WP2: Review of advances in toxicogenomics with relevance for the reduction of uncertainties in EMF related cancer risk assessment

Top-down approach: Overall assessment of the potential contribution of toxicogenomics (focused on proteomics and metabolomics) to close knowledge gaps in current EMF cancer risk assessment

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1 Introduction – strategy

IMBA (Implications of Biomedicine for the Assessment of Human Health Risks) is a health technology project that will assess how new developments in biomedicine can be applied in risk management. As a sub-discipline of toxicology 'toxicogenomics' promises to provide new opportunities for a better risk assessment. The goal of work package 2 (WP2) is to review the contribution of toxicogenomics for improving EMF (electromagnetic field) cancer risk assessment. The following review will assess in a so-called "top-down approach" current and future –omics approaches that could find application in EMF cancer risk assessment.

The complementary use of genomic, transcriptomic, proteomic and metabolomic approaches in toxicogenomics has been widely appreciated. The ideal case of a complete toxicity profiling would comprise all changes in the genome (DNA), transcriptome (mRNA), proteome (proteins) and metabolome (intermediary metabolites, small molecules, peptides) after exposure to a toxic substance. Since toxicogenomics is a broad field, the following report focuses on issues regarding primarily proteomics and metabolomics in EMF cancer risk assessment. This does not mean that cancer is the only health risk emanating from mobile phone radiation. However, the emphasis of this project is confined to survey possibilities for toxicogenomics in cancer risk assessment in order to maintain the research complexity to a manageable level. In addition, EMF is considered to be a good example because ELF-EMF are classified by the International Agency for Research on Cancer (IARC) as a possible carcinogen, and in the REFLEX study (2004) evidence is given that RF-EMF might cause genotoxic effects.

To evaluate whether proteomic and metabolomic approaches have the potential to contribute to EMF cancer risk assessment the following research steps have been conducted. Initially, a literature search and review of the available literature in the field of proteomics and EMF was performed. Following the few available research studies on proteomics and EMF we developed a strategy that allows to make a statement of the possible contributions by conducting a review of available literature whether proteomic approaches are able to indicate the cancer risk of a specific environmental factor in general. By using this assessment as a reference, the question if this is also applicable to EMF cancer risk assessment can be considered and discussed.

2 Proteomics and metabolomics

2.1 Concepts and definitions

The term 'proteome' was used for the first time in 1994 to describe the set of proteins encoded by the genome. Compared to the genome, which is a static collection of genes, the proteome is rather dynamic representing a particular protein profile in a given cell, tissue or organism under well-defined conditions. The proteome differs from cell to cell, at different stages of the life cycle and under different environmental conditions. Compared to the genome the proteome shows an extraordinary complexity. In the human organism, there are about 20,000 genes and more than 500,000 proteins originating from these genes in consequence of various post-transcriptional and post-translational modifications like for example alternative splicing, protein modification (phosphorylation, glycosylation, ubiquitination) or protein degradation. The study of proteins is a thrilling task because, rather than genes or mRNA, proteins do most of the work in the cell. As major components of the metabolic pathways, proteins are mainly responsible for cellular responses, and in addition, they comprise much of the structural components of cells, tissues and organs.

Proteomics is a global protein analysis mode that includes the separation and identification of proteins to create protein profiles or differential protein display with the potential to attribute a function to a given biological phenomenon. One can distinguish between differential proteomics, implying the comparison of the protein expression of tissue with pathological changes and control tissue or the comparison of treated versus normal conditions. On the other hand, functional proteomics, contribute to the elucidation of protein interactions, protein complexes and protein pathways.

Metabolomics is the newest –omics science representing the metabolic status of living systems. The metabolome is defined as a highly complex and organized biochemical network in which small molecules like metabolite substrates and products, lipids, small peptides, vitamins and other protein co-factors act with each other or with other biologic macromolecules. Like the proteome, the metabolome is dynamic, reflecting the continuous variability of metabolic and signalling pathways. However, compared to the proteome or transcriptome, the metabolome is more diverse concerning chemical and physical properties due to the larger variations in atomic arrangements. Metabolomics can be very easily applied to large populations in studies on the influence of stress on animals, plants or microbes, because it tends to be of higher throughput and generally lower cost than transcriptomics and proteomics. It also provides evidence of which area of metabolism may be affected by external perturbation.

2.2 Techniques & Methods – feasibility of -omics techniques

As proteomics claims to elucidate the biological significance of changes in the proteome, the initial challenge is to resolve the proteins for a subsequent characterization of the individual components. One of many approaches in proteomics is to use 2D gel electrophoresis (2 DE) to separate mixtures of proteins on the basis of charge in the first dimension and mass in the second. By visualizing and comparing the intensities of spots, conclusions of changes in the protein pattern can be drawn. Individual spots, containing one or a few proteins, are excised and subjected to mass spectrometric analysis and subsequent peptide mass fingerprint (PMF) search for identification. The 2DE/PMF approach has the advantage as it allows the resolution of more than 2,000 proteins and a direct comparative proteome mapping. Moreover, 2DE in particular plays a major role in the separation and quantification of post-translationally modified proteins. On the other hand, 2DE has its limitations. Due to a narrow dynamic range, quantitative measurements are limited. The sensitivity for low-abundance proteins is poor and they are in many cases masked by common 'housekeeping' proteins. Additionally, 2DE cannot deal with very large proteins or proteins that have an extreme isoelectric point as well as hydrophobic proteins including membrane or membrane-associated proteins.

An alternative strategy in proteomics is the identification of proteins by nano-LC-tandem mass spectrometry (MS/MS) techniques. MudPIT (Multidimensional Protein Identification Technology) is a technique for the identification of complex protein mixtures that implies extensive separation steps on the peptide level. Rather than using high resolution protein separation by 2D gel electrophoresis, MudPIT separates peptides in multidimensional liquid chromatography steps. In this way, the separation can be interfaced directly with the ion source of a mass spectrometer. An alternative technique is 1DE protein separation in combination with LC-MS/MS.

With respect to risk assessment a method that provides a quantitative comparison of proteins in two samples, for example, normal versus tumour or control versus treated, is most promising. Since mass spectrometry is not inherently quantitative, a common strategy is to employ differential stable isotope labeling for the quantification of differences between physiological states of a biological system. The mass spectrometer recognizes a specific mass tag which is the basis for the quantification. However, the identification and quantification of all of the proteins in a biological system is still an impracticable technical problem. The percentage of proteins that can be analysed by proteomic analysis rarely exceeds 10% of the complete proteome and, moreover, for protein quantification this amount can hardly be

realized because more comprehensive data is required for quantification than for protein identification.

The proteomic approach using LC-tandem mass spectrometry and stable isotope labeling for quantification has the advantage that more than 200 different proteins can be identified in one LC-MS sample resulting in up to 10,000 proteins in one experiment. Due to the MS/MS-fragmentation the reliability of protein identification is high. Compared to the traditional 2DE/PMF the sensitivity and dynamic range is higher as it is determined by the mass spectrometry method. Moreover, LC-tandem mass spectrometry enables the identification of membrane and very large proteins. A drawback of this technique is the differentiation between protein isoforms because a covering of the complete sequence is not possible. In addition, post-translational modifications might be undetected since modified peptides are often lost. In Table 1 the benefits and limitations of the techniques used in proteomics are summarized.

Table 1: Benefits and limitations of techniques used in proteomics

Technique	Benefits	Limitations
2D gel electrophoresis	Resolution of more than 2,000 proteins	Narrow dynamic range → quantitative measurements are limited
	Direct comparative proteome mapping	Sensitivity for low-abundant proteins is poor
	Separation and quantification of post-translationally modified proteins	Problematic with very large proteins, hydrophobic (membrane/membrane-associated) proteins and proteins that have an extreme isoelectric point
Nano-LC-tandem mass spectrometry	More than 200 different proteins can be identified in one MS-sample → up to 10,000 proteins in one experiment	Differentiation between protein isoforms is difficult
	Reliability of protein identification is high	Undetected post-translational modifications
	Higher sensitivity and dynamic range that as it is determined by the mass spectrometry method	
	Identification of membrane and very large proteins possible	

Whereas the genome and proteome are generally studied using microarrays and 2D gels or mass spectrometry, no standard procedure exists for metabolic identification, yet. To date, primarily nuclear magnetic resonance (NMR) or mass spectrometry coupled to chromatography are used in metabolomic studies.

2.3 Application of –omics in toxicology

Toxicoproteomics, as part of the larger field of toxicogenomics, seeks to identify critical proteins and pathways in biological systems that are affected by and respond to adverse chemical and environmental exposures using global protein technologies (Wetmore et al., 2004). A major goal of toxicoproteomics is to identify new biomarkers and signatures of toxicity for classifying toxicants for health risk and for observing toxicity. It aims to better understand both, the consequences of acute exposure to toxicants as well as long-term development of diseases. The determination of individual proteins or groups of proteins associated with the exposure to toxic substances could be reflective of a common mechanism of toxicity.

The carcinogenic potential of a new compound can be investigated by comparing effects on the proteome against the changes in the protein expression pattern caused by a known carcinogen. This carcinogen should have been analyzed and classified in previous proteomic approaches. The larger the collection of data points is, the more confidence can be gained in the predictive value of biomarkers. Furthermore, numerous studies have been made for the identification of alterations in the proteome that occur in cancerous cells. Possibly, some of these markers are also affected following treatment with carcinogenic agents and could be used as early indicators of tumourigenesis.

Many diseases are a result of metabolic disorders, and it makes sense to measure metabolites directly to predict toxicity. In this regard, metabolomics is gaining increasing interest. Once key metabolite markers are identified this will likely lead to a better understanding of the disease process and could be implemented into a risk assessment scheme. However, the current situation reflects the very beginning of a developing field. The techniques and the methodology are far away from detecting the whole metabolome. In addition, the situation at the metabolic level is characterized by an even faster change of the components than at the protein level.

So far (January 2008) nobody has tried to apply metabolomic approaches in the field of EMF research, and a lot of technical improvements have to be achieved before metabolomic profiling can be applied in risk assessment. Hence, no metabolomics studies were considered in the following assessment on –omic approaches in EMF cancer risk assessment.

3 Proteomics and Risk assessment

3.1 Research Strategy

Within the evaluation if –omic approaches have the potential to contribute to EMF cancer risk assessment, a literature search with respect to EMF and proteomics/metabolomics was done, in order to get an idea to what extent research has been done in this field so far. The literature search was carried out using Pubmed and the database provided by the EMF-portal (www.emf-portal.de) and includes papers published up to February 2008. Concerning EMF, both, radiofrequency electromagnetic fields (RF-EMF) utilized in mobile telephony as well as extremely low frequency electromagnetic fields (ELF-EMF) such as those generated, for example, by high tension electrical distribution networks were considered. The following keywords were used in different combinations to search the databases: magnetic field, RF, ELF, EMF, 900 MHz, 50 Hz, mobile phone radiation, electromagnetic fields, GSM, global system for mobile communication, radiofrequency. Taken together, the findings with respect to proteomics/metabolomics and EMF were rather poor because only 10 studies were found, implying 5 on RF-EMF and 5 on ELF-EMF. The assessment if proteomic approaches can contribute to EMF cancer risk assessment turns out to be unfeasible using these data only. Consequently, we developed a strategy to provide a basis for the assessment if toxicoproteomics have the potential to close knowledge gaps in current EMF cancer risk assessment.

Initially, we evaluated the general contribution of proteomics to cancer risk assessment. We focussed on risk factors that are already well-known to cause cancer and discussed and assessed concrete and comprehensive examples of studies in which proteomic approaches had been applied. For the sake of clarity we only included chemical carcinogens as specific environmental risk factors. Eventually, conclusions were drawn if the findings regarding chemical carcinogenic substances and proteomics are also applicable to EMF cancer risk assessment and what kind of specifics and restrictions have to be considered dealing with the case of EMF.

Within the assessment, whether proteomics add value to cancer risk assessment of specific environmental risk factors a comprehensive literature search on proteomics in research with chemical carcinogens was conducted using various libraries and databases. Additionally, proteomics-platforms and publications of public authorities, institutions, agencies etc. were included. Table 7 summarizes the different sources.

The search was initiated by looking for any entry dealing with proteomics/proteome, and if so, expanded using additional keywords including carcinogen, cancer, carcinogenesis,

chemical, tumourigenic and tumour. As the literature search for studies on proteomics and chemical carcinogenic agents emerged to be most promising using Pubmed, it was intensified by successively using the following proteomics-keywords (proteom*, two-dimensional, 2 DE, ms, mass spectrom*, array) in combination with a selected chemical carcinogen that is well-known to cause tumours in either liver or brain. In Table 8 the selection of the included chemical carcinogenic agents is shown. The focus of the review was directed on chemical carcinogens inducing tumours particularly in liver and brain. Chemical carcinogens inducing tumours in liver were included because there is extensive experimental data available on hepatocarcinogenesis. Moreover, the liver is the organ which carries out metabolism and detoxification of chemical substances and is primarily affected by many toxic compounds. Additionally, chemical carcinogens associated with brain tumours were incorporated as one can assume that exposure to the emission of RF-EMF occurs almost exclusively to the head region because until now mobile phones are commonly held to one side of the head during a talk.

3.2 Proteomics in research with carcinogenic agents

The literature search through Pubmed revealed that no study on proteomics and any chemical carcinogen inducing tumours in brain have been published so far (December 2007). On the other hand 30 studies dealing with proteomics and different chemical agents leading to hepatocarcinogenesis were found. A summary of these studies is shown in Table 2. The studies were published by 20 different authors in the period between 1987 and 2007. The studies on proteomics and chemical carcinogens inducing tumours particularly in liver clearly showed that proteins were deregulated in consequence of the application of the substance. However, in eight studies no information about the identity of the deregulated proteins were given at all. It was just mentioned that protein spots were either up- or down-regulated however an identification of these proteins was not performed. In 15 studies only a small number of deregulated proteins was identified as the authors were interested to determine whether particular proteins, e. g. variants of glutathione S-transferase (GST) were affected. Moreover, in some studies methods other than mass spectrometry were used for protein identification. Compared to mass spectrometric analysis, which is a high throughput screening method providing comprehensive lists of protein identities, the chosen technologies, e. g. N-terminal Edman sequencing for amino acid analysis, are more time-consuming and labour-intensive as they are only suitable for a selective identification of proteins. In seven studies

substantial lists of deregulated proteins were supplied as extensive mass spectrometry analysis for protein identification was performed.

Table 2: Studies on proteomics and different chemical agents causing hepatocarcinogenesis

Study	Chemical carcinogen	Alterations in protein expression level	Identities of deregulated proteins
Anderson, NL (1987)	Aroclor1254 Phenobarbital Ibuprofen Cycloheximide Carbon tetrachloride	Quantitative changes of more than 70 proteins	No proteins were identified
Beyer, S (2006)	Nitrosomorpholine (NNM)	9 differentially expressed m/z values	No proteins were identified
Chu, R (2002)	Wy14,643	• a total of 40 protein peaks showed more than twofold changes using SELDI technology • using 1D-SDS-PAGE seven bands could be identified as differentially expressed proteins	Examples of identified proteins (more details see paper): • *Up-regulated*: peroxisomal bifunctional enzyme 3-hydroxyacyl-CoA dehydrogenase, peroxisomal 3-ketoacyl-CoA thiolase, GST-GT8.7, triosephosphate iosomerase, antioxidant protein2 • *Down-regulated*: carbamoyl-phosphate synthase mitochondrial precursor (CPSASE I), GST-P1, major urinary protein (MUP), bilverdin reductase B, membrane associated progesterone receptor component, proteasome subunit alpha type 7, carbonic anhydrase 3 •
Cunningham, ML (1995)	Methapyrilamine (MPH) Wy-14,643	• 32 proteins were induced by MPH • 39 changes induced by Wy-14,643	No proteins were identified

Table 2: Continued

Fella, K (2005)	Nitrosomorpholine (NNM)	Differentially expressed proteins after • One day: 13 • Three weeks: 66 • Week 25: 47 Overlap of deregulated proteins of week 3 and week 25: 18	Examples of identified proteins (more details see paper): deregulated proteins of week 3 and week 25 • *Up-regulated*: annexin A5, adenine phosphoribosyltransferase • *Down-regulated*: ornithine aminotransferase, succinyl-CoA ligase, fructose-1,6-bisphosphatase, major urinary protein precursor, estrogen sulfotransferase
Giometti, CS (1991)	Clofibrate Ciprofibrate Wy-14,643 Di(2-ethylhexyl)-phthalate (DEHP)	259 proteins were altered by one or more of the treatment	No proteins were identified
Giometti, CS (1998)	Wy14,643	• 49 mouse liver proteins: 47 down-regulated 2 up-regulated • 35 hamster liver proteins: 27 down-regulated 8 up-regulated ➢ 14 mouse proteins were identified	Examples of identified proteins (more details see paper): • *Up-regulated*: acyl CoA thioesterase, • *Down-regulated*: glucose responsive protein 78, 10- formyltetrahydrofolate dehydrogenase, selenium-binding liver protein, pobable protein disulfide isomerase ER-60 precursor, selenium-binding liver protein, actin β-subunit, senescence marker protein-30, ubiquinol cytochrome C reductase, arginase, ornithine aminotransferase, purine nucleotide phosphorylase, nonselenium glutathione peroxidase apolipoprotein A1
Glückmann, M (2007)	Nitrosomorpholine (NNM)	23 down-regulated and 42 up-regulated proteins were identified	Examples of identified proteins (more details see paper): • *Up-regulated*: annexinA5, HSP90, retinol-binding protein I • *Down-regulated*: senescence marker protein-30, ornithine aminotransferase, major urinary protein

Table 2: Continued

Iida, M (2003)	Oxazepam, Wyeth (Wy)-14,643	• 39 proteins were altered in liver cytosol • 28 proteins were altered in the microsomal fraction • 15 proteins were altered in the nuclear fraction ➢ Selection of 17 proteins were identified	Examples of identified proteins (more details see paper): • *Up-regulated (Oxazepam)*: Cyp2b20, growth arrest- and damage-inducible gene beta (Gadd45beta), tumor necrosis factor alpha-induced protein 2 and insulin-like growth factor binding protein 1 (Igfbp5); • *Up-regulated (Wy-14,643)*: Cyp2b20, cyclin D1, proliferating cell nuclear antigen, Igfbp5, Gadd45beta and cell death-inducing DNA fragmentation factor alpha subunit-like effector A exhibited higher expression
Kitahara, A (1984)	Diethylnitrosoamine (DEN) + 2-acetylaminoflourene (AAF)	Changes in molecular forms of hepatic cytosolic glutathione S-transferase (GST) during rat chemical hepatocarcinogenesis were investigated	GST-A (YbYb) was markedly increased, GST-B (YaYc) increased, GST-C (YbYb') and –D (Yb'Yb') changed little, GST-P was markedly induced
La porta, CA (1995)	Diethylnitrosamine (DEN) + 2-acetylaminofluorene (AAF)	Numerous acidic as well as basic membrane- and cytosolic phosphorylated proteins of high M_r appear	No proteins were identified
Léonard, JF (2006)	Clofibrate	• 77 proteins were up-regulated • 27 proteins were down-regulated	Examples of identified proteins (more details see paper): • *Up-regulated*: acyl-CoA dehydrogenase, carnitine O-palmitoyltransferase, fructose-1,6-bisphosphatase, vimentin • *Down-regulated*: arginase 1, malate dehydrogenase, 10-formyltetrahydrofolate, senescence marker protein-30, apolipoprotein A-IV precursor

Table 2: Continued

Macdonald, N (2001)	Diethylhexylphthalate (DEHP)	542 statistically significant, treatment-dependent differences in protein expression were detected ➢ 111 of these proteins were identified	Examples of identified proteins (more details see paper): Apolipoprotein A1 precursor, selenium binding protein 2, GST-P1/P2, senescence marker protein-30, superoxide dismutase, contrapsin precursor, phosphoglycerat mutase, HSP 60
Mairesse, N (1990)	Diethylnitrosamine (DEN)	Three proteins were increased ➢ only one could be identified	27 kD heat shock protein
Ramagli, LS (1985)	Acetylaminofluorene (AAF) Diethylnitrosamine (DEN)	• 51 qualitative changes • 7 proteins were induced • 3 proteins disappeared	No proteins were identified
Raymondjean, M (1985)	Diethylnitrosamine (DEN)	Increased synthesis of several peptides	No proteins were identified
Strathmann, J (2007)	N-nitrosodiethylamine (DEN) + polychlorinated biphenyl PCB77 N-nitrosodiethylamine (DEN) + polychlorinated biphenyl	98 deregulated proteins: • PCB77 -> 56 proteins • PCB153 -> 44 proteins	Examples of identified proteins (more details see paper): *Proteins deregulated in both*: argininosuccinate lysate, carboxylesterase 3 precursor, formimidoyltransferase cyclodeaminase, 10-formyl-tetrahydrofolate dehydrogenase, glycine-N-methyltransferase, liver carboxylesterase 31, cytokeratin-8, serotransferrin (siderophilin), 3-alpha-hydroxysteroid dehydrogenase type 1, arginase-1, GST P1
Sugioka, Y (1985)	2-acetylaminofluorene (AAF) Diethylnitrosamine (DEN) 3'-methyl-4-dimethylaminoazo-benzene (3'-ME-DAB)	several proteins either appear new or increase not carcinogen dependent: • 1 spot appeared was induced • 5 polypeptides increased ➢ 2 proteins were identified	• *AAF:* GST-A was up-regulated in HPN and HCN • *AAF, DEN, 3'-ME-DAB*: GST-P was up-regulated in HPN and HCC

Table 2: Continued

Teufelhofer, O (2006)	N-nitrosomorpholine (NNM)	several proteins were up-regulated or down-regulated ➢ some proteins were identified	• *Up-regulated:* 10-formyl tetrahydrofolate dehydrogenase, annexinA3 • *Down-regulated*: glutathione synthetase, contrapsin-like protease inhibitor 1
Williams, KE (2002)	(14)C benzene	Identifcation of proteins that are covalently modified by benzene	Several histones were attacked, but the resulting modifications appeared to be largely nonspecific
Wirth, PJ (1986)	Diethylnitrosamine (DEN) + 2-acetylaminofluorene (AAF)	4 different polypeptides were induced	*Up-regulated*: Ya subunit of GST B Yb subunit of GST-A 3 isoelectric point variants of the Yp subunit of GST-P DT-diaphorase
Wirth, PJ (1987)	Diethylnitrosamine (DEN) + 2-acetylaminofluorene (AAF) Ciprofibrate	• 34 polypeptides were increased • 27 polypeptides were decreased ➢ some proteins were identified	• *DEN + AAF*: DT-diaphorase, Ya subunit GST-B, Yb subunit of GST-A and Yp subunit of GST-P were up-regulated • *Ciprofibrate*: Yb subunit GST-A was up-regulated
Wirth, PJ (1992)	Aflatoxin BI	Some proteins were deregulated ➢ some proteins were identified	*Down-regulated*: fibronectin, tropomyosin 6 *Up-regulated*: tropomyosin 1
Wirth, PJ (1994)	Aflatoxin BI	19 proteins were modulated ➢ some proteins were identified	*Down-regulated*: fibronectin, tropomyosin 6 *Up-regulated*: tropomyosin 1
Witzmann, F (1996)	Perfluoro-n-octanoic acid Perfluoro-n-decanoic acid Di(2-ethylhexyl)phthalate (DEHP) Clofibrate	Effect on protein patterns of sulfotransferases (ST1A1, ST1C1, ST2A1) was investigated	ST1A1 and ST1C1 were down-regulated

Table 2: Continued

Zeindl-Eberhart, E (1992)	N-methyl-N-nitrosourea (MNU)	• 6 proteins were up-regulated • 18 proteins were down-regulated • 6 proteins were induced	No proteins were identified
Zeindl-Eberhart, E (1994)	N-methyl-N-nitrosourea (MNU)	Several tumour-associated protein variants were detected ➢ 2 proteins were identified	*Up-regulated*: GST-P, aldose reductase
Zeindl-Eberhart, E (1994)	N-methyl-N-nitrosourea (MNU)	8 proteins were chemically induced ➢ only 1 protein was identified	Aldose reductase
Zeindl-Eberhart, E (1997)	N-methyl-N-nitrosourea (MNU)	30 changed protein variants were detected ➢ only 1 protein was identified	*Up-regulated*: GST-P
Zeindl-Eberhart, E (2001)	N-methyl-N-nitrosourea (MNU), Diethylnitrosamine (DEN), N-Nitrosomorpholine (NNM), Nafenopin (NAF)	Independent of the substance: 24 proteins spots were detected as tumour-associated variant proteins ➢ 17 spots were identified	• *Rat aldose reductase-like protein-1 (rARLP-1)*: all four rAJPs induced by nitroso compound • *rat aldo-keto reductase protein-c (Rak-c)*: induced by MNU • *3alpha-hydroxysteroid dehydrogenase (3alpha-HSD):* reduced by all • *delta4-3-ketosteroid-5beta-reductase (5beta-Red)*: reduced by all

A comparison of the proteins found to be deregulated in the different studies should indicate if substances with a general risk leave a specific fingerprint in the protein patterns. However, direct comparison of the proteome analysis experiments is problematic as samples were not taken at identical time points throughout the studies. Uniformity of the 'sampling' was not given. In order to compare the proteins in spite of the divergency of the data, we established a system in effort to cluster the 'sampling' that had been applied in the different experiments.

We clustered the samples from different time points by assigning them to distinct groups which represent an early, advanced or late stage of carcinogenesis. If possible, the clustering was made on the basis of the histopathological features (Table 3). Otherwise, the duration of the application of the chemical carcinogen was considered.

Table 3: Clustering of the 'sampling' on the basis of histopathological feature

preneoplastic nodules	neoplastic nodules hyperplastic nodules (HNP) putative precancerous lesion	hepatocellular carcinom (HCC) hepatoma tumour

A summary of the proteins that were deregulated due to the application of chemical carcinogens reveals, that numerous proteins were either up- or down-regulated consistently in independent studies. For example, up-regulation of acyl CoA thioesterase was found in three different studies (Giometti et al., 1998; Strathmann et al., 2007; Léonard et al., 2006) induced by diverse chemical carcinogens (Wy14,643, DEN + PCB153, clofibrate). The studies showed that acyl CoA thioesterase was up-regulated after 2 weeks (Giometti et al., 1998) as well as in tumours (Strathmann et al., 2007), representing an early and late stage of carcinogenesis, respectively. Moreover, up-regulation of acyl CoA thioesterase seems to be species-independent as it was found in mouse (Strathmann et al., 2007) and in rat (Léonard et al., 2006). In addition, the example of GST-P shows that it was not only up-regulated due to the application of 5 different chemical carcinogens, but the up-regulation was also detected almost consistently in the same stages of carcinogenesis, namely in hyperplastic nodules and carcinoma. In Table 4 some examples of consistently deregulated proteins are summarized.

Table 4: Summary of consistently deregulated proteins due to application of carcinogenic agents causing hepatocarcinogenesis

Acyl CoA thioesterase: up-regulated in 3 studies			
Wy14,643	mouse	up-regulated after 2 weeks	Giometti, CS (1998)
DEN + PCB153	mouse	up-regulated in tumours	Strathmann, J (2007)
clofibrate	rat	up-regulated after 7 days	Léonard, JF (2006)
10-formyl tetrahydrofolate dehydrogenase: down-regulated in 4 studies			
NNM	rat	down-regulated after 3 weeks	Fella, K (2005)
Wy14,643	mouse	down-regulated after 2 weeks	Giometti, CS (1998)
clofibrate	rat	down-regulated after 7 days	Léonard, JF (2006)
DEN + PCB77	mouse	down-regulated in tumours	Strathmann, J (2007)
Arginase-1: down-regulated in 4 studies			
Wy14,643	mouse	down-regulated after 2 weeks	Giometti, CS (1998)
DEN + PCB153	mouse	down-regulated in tumours	Strathmann, J (2007)
DEN + PCB77	mouse	down-regulated in tumours	Strathmann, J (2007)
clofibrate	rat	down-regulated after 7 days	Léonard, JF (2006)
Glutathione S-transferase A (GST-A): up-regulated in 3 studies			
DEN + 2-AAF	rat	up-regulated in hyperpl. nodules/carcinoma	Kitahara, A (1984)
AAF	rat	up-regulated in hyperpl. nodules/carcinoma	Sugioka, Y (1985)
DEN + 2-AAF	rat	up-regulated in preneopl./neopl. nodules	Wirth, PJ (1985)
Glutathione S-transferase B (GST-B): up-regulated in 3 studies			
Ciprofibrate	rat	up-regulated in preneopl./neopl. nodules	Wirth, PJ (1987)
DEN + 2-AAF	rat	up-regulated in preneopl./neoplc nodules	Wirth, PJ (1986)
DEN + 2-AAF	rat	up-regulated in hyperpl. nodules	Kitahara, A (1984)
Glutathione S-transferase P (GST-P): up-regulated in 6 studies			
DEN + 2-AAF	rat	up-regulated in hyperpl. nodules/carcinoma	Kitahara, A (1984)
AAF	rat	up-regulated in hyperpl. nodules/carcinoma	Sugioka, Y (1985)
DEN	rat	up-regulated in hyperpl. nodules/carcinoma	Sugioka, Y (1985)
3‘-(ME-DAB)	rat	up-regulated in hyperpl. nodules/carcinoma	Sugioka, Y (1985)
DEN + 2-AAF	rat	up-regulated in preneopl./neoplastic nodules	Wirth, PJ (1987)
MNU	rat	up-regulated in hyperpl. nodules/carcinoma	Zeindl-Eberhart, E (1994)

AnnexinA5: up-regulated in 3 studies			
NNM	rat	up-regulated in neopl. nodules/carcinoma	Fella, K (2005)
NNM	rat	up-regulated after 3 weeks	Glückmann, M (2007)
Den + PCB77	mouse	up-regulated in tumours	Strathmann, J (2007)
Senescence marker protein-30: down-regulated in 3 studies			
Clofibrate	rat	down-regulated after 7 days	Léonard, JF (2006)
Wy14,643	mouse	down-regulated after 2 weeks	Giometti, CS (1998)
NNM	rat	down-regulated after 3 weeks	Glückmann, M (2007)

On the other hand proteins also showed inconsistence in their deregulation in independent studies. A summary of inconsistently deregulated proteins is shown in Table 5.

Table 5: Summary of inconsistently deregulated proteins due to application of carcinogenic agents causing hepatocarcinogenesis

Apolipoprotein A1: down-regulated in 3 studies, up-regulated in 1 study			
	mouse	down-regulated after 2 weeks	Giometti, CS (1998)
Wy14,643	rat	up-/down-regulated after 3 weeks	Fella, K (2005)
NNM	rat	down-regulated after 7 days	Léonard, JF (2006)
clofibrate			
Rho-GDI-dissociation inhibitor: up-regulated in 3 studies, down-regulated in 1 study			
NNM	rat	up-regulated after 3 weeks	Glückmann, M (2007)
DEN + PCB77	mouse	up-regulated in tumours	Strathmann, J (2007)
NNM	rat	up-regulated after 3 weeks	Fella, K (2005)
NNM	rat	down-regulated in neopl. nodules/carcinoma	Fella, K (2005)
Fructose-1,6-bisphosphatase: up-regulated in 2 studies, down-regulated in 3 studies			
clofibrate	rat	up-regulated after 7 days	Léonard, JF (2006)
DEN + PCB153	mouse	down-regulated in tumours	Strathmann, J (2007)
NNM	rat	down-regulated after 3 weeks	Glückmann, M (2007)
NNM	rat	up-/down-regulated in neopl. nod./carcinoma	Fella, K (2005)
Actin beta-subunit: down-regulated in 1 study, up-regulated in 2 studies			
Wy14,643	mouse	down-regulated after 2 weeks	Giometti, CS (1998)
clofibrate	rat	up-regulated after 7 days	Léonard, JF (2006)
DEN + PCB77	mouse	up-regulated in tumours	Strathmann, J (2007)

Succinyl-CoA ligase: down-regulated in 1 study, up-regulated in 2 studies			
NNM	rat	down-regulated in neopl. nodules/carcinoma	Fella, K (2005)
clofibrate	rat	up-regulated after 7 days	Léonard, JF (2006)
NNM	rat	up-regulated after 3 weeks	Glückmann, M (2007)
Ubiquinol cytochrome c reductase: down-regulated in 2 studies, up-regulated in 1 study			
Wy14,643	mouse	down-regulated after 2 weeks	Giometti, CS (1998)
NNM	rat	down-regulated after 3 weeks	Fella, K (2005)
clofibrate	rat	up-regulated after 7 days	Léonrad, JF (2006)

Strathman et al. (2007) indicated that Rho-GDI-dissociation inhibitor was up-regulated in tumours whereas the study published by Fella et al. (2005) revealed, that this protein was down-regulated in neoplastic nodules and carcinoma.

To summarize, due to the application of chemical carcinogens proteins are either up- or down-regulated. On the one hand proteins are deregulated consistently and on the other hand proteins show inconsistence in their deregulation in different studies. Nevertheless, the survey on proteomics research with chemical carcinogenic substances provides an evidence of re-occurring protein patterns in studies of different authors although no validated data is available.

The usefulness of proteomics to identify toxicity-related protein expression profiles has been demonstrated in numerous studies focused on liver as a major target of general toxicity. The paper of Oberemm et al. (2005) is an example showing that 2-DE based proteomics can be applied for the detection and application of low-dose effects of toxic substances. The study demonstrates changes of protein expression in liver and thymus of marmosets following the application of a single dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) although no significant findings in pathology and histopathology were detected. Proteins were resolved by 2D gel electrophoresis and analyzed on the basis of fluorescence labeling. Selected spots were excised, proteins digested and peptides subjected to mass spectrometry for protein identification. In the liver and in the thymus of treated animals, protein expression was altered significantly. In the thymus, 13 up-regulated proteins were identified, among others, proteins that are known as toxicologically relevant factors like chaperones, glycerol-3-phosphate dehydrogenase and adseverin. Otherwise, 6 proteins were down-regulated including vimentin, Ca-dependent protease and protein disulfide isomerase. In the liver, 8 proteins were up-regulated (Lamin A, voltage-dependent anion selective channel 1, hsp70) and, moreover, 7 down-regulated proteins were identified, for example, thymidine phosphorylase. A

comparison of the deregulated proteins in the liver as well as in the thymus showed a pattern of related functions. The found marker proteins fit well into the existing knowledge of the toxic processes and mechanisms mediated by TCDD. Moreover, new proteins of known function have been identified which may play a role in TCDD-response.

3.3 Assessment of the potential of proteomics to add value to cancer risk assessment

People are exposed to diverse chemical and physical agents in the environment, some of which may adversely affect human health. To assess the toxicological effects of such substances on living systems efficient and accurate approaches are necessary. Due to the complexity of human and animal physiology and individual variations no single approach exists to analyze the responses to toxicologic factors. For the majority of risk factors no or little epidemiological data is available. Traditionally, toxicologists have defined the risk of a new compound to human safety using animal studies together with histopathological and biochemical techniques. A number of recently developed molecular approaches that include studying gene and protein activity and other biological processes have led to a new sub-discipline of toxicology, toxicogenomics, that could aid to characterize toxic substances and their potential risk. A major tenet of toxicogenomics is that signature patterns and biomarkers of gene/protein expression exist that are associated with health, disease or toxicity. Concerning risk assessment a biomarker is a recognizable sign of changes in the expression level of proteins that is correlated with a certain risk of disease like cancer.

The contribution of proteomics in cancer risk assessment boils down to the establishment of biomarkers that indicate a possible risk. In the past years proteomic approaches have been successfully employed in the development of potential biomarkers of human diseases. As the overlap of genes and their corresponding proteins in transcriptome and proteome analysis is quite small, the complementary use of genomic and proteomic approaches in the identification of biomarkers that reflect toxicant exposure is considered indispensable. Changes in transcription rates do not necessarily correlate with protein expression or protein activity. The latter, e. g., can be determined by post-translational modifications. Proteins can be regulated in response to a carcinogen treatment but corresponding changes in the mRNA profile may not occur. Moreover, for almost all types of cancer, it seems as if the transition from a normal, healthy cell to a cancer cell is a step-wise progression that requires genetic changes in several different oncogenes and tumour suppressors. Mutations in key regulatory genes alter the behaviour of cells and can potentially induce the unregulated growth seen in cancer. However, mutations do not necessarily lead to the formation of cancer. When damage

occurs to the DNA a number of repair processes are started in order to prevent mutations. Although mutations might not take place due to an efficient repair process, changes in the expression of proteins involved in repair might be detectable. Changes in the expression patterns of proteins, e.g. representing DNA repair processes, indicate a potential risk. Therefore, if compared to the conventional development of biomarkers on the basis of transcriptome analysis, proteomics holds promise for the identification of additional biomarkers. Proteomics approaches might easily identify biomarker candidates that could be validated in further experiments, e. g. comprehensive microarray studies. The latter are expensive and time-consuming, thus proteomics can make a substantial contribution in advance.

An additional proteomic approach to biomarker development presents several advantages over the single use of genomics methods, including the important issue of improved sample accessibility. A particular advantage of proteomics is, that numerous proteins are in many times secreted into body fluids in response to a particular physiological state. The deranged signalling pathways in cancer extend the tumour-host interface by generating cascades of enzymatic cleavage, shedding and sharing of growth factors that could be a source of low molecular weight biomarkers. Protein or protein fragments produced by cancer cells or their microenvironment can diffuse into the circulation during tumour development and progression, enabling early disease detection and risk assessment. As protein biomarkers are available from body fluids, like serum, urine or saliva samples can be obtained by non-invasive methods. A potentially more hazardous and expensive collection by biopsy is thus dispensable.

Usually, substantial data for the assessment of the preliminary risk of a new compound is obtained from 2-year cancer bioassays in rodents. A drawback of this approach is that the assessment of a cancer risk to humans of a low (environmental) dose is based on data from high-dose experiments. The extrapolation from tumour data obtained at relative high doses to low (environmental) doses requires the use of surrogate biomarkers of tumours. The highly sensitive mass spectrometry techniques in combination with the global measurements used in proteomics can potentially detect toxic effects at lower doses than conventional methods used in risk assessment. The ability of proteomics to identify biomarkers indicating an early stage of carcinogenesis points out another benefit proteomic approaches can add to cancer risk assessment, since in the analysis of tumours, the sensitivity is not sufficient to detect small increases over background. Moreover, in this regard, proteomic approaches may contribute to the reduction of animal experiments.

Regardless of several positive aspects proteomics can add to cancer risk assessment there are also some limitations that come along with proteomic approaches. In spite of progressive improvements in mass spectrometry and peptide separation techniques, the identification and quantification of all the proteins in a biological system is still an impracticable technical challenge. Every proteomic approach surveys only a small subset of the proteins actually occurring in a sample. Moreover, the amount of proteins that can be quantified is even smaller because for quantification more comprehensive data than for protein identification is required. Figure 1 is a schematic representation of the protein fraction that can be identified and quantified by mass spectrometry based proteomics indicating that numerous potential biomarkers are not detectable up to now.

The specific challenges for quantitative proteomics still remain, claiming further improvements of the mass spectrometry instrument performance with respect to sensitivity.

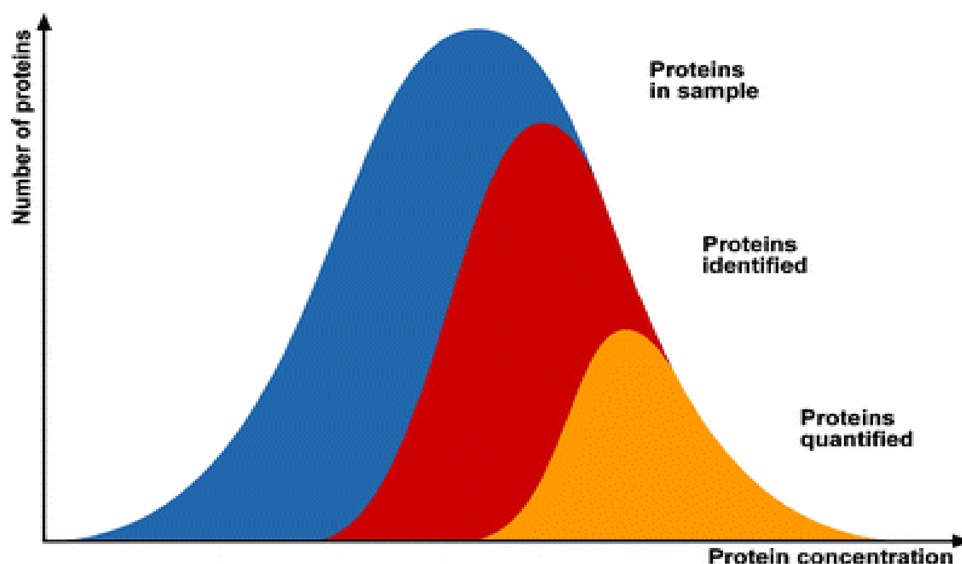

Figure 1: Protein fractions that can be identified and quantified by mass spectrometry based proteomics. In: Bantscheff et al. (2007)

For the quantification of potential biomarkers that are low-abundant, the dynamic range has to be increased at least 100-fold. An increase in specificity could be realized by doing a more extensive sample fractionation what is, on the other hand, incompatible with high throughput screening approaches.

Another drawback of proteomic approaches is the comparability of the data. As explained earlier, a proteome is only a subset of the entire protein pool under precisely defined conditions. Thus, the smallest experimental change can result in a different protein expression pattern. Consequently, to get reliable and reproducible results the conditions an experiment is performed at have to be identical all the time.

As our survey shows, a large collection of data is necessary to determine protein profiles of predictive value. Additionally, a correlation of the risk factor and a change in the expression level is not necessarily given. Consequently, the interpretation of the cause and consequences and the biological meaning of changes in the protein composition is a challenge.

Proteomics is a rapidly developing and promising technology, that could provide a lot of benefits to cancer risk assessment. Automated image analysis and powerful mass spectrometry tools allow to detect changes of protein expression over a growing dynamic range. It should be possible to screen toxic effects more rapidly with newer proteomic methods than with conventional methods. Our survey on proteomics in research with carcinogenic substances demonstrates re-occurring patterns in the deregulation of proteins in independent studies. Moreover, the carcinogenic potential of a new compound can be assessed by comparing effects on the proteome against a panel of known toxins that are already analyzed and categorized. Biomarkers reflecting positive correlations could serve as an early warning of toxicity for further studies. The example of the study of Oberemm et al. (2005) about the effects of a TCDD application on the protein expression in liver and thymus showed that marker proteins could be found that are consistent with known toxic processes and mechanisms mediated by TCDD. These data underline that proteomics could play a role in predictive toxicology to establish relationships between toxic effects and protein markers. However, the value proteomics can add to cancer risk assessment is highly dependent on the accuracy and reliableness of the established biomarkers.

For the establishment of biomarkers previously unrecognized compounds and/or associations that prove to be both specific and sensitive for disease have to be identified from the thousands of peptides and proteins present in a biological sample. Numerous experiments have been performed to identify proteomic changes that occur in cancerous cells. It is possible that some of these proteins could be used as markers for an early indication of tumourigenesis. The establishment of a biomarker is a comprehensive and complex process including several steps from the discovery of a putative biomarker to the development, validation and application of a well-established biomarker. Initially, exploratory studies for the identification of potentially useful biomarkers are required. Comprehensive and precise

analysis of biological samples are performed with the aim to identify the largest possible set of proteins that appear to be deregulated in tumour tissue relative to control tissue. Once a putative biomarker has been identified, carefully designed validation studies are necessary. Critical issues that need to be addressed for the validation studies include the specificity and reproducibility of the marker. The development of a clinical assay that is usually based on a sample that can be obtained non-invasively e. g. from blood. The procurement of organ tissue is problematic because multiple tissue sampling is not always feasible. The validation of biomarkers comprises additional measurements and compound identification. An immunoassay analyzing the response to a protein uniquely expressed in tumour specimen and measured with antibodies would be an example of such an assay. Finally, biomarkers are studied to determine their capacity for distinguishing between people with cancer and those without.

As current clinical and pathological markers poorly predict early disease development and response to treatment standard diagnostic methods shift rapidly toward molecular methods due to the rapid progress in proteomic instrumentation

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4 Assessment of the potential of proteomics to add value to EMF cancer risk assessment

Chapter 2.3 illustrates that proteomics can add value to investigate the effects of carcinogenic agents. Therefore it is tempting to presume that proteomics can add value to EMF cancer risk assessment provided that sound biomarkers relating to EMF have been developed. However, for the establishment of specific biomarkers comprehensive data have to be considered. A literature search concerning electromagnetic fields and proteomics demonstrates that a limited number of studies have been published in this field up to now. As shown in Table 6 a total of 10 studies were found, implying 5 on RF-EMF and 5 on ELF-EMF, respectively. Zeng et al. (2006), Sinclair et al. (2006) and Nakasano et al. (2000, 2003) found no convincing evidence that exposure to electromagnetic fields produces distinct effects on the expression of proteins. Li et al. (2005) identified three proteins whose expression changed significantly and reproducibly following the exposure to ELF-EMF as RNA binding protein regulatory subunit, proteasome subunit beta type 7 precursor and translationally controlled tumour protein.
Concerning the study of Pipkin et al. (1999) hsp27 and hsp90 were significantly increased due to ELF-EMF exposure. The paper of Leszczynski et al. (2002) reports an increase in phosphorylation of hsp27 together with changes in the protein expression levels of p38MAPK. Nylund et al. (2004) identified four proteins (two isoforms of vimentin, isocitrate dehydrogenase 3(NAD^+)α, heterogeneous nuclear ribonucleoprotein H1) that significantly changed their expression level following exposure to RF-EMF. In a second study by Nylund et al. published in 2006 the effects of RF-EMF on the protein expression in two closely related human endothelial lines were examined. The comparison revealed that the protein expression profiles in both cell lines were different. However, in this paper no protein list of the comparison is given.
Taken together, only 4 out of 10 studies published on EMF and proteomics actually provide identified proteins. A direct comparison of these studies is exceedingly difficult as the experimental conditions were completely different. Except for the studies of Leszczynski et al. (2002) and Nylund et al. (2004), belonging to the same group, different cell systems and exposure conditions were used.
To summarize, only a few investigations using proteomic approaches have been done to estimate the influence of EMF exposure to biological systems so far. Therefore, comprehensive data is not available and at least for now, a determination of biomarkers is unfeasible. The establishment of specific biomarkers postulates the existence of a defined

effect or health risk of EMF exposure. The major requirement for integrating proteomics in EMF cancer risk assessment is a mode of action on the protein level. However, until now, it was not possible to determine such mode of action. As long as no compelling evidence for a mode of action of EMF on the protein level has been determined, the application of proteomic approaches in EMF cancer risk assessment is difficult. Nevertheless, proteomics could add value to EMF research by disclosing the complexities of processes that are initiated following exposure to EMF.

Although the use of proteomics in EMF research is still in its infancy, its application in EMF cancer risk assessment should be discussed under the assumption that the limitations that come along with proteomic approaches will be addressed in the near future and sound biomarkers can be developed. The evaluation of new frequencies is an application proteomics could add value in terms of cancer risk assessment. As no linear correlation between field strength and increase of temperature exists, the evaluation of new frequencies based on energy is difficult. The comparison of characteristic proteome profiles might provide additional information on how new frequencies should be assessed. One could examine whether ranges of frequencies can be grouped for their ability to produce distinct protein patterns in proteomic approaches. In case these patterns include biomarkers that indicate a potential health risk, a new frequency that displays a certain pattern should be dealt with caution.

Table 6: Summary of studies on the effects of EMF exposure using proteomic approaches sorted by the field frequency

Study	Field frequence	Type of sample	Findings
Karinen, A (2008)	RF-EMF (900 MHz)	Biopsoy of small area of forearm's skin of female volunteers	no list of identified proteins available
Leszczynski, D (2002)	RF-EMF (900 MHz)	EA.hy926 (human endothelial cell line)	increase in phosphorylation of hsp27 and p38MAPK
Nylund, R (2004)	RF-EMF (900 MHz)	EA.hy926 (human endothelial cell line)	Increase of 2 isoforms of vimentin, decrease of isocitrate dehydrogenase and heterogeneous nuclear ribonucleoprotein H1
Nylund, R (2006)	RF-EMF (900 MHz)	EA.hy926, EA.hy926n1 (human endothelial cell lines)	no list of identified proteins available
Zeng, Q (2006)	RF-EMF (1800 MHz)	MCF-7 (human breast cancer cell line)	no convincing evidence that RF-EMF exposure can produce distinct effects on protein expression
Li, H (2005)	ELF-EMF (50 Hz)	MCF-7 (human breast cancer cell line)	increase of RNA binding protein regulatory subunit, decrease of proteasome subunit beta type 7 precursor and translationally controlled tumour protein
Nakasono, S (2000)	ELF-EMF (50 Hz)	*Saccharomyces cerevisiae*	no reproducible changes
Nakasono, S (2003)	ELF-EMF (5-100 Hz)	*E.coli*	no reproducible changes
Pipkin, JL (1999)	ELF-EMF (60 Hz)	HL-60 (human leukaemia cells)	increase of hsp27 and hsp90
Sinclair, J (2006)	ELF-EMF (50 Hz)	*Schizosaccharomyces pombe* (s Sty1p deletion mutant)	no convincing evidence that ELF-EMF can produce distinct effects on protein expression

5 Assessment of the potential of proteomics to close knowledge gaps

As in the course of WP1 of the IMBA project knowledge gaps existing in EMF cancer risk assessment have been defined, the question arises if proteomics could contribute to close some of these gaps.

Proteomics could contribute to close number 5 and 8 of the knowledge gaps in genotoxicity studies regarding RF-EMF cancer risk assessment under the assumption that sound biomarkers indicating effects of EMF exposure have been established. Knowledge gap 5 points out that controversial results on genotoxic action of RF-EMF might be a consequence of differences in the used cell types. Proteomic profiling may provide additional data that is in conformity with findings on the DNA level. One might be able to decide whether different cell types differ in their sensitivity to RF-RMF. In toxicology uncertainties exist if RF-EMF and chemical mutagens like mitomycin C act synergistically (knowledge gap 8). Proteomic approaches on the basis of available biomarkers could contribute to decide if the simultaneous application of RF-EMF and chemical mutagens cause synergistic effects in comparison to their single use.

Proteomics could reduce uncertainties in animal studies regarding RF-EMF cancer risk assessment that are pointed out in knowledge gap 8 and 9. These knowledge gaps allude to the question how to extrapolate toxicity findings from one frequency to another as well as from one type of signal modulation to another, because different mechanisms of action might be involved. Once well-founded protein profiles characterizing effects of EMF exposure are established, they might lead to the disclosure of protein patterns caused by different frequencies or signal modulations. As already mentioned in chapter 3 one could examine whether ranges of frequencies or different types of signal modulation can be grouped for their ability to produce distinct protein patterns in proteomic approaches. In case these patterns include biomarkers that indicate a potential health risk, a new frequency that displays a certain pattern should be dealt with caution.

Proteomic approaches could contribute to the development and application of a guideline that outlines the minimum toxicity testing required before an exposure to a given EMF source can be considered as safe, as it is demanded with respect to knowledge gap 14. Different EMF frequencies used in mobile phone communication require specific toxicity testing because different mechanisms of action might be involved. An extrapolation of toxicity findings from one frequency range to another is not possible. Once an EMF source is considered to be safe, the protein expression profile representing aspects of the corresponding mode of action could be used as a guideline. This guideline should serve to classify EMF devices the possible

hazard of which has not been determined so far. If it were possible to get consistent protein patterns, the corresponding exposure setups could be included in “minimum toxicity testing” of new EMF devices. Even more, new frequencies and signal modulations could be assessed.

It is assumed that a lack of plausible mode of action (MOA) of RF-EMF with living systems might limit the design of sound carcinogenicity studies (knowledge gap 4). Dr. Jochen Buschmann suggests in “knowledge gaps in animal studies regarding RF-EMF cancer risk assessment” that knowledge about the precise MOA of RF-EMF benefits the design of animal studies that enable the addressing of specific cancer endpoints, especially for the design of mechanistic studies. This applies to proteomic approaches as well. The major requirement for integrating proteomics in EMF cancer risk assessment is a mode of action on the protein level. A reasonable development of biomarkers is based on reproducible protein profiles that can be directly correlated with EMF exposure. However, as long as no compelling evidence for a mode of action of RF-EMF on the protein level has been determined, the application of proteomic approaches in EMF cancer risk assessment is questionable.

6 Future prospects and conclusions

Proteomics is a powerful and promising technique with the potential to add considerable value to cancer risk assessment. The usefulness of proteomics to identify toxicity-related protein expression profiles has been demonstrated in numerous studies focused on liver as a major organ of general toxicity as our survey on –omics in research with chemical carcinogens showed. In complementary use in toxicogenomics together with genomics, transcriptomics and metabolomics, proteomics can provide additional information that are characteristic for this technique. Although the interpretation of the causes and consequences of changes is challenging in proteomics, the analysis of the protein expression by proteomic approaches directly points out the effect where it takes place.

Above all, proteomic approaches can add value to biomarker discovery as proteomics addresses alternative slicing and post-translational modifications, which are seminal events in complex biological processes associated with cancer. Blood is the most-used specimen for monitoring biomarkers because its procurement is non-invasively. Many organs secret proteins into the bloodstream constituting a molecular fingerprint that reflects a specific physiological state. However, current proteomics technologies have their limitations concerning the identification of low-abundance biomarkers in the blood proteome. Blood proteins exhibit an extra-ordinary dynamic range in their abundance, from 40 mg/ml (albumin) to 5 pg/ml (cytokines). Given that only 22 proteins account for 99% of the blood protein content, the discovery of low abundant biomarkers is a challenge. Due to the anticipated low abundance of interesting biomarkers, efficient strategies have to be considered. If biomarkers are to be found in the bloodstream the development of additional separation and enrichment technologies is required to reach low abundance components. An emerging method for biomarker discovery in plasma proteomes is to use a combination of 'peptidomics' and 'differential mass spectrometry' to overcome the protein abundance dynamic range issue associated with complex proteomes. This technology helps to enrich for the low-molecular weight protein/peptide fragments (< 20 K) and determines changes in peptides abundances based on alignments of mass spectra.

Another strategy is to carry out the initial search for biomarkers on a complete cellular extract in a cellular model of the disease of interest, e.g. cancer cell lines have been used as surrogates defining potential biomarkers. When a differentially expressed protein has been identified, its presence is searched in blood. It is checked to be correlated with the disease of interest.

It has been hypothesized that concentrations of potential biomarkers are highest in the tumour and its immediate microenvironment. Other specimen such as tumour biopsy tissues are currently considered as alternative sources for biomarker discovery. In order to reduce microheterogeneity within tumour tissues, manual microdissection or laser capture microdissection (LCM) can be used for selecting tumour cells. In combination with the two-dimensional fluorescence difference gel electrophoresis (DIGE) saturation labelling technique LCM can be applied for standard 2-DE-based proteomics profiling. Another alternative would be a direct protein profiling of the tumour microenvironment tissue interstitial fluid.

However, the difficulties to determine cancer biomarkers is not necessarily due to the lack of proteomics-based candidate biomarker discovery as proteomics has yielded plenty of candidates. The major limitation is to connect biomarker discovery with the necessary verification, assay optimization, validation and commercialization. For sensitive clinical blood tests like enzyme-linked immunoabsorbent assays (ELISA) or flow cytometry-based assays antibodies need to be produced which is time-consuming and expensive for a large range of candidate proteins (i. e. several hundred). Newer orthogonal MS-based clinical assays (multiple-reaction-monitoring, MRM-MS) could target a number of candidate biomarkers simultaneously.

The establishment of reliable and sound biomarkers is crucial in cancer risk assessment. This implies a defined health risk as well as a precise correlation of the risk factor and a change in the expression level of proteins. Quantitative risk assessment using proteomics requires knowledge of the key events leading to tumour induction. With respect to EMF the establishment of biomarkers is challenging because no compelling evidence for a mode of action of EMF on the protein level has been discovered up to now. This might be one reason why in many current EMF/proteomics studies no reproducible changes of protein patterns or convincing evidence that EMF can produce distinct effects on the protein expression have been found.

Proteomics has the potential to add value to EMF cancer risk assessment and could contribute to close some of the knowledge gaps defined in WP1 provided that sound biomarkers indicating an actual effect of EMF exposure on the protein level can be established and the limitations that come along with proteomic approaches will be addressed in the near future.

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8 Appendix

Table 7: Summary of the sources used for the survey on –omics in research with carcinogenic agents

Database	contents	provided by	link
German National Library of Medicine – ZB Med	e-journals and databases (ordered by subjects)	Deutsche Zentralbibliothek für Medizin, Cologne	www.zbmed.de
German Institute of Medical Documentation and Information – DIMDI	databases (ordered by subjects)		www.dimdi.de
Scirus	science-specific search engine that searches over 450 million science-specific webpages	Elsevier, Amsterdam	www.scirus.com
Google scholar	allows to broadly search through: peer-reviewed papers, theses, books, abstracts and articles, from academic publishers, professional societies, preprint repositories, universities and other scholarly organizations		www.scholar.google.com
TOXNET	databases on toxicology, hazardous chemicals, environmental health, and toxic releases	U.S. National Library of Medicine	www.toxnet.nlm.nih.gov
forschungsportal.net	provides material of servers of government-funded science organizations, about 20,000 web servers are currently included	Federal Ministry for Education and Research	www.forschungsportal.net
GEPRIS	is the information system on DFG (German Research Foundation) projects, contains all projects funded by the DFG	Federal Ministry for Education and Research	
Chemical Effects in Biological Systems database – CEBS	is an integrated public repository for toxicogenomics data, including the study design and timeline, clinical chemistry and histopathology findings and microarray and proteomics data	National Institute of Environmental Health Sciences – NIEHS	http://cebs.niehs.nih.gov

medpilot	a medical information portal that enables fast, user-oriented, cross-database access to specialised medical information	Deutsche Zentralbibliothek für Medizin, Cologne	www.medpilot.de
www.vascoda.de	German internet-portal that provides access to scientific information		www.vascoda.net
BioMed Central	is an independent publishing house committed to providing immediate open access to peer-reviewed biomedical research		www.biomedcentral.com
World Health Organization Library Information System – WHOLIS	indexes all WHO publications from 1948 onwards and articles from WHO-produced journals and technical documents from 1985 to the present	WHO	www.who.int/library/databases/en/
PubMed	includes over 16 million biomedical journal abstracts back to the 1950s	U.S. National Library of Medicine	www.ncbi.nlm.nih.gov

Proteomics-platforms	**contents**	**link**
Human Proteome Organization – HUPO	an international scientific organization representing and promoting proteomics through international cooperation and collaborations	www.hupo.org
German Society for Proteome Research – DGPF	a platform for bringing proteomics activities in Germany at different locations below one roof, for promoting proteome research by national and international initiatives	www.dgpf.com
Human Metabolome Project – hmp	the purpose of the project is to facilitate metabolomics research through several objectives to improve disease identification, prognosis and monitoring, it provides inside into drug metabolism and toxicology	www.hmdb.ca
proteomicssurf	provides web information on proteomics technologies and applied proteomics	www.proteomicssurf.com
proteinscience	serves as an international forum for publishing original reports on proteins	www.proteinscience.com
proteomecommons	a public repository for digital content relating to proteomics, and a foundation for building a community around such content, public access to free, open-source proteomics-related projects both new and old	www.proteomecommons.org

German public authorities/institutions	link
Federal Institute for Risk Assessment	www.bfr.bund.de
National Research Centre for Environment and Health	www.gsf.de
Ministry of Health	www.bmg.bund.de
Federal Institute for Drugs and Medical Devices	www.bfarm.de
The Federal Office of Consumer Protection and Food Safety	www.bvl.bund.de
Federal Centre for Health Education	www.bzga.de
Federal Ministry for Education and Research	www.bmbf.de

International public authorities/institutions	task	link
European Chemicals Bureau – ECB	development and harmonisation of testing methods, classification and labelling of hazardous chemicals, coordination of the EU risk assessment reports on priority chemicals	http://ecb.jrc.it
International Agency for Research on Cancer – IARC	coordinates and conducts research on the causes of human cancer, the mechanisms of carcinogenesis, and develops scientific strategies for cancer control, is involved in laboratory research and disseminates scientific information through publications, meetings, courses, and fellowships	www.iarc.fr
National Cancer Institute – NCI	conducts and supports research, training and health information dissemination with respect to the cause, diagnosis, prevention, and treatment of cancer	www.cancer.gov
National Toxicology Program – NTP	evaluates agents of public health concern by developing and applying tools of modern toxicology and molecular biology	http://ntp.niehs.nih.gov
U.S. Food and Drug Administration – FDA	assures the safety, efficacy, and security of drugs, biological products, medical devices, nation’s food supply, cosmetics, and products that emit radiation	www.fda.gov
National Institute of Environmental Health Sciences – NIEHS	reduces the burden of environmentally associated disease and dysfunction by defining, how environmental exposures affect our health, how individuals differ in their susceptibility to these exposures and how these susceptibilities change over time	www.niehs.nih.gov

Table 8: Selection of chemical carcinogens inducing tumours in either liver or brain that were

Liver*	**Brain****
1,4-Dichlorobenzene 2-Acetylaminoflouren (2-AAF) 3’-methyl-4-dimethyl-aminoazobenzene (3’-ME-DAB) Aflatoxin Alkylbenzene Chlorendic acid Cinnamyl anthranilate Ciprofibrate (PP) Clofibrate (CF) (PP) Diethylhexyl phthalate Ethylene thiourea IQ (2-amino-3-methylimidazo[4,5-]quinoline) MeIQx (2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline) Nafenopin (NAF) (non-genotoxic) N-methyl-N-nitrosourea (MNU) (genotoxic) Diethylnitrosamine (DEN) (genotoxic) N-nitrosomorpholine (NNM) (genotoxic) N-nitrosopyrrolidine Tamoxifen Trichloroethylene Oxazepam (non-genotoxic) Phenobarbital (PB) Trichloroethylene Vinyl chloride Wy-14,643 (non-genotoxic) (PP)	1,3-Propane Sultone (Glioma) 3,3-Dimethyl-1-Phenyltriazene Acrylonitrile (Ear) Aflatoxin (Olfactory bulb) Azoxyethane (Olfactory bulb) Cyclophosphamide (Periphal neurogenic sacromas) Dacarbazine (Ependymoma) Dimethyl sulfate Ethylene oxide (Glioma) Glycidol (Glioma) Lead subacetate (Glioma) N,N’-Dimethyl-N-Nitrosourea N-ethyl-N-nitrosourea (NEU) (Glioma) N-ethyl-N‘-(2-hydroxyethyl)-N-nitrosourea (Tumor) N-methyl-N-nitrosourea (MNU) (Glioma) Procarbazine Hydrochloride (Olfactory neuroblastomas) Propyleneimine (Glioma) Vinyl chloride (Neuroblastoma)

included in the literature search for studies on proteomics and carcinogenic agents

* In: Gold, LS *et. al.* (2001): Compendium of Chemical Carcinogens by Target Organ: Results of Chronic Bioassays in Rats, Mice, Hamsters, Dogs, and Monkeys. *Toxicologic Pathology*, 26(6), 639-652.

** In: CCRIS (Chemical Carcinogenesis Research Information System), a database sponsored by the National Cancer Institute (NCI) containing data and information on carcinogens, mutagens and tumor promotors. Sources include journals, books, research reports, and governmental reports (USA). Contains critically reviewed tests and fully referenced results on the carcinogenic, tumor promoting, and mutagenic activity of chemical substances in man, animals, and various test systems.