Chapter 7

Haptoglobin in lacunar stroke: a SELDI-TOF-MS study with subsequent phenotyping

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Submitted

Abstract

Background
Using Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry, we aimed to detect differences in protein expression profile in serum samples of two lacunar stroke subtypes.

Methods
Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry, followed by protein identification, was performed in samples of eight first-ever lacunar stroke patients with MR imaging showing a single symptomatic lacunar lesion (type I), and eight with multiple additional "silent" lacunar lesions and extensive white matter lesions (type II).

Results
A 16 kDa protein, identified as alpha-2-chain of haptoglobin (Hp), was up-regulated in type I compared to type II (peak intensity 12.5 vs. 5.0; p=0.02). A polymorphism with two alleles, Hp-1 and Hp-2, determines the presence of alpha-1 and/or alpha-2-chains in the Hp-molecule. Therefore, Hp phenotypic analysis was performed. Hp-1 : Hp-2 allele frequency was 0.562 : 0.438 in type I and 0.812 : 0.188 in type II (population reference ~0.4 : 0.6).

Conclusions
The up-regulation of the alpha-2-chain in type I is (partly) related to a higher Hp-2 allele frequency. Yet, compared to population reference, the phenotype distribution in both lacunar stroke patient groups deviates towards a high Hp-1 allele frequency, suggesting a role for the Hp gene in the etiology of cerebral small vessel disease. The even higher Hp-1 allele frequency in type II than in type I implies a promoting role for Hp-1 in developing multiple silent lacunar lesions and white matter lesions and could be a reflection of a difference in underlying vascular pathology between the two types, but needs confirmation in larger series.

Introduction

Lacunar infarcts are small, deeply in the brain located infarcts, mostly caused by occlusion of a small perforating artery[1]. They can present with an acute lacunar stroke syndrome, but can also remain asymptomatic. Lacunar strokes account for about 25% of all ischemic strokes. Lacunar stroke patients in whom cerebral imaging shows multiple additional "silent" lacunar lesions, differ clinically from those patients with only a single symptomatic lesion in that they show more extensive cerebral white matter lesions (WML) on neuro-imaging, have more often hypertension, and have worse prognosis on functional outcome, a higher stroke recurrence rate, higher short- and long-term mortality and higher rate of asymptomatic lesion progression[2-6]. Whether these two types of small vessel disease (SVD) are both ends of one pathogenetic spectrum, or represent two pathogenetically different entities, remains subject of debate. Research into the underlying pathogenetic mechanisms thus far focused on known proteins that are thought to be involved in vascular pathogenesis, such as coagulation and inflammation factors[7-10].

To obtain new insight in the underlying pathogenesis of lacunar stroke, we set out to look for possible differences in protein expression profile in lacunar stroke patients with or without extensive concomitant silent cerebral SVD using Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS) in serum samples. This technology provides a rapid method to detect expressed proteins in a biological sample. It uses chip surfaces, which bind a subset of proteins from a sample. An energy-absorbing matrix is added so that when laser energy is applied to the chip the proteins become ionized, enabling their mass to be measured by time-of-flight mass spectrometry. Protein profiles of different patient groups can be compared to find distinctive patterns.

This proteomic approach led us to the finding of a difference between the two lacunar stroke types in the expression of a 16 kDa protein, which was identified as alpha-2 chain of haptoglobin (Hp). These results brought us to the analysis of Hp 1/2 polymorphism.

Materials and methods

Study population

From May 2003 on, all patients who present with a first-ever stroke are registered in the Maastricht Stroke Registry, a prospective stroke database at University Hospital Maastricht, the Netherlands. Lacunar stroke is defined as an acute stroke syndrome with an MRI finding compatible with the clinical findings and consisting of a T2-weighted hyperintense subcortical small lesion of less than 20 mm in

diameter; or an acute stroke syndrome compatible with one of the lacunar syndromes in the absence of such MRI lesion[11]. With informed consent, these patients participate in an ongoing lacunar stroke research project for which the local Medical Ethical Committee gave approval. From this cohort we carefully selected two groups of each eight Caucasian patients, according to pre-defined criteria. Group 1 were patients in whom brain MRI showed only one single symptomatic lacunar lesion (type I). Group 2 consisted of eight patients in whom brain MRI additionally showed multiple (four or more) asymptomatic lacunar lesions as well as extensive WML (type II). Extensive WML was defined according to Fazekas' classification as confluent deep white matter hyperintensities and irregular periventricular hyperintensities extending into the deep white matter[12]. Patients had no potential source of cardiac embolism, diabetes mellitus, ischemic heart disease *(*known or treated angina pectoris, or a history of myocardial infarction), peripheral arterial vessel disease or carotid stenosis. Hypertension was defined as known and treated hypertension, or blood pressure recordings higher than 140/90 mmHg on at least two separate occasions before or at least two weeks after stroke. Patient baseline characteristics are shown in Table 7.1. Fasting blood samples were taken at least three months after stroke. Collection of serum samples was standardized by using a clotting time of 30 min at room temperature, spinning for ten min at 4000 RPM (3000 g) and storage in aliquots within one hour after blood collection at -80°C.

Table 7.1 Patient baseline characteristics

Lacunar stroke	Type I	Type II
Total number of patients	8	8
Age (median in years)	57 (46-80)	67 (44-79)
Male (absolute number)	4	4
Hypertension (absolute number)	6	6
Smoking (absolute number)	2	1
Number of lacunes (median)	1	5.5 (5-11)

SELDI-TOF-MS analysis

Anion exchange fractionation: Serum samples were pre-treated using an anion exchange procedure, which allowed high-throughput fractionation of all 16 serum samples based on the biophysical properties of proteins, as described by Solassol et al.[13]. Serum was separated into six different fractions (pH9, pH7, pH5, pH4, pH3, and organic wash). The anion exchange fractionation was standardized according to the Ciphergen protocols. All fractionated serum samples had only two thaw steps from collection to analyzing. The anion exchange procedure is illustrated in Figure 7.1. After this fractionation the samples were applied on the ProteinChips.

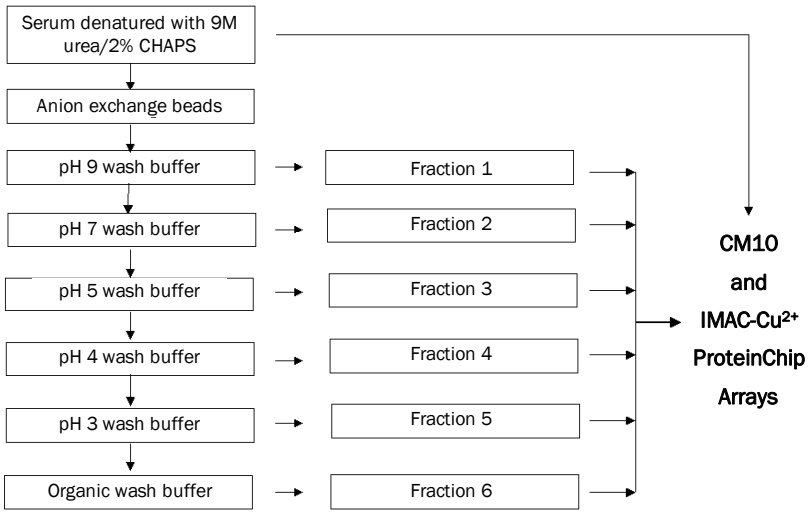

Figure 7.1 Schematic view of the anion exchange fractionation procedure: Anion exchange beads were supplied in a 96-well filtration plate. Samples were added to the plate and eluted in a stepwise manner, by altering the pH of the wash buffer. Serum was separated into six different fractions (pH9, pH7, pH5, pH4, pH3, and organic wash).

ProteinChip arrays: In the screening experiment, denatured serum and six fractions of all 16 patients were applied to two different array surfaces (Ciphergen Biosystems Inc., Fremont, CA, USA) to find out the optimal array and fraction. The weak cation exchange (CM10) and the immobilized metal affinity capture coupled with copper (IMAC-Cu^{2+}) ProteinChip arrays were used. The CM10 and IMAC-Cu^{2+} experiments were performed according to the Ciphergen protocols. The ProteinChip arrays were read on a Protein Biosystem IIc (Ciphergen Biosystems Inc.) instrument. A well defined protocol for calibration of the PBS IIc, implementation of quality control (QC) samples and acceptance criteria for QC were used in this study[14].

Data Analysis

Serum protein profiles of both lacunar stroke groups were compared. Peaks were identified after mass calibration, background subtraction, and normalization using Ciphergen Express Data manager 3.0.6 (Ciphergen Biosystems, Inc.). Cluster analysis was performed using the Biomarker Wizard in the Ciphergen Express Software. The biomarker Wizard parameters were as follows: signal-to-noise ratio (S/N) for the first pass: 5.0 (peak height) and 3.0 (valley depth) and for the second pass: 3.0 (peak height) and 1.0 (valley depth). The minimal peak threshold was 50% of all spectra. The cluster mass window was set at 1.0 peak width and the

specified *m/z* range was 2000 to 30,000 for the low range, 5000 to 100,000 for the mid range, and 10,000 to 200,000 for the high range. The Mann-Whitney Wilcoxon test (U-test) was used to detect significant differences in the intensities of the *m/z* values between both groups.

Identification

Protein identification was performed by one-dimensional gel electrophoresis (1-DE) and two-dimension gel electrophoresis (2-DE) followed by Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) as described in the studies of Bouwman et al. with minor modifications[15,16]. Two patient samples of each group were chosen which presented a clear up- or down-regulation in the protein spectra. To identify the 16.1 kDa marker, 10 µl of denatured serum sample was diluted in 90 µl Milli-Q. Ten µl of this diluted serum sample was subsequently diluted with 2 µl 6 x sample buffer (10% SDS, 0.6 M DTT, 30% glycerol, 0.012% bromophenol blue, 0.35 M Tris) and loaded on 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. For the 2-DE, 10 µl of the denatured serum sample was diluted in 190 µl 2-DE buffer (6 M urea, 30% glycerol, 2% SDS and 50 mM Tris-HCL) and was loaded for the first dimension.

Total Hp concentration and Hp phenotyping

Because of the results of SELDI analysis and protein identification, we measured total Hp concentration, using particle-enhanced immunonephelometry on the BN Prospec (Dade Behring Inc., Deerfield, IL, USA). Hp 1/2 phenotyping was carried out using starch gel electrophoresis according to Smithies et al.[17].

Results

SELDI-TOF-MS analysis

Although the anion exchange fractionating procedure was performed, the best distinctive pattern was found with denatured serum on the IMAC-Cu^{2+} ProteinChip array. Three peaks were significantly different between both groups. The peak at *m/z* 16,122 was the most prominent discriminating peak. The peak at *m/z* 16,122 was up-regulated in lacunar stroke type I versus type II with mean intensities of 12.5 and 5.0 for type I and type II patients, respectively (p=0.02). The clusterplot and protein spectra are illustrated in Figure 7.2 and 7.3.

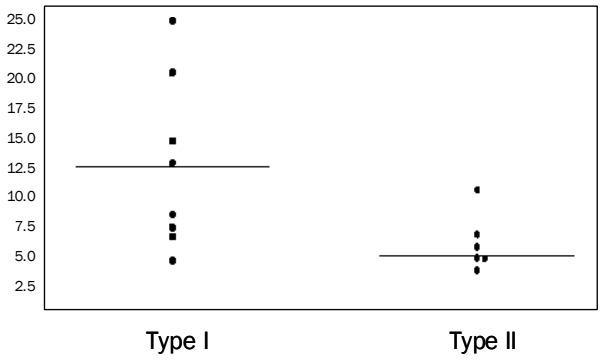

Figure 7.2 The cluster plot of the peak at *m/z* 16,122. The lacunar groups are indicated on the x-axis and the normalized intensities are indicated on the y-axis. The horizontal bars present the mean intensities for the type I and type II group.

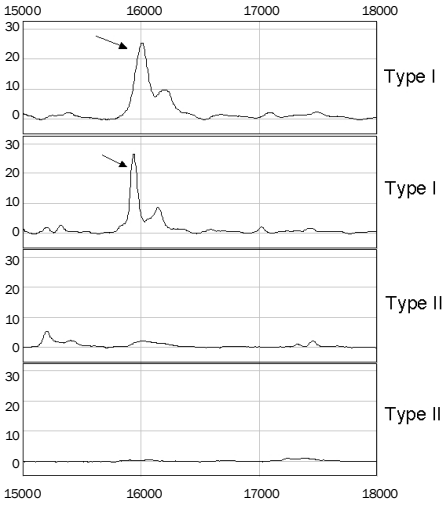

Figure 7.3 SELDI mass spectra of the marker at *m/z* 16.122. Protein spectra of two type I and two type II patient samples are illustrated. The marker is indicated with an arrow. The mass is given as *m/z* values on the x-axis and the intensity is displayed along the y-axis.

Identification

1-DE and 2-DE were used for isolation and purification of the *m/z* 16,122 marker, followed by MALDI-TOF-MS analysis, which was used for identification. Denatured serum samples of two type I patients which showed a high peak intensity at *m/z* 16,122 on the IMAC-Cu^{2+} ProteinChip arrays and denatured serum samples of two type II patients which showed a low peak intensity were used.
Figure 7.4 illustrates that the band at a molecular weight (MW) of 16 kDa, which

represents the peak at *m/z* 16,122, is clearly visible in the two type I patient samples and absent in the two type II samples. This agrees with the SELDI-TOF-MS results. After excising the 16.1 kDa peak out of the SDS-PAGE gel, identification with the Mascot search engine against the Swiss-Prot database revealed the following protein as the best candidate of database searches: alpha-chain of Hp (P00738) with a probability Mascot score (probability based mowse score) of 68. Protein scores >67 are significant ($p<0.05$). The alpha-chain of Hp exists in two variants, alpha-1 (8.9 kDa) and alpha-2 (16 kDa), the latter being compatible with the marker at *m/z* 16,122.

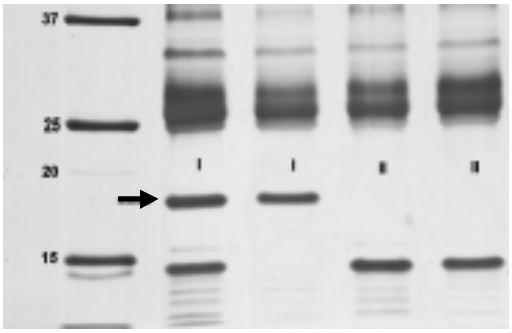

Figure 7.4 Silver stained band pattern of two type I and two type II patient samples separated by SDS-PAGE. There was one band at 16 kDa present for the type I patient samples, which was absent in the type II patient samples. The bands are indicated with an arrow.

There were three protein spots on 2-DE gels horizontally surrounding the 16 kDa spot which were clearly present in the type I patient samples and absent in the type II patient samples. All spots were successfully identified as alpha-chain of Hp (Probability Mascot scores: 69-75). These spots are probably isoforms of the alpha-chain, with the same MW, but different pI value (pI 4-7).

Haptoglobin

Because the peak of the marker at *m/z* 16,122 was identified as the alpha-2-chain of Hp, total Hp concentration and phenotype distribution were measured. Results are presented in Table 6.2. Mean Hp concentration in lacunar stroke type I was not significantly different from that in type II (t-test; $p=0.62$). Hp phenotype distribution and Hp-1:2 allele frequency are presented with a western European reference population.[18]

Table 7.2 Results of the SELDI-TOF-MS analysis, total haptoglobin (Hp) concentration measurement, and Hp phenotyping.

	Type I	Type II	*reference population*
16 kDa peak intensity	12.5	5.0	
Mean Hp (g/L)	1.69 ± 0.47	1.81 ± 0.51	0.25 - 1.90
Hp phenotype (1-1 : 2-1 : 2-2)[a]	2 : 5 : 1	5 : 3 : 0	15 : 50 : 35 [18]
Hp-1 : Hp-2 allele frequency[b]	0.562 : 0.438	0.812 : 0.188	0.40 : 0.60 [18]

[a] absolute numbers in type I and II, percentages in reference population
[b] χ^2 test : p=0.13 in type I vs. type II; p=0.02 in lacunar stroke (type I and II combined) vs. reference population

Discussion

The first important finding of our study is that we detected differences in protein profile between lacunar stroke patients with or without concomitant signs of silent cerebral SVD using SELDI-TOF-MS. A protein at *m/z* 16,122 was successfully identified as the alpha-2-chain of Hp. It was found up-regulated in patients without silent lesions (type I) compared to those with additionally multiple silent lacunar lesions and extensive WML (type II).

Hp is a plasma protein synthesized in the liver. It has hemoglobin-binding capacity and can be regarded as a major antioxidant. It also plays a role in inflammatory reactions, being an inflammation-sensitive protein. Its production is induced by cytokines[18]. There is a plausible role for Hp in vascular disease as both oxidative and inflammatory factors are important in the pathogenesis of atherosclerosis, and its presence has been shown in atherosclerotic coronary lesions[19,20]. High levels of Hp have been associated with the incidence of stroke[21,22].

Hp consists of alpha- and beta-polypeptide chains, linked by disulfide bonds[18]. Three Hp phenotypes, Hp 1-1 [structural formula $(\alpha^1\beta)_2$], Hp 2-1 [$(\alpha^1\beta)_2 + (\alpha^2\beta)_n$] and Hp 2-2 [$(\alpha^2\beta)_n$], are genetically determined by a polymorphism with two alleles: Hp-1 and Hp-2. This polymorphism results in variants in alpha-chains: alpha-1 (8.9 kDa) and alpha-2 (16 kDa)[18]. The beta-chain (40 kDa) is identical in all Hp phenotypes. One explanation for an up-regulation of the alpha-2-chain would be a higher total Hp concentration, however, total Hp concentration did not differ significantly between our two lacunar groups. Another explanation could be that the multimeric Hp-2 proteins are larger sized in type I. This was not further explored. Thirdly, it could represent a higher Hp-2 allele frequency. Indeed Hp phenotypic analysis showed a higher Hp-2 allele frequency in type I. We conclude that the up-regulation of the alpha-2-chain in type I compared to type II is (partly) related to a higher Hp-2 allele frequency in the former. Yet, in contrast to a reference population[18], in both lacunar stroke groups Hp-1 outweighed Hp-2 allele frequency.

This brings in the second important finding of our study: Hp-1 is associated with cerebral SVD. Understanding the genetic basis of lacunar stroke may help clarifying

the aetiology and pathogenesis. However, knowledge about genetic factors modulating differences in lacunar stroke types is limited. Our study brings in a new candidate gene. The association between Hp-1 and SVD can be viewed in just a quantitative way, given the even higher Hp-1 allele frequency in type II than type I (although not significantly, due to small sample groups), suggesting a role for Hp-1 in developing more extensive disease. However, based on the pathology work of Fisher and Lammie, we hypothesized earlier that the two lacunar types that we distinguish on neuro-imaging and clinical grounds, represent distinct vascular pathologies, namely microatheromatosis and lipohyalinosis (also called arteriolosclerosis)[1,2,4,23]. The influence of the Hp phenotype may thus be relevant in predisposing to one type of small vessel pathology more than the other.

The mechanism underlying the association between Hp phenotype and SVD is speculative but could be related to blood pressure regulation. Hp levels have been associated with systolic blood pressure[21]. Weinberger et al. reported a relationship between Hp-1 and sodium-sensitive hypertension[24]. A more recent study by Depypere et al. associated Hp-1 with more severe hypertension in preeclamptic women[25]. Hypertension is an important risk factor for both lacunar stroke and WML, whereas multiple silent lacunar lesions are more often associated with hypertension than single lacunar lesions[2]. Hypertension was equally present in both our patient groups. However, there are indications that some other characteristics of the 24-hour blood pressure profile, such as nocturnal dipping, may be more important in small vessel pathogenesis than just the label “hypertension”, which is defined at an arbitrarily fixed value[26].

It was suggested that blood-brain barrier failure with leakage of plasma components into brain tissue might be an early stage of the process that ultimately leads to lacunar infarcts and WML[27]. Hp-1 passes the blood-brain barrier, while Hp-2 may pass to a far less extent due to its larger size. In barrier dysfunction the passage is increased[28]. Hp phenotype may play a role in determining the severity of SVD once a barrier defect has developed.

As opposed to our findings, studies on Hp polymorphism in extracerebral large vessel atherosclerotic disease show Hp-2 to be a risk factor in developing peripheral vascular disease and coronary artery disease[29,30]. In the Strong Heart Study Hp-2 was a strong predictor for cardiovascular disease, including stroke, in diabetic patients[31]. However, lacunar strokes were not separately distinguished. Hp polymorphism has also been studied in the development of extracerebral small vessel vasculopathy: the Hp-1 allele may protect against nephropathy and retinopathy in diabetic patients[32,33]. As the distribution of the Hp-2 protein in extravascular fluids is restricted by its larger molecular mass, its antioxidative capacity is lower[18].

A drawback of our study is the small sample size of 16 patients. However, one needs to remind that the study was not designed primarily with the intent to study polymorphisms; haptoglobin phenotypic analysis arose from the results of the

SELDI-TOF-MS analysis. The small sample size followed from the strictly chosen predefined inclusion criteria. In many patients MR imaging shows some silent lesions that are sometimes difficult to discern from dilated perivascular spaces. To increase the chance of finding a difference in protein expression profile we avoided this in-between area and included only the extreme ends of the spectrum: those with just a single symptomatic versus those with multiple concomitant silent lesions. Because of its possible confounding influence, we also excluded patients with coexisting large vessel disease. Although some degree of subclinical vascular disease may still have been present, we do not expect it to be overrepresented in one of the groups.

In conclusion, the SELDI-TOF-MS technique appeared to be useful to detect differences in protein expression between two lacunar stroke types which leads to new ideas about cerebral small vessel pathogenesis and its genetic basis. If a larger study would confirm our results, the association between Hp-1 and lacunar stroke signifies a new candidate gene involved in cerebral SVD etiology. The trend for a difference in Hp-1 association between two lacunar stroke types could be a reflection of a difference in underlying vascular pathology.

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