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Thesis

Chronic Fatigue Syndrome: A Proteomic Approach

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I was like a child, I was lying strong
And my father lifted me up there
Took me to a place where they checked my body
My soul was floating in thin-air

I clung to the bed and I clung to the past
I clung to the welcome darkness
But at the end of the night there's a green green light
The quiet before the madness

There was a girl that sang like the chime of a bell
And she put out her arm, she touched me when I was in hell
When I was in hell
[…]
Lying on my side you were half awake
And your face was tired and crumpled
If I had a camera I'd snap you now
Cause there's beauty in every stumble

*(**Belle and Sebastian** – Nobody's Empire)*

لله الحمد

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Introduction

One of the definitions of fatigue is the decrease, perceived and objectively measured, of the physiological capacity to perform a job, however fatigue is a common symptom in the community (with up to half of the general population reporting fatigue in large surveys) [1, 2].

When fatigue loses some of its typical features like: transience, is no longer self-limiting, and its onset cannot be explained by prevailing circumstances, then it is possible to speak of chronic fatigue syndrome.

The first to describe a disorder similar was R. Manningham in 1750 using the word "febricula" (or even "little fever"); G.M. Beard in 1869 coined the term "neurasthenia" to describe a syndrome characterized by physical fatigue and mental exhaustion, associated with difficulty in concentrating, impaired memory and reduction of interest [3].

In 1889 Charcot has succeeded to give to the symptoms of the “neurasthenia”, described by Beard, a pathognomonic significance. Typical symptoms were neuromuscular asthenia, chronic headache, dyspepsia with sleepiness associated to a morbid state of mind characterized by intellectual weakness with impaired memory and depressed and irritable mood.

Studies Charcot allowed to Ballet, in 1908, to classify the “neurasthenia” in 4 different types: genital, traumatic, cerebrospinal and feminine [3].

Only in 90’s the term “neurasthenia” was replaced with Chronic Fatigue Syndrome (CFS); precisely in 1988 the Centres for Disease

Control and Prevention (CDD) described an illness of debilitating fatigue accompanied by a various combination of symptoms using the term Chronic Fatigue Syndrome/Myalgic Encephalomylites [4, 5] and thanks to Fukuda have specific criteria that allow us to perform a more precise diagnosis [6].

Since 1988 until recent years Fukuda and other researchers have produced several well – established case definitions for CFS which has summarized in table below [7]:

Criteria	*CDC 1988*	*CDC 1994*	*London 1990*	*Australian 1990*	*Oxford 1991*	*Canadian Conseus 2003*
Minimum duration (months)	6	6	6	6	6	6
Functional Impairment	50% decrease in activity	Substantial	Not specified	Significant disruption of usual activities	Disabling	Substantial
Symptoms and their requirements by definition	Sore throat • Painful cervical or auxiliary lymph nodes • Muscle pain • Arthralgia without joint swelling or redness • New headaches • Sleep disturbance • Generalized fatigue lasting 24 hours or more following exercise that in a patients premobid state would have been easily tolerated • Mild fever • Generalized muscle waelness • Main symptom complex must have developed over a few hours/ days	Requires four • Sore throat • Tender lymph nodes • Muscle pain • Arthralgia without joint swelling or redness • New headaches • Unrefreshing sleep • Post-exertional malaise lasting more than 24 hours	The following three symptoms must be present • Symptom fluctuation brought about and precipitated by physical or mental exercise • Exercise-induced fatigue • Impairment of short-term memory and loss of powers of concentration	The following two symptoms must be present • Chronic persisting/ relapsing fatigue • Fatigue is exacerbated by minor exercise	None required symptoms of muscle pain, mood and sleep disturbance	The following three symptoms must be present • Significant muscle pain • Post-exertional malaise and/or fatigue lasting 24 hours or more • Sleep dysfunction A further one symptom from two of the following categories of • Immune • Neuroendocrine • Autonomic symptoms is required
Cognitive and Neuropsychiatric Symptoms	Requires one or more of symptoms	Requires • Impairment of short-term	Requires • Short-term memory	Requires • Neuropsychiatric dysfunction which	Requires • Mental fatigue	Requires two or more symptoms of

	• Forgetfulness • Irritability • Confusion •Concentration difficulties • Depression • Photophobia • Difficulty thinking	memory • Concentration difficulties	difficulties • Concentration difficulties • Emotional liability • Nominal dysphasia	includes new onset memory impairment		• Confusion • Short-term memory problems • Concentration difficulties • Disorientation • Information processing problems • Word retrieval problems • Perceptual and sensory disturbances • Ataxia • Photophobia • Hypersensitivity to noise/emotion
New Onset	Required	Required	Not Required	Not Required	Required	Required
Medical Exclusion	Extensive list of known medical causes	Clinically Important	Extensive list of known medical causes	Known physical causes	Known physical causes	Extensive list of known medical causes
Psychiatric Exclusion	Bipolar disorder • Substance abuse • Schizophrenia • Anxiety disorder • Depressive disorder	Bipolar disorder • Substance abuse • Eating disorder • Schizophrenia • Severe melancholic or Psychotic depression • Dementia • Delusional disorder	• Primary depressive illness • Anxiety neurosis	• Bipolar disorder • Substance abuse • Eating disorder • Psychosis	Bipolar disorder • Substance abuse • Eating disorder • Psychosis	• Primary psychiatric disorders • Substance abuse

Table 1*: Variability of case definition criteria for CFS [7]*

The most prominent similarity between the definitions shown in the table was the necessity for symptoms to be present for 6 months or more. Almost all included a requirement for fatigue to be present, with the exception of the 2003 Canadian definition [8] which focused not on fatigue but on post-exertional malaise [7].

CFS, like many chronic illnesses, is proving difficult to define and despite more than 30 years of research, continues to be a poorly understood and controversial syndrome [9].

Between all the case definitions views, to date, with exception, that of Fukuda of 1994, is the most the most frequently used and has created a unified approach to the definition and study of CFS [7, 10].

DEFINITION CRITERIA

Clarification of the relation between the chronic fatigue syndrome and the neuropsychiatric syndromes is particularly important.[6]

In order to guide the development of studies relevant to the CFS, Fukuda proposed a framework the clarified the need to compare population defined by chronic fatigue syndrome with several other populations in case – control and cohort studies.[6]

Figure 1 show a conceptual framework of abnormally fatigued populations, including those with the CFS and overlapping disorders.

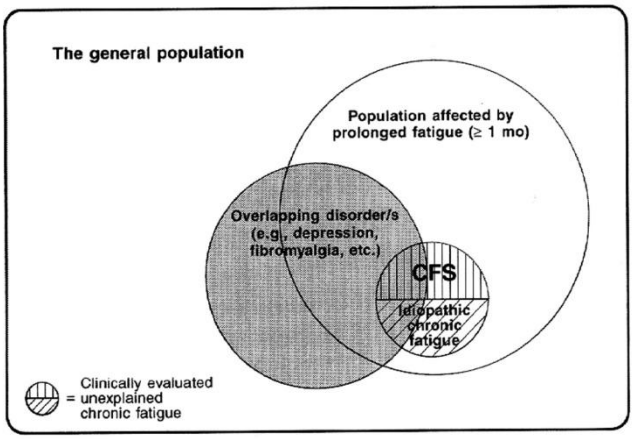


Figure 1: ***A conceptual framework of abnormally fatigued populations, including those with the chronic fatigue syndrome (CFS) and overlapping disorders [6]***

Thanks to Fukuda studies now we have an evaluation and classification of unexplained chronic fatigue (figure 2)

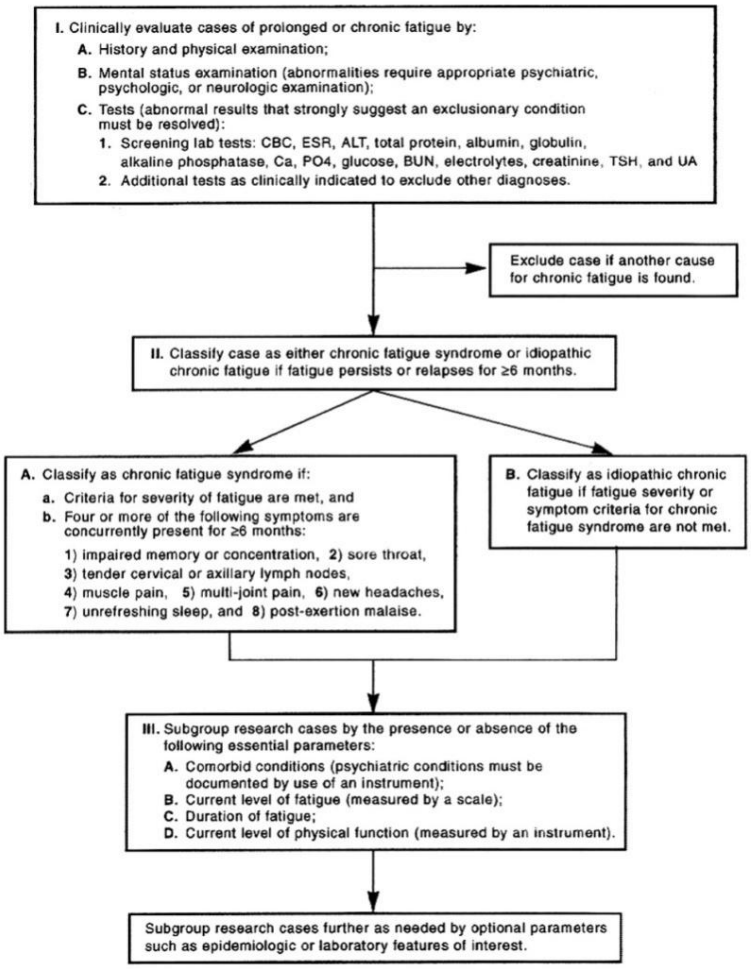


Figure 2: ***Evaluation and classification of unexplained chronic fatigue; ALT:*** *alanine aminotransferase;* ***BUN****: blood urea nitrogen;* ***CBC****: complete blood count;* ***ESR:****: erytrocite sedimentation rate;* ***PO4****: phosohorus;* ***TS****: thyroid stimulating hormone;* ***UA****: urinalysis [6]*

Summarizing the data in the chart above a case of CFS is defined by the presence of the following:

1. Clinically evaluated, unexplained, persistent on relapsing chronic fatigue that is of new ore definite (as note been lifelong); in not the result of ongoing exertion: is not substantially alleviated by rest; and results in substantial reduction in previous levels of occupational, educational, social or personal activities.
2. The concurrent occurrence of four or more of symptoms (like sore throat, tender cervical, muscle pain, headaches) all of which must have persisted or recurred during 6 or more consecutive months of illness and must not have predated the fatigue[6].

Characterised by persistent or relapsing unexplained chronic fatigue

- Fatigue lasts for at least 6 months
- Fatigue is of new or definite onset
- Fatigue is not the result of an organic disease or of continuing exertion
- Fatigue is not alleviated by rest
- Fatigue results in a substantial reduction in previous occupational, educational, social, and personal activities
- Four or more of the following symptoms, concurrently present for ≥6 months: impaired memory or concentration, sore throat, tender cervical or axillary lymphnodes, muscle pain, pain in several joints, new headaches, unrefreshing sleep, or malaise after exertion

Exclusion criteria

- Medical condition explaining fatigue
- Major depressive disorder (psychotic features) or bipolar disorder
- Schizophrenia, dementia, or delusional disorder
- Anorexia nervosa, bullaemia nervosa
- Alcohol or substance abuse
- Severe obesity

Panel 1: ***1994 case definition for CFS from US Centers for Disease Control and Prevention [11]***

Another interesting algorithm that allows us to make a diagnosis of CFS is given by Barbado [12].

We can observe by this scheme that the conditions that exclude diagnosis of CFS are: psychiatric disorders, such as a major

depression, schizophrenia eating disorders (anorexia bulimia) bipolar disorders, alcohol or other substances abuse, in addition to morbid obesity, and active medical diseases, either non treated or without a completely established resolution [13].

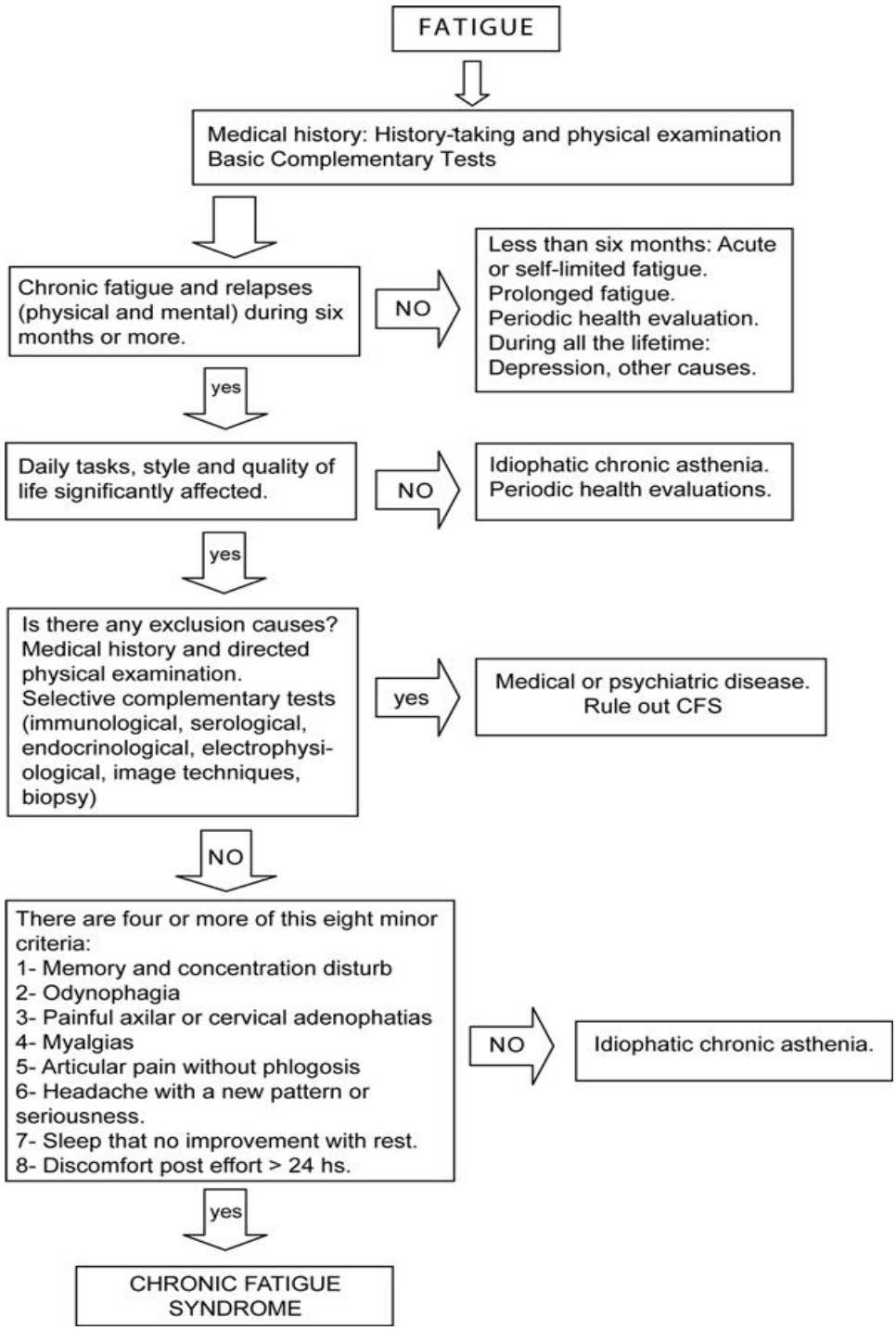


Figure 3: ***diagnostic protocol for patients with suspected CFS [13]***

Core symptoms found by Fukuda were included in 2003 in the Canadian Consensus Criteria [4, 14]

Its application in research however, suggests that patients fulfilling the Canadian definition have more severe impairment to their physical functioning and cognition than 1994 CDC defined patients [15, 16].

The Canadian definition was revised in 2011 and renamed the International Consensus Criteria (ICC) [17]

A major criticism of the 1994 CDC definition is that it has remained the most common criteria for CFS/ME due to consensus [18].

The ICC was proposed based on collective empirical findings on dysfunction found in CFS/ME patients fulfilling broader definitions of the illness [19-29].

These findings, however, may be more prominent in a more homogenous sample. The potential of the ICC to identify a distinct subgroup of CFS/ME may, therefore, enhance the opportunity of discovering a unique biological marker for the illness [4].

EPIDEMIOLOGY

The difficulties with definitions have affected the results of epidemiological studies and in this way prevalence varies widely [11] and it is known that knowing the prevalence of a disease allows us to understand its impact on public health, to plan resources based on its expected prevalence and program based on its temporal variation.

Estimates of the prevalence of CFS have varied depending on which definition was used, the type of population that was surveyed, and the study methods [30].

Unfortunately few reliable and valid epidemiological data on CFS are available [11]. People of every age, gender, ethnicity and socioeconomic group can have CFS but it affects women at six times the rate of men [31].

The estimated prevalence of CFS is much lower among children and adolescents than among adults [32-34].

Some demographic data show that in most studies 75% or more of patients with CFS are female; early reports from tertiary clinics suggested that CFS affected primarily young, white, successful women. The mean age at onset of CFS is between 29 years and 35 years. The mean illness duration ranges from 3 years to 9 years [3, 35-37].

In the Chicago community-based sample, gender predicted fatigue severity, with women exhibiting higher fatigue scores than men. Also, within this sample women had significantly poorer physical functioning, more bodily pain, poorer emotional functioning, significantly more severe muscle pain, and significantly greater impairment of work activities [38, 39]. More than 80% of CFS sufferers go undiagnosed [40].

However, community surveys have found that white individuals have a lower risk of CFS, compared with Latinos [36], African Americans . [37, 41], and Native Americans [36]; nevertheless, ethnic minority status does not always confer a risk and although CFS show an association with ethnicity, fatigue does not.

Nevertheless this result needs to be replicated in future research as there were only two studies for CF and only one study for CFS that compared Native American and African American with White people

and all three studies found a higher prevalence of CF and CFS in ethnic minority groups [42].

These disparate findings suggest that the increased prevalence of this syndrome among whites in clinic populations is most likely the result of a bias attributable to health care access and utilization [3].

AETIOLOGY

Many studies have investigated the aetiology and pathogenesis of CFS; most of those carried out between 1980 and 1995 concentrated on the physical aetiology of CFS, with a slight shift towards psychological and psychiatric research in the next few years [43].

Despite all of these studies, however, the real aetiology of chronic fatigue syndrome remains elusive [3].

As the criteria for CFS diagnosis are not based on the understanding of aetiopathogenic mechanisms, some patients present similar clinical manifestations but are diagnosed with other conditions because fatigue is not the primary symptom. Some of those conditions are fibromyalgia, irritable bowel syndrome, and temporomandibular joint syndrome. Furthermore, in addition to sharing several symptoms with CFS, currently available evidence suggests that those diseases also share similar pathophysiologic mechanisms [44, 45].

Although the aetiology and the pathogenic mechanisms of CFS are not fully understood, several hypotheses have been postulated, being the disorders of the central nervous system neuromodulator the one

supported by more evidence to explain the possible pathogenic mechanisms involved in CFS [46].

Many somatic and psychosocial hypotheses on the aetiology of CFS have been explored. Explanations for CFS were sought in viral infections, immune dysfunction, neuroendocrine responses, dysfunction of the central nervous system, muscle structure, exercise capacity, sleep patterns, genetic constitution, personality, and (neuro)psychological processes [11].

Although several studies found abnormalities, only a few were diagnosed in large groups of patients with CFS and were independently confirmed in well-controlled studies, an exception being the subtle changes in the hypothalamopituitary-adrenal axis [47].

The aetiology and pathogenesis are generally believed to be multifactorial [48].

Distinction between categories of predisposing, precipitating, and perpetuating factors is useful for understanding of this complex disorder. The assumption is that one or more factors of each of these categories is conditional but insufficient for development of CFS [49-53].

Predisposing Factors

Some studies have shown that personality and lifestyle can affect the vulnerability of the subjects to the CFS; neuroses and introversion have been reported as risk factors for the disorder [54].

In adults, the risk of CFS increases if high inactivity in childhood or after mononucleosis [53, 55].

Also genetics may play a role predisposing, since women are more prone to CFS than men at the same time, however, studies of twins have shown a predisposition familiar but no genetic abnormality [56-58].

Precipitating Factors

The onset of CFS can be due to several factors: acute physical or psychological stress [59]; for three-quarters of patients the trigger of the disease was identified in infection, like a, flu-like illness cold, or infectious mononucleosis [59, 60]. A causal relationship was also found with infectious mononucleosis [49, 61-63] and finally found high rates of chronic fatigue after Q fever and Lyme disease [63].

However, differences were found in the load of Epstein-Barr virus and immunological reactivity among individuals who developed CFS and those that have not done so [64, 65].

Precipitating somatic events, such as serious injury, surgery, pregnancy, or labor, which are reported as the onset of CFS by patients, have not been studied systematically. Psychological stress as a trigger of CFS has also been studied.

Severe life events, such as the loss of a loved one or a job, and other stressful situations were found to precipitate the disorder [66, 67].

Perpetuating Factors

Once CFS has developed, several maintaining factors can impede recovery. Psychological processes seem to be involved in the perpetuation of complaints in patients with CFS. These processes involve ideas or cognitions of patients about complaints and behavioural factors such as persistent avoidance of activities associated with an increase in symptoms [11]. A strong belief in a physical cause of the illness, a strong focus on bodily sensations, and a poor sense of control over complaints contribute to an increase in fatigue severity and functional impairment [68-71].

Other perpetuating CFS factors that have been identified are social processes ranging from solicitous behaviour [72] to lack of social support [73].

Illness perceptions and illness behaviour can be reinforced by people in the patient's environment, such as a partner or family [74-76].

Practitioners can contribute to the persistence of CFS by continuously encouraging unnecessary medical diagnostic procedures, by persistently suggesting psychological causes, or by not acknowledging CFS as a diagnosis, thus causing communication problems [77, 78].

Apart from the many disadvantages, long-lasting illness can also have more desirable consequences, such as care, attention, disengagement, or even financial benefits, which might also be considered perpetuating factors [72, 79].

NEUROENDOCRINOLOGICAL THEORY

In patients with fibromyalgia, the research on neurotransmitter disorders has started to yield positive findings, and it is known that different clinical manifestations will appear according the type and the site of action of affected neurotransmitters [44, 80]

Other studies reported that abnormalities in the hypothalamic-pituitary-adrnal (HPA) axis and serotonin pathways gave been identified in CFS patients, suggesting an altered physiological response to stress [81, 82].

CFS seems to also affect the production of some neurotransmitters: About one-third of patients have been shown to exhibit hypocortisolism, which appears to originate from a CNS source rather than a primary adrenal site [83].

In addition, studies have demonstrated abnormalities of CNS serotonin physiology in patients with chronic fatigue syndrome [81].

The studies of abnormalities in HPA function, hormonal stress responses, and serotonin neurotransmission in CFS patients have generated the most reproducible and robust findings reported to date [3].

ROS THEORY

Several groups of investigators assume that a defective oxidative phosphorylation and subsequent free radical production and oxidative stress play an important role in the pathophysiology of CFS/ME [27, 28, 84-91].

Moreover, the level of oxidative stress correlates with the severity of symptoms [91].

One research found increased levels of MDA (malondialdehyde, a potential biomarker of oxidative damage and disease severity) and CO levels in the CFS group and that the oxidative stress was present in their group of female patient [92]. Another one shown that CFS patients have a lipid profile and oxidant biology that is consistent with cardiovascular risk [93]

INFECTUS THEORY

As for this theory literature expresses different opinions; on one hand, in fact, Epstein-Barr virus, human herpesvirus 6, group B coxsackie virus, human T-cell lymphotrophic virus II, hepatitis C, enteroviruses, and retroviruses have been proposed as etiological agents in chronic fatigue syndrome. Research is focusing on a potential marker for viral infection [94]. On the other side, there has been no consistent evidence to date that chronic fatigue syndrome results from a specific infection [3]. According to Engleberg the pathogenic relationship between infection and the CFS has not been demonstrated [95].

There is also disagreement about the function of some vaccines; vaccines have been depicted by some researchers as playing an important role in CFS onset [96, 97], while on the other hand, others scholars have rejected this possibility, affirming that the vaccine is safe [98, 99].

GENETIC AND TWIN STUDIES

We have seen how difficult it is to prove the exact aetiology of this syndrome; to understand the relative importance of genetic and environmental influences on the development of this disorder, investigators researchers have tried to demonstrate its heritability and its familiarity with different types of studies (family, adoption, or twin studies).

Several other investigations have suggested that a combination of host and environmental factors may be involved in the etiology of CFS [58].

However, up to now have been few studies of this type (we just know that it was not carried out any study of adoption) [3].

In the family history study the results, based on subjects'reports of illness in family members, suggested that within relatives of patients with chronic fatigue syndrome there were significantly higher rates of the same syndrome than in relatives of comparison subjects [100].

Twin studies have been useful in elucidating the relative contributions of genetic and environmental factors to numerous medical and psychiatric disorders, and they are especially helpful for the study of diseases of unknown cause and have been widely used to estimate the

heritability of numerous complex disorders and conditions [58, 101].

Investigators have used twin methodology to determine how much of the family aggregation described is due to genetic factors and how much to the environment. Twin studies in adults have shown consistently higher concordance rates in monozygotic twins compared to dizygotic twins for CFS/ME, with the monozygotic correlation usually at least twice that of the dizygotic correlation [58, 102-105].

Despite different populations, highly variable case definitions of fatigue and few data available, these type of study found evidence to support a familial effect [58].

In summary, there is now increasingly strong evidence that CFS/ME is heritable. It seems likely that the heritability contributes to the experience of fatigue as well as to the development of CFS/ME itself.

Although there is some agreement that a model involving the joint action of genes and environment is required, there is currently little agreement on the actual genes and environmental factors involved [102].

An important observation reported by Buchwald in his study is that heritability increased our definition of chronic fatigue has been made more stringent. This suggests that future research would benefit from distinguishing CFS from milder forms of chronic fatigue [58].

COMMORBIDITY

The difficulty during these years of research to find a physiological, stable and consistent marker for this syndrome has pushed some scientist to say that CFS is primarily a psychiatric disorder [3, 106, 107].

In several research Chronic Fatigue Syndrome is described in relation to psychological stress as a work that causes abnormal nictemerale rhythm, or works that cause restrictions. The presence of a physical and / or psychological stress seems to be of great importance as a causative factor in the pathogenesis of CFS cases [3, 108].

Other studies reported that persons with CFS experienced significantly more stressful life events in the year before illness onset than controls during the same calendar time-period [109-112].

Several stress research has repeatedly shown that the qualitative evaluation of a life event as negative is crucial in its subsequent effect on health and no studies of which we are aware have measured stress perception, occurrence of stressful life events and stress levels within the same study [112].

Investigation conducted by Nater compared subjects with CFS with healthy subjects and showed that exposure to stressors was significantly more common in persons with CFS compared to the healthy controls; those with CFS reported experiencing significantly higher levels of psychological distress; and, post-traumatic stress disorder (PTSD) was more common in people with CFS [112].

Another study found that a lifetime diagnosis of CFS was strongly associated with both lifetime PTSD and current traumatic symptoms, as measured by the IES (impact of events scale), in a large community-based twin registry. A role in the association between CFS and PTSD can be play by the stress-response system in general, and the hypothalamic-pituitary-adrenal (HPA) axis in particular [113].

Dansie et al. demonstrated that both animal and human studies have shown that traumatic early life stressors may permanently change the stress response system in ways that leave affected individuals more prone to the effects of other stressors and to stress-related disorders [113-116].

Results obtained by Dansie are very interesting and have substantial relevance to practitioners and researchers alike because support previous results on the comorbidity of CFS with PTSD, and they extend the literature to include the potential role of familial and unique environmental factors in the link between these two conditions. Dansie argue that clinicians should carefully and thoroughly consider the complex nature of CFS as it relates to other conditions, and follow a multifaceted treatment approach that addresses the potential common mechanisms, such as the hormonal changes previously discussed, which underlie both CFS and stress-related conditions. [113].

Panic disorder and generalized anxiety disorder are also common comorbid conditions among those with chronic fatigue syndrome, although the syndrome is differently characterized across studies. Some articles points to an overlap between CFS and anxiety. The simple comorbidity of chronic fatigue syndrome and anxiety

disorders, however, does not suggest that chronic fatigue syndrome is a physical manifestation of an anxiety disorder [3].

A study carried out by Chan and his colleagues. on effects of Qigong Exercise on patients affected by CFS demonstrated that the participants' anxiety symptoms were significantly improved in both groups compared with baseline values [117].

Despite several limitations, this study also showed a significant correlation between alleviation of depression and fatigue reduction, as well as reduced anxiety following Qigong exercise; furthermore Qigong exercise may be effective in reducing fatigue symptoms and alleviating depressive symptoms for patients with CFS-like illness and that the improvement of fatigue symptoms may predict the alleviation of depressive symptoms after Qigong intervention. The most interesting part of this work concerns the association between fatigue symptoms and psychiatric disorder, association confirmed by results [117-120]

Another example of commorbidy concerns sleep disorders.

Indeed, CFS patients report more difficulty falling asleep, more interrupted sleep, and more daytime napping than healthy or chronically ill comparison subjects[3, 121]. However, it is unclear whether they also experience excessive daytime sleepiness (EDS) [3]. To date this correlation is very unclear: the semiological distinction of fatigue and sleepiness is difficult both for patients and clinicians, leading to possibly imprecise diagnostic formulations [122]. Moreover the relationships of sleepiness or fatigue to sleep remain insufficiently understood [122, 123].

Complaints of fatigue and sleepiness can overlap in patients, but should be taken into account separately as they may

express very different etiologies and may imply different treatment considerations [124].

Persons with chronic fatigue syndrome have high rates of current and lifetime major depression, which has been taken as evidence that chronic fatigue syndrome is an atypical manifestation of major depression. On the other hand, the high rates of depression in this syndrome could be a result of overlapping symptoms, (emotional response to disabling fatigue, viral or immune changes, alterations in brain physiology).In fact, several lines of research have suggested that CFS and major depression are possibly distinct entities [3]

TREATMENT AND RECOVERY

We have seen in previous sections that CFS is a chronic process that becomes a social disease due to the incapacity that it causes in the person who suffers to continue to fulfill their work, social and family responsibilities. The specific characteristics of the symptomatology of patients with CFS require a rapid adaptation of the educational, healthcare and social systems to prevent the problems derived from current systems.

At present, no curative treatment exists for patients with CFS. Treatment objectives must be focused on improving the clinical manifestations, maintaining the functional capacity and quality of life, and developing a tailored programme, providing each patient with the maximum perception of improvement. Patients with CFS require multidisciplinary management due to the multiple and different issues affecting them. This multidisciplinary management requires coordination between the different specialists, which leads to the need for the existence of an Action Protocol to establish the intervention procedure according to the needs of each patient. As mentioned above, CFS is disabling in some patients [13]

CSF remains a difficult disease to be treated effectively [125]; according Arroll individualised treatment protocols which include a range of tailored strategies are a favourable direction for dealing with a complex and multisystem disorder such as ME/CFS [126].

The subtle changes found in the hypothalamopituitary-adrenal axis have led to two randomised controlled trials, on the basis of which the

researchers have not concluded that steroids are the treatment of choice [127, 128]. For immunological interventions such as immunoglobulin, the evidence has been inconclusive. There has also been insufficient evidence of the effectiveness of pharmacological, supplementary, complementary, and other interventions [129].

According to some studies, cognitive behaviour therapy (CBT) and graded exercise therapy (GET) are the only interventions found to be beneficial. This therapy is a general form of psychotherapy directed at changing condition-related cognitions and behaviours. Central CBT components for CFS include explanation of the aetiological model, motivation for CBT, challenging and changing of fatigue-related cognitions, achievement and maintenance of a basic amount of physical activity, gradual increase in physical activity, and planning work rehabilitation or rehabilitation in other personal activities. CBT teaches patients with CFS how to acquire control over symptoms. CBT for CFS is based on a behavioural model of avoidance and always includes a graded activity programme. GET is based on a physiological model of deconditioning and has no intention of explicitly treating cognitions [11].

In adolescents, cognitive behaviour therapy combined with other group therapies that promote treatment compliance and sharing experiences or thoughts with other adolescents is very useful, even when they do not have identical pathologies. It must be noted that there is very limited evidence on cognitive therapy in adolescents.

Some non-controlled studies suggest that this therapy reduces fatigue in young people [130, 131] with early intervention [13].

However, not all patients benefit from CBT or GET [11].

Results obtained by Tirelli, instead showed that a significant number of patients treated with antiviral/immunoglobulin approaches have a long positive disease free survival in comparison with other patients treated with the other approaches (i.e., antidepressants, corticosteroids, and supplements) [125].

Some studies in the past have tried to show if complementary and alternative medicine (CAM) were effective against chronic fatigue; however, was never reached a sufficient number of tests to determine their effectiveness in a definitive way [132].

A recent systematic review of the prognosis of CFS showed that full recovery without treatment is rare. [35].

Most prognostic studies were done in specialist centres with a bias towards severe cases. The duration of follow-up of prognostic studies ranged from 1 year to 5 years.
The median recovery rate was 5% (range 0–31%) and the median improvement rate 39·5% (range 8–63) [11].

The relatively small proportion of recovered patients may reflect the heterogeneity of CFS [133].

One last alternative therapy was provided to us by Chan; his results on the Qigong suggest that the exercise of this ancient self-healing is an ancient mind-body exercise can be used as an alternative and complementary therapy or rehabilitation program for patients with CFS-like illness [117].

PROTEOMIC STUDIES

We have seen how difficult it is to define aetiology of chronic fatigue syndrome; for this reason, in recent years, several studies have been conducted proteomic type that would allow the identification of biomarkers useful for research and therapy.

A study of Schutzer et al. that has compared CFS and Neurologi Post Treatment Lyme disease Syndrome (nPTLS), in addition to demonstrating that the two syndrome there is no correlation between the proteomes despite similar symptoms, showed that CFS proteome analysis can provide important and meaningful insights into biological processes modulated as a function of the disease and facilitate the identification of protein candidates for further investigation [134].

Another study instead demonstrated that the CFS, PGI and FM subjects had a significant overlap between their syndromes. Despite the differences in their original case designations, they had very similar responses on questionnaire, quality of life and nociceptive measures. Again, despite the differences in the diagnostic label applied to them for study entry, their cerebrospinal fluid proteome demonstrated reproducible constituents [135].

Instead, the study of Ciregia correlates proteomic and twin studies. This research started from a previous study on monozygotic twins discordant for CFS, that did not identify a biomarker for CFS in the transcriptome of peripheral blood leukocytes, and moved to the study of proteins, using a proteomic approach to investigate the proteomics salivary profile in a couple of monozygotic discordant for CFS [136, 137].

Aim of Work

Our study moves from the previous research conducted by Ciregia which, however, was limited to only a couple of monozygotic twins discordant for CFS. The study of 2013 pointed out some proteins which are useful both to define a panel of potential diagnostic biomarkers and to shed new light on the comprehension of the pathogenetic pathways of CFS [136].

Our research was born, then, from the need to extend the results obtained from the twin study on a larger number of subjects with similar medical history and continue the search for biomarkers useful for both diagnosis and therapy of this syndrome.

Using different techniques (2-DE; western blot and ELISA – kit), with a proteomic approach, we tried to confirm the biomarkers found in past studies and to seek new ones.

Methods

SUBJECTS

A consecutive sample of 41 subjects with a diagnosis of CFS was recruited at the Rheumatology outpatient Unit of the University of Pisa between June 2010 and January 2011, The diagnosis of CFS and FM was made according to the ACR criteria by a rheumatologist.

Subjects included new and continuing patients, of at least 18 years of age, according to the classification criteria of Fukuda of 1994. The Ethics Committee of the Azienda Ospedaliero-Universitaria Pisana approved all recruitment and assessment procedures. All patients interested in take part in the study underwent a psychiatric assessment after a routinely appointment. Eligible subjects provided written informed consent after receiving a complete description of the study and having an opportunity to ask questions.

Medical history of each patients was carefully recorded focusing in particular on comorbidities and concomitant medications. Moreover, as far as serological data is concerned, complete blood tests, non organ specific auto-antibodies (Antinuclear antibodies, ENA, Ra-test and ACLA) and thyroid hormones and thyroid specific autoantibodies were detected in all the cases. Hepatitis B and hepatitis C infections were excluded as well in all the participants. Finally, every patient had a psychiatric specialist evaluation to assess psychiatric concomitant disorders. The TP count was determined by

the number of TPs that had a threshold of 4 kg/cm². Each positive TP had a pain score between 0 and 10. Psychiatric diagnoses were made through the Structured Clinical Interview for the DSM-IV Axis I disorders (SCID-I/P) (129), administered by psychiatrists trained and certified in its use at the "Dipartimento di Psichiatria, Neurobiologia, Farmacologia e Biotecnologie" of the University of Pisa, Italy.

The following questionnaires were also administered: the MOODS-SR lifetime version [138], the PAS-SR lifetime version [139], the Software CNS Vital Signs, tests for the assessment of neurocognitive tasks [140] and the Fibromyalgia Impact Questionnaire (FIQ) [141].

The FIQ (Fibromyalgia Impact Questionnaire) [141-144], is a questionnaire of 10 questions that assess the physical, occupational, depression, anxiety, sleep, pain, stiffness, fatigue and well-being in patients with FM. The higher scores indicate a worse quality of life. The HAQ ("Health Assessment Questionnaire") [145, 146] is used for patients with various rheumatic diseases: disability is assessed by 8 categories of questions designed to verify if the patient has difficulty in dressing, arising, eating, walking, bathing, reaching objects, shaking objects and doing activities.

The FACIT-Fatigue Scale questionnaire (Functional Assessment of Chronic Illness Therapy-Fatigue Scale) [147] is a collection of questions related to the quality of life aimed at chronic disease management. It consists of 13 questions that investigate the fatigue, the ability to perform everyday skills, the need for sleep, the need of help to perform daily activities, and how physical state affects the psychic state.

Socio-demographic data were collected using interviewer-administered questionnaires. A structured interview format was used to record sex, age, educational level, marital status, employment and duration of illness (table 2).

Were enrolled in the study 41 healthy subjects with similar mean age and demographic characteristics.

FACIT	30,38
FIQ	54,61
VAS pain	3,51
VAS fatigue	7,67
VAS sleep	7,35
HAQ	0,41
SF 36	
Physical functioning	60,28
Role Physical	16,67
Bodily Pain	47,72
General health status	35,67
Vitality	25,83
Social activities	33,17
Mental health	60,22
Role Emotional	42,33

Table 2*: Questionnaire score and scales of CFS patient:* *mean of various parameters of 41 subjects affected by CFS*

WHOLE SALIVA SAMPLES: COLLECTION AND PREPARATION

We decided to use whole saliva (WS) because recently, the number of publications related to salivary proteome have significantly increased suggesting human saliva as a biological fluid with an enormous potential to reflect systemic health conditions. Saliva has many advantages in terms of low invasiveness, minimum cost, and easy sample collection and processing. Moreover, saliva has a less complex protein composition than serum or plasma reducing the risk of non specific interactions, and at the same time it represents a useful diagnostic tool since about 30% of blood proteins are also present in saliva [136, 148].

Unstimulated whole saliva samples were collected early in the morning (between 8 and 10 a.m.) in standard conditions, i.e. all the subjects were asked to be on an empty stomach, without having assumed any drinks or any kind of food (including gum or candies) since the night before. In order to minimize the degradation of the proteins, the samples were processed immediately and kept on ice during the process. Between 1-3 ml saliva was obtained from each subject. To remove the debris and the cells, a centrifugation at 14000 g for 30 min at 4 °C, was performed and the protein amount of resulting supernatants was estimated using a DC protein assay from Bio-Rad. Bovine serum albumin (BSA) was used as a standard. All subjects (CFS and healthy) were randomly pooled in order to obtain 6 pools for each class; 200 µg for each pool were solubilised with rehydration solution

(*Urea* 7M – *Tiourea* 2M, *Chaps* 4%, *Ditiotreitolo* -DTT- 60mM, *Blu di Bromofenolo* 0,002%) filled up to 400µl and supplemented with 1.2% *IPG Buffer pH3-10.*

Each experiment were performed in duplicate.

2-DE ANALYSIS

Isoelectrofocusing (IEF) was carried out by using 18cm Immobiline Dry-Strips (GE-Healthcare) with a linear pH 3-10, gradient. IEF was performed at 16°C on a Ettan IPGphor II apparatus (GE Health Care) according to the following schedule: the strips were swollen for 24h onto Reswelling tray IPG, then they were moved on manifold and the voltage was linearly increased from 200 V to 5000 V during the first 4 h, and then stabilized at 8000 V for 10 h. Before the second dimension, IPG strips were equilibrated for 15 min in 50 mM TrisHCl, pH 8.8, 6 M Urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 1% DTT, and subsequently for 10 min in the same buffer solution but substituting the 1% DTT with 2.5% IAA. The equilibrated strips were applied to the top of 12.5% SDS-PAGE gels (21cmx20cmx1.5mm) and electrophoresis was performed using the PROTEAN Plus-Dodeca Cell (Bio-Rad) with constant amperage at 192mA and 15 °C for 14h (over-night) applying a continuous buffer system.

Staining and image analysis

Gels were stained with ruthenium II tris (bathophenanthroline disulfonate) tetrasodium salt (Sunatech Inc.) essentially as described by Rabilloud et al. [99]. Briefly, after electrophoresis, gels were fixed in 1% phosphoric acid (v/v) and 30% ethanol for 1h at room temperature, then were stained overnight with 1mM ruthenium complex (RuPB) in 1% phosphoric acid (v/v) and 30% ethanol. The day after the gels were destained for 4-6 hours in 1% phosphoric acid and 30% ethanol and rinsed in water prior to acquisition on fluorescence by "ImageQuant LAS4010" (GE-Healthcare). The images were analyzed with the Progenesis SameSpot (Non linear - Dynamics) software program.

Statistical analysis

Anova test has been used to explore qualitative and quantitative differences in the protein expression between CFS and health patients. We selected only proteins whose expression showed over 2-fold spot quantity change in FM samples. The significance of the differences was expressed by p-value <0.05.

WESTERN BLOT

Proteins of interest detected by MS/MS were also identified by Western Blotting (WB). WS samples were mixed with SDS sample buffer (Laemmli solution) and heated at 100°C for 5 min. Amounts of the samples, corresponding to 50 µg of proteins for transaldolase and 2,5 µg for PGAM1, were run on 12 % SDS-PAGE gels and transferred onto nitrocellulose membranes (0.2 µm) using a voltage of 100 V for 30 min (Criterion Blotter, Biorad). Non-specific binding was prevented by blocking the membranes with 3% low fat dried milk, 0.2% (v/v) Tween 20 in PBS (10 mM NaH2PO4, pH 7.4, 0.9% NaCl) (PBS/milk) overnight at 4°C. After blocking, the membranes were incubated for 2 h at RT in PBS/milk containing primary antibody After 4 washes with PBS/milk, we incubated the membranes with peroxidase-labeled secondary antibody. Proteins were revealed with an enhanced chemiluminescence detection method according to the manufacturer's instructions (PerkinElmer).

ELISA KIT

The concentration of proteins was also determined using commercial ELISA kit (USCN Life Science Inc., Wuhan, China) according to the manufacturer's instruction.

Amounts of 100ul of sample, whole or diluted saliva, were assayed; for the dilution of WS samples we used a phosphate buffer saline PBS 20mM pH 7.15.

Absorbance values were measured spectrophotometrically at a wavelength of 450nm by Wallac Victor 2, 1420 label (Perkin Elmer).

Results and Discussion

PROTEOMIC ANALYSIS OF HEALTHY AND CFS WHOLE SALIVA SAMPLES

A comparative proteomic analysis was performed on CFS and healthy samples using 2D followed by nanoLC-ESI-MS/MS. Typical 2D gel image of salivary proteins profiles is shown in Fig. 1A and B for CFS and healthy subjects respectively. By computational 2D gel image comparison, 52 protein spots were found to be differentially expressed exhibiting ≥1,5 fold-change of mean value spot intensities in the CFS with respect to control samples. Only the spots that showed p values < 0.01 (13 spots) were cut off and subjected to nanoLC-ESI-MS/MS analysis for the identification. These protein spots collapsed into the identification of 11 different proteins.
A list of identified proteins, MW, pI, score and coverage values of MS/MS, are shown in Table 3. The statistical analysis (mean ± SEM) with p-value and fold-change in expression levels between CFS and control is given in Table 4.

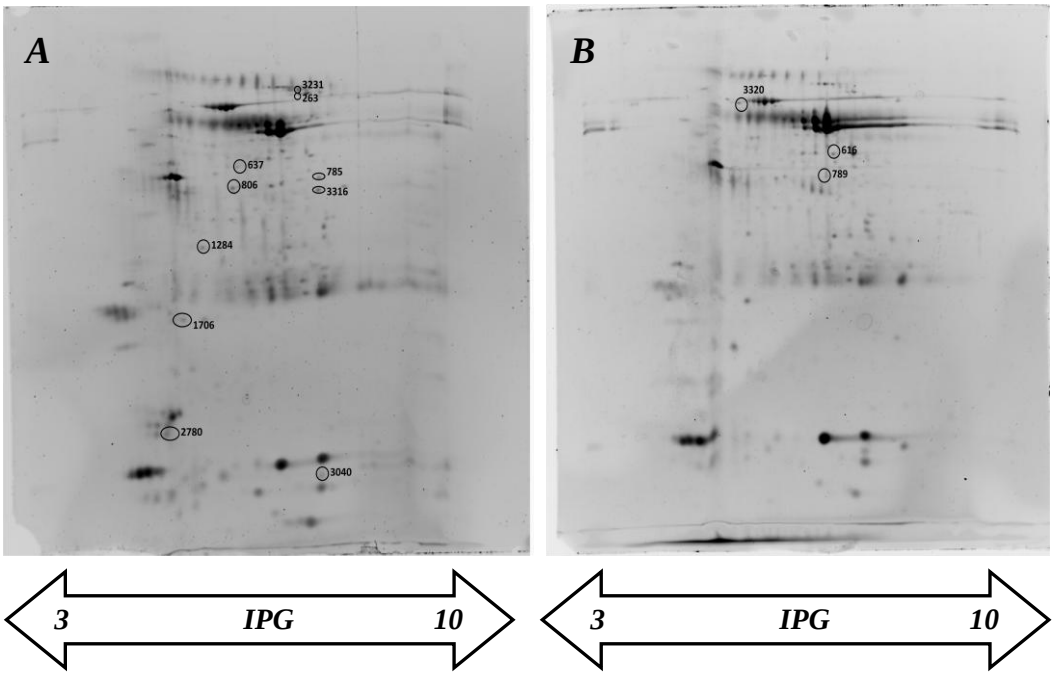


Figure 4*: 2DE patterns of human salivary pool: CFS subjects (A) and healthy subject (B).* *A total of 200 µg of proteins was separated by 2DE using 18 cm pH 3-10L strips and 12.5% SDS-PAGE. The 13 spots differently expressed in CFS in respect to control are indicated.*

Spot #	*Protein name*	ID	Gene Name	MW KDa		pI		Matched peptides	Coverage %	Best ion score.
				obs	th	obs	th			
263	***Serotransferrin***	P02787	TF	72	77,06	6,52	6.81	19	32	*98,0*
616	***Alpha Enolase***	P06733	ENO1	48	47,17	6,43	7,01	11	33	103,5
1284	***Zinc-alpha-2-glycoprotein***	P25311	AZGP1	37	34,26	5,35	5,71	9	31	81,5
3040	***Cystatin-B***	P04080	CSTB	15	11,4	6,63	6,96	4	70	95,9
806	***SerpinB1***	P30740	SERPINB1	42	42,74	5,7	5,9	14	37	102,5
3231	***Serotransferrin***	P02787	TF	76	77,07	6,6	6,81	18	27	97,1
637	***Ig alpha-1 chain C region***	P01876	IGHA1	46	37,65	5,75	6,08	2	8	95
1706	***Glutathione S-transferase P***	P09211	GSTP1	30	23,35	5,35	5,43	7	48	145,6
2780	***Prolactin-inducible protein***	P12273	PIP	18	16,57	4,83	8,26	2	16	61
3320	***Polymeric immunoglobulin receptor***	P01833	PIGR	67	83,28	5,07	5,59	2	2	35,9
789	***Carbonic anhydrase 6***	P23280	CA6	42	35,36	6,34	6,21	3	10	70,2
785	***SerpinB1***	P30740	SERPINB1	42	42,74	6,64	5,9	8	26	110,2
3316	***Alpha Enolase***	P06733	ENO1	41	47,17	6,63	7,01	10	26	116,3

Table 3: ***MS/MS data of salivary proteins found differentially expressed between pool of whole saliva of CFS patient and healthy patients – ID:*** *SwissProt accession number of the protein;* ***Gene name****: name of the corresponding gene;* ***Matched peptides****: the number of unique peptides on which the protein identification is based;* ***Coverage****: the percentage of the protein's sequence represented by the peptides identified by MS;* ***Best Ion Score****: it is a measure of how well the observed MS/MS spectrum matches to the stated peptid (obs.: observed; th.: theoretical).*

Spot #	*Protein name*	p - Value	Fold	CFS ± SD	Healthy ± SD
263	***Serotransferrin***	0,0001	- 2,3	5,11E+06 ± 1,44E+06	2,20E+06 ± 8,16E+05
616	***Alpha Enolase***	0,006	- 1,4	1,64E+06 ± 3,62E+05	2,31E+06 ± 4,24E+05
1284	***Zinc-alpha-2-glycoprotein***	0,004	- 1,5	3,68E+06 ± 9,85E+05	2,43E+06 ± 7,14E+05
3040	***Cystatin-B***	0,01	1,3	2,91E+06 ± 8,48E+05	3,92E+06 ± 1,05E+06
806	***Serpin B1***	0,02	- 1,3	1,89E+06 ± 3,33E+05	1,40E+06 ± 4,41E+05
3231	***Serotransferrin***	$3,32e^{-6}$	- 2,4	2,68E+06 ± 7,00E+05	1,10E+06 ± 3,20E+05
637	***Ig alpha-1 chain C region***	0,0027	- 2,5	5,91E+05 ± 3,16E+05	2,36E+05 ± 5,51E+04
1706	***Glutathione S-transferase P***	0,01	1,5	1,98E+06 ± 3,56E+05	2,88E+06 ± 1,08E+06
2780	***Prolactin-inducible protein***	0,0001	- 3,6	2,72E+06 ± 1,24E+06	7,47E+05 ± 3,18E+05
3320	***Polymeric immunoglobulin receptor***	0,003	1,5	7,97E+05 ± 1,88E+05	1,18E+06 ± 2,65E+05
789	***Carbonic anhydrase 6***	0,01	1,4	2,92E+06 ± 8,05E+05	3,97E+06 ± 1,16E+06
785	***SerpinB1***	0,0003	- 2,7	1,08E+06 ± 5,34E+05	4,01E+05 ± 1,65E+05
3316	***Alpha Enolase***	0,003	- 2,4	9,33E+05 ± 5,70E+05	3,90E+05 ± 2,55E+05

Table 4*: Results of 2DE statistical analysis*

VALIDATION OF PROTEIN EXPRESSION IN CFS AND CONTROL SAMPLES BY USING WESTERN BLOT ANALYSIS AND/OR ELISA ASSAY

We used western blot analysis and/or ELISA assay to validate the expression changes of some of the proteins identified by 2D. We selected a subset of 3 candidate proteins for validation by immunoassays, namely we confirmed the different expression of Zn-Alpha2, Alpha-Enolase and Serpin-B1. Moreover, we performed a validation of other three proteins that we previously found differentially expressed in the study of twins discordant for CFS [136] namely Cyclophilin, Psoriasin and Cystatin-C.

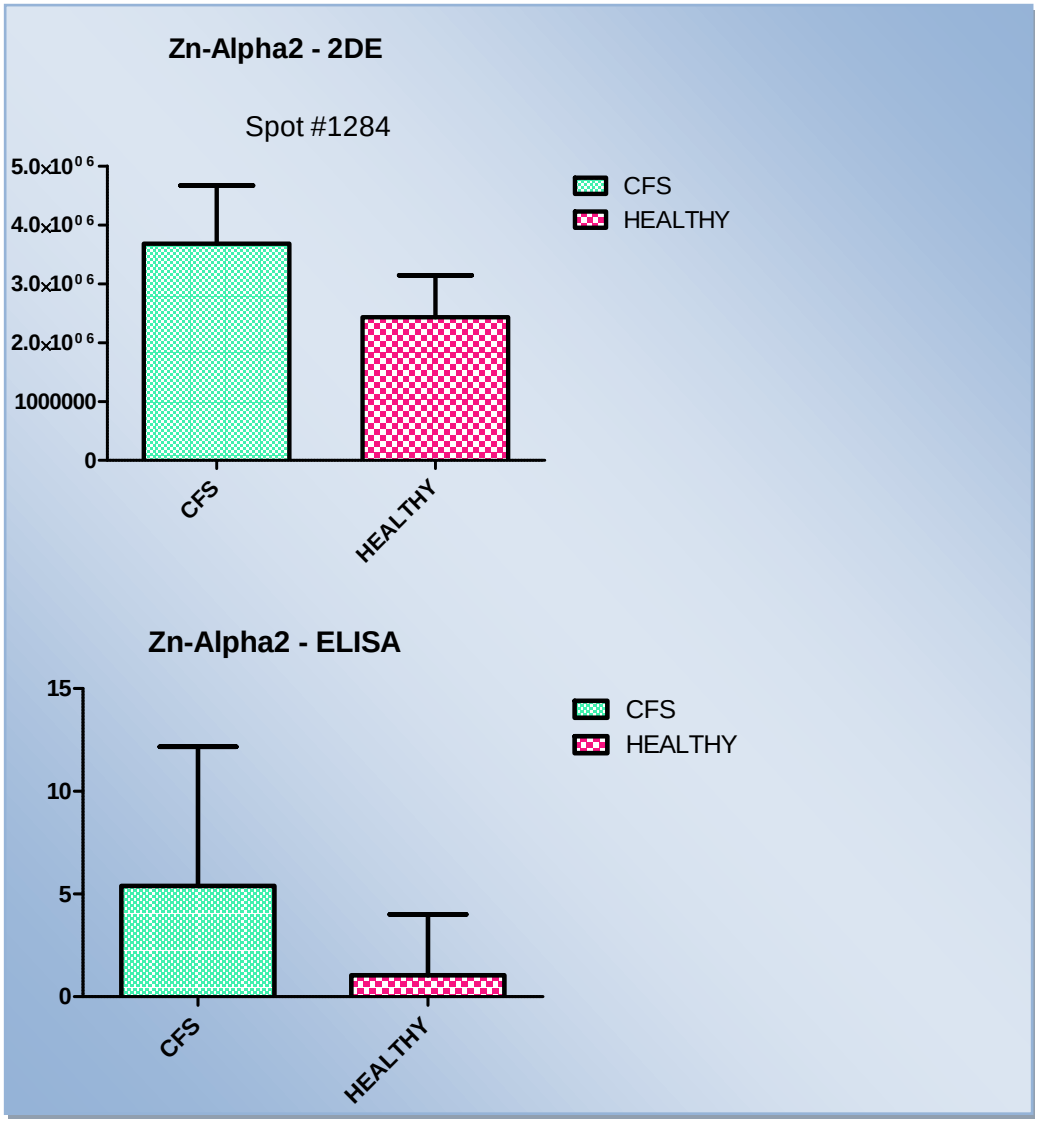
Protein validation was performed on 41 CFS and 40 control saliva samples. For each tested protein the optical density of specific immune reaction was determined and the resulting mean values ± SEM were compared.

Histograms show the volumes of the proteins found in significantly different quantities in WS of patients with respect to the healthy subjects. For the 2DE analysis the bar chart represents the mean±SD of optical density of each spot. Significant differences are based on unpaired t test; (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

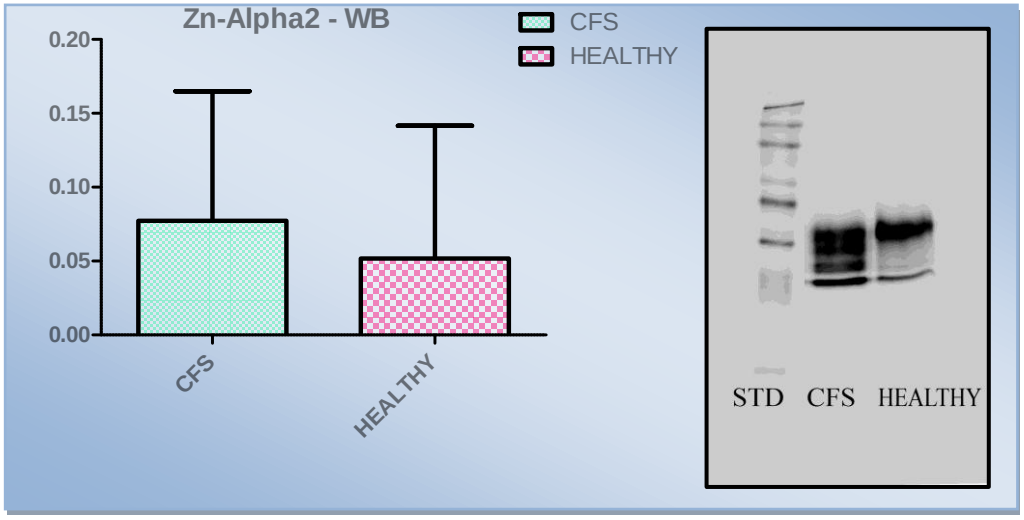
PROTEINS

Zn-Alpha 2

According with 2DE analysis, both western blot analysis and ELISA assay confirmed the up-regulation of this protein in our saliva samples of CFS patients with respect to healthy subjects. (Graph 3).



Graph 3: histograms of the normalized volume (M±SD) obtained with 2DE and ELISA-Kit for this protein.



Graph 4: Western blot analysis of and the respective histograms of the optical density (OD). Each bar represents the mean±SD

Zinc α2-glycoprotein (ZAG) is a protein of interest because of its ability to play many important functions in the human body, including fertilization and lipid mobilization. Its expression is regulated by glucocorticoids. Due to its high sequence homology with lipid-mobilizing factor and high expression in cancer cachexia, it is considered as a novel adipokine. On the other hand, structural organization and fold is similar to MHC class I antigen-presenting molecule; hence, ZAG may have a role in the expression of the immune response [149].

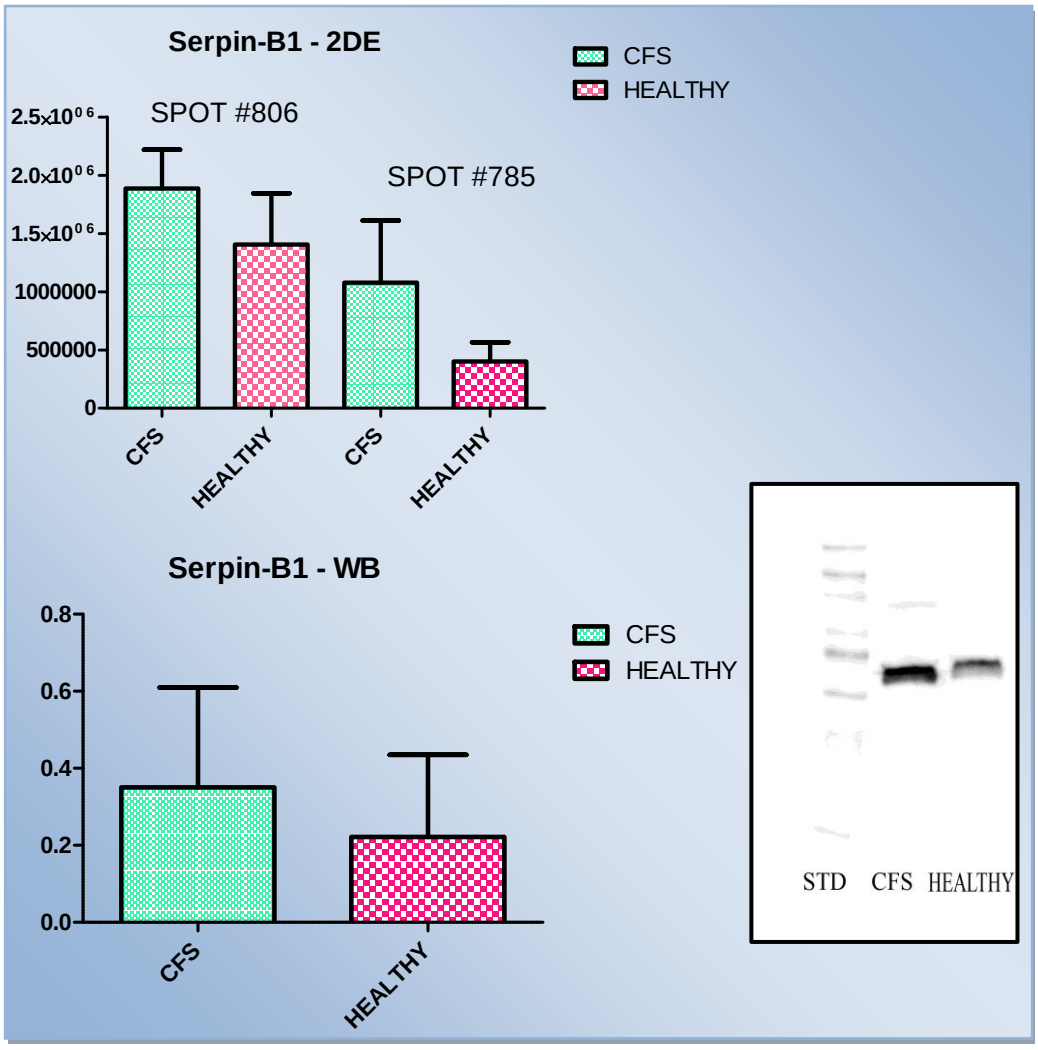
In addition, ZAG is used as biomarker for identification of female breast and male prostatic tumors. The transcriptional regulation of ZAG by androgen reveals its direct role in tumor progression as observed for other tumor-proliferating proteins such as prostate-specific antigen, prostatic acid phosphatase, apolipoprotein D, and progastriscin. The potential multiple functions of ZAG are not only a sensitive biomarker of tumor proliferation but it is also attributed to several functions such as regulation of melanin production, RNase

activity, and transport. Moreover, ZAG may be a potential therapeutic target, with its over-expression and under-expression being either beneficial or deleterious [149].

Beyond this action other study observed a role of ZAG in the activation of AMP kinase (AMPK), an important regulator of energy metabolism, in human skeletal muscle cells [150]. The mechanism may be involved in mediating the effects of ZAG in relation to increased energy utilization. This is interesting if we consider that several studies suggest an organic cause for CFS related to defects in oxidative metabolism. In particular, individuals with CFS reach exhaustion (in the presence of reduced sarcoplasmic ATP concentrations) much more rapidly in respect to healthy subjects with resultant acceleration of glycolysis in working skeletal muscles [151]. Moreover, one of the primary symptoms of CFS is muscle pain and weakness; this process is probably the result of cellular changes [151] such as reduction in the number of motor units and atrophy due to disuse. Considering that Russell and colleagues have demonstrated the ability of ZAG to reduce reactive oxygen species (ROS) production and to counter muscle atrophy associated with insulin resistance and other catabolic conditions [152].

Serpin-B1 (Leukocyte Elastase Inhibitor)

Western blot analysis also confirmed the increase of expression found by 2DE of Serpin-B1, in CFS when compared with control samples (graphs below).



Graph 5: histograms of the normalized volume (M±SD) obtained with 2DE featuring Western blot analysis and the respective histograms of the optical density (OD).
Each bar represents the mean±SD

Serpin B1 also known as Leukocyte elastase inhibitor is a protein that in humans is encoded by the SERPINB1 gene; is a cytoplasmic serine protease inhibitor of polymorphonuclear neutrophils. Among other

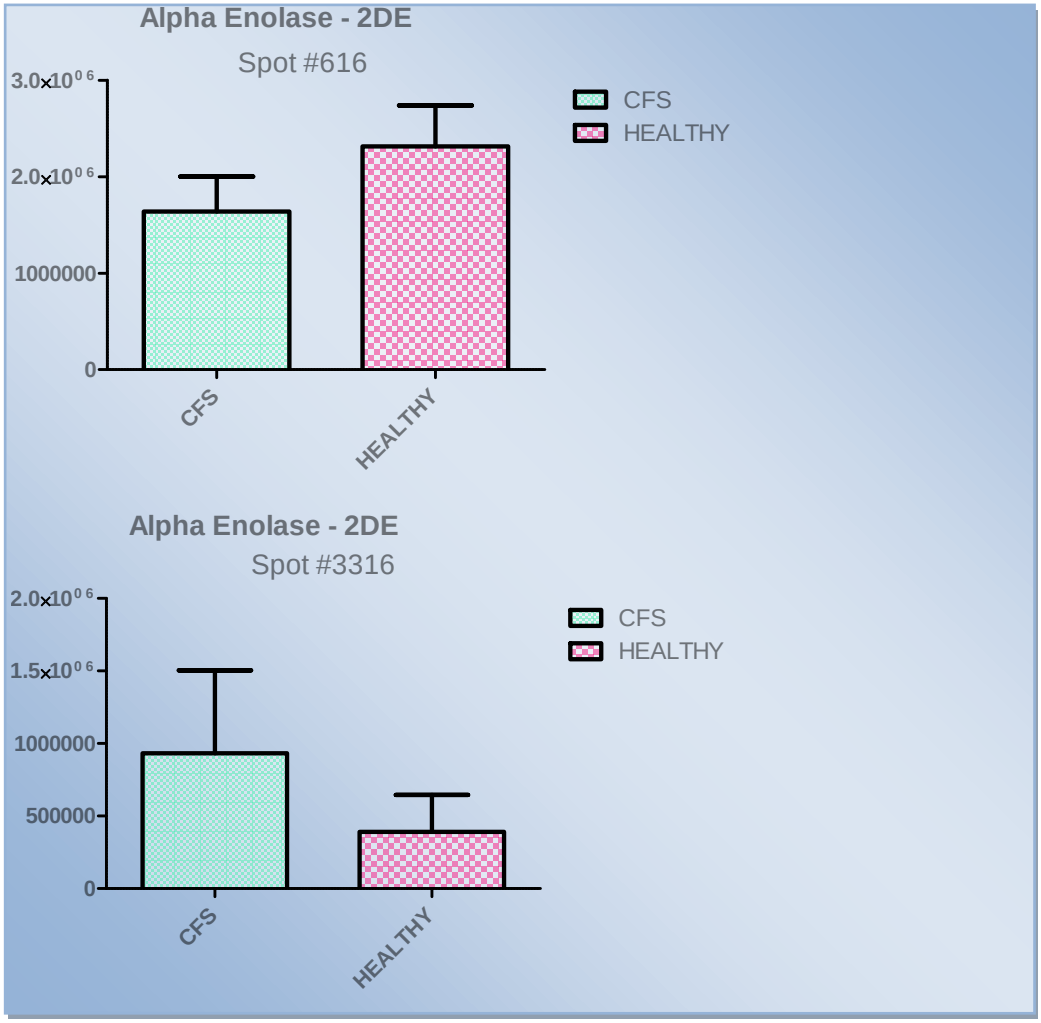
serine proteases, it specifically inhibits neutrophil elastase, PR3 and cathepsin G, all found in neutrophil granules, by a suicide inhibition mechanism.

Recent studies suggest that Serpin B1 could provide protection in the airways by regulating excess protease activity associated with inflammatory lung disorders [153].

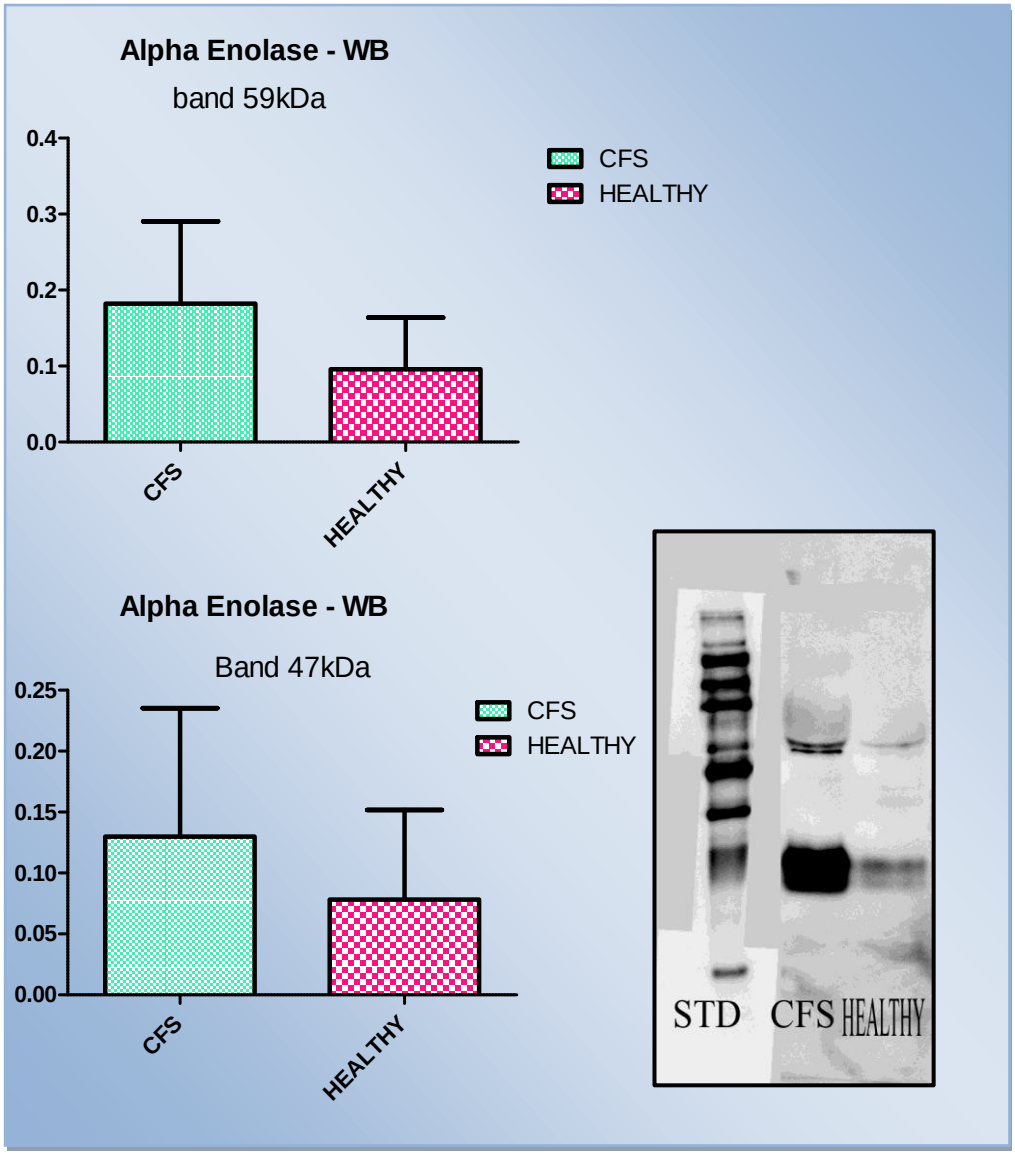
Uchiyama K, in addition, founded that SerpinB1 may be a novel marker of active ulcerative colitis and may play an important role in the pathogenesis of inflammatory bowel disease [154].

Alpha Enolase

As shown in the Table 3 two spots with different observed MW and/or pI are present for Alpha enolase suggesting the existence of protein isoforms or post-translational modifications for this protein in our samples. Comparative analysis suggested different level of expression of these spots in CFS. Western blot analysis evidenced the presence of two immunoreactive bands at different MW (band1=59kDa and band2=47kDa) as suggested by 2DE. Nonetheless, the analysis of optical densities of all samples suggested a significant increase of expression for both two bands in CFS with respect the control (see figures)



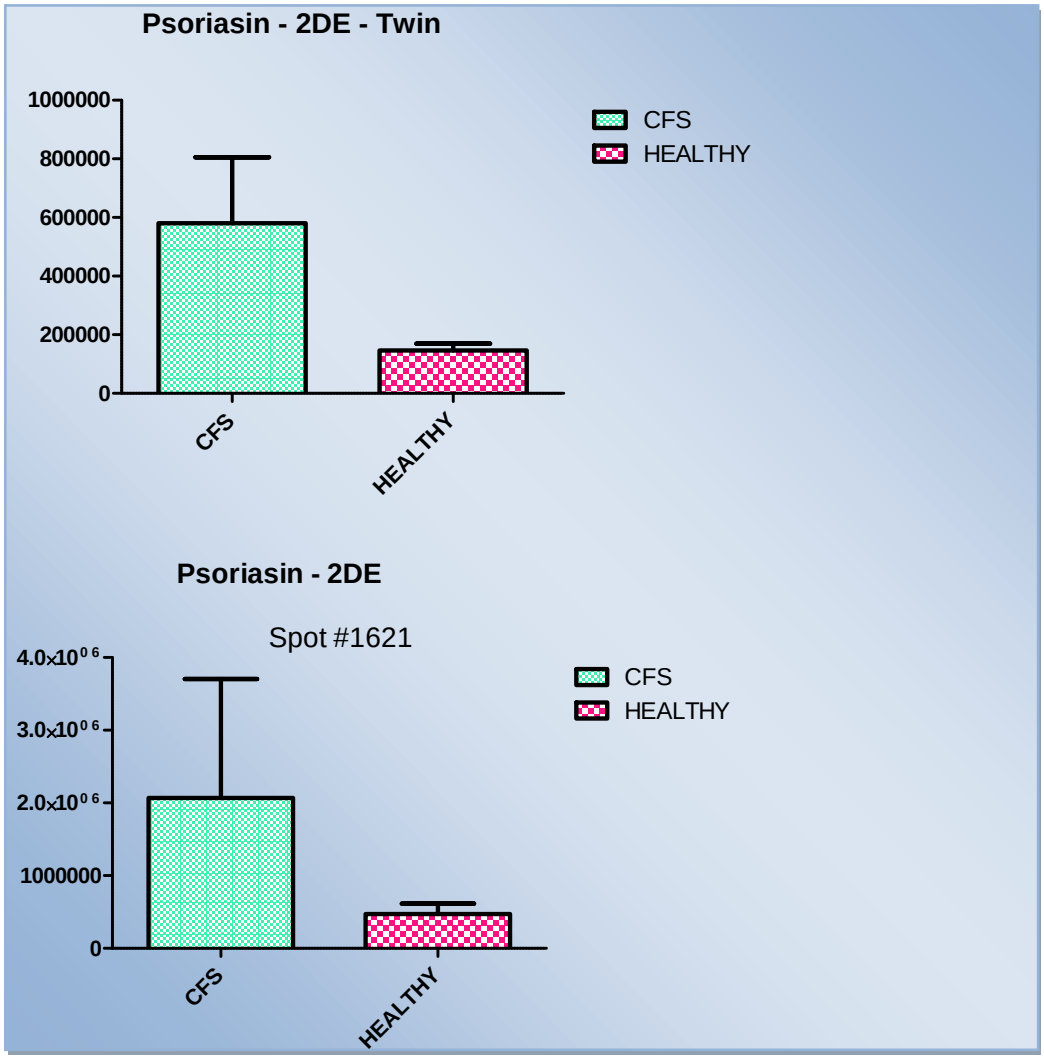
Graph 1: histograms of the normalized volume (M±SD) obtained with 2DE analysis for these proteins for the two spots of interests.



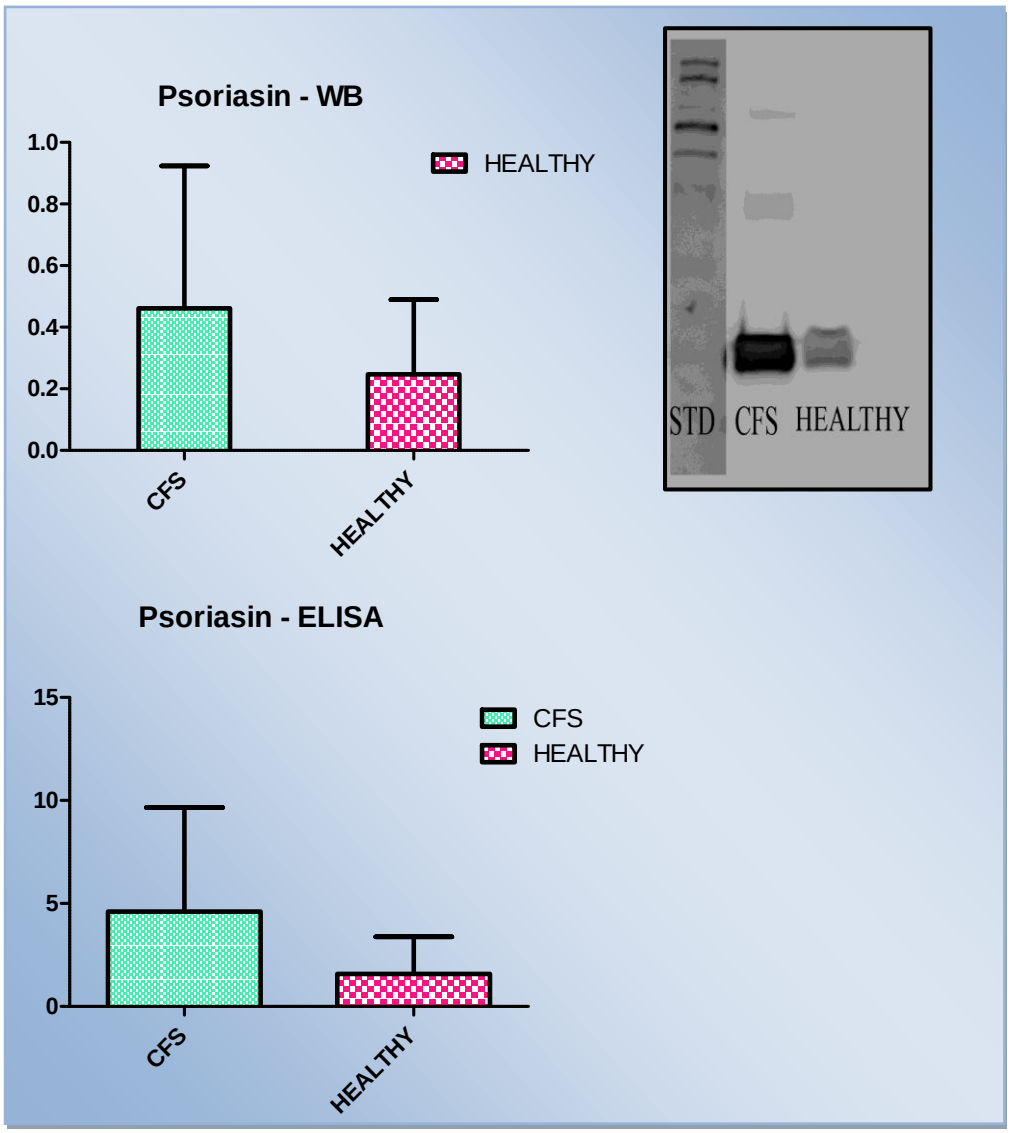
Graph 2: Western blot analysis of and the respective histograms of the optical density (OD). Each bar represents the mean±SD.

Psoriasin

As far as psoriasin is concerned increase of expression was suggested after comparison of twins discordant for CFS. Using Western Blot analysis and ELISA assay the level of expression of this protein was also investigated in our cohort of CFS patients. The results obtained are reported below. The psoriasin up-regulation is confirmed significant with both the approaches.



Graph 6: histograms of the normalized volume (M±SD) obtained with 2DE in Twin and in our subjects. Each bar represents the mean±SD



Graph 7: Western blot analysis and the respective histograms of the optical density (OD); histograms of the normalized volume (M±SD) obtained with ELISA-Kit for this protein.
Each bar represents the mean±SD

Psoriasin is a relatively new member of the S100 gene family that was first identified as a 11.4 kDa protein induced in squamous epithelial cells of the epidermis isolated from skin involved by psoriasis. The normal function of this protein is unclear it might be deduced from its close relatives. The S100 proteins are believed to infuence calcium

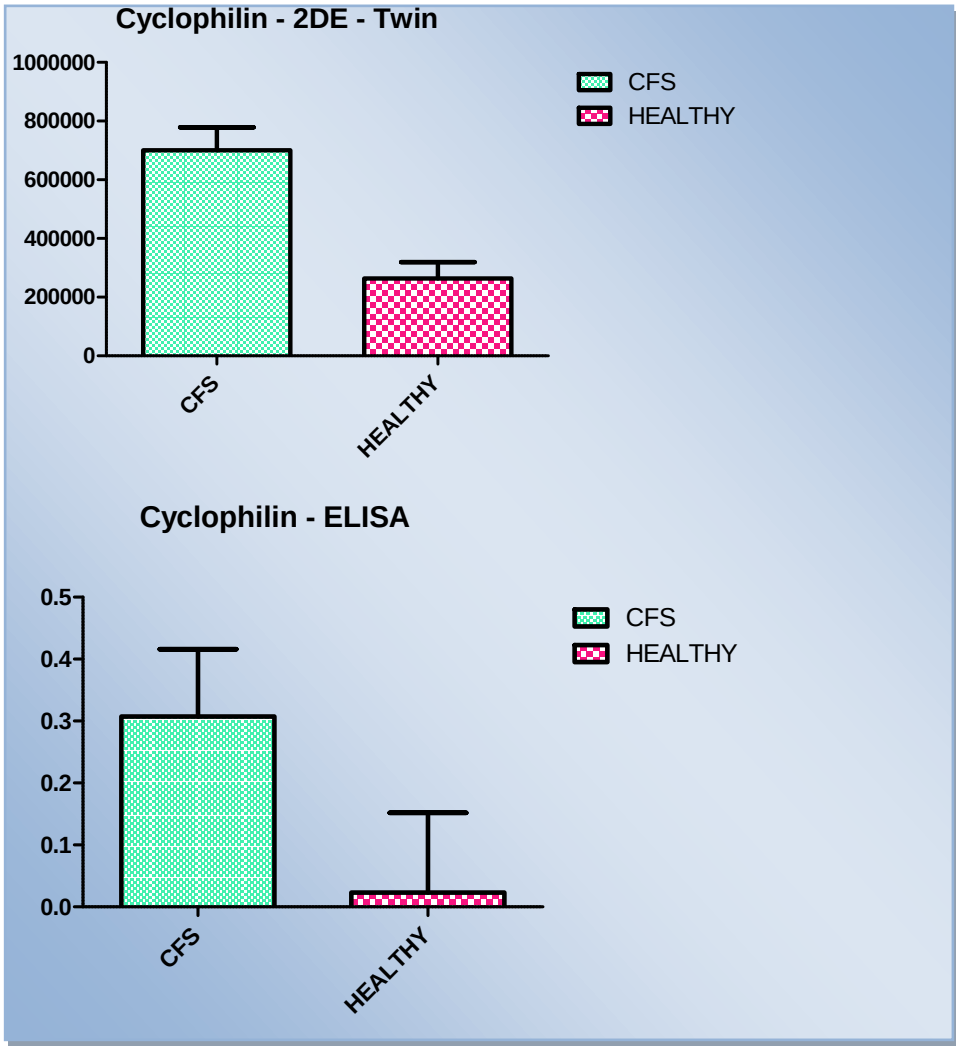
mediated signal transduction and cellular events through direct target protein interactions, as opposed to a function as mere storage buffers [155].

More recently, Psoriasin and the related S100 A8 and A9 proteins have also been found in other abnormal epithelia, including neoplastic breast ductal epithelium and bronchial epithelium in individuals with cystic fibrosis. Psoriasin might exert an epithelial migration inhibitory function in in-situ breast carcinoma cells that once lost could contribute to the onset of successful invasion [155].

Furthermore S100A7 is up-regulated in the saliva of patients with systemic sclerosis when compared to healthy individuals and has been proposed as a potential biomarker for systemic sclerosis with pulmonary involvement as demonstrated by studies conducted by Giusti and Baldini [156, 157].

Cyclophilin

Similarly to psoriasin, the increase of expression of Cyclophilin found in twin affected by CFS was confirmed in our cohort of patients Results are showed below.



Graph 8: histograms of the normalized volume (M±SD) obtained with 2DE and ELISA-Kit for this protein. Each bar represents the mean±SD

Cyclophilins (CyPs) are a family of ubiquitous proteins evolutionarily well conserved and present in all prokaryotes and eukaryotes [158]. They have peptidyl prolyl isomerase activity, which catalyzes the isomerization of peptide bonds from trans form to cis form at proline residues and facilitates protein folding [159].

Human CyPs consist of 16 family members that are structurally distinct. Among them, the most abundant member is CyPA, which makes up B 0.1–0.6% of the total cytosolic proteins [158, 160] CyPA was initially purified from bovine thymocytes and identified as the primary cytosolic binding protein of the immunosuppressive drug cyclosporin A (CsA) [161].

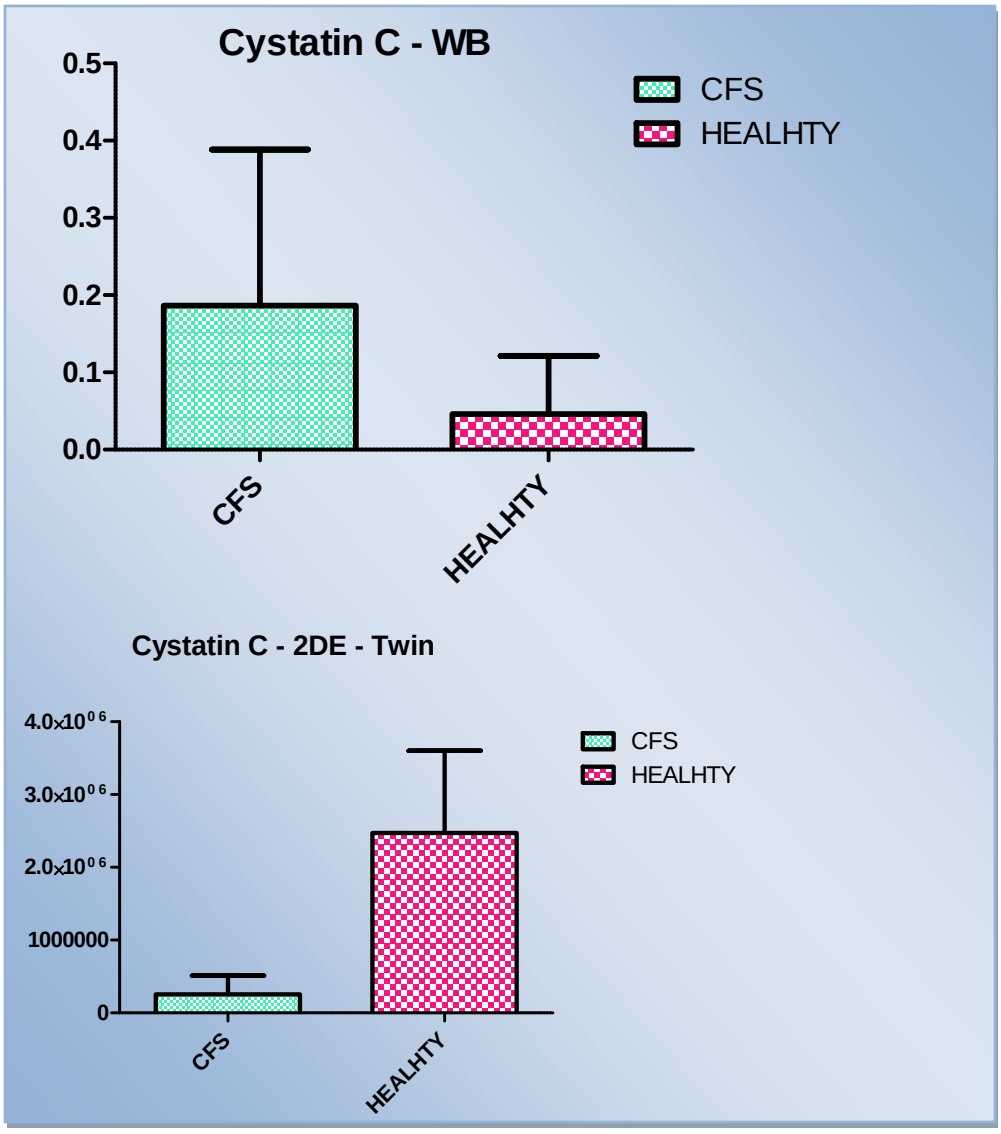
CyPA is believed to have important roles in many biological conditions including protein folding, trafficking, and T-cell activation [162] CyPA is involved in several human disease. One of these is the viral infections; in this kind of disease have been discovered to promote the secretion of CYPA [163-165] supporting the hypothesis that a persistent viral infection may contribute to the pathogenesis of CFS [166].

In addition, CyPA is involved in development of Rheumatoid Arthritis (RA). Previous study have shown that CyPA was increased in the synovial fluids of RA patients compared with knee osteoarthritis patients [167]. Influence of this protein in RA was also demonstrated by Won-Ha Lee which demonstrated that macrophages of the synovial lining layer constitute the major source of CyPA. In addition, they showed that stimulation of monocytes with CyPA results in increased production of inflammatory cytokines [168].

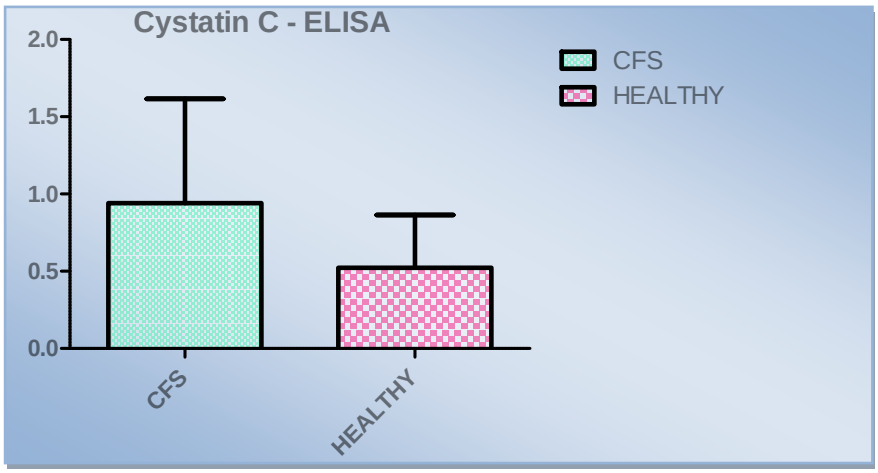
Cystatin C

In twin study Cystatin C expression, unlike Psoriasin and Cyclophilin, is down-regulated in CFS twin with respect the healthy.

Validation performed in the cohort of CFS patients showed a discordant result as showed in the figure. In fact a significant increase of Cystatin C was observed in CFS patients with both western blot and ELISA kit approaches.



Graph 9: histograms of the normalized volume (M±SD) obtained with 2DE in the twin studty for this protein. Each bar represents the mean±SD



Graph 10: histograms of the normalized volume (M±SD) obtained with WB and with ELISA-Kit fot this protein. Each bar represents the mean±SD

Cystatin C belongs to the cystatin superfamily 2 and is a secreted protein, produced by most nucleated cell types; hence, it is present in all investigated biological fluids. Since cystatin C is a secreted protein, its major site of function is in the extracellular compartment [169, 170]. Cystatins are Proteins with a role in protein catabolism, in regulation of hormone processing and bone resorption, in inflammation, in antigen presentation and T-cell dependent immune response as well as resistance to various bacterial and viral infections [136].

In relation to arthritis, cystatin C has been found to be the most prominent cystatin in synovial fluid of RA patients and that RA patients have significantly lowered levels of cystatin C in circulation [171]. In addition, cystatin C has been shown to enhance fibroblast and smooth muscle cell proliferation and neutrolphil function [172-174].

Furthermore it was demonstrated that the two types of cystatins (type I and II). different functions, e.g., type I cystatins are up-regulated in tumor tissue while type II cystatins are generally downregulated in

tumors [175]. Therefore, results obtained by Ciriega could suggest that the balance between cysteine proteinases and their inhibitors is impaired in CFS [136].

Conclusion

Overall our results suggest the applicability of a proteomic approach on whole saliva to search potential biomarkers of CFS. The pilot study performed in a couple of omozygotic twins discordant for CFS suggested a list of proteins of interest as potential biomarkers.

In this work we applied the proteomic analysis of whole saliva in a large cohort of patients affected by CFS and healthy subjects as control and we confirmed the potential role of some proteins such as Psoriasin, SerpinB1 and Cyclophilin as potential marker of this disease.

Discordant results found for the Zn-alpha2, Cystatin-C and Alpha Enolase expression in our study with respect the twin study could be due to different clinical parameters.

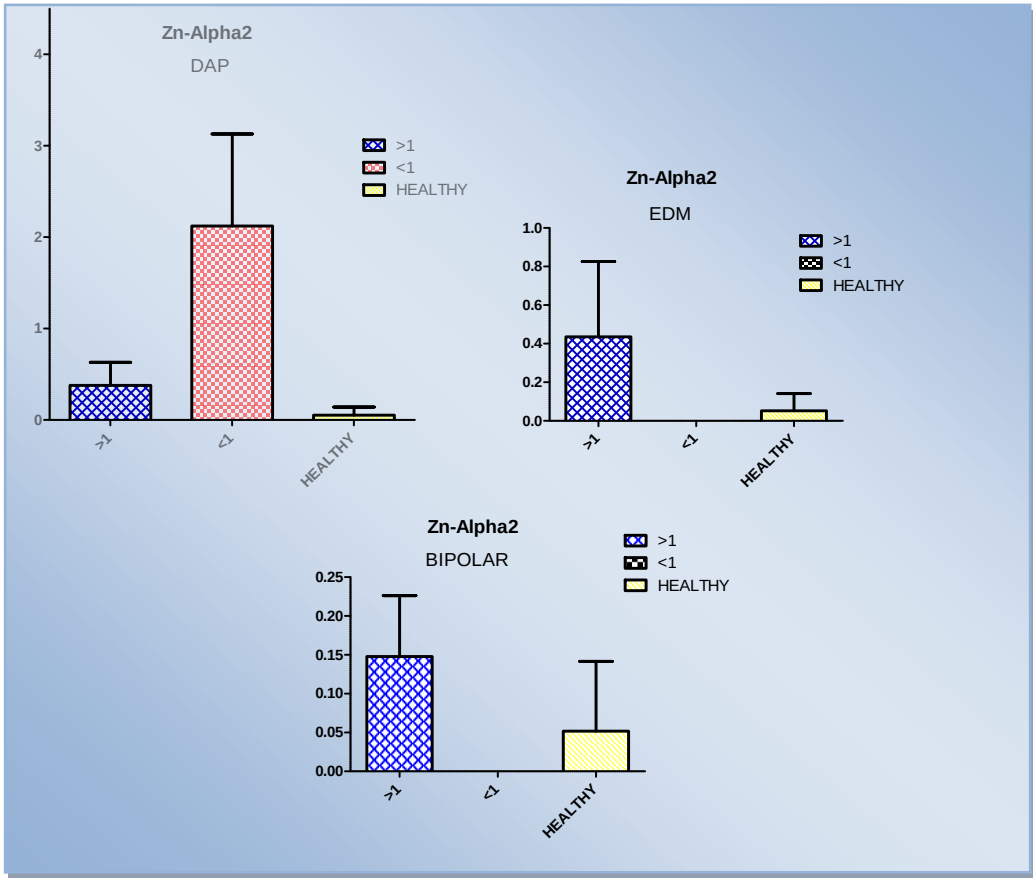
We investigated if the trend difference observed for Zn-alpha2 and Cystatin-C in CFS patient population were correlated with some clinical parameters of the patients.

Indeed, we can definitely say, thanks to past research, that an important aspect that characterizes CFS patients is their psychiatric profile.

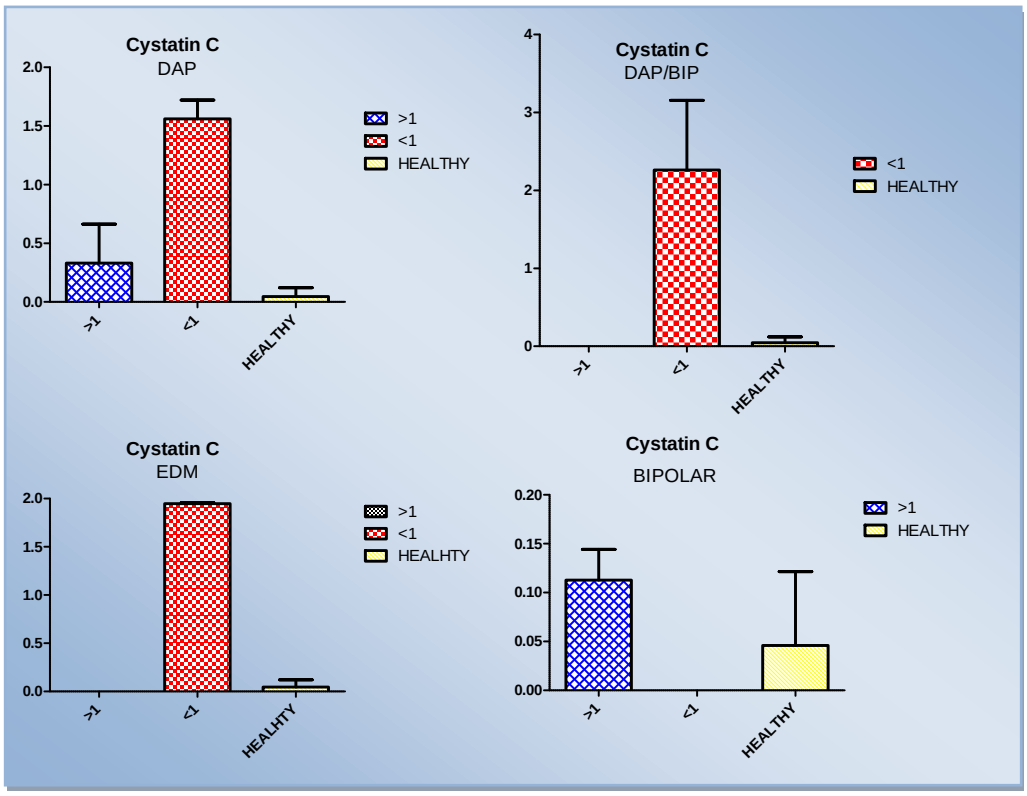
A first analysis performed on our patients divided by class DAP (*Panic Attack Disorder*), EDM (*Major Depressive Episode*), BIPOLAR and DAP/BIP you may notice discordant patterns of the same protein in the different classes of psychiatric illness suggesting a possible correlation with this profile for both proteins (graph 11 and 12).

Another important aspect is the correlation with the clinical parameters such FIQ, VAS (*pain*, *fatigue*, *sleep*) FACIT etc.

From a first analysis it is possible to note how an opposing trend (reduction of the one part and the other increase) in the expression of Zn-Alpha2 then corresponds to values rather various parameters such as physical role, vitality, body pain, social activities.
Same for Cystatin-C about the parameters: social activities and physical role (table 5).



Graph 11: Histograms for the 3 psychiatric parameters (DAP, EDM and BIPOLAR). For every parameter We subdivided patients into three groups, based on the ratio of the volume of the band on the total average. Finally, I 2 groups, those with a ratio greater and smaller than 1, are compared with the average of the healthy subjects.



Graph 12: histograms for the 4 psychiatric parameters (DAP, DAP/BIP, EDM and BIPOLAR). We subdivided patients into three groups, based on the ratio of the volume of the band on the total average. Finally, I 2 groups, those with a ratio greater and smaller than 1, are compared with the average of the healthy subjects.

	Zn-Alpha 2 mean <1	*Zn-Alpha 2 mean >1*	*Cyst 3 mean <1*	*Cyst 3 mean >1*
FIQ	51,4	60,7	55,9	52,6
VAS Pain	3,3	4,0	3,8	3,1
VAS Fatigue	7,7	7,6	8,0	7,2
VAS Sleep	7,2	7,6	7,7	6,8
FACIT	29,2	32,5	31,2	29,0
SF-36				
Physical Functioning	65,8	46,0	57,5	62,5
Role Physical	20,0	6,0	9,4	22,5
Bodily Pain	42,0	69,0	42,5	51,9
General Health Status	37,1	32,0	33,1	37,7
Vitality	28,0	18,0	24,4	27,0
Social Activities	40,2	14,8	23,4	41,0
Role Emotional	48,5	26,4	41,3	43,2
Mental Health	60,9	58,4	58,5	61,6

Table 5: Clinical Parameters for CFS subjects divided in two different group.
First group contains subjects whose volumes, found via WB, are below average; in the other one, those whose volumes are superior.

Results found in this study confirm presence of possible biomarkers for the identification of CFS and the subsequent use of these for any future treatment.

But what follows from this work is that the wide variety of psychiatric subjects involved in the study does not allow us to draw general conclusions.

Certainly more research will be needed, with an expansion of the number of parties involved, both sick and healthy ones, and for both is required a clinical psychiatric-as complete as possible in order to compare the best the two types of subjects.

Future studies can fully examine the genetic, familial, and unique environmental mechanisms that link these conditions, especially with regard to the social support network available to individuals with these conditions and to potential differences between men and women [113].

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