Investigation of anti-neurofascin and anti-myelin oligodendrocyte glycoprotein antibodies in patients with chronic inflammatory demyelinating polyneuropathy

by

Ali Burak Kızılırmak

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Koç University

Graduate School of Health Sciences

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Ali Burak Kızılırmak

and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
examining committee have been made.

Committee Members:

_____

Assoc. Prof. Atay VURAL, M.D. PhD. (Advisor)

_____

Prof. Yasemin GÜRSOY ÖZDEMİR, M.D. PhD.

_____

Prof. Erdem TÜZÜN, PhD.

Date: _____

To Meryem Kızılırmak

ABSTRACT

Investigation of antibody positive CIDP patients in the Turkish cohort

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Chronic inflammatory demyelinating polyneuropathy (CIDP) is a chronic immune-mediated peripheral neuropathy that can affect people of all ages. Walking difficulty, arm and leg weakness, numbness and tingling and tremor can be seen in CIDP. In around 2-18% of patients diagnosed with CIDP, antibodies against paranodal antigens, including Neurofascin 155 (NF155), Neurofascin 186 (NF186), Contactin1 and Caspr1 can be detected in the serum, identifying this subgroup as seropositive CIDP. Treatment approach can be different is this subgroup compared to common CIDP, therefore, it is important to identify these patients. However, antibody tests against the paranodal antigens are not available in Turkey.

In this study, we first aimed to establish a live cell-based assay to test the presence of antibodies against NF-155, NF-186 in the serum and cerebrospinal fluid of patients. Then we aimed to test the sera obtained from a nationwide cohort of CIDP patients for these antibodies, as well as anti-MOG antibodies to identify the frequency and characteristics of anti-NF155 antibody positive CIDP in Turkey.

The live cell base assay method was applied to live, unfixed cells to preserve the structural integrity of the NF protein. Initially, plasmids containing NF155-EGFP, NF186-EGFP or EGFP (as control) gene was transfected into HeLA cells using a transfection reagent. Then, patient serum or CSF was incubated the transfected cells followed by incubation with a biotin-conjugated secondary antibody. Finally, Alexa fluor-647 conjugated streptavidin was added to detect antibody binding by flow cytometry. Delta and ratio of mean fluorescent intensity (MFI) was calculated by using the data from protein and GFP-only expressing cells. A cut-off value was determined by using sera from healthy controls.

We tested sera from 196 CIDP patients. The patients were 38.2 (21.3) years old on average, with a male to female ratio of 1:1.3. 161 patients were adult, and 35 patients were pediatric. A total of 11 samples were positive for NF-155-IgG. Among these samples, 7/11 had IgG4 as the dominant isotype, verifying anti-NF155 antibody positivity. Detailed clinical information was present in five patients, that was compatible with the clinical characteristics of seropositive CIDP patients previously reported in the literature.

Among seven NF155-IgG4+ patients six were adults and one was a pediatric patient. IgG2 and IgG3 antibodies were also present in addition to IgG4 subtype in three patients.

The frequency of anti-NF155 antibody positivity in the Turkish cohort was found to be 3.5%. In the rest of the four patients no IgG positivity was detected and low IgM positivity was detected in three samples.

CSF from one NF155 IgG4 positive patient was also analyzed and showed the presence of antibodies in the CSF, as well. In another 13 CSF samples tested from CIDP patients one samples had low titers of NF155-IgG, NF186-IgG and MOG-IgG. This patient's serum was negative for these antibodies. Among 12 Guillain-Barre Syndrome (GBS) CSF samples tested, two patients had low titers of NF155-IgG. Among eight MS patients and 55 patients with other neurological disorders, none of the CSF samples tested positive for NF155-IgG or NF186-IgG.

In addition, we analyzed the presence MOG antibodies in CIDP patients. Serum tests were positive for MOG antibodies in nine patients. Two of the patients were children. Pediatric patients were diagnosed with CIDP and combined central and peripheral demyelination syndrome (CCPD). Adult patients comprised the remaining seven cases, of which two were diagnosed with CCPD and the remaining five with CIDP. In the nine patients with positive serum MOG-IgG, NF155 and NF186 antibodies were negative. Analysis of 16 CSF samples obtained from CIDP and GBS patients revealed that, five patients had low titers of MOG-IgG. Serum MOG-IgG was found to be borderline positive in two of these patients. In the other three patients, serum positivity was not detected. Among patients with both serum and CSF MOG-IgG positivity, one patient had CCPD and the other had CIDP.

In conclusion, we have established a live cell-based assay to test anti-NF155 and anti-NF186 antibodies from serum and CSF. We further identified the frequency of these antibodies in a large cohort of Turkish CIDP patients containing adult and pediatric patients, together with their immunological and clinical characteristics. Additionally, we found that MOG-IgG can also be present in the serum and CSF of CIDP patients as well as CCPD patients.

Anahtar Kelimeler:
CIDP, CCPD, autoantibody, neurofascin, MOG

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ABBREVIATIONS

AIDP	Acute Inflammatory Demyelinating Polyradiculoneuropathy
ALS	Amyotrophic Lateral Sclerosis
Caspr-1	Contactin Associated Protein Receptor-1
CCPD	Combined Central and Peripheral Demyelination

CIDP	Chronic Inflammatory Demyelinating Polyneuropathy
CMV	Cytomegalovirus
CNTN-1	Contactin-1
DADS	Distal Acquired Demyelinating Symmetric Neuropathy
EBV	Epstein-Barr Virus
EMP	Extracellular Matrix Proteins
FBS	Fetal Bovine Serum
GBS	Guillain-Barré syndrome
HMN	Hereditary Motor Neuropathy
HSP	Hereditary Spastic Paraplegia
IVIG	Intravenous Immunoglobulin
MADSAM	Multifocal Acquired Demyelinating Sensory and Motor Neuropathy
MMN	Multifocal Motor Neuropathy
MOG	Myelin Oligodendrocyte Glycoprotein
ON	Optic Neuritis
PNS	Peripheral Nervous System
P/S	Penicillin/Streptomycin
SMA	Spinal Muscular Atrophy
TM	Transverse Myelitis

Chapter 1:
INTRODUCTION

1.1 Polyneuropathy

Polyneuropathy is the term for damage to many PNS nerves at once. Patients with polyneuropathy may have numbness, burning sensation, and pain in their hands or feet. In addition, these main symptoms are related to an important neurological disorder that occurs with muscle strength, numbness, weakness, and impaired sensory functions (Claudia Sommer et al., 2018). Polyneuropathy is the term used when they are linked to simultaneous injury to several nerves.

A variety of factors and diseases can contribute to polyneuropathy, which is not a single condition. Consequently, there are numerous varieties. Several possible sources of polyneuropathy include genetic, toxin, immune system, cancer, viral, metabolic, infectious, and lymphoproliferative conditions. One or more metabolic diseases that might cause neuropathies include diabetes, kidney illness, and neuropathies brought on by a lack of a certain vitamin. In addition, toxins, toxic metals, and several chemotherapeutic drugs are recognized causes of neuropathies. In terms of heredity, Motor neuron diseases (SMA, ALS, X-linked bulnospinal muscular atrophy, HMN, HSP etc.) can cause neuropaties (Barohn & Amato, 2013; Claudia Sommer et al., 2018). The term "Immune-mediated polyneuropathy" refers to neuropathies that are caused by and connected to the immune system. Examples include GBS, CIDP, DADS and etc.

1.2 Classification of immune mediated Polyneuropathy

Inflammatory neuropathies are peripheral neuropathies characterized by the body's inflammatory response in peripheral nerves. Neuropathies developing due to immune disorder are called especially GBS, CIDP and CIDP variants, DADS (anti-MAG positivity), MMN (Multifocal motor neuropathy) and Vasculitis. On the other hand, infections that cause infectious neuropathies could be related to hepatitis (A,B,C), Lyme, HIV, CMV, EBV. Polyneuropathies associated with cancer and lymphoproliferative diseases can be lymphoma, myeloma, paraneoplastic subacute sensory neuropathies etc. The thesis will later explain CIDP-GBS.

1.3 CIDP disease

The PNS is affected by the trouble inflammatory disease which is known as CIDP. In addition to CIDP being the most prevalence of chronic immune-mediated peripheral neuropathy, the incidence of CIDP has been known to be approximately 1 to 8 /100.000 in the literature (Kira, 2021) (MCOMBE, POLLARD, & MCLEOD, 1987) . CIDP can affect people of all age, although men and adults in their middle years are substantially more likely to develop it. The CIDP's clinical progression can be extremely severe between the ages of 20 and 40; however, a senior population shows a more progressive course. Walking difficulties, arm and leg weakness, state of numbness and small or continiously tingling sensation in the fingers and toes , as well as feeling unwell in the body, are all signs of CIDP. Within a few months, these symptoms can appear, and they might get worse with time.

Correspondingly, we aimed to look at patients clinics who had positive NF155-NF186 autoantibodies as part of our research. This is primarily since some subgroups of CIDP patients experience antibody positivity, and research from the literature point out that this subset of individuals needs a particular course of care. The identification of autoantibodies facilitates diagnosis and expands therapeutic options. Especially patients with seropositive-CIDP have autoantibodies against Neurofascin-155 and Neurofascin-186 in particular. There are many clinically distinct subtypes of CIDP, and seropositive patients may exhibit unique clinical characteristics from other CIDP subtypesParticularly in persons who have antibodies to NF155-IgG4, the illness is usually referred to as paranodopathy or nodopathy. The main role of NF155-IgG4 is to block interactions between paranodal proteins, particularly in patients who are IgG4 positive typically resistant to conventional immunotherapies. Additionally, IgG4 does not activate complement, so C1q cannot bind to IgG4'. Also, IgG4 exhibits some FcR site binding, but not as much as FcR and C1q binding, which are indispensable for the IgG1 and IgG3 antibodies effector functions. By preventing protein interactions, such as the interaction between NF155-CNT1/CASPR, IgG4 antibodies can make protein junctions dysfunctional and impair their ability to function. Additionally, IgG4 is dysfunctional for internalizing antigens and exists in living things in a bispecific form (J. I. Kira, R. Yamasaki, & H. Ogata, 2019). A disorder of nerve conduction may result from this. The fact that IgG4 titers sharply decrease in response to rituximab therapy suggests that,

unlike IgG1 antibodies, IgG4- generative cells do not survive as long as plasma cells (Vural, Doppler, & Meinl, 2018). IgG subtyping changes as a result of B cell activation and the following response in the germinal center with T helper cell assistance. There is a progressive shift toward IgG3>IgG1>IgG2>IgG4 with enhanced somatic hypermutation and ongoing functionally hetero-bispecific half-antibody exchange. IgG4 cannot efficiently crosslink target antigens. Response to rituximab therapy results in a sharp decline in IgG4 antibody titer. IgG4 demonstrates minimal FcR binding, C1q cannot be bound, and the complement system cannot be activated (Vural et al., 2018). On the other hand, autoantibody testing for the majority of CIDP patients is negative. Patients who are antibody positive display distinctive traits, such as a poor response to high doses of IVIg, and their clinical manifestations are extremely different from those of patients who are antibody negative for CIDP (Mathey et al., 2007), (Doppler et al., 2016).

In addition to, IgG1 subtyping was found in 3 patients of 4 positive GBS cases with NF155 antibodies, whilst IgM was found in 1 patient (Burnor et al., 2018; Ng et al., 2012; Ogata et al., 2015).

In addition, we concentrate our research on CIDP patients who have MOG positive antibodies (Part 1.6). Evidence of CNS involvement has been identified in MOGAD patients who have Mog antibodies. MOG antibodies are infrequent in PNS involvement patients. According to studies, MOG antibodies have been discovered in some patients with atypical inflammatory neuropathy, CCPD, and other inflammatory neuropathies (Vazquez Do Campo, Stephens, Marin Collazo, & Rubin, 2018), (Rinaldi et al., 2021), (Dinoto et al., 2022a). Taking into account these outcomes and results, we sought to identify MOG-positive patients in our Turkish cohort.

1.3.1 Clinical

Between the ages of 20 and 40, the disease takes on a much more severe course. It does, however, this illness manifests itself in progressive a gradual manner among the elderly. Patients' general complaints include difficulty walking, which starts and progresses over a few months, state of numbness, feeling tingling in their body, and lacking feeling and sensation in the fingers and toes, and weakness in the extremities, which is usually symmetrical.

Depending on which part of the nerve is affected by the polyneuropathy, there are 'demyelinating' polyneuropathies due to damage to the sheath surrounding the nerve or 'axonal' polyneuropathies wherefore in light of harm to the primary trunk of the nerve . Polyneuropathies are sometimes characterized as acute or chronic depending on how quickly the condition develops. Acute occur rapidly developing disease and chronic occur slowly developing disease. Acute Inflammatory Demyelinating Polyradiculoneuropathies are called AIDP. Chronic Inflammatory Demyelinating Polyneuropathy is called CIDP. The distinction of CIDP from AIDP is due to their temporal profile and long-term consequences. Symptoms and neurological problems seen in AIDP also improve within weeks. Then the gradual recovery routine continues. In CIDP symptoms and neurological problems develop slowly over months (at least 2 months) (Mansour et al., 2020; Peltier & Donofrio, 2012).

Autoimmune demyelinating polyneuropathies are caused by immune system illnesses that destroy the myelin sheath around peripheral nerve fibers. This category of disorders is both acquired and treatable. In this group, It includes CIDP, DADS, MADSAM and MMN. Anti-GM1b antibodies can be applied to aid the identification of MMN, as well as anti-MAG antibodies in the determination and diagnosis of DADS (Cats et al., 2010; Menon, Katzberg, & Bril, 2021).

1.3.2 Immunopathogenesis and detection antibodies

In immune-mediated diseases like CIDP, which is a polyneuropathy, it is hypothesized that the pathophysiology of CIDP disease is influenced by cell- and humoral-mediated pathways (Mathey et al., 2015). Polyneuropathies come in two different forms: "demyelinating" polyneuropathies, which happen when the sheath encasing the nerve is harmed, and "axonal" polyneuropathies, which occurs when the nerve's primary trunk is compromised and damaged. According to some research, CIDP nerve biopsy tests have revealed T cell infiltrates and demyelination caused by macrophages. (Schmidt et al., 1996). CIDP patient sural nerve biopsy samples showed cell infiltrates. The cell types commonly displayed in these cell infiltrates were macrophages, CD4 T and CD8 T cells (Schneider-Hohendorf et al., 2012) (Schmidt et al., 1996),(Cornblath, Griffin, Welch, Griffin, & McArthur, 1990).

With the recent identification of various antibodies against cell adhesion molecules which are node-paranodal region, they have been identified as biomarkers related to the etiology of CIDP (Wang et al., 2022). In autoimmune diseases, different autoantibodies that target myelin proteins have also been discovered. In recent years, research has concentrated on antibody-mediated autoimmune illnesses, as these antibodies are thought to be essential to the disease's etiology (Vural et al., 2018). Recent researchs have revealed that antibodies to these antigenic PNS components known as neurofascin (NF) NF155, NF186, CNTN1, Caspr autoantigens are present in about 5.5% of CIDP patients (Cortese et al., 2020), (Kira, 2021).

Some part of the patients have autoimmune antibodies, which influence the etiology of the disease. The majority of antibody-positive patients showed antibodies to the NF155 protein, with IgG4 being the most prevalent subtyping (J.-i. Kira, R. Yamasaki, & H. Ogata, 2019). In GBS cases, antibody positivity is also extremely uncommon (Devaux, Odaka, & Yuki, 2012). In one of the two studies that reported anti-NF186 positivity, anti-NF186 was found positive in 5 patients in a large patient group with 246 CIDP cases in Europe (Delmont et al., 2017; Ng et al., 2012). These antibodies were discovered to have similar reactivity to NF155 and NF186 proteins (Desmazieres et al., 2014). The patients exhibited a more severe clinical presentation than the antibody-negative CIDP cases, and their IVIg response was superior to the anti-NF155-positive cases. There have been some claims that antibodies may be cross-reacting against NF-155 and NF-186, and investigations have revealed that when the binding sites of antibodies that recognize these two isoforms are studied, they detect Ig-domains, which are shared structures in the isoform. The bulk of antibodies specific for NF155, nonetheless, it has been established that they are IgG4 and generated exclusively for the FN3-FN4 domain of the neurofascin protein, i.e., specific to it (J.-i. Kira et al., 2019). In another research where anti-NF186 positivity was found, a 50-year-old male case with a form of CIDP nearly as severe as "locked-in," recognizing all three NF isoforms, but with the highest titer of anti-NF186 positivity, was reported. In some extensive studies on pan-NF antibodies, IgG1-panNF has been linked to very troubled and severe disease progression and, more importantly, mortality, and in some cases, people with pan-IgG1 antibodies have also been found to be bind to rapidly progressive tetraplegia. Eight individuals suffered very severe neuropathy, and this severity was linked to pan IgG1. Four of the eight patients in this study died because of the investigation, which was

reported in a disease course. Whether used separately or in combination, IVIG, steroids, and plasmapheresis did not reduce the patients' mortality significantly. Only IgG1 was positive in all 8 patients; other subtyping were negative (Fehmi et al., 2021). When IgG1-pan NF antibodies were identified in 100% of cranial nerve deficiency positive patients, 28% of seronegative patients; also, in addition to 75% of autonomic dysfunction IgG1-pan positive patients, and 13% of seronegative patients when they were compared to seronegative patients. Additionally, pulmonary involvement was seen in 88% of individuals with pan NF antibody positivity compared to 14% of patients with seronegative antibodies. In contrast, a cross reaction has been put forth as a theory to account for the existence of antibodies that bind to all three pan-NF155,186,140 antibodies (Fehmi et al., 2021).

In the majority of Pan-NF patients, atypical-CIDP has been noted. Additionally, individuals with the most recent diagnoses of GBS and CIDP had pan-NF antibodies found in their bodies. All IgG3,2,4,1 subtypings may be detected in pan-NF, and these patients had positive results for all three neurofascin isoforms. These patients were also shown to have rather severe symptoms. In Appeltshauser's study from 2022, 11 patients had antibodies to all three NF isoforms. Particularly regarding complement binding and cytotoxic impact, IgG3 has been associated (Appeltshauser et al., 2022).

The Fibronectin-fibronectin domain is only detected by the antibody of NF155 positive patients, while the mucin-FN5 domain is only recognized by the antibody of NF186 positive patients (Ng et al., 2012). Pan-NF antibodies were identified to bind to the nf protein's Ig domain (Fehmi et al., 2021). Additionally, they contrasted panNF antibodies with those that were only focused on NF155. In comparison to samples associated simply with NF155, the rare modified Rankin scale (mRS) is much greater. While ataxia (3/8) and neuropathic pain (4/8) are occasionally (rarely) present in Pan NF patients, CNT1, CASPR were substantially less common in antibody positive samples (Fehmi et al., 2021). One analysis revealed that 2 out of 7 patients had axonal binding while 5 out of 7 patients had nodal binding. Here, it is demonstrated that there is axonal binding (a distribution function), but there is neither infiltration nor significant demyelination. In conclusion, neuropathies resembling nodo-paranodapathy rather than demyelination are more prevalent in pan-IgG1 individuals (Fehmi et al., 2021). To

summarize, in nowadays only non-IgG4 individuals could not be discriminated from seronegative ones (Cortese et al., 2020). It was later found out that there may be a different clinic other than IgG4 positive patient and antibody negative patient. There is a high possibility that GBS will be detected as the initial diagnosis in this technique due to significant differences in clinical features, disease severity, and mortality between seropositive and panNF-IgG1 patients. Because NF155 expression occurs in both PSS Schwann cells and CNS oligodendrocytes, it causes mixed demyelination. Demyelination in combination is referred to as CCPD (Ogata et al., 2016). In a study, the percentages of patients in each disease category who had anti-neurofascin antibodies that were positive were calculated as follows. Out of 16 patients with a final diagnosis of CIDP, 4 patients had antibodies found, compared to 45-86% of patients with CCPD. Additionally, antibodies were found in 2/10 patients (20%) with AMAN and 1/7 patients (14.3%) with AIDP (Kawamura et al., 2013). In contrast to this study, other studies have found the reverse. This could be due to CCPD disease or poor patient cohort identification criteria, as well as the cohort's broad ethnic origin (Vural et al., 2018).

On the other hand, several autoimmune illnesses have begun to take molecular mimicry and related factors into account. Even though 4–30% of patients report recent infections before experiencing neurological symptoms, the cause of the autoimmune response and any infectious agent directly linked to the illness are yet unknown. The main inflammatory cells infiltrating nerve biopsies in CIDP are macrophages and form clusters around endoneuria vessels (C. Sommer et al., 2005). As an innate system component, macrophages deliver the antigen and release proinflammatory cytokines extracellularly during the immune response. By phagocytosis, macrophages and microglia participate in the demyelination of the central and peripheral systems, the peeling of the myelin sheath (Kiefer, Kieseier, Stoll, & Hartung, 2001). Axonal degeneration is more frequent, CIDP negative individuals have more demyelination and inflammation, while antibody positive patients occasionally have less demyelination and inflammation (J. I. Kira et al., 2019).

Also, In a European study on HLAs, it was stated because of the studies that the HLA type2 allele had a significant relationship in anti-NF155 CIDP patients from that

cohort. In addition, another study strongly associated HLA-DRB1*15 present in a proportion of patients with CIDP who had NF155 antibodies (Martinez-Martinez et al., 2017).

In a 2007 study, it was discovered that passive transfer of NF antibodies to the animal in the EAE model created by administering mog-specific T cell rats exacerbated the severity of the disease. Therefore, it was thought that NF antibodies facilitate axonal pathology in chronic progressive MS (J. I. Kira et al., 2019). Also, in another study, NF pan antibodies in the EAN model increased the severity of the model and produced prolong neurites. Another more important finding was that NF antibodies were not pathogenic on their own. Edgar Meinl in 2012 article, AntipanNF was added to the EAN model that the P2 peptide caused, and it was discovered that NF targeting made diseases worse (Ng et al., 2012).

In addition to all these, especially in IgG4 NF155 antibody positive paranodopathies, diffuse spinal root hypertrophy as well as a significant and clear increase in CSF protein occur. These conditions suggest that patients with these antibodies have extreme nerve root inflammation. Increased CSF protein level and proinflammatory cytokines were found in IgG4 anti NF155 patients. It is thought that the cells producing these cytokines may be T cells specific for NF155, and they may play an active role in inflammation of the spinal root and CNS (J. I. Kira et al., 2019).

1.3.3 Treatment

IVIg or corticosteroids are utilized in treatment, either alone or in combination with immunosuppressive medications (most commonly azathioprine). Plasma exchange and other immunosuppressive drugs are used in unresponsive cases. There is no benefit against steroid therapy in MMN, therefore IVIg therapy is primarily administered for MMN patient. In a study of five MMN cases, none of the patients reacted to PE, cyclophosphamides, or steroids. However, all of these five patients responded quite well to IVIg therapy (Giancarlo Comi, 1994).

According to reports, glucocorticoids work well for CIDP (Austin, 1958). CIDP treatments may be like in GBS. Corticosteroids, intravenous immune globulin, and plasmapheresis have been shown to improve in approximately 65 to 70% of patients

(Dalakas, 2011). IVIg used in classical treatment. IVIg affects modulating macrophage Fc receptors, amount of adhesion molecules, arrangement of remyelination, complement protein regulation, and other processes (Kira JI et al., Front Neurol. 2021 ; (Peltier & Donofrio, 2012). These variants, however, include neurofascin 155 and CASPR1, which form a subset of inflammatory neuropathies with IgG4 autoantibodies against paranodal proteins. These situations are special because of their pathophysiology and the fact that they do not improve with traditional treatment (Doppler & Sommer, 2017). Given the exceedingly poor IVIG response in CIDP patients with positive NF155 antibodies, it is safe to presume that these individuals are IVIG resistant (Devaux et al., 2016). Rituximab and steroids work better on IgG4 positive patients than IVIg, it has been found (Wang et al., 2022).

Although IVIg inhibits the complement system, it also reduces the fc receptor of macrophages and is responsible for the regulation of the immune system by reducing the production of adhesion molecules, it was observed that IVIg was insufficient in NF155-IgG4 antibody positive cases. It has been interpreted as inhibiting protein function and inhibiting its binding in antibody positive cases. Rituximab provided significant improvements in the NF155+ CIDP group with poor response to IVIG treatment. (Querol L et al., Devaux JJ et al. Shimizu S et al.). In short, Rituximab is a very good option for patients with CIDP who have autoantibodies to nodal/paranodal proteins resistant to conventional therapies. Weakness of IVIg response in IgG4 positive patients is under investigation, and one of the views from the research is that IgG4 does not activate complement system molecule cascade ; so steroids, plasmapheresis or B cell depleting agents such as Ritiksumab should be used in IgG4 positive patients (Wang et al., 2022). For this reason, autoantibodies should be investigated in patients unresponsive to conventional therapy.

1.4 Seropositive cidp

As indicators connected to the etiology of CIDP, certain antibodies against nodal-paranodal cell adhesion molecules have been identified. Low antibody seropositivity and NF, Contactin1, and Caspr are all of them related with CIDP illness. In contrast to seronegative CIDP, NF155-positive patients typically have IgG4 subtyping, are

clinically substantially different, and have a significantly lower rate of demyelination and overt inflammation. More axonal dysfunction and, ultimately, axonal degeneration, occur in antibody positivity. In other words, the dissection of the myelin loops in the paranode of the axons characterizes the pathology brought on by antibodies, and the axonal degeneration that takes place there is categorized as nodopathy and paranodopathy. Age at illness beginning varies between 50 and 70 years for patients with seronegative and NF186-positive status and between 20 and 30 years for those with NF155 seropositive status. Tremor, sensory ataxia, and an inability to respond to IVIg are more prevalent in NF155 seropositive patients. Clinically, patients with NF155 antibody positivity may also have a substantial rise in CSF proteins and an increase in the nerve roots of the MR contrast agent gadolinium. Additionally, the CNS may exhibit 5–15% demyelination in NF155 seropositive patients (Vural et al., 2018).

When subtyping is carried out on NF186 positive patients, it has been demonstrated that they are typically IgG3 and IgG4 antibodies (Vural et al., 2018). Also, Patients who result positive for the anti-NF155 antibody typically have a few common clinical problems. The majority of individuals with this antibody appear to have IgG4 that does not activate complement, and these symptoms include tremors, sensory ataxia, swollen nerve roots, high levels of bos protein, and expanded nervesThese patients responded very poorly to IVIg. According to some research, patients who test positive for IgG4 respond better to rituximab and steroids than to IVIg. The pathophysiology of CIDP disease is thought to be greatly influenced by autoantibodies against NF that trigger a humoral response, and later research has revealed that isotypes of IgG4 in NF155 have distinct clinical characteristics. Neuropathy in IgG4-positive patients is not leaded to by macrophage-mediated demyelination but by humoral immune-mediated paranodal dissection (Wang et al., 2022). W. Wang, 2022; In that study, 11 of 17 patients had IgG4, while IgG3 and IgG1 were detected in the remaining 6 patients. Furthermore, it was discovered in this study that individuals with this distinct isotype differ clinically from individuals with the IgG4 isotype in a variety of ways (Wang et al., 2022). In addition, IgG4-NF155 antibodies prevent paronodal complex formation and are thought to exacerbate demyelination in this way. Given everything above, it is claimed that nerve root hypertrophy causes elevated protein levels in the CSF. Patients with antibody-positive IgG4 respond poorly to IVIg due to the lack of macrophage ,

complement activation (Nirula, Glaser, Kalled, & Taylor, 2011). Plasmapheresis and corticoids are effective (Ogata et al., 2015). Due to its potent role in B-cell depletion, rituximab has proven beneficial in IgG4 patients resistant to other drugs (Wang et al., 2022).

1.4.1 Differences of CNS between PNS

The PNS and CNS are different in some ways, despite their same basic structure and constituent parts. In PNS, the microvilli that come from the Schwann cells occupy the nodal space. Schwann cells at PNS nodes release trimers of gliomedin into the nodal region. The microvillar membrane's NrCAM is bound by these trimers. By establishing an NF186-binding site with high affinity, this complex encourages their aggregation at the node. Oligodendrocytes do not produce microvilli in the CNS; instead, extracellular matrix proteins occupy the nodal space. Versican, Brevican, and other EMP are among those with which NF186 interacts in this region. The only difference in the paranode that has been identified so far is the interaction between NF155 and ankyrin G in the CNS, when it is interaction ankyrin B in the PNS. Additionally, there are variations in tight junctions.

1.4.2 Protein structure of nf155/186

Neurofascin protein is consist of 4 main isoform which are NF155, NF186, NF180, NF166. The dominant isoforms in the mature nervous system are NF155 and NF186 (Kira JI et al. Front Neurol. 2021). NF155 and NF186 is located in myelin protein. There are four components that consist of the myelinated structure: nodes, paranodes, juxta-paranodes, and internodes. While Schwann cells, which create neurofascin in the PNS, oligodendrocytes create it in the CNS. NF155, which can be found in a paranode. On the other hand, NF186 is found in the node and the initially axon segments.

Neurofascin proteins are polypeptides composed of six Ig-like domains, up to five type III fibronectin domains (FNIII domain), a domain of a transmembrane part, and a small cytoplasmic domain. Two major isoforms of neurofascin occur: neurofascin-isoform 186 (NF186) and neurofascin-isoform 155 (NF155). A transmembrane protein called neurofascin 186 has four Fn-domains, one mucin domain, and six Ig-like domains. While proper paranodal junction formation necessitates the presence of NF155

at the paranode, NF186 expressed at the node is essential for Na+ channel clustering (Ng et al., 2012). Ankyrin G is a molecule associates with Na+ channel and NF186. And in the cytoplasm, ankyrin G interacts with βIV-spectrin, thus grouping all molecules. In the PNS, extracellular NF186 interaction has been connected to soluble gliomedin. Moreover, In PNS, NF186 association with cell adhesion molecule NrCAM that is placed on the Schwann cell microvilli which fills the space of nodal area while NF186 associates with EMP in the CNS (Kira, 2021). The mucin molecule connects the FN-4 and FN-5 domains in with NF186.

The NF186, known as isoforms of neurofascin 155, lacks the mucin domain and possesses a Fn3 domain rather than a Fn5 domain (Liu, Focia, & He, 2011). NF155 engages in interaction with CNTN1 via its Ig domains. Through its cytoplasmic domain, the NF155 engages in interaction with the ankyrin B proteins (Thaxton et al., 2010), (Susuki, Otani, & Rasband, 2016), (Ng et al., 2012), (Devaux et al., 2016).

Three distinct fibronectin type III domains, FN1, FN2, and FN4, are present in NF166, along with additional isoforms, but no musin domain. Six immunoglobulin (Ig)-like domains are also present in it. NF180 has three FN1, FN2, and FN4 fibronectin type III domains in addition to six Ig-like domains and one mucin domain (Kira, 2021).

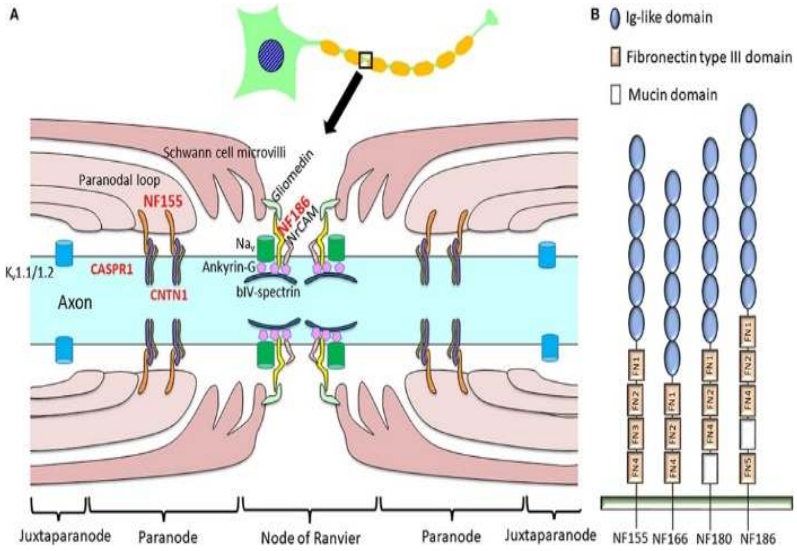

Figure 1-1.1: Neurofascin structure and location (Kira, 2021).

1.5 Guillain-Barré syndrome (GBS)

Acute immune-mediated neuropathy with a increase risk of morbidity and mortality, GBS is classified as such. Patients with fast progressing disease may experience severe symptoms such sensory paresis of both hind legs, autonomic and cranial nerve involvement, and occasionally respiratory failure. However, this disease has a diverse etiology and own special clinical manifestations (Appeltshauser et al., 2022).

The most extensively type of GBS is AIDP. In AIDP disease, the myelin sheath is damaged and as a result of this damage, signal transmission between neurons is interrupted, which causes muscle weakness, numbness in some parts of the PNS, and even temporary paralysis in patients. Other types of GBS are consist of AMAN, AMSAN, MFS. GBS spreads quickly and begins in the feet and legs before moving up to the upper body. All age groups are susceptible to GBS, however it appears as a disease with a higher risk as you get older. Also, Studies examining prevalence by gender reveal that men experience this condition more frequently than women.

Some infective causes that trigger GBS are as follows; Infection with Campylobacter, Influenza virus, AIDS, CMV, EBV, various Hepatitis. In addition, diseases such as Traumas, Surgeries, Hodgkin Lymphoma can trigger GBS.

Majority of GBS are AMAN and AIDP. AMAN affects only motor nerves and axonal distraction occurs, however, there are no sensory signs. In AIDP, demyleization occurs. The antibodies found in AMAN are GD1a, Ga1Nac-GD1a , GM1a, GM1b. The difference between chronic inflammatory demyelinating polyneuropathy and GBS is that although CIDP is similar to Guillain Barre Syndrome, it progresses more slowly. It's crucial to quicken the healing process, and plasmapheresis is recommended and preferred (Peltier & Donofrio, 2012). In this method, the blood in your body is taken, the plasma part is separated from the blood cells and the blood cells are put back into your body. Thus, the antibodies in the immune system that attack the nerve cells are reduced. These are used to reduce the attack of your immune system on your body. NF155 positivity is found in AIDP subtype of GBS and in CIDP patients. NF155 antibodies are responsible for the enhance and prolong disease of the disease. IgG1,3 was generally found in AIDP-associated NF155 positivity. In the article published by Edgar Meinl in 2012, it was found that the OD value decreased in the patient with plasma exchange, and the titration of the antibody decreased (Ng et al., 2012). In addition, 3 patients of 4 positive GBS cases with

NF155 antibodies had IgG1 subtyping, while 1 patient had IgM (Burnor et al., 2018; Ng et al., 2012; Ogata et al., 2015).

By examining patient spinal fluid, they can ascertain the level of protein in the CSF (CSF test) (Illes & Blaabjerg, 2017). It is widely accepted that CSF protein levels increase with patient aging under physiological conditions. It has been demonstrated that it is critical to include this protein increase with aging when interpreting CSF protein testing (Hegen et al., 2021). The protein level of people with GBS is high, or by placing thin needle electrodes on the muscles they want to examine with EMG, their activity can be measured (Hegen et al., 2021).

1.6 Mog antibody positive in PNS

CNS involvement has been discovered in MOGAD individuals who have reportedly tested positive for Mog-ab (Rinaldi et al., 2021). ON and TM are among the CNS demyelination conditions that are accompanied with antibodies to MOG. Also, MOG-ab may rarely be associated with PNS involvement (Dinoto et al., 2022b). Resently study have been reported that some patients have got with CCPD have been found to have MOG antibodies. CCPD patient's high serum MOG-IgG1 point to a potential function for these antibodies in CCPD symptoms (Vazquez Do Campo et al., 2018). Inflammatory neuropathies, mixed central and peripheral demyelination disease (CCPD), and myeloradiculitis may be linked to MOGAD and may respond to immunotherapy. In this study, they also evaluated 19 patients in the MOGAD-PNS cohort, and one of these 19 patients had NF155 antibody positivity. This patient was NF155 positive, and both the PNS and CNS were affected (Rinaldi et al., 2021). Other study prescribed that some individuals with isolated Peripheral Neuropathy or CCPD have MOG positive sera. In this study, 3 percent or so of the peripheral neuropathy patients tested result in positive for MOG-IgG. They proposed that these MOG-IgG positive patients might exhibit CCPD and isolated PNS involvement, but more significantly, they explained that in a study related to our thesis study, they had found that the most prevalent clinical phenotype of MOG antibody positive patients, which was found in a small number of peripheral neuropathy patients, was **atypical** form of CIDP (Dinoto et al., 2022a).

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Chapter 2:
METHODOLOGY

2.1 Sample Preperation

People's blood was collected from about 30 different hospital facilities. 15 minutes were spent centrifuging blood samples at 2000 g. Centrifugation resulted in the separation of serum samples and kept in our refrigerators at -80 0°C. At room temperature, a 15-minute centrifugation of the 400-g CSF sample was performed. Take 1-2 mL of CSF and put it in a cryovial to keep it frozen at -80°C (Fig 2-1).

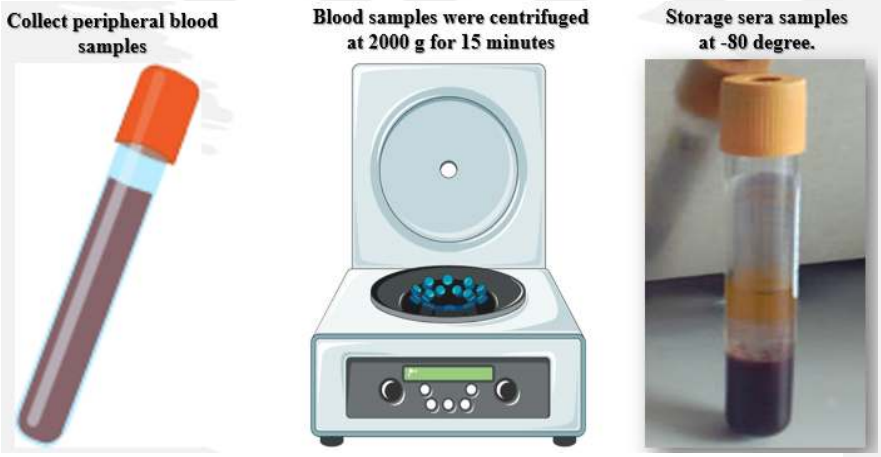


Figure 2-1:Stages of separation of sera from blood samples.

2.2 Patient

Our sera samples came from 29 research centers, these centers:
Akdeniz University, Ankara University Adult-Ped Neurology, Aydın Adnan Menderes University, Bakırköy Mazhar Osman Neurological Diseases Education and Research Hospital, Baskent University Faculty of Medicine, Bezmialem University, Dicle University, Çapa DETAM, Dokuz Eylül University, Düzce University, Ege University Medical Faculty Hospital, Erciyes University, Gaziantep University, Hacettepe Ped Neurology, Haydarpaşa Numune Hospital, Istanbul Medical Faculty (ITF), İzmir Dr.

Behçet Uz Training and Research Hospital, Kartal Hospital, Karadeniz Technical University Hospital, Koc University, Marmara Hospital, Prof.Dr.Cemil Topçuoğlu City Hospital, Sakarya University, Sami Ulus Hospital, Samsun OMU Faculty of Medicine, Sancaktepe Hospital, Sultan Abdulhamid Han Hospital, Tokat Gop University, Trakya University.

264 patients' serum samples were examined. Among the 196 patients, 19 had GBS, 196 had CIDP, 14 had CCPD, 20 had other neurological conditions, and 22 had HC. (Table 2-1). The patients were 38.2 (21.3) years old on average, with a male to female ratio of 1:1.3. 35 patients were children, and there were 161 adult patients. A total of 22 healthy control (+22 MS control group) sera samples were used.

There were 26 individuals analyzed from the CSF samples, 13 of whom were children. MS and other diseases were chosen as the healthy control. non-GBS, CIDP, CCPD, and disorders that are under treatment in CIDP patients' CSF samples. 55 samples of CSF from completely healthy controls. We studied 12 CSF samples from GBS patients and 14 from CIDP patients.

Table 2-1: The distribution of Sera and CSF samples studied with Cell based assay according to diseases is shown.

Live Cell Based Assay	CIDP NF assay	GBS NF assay	CCPD NF assay	MS NF assay	Control NF assay	Control Mog assay	CIDP/GBS Mog assay	CCPD Mog assay	OND Mog assay	MS Mog assay
Sera sample	196	19	14	22	22 HC	32	114 CIDP 7 GBS	12	15	
CSF sample	14	12	-		55	27	16			15

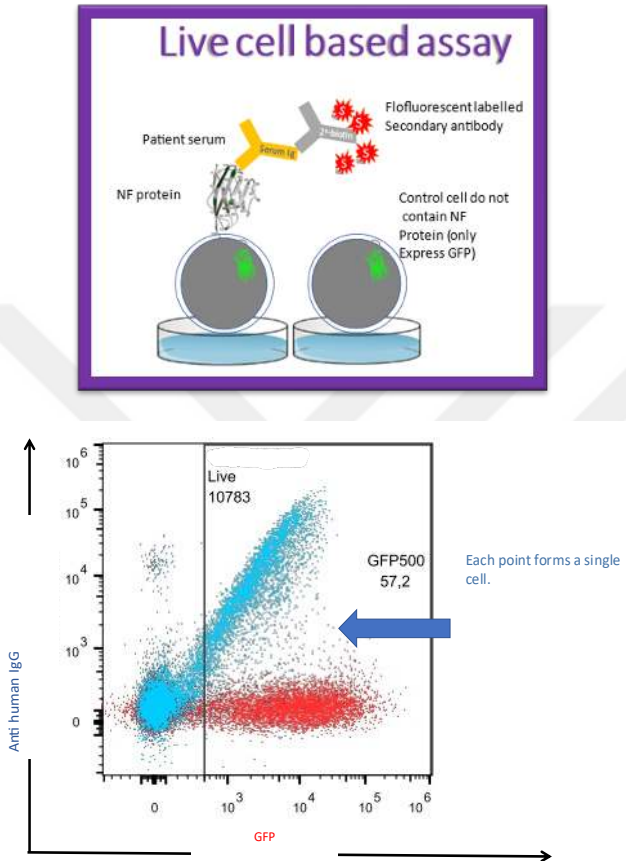


Figure 2-2:The antibody for NF protein assay.

The live cell base assay method has recently become the antibody for neurofascin protein analysis. It was applied to living, unfixed cells to safeguard the NF protein's structural integrity. Initially, The NF protein gene was transfected into HELA cells ,using a transfection agent like Fu-gene or PEI (a chemical agent). Patient serum was used to incubate the transfected cells. Then a secondary antibody called biotin was added. One Biotin is bound by a dye containing four streptavidin. Streptavidin was conjugated with AF647 fluorochrome. The molecule AF647 was the one that radiated in the flow device, allowing analysis to be done there. The NF protein does not exist in control cells. Control HELA cells only express the GFP proteins. The blue peak in the figure indicates the patient's positive result. If this blue image were almost red, we would say the patient does not have any NF antibodies.

2.3 Live cell based assay

The goal of our research study was to examine and characterize individuals who were either seropositive or seronegative for Neurofascin-Ab 155 or 186 utilizing a live cell-based test carried out in our laboratory. The diagnostic significance of these antibodies was evaluated for further research by evaluating the IgG antibody subclass level in seropositive individuals. For this investigation, sera from people with suspected CIDP were collected from several locations. A live cell-based test was the primary methodology, and a flow cytometer was used to assess the results. Hela cells were used in the investigation. Hela cells were transfected using the Neurofascin-155 and Neurofascin-186 plasmids. The cationic polymer polyethyleneimine (PEI) and FuGene HD were utilized in the transfection stage. The day following transfection we placed 50,000 Hela cells per well in a 96-well plate. PBS was used to dilute sera samples by 1/50 and the CSF sample by a 1:1 ratio. The secondary antibody was used Biotin- (Jackson Immuno-Research Lab, INC.-Code Number: 109-066-098, Lot Number: 140683). For analysis in the flow device, we used streptavidin, which binds to biotin and emits alexa Fluor 647. (Alexa Fluor® 647-conjugated Streptavidin- Jackson Immunoresearc lab,INC-Lot Number: 142388, Code Number: 016-600-084). Additionally, four streptavidin are coupled to one biotin. As a result, there is a greater fluorescent shine. Thus, keeping track of slightly positive readings was the aim. The viability of the cells was determined using the PI (propidium iodide) dye. (Fig 2-3).

A cell-based assay test was administered to 235 people who had CIDP suspicions, and the results were subsequently assessed with a flow cytometer. The cut-off was determined by comparing the results of 22 HC and 22 MS (healthy cut off—total 44 person) Health Control individuals. The cut-off was established using the formula "mean + 5*SD" applied to the information collected from the flow device. Serum samples from patients who had positive results were examined at least three more times. Additionally, by evaluating the IgG antibody subclass level in seropositive patients, the diagnostic value of these antibodies will be assessed. On samples that tested NF positive, subtyping was done. IgM, IgG1, IgG2, IgG3, and IgG4 were each evaluated. IgM is diluted 1:100, IgG1 is diluted 1:500, IgG2 is diluted 1:500, IgG3 is diluted 1:500, and IgG4 is diluted 1:100. (Fig 2-4).

Figure 2-3: Dilution rates for subtyping.

Previous studies used Western blotting and peptide-based ELISA to achieve that goal, however it was found that their specificity was restricted. This makes sense because in many investigations, peptide fragments or denatured proteins have been used rather than native proteins. Antibodies to protein epitopes that are present in their original form can be quickly and easily found using CBA.

2.4 Prepare of Bacteria growth medium:

2.5.1 Tryptic Soy Agar: (Lot: BCBQ1774V)

40g of the dried medium must be dissolved in 1 liter of filtered, sterile water. Sterilize for 15 to 20 minutes at 121 °C. To cool, use 45–60 °C. (This part is autoclaved at 121 degrees, solid mode, for 15 or 20 minutes.)

2.5.2 Tryptic soy broth (Lot:BCBP7923V)

30g of dried media should be dissolved in 1 liter of filtered, sterile water. Stir frequently while heating, then boil for one minute. Sterilization in an autoclave at 121 °C for 15 to 20 minutes. Prior to use, cool to 45 to 50 °C.

2.5 Obtaining Competent Cell:

It was combined with the highly suited for transformation DH5 strain of E. coli to produce our plasmids. However, I would choose the BL21 strain of E. coli if I wanted to see protein expression. The main difference between BL21 and DH5-Alpha is that BL21 is a competent E. coli cell created through genetic engineering that has the recA1 mutation and is primarily used for plasmid transformation, whereas DH5-Alpha is a competent E. coli cell created through genetic engineering that lacks the protease gene.

After defrosting, we removed a DH5-alpha strain cell from -80 °C. Spread out on the first piece of solid LB agar were the bacteria that were kept at -80°C. Each bacterial sample was prepared for use at -80°C by distributing the bacteria in the first solid LB since we could not determine if there was contamination in our bacterial stock at -80°C in liquid LB. As a result, the bacteria are seeded onto solid LB agar to see the contamination. (For instance, fungus contaminations). It stayed in the incubator at 37°C for 24 hours in solid LB. The next day it was started for starter bacteria culture. For starter culture, it was taken one colony from the colonies that appear on solid LB agar and was started starter culture in liquid LB medium (without antibiotics). (**Starter Culture: It will reproduce one or more of our colonies in 4-5ml liquid culture. For one colony, one day was required; for three to four colonies, five to six hours should be sufficient. According to the blurring of 4-5 ml of medium in the 15 falcon, it was deduced. Starter Culture can be greatly scaled up using it. A 250 ml flask was filled with 50 ml of LB liquid medium devoid of antibiotics. In the bacteria incubator with a shaker, 500 µl of bacteria that had been produced in the beginning culture were added. It can be stock the remaining 3ml of starter culture. For this, it was divided into three Eppendorf tubes, each holding one milliliter, and 2-3 drops of glycerol were added before it was heated to -80 °C. (Glycerol: Sigma-49782). It was important to add +500 µl of starter culture to 250 ml of medium on a daily basis in order to control the growth of the culture you generated. It should be removed from the culture after there is a sufficient density of cell growth visible. The following step is to distribute 50 ml of liquid medium to 50 falcons. The centrifugation stage comes next. 15 minutes of centrifuging at 3000 g. Once centrifugation has finished, pour off the supernatant. The calcium chloride technique is what we'll use. (CaCl2). 10 milliliters (ml) of 100 millimolar (mM) CaCl2 were diluted

with ice and held for at least two hours and up to a day. (***NOTE: All processes must be performed on ice and in the cold after applying CaCl2).

The following phase involved centrifuging at +4 degrees for 3000g for 15 minutes. Remove the pellet, then throw away the supernatant. Glycerol was added to CaCl2 to make it 15% glycerol. On top of the 10ml of take-15 falcon, 1.5ml of 100% glycerol was added. It was thoroughly mixed together to make the mixture. Resuspend the pellet by adding 3ml of the 5ml mix combination on top of it. (We still carry out the procedure on ice). The 200 µl of Eppendorf are distributed. (Ice was utilized with an iron rack.) It was swiftly taken after the aliquot and frozen in liquid hydrogen. It was then raised to -80°C.

2.6 Transformation of competant bacterial cells by heat shock

Prior to beginning the transformation, our heating was set to 42 degrees. Our plasmids had been frozen. It was extracted from the competent cells that we had previously grown at -80 degrees and placed on ice. A 1.5 cc Eppendorf tube that was empty was then placed on ice to cool. The competent cells were first collected in 25 microliters and placed in an empty tube on ice for transformation. 10% of the competent cell (2.5-1 microliter) from the plasmid was added. It was pipetted 4-5 times, and it was kept on ice for 15-20 minutes. After 20 minutes of incubation, the tube was quickly placed into the incubator, which had been preheated to 42 degrees for 90 seconds, and then quickly taken it out and put our sample on the ice. After 5 minutes on ice, 1 ml of fresh, antibiotic-free (LB-liquid) media was added to the same ependorphin. After that, place the Eppendorf in a 37 degree shaker incubator for 1-3 hours. Let the bacteria grow for a little while, then spread 100 microliters of the mixture of solid agar and antibiotic on antibiotic agar. For one day, incubate at 37 degrees. One day after the seed was placed on solid agar with antibiotics, some colonies were anticipated to appear in the solid bacterial plaque. The only colony on the plate was picked, and started as a starter culture in 5 ml of liquid broth containing antibiotics. Our plasmid, which only contains the GFP protein, has a kanamycin resistance gene, but the resistance genes for both of our NF155,186 proteins are ampicillin. This experiment used antibiotic rate in liquid media which was 100µg /ml. It was added our appropriate antibiotics as 100µg /ml to 5ml broth and waited for the growth of resistant bacteria in an incubator at 37 degrees for 3-4 hours until the

liquid became cloudy. The medium was set at +4 degrees when it started to become cloudy. It will be produced on a massive scale the next day. It will be produced in large amounts in 100 ml LB medium (1 liter tubes) with antibiotic broth containing 100µg /ml. Large-scale transplantation used 2 ml of 5 ml of altered cells. After a day of incubation, the plasmid isolation procedure was the next step.

2.7 Plasmid isolation with NucleoBond® Xtra Midi EF / Maxi EF kits:

The bacterial strain was taken in the incubator and separated into falcons. Centrifugation was performed at 6000g at +4 degrees for 10 minutes with ultracentrifuge. The column was rinsed with 15 ml of equilibration buffer during centrifugation (EQU). Waited for the liquid to completely pass through the colon. Then, discarded the supernatant after ultracentrifugation. RNase-A used; In the kit protocol, it was written to use 6mg of RNase-A for 10 samples. Poured 8ml (RES+RNase-A mix) onto each pellet, vortexed the pellet well, and placed on ice. Added 8ml Lyziz Buffer on it. It progressively turn into of rotation five times while incubating for five minutes at room temperature. Neutralization buffer (8 ml) was added and up and down 6-9 times until our sample turned white. After that, it was incubated for 5 minutes on ice. After incubation up and down 3 times, transferred to the colon and waited for all the liquid to go away. Slowly pour 5 ml of "Wash Buffer FIL-EF" from the edge of the column. Discard the upper colum and discern the fluid under the falcon. Slowly add 35 ml of Endo-EF from the side onto the remaining filter. (Dropping on the filter suddenly). Wait for 15 ml buffer wash-EF flow on it. Get a new falcon, throw the old one away, and give the old falcon's filter to the new falcon. We put 5 ml of ELU-EF. It was waited for the liquid to flow, then we pressed the filter tube with a syringe. Add 3.5 ml of isopropanol, vortex, then ultracentrifuge 12,000 g for 30 minutes at 4 degrees. Remove gently from the centrifuge, do not shake the tube. It was seen the pellet, very gently aspirate the liquid and try not to touch the pellet while pulling the liquid. After withdrawing the liquid, it was added 2 ml of 70% ethanol. Pipette all the liquid into the Eppendorf. Spin at 12.000g for 5 minutes, extract the ethanol after centrifugation and evaporate the remaining ethanol. In the last step, dissolve in 50 µl of dH2O and measure the concentration and purity in the nanodrop.

2.8 Cell culture of HeLa cells

At 37 °C and 5% CO 2 , HeLa cells were seeded, seeded in T25 and then passaged into T75 flasks and grown sterile under conditions suitable for cell culture. Every 48 or 72 hours, cells were passaged through at a ratio of 1:10. To remove the cell culture medium, wash the cells with 10 ml PBS (Invitrogen), and then add 3.5 ml Trypsin-EDTA (Invitrogen). The added 7 ml complete medium for trypsin inhibition after 4 minutes at 37 °C of incubation. Complete medium high glucose-modified Dulbecco's Eagle Medium (DMEM) for HeLa culture 10% FBS (Invitrogen) and 1% P/S (Gibco).

2.9 Transfection of HeLa cells with PEI optimisation

It have been used cells with 15th passage that passage that it had been incubating for two days, and the cells were examined. Cells were used for PEI reagent transfection when they were 80% confluent. It was necessary to seed the cells in a 6-well plate for the transfection, with each well containing about $300x10^3$ cells. After that, the 6-well plate was placed in an incubator for 24 hours to achieve an efficiency of 80–90%. It was necessary to quantify the quantity of the DNA stock that was stored in the -80 to prepare the precise amount of the DNA concentration. Finally, depending on the amount of stock for GFP+NF and only for GFP, the amount of DNA for each sample was determined. The PEI vial should be at room temperature before usage. either momentarily vortexing or inverting to combine (10seconds). Add various volumes of PEI "Max" to 150 volumes of Opti-MEM to dilute. Variations in PEI content are possible. Dilute 3 µg of DNA into a total volume of 150 µl of Opti-MEM. Mix the diluted DNA with PEI "Max" at a variable ratio. At room temperature, vortex and incubate for 30 minutes. A well of adhering cells should be filled with the PEI-DNA mixture carefully. To avoid damaging the adhering cells, gently pipette the solution down the side of the well rather than on top of them. Incubate for 24 hours after returning the dish to the 5% CO2 incubator.

When I tested all the transfected cells on the first day after transfection, the confluency was 100% overflow and there were approximately 32% dead cells. According to flowcytometry analysis of all transfected cell ratios, we had about 45% of transfected cells in the 1:3 ratio, 40% of transfected cells in the 1:4 ratio, and 20% of

transfected cells in the 1:6 ratio. Therefore, it was decided to use a DNA/PEI ratio of 1:3 or 1:4, and then it was optimized for the number of cells to seed. And then it was prepared this optimization protocol. It will be tested 3 µg DNA & PEI 9- PEI 12 µg (ratio1:3 and 1:4). And we tried different cell amounts which are $150x10^3$ -$200x10^3$-$250x10^3$ cells.

Fluorescence microscopy was used to examine every transfected cell on the first day after transfection, the confluency of the cells in the $150x10^3$ well was around 70%, and in other wells containing 200x103 cells, it was approximately 80%. Additionally, the amount of dead cells was quite high in two other wells that each contained 250x103 cells, and the confluency of the cells was overloaded. When we used flow cytometry to assess all the transfected cell ratios, we discovered that there were roughly 60% GFP, 34% NF155, and 44% NF186 plasmid transfected cells in the 150x103 1:3 PEI ratio. In comparison to lesser concentrations, when we had roughly 33% plasmid of GFP in the 200x103 and 30% plasmid of GFP in the 200x103, it was observed that the transfection ratio of the cells was observed to decrease with increasing cell confluency.

Table 2-2: It was tested 3 µg DNA & PEI different ratio (ratio1-1; 1-4; 1-6):

	$300x10^3$ CELLS		
	3 PEI	4 PEI	6 PEI
3 µg DNA	150µL Opti-MEM 3 µg DNA 9µL PEI	150µL Opti-MEM 3 µg DNA 12µL PEI	150µL Opti-MEM 3 µg DNA 18µL PEI
3 µg DNA	150µL Opti-MEM 3 µg DNA 9µL PEI	150µL Opti-MEM 3 µg DNA 12µL PEI	150µL Opti-MEM 3 µg DNA 18µL PEI

Table 2-3:It was tested 3 µg DNA & PEI 9 µg (ratio1,3 and 4):

Cells Amount	**150x10^3**	**200x10^3**	**250x10^3**
DNA Amount	3 µg DNA	3 µg DNA	3 µg DNA
3 PEI	150µL Opti-MEM 3 µg DNA 9µl PEI	150µL Opti-MEM 3 µg DNA 9µL PEI	150µL Opti-MEM 3 µg DNA 9µL PEI
4 PEI	150µL Opti-MEM 3 µg DNA 12µL PEI	150µL Opti-MEM 3 µg DNA 12µL PEI	150µL Opti-MEM 3 µg DNA 12µL PEI

Figure 2-4: Transfection with PEI solution in our experiment.

2.9.1 PEI transfection in HeLa cells

When transfection experiments first began, transfection was performed at a 60% to 80% confluence stage in a 100mm dish or 6-well plate. Before using the PEI, prepare it to room temperature. Prior to applying the PEI solution, perform up-and-down (or 10 seconds of vortex). 150 µl of Opti-MEM should include 9 µg of PEI "Max" in total. for making 9 µg of PEI transfection reagent from a stock of 1µg / µL. The components for

a 6 well transfection are as follows: 54 µl PEI SOLUTION + 900 µl Opti-MEM + 18 µg DNA. Followed by a 30-minute vortex and incubation period. Distribute 155µl into 6 wells after 30 minutes of incubation, and then stir the wells four or five times with a thousand-pipette. There is no need to alter culture conditions or remove serum. Incubate for 24 hours or until the results can be measured.

2.10 FuGENE-based transfection optimization in HeLa cells

Transfection was first carried out at 70–80% confluence when the transfection experiments were first initiated. To determine the ideal transfection rate, we first performed transfection optimization. We planned the FugeneHD protocol optimization according to the cell type and plate that we were using. The table below provides a detailed explanation of the optimization's specifics. The suggested range for DNA concentration is 0.4–2ug. We used 1ug and 1.75ug of DNA concentration in our experiment. The ratio of DNA to fugene was our initial objective in this. We increased the amount of DNA when we found it. 1. Finding the ratio was the first step, and we discovered that it was 3:1. Next, we looked at how much DNA was being transfused and its impact on the rate of transfection. (DNA ratio: 0.75 – 1- 1.5- 1.75 ug)

Table 2-4: The first step optimization for FuGENE solutions.

	1µg DNA of NF155+GFP	**1µg DNA of NF155+GFP**	**1µg DNA of NF155+GFP**
150.000 cells	89µL Opti-MEM 1µg DNA 4 µL Fu 4:1 ratio Total 100 µL	89µL Opti-MEM 1 µg DNA 3.5 µL Fu 3.5:1 ratio Total 100 µL	89µL Opti-MEM 1 µg DNA 3µL Fu 3:1 ratio Total 100 µL
	89µL Opti-MEM 1 µg DNA 2.5µL Fu 2.5:1 ratio Total 100 µL	89µL Opti-MEM 1 µg DNA 2µL Fu 2:1 ratio Total 100 µL	89µL Opti-MEM 1 µg DNA 1.5µL Fu 1.5:1 ratio Total 100 µL

200.000 cells	**1µg DNA of NF155+GFP**	**1µg DNA of NF155+GFP**	**1µg DNA of NF155+GFP**
	89µL Opti-MEM 1µg DNA 4 µL Fu 4:1 ratio Total 100 µL	89µL Opti-MEM 1 µg DNA 3.5 µL Fu 3.5:1 ratio Total 100 µL	89µL Opti-MEM 1 µg DNA 3µL Fu 3:1 ratio Total 100 µL
	89µL Opti-MEM 1 µg DNA 2.5µL Fu 2.5:1 ratio Total 100 µL	89µL Opti-MEM 1 µg DNA 2µL Fu 2:1 ratio Total 100 µL	89µL Opti-MEM 1 µg DNA 1.5µL Fu 1.5:1 ratio Total 100 µL
150.000 cells	**1.75µg DNA of NF155+GFP**	**1.75µg DNA of NF155+GFP**	**1.75µg DNA of NF155+GFP**
	89µL Opti-MEM **1.75µg DNA** 7 µL Fu 4:1 ratio Total 100 µL	89µL Opti-MEM **1.75µg DNA** 6.2 µL Fu 3.5:1 ratio Total 100 µL	89µL Opti-MEM **1.75µg DNA** 5.25µL Fu 3:1 ratio Total 100 µL
	89µL Opti-MEM **1.75µg DNA** 4.37µL Fu 2.5:1 ratio Total 100 µL	89µL Opti-MEM **1.75µg DNA** 3.5µL Fu 2:1 ratio Total 100 µL	89µL Opti-MEM **1.75µg DNA** 2.6µL Fu 1.5:1 ratio Total 100 µL

200.000 cells	**1.75µg DNA of NF155+GFP**	**1.75µg DNA of NF155+GFP**	**1.75µg DNA of NF155+GFP**
	89µL Opti-MEM **1.75µg DNA** 7 µL Fu 4:1 ratio Total 100 µL	89µL Opti-MEM **1.75µg DNA** 6.2 µL Fu 3.5:1 ratio Total 100 µL	89µL Opti-MEM **1.75µg DNA** 5.25µL Fu 3:1 ratio Total 100 µL
	89µL Opti-MEM **1.75µg DNA** 4.37µL Fu 2.5:1 ratio Total 100 µL	89µL Opti-MEM **1.75µg DNA** 3.5µL Fu 2:1 ratio Total 100 µL	89µL Opti-MEM **1.75µg DNA** 2.6µL Fu 1.5:1 ratio Total 100 µL

Table 2-5: The second step optimization for FuGENE solutions

3:1 ratio	**0.75µg DNA of NF155+GFP**	**0.75µg DNA of NF155+GFP**	**0.75µg DNA of NF155+GFP**
	89µL Opti-MEM 2.25 µL Fu 120.000cells Total 100 µL	89µL Opti-MEM 2.25µL Fu 150.000cells Total 100 µL	89µL Opti-MEM 2.25µL Fu 175.000cells Total 100 µL
	89µL Opti-MEM 2.25µL Fu 200.000cells Total 100 µL	89µL Opti-MEM 2.25µL Fu 230.000cells Total 100 µL	89µL Opti-MEM 2.25µL Fu 250.000 cells Total 100 µL
3:1 ratio	**1 µg DNA of NF155+GFP**	**1µg DNA of NF155+GFP**	**1µg DNA of NF155+GFP**
	89µL Opti-MEM 3µL Fu 120.000cells Total 100 µL	89µL Opti-MEM 3µL Fu 150.000cells Total 100 µL	89µL Opti-MEM 3µL Fu 175.000cells Total 100 µL
	89µL Opti-MEM 3µL Fu 200.000cells Total 100 µL	89µL Opti-MEM 3µL Fu 230.000cells Total 100 µL	89µL Opti-MEM 3µL Fu 250.000 cells Total 100 µL

3:1 ratio	**1.5µg DNA of NF155+GFP**	**1.5µg DNA of NF155+GFP**	**1.5µg DNA of NF155+GFP**
	89µL Opti-MEM 4.5µL Fu 120.000cells Total 100 µL	89µL Opti-MEM 4.5µL Fu 150.000cells Total 100 µL	89µL Opti-MEM 4.5µl Fu 175.000cells Total 100 µL
	89µL Opti-MEM 4.5µL Fu 200.000cells Total 100 µL	89µL Opti-MEM 4.5µL Fu 230.000cells Total 100 µL	89µL Opti-MEM 4.5µL Fu 250.000 cells Total 100 µL
3:1 ratio	**1.75µg DNA of NF155+GFP**	**1.75µg DNA of NF155+GFP**	**1.75µg DNA of NF155+GFP**
	89µL Opti-MEM 5.25µL Fu 120.000cells Total 100 µL	89µL Opti-MEM 5.25µL Fu 150.000cells Total 100 µL	89µL Opti-MEM 5.25µL Fu 175.000cells Total 100 µL
	89µL Opti-MEM 5.25µL Fu 200.000cells Total 100 µL	89µL Opti-MEM 5.25µL Fu 230.000cells Total 100 µL	89µL Opti-MEM 5.25µL Fu 250.000 cells Total 100 µL

2.10.1 Transfection of HeLa cells with FuGENE

Transfection was first carried out at 70–80% confluence when the transfection experiments were first initiated. The FugeneHD methodology was planned and optimized based on the cell type and plate that we used. For our experiment, a 100mm dish plate requires a total of 8 ug of plasmid. First, 712 microliters were placed in an empty tube. Then Fugene-HD solutions were added, followed by a vortex. Afterward, incubation at room temperature for 5 minutes. Then, 8 µg of plasmid DNA and a vortex were introduced. Fugene-DNA was employed at a 3:1 ratio. 15 minutes of room temperature incubation follow. The 100mm dish containing mix solution and 1.8–2 x 10^6 cells are inserted as the final step.

2.11 Calculation of MFI: (MFI-Ratio vs Delta MFI):

The term "MFI" stands for mean fluorescence intensity. The software FlowJo provides choices to determine the Mean, Median, Mode, and Geometric Mean when you analyze your data. Geometric Mean is used in this antibody assay. Geometric mean: the best choice of describing the MFI of a logarithmic histogram. The geometric mean is found by taking the product of these numbers for a sequence of numbers and taking the root of the number obtained by the number of sequences. In my data analysis, we use the geometric mean in statistical analysis of the data we acquired from our flow cytometry data. Additionally, after determining its geometric mean value, the MFI-ratio and Δ MFI are used to assess it. We use the flow-jo program to analyze the data from the flow cytometry in order to calculate the MFI. Geometric mean value was employed. Excel file with our info transmitted. The delta-MFI formula Δ MFI (= MFI nf155- MFI gfp or =MFI nf186- MFI gfp) should then be written in the excel file. The MFI ratio can also be calculated using the following formulas: MFI nf155/MFI gfp or =MFI nf186/MFI gfp. After this calculation, the sample's positivity or negativity is detected.

DeltaMFI = MFI nf155- MFI gfp or MFI nf186- MFI gfp

MFI ratio = MFI nf155/ MFI gfp or MFI nf186/ MFI gfp

Figure 2-5: Calculation of DeltaMFI and MFI ratio.

2.12 Flow Cytometry Methods

2.12.1 Antibody detection with flow cytometry

The patient serum was transfected into the cells after being combined with the patient's serum samples, and the patient sera were then treated with biotin. Four streptavidin molecules that attach to one biotin allowed for the development of a method that can determination of low positives. Due to the AF-647 fluorochrome linked to the

streptavidin dye in this approach, it was picked up in the flow cytometry instrument. We were able to recognize our positive samples with clarity this manner samples (Figure 2.3).

Table 2-6: Voltages and flow device channels used in antibody detection testing.

Flow settings	**FSC: 80 Voltage**	**SSC: 280 Voltage**
BL1(Gfp): 220 Voltage	**RL1(APC):300 Voltage**	

FSC separates cells based on size, while SSC separates cells based on granule. We adjusted and optimized our voltages to be 80 Volts FSC and 280 Volts SSC according to the HELA cell. The gfp channel used to look at the transfected cells is BL1 and the voltage of this channel is 220 Volts. AF647 dye conjugated to Streptavidin is viewed from the RL1 channel and its voltage is 300 Volts (Table 2-6).

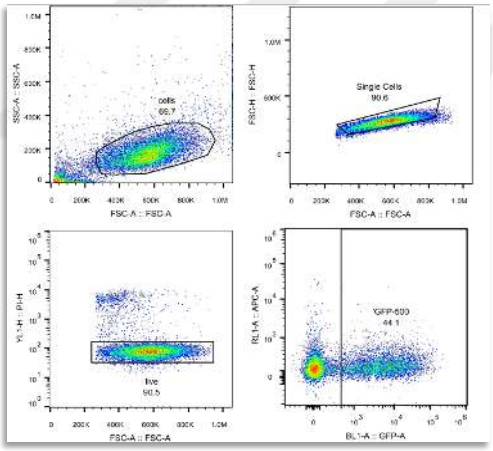


Figure 2-6:Live cell based assay gating strategy is explained.

Chapter 3:
RESULTS

3.1 Live cell based assay cut off for sera sample:

The cut-off was established using healthy Control subjects. Thanks to the data obtained from the flow device, although the cut-off point was established by using "mean + 3*SD", "mean + 4*SD" and "mean + 5*SD" over the MFI ratios in these data, mean+5, which is applicable for the NF test, from these values We set it to SD. In the CIDP, we will employ two separate healthy controls, consisting of 22 healthy people and 22 people with MS. 44 individual sera are used as the healthy control sample.

The cut-off was determined and defined using "mean + 5*SD" over the values obtained from the flow device. We used control which is only 22-healty person for healthy control in CIDP. We avoid using MS control since its cut-off is lower than the outcomes of typical health control. So, we made the decision to exclusively use data from healthy controls.

On the other hand, 32 health controls were used to determine the cut-off of MOG antibodies in serum and mean+3 SD was used from these values, and when determining positive samples, samples in which both delta and ratio were positive were selected. 27 CSF samples were used to determine the cut-off of mog antibody in CSF. 27 CSF samples are in OND (other neurological disease) group. The mean+3SD cut off was determined as found in mog samples in serum. The CSF part is explained in 3.5.

Table 3-1: Cut-off values for 3 different groups as MS group, healthy people and CIDP NF-assay were established. It was used 32 healty sample for MOG assay. We used sera sample from Human and analyse flow devices.

CUT OFF	***NF155***		***NF186***	
44 SAMPLE	**Delta**	**Ratio**	**Delta**	**Ratio**
mean	**154.83**	**1.65**	**15.13**	**1.11**
SD	**66.610**	**0.39**	**64.71**	**0.26**
mean+3SD	**354.66**	**2.84**	**209.28**	**1.91**
mean+4SD	**421.27**	**3.24**	**274.0**	**2.18**
Mean+5SD	**487.88**	**3.64**	**338.72**	**2.45**

ONLY HC CUT OFF	***NF155***		***NF186***	
22 SAMPLE	**Delta**	**Ratio**	**Delta**	**Ratio**
mean	**97.25**	**1.66**	**41.35**	**1.33**
SD	**88.18**	**0.50**	**66.05**	**0.49**
mean+3SD	**361.75**	**3.19**	**239.6**	**2.80**
mean+4SD	**450**	**3.70**	**305.50**	**3.3**
mean+5SD	**538.17**	**4.21**	**371.6**	**3.79**

MS HC CUT OFF	***NF155***		***NF186***	
22 SAMPLE	**Delta**	**Ratio**	**Delta**	**Ratio**
mean	**154.41**	**1.63**	**27.5**	**1.15**
SD	**68.65**	**0.23**	**64.69**	**0.31**
mean+3SD	**360.37**	**2.34**	**221.57**	**2.10**
mean+4SD	**429.03**	**2.58**	**286.27**	**2.41**
mean+5SD	**497.69**	**2.82**	**350.96**	**2.73**

Healty Control sera	**MOG Antibody**	
32 sample	**Delta**	**Ratio**
Mean	106	1.8
sd	56.0	0.6
mean+3*sd	**274.6**	**3.5**
mean+4*sd	330.6	4.0

3.2 The results of the antibody assay by flow cytometry:

Clinical, pathological, prognostic, and radiological variations exist among demyelinating disorders. As a result, these data should also be used to evaluate the outcomes of the live cell-based experiment. In 4–20% of individuals with CIDP, antibodies against the paranodal neurofascin155 protein are found.

These patients' clinical features differ from cases of seronegative CIDP. In our country, the frequency of NF IgG positivity is unknown. The goal was to create a live cell-based test for the diagnosis of antibodies in CIDP patients and to measure the prevalence of NF155-IgG and NF186-IgG positivity in our country by testing a sizable cohort. To express these proteins, plasmids containing NF155, NF186, and green fluorescent protein were transfected into HeLa cells. After incubation with patient samples, they were labeled first with biotin and then with streptavidin and analyzed using flow cytometry measurement. Calculating the mean fluorescence intensity difference and ratio allowed us to determine the positive of NF155-IgG and NF186-IgG. The cut-off point was established using healthy control sera.

264 patients' serum samples were analyzed. There are 196 cases of CIDP, 19 cases of GBS, 14 cases of CCPD, 22 cases of HC, and 20 cases of other neurological illnesses. The patients were 38.2 (21.3) years old on average, with a male to female ratio of 1:3. NF155-IgG positive was present in 6/196 CIDP patients. In these 6 patients, high NF155 antibodies were found to be predominantly of the IgG4 type. Low Positive NF155 samples are predominantly IgM positive sera samples. Moreover, a low titer positive result was found in 1 CCPD patient. Antibodies to NF186 were also positive in this case. On the other hand, NF186-IgG was high positive in two CIDP patients. High NF186 antibodies were discovered to be predominantly of the IgG3 type in these two patients.

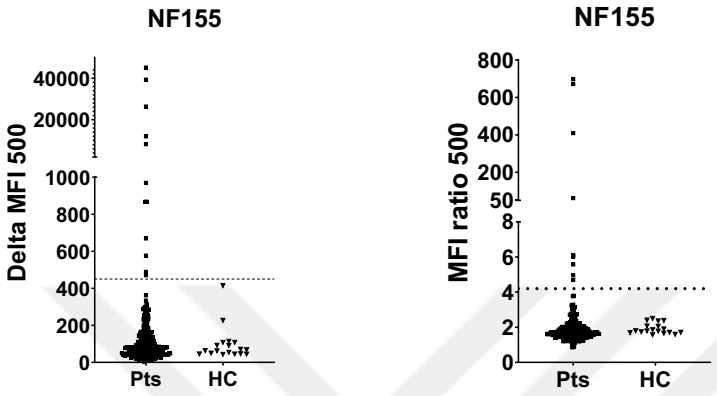


Figure 3-1: NF-155 was subjected to flow cytometry analysis in accordance with DELTA-MFI and MFI-ratio values.

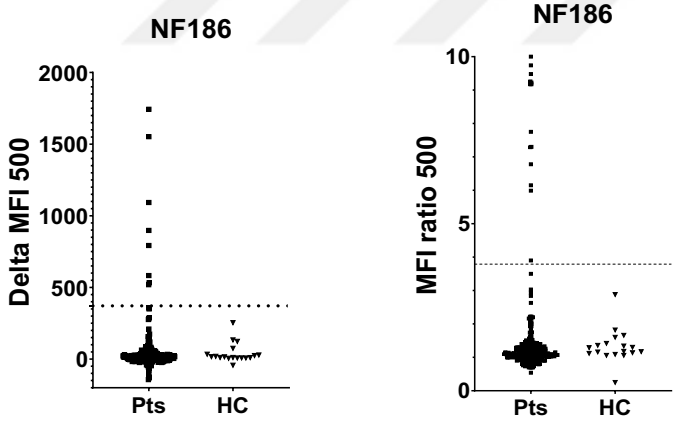


Figure 3-2: NF-186 was subjected to flow cytometry analysis in accordance with DELTA-MFI and MFI-ratio values.

3.3 Diversity of patients tested for NF155 antibody:

NF155-IgG was highly positive in 6/196 CIDP patients. NF155-IgG was low positive in 3/196 CIDP patients. In addition, low titer positivity was detected in 1 CCPD patient. This patient was also positive for NF186 antibodies.

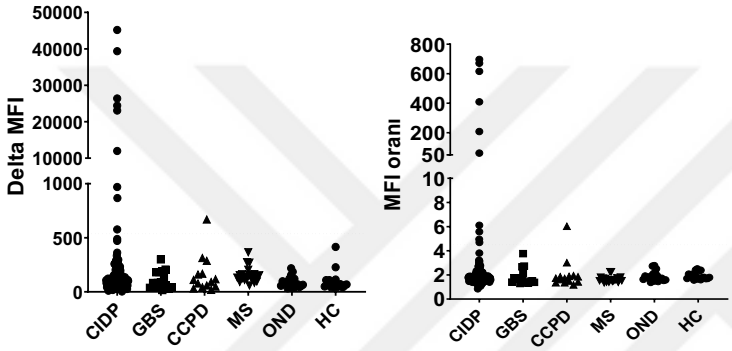
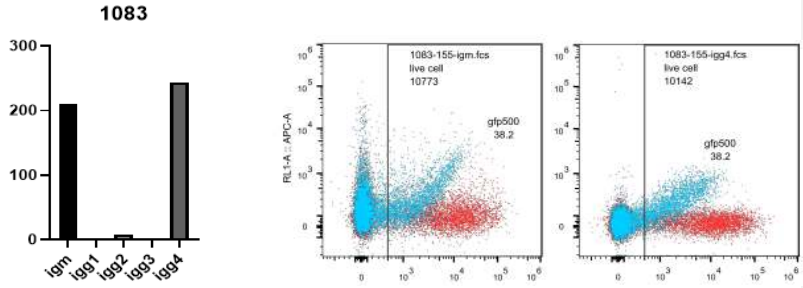


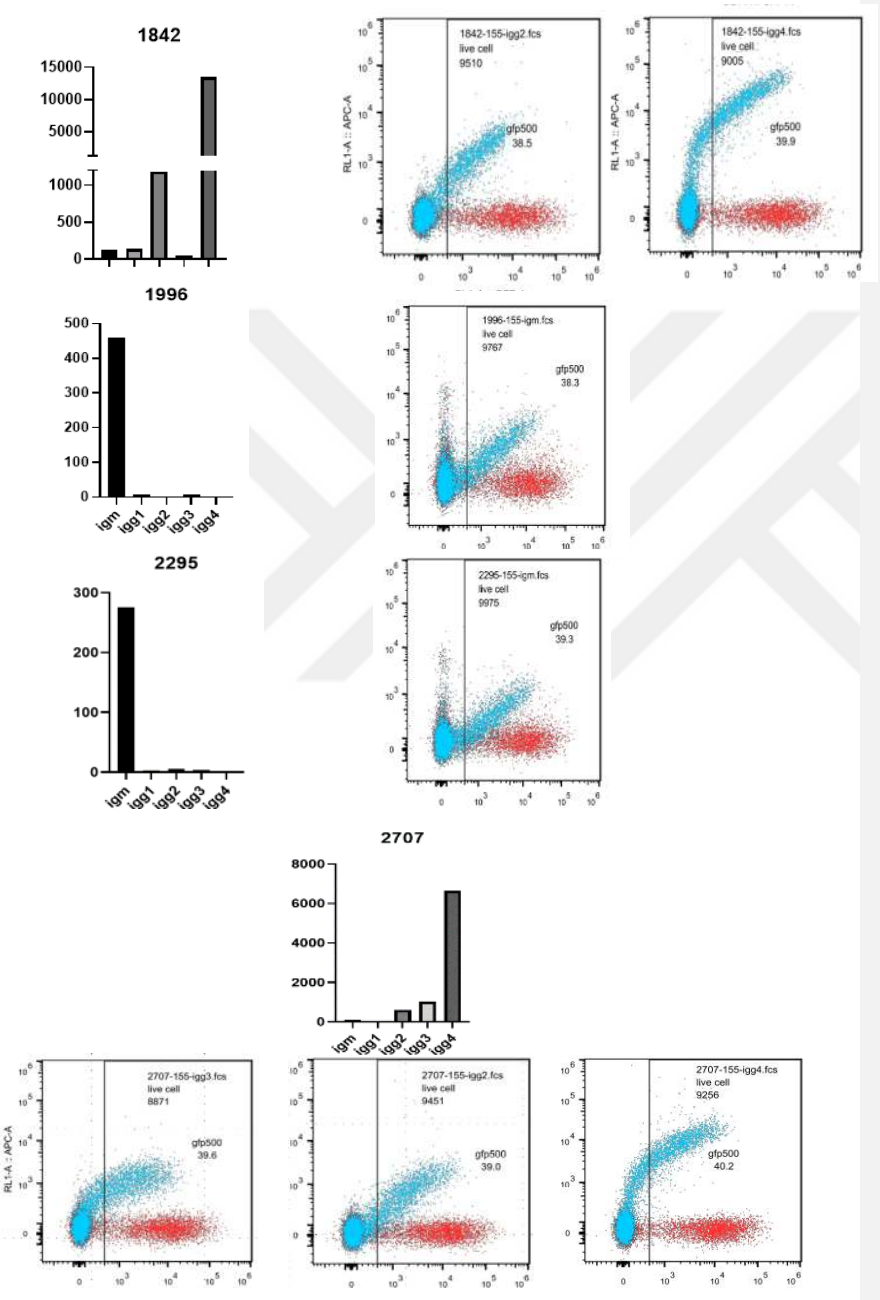
Figure 3-3:Diversity of patients tested for NF155 antibody : 196 cases of CIDP, 19 cases of GBS, 14 cases of CCPD, 20 cases of other neurological disease, and 22 cases of HC.

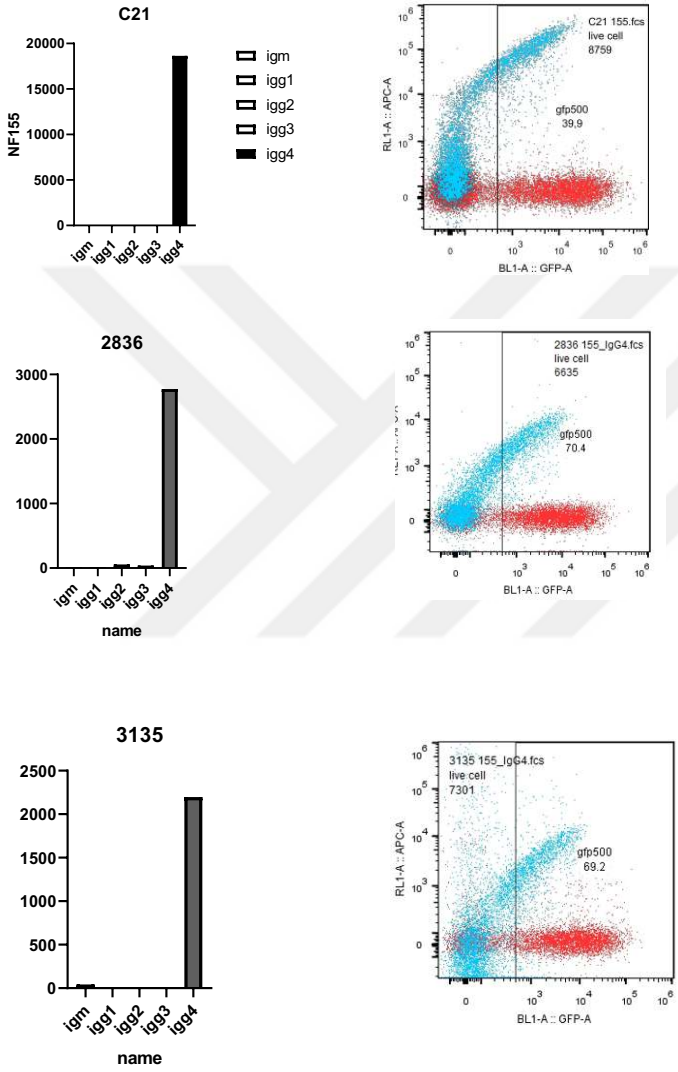
(OND-other nörolojik diseases)

3.4 SUBTYPING

3.4.1 NF155 SUBTYPING




1842
15000
10000
5000
1000
500
0
igm
igg1
igg2
igg3
igg4
1842-155-igg2.fcs
live cell
9510
gfp500
38.5
1842-155-igg4.fcs
live cell
9005
gfp500
39.9
1996
500
400
300
200
100
0
1996-155-igm.fcs
live cell
9767
gfp500
38.3
2295
300
200
100
0
2295-155-igm.fcs
live cell
9975
gfp500
39.3
2707
8000
6000
4000
2000
0
2707-155-igg3.fcs
live cell
8871
gfp500
39.6
2707-155-igg2.fcs
live cell
9451
gfp500
39.0
2707-155-igg4.fcs
live cell
9256
gfp500
40.2
RL1-A :: APC-A


C21
NF155
20000
15000
10000
5000
0
igm
igg1
igg2
igg3
igg4
C21 155.fcs
live cell
8759
gfp500
39.9
RL1-A :: APC-A
BL1-A :: GFP-A
2836
3000
2000
1000
0
name
2836 155_IgG4.fcs
live cell
6635
gfp500
70.4
3135
2500
2000
1500
1000
500
3135 155_IgG4.fcs
live cell
7301
gfp500
69.2

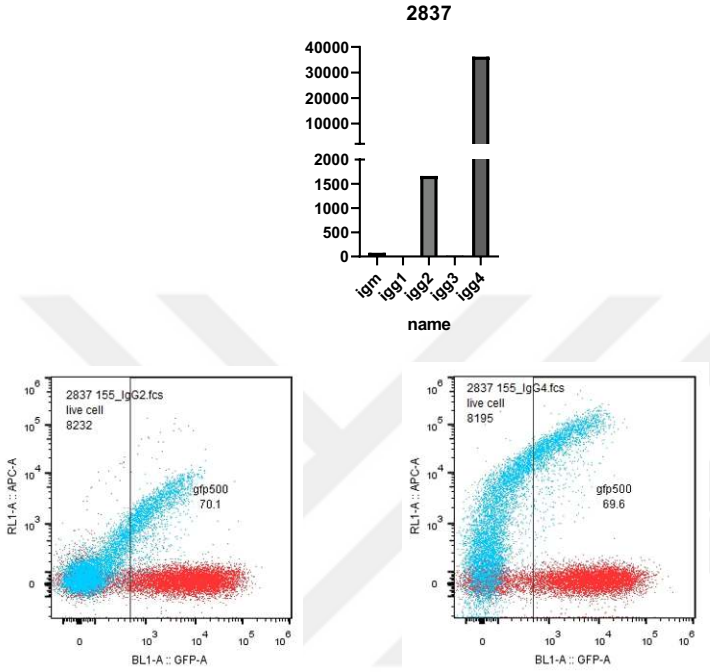


Figure 3-4: NF155 subtyping results.

The samples that we thought to be positive for NF155 were subtyped. IgM and IgG4 positive were found in patient 1083. IgG2 and IgG4 positive was found in patient 1842. The only IgM-positive patients were Patients Number 1996 and 2295. One of our high-positive samples was Patient Number 2707, and IgG4 was the subtyping result. Additionally, patient 2707 also had IgG2 and IgG3 detected. Another high positive NF155 sample was IgG4 and also had IgG2 positive. Finally, we found high IgG4 positivity in the c21, 2836, 3135 sera sample.

3.4.2 NF186 SUBTYPING

It was done for subtyping from samples that tested positive for NF186, which we believed to be positive. In two of our samples, IgG3 was positive. In addition, 1 CCPD patient had NF155 low titer positive. This patient was also positive for NF186 antibodies.

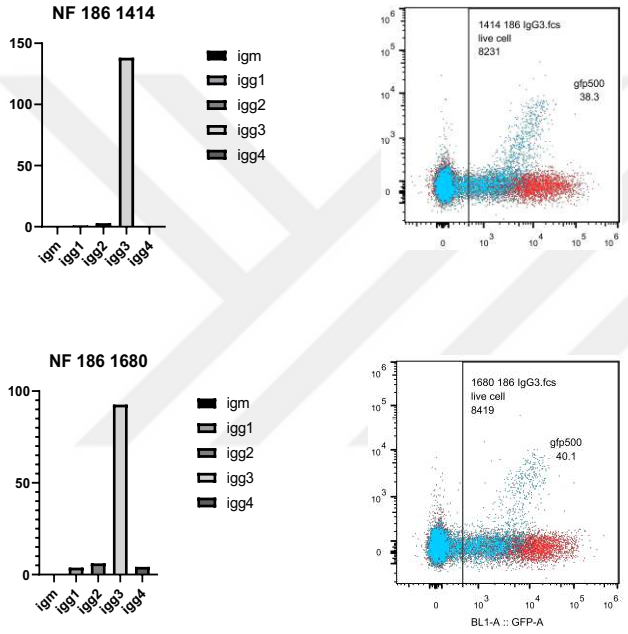
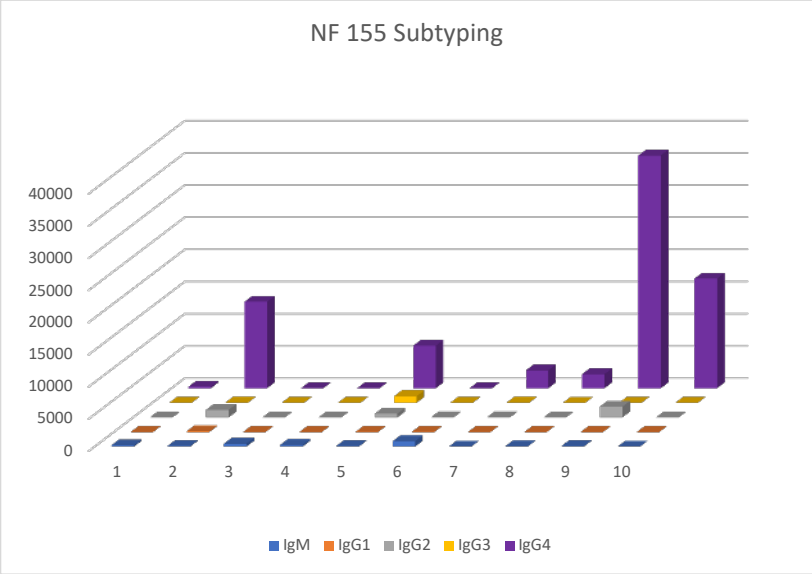
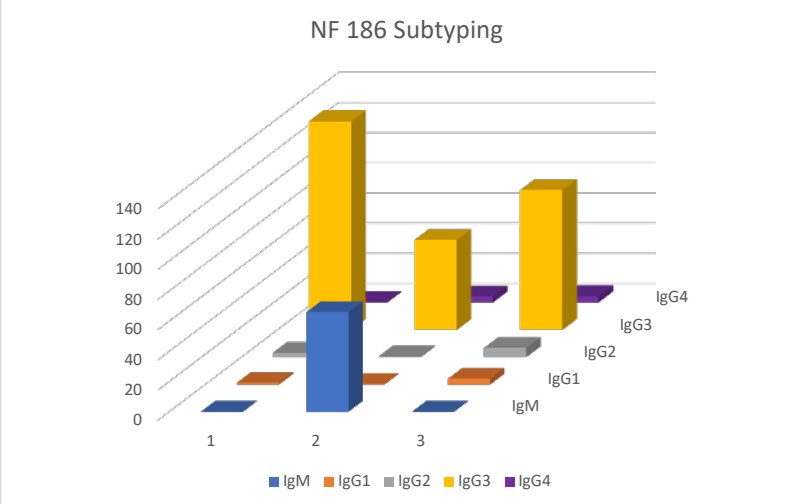


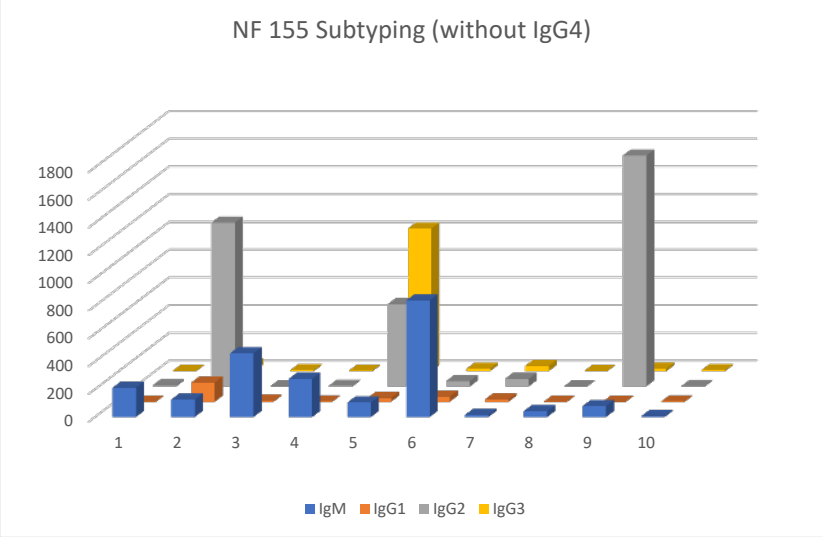
Figure 3-5:NF186 subtyping results.


NF 155 Subtyping
40000
35000
30000
25000
20000
15000
10000
5000
0
1
2
3
4
5
6
7
8
9
10
IgM
IgG1
IgG2
IgG3
IgG4


NF 186 Subtyping
140
120
100
80
60
40
20
0
1
2
3
IgM
IgG1
IgG2
IgG3
IgG4

According to the subtyping result of antibodies for NF155, it was determined that patients with high NF155 positive in serum samples taken from our patients were especially IgG4 positive. 6 NF155-IgG4 positive patients were identified. Other subtypings were also detected in 4 of these 6 patients. In addition, IgG3 was detected as a result of subtyping in NF186 positive patients. These two patients were also low NF155 positive. In one patient, low IgM positivity was detected in addition to IgG3.

35 pediatric patients from our cohort had serum samples analyzed for the presence of the NF155 and NF186 antibodies. The test revealed that one patient had both NF-155 IgG4 antibodies and NF186 antibodies, while the second patient had only NF-186 antibodies. The remaining patients tested negative for NF.



In this graph, subtypings apart from IgG4 positivity are shown. IgG4 positive patients are patients with numbers 2,5,7,8,9,10. In patient 2, high IgG2 and low IgM and IgG1 were detected, except for IgG4. Apart from IgG4, high IgG3 and low IgG2, IgM were detected in patient 5, and finally, high IgG2 other than IgG4 was detected in IgG4

high positive patient. Antibodies other than IgG4 were not detected in 3 of the high IgG4 positive patients, low IgM was detected in only 1,2,4 patients from the IgG4 negative patients.

3.5 Live cell based assay cut off for CSF sample:

Using healthy Control participants, the cutoff was established. The MFI data acquired from the flow device were multiplied by "mean + 3*SD," "mean + 4*SD," and "mean + 5*SD" to determine a breakpoint. We will use healthy control which are MS and other diseases CSF sample (non-GBS and non-CIDP) diseases person which are healthy control in CIDP. Totally healthy control is 55 samples of CSF.

The cut-off was determined for CSF sample with defined "mean + 5*SD over the values obtained from the flow device. It was used control CSF sample which is 55 person for healty control in CIDP . According to Mean+ 5SD, the DeltaMFI value of NF155 is 57.41 , while it is 77.95 for NF186. According to Mean+ 5 SD, the MFI ratio value of NF155 is 2.03 and NF186 is 2.29.

A cut-off was created for our MOG-CSF experiment from 27 patients with CIDP GBS and other non-MOGAD neurological disorders. As in the MOG serum sample, our positive samples were determined based on mean+3 SD values. While the numerical value of the cut-off in Delta MFI for Mog antibodies in CSF was determined as 66.2, the MFI ratio ratio was determined as 1.82.

As the positivity of MOG was determined in the serum, samples in which both delta MFI and MFI ratio were positive were considered positive.

Table 3-2:Cut-off values were defined for NF155 and NF186 and MOG. We used CSF sample from Human and analyse flow devices.

CSF	NF155		NF186		MOG	
	Delta	Ratio	Delta	Ratio	Delta	Ratio
mean	6.29	1.14	-1.17	1.00	11.4	1.15
SD	10.2	0.18	15.8	0.25	18.3	0.22
mean+3SD	**36.96**	**1.67**	**46.86**	**1.77**	**66.2**	**1.82**
mean+4SD	**47.18**	**1.85**	**62.13**	**2.03**	**84.5**	**2.04**
mean+5SD	**57.41**	**2.03**	**77.95**	**2.29**	**102.76**	**2.27**

3.6 Results of NF antibody assays on CSF samples:

For CSF samples, 9 MS and 22 OND control, 13 CIDP and 13 GBS samples were used. When 57 samples were analyzed in CSF samples, delta MFI was positive in 5 samples for NF155 and only 4 of them had MFI ratio positive. These 4 samples were considered positive and 2 of these 4 samples were patients with GBS while the other two were patients with CIDP. When we evaluate it in terms of NF186, we have 2 positive patients, one of whom was diagnosed with GBS and the other with a diagnosis of CIDP. These patients were also positive for NF155. In other words, NF 155 and NF 186 positivity were detected in one of the 2 GBS patients who were considered positive. Likewise, NF155 and NF186 positivity were detected in CSF in one of the 2 CIDP patients who were considered positive.

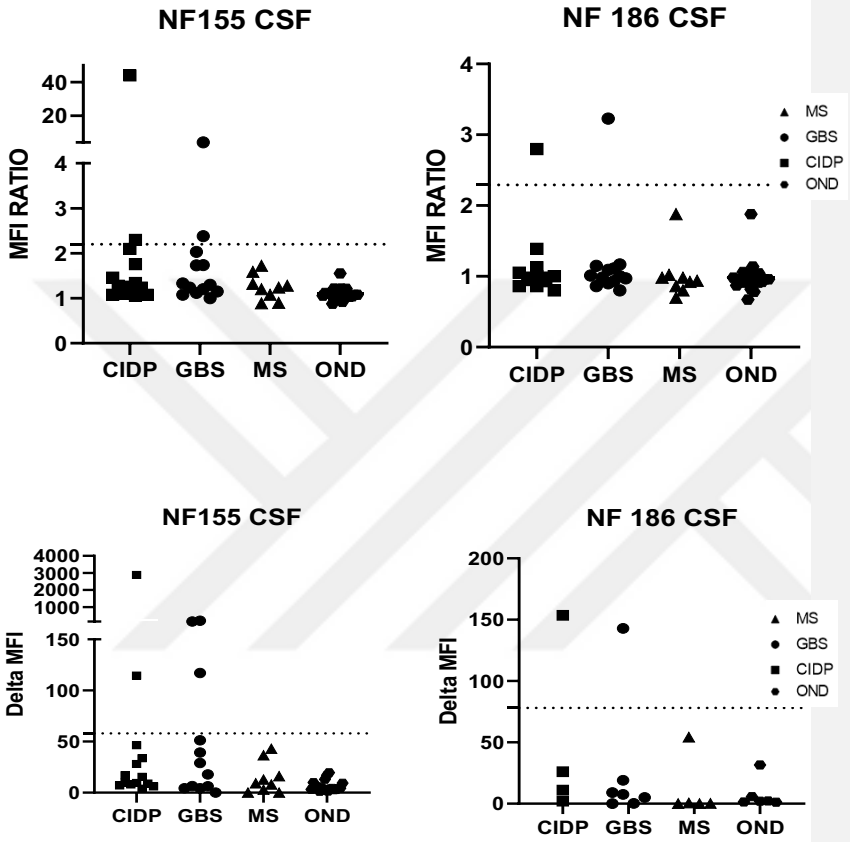
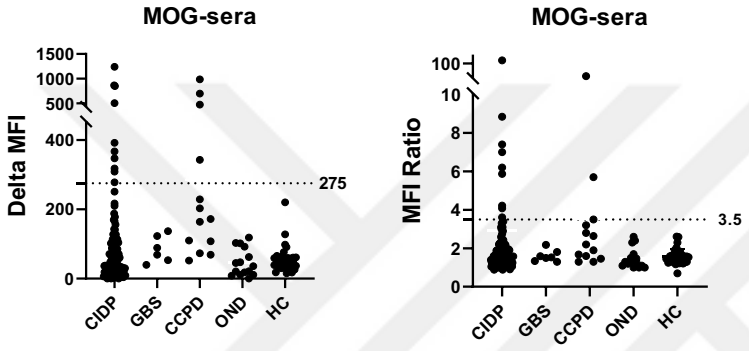


Figure 3-6:Flow cytometry analysis was performed for antibodies of NF-155, NF186, MOG according to DELTA-MFI and MFI-ratio values in CSF sample.

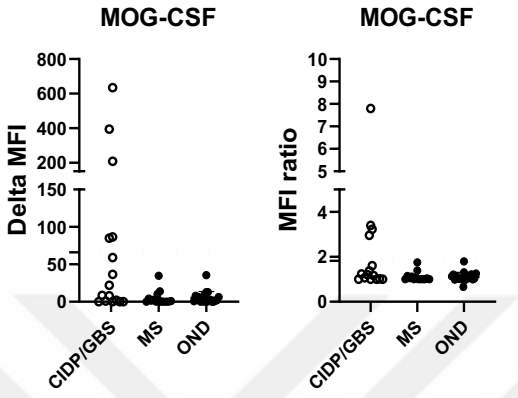
3.7 Mog assay our Cohort patient:

For Mog assays, a live based assay, which was also used in the neurofascin experiment, was used. In this mog assay, it was tested using serum and control samples. In order to detect positive samples, we established our cut-off values for Mog at 3.1 for serum samples and 3.5 for CSF samples. Patients with positive Delta MFI and MFI ratio results are regarded as positive for the Mog test.

In serum samples, Delta MFI result was positive in 12 patient samples and MFI ratio was positive in 9 samples. As a result of our experiment, we searched for samples with both positive Delta MFI and MFI ratios, so the number of double positive samples is 9. While the amount of serum with positive both Delta MFI and MFI ratio detected in patients with CIDP was 6, mog positivity was detected in 3 patients with CCPD.



16 CIDP-GBS samples were tested in CSF samples. Among these samples, Delta MFI result was positive in 5 samples, while MFI ratio positivity was also detected in the same samples. Due to these results, we accepted our 5 samples as positive. Five of 16 inflammatory neuropathy patients had positive MOG antibodies in the CSF. As a result of the tests, we performed in two of these patients, there was also borderline positivity in the serum. However, while the serum samples of the other 3 patients were negative, mog positivity was detected in the CSF samples. While 1 of these 5 samples was definitively diagnosed as CCPD, 3 samples belonged to a CIDP patient, while a single positive sample of a GBS patient was detected. In addition, 13 MS patients and all 20 ONDs were mog negative in CSF.


MOG-CSF
Delta MFI
800
600
400
200
150
100
50
0
CIDP/GBS
MS
OND
MOG-CSF
MFI ratio
10
9
8
7
6
5
4
2
0
CIDP/GBS
MS
OND

Chapter 4:
DISCUSSION

We validated our test with cell-based assay for the first time in our country and defined the frequency of NF antibodies in the Turkish cohort. In our patients with NF155 high titer positive, especially IgG4 isotype was found prominently. It has been demonstrated once more that NF155-IgG4 positive individuals vary clinically from antibody negative patients, which is consistent with the literature. We now base our study on the patients' current diagnoses; however, if more clinical data becomes available, the precise diagnoses of the patients may change, something we are unable to currently detect.

Among seven NF155-IgG4+ patients six were adults and one was a pediatric patient. IgG2 and IgG3 antibodies were also present in addition to IgG4 subtype in three patients. The frequency of anti-NF155 antibody positivity in the Turkish cohort was found to be 3.5%. In the rest of the four patients no IgG positivity was detected and low IgM positivity was detected in three samples.

CSF from one NF155 IgG4 positive patient was also analyzed and showed the presence of antibodies in the CSF, as well. In another 13 CSF samples tested from CIDP patients one samples had low titers of NF155-IgG, NF186-IgG and MOG-IgG. This patient’s serum was negative for these antibodies. Among 12 Guillain-Barre Syndrome (GBS) CSF samples tested, two patients had low titers of NF155-IgG. Among eight MS patients and 55 patients with other neurological disorders, none of the CSF samples tested positive for NF155-IgG or NF186-IgG.

In addition, we analyzed the presence MOG antibodies in CIDP patients. Serum tests were positive for MOG antibodies in nine patients. Two of the patients were children. Pediatric patients were diagnosed with CIDP and combined central and peripheral demyelination syndrome (CCPD). Adult patients comprised the remaining seven cases, of which two were diagnosed with CCPD and the remaining five with

CIDP. In the nine patients with positive serum MOG-IgG, NF155 and NF186 antibodies were negative. Analysis of 16 CSF samples obtained from CIDP and GBS patients revealed that, five patients had low titers of MOG-IgG. Serum MOG-IgG was found to be borderline positive in two of these patients. In the other three patients, serum positivity was not detected. Among patients with both serum and CSF MOG-IgG positivity, one patient had CCPD and the other had CIDP.

It was found that IVIg was insufficient in cases where the NF155-IgG4 antibody was positive, despite the fact that IVIg suppresses the complement system, lowers the fc receptor of macrophages, and controls the immune system by limiting the synthesis of adhesion molecules. According to one interpretation, it prevents proteins from functioning and, in circumstances when an antibody is present, from binding.

IgG4 was negative and low titer IgM was only seen in these two patients in adult patients with low NF155 positive subtyping study. She was a 56-year-old woman.

In the progression stage, the male patient, who was 31 years old, displayed fulminant subacute progression. Weakness in the hands, bilateral atrophy, slight axonal neuropathy, loss of strength in the hands during the patient's hospitalization, and weakness in the feet were also seen in our male patient. In Bos, the protein content is a little higher. The patient lost a lot of weight quickly, was admitted to the intensive care unit, and passed away soon after being placed on an intubation machine.

Patients with pediatric onset CIDP less than 19 years of age are known to exhibit different clinical characteristics from those of adults, including a faster rate of disease progression, dominance of the motor system, more recurrences, and a better prognosis, independent of gender (Hattori et al., 2001). When the pediatric patients in our Turkey-cohort were examined, NF155 and NF186 antibodies were tested on serum samples of 35 pediatric patients. The test revealed that one patient had both positive NF-155 antibodies and positive NF186 antibodies, while the second patient had only positive NF-186 antibodies. The remaining pediatric patients' results from the NF-assay are entirely negative. When he was nine years old, a male patient in his mid-twenties who

had tested positive for NF155 came to the doctor with complaints of frequent falls and weakness in his upper and lower extremities. He was identified as having CIDP after the neurological evaluation revealed tremor and ataxia symptoms. He was further diagnosed with CIDP based on additional clinical abnormalities and the EMG data. Due to the side effects that followed, the initial IVIg and steroid combined treatment was stopped, and only IVIG was used to continue the therapy. In both our NF-155 and NF-186 low positive pediatric patient, low IgM and IgG4 antibodies were detected as a result of subtyping analysis of NF155.

Another 16-year-old female patient was IgG3-NF186 positive and her values were determined as negative for NF155 antibody using a live cell based assay. The initial complaints of our female patient were severe loss of strength and weakness in the extremities, and the other complaint was muscle pain. During the examination, the neurological doctor noticed feblesse in the all extremities. Additionally, an MRI revealed nerve root thickness and some spinal nerve enhancement. Clinical, EMG, and MRI results led to the patient's CIDP diagnosis. Only NF186 antibody positivity, with IgG3 being the predominate Ig subtype, was discovered in the patient's serum test.

Following confirmation of the prevalence of NF-antibodies in CIDP patients, MOG-antibody positivity in CCPD and CIDP patients was also investigated, and a limited number of patients had mog-antibody positivity. We presented mog positivity in CIDP for the first time, and it was also seen in patients with CCPD. Even though one of the adult patients with CCPD was mog negative, both NF antibodies were also detected at low titers. IgG4 was negative when NF antibody subtyping was done on our adult CCPD patient, a 29-year-old, but IgM antibody was found at a low titer. The clinical significance of the mog antibody found in patients with CIDP is one of the limitations of our investigation because there is a dearth of clinical data in this area.

Nine patients had positive serum tests for the mog antibodies. Children make up two of the patients. The pediatric patients were given CIDP and CCPD diagnoses. Adult patients made up the remaining 7 cases, 2 of them were diagnosed with CCPD and the remaining 5 with CIDP. All of the NF antibodies in these 9 patients with positive serum mog were negative.

It should be highlighted that there may be a patient who is positive for antibodies but may not be discovered since our patient is receiving therapy, given that a major portion of our patients are receiving treatment.

In conclusion, we have established a live cell-based assay to test anti-NF155 and anti-NF186 antibodies from serum and CSF. We further identified the frequency of these antibodies in a large cohort of Turkish CIDP patients containing adult and pediatric patients, together with their immunological and clinical characteristics. Additionally, we found that MOG-IgG can also be present in the serum and CSF of CIDP patients as well as CCPD patients.

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