Determination of Homocysteine in Cerebrospinal Fluid as an Indicator for Surgery Treatment in Patients with Hydrocephalus

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Short title: Homocysteine in cerebrospinal fluid at hydrocephalus

Summary

Increased homocysteine levels in serum are typical features of neurodegenerative brain diseases including hydrocephalus. The most frequent therapeutic approach consists of the insertion of a shunt, connecting the brain ventricles to an alternative drainage site. To decide whether the patient should undergo this, the lumbar drainage test is usually carried out to distinguish patients who can benefit from the shunt insertion. In searching for other potential biochemical markers for shunt indication we determined homocysteine levels in CSF during the lumbar drainage test.

Homocysteine in CSF was measured during the 5-day lumbar drainage test in 27 patients with normal-pressure hydrocephalus (NPH) and in 25 patients with excluded hydrocephalus. A novelized gas chromatography method with flame ionization detection (GC-FID) was developed and evaluated.

During the first two days of lumbar drainage, the levels of CSF homocysteine in NPH patients were significantly higher compared to the controls, while on the fifth day, the homocysteine levels in patients with hydrocephalus reached the level of controls.

Determination of CSF homocysteine in patients with confirmed or suspected hydrocephalus may serve as an independent marker for deciding on their further treatment strategy.

Keywords: Homocysteine; cerebrospinal fluid; hydrocephalus; shunt.

Introduction

One of the common features of neurodegenerative brain diseases such as Alzheimer´s and other dementias, Parkinsonism or stroke, is the increased formation of homocysteine (HCy) to the detriment of physiological methionine metabolism. Homocysteine metabolism depends on folate, vitamin B12 and vitamin B6 and therefore its concentrations in body fluids are increased under the latter vitamins deficiency (Stanger *et al.* 2009). Homocysteine was measured in blood plasma, urine and also in cerebrospinal fluid (CSF). Elevated HCy in CSF follows its plasma levels in above-mentioned neurodegenerative diseases and was recommended as a biomarker not only for their diagnostics, but also for the monitoring of their treatment. The time course of HCy changes may predict the decline of cognitive functions in the elderly as recorded by memory scores (Herrmann and Obeid 2011; Obeid and Herrmann 2006).

In addition, HCy itself is neurotoxic due to its pleiotropic actions on nervous system, as reviewed in (Obeid and Herrmann 2006). In brief, HCy and its oxidative product homocysteinic acid interact as agonist with glutamate receptors, namely its NMDA subtype through its binding to the glutamate site and, at the same time, by blocking the glycine site, both resulting in an increase of calcium influx. It enhances oxidative stress and the formation of reactive oxygen species. Its competition for the methyl group from its donor S-adenosyl methionine leads to impairment of methylation systems, resulting in DNA hypomethylation. Reduced methyl group availability may cause brain white matter damage (Rossi *et al.* 2001). HCy also accelerates dementia by stimulating amyloid-beta deposition in the brain, leading to decreased CSF levels of this protein (Obeid and Herrmann 2006).

Hydrocephalus is also a severe neurodegenerative brain disease. This is generally the result of an imbalance between the formation and resorption of CSF. We usually distinguish non-obstructive hydrocephalus caused by impaired cerebrospinal fluid resorption in the absence of CSF-flow obstruction, while the obstructive form is a result of a CSF-flow obstruction ultimately preventing CSF from flowing into the subarachnoid space; for a review see e.g. (Tanaka *et al.* 2009). The most frequent therapeutic approach to hydrocephalus is surgical treatment, consisting mostly of a surgical insertion of a shunt that connects the ventricles of the brain to an alternative drainage site, usually the abdominal cavity (Iseki *et al.* 2009). The patient´s condition improves and he or she attends the hospital for periodic controls, at which CSF can be easily and repeatedly collected. Unfortunately, the effect of the operation is not sustainable and sooner or later many of the patients develop dementia, mostly of Alzheimer´s type (Tanaka et al. 2009).

The diagnosis of hydrocephalus is difficult, usually based on history, clinical testing and imaging methods (CT, ultrasound, NMR). Symptoms are difficult to distinguish from Alzheimer's disease and Parkinson's disease, typical manifestation of disease – Hakim trias – gait disturbance, urinary incontinence and dementia development. Hydrocephalus is one of the most misdiagnosed diseases. The worldwide prevalence is still not clear yet. The most recent studies show that the prevalence of hydrocephalus differs between regions. Two studies from Japan presented a 1.4% prevalence for patients over 65 years (Tanaka et al. 2009) and 0.51% for patients over 61 years (Iseki et al. 2009). A study from Norway published the prevalence 0.18% and 0.93% for patients 70–79 and over 79 years, respectively. These results also show that only 20% of patients are treated correctly (Brean and Eide 2008; Brean *et al.* 2009).

To decide whether shunt insertion is appropriate for a patient, dynamic liquor tests (lumbar drainage test or lumbar infusion test) are usually used. A principle for the lumbar drainage test is the continual lowering of CSF volume via the lumbar drainage during 3 – 5 days (Haan and Thomeer 1988; Marmarou *et al.* 2005b). With this test we can evaluate patients who can benefit from the shunt insertion. In patients with hydrocephalus, the lumbar drainage simulates the shunt ingestion and results in amelioration of their stage. (Marmarou *et al.* 2005a). Patients without hydrocephalus do not show amelioration of their state during the lumbar drainage.

Various biochemical markers in blood plasma as well as in the CSF were therefore searched to predict the fate of the patients, some of which seem to be promising, but further studies are needed (Sosvorová *et al.* 2012; Tarnaris *et al.* 2011; Tarnaris *et al.* 2009a; Tarnaris *et al.* 2009b). With respect to the above-mentioned alterations in neurodegenerative brain diseases, a potential biomarker of the patient´s development after surgery may be HCy. Indeed, increased homocysteinemia as well as homocysteinuria were repeatedly found in patients with hydrocephalus (Baethmann *et al.* 2000; Biancheri *et al.* 2001; Cerbo *et al.* 2010; Rossi et al. 2001), but, to the best of our knowledge, not in CSF.

We are presenting here the first results of HCy determination in CSF in a group of patients with hydrocephalus indicated for the shunt insertion, and in control subjects without diagnosed hydrocephalus.

For this purpose, a novelized GC-FID method for HCy determination in CSF was developed and evaluated.

Materials and Methods

Subjects

The patient group consisted of 10 males and 17 females with non-obstructive idiopathic normal-pressure hydrocephalus diagnosed on the basis of a combination of NMR imaging and a dynamic liquor test. Cerebrospinal fluid was collected during a five-day lumbar drainage test. The shunt was introduced to all the patients diagnosed with hydrocephalus after the lumbar drainage test was finished. The shunt implementation led to an improvement of the all Hakim trias symptoms in all patients. The control group (9 males and 16 females) consisted of patients tested for suspected hydrocephalus, in which, however, this diagnosis was excluded on the basis of the above-mentioned combination of NMR and the dynamic liquor test. They underwent cerebrospinal fluid collections as patients with diagnosed hydrocephalus. Samples were collected in plastic tubes, subsequently frozen and stored at -79°C. The protocol was approved by the Ethical Committee of the Institute of Endocrinology. Informed and written consent with the use of biological materials for research reasons was obtained from all patients participating to the project.

Chemicals and solutions

Homocystine standard, dithiothreitol, pyridine, p-fluoro-DL-phenylalanine, ethyl chloroformate, isooctane, ethanol, chloroform, sodium chloride, hydrochloric acid and potassium oxalate monohydrate were purchased from Sigma-Aldrich (St. Louis, USA). 50µL Sorbent tips for physiological amino acid profiling or protein hydrolyzates were supplied by Phenomenex (Torrance, CA, USA). PD (positive displacement) tips of 0.5 and 1.25 mL volume were obtained from Brand (Wertheim, Germany). Adjustable 100 µL transfer-pettor pipettes with glass capillary were supplied by Merck AG (Darmstadt, Germany). Tapered

polypropylene (PP) 1.1 mL reaction vials came from Continental Laboratories (San Diego, CA, USA).

Working solutions for the reduction and derivatization steps were prepared as follows: The concentration of p-fluoro-DL-phenylalanine in the stock solution of internal standard was 20 mmol/l. The reducing solution, i.e. 50 mmol/l dithiothreitol (0.8%) plus 10 mmol/l (0.2%) potassium oxalate, was mixed daily with an internal standard stock solution in a ratio of 49:1. The eluting medium was a mixture of 1% aqueous sodium chloride, ethanol and pyridine in a ratio of 75:40:10, the reactive medium consisted of isooctane, chloroform and ECF in a ratio of 12:4:1.

Sample preparation

Samples were processed according to the Husek *et. al* method for determining plasma homocysteine (Hušek *et al.* 2003) with minor modifications. 500 µL of cerebrospinal fluid and 100 µL of the reducing solution (spiked with internal standard) were added into the vial. The solution was gently mixed and left to react for 2-3 minutes. A sorbent tip was attached to a 1.25 mL PD tip and the fluid content was sucked slowly through the exchanger bed. After passing the fluid through the sorbent completely, 150 µL of water-ethanol (2:1) were added and passed through the sorbent bed. The liquid was completely drained from the sorbent tip, the PD tip was removed and its content discarded. A 0.5 mL PD tip was then attached to a sorbent tip. After adding 150 µL of the eluting medium into the same working vial, the fluid was sucked into the resin bed until it reached the filter, and the resin was then transferred into the vial. Subsequently, 150 µL of the reactive medium was added into the vial. The content was mixed by vortex for about 15 seconds, until the upper organic layer became clean. Following the pyridine-scavenge step accomplished by addition of 100µL 1M

hydrochloric acid and brief vortexing, an aliquot of the upper organic phase was transferred into an autosampler vial using a microcapillary tip.

GC-FID analysis

The GC analysis employed AutoSystem XL with FID detector from Perkin Elmer (Waltham, MA, USA). A ZB-AAA fused silica capillary column 10m x 0.25 mm from Phenomenex, (Torrance, CA, USA) was used. The chromatography separation runs with a temperature program of 150-300°C at 25°C/min under a constant velocity mode. The injector tempered at 250°C FID temperature was 320°C. Split injection (2 µL) was performed (approximate split ratio 1:10). A Siltek-deactivated split liner (I.D. 4mm) with Siltek wool supplied by Restek (Bellefonte, PA, USA) was employed. Clarity software version 3.0 DataApex (Prague, Czech Republic) was applied for the evaluation of chromatographic data.

Quantification

Homocysteine was quantified by means of calibration curves made on the basis of known concentrations in the mixtures of analyzed standard with a constant level of the internal standard p-fluoro-DL-phenylalanine (80 µmol/l). The calibration lines were obtained by plotting the response factor (analyte area/internal standard area) against the concentration of the calibration standard. The values were corrected for procedural losses according to yields of internal standard.

Statistical evaluation of the data

The relationships between dependent variables and effects of hydrocephalus and stage of the experiment were evaluated using a repeated measures of the ANOVA model consisting

of factors Status (patients with NPH vs. controls), Stage (1^{st}, 2^{nd}, 3^{rd}, 4^{th} and 5^{th} day of lumbar drainage test), Subject (explaining inter-individual variability) and Status × Stage interaction. The ANOVA model was followed by least significant difference (LSD) multiple comparisons. To eliminate skewed data distribution and heteroscedasticity, the original data were transformed to a Gaussian distribution before further processing using a power transformation. Statistical software Statgraphics Centurion, version XV from Statpoint Inc. (Herndon, Virginia, USA) was used for data processing.

Results

Evaluation of the method

Matrix effect

When the standards in pure solvents were used for the calibration, the actual concentrations could be affected. To assess these matrix effects, the calibration curves were constructed both for the solvent standard solution and matrix-matched standards. Matrix-matched standards were prepared from cerebrospinal fluid with a low concentration of homocysteine. When compared with the calibration curve slopes, no significant matrix effects were found.

Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) were defined as concentrations at which the signal-to-noise ratio (S/N) were 3 and 10, respectively. They were determined by repetitive analyses (n=6) of low levels of solvent solutions. The values of LOD and LOQ for homocysteine were in the range of 0.08 and 0.28 µmol/l, respectively.

Recovery of the method

The recovery of the analyte was determined by spiking pooled cerebrospinal fluid samples with six concentrations of homocysteine, 0.3 µmol/l, 0.5 µmol/l, 1 µmol/l, 1.5 µmol/l, 2 µmol/l and 3 µmol/l. The spiked samples were processed in the same way as the other samples. As shown in Table 1, the recovery of homocysteine in cerebrospinal fluid ranged from 84% to 108%.

Repeatability

The repeatability, expressed as a relative standard deviation (RSD), was determined by repetitive (n=5) sample analyses. The intra-assay and inter-assay coefficients of variation ranged from 2.6 – 9.9% and 3.6 – 5.9%, respectively. The analytical criteria (validation parameters) of the method are summarized in Table 2.

Homocysteine levels in cerebrospinal fluid

Repeated measures ANOVA ($p<0.0001$; Figure 1) demonstrates differences between means CSF homocysteine levels in patients with NPH and controls obtained in the individual days of 5-day lumbar drainage test. The interaction effect was significant ($p=0.0396$) indicating opposite pattern of CSF homocysteine levels with time between both groups of subjects in individual stages (lumbar drainage test day). As may be seen, the levels of CSF homocysteine are on the first day ($p< 0.01$) and second day ($p<0.05$) of lumbar drainage considerably higher in patients with NPH compared to the controls. During following days, the levels in patients with hydrocephalus decreased continuously and on the fifth day of lumbar

drainage, reached the levels of controls, which did not change significantly with time. The decrease in CSF homocysteine in patients with hydrocephalus reflects the amelioration of their state.

The individual changes of homocysteine levels during succeeding lumbar drainages are shown in Figure 2.

In Table 3 several data (means, medians, standards deviations) of homocysteine concentrations are provided from patients with hydrocephalus indicated to the shunt insertion and in control group on the first and on the fifth day of lumbar drainage test.

Discussion

The aim of our study was to test homocysteine as an independent laboratory marker that would be helpful in the neurosurgeon´s decisions concerning the indication for shunt insertion.

Several methods has been developed to determine homocysteine in CSF, as a laboratory diagnostic tool for status assessment of various neuropsychiatric diseases such as Alzheimer´s and other dementias, Parkinson disease or multiple sclerosis (Linnebank *et al.* 2010; Smith *et al.* 2012; Valentino *et al.* 2010), but, so far, not for hydrocephalus. An important issue for the clinician concerning the hydrocephalus patient is to decide whether to insert a shunt or, in other words, if this treatment would bring him/her a benefit. The usual approach consists of a lumbar drainage test followed by clinical evaluation of the patient´s state. As such, however, the test may not be fully objective. In this study we tested whether determining homocysteine in CSF from these patients could serve as a potential marker for this purposes. Therefore we have developed and evaluated a novelized GC

method tailored for assessment of small changes in HCy concentrations in CSF during the lumbar drainage. The method fulfills the analytical criteria and we also plan to use it for HCy determination in samples collected after longer period from shunt insertion.

As demonstrated in Table 3 and on Figures 1 and 2, the lumbar drainage led to a decrease in HCy concentration and, in all patients diagnosed with hydrocephalus, decreasing levels corresponded to amelioration of the clinical state, while no changes were observed in the controls. On the first day of the lumbar drainage test, the levels of homocysteine in cerebrospinal fluid were significantly higher in patients with hydrocephalus compared to controls. On the basis of these results, we can suggest the level 1.25 μmol/l as the border line for selective identification of patients who will have benefit from shunt implantation.

These first results show that determination of HCy in CSF patients with confirmed or suspected hydrocephalus may indeed serve as a possible independent marker for an important decision about their further treatment strategy.

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Legend to Figures and Tables

Fig. 1. A schematic graph of CSF homocysteine levels in patients with hydrocephalus (n=27) and controls without diagnosed hydrocephalus (n=25). The mean values from each patient from the 1st to 5th lumbar drainage (LD) were calculated and the obtained data were further compared by ANOVA test. Triangles: patients with hydrocephalus, circles: controls. The figure shows average values from both groups of patients ± STD in individual days of 5-day lumbar drainage test.

Fig. 2. Individual values of CSF homocysteine levels in patients and controls during lumbar drainage (LD). Triangles show individual values from patients with hydrocephalus, circles from controls. Full lines show average values ± STD from patients with hydrocephalus, open lines from controls.

Tab. 1. The recovery study of homocysteine in cerebrospinal fluid performed by the gas chromatography with flame ionization detector (GC-FID).

Tab. 2. The intra-assay and inter-assay coefficients of variability (CV) for homocysteine in cerebrospinal fluid measured by GC-FID method.

Tab. 3. Means, standard deviations and medians of homocysteine in CSF during individual days of the lumbar drainage test in patients with hydrocephalus and controls.

Fig. 1.

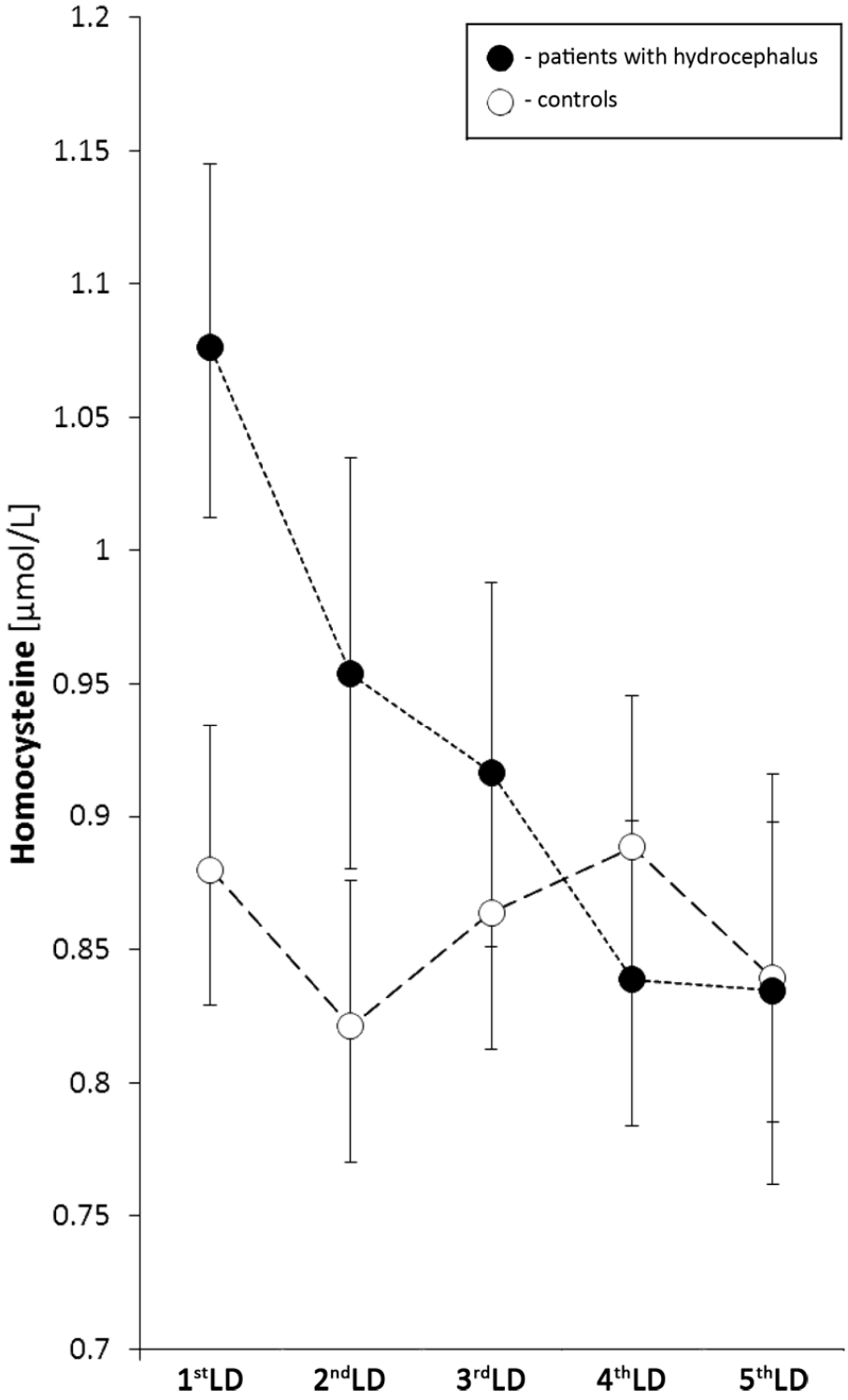
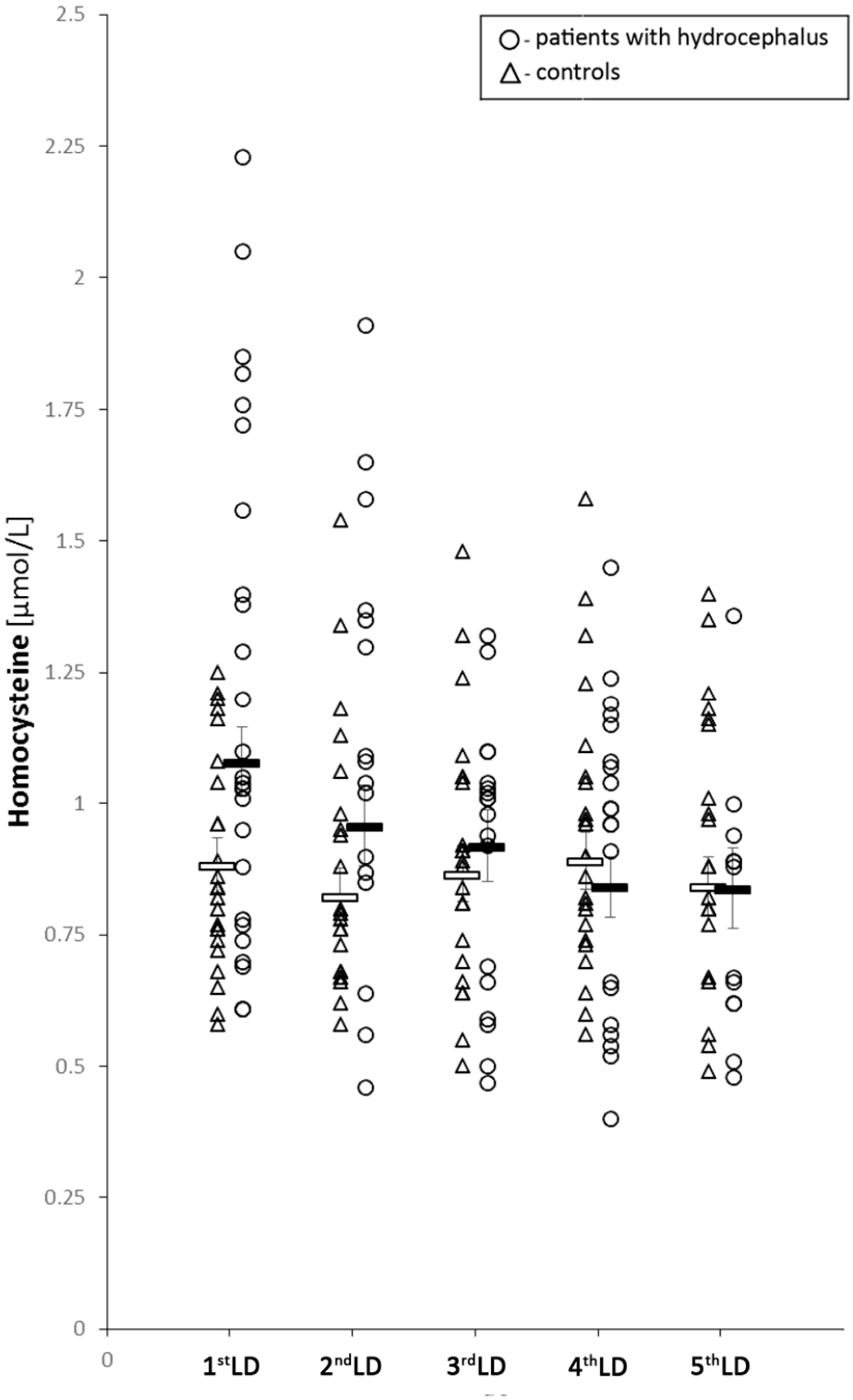

- patients with hydrocephalus
- controls
Homocysteine [µmol/L]
1.2
1.15
1.1
1.05
1
0.95
0.9
0.85
0.8
0.75
0.7
1stLD
2ndLD
3rdLD
4thLD
5thLD

Fig. 2.


○ - patients with hydrocephalus
△ - controls
Homocysteine [µmol/L]
2.5
2.25
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
0
1stLD
2ndLD
3rdLD
4thLD
5thLD

Tab. 1.

Homocystine added (µmol/l)	Recovery (%)
0.3	100.7
0.5	96.0
1	108.0
1.5	83.6
2	87.0
3	98.1

Tab. 2.

Sample	Intra-assay		Inter-assay	
	Mean (µmol/l)	CV (%)	Mean (µmol/l)	CV (%)
1	0.454	9.3	0.512	5.7
2	0.934	7.7	0.928	4.5
3	1.534	4.8	1.452	3.6
4	1.708	4.9	1.858	5.9
5	2.194	3.7	2.3	3.7

Tab. 3.

Day of lumbar drainage	Homocysteine in cerebrospinal fluid (µmol/l)					
	Patients with hydrocephalus			Controls		
	Mean	STD	Median	Mean	STD	Median
1st	1.12	0.39	1.04	0.89	0.20	0.84
2nd	1.05	0.34	1.04	0.85	0.20	0.79
3rd	0.90	0.25	1.00	0.89	0.24	0.89
4th	0.89	0.28	0.96	0.90	0.22	0.88
5th	0.79	0.24	0.78	0.88	0.24	0.85