Diagnostic Importance of Circulating Neurofilaments in Multiple Sclerosis

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Abstract

Background: In adults as young, sclerosis as multiple considered as most cause common of disability as neurologic. MS is characterized by demyelination, inflammation, and injury of neuro-axonal at nervous central system causing long-term developing disability. At the current time no definitive single exam for MS diagnosis and monitoring. Objective: The aim of present study is to find out a non-invasive diagnostic neurofilaments biomarker (Neurofilaments light chain, Phosphorylated Neurofilament, Heavy Polypeptide) (NFL-L and P-NFL-H) for multiple sclerosis with high sensitivity and specificity. Patients and methods: A case control study based on three groups. first group 48 patients with previously diagnosis as MS and under treatment which include (11 male and 39 female), also second group was 20 patients new cases without treatment or who have sign of MS or suspected MS which include 7 male and 13 female who were observation in Middle-Euphrates-Neuroscience-Center in Anajaf Al-Ashraf/ Iraq. Third group was include 20 healthy volunteers (non-MS). Samples of blood (5 ml) were taken via venipuncture out of venous from such groups drawed via syringes being disposable at technique of aseptic. Every sample of 3 groups were taken in free plane EDTA tube, permitted for clotting then separation to serum was done via centrifuge of 13000 rpm for 5 min and then stored at -20C. Which was further used to identify NFL-L and P-NFL-H levels by used ELISA technic. Conclusion: neurofilaments consist of numerous properties of to be exceptional biomarker, such as neurofilaments protein only found in neural cells, also during neurodegenration processes this protein can be released in CSF and plasma. Therefore we can get a good biomarker (non –invasive sampling way) for diagnosis and monitoring of MS disease by measurements of serum neurofilaments level in patients MS and compared with healthy control subjects.

Key Words: Health, Neurosystems, Circulating Neurofilaments, Diagnostic.

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Introduction

In adults as young, sclerosis as multiple is considered as most cause being common of disability as neurologic. MS is characterized by demyelination, inflammation, and injury of neuro-axonal at nervous central system causing long-term developing disability. (Nicholas, R.R.W. 2013).

MS clinical phenotypes are including relapsing-remitting MS (RRMS), and developing forms: secondary developing MS (SPMS) and primary developing MS (PPMS). RRMS is prevailed mostly as subtype, containing more than 70% cases. Disease onset for those of 10–15 years, patient's majority of RRMS transfer to SPMS, disease phase defined through worsening as gradual clinically which is not responding to any treatment available. PPM is indistinguishable clinically from SPMS, excluding that it manifests de novo, without a preceding phase as relapsing-remitting (Lublin, F.D. et al. 2014).

At the current time, there is no one definitive test for assessment of MS; monitoring of disease and diagnosis depends on multiple parameters as clinical include magnetic resonance imaging, clinical examination, electrophysiology and assessment of cerebrospinal fluid.

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These investigations over disease course protracted are costly with limited utilization in distinguishing developing disease from active RRMS (Housley, W.J et al. 2015). Many recent studies have reported the existence and the prominence of circulating Neurofilaments in the CSF and serum or plasma. NFs are proteins of cytoskeletal with axons stabilizing role, axon diameter determination and axonal transport participation (C.E. Teunissen, 2009). Since NFs are exclusively found within neurons, detection them in CSF or blood thus reflects axonal and neuronal damage (M.M. Gresle, 2008). NFs of mammalian have various subunits: NF-light chain (NF-L) that considered as NF- intermediate chain backbone for (NF-M) as well as copolymerise heavy chain (NF-H) (C.E. Teunissen, 2005). The most abundant soluble NF subunit and smallest is NF-L but proteases susceptible (A. Petzold, 2005). However, HF-H is molecule being larger as well as with higher proteases resistant when phosphorylated (M.E. Goldstein 2007). NF molecules phosphorylated parts are abundant mostly within subunits of NF-H and HF-M (J.P. Julien and W.E. Mushynski, 2007) where phosphorylation state affects diameter of axonal (E. Fuchs and D.W. Cleveland, 2008). Phosphorylated NF-H being high are usually present in NFs axonal only where such marker is believed to mention degeneration and/or injury of axonal (M.M. Gresle, 2008), while NF-L comprises only dendrites and neuronal body cell minor part relative to axons. (J.N. Lycke, 2008).

Materials and Methods

Patients and Sample Collection

A case control study based on three groups. first group 48 patients with previously diagnosis as MS and under treatment which include (11 male and 39 female), also second group was 20 patients new cases without treatment or who have sign of MS or suspected MS which include 7 male and 13 female who were observation in *Middle-Euphrates-Neuroscience-Center in Anajaf Al-Ashraf/ Iraq.*

This study was performed during Jan. 2018 – Feb. 2019 under specialist's supervision in internal medicine where data collected from patients as well as the investigation that have been done in center departments. Such as (MRI, EMG, and Laboratories assay including CSF analysis, auto immune screen test and in addition routine blood test. Third group was include 20 healthy volunteers (non-MS). Samples of blood (5 ml) were taken via venipuncture out of venous from such groups drawed via syringes being disposable at technique of aseptic. Every sample of 3 groups were taken in free plane EDTA tube, permitted for clotting then separation to serum was done via centrifuge of 13000 rpm for 5 min and then stored at -20 ^{0}C. Serum separated was then gathered in Eppendrof tubes and stored at -20C for further usage in NFs measurements in ELISA method. This ELISA kit applies to the in vitro quantitative determination of human NFs concentration in serum, plasma and other biological fluids.

Such kit of ELISA utilizes principle of ELISA–Sandwich. In this kit, provided micro-plate was pre-coated by specific antibody to NEFL of human. Samples or standards to micro-plate wells of ELISA were added as well as combined along antibody specific. Following that, Human NFs as biotinylated detection specific antibody as well as conjugate of Avidin –Horseradish peroxidase (HRP) were successively added to every well. Wells containing biotinylated detection antibody, Avidin-HRP conjugated, and Human NFs show blue color. Reaction of enzyme-substrate is terminated via stop solution addition and color changes to yellow. OD is spectrophotometrically measured at 450nm ± 2nm wavelength. Value of OD is proportional to Human NFs concentration. We can measure Human NFs sample concentration via comparing standard curve to sample OD.

Results Calculation

Readings as duplicate average for every sample and standard was measured as well subtract average 0 standards OD. Plot 4 parameters curve of logistic on paper as graph of log-log, where OD values on the y-axis and standard concentrations on the x-axis (Figures not shown).

Discussion

NF-L and NF-H serum level in patients with MS compared to healthy individuals, NfL has been suggested both as neurodegeneration standalone biomarker and as composite 'treatment target' measure component where no activity of disease evidence. (Thompson AJ et al., 2018). NfL levels role in clinical practice (as a measure of inflammatory activity or neurodegeneration, as DMT efficacy monitoring tool or as biomarker of prognostic is still of determination while capability for measuring levels of blood elevates considerably their utility as clinical (Martin et al., 2019).

The NF-L and NF-H serum levels in patients with

MS compared to healthy individuals are shown in table 1 and figures 1 and 2.

There was highly significant difference in serum level of NF-H among study groups ($P < 0.001$); the level was highest in MS on treatment followed by MS without treatment and lastly by control group; the difference between both MS patients.

In addition, there was significant difference in the serum level of NF-L among study groups ($P = 0.031$); the level was highest in MS without treatment followed by MS on treatment and lastly by control group; the difference between both MS patients.

Correlations of serum neurofilament- heavy (NF-H) and serum neurofilament- light (NF-L) to clinicopathological parameters are shown in table 2. Neither serum neurofilament- heavy (NF-H) nor serum neurofilament- light (NF-L) showed significant correlation to any of clinicopathological parameters, table 2.

CSF biomarkers utility as clinical is restricted via invasiveness, acceptability lack, and posed risks through repeated puncture lumbar. Thus, axonal degeneration blood biomarkers in MS could serve a major advance in disease activity monitoring. Methods development in serum for biomarkers measurement is complicated due to blood brain barrier potential influence on release dynamics of such proteins into blood and blood complexity and higher protein concentration compared to CSF. Furthermore, proteins releasing in blood might exposed to proteolysis and might link to components of blood which cleared actively out of blood. Nevertheless, blood brain barrier compromise seems as MS disease early feature process as well as biomarker proteins being potential axonal released into the lesions extracellular space that of ready access into blood from active MS sites pathology. Few relatively studies have estimated biomarkers of blood as neurodegeneration potential measures in MS. (A. Petzold et al., 2004; Gresle et al., 2011).

Evidence as accumulating shows that axonal neurodegeneration and damage already emerge in MS early phases (Kuhlmann T et al., 2002). Degeneration as axonal in MS is believed as disease development pathologic basis (Semra YK et al., 2002), and such damage seems to correlates along clinical disability degree (Trapp BD et al., 1999).

Protein of NFL chain in patients of MS is myelinated axons cytoskeletal protein major part, and NFL levels increased mention axonal continuous damage (Malmeström C et al., 2003). The high NFs intraneuronal levels of are of great importance both for therapeutic effect and diagnosis in medicine as clinical. According to previous obtained data, levels of NFL are increased through all MS stages, especially in relapsing-remitting one, and in progressive one, while NFL levels declined toward typical one through intervention along therapies of disease-modifying, (Kuhle J et al., 2015), proposing that NFL in respect to various processes of pathology involving in MS reflects activity of disease, disease development, and efficacy of treatment (Novakova L et al., 2017; Cai, and Huang, 2018). Neurofilament-H is a major axonal protein cytoskeletal. After injury as axonal, they are releasing into the compartment of extracellular, and as subsequence into peripheral blood and CSF, hence, serum levels of neurofilaments and CSF analysis might present tool being valuable to extent of axonal damage estimating in MS patients (Teunissen et al., 2005).

Another study by Brettschneider and colleagues investigated heavy chain of neurofilament as a axonal damage marker. They concluded that neurofilament heavy chain might increase sensitivity for predicting conversion to clinically definite MS during a follow up time of 48 months. The relation of neurofilament heavy chain with disease progression suggests that high neurofilament heavy chain reflects late stages of neuronal damage (Brettschneider et al., 2005). present result is consistent with Petzold and colleagues, where 34 patients with clinically definite MS were followed up after 3 years. MS patients were categorized as having developing disease and relapsing remitting. A significant increase in neurofilament heavy chain from baseline to follow-up was observed in a higher proportion of patients with primary developing MS. The marked increase of neurofilament heavy chain levels suggests that damage as axonal is a gradual process being cumulative through disease course and neurological function losing is axonal injury direct consequence (Scalfari et al., 2014). Petzold found that phosphorylated neurofilament heavy chain were higher for patients of all MS subtypes. Such studies propose that PNF-H might useful more as ongoing activity as neurodegenerative measure in MS patients that render such protein a candidate potential to be used as marker of surrogate for treatment assessment aiming to reduce axonal injury (Scalfari et al., 2014).

On contrary to NF-L observations, pNF-H median levels were highest for developing disease patients

(Jones J, and Coles A, 2010). Study as retrospective consisted 30 patients already has been conducted to assess if plasma levels of pNF-H may used for measuring response to treatment of interferon-β. In spite of plasma levels of pNF-H is higher in patients who are not well responding to treatment of Interferon-β, such is of no significance, due to possibly small size sample. Thus, more studies are needed to determine CSF or blood pNF-H levels utilization as disease activity indicator and, therapeutic efficacy potentially (Massoud et al., 2017).

Table 1. Serum Level of Neurofilament Heavy (NF-H) and Neurofilament Light (NF-L) in MS Patients with Compared to Healthy Individuals

Characteristic	Control group *n* = 20	MS without treatment *n* = 20	MS on treatment *n* = 48	*P*
Serum NF-H level				
Median (IQR)	117.50 (26.75) C	3291.00 (542.75) B	3775.00 (817.25)A	< 0.001
Range	104.00 -208.00	2029.00 -4455.00	1880.00 -4327.00	KHS
Serum NF-L level				
Median (IQR)	99.00 (18.25) B	179.50 (106.00) A	137.50 (111.50) A	0.031
Range	86.00 -126.00	79.00 -226.00	23.00 -782.00	KS

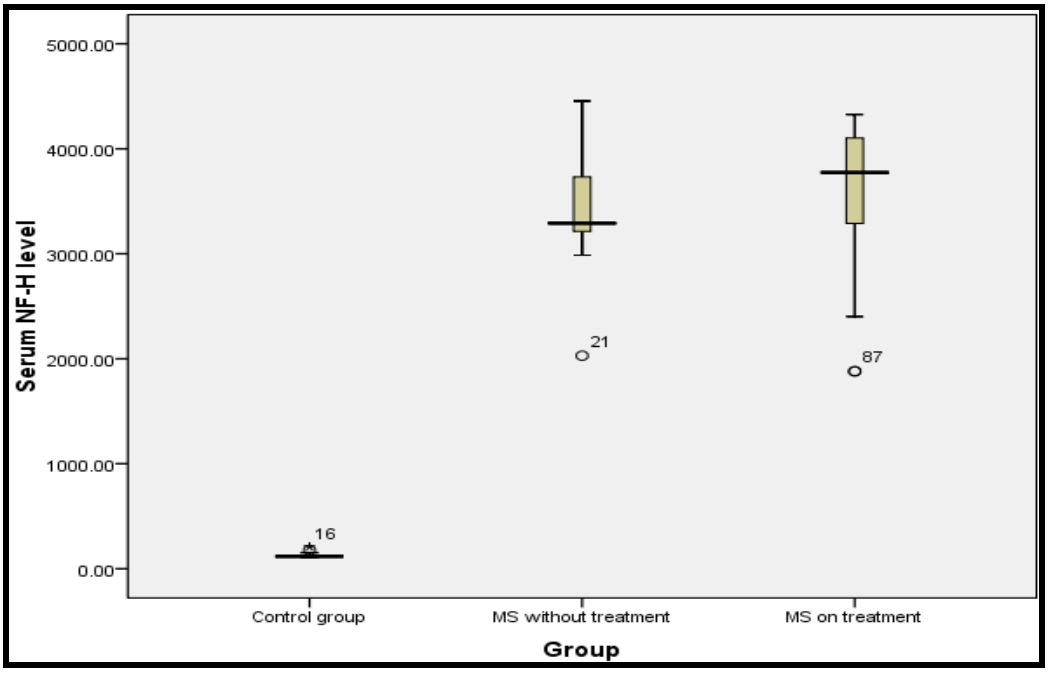

Figure 1. Serum Level of Neurofilament Heavy (NF-H) in MS Patients with Compared to Healthy Individuals

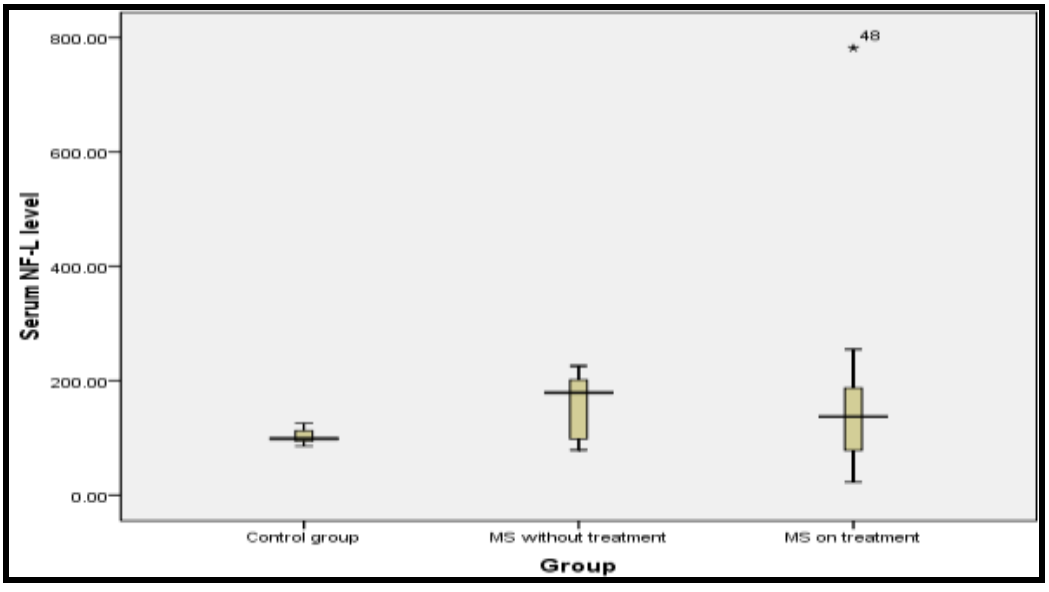

Figure 2. Serum Level of Neurofilament Light (NF-L) MS Patients with Compared to Healthy Individuals

In spite of enhancing lesions patients have higher levels of NfL, levels NfL for patients of no lesions of Gd+ significantly still higher compared to healthy individuals levels. Such finding proposes that levels of NfL as well reflecting damage of ongoing neuronal and detectable activity as inflammatory losing independent, probably taking place in usual-appearing white and gray matter. Such propos is more confirmed through association among levels of NfL and an objective of neuroaxonal damage measure, spinal cord and brain volume losing in study recently performed via our group (Barro C et al., 2018), and via baseline NfL predictive value for brain volume losing after two years on FREEDOMS trial. Such results propose that NfL in blood seem to reflect an integral ongoing neuronal damage and recent measure (over weeks, as well months). Such damage as neuronal is lesions relate in gray and white matter regions but as well the extra-lesional (normal-appearing) gray cortical matter MS pathology that manifests as density reduction in oligodendroglia and neurons in lesions areas outside. High plasma NfL levels correlation along lesional MRI activity noticed in the current study is in agreement with the recent observational results where patients of T2 high numbers or contrast-enhancing lesions spinal cord in or brain with higher NfL levels of serum. This MRI activity correlation along a specific neuroaxonal damage marker serves clinical-radiologic paradox understanding in MS—MRI activity low correlation with disability is being clinical (Barkhof F, 2002). T2 lesion load identification and Gd+ lesions presence as later brain volume loss strong predictors (Radue EW et al., 2015). Due to their neurons specificity, elevated levels of NfL prove that active lesions as inflammatory are correlated to damage of neuroaxonal. Studies of neuropathology show a higher axonal damage rate in early/relapsing compared to progressive MS (Kuhlmann T et al., 2002; Kuhle et al., 2019). current data are resemblances to natalizumab study that levels of CSF NF-L declined to level as same as the healthy ones following treatment of six or twelve months (Gunnarsson M et al., 2011), as well studies invistigating treatment of fingolimod and levels of CSF NF-L (Kuhle J et al., 2015; Novakova L et al., 2017). Present results are in agreement with CSF NFL lower levels for patients treated with rituximab and mitoxantrone. According to the obtained results, we might suggest that NF-L may regard as disease activity biomarker and on reflects response of treatment.

Current data show no association significantly with activity of MRI or score of EDSS is confirmed recently where no difference of significance in levels of CHI3L1 and activity of disease (Matute-Blanch C et al., 2017). Indeed, their study proposed that CHI3L1 might reflect IFNB-1a response to treatment in RRMS patients. Present study did not confirm such conclusion. There are some limitations to the current study; 1st, scans MRI in the study does not frequently conducted in comparison to clinical practice which influence results generalization. Furthermore, follow-up limited of this study might influence power for detecting associations among outcomes as clinical and NF-L (Matute-Blanch C et al., 2017; Varhaug ey al., 2019).

Evidence exist to confirm correlation between EDSS and level of pNF-H at follow-up, as well a propensity of potential for pNF-H patients of high levels at baseline to show EDSS disease progression at follow-up. Such analyses, however of no significance, mostly due to limited size sample. Such observations were supported where levels of CSF pNF-H be weakly correlateded with EDSS in a larger patients cohort. As far the current study, CSF pNF-H average levels were higher in patients with all MS subtypes compared to controls. Levels of CSF pNF-H on average were 1.5- fold higher for PP-MS and SP-MS cases compared to RR-MS ones. In contrary to NF-L observations, CSF pNF-H levels in cases of CIS not predicting change to definite MS clinically. Such studies proposed that pNF-H might of more benificial as ongoing activity being neurodegenerative measure in MS patients, that renders protein a candidate potential to be used as marker surrogate for treatments assessing aimed to reduce injury of axonal (Massoud et al., 2017).

Regarding the relation between PNF-H and expanded disability status scale, the present study showed that the level of PNF-H significantly increased with the progression of MS as measured by expanded disability status scale (Gresle M et al., 2014). In another study done by Eikelenboom and colleagues, patients with MS mostly had EDSS developed clinically. At follow-up, difference among groups of MS for score of EDSS. Progression disability seemed to be correlated with higher levels of serum for phosphorylated neurofilament (Eikelenboom M, and Petzold A, 2011; Massoud et al., 2017).

The study limitations are varying treatments, sample size, and MRI lack data out of healthy individuals. Varying regimens treatment could

lower the baseline levels of biomarker prognostic value. Nevertheless, in spite of that few patients received disease drugs modifying through follow-up.

Table 2. Correlations of Serum Neurofilament- heavy (NF-H) and Serum Neurofilament- light (NF-L) to Clinic-pathological Parameters

Characteristic	Serum NF-H level		Serum NF-L level	
	r	*P*	*r*	*P*
MRI	-0.040	0.785	0.219	0.135
Severity	-0.069	0.641	0.151	0.307
relapse	-0.019	0.896	0.075	0.613
Gender	0.074	0.615	0.058	0.694
Duration	-0.086	0.562	-0.055	0.709
Class	-0.061	0.682	-0.004	0.981
EDSS	0.033	0.824	-0.125	0.399

MRI: magnetic resonance imaging; EDSS: The Expanded Disability Status Scale; *r*: correlation coefficient.

Performance of ROC analysis revealed that a NFH cutoff value of >208 was highly accurate since the AUC was 1.000 (> 0.7) resulting in excellent specificity and sensitivity, 100 % and 100 %, respectively, table 3 and figure 3; whereas, NFL has poor predicative cutoff value of >126 with an AUC of 0.657, poor sensitivity of 55.9 % and excellent specificity of 100 %, table 3 and figure 4.

Table 3. Characteristics of ROC curve analysis for serum NFH, and NFL Chain

Characteristics	NFH	NFL
Cutoff value	>208	>126
AUC	1.000	0.657
95 % CI	0.959 to 1.000	0.548 to 0.755
P-value	< 0.001	0.005
Sensitivity %	100.0	55.9
Specificity %	100.0	100.0
Accuracy %	100.0	65.7

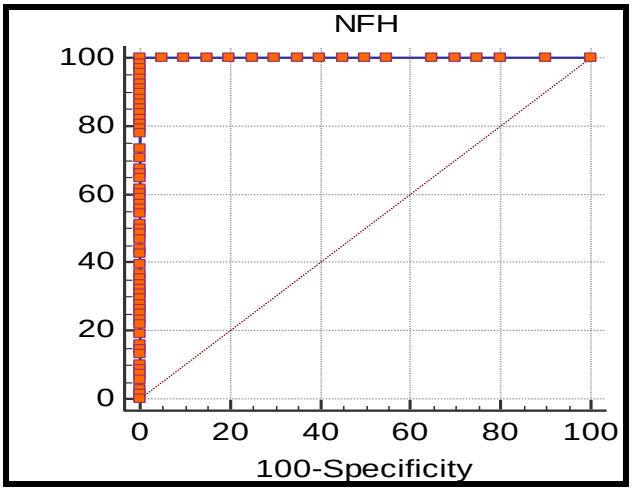

Figure 3. ROC Curve Analysis to Find Best NFH Cutoff Value that Predict Diagnosis of Multiple Sclerosis

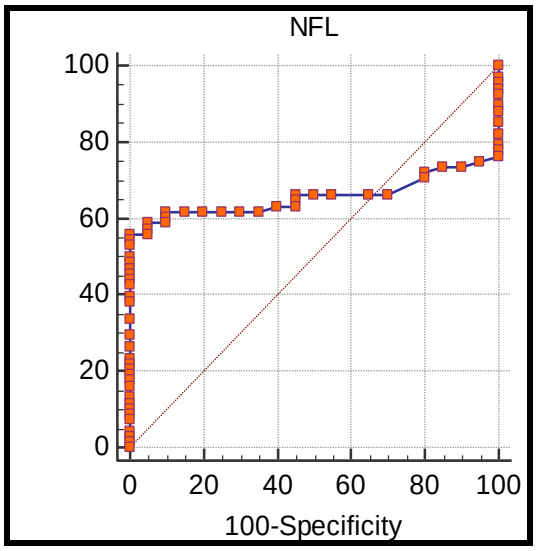

Figure 4. ROC curve analysis to find best NFL cutoff value that predict diagnosis of multiple sclerosis

A previous study that have been done by Novakova et al., analysis revealed that serum NfL have (95% CI with, 45% sensitivity and 80% specificity), and the CSF NFL for AUC was 0.774 (95% CI with 75% specificity and 67% sensitivity). Disease age, duration, and sex did not influence significantly serum NFL concentrations or CSF (Novakova et al., 2017).

In the ROC analysis, with another study show that, patients of subsequently activity of disease had higher significant CSF NfL baseline values in comparison to radiologically and clinically patients being stable (median 812.5 pg/mL, vs 329.5 pg/mL, p=0.002). A CSF NfL value cut-off of 500 pg/mL discriminated significantly 2 patients groups along specificity of 83.3% and sensitivity of 90% (Gaetani. et al., 2019).

The discrepancy between these previous studies and present study concerning Cut Off value in addition to sensitivity and specificity of NFL chain is might be beyond type of sample since current study depend on serum samples while the above mentioned previous studied NFL chain detection have been done at CSF level,

In terms of appropriate cut-off values, NfL levels in healthy have been reported as ranging from 16 to 20 pg/ml. In a longitudinal study of healthy individuals (median age 44.3), median NfL levels increased by 1.8 % over one year and 2.2% for each additional year (Varhaug et al., 2019). Sex does not affect NfL levels. There appears to be a small contribution of body mass index (BMI), with a unit rise in BMI associated with a decrease of 0.02 pg/ml in serum NfL levels (Manouchehrinia et al., 2020). This is probably because of dilution and volume of distribution. Although the influence of

age and BMI are significant, they contribute to less than 2.5% percent of the variability in NfL levels. (Gaiottino et al., 2013).

Findings of the comparative current study as primary revealed that NfH is considered to represent the most abundant and also the most analyte being stable, as well as system assay utilized for NfH is sensitive more compared to NfL.

Previous studies mentioned that, NfL is considered to represent subunit as soluble mostly, with concerns regarding proteases susceptibility, particularly blood or protease-rich CSF (Koel-Simmelink et al., 2011). Various groups include current study; have thus focused on NfH being axonal damage biomarker, as the state phosphorylation thought to be stable more (Petzold et al., 2003).

In CSF, levels of NfL were proposed being higher in various MS stages in comparison to healthy individuals and in remissions versus relapses (Norgren et al., 2003). Other studies also show relatively NfL levels weak associations along EDSS (Norgren et al., 2004).

Taken together, present study proved and expanding the foregoing findings prescribing Nf being neurodegeneration markers as quantitative for serum. Currenty present data showed NfH and NfL are significant biomarkers prerequisite. Generally, it is significant to observe according to such findings, one cannot conclude NfHis is analyte superior compared to NfL. Few of present results are reflect likely used assays properties, but not such proteins properties as whole. All current analyses were performed on serum for both Nf and NfH, these measurements considered as mostly relevant being practice clinically, which appeared approach of promise aiming towards measurement of NfH and NfL in plasma/serum samples. Thus, present results confirm Nf role as a neurodegeneration useful measurement and their usefulness as potential for surrogate measurement in treatment of MS (Kuhle et al., 2010).

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