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Homovanillic acid in CSF of mild stage Parkinson's disease patients correlates with motor impairment

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ABSTRACT

In Parkinson's disease (PD), several efforts have been spent in order to find biochemical parameters able to identify the progression of the pathological processes at the basis of the disease. It is already known that advanced PD patients manifesting dyskinesia are featured by the high homovanillic acid (HVA)/dopamine (DA) ratio, suggesting the increased turnover of DA in these patients. Less clear is whether similar changes affect mild and moderate stages of the disease (between 1 and 2.5 of Hoehn & Yahr –H&Y- stage). Hence, here we tested whether cerebrospinal fluid (CSF) concentrations of DA and its major metabolites, either 3,4-dihydroxyphenylacetic acid (DOPAC) or HVA, correlate with motor performance in mild and moderate PD patients. CSF samples were collected after 2 days of anti-PD drugs washout, via lumbar puncture (LP) performed 130 minutes following administration of oral levodopa (LD) dose (200 mg). LP timing was determined in light of our previous tests clarifying that 2 hours after oral LD administration CSF DA concentration reaches a plateau, which was un-respective of PD stage or duration. DA, DOPAC and HVA were assayed by high performance liquid chromatography in a group of 19 patients, distributed in two groups on the basis of the H&Y stage with a cut-off of 1.5. In these PD patients, HVA was correlated with DOPAC (R=0,56, $p < 0,01$) and both HVA and DOPAC CSF levels increased in parallel with the motor impairment. More importantly, HVA correlated with motor impairment measured by the Unified Parkinson's Disease Score –III (UPDRS) (R = 0.61; $p<0.0001$).

The present findings showed the early alteration of the DA pre-synaptic machinery, as documented by the progressive increase of CSF HVA concentrations, which also correlated with PD motor impairment. Therefore, we suggest the potential use of measuring the CSF HVA level as a possible biomarker of PD stage changes in order to monitor the effectiveness of PD-modifying pharmacological therapies.

Highlights

- Homovanilic acid (HVA) concentration increases in CSF along with the disease progression, also in the early and moderate stages of the disease.

- Increased CSF HVA levels are related to the degree of motor impairment in PD patients.

- CSF HVA levels correlated with CSF DOPAC levels, but not with CSF DA concentration.

INTRODUCTION

Analysis of catecholamine levels in the cerebrospinal fluid (CSF) of Parkinson's disease (PD) patients began over thirty years ago, when the DATATOP trial demonstrated a correlation between reduced levels of endogenous dopamine (DA) and motor impairment (Ebinger et al., 1987; Lewitt et al., 1992). Unfortunately, despite several investigations aimed to identify CSF biomarkers reflecting the DA denervation and/or compensatory mechanisms (Goldstein et al., 2012), objective instruments capable of monitoring PD staging nowadays still lack.

An opinion leader in looking for advanced approaches as metabolomic analysis inferred that the "investigations of dopamine metabolism have been relatively uninformative" (Lewitt, 2012); however, the same author stated that "indexing homovanillic acid (HVA) concentration to that of the purine metabolite xanthine permitted differentiation of PD specimens from healthy controls ($p < 0.0016$)" (Lewitt et al., 2011). Metabolites of DA are produced along with DA degradation and are used to assess central DAergic function (Thiffault et al., 2003). Previous studies showed the reduction of CSF HVA levels in patients with PD (Abdo 2004; Mann et al., 1983). Nevertheless, the relationship between CSF HVA concentration and clinical disability or disease progression is so far unclear, since several studies reported different and conflicting results about the interplay between CSF HVA levels and disease duration (Chia et al., 1993; Gibson et al., 1985; Tohgi et al., 1990).

It has been documented that DA decreases early in the CSF of PD patients along with the disease progression (DATATOP). Indeed, the decrease of DA levels in the CSF occurs prior to the development of dyskinesia in PD patients. Moreover, a previous report by our group documented that advanced PD patients are affected by DA metabolism dysregulation, not only by decreased DA CSF levels (Lunardi et al., 2009). In fact, the increase of DA metabolism, featured by the higher HVA/DA ratio, has been related to the occurrence of dyskinesia in advanced PD patients (Lunardi et al., 2009). However, not only DAergic system but also serotoninergic, cholinergic, noradrenergic, opioid and other central neurotransmitter systems are impaired in PD pathology, which is actually

considered a multisystem degenerative disease (Brooks and Pavese, 2011). Although nigrostriatal DAergic denervation remains the main pathological hallmark of PD, neuropathological, biochemical and neuroimaging studies in PD patients have proved the occurrence of concomitant degeneration in non-dopaminergic circuitries, mainly serotonin, noradrenaline, and acetylcholine neuronal systems (Morrish et al., 1998; Liguori et al., 2015; Buddhala et al., 2015). Dysfunction of these neurotrasmitter systems could participate in the development of both motor and non-motor symptoms in PD patients (Brooks and Pavese, 2011). In particular, several neuropathological studies demonstrated the involvement of locus coeruleus (LC) in PD pathology (Mann and Yates, 1983; Ehringer and Hornykiewicz, 1998). LC dysfunction in PD patients has been clinically related to the occurrence of movement initiation and gait dysfunctions, since the LC, the major structure for brain synthesis of noradrenaline (NA), projects to the sub-thalamic nucleus thus negatively influencing the extrapiramidal network (Albares et al., 2015; for a recent review on the NA involvement in different neurodegenerative disease including PD consider Vermeiren et al., 2016 **).** On the other hand, also the serotonin circuitry inefficiency has been hypothesized representing the fitting partner of the dopaminergic deficit in PD, since the serotonergic transmission dysfunction is actually evident in PD pathology at all stages of disease. (Olivola et al., 2014; Qamhawi et al., 2015; Liguori et al., 2015).

Inconsistencies in chemical detection have discouraged the required investments in dosing DA and its metabolites in the CSF of PD patients. However, new investigations have recently fuelled the proposition to use CSF analysis as potential clue in understanding PD pathogenesis (Goldstein et al., 2014, Stefani et al., 2012). Moreover, the reliability of the modern chromatographic approach is refreshing the research on catecholamine and their metabolites in human fluids, such as CSF (Herbert et al., 2013; Ishibashi et al., 2010; Olivola et al., 2014; Van Dam et al., 2014; Stefani et al., 2015;). Accordingly, high performance liquid chromatography (HPLC) with electrochemical detection is a technique providing the simultaneous, accurate and precise measurement of DA and its major metabolites in CSF even on small samples.

Therefore, since clinical motor parameters are incomplete tools to monitor PD progression or to identify disease-modifying effects, the development of reliable approaches based on valid biochemical markers has recently received a great deal of attention in the field of clinical trials (Kazmerova et al., 2013; Jenner and Langston, 2011).

On these bases, the previous paper by our group (Lunardi et al., 2009) was primarily focused in understanding the CSF changes of DA and its metabolites (HVA and 3,4-dihydroxyphenylacetic acid -DOPAC) in the advanced phases of PD. In the present study we aimed to investigate whether CSF concentration of DA, DOPAC, and HVA correlates with motor performance, as quantified by Unified Parkinson's Disease Rating Scale-III (Fahn et al 1987; UPDRS-III), in mild PD stages, defined by the Hoehn & Yahr (H&Y) stages between 1 and 2.5 (Hoen and Yhar 1967).

In particular, the present study takes advantage from the un-published findings of the previous one (Lunardi et al., 2009, for specific methods see legend fig. 1). This investigation in fact allowed understanding the basic kinetics of DA after levodopa (LD) 200 mg oral administration (Figs 1 and 2). It was determined that the CSF DA concentration reaches a plateau at 90-150 minutes after administration, despite inter-patient variability (Fig 1). To note, no differences in CSF DA levels after oral LP administration between untreated and moderately advanced non-dyskinetic PD patients were observed, thus suggesting that CSF DA peaks are not influenced significantly by therapeutic history and disease duration (Fig 1). More importantly for the present study, HVA concentrations, despite its rather complex kinetics, variable from patient to patient, reached a sort of "plateau" (at least 50% of the normalized HVA mean concentration), detectable at 120-150 min (Fig. 2).

Material and Methods

The present study, as presented in the introduction, is based on the previous published study protocol approved by Ethical Committee of the University of Rome "Tor Vergata", and designed to

assess CSF DA kinetics in PD patients by means of repeated consecutive samples after a prolonged pharmacological washout - 2 weeks (data presented in Figures 1 and 2) (Lunardi et al., 2009).

On the basis of this previous experience, which suggested a consistent plateau of CSF HVA concentration around 120 min following oral LP, we decided to perform LP 120-130 min after LD oral administration in order to catch the more accurate CSF concentrations of DA and its metabolites in PD patients.

Here, we enroll a population of PD patients referred to our Movement Disorder Centre, at the University of Rome "Tor Vergata". All patients received the diagnosis of idiopathic PD according to the UK Parkinson's Disease Society Brain Bank criteria (Hughes et al., 1992) PD patients underwent a standard protocol counting neurological examination, neuropsychological assessment, blood screening, brain neuroimaging (computerized tomography and/or magnetic resonance imaging) and lumbar puncture (LP). Motor severity and disease stage were assessed using the motor examination section of the Unified Parkinson's Disease Rating Scale (UPDRS-III) (Fahn et al 1987) and the modified H&Y staging (Hoen and Yhar 1967). PD patients have to meet the following inclusion criteria: i) being treated with a monoamine oxidase (MAO) inhibitor (MAO-I) plus LD and/or immediate release dopamine-agonists therapy; ii) manifesting a disease severity equal or below 2,5 at the H&Y staging; iii) an improvement ≥30% at the UPDRS-III to the acute challenge of fasting oral dose LD/carbidopa (Merello et al., 2002). Exclusion criteria for PD patients were: i) cognitive impairment and dementia; ii) relevant systemic diseases; iii) abnormal CSF cell count.

In particular, at the time of enrollment patients' clinical disability was quantified using the motor examination section of the UPDRS. LD acute dose was set at 200 mg to minimize the risk of generating disable dyskinesia during lumbar puncture (LP).

All participants were adequately informed on all aspects of the study and gave written consent to the pharmacological protocol and the CSF analysis, after receiving full disclosure of study purposes and risks.

LP was performed in all the PD patients included 48 hours after immediate release dopamine-agonists treatment stop. However, although we are aware that mono-amino-oxidase inhibitors (MAO-I) are known to decrease CSF levels of DOPAC (Eisenhofer et al., 1986), we cannot hypothesize a drug suspension of three weeks (necessary for nullify the influence of MAO-I on CSF DOPAC levels) for ethical reasons, and then all patients were under MAO-I during the protocol procedures. Moreover, the last dose of LD therapy was administered the night before LP (CAPIT). CSF samples were obtained by LP performed in decubit-position, between 8:00 and 9:00 AM after overnight fasting, using an a-traumatic needle. For LP protocol see some of our previous contributions (Lunardi et al., 2009; Olivola et al., 2014; Stefani et al., 2015). As previously suggested, LP was done 130 minutes after having administered a fasting oral dose LD/carbidopa 200/50 mg. The first 4 ml sample of CSF was used for routine analyses. The following 5th and 6th ml were protected from light for catecholamine analysis, with the respect to the concentration gradient (Lewitt et al., 1992). 200 µl aliquots were collected, acidified by adding 4 µL of perchloric acid (0.01 M) and immediately frozen at -80 °C for subsequent HPLC analysis coupled with electrochemical detection. The chromatographic method (Alexys monoamine analyzer-Decade II) was optimised for small sample volumes (5-12 µL injection) with a detection limit below 50 pmol/L for the biogenic amines (see legend Fig 1). Linear calibration graphs of the peak-area ratio of each compound to the internal standard versus the concentration of the compound studied were constructed. By mean of a dual loop 10-port valve a single injection was loaded on two LC-flow paths simultaneously. For separation of the components of interest, a suitable combination of column (type Antec ALF -115 column, 150x1.0mm ID, 3 µm C18 and ALF-105 column, 50x1.00mm ID 3 µm C18) and mobile phase composition was used. The composition of mobile phase for column 1 was performed using the following: 50 mM phosphoric acid, 8 mM NaCl, 0.1 EDTA, 12.5% methanol, 500 mg/L OSA and pH 6.0 (to note, column 1 merely detected DA, noradrenaline and serotonin); instead, the mobile phase composition for column 2, which is devoted for measuring DA metabolites and, in particular, HVA and DOPAC, is composed by: 50 mM

phosphoric acid 8 mM, NaCl, 0.1 EDTA, 50 mM citric acid, 10 % methanol 500 mg/L OSA and pH 3.25 (analyses were at a standard detection potential around 550-560 mV).

Statistical analysis

For the statistical analysis, we used commercial software (Statistica 10.0 program; Statsoft Inc, Tulsa, OK, USA).

For each biochemical variable, both median and mean values are reported. Median values of ratios are reported. Since data were not normally distributed we used non-parametric statistics. Correlations between CSF and clinical data were analyzed by the Spearman's test. Thereafter, the PD patients group was dichotomized on the basis of the H&Y stage in two subgroups with the cut-off set at 1.5. Therefore, the two subgroups were compared by using the Mann-Whitney *U* test. P value < 0.05 was considered to be statistically significant.

Results

Twenty-one PD patients were included in the study. We analyzed CSF data from 19 out of 21 PD patients, since 2 patients did not show at least the 30% improvement of the UPDRS-III score and thus were excluded. Patients' demographic and clinical data are summarized in Table 1.

The mean CSF concentration of DA, HVA and DOPAC following LD/carbidopa 200/50 mg in the whole group were, respectively (in nmoles/L): 0.44 ($\pm$ 0.38); 11.91 ($\pm$ 8.27); 2.03 ($\pm$ 1.83).

CSF HVA levels correlated with DOPAC CSF levels (R=0.56, p<0.01), but not with DA CSF concentration. Also DOPAC CSF levels did not correlated with DA CSF concentrations.

Considering the correlations between CSF and clinical data, we observed a significant positive correlation between UPDRS-III scores and CSF HVA concentrations (R= 0.61; p<0.0001, Figure 3). On the other hand, UPDRS-III scores did not correlate with DOPAC (R=0.05) or DA (R=0.31).

Moreover, CSF HVA, DA and DOPAC concentrations did not correlate with PD disease duration (R=0.22). However, UPDRS-III scores correlated with disease duration (R=0.39, p<0.05).

Dividing PD patients group into 2 subgroups based on H&Y stage (≤1.5 and >1.5 stage), we documented the significant increase of CSF HVA levels in PD patients showing a greater H&Y stage (>1.5 and <2.5, n=10) compared to PD patients with a H&Y stage ≤1.5 (n=8) (15.28±12.08 vs 5.75±2.16, p<0.05; Figure 4).

Discussion

This study showed that in PD patients CSF HVA levels correlate with motor impairment, as measured by the UPDRS motor score. This relationship, already evident in the advanced phases of the disease, is present also in the mild stages of PD pathology. In particular, we demonstrated that HVA trends in increasing its CSF concentrations from the early stages of the disease and along with the progression of PD pathology.

In PD, considerable efforts have been spent in seeking reliable markers of disease pathogenesis (Parnetti et al., 2013; Noyce et al., 2016). Up to now, the most reliable findings, based on the investigation of CSF Alzheimer's Disease biomarkers in PD patients, have suggested that a specific CSF pattern, characterized by the reduction of CSF beta-amyloid levels, correlates with future development of dementia [Siderowf et al., 2010, 2014]. The researches of other biomarkers have been inconsistent or debatable. Accordingly, studies focused on the investigation of CSF markers linking PD pathology progression to motor and non-motor symptoms have reported different and sometimes controversial results.

It is our contention that adequate biomarkers of disease stage would be extremely valuable, for monitoring disease progression and treatment efficacy. This issue is especially pertinent in attempts to identify "disease-modifying" strategies during relatively brief trials (adding some objective tolls to merely clinical, frequently subjective, evaluation scores). In addition, it is worth considering that the failure of several trials could be attributed, at least in part, to the initial enrollment of a heterogeneous populations of patients, apparently sharing similar motor features but affected by profoundly different impairment of the endogenous dopamine machinery.

As regards CSF biomarkers, the previous study conducted in our site found altered concentrations of DA metabolites in more advanced PD stages (Lunardi et al., 2009). In that study, we demonstrated that metabolite/DA ratios, evaluated from basal CSF samples collected in off-therapy condition and after a prolonged washout, was significantly higher in advanced and dyskinetic patients compared to untreated ones.

The present report demonstrates that significant changes in CSF DA metabolites, HVA in particular, may accompany also modest motor impairment in PD patients with relatively short disease duration, and manifesting neither fluctuations nor dyskinesia. To note, in this study, the LD test preceding the LP was performed in order to increase the sensitivity in detecting significant correlation between CSF data and modest motor changes.

The concentration here calculated: a) are different from "basal" levels (Ueda et al., 2001) and b) reflects (at 130 min) a first-round passage before subsequent accumulations. Therefore, the HVA "plateau" detectable at 120-130 min (Figures 1 and 2) may represent a reliable index of a first-round DA metabolism showed by any patient.

The correlation between CSF HVA and motor clinical scores, as here described, might be further validated with studies including CSF samples obtained several hours after LD intake, since HVA formation obeys to complex kinetics (in theory, a full scenario of HVA concentrations until full catabolism would require prolonged sampling up to seven hours, performed in the past as revealed in Figures 1 and 2, but considered not ethical nowadays, and thus not performed in our present study).

Some degree of caution is advisable, since "lumbar" CSF titles do not reflect closely the activity of the DA systems in the brain. However, we have analysed consistently only samples after the first 4 ml, in respect of the well-known rostro-caudal gradient (Lewitt et al., 1992); besides, CSF metabolites should represent central DAergic metabolism, and negligible appears the potential contribution of peripheral sources.

Moreover, our system revealed high sensitivity; besides, this is a pilot study and we wish to replicate the same test in the same subjects, acquiring two CSF titles along with the disease progression. So far, this sort of repeated samples is usually not permitted; however, few recent investigations utilized longitudinal measurements (Siderowf et al., 2010; Hall et al., 2015), obtained at baseline and after 3 years, in order to study if changes of CSF biomarkers correlate with motor progression.

A possible pitfall of our study regards the naturalistic design, the fact that patients were admitted on a consecutive base and, more critically, without a rather extended washout (as performed, for instance, by pivotal investigation in US or Italy, in which CSF samples had been collected following 2-5 or weeks of drug-suspension (Lewitt et al., 1992; Lunardi et al., 2009). The present washout procedure might be too short, since HVA levels might still be unpredictable or still high, despite two days washout, before the LD challenge. Another limitation is related to the ongoing treatment with MAO-I, which cannot be stopped prior to LP for ethical reasons. Nevertheless, if pharmacodynamics factors are playing such a relevant role in influencing our data, it would be quite surprising to detect, as here, such a significant correlation. To note, the CAPIT protocol (at least for LD) is the common regimen utilized as "worse-OFF" for several on-going trials. In addition, CAPIT (plus the 2 days withdrawal from "fast release" agonists) may keep low patients discomfort; otherwise, prolonged washout might actually introduce art-factual changes in catecholamine concentrations (placebo/lessebo factors see Mestre et al., 2015]. It is to note that patients were constantly under our supervision in order to avoid spontaneous rescue doses and to ensure nursery assistance, and that no patient was taking extended release DA-agonists.

We are aware that DA individual concentration, in any given patient, may vary profoundly in light of more or less controllable factors (craving for drug assumption blind to the scorer, peculiar dietary regimens). In this respect, the present study does not provide a complete determination of CSF DA concentration. Although this issue was not directly tested (by a blind assessment of, i.e., 3 days

versus 2-3 weeks washout) data presented in figure 1-2 show that a mean estimation of LD and HVA concentrations, following 2 hours from a single LD dose, is feasible. In other words, we are confident that the determination of metabolites concentration under LD challenge may somehow minimize the individual catabolic features.

Our data suggest that, when the DA machinery is challenged with LD, HVA concentration in CSF regains a significant correlation with motor impairment. Putative mechanisms include a likely compensatory change in DA turnover, as inferred from multi-tracer positron emission tomography showing up-regulation of DA synthesis and down-regulation of DA transporter in PD "early stages" (Nandhagopal et al., 2011) and/or a plausible hyperactivity of DA.

Conclusions

CSF levels of the DA metabolite HVA may be a suitable marker of PD stage. This finding, when validated in large cohort, may translate into quantitative endpoints in future trials of disease-modifying therapy. Of course, in a multisystem disease as PD, it is expected that a concomitant alteration of other biogenic amines occurs, as already inferred in patients afflicted by different types of dementia (Vermeiren et al., 2013). For the time being, if an altered concentration of metabolites and/or DA/HVA ratio is recognized along subtle disease progression, we might utilize those indexes as biomarkers to regroup patients as sharing a similar impairment of the DA machinery.

Acknowledgements

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Figure legends

Figure 1. Consistency of LD CSF peak in PD patients, independent from disease duration and severity.

Filled triangles = mean (n = 15) normalized LD concentrations in untreated PD patients; Filled squares = mean (n = 13) normalized LD concentration in moderately advanced – but not dyskinetic – " PD patients.

Each point represents a CSF sample (every 30 min - or 60 min after 3 hours-).

(These data belong to a pilot experimental protocol perfomed in late 2000s, as detailed in the methods section. Briefly, this study counted the assessment of DA, DOPAC, and HVA cconcentrations in CSF samples determined by HPLC analysis coupled with electrochemical detection (Model 5100A with a 5014B analytical cell, ESA, USA). In order to assess CSF, DOPAC and HVA levels, samples were loaded into a C18 reverse-phase column (Atlantis dC18 3 mm 4.6 _ 150 mm, Waters, Ireland); for mobile phases, see Lunardi et al.,2009). Detector gain was adjusted to obtain a detection limit of 50 pg/mL for DOPAC, whose concentration in CSF samples was markedly lower than that of HVA).

Figure 2. HVA CSF "plateau" in PD patients, independent from disease duration and severity.

Filled triangles = mean (n = 15) normalized HVA concentrations in untreated PD patients; Filled squares = mean (n = 13) normalized HVA concentration in moderately advanced – but not dyskinetic – " PD patients.

Each point represents a CSF sample (every 30 min - or 60 min after 3 hours-).

(These data belong to a pilot experimental protocol perfomed in late 2000s, as detailed in the methods section)

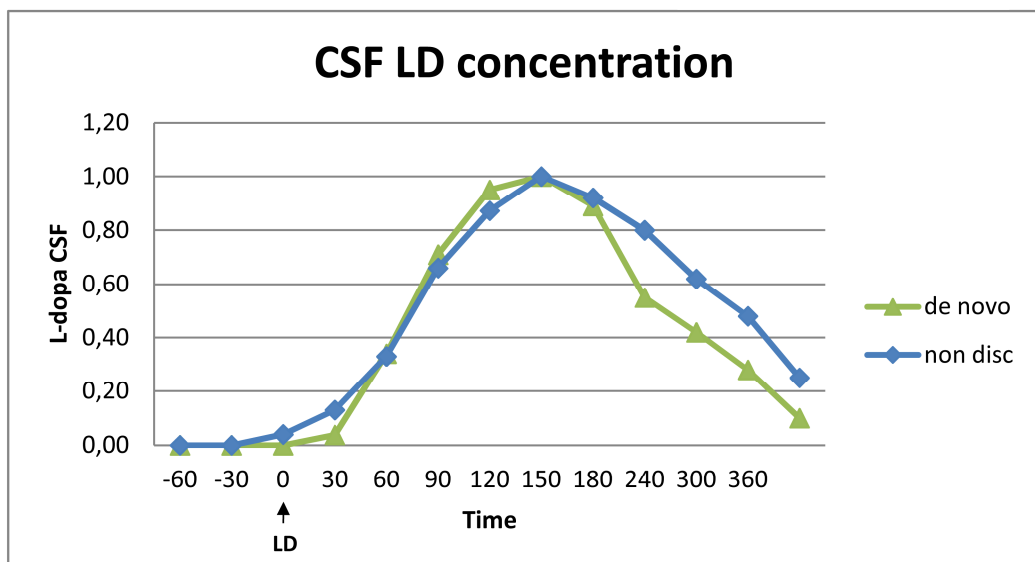**Figure 3. Correlation of CSF HVA with UPDRS-III score.**

CSF HVA concentration (nM/L) correlates with UPDRS-III score rated before LP (R=0.61). Data refer to 19 PD patients manifesting > 30% UPDRS amelioration following 200 mg LD.
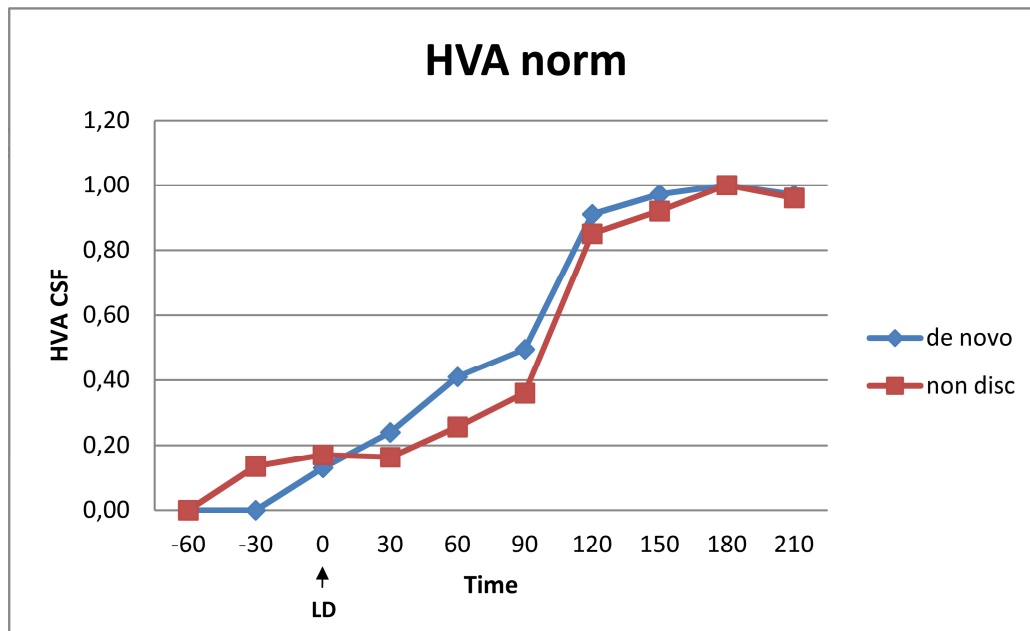
Figure 4. CSF HVA levels in patients divided by Hoehn & Yahr (H&Y) stage. Boxplots showing CSF HVA levels in PD patients subgroups (group 1, on the left, H&Y ≤ 1.5; group 2, on the right, H&Y > 1.5).
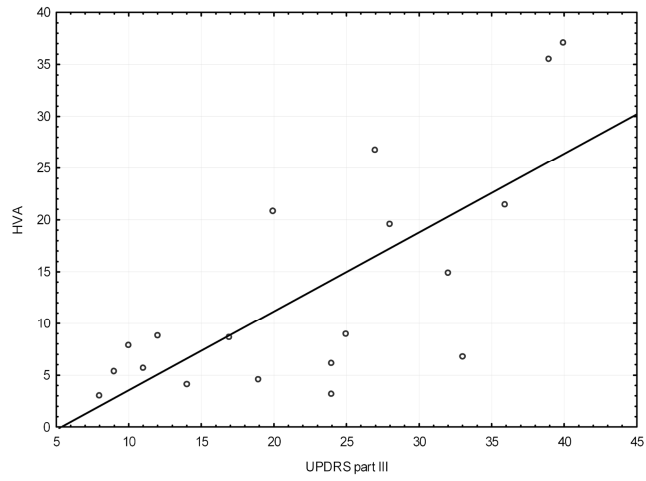
Table 1. Demographic and clinical data of PD patients
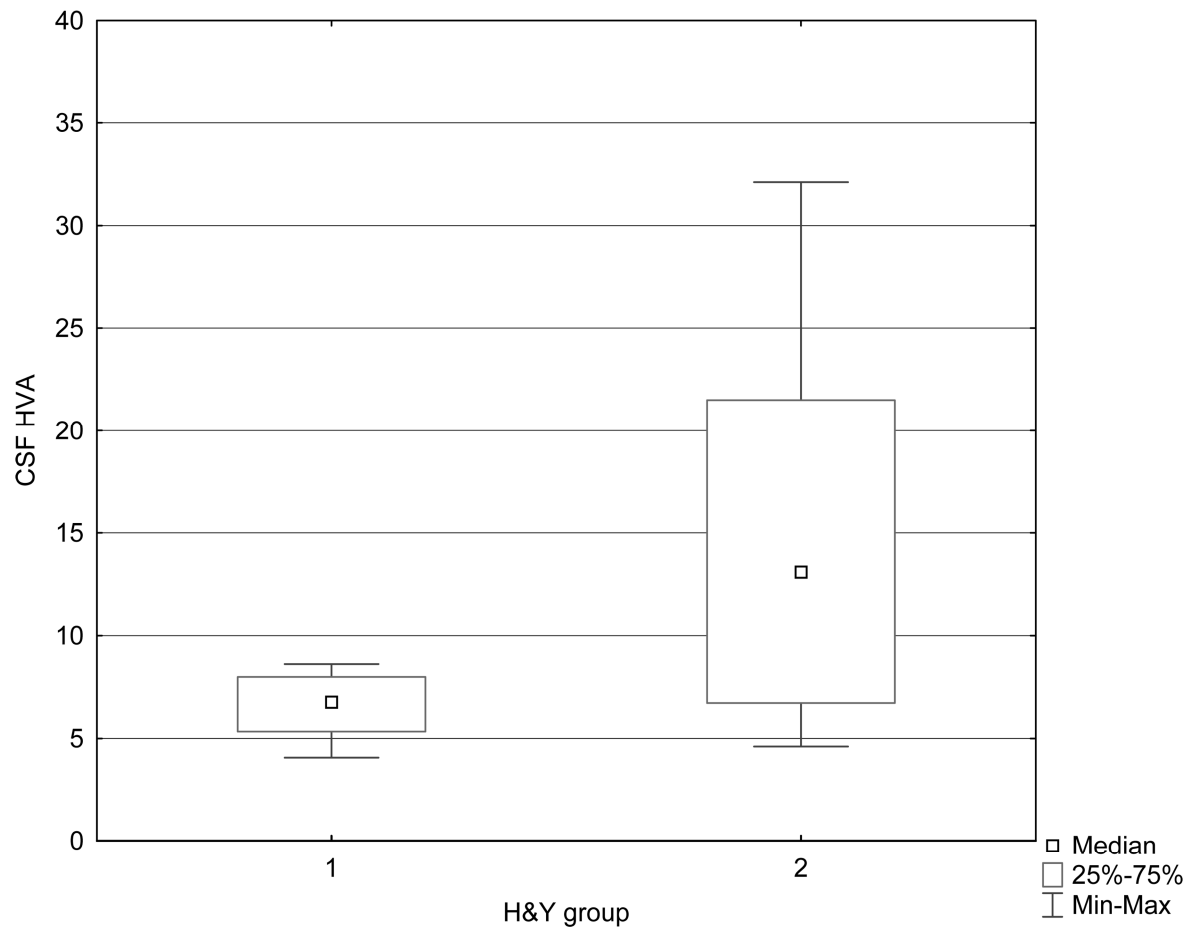submitted to "LD-challenged" LP

N	19
Male/Female ratio	10/9
Age (years)	60,3 ± 9,1
MMSE score	26,5 ± 2,4
UPDRS part III	25,1 ± 15,1
LEDD	510 mg
Disease duration (months)	27,5 ± 17,6

PD: Parkinson Disease; UPDRS: Unified
Parkinson Disease Rate Scale; MMSE: Mini
Mental State Examination; LEDD: levodopa
equivalent doses.

CSF LD concentration

Highlights

- Homovanilic acid (HVA) concentration increases in CSF along with the disease progression, also in the early and moderate stages of the disease.

- Increased CSF HVA levels are related to the degree of motor impairment in PD patients.

- CSF HVA levels correlated with CSF DOPAC levels, but not with CSF DA concentration.