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IMMUNOPHENOTYPING PREDICTIVE OF MYCOPLASMA INFECTION IN PATIENTS WITH CHRONIC FATIGUE SYNDROME?

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Abstract

An impaired immune system and opportunistic infections are 2 main characteristics of the pathophysiology of Chronic Fatigue Syndrome (CFS). This study therefore aimed at comparing immunophenotyping among healthy subjects (N=35) and two subsets of CFS: patients with no *Mycoplasma* detected (N=55) and patients with evidence of a *Mycoplasma* infection in their blood (N=131). Immunophenotyping was performed using immunofluorescence with the appropriate combination of monoclonal antibodies. *Mycoplasma* detection *(Myc. hominis, Myc*. *fermentans*, *Myc. pneumoniae* and *Myc. penetrans*) was performed with forensic polymerase chain reaction on peripheral blood samples. Both patients groups presented with significantly elevated CD25+ (activated) cells as compared to healthy volunteers. CFS patients without evidence of *Mycoplasma* infection had increased amounts of CD5+ B-cells. Stepwise discriminant analysis indicated the number of activated cells, the number of memory CD4+ cells and the percentage of suppressor T-cells (lower in Mycoplasma+ patients as compared to Mycoplasma- patients) as the discriminant variables. A classifiaction tree, for predicting the presence of *Mycoplasma* species in CFS patients, was constructed. Taken together, these data confirm earlier reports on immune activation among CFS patients, but deny this to be specific for *Mycoplasma* infected CFS patients. Immune reaction appears to be less controlled in *Mycoplasma* infected CFS patients.

Introduction

Chronic Fatigue Syndrome (CFS), named Chronic Fatigue Immune Dysfunction Syndrome in the United States, is a chronic debilitating disease of unknown cause. However, *Mycoplasma* infections may serve as primary or secondary etiological factors. Mycoplasmas are prokaryotes containing circular DNA and some ribosomes (12), while lacking a cell wall and certain cellular

organelles. The high prevalence of *Mycoplasma* infections in CFS has been discussed at length in the scientific literature (3-8). The presence of opportunistic infections in CFS suggests an impaired immune system in these patients. Indeed, a deregulated 2'5' oligoadenylate synthetase ribonuclease antiviral pathway (9-11) and a reduced natural killer cell function (13-15) are some of the characteristics of the impaired immunity in at least subsets of CFS. Some species of *Mycoplasma* are part of the normal human flora and are capable of inducing complex systemic infections in immunocompromised hosts, as seen in HIV-AIDS (16) and CFS.

Immunogenic properties of *Mycoplasma* species have been studied extensively. *Myc. fermentans* has been shown to affect the immune system via T- or B-cell activation and macrophage stimulation (reviewed in 2). A membrane-associated C3 (the third component of the complement system) activating protein (M161Ag), originating from *Myc. fermentans*, efficiently promotes the production of nitric oxide (NO), interleukin 1β (IL-1β), tumour necrosis factor α (TNF-α), IL-10 and IL-6 in human peripheral blood monocytes (2,17,18, reviewed in reference 19). Activation of the complement system by *Myc. fermentans* implicates rapidly marking of infected cells with C3, which in turn stimulates the release of C5a chemotactic factor and enhances the phagocytic activity. Kikkawa and co-authors have demonstrated that the human complement system was able to clear *Myc. fermentans* from the surface of infected cells, but was not able to prevent persisting low-grade infection in human tumour cell lines probably due to rapid invasion of the human cells and tissues (17).

Fewer data describing host defence action against respiratory mycoplasmosis (*Myc. pneumoniae* and *Myc. pulmonis*) are currently available. Both the innate (alveolar macrophages) and humoral immunity appear to be of prime importance in the defence action against *Myc. pneumoniae* and *Myc. pulmonis* (20). Moreover, T-cell responses in respiratory mycoplasmosis infection can cause exacerbation of lung lesions, while innate immunity is crucial in defence of the lungs and humoral immunity in preventing dissemination of infection to extrapulmonary sites (20). In accordance to these observations, a recent research report provides an explanation for the characteristic chronic nature of *Mycoplasma* infection. Indeed, *Myc. pulmonis* infection induced a transitional shift of the T_H1 (T helper cells type 1) – T_H2 balance in favour of humoral immunity, which illustrated the immunological incompetence that allows the micro organism to survive (21). The exact role of cell-mediated immunity however, still needs to be established.

To our knowledge, no attempt has been made so far to examine the effect of Mycoplasmas on immune cells in CFS patients. This study aimed at comparing immunophenotyping among CFS patients with Mycoplasmal infection, CFS patients with no *Mycoplasma* detected and healthy volunteers.

Methods

Sample

The study was conducted in Brussels, at a university-based outpatient clinic (Vrije Universiteit Brussel). One hundred and eighty-five consecutive patients seeking care for prolonged fatigue as major complaint, and who complied with the 1994 CDC case definition (1), were enrolled. Thirty-five age matched healthy volunteers were recruited among university students, health care professionals and hospital employees and served as control subjects.

To fulfil the CDC criteria for CFS, clinically evaluated, unexplained, persistent or relapsing chronic fatigue that is of new or definite onset, should result in a substantial reduction in previous levels of occupational, educational, social, or personal activities (1). Additionally, at least four of the following symptoms must have persisted or recurred during 6 or more consecutive months and must have not predated the fatigue: impairment in short-term memory or concentration, tender cervical or axillary lymph nodes, muscle pain, multi-joint pain, headache, unrefreshing sleep and post-exertional malaise > 24 hours (1). Any active medical condition that may explain the presence of chronic fatigue prohibits the diagnosis of CFS. Therefore, all subjects underwent an extensive medical evaluation, consisting of a standard physical examination, medical history, exercise capacity test and routine laboratory tests. The laboratory tests included a complete blood cell count, determination of the erythrocyte sedimentation rate, serum electrolyte panel, measures of renal, hepatic and thyroid function, as well as rheumatic and viral screens. When judged necessary, a structured psychiatric interview was performed. In a number of cases further neurological, gynaecological, endocrine, cardiac and / or gastrointestinal evaluations were performed. The medical records were also reviewed to determine if patients suffered from organic or psychiatric illnesses that could explain their symptoms.

Control subjects were questioned about their health status. They had to be free of disease at least three months prior to data collection. All subjects were excluded if they were < 18 or ≥ 60 years of age, or if they had received antibiotics two months prior to phlebotomy. All patients and controls were Caucasian. Demographic features of the data sample are presented in Table 1.

Immunophenotyping

Anticoagulated blood (EDTA) was collected between 9 and 11 a.m. and used for white blood cell enumeration, differential counts (Celldyn 4000, Abbott Laboratories, Abbott Park, IL 60064, USA) and flow cytometric studies. Lymphocyte populations were analysed with dual colour direct immunofluorescence on a EPICS® xl flow cytometer (Coulter, Miami, Florida, USA), with aid of the System I ™ computer software. One hundred μl of whole blood was incubated, using the appropriate combination of monoclonal antibodies for 25 minutes at 4°C. Then, red cells were lysed using lysis buffer (Becton Dickinson) for 7 minutes, spun down and washed once with 2 ml phosphate buffered saline (PBS). Resuspension was immediately followed by cell analysis. Commercially available (Becton-Dickinson) phycoerythrin (PE) or fluorescein isothiocyanate (FITC) monoclonal antibodies were used and are listed in Table 2. Estimates of absolute numbers of lymphocyte subsets were determined by multiplying peripheral lymphocyte counts by the percentage of each surface marker.

Mycoplasma detection: Forensic PCR

Collection of Blood

Subjects' blood was collected between 9.00 and 11.00 A.M. at the Chronic Fatigue Clinic of the academic hospital of the Vrije Universiteit Brussel. Blood was collected in EDTA-containing tubes and immediately brought to ice bath temperature and flash frozen as described previously (3,7). Samples were shipped with dry ice by air courier to the Institute for Molecular Medicine for analysis. All blood samples were blinded. Whole blood (50 μl) was used for preparation of DNA using Chelex (Biorad, Hercules, USA) as follows. Blood cells were lysed with nano-pure water (1.3 ml) at room temperature for 30 min. After centrifugation at 13000 x g for 2 minutes, the

supernatants were discarded. Chelex solution (200 μl) was added, and the samples were incubated at 56°C and at 100°C for 15 minutes each. Aliquots from the centrifuged samples were used immediately for PCR or stored at –70°C until use. Multiple *Mycoplasma* tests were performed on all patients.

Amplification of Gene Sequences

Amplification of the target gene sequences (Table in Nasralla et al. (3)) was performed in a total volume of 50 μl PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 9) containing 0.1% Triton X-100, 200 μm each of dATP, dTTP, dGTP, dCTP, 100 pmol of each primer, and 0.5 – 1 μg of chromosomal DNA. Purified mycoplasmal DNA (0.1 – 1 ng of DNA) was used as a positive control for amplification. The amplification was carried out for 40 cycles with denaturing at 94°C and annealing at 60°C (genus-specific primers and *Myc. penetrans*) or 55°C (*Myc. pneumoniae, Myc. hominis, Myc. fermentans*). Extension temperature was 72°C in all cases. Finally, product extension was performed at 72°C for 10 min. Negative and positive controls were present in each experimental run.

Southern Blot Confirmation

The amplified samples were run on a 1% agarose gel containing 5 ml / 100ml of ethidium bromide in TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA, pH 8.0). After denaturation and neutralization, Southern blotting was performed as follows. The PCR product was transferred to a Nytran membrane. After transfer, UV cross-linking was performed. Membranes were prehybridized with hybridisation buffer consisting of 1x Denhardt's solution and 1 mg/ml salmon sperm DNA as blocking reagent. Membranes were then hybridized with ^{32}P-labeled internal probe (10^7 cpm per bag). After hybrization and washing to remove unbounded probe, the membranes were exposed to autoradiography film for 1-2 days at –70°C.

Statistics

Subjects' age characteristics were analysed using descriptive statistics and Bonferroni multiple comparisons. Sex differences were assessed using binomial nonparametric testing and Chi-Square tests. Descriptive statistics were computed for all variables among different groups (Mycoplasma infected CFS patients, CFS patients with no Mycoplasma detected and healthy volunteers). Uni-variant group differences were assessed on untransformed data using one-way ANOVA (analysis of variances), followed by pair wise multi-comparison Bonferroni test (t-test). As for a few variables heterogeneity of variances was observed (Levene's test), it was decided to double-check the ANOVA-results by non-parametric Kruskal-Wallice test. The significance level of the different tests was at 0.05. The data were processed using SPSS 10.0© for Windows (Prentice Hall). Discriminant variables were determined using stepwise linear discriminant analysis of the differences between the 3 groups. At each step, the variable that minimizes the overall Wilks' Lambda is entered (the maximum significance of F to enter is 0.20; the minimum significance of F to remove is 0.25).

Classification trees were originally invented by Brieman and colleagues (22). Classification trees (CT) classify the groups of a single discrete variable (e.g. types of *Mycoplasma*) using explanatory variables, which may be numeric or categorical. This is done by growing a tree structure, which divides the data into mutually exclusive groups, of which has similar values for the response variable. Starting with all the data, which are represented by a single node at the top of the tree, the tree is grown by repeated binary splitting of the data. Each split defines a simple rule and forms two nodes. Rules are usually based on a single explanatory variable, and the form of the rule depends on the type of explanatory variable. For numeric explanatory variables, a split is defined by values less than and greater than a certain value. The terminal nodes (i.e. unsplit nodes) represent the groups of data formed by the tree and are also called the leaves of the tree.

Splits are (generally) chosen to maximise the homogeneity (minimise the impurity) of the resulting two nodes. Thus for each split a search is made through all possible splits of all explanatory variables and the best is selected. The impurity of a node can be defined in many ways. For

classification trees the impurity is the proportion of cases from other groups present in the node together with predominant group. Total purity means that a node contains only cases from one group. The splitting procedure is continued until an over large tree is grown, which is then pruned back to the desired size. The size may depend on the objective of the analysis: be it exploration, description or prediction. For prediction we select the size that is the most accurate predictor. Often cross validation is used to select the tree size, with the chosen tree having the lowest misclassification rate. This tree can be thought of as the best predictive tree in the sense that on average it should give the most accurate predictions of all possible trees obtainable by pruning back the over large tree.

Results

Mean age characteristics ranged between 34 and 36 years for the 3 groups. Statistical analysis revealed no significant differences in mean age between the 3 groups. No sex differences between the 2 patients' groups (CFS patients with or without *Mycoplasma* detected) were found. Significantly more female subjects were present in the control sample as compared to both patients groups. All demographic data are presented in Table 1.

One-way ANOVA (Table 3) revealed significant differences among groups for the number of CD25+ cells (activated cells) ($p = 0.007$). Looking at the Bonferroni analysis, it was concluded that the number of activated cells was significantly elevated in both CFS-groups (*Mycoplasma+* as well as *Mycoplasma–* CFS patients) compared to healthy volunteers (see Table 4). Bonferroni analysis can be used to analyse this variable, because Levene's test suggested homogeneity of variances for the number of activated cells (data not shown). No differences between the two patients' groups were observed concerning this variable. Kruskal-Wallice test confirmed the differences in CD25+ count and revealed significant differences in the number ($p = 0.009$) as well as the percentage ($p = 0.002$) of CD5+ B-cells (CD19+CD5+) (Table 5). These observations were confirmed by Tanhame's pair wise multi-comparisons, indicating significantly increased CD19+CD5+ cells between CFS with no *Mycoplasma* detected and healthy volunteers ($p < 0.001$) (Table 4). No such observations were done between other groups.

Stepwise linear discriminant analysis of the differences between the three groups indicated the number of activated cells as the primary discriminant variable (Wilks'$\lambda = 0.953$, $F = 5.254$, $p = 0.006$), followed by the number of memory CD4+ cells (CD4+CD45RA-) (Wilks'$\lambda = 0.922$, $F = 4.411$, $p = 0.002$) and the percentage of suppressor T-cells (Wilks'$\lambda = 0.908$, $F = 3.507$, $p = 0.002$)(Table 6). In comparison to healthy volunteers, this sample of CFS patients had elevated numbers of activated cells and lower numbers of memory CD4+ cells. Additionally, among these CFS patients, *Mycoplasma* infected subjects presented with lower numbers of suppressor cells compared to those with no *Mycoplasma* detected.

The classification tree for predicting the presence of a *Mycoplasma* infection in CFS patients using immunophenotyping data is presented in Figure 1; corresponding probabilities of the root endings are listed in Table 7. It is impossible to explain the entire classification tree in this manuscript, we therefore chose to write down a single case report of a patient that did not participate in the trial. An Endocrinologist referred a 54-year old woman to the Chronic Fatigue Clinic of the Vrije Universiteit Brussel (VUB). Her current health problems had started 15 years ago with an autoimmune disorder of the thyroid gland. At the time she visited the fatigue clinic this patient complained of severe fatigue, especially following exercise, and many other symptoms (myalgia, arthralgia, non-refreshing sleep, neurocognitive impairments…). Blood was collected for routine laboratory tests, for immunophenotyping and for *Mycoplasma* PCR detection. According to the classification tree, the CD5+ B-cell (# = 77) count was checked first and exceeded the threshold value of 75.04. We were therefore guided into the right part of the tree, with the number of CD3+CD16CD56+ (subset cytotoxic T-cells; # = 103) being the next discriminating variable. Because this parameter did not fulfil the tree equation, the cytotoxic T-cells count (CD8+CD11b-) was screened and it exceeded the tree threshold value (step 3: 515 > 283.5). Next, the B-cell count fulfilled the equation (335 < 587) as well as the percentage virgin CD4-cells in step 5. This way we were quite sure this was a CFS patient, because the next step consists out of a binary split into 2 root endings suggesting CFS. The patient' immunophenotyping profile revealed a slightly increased number of activated T-cells (232 exceeded the threshold value in the tree and exceeded the reference range used in the laboratory (see Table 3)). This way, according to the classification tree, the patient was judged to have a 100 % probability (see Table 7 for the probability data for each root ending) for being a *Mycoplasma* infected CFS patient. Indeed, this patient fulfilled both the 1988 (23) and the 1994 (1) CDC case definitions for CFS and was diagnosed as having a *M. hominis* infection. Interestingly, the *Mycoplasma* PCR was performed at a distinct laboratory (RED laboratories Zellik, Belgium) than the analyses used for the construction of the classification tree.

Discussion

A significant increase in activated cells ($p = 0.007$ in the one-way ANOVA) was observed in these CFS patients as compared to healthy controls, and the stepwise linear discriminant analysis indicated this as the primary discriminant variable of the differences between the 3 groups. These data are consistent with earlier reports on immune activation (24-26). To our knowledge however, a manuscript providing evidence of immune activation using CD25+ cells is currently unavailable. Both the CFS patients with and without a *Mycoplasma* infection presented with significantly elevated CD25+ cells, suggesting an immune response against another pathogen in the *Mycoplasma* negative patients. Immune activation appears to be less controlled in *Mycoplasma*-infected CFS patients in comparison to *Mycoplasma* negative CFS patients, because the former group presented with a lower percentage of suppressor cells. Both increased (27) and reduced numbers (28) of CD8+CD11b+ cells have been reported in CFS as compared to healthy controls, the lack of sub-grouping in these research reports may account for this discrepancy.

The *Mycoplasma* PCR negative CFS patients presented with significantly elevated numbers of CD5+ B-cells compared to healthy volunteers (Tanhame's pair-wise multi-comparisons: $p < 0.001$), which was not observed in *Mycoplasma* infected CFS patients. This obviously explains why this marker is used on top in the classification tree. The CD5+ surface marker was originally found only on T-cells; its function on B-cells in uncertain. It may be involved in the regulation of the B-cell activation and is mainly displayed on B-1 cells, which are biased towards auto antigens and bacteria (29). Mycoplasmas have been reported to activate B-cells (2,20), which may account for the increased however non-significant numbers of CD19+CD5+ cells observed in these *Mycoplasma*+ CFS patients as compared to healthy controls. Tirelli and colleagues (1994) found increased numbers of CD19+ cells in CFS as compared to asymptotic volunteers (30). Interestingly, in 11 out of 30 examined CFS patients the increase in B-lymphocytes was sustained by the expansion of the CD5+ subset of B-cells (30). Klimas et al (1990) reported additional evidence for a high proportion of CD5+ bearing B-cells in CFS (31), while this was refuted by others (32).

The data presented in this manuscript suggest an important discriminating role for memory CD4+ cells as well (Wilks'$\lambda = 0.922$, $F = 4.411$, $p = 0.002$). The lower numbers of memory cells as observed in these CFS patients compared to healthy controls may not be valid because of the high number of hospital employees in the control group.

In conclusion, analysing these data using 'classical' statistics did not reveal major differences in immunophenotype profile between *Mycoplasma*+ and *Mycoplasma*- CFS patients. One could state immunophenotyping not to be indicative of *Mycoplasma* infection in CFS. We therefore chose to reanalyse these data using a recently developed statistical method (the construction of a classification tree). The classification tree (Figure 1 and Table 7) enables one to use immunophenotyping for a prediction of a *Mycoplasma* infection in CFS patients. In the presented case report, the classification tree was able to predict the presence of a *Mycoplasma* infection. A *Mycoplasma* PCR was still required because the classification tree is unable to distinguish between different *Mycoplasma* species, which has important therapeutic implications. The classification tree should not be used to diagnose CFS, as suggested in the case study. It can only be utilised as a differential diagnostic aid for predicting *Mycoplasma* infection in case of CFS.

These data should be interpreted with caution because no attempt was made to monitor *Mycoplasma species* in the control group and a high number of health care workers were selected for the control group. The main goal of this study however, was to examine immunopathology of Mycoplasmas in patients with CFS and consequent subgrouping of the CFS population. Moreover, the prevalence of mycoplasmal infections among asymptomatic individuals ranges between 5 and 15 % (reviewed in (6)), while in a previous study among European citizens 2 out of 36 (5.6 %) healthy control subjects were infected by one species of *Mycoplasma* (unpublished observations). Additionally, the control subjects differed in sex distributions as compared to both patients' groups. This may well have biased these results. On the other hand, this classification tree should aid in predicting the presence of *Mycoplasma* infections in CFS

patients rather than differentiating between patients and controls. Finally, this sample was not randomly selected. A sample consisting out of consecutive patients visiting a clinic however is more likely to represent routine clinical practice. Although much work was done to validate the methods used for the construction of the classification tree, the usage of this tree still needs to be validated in clinical situations (in accordance to the presented case report).

In this research report, minor differences in immune cells were observed between healthy volunteers, *Mycoplasma*+ and *Mycoplasma*- CFS patients. The number of CD25+ activated cells, the number of memory CD4+ cells and the percentage of suppressor T-cells were identified as the primary discriminant variables. Nevertheless, the construction of this classification tree should enable physicians to predict the presence of a *Mycoplasma* infection in CFS more accurately and consequently reduce the number and costs of laboratory tests.

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Table 1. Demographic features

Subgroup	N	Mean age in years (SD)	Number of females	Females (%)	Number of males	Males (%)
***Mycoplasma* infected CFS patients**	131	35.7* (9.6)	113**	86.3	18**	13.7
CFS patients no *Mycoplasma* detected	55	36.2* (10.6)	50**	90.9	5**	9.1
Healthy controls	35	34.2* (11.5)	26**	74.3	9**	25.7

* Bonferroni multiple comparisons revealed no statistical significant differences in mean age between the 3 groups.

** Binomial nonparametric testing and Chi-Square tests revealed no sex differences between CFS with or without *Mycoplasma* detected, but did show statistical significant sex distributions between the healthy volunteers and both patients groups.

Table 2.
Monoclonal antibodies used for immunophenotyping

Complementarity Determination	Monoclonal antibodies	Subset
CD2+	Leu5b+FITC	E-rosette receptor
CD3+	Leu4+FITC	T-cells
CD3+HLADR+	Leu4+HLADR+PE	Activated T-cells
CD25+	IL2R1-PE	Activated cells
CD4+	Leu3a+FITC	Helper/inducer T-cells
CD4+CD45RA-	Leu3aLeu18-PE	Memory CD4-cells
CD4+CD45RA+	Leu3aLeu18+	Virgin CD4-cells
CD8+	Leu2a+FITC	Cytotoxic/Suppressor T-cells
CD8+CD11b+	Leu2aLeu15+PE	Suppressor cells/NK-subset
CD8+CD11b-	Leu2aLeu15-	Cytotoxic T-cells
CD19+	Leu12+PE	B-cells
CD19+CD5+	Leu1+Leu12+	Mature B-cells
CD3-CD16+CD56+	Leu4-Leu16+PE Leu19+PE	NK-cells
CD3+CD16+CD56+	Leu4+Leu16+Leu19+	Subset cytotoxic T-cells

Table 3. The descriptive and ANOVA analysis of the differences in the immunophenotype profile in the CFS patients with and without *Mycoplasma* infection and healthy controls.

Variable	Mean (SD)	Range	Reference range*	ANOVA *p*-value
CD4+/CD8+ ratio	1: 1.61 (.56) 2: 1.68 (.72) 3: 1.72 (.68)	0.47 – 2.65 0.70 – 4.71 0.57 – 4.77		.686
% CD2+	1: 84.03 (4.13) 2: 82.29 (5.29) 3: 81.91 (.44)	76.00 – 92.00 69.00 – 93.00 69.00 – 93.00	76-91	.084
CD2+	1: 1930.66 (491.78) 2: 1921.04 (602.72) 3: 1881.17 (590.33)	782.00 – 3019.00 655.00 – 3631.00 11.00 – 3370.00	1158-2680	.858
% CD3+	1: 74.77 (5.19) 2: 73.37 (8.21) 3: 73.19 (7.86)	65.00 – 87.00 53.00 – 89.00 46.00 – 90.00	66 - 83	.545
CD3+	1: 1710.86 (461.17) 2: 1717.49 (586.06) 3: 1687.44 (581.36)	763.00 – 2700.00 554.00 – 3453.00 10.00 – 3248.00	982 – 2508	.938
% CD3+HLADR+	1: 5.09 (2.76) 2: 5.54 (2.31) 3: 5.34 (3.04)	1.00 – 14.00 1.00 – 10.00 1.00 – 19.00	2 – 9	.762
CD3+HLADR+	1: 115.14 (67.82) 2: 128.55 (70.42) 3: 119.85 (77.50)	24.00 – 310.00 14.00 – 353.00 1.00 – 420.00	28 – 197	.667
% CD25+	1: 14.20 (4.46) 2: 16.68 (6.46) 3: 16.92 (6.34)	8.00 – 24.00 4.00 – 41.00 8.00 – 38.50	8 – 22	.064
CD25+	1: 318.89 (138.91) 2: 448.40 (237.61) 3: 442.08 (216.71)	106.00 – 718.00 176.00 – 1337.00 2.00 – 1488.00	142 – 493	**.007**
% CD4+	1: 46.66 (8.66) 2: 47.35 (7.83) 3: 47.96 (7.11)	24.00 – 61.00 33.00 – 72.00 29.00 – 65.00	33 – 61	.638
CD4+	1: 1067.49 (326.50) 2: 1108.04 (403.28) 3: 1101.36 (385.69)	357.00 – 1847.00 338.00 – 2205.00 5.00 – 2270.00	495 – 1652	.873
% CD4+CD45RA-	1: 28.71 (9.00) 2: 25.65 (9.66) 3: 26.72 (7.97)	4.00 – 49.00 3.00 – 43.00 0.00 – 44.00	18 – 45	.258
CD4+CD45RA-	1: 652.71 (254.34) 2: 586.38 (252.62) 3: 610.09 (261.09)	109.00 – 1292.00 52.00 – 1148.00 0.00 – 1541.00	181 – 1092	.493
% CD4+CD45RA+	1: 17.89 (8.17) 2: 21.69 (10.55) 3: 21.18 (8.37)	6.00 – 43.00 4.00 – 52.00 6.00 – 46.00	7 – 30	.107
CD4+CD45RA+	1: 413.39 (252.63) 2: 522.76 (341.46) 3: 490.52 (242.78)	144.90 – 1207.68 50.00 – 1702.00 1.00 – 1388.00	139 - 529	.174
% CD8+	1: 31.34 (7.44) 2: 30.76 (7.01) 3: 30.47 (7.69)	21.00 – 51.00 12.00 – 46.50 13.00 – 64.00	23 – 41	.826
CD8+	1: 731.01 (288.91) 2: 713.07 (263.90) 3: 699.70 (286.40)	372.60 – 1426.92 233.00 – 1492.00 6.00 – 1908.00	376 – 1119	.834
% CD8+CD11b+	1: 7.23 (4.24) 2: 8.59 (5.84) 3: 6.97 (3.72)	1.00 – 18.00 1.00 – 30.00 1.00 – 20.00	1 – 16	.074
CD8+CD11b+	1: 169.20 (109.82) 2: 191.95 (135.41) 3: 159.17 (95.49)	16.00 – 437.00 24.00 – 787.00 1.00 – 465.00	18 – 330	.178
% CD8CD11b-	1: 24.03 (6.76) 2: 22.15 (7.26) 3: 23.50 (7.11)	8.00 – 39.00 3.00 – 34.00 9.00 – 62.50	15 – 36	.390
CD8+CD11b-	1: 560.34 (238.56) 2: 521.20 (250.19)	151.00 – 1065.00 53.00 – 1359.00	286 – 826	.757

	3: 540.32 (245.31)	4.00 – 1555.0		
% CD19+	1: 11.65 (4.07) 2: 12.62 (5.05) 13.65 (5.44)	6.00 - 22.00 4.00 – 27.00 0.00 – 27.00	6 – 20	.101
CD19+	1: 261.95 (103.17) 2: 294.75 (139.09) 3: 315.05 (165.13)	102.96 – 574.08 37.00 – 606.00 0.00 – 916.00	111 – 401	.177
% CD19+CD5+	1: 2.00 (0.85) 2: 4.81 (11.25) 3: 3.56 (2.70)	0.00 – 4.00 1.00 – 84.00 0.00 – 20.00	1 – 4	.100
CD19+CD5+	1: 44.91 (19.83) 2: 117.09 (303.10) 3: 82.42 (73.41)	0.00 – 73.08 15.00 – 2268.00 0.00 – 540.00	12 – 73	.121
% CD3+CD16CD56+	1: 87.63 (92.08) 2: 104.62 (103.76) 3: 89.15 (70.42)	0.00 – 486.00 20.00 – 542.00 0.00 – 323.00	1 – 9	.477
CD3+CD16CD56+	1: 3.77 (3.37) 2: 4.56 (4.45) 3: 3.78 (2.50)	0.00 – 16.00 1.00 – 27.50 1.00 – 12.00	20 – 113	.299
% CD3-CD16CD56+	1: 11.51 (5.35) 2: 12.26 (6.99) 3: 11.15 (6.70)	3.00 – 23.00 3.00 – 34.00 0.00 – 30.00	4 – 22	.577
CD3-CD16CD56+	1: 266.06 (137.26) 2: 275.71 (180.18) 3: 250.09 (161.88)	29.00 – 628.00 60.00 – 1032.00 0.00 – 796.00	64 – 437	.599

Legend Table 3.

* reference range used by the department of haematology and immunology at the Academic Hospital Vrije Universiteit Brussel (VUB)
1 = healthy controls
2 = *Mycoplasma* infected CFS patient
3 = CFS patient with no *Mycoplasma* detected

Table 4. The Bonferroni and Tamhane analysis (multiple comparisons) of the differences in the immunophenotype profile in the CFS patients with and without *Mycoplasma* infection and healthy controls.

Dependent Variable	Type of analysis	(I) GROUP	(J) GROUP	Mean Difference (I-J)	*p*-value
CD4+/CD8+ ratio	Bonferroni	1.00	2.00	-7.1948E-02	1.000
			3.00	-.1098	1.000
		2.00	1.00	7.195E-02	1.000
			3.00	-3.7832E-02	1.000
		3.00	1.00	.1098	1.000
			2.00	3.783E-02	1.000
	Tamhane	1.00	2.00	-7.1948E-02	.934
			3.00	-.1098	.701
		2.00	1.00	7.195E-02	.934
			3.00	-3.7832E-02	.982
		3.00	1.00	.1098	.701
			2.00	3.783E-02	.982
% CD2+	Bonferroni	1.00	2.00	1.7377	.323
			3.00	2.1193	.079
		2.00	1.00	-1.7377	.323
			3.00	.3817	1.000
		3.00	1.00	-2.1193	.079
			2.00	-.3817	1.000
	Tamhane	1.00	2.00	1.7377	.235
			3.00	2.1193	.038
		2.00	1.00	-1.7377	.235
			3.00	.3817	.957
		3.00	1.00	-2.1193	.038
			2.00	-.3817	.957
CD2+	Bonferroni	1.00	2.00	9.6208	1.000
			3.00	49.4866	1.000
		2.00	1.00	-9.6208	1.000
			3.00	39.8658	1.000
		3.00	1.00	-49.4866	1.000
			2.00	-39.8658	1.000
	Tamhane	1.00	2.00	9.6208	1.000
			3.00	49.4866	.943
		2.00	1.00	-9.6208	1.000
			3.00	39.8658	.967
		3.00	1.00	-49.4866	.943
			2.00	-39.8658	.967
% CD3+	Bonferroni	1.00	2.00	1.3987	1.000
			3.00	1.5860	.822
		2.00	1.00	-1.3987	1.000
			3.00	.1873	1.000
		3.00	1.00	-1.5860	.822
			2.00	-.1873	1.000
	Tamhane	1.00	2.00	1.3987	.692
			3.00	1.5860	.405
		2.00	1.00	-1.3987	.692
			3.00	.1873	.999
		3.00	1.00	-1.5860	.405
			2.00	-.1873	.999
CD3+	Bonferroni	1.00	2.00	-6.6338	1.000
			3.00	23.4153	1.000
		2.00	1.00	6.6338	1.000
			3.00	30.0490	1.000
		3.00	1.00	-23.4153	1.000
			2.00	-30.0490	1.000
	Tamhane	1.00	2.00	-6.6338	1.000
			3.00	23.4153	.992
		2.00	1.00	6.6338	1.000
			3.00	30.0490	.984
		3.00	1.00	-23.4153	.992
			2.00	-30.0490	.984
% CD3+HLADR+	Bonferroni	1.00	2.00	-.4506	1.000
			3.00	-.2527	1.000
		2.00	1.00	.4506	1.000

			3.00	.1979	1.000
		3.00	1.00	.2527	1.000
			2.00	-.1979	1.000
	Tamhane	1.00	2.00	-.4506	.810
			3.00	-.2527	.953
		2.00	1.00	.4506	.810
			3.00	.1979	.950
		3.00	1.00	.2527	.953
			2.00	-.1979	.950
CD3+HLADR+	Bonferroni	1.00	2.00	-13.4026	1.000
			3.00	-4.7099	1.000
		2.00	1.00	13.4026	1.000
			3.00	8.6927	1.000
		3.00	1.00	4.7099	1.000
			2.00	-8.6927	1.000
	Tamhane	1.00	2.00	-13.4026	.751
			3.00	-4.7099	.979
		2.00	1.00	13.4026	.751
			3.00	8.6927	.841
		3.00	1.00	4.7099	.979
			2.00	-8.6927	.841
% CD25+	Bonferroni	1.00	2.00	-2.4818	.186
			3.00	-2.7169	.062
		2.00	1.00	2.4818	.186
			3.00	-.2351	1.000
		3.00	1.00	2.7169	.062
			2.00	.2351	1.000
	Tamhane	1.00	2.00	-2.4818	.099
			3.00	-2.7169	.015
		2.00	1.00	2.4818	.099
			3.00	-.2351	.994
		3.00	1.00	2.7169	.015
			2.00	.2351	.994
CD25+	Bonferroni	1.00	2.00	-129.5143	**.016**
			3.00	-123.1918	**.008**
		2.00	1.00	129.5143	**.016**
			3.00	6.3225	1.000
		3.00	1.00	123.1918	**.008**
			2.00	-6.3225	1.000
	Tamhane	1.00	2.00	-129.5143	**.005**
			3.00	-123.1918	**.000**
		2.00	1.00	129.5143	**.005**
			3.00	6.3225	.998
		3.00	1.00	123.1918	**.000**
			2.00	-6.3225	.998
% CD4+	Bonferroni	1.00	2.00	-.6974	1.000
			3.00	-1.3044	1.000
		2.00	1.00	.6974	1.000
			3.00	-.6070	1.000
		3.00	1.00	1.3044	1.000
			2.00	.6070	1.000
	Tamhane	1.00	2.00	-.6974	.973
			3.00	-1.3044	.801
		2.00	1.00	.6974	.973
			3.00	-.6070	.946
		3.00	1.00	1.3044	.801
			2.00	.6070	.946
CD4+	Bonferroni	1.00	2.00	-40.5506	1.000
			3.00	-33.8786	1.000
		2.00	1.00	40.5506	1.000
			3.00	6.6720	1.000
		3.00	1.00	33.8786	1.000
			2.00	-6.6720	1.000
	Tamhane	1.00	2.00	-40.5506	.937
			3.00	-33.8786	.937
		2.00	1.00	40.5506	.937
			3.00	6.6720	.999
		3.00	1.00	33.8786	.937
			2.00	-6.6720	.999

% CD4+CD45RA-	Bonferroni	1.00	2.00	3.0597	.302
			3.00	1.9912	.673
		2.00	1.00	-3.0597	.302
			3.00	-1.0685	1.000
		3.00	1.00	-1.9912	.673
			2.00	1.0685	1.000
	Tamhane	1.00	2.00	3.0597	.343
			3.00	1.9912	.561
		2.00	1.00	-3.0597	.343
			3.00	-1.0685	.852
		3.00	1.00	-1.9912	.561
			2.00	1.0685	.852
CD4+CD45RA-	Bonferroni	1.00	2.00	66.3325	.707
			3.00	42.6213	1.000
		2.00	1.00	-66.3325	.707
			3.00	-23.7112	1.000
		3.00	1.00	-42.6213	1.000
			2.00	23.7112	1.000
	Tamhane	1.00	2.00	66.3325	.544
			3.00	42.6213	.768
		2.00	1.00	-66.3325	.544
			3.00	-23.7112	.918
		3.00	1.00	-42.6213	.768
			2.00	23.7112	.918
% CD4+CD45RA+	Bonferroni	1.00	2.00	-3.8052	.150
			3.00	-3.2966	.162
		2.00	1.00	3.8052	.150
			3.00	.5086	1.000
		3.00	1.00	3.2966	.162
			2.00	-.5086	1.000
	Tamhane	1.00	2.00	-3.8052	.165
			3.00	-3.2966	.114
		2.00	1.00	3.8052	.165
			3.00	.5086	.985
		3.00	1.00	3.2966	.114
			2.00	-.5086	.985
CD4+CD45RA+	Bonferroni	1.00	2.00	-109.3702	.194
			3.00	-77.1260	.416
		2.00	1.00	109.3702	.194
			3.00	32.2443	1.000
		3.00	1.00	77.1260	.416
			2.00	-32.2443	1.000
	Tamhane	1.00	2.00	-109.3702	.234
			3.00	-77.1260	.300
		2.00	1.00	109.3702	.234
			3.00	32.2443	.894
		3.00	1.00	77.1260	.300
			2.00	-32.2443	.894
% CD8+	Bonferroni	1.00	2.00	.5792	1.000
			3.00	.8721	1.000
		2.00	1.00	-.5792	1.000
			3.00	.2929	1.000
		3.00	1.00	-.8721	1.000
			2.00	-.2929	1.000
	Tamhane	1.00	2.00	.5792	.977
			3.00	.8721	.905
		2.00	1.00	-.5792	.977
			3.00	.2929	.992
		3.00	1.00	-.8721	.905
			2.00	-.2929	.992
CD8+	Bonferroni	1.00	2.00	17.9384	1.000
			3.00	31.3135	1.000
		2.00	1.00	-17.9384	1.000
			3.00	13.3751	1.000
		3.00	1.00	-31.3135	1.000
			2.00	-13.3751	1.000
	Tamhane	1.00	2.00	17.9384	.987
			3.00	31.3135	.921
		2.00	1.00	-17.9384	.987

			3.00	13.3751	.986
		3.00	1.00	-31.3135	.921
			2.00	-13.3751	.986
% CD8+CD11b+	Bonferroni	1.00	2.00	-1.3623	.466
			3.00	.2563	1.000
		2.00	1.00	1.3623	.466
			3.00	1.6186	.071
		3.00	1.00	-.2563	1.000
			2.00	-1.6186	.071
	Tamhane	1.00	2.00	-1.3623	.496
			3.00	.2563	.984
		2.00	1.00	1.3623	.496
			3.00	1.6186	.173
		3.00	1.00	-.2563	.984
			2.00	-1.6186	.173
CD8+CD11b+	Bonferroni	1.00	2.00	-22.7455	1.000
			3.00	10.0295	1.000
		2.00	1.00	22.7455	1.000
			3.00	32.7749	.190
		3.00	1.00	-10.0295	1.000
			2.00	-32.7749	.190
	Tamhane	1.00	2.00	-22.7455	.767
			3.00	10.0295	.947
		2.00	1.00	22.7455	.767
			3.00	32.7749	.288
		3.00	1.00	-10.0295	.947
			2.00	-32.7749	.288
% CD8CD11b-	Bonferroni	1.00	2.00	1.8740	.669
			3.00	.5301	1.000
		2.00	1.00	-1.8740	.669
			3.00	-1.3439	.720
		3.00	1.00	-.5301	1.000
			2.00	1.3439	.720
	Tamhane	1.00	2.00	1.8740	.520
			3.00	.5301	.969
		2.00	1.00	-1.8740	.520
			3.00	-1.3439	.577
		3.00	1.00	-.5301	.969
			2.00	1.3439	.577
CD8+CD11b-	Bonferroni	1.00	2.00	39.1429	1.000
			3.00	20.0250	1.000
		2.00	1.00	-39.1429	1.000
			3.00	-19.1178	1.000
		3.00	1.00	-20.0250	1.000
			2.00	19.1178	1.000
	Tamhane	1.00	2.00	39.1429	.842
			3.00	20.0250	.962
		2.00	1.00	-39.1429	.842
			3.00	-19.1178	.951
		3.00	1.00	-20.0250	.962
			2.00	19.1178	.951
% CD19+	Bonferroni	1.00	2.00	-.9711	1.000
			3.00	-1.9999	.136
		2.00	1.00	.9711	1.000
			3.00	-1.0287	.649
		3.00	1.00	1.9999	.136
			2.00	1.0287	.649
	Tamhane	1.00	2.00	-.9711	.689
			3.00	-1.9999	.061
		2.00	1.00	.9711	.689
			3.00	-1.0287	.523
		3.00	1.00	1.9999	.061
			2.00	1.0287	.523
CD19+	Bonferroni	1.00	2.00	-32.7960	.959
			3.00	-53.0971	.207
		2.00	1.00	32.7960	.959
			3.00	-20.3011	1.000
		3.00	1.00	53.0971	.207
			2.00	20.3011	1.000

	Tamhane	1.00	2.00	-32.7960	.501
			3.00	-53.0971	.067
		2.00	1.00	32.7960	.501
			3.00	-20.3011	.777
		3.00	1.00	53.0971	.067
			2.00	20.3011	.777
% CD19+CD5+	Bonferroni	1.00	2.00	-2.8148	.099
			3.00	-1.5581	.535
		2.00	1.00	2.8148	.099
			3.00	1.2567	.590
		3.00	1.00	1.5581	.535
			2.00	-1.2567	.590
	Tamhane	1.00	2.00	-2.8148	.203
			3.00	-1.5581	**.000**
		2.00	1.00	2.8148	.203
			3.00	1.2567	.806
		3.00	1.00	1.5581	**.000**
			2.00	-1.2567	.806
CD19+CD5+	Bonferroni	1.00	2.00	-72.1864	.127
			3.00	-37.5124	.688
		2.00	1.00	72.1864	.127
			3.00	34.6740	.559
		3.00	1.00	37.5124	.688
			2.00	-34.6740	.559
	Tamhane	1.00	2.00	-72.1864	.239
			3.00	-37.5124	**.000**
		2.00	1.00	72.1864	.239
			3.00	34.6740	.794
		3.00	1.00	37.5124	**.000**
			2.00	-34.6740	.794
% CD3+CD16CD56+	Bonferroni	1.00	2.00	-16.9896	1.000
			3.00	-1.5187	1.000
		2.00	1.00	16.9896	1.000
			3.00	15.4709	.753
		3.00	1.00	1.5187	1.000
			2.00	-15.4709	.753
	Tamhane	1.00	2.00	-16.9896	.804
			3.00	-1.5187	1.000
		2.00	1.00	16.9896	.804
			3.00	15.4709	.679
		3.00	1.00	1.5187	1.000
			2.00	-15.4709	.679
CD3+CD16CD56+	Bonferroni	1.00	2.00	-.7922	.773
			3.00	-1.1648E-02	1.000
		2.00	1.00	.7922	.773
			3.00	.7806	.403
		3.00	1.00	1.165E-02	1.000
			2.00	-.7806	.403
	Tamhane	1.00	2.00	-.7922	.714
			3.00	-1.1648E-02	1.000
		2.00	1.00	.7922	.714
			3.00	.7806	.536
		3.00	1.00	1.165E-02	1.000
			2.00	-.7806	.536
% CD3-CD16CD56+	Bonferroni	1.00	2.00	-.7457	1.000
			3.00	.3666	1.000
		2.00	1.00	.7457	1.000
			3.00	1.1123	.884
		3.00	1.00	-.3666	1.000
			2.00	-1.1123	.884
	Tamhane	1.00	2.00	-.7457	.920
			3.00	.3666	.981
		2.00	1.00	.7457	.920
			3.00	1.1123	.684
		3.00	1.00	-.3666	.981
			2.00	-1.1123	.684
CD3-CD16CD56+	Bonferroni	1.00	2.00	-9.6519	1.000
			3.00	15.9641	1.000
		2.00	1.00	9.6519	1.000

			3.00	25.6161	.992
		3.00	1.00	-15.9641	1.000
			2.00	-25.6161	.992
	Tamhane	1.00	2.00	-9.6519	.989
			3.00	15.9641	.915
		2.00	1.00	9.6519	.989
			3.00	25.6161	.745
		3.00	1.00	-15.9641	.915
			2.00	-25.6161	.745

Legend Table 4.

1 = healthy controls
2 = *Mycoplasma* infected CFS patient
3 = CFS patient with no *Mycoplasma* detected

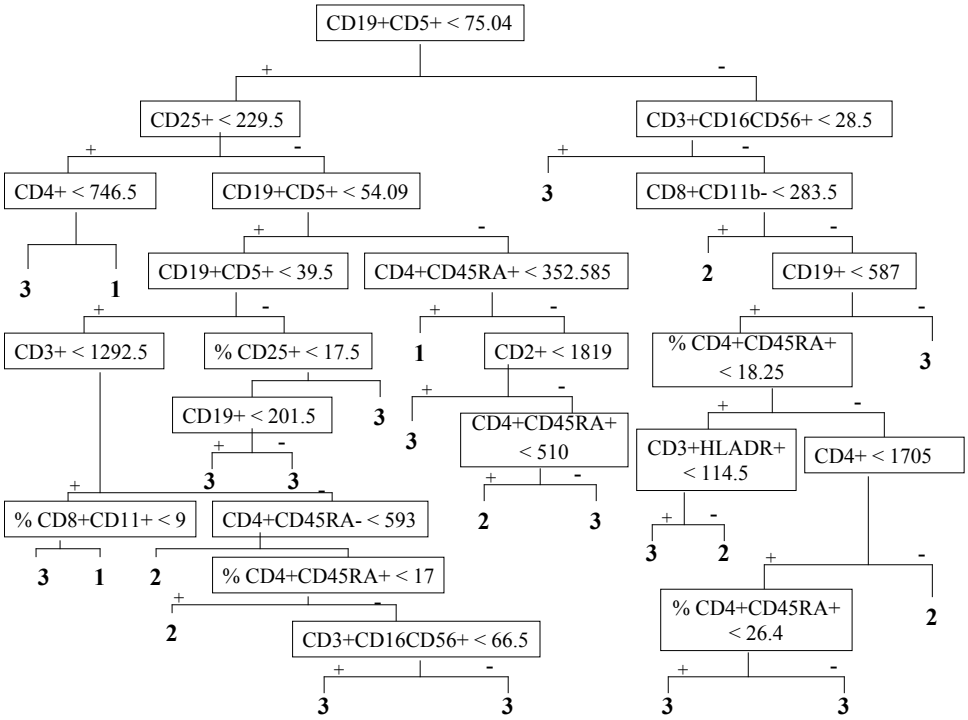
Table 5. The Kruskal-Wallice (non-parametric) test for the differences in the immunophenotype profile in the CFS patients with and without *Mycoplasma* infection and healthy controls.

Variable	χ	*p*-value
CD4+/CD8+ ratio	0.317	0.853
% CD2+	3.972	0.137
CD2+	0.348	0.840
% CD3+	0.812	0.666
CD3+	0.148	0.929
% CD3+HLADR+	2.772	0.250
CD3+HLADR+	2.168	0.338
% CD25+	5.388	0.068
CD25+	11.337	**0.003**
% CD4+	0.778	0.678
CD4+	0.167	0.920
% CD4+CD45RA-	2.026	0.363
CD4+CD45RA-	1.401	0.496
% CD4+CD45RA+	5.355	0.069
CD4+CD45RA+	5.545	0.063
% CD8+	0.384	0.825
CD8+	0.241	0.887
% CD8+CD11b+	2.466	0.291
CD8+CD11b+	2.184	0.336
% CD8CD11b-	0.492	0.782
CD8+CD11b-	0.436	0.804
% CD19+	4.509	0.105
CD19+	2.749	0.253
% CD19+CD5+	12.584	**0.002**
CD19+CD5+	9.354	**0.009**
% CD3+CD16CD56+	1.540	0.463
CD3+CD16CD56+	1.538	0.463
% CD3-CD16CD56+	1.472	0.479
CD3-CD16CD56+	1.571	0.456

Table 6. The stepwise lineair discriminant analysis of the differences between the 3 groups.

Step	Variables Entered	Wilks' Lambda	Exact F Statistic	*p*-value
1	C25	.953	5.254	.006
2	C4C45M	.922	4.411	.002
3	C8C11P	.908	3.507	.002

Figure 1.
Classification tree Immunophenotyping and Mycoplasmas



Legend Figure 1

1 = healthy controls 2 = *Mycoplasma* infected CFS patient 3 = CFS patient with no *Mycoplasma* detected CD19+CD5+ = CD5+ B-cells CD25+ = activated cells CD4+ = helper / inducer T-cells CD4+CD45RA+ = virgin CD4-cells % CD4+CD45RA+ = percentage virgin CD4-cells CD4+CD45RA- = memory CD4-cells CD3+ = T-cells % CD25+ = percentage CD25+ cells CD2+ = E-rosette receptor % CD8+CD11b+ = percentage suppressor cells / NK-subset CD8+CD11b- = cytotoxic T-cells CD19+ = B-cells CD3+CD16CD56+ = subset cytotoxic T-cells CD8+CD11b- = cytotoxic T-cells CD3+HLADR+ = activated T-cells

Table 7.
Probability of the root endings in the classification tree

Root ending	Subset	Probability for being healthy (%)	Probability for being a *Mycoplasma* infected CFS patient (%)	Probability for being a CFS patient (no *Mycoplasma* detected) (%)
CD4+ < 746.5	3	20.00	10.00	**70.00**
CD4+ > 746.5	1	**84.62**	0.00	15.38
%CD8+CD11b+ < 9	3	0.00	0.00	**100.00**
%CD8+CD11b+ > 9	2	0.00	**62.50**	37.50
CD4+CD45RA- < 593	2	20.00	**80.00**	0.00
%CD4+CD45RA+ < 17	2	33.33	**50.00**	16.67
CD3+CD16CD56+ < 66.5	3	28.57	14.29	**57.14**
CD3+CD16CD56+ > 66.5	3	0.00	0.00	**100.00**
CD19+ < 201.5	3	0.00	0.00	**100.00**
CD19+ > 201.5	3	0.00	41.67	**58.33**
%CD25+ > 17.5	3	0.00	0.00	**100.00**
CD4+CD45RA+ < 352.585	1	**90.00**	0.00	10.00
CD2+ < 1819	3	0.00	0.00	**100.00**
CD4+CD45RA+ < 510	3	11.11	11.11	**77.78**
CD4+CD45RA+ > 510	2	42.86	**57.14**	0.00
CD3+CD16CD56+ < 28.5	3	0.00	0.00	**100.00**
CD8+CD11b- < 283.5	2	0.00	**85.71**	14.29
CD3+HLADR+ < 114.5	3	0.00	33.33	**66.67**
CD3+HLADR+ > 114.5	2	0.00	**100.00**	0.00
%CD4+CD45RA+ < 26.4	3	0.00	0.00	**100.00**
%CD4+CD45RA+ > 26.4	3	0.00	25.00	**75.00**
CD4+ > 1705	2	0.00	**66.67**	33.33
CD19+ > 587	3	0.00	0.00	**100.00**

Legend Table 7.

Subsets: 1 = healthy controls
2 = *Mycoplasma* infected CFS patient
3 = CFS patient with no *Mycoplasma* detected