Am J Cardiovasc Dis 2015;5(3):140-145
www.AJCD.us /ISSN:2160-200X/AJCD0009253

Review Article

Proteomic approaches to identify circulating biomarkers in patients with abdominal aortic aneurysm

Dan Bylund[1], Anders E Henriksson[1,2]

[1]*Department of Natural Sciences, Mid Sweden University, Sundsvall, Sweden;* [2]*Department of Laboratory Medicine, Sundsvall County Hospital, Sweden*

Received April 17, 2015; Accepted September 2, 2015; Epub September 15, 2015; Published September 30, 2015

Abstract: Abdominal aortic aneurysm (AAA) is a common condition with high mortality when ruptured. Most clinicians agree that small AAAs are best managed by ultrasonographic surveillance. However, it has been stated in recent reviews that a serum/plasma biomarker that predicts AAA rupture risk would be a powerful tool in stratifying patients with small AAAs. Identification of such circulating biomarkers with traditional hypothesis driven studies has been unsuccessful. In this review we summarize six studies using different proteomic approaches to find new, potential plasma AAA biomarker candidates. In conclusion, by using proteomic approaches novel potential plasma biomarkers for AAA have been identified.

Keywords: Aortic aneurysm, biomarker, proteomics

Introduction

Abdominal aortic aneurysm (AAA) is a common condition with a prevalence of around 5% in men over 50 years of age [1, 2]. The condition is often asymptomatic until the catastrophic onset of hemorrhagic shock due to aneurysm rupture. Despite advances in surgical and anesthetic techniques the perioperative mortality remains high in patients with ruptured AAA [1]. A presymptomatic elective repair in appropriately selected individuals will prevent rupture and thereby increase life expectancy. AAA screening programs have been introduced in an attempt to reduce mortality due to ruptured AAAs in the general population. However, most clinicians agree that due to a very low rupture rate, small AAAs are best managed by ultrasonographic surveillance and that an AAA diameter above 5.0-5.5 cm generally justifies elective repair. Two studies have shown the safety of surveillance until a diameter of the AAA reaches 5.5 cm among male patients [3, 4]. The expansion pattern of AAAs is estimated to be about 10% per annum [1]. However, there are large individual variations in expansion patterns. Episodes of rapid expansion may be followed by periods of slower, or even cessation of, expansion [1, 2]. Thus, finding a biomarker for identifying aneurysms with progressive growth, that indicates a necessity for treatment, seems important. The development of plasma biomarkers is attractive due to simple and minimally invasive sample collection [5]. Such a biomarker discovery will allow identification of aneurysms more likely to rupture and stratify high-risk patients. Identification of such circulating biomarkers with traditional hypothesis driven studies however has been unsuccessful. Previous AAA biomarker research has focused on one or few possible markers in each study [6-8]. Screening for new biomarker candidates by a proteomic approach allows a simultaneous detection of changes in hundreds of proteins in each study [9]. This strategy has given the scientists new hope of discovering blood biomarkers useful in the management of AAA. Furthermore, proteomic analysis is a convenient method to monitor changes in protein expression without prior knowledge of what those changes might be. Plasma is the most complex human-derived sample for proteomic analysis because it contains the widest dynamic range of cellular proteins [9]. However, recent advances in proteomic technologies, including two-dimensional differential in-gel electropho-

resis (2D-DIGE) and improved mass spectrometry (MS), have provided new opportunities for biomarker discovery [9, 10]. Moreover, studies that focus on combining fractionation with gel electrophoresis and liquid chromatography (LC) coupled to MS have shown improved protein identification [10].

In this paper, we first give a brief survey of current methodology in proteomic research. Secondly, we review results from six recent studies [11-16] using a MS-based proteomic strategy for identification of circulating biomarker candidates for AAA. Finally, we suggest a proteomic strategy for future studies to identify clinical valuable circulating biomarkers in patients with AAA.

General principles in MS-based proteomic research

Subjects and blood sampling

A longitudinal population-based study design is preferred in studies attempting to identify useable, novel biomarkers for screening of the expansion patterns for AAA [17]. However, since AAA develop over years or even 2-3 decades the scientists are often restricted to case-control studies. Male gender, increasing age, and smoking are the dominant risk factors for AAA [1]. Owing to this fact it is important to use a control group matched by age, gender and smoking habits to the AAA patient group in case-control studies to eliminate possible bias in accordance with the guidelines given by Grimes and Schulz [18]. Furthermore, preanalytical characteristics such as sample handling and storage have to be considered [19]. For instance, plasma instead of serum has been recommended in proteomic studies since blood plasma is a more stable protein suspension than serum [11, 20].

Sample preparation

It has been hypothesized that disease-specific biomarkers are present in one percent of proteins that make up the low-abundance component of plasma or serum. A major problem in proteome studies using plasma or serum samples is that high abundance proteins may mask these low abundance proteins [21]. The dynamic range of protein concentration between the low- and high-abundance proteins is thought to span approximately ten orders of magnitude, which is much greater than the 2-4 orders of magnitude that MS measurements are normally constrained to [9]. However, several depletion and fractionation technologies have been developed to remove highly abundant proteins such as albumin, haptoglobins, transferrins, and immunoglobulins prior to MS. The development of immunodepletion techniques, i.e. multiple affinity removal system (MARS), that utilizes antigen-antibody interactions to remove high-abundant plasma proteins have contributed to a better assessment of low abundant proteins. Recently, a novel sample enrichment tool (ProteoMiner) has been proposed as a promising and powerful alternative to common immuno-subtraction methods [21]. This protein enrichment tool is based on the interaction of complex protein sample with a large, highly diverse library of hexapeptides bound to a chromatographic support where each unique hexapeptide binds to a unique protein sequence. Treatment of samples with the ProteoMiner kit causes partial depletion of high-abundance proteins and simultaneous up-concentration of low-abundance proteins, resulting in dynamic range compression of samples [21]. This reduces the dynamic range of protein concentrations while maintaining representatives of all proteins within the original sample.

Protein separation

Two-dimensional electrophoresis and image analysis: Two-dimensional (2-D) gel electrophoresis is a well-established tool for initial separation (or fractionation) of plasma proteins. However, despite a wealth of experimental progress, critical limitations, such as discrimination against low-abundance and highly hydrophobic proteins, are still a problem in standard 2-D gel electrophoresis methods such as 2D polyacrylamide gel electrophoresis (2D-PAGE). The introduction of differential in-gel electrophoresis (DIGE), which allows separation of two sets of protein mixtures from different sources (e.g., small AAA and controls without aneurysm), has minimized previous difficulties with reproducibility associated with 2-D gels [22]. 2D-DIGE differs from 2D-PAGE in that each sample is pre-labelled with a fluorescent dye prior to isoelectric focusing. Three dyes (Cy2, Cy3 and Cy5) are commercially available and hence up to three samples can be run simultaneously on the same gel. The ability to pre-label

each sample with different dyes and run them together on the same gel makes 2D-DIGE a much more powerful technique than running single samples on individual 2D-PAGE gels and staining with silver or Coomassie blue stain. Analysing three samples on one 2D-DIGE rather than three individual 2D-PAGE gels reduces the experimental gel-to-gel variation that is often observed in gels cast in-house rather than those bought commercially. Traditionally, 2D-PAGE gels are stained using Coomassie blue or the more sensitive silver staining procedures. Gels are then compared using computer software for changes in protein expression. However, the degree of linearity of both silver and Coomassie blue stains are limited compared to the four orders of magnitude possible using CyDye technology. The CyDye technique is a much quicker and sensitive method for the detection of proteins (low nanogram) than silver and Coomassie blue. With silver and Coomassie staining, gels require time consuming fixation, staining and destaining techniques. However with DIGE, immediately after completion of electrophoresis each gel is scanned three times, at three different wavelengths (red, blue and green) and the analyses is complete. The three samples can then be compared and analysed using sophisticated computer software and subtle changes in protein expression detected very accurately. Furthermore, by allocating one of these three samples to an internal standard (normally a pooled sample), the influence of gel-to-gel variation in larger DIGE studies can be even more reduced [23].

Protein identification

Prior to the protein identification by MS the selected protein spots are picked and digested with proteolytic enzymes to produce peptide fragments more or less specific to each protein. The most common method of choice for in-gel protein digestion is with trypsin as described by Shevchenko et al. [24]. The current most widely adopted mass spectrometric strategy is then to use liquid chromatography (LC) to separate the peptides, and couple the LC on-line to a mass spectrometer operating with two mass analyzers in sequence (tandem MS or MS/MS), applying collision induced dissociation of the peptides. The resulting MS/MS spectra can be evaluated manually for sequence tags or, more commonly, with the aid of computer algorithms such as MASCOT or BLAST finally feeding the resulting data into databases for protein identification.

In the last few years, substantial progress has been made in LC-MS instrumentation and improved database searching methods. Moreover, isobaric labelling techniques have recently been developed as a MS strategy for quantitative proteomics. Basically this strategy relies on isotope labelling of peptide/protein instead of separation of plasma proteins by electrophoresis. Currently, there are two types of isobaric labelling techniques commercially available; tandem mass tags (TMT) [25] and isobaric tags for relative and absolute quantitation (iTRAQ) [26, 27].

Statistical considerations

Depending on the number of patient groups included in the study, ANOVA or t-tests can be applied to evaluate the significance of the differences in protein expression between the patient group (s) and the control group [28]. Data pre-treatment, such as log-transformations, may be necessary in some cases to improve normality. Non-parametric alternatives, such as the Mann-Whitney U-test, can also be applied if the data set deviates too much from the normal distribution [28, 29].

Due to the large amount of proteins analyzed, proteomic studies have an inherent risk for mass significance, i.e. production of a large number of false positives as a result of the multiple comparisons performed [30, 31]. The number of potential biomarkers found can be reduced by changing the significance level, for example by a Bonferroni correction. On the other hand, this correction may be too conservative, especially as the different tests are not expected to be fully independent of each other, thereby producing large numbers of false negatives instead.

In order to incorporate biological/clinical significance, the fold change, i.e. the relative difference in protein expression between the patient group and the control group, is often used as a complementary measure to the *p*-values obtained by the above mentioned statistical tests. A certain limit in the fold change (often a factor 2) can also be used as a first cut-off, minimizing the number of proteins necessary to identify by MS, and thereby also limiting the cost and work-load of the study.

Table 1. Methods used in six mass-spectrometry based proteomic studies

Study Author, year	Subjects (n) AAAs/Controls	Sample	Depletion kit	Protein separation	Protein identification
Nordon, 2010	20/20	Serum	IgY-12	2D-PAGE	LC-MS/MS
Gamberi, 2011	8/6	Plasma	Not used	2D-PAGE	MS/MS
Pulinx, 2011	24/0	Serum	Not used	2D-DIGE	MS/MS
Acosta-Martin, 2011	17 (pooled)/17 (pooled)	Plasma	MARS-14	TMT	LC-MS/MS
Wallinder, 2012	4/4	Plasma	ProteoMiner	2D-DIGE	LC-MS/MS
Spadaccio, 2012	20/20	Serum	PROT-BA	2D-PAGE	MS

Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; 2D-DIGE, two-dimensional differential in-gel electrophoresis. LC, liquid-chromatography; MS, mass-spectrometry; MS/MS, tandem mass-spectrometry; TMT, tandem mass tags.

Multivariate tools are also often used in the analysis of proteomic data sets [28, 29], although not applied in the reviewed studies on AAA biomarkers. Principal component analysis can be applied to identify outliers and clusters in the data, while supervised methods, such as partial least squares discriminant analysis can be used to identify potential biomarkers. Furthermore, multiple regression models can be used to incorporate covariates, reducing the effects of poorly matched patient and control groups.

Results and potential biomarkers from six MS-based proteomic studies

Six MS-based proteomic studies were reviewed [11-16]. The methods used in these studies showed different study-design according to subjects (selection, number and control-matching), sampling, sample preparation, protein separation and identification (**Table 1**). Not surprisingly, the reviewed studies show quite different results.

Nordon et al. In this study three highly abundant proteins (albumin, fragments of hemoglobin, and apolipoprotein C-II precursor) were identified, but none were deemed as plausible potential biomarkers of aneurysmal disease.

Gamberi et al. This study confirmed a number of biomarkers (for instance; fibrinogen, alfa-1 antitrypsin, haptoglobin were up-regulated and vitamin D-binding protein was down-regulated in AAA) associated with AAA that have been previously identified by various authors. However, none of the identified biomarkers in this study has the biologic plausibility to be used singularly as a biomarker for aneurymal disease due to inadequate specificity.

Pulinx et al. In this study five proteins (Albumin, complement C3, alfa-1 antitrypsin, factor XII, Ig kappa chain C region) were significantly differentially expressed between three AAA subgroups. These differential proteins are involved in previous well-known pathophysiological key processes of AAA, such as inflammation, extracellular matrix remodeling or coagulation and fibrinolysis.

Acosta-Martin et al. In this study the biomarker screen was made by a gel-free TMT method on pooled plasma samples from 17 AAA and 17 control patients. This group found five potential biomarkers for early AAA diagnosis (i.e. adiponectin, extracellular superoxide dismutase, kallistatin, carboxypeptidase B2, and protein AMBP). None of these five proteins have been previously proposed as potential biological markers for AAA diagnosis.

Wallinder et al. In this study the ProteoMiner technology was used for plasma enrichment followed by 2D-DIGE analysis combined with LC-MS/MS for detection of differences in the protein profile between four patients with small AAA and four controls without aneurysm. This study provides evidence of the enzyme glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD) as a possible biomarker for AAA. However, GPI-PLD as a potential biomarker was not confirmed in a recent study [32].

Spadaccio et al. The investigators found four potential biomarker candidates (Hemopexin, vitamin D-binding protein, and serum amyloid P were up-regulated while apolipoprotein A-1 was down-regulated). The significance and clinical use of the four potential biomarker candidates needs further evaluation.

Conclusion and suggested future proteomic research strategies

The pathophysiology behind the onset, progress and rupture of AAA is still not completely

understood. Furthermore, prevention or non-surgical management strategies do not yet exist. Human plasma is one of the most important proteomes from a clinical and medical point of view [9]. Recent reviews also state that a blood plasma biomarker predicting aortic rupture risk would be a powerful tool to stratify patients with small screen detected aneurysms [6-8]. Identification of such circulating biomarkers with traditional hypothesis driven studies has so far been unsuccessful. The present review has shown that studies using non-hypothesis-driven MS-based proteomic approaches to find new clinically useful biomarkers for AAA have emerged during the last years. However, the method of choice has not yet been established. Finally, we currently suggest iTRAQ [26, 27] as the next step to screen for new biomarker candidates for AAA management.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Anders E Henriksson, Department of Laboratory Medicine, Sundsvall County Hospital, SE-851 86 Sundsvall, Sweden. Tel: +46 60 181379; Fax: +46 60 182230; E-mail: anders.henriksson@lvn.se

References

[1] Sakalihasan N, Limet R, Defawe OD. Abdominal aortic aneurysm. Lancet 2005; 365: 1577-1589.

[2] Lederle FA, Johnson GR, Wilson SE, Chute EP, Hye RJ, Makaroun MS, Barone GW, Bandyk D, Moneta GL, Makhoul RG. The aneurysm detection and management study screening program. Arch Intern Med 2000; 160: 1425-1430.

[3] Mortality results for randomised controlled trial of early elective surgery or ultrasonographic surveillance for small abdominal aortic aneurysms. The UK Small Aneurysm Trial Participants. Lancet 1998; 352: 1649-1655.

[4] Lederle FA, Wilson SE, Johnson GR, Reinke DB, Littooy FN, Acher CW, Ballard DJ, Messina LM, Gordon IL, Chute EP, Krupski WC, Busuttil SJ, Barone GW, Sparks S, Graham LM, Rapp JH, Makaroun MS, Moneta GL, Cambria RA, Makhoul RG, Eton D, Ansel HJ, Freischlag JA, Bandyk D; Aneurysm Detection and Management Veterans Affairs Cooperative Study Group. Immediate repair compared with surveillance of small abdominal aortic aneurysms. N Engl J Med 2002; 346: 1437-1444.

[5] Beretta L. Proteomics from the clinical perspective: many hopes and much debate. Nat Methods 2007; 4: 785-786.

[6] Urbonavicius S, Urbonaviciene G, Honoré B, Henneberg EW, Vorum H, Lindholt JS. Potential circulating biomarkers for abdominal aortic aneurysm expansion and rupture--a systematic review. Eur J Vasc Endovasc Surg 2008; 36: 273-280.

[7] Golledge J, Tsao PS, Dalman RL, Norman PE. Circulating markers of abdominal aortic aneurysm presence and progression. Circulation 2008; 118: 2382-2392.

[8] Nordon I, Brar R, Hinchliffe R, Cockerill G, Loftus I, Thompson M. The role of proteomic research in vascular disease. J Vasc Surg 2009; 49: 1602-1612.

[9] Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics 2002; 11: 845-867.

[10] Wilkins MR, Appel RD, Van Eyk JE, Chung MCM, Görg A, Hecker M, Huber LA, Langen H, Link AJ, Paik YK, Patterson SD, Pennington SR, Rabilloud T, Simpson RJ, Weiss W, Dunn MJ. Guidelines for the next 10 years of proteomics. Proteomics 2006; 6: 4-8.

[11] Nordon IM, Brar R, Hinchliffe RJ, Cockerill G, Thompson MM. Proteomics and pitfalls in the search for potential biomarkers of abdominal aortic aneurysms. Vascular 2010; 18: 264-268.

[12] Gamberi T, Puglia M, Guidi F, Magherini F, Bini L, Marzocchini R, Modesti A, Modesti PA. A proteomic approach to identify plasma proteins in patients with abdominal aortic aneurysm. Mol Biosyst 2011; 7: 2855-2862.

[13] Pulinx B, Hellenthal FA, Hamulyák K, van Dieijen-Visser MP, Schurink GW, Wodzig WK. Differential protein expression in serum of abdominal aortic aneurysm patients - a proteomic approach. Eur J Vasc Endovasc Surg 2011; 42: 563-570.

[14] Acosta-Martin AE, Panchaud A, Chwastyniak M, Dupont A, Juthier F, Gautier C, Jude B, Amouyel P, Goodlett DR, Pinet F. Quantitative mass spectrometry analysis using PAcIFIC for the identification of plasma diagnosic biomarkers for abdominal aortic aneurysm. PLoS One 2011; 6: e28698.

[15] Wallinder J, Bergström J, Henriksson AE. Discovery of a novel circulating biomarker in patients with abdominal aortic aneurysm: a pilot study using a proteomic approach. Clin Trans Sci 2012; 5: 56-59.

[16] Spadaccio C, di Domenico F, Perluigi M, Lusini M, Giorgi A, Schininà ME, Blarzino C, Covino E, Chello M, Coccia R. Serum proteomics in patients with diagnosis of abdominal aortic aneurysm. Cardiovasc Pathol 2012; 21: 283-290.

[17] García-Closas M, Vermeulen R, Cox D, Lan Q, Caporaso NE, Rothman N. Population-based study designs in molecular epidemiology. IARC Sci Publ 2011; 163: 241-259.
[18] Grimes DA, Schulz KF. Compared to what? Finding controls for case-control studies. Lancet 2005; 365: 1429-1433.
[19] Christenson RH, Duh SH. Methodological and analytic considerations for blood biomarkers. Prog Cardiovasc Dis 2012; 55: 25-33.
[20] Luque-Garcia JL, Neubert TA. Sample preparation for serum/plasma profiling and biomarker identification by mass spectrometry. J Chromatogr A 2007; 1153: 259-276.
[21] Boschetti E, Lomas L, Citterio A, Righetti PG. Romancing the "hidden proteome", Anno Domini two zero zero seven. J Chromatogr A 2007; 1153: 277-290.
[22] Marouga R, David S, Hawkins E. The development of the DIGE system: 2D fluorescence difference gel analysis technology. Anal Bioanal Chem 2005; 382: 669-678.
[23] Alban A, David SO, Bjorkesten L, Andersson C, Sloge E, Lewis S, Currie I. A novel experimental design for comparative two-dimentional gel analysis: two-dimentional difference gel electrophoresis incorporating a pooled internal standard. Proteomics 2003; 3: 36-44.
[24] Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 1996; 68: 850-858.
[25] Thompson A, Schäfer J, Kuhn K, Kienle S, Schwarz J, Schmidt G, Neumann T, Johnstone R, Mohammed AK, Hamon C. Tandem mass tags: A novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. Anal Chem 2003; 75: 1895-904.
[26] Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, Pappin DJ. Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol Cell Proteomics 2004; 3: 1154-1169.
[27] Mertins P, Udeshi ND, Clauser KR, Mani DR, Patel J, Ong S, Jaffe JD, Carr SA. iTRAQ labeling is superior to mTRAQ for quantitative global proteomics and phosphoproteomics. Mol Cell Proteomics 2012; 11: 1-12.
[28] Marengo E, Robotti E. Biomarkers for pancreatic cancer: recent achievements in proteomics and genomics through classical and multivariate statistical methods. World J Gastroenterol 2014; 20: 13325-13342.
[29] Smit S, Hoefsloot HC, Smilde AK. Statistical data processing in clinical proteomics. J Chromatogr B Analyt Technol Biomed Life Sci 2008; 866: 77-88.
[30] Franceschi P, Giordan M, Wehrens R. Multiple comparisons in mass-spectrometry-based *-omic*s technologies. Trends Anal Chem 2013; 50: 11-21.
[31] Broadhurst DI, Kell DB. Statistical strategies for avoiding false discoveries in metabolomics and related experiments. Metabolomics 2006; 2: 171-196.
[32] Lindqvist M, Wallinder J, Bergström J, Henriksson AE. Plasma glycosylphosphatidylinositol phospholipase D (GPI-PLD) and abdominal aortic aneurysm. Int J Clin Exp Med 2012; 5: 306-309.