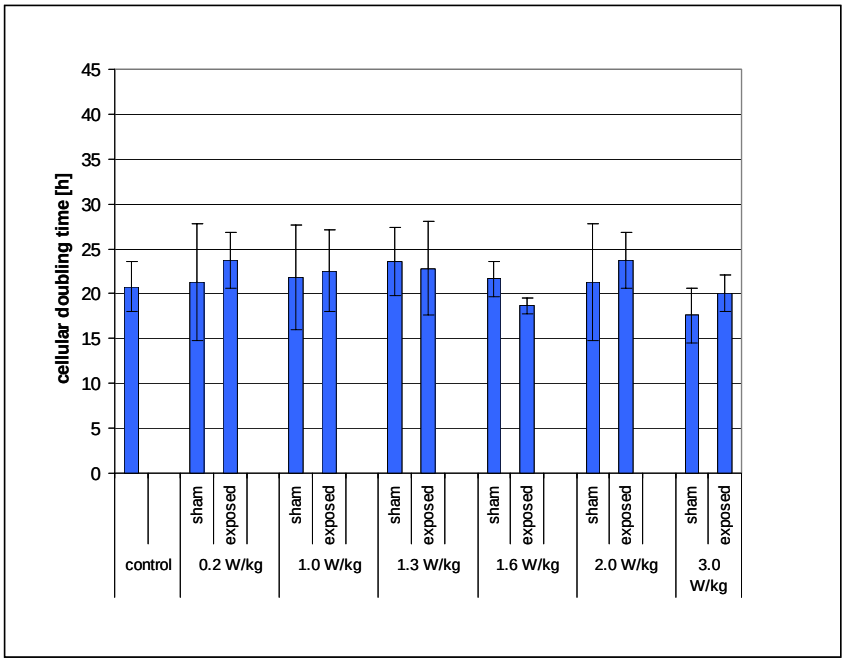


Figure 102. Effect of RF-field exposure (1800 MHz, continuous wave, SAR 0.2, 1.0, 1.3, 1.6, 2.0 and 3.0 W/kg, 24h) on HL-60 cell growth with respect to growth velocity compared to control and sham-exposure, determined by the cellular doubling time. Each bar represents the mean ± SD of results obtained in at least three independent experiments, except for control (n=6) and SAR 1.3 W/kg (n=6).

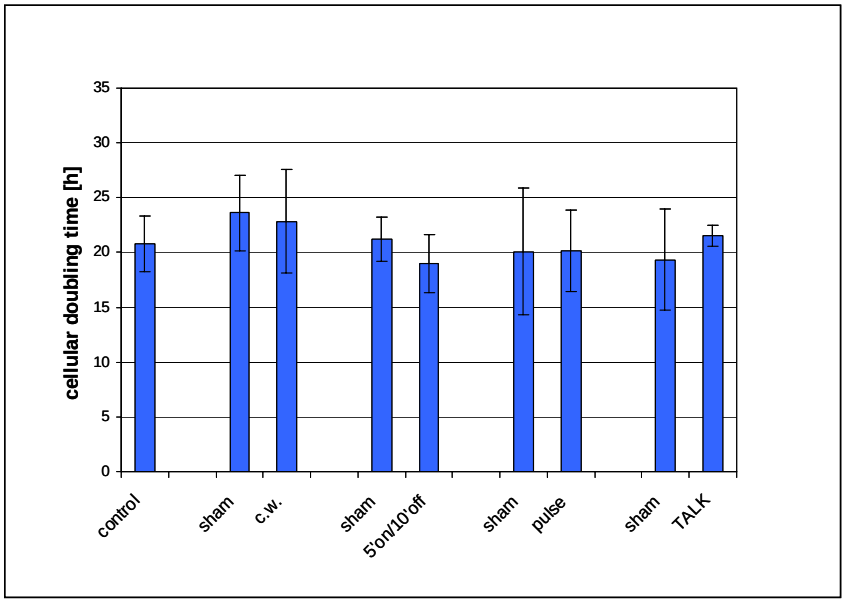


Figure 103. Effect of RF-field exposure (1800 MHz, different signal modulations, SAR 1.3 W/kg, 24h) on HL-60 cell growth with respect to growth velocity compared to control and sham-exposure, determined by the cellular doubling time. Each bar represents the mean ± SD of results obtained in at least three independent experiments, except for control (n=6), continuous wave (n=6) and GSM-Talk (n=2).

Thymidine kinase (TK) activity

Intracellular thymidine kinase (TK) activities were determined by radioenzyme assay with ^{125}I-deoxyuridine monophosphate as substrate (Prolifigen® TK-REA, AB Sangtec Medical, Bromma, Sweden). The level of radioactivity is directly proportional to the enzyme activity, the TK value is calculated from the standard curve and expressed as U/l.

TK activities of HL-60 cells following RF-field exposure (1800 MHz, continuous wave, 1.3 W/kg, 24 h) was compared to control and sham-exposure. Table 24 represent levels of thymidine kinase activities for two independent experiments. No changes in intracellular TK activities were found in HL-60 cells following RF-field exposure compared to control and sham-exposure. In summary, the growth behaviour of HL-60 cells with respect to growth velocity and DNA synthesis are not altered by RF-EMF exposure compared to control and sham-exposure using the assays described above.

Table 24. Thymidine kinase (TK) activity in HL-60 cells after exposure to RF-field (1800 MHz, continuous wave, 1.3 W/kg, 24h), compared to control and sham-exposed cells

Group	thymidine kinase activity [U/l]	
	exp. 1	exp. 2
control	121.4	126.8
sham	151.1	116.3
RF-field	121.9	118.4

3.2.2.4 Mouse embryonic stem cells (Participant 4)

RF-EMF did not induce cardiac differentiation of R1 ES cells and cardiac differentiation and proliferation of P19 EC cells, but may affect the bcl-2 mediated apoptotic pathway in ES-cell derived neural progenitors and neuronal differentiation by inhibiting nurr-1 and TH transcription.

See 3.2.4.1

3.2.2.5 Summary (Participant 1)

Our data did not reveal a significant effect of RF-EMF on proliferation and differentiation of various cell systems such as neuroblastoma cells (NB69) (see 3.2.2.1), R1-embryonic stem cells and embryonic cancer cells (P19) (3.2.2.4 and 3.2.4.1), human lymphocytes and human thymocytes (3.2.2.2) and HL-60 cells (3.2.2.3), though some effects on the differentiation process in neural stem cells were observed (see 3.2.2.1). These effects may be of indirect nature possibly through modulation of the expression of various genes and proteins. With respect to neural stem cells, RF-EMF may affect proliferation and differentiation via up-regulation of bcl-2 which mediates the apoptotic pathway and via inhibiting nurr-1 and TH transcription (see 3.2.2.4 and 3.2.4.1)

3.2.3 Apoptosis

3.2.3.1 Brain cells of different origin and human monocytes (Participant 9)

RF-EMF did not affect apoptosis in neuronal cells.

Spontaneous apoptosis was found higher in sensitive primary nerve cells than in the human neuroblastoma cell line SH-SY5Y) (around 20% versus 10% using the $DiOC_6$ dye). A high percentage of spontaneous apoptosis in granule cells was found using Annexin V staining compared to $DiOC_6$ staining. This observed difference seems to be cell type-dependent and it appeared difficult, in this case, to

correlate information given by these two dyes. Besides this technical consideration, exposure of primary granule cells to GSM-900 at 2.0 W/kg for one hour did not induce apoptosis as shown by the time-kinetics up to 24 hours after exposure (Figure 104). The same observation was made with the SH-SY5Y cell line. To test longer time exposure, we decided to expose granule cells and SH-SY5Y to GSM-900 during 24 hours and to quantify apoptosis at the end of exposure. In these exposure conditions, no significant difference was observed between sham and exposed cells in both cases (Figure 105).

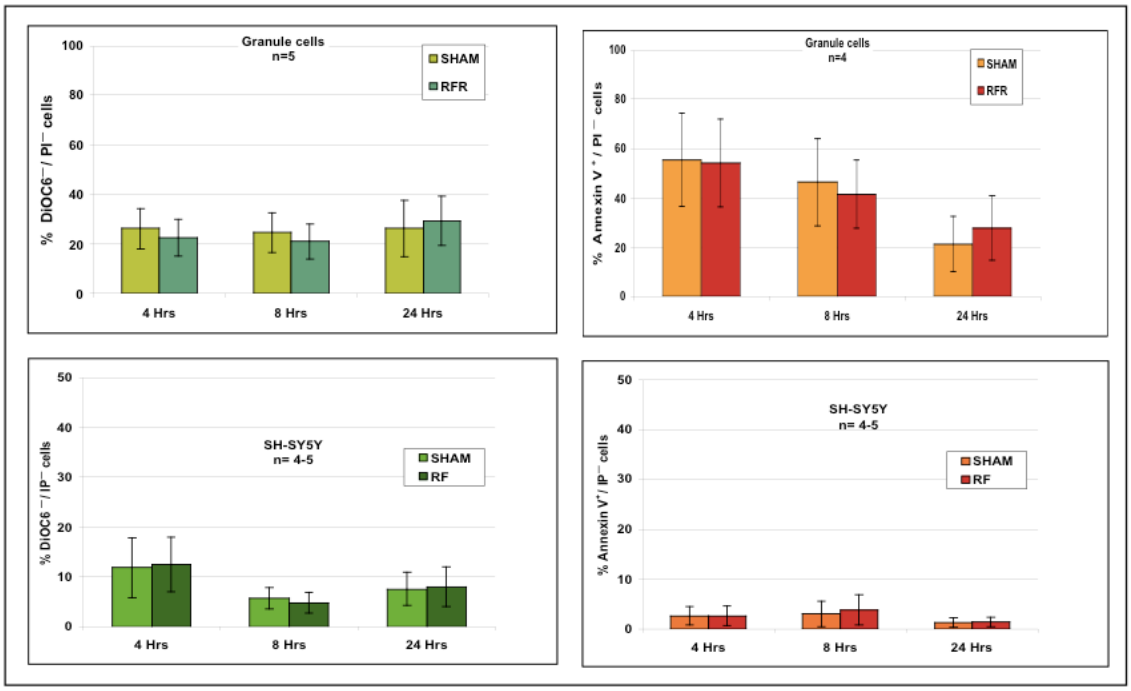


Figure 104. Effect of a one-hour exposure to GSM-900 on apoptosis in nerve cells. Results are expressed as the percentage of cells with depolarised mitochondrial transmembrane potential ($DIOC6^-/PI^-$, left panel) and apoptotic cells (ANX^+/PI^-, right panel) after exposure of primary granule cells (upper panels) and human neuronal cells (lower panels) to frame GSM-900 signal at 2.0 W/kg for 1 hour. Apoptosis was measured 4, 8 and 24 hours after the exposure began. Data are presented as the mean ± SEM of 4 to 5 independent and blind experiments.

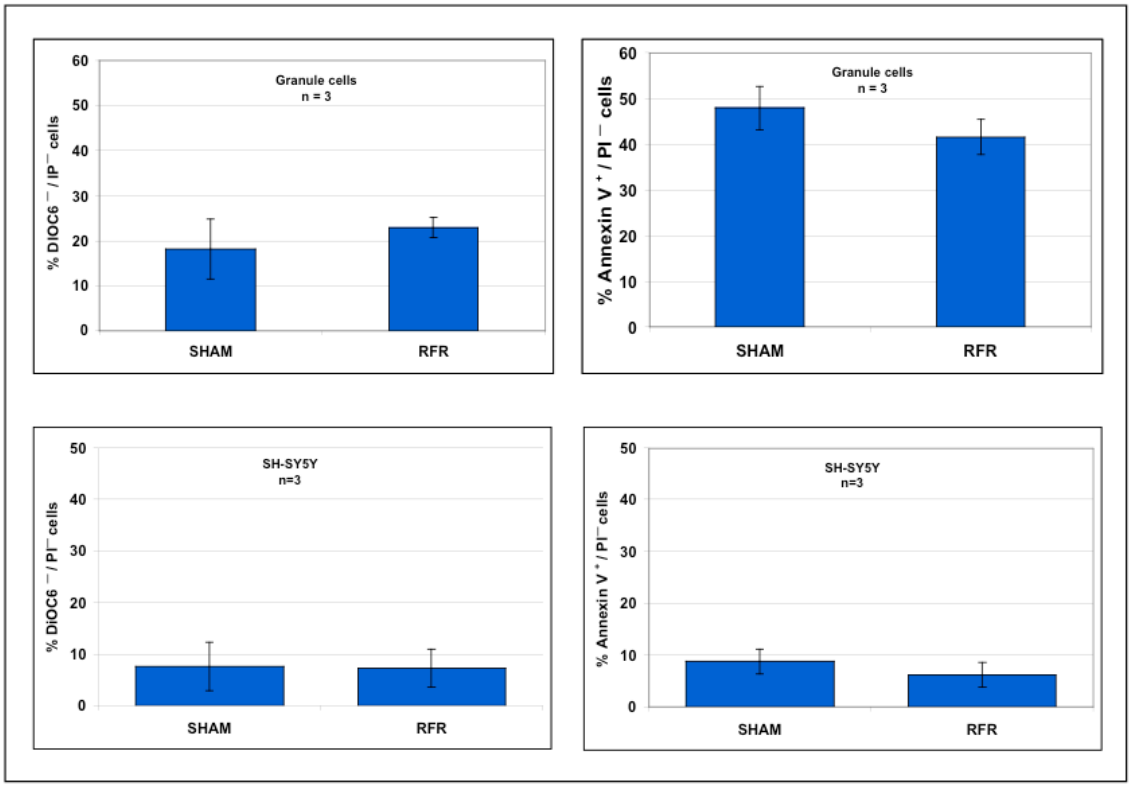


Figure 105. Effect of a 24-hour exposure to GSM-900 on apoptosis in nerve cells. Results are expressed as the percentage of cells with depolarised mitochondrial transmembrane potential ($DIOC6^-/PI^-$, left panel) and apoptotic cells (ANX^+/PI^{--}, right panel) after exposure of rat primary neurons (upper panels) and human SH-SY5Y neuroblastoma cells (lower panels) to frame GSM-900 signal at 2.0 W/kg for 24 hours. Apoptosis was measured immediately after exposure. Mean ± SEM of 3 independent, blinded experiments are presented.

We conclude from our results that granule cells and SH-SY5Y cells are not sensitive to GSM-900 exposure for up to 24 hours.

RF-EMF did not affect apoptosis in astrocytic cells.

Primary cultures of astrocytes and human U87 glioblastoma cells were sham-exposed or exposed to GSM-900 for one hour at 2.0 W/kg and apoptosis was followed up during 24 hours in the conditions described previously. Figure 105 shows the data obtained using the primary culture and the U87 cell line. In the two cell types, no difference in the number of cells with depolarised mitochondrial potential or in AnnexinV-positive cells could be evidenced after exposure to GSM-900. An increase in apoptotic astrocytic cell population measured with $DiOC_6$ was noticed, correlated to the time spent in culture. Nevertheless, no significant difference was observed between sham- and GSM-900 exposed cells. In the exposure conditions tested, no demonstration of a significant effect of GSM-900 signal on primary astrocytes or glioblastoma U87 cells could be made.

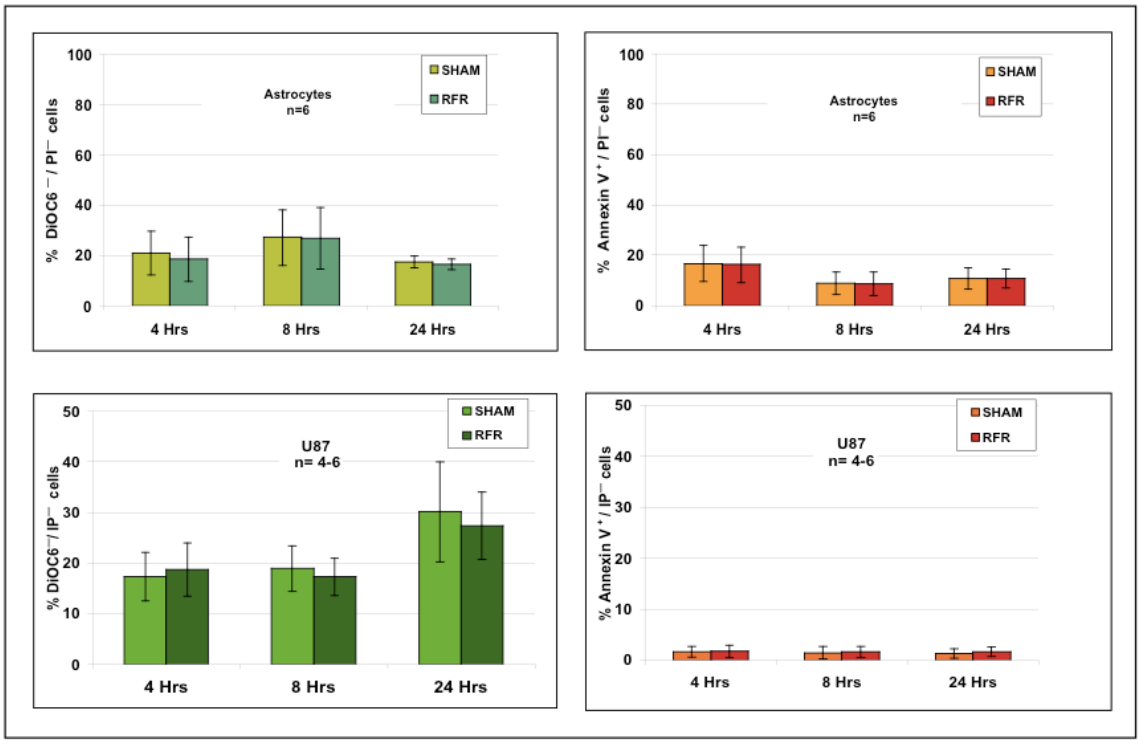


Figure 106. Effect of a 1-hour exposure to GSM-900 on apoptosis in astrocytic cells. Results are expressed as the percentage of cells with depolarised mitochondrial transmembrane potential ($DIOC6^-/PI^-$, left panel) and apoptotic cells (ANX^+/PI^-, right panel) after exposure of primary astrocytes (upper panels) and human astrocytic cells (lower panels) to GSM-900 signal at 2.0 W/kg for 1 hour. Apoptosis was measured 4, 8 and 24 hours after the exposure began. Data from 4 to 6 independent experiments are presented as the Mean ± SEM.

In summary, an immediate or delayed effect of RF-EMF on apoptosis in rat primary cells and human cell lines could not be demonstrated.

RF-EMF did not influence apoptosis in immune cells.

The ability of the human U937 promyeloma cells to undergo apoptosis was tested by using camptothecin as a positive control (Figure 107). We show a significant 4- to 8-fold increase ($p<0.01$), depending of the marker of apoptosis used, in U937 cells treated for 4 hours with CPT (4 µg/ml).

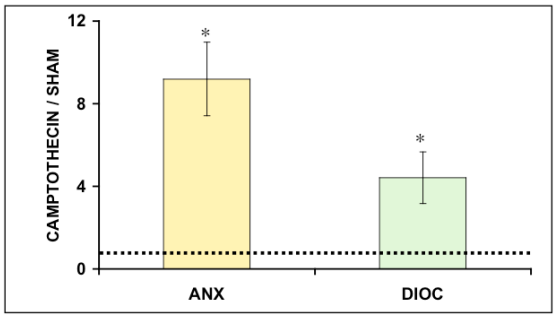


Figure 107. Effect of camptothecin treatment on apoptosis in human U937 cells. The results are expressed as the ratio of apoptotic cells (ANX^+/PI^-, left panel) and cells with depolarised mitochondrial transmembrane potential ($DIOC^-/PI^-$, right panel) in camptothecin-treated versus sham-exposed U937 promyeloma cells. U937 cells were sham-exposed for 1 hour and then treated for 4-hours with camptothecin (4 µg/ml). Data are presented as the mean ± SEM of 5 independent experiments. *=$p<0.01$

Sham-sham experiments (n=3-4) showed that inter-incubator (incubator used for RFR exposure versus incubator used for sham-exposure) variation of U937 apoptotic cells was around 1.1 ± 0.5 after one hour and 1.2 ± 0.6 when the cells were placed back in a control incubator for 4 hours after the one hour spent in the dedicated incubators (Figure 108). The effect of CPT treatment was very similar when the samples were put for one hour in either dedicated incubator. We therefore concluded that both dedicated incubators were equivalent within a range of 50% of ratio variation and that an at least two-fold increase in apoptosis in RFR-exposed cells could be considered as significant.

No statistically significant influence of GSM-900 could be evidenced on spontaneous apoptosis of U937 cells (Figure 109), when they were exposed for one hour at a SAR of 0.7 W/kg and even at the highest SAR tested (2.0 W/kg). No difference could be detected either immediately after exposure or after a 4-hour resting period in a control incubator. Hence, no delayed effect of GSM-900 on apoptosis could be evidenced. A longer exposure duration, i.e. 48 hours, to GSM-900 at the lowest SAR tested (0.7 W/kg) was also not able to alter spontaneous apoptosis in the human cell line.

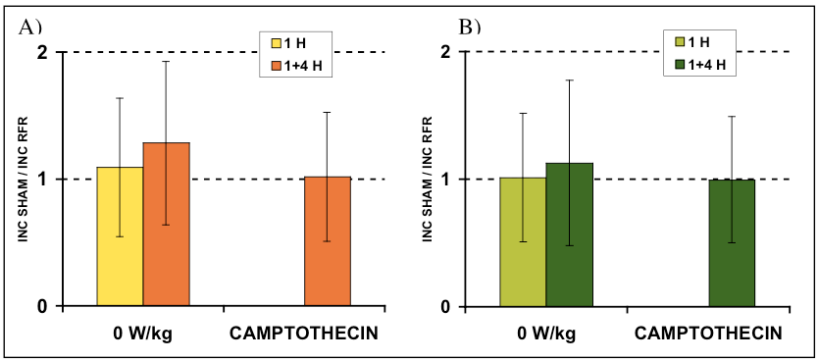


Figure 108. Comparison of occurrence of apoptosis in U937 cells in both dedicated incubators. Results are expressed as the ratio between apoptotic U937 cells in the incubator used for RFR exposure and apoptotic U937 cells in incubator used for sham-exposure. Sham-sham exposure lasted 1 hour and cells were harvested either immediately (1 H) or after an additional 4-hour resting period or campthotecin treatment (1+4 H). A) apoptotic U937 cells (ANX^{+}/PI^{-}) and B) U937 cells with depolarised mitochondrial transmembrane potential ($DIOC6^{-}/PI^{-}$). Data are presented as the mean ± SEM of 3 to 4 independent experiments.

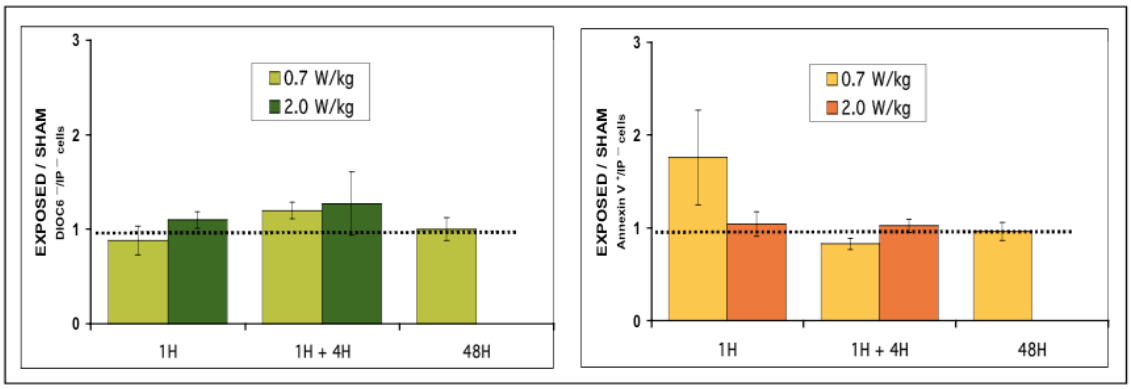


Figure 109. Effect of GSM-900 exposure on apoptosis in human U937 cells. Results are expressed as the ratio of cells with depolarised mitochondrial transmembrane potential ($DIOC6^{-}/PI^{-}$, left panel) and apoptotic cells (ANX^{+}/PI^{-}, right panel) in GSM-900- versus Sham-exposed U937 cells. U937 cells were exposed to GSM-900 at 0.7 W/kg (n=7) and 2.0 W/kg (n=6) for 1 hour, for 1 hour followed by a 4-hour resting period or for 48 hours. Data are presented as the mean ± SEM.

RF-EMF did not influence chemically-induced apoptosis in immune cells.

When possible interaction between RFR and camptothecin was tested, we show that a one-hour treatment with GSM-900 at either 0 .7 or 2.0 W/kg was not able to influence camptothecin-induced apoptosis (Figure 110).

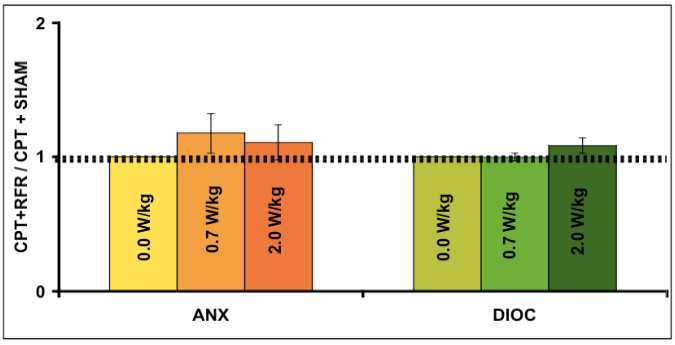


Figure 110. Effect of GSM-900 exposure on camptothecin (CPT)-induced apoptosis. The results are expressed as the ratio of apoptotic cells (ANX $^{+}$/PI $^{-}$, left panel) and cells with depolarised mitochondrial transmembrane potential (DIOC6 $^{-}$/PI $^{-}$, right panel) in GSM-900 plus camptothecin-treated versus GSM-exposed U937 promyeloma cells. U937 cells were sham-exposed or exposure to GSM-900 for 1 hour and then treated for 4-hour with camptothecin (4 µg/ml). The SAR level was 0.7 W/kg (n=7) or 2.0 W/kg (n=6). Data are presented as the mean ± SEM.

In summary, we showed no evidence for an immediate, cumulative or delayed effect of RF-EMF on apoptosis in a human monocytic cell line. We conclude from our results that U937 cells are not sensitive to GSM-900 exposure for up to 48 hours. Taken together, our results strongly suggest that the apoptotic process is not a major biological target for GSM mobile telephony-related signals.

3.2.3.2 Human lymphocytes (Participant 8)

RF-EMF did not affect apoptosis in human lymphocytes.

Negative results were obtained studying spontaneous or dRib-induced apoptosis, when PBMCs were exposed at all the three signal modulations. Moreover, PBMCs from old donors were exposed to Talk modulated RF, but also in this case no effects on spontaneous or oxidative stress-induced apoptosis were found. These results were also confirmed by mitochondrial membrane polarisation, since no differences were noticed in dependence of the exposure and age of donor.

RF-EMF did not increase the Hsp70 level in human lymphocytes after induction of apoptosis.

dRib induces an increase of hsp70 in treated cells in comparison with untreated cells already detectable after 3h of treatment (unpublished data) up to 44 hours of treatment. When we studied RF-EMF effects (1800 MHz, GSM talk signal, 2 W/kg), we did not found any alteration of hsp70 gene product, after 44 hours of intermittent exposure, in PBMCs from 7 young donors, as showed in Figure 111. In this figure it is possible to notice the significant difference between the level of hsp70 in untreated cells versus the dRib-treated cells. Additional analysis were performed in separated lymphocytes and monocytes, but no differences were found between RF-exposed and sham-exposed cell populations.

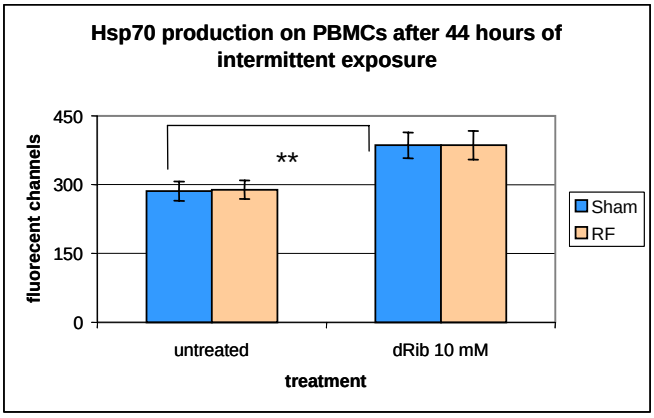


Figure 111. Hsp70 production in untreated and dRib treated PBMCs after 44hours of intermittent exposure. PBMCs were obtained from 7 donors and data are represented as mean of fluorescent channels ± s.e. ** = p< 0.01, untreated PBMCs versus dRib treated PBMCs.

RF-EMF did not affect apoptosis in thymocytes.

Thymocyte apoptosis was assessed with two different methods, in the same conditions, but the data obtained from 6 human thymus on thymocyte apoptosis did not suggest positive results on both the endpoints. Actually, a small increase (4%) of double positive thymocytes (CD4+CD8+) was found in RF-EMF-exposed cultures in comparison with sham-exposed tissue fragments. Also in this case the effect is of the order of the standard error thus we consider these results irrelevant.

3.2.3.3 Human promyelocytic cell line HL-60 (Participant 2)

RF-EMF did not affect apoptosis in HL-60 cells as shown by flow cytometric analysis and the Annexin V and TUNEL assay.

See 3.2.1.1

3.2.3.4 Embryonic stem cells of mice (Participant 4)

RF-EMF exposure may influence the bcl-2 mediated apoptotic pathway in ES-cell derived neural progenitors.

See 3.2.4.1

3.2.3.5 Human endothelian cell lines (Participant 6)

The RF-EMF-induced enhancement of hsp27 phosphorylation as well as the concomitantly RF-EMF-induced down-regulation of proteins of Fas/TNFα suggest that the anti-apoptotic pathway in RF-EMF exposed cell systems may be modified.

See 3.2.4.6

3.2.3.6 Summary (Participant 1)

Our data did not reveal a significant effect of RF-EMF on apoptosis in various cell systems such as brain cells and human monocytes (see 3.2.3.1), human lymphocytes and thymocytes (see 3.2.3.2), human endothelial cells (3.2.4.6) and HL-60 cells (see 3.2.3.3). On the other hand, an indirect effect on apoptosis through modulating the expression of various genes and proteins cannot be excluded at present. Up-

regulation of bcl-2 in differentiating embryonic stem cells (see 3.2.3.4 and 3.2.4.1) and of hsp27 in endothelial cells (see 3.2.4.6) both of which may affect the apoptotic process support such an assumption.

3.2.4 Gene and protein expression

3.2.4.1 Mouse embryonic stem cells (Participant 4)

Loss of p53 function rendered pluripotent ES cells sensitive to RF-EMF after prolonged exposure.

Exposure of undifferentiated cells to GSM-217 signals for 6 hours (Table 25) did not evoke any short-term modification of gene expression patterns neither in wt nor in p53-/- ES cells (Figure 112A). On the other hand, long-term (48 hours) GSM-217 exposure of p53-/- cells during EB development resulted in the up-regulation of transcript levels of 4 out of 6 analysed genes. Whereas c-jun, p21 and c-myc mRNA levels were only transiently up-regulated at early stages (days 2, 5 and 5+2 of EB differentiation, Figure 112B, a prominent induction of hsp70 levels in p53-/- cells was observed throughout the differentiation period. The same experimental protocol was applied to analyse the influence of GSM signals simulating talking and listening phases during a typical conversation (GSM-Talk). However, no changes of gene expression patterns in both wt and p53-/- cells were observed upon the exposure to GSM-Talk signals regardless of the protocol used. These observations indicate that the genetic constitution of cells determined by the p53 function affected cellular responsiveness to GSM-modulated EMF, whereas low frequency components characteristic for GSM-Talk modulation were not responsible for these effects.

Table 25. Conditions of the exposure of p53-proficient and deficient pluripotent embryonic stem cells to RF-EMF and summary of the effects on transcript levels of regulatory genes.

GSM-Basic			
1.5 W/kg (5min on/30 min OFF)			
$p53^{+/+}$ ES cells		**$p53^{-/-}$ ES cells**	
6h, (5min ON/30 min OFF) (n=3)	no EMF effect	6h, (5min on/30 min OFF) (n=3)	no EMF effect
48h, (5min on/30 min OFF) (n=3)	no EMF effect	48h, (5min on/30 min OFF) (n=3)	upregulation of hsp70, c-jun,c-myc and p21

GSM-Talk (33% GSM-Basic, 66% DTX)			
0.4 W/kg (5min on/30 min OFF)			
$p53^{+/+}$		**$p53^{-/-}$**	
6h, (5min on/30 min OFF) (n=3)	no EMF effect	6h – (5min on/30 min OFF) (n=3)	no EMF effect
48h,(5min on/30 min OFF) (n=3)	no EMF effect	48h (5min on/30 min OFF) (n=3)	no EMF effect

GSM – DTX (100% DTX)			
0.11W/kg			
$p53^{+/+}$		**$p53^{-/-}$**	
6h, (5min on/30 min OFF) (n=3)	no EMF effect	6h, (5min on/30 min OFF) (n=3)	no EMF effect

n- number of experiments

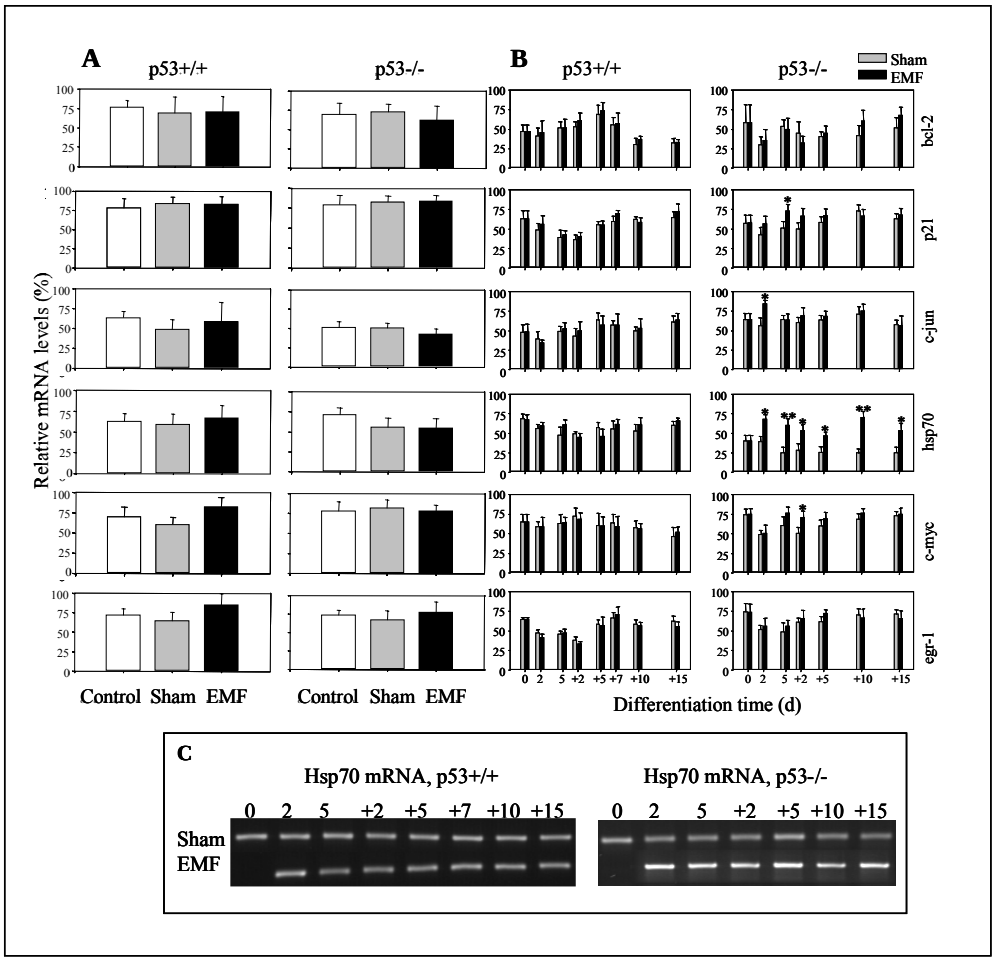


Figure 112. Relative mRNA levels of genes encoding bcl-2, p21, c-jun, hsp70, c-myc and egr-1 in p53-deficient ($p53^{-/-}$) and wild-type ($p53^{+/+}$) D3 ES cell-derived EBs after 6 (A) and 48 (B) hours GSM-217 Hz exposure. GSM-217 exposure resulted in a significant and long-lasting upregulation of hp70 transcripts (C) paralleled by a temporary upregulation of c-myc, c-jun and p21 levels in differentiating $p^{-/-}$ ES cells after 48h exposure (B), but not after 6h (A) exposure. Error bars represent standard deviations. Statistical significance was tested by the Student's t-test for significance levels of 1 and 5% (**, p<0.01, *,<0.05)

RF-EMF did not influence cardiac differentiation and gene expression levels in R1 ES cells.

For the evaluation of embryotoxic effects of chemical compounds in vitro, the mouse embryonic stem cell test, EST (Spielmann 1997), studying cardiac differentiation of ES cells as endpoint has been established. Therefore, we further analysed effects of EMF on cardiac differentiation in R1 ES and P19 EC cells. R1-derived EBs were exposed to EMF for 5 days and the cells were analysed after further differentiation. Similarly to D3 wt cells, no effect of GSM-217 signals on the expression of the regulatory genes including p53 was observed in R1 wt cells. Moreover, no significant differences in cardiac differentiation were found between sham- and EMF-exposed variants during EB differentiation. The results of morphological investigations were confirmed by RT-PCR analyses, where no differences in cardiac α-MHC mRNA levels between sham- and EMF-exposed variants were observed.

RF-EMF did not induce cardiac differentiation and gene expression and the proliferation of P19 EC cells.

Contrary to ES cells, which spontaneously differentiate into the cardiac lineage, differentiation of P19 cells has to be stimulated by external differentiation factors, i.e. DMSO or retinoic acid. To elucidate, whether EMF signals interfere with DMSO-induced cardiac differentiation, undifferentiated P19 EC cells were exposed to GSM-217 signals for 22 and 40h, respectively, and differentiated in the absence or

presence of 1% DMSO. The mRNA levels of regulatory genes were not affected by EMF exposition, and no significant differences were found between EMF-, sham-exposed and control variants of undifferentiated P19 cells, respectively.

Without DMSO induction, only low levels (5-10%) of spontaneous cardiac differentiation were found. By differentiation induction with DMSO during the first 48 hours of EB development, the differentiation of spontaneously beating cardiac cells was increased to a maximum level of 90% in both variants. Exposure of P19 cells to GSM-217 signals for 22 and 40 hours did not result in significant changes of cardiac differentiation suggesting that EMF had no effects on spontaneous or DMSO-induced cardiogenesis. Furthermore, undifferentiated EC cells were EMF- (GSM-217) and sham-exposed at a SAR value of 2.0 W/kg for 22 or 40 hours, and the lengths of cell cycle phases were analysed by flow cytometry. In both cases, no differences in the distribution of cells in G1, S or G2/M phases were observed between sham- and EMF-exposed variants.

RF-EMF exposure may affect the bcl-2 mediated apoptotic pathway in ES-cell derived neural progenitors and neuronal differentiation by inhibiting nurr-1 and TH transcription.

We used an experimental protocol that has been shown to be efficient for differentiation induction of ES cells into the neural lineage (Figure 6) for our experiments aimed at defining the influence of RF-EMF (1.71 GHz, 1.5 W/kg, 5 min on/30 min off) as shown in Table 26. Neural progenitors were analysed at stage 4+4d to 4+6d, where nestin-positive cells were detected in ca. 60-80% of the cells. The exposure conditions applied for 6 or 48 hours are shown in Table 23. The analysis of hsp70, bax and p21 mRNA levels after RF-EMF exposure of neural progenitor cells did not provide evidence of gene expression changes. Bcl-2 was significantly up-regulated after the RF-EMF exposure at the terminal stage 4+23d (Figure 109, $p<0.01$). In addition, we investigated the effect of RF-EMF exposure on GADD45 transcript levels and found that GADD45 mRNA levels were significantly up-regulated at the same stage of differentiation at 4+23d (Figure 113, $p<0.05$). The data on the influence of RF signals on gene expression pattern in differentiating neuronal cells revealed statistically significant down-regulation of nurr-1 at stage 4d+11 and TH at terminal stage 4d+23d, but no clear shifts in transcript levels of the tissue specific genes GFAP, Nestin and En-1 (Figure 113). Quantitative RT-PCR with specific primers and TaqMan probe confirmed the up-regulation of GADD45 at 4+23d stage, but not for bcl-2. According to the Q-RT-PCR data nurr-1 was down-regulated both at stage 4+7d and 4+11d, but the decrease in mRNA levels was statistically significant for stage 4+7d (Figure 114).

Table 26. Conditions of the exposure of neuronal progenitor cells to PL-MF or RF-EMF.

Intermittent exposure (5min on/30 min off)			
48h, GSM 217 Hz (1.71 GHz)		**6h, GSM 217 Hz (1.71 GHz**	
1.5 W/kg	Alterations of GADD45 transcript levels Down-regulation of nurr-1. No effect on DNA break induction (n=7)	1.5 W/kg	Low, but statistically significant induction of double-strand DNA breaks (n=7)

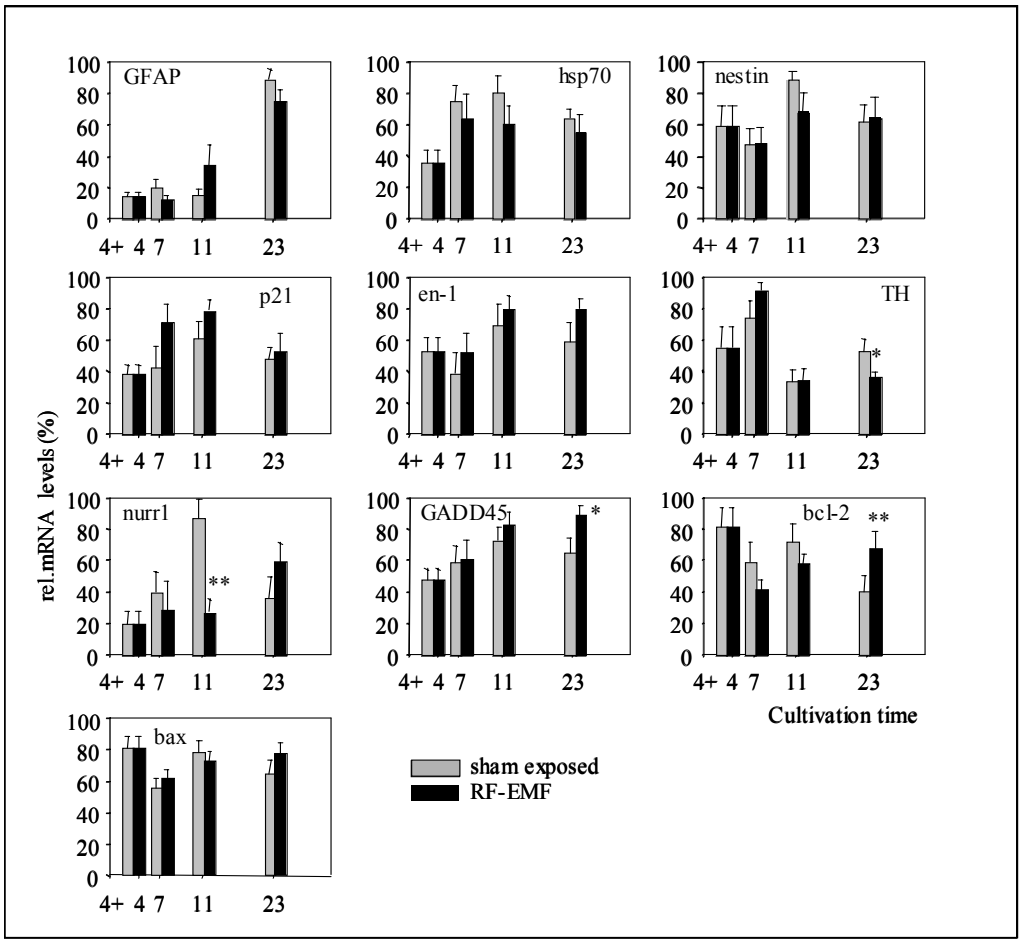


Figure 113. Relative mRNA levels analysed by semi-quantitative RT-PCR of genes encoding bcl-2, p21, c-jun, hsp70, c-myc and egr-1 in p53-deficient (p53-/-) and wild-type (p53+/+) D3 ES cell-derived EBs and EB outgrowths after 6 (A) and 48 hours (B) exposure to GSM-Talk. No effects of GSM-Talk on gene expression levels in ES cells were observed.

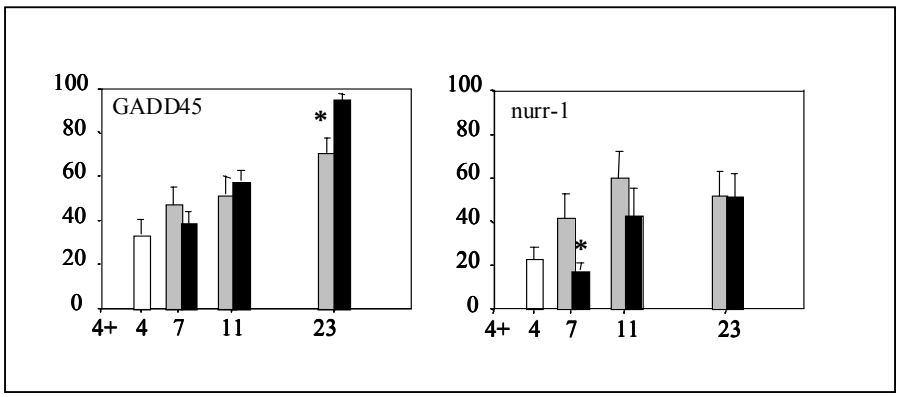


Figure 114. Quantitative RT-PCR with specific primers and Taqman probe for estimation of relative mRNA levels of the growth arrest and DNA damage inducible gene GADD45 and the nurr-1 gene involved in neuronal differentiation in ES-derived neural progenitors after 48 hours RF - EMF (GSM signal 217-Hz) exposure (1.5 W/kg, 1.71 GHz, intermittency 5 min ON/30 min OFF), at stage 4+4d - 4+6d. EMF exposure resulted in a significant transcript down-regulation, followed by up-regulation of GADD45 and down-regulation of nurr-1 at stage 4+7d. Error bars represent standard deviations. Statistical significance was tested by the Student's two-tailed paired t-test for a significance level of 5% (*, $p < 0.05$).

3.2.4.2 Human neuroblastoma cell line NB69 and neural stem cells (NSC) (Participant 5)

RF-EMF (GSM-CW and GSM-Basic) interfered with the expression of FGF receptors in NB69 human neuroblastoma cells.

In a first experiment the cellular response to a chemical promoter of differentiation was characterised. Immunocytochemical staining using antibodies against phenotype-specific antigens revealed that NB69 cells contain the neuroblast-specific protein ßIII-tubulin, but not the neuroepithelial marker nestin, which is present in immature progenitors and in some neuroblastoma cells (Kashima et al. 1995). Untreated NB69 cells remain in an undifferentiated state. Immunocytochemistry for FGFR1-3 revealed the three types of receptors in the human neuroblastoma cell line. On day 3 after plating approximately 70% of cells expressed R1, whereas FGFR3 and FGFR2 were present in a smaller proportion of cells, 30% and 20%, respectively (Figure 115, grey colour). Basic fibroblast growth factor (bFGF), which induces morphological changes including neurite extension at a 20-ng/ml concentration, was used as a positive control for the subsequent EM treatments. This growth factor reduced the number of NB69 cells expressing FGF receptors R1, R2 and R3. Such an effect was accompanied with changes in the cellular morphology associated to differentiate phenotypes. These changes included increased neural outgrowth, neural microtubule network and cell surface. As shown in this Figure 115, the 24-hour treatment with bFGF induced a consistent reduction in the percent of FGFR positive cells for the 3 receptors tested ($p<0.0001$; ANOVA).

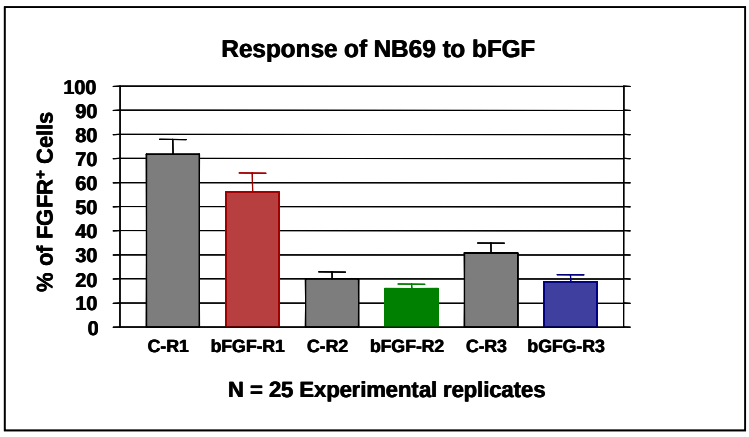


Figure 115. Expression of FGF Receptors 1, 2 and 3 in NB69 cells after a 24-hours treatment with 20-ng/ml bFGF. See text for details.

In the following experimental series, the cellular responsiveness to a differentiation-promoter was confirmed through treatment with bFGF, which induced a reduction of the percent of cells expressing FGF receptors R1, R2 and R3. Such an effect was associated with cell enlargement and neurite arborisation. In contrast, the treatment with GSM-Talk (SAR of 2 W/kg) signal alone (N= 4 experimental replicates), does not modify significantly the normal expression of the FGF protein receptors R1, R2 and R3 in NB69 cells (Figure 116). However, the results of the combined treatment bFGF + GSM-Talk signal show that the EMF seems to antagonize the significant reduction of FGFR-2 expression induced by bFGF. This indicates that the GSM-Talk signal might interfere with some of the cellular responses to bFGF.

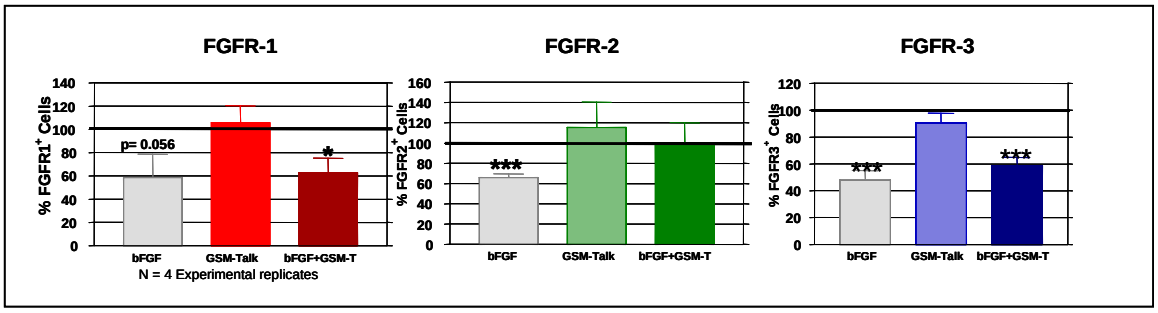


Figure 116. Response of NB69 cells to GSM-Talk signal. Percent of cells expressing FGF Receptors R1, R2 and R3. The treatment with bFGF induced significant reductions in the percent of cells expressing the receptors. Data normalised over the respective controls. (*, p<0.05; **, p<0.01; ANOVA followed by Student's T test).

When administered alone, the exposure to GSM 1800-Basic signal at a 2 W/kg SAR was found to induce a decrease in the number of cells expressing the FGFR-1 (15% reduction vs. controls, Figure 117) and photomicrographs of NB69 cell cultures) without affecting significantly the number of cells expressing receptors R2 and R3. The magnitude of the effect on R1 was equivalent to that induced by bFGF at a 20 ng/ml concentration. However, unlike bFGF, the exposure to GSM-Basic alone did not provoke changes in the cellular morphology. Provided that, as described previously, the GSM-Basic treatment does not induces significant changes in the total cell number or the cell viability, the present results indicate that the GSM-Basic-induced effect on FGFR-1 is not due to a reduction in the number of cells, but to a loss of expression of this receptor.

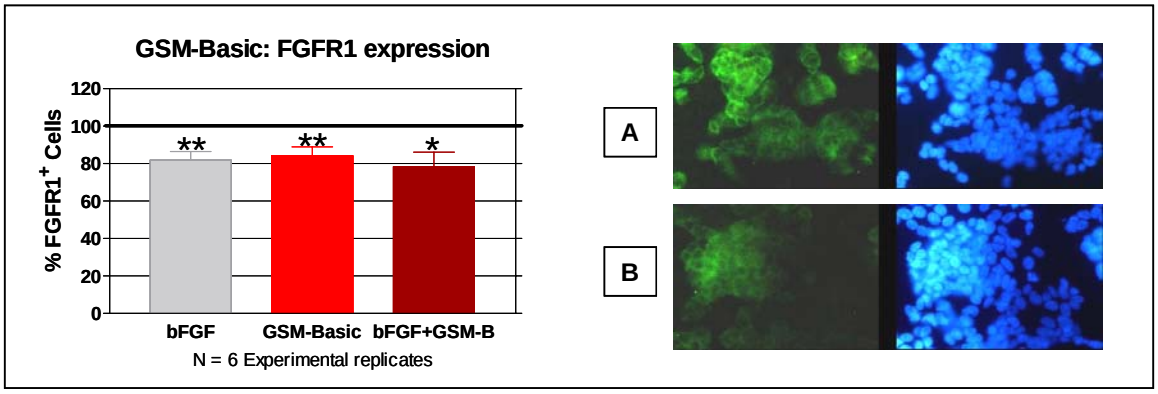


Figure 117. Photomicrographs of NB69 cell cultures: A, control; B, exposed to GSM-Basic signal. Immunocytochemistry for FGFR1. The image analysis showed a statistically significant reduction in the protein expression in the exposed cells (left). The matched fields (right) show the total cells stained with Hoechst.

The treatment with RF-CW signal (SAR 2 W/kg) induced effects on the expression of FGFR-1 equivalent to those induced by the GSM-Basic signal (Figure 118A), whereas the exposure to GSM-DTX signal at a lower SAR (1 W/kg) did not modify significantly the normal expression of the FGF protein receptors R1 in NB69 cells (Figure 118B).

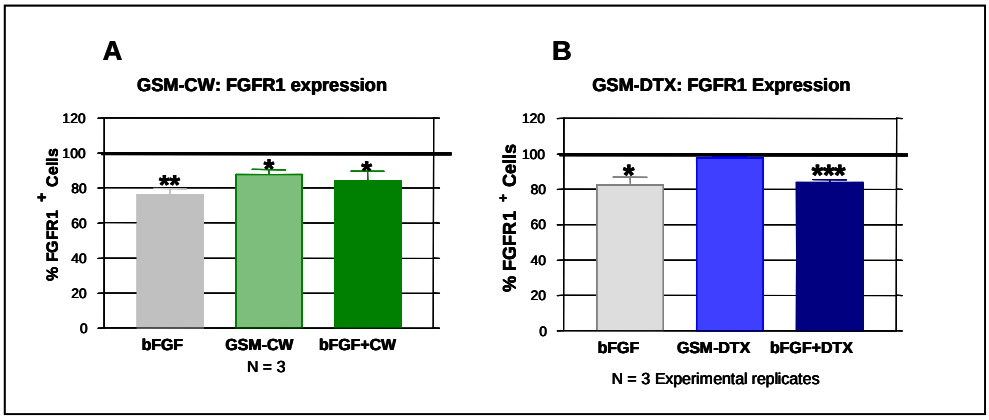


Figure 118. FGFR1 expression in NB69 cells after exposure to: (A) RF-CW signal, 2 W/kg and (B) GSM-DTX signal, 1 W/kg. Only RF-CW induced a significant response. *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$ (ANOVA followed by Student's T test for unpaired data).

Taken together, the results on the expression of FGFRs in NB69 cells exposed to GSM-1800 MHz signals and RF-CW signals suggest that (1) these cells are sensitive to low-SAR signals, (2) the cellular response does not seem to be dependent on the tested low-frequency modulation and (3) the observed response on FGFR1 could be indicative of a EMF-induced promotion of cell differentiation. However, additional studies on the expression of differentiation markers have to be done to confirm this hypothesis. (4) The GSM signal does not seem to interfere significantly with the cellular response to bFGF.

RF-EMF affected the expression of FGF receptors in neural stem cells (NSC).

In order to enhance expansion of NS precursor cells, neurospheres were seeded onto adherent substrate and treated with the mitogen epidermal growth factor (EGF) during the first 3 days in culture. After this period the EGF was withdrawn, and cells grew in a defined medium, which promoted differentiation processes to neurons, astrocytes and oligodendrocytes. Between 2 h and 3 days cultures comprised mainly nestin-positive, undifferentiated precursors. At later stages, the total number of cells dropped, paralleling to a gradual loss of nestin content, and an enhancement in the differentiation processes of neurons, oligodendrocytes and astrocytes.

Immunocytochemistry for FGFR1-3 identified the three types of receptors in the progeny of EGF-expanded NSCs. During the first day after plating, approximately 70% and 50% of the precursors expressed FGFR1 and FGFR2, respectively, whereas FGFR3 was restricted to a less abundant population. At 3 days and thereafter the number of cells exhibiting FGFR1 and FGFR2 decreased gradually, so that at 3 days the percent of FGFR-1 was 35% and at 9 days postplating only approximately 15% of the cell population was immmunopositive for this receptor. FGFR1 immunostaining was preferentially localised in the cytoplasmic compartment, FGFR-3 was found in the cytoplasmic and/or nuclear compartments, and FGFR2 was frequently confined to the nucleus. In situ hybridisation studies on the third day postplating showed high levels of FGFR1 mRNAs in NSC.

On the basis of our previous data showing that the GSM-Basic signal induces a reduction in the percent of cells expressing FGF-R1 in human neuroblastoma cells, we tested firstly this signal (2 W/kg) on NSC. Like in NB69 cells, a significant decrease in FGFR1-positive NS cells was also observed after exposure to the GSM-Basic signal (50% reduction with respect to controls, Figure 119). Western blot analysis for FGFR1 confirmed this effect (data not shown). The study also indicates that the response of the neural stem cells seems to be dependent on the age of the culture (Figure 120).

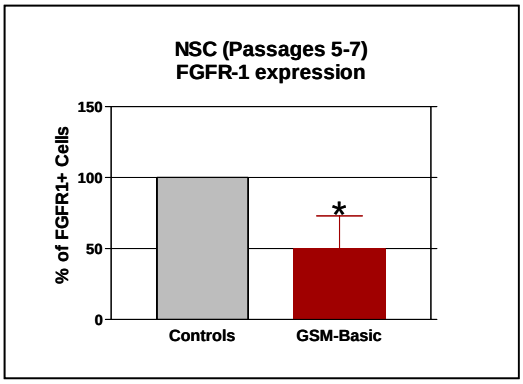
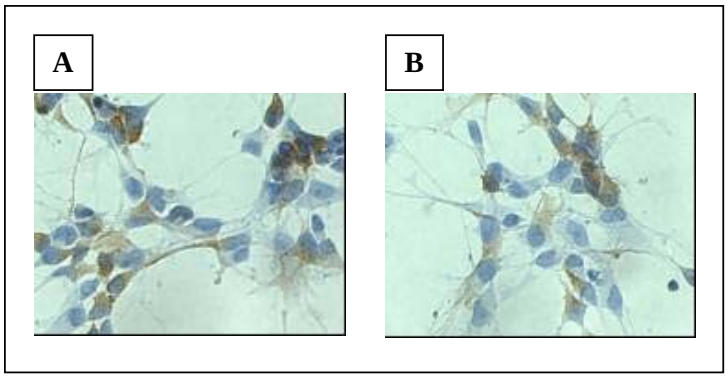


Figure 119. Percent of FGFR-1 positive labelling vs. total cell number normalised over their controls, quantification by the program for image analysis (IPWIN-3). Data represent the mean ± SEM of 3 independent experiments, done in duplicate (two coverslips), for the different treatment conditions. Student T-test *p< 0.05.



Photomicrographs of Neural Stem Cells, A, control and B exposed to GSM-Basic signal. Inmunocytochemistry for FGFR1-positive cells (brown).

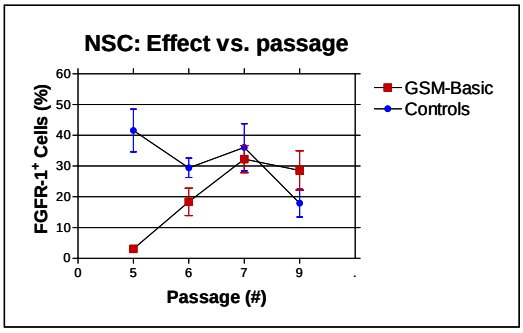


Figure 120. NSC exposed to GSM-Basic signal. The percent of FGFR-1 positive cells seems to be dependent on the passage number.

RF-EMF did not affect gene expression of FGF Receptor-1 in NB69 neuroblastoma cells and in neural stem cells (NSC)

In situ hybridisation studies were conducted on neural stem cells and in NB69 cells exposed for 21 hours (5 min on/10 min off) to the GSM-Basic signal. The objective is to evaluate potential EMF effects on gene expression of FGFR1. All experiments were conducted following blind protocols. The results on both cell types showed no differences in FGFR1 mRNA-expression between controls and exposed samples. An image-analysis study confirmed this result in NSC (Figure 121). Taken together, the

described effects on NB69 and NSC (FGFR1 protein-expression and FGFR1 mRNA-expression), suggest that the GSM-Basic signal can modulate FGFR1 protein translation without affecting protein transcription.

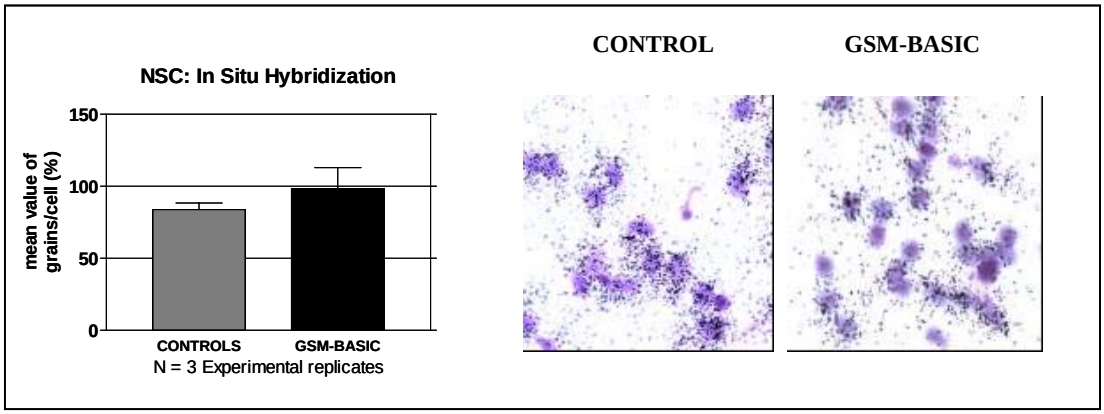


Figure 121. Quantification of in situ hybridisation staining by image-analysis technique (IPWIN 3.0) of the number of grains/cell. The photomicrographs show the developmental pattern of FGFR1 in NSC processed with Radiolabelled Probe Specific for Transcript of FGFR1.

3.2.4.3 Human promyelocytic cell line HL-60 (Participant 2)

RF-EMF exposure reproducibly up- and down-regulated protein expression in HL-60 cells (41 proteins showed to be up-, 1 protein to be down-regulated and 14 proteins appeared to be de-novo expressed).

The proteome screening approach included analysis of the entire HL-60 protein expression pattern by means of 2-D polyacrylamide gel electrophoresis (2D-PAGE). After having established the technique for HL-60 cells, cells were exposed to RF-fields at selected conditions in repeated independent experiments in order to obtain reproducible information on changes in the cellular protein pattern, correlated with RF-EMF-exposure.

HL-60 cells were exposed at 1800 MHz, continuous wave, SAR 1.3 W/kg, 24h, or were sham exposed in repeated independent experiments. Additionally, incubator controls were run and analysed for their protein expression pattern by 2D-PAGE. Cell samples were partly analysed as described above, partly stored at -80 °C for further analyses. Comparison of protein pattern after 2D-PAGE showed that optimal reproducibility is achieved when the 2-D separation step is performed in one series with identical reagent batches. In order to be able to perform statistics, appropriate numbers of comparable 2D-gels are required, also to have enough material for protein identification.

Figures 122, 123, and 124 show representative high-resolution 2-dimensional polyacrylamide gels (23 x 30 cm) for each of the conditions described above (incubator control, sham-exposure and RF-field exposure).

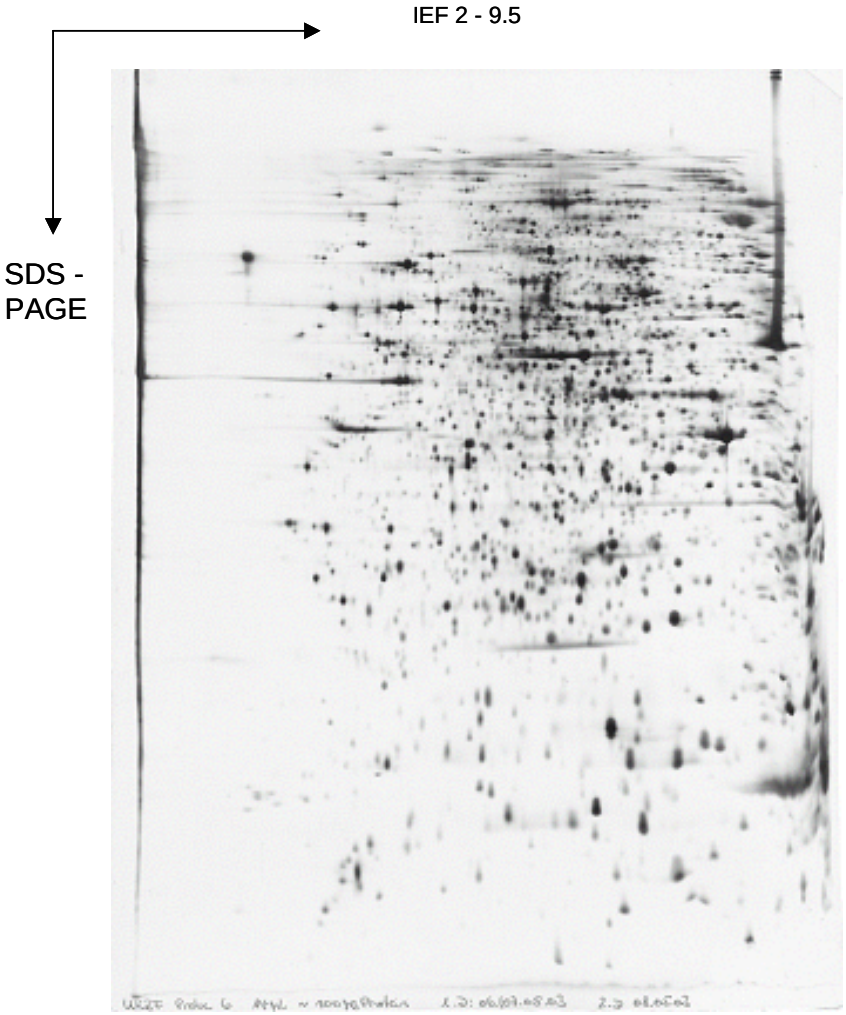


Figure 122. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) profile of incubator control HL-60 cells (whole cell lysate). Incubation time: 24h. First dimension (isoelectric focussing): pH-gradient 2-9.5. Second dimension: 12.5% polyacrylamide gel, silver stain.

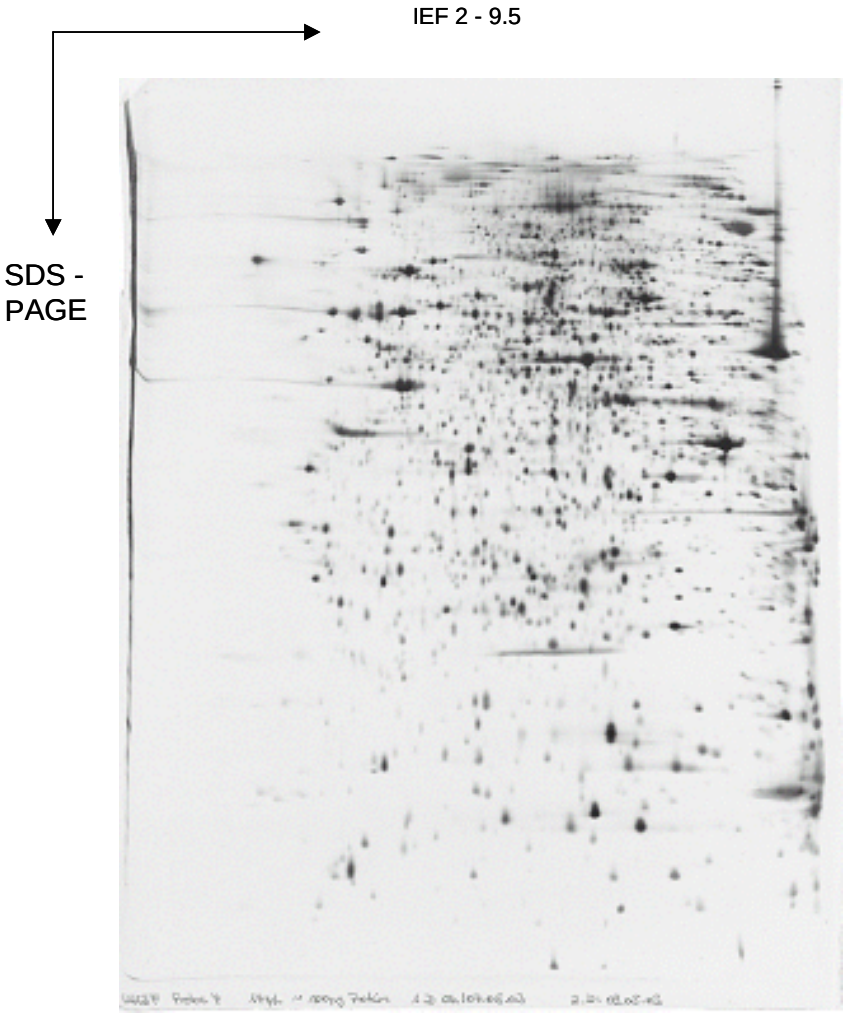


Figure 123. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) profile of sham-exposed HL-60 cells (whole cell lysate). Exposure time: 24h. First dimension (isoelectric focussing): pH-gradient 2-9.5. Second dimension: 12.5% polyacrylamide gel, silver stain.

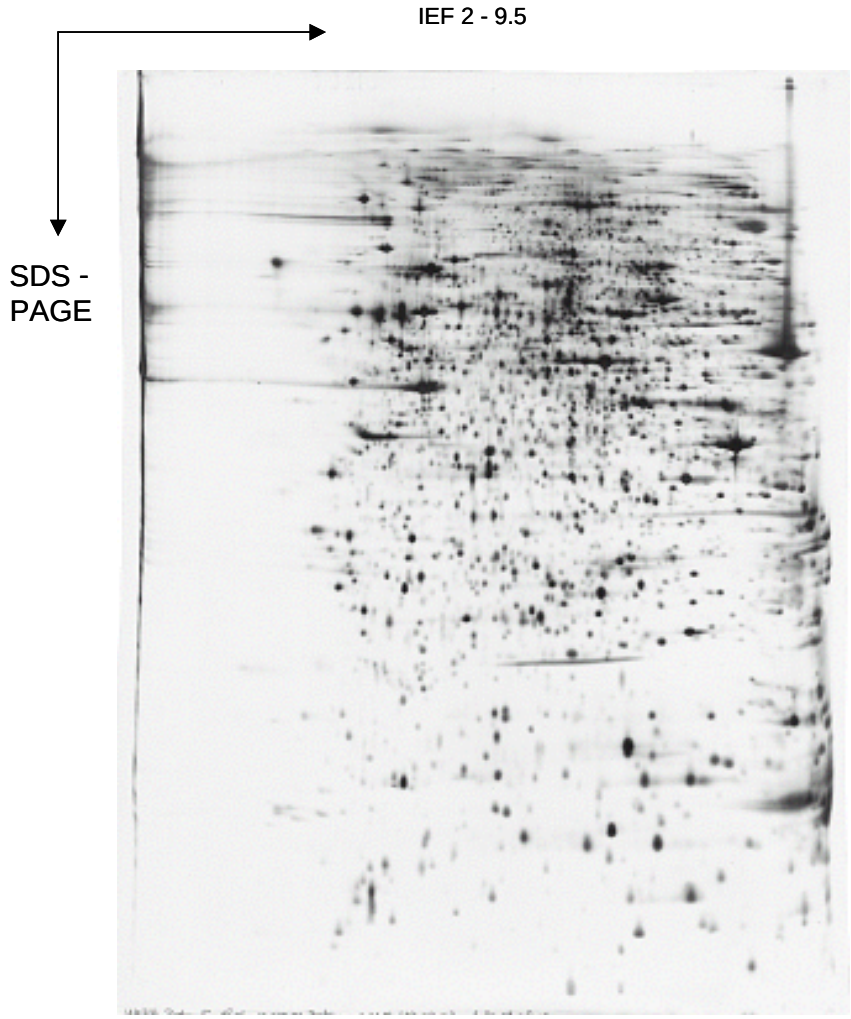


Figure 124. Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) profile of RF-exposed HL-60 cells (whole cell lysate). Exposure characteristics: 1800 MHz, continuous wave, 1.3 W/kg, 24h. First dimension (isoelectric focussing): pH-gradient 2-9.5. Second dimension: 12.5% polyacrylamide gel, silver stain.

Following digitalisation, in a second analysis step the qualitative and quantitative comparison of protein expression was performed by use of Proteom Weaver image analysis program. Figure 125 shows a representative comparative 2D-gel, in which expression differences between RF-field exposed and sham-exposed HL-60 cells are marked. Expression differences were quantified. In Table 27 (a-c) proteins up-or down-regulated and those that have disappeared after RF-field exposure are listed.

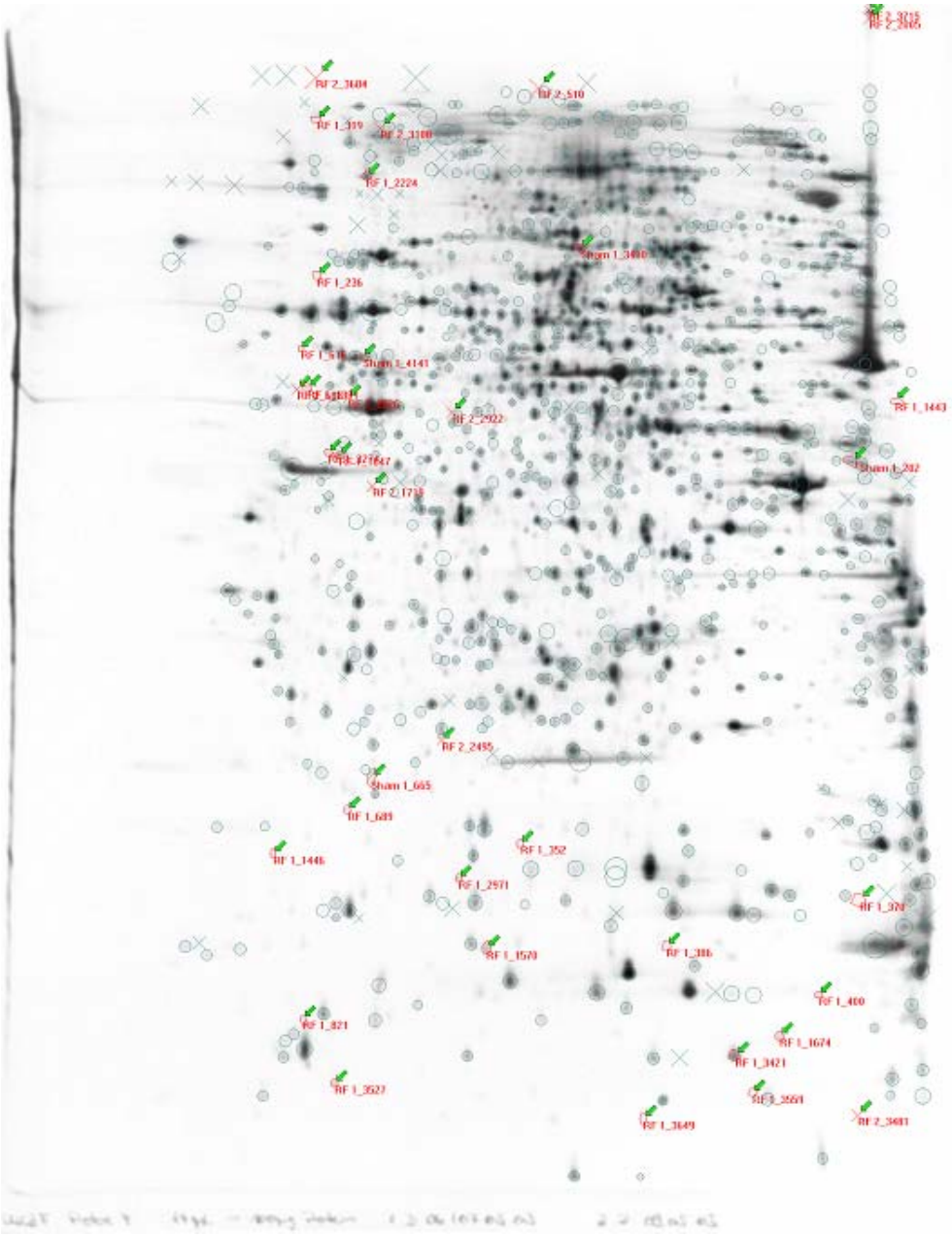


Figure 125. Representative qualitative and quantitative comparison of two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) profiles of sham-exposed and RF-exposed HL-60 cells (whole cell lysate).

Exposure conditions: see above. Green arrows mark different proteins of sham-exposed cells compared to RF-field exposed cells (green arrows and red circles: up-regulated proteins; green arrows and red crosses: newly expressed or disappeared proteins).

Table 27. List of proteins in 2-DE patterns of HL-60 cells, differing between RF-field exposed and sham exposed cells. Exposure conditions: see above

a: List of proteins up-regulated in RF-field exposed HL-60 cells (average intensity see arrow) as compared to sham-exposed cells (average intensity see arrow).

				sham				⇩		RF-field			⇩
ID	ID	Name	Freq	X	Y	I	/	Avg	X	Y	I	/	Avg
1	1_3559	RF 1_3559	2	1163	1684	0,039	N.R.	0,039	1183	1726	0,278	7,1924	0,278
2	1_1443	RF 1_1443	2	1384	614	0,058	N.R.	0,058	1399	628	0,389	6,649	0,389
3	1_400	RF 1_400	2	1266	1533	0,055	N.R.	0,055	1278	1562	0,354	6,3801	0,354
4	1_689	RF 1_689	2	536	1247	0,048	N.R.	0,048	574	1271	0,267	5,5816	0,267
12	1_616	RF 1_616	2	464	533	0,031	N.R.	0,031	512	546	0,137	4,404	0,137
15	1_386	RF 1_386	2	1030	1458	0,057	N.R.	0,057	1056	1491	0,247	4,3329	0,247
16	1_1446	RF 1_1446	2	421	1315	0,046	N.R.	0,046	464	1345	0,193	4,2311	0,193
17	1_236	RF 1_236	2	489	421	0,047	N.R.	0,047	538	431	0,198	4,227	0,198
22	1_1391	RF 1_1391	2	474	594	0,094	N.R.	0,094	522	607	0,354	3,7725	0,354
24	1_821	RF 1_821	2	467	1570	0,042	N.R.	0,042	507	1611	0,157	3,7049	0,157
27	1_3274	RF 1_3274	2	506	694	0,067	N.R.	0,067	549	708	0,229	3,4382	0,229
28	1_1674	RF 1_1674	2	1205	1597	0,117	N.R.	0,117	1220	1631	0,394	3,3527	0,394
35	1_3649	RF 1_3649	2	994	1725	0,048	N.R.	0,048	1017	1778	0,15	3,102	0,15
36	1_370	RF 1_370	2	1329	1387	0,084	N.R.	0,084	1338	1406	0,261	3,0949	0,261
37	1_352	RF 1_352	2	804	1299	0,043	N.R.	0,043	834	1330	0,132	3,0908	0,132
41	1_1647	RF 1_1647	2	523	698	0,199	N.R.	0,199	565	712	0,59	2,9714	0,59
43	1_2971	RF 1_2971	2	708	1353	0,075	N.R.	0,075	743	1385	0,221	2,9515	0,221
45	1_2224	RF 1_2224	2	565	267	0,345	N.R.	0,345	607	275	1,016	2,9415	1,016
48	1_319	RF 1_319	2	489	178	0,058	N.R.	0,058	536	186	0,168	2,9054	0,168
49	1_3421	RF 1_3421	2	1136	1628	0,597	N.R.	0,597	1159	1669	1,728	2,8956	1,728
54	1_1570	RF 1_1570	2	751	1462	0,231	N.R.	0,231	784	1499	0,63	2,7259	0,63
61	1_3527	RF 1_3527	2	516	1670	0,075	N.R.	0,075	552	1717	0,187	2,5069	0,187

b: List of proteins down-regulated in RF-field exposed HL-60 cells (average intensity see arrow) as compared to sham-exposed cells (average intensity see arrow).

				sham					RF-field				
ID	ID	Name	Freq	X	Y	I	/	Avg	X	Y	I	/	Avg
13	1_3400	Sham 1_3400	2	897	378	0,345	N.R.	0,345	927	389	0,12	0,3489	0,12

c: List of newly expressed or disappeared proteins in RF-field exposed HL-60 cells (average intensity see arrow) as compared to sham-exposed cells (average intensity see arrow) .

				sham					RF-field				
ID	ID	Name	Freq	X	Y	I	/	Avg	X	Y	I	/	Avg
1	1_202	Sham 1_202	1	1317	708	0,417	N.R.	0,417	1326	716	X	N.A.	N.A.
2	2_2005	RF 2_2005	1	1343	21	X	N.R.	N.A.	1358	29	0,546	N.A.	0,546
3	2_1719	RF 2_1719	1	574	746	X	N.R.	N.A.	614	761	0,198	N.A.	0,198
4	2_3100	RF 2_3100	1	586	191	X	N.R.	N.A.	628	196	0,424	N.A.	0,424
5	2_688	RF 2_688	1	458	595	X	N.R.	N.A.	506	609	0,133	N.A.	0,133
6	2_2495	RF 2_2495	1	682	1138	X	N.R.	N.A.	718	1160	0,142	N.A.	0,142
7	1_4141	Sham 1_4141	2	560	546	0,158	N.R.	0,158	600	558	0,468	2,9721	0,468
8	2_2922	RF 2_2922	1	697	631	X	N.R.	N.A.	735	643	0,222	N.A.	0,222
9	2_3481	RF 2_3481	1	1325	1718	X	N.R.	N.A.	1337	1750	0,101	N.A.	0,101
10	2_510	RF 2_510	1	832	129	X	N.R.	N.A.	868	135	1,007	N.A.	1,007
11	2_3684	RF 2_3684	1	487	114	X	N.R.	N.A.	533	117	1,457	N.A.	1,457
12	2_2826	RF 2_2826	1	537	609	X	N.R.	N.A.	582	622	0,146	N.A.	0,146
13	2_3715	RF 2_3715	1	1343	8	X	N.R.	N.A.	1358	16	0,199	N.A.	0,199
14	1_665	Sham 1_665	2	572	1199	0,124	N.R.	0,124	609	1227	0,13	1,0504	0,13

Overall, 56 polypeptides of HL-60 cells are influenced in their expression under RF-EMF. Reproducibly, 41 proteins showed to be up- and 1 protein to be down-regulated following RF-field exposure. 14 proteins appeared to be de-novo expressed after RF-field exposure of HL-60 cells.

By use of these lists identification strategies are further performed. They will include in gel-cleavage, identification of selected proteins by mass spectrometry (MALDI-TOF) and mass spectrometric sequencing (ESI-MS/MS). Further identification of selected proteins will include immunoblotting and functional protein assays.

3.2.4.4 Human lymphocytes (Participant 8)

RF-EMF did not affect gene expression in human lymphocytes.

T lymphocyte gene expression analysis was performed in collaboration with Participant 12 and Dr. Daniel Remondini in Bologna. The results suggest that no differences in gene expression are found between

quiescent T lymphocytes exposed to RF-EMF (DTX only) in comparison with sham-exposed cells. This finding did not suggest any significant interaction of RF-EMF with gene profile expression.

3.2.4.5 Brain cells of different origin (Participant 9)

RF-EMF exposure did not affect expression and activity of the inducible nitric oxide synthase (iNOS or NOS2) in nerve cells.

A basal level of NOS_2, probably due to the SVF deprivation, was detected in C6 cells, although inter-experiment's variation could be seen. A 48-hour treatment with LPS plus CK increased the expression of the enzyme by a factor 5 (Figure 126)

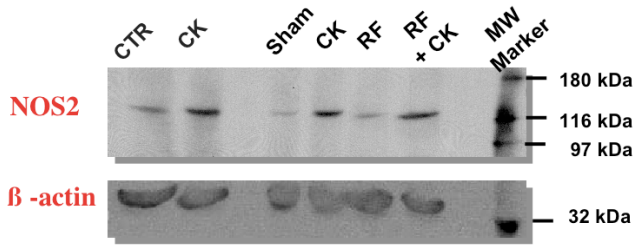


Figure 126. Representative blot for the detection of NOS_2 (upper blot) and ß-actin (lower blot) proteins.

Sham/sham experiments showed that a 15-20% inter-incubator variation had to be expected, so that a more than a 30% difference between sham- and RF-exposed data would be considered as a significant biological effect for both NOS_2 expression and nitrite accumulation (Figure 127).

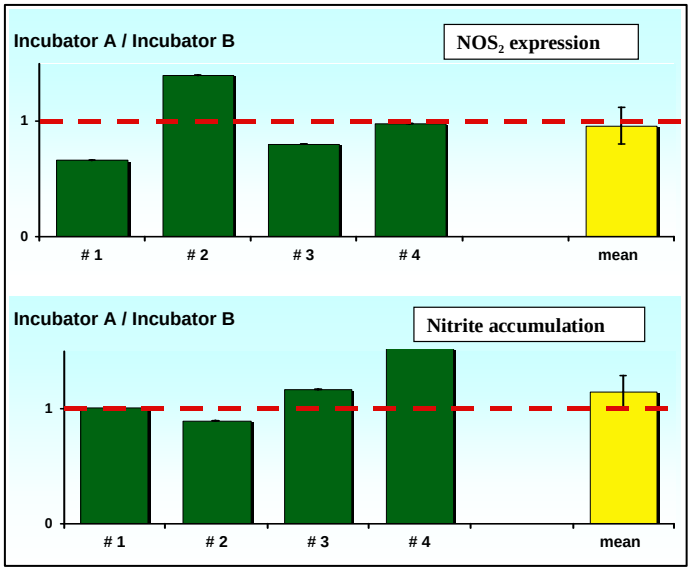


Figure 127. Expression of NOS_2 protein and nitrite accumulation (culture medium) in C6 cells in four sham/sham experiments (#1 to #4). Data are given as the ratio ± SEM between the levels found in the incubator A used for RF exposure and incubator B used for sham-exposure.

Exposure to GSM-900 at 0.2 W/kg and 2 W/kg for 48 hours was shown to not alter the expression of NOS_2 compared to sham exposure. Co-exposure to GSM and LPS plus cytokine was ineffective in modifying the effect of LPS plus cytokine treatment on NOS_2 expression (Figure 128).

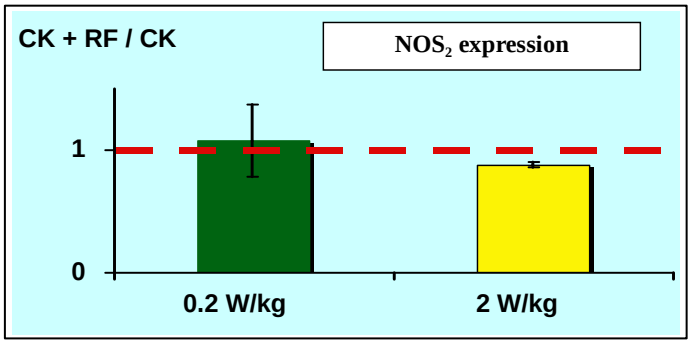


Figure 128. Expression of NOS_2 protein in C6 cells exposed to frame-GSM-900 at 0.2 and 2 W/kg for 48 hours. Data are given as the ratio ± SEM between the levels found in samples treated with CK+LPS (CK) and exposed to RF and those treated with CK alone.

Nitrite accumulation in culture medium was used as a marker of NOS_2 activation. No nitrite accumulation was shown in sham-exposed samples. Although inter-experiment variability, treatment with the cocktail of LPS plus CK led to a significant nitrite accumulation ($p<0.001$). As shown in Figure 129, a mean 20-fold increase in nitrite accumulation was measured after 48 hours of LPS plus CK treatment.

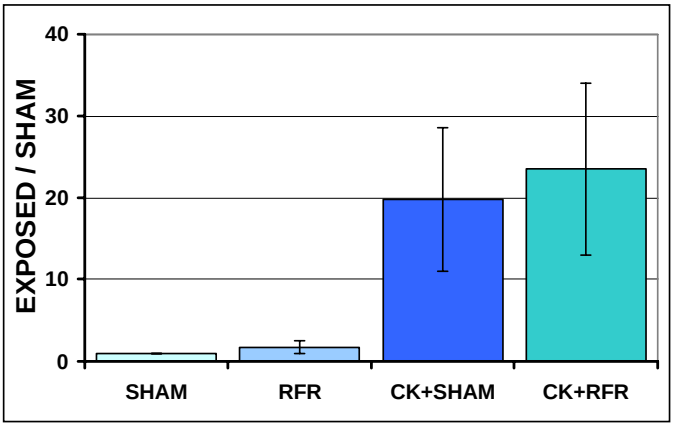


Figure 129. Nitrite accumulation in culture medium of C6 cells sham-exposed or exposed to GSM-900 at 0.2 W/kg and /or treated with CK+LPS (CK). In all cases, treatment duration was 48 hours. Data are given as the ratio ± SEM between the levels found in treated- versus sham-exposed samples.

No significant effect of GSM-900 exposure was detected on nitrite accumulation. When co-exposures to GSM-900 and LPS + CK treatment were performed, no modulation of chemically-induced NOS_2 expression. Overall, exposure to GSM-900 did not modulate CK+LPS-induced nitrite accumulation (Figure 130).

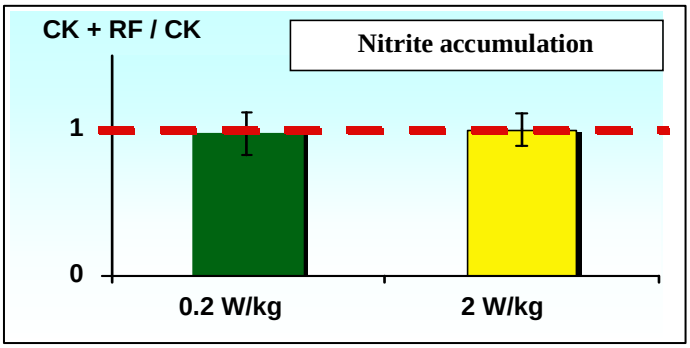


Figure 130. Nitrite accumulation (culture medium) in C6 cells exposed to frame-GSM-900 at 0.2 W/kg and 2 W/kg for 48 hours. Data are given as the ratio ± SEM between the levels found in samples treated with CK+LPS (CK) and exposed to RF and those treated with CK alone.

No evidence of an effect of RF-EMF (GSM-900) exposure on spontaneous expression and activity in C6 cells was obtained from our experiments. However, one can note that only strong treatments (serum deprivation plus long duration of chemical treatment) are shown to increase NOS_2 expression in C6 cells. It is noteworthy that most papers in the literature looked at the mRNA but not at the *protein* level as we did in the present work. Two SAR levels of GSM-900 were tested. Even at the highest SAR level of 2 W/kg corresponding to the public exposure limit recommended by the EU Commission, GSM-900 exposure was not shown able to influence NOS_2 expression or activity in activated C6 cells. Taken together, RF-EMF at a low SAR level were not identified as a stimulus for C6 cells activation.

RF-EMF (GSM-900 signals) did not affect heat shock protein expression in nerve cells.

When used as a positive control, heat shock (43°C for 20 min) increased expression of hsp70 in all nerve cell cultures, i.e. human U87 astrocytoma cells, rat C6 glioma cells and human SH-SY5Y neuroblastoma cell lines. However, when exposed to GSM-900 for 24 hours, none of the cell line showed a significant change in expression of hsp70 (Figures 131, 132). Altogether, our data show that exposure to ELF-EMF does not seem to be able to induce Hsp70 expression in rat and human nerve cells.

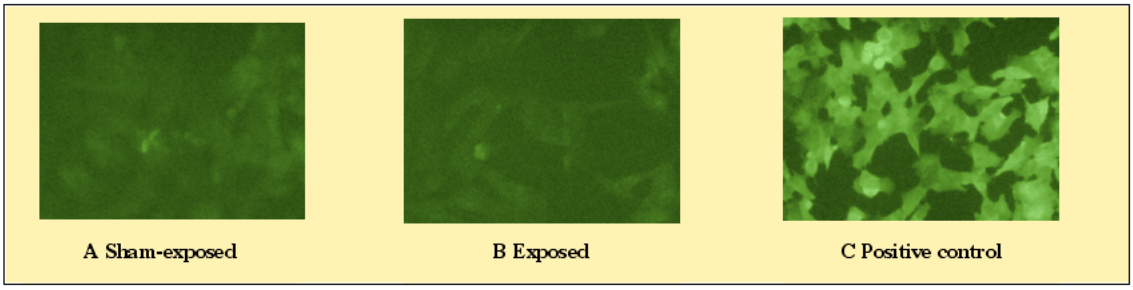


Figure 131. Fluorescent Hsp70 immunolabelling after sham-exposure or exposure of human SH-SY5Y neuroblastoma cells to frame GSM-900 signal at 2 W/kg for 24 hours (A and B) or to heat shock (43°C, 20 min) (C).

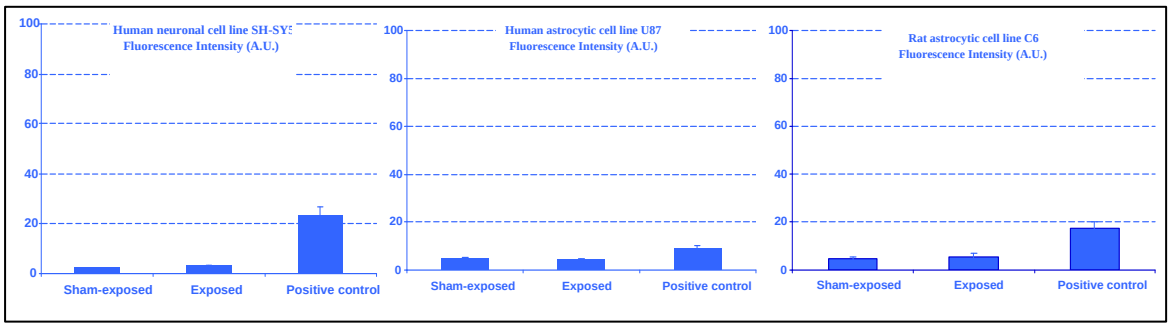


Figure 132. Effect of GSM-exposure (2 W/kg, 24h) or Heat Shock exposure (43°C, 20 min) (Positive control) on hsp70 expression in three different cell lines. Results are expressed in Fluorescence Intensity (A.U). Data from 3 independent experiments are presented as the Mean ± SEM

GSM-900 microwave exposure did not affect hsp27 expression in human endothelial cell line EA.hy926.

The hsp27 expression was measured using the immunofluorescence technique. Qualitative analysis did not allow for detecting any difference in fluorescence intensity in RFR exposed cells versus sham cells. Our quantitative results obtained after fluorescence image analysis of hsp27 expression in EA-hy926 revealed that no significant difference was observed between sham-or exposed cells in both cell lines (Figures 133, 134)

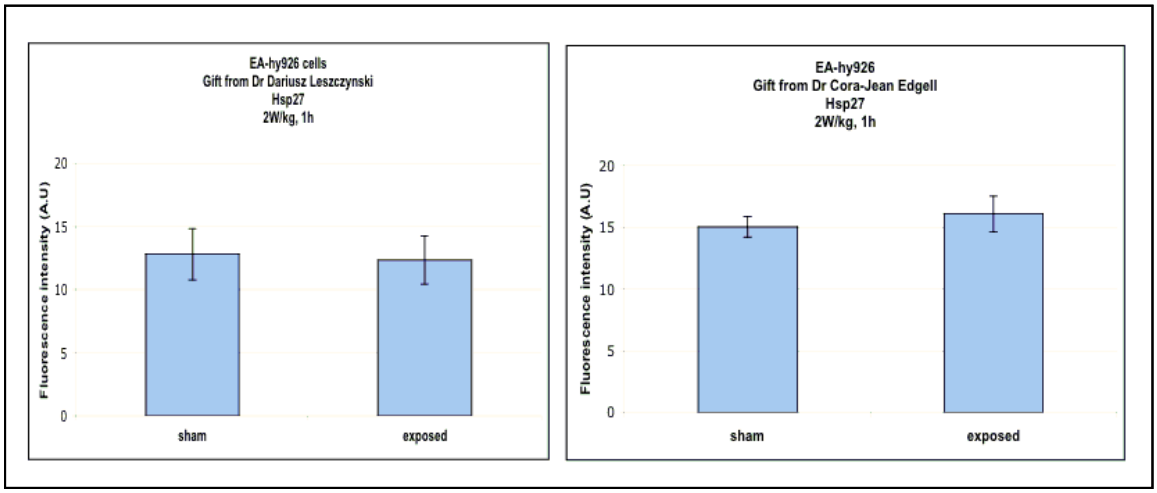


Figure 133. Effect of GSM-exposure (2 W/kg, 1 hour) on hsp27 in EA-hy926 cell lines given by Participant 6 and Dr Cora-Jean Edgell. Results are expressed as the fluorescence Intensity (A.U). Data from 5 independent experiments are presented as the Mean ± SEM.

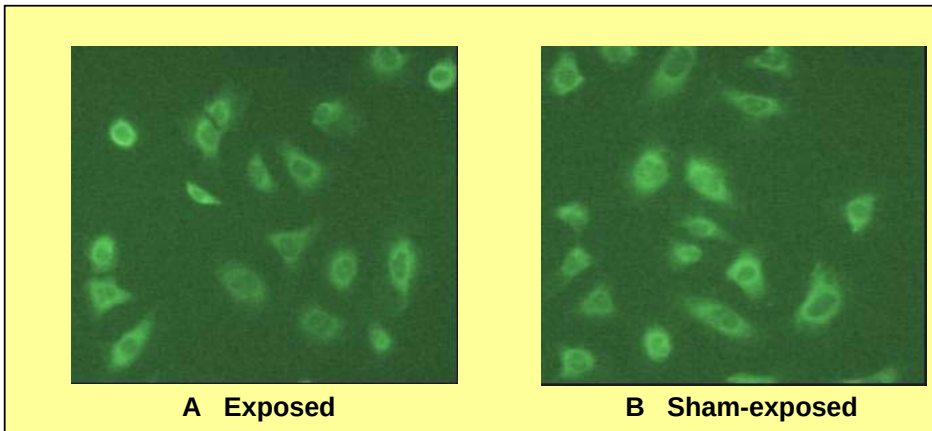


Figure 134. Fluorescent hsp27 immunolabelling after exposure(A) or sham-exposure(B) of EA-hy926 cell lines given by Participant 6 to GSM-900 signal at 2 W/kg for 1 hour.

A third method using Elisa test will allow us to quantify precisely if RFR are able to induce changes in hsp27 expression. However, with our method we were unable to confirm previous data on hsp27 expression in endothelial cell lines (Leszczynski et al., 2002). Therefore, we can not conclude that RFR induce stress response.

No conclusive data was obtained on the effect of RF-EMF exposure on Hsp27 expression in rat brain.

Table 28 shows results obtained on hsp27 expression in rat brains in the pilot experiment.

Results obtained on samples treated without perfusion show non-specific labelling disturbing the analysis. Results obtained on perfused brains show acceptable background noise. Image analysis obtained on perfused brains reveal conflicting and opposite results within groups preventing to draw conclusions.

Table 28. Hsp27 expression in rat brains

Experimental procedure	Background noise	Hsp27 labelling	Exposure conditions
Perfusion	-	+	Control
Perfusion	+	-	Control
Perfusion	-	-	Sham-exposed
Perfusion	-	+	Sham-exposed
Perfusion	-	-	Exposed
Perfusion	+	+/-	Exposed
Without perfusion	+	+	Control
Without perfusion	+	+	Control
Without perfusion	+	+/-	Sham-exposed
Without perfusion	+	++	Sham-exposed
Without perfusion	+	+	Exposed
Without perfusion	+	+/-	Exposed

– : negative labelling; + : positive labelling; +/- : negative or positive labelling depending of the area; +/+ : clear positive labelling

This pilot study did not allow us to draw conclusion on results obtained *in vitro* on hsp27 expression but it gave information on technical methodologies and on the number of animals to use.

RF-EMF (GSM-900) exposure weakly affected gene expression in immune cells.

This investigation was carried out in cooperation with Participant 12. Criteria for the selection of significantly altered gene expression was an exposed over sham ratio less than 0.5 for a significant decrease and more than 2.0 for a significant increase. Using these criteria, over 15588 human genes were detected, changes in expression of about 50 genes were significant corresponding to 0.3% of total number of detectable genes. Genes shown to be altered after RF-EMF exposure (increase or decrease) are known to be involved in signal transduction, ion electron transport, metabolism of energy and proteins, cell proliferation, apoptosis or differentiation, immune answer, inflammation, stress answer, extracellular matrix, cytoskeleton, adhesion and DNA repair. The largest modification in RNA expression corresponded to genes related to signal transduction (linked with GTP or calcium) and energy metabolism. Only a few genes involved in apoptosis or stress response were detected and show no significant sensitivity to RFR exposure. Concerning our purpose to investigate modification of genes involved in inflammatory response and processes, one gene corresponding to a component of major histocompatibilty complex class II and another acting as plasminogen activator were altered by RF-EMF. Finally, the largest increase of expression (30 fold increase), after mobile phone exposure, concerned genes described to participate in amine oxidase (copper containing) activity. This enzyme is involved in cell growth and proliferation but also in immune regulation.

3.2.4.6 Human endothelial cell lines EA.hy926 and EA.hy926v1 (Participant 6)

It has been suggested that high-throughput screening techniques (HTST) of transcriptomics and proteomics could be used to rapidly identify broad variety of potential molecular targets of RF-EMF and generate variety of biological end-points for further analyses (Leszczynski 2001). Combination of data generated by transcriptomics and proteomics in search for biological effects is called the “discovery science”. This term has been coined-in by Aebersold and co-workers (Aebersold et al. 2000) to define the new approach that will help in revealing biological mechanisms, some of which might be unpredictable using the presently available knowledge. This approach seems to be particularly suited for elucidation RF-EMF health hazard issue because it might reveal effects that are not possible to predict based on the present knowledge about the biological effects of RF-EMF. However, before committing large funds that are needed for HTST studies it is necessary to determine whether indeed this approach will be successful in unravelling physiologically significant biological events induced by RF-EMF. Due to their high sensitivity HTST are able to pick-up very small changes in protein or gene expression which changes might be of insufficient magnitude to alter cell physiology. Thus, although using HTST it might be possible to find biological effects induced by RF-EMF these effects might be of limited or no significance at all, from the physiological stand point. Therefore, to determine the usefulness of HTST approach to the issue of bio-effects induced by RF-EMF, we have performed a 5-step feasibility study and have shown that HTST might indeed help to identify experimental targets for physiological studies of RF-EMF-induced biological responses (Figure 135).

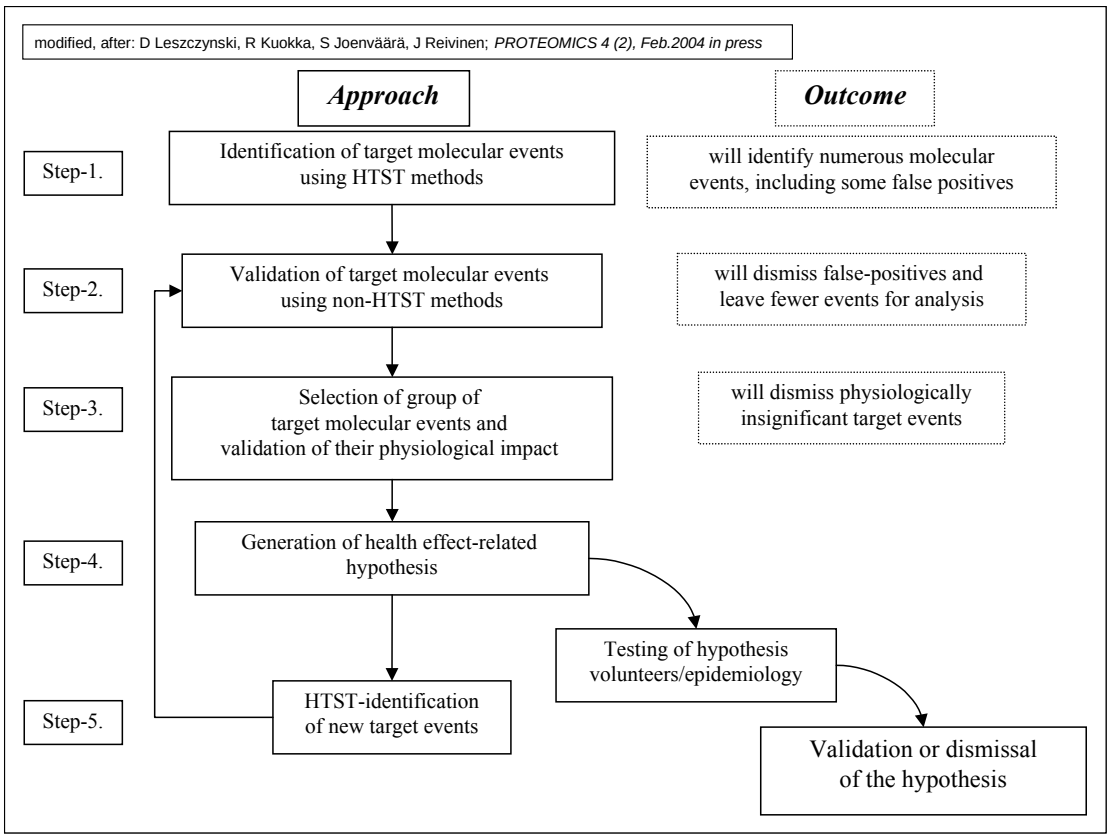


Figure 135. Scheme of experimental procedure which execution will elucidate new RF-EMF induced molecular events that might affect cell physiology. Events of magnitude sufficient to alter cell physiology could be then examined for their potential impact on the organ/whole body physiology in attempt to predict the extent of eventual health hazard.

A. The 5-step feasibility study

Step-1: HTST-identification of target molecular event

Firstly, we have determined the extent of cell response to RF-EMF (Leszczynski et al. 2002). This has been done by analysing global changes in the pattern of protein phosphorylation. As an experimental model we have used cultures of human endothelial cell line EA.hy926 (Edgell et al. 1983). Cells were exposed for 1 hour to 900MHz GSM mobile phone simulating signal at an average specific absorption rate (SAR) of 2.4 W/kg (Deli et al. 1995) that is slightly above the European safety limit (SAR=2.0 W/kg). In order to be able to determine changes in protein phosphorylation, the ^{32}P-labelled orthophosphate was present in the cell cultures during the 1 hour RF-EMF exposure period. Immediately after the end of exposure cells were harvested; proteins extracted and separated using standard two-dimensional electrophoresis (2-DE). Using PDQuest software (Bio-Rad, UK), some 1266 different protein spots were identified in silver-stained 2-DE gels (Figure 136A). Using autoradiography it was possible to determine that among the 1266 proteins spots, in non-irradiated control exposed cells, were detected some 110 phosphoproteins (Figure 136B), whereas in exposed cells were detected some 372 phosphoproteins (Figure 136C). The observed broad change in the pattern of global protein phosphorylation has suggested that cells respond to RF-EMF and that possibly any of the hundreds of phosphoproteins that have altered their phosphorylation status could, at least potentially, affect cell physiology. By using western blot or mass spectrometry, to identify the phosphoproteins present in the 2DE spots, it might be possible to find variety of protein targets that could be used in examining effects of mobile phone radiation on cell physiology. With this approach, the selection of molecular targets for further studies would not be based only on deduction of potentially affected events but on the knowledge of the identities of proteins that indeed respond to RF-EMF. Thus, in the continuation of Step-1, using

simple western blot screening with antibodies directed against various stress response proteins, we have identified hsp27 as one of the phosphoproteins responding to RF-EMF. Hsp27 is continuously expressed in endothelial cells (Edgell et al. 1983). In 2DE-western blots it appeared as two spots of 27kDa molecular weight but with different isoelectric points (pI=5.7 and pI=6.1) (Figure 136D). Only the $hsp27_{pI=5.7}$ isoform was phosphorylated and, following exposure to RF-EMF, the size of $hsp27_{pI=5.7}$-spot has increased (Leszczynski et al. 2002).

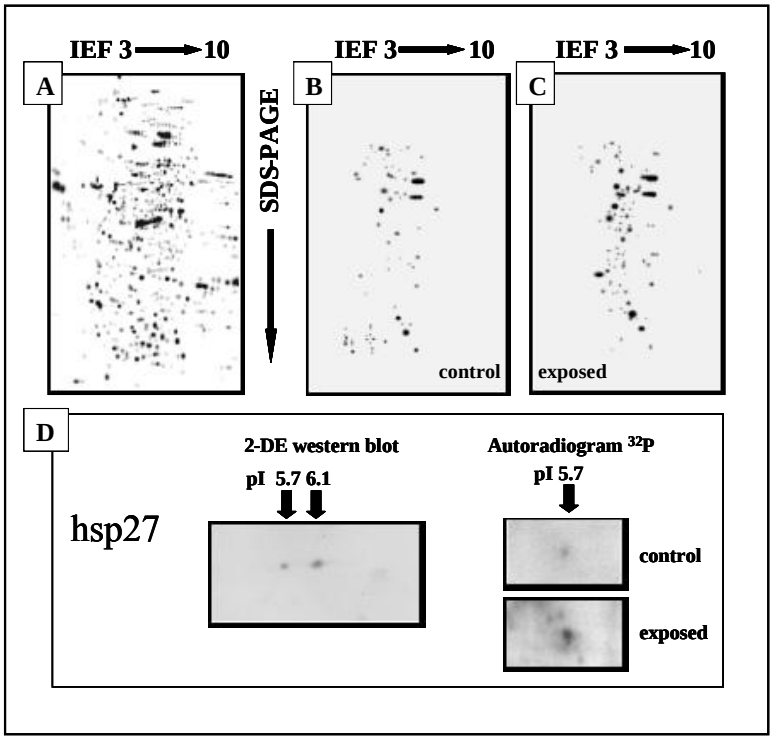


Figure 136. Identification of target molecular event for further studies – Step-1. Pattern of protein expression in EA.hy926 human endothelial cell line as determined by 2DE (panel A). Pattern of expression of ^{32}P-labeled phosphoproteins in control (panel B) and in exposed (panel C) cells. Hsp27 protein was identified, using 2DE western blot, as existing in EA.hy926 cells in two isoforms with different pI values (panel D-left). The pI 5.7 form was phosphorylated and its phosphorylation level has increased after RF-EMF exposure (panel D-right). For experimental details see Material and Methods section in Leszczynski et al. 2002.

Step-2: Validation of target molecular event

The change in phosphorylation status of hsp27 was confirmed in several ways to assure the validity of this observation (Leszczynski et al., 2002):

- immunoprecipitation of phosphorylated hsp27 (Figure 137A),
- immunoprecipitation of p38MAPK (Figure 137B), an up-stream kinase indirectly involved in phosphorylation of hsp27,
- inhibition of hsp27 phosphorylation by introduction to cell cultures of inhibitor of hsp27-up-stream kinase p38MAPK (SB203580) and determining hsp27 phosphorylation status by immunoprecipitation (Figure 137C).

Thus, in the Step-2 was confirmed that hsp27 is the valid molecular target event of the RF-EMF and that it is justified to further examine impact of this change on cell physiology.

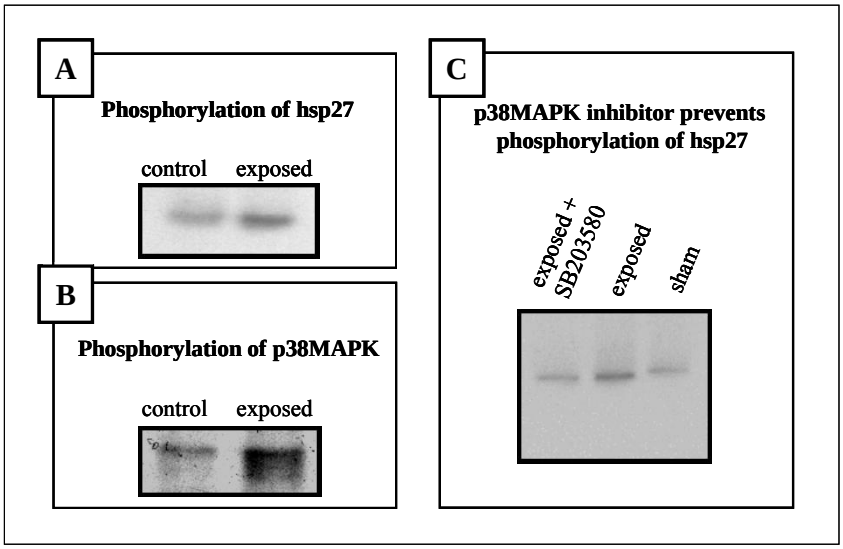


Figure 137. Validation of the target molecular event – Step-2. Increase in phosphorylation of hsp27 was confirmed by immunoprecipitation (panel A). Effect of p38MAP kinase on the RF-EMF-induced hsp27 phosphorylation was confirmed by determining, by immunoprecipitation, that p38MAPK is also activated by the RF-EMF exposure (panel B). As expected, presence of p38MAPK inhibitor, SB203580, in the culture medium during the exposure to RF-EMF, has prevented phosphorylation of hsp27 (panel C). For experimental details see Material and Methods section in Leszczynski et al. 2002.

Step-3: Cellular response – validation of the physiological event

Observed by us phosphorylation and increase in expression of hsp27 (Leszczynski et al. 2002) is a well-established mechanism of cell response to a broad variety of stress stimuli (Rogalla et al. 1999). Therefore, the observed by us doubling of Hsp27 expression and 2- to 7-fold increase in amount of phosphorylated hsp27 in cells (Leszczynski et al. 2002) have suggested that EA.hy926 cells have recognised RF-EMF as an external stress factor and that they have launched an hsp27-dependent counter response. Phosphorylation of hsp27 has been shown to regulate polymerisation of F-actin and stability of made of this protein - stress fibers (Landry and Huot 1995). Thus, we have examined status of the stress fibers in exposed cells by staining F-actin with AlexaFluor-labelled phalloidin. As shown in Figure 136A, RF-EMF exposure has caused increase in cellular staining with phalloidin what indicates increase in stability of F-actin stress fibers. The stability of stress fibers, as determined by the pattern of staining with phalloidin-AlexaFluor, increased after 1 hour irradiation and did not decline during the 1 hour of post-irradiation incubation. Induction of the stability of stress fibers caused cells to shrink and visible cell shrinking was observed among the cells brightly stained with AlexaFluor-phalloidin (Figure 138A; middle and right panels). The increase in the stability of stress fibers was prevented in the presence of p38MAPK inhibitor SB203580 (Figure 138B). Also it was possible to observe that in cells expressing high levels of hsp27 (Figure 138C), the cell edges were brightly stained with phalloidin-AlexaFluor, what indicates re-localisation of F-actin stress fibers to cell ruffles whereas in cells expressing low levels of hsp27, network of stress fibers was seen throughout the whole cytoplasm but not in the ruffles. Such behaviour of hsp27 and stress fibers in cells exposed to RF-EMF is in agreement with the general pattern of cellular response to stimuli that activate hsp27-dependent stress response (Landry and Huot 1995).

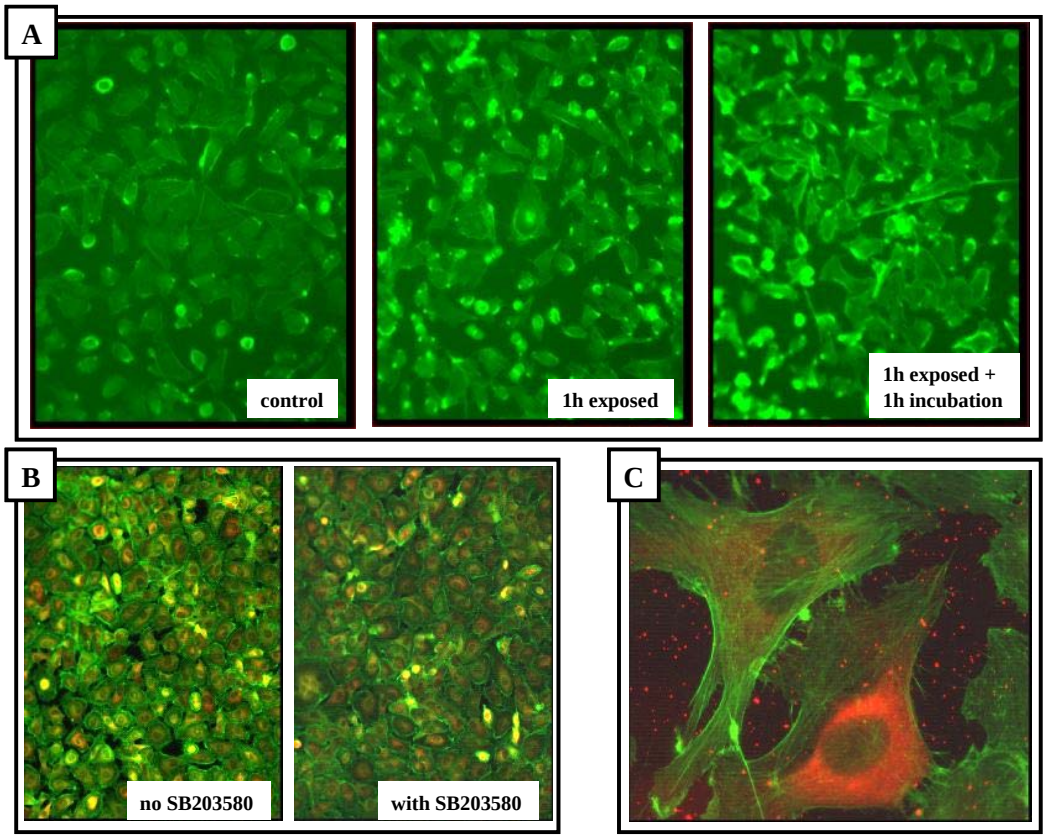


Figure 138. Cellular response to RF-EMF – validation of physiological event – Step-3.Exposure of cells to RF-EMF has caused increase in cell staining with AlexaFluor-labelled phalloidin (panel A). This suggests the increase in the expression/stability of F-actin, protein that forms cellular stress fibers. Rounding up is visible among the cells expressing highest F-actin content (the brightest staining with AlexaFluor-phalloidin). This effect persisted during the 1-hour post-exposure incubation of cells in control conditions. Presence of p38MAPK inhibitor, SB203580, in cell culture medium has prevented increase in AlexaFluor-phalloidin staining (panel B). Large magnification of cells shown in panel C demonstrates difference in distribution of AlexaFluor-phalloidin stained stress fibers (green colour) in cells with high (cell on the right) and low (cell on the left) content of hsp27 protein (indirect immunohistochemical staining; red fluorescence).

Step-4: Generation of hypothesis based on molecular and physiological events

The above results (Leszczynski et al. 2002) have formed basis and support for our working hypothesis (Figure 132, Step-4). Stabilisation of stress fibers and caused by it cell shrinking, when occurring in endothelial cells lining brain's capillary blood vessels, might be of importance for the functioning of blood-brain barrier. Stabilisation of stress fibers and cytoplasmic distribution of F-actin was previously shown to cause: (i) cell shrinkage (Landry and Huot 1995; Piotrowicz and Levin 1997a), that might lead to opening of spaces between cells, (ii) increase in the permeability and pinocytosis of endothelial monolayer (Deli et al. 1995; Lavoie et al. 1993), (iii) increase in formation of the so called "apoptosis-unrelated" blebs on the surface of endothelial cells (Becker and Ambrosio 1987), which eventually might obstruct blood flow through capillary blood vessels, (iv) stronger responsiveness of endothelial cells to estrogen and, when stimulated by this hormone, to secrete larger than normally amounts of basic fibroblast growth factor (bFGF) (Piotrowicz et al. 1997b) which might, in endocrine manner, stimulate de-differentiation and proliferation of endothelial cells and possibly led to the associated with cell's proliferative state - cell shrinkage and unveiling of basal membrane. Also, the activated (phosphorylated) hsp27 has been shown to inhibit apoptosis by forming complex with the apoptosome (complex of Apaf-1 protein, pro-caspase-9 and cytochrome *c*), or some of its components, and preventing proteolytic activation of pro-caspase-9 into active form of caspase-9 (Pandey et al. 2000; Concannon et al. 2001). This, in turn, prevents activation of pro-caspase-3 which is activated by caspase-9. Thus, induction of the

increased expression and phosphorylation of hsp27 by the RF-EMF exposure might lead to inhibition of the apoptotic pathway that involves apoptosome and caspase-3. This event, when occurring in RF-EMF exposed brain cells that underwent either spontaneous or external factor-induced transformation/damage, could support survival of the transformed/damaged cells. Therefore, based on the known cellular role of over-expressed/phosphorylated hsp27 we have proposed a hypothesis (Leszczynski et al. 2002) that: the activation (phosphorylation) of hsp27 by mobile phone radiation might be the molecular mechanism (i) regulating increase in blood-brain barrier permeability, which would explain, observed in some animal experiments, increase in blood-brain barrier permeability, and (ii) regulating apoptosis through interference with the cytochrome c/caspase-9/caspase-3 pathway (Figure 139). Thus, it is possible that the RF-EMF might have effect on cytoskeleton-related and on the apoptosis-related cell functions. This notion supports and justifies further examination of cytoskeleton and apoptosis related properties of RF-EMF exposed endothelial cells.

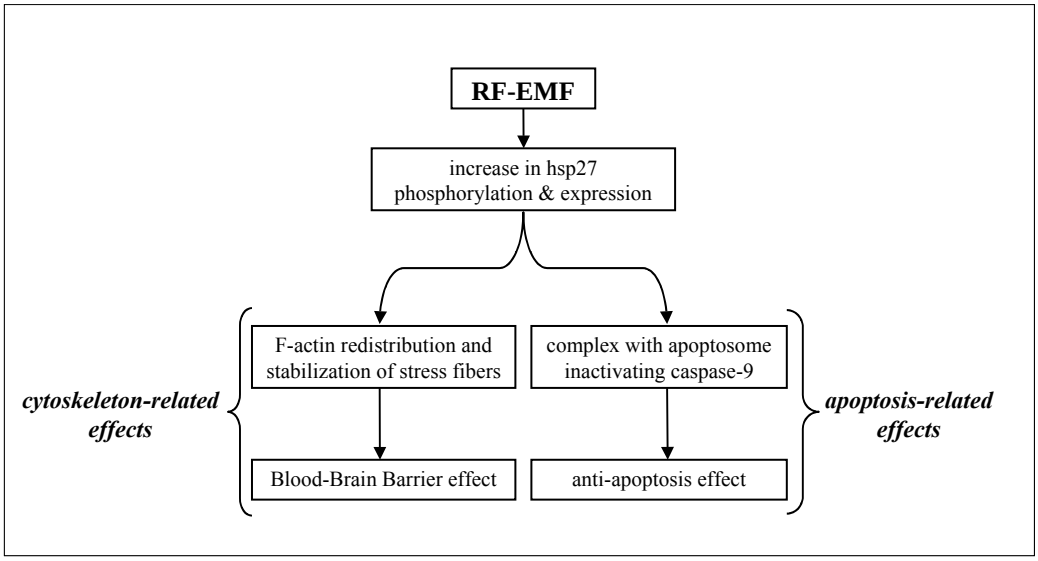


Figure 139. Hypothesis based on the molecular and physiological events – Step-4. Based on the known functions of hsp27 we have proposed that RF-EMF induced hsp27 phosphorylation might affect cell cytoskeleton and cell apoptosis. For full scheme and description of hypothesis see reference Leszczynski et al. 2002.

Step-5: HTST-identification of new target events, with support of hypothesis

Further experiments using HTST have revealed additional information pertinent to the cytoskeleton and apoptosis related properties of RF-EMF exposed endothelial cells.

The suggested changes in the cytoskeletal proteins were detected using 2-DE separated proteins. Approximately 1300 protein spots were detected 2-DE. Comparison of the control and exposed samples revealed some 49 protein spots which were statistically significantly (student T-test, $p<0.05$, $n=10$) affected by the exposure (increased or declined expression). Few of the spots were selected for the mass spectrometry identification using the following criteria: spots needed to be (i) enough separate from the adjacent spots, (ii) sufficiently large and (iii) well focused in all dimensions. Cytoskeletal proteins vimentins (Figure 140) and tubulin (not shown) were identified by mass spectrometry among the proteins that responded to RF-EMF. The suggested interference with apoptosis was further examined using cDNA Expression Arrays (Clontech) and screening expression of 3600 different genes. Among the genes that were down-regulated in cells exposed to RF-EMF were numerous genes encoding proteins of Fas/TNFα-apoptotic pathway (Figure 141). This pathway was suggested as target for the RF-EMF induced phosphorylated hsp27. Therefore, concomitantly observed increase in hsp27 phosphorylation, that is anti-apoptotic event, and down-regulation of proteins of Fas/TNFα apoptotic pathway suggest that further studies aiming at elucidation of RF-EMF effect on cell apoptosis are justified.

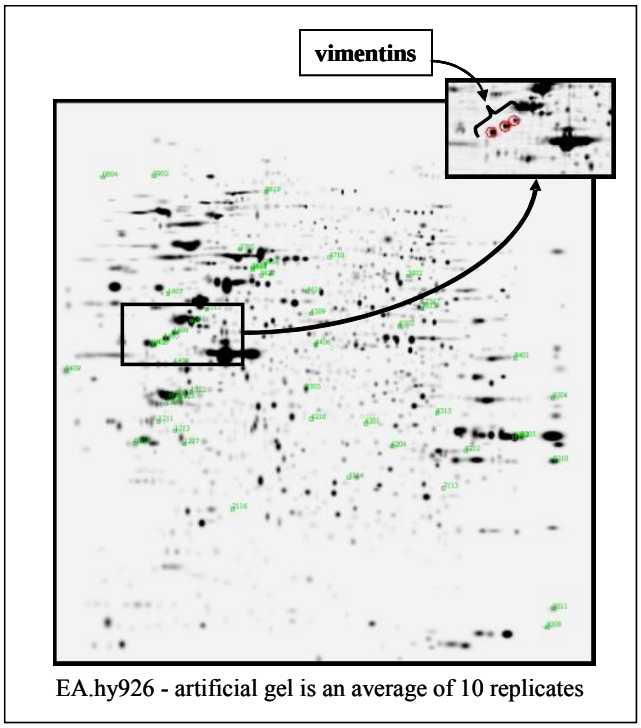


Figure 140. HTST-identification of new target events – cytoskeleton-related – Step-5. Using larger 2-DE gels (20x20 cm) and 10 replicates of each run we have identified some 49 proteins that, in statistically significant fashion, have altered their expression following RF-EMF exposure. Among the mass spectrometry identified spots were cytoskeletal proteins vimentins (inset) and tubulin (not shown).

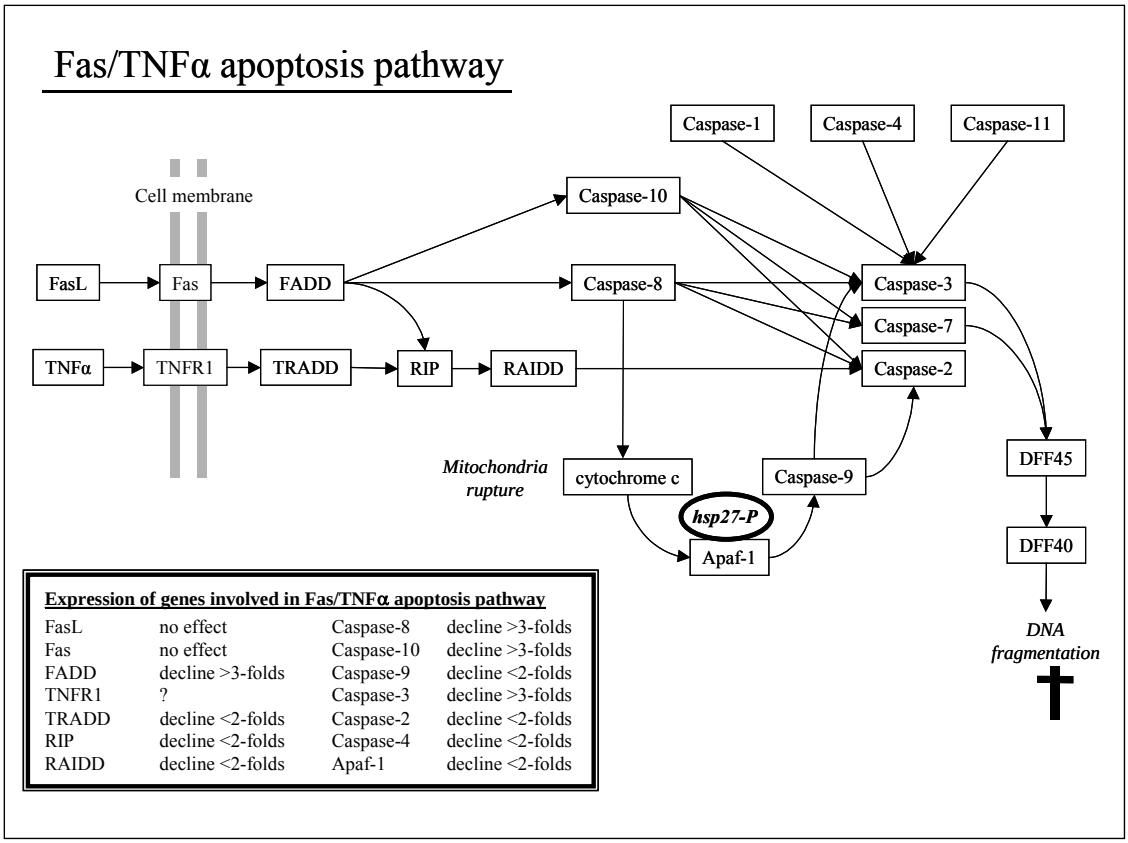


Figure 141. HTST-identification of new target events – apoptosis-related – Step-5. Analysis of RF-EMF-induced expression changes, using cDNA Expression Array for 3600 tumour-related genes, has revealed that the majority of genes that encode proteins forming Fas/TNFα apoptotic pathway are down-regulated (Table inset).

B. Genotype-dependent cell response to 900 MHz GSM radiation

We have compared response to mobile phone radiation of two human endothelial cell lines: fast proliferating EA.hy926 (Edgell et al., 1983) and its slow proliferating variant EA.hy926v1 (derived by sub-cloning from the EA.hy926 cell line).

Proteomics approach

Using 2-DE and MALDI-MS proteomics approach we have determined what proteins respond to the mobile phone radiation. Using PDQuest 6.2 software (Bio-Rad, UK) the 2-DE artificial gels (Figure 135) were generated from 10 independent protein samples from ten independent replicates of controls and irradiated cell cultures. The protein expression pattern in ten replicate control samples was then compared with the protein pattern in ten replicate irradiated samples. The normalised spot volumes of the proteins from control and exposed sample gels were statistically analysed using student t-test at the confidence level of 95%. The most striking observation was that the comparison in-between the two cell lines showed that their protein expression patterns are very different in spite of the closely related origin of both cell lines (Figures 142A, 142B). Only approximately half of all of the protein spots could be matched confidently between the cell lines. This difference in protein expression pattern might explain the observed differences in the growth rate between the cell lines. Because of the observed differences in the protein expression and proliferation between the cell lines, it was not a surprise that the response to the mobile phone radiation also varied between EA.hy926 and EA.hy926v1 cell lines. The comparison of the exposed and control samples has shown several tens of protein spots with radiation-induced statistically significant change in expression levels (t-test $p<0.05$). In EA.hy926 cell line there were 38 of protein spots which expression was altered by the radiation exposure (Figure 142A) whereas in EA.hy926v1 cell line there were 45 differentially expressed protein spots (Figure 142B). The identity of

the all radiation-responding protein spots is being determined by MALDI-MS and will be reported in due time.

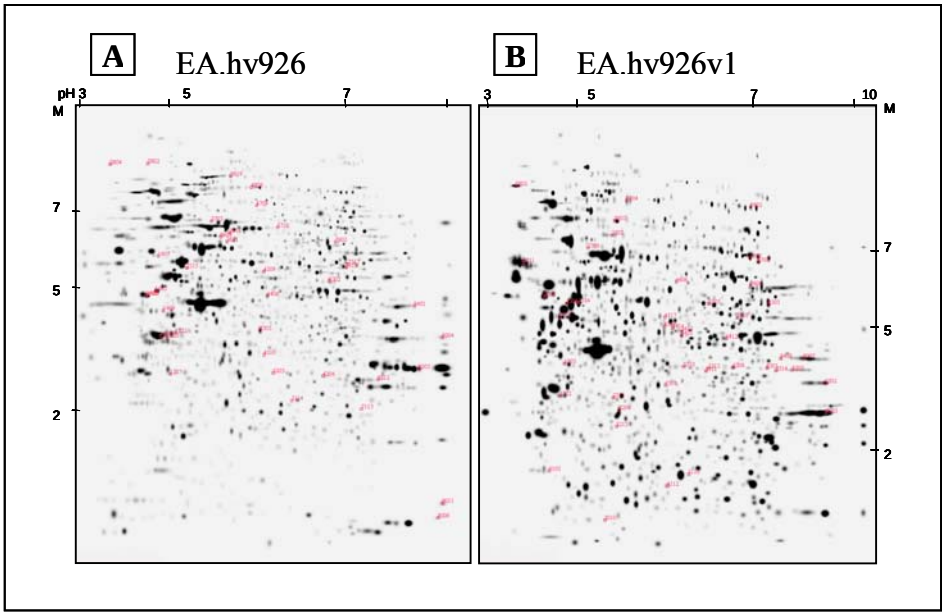


Figure 142. 2-DE gels of proteins extracted from human endothelial cell lines; EA.hy926 (A) and EA.hy926v1 (B). The 1st dimension IEF using pH gradient 3-10 NL, 2nd dimension 8% SDS-PAGE. Statistically significantly (t-test $p<0.05$) differing spots in the cell lines are numbered using PDQuest SSP numbers.

(A) EA.hy926 cell line - 38 statistically significantly differing spots. Four spots: vimentin (1402 and 1405), isocitrate dehydrogenase 3 (NAD+) alpha (4305), and heterogeneous nuclear ribonucleoprotein H1 (4406), were identified using mass spectrometry.

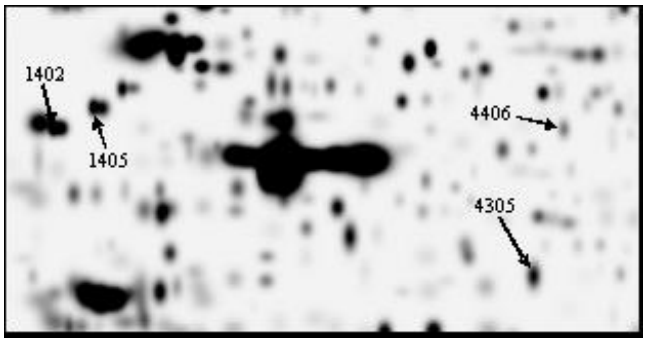
(B) EA.hy926v1 cell line; 45 statistically significantly differing spots.

Few of the protein spots, which expression was statistically significantly altered by the irradiation, were identified using MALDI-MS (Figure 143). In order to increase probability of a single protein present in the single spot, the protein spots that were selected for MALDI-MS analysis had to fulfil the following requirements: (i) spots were well separated from other spots in both 2-DE dimensions, (ii) spots were sufficiently large (Figure 143A). The MALDI-MS analysis service was purchased from the Protein Chemistry Laboratory of the Institute of Biotechnology at the Helsinki University, Finland. The selected spots were reduced with DTT and alkylated with iodoacetamide before overnight digestion with a sequence-grade modified trypsin (Roche, France). The peptide mixture was concentrated and desalted using Millipore ZipTip™ μ-C18 pipette tips. The peptide mass fingerprints were measured with Bruker Biflex™ MALDI-ToF mass spectrometer in a positive ion reflector mode using α-cyano-4-hydroxycinnamic acid as a matrix. The database searches were performed using ProFound and Mascot searches. The protein spots that were identified with MALDI-MS were as follows (Figure 143):

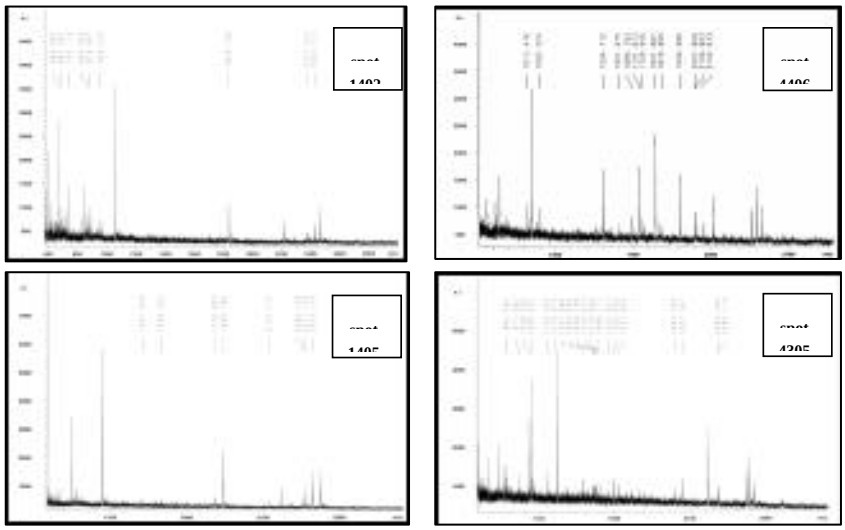
- Protein spot 4305 - isocitrate dehydrogenase 3 (NAD+) alpha (Kim et al. 1995) is a subunit of the mitochondrial enzyme, which catalyses the conversion of isocitrate to 2-oxoglutarate in the citric acid cycle. The expression level of this protein was moderately down-regulated in the exposed samples having a ratio exposed vs. control 0.72 with the p-value of 0.03. The down-regulation of this protein might affect cellular energy production.
- Protein spot 4406 - heterogeneous ribonucleoprotein H1 (Honore et al. 1995) is a component of the heterogeneous nuclear ribonucleoprotein (HNRNP) complexes which provide a substrate for the processing events which pre-mRNAs go through before becoming functional mRNAs in the cytoplasm. The expression level of this protein is slightly down-regulated in the exposed samples with a ratio exposed vs. control 0.61 with the p-value 0.03. The potential down-regulation of this protein might affect protein translation process.

- Protein spots 1402 and 1405 - vimentin (Ferrari et al. 1986) is a protein component of class III-intermediate filaments. In EA.hy926 cells it was found to be expressed in at least two different iso-forms differing in molecular weight and isoelectric point. Both vimentin iso-forms were up-regulated; spot 1402 (experimental MW/pI ca. 47 kDa/4.4) by 2.5-fold with p-value of 0.006 and spot 1405 (experimental MW/pI ca. 48kDa/4.8) by 2.2-fold with p-value of 0.02.

A



B



C

SSP	Protein name	Swiss-prot ID code	Sequence coverage	Swiss-prot annotations
4305	Isocitrate dehydrogenase 3 (NAD+) alpha	P50213	21%	component of enzyme complex catalysing conversion of isocitrate to 2-oxoglutarate
4406	Heterogeneous nuclear ribunocleoprote in H1	P31943	34%	component of HNRNP complex providing a substrate for pre-mRNA processing
1402	vimentin	P08670	19%	class III-intermediate filament
1405	vimentin	P08670	14%	class III-intermediate filament

Figure 143. (A) Fragment of the 2-DE gel of EA.hy926 cells with marked spots that were identified with MALDI-MS. (B) MALDI-MS spectra showing peptide finger prints of the four identified protein spots. (C) Table summarising the properties of the identified proteins**.**

Alterations in the vimentin expression suggest that some form of cytoskeletal response might be taking place in cells exposed to the mobile phone radiation. This notion agrees with our earlier observation of the effect of the mobile phone radiation on the stability of F-actin stress fibers (Leszczynski et al. 2002; Leszczynski et al. 2004). Changes in the vimentin expression observed in 2-DE were further confirmed by SDS-PAGE and western blotting and by cell staining using indirect immunofluorescence method. For SDS-PAGE/western blotting a standard protocol was used. Briefly, the cell lysates were separated using 7.5% SDS-PAGE, blotted to PVDF-membrane, blocked with 5% non-fat dry milk and exposed to the primary vimentin antibody (Zymed, USA) and the secondary antibody containing a HRP-conjugate (Dako, Denmark). The signal was detected using enhanced chemiluminescence (Pierce, UK). For immunocytochemistry cell were fixed in 3% paraformaldehyde, membranes were permeabilised in 0.5% Triton X-100 and as a primary antibody was used vimentin antibody (Zymed, USA) and the secondary antibody was TRICT-conjugated (Dako, Denmark). The images were captured using a Leitz fluorescence microscope and computerised image acquisition system (Metafer, Germany).

SDS-PAGE and western blot have confirmed that EA.hy926 cells express two iso-forms of vimentin. The higher molecular weight form (experimental MW ca. 57 kDa) was present both in control and in irradiated cells and its expression was not affected by the irradiation (Figure 144A). The lower molecular weight vimentin (experimental MW ca. 48 kDa) was not detectable in the non-irradiated cells but was expressed in the irradiated cells (Figure 144B). Indirect immunohistochemistry staining of vimentin has shown the change in the distribution pattern of the vimentin filaments after the exposure to the mobile phone radiation (Figure 143C, 143D). Together, the observed changes in the vimentin expression suggest that the mobile phone radiation might potentially alter cell physiology by affecting cellular cytoskeleton.

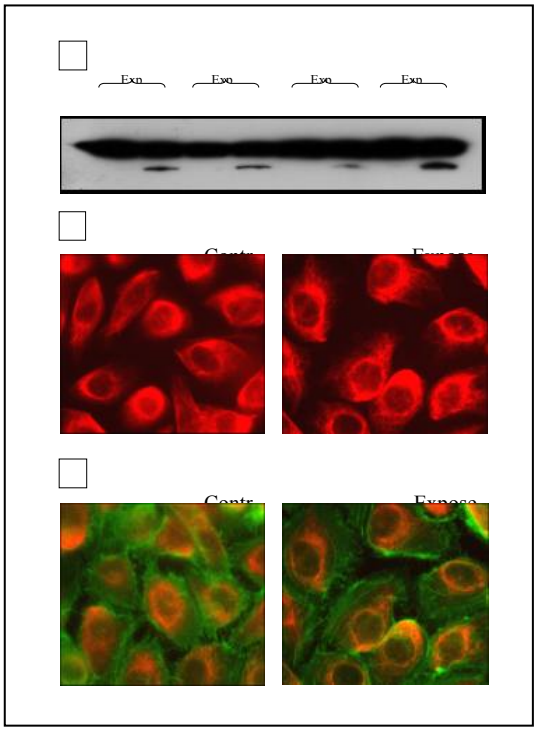


Figure 144. (A) Western blot-detected expression of vimentin in non-irradiated EA.hy926 cells (C-lanes) and in exposed cells (RF-lanes). MW ca. 57 kDa and ca. 48 kDa. Four separate experiments are shown. (B) Immunostaining of vimentin in non-exposed and in exposed EA.hy926; red colour – vimentin. Note diffuse-like staining for vimentin in non-exposed cells as compared with more filament-like expression in exposed cells.(C) Immunostaining of vimentin (red colour) and F-actin stress fibers (green colour) in non-exposed and in exposed EA.hy926. F-actin was detected with phalloidin-AlexaFluor. Note diffuse-like staining for both vimentin and F-actin in non-exposed cells as compared with more filament-like expression in exposed cells.

Transcriptomics approach

Using cDNA Expression arrays (Clontech, USA) we have determined that number of genes increased/declined expression in both cell lines following the exposure to mobile phone radiation (900 MHz GSM). Most strikingly, genes that were up regulated in one of the cell lines were down-regulated or not affected in the other cell line (Tables 29, 30, and 31). It suggests that the cell response might depend on the genotype.

Table 29. Genes which were up-regulated in EA.hy926 cell line following to the exposure to the 900 MHz GSM.

Gene name	**EA.hy926 (*fast proliferating*)**		**EA.hy926v1 (*slow proliferating*)**	
Increased expression in EA.hy926	**Ratio RF/sham**	**Difference RF-sham**	**Ratio RF/sham**	**Difference RF-sham**
proliferating cell nucleolar antigen P120; NOL1	**29,71**	**18734**	**0,38**	**-6343**
Homo sapiens mRNA for beta 2-microglobulin	**14,70**	**3895**	**0,50**	**-5312**
MCM7 DNA replication licensing factor; CDC47 homolog; p1.1-MCM3	**12,18**	**9029**	**0,98**	**-172**
zinc-finger protein (ZNFPT7) (fragment).	**8,43**	**15053**	**0,46**	**-2512**
chloride conductance regulatory protein ICLN; nucleotide-sensitive chloride channel 1A; chloride ion current inducer protein (CLCI); reticulocyte PICLN	**5,37**	**16944**	**0,79**	**-1686**
HHR23A; UV excision repair protein protein RAD23A	**4,42**	**20499**	**0,66**	**-7485**
ferritin heavy chain (FTH1); FTHL6	**4,30**	**6961**	**0,93**	**-2435**
CD166 antigen precursor (activated leukocyte-cell adhesion molecule) (ALCAM).	**4,09**	**3184**	**0,70**	**-2869**
nucleolar phosphoprotein B23; nucleophosmin (NPM); numatrin	**3,89**	**50540**	**0,97**	**-1699**
annexin IV (ANX4); lipocortin I; calpactin II; chromobindin 9; phospholipase A2 inhibitory protein	**3,68**	**7176**	**4,20**	**15254**
N4-(beta-N-acetylglucosaminyl)-L-asparaginase precursor (EC 3.5.1.26) (glycosylasparaginase) (aspartylglucosaminidase) (N4-(N-acetyl-beta-glucosaminyl)-L-asparagine amidase) (AGA).	**3,63**	**13266**	**0,55**	**-1060**
glial growth factor 2 precursor (GGFHPP2); neuregulin; heregulin-beta3 + neu differentiation factor + heregulin-alpha	**3,53**	**21908**	**0,70**	**-3070**
serine/threonine protein phosphatase PP1-alpha 1 catalytic subunit (PP-1A)	**3,31**	**7521**	**1,08**	**1402**
flavin reductase (EC 1.6.99.1) (FR) (NADPH-dependent diaphorase) (NADPH-flavin reductase) (FLR) (biliverdin reductase B) (EC 1.3.1.24) (BVR-B) (biliverdin-IX beta-reductase) (green heme binding protein) (GHBP)	**3,17**	**15395**	**0,77**	**-2827**
cytochrome c	**2,97**	**12337**	**0,82**	**-2047**
DNA-directed RNA polymerase II 19 kD polypeptide (EC 2.7.7.6) (RPB7).	**2,88**	**12043**	**0,88**	**-1360**

Table 30. Genes which were down-regulated in EA.hy926 cell line following to the exposure to 900 MHz GSM.

Gene name	EA.hy926 (*fast proliferating*)		EA.hy926v1 (*slow proliferating*)	
Decreased expression in EA.hy926	**Ratio RF/sham**	**Difference RF-sham**	**Ratio RF/sham**	**Difference RF-sham**
pyruvate kinase M2 isozyme (PKM2)	**0,33**	**-6273**	**0,90**	**-2490**
RAD51-interacting protein	**0,31**	**-3405**	**0,79**	**-510**
glutathione S-transferase mu1 (GSTM1; GST1); HB subunit 4; GTH4	**0,29**	**-3486**	**1,59**	**599**
glutathione S-transferase A1 (GTH1; GSTA1); HA subunit 1; GST-epsilon	**0,29**	**-4095**	**0,36**	**-2545**
early growth response protein 1 (hEGR1); transcription factor ETR103; KROX24; zinc finger protein 225; AT225	**0,28**	**-4223**	**0,54**	**-1932**
caspase-3 (CASP3); apopain precursor; cysteine protease CPP32; YAMA protein; SREBP cleavage activity 1; SCA-1	**0,27**	**-5844**	**1,04**	**79**
calpain 2 large (catalytic) subunit; M-type calcium-activated neutral proteinase (CANP)	**0,27**	**-3464**	**0,95**	**-1210**
ras-related C3 botulinum toxin substrate 1; p21-rac1; ras-like protein TC25	**0,26**	**-6905**	**1,10**	**730**
alpha-actinin 1 cytoskeletal isoform; F-actin cross linking protein	**0,25**	**-5276**	**0,82**	**-2597**
ras-related protein RAB-11B; YPT3	**0,24**	**-2870**	**0,79**	**-3643**
DNA ligase I; polydeoxyribonucleotide synthase (ATP) (DNL1) (LIG1)	**0,22**	**-2969**	**0,38**	**-2497**
ATP synthase lipid-binding protein P2 precursor (EC 3.6.1.34) (ATPase protein 9) (subunit C)	**0,19**	**-4520**	**0,80**	**-1486**
EDF-1 protein	**0,14**	**-6951**	**0,68**	**-2931**
coatomer delta subunit; delta-coat protein; delta-COP; archain (ARCN1)	**0,14**	**-4280**	**n/a**	**n/a**
nuclear transport factor 2 (NTF-2) (placental protein 15) (PP15).	**0,14**	**-2836**	**0,65**	**-2155**
fascin (actin bundling protein).	**0,14**	**-16072**	**0,54**	**-6852**
neurogranin (NRGN); RC3	**0,11**	**-18728**	**0,86**	**-2175**
MYLE	**0,11**	**-4959**	**0,81**	**-539**
sepiapterin reductase (EC 1.1.1.153) (SPR).	**0,10**	**-3467**	**0,63**	**-1123**
caspase-8 precursor (CASP8); ICE-like apoptotic protease 5 (ICE-LAP5); MORT1-associated CED-3 homolog (MACH); FADD-homologous ICE/CED-3-like protease (FADD-like ICE; FLICE); apoptotic cysteine protease MCH-5	**0,05**	**-3359**	**1,90**	**1080**

Table 31. Genes which were affected in EA.hy926v1 cell line following to the exposure to 900 MHz GSM.

Gene name	EA.hy926v1 *(slow proliferating)*		EA.hy926 *(fast proliferating)*	
Increased expression in EA.hy926v1	**Ratio RF/sham**	**Difference RF-sham**	**Ratio RF/sham**	**Difference RF-sham**
procollagen C-proteinase enhancer protein precursor.	**9,58**	**10373**	**1,09**	**5589**
HOMER-3.	**9,45**	**3027**	**0,98**	**-25**
T-lymphoma invasion and metastasis inducing TIAM1	**5,55**	**6554**	**0,01**	**-1335**
elafin precursor (elastase-specific inhibitor) (ESI) (skin-derived antileukoproteinase) (SKALP).	**5,33**	**10720**	**1,53**	**12919**
mitochondrial matrix protein P1 precursor; p60 lymphocyte protein; chaperonin homolog; HUCHA60; heat shock protein 60 (HSP-60); HSPD1	**4,43**	**8459**	**1,00**	**-89**
proteasome component C8; macropain subunit C8; multicatalytic endopeptidase complex subunit C8	**4,06**	**5029**	**1,10**	**5254**
special AT-rich sequence binding protein 1 (SATB1); MAR/SAR DNA-binding protein	**3,48**	**11151**	**0,99**	**-222**
HLA class I histocompatibility antigen C-4 alpha subunit (HLAC)	**3,12**	**2805**	**0,64**	**-1344**
ras-related protein RAP-1B; GTP-binding protein SMG p21B	**3,04**	**4820**	**63,80**	**2512**
phospholipase A2; tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ); 14-3-3 protein zeta/delta; protein kinase C inhibitor protein 1 (KCIP1); factor activating exoenzyme S (FAS)	**2,93**	**4033**	**1,14**	**767**
Decreased expression in EA.hy926v1				
tuberin; tuberous sclerosis 2 protein (TSC2)	**0,33**	**-7082**	**0,42**	**-16846**
KIAA0115; dolichyl-diphosphooligosaccharide protein glycosyltransferase 48-kDa subunit precursor; oligosaccharyl transferase 48-kDa subunit; HA0643	**0,27**	**-3494**	**0,19**	**-1968**
sodium channel beta-1 subunit precursor (SCN1B)	**0,27**	**-2842**	**0,00**	**-1846**
embryonic growth/differentiation factor 1 (GDF1) + UOG-1	**0,21**	**-3542**	**0,00**	**-702**
SH3P18 SH3 domain-containing protein	**0,05**	**-3389**	**0,00**	**-949**

C. Comparison of the effect of CW and modulated RF-EMF on protein expression

Using cICAT method combined with liquid-phase chromatography and mass spectrometry we have compared protein expression changes in cells exposed either to continuous wave or to radiofrequency modulated ("talk" signal) RF-EMF (1800 MHz GSM). The cICAT reagent labelled samples were analysed using an automated mass spectrometric approach in which those proteins showing abundance differences between the two conditions being compared were selectively identified. In total, 58 unique proteins were identified and determined to show significant changes in abundance using this approach. These proteins were selected for identification by MS/MS analysis based upon the criteria that the measured abundance ratios ($C^{13}(0)/C^{13}(9)$) were either >1.7 or <0.60. The average abundance ratio for all detected cICAT reagent labelled peptide pairs (n=1476) was 1.26 +/-0.38, indicating that the vast majority of proteins within the two samples did not change in abundance. Peptides detected as singlets (i.e. having no corresponding $C^{13}(0)$ or $C^{13}(9)$ signal) were also selected for MS/MS analysis. The threshold abundance ratio values were selected based on the following criteria: 1) In relation to previously described errors of quantitative measurements using the ICAT reagent, these values represent conservative estimates of significant abundance changes; 2) These values are significant outliers relative to the average and median $C^{13}(0)/C^{13}(9)$ values for this dataset. The average $C^{13}(0)/C^{13}(9)$ value for all detected cICAT reagent labelled peptides (n = 1476) was 1.26 +/-0.38, and the median was 1.19. The threshold values for significant changes in abundance are therefore well outside the standard deviation for this dataset. Furthermore, the fact that the average and median values are close to one indicates the accuracy of the quantitative measurements used here, as it is expected that the majority of proteins will be constitutively represented, giving ratios close to one.

In conclusion it appears that the "talk" signal has caused increase in expression of a variety of proteins whereas CW did not (Table 32). It suggests that the modulation might have impact on cell response to RF-EMF.

Table 32. List of proteins induced by “talk” signal but not by the CW signal.

Protein name	Accession No.	$C^{13}(0)/C^{13}(9)$	Confidence score
serine-threonine protein kinase	NP_055212	1.9	0.996
GP:AF090929_1		1.8	0.912
RING finger protein 20	AAK58539	2.0	0.958
Homo sapiens cDNA FLJ20303	AK000310	2.4	0.957
hypothetical protein FLJ20420	NP_060282	2.0	0.984
fatty-acid synthase	PIR:G01880	2.2	0.993
hypothetical protein DKFZp434G171.1	T42678	1.9	0.855
hypothetical protein DKFZp564N1563.1 (2)	T46270	0.5	0.921
Serine/threonine protein phosphatase 2A	Q15173	1.8	0.950
Beta-adaptin 1	Q10567	2.0	0.813
Actin-like protein 2	O15142	2.0	0.993
Sarcoplasmic/endoplasmic reticulum calcium ATPase 2 (2)	P16615	1.9	0.944
CD59 glycoprotein precursor (6)	P13987	2.4	0.995
Chloride intracellular channel protein 1	O00299	2.7	0.997
Cellular nucleic acid binding protein (2)	P20694	2.2	0.980
Cofilin, non-muscle isoform	P23528	1.7	0.990
Coatomer alpha subunit	P53621	2.4	0.997
Coatomer beta subunit	P53618	2.0	0.993
Cleavage and polyadenylation specificity factor	Q9P2I0	1.9	0.994
Cyclophilin A (3)	P05092	2.2	0.975
Destrin (Actin-depolymerizing factor)	P18282	1.7	0.990
Aspartyl aminopeptidase	Q9ULA0	1.8	0.993
D-dopachrome tautomerase	P30046	1.7	0.952
Elongation factor 2	P13639	2.0	0.982
Alpha enolase	P06733	2.1	0.981
Fatty acid synthase (2)	P49327	2.1	0.975
Filamin A (2)	P21333	2.5	0.975
FL cytokine receptor precursor	P36888	1.9	0.970
Follistatin-related protein 1 precursor Q12841	Q12841	1.7	0.942
PROTEIN KINASE C SUBSTRATE	P14314	1.7	0.984
Transducin beta chain 1	P04901	1.9	0.993
Transducin beta chain 2	P11016	1.8	0.964
Guanine nucleotide-binding protein beta subunit-like protein (3)	P25388	2.9	0.998
Stress-induced-phosphoprotein 1	P31948	2.2	0.993
Pyruvate kinase, M1 isozyme	P14618	1.9	0.983
LAM2_HUMAN, partial CDS	AAC34573	0.6	0.994
Galectin-1 (3)	P09382	1.9	0.996
Myosin heavy chain, nonmuscle type A	P35579	0.6	0.996
Myoferlin (2)	Q9NZM1	2.9	0.940
NHP2-like protein 1 (3)	P55769	3.6	0.980
Nitric-oxide synthase, endothelial (2)	P29474	2.0	0.990
Purine nucleoside phosphorylase (2)	P00491	1.9	0.997
40S ribosomal protein S27a	P14798	3.0	0.974
Heterogeneous nuclear ribonucleoprotein D0	Q14103	3.0	0.984
Heterogeneous nuclear ribonucleoprotein K	Q07244	2.6	0.880
Heterogenous nuclear ribonucleoprotein U	Q00839	2.1	0.992
ribosomal protein S2 (2)	P15880	1.8	0.940
40S ribosomal protein S3a	P49241	2.1	0.991
Splicing factor, arginine/serine-rich 9	Q13242	2.0	0.986
Tubulin beta-2 chain	P05217	2.8	0.998
T-complex protein 1, theta subunit (2)	P50990	4.9	0.995
Transcription intermediary factor 1-beta	Q13263	2.3	0.995
Thioredoxin	P10599	2.1	0.935
Hypothetical UPF0123 protein BK223H9.2	Q9UH06	2.1	0.994
Splicing factor U2AF 35 kDa subunit (2)	Q01081	2.4	0.945
Ubiquitin-activating enzyme E1	P22314	1.8	0.963
Zinc finger protein 147	Q14258	3.7	0.995
Nuclear pore complex protein Nup133	Q8WUM0	2.3	0.914

3.2.4.7 Whole-genome analysis of various cell lines exposed to RF-EMF (Participant 12)

Altogether, 58 whole-genome analyses of 10 different cell lines (sham-exposed cells and control cells) were performed (Table 1). After primary data analysis, we only worked on genes which were reproducibly regulated in several experiments (see materials and methods) and which belonged to certain gene families (Table 33). We defined gene families which are potentially relevant for the cellular answer on EMF exposure: signal transduction, ion/electron transport, metabolism of energy/proteins, cell proliferation/apoptosis, immune answer/inflammation and extracellular matrix/cytoskeleton. Each gene family was sub-divided in subgroups again, e.g. GTP proteins in the signal transduction family (Tables 33, 11). In a first step, we did not go into single genes, but simply counted genes up- or down-regulated in the different gene families. The total number of regulated genes in a certain gene family is not very meaningful, because the sizes of the gene families are of course very different. Therefore, the total numbers of genes on the human array belonging to a gene family are shown in the first column of Tables 33 and 11. Although a single gene might appear in different categories (e.g. all small G proteins are GTP binding proteins), the tables give a good overview on what might happen in the cells after EMF exposure on the molecular level.

Although appearing regulated in all experiments, mitochondrial genes, ribosomal genes and cell cycle genes especially showed a high rate of regulation in the some RF-EMF experiments (U937 human monocytic cells and microglia cells, Participant 9; HL-60 human hematopoietic leukemia cells, Participant 2).

Moreover, the bio-statistical analysis of RF-EMF experiments (Participant 8, Dr. Remondini) allowed some interesting conclusions from the experiments with HL-60 cells (Participant 2), endothelial cells (Participant 6) and U937 cells (Participant 8, Table 34). Again, the regulation of mitochondrial and ribosomal genes was evident with this analysis. Most of the regulated genes in endothelial cells appear in the groups of ATP-associated genes (energy metabolism), transcription, and cytoskeleton. Remarkably, compared to ELF-EMF experiments, we find more up-regulated genes in RF-EMF experiments (Table 34, see also Table 12). However, the results do have to be interpreted in more detail, because down-regulation of a special gene does not mean that the respective process is down-regulated as well (for example, down-regulation of Bcl-2 might lead to up-regulation of apoptosis).

For T-lymphocytes (Participant 8) and microglia cells (Participant 9) the bio-statisitical analysis did not reveal significant data.

In detail, the following genes were extracted by bio-statistics so far:

Actin associated proteins (belong to cytoskeleton):
- Caldesmon (tropomysin binding, actin binding. Activation of ERK MAP kinases lead to phosphorylation of caldesmon. Regulatory protein of the contractile apparatus): down-regulated (endothelial cells, Participant 6).
- Gamma-actin: down-regulated ((endothelial cells, Participant 6, and U937 cells, Participant 8)
- “coactosin-like”: down-regulated (endothelial cells, Participant 6)
- “actin-binding”: down-regulated (endothelial cells, Participant 6)
- “procollagen-proline 2”: down-regulated (endothelial cells, Participant 6)
- “actin modulating activity”: up-regulated (endothelial cells, Participant 6)
- “actin-binding, calcium ion binding”: down-regulated (endothelial cells, Participant 6)
- CD2-associated protein, actin binding: down-regulated (endothelial cells, Participant 6)
- Tropomodulin 3: actin binding down-regulated (endothelial cells, Participant 6)

Calcium (Ca2+)-associated proteins:
- Ca: “hypothetical protein” (actin-binding): down-regulated (endothelial cells, Participant 6)
- “hypothetical protein”: down-regulated (endothelial cells, Participant 6)
- voltage-gated Ca channel: up-regulated (perhaps up-regulated, because Ca goes down? Endothelial cells, Participant 6)

Cytoskeleton (compare also actin and calcium-associated proteins):
- “hypothetical protein”: down-regulated (endothelial cells, Participant 6)
- “protein phosphatase 4, caldesmon): down-regulated (endothelial cells, Participant 6)
- “SH3 protein interacting with Nck”: down-regulated (endothelial cells, Participant 6)
- “in kinesin complex”: down-regulated (endothelial cells, Participant 6)

Table 33. Numbers of genes regulated within different gene families

Gene Family	total number of clones in Human Unigene RZPD-2	partner 8 U937 monocytes RF up-regulated genes	partner 8 microglia cells RF up-regulated genes	partner 8 U937 monocytes RF down-regulated genes	partner 8 microglia cells RF down-regulated genes	partner 6 endothelial cellsPr1 RF 900MHz up-regulated genes	partner 6 endothelial cells RF 1800 MHz Exp1 up-regulated genes	partner 6 endothelial cells RF 1800 MHz Exp2 up-regulated genes	partner 6 endothelial cellsPr1 RF 900MHz down-regulated genes	partner 6 endothelial cells RF 1800 MHz Exp2 down-regulated genes	partner 6 endothelial cells RF 1800 MHz Exp2 down-regulated genes	partner 2 HL-60 RF ON/OFF up-regulated genes	partner 2 HL-60 RF continuous waves Exp1 up-regulated genes	partner 2 HL-60 RF continuous waves Exp2 up-regulated genes	partner 2 HL-60 RF ON/OFF down-regulated genes	partner 2 HL-60 RF continuous waves Exp2 down-regulated genes	partner 2 HL-60 RF continuous waves Exp2 down-regulated genes	Gene "Superfamily"
Signal	2528	149	40	##	45	190	176	92	160	149	149	91	153	162	190	155	72	signal transduction
GTP	560	37	15	45	14	42	58	24	49	40	51	20	43	51	49	38	18	signal transduction
Small G	235	16	5	20	3	14	17	10	23	15	19	8	17	18	21	18	7	signal transduction
Jak	23	2	0	1	1	1	4	2	1	4	0	1	4	2	1	1	0	signal transduction
Rab	80	3	1	11	3	6	6	4	6	4	9	0	5	5	6	3	5	signal transduction
Ras	66	4	2	7	0	4	4	1	6	4	7	2	6	6	7	2	2	signal transduction
wnt	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	signal transduction
phosphatase	334	24	6	21	7	24	31	17	23	20	25	19	18	26	19	29	9	signal transduction
protein kinase	304	19	6	19	4	19	24	16	27	15	16	18	16	25	23	11	13	signal transduction
phospholipase	72	6	1	7	1	6	4	5	1	2	4	1	3	7	7	5	2	signal transduction
calcium	715	40	6	39	14	56	51	27	45	30	45	20	45	44	45	35	13	signal transduction
calmodulin	131	8	2	6	1	8	6	1	11	8	9	6	5	8	11	10	4	signal transduction
channel	348	12	1	18	7	12	12	8	16	12	11	12	28	11	16	13	6	ion/electron transport
voltage-gated	164	3	0	6	2	7	5	3	3	5	4	5	12	9	7	2	3	ion/electron transport
electron transport	423	25	11	29	6	35	44	20	37	17	34	25	19	36	26	18	15	ion/electron transport
ion transport	501	22	8	32	11	35	21	13	25	18	21	26	39	29	27	37	14	ion/electron transport
metaboli	1241	80	21	81	15	98	124	68	91	71	86	66	87	110	114	64	39	metabolism of energy/proteins
ATP	1234	81	27	92	24	75	116	49	111	77	83	86	102	104	111	82	47	metabolism of energy/proteins
mitochon	574	50	10	63	19	55	61	32	67	46	55	49	51	76	64	66	24	metabolism of energy/proteins
ribosom	254	39	14	39	19	32	35	15	30	25	32	33	31	23	26	37	13	metabolism of energy/proteins
translation	168	21	2	13	9	18	14	12	25	14	18	18	12	12	21	14	17	metabolism of energy/proteins
transcript	1991	116	39	##	41	172	142	78	144	120	129	122	143	138	138	130	75	metabolism of energy/proteins
cell cycle	478	34	11	39	14	42	46	28	46	37	39	44	49	42	46	22	23	cell proliferation/apoptosis/differentiation
apoptos	373	29	8	26	10	34	36	12	18	31	29	18	30	39	32	30	10	cell proliferation/apoptosis/differentiation
differentiat	177	17	2	20	1	14	11	8	5	6	13	6	7	9	12	7	4	cell proliferation/apoptosis/differentiation
immun	390	19	5	26	7	22	44	21	25	19	24	19	31	30	29	17	18	Immune answer/inflammation/stress answer
inflamma	184	8	1	6	3	15	15	8	13	13	9	10	11	6	12	14	2	immune answer/inflammation/stress answer
stress	118	5	6	7	2	8	16	5	8	5	13	5	8	12	10	11	4	immune answer/inflammation/stress answer
peroxidase	32	2	2	7	0	3	4	2	5	3	16	1	4	4	4	5	3	immune answer/inflammation/stress answer
heat shock	188	2	2	4	2	3	5	4	6	6	2	3	3	2	4	6	1	immune answer/inflammation/stress answer
DNA repair	154	10	3	17	4	11	15	6	13	16	15	7	13	12	17	7	8	immune answer/inflammation/stress answer
early	8	0	0	1	0	0	1	0	0	1	2	1	0	0	2	1	1	immune answer/inflammation/stress answer
adhesion	573	30	5	28	9	42	44	19	42	28	38	19	34	31	32	28	14	extracellular matrix/cytoskeleton/adhesion
extracellular matrix	226	14	4	7	5	12	12	7	7	8	8	5	11	10	11	10	8	extracellular matrix/cytoskeleton/adhesion
cytosk	529	33	9	36	21	39	41	19	33	37	43	24	30	46	48	32	21	extracellular matrix/cytoskeleton/adhesion
junction	129	0	3	11	5	3	10	2	9	3	10	5	5	7	4	8	2	extracellular matrix/cytoskeleton/adhesion
actin	494	35	7	35	19	42	39	24	38	41	40	28	30	34	44	40	21	extracellular matrix/cytoskeleton/adhesion

Table 34. Numbers regulated genes in different expression profiling experiments (bio-statistical analysis by Dr. Remondini/Participant 8)

GeneFamily	total number of clones in Human Unigene RZPD-2	partner 6 endothelial cells RF up-regulated genes	partner 6 endothelial cells RF down-regulated genes	partner 2 HL-60 cell RF up-regulated genes	partner 2 HL-60 cell RF down-regulated genes	partner 8 U937 cells RF up-regulated genes	partner 8 U937 cells RF down-regulated genes	Gene "Superfamily"
Signal	2528	4	9	1	0	1	1	signal transduction
GTP	560	1	2	1	0	0	0	signal transduction
Small G	235	0	1	0	0	0	0	signal transduction
Rab	80	0	1	0	0	0	0	signal transduction
Ras	66	0	0	0	0	0	0	signal transduction
phosphatase	334	0	1	0	0	1	0	signal transduction
protein kinase	304	0	2	0	0	0	0	signal transduction
calcium	715	1	2	0	0	0	0	signal transduction
calmodulin	131	0	1	0	0	0	0	signal transduction
channel	348	1	0	1	0	1	0	ion/electron transport
voltage-gated	164	1	0	0	0	0	0	ion/electron transport
ion transport	501	1	0	0	0	0	1	ion/electron transport
electron transport	423	1	1	0	0	1	0	ion/electron transport
metaboli	1241	3	0	0	0	2	0	metabolism of energy/proteins
ATP	1234	1	4	0	0	0	1	metabolism of energy/proteins
mitochon	574	2	1	0	0	0	0	metabolism of energy/proteins
ribosom	254	0	1	0	0	1	4	metabolism of energy/proteins
translation	168	0	0	0	0	0	0	metabolism of energy/proteins
transcript	1991	1	6	3	0	2	0	metabolism of energy/proteins
cell cycle	478	0	3	1	0	0	0	cell proliferation/apoptosis/differentiation
apoptos	373	1	0	0	0	0	0	cell proliferation/apoptosis/differentiation
differentiat	177	0	1	1	0	0	0	cell proliferation/apoptosis/differentiation
immun	390	0	0	0	0	0	0	immune answer/inflammation/stress answer
DNA repair	154	0	1	0	0	0	0	immune answer/inflammation/stress answer
inflamma	184	0	0	0	0	1	0	immune answer/inflammation/stress answer
adhesion	573	0	1	0	0	1	0	extracellular matrix/cytoskeleton/adhesion
extracellular matrix	226	1	0	0	0	1	0	extracellular matrix/cytoskeleton/adhesion
cytosk	529	0	5	0	0	1	1	extracellular matrix/cytoskeleton/adhesion
actin	494	0	4	0	0	1	1	extracellular matrix/cytoskeleton/adhesion
junction	129	0	1	0	0	1	0	extracellular matrix/cytoskeleton/adhesion

3.2.4.8 Summary (Participant 1)

Our data indicate an effect of RF-EMF on gene and protein expression in various cell systems. This conclusion is based on the following findings:

- RF-EMF exposure at a SAR value of 1.5 W/kg caused a transient up-regulation of p21 and c-myc genes and a long-term up-regulation of the stress response gene hsp70 in embryonic stem cells deficient of the p53 gene (3.2.4.1)
- RF-EMF exposure at a SAR value of 2 W/kg reduced the expression of the receptor FGFR1 of fibroblast growth factor (FGF) in human neuroblastoma cells (NB69) and in neural stem cells of rats (3.2.4.2).
- RF-EMF exposure at a SAR value of 1.3 W/kg up- or down-regulated the expression of various genes and proteins in HL-60 cells and in endothelial cells of human origin (3.2.4.3, 3.2.4.6, 3.2.4.7).
- RF-EMF exposure at a SAR value of 2.4 W/kg activated the p38MAPK/hsp27 stress response pathway in endothelial cells of human origin (3.2.4.6).
- RF-EMF exposure at a SAR value of 2.4 W/kg changed the global pattern of protein phosphorylation in endothelial cells of human origin with possible consequences for the signal transduction pathway (3.2.4.6).
- RF-EMF exposure at a SAR value at 2 W/kg did not significantly affect gene expression in human lymphocytes, although a few genes among several thousand tested with the micro-array system were found altered in two human immune cell lines (3.2.4.4, 3.2.4.5).
- RF-EMF exposure at a SAR value of 2 W/kg did not affect the expression and activity of the inducible nitric oxide synthase (iNOS) and the expression of hsp27 and hsp70 in nerve cells (3.2.4.5).
- The increased expression of hsp27 in endothelial cells (EA.hy926) after RF-EMF exposure as described in 3.2.4.6 could not be reproduced in another laboratory where slightly different methods were used (3.2.4.5).

4.0 DISCUSSION

4.1 Results obtained after ELF-EMF exposure

4.1.1 Genotoxic effects

4.1.1.1 Human fibroblasts, lymphocytes, monocytes, melanocytes and muscle cells and granulosa cells of rats (Participant 3)

Intermittent ELF-EMF exposure generated DNA strand breaks in various but not all cell lines.

Our results show, that intermittent exposure to a 50 Hz magnetic field causes a reproducible increase in DNA strand breaks in cultured human cells. These findings are in accordance with some recent studies with whole-body exposure of rodents to ELF-EMF which revealed DNA single- and double-strand breaks in the brain (Lai and Singh 1997c; Singh and Lai 1998; Svedenstal et al. 1999a/b). However, the majority of the studies investigating genotoxic effects of 50/60 Hz electromagnetic fields (McCann et al. 1993, 1998; Murphy et al. 1993; Moulder 1998) have reported a negative outcome on genotoxicity. Our results from tests with continuous exposure of fibroblasts to EMF corroborate these findings. Subjecting cells continuously to a constant field probably may induce adaptive mechanisms, protecting the genome from harmful influences. A regular change of environmental conditions might interfere with such mechanisms and lead to DNA damage. The extent of damage would depend on the duration of exposure and the time of recovery.

It is highly unlikely, that the observed genotoxic damage is caused non-specifically by spots of increased temperature within the cell layer as a secondary effect of the electromagnetic field. If so, the damage would increase with a prolongation of on-time during the intermittent exposure and would be largest at continuous exposure. The largest effects, however, are obtained at 5 min on/10 min off cycles, and continuous exposure had no effect at all. Therefore, we conclude, that the observed induction of DNA-single and double strand breaks is a direct consequence of an intermittent exposure to ELF-EMF.

Environmental exposure to continuous ELF-EMF is rather exceptional. Different electrical household devices (hair dryer, razor, vacuum cleaner) reaching peak values up to 1 mT are often used for a short period of time (5-10 min), producing a variety of exposure levels. To date, we could make out only one study dealing with genotoxic effects of ELF-EMF at intermittent exposure. This was done by Nordenson et al. (1994), who found a significant increase of chromosome aberrations in human amniotic cells (50 Hz, 30 µT, 20 s on-off). However, these results have not been corroborated by other studies as yet.

Genotoxic effects of ELF-EMF varied with exposure time.

We observed a time dependent increase of DNA breaks up to 15 to 19 hours of ELF-EMF exposure and then a decline to a steady state level of about 1.5 fold of the base line. This unexpected finding can be explained, if the exposure activates DNA repair processes and this activation takes a time of 10 to 12 hours. After this time the DNA damage is repaired then at an enhanced rate, which leads to a reduction of DNA breaks albeit not to a normalisation. This explanation is experimentally supported by the observation, that the single strand DNA breaks (alkaline conditions) are repaired after approximately 30 minutes, and double strand breaks 7 to 9 hours after finishing the exposure. Removal of damaged DNA-bases by induced repair enzymes (glycosylases) may lead to a temporary increase of abasic sites in the DNA (Friedberg et al. 1995). Abasic sites (alkali-labile sites) result in DNA single strand breaks after alkaline treatment (Tice et al. 2000). The alkali-labile sites generated after ELF-EMF exposure are therefore detected as peak at hour 12 to 17 at Comet assay conditions of pH > 13, but not of pH 12.1, the latter not being able to cleave the alkali sensitive sites.

It is well known, that the repair of single strand breaks is a fast and almost error free process, while the repair of more complex DNA damage (i.e. DNA double strand breaks) by homologous recombination, single strand annealing or non-homologous end joining require more time and are error prone in part (Van den Bosch et al. 2002). Therefore DNA double strand breaks may affect the integrity of the genome leading to cell death, uncontrolled cell growth or cancer (Van Gent et al. 2001).

Our results show, that intermittent exposure to a 50 Hz magnetic field causes a time dependent increase in micronuclei in cultured human fibroblasts. These findings are in accordance with Simko et al. (1998a/b), who could demonstrate an ELF-EMF-induced formation of micronuclei in human amnion and in human squamous cell carcinoma cells. In contrast, the greater part of studies performed as yet using different cell types did not point to direct clastogenic effects of ELF-EMFs (Livingston 1991; Scarfi et al. 1991, 1994; Paile et al. 1995), but they propose epigenetic or co-clastogenic mechanisms in combination with other genotoxic exposures (Lagroye et Poncy 1997; Cho and Chung 2003; Simko et al. 2001b). Micronucleus formation can either result from chromosomal non-disjunction due to damage of kinetochore proteins or from acentric fragments secondary to DNA double strand breaks. Since we did not use kinetochor antibodies to differentiate between these two possible mechanisms, the cause for the micronuclei induction remains an open question. At extended exposure times micronucleus frequencies reached a constant level, which is not in contrast to the results found in Comet assay since micronuclei cannot be repaired.

ELF-EMF produced DNA strand breaks in human fibroblasts in a dose dependent way.

We could demonstrate a dose dependent relationship between alkaline and neutral Comet assay tailfactors and applied magnetic flux density. The guidelines of the International Commission on Non-Ionizing Radiation Protection (ICNIRP 1998) are 500 µT during workday for occupational exposures and 100 µT for 24 h/day for the general population. The on-set of genotoxic effects in our tests was at a magnetic flux density as low as 35 µT at 15 hours and 70 µT at 24 hours of exposure, being well below these proposed guideline values. Moreover, these guidelines are dealing with continuous EMF exposure. No proposal how to handle intermittent exposures has been made by the ICNIRP as yet.

Generation of DNA strand breaks in human fibroblasts through ELF-EMF was related to the age of the donors.

Our findings of significant differences in basal DNA single and double strand break levels in fibroblasts of donors of different age are consistent with studies of several species and tissues (Mullaart et al. 1988; Holmes et al. 1992; Zahn et al. 1996; Diem et al. 2002). In addition, we here report differences in response to ELF-EMF exposure in relation to donor age, which point to a higher susceptibility of older donors to the genotoxic action of ELF-EMF. This could be interpreted by a later on-set of DNA repair. These findings are in agreement with age-related increases of DNA damage and mutations as a result of a reduced DNA repair capacity (Wolf et al. 2002; Bohr 2002; Cabelof et al. 2002; Ben Yehuda et al. 2000; Goukassian et al. 2000). Observations of an altered gene activity during ageing were reported for the rat brain, heart, and liver (Salehi et al. 1996; Goyns et al. 1998) and human fibroblasts (Linskens et al. 1995). This decline may be due to a reduction in chromatin associated RNA polymerase II activity (Rao and Loeb 1992), to mutation-induced changes in binding activity of transcription factors (Sheerin et al. 2001), or due to a decline in protein synthesis secondary to a decrease in the amount and activity of certain elongation factors (Shikama et al. 1994). Changes in the availability of proteins or enzymes may be critical if proteins of DNA repair machinery are affected.

Effects of ELF-EMF were cell type specific.

Our results point to cell type specific reaction and to differences in sensitivity of different tissues to ELF-EMF exposure. We could identify three responder (human fibroblasts, human melanocytes, transformed rat granulosa cells) and three non-responder cell types (human lymphocytes, monocytes and skeletal muscle cells). Up to date a plausible mechanism for these findings is mere speculation, but these data propose an epigenetic, indirect action of intermittent ELF-EMF. The observed cell specific response can not be explained by age-related effects, since the non-responding skeletal muscle cells are derived from the oldest donor.

In our experiments we exposed dividing and quiescent lymphocytes to ELF-EMF, and in both cases no induction of DNA strand breaks could be observed. Isolated monocytes did not respond either. The other cell types used were cells in the log-growing phase and some of them showed genotoxic effects, whereas dividing skeletal muscle cells did not react. Therefore, it is not likely that the observed effects could be due to differences in response between proliferating and non-proliferating cells. In addition, the observed effects can not be attributed to differences between adherent cells or suspension cultures, since there are non-responder cell types in both cases.

Based on the results with human fibroblasts, which suggest an induction of DNA repair upon intermittent ELF-EMF exposure, we speculate, that the effects reported here, may reflect differences in DNA repair

capacities between different tissues. This explanation, however, requires further assessment, e. g. evaluation of repair kinetics.

Generation of DNA strand breaks in human fibroblasts through ELF-EMF and their repair were modified by UVC or heat stress.

During ELF-EMF exposure UVC induced DNA-damage was repaired very slowly, although the maximum at 15h ELF-exposure could not be detected any more. The results were similar with the neutral Comet assay, but DNA damage (DNA double strand breaks) was repaired within a shorter time. These results suggest that ELF-EMF-exposure might impair and/or delay the onset of repair of DNA damage.

In regard to studies on repair kinetics, the exposure time dependent extent of DNA damage implicated an induction of DNA repair upon intermittent ELF-EMF exposure. We concluded that pre-exposure to intermittent ELF-EMF would have a protective effect and reduce genotoxic actions of additional exposures. In contrast to our assumption, pre-exposure to intermittent ELF-EMF for 20 hours resulted in an additive genotoxic effect of combined exposures and a reduced repair rate of UVC or heat stress induced DNA damage. A protective effect of ELF-EMF exposure could not be confirmed by these results. In contrast, they suggest an impairment or delay of DNA repair mechanisms due to ELF-EMF exposure.

Recently, Robison et al. (2002) have demonstrated that pre-exposure to ELF-EMF for 4 to 24 hours can decrease DNA repair rate and protect human HL-60 cells from heat induced apoptosis. Miyakoshi et al. (2000) showed that strong ELF-EMF for 2 hours can potentiate X-ray-induced DNA strand breaks in human malignant glioma cells, whereas others (Whitson et al. 1986; Frazier et al. 1990; Cantoni et al. 1996) found no evidence that ELF-EMF could inhibit repair of DNA damage induced by ionising radiation or UV light using different human cell types. However, in these experiments ELF-EMF exposure was not performed prior to UV or X-ray exposure, but afterwards. ELF-EMF preconditioning of cells may evoke different reactions. In addition, responses of the cells could differ with ELF-EMF exposure duration, applied exposure protocol (continuous vs. intermittent) or used cell type. Anyhow, these experiments may not overrule our theory of an induction of DNA repair upon ELF-EMF exposure, since repair processes are very complex and different mechanisms may engaged in the repair of UV or thermal stress induced DNA damage.

Generation of DNA strand breaks in human fibroblasts through ELF-EMF was dependent on the genetic background of cells.

We concluded that the cupola-shaped time dependent pattern of DNA breaks in the Comet assay mirrors the action of repair processes. This is supported by the more than two fold increased rate of DNA breaks in DNA repair deficient fibroblasts from a patient with Ataxia Telangiectasia after 24 hours of exposure. The increased DNA breaking rate seen in fibroblasts from this patient and in fibroblasts from older donors points to the significance of the genetic background regarding the response to ELF-EMF-exposure.

Generation of DNA strand breaks in human fibroblasts by ELF-EMF was dependent on the frequency of ELF-EMF.

Although intermittent ELF-EMF induced DNA strand breaks in the Comet assay at a broad frequency range between 3 and 550 Hz, there are noteworthy peak effects at 50 Hz and 16.66 Hz, these representing the commonly used frequencies of alternating current in Europe. However, this has been tested as yet at intermittent 5 min on /10 min off cycles only and may be different under changed intermittent conditions.

ELF-EMF generated chromosomal aberrations in human fibroblasts.

Structural chromosome aberrations result from breakage and abnormal rearrangement of chromosomes. They can be classified either to stable or unstable aberrations, depending upon their ability to persist in dividing cell populations. Unstable aberrations are ring chromosomes, dicentric chromosomes or acentric fragments, whereas stable aberrations, which result from repair processes, consist of balanced translocations or other symmetrical rearrangements. At exposure conditions producing maximum effects in micronucleus test and in Comet assay, we observed significant increases in gaps, breaks, ring chromosomes, dicentric chromosomes and acentric fragments, but not of translocations. These results are in accordance with studies performed by Nordenson et al. (1994) and Khalil and Quassem (1991), who applied intermittent or pulsed field ELF-EMF exposure. Several other studies performed at continuous ELF-EMF exposure could detect an increase in chromosomal damage (Jacobson-Kram et al. 1997; Galt et al. 1995; Scarfi et al. 1991; Paile et al. 1995).

The fate of a cell carrying a chromosomal aberration is crucial for the assessment of a possible cancer risk. Cells with unstable aberrations like rings, dicentrics or acentric fragments will be committed to apoptosis or cell death, whereas cells with repairable DNA damage like chromosomal gaps or breaks may survive. The repair process itself can lead to translocations, thereby creating a stable mutation. Surprisingly this could not be detected in 24-times 1,000 metaphases, when each chromosome had been separately painted.

Although no significant differences in cell numbers could be detected between ELF-EMF exposed and sham-exposed cells, a possible elimination of cells carrying non-stable chromosomal aberrations is not contradictory to these previous findings. Cell numbers were assessed directly after ELF-EMF exposure termination, whereas for evaluation of chromosomal aberrations, cells were maintained in culture. In addition, the total fraction of cells with non-stable aberrations in exposed cells was 0.8%. The method used for assessment of cell numbers (Coulter counter) is to imprecise to detect such o low number of cells.

Since experimental analyses have shown, that DNA double strand breaks are the principal lesions to produce chromosomal aberrations (Bryant 1998; Natarajan and Obe 1978; Obe et al. 1992), the induction of micronuclei and chromosomal aberrations is in good agreement with the previous demonstration of DNA strand breaks.

ELF-EMF did not influence the mitochondrial membrane potential.

Hitherto, response of $\Delta\Psi_m$ to ELF-EMF exposure has not been assessed and no data on EMF-induced modifications of the membrane potential of cells are available. Effects of electric fields on membrane ATPases (optimal ranges: 5 - 30 Vcm^{-1}, 10 Hz - 1 MHz) have been reported by several groups (Tsong 1992). Short pulses of electric field (100 µs decay time) of several $kVcm^{-1}$ have been used to trigger ATP synthesis in rat liver submitochondrial particles. The electric field-induced ATP synthesis was abolished by inhibitors of the F_0F_1-ATPase, oligomycin, *N,N´*dicyclohexylcarbodiimide, venturicidin and aurovertin, but occurs independently of components of the mitochondrial electron transport chain. In low field experiments (<75 Vcm^{-1}) Tsong showed a dependence of ATP yield on the field strength and frequency of the alternating current (AC) field. Effects of AC fields on the activity of Na^+,K^+-ATPase, the enzyme principally responsible for establishing ion gradients across the cell membrane, have also been reported by Blank (1992). Under normal conditions *in vitro*, the enzyme in weak electric fields has a decreased ability to split ATP (100 Hz, threshold for effects of 5 μVcm^{-1} estimated by extrapolation). When the enzyme activity was inhibited to less than half its optimal level by ouabain or low temperature, an increase in ATP splitting was observed. The greatest effects appear to be in the extremely low frequency range that includes 50 Hz.

Our data do not indicate an influence of intermittent ELF-EMF exposure of human fibroblasts on $\Delta\Psi_m$. Although these results cannot rule out rapid alterations of $\Delta\Psi_m$, we consider it to be unlikely that ELF-EMF-induced formation of DNA strand breaks is mediated via significant intracellular changes which affect $\Delta\Psi_m$.

4.1.1.2 Human fibroblasts and granulosa cells of rat (Participant 7)

The genotoxic effects induced by ELF-EMF are not reflected by physiological functions like volume regulation and free cytoplasmic Ca^{2+}-concentration.

The experiments have been performed on two different cellular levels, the genomic and the cellular level using cultured granulosa cells of rat (GFSHR-17) and human fibroblasts. On the genomic level the neutral and alkaline Comet assay has been applied to evaluate ELF-EMF (5min on/10 min off, 1 mT) induced effects on DNA single- and double-strand breaks. In parallel, the effects were compared with those obtained on the cellular level by analysis of volume regulation (Ngezahayo et al., 2003) and cytoplasmic free Ca^{2+} concentration (Pilger et al., submitted). Since Participant 3 observed no effect during permanent ELF-EMF exposure on the occurrence of DNA strand-breaks, but for intermittent exposure (Ivancsits et al. 2002a; Ivancsits et al. 2003b), we followed this exposure protocol.

The results of the alkaline Comet assay indicate that intermittent exposure to ELF-EMF induced a significant increase of single- and double-strand breaks in rat granulosa cells (Figure 26) with a maximum after exposure for 16h to 18h. The maximum is followed by a decline of DNA damage in the time range

of a few hours which can be attributed to the onset of DNA repair mechanisms (Ivancsits et al. 2002a). It should be noted that a similar time course of DNA damage was observed at an exposure frequency of 16 2/3 Hz for rat granulosa cells. The time course of DNA damage on rat granulosa cells is similar as reported for cultured human fibroblasts (Ivancsits et al. 2002a; Ivancsits et al. 2003b), CHO and HeLa cells, but appears to be more pronounced. Therefore, it seems to be reasonable to suggest that the sensitivity of cultured cell lines to ELF-EMF (intermittently applied) depends significantly on the cell type.

In addition the frequency dependence of DNA damage was studied. The quantity of DNA single-strand breaks appears to depend on the frequency of the applied ELF-EMF with a maximum at lower frequency within the applied sequence of frequencies (8 Hz, 16 2/3 Hz, 50 Hz, 1000 Hz) (Figure 27). Surprisingly, virtually no frequency dependence is found for the results of the neutral Comet assay (Figure 28).

The important question arises whether the ELF-EMF effects observed on the genomic level are reflected in a change of the macroscopic cellular behaviour, especially in basic regulatory physiological functions. As marker of physiological cell functions the regulatory volume decrease/increase of rat granulosa cells was considered. In response to a constantly applied hypotonic shock rat granulosa cells swell due to water influx like an osmometer and shrink thereafter to the original cell volume within the time scale of an hour. According to this physiological regulatory behaviour a hypertonic shock causes cell shrinkage. The results show no significant influence of ELF-EMF exposure at the additional stress condition caused by a non-isotonic bath medium (Figure 69). It could be argued that, since for technical reasons the regulation of cell volume was studied 10 min after the end of ELF-EMF exposure for 18h, the DNA repair mechanisms act significantly and thereby bias the results. But the time span for DNA repair after an exposure time of 18h occurs in the range of hours (Figures 16, 17), whereas the experiments focused to volume regulation were started 10 min after end of ELF-EMF exposure. Therefore it can be concluded that a significant increase of DNA single- and double-strand breaks by ELF-EMF exposure is virtually not reflected in a change of regulatory volume decrease/increase of granulosa cells. As second macroscopic cellular parameter the time course of free cytoplasmic Ca^{2+}-concentration ($[Ca^{2+}]_i$) was studied by fluorescence-spectroscopy after ELF-EMF exposure for 5h, 6h, 7h, 8h, and 18h. $[Ca^{2+}]_i$ was recorded in the absence (Figure 67, Table 9) and presence of a further stress factor, the exposure to H_2O_2 containing bath media (Figure 68, Table 10)). Also for this cellular parameter no significant influence of ELF-EMF exposure could be observed. In co-operation with Participant 3 a further cell-culture system, human fibroblasts, were used. In parallel to the results showing ELF-EMF induced DNA strand-breaks, the corresponding free Ca^{2+}-concentration was recorded. Also for this cell system the observed ELF-EMF induced DNA strand-breaks are not reflected in a change of the cellular level of free cytoplasmic Ca^{2+} (Figures 66, 67) or the mitochondrial potential (see also ref. Pilger et al., submitted). In contrast to our findings Tonini et al. (2001) reported a fast, within the time range of minutes, and significant increase of $[Ca^{2+}]_i$ in a cultured neuroblastoma cell line by ELF-EMF exposure at 50- to 60-Hz and 0.12 µT (0.24 µT). Surprisingly, the observations were made at continuous ELF-EMF exposure. The various findings could be related to the specific cell type, the different set-up used for ELF-EMF exposure or the method applied for analysis of $[Ca^{2+}]_i$.

4.1.1.3 Mouse embryonic stem cells (Participant 4)

ELF-EMF did not induce the formation of DNA strand breaks in embryonic stem cells.

The potential to induce primary DNA damage by ELF-EMF was analysed by the Comet assay, as a consequence of up-regulation of the DNA-damage inducible gene GADD45 after ELF-EMF exposure (4.1.3.1). In addition, it was shown by Participant 3 that the exposition of human fibroblasts to ELF-EMF results in the increase in DNA breaks suggesting a possible direct mutagenic effect (Ivancsits et al., 2002). A correlation has been described between up-regulation of GADD45, bcl-X_L, and increased DNA damage as determined on the basis of the alkaline Comet assay in human preneuronal cells (Santiard-Baron et al. 2001). However, we did not observe significant effects of 6h or 48h intermittent ELF-EMF exposure on single- and double-strand DNA break induction in the alkaline and neutral Comet assay. One reason for our negative results (as compared to the data presented by Participant 3 on human fibroblasts, e.g.) could be the different intermittency scheme of exposure, which was applied (5 min on/30 min off by Participant 4 vs. 5 min on/10 min off by Participant 3). Actually Ivancsits et al. did not observe effect by using an intermittency scheme of 5 min on/25 min off, but only for shorter off time durations. However, by RF-EMF exposure of ES cells using 5 min on/30 min off cycles, we found a low, but significant

increase in double-strand DNA breaks, suggesting that the EMF frequency has significance for the DNA damaging effects.

4.1.1.4 Summary (Participant 1)

As discussed by Participant 3 there has been sporadic literature concerning *in vitro* studies which demonstrate that ELF-EMF may possess a genotoxic potential (Lai and Singh 1997c; Singh and Lai 1998; Svedenstal et al. 1999a/b). However, the energy impact to the genome of livings cells exposed to ELF-EMF had been calculated to be too low to generate DNA damage. Since the mainstream literature contradicted the assumption of genotoxic effects (McCann et al. 1993; McCann et al. 1998; Murphy et al. 1993; Moulder 1998), these sporadic findings were considered more or less meaningless. Opposite to this widely accepted view, the data of the REFLEX study which were systematically investigated and confirmed in 4 laboratories, of which two were not members of the REFLEX consortium, support the view that ELF-EMF causes genotoxic effects in certain, but not all cell systems.

Based on the methodology used and the data obtained in the REFLEX study, the findings of genotoxicity caused by ELF-EMF are hard facts. DNA single and double strand breaks were observed in human fibroblasts exposed to ELF-EMF at a flux density as low as 35 µT, which is far below the presently valid safety limit. Increases in micronuclei and chromosomal aberrations were found at higher flux densities (3.1.1.1). These effects, although striking in fibroblast from normal donors and donors with a known repair deficiency, were not observed consistently in all cell types, e.g. in human lymphocytes. This suggests that the genetically determined defence mechanisms of cells play a decisive role as to whether or not the cells respond to ELF-EMF exposure. The question arises why the genotoxic potential of ELF-EMF was not confirmed many years ago when suitable biochemical methods became available the first time. One explanation may be that most of the experiments were carried out with lymphocytes which seem to be resistant to ELF-EMF, and that in experiments with different cell systems the exposure time and the exposure conditions may have been inadequate.

As already stated, for energetic reasons ELF-EMF can neither denature proteins nor damage cellular macromolecules directly. If the energy impact on the genome of living cells exposed to ELF-EMF is too low for damaging their DNA, the genotoxic alterations observed in the REFLEX project must be produced indirectly through intracellular processes. Participant 3 observed in its most recent experiments that the increase of DNA strand breaks in human fibroblasts after ELF-EMF exposure can partly be inhibited by oxygen radical scavengers. This finding speaks for the assumption that the observed DNA damage may be caused by free oxygen radicals which are released by ELF-EMF. This assumption is further supported by results obtained by Simko et al., who measured an increase of free oxygen radicals in macrophages derived from murine bone marrow after exposure to ELF-EMF at a flux density of 1 mT (Simko et al. 2001) and by Lupke et al. who observed an increase of free oxygen radicals in monocytes derived from umbilical cord blood and in a human monocytic leukaemia cell line also after exposure to ELF-EMF (50 Hz) at a flux density of 1 mT (Lupke et al. 2004). Into the same direction hint the results of Zymslony et al., who assessed the effects of ALF-EMF (50 Hz, 40 µT) on the oxidative deterioration of DNA in rat lymphocytes after *in vitro* irradiation by UVA (Zymslony et al. 2004). The free radical hypothesis is further supported by the studies of Lai and Singh (2004) who found that brain cells of rats after whole body-exposure to ELF-EMF (60 Hz) at very low flux densities (0.01-0.25 mT) for 2-48 hrs showed increases in DNA single and double strand breaks, and that these increases could be blocked by pre-treating the animals with the free radical scavengers melatonin, N-tert-butyl-α-phenylnitrone and Trolox (a vitamin E analogue). The work of Lai and Singh, which must still be reproduced by other independent research groups, deserves special attention, since the DNA damage reported by them was observed in the brain of whole-body exposed animals, not in isolated cells as in the REFLEX study.

Based on the data of the REFLEX project it must be assumed that ELF-EMF is able to damage the genome in certain, but not all cell systems after exposure *in vitro*. The work of Lai and Singh suggests that these effects might also be seen after exposure *in vivo*. The genotoxic effects of ELF-EMF may be best explained by an ELF-EMF induced increase of intracellular free radicals within the exposed cells and by the genetic background of the exposed cells. It is well known that a balanced free radical status is the prerequisite for maintaining health and that an unbalanced free radical status promotes the process of ageing and the development of chronic diseases such as cancer and neurodegenerative disorders. Whether the balance of free oxygen radicals can also be impaired through ELF-EMF *in vivo* as suggested by Lai and Singh (2004) needs to be further clarified.

4.1.2 Cell proliferation and differentiation

4.1.2.1 Human neuroblastoma cells (NB69 cell line) (Participant 5)

ELF-EMF enhanced proliferation and reduces spontaneous apoptosis of NB69 neuroblastoma cells.

The described results indicate that 42- or 63-hour exposure to 50 Hz magnetic fields at 10 or 100 µT can increase proliferation and reduce spontaneous apoptosis in human neuroblastoma cells. Initial evidence obtained through cell counting (Trypan blue exclusion) was subsequently confirmed through PCNA labelling, 5-bromo-2'-deoxyuridine (BrdU) labelling for identification of DNA-synthesizing cells, and flow cytometry. The modest, though statistically significant increase in the total number of cells in response to a 100-µT field estimated by the Trypan blue exclusion is consistent with the observed increase in the number of PCNA positive cells. This is also consistent with the increased numbers of cells in G2-M phase and of BrdU positive cells observed 24 hours before the increase in the number of cells was detected. The present data also indicate that a 50-Hz EMF at 100 µT can induce changes in the activation of the transcriptional factor CREB in a time-dependent manner.

A number of experimental studies investigating proliferative effects of EMF using in vitro or in vivo models (Kavet 1996), have provided limited evidence that ELF-EMF can represent a growth stimulus. Kwee and Raskmark (1995) have reported that a 24-h exposure to 50 Hz MF at 80 µT significantly increases the proliferation of transformed human epithelial amnion cells and K14 skin fibroblast cells. Wei et al. (2000) have reported that 60 Hz MF (30-120 µT, 3-72 h exposure) can induce proliferation in human astrocytoma cells and strongly strengthen the effect of two chemical agonists.

Some studies, however, have reported effects that are in apparent contrast to those described above. For instance, Conti et al. (1983) and Cleary (1993) have reported reduced ^{3}H-thymidine incorporation into lymphocyte DNA after exposure to 2.0 - 7.0 mT, 50-Hz magnetic fields. It has been proposed that several physical and biological variables, including different field parameters, exposure protocols, cell types and physiological conditions (degree of differentiation or activation) may account for the conflicting results reported in the literature (see for instance Schimmelpfeng and Dertinger 1997). In fact, the cellular response to the fields seems to be strongly dependent on biological parameters (Simko et al. 1998a/b; Wei et al. 2000). In addition, there is experimental evidence that specific combinations of AC/DC fields interact with biological systems (Blackman et al. 1994; Trillo et al. 1996) and the key to affecting proliferation of cells in a consistent manner might lie in the simultaneous control of the AC field amplitude and frequency, and the AD/DC field intensity ratio (Yost and Liburdy 1992; Blackman et al. 1985a,b; Trillo et al. 1996; Bauréus Koch et al. 2003).

Also, a recent study by Pirozzoli et al., (2003) has shown that the apoptosis induced by camptothecin in neuroblastoma cells (LAN-5) can be prevented by a 24-h exposure to 50 Hz, 1 mT MF. In addition, the cells respond to the stimulus with an increase in the proliferation index after seven days of continuous exposure to the field. In our cellular model (NB69) and under our experimental conditions, a 63-h exposure to 50 Hz, 100 µT MF significantly reduces the spontaneous rate of apoptosis while increasing proliferation in an extent that is similar to that reported by Pirozzoli et al.

The present data on PCNA, a protein that has been reported to be peak in proliferating cells at late G1 and S phases (Oue et al. 1995), indicate that the normal regulation of the PCNA positive cells is altered by the exposure to 50 Hz, 100 µT MF. In the MF-exposed samples the percent of PCNA-positive cells does not differ significantly from that at day 5 post plating, while in the control groups a significant reduction of PCNA positive cells was observed on day 6. These data are consistent with previous results reported by Cridland et al. (1999) on normal human fibroblasts showing a modest though significant increase in the length of the G1 phase when exposed to 50 Hz, 20 and 200 µT MF

The mechanism of interaction between ELF-EMF and NB69 neuroblastoma cells is not known yet.

Regarding the mechanism of interaction of magnetic fields that could underlay the herein described responses of NB69, the mobilization of cellular Ca^{2+} or some Ca^{2+}-regulatory process have been proposed as pre-eminent targets of the MF stimuli (Tonini et al. 2001). Also, in a recent work Zhou et al. (2002) have reported that ELF MF at 100 µT induced a time-dependent activation of CREB DNA binding in HL-60 cells. The effect was dependent on both the extracellular and intracellular Ca^{2+}, which suggests that ELF-EMF can activate CREB DNA binding through calcium-related signal transduction pathways. Similarly, in the present study, the activation of CREB was found to be influenced by the MF stimulus in

a time-dependent manner. Although additional research is needed to determine whether or not calcium is involved directly in the observed response of NB69 cells to 50-Hz MF, the present results are consistent with such a possibility. Further work is also necessary to determine the gene transcription pattern resulting from the increase of CREB activation after exposure to MF. Such an information would be crucial to identify the mechanism(s) by which MF interact with human neuronal cells in vitro. This hypothesis is not in contradiction with the recent results reported by Ivancsits et al. (2002b) and the studies by this group included in the REFLEX project. Their results showed that 50 Hz MF induced a dose dependent and time dependent DNA-single and double-strand breaks, with responses at a magnetic flux density as low as 35 µT.

4.1.2.2 Mouse embryonic stem cells (Participant 4)

ELF-EMF did not exert any influence on neuronal differentiation of embryonic stem cell.

We could not find evidence that under our experimental conditions, ELF-EMF exposure of ES cell derived neural progenitors affected the neural differentiation process, because we did not observe effects on transcript levels of genes involved in neuronal and glial differentiation (nestin, en-1, nurr1, tyrosine-hydroxylase and GFAP). Immunofluorescence analysis did not show any changes in the intracellular distribution and number of cells expressing neuronal markers (ßIII-tubulin, TH, GFAP).

4.1.2.3 Human lymphocytes and embryonic stem cells (Participant 8)

ELF-EMF did not affect proliferation, cell cycle and activation of lymphocytes.

Since the immune System has a key role in contrasting diseases, possible damages induced by exposure of immune cells, such as lymphocytes, could represent a great risk for human health. Thus, the objectives were to determine if different EMF exposures were able to modify human lymphocytes functionality and gene expression using appropriate in vitro tests. Moreover, since immune system efficiency is modified with ageing, a group of elderly donors was enrolled in order to study possible EMF effects age-related. On the whole, the results obtained show no differences between sham- and ELF-EMF exposed lymphocytes for most of the endpoints studied. Obviously, ELF-EMF is not able to modify proliferation, cell cycle and cell activation, which are fundamental phases of lymphocyte function. Negative results are extremely important for evaluations on human health risk.

ELF-EMF activated the expression of cardiac genes in embryonic stem cells thus enhancing their cardiac differentiation.

ELF - EMF were able to promote the differentiation of mice embryonic stem cells into a specific cardiac cell lineage, selectively promoting the expression of fundamental genes involved in the orchestration of cardiac differentiation. At the end of the differentiation process the expression of typical cardiac genes revealed that a specific direction of differentiation into a cardiac phenotype took place, which was also demonstrated by lack of expression of genes related to other cell lineages (e.g., skeletal muscle cells, neuronal cells, etc.).

4.1.2.4 Summary (Participant 1)

As discussed by Participant 5 (4.1.2.1) the findings reported in the literature about a possible influence of ELF-EMF on the proliferation and differentiation of various cell systems in vitro are controversial. Just recently, Lisi et al. (2004) demonstrated that exposure to ELF-EMF (50 Hz, 1 mT) triggered the differentiation of human pluripotent embryonic stem cells. In the REFLEX project, no data were obtained which suggest a major effect of ELF-EMF on cell proliferation and differentiation in human fibroblasts (4.1.1.1), embryonic stem cells (4.1.2.2), human lymphocytes (4.1.2.3) or neuroblastoma cells (3.1.4.2). On the other hand, some influence of ELF-EMF on proliferation and differentiation in certain cell systems cannot be excluded (4.1.2.1).

Participant 5 (3.1.2.1, 4.1.2.1) observed an inhibition of the spontaneous apoptosis in neuroblastoma cells which was followed by an increase of the proliferation rate, when the cells were exposed to ELF-EMF for 63h at a flux density of 50 or 100 µT. This observation is in line with the results of a recent study by Tokalov et al. (2003) and Tokalov and Gutzeit (2003) who reported that ELF-EMF alone does not have

any effect on the proliferation of HL-60 cells, while it protects heat shock treated HL-60 cells from becoming apoptotic. Quite obviously, ELF-EMF enabled heat shock treated HL-60 cells to escape the cell cycle arrest and to re-enter the normal cell cycle thus allowing the cell to continue the proliferation process. The authors explained this phenomenon by an ELF-EMF induced release of hsp-proteins which are thermo- or cytoprotective.

An answer of what may be the reason for the sporadically observed, but until now not unambiguously confirmed influence of ELF-EMF on cell proliferation and differentiation, may be provided by the REFLEX findings on gene and protein expression. As found by Participant 8 (3.1.4.3, 4.1.2.3), ELF-EMF accelerated the cardiac differentiation of embryonic stem cells through enhanced expression of cardiac genes. Further evidence for the validity of such an assumption comes from Participants 3 and 12 (3.1.4.5), who observed in human fibroblasts a remarkable influence of ELF-EMF on the expression of various genes, among them genes regulating Ca-metabolism, cell cycle, apoptosis, extracellular matrix, and cytoskeleton.

Of course, even if there is a relationship between ELF-EMF exposure and an acceleration of cell proliferation and differentiation in in vitro studies through the proposed mechanisms, it is at present not possible to draw any conclusion for the in vivo effects on man and animal.

4.1.3 Apoptosis

4.1.3.1 Mouse embryonic stem cells (Participant 4)

ELF-EMF altered the expression of bcl-2, bax and GADD45 gene in ES-cell derived neural progenitor cells.

In our experiments with ELF-EMF exposed wild-type mouse ES derived neural progenitors, we showed by Q-RT-PCR analysis significant changes in the transcript levels of the anti-apoptotic bcl-2 gene and the related pro-apoptotic bax gene. The biological significance of this finding and its relevance to the in vivo situation is not yet known. Apoptotic cell death is regulated by members of the bcl-2 family for differentiating mouse embryonic stem cells (Sarkar and Sharma 2002). Apoptosis plays an important role during embryonic development, including the development of the nervous system. Bcl-2 over expression was also reported to eliminate deprivation-induced cell death of brainstem auditory neurons (Mostafapour, 2002). Bcl-2 and bax mRNA transcripts in the hippocampus were significantly but transiently upregulated following the administration of the potent neurotoxin domoic acid (Ananth et al. 2001).

Another gene, whose expression was affected after ELF-EMF exposure, was the 'growth arrest DNA-damage' inducible GADD45 gene. The members of the GADD protein family are considered to play an important role in maintaining genomic stability and to regulate cell cycle activity (Chung et al. 2003).

Our results, which demonstrate changes of bcl-2, bax and GADD45 transcript levels indicate that ELF-electromagnetic signals could be perceived in ES cell-derived neural progenitors as environmental stress signals. These signals may trigger cellular responses for the maintenance of cellular homeostasis via alterations of genes that control cell cycle and apoptotic cell death.

In summary, we may conclude that exposure of ES-derived neural progenitor cells to magnetic fields simulating 50Hz power line ELF-EMF may influence transcript levels of genes encoding proteins of the bcl-2 family involved in apoptosis and the p53 responsive growth arrest and DNA damage inducible GADD45 gene. Since the fundamental processes of programmed cell death and cell cycle regulation are closely related to processes underlying cell transformation, the association of ELF-EMF with early stages of carcinogenesis cannot be excluded yet. Further investigations in vivo using genomics analyses and animal studies after EMF exposure have to be performed.

4.1.3.2 Neuroblastoma cells (NB69 cell line) (Participant 5)

ELF-EMF inhibited spontaneous apoptosis in neuroblastoma cells.

Environmental electromagnetic fields (EMF) such as those from electric power transmission and

distribution lines have been associated with increased risk of childhood leukaemia, cancer of the nervous system and lymphomas (Ahlbom et al. 2001; De Roos et al. 2001). In vitro studies of EMF effects have attempted to find an explanation to the epidemiological data and to determine the possible mechanism for cancer risk. Recent evidence has suggested that a common property shared by a number of known and suspected tumour promoters, is their ability to block the process of apoptosis (Jaattela et al., 1999). Therefore, one possible mechanistic explanation for the apparent effect of weak ELF magnetic fields would be their expression of tumour-promoting activities by interfering with the regulation of apoptosis. We have addressed this hypothesis by testing the effects of a 50 Hz 100 μT MF on apoptosis in the human neuroblastoma cell line.

Our data indicate that the field exposure can significantly inhibit spontaneous apoptosis of NB69 cells as revealed through TUNEL assay. This response was associated with significant increase in the number of cells as well as in BrdU incorporation into ADN. Besides, the immunoreactivity for Bcl-2 protein in exposed samples was also significantly increased at 60 min of exposure with respect to controls (data not shown). Regulation of apoptosis is delicately balanced by signalling pathways between apoptosis-promoting factors such as p53 and caspases, and antiapoptotic factors such as Bcl-2 and MDM2. Several lines of evidences have shown that the functional interaction between these factors play important roles in the control of cell growth and apoptosis.

Previous studies investigating changes in susceptibility to apoptosis after EMF exposure have reported both reduced (Simko et al. 1998b; Fanelli et al. 1999; Ding et al. 2001; Kumlin et al. 2002; Robison et al. 2002) and increased susceptibility (Ismael et al. 1998; Tofani et al. 2001; Mangiacasale et al. 2001; Liu et al. 2003). Other studies concerning DNA repair after EMF exposure have reported no effects (Cossarizza et al. 1989a; Frazier et al. 1990; Cantoni et al. 1996). In the majority of the studies reporting effects on apoptosis, cancer cells were exposed to MF (B>100 μT) after apoptosis induction by radiation or chemical treatments. The cellular susceptibility to such MF-driven apoptosis has been reported to be dependent on the cell type, the presence of genetic abnormalities, cell physiology and the MF exposure time. Cancer cells frequently have decreased cell death as a primary mode of increased cell proliferation. Attention has been focused on the expression of the p53 gene, which induces either a stable arrest of cell growth or apoptosis. The final outcome of the different mechanism of action of p53 is to maintain the genomic stability of the cell. Thus, the absent of this protein or their inactivation contributes to genomic instability, the accumulation of mutations and increased tumorigenesis. In the study by Czyz et al. (2004a, included in the present report) the exposure to 50 Hz EMF at 2.3 mT results in up-regulation of egr-1, c-jun and p-21 transcript levels in p53-deficient, but not in wildtype embryonic stem cells. These data indicate that loss of p53 may also affect the sensitivity of cells to external stress factors, such as EMF.

On the other hand, it has been reported (Tian et al. 2002b) that X-ray irradiation followed by 60 Hz EMF exposures can affect cell cycle distribution and transiently suppress apoptosis in xrs5 cells, which show a defect in rejoining of DNA double-strand breaks. The effect has been proposed to be exerted through EMF-induced decrease in the levels of caspase-3, p21, p53 and phospho-p53 and by increasing Bcl-2 expression. Our present results show that a 50 Hz 100 μT MF induces changes in the cell cycle together with a reduction of spontaneous apoptosis associated with increased Bcl-2 expression in NB69 cells. It is possible that a MF action on p53 and Bcl-2 is responsible for the effects on growth and apoptosis observed in our study. In addition we have investigated possible EMF-induced changes in the activation of the phosphorylated cyclic adenosine monophosphate response-element binding protein (p-CREB). CREB appears to be a primary transcriptional activator of the antiapoptotic gene Bcl-2 (Francois et al. 2000). Inhibition of CREB activity induces apoptosis in sympathetic neurones (Riccio et al. 1997) while CREB overexpression inhibits apoptosis induced by okadaic acid (Walton et al. 1999). Our data show that EMF exposure significantly increases the percent of p-CREB positive cells after 60-minute exposures. These results suggest that CREB may also be involved in the above-described effects of 50 Hz, 100 μT EMF on growth/apoptosis of NB69 cells.

4.1.3.3 Human fibroblasts (Participant 3)

ELF-EMF may not affect the apoptotic process in human fibroblasts after intermittent exposure for 24 hours at a flux density of 1 mT.

No differences in cell count between exposed and sham exposed human fibroblasts after any exposure duration could be detected. Therefore, an elimination of cells by apoptosis and cell death during ELF-EMF exposure can probably be ruled out (3.1.1.1).

4.1.3.4 Summary (Participant 1)

As discussed by Participant 5 (4.1.3.2), data reported in the scientific literature on possible effects of ELF-EMF on the apoptotic process are inconsistent. In many studies available to date, inhibition of apoptosis, enhancement of apoptosis and no effect at all have been reported. Most recently, Lai and Singh (2004) found a significant increase both in apoptosis and in necrosis in brain cells of rats after in vivo exposure to ELF-EMF which they explained by an increase in free radicals. Kim et al. (2004) demonstrated that apoptosis in testicular germ cells of mice can be induced by continuous exposure to ELF-EMF (60 Hz, 0.1 and 0.5 mT). The REFLEX findings did not show a significant effect of ELF-EMF on apoptosis in human fibroblasts (3.1.1.1, 4.1.3.3), embryonic stem cells (3.1.4.1, 4.1.3.1), human lymphocytes (3.1.2.3) and neuroblastoma cells (3.1.2.4).

On the other hand, some influence of ELF-EMF on the apoptotic process cannot be excluded at present. Participant 5 observed an inhibition of the spontaneous apoptosis in neuroblastoma cells which was followed by an increase of the proliferation rate, when the cells were exposed for 63 hours to ELF-EMF at a flux density of 50 or 100 µT (3.1.2.1, 4.1.2.1). A similar phenomenon was also reported by Tokalov and Gutzeit (2003) and Tokalov et al. (2003), who did not observe any direct effect of ELF-EMF on apoptosis in HL-60 cells either, while ELF-EMF protected heat shock treated HL-60 cells from becoming apoptotic, thus enabling cells arrested in the cell cycle to continue the proliferation process.

An answer of what may be the cause for the sporadically observed, but probably not systematically enough studied influence of ELF-EMF on apoptosis may be provided by the REFLEX findings on gene and protein expression. As found by Participant 4 (3.1.4.1, 4.1.3.1), ELF-EMF at a flux density of 2 mT up-regulated in neural progenitor cells the transcript levels of the bcl-2 and the GADD45 gene and down-regulated the transcript levels of the bax gene thus influencing cellular processes, which may result in an enhancement of the anti-apoptotic pathway. Further evidence for the validity of such a hypothesis comes from Participants 3 and 12 (3.1.4.5), who observed a remarkable influence of ELF-EMF on the expression of various genes, including those that regulate cell cycle and apoptosis.

From the physiological point of view, inhibition as well as promotion of apoptosis may be induced by ELF-EMF dependent on the type of cell exposed, its genetic background, its immediate metabolic stage and the pattern of exposure. The mechanisms may follow different routes. It may be possible, that two counteracting mechanisms balance out each other which would result in a zero outcome. Taken together, even if a relationship between ELF-EMF exposure and an inhibition or promotion of apoptosis in in vitro experiments were proven, it would in no way be possible to draw any conclusion for the in vivo situation in man and animal.

4.1.4 Gene and protein expression

4.1.4.1 Mouse embryonic stem cells (Participant 4)

Short-term high intensity exposure to ELF-EMF signals may cause a transient up-regulation of immediate early response and regulatory genes in p53-deficient ES cells.

It was found that a high flux density of 2.3 mT of 50 Hz ELF-EMF signals applied to p53-deficient ES cells at an intermittency scheme of 5 min on/30 min off induced a significant up-regulation of transcript levels of the immediate early growth response gene egr-1. This upregulation was paralleled by a transient upregulation of mRNA levels of the cyclin kinase inhibitor p21 and the AP-1 component c-jun in p53-deficient, but not in wt ES cells. This finding confirms our observation that loss of p53, affects the sensitivity of cells to external stress factors, such as GSM-signals. A correlation between loss of p53

function and external stress-induced expression of egr-1 has also been described by Zhang and Chen (2001), who reported experimental evidence for UV-induced egr-1 expression in p53-deficient mouse cells, whereas the effect was suppressed by functional p53. Our data indicate that a similar egr-1-dependent pathway may be triggered upon ELF-EMF exposure.

The role of c-jun and p21 in these processes has not been clarified so far. Egr-1 cooperates with c-jun in the regulation of DNA synthesis and cell survival in response to ionizing radiation (Hallahan et al. 1995). p21 is implicated in G1 arrest following ionizing radiation-induced DNA damage (Brugarolas et al. 1995). Therefore, one could speculate that the tumor suppressor p53 may be involved in the maintenance of cellular homeostasis of ES cells in response to external stress. However, there are also other data showing that despite abundant quantities of p53 in ES cells, the p53-mediated response is inactive, because of a predominantly cytoplasmic localisation and sequestration of p53 (Aladjem et al. 1998). In spite of this, undifferentiated ES cells are sensitive to DNA damage, because they activate a p53-independent apoptotic response. According to Sabapathy et al. (Sabapathy et al. 1997), the balance between positive and negative regulators of the cell cycle is critical for ES cell differentiation and, if disturbed by exogenous factors, this could lead to the activation of a tumorigenic pathway.

The nature of gene-expression responses to ELF-EMF was short-term only.

In our experiments, cellular responses to ELF-EMF signals were observed only immediately after the end of the 6h exposure and disappeared after an 18h recovery time. Similarly, a 48h exposure to ELF-EMF did not result in gene expression-related responses throughout the differentiation process. These results indicate a short-term nature of cell responses to ELF-EMF and the existence of pathways compensating potential stress-evoked effects of ELF-EMF.

There is some indication that threshold of field flux density exists for ELF-EMF biological effects.

We further investigated the influence of the signal strength and the quality of ELF-EMF exposure on cellular reactions in the ES cell system. Our data indicate the existence of threshold values of field flux density that are needed to evoke biological effects by ELF-EMF. Modifications of transcript levels in p53-deficient cells were observed only upon exposure to ELF-EMF signals applied at a high (2.3 mT) flux density, whereas weaker fields did not cause gene expression-related responses.

ELF-EMF effects in p53-deficient cells were dependent on intermittency cycles (on/off cycle duration).

The exposure protocols of ELF-EMF signals involving either intermittent (on/off cycles) or continuous exposure affected the responses of ES cells. Only an intermittency scheme of 5 min on/30 min off ELF-EMF signals exerted effects on transcript levels, whereas intermittency signals of 5 min on/10 min off exposure or continuous exposure showed no effects on transcript levels of ES cells. These findings demonstrate that a specific intermittency scheme of ELF-EMF exposure may be a critical factor to determine the interference of electromagnetic fields with biological systems (Murphy et al. 2002).

The mechanism of action induced by ELF-EMF exposure of living cells is not yet known.

Several hypothetical models have been proposed to explain the mechanisms of interference of ELF-EMF with biological systems, such as an induction of electric currents by acceleration of ions, resonant interactions involving driving vibrations or orbital transitions in biomolecules (Valberg et al. 1997), biochemical reactions involving free radicals (Brocklehurst and McLauchlan 1996; Eveson et al 2000) or direct interactions of EMF with moving electrons within DNA (Blank 1997). It was also suggested that external oscillating fields cause forced vibrations of free ions of the cellular surface and distort the gating of electro-sensitive channels on the plasma membrane. This would explain, why pulsed electromagnetic fields could have a higher biological activity than continuously applied fields (Panagopoulos et la. 2000, 2002). According to another model (Binhi and Goldman 2000), specific ‘windows’ of the electric-field frequency and amplitude might be predicted. These properties of ELF-EMF could explain the positive results of certain exposure schemes with a specific on/off cycle (in our case, 5 min on/30 min off) and the lack of biological effects at other experimental conditions.

4.1.4.2 Neuroblastoma cells (SY5Y cell line) (Participant 11)

The function of neuronal nicotinic receptors in the brain

Neuronal nicotinic receptors (nAchRs) are a family of ligand-gated cationic channels expressed both in the peripheral and central nervous system where they play a fundamental role in synaptic transmission. At the periphery nAchRs are expressed in post-ganglionic neurons of the autonomic nervous system (Wang et al. 2002 and references therein). In the CNS they seem to be located predominantly at the presynaptic and preterminal parts of the axons where they control the release of a number of different neurotransmitters, such as glutamate, GABA and dopamine (Wonnacott 1997).

nAchRs are composed of different subunits: so far nine ligand binding subunits, alpha 2 - alpha 10, and three structural subunits, beta2 - beta4, have been cloned from different species (Wang et al. 2002). Different combinations of alpha and beta subunits can form different receptor subtypes with their own pharmacological and biophysical characteristics. Neuronal nAchRs are involved in a number of functional processes including cognition, learning and memory (Jones et al. 1999). Alterations in the expression and/or activity of nAchRs have been implicated in different neurological disorders. For instance, mutations in the alpha4 or beta2 subunits produce in humans the autosomal dominant nocturnal frontal lobe epilepsy (Steinlein et al. 1995; De Fusco et al. 2000). Roles for the alpha7 subunit have been suggested in Alzheimer's disease (Dineley et al. 2001) and schizophrenia (Lindstrom 1997; Freedman 1999; Freedman et al. 2000). It has been recently shown that the expression of alpha7 is increased in a well-established mouse model of Alzheimer's disease, whereas the beta-Amyloid (1-42) peptide binds with high affinity to alpha7, suggesting a pathogenetic role for this receptor subtype (Grassi et al. 2003).

Insights into the functional role of nAchRs and their possible involvement in neurological disorders have been obtained by means of knock-out mice (Cordero-Erausquin et al. 2000). By this approach, it has been possible to show that the absence of the beta2 subunit as well as the hyperactivity of the alpha7 subunit are conditions sufficient to promote neurodegeneration. Epidemiological studies have shown that exposure to electromagnetic fields (EMF) might be responsible for neurodegenerative diseases such as Alzheimer's (Sobel et al. 1995, 1996). In light of the role of nAchRs in physiological and pathological conditions, we wondered whether EMF might affect the expression of these molecules. With this aim, we have characterized some neuronal cell lines for their ability to express nAchRs. We have identified some human neuroblastoma cell lines that are currently used to evaluate whether extremely low frequency EMF (ELF-EMF) can interfere with the expression of alpha3, alpha5 and alpha7 nAchR subunits. The expression of these subunits has been studied both at mRNA level by Northern blotting, and at protein level by radioligand assays, upon exposure to different protocol settings.

The function of the cathecolaminergic system in the brain

The cathecolaminergic system is very relevant for many brain functions. Moreover, in the periphery, cathecolamines, in particular norepinephrine, are released by the post-ganglionic neurons of the autonomic nervous system, representing the main neurotransmitters of the ortosympathetic division. In collaboration with Participant 1, we decided to investigate the expression of Dopamine beta-hydroxylase (DßH), the limiting enzyme for the synthesis of norepinephrine, in order to investigate whether ELF-EMF might modify its expression, therefore interfering with autonomic functions as it has been reported in some papers (Kim et al. 2002). With this aim, we carried out Northern blot analyses with RNA extracted from neuroblastoma cells exposed to ELF-EMF.

Finally, we have also been investigating the effects of ELF-EMF on the expression of two transcription factors, Phox2a and Phox2b. These homeodomain proteins are the main regulators of the expression of Dopamine beta-hydroxylase (Yang et al. 1998). In particular, they are responsible for the development of all three divisions of the autonomic nervous system (Lo et al. 1999; Stanke et al. 1999). Indeed Phox2b KO mice fail to develop the whole autonomic nervous system (Pattyn et al. 1999), whereas Phox2a mice show an apparently less severe phenotype, but die the day of birth (Morin et al. 1997). Furthermore, preliminary results from our laboratory have shown that they seem to play a role in the regulation and maintenance of the expression of nAchR alpha3 subunit gene (Flora, personal communication). In order to understand whether ELF-EMF can interfere with the expression of these transcription factors, therefore affecting the formation and function of the autonomic nervous system, we have been carrying out Northern blot experiments to evaluate possible variation in the expression of Phox2a and Phox2b mRNA.

ELF-EMF did not affect the expression of neuronal genes such as nAchRs, DβH, Phox2a and Phox2b, either at mRNA or protein level.

A human neuroblastoma cell line, SY5Y, was used in all the experiments, as it expresses the ganglionic-type nAchR subunits alpha3, alpha5 and alpha7 as well as DßH, Phox2a and Phox2b genes. Cells were

exposed by means of the ELF-EMF generator, setup by Participant 10, under different exposure protocol. The intensity of the electromagnetic field applied was always higher (2 mT and 1 mT) than that of a real life situation, in order to highlight possible, if any, macroscopic effect on gene expression due to ELF-EMF exposure. The duration time of the exposure varied from a relatively short period of time (16h) to a longer period (48h), in order to investigate a time-dependent effect upon exposure to EMF. Finally, the type of exposure, intermittent (5 min on/5 min off) rather than continuous was chosen in order to mimic different kinds of situations that may be encountered during the life-time of an individual. The cells were always collected immediately after the end of the exposure for gene expression analysis, except in one case (1 mT continuous exposure for 48h), when the cells were harvested 48 hours after the end of the exposure, in order to investigate possible indirect effects on the expression of nAchR subunits, DßH, Phox2a and Phox2b, due to the activation of second messenger cascades. We found that exposure of human neuroblastoma cells to continuous (magnetic field intensity of 2 mT and 1 mT) and intermittent (2 mT and 1 mT) low-frequency EMF either for a relatively short period (16h) as well as a longer period (48h) does not seem to influence the expression of neuronal genes for nAchRs, DßH, Phox2a and Phox2b, either at mRNA or protein level. In order to validate these negative results, every exposure condition was tested in at least three to nine independent experiments.

4.1.4.3 Embryonic stem cells of mice during cardiac differentiation (Participant 8)

ELF-EMF up-regulated the expression of cardiac specific genes thus promoting cardiogenesis.

The exposure of EC (P19) cells to ELF-EMF yielded conflicting results and poor reproducibility of the data. On the contrary, the development of a model of in vitro cardiogenesis based on “gene trapping” selection of cardiomyocytes from pluripotent (GTR1) cells provided a potentially homogenous and reproducible approach to assess whether ELF-EMF may afford developmental decisions (i.e. cardiogenesis) in ES cells. In this ES cell model, ELF-EMF afforded a consistent increase in the expression of genes tightly involved in coaxing ES cells to the cardiac lineage. As shown by in vitro run-off analyses, ELF-EMF affected the transcriptional machine of ES cells. These responses led to the expression of cardiac specific genes and ultimately ensued into a high-throughput of cardiogenesis, as shown by the increase in the number of spontaneously beating colonies in ELF_EMF-exposed cells. Failure of ELF-EMF to affect the transcription of a gene promoting skeletal muscle determination and the faint effect on neuronal specification seem to exclude a generalized activation of repressed genes and suggests that coupling of ELF-EMF with GATA-4, Nkx-2.5 and prodynorphin gene expression may represent a mechanism pertaining to ES cell cardiogenesis.

4.1.4.4 rCx46 in oocytes of *Xenopus laevis* (Participant 7)

The influence of ELF-EMF exposure on the expression of rCx46 in single and paired oocytes of *Xenupous laevis* was analysed. Especially the expression level as well as the corresponding regulatory properties of conducting hemi-channels and cell-to-cell channels (Bruzzone et al., 1996) were studied. ELF-EMF exposure neither significantly influenced the expression level of conducting hemi-channels composed of rCx46 (Figures 59 to 61), nor their gating properties by voltage, pH, Ca2+ (Figure 64). A similar result was found for cell-to-cell channels, which could be formed by pairing of oocytes expressing rCx46 (Figure 65). This finding is in contrast to the observation that in general ELF-EMF exposure causes a decrease of cell-to-cell coupling (Hu et al. 2001; Lohmann et al. 2003; Trosko and Ruch 1998; Vander Molen et al. 2000; Yamaguchi et al. 2002; Zeng et al. 2003), but different regulatory mechanisms were suggested. It was proposed that ELF-EMF increases Ca^{2+}-influx which in turn inhibits gap junctional coupling in synovial fibroblasts (Marino et al. 2003). But in osteoblast like cells such an increase of Ca^{2+} was not observed, despite the finding that ELF-EMF induced a decrease of gap junctional coupling (Yamaguchi et al. 2002). In contrast to further reports (Lohmann et al. 2003; Zeng et al. 2003) the authors showed that ELF-EMF does also not effect the distribution of the corresponding membrane protein connexin (Cx43) between the cytoplasmic and the membrane pool. Therefore, a change in the state of Cx-phosphorylation was considered as target of ELF-EMF exposure causing a decrease of cell-to-cell coupling (Yamaguchi et al. 2002, see also Lacy-Hulbert et al. 1998). By ELF-EMF exposure of oocytes expressing rCx46 cytoplasmic free Ca^{2+} and/or signal transduction pathways involved in protein phosphorylation also of rCx46 virtually remain unchanged. This conclusion can be drawn from the unchanged behaviour of the leak-current of the oocytes in the absence and presence of

ELF-EMF (Figure 58). The leak-current includes the sum of all electrogenic transport systems which are known to partially depend on cytoplasmic free Ca^{2+} and protein phosphorylation. At present the origin for the different response of cell systems expressing Cx43 (Hu et al. 2001; Lohmann et al. 2003; Trosko and Ruch 1998; Vander Molen et al. 2000; Yamaguchi et al. 2002; Zeng et al. 2003) and oocytes expressing rCx46 at ELF-EMF exposure remains unsolved.

4.1.4.5 Whole-genome analysis of various cell lines exposed to ELF-EMF (Participant 12)

If we look on the numbers in Table 12, it is obvious that members of some gene families are regulated predominantly. Moreover, repetitions of experiments with the same cell line and the same exposure conditions look more similar than repetitions with different cell lines or different exposure conditions. This might tell us that obviously something is happening on the gene-expression level after ELF-EMF exposure. Otherwise, if we only would see experimental variances (differences in experimental procedures, cell cycle stages, etc.), we would expect about the same numbers in each experiment, or higher similarities between same experiments as between different cell lines.

The results with the different cell lines obviously have not the same quality. For example, the results of profiling 1 and 2 of the fibroblasts (Participant 3) seem to be more similar than between the experiments between cells with differentiating potential. Also genes of different gene families react differently on certain influences. Whereas for example structural proteins like cell adhesion proteins are regulated slowly, certain proto-oncogenes like c-fos, c-fos or actin can be regulated within 10 to 30 min of growth factor addition (Quantin and Breathnach 1988). Moreover, the situation is different here from, for example, a disease situation with a certain defect in a single gene. We deal with environmental influences here, which are complex and variable. Even adaptation to the electromagnetic fields after some hours due to changes in gene expression cannot be excluded.

How the potential molecular changes after ELF-EMF exposure are regulated, remains speculative. However, if we look on the genes extracted by the bio-statistical analysis in more detail, some interesting points become obvious: A remarkable number of members of the actin cytoskeleton and associated proteins are down-regulated (also in RF-EMF experiments). Remarkably, in ELF-EMF treated cells (Participant 3) the actin-associated proteins obviously down-regulated seem to be regulated by Ca, and several Ca regulators were also down-regulated in our experiments. This would mean that the actin cytoskeleton as far as regulated by Ca is down-regulated. In addition to Ca^{2+}-associated proteins, proteins associated with other cations like Fe+, K+, and H+ are down-regulated. More experiments will be necessary for showing if these proteins might be involved in signalling or energy metabolism after ELF-EMF exposure.

4.1.4.6 Summary (Participant 1)

From the REFLEX data, the conclusion must be drawn that ELF-EMF may affect gene and protein expression in various cell systems. Based on the results of the genome analysis of human fibroblasts as carried out by Participant 12 (3.1.4.5), ELF-EMF appears to regulate the expression of a series of genes and proteins such as mitochondrial and ribosomal genes and Ca-, cell cycle-, apoptosis-, extracellular matrix-, and cytoskeleton-related genes. In particular, a number of G proteins and calcium associated proteins involved in signal transduction seem to be strongly regulated by ELF-EMF. Since the variances between the experiments were high, the significance of these findings is limited. Participant 4 observed a transient up-regulation of early response and regulatory genes only in embryonic stem cells deficient for p53 and not in wild type cells after ELF-EMF exposure. This suggests that the genetic background affects the responsiveness of the cells (3.1.4.1, 4.1.4.1). Participant 8 found that ELF-EMF up-regulates the expression of cardiac specific genes in cardiomyocytes derived from embryonic stem cells thus promoting cardiogenesis (3.1.4.3, 4.1.4.3). All these findings were obtained after ELF-EMF exposure at rather high flux densities. It remains, therefore, an open question whether or not these in vitro results are of any significance for the real life exposure of man and animal.

The REFLEX data on gene and protein expression due to ELF-EMF exposure are in line with the results of a series of studies already published in the literature. Goodman et al. (1994) and Lin et al. (2001) reported increased hsp70 transcript concentrations in HL-60 cells after exposure to weak ELF-EMF (60 Hz). Tokalov and Gutzeit (2004) observed an increase in several heat shock proteins in HL-60 cells after

exposure to ELF-EMF (50 Hz, 60 µT, 30 min), which was comparable to that after exposure to heat (41°C, 30 min) or X-ray (200 kV, 5 Gy). Most recently, Zeng et al. (2004a) and Xu et al. (2004) demonstrated that ELF-EMF (50 Hz, 0.4 mT, 24h) altered the signal transduction-related protein expression in human breast cancer cells (MCF-7). Mannerling et al. (2004) who studied the hsp70 expression in several human cell lines reported an increased expression after exposure to ELF-EMF (50 Hz, 0.1 or 0.2 mT, up to 24h). Of course, the expression of genes and proteins induced by ELF-EMF may again be dependent on the type of cell exposed, its genetic background and its immediate metabolic stage and, of course, the pattern of exposure. The available data indicate that the flux density (threshold) at which effects on gene and protein expression are at first found is in the range or not far above the presently valid safety levels of 100 µT for the general public or 400 µT for the workplace.

4.2 Results obtained after RF-EMF exposure

4.2.1 Genotoxic effects

4.2.1.1 Human promyelocytic cell line HL-60 (Participant 2)

Discussion on potential health effects of using mobile telephones has focused on possible cancer-enhancing effects. It seems quite clear that any cancer-related effects of radiofrequency electromagnetic waves cannot be based on direct genotoxic effects, since the energy level is not high enough to damage DNA. Most of RF-field studies concluded that RF-field exposure is not genotoxic or mutagenic. With respect to DNA strand breaks, there is no replicated evidence for DNA and/or repair damage due to RF-field exposure (Lai versus Malayapa, examples see literature cited). On the other hand, some studies have shown that radiofrequency-field/microwave (RF-fields/MW) radiation and extremely low frequency (ELF) fields cause increased DNA strand breakage and chromosome aberrations. This has been shown in cell lines (Phillips et al. 1998), human blood (Verschaeve et al. 1994), animals (Lai and Singh 1995, 1996a/b, 1997a/b/c, 2004) and living human beings (Fucic et al. 1992; Garaj-Vrhovac 1999). The basic strategy in our studies was to test whether RF-EMF are able to alter DNA integrity (MN induction and DNA strand breakage), cell proliferation, cell cycle kinetics and/or apoptosis using the promyelocytic leukaemia cell line HL-60 testing different SAR levels, exposure times and signal modulations. For the experiments a highly standardized exposure system setup was provided by Participant 10. This setup enabled the exposure of suspensions of cells with a highly standardized temperature constancy, an inhomogeneity of SAR of less than 30% and an efficiency of more than 20 W/kg per Watt input power. All experiments were performed blinded, i.e. not knowing, which of the waveguides was exposed to the RF-field and which was the sham control.

RF-EMF exposure for different SAR and different exposure times (1800 MHz, continuous wave) led to the induction of single and double DNA strand breaks.

DNA damage through RF-EMF was evaluated immediately after exposure using the alkaline single cell gel electrophoresis assay (Comet assay). RF-fields at 1800 MHz, continuous wave exposure for different exposure times caused the induction of single and double DNA strand breaks in HL-60 cells. No significant difference was seen between exposed and sham exposed cells at a SAR of 0.2 to 1.0 W/kg. An increase in the steepness of the dose response relation is observed between SAR 1.0 W/kg and 1.3 W/kg. A less expressed increase is observed between 1.6 W/kg and 3.0 W/kg (Figure 73).

Two other laboratories have recorded that RF-field/MW produced significant DNA stands breaks. Verschaeve et al. (1994), who used a GSM cell phone signal to expose human and rat peripheral blood lymphocytes, found significantly increased strand breaks at high, but non-thermal exposure levels. Phillips et al. (1998) exposed Molt-4 T-lymphoblastoid cells with cell phone radiation in the SAR range 0.0024 W/kg to 0.026 W/kg. A 2-hour exposure to these low levels of cell phone radiation significantly increased or decreased the DNA damage. Decreased DNA damage is evidence of increased repair that is, of course, evidence of damage (Meltz 1995). In some other studies the observations of significant increase in DNA single and double strand breaks in brain cells of rats whole body exposed to 2.45 GHz RF-field (Lai and Singh 1995, 1996a/b, 1997a/b) were not confirmed using rodent and human cells

exposed in vitro and in vivo to RF-fields (Malayapa et al. 1997, 1998; Maes et al., 1997; Vijayalaxmi et al., 2000; Li et al., 2001).

RF-EMF exposure for different SAR and different exposure times (1800 MHz, continuous wave) led to an increase in micronuclei.

Micronuclei are easily measured under day light microscopy. They consist of small amounts of DNA that arise in the cytoplasm when chromatid/chromosomal fragments or whole chromosomes are not incorporated into daughter nuclei during mitosis. We have used the conventional cytokinesis-block MN assay to assess induction of cytogenetic damage in HL-60 cells after exposure to RF fields. RF-EMF exposure on HL-60 cells at 1800 MHz, 24h, continuous wave, at the given experimental conditions, caused a significant increase of micronuclei induction in the same SAR-dependent manner as observed for the induction of DNA strand breaks. Whereas at a SAR of 1.0 W/kg no significant difference of micronuclei frequencies was noted compared to sham controls, a clear increase was observed at SAR of 1.3 W/kg and 1.6 W/kg, and, less expressed at a SAR of 2.0 W/kg and 3.0 W/kg (Figure 72).

Induction of both, micronuclei and Comet formation, by RF-EMF was dependent on the time of exposure. A short exposure period of 6 hours caused no increase in MN frequencies compared to longer exposure times of 24 and 72 hours, respectively. Exposure to a 1800 MHz magnetic field at SAR of 1.3 W/kg for 72 hours produces a similar micronucleus frequency in HL-60 cells as that caused by 0.5 Gy ionising radiation (exposure time: 5.2 s), i.e., an average of 22 MN per 1000 BNCs (Figures 75, 76). However, it is not likely that the two entities cause MN induction by similar mechanism and produce the same types of DNA damage. In contrast to these findings, Comet formation already started after short exposure periods of 2 and 6 hours, respectively, with a maximum after 24 hours, and a clear decline occurred towards a longer exposure period of 72 hours. Mechanistically, this finding may be explained by DNA repair phenomena in the case of the DNA strand breakage in contrast to MN induction.

On the other hand, data from several other studies have indicated in primary human lymphocytes an absence of significant differences in the incidence of CA, SCE and MN between RF-EMF-exposed and sham-exposed cells (Vijayalaxmi et al. 1997, 2001a/b; Bisht et al. 2002). The significant increase and a weak effect on sister chromatid exchanges in RF-field exposed human blood lymphocytes reported by Maes et al. (1996, 1997) was not confirmed in their own subsequent investigation (Maes et al. 2001). Some positive findings occurred under conditions in which RF exposure elevated the temperature (Manikowska-Czerska et al. 1985; Sarkar et al. 1994; Varma and Traboulay 1997).

RF-EMF-associated increase of DNA strand breaks and micronuclei (1800 MHz, 1.3 W/kg, 24h) in HL-60 cells was signal-independent.

Interestingly, DNA strand breaks and MN induction were similarly induced by different RF-EMF signal modulations including CW exposure, CW intermittent exposure (5 min on/10 min off), 217 Hz pulse modulation and GSM Talk each at 1800 MHz, SAR 1.3 W/kg for 24h (Figure 77, 78).

RF-EMF induced formation of reactive oxygen species as shown by flow cytometric detection of oxyDNA and rhodamine fluorescence.

ROS, including superoxide anion (O^-_2), hydrogen peroxide (H_2O_2), hydroxyl free radical (OH·) and singlet oxygen ($^1O_2$), continuously generated from the mitochondrial respiratory chain, own a powerfully oxidative potential. ROS are capable of attacking lipids, nuclear acids and proteins, resulting in certain degrees of oxidative damage. The total ROS level in resting HL-60 cells, however, was directly measured in the present study, by flow cytometric detection of Rh123 and the oxidized nucleotide 8-oxoguanosine (Figures 86, 87). Detecting the ROS level by flow cytometry has been a novel approach with the characteristic of fastness, convenience and reproducibility and, to our knowledge, has not been frequently reported before. DHR123, one of common ROS captures, is membrane permeable. It is oxidized intracellularly by ROS to become fluorescent Rh123, which is pumped into mitochondria and remains there. After a period of accumulation it is then detectable by flow cytometry (e.g., Gao et al., 2002) The probe used in the Calbiochem OxyDNA Assay kit is specific for 8-oxoguanine, which, as part of the oxidized nucleotide 8-oxoguanosine, is formed during free radical damage to DNA and is thus a sensitive marker for differences of ROS levels (de Zwart et al. 1999; Kasai 1997; Cooke 1996) in HL-60 cells after exposure to RF-EMF compared to control and sham-exposed cells (Figure 86).

If an involvement of free radicals in the mechanism of RF-EMF induced DNA strand breaks in HL-60 cells could be shown, this would have an important implication on effects to cell integrity due to RF-EMF

exposure. The “free radical hypothesis” stating that EMF increase free radical activity has been proposed by various researchers (Grundler et al. 1992; Reiter 1997; Lai and Singh 2004). Involvement of free radicals in human diseases, such as cancer and atherosclerosis, have been suggested (Beckmann and Ames 1997). Free radicals also play an important role in aging processes (Reiter 1995).

Co-administration of ascorbic acid, a free radical scavenger, inhibited the effects of RF-EMF on HL-60 cells and may, thus, decrease DNA damage without affecting cellular growth.

Two plausible biological mechanisms involving free radicals have to be discussed for the RF-EMF effect. The first involves increased free radical formation and activity and genetic damage as a response to RF-field exposure. The second involves increased free radical activity and genetic damage because of an induced reduction of free radical scavenger, e.g. reduced SOD activity or melatonin (Reiter 1994). Indications were found in our investigations for increased free radicals activities and a correlation with genetic damage (Figures 86 to 89). Cells possess efficient antioxidant defence systems, mainly composed of the enzymes such as superoxide dismutase, glutathione peroxidase, and catalase, which can scavenge the ROS excessive to cellular metabolism, and make ROS level relatively stable under physiological conditions. Under the conditions used in our experiments, endogenous antioxidant enzyme activities of HL-60 cells (SOD and GPx activity) did not show pronounced alterations following RF-field exposure as compared to sham-exposure. Therefore, the first proposed mechanism mentioned above seems to be dominant.

In summary, the findings of an increase of micronuclei induction as well as Comet formation in HL-60 cells after exposure to RF-EMF at the conditions stated above indicate, that RF-EMF might generate genotoxic effects. The results obtained clearly show that RF-EMF under distinct exposure conditions cause DNA damage in human HL-60 cells. Since on the basis of these data RF-EMF have to be regarded as potentially genotoxic, it is pivotal to clarify first the molecular mechanisms involved in these potentially clastogenic effects in forthcoming experiments and secondly the biological consequences of DNA damage induced by RF-EMF, in particular the relevance for inducing mutations and changes in cellular signalling cascades. Responsive to the European Commission´s suggestions, additional independent verification experiments of the results obtained so far have to be conducted in the same cell line and other cell types, which are normal or similar in the metabolic process. Studies on indirect genotoxicity (e.g., reactive oxygen species, oxy-DNA, DNA repair) of RF-EMF on HL-60 cells, have to be extended. Studies on potential changes in gene expression profiles with respect to DNA repair have to be continued in co-operation with other groups.

4.2.1.2 Human fibroblasts and granulosa cells of rats (Participant 3)

RF-EMF generated DNA strand breaks in granulosa cells of rats and DNA strand breaks and chromosomal aberrations in human fibroblasts.

We could demonstrate an induction of DNA single and double strand breaks upon RF-EMF exposure in human diploid fibroblasts and in rat granulosa cells in culture. This induction depended on exposure duration as well as on the applied signal and could be determined in cells of different tissues. Based on the findings which we obtained with ELF-EMF, we also used for the intermittent RF exposure an “on” duration time of 5 minutes and an “off” duration time of 10 minutes. In contrast to ELF-EMF, RF-EMF induced DNA strand breaks also under continuous exposure conditions. However, the effects were more pronounced under intermittent exposure conditions at 5 min on /10 min off cycles.

The identification of the processes which lead to this DNA breakage will help to determine the extent of biological effects induced by RF-EMF exposure. Importantly, cellular effects observed in this study started already at an SAR of 0.3 W/kg which is far below 2 W/kg, the highest level allowed by the European safety limits. This suggests that the currently allowed radiation emission levels for the mobile phones, are clearly not sufficient to protect from biological effects. We have demonstrated, that the effect of ELF-EMF depends on the cell type and the on and off duration times used in research. The negative effects of RF-EMF reported in the literature (McNamee et al. 2002a, b; Tice and Hook 2002; d'Ambrosio and Scarfi 2002a), however, are based on lymphocytes and continuous exposure.

As with Elf-EMF, RF-EMF exposure of human fibroblasts induced also an increase in micronuclei and an even higher incidence of chromosome gaps, chromosome breaks, dicentrics and acentric fragments, which was 10-fold after ELF-EMF exposure as compared to control cells and 100-fold after RF-EMF

exposure. The RF-EMF results regarding chromosomal aberrations are of preliminary nature, but they are in line with the results obtained after ELF-EMF exposure. The evaluation of the micronulei carried out in our laboratory was reproduced blindly with coded slides in two independent laboratories that do not belong to the REFLEX consortium (Universities of Ulm and Kaiserslautern, Germany).

RF exposure revealed a significant decrease in the mitochondrial membrane potential in one experiment, which could not be reproduced. The RF induced formation of DNA strand breaks could not be related to changes in the membrane potential.

4.2.1.3 Mouse embryonic stem (ES) cells (Participant 4)

RF-EMF exposure of ES-derived neural progenitor cells induced a low transient increase of double DNA strand breaks measured by the neutral Comet assay.

Since we observed an up-regulation of GADD45, which is a DNA-damage inducible gene, it was logical to test the induction of primary DNA damages. It has been shown previously that EMF exposition of human HL-60 cells resulted in an increase of DNA breaks, suggesting a direct mutagenic effect (Ivancsits 2002). In addition, a correlation was found between up-regulation of GADD45, of the bcl-2 family member bcl-$X_{L,}$ and an increased amount of early DNA damage measured by the alkaline Comet assay in human preneuronal cells exposed to the amyloid protein (Santiard-Baron 2001). Therefore, we used the alkaline and neutral Comet assay to detect single, and double-strand DNA breaks, resp., in neural progenitors derived from murine pluripotent ES cells after RF-EMF exposure. Under our experimental conditions, 6 hours exposure to GSM signals induced a low transient increase of double-strand DNA breaks, whereas ELF-EMF did not induce a significant DNA damage. Our finding suggests that genotoxic effects of RF-EMF, at least *in vitro*, could not be excluded.

4.2.1.4 Summary (Participant 1)

As discussed by Participant 2 (4.2.1.1) there is sporadic literature about in vitro studies demonstrating that RF-EMF may possess a genotoxic potential (The Royal Society of Canada 1999; Stewart Report 2000). Since the energy impact on the genome of livings cells exposed to RF-EMF was calculated to be too low to cause DNA damage and since the mainstream literature contradicted the assumption of genotoxic effects (Moulder et al. 1999; Meltz 2003), these sporadic findings were considered more or less meaningless. Opposite to this widely accepted view, the data of the REFLEX study which were elaborated in a hitherto unknown systematic approach and confirmed in four laboratories, of which two were not members of the REFLEX consortium, support the view that RF-EMF causes genotoxic effects in certain, if not all cellular systems.

Based on the methodology used and the data obtained in the REFLEX study, the findings on genotoxicity caused by RF-EMF are hard facts. RF-EMF exposure at a SAR value below 2 W/kg induced an increase in DNA single and double strand breaks as well as in micronuclei in HL-60 cells. The DNA damage was dependent on the time of exposure, the field strength of RF-EMF and the type of RF-EMF signals. There is some indication that the effects may be caused via an increase in free oxygen radicals generated by RF-EMF (3.2.1.1, 4.2.1.1). RF-EMF exposure between SAR values from 0.3 to 2.0 W/kg made also DNA single and double strand breaks in human fibroblasts and in granulosa cells of rats dependent on the exposure time and the type of signals. This increase of DNA-strand breaks in human fibroblasts was accompanied by an increase in micronuclei and in chromosomal aberrations thus demonstrating that the DNA repair was not error-free (3.2.1.2, 4.2.1.2. In addition, RF-EMF exposure at a SAR value of 1.5 W/kg caused a slight, but significant increase in DNA double strand breaks in embryonic stem cells of mice (3.2.1.3, 4.2.1.3).

As already stated, for energetic reasons, RF-EMF can neither denature proteins nor damage cellular macromolecules directly. If the energy impact on the genome of living cells exposed to RF-EMF is too low for a DNA damage, the genotoxic alterations observed in the REFLEX project must be produced indirectly through intracellular processes in the course of RF-EMF exposure. In their experiments Participant 2 observed an increase of free radicals in HL-60 cells after RF-EMF exposure. With the oxygen radical scavenger ascorbic acid was it possible to inhibit the generation of DNA strand breaks and of micronuclei during RF-EMF exposure (3.2.1.1, 4.2.1.1). This findings support the assumption that the observed DNA damage may be caused by free oxygen radicals which are released by RF-EMF during

exposure. This possibility is further strengthened by the observation of Lai and Singh (1997a,b), who demonstrated that the increase in single and double DNA strand breaks in brain cells of RF-EMF exposed rats can be blocked with radicals scavengers. A final conclusion whether or not this finding is indisputable is still pending, since an increase in DNA strand breaks at the same model could not be confirmed by another research group (Malyapa et al. 1997, 1998).

Taken together, RF-EMF is able to damage the genome at least in certain cell systems after exposure in vitro. As with ELF-EMF, the genotoxic effects of RF-EMF may be best explained indirectly by an RF-EMF induced intracellular increase in free radicals. It is well known that a balanced free radical status is the prerequisite for maintaining health and that an unbalanced free radical status promotes the process of ageing and the development of chronic diseases such as cancer and neurodegenerative disorders. Whether the balance of free oxygen radicals can also be impaired through RF-EMF in vivo as suggested by the work of Lai and Singh (1997a,b) needs further clarification.

4.2.2 Cell proliferation and differentiation

4.2.2.1 NB69 neuroblastoma cells and neural stem cells (NSC) (Participant 5)

RF-EFM did not affect cell growth of NB69 and neural stem cells.

A short-tem (24h) exposure to the GSM-Basic signal does not modify the cell growth of NB69 cells and NSC. However, as described in 4.2.4.2, this signal induced in both cell a reduction in the proportion of cells expressing FGFR1. Signalling through fibroblast growth factor receptors (FGFRs) is essential for many cellular processes, including proliferation and differentiation (Kovalenko et al., 2003) and nervous system development (Oh et al. 2003). Our results indicate that in the selected exposure conditions, the GSM-basic signal does not induce changes in cell proliferation. Also, the short-term response induced by this GSM-signal on FGFR1 does not seem to be related to changes in cell growth.

4.2.2.2 Human lymphocytes and thymocytes (Participant 8)

RF-EMF may not affect proliferation, cell cycle, apoptosis and activation of human lymphocytes and thymocytes.

The immune system plays a decisive role in health and disease. Therefore, it was important to find out whether or not RF-EMF affect the immune system. Lymphocytes were exposed to RF-EMF at 1800 MHz with three different signals, such as GSM basic, Talk modulated and DTX only (SAR 1.4 - 2 W/kg).The in vitro tests were chosen in order to study the following endpoints: 1) cell proliferation; 2) cell cycle; 3) expression of membrane receptors on T lymphocytes, 4) spontaneous and induced apoptosis; 5) mitochondrial membrane potential (MMP) modifications in induced and spontaneous apoptosis ; 6) cytokine production; 7) Hsp70 levels in induced and spontaneous apoptosis; 8) thymocyte development and apoptosis; 9) T lymphocyte gene expression.

On the whole, the results obtained suggest that no differences exist for the most endpoints studied in RF-EMF exposure. Only some slight differences were observed in PBMCs; in particular, CD8+CD28+ appeared increased in exposed cultures, but the difference (3%) of the order of the calculated standard error did not indicate a relevant effect from a biological point of view. Actually, in a previous work we found that 900 MHz (SAR 76 mW/kg) RF seem to slightly decrease lymphocyte proliferation when these cells are low-stimulated (Capri et al., accepted 2004); thus our results suggest that RF effects on lymphocyte proliferation are frequency-dependent. However, the literature on this field is still scanty. Some groups showed different effect on cytolitic T lymphocyte proliferation (Cleary et al. 1996) and some groups did not found significant effects on mitotic indices between RF-exposed and sham-exposed lymphocytes (Vijayalaxmi et al. 1997).

A more interesting result appears the decrease of CD95 molecules on membrane surface of stimulated CD4 helper T cells, from elderly donors, which was found when cells were exposed to Talk modulated RF in comparison with sham exposed cells. Due to the importance of this receptor in the regulation and homeostasis of immune response, these results deserve further evaluations to confirm this decrease (around 9%) on CD^{4+} helper T lymphocytes from elderly and not from young donors.

An important observation was the observed decrease (around 13%) of IL-1 b production; this effects was found only in low-stimulated PBMCs exposed to DTX RF and suggest that a possible cell target of RF-EMF are monocytes rather than lymphocytes. Also this effect deserves further investigation in order to confirm possible interactions of RF-EMF exposure with human monocytes. Data in the literature are really scanty. A recent study, performed in vivo, demonstrated a transient increase of interferon-g (IFN-g) in mice exposed to GSM-modulated 900 MHz in mice exposed 2 hours/day for 1, 2 and 4 weeks in a TEM cell (Gatta et al. 2003)

Negative results are extremely important for evaluations on human health risk. RF-EMF exposure is obviously not able to interfere with cell cycle, spontaneous or chemically- induced apoptosis, mitochondrial membrane polarisation and cell activation. Negative results were also obtained on thymocyte development. This last result is extremely important, since it was observed in conditions very near to what happens in vivo. Moreover, results from gene expression of quiescent T lymphocyte confirm the absence of significant changes due to RF-EMF exposure.

4.2.2.3 Human promyelocytic cell line HL-60 (Participant 2)

RF-EMF generated genotoxic effects in HL-60 cells within a narrow energy window without affecting cell proliferation, cell progression and apoptosis.

Using the MTT assay, the annexin V assay, the TUNEL assay, cell counting, determination of cellular doubling time and thymidine kinase activity, it could be shown that the RF-field at 1800 MHz, SAR 1.3 W/kg and 24h exposure did not effect cell viability and cell growth, and did not induce apoptosis in HL-60 cells. These findings are in substantial agreement with previous literature reports on effects of RF-EMF in HL-60 cells and other human cells (e.g., Hambrook et al. 2002, Higashikubo et al. 2001). In contrast to the present results, induced cell proliferation and apoptosis have been reported in various other cell types after exposure to EMF (Blumenthal et al. 1997, Philips et al. 1997, Ismael et al. 1998, Kwee and Raskmark 1998, Simko et al. 1998, Velizarov et al. 1999).

4.2.2.4 Mouse embryonic stem (ES) cells (Participant 4)

RF-EMF exerted no influence on ES-derived cardiogenesis and did not affect DMSO-induced cardiac differentiation, proliferation and expression of regulatory genes in P19 EC cells.

Several in vitro studies report negative effects of high frequency EMF on cell cycle, gene expression and differentiation (Fritze et al. 1997; Cain et al. 1997; Goswami et al. 1999; Ivaschuk et al. 1997), DNA and chromatin structure (ICNIRP 1996; Repacholi 1998) and rat embryo development (Klug et al. 1997). In contrast, several reports described positive effects by high-frequency EMF exposure on the length of cell cycle phases, proliferation and gene expression levels in mammalian cells (Cleary et al. 1996; Czerska et al. 1992; Goswami et al. 1999; Lai and Singh 1996a; Sarkar et al. 1994). These studies, however, were performed with different experimental models, carrier frequencies (835 MHz to 2.45 GHz versus 1.71 GHz used in our study) and modulation schemes, and therefore, are not comparable. Moreover, positive RF-EMF effects were often observed at relatively high average SAR values (Cleary et al. 1996; Czerska et al. 1992; Fritze et al. 1997), which suggests that they could arise from RF-EMF-evoked thermal effects. In our studies, GSM signals were applied under conditions of the ICNIRP safety limit using an experimental set-up that enabled precise temperature control (Schönborn et al. 2000), and any temperature increase as a consequence of EMF exposure (Laurence et al. 2000) can be excluded.

For the evaluation of embryotoxic effects of chemical compounds in vitro, the mouse embryonic stem cell test (EST, (Spielmann et al. 1997) using cardiac differentiation of ES cells as endpoint has been established. Therefore, for a further specification of the effects of GSM-217 signals, we analysed EMF exposure during the process of cardiac differentiation. GSM-217 EMF exerted no influence on ES-derived cardiogenesis and did not affect DMSO-induced cardiac differentiation, proliferation and expression of regulatory genes in P19 EC cells. These data present evidence that wild-type ES cells are not sensitive during cardiac differentiation to EMF. However, this finding is in contrast to EMF-induced effects in ES-derived neural progenitors.

The differentiation process in cells is affected by RF-EMF exposure, when applied at the neural progenitor stage.

The intact nervous system might be very sensitive to induced electric fields and currents, due to the high level of spontaneous activity and the greater number of interacting neurons. It has been suggested that induced current densities above 10 mA/m^2 may have effects on some central nervous system functions (Saunders and Jefferys 2002). Because of the special public concern for neurotoxicity due to EMF exposure, we used an experimental protocol successful at selectively differentiating ES cells into the neural lineage (Rolletschek et al. 2001). It provides a tool to investigate in vitro neuropathogenic effects of environmental factors during early development. We exposed the cells to EMF during the differentiation stage when the first neural nestin-positive progenitors appear. This developmental stage is presumably very sensitive to environmental factors. In our experiments, we observed RF-EMF effects on neural differentiation. Among the investigated transcripts (the mRNA levels of the neuronal genes TH, Nurr1 and en-1, and the astrocyte-specific gene GFAP) we observed a statistically significant down-regulation of nurr at 4d+11d and TH at the terminal stage 4d+23d. This might indicate a delayed neural differentiation and would correlate with the up-regulation of the growth arrest gene GADD45 at terminal stage. The significant up-regulation of GADD45 at the terminal stage 4d+23d was also confirmed by quantitative RT-PCR with TaqMan probe. Bcl-2, whose transcript levels were found increased in our study, has also been shown to be involved in neuronal differentiation and axonal regeneration (Daadi et al. 2001). Human teratocarcinoma-derived neurons expressed bcl-2 in 85% of the implanted neurons after transplantation into the rat striatum. In addition, the in vitro induction into the neuronal lineage resulted in an up-regulation of bcl-2 expression. The authors suggested that neuronal differentiation could be mediated at least partially by bcl-2 (Daadi et al. 2001).

Since we observed an up-regulation of GADD45, which is a DNA-damage inducible gene, it was logical to measure the eventual induction of primary DNA damages (4.2.1.3)

4.2.2.5 Summary (Participant 1)

As discussed by Participant 4 (4.2.2.4), the results on possible effects of RF-EMF on cell proliferation and differentiation in vitro which are reported in the literature (The Royal Society of Canada 1999; Stewart Report 2000) are controversial. The REFLEX data do not reveal a significant effect of RF-EMF on proliferation and differentiation of various cell systems such as neuroblastoma cells (NB69) and neuronal stem cells (3.2.2.1, 4.2.2.1), embryonic cancer cells (P19) (3.2.4.1, 4.2.2.4), human lymphocytes and human thymocytes (3.2.2.2, 4.2.2.2) and HL-60 cells (3.2.2.3, 4.2.2.3). In neural progenitor cells only some effect on the differentiation process was observed at a SAR level of 1.5 W/kg (3.2.4.1, 4.2.2.4). Quite obviously, whether or not living cells respond to RF-EMF exposure in vitro may depend on the type of the cell, its genetic background, its metabolic state and, of course, on the exposure conditions.

An answer of what may be the reason for the sporadically observed, but until now not confirmed influence of RF-EMF on cell proliferation and differentiation may be provided by the REFLEX findings on gene and protein expression. As shown by Participant 5, RF-EMF reduced the expression of the receptor FGFR1 of fibroblast growth factor (FGF) in the human neuroblastoma NB69 cell line and in neural stem cells from rat embryonic nucleus striatum (3.2.4.2, 4.2.4.2). Participant 3 (3.2.4.3, 4.2.4.3), Participant 6 (3.2.4.6, 4.2.4.6), and Participant 12 (3.2.4.7, 4.2.4.7) observed, that RF-EMF enhanced the expression of various genes among them ribosomal and mitochondrial genes, ATP related genes and genes encoding calcium-associated proteins and cell cycle proteins.

Of course, the relationship between RF-EMF exposure and the acceleration or inhibition of cell proliferation and differentiation in vitro caused by alteration of gene and protein expression is not proven yet. Should that be shown one day, it is to be found out, whether such cellular events occur also in vivo in RF-EMF exposed man and animal. The most recent data of Weisbrot et al. (2003), who observed an increase in the numbers of off-springs, an elevation of the hsp70 levels, an increase in serum response element (SRE) DNA-binding and an induction of the phosphorylation of the nuclear transcription factor, ELK-1, in Drosophila melanogaster after RF-EMF discontinuous exposure (900/1900 MHz, 1.4 W/kg) during the 10 day developing period, speak in favour of such an assumption.

4.2.3 Apoptosis

4.2.3.1 Brain cells of different origin and human monocytes (Participant 9)

There is no indication that apoptosis is affected in nerve and immune cells after exposure to GSM-like RF-EMF.

Beside the importance of the apoptotic process in cellular homeostasis, only a few papers are available in the literature on the effects of ELF-EMF on apoptosis and almost no data were published on the interaction of RF fields with the apoptotic process. Thus, one of our objectives within the REFLEX programme was to investigate the potential role of environmental electromagnetic fields, specifically GSM-900 radiofrequency radiation (RFR) on the apoptotic process in critical cell types.

Briefly, apoptosis or programmed cell death plays a central role both in development and homeostasis of multicellular organisms (Skulachev 2002). A dual physio-pathological role of cellular apoptosis has been described (Rossi and Gaidano 2003). On the one hand, apoptosis is a major mechanism of protection against genotoxic agents since potential cancer cells are removed by apoptosis. On the other hand, dysregulation in the apoptotic pathways is involved in different pathologies since excessive apoptosis can contribute to diseases such as AIDS or neurodegenerative diseases (Olney 2003) whereas default in apoptosis is involved in cancer or autoimmune diseases (Burns and el-Deiry 2003). Moreover, inducing apoptosis in apoptosis-resistant tumour cells may lead to therapeutic applications (Tolomeo and Simoni 2002) while preventing apoptosis in apoptosis-sensitive cancer cells may be deleterious.

Because the phone is close to the head when in use, brain cells represent a major potential target for RFR emitted by the phones. Furthermore, one of the most critical cell types in the central nervous system is primary neurons. For our studies, we chose rat granule cells. Granule cells of the cerebellum constitute the largest homogeneous neuronal population of mammalian brain. Cerebellar granule cells are a model of election for the study of cellular and molecular correlates of mechanisms of survival/apoptosis and neurodegeneration/neuroprotection (Contestabile 2002). We failed to detect any influence of GSM-900 exposure on apoptosis in this highly critical cell type. All other nerve cell types tested, i.e. SH-SY5Y neuroblastoma cells, human U87 astrocytoma cells and rat C6 glioma cells - a priori less critical than granule cells - were not shown to be sensitive to GSM-900 exposure for up to 24 hours. Hence, no demonstration of an immediate or delayed effect of RFR on apoptosis in nerve cells has been made in rat primary cells and human cell lines. We conclude from our results that nerve cells do not represent a major target, in terms of apoptosis, for RFR emitted by mobile phones.

Because of the role of the immune system for cell homeostasis, cells from the immune system were to be tested. No evidence for an immediate, cumulative or delayed effect of RFR on apoptosis was shown in a human monocytic cell line. Gene expression experiments gave some confirmation that RF-EMF (GSM-900) had no influence on apoptosis in U937 cells as no significant effect was demonstrated on genes involved in apoptosis. We conclude from our results that U937 cells are not sensitive to GSM-900 exposure for up to 48 hours. Taken together, in our experiments, no substantial effect of exposure to RF-EMF (GSM-900) on spontaneous apoptosis of nerve and immune cells was found. No delayed effect could be evidenced either. When tested, interaction between GSM-900 exposure and pro-apoptotic chemicals could not be evidenced.

The results from the REFLEX programme strongly suggest that the apoptotic process may not be a major biological target for GSM mobile telephony-related signals. The REFLEX programme is contributing to most of our current knowledge on the effects of RF fields on cellular apoptosis. Two papers have been very recently published on that topics. Hook et al. (2004) found no evidence of programmed cell death in Molt 4 human lymphoblastoid cells after exposure to 4 different American signals for up to 24 hours at SAR ranging from 0.0024 and 3.2 W/kg. Markkanen et al. (2004) in Finland reported that 900-MHz CW or GSM-modulated RF fields at a SAR of 0.6 W/kg did not induce apoptosis in a control yeast strain and in its temperature-sensitive mutant of cdc48 (apoptosis strain). When yeast strains were pre-exposed to UV, GSM-900 only was able to enhance the UV-induced apoptosis in the mutant yeast strain only. In the REFLEX programme, no significant effect on spontaneous apoptosis was detected in cells from the immune system (human peripheral blood mononuclear cells, human U937 cells) and in EA.hy926 human endothelial cells exposed to RFR-fields (GSM-900 and GSM-1800). No delayed effect (time kinetics) after RFR exposure was demonstrated.

In the present body of work, the status (transformed or non-transformed) of cells used did not influence the effect of RF-fields. The signalling pathways involving bcl-2 was not affected in either $p53^{+/+}$ or $p53^{-/-}$ embryonic stem cells tested after exposure to RFR-fields. The activity of caspase3 was not altered in EA.hy926 cells. In all systems tested, intermittence in the signal did not elicit apoptosis. Data also suggest that for the exposure conditions tested, field effects were not substantially affected by the cell genetics (embryonic stem cells), or the age of the donor (human peripheral blood mononuclear cells).

Hence, no effects of GSM signals (GSM-900 and GSM-1800) have been detected on spontaneous apoptosis of mature and embryonic stem cells in the various groups involved in REFLEX, even in conditions reported to modify other biological endpoints (for instance, an increase of hsp27 expression was detected in EA.hy926 human endothelial cells, 4.2.4.6). However, the expression of the bcl-2 anti-apoptotic gene was shown to increase in murine differentiating embryonic stem cells after exposure to GSM-1800, which correlated with changes in the process of neural differentiation (down-regulation of certain neuronal genes, 4.2.2.4). This needs to be further investigated in order to understand the potential relevance for human health.

We then focused our research on the investigation of some interaction between RF-EMF and known pro-apoptotic drugs. Extension of studies on the expression of apoptosis-related genes was also performed. The data show that cells from the immune and the nervous systems did not exhibit any sensitivity in a concomitant or successive treatment with apoptogenic chemicals and GSM signals, by contrast to cells from the endothelium. However, even in that cell type, GSM-900 was shown to interact with only one chemical (polyHema) over the two chemicals tested. The effect observed was a partial prevention of chemically-induced apoptosis. In this cell line, a weak decrease in pro-apoptogenic genes after exposure to GSM-900 was correlated with the former effect observed. These data on endothelial cells have still to be independently replicated.

Moreover, apoptosis-related genes were shown only weakly affected after exposure to RFR when compared to other gene families such as the ribosomal-related genes (Participant 12). Compared to data of the Juutilainen group, experiments performed within the REFLEX consortium used mammalian cells instead of yeast cells. This, as well as the nature of apoptogenic agent (chemical versus physical), may account for the discrepancy observed in the interaction experiments.

These data suggest that, except for murine differentiating stem cells, low-level RFR are not able to interfere with the spontaneous integrative apoptotic process. If confirmed, interaction with pro-apoptotic chemicals is suggested to be highly dependent of the cell type and the chemical agent used.

4.2.3.2 Human lymphocytes (Participant 8)

RF-EMF may not affect apoptosis in human lymphocytes.

1800 MHz RF (GSM basic, Talk and DTX modulated; SAR 1.3 - 2 W/kg) is not able to modify spontaneous and chemical-induced apoptosis, when human PBMC were exposed 10 min on and 20 min off for 44 hours. This result was also confirmed using cells from old donors (GSM basic, SAR 2 W/kg), since their cells could result differently susceptible to undergo apoptosis (Salvioli et al. 2003). Data were further confirmed by the analysis of mitochondrial membrane potential, which was not affected by RF in all the conditions tested. Negative results were also obtained analysing thymocyte apoptosis during their differentiation. On the basis of these data we can conclude that these types of exposures do not affect apoptotic process, even if this is not established for longer or chronic exposures. (4.2.2.2)

4.2.3.3 Human promyelocytic cell line HL-60 (Participant 2)

Using the annexin V assay and the TUNEL assay, it could be shown that RF-EMF at 1800 MHz, SAR 1.3 W/kg and 24h exposure did not affect cell viability and cell growth, and did not induce apoptosis. (4.2.2.3**)**

4.2.3.4 Mouse embryonic stem (ES) cells (Participant 4)

RF-EMF affected the bcl-2 –mediated anti-apoptotic pathway in differentiating embryonic stem cells.

An up-regulation of bcl-2, bax and GADD45 transcript levels was observed after exposure of ES-derived neural progenitors at specific stages of differentiation to high frequency and extremely low frequency electromagnetic field. We studied the gene expression levels of regulatory genes like hsp70, p21 and apoptosis–related genes of the bcl-2 family (the anti-apoptotic bcl-2 and the pro-apoptotic bax gene). These regulatory genes were pre-selected after our previous experiments with undifferentiated p53-deficient ES cells, where we found significantly increased transcript levels of p21 and hsp70 (Czyz 2004a) after RF-exposure and of p21 after ELF-EMF exposure (50 Hz Powerline) (Czyz 2004b). Therefore, we analysed the effects of EMF on apoptosis-related genes in wild-type (wt) embryonic stem cells at the stage of neural differentiation. Our data demonstrated up-regulation of the transcript levels of bcl-2 in neuronally differentiated ES cells at terminal stages for RF-EMF. However, the biological significance of this finding and its relevance to the situation in vivo has to be clarified.

Apoptotic cell death is executed by caspases and can be regulated by members of the bcl-2 family as reported for differentiating murine embryonic stem cells (Sarkar and Sharma 2002). Apoptosis plays an important role during embryonic development, including the development of the nervous system. Studies applying the model of central axotomy in mouse have shown a degeneration of up to 70% of nigral neurons post transection due to the activation of c-jun, but bcl-2 over-expression leads to a reduced phosphorylation state of c-jun in transected neurons and protection against cell death (Winter et al. 2002). Bcl-2 over-expression was also reported to eliminate deprivation-induced cell death of brainstem auditory neurons (Mostafapour et al. 2002). In other studies, in situ hybridisation revealed a rapid and transient increase in bcl-2 mRNA in neurons following de-afferentation (Wilkinson er al. 2002).

In the RF-EMF experiments, we extended our study by including the analysis of mRNA levels of the growth arrest DNA-damage inducible (GADD45) gene and found a significant up-regulation at the terminal stage of differentiation (at 4+23d). The members of the GADD protein family are considered to play important roles in maintaining genomic stability and in regulating the cell cycle (Chung et al. 2003). The phenotype of GADD45-deficient mice is similar to the phenotype of p53-deficient mice, including genomic instability and sensitivity to radiation induced carcinogenesis (Hollander et al. 1999). GADD45 was found to promote G2/M arrest thus inhibiting entry of cells into S-phase and allowing genomic DNA repair in keratinocytes (Maeda et al. 2002). These findings suggest that GADD45 is a component of the p53 pathway that maintains genomic stability, albeit damage-induced transcription of the GADD45 gene is supposed to be mediated by both p53-dependent (Kastan et al. 1992) and p53–independent mechanisms (Jin et al.2001). Our results, which demonstrate an up-regulation of bcl-2 and GADD45 mRNA levels indicate, that electromagnetic signals are, probably, perceived in embryonic stem cell-derived neural progenitors as environmental stress signals at defined stages of differentiation. Such signals may trigger cellular responses for maintenance of the cellular homeostasis via mobilization of the mechanisms of DNA repair and protection against apoptotic cell death.

4.2.3.5 Human the endothelial cell lines EA.hy926 and EA.hy926v1 (Participant 6)

RF-EMF may affect the hsp27 mediated anti-apoptotic pathway in human endothelial cells.

Stress proteins are known to regulate cell apoptosis (Pandey et al. 2000; Mehlen et al. 1996; Creagh et al. 2000). RF-EMF-induced deregulation of apoptotic process might be a risk factor for tumour development because it could lead to the survival of cells that “should” die. This notion was suggested in the hypothesis presented recently by French et al. (2001). We suggest that the apoptotic pathway regulated by hsp27/p38MAPK might be the target of RF-EMF radiation (6.2.4.6).

4.2.3.6 Summary (Participant 1)

As discussed by Participant 9 (4.2.2.4), knowledge on a possible influence of RF-EMF on the apoptotic process in living cells *in vitro* is rather poor. In the two most recent studies no such effect of RF-EMF was observed (Hook et al. 2004; Markkanen et al. 2004). The REFLEX data did not reveal a significant influence of RF-EMF on apoptosis of various cell types such as brain cells and human monocytes (see 3.2.3.1 and 4.2.31), human lymphocytes (3.2.3.2, 4.2.3.2) and HL-60 cells (3.2.1.1, 4.2.3.3). On the other

hand, an indirect effect on apoptosis via the bcl-2 or hsp27 mediated anti-apoptotic pathway which was detected in differentiating embryonic stem cells (3.2.4.1, 4.2.3.4) and in endothelial cells, respectively (3.2.4.6, 4.2.3.5), cannot be excluded at this time.

Of course, based on the data on gene and protein expression obtained in the REFLEX project, an effect of RF-EMF on the apoptotic pathway, either through inhibition or promotion, seems to be possible. Whether or not cell cultures respond to RF-EMF may depend on the type of cell exposed, their genetic background, their metabolic state and, of course, on the pattern of exposure. But taken together, even if a relationship between RF-EMF exposure and an inhibition or promotion of apoptosis in *in vitro* experiments were proven, it would in no way be possible to draw any conclusion for the *in vivo* situation in man and animal.

4.2.4 Gene and protein expression

4.2.4.1 Mouse embryonic stem (ES) cells (Participant 4)

The genetic constitution of early differentiating embryonic stem cells may play a role on their responsiveness to differently modulated RF-EMF.

In ES cells deficient for the tumour promoter p53, a permanent up-regulation of mRNA levels of the stress response gene hsp70 paralleled by a slight and temporary increase of p21, c-jun and c-myc expression was found in response to GSM-217- but not GSM-Talk-modulated signals characterized by the presence of low frequency components. On the other hand, wt ES and EC cells exposed to GSM-217 signals revealed no effects on gene expression, cardiac differentiation and proliferation. This would indicate, that the genetic background of stem cells may potentially influence the response of early differentiating cells to GSM signals dependent on the modulation schemes, whereas wt cells analysed in this study remained insensitive to GSM-modulated EMF.

p53-/- mice are highly susceptible to the development of spontaneous tumours, in particular, of malignant lymphomas at early age (Attardi and Jacks 1999; Sigal and Rotter 2000). p53 is required for G1 arrest in response to DNA damage and is involved in apoptosis (Attardi and Jacks 1999) via modulating versatile regulatory genes. In our studies using mouse $p53^{-/-}$ ES cells, hsp70 mRNA levels were continuously elevated after 48h EMF exposition. Heat shock proteins act as chaperons whose expression is activated or up-regulated in response to external stress (Beere and Green 2001). Hsp70 has also been defined to regulate homoeostasis in response to external stress during early embryo development (Luft and Dix 1999), while up-regulated hsp70 levels were observed in tumour cells and are correlated with metastases and poor prognosis (Zylicz et al. 2001). A potential involvement of heat shock proteins in cell responses to EMF was reported recently: up-regulated hsp27 protein levels and a transient increase of hsp27 phosphorylation were found in human endothelial cells (Leszczynski et al. 2002).

However, if EMF act as inducers of cellular transformation processes, effects on expression levels of other early response genes should be expected. Indeed, we observed an up-regulation of c-myc, c-jun and p21 mRNA levels in $p53^{-/-}$ cells upon GSM-217 exposure. The same genes were previously shown to be affected by environmental factors, such as UV or X-irradiation in various systems (Amati et al. 1993; Angel et al. 1988; Jean et al. 2001). Because in our model, the shifts in gene expression were low and transient, it is conceivable that EMF signals, while affecting gene expression pattern in $p53^{-/-}$ cells, do not induce permanent cellular transformations in wt cells.

The response of early differentiating cells to RF-EMF is dependent mainly on the carrier frequency of the modulation schemes.

Contrary to GSM-217 signals, which elicited cellular responses in $p53^{-/-}$ ES cells, GSM-Talk modulation exerted no effects on gene expression in our model. This indicates that low frequency components generated by GSM-Talk (2 and 8 Hz) do not promote the action of EMF signals in our cell system. In contrast, time-averaged SAR values (1.5 W/kg for GSM-217 vs. 0.4 W/kg for GSM-Talk) may comprise a factor determining the biological activity of EMF. Furthermore, it cannot be excluded that the modulation scheme (time distribution of high SAR pulses) may also play a role in evoking biological

responses, because slot-averaged SAR values remained similar between the analysed modulation schemes.

The exposure duration may also influence the biological responses to RF-EMF.

In our in vitro studies, up-regulated transcript levels of regulatory genes in $p53^{-/-}$ ES cells were observed after 48 hours exposure to GSM-217 signals, whereas a short-term 6h exposure exerted no effects.

The parameters of genetic constitution, carrier frequency and exposure duration in determining the response of biological systems to RF-EMF have been proposed by in vivo studies using Eμ-Pim1-transgenic mice predisposed to develop spontaneous lymphomas. Repacholi et al. observed an increase in tumour formation after long-term, 18 months exposure of Eμ-Pim1-transgenic mice to 900 MHz EMF (Repacholi et al. 1997), which positive data suggest that mobile phone radiation-induced events may be hazardous to cells deficient in cell repair when occurring frequently over long periods.

It has been shown previously that the exposition of human HL-60 cells resulted in an increase in DNA breaks, suggesting a possible direct mutagenic effect (Ivancsits et al. 2002). In addition, there is a report about a correlation between up-regulation of GADD45, of the member of the Bcl-2 family bcl-$X_{L,}$ and an increased amount of early DNA damage as measured by the alkaline Comet assay in human preneuronal cells exposed to the amyloid protein (Santiard-Baron et al. 2001). Therefore, we used the alkaline and neutral COMET assay to detect single, and double-strand DNA breaks respectively, in the neuronal progenitors derived from murine pluripotent ES cells after ELF or RF-EMF exposure. Under our experimental conditions, however, we could not find a clear evidence of increased single-strand DNA break induction in the alkaline Comet assay. A low but significant increase in double-strand DNA breakage was observed only after a short (6h) RF-EMF exposure in the neutral Comet assay.

In summary, we found that RF-EMF simulating GSM signals caused a transient up-regulation of p21 and c-myc genes and a long-term up-regulation of the stress response gene hsp70 in ES cells deficient for p53 in response to GSM-217- but not GSM-Talk-modulated signals characterized by the presence of low frequency components. Here again we found that the genetic constitution (loss of p53 function) could alter the responsiveness of ES cells and render them sensitive to high frequency EMF, while wild-type cells were irresponsive. However, we did not observe any distinct direct genotoxic effects as measured by the Comet assay.

In ES cell-derived neuronal progenitors we found indication of growth arrest and effects on apoptosis (a significant up-regulation of the growth arrest and DNA damage inducible gene GADD45, the proapoptotic bax and the antiapoptotic gene bcl-2 mRNA levels), which correlated with changes in the process of neural differentiation (down-regulation of the neuronal genes Nurr1 at stage 4d+11d and TH at 4d +23d).

4.2.4.2 NB69 neuroblastoma cells and neural stem cells (NSC) (Participant 5)

RF-EMF reduced the expression of the receptor FGFR1 of fibroblast growth factor (FGF) in the human neuroblastoma NB69 cell line and in neural stem cells from rat embryonic nucleus striatum.

In NB69 cells, this response is similar to that induced by exogenous treatment with 20 ng/ml of bFGF, and by the combined treatment with bFGF plus EMF. In this biological system, an induction of morphological changes (increases in cell size and cell extensions) is also observed after 24 hours of treatment with bFGF. Such morphological changes are accompanied with a reduction in the proportion of cells expressing FGFR1-3 receptors. However, in cultures exposed to the GSM-Basic for 24 hours the effect on FGFR1 was not associated to changes in the cells′ morphology. Evidence exists that treatment of olfactory neuroblastoma cells with bFGF deregulates FGFR1 prior to differentiation (Nibu et al. 2000). It is possible that in our NB69 cells the response induced the GSM-Basic signal on FGFR1 can also be associated to a promotion of differentiation in long-term cultures. Additional work using differentiation markers for neuronal NB69 cells has to be done to verify the above hypothesis.

On the other hand, in neural stem cells, the 24h-exposure also induced a reduced expression of receptor FGFR1, and further significant changes in the cell morphology were observed six days later, in the absence of the GSM-exposure. The oligodendrocytes showed an advanced developmental stage with respect to controls. Similarly, the astrocytes showed longer cell-processes. The morphology of the neuronal progeny of NSC was not significantly changed by the exposure to the GSM-Basic signal.

Evidence exists that treatment of a human astrocytoma cell line, U-87 MG, with 835 MHz electromagnetic radiation induces alterations in F-actin distribution and cell morphology (French et al. 1997). Their astrocytes showed a similar response to that observed in our precursors lineage exposed to the GSM-signal: an increased cell spreading. Taken together, the present data suggest that the reduction of cells expressing FGFR1 induced by signal could be linked to a promotion of the differentiation of non-neuronal populations.

The changes in FGFR1 induced by RF-EMF are dependent mainly on the carrier frequency.

Both, basic and CW signal induced similar changes on FGFR1. This suggests that the cellular response is not dependent on the tested low-frequency modulation, but on the carrier frequency. The signals used in advanced telecommunication systems such as global system for mobile communications (GSM) and universal mobile communications system (UMTS) include extremely low frequency (ELF) amplitude modulation or pulse modulation components. There is a lack of scientific data on the possible health implications of such modulations. A recent study (Huber et al. 2002) has reported that a 30-minute exposure to 900 MHz, 1 W/kg GSM signal can influence the electrical activity of the brain, both before and after sleep onset, in young male test subjects. Both sleep and waking EEG changes were observed only with pulse modulated-EMF. Also, it has been reported a statistically significant micronucleus effect in peripheral blood cultures following 15-minute exposure to phase modulated field (Gaussian minimum shift keying, GMSK), 1.748 GHz, at SAR ≤ 5 W/kg (d'Ambrosio et al. 2002b). However, the micronucleus frequency result was not affected by CW exposure. No changes were found either in cell proliferation kinetics after exposure to both CW and phase modulated fields. In our present work we investigated whether fibroblast growth factor receptors (FGFR) could be influenced by the modulation of the GSM-signals. NB69 cells were exposed to GSM- Talk, GSM-Basic and CW signals, 2 W/kg SAR or to DTX-signal, 1W/kg SAR. The exposure to GSM 1800-Basic signal at a 2 W/kg SAR was found to induce a significant decrease in the number of cells expressing the FGFR-1 (15% reduction vs. controls) without affecting significantly the number of cells expressing receptors R2 and R3. The effect on R1 was equivalent to that induced by basic fibroblast growth factor (bFGF) at a 20 ng/ml concentration. The exposure to RF-CW signal (SAR 2 W/kg) induced effects on the expression of FGFR-1 equivalent to those induced by the GSM-Basic signal, whereas the exposure to GSM-Talk signal at the same SAR (2 W/kg) or to DTX-signal (1 W/kg SAR) did not modify significantly the normal expression of the FGF protein receptors R1. Our results indicate that the ELF modulation components resulting from the GSM signals shape (2, 8 and 217 Hz) and higher harmonics are not critical for the EMF-induced changes in FGFR-1 expression. Provided that the Talk mode is a temporal change between GSM Basic (66%) and DTX only (34%) our data together with those from Partner 4 indicate that the exposure duration could also be a critical factor for the herein described response. Future studies may also examine dose-response relationships by varying the exposure time and the specific absorption rate.

4.2.4.3 Human promyelocytic cell line HL-60 (Participant 2)

RF-EMF modulates the gene and protein expression in HL-60 cells.

Applying high resolution two-dimensional polyacrylamide gel electrophoresis to the HL-60 cell system, more than 4000 protein spots can be differentiated on the silver stained protein map. These spots were detected and the master gel image was calibrated. Clear differences in protein expression have been found for RF-field exposed HL-60 cells as compared to control and sham-exposed cells. The quantitative comparison has been completed. Further strategies in the future will include structural and biochemical identification of proteins significantly altered following RF-field exposure, beside mass spectrometry (MALDI-TOF) and mass spectrometric sequencing (ESI-MS/MS) and immunoblotting/functional protein assays, also by comparative studies with reference databases. Clarification of changes in protein expression after exposure to RF-fields will help to understand molecular pathomechanisms.

4.2.4.4 Human lymphocytes (Participant 8)

RF-EMF did not affect gene expression in human lymphocytes.

1800 MHz RF (DTX modulated, SAR 1,4 W/kg) is not able to modify gene expression profile when quiescent T lymphocyte are exposed 10 min on/20 min off for 44 hours. This result, obtained in collaboration with Participant 12 by means of micro-array technique, was expected because lymphocytes

were not stimulated and represents a first step toward a further evaluation in low-stimulated and RF-exposed T lymphocytes; future analyses should clarify the presence of potential gene targets for RF exposure in primary human cells.

4.2.4.5 Brain cells of different origin, human immune cells and human endothelial cell lines (Participant 9)

There is no indication that expression and activity of the inducible Nitric Oxide Synthase (iNOS or NOS_2) is affected in nerve cells after exposure to RF-EMF.

Under pathological conditions, nitric oxide, NO, can act as a neurotoxic agent (Leist and Nicotera 1998; Brown and Bal-Price 2003). A variety of stresses are known to induce neuronal cell death via NOS_2 (or inductible NOS, iNOS) activation and NO production in stimulated astrocytic cells. Hence, activated astrocytes may be involved in the pathogenesis of neurodegenerative diseases. Our goal was to determine whether exposure to a GSM-900 signal could activate C6 glioma cells by increasing the activity of the iNOS enzyme. A potential synergistic effect of such radiofrequency radiation (RFR) on cytokine-induced NO production was also investigated.

A few data are available in the literature on the effect of RF-EMF on nitric oxide, NO, production. Over three identified papers, only one used RF-EMF compatible with mobile telephony. Miura et al. (1993) reported that 10 MHz RFR (10 kHz bursts) caused an increase in NO production in rat cerebellum extracts. Using ultra-wideband pulses, Seaman et al. (2002) recently showed no influence of RFR in the Ultra Wide Band (UWB) range in RAW 264.7 macrophages except when nitrate was added to the culture medium. Paredi et al. (2001) showed a tendency for higher nasal NO levels in humans exposed to GSM-900 for 30 minutes that was due to skin heating experienced by the phone's users. As it has been clearly shown that heating from mobile phone microwaves is negligible, this effect was probably due to the battery's heating. Based on our data, GSM-900 did not appear to be able to alter chemically- induced activation in mammalian astrocytes and thus appeared unlikely to influence tumour cells characteristics and neuronal cells' viability via NO pathways.

There is no indication that expression of heat shock proteins is affected in nerve cells after exposure to RF-EMF.

The first objective was to determine whether exposure to GSM-900 microwaves could influence the expression of hsp70 proteins in neuronal and glial cell lines as reported in an endothelial cell line by Participant 6.

In response to environmental disturbances, cells respond by expressing heat shock proteins. Our study focuses on the 70-kDa family, which is the major form of stress proteins found in the brain (Pavlik et al. 2003) and on hsp27 that is expressed in endothelial cells (Loktionova et al. 1996).

Our data showed that exposure to GSM-900 microwaves were not able to induce hsp70 expression in rat and human nerve cells. These data are not in agreement with results of recent research showing that the expression of heat shock protein (hsp) may be induced in response to radiofrequency radiation exposure at non-thermal levels in different models (de Pomerai et al. 2000, in worms; Kwee et al. 2001, in human amnion cells; Leszczynski et al., 2002 in human EA-hy926 endothelial cells; Weisbrot et al. 2003, in Drosophilae). Thus, a common feature on the effect of GSM-900 at low SAR (about 2 W/kg) on the expression of hsp cannot be drawn. Different cell types could behave differently to exposure to a GSM-900 signal or different members of the hsp family could show a different sensitivity to exposure to GSM signals. Moreover, we can note that, so far, none of the "positive" effects have been independently replicated.

We failed to independently confirm that expression of heat shock proteins is affected in EA-hy926 cells after exposure to GSM-like RF-EMF.

The second objective was to confirm the data of Participant 6 on hsp27 in the EA-hy926 cells. Hsp27 is indeed the major form of stress proteins that is expressed in endothelial cells (Loktionova et al. 1996). Our results obtained by fluorescent image analysis in the two cell lines tested differed from those obtained in one of them by Participant 6 after western blotting experiments. A third method using Elisa test will allow us to quantify precisely if RFR are able to induce changes in hsp27 expression since the sensitivity reported is 1-10 ng for Western Blot and less than 1 ng for ELISA.

Here, with our semi-quantitative method and statistical analysis we were unable to confirm previous data on hsp27 expression in endothelial cell lines. Exposure set-up used in both groups also differed (water bath versus air cooling, homogeneity of SAR distribution at the cells level, …etc), which probably imply different dosimetric features. Whether this could account for the discrepancies observed could be determined (Participant 10). Meanwhile, we cannot conclude yet that RFR induce stress response. Hence, no implication for health hazard can be drawn at the moment based on Hsp expression in mammalian cells after low level RFR exposure.

The possible effect of low-level RFR on the expression of hsp is quite controversial. While some laboratories reported effects in mammalian cells (Leszczynski et al. 2002; Kwee et al. 2001) or simple organisms (de Pomerai et al. 2000; Weisbrot et al. 2003) as stated above, we (present work), as other groups (Cleary et al. 1997; Tian et al. 2002a; Miyakoshi et al. 2003) could not observe any effect in mammalian cells at SAR up to 10 W/kg. To date, no clear and satisfactory explanation can be given. However, we do think that the investigation of hsp expression after RFR exposure in *in vivo* mammalian models will help and enlighten the debate. Some but still sparse data are available, showing that heat shock proteins could not be induced in rat brain below 7 W/kg (Fritze et al., 1997).

There is some indication that gene expression is affected in immune cells after exposure to RF-EMF.

These data were obtained in collaboration with Participant 12 (4.2.4.7). First, these results gave a confirmation that GSM-900 had no influence on apoptosis in U937 cells as no significant effect was demonstrated on genes involved in apoptosis. Then, only a few genes among several thousand tested genes were shown altered after RFR exposure (increase or decrease) in two human immune cell lines. The largest modification in RNA expression corresponded to genes related to signal transduction and energy metabolism. Finally, amine oxidase activity-related genes experienced the largest changes after exposure to mobile phone-like RFR. This later gene is coding for an enzyme which is involved in cell growth and proliferation but also in immune regulation. This gene-profiling analysis showed that RFR can influence some biological processes and gave us trails for further investigations such as looking at energy metabolism in cells exposed to RFR using spectroscopic NMR. Comparing gene profiling obtained in different cell types may provide a “signature” for environmental RF-EMF exposure. It is however still unclear if and how those changes in gene expression can be related to human health.

Reported gene profiling after ethanol stress in yeast (Alexandre et al. 2001) showed that about 6% of the yeast genome were experiencing changes (about 3% upregulated and 3% downregulated). This represents 20 fold more genes than what was found affected after exposure to RF-EMF (0.3%). Genes identified were mainly involved in energetic metabolism, protein destination, ionic homeostasis and stress response with more than 10 hsp members. Stronger criteria were used in Alexandre et al. (2001) for significance of changes in gene expression compared to ours. When compared with the effect of a strong stress agent such as ethanol (in yeast), RF-EMF affected only a few genes in the human immune cell lines we tested. Whereas genes involved in energy metabolism seem to be a common feature for both types of exposure, none of the genes identified in human immune cells after RFR exposure belonged to the stress response family. Based of these comparisons, RF-EMF may be identified as a weak environmental stress, if any.

4.2.4.6 Human endothelial cell lines EA.hy926 and EA.hy926v1 (Participant 6)

RF-EMF induce cellular stress response.

Observed in this study changes in protein phosphorylation and activation of p38MAPK/hsp27 stress response pathway agree with the earlier studies suggesting that mobile phone RF-EMF radiation induces cellular stress response at non-thermal power level. In vitro, Cleary et al. (1997) claimed that RF-EMF exposure has no effect on stress proteins. However, because identification of stress proteins was based solely on their molecular weight in liquid chromatography, the exact identity of proteins claimed to be stress proteins remains unclear. Fritze et al. (1997b), using rat model, have shown increase in expression of stress protein hsp70 in brains of animals exposed for 4 hours to RF-EMF (890-915 MHz) at SAR of 1.5 W/kg. Daniells et al. (1998) and de Pomerai et al. (2000) have shown that overnight in vivo irradiation of nematode worms with RF-EMF (750 MHz) at SAR of 0.001 W/kg cause increase in expression of heat shock protein. Kwee et al. (2001) have shown induction of stress protein hsp70, but not hsp27, in cultures of transformed human epithelial amnion cells exposed for 20 min to RF-EMF (960 MHz) at SAR of 0.0021W/kg.

Because of the known broad spectrum of physiological processes that are regulated by stress proteins (Tibbles and Woodgett, 1999), and by hsp27 in particular, it is here hypothesized that mobile phone radiation-induced activation of hsp27/p38MAPK-dependent cellular stress response might: (i) lead to the development of brain cancer due to inhibition of cell apoptosis and (ii) cause increased permeability of blood-brain barrier due to stabilization of endothelial cell stress fibers (Figure 138).

Stress proteins are known to regulate cell apoptosis (Pandey et al. 2000; Mehlen et al. 1996; Creagh et al. 2000). RF-EMF-induced deregulation of apoptotic process might be a risk factor for tumour development because it could lead to the survival of cells that "should" die. This notion was suggested in the hypothesis presented recently by French et al. (2001). We suggest that the apoptotic pathway regulated by hsp27/p38MAPK might be the target of RF-EMF radiation. Hsp27, stress protein shown in this study to be affected by mobile phone radiation exposure, is a member of a family of small heat shock proteins that is ubiquitously expressed in most of cells and tissues under normal conditions in form of large-molecular complexes. In response to stress occurs rapid phosphorylation of hsp27 on serine residues (in human cells Ser-78 and Ser-82) what leads to dissociation of the large-molecular complexes into smaller units (Kato et al. 1994). Various stress factors have been indicated as inducers of changes in expression (accumulation) and/or phosphorylation (activity) of hsp27 (Ito et al. 1995; Deli et al. 1995; Garrido et al. 1997; Huot et al. 1997; Tibbles and Woodgett 1999). Activated (phosphorylated) hsp27 has been shown to inhibit apoptosis by forming complex with the apoptosome (complex of Apaf-1 protein, pro-caspase-9 and cytochrome *c*), or some of its components, and preventing proteolytic activation of pro-caspase-9 into active form of caspase-9 (Pandey et al. 2000; Concannon et al. 2001). This, in turn, prevents activation of pro-caspase-3 which, in order to become active, has to be proteolytically cleaved by caspase-9. Thus, induction of the increased expression and phosphorylation of hsp27 by the RF-EMF exposure might lead to inhibition of the apoptotic pathway that involves apoptosome and caspase-3. This event, when occurring in RF-EMF exposed brain cells that underwent either spontaneous or external factor-induced transformation/damage, could support survival of the transformed/damaged cells what, in favourable circumstances, could help clonal expansion of the transformed/damaged cells - a prerequisite for the tumour development. Furthermore, hsp27 in particular was shown to be responsible for the induction of resistance of tumour cells to death induced by anti-cancer drugs (Huot et al. 1996; Garrido et al. 1997). Thus, it appears possible that RF-EMF induced changes in hsp27 phosphorylation/expression might affect not only tumour development but also its drug-resistance.

Induction of the increase of the permeability of blood-brain barrier by RF-EMF exposure, which has been suggested by some animal and in vitro studies, is one of the controversial health issues that came up in relation to the use of mobile phones. It has been already established that, at thermal levels of exposure, microwave radiation causes increase in the permeability of blood-brain barrier (for review see Jokela et al. 1999; The Royal Society of Canada Report 1999, Stewart Report 2000, Zmirou Report 2001). However, the effect of non-thermal RF-EMF exposure on blood-brain barrier is still unclear. Some studies have suggested that mobile phone radiation, at non-thermal exposure levels, increases permeability of blood-brain barrier *in vivo* (Salford et al. 1994) and *in vitro* (Schirmacher et al. 2000), whereas others suggested lack of such effect (Fritze et al. 1997a; Tsurita et al. 2000). However, the no-effect claimed by Fritze et al. (1997a) is not so straight forward as suggested by the authors because they reported induction of stress response and increased permeability of the blood-brain barrier immediately after the end of irradiation. This effect was short lasting and, because of it, was considered by the authors as insignificant. Also, it remains unclear what would be the blood-brain barrier response to the repeated exposures to mobile phone radiation because the effect of repeated exposures was not examined. The increased blood-brain barrier permeability due to increase of endothelial pinocytosis was suggested by Neubauer et al. (1990) who have demonstrated increase in pinocytosis of cerebral cortex capillaries that were exposed to 2.45 GHz microwave radiation. Finally, the recently reported study by Töre et al. (2001) has shown that 2 hour exposure of rats to RF-EMF (900 MHz) at SAR of 2W/kg (averaged over the brain) causes increase in the permeability of blood-brain barrier. However, the molecular mechanism and the cellular signalling pathways that are involved in the induction of blood-brain barrier permeability are still unknown. We propose that the induction of hsp27 phosphorylation and increased expression by RF-EMF exposure, shown in this study to occur in vitro in human endothelial cells, might be the molecular signalling event that triggers the cascade of events leading to the increase in blood-brain barrier permeability. Phosphorylated hsp27 has been shown to stabilize endothelial cell stress fibers due to the increased actin polymerisation (for review see Landry and Huot 1995). The stabilisation of stress fibres was shown to cause several alterations to endothelial cell physiology: (i) cell shrinkage and opening of spaces between cells (Landry and Huot 1995; Piotrowicz and Levin 1997), (ii) increase in the

permeability of endothelial monolayer (Deli et al. 1995), (iii) increase in pinocytosis (Lavoie et al. 1993), (iv) formation of apoptosis-unrelated blebs on the surface of endothelial cells which may obstruct blood flow through capillary vessels (Becker and Ambrosio, 1987), (v) stronger responsiveness of endothelial cells to estrogen and, when stimulated by this hormone, secretion of larger than normally amounts of basic fibroblast growth factor (bFGF) (Piotrowicz et al. 1997) which could, in endocrine manner, stimulate de-differentiation and proliferation of endothelial cells leading to, the associated with proliferative state - cell shrinkage and unveiling of basal membrane. Occurrence of these events in brain capillary endothelial cells could lead to de-regulation of the mechanisms controlling permeability of blood-brain barrier. Furthermore, in addition to blood-brain barrier effects, the stabilization of stress fibres in endothelial cells may affect apoptotic process - it has been shown that the apoptosis-related cell surface blebbing is prevented by the stabilised stress fibres (Huot et al. 1998).

The proposed hypothetical molecular mechanism for the possible role of mobile phone radiation in development of brain cancer and in increasing permeability of the blood-brain barrier, although a hypothesis, it is reasonably supported by the evidence concerning both effects of microwaves on stress response and effects of hsp27 (increased expression and activity) on cell physiology. Proving or disproving of this hypothesis using in vitro and in vivo models will provide evidence to either support or to discredit the existence of some of the potential health risks that were suggested to be associated with the use of mobile phones.

The recently published hypothesis of French et al. (2001) of the possible effect of chronic/frequent exposure to mobile phone radiation that would induce abnormally high levels of stress proteins in cells still requires experimental confirmation that, indeed, repeating exposures to RF-EMF radiation could cause such an increase. On the other hand, proposed by us hypothetical mechanism of the mobile radiation effect on the brain relies on the single-exposure-induced transient increases in hsp27 phosphorylation and expression. We suggest that the transient effects, induced by repeated exposures, might, by chance of timing coincidence, led to survival of damaged/transformed cells and temporarily increase permeability of the blood-brain barrier. These events, when occurring repeatedly (on daily basis) over the long period of time (years) could become a health hazard because of the possibility of accumulating of brain tissue damage. Furthermore, our hypothesis suggests that other, than RF-EMF, cell-damaging factors might play a co-participating role in the tumour development caused by mobile phone radiation.

Finally, in addition to the p38MAPK/hsp27 stress pathway-induced effects, the extent of the global change of the pattern of protein phosphorylation observed in our study suggests that it is likely that multiple signal transduction pathways might be affected by the RF-EMF exposure. Identification of these pathways will help to determine the extent of biological effects induced by RF-EMF exposure. Importantly, cellular effects observed in this study were induced by RF-EMF irradiation at non-thermal levels, with SAR values set at the highest level that is allowed by the European safety limits. This suggests that the presently allowed radiation emission levels for the mobile phones, although low, might be sufficient to induce biological effects. However, determination of whether these effects might cause any significant health effects requires further studies.

5-step feasibility study of applying proteomics/transcriptomics to mobile phone research.

It has been suggested that high-throughput screening techniques (HTST) of transcriptomics and proteomics could be used to rapidly identify broad variety of potential molecular targets of RF-EMF and generate variety of biological end-points for further analyses (Leszczynski et al. 2004). Combination of data generated by transcriptomics and proteomics in search for biological effects is called the “discovery science”. This term has been coined-in by Aebersold et al. (2000) to define the new approach that will help in revealing biological mechanisms, some of which might be unpredictable using the presently available knowledge. This approach seems to be particularly suited for elucidation RF-EMF health hazard issue because it might reveal effects that are not possible to predict based on the present knowledge about the biological effects of RF-EMF. However, before committing large funds that are needed for HTST studies it is necessary to determine whether indeed this approach will be successful in unravelling physiologically significant biological events induced by RF-EMF. Due to their high sensitivity HTST are able to pick-up very small changes in protein or gene expression which changes might be of insufficient magnitude to alter cell physiology. Thus, although using HTST it might be possible to find biological effects induced by RF-EMF these effects might be of limited or no significance at all, from the physiological stand point. Therefore, to determine the usefulness of HTST

approach to the issue of bio-effects induced by RF-EMF, we have performed a 5-step feasibility study and have shown that HTST might indeed help to identify experimental targets for physiological studies of RF-EMF-induced biological responses. The obtained by us results clearly demonstrate that by using HTST it is possible to identify RF-EMF-induced molecular events that might alter cell physiology. Even though the increase in expression/phosphorylation of the examined hsp27 protein was very modest (ca. 2-3 folds increase) it was possible to determine impact of this event on cell physiology. Whether any impact on organ (e.g., brain) or whole body will be exerted by this change remains to be determined by in vivo studies. Although the use of discovery science-approach employing HTST will not provide direct evidence of health hazard or its absence, it will be essential in unravelling of possibly all biological effects exerted by RF-EMF exposure. Further elucidation of the physiological significance of these biological effects for the health and well-being, in short- and long-term exposure conditions, will allow determination whether any health hazard might be associated with the use of mobile phones at the presently allowed radiation safety levels.

Use of HTST to determine genotype-dependent and modulation-dependent cellular responses.

Our study has shown that proteomics transcriptomics and might be an efficient tool when searching for the proteins and genes responding to a weak stimulus, like the mobile phone radiation. In this pilot study we have found several tens of protein and gene targets of the mobile phone radiation. Functions of the few of the MALDI-MS-identified protein spots suggest possibility of the effects of the mobile phone radiation on such physiological functions as (i) cellular energy production, (ii) protein translation, and (iii) cytoskeleton-dependent processes (e.g. cell size, shape and cell-cell interactions). Potential effects on these processes were supported by the evidence gained with cDNA arrays. Further studies will be needed to determine whether there is any impact of these changes on cell physiology.

The other major finding of the study is the observation that the exposure of cells to the continuous-wave microwaves ("CW-signal" 1800 MHz GSM) does not induce changes in protein expression whereas radiofrequency modulated microwaves ("Talk-signal" 1800 MHz GSM) induces broad changes in protein expression. Analysis of changes in expression of some 1500 proteins using cICAT method combined with liquid chromatography and MS/MS identification of proteins has revealed several tens of affected proteins. Importantly, using other methods such as 2-DE and cDNA arrays the same cytoskeleton-related genes/proteins were detected as being affected by RF-EMF exposure up. It means that with two different proteomics approaches we have observed similar protein changes what strengthens the validity of our observations.

4.2.4.7 Effects of RF-EMF on gene expression in human cells analysed with the cDNA array (Participant 12)

The elevated turnover of ribosomal proteins and proteins involved in energy metabolism allows the hypothesis that the cellular turnover is increased after RF-EMF exposure. To prove this hypothesis for RF-EMF treated HL-60 cells (Participant 2), a very interesting additional comparison was performed: In the 1800 MHz continuous wave experiments (2 expression profiles, 4 hybridisations) we used two controls instead of one: One control in each experiment was a sham-exposed control as usual. Cells from another incubator, neither exposed nor sham-exposed, served as a second control. After both experiments we performed comparisons between both controls as well as between sham-exposed and RF-exposed cells (each comparison with 8 data points per gene, from 2 expression profiling experiments, 4 hybridisations). After going through all investigated gene families listed in Tables 30 and 31, with some gene families we found remarkable differences between the control comparison and the sham-RF comparison (Figure 144). As expected, again ribosomal and mitochondrial genes are much more upregulated in the sham-RF comparison than in the "blinded" comparison (Sham-Ctrl). But there are also other gene families showing the same tendency, as for example ATP related genes, genes encoding calcium-associated proteins and cell cycle proteins.

The increasing ribosomal turnover might lead to cell growth and, in the end, to mitosis and cell proliferation, respectively. This hypothesis has not been confirmed so far by the BrdU-incorporation assay, because no significant increase of DNA synthesis, and therefore, an increase in cell proliferation, could not detected (Participant 2). The same is true for the analysis of protein mass and the MTT assay for the detection of mitochondrial activity (Participant 2).

However, the changes in ribosomal protein synthesis are not so strong that we would expect a very strong increase in cell cycle progression. Ribosomal transcription rates have been found to vary by up to a factor of four (Derenzini et al. 2001; Leary and Huang 2001). It is known from stimulation by growth factors, that an increase of ribosomal activity not necessarily leads to significant changes in cell cycle (Stefanovsky et al. 2001; Bodem et al, 2000). We should investigate the cell cycle distribution after RF-EMF exposure in more detail. Also the analysis of the 45S precursor rRNA by real-time RT PCR or the analysis of the RNA polymerase I and associated proteins would help us to figure out if ribosomal transcription is elevated, which is a pre-requisite for an increase of ribosomal proteins (Stefanovsky et al. 2001; Jacob and Gosh 1999). Compared to the ELF-EMF results (Participant 3) the results with RF-EMF seem to be more uniform. The comparison of HL-60 profiling 2 and 3 for example (Participant 2), shows a much lower number of reproducibly regulated genes than after the analysis of only one experiment (compare Figure 139). If the reason for this is the differentiating potential of the cells (U937 cells of Participant 9, HL-60 cells of Participant 2, T-lymphocytes of participant 8), or the exposure conditions (homogenous ELF-EMF field with or without on/off cycles versus RF-EMF GSM talk signal), or both, remains to be elucidated. More cell lines, each exposed to different fields (ELF, RF), would have to be investigated to draw more reliable conclusions.

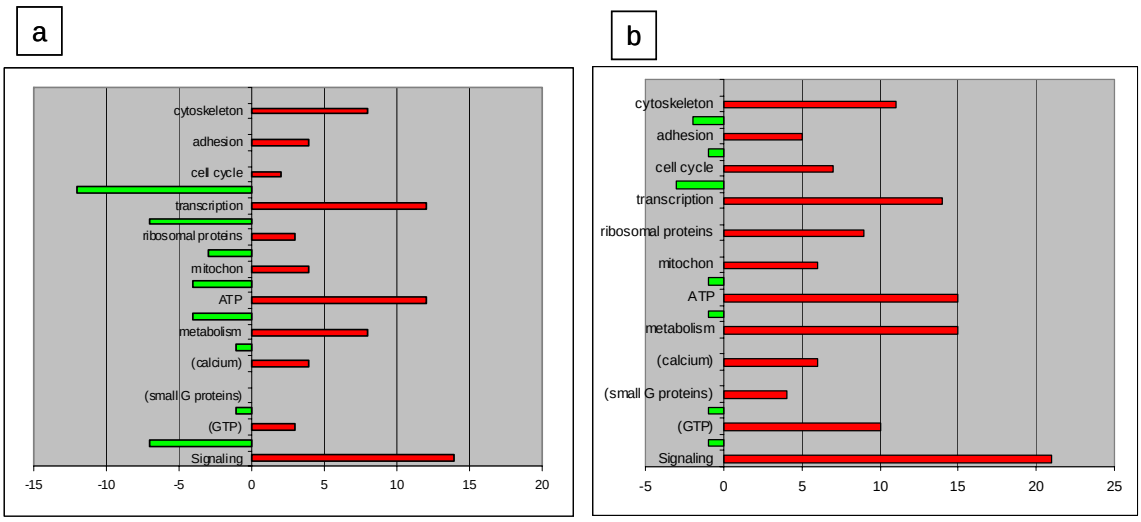


Figure 144. Numbers of regulated genes after RF-EMF exposure sorted according to different gene families. a, comparison between batches of control HL-60 cells (not exposed vs. sham exposed). Red: Genes appearing up-regulated in sham-exposed. Green: Genes appearing down-regulated in sham-exposed. b, comparison between sham-exposed (ctrl) and RF-EMF exposed HL-60 cells. Red: Genes showing up-regulation in RF-EMF. Green: Genes showing down-regulation in RF-EMF.

4.2.4.8 Summary (Participant 1)

Scientific work on gene and protein expression due to RF-EMF exposure using in vitro cell cultures and animal models is still in its early stages and as far as already published difficult to interpret (Independent Expert Group on Mobile Phones 2000). From the REFLEX data the conclusion can be drawn that RF-EMF may affect the gene and protein expression in various cell systems. RF-EMF exposure at a SAR value of 1.5 W/kg caused a transient up-regulation of the p21 and c-myc genes and a long-term up-regulation of the hsp70 gene in p53 deficient embryonic stem cells (3.2.4.1, 4.2.4.1). RF-EMF exposure at a SAR value of 2 W/kg reduced the expression of the receptor FGFR1 of fibroblast growth factor (FGF) in human neuroblastoma cells (NB69) and in neural stem cells of rats obviously without affecting protein transcription (3.2.4.2, 4.2.4.2). RF-EMF exposure up- or down-regulated the expression of various genes and proteins in HL-60 cells (1800 MHz, 1.3 W/kg) and in endothelial cells of human origin (900 MHz, 2.0 W/kg) (3.2.4.3, 3.2.4.6, 3.2.4.7, 4.2.4.3, 4.2.4.6, 4.2.4.7). RF-EMF exposure at a SAR value of 2.0 W/kg activated the p38MAPK/hsp27 stress response pathway and changed the global

pattern of protein phosphorylation in endothelial cells with possible consequences for the signal transduction pathway (3.2.4.6, 4.2.4.6).

Not unexpected, the available literature is controversial (Stewart Report 2000). While Lee et al. (2004) observed an alteration of gene expression in HL-60 cells after exposure to RF-EMF of 2450 MHz and Zeng et al. (2004a) an alteration of protein expression in human breast cancer cells (MCF-57) after exposure to RF-EMF of 1800 MHz, no such effects were found by Miyakoshi et al. (2004), who studied the influence of RF-EMF (1950 MHz) on the expression of hsp27 and hsp70 in human glioma cells (MO54). Opposite to the finding of Participant 6 (3.2.4.6, 4.2.4.6), no significant increase in hsp27 expression in endothelial cells was observed by Participant 9 who used a slightly different method (3.2.4.5, 4.2.4.5). Since the hsp27 expression was significantly increased in one laboratory, while this increase was near to significance in the other laboratory, this discrepancy seems to be neglectable. Furthermore, RF-EMF exposure (1800 MHz) at a SAR value of 1,4 W/kg did not affect gene expression in human lymphocytes (3.2.4.4, 4.2.4.4) and after RF-EMF exposure (900 MHz) at a SAR value of 2 W/kg only a few genes among several thousand tested with the micro-array system were found altered in two human immune cell lines (3.2.4.5, 4.2.4.5). Finally, RF-EMF exposure did not affect the expression and activity of the inducible nitric oxide synthase (iNOS) in nerve cells (3.2.4.5, 4.2.4.5).

The outcome of experiments following the genomics and proteomic approach may essentially depend on the cell system investigated and the RF-EMF signal used. Of course, the question remains as to whether or not these alterations in gene and protein expression are within the normal physiological range and if that is the case, they are without any biological relevance.

5.0 CONCLUSIONS

5.1 Conclusions based on the findings obtained in ELF-EMF research

5.1.1 Human fibroblasts, human lymphocytes, human monocytes, human melanocytes, human muscle cells and granulosa cells of rats (Participant 3)

These are the conclusions that Participant 3 draws from their findings:

1. The data strongly indicate a clastogenic potential of intermittent electromagnetic fields, which may lead to considerable chromosomal damage in dividing cells. However, the induced DNA damage did not persist in form of stable translocations.
2. The induced DNA damage was not based on thermal effects and arouses consideration about environmental safety limits for ELF-EMF exposure.
3. The effects were clearly more pronounced in cells from older donors, which could point to an age-related decrease of DNA repair efficiency of ELF-EMF induced DNA strand breaks.
4. In addition, three responder and three non-responder cell types could be identified, which could in part explain different results in reaction to ELF-EMF reported in the literature so far.
5. Fibroblasts from a donor with the genetically DNA repair defect Ataxia Telangiectasia had a more than two fold increase rate of ELF-EMF induced DNA breaks.
6. Between 3 and 550 Hz the largest DNA breaking effects were seen at 16.66 and 50 Hz, the most commonly used frequencies of alternating current in Europe.
7. Taken together, the results suggest that the observed effects of EMF exposure are caused by indirect mechanisms and are not inflicted due to changes in mitochondrial membrane potential.

5.1.2 Human neuroblastoma cell line NB69 and human hepatocarcinoma cell line HepG2 (Participant 5)

Our present results confirm preliminary observations that a 42- or 63-hour exposure to 50 Hz, sine wave MF at 10 or 100 µT (3 hours on/3 h off exposure cycle) can induce changes in the cell growth of NB69 human neuroblastoma cells. The data indicate that such an effect is exerted through an increase in cell proliferation, as revealed by BrdU-incorporation and flow cytometry.

1. In contrast, a 50-Hz MF at 2000 µT magnetic flux density, 5 min on/30 min off exposure cycle, did not affect significantly cell growth on the NB69 line. Thus, our cells were not responsive to these exposure parameters, which have been reported to be effective on differentiating neural embryonic stem cells (Participant 4). Additional experiments exposing NB69 cells to a 100 µT field in a 5 min on/30 min off cycle showed no significant responses. This indicates that the exposure cycle is crucial to eliciting a detectable cellular response.
2. In the NB69 line, the results on PCNA labelling show that at day 6 post-plating the percent of PCNA-positive cells in samples exposed to a 50-Hz, 100-µT field is significantly increased when compared to controls. Actually, the percent of PCNA positive cells significantly decreases in controls between the days 5 and 6 post-plating, whereas such a decrease did not occur in exposed cells. The results suggest that the MF could impair the normal cell cycle regulation through alterations in the late G1 and S-phases.
3. We have also investigated the response of a different human cancer line, the HepG2 human hepatocarcinoma cell line (data not shown), with a growth pattern different from that of the NB69 line. Fifty-Hertz magnetic fields at 10 or 100 µT elicited similar responses in both cell lines, consisting of significant increase in the number of cells at days 5 postplating. In HepG2, the melatonin, at a 10 nM concentration, inhibited the growth-promoting effect induced by the field (Cid et al., 11th International Congress of IRPA, 2004). In the HepG2 line, the growth effect became even stronger when the exposure was maintained until day 7 post-plating, whereas in the NB69 line, an equivalent extension of the exposure period results in a loss of the effect. The differential responses in both cell lines could be due to the fact that, in control conditions, NB69 cultures become saturated at day 7 post-plating and, consequently, their capability to respond to any stimulus is strongly impaired.

4. A 50-Hz, sine wave MF at 100-µT (3h on/3h off exposure cycle) induces a significant reduction in the spontaneous apoptosis of the human neuroblastoma cell line NB69. This response was associated to an increase in the total number of cells. The data suggest that both responses are a consequence of an effect of the field on cell cycle regulation.
5. In NB69 cells, a 50-Hz, sine wave MF at 100-µT (3h on/3h off exposure cycle) alters the activation of the phosphorylated cyclic adenosine monophosphate response-element binding protein (p-CREB) in a time-dependent manner. The results suggest that the activation of p-CREB is involved in the above described effects of this field on cell growth/apoptosis.

5.1.3 Human lymphocytes (Participant 8)

On the whole, data obtained indicate no response of human PBMCs to ELF-EMF exposure. Thus the conclusions are that ELF-EMF do not affect proliferation and cell activation, two fundamental phases of lymphocyte function. Since previous works indicated that pulsed ELF-EMF may interfere with human lymphocyte functionality (Cossarizza et al. 1989a/b, 1991, 1993), future experiments could be addressed to investigate the role of pulsed signal in biological systems in comparison with the negative results obtained with A.C. 50Hz ELF-EMF.

5.1.4 Mouse embryonic stem cells (Participant 4)

1. ELF-EMF signals at a high flux density are capable to transiently increase transcript levels of the regulatory genes egr-1, p21 and c-jun in ES cells deficient for the tumour suppressor p53.
2. The intermittency scheme of the ELF-EMF signals may play a critical role for changes in transcript levels of some regulatory genes.
3. The genetic constitution of pluripotent embryonic stem cells determined by loss of p53 function can influence ELF-EMF-related cellular responses, whereas wild-type cells are insensitive. It remains to be elucidated, whether ELF-EMF-induced changes of expression levels of regulatory genes may be compensated or normalized, or would result in sustained biological effects in vivo.
4. ELF-EMF exposure of ES-derived neural progenitor cells may influence transcript levels of genes of the bcl-2 family and the p53-responsive growth arrest and DNA damage inducible gene GADD45. This finding is an indication that ELF-EMF may affect, at least transiently, fundamental cellular processes including programmed cell death and cell cycle regulation.
5. Alkaline and neutral Comet assay failed to demonstrate a clear effect on the induction of single- and double-strand DNA breaks after ELF-EMF exposure of ES cell derived neural progenitors.

5.1.5 Experiments with embryonic stem cells of mice during cardiac differentiation (Participant 8)

In the ES cell model (GTR 1), ELF-EMF afforded a consistent increase in the expression of genes tightly involved in coaxing ES cells to the cardiac lineage. As shown by in vitro run-off analyses, ELF-EMF affected the transcriptional machine of ES cells. These responses led to the expression of cardiac specific genes and ultimately ensued into a high-throughput of cardiogenesis, as shown by the increase in the number of spontaneously beating colonies in ELF-EMF-exposed cells. Failure of EMF to affect the transcription of a gene promoting skeletal muscle determination and the faint effect on neuronal specification seem to exclude a generalized activation of repressed genes and suggests that coupling of MF with GATA-4, Nkx-2.5 and prodynorphin gene expression may represent a mechanism pertaining to ES cell cardiogenesis. This work represents, in our opinion, a first step toward an extensive investigation concerning the influence of EMF on the expression of a sequence of genes specifically involved in cell differentiation, and in particular the differentiation into a cardiac phenotype, using genomic and post-genomic techniques.

5.1.6 Experiments with the human neuroblastoma cell line SY5Y (Participant 11)

The results clearly demonstrate that, under the discussed exposure conditions, the expression of major components of the cholinergic and catecholaminergic systems is unresponsive to environmental exposure to ELF-EMF.

5.1.7 *Xenopus laevis* oocytes, human fibroblasts and granulosa cells of rats (GFSHR-17 cell line) (Participant 7)

1. For the applied three exposure protocols (50-Hz powerline, 1.0 mT or 2.3 mT continuously applied for 16 h; 50-Hz powerline, 1.0 mT and 2.3 mT intermittently (on/off: 5 min/10 min) applied for 16h the data indicate that the expression level as well as the voltage dependent gating of rCx46-connexons is not significantly affected. Since we could previously show that protein kinase C dependent phosphorylation processes affect the voltage-dependent gating of rCx46-connexons (Ngezahayo et al. 1998), a significant interaction of ELF-EMF on proteinphosphorylation can be neglected. The formation of cell-to-cell channels composed of two rCx46-hemi-channels, respectively, between a pair of mechanically contacting oocytes indicates an effect of ELF-EMF exposure. Exposure virtually suppresses the formation of cell-to-cell channels, but the effect is not significant on the level of three experiments analysed so far. The known $[Ca^{2+}]_o$-dependent gating property of hemi-channels appears not to be influenced by ELF-EMF exposure.
2. Continuous ELF-EMF exposure at high flux intensity 2.3 mT for 30 min did not significantly influence gap junctional coupling (cell-to-cell channels) of cultured pairs of rat granulosa cells as explored by the double whole cell patch-clamp technique.
3. The presented data indicate that intermittent exposure (5 min on / 10 min off) to ELF-EMF (50 Hz, 1 mT) neither generates a long lasting effect on the time course of $[Ca^{2+}]_i$ in cultured fibroblasts nor granulosa cells. This finding appears to be independent of an exposure for 5 to 18h. The corresponding observation of Ivancsits et al. (2003b), of a time dependent increase/decrease of DNA strand breaks with a maximum at about 15h, therefore seems not to be reflected in a corresponding long lasting change of $[Ca^{2+}]_i$. It is interesting to note that such a long lasting effect is also not found for the mitochondrial potential of fibroblasts. ELF-EMF exposure followed by exposure to further stressors, like 200 µM H_2O_2 or 30 mM KCl, also caused no significant change of $[Ca^{2+}]_i$.
4. Exposure experiments show no significant influence on volume regulatory mechanisms of granulosa cells. Further studies of intracellular signal transduction pathways should allow to understand the unsolved question whether significant effects of ELF-EMF on the genomic level are reflected on the cellular level. At present significant changes of cellular properties could not be derived from the analysed cellular parameters.
5. ELF-EMF exposure of cultured granulosa cells shows a significant time dependent increase of double DNA strand breaks with a maximum at about 18 h as observed by the neutral comet assay. This time dependence was also observed at 8 Hz, 16.66 Hz, 30 Hz, 50 Hz and 300 Hz. Therefore, it appears likely that for the chosen ELF-EMF exposure protocol the observed increase of double DNA strand breaks is not frequency dependent. But, the results of the alkaline comet assay indicate a frequency dependent effect of ELF-EMF exposure on the sum of double and single DNA strand breaks. The data obtained for the granulosa cells, after ELF-EMF exposure at 50 Hz, by the alkaline comet assay are comparable with those obtained by participant 3.

5.1.8 Effects of ELF-EMF on gene expression in human cells analysed with the cDNA array (Participant 12)

The gene expression analyses presented here make it very likely that EMFs – RF-EMF and ELF-EMF – can change gene expression in human cells. Although the in vitro studies do not allow any conclusions concerning health risk, the results are an important pre-requisite for further experiments to elucidate the detailed molecular changes in a cell, caused by EMFs.

The most obvious changes have been detected in the expression of genes involved in ribosomal biogenesis and energy metabolism. If the effects are momentary or lead to more dramatic changes like increase of cell proliferation has to be further investigated by molecular assays. The same is true for first ideas how the signalling after EMF exposure could work, referring to the bio-statistic analysis: The Ca-pathway (PIP3, PKC, ERK MAP and other pathways might be involved, but this is not obvious after our analysis) might be involved in regulation after EMF exposure. The actin cytoskeleton (e.g. stress fibers) and ECM possibly is down-regulated, which might lead to dedifferentiation of cells, again important for growth and proliferation of cells. The actin cytoskeleton behaves different in different cell types (adhesive, non-adhesive cells, cell migration etc.), and therefore also has to be investigated with the help of more specific assays.

5.1.9 Summary (Participant 1)

The ELF-EMF data obtained in the course of the REFLEX project allow the following conclusion:

1. ELF-EMF had genotoxic effects on primary cell cultures of human fibroblasts and on other cell lines. These observations were made in two laboratories within the REFLEX consortium (Participants 3 and 7) and confirmed by two other laboratories from outside the REFLEX project. ELF-EMF generated DNA strand breaks at a significant level at a flux density as low as 35 µT. A strong positive correlation was observed between both the intensity and duration of exposure to ELF-EMF and the increase in single and double strand DNA breaks and micronuclei frequencies. Surprisingly this genotoxic effect was only found when cells were exposed to intermittent ELF-EMF, but not to continuous exposure. Responsiveness of fibroblast to ELF-EMF increased with the age of the donor and in the presence of specific genetic repair defects. The effect also differed among the other types of cells examined. In particular, lymphocytes from adult donors were not responsive. Chromosomal aberrations were also observed after ELF-EMF exposure of human fibroblasts.
2. ELF-EMF at a flux density of 10 and 100 µT increased the proliferation rate of neuroblastoma cells (Participant 5) and at a flux density of 0.8 mT it enhanced the differentiation of mouse stem cells into cardiomyocytes (Participant 8). In contrast to these results, no clear-cut and unequivocal effects of ELF-EMF on DNA synthesis, cell cycle, cell differentiation, cell proliferation and apoptosis were found in the many other cell systems under investigation.
3. Elf-EMF inhibited the spontaneous apoptosis in neuroblastoma cells which was followed by an increase of the proliferation rate, when the cells were exposed for 63 hours to ELF-EMF at a flux density of 50 or 100 µT (Participant 5). In contrst to these results, no clear-cut and unequivoval effects of ELF-EMF on the apoptotic process were found in the many other cell systems under investigation.
4. ELF-EMF at a flux density of about 2 mT up-regulated the expression of early genes, such as p21, c-jun and egr-1, in p53- deficient mouse embryonic stem cells, but not in healthy wild-type cells (Participant 4) and, in addition, may affect the expression of genes and proteins in a variety of other cell systems. The results of the whole genome cDNA micro-array and proteomic analyses indicate that EMF may activate several groups of genes that play a role in cell division, cell proliferation and cell differentiation (Participant 12).

Taken together, the results of the REFLEX project were exclusively obtained in in vitro studies and are, therefore, not suitable for the conclusion that ELF-EMF exposure below the presently valid safety limits causes a risk to the health of people. They move, however, such an assumption nearer into the range of the possible. Furthermore, there exists no justification anymore to claim, that we are not aware of any pathophysiological mechanisms which could be the basis for the development of functional disturbances and any kind of chronic diseases in animal and man.

5.2 Conclusions based on the findings obtained in RF-EMF research

5.2.1 Human promyelocytic cell line HL-60 (Participant 2)

1. Different SAR levels have been examined with respect to the effect on comet formation and micronuclei induction in HL 60-cells. Comparing RF-EMF exposure (1800 MHz, continuous wave, 24h) at SAR levels ranging from 0.2 W/kg to 3.0 W/kg indicate that both effects appear to be energy dependent. Whereas at SAR of 0.2 W/kg and 1.0 W/kg both, comet formation and micronucleus frequency, were not significantly different from that observed in sham-exposed control cells, comet formation as well as micronucleus frequency were significantly increased at SAR of 1.3 W/kg, 1.6 W/kg and 2.0 W/kg. The maximal effect was observed at a SAR of 1.3 W/kg. At higher SAR levels from 2.0 to 3.0 W/kg micronucleus frequencies and comet formation were less expressed as compared to the effect noted at a SAR of 1.3 W/kg.
2. In order to extend the statistical basis of evaluation average numbers of micronuclei (micronuclei per 1000 BNC) in different experimental groups were calculated comparing cells exposed either at (i) all SAR tested (0.2 W/kg, 1.0 W/kg, 1.3 W/kg, 1.6 W/kg, 2.0 W/kg, 3.0 W/kg, (ii) higher SAR (1.0 W/kg, 1.3 W/kg, 1.6 W/kg, 2.0 W/kg, 3.0 W/kg) or (iii) lower SAR of 0.2 W/kg or 1.0 W/kg. In both groups (i) and (ii) the number of micronuclei was increased at a significant level ($p<0.001$) as

compared to sham-exposed controls, while in group (iii) micronuclei numbers per 1000 BNC were not significantly different from that observed in sham-exposed controls.

3. Likewise, in order to extend the statistical basis of evaluation average values of Olive Tail Moments as a measure of comet formation were calculated in different experimental groups comparing cells exposed either at (i) all SAR tested (0.2 W/kg, 1.0 W/kg, 1.3 W/kg, 1.6 W/kg, 2.0 W/kg, 3.0 W/kg, (ii) higher SAR (1.0 W/kg, 1.3 W/kg, 1.6 W/kg, 2.0 W/kg, 3.0 W/kg) or (iii) lower SAR of 0.2 W/kg or 1.0 W/kg. In both groups (i) and (ii) the comet formation was increased at a significant level (group (i) $p<0.01$; group (ii) $p<0.001$) as compared to sham-exposed controls, while in group (iii) comet formation was not significantly different from that observed in sham-exposed controls.
4. Experiments on the influence of the duration of exposure showed that short exposure period (6h) caused no (MN) or less (Comet) pronounced effects on micronuclei induction and comet formation as compared to longer exposure periods of 24h. While micronucleus frequencies were further increased after exposure for 72h, comet formation after 72h of exposure was less expressed as compared to 24h exposure.
5. Experiments on the influence of RF-signals showed that at a SAR level of 1.3 W/kg all RF-signals tested, i.e. continuous wave (C.W.), C.W. 5 min on/10 min off, GSM-217 Hz, and GSM-Talk exhibited similar effects on micronuclei induction and on comet formation.
6. By applying sequential approaches for the detection of reactive oxygen species (ROS) in HL-60 cells, an increase in the intracellular generation of free radicals accompanying RF-EMF exposure could be clearly demonstrated by flow cytometric detection of the oxidized nucleotide 8-oxoguanosine (oxy-DNA assay) and the fluorescent Rhodamine 123 (DHR 123 assay), respectively.
7. RF-EMF exposure (1800 MHz, 1.3 W/kg, 24h) had no effect on the cellular doubling time and the activity of the enzyme thymidine kinase of HL 60 cells, indicating that RF-EMF exposure does not influence cellular growth rates.
8. RF-EMF exposure (1800 MHz, 1.3 W/kg, 24h) did not induce apoptosis in HL-60 cells.
9. Within the investigated SAR energy ranges RF-EMF under the in-vitro conditions used are genotoxic in HL-60 cells without affecting cell-cycle distribution cell proliferation or cell progression.
10. The partial-body SAR for any 10-gram tissue like for example the head as exposed region to mobile phone electromagnetic fields should not exceed 2 W/kg according to the Radio-Radiation Protection Guidelines. Notably, our findings on genotoxic effects of RF-fields in HL-60 cells have been shown for SAR levels below these acceptable partial-body SAR levels.
11. These results on genotoxicity in the HL-60 cell line cannot be transferred automatically to other cells, especially to primary cells, and definitely not to whole organism.
12. Clear differences in protein expression have been shown for RF-exposed HL-60 cells as compared to control and sham-exposed cells. This indicates that, as also demonstrated by genetic profiling, RF-EMF exposure has an influence on as well the transcriptional as the translational level in these cells. Clarification of changes in protein expression with respect to functional analysis will help to understand molecular pathomechanisms.

5.2.2 Human fibroblasts and granulosa cells of rats (Participant 3)

Our results imply a genotoxic action of RF-EMFs below proposed radiation safety levels.

1. RF-EMFs were able to induce DNA single and double strand breaks in human fibroblasts and SV40 transformed rat granulosa cells. In contrast to ELF-EMF, genotoxic effects were also observed at continuous exposure.
2. In addition, the decline of DNA strand break levels at elongate exposure (16-24 h), which was found in ELF-EMF exposed cells, could not be demonstrated after RF-EMF exposure. These results could point to differences in mechanisms between the genotoxic action of RF and ELF-EMF
3. Differences in genotoxic effects between different cell types after EMF exposure could be found in RF as well as in ELF-EMF exposed cells.
4. RF-EMF exposure of human fibroblasts was able to induce higher incidences of chromosome aberrations than which was found in ELF-EMF exposed cells.
5. No effects of RF-EMF exposure on mitochondrial membrane potential could be observed. These findings are in accordance with the results obtained with ELF-EMF.

5.2.3 Human lymphocytes and thymocytes (Participant 8)

On the whole, the data obtained indicate a very low response of human PBMCs and no response of thymocytes to RF-EMF exposure. Concerning PBMCs, some results suggest a possible effect on the number of CD95 surface molecules in stimulated T lymphocytes from aged donors. Moreover, other results seem to indicate a greater susceptibility to RF of monocytes with respect to lymphocytes, as demonstrated by a decrease of IL-1 b cytokine, specifically produced by monocytes, in RF-exposed cultures. Future work could be addressed to analyse further effects on these type of human cells.

5.2.4 Human neuroblastoma cell line NB69 and neural stem cells (Participant 5)

1. When administered alone, the exposure to the GSM-Basic signal at a 2W/kg SAR induced a decrease in the number of cells expressing the fibroblast growth factor receptor-1 (FGFR-1), both in NB69 cells and NSC, without affecting significantly the number of cells expressing receptors R2 and R3. The magnitude of the effect on R1 was equivalent to that induced by 20 µg/ml bFGF. Since the GSM-Basic treatment did not affect significantly the total cell number or the cell viability, the above data indicate that RF-induced effect in FGFR-1 is not due to a reduction in the number of cells, but to a loss of the cellular expression of receptor-1.
2. The results also indicate that the exposure to GSM 1800-CW signals at a 2 W/kg SAR induced effects on the expression of FGFR-1 equivalent to those described above for the GSM 1800-Basic signal. No significant effects on the expression of FGFR-1 were observed after exposure to GSM 1800-Talk and DTX signals at 2 W/kg SAR and 1 W/kg SAR, respectively. The data obtained with the different GSM-signals suggest that the cellular response is not dependent on the tested low-frequency modulation.
3. The exposure to the GSM-basic signal induces specific, morphological changes in oligodendrocytes and astrocytes derived from neural stem cells, at day 9 post-plating. These results are indicative that GSM-basic radiation at SAR = 2 W/kg can promote differentiation in NSC. The effect would be exerted through short-term changes in the expression of FGF receptor-1. In contrast, the GSM-Basic signal does not influence cytodifferentiation in NB69 cells or in the neuronal progeny of NSC, as revealed with anti-beta-tubulin antibody.

5.2.5 Brain cells of different origin and human monocytes (Participant 9)

1. Our results strongly suggest that the spontaneous apoptotic process is not a biological target for GSM mobile telephony-related signals. This was shown in different primary cells and cell lines from both nerve and immune systems.
2. Based on the expression and activity of inducible nitric oxide synthase (NOS2) in an astrocytic cell line, GSM-like signals did not “activate” the inflammatory process in nerve cells.
3. No evidence was found of effects of GSM-like signals on heat shock proteins in different mammalian nerve cells. Replication of the previously reported increase in hsp27 expression in a human endothelial cell line after exposure failed.
4. Based on the whole data set, our conclusion is that exposure to low-level GSM-900 signal is unlikely to lead to neurodegeneration or to favour tumour development via pathways involving apoptosis, nitric oxide or heat shock proteins.

5.2.6 Mouse embryonic stem cells (Participant 4)

1. Our present data suggest that currently applied GSM radiation levels under certain circumstances might induce biological effects, at least in cells generated from embryonic stem cells in vitro.
2. The genetic constitution of pluripotent embryonic stem cells determined by loss of p53 function influences RF-EMF-related cellular responses at the level of gene expression, whereas wild-type cells are insensitive. It remains to be elucidated, whether RF-EMF-induced changes of mRNA levels of regulatory genes may be compensated or normalized, or would result in sustained biological effects in vivo.
3. RF-EMF exposure of ES-derived neural precursor cells influences the bcl-2 mediated anti-apoptotic pathway, affects the growth arrest and DNA damage inducible gene GADD45 and the neuronal differentiation by inhibition of Nurr1.

4. Short exposure to RF-EMF could induce double-strand DNA breaks in ES-derived neural progenitor cells (as measured by the neutral Comet assay).

5.2.7 Human the endothelial cell lines EA.hy926 and EA.hy926v1 (Participant 6)

1. RF-EMF appears to be recognized by the cells as an external stress factor because it in response to exposure phosphorylation status of several hundreds proteins was altered either up or down; identification of these proteins will be done in due time.
2. RF-EMF appears to be a weak inducer of cellular stress response because it increases expression and phosphorylation of heat shock protein-27 (hsp27) - a known marker of cellular stress response.
3. RF-EMF induced phosphorylation of Hsp27 appears to be regulated by the activation of up-stream stress kinase p38MAPK.
4. RF-EMF-induced hsp27 activation appears to affect down-stream physiological processes in cell - stabilization of F-actin stress fibers what, in turn, alters cell size and shape (causes rounding-up of cells).
5. Using cDNA Expression Arrays and protein separation by 2-dimensional electrophoresis followed by mass spectrometric identification of individual proteins we have determined that the cellular skeleton appears to be a target of RF-EMF exposure as changes in gene/protein expression of some dozen cytoskeletal proteins were induced by RF-EMF exposure.
6. RF-EMF-induced phosphorylation of hsp27 is followed by translocation of hsp27 to cell nucleus where it appears to interfere with the gene expression processes.
7. RF-EMF causes changes in the expression of several tens of genes and proteins as determined by high-throughput screening technologies - cDNA Expression Arrays and protein separation by 2-dimensional electrophoresis followed by mass spectrometric identification of individual proteins.
8. RF-EMF-induced changes in gene and protein expression appear to be dependent on the cell genotype/phenotype what suggests that some cell types might be more and some less responsive to RF-EMF exposure.
9. RF-EMF induced changes in protein expression appear to be modulation dependent since RF-EMF exposure caused changes whereas CW-EMF did not.
10. the ability of RF-EMF to induce cellular stress response indicates that cells recognize this radiation in spite of it low energy but the induction of stress response per se can not be considered as any indicator of potential health risk.
11. We have practically demonstrated that the use of high-throughput screening methods of transcriptomics and proteomics is useful tool in determining the potential targets of RF-EMF exposure in cells.

5.2.8 Effects of RF-EMF on gene expression in human cells analysed with the cDNA array (Participant 12)

The gene expression analyses presented here make it very likely that EMFs – RF-EMF and ELF-EMF – can change gene expression in human cells. Although the in vitro studies do not allow any conclusions concerning health risk, the results are an important pre-requisite for further experiments to elucidate the detailed molecular changes in a cell, caused by EMFs.

The most obvious changes have been detected in the expression of genes involved in ribosomal biogenesis and energy metabolism. If the effects are momentary or lead to more dramatic changes like increase of cell proliferation has to be further investigated by molecular assays. The same is true for first ideas how the signalling after EMF exposure could work, referring to the bio-statistic analysis: The Ca-pathway (PIP3, PKC, ERK MAP and other pathways might be involved, but this is not obvious after our analysis) might be involved in regulation after EMF exposure. The actin cytoskeleton (e.g. stress fibers) and ECM possibly is down-regulated, which might lead to dedifferentiation of cells, again important for growth and proliferation of cells. The actin cytoskeleton behaves different in different cell types (adhesive, non-adhesive cells, cell migration etc.), and therefore also has to be investigated with the help of more specific assays.

5.2.9 Summary (Participant 1)

The RF-EMF data obtained in the course of the REFLEX project allow the following conclusion:

1. RF-EMF produced genotoxic effects in fibroblasts, HL-60 cells, granulosa cells of rats and neural progenitor cells derived from mouse embryonic stem cells (Paticipants 2, 3 and 4). Cells responded to RF-EMF exposure between SAR levels of 0.3 and 2 W/kg with a significant increase in single and double strand DNA breaks and in micronuclei frequency (Participants 2 and 3). Chromosomal aberrations in fibroblasts were also observed after RF-EMF exposure (Participant 3). In HL-60 cells an increase in the intracellular generation of free radicals accompanying RF-EMF exposure could clearly be demonstrated (Participant 2).
2. No clear-cut and unequivocal effects of RF-EMF on DNA synthesis, cell cycle, cell proliferation, cell differentiation and immune cell functionality were found in the cell systems under investigation. (Participants 2, 3, 4, 5, 6, 8). There is some indication that RF-EMF may affect the growth arrest and DNA damage inducible gene GADD45 and the neuronal differentiation by inhibition of Nurr1 in neural progenitor cells (Participant 4).
3. No clear-cut and unequivocal effects of RF-EMF on apoptosis were fond in the cell systems under investigation was observed (Participants 2, 3, 4, 5, 6, 8 and 9). There is some indication that RF-EMF may have some influence on the bcl-2 mediated anti-apoptotic pathway in neural progenotor cells (Participant 4) and on the the p38MAPK/hsp27 stress response pathway in endothelial cells of human origin (Participant 6) which may in turn exert an inhibitory effect on apoptosis.
4. RF-EMF at a SAR of 1.5 W/kg down-regulated the expression of neuronal genes in neuronal precursor cells and up-regulated the expression of early genes in p53-deficient embryonic stem cells, but not in wild-type cells (Participant 4). Proteomic analyses on human endothelial cell lines showed that exposure to RF-EMF changed the expression and phosphorylation of numerous, largely unidentified proteins. Among these proteins is the heat shock protein hsp27, a marker for cellular stress responses (Participant 6). The results of the whole genome cDNA micro-array and proteomic analyses indicated that EMF may activate several groups of genes that play a role in cell division, cell proliferation and cell differentiation (Participants 2, 6 and 12).

Taken together, the results of the REFLEX project were exclusively obtained in in vitro studies and are, therefore, not suitable for the conclusion that RF-EMF exposure below the presently valid safety limits causes a risk to the health of people. They move, however, such an assumption nearer into the range of the possible. Furthermore, there exists no justification anymore to claim, that we are not aware of any pathophysiological mechanisms which could be the basis for the development of functional disturbances and any kind of chronic diseases in animal and man.

6.0 EXPLOITATION AND DISSEMINATION OF RESULTS

6.1 Coordination (Participant 1)

a. Scientific publications, meetings, interviews, and round tables

Adlkofer F et al.: Brochure presenting the REFLEX project, June 2001 (*for distribution*)

Adlkofer F et al.: Oral presentation of results. Bundesamt für Strahlenschutz, Salzgitter/Germany, June 21-22, 2001, p. 18-19

Adlkofer F et al.: Oral presentation of the project. EBEA, Helsinki/Finland, Sep 6-8, 2001. Proceedings, p. 54-56, 269-270

Adlkofer F et al.: Oral presentation of results. EU/Japan/Korea/US workshop on EMF, mobile telephony and health. Brussels/Belgium, Oct 29-30, 2001, Proceedings, p. *(not numbered)*

Adlkofer F et al.: Oral presentation of results. Institut für Zoologie, Technical University of Dresden/ Germany, April 2, 2002

Adlkofer F et al.: Oral presentation of results. COST281/EBEA Forum, Rome/Italy, May 2-5, 2002

Adlkofer F et al.: Oral presentation of the project.. 24th BEMS Meeting, Quebec City/Canada, June 23-27, 2002, Proceedings, p. 91-92, 95, 98-100

Adlkofer F et al.: Oral presentation of the project and of results. PIERS, Cambridge/Mass/USA., July 1-5, 2002. Proceedings, p. 498

Adlkofer F et al.: Oral presentation of results. Cursos de Verano Universidad de Malaga, Ronda/Spain, July 22-26, 2002

Adlkofer F et al.: Oral presentation of the project. Biological Effects of EMF, Rhodes/Greece, Oct 7-11, 2002. Proceedings, p. 514-522

Adlkofer F, Rüdiger HW, Wobus AM: DNA-Doppelstrangbrüche bei intermittierender Exposition. Diskussionsbeitrag. Deutsches Ärzteblatt, Nov 15, 2002, p. 3114-3115

Adlkofer F et al.: Oral presentation of results. Elektromagnetische Felder in der Umwelt, Umwelt-ministerium Nordrhein-Westfalen, Dortmund/Germany, Nov 28, 2002

Adlkofer F et al.: Oral presentation of results. The EMF Biological Research Trust, London/UK, Jan 16, 2003

Adlkofer F et al.: Oral presentation of results. WHO EMF Project, Research Coordination Meeting, Geneva/Switzerland, June 12-13, 2003

Adlkofer F et al.: Oral presentation of the project. 25th BEMS Meeting, Maui/Hawaii, June 22-27, 2003. Proceedings, p. 127, 135 - 136

Adlkofer F: Interview. Television feature on “Elektrosmog”, ARD Germany, Aug 7, 2003

Adlkofer F et al.: Oral presentation of results. 3rd Int. EMF Seminar, Guilin/China, Oct 13-17, 2003. Proceedings, p. 23 - 24

Adlkofer F et al.: Oral presentation of results. O_2 Telecommunication Company, München/Germany, Oct 22, 2003

Adlkofer F et al.: Oral presentation of results. Die Umwelt-Akademie e.V., München/Germany, Dec 5, 2003

Adlkofer F: Interview. Bayer. landwirtschaftliches Wochenblatt, Heft 5, Jan 2004, p. 48

Adlkofer F: Interview. life + sciences, Heft 1, Feb - April 2004, p. 30-31

Adlkofer F et al.: Oral presentation of results. Bündnis 90/Die Grünen, Bavarian State Parliament, München/Germany, April 2, 2004

Adlkofer F: Round Table Discussion. Bayer. Akademie der Wissenschaften, München/Germany,

April 29, 2004

Adlkofer F et al.: Oral presentation of results. EMF-NET, Brussels/Belgium, April 30, 2004

Adlkofer F et al.: Oral presentation of results. BUND, 3. Rheinland-Pfälzisch-Hessisches Mobilfunk-symposium, Mainz, June 12, 2004. Tagungsband, p. 33 - 49

b. Posters

Adlkofer F et al.: Poster presentation. EBEA, Helsinki/Finland, Sep 6-8, 2001

Adlkofer F et al.: Poster presentation. An Environment for Better Health Conference, Arhus/Denmark, May 8-11, 2003

6.2 Experiments with the human promyelocytic cell line HL-60 (Participant 2)

Research performed is basic research with relevance for life science and techniques, respectively. The results obtained by participant 2 have been subsequently actualised and reported in the usual scientific manner. These reports included confidential Annual Reports as progress reports to the European Commission (1st, 2nd, 3rd Annual Report) and public presentations at the following scientific meetings: BEMS 2002 (Radiofrequency EMF and DNA strand breaks), BEMS 2002 (RF-EMF genotoxic effects), PIERS 2002 (1800 MHz radiofrequency exposition of human HL-60 cells induces DNA strand breaks as measured by the alkaline comet assay), BEMS 2003 (Genotoxic effects of RF-EMF on cultured cells in vitro), Deutscher Ärztekongress 2002 (Workshop in German), Deutscher Ärztekongress 2003 (Workshop in German). Peer-reviewed publications have been prepared and will be submitted after the end of the project.

a. Scientific papers

in preparation:

Schlatterer K., Gminski R., Tauber R., Fitzner R. (2004) Radiofrequency (1800 MHz) electromagnetic fields cause DNA strand breaks and micronuclei formation in HL-60 human promyelocytic cells.

b. Scientific meetings

Fitzner R, Gminski R, Schlatterer K (2004) 1800 MHz radiofrequency electromagnetic fields cause energy-dependent genotoxic effects in human promyelocytic HL-60 cells. Session 14: Non thermal biological effects of EM Fields used for mobile communication. Progress In Electromagnetic Research Symposium (PIERS 2004), Pisa, March 28-31, 2004 (oral presentation)

Fitzner R (2004) In-vitro-Untersuchungen an HL-60-Zellen – Einfluss niederfrequenter Magnetfelder (50 Hz; 162/3 Hz). Umweltmedizin – Elektromagnetfelder, Zellen, Gesundheit, 53. Deutscher Ärztekongress, 3.-5. Mai, Berlin 2004 (oral presentation)

Schlatterer-Krauter, K (2004) Einfluss hochfrequenter Elektromagnetfelder des Mobilfunks – Erbgut-veränderungen direkt oder indirekt verursacht. Umweltmedizin – Elektromagnetfelder, Zellen, Gesundheit, 53. Deutscher Ärztekongress, 3.-5. Mai, Berlin 2004 (oral presentation)

c. Posters

Fitzner R, Gminski R, Schlatterer K (2004) Radiofrequency electromagnetic fields (1800 MHz) induce elevated production of reactive oxygen species in human promyelocytic HL-60 cells. Poster presentation. Bioelectromagnetics Society 26th Annual Meeting, 20-24th June, Washington, 2004

6.3 Experiments with human fibroblasts, human lymphocytes, human monocytes, human melanocytes, human muscle cells and granulosa cells of rats (Participant 3)

a. Scientific papers

published:

Ivancsits S, Diem E, Rüdiger HW, Jahn O (2002) Induction of DNA strand breaks by intermittent exposure to extremely-low-frequency electromagnetic fields in human diploid fibroblasts. Mutation Res 519: 1-13

Ivancsits S, Diem E, Jahn O, Rüdiger HW (2003) Intermittent extremely low frequency electromagnetic fields cause DNA damage in a dose dependent way. Int Arch Occup Env Health 76: 431-436

Ivancsits S, Diem E, Jahn O , Rüdiger HW (2003) Age-related effects on induction of DNA strand breaks by intermittent exposure to electromagnetic fields. Mech Age Dev 124: 847-850

submitted:

Diem E, Jahn O, Rüdiger HW. Non-thermal DNA breakage by mobile phone radiation in human fibroblasts and transformed GFSH-R17 (rat granulosa) cells in vitro. Mutation Research

Ivancsits S, Diem E, Jahn O, Rüdiger HW. Chromosomal damage in human diploid fibroblasts by intermittent exposure to extremely low frequency electromagnetic fields. Mutation Research

Ivancsits S, Diem E, Jahn O, Rüdiger HW. Cell type specific genotoxic effects of intermittent extremely low frequency electromagnetic fields. Mutation Research

Ivancsits S, Diem E, Jahn O, Rüdiger HW. Intermittent exposure to extremely low frequency electromagnetic fields increases the genotoxic sensitivity to UV-light or mild thermal stress in cultured human fibroblasts. J Toxicol Envir Health

Pilger A, Ivancsits S, Diem E, Steffens M, Kolb HA, Rüdiger HW. No long-lasting effects of intermittent 50 Hz electromagnetic field on cytoplasmic free calcium and mitochondrial membrane potential in human diploid fibroblasts. Radiat Envir Biophysics

b. Scientific meetings

Wiener Forum Arbeitsmedizin, April 2000 Vienna,"Elektrosmog – Neue Untersuchungen zur gentoxischen Wirkung elektromagnetischer Felder" Oswald Jahn Oral presentation

26th International Conference of Occupational Health (ICOH), 27th August-1st September2000, Singapore, In vitro evaluation of genotoxic potential of low EMF of 50 Hz" Oswald Jahn, Eva Valic, Elisabeth Diem, Hugo W. Rüdiger Oral presentation

Tagung der Österreichischen Gesellschaft für Arbeitsmedizin (ÖGAM), 27-28th September 2002, Vienna. Sabine Ivancsits, Elisabeth Diem, Hugo W. Rüdiger and Oswald Jahn „Biologische Wirkung elektromagnetischer Felder" Oral presentation

Bioelectromagnetics Society 24th Annual Meeting, 23rd-26th June, Quebec, 2002

H.W. Rüdiger, S. Ivancsits, E. Diem, A. Pilger, F. Bersani, O. Jahn. „Genotoxic effects of extremely-low-frequency electromagnetic fields on human cells in vitro" Oral presentation

Gesellschaft für Umwelt und Mutationsforschung 20. Jahrestagung, 17-20th March 2003, Mainz. Sabine Ivancsits, Elisabeth Diem, Oswald Jahn and Hugo W. Rüdiger „Dosisabhängige Induktion von DNA-Strangbrüchen nach niederfrequenter elektromagnetischer Bestrahlung" Oral presentation

Bioelectromagnetics Society 25th Annual Meeting, 22-27th June, Maui, 2003

H.W. Rüdiger, S. Ivancsits, E. Diem, O. Jahn. „Genotoxic effects of extremely-low-frequency electromagnetic fields on human cells in vitro" Oral presentation

Tagung der Österreichischen Gesellschaft für Arbeitsmedizin (ÖGAM), 19-20th September 2003, St. Pölten.. Sabine Ivancsits, Elisabeth Diem, Oswald Jahn and Hugo W. Rüdiger „Induktion von chromosomalen Aberrationen durch niederfrequente elektromagnetische Felder" Oral presentation.

6th Congress of European Bioelectromagnetics Association (EBEA), November 13–15th 2003, Budapest. S. Ivancsits, E. Diem, O. Jahn, H.W. Rüdiger "In vitro genotoxic effects of extremely-low-frequency electromagnetic fields" Oral presentation

c. Poster

Tagung der Österreichischen Gesellschaft für Arbeitsmedizin (ÖGAM), 28-29th September 2001, Salzburg, Sabine Ivancsits, Elisabeth Diem, Hugo W. Rüdiger and Oswald Jahn „Gentoxische Wirkung

von elektromagnetischen Feldern“ Poster presentation

Conference on RF interactions with Humans: Mechanisms, Exposure and MedicalApplications, 27-28th February 2003, London. E. Diem, S. Ivancsits, H.W. Rüdiger “Non-thermal DNA breakage by mobile phone radiation in human fibroblasts and transformed GFSH-R17 (rat granulosa) cells” Poster presentation

Gesellschaft für Umwelt und Mutationsforschung 20. Jahrestagung,17-20th March 2003, Mainz. E. Diem, S. Ivancsits, H.W. Rüdiger “Non-thermal DNA breakage by mobile phone radiation in human fibroblasts and transformed GFSH-R17 (rat granulosa) cells” Poster presentation

4. Gemeinsame Jahrestagung der Österreichischen und Deutschen Gesellschaft für Arbeitsmedizin, April 22-24th 2004, Innsbruck. S. Ivancsits, E. Diem, O. Jahn, H.W. Rüdiger “Induktion von chromosomalen Schäden durch niederfrequente elektromagnetische Felder“ Poster presentation

4. Gemeinsame Jahrestagung der Österreichischen und Deutschen Gesellschaft für Arbeitsmedizin, April 22-24th 2004, Innsbruck. A. Pilger, S. Ivancsits, E. Diem, M. Steffens, H.A. Kolb, H.W. Rüdiger „Intermittierende Belastung mit 50 Hz ELF-EMF bewirkt keine Veränderungen des mitochondriellen Menbranpotentiales und freien Kalziums in humanen Fibroblasten“ Poster Presentation

6.4 Embryonic stem cells (Participant 4)

a. Scientific papers

published:

Jaroslaw Czyz, Kaomei Guan, Qinghua Zeng, Teodora Nikolova, Armin Meister, Frank Schönborn, Jürgen Schuderer, Niels Kuster, and Anna M. Wobus. High frequency electromagnetic fields affect gene expression levels in tumor suppressor p53-deficient embryonic stem cells. Bioelectromagnetics (2004) 25: 296-307.

Jaroslaw Czyz, Teodora Nikolova, Jürgen Schuderer, Niels Kuster, and Anna M. Wobus. Non-thermal effects of power-line magnetic fields (50 Hz) on gene expression levels of embryonic stem cells – the role of tumour suppressor p53. Mutation Research (2004) 557(1): 63-74.

submitted:

Teodora Nikolova, Jaroslaw Czyz, Alexandra Rolletschek, Przemyslaw Blyszczuk, Jürgen Schuderer, Niels Kuster, and Anna M. Wobus. Electromagnetic fields affect the transcript levels of apoptosis-related genes in embryonic stem cell-derived neural progenitor cells. Submitted to Environ Health Persp.

Part of our results were presented by Participant 8 of the REFLEX Project at the International BEMS 2003 Conference in June, 2003 in Maui, Hawaii.

6.5 Experiments with the human neuroblastoma cell line NB69 and neural stem cells (Participant 5)

For our REFLEX studies a specific software for the analysis of immunocytochemical images was developed in collaboration with Escuela Universitaria de Ingeniería Técnica Industrial (Erasmus-Socrates arrangement between Madrid-Belgium).

Part of the results has been presented in different meetings: BEMS, EBEA, and 2nd International Workshop on Biological Effects of EMF.

a. Scientific papers:

published:

Carlos Platero, Kristof Verbiest, Alejandro Úbeda, M-Angeles Trillo, Jaime Gosalvez, and Javier Bartolomé. Platform opened for the processing and management of biomedical images. XXI Jornadas of

Automática 1-7. ISBN: 84-699-3163-6 (2000)

Carlos Platero, M-Angeles Trillo and Alejandro Úbeda. Processing of biomedical images for the study of the potential influence of GSM electromagnetic radiation on neural stem cells. XXIII Jornadas of Automática 1-7. ISBN: 84-699-8916-2 (2002)

in preparation:

M-Angeles Trillo, M-Antonia Cid, M-Antonia Martinez, Vicente-J. Garcia, Alejandro Úbeda and Jocelyne Leal. Influence of 50 Hz magnetic fields on the proliferation and apoptosis of human neuroblastoma cells *in vitro"*.

M.A.Trillo, G. Alegría, M.A. Martínez, D. Reimers, E. Bazán, A. Úbeda, Jürgen Schuderer and J. Leal. Influence of RF fields (GSM signals, 1800 MHz) on the expression of FGFR1 by NB69 human neuroblastoma cell line and neural stem cells from rat embryonic nucleus striatum".

M.A. Trillo, M.A. Martínez, M.A. Cid A. Úbeda and J. Leal. 50 Hz sinus wave magnetic field at 100 µT activates phosphorilated cyclic adenosine monophosphate response-element binding protein (P-CREB) in NB69 human neuroblastoma cell line.

6.6 Human the endothelial cell lines EA.hy926 and EA.hy926v1 (Participant 6)

a. Scientific papers

published:

Leszczynski D, Joenväärä S, Reivinen R, Kuokka R. Non-thermal activation of hsp27/p38MAPK stress pathway by mobile phone radiation in human endothelial cells: Molecular mechanism for cancer- and blood-brain barrier-related effects. Differentiation 70, 2002, 120-129

Leszczynski D, Nylund R, Joenväärä S, Reivinen J. Applicability of Discovery Science-Approach to Determine Biological Effects of Mobile Phone Radiation. Proteomics 4, 2004, 426-431

Nylund R, Leszczynski D. Proteomics analysis of human endothelial cell line EA.hy926 after exposure to GSM 900 radiation. Proteomics, 4, 2004, 1359-1365

submitted:

Leszczynski D. Mobile phone radiation and blood-brain barrier: The available scientific evidence is insufficient to support or dismiss claims of an effect.

in preparation:

Nylund R, Griffin T, Maercker Ch, Schuderer J, Kuster N, Aebersold R, Leszczynski D. Effect of low-energy microwaves on protein expression in human endothelial cell line might be frequency modulation-dependent

Nylund R, Toivo T, Sihvonen AP, Jokela K, Schuderer J, Kuster N, Landry J, Leszczynski D. Mobile phone radiation-induced activation of cellular stress response induces cytophysiological effects.

Nylund R, Reivinen J, Leszczynski D. Cellular response to mobile phone radiation is proteome- and genotype-dependent

Leszczynski D. Induction of Cellular Stress Response by Mobile Phone Radiation: Possible mechanism behind the effects – a molecular biologists perspective. invited review for IEEE Transactions

Nylund R, Toivo T, Sihvonen AP, Schuderer J, Jokela K, Kuster N, Leszczynski D. Mobile phone radiation-induced Hsp27 stress response in human endothelial cell line EA.hy926 is a non-thermal effect.

b. Scientific meetings and reports for the media

invited lectures:

Harvard University, Boston, MA, USA, 15.11.2000, Proteomics: a novel approach to determine health effects of mobile phone radiation.

Centre for Immunology at St. Vincent's Hospital, Sydney, Australia, 11.02.2002, Possible Effects of Mobile Phones on Brain - Should We Be Afraid?

Telstra Laboratories, Melbourne, Australia, 12.02.2002, Mobile Phones and Health Risk: Why Do We Know So Little?

Department of Physics, Sydney University, Sydney, Australia, 15.02.2002, Mobile Phones, Cancer and Blood-Brain Barrier: A Possible Molecular Mechanism.

Zheijang University, School of Public Health, Hangzhou, China, 10.10.2003; Biological effects of mobile phone radiation.

Brooks AFB, San Antonio, TX, USA, 3.12.2003; Application of transcriptomics and proteomics in search for the potential health effects of EMF.

invited presentations at the conferences:

24th Annual Meeting of Bioelectromagnetics Society, Quebec City, Canada, 23-27.06.2002, Effect of mobile phone radiation on gene and protein expression.

27th General Assembly of the International Union of Radio Science (URSI), Maastricht, The Netherlands, 17-24.08.2002, Effect of GSM mobile phone radiation on blood-brain barrier: Use of proteomics approach to define the hypothetical molecular mechanism.

COST 281 Seminar "Subtle Temperature Effects of RF-EMF", 12-13.11.2002, London, UK, Indirect evidence of non-thermal biological effects induced by mobile phone radiation in vitro.

FGF & COST 281 Workshop on "Genetic and Cytogenetic Aspects of RF-Field Interaction", 24-27.11.2002, Löwenstein, Germany, Mobile phone radiation-induced gene expression might be cell genotype-dependent.

Proteomica Symposium, University of Madrid, 4-8.02.2003, Cordoba, Spain, Use of discovery science-approach to elucidate bio-effects of electromagnetic fields.

25th Annual Meeting of Bioelectromagnetics Society, Maui, HI, USA, 23-27.06.2003, Use of discovery science-approach to elucidate bio-effects of electromagnetic fields. (Plenary talk)

25th Annual Meeting of Bioelectromagnetics Society, Maui, HI, USA, 23-27.06.2003, Cellular response to mobile phone radiation appears to be cell genotype-dependent.

WHO & ICNIRP & China Health Ministry, 3rd International EMF Seminar in China: Electromagnetic Fields and Biological Effects, 14-17.10.2003, Guilin, China, Discovery science and mobile phone safety: a need for the new research approach. (Keynote talk)

FGF & COST281 Workshop "The Blood-Brain Barrier (BBB) - Can it be influenced by RF-field interactions?", 3-6.11.2003, Reisensburg, Germany, Mobile Phone Radiation and Blood-Brain Barrier: The available scientific evidence is insufficient to dismiss or to support claims of a health risk in humans.

6th Meeting of the European BioElectromagnetics Association, Budapest, Hungary, 12-16.11.2003, New research approach in EMF research - proteomics and transcriptomics (Plenary talk)

IEEE ICES (SCC-28) meeting, San Antonio TX, USA, 4-7.12.2003; Use of high-throughput screening techniques to determine biological effects of mobile phone radiation.

26th Annual Meeting of the Bioelectromagnetics Society, Washington, DC, USA, 20-25.06.2004; Biological effects of EMF: do they exist and what might be their biophysical mechanism - a molecular biologists perspective.

6.7 rCx46 in oocytes of *Xenopus laevis* and human fibroblasts and granulosa cells of rats (Participant 7)

a. Scientific papers

submitted:

Pilger A., Ivancsits S., Diem E., Steffens M., Kolb H-A., Rüdiger H. W. No long-lasting effects of intermittent 50 Hz electromagnetic field on cytoplasmic free calcium and mitochondrial membrane potential in human diploid fibroblasts Radiation and Environmental Biophysics.

in preparation:

Steffens M., Enders O., Behnsen J., Kolb H.-A. Effects of intermittent 50 Hz electromagnetic field on gap junctional coupling of paired *Xenopus laevis* oocytes expressing rCx46.

Steffens M., H.-A. Kolb; Effects of intermittent 50 Hz on conducting connexons of rCx46 expressed in oocytes of *Xenopus laevis*.

Steffens M., Kolb H.-A. Frequency dependent induction of DNA strand breaks by intermittent exposure to extremely low frequency electromagnetic field in various cultered cell lines.

b. Scientific meetings and reports

Steffens M., Kolb, H.-A. Gene expression of rCx46 in *Xenopus* oocytes is not affected by 50 Hz electromagnetic radiation. Pflügers Arch. 443 (Plenary Lectures, Oral Sessions, Poster Sessions, Symposia) : S280 (2002)

Wobus A.M., Trillo M.A., Ubeda A., Kolb H.-A. Effects of ELF-and RF-EMF on cell proliferation and cell differentiation. 25th Annual Meeting of the BEMS, Maui, USA (June 2003). Proceedings: p. 133

Kolb, H.-A. Die Angst vor dem Strom. Neue Presse. Hannover (September 05, 2003)

Kolb, H.-A. Biologische Wirkungen ELF- und RF- elektromagnetischer Felder (EMF) (Nicht-ionische Wirkungen). 1.Nationaler Kongress Elektrosmog-Betroffener, Biel, Switzerland (22.11.2003)

Kolb, H.-A. Interview with Südwestfunk: Wirkung von EMF auf biologische Systeme. Hannover (July 07, 2004)

c. Poster

M. Steffens, H.-A. Kolb; Gene expression of rCx46 in *Xenopus* oocytes is not affected by 50 Hz electromagnetic radiation. The Physiological Society Scandinavian Physiological Society, Deutsche Physiologische Gesellschaft (81st Annual Meeting), Tübingen, Germany (15–19 March 2002)

6.8 Experiments with human lymphocytes and thymocytes and with mice embryonic stem cells during cardiac differentiation (Participant 8)

a. Scientific papers

published:

Capri M, Scarcella E, Bianchi E, Fumelli C, Mesircas P, Agostini C, Remondini D, Schuderer J, Kuster N, Franceschi C, Bersani F (2004) 1800 MHz radiofrequency (mobile phones, different Global System for Mobile communication modulations) does not affect apoptosis and heat shock protein 70 level in peripheral blood mononuclear cells from younf and old donors. Int J Radiat Biol, Vol 80, No 6: p. 389 - 397

Ventura C, Maioli M, Asara Y, Santoni D, Mesirca P, Remondini D, Bersani F (2004) Turning on stem cell cardiogenesis with extremely low frequency magnetic fields. The FASEB J, published online Oct 26, 2004.

in preparation:

Magnetic fields and cell fate specification in embryonic stem cells. In preparation for Science

b. Scientific meetings

Effects of ELF-EMF on gene expression of various cell lines. 25th Annual BEMS Meeting 2003, Abstract book, p. 131

6.9 Experiments with brain cells of different origin and human monocytes (Participant 9)

a. Scientific papers

in preparation:

Poulletier de Gannes F. et al., Effects of GSM-900 radiofrequency radiation on apoptosis in brain cells. (Ready to be submitted to Radiation Research)

Lagroye I. et al., GSM-900 signal does not affect iNOS expression in rat C6 glioma cells (in preparation for Radiation Research)

Lagroye I. et al., Apoptosis in U937 after exposure to 217 Hz-modulated GSM-900 radiofrequency radiation. (in preparation for Bioelectromagnetics)

Poulletier de Gannes F. et al., Expression of heat shock proteins in brain cells after exposure to GSM-900 radiofrequency radiation (in preparation for International Journal of Radiation Biology)

b. Scientific meetings

Lagroye I., E. Haro, P.-E. Dulou, B. Billaudel, B. Veyret. Effect of GSM-900 exposure on NOS-II expression in rat C6 glioma cells. 24st Annual Meeting of the BEMS, Quebec, Canada (June 2002)

Lagroye I., E. Haro, P.-E. Dulou, B. Billaudel, B. Veyret. Effect of GSM-900 exposure on NOS-II expression in rat C6 glioma cells. 24st Annual Meeting of the BEMS, Quebec, Canada (June 2002).

Leszczynski, D., Billaudel, B., Czyz, J., Dulou, P-E., Guan, K., Haro, E., Joenväärä, S., Kuokka, R., Lagroye, I. , Meister, A., Reivinen, J., Veyret, B., Wobus, A.M., Zeng. Q. Effects of mobile phone radiation on gene and protein expression in vitro. 24st Annual Meeting of the BEMS, Quebec, Canada (June 2002).

Lagroye I, Bersani F., Billaudel B., Capri M., Czyz J., Dulou P-E., Guan K. Haro E., Joenväärä S., Kuokka R., Kuster N.,., Leszczynski D., Meister A., Reivinen J., Schuderer J., B. Veyret, A.M. Wobus, Q. Zeng. Do ELF or RF fields affect the apoptotic process? Data from the REFLEX programme. 24st Annual Meeting of the BEMS, Québec, Canada (June 2002).

Lagroye I., Poulletier de Gannes F., Haro E., Billaudel B., Dulou P.E., Veyret B. Effect of GSM-900 radiofrequency on apoptosis of immune and nervous cells. 27ème assemblée générale de l'URSI, Maastricht, Pays-Bas, (August 2002).

Lagroye I., Bersani F., Agostini C., Bianchi E.,Billaudel B., Capri M., Dulou P.E., Fumelli C., Haro E., Mesirca P., Poulletier de Gannes F., Scarcella E., Veyret B., Do GSM signals induce apoptosis in mammalian immune and nervous cells? 2nd International Workshop on Biological effects of EMF's, Rhodes, Crète, (October 2002).

Lagroye I., Bersani F., Agostini C., Bianchi E., Billaudel B., Capri M., Dulou P.E., Fumelli C., Haro E., Mesirca P., Poulletier de Gannes F., Scarcella E., Veyret B. Do GSM-900 signals induce apoptosis in mammalian immune and nervous cells? 2nd International Workshop on Biological Effects of Electromagnetic Fields, Rhodes, October 7-11, 2002, page 404-408.

Adlkofer F., R. Tauber, H.W. Rüdiger, A.M. Wobus, A. Trillo, D. Leszczynski, H.-A. Kolb, F. Bersani, I. Lagroye, N. Kuster, F. Clementi, C. Maercker, Risk Evaluation of Potential Environmental Hazards from Low Energy Electromagnetic Field Exposure Using Sensitive in vitro Methods (REFLEX), 2nd International Workshop on Biological effects of EMF's, Rhodes, Crète, (October 2002).

Poulletier de Gannes F., I. Lagroye, E. Haro, P.E. Dulou, B.Billaudel, B. Veyret. Heat shock proteins as sensors of nonthermal effects? Subtil effects of temperature, Cost 281 meeting, London, UK, (november 2002).

Lagroye I., Bersani F., Billaudel B., Capri M., Czyz J., Dulou P-E., Guan K., Haro E., Joenväärä S., Kuokka R., Kuster N., Leszczynski D., Meister A., Poulletier de Gannes F., Reivinen J., Schuderer J., Veyret B., Wobus A.M., Zeng Q. Effects of ELF- and RF-EMF on the apoptotic process. Abstract for the BEMS 25th annual meeting - Maui, Hawaii, June 22-27, 2003, page 134.

Poulletier de Gannes F., I. Lagroye, E. Haro, M. Taxile, P.E. Dulou, B. Billaudel, B. Veyret, Effects of GSM-900 on apoptosis in brain cells. 6th International Congress of the European BioElectromagnetics Association, 2003, Budapest, Hongrie (November 2003).

Lagroye I., Haro E., Billaudel B., Veyret B. The effect of GSM-900 radiofrequency radiation on camptothecin-induced apoptosis in human U937 lymphoblastoma cells. 6th International Congress of the European BioElectromagnetics Association, 2003, Budapest, Hongrie (November 2003).

Poulletier de Gannes, F., Sanchez, S., Lagroye, I., Haro, E., Dulou, P.-E., Billaudel, B., Veyret. B. In vitro and in vivo studies of the effects of GSM-900 microwave exposure on heat shock proteins in the brain and skin. 25th Annual Meeting of the BEMS, Maui, USA (June 2003).

Lagroye I, Bersani F, Billaudel B, Capri M, Czyz J, Dulou P-E., Guan K., Haro E., Joenväärä S., Kuokka R., Kuster N., Leszczynski D., Meister A., Poulletier de Gannes F. Reivinen J., Schuderer J., B. Veyret, A.M. Wobus, Q. Zeng. Effects Of ELF And RF Fields On Apoptosis In Different Cell Lines. . 25th Annual Meeting of the BEMS, Maui, USA (June 2003).

F. Poulletier de Gannes, S. Sanchez, I. Lagroye, E. Haro, B. Billaudel, B. Veyret. Effects of GSM-900 microwave exposure on heat shock proteins: *in vitro* and *in vivo* studies on different models in PIOM laboratory. COST 281bis workshop on "Influence of RF Fields on the Expression of Stress Proteins". April 28-29 2004, STUK Helsinki, Finland.

F. Poulletier de Gannes, I. Lagroye, S. Sanchez, B. Billaudel, B. Veyret. Effect of GSM-900 exposure on hsp27 expression in EA-hy926 endothelial cells: a replication study. 26th Annual Meeting of the BEMS, Washington DC, USA (June 2004).

6.10 Provision of exposure setups and technical quality control (Participant 10)

Exploitation

The success of the exposure setups developed under the umbrella of REFLEX have resulted in additional demands for similar setups being used in further European research programs, e.g. PERFORM B.

Dissemination

a. Scientific papers

published:

J. Schuderer, T. Schmid, G. Urban, N. Kuster, “Novel High Resolution Temperature Probe for RF Dosimetry”, Physics in Medicine and Biology, vol 49, pp. N83-N92, 2004.

J. Schuderer, T. Samaras, W. Oesch, D. Spät, N. Kuster, “High Peak SAR Exposure Unit with Tight Exposure and Environmental Control for In Vitro Experiments at 1800 MHz”, IEEE Transactions on Microwave Theory and Techniques, vol 52, No 8, 2004: 2057-2066

J. Schuderer, D. Spät, T. Samaras, W. Oesch, N. Kuster, “In Vitro Exposure Systems for RF Exposures at 900 MHz”, IEEE Transactions on Microwave Theory and Techniques, vol 52, No8, 2004: 2067-2075

J. Schuderer, W. Oesch, N. Felber, N. Kuster, “In Vitro Exposure Apparatus for ELF Magnetic Fields”, Bioelectromagnetics, in press, 2004.

J. Schuderer, "EMF Risk Assessment: *In Vitro* Research and Sleep Studies", Dissertation, ETH, 2003

J. Schuderer, N. Kuster, “The Effect of the Meniscus at the Solid/Liquid Interface on the SAR Distribution in Petri Dishes and Flasks”, Bioelectromagnetics, vol. 24, pp.103-108, 2003.

submitted:

J. Schuderer, U. Lott, N. Kuster, “UMTS In Vitro Exposure System and Test Signal for Health Risk Research”, Bioelectromagnetics, submitted 2004

in preparation:

W. Oesch, J. Schuderer, N. Kuster, R. Mertens, R. Adey, "Selection of Specific EMF Exposure Conditions for Bioexperiments in the Context of Health Risk Assessments", in preparation, 2004.

b. Scientific meetings

J. Schuderer, T. Samaras, W. Oesch, N. Nikoloski, D. Spät, N. Kuster, "Electromagnetic Field Exposure of Cells at 900 and 1800 MHz: Requirements, Dosimetry and Performance Comparison of Different Setups", FGF & COST281 Workshop on the Influcence of RF Fields on the Expression of Stress Proteins, April, Helsinki, Finland, pp. 21-22, 2004.

J. Schuderer, W. Oesch, U. Lott, N. Kuster, "In Vitro Exposure Setup for Risk Assessment Studies with UMTS Signal Schemes at 1950 Mhz", 25th Annual Meeting of the Bioelectromagnetics Society, June, Maui, USA, p. 68, 2003.

J. Schuderer, W. Oesch, R. Mertens, U. Frauenknecht, N. Kuster, "Exposure Systems, Dosimetry and Quality Control", 25th Annual Meeting of the Bioelectromagnetics Society, June, Maui, USA, pp. 127-128, 2003.

T. Samaras, J. Schuderer, N. Kuster, "Temperature Distributions Inside Cell Cultures Exposed to Electromagnetic Fields In Vitro", Cost 281 Management Committee Meeting, London, GB, Nov. 12-13, 2002.

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N. Kuster, W-R. Adey, "Criteria for Selecting Specific EMF Exposure Conditions for Bioexperiments in the Context of Health Risk Assessments", 23rd Annual Meeting of the Bioelectromagnetics Society, St. Paul, Minnesota, USA, p. 24, 2001.

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c. Posters

W. Oesch, H-U. Gerber, N. Kuster, "Requirements for Controlling & Monitoring Software of Expoure Systems in (Double-)Blinded Bio Experiments", 24th Annual Meeting of the Bioelectromagnetics Society, June, Quebec, Canada, pp. 152-153, 2002.

d. Reports in the general media

J. Schuderer, "EMF Risk Assessment: In Vitro Research and Sleep Studies", Diss. ETH 15347, 2003.

N. Kuster, "Latest Progress in Experimental Dosimetry for Human Exposure Evaluations and for Characterization and Optimization of Exposure", in "Communication Mobile – Effects Biologique", ed. Claude Legris, CADAS, Académie des Sciences, Paris, France, pp. 63-69, 2001.

6.11 Experiments with the human neuroblastoma cell line SY5Y (Participant 11)

a. Scientific papers

The results described in this final report will be *submitted* for two publications in international scientific journals.

Benfante R., Antonini R.A., Gotti C., Moretti M., Kuster N., Schuderer J., Clementi F, and Fornasari D. – "Extremely low-frequency electromagnetic field (ELF-EMF) does not affect the expression of α3, α5 and α7 nicotinic receptor subunit genes in SY5Y neuroblastoma cell line". Manuscript in preparation

Antonini R.A:, Benfante R., Flora A., Kuster N., Schuderer J., Adlkofer F, Clementi F., and Fornasari D. – "The expression of DβH (dopamine-β-hydroxylase) and noradrenergic phenotype specifying genes Phox2A and Phox2B is unresponsive to exposure to extremely-low-frequency electromagnetic field (ELF-EMF)". Manuscript in preparation

6.12 Effects of EMF on gene expression in human cells analysed with the cDNA array (Participant 12)

a. Scientific publications

submitted:

Schlatterer K, Gminski R, Hermann S, Tauber R, Fitzner R, Maercker C. Gene expression profiling identifies differences in ribosome biogenesis of human promyelocytic leukemia HL-60 cells following exposure to 1800 MHz radiofrequency electromagnetic fields.

in preparation:

Remondini D, Leszczynski D, Nylund R, Ivancsits S, Rudiger HW, Bersani B, Maercker C. The bio-statistical analysis of micro-array data give indications for the induction of calcium-related signaling pathways after exposure of primary fibroblasts and endothelial cells to electromagnetic fields.

Kuokka R, Griffin T, Maercker C, Schuderer J, Reivinen J, Kuster N, Aebersold R, Leszczynski D. Effect of low-energy microwaves on protein expression in human endothelial cell line: Microwave modulation might be the cause of biological response.

b. Oral presentations at scientific meetings and round tables

Maercker C, Czyz J, Ivancsits S, Ruediger HW, Jahn O, Diem E, Pilger A, Rolletschek A, Schuderer J, Kuster N, Guan K, Trillo A, Bazán E, Reimers D, Fornasari D, Clementi F, Schlatterer K, Tauber R, Fitzner R, Adlkofer F, Wobus AM (2002) Gene expression profiling studies on global cDNA arrays show sensitivity of human and mouse cell lines to extremely-low frequency (ELF-EMF) and radiofrequency (RF-EMF) exposure. 24th annual BEMS Meeting, Quebec, Canada.

Maercker C, Wobus AM, Huber W, Poustka A, Ivancsits S, Rüdiger HW, Jahn O, Diem E, Schuderer J, Kuster N, Fornasari D, Clementi F, Schlatterer K, Tauber R, Fitzner R, Reivinen J, Adlkofer F, Leszczynski D (2002) An EU-wide initiative to characterize the biological effects of EMF on human and mouse cell lines by gene expression profiling. 2nd Int. Workshop on Biolocical Effects of Electromagnetic Fields, Rhodes, Greece. Proceedings, pp. 588-594.

Maercker C (2003) In-vitro investigation of molecular effects of electromagnetic fields by high-throughput techniques. Deutscher Ärztekongress, Berlin, Germany.

Maercker C, Schlatterer K, Gminski R, Schuderer J, Kuster N, Adlkofer F, Fitzner R, Tauber R (2003) RF-EMF exposure increases protein synthesis in human promyelocytic cells. 25th annual BEMS Meeting, Hawaii, USA.

Maercker C (2003) Effects of electromagnetic fields on the human genes – expererimental results and assessment of the potential of molecular biology in environmental research. Workshop of the ministry for environment in Nordrhein-Westfalen, Universität Witten-Herdecke, Germany.

Maercker C, Schlatterer K, Gminski R, Schuderer J, Kuster N, Adlkofer F, Fitzner R, Tauber R (2003) In vitro studies on promyelocytic cells with the help of gene expression profiling on cDNA microarrays show an increase of protein synthesis after RF-EMF exposure. EBEA2003 meeting, Budapest, Hungary.

Maercker C (2004) Genomics and proteomics approaches in EMF research. Erice School in Bioelectromagnetics, Erice, Italy.

Maercker C (2004) Invited participant in a round table discussion about research needs in European EMF research. Erice School in Bioelectromagnetics, Erice, Italy.

Maercker C (2004) Do electromagnetic fields induce stress responses? A whole-genome approach helps to identify cellular pathways modulated by RF-EMF and ELF-EMF" COST Workshop "Influence of RF Fields on the Expression of Stress Proteins" Helsinki, Finland.

Maercker C, Schlatterer K, Gminski R, Remondini D, Leszczynski D, Rüdiger H, Bersani F, Fitzner R, Tauber R (2004) The whole-genome gene expression analysis as a powerful method to detect molecular effects of electromagnetic fields. Deutscher Ärztekongress, Berlin, Germany.

Maercker C, Remondini D, Nylund R, Lezczynski D, Schlatterer K, Fitzner R, Tauber R, Ivancsits S, Rudiger H, Bersani F (2004) Analysis of gene expression in EMF research. 26th Annual Meeting of the Bioelectromagnetic Society (BEMS), Washington, USA.

Maercker C (2004) Molecular investigation of the effects of electromagnetic fields. T-Mobile GmbH, Darmstadt, Germany.

c. Posters

Maercker C, Czyz J, Wobus AM, Huber W, Poustka A, Ivancsits S, Ruediger HW, Reivinen J, Leszczynski D, Schlatterer K, Tauber R, Fitzner R, Fornasari D, Clementi F, Schuderer J, Kuster N, Adlkofer F (2002) An EU-wide initiative to characterize the biological effects of electromagnetic fields on different human and mouse cell lines by gene expression profiling. Annual Meeting of the "Nationales Genomforschungsnetz" (NGFN) in Berlin, Germany.

Maercker C, Kuokka R, Reivinen J, Ivancsits S, Ruediger HW, Schuderer J, Kuster N, Fornasari D, Clementi F, Schlatterer K, Tauber R, Fitzner R, Adlkofer F, Leszczynski D (2003) Whole-genome gene expression profiling: a big challenge to find out the molecular answer to EMF exposure. 25th annual BEMS Meeting, Hawaii, USA.

Lupke M, Maercker C, Simkó M (2004) Alteration in gene expression after 50 Hz ELF-MF exposure in human umbellical cord blood-derived monocytes. 3nd Int. Workshop on Biolocical Effects of Electromagnetic Fields, Kos, Greece.

7.0 POLICY RELATED BENEFITS

7.1 Studies on the human promyelocytic cell line HL-60 (Participant 2)

Research performed is basic research with relevance for life science and techniques, respectively. Our results obtained with human promyelocytic HL-60 cells have made a substantial addition to the data base relating to genotoxic and phenotypic effects of RF-EMF in vitro. Its value lies in providing new data that will enable mechanisms of RF-EMF effects to be studied more effectively (e.g. ROS effects) than in the past. Proteomics studies should be extended to identify possible, potential biological and molecular markers. Furthermore, our data provide new information that will be used for risk evaluation by WHO, IARC and ICNIRP.

7.2 Studies on human fibroblasts, human lymphocytes, human monocytes, human melanocytes, human muscle cells and granulosa cells of rats (Participant3)

Based on our findings we propose the suitability of the comet assay, micronucleus test and evaluation of chromosomal aberrations for monitoring and surveillance of EMF exposed subjects. Due to possible cell specific differences in response to EMF the biological material chosen for biomonitoring could be crucial. Our findings arouse concern about environmental threshold limit values and protective measures regarding EMF exposure in particular with respect to older individuals or people suffering from repair syndroms. The observed activation of DNA repair could display beneficial health effects and could be applied for medical treatment.

7.3 Studies on mouse embryonic stem cells (Participant 4)

Our research results confirmed subtle biological effects emanating from both extremely low frequency fields (simulating the magnetic components of 50 Hz power line fields) and high-frequency (RF) electromagnetic fields (EMF) simulating GSM-modulated schemes. The effects were dependent on the genetic constitution of the cells, and especially the transcription of apoptotic/anti-apoptotic related genes was shown to be affected. Neural progenitor cells appeared to perceive EMF at certain stages of differentiation as external stress signals, which may activate at least, a bcl-2 mediated anti-apoptotic pathway.

1. Important for the quality of life and for human health could be the improvement of products emitting EMF. In this context, 'no effect-levels' were observed with regard to flux density, which can be used to determine threshold values more precisely.
2. The analysis of transcript levels affected by EMF should be extended by genomics and proteomics studies in animals, but also in human populations, to identify further potential molecular markers which may serve as "EMF-responsive bio-indicator".
3. Cell biological studies should be continued to elucidate the molecular processes that may be affected by EMF, especially in the context of carcinogenesis.

7.4 Studies on the human neuroblastoma cell line NB69 and neural stem cells (Participant 5)

The described results indicate that a human neuroblastoma cell line can be sensitive to the in vitro exposure to power frequency, sine wave EM fields at magnetic flux densities that are equal to or lower than the exposure threshold (100 μT) recommended by ICNIRP and UE for the general public. The effects, which include changes in the cells' proliferation, apoptosis or the cellular response to growth factors, among others, were found to be dependent on the ELF-EMF density, the exposure time, the cell passage or the cell cycle. The same human cancer cell line, as well as neural stem cells from rat's embryonic nucleus striatum, was found to be sensitive to the in vitro exposure to GSM-1800 signals at a SAR of 2 w/kg, the exposure threshold recommended by ICNIRP and UE for the general public. The effects included changes in the expression of fibroblast growth factor receptor-1, accompanied or not with changes in the cellular morphology linked to a potential promotion of non-neuronal precursors of NSC' progeny. No differential responses were detected when the cells were exposed to GSM signals with

different ELF modulation patterns, suggesting that the observed effects reflect a cellular sensitivity to the RF carrier wave, rather than to the ELF modulation.

1. These data identify cellular mechanisms of response to specific parameters of exposure to ELF and RF electromagnetic fields that are ubiquitous in today's human environment. Such information can significantly contribute to the establishment of adequate strategies for the protection against non-ionising radiation in public, residential or occupational environments.
2. The study of the cellular mechanisms of response to ELF and RF EMF should be extended to properly identify the biophysical phenomena underlying the potential health effects of the exposure to environmental, non-ionising radiation. In this context, two types of studies are of crucial interest. A) Studies aimed to identify potential markers or bio-indicators of EM sensitivity; B) Studies on human cancer cells and the in vitro response to chemicals that could prevent the EMF effects.

7.5 Studies on the human endothelial cell lines EA.hy926 and EA.hy926v1 (Participant 6)

We have found that 900 and 1800 MHz GSM radiation at SAR of 2.0-2.4W/kg causes activation of stress response in human endothelial cell line. The stress response was followed by a physiological response on a single cell level. Cellular stress fibers were stabilized what was followed by the changes in cellular size and shape (cells contraction). Also we have observed effect on other cytoskeletal proteins, in particular on vimentin and formed of it - vimentin filaments. In respect of cell apoptosis we have observed decline in the expression of nearly all proteins involved in Fas/TNFα-dependent apoptosis pathway. This suggests that the mobile phone radiation might have some potential to prevent apoptosis of cells - a possibility that is currently being explored in further research. All-in-all, results of our research suggest that cells recognize mobile phone radiation as an external stress and this in spite of the very low energy of the exposure. Part of the results of our research has been already published in three articles (one in Differentiation 2002; two in Proteomics 2004). These results, although directly can not be used for prediction of any health hazard, they are available for the scientific evaluation of the potential risks associated with the use of mobile phones and for recommendations of further research needs. The remaining experimental data obtained by us within REFLEX is in process of submission for publication (3 manuscripts) and will be available for the scientific community in 2005.

7.6 Studies on rCx46 in oocytes of Xenopus laevis and human fibroblasts and granulosa cells of rats Participant 7)

On the level of DNA we found significant evidence for DNA damage by ELF-EMF for cultured human fibroblasts and granulosa cells of rats. The findings were strongly related to the exposure protocol. At intermittent ELF-EMF exposure maximal effects were observed after 16-18 h of exposure independently on the applied frequency in the range of 8 Hz to 300 Hz.

The effects appeared not to be reflected on the cellular level of free cytoplasmic calcium. Also cellular studies on the expression level of connexin 46 in oocytes of *Xenopus laevis* showed no significant effect on ELF-EMF exposure. Therefore, it is tempting to suggest that significant effects by ELF-EMF exposure on the genomic level appear not to be reflected on the cellular level. But is has to be taken into account that the methods which are applied to study DNA damage are quite different and most probably more sensitive than those for studying cellular parameters.

7.7 Studies on embryonic stem cells during cardiac differentiation and human lymphocytes and thymocytes (Participant 8)

Studies on embryonic stem cells open a totally new perspective: on one side, the possibility to study in a reproducible way the effects of ELF-EMF on cell differentiation and in particular on cardiogenesis; on the other side, the possibility to direct in some way the differentiation processes of stem cells into specific cell phenotypes.

The more or less negative results studies on human lymphocytes and thymocytes are of paramount importance for risk evaluation since they show that the immune system cells are nearly insensitive to ELF and RF EMF exposure.

7.8 Studies on brain cells of different origin and human monocytes (Participant 9)

We have found no evidence of biological effects of GSM-like signals on mammalian immune and nerve cells. The endpoints were apoptosis and the expression of stress- or inflammation induced proteins. Protocols included exposures at SAR levels corresponding to the public limit for local exposure (2 W/kg) and prolonged exposure duration (24 to 48 hours) that represent a "worst-case" exposure condition. These in vitro results will add to the database, on which the next scientific evaluation of RF-EMF health effects will be based. Although the present findings do not suggest a need for a revision of the local exposure limits to RF-EMF (1999/519/CE), more investigations on animal models of neurodegenerative diseases are needed.

7.9 Provision of exposure set-ups and technical quality control (Participant 10)

Our research is basically aimed at guaranteeing appropriate exposure setups and thorough quality control of the engineering aspects of the various experiments. High technical quality control is of special interest, since the variability of experiments within REFLEX is rather broad. Solid risk assessment will contribute to the future development of new communications technologies.

7.10 Studies on the human neuroblastoma cell line SY5Y (Participant 11)

Our results fit in a scientific debate around the contribution of ELF-EMF on brain neurodegenerative diseases, with particular emphasis on Alzheimer's disease (AD). Epidemiological studies showed that workers with likely electromagnetic field exposure may have an elevated risk of AD. On the other hand, experimental studies employing animal models failed to confirm these observations. In our studies we demonstrate that, at molecular level, the cholinergic system, which is one of the most affected neurotransmission system in AD, did not undergo any modification in the expression of relevant nAChR subtypes.

7.11 cDNA array analysis (Participant 12)

The micro-array technique is a state-of-the-art tool to investigate changes in gene expression and therefore molecular defects in human cells. Whereas in the field of medicine this kind of technique is on the way to get a diagnostic standard for certain diseases (e.g. cancer), it is not common so far for the detection of environmental effects. With our study we have shown that the whole-genome analysis is a suitable method to detect potential molecular effects of EMF. Since the different labs participating in the REFLEX project have worked with the same exposure setups (Participant 10) and we have done all hybridisations and data analyses in the same way, we have created a platform which can work with comparable material (RNA) of different cell lines and different experimentators. A quality control of the RNA (test for degradation, concentration) allowed us to make experiments with very different cell lines under comparable conditions and therefore to produce reliable results. This is a big difference to other assays (e.g. microscopical analysis), which strongly depend on people and software. In the near future, whole-genome approaches might support or even replace other measurements of the effects of electromagnetic fields or related environmental influences. Since the technique is very sensitive and specificly applicable for human cells, it also should be applied for in vivo studies in upcoming projects.

7.12 Summary (Participant 1)

The policy related benefits of the REFLEXproject consist in the fact that new knowledge has been generated independent of whether one likes it or not. Biological effects of extremely low-frequency (ELF) and radio-frequency (RF) electromagnetic fields (EMFs) the exposure to which is constantly increasing especially in Europe with its high density of population and industry and with the omnipresence of EMFs in infrastructures and consumer products have become a topic of public concern. This is due to the fear of people that based on the many conflicting research data a risk to their health cannot be excluded with some certainty. Therefore, the overall objective of REFLEX was to find out whether or not the fundamental biological processes at the cellular and molecular level support such an assumption. For this purpose, possible effects of EMFs on cellular events controlling key functions, including those involved

in carcinogenesis and in the pathogenesis of neurodegenerative disorders, were studied through focussed research. Failure to observe the occurrence of such key critical events in living cells after EMF exposure would have suggested that further research efforts in this field could be suspended and financial resources be reallocated to the investigation of more important issues. But as clearly demonstrated, the results of the REFLEX project show the way into the opposite direction.

The REFLEX project has made a substantial contribution to the data base on biological effects of both ELF-EMF and RF-EMF on in vitro cellular systems. The study was designed to investigate whether or not EMF exposure below the energy density reflected by the present safety levels generates in vitro critical cellular events. Gene mutations, deregulated cell proliferation and suppressed or exaggerated programmed cell death (apoptosis) that are caused by or result in an altered gene and protein expression profile are such critical events, the convergence of which is required for the development of chronic diseases. Genotoxic effects and a modified expression of numerous genes and proteins after EMF exposure could be demonstrated with great certainty, while effects on cell proliferation, cell differentiation and apoptosis were much less conclusive. Since all these observations were made in in vitro studies, the results obtained neither preclude nor confirm a health risk due to EMF exposure, but they speak in favour of such a possibility. Because of their fundamental character the findings will be presented to WHO, IARC and ICNIRP. It will be up to these organisations to make use of them for risk evaluation, in combination with findings from animal and epidemiological studies.

A major European added value of REFLEX consists also in the fact that the need for further research and especially how it should look alike have clearly been demonstrated. Furthermore, the outcome of the project should stimulate the research and development departments of the electrical, electronic, and telecommunication industry to make use of the methods developed in order to better adjust the state of technology to the conditions of life, and prompt the European governments to ensure multidisciplinary EMF research in order to take care, that the solution of the presently existing problem of uncertainty about a possible health risk for the people in Europe and beyond due to EMF exposure will not be postponed in the far future.

8.0 References

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ANNEX I - Results submitted after deadline

A) ELF-EMF

1) Re-evaluation of micronucleus frequencies on slides prepared by Participant 3 in two additional laboratories which are not members of the REFLEX consortium

Micronucleus frequencies in fibroblasts which were exposed to ELF-EMF (50 Hz, 1 mT, 15h, 5 min on/10 min off) or sham-exposed in the laboratory of Participant 3 (Vienna, see 2.2 and 3.1.1.1) were re-evaluated under blinded conditions.

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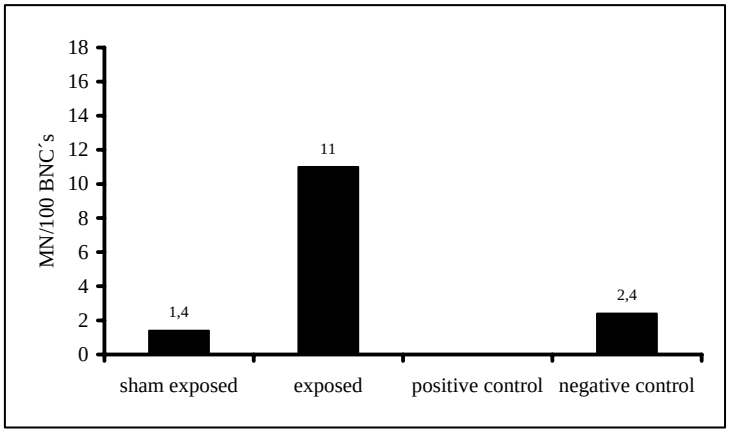

Figure 1: Micronucleus frequencies in ELF-EMF exposed cultured human fibroblasts (50 Hz, 1 mT, 15h, 5 min on/10 min off) and in control cells. Bleomycin treated cells (10µg/ml) were used as a positive control.

b) Prof. Heinrich Zankl, Fachbereich Biologie der Technischen Universitaet Kaiserslautern, Paul-Ehrlich-Strasse 7, 67663 Kaiserslautern, Germany

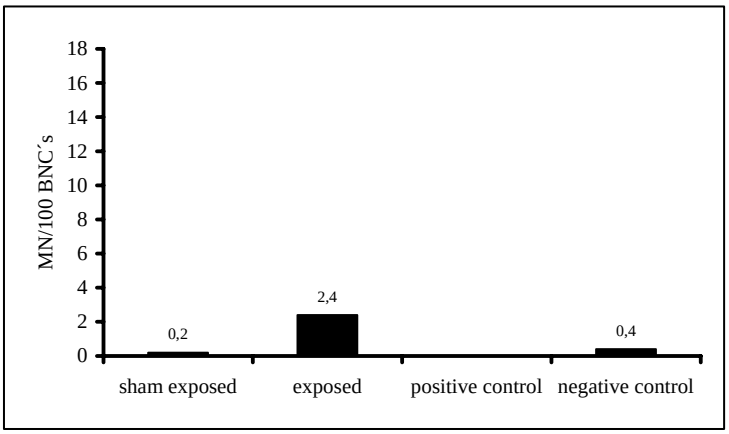

Figure 2: Micronucleus frequencies in ELF-EMF exposed cultured human fibroblasts (50 Hz, 1 mT, 15h, 5 min on/10 min off) and in control cells. Bleomycin treated fibroblasts (10 µg/ml) were used as a positive control.

ELF-EMF exposed cells showed an increase in micronucleus frequencies, which was about 6 to 10-fold as compared to sham exposed cells and negative controls. Although basal levels of micronuclei correlated well with the results obtained in the laboratory of Participant 3, exposed cells showed definitely higher values. The variability in micronuleus frequencies between the laboratories maybe due to the fact that different staining techniques were applied: Vienna and Ulm used a more sensitive fluorescent dye (DAPI), whereas the laboratory in Kaiserslautern stained the slides with GIEMSA. In addition, the laboratories in Ulm and Kaiserslautern scored 500 binucleated cells only instead of 2,000 processed in Vienna.

In conclusion, in three independent laboratories micronucleus frequencies showed a consistent increase in cultured human fibroblasts after ELF-EMF exposure. Differences in micronucleus frequencies between the laboratories can be attributed to different staining techniques and the different a numbers of scored cells.

2) Evaluation of genotoxic effects on human fibroblasts following intermittent exposure to 50 Hz powerline magnetic fields by Participant 8 (Bologna/Naples)

Some experiments related to the evaluation of the induction of genotoxic effects following intermittent (5 min field on/10 min field off) exposures to 50 Hz ELF magnetic fields, 1 mT field intensity, were carried out on human diploid fibroblasts (ES-1). In particular, to evaluate the induction of DNA single strand breaks the alkaline comet assay was applied following 15, 18 and 24 hours exposures, while the induction of micronuclei (MN) was measured following 24 h exposure.

Moreover, positive controls were also provided by treating cells with hydrogen peroxide (Alkaline Comet Assay) or Mitomycin-C (MMC; micronucleus assay) at several concentrations.

The results obtained do not indicate induction of genotoxic effects, neither in terms of comets induction nor in terms of MN frequency increase. On the contrary, positive controls showed an increase in DNA damage, as expected.

Experimental conditions and cytogenetic analysis

Human diploid fibroblasts (ES-1) were cultured in DMEM containing 10% FBS, 20 mM Hepes buffer, 2mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Cells were supplied with fresh culture medium every 48 h and splitted once a week. Cells were received at passage 6 and a master bank was established with cells at passage 8. For experiments, cells from passages 10-16 were used.

Preliminary experiments were devoted to find the dose of chemicals to be used to provide positive controls for both the assays employed. Dose-response curves were set up by treating ES-1 cells with H_2O_2 ranging from 25-100 µM final concentration for 30 minutes (alkaline comet assay) and with MMC ranging form 0,005 – 0,05 µg/ml final concentration for the whole culture period (MN assay). For both chemicals a dose-dependent increase in DNA damage was detected, even for the lowest doses tested. However, the best compromise between induced damage and cell survival was found at 50 µM H_2O_2 and 0,025 µg/ml MMC and these concentrations were used for the experiments.

A second set of preliminary experiments was devoted to define the cell cycle duration of ES-1 cells to block cytokinesis for micronucleus test: it resulted of 28 h (data not shown).

The exposures were carried out at 50 Hz (1 mT field intensity) and the signal used was powerline. Several exposure durations were tested, as reported below.

Alkaline Comet Assay

Ten independent experiments (5 by exposing cells for 24 h, 2 by exposing cells for 18 h, 3 by exposing cells for 15 h) were carried out by setting up 8 cultures each: 4 to be exposed and 4 to be sham exposed.

Moreover, positive controls were provided by treating cultures for 30 min with hydrogen peroxide (H_2O_2, 50 µM). 24 hours before the experiments, 50000 cells/3 ml complete medium were seeded into 35 mm Petri dishes (Corning, cat. 430165). Following the intermittent exposure cultures were processed for the comet assay as described in details in (1). Slides were stained, just before the analysis, with ethidium

bromide (12 µg/ml) and images of 1000 randomly selected cells (250 from each of four replicated cultures) were analyzed by a computerized image analysis system (Delta Sistemi, Rome, Italy) fitted with a Leica DM BL fluorescence microscope at 250 X magnification. This system acquires images, computes the integrated intensity profile for each cell, estimates the comet cell components, head and tail, and evaluates a range of derived parameters. DNA damage was evaluated by calculating the tail factor, as reported in (1). Moreover, tail moment, comet moment and percentage of tail DNA were also measured.

Cytokinesis-block Micronucleus Assay

4 independent experiments were carried out by exposing cells for 24 h. For each experiment 4 cultures were set up: 2 to be exposed and 2 to be sham exposed. Positive controls were provided by adding MMC (0,025 µg/ml) 24 h after the seeding.

50000 cells/5 ml complete medium were seeded in slide flasks (NUNCLON, cod. 170920, 9 cm^2 growth area) and recovered for 24 h. To block cytokinesis, 4 hours before the end of the first cycle (48 h after seeding), Cytochalasin-B (3 µg/ml final concentration) was added and at the end of the second replication cycle (80 h after seeding), cells were incubated for 30 min at 37°C with hypotonic solution (KCl 0,075 M) and fixed for 10 min (methanol 80% in distilled water). Air dried slides were stained for 8 min (10% Giemsa in phosphate buffer pH 6.8). The intermittent MF exposure was carried out during the first 24 hours following the recovery.

MN were scored in binucleated cytokinesis-blocked (CB) cells with well preserved cytoplasm by using a light microscope, and for each experiment their frequency was evaluated in 2000 cells (1000 cells for each duplicate slide). The results were expressed as micronucleated binucleated (MNBN) cells per thousand binucleated cells.

The morphological criteria for MN scoring in binucleated cells were similar to those reported by Fenech for human lymphocytes (2). By classifying 500 cells according to the number of nuclei, the binucleate cell index (BCI) and the cytokinesis-block proliferation index (CBPI) were evaluated for each culture, as reported in (3) and (4), respectively.

Statistical analysis

Differences between treated and untreated samples (sham exposed vs. exposed; sham exposed vs. positive controls) were tested by using the two tailed paired Student's t test. P values lower than 0.05 were considered as statistically significant.

RESULTS

Comet assay

Following 24 h intermittent exposure (5 min on/10 min off) to 50 Hz powerline MF, human diploid fibroblasts did not show statistically significant differences in all the parameters investigated when sham-exposed samples were compared to exposed ones. The results obtained are reported in table 1 as mean ± standard error of 5 independent experiments. Same results have been obtained when cultures exposed for 18 h (2 experiments) and 15 h (3 experiments) were compared to their own sham exposed cultures, as shown in table 2 and 3, respectively.

On the contrary, when sham exposed cultures were compared to H_2O_2 -treated samples, a significant increase in all the parameters investigated was detected following treatments of 30 minutes ($p<0.05$). The results obtained are reported in figure 1 as mean ± standard error of all the parameters investigated.

Micronucleus assay

Following 24 h exposure no genotoxic effects were detected by comparing sham-exposed with exposed samples. The proliferation index (CBPI) also resulted not affected by the exposure. The results are reported in table 4 (4 independent experiments), where data related to MMC treatments are also shown. By comparing sham-exposed with MMC treated cultures a statistically significant increase in MN frequency was detected, together with a decrease of cell proliferation and of percentage of binucleated cells ($p<0.05$ in all cases).

DISCUSSION

The data here reported do not support the hypothesis that intermittent exposures to 50 Hz MF (powerline signal) induce genotoxic effects in human diploid fibroblasts.

Concerning the alkaline comet assay, our finding is also supported by the results obtained by treating cells with hydrogen peroxide as positive control, where an increase in all the comet parameters investigated has been detected. Moreover, the cells investigated showed an high sensitivity to 30 min treatment already at 25 µM final concentration (data not shown), while most of the data reported in literature on several cell types indicate that 30 min treatments at concentrations between 50 and 100 µM are needed to induce a statistically significant effect.

Concerning the data on the induction of micronuclei (MN), also in this case we have not found genotoxic effects induced by the field. On the contrary, MMC treatments at doses of 0,025 µg/ml resulted cytotoxic (CBPI: 1,18 vs. 1,45 in treated and untreated cultures, respectively) while a dose of 0,033 µg/ml is necessary to induce MN increase in human peripheral blood lymphocytes without affecting cell proliferation.

The data here reported refer only to the exposures performed with the powerline signal: experiments devoted to test the sinusoidal signal are in progress.

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Table 1 – mean values ± standard error of the parameters investigated following alkaline comet assay in ES-1 human fibroblasts exposed for 24 h to 50 Hz powerline magnetic field. Results of 5 independent experiments (1000 cells/treatment investigated)

Parameters	Sham-exposed	MF-exposed
Tail Factor (%)	5.65 ± 0.98	6.36 ± 0.93
Comet Moment	1.86 ± 0.43	2.01 ± 0.49
% DNA	3.8 ± 0.97	4.12 ± 1.12
Tail Moment	1.10 ± 0.32	1.29 ± 0.36

Table 2 – mean values ± standard error of the parameters investigated following alkaline comet assay in ES-1 human fibroblasts exposed for 18 h to to 50 Hz powerline magnetic field. Results of 2 independent experiments (1000 cells/treatment investigated)

Parameters	Sham-exposed	MF-exposed
Tail Factor (%)	6.24 ± 0.25	6.11 ± 0.26
Comet Moment	2.32 ± 0.04	2.25 ± 0.04
% DNA	4.37 ± 0.22	4.20 ± 0.22
Tail Moment	1.56 ± 0.05	1.54 ± 0.11

Table 3 – mean values ± standard error of the parameters investigated following alkaline comet assay in ES-1 human fibroblasts exposed for 15 h to to 50 Hz powerline magnetic field. Results of 3 independent experiments (1000 cells/treatment investigated)

Parameters	Sham-exposed	MF-exposed
Tail Factor (%)	5.23 ± 0.26	5.20 ± 0.63
Comet Moment	1.76 ± 0.08	1.68 ± 0.18
% DNA	3.07 ± 0.29	3.08 ± 0.68
Tail Moment	0.89 ± 0.03	0.88 ± 0.15

Table 4 – MN frequency, proliferation index (CBPI) and binucleate cell index (BCI) in cultures sham-exposed, exposed for 24 h and positive controls. Data are reported as mean ± SD of 4 independent experiments. * Sham-exposed vs. MMC treated cultures: $p<0.05$.

Parameters	Sham-exposed	MF-exposed	MMC (0.025 µg/ml)
MN/1000 CB cells	0.45±0.06	0.43±0.07	2.35±0.35*
CBPI	1.45±0.02	1.43±0.02	1.15±0.01*
% BCI	45.2±2.45	43.3±2.01	15.0±1.41*

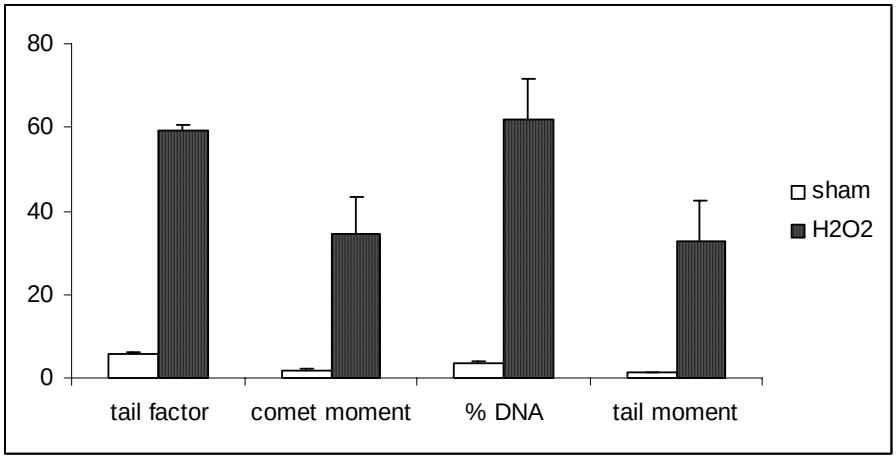

Figure 1 – mean values ± standard error of the parameters investigated following alkaline comet assay in ES-1 human fibroblasts treated for 30 min with 50µM hydrogen peroxide compared to sham-exposed samples.

B) RF-EMF

1) Re-evaluation of micronucleus frequencies on slides prepared by Participant 3 in two additional laboratories which are not members of the REFLEX consortium

Micronucleus frequencies in fibroblasts which were exposed to RF-EMF (GSM basic 1950 MHz, 15h, 2 W/kg) or sham-exposed in the laboratory of Participant 3 (Vienna, see 2.2 and 3.2.1.2) were re-evaluated under blinded conditions.

a) Prof. Guenter Speit, Medizinische Fakultaet/Humangenetik, University of Ulm, Albert-Einstein-Allee7, 89081 Ulm, Germany

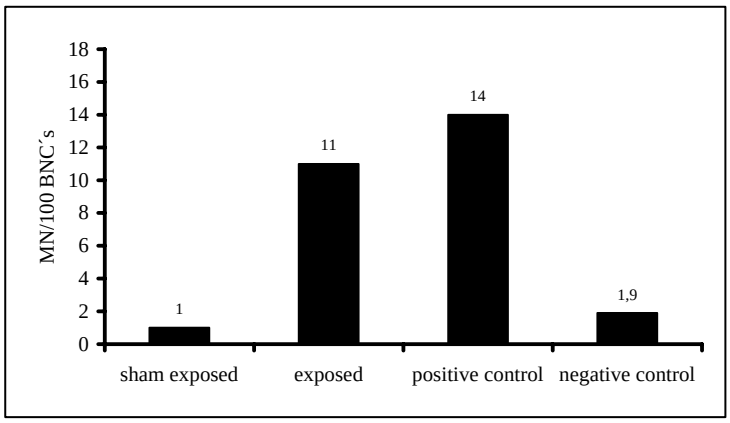

Figure 1: Micronucleus frequencies of RF-EMF exposed (GSM basic 1950 MHz, 15h, 2 W/kg) cultured human fibroblasts and control cells. Bleomycin-treated cells were used as a positive control.

b) Prof. Heinrich Zankl, Fachbereich Biologie der Technischen Universitaet Kaiserslautern, Paul-Ehrlich-Strasse 7, 67663 Kaiserslautern, Germany

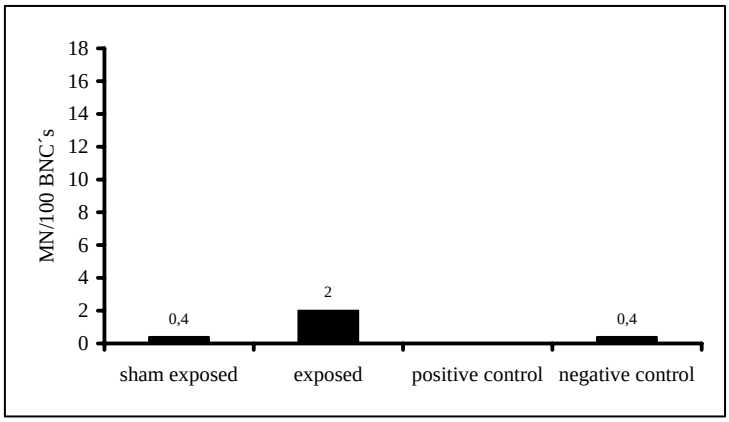

Figure 2: Micronucleus frequencies of RF-EMF exposed (GSM basic 1950 MHz, 15h, 2 W/kg) cultured human fibroblasts and control cells. Bleomycin-treated cells were as a positive control.

RF-EMF exposed cells showed an increase in micronucleus frequencies, which was about 5-fold compared to sham exposed cells and negative controls. Although, basal levels of micronuclei correlated well with the results obtained in the laboratory of Participant 3, exposed cells showed definitely lower levels. These variability could be attributed to different staining techniques. Laboratories in Vienna and Ulm used a more sensitive fluorescent dye (DAPI), whereas the laboratory in Kaiserslautern stained the slides with GIEMSA. Positive controls could not be evaluated, due to a too low number of assessable cells.

In conclusion, in three independent laboratories micronucleus frequencies showed a consistent increase in cultured human fibroblasts after RF-EMF exposure. Differences in micronucleus frequencies between the laboratories can be attributed to different staining techniques and a various number of scored cells.

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ANNEX II

Technical support (Participant 10)

OBJECTIVES

To be able to compare the results of investigations carried out in the different laboratories and to ensure the conclusiveness of the data obtained in the studies, it is of the utmost importance that the conditions of exposure to EMFs be strictly controlled. Therefore, the objective are:

- Evaluation of existing setups
- Development of an optimised ELF setup
- Development of an optimised RF setup (900 MHz)
- Development of an optimised RF setup (1800 MHz)
- Comprehensive dosimetry ELF setup
- Comprehensive dosimetry RF setups
- Technical quality control during the entire period of exposure

Detailed information about the applied exposure systems and their dosimetry are given in

- [Schuderer et al., 2004a] for the ELF setup
- [Schuderer et al., 2004b] for the RF setup (GSM)
- [Schuderer et al., 2003] for the RF setup (UMTS)
- [Schönborn et al., 2000] for the waveguide setup of Participant 4
- [Laval et al., 2000] and [Schuderer et al., 2004c] for the Wire-patch cell of Participant 9
- [Toivo et al., 2001] and [Schuderer et al., 2004c] for the resonator setup of Participant 6
- [Kuster et al., 2002] for the choice of the exposure signals

REQUIREMENTS

The requirements for the exposure systems are formulated in Kuster and Schönborn (2001). In particular, the following parameters need to be fulfilled:

ELF setup:

- large loading volume with uniform exposure
- high dynamic (µT - mT) and frequency ranges (subHz - kHz)
- enabling complex signals and intermittent exposure
- good isolation between exposure and sham
- identical atmospheric parameters for exposed and sham cells (preferably placed in the same incubator)
- blinded exposure protocols by a computer-controlled random decision maker
- continuous monitoring of all environmental and technical parameters in order to detect any malfunctions
- evaluation of possible artefacts such as parasitic E-fields, temperature loads, vibrations, etc.

RF setup:

- peak SAR exposure: >100 W/kg
- maximum time averaged SAR exposure for thermal load < 0.1°C: > 2 W/kg
- deviations from uniformity of exposure: < 30%
- variability of exposure: < 10%
- loading volume with uniform exposure: min. 50 cm^2
- flexible signal unit with high dynamic range enabling complex modulation such as:
  - continuous wave (CW)
  - pulse or sinusoidal modulation at any frequency and repetition rate
  - GSM DTX (discontinuous transmission mode)
  - GSM non-DTX
  - GSM talk (temporal changes between non-DTX and DTX)
  - UMTS signal schemes
- flexible intermittent exposure protocols: seconds to hours
- good isolation between exposure and sham: > 30 dB
- identical atmospheric parameters for exposure and sham: µT < 0.1°C
- blinded exposure protocols
- continuous monitoring of exposure and environmental parameters
- detailed numerical modelling including meniscus
- evaluation of SAR distribution and experimental verification by dosimetric measurements
- uncertainty and variability analysis for SAR
- evaluation of the temperature load
- reliability, user-friendliness and self-detection of malfunctions
- minimal setup cost

MATERIAL AND METHODS

Overview

High-end exposure setups for ELF, RF-GSM and RF-UMTS were developed and fully characterized with respect to exposure parameters, design and possible artifacts.

Since the budget did not allow to equip every laboratory with these setups, existing setups were evaluated with respect to their suitability.

Evaluation of Existing Setups

In order to assess the performance of the already existing setups, the exposure systems in the laboratories of Participants 2 - 9 were evaluated with respect to dosimetric performance and characterization. Possible modifications to improve the performance were also evaluated.

In addition to the newly developed exposure systems, it was decided to use five existing setups (Participants 4, 5, 6, 8, 9) and to modify and improve the two RF setups of Particioants 4 and 9. Similar methods for dosimetry and characterization as described for the newly developed RF setups were used.

ELF setups:

- *Helmholtz coils (Participant 5)*: A pair of Helmholtz coils is placed inside a µ-metal shield; exposure and sham are kept in different incubators; and sinusoidal B-fields (50 Hz) up to 0.1 mT can be applied.
- *4-coil system (Participant 8)*: Two unshielded 4-coil systems are arranged in the same incubator; B-fields up to 1 mT (50 Hz) can be applied.

Both setups provide acceptably uniform B-fields, and no further modification and optimisation was applied.

RF setups:

- *Waveguide setup (Participant 4)*: The waveguide setup of Participant 4 is operated at 1710 MHz and allows the exposure of eight 60 mm diameter Petri dishes. The original system [Schönborn et al., 2000] was enhanced with
  - a new signal unit, allowing complex GSM modulation
  - field sensors to monitor the exposure
  - temperature sensors to monitor the incubator environment
  - an optimised air flow system to reduce temperature differences between both chambers
- *Wire-patch cell (Participant 9)*: The Wire-patch cell is an open radiating setup operated at 900 MHz. The setup is based on a 150 mm x 150 mm parallel plate configuration (distance 29 mm), short-circuited at the edges by four plots [Laval et al., 2000]. Eight 35 mm Petri dishes (placed inside a 60 mm dish with distilled water) are arranged symmetrically around the central coaxial feed. Since the WP cell is an open setup, exposure and sham groups need to be placed in two different incubators. For the purpose of REFLEX, the original system was enhanced by
  - distance keepers for the Petri dishes,
  - optimised Petri dish loading (to reduce the thermal load, distilled water instead of cell medium is used for the 60 mm dish),
  - E-field sensors for monitoring and regulation
  - A computer-controlled signal unit, allowing complex GSM modulation
- *Resonator setup (Participant 6)*: The resonator setup consists of a short-circuited waveguide chamber at 900 MHz [Toivo et al., 2001]. Four 60 mm diameter Petri dishes are exposed in a standing wave E-field maximum in E-polarization. The dishes are placed on a glass plate which is water-cooled from below. In this way a temperature stability of ± 0.3°C over the range from 0 – 10 W/kg average SAR is achieved. No modification of the resonator setup was performed.

Development of ELF Exposure Setup

An ELF setup was developed and four copies were installed in the laboratories of Participants 3, 4, 7 and 11. The following methods have been applied to achieve an optimised design for the ELF exposure system:

- Two coil chambers are placed inside the same incubator to guarantee identical environmental parameters for exposure and sham groups. A fan system serves for enhanced atmospheric exchange between coil chambers and incubator.
- µ-metal shielding is applied for the coils in order to provide sufficient sham isolation.
- E-field shielding of the exposure area is applied to remove parasitic E-fields, generated by the voltage drop over the inductive coils.
- Elastically damped dish holders are used to minimize the coupling of the mechanical vibrations to the Petri dishes.
- Numerical field calculation was used to optimise the 4-coil system within the µ-metal shielded exposure chamber. Optimisation parameters were size of the coils, number of windings and distance between the windings. B-field uniformity was used as an optimisation target.
- Exposure control is realized by monitoring and feedback regulation of the coil currents.
- Complex ELF signals can be applied by using an arbitrary function generator together with a custom-made current source to generate any signal with a point length of 16000 points, a point resolution of 12 bit and frequencies up to 1.5 kHz.
- A power-line signal was defined as the maximum accepted distortion for low- to medium-voltage power systems by the IEC (spectral components up to 1250 Hz are present).
- Environmental monitoring is applied with temperature sensors inside the chambers and by controlling the fan system (current measurement).
- Computer control allows blind protocols and easy handling of the system.

Development of RF Exposure Setup (GSM)

An RF setup (GSM) was developed and four copies were installed in the laboratories of Participants 2, 5, 6 and 8. The following methods have been applied to achieve an optimised design for the RF exposure system:

- The study from Schönborn et al. (2001) showed that the most suited setup with respect to highly uniform cell monolayer exposures should be based on waveguides.
- A waveguide setup was numerically analysed by using the FDTD method. The length of the waveguides was optimised, so that the system is operated at a fundamental resonator mode at 1800 MHz. In this way, superior power efficiency can be achieved.
- The required uniformity and maximum SAR is achieved for a fan-cooled cell monolayer exposure in E-polarization, which provides minimum temperature load.
- Exposure control is realized by field sensors.
- Environmental control is realized by placing the waveguides inside an incubator and using fans for atmospheric exchange. A common air inlet of the fan system was realized in order to reduce temperature differences between both waveguides.
- Environmental monitoring is applied with air temperature sensors and fan monitoring.
- Low variability is realized with a field sensor feedback regulation and by using dish holders together with distance keepers to provide defined positions of the Petri dishes with respect to the incident fields.
- Flexible signal schemes and blinded protocols are realized with a computer-controlled signal unit. AM modulation of the RF generator is applied via an arbitrary function generator and additionally via software commands. Temporal changes between different modulation modes like GSM DTX and non-DTX is realized with a GSM frame unit, blanking the output of the RF amplifier.

Development of RF Exposure Setup (UMTS)

An RF setup (UMTS) was developed and installed in the laboratory of Participant 3. Similar methods as for the RF setup (GSM) have been applied to achieve an optimised design for the UMTS exposure system:

- Similar to the GSM setup, two waveguides are used, equipped with field and temperature sensors and an optimized fan cooling system.
- Due to the different carrier frequency at 1950 MHz, new positions for the Petri dishes inside the waveguide are necessary. Because of the 5 MHz bandwidth of the UMTS signal, a broadband coax-to-waveguide adapter was required.
- The signal unit was updated by a UMTS signal generator. The fast power control of the signal is realized by AM modulation of the RF generator with a fading function stored on the arbitrary function generator.
- A UMTS test signal was defined, which represents worst-case exposure with respect to ELF spectral content. The signal is based on closed loop power controlled fades and additionally covers compressed mode and an open loop power controlled sequence for the physical random access channel.

Dosimetry ELF Exposure Setup

The following methods for dosimetry of the ELF setup were applied:

- *Numerical B-field characterization*: Mathematica V4.1 was used for analytical calculation of the B-field distribution as resulting from the 4-coil configuration.
- *Experimental B-field characterization*: A 3-axis Gaussmeter (FH49, Magnet-Physik, Germany) was used to measure the B-field distribution inside the exposure chamber.
- *Uncertainty and variability*: Uncertainty of the dosimetric assessment and exposure variability were analyzed for the applied numerical and experimental methods.
- *Induced E-field characterization*: The distribution of the induced E-fields within the cell medium was assessed by calculation.
- *Artefact characterization*:
  - *Parasitic E-fields*: A Wandel and Goltermann EFA-3 sensor system was used to determine the electric fields inside the exposure chamber as produced by the setup.
  - *Temperature*: Temperature was measured inside the cell medium with a SPEAG T1V3 probe as well

as for several positions inside the exposure chamber with Thermometrics Pt100 temperature sensors.

- *Vibrations*: To assess the acceleration resulting from coil vibrations, a Wilcoxon Research accelerometer Model 728T equipped with an amplifier unit P704T was applied.

Dosimetry RF Exposure Setup (GSM, UMTS, Wire-Patch Cell)

The following methods for dosimetry of the RF setups were used:

- *Numerical field simulation*: The FDTD simulation platform SEMCAD was applied for a full 3D electromagnetic field analysis.
- *Numerical modelling*: High resolution numerical models including menisci at the solid/liquid interfaces have been used to achieve realistic modelling. SAR extrapolation to the monolayer was applied to compensate discretization error in the strong SAR gradients.
- *Experimental verification*: Simulation was experimentally verified using the DASY3 near-field scanner equipped with 3-axis free space E- and H-field probes (SPEAG EF3DV2, H3DV6) and a dosimetric E-field probe with a diameter of only 1 mm [Pokovic et al., 2000].
- *Uncertainty and variability*: The uncertainty of the SAR assessment was evaluated with respect to the applied numerical and experimental methods. Possible variability of SAR values was additionally evaluated.
- *Thermal load*: The thermal load for the exposed group was assessed by measurement and simulation: A SPEAG T1V3 temperature probe was used for a single-point measurement of the temperature response of the medium (probe was fixed in the temperature maximum). Additionally, a coupled electro-thermal FDTD simulation was used for the 3D assessment of the temperature distribution as a function of exposure duration.

Quality Control and Maintenance

Quality control is ensured by the analysis of the exposure data as recorded from the monitoring unit and stored within an encoded file. All experimental settings and software commands are saved together with the sensor data for field exposure and environment. The monitoring sampling rate is 0.1 Hz. Decoding of the data files can only be provided by a dedicated software and is done by the quality assurance group after biological evaluation. A detailed report of all exposure parameters is then provided.

Furthermore the controlling and monitoring software is able to self-detect malfunctions and responds with warnings or abortions if required (tracing and handling of 60 errors).

For the RF setups, the ambient ELF-fields in the different laboratories were determined for several positions within the incubator and laboratory using a Wandel & Golterman EFA-3 sensor system.

RESULTS

Dosimetry ELF Exposure Setup

The performance of the ELF setup can be summarized by:

- Dynamic range for B-field amplitude (50 Hz): 0.02 – 3.6 mT_{rms}
- Dynamic range for B-field frequency: mHz –1500 Hz
- Nonuniformity of B-field: < 1%
- Uncertainty for B-field assessment: 4.3%
- Variability of exposure: 1.6%
- Loading volume: 3500 cm^3
- Parasitic E-fields (50 Hz): < 1 V/m
- Vibrations:
  - < 0.1 m/s^2 for elastically damped holder
  - < 1 m/s^2 for non-damped holder

- Signal schemes:
  - Sinusoidal 3 –1000 Hz
  - 50 Hz power-line signal (components up to 1250 Hz)
  - Arbitrary intermittency
- Exposure control and monitoring: provided by current measurements (sampling rate 0.1 Hz)
- Environmental control: provided by incubator and fan system (air temperature difference between exposure and sham: < 0.1°C)
- Environmental monitoring: provided by temperature probes and fan current sensing (sampling rate 0.1 Hz)

Dosimetry RF Exposure Setup (GSM)

The performance of the RF setup (GSM) can be summarized by:
- Dynamic range for peak SAR: 0.01 W/kg to > 100 W/kg
- Nonuniformity of SAR: < 30%
- Thermal load: < 0.03 °C / (W/kg)
- Uncertainty of SAR assessment: 20%
- Variability of exposure: 5.1%
- Loading surface for cell monolayers: 60 cm^2
- Signal schemes:
  - Continuous wave
  - 217 Hz pulse modulation
  - GSM non-DTX
  - GSM DTX
  - GSM Talk
  - Arbitrary intermittency
- Exposure control and monitoring: provided by field sensor (sampling rate 0.1 Hz)
- Environmental control: provided by incubator and fan system (air temperature difference between exposure and sham: < 0.1°C)
- Environmental monitoring: provided by temperature probes and fan current sensing (sampling rate 0.1 Hz)

Dosimetry RF Exposure Setup (UMTS)

The performance of the RF setup (UMTS) can be summarized by:
- Dynamic range for peak SAR: 0.01 W/kg to > 200 W/kg
- Nonuniformity of SAR: < 26%
- Thermal load: < 0.03 °C / (W/kg)
- Uncertainty of SAR assessment: 18%
- Variability of exposure: 1.9%
- Loading surface for cell monolayers: 60 cm^2
- Signal schemes:
  - Continuous wave
  - UMTS test signal (maximized ELF spectral content)
  - 217 Hz pulse modulation
  - GSM DTX
  - GSM non-DTX
  - GSM Talk
  - Arbitrary intermittency
- Exposure control and monitoring: provided by field sensor (sampling rate 0.1 Hz)

- Environmental control: provided by incubator and fan system (air temperature difference between exposure and sham: < 0.1°C)
- Environmental monitoring: provided by temperature probes and fan current sensing (sampling rate 0.1 Hz)

Quality Control and Maintenance

Quality Control:

- In the course of the REFLEX project, approximately 1800 *in vitro* experiments have been performed. Each of these experiments is documented with a dosimetric evaluation report covering the time courses and statistics for the field values, air temperatures and fan currents as well as all experimental settings.
- Average ambient ELF B-fields in the incubators of laboratories 2, 3, 4, and 8 are:
  - Participant 2: B = 0.3 ± 0.2 μT_{rms}
  - Participant 4: B = 3.2 ± 2.0 μT_{rms}
  - Participant 5: B = 3.5 ± 2.2 μT_{rms}
  - Participant 8: B = 2.8 ± 1.9 μT_{rms}

Maintenance:

Maintenance and assistance was provided in the course of the project for:

- installation of the setups
- handling of the setups
- exchange of several Pt100 temperature probes
- exchange of some RF dish holders
- exchange of one malfunctioning ELF current source
- exchange of one malfunctioning RF generator
- provision of software updates
- evaluation of data files

DISCUSSION

All tasks except development of an optimised RF setup at 900 MHz have been fully solved. More setups than initially planned needed to be developed, since the quality of the setups available in the laboratories were not sufficient to meet the requirements of the project. In order to stay within the budget, the consortium decided to develop a new RF setup only for 1800 MHz and use already available setups for 900 MHz (setups of Participants 6 and 9). Furthermore, the setup of Participant 9 was updated to allow complex GSM modulation. In addition to the required deliverables, a novel UMTS exposure system was developed.

CONCLUSIONS

High-end exposure systems for conducting *in vitro* laboratory studies in several European research institutes were realized and characterized. These systems have already become standard exposure setups for bioexperiments around the world.

An ELF exposure system that allows flexible signal and intermittent exposure schemes has been developed and characterized. It is easy to handle due to automated software control. Coil currents, chamber temperatures and fan speed are continuously monitored and allow the experimental history to be traced with 10 s resolution. B-field and E-field distributions were characterized. The B-field shielding of the 4-coil configuration considerably enhances the uniformity of the field distribution, and a highly efficient E-field shielding inhibits strong parasitic electric fields generated by the coils. Temperature differences between exposed and sham-exposed cells are kept below 0.1°C. The vibration load on the

exposed Petri dishes is sensitive to mechanical resonance; however, a mechanically isolated and elastically damped dish holder limits this to less than $0.1 m/s^2$, which is no more than twice the background vibration of the sham setup.

The waveguide-based, computer-controlled RF (GSM) setup enables the exposure of cell monolayers with excellent efficiency > 20 W/kg /W. The flexible signal unit allows the generation and control of complex modulated signals, e.g. temporal changes between different GSM operation modes (DTX/non-DTX). Exposure field strength and environmental parameters (air temperature, fan system) are continuously monitored. Due to the field regulation, exposure variability is kept below 10%. A coupled electro-thermal FDTD analysis was performed and resulted in a nonuniformity of SAR of < 30%. The temperature load was assessed by measurement and simulation, and a maximum temperature increase of less than 0.03°C was found. No localized temperature hot "spots" are generated within the cell medium. All simulations were verified by dosimetric measurements.

An exposure setup allowing the blinded exposure of cell monolayers to UMTS signal schemes was developed and dosimetrically analysed. Cells can be exposed to up to 17 W/kg/W with less than 26% nonuniformity of SAR. The temperature load for the exposed cells is less than 0.03°C /(W/kg). The UMTS specifications have been analysed in order to identify ELF spectral components in the signal. These mainly result from inner loop power control; however, pulsed signal structures due to compressed mode and PRACH/PCPCH procedures also contribute to the ELF components. A test signal is proposed which is compliant to the 3GPP FDD modulation specifications and is optimised for maximized ELF spectral power (1 Hz harmonics).

Quality control for the entire duration of the project is ensured due to automatically generated data files. Exposure field strength, temperature, fan currents and all settings and computer commands are stored in the data files with a sampling rate of 0.1 Hz. Evaluation reports are available for every experiment performed in the REFLEX consortium.

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