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ORIGINAL ARTICLE

Extra-low-frequency magnetic fields alter cancer cells through metabolic restriction

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Abstract

Background: Biological effects of extra-low-frequency {ELF) magnetic fields {MFs) have lacked a credible mechanism of interaction between MFs and living material. *Obiectives:* To examine the effect of ELF·MFs on cancer cells. *Methods:* Five cancer cell lines were exposed to ELF·MFs within the range of0.025-Sf.1T, and the cells were examined for karyotype changes after 6d. *Results:* All cancer cells lines lost chromosomes from MF exposure, with a mostly flat dose·response. Constant MF exposures for three weeks allow a rising return to the baseline, unperturbed karyotypes. From this point, small MF increases or decreases are again capable of inducing karyotype contractions {KCs). Our data suggest that the KCs are caused by MF interference with mitochondria's adenosine triphosphate synthase {ATPS), compensated by the action of adenosine monophosphate-activated protein kinase {AMPK). The effects of MFs are similar to those of the ATPS inhibitor, oligomycin. They are amplified by metformin, an AMPK stimulator, and attenuated by resistin, an AMPK inhibitor. Over environmental MFs, KCs of various cancer cell lines show exceptionally wide and flat dose-responses, except for those of erythroleukemia cells, which display a progressive rise from 0.025 to 0.4 f.1T. *Conclusions:* The biological effects of MFs are connected to an alteration in the structure of water that impedes the flux of protons in ATPS channels. These results may be environmentally important, in view of the central roles played in human physiology by ATPS and AMPK, particularly in their links to diabetes, cancer and longevity.

Keywords

AMP-activated protein kinase, ATP synthase, chromosome instability, extra-low-frequency, magnetic field

History

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Introduction

Since the Wertheimer and Leeper (1979) article l:inking wire codes to childhood cancer, the relation between cancer and power-frequency magnetic fields (MFs) has been under investigation (Heroux, 1991). Population, *in vivo* and *in vitro* studies have falled to provide a clear link. The exception is childhood leukemia (Ahlbom et al., 2000), leading the International Agency for Research on Cancer to attach the class 2B carcinogen designation to MFs in June 2001 (IARC, 2002).

It has been argued that enviroru nental 60-Hz lviFs, as non-ionizing radiation and incapable of raising tissue temperatures, could not have significant impacts on cells. But effects on breast cancer cells, MCF-7, were confirmed by a number oflaboratories near 1.2 T (Ishido et al, 2001). Many have also reported a diversity of effects above 2.5 T, higher than common environmental exposures. These include lengthened mitotic cycle and depressed respiration (Goodman et al., 1979), increased soft agar colony formation (Phillips et al., 1986), inhibition of differentiation with increased cell proliferation (Chen et al., 2000) as well as DNA breaks with apoptosis and necrosis (Lai and Singh, 2004).

In the early days of extra-low-frequency (ELF)-MF research, Senllkhina et al. (Sem.ild:rina et al., 1988; Semikhina and Kiselev, 1981) documented by electrical dissipation factor (roRC, also known in electrical engineering as *tg* o) and optical measurements (the dimerization of dilute rhodamine 60 solutions) that alternating lviFs in the range 25nT-879 T disrupt the anangement of water molecules, particularly under *high concentrations of hydrogen bonds and protons.* The effects were absent above 40-50°C, as water structure changes. The maximum effect was detected at 156.2Hz and 15.45 T for 70C pure water. NatTow resonances were observed, easily broadened by the presence of even small levels of impurities. The MF effects on water progressed over 5h and dissipated over 2h after the field was turned off.

Interestingly, when alternating lviFs were kept below 25nT, an influence of static MFs on water could also be detected. Removing the static MF acted on water variables (dissipation factor and optical measurements) in a direction opposite to the application of ELF-lviFs larger

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than 25 nT. Thus, it seemed that elimination of both ELF and static MFs allowed water to "opti.rnlze" its molecular arrangement.

These observations created ground to attempt an interpretation of ELF-MF health effects based on water structure alterations brought about by the MF itself, as opposed to magnetically induced currents. We investigated this possibility by setting up baseline cancer cell lines maintained under power-frequency MFs lower than 4 nT, and also under anoxia.

As 82% of oxygen readings in solid tumors are less than 0.33% (Kizaka-Kondoh et al., 2003), and stem cells are hosted in niches that are very low in oxygen (Hill et al., 2009), anoxia is a better simulation of the tumor environment than routinely used 21% oxygen. Our cells are also hyperploid, displaying a range of chromosomes numbers larger than 46, as a result of the enhanced metabolism typical of cancer cells. The absence of oxygen reduces chromosome numbers to some extent, but not back to normal, and also narrows their range (Li et al., 2012).

Metabolic restrictors, chemicals that impair oxygen metabolism, adenosine triphosphate (ATP) synthesis or ATP use, can bring back chromosome numbers in cancer cell lines even closer to their original46 than anoxia alone, an effect we labeled *karyotype contraction* (KC). KC is a rapid and reversible loss of clu·omosomes resulting from metabolic restriction (Li et al., 2012).

A critical enzyme in ATP production is ATP synthase (ATPS).The structure of ATPS is documented in detail (Boyer; 2002; Sasada and Marcey, 2010) as a rotating motor-generator structure activated by the trickle of high-density protons from the inter-membrane space into the matrix of mitochondria. Proton diffusion along the 15nm thick inter-membrane space does not limit their transit time of 1-2 JlS (Procopio and Fornes, 1997). Protons enter the Fo of ATPS along an *entry half-channel* made of four hydrophilic ex-helices, to reach a rotating helix. After rotation, protons flow out through a similar *exit half-channel*.The rotation is used by the F_1 segment of ATPS to produce ATP (Procopio and Fornes, 1997).

These hydrophilic channels (Fillingame etal., 2003) provide a high density of hydrogen bonds, while the mitochondrial inter-membrane space feedsATPS a high-density of protons, as required for maximum MF effect on water by Semikhina et al. (Semikhina et al., 1988; Semikhina and Kiselev, 1981). The high-density protons (pH 1; Procopio and Fornes, 1997) are driven through the half-channels by a 180kV/cm electric field (Zorov et al., 2009) across the inner membrane (Mitchell, 1966). In this study, we assess the ability of 1-fFs at common environmental levels to induce KCs.

Methods

Cells and culture conditions

The cell lines, K562 and HEL 92.1.7 (erythroleukemias), MCF7 (breast cancer), NCI-H460 (lung cancer) and COL0320DM (colon cancer) were obtained from ATCC (Manassas, VA). Cells are maintained under 5% carbon dioxide and 90% humidity, and grown in synthetic culture medilll11. because changes in serum can alter chromosome counts. The medium is RPMI-1640 with L-glutamine (Sigma 61-030-RM), sodium selenite, 20nM (Sigma S-5261), bovine insulin 1mgll (Sigma15500), iron saturated bovine transferrin 25mgll (Sigma T1408), sodium bicarbonate 2g/1 (SigmaS-6014) and bovine serum albumin 4g/1 (Sigma A3311, Oakville, Canada). Vented T-25s (Sarstedt 83.1810.502, Ntirnbrecht, Germany) and T-12s (Falcon 353018, BD, Franklin Lakes, NJ) were used for experiments, and cells are seeded at 5000/cm^2 and kept in the same medium for 6 d. In longer tests (3 weeks), new medium is added weekly. Oxygen was eliminated by enclosing T-25s and T-12s in large polycarbonate containers (1.61, Starfrit Lock & Lock, Longueuil, Canada) flushed with medical-grade nitrogen (95%) and CO_2 (5%). pH readings were conducted under isothennal conditions (water bath) for samples as well as calibration buffers, using Corning 445 meters (Corning, NY).

Magnetic fields

Unexposed cells for experiments are kept in T-12 or T-25 culture flasks under anoxia and MFs below 4 nT.Three 6.3 mm thick layers of structural steel reduce ELF-MFs from incubators and the environment. Culture vessels are centered in a rectangular st.ructw:al steel pipe 5.1 x 7.6 em, itself contained in a7.6 x 10.2mm pipe, both 20 em long.These two shields are placed in a 15.2 x 24.5 x 36 em long pipe. This reduces 60-HzMFs by a factor of 144, providing unexposed cells with a MF environment of 3nT, slightly below the measurement floor (5 nT at 60Hz) of our Narda EPA-300 instrument (Hauppauge, NY).The incubator is a Forma 3310 (ThermoFisher, Waltham, MA), with low average MF (0.4).11).

MFs are applied by rectangular coils (19 x 25.6 em) with 20-50 turns of #25 AWG varnished copper wire wound on 13mm polycarbonate, providing8 ft The coil is under the two irmer shields and over an ac1ylic spacer at the bottom of the outer shield. 60-Hz fields above 0.4 JlT are from sector-cormected variable transformers fitted with a passive low-pass capacitive filters, with all harmonics at less than 20 dB. Smaller 60-Hz fields and other frequencies were generated with computer-based synthesizers with a background noise at less than 40dB. MFs are within 10% of nominal in the whole cell culture area.

The "NIM" shield cancelling both alternating and static MFs is an acrylic cylinder 5.7 em in internal diameter 'vith a 0.38 em wall and 38 em in length, covered by six layers of 0.4mm nickel-iron-molybdenum foil (ASTM A753 Type 4) wound in a spiral, together with a 1.6 mm neoprene membrane spacer (Futurplast, St-Laurent, Canada).

60-Hz 5 JlT exposures produce no measurable temperature rises. K562 is a good the1mal sentinel, hype1ihermia being detectable from larger cell sizes at +0.5 K, while +1K seriously impairs proliferation and +2K over a few days is lethal.

Chromosome, cell and adenosine monophosphate-activated protein kinase assays

Metaphase preparation and cytogenetic analysis were performed according to the standard trypsin-Gien1sa banding technique. Karyotypes are obtained using x100 oil immersion, a Laborlux D (Leitz, Leica, Wetzlar, Germany) microscope, and an Infinity X (21 Mpixels) CMOS camera (Lumenera, Ottawa, Canada).

Cell proliferation and cell size histograms of are *fi:om* a Scepter automated cell counter (Millipore, Billerica, MA). Metformin was obtained from Sigma (D150959) and resistin from Prospec Protein Specialists, East Bnmswick, NJ.

Results

Because of the controlled MFs and of anoxia, our reference K562 cultures are karyotypically and otherwise exceptionally stable. Seventy-five percent of the cells have just t\vo chromosome numbers, 62 and 61, compared to a wider range under 21% oxygen (Li et al., 2012). The stability of chromosome numbers in baseline anoxic K562 has been pe1iodically confirmed in our laboratory over 5 years. These cultures provide an extremely precise reference point, as shown in the nanow baselines of Figures 1, 2 (top) and 4. This is of great advantage in obtaining statistical significance in our data. Figure 1 sho\-vs little overlap between baseline and exposed data, yielding small numbers in Student's t-tests. In Figure 2 (top), the *p* value between baseline and 0.025 J..LT is 0.00012. In Figure 3, even when using 21% oxygen, the large number of karyotypes performed, and the strong shifts in average chromosome numbers produced by MFs result in extremely small *p* values (0.000006 for MCF7).

Induced currents

\\'hether biological effects of power-frequency MFs relate to the MF itself, or to the CUlTents induced in tissues by the fields, has been a perennial question. Many think that effects occur through potentials produced by magnetically induced currents blocked by the thin membranes, within or bordering living cells. Such cments and membrane potentials are familiar to conventional electrophysiology.

In the results of Figure 1, one aliquot of an anoxic K562 cell culture is placed in a vertical and the second in a horizontal MF exposure system. At the same MF, the horizontal coil induces cments six times larger because the exposed culture dish area is 34 x 34 mm for the horizontal coil, compared to 5.8 x 34mm for the vertical coil. As KCs after 6 d at 1J..LT come out similarly for both orientations (Figure 1), we conclude that the effect on chromosome numbers are dependent on the MF itself. \Ve assume direct MF, rather than induced cment action on the basis that variations of cunent density by a factor of 6 do not affect KC. But this would also occur if induced cments had a flat dose-response, already saturated at the lower current. Furthemwre, direct MF action on KC does not preclude that other effects of MFs may depend on induced currents.

Dose-response

Figure 2 (top) shows the chromosome number losses experienced by previously shielded anoxic K562 cells after 6 d in various MFs. Under any exposure, the nanow baseline expands, and there are substantial KCs.

Three features are of importance. First, a no-effect-level lower than 25nT. Second, a progression of KCs up to 0.4 J..LT. Third, the relatively flat dose-response bet\veen 0.1 and 1.5J..LT.

The graph spans time-averaged MFs representing domestic (0-0.2J..LT), commercial (0.07-0.5 J..LT) and occupational (0.1-1J..LT) environments (Heroux, 1987).

Later sections of this article will argue that MFs act most directly on the structure of water, leading to an alteration in proton mobility. As proton mobility is tightly related to pH, a measurement of hydrogen ion activity, some perturbation of pH values might be expected. A lasting effect of MFs on aqueous fluids is actually observable from pH measurements in cell culture media, which turn slightly more acidic under short MF exposures. After 20h, there is a difference of 0.09 pH units with a 95% confidence interval of 0.045 between unexposed versus 5 J..LT 60-Hz exposed media (Figure 2; bottom) for the widely used RPMI-1640

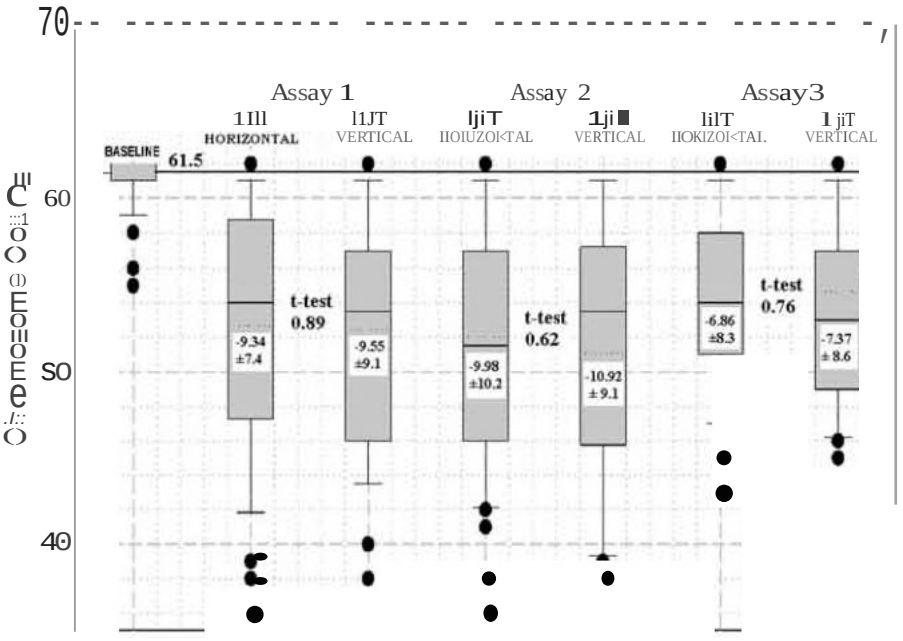

Figure 1. Baseline anoxic K562 cells at less tl1an 4nT (60Hz) with an average of 61.5 chromosomes (horizontal line) and a very narrow distribution (at left) are simultaneously transferred for 6 d to 11-1T MFs applied eitl1er horizontally or vertically. Three independent 6-d assays show the resulting chromosome numbers. Box plots show median (solid), average (dotted), 25 and 75% (box), 10 and 90% limits (whiskers) and outside values (dots). Fifty-si'< (assay 1), 50 (assay 2) and 51 (assay 3) metaphases were karyotyped in each orientation. Inside the box plots are average chromosome losses. TI1e Student's t-test results quantify the probability that the horizontal and vertical results are identical.

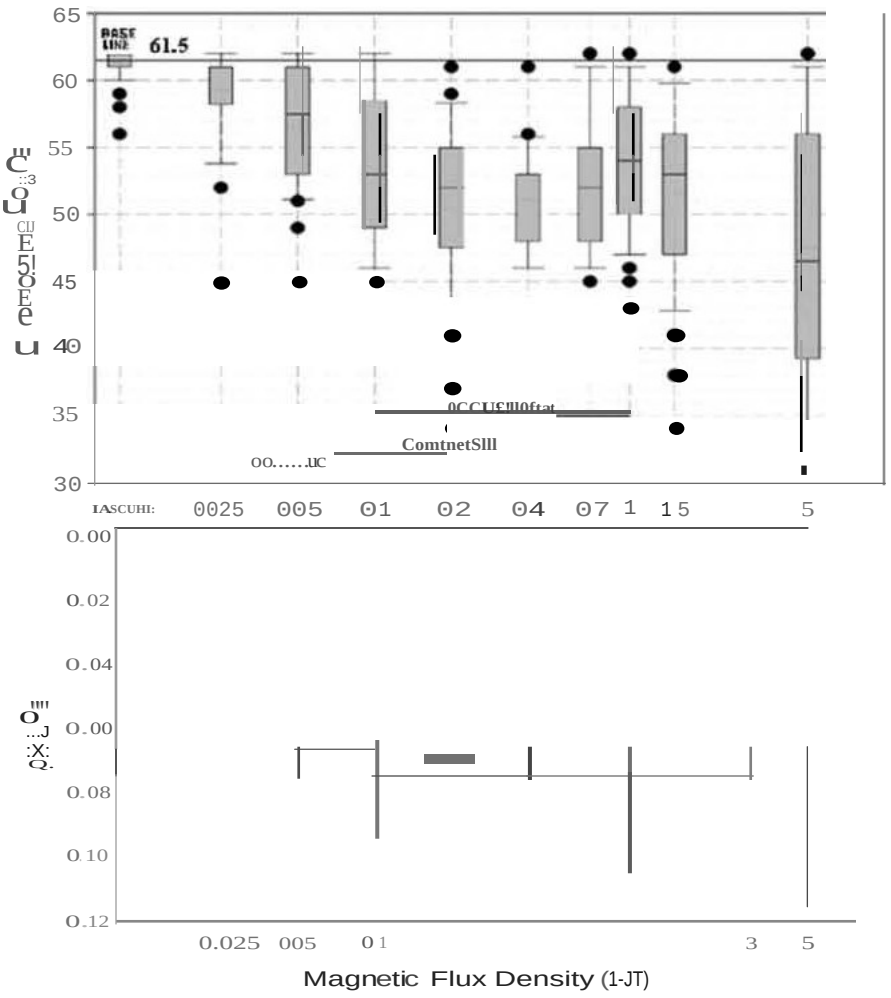

Figure 2. Top: K562 chromosome numbers as a function of 60Hz magnetic flux density. Six-day assays with, in sequence, 65, 28, 50, 77, 46, 33, 65, 102, 56 and 50 metaphases. 1\vo to *6* experiments at each MF. Approximate ranges for domestic, commercial and occupational exposures are shown. Bottom: pH differences between two cell medium aliquots, one exposed for 20h to <4nT at 60Hz and the second to the MF density in the figure. Medium is RPMl-1640 with 10% FBS. Isotherm measurements using auto read were made with the same probe, alternating between the two aliquots. TI1ree measurements for each aliquot and three repeats at each MF density.

with 10% serum. The pH shift was confirmed for a variety of cell culture media

Across cell lines

Beyond K562, we investigated four more hyperploid cancer cell lines to determine the generality of KC by MFs. Over two orders of MF magnitude, erythroleukemia (HEL 92.1.7), breast (l\1CF7) and lung (NCI-H460) cancer cells lose between 8 and 13 chromosomes (Figure 3). BEL, our second erythroleukemia cell line, shows fewer losses at lower fields, similar to K562. Three of the four results reported in Figure 3 were obtained under standard (21% oxygen) culture conditions.

Classical toxicology and epidemiology, where smoothly climbing dose-responses are justified by binding chemistry and the central tendency theorem. do not expect the flat dose-responses observed in Figures 2 (top) and 3. The effects found for different cell types are strikingly similar, with similar low-field deviations in the two erythroleukemia lines, suggesting common, basic mechanisms.

Differential action

K562 cells with magnetic KCs, such as in Figure 2 (top), progressively recover their original chromosome numbers after 3 weeks, even as the :MF is maintained at a constant level (Figure 4; top). Surprisingly, in cells recovering over 3 weeks from a MF disturbance, the deviation of chromosome numbers is even less than what is observed in the long-term baseline culture, as shown in the last measurement of Figure 4 (top) and in the central measurements of Figure 4 (middle and bottom). Chromosome numbers restore earlier than chromosome number dispersions.

After 3 weeks, if the MF is altered by a small percentage of the original value, either positively or negatively, KCs are again observed, as shown in Figure 4 (middle and bottom). Starting from low (middle, 0.1 T) or high (bottom. 1 T) baselines, symmetrical KCs are observed. This bilateral sensitivity to changes is unforeseen by conventional toxicological principles. KC is also observed when fields are reduced from 50 to 4nT (not shown).

The KCs will be interpreted below as caused by magnetically induced perturbations in intra-cellular AlP levels. These results cast doubt on the stability of cancer cell models housed in incubators with MFs that are highly variable over space and time (l\1ild et al., 2009; Su and Heroux, 2012).

Over frequency

We measured in anoxic K562 6-d tests at 1 T, the average KCs over frequency as follows:

3.6 ±0.79 at 50-Hz, 9.36 ± 1.06 at 60-Hz,
12.71 ±1.82 at 120-Hz and 9.8 ±1.31 at 155-Hz.

A polynomial fit predicts maximum KC at 113Hz for 1T.

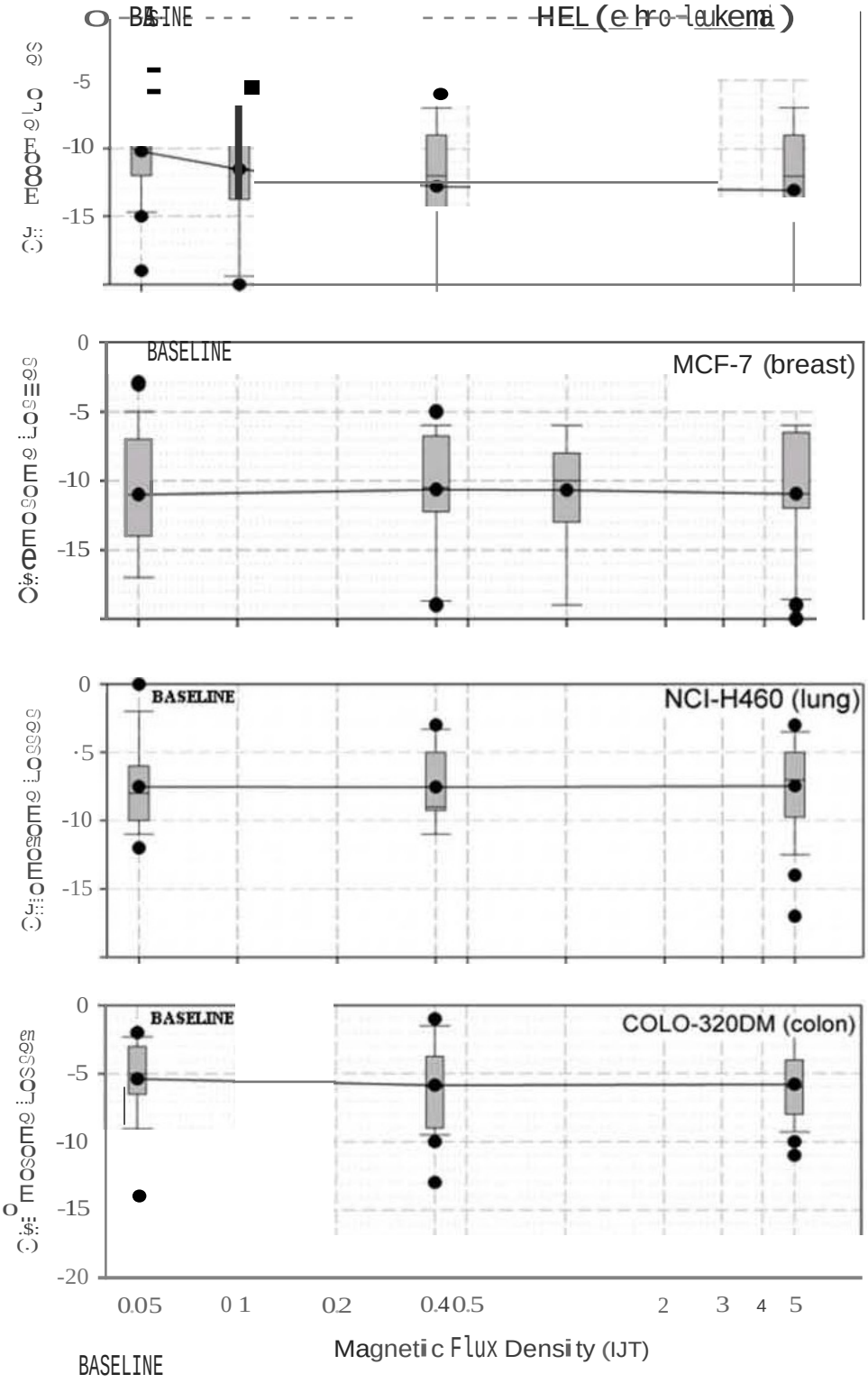

Figure 3. Average chromosome losses in erythroleukemia, breast, lung and colon cancer cells as a function of 60Hz magnetic flux density. The cllromosome number baseline(''0'') averages for <4nT cells at 60Hz, 80% range and metapllase number are: HEL: 66, 62-67 and 32; MCF7: 74, 61-75 and 30; NO-H460: 57,53-65 and 30; and COLO 320DM: 54,49-61 and 30. Six-day assays witll, in sequence, 32, 22, 29, 32; 19, 22, 19, 21; 29, 22, 24; 22, 34 and 46 metapllases. Two to five experiments at each MF. HEL, NO-H460 and COL 320DM assays used 21% oxygen, ratller tl1an anoxic conditions, as some anoxic karyotype modes are too close to 46 to allow easy statistical separation from MF-exposed samples.

Static MF removed

The :influence of the static MF was investigated by observing K562 cells transferred from a steel shield that eliminated ELF MFs (to less than 5n'I), but had a static field of 74 T, to a second shield (NIM) that attenuated both the ELF-MF (less than 5n'I) and the static field to 3 T. Karyotyping revealed a very slow drift downward, but a strong effect on proliferation rate was observed. After 4 d, cell numbers in the NIM shield were increased by a factor of 2.05 ± 0.13 (SD) over cells kept in the steel shield, indicating enhanced metabolism. The effect is persistent over time.

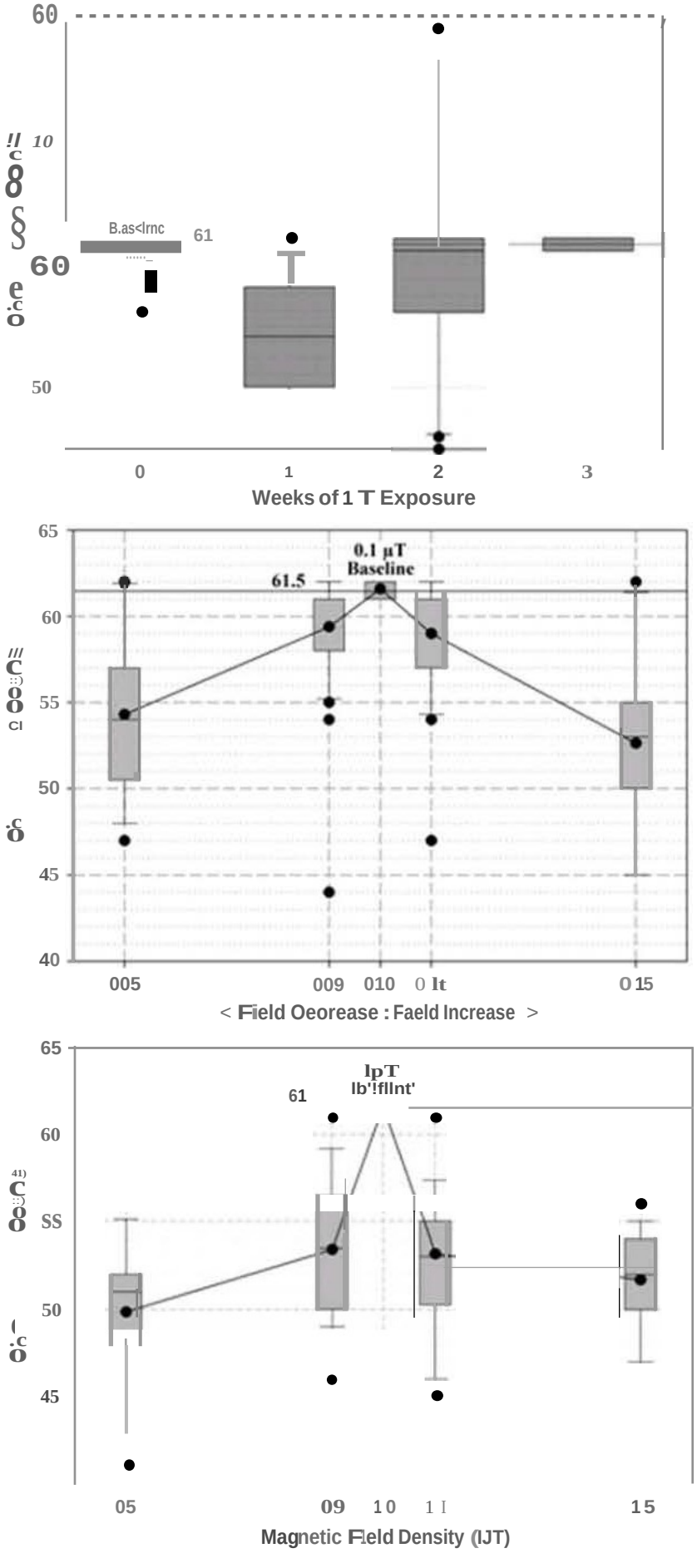

Figure 4. Top: K562 chromosome numbers return to baseline after 3 weeks of continuous 1f.lT lVIF exposure. Sixty -five, 102, 50 and 37 metaphases. Two experiments at each lVIF. Middle: K562 chromosome numbers obtained after 6 d by altering baseline lVIFs of 0.1f.lT. Twenty, 31, 37 (baseline), 31 and 35 metaphases. Three to six e..-xperiments at each lVIF. Bottom: For 1f.lT, 28, 28, 37 (baseline), 28 and 28 metaphases. Three experiments at each lv!F. Although the symmetry of the chromosome numbers is strong, there is more cell decay with increased than with reduced fields.

MF and oligomycin

Previous experiments (Li et al., 2012) on the five cancer cell lines used in this article show a link between metabolic restriction and KC. Anoxia alone induces partial KCs of 6-8 chromosomes. Deeper contractions, almost to normalization of the karyotypes to 46, are produced by IC_{50} doses (allov.ring 50% of the nonnal cell division rate) of the metabolic restrictors oligomycin and imatirrib. Similar KCs are produced by physiological levels of melatonin and vitamin C together.

We believed that comparison of metabolically restricted cultures with MF-exposed cultures could provide clues on action mechanisms, as the different metabolic restrictors mentioned above have different sites of action. Figure 5 (top), displays the similarity in cell size distribution after 6-d between two of seven anoxic K562 assays, one exposed to a very effective MF, 0.4)IT at 60-Hz, and the second to oligomycin at IC_{50} (2.5 ng/mJ). TI1e two distributions stand apart, with smaller cell diameters and higher ratios of cells-to-objects below 11J.IIII, the decay particles and apobodies. This suggests that lYIFs and oligomycin share a common mode of action. Despite the closeness between oligomycin and MF assays in Figure 5 (top), oligomycin is faster-acting than 0.4)lT: changes in cell size, revealing of KC, are visible at 1d, em·lier than for the IVIF. But more efficant MFs, such as 5)IT at 60-Hz or 1)IT at 120-Hz, show effects earlier (not shown).

MF and adenosine monophosphate-activated protein kinase

The similarity between 0.4)IT and oligomycin suggests that the MF may be an inhibitor of ATPS, as oligomycin is a highly specific inhibitor of ATPS. **If** this were the case, inhibition of mitochondrial ATPS by lYIFs would activate adenosine monophosphate-activated protein kinase (AMPK), because healthy cells must maintain a high level of phos-phorylation capacity (ATP:ADPl10) to function well (Hardie and Hawley, 2001). AMPK is a sensitive ATP regulator that switches *on* catabolic pathways and *off* many ATP-consmning processes, both acutely and clu·orrically, through gene expression.

The MF>ATPS>AMPK pathway was investigated using metfonrrin and resistin. Metfonnin is a diabetes drug that activates AMPK, leading to reduced glucose production in the liver and reduced insuJin resistance in muscle. **It** is an attractive anti-aging drug that usually causes weight and appetite loss.

Resistin, a product of the *RSTN* gene, is a 9.9 kDa protein containing 93 alllino acid residues which, at 20ng/rn1 or more, inhibits AMPK. **It** interferes \vlth phosphorylation of **Akt** (serine/threonine protein kinase), active in multiple cellular processes such as glucose metabolism, cell proliferation, apoptosis, transcription and cell migration.

Metfonrrin (0.01mg/1) and resistin (40ngll) alone for 3d induce average KCs of 9 and 10, respectively, in K562.

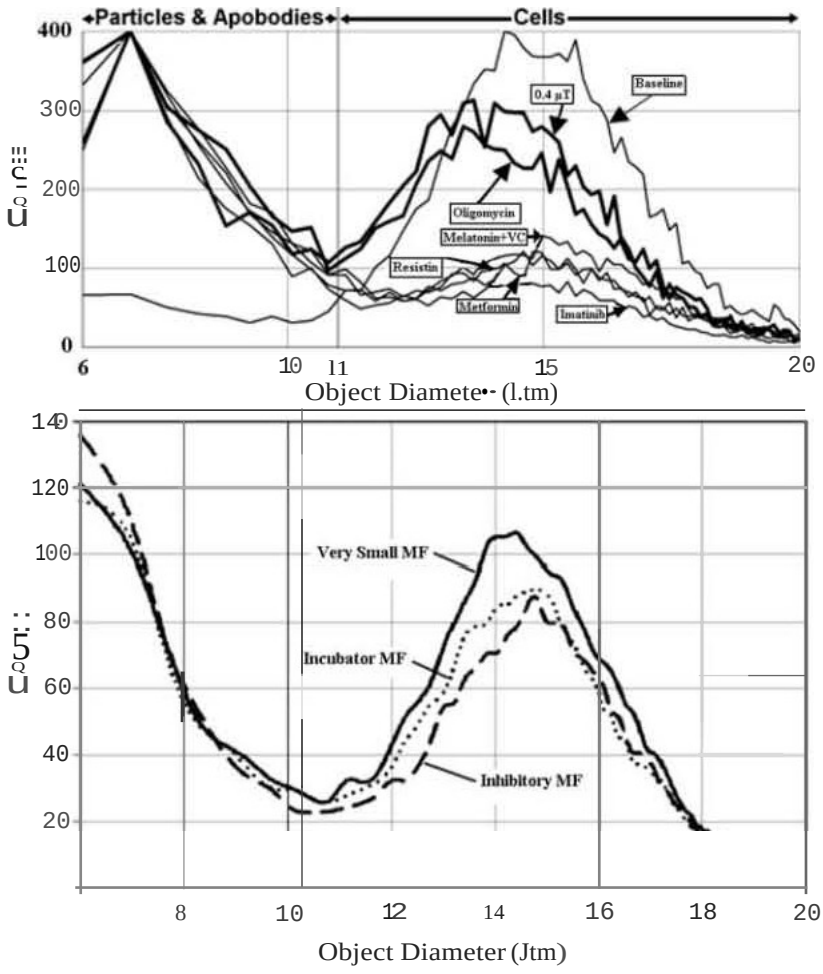

Figure 5. Top: Object diameter histograms for 6-d anoxic exposures of K562 cultures to 0.4 f!T MF at 60-Hz and oligomycin at *ICso* (2.5 ngfml). The lower four *ICso* curves are: imatinib (0.04 J!gfml), resistin (40ngtml), metfonnin (0.01 mg/ml) and melatonin-vita-min C (0.3 J.lg!ml and 26 J.lg!ml). All cultures are adjusted to a common small particle count maximum. Bottom: Object diameter histograms for 7-h 21% oxygen exposures of three K562 cultures under typical incubator MF. Aliquots of RPMl-1640 witl1 10% FBS medium exposed for 15h to very small MF (<4nT at 60Hz, 3 f!T static), incubator MF (2-2.7 f!T at 60Hz) or Inhibitory MF (0.62 f!T at 120Hz) were seeded with cells at time 0 and measured with a lvfillipore Scepter at 7h. Average of three repeats for each condition. TI1e p value between average levels (l2-16J!m) for very small MF and inhibitory MF is 0.001 (n=4).

\\'hen, in the 6-d trials routinely used for MF tests, 1T is combined with metfornrin, even *larger* KCs are observed (9 becomes 11±0.34). \\'hen 1Tis combined with resistin, the KC of resistin *reduces* from 10 to 4±0.46, aJso less than the KC of 1T alone, at 7.5. The conclusion from summary of Table 1 is that l\.1Fs enhance the action of metformin, but neutralize the effect of resistin, again suggesting a connection between l\.1Fs and ATPS.

Experiments on medium alone

Cells grown for 7 h nnder identical *incubator MF* (2-2.7 Tat 60-Hz) conditions fared differently according to whether the culture medium added at Oh originated from closed flasks exposed for 15 h to: *very small MF* (<4 nT at 60-Hz, 3 T static), *incubator MF* (2-2.7T at 60-Hz) or *inhibitory MF* (0.62T at 120Hz).

After the sealed flasks v.>ith media (only) are exposed to their respective MFs, cell culture aliquots are introduced into each flask, and incubated for 7 h nnder *incubator iv!F* conditions. Measurements of cells numbers of each S1Ze are acquired at 0h, as well as at 7 h for each flask.

Table 1. K562 karyotype contractions under the action of AMPK modulators and MFs.

Agent	Concentration/intensity	Chromosomes lost
Metformin (activator)	O.Olmgll	-9
Resistin (inhibitor)	40ngll	-10
Metformin + MF	O.Olmgll+lJ.lT	-11±0.34
Resistin + MF	40ngll + lJ.lT	-4±0.46
MF	lJ.lT	-7.5

There is an increase (Figure 5; bottom) in the number of living cells observed nnder the *very small MF* condition, compared to the *inhibitory MF* condition, with the *incubator MF* condition rating in between.

When stressed cells from a culture with depleted medium (lower pH) were used, the *inhibitory MF* had the effect of increasing the level of decay products (object diameters less than 11)in the culture (not shown).

NCI-H460 and MCF7 proliferation

Beyond strong effects on cancer cells karyotypes, MFs also impact proliferation rate, adhesion and cell shape, but in specific cases, such as for lnng (NCI-H460) and breast cancer (MCF7), effects are particularly striking. Figure 6(C) illustrates that the cell connts of lnng cancer cells after 4 d in our synthetic medium at 50nT, 400nT and 5Tare 8, 9.2 and 14.8, respectively, times larger than those of nnexposed (4 n'I) cells. NCI-H460 and MCF7 cells grown at less than 4nT in our synthetic medium do not attach to the growth surface, but do so nnder any MF exposure. Figure 6 illustrates that the appearance of MCF7 cells after 4 d.is completely different at <4nT (A) than at 5T (B). These MF-related differences in adhesion, appearance and proliferation last over two passages, but finally return to the (A) characteristic, the "non-adherent" style, which is coherent with the restoration of chromosome numbers nnder constant l\.1F exposures displayed in Figure 4 (top). The proliferation results for NCI-H460 are also compatible with our metfornrin and resistin experiments, which suggest that MFs stimulate Akt, which in turn activates protein synthesis and apoptosis inhibition in NCI-H460 (Hovelmann et al., 2004).

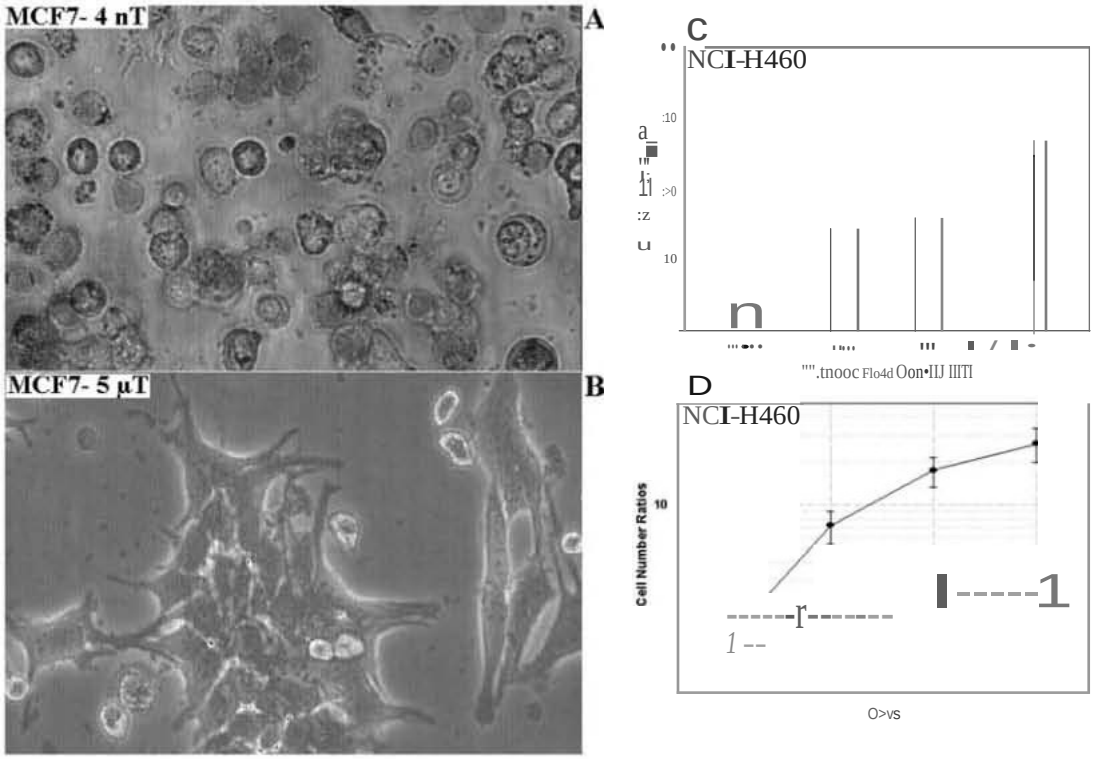

Figure 6. EffectofMFs on MCF7 adhesion and shape and NCI-H460 proliferation. MCF7 (breast cancer) a:nd NO-H460 (lwlg cancer) cells originally under shielded levels (4nT). (A) MCF7 exposed to 4nTfor 4d. (B) MCF7 exposed to 5 J.lT for4 d. (C) Proliferation ofNCI-H460 over4 d as a function of MF density. (D) Evolution of NO-H460 over 8d for 4nT and 5 J.lT exposures. Three experiments per determination.

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Discussion

Possible site of action of Mfs

There are documented examples of hydrogen bonding impacting biological processes. The hydrogen bond is 4% longer for heavy water than for light water, which results in lethal rnamrnalian toxicity at about 50% heavy water (Kushner et al., 1999). Small changes in hydrogen bond lengths and angles are critical in determining how binding pockets react, as demonstrated in the selective uptake of phosphorous over arsenic in bacteria (Elias et al, 2012).

The involvement of water structure disruptions through alterations in hydrogen bonding was also anticipated by recent views on EMF bioeffects (De Ninno and Castellano, 2011; Novikov et al., 2010).

If the effect on water described by Semildrina and Klselev is involved, it would be more prominent "the higher the concentrations of hydrogen bonds and proton containing groups in aqueous systems" (Semikhina et al., 1988). The only known location in the biota where these two conditions are met with such intensity are the entry and exit water channels of ATPS. That protons can proceed through water channels by tunneling bas been confirmed in water-filled nanotube models where neutron Compton scattering has detected as double wells the quantum delocalization of protons (Reiter et al, 2011). Proton movement is sensitive to the global configuration of hydrogen bonds.

The intense electric field (Zorov et al., 2009) applied to ATPS Fo in mitochondria lowers the potential barriers inhibiting proton movement. In low-barrier hydrogen bonds, hydrogen is free to move between two oxygen atoms (Cleland, 2000). According to this mechanism, MFs would impede the quantum mechanical proton flow through the hydrophilic channels, and MF removal would improve proton flow, directly impacting ATPS efficiency. The dose-responses of Figures 2 (top) and 3 would be determined by rising proton impedance (decreased soliton tunneling) through ATPS half-channels.

The involvement of protons relieves the area of EMF bioeffects of the "kT problem", because EMF would act not on molecules, but on particles (protons and electrons), wbich, as ferrnions, do not follow Maxwell-Boltzmann statistics, but Fermi-Dirac statistics, and are governed by quantum electrodynamics.

Numerous elements documented by Russian physicists in their studies of :MF's on water (Semikhina et al, 1988; Senrikbina and Kiselev, 1981) are compatible with our own biological observations.

It is particularly notable that the KC threshold of 25nT in Figure 2 (top) falls in line with the water effect threshold detected by Russian physicists (Semikhina et al., 1988). The extended, flat response is also compatible with their obser- vations, and there is an interesting biological aspect to this flat dose-response. Quickly saturating dose-responses are observed in endocrine disrupters that attach to a limited number of binding sites, but still manage to elicit a strong response (Vandenberg et aL, 2012). In our case, the "binding 2008; Zorov et al., 2009). The transitions between *para* and *ortho* forms are forbidden by quantum mechanics. They rarely occur during random thermodynantic fluctuations as a proton drifts away from its OH radical, releases Pauli exclusion principle constraints and allows spin flipping according to an external MF. The *ortho* state is sensitive to the l\.1F. This dependence of the transitions on thermodynantic instability is convenient to explain the slow rate of action ofMFs on water. The detailed physics within the water twmel is complex because proton tunneling is probably coordinated with electron tunneling (Gray and Winkler, 1996; Moser et al., 1992). Proton-coupled electron transfer underpins many biological reactions and may occur as unidirectional or bidirectional, and synchronous or asynchronous, transfer of protons and electrons (Reece and Nocera. 2009). Most enzymatic reactions would involve a single such transfer, wbile ATPS is exquisitely sensitive to MFs because its structure is dependent on a serial layout of such transfers.

Finally, the metabolic "restriction" that we attribute to the action of MFs on ATPS could be labeled as a metabolic "disruption" and bas at least three characteristics in common with endocrine disruption.

First, dependence on very specific receptors, namely ATPS channels of mitochondria for the MF, and ligand-receptor systems in the cell membrane, cytosol or nucleus for endocrine disrupters. Second, the quickly saturating dose-response curves observed in Figures 2 (top) and 3 are similar, for example, to the effects of atrazine on the size of the larynx. Atrazine does not shrink the larynx, but it inhibits the androgen stimulus (Hayes et al., 2002). Third, thresholds of action at very low levels: 25nT for l\.1Fs and pico-molar to nano-molar levels for endocrine-disrupting chemicals (Vandenberg et al, 2012).

The increased metabolism observed when alternating and static MFs are removed. and the ability of MF-conditioned culture media to influence cellular development are aD compatible with Russia11 water data. The presence of a KC resonance wider tha11that observed for pure water by Russian physicist adds support. Finally, the ratio between frequency and field intensity (fiB) for maximum biological effects is suggestive of a coupling with the gyromagnetic ratio of the proton.

In tbis context, similar fingerprints between the 0.4}-LT and oligomycin (Figure 5; top), known to inhibit ATPS by binding to the 0 subunit of the Po segment of ATPS (also named *oligomycin sensitivity conferral protein)* comes as no surprise. Another intriguing link between MFs and ATPS is provided by the fact that rhodamine 6 G, used by Semikbina to detect MF effects on water, also happens to inhibit the F_O segment of ATPS.

KC,AMPK and diabetes

Perturbations of *A1P* concentrations trigger AMPK, which activates p53 and reduces both ATP consumption and DNA synthesis (Jones et al., 2005; Motoshima et al., 2006). The suppression ofDNA synthesis, part of AMPK's catabolic control, leads to KCs through suppression of chromosome endoreduplication, the mechanism probably responsible for rapid chromosome number increases :in cancer cells (Li et al., 2012).

Two unusual aspects of MF action, adaptation to a stable field over three weeks (Figure 4; top) and the unusual shorter-term sensitivity to small MF increases and decreases (Figure 4; middle and bottom) are compatible with AMPK physiology. As far as we know, this is the first example of an agent presenting this kind of symmetry, making it possible to sustain KCs indefinitely by judicious selection of MF sequences. AlYIPK is easily triggered by small changes in A1P levels (Hardie and Hawley, 2001), and also controls long-term dynamic adaptation in muscle (Winder et al., 2000). The connection between metabolic restrictors, including MFs, and KC can be explained by AMPK physiology.

The MF >A1PS> AlYIPK pathway is easily detectable in cancer cells because of KC, but there is no reason to think that the A1PS of normal cells is spared under MF exposure. *A* major regulator of metabolism (Liu et al., 2006), AMPK modulates insulin secretion by pancreatic beta-cells (Winder and Hardie, 1999) and is investigated for the treatment of diabetes (Viollet et al., 2009). AMPK is tied with body weight (Kim et al., 2004) as well as with immWle cell behavior (Kanellis et al., 2006). Type 2 diabetes has been linked to prolonged and persistent exposures to endocrine disruptors (Lee et al., 2010).

KCs and cancer

Cancer cells depend on glycolysis and significantly upregulate it when respiration is inhibited. The Warburg effect manifests as increased glycolysis and reduced mitochondrial respiration (Jezek et al., 2010; Wu et al., 2006). These capabilities of cancer cells allow growth under metabolic restriction by concentration of their resources on bio-synthesis through the elimination of detoxification mechanisms associated with oxygen exposure, such as glutathione-S-transferase and CYP3A4 expression (Nagai et al., 2004). The smaller karyotypes maintained under metabolic restriction contribute to tumor core expansion, as fewer chromosomes can be more rapidly duplicated. The survival of twnors could thus be enhanced by certain levels of chronic metabolic restrictions from hypoxia, oligomycin or MFs.

It has been repeatedly confirmed that cancer cells become more malignant under metabolic restriction (Hill et al., 2009; Rockel and Vaupel, 2001; Jogi et al., 2003) *in vitro* (Anderson et al., 1989) and in the clinic (Brizel et al., 1996; Nordsmark et al., 1996), to the point where it has become a central issue in twnor physiology and treatment (Rockel and Vaupel, 2001). From our data, it is logical to conclude that KC observed under metabolic restriction is a possible indicator of meta-genetic promotion in cancer cells (Li et al., 2012).

Mfs and cancer epidemiology

For many cancer cell types, the dose-response of KC versus MFs is remarkably flat (Figure 3). The deviation from flatness in erythroleukemia cells (Figures 2 (top) and 3; HEL) is due, we suspect, to extra-mitochondrial A1P secretion in the cell membrane (Arakaki et al., 2003) where pH is at a physiological 7.3 rather than 1, a probable feature of this cell type (Das et al., 1994).

If KC is indeed a marker of increased malignancy, there is a possibility of carcinogenicity from MF exposures. fu such a case, the phenomenon would not be easy to document through epidemiology. First, the threshold for the effect (25n1) is very low, which means that *all* the population is "exposed". Second, the dose-response is unusually flat (Figure 3), such that useful low and high exposure groups \vlth otherwise similar characteristics would be difficult to assemble. Third, the differential action of MFs may confuse conventional exposure analysis.

Occupational studies are often at the forefront of epidemiological discovery because of their higher and better docwnented exposures. According to Figure 2 (top), occupational populations of low (0.1JlT) and high exposures (1)l1) have a KC difference of "1 chromosome" between them. Domestic MF epidemiology on leukemia may have been successful (Ahlborn et al., 2000; Svendsen et al., 2007) because it benefited from a KC of "10 chromosomes" between 0 and 0.4 *JlT* (Figure 2; top).

The increased proliferation rates reported for lung cancer cultures may also be important. Lung cancer was pointed in at least three studies related to EMFs (Armstrong et al., 1994; l'vfiller et al., 1996; Vagero and Olin, 1983).

Conclusion

The following evidence swpmt the inhibition of A1PS byMFs.

Mfs alter metabolism

(1) MFs induce KCs in five cancer cell lines, as do other metabolic restrictors (Li et al., 2012).
(2) MFs interact with metformin and resistin as would an AMPK activator.
(3) Elimination of alternating *and* static MFs produces a durable increase in cell proliferation.

Mfs alter water

(4) The KC threshold (25 n1), as well as its extent over two orders of magnitude, is predicted by the work of Russian physicists on water (Semikhina and Kiselev, 1981). Lack of sensitivity to MF intensity or to cell type suggest tl1e knockout of a biological enzyme by physics.
(5) MF-exposed culture meditun. without cells, is a vector of MF action (proliferation and cell decay).
(6) Measured changes in the pH of cell culture media from MF exposures.
(7) *A* wide KC resonance (113Hz at 1)l1) is compatible with the work of Russian physicists on water (Semikhina and Kiselev, 1981).

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(8) KC is maximized at specific frequency-amplitude (fiB) combinations (Semikhina and Kiselev, 1981), suggesting an interaction with water's protons.

Mfs alter ATPS Fo

(9) ATPS Fo is the only site in the biota where conditions for maximum sensitivity to MF action (Semik:hina et al., 1988) happen together: high concentrations of protons and hydrophilic bonds in a narrow channel.
(10) Strongly acting MFs induce cell cultme characteristics (Figure 5; top), closely matching those of a specific ATPS Fo inhibitor, oligomycin.
(11) 1-IF activation of AlVIPK implies a perturbation to ATP levels, thus a change in ATPS performance.
(12) Rhodamine 6 G, used by Russian physicists (Semik:hina and Kiselev, 1981) to detect MF effects, is also an inhibitor of ATPS Fo.

Environmental :rv!Fs act on the core of human metabolism. Past evaluations of MF bio-effects were at a serious disadvantage because of traditional toxicological and epidemiological assumptions, that larger exposures induce larger responses. The controls of *in vitro* scientists were ah·eady randomly exposed by the :rv!Fs of their incubators. The flatness ofMFs' dose-response impaired epidemiological work, as most studies, except for domestic leukemia, used tainted controls (Milham, 2010). The interaction between power-frequency :rv!Fs and living cells may have been underestimated for a long time, because of these unexpected characteristics.

Some diseases appear to have strengthened, with no clear causation, as more advanced technology, in great part based on electricity, has expanded. Chronic diseases that increased or decreased in the past century, and that are connected to ATP metabolism, should be examined for a link with MFs. But our understanding of AMPK and metabolism is incomplete (Jones and Thompson, 2009), making a link between MFs and any specific disease, such as diabetes, uncertain.

According to our data, MFs are physiological agonists of metformin, perhaps through inhibition of ATPS. But metformin is a knovm geroprotector (Bulterijs, 2011). Beyond the anti-oxidants we investigated and :rv!Fs, there are other agents known to impair the use of oxygen. H_2S, for example, inhibits the cytochrome enzymes of mitochondria, reversibly slowing the metabolism of mice, lowering their body temperatme, breathing rate and use of oxygen. Ftuthermore, *Caenorhabditis elegans*, when grown in 50 ppm H_2S, lives 70% longer than in normal air (Miller and Roth, 2007). Such models suggest that the ability of MFs to suppress ATPS (Hootkooper et al., 2013) may have played a role in the increased lifespan observed in developed countries in the past century.

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Declaration of interest

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