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RESEARCH ARTICLE

Molecular biomarkers in Electrohypersensitivity and Multiple Chemical Sensitivity: How They Can Help Diagnosis, Follow-Up, and in Etiopathologic Understanding.

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ABSTRACT:
Electrohypersensitivty (EHS) and multiple chemical sensitivity (MCS) are new worldwide emerging neurologic disorders in the framework of sensitivity-related environmental pathology. We have recently extended and confirmed our previous observation showing that EHS and MCS share clinically identical symptoms and may co-exist as a unique, common, sensitivity-related neurologic syndrome in 25% of the cases. There is presently no published biological study of these disorders, except the one we have previously published as preliminary. In the present study, we show that EHS and MCS and the combined syndrome share identical biochemical changes. More precisely, by measuring levels of peripheral blood and urine molecular biomarkers in a cohort of 2,018 consecutive cases, we show that both disorders and the combined syndrome can be objectively characterized, in about 90% of the cases, by a decrease in the production of 6-hydroxymelatonin sulfate in urine, while in 30-50% they are characterized by increased levels of histamine and of heat shock proteins (HSP) 27 and/or 70, and of protein S100B and nitrotyrosine in the peripheral blood. Increased levels of histamine and HSP are indicators of low grade inflammation while increased levels of protein S100B and nitrotyrosine are indicators of blood-brain barrier disruption/opening. In addition, we show that in about 15% of the cases anti-myelin autoantibodies can be detected in the peripheral blood, accounting for the occurrence of an autoimmune response. Sensitivity, specificity and reproducibility of the biochemical tests are discussed, as well as the role of these indicators used as biomarkers for the diagnosis and follow-up of patients. We also discuss cases with undetectable biological change for which they can be nevertheless diagnosed by cerebral neurotransmitters analysis in urine and brain imaging. On the basis of these biological data it is suggested that EHS and/or MCS are new brain disorders, generated via a common etiopathogenic mechanism.

Keywords: sensitivity-related neurologic diseases; electromagnetic field; electrohypersensitivity; idiopathic environmental intolerance; multiple chemical sensitivity.

Abbreviations:

6-OHMS, Hydroxy-melatonin sulfate; BBB, Blood Brain Barrier; CNS, Central Nervous System; EHS, electrohypersensitivity; EMF, electromagnetic field; hs-CRP, High sensitivity-C reactive protein; HSP27, Heat shock protein 27; HSP70, Heat shock protein 70; IEI-EMF, Idiopathic Environmental Intolerance attributed to Electromagnetic Fields; IgE, Immunoglobulin E; MCS, multiple chemical sensitivity; NOS, nitroso-oxidative stress; NTT, Nitrotyrosine; TDU, transcranial Doppler ultrasound; UCTS, ultrasonic cerebral tomosphygmography; WHO, World Health Organization; WiFi, Wireless Fidelity.

1. Introduction

Multiple Chemical sensitivity (MCS) and electrohypersensitivity (EHS) are new worldwide emerging neurological disorders in the framework of acquired sensitivity-related environmental pathology. Both disorders were defined as the occurrence of symptomatic manifestations for levels of environmental exposure lower than commonly tolerated. MCS was first described in 1962 by the American allergist Theron G Randolph as a disorder caused by exposure to low concentration of multiple exogenous chemicals[1]. Then EHS was experimentally identified in 1991 by William Rea and defined similarly as a sensitivity-related pathological disorder resulting from *low intensity* electromagnetic field (EMF) exposure[2]. As reported, the experimental provocation investigation was done under totally environmentally controlled conditions using a series of electromagnetic field (EMF) challenges, ranging 0 to 5 MHz in frequency. Both the blank placebo controls and the normal healthy controls had no symptoms, whereas 64% of the EMF sensitive patients had symptoms.

Since Randolph's seminal description, MCS has been acknowledged as an acquired environmental intolerance to low level exposure to multiple chemicals during the 1996 Berlin World Health Organization (WHO)-sponsored workshop[3], and has been further identified and more precisely characterized during a 1999 consensus meeting in Atlanta (USA)[4].

Regarding EHS, there remains in the scientific literature a persisting confusion between EHS and the clinically described pathological disorder called idiopathic environmental intolerance attributed to EMF' (IEI-EMF), which both have been reported and recognized by WHO [5-6].

Following our initial attempt to characterize EHS in 2015[7], we have shown that EHS can be identified as a distinct medical disorder[8]; and more recently that it may be caused by exposure to anthropogenic EMF (i.e. by exposure to man-made as opposed to natural EMF) and possibly in some cases by environmental chemicals as in MCS[9]. Moreover we have shown that EHS and MCS share identical clinical symptoms and may coexist as a unique common sensitivity-related neurologic syndrome in 25% of cases[7,10]. In that attempt we had shown that both EHS and MCS and the combined EHS/MCS syndrome could be characterized in the peripheral blood by decreased level values of Vitamin D and increased level values of high sensitivity-C reactive protein (hs-CRP) and Immunoglobulin E (IgE), and more characteristically, by increased level values of histamine, protein S100B, heat shock protein (HSP) 27 and/or 70, Nitrotyrosine (NTT) and anti-myelin P0 autoantibodies, while these disorders could be also characterized by a decrease of 6-hydroxy-melatonin sulfate (6-OHMS) in the 24h urine.

In this study, we would like (1) to critically re-assess the results obtained previously by investigating more patients; (2) to compare the results obtained in EHS patients with those obtained in patients with MCS or with the combined syndrome; (3) to discuss whether the previous investigated biomolecules used as indicators could be used as biomarkers for the objective diagnosis and follow-up of patients; (4) and finally, to estimate how these biomarkers could provide some insight into the etiopathogenic mechanisms involved in both sensitivity-related neurologic disorders.

2. Material and methods

In this study we used a cohort study in which patients with EHS, MCS or both disorders were divided into three groups respectively according to their clinical presentation. For these different groups, we measured as previously reported the molecular indicators listed in Table 1.

2.1. PATIENT ACCRUAL

Patients were not actively recruited. Accrual was due to the fact that in France there are not yet medical doctors specialized in the care of EHS and MCS patients. All patients were thus spontaneously referred to one of us (DB) by following their own information. Since all patients were informed by EHS and MCS patients charity organizations that we medically took care of both disorders, and as all consecutive cases were included in the study, we reasonably believe that accrual did not suffer from selection bias.

2.2. INCLUSION CRITERIA

Since there is as yet no available published biological characterization of EHS and MCS

showing that these disorders can be defined by biological criteria, inclusion criteria for MCS were those internationally recognized by the 1999 Atlanta consensus meeting[4] which were based on the fact that the patients report to be clinically intolerant to low exposure to exogenous chemicals (i.e. to lower exposure than normally tolerated).

Inclusion criteria for EHS patients were those proposed by WHO[11]. They were similar to those recommended by the 1999 Atlanta consensus meeting for MCS, adapted to EHS[7]. They concern the reproducibility of symptom occurrence as in MCS under lower exposure than normally tolerated of presumed EMF sources, such as mobile phones, wireless fidelity (WiFi), powerful lines, smart meters, etc.[8]. They concern also the regression or disappearance of symptoms when presumed incitants were removed. In addition to these clinical criteria provided by the patients questioning, special attention was paid to the absence of known preexisting and/or coexisting pathologies that could account for the observed clinical symptoms such as atherosclerosis, diabetes, neurodegenerative or psychiatric diseases, which would render the interpretation of biological data difficult. This was particularly true for Alzheimer disease which has been reported to be caused by EMF exposure[12,13]. To this end, we used suitable standard tests to avoid these pathologies (section 2.4)

We emphasize that our inclusion criteria and symptomatic description of cases were not just based on the subjective claims by the patients, but on a careful clinical analysis of the medical anamnesis, a systematic face-to-face questioning, and on a physical examination of patients to avoid unrelated pathologies and to make the diagnosis of EHS and/or MCS, as clearer as possible.

2.3. PATIENT INCLUSION

This study derives from an analysis of the patients registered in the database that we have constituted and have prospectively maintained since 2009 in France, which presently contains more than two thousand EHS and/or MCS cases. It appears to be the most important series of such patients worldwide. This database was referred to the French Committee for the Protection of Persons with registration number 2017-A02706-47, and is also registered in the European Clinical Trials Database (EudraCT), with registration number 2018-001056-36.

All included patients gave their informed consent for clinical and biological research investigation, and were anonymously registered. For registration, as indicated above, we did not use telephone interviews or internet-based questionnaire surveys, but rather face-to-face interviews and medical examinations, a method which minimizes patient-dependent subjective biases or imprecise analysis. This present study is based on the analysis of 2,070 cases registered from 2009 to 2021, from which 2,018 cases are evaluable for determination of the association of EHS with MCS. They were 1,428 cases with EHS, 85 with MCS and 505 (25%) with the combined syndrome. Biological changes in patients with this combined syndrome have been compared to those bearing MCS or EHS alone. Patients have been also compared with a control group of apparently healthy subjects.

2.4. LABORATORY TESTING

Analysis of all biomolecules used as indicators were performed in a single laboratory in Paris, France. In most cases, sampling were done before any patient's treatment. Laboratory normal limit reference values were checked by investigating a group of normal subjects without EHS nor MCS. This group of apparently normal healthy volunteers was used as a control group (section 3.1).

The number of evaluable cases with EHS and/or MCS was dependent on the type of biomolecules investigated. Not all of them could be investigated in all patients, mainly due to monetary reasons (no reimbursement of several tests by the French social security system). 672 cases were nevertheless fully evaluable for the seven more characteristic biomolecules (section 3.2.5).

All registered patients were first investigated with hematological metabolic, hepatic and renal standard laboratory tests, to eliminate unrelated pathologies. Then more characteristic peripheral blood and urine tests were done. They are listed in Table 1. Each assay was performed according to the recommended manufacturer's method. Hs-CRP, vitamin D2-D3 and IgE were measured by using a classical automated immunoassay [Architect Ci 4100 (Abbott Laboratories, Abbott Park, Chicago, IL, USA)]; while for Histamine we used an ELISA specific test (IBL International Gmbh, Hamburg, Germany); for protein S100B, a quantitative automated chemiluminescent immunoassays [Liason S100 (DiaSorin Deutschland GmbH, Dietzenbach, Germany)]; for NTT, a competitive ELISA test (Cell Biolabs Inc., San Diego, CA, USA); for Anti-myelin P0 protein autoantibodies detection, a Western Blot qualitative analysis (IMMCO Diagnostics, Buffalo, NY, USA); for chaperone proteins HSP 27 and HSP 70, a specific high sensitivity enzymatic immunoassays (Stressgen Biotechnologies Corporation, San Diego, CA, USA); and for 6-OHMS measurement in urine, an ELISA test (IBL International GmbH, Hamburg, Germany).Table 1

indicates the different biomolecules investigated, the methods for their measurement and the normal reference range values used by the laboratory, which our control group of healthy individuals fell within.

Table 1. Tests used routinely for the measurement of the investigated biomolecules. Methods of measurement and normal range.

Biomarker	Author, year	Sample type	Normal reference values*	Control values +/- SE**
Low grade inflammation				
High-sensitivity C reactive protein (hs-CRP)	Pearson et al., 2003[14]	Serum	≤ 3 mg/l	0.57 +/- 0.04
Vitamin D2-D3	Belsey, Deluca and Potts 1971[15]	Serum	≥ 30 ng/ml	31.58+/-0.96
Histamine	Lebel et al., 1996[16]	Plasma	≤ 10 nM	4.96+/-0.03
IgE	Dessaint et al., 1975[17]	Serum	≤ 100 U/ml	46.32+/-3.75
Heat shock protein 27 (HSP27)	De and Roach, 2004[18]	Serum	≤ 5 ng/ml	1.99+/-0.08
Heat shock protein 70 (HSP70)	Pockley, Shepherd and Corton 1998[19]	Serum	≤ 5 ng/ml	2.43+/-0.09
Blood brain barrier disruption/opening				
Nitrotyrosine (NTT)	Ischiropoulos et al., 1992[20]	Serum	≥ 0.6 µg/l and ≤ 0.9 µg/ml	0.73+/-0.01
Protein S100B	Smit, Korse and Bonfrer. 2005[21]	Serum	≤ 0.105 µg/l	0.06+/-0.01
Autoimmune response				
Anti-myelin P0 protein autoantibodies	Arnold, Pfaltz and Altermatt 1985[22]	Plasma	Negative	Negative
Melatonin metabolites				
Hydroxy-melatonin sulfate (6-OHMS)	Schumacher, Nanninga, and Leidenberger 1989[23]	Urine	≥ 5 ng/l and ≤ 40 ng/l	33.96 +/-1.93

*These normal reference values correspond to those used in this study. They were provided by the laboratory in which the chemical tests were done. The normal reference values were those coming from the tests manufacturers (section 2.4) which were confirmed by the analysis of a normal control group (section 3.1).

**Values with standard error obtained from 80 normal subjects.

2.5. CHOICE OF BIOMOLECULES USED AS PATHOLOGICAL INDICATORS

Our search for biomolecules used as indicators for the characterization of EHS and/or MCS and their scientific justification has been reported previously[7,10]. We looked for biological changes caused in humans by brain injury and in animals by man-made radiation or exogenous chemicals; then we chose several biomolecules which could be referred as biomarkers as defined by international scientific consensus[24,25] (section 4.2).

In brief, we routinely measured the hs-CRP; and the secosteroid 25 hydroxy-vitamin D, as it has been suggested that the high level of hs-CRP and low levels of vitamin D in the peripheral blood of patients are associated with inflammatory and/or autoimmune processes[26]. Since upon brain injury the inflammatory response may trigger degranulation of mast cells, leading to a massive release of histamine in the blood[27-28], we also measured the levels of histamine in the peripheral blood of patients. In addition, as the best known mast cell degranulation mechanism may involve the crosslinking of IgE to its high affinity specific cell surface receptor[29] we also measured the total IgE levels in the peripheral blood.

Furthermore, since histamine is able to increase permeability of the blood brain barrier (BBB) through nitroso-oxidative stress (NOS)[30-31], we looked for possible NOS-related indicators that could be involved in BBB disruption/opening. We identified NTT, because it results from the toxic effects of peroxynitrite (ONOO-) on proteins, and is considered as an indicator of BBB disruption/opening[32-35]. In addition, we considered the calcium-binding protein S100B, produced and released predominantly by perivascular astrocytes, since increase in this biomolecule in the peripheral blood has been shown to be associated with BBB disruption/opening[36-38], although it is not specific and probably not dependent on NOS.

We also considered that non-thermal EMF exposures could induce a repetitive cellular stress,

leading to continuous HSP over-expression and release in exposed tissues, particularly in the brain[39-43]. It is well known that under inflammatory-related cellular stress conditions the HSP over-expression promotes an anti-inflammatory response[44-46]. We thus hypothesized that the major inducible stress protein HSP70, which was shown to oppose neuronal apoptosis[47-48] and BBB disruption/opening[47,49], could be involved; as well as the anti-inflammatory HSP27 stress protein[50]. We thus systematically measured HSP70 and HSP27 levels in the peripheral blood of patients to determine whether these chaperone proteins could be associated with some low grade inflammation, as has been evidenced in experiments using non-thermal EMF exposure[39-42,51]. Moreover, during NOS, proteins may be extensively modified and denatured, acquiring new epitopes which can result in their loss of specificity and biological activity; hence in the synthesis of autoantibodies[52-53]. We consequently hypothesized that under exposure to environmental stressors, proteins such as myelin protein zero (myelin P0)–the most abundant protein in the nervous system–may be denatured to such a degree that it acquires auto-antigenic properties. We thus systematically searched for the presence of autoantibodies against myelin P0 in the blood of patients.

Finally, as previously reported[7,10] since many EHS and/or MCS patients had sleep disturbances, and these effects are reported to be associated with EMF exposure and mediated by the pineal hormone melatonin[54], we systematically measured the 6-Hydroxymelatonin Sulfate (6-OHMS) melatonin-derived metabolite in the 24h urine of patients[55]. We also calculated the 6-OHMS/creatinine ratio in 24-hour urine collection, to reduce the variability of 6-OHMS measurement attributed to urine dilution.

2.6. URINE CEREBRAL NEUROTRANSMITTERS AND CEREBRAL IMAGING

In a limited number of cases we have also searched for cerebral neurotransmitters in the urine[8,10]. This was particularly done in EHS cases with no molecular change detected by the routine tests. In such cases as well as in others, we used cerebral imaging techniques to make or confirm the diagnosis of EHS and/or MCS, as we and others have reported in previous studies[8,56-57]. To this end, we used ultrasonic cerebral tomosphygmography (UCTS), transcranial Doppler ultrasound (TDU), cerebral functional magnetic resonance imaging and/or positron emission tomography (data not shown). As previously emphasized, these imaging investigations were systematically performed in case of negative routine biomarker results and carefully interpreted.

2.7. STATISTICAL ANALYSIS

Four different statistical tests were used: i) The two-tailed Student's t-test, for comparison between patients values; ii) the chi-squared test for analyzing different frequency distributions; iii) the Pearson's product-moment correlation test to search for correlation between two quantitative variables; and iv) the Kruskal–Wallis test to assess whether samples originate from the same distribution. All statistical analysis were performed using the XLSTAT software (XLSTAT 2018.1.49725; Addinsoft; https://www.xlstat.com).
Considering the fact that the two-tailed Student's t-test was used to perform two comparisons (MCS patients versus EHS patients and EHS/MCS patients versus EHS patients), the Bonferroni correction was applied, which sets the α cut-off of significance at 0.05/2, i.e. 0.025. On the other hand, the statistical analysis using the chi-squared test used a cut-off value of $\alpha=0.05$.

3. Results

3.1. ROUTINE BIOMOLECULAR TESTS USED IN HEALTHY SUBJECTS

The 10 biomarkers selected by our initial approach were tested in 80 apparently normal subjects to test the validity of the normal limit reference range values used by the laboratory in which all samples were analyzed. All subjects entered into this control study had no EHS and/or no MCS clinical symptoms and a normal UCTS. Globally we confirmed the validity of the normal reference range values used by this laboratory. Table 1 summarizes the normal limit reference values recommended by the laboratory which were validated by the analysis of our control group.

3.2. BIOMOLECULES MEASUREMENT IN EHS AND/OR MCS PATIENTS

We routinely used the 10 different peripheral blood and urine biomolecules listed in Table 1.

3.2.1. Low grade inflammation

An increase in hs-CRP levels was found in about overall 15 % of the cases, more precisely in 14.9%, 15 % and 15.7%, of the EHS, MCS and EHS/MCS groups respectively without significant difference (Table 2).

Table 2. Frequency of presumed low-grade inflammation measured in the peripheral blood of patients with EHS and/or MCS as indicated by hs-CRP, histamine, IgE and HSP chaperone proteins.

Biomolecules Normal Values	Evaluable cases above normal						p*	p**	p***
	EHS		MCS		EHS/MCS				
	Ratio	%	Ratio	%	Ratio	%			
hs-CRP < 3 mg/l	185/1245	14.9	12/80	15	67/441	15.7	0.99	0.93	1
Histamine < 10 nmol/l	455/1332	34.2	21/80	26.25	122/481[b]	25.4	0.18	**0.0004**	0.97
IgE < 100 U/ml	255/1278	20	14/80	17.5	102/456	22.4	0.69	0.30	0.41
HSP 70 < 5 ng/ml	131/821	16	7/57	12.3	52/350	14.9	0.58	0.69	0.76
HSP 27 < 5 ng/ml	159/780	20.4	8/56	14.3	61/331	18.4	0.35	0.51	0.57
HSP27 and/or HSP70	205/776	26.42	12/56	21.4	54/313	17.25	0.51	**0.001**	0.57

We used the Pearson's Chi-squared test for distribution comparison.
*Comparison between the EHS and MCS groups. ** Comparison between the EHS and EHS/MCS groups.
*** Comparison between the EHS/MCS and MCS.

These increased Hs-CRP mean level values did not differ significantly between the three groups of patients (Table 3). We thus systematically looked for unrelated causes of inflammation/infection in these patients, and did not find any. Furthermore, since hs-CRP is considered to be a biomarker of age-related cognitive decline or dementia, and more particularly of Alzheimer's disease[58-59], we systematically looked for Alzheimer's disease in these patients. In three cases, Alzheimer's disease was discovered during the follow-up of the patients. This may be possibly due to previous excessive EMF exposure[12-13], but this was not proven as specifically related to EHS. We thus considered these three cases as non-evaluable cases. Our data thus suggests that in each of the different groups a limited number of patients may display some type of systemic inflammation.

Table 3. Mean level values of low-grade inflammation-related biomolecules in the peripheral blood of patients with EHS and/or MCS.

Biomarker Normal Values	EHS Mean±SE	MCS Mean±SE	EHS/MCS Mean±SE	*p* *	*p* **	*p* ***
hs-CRP < 3 mg/l	8.48+/-0.92	5.66+/-0.33	9.10+/-1.41	0.44	0.72	0.31
Histamine < 10 nmol/l	24.49+/-1.04	18.87+/-2.29	20.83+/-1.49	0.25	0.09	0.59
IgE < 100 U/ml	416.13+/-31.95	304.64+/-97.49	312.99+/-26.31	0.42	0.06	0.91
HSP 70 < 5 ng/ml	8.11+/-0.23	6.93+/-0.36	7.88+/-1.19	0.24	0.63	0.09
HSP 27 < 5 ng/ml	7.65+/-0.18	7.26+/-0.22	7.21+/-0.16	0.64	0.23	0.91

SE: standard error. p: probability that difference is due to random variation.
*Comparison between the EHS and the MCS groups of patients for mean level values using the two-tailed t-test ($\alpha = 0.025$). **Comparison between the EHS and the EHS/MCS groups of patients for mean level values using the two-tailed t-test ($\alpha = 0.025$). ***Comparison between the EHS/MCS and the MCS groups of patients for mean level values using the two-tailed t-test ($\alpha = 0.025$).

We also found a decrease in Vitamin D level in the peripheral blood of a majority of patients. Tables 4 and 5 depict the results we have obtained for Vitamin D. As indicated in Table 4 mean Vitamin D level values were found to be decreased in all groups of patients without significant difference.

Table 4. Mean Vitamin D levels in the peripheral blood of patients with EHS and/or MCS.

Normal Values	**EHS** Mean±SE	**MCS** Mean±SE	**EHS/MCS** Mean±SE	***p*** *	***p*** **	p ***
Vitamin D >30 ng/ml	20.22+/-0.22	18.95+/-0.86	19.88+/-0.39	0.14	0.44	0.32

SE: standard error. p: probability that difference is due to random variation.
*Comparison between the EHS and the MCS groups of patients for mean level values using the two-tailed t-test (α = 0.025). **Comparison between the EHS and the EHS/MCS groups of patients for mean level values using the two-tailed t-test (α = 0.025). ***Comparison between the EHS/MCS and the MCS groups of patients for mean level values using the two-tailed t-test (α = 0.025).

However, as indicated in table 5, this decrease in Vitamin D levels was found in about 66%, 82% and 62% of cases in the EHS, MCS and the combined groups respectively, with a significantly more number of patients with decreased vitamin D level in the MCS than in the EHS and the combined groups for which we found no significant difference. This suggests that chemical exposure induces a more frequent deficiency in vitamin D than exposure to EMF does.

Table 5. Frequency of abnormal Vitamin D level values in the peripheral blood of patients with EHS and/or MCS.

	Evaluable cases below normal						**p***	**p****	**p*****
	EHS		**MCS**		**EHS/MCS**				
	Ratio	%	**Ratio**	%	**Ratio**	%			
Vitamin D >30 ng/ml	858/1293	66.36	61/74	82.43	291/467	62.30	**0.006**	0.13	**0.001**

We used the Pearson's Chi-squared test for distribution comparison.
* Comparison between the EHS and MCS groups. ** Comparison between the EHS and EHS/MCS groups.
*** Comparison between the EHS/MCS and MCS.

Increased level of circulating IgE was detected in 20%, 17.5% and 22.4% of the EHS, MCS, and MCS/EHS groups respectively, with no statistically significant difference between the three groups (Table 2). For IgE, the mean increased values were not significantly different between the three investigated groups (Table 3). This was not associated with the coexistence of unrelated allergy. Since histamine release from mast cells involves the high affinity of IgE with its membrane receptor situated at the surface of mast cells[29,60], we searched for a correlation between histamine and IgE levels in the peripheral blood of the patients. However, such a correlation was not found.

A major finding in our study is that histamine in the peripheral blood was found to be increased overall in 32% of the cases, i.e. in 25 to 34% of the patients, depending of the investigated group, meaning that this pathological abnormality concerned all groups of patients. This was confirmed by evidencing that for the patients having such increased values the increased mean peripheral blood level values did not significantly differ between the 3 groups (Table 3). However, we found a significant greater number of patients with increased histamine levels in the EHS group than in the combined group; while the frequency of patients with increased histamine levels in the MCS group tends to be less frequent than in the EHS group but with no significant difference.

As indicated in Table 2, depending on the group considered, increased levels in HSP70 and HSP27 chaperone proteins were detected in the peripheral blood in 12%–16%, and 14%–20% of the cases respectively; with no significant difference for frequency between the 3 groups. However, collectively, i.e. for the 17–26% of the patients with increased levels of HSP70 and/or HSP27, there was a significantly more number of patients with either or both increased chaperone proteins in the EHS group than in the combined syndrome group, but there was no significant difference between the MCS group and the EHS group, and between the MCS group with the combined syndrome group (Table 2). For the patients with either HSP70 or HSP27 increase, the increased mean level values were not different between the three groups (Table 3). Therefore these data which show that the acquired histamine-related low-grade inflammation is more frequent in the EHS than in the EHS/MCS group of patients indicate that EHS is associated with a more frequent HSP70-27-related

anti-inflammation response while this response is less frequent in the MCS and the combined groups of patients, which show no difference for frequency between these two last groups.

3.2.2. Blood brain barrier disruption/opening

As emphasized above, proteins S100B and NTT have been shown to be presumably indicators of BBB disruption/opening[32-37]. We aimed to look for a statistical correlation between protein S100B and NTT in 1487 evaluable cases, we used the Pearson's product-moment correlation test, and found that protein S100B and NTT were weakly but nevertheless positively correlated one each other (p-value: $6.55.10^{-22}$ and rho: 0.2459).

As indicated in Table 6, the levels of circulating protein S100B were found to be increased overall in 25% of the investigated cases, i.e. in 23.2 to 26.9 % of the patients, with no significant difference between the three groups. Similarly, an increase in free or protein combined NTT blood levels was found in about 26% of all investigated patients, i.e. overall in 25.6 to 27.7% of the cases, with no statistical difference between the three groups.

Moreover, Table 7 indicates that for NTT and protein S100B we found no significant difference in mean level values between the three groups.

Table 6. Frequency of abnormal S100B and NTT values in the peripheral blood of EHS and/or MCS patients.

Normal Values	**Evaluable cases above normal**						**p***	**p****	**p*****
	EHS		**MCS**		**EHS/MCS**				
	Ratio	%	**Ratio**	%	**Ratio**	%			
S100B < 0.105 µg/l	318/1370	23.2	22/83	26.5	131/487	26.9	0.58	0.12	0.99
NTT >0.3 and < 0.9 µg/ml	270/1034	26.1	18/65	27.7	117/458	25.6	0.89	0.86	0.82
Increased NTT and/or S100B	354/923	38.3	24/49	49	169/405	41.7	0.18	0.27	0.41

We used the Pearson's Chi-squared test for distribution comparison.
* Comparison between the EHS and MCS groups. ** Comparison between the EHS and EHS/MCS groups. *** Comparison between the EHS/MCS and MCS.

Table 7. Mean level values of S100B and NTT in the peripheral blood of EHS and/or MCS patients.

Normal Values	**EHS** Mean±SE	**MCS** Mean±SE	**EHS/MCS** Mean±SE	***p*** *	***p*** **	***p*** ***
S100B < 0.105 µg/l	0.20+/-0.02	0.20+/-0.03	0.22+/-0.03	0.94	0.55	0.75
NTT >0.3 and < 0.9 µg/ml	1.44+/-0.07	1.30+/-0.12	1.32+/-0.04	0.63	0.31	0.85

SE: standard error. p: probability that difference is due to random variation.
*Comparison between the EHS and MCS groups for mean level values by the two-tailed t-test (α = 0.025). **Comparison between the EHS and EHS/MCS groups for mean level values by the two-tailed t-test (α = 0.025). ***Comparison between the EHS/MCS and MCS groups for mean level values by the two-tailed t-test (α = 0.025).

Finally, it appears that increased levels of protein S100B and/or NTT can be detected in 38 to 49% of the cases, without significant difference between the three groups, when one or the other or both indicators are measured (Table 6). This confirms our previous publication having shown that in addition to increased histamine levels increased levels of these two biomolecules may reflect major change in the peripheral blood of patients with EHS and/or MCS[7]. Since, as indicated above, protein S100B and NTT are potentially indicators of BBB disruption/opening, we consider that such BBB permeability may be detected in 40 to 50% of the cases, regardless the EHS and/or MCS etiopathogenic presentation.

3.2.3. Anti-myelin P0 autoimmune response

As indicated in Table 8, we detected autoantibodies against myelin P0 in 14% to nearly 17% of the patients, with no statistically significant difference between the three investigated groups; suggesting that, in these patients, EHS and/or MCS

are associated with some type of autoimmune response against myelin P0. Additionally, it has been shown that autoimmune process may result from long-term exposure to man-made EMFs[61].

Table 8. Number and percentages of EHS and/or MCS patients with positive test for myelin P0 protein autoantibodies detection.

	Evaluable cases above normal						p*	p**	p***
	EHS		MCS		EHS/MCS				
	Positive Ratio	Positive %	Positive Ratio	Positive %	Positive Ratio	Positive %			
Auto-antibodies against myelin P_0 protein (qualitative test)	204/1204	17	10/77	13	66/446	14.8	0.37	0.29	0.68

We used the Pearson's Chi-squared test for distribution comparison. * Comparison between the EHS and MCS groups. ** Comparison between the EHS and EHS/MCS groups. *** Comparison between the EHS/MCS and MCS groups.

3.2.4. Production of 6-Hydroxymelatonin Sulfate in urine

6-OHMS and creatinine were measured in the 24-hour urine samples in all evaluable patients from the three EHS, MCS, and EHS/MCS groups. Overall, 86-92% had a decrease in both the 6-OHMS and the 6-OHMS/creatinine ratio, without statistically significant difference between the three groups; suggesting that these patients have a decrease of melatonin-related antioxidant defenses[62-63]; thus, they may be at higher risk of NOS-associated chronic diseases. Since it has been shown that decreased melatonin production is associated with sleep disturbances[64], this may explain why EHS and/or MCS patients may also present sleep disturbance[10]. However, in about 8-14% of cases an increase in 6-OHMS levels in urine, and consequently in the 6-OHMS/creatinine ratio were observed in the three groups of patients with no significant difference. We have presently no clear explanation of this increase in 6-OHMS urine production. It may be due to an unexplained EMF and/or chemical-induced melatonin over-production by the pineal gland, and/or a consequent increase in NOS-related melatonin consumption.

3.2.5. Overall Biomolecular change detection

Figure 1 summarizes the overall results obtained from the routine use of the investigated biomolecules, in the EHS and MCS and in the combined syndrome groups. In this analysis, we did not consider hs-CRP, vitamin D and IgE because of their extreme lack of specificity. Figure 1 reports the percentage and number of cases according to the number of the seven characteristic biomolecules investigated. As it was expected, not all abnormal changes were found in the patients, some having only one abnormal change detected, others two or more. According to the Kruskal-Wallis rank sum test with a $p=0.96$, the distribution is the same in the three groups of patients considered. A majority of cases had one detected peripheral blood abnormal change, while depending on which group of patients considered, 14 to 24% of the cases had no abnormal level of biomolecules detected; meaning that in such cases measurement of these routine indicators was not sufficient to biologically characterize EHS and/or MCS.

3.2.6. Symptomatic presentation without abnormal biomolecular change

The above-described biochemical investigation has objectively characterized EHS, MCS and the combined syndrome in 76-86% of the cases (Fig. 1). No particular different clinical symptoms could be identified to distinguish the 14 to 24 % non-contributing cases (i.e., cases with normal biomolecules level values) from the 76-86% cases with biologically characterized EHS and/or MCS (data not shown). However, as indicated above (section 2.6), brain neurotransmitter measured in urine and cerebral imaging were used, to nevertheless provide the diagnosis of EHS and/or MCS in such non contributive cases[8] (section 8 3.2.7.- Table 9).

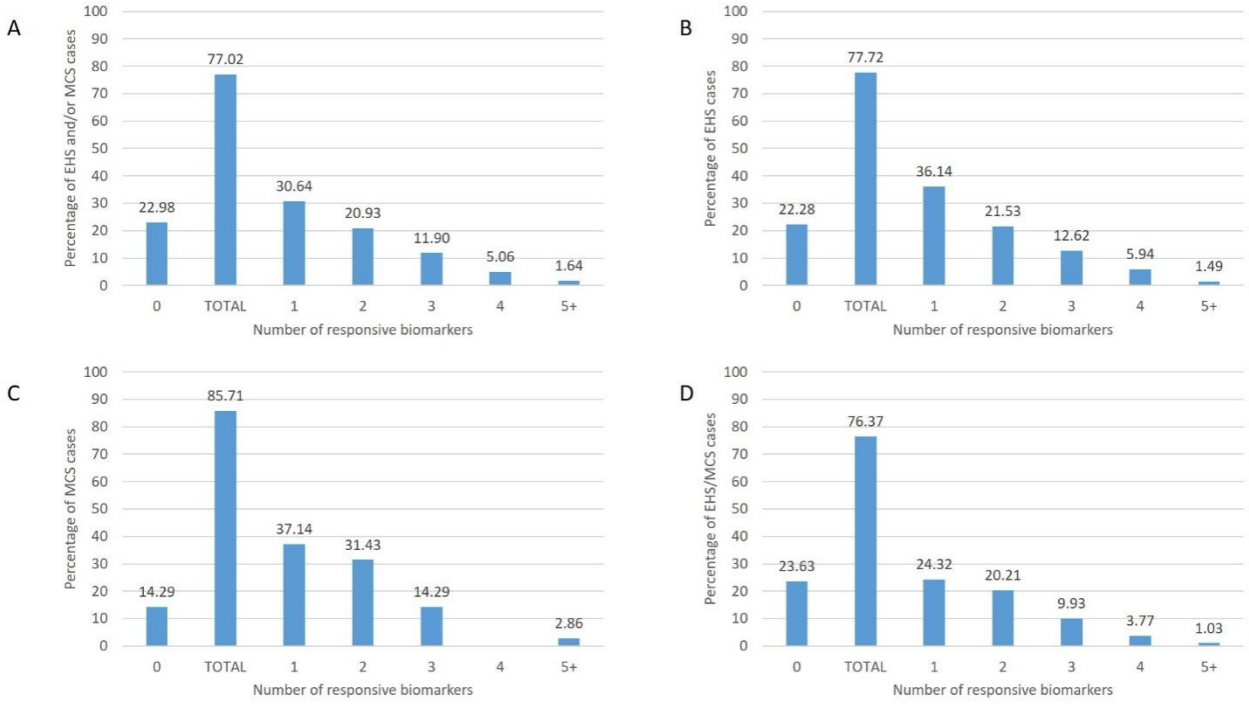

Figure 1. Percentages and numbers of EHS and/or MCS patients (total number=672) according to the number of indicators detected (included histamine, 6-OHMS, HSP 27/70, autoantibodies to myelin P0, S100B and NTT) in the overall population studied (A), in the EHS group (B), in the MCS group (C) and in the EHS/MCS group (D). The bar called "total" corresponds to the percentage of patients with one or several detectable indicators. The bar called 0 corresponds to the percentage of patients with no detectable abnormal level values of biomolecules.

3.2.7. Neurotransmitters urine detection

Table 9 summarizes our data. We have shown for the first time that EHS is associated with changes in the normal levels of cerebral neurotransmitters in urine; and that various abnormal neurotransmitter profiles may exist among patients, i.e. that levels of neurotransmitters differ from one patient to another; a finding for which we have presently no clear explanation, but which overall means that EHS is a brain disorder[8].

Table 9. Ratio and Percentage of EHS patients with altered levels of various neurotransmitters and their metabolites in urine[8].

Neurotransmitter	Patients	Percentage (%)
3-4 DOPAC decrease	18/42	43
Dopamine increase	17/42	41
Adrenaline decrease	12/42	28
Noradrenaline increase	11/42	26
Adrenaline increase	8/42	19
Serotonin decrease	5/42	12
Serotonin increase	4/42	9.5

3-4 DOPAC: 3,4-Dihydroxyphenylacetic acid.

4. Discussion

Using the aforementioned internationally-recognized Atlanta criteria for MCS and similarly, WHO-recognized criteria for EHS, we have provided for the first time a biological analysis of EHS and/or MCS, and show that these two disorders can be characterized objectively by identical biological abnormalities. In particular we showed that whatever their etiopathogenic presentation, EHS, MCS and the combined syndrome are associated with a decreased production of 6-OHMS in the 24 hour urine in 86-92% of the cases, while in the peripheral blood there is an increase in histamine and HSP 27 and/or 70 in about 30% and 20% respectively, and an increase of protein S100B and/or NTT in about 40 to 50% of cases. Increased histamine and HSP are presumably indicators of low grade inflammation, while increased protein S100B and NTT are presumably indicators of BBB disruption/opening. In addition, we were able to detect antimyelin P0 autoantibodies in 15-17% of the cases meaning that these disorders are also associated with an autoimmune response. This means that such peripheral blood and urine biomolecular indicators can objectively characterize these disorders.

There are three points to discuss: A major point is whether the biomolecules we have investigated can be considered as disease biomarkers and thus may help diagnosis making, treatment assessment and follow-up of patients. Second, we would like to discuss how our data may shed some light on the hypothesis of a common etiopathogenic mechanism for EHS and MCS. Finally, there are some limitations of our study to discuss.

4.1. ARE THE BIOMOLECULES INVESTIGATED BIOMARKERS WHICH CAN HELP DIAGNOSIS AND FOLLOW-UP OF PATIENT?

A symptomatic clinical approach is necessary but quite insufficient to make objective diagnosis of any disease including EHS and/or MCS. The identification and measurement of reliable biomarkers is indeed a critical preliminary clinical step for identifying and characterizing disease. Surprisingly, such a bioclinical objective was not previously considered in setting-up diagnosis criteria to identify EHS and/or MCS[65]. Hence there is a need for using complementary suitable biomarkers and imaging techniques, as was recently confirmed in the scientific multinational consensus report on EHS and MCS that we have recently initiated[66].

In our study, we found that usual standard blood tests in EHS and/or MCS patients were typically normal; with the exception of non-specific biological thyroid and liver dysfunctions in several cases[67]. These latter might be causally associated with EMF exposure or other causes, rather than being biological characteristics of EHS or MCS (data not shown). We therefore searched for characteristic biomolecules from previously reported in vitro and in vivo experimental data obtained from EMF- or chemical-exposed animal studies or from human clinical reports (section 3.4), and chose some molecules which could be measured routinely and repeatedly for a suitable characterization of the patients with EHS and/or MCS (Table 1).

Indeed, the choice of biomolecules used in our study and their role as biomarkers deserve discussion. Biomarkers for clinical practice and research have been defined by the Biomarker Definition Working Group of the US National Institute of Health (NIH)[24], and more recently by the Food and Drug Administration (FDA)-NIH biomarker Working group[25], as objectively measurable indicators that can be routinely and repeatedly used to assess disease presentation and evolution. In patients for whom they can be evidenced, biomarkers can help not only establish objective diagnosis of diseases, but also provide assessment of therapeutic interventions, and follow up of patients[10].

There are in fact three arguments justifying that the biomolecules we have investigated can be considered as biomarkers. First, as indicated in this study, we have observed that changes on levels of these biomolecules were associated with the EHS and/or MCS clinical presentation that we had previously shown to be similar in the three individualized groups of patients, whereas levels of these biomolecules were normal in all the healthy individuals we have so far investigated as controls. Second, we have previously shown that in EHS patients, histamine, protein S100B and HSP27-70 increased levels were significantly correlated with response to treatment (the use of Fermented Papaya Preparation)[68], i.e. that a normalization of level values of these biomolecules could be obtained when we observed disappearance of all clinical symptoms (complete response); whereas increased level values of these biomolecules persisted or even were growing, in case of therapeutic failure (progressive or stable response). Thirdly, we were able to diagnose a clinical relapse in cases of EHS and combined syndrome during the follow-up of patients mainly by using repetitive measurement of protein S100B, which appears to be the best biomarker to follow such patients (data not shown).

Therefore, it appears that the medical use of biomarkers investigated in our study can contribute

to the identification of EHS and/or MCS as disorders, but not as a mean to establish their causal origin, nor their (hyper)sensitivity-associated properties[69]. They can however help diagnose these disorders objectively and follow-up patients. In addition, they can provide insight into some aspects of their pathophysiological process.

4.2. TOWARD A COMMON ETIOPATHOGENIC PROCESS

On the basis of this biological study, including our neurotransmitter findings, we confirm that EHS and MCS are objective brain somatic disorders[7,8,70]. Indeed, contrary to other reports[71-72] these disorders cannot be hypothesized to be of pure psychologic or psychiatric origin, nor be considered as a vague undefined functional impairments[69-70]. To the contrary as we have previously shown they may be caused by EMF and/or chemical exposure[9,10]. This does not exclude however that risk factors such as trauma or infectious diseases may help trigger the occurrence of these disorders[73], but these risk factors should not be confused with the man-made EMF and/or environmental chemicals as direct primary causes or with the types of intolerance associated with these disorders.

As reported above, we show in the present study that EHS and MCS and their combination are characterized by identical biological abnormalities, meaning that symptoms occurrence may share a common pathophysiological mechanism triggered by exposure to EMF and/or environmental chemicals. This interpretation is supported by our observation of the association of EHS with MCS in the same patients in the framework of a common pathological syndrome[7,74]. Thus not just EHS but MCS should be studied clinically and biologically, to clearly identify EHS and enlighten the presumed common pathophysiological mechanism involved in both disorders.

A major finding from our study is that histamine seems to play a critical role both in EMF- and/or chemicals-induced pathological changes, since it is found increased in the peripheral blood of about 30% of EHS and/or MCS patients. This molecule plays a critical pathophysiological role as a neurotransmitter in the brain. For example, neuronal histamine has been shown to be involved in the sleep cycle, motor activity, synaptic plasticity, and memory[27,75-78]. As we have previously shown[9,10,79] all these functions have been found to be affected in EHS and/or MCS patients. Furthermore, this molecule is not just a neurotransmitter produced by and released from the central nervous system (CNS), but is also an inflammatory mediator produced by and released from mast cells in allergic disorders[27], and otherwise in non-allergic neuro-inflammatory processes[29].

Mast cells are critical regulators in the pathogenesis of CNS diseases[80-81], and brain mast cells are involved in BBB disruption/opening and may contribute to local cerebral changes due to the release of angiogenic mediators stored in their granules.

By using S100B and NTT biomarkers, we showed the possibility of BBB disruption/opening in about 40-50% of patients whatever the EHS, MCS and combined EHS/MCS investigated groups of patients.

There is evidence that anthropogenic (i.e. man-made) EMFs can disrupt/open the BBB and consequently render the brain unprotected from blood toxic substances[82-85]. This has particularly been shown experimentally in rats exposed to anthropogenic EMF[86-90]. Furthermore, by using imaging techniques we have previously shown that EHS may be associated with several cerebral vascular changes[57]. The present finding confirms other previously reported data showing that the glia-derived S100B protein may be not only a biomarker of BBB opening/disruption, but also a marker of hypoperfusion-associated brain damage/dysfunction[91,92]. This is not specific since it has been shown to occur particularly in neurodegenerative diseases such as Alzheimer's disease[93] and amyotrophic lateral sclerosis[94].

On the basis of the present study, and on our previous study showing that EHS is associated with NOS in 70-80% of cases[95], we propose a hypothetical pathophysiological model in which the presumed deleterious role of EMF and/or chemicals on the brain involves two main steps: a) *a primary local NOS-related inflammatory step* (with histamine and/or angiogenic mediators release from mastocytes and/or from other reactive proliferating glia cells); and b) *a secondary NOS-related neuro-inflammatory amplification step*, with BBB disruption/opening and transmigration of circulating inflammatory cells into the brain.

4.3. STUDY LIMITATIONS

There are several limitations to our study. First, we did not correlate the biological presentation of these disorders with a simultaneous measurement of EMF and/or chemical exposure. This resulted in the inclusion of patients into the different groups on the basis of previous internationally recognized clinical criteria, but not on objective measurements of specific intolerance to environmental stressors. In

addition, although we have interviewed and physically examined all included patients, we did not measure systematically EMFs and/or chemical exposure of patients, so we were not able to evidence objectively any biological change related to low level exposure to EMFs and/or chemicals which are characteristic properties of EHS and MCS. Note that these two study limitations were due to the present ubiquitous and multiform pollution of our environment which renders any distinctive measurement of the environmental stressors involved in each patient quite impossible.

Furthermore, whatever the three investigated groups, by using routine tests we were unable to detect any abnormal biological change in 14-24% of cases which however could not be clinically distinguished from cases with biological changes. We have however proved these non-contributing cases to be true EHS and/or MCS on the basis of their similar clinical picture, TDU and UCTS imaging and neurotransmitter urine analysis. We have presently no clear explanation. They may involve other as yet unknown released inflammatory molecules. Also, the biomarkers we used routinely (notably histamine) may be not released into the blood from the tissues where they are locally produced: they may be labile, making them not detectable at the time of testing.

Except for the decreased 6-OHMS urine production in about 90% of cases, all biomolecular changes we were able to measure were detected in a relatively limited number of cases. Consequently, one should consider that it is not only one biomarker in particular which should be used to objectively diagnose these disorders, but several of them.

Finally, we would like to discuss the sensitivity, specificity and reproducibility of the biochemical tests we have used for all biomarkers investigated (Table 1). Regarding specificity, a major consideration is that all biomarkers investigated are not specific; since for example, increase in histamine, protein S100B and NTT, and anti-myelin P0 autoantibodies in the peripheral blood, and decrease in 6-OHMS production in urine are biological changes found in many chronic neurodegenerative diseases, or disorders[96-97]. For sensitivity, as indicated in Fig 1, the use of these biomarkers was not able to identify and characterize EHS and/or MCS cases in 14 to 24% of the cases. Yet we have emphasized the utility of brain neurotransmitters analysis and of cerebral imaging techniques to diagnose objectively such non contributive cases[8,57]. As to reproducibility, these biochemical tests remain to be reproduced by other laboratories, as our results were provided by a single biological laboratory in Paris (France). Overall, we must emphasize that the sensitivity and specificity of the methodology used should be improved by the use of other biomarkers.

5. Conclusion

We have shown that EHS, MCS and the combined syndrome can be objectively characterized in about 90% of the cases by a decrease in the production of 6-OHMS in the urine; and in about 30% and 20% by increased values of histamine and of HSP27 and/or HSP70, respectively; and of protein S100B and/or NTT in 40-50% in the peripheral blood. This suggests that these disorders, whatever their etiopathogenic presentation, may be associated with both low-grade inflammation (increased histamine and HSP27/70) and BBB opening/dysfunction (increased protein S100B and/or NTT). In addition, we found a profound decrease in vitamin D in the peripheral blood of patients as well as antimyelin P0 autoantibodies in about 15% of patients, indicating an autoimmune response in such cases.

In summary, we have shown that for patients with increased values, the increased mean values of all biomarkers investigated do not differ between the three EHS, MCS and EHS/MCS groups of patients, meaning that these pathological disorders are similar not only clinically[74] but on the basis of their biological change. There are however several differences between the three groups for the frequency of patients having such abnormal level values. For Vitamin D, the decrease was found to be significantly more frequent in the group of EHS patients than in the other groups. This suggests that this alteration may be mainly caused by chemicals. For histamine, we found that its increase was significantly more frequent in the EHS group than in the EHS/MCS combined group, a result which is coherent with the fact that the EHS group was found to be associated with a significantly increase in HSP 27-70 anti-inflammatory response in comparison with the other groups. For protein S100B and NTT we found no significant difference in frequency of involvement in the three groups, meaning that these biomarkers are indicators of these disorders whatever their etiopathogenic presentation.

Finally, it is concluded from our study that measurement of biomarkers may contribute to objective diagnosis, therapeutic assessment, follow-up of patients and etiopathogenic understanding. This opens a new way for research in EHS and/or MCS. Further research is however needed, to validate the pertinence of such a combined clinical and biological approach.

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