IL-6 increase in CSF as additional diagnostic criteria for definite neuro-behcet disease

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Research Article

Keywords: Behçet's disease, central nervous system, cerebrospinal fluid, cytokine

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Abstract

Background: When the central nervous system (CNS) is the primary affected site in an initial attack of Behcet's disease (BD), the differential diagnosis is particularly challenging. Some cases remain unclassified or qualified as probable Neuro-Behçet disease (NBD). Moreover, it was demonstrated that cytokines play a crucial role in the pathogenesis of NBD. We therefore studied peripheral and cerebrospinal inflammatory profile of these patients.

Methods: Twenty two parenchymal NBD patients diagnosed according to the international consensus recommendation criteria and classified into definite (d-NBD; n= 13) and probable (p-NBD ; n=9) were sampled at their first neurological symptoms and compared with healthy control subjects (n=10). Oligoclonal bands of IgG were detected by isoelectric focusing on agarose and immunoblotting of matched serum and Cerebrospinal fluid (CSF) sample pairs. Cytokines and transcription factors related to TH1, TH2, TH17 and T regulatory populations were studied by quantitative RT-PCR in the CSF.

Results: Oligoclonal bands (OCB) were present in only 1/22 patients. Two d-NBD patients had OCB in the CSF showing pattern 4. In NBD CSF samples, INF gamma, IL-17 and IL-10 expressions were significantly elevated compared with controls, however no difference in those cytokine expressions was observed between d-NBD compared to p-NBD. The most stricking finding was the significant increase of CSF IL-6 in d-NBD compared to p-NBD.

Conclusion:These results indicate the rare presence of OCB in parenchymal NBD patients. Additionally, CSF IL-6 could help us to identify definite NBD.

Keywords: Behçet's disease, central nervous system, cerebrospinal fluid, cytokine.

Background

Behçet disease (BD) is a chronic relapsing, multisystem vasculitis. Diagnosis is based on the International BD Study Group (ISG) Criteria (1). It requires recurrent oral aphthous ulcerations and two of the following: genital ulcerations, skin lesions, eye lesions, and positive pathergy test.

One of the particular forms of this disease is neuro-Behcet disease (NBD) which is characterized by neurological involvement and is one of the most serious causes of long-term morbidity and mortality (2). Among the NBD patients, parenchymal and non parenchymal central nervous system (CNS) involvement has been described as two separate entities. Parenchymal NBD is the most commonly seen form comprising 60 to 75% cases and affecting the brainstem, mesodiencephalic junction, cerebellar peduncies and cerebral hemispheres. Lesions are typically characterized by areas of T2 prolongation on brain magnetic resonance imaging (MRI) that have been suggested to represent a small vessel vasculitis(3).

The prevalence of CNS involvement varies widely from 1.3% to 59% depending on diagnosis criteria and ethnic populations (4,5). Epidemiological studies show that BD is the most frequent vasculitis in Tunisia (6) and the frequency of NBD is 28.1% (7). It appears about 5 years after the onset of BD symptoms. Although, neurological signs may be the first manifestations or even precede other manifestations of the disease by several years. These particular forms can mimic other CNS inflammatory diseases especially multiple sclerosis which is also prevalent in our country (8). Due to the absence of specific biomarkers, the diagnosis of definite NBD requires a high index of clinical suspicion, laboratory and neuroimaging findingsin an individual who fulfils the diagnosis criteria for BD, after exclusion of other possible causes (9). However, diagnosis remainsprobable according to the international consensus recommendation **(**ICR) (9) in tow cases: suggestive neurological syndrome as in definite NBD, with systemic BD features but not satisfying ISG criteria or a non characteristic neurological syndrome occurring in the context of ISG criteria-supported BD. In these cases, treatment is challenging due to the occurrence of neuronal loss at early stages of these potentially disabling diseases.

NBD etiology remains unclear and it is likely that genetic, environmental and immunological factors play a role in the development of the disease. In this context, pro-inflammatory and anti-inflammatory cytokines are believed to play a prominent role in modulating the inflammatory cascade in NBD (10,11,12,13).However data concerning peripheral blood and CSF levels of cytokines in NBD patients are limited (10, 11,12,13). Borhani and colleagues show different patterns between parenchymal and non-parenchymal subdivisions of NBD (14). Cytokines differences were also described between acute and chronic progressive parenchymal NBD (10,15). Furthermore, IL-6 levels have been reported to correlate with NBD activity (16). Moreover, to our knowledge, whether cytokine levels significantly differ in definite versus probable NBD patients was never been studied.

We propose to investigate the profile of cytokines in PBMCs and CSF of NBD patients classified into definite and probable. Peripheral and cerebrospinal pro and anti-inflammatory cytokines expression associated with T cell subsets of patients with NBD will be evaluated in order to define the immunological pattern among Tunisian population. The specific aim of this study is to identify an inflammatory marker which could confirm definite NBD diagnosis compared to a group of probable NBD and controls.

Methods

Patient selection

22 patients with a diagnosis of NBD according to International Consens Criteria ICR (9) were enrolled at the Neurological department of Charles Nicolle Hospital, during 2013-2015. **Figure1** shows a flowchart for the selection of the study population.

Inclusion criteria were the following: age≥18 years old; diagnosis of parenchymal forms of NBD classified into definite (n= 13) and probable (n=9). Patients with vascular-NBD as well as subjects with comorbid/coexisting disorders were excluded from this study. Patients with relapses treated with high doses of steroids within one month preceding enrollment were excluded. No patient had received any

immunomodulatory or immunosuppressive treatment for at least 3 months prior study. No patient had history of recent vaccination before inclusion in the study. Meningoencephalitis and other CNS infections were excluded.

Definite NBD (d-NBD) are patients with BD (ISG+) under colchicine treatment who developed neurological signs (n=6) or patients with first neurological symptoms suggestive of NBD who developed later systemic signs fulfilling ISG criteria (ISG+) (n=7). Probable NBD (p-NBD) are patients with first neurological symptoms suggestive of NBD but remaining (ISG-) after 3-5 years of follow up (n=7) or patients (ISG+) whodeveloped neurological symptoms but non suggestive of NBD (n=2). All these patients are diagnosed and sampled at the active stage of the disease (at the onset of neurological features). Then, patients were treatedwith high doses of steroids and pulses of cyclosphamide relayed 6 months later by Azathioprine except the two p-BD patients with non-suggestive neurological signs.

A full review of each patient's clinical history was conducted. Demographic, epidemiological and clinical data, as well as neuroimagery, laboratory tests and therapeutic management, were recorded. Other possible etiologies had been excluded by immunologic testing, serology, and neuroimaging.

Control group consisted of 10 healthy individuals with persistent headaches requiring lumbar puncture to exclude meningitis or meningeal hemorrhage with normal magnetic resonance imagery (MRI).

The project was approved by the Ethics Committee of Pasteur Institute of Tunis. A written informed consent was obtained from all participants before the inclusion in the study.

Blood and CSF samples

Blood samples were collected from 32 subjects (13 d-NBD, 9 p-NBD and 10 controls). Clear specimens of CSF (10 cm3) were taken from all participants. One-fifth of the fresh samples were used for cell counting, glucose and protein analysis, tow fifth were used for immunoelectrophoresis and the remaining were stored at -80° C immediately after centrifugation for subsequent cytokine analysis.

CSF albumin and Immunoglobulin analysis

Albumin and Ig G concentration were measured in the CSF and sera. Ig G index albumin ratios were calculated as following: (IgG CSF/IgG serum)/(Albumin CSF/Albumin serum). Isoelectric focusing on agarose and immunoblotting of matched serum and CSF samples pair identify 4 characteristic oligoclonal band (OCB) patterns. Type1 is a normal pattern where no bands are identified. Type 2 indicates intrathecal synthesis, where bands are seen only in the CSF. When the pattern of bands seen is identical in both sera and CSF, a « mirrored » type 4 is recorded. Identical shared bands but additional CSF -specific bands indicates a type 3pattern.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

After extraction of total CSF RNA , the first strand cDNA was synthesized using the reverse transcriptase system (invitrogen). PCR chain reaction were perrfomed using SYBR green and relative quantification of mRNA levels was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous reference. CSF levels of some major pro-inflammatory cytokines (INF gamma, IL-17 and IL-6), transcription factors (T-bet, GATA-3, RoR-γt and Foxp3) and anti-inflammatory cytokines (IL-4 and IL-10) were assessed.

Statistical Analysis

All Data analysis was performed using Prism software version 5 (GraphPad, San Diego, tailed unpaired CA). Two-tailed unpaired Mann-Whitney U test was performed when comparing two groups . All p values < 0.05 were considered statistically significant.

Results

Demographic features

Data concerning demographic and clinical characteristics of subjects included in the study are summarized in **table 1**. There were no significant differences in age or gender distribution among the 3 groups, although there was a tendency for male predominance in all the 3 groups. There were no differences in age at onset of neurological signs between d- and p- NBD. The mean interval between neurological and systemic signs, if coming, was 5.57 months. Neurological signs were developed after a mean interval of 30 months in patients classified BD without statistical differences between d- and p-NBD. The mean period of follow up of all NBD patients was 51 months.

Neurological characteristics, MRI findings and CSF analysis

Neurological characteristics, MRI findings and CSF analysis of patients are reported in **table2**. Among a variety of neurological manifestations, motor and pyramidal signs were the most frequent signs in the 2 groups. Isolated supratentorial location was present in 59% of all NBD patients. Brainstem lesions were more observed ind-NBD patients. Myelitis was observed only in one case ofp-NBD. There was an increase in the median of CSF protein level in the two groups (d-NBD= 0.64 g/l; p-NBD= 0.67g/l) . Wealso noted an elevated CSF cell count in the 2 groups compared to controls (d-NBD=34.8/mm3; p-NBD=23/mm3). None of patients had increased IgG index. However; OCB were present only in one patient with p-NBD disease (profile 2) and in two d-NBD patients (profile 4).

Cytokine expressions

In order to evaluate a different cytokine profile in the two groups: d-NBD and p- NBD, we compared the expression of different pro-inflammatory and anti-inflammatory cytokines in PBMCs and CSF of d-NBD (Group1) p-NBD (Group2) patients and a group of controls (**Table3).** In PBMCs samples, we noticed no significant difference in the mean of T-bet, RoR-γt, IFN-γ, IL-17, IL-6 and IL-10 mRNA between the two groups of NBD patients. However, foxp3 median (M) gene relative expression was significantly decreased

in d-NBD (M=2.21) patients versus p-NBD (M=7.37) and controls (4.77) (p□0.0001). In the CSF compartment, comparisons among the three different groups indicated that IFN-γ and IL-17 expression showed a significant increase in NBD groups compared to controls but failed to reach significance between p-NBD and d-NBD.However, concerning IL-6 expression we found a significant increase in all NBD patients compared to controls (p=0,0034 for d-NBD and p=0,015). Interestingly, CSF IL-6 expression was higher in d-NBD patients as compared to p-NBD patients (p = 0.047)**(Figure 2).**

We also evaluated IL-10 expression as an anti-inflammatory cytokine, in the CSF of the three studied groups.Our results show that this cytokine was significantly increased in the two groups of NBD compared to the healthy control group (p<0,5) but no significant difference between p-NBD and d-NBD was observed (p>0,5). TH2 and T reg (GATA3, IL-4 and Foxp3 respectively) markers were also investigated but we didn't detect any significant difference (p>0,5).

Discussion

The main aim of the present study was to evaluate a differential profile in PBMCs and CSF of NBD classified as definite and probable parenchymal NBD. The most important conclusions that can be draw from this study are first the absence of OCB in CSF of NBD patients and second the higher IL-6 expression which plays a crucial role in the pathogenesis of NBD. Although the key role ofIL-6 has been already pointed by several investigations (11,17,18).The result of the present study show that IL-6 expression in CSF can support the definite NBD diagnosis.

Our findings concerning demographic and clinical characteristics are in line with previous studies (19, 20, 21). Indeed NBD was reported 2-8 times more prevalent in men than women (2). The mean duration of BD before neurological manifestation onset was 2.5 years (30 months) for our patients and ranged from 3 to 6 years in two major studies (22,23). However, neurological presentation might coincide with the first systemic symptoms of BD or precede them (6% of patients) (24,25). In such cases, and in high prevalence areas, diagnosis of p-NBD can be advanced and treatment with immunosuppresive drugs can be started. In 14 cases of inaugural neurological signs, 50% of patients (N=7) developed systemic signs such buccal and genital aphtosis, pseudofolluculitis, erythema nodosa,fulfilling ISG criteria, in a mean period of 5.57 months and become d-NBD. The other half (N=7) never developed systemic signs suggesting BD during 3-5 years of follow-up, since all patients included in this study are treated in the same way, except the two BD patients with non-suggestive neurological signs. Knowing that in the presence of early high suggestive neurological signs and in the absence of ISG criteria of BD, patients can be treated as d-NBD after expert opinion.

Neuroimaging studies in CNS NBD have shown that lesions are generally located within the brainstem, with extension to the diencephalon and less commonly within the periventricular and subcortical white matter (2,3). In Contrary, 8/13 (61,5%) of d-NBD patients included in our study, had isolated supratentorial location and only 5/13 (38.5%) d-NBD patients had brainstem lesions.

There are very few studies regarding the CSF immunoelectrophoretic data. Intrathecal oligoclonal Ig G bands are infrequently found in NBD. Only 8/121 consecutive NBD had OCB in the CSF showing pattern 2 in a Turkich Study (26). All these positive cases had parenchymal NBD. In our study, only one patient had OCB in the CSF showing pattern 2. This patient is classified into p-NBD and never developed BD signs or the ISG criteria. 19/22 (86.36%) had pattern 1 and two d-NBD patients had pattern 4 meaning that OCB synthesized in the blood and migrated across impaired blood-CSF barriers showing mirror pattern. The most important differential diagnosis in this case is chronic infectious disease like Tuberculosis or Brucellosis especially in endemic areas. These two patients with profile 4 had a non infectiousmeningoencephhalis like presentations.

CNS damage in NBD disease is associated to T and B cell infiltration however data on CSF levels of cytokines in patients with NBD are limited. Since 2006, IL-6 was found in autopsic cases of NBD with multifocal neutrophic perivascular inflammation (27). A. Borhani and al shows elevated IL-6 levels in parenchymal NBD patients compared to headache attributed to BD and healthy controls (13). Hirohata et al had demonstrated that both CSF IL-6 and IL-8 levels were elevated in patients with acute NBD compared to chronic progressive NBD (10). The same authors showed significant elevation of CSF IL-6 in patients with progressive NBD compared to patients with active BD without neurological manifestations (18). Wang et alshowed that IL-6 level, which was higher in CSF of NBD patients, dropped when the disease activity subsided (16). IL- 6 was also used as a marker of therapeutic response in a Japanese center (28) and was shown to be significantly lower in patients with more favorable outcomes (29). In previous Tunisian studies, Hamzaoui et al. showed elevated CSF levels of IL-15 in patients with NBD in comparison with non inflammatory neurological disease patients (30). Another study of the same author pointed to the possible inflammatory role of IL-33 in the CSF of NBD patients (31).

Collectively, these results showed elevated expression of IL-6, IL-17, IFN-γ and IL-10 in CSF NBD patients.Furthermore, our data indicate that IL-6 can be a reliable biomarker of NBD. The absence of IL-6 in patients with neurological signs highly suggestive of NBD can advance two major hypotheses : first, these patients are really NBD IL-6 (-) and never developed systemic signs since they are already under immunosuppressive therapy ; second we can discuss the possibility of anotherneuroinflammatory disease mimiking NBD but having a different pathogenesis requiring more research.

Conclusions

The current study, clearly demonstrate two major points : First ; the absence of OCB in d-NBD, if present, the profile is type 4; second among the pro-inflammatory cytokines, Il-6 is an excellent surrogate marker of NBD, as is consistent with the results of previous studies. Better understanding of pathogenesis of borderline forms is needed among immunologic comparative studies between NBD and other CNS inflammatory disease. Our findings not only shed new light on NBD pathogenesis but also emphasize the potential benefits of antibody-depleting treatment methods for NBD patients. Further studies with a wider range of patient samples are needed to determine the impact of treatment on disease progression and the secretion of these mediators.

Abbreviations

CNS: central nervous system; BD: Behcet disease; NBD: Neurobehcet disease; d-NBD: Definite Neurobehcet disease; p-NBD: probable Neurobehcet disease; CSF: cerebral spinal fluid; OCB: oligoclonal bands; ISG: International study group; ICR: International Consens Criteria; MRI: Magnetic resonance Imagery, PCR: Polymerase Chain Reaction, INF: Interferon; IL: Interleukine; GAPDH: glyceraldehyde-3-phosphate dehydrogenase

Declarations

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Pasteur Institute of Tunis

Informed Consent: Written informed consent was obtained from all participants who participated in this study.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

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Authors Contributions: MK conceived the study, participated in its design and coordination and helped to draft manuscript. MB participated in the design of the study and performed the experiments. KB participated in the qRT-PCR assays. AC and OM performed the statistical analysis. SB and RB conceived and designed the experiment and wrote the manuscript. All authors read and approved the final manuscript.

Conflict of Interest: No conflict of interest was declared by the authors.

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Tables

Table 1: Demographic and clinical characteristics of subjects included in the study

	Overall (patients)	Definite NBD	Probable NBP	Controls	p value
N= 32	N= 22	N=13	N=9	N=10	-
Gender (F/M)	10/12	6/7	4/5	4/6	-
Age (mean± SD)	41.81 (±10.66)	40.46 (±9.32)	43.77 (±12.67)	40.23 (±11.62)	0.43
*** Patients with BD first (ISG+)**	N= 8	N=6	N=2		-
Interval between BD and onset of neurological signs (months)	30 (±22.02)	34 (±22.01)	18.5 (±24.74)		0.05
***Patients with neurological signs first**	N=14	N=7	N=7		-
Interval between neurological signs and BD (months)	5.57 (±3.95)	5.57 (±3.95)	-		-
Age at onset of Neurological signs **(mean± SD)**	37.95 (10.74)	36.46 (9.12)	40.11 (13.00)	-	0.61
Follow up period (months) **(mean± SD)**	51 (±14.31)	51.76 (±15.39)	50 (±13.41)	-	0.81

Table 2: Neurological characteristics, MRI findings and CSF data of subjects included in the study

	Overall patients (N=22)	Definite NBD (N=13)	Probable NBD (N=9)	Controls (N= 10)
Neurologicalcharacteristics :				-
Headaches	6	2	4	
Hemiparesis/pyramidal Signs	17	11	6	
Sensorysymptoms	11	5	6	
Cranial nerve involvement	5	2	3	
Ataxia	3	2	1	
psychiatricsigns	3	0	3	
Seizures	1	1	0	
MRI findings				-
Isolatedsupratentorial	13	8	5	
location	5	3	2	
Basal ganglia	5	2	3	
Internal capsule	6	5	1	
Brainstemlesions	1	0	1	
myelitis	2	0	2	
No abnormalities				
CSF analysis				
CSFprotein concentration	-	0.64 (±	0.67 (± 0.19)	0.39 (±0.13)
(g/l)	-	0.21)	23 (±7.66)	4,3 (±5.6)
Cell count (/mm3)	0.50 (±0.05)	34.8 (±	0.53 (±0.07)	-
Ig G Index		8.22)		
IEPP	19	0.50 (±0.05)	8	10
Profile type1 (N)	1		1	0
Type 2 and 3 (N)	2	11	0	0
Type 4 (N)		0		
		2		

Table 3: Median of peripheral blood and CSF expression of transcription factors and cytokines in NBD patients

		IL-17	INF-γ	IL-10	IL-4	GATA3	T-bet	RoR-γt	FOXP3	IL-6
PBMC	d-NBD	7,48	6,707	6,53	5,95	6,84	7,028	6,96	2,21	4,77
	p-NBD	6,63	6,04	5,082	6,93	6,91	6,902	8,051	7,37	5,039
	p value	0,51	0,74	0,66	0,29	0,81	0,86	0,58	0,39	0,96
	Controls	5,49	4,07	4,54	6,25	3,98	3,93	3,73	5,015	3,29
CSF	d-NBD	3,65	3,95	4,4	2,97	2,32	4,15	4,67	0	2,54
	p-NBD	2,39	6,65	3,63	1,59	4,40	2,17	5,34	0	0
	p value	0,32	0,76	0,72	0,68	0,113	0,27	0,79	0,95	0,047
	controls	0	1,14	1,42	3,26	4,30	3,28	4,38	0,073	0

Figures

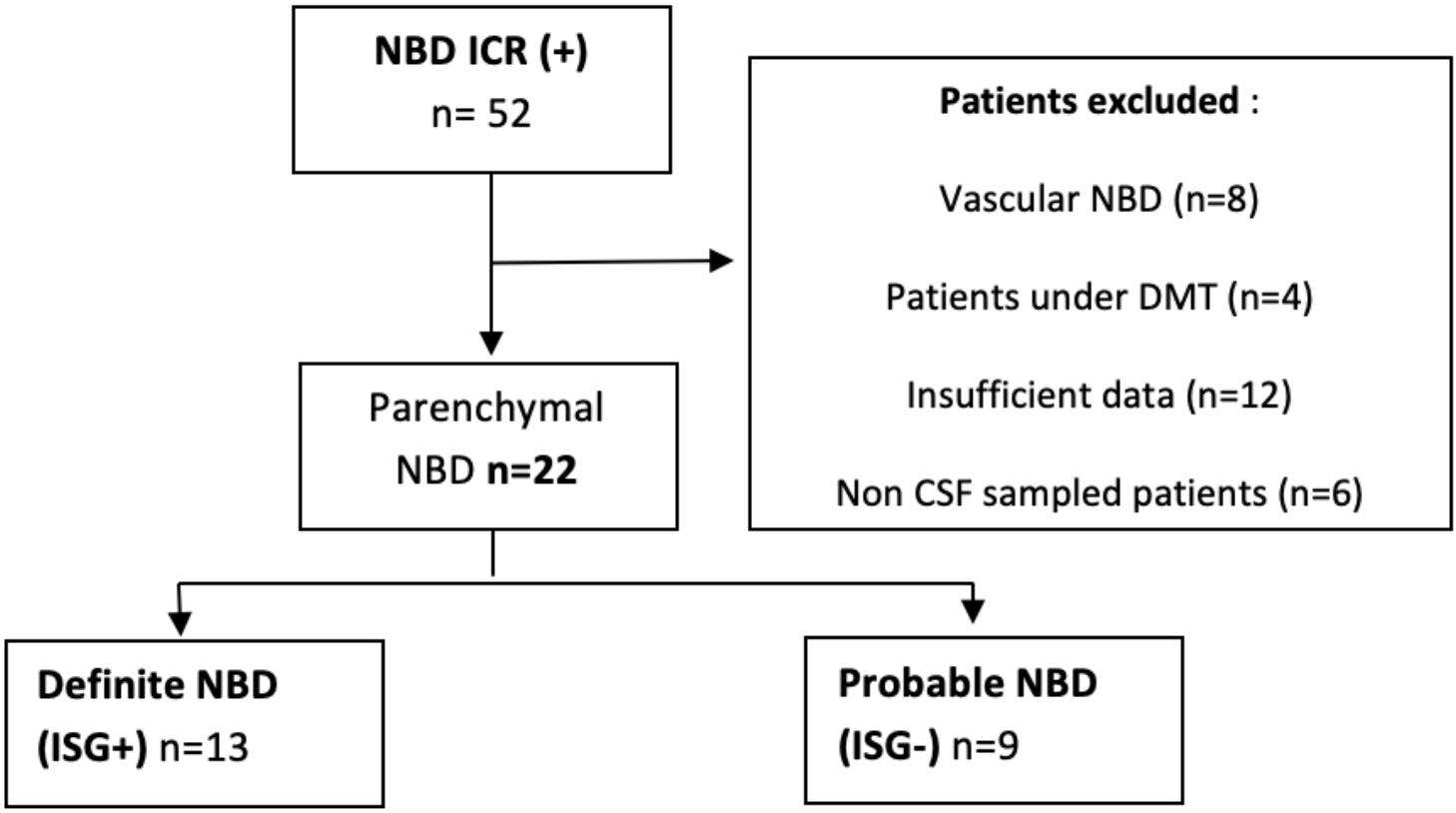

Figure 1

Flow chart showing the distribution of the study population - DMT : Disease modifying therapy, CSF : cerebro spinal fluid, ISG : International Study Group.

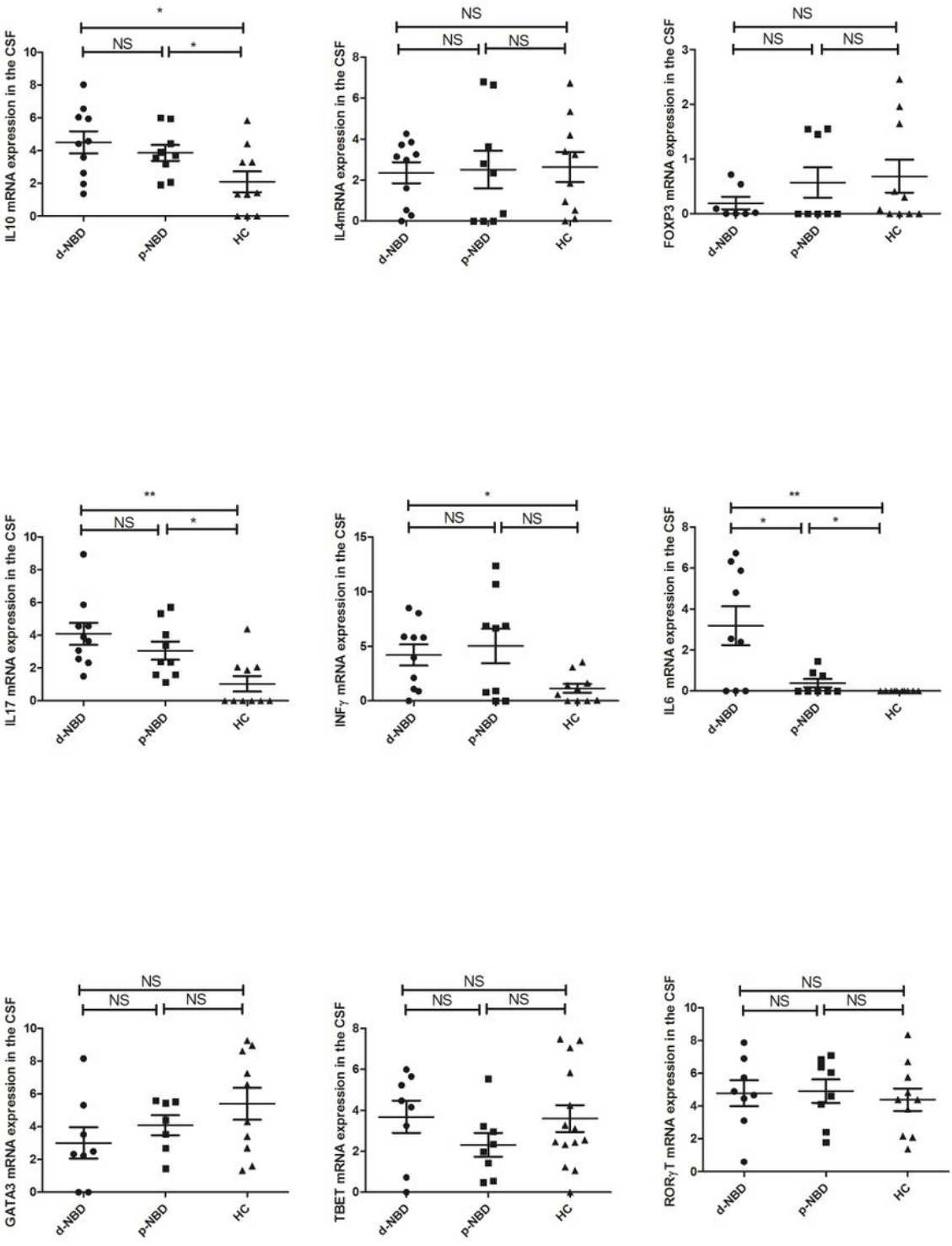
IL10 mRNA expression in the CSF
IL4mRNA expression in the CSF
FOXP3 mRNA expression in the CSF
IL17 mRNA expression in the CSF
INFγ mRNA expression in the CSF
IL6 mRNA expression in the CSF
GATA3 mRNA expression in the CSF
TBET mRNA expression in the CSF
RORγT mRNA expression in the CSF
d-NBD
p-NBD
HC
NS
*
**

Figure 2