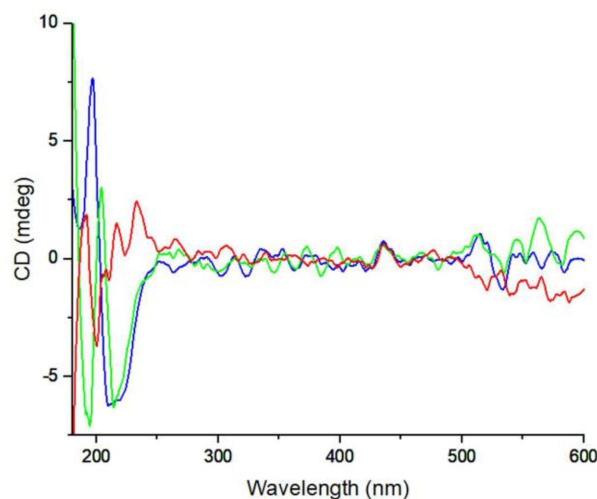

Figure 2. CD spectra of A2MG in phosphate buffer (blue), after addition of DTT and heating (green), and after digestion (red). The changes in secondary structure suggest the transition from unfolding to complete digestion.

Targeted MS method was developed to profile peptides for A2MG. Thirteen tryptic peptides representing A2MG were used for the determination of A2MG. Peptides were digested from the standard protein. Predominant charges and transitions were screened to identify qualifier and quantifier transitions. One out of thirteen peptides, QTVSWAVTPK showed poor ionization. Thus, it was not

included in the study. Remaining twelve peptides were further investigated to ensure reliable and reproducible measurements. Peptide fragmentations were optimized to yield specific and sensitive measurement.

The chromatographic separation for 12 A2MG peptides is shown in Figure 3. The method enables simultaneous quantification at different time windows. Overlapping peaks correspond to fragments of each peptide that can be used as qualifier transitions. First standard A2MG protein was digested to optimize the instrumental parameters. Figure 3a represents the corresponding peptide separation. Then the same method was applied to the serum digest to identify potential interferences that might come from biological matrix (Figure 3b). The most intense and interference free transitions were used for quantification purposes. Table 1 lists the specific precursors, charge states of each peptide, qualifier and quantifier transitions shown in Figure 3c. The same unique peptides were identified in both standard protein and serum samples however, peptide profiles were slightly different. The difference could be related to the sample matrix. Total protein concentration in serum is higher than that of pure protein standard. Therefore, digestion efficiency might be different. Future studies should focus on quantitative protein-unique peptide relationship.

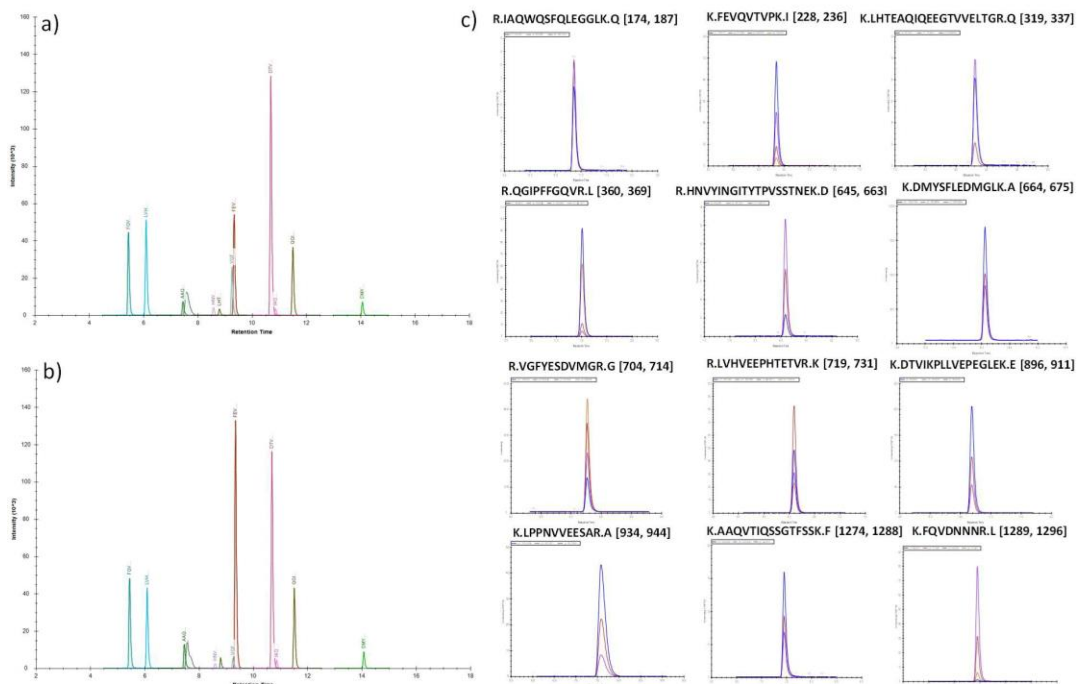

Figure 3. Chromatographic separation of A2MG peptides in the a) A2MG standard b) standard human serum digest and c) qualifier and quantifier transitions for 12 target peptides.

The reproducibility of peptide measurements was further investigated. Table 2 lists the replicates (N=6) log 2 normalized peak intensities for all 12 A2MG unique peptides.

Relative peptide abundances of A2MG proteins in the protein standard and human serum are shown in Figure 4. Each peptide was color coded and were normalized based on total intensities. All twelve A2MG peptides were detected in both samples. Peptides DTVIKPLLVEPEGLEK, FEVQVTVPK, FQVDNNNR, LPPNVVEESAR, LVHVEEPHTETVR and QGIPFFGQVR are the most intense peptides in all samples. However, relative abundances showed discrepancy between the standard protein and human serum. While the commonly used DTVIKPLLVEPEGLEK containing 1-miscleavage is the most abundant in the pure protein, FQVDNNNR is the dominant peptide in human serum.

A2MG is a clinically relevant protein associated with neurological diseases, liver fibrosis, and many inflammatory diseases. It is an abundant protein secreted in blood. Thus, it has a great potential to be utilized in clinical studies.

MS is emerging as a standard analytical method to quantify proteins in complex biological samples. The bottom-up approach involves protein digestion and quantification based on proteolytic peptides. In order to ensure the specificity of the measurements, only unique peptide(s) are used for this purpose. However, the number of unique peptides for each target protein varies depending on protein size and sequence. Often a limited number of peptides are used in clinical studies. Recently, Plubell et al. disputed using peptides from a protein can be misleading as an important biological difference may be lost while focusing on a single unit. The peptide-centric approach involves protein denaturation and proteolytic digestion using specific proteases such as trypsin. In order to verify the change on protein structure after denaturation and digestion, a CD analysis was performed. The DTT and heat treatment caused A2MG to unfold from its native state. The completeness of digestion was confirmed by measuring the CD spectrum after trypsin digestion. The spectrum showed signature signals demonstrating the degradation of the protein into peptides.

The abundance of specified fragment ions is indicative of the abundance of peptides and proteins in the sample. As there is often one unique peptide for each protein, it is important to monitor all to identify the most relevant one(s). There are two fundamental parameters affecting the peak intensities; proteolytic digestion and peptide ionization. Both parameters were investigated in this study by replicate measurements of standard A2MG protein and human serum samples. A reproducible digestion was observed in all samples. The ionization of a peptide depends on the size and the amino acid composition. The QTVSWAVTPK, GGVEDEVTLSAYITIALLEIPLTVTHPVVR and ALLAYAFALAGNQDK peptides showed poor ionization and were excluded from the method.

Table 1. Transition list and method parameters for A2MG

Peptide	Precursor Ion	MS1 Res	Transition	MS2 Res	Dwell	Fragmentor	Collision Energy	Polarity
IAQWQSFQLEGGLK	535.62	Unit	744.43	Unit	20	130	14.5	Positive
IAQWQSFQLEGGLK	535.62	Unit	616.37	Unit	20	130	14.5	Positive
IAQWQSFQLEGGLK	535.62	Unit	616.31	Unit	20	130	14.5	Positive
FEVQVTVPK	523.80	Unit	770.48	Unit	20	130	14.1	Positive
FEVQVTVPK	523.80	Unit	671.41	Unit	20	130	14.1	Positive
FEVQVTVPK	523.80	Unit	543.35	Unit	20	130	14.1	Positive
FEVQVTVPK	523.80	Unit	704.36	Unit	20	130	14.1	Positive
LHTEAQIQEEGTVVELTGR	704.03	Unit	931.52	Unit	20	130	20.5	Positive
LHTEAQIQEEGTVVELTGR	704.03	Unit	773.45	Unit	20	130	20.5	Positive
LHTEAQIQEEGTVVELTGR	704.03	Unit	793.42	Unit	20	130	20.5	Positive
LHTEAQIQEEGTVVELTGR	704.03	Unit	832.91	Unit	20	130	20.5	Positive
QGIPFFGQVR	574.81	Unit	850.46	Unit	20	130	15.9	Positive
QGIPFFGQVR	574.81	Unit	753.40	Unit	20	130	15.9	Positive
QGIPFFGQVR	574.81	Unit	606.34	Unit	20	130	15.9	Positive
QGIPFFGQVR	574.81	Unit	425.73	Unit	20	130	15.9	Positive
HNVYINGITYTPVSSTNEK	713.02	Unit	962.48	Unit	20	130	20.9	Positive
HNVYINGITYTPVSSTNEK	713.02	Unit	861.43	Unit	20	130	20.9	Positive
HNVYINGITYTPVSSTNEK	713.02	Unit	431.22	Unit	20	130	20.9	Positive
HNVYINGITYTPVSSTNEK	713.02	Unit	638.82	Unit	20	130	20.9	Positive
DMYSFLEDMGLK	724.83	Unit	1039.51	Unit	20	130	21.3	Positive
DMYSFLEDMGLK	724.83	Unit	952.48	Unit	20	130	21.3	Positive
DMYSFLEDMGLK	724.83	Unit	805.41	Unit	20	130	21.3	Positive
VGFYESDVMGR	630.29	Unit	1160.50	Unit	20	130	17.9	Positive
VGFYESDVMGR	630.29	Unit	956.41	Unit	20	130	17.9	Positive
VGFYESDVMGR	630.29	Unit	793.35	Unit	20	130	17.9	Positive
VGFYESDVMGR	630.29	Unit	664.31	Unit	20	130	17.9	Positive
LVHVEEPHTETVR	515.94	Unit	667.33	Unit	20	130	13.8	Positive
LVHVEEPHTETVR	515.94	Unit	598.80	Unit	20	130	13.8	Positive
LVHVEEPHTETVR	515.94	Unit	420.22	Unit	20	130	13.8	Positive
LVHVEEPHTETVR	515.94	Unit	707.37	Unit	20	130	13.8	Positive
DTVIKPLLVEPEGLEK	594.01	Unit	672.36	Unit	20	130	16.6	Positive
DTVIKPLLVEPEGLEK	594.01	Unit	782.47	Unit	20	130	16.6	Positive
DTVIKPLLVEPEGLEK	594.01	Unit	732.94	Unit	20	130	16.6	Positive
DTVIKPLLVEPEGLEK	594.01	Unit	336.68	Unit	20	130	16.6	Positive
LPPNVVEESAR	605.82	Unit	1000.51	Unit	20	130	17.0	Positive
LPPNVVEESAR	605.82	Unit	690.34	Unit	20	130	17.0	Positive
LPPNVVEESAR	605.82	Unit	549.28	Unit	20	130	17.0	Positive
LPPNVVEESAR	605.82	Unit	500.76	Unit	20	130	17.0	Positive
AAQVTIQSSGTFSSK	756.39	Unit	1142.57	Unit	20	130	22.4	Positive
AAQVTIQSSGTFSSK	756.39	Unit	1041.52	Unit	20	130	22.4	Positive
AAQVTIQSSGTFSSK	756.39	Unit	928.44	Unit	20	130	22.4	Positive
AAQVTIQSSGTFSSK	756.39	Unit	800.38	Unit	20	130	22.4	Positive
FQVDNNNR	503.74	Unit	859.40	Unit	20	130	13.3	Positive
FQVDNNNR	503.74	Unit	731.34	Unit	20	130	13.3	Positive
FQVDNNNR	503.74	Unit	632.27	Unit	20	130	13.3	Positive
FQVDNNNR	503.74	Unit	517.25	Unit	20	130	13.3	Positive

Table 2. Replicate A2MG peptide measurements (N=6) Log 2 normalized peak intensities for all 12 unique peptides.

A2MG Peptides	**Area (Log2)**		
Peptide Sequence	**Mean**	**Stdev**	**CoV**
AAQVTIQSSGTFSSK	15.24	0.22	1.5
DMYSFLEDMGLK	15.15	0.27	1.8
DTVIKPLLVEPEGLEK	15.05	0.26	1.7
FEVQVTVPK	14.95	0.37	2.4
FQVDNNNR	14.91	0.38	2.5
HNVYINGITYTPVSSTNEK	14.87	0.37	2.5
IAQWQSFQLEGGLK	14.84	0.37	2.5
LHTEAQIQEEGTVVELTGR	14.76	0.27	1.8
LPPNVVEESAR	14.71	0.22	1.5
LVHVEEPHTETVR	14.65	0.15	1.1
QGIPFFGQVR	15.09	1.33	8.8
VGFYESDVMGR	15.52	1.73	11.2

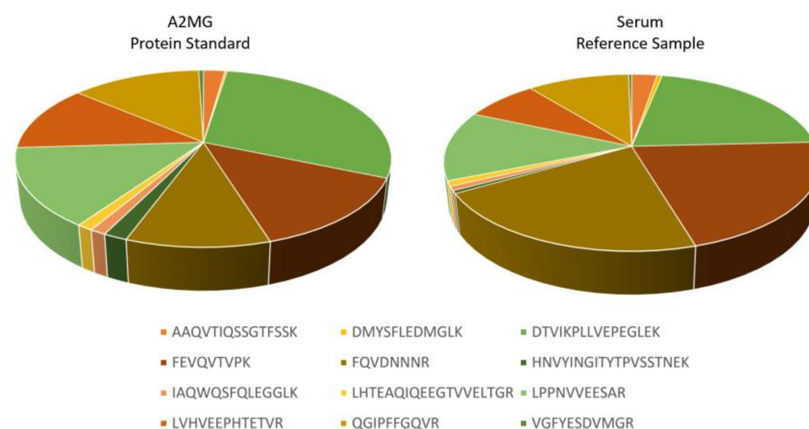

Figure 4. Relative peptide abundance of A2MG peptides in serum and protein standard respectively.

The MS method performance was developed and optimized for 12 peptides that are all specific to A2MG.The method performance was tested against a standard human protein and a reference serum sample. The comparison showed that although the top 6 peptides, DTVIKPLLVEPEGLEK, FEVQVTVPK, FQVDNNNR, LPPNVVEESAR, LVHVEEPHTETVR and QGIPFFGQVR, are the most abundant in both samples, the relative intensities of those showed differences. The difference could be associated with the sample matrix. While a standard protein is relatively simple as it is isolated and purified, the human serum involves hundreds of proteins. Therefore, the digestion performance could be different, Zhang et al. previously stated that the different digestion protocols may also affect the targeted quantification from protein-protein interaction analysis [23-24]. The change in peptide intensities should be further investigated to understand the root cause of the observed differences. Conventional methods used in clinical proteomics target either specific binding point or single/limited unique peptide(s). Proposed approach offers comprehensive screening by monitoring multiple unique

peptides for A2MG. Although results indicate reproducible measurements between replicate samples for each peptide, the relative peptide abundances show different pattern in reference serum than that of protein standard. Therefore, the variation in unique peptide responses raise a number of important questions; i) is the variation due to matrix affect? ii) are digestion efficiencies different in the standard and serum? iii) Is protein concentration directly proportional to individual peptides? iv) Are all unique peptides correlated to each other? v) If not, which unique peptide gives more reliable information and above all iv) Are the differences related to the biological complexity considering the presence of different protein forms. Consequently, monitoring multiple unique peptides for a single protein offers great advantages and provide comprehensive information. The approach can easily be implemented to other proteins and clinical studies. Future studies should focus on the gaps described above.

Here, a new method was developed to profile all the unique peptides isolated from A2MG.This method is the most comprehensive method available to date for the determination of A2MG. Monitoring multiple peptides for a single protein enables to observe unseen differences at peptide level. The concept of targeted-MS provides an ideal quantification and validation platform for clinical applications.

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AUTHOR CONTRIBUTIONS

Concept: *S.O.K.*; Design: *S.O.K.*; Control: *S.O.K.*; Sources: *S.O.K.*; Materials: *S.O.K.*; Data Collection and/or processing: *S.O.K.*; Analysis and/or interpretation: *S.O.K.*; Literature review: *S.O.K.*; Manuscript writing: *S.O.K.*; Critical review: *S.O.K.*; Other: -

CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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