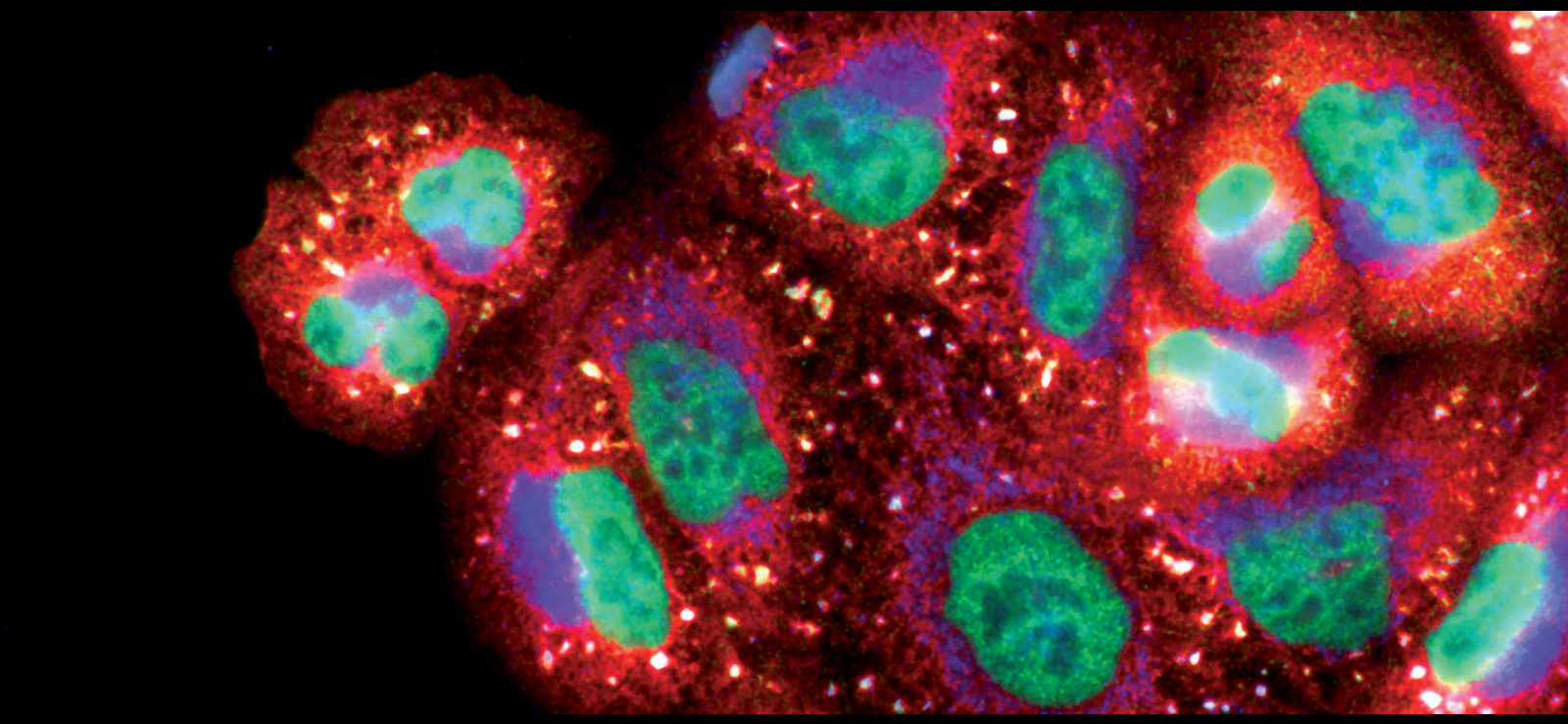
Oxidative Medicine and Cellular Longevity

Innovative Approaches in Environmental Medicine: Redox/Detoxification Biomarkers in Environmental Intolerances

Guest Editors: Giuseppe Valacchi, Daniela Caccamo, Edward Pelle, and Chiara De Luca



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Editorial Board

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Editorial

Innovative Approaches in Environmental Medicine: Redox/Detoxification Biomarkers in Environmental Intolerances

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The increase of old-and-new pollutants heavily conditions quality of civil and working life. Concern about the exponential increase of both allergic and nonallergic environmental sensitivity-related illnesses (SRI) has brought into focus a new inhomogeneous cluster of adverse—often socially and professionally disabling—clinical multiorgan conditions, still lacking a satisfactory nosologic classification. These comprise the prototypical multiple chemical sensitivity, along with fibromyalgia, chronic fatigue syndrome, sick building/house syndrome, hypersensitivity to electromagnetic fields, and other syndromes, commonly so far considered as "idiopathic" by medical community. They are elicited by exposure to low-to-negligible doses of diverse, multiple, environmental-borne physical, chemical, and biological triggers, innocuous to the general population, that is, xenobiotic chemicals, drugs, and metals, electro-magnetic or nuclear radiations, and new iatrogenic factors like biocompatible implants, specific food, or microbial allergens.

With this special issue we attempted the challenging effort to gather experts in the field as to contribute to the scant but constantly growing laboratory and epidemiologic studies highlighting common molecular features of SRI, on a genetic or metabolic base. The still open issues here addressed were (a) identifying distinctive biologic and clinical effects of different categories of environmental/nutritional stressors that may be relevant to better describe molecular mechanisms and natural history of these emerging disease conditions, (b) searching reliable biomarkers of diagnostic value for thorough disease classification, and (c) evaluating all available evidence-based information to produce suitable clinical protocols applicable for prevention and treatment, in both the civil and working population at risk of environmental hypersensitivities.

Within this frame, the first two papers contributed significant *in vitro* pieces of evidence on the biological efficacy of very low-dose electromagnetic radiation in cell structure and function derangement. E. Calabro' et al. described lipid and protein alterations in SH-SY5Y neuroblastoma cell membranes, with mitochondrial transmembrane potential alterations and significant loss of cell viability, whilst F. Cervellati et al. addressed the often raised issue of teratogenic risks, demonstrating that 17-β-estradiol modulates connexins and integrins, and ER-β expression induced by high-frequency electromagnetic fields, with hypothesized consequences on trophoblast-derived HTR-8/SVneo cells differentiation and migration. J. V. Gruber and R. Holtz evidenced molecular effects of three commonly used skin chemical lighteners

on keratinocytes and melanocytes, especially highlighting the role of ferritin and iron in skin melanization. The following two papers evidenced the role of exogenous nutritional stressors in epigenetic and metabolic alterations leading to obesity. A. Muňoz and M. Costa reported on the updated knowledge about epigenetic changes measurable in both adipocyte and peripheral blood mononuclear cells by chronic inflammatory stimuli deriving from hypercaloric diet and specific prooxidant nutritional components, that may be selectively counteracted by antioxidant-rich foods like Mediterranean or fish/vegetable-based dietetic regimens. The paper by C. Lubrano et al. reviewed the more and more compelling pieces of evidence linking obesity with endocrine disruptors of environmental origin, focusing on the relevance of individually-impaired detoxification of organic chemicals and heavy metals.

In line, the importance of metal sensitivity in multiple chemical sensitivity was addressed by P. Pigatto et al. in the clinical-diagnostic setting, with a study demonstrating the prevalence of mercury traces in biological matrices from subjects with MCS bearing dental amalgams, producing convincing indications on the validity of noninvasive *in vivo* and *ex-vitro* testing of metals for the diagnosis of MCS. A common leading feature of SRI is undoubtedly the severe imbalance in the capability to modulate and detoxify endogenous and environmental-driven free radical formation, accompanied by specific profiles of altered inflammatory cytokines, of detoxifying and antioxidant capacities, and of oxidative stress markers currently under intense observation (i.e., genotoxic aldehydes, 4-hydroxynonenal, isoprostanes, etc.). The Italian group who previously first described a specific combination of metabolic alterations of diagnostic value for multiple chemical sensitivity here contributed, with the paper by P. Caccamo et al., to the strongly debated question of genetic alterations in MCS and fibromyalgia, showing a significant prevalence of gene mutations of phase I metabolizing genes and environmental-sensing receptors in patients suffering MCS, fibromyalgia, and/or chronic fatigue syndrome symptoms, which is possibly also useful for differential diagnosis among SRIs.

Hopefully, the results presented in this issue concerning the complex, uncompleted SRI models, which stand far away from classical toxicology approaches, will have contributed innovative solutions applicable to environmental toxicology and medicine, through modern comprehensive protocols of genomic, epigenomic, and metabolomic diagnostics, complying to good practice criteria, validated for clinical use. In association with toxico- and pharmacogenomics, these are bound to offer a solid rationale for still-to-come, evidence-based individualized therapy of SRI based on antioxidant/chelator/natural immunomodulating treatments.

Papers gathered in the issue will hopefully stimulate the ongoing efforts to identify specific biomarkers of environmental hypersensitivity to be measured in the clinical setting, especially applicable for professionally borne environmental-connected disorders, which are here extensively approached in the paper by A. Martini et al. They may in perspective also provide mechanistic insights, prognostic and therapeutic indicators, and nutritional/lifestyle recommendations for other recognized, difficult-to-cure, and chronic inflammatory conditions with suspected environmental-borne etiological cofactors, like atopic or autoimmune skin pathologies or metabolism diseases.

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Clinical Study

Allergological and Toxicological Aspects in a Multiple Chemical Sensitivity Cohort

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Background. Multiple chemical sensitivity (MCS) is a chronic condition characterized by an exaggerated response to toxicants. We ascertained the prevalence of allergy to metals and toxicological aspects in MCS patients. *Methods*. We conducted a retrospective review of medical records of 41 patients with MCS. We performed patch testing ($n = 21$) for dental series and did lymphocyte transformation test ($n = 18$) for metals. We measured mercury in samples of blood ($n = 19$), urine ($n = 19$), saliva ($n = 20$), and scalp hair ($n = 17$) to investigate the association between mercury levels and cases of MCS. *Results*. The prevalence of metal immune hypersensitivity in a subset of 26 patients was 92.3 percent. Elevations of mercury occurred in 81.2 percent (26 of 32). The mean (±SD) in blood concentrations of mercury was 7.6 ± 13.6 μg/L; mean in urine was 1.9 ± 2.5 μg/L; mean in scalp hair was 2.2 ± 2.5 μg/g; mean in saliva was 38.1 ± 52.1 μg/L. Subgroup analyses showed that elevation of mercury levels in biological matrices were associated with mercury amalgams in patients with MCS (22 patients), compared with controls (8 patients) (odds ratio 11 : 95 percent confidence interval 1.5 to 81.6; $P = 0.023$). *Conclusions*. Our data show an increased prevalence of metal allergy and elevation of mercury levels in bioindicators among patients with MCS.

1. Introduction

Multiple chemical sensitivity (MCS)—also termed idiopathic environmental intolerance (IEI)—is a chronic condition characterized by an exaggerated body response to chemical toxicants, especially organic solvents [1–6]. The symptoms experienced and reported by patients are usually of the respiratory, musculoskeletal [7], and gastrointestinal tracts [8–12]. Symptoms of hyperosmia [13] of the brain are commonly described in patients with MCS when exposed to chemical substances [14, 15]. Thus, it has long been thought that MCS has a strong environmental component [16–20]. The prevalence of MCS is reported to range from 10 to 15 percent in the general population [21, 22] and its pathogenesis remains elusive. Numerous mechanisms have been implicated in the etiologic process of MCS, including *N*-methyl-D-aspartate (NMDA) sensitization [23, 24], peroxynitrite and nitric oxide elevation [24, 25], oxidative stress [26, 27], proinflammatory cytokines [26, 27], altered redox enzymes [26, 27], cytochrome P450 metabolism [26–28], hypoxia [29], serotonin receptors [30], neural sensitization [17, 31–35], and neurogenic inflammation [36]. As a consequence, various studies have suggested that persistent symptoms of MCS impaired health-related quality of life in these patients [37, 38]. And there is evidence that mercury exposure may cause symptoms that clinically overlap with MCS. To test this hypothesis,

we conducted a retrospective cohort study to determine (i) whether patients with MCS had detectable levels of mercury due to chronic environmental metal exposure and (ii) whether patients with MCS had allergy to metals since we hypothesized a priori that these exposures might play a causal role in MCS. Our aim, therefore, was to measure the levels of mercury in human biological matrices. We also sought to determine the outcome of allergic reactions to metals by using *in vivo* patch testing and/or *in vitro* lymphocyte transformation test (LTT).

2. Materials and Methods

2.1. Study Oversight. We evaluated 41 consecutive patients who had been referred for MCS (39 women and 2 men); mean [±SD] age of the patients at their first study visit was 44.8 ± 11.2 years. To study metal immune hypersensitivity, we did *in vivo* patch testing for dental series in 21 subjects, and we also did *in vitro* lymphocyte transformation test (LTT) for 20 metal allergens in 18 case subjects. We analyzed for concentrations of total mercury (elemental, inorganic, and organic mercury) in samples of peripheral whole blood, urine, saliva, and scalp hair and we tested for an association between alterations in the levels of mercury and MCS by means of inductively coupled plasma mass spectrometry method and/or atomic absorption spectrometry. The total number of silver-mercury amalgam restorations was charted, and we recorded when amalgam restorations were placed in teeth in order to correlate approximately the median duration of dental amalgam exposure (Table 1). None of the patients had been occupationally exposed to mercury.

2.2. Definition and Clinical Diagnostic Criteria of MCS. MCS is characterized by adverse health effects related to—or exacerbated by—exposure to chemical substances. The most common chemicals triggers of MCS are organic solvents, metals, volatile organic compounds (VOCs), chlorine, drugs, perfumes, hairsprays, diesel exhaust fumes, and pesticides. MCS was defined by the presence of following MeSH code: D018777. Even though an accurate classification remains difficult, criteria for entry in our cohort were similar to those described by Cullen [1, 2, 39]. Of the 53 patients evaluated for the study, 41 were eligible and we excluded 12 patients (12 of 53, 41) who had reported a probable MCS but they did not meet the above classification criteria [1, 2, 39].

2.3. Immunological Assay and Immune Markers. Allergic sensitization to metals contained in mercury dental amalgam restorations was ascertained on the basis of patch testing done on the upper back. Most of the patients ($n = 21$) were patch tested with the Italian dental screening series (SIDAPA) obtained from Chemotechnique Diagnostics AB (Malmöe, Sweden). Readings were taken on day 2 (48 hours) and day 4 (96 hours). With regard to the pathophysiology of allergic reactions induced by metal allergens, they are most frequently mediated by type IV immune reactions (delayed-type hypersensitivity reaction), according to the Gell and Coombs classification. Patch testing was integrated with the lymphocyte transformation test (LTT), a noninvasive test *in vitro* to determine cell-mediated immunological responses to metals and metalloids [40]. In most of the patients ($n = 18$), we used a highly sensitive and optimized LTT method in human lymphocytes (LTT-MELISA® - Memory Lymphocyte Immune Stimulation Assay) [41]. Data were expressed as a stimulation index (SI), which was calculated from the quotient of test counts per minute (cpm) and the average cpm from three negative controls [40, 41]. No corticosteroids and/or immunosuppressive drugs were taken for at least two months before skin contact patch-test allergens and/or the LTT. No subjects had used nonsteroidal antiinflammatory drugs (NSAIDs), antibiotics, and H_1-anti-histamine agents within the previous three weeks before patch testing and/or the LTT. None of these medications were permitted for all routes of administration (enteral, parenteral, topical, and/or by inhalation), which may alter the immunological response to metals.

Table 1: Distribution of patients as stratified according to study cohort and the total number [mean (±SD)] of mercury-containing dental amalgam fillings.

Sex - no. (%)		
Female	39	95.1%
Male	2	4.9%
Patients with dental amalgam fillings	27	65.9%
Mean age	42.4 ± 10.5	
No. mercury amalgam fillings		
Mean	3.8 ± 2.7	
Patients without dental amalgam fillings	14	34.1%
Mean age	49.3 ± 11.7	

2.4. Sampling and Mercury Analysis of Biological Specimens. We have measured total mercury concentrations in whole blood samples and urine samples in 19 of 41 patients (46.3%) with MCS (Table 3). At the time of admission, fasting morning venous peripheral whole blood samples (4 milliliters) were collected in mercury-free polypropylene tubes containing potassium EDTA (K_2-EDTA), as an anticoagulant. First morning urine specimens (100 milliliters) and/or 24-hour urine collection specimens were obtained and stored at +4°C until mercury analysis. All blood and urine samples were delivered immediately to the laboratory of toxicology for mercury analysis and were processed within 24–72 hours after collection. Concentrations of total mercury in whole blood, urine, and chewing gum-stimulated whole saliva samples in our cohort were measured by cold vapor atomic absorption spectrometry (CVAAS) [42, 43] and/or ICP-MS (inductively coupled mass spectrometry) methods [43]. The lower limit of detection (LD) for total mercury in both blood and urine was 0.05 micrograms per liter. The intraassay and interassay coefficients of variation, determined at various concentrations, were 2 percent and 5 percent, respectively. External and internal quality-control procedures were made. We also determined total mercury in head hair in a small subgroup of 17 subjects (17 of 41, 41.4 percent), and human scalp

hair samples were taken from the occipital region of the head with sterile stainless steel surgical scissors. Each specimen of head hair was collected from the first three centimeters (3 centimeters hair segments) in length next to the scalp, and it was weighed. The overall mean [±SD] weight of the hair specimens was approximately 220 ± 110 milligrams. Mercury hair analysis is proper indicator medium for determining organic mercury, and it reflects exposure that occurred during the last few months. Total mercury in strands of scalp hair was measured with ICP-MS system. The detection limit (DL) of mercury in scalp hair was 0.07 micrograms per gram [44]. To avoid loss of mercury in head hair, none of the 17 patients received hair dyes three months before the hair sampling procedure. Toenails samples were collected from only one subject to confirm the level of exposure to mercury (data not shown). To examine whether there were significant elevations in total mercury levels in saliva, we collected stimulated saliva specimens after chewing a sugar-free gum for 5–10 minutes before collection, and subsequently mercury content was quantified by atomic absorption spectrometry (AAS) [45]. The operational lower limit of detection (LD) for mercury in saliva was 0.1 micrograms per liter [46]. To assess average fish or seafood consumption, we used a semiquantitative food-frequency questionnaire, which included distinct questions about other dietary variables (i.e., alcohol and—as contribution to the total caffeine intake—coffee, tea, and chocolate). We also estimated modifiable risk factors (i.e., smoking) for MCS among patients, using data derived from interviews, questionnaire [47], and the medical records. All subjects gave oral and written informed consent. This retrospective observational case series study was conducted from 2001 to February 5, 2013, which was the cutoff date for analyses of all cases of MCS. The corresponding author gathered the data and vouch for its accuracy.

2.5. Interventions. Our team developed a new method to remove mercury amalgam fillings by using the *en bloc technique* [48]. Treatment method includes complete dental amalgam removal. It is good clinical practice to avoid unnecessary overexposure to mercury vapor during the removal of dental amalgam [48]. The dentist was well trained and performed at least 80 mercury amalgam removal annually.

2.6. Statistical Analysis. All statistical tests, performed with the use of SPSS software, version 19, were two sided, and a P value of less than 0.05 was considered to indicate statistical significance. The statistical tests used were chosen after confirmation of the distribution of normality of the sample with the Kolmogorov Smirnov test. Comparison of continuous variables between the two groups was conducted with the use of the Mann-Whitney U test for variables with a non-normal distribution. Chi-square tests were used for the comparison of categorical variables and the Fisher's exact test was used where appropriate. The association between two casual variables has been detected with the Correlation Pearson (r) test. All data are expressed as means ±SD.

Table 2: Distribution of 41 cases of multiple chemical sensitivity in the Italy cohort. The MCS cohort contained 39 (95.1 percent) women and 2 (4.9 percent) men.

Regions of Italy	Patients no.	% of total
Lombardy	23	56.10
Emilia-Romagna	4	9.75
Lazio	5	12.19
Trentino-Alto Adige/Südtirol	3	7.32
Veneto	3	7.32
Apulia	1	2.44
Campania	1	2.44
Calabria	1	2.44

3. Results

3.1. Patients. The total of 41 patients with MCS were of white race. Patients were of Caucasian adult origin and were identified as Italy-born persons. Most patients were from Northern Italy (80.5 percent), Central Italy (12.2 percent), and Southern Italy (7.3 percent) (Table 2). The mean age of patients with MCS was 44.8 ± 11.2 years; 95.1 percent (39 patients) were female and 2 were men (Table 3). Female mean age was 45 ± 11.3 years and men mean age was 36 years. Details of marital status are shown in Figure 1. The acquisition of MCS was largely associated with female sex; female to male ratio was 19.5 : 1.

3.2. Prevalence of Allergy to Metals among Patients with MCS. In the cohort of patients screened with both patch testing and the lymphocyte transformation test (LTT), the cumulative prevalence of allergic sensitization to metals in 26 patients (26 of 41, 63.4 percent) was 92.3 percent. In order to compare the frequency of allergy to metals using the two methods, patch testing for dental series was assessed in 21 of our series of 41 patients (51.2 percent). Positive allergic patch test reactions to metal allergens were noted in 17 of 21 participants (80.9 percent). The prevalence of positive reactions to metals by the lymphocyte transformation test (LTT) was 94.4 percent (17 of 18). 5 patients (5 of 18, 27.7 percent) refused patch testing for contact allergy procedures to establish possible sensitization to dental materials because of contact with metal allergens and the subsequent risk of flare-up of MCS symptoms, therefore, they choose to use another laboratory test: an *in vitro* testing, the lymphocyte transformation test (LTT). In 13 patients (13 of 18, 72.2 percent) sensitization to metals was reported with the use of both methods—skin patch testing and the LTT. When assessed according to mercury-compound allergens by patch testing with a dental screening series (in aggregate; metallic mercury, ammoniated mercury, thimerosal, phenyl mercury, and mercury dental amalgam, all of which in petrolatum) and/or the lymphocyte transformation test (LTT), allergy to mercury was diagnosed in 13 patients of 26 (50 percent). We also found that 22 patients of 26 (84.61 percent) had allergy to other metallic components of dental amalgam, which is the metal-matrix alloy of dental amalgam. By excluding mercury compound allergens,

Table 3: Mean [±SD] total mercury concentrations in biological matrices (whole blood, urine, saliva, and scalp hair).

Characteristics	Total cohort with MCS patients		Concentrations of total mercury in biological matrices micrograms/liter	Reference range micrograms/liter
	41			
Sex - no. (%)				
Female	39	95.1%		
Male	2	4.9%		
Age at diagnosis of MCS				
Mean	44.8 ± 11.2			
Range (min–max)	25–65			
Total cases of Hg in blood	19			
Mean			7.6 ± 13.6	≤2.0
Total cases of Hg in urine	19			
Mean			1.9 ± 2.5	≤2.0
Total cases of Hg in saliva[§]	20			
Mean			38.1 ± 52.1	≤2.7
Total cases of Hg hair analysis	17			
Mean			2.2 ± 2.5	≤2.0[*]

[§]Total mercury was measured in gum-stimulated saliva samples.
[*]Total mercury in scalp hair was expressed in micrograms per gram.

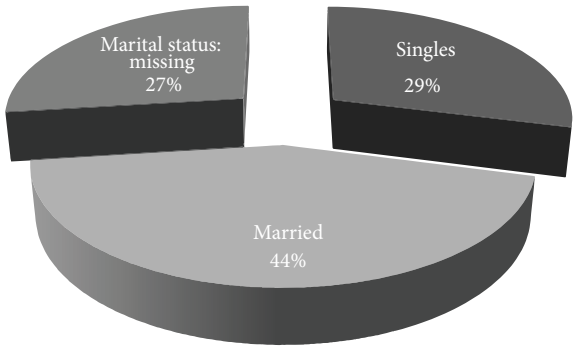

Figure 1: Marital status of the cohort of MCS patients.

the most common metal immune hypersensitivity reactions associated with amalgam metal-matrix alloy were—listed in decreasing order of frequency—nickel, cadmium, palladium, gold, chromium, and silver.

3.3. Mercury Analyses. Of 19 patients who could be evaluated, in 19 of 41 (46.3 percent) the mean mercury whole blood levels was 7.6 ± 13.6 micrograms per liter (range from 0.5 to 59.4 micrograms per liter); normal range from 0 to 2. Of 19 patients considered, in 19 of 41 (46.3 percent), the mean of urine mercury levels was 1.9 ± 2.5 micrograms per liter (range from 1 to 10 micrograms per liter); normal range from 0 to 2. Of 17 patients evaluated, in 17 of 41 (41.5 percent), the mean of total mercury accumulated in scalp hair was 2.2 ± 2.5 micrograms per gram (range from 0.06 to 8.45 micrograms per gram); normal range from 0 to 2. Of 20 patients evaluated, 20 of 41 (48.8 percent), the mean of salivary mercury levels in chewing gum-stimulated whole saliva was 38.1 ± 52.1 micrograms per liter (range from 0.1 to 168 micrograms per liter); upper limit value <2.7 (Table 3). In a subgroup analysis of 24 patients, 22 patients with mercury dental amalgam fillings (91.7 percent), levels of total mercury in human biological matrices (in aggregate; whole blood, urine, scalp hair, and saliva) correlate with the total number of mercury dental amalgam tooth fillings, as compared with 4 patients (50 percent) without mercury dental amalgams (odds ratio, 11; 95 percent confidence interval, from 1.5 to 81.6; $P = 0.023$) (Table 4). In a subgroup of 27 (27 of 41, 65.8 percent) patients with MCS who carry dental amalgam fillings, we also observed a strong correlation between the number of mercury-containing dental amalgam fillings and the concentrations of total mercury in urine ($r = 0.71$, $P = 0.002$). No other variables were associated with a statistically significant increase in level of total mercury in biological matrices (including fish consumption).

3.4. Mercury Amalgam Tattoos and MCS. The prevalence of amalgam tattoo of human oral mucosa at the time of first visit was 17 percent (7 of 41), which was more than twice as high as the prevalence among a sample in the Swedish population in whom the prevalence of amalgam tattoo is remarkably high [49]. Intraoral mercury amalgam tattoos are known to have the highest levels of total mercury (inorganic mercury along with organic mercury) compared to any other tissues and organs in humans [50]. Previous estimates of the prevalence of amalgam tattoo ranging from 0.4 to 8.2 percent in the general population [49]. Amalgam tattoo should be surgically removed in case of allergy to mercury.

Table 4: Increased levels of mercury in biological matrices in patients with MCS who have mercury dental amalgams.

	Patients with mercury amalgam fillings	Patients without mercury amalgam fillings	Total patients	*P* value	Odds ratio
Number	24	8	32	0.023	11
Yes	22 (91.7%)	4 (50.0%)	26 (81.3%)		
No	2 (8.3%)	4 (50.0%)	6 (18.7%)		

In a subgroup analysis of 24 patients, 22 patients with dental amalgam (91.7 percent), levels of total mercury in biological matrices (in aggregate; whole blood, urine, scalp hair, and saliva) correlate with the number of mercury dental amalgam tooth fillings, as compared with 4 patients (50 percent) without dental amalgams (odds ratio, 11; 95 percent confidence interval: from 1.5 to 81.6; $P = 0.023$).

Table 5: Adverse health effects occurring in 33.3 percent of patients (11 of 33) who underwent mercury dental amalgam removal without adopting safety measures.

Patient no.	Sex	Age at diagnosis	No. of total mercury amalgam fillings	Reported adverse events	Allergy to metals*	Blood mercury levels μg/L	Urine mercury levels μg/L	Scalp hair mercury levels μg/g	Saliva mercury levels μg/L
1	F	52	PR‡	Fever of unknown origin	Thimerosal	0.5	0.5	0.06	NA**
2	F	31	2	Asthma attacks	Nickel	2.7	0.7	NA	0.7
3	F	27	2	Asthma attacks	Thimerosal nickel	59.4	4	8.2	1.3
4	M	36	PR	Tunnel vision, trigeminal neuralgia, metallic taste	Inorganic mercury	NA	NA	0.2	0.2
5	F	39	PR	Hyperosmia, asthma attacks	NA	NA	NA	NA	NA
6	F	29	5	Fever of unknown origin	NA	1.1	0.3	NA	2.4
7	F	61	2	Atypical facial pain	NA	NA	NA	2.8	2
8	F	31	PR	Fatigue, muscle pain	Nickel	NA	NA	NA	NA
9	F	58	1	Facial paresthesia, metallic taste, ocular inflammation	NA	7.6	0.5	4.03	0.7
10	F	47	2	Vertigo, asthma attacks, pricking pain in arms	NA	2.7	0.5	1.3	NA
11	M	36	2	Fatigue, muscle pain/weakness	Inorganic mercury	3.5	0.5	NA	9.2

*Allergy to mercury: patients were tested with skin patch testing and/or lymphocyte transformation test (LTT).
‡PR: previously removed, number of mercury amalgam undefined.
**NA: not analyzed.

3.5. Mercury Dental Amalgam as a Risk Factor. 41 patients with MCS were evaluated for appropriate treatment of adverse events to dental materials, particularly to exposure to dental metal alloys (i.e., mercury-containing dental amalgam fillings). 27 of 41 patients (65.9 percent) had mercury dental amalgam fillings and 14 of 41 (34.1 percent) did not have mercury amalgam tooth fillings. The average number of mercury amalgam restorations of persons in the cohort who carry dental amalgam was 3.8 ± 2.7, ranging from 1 to 10. In 18 of 27 (66.6 percent) patients with mercury amalgam, the mean duration of exposure to mercury amalgam tooth fillings was 25.1 ± 9.5 years before the onset of definite clinical manifestations of micromercurialism after long-term exposure to mercury dental amalgam.

3.6. Adverse Events after Mercury Amalgam Removal. A total of 11 patients (11 of 33, 33.3 percent) of the cohort reported having had at least one major adverse outcome related to dental amalgam removal without safe procedures (Table 5). All 11 patients underwent dental amalgam removal at the various dental centers. Clinical manifestations and adverse outcomes that were considered to be related to toxic effects of acute overexposure to mercury vapors during amalgam-removal treatment were as follows: dysgeusia (metallic taste), constriction of the visual fields (tunnel vision), trigeminal neuralgia, atypical facial pain, burning mouth disorder (BMS), cervical lymphadenopathy, axillary lymph nodes enlargement, bronchial hyperresponsiveness and asthma attacks, skin rashes (salmon-colored and/or pink'rash), headache, lightheadedness, weight loss, vertigo, muscle pain/weakness, fatigue, fever of unknown origin (FUO-body temperature $\geq 37.5^{\circ}$C). The adverse events reported in each patient are listed in Tables 5 and 6. In the Table 6, these adverse events were associated with long-term exposure to mercury amalgam and/or due to high levels of mercury vaporization emitted during amalgam removal by standard drill-out method, which is no longer recommended [48].

3.7. Treatment and Mercury Amalgam Replacement. Elemental and inorganic mercury's biological half-life is rather

Table 6: New and classical systemic signs and symptoms associated with mercury exposure among MCS patients cohort.

Signs	
(i) Angioedema	N
(ii) Cervical and axillary lymph nodes swollen	N
(iii) Dermographism	C
(iv) Enlargement of thyroid	C
(v) Eyelid myokymia (eyelid tremors)	N
(vi) Gastrointestinal malabsorption	N
(vii) Gingivitis - Stomatitis	C
(viii) Lichenoid contact stomatitis	N
(ix) Low-grade fever (fever of unknown origin—FUO)	N
(x) Muscle atrophy	N
(xi) Muscle fasciculations	C
(xii) Non-allergic rhinitis/vasomotor rhinitis-like	N
(xiii) Peripheral neuropathy	C
(xiv) Salmon-colored and/or pink' rash	C
(xv) Sialorrhea (hypersalivation)	C
(xvi) Spasms	C
(xvii) Systemic contact dermatitis	N
(xviii) Tremors (upper limb, hands, fingers, face, eyelids, and lips)	C
(xix) Urticaria	N
(xx) White matter hyperintensity (by brain MRI)	N
(xxi) Xerostomia (dry mouth)	C
Symptoms	
(i) Abdominal cramps	N
(ii) Anorexia	C
(iii) Atypical facial pain (persistent idiopathic facial pain)	N
(iv) Burning mouth syndrome (BMS)	N
(v) Burning pain (neuropathic)	C
(vi) Chemical odor intolerance	N
(vii) Chest pain (anterior or posterior, on the left side)	N
(viii) Confusion	C
(ix) Depression	C
(x) Dysesthesia	N
(xi) Fatigue	C
(xii) Flu-like symptoms	N
(xiii) Headache	C
(xiv) Insomnia	N
(xv) Intestinal movement disorders	N
(xvi) Intolerance to odors	N
(xvii) Itching (neuropathic)	N
(xviii) Muscle weakness	C
(xix) Nausea	C
(xx) Noise sensitivity	N
(xxi) Paresthesia	C
(xxii) Photophobia	N
(xxiii) Recurrent infections	N
(xxiv) Short-term memory disturbances	C
(xxv) Tachycardia	C
(xxvi) Thermal regulation disorders (low cold tolerance)	N
(xxvii) Trigeminal neuralgia	N
(xxviii) Vertigo	C

The table lists signs and symptoms triggered by mercury amalgam exposure and also noting those defined as "new" and "classical" signs and symptoms related to exposure to mercury amalgam in a cohort of patients with MCS. (N) and (C) denote "new" and "classical" signs and symptoms.

protracted and ranged from 30 to 90 days, averaging 60 days [51]. Most patients who develop MCS following exposure to mercury amalgams show improvement within one year (mean time to resolution = 6 months), after the removal of the remaining mercury dental amalgams. Usually, best results appear to be achieved one year after the last dental amalgam replacement.

Table 7: Recommended threshold levels in matrices for biological monitoring of total mercury in humans after complete mercury dental amalgam removal. Very low level of mercury in bioindicators are able to reverse clinical manifestations as well as abnormal laboratory values associated with mercury amalgam exposure.

Mercury levels	Threshold limit values	Unit of measurements
Total Hg in scalp hair	≤0.5	micrograms/g
Total Hg in whole blood	<1.5	micrograms/L
Total Hg in serum	<1.5	micrograms/L
Total Hg in plasma	<1.5	micrograms/L
Total Hg in urine	≤1.0	micrograms/L
Total Hg in saliva	≤0.5	micrograms/L
Total Hg in breast milk	≤0.5	micrograms/L
Total Hg in nails	≤0.5	micrograms/g
Total Hg in intraoral cavity	≤1.5	micrograms/m^3

3.8. Outcomes. 16 of 41 (39 percent) patients with MCS were treated by our dental team, in accordance with the *en bloc technique*, in which we do not touch the mercury amalgam filling with tungsten burr [48]. The level of whole blood total mercury, the level of urinary total mercury, and the concentrations of total mercury in saliva all decreased significantly—fell below measurable levels—following mercury amalgam-replacement within 6 to 12 months in 6 patients. After a mean follow up of 41.3 months (ranged from 13 to 130 months), at the end of total mercury amalgam removal, 10 of 16 (62.5 percent) patients reported that their symptoms had improved markedly (>50 percent), according to the patient's subjective assessment. In 37.5 percent (6 of 16) the condition of these patients improved only moderately (<50 percent) after receiving dental amalgam replacement (Table 7). The patients with allergy to metals had the better prognosis and control of symptoms. No side effects were reported with a safe and effective dental amalgam removal.

3.9. Body-Mass Index and MCS. The body-mass index—the weight (kg) divided by the square of the height (m)—did not differ significantly in MCS patients. The overall average body mass index (BMI) of 38 female patients and 2 male patients with MCS was 21.3 ± 3.28 (range min–max: 17–30).

3.10. Hormonal Risk Factors and MCS. A subgroup of 13 subjects (13 of 41, 31.7 percent) showed hormone disorders, and—of the patients who could be evaluated—an elevated serum prolactin level was detected in 4 of 13 patients (30.8 percent), which means that the endocrine system was found to be deregulated. This corroborates other research suggesting that

there was a positive association between the increase in circulating serum prolactin levels and exposure to mercury. 2 of 4 of these patients had allergy to mercury (metallic mercury and mercury dental amalgam, resp.), and they had elevated levels of mercury in saliva samples (17.6 and 49.6 micrograms per liter, resp.). 5 of 13 patients (38.5 percent) had hypothyroidism. 3 of 13 patients (23.1 percent) had hyperthyroidism. Adrenal gland disorders were seen only in one female patient.

3.11. MCS, Fibromyalgia, Chronic Fatigue Syndrome, and EHS. Chronic fatigue syndrome (CFS) as well as fibromyalgia were the most common coexisting conditions (fibromyalgia: 11 of 41, 26.8 percent; CFS: 11 of 41, 26.8 percent, resp.). Of 27 patients evaluated, in 14 of 27 (51.9 percent), electromagnetic hypersensitivity symptoms (EHS) were self-reported.

3.12. Demyelinating Disorders and MCS. Two patients (2 of 41, 4.9 percent) received a diagnosis of demyelinating disorders within a few months either before or after the diagnosis of MCS.

3.13. Dietary Variables and MCS. In 27 patients of 41 (66 percent) the mean consumption level of fish and/or seafood was 1.7 (8.5 oz) fish serving meal per week. It has been reported that foods and beverages (alcoholic) may alter the level of exposure to elemental mercury (Hg^0) emitted from amalgams in humans. Of 32 patients evaluated, in 11 of 32 (34.4 percent) the level of alcohol consumption per week was 2.43 ± 1.9. Of 32 patients evaluated, 20 of 32 (62.5 percent), the average of coffee consumption per week was 14.7 ± 7.7. Of 32 patients evaluated, 10 of 32 (31.2 percent), the mean number of tea intake weekly was 3.1 ± 2.5. Of 27 patients evaluated, 11 of 27 (40.7 percent) patients reported that consumption per week of chocolate was 1.1 ± 1. The role of dietary factors (i.e., alcohol, coffee, and tea) in the development of MCS remains to be elucidated.

3.14. Smoking Status. Of the 35 subjects who were evaluated for smoking, 4 of 35 (11.4 percent) were current tobacco smokers, 9 were former smokers (25.7 percent), and 22 were nonsmokers (62.9 percent).

3.15. Other Laboratory Features. It has been suggested that mercury is able to induce a hematologic immunotoxicity. To address this, we estimated total white blood cells (WBCs) count as a subclinical index of mercury immunotoxicity. Of 19 patients who could be evaluated, 6 of 19 (31.6 percent), the average of white blood cells (WBCs) was $5.9 \pm 1.5\ 10^8$ per liter. There was no evidence of the reduction in leukocyte count. We also tested plasma homocysteine concentrations as a subclinical index of mercury vasculotoxicity. Of 15 patients evaluated, 7 of 15 patients (46.7 percent), the average of serum level of homocysteine was borderline low at $13.8 \pm 2.7\ \mu$mol per liter; normal level, <14. The immunoglobulin E (IgE) level was mildly increased in 16 patients assessed, 6 of 16 (37.5 percent), the average of IgE was 350.1 ± 181.9 UI per milliliter (normal range 0–100 UI per milliliter). These results

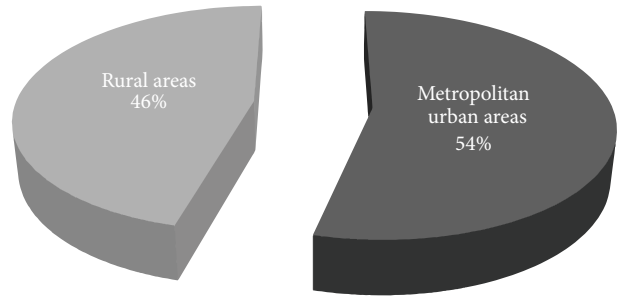

Figure 2: MCS in urban and rural areas.

are consistent with previous studies that showed a role of mercury in immune activation, enhancing B-cell IgE polyclonal production. Biochemical changes, including changes in the serum (liver) alanine aminotransferase (ALT) levels and aspartate aminotransferase (AST) levels (the latter is more reliable indicator for mercury-induced liver injury), were also not significantly different from normal range in our cohort (data not shown). No significant changes in lead blood concentrations (possible plumbism) were found in 10 (10 of 41, 24.3 percent) patients, and no interaction between mercury and lead blood levels was seen in these patients (data not shown).

3.16. Rural-Urban Differences and the Prevalence of MCS. 22 of 41 (53.6 percent) of MCS patients were located in large metropolitan urban areas, where exposure to fine-particulate air pollution has been associated with increased morbidity. 19 of 41 (46.3 percent) were patients who live in rural areas (Figure 2). There was no a statistical difference in the number of patients living in urban compared with rural areas. In two urban Milan (Italy) sites, we measured real-time ambient air mercury vapor concentrations (outdoor Milan atmospheric Hg^0) and was approximately 50 nanograms per cubic meter, and it was detected by cold vapor atomic fluorescence spectroscopy (CVAFS). Outdoor air levels of mercury in urban area were considerably lower than those released from dental amalgam into the oral cavity in a case series of our patients with mercury amalgam fillings (i.e., 50 nanograms per cubic meter *versus* 15–30 micrograms per cubic meter, during mastication).

3.17. Exposure to Pesticides. Some studies have previously highlighted a link between pesticides exposures and the development of MCS [20, 22, 24, 52–55]. We were surprised to see that some of our patients applied in-home and/or outdoor pesticides at their residence, even after the diagnosis of MCS.

3.18. Exposure to Pets. Of 28 patients interviewed about lifetime indoor pet exposure, 10 of 28 (35.7 percent) reported exposure to pets, mainly cats (90 percent).

3.19. Exposure to Solvents and MCS. Thirty-nine percent (16 of 41, 39 percent) of the cohort reported having had adverse events to organic solvents. In some instances, we witnessed

episodes of clinical important aversive response to organic solvents involving respiratory system (bronchial hyperreactivity and asthma attacks) and central nervous system (vasovagal response: fainting and/or near-fainting, dizziness, vertigo, low blood pressure, and tremor) triggered by acute (short-term) inhalation exposures to organic solvents. Of the 28 patients who could be evaluated, (28 of 41, 68 percent) chemical odor intolerance (i.e., hyperosmia, cacosmia, and dysosmia) was self-reported in 24 of 28 (85.7 percent). In some patients the perception of chemical odor (i.e., perfumes, fragrances, hairsprays, cigarette smoke, and diesel fumes) persists some days after exposure.

3.20. Dietary Supplements and Antioxidant Supplements. Professionals usually recommended supplements as a supportive therapy to reduce mercury burden. In theory, these supplements would bind and detoxify mercury that can be deposited in parenchymal pattern, reestablishing and maintaining the hepatic glutathione stores (i.e., *N*-acetylcysteine (NAC), selenium, and reduced glutathione, GSH). By contrast, there is no documented evidence either in animal model or in outcome studies that support therapy with supplements is able to remove mercury from human tissues [56]. Our observations raise the possibility that treatment with vitamins, minerals, and antioxidants did not ameliorate the symptoms in most patients with MCS. In particular, we suggest that important clinical adverse events associated with oral supplements should be carefully evaluated when these nonspecific supportive therapies are prescribed to persons who have a clear history of mercury amalgam exposure, for example, ascorbic acid and thiol (sulfur derivatives) agents. Vitamin C (ascorbic acid) was not able to mobilize and remove mercury from tissues in both human and animal studies [56, 57]. Rather, some antioxidants worsen the retention kinetics of mercury in patients exposed to dental amalgam. 5 of 41 (12.2 percent) patients received secondary supportive therapy: 3 patients received selenium, 1 patient received alpha lipoic acid (ALA), and 1 received *N*-acetylcysteine (NAC). Adverse health effects occurred in 3 of 5 (60 percent). In two (2) of them, supplementation with selenium has been implicated in an elevation of the levels of serum antinuclear antibodies (ANAs) and one patient developed severe major aphthous stomatitis, whereas panic attacks were associated with the oral administration of alpha lipoic acid (ALA). 7 of 10 patients who have received intravenous (iv) administration of reduced glutathione (GSH) by continuous infusion, 5 of 7 (70 percent) patients had various adverse events while receiving GSH, including urticaria, asthma attacks, worsening of MCS symptoms, and cheilitis.

3.21. Chelation Therapy and MCS. Chelating agent-related toxic effect was reported to us by one patient who underwent a tentative treatment of mercury-chelation therapy. This patient, one of 41 (2.4 percent) received calcium EDTA (ethylenediaminetetraacetic acid—EDTA) by intravenous (iv) continuous infusion [57]. This approach with chelating agents for "detoxification" of mercury was suggested by other physicians, and in our view, it should be used with great caution. Chelation and mobilizing agents are usually contraindicated in patients with mercury-containing dental amalgam fillings [58, 59].

3.22. Interactions between Mercury and Other Metals

3.22.1. Mercury and Chromium. In our accumulated clinical experience, chromium allergy and/or chromium exposure appear to confer a considerable susceptibility to mercury exposure. The relation between such biochemical interaction in humans is currently unclear. In our case series of 41, in 4 of 26 patients the point prevalence of allergy to chromium was 15.4 percent.

3.22.2. Mercury and Titanium. We also observed a potential interaction between mercury amalgam restorations and endosseous dental titanium implants among 4 patients in our group with MCS (9.8 percent, 4 of 41). In these 4 patients, we have noted important neurological adverse events (i.e., persistent idiopathic facial pain).

3.22.3. Mercury and Gold. Both metallic mercury (Hg^0) and gold may elicit autoimmunity in humans and experimental animals. Some early studies suggest that after the removal of mercury dental amalgam serum antinuclear antibodies (ANAs) frequently reverted to negative [60].

4. Discussion

A surprisingly very high prevalence of allergic sensitization to metals (92.3 percent) was detected among 26 of 41 patients with MCS in our cohort. Consistent with our findings, other studies have reported a high prevalence of allergy among persons with a diagnosis of MCS [61]. In this study, patients with MCS had significantly higher prevalence of allergy and/or immune sensitization to metals with respect to the general population and were more likely to have both fibromyalgia (FM) and chronic fatigue syndrome (CFS) [62].

Combined skin patch testing and lymphocyte transformation test (LTT) were sufficiently sensitive and specific to provide important clinical guidance.

Importantly, mercury-containing dental amalgam filling was associated with increased odds of elevation of mercury in biological indicator media (blood, urine, saliva, and scalp hair). An unexpected finding of our study is the very high salivary mercury levels (mean 38.1 ± 52.1 micrograms per liter, threshold limit values <2.7) in MCS patients who have mercury-containing dental amalgam. It appears that saliva mercury levels were significantly higher in individuals affected by MCS, as compared with other cohorts. Mercury amalgam tattoo—the clinical hallmark of dental amalgam—was more prevalent among patients with MCS than the population-based prevalence (17 *versus* 8 percent) [49]. The potential toxicity of mercury-containing dental amalgam has been underestimated for a long time in patients with

underlying MCS. Nearly 33 percent of the patients were given a diagnosis of MCS after dental amalgam removal treatment, thus, there is a clear evidence that unsafe and inaccurate removal of mercury amalgam is a major risk factor for MCS. According to our experience and on the basis of data from the literature [63], many patients reported symptomatic improvement of MCS after complete removal of mercury-containing dental amalgam as well as other dental alloy restorations (especially palladium- and gold-based alloys) [63].

4.1. Mercury Toxicity. Mercury has no known biological role in normal human metabolism. In all of its chemical forms, mercury is able to increase the production of reactive oxygen species (ROS) and the subsequent oxidative stress, potentially causing DNA damage [64]. Mechanisms of mercury toxicity also include inactivation of enzymes (mainly sulfhydryl groups—SH), disruption of membranes, and altered neurotransmitters [56]. A well-known toxic endpoint of mercury is the immune system, in fact, immunotoxic effects on cytokines production (elevated serum interleukin-2 receptor) and autoimmune disorders have been described in the literature [56, 60]. Mercury amalgam is a direct toxicant but it is also a health hazard because of its conversion (biomethylation), owing to oral bacteria biotransformation from inorganic mercury to organic mercury [65]. Experimentally, three chemical species of mercury are present in saliva specimens in individuals carrying mercury-containing amalgam fillings: metallic mercury (Hg^0), inorganic mercury (mainly inorganic divalent mercury Hg^{2+}), and organomercury compounds (as mono methyl mercury—CH_3Hg^+—and ethyl mercury—$CH_3CH_2Hg^+$) [65–67]. Consequently, adverse events to inorganic and/or organic mercury content in saliva may involve both immune and non immune mechanisms.

4.2. Caveats about Mercury Biomonitoring. Whole blood and urine mercury levels are believed to be a reliable marker for recent exposure to inorganic and elemental mercury (Hg^0). Therefore, monitoring blood and urine is valuable for identifying patients with acute exposure to mercury. With ongoing exposures, however, tissue levels of mercury in humans are increased due to accumulation [68], especially in brain (pituitary gland and cerebral cortex), central nervous system, thyroid, and kidneys, as established in previous studies from postmortem examinations [59, 68, 69]. This may elucidate why monitoring blood concentrations of total mercury is of questionable clinical relevance as indicator of tissues body burden of mercury released from dental amalgam in humans. Consistently, preclinical studies have suggested direct evidence that low circulating mercury levels could reflect mercury disposition and redistribution to target organs, at least in adult sheep model [68]. Hence, concentrations of mercury in blood and urine may underestimate retention toxicity of mercury in the tissues and organs. In other words, there is the possibility that measurements of mercury in blood and urine do not fully reflect the actual mercury amalgam burden in humans [59].

4.3. Clinical Toxicology of Mercury. Patients with clinical signs and symptoms of unrecognized chronic mercury exposure from dental amalgam fillings—which is frequently overlooked on physical examination—are likely to have a misdiagnosis of postviral syndrome, endocrine disorders, or psychiatric dysfunction [4]. Therefore, due to a lack of specificity, particularly early signs of mercury toxicity, a delay in diagnosis of "micromercurialism" (also known as the "asthenic-vegetative syndrome") is common, as observed within our cohort. None of the 41 subjects in our present study received a diagnosis of celiac disease (or non-celiac gluten sensitivity) whose condition is associated with increased levels of mercury in both blood and urine [70]. A transient, acute overexposure to mercury vapors (Hg^0) released from mercury amalgam during drilling cannot rule out the likelihood of long-term health risks [48]. Of note, signs and symptoms caused by chronic exposure to various forms of mercury generated from amalgam are characterized by a very long-lasting latency period of more than 5 or 7 years, as previously investigated [71]. Excluding patients in whom the initiation of MCS symptoms was related to mercury amalgam removal (11 of 33, 33.3 percent), the median times of exposure to mercury amalgam restorations were approximately 20–25 years before the onset of adverse health outcome [72]. With regard to the chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME) and fibromyalgia conditions (FM), our results are similar to those reported previously, in which several small studies have documented an association between mercury amalgam exposure and CFS/FM [50, 73–76]. In most reported cases of CFS in patients in our series, the features of CFS and the "*asthenic-vegetative syndrome*" caused by inhalation of mercury vapor overlap significantly.

4.4. Electromagnetic Hypersensitivity: EHS. The causes of electromagnetic hypersensitivity are not clear and are probably multifactorial, however, we speculate that tissues and organs of the body respond to cellular retention and injury of mercury with inflammation (proinflammatory microenvironment). Exposure to electromagnetic field might increase mercury mobilization of metal ions from tissues, which in turn increases inflammation and oxidative damage to cellular level [77–81].

4.5. Mercury in Fish. Additional pathway of exposure to mercury in humans derived from fish consumption, mainly as organic mercury (CH_3Hg^+). But although we recognize the potential benefits of eating fish, we are concerned about the possible overlapping toxic effects of elemental mercury (Hg^0) emitted from dental amalgams and organic mercury exposure (CH_3Hg^+) from fish intake. Patients who already have mercury overload from amalgam fillings may be the least able to tolerate additional mercury burden from dietary fish intake and should not be further exposed to organic mercury from fish consumption during amalgam-treatment replacement [82].

4.6. Limitations of the Study. There are notable limitations to our study. We did not perform mercury assessments in

all the patients and the central limitation is the quantity of missing data. Further, being a retrospective study and as the sample size is fairly small (forty-one case subjects), it should be interpreted with caution.

4.7. Interpretation. Studies have documented the increased risks of adverse events associated with exposure to chemical substances among patients with MCS [16, 18–20, 83, 84]. A substantial proportion of our MCS patients (81.2 percent, 26 of 32) had an elevation in the total mercury levels at the time of the first study visit, and they correlated significantly with the total number of mercury-containing dental amalgam fillings. As far as we are aware, this is the first observation of quantitative assessment of the association between increased elevations of mercury in biological indicator media and risk of MCS. It is therefore reasonable to assume that exposure to mercury might contribute to the observed symptoms among patients affected by MCS [53, 54, 85] and that measurements of mercury levels may be clinically useful. It is also not inconceivable that previous exposure to organic solvents (and VOCs) may determine susceptibility to metals in humans, but the mechanisms are not clear. Further studies are needed to document that levels of mercury are increased among individuals with MCS and are able to trigger the disease process.

5. Conclusions

This study indicates that there is an increased prevalence of allergy to metals among patients with MCS, whereas a higher level of mercury in biological matrices is associated with the presence of mercury-containing dental amalgam fillings.

Conflict of Interests

The authors have no conflict of interests connected with this work.

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Research Article

50 Hz Electromagnetic Field Produced Changes in FTIR Spectroscopy Associated with Mitochondrial Transmembrane Potential Reduction in Neuronal-Like SH-SY5Y Cells

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SH-SY5Y neuroblastoma cells were used as an experimental model to study the effects of 50 Hz electromagnetic field, in the range from 50 μT to 1.4 mT. Fourier transform infrared spectroscopy analysis evidenced a reduction in intensity of the amide A band and a slight increase of vibration bands at 2921 cm^{-1} and 2853 cm^{-1} corresponding to methylene groups. A further increase of the magnetic field intensity of exposure up to 0.8 mT and 1.4 mT produced a clear increase in intensity of CH_2 vibration bands. Moreover, it has been observed some alterations in the amide I region, such as a shifted peak of the amide I band to a smaller wavenumber, probably due to protein conformational changes. These results suggested that exposure to extremely low electromagnetic fields influenced lipid components of cellular membrane and the N–H in-plane bending and C–N stretching vibrations of peptide linkages, modifying the secondary structures of α-helix and β-sheet contents and producing unfolding process in cell membrane proteins. The observed changes after exposure to 50 Hz electromagnetic field higher than 0.8 mT were associated with a significant reduction of cell viability and reduced mitochondrial transmembrane potential.

1. Introduction

Although 50/60 Hz EMF seems to not directly lead to genotoxic effects, it is possible that certain cellular processes altered by exposure to ELF-EMFs indirectly affect the structure of DNA, causing strand breaks and other chromosomal aberrations [1].

Several epidemiological studies reported a relationship between an increase of risk of cancer and the exposure to ELF-EMF. In particular three studies of the World Health Organization (WHO) on EMF evidenced possible health effects from exposure to static and ELF-EMFs [2–4].

Environmental Health Criteria (EHC) delineated the main objectives to review the scientific literature on the biological effects of exposure to ELF-EMFs to use this health risk assessment to make recommendations to national authorities on health protection programs.

EMFs are generated everywhere in our living environment by modern electrical systems such as power lines, electric generators and motors, electrical wiring, home electronic devices, and wireless communication systems [5].

The electric field inside the body is normally five to six orders of magnitude smaller than the external electric field, whereas the permeability of tissue relative to magnetic fields is the same as that of air, so that the field in organic system is the same as the external field. From this evidence many investigations emphasized the effects of magnetic field component with respect to those due to electric component effects.

In the close proximity of certain home appliances, the magnetic-field intensities can be as much as few hundred microteslas, whereas in some workplaces they can reach 10 mT, leading us to investigate the range of exposure to magnetic field around a few milliteslas.

The exposure of organic systems to ELF-EMF can interfere with some metabolic processes, modifying intracellular enzymatic pathways and causing an increase in the production of free radicals which may alter or interfere with DNA reparation or replication mechanisms and protein and lipid-containing structures [1].

The redox status can be abled to regulate gene expression determining changes in cell growth, differentiation, proliferation, and stress response.

Thus, EMF might be a stimulus to induce an activated state of the cell such as phagocytosis, which then enhances the release of free radicals, in turn leading to genotoxic events. EMF exposure can cause both acute and chronic effects that are mediated by increased free radical levels.

An increase in the lifetime of free radicals by EMF leads to persistently elevated free radical concentrations. In general, reactions in which radicals are involved become more frequent, increasing the possibility of DNA damage.

Several studies have shown no genotoxic effects after exposure to ELF-EMFs in several types of mammalian cells [6–9].

In contrast, other studies demonstrated genotoxic damage in various cell models [10–12]. In this regard, lymphocytes first exposed to the ELF field for 24 h at 80 or 800 μT showed significant increases in the frequency of micronuclei and apoptosis after incubation with different doses of vinblastine [13]. Other authors investigated the effect of ELF-EMF on the expression of heat shock proteins (HSPs). Hsp proteins are inducible after the cells have been exposed to a wide range of stress signals and have also been shown to be expressed at atypical levels in tumour cells or tissue. Such observations have led to suggestions that HSPs could be used as biomarkers for cellular stress in general.

The transcription of the heat shock genes hsp70 was shown to increase because of the exposure to a 60 Hz, 8 μT magnetic field [14]. Pipkin et al. [15] showed that inducible hsp70 (hsp70B) was overexpressed after 60 Hz ELF magnetic field exposure at 1 mT.

In neuroblastoma cells, no effects were found in nerve cells, but a decrease in the conductance of gap junction channels under exposure to 20 mA m-2 at 60 Hz and a significant increase in intracellular Ca^{2+} at current densities of more than 10 mA m-2 were found [16].

According to previous observations, in the present study the parameters of time exposures and EMF intensities were 5–24 hours and 50 μT–1 mT, respectively.

This study was aimed to investigate whether the exposure to electromagnetic fields at the frequency of 50 Hz may be considered as an environmental insult by examining mitochondrial transmembrane potential in SH-SY5Y neuroblastoma cells, differentiated to dopaminergic neuron-like cells. Further FTIR spectroscopy was used to study the relationship between conformational changes of protein and DNA structures and cell damage.

2. Materials and Methods

The human neuroblastoma cell line SH-SY5Y (CRL-2266) was purchased from the American Type Culture Collections (ATCC) (Rockville, MD, US). Fetal bovine serum (FBS), antibiotics, minimum essential medium (MEM) eagle (M5650), Nutrient Mixture F-12 Ham (M4888), all-trans retinoic acid (RA), sodium pyruvate, phosphate buffered saline (PBS) solution, and other chemicals of analytical grade were from Sigma, Milan, Italy.

2.1. Cell Culture and Treatment. Human neuroblastoma cell line SH-SY5Y cells were cultured in a 1 : 1 mixture of MEM and Ham's F-12 medium containing 10% (v/v) heat inactivated FBS, L-glutamine (2 mM), and sodium pyruvate (1 mM) and maintained at 37°C in a humidified incubator with 5% CO_2 and 95% air.

Subconfluent cells were washed twice with PBS then incubated in MEM/Ham's F-12 medium containing 10 μM RA (10 mM in dimethyl sulphoxide (DMSO) stock solution), 1% FBS, L-glutamine (2 mM), and sodium pyruvate (1 mM). The medium was renewed every two days.

After 5 days of 10 μM RA exposure, differentiated SH-SY5Y cells were exposed to magnetic field.

2.2. Experimental Design. The exposure system consisted of a couple of Helmholtz coils, with pole pieces of round parallel polar faces, to produce a uniform magnetic field at the center of the coils distance.

This device was used to generate time-varying electromagnetic fields at the frequency of 50 Hz by means of a AC voltage, which enabled us to change the magnetic flux density between the polar faces of the coils. RA differentiated SH-SY5Y cells, grown in both 25 cm^2 culture flasks or in 96-well plates, were exposed to magnetic field. Samples were placed at the centre of a uniform field area between the coils.

The coils aligned on a common axis were wound in the same sense and connected in series.

The value of the coil spacing was assumed to be equal to the coils radius $R = 150$ mm, because this experimental setup showed that the coils fields add in such a way that there is a region around the geometric centre where the magnetic field has constant magnitude and angle.

This experimental setup provided that the magnitude of the magnetic field is linearly proportional to the applied current through the two coils and it is given by $B = (4/5)^{(3/2)}(\mu_o NI)/R$, where N is the number of turns per coil ($N = 124$), $\mu_o = 4\pi^* 10^{-7}$ T·m/A is the magnetic permeability, and I is the coil current.

However, the uniformity of the magnetic field intensity was continuously monitored by the magnetic field probe GM07 Gaussmeter (HIRST-Magnetic Instruments Ltd, Falmouth, Cornwall, UK) within a range of 2 cm around the centre of the coils distance, where samples of human SH-SY5Y neuroblastoma cells were placed.

The coils were located into a incubator in a 5% CO_2/95% air humidified at the temperature of 37.1°C (incubator series 5400-115 V models, Thermo Electron Corporation, Winchester, VA, USA).

Not exposed samples were placed into another incubator of the same model, at the same physical conditions, maintained rigorously at the values of air humidified incubator and temperature previously reported. Preliminary experiments for thermal simulation were performed. Inside the culture

medium, temperatures are monitored with accurate Pt100 probes, using hand-held thermometer model CTH 6200 (from Wika Wiegand GmbH & Co., Klingenberg, Germany). During the exposure, no significant increase in the temperature (±0.1 T°C) was observed.

2.3. Infrared Spectroscopy. FTIR spectra of SH-SY5Y neuroblastoma cells, exposed and not exposed, were recorded at room temperature by a spectrometer Vertex 80 v from Bruker Optics.

For FTIR analysis cellular cultures were disaggregated from the culture medium using a trypsin solution to form single-cell suspensions [17, 18] and were placed upon CaF_2 windows for FTIR measurements.

The attenuated total reflection (ATR) method was chosen for spectrum collection.

In fact, ATR for cells spectra collection in FTIR was improved, because it was no dependent on sample thickness [19].

Furthermore, ATR technique is the desired method to overcome solvent masking since the penetration depth of infrared light is inherently limited to a fraction of the wavelength estimated to be $\lambda/10$, permitting rapid secondary structure analysis on small volumes [20].

For each spectrum, 128 interferograms were collected and coadded by Fourier transformed employing a Happ-Genzel apodization function to generate a spectrum with a spectral resolution of 4 cm^{-1} in the range from 5000 cm^{-1} to 1000 cm^{-1}.

IR spectra of water solution were subtracted from acquired spectra at the corresponding temperature. Each measure was performed under vacuum to eliminate minor spectral contributions due to residual water vapor, and a smoothing correction for atmospheric water background was performed.

The IR spectra were baseline-corrected by means of automatic baseline scattering correction function, to subtract baselines from spectra, which allows getting spectra with band edges of up to the theoretical baseline.

The spectra were successively area-normalized for exposed cells and control samples, and vector normalization was used, calculating the average value of the spectrum and subtracting from the spectrum decreasing the mid spectrum. The sum of the squares of all values was calculated, and the spectrum was divided by the square root of this sum.

Interactive baseline rubberband correction was used to minimize the water band contribution to spectra. This method also uses a rubber band which is stretched from one spectrum end to the other, and the band is pressed onto the spectrum from the bottom up with varying intensity. This method performs iteratively, depending on the number of iterations in the algorithm and the baseline as a frequency polygon consisting of n baseline points. The resulting spectrum will be the original spectrum minus the baselines points manually set and a subsequent concave rubberband correction. We used the default value of $n = 64$ baseline points and a number of 60 iterations.

ATR spectra were smoothed by the Loess algorithm, and the deconvolved spectra were fitted with Gaussian band profiles.

Second-derivative analysis of infrared spectra was performed to enhance spectral features [21, 22].

Both exposed and control samples were located in the same room at a temperature of 20°C.

2.4. Evaluation of Cell Viability. Cell viability was evaluated by an MTT quantitative colorimetric assay. After exposure to magnetic field, RA differentiated SH-SY5Y cells, grown in 96-well culture plates at a density of 5×10^4 cell/well, were incubated with fresh medium containing MTT (0.5 mg/mL) at 37°C for 4 h. Then, insoluble formazan crystals were dissolved in 100 μL of a 10% (w/v) sodium dodecyl sulfate solution in HCl 0.01 M for 10 min. The optical density in each well was evaluated by spectrophotometrical measurement. Absorbance was determined at 570 nm using a microplate reader (Tecan Italia, Cologno Monzese, Italy).

2.5. Measurement of Mitochondrial Transmembrane Potential ($\Delta\psi_m$). Alterations in mitochondrial transmembrane potential ($\Delta\psi$m) were assayed by the incorporation of a cationic fluorescent dye rhodamine 123. After treatments as previously described, the cells ($2.5 \cdot 10^5$ cells/mL in 6-well plates) were changed to fresh medium containing 10 μM rhodamine 123 and incubated for 15 min at 37°C.

The cells were then collected and washed twice with PBS (pH 7.4), and the fluorescence intensity was analyzed at wavelength of 488 nm excitation and 525 nm emission under fluorescein optics.

2.6. Statistical Analysis. All values are expressed as mean ± standard error of the mean (SEM). Statistical analysis was carried out using Student's t-test for comparisons between two groups, with P values less than 0.05 considered significant.

3. Results and Discussion

The range of exposure of the magnetic flux density was chosen from 50 μT to 1.4 mT, following values of exposure used in previous research, as reported in the first section of this paper. Otherwise, the values selected for this study are around the reference, based on the guidelines of the International Commission of Non-Ionizing Radiation Protection [23].

FTIR techniques can easily investigate the spectral region covering the range between 14000 and 20 cm^{-1}, but only in the midinfrared region, 200 to 4000 cm^{-1} is used for analysis of biological materials [24], where absorption spectra of the compounds are characterized by the functional group frequencies of the molecules, which are sensitive to any environmental changes or the changes in their structures and conformations.

Previous FTIR spectroscopic analysis of dying cells has shown that two main characteristic spectral signatures can be assumed as indicative of death [18, 25].

In particular, we observed the shift down of the protein amide I and amide II peak's centroid, indicating a change in

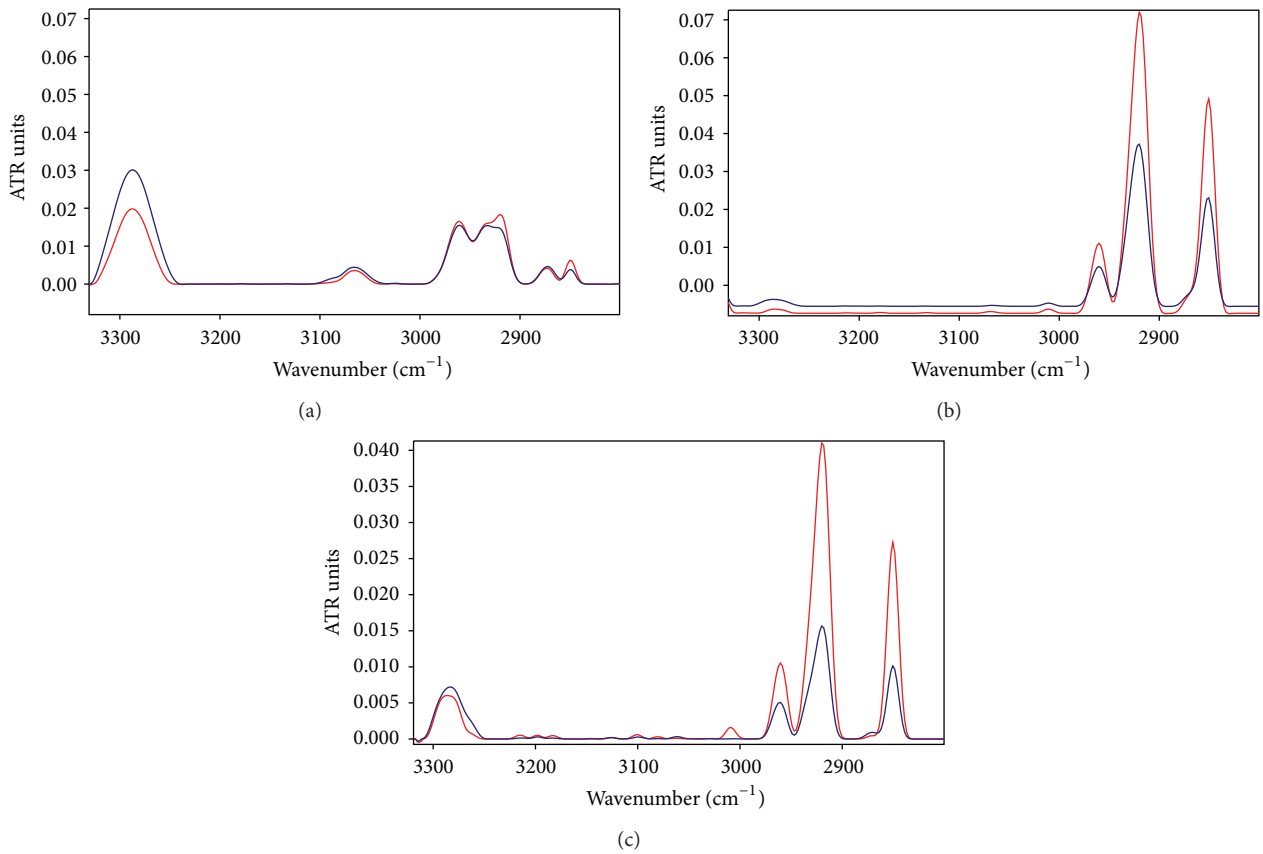

FIGURE 1: ATR spectra of neuroblastoma cells in the spectral IR region from 3300 to 2800 cm^{-1}: (a) after 4 h of exposure to 50 Hz EMF at 50 μT; (b) after 4 h of exposure to 50 Hz EMF at 0.8 mT; (c) after 4 h of exposure to 50 Hz EMF at 1.4 mT. The increase in intensity of CH_2 asymmetric and symmetric stretching appeared clearly after exposure to the magnetic flux densities of 0.8 mT and 1.4 mT.

the overall proteins conformational states within the cell and an increase in the vibration band at 1740 cm^{-1}.

The exposure to ELF-EMF at the low intensity of 50 μT for 4 h produced a decrease in the amide A intensity vibration band around 2995 cm^{-1} (Figure 1(a)), whereas no changes was produced in amide I and amide II bands (Figure 2(a)).

The amide A band, due to the peptide linkage N–H stretching mode, can be considered a useful marker of secondary structure, which often appears as a doublet band arising from two different structures, the stronger of the two components being associated with the standard α-helical structure in the chain [26, 27].

Previous research showed that local environments and hydrogen bonding configurations can play a role in determining the line shape of amide A vibration [28–30].

A decrease of amide A after exposure to ELF-EMF was already observed in FTIR spectra of haemoglobin aqueous solution after 3 hours of exposure to 50 Hz frequency EMF at 1 mT [31].

In addition, a low increase of the vibration bands at 2921 and 2853 cm^{-1} occurred after exposure to 50 Hz EMF at 50 μT, (Figure 1(a)).

Further exposures of 4 h at the magnetic flux densities of 0.8 mT and 1.4 mT were carried out, whose representative spectra in the same region 3300–2800 cm^{-1} are represented in Figures 1(b) and 1(c), respectively, showing a relevant increase in intensity of CH_2 group at 2921 and 2853 cm^{-1} of exposed samples.

The band near 2853 cm^{-1} is due to the symmetric $^{s}CH_2$ stretching of the methylene chains in membrane lipids or proteins; the peak around 2925 cm^{-1} is due to the asymmetric $^{as}CH_2$ stretching [32, 33]. Otherwise, the bands at 2961 and 2871 cm^{-1} can originate, respectively, from the asymmetric $^{as}CH_3$ and the symmetric stretching vibrations $^{s}CH_3$ of CH_3 methyl groups of lipids or protein side chains [34, 35].

In order that CH_2/CH_3 ratio was quantified, the ratio between the integrated area of 2925 cm^{-1} $^{as}CH_2$ band (computed from 2945 to 2910 cm^{-1}) and the integrated area of 2961 cm^{-1} $^{as}CH_3$ (computed from 2980 to 2945 cm^{-1}) of exposed samples and that relative to not exposed samples were calculated.

Analogue calculation was carried out as to the ratio between the integrated area of 2853 cm^{-1} $^{s}CH_2$ band (evaluated from 2835 to 2875 cm^{-1}) and the same integrated area of 2961 cm^{-1} $^{as}CH_3$.

These computations have been summarized in Table 1.

Statistical analysis established significant difference in comparison to controls for the CH_2/CH_3 changes after

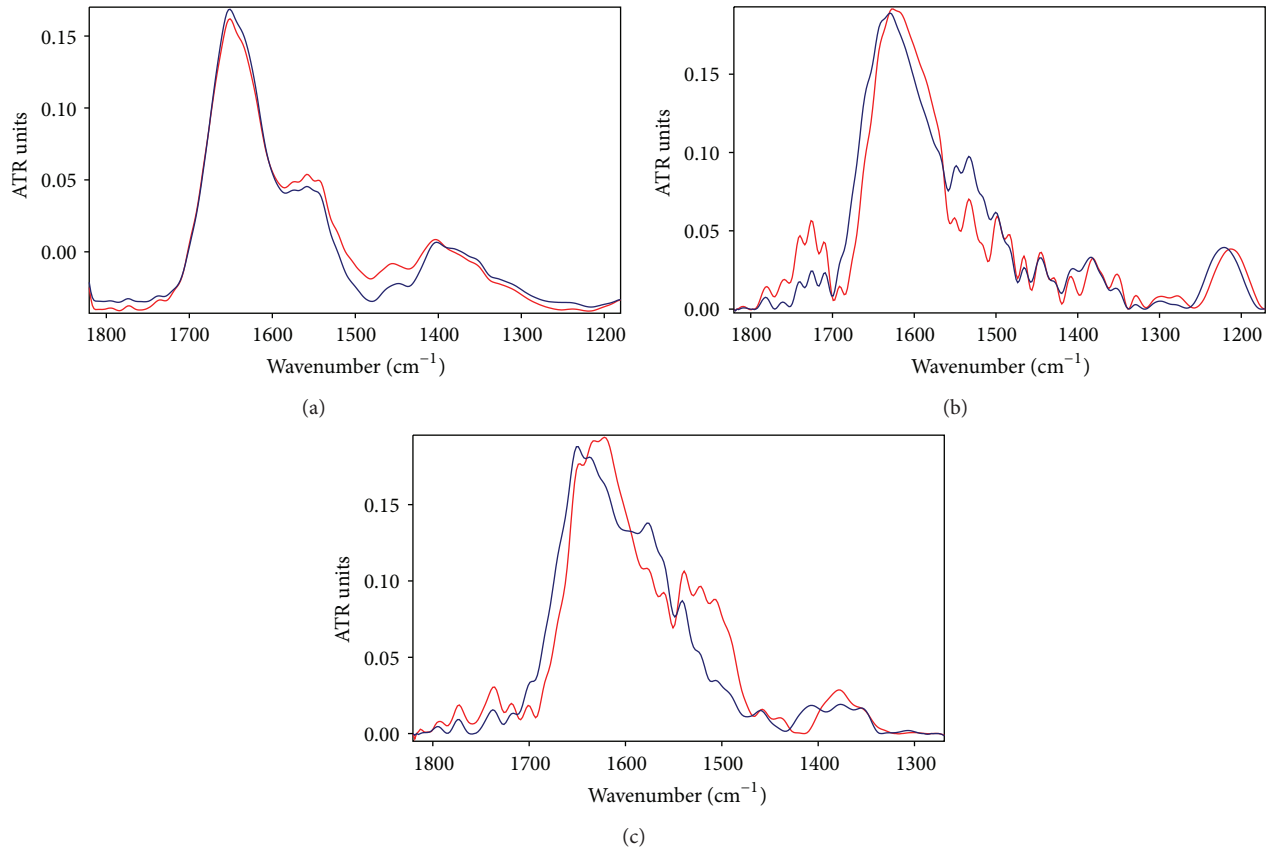

Figure 2: ATR spectra of neuroblastoma cells in the spectral IR region from 1800 to 1250 cm^{-1}. (a) after 4 h of exposure to 50 Hz EMF at 50 μT; (b) after 4 h of exposure to 50 Hz EMF at 0.8 mT; (c) after 4 h of exposure to 50 Hz EMF at 1.4 mT. The increase in β-sheet content with respect to α-helix component in amide I region and the increase at 1740 cm^{-1} vibration band in the amide I region appeared clearly after the exposure to 50 Hz EMF at the intensity of 0.8 mT. The β-sheet content increased more after exposure to 1.4 mT, as reported in Table 2.

the exposures to 50 Hz EMF at the intensity of 0.8 mT, ($P < 0.001$).

However, the change observed in the CH_2/CH_3 ratio must not be necessarily associated to apoptosis, as it could be produced by cell growth, or a decrease in cellular volume to which would correspond an increase in the surface area. This may enrich the overall cellular content with fatty acids and phospholipids and a relative increase of CH_2 as to CH_3 groups.

Exposure to ELF-EMF at 0.8 mT produced evident changes in amide I and amide II regions as represented in Figure 2(b) in which these regions were zoomed in, whereas no appreciable changes occurred in these regions after exposure at the low intensity of 50 μT (see Figure 2(a)).

The peaks observed around 1650 cm^{-1}, 1635 cm^{-1}, 1675 cm^{-1}, and 1640 cm^{-1} in the amide I region can be assigned to α-helices, β-sheets, turns, and random coil, respectively [36].

The analysis of exposed spectra at the intensity of 0.8 mT revealed a loss of α-helical and short segment connecting α-helix segments content and an increase of the β-sheet component at 1635 cm^{-1} relative to the α-helix, as reported in Table 1.

The β-sheet/α-helix ratio of the integrated areas of their vibration bands for the exposed samples was higher in comparison to controls confirming a significant increase of the β-sheet content (1.29 ± 0.05) with respect to the α-helix (1.13 ± 0.04) ($P < 0.05$) in the proteins secondary structure.

These results indicated changes in the overall protein conformational state within the cell, which could be due to denaturation of protein during apoptosis or, alternatively, to different distributions of proteins and unfolding process and formation of aggregates [37].

In the amide II region the absorption band around 1545 cm^{-1} can be assigned to α-helix structure, and vibration bands close to 1525 and 1535 cm^{-1} may be attributed to β-sheet and random coil, respectively [22].

However, the relative increase of β-sheet content as to the α-helix component in this region after exposure was lesser evident than that occurred in amide I region.

The peak at 1740 cm^{-1} assigned to nonhydrogen-bonded ester carbonyl C=O stretching mode within phospholipids increased heavier after the exposure, as can be observed in Figure 2(b), giving further evidence of a dying cell state as suggested by [38, 39]. The integrated area of 1740 cm^{-1} band (computed from 1725 to 1760 cm^{-1}) of exposed samples

Table 1: Integrated areas of representative vibration bands computed for exposed and not exposed samples, whose limits of integration were 3340–3260 cm^{-1}, 2980–2947 cm^{-1}, 2945–2900 cm^{-1}, 2865–2835 cm^{-1}, 1750–1730 cm^{-1}, 1730–1710 cm^{-1}, 1660–1640 cm^{-1}, 1640–1610 cm^{-1}, 1250–1180 cm^{-1}, and 1120–1010 cm^{-1}, respectively. Each value reported was averaged over a number of 26 spectra acquired at successive exposures. The cells of the table without values (n.d.) correspond to no appreciable change between exposed and not exposed samples or to not detected vibration band in the relative spectrum. The exposure levels refers to a time exposure of 4 h. $^{*}P <, 0.05$, $^{**}P < 0.005$ and $^{***}P < 0.001$ significant differences in comparison to control cells.

Exposure levels (mT)	Amide A 3295 cm^{-1}	$^{as}CH_3$ 2961 cm^{-1}	$^{as}CH_2$ 2925 cm^{-1}	$^{s}CH_2$ 2853 cm^{-1}	1740 cm^{-1}	1725 cm^{-1}	β-Sheet — 1635 cm^{-1}	α-Helix — 1652 cm^{-1}	$^{as}PO_2^-$ 1235 cm^{-1}	$^{s}PO_2^-$ 1080 cm^{-1}
0.00	1.00 ± 0.1	1.00 ± 0.07	1.00 ± 0.1	1.00 ± 0.1	1.00 ± 0.08	1.00 ± 0.1	1.00 ± 0.09	1.00 ± 0.1	1.00 ± 0.07	1.00 ± 0.07
0.050	0.67 ± 0.05	1.15 ± 0.06	1.26 ± 0.04	1.76 ± 0.03	1.55 ± 0.10	0.57 ± 0.05	n.d.	n.d.	n.d.	n.d.
0.81	0.32 ± 0.04	1.54 ± 0.15	1.84 ± 0.07***	2.02 ± 0.08***	2.03 ± 0.15*	1.25 ± 0.10	1.29 ± 0.05	1.13 ± 0.04	0.78 ± 0.07*	0.64 ± 0.06*
1.41	0.64 ± 0.04	1.67 ± 0.20***	2.69 ± 0.15***	3.08 ± 0.15***	2.88 ± 0.07	1.14 ± 0.04	1.95 ± 0.10	1.31 ± 0.08	n.d.	n.d.

Table 2: Mitochondrial transmembrane potential (exposed/control) ΔΨm and MTT test for cell viability, relative to three different magnetic flux density values after 4 h of exposure. $^{*}P < 0.05$ and $^{**}P < 0.01$ significant differences in comparison to control cells.

Exposure levels	0.00 mT	0.63 mT	0.81 mT	1.41 mT
ΔΨm (exposed/control)	1.00 ± 0.06	0.91 ± 0.06	0.82 ± 0.06	0.53 ± 0.05**
MTT (exposed/control)	1.00 ± 0.03	0.87 ± 0.03*	0.87 ± 0.02**	0.85 ± 0.02**

and that relative to not exposed samples were computed and reported in Table 1, providing that the 1740 cm^{-1} band increased significantly with respect to the band at 1725 cm^{-1} (2.03 ± 0.15 versus 1.25 ± 0.10) after the exposure ($P < 0.005$).

This result suggest that the C=O ester carbonyl groups of lipids in the cell are becoming predominantly nonhydrogen bonded, which would be in agreement with occurring oxidative damage. Indeed, apoptosis should be associated with increased oxidative damage [40, 41].

The influence of exposure to ELF-EMF on the DNA of treated cells can be observed from IR bands due to vibrations of various structural groups in DNA such as the two phosphate absorption bands around 1235 cm^{-1} and 1080 cm^{-1}, that correspond to the asymmetric stretching phosphate mode $^{as}PO_2^-$ and symmetric stretching phosphate mode of phosphodiester bonds $^{s}PO_2^-$ in nucleic acids, respectively [32, 33].

The integrated area of $^{as}PO_2^-$ and $^{s}PO_2^-$ of exposed samples in comparison to control samples, computed from 1260 to 1200 cm^{-1} and from 1120 to 1020 cm^{-1}, respectively, decreased significantly ($P < 0.05$) after the exposure to ELF-EMF at 0.8 mT, as reported in Table 1.

In addition, the peak's centroid of $^{as}PO_2^-$ and $^{s}PO_2$ bands shifted 9 cm^{-1} and 3 cm^{-1}, respectively after exposure, as can be observed in Figure 2(b).

Dependent on EMF field intensity, we observed a significant reduction of the levels of the bands suggesting a decrease in DNA content in the cells exposed to ELF-EMF.

Further exposure of 4 h to 50 Hz EMF at the magnetic flux density of 1.4 mT produced heavier significant increases of CH_2/CH_3 ratio ($P < 0.001$), as shown in Figure 1(c) and reported in Table 1.

In addition, the β-sheet/α-helix ratio in amide I and the 1740 cm^{-1}/1725 cm^{-1} ratio increased getting to the values of 1.95 ± 0.10 versus 1.31 ± 0.08 and 2.88 ± 0.07 versus 1.14 ± 0.04, respectively, as represented in Figure 2(c) and reported in Table 1, providing further evidence for the dying cells state, which was confirmed by MTT analysis (Table 2).

As reported in Table 2, the 4 h exposure to ELF-EMF (1.4 mT) produced a reduction by 15% of cell viability ($P < 0.01$). According to these results, significant decreases in cells viability of neuronal-like were already observed after exposures of 2 h and 4 h to EMF at 1800 MHz [42].

Furthermore, after 4 h exposure to 50 Hz EMF at 1.4 mT heavy reduction (53%) of mitochondrial transmembrane potential was observed (Table 2). The results confirm several observations suggesting that mitochondria can be the source of energy as well as the source of signals that initiate apoptotic cell death.

A significant loss of ΔΨm renders cells depleted of energy; given the energy needs of neurons, defects in mitochondrial dynamics lead to neuronal cell death.

4. Conclusions

To summarize, the exposure to 50 Hz electromagnetic field higher than 0.8 mT was able to produce conformational changes in different biological structures as evidenced by FITR spectroscopy, and these alterations can be associated to reduction of mitochondrial transmembrane potential and cell viability.

Conflict of Interests

All authors further confirm that do not have a conflict of interest.

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Research Article

Xenobiotic Sensor- and Metabolism-Related Gene Variants in Environmental Sensitivity-Related Illnesses: A Survey on the Italian Population

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In the environmental sensitivity-related illnesses (SRIs), multiple chemical sensitivity (MCS), chronic fatigue syndrome (FCS), and fibromyalgia (FM), the search for genetic polymorphisms of phase I/II xenobiotic-metabolizing enzymes as suitable diagnostic biomarkers produced so far inconclusive results, due to patient heterogeneity, geographic/ethnic differences in genetic backgrounds, and different methodological approaches. Here, we compared the frequency of gene polymorphisms of selected cytochrome P450 (CYP) metabolizing enzymes and, for the first time, the frequency of the xenobiotic sensor Aryl hydrocarbon receptor (AHR) in the three cohorts of 156 diagnosed MCS, 94 suspected MCS, and 80 FM/FCS patients versus 113 healthy controls. We found significantly higher frequency of polymorphisms CYP2C9*2, CYP2C9*3, CYP2C19*2, CYP2D6*4 and CYP2D6*41 in patients compared with controls. This confirms that these genetic variants represent a genetic risk factor for SRI. Moreover, the compound heterozygosity for CYP2C9*2 and *3 variants was useful to discriminate between either MCS or FM/CFS versus SMCS, while the PM *41/*41 genotype discriminated between MCS and either SMCS or FM/CFS. The compound heterozygosity for CYP2C9 *1/*3 and CYP2D6 *1/*4 differentiated MCS and SMCS cases from FM/CFS ones. Interestingly, despite the distribution of the AHR Arg554Lys variant did not result significantly different between SRI cases and controls, it resulted useful for the discrimination between MCS and SMCS cases when considered within haplotypes in combination with CYP2C19 *1/*2 and CYP2D6 *1/*4. Results allowed us to propose the genotyping for these specific CYP variants, together with the AHR Arg554Lys variant, as reliable, cost-effective genetic parameters to be included in the still undefined biomarkers' panel for laboratory diagnosis of the main types of environmental-borne SRI.

1. Introduction

In the last years, much attention has been paid to a variety of pathological conditions sharing the common feature of an aberrant response triggered by airborne or other routes of exposure to low doses of environmental pollutants or xenobiotics, such as chemicals, drugs, heavy metals, electromagnetic, or nuclear radiations, in concentrations far below average reference levels admitted for environmental toxicants [1–4]. Indeed, the World Health Organization has collectively defined these conditions as "idiopathic environmental intolerances"(IEIs), namely multiple chemical sensitivity (MCS), fibromyalgia (FM), chronic fatigue syndrome (CFS), dental amalgam disease, and others, among which the intolerances to microbial and environmental allergens or toxins, drugs, vaccines, specific foods, synthetic implants, and possibly new biomaterials are also included [5–7]. In view of the progressively increasing knowledge and awareness regarding these diseases, they are presently better described collectively as "sensitivity-related illnesses" (SRIs) [7, 8].

SRI symptoms appear mainly in adult life, with higher prevalence in women, although a growing number of pediatric cases have been recently registered, and evidence is accumulating for a role of *in-utero* sensitization [6, 9]. For the prototypical MCS, the disease onset is commonly self-reported as a single precipitating event following a severe intoxication or as a chronic exposure to lower doses of an environmental pollutant [10, 11], either in the occupational or in the domestic setting. Then, the intolerance becomes chronic, with symptoms elicited not only by the same incitant but also by different, multiple incitants, resolving at their removal. Challenges for establishing differential diagnostic criteria for various SRI lie in: (i) the absence of consensus on case definition in all SRI conditions, with the exception of FM [12]; (ii) the wide variety of multiorgan symptoms, including psychosomatic, neurological, memory loss, mood disorders, posttraumatic distress, chronic muscular fatigue, chronic bronchitis and asthma, eye-nose-throat, gastrointestinal, cardiac, and autoimmune disorders; (iii) the variable individual sensitivity and genetic predisposition; (iv) the absence of clearly defined pathogenic mechanisms; (v) the variety of potential triggers; (vi) the absence of dose-dependent responses, even after provocation; and (vii) common comorbidity features with known autoimmune diseases like systemic lupus erythematosus, rheumatoid arthritis, or vitiligo [13–18].

To date, the diagnosis of MCS is based on the compliance to Cullen's inclusion anamnestic criteria [19] and on the score resulting from the Quick Environmental Exposure and Sensitivity Inventory (QEESI) [20, 21]. QEESI is a multistep questionnaire determining the levels of sensitization to chemical environmental triggers, scoring the type, localization and severity of symptoms after exposure, and the life impact. It was first validated by Miller and Prihoda (1999) [9] in the USA, adapted on the German population by Fabig (2000) [21], and successively validated in different national contexts, Japan, Sweden, Denmark [8], and, more recently, also in Spain [22].

The classification as "idiopathic" for these environmental intolerances arises from the poor current knowledge of their etiology and pathogenesis and the absence of recognized genetic and metabolic markers [8]. However, recent advances in toxicogenomics and metabolomics have highlighted the role of inherited or acquired impairment of xenobiotic metabolism in the individual hypersensitivity to both xenobiotics and toxic endogenous metabolites [14, 23–25]. Alterations of this system might lead to incomplete detoxification of exogenous/endogenous toxins or/and to excessive generation of toxic by-products, in the case of "poor metabolizers" (PM) or to higher-than-normal rates of metabolization in subjects with hyperfunctional genes, possibly duplicated or multiplicated, termed as "extensive metabolizers" (EMs) [11, 17, 26].

Leading studies on chemically hypersensitive individuals in European and North American populations have been so far focused on single-nucleotide polymorphisms (SNPs) in genes coding for a variety of phase I and phase II metabolizing enzymes and their receptors [17, 25, 27–32]. However, the conclusions obtained were limited and contradictory, mainly due to inhomogeneous and insufficient size of the patient and control groups and due to different and non standardized patient cohort inclusion criteria adopted by the different studies. Thus, the search for genetic markers distinctive of SRI remains under question, awaiting for conclusive evidence of their diagnostic value and cost-effectiveness.

Moreover, the identification of genetic markers specific for different SRI would be extremely useful for a better diagnosis assessment of MCS, FM, CFS and other idiopathic environmental intolerances. this work was aimed at assessing the distribution of some selected gene polymorphisms in representative groups of Italian hypersensitive individuals fully or partially diagnosed with MCS, or affected by its more common comorbidities, FM or FCS, as compared with sex- and age-matched healthy controls.

In these groups, we analyzed the frequency of some well-known polymorphic variants of cytochrome P450 (CYPs) enzymes, namely, (CYP2C9, CYP2C19, and CYP2D6), commonly investigated in different clinical conditions including MCS [29, 33, 34]. CYP family of phase I enzymes is essential for drug metabolism and bioactivation [35]. We then analyzed for the first time, in the same cohorts, the frequency of selected gene polymorphisms of the ligand-activated transcription factor Aryl hydrocarbon receptor (AHR) [36]. AHR was first known as the main controller of the expression of several classes of xenobiotic metabolizing enzymes (XMEs) [37] and then, more extensively, of a wide array of cell responses to organic chemical xenobiotic and UV environmental stressors [38] through multiple signal transduction pathways (recently reviewed in [39]).

Because of their critical role in the xenobiotic-induced toxicity, the potentially wide array of altered gene polymorphisms of the complex drug-metabolizing network in individuals affected by environmental intolerances so far not conclusively demonstrated in spite of rather extensive investigations, still remains of utmost interest for patient management and possibly also for therapeutic approaches.

2. Methods

2.1. Patients. Patients of case cohorts were selected on the basis of their diagnosis made according to Cullen's criteria [19] and QEESI questionnaire [21]. A modified QEESI score of 10 common environmental exposures and 10 major symptoms allowed to diagnose MCS ($20 \leq \text{score} \leq 30$) or SMCS (suspected MCS) ($10 \leq \text{score} \leq 20$) or others to be excluded from enrollment ($0 \leq \text{score} \leq 10$) [8].

One hundred and fifty-six Italian consecutive MCS patients (87 F/14 M, 49 ± 11 years), 94 consecutive SMCS patients (79 F/15 M; 49 ± 12 years), partly corresponding to the diagnostic criteria reported above, and 80 consecutive patients (61 F/19 M; 47 ± 10 years) presenting either fibromyalgia or chronic fatigue syndrome (FM/CFS group), were enrolled for this study by specialized clinicians, who performed anamnestic and lifestyle data collection, at Istituto Dermopatico dell'Immacolata, IRCCS, according to a study protocol approved by IDI Ethical Committee (no. 121/CE/2008).

One hundred and thirteen healthy Italian subjects (90 F/23 M, 53 ± 12) matched for sex and age with patient group were selected as controls (Ctr) at Istituto Dermopatico dell'Immacolata (IDI IRCCS) and Messina University according to the established criteria of (i) an absence of any clinically diagnosed disease, in particular allergic or immunologic disturbances, and (ii) no drug or nutraceutical supplement since at least six weeks at the time of blood sampling, (iii) whole blood total production of reactive oxygen and nitrogen species (ROS/RNS) below 650 cps/μL, as determined by luminol-dependent chemiluminescent response to phorbol 12-myristate 13-acetate (PMA) [40] (IDI study protocol approval no. 52/CE/2010). Nonsmokers in the patient group were 70.4% (MCS), 82.2% (SMCS), and 84.2% (FM/FCS); smokers were 8.2% (MCS), 17.8% (SMCS), and 12.0% (FM/FCS). Patients with undetermined smoking habits were 21.4% (MCS) 0% (SMCS), and 3.8% (FM/FCS). Nonsmokers in the Ctr group were 85.2%. No alcohol or drug abusers were present neither in patients' nor in control groups.

Patients and controls were selected from different Italian regions in order to partially overcome the historical genetic variability in this country [41].

All subjects provided written informed consent to blood sampling and anamnestic data collection.

2.2. Genotyping for SNPs in Drug Metabolism-Related Enzymes. Genomic DNA was purified from 400 μL of human whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was quantified spectrophotometrically at 260 nm, aliquoted, and stored at −20°C until assayed.

Genotyping of SRI patients and control subjects for eight single nucleotide polymorphisms in drug metabolism- and inflammation-related genes was carried out by real-time PCR allelic discrimination using predesigned TaqMan SNP genotyping assays available from Applied Biosystems (Applera Italia, Monza, Italy). The polymorphisms analyzed were those of genes coding for: cytochrome P450 (CYP), family 2, subfamily C, polypeptide 9 and 19, namely, CYP2C9*2 (C > T, rs1799853; assay ID: C_25625805_10), CYP2C9*3 (A > C, rs1057910; assay ID: C_27104892_10), and CYP2C19*2 (G > A, rs4244285; assay ID: C_25986767_70); CYP2 subfamily D, polypeptide 6, namely, CYP2D6*4 (1846G > A, rs3892097; assay ID: C_27102431_D0) and CYP2D6*41 (C > T, rs28371725; assay ID: C_34816116_20); and aryl hydrocarbon receptor (AHR) Arg554Lys variant (G > A, rs2066853; assay ID: C_11170747_20).

Genotyping reactions were set up in a 96-well plate on a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA), and were carried out in a final volume of 20 μL containing 1x TaqMan Genotyping Master Mix, 1x TaqMan-specific assay, and 10 ng genomic DNA, using thermal cycling conditions suggested by manufacturer's protocols.

2.3. Statistical Analysis. Allele and genotype frequencies obtained on patients' and control groups were compared by Fisher test analysis. Software employed was GraphPad Prism 4 (San Diego, CA, USA). Values of $P \leq 0.05$ were considered as significant.

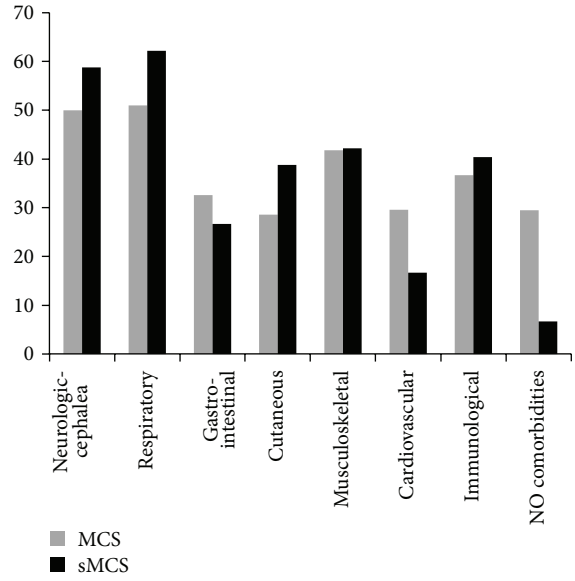

Figure 1: Comorbidities registered in the MCS group (n = 156; BMI = 23.4 mean ± 5.4 SD) and the sMCS group (n = 94; BMI = 23.8 mean ± 4.8 S.D.) through evaluation of anamnesis. Data are expressed as percentage of patients affected by each single category of organ pathologies. Abbreviations: body mass index (BMI); multiple chemical sensitivity (MCS); and suspected multiple chemical sensitivity (sMCS).

3. Results

The main categories of co-morbidities resulting from anamnestic analysis are compared in MCS and SMCS patients and reported in Figure 1.

Genotype and allele frequencies of selected polymorphisms in drug metabolism-related genes examined in SRI patients (MCS, SMCS, and FM/CFS) and controls are shown in Table 1. Genotype frequencies among cases and controls were in Hardy-Weinberg equilibrium, and, similarly, allele frequencies among cases and controls were within the 95% confidence.

3.1. Analysis of CYP2C9 Genetic Background and Risk for Disease. The analysis of the distribution of CYP2C9 gene variants showed that the allele CYP2C9*2 was more present than the CYP2C9*3 both in MCS as well as in SMCS patients and in healthy subjects but not in FM/CFS group. However, both mutated alleles were more frequent in cases than in controls as the prevalence of CYP2C9*2 was around 1.5-fold higher in patients than in controls and that of CYP2C9*3 was 7-fold, 11-fold, and 21-fold higher in MCS, SMCS, and FM/CFS patients, respectively, than in controls (Table 1). However, significant differences were found only for the frequency of *2 allele between controls and other patients and for the frequency of *3 allele among all cases and controls

Table 1: Allele and genotype frequencies of some drug metabolism-related gene variants in SRI patients (MCS: multiple chemical sensitivity; sMCS: suspected multiple chemical sensitivity; FM/CFS: fibromyalgia/chronic fatigue syndrome) and healthy subjects (Ctr: controls).

Genotype	MCS	SMCS	FM/CFS	Ctr
CYP2C9	$N = 101$	$N = 94$	$N = 80$	$N = 90$
Wt	66.3%	66%	55%	84.4%
Ht*1/*2	23.8%†	18.1%	15%	12.2%
Ht*1/*3	6.9%†,§§	11.7%††	20%††††	1.1%
PM*2/*2	0.9%	2.1%	3.75%	2.2%
PM*2/*3	0.9%	—	2.5%	—
PM*3/*3	0.9%	2.1%	3.75%	—
*2 allele frequency	0.132§§	0.111§	0.125†	0.083
*3 allele frequency	0.048††	0.079†††	0.15††††	0.007
CYP2C19	$N = 101$	$N = 93$	$N = 80$	$N = 62$
Wt	61.4%	84.8%	78.8%	80.3%
Ht*1/*2	36.6%$^{\dagger\dagger,\#\#\#,\S}$	14.1%	20.0%	16.7%
PM*2/*2	2.0%	1.1%	1.3%	3.0%
*2 allele frequency	0.203$^{\dagger,\S,\#\#}$	0.081	0.113	0.113
CYP2D6	$N = 55$	$N = 93$	$N = 75$	$N = 113$
Wt	40%	52.7%	40.7%	77.9%
Ht*1/*4	36.4%††	25.8%†	28.5%†	18.6%
Ht*1/*41	14.8%††††	5.4%††††	7.4%††††	—
PM*4/*4	5.5%	4.3%	8.5%	3.5%
PM*4/*41	3.6%	7.5%	7.4%	—
PM*41/*41	—	4.3%	7.4%	—
*4 allele frequency	0.255†††	0.209††	0.314††††	0.128
*41 allele frequency	0.092††††	0.107††††	0.148††††	—

$^{\dagger}P < 0.05$ versus Ctr, $^{\dagger\dagger}P < 0.01$ versus Ctr; $^{\dagger\dagger\dagger}P < 0.001$ versus Ctr, $^{\dagger\dagger\dagger\dagger}P < 0.0001$ versus Ctr; $^{\S}P < 0.05$ versus FM/CFS; $^{\S\S}P < 0.01$ versus FM/CFS; $^{\#}P < 0.05$ versus SMCS; $^{\#\#}P < 0.01$ versus SMCS; $^{\#\#\#}P < 0.001$ versus SMCS.

and also between MCS patients and FM/CFS ones, as well as SMCS and FM/CFS patients (Table 1). Both CYP2C9*2 and CYP2C9*3 alleles were almost entirely represented by the heterozygous genotype, given that poor metabolizer homozygous individuals, either PM *2/*2 or PM *3/*3, were very few in each subgroup of patients and almost absent in the control population. The frequency of the CYP2C9*2 heterozygous genotype (Ht *1/*2) was higher in the MCS subgroup than in all other groups but reached a statistically significant difference only in comparison with control group. Notably, the frequency of the CYP2C9*3 heterozygous genotype (Ht *1/*3) was significantly higher in all groups of patients than in controls and in the group of FM/CFS patients compared with MCS one. Interestingly, compound heterozygotes for both CYP2C9 gene variants (PM *2/*3) were generally poorly represented in cases and absent in the SMCS subgroup and in healthy subjects (Table 1).

The calculation of odds ratio (ORs) showed that the presence of genotype Ht *1/*2 increases 2.4-fold the risk for developing MCS, while having the genotype Ht *1/*3 causes a 7.5-, 12-, and 26-fold increase of the risk for developing MCS, SMCS, and FM/CFS, respectively.

3.2. Analysis of CYP2C19 Genetic Background and Risk for Disease. Genotyping for the CYP2C19 gene variants showed that the frequency of the mutated *2 allele was significantly higher in MCS cases than in all other groups. Interestingly, the lowest frequency for this allele was observed in SMCS cases; moreover, healthy subjects and FM/CFS patients had a similar *2 allele frequency (Table 1). The CYP2C19*2 allele was almost entirely represented by the heterozygous genotype in all sampled subgroups and showed a significantly higher prevalence in MCS cases than in all other groups, while the frequency of homozygotes PM *2/*2 was similar among all subgroups (Table 1).

OR calculation showed that a mutated CYP2C19* background was associated with a threefold increased risk for developing MCS.

3.3. Analysis of CYP2D6 Genetic Background and Risk for Disease. The analysis of genetic background at the CYP2D6 gene locus showed that the CYP2D6*4 was the most represented gene variant in all subgroups of SRI cases compared with the CYP2D6*41; moreover, this latter variant was absent in the control population.

The comparison of allele frequencies showed that CYP2D6*4 allele had a highly significant, different frequency among all cases compared with controls, being two-threefold higher in patients than in healthy subjects. The CYP2D6*41 allele, that was absent in control population, had the highest frequency in FM/CFS group.

Table 2: Allele and genotype frequencies of AhR-related gene variants in SRI patients (MCS: multiple chemical sensitivity; sMCS: suspected multiple chemical sensitivity; FM/CFS: $fibromyalgia/chronic fatigue syndrome) and healthy subjects (Ctr: controls).

Genotype	MCS	SMCS	FM/CFS	Ctr
AHR	$N = 156$	$N = 56$	$N = 32$	$N = 54$
Arg554Arg	80.1%	82.1%	68.7%	77.8%
Arg554Lys	19.9%	17.9%	31.3%	16.7%
Lys554Lys	—	—	—	5.6%
A allele frequency	0.099	0.089	0.156	0.139

Both CYP2D6*4 and CYP2D6*41 alleles were largely represented by heterozygous individuals but displayed a different variability among the sampled subgroups. The CYP2D6 *1/*4 heterozygous genotype was more frequent in MCS cases than in either SMCS or FM/CFS patients and controls. However, the frequencies of Ht *1/*4 genotype in all three patient groups resulted significantly higher than in healthy subjects. Poor metabolizers, homozygotes *4/*4 were more frequent in the FM/CFS patients than in all other groups (Table 1), showing nonsignificant differences among them and tended to statistically significant difference ($P = 0.06$) in comparison with controls.

The CYP2D6 *1/*41 heterozygous genotype had a higher frequency in MCS group than in the other patients, having a similar distribution, and was absent in the control population, reaching a statistically significant difference. Poor metabolizers, compound heterozygotes *4/*41, and homozygotes *41/*41 were more frequent in FM/CFS patients than in SMCS as well as MCS cases, these latter lacking of the PM *41/*41 genotype, and both were absent in the control population (Table 1).

The calculation of odds ratio indicated that a genetic background positive for the presence of genotype CYP2D6 Ht *1/*4 increased three-fold and two-fold the risk for developing MCS and either SMCS or FM/CFS, respectively, while the same risk was 52-fold, 38-fold, and 36-fold increased by the presence of CYP2D6 Ht *1/*41.

3.4. Analysis of AHR Genetic Background and Risk for Disease. Genotyping for the Arg554Lys variant of AHR gene showed for the first time that the Lys-mutated variant was less frequent in MCS and SMCS cases, displaying similar distributions, than in FM/CFS patients and controls, and was entirely represented by the heterozygous genotype in all subgroups of cases, while the control population included both heterozygotes and homozygotes. Moreover, the frequency of heterozygotes was higher in the FM/CFS patients than in MCS, SMCS, and control groups, all showing similar frequencies (Table 2). However, these differences did not reach statistical significance.

3.5. Analysis of Haplotype Distribution. The analysis of the distribution of two or more combined mutant alleles, that is, the frequency of given haplotypes in the various sampled population, is reported in Table 3. It was shown that very few individuals were carriers of more than one mutation in the examined CYP isoforms. This was true both considering the occurrence of more than one mutation in the same gene, that is, the presence of both CYP2C9 variants and both CYP2D6 variants, and the occurrence of more than one mutation in different genes.

The most frequent haplotype in MCS cases was the CYP2C19 Ht *1/*2 - CYP2D6 Ht *1/*4- AHR Arg554Lys (9.1%), that was not present neither in SMCS cases nor in other patients ($P < 0.001$).

In the SMCS cases the most represented haplotype was the CYP2C9 Ht *1/*2-CYP2D6 Ht *1/*4, that was absent in the group of other patients and had a significantly lower frequency in MCS ($P < 0.05$).

In the subgroup of FM/CFS patients the most frequent haplotype was the CYP2C19 Ht *1/*2 - CYP2D6 Ht *1/*4, that showed a significantly higher distribution compared with SMCS cases ($P < 0.01$). Moreover, the haplotype CYP2C9 Ht *1/*3 - CYP2D6 Ht *1/*4 was only represented in MCS cases and SMCS, while the haplotype CYP2C9 Ht *1/*3- CYP2D6 Ht *1/*4 was only present in MCS cases. On the contrary, the combination of four mutated alleles either in heterozygosis or homozygosis was only observed in the subgroup of FM/CFS patients.

4. Discussion

To date a commonly agreed-upon set of laboratory metabolic parameters to be used worldwide for the classification of environmental-borne multiorgan syndromes, such as multiple chemical sensitivity is not available [6, 42]. Based on a consistent body of data on oxidative stress markers in fibromyalgia and chronic fatigue syndrome [8], previous reports by our research group have highlighted the validity, for the laboratory confirmation of clinical MCS diagnosis of an extensive biochemical characterization based on metabolic and immunological markers. These include alterations of erythrocyte catalase, glutathione peroxidase and transferase activities, glutathione depletion, and the polyunsaturated fatty acid-depleted profile of erythrocyte membrane, combined with specific plasmatic patterns of proinflammatory cytokine alterations [17, 25]. However, despite these available biological profiles, a reference panel useful for the unequivocal laboratory discrimination of MCS from other SRI, such as FM, CFS or for the confirmation of suspected MCS is still under construction.

Until now, the search for SRI biomarkers of disease has been centered on genetic determinants, including polymorphic variants of phase I/phase II detoxification enzymes, mainly analyzed in MCS subjects, with the study of a variety of cytochrome P450 isoenzymes (CYPs), glutathione-S-transferases (GSTs), UDP-glucuronosyl transferases (UGTs), catechol-O-methyltransferases (COMTs), N-acetyl transferases (NATs), paraoxonase 1 and 2 (PON1, PON2), methylenetetrahydrofolate reductase (MTHFR), and of cholecystokinin 2 receptor (CCKR2) [25, 27–32], although data are available also for FM [34, 43] and CFS [44].

Table 3: Haplotype frequencies of CYP2C9, CYP2C19, CYP2D6 and AHR gene variants in SRI patients (MCS: multiple chemical sensitivity; Smcs: suspected multiple chemical sensitivity; other patients: FM/CFS-fibromyalgia/chronic fatigue syndrome).

Haplotype	Haplotype frequency (%)		
	MCS	SMCS	Other patients
CYP2C19 Ht*1/*2 - CYP2D6 Ht*1/*4 - AHR Arg554Lys	9.1	—	—
CYP2C19 Ht*1/*2 - CYP2D6 Ht*1/*4	5.5	1.1	6.25
CYP2C9 Ht*1/*2 - CYP2D6 Ht*1/*41	5.5	—	—
CYP2C9 Ht*1/*2 - CYP2D6 Ht*1/*4	3.6	6.4	—
CYP2C9 Ht*1/*3 - CYP2D6 Ht*1/*4	3.6	2.1	—
CYP2C9 Ht*1/*3 - CYP2D6 Ht*1/*41	3.6	—	—
CYP2C9 Ht*1/*3 - CYP2C19 Ht*1/*2 - CYP2D6 Ht*1/*4	1.8	—	—
CYP2C9 Ht*1/*3 - CYP2C19 Ht*1/*2 - CYP2D6 Ht*1/*4 - Arg554Lys	—	—	1.25
CYP2C9 Ht*1/*2 - CYP2C19*1/*2	1.8	—	—
CYP2C9 Ht*1/*2 - CYP2C19 Ht*1/*2 - AHR Arg554Lys	—	1.1	—
CYP2C9 Ht*1/*2 - CYP2C19 Ht*1/*2 - CYP2D6 PM*4/*4 - AHR Arg554Lys	—	—	1.25
CYP2C9 Ht*1/*3 - CYP2C19 Ht*1/*2	—	1.1	—
CYP2C9 Ht*1/*2 - CYP2D6 Ht*1/*41 - AHR Arg554Lys	—	1.1	—
CYP2C19 Ht*1/*2 - CYP2D6 Ht*1/*41	—	1.1	2.5
CYP2C9 Ht*1/*2 - CYP2D6 PM*4/*4	—	1.1	—
CYP2C9 Ht*1/*2 - CYP2D6 PM*4/*41	—	1.1	—
CYP2C9 Ht*1/*2 - CYP2D6 PM*41/*41 - Arg554Lys	—	—	1.25
CYP2C19 Ht*1/*2 - CYP2D6 PM*4/*4	—	1.1	1.25
CYP2C19 Ht*1/*2 - CYP2D6 PM*4/*4 - AHR Arg554Lys	—	—	1.25
CYP2C19 Ht*1/*2 - CYP2D6 PM*4/*41	—	2.1	—
CYP2C19 Ht*1/*2 - CYP2D6 PM*41/*41	—	1.1	—
CYP2C9 Ht*1/*3 - CYP2D6 PM*4/*41 - AHR Arg554Lys	—	—	1.25
CYP2C9 PM*2/*2 - CYP2D6 Ht*1/*41	1.8	—	—
CYP2C9 PM*3/*3 - CYP2C19 PM*2/*2	1.8	—	—
CYP2C9 PM*2/*2 - CYP2D6 PM*4/*41	—	1.1	—
CYP2C9 PM*3/*3 - CYP2D6 PM*41/*41	—	1.1	—
CYP2C19 PM*2/*2 - CYP2D6 Ht*1/*4	—	1.1	—
CYP2C19 PM*2/*2 - CYP2D6 Ht*1/*4 - AHR Arg554Lys	—	—	1.25

Results, as a whole, have not been encouraging, given that the complexity and high costs of the investigations in that contrasting results were reported by different groups and the differences in genotype distribution between cases and controls were often not reaching statistical significance. However, a reliable between-groups comparison of outcomes from these studies was hard, due to the lack of homogeneity with regard to sample size, gender, and ethno-geographical features, of case/control populations examined. Furthermore, in general, positive findings from genetic association studies seem to be difficult to replicate and possibly inflated by chance [45].

Another critical issue is the heterogeneity of SRI, most of which exhibit overlapping symptoms with other common pathological conditions. Indeed, a diagnostic panel including defined criteria for diagnosis assessment, would be very important for etiological studies. Even though the MCS case definitions and the grading of chemical sensitivity in general populations used in the previous studies cited appear to be very similar, the underlying classification differences cannot be ruled out as they contribute to the inconsistency among published studies. In this regard, all patients included in our study have been diagnosed consecutively according to Cullen's criteria and adapted QEESI questionnaire scoring [19, 20, 25], by the same team of specialists, thus minimizing selection bias in the recruiting of patients and securing the uniform group as much possible.

Importantly, an additional critical issue is the use of genotyping techniques having 100% sensitivity and specificity for the detection of heterozygous genotypes, the most common form by which a mutant allele is present in a given population. In this regard, most of the published genetic investigations on MCS patient, including our previous report on 110 Italian subjects [25], employed the PCR-RFLP technique which, lacking of the required sensitivity, often provides a misrepresentation of genotype distribution in the population, specifically with regard to the frequency of heterozygotes [46].

Here, we investigated the distribution of some gene variants of clinically relevant drug metabolism-related proteins, that is, selected CYP enzyme isoforms, namely, CYP2C9*2, CYP2C9*3, CYP2C19*2, CYP2D6*4, and CYP2D6*41 already addressed in previous studies producing conflicting

data. We reported here for the first time that the examined variants of CYP isoforms have a significantly different distribution in SRI patients versus controls. Most importantly, some of these variants may be useful for an objectively based discrimination between different types of SRI, given the observed significant differences after between-groups comparison of allele and genotype frequencies. In particular, allele and heterozygous genotype frequencies observed in all subgroups of SRI patients for the variant CYP2C9*3 showed significant or even highly significant differences with those observed in healthy subjects. These differences allowed us to propose the presence of the genetic variant CYP2C9*3 as a candidate marker of disease state with specific reference to SRI, as also confirmed by calculation of disease risk by Odds Ratio (OR), that was increased in a range from 7- to 26-fold for MCS, SMCS, and FM/CFS conditions, respectively. In particular, highly discriminant between pathological condition and healthy state was the presence of the homozygous genotype PM *3/*3 and the compound heterozygous one PM *2/*3 that was not recorded in control subjects. Moreover, the presence of the heterozygous genotype Ht *1/*3 may be useful for discrimination between MCS and FM/CFS patients, as the difference in genotype frequencies are highly significant.

The examination of genetic background for CYP2C9*2 showed that the allele *2 frequency may discriminate MCS patients and SMCS patients from FM/CFS ones and that the frequency of genotype Ht*1/*2 differentiates MCS patients from controls. As a whole, the search for this CYP2C9 variant may be quite useful in population-based studies for the classification of different SRI.

Similar results were obtained after comparison of allele and genotype frequencies for CYP2D6 variants in patients and controls, even though variable significance level for differences wre found. Both allele and heterozygous genotype frequencies for CYP2D6*4 and *41 resulted useful for discriminating between intolerance syndromes and healthy state. In particular, a genetic background positive for the presence of either genotype CYP2D6 Ht *1/*4 or Ht *1/*41 increased up to 3-fold and 50-fold the risk for developing SRI (see Table 1). Notably, the presence of poor metabolizer individuals, having either the homozygous genotype PM *41/*41 or the compound heterozygous one PM *4/*41, was not recorded in control subjects. These results, at least those demonstrating the association of CYP2D6 genetic variants with MCS state, are in agreement with those reported in a previous study on a Canadian population [29], where a relationship was found between MCS and CYP2D6 activity, with an OR of 2.49 for intermediate metabolizers and 3.36 for extensive metabolizers, while ultrarapid metabolizers were not examined. Finally, neither allele or genotype frequencies for both CYP2D6 variants may be regarded as differential markers between various disease states, given the observed between-groups similarities.

Interestingly, the analysis of allele and genotype distribution for the CYP2C19 variants showed that the examination of genetic background for this CYP isoform may be highly useful to diagnose MCS patients compared with healthy subjects, since individuals carrier of the heterozygous genotype CYP2C19 Ht *1/*2 has a threefold increased risk of developing MCS. Most importantly, the presence of genotype Ht *1/*2 is highly discriminant between MCS and either SMCS or FM/CFS patients.

Allele and genotype distribution for different CYP2C9 and CYP2D6 variants as well as for the CYP2C19 variant in MCS patients and healthy subjects recruited for this study resulted different, though not statistically significant from previously reported frequencies in Italian MCS patients by our group [25] and from both Caucasian and Italian general populations [26, 47]. In general, this may be prevalently due to the different technical approaches employed, since previous studies were based on the use of less sensitive PCR-RFLP technique. Additionally, and specifically with regard to the population of the affected individuals, the different frequencies observed in our previous study [25] could be derived from grouping together MCS individuals affected by different types of SRI comorbidities, including FM and CFS. Indeed, results obtained in the present study and discussed above, clearly show that allele and genotype frequencies for CYP2C9 variants as well as for the other examined CYP variants, are variable and reach statistically significant differences between the various disease states (MCS, SMCS, and FM/CFS). Thus, putting together affected individuals exhibiting different genetic backgrounds in one "disease" group may have generated a bias in those previously carried-out genetic studies.

Finally, some gene variants, both single or combined together, may be useful for the discrimination between different types of SRI or for the confirmation of clinically suspected MCS cases. In particular, the compound heterozygosity for CYP2C9 *2 and *3 variants may discriminate between the MCS and "other patients" groups versus SMCS, and the PM *41/*41 genotype may discriminate between MCS versus SMCS as well as versus patients with other pathologies. The compound heterozygosity for CYP2C9 Ht *1/*3 and CYP2D6 Ht *1/*4 may discriminate MCS cases as well as SMCS versus patients with other pathologies.

As a new direction of investigation, we here inquired if a described gene polymorphism of the transcription factor AHR - AHR Arg554Lys may be present with significant frequencies in a population of patients affected by specific SRI categories, fully diagnosed MCS, suspected MCS, and the FM/CFS group, in comparison with healthy individuals. Interestingly, although the distribution of the AHR Arg554Lys gene variant did not result significantly different between SRI cases and controls (Table 2), it proved indeed to be useful for the between-groups discrimination, when considered within different haplotypes (Table 3). In particular, we found that the haplotype CYP2C19 Ht*1/*2 - CYP2D6 Ht *1/*4- AHR Arg554Lys was only present in MCS cases, and the presence of the AHR variant was useful to differentiate MCS cases from SMCS patients, who showed also a high frequency of the haplotype CYP2C9 Ht *1/*2-CYP2D6 Ht *1/*4; this latter is absent in the group of "other patients." On the contrary, the various combinations of mutant alleles of the three CYP isoforms with heterozygosity for the AHR Arg554Lys gene variant were only observed in the subgroup of the "other patients."

In conclusion, on the basis of these observations, we propose the genotyping for CYP2C9*2 and *3, CYP2C19*2, and CYP2D6 *4 and *41 variants, together with the AHR Arg554Lys variant to be included in the panel of diagnostic biomarkers under construction aimed at the laboratory diagnosis of SRI and at the discrimination of the different specific conditions grouped under the collective definitions of "environmental-borne sensitivity-related illnesses," more largely known as "idiopathic environmental intolerances".

A further step of our study design will be the correlation between a given genotype and the corresponding biochemical profile as described by De Luca et al. [25], both to be also carefully correlated with assessed individual exposure levels to chemical toxicants, substrate of the examined receptor/enzyme-metabolizing actions. Given the gene-environment interactions and the fact of depending on the type of chemical toxicant and its metabolites, a given metabolizer phenotype may either confer protection or increase the risk of harmful effects; ruling out the effects of genetic background on the hypersensitive phenotype is expected to be very useful for clinical management.

Conflict of Interests

Authors declare no conflict of interests.

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Review Article

Multiple Chemical Sensitivity and the Workplace: Current Position and Need for an Occupational Health Surveillance Protocol

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Multiple chemical sensitivity, commonly known as environmental illness, is a chronic disease in which exposure to low levels of chemicals causes correlated symptoms of varying intensity. With the continuous introduction of new substances, people with MCS suffer significant limitations to their living environment and frequently to their workplace. This paper describes the current situation as regards MCS and the critical points in its case definition, which is still not generally agreed upon; this makes it difficult to recognize with certainty, especially, its precise relationship with work. Other problems arise in relation to the occupational physician's role in diagnosing and managing the worker with the disorder, the question of low levels of exposure to chemicals, and the best measures possible to prevent it. A diagnostic "route" is proposed, useful as a reference for the occupational physician who is often called in first to identify cases suspected of having this disease and to manage MCS workers. Work-related problems for people with MCS depend not only on occupational exposure but also on the incompatibility between their illness and their work. More occupational physicians need to be "sensitive" to MCS, so that these workers are recognized promptly, the work is adapted as necessary, and preventive measures are promoted in the workplace.

1. Introduction

Multiple chemical sensitivity (MCS), often referred to as environmental illness (EI), is an acquired chronic disorder in which exposure to low levels of chemicals causes related symptoms of varying intensity, from mild to totally disabling. Symptoms can affect multiple organs or systems: nervous, cardiovascular, gastrointestinal, respiratory, genitourinary and skeletal-muscular systems, skin, and ocular epithelia [1–4].

The etiology and pathogenesis of MCS is still not clear and it is hard to estimate its prevalence on account of numerous factors. For instance, (a) the various names given to the disorder and the fact that a single term can often comprise several pathological pictures mean it is difficult to find pertinent published studies; (b) there still seems to be no case definition accepted by all healthcare workers; (c) most reports do not list in full the criteria used to define cases; and (d) the various studies often use different diagnostic tools and investigation strategies (telephone interviews, hospital diagnoses, etc.).

Often the prevalence rates in the literature are "self-reported," with substantial differences between the percentages of self-reported cases and those diagnosed by medical staff, particularly by occupational physicians. Between 13% and 33% of people in various populations consider themselves to be "unusually" sensitive to certain common environmental chemicals [4–11].

The literature review for the preparation of the *Documento de Consenso sobre Sensibilidad Quimica Multiple* (Consensus Document on Multiple Chemical Sensitivity, based on the best available scientific evidence, is intended to help healthcare workers make decisions on diagnosis, treatment, prevention, and other aspects of MCS) shows a difference between the percentage of people who consider themselves ill (0.48–15.9%) and those diagnosed by physicians (0.5–6.3%) [12].

In a US study in 2003 on a sample of the urban population of Atlanta, self-reported MCS was 12.6%, while medical diagnosis is found only 3.1% [13]. In a study a year later on the entire US population, the prevalence of self-reported MCS was 11.2%, while medical diagnosis gave a figure of 2.5% [9]. A study in Germany in 2005 found a prevalence of self-reported MCS of 9% while the prevalence from medical diagnosis was 0.5% [14].

Different data collection methods might partially explain the differences in prevalence of MCS. On the other hand, since MCS is underdiagnosed, it is probably more useful to rely on epidemiological surveys.

In the US it is estimated that, respectively, 12%, 16%, and 18% of the local population in Atlanta, California, and North Carolina are particularly sensitive to chemicals [9, 10, 15].

A study by Caress and Steinemann in the US population found 11.6% of people reporting adverse effects from exposure to perfumed products [16]. A study funded by the Ontario Ministry of Health found that 3.1–6.3% of the Canadian population reported diagnosis of MCS [17].

A survey in Nova Scotia, Canada, showed that 3% of the Canadian population had had a diagnosis of environmental illness, but also that one in eight adults had complained of symptoms, gone absent from work, and complained of impaired ability to work due to exposure to "normally safe" levels of some common chemicals [18].

A Canadian Community Health Survey (2005) reported the prevalence of MCS in a target population (excluding Canadians living in institutions, native Canadians living on reserves, full-time members of the armed forces, and Canadians living in remote regions) by age and sex; the total prevalence was 2.5% of adult Ontarians, rising with age, and peaking at 5.8% in women between the ages of 60 and 64 years [11].

As regards the sex distribution of MCS, in all studies women were the most affected. Proportions were between 55 and 100%, with a mean of 81.5% [19]. Other studies too found a larger number of women with MCS, with 60.7% and 86.2% [9, 20]. Women may be more vulnerable because of exposure to chemicals at home and other indoor workplaces such as offices, hospitals, or schools. Even biological and hormonal differences make women more vulnerable. Many compounds in pesticides and plastics are endocrine-disrupting chemicals (EDCs) that can copy or imitate natural hormones. EDCs tend to accumulate in fat, interfere with the function of hormones in the body, and can cause other health problems, even at low levels of exposure [21].

2. Case Definition

The case definition has seen changes over time. In 1987, Cullen identified MCS as "an acquired disorder characterized by recurrent symptoms, referable to multiple organ systems, occurring in response to demonstrable exposure to many chemically unrelated compounds at doses far below those established in the general population to cause harmful effects. No single widely accepted test of physiological function can be shown to be correlated with the symptoms" [22].

In February 1996, the invited experts forming a workshop organized by the International Program on Chemical Safety (IPCS) of the WHO, the United Nations Environment Program (UNEP), and the International Labor Organization (ILO) recommended a new name: idiopathic environmental intolerances (IEI) because the term MCS "makes an unsupported judgment on causation" (i.e., environmental chemicals). This concept was taken from Sparks (2000), who defined IEI as a chronic recurrent condition, caused by a person's inability to tolerate an environmental chemical or a class of exogenous chemicals [23–26].

IEI, according to the proponents, is a complex gene-environment interaction, whose real cause is not known, for which it is possible—though not always—to identify a triggering event (e.g., sniffing a substance) and a response involving one or more organs or systems. Depending on its characteristics (i.e., the prevalence of somatic or psychological disorders) it can be confused with allergic reactions or psychiatric illness [27, 28].

However, multiple chemical sensitivities (MCS) is still the term most widely used to describe the complex syndrome; it presents as a chain of symptoms linked to a wide variety of environmental agents and components, at levels normally tolerated by most people [11].

The first attempt to establish some criteria for the standardization of the symptoms and their classification was proposed in 1987 and has to do with the compatibility between symptoms and exposure to chemicals, the supposed relationship between exposure and the onset of symptoms, and the exclusion of other known diseases. Lax and Henneberger (1995), analyzing the data for hundreds of individuals considered to have MCS, showed that only 6.4% met the diagnostic criteria of Cullen [29].

Currently, the most widely adopted criteria for the recognition of MCS are proposed in a consensus document [30, 31]:

(1) the symptoms are reproducible with (repeated chemical) exposure;

(2) the condition is chronic;

(3) low levels of exposure (lower than previously or commonly tolerated) result in manifestations of the syndrome;

(4) the symptoms improve or resolve when the incitants are removed;

(5) there are responses to multiple chemically unrelated substances;

(6) symptoms involve multiple organ systems (added in 1999).

This international document, published in 1999, was the product of a multidisciplinary study conducted by 89 clinicians and researchers with broad experience in the field. There were 36 allergologists, 23 occupational physicians, 20 clinical ecologists, and 10 internal medicine and ENT—ear, nose, and throat specialist, with the aim of establishing a case definition for MCS.

In 2005 Lacour et al. [32] proposed extensions to the definition criteria, including the following:

(1) chronic condition lasting more than six months and causing deterioration of lifestyle and body functions;

(2) symptoms recur reproducibly and affect the nervous system, with a characteristic hypersensitivity to odors;

(3) continuous involvement of the central nervous system and of at least one other apparatus;

(4) responses induced after low levels of exposure;

(5) responses to multiple unrelated chemicals;

(6) improvement or resolution after removal of exposure.

The wide range of symptoms with which MCS manifests and the difficulties of differentiating them from other pathologies—immunologic, digestive, cardiac, respiratory, psychiatric, neurologic, endocrine, and so forth—make it hard to develop a diagnostic tool that specifically identifies patients with MCS. The 1999 Consensus Document suggests using the Environmental Exposure and Sensitivity Inventory (EESI) to investigate patients for MCS. The authors subsequently modified this for faster, more widespread use, as the Quick Environmental Exposure and Sensitivity Inventory (QEESI). Some investigators have used the questionnaire in its original form but modified or adapted to take account of geographical differences [3, 33–41].

The QEESI was developed as a screening questionnaire for multiple chemical intolerances (MCI). The instrument has four scales (symptom severity, chemical intolerances, other intolerances, and life impact) and can be used for the following applications:

(1) research: to characterize and compare study populations and to select subjects and controls;

(2) clinical assessment: to obtain a profile of patients' self-reported symptoms and intolerances; patients can be asked to complete a QEESI at intervals in order to follow the course of their illness over time or in response to treatment or exposure avoidance;

(3) workplace or community investigations: to identify and provide self-assessment information to individuals who may be more susceptible or who report new intolerances; affected employees should have the opportunity to discuss the results with investigators or their personal physicians.

A simplified version of this questionnaire was employed in a study by Fabig for screening MCS patients. The first part focuses on the type(s) of substance with which the patient might have contact and the intensity of the disorders present. The second part examines the type and severity of the disorders the patient suffers after exposure to the culprit substance(s).

In the original QEESI there were from 0 to 10 responses for each substance. The modified version avoids this "excessive detail of subjective evaluations," with three possible answers to each question on the level of the disorders related to ten types of exposure. The minimum score in the QEESI modified according to Fabig is 10 (no disorder), and the maximum is 30 (serious disorders after exposure to all the substances listed). A score between 10 and 20 indicates a normal situation, while 21–30 suggests MCS [42].

There are other questionnaires too, to help in diagnosis. One of these is the University of Toronto Health Survey (UTHS) which starts with various case definitions for MCS. It then identifies a series of symptoms related to low-dose exposure. The reproducibility of the UTHS is evaluated in relation to the seven case definitions. Another diagnostic aid is the Idiopathic Environmental Intolerance Symptom Inventory (IEISI) which investigates the frequency of symptoms in MCS patients [22, 30, 37, 43–46].

Other questionnaires investigate the severity of the environmental chemical sensitivity [8, 11, 36, 47–49].

3. MCS and Work

3.1. Work-Related MCS and Workers with MCS. A definition of work-related MCS was introduced in 1987 [22]. This definition, subsequently amended, includes a number of health effects observed in workers who had been exposed to low levels of different chemicals. With the continuous introduction of new substances, both indoors and outdoors, people with MCS suffer significant limitations in their living environment [50] and frequently in their work environment.

There are more than 70 million unique chemical substances, organic and inorganic, on the market, such as alloys, coordination compounds, minerals, mixtures, polymers, and salts. Every day about 15,000 new substances are added [51]. The chemicals are found in many products of daily use, such as detergents, textiles, clothing, and furniture. They are employed not only by workers in the industries that produce them, but also—widely—in other industries: construction, metalworking, woodworking, automotive, textile, food, agriculture, information technology, waste management, cleaning, and so forth.

The work-related problems for people with MCS do not depend only on occupational exposure but also on the incompatibility between their illness and their work [52]. A study of people with self-reported MCS found that three-quarters of the 268 respondents had lost or had had to leave their jobs because they did not tolerate exposure to chemicals present in the environment. The Human Ecology Action League (HEAL) survey of 269 people with MCS showed that 45% had lost their jobs [53]. Caress and Steinemann found that 1.8% of their random community sample of 1582 people had lost their jobs on account of hypersensitivity to common chemicals [12].

3.2. Categories of Workers at Higher Risk. It is usual to define as "sensitive" an individual who responds adversely to low exposures to chemicals. While a person sensitive to chemicals can be found in any group, a classification of the job categories most at risk has been attempted several times. At first, in studying the relation between MCS and work, attention focused mainly on patients who were industrial workers, initially suggesting that MCS may be linked to

Table 1: Classification of exposure conditions and demographics by Ashford and Miller (amended and supplemented by Winder).

Group	Nature of exposure	Demographics
Industrial workers	Acute or chronic exposure to industrial chemicals	Primarily males; 20–65 years old
Office workers (in "tightly closed buildings")	Inadequate ventilation. Offgassing from construction or refurbishment materials or from office equipment. Tobacco smoke	More females than males. White-collar workers. 20–65 years old. School children
Contaminated communities	Toxic waste sites. Contamination by nearby industry sites. Aerial pesticide spraying. Groundwater contamination. Other community exposures	Low to middle classes. All ages, male and female. Children or infants affected first or most; possible effects in pregnant women
Individuals	Heterogeneous. Indoor air (domestic). Pesticides, consumer products, and drugs	White middle to upper classes, primarily females, 30–50 years old

occupational, therefore potentially intense, chemical exposure. A subsequent study by Cullen and coworkers of all MCS patients seen at Yale University Occupational Medical Clinic between 1986 and 1992 found only low rates of MCS in industrial sectors, associated with the highest rates of chemical and physical injuries. Only about 27% of patients with MCS were occupationally exposed to chemicals present in the construction and manufacturing sectors, paradoxically suggesting that exposure backgrounds with low levels of chemical exposure are more likely to be associated with MCS than those with high exposure [54].

After some time, similar problems were described in occupants and workers in "tight" (tightly closed) buildings, residents of communities whose air and water were contaminated by chemicals and persons who had experienced personal exposure to various chemicals in domestic indoor air [55]. The classification of Ashford and Miller was further amended and supplemented by Winder [56], as shown in Table 1, outlining the exposure conditions and demographics:

(i) workers who are occupationally exposed to chemicals as part of their everyday activities;

(ii) employees who work in tightly closed buildings;

(iii) individuals working in contaminated areas;

(iv) people who, for one reason or another, were unexpectedly exposed to a chemical substance.

The authors described various demographic characteristics of these groups. For example, industrial workers are predominantly male, whereas those with chemical sensitivity from tightly closed buildings and those with "personal and unique" chemical exposures are a heterogeneous group, though predominantly female, white-collar, or professional.

Similarly, of 200 individuals with MCS (case definition not mentioned), observed at an environmental health center in Dallas, USA, less than 5% were workers, and the highest percentage (25%) were housewives, suggesting an association between certain domestic chemical exposure events and MCS. Just like the demographic findings of other studies, most of these MCS patients were women, who presented themselves for examination mainly at an age of around 30 to 40 [57].

Lax and Henneberger in 1995 [29] identified 35 of the 605 new patients who presented for visits between 1989 and 1991 as meeting a case definition similar to that proposed by Cullen [22]. In this study, 54% of the non-MCS patients had worked in sectors considered to be at greater risk of dangerous exposure to chemicals than other workplaces. In contrast, only 26% of patients with MCS were employed in the more risky sectors [58].

The US Environmental Protection Agency (EPA) reported that about one-third of people employed in a closed work environment reported particular sensitivity to one or more common chemicals [59]. In fact, supporters of the existence of MCS have described a greater spread among women, aged between 25 and 50, who spend many hours inside sealed or otherwise closed buildings, those living and working in cities with high pollution, and among the users of deodorants, perfumes, detergents, insecticides and herbicides [9].

In the work environment, Watanabe et al. identified as at-risk categories users of chemicals, especially volatile compounds such as organic solvents, or workers belonging to certain categories such as farmers, construction workers, urban policemen, and hairdressers, but especially housewives [60]. Lucchini et al. identified the professional categories most frequently affected by the syndrome as workers in industry in general (where it is easier to come into contact with chemicals) and in particular where solvents are used, as well as farmers, construction workers, policemen, hairdressers, housewives, and office workers [61]. A 2008 study examined pest controllers frequently exposed to pesticides, a class of chemicals commonly associated with MCS. There was an increased risk of development of MCS in this category [62].

In 2005, a South Australian parliamentary inquiry into MCS collected data from healthcare professionals caring for patients suffering from the disorder, which showed the role played by certain chemicals such as detergents, glutaraldehyde, and formaldehyde in triggering MCS. In support of this theory, in 1998 a national support group was established for individuals who suffer from health problems apparently related to exposure to hazardous chemicals in the workplace, including glutaraldehyde. So the Glutaraldehyde-Affected Support Persons Injured Nurses Group (GASP-ING), which evolved primarily as a network of shared experience, identified glutaraldehyde as a chemical of particular concern for healthcare workers [58].

The oils and hydraulic fluids used in aircraft engines can be toxic, and specific ingredients of oils can be irritating,

Table 2: Categories at high risk of MCS.

Industrial workers	Workers with acute or chronic exposure to industrial chemicals
Other workers	Farmers
	Hairdressers
	Healthcare workers with specific activities (e.g., radiographers, anesthetists)
	Urban policemen
	Flight crew
	Cabin crew
	Swimming pool workers
People who live or work indoors	Teachers
	Students
	Office employees
	Housewives
	Construction workers
	House painters
People who might be exposed to toxic chemicals only once	Workers with exposure to pesticides
	Workers with exposure to drugs
	Victims of industrial accidents
	Victims of chemical accidents
Office workers	Office workers in tightly closed buildings

sensitizing, and neurotoxic. In fact, flight crews and cabin crews have identified exposure to engine oil or hydraulic fluid leaks as a cause of their diagnosis of MCS [63].

A report from the Danish Environmental Protection Agency in 2005, that reviewed the state of knowledge regarding MCS, reported cases in Denmark among people exposed to organic solvents or pesticides at work [64]. A group from the Division of Environmental Medicine in Stockholm reported a higher frequency of MCS-like symptoms among housepainters than other job categories [65]. In a Danish study, two cases of MCS were described among workers employed in public swimming pools. Chlorine vapors, which are formed in special circumstances, caused the onset of symptoms (e.g., trihalomethanes and chloramines). In both cases, the patients had to leave work and go through reeducation. The Danish consensus document indicates that a wide range of people from different professional groups display symptoms of MCS: healthcare, aviation, farmers, mechanics, and aluminum workers at Alcoa Wagerup [64].

An analysis of the literature shows that there are numerous categories at risk of developing MCS (Table 2).

3.3. Chemicals Related to MCS. Many substances have been called into question in the onset of MCS. It is difficult to classify them comprehensively and systematically, partly because of the continuous introduction of new chemicals on the market. There are, however, numerous extensive lists in several publications and articles.

Ashford et al. in the European report of 1994 proposed a classification of chemical compounds associated with MCS, grouped according to the source of exposure [66]:

(i) external contamination: pesticides, volatile solvents, and paint fumes;

(ii) fuels, combustion products, tars, emissions from diesel and gasoline engines and air of industrial areas;

(iii) indoor air pollution at home and at work, especially in confined spaces: products of gas combustion and domestic heating, synthetic sponges, plastics, pesticides, perfumes, deodorants, detergents, cleaning products, disinfectants, ink of newspapers and other printed materials, fabrics, curtains, rugs, odors of petroleum derivatives, wood, and cooked food;

(iv) food additives and contaminants, such as corn and sugar, residues of pesticides, fungicides, artificial colors, preservatives, food sweeteners, protective waxes, and packaging materials;

(v) water contaminants and additives ingested in drinking water;

(vi) drugs and consumer products such as aspirin, barbiturates, sulfa drugs, diluents, flavorings, preservatives, mineral oils, lotions, laxatives, synthetic vitamins, adhesive tape, cosmetics, perfumes, shampoos, personal hygiene products, dental adhesives, salts and bath oils, water beds, pens, polishes, chlorinated pools, radiographic contrast medium, contact lenses, plastic components, and medical equipment.

Ziem in 1999 identified substances that can cause MCS generally after repeated exposure to low doses: pesticides, solvents, combustion products, renovating "sick" buildings, carbonless copy paper, other irritants, and petrochemical products [67].

In the USA in 2003 a list of twelve chemicals that trigger symptoms was published—substances which in a population study were most frequently associated with MCS. The list of chemicals included cleaning products, perfumes, pesticides, traffic fumes, products used in beauty and hair salons, carpets, furniture, chlorine in drinking water, and fresh ink markers [19].

In general, perfumes are frequently signaled as chemical compounds of interest (82.5%), followed by tobacco smoke, new housing, pesticides, petroleum products, fumes from combustion engines, and other chemicals [68]. Table 3 lists the agents recognized as related to MCS proposed in the Spanish Consensus Statement of 2011 [69].

A new group of people were described only a few years ago, with particular symptoms associated with exposure to electromagnetic radiation in connection with the use of electromagnetic devices. There is currently no scientific evidence that these symptoms are related to MCS [12, 27].

4. Primary and Secondary Prevention of MCS at Work

As the etiopathology of MCS is still not clear, the most effective approach to manage the disorder appears to be avoidance of triggering factors or situations in which the problem might arise. Consequently the reduction of environmental

Table 3: Agents related to MCS.

(i) Organic solvents, paints, and lacquers for finishes (xylene, methylene chloride, petroleum distillates, glycol ethers, and trichloroethane)
(ii) Pesticides (diazinon, azinphos-methyl [Guthion], and other organophosphates)
(iii) Smoke and fumes from welding
(iv) Metals (nickel, lead)
(v) Various chemicals (formaldehyde, freon, ethanol, nitric acid, hydrochloric acid, and toluene)
(vi) Powder and dust (wood, beet sugar)
(vii) Food
(viii) Certain diseases (scabies, herpes zoster)
(ix) Perfume and air fresheners (shampoo, nail varnish and nail varnish remover, colognes, shaving lotions, various cosmetics, deodorants, etc.)
(x) Furniture
(xi) Paper
(xii) New buildings

chemical exposures, particularly to pesticides and petroleum derivatives, is extremely important to reduce episodes of the disease. As this type of exposure seems to be associated with MCS, its reduction could have a wider preventive impact, for the general population too. Many of the chemicals involved have doubtful social utility, so reducing their use is anyway recommendable [11, 70].

The European legislation on chemicals has been "updated" and involves an integrated system of registration, evaluation, authorization, and restriction of chemicals—REACH—with the aim of protecting human and environmental health, and the European Chemicals Agency now deals with everyday questions relating to the REACH requirements, but it is still proving difficult to revise the exposure levels accepted to date [71].

It would be useful to have further knowledge of MCS and possible preventive approaches to support the coordination of national health surveillance projects and control of the use of chemicals as a result of application of this legislation throughout Europe. The findings of biomonitoring programs on persistent organic compounds could be integrated with data on the levels of human exposure to numerous other chemicals and the exposure situations that come to light from MCS research.

The main triggers may involve single exposure to high doses or multiple exposures to one or more substances. It is not always simple to verify the latter situation because the people concerned cannot always reconstruct their personal and/or occupational history. In addition, exposure may arise in different circumstances—at work, at home, accidents, food, and so forth. Since the risk of exposure to dangerous substances is theoretically universal, the persons involved would have to be isolated in order to avoid it. Clearly, this is not often feasible because it would be incompatible with work and daily life. Then too, with our still limited knowledge in this field, we have no means of creating public spaces where people would be fully protected from exposure.

Since it is prudent to avoid reexposure to triggering factors, people are advised to modify their usual habits, ventilating their premises thoroughly at home and at work, avoiding damp places, avoiding exposure to irritants such as gas or vapors, and following an appropriate diet, as far as possible. Avoiding exposure in daily activities and at work and changing lifestyle as necessary are in fact more effective measures than any therapy. Undeniably, however, these measures may limit a person's relations with other people, their access to work, and recreation.

At work, establishing an environment suitable on the whole for someone's health may require significant changes, which may need the cooperation of the people responsible for prevention—the occupational physician and occupational psychologist.

If the safety and prevention officers at work can identify the source of the problem at an early stage, this may be helpful in preventing the sensitivity mechanism from spreading and becoming chronic. It will be their task, with the occupational physician, to assess any adaptations or job change, considering the persons with MCS as sensitive workers. Workers who suspect they have MCS symptoms or their symptoms have got worse after exposure at work should therefore always consult the occupational physician [11].

An American study examined 605 patients at the Central New York Occupational Health Clinical Center in Syracuse, New York, between 1989 and 1991, to identify any who had a possible, probable, or definite diagnosis of MCS; 7.9% had a diagnosis, and the criteria for admitting them as MCS patients in the study required them to be defined as MCS cases, with evidence of exposure in the workplace. The identification of the pathology as work related relied on the clinical judgment of the physician who had examined the patient was based on several criteria: onset of symptoms following specific exposure in the workplace, worsening of the symptoms at work improvement when not there, no evidence of significant exposure outside the workplace, and symptoms among workmates [29].

People with MCS/EI may present some limitations, varying in severity from one individual to another. Not all of them need to make adaptations in order to work; some may only need minor changes. Adaptations may involve the ventilation system and quality of indoor air, the lighting, or aspects of the building, renovation work, and cleaning in the premises [72].

For example, in June 2009, the CDC put on its internal website an Indoor Air Environmental Quality Policy intended to maintain good indoor air quality in buildings where its employees work. Among other things, the policy states that scented or fragranced products are prohibited at all times in all interior space owned, rented, or leased by the CDC. This includes the use of the following products:

(1) incense, candles, or reed diffusers;

(2) fragrance-emitting devices of any kind;

(3) wall-mounted devices, similar to fragrance-emitting devices, that operate automatically or by pushing a button to dispense deodorizers or disinfectants;

(4) potpourri;

(5) plug-in or spray air fresheners;

(6) urinal or toilet blocks;

(7) other fragranced deodorizer/reodorizer products.

Personal care products (including colognes, perfumes, and essential oils) should not be applied at or near actual workstations, restrooms, or anywhere in CDC-owned or leased buildings. In addition, the CDC encourages employees to be as fragrance-free as possible when they arrive in the workplace. Fragrance is not appropriate for a professional work environment, and the use of some products with fragrance may be detrimental to the health of workers with chemical sensitivities, allergies, asthma, and chronic headaches and migraines [73].

According to "A Guide for the Workplace" by Sine et al. a "no-scent" policy includes perfume, cologne, and aftershave and scented personal care products such as deodorants, shampoos, hair products, cosmetics, soaps, hand creams, laundry detergents, and fabric softeners. Smoke-laden and dry-cleaned clothing must be aired well before wearing. Avoid scented laundry detergents and all fabric softeners [74].

The CDC Indoor Air Quality Policy is a very important document and provides an example of what we should be doing in every workplace. All workplaces should be fragrance-free. The number of people who are chemically sensitive and/or have been diagnosed with MCS is increasing daily. This problem is very similar to those faced by workers when smoking was allowed in the workplace. The implementation of a smoke-free workplace policy by the Occupational Safety and Health Administration and other regulatory agencies has been very important in preserving the health of workers in those workplaces.

A fragrance-free policy allows individuals who are chemically sensitive to continue their employment. As a result, they do not have to turn to Social Security Disability for income. Those who are not the beneficiaries of a fragrance-free policy are often unable to work and do find themselves on Social Security Disability [73].

5. The Occupational Physician's Role in MCS Risk Management

In accordance with current European regulations, the occupational physician cooperates with the employer and the prevention and protection service in assessing risk in the workplace; when necessary this may involve establishing and implementing general health measures to safeguard workers' health and safety.

The general measures for this purpose include health surveillance, which the physician must plan and carry out in relation to the specific risks found in each workplace on the basis of the risk assessment, and in the light of the latest scientific information. Since it is the occupational physician who cooperates in drafting a risk assessment report—with its implications for health surveillance—his/her contribution is particularly important in assessing chemical risk. Often this type of risk assessment is based on algorithms and software that automatically quantify the secondary risk deriving from the use of dangerous substances (chemical risk) but which overlooks some of the fundamental principles of occupational medicine.

Software and algorithms can take into account numerous variables to establish how "important" a risk for health is: the amounts used, acceptable limits, and so forth. Some substances or classes of chemicals, however, need closer analysis because they can have an effect—causing harm—with no dose relation. The occupational medicine physician's contribution to any assessment of chemical risk is therefore a basic starting point, particularly as regards the idea of "low doses."

The dose, or concentration, of a substance is considered low when—unlike a high dose—it has no toxic effects, is not measurable (it is below the level of detection), or does not significantly differ from the values found in the nonexposed population (reference value); it may be well below the limits allowed in a workplace.

The relation between dose and effect, or response, is the basic principle for assessing toxic effects in general and those of chemicals in particular. The effect, however—or better, the probability of the effect (the risk)—does not depend solely on exposure, where the dose or concentration is the main quantitative variable; it depends on two other variables—the type of risk factor and the individual susceptibility of the person exposed. In other words some people may show high susceptibility to a certain chemical, either genetic or acquired, and cannot be protected even from low- or very low-dose exposure, for acute or chronic effects.

Hypersusceptibility causes an abnormal reaction to concentrations of a substance that would have no effect in most people; most members of a population would show no change in their health after exposure to a certain concentration of the substance, but a small proportion would suffer health problems—of varying severity—when exposed to the same concentration. Ideally, the occupational physician's checkup before someone is hired should aim to find out whether the candidate has any conditions and/or contraindications to the work proposed (e.g., hypersusceptibility) so that workers can be placed in an environment suited to their physiological and psychological capacities. Another aim of the prehiring checkup is to see whether the candidate is fit for the work proposed without posing any risk to him/herself or others [75].

When a person starts a job s/he must be questioned to detect any congenital or acquired conditions that might influence his/her susceptibility in the specific job. It is not a good idea to employ only hyposusceptible workers in a polluted workplace instead of taking all possible measures to clean up the environment; efforts should be made in the exact opposite direction—try to reduce the pollution so that even hypersusceptible people can work there.

It is a widespread idea in occupational medicine that the physician has detailed knowledge of each job—the work done, work cycles/shifts, workplaces, procedures, machinery, equipment, chemicals employed, and so forth. S/he must also be familiar with the workers' health and must have the tools to protect it. The occupational physician must assess a worker's

clinical situation at the work station and judge whether that person is fit for the specific task, so as to safeguard workers with contraindications to the tasks required of them. Should the physician decide that a worker is not fit, on account of a disorder that has just become evident, s/he is obliged to assess whether the pathology is related to occupational exposure and, therefore, whether it calls for reexamination of current preventive measures [76].

Formulating a hypothetical diagnosis of MCS related to occupational conditions is part of this examination, but the facts that there is still no agreed definition of the disorder and MCS is not even universally recognized are important barriers to any evaluation. The following criteria, however, may be useful for formulating a diagnosis: (1) symptoms start after specific exposure in the workplace; (2) the symptoms become worse during work and may improve when the person moves away from the source of exposure; (3) there is no significant evidence of exposure outside work; and (4) colleagues at work show none of the symptoms [29].

One of the occupational physician's constant worries is that a worker will develop functional alterations that mean s/he cannot do the job; this calls for an opinion on the worker's temporary or permanent unfitness for the work. It is worth recalling that a person with MCS may usefully be moved temporarily from the job or given an alternative job for a short while; it is equally important, if s/he shows improvement, to give the worker a chance to return to work, if necessary with retraining.

The occupational physician faced with the possibility of a diagnosis of MCS may wish to work on the following considerations:

(1) MCS is caused by occupational exposure: MCS subjects should be overrepresented in occupations with high relevant exposure (e.g., neurotoxic);

(2) MCS is not caused by occupational exposure: the occupational distribution of MCS subjects should not differ from the general working population;

(3) MCS is a chance effect, caused by strictly individual health problems: the occupational distribution of MCS subjects should not differ from the general working population;

(4) MCS is an (acquired, genetic) susceptibility effect: the occupational distribution of MCS subjects should not differ from the general working population (unless susceptibility determines choice of career);

(5) MCS is an oversensitivity plus selection effect (e.g., an accumulation of oversensitive individuals in underexposed intellectual occupations) MCS subjects should be underrepresented in occupations with high relevant exposure (e.g., neurotoxic);

(6) MCS is a selective perception effect (e.g., different attitudes about "complaining" of exposure): the occupational distribution of MCS subjects should differ from the general working population, without MCS correlating with exposure.

As we have already noted, the MCS subject/worker develops symptoms at exposure levels well below official limits for the workplace, and sometimes in response to substances or preparations used, or commonly present, in any workplace's new furniture, cleaning products, printed paper, fragrances and perfumes, and so forth. The occupational physician must therefore find out what substances and preparations are used, based on information gained during scheduled inspections of workplaces, from prevention and protection services, and from talking to individual workers. S/he should also—as the CDC policy recommends—ask to be informed of the introduction of new chemicals in the work cycle, before they start to be used and of "renovation work or installation of new equipment" before it is done in the workplace. This practice would enable people with MCS and other chronic diseases such as asthma, allergies, and chronic obstructive lung disease to make other arrangements while the renovation work is going on, or the new carpeting, for example, is being laid. It also would allow them to discuss with their supervisors new chemicals that are being introduced into the work environment because often people who are chemically sensitive know about alternatives that are less toxic, not only for them but also for other people who may be affected [73].

Hygiene and safety rules at work do not only include health surveillance as one of the general measures for protecting workers' health, but expect it to be preceded by measures that give priority to eliminating or reducing risk [11].

5.1. Workers with MCS: Occupational Health Surveillance Protocol. The occupational physician's main responsibility is, as we have already said, to assess each worker's clinical status and, as required by local regulations, to establish a protocol for health surveillance. Its main aim would be to verify that there are no contraindications to a worker doing the job he is expected to do, to formulate a judgment that the worker is fit for that specific task, and to keep an eye on workers' health in the longer term. The physician has to plan and conduct this health surveillance following health protocols drawn up to take account of the specific risks in each case, in the light of the latest scientific knowledge.

Traditionally, the application of medical principles was a static process, modified on occasion by the practitioner's experience. In recent years, however, partly fuelled by computer-accessible databases, techniques for systematic review of clinical guidelines and economic analyses have become more widespread. For a variety of reasons, these evidence-based methods have only recently been applied to occupational health risks and interventions. As noted by Carter, the application of these methods in occupational medicine would likewise "improve the quality of prevention and would also enable practitioners to give more soundly based advice and to secure their professional positions as providers of quality-assured information" [77].

The analysis of the literature shows there is no single test yet that has proved 100% effective in diagnosing all MCS patients. MCS also does not meet the classic definition of an occupational disease which establishes a link between a specific condition and a specific exposure, as is the case of asbestosis and asbestos exposure. In fact exposure to a wide variety of substances can trigger a broad range of symptoms

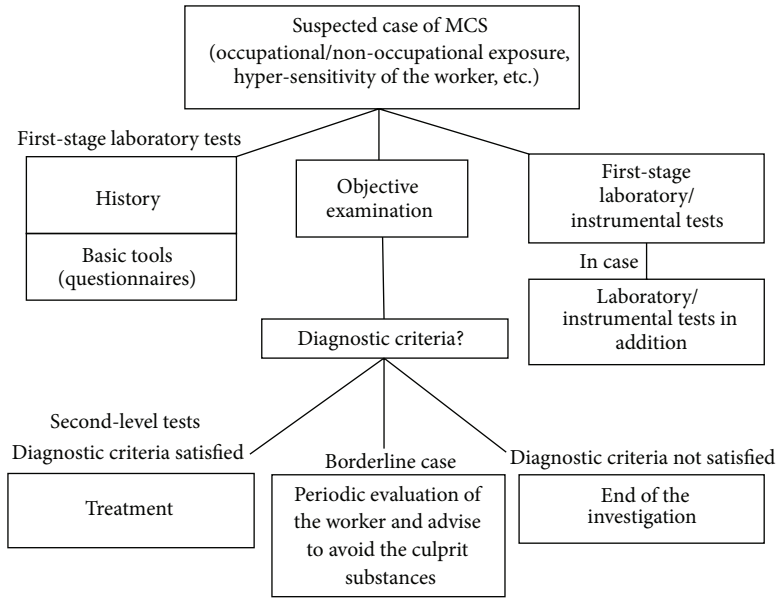

Figure 1: Flow chart-proposal of diagnostic protocol.

in MCS sufferers. A different approach must therefore be used to study the disease which is why there is no single known symptom or any definite test to establish an MCS diagnosis. The only sure element a physician can rely on is the person's environmental and medical history, combined with routine laboratory tests, which are intended to exclude other diagnoses [11].

We outline a diagnostic strategy here for MCS, which may be useful to occupational physicians (Figure 1).

Starting from the suspicion of MCS, based on various indications, episodes of exposure at work or outside, and/or hypersensitivity, the worker's history is collected in a detailed interview aimed at getting information on the chemical features of the environment where the disorder first presented itself, the setting where it developed, and the (work or personal) environment where the worker now spends his/her time; data must be recorded on environmental exposure times to chemicals, odor producing or not, indoors (household cleaning products, fitted carpets, etc.) and outdoors (nearby industrial plants, potential sources of pollution, etc.). Note when the indicative signs or symptoms appeared using a specific, detailed questionnaire, and record any adverse reactions to foods or drugs.

The objective examination must focus in particular on the worker's organs and systems related to the symptoms reported and on any signs that appeared subsequent to the exposure to chemicals.

Basic tools in this phase are questionnaires (QEESI, UTHS, IESI, etc.), to be used before verifying that recognized diagnostic criteria are met. Tests may then be scheduled on the basis of each individual's history, the objective findings and/or any suspicion of associated pathologies, so as to exclude any other disorders.

The following may be useful first-stage laboratory tests:

(i) complete blood count;

(ii) serum iron, transferrin and ferritin;

(iii) glycemia;

(iv) immunoglobulins (IgG, IgM, IgA, and IgE);

(v) proteinemia and protein electrophoresis;

(vi) electrolytes (Na, K, Cl, and Mg);

(vii) lymphocyte typing;

(viii) inflammatory indices (ESR—erythrocyte sedimentation rate, CRP—C reactive protein);

(ix) liver function (GOT—glutamate oxaloacetate transaminase, GPT—glutamate pyruvate transaminase, gamma-GT—gamma-glutamyl transpeptidase, total and fractionated bilirubin total cholesterol, LDL—low density lipoprotein, and HDL—high-density lipoprotein, triglycerides);

(x) renal function (creatininemia, BUN—blood urea nitrogen).

In addition to laboratory tests, it may be useful to do an instrumental test such as global spirometry, which is fairly frequently used in occupational medicine.

Other tests may be added, on the basis of the worker's history, the objective findings, and the indications of each case:

(i) protein kinase C and isoenzymes;

(ii) thyroid function (FT3—free triiodothyronine, FT4—free tetraiodothyronine, TSH—thyroid-stimulating

hormone, antibody antithyreoperoxidase, antibody antithyroglobulin);

(iii) blood coagulation picture (PT—prothrombine time, aPTT—activated partial thromboplastin time, fibrinogen, and homocysteine);

(iv) antibody picture (ANA—antinuclear antibody, ENA—extractable nuclear antibody, antids-DNA—antidouble-stranded deoxyribo-nucleic acid, AMA—antimitochondrial antibody, ASMA—anti smooth muscle antibody);

(v) hepatitis virus markers (HbsAg—hepatitis B surface antigen, anti-HCV antibodies—antihepatitis C virus antibodies, and anti-HAV antibodies—antihepatitis A virus antibodies);

(vi) serum tests for CMV—Cytomegalo virus, EBV—epstein-Barr virus;

(vii) VDRL test—Venereal Disease Research Laboratory;

(viii) urine tests and urine culture;

(ix) screening for celiac disease (antiendomysium, antitransglutaminase, and antigliadin antibodies);

(x) breath test (urea, lactose, and lactulose);

(xi) glutathione transferase and catalase activities;

(xii) vitamins B1, B6, B12, folates, vitamin C, D3, E, and coenzyme Q10.

Second-level tests are indicated when the criteria such as the following are met:

(1) suspicion of a history of MCS but no other pathologies that might explain the patient's symptoms;

(2) substantial impairment of activities of daily life as a result of exposure to the chemicals;

(3) two or more organs or systems affected after exposure to known chemicals;

(4) questionnaire scores 21 or higher (the QEESI version modified by Fabig is recommended for its high sensitivity and specificity).

The following second-level tests may be useful:

(i) psychological assessment: personality questionnaires, self-assessment of symptoms of mental distress and quality of life (MMPI 1—Minnesota Multiphasic Personality Inventory 1, MMP2—Minnesota Multiphasic Personality Inventory 2, Rorschach test, and Zulliger test);

(ii) neurophysiologic test: reaction times (simple and/or selected), balance, visual contrast, color, and vibration tests;

(iii) allergy tests: skin reaction, patch test, specific and total IgE, eosinophilic cationic protein (ECP), and tryptase; oral exposure to drugs, foods, and additives; hypoallergenic diets;

(iv) genetic polymorphisms: tests for genes implicated in oxidation (PON1—paraoxonase 1, CYP2D6—cytochrome P450 2D6, NAT2—N-acetyltransferase 2, GSTM1—glutathione S-transferase M1, GSTT1—glutathione S-transferase theta 1, GSTP1—glutathione S-transferase P1, and CAT—catalase) and assay of plasma proinflammatory cytokines;

(v) assays in biological samples to check for chemicals, metals, and/or their metabolites;

(vi) metabolism and detoxification investigations.

If the worker fulfils the diagnostic criteria and further tests confirm the diagnosis of MCS, s/he should be sent for treatment. If it appears to be a borderline case, when the criteria are not all met but the clinical history suggests the problem may be there, further investigation is indicated, and the worker should be advised to avoid the culprit substances [11, 78, 79].

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Review Article

Nutritionally Mediated Oxidative Stress and Inflammation

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There are many sources of nutritionally mediated oxidative stress that trigger inflammatory cascades along short and long time frames. These events are primarily mediated via NFκB. On the short-term scale postprandial inflammation is characterized by an increase in circulating levels of IL-6 and TNF-α and is mirrored on the long-term by proinflammatory gene expression changes in the adipocytes and peripheral blood mononuclear cells (PBMCs) of obese individuals. Specifically the upregulation of *CCL2*/MCP-1, *CCL3*/MIP-1α, *CCL4*/MIP-1β, *CXCL2*/MIP-2α, and *CXCL3*/MIP-2β is noted because these changes have been observed in both adipocytes and PBMC of obese humans. In comparing numerous human intervention studies it is clear that pro-inflammatory and anti-inflammatory consumption choices mediate gene expression in humans adipocytes and peripheral blood mononuclear cells. Arachidonic acid and saturated fatty acids (SFAs) both demonstrate an ability to increase pro-inflammatory IL-8 along with numerous other inflammatory factors including IL-6, TNFα, IL-1β, and CXCL1 for arachidonic acid and IGB2 and CTSS for SFA. Antioxidant rich foods including olive oil, fruits, and vegetables all demonstrate an ability to lower levels of IL-6 in PBMCs. Thus, dietary choices play a complex role in the mediation of unavoidable oxidative stress and can serve to exacerbate or dampen the level of inflammation.

1. Introduction

There are many sources of nutritionally mediated oxidative stress that trigger inflammation along short and long time frames. In order to focus the discussion of this topic this review will address how consumption of food, the quantity of food, and the macronutrient constituents serve as sources of oxidative stress and inflammation (Figure 1). On the short-term scale postprandial mitochondrial oxidative stress leads to inflammation, a process that is most strongly influenced by quantity and is mediated primarily by nuclear factor κB (NFκB), and on the long-term scale chronic overconsumption leads to obesity, which induces more permanent states of inflammation through the generation of white adipose tissue which secretes proinflammatory factors. Gene expression changes associated with obesity serve as a lens through which to view the short- and long-term consequences of nutritionally mediated oxidative stress and inflammation. There are additional mechanisms through which fats and glucose mediate inflammation, and these will be briefly discussed. Numerous human intervention studies have implemented various strategies to ameliorate the impact of nutritionally mediated inflammation changes in gene expression. Review of these studies highlights how pro-inflammatory and anti-inflammatory consumption choices mediate expression of a similar set of genes in post-prandial inflammatory states and in chronic inflammatory states in obese individuals and that such changes can be observed in both human adipocytes and peripheral blood mononuclear cells.

2. Oxidative Stress as a Diet-Induced Condition

2.1. From Nutrient Overload to Oxidative Stress to Inflammation. Overconsumption of food leads to dysmetabolism a state where energy intake exceeds energy expenditure, and cellular oxidative stress ensues [1, 2]. The increase in oxidative stress leads to numerous downstream effects including the

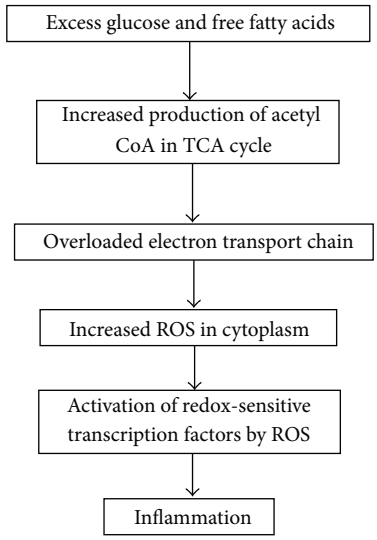

Figure 1: Downstream effects of nutrient overload. Overview of the downstream effects mediated by nutrient overload. Excess glucose and free fatty acids overwhelm the tricarboxylic acid (TCA) cycle which leads to an increase in the production of acetyl CoA. Excess acetyl CoA stimulates the mitochondria to produce excess superoxide in the electron transport chain, and the subsequent conversion of superoxide to hydrogen peroxide results in an increase of reactive oxygen species (ROS) within the cell. This change in redox status activates numerous redox-sensitive transcription factors, including NFκB, which is the main mediator of inflammatory responses.

induction of inflammatory cascades [1–3]. Figure 1 provides a simplified overview of the events that link overconsumption and inflammation. This process begins with mitochondrial overload of free fatty acids and glucose, which results in an increase in the production of acetyl coenzyme A (acetyl CoA), an enzyme important in cellular metabolism [4]. Higher levels of acetyl CoA result in an increase in reduced nicotinamide adenine dinucleotide (NADH) generation from the tricarboxylic acid (TCA) cycle. Increased availability of NADH increases electron generation by complex I of the mitochondrial electron transport chain and elevates membrane potential to the extent that complex III is stalled resulting in a longer half-life for coenzyme Q. Increased availability of coenzyme Q leads to an increased reduction of oxygen to superoxide ($O_2^{\bullet -}$). Thus the main impact of overconsumption of free fatty acids and glucose is higher levels of superoxide in the mitochondria [5]. Superoxide is a relatively unstable intermediate and in large part is converted to hydrogen peroxide in the mitochondria by superoxide dismutase. The newly formed hydrogen peroxide can then undergo a Haber-Weiss or Fenton reaction, yielding a highly reactive hydroxyl radical ($^{\bullet}$HO), which can oxidize mitochondrial proteins, DNA, and lipids and amplify the effects of the superoxide-initiated oxidative stress [6, 7]. The generation of highly reactive oxygen radicals can activate redox-sensitive transcription factors and result in numerous downstream effects, including triggering inflammatory cascades and increasing ROS production. Questions remain regarding the permeability of the mitochondrial inner membrane to superoxide, and there is some evidence suggesting that superoxide can permeate anion channels, which may serve as an additional source of oxidative stress in the cytoplasm [8]. Depending on the cell types, the impact of this oxidative stress can result in various forms of dysfunction, making this a complex system to understand and track [9].

It is important to note that there are additional mechanisms that can induce oxidative stress and inflammation both for glucose and free fatty acids, and those will be addressed in more detail later in this review. For now, the focus will remain on the oxidative stress induced by an overloaded TCA cycle.

2.2. Consequences of Oxidative Stress. A strong theory has emerged in the literature that supports the idea that the excess generation of superoxide in the mitochondria and the subsequent generation of reactive oxygen species lead to the cell's inability to deal with chronic mitochondrial oxidative stress and that the consequences of such stress in various cell types are responsible for several conditions including cardiovascular disease, type 2 diabetes mellitus, obesity, and metabolic syndrome. The sequelae of events that lead to these conditions have been detailed in the comprehensive review provided by Ceriello and Motz [5]. In short, the oxidative stress impacts pancreatic β cells by leading to the decreased expression of glucose transporter type 4 (GLUT4), which over time can support the onset of type 2 diabetes mellitus. In endothelial cells the oxidative stress primarily impacts cell function through peroxynitrite formation, a highly favorable reaction that reduces nitric oxide (NO) availability, resulting in defective endothelial dependent vasodilation, which on the long-term scale leads to cardiovascular disease [10].

Nutritionally mediated oxidative stress may also play a role in cancer development. Oxidative stress can alter the epigenetic program by interacting with the activity of the dioxygenase family of enzymes and in turn can lead to changes in histone methylation which alters gene expression [11]. Epigenetic changes induced by oxidative stress can promote the progression of gene expression changes that have been associated with the progression of cancer [12].

Overloading of the TCA cycle may also result in additional epigenetic responses due to fluctuations in the steady-state dynamics of cellular metabolism and the reliance of histone modifying enzymes on acetyl CoA. Histone acetyltransferases (HATs) utilize the acetyl group in acetyl CoA to acetylate the lysine residue on the N-terminal of histones, which serves to promote a more open chromatin formation and increased gene expression, while histone deacetylases (HDACs) reverse this process and promote a more closed chromatin formation that reduces gene expression [13]. HATs and HDACs rapidly cycle in applying and removing acetyl groups, and it was recently demonstrated that the regulation of HATs and HDACs occurs in response to changes in the steady-state dynamics of metabolic products and is responsive to intracellular pH [14]. *In vitro* assessments have demonstrated that coenzyme A (CoA) derivatives, including acetyl CoA, butyryl CoA, malonyl CoA, and NADPH stimulate class I HDACs on histones, while free CoA inhibits

HDAC activity [15]. Thus changes in the steady-state of acetyl CoA brought on by an overloaded TCA cycle may also impact histone acetylation dynamics and lead to changes in histone acetylation and gene expression.

Oxidative stress can also serve to promote cancer by influencing telomere length. A study conducted on men from the Framingham study found that oxidative stress and insulin resistance are inversely associated with telomere length [16]. Telomere length then reflects the lifelong burden of oxidative stress and its cumulative impact on insulin resistance. Because long telomeres are an important barrier against aberrant segregation events in mitosis, which protects the cell from aneuploidy, a hallmark of cancer cells [17], this finding further underscores the importance of minimizing oxidative stress generated by mitochondrial overload to protect against cancer.

3. Obesity and Inflammation

3.1. From Oxidative Stress to Inflammation. The food-induced increase in oxidative stress also corresponds to an increase in inflammation (Figure 1), and this increase in inflammation can be observed through alterations in numerous signaling pathways and immune system processes. Oxidative stress can modulate numerous redox-sensitive transcription factors including NFκB, activator protein 1 (AP-1), and early growth response 1 (EGR1), which can collectively engage cellular and systemic inflammation in a strong feed-forward process [18, 19]. The NFκB mediated release of inflammatory cytokines (tumor necrosis factor alpha (TFNα) and interleukin-6 (IL-6)), and acute phase reactants (C-reactive protein (CRP)) are the most commonly addressed pathways linking food consumption and inflammation in human studies.

On longer-time scales excess free fatty acids are stored as triglycerides in adipocytes. Brown adipocytes primarily serve to promote thermogenesis and play a major role in the formation of "baby fat," while white adipocytes regulate endocrine function with the secretion of the hormone leptin [20]. In the onset of obesity the accumulation of white adipose tissue generates an additional set of factors that contribute to inflammatory cascades. For instance, adipose tissue often exhibits hypoxia which leads to induction of hypoxia inducible factor 1 alpha (HIF-1α) and the expression of inflammation-related adipokine genes including leptin, vascular endothelial growth factor (VEGF), and angiopoietin-like protein 4 (ANGPTL4) that serve to perpetuate the state of inflammation [21]. Changes in the white adipose tissue promote local and systemic inflammation and will be discussed in more detail in "Obesity and Inflammation" below.

3.2. Postprandial Inflammation. On a short-term scale the consumption of food leads to certain levels of oxidative stress and inflammation after every meal as discussed via overloaded mitochondrial metabolism (Figure 1). Studies that assess postprandial gene expression support the idea that food consumption increases inflammation and have determined that the level of inflammation can be impacted by the amount of calories consumed at a sitting, as well as the glycemic index and the fatty acid profile of the meal [22]. Postprandial inflammation is triggered by blood glucose levels, which act on inflammatory processes in a dose-dependent manner such that meals with higher glycemic index induce increased inflammatory response relative to meals with lower glycemic index [23]. Thus the magnitude of the blood glucose peak is not only strongly influenced by the macronutrient composition of the meal, but it is also influenced by the amount of the food consumed such that a well-balanced meal may still cause a substantial peak if the serving size is excessive [2]. Human intervention studies assessing postprandial inflammation have found that reducing the glycemic index [23, 24] of a meal and caloric restriction [25, 26] result in downregulation of immunological genes and their inflammatory processes.

3.3. Obesity and Inflammation. Macronutrient consumption and habitual overconsumption of food have the consequence of producing chronic levels of inflammation and the upregulation of adhesion molecules, leading to infiltration of the adipose tissue with macrophages. Over time the accumulation of macrophages and monocytes in the tissue alter the nature of the tissue, and the extensive tissue remodeling turns the adipose tissue into an endocrine organ that can mediate further levels of inflammation [18]. Adipocytes found in white adipose tissue exhibit altered physiology due to excess fat storage and release numerous pro-inflammatory cytokines and chemokines including TNF-α, IL-6, leptin, resistin, visfatin, adiponectin, monocyte chemotactic protein-1 (MCP-1), and plasminogen activator inhibitor-1 (PAI-1), which serve to recruit additional immune cells and promote infiltration of macrophages, leading to a strong inflammatory cycle and eventually to insulin resistance at local and systemic levels [3, 18, 27]. Circulating levels of IL-6 and TNFα are strongly correlated with increasing adipose mass [28]. There is also evidence to support the idea that peripheral blood mononuclear cells (PBMCs) may also mediate the increase of the pro-inflammatory cytokines in obese states [29].

Later studies in this area have confirmed the pro-inflammatory state and further characterized the monocyte-macrophage system, where two types of macrophages mediate the inflammatory profile. In obese subjects pro-inflammatory macrophages (M1) predominate over anti-inflammatory macrophages (M2) [30, 31]. The M2 macrophages are alternatively activated by interleukin-4 (IL-4) stimulation and the peroxisome proliferator-activated receptor gamma (PPARγ) receptor and have been demonstrated to protect against the metabolic consequences of obesity in mice [32]. In humans, there is evidence to suggest that (PPARγ) upregulation coincides with increased expression of interleukin-10 (IL-10), an anti-inflammatory cytokine and M2 marker, suggesting that IL-10 expression and M2 dominance are correlated [33]. Expression of IL-10 appears to be complex, such that individuals exhibiting symptoms of metabolic syndrome, whether obese or nonobese, exhibit lower levels of IL-10 compared to their obese and nonobese counterparts [34], possibly due to the

distribution of M1/M2 macrophages. Moreover, levels of IL-10 in nonobese but overweight female adolescents have been correlated with levels of TNFα and IL-6 suggesting that, in more healthy but still overweight phenotypes, IL-10 is upregulated to suppress inflammation [35].

3.4. Obesity-Linked Changes in Gene Expression. Obesity-linked changes in gene expression are important to note as they are strong markers for the long-term consequences of nutritionally mediated inflammation. In large part these changes are likely mediated by the hormone leptin, which is released by the adipose tissue and plays various complex roles in the body including acting as an immunomodulating and pro-inflammatory agent [36].

Changes in gene expression resulting from obesity-linked inflammation are observed in both adipocytes and in peripheral blood mononuclear cells. In a microarray study that compared the gene expression profile of adipocytes of obese and nonobese Pima Indians, the major changes in gene expression profiles were observed in relation to inflammation related genes. The majority of the differentially expressed inflammation related genes (52/54) were upregulated in the adipocytes including chemokines monocyte chemoattractant protein-1 (MCP-1/CCL2), macrophage inflammatory protein (MIP-1α/CCL3), MIP-1β/CCL4, chemokine (C-X-C motif ligand 1 (CXCL1), macrophage inflammatory protein 2α (MIP-2α/CXCL2), MIP-2β/CXCL3, and stromal cell-derived factor 1 (SDF-1/CXCL12) [37]. Elevated levels of MCP-1 and MIP-1α serve to attract monocytes and macrophages to adipose tissue, and their presence is supported by numerous studies, which indicate that the percentage of adipose tissue comprised of macrophages is correlated with obesity [27]. TNFα was excluded from the list of differentially expressed genes because it did not pass with FDR correction, though phosphatidylinositide 3-kinase (PI3K), a member of a downstream pathway associated with TNFα, was significantly overly represented in gene ontology (GO) terms. There was also an upregulation of interferon-induced genes.

A pro-inflammatory state has also been observed in the PBMCs of obese individuals. This state is characterized by an increase in NFκB binding activity in the nucleus and p65 expression, as well as a decrease in I kappa B kinase subunit b (IKKB-B) in the mononuclear cells. Additionally, NFκB regulated genes also exhibit up-regulation in this state and include *TNFα*, *IL-6*, migration inhibitory factor *(MIF)*, and matrix metallopeptidase 9 *(MMP-9)* [29].

The use of microarray studies in this area is still being established, and there is debate in the field as to whether it is more appropriate to assess changes via gene expression patterns found in subcutaneous adipose tissue or in peripheral blood mononuclear cells. The ease of collection for PBMCs is favorable for study implementation, but the extent to which patterns are consistent between adipose tissue and PBMCs requires additional study. One study which evaluated the expression of inflammatory cytokines associated with truncal fat found a strong correlation between the level of truncal fat and the mRNA levels in PBMCs of various inflammatory markers [38].

4. Lipid and Glucose Specific Pathways to Inflammation

4.1. Ω-6 Fatty Acids. Fatty acids and their derivatives eicosanoids can serve as signaling molecules that interact with numerous transcription factors to promote downstream effects. Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that serve as sensors of lipid levels. Fatty acids and various fatty acid derived compounds can serve as ligands, and among them PPARs demonstrate a general preference for long-chain polyunsaturated fatty acids (PUFAs) [39, 40]. Dietary PUFAs also interact with sterol regulatory element binding protein (SREBP), and their transcription in the liver is involved in the regulation of genes related to synthesis and uptake of cholesterol, fatty acids, and phospholipids, and in addition SREBPs are implicated as early mediators of insulin responses [41]. *NF-E2 related factor-2 (NRF2)*, which serves widely as an oxidative stress response factor, exhibits up-regulation in response to the oxidized products of eicosapentaenoic acid ((EPA) 20 : 5 ω-3) and docosahexaenoic acid ((DHA) 22 : 6 ω-3), thereby mediating oxidative stress responses and providing experimental support to the idea that the oxidative quality of fat supplements requires careful regulation [40, 42].

Arachidonic acid ((AA) 20 : 4n-6) is a PUFA that has substantial evidence supporting its role in pro-inflammatory conditions. Arachidonic acid is widely available for intake and can be found in high quantities in many food items including fish, white meat, red meat, eggs, and dairy and also in vegetable oils such as peanut oil, canola oil, and sesame oil. The term "Western diet" is typically used to describe the modern diet that is a product of the industrial and agricultural revolutions. Western diets are characterized by consumption of an increased proportion of fat and refined sugar, reduced proportion of complex carbohydrate and fiber intake, and reduced proportion of fruit and vegetable consumption [43]. The proportions of consumption in the Western diet are often highlighted as a contrast from more traditional diets, such as the Okinawan diet, which includes higher consumption of complex carbohydrates, fiber, fruits, and vegetables and lower consumption of animal products and their fats [44]. Therefore in Western diets AA is a major source of PUFA as it is found in eggs, dairy, fish, and meats.

AA is a key player in promoting inflammation, because it is the precursor for numerous eicosanoids, which are fatty acid derived molecules that mediate inflammatory responses [45]. Eicosanoids which include prostaglandins (PGs), thromboxanes, and leukotrienes are derived from 20 carbon PUFAs, and inflammatory cells are dominated by the presence of ω-6 20 carbon PUFAs making AA metabolism central to inflammation and pharmacological approaches aimed at reducing inflammation. AA is converted by the enzymes cyclooxygenase- (COX-) 1 and COX-2, the inducible form, to PGs of which prostaglandin E2 (PGE_2) is primarily known for its pro-inflammatory effects, and also has less well-known anti-inflammatory effects such as its ability to inhibit the pro-inflammatory cytokines TNF-α and interleukin-1 (IL-1) which were demonstrated in *in vitro* [46]. The evidence supporting AA's role as a key player in inflammation and

disease however is strong, and COX-2 up-regulation occurs during NFκB activation and in response to IL-1B [47, 48]. *In vitro* studies utilizing human prostate cancer cells have found that AA induces COX-2, which is significant because prostate cancer and colorectal cancers consistently exhibit increased levels of COX-2 and PGE_2 [49, 50]. In addition, AA has been shown to induce 11 genes regulated by NFκB in a human prostate cancer cell line PC-3 including *COX-2*, *IκBa*, *NFκB*, granulocyte macrophage stimulating factor *(GM-CSF)*, *IL-1B*, *CXCL-1*, *TNF-α*, *IL-6*, *LTA*, *IL-8*, *PPARγ*, *PPARδ*, and intercellular adhesion molecule 1 *(ICAM-1)*. AA's effects begin as early as five minutes when added *in vitro* to prostate cancer cells at which time PI3K exhibits significant activation with activation of Akt and nuclear translocation of NFκB following at 30 minutes [51].

4.2. Saturated Fatty Acids. While there is substantial evidence to suggest that saturated fatty acids (SFAs) can induce pro-inflammatory signaling, the interactions of saturated fatty acids are still rather ambiguous in many areas, and care needs to be taken when addressing their effects as the lengths of saturated fatty acid chains can produce varying physiological effects and many mechanisms are still debated [52]. Long-chain saturated fatty acids are typically cited for their harmful effects to endothelial cells and include acids such as myristate and palmitate which can induce apoptosis via NFκB induction in human coronary artery endothelial cells (HCAECs) [53]. Further studies in this area have indicated that long-chain SFA can induce pro-inflammatory endothelial cell phenotypes via incorporation into endothelial cell lipids and that short- and medium-chain SFAs do not incorporate or cause lipotoxicity. Specifically, stearic acid induced an up-regulation of *ICAM-1* human aortic endothelial cells (HAECs) in an NFκB dependent manner [54].

One area of contention surrounds the question of whether SFAs mediate NFκB inflammatory effects and the induction of COX-2 through the Toll-like receptors (TLRs). Controversy in this area arises from technical issues of contamination that may occur from endotoxins which are capable of activating TLRs. Recent studies in this area have taken care to purify reagents and, despite these precautions, have still produced conflicting reports. In one study investigators found that SFA (lauric acid and palmitic acid) did not activate TLR2 and TLR4 [55] in HEK-Blue cells transfected with TLR2 and TLR4, but in another study investigators found that SFA (lauric acid and palmitic acid) did activate TLR2 and TLR4 in RAW264.7 macrophages and transiently transfected THP-1 monocytes [56].

Human studies assessing the impact of SFA on gene expression are limited, but there are numerous epidemiologic studies, which assess the relationship between SFA intake and cardiovascular disease, an inflammatory condition. Meta-analyses of prospective studies assessing the association between cardiovascular disease and saturated fat found a consistent lack of an association, and metaregressions performed on randomized trials that substituted PUFA for SFA found there was no change in risk for cardiovascular disease with the fat substitution [57]. Lack of conclusiveness in these studies may result from the fact that SFAs are generally grouped together, and medium-chain SFAs have been shown to provide beneficial health effects including suppression of body fat accumulation and obesity [58, 59]. One human study which aimed to assess the impact of a SFA diet versus a monounsaturated fatty acids (MUFAs) diet on gene expression in adipose tissue found that the SFA diet led to an overexpression of genes involved in inflammatory processes. They found that the gene expression profile included upregulation of cathepsin S *(CTSS)* interleukin-8 *(IL-8)*, integrin beta 2 *(ITGB2)* in moderately overweight individuals and that the profile was similar to that found in obese Pima Indians, concluding that changes were associated with diet-induced changes rather than due to obesity [60].

4.3. Glucose. Postprandial hyperglycemia provides another series of mechanisms through which consumption of food can induce inflammatory cascades and which over time can lead to the inflammation related condition of type 2 diabetes [61]. The results of these glucose excursions are mediated via an increase in oxidative stress likely initiated by the same mitochondrial overload previously discussed, but glucose provides an additional set of mechanisms with which the oxidative stress and associated inflammation can manifest. For instance, oxidative stress in the presence of intracellular hyperglycemia results in the production of reactive intracellular dicarbonyls which react with amino acids to form advanced glycation end (AGE) products that go on to bind AGE receptors and induce expression of inflammatory cytokines in macrophages and procoagulatory and proinflammatory molecules in endothelial cells [62].

Acute hyperglycemia results in elevated levels of circulating inflammatory cytokines including TNFα, IL-6, and IL-18 and more extreme responses in these parameters are observed when glucose spikes; this response is attenuated by administration of glutathione confirming the presence of an oxidative stress-related mechanism [1]. Individuals with diabetes are particularly susceptible to postprandial glucose spikes and these peaks spike oxidative stress to a greater degree than sustained hyperglycemia [4]. Additionally, even in normal subjects hyperglycemia induces an increase in circulating levels of serum-soluble intercellular adhesion molecule-1 (sICAM-1) indicating that glucose excursions can initiate atherogenic events in nondiabetic individuals [63].

5. Nutritional Strategies to Ameliorate Inflammation

While it is evident that high levels of consumption of macronutrients can increase oxidative stress and produce inflammation through NFκB mediated pathways, as well as via alternative mechanisms, such as through excessive ω-6 stimulated inflammation, there are other dietary choices that can simultaneously reduce inflammation. Much information about these dietary choices comes from epidemiologic evidence, which indicates that the Mediterranean and Okinawan diets of the Greek and Japanese populations, respectively, are

associated with significantly lower levels of type 2 diabetes, cardiovascular disease, metabolic syndrome, and cancer [44, 64]. The Okinawan diet, in particular, is marked by consumption of minimally processed foods that are rich in antioxidants, have low glycemic index, and are supported culturally by smaller portion sizes. The Okinawan diet is rich in vegetables, low glycemic index beans, and sweet potatoes and contains small amounts of fish and lean meats [44, 65]. Each of these diets is also rich in virgin olive oil or fish oil, which play critical roles in dampening inflammation and will be discussed in detail here. Thus these diets promote less inflammation due to consumption of smaller meals comprised of minimally processed foods (e.g., vegetables and legumes) and include foods that dampen inflammation, such as healthful fats and antioxidants.

5.1. Caloric Restriction and Macronutrient Balance. The extent to which macronutrient composition and caloric restriction independently affect gene expression patterns is unclear as most studies implement both strategies in their interventions. One study that independently assessed the impact of a macronutrient balanced diet found that the intervention diet (30 : 30 : 40 energy percent from carbohydrates, proteins, and fat, resp.) which had higher protein and less fat than the prestudy diet (41 : 19 : 40) yielded immediate and persistent downregulation in immunological genes in PBMCs [66]. Attempts to sort out the key signal have been made, and one study which assessed the impact of both factors in gene expression of adipose tissue in obese women found that caloric restriction had a more profound impact on adipose tissue gene expression than macronutrient composition [67]. The effects of caloric restriction on inflammatory profiles have been well documented but typically take time to shift the profile, likely due to its impact on weight loss and the adipocyte generation of inflammation. Obese women undergoing intense caloric restriction for 28 days exhibited an improvement in the inflammatory profile of 100 transcripts in subcutaneous adipose tissue, including downregulation of inflammatory markers including acute phase reactants and TNF-related proteins, as well as a simultaneous upregulation of anti-inflammatory markers such as IL-10 and IL-1. These changes were only observed after a 28-day period and not after 2 days [25]. Similarly, gene expression evaluation by microarray in PBMCs demonstrated that caloric restriction downregulates genes involving oxidative phosphorylation such as NDUFS2 (NADH-coenzyme Q reductase) and inflammatory cytokines, including IL-8 [26]. In another study that evaluated the long-term effects of caloric restriction, which was implemented via gastric bypass surgery, microarray analysis of gene expression in adipose tissue also indicated a significant down-regulation of numerous inflammatory markers including *IL-6*, *IL-8*, *IL-1B*, *CCL2*/MCP1, *HIF1α*, and *PTGS2*/COX-2. In addition there was a significant up-regulation of homeobox transcription factors (*HOXA5*, *HOXA9*, *HOXB5*, and *HOXC6*) that may be involved in a metabolically favorable remodeling of adipose tissue after fat loss, however, because downstream targets of homeobox genes have not yet been identified their exact role in this process or relationship to fat loss remains unknown [68].

5.2. Fish Oil. Evolutionary evidence suggests that humans evolved eating a diet where the ratio of ω-6 to ω-3 was approximately 1, and over the last 50 years the ratio has increased from 2 : 1 to 25 : 1, and thus the idea that greater incorporation of ω-3 into the diet is important for health has gained general acceptance [69]. However it is specifically the ω-3s found in fish oil, DHA and EPA, that are implicated in improved health through numerous epidemiologic studies. A hallmark study in the field of ω-3 fatty acids was the 1970s epidemiologic study, which found that Inuit consumed more than 14 g per day of ω-3 fatty acids and that their rate of myocardial infarction was 10 times lower than the rate among Danes who consumed only 3 g per day [70]. Many studies have investigated this relationship, and there is substantial evidence in support of it, though there are some conflicting reports [71]. The complexity of this issue may be part of the cause of such confusion because the key factors in understanding the role of ω-3s in the diet have yet to be fully elucidated. The concept that is mostly unclear is whether it is the total ratio of ω-6 to ω-3 PUFA, the ratio of long-chain ω-6 to ω-3 PUFA, or the presence of high concentrations of ω-3 PUFA that is the key factor [72]. What has been made clear through studies focused on cardiovascular disease, as well as other inflammatory conditions, is that, unlike Inuit diets that are rich in ω-3, Western diets are typically rich in ω-6 PUFA and exhibit ratios of ω-6 to ω-3 that are well beyond recommended ratios. Associated with these ratios and low levels of ω-3 are a host of diseases including autoimmune diseases, allergies, asthma, and cancer [69].

Numerous human studies have observed that ω-3 PUFAs found in fish oil have the capacity to produce therapeutic effects on a number of diseases including cardiovascular disease [71, 73] and rheumatoid arthritis [74]. Increased consumption of ω-3 fatty acids is associated with anti-inflammatory effects that result from reduced AA-derived eicosanoids due to competitive inhibition for enzymes, reduced triglyceride levels, and inhibition of platelet aggregation [71]. In a human intervention study, healthy individuals were placed on diets that controlled for caloric intake, as well as fat intake, for 1 week and then were provided with supplements of fish oil containing long-chain EPA and DHA and borage oil containing short-chain gamma linolenic acid (GLA) for 4 weeks. There was an observed decrease in the levels of PI3Kα and -γ but not in its downstream effectors AKT/NFκB. PI3Kδ and PI3kγ are thought to be involved in the inflammatory response [75, 76].

The relationship between fish oil supplementation and fluctuations in IL-10 is another interesting point of interaction. In one human study supplementation with a combination of fish oils and borage oil significantly decreased expression for *IL-1B*, *IL-10*, and *IL-23*. *IL-5* and *IL-17* exhibited strong but not significant down-regulation as well. No effect was observed on a number of enzymes involved in leukotriene production suggesting that the observed changes were caused by substrate availability [72]. In another human

study involving obese patients, supplementation with EPA increased *IL-10* levels [33]. The discrepancy between the direction of change for the *IL-10* expression in these studies may be the result of supplementation in obese versus normal weight patients, suggesting that there are nuanced regulatory mechanisms in place. A nuanced regulation may mediate responses to EPA supplementation in relation to the given phenotype (obese or normal). Such a nuanced response would support the underlying logic that EPA may shift macrophage dominance in obese patients from the more pathologic M2 state to a more healthful M1 state in which IL-10 is initially upregulated in the M2 state to suppress excess inflammation and then downregulated when the M1 state is achieved.

5.3. Extra Virgin Olive Oil. The Mediterranean diet has been associated with lower incidence of cardiovascular events, obesity, diabetes, and cancer [64, 77, 78]. One of the key components of a Mediterranean diet is high consumption of extra virgin olive oil (VOO), which can be rich in oleic acid and phenolic compounds that contain antioxidant and anti-inflammatory capabilities. In a post-prandial state consumption of VOO has been shown to reduce inflammatory markers and improve levels of antioxidants in serum [79]. Other studies have demonstrated that in a postprandial state VOO can reduce the NFκB inflammatory response in PBMCs compared to diets enriched with fat from butter and walnuts [80]. In a microarray study assessing the impact of acute early morning VOO consumption in obese individuals, VOO was found to downregulate genes in the NFκB pathway and specifically down-regulate the expression of multiple inflammatory genes including *PTGS2, IL1B, CCL3, CXCL1, CXCL2, CXCL3, CXCR4, IL-6,* and oncostatin M *(OSM)* [81].

5.4. Dietary Antioxidants and Phytonutrients. Another intervention that can attenuate diet-induced oxidative stress is the inclusion of dietary antioxidants and phytonutrients, which dampen down the oxidant stress that is generated during metabolism of glucose or fatty acids in the TCA cycle during any meal. Deeply pigmented foods such as berries, red wine, dark chocolate, tea, and pomegranates are rich sources of antioxidants that are shown to mitigate the effects of oxidant production and protect the vascular endothelium [2]. Evidence from a human study indicates that oxidative stress of a high-fat meal can be mitigated by coconsumption of dietary antioxidants with the high-fat meal [82]. The inclusion of cinnamon in a glucose-rich meal delays gastric emptying and significantly reduces the postprandial glucose excursion which aids in dampening inflammation [83, 84] but does not have this effect following a high-fat meal [85].

The phytonutrients and antioxidants found in fruits and vegetables have been demonstrated in human studies to impact inflammatory markers in the PBMCs of young adults. After adjustment for possible confounding factors including age and fiber intake, the highest tertile of fruit and vegetable consumption was found to be associated with the lowest levels of CRP, homocysteine, *ICAM1*, interleukin receptor 1 (*ILR1*), *IL6, TNFα*, and *NFκB* gene expression in young adults [86].

6. Conclusion

The consumption of food and the subsequent cellular metabolism of fatty acids and glucose produce, even under normal circumstances, oxidative stress which triggers an NFκB mediated response that invokes inflammatory factors. Post-prandial inflammation is characterized by an increase in IL-6 and TNF-α in both normal individuals and those with diabetes. Obese individuals have chronically elevated levels of IL-6 and TNFα, and their adipose tissue and PBMCs exhibit increased expression of inflammatory genes. Specifically, *CCL2*/MCP-1, *CCL3*/MIP1α, *CCL4*/MIP-1β, *CXCL2*/MIP-2α, and *CXCL3*/MIP-2β are noted because these have been observed to be elevated in both adipocytes and PBMCs of obese humans.

AA and SFA both demonstrate an ability to increase *IL-8* along with numerous other inflammatory factors including *IL-6*, *TNFα*, *IL-1β,* and *CXCL1* for ω-6 AA, and *IGB2* and *CTSS* for SFA. Dietary strategies aimed at reducing chronic levels of inflammation prove effective and are centered around caloric restriction and inclusion of foods, which either dampen oxidative stress through the use of antioxidants and/or mediate anti-inflammatory signaling. Caloric restriction demonstrates an ability to reduce the level of the proinflammatory *IL-8* in PBMCs and is most effective when weight loss ensues. Notably antioxidant rich foods including olive oil, fruits, and vegetables all demonstrate an ability to lower levels of *IL-6* in PBMCs.

Thus, dietary choices play a complex role in the mediation of unavoidable oxidative stress, and certain choices can either exacerbate or dampen that process as downstream interactions lead to transcription of pro- or anti-inflammatory factors. Moreover, the cumulative impact of long-term oxidative stress that leads to inflammatory conditions such as obesity are increasingly recognized as central factors in the development of cancer. While the full spectrum of mechanisms which link cancer and obesity is not fully elucidated and may include emerging factors such as microbiome composition [87], there is strong epidemiological evidence to support the risk of several types of cancer, such as colon, breast, endometrium, liver, kidney, gastric, gallbladder, and others with obesity, and mechanistic evidence to support the role of inflammation in this process [88].

Abbreviations

acetyl CoA:	Acetyl coenzyme A
AP-1:	Activator protein 1
AGE:	Advanced glycation end
ANGPTL4:	Angiopoietin-like protein 4
AA:	Arachidonic acid
CTSS:	Cathepsin S
CRP:	C-reactive protein
COX:	Cyclooxygenase
DHA:	Docosahexaenoic acid
EGR1:	Early growth response 1
EPA:	Eicosapentaenoic acid
GLA:	Gamma linolenic acid

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GLUT4: Glucose transporter type 4
GM-CSF: Granulocyte macrophage stimulating factor
HATs: Histone acetyltransferases
HDACs: Histone deacetylases
HIF-1α: Hypoxia inducible factor 1 alpha
IKKB-B: I kappa B kinase subunit b
iNOS: Inducible nitric oxide synthase
ITGB2: Integrin beta 2
ICAM-1: Intercellular adhesion molecule 1
IL: Interleukin
MIP: Macrophage inflammatory protein
MMP-9: Matrix metallopeptidase 9
MIF: Migration inhibitory factor
MCP-1: Monocyte chemotactic protein-1
MUFAs: Monounsaturated fattyacids
NADH: Nicotinamide adenine dinucleotide
NO: Nitric oxide
NFκB: Nuclear factorκB
NRF2: Nuclear factor-E2 related factor-2
PPAR: peroxisome proliferator-activated receptor
PI3K: Phosphatidylinositide 3-kinase
PAI-1: Plasminogen activator inhibitor-1
PARP: Poly-ADP ribose; polymerase
PUFA: Polyunsaturated fatty acid
PGE_2: Prostaglandin E2
PKC: Protein kinase C
SFAs: Saturated fatty acids
sICAM-1: Serum-soluble intercellular adhesion molecule-1
SREBP: Sterol regulatory element binding protein
SDF: Stromal cell-derived factor
TLRs: Toll-like receptors
TFN-α: Tumor necrosis factor alpha
TCA: Tricarboxylic acid
VEGF: Vascular endothelial growth factor
VOO: Virgin olive oil.

Conflict of Interests

The authors have no actual or potential conflict of interests.

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Research Article

17-β-Estradiol Counteracts the Effects of High Frequency Electromagnetic Fields on Trophoblastic Connexins and Integrins

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We investigated the effect of high-frequency electromagnetic fields (HF-EMFs) and 17-β-estradiol on connexins (Cxs), integrins (Ints), and estrogen receptor (ER) expression, as well as on ultrastructure of trophoblast-derived HTR-8/SVneo cells. HF-EMF, 17-β-estradiol, and their combination induced an increase of Cx40 and Cx43 mRNA expression. HF-EMF decreased Int alpha1 and β1 mRNA levels but enhanced Int alpha5 mRNA expression. All the Ints mRNA expressions were increased by 17-β-estradiol and exposure to both stimuli. ER-β mRNA was reduced by HF-EMF but augmented by 17-β-estradiol alone or with HF-EMF. ER-β immunofluorescence showed a cytoplasmic localization in sham and HF-EMF exposed cells which became nuclear after treatment with hormone or both stimuli. Electron microscopy evidenced a loss of cellular contact in exposed cells which appeared counteracted by 17-β-estradiol. We demonstrate that 17-β-estradiol modulates Cxs and Ints as well as ER-β expression induced by HF-EMF, suggesting an influence of both stimuli on trophoblast differentiation and migration.

1. Introduction

Broadcasting systems and mobile phones generate high-frequency electromagnetic fields (HF-EMFs) ranging from 30 kHz to 300 GHz. As a consequence of their widely increasing diffusion human beings are today chronically exposed to such sources of energy, whose influences on physiological responses have not been yet exhaustively investigated. Data regarding the effects of these fields on human health are conflicting [1, 2]. Interestingly, *in vivo* human evaluation of brain glucose metabolism showed a significant increase upon acute cell phone radiofrequency signal exposure [3, 4].

As far as reproductive function is concerned, based on postnatal evaluation, no significant increase of reproductive risk was found in the rat following irradiation [5, 6]. However, a decrease in the number of mouse offspring, a prevalence of males over females, and an increase of stillbirth were also reported [7]. More recently it has been reported that the use of mobile phone decreases the human sperm count, motility, viability, and normal morphology [8, 9] probably due to oxidative stress [10].

During the first trimester of human pregnancy, extravillous trophoblast (EVT) cells invade the uterine spiral arteries generating a low-resistance, high-capacity uteroplacental circulation that ensures the success of gestation [11]. EVT cell functions are tightly regulated by multiple factors such as gap-junctional intercellular communication (GJIC) [12–14] and integrins [15, 16].

Gap junctions are membrane channels constituted by the association of two hemi-channels, termed connexons,

each composed of six connexin (Cx) subunits. Gap junctions provide not only a pathway for the exchange of signaling molecules, but they are recognized as true signaling complexes regulating cell function and transformation [17]. Indeed, Cxs influence cell growth, development, and differentiation both in normal and pathologic conditions [17].

Integrins (Ints) are heterophilic cell adhesion molecules consisting of noncovalently connected α and β chains that together determine ligand-binding specificity and intracellular coupling. Thereby, Ints are the most important cell surface receptors for cell interactions with the extracellular matrix structures [18].

Changes in both Cxs and Ints [13, 16, 19, 20] expression have been reported during trophoblast differentiation to EVT.

Environmental stresses, including chemical pollutants [21], ionizing radiations [22], and oxidative stress [23], influence the expression of both Cxs and Ints. Moreover it is well documented that, in trophoblast tissue, the expression of these adhesion molecules is modulated by several hormones including estradiol [15, 24, 25]. This growth-promoting hormone affects placental function and embryo development both in primates and humans [26]. In addition it has been found to be involved in cytotrophoblast cell differentiation towards syncytiotrophoblast [26–29].

On the other hand, clinical effects of HF-EMF exposure on pregnancy are likely to occur, since it has been demonstrated that it results in increased levels of heat-shock protein 70 (HSP 70) in human amnion cells *in vitro* [30], although it does not influence the expression of this protein in human first-trimester extravillous-derived HTR-8/SVneo cells [31]. This cell line, derived from first-trimester human EVT, preserves all of their parental markers, as well as their responsiveness toward factors known to control EVT cell functions [32], thus representing a suitable model for the experimental study of early placentation process. In this experimental model we have shown that one hour exposure to GSM-217 Hz signals selectively modifies Cx mRNA expression pattern and protein localization [33].

The aim of the present work was to investigate whether HF-EMFs and 17-β-estradiol regulate cell-cell and cell-extracellular matrix interactions. To answer this question, we analyzed the effect of HF-EMFs, 17-β-estradiol, and their combination on both Cx and Int expressions inHTR-8/SVneo cells. Moreover, we studied the effect of HF-EMFs, 17-β-estradiol, and their combination on the estrogen receptor expression and immunofluorescence localization. Under the same experimental conditions, ultrastructural features were also evaluated.

2. Materials and Methods

2.1. Cell Cultures. The HTR-8/SVneo cell line was kindly provided by Doctor CH Graham of Queen's University, Kingston, ON, Canada. Cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen Paisley, Scotland, UK). Cells were maintained at 37°C in normal atmosphere containing 5% CO_2. For the experiments, cells were treated with trypsin, removed from culture flasks, and then seeded at a density of 1×10^6 cells per 35 mmdiameter Petri dish. After 24–48 h culture, semiconfluent monolayers were exposed to treatments.

2.2. Chorionic Villi. First-trimester human chorionic villi were obtained from consenting patients undergoing chorionic villous biopsy for prenatal diagnosis at the 11th week of gestation. Only tissues from physiological pregnancy were included in the study.

For RNA isolation, tissues were immediately frozen in liquid nitrogen and stored at −80°C.

2.3. Cell Viability Assay. Cell viability was assessed by MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma Chemical Co., St. Louis) assay in 0.01 M phosphate-buffered saline (pH 7.2). After 1 h cell incubation at 37°C, the formazan formed was extracted in 1 mL of DMSO for 1 h at 37°C and its absorbance measured at 510 nm in a spectrophotometer against a DMSO blank. The mean absorbance values obtained from 3 replicates were compared with controls. Inhibition of the MTT reduction potential of the HTR-8/SVneo cells was calculated and expressed as a percentage of control values following the method described [35], data not shown.

2.4. HF-EFM Exposure. All experiments consisted of control samples kept at 37°C and 5% CO_2 in a Forma thermostat. Sham- and HF-EMF-exposed samples were kept in identical Forma thermostats which also housed the GSM-exposure system. Cells were exposed for 1 h to a 1.8 GHz sinusoidal wave, whose amplitude was modulated by rectangular pulses with a repetition frequency of 217 Hz [36] applied at time-averaged SAR values of 2 W/Kg.s, the safety limit for mobile phone emission according to INCIRP (International Commission on Non-Ionizing Radiation Protection). The exposure system was developed and built by the Foundation for Research and Information Technologies in Society (IT'IS Foundation, Zurich, Switzerland) following the specifications outlined in 5 [36] and extensively described in [37]. The system consisted of two $128.5 \times 65 \times 424\ mm^3$ brass single-mode waveguide resonators operated inside the Forma thermostat. Each resonator was equipped with a plastic holder hosting six 35 mm Petri dishes arranged in two stacks. The carrier frequency, modulation, SAR level, and the periodicallyrepeated on and off exposure times were controlled by a computer. The exposure/sham conditions were assigned to the two waveguides by the computer-controlled signal unit. All exposure conditions and monitor data were encrypted in a file, which was decoded only after data analysis in order to ensure blind conditions for the experiment. Dosimetric field and temperature probes ensured that the temperature differences between sham (cells incubated into the waveguide resonator not selected for irradiation) and exposed cells at the standard condition of incubation was less than 0.1°C, ensuring no untoward thermal influence.

2.5. Estradiol Treatment. HTR-8/SVneo cells were treated for 24 h with 17-β-estradiol (Sigma Chemical Co., St. Louis, MO). Control and treated cells were maintained at 37°C by

Table 1: Primer sequences and PCR condition.

Gene	Primer sequence	T_a °C	Product length (bp)	QPCR amplification efficiency* (%)	No. of cycles	Reference primer bank
Cx40	F: 5′-tcctggaggaagtacacaagc-3′ R: 5′-atcacaccggaaatcagcctg-3′	60.1	137	97.2	39	GenBank Accession NM 181703
Cx43	F: 5′-tcaagcctactcaactgctgg-3′ R: 5′-tgttacaacgaaaggcagactg-3′	60.4	125	98.4	39	GenBank Accession NM 000165
Cx45	F: 5′-atgagttggagctttctgactcg-3′ R: 5′-cggctgttctgtgttgcac-3′	60.4	174	94.5	39	GenBank Accession NM 005497
Int α1	F: 5′-tgctgctggctcctcactgttgtt-3′ R: 5′-gggcccacaagccagaaatcct-3′	60.6	354	95.8	39	GenBank Accession NM 181501.1
Int α5	F: 5′-gaaccagagccgcctgctgg-3′ R: 5′-gagcctccacggagagccga-3′	60.8	215	95.8	39	GenBank Accession NM 002205.2
Int β1	F: 5′-acgccgcgcggaaaagatgaatt-3′ R: 5′-acccacaatttggccctgcttg-3′	60.5	155	95.4	39	GenBank Accession NM 002211.3
RPL13A	F: 5′-cctaagatgagcgcaagttgaa-3′ R: 5′-ccacaggactagaacacctgctaa-3′	60.2	203	97.3	39	Pattyn et al. 2006 [34]
RPL11A	F: 5′-tgcgggaacttcgcatccgc-3′ R: 5′-gggtctgccctgtgagctgc-3′	60.1	108	96.5	39	GenBank Accession NM 000975.2
GAPDH	F: 5′-tgacgctggggctggcattg-3′ R: 5′-ggctggtggtccaggggtct-3′	60	134	94.6	39	GenBank Accession NM 002046.3

Data calculated by OpticonMonitor 3 Software (Bio-Rad).

Forma thermostat in normal atmosphere containing 5% CO_2 and stabilized in serum-free medium for 1 h before HF-EFM exposure. The optimal concentration of 10^{-6} M 17-β-estradiol was chosen on the basis of a dose-response curve carried out in preliminary experiments (data not shown).

2.6. RT-qPCR (Reverse Transcription Quantitative Real-Time PCR). Total RNA from 2 × 106 HTR-8/SVneo cells for each experimental condition was extracted with the AURUM total RNA Mini Kit with DNAse digestion (Bio-Rad, Laboratories, Inc., USA), according to the manufacturer's recommended procedure. After solubilization in RNAase-free water, total RNA was quantified by Bio-Rad SmartSpec Plus spectrophotometer (Bio-Rad, Laboratories, Inc., USA). First-strand cDNA was generated from 1 μg of total RNA using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., USA). As shown in Table 1, primer pairs were obtained from Primer Bank from the Real-Time PCR Primer and Probe Database, RT primerDB [34], to hybridise to unique regions of the appropriate gene sequence. The reverse transcriptase (RT-) PCR reactions were carried out using 1 μL of cDNA in a 15 μL total volume of PCR buffer (Invitrogen, Milan, Italy), containing 3 mM $MgCl_2$, 300 μM dNTPs, and 300 nM of appropriate primers. Taq polymerase (0.35 U) was also added. The amplification reactions were carried out in a thermal gradient cycler (Bio-Rad Laboratories, Inc., USA) for 40 cycles. Each cycle consisted of denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C. A final extension step at 72°C for 5 min terminated the amplification. For each amplification, two types of controls were performed: (i) RT-PCR mixture with no reverse transcriptase to control for genomic DNA contamination and (ii) PCR mixture with no cDNA template, to check for possible external contamination. A 5 μL sample of the PCR reaction was electrophoresed on an ethidium bromide-containing 2% agarose gel by the use of the Bio-Rad Subcell GT system.

Quantitative Real-Time PCR (qPCR) was performed using SYBR Green on iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., USA). The final reaction mixture contained 1 μL of cDNA, 300 nM of each primer, 7.5 μL of iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., USA), and RNAse-free water to complete the reaction mixture volume to 15 μL. All reactions were run as triplicates. The QPCR was performed with a hot-start denaturation step at 95°C for 3 min and then was carried out for 40 cycles at 95°C for 10 s and at 60°C for 20 s. The fluorescence was read during the reaction by the Opticon Monitor 3 software (Bio-Rad Laboratories, Inc., USA), allowing a continuous monitoring of the amount of PCR products. Primers have been initially used to generate a standard curve over a large dynamic range of starting cDNA quantity which allows to calculate the amplification efficiency (a critical value for the correct quantification of expression data) for each of the primer pairs. The melt curve analysis was performed at the end of each experiment to verify that a single product for primer pair was amplified (data not shown). As to control experiments, gel electrophoresis was also performed to verify the sizes of the amplified QPCR products. Ribosomal protein L13a (RPL13a), L11a (RPL11a), and GAPDH were used in our experiments as internal standards. As previously described, samples were compared using the relative cycle threshold (CT) method [38]. The fold increase or decrease was determined relative to a control after normalising to RPL13a (internal standard). The formula $2^{-\Delta\Delta CT}$ was used, where ΔCT is (gene of interest CT) − (RPL13A CT), and $\Delta\Delta$CT is (ΔCT experimental) − (ΔCT control).

All the primers for qPCR analyses were GenBank obtained from Invitrogen (Invitrogen, Carlsbad, CA), and the sequences are listed Table 1.

2.7. Western Blot. After the experimental treatments, cells were washed with ice-cold phosphate-buffered saline solution (PBS), detached by scraping and transferred to eppendorf tubes. After 10 min centrifugation at 800 ×g at 4°C, the pellet was resuspended in ice-cold 10 mM Na-phosphate buffer, pH 7.4, containing 1% Nonidet-P40, 0.5% Na deoxycholate, 0.1% SDS, 1 μg/mL of pepstatin A, E-64, bestatin, leupeptin and aprotinin, and 25 μg/mL of PMSF. After 30 min on ice samples were centrifuged at 9,000 ×g at 4°C for 20 min. The supernatant was diluted 1.5 times with Laemmli buffer, boiled for 5 min, and kept at –20°C until use. Sample proteins were assessed according to Lowry et al. [39] using bovine serum albumin as standard. Western blotting procedures were carried out as we previously reported [29]; briefly, electrophoresis was carried out with a Mini Protean III apparatus (28 mA, 2 hours at 4°C), and the resolved proteins were transferred onto a nitrocellulose membrane (300 mA, 1 h at 4°C). Connexin, integrin, and estrogen receptor proteins were assessed by using Cx40, Cx43, Cx45, Int α1, Int α5, Int β1, and ER-β rabbit polyclonal antibodies (Santa Cruz Biotechnology Inc., CA, USA) against Cx, integrin and estrogen receptor β proteins were assessed by using rabbit polyclonal antibodies (Santa Cruz Biotechnology Inc. CA, USA) against proteins of human origin as primary antibodies (1 : 200). All antibodies were incubated overnight and, after washings, with goat anti-rabbit IgG colorimetric kit (Invitrogen, Carlsbad, CA) for 1 h. Immunoblots were developed by enhanced colorimetric reagent kit (Invitrogen, Carlsbad, CA), and a densitometric analysis of the band intensities was performed by the Gel Doc 2000 video image system (Bio-Rad Laboratories, Hercules, USA). Actin polyclonal antibody (Santa Cruz Biotechnology Inc., CA, USA) was used as an endogenous control for normalization. Values within each experiment were normalized to the control sample.

2.8. Indirect Immunofluorescence Staining of ERβ. HTR-8/SVneo cells on coverslips were stained using rabbit polyclonal antibodies (Santa Cruz Biotechnology) raised against the human ERβ protein (H-150, working dilutions 1 : 200 in PBS containing 0.05% BSA and 0.1% sodium azide). Cells were incubated with the primary antibodies for 1 h at room temperature (RT) and with secondary FITC-labelled goat anti-rabbit IgG serum (Santa Cruz Biotechnology) diluted 1 : 100 in PBS, for 1 h at RT in the dark. Slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) antifading and examined using an Epifluorescence microscope (Nikon Eclipse E800; Nikon Corporation, Surrey, UK) equipped with a plan apochromat 100 × 0.5–1.3 oil immersion objective and a mercury lamp source. Amplifier and detector optimising parameters were maintained constant for all the experiments.

2.9. Ultrastructural Study. Cells were scraped and collected in 0.1 M cacodylate buffer (pH 7.4) and then spun in 1.5 mL tubes at 2,000 ×g for 5 min. Pellets were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 4 h at 4°C. They were then washed with 0.1 M cacodylate buffer (pH 7.4) three times and postfixed in 1% osmium tetroxide and 0.1 M cacodylate buffer at pH 7.4 for 1 h at room temperature. The specimens were dehydrated in graded concentrations of ethanol and embedded in epoxide resin (Agar Scientific, 66A Cambridge Road, Stansted Essex, CM24 8DA, UK).

Cells were then transferred to latex modules filled with resin and subsequently thermally cured at 60°C for 48 h.

Semithin sections (0.5–1 μm thickness) were cut using an ultramicrotome (Reichard Ultracut S, Austria) stained with toluidine blue, and blocks were selected for thinning. Ultrathin sections of about 40–60 nm were cut and mounted onto formvar-coated copper grids. These were then double-stained with 1% uranyl acetate and 0.1% lead citrate for 30 min each and examined under a transmission electron microscope, Hitachi H-800 (Tokyo, Japan), at an accelerating voltage of 100 KV.

2.10. Statistical Analysis. All data were subjected to statistical analysis using PRISM software (version 2.1, Graph Pad Inc.). Data were examined by Bonferroni's posttest (2-way ANOVA). Specifically, tests were performed to ensure that the sham-exposed samples were not significantly different from one another. When these conditions were met, a second 1-way ANOVA was performed on the data from the sham and HF-EMF exposed groups. In all cases statistically significant difference was accepted when $P < 0.05$.

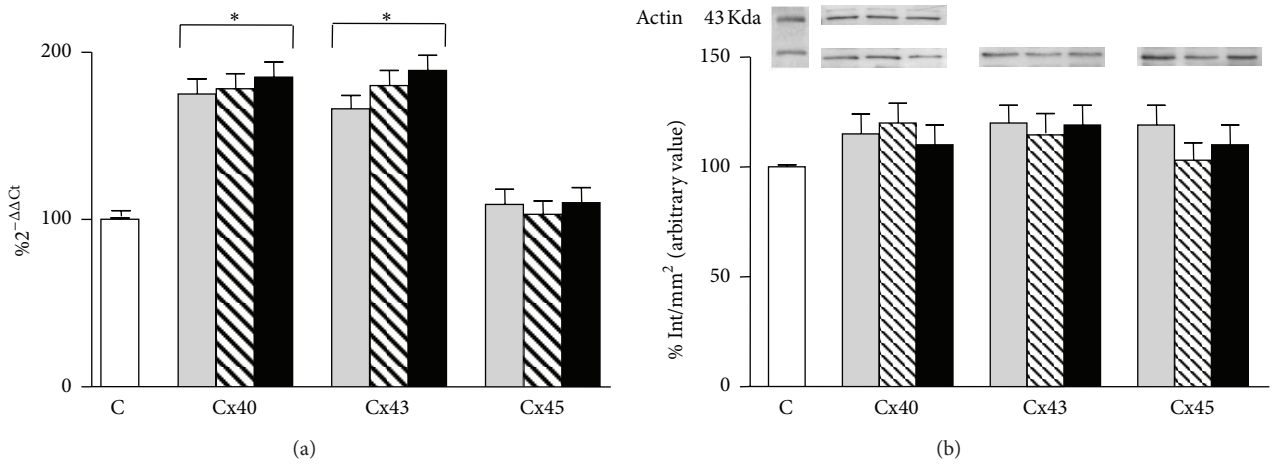

Figure 1: (a) Effect of HF-EMF (grey bar), 10^{-6} M 17-β-estradiol (hatched bar), and their combination (black bar) on the expression of Cx40, Cx43, and Cx45 mRNA in HTR-8/SVneo cells. Results are expressed in % $2^{-\Delta\Delta CT}$ with respect to the control value (white bar). (b) Western blot detection of CXs protein expression in HTR-8/SVneo cells HF-EMF exposed. Representative immunoblots of Cx40, Cx43, and Cx45 are shown. Results are the means ± SEM of three independent experiments, each analysed in triplicate. Data are means ± SEM of at least three independent experiments. $^{*}P < 0.001$ versus sham-exposed cells (one-way ANOVA followed by Bonferroni's posttest).

3. Results

The aim of this work was to investigate the effects of electromagnetic fields on cell-cell and cell-matrix interactions in the first trimester of pregnancy and the control of these effects by the 17-β-estradiol. To answer this question, we analyzed the effect of HF-EMF, 17-β-estradiol, and their combination on the expression of Cx and integrins in HTR-8/SVneo cells.

3.1. Viability of HTR-8/SVneo Cells. As revealed by MTT test, there was no significant difference in cells viability between the negative control (incubator) and the sham-exposed cells. Viability of exposed samples (1 h to GSM-217 Hz signal) was always greater than 98% with respect to sham-exposed samples data not shown.

3.2. Effect of HF-EMF, 17-β-Estradiol, and Their Combination on Cx Expression in HTR-8/SVneo Cells. As already reported by Cervellati et al. [33], 1 h exposure to GSM-217 Hz signals significantly increased Cx40 (175%; $P < 0.001$) and Cx43 (166%; $P < 0.001$) mRNA expression as compared to sham-exposed cells, whereas it did not change expression levels for Cx45 gene product (Figure 1(a)).

Treatment with 10^{-6} M 17-β-estradiol for 24 h significantly increased mRNA expression of Cx40 (178%; $P < 0.001$) and Cx43 (180%; $P < 0.001$) with respect to sham-exposed cells, whereas Cx45 mRNA remained unmodified (Figure 1).

When cells pretreated with 10^{-6} M 17-β-estradiol for 24 h were exposed to 1 h HF-EMF, we observed an increase for Cx40 and Cx43 (185%; $P < 0.001$, 189%; $P < 0.001$, resp.) mRNA expression similar to that found in the presence of HF-EMF or steroid hormone alone. As for Cx45 mRNA, also the combination of the two treatments did not induce any significant change (Figure 1(a)).

All treatments produced no effect on protein expression, at the same experimental conditions (Figure 1(b)).

3.3. Effect of HF-EMF, 17-β-Estradiol, and Their Combination on Integrin Expression in HTR-8/SVneo Cells. HF-EMF exposure for 1 h significantly decreased α1 subunit (55%; $P < 0.001$) and β1 subunit (25%; $P < 0.001$) mRNA levels, but it significantly enhanced α5 subunit (+50%; $P < 0.001$) mRNA expression with respect to sham-exposed cells (Figure 2(a)).

All the integrin subunit mRNA expressions were significantly increased (230%, $P < 0.001$ for α1; 167%, $P < 0.001$ for α5; 127%, $P < 0.001$ for β1) by 24 h treatment with 10^{-6} M 17-β-estradiol (Figure 2(a)).

An effect similar to that obtained in the presence of 17-β-estradiol treatment was found when the cells were pretreated with the hormone for 24 h and then exposed to 1 h HF-EMF. In fact mRNA expression for all integrins tested significantly augmented with respect to sham-exposed cells (203%, $P < 0.001$ for α1; 160%, $P < 0.001$ for α5; 118%, $P < 0.001$ for β1) (Figure 2(a)).

All treatments produced no effect on protein expression, at the same experimental conditions (Figure 2(b)).

3.4. Expression of ER Subtypes in Human Chorionic Villi and HTR-8/SVneo Cells. Both ER-α and ER-β subtypes were expressed in human chorionic villi, as previously reported [26]. In HTR-8/SVneo cells, instead, only ER-β isoform was present whereas ER-α isoform was not detectable (Figure 3).

3.5. Effect of HF-EMF, 17-β-Estradiol, and Their Combination on ER-β Expression in HTR-8/SVneo Cells. The 1 h exposure to GSM-217 Hz signal significantly reduced mRNA expression of ER-β (35%, $P < 0.001$) whereas 10^{-6} M 17-β-estradiol for 24 h significantly increased the receptor mRNA expression (210%, $P < 0.001$), with respect to sham-exposed cells. A stimulatory effect (180%, $P < 0.001$) on ER-β mRNA level was also found when cells pretreated with the steroid hormone for 24 h were then exposed for 1 h to HF-EMF (Figure 4(a)).

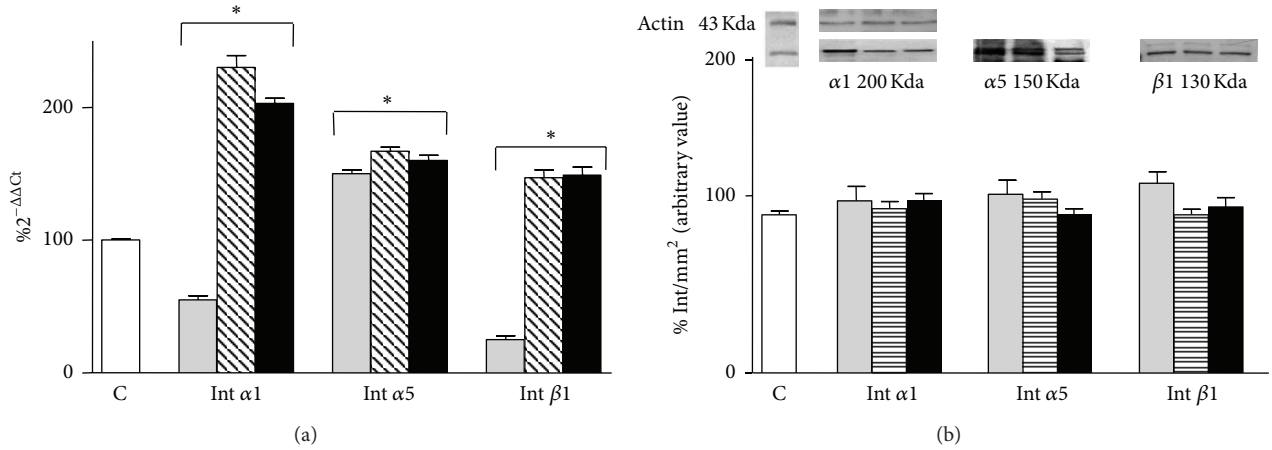

Figure 2: (a) Effect of HF-EMF (grey bar), 10^{-6} M 17-β-estradiol (hatched bar), and their combination (black bar) on the expression of Int α1, Int α5, and Int β1 mRNA in HTR-8/SVneo cells. Results are expressed in % $2^{-\Delta\Delta CT}$ with respect to the control value (white bar). (b) Western blot detection of Integrins Int α1, Int α5, and Int β1 subunit proteins expression in HTR-8/SVneo cells HF-EMF exposed. Results are the means ± SEM of three independent experiments, each analysed in triplicate. Data are means ± SEM of at least three independent experiments. $^{*}P < 0.001$ versus sham-exposed cells (one-way ANOVA followed by Bonferroni's posttest).

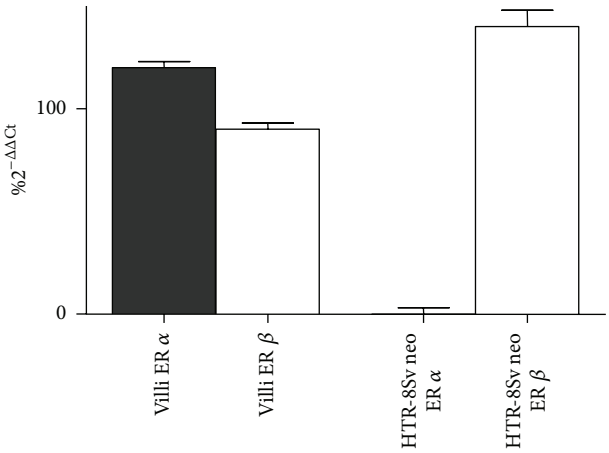

Figure 3: Expression of ER in first trimester human villi and in HTR-8/SVneo cells. Results are expressed in % $2^{-\Delta\Delta CT}$ with respect to the housekeeping gene. Data are means ± SEM of at least three independent experiments. $^{*}P < 0.001$ versus sham-exposed cells (one-way ANOVA followed by Bonferroni's posttest).

All treatments produced no effect on protein expression, at the same experimental conditions (Figure 4(b)).

3.6. Effect of HF-EMF, 17-β-Estradiol, and Their Combination on ER-β Immunolocalization in HTR-8/SVneo Cells. In sham-exposed cells we found punctuate fluorescence for ER-β predominantly in the cytoplasm (Figure 5(a)). The 1 h HF-EMF irradiated cells showed a fluorescence distribution for ER-β comparable to that observed in sham-exposed cells (Figure 5(b)). When the cells were treated with 10^{-6} M 17-β-estradiol for 24 h, a nuclear translocation of ER-β fluorescence was observed (Figure 5(c)). A similar effect was induced by pretreatment with 10^{-6} M 17-β-estradiol for 24 h followed by 1 h exposure to HF-EMF (Figure 5(d)).

3.7. Effect of HF-EMF, 17-β-Estradiol, and Their Combination on Ultrastructural Features in HTR-8/SVneo Cells. As already reported [33], electron microscopy examinations of selected areas of sham-exposed HTR-8/SVneo cells showed neighbouring cells in apposition with each other (Figure 6(a)). A decrease in cellular adhesion was found when cells were exposed to 1 h HF-EMF (Figure 6(b)). On the contrary, following 10^{-6} M 17-β-estradiol treatment for 24 h, HTR-8/SVneo cells formed tightly adherent cellular islets (Figure 6(c)). Cells exposed for 1 h to HF-EMF and pretreated with the steroid hormone for 24 h did not show ultrastructural morphological changes in comparison with cells treated with 10^{-6} M 17-β-estradiol alone (Figure 6(d)).

4. Discussion

Human placental development critically relies upon the differentiation of cytotrophoblast stem cells towards the villous and the invasive extravillous pathways. During this process a pivotal role is played by interactions between trophoblast cells and extracellular matrix, mediated by various kinds of adhesion molecules such as Cxs and Ints [16]. For instance, trophoblast cells lose $\alpha6\beta4$ and gain $\alpha5\beta1$ and $\alpha1\beta1$ Ints [40], and they decrease Cx40-containing gap junctions, while increasing α1 integrin expression [13, 41]. Expressions of both Cxs and Ints are affected by hormones [15] as well as by environmental stresses [42, 43].

As for hormonal regulation of Cx expression, estrogens were reported to increase Cx43 and cell to cell communication in human myometrial cultured cells [44]. Moreover, they induce Cx26 and Cx43 in rat endometrium during preimplantation, implantation, and decidualization [45]. In human trophoblast 17-β-estradiol regulates the expression of Cx43 that is involved in differentiation from cyto- to syncytiotrophoblast, as well as EVT from the proliferative to the invasive phenotype [12]. In the present work, using a

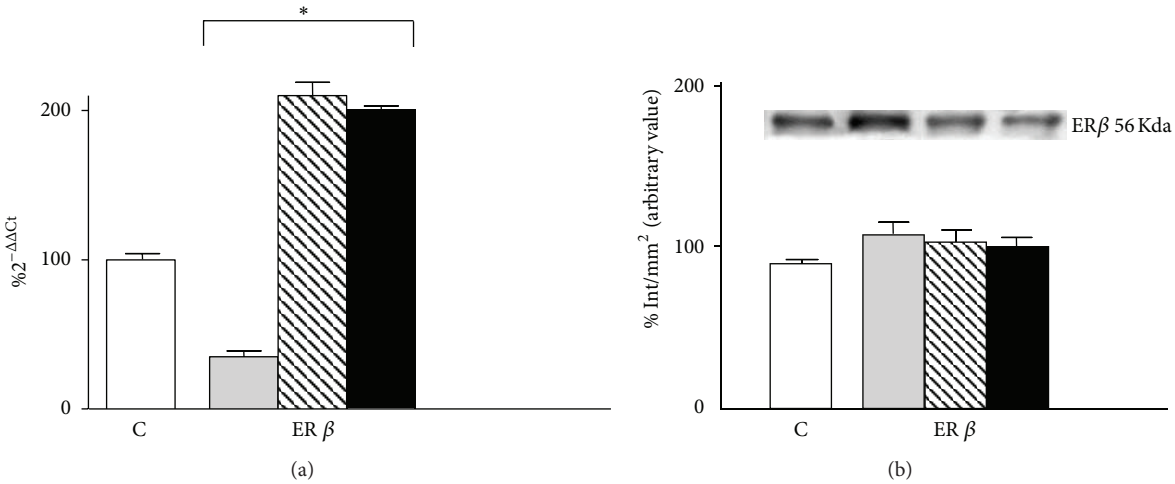

Figure 4: (a) Effect of HF-EMF (grey bar), 10^{-6} M 17-β-estradiol (hatched bar), and their combination (black bar) on the expression of ERβ mRNA in HTR-8/SVneo cells. Results are expressed in % $2^{-\Delta\Delta CT}$ with respect to the control value (white bar). (b) Western blot detection of ERβ protein expression in HF-EMF-exposed HTR-8/SVneo cells. Results are the means ± SEM of three independent experiments, each analysed in triplicate. Data are means ± SEM of at least three independent experiments. $^{*}P < 0.001$ versus sham-exposed cells (one-way ANOVA followed by Bonferroni's posttest).

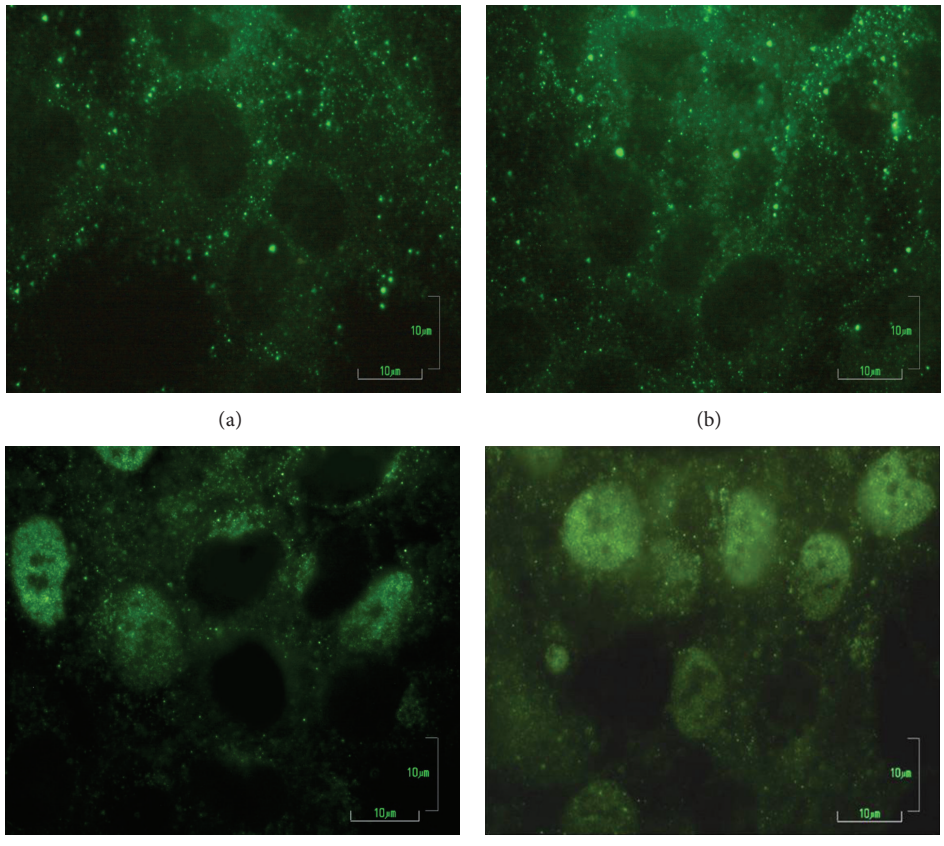

Figure 5: Indirect immunofluorescence staining of ERβ in HTR-8/SVneo cells. (a) Sham-exposed cells; (b) HF-EMF-exposed cells; (c) sham-exposed + 10^{-6} M 17-β-estradiol; (d) HF-EMF + 10^{-6} M 17-β-estradiol exposed cells. Scale bars = 10 μm.

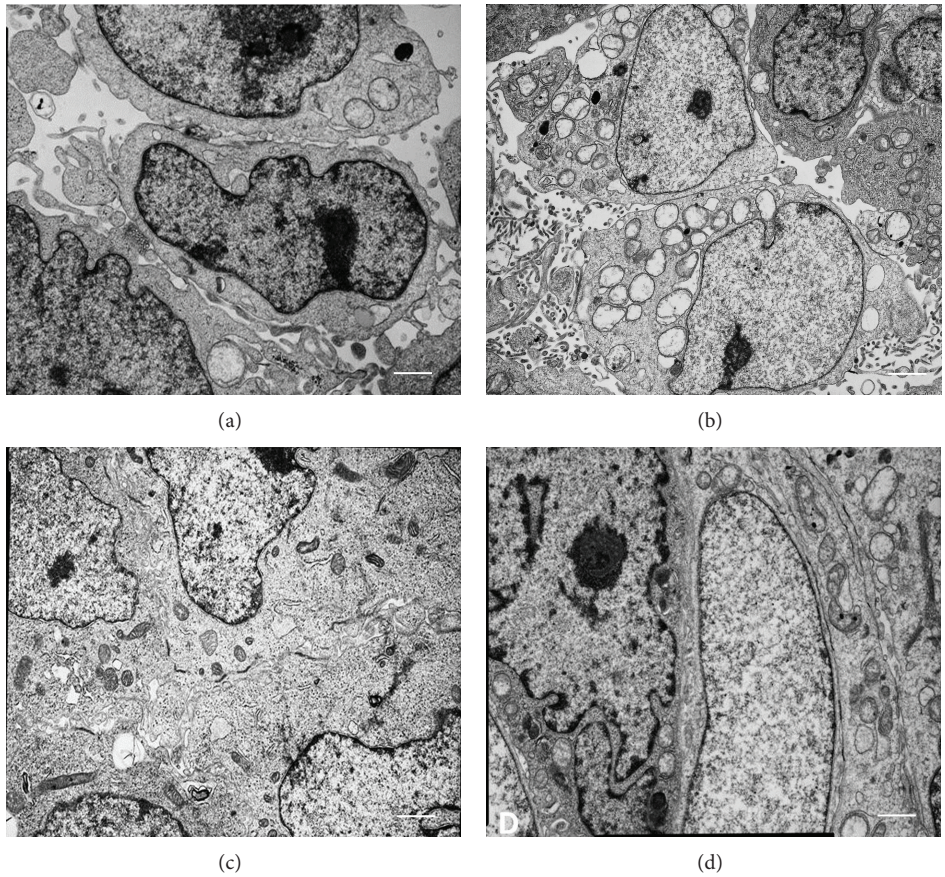

Figure 6: Electron microscopy of HTR-8/SVneo confluent culture cells under different experimental conditions. (a) Sham exposed-cells; (b) HF-EMF exposed cells; (c) 10^{-6} M 17-β-estradiol; (d) 10^{-6} M 17-β-estradiol + HF-EMF exposed cells. Bars = 2 μm.

well-characterized model of human EVT, the HTR-8/SVneo cell line, we investigated the effect of HF-EMF, estradiol, and their combination on Cx, Int, and ER expression as well as on cell ultrastructure. In our cell line either HF-EMF or 17-β-estradiol, both alone or in combination, increased Cx40 and Cx43 mRNA expression leaving unaltered Cx45 transcript. As already showed in our foregoing paper [33] with regard to HF-EMF effect on Cx protein expression, in the present study 17-β-estradiol and its combination with HF-EMF did not induce any change in Cx protein levels. Accordingly, a discrepancy between mRNA transcript and protein expression had already been reported both in EVT cells and other cell types regarding different genes and proteins [46, 47].

As for Ints, the expressions of α1, α5, and β1 were already shown in HTR-8/SVneo cells [48, 49].

Very little is known, however, about the effect of HF-EMF on Int expression. Pulsed electromagnetic fields had no effect in osteosarcoma cell line [50], but extremely low frequency magnetic fields induced a segregation of α4 integrin in human keratinocytes, suggesting an interference with cellular adhesion [51]. In human decidua during early pregnancy the regulation of extracellular matrix remodeling as well as integrin switching is at least partially modulated by reproductive hormones [15, 52].

In our study we demonstrate, for the first time, that both HF-EM and 17-β-estradiol were able to modulate the expression of these adhesion molecules. In fact 1 h exposure to HF-EMF decreased α1 and β1 Int subunit mRNA levels, while increasing α5 transcript. On the contrary 17-β-estradiol induced an enhancement of all the Int subunit mRNA expressions. These data suggest that the hormone may exert an action promoting trophoblast differentiation along the invasive pathway, contrary to HF-EMF. Moreover, 17-β-estradiol effect seemed to prevail over the electromagnetic field one, since the treatment with both agents provoked results comparable to those obtained in the presence of the estrogen alone. However, once again, no effect on protein expression was detected in any experimental condition. Accordingly, 24 h estradiol treatment did not affect Int α5 and Int β4 proteins in human decidua [52]. Nevertheless, integrin protein expression enhancement by estradiol was reported in other experimental conditions [15, 53].

Conflicting results have been reported on estrogen receptor in human placenta [15, 26, 54], although the α isoform has been found in first trimester human chorionic villi [55].

However, in our study, we detected the presence of the ER-β isoform, while ER-α isoform was undetectable either in basal or in stimulated conditions, thus suggesting a mature state of differentiation for HTR-8/SVneo cells [26]. Therefore, 17-β-estradiol stimulatory action on both Cx and Int mRNA levels should be mediated by the ER-β isoform receptor. In our data ER-β receptor subtype mRNA was reduced by HF-EMF exposure but enhanced by 17-β-estradiol treatment.

Also in this case the HF-EMF reductive effect was blinded by 24 h hormone pretreatment. These data suggest a putative autocrine action of estrogen on its own receptor in HTR-8/SVneo cells, as already reported in placental cells with regard to ER-α [15]. Although no significant modifications at the protein level were found, localization of ER-β isoform was notably influenced by hormonal treatment. In fact a nuclear translocation of ER-β fluorescence became evident after estrogen exposure, also in the presence of HF-EMF which, per se, did not alter the cytoplasmic localization.

Ultrastructural observation seemed to reflect the results found at the Int mRNA level. In fact, hormone treatment ameliorated adhesion between neighboring cells, favoring the formation of compact cellular islet. HF-EMF exposure, instead, seemed to increase the distance between adjacent cells. Moreover, estradiol was able to preserve the ultrastructural features of HTR-8/SVneo cells also in the presence of the electromagnetic field.

Thus it can be hypothesized that estradiol may facilitate decidual stroma invasion reinforcing adhesion between cells and extracellular matrix, probably through a modulation of Int subunits, even if we were not able to detect any effect on Int protein expression. This may be due to translational and posttranslational regulation of these adhesion molecules [56]. It is well established that antibodies that interfere with integrin ligand occupancy not only inhibit cell attachment to the ECM but also inhibit cell movement [57].

At this regard it is important to consider that a dysregulation of the previously mentioned molecules is associated to pregnancy disorders such as preeclampsia, IUGR, and preterm labour [16]. As for the mechanism of 17-β-estradiol action, the protective efficacy of the hormone against oxidative stress can be hypothesized. Indeed, in ARTE-19 cells it has been shown to exert an ER-β-mediated cytoprotection through the preservation of mitochondrial function, reduction of reactive oxygen species production, and induction of cellular antioxidant genes [58].

5. Conclusions

Growing attention is devoted today, even from international political institutions, to the influence of HF-EMF on human health, in particular following the recent report that exposure to cell phone radiofrequency signal increases brain glucose metabolism [59]. In the context of human pregnancy protection, it appears mandatory not only to investigate the effects of HF-EMF on implantation, morphogenesis, and fetal development, but also to ascertain the possible existence of protective physiological control mechanisms. At this regard, our study shows, for the first time, that 17-β-estradiol is able to counteract the effects of HF-EMF on trophoblastic Cx, integrins, and ER.

Conflict of Interests

On the behalf of all the authors, we declare that there is no conflict of interests.

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Research Article

Examining the Impact of Skin Lighteners *In Vitro*

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Three cosmetically important skin lightening agents, hydroquinone (HQ), kojic acid (KA), and niacinamide (NA), consume the bulk of successful skin lightening ingredients in cosmetic applications. However, the mechanisms by which these ingredients work are still unclear. In this study, melanocytes and keratinocytes were treated with high, nontoxic doses of HQ, KA, and NA and the cells were examined by human microarrays and protein assays for several important targets including cytotoxicity, melanin expression, tyrosinase gene (TYR) and protein expression, melanocortin-1 receptor (MC1R) gene and protein expression, cytochrome c oxidase-1 (COX1) gene and protein expression, and ferritin (FTH1) gene and protein expression. It was found that all the skin lighteners examined showed marked increases in TYR, COX1, and FTH1 gene and protein expression, but not in MC1R expression in melanocytes. Upregulation of COX1 and FTH1 genes and proteins was common across both cell lines, melanocytes and keratinocytes. The results of the tyrosinase expression were somewhat unexpected. The role of iron in the expression of melanin is somewhat unexplored, but common and strong upregulation of ferritin protein in both types of cells due to the treatments suggests that iron plays a more pivotal role in melanin synthesis than previously anticipated.

1. Introduction

Mechanisms of melanogenesis are complex and a number of recent publications have summarized the current understanding of the process of melanin production in the skin [1–5]. It is well understood that the rate controlling enzyme for melanin formation is tyrosinase (TYR) which is responsible for several oxidative steps in the synthesis of melanin [6, 7]. Melanocortin-1 receptor (MC1R) is responsible for binding melanocyte stimulating hormone (MSH), expressed by stressed keratinocytes, and initiating the cascade of melanogenesis [8]. The role of cytochrome c oxidase in melanin synthesis has been suggested to principally occur through the cytochrome c/H_2O_2 oxidation of catecholamines [9]. Likewise, the role of iron in the skin pigmentation process is also somewhat vague. Perhaps the best work in this area was conducted by Palumbo et al. who demonstrated that ferrous ions can help drive tyrosinase activity in melanocytes [10, 11].

In this work, melanocytes were treated with three well established skin lighteners, hydroquinone (HQ), kojic acid (KA), and niacinamide (NA) [12–15]. The highest dosage of each of the ingredients that was noncytotoxic was established using the MTT assay. This established the levels of treatment on normal melanocytes used in the DNA microarray analyses. While multiple genes were influenced by the skin lightening ingredients, four genes of particular interest were chosen to be examined in greater detail because the genomic assays results presented unusual or unanticipated responses. These genes transcribe several proteins which would not have been anticipated to be upregulated by these skin lighteners including tyrosinase (TYR), cytochrome c oxidase (COX1) (not to be confused with the protein cyclooxygenase-1 identified as COX-1), melanocortin-1 receptor protein (MC1R) and ferritin (FTH1). To further elucidate what might be happening to these four cellular markers, melanocytes were further tested with these ingredients at the same noncytotoxic concentrations and protein expression was examined using immunoblot assays. In addition, the cells were examined for their ability to express melanin. The studies were further extended by examining the genomic and protein impact of the same skin lighteners on normal human epidermal

keratinocytes (NHEK) examining two of the key markers including COX1 and FTH1.

2. Methods and Materials

Samples of hydroquinone, kojic acid, and niacinamide were purchased from Sigma Chemical and were prepared directly in cell culture media.

2.1. Melanocyte Cell Culture. Human epidermal melanocytes were obtained from Cascade Biologics (obtained from a single darkly pigmented donor) and grown in M254 media (supplemented with bovine pituitary extract (BPE) (0.2% v/v), fetal bovine serum (0.5% v/v), insulin (5 μg/mL), transferrin (5 μg/mL), basic fibroblast growth factor (3 ng/mL), hydrocortisone (0.18 μg/mL), heparin (3 μg/mL), phorbol 12-myristate 13-acetate (PMA) (10 ng/mL), and l-tyrosine (0.2 mM)). The cells were seeded into T-25 flasks for the array work, or in well plates for the cytotoxicity, melanin, and protein expression assays, and grown at 37 ± 2°C and 5 ± 1% CO_2 until confluency. Upon reaching confluency the cells were treated with the various test materials for 24 hours for the array work and the cytotoxicity assay and for 48 and 96 hours for the melanin and protein expression assays.

2.2. Keratinocyte Cell Culture. Human epidermal keratinocytes were obtained from Cascade Biologics and grown in Epilife media (supplemented as per the manufacturer's recommendation). For the DNA microarray work the cells were seeded into T-25 flasks, while for the protein expression work the cells were seeded in 24-well plates. In both cases the keratinocytes were grown at 37 ± 2°C and 5 ± 1% CO_2 until confluency and then treated with the test materials. The treatment time was 24 hours for the array work while 48 hours were used for the protein expression work.

2.3. DNA Microarray. After the 24-hour treatment, total RNA was isolated using an RNAqueous Kit (Ambion) as per the manufacturer's instructions. After purification, the total RNA was prepared for array use by first amplifying the RNA using a MessageAmp aRNA Kit (Ambion) and then fluorescently labeling the aRNA with Cy3 or Cy5 using an ASAP Labeling Kit (Perkin Elmer), both as per the manufacturer's instructions. To purify the fluorescently labeled aRNA, a microcon M-30 filter column was inserted into a collection tube and filled with 400 μL of TE buffer. The Cy3 and Cy5 probes were combined and then added to the microcon filter and thoroughly mixed with the TE buffer. The filter was centrifuged at 12,000 RPM for 8 minutes and the flow-through was discarded. The column was then washed twice with 400 μL of TE buffer, discarding the flow-through each time. After the final wash the filter column was inverted, placed into a new collection tube, and centrifuged at 2,000 RPM for 2 minutes to collect the probe.

The fluorescently labeled aRNA was applied to the DNA microarray chips (Agilent Technologies) and the chip was hybridized overnight and washed as per the manufacturer's recommended protocol. After washing the microarrays were scanned with an Axon GenePix 4100A Scanner with the scanning resolution set to 5 μm and analyzed with GenePix Pro software. During the initial scan the PMT gains for the scanner were adjusted such that the cy5/cy3 image count ratios are between 0.88 and 1.12.

Fluorescence intensities for the microarrays were subjected to global normalization. The total fluorescent signal for both dyes was normalized with a correction factor that would make the ratio of total intensities for both dyes equal to one. For this study a Cy5/Cy3 (treated/untreated) fluorescence intensity ratio greater than 1.3 or less than 0.7 (this relates to a change in gene expression of at least ±30%) was used as the cutoff for- up and down-regulated genes, respectively.

2.4. Melanin Assay. On the final day of treatment the cells used for the melanin assay were washed with PBS and lysed with 100 μL of 1 N NaOH. The well plate was gently rocked to ensure that the 1 N NaOH covered the entire well and complete cell lysis was confirmed via microscopic examination. After the cells were lysed, 100 μL of ultrapure water was added to each well to reduce the concentration of NaOH to 0.5 N. After mixing, 150 μL of each cell lysate was transferred to a 96-well plate. In addition, 150 μL of melanin standards (synthetic melanin obtained from Sigma Chemical, prepared in 0.5 N NaOH) was also transferred to the 96-well plate (in duplicate). The well plate was read at 405 nm using a plate reader. After the melanin assay a 10 μL aliquot of each sample was used to determine the protein concentration of the melanocyte lysate via a BCA Protein Assay.

2.5. Melanocyte Lysates: Protein Expression Assays. On the final day of treatment the cells used for the protein expression assay were washed with PBS and then lysed in 200 μL of lysis buffer (1 mM EDTA, 0.5% Triton X-100, 10 mM NaF, 150 mM NaCL, 20 mM β-glycerophosphate, 1 mM DTT, 10 μg/mL leupeptin, 10 μg/mL pepstatin, and 3 μg/mL aprotinin prepared in phosphate-buffered saline) on ice for 15 minutes. The protein concentration of the melanocyte lysates was then determined via a BCA Protein Assay.

2.6. Bicinchoninic Acid (BCA) Protein Assay. Fifty volumes of Reagent A (BCA solution) was combined with 1 volume of Reagent B (4% (w/v) $CuSO_4$–$5H_2O$) in a 15 mL centrifuge tube. Two hundred microliters of this combined reagent was then dispensed into a 96-well plate. Next, 10 μL of each of the standards (bovine serum albumin) or cell lysate sample was added to respective wells. The plate was then covered and incubated at 37 ± 2°C for 30 ± 5 minutes and then read at 540 nm using a microplate reader.

2.7. Tyrosinase, MC1R, COX1, and Ferritin Expression: Microfiltration Blotting of Cell Lysate and Immunodetection. A membrane was equilibrated in Tris Buffered Saline (TBS: 20 mM Tris, pH 7.5, 150 mM NaCl) and assembled into a Bio-Dot microfiltration apparatus. After assembly, 200 μL of TBS was added to each well used in the Bio-Dot and the vacuum was applied to ensure that there was adequate flow through all of the wells. Next, each cell lysate sample

(approximately 5 μg) was assigned a well in the apparatus and was applied to the appropriate well. The samples were filtered under low vacuum. TBS was added to wells not assigned a sample to ensure that the membrane did not dry out during the procedure. At the end of the blotting procedure an additional 200 μL was applied and filtered through each well. The membrane was then removed from the Bio-Dot apparatus, washed in TBS for 5–10 minutes and then placed into blocking solution (Tris Buffered Saline (20 mM Tris, pH 7.5, 150 mM NaCl, 1% nonfat milk powder)) and allowed to incubate for at least 1 hour at room temperature on a rocking platform.

2.8. Antibody Incubation and Detection. After blocking, the membrane was transferred to 20 mL of TBST (TBS with 0.1% Tween-20) and 0.1% nonfat powdered milk with the appropriate primary antibody (all antibodies were obtained from Santa Cruz Biotechnology, Inc., and used at a 1 : 1000 dilution) was added and allowed to incubate overnight at 4°C on a rocking platform. After this incubation the membrane was washed 3 times (1x for 15 minutes and 2x for 5 minutes) in TBST. A fluorescently conjugated secondary antibody (diluted 1 : 2500) was then incubated with the membrane in 15 mL of TBST with 0.1% nonfat powdered milk for 1 hour at room temperature and then washed 3 times with TBS (1x for 15 minutes, and 2x for 5 minutes).

After the final wash, the membrane was placed into a Bio-Rad Molecular Imager FX and scanned using an excitation laser and emission filter combination appropriate for the fluorophore. Images produced by the scanner were then analyzed using ImageJ image analysis software.

3. Calculations

3.1. Melanin and Protein Assays. The mean absorbance values for the standards were calculated and then used with the standard concentrations to generate an equation for a standard curve using regression analysis. This equation was then used to determine melanin and protein concentrations in the cell lysate samples.

3.2. Image Analysis. Fluorescence intensity measurements were expressed in relative fluorescence units (RFUs). RFUs for the target protein of interest were then normalized to GAPDH. Mean normalized RFU values for each treatment were then calculated and treatments were compared using a one-way ANOVA.

4. Results and Discussion

Results from the MTT assays are shown in Figure 1. It was found that while hydroquinone was nontoxic at 0.0001% when used for a 24-hour incubation in the gene array studies, this concentration was not tolerated for longer incubation periods used for the protein expression assays. Therefore, hydroquinone was also tested in protein assays at 0.00001% and at 0.000001% in the ferritin assays. The shorter timeframe for genomic testing (24 hrs) versus protein assays (48–96 hours) allows for higher concentrations to be tested in the genomic assays without cytotoxic results.

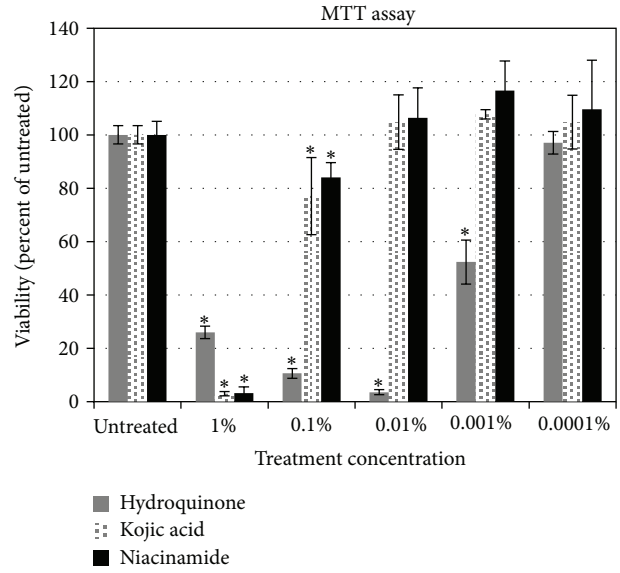

Figure 1: Cell viability assays (MTT) of three skin lightening ingredients on melanocytes.

Table 1: Summary of gene expression indicated by ratio of medians for three skin lightening agents, Hydroquinone (0.0001%), kojic acid (0.01%), and niacinamide (0.01%) looking at tyrosinase (TYR), cytochrome c oxidase-1 (COX1), melanocortin-1 receptor (MC1R), and ferritin (FTH1) expression. Ratios of medians higher than 1.3 typically indicate a statistically significant upregulation of the gene. Values less than 0.7 typically demonstrate a statistically significant downregulation of the gene.

Gene symbol	Hydroquinone	Kojic acid	Niacinamide
TYR	1.528	1.61	2.219
COX1	2.538	2.235	2.696
MC1R	0.438	0.716	0.879
FTH1	2.999	2.133	2.92

Results from the human microarrays for the four genes mentioned previously are shown in Table 1 showing ratio of medians for each ingredient after 24 hours at the highest noncytotoxic dosages possible (dosages are shown next to each ingredient). It was found that all three skin lighteners upregulated TYR, COX1, and FTH1, but not MC1R. All three skin lighteners appear to have little effect on MC1R expression at the genomic level within the 24-hour timeframe of the arrays and hydroquinone may slightly suppress MC1R gene expression in the 24-hour timeframe. The genes for COX1 and FTH1, however, were quite strongly upregulated in these assays by all three skin lighteners.

Results from the melanin assays are shown in Figure 2. It appears that while HQ and KA both significantly suppressed melanin expressions, NA did not. However, this is consistent with results shown by Boissy and Hakozaki et al. in similar models [14, 15]. Niacinamide does not appear to suppress direct melanin expression but has been suggested to instead

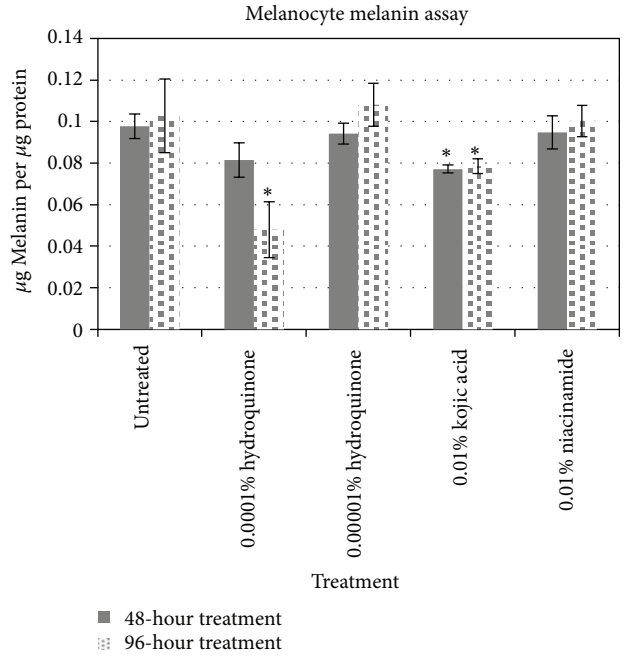

Figure 2: Results of melanin assay results looking at three skin lighteners.

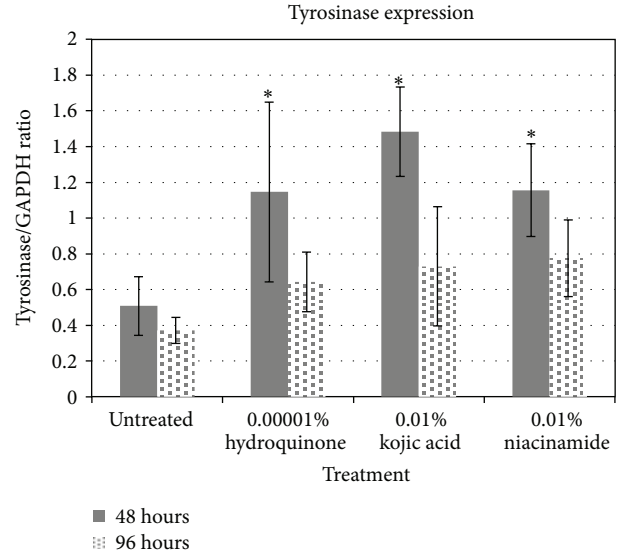

Figure 3: Results from tyrosinase protein expression assays for three skin lighteners.

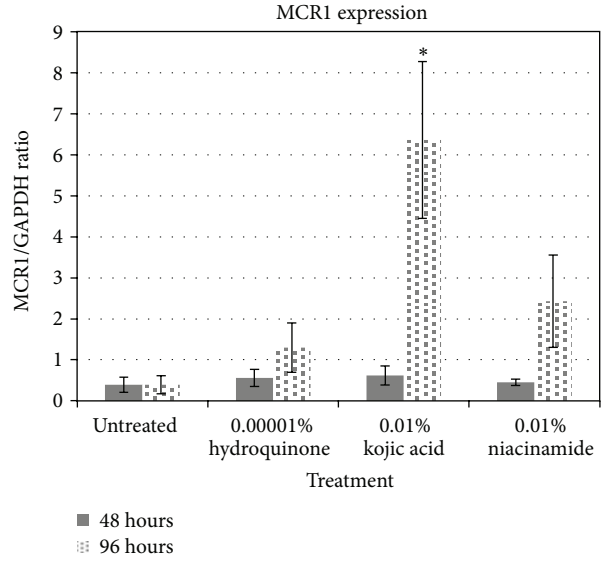

Figure 4: Melanocortin-1 receptor protein expression assay results.

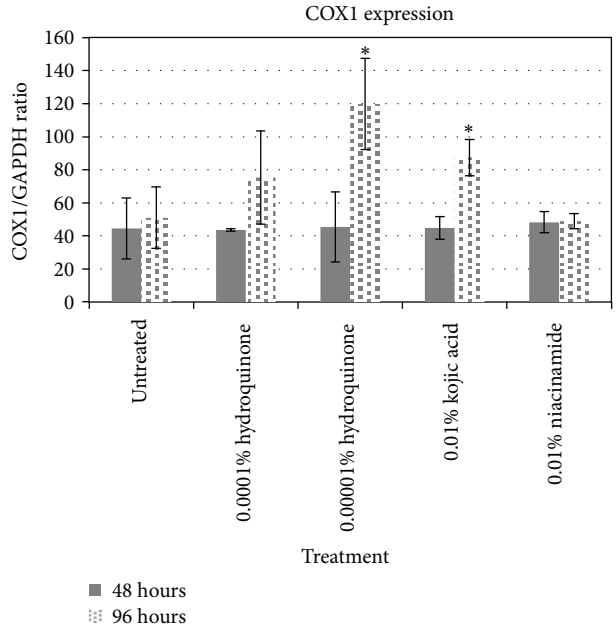

Figure 5: Cytochrome c oxidase-1 protein expression assay results.

slow transfer of melanin from the melanocytes to the keratinocytes. This effect cannot be detected in melanocyte cell cultures.

Results from the expression of tyrosinase are shown in Figure 3. It appears that all three ingredients seem to upregulate tyrosinase expression consistent with the results from the arrays. The upregulation of tyrosinase was somewhat unexpected and is difficult to correlate to products that are supposed to be skin lightening. However, the high levels of tyrosinase in the melanocytes after treatment with skin lightening agents might explain why many people experience hyperpigmentary reactions after ceasing use of skin lightening agents.

MC1R protein expression (Figure 4) was increased by all three skin lighteners, but the effect did not become apparent until 96 hours into the assay. At 48 hours, MC1R was not upregulated in melanocytes by any of the skin lighteners, consistent with the results from the genomic microarrays.

Of most particular interest were the results from the COX1 (Figure 5). HQ and KA both had very strong upregulating effects on expression of cytochrome c oxidase, consistent

with the genomic data. However, the upregulation of COX1 does not become statistically significant until 96 hours of treatment. It suggests that while gene expression can be noted relatively early, resulting protein expression appears to be delayed by many hours. However, NA, while showing a strong genomic effect, did not increase COX1 protein expression at any time. Thus, the one ingredient that did not show direct melanin suppression in melanocytes also did not show direct upregulation of COX1 protein expression. Whether there is a correlation between COX1 and the ability for melanocytes to move melanin to the keratinocytes is something that would need to be confirmed.

The expression of ferritin protein mirrored nicely the genomic data, showing upregulation by all three skin lighteners with 48 hours (Figure 6). The expression of ferritin then diminished after 96 hours for all the treatments.

Presently, the role of iron in the tanning process is relatively unexplored. Jimbow demonstrated that exposure of skin to UV light increases expression of both ferrous (Fe^{+2}) and ferric (Fe^{+3}) ions in melanosomes [16]. Palumbo et al. has demonstrated, however, that it appears that ferrous ions are the critical oxidative iron species that influence tyrosinase activity by upregulating the enzyme's activity [10, 11]. Ros et al. have suggested that ferrous ions may act to accelerate the hydroxylation of tyrosine which can increase the functionality of tyrosinase to form DOPA [17]. However, ferritin principally binds ferric ions, keeping such ions in a nontoxic state within the cells. Maresca et al. has demonstrated that ferritin downregulation caused melanin suppression in melanoma cells [18]. The extensive upregulation of ferritin within cells treated with skin lightening agents suggests that the cells are accumulating high levels of ferric ions at the expense, perhaps, of ferrous ions. This would be consistent with the observed reductions in melanin and suggests that the role of ferrous ions in skin lightening is presently an overlooked pathway to skin lightening. Applegate et al. has reported that ferritin expression is increased in melanocytes exposed to UVB radiation and that expression of ferritin in radiation-stressed skin cells should be considered a defensive response to oxidative stress [19]. This suggests, counterintuitively, that the skin lighteners examined here are offering a UV-type stress to the melanocytes, as measured by ferritin increase, and yet they are causing reductions in melanin production which does not happen with UV radiation.

The influx of iron appears to occur quickly and then to subside as the levels of ferritin are high within 48 hours, but begin returning to more normal levels within 96 hours. This protein expression behavior seems to mirror pretty closely the expression of tyrosinase as well. It would seem that when tyrosinase expression is upregulated, it is important for ferritin expression to also be upregulated. This would suggest that the interplay between tyrosinase activity and ferrous ions should be explored more closely.

To extend the study of the skin lighteners to additional skin cells, each skin lightener was also added to normal human epidermal keratinocytes (NHEK) at comparable concentrations as those used on melanocytes. The results of the gene expression for two key genes of interest, COX1 and FTH1, are shown in Table 2. It can be noted that comparable to the results from the melanocyte array results, there is a pronounced influence of these skin lighteners on COX1 and FTH1 expression. These results were further confirmed by protein assays as shown in Figures 7 and 8.

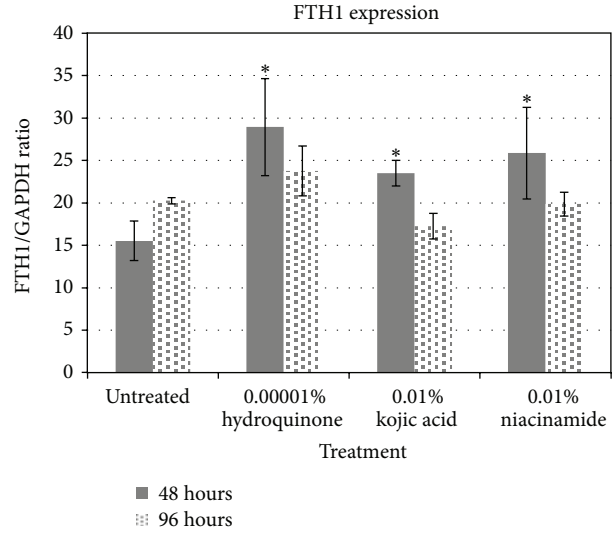

Figure 6: Ferritin protein expression assay results.

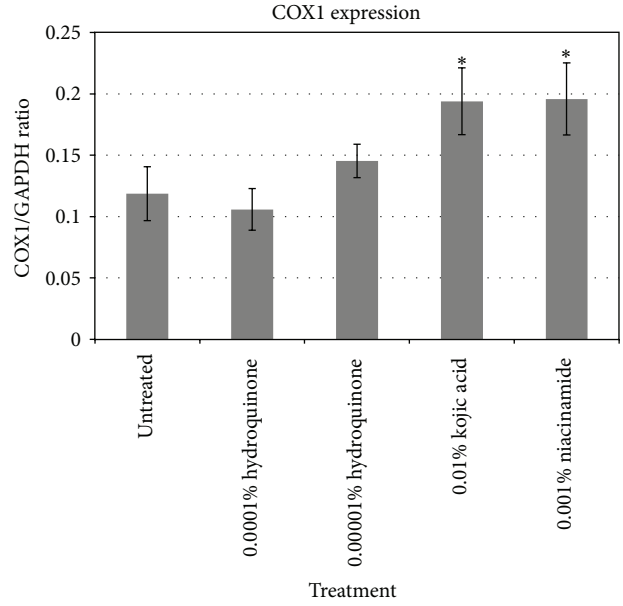

Figure 7: Expression of cytochrome c oxidase in treated keratinocytes.

What is very interesting is that all these skin lighteners also show pronounced upregulation of the ferritin gene and ferritin protein in keratinocytes indicating that in both melanocytes and keratinocytes the expression of ferric ion appears to be significant upon addition of these well established skin lighteners to both cell lines. The apparent upregulation of ferritin and cytochrome c oxidase in keratinocytes treated with skin lighteners again suggests a stress response

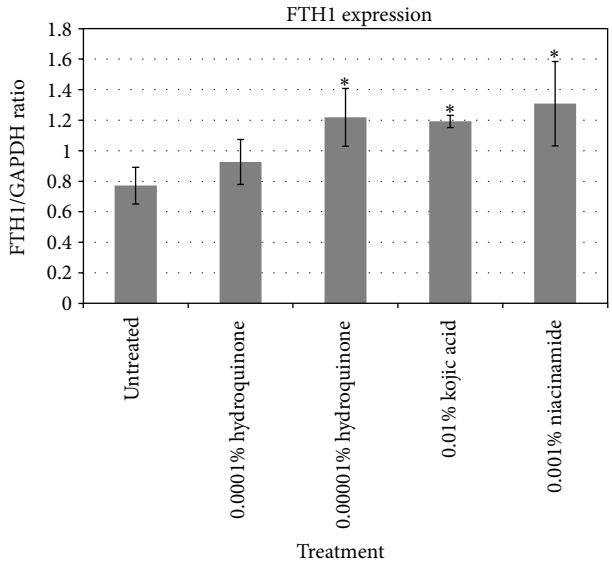

Figure 8: Expression of ferritin protein in treated keratinocytes.

Table 2: Summary of gene expression indicated by ratio of medians for three skin lightening agents, hydroquinone (0.0001%), kojic acid (0.01%), and niacinamide (0.01%) looking at cytochrome c oxidase-1 (COX1) and ferritin (FTH1) expression on normal human epidermal keratinocytes. Ratios of medians higher than 1.3 typically indicate a statistically significant upregulation of the gene. Values less than 0.7 typically demonstrate a statistically significant downregulation of the gene.

Gene symbol	Hydroquinone	Kojic acid	Niacinamide
COX1	1.782	4.347	1.529
FTH1	2.478	3.295	5.896

similar to UV radiation. Applegate et al. has demonstrated that in skin cells exposed to UV radiation upregulation of ferritin is a stress response [20]. The consistency of ferritin and cytochrome c oxidase response in two cell lines exposed to well-established skin lighteners suggests that the mechanisms by which these skin lighteners function may still be vaguely understood. Nevertheless, the apparent responses indicate that the skin cells are responding to these ingredients as though they have been stressed and yet, in cell culture as well as *in vivo*, these ingredients are demonstrated to be successful skin lightening ingredients.

Conflict of Interests

The authors report no conflict of interests.

Acknowledgment

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Review Article

Obesity and Metabolic Comorbidities: Environmental Diseases?

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Obesity and metabolic comorbidities represent increasing health problems. Endocrine disrupting compounds (EDCs) are exogenous agents that change endocrine function and cause adverse health effects. Most EDCs are synthetic chemicals; some are natural food components as phytoestrogens. People are exposed to complex mixtures of chemicals throughout their lives. EDCs impact hormone-dependent metabolic systems and brain function. Laboratory and human studies provide compelling evidence that human chemical contamination can play a role in obesity epidemic. Chemical exposures may increase the risk of obesity by altering the differentiation of adipocytes. EDCs can alter methylation patterns and normal epigenetic programming in cells. Oxidative stress may be induced by many of these chemicals, and accumulating evidence indicates that it plays important roles in the etiology of chronic diseases. The individual sensitivity to chemicals is variable, depending on environment and ability to metabolize hazardous chemicals. A number of genes, especially those representing antioxidant and detoxification pathways, have potential application as biomarkers of risk assessment. The potential health effects of combined exposures make the risk assessment process more complex compared to the assessment of single chemicals. Techniques and methods need to be further developed to fill data gaps and increase the knowledge on harmful exposure combinations.

1. Introduction

Obesity is an increasing health problem; more than half of the European population is overweight and up to 30% is obese and its prevalence worldwide doubled since 1980 (World Health Organization 2011) [1]. Similarly, increased body weights have also been reported in pets and laboratory animals over the past decades [2]. Obesity is a condition characterized by significant clinical implications, such as comorbidities and somatic fragility, which seriously affect independence, psychological wellbeing, and overall quality of life [3, 4]. Obesity is associated with type 2 diabetes mellitus (DM), dyslipidemia, cardiovascular disease, cancer, and obstructive sleep apnea [5, 6]. Medical treatments are often ineffective and bariatric surgery is the only available therapeutic modality associated with clinically significant and relatively sustained weight loss in subjects with morbid obesity [7–9].

Proinflammatory factors are increased in obesity and DM, and the prevalent metabolic state is defined by the term "glucolipotoxicity," in which excess extracellular glucose and fatty acids exert various damaging effects. Obesity and DM-associated oxidative stress eventually lead to systemic inflammation and endothelial cell dysfunction, central to the development of cardiovascular diseases and metabolic syndrome [10, 11].

Excess caloric consumption and a sedentary lifestyle are the only recognized risk factors for obesity and DM but alone do not account for the current worldwide obesity epidemic. New hypotheses are emerging to explain the etiopathogenesis of these conditions, including environmental chemicals, stress, immunological alterations, micronutrient deficits, and gut microbiota [12, 13]. Genetic modifications could be involved in predisposition to obesity; however the human genome has not undergone significant modifications over the last years. On the contrary, the correlation between

accumulation of synthetic chemicals and increase of obesity prevalence might not be a random event. This dramatic change in the environment has led to the hypothesis that some environmental pollutants act as Endocrine Disrupting Chemicals (EDCs), interfering with various aspects of metabolism and of energy balance [14].

2. Endocrine and Metabolic Disruption

2.1. EDCs. EDCs have been defined as exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)populations. (International Programme for Chemical Safety—IPCS). EDCs can mimic the action of natural hormones or interfere with their production, release, metabolism, and elimination [15]. These substances may derive from natural, animal, human, or plant sources. Phytoestrogens are EDCs found naturally in certain plants, including foods like whole grains, leafy greens, beans, and garlic, and can mimic the action of estrogen, showing some beneficial effect on bone mineral density, insulin resistance, and cardiovascular risk factors in women after menopause [16–20]. Another class of EDCs belongs to the group of heavy metals (i.e., cadmium, mercury, and arsenic). These metals may cause occupational or residential exposure [21]. Heavy metal toxicity can result in reduced mental and central nervous function and damage to blood composition, lungs, kidneys, liver, breast, and other vital organs [22, 23]. Allergies are not uncommon and repeated long-term exposure with some metals may even cause cancer [24, 25]. Major concerns, however, are currently focused on industrial products. In fact, most EDCs are synthetic chemicals designed for use in a variety of industries. Classes of these chemicals are solvents or lubricants and their byproducts (polychlorinated biphenyls (PCBs) and dioxins), plastics, plasticizers (bisphenol A (BPA)), phthalates; pesticides (methoxychlor, chlorpyrifos, DDT) fungicides (vinclozolin), herbicides (atrazine), and antibacterials (triclosan). There are several reports of pollution from PCBs and dioxins that show a direct causal relationship between a chemical and the manifestation of an endocrine, immunological, or metabolic dysfunction in humans and in wildlife [26–29] but the more common event is the widespread persistent exposure to a broad mix of chemicals. Table 1 shows a brief summary of the principal incidents caused by dioxins contamination in the last fifty years.

2.2. Mechanisms of Action. Some EDCs were designed to have long half-lives and therefore are persistent contaminants, do not decay easily, may not be metabolized, or may be broken down into more toxic compounds [30]. Others, such as BPA, although not very persistent in the environment, are so widespread in their use that there is a prevalent human exposure [31]. Humans and wildlife are exposed daily to a variety of compounds, and it is thus likely that even if none reach an effective level, the combination or mixture of chemicals may become dangerous. These chemical mixtures enter the food chain and accumulate in animals up to humans. Exposure occurs also through drinking contaminated water, breathing contaminated air, or contacting contaminated surfaces. They may exert nontraditional dose-response curves, the so-called U-shaped or inverted U-shaped dose-response curve. As a consequence, any level of exposure may cause endocrine or metabolic abnormalities, particularly if the exposure occurs during a critical developmental period, and low doses may even be more potent than high doses [32, 33]. The age of exposure is important [34], since the environment to which a developing organism (fetal life, childhood) is exposed interacts with the individual's genes to determine the propensity to develop a disease later in life. The majority of environmental factors and toxicants do not alter DNA sequence or promote genetic mutations. Therefore, they may promote abnormal phenotypes or disease through modifications of factors that regulate gene expression such as DNA methylation and histone acetylation [35–39].

EDCs can bind and activate multiple hormone Nuclear Receptors (NRs). Various EDCs share receptors, and thus additive or even synergistic effects may be observed [40]. Among NRs, Estrogen Receptors (ERs) regulate many aspects of metabolism, including glucose transport, glycolysis, mitochondrial activity, and lipid metabolism [41]. It is likely that ER activation modulates neural networks controlling food intake and adipose tissue [42, 43]. Male and female ER knock-out mutant mice show increased insulin resistance and impaired glucose tolerance [44]. Neonatal exposure to a low dose of the estrogenic drug diethylstilbestrol (DES) stimulated a subsequent increase in body weight and an increase in body fat in mice [45]. BPA, a breakdown product of coatings in food and beverage containers, may act as an ER agonist. In the US population, exposure is nearly ubiquitous, and BPA has been detected in fat, blood, and urine [46]. Short exposure to BPA provokes chronic hyperinsulinemia, with perturbations of glucose and insulin tolerance tests [47]. Furthermore, high- or low-dose exposure to BPA during gestation up to puberty leads to hyperlipidemia with increased body and adipose tissue weight in both sexes [48, 49]. BPA exposure has been shown to disrupt multiple metabolic mechanisms, suggesting that it may contribute to obesity in humans [50–52]. Other studies have demonstrated associations between urinary BPA concentration and adult DM, cardiovascular diseases, obesity, and abnormalities in liver function [53, 54] A longitudinal study of apparently healthy adults showed an association between baseline urinary BPA concentration and later-life coronary artery disease [55].

EDCs may also modulate other hormone NRs, particularly thyroid hormone receptor (TR) and glucocorticoid receptor (GR). BPA acts as a TR antagonist in vitro, increases serum thyroxin, and alters RC3/neurogranin expression in the developing rat brain [56]. Brominated Flame Retardants (BFRs) also disrupt the TR pathway, and exposure of rats to Polybrominated Diphenyl Ethers (PBDEs) resulted in a significant increase in lipolysis and a significant decrease in glucose oxidation [57]. Organotins and PCBs can bind GR and alter 11beta-hydroxysteroid dehydrogenase type 2 activity [58].

The body is protected from the accumulation of toxic chemicals by the expression of drug-metabolizing enzymes

Table 1: Examples of dioxin contamination incidents.

Year	Country	Type of accident	Cause
1960–1975	Vietnam	Contamination by a defoliant, agent orange	Contaminated agent orange
1976	Italy (Seveso)	Contamination by a cloud of toxic chemicals of an area of 15 square kilometers	Accident at a chemical factory
1998	Germany	Contamination of milk	Contaminated citrus pulp pellet from Brazil
1999	Belgium	Contamination of poultry and eggs	Animal feed contaminated with illegally disposed PCB-based waste industrial oil
2004	The Netherlands	Contamination of milk	Contaminated clay in animal feed
2007	Europe	Contamination of guar gum (food additive)	Guar gum from India contaminated by pentachlorophenol that contains dioxins as further contamination
2008	Ireland	Contamination of pork meat and products	Contaminated animal feed

and transporters. This adaptive response incorporates at least three NRs: pregnane X receptor (PXR), constitutive androstane receptor (CAR), and aryl hydrocarbon receptor (AhR), as well as xenobiotic metabolic and transporter systems. PXR and CAR are members of the NR super family of sensor receptors and contribute to fatty acid, lipid, and glucose metabolism, and CAR seems to be an anti-obesity NR that ameliorates DM and fatty liver accumulation [59–61]. Endogenous ligands of PXR and CAR include some bile acid derivatives, pregnanes, androstane metabolites, and other metabolic products of steroids; exogenous compounds include herbal medicines pharmaceutical drugs and synthetic steroid hormones. A number of EDCs activate both PXR and CAR: nonylphenol, Di (2-Ethylhexyl) Phthalate (DEHP), Mono-(2-Ethylhexyl) Phthalate (MEHP), BPA, some PCBs perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), and the organochlorine methoxychlor [62]. AhR is a xenosensor that mediates the biological response to a wide spectrum of xenobiotics; in particular, AhR mediates the toxic effects of dioxins [63]. Endogenous molecules that bind AhR are lipoxin 4, leukotriene derivatives, biliverdin, and bilirubin. Xenobiotics that activate AhR include various dietary phytochemicals, some PCBs, and 2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD). The mechanisms through which AhR regulates energy metabolism are not clearly established; however, crosstalk with ER may be involved. In addition, AhR also indirectly affects adipogenesis through inhibition of Peroxisomal Proliferator-Activated Receptor (PPAR)γ expression [64].

2.3. Health Effects. All major endocrine organs are vulnerable to endocrine disruption, including the Hypothalamus-Pituitary-Adrenal axis, reproductive organs, the pancreas, and the thyroid gland [65, 66]. For example, it has been reported that EDCs, and estrogen mimicking agents among them, prolong the proliferation phase of immature Leydig cells, prior to their maturation [67], and can interfere with peritubular myoid cells [68], and it is known that estrogens are associated with Leydig cell tumorigenesis in mice [69–71]. The declining level of androgen during aging, associated with an increasing level of estrogen, has been hypothesized to be important in the development of benign prostatic hyperplasia [72–74], and oral exposure to low-dose BPA seems to aggravate testosterone-induced benign hyperplasia prostate in rats [75]. Several studies have suggested that estrogen exposure may increase the risk of prostate cancer [76], and various hormones such as androgens and gonadotropin-releasing hormone may play a role in prostate cancer cell growth [77, 78]. BPA and dibutyl phthalate (DBP) seem to be able to stimulate the growth of prostate cancer cells [79]. Thyroid carcinoma is the most common endocrine malignancy being about 3 times more common in women than in man [80]. Analogously, in differentiated thyroid cancer (DTC), EDCs with estrogen-like activity may be suspected to play a role in disease progression. Experimental evidence demonstrated that estrogen, but not testosterone, promotes DTC cell proliferation and that this effect could be attenuated by tamoxifen [81, 82].

In 2006, Bruce Blumberg developed the "obesogen hypothesis", to explain the weight gain effects of certain chemicals. This hypothesis is supported by laboratory and animal research as well as epidemiological studies that shown that a variety of EDCs can influence adipogenesis and obesity [83–86]. EDCs are also known to impact hormone-dependent metabolic systems and brain functionand can be easily found in human blood and urine and epidemiological literature on associations between EDC exposure and body weightis increasing [87–89].

These substances target various endocrine axes and affect adipocyte physiology and more generally the regulation of energy homeostasis [90]. Several persistent organochlorine pesticides and fungicides have been implicated in obesity [91, 92]. Metabolic alterations like metabolic syndrome and type 2 DM are recognizedas obesity comorbid conditions, and EDCs exposure may be involved in their pathogenesis. Such contribution was revealed by comprehensive studies of large and well-characterized cohorts, such as the cohort used for the NHANES (National Health and Nutrition Examination Survey) project [93, 94].

Epidemiological studies show an association between dioxins exposure and type 2 DM [95]. High and low

doses of dioxins affect genes linked with hepatic circadian rhythm, cholesterol biosynthesis, fatty acid synthesis, glucose metabolism, and adipocyte differentiation, in an AhR-dependent manner [96, 97]. Weight control and intermediate metabolism require a precise balance between energy input, storage, and consumption; several NRs are involved. The PPARs act as lipid sensors that act in different organs to adapt gene expression to a given metabolic status [98]. Plasticizers, surfactants, pesticides, and dioxins can modulate PPARs activity, and the phthalates are a group of well-characterized peroxisome proliferators. Mono-(2-Ethylhexyl) Phthalate (MEHP) is proadipogenic in a cell culture model, suggesting that it may act as a metabolic disruptor and may promote obesity in vivo [99, 100]. Kanayama et al. [101] showed that among 40 EDCs, organotins such as tributyltin (TBT) and bis (triphenyltin) oxide (TPTO) are activators of human PPARγ [102].

3. Oxidative Stress and Mitochondrial Dysfunction

An increase in oxidative stress-associated inflammation has been hypothesized to be a major mechanism in the pathogenesis of obesity-related diseases. Additionally, a rise in inflammatory cytokine levels might drive a further increase in oxidative stress, setting up a vicious cycle [104, 105]. When perturbed, the mitochondrial system alters the output of matter and energy and this may result in a pathological phenotype, such as that of obesity, dyslipidemia, metabolic syndrome, hypertension, and cancers. The failure of the skeletal muscle mitochondria to oxidize fat properly leads to ectopic lipid deposits. Cellular infiltration by excess triglycerides can impair cellular function and can also lead to oxidative stress through increased ceramide formation, increased lipid peroxidation, inflammatory cytokine production, and excess Reactive Oxygen Species (ROS) formation [106, 107]. When ROS production is increased, the disturbed balance results in a prooxidative condition. This oxidative stress can then damage various cellular structures and triggers an inflammatory response associated with adiposity, insulin resistance, and metabolic syndrome, suggesting that oxidative stress could be an early event in the pathology of these chronic diseases. [108, 109]. Recently, much evidence has emerged showing that environmental toxins, including Persistent Organic Pollutants (POPs), can affect mitochondrial function and subsequent insulin resistance. In this regard, many herbicides, insecticides, rodenticides, industrial products, and industrial toxic wastes might affect mitochondrial function and cause pro-oxidative conditions [110–112].

4. Exposure Monitoring

Various molecules, involved in antioxidant and detoxification pathways, have potential application as biomarkers in biomonitoring and risk assessment.

The National Academy of Science, in 1987, defined a biomarker as "a xenobiotically induced variation in cellular or biochemical components or processes, structures, or function that is measurable in a biological system (body fluids, cells, or tissues)" [113]. Biomarker responses in fish are routinely used to assess exposure of anthropogenic chemicals in the aquatic environment. The use of biomarkers could complement the current methods used to determine the presence of environmental pollutants and might also help to predict human health risks [114]. Among the various types of biomarkers in ecotoxicological studies are the following: cytochrome P450 activity (an indicator of the exposure and effect of organic contaminants, such as polycyclic aromatic hydrocarbons (PAHs), PCBs, and pesticides), the inhibition of Acetyl cholinesterase (AChE) activity (a biomarker of the exposure and effect of organophosphate (OP) and carbamate (CAR), metallothionein synthesis in hepatic and other tissues (exposure to the metals Zn, Cu, Cd, Hg, and Fe and some pesticides), antioxidant enzymes such as superoxide dismutase, catalase, and glutathione transferase (exposure to ROS, free radicals, and pollutants causing oxidative stress and lipid peroxidation, such as pesticides and metals), and vitellogenin induction (estrogenic substances) [115–121]. Recently, redox markers have been used to biologically define Multiple Chemical Sensitivity (MCS) [122]. The development and use of biomarkers in ecotoxicology for providing sensitive early warning signals of incipient ecological damage is motivated by the inherent instabilities of many EDCs and the chemical specificity of some biomarkers on underlying mechanisms of toxic action. However, little is known about how cocktail effects affect these biomarker responses, and chemical safety-levels are traditionally based on experiences from lab studies with single chemicals, which are unfortunate as a chemical can be more toxic when it is mixed with other chemicals, because of the cocktail effect, for example, if there is a risk for increased bioavailability of certain pollutants that can result in harmful bioaccumulation or if there is an increased risk for accumulation of toxic metabolites or if there is an increased risk for depletion of endogenous hormones. The possible involvement of receptor crosstalks, inhibition, or activation on key biotransformation enzyme and transporter proteins such and multidrug resistance-associated proteins needs to be addressed for estimation of possible adverse pharmacokinetic interactions [123].

5. Concluding Remarks

EDCs clearly contribute to diverse male and female human health problems such as decreased male sperm counts, increased incidence of hypospadias and cryptorchidism, altered male: female birth sex ratios, decreased fertility, and increased incidence of breast and testicular cancers and may be responsible for neurodevelopmental deficits in children. Recently, human exposure to EDCs has been associated with the development of some of the main diseases of the industrialized world, particularly metabolic disorders like obesity, diabetes, and metabolic syndrome. POPs such as organochlorine pesticides, dioxins, and polyfluoroalkyl compounds and no persistent pollutants such as BPA and several phthalates are endowed with metabolic disruption activity. The European Union has sponsored several international

Table 2: Examples of ongoing European research projects on endocrine disrupters [103].

Project acronym and duration	Project title	Research team	Focus
CONFFIDENCE (2008–2012)	Contaminants in food and feed: inexpensive detection for control of exposure	Participating laboratories: 17 (NL, CZ, ES, DE, DK, BE, UK, IT, FI, CH)	The main aim is to further improve food safety in Europe by the development of faster and more cost-efficient methods for the detection of a wide range of chemical contaminants (persistent organic compounds, perfluorinated compounds, and heavy metals) in different food and feed commodities
ARCRISK (2009–2013)	Arctic health risks: Impacts on health in the Arctic and Europe owing to climate-induced changes in contaminant cycling	Participating laboratories: 22 (NO, SE, DK, FI, DE, UK, ES, SI, CH, CZ, RU, CA)	The main aim will be to study the influence of climate change on contaminant spreading and transfer and the resultant risk to human populations in the Arctic and other areas of Europe
COPHES (2009–2012)	European coordination action on human biomonitoring	Participating laboratories: 35 (BE, DE, ES, UK, FR, DK, IT, EE, SI, NL, AT, RO, LT, HR, EL, CY, PT, SE, NO, HU, CH, SK, PL, CZ, IE, FI, LU)	The main goal is to develop a coherent approach to human biomonitoring in Europe, addressing the aims of Action 3 of the European Environment and Health Action Plan.
ENFIRO (2009–2012)	Life cycle assessment of environment-compatible flame retardants (prototypical case study)	Participating laboratories: 12 (NL, UK, SE, DE, IT)	ENFIRO will offer a prototypical case study on substitution options for BFRs resulting in a comprehensive dataset on viability of production and application, environmental safety, and a complete life cycle assessment
OBELIX (2009–2013)	Obesogenic endocrine disrupting chemicals: linking prenatal exposure to the development of obesity later in life	Participating laboratories: 7 (NL, BE, NO, FR, SK)	Examination of the hypothesis that prenatal exposure to endocrine disrupting compounds in food plays a role in the development of obesity later in life

research project to investigate various obesity-related effects from EDCs exposure (Table 2), regarding the diverse types of chemical compounds involved, the influence of climate changes on contaminant spreading, the age of exposition (developmental basis of adult diseases), food safety, and human bio monitoring. A greatest challenge in environmental toxicology is to understand effects of mixture toxicity (cocktail effects) in humans and in wildlife. Considering that metabolic perturbations are only one small aspect of the EDCs-related problems to be solved and that we know only the tip of the iceberg, new integrative approaches are required to understand the complexity of the cocktail effect and its consequences when exposure occurs at various life stages [124]. Procedures for risk assessment of chemical mixtures, combined, and cumulative exposures are under development, but the scientific database needs considerable expansion [114]. In particular, there is a lack of knowledge on how to monitor effects of complex exposures. As described here, solid evidence shows that endocrine disrupters can interact and even produce synergistic effects. They may act during sensitive time windows and biomonitoring their effects in epidemiological studies is a challenging task. The potential health effects of combined exposures make the risk assessment process more complex compared to the assessment of single chemicals. Techniques and methods need to be further developed to fill data gaps and increase the knowledge on harmful exposure combinations.

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