



## NLRP 3 INFLAMMASOME CSF EXPRESSION AND NEUROPSYCHOLOGICAL CHANGES IN IDIOPATHIC NORMAL PRESSURE HYDROCEPHALUS.

### Neurology

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### ABSTRACT

**Object.** Inflammasomes are multiprotein complexes involved in the pathogenesis of neurodegenerative diseases. Aim of this study is to evaluate the expression of Nod-like receptor protein 3 (NLRP3) in cerebrospinal fluid (CSF) of idiopathic normal pressure hydrocephalus (iNPH).

**Patients and methods.** Eleven patients with iNPH (*iNPH group*), 8 patients excluded for iNPH criteria (*non iNPH group*) and 11 controls were enrolled. CSF samples were collected before and after 48 hours of extended lumbar drainage (ELD). Western blot analysis was performed for NLRP3, caspase-1 and interleukin (IL)-1b.

**Results.** Results demonstrated that NLRP3 was upregulated iNPH group and non iNPH group, compared to controls. Expression of NLRP3 was significantly increased in iNPH group. CSF removal led to the reduction of NLRP3 expression in both group of patients. This downregulation was more significant in iNPH group. Pearson analysis evidenced a negative correlation between NLRP3 expression and gait, balance and neuropsychological parameters in iNPH group. NLRP3 levels were significantly higher in CSF of patients with worse scores, as well as the post ELD NLRP3 reduction was associated with an improvement of outcome. A marked decrease of caspase-1 and IL-1b levels, NLRP3 downstream mediators was observed after ELD.

**Conclusions.** Our study suggests that NLRP3 inflammasome is involved in iNPH pathogenesis and might be a predictive biomarker of shunt response in iNPH patients.

### KEYWORDS

Inflammasome, NLRP3, CSF, iNPH, neurodegenerative disease

### Introduction

Idiopathic normal pressure hydrocephalus (iNPH) is a common form of reversible condition which accounts for 2-10% of all forms of dementia and 40% of hydrocephalus in adults.<sup>1</sup> Gait, balance and continence impairment characterize the classical triad. Despite in the last decades several authors published a large amount of guidelines in order to achieve a correct identification of iNPH patients<sup>2</sup> the rate of misdiagnosis remains still significant, considering the features shared with other neurodegenerative diseases. Nowadays, in clinical practice

the use of biomarkers in iNPH is less applied due to the controversial data reported, and to the presence of common cerebrospinal fluid CSF profile between iNPH and other disorders. Identification of specific biomarkers could improve diagnostic and selection processes of this syndrome<sup>3-6</sup> Neuroinflammation exerts a key role in the development and progression of neurodegenerative pathologies<sup>7,9</sup>, mainly through the production of inflammatory mediators such as interleukins (IL), and tumor necrosis factor-alpha (TNF- $\alpha$ )<sup>8-13</sup>. Moreover, studies on CSF of iNPH patients evidenced high levels of proinflammatory cytokines,

suggesting that neuroinflammation is strictly involved in iNPH pathophysiology<sup>14-18</sup>. However, none of the investigated biomarkers has been validated for diagnosis. In the last years, a large amount of evidence demonstrated that inflammasomes play a central role in neuroinflammation and neurodegeneration<sup>19-24</sup>. Inflammasomes are multiprotein complexes formed by the interaction of a Nod-like receptor protein (NLRP) with caspase-1 and the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain)<sup>25</sup>. Upon initiation, NLRPs lead to the recruitment of ASC which interacts with the pro-caspase-1 that is converted in caspase-1, and activated. This complex triggers a network of downstream mediators, as IL-1 $\beta$ , IL-18 and IL-33, sustaining and amplifying inflammation process<sup>26</sup>. In brain microenvironment, the activation of NLRPs inflammasomes has been associated with cellular damage and pyroptosis, and it has been demonstrated playing a crucial role in neuroinflammatory and neurodegenerative conditions, such as Alzheimer disease (AD), multiple sclerosis (MS) and depression<sup>27-29</sup>. The NLRP3 inflammasome is one of the most studied and well characterized inflammasome<sup>30</sup>. Although NLRP3 has been well investigated in neurodegenerative disorders<sup>19,24,31</sup>, no reports have highlighted the activation of inflammasome in iNPH. The purpose of this study is to evaluate the expression of NLRP3 and its downstream mediators in CSF of iNPH patients, before and after the extended lumbar drainage, and its correlation with clinical and neuropsychological performances.

## Materials and Methods:

### Patient characteristics

A total of 19 patients and 11 controls were prospectively enrolled in this study from January 2014 to January 2016. The Ethical Institutional Board of the University of Messina approved this study, and an informed consent was obtained from each patient and/or relatives. The total cohort was subdivided in two groups, *iNPH* group (n=11, nine males and two females; mean age 70 $\pm$ 5) with specific features of probable iNPH<sup>32</sup>, and *non iNPH* group (n=8, five males and three females; mean age 72 $\pm$ 8) suffering from neurodegenerative diseases such as Alzheimer's disease, neurovascular disturbances, frontotemporal dementia, and excluded for iNPH. Eleven controls (six males and five females; mean age 69 $\pm$ 9) were selected from subjects tested for suspected neurodegenerative disorders, in which this diagnosis was subsequently excluded. The mean duration of symptoms in *iNPH* and *non iNPH* groups was 16 $\pm$ 5 months. All patients underwent preoperative neuroimaging to estimate the ventriculomegaly and other specific MR features. The following indexes were estimated: Evans Index (EI) > 0.3 was considered indicative of hydrocephalus; Disproportionately Enlarged Subarachnoid Spaces (DESH), due to CSF flow impairment and brain tissue atrophy, was estimated as mild, moderate and severe; Callosal Angle (CA), representing the angle measured on coronal MR T1 weighted or FLAIR images at the level of posterior commissure, was considered indicative of iNPH when ranged between 66 $\pm$ 14 degrees<sup>33</sup>.

### Clinical and neuropsychological assessments

The clinical assessment was performed according to the Hellstrom iNPH severity and outcome scale divided into the following domains: gait, balance and continence<sup>34</sup>. The evaluation assessed the ordinal rating of gait and number of steps and seconds in 10 meters walk, the balance through the observation of patients standing on one or both legs, and the continence including six steps of severity. The results of each examination were converted in domain scores in order to identify the severity of clinical status. The neuropsychological assessment was performed through quantitative and qualitative analysis as previously reported<sup>35</sup>. The former was devoted to assess general cognitive status, short and long term memory, episodic memory, immediate visual memory, constructive praxia, reasoning, executive functions. The batteries applied were: Mini Mental State Examination (M.M.S.E), Mental Deterioration Battery (M.D.B.), including seven subtests for immediate and delayed recall, as Rey Auditory Verbal Learning Test (RAVLT), semantic and phonological fluency, phrase construction, immediate visual memory, as Raven's Colored Progressive Matrices (PM 47), copying drawings, and copying drawings with landmarks, Stroop Color Word test, Deux Barrage Test, Frontal Assessment Battery (FAB), Grooved Pegboard, Rey-Osterrieth Complex Figure. The qualitative assessment was based on the identification of the following validated markers in order to distinguish cognitive profile between iNPH and AD: absence of the primacy effect in Rey's Auditory verbal learning test, absolute decay of memory trace, and tendency to produce false alarms during delayed recognition of the

same word list, tendency to choose globalistic or odd responses in Raven's Colored Progressive Matrices test, closing in phenomenon in copy drawings test, inaccuracy in tasks execution of Deux Barrage test, positional preference mistakes in Raven's Colored Progressive Matrices test.

### Sample collection and protein quantification

CSF samples were collected at the moment of lumbar puncture and 48 hours after ELD positioning. Samples were centrifuged at 2000  $\times$  g for 10 min at 4°C and the supernatant was aliquoted and stored at -80 °C until analysis. Total protein concentration of CSF samples was evaluated using the Bradford assay (Bio-Rad, CA, USA).

### Western blot analysis

Western blot was used to examine the protein expression of NLRP3, caspase-1 and IL-1 $\beta$  in all CSF before and after 48 hours. Protein samples were denatured in reducing buffer (62-mmol/L Tris [pH 6.8], 10% glycerol, 2% sodium dodecyl sulfate [SDS], 5%  $\beta$ -mercaptoethanol, and 0.003% bromophenol blue) and separated by electrophoresis on a polyacrylamide gel. The proteins were transferred on a PVDF membrane (Amersham, GE Healthcare Europe GmbH) at 100V for 1 hour. The membranes were then blocked with 5% nonfat dry milk in Tris-buffered saline (TBS)-0.1% Tween for 1 hour at room temperature and incubated with a primary antibody for NLRP3 (St John Lab, London, UK), Caspase-1 (Cell Signaling Technology, CST) or IL-1 $\beta$  (Santa Cruz technologies, USA) in TBS-0.1% Tween overnight at 4°C. The membranes were incubated with a secondary antibody (Pierce, Chester, UK) for 1 hour at room temperature, and visualized by the enhanced chemiluminescence using the ECL Prime Western blocking detection system according to the manufacturer's protocol (Amersham, GE Healthcare Europe GmbH). Equal loading of protein was assessed on stripped blots by immunodetection of GAPDH with a rabbit monoclonal antibody (Santa Cruz, USA) and peroxidase-conjugated goat anti-rabbit immunoglobulin G (Pierce, Chester, UK). The protein signals were quantified by scanning densitometry using a bioimage analysis system (Bio-Profil, Celbio, Milan, Italy).

### Statistical Analysis

Statistical analysis was performed with GraphPad Prism Software 5.0 (GraphPad Software, Inc., CA, USA). ANOVA test was used to assess the differences of NLRP3 expression before and after ELD. Bonferroni's multiple comparison test was used for statistical analysis between each group. Pearson test was used in correlation analysis between CSF NLRP3 expression and clinical and neuropsychological scores. A p value < 0.05 was considered statistically significant.

## Results

### Clinical and neuroradiological profile

Clinical and neuroradiological data are reported in table 1. In *iNPH* group EI was 0.35 $\pm$ 0.03 and CA was 74.31 $\pm$ 20.5. In *non iNPH* group EI was 0.31 $\pm$ 0.07 and CA was 106.48 $\pm$ 17.63. The two groups, *iNPH* and *non iNPH*, underwent lumbar puncture with pressure opening measurement and removal of 20 cc of CSF for biomarker analysis, and an extended lumbar drainage (ELD) was positioned and maintained for 48 hours. In *iNPH* group the pressure opening was 11.19 $\pm$ 2.5 whereas in *non iNPH* group the pressure opening was 11.67 $\pm$ 2.75. Among the eleven patients of *iNPH* group, seven patients underwent ventriculo-peritoneal shunt (VPS) and new clinical and neuropsychological assessments were repeated 1 week and 6 months postoperatively.

### NLRP3 inflammasome is highly activated in iNPH patients

The expression of NLRP3 protein was investigated by Western Blot analysis in CSF of all patients and controls. Results obtained evidenced that NLRP3 was expressed in CSF of both *iNPH* and *non iNPH* group (Figure 1). Densitometric analysis showed that NLRP3 expression was significantly higher in pre ELD CSF samples of *iNPH* compared to *non iNPH* group and controls (Figure 1). A marked reduction of NLRP3 expression was observed in CSF of both *iNPH* and in *non iNPH* patients compared to controls, after 48 hours of ELD. The reduction was more significant in *iNPH* group (Figure 1).

### Downstream mediators of NLRP3 are upregulated in iNPH

Additionally, we explored the role of NLRP3 inflammasome activation in the pathogenesis of iNPH analyzing the levels of caspase-1 and IL-1 $\beta$ , known effectors of NLRP3 cascade. As expected, NLRP3 activation led to a remarkable elevation of caspase-1 (Figure 2A) expression with the concomitant enhancement of IL-1 $\beta$  levels (Figure

2B) in pre ELD CSF samples of *iNPH* and *non iNPH* group compared to controls. Upregulation of caspase-1 and IL-1b was significantly increased in *iNPH* than in *non iNPH* group (Figure 2 A and B). Moreover, CSF samples obtained 48 hours after ELD positioning showed a significant reduction of IL-1b and caspase-1 levels in both groups, compared to controls (Figure 2 A and B). These data proved that NLRP3 inflammasome is involved in the pathophysiology of *iNPH*.

#### Correlation between continence, balance, ordinal rate of gait, neuropsychological profile and NLRP3 activation

Furthermore, in order to investigate the impact of NLRP3 activation on *iNPH*, we correlated the expression level of NLRP3 with clinical and neuropsychological performances. Specifically, we analyzed the correlation between protein expression and gait domains, balance, continence, and neuropsychological scores (Table 2). Pearson analysis evidenced a significant negative correlation between NLRP3 expression and clinical parameters in *iNPH* group pre ELD (Table 2. Continence:  $r=-0.653$ ,  $p<0.05$ ; balance:  $r=-0.52$ ,  $p<0.05$ ; ordinal:  $r=-0.78$ ,  $p<0.01$ ), post ELD (Table 2. Continence:  $r=-0.623$ ,  $p<0.05$ ; balance:  $r=-0.52$ ,  $p<0.05$ ; ordinal:  $r=-0.54$ ,  $p<0.05$ ), 1 week postoperatively (Table 2. Continence:  $r=-0.85$ ,  $p<0.001$ ; balance:  $r=-0.62$ ,  $p<0.05$ ; ordinal:  $r=-0.639$ ,  $p<0.05$ ) and 6 months postoperatively (Table 2. Continence:  $r=-0.71$ ,  $p<0.01$ ; balance:  $r=-0.54$ ,  $p<0.05$ ; ordinal:  $r=-0.61$ ,  $p<0.05$ ). In contrast, no correlation was observed between NLRP3 expression and clinical and cognitive scores in *non iNPH* group. Additionally, Pearson correlation coefficients evidenced a significant correlation between NLRP3 expression and the following neuropsychological parameters in *iNPH* group: verbal fluency (Table 2. pre ELD:  $r=-0.606$ ,  $p<0.05$ ; post ELD:  $r=-0.627$ ,  $p<0.05$ ; 1 week post operatively:  $r=-0.55$ ,  $p<0.05$ ; 6 months post operatively:  $r=-0.65$ ,  $p<0.05$ ) and RAVLT immediate (Table 2. post ELD:  $p=0.06$ ; 1 week post operatively:  $r=-0.58$ ,  $p<0.05$ ; 6 months post operatively:  $r=-0.635$ ,  $p<0.05$ ). No others significant correlations were demonstrated between NLRP3 expression and clinical and neuropsychological scores.

#### Discussion

Neuroinflammation is strongly involved in the development of neurodegenerative diseases, and it is mainly mediated by inflammasomes. Inflammasomes are multiprotein complexes that upon activation, initiate a complex cascade which leads to the activation of caspases and the release of proinflammatory cytokines, promoting inflammation process and contributing to the clinical onset of disease<sup>19-23</sup>. Although NLRP3 is one of the most studied inflammasomes and has been investigated in various neuroinflammatory and neurodegenerative disorders<sup>26-40</sup>, its involvement in idiopathic normal pressure hydrocephalus has not yet been reported. This is the first study which highlighted that NLRP3 inflammasome is elevated in CSF of *iNPH* patients, and postulated its contribution to the pathogenesis of this syndrome. Considering the similar hallmarks between *iNPH* and other neurodegenerative disorders, these results are not surprising since they are in agreement with those reported in previous researches, demonstrating the activation of inflammasomes in AD<sup>38</sup>, and suggesting a similar molecular pattern for these conditions. Interestingly, a marked reduction of NLRP3 48 hours after ELD, has been observed in our cohort of *iNPH* patients. The activation of NLRP3 inflammasome in *iNPH* is further confirmed by the significant enhancement of its downstream effectors, caspase-1 and IL-1b, revealed in CSF. Generally, low cytokines levels are required for normal brain physiology; however an upregulation of proinflammatory cytokines has been related to neuroinflammation and neurodegeneration<sup>41,42</sup>. As matter of fact, high levels of proinflammatory cytokines have been demonstrated in many brain diseases, including AD, Parkinson disease and age related dementia as well as in *iNPH*<sup>18,41-44</sup>. Accordingly, in our study, the marked increase of IL-1 $\beta$  and caspase-1 in *iNPH* group, might reflect the strong activation of NLRP3 inflammasome and the extent of neuroinflammation in the chronic process of normal pressure hydrocephalus. We further highlighted that the expression of NLRP3 inflammasome in *iNPH* is related to the severity of clinical and neuropsychological parameters, as patients with higher NLRP3 levels had worse scores. Indeed, correlating the clinical status with NLRP3 inflammasome expression, we evidenced a significant negative correlation with continence, balance and ordinal rating of gait in *iNPH* group. Additionally, the correlational analysis showed a significant relationship between NLRP3 expression and neuropsychological test as the verbal fluency and the RAVLT immediate. In a previous report, Sosvorova and coauthors reported the reduction of inflammatory

cytokines levels in *iNPH* after CSF removal<sup>18</sup>. Interestingly, in our study CSF removal through ELD, reducing NLRP3 activation conducted to a parallel decrease of IL-1b levels and caspase-1, and to a concomitant improvement of clinical and neuropsychological scores. The tight association between NLRP3 levels and the clinical and neuropsychological parameters reinforced the assumption of the possible role of inflammasome in *iNPH* pathogenesis. Normal pressure hydrocephalus is often misdiagnosed given the similarities with other neurodegenerative disorders, primarily AD, making difficult the correct selection of patients to VP shunt<sup>45-47</sup>. Differently from AD, *iNPH* is a reversible dementia in which a significant improvement of signs and symptoms occurs after CSF removal. The reversibility of clinical and neuropsychological patterns in *iNPH* observed in our cohort could be strongly related to the reduction of NLRP3 levels after ELD positioning, in light of the role of neuroinflammation in the pathophysiology of this disorder. Several functional and metabolic changes arise in hydrocephalus and could explain its clinical manifestations. As suggested by Kondziella and coauthors<sup>19</sup> in their experimental model, two phases lead to NPH. During the acute phase of hydrocephalus, in which an increase of intracranial pressure is registered, the main brain alteration is the edema concentrated on the periventricular regions due to both decrease of cerebral blood flow (CBF) and CSF impairment, resulting into the typical ventriculomegaly. Conversely, the chronic phase is characterized by the partial resolution of the edema and by the establishment of gliosis and neuronal degeneration, maintained by the increase of ventricular sizes. During the first phase, the triggering of inflammation cascade lead to the NLRP3 activation and to the inflammatory response with subsequent neuronal damage and pyroptosis. The maintenance of inflammation process in the chronic phase, due to specific alterations of neuronal structures, conduces to the impairment of specific pathways involved into the clinical and cognitive dysfunction of *iNPH*. As previously reported, the neurocognitive profile of *iNPH* patients is mainly characterized by the dysfunction of executive domains and short term memory. In our investigation a significant improvement of neuropsychological performances, after ELD, has been demonstrated for RAVLT immediate, RAVLT delayed, immediate visual memory, and coloured progressive Raven's matrices in *iNPH* group compared to non *iNPH* group. An improvement, also if not statistically significant, was observed in *iNPH* group in Mini Mental State Examination (MMSE). Few studies have been investigated the relationship between clinical and cognitive performances and biomarker profile<sup>16</sup>. The decrease of NLRP3 levels after ELD, and the consequent improvement of clinical and cognitive performances, could be the expression of the possible temporary attenuation of the inflammation process induced by CSF removal. This series of events could be linked to intracranial hypertension, CSF and CBF impairment, and related alterations, that acting as stimulus, determine the assembly and the activation of NLRP3 cascade. Indeed, the CSF removal, reducing the inflammatory stimulus leads to the reduction of NLRP3 levels and inflammasome cascade, and subsequently to the restoration of the clinical and neuropsychological functions. In our study we found significant lower levels of NLRP3 and its downstream mediators in *iNPH* group after ELD and the improvement of ordinal rating of gait, balance, continence and cognitive domains explored. Thus, among the eleven patients of *iNPH* group, showing a more significant increase of NLRP3 and its mediators pre ELD, and a marked reduction after ELD, seven patients underwent ventriculo-peritoneal shunt (VPS) and a new clinical and neuropsychological assessment was repeated 1 week and 6 months postoperatively. Notably, patients underwent surgery, with lower post ELD NLRP3 levels revealed a more significant improvement of clinical and cognitive status 1 week and 6 months postoperatively. These data prompted us to speculate that NLRP3 contributes to the inflammatory network of *iNPH* and might be a potential predictive biomarker of shunt response.

#### Conclusions

In conclusion, our preliminary data could represent the first step to understand the molecular features involved in the pathogenesis of *iNPH*. We preliminarily demonstrated that NLRP3 inflammasome is a potential reliable predictive biomarker of positive shunt response in *iNPH* syndrome. However, the small cohort of patients, the heterogeneity of non *iNPH* group and the short follow up do not allow us to confirm this hypothesis. Future studies, focusing on the mechanisms involved in NLRP3 activation in imp, are required to elucidate and clarify the role of NLRP3 inflammasome in this neurodegenerative disorder.

**Conflict of interest**

The authors declare that there is no conflict of interest regarding the publication of this paper.

**Figure legends**

**Figure 1.** Western blot analysis of NLRP3 expression in CSF of patients with *iNPH* and *non iNPH* pre ELD and post ELD. *Upper panel:* Representative autoradiography highlights NLRP3 and GAPDH expression. a=CTRL, b=*non iNPH* pre ELD, c=*non iNPH* post ELD, d=*iNPH* pre ELD, e=*iNPH* post ELD *Lower panel:* Quantitative data represent represents the mean ±SD of relative expression. \*p<0.05 vs CTRL, #p<0.05 *non iNPH* pre ELD vs *non iNPH* post ELD, §p<0.001 vs CTRL and non *iNPH*, Φp<0.01 vs *iNPH* pre ELD.

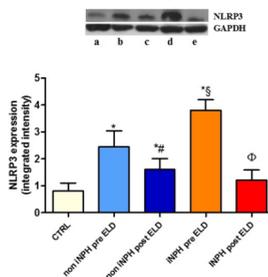
**Figure 2.** Western blot analysis of Caspase-1 (A), IL-1β (B) expression in CSF of patients with *iNPH* and *non iNPH*, pre ELD and post ELD. *Upper panel:* Representative autoradiography highlights Caspase-1 or IL-1β and GAPDH expression; a=CTRL, b=*non iNPH* pre ELD, c=*non iNPH* post ELD, d=*iNPH* pre ELD, e=*iNPH* post ELD. *Lower panel:* Quantitative data represent represents the mean ±SD of relative expression. \*p<0.05 vs CTRL, Φp<0.05 *non iNPH* post ELD vs *non iNPH* pre ELD, §p<0.001 *iNPH* pre ELD vs *non iNPH*, #p<0.01 *iNPH* post ELD vs *iNPH* pre ELD.

	Sex	Mean Age	Evans' Index	Callosal Angle	Pressure Opening
<i>iNPH</i>	9 M; 2 F	70±5	0,35±0,03	74,31±20,5	11,19±2,5
<i>non iNPH</i>	5 M; 3 F	72±8	0,31±0,07	106,48±17,63	11,67±2,75

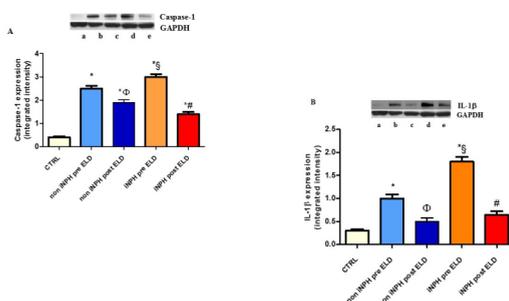
**Table 1.** Clinical and neuroradiological profile of *iNPH* and *non iNPH* patients. Abbreviations: M= male; F=female.

	pre ELD	post ELD	1 week post operatively	6 months post operatively
<i>NLRP3/Continence</i>	p<0.05 r= -0.653	p<0.05 r= -0.623	p<0.05 r= -0.85	p<0.01 r= -0.71
<i>NLRP3/Balance</i>	p<0.01 r= -0.52	p<0.02 r= -0.521	p<0.05 r= -0.62	p<0.05 r= -0.54
<i>NLRP3/Ordinal</i>	p<0.01 r= -0.78	p=0.01 r= -0.54	p<0.05 r= -0.639	p<0.05 r= -0.61
<i>NLRP3/Verbal fluency</i>	p<0.05 r= -0.606	p<0.05 r= -0.627	p<0.05 r= -0.55	p<0.05 r= -0.65
<i>NLRP3/RAVLT immediate</i>	p=0.09	p=0.06	p<0.05 r= -0.58	p<0.05 r= -0.635

**Table 2.** Pearson correlational analysis between NLRP3 levels and clinical and neuropsychological assessments in *iNPH* patients pre ELD, post ELD and 1 week and 6 months post operatively. A p value <0.05 was considered statistically significant.



**Table 1**



**Table 2**

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