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SELDI-TOF proteomic profiles to predict atherosclerotic plaque rupture

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Introduction

Cardiovascular diseases (CVD) remain the leading cause of death (34% in the Netherlands), mainly due to clinical complications such as myocardial infarction or stroke (1). Atherosclerosis, a progressive disease, is the main underlying pathology of CVD and is characterised by a build-up of lipids, cells and fibrous elements in the vessel wall of large arteries (2). Such plaques can be stable, or ruptured. Stable plaques have an intact fibrous cap, whereas ruptured plaques expose their contents to the bloodstream, which can lead to thrombus formation. Plaque rupture and subsequent thrombus formation are important clinical complications resulting in ischaemic heart diseases (33%), or cerebrovascular diseases (25%) (1). Atherosclerosis is a very complex disease for which the available biomarkers still lack the specificity required for reliable diagnosis and follow-up. Three years ago, Surface Enhanced Laser Desorption/Ionisation Mass Spectrometry (SELDI-TOF-MS) was shown to be a valuable tool in the discovery of new potential biomarkers (3). The search for new biomarkers using SELDI-profiles was originally mainly focused on cancer research, but can of course be used as a general approach in the search for new biomarkers. A pattern of multiple markers may be even more accurate and specific compared to the single marker approach.

In this pilot study we investigated the use of SELDI-TOF-MS to discriminate stable plaques from thrombus containing plaques based on differences in serum proteomic patterns of histologically confirmed patients.

Materials and Methods

Study population

After written informed consent, serum samples were obtained from patients undergoing carotid endarterectomy or femoral artery bypass, centrifuged at 4000 rpm for 15 minutes and stored in aliquots at -20°C. Plaque specimens were divided into 3 mm thick slices, formalin-fixed, processed for histological analysis and classified according to Virmani et al. (4). Using this classification, two groups of patients were selected. One group (S) with stable plaques (n=10) and one group (T) with plaques containing a thrombus (n=11).

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SELDI-TOF-MS

Serum samples were denatured by diluting 10 µL serum in 90 µL 9 M urea with 2% CHAPS. Each sample was applied to the ProteinChip® arrays (Ciphergen Biosystems) in duplo. Optimal conditions of buffer and array type were investigated in a screening experiment. We used cation exchange (CM10) arrays with a binding buffer of pH 4, 5 (50 mM sodium acetate) or 7 (50 mM sodium phosphate), anion exchange (Q10) arrays with a binding buffer of pH 5 (50 mM sodium acetate), 7 or 9 (50 mM Tris-HCl), hydrophobic (H50) arrays with a 100 mM sodium phosphate buffer containing 10% acetonitrile and metal affinity (IMAC30-Cu^{2+}) arrays with a 100 mM sodium phosphate binding buffer. Protocols from the manufacturer were followed for applying and washing samples. All samples were analysed at low and high laser intensities. Sinapinic acid was used as the energy absorbing matrix.

The arrays were analysed in the PBSIIc mass spectrometer (Ciphergen Biosystems) with a laser intensity of 170, detector sensitivity of 6 and high mass of 200 kDa, optimised from 1,500 - 15,000 Da. Peak analysis, baseline subtraction, total ion current normalisation, peak alignment and statistical analysis (Mann-Whitney-U non-parametric test) were performed using ProteinChip® software version 3.2 and Ciphergen Express software version 2.1. For peak clustering the following parameter settings were used: first pass S/N ratio 5, minimal peak threshold 50%, cluster mass window 0.3%, second pass S/N ratio 2 and m/z range of 2 - 200 kDa.

Results

We used four serum samples (two from each group) to determine the optimal analysing conditions. Based both on the number of significant clusters and the total number of clusters found by the software, the IMAC30-Cu^{2+} array combined with a 100 mM sodium phosphate binding buffer achieved the best results.

The inter-spot and inter-array variation was determined by applying one serum sample onto all 24 spots of three IMAC-Cu^{2+} arrays. The variation in peak intensity, which is used in the statistical analysis, was calculated from 25 randomly selected peaks across the whole m/z range. The inter-spot coefficient of variation (CV) was 15.5%, 22.0% and 20.7% for the three arrays respectively. The inter-array variation was 20.1%.

With the settings mentioned earlier, we compared 10 stable plaques with 11 plaques containing a thrombus

Table 1. Average mass-to-charge (m/z) ratios of eight significant clusters discriminating patients with a stable atherosclerotic plaque (S) from patients with a plaque containing a thrombus (T). The right column indicates the relative abundance.

m/z ratio	p value	Relative abundance
4302.7	0.006	S>T
4284.8	0.006	S>T
3975.5	0.007	S>T
3276.7	0.020	S>T
3145.6	0.024	S>T
4468.9	0.035	T>S
3958.5	0.049	S>T
8936.3	0.049	T>S

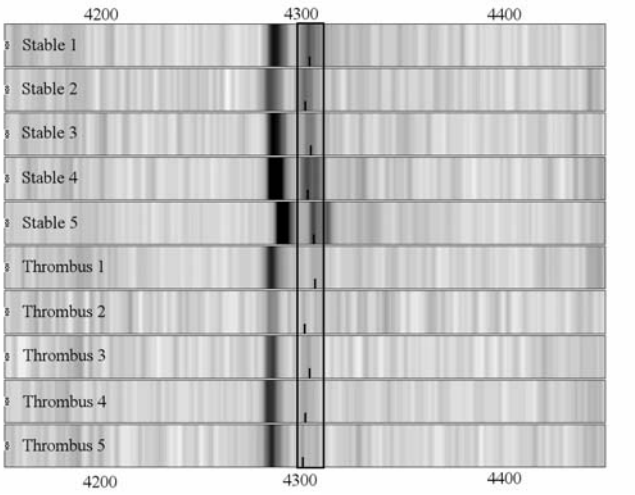

Figure 1. Proteomic patterns (gel view) of five patients with a stable plaque and five patients with a plaque containing a thrombus. Ticks represent the top of the peaks. In the boxed cluster at m/z 4302.7 ± 4.3 the peak was expressed significantly different between both groups (p=0.006), with a lower expression in the patients with a plaque containing a thrombus. Spectra were normalised for total ion current and baseline was subtracted.

and found eight clusters that differed significantly between both groups (see table 1). Six out of these eight clusters were more abundant in serum samples from patients with stable plaques compared to the group with thrombus containing plaques. Figure 1 shows an example of the most significant cluster. It is clear that the peak in the cluster at m/z 4302.7 is more abundant in the group with a stable plaque.

Discussion

We have shown that SELDI-TOF-MS is a powerful new tool for discovering potential biomarkers for the progression of atherosclerosis. However, there are some points of concern to be addressed.

(a) Reproducibility. Recently Semmes et al. addressed this topic and concluded that if protocols are adhered to, SELDI-TOF-MS profiling can provide a reproducible diagnostic assay platform. For three peaks they found CV values of 15-36% for the intensity (5).

(b) Pre-analytical sample handling. It is very important to follow identical protocols for all samples in all groups. Bias is easily introduced. Freezing temperature, number of freeze-thaw cycles and time between blood draw and freezing should all be standardised.

(c) Post-analytical analysis. Most studies describe that data analysis was done with "home-made" software, making it almost impossible to check and reproduce these data. Using standard Ciphergen software, we hope to have overcome this issue.

In this pilot study we evaluated whether it is possible to discriminate serum protein profiles from patients with a stable plaque from patients with a thrombus containing plaque using SELDI-TOF-MS and proteomic pattern analysis. We found eight clusters significantly different between both groups. Note that this was a low number study that needs to be repeated with larger sample sizes. Future research is aimed at analysing larger groups of patients. After establishing a model based on proteomic patterns for the discrimination of both groups, this model will be validated with samples of unknown pathology. This will allow us to determine the diagnostic value of the obtained protein profiles to detect atherosclerotic plaque rupture.

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