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of electroMagnetic exposures with Organisms for Risk Assessment

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Periodic Report

PROJECT PERIODIC REPORT

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1. Publishable summary

Summary description of project context and objectives

Research on exposure to extremely low frequency magnetic fields (ELF-MF) as a potential risk factor for childhood leukemia has been conducted since the late 1970s, and more than 30 epidemiological studies have been published since then. However, even though the International Agency for Research on Cancer (IARC) classified ELF MF as possibly carcinogenic in 2001, animal and cellular studies have provided only weak support for a causal relationship between sub-millitesla-level ELF-MFs and malignant diseases. As biophysical studies also do not support such a relationship, it remains difficult to rule out chance and experimental bias as possible explanations for the detected association between childhood leukemia and exposure to ELF MF. The ARIMMORA (Advanced Research on Interaction Mechanisms of electroMagnetic exposures with Organisms for Risk Assessment) project aims to resolve this controversy by conducting a sound risk assessment on the association between childhood leukemia and ELF-MF exposure. This task requires a better understanding of the levels and temporal patterns of ELF-MF exposures of children in their daily lives and normal activities; extensive biological investigations to detect possible interaction mechanisms at the cellular and sub-cellular levels; and advanced biophysical simulations with efficient numerical solvers to support the experiments. The impact of ELF-MF exposure is investigated for four processes in particular: 1) the epigenetic dynamics associated with hematopoietic cell lineage commitment and differentiation; epigenetic signatures are monitored genome-wide, and mechanisms underlying eventual “misprogramming” are addressed in gene promoter models; 2) the alteration of signalling processes in cells; 3) the induction of possible cytotoxic effects on CD8-positive T-cells; and 4) the genesis and evolution of childhood leukemia in advanced genetically-modified animal models. In a final phase, the results of ARIMMORA and other projects will be combined, and a risk assessment will be performed by adapting and applying the procedures outlined by the IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Research on exposure to extremely low frequency magnetic fields (ELF-MF) as a potential risk factor for childhood leukaemia has been conducted since the late 1970s, and more than 30 epidemiological studies have been published since then. However, even though the International Agency for Research on Cancer (IARC) classified ELF-MF as possibly carcinogenic in 2001, animal and cellular studies have provided only weak support for a causal relationship between sub-millitesla-level ELF-MFs and malignant diseases. As support from biophysical studies for such a relationship is also insufficient, it remains difficult to rule out chance and experimental bias as possible explanations for the detected association between childhood leukaemia and exposure to ELF-MF.

The ARIMMORA (Advanced Research on Interaction Mechanisms of electroMagnetic exposures with Organisms for Risk Assessment) project aimed to resolve this controversy by conducting a sound risk assessment on the association between childhood leukaemia and ELF-MF exposure. This task required a better understanding of the levels and temporal patterns of ELF-MF exposures of children in their daily lives and normal activities; extensive biological investigations to detect possible interaction mechanisms at the cellular and sub-cellular levels; and advanced biophysical simulations with efficient numerical solvers to support the experiments. The impact of ELF-MF exposure was investigated for four processes in particular: 1) the epigenetic dynamics associated with hematopoietic cell lineage commitment and differentiation; epigenetic signatures were monitored genome-wide, and mechanisms underlying possible “misprogramming” were addressed in gene promoter models; 2) the alteration of signalling processes in cells; 3) the induction of possible cytotoxic effects on CD8-positive T-cells; and 4) the genesis and evolution of childhood leukaemia in advanced genetically-modified animal models. In the final phase, the results of ARIMMORA and other projects were combined, and a risk assessment was performed by adapting and applying the procedures outlined by the IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. The ARIMMORA consortium consisted of ten world-renowned research groups – from Switzerland, Germany, Spain, Israel, France, and Italy – with expertise ranging from engineering to biology. This three and a half year project was coordinated by Prof. Niels Kuster, Director of the IT'IS Foundation for Research on Information Technologies in Society in Zurich, Switzerland.

Description of work performed and main results

In work package WP1 (Literature Database), a complete cross-disciplinary database covering all the

scientific and engineering aspects relevant to the development of the ARIMMORA project was created (<http://arimmora-fp7.eu/refbase/>) and has been continually updated during the course of the project. This database was available to all project partners, and it was particularly relevant for the preparation of WP9 (Risk Assessment).

Work package WP2 (Exposure Matrix for Children Including Power Lines, Transformers & Near-Field Sources) aimed to broaden the knowledge base on ELF-MF exposure in children from prenatal life to adolescence (0 – 16 years old) by conducting personal exposure assessments, by determining the contributions of near-field sources in comparison to power line fields, and by translating the incident fields into field quantities in tissues and organs..

The near-field sources to which children and pregnant women are exposed were measured with the novel instrument developed in WP4, and the data was analysed. These data were used to validate the developed transformation matrices of gradient measurements to induced fields that are used in WP4. The posture tool has been extended to enable the generation of child models in realistic postures. This achievement allowed the analysis of the exposure variations in children to be completed. A manuscript on this work is now being submitted for publication.

Furthermore, all knowledge generated in WP2 about exposure and exposure assessments have been disseminated to the relevant standardization groups in the course of WP8.

The task of WP3 (Exposure systems, dosimetry & quality control) was to provide well-characterized exposure equipment for the biological experiments of WP5 and WP6. Two in vitro systems sXcELF were adapted to the needs of ARIMMORA and installed in the partners' laboratories. A novel high-performance in vivo exposure system (sXvELF) was developed, manufactured, validated, and installed at the CBM animal facility in Madrid, Spain. In addition, field and environmental monitoring systems were installed at the University of Veterinary Medicine in Hannover, Germany. Importantly, numerical dosimetry to relate the induced fields and currents in rats and mice to those in children was performed.

The objective of WP4 (Development of Instrumentation for Exposure Assessment and Surveillance) was to close the gaps regarding the sound assessment of ELF-MF exposure, measurement equipment, and extensions of numerical tools. The instrumentation requirements were evaluated by conducting measurements and simulations in various exposure scenarios. The second prototype of a novel measurement equipment was developed to determine the magnitude and spatial gradient of the incident magnetic fields simultaneously. Numerical postprocessing tools for the evaluation of quantities requested by the standards agencies were developed, and the data of WP2 was used to determine the preliminary transformation coefficient functions (TCF) for the most important exposure scenarios reflecting the entire user population as a function of usage and distance. The validation and calibration prototype has been finalized and tested.

In WP5 (ELF-MF Perception, Signalling, and Impact on Epigenetic Stability), the hypothesis that ELF-MFs may induce MAPK signalling and impact downstream cellular processes was tested. A series of cell lines for the induction of ERK1/2 phosphorylation were screened. Statistically significant activation of ERK1/2 phosphorylation was observed in non-transformed cell lines, whereas cancer-derived cells responded less or not at all. Dose response experiments indicated that the signal reached the maximum at $\sim 7 \mu\text{T}$, and remained high at 1 mT. Exposure to weaker ELF-MF intensities (10, 1, 0.5, and 0.15 μT) revealed ERK1/2 phosphorylation in some cell lines already at 0.15 μT . The observed activation was generally weak and delayed when compared to effects observed with canonical mitogens. Although the genetic dysregulation of the ERK1/2 cascade is well linked to cancer, it is uncertain whether and how a transient activation by EMF-MF impacts the process of cell transformation. Therefore, it is too early to speculate whether the observed low activation of the ERK1/2 cascade is related to possible EMF-EF related induction of leukaemia. To resolve these issues, the project was extended with internal funding from the partners, the final results of which will become available only after the conclusion of the ARIMMORA project. In an additional experiment, a hypothesis regarding the possible involvement of cryptochromes and the radical pair mechanism in the cellular responses to ELF-MF, was tested. The involvement of cryptochrome was supported by results showing that the stimulation by blue light reduces ELF-MF-induced ERK1/2 phosphorylation, while knocking down cryptochromes (CRY1/2) prevented this reduction,

Genome-wide analysis of the epigenetic landscape of leukemic cells intermittently exposed to a 50 Hz ELF-MF for about 3.5 cell cycles did not reveal an ELF-MF disturbance of cell growth or viability nor any substantial epigenetic alterations. Hence, genomic regions responding to ELF-MF exposure could not be identified and/or validated as a potential biomarker for exposure.

To address the impact on epigenetic programming, an *in vitro* haematopoietic differentiation protocol was established. This protocol was used to generate highly reproducible and informative snapshots of the epigenetic landscape as it changes during the differentiation of human hematopoietic stem cells into neutrophilic granulocytes. These epigenetic profiles will advance the current understanding of haematopoiesis and may lead to the identification of key regulatory processes and factors. While the number of differential histone modifications (H3K27me3 and H3K4me2) between differentiation states was rather large, as expected, the effect of ELF-MF exposure (50 Hz with power line harmonics) was detectable but much less pronounced in both numbers and extent. These results indicate that, unlike in the leukaemia cell lines tested, ELF-MF exposure may generate a localized impact on the epigenetic programming in differentiating haematopoietic cells, hence potentially affecting the lineage commitment. Yet, although minor differences in apoptosis and cell cycle progression were observed, lineage commitment was not notably affected in the experimental setup used. These proof-of-concept experiments for an ELF-MF effect on genome-wide epigenetic profiling hence generated a valuable dataset that can be explored towards the identification of a potential epigenetic footprints of ELF-MF exposure and provide information for future functional studies as well as the establishment of biomarker panels for biological exposure assessment. The overall objective of WP6 (Mechanisms and Animal Models) was to gain mechanistic insights on the impact of ELF-MF exposure in animal models by applying three different strategies.

1) In the CD1 mouse study, animals were exposed to continuous linear sine-shape 50 Hz electromagnetic fields of 10 μ T, 1 mT, or 10 mT (negative controls were sham-exposed) for 20 hours/day, 7 days/week, starting from pregnant dams and continuing to F1 offspring up to 90 days of age.

The *in vivo* findings did not show any damaging effects of ELF-MF signals on the health of animals. The results did not demonstrate any significant and biological relevant effect on the (routine) haematology of the ELF-MF exposed CD-1 mice up to the age of 90 days. Single significant results did not show any relation to dose and are therefore considered as biological irrelevant. In addition, none of the tested doses of the ELF-MF demonstrated a significant induction of micronuclei in the peripheral blood erythrocyte fraction, irrespective of the time point (4 days, 28 days, 18 months) of analysis. Finally, some effects on haematopoiesis and on the juvenile immune system were demonstrated. On day 90, the number of CD4+ and CD3+ T-lymphocytes was reduced in the 1 mT group. IgM and IgG were determined in blood samples of exposed animals. IgG was significantly increased in 28-day-old animals after exposure to 10 mT ELF-MF and in 90-day-old mice also after exposure to 1 mT.

Most importantly, on day 28, a diminished number of CD8+ cytotoxic T-lymphocytes (CTL) was seen in peripheral blood in all ELF-MF groups (10 μ T, 1 mT, and 10 mT). Although the effect was moderate, it was significant but not dose-dependent. This significant reduction was not observed after 60 and 90 days. Furthermore, the number of B-lymphocytes was significantly increased in the blood of CD-1 female mice after 60 days exposure in those groups receiving 1 mT and 10 mT, and the number of monocytes was diminished after exposure to 10 mT.

Gene expression profiling showed clues that ELF-MF at high dose may pose certain risk at the level of the individual, and this risk could depend on the complex interplay among environmental, genetic and epigenetic factors.

2) In the course of the ARIMMORA project, a new transgenic mouse model of childhood B acute lymphoblastic leukaemia (B-ALL) has been generated in which the human B-ALL-associated first genetic lesion, TEL-AML1, is expressed in the stem/progenitors compartment of the haematopoietic system. Breeding pairs of these transgenic mice are being exposed to a 50 Hz magnetic field of 1.5 mT with both fundamental and harmonic content, with and an on/off cycle of 10 min/5 min, for 20 hours per day. Analysis of the bone marrow of unexposed mice showed that, at 6 months of age, specific alterations in B-cell development could already be detected in the form of an increase in BM pro/pre-B-cells and immature B-cells. However, like in humans, the leukaemic process in the mouse model does not develop very rapidly. Moreover, the appearance of this fusion protein in the mouse model does not commit the premalignant target cells to develop malignant disease, as a significant proportion of the model animals developed no alterations, similar to observations in children who harbour TEL-AML1 fusion gene but never develop B-ALL, indicating that secondary cooperative changes in the mouse genome seem to be necessary for disease expression. Thus, the transgenic mouse model generated is ideal for *in vivo* modelling of possible ELF-MF effects in B-ALL. Analysis of the peripheral blood of ELF-MF-exposed Sca1-ETV6-RUNX1 transgenic mice (“exposed mice”) or unexposed control Sca1-TEL-AML1 (“unexposed young mice”) was performed at different time points: 1 month, 2, 4, 6, 9, 12, 15, 18, and 21 months. A first relevant finding was

the existence of a small, but statistically significant decrease in the numbers of CD8+ T-cells in the exposed mice at 60 days of age. This is in good correlation with the previous findings with CD1 mice. Taken together, these data would suggest that a decrease in the number of CD8+ T-cells could be considered as a biomarker of early exposure to ELF-MF fields of different intensities and characteristics, in mice of different genetic backgrounds. Of the remaining parameters measured, also total B-cell numbers and mouse weight presented small differences between EMF-exposed and unexposed mice. Overall survivals of the exposed and unexposed populations were not statistically different, with almost 65% of the mice being still alive at 24 months of age. Of the dead animals, one of the exposed transgenic mice developed a B-ALL at 14 months.

3) In another study, two inbred rat strains, Lewis and Fischer 344 (F344), which are genetically related to the same background strain but differ in their sensitivities to stress, carcinogens, and ELF-MF exposure, were used to investigate the ELF-MF effects on the haematopoietic system. The findings indicated that ELF-MF exposure has a functional impact on rat haematopoietic cells by affecting the proliferative capacity of lymphocytes from spleen and bone marrow *ex vivo*. The observed effects revealed sex and strain differences, and the data suggest that B- and T-cells as well as their interactions might be affected by ELF-MF exposure. The functionality of bone marrow cells from female rats, particularly of the F344 strain, was predominantly decreased after exposure to 100 μ T, indicating a lower responsiveness of lymphocytes and probably immunosuppressive effects in these rats. In contrast, increased functionality was observed, e.g., in spleen cells from male Lewis rats after ELF-MF exposure, revealing enhanced lymphocyte sensitivity. The evaluation of differential blood counts showed alterations in exposed F344 in terms of increased proportions of neutrophil granulocytes, decreased lymphocytes, and an increased ratio of neutrophils to lymphocytes, which is an indicator for inflammation and certain diseases, including cancer. Furthermore, investigations of apoptosis were performed by determining two markers of apoptosis, Annexin V and caspases. In addition to directly addressing apoptosis, a screening assay was performed by simultaneously determining 90 different rat cytokines in the cell culture medium to examine differences in cellular signalling after *in vivo* ELF-MF exposure associated with cell death, immune cell suppression, and activation.

Ex vivo studies were performed to investigate novel possible targets of ELF-MF mechanisms, which have not been yet examined in this context. Recent studies suggest that adrenergic receptors are involved in cell proliferation and cancer development and are considered as new targets for tumour treatment. Therefore, we investigated the binding to adrenergic receptors in primary spleen and bone marrow cells after *in vivo* exposure of Lewis and F344 rats. Data showed alterations of beta2- and alpha2-adrenergic receptors after ELF-MF exposure *in vivo*, suggesting a modulation of immune functions and apoptosis. The observed changes were predominantly strain-dependent.

Work package WP7 (Biophysical Modelling) combined all tasks related to biophysical modelling with the objective to determine interaction sites and mechanisms. The first task was to determine the induced magnetic and electric fields at a macroscopic level for the setups and various cell dishes for field directions developed in WP3, i.e., averaged fields induced in the cell medium of the *in vitro* studies (WP5) and in specific tissues examined in the *in vivo* experiments (WP6), that can be correlated to those determined in WP2 for children. Mappings between child age or weight and the experimental rats and mice were developed such that the induced fields over the course of the experiments could be related to childhood exposure. Organ specific dosimetry for mice, rats, and children was performed. The comprehensive literature review of microdosimetry confirmed that it is unlikely that interactions occur via the induced electric fields but rather via direct interaction with the magnetic fields. This was also supported by the third task on potential mechanisms. Based on our two literature reviews on 1) the reported positive effects of low-level EMF and 2) a potential interaction mechanism, we concluded that the radical pair mechanism is the most plausible model. This was followed by a more focused literature review to collect specific evidence and the most effective exposure conditions to confirm the radical repair mechanism for the cells employed within ARIMMORA. Results of WP5 showing that the stimulation by blue light reduces ELF-MF-induced ERK1/2 phosphorylation, while knocking down cryptochromes (CRY1/2) prevented this reduction, supports the hypothesis of a possible involvement of cryptochromes and the radical pair mechanism in the process of cellular response to ELF-MF. The analysis of all activities could be completed and most of those will be submitted to appropriate journals within the next weeks.

Based on the project achievements, WP8 (Dissemination to Standards) dealt with the dissemination of the results to the relevant standards committees. The final evaluation report was sent to all currently active standardization bodies including WHO, ICES, ICNIRP, Industry Canada, FDA, etc.

The insight gained in WP2 on methods of exposure assessment and the novel instrumentation developed in WP4 have also been discussed inside standardization committees during the reporting period as well as with NIOSH and Industry Canada, who are interested in utilizing the instrument developed in WP4.

The project was finalized with the risk assessment workshop of WP9 (Risk Assessment). The risk assessment was conducted by evaluating the carcinogenicity of ELF-MF based on studies conducted within this project and to compare the results with those from recent studies conducted outside the consortium, and by adapting and applying procedures as outlined by the IARC Monographs Program on the evaluation of carcinogenic risks to humans. The 5-day workshop was performed in Lyon, France, in March 2015. The ARIMMORA consortium and external experts were involved in the evaluation.

Work package 10 (Management) was dedicated to the management of the ARIMMORA project. Special emphasis has been placed on dissemination, which includes the creation and maintenance of the attractive project website, the distribution of informative leaflets, the unique workshop organized on Monte Verita 'EMF Health Risk Research - Lessons Learned and Recommendations for the Future – 7 Years Later.' And last but not least, the risk aforementioned assessment workshop was performed at the end of the ARIMMORA project.

Expected final results and potential impacts

1. Impact on Science

While the overall evidence has not changed compared to the previous assessments by the IARC in 2001 and the three SCENIHR opinion statements from 2007, 2009, and 2015, the ARIMMORA project still added important knowledge on the possible carcinogenicity of exposure to ELF-MF. Most importantly, a mouse model for predisposed childhood leukaemia has been successfully established and used. Although the impact of EMF-MF exposure on the development of leukaemia is uncertain at this point, this achievement is considered an important step towards providing a relevant disease model. If used with larger numbers of mice and with several different exposure levels, this animal model has the potential to provide major future insights. Mechanistic findings related to the immune system also have the potential to provide a future insight if a role in the development of childhood leukaemia is identified. Other mechanistic effects were observed at the levels of cell signalling and epigenetic programming. Although their biological significance and contribution to carcinogenicity is currently unclear, the findings implicate novel, as yet unexplored, mechanisms of interaction of EMF-MF with cell behaviour.

2. Impact on Risk

The findings of ARIMMORA are insufficient to impact decisions on safety policy at present but mandate that the research effort be accelerated as they point to future research that could provide a step-change in future assessments. The consortium recommends that future investigations be addressed with one or two 3-year research programs of similar size.

The results also encourage and reinforce the current policy of 'prudent avoidance'. The policy in view of the current state of knowledge regarding the risk of childhood leukaemia from ELF-MF exposure might include deciding to locate newly built schools at sufficient distance from high voltage power lines, or, conversely new lines far from existing childcare centres, kindergartens, and schools. However, there is as yet insufficient scientific evidence to justify the relocation of existing institutions and power lines.

1.3. Impact on Measurement Standards

Measurement standards are likely to change as the instrumentation and procedures developed in ARIMMORA empower more reliable demonstration of compliance in the close vicinity of ELF-MF sources with the basic restrictions. This can be considered a breakthrough, in particular in the context of with the EU directives on EMF exposure. However, also Industry Canada and the National Institute for Occupational Safety and Health (NIOSH) of the United States Centers for Disease Control have shown great interest in the newly developed device for exposure measurement.