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RESEARCH ARTICLE

Multivariate Entropy Analysis of Oxidative Stress Biomarkers Following Mobile Phone Exposure of Human Volunteers: A Pilot Study

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Abstract—A multidisciplinary project was conducted to study the possible biological impact of mobile phone emissions. As part of that project, we conducted a pilot study on 18 human volunteers, with the treatment being GSM mobile phone exposure. The volunteers were randomized and the study was a double-blind, crossover design. Two categories of oxidative stress biomarkers were followed and measured in blood and exhaled air: those assessing oxidative attacks of cell membrane lipids (malondialdehyde, exhaled alkanes, aldehydes, and isoprene) and those accounting for the

organism's antioxidant defense systems (superoxyde dismutase, glutathion peroxydase, and exhaled halogenated alkanes). The overall entropy of the system with and without GSM exposure was then calculated for each volunteer, using a statistical approach based on the global entropic difference of raw data. A significant modulation of organization of the biomarkers after 30 minutes of mobile phone exposure was found, as evidenced by a decreased entropy of the dataset associated to the emitting mobile phone condition. While these results illustrate neither deleterious effects nor the innocuity of mobile phone use, they nonetheless constitute evidence of actual interactions of these wavelengths with complex biological systems. These results will need to be confirmed in larger, future studies.

Introduction

Despite much contrary evidence, there is continued concern that electromagnetic fields (EMF) might affect human health (Kheifets, Swanson, Kandel, & Malloy 2010, Repacholi, Saunders, van Deventer, & Kheifets 2005). In addition to the extremely low frequency EMF (ELF) emitted by domestic electrical apparatus and supply lines, mobile phones are the most common source of daily exposure to EMF. The GSM ("Global System for Mobile") network and its more recent evolutions, emitting fields from 900 to about 2,000 MHz, are the most widespread form of mobile communications (http://www.gsm.org). The rapid expansion of these devices and dedicated infrastructures very legitimately raised questions about the effects of their associated radiofrequencies (RF) on human health, making this topic the subject of much public health debate. As of today, research has been very active but failed to reach consensus, as data and conclusions remain anything but clearcut (Genuis 2008, Carpenter, & Sage 2008, Otto & von Muhlendahl 2007).

While numerous studies conclude that there is a lack of a biological effect or association with pathology, other results from both in vitro or in vivo animal studies point to possible effects (Lerchl, Kruger, Niehaus, Streckert, Bitz, et al. 2008, Panagopoulos, Chavdoula, Nezis, & Margaritis 2007, Ammari, Lecomte, Sakly, Abdelmelek, & De Sèze 2008, Del Vecchio, Giuliani, Fernandez, Mesirca, Bersani, et al. 2009, Ragbetli, Aydinlioglu, Koyun, Ragbetli, Bektas, et al. 2010). Physiological biomarkers–based studies on human volunteers exposed in vivo are not as widespread (Soderqvist, Carlberg, Hansson Mild, & Hardell 2009), in contrast to studies centered on behavioral and cognitive endpoints (Panda, Jain, Bakshi, & Munjal 2010, van Rongen, Croft, Juutilainen, Lagroye, Miyakoshi, et al. 2009), studies describing ex vivo exposure of human samples (Belyaev, Markova, Hillert, Malmgren, & Persson 2009), or classical epidemiology/meta analysis studies (Makker, Varghese, Desai, Mouradi, & Agarwal

2009). Importantly, RF and sham exposures of humans require strong multidisciplinary and interdisciplinary control and knowhow, ranging from the exposure setup itself to the biomarkers and the statistical analysis. Based on the putative effects of EMF on cellular components, some of these studies, alongside numerous animal studies, have focused on the potential influence on oxidative stress (Moustafa, Moustafa, Belacy, Abou-El-Ela, & Ali 2001, De Iuliis, Newey, King, & Aitken 2009, Agarwal, Desai, Makker, Varghese, Mouradi, et al. 2009, Tomruk, Guler, & Dincel 2010, Dasdag, Akdag, Ulukaya, Uzunlar , & Ocak 2009). Indeed, the equilibrium between oxidant and reductive species is precisely regulated, making this balance a suitable and important target for the investigation of possible effects of EMF. The usual ways to investigate this phenomenon rely on either monitoring the activities of the antioxidant defense systems per se or, alternatively, examining the presence of the free radicals directly or that of byproducts of their interactions with biological macromolecules.

In the present study, the activity of superoxide dismutase (SOD), which specifically removes superoxide anion and that of glutathion peroxidase (GPx), scavenging various peroxides via concomitant oxidation of glutathione, were monitored as prominent actors in the enzymatic antioxidant system (Gelain, Dalmolin, Belau, Moreira, Klamt, et al. 2009, Giustarini, Dalle-Donne, Tsikas, & Rossi 2009). On the other hand, volatile organic compounds (VOCs) such as alkanes (in native or halogenated states, referred here to as, respectively, BAA and BHA), aldehydes and isoprene found in the breath, and malondialdehyde (MDA) in erythrocytes were assayed as products of degraded lipids, downstream of the initial oxidizing attack of the free radicals (Phillips 1997, Phillips, Herrera, Krishnan, Zain, Greenberg, et al. 1999, Phillips, Cataneo, Greenberg, Grodman, Gunawardena, et al. 2003, Phillips, Cataneo, Ditkoff, Fisher, Greenberg, et al. 2003).

We evaluated the biomarkers' modifications in the course of the study with standard ANOVA tests, but more importantly using an innovative statistical analysis strategy consisting of entropy calculation to measure the degree of organization of the whole system (Durbin, Eddy, Krogh, & Mitchison 1998). The portions of the global dataset (comprising every single measurement of the study) corresponding to either the treated or the untreated conditions could then be compared to highlighted changes in their respective entropy. Our global effect analysis is expected to remain robust and effective in addressing questions such as the present one where wide inter-individual and even intra-individual variations are described, which might otherwise hide finer effects if any are present. The entropy calculation method is thus central to the present study. Noteworthy, the biomarkers were collected for

24 human volunteers before and after a single exposure to a 30-minute GSM emission (900 MHz, SAR [specific absorption rate] 0.3 W/kg). The phone was positioned with respect to each volunteer's head as it would be during regular operation, albeit through the use of a hands-free attachment system eliminates the need to actually grasp and hold the device.

Importantly, our results indicate a significant change in the organization of the biomarker panel constituted by the totality of each sample and individual systemic redox-related species analyzed. As such and under carefully controlled conditions, this work illustrates biological modulations that can only be attributed to a non-thermal effect of GSM exposure. Naturally, these studies need to be confirmed with a greater number of volunteers and integrated in a wider research project on the effect of mobile phone emissions on human biomarkers using lifelike (typical) exposure.

Materials and Methods

Ethics Statement

Each detail of the investigation was approved by the Comité de Protection des Personnes (CPP), the French commission in charge of ethical questions and agreements related to studies on human volunteers. The latter were made fully aware of the experimental procedure using a walkthrough document prior to giving their written consent.

Subjects

Healthy adult individuals (12 women and 12 men, age between 20 and 35 years) volunteered to participate in this study. 3 volunteers out of 24 were excluded from this study because they displayed high rates of C-reactive protein, a biomarker of inflammation (Elkind 2010, Genest 2010, Kaysen 2009), and three others did not participate integrally, not allowing full collection of the required samples. Eventually, 18 volunteers fully completed the study. All subjects were non-smoking, were not taking any medication, and did not regularly engage in intensive physical activities. There were no detectable caries in their mouths nor were there any signs of inflammation before exposure. On the day of the experiment, volunteers were kept 1 hour at rest before installation of a collection catheter, experimental samplings, and mobile phone exposure, "On" or "Sham".

Mobile Phone Exposure

Each volunteer participated in 2 one-day sessions, separated by a week, one including an actual EMF exposure (test) and the other a simulated (SHAM)

pseudo-exposure without any kind of emission; the day of actual exposure was randomly distributed among volunteers. A Motorola dual-band mobile phone, M3688 (900–1800 MHz), was used and located near the right ear using a PVC head holder, as previously used in the COMOBIO study of Maby, Le Bouqin, Faucon, Liegeois-Chauvel, and De Sèze (2005). This system guaranteed good reproducibility of phone positioning. During the actual exposure session, participants underwent a 900 MHz radiofrequency field for 30 min, pulsed with a repetition rate of 217 Hz with a pulse width of 0.576 ms at 250 mW mean full power. The SAR over 10 g of tissue, calculated and measured as specified on the IEC 85-214 standard, was 0.3 W/kg for the actual emission. The EMF exposure was carried out under double-blind conditions: Phones were activated through a test card controlled by codes on the keyboard by one experimenter, but on half of the phones the RF signal was routed to an internal resistance. A second investigator handled and installed the phone on each volunteer, ensuring the local experimenter and volunteers were not aware of the actual status of emission.

Exposure Control

Continuous monitoring of all exposures was performed through 2 PMM 8053 recorders during the experiments. The recorders were hidden so that volunteers could not see them nor be troubled by unknown devices. The electric field was measured every 10 seconds during the 4 hours of one single session for 4 volunteers. Each day, recordings were downloaded on a PC and sent to external collaborators who checked the correct course of the study. Samples from blood and expired air were collected in the morning and in the afternoon (three time-points before exposure and three time-points after exposure). Three markers were measured in blood: GPX, SOD, MDA. Four markers were measured in expired air: BAA, BHA, Aldehyde, Isoprene. The original design was evenly randomized in order to separate exposure and day effects. The exposed or non-exposed status was disclosed to the experimenters only after the data was statistically analyzed.

Sampling

A first batch of blood and exhaled air samples was collected during a 1-hour window prior to exposure. After the 30 minutes of mobile phone exposure, three additional samplings were performed, three exhaled air samples were collected during the first hour following exposure and three blood samples collected within two hours following exposure. Logistics and apparatus requirements made simultaneous cohort-wide sampling impossible, as such

samplings were individually scheduled to ensure volunteers had comparable time points on both experimental days.

Peripheral blood was drawn from an intravenous catheter (Jelco 18G, Rossendale, UK) with subjects in a sitting position. 5 mL of whole blood was collected in a lithium heparin tube (BD Vacutainer LH 119IU, Plymouth, UK), inverted 3 times, and spun immediately in a centrifuge (2,000×g, 15 minutes at room temperature). Supernatant was discarded and red blood cells were washed 2 times with a cold isotonic NaCl 0.9% solution by inversion and centrifugation (2,000×g, 5 minutes at room temperature). After each centrifugation, the supernatant and white blood cells interface were removed. Erythrocytes were stored in 500 µL aliquots at –20 °C prior to analysis.

Collection and gas chromatography–mass spectrometry (GC–MS) of the volatile organic compounds in human alveolar breath were performed using a dedicated, transportable apparatus (Exp'Air, AR2i, France) that specifically sampled alveolar breath. Briefly, the technique carried out in this study is a concentration method using a pump and active carbon-containing cartridges. Subjects are breathing room air through the apparatus via a set of valves, but upon reaching the end of each breath a procedure is carried out to isolate the final exhalation corresponding to the alveolar breath. This enabled collection of a concentrated alveolar breath sample through the Carbotrap 200 glass tube (Supelco, Sigma-Aldrich, France) across 2 minutes of effective pump work. After collection using this transportable system, the adsorptive trap was removed and sealed in a screwtop glass storage container prior to GC–MS analysis for volatile compounds.

Biomarkers Analysis

SOD: Superoxyde dismutase activity (SOD Cu/Zn) was measured with a commercial kit (RANSOD, Randox Laboratories, Ardmore, UK) based on the method developed by McCord and Fridovich (1969).

GPx: Glutathion peroxidase GPx activity was determined with a commercial kit (RANSEL, Randox Laboratories, Ardmore, UK) using a method based on that developed by Paglia and Valentine (1967).

MDA: A fluorimetric method, developed by Conti, Morand, Levillain, and Lemonnier (1991), was used to determine total MDA in erythrocytes. Briefly, 50 µL of erythrocytes solution was added to 1 mL of 10 mmol/L diethylthiobarbituric acid (DETBA) in phosphate buffer (0.1 mol/L, pH 3). The mixture was mixed for 5 s and incubated for 60 min at 95 °C. Samples were placed in ice for 5 min and then 5 mL of butanol was added. The DETBA-MDA adduct was extracted by shaking for 1 min, then centrifuged at 1500×g for 10 min at 4 °C. The supernatant was collected

and the fluorescence intensity measured at 553 nm. Results were quantified by comparison with the standard curve obtained with standard solutions prepared in the same conditions.

Expired alkanes, halogenated alkanes, aldehydes, and isoprene: The samples were thermally desorbed, and alkanes and halogenated alkanes were analyzed using a gas chromatograph GC 8000 TOP coupled with the mass-spectrometric detector device Automass II (electron ionization) led by Xcalibur software (Finnigan Corporation, Quad Services, Poissy, France). Compounds were identified by reference to a computer-based library of mass spectra.

Statistical Analysis

A multiple testing strategy (multivariate and univariate) was planned at the outset of the conception of this study, based on observations derived from a similar but unrelated in-house study. This resulted in one single multivariate p-value associated with entropy calculation along with marker-specific univariate p-values. As a consequence of that configuration, the single multivariate p-value was multiplied by two to adjust for test multiplicity, while univariate p-values were corrected for multiplicity using Bonferroni correction and then multiplied by two as well.

ANOVA test: For each volunteer on each day, there are 6 values for each biomarker, 3 samples collected before exposure (T01–T03) and 3 samples collected after (T1–T3). For each day and biomarker, the values T01, T02, and T03 were averaged, lending a single value. 3 ratios were then computed by dividing each of the 3 post-exposure measurements (T1, T2, and T3) by this average. As a consequence, the final dataset for each of the latter comprise 6 entries: 3 ratios of sham exposure and 3 ratios of actual GSM exposure (at this point the type of exposure is still undisclosed; only exposure days are known). A global mean effect was eventually calculated based on these 6 entries and subtracted from each individual ratio, leading to the final dataset suited for ANOVA analysis. A regular two-way ANOVA was computed, including correction multiplicity targeting "group" and "volunteer" effects on the ratio of afternoon measurements over the average of morning measurements. There are 7 biomarkers under consideration and a multiple testing strategy (multivariate and univariate), so a Bonferroni factor of 14 was used for multiplicity correction. Information about the nature of exposure for all volunteers on each day is then disclosed for final presentation of results.

Multivariate analysis: To perform multivariate entropy analysis, a table was set up with 126 columns (18 volunteers × 7 biomarkers) by 12 rows (6 samples × 2 days). The first six rows of non-exposure (sham)

were labeled "A" and referred to as *dataset OFF*, while the last six rows of EMF treatments were noted "B" and referred to as *dataset ON*. Note that exposure was randomized so as to distribute the actual or sham exposure on different days for the different volunteers.

Entropy calculation: Entropy is a statistical measure of randomness that can be used to characterize the texture of the input image. Entropy is defined as: $-\Sigma(p*\log(p))$ where p contains the histogram of the gray levels used in the image. Each column of the dataset ON or OFF was scaled (divided by its average) to put columns on a comparable scale. The entropy calculation depends on resolution, the number of bins in the histogram. An insufficient resolution results in a sharp decrease in the entropy, merely indicating that substantial information has been lost. The entropy achieved with three or four decimal points is still close to the maximum reached when the number of decimal points exceeds five, so we decided to run the permutation test with three and four decimal points and considered the mean p-value.

Results and Discussion

18 of the recruited 24 volunteers completed the study. The 30-minute EMF duration was chosen since it represented a significant exposure while remaining plausibly close to normal usage. Figure 1 illustrates the whole sampling and exposure strategy, described further here. Following their inclusion in the study, volunteers committed to following strict guidelines at least for one whole day (or more, whenever possible and if desired) before participation in the experiments. The latter included avoiding use of personal mobile phones, alcohol and pharmaceutical drugs intake, intense physical activity, and any possibly stressful situation. Upon their arrival by means of non physically exerting transportation means on the day of the experiments, volunteers were first invited to rest for one hour in a relaxed sitting position to reduce extraneous stress and to allow for gentle adaptation to the conditions of the study. As Figure 1 indicates, the first collection of both breath and blood samples took place after this resting period: Over the span of another hour, 3 breath and 3 blood samples were collected for each volunteer as described in Materials and Methods. These samples thus constituted the resting, pre-exposure status for all biological parameters explored, referred to as "T0". Volunteers then underwent either the sham or the actual GSM exposure depending on the group they were assigned to. Indeed, the two different modes of exposure took place for each participant on two different days separated by a whole week and distributed in a double-blind, randomized fashion. Thus, one half of the volunteers received a sham exposure while the other half underwent actual exposure

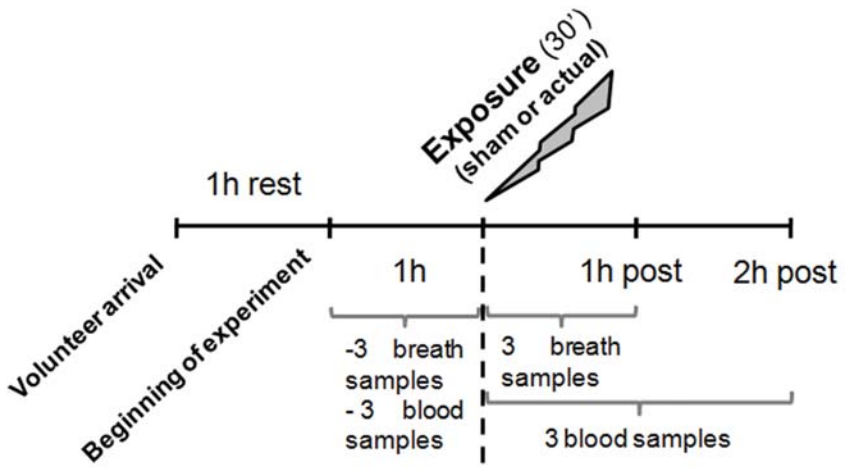

Figure 1. Sampling protocol implemented during each experimental day. Two similar experimental days took place across two consecutive weeks, with each volunteer randomly ascribed either to the sham or actual GSM exposure group for any given day. Each volunteer experienced the two types of exposures at the end of the experimental campaign.

on day 1 and vice-versa on day 2, enabling us to cancel out the day effect. In between the two experimental days, separated by exactly one week, the participants were asked to abide by the aforementioned daily-life behaviors as thoroughly as possible to reduce possible impact of these activities on any subsequent measurements. The experimenters made sure to carefully monitor that over the duration of the study no volunteer developed skin redness, rashes, or expressed discomfort or illness of any kind at any time. Local heating due to the exposure device (mainly the phone itself but also to the bands and supports used to reproductively position it against the volunteers' heads) was virtually non-existent regardless of the type of exposure, as were the local thermal effects expected from the actual GSM emission. Hence at the end of the study, each participant had undergone the two types of exposures, sham and GSM, without themselves or the operators being aware of which exposure was applied at any given moment. Disclosure of the exposure pattern and data assignation was carried out only after all statistical analyses were performed. The study was triple-blind; neither volunteer, experimenter, nor the statistician knew the treatment until after the analysis was entirely computed and results gathered. As again illustrated in Figure 1, 3 breath samples were then collected in the span of the first hour immediately following either treatment, while 3 blood samples were drawn in parallel, but on a slightly longer time interval of two

TABLE 1
Mean Blood Antioxidant Enzymatic Activities and Breath Oxidative Metabolism Markers Before (T0) and After (T) Sham or Actual EMF Exposure as Measured in the Entire Volunteer Panel

		Erythrocytes			Breath			
		GPx (U/g Hb*)	SOD (U/g Hb*)	MDA (nmol/g Hb*)	BAA (index)	BHA (index)	Isoprene (ng)	Aldehyde (ng)
Sham	T0	57.5 ± 3.0	1198 ± 15	25.5 ± 1.0	6.3 ± 0.6	6.3 ± 2.0	1302 ± 161	439 ± 36
	T	57.2 ± 3.2	1186 ± 15	24.9 ± 0.9	6.1 ± 0.5	4.0 ± 1.1	1421 ± 180	417 ± 35
EMF	T0	58.1 ± 3.1	1200 ± 12	25.4 ± 1.0	5.2 ± 0.4	3.3 ± 0.7	1093 ± 131	424 ± 33
	T	56.9 ± 3.1	1188 ± 14	24.9 ± 1.0	6.3 ± 0.4	3.7 ± 0.6	1512 ± 140	486 ± 38

Values are means ± S.E.M.
Hb = Hemoglobin, GPx = Glutathion Peroxidase, SOD = Superoxide Dismutase, MDA = Malondialdehyde, BAA = alkanes, BHA = Halogenated alkanes.

hours. These samples were denoted T1–T3, and refer to the post-exposure status of all parameters followed. This slight offset in sampling time reflects the respective expected onsets of the various biomarkers monitored: Breath markers of oxidative stress are known to appear relatively early while blood antioxidant enzymatic activities or peroxylipids were expected somewhat later, although they also are known to be rather quick-responding systems (Larstad, Toren, Bake, & Olin 2007, Corradi, Alinovi, Goldoni, Vettori, Folesani, et al. 2002). Table 1 lists the results of the measurements of blood antioxidant enzymatic activities and breath oxidative metabolism markers thus obtained, as described in more detail in Materials and Methods. All values obtained for the 3 samples of either matrix, both before and after exposure and for every volunteer, were averaged in the present set. Intuitively, the results therein do not seem to point to an obvious influence of the exposure on whatever biomarker was considered. However and importantly, it should be noted that these calculations are, in the present form, not intended nor suited for actual statistical analysis, as they lack crucial computations such as subject effect and confidence interval among other things. Table 1 is given here for reference and serves to ascertain that the measured levels of each parameter are in line with those expected (simultaneously shedding light on sampling and measurement quality).

That said, application of the entropy analysis itself was done on the global dataset comprising every single measurement / experimental point taken individually, as described in Materials and Methods. This process resulted in two datasets: The ON dataset corresponded to the matrix of 126 columns (7 markers × 18 individuals) by 6 rows (3 T0 + 3 T1 values measured during exposure day), whereas the OFF dataset corresponded to the similar matrix of 126 × 6 values measured during non-exposure day. In order to make a quantitative assessment of the level of contrast in the ON dataset, we calculated its entropy. Intuitively, the most organized dataset has the lowest entropy. Now, the question of how low the entropy should be to prove significantly low can be answered by applying a resampling-based testing strategy (Westfall & Young 1993): For this we compared the entropy of the dataset ON with the entropies of thousands of permuted datasets obtained through random selection of eight individuals arbitrarily exposed to GSM emission on day 1, whereas the ten remaining individuals were arbitrarily exposed on day 2. Specifically, each permuted dataset was obtained by: (i) picking up at random 8 individuals among the 18 individuals, (ii) labeling them as exposed to GSM emissions on day 1 (independently of the actual day of exposure for each individual), (iii) labeling the 10 other individuals as exposed to GSM on day 2 (again independently of the actual day of exposure for each individual). The number 8 was chosen to reflect the fact that due to the initial randomization of exposure sequences on the 24 starting volunteers and the completion of the study by only 18 of them, only 8 volunteers were exposed to the actual GSM emission on day 1. In these conditions, there were 43,758 possible permutations, one of them resulting in the particular dataset ON. We then counted the number of the random datasets generated, which entropies prove lower than the tested ON dataset. The ratio of this number over the total number of random datasets constitutes the significance (p-value) of the entropy of the tested dataset.

Another important point to be noted is that null values for BHA and Isoprene levels were naturally found in numerous samples because of the known detection limit of the technology used and with regard to common physiological levels. However, these measurement values could not be simply set arbitrarily at the detection limit in the original dataset because of the important number of identical values it would have generated. Indeed, random permutations that would assign values that are all equal to the detection limit into the same dataset would artificially present much lower entropy, and background noise of such datasets would be grossly underestimated. Thus, in order to prevent a bias in our multivariate test, we imputed random values for samples measured below the detection limit using a uniform distribution (in order to minimize assumptions on the error

TABLE 2
Significance of the Entropy Represented by the p-Value of the Tested Sub-datasets ON and OFF

	Sub-dataset ON	Sub-dataset OFF
Raw data	0.0202 *	ns
Raw data without outliers [a]	0.0169 *	ns
Raw data without outliers lines [b]	0.0205 *	ns

* significant ($p < 0.05$).
ns: not significant.
[a] 7 outliers out of 864 values.
[b] 28 outliers out of 864 values.

model). Since ON and OFF state values could fall below the measurement threshold, both types of measurements could result in missing values. Consequently, our test statistic is not biased by imputation.

Nevertheless, the entropy reduction might turn out to be unduly sensitive to the model used to fill in these missing values. Therefore, to ensure that our multivariate test stood robust to this imputation, a spectrum of 30 different datasets was created, each with imputed values below detection limit, prior to application of the multivariate test on each of these datasets. Eventually, for 23 out of the 30 tests, entropy of dataset ON was found to be significantly lower than the usual level of 0.05. Specifically, p-values ranged from as low as 0.005 up to 0.074, with median p-value equal to 0.0202. Since we have a multiple testing strategy (multivariate and univariate), we applied a simple multiplicity adjustment by multiplying the p-value by two. Even then, the median p-value was still lower than 0.05, showing that knowledge of exposure status while constructing the dataset resulted in a significant decrease in the entropy of dataset B. The "cleaner," more homogenous organization of dataset ON could only be attributed to an event taking place between sample collection in the morning and samples collected in the afternoon, hence most probably the exposure to the radiofrequencies. Indeed and importantly, note that if this greater organization were just a time-of-day effect, we would have seen more organization on the sham treatment day as well (dataset OFF). On the contrary, the p-value for dataset OFF always proved insignificant for any of the created datasets (p-value > 0.5).

TABLE 3
ANOVA Calculation and Biomarkers Expression Comparison between Sham or Actual EMF Exposure as Measured in the Entire Volunteer Panel

	Erythrocytes			Breath			
	GPx	SOD	MDA	BAA	BHA	Isoprene	Aldehyde
Sham exposure [a]	0.98	0.99	1.00	1.02	1.36	2.13	1.03
EMF exposure [a]	0.99	0.99	1.00	1.50	2.81	2.44	1.24
% Change [a]	1%	0	0	+47%	+107%	+15%	+24%
p-value	0.8462	0.9037	0.9179	0.0013 [b]	0.0333 [b]	0.6516	0.0932
Corrected p-Value [d]	1	1	1	0.0182 [c]	0.4662	1	1

[a] Values are least square means, percent change calculated as increase of EMF mean vs. Sham mean.
[b] Significantly different from sham exposure using ANOVA test ($p < 0.05$).
[c] Significantly different from sham exposure after adjustment for test multiplicity ($p < 0.05$).
[d] Bonferroni correction using a factor of 14 (7 biomarkers and both multi- and univariate testing strategies).

Additionally, we applied the multivariate testing strategy to the dataset cleared from outliers after implementation of a dedicated statistical analysis. Results became even more significant: Depending on whether we either mathematically removed only the seven found outliers or even more stringently the entirety of measurements datamined from breath-collection cartridges displaying such outliers, median p-values were found to be equal to 0.0169 and 0.0205, respectively. Regardless, all p-values based on different imputations remained below 0.05. The integrality of these results is summarized in Table 2.

We also applied more classical statistical tools such as least square means and ANOVA calculation to the measurements obtained. As shown in Table 3, notable increases were only evidenced in all expired air markers, albeit only the BAA increase eventually proved significant, with a p-value of 0.0013, and remained so even after stringent multiplicity correction, with a final p-value of 0.0182. These observations are paramount in the sense that they vouch for the superior relevance of the entropy calculation strategy in the context of extended, wildly varying datasets. These may indeed present minute variations across the board (something which is expected for the complex networks and equilibriums of physiological systems), albeit with no one parameter individually shifting enough to register positive via classical approaches, even though this was not the case here as illustrated by the BAA. Of note, all the permutations and corrections implemented

during entropy assessment ensured that this single result alone could not "weigh in" enough to explain the observed modulations, which is owed to the global evolution of the entire dataset versus the impact of only a handful of measurements.

Conclusion

The results of the present study, at least in our experimental conditions, support the hypothesis of non-thermal effects of mobile phone-emitted electromagnetic fields on biological tissues and/or functions. As such, the data illustrate modulations of chosen biomarkers associated with or directly implicated in oxidative stress response/onset in human organisms. This is in agreement with observations reported in other studies, although in very different experimental conditions, which in the end tends to enforce the initial statement (Xu, Zhou, Zhang, Yu, Zhang, et al. 2009, Ozgur, Guler, & Seyhan 2010, Kovavic & Somanathan 2010).

The manifestations of oxidative stress-related events were chiefly evidenced by a significant increase of alkanes in the breath of volunteers (at the exclusion of all other pro-oxidant actors monitored) who participated in the study. In parallel, the activities of the antioxidant enzymes SOD and GPX did not seem to be modified in erythrocytes. However, it should be noted here that the aforementioned results were obtained through a classical ANOVA testing that, in essence, singles out each biomarker and the various measurements associated with them. Interestingly, the implementation of the multivariate, entropy-based analysis of the dataset taken as a whole (as opposed to segmented into each individual biomarker and value) painted a much different picture. The latter revealed global modulations across the board, modulations that appeared individually, with the exception of BAA, too minute to score significantly for a given parameter but in the end confirming a global interaction of the applied electromagnetic fields upon the entirety of the data gathered.

Although it is likely, still, that the overall oxidative balance was not dramatically offset by the mobile phone exposure in the end, it is important to remember that all biomarkers chosen were systemic and monitored in the complex compartments and interfaces that blood and breath constitute. Hence, the potential relevance in terms of biological effects, while impossible to ascertain based solely on these observations, should not be too hastily ignored.

In conclusion, these data illustrate interactions of EMF emitted by mobile phones with whole biological systems, in a context that still presents no clear consensus (Verschaeve, Juutilainen, Lagroye, Miyakoshi, Saunders, et al. 2010, Gaestel 2010, Ziemann, Brockmeyer, Reddy, Vijayalaxmi, Prihoda, et

al. 2009, Kundi & Hutter 2009) regarding that particular issue. Results must naturally be confirmed with more subjects in a similar experimental setup; nevertheless, the design proposed here undoubtedly allows for appropriate statistical robustness and relevance of exposure and readouts in its present form. It would surely prove very enlightening to extend this investigation to chronic EMF exposures in an identical context in order to explore potential, longer-term effects of the use of cellular phones in human organisms.

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References Cited

Agarwal, A., Desai, N. R., Makker, K., Varghese, A., Mouradi, R., et al. (2009). Effects of radiofrequency electromagnetic waves (RF-EMW) from cellular phones on human ejaculated semen: An in vitro pilot study. *Fertility and Sterility*, *92*, 1318–1325.

Ammari, M., Lecomte, A., Sakly, M., Abdelmelek, H., & De Sèze, R. (2008). Exposure to GSM 900 MHz electromagnetic fields affects cerebral cytochrome c oxidase activity. *Toxicology*, *250*, 70–74.

Belyaev, I. Y., Markova, E., Hillert, L., Malmgren, L. O., & Persson, B. R. (2009). Microwaves from UMTS/GSM mobile phones induce long-lasting inhibition of 53BP1/gamma-H2AX DNA repair foci in human lymphocytes. *Bioelectromagnetics*, *30*, 129–141.

Carpenter, D. O., & Sage, C. (2008). Setting prudent public health policy for electromagnetic field exposures. *Reviews on Environmental Health*, *23*, 91–117.

Conti, M., Morand, P. C., Levillain, P., & Lemonnier, A. (1991). Improved fluorometric determination of malonaldehyde. *Clinical Chemistry*, *37*, 1273–1275.

Corradi, M., Alinovi, R., Goldoni, M., Vettori, M., Folesani, G., et al. (2002). Biomarkers of oxidative stress after controlled human exposure to ozone. *Toxicology Letters*, *134*, 219–225.

Dasdag, S., Akdag, M. Z., Ulukaya, E., Uzunlar, A. K., & Ocak, A. R. (2009). Effect of mobile phone exposure on apoptotic glial cells and status of oxidative stress in rat brain. *Electromagnetic Biology and Medicine*, *28*, 342–354.

Del Vecchio, G., Giuliani, A., Fernandez, M., Mesirca, P., Bersani, F., et al. (2009). Continuous exposure to 900MHz GSM-modulated EMF alters morphological maturation of neural cells. *Neuroscience Letters*, *455*, 173–177.

Deluliis, G. N., Newey, R. J., King, B. V., & Aitken, R. J. (2009). Mobile phone radiation induces reactive oxygen species production and DNA damage in human spermatozoa in vitro. *PLoS One*, *4*, e6446.

Durbin, R., Eddy, S., Krogh, A., & Mitchison, G. (1998). *Biological Sequence Analysis. Probabilistic Models of Proteins and Nucleic Acids*. Cambridge University Press. pp. 305–311.

Elkind, M. S. (2010). Impact of innate inflammation in population studies. *Annals of the New York Academy of Science*, *1207*, 97–106.

Gaestel, M. (2010). Biological monitoring of non-thermal effects of mobile phone radiation: Recent approaches and challenges. *Biological Reviews of the Cambridge Philosophical Society*, *85*, 489–500.

Gelain, D. P., Dalmolin, R. J., Belau, V. L., Moreira, J. C., Klamt, F., et al. (2009). A systematic review of human antioxidant genes. *Frontiers in Bioscience*, *14*, 4457–4463.

Genest, J. (2010). C-reactive protein: Risk factor, biomarker and/or therapeutic target? *The Canadian Journal of Cardiology*, *26*(Supplement A), 41A–44A.

Genuis, S. J. (2008). Fielding a current idea: Exploring the public health impact of electromagnetic radiation. *Public Health, 122*, 113–124.

Giustarini, D., Dalle-Donne, I., Tsikas, D., & Rossi, R. (2009). Oxidative stress and human diseases: Origin, link, measurement, mechanisms, and biomarkers. *Critical Reviews in Clinical Laboratory Sciences*, *46*, 241–281.

Kheifets, L., Swanson, J., Kandel, S., & Malloy, T. F. (2010). Risk governance for mobile phones, power lines, and other EMF technologies. *Risk Analysis.*

Kaysen, G. A. (2009). Biochemistry and biomarkers of inflamed patients: Why look, what to assess. *Clinical Journal of the American Society of Nephrology*, *4*(Supplement 1), S56–S63.

Kheifets, L., Swanson, J., Kandel, S., & Malloy, T. F. (2010). Risk governance for mobile phones, power lines, and other EMF technologies. *Risk Analysis.*

Kovacic, P., & Somanathan, R. (2010). Electromagnetic fields: Mechanism, cell signaling, other bioprocesses, toxicity, radicals, antioxidants and beneficial effects. *Journal of Receptors and Signal Transduction Research*, *30*, 214–226.

Kundi, M., & Hutter, H. P. (2009). Mobile phone base stations—Effects on wellbeing and health. *Pathophysiology*, *16*, 123–135.

Larstad, M. A., Toren, K., Bake, B., & Olin, A. C. (2007). Determination of ethane, pentane and isoprene in exhaled air—Effects of breath-holding, flow rate and purified air. *Acta Physiologica* (Oxford, England), *189*, 87–98.

Lerchl, A., Kruger, H., Niehaus, M., Streckert, J. R., Bitz, A. K., et al. (2008). Effects of mobile phone electromagnetic fields at nonthermal SAR values on melatonin and body weight of Djungarian hamsters (*Phodopus sungorus*). *Journal of Pineal Research*, *44*, 267–272.

Maby, E., Le Bouquin, J. R., Faucon, G., Liegeois-Chauvel, C., & De Sèze, R. (2005). Effects of GSM signals on auditory evoked responses. *Bioelectromagnetics*, *26*, 341–350.

Makker, K., Varghese, A., Desai, N. R., Mouradi, R., & Agarwal, A. (2009). Cell phones: Modern man's nemesis? *Reproductive Biomedicine Online*, *18*, 148–157.

McCord, J. M., & Fridovich, I. (1969). Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *The Journal of Biological Chemistry*, *244*, 6049–6055.

Moustafa, Y. M., Moustafa, R. M., Belacy, A., Abou-El-Ela, S. H., & Ali, F. M. (2001). Effects of acute exposure to the radiofrequency fields of cellular phones on plasma lipid peroxide and antioxidase activities in human erythrocytes. *Journal of Pharmaceutical and Biomedical Analysis*, *26*, 605–608.

Otto, M., & von Muhlendahl, K. E. (2007). Electromagnetic fields (EMF): Do they play a role in children's environmental health (CEH)? *International Journal of Hygiene and Environmental Health*, *210*, 635–644.

Ozgur, E., Guler, G., & Seyhan, N. (2010). Mobile phone radiation-induced free radical damage in the liver is inhibited by the antioxidants N-acetyl cysteine and epigallocatechin-gallate. *International Journal of Radiation Biology*, *86*, 935–945.

Paglia, D. E., & Valentine, W. N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *Journal of Laboratory and Clinical Medicine*, *70*, 158–169.

Panagopoulos, D. J., Chavdoula, E. D., Nezis, I. P., & Margaritis, L. H. (2007). Cell death induced by GSM 900-MHz and DCS 1800-MHz mobile telephony radiation. *Mutation Research*, *626*, 69–78.

Panda, N. K., Jain, R., Bakshi, J., & Munjal, S. (2010). Audiologic disturbances in long-term mobile phone users. *Journal of Otolaryngology Head & Neck Surgery*, *39*, 5–11.

Phillips, M. (1997). Method for the collection and assay of volatile organic compounds in breath. *Analytical Biochemistry*, *247*, 272–278.

Phillips, M., Herrera, J., Krishnan, S., Zain, M., Greenberg, J., et al. (1999). Variation in volatile organic compounds in the breath of normal humans. *Journal of Chromatography B: Biomedical Sciences and Applications*, *729*, 75–88.

Phillips, M., Cataneo, R. N., Ditkoff, B. A., Fisher, P., Greenberg, J., et al. (2003). Volatile markers of breast cancer in the breath. *The Breast Journal*, *9*, 184–191.

Phillips, M., Cataneo, R. N., Greenberg, J., Grodman, R., Gunawardena, R., et al. (2003). Effect of oxygen on breath markers of oxidative stress. *European Respiratory Journal*, *21*, 48–51.

Ragbetli, M. C., Aydinlioglu, A., Koyun, N., Ragbetli, C., Bektas, S., et al. (2010). The effect of mobile phone on the number of Purkinje cells: A stereological study. *International Journal of Radiation Biology*, *86*, 548–554.

Repacholi, M., Saunders, R., van Deventer, E., & Kheifets, L. (2005). Guest editors' introduction: Is EMF a potential environmental risk for children? *Bioelectromagnetics Supplement, 7*, S2–S4.

Soderqvist, F., Carlberg, M., Hansson Mild, K., & Hardell, L. (2009). Exposure to an 890-MHz mobile phone-like signal and serum levels of S100B and transthyretin in volunteers. *Toxicology Letters*, *189*, 63–66.

Tomruk, A., Guler, G., & Dincel, A. S. (2010). The influence of 1800 MHz GSM-like signals on hepatic oxidative DNA and lipid damage in nonpregnant, pregnant, and newly born rabbits. *Cell Biochemistry and Biophyics*, *56*, 39–47.

van Rongen, E., Croft, R., Juutilainen, J., Lagroye, I., Miyakoshi, J., et al. (2009). Effects of radiofrequency electromagnetic fields on the human nervous system. *Journal of Toxicology and Environmental Health Part B Critical Reviews*, *12*, 572–597.

Verschaeve, L., Juutilainen, J., Lagroye, I., Miyakoshi, J., Saunders, R., et al. (2010). In vitro and in vivo genotoxicity of radiofrequency fields. *Mutation Research*, *705*, 252–268.

Westfall, P. H., & Young, S. S. (1993). *Resampling-Based Multiple Testing*. New York: Wiley.

Xu, S., Zhou, Z., Zhang, L., Yu, Z.., Zhang, W., et al. (2009). Exposure to 1800 MHz radiofrequency radiation induces oxidative damage to mitochondrial DNA in primary cultured neurons. *Brain Research, 1311*, 189–196.

Ziemann, C., Brockmeyer, H., Reddy, S. B., Vijayalaxmi, Prihoda, T. J., et al. (2009). Absence of genotoxic potential of 902 MHz (GSM) and 1747 MHz (DCS) wireless communication signals: In vivo two-year bioassay in B6C3F1 mice. *International Journal of Radiation Biology*, *85*, 454–464.