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1. INTRODUCTION

Food handling has evolved over the years from a short line of trading between producer and consumer to a complex chain involving several different parties. Today, food consumption includes large-scale production, time-efficient handling, transport and packaging of food. Before the final products reach the consumer, many persons, different handling procedures and industrial processes have been involved, which may alter food composition and quality. Food consumption is a global issue and so are the adverse health effects coupled to food consumption. Depending on the quality of dietary products, this could be a matter of improving human health on a global scale.

The importance of a well-composed diet has been known for decades. In recent years, novel functional food has been introduced as a step to improve the health of consumers and well-being and the market is bursting with lean, green and supplementary food products.

However, what about the effects of unwanted chemicals present in food? What risk do they pose to consumers? The manner in which food is handled today inevitably leads to the presence of non-food related compounds in the diet. Even though the levels of exposure are generally low, it is important to remember that exposure of consumers to these compounds persists for a long time and therefore any effects on health may appear late in life.

Chemical residues from different stages in the production are present in the ultimate product and can potentially affect the health of consumers. For the general consumer, food represents a major route of exposure to a broad variety of chemical residues and environmental pollutants.
In addition, recent estimates suggest that over 80% of total body burden of chemical contaminants will occur during the first 5 years of life, clearly demonstrating that dietary exposure affects consumers from the earliest stages of life and onwards. This is a distressing scenario as exposure to chemical contaminants may affect humans at all stages during development. Since most environmental chemicals persist and accumulate in the body for long time periods, the effects may be delayed and not become apparent until later stages in life. This could potentially mean that exposure during early age to different chemical residues in food may predispose them to disease in adulthood.

1.1 Health impacts of diet-derived chemicals

Many studies have coupled food consumption to different types of diseases or, in some cases, to protection from different diseases. For example, Asian populations seem to be protected from certain types of tumor diseases due to a high intake of soy products. The protective effect against tumors has been attributed to the high content of hormonally active phytoestrogens present in soy. Thus, it seems likely that the general health status of a population can be influenced through diet. There are also cases of the opposite, where dietary intake of certain compounds results in health hazards. Currently, few scientific studies have attempted to link chemical contaminants in food to human diseases. However, the scientific challenge to identify these effects is extreme. In essence, food is a highly complex mixture of a large number of compounds and the interplay between these compounds is difficult to characterize. Another difficulty lies in discriminating the effects stemming from natural food components from those of man-made compounds and to understand how this complex interplay will affect human health. Importantly, it has been suggested in epidemiological studies that different disease conditions, such as several types of cancer, can be coupled to food consumption patterns and therefore possibly to the presence of chemical contaminants in food.

1.2 Societal and research needs in the area of food and chemical contaminants

The presence of chemical contaminants in food, and the lack of sufficient scientific evidence regarding the possible adverse health outcomes of this contamination, is an increasing concern among European consumers. In addition, the health implications of this exposure have given rise to concerns among different authorities regarding the future costs of disease. Food is a global commodity and all societal sectors are exposed to the compounds, thus the risk of exposure and the costs associated with disease are likely to be considerable.

National governments and the European Union have made significant investments to better understand the risks associated with exposure to chemical food contaminants. Still, a comprehensive review of the European research funding during the transition period between the 5th and 6th Framework Programme identified several structural problems associated with EU funded research projects. In general, the evaluation stated that European research projects lacked the means to provide substantial support to policy development due to their relatively small size. Other gaps that were identified demonstrated that there is little or no integration between scientific disciplines and that there is a major gap between research providers and consumers.
To address these shortcomings, the European Union decided to launch two fundamentally new types of instruments, namely Integrated Projects and Networks of Excellence.

1.3 The CASCADE Network of Excellence

The main objective of CASCADE as a Network of Excellence is to act as an integrated research network in the field of chemical contaminants in food. Research in this particular area displayed several of the shortcomings that were highlighted in the evaluation during late phases of the 5th framework programme. CASCADE was established as a multidisciplinary network of experts with a wide European identity.

The CASCADE Network of Excellence was originally composed of 19 partner universities and institutes but has been additionally expanded through the introduction of new partners. CASCADE is today a highly multidisciplinary network, featuring a wide array of scientific disciplines including risk assessment, toxicology, biochemistry, molecular biology, mouse genetics, in-silico and in-vitro methodologies and several additional research areas. In addition, from the start the

CASCADE NoE developed an extensive training and dissemination scheme. All these activities have been glued together by a robust and highly efficient management component that is designed to both administer the "day to day" activities of CASCADE but also to launch long term strategic actions to further develop and strengthen the durability of the network.

A key feature of NoEs and CASCADE is the requirement to become established in such a fashion that network activities would remain active even after the EC funding period. This requirement is thus a major difference between NoEs and other community funding instruments. In addition, Networks of Excellence are not per se research projects; their main aim is to achieve durable and sustainable integration among the partner institutes and to serve as links between research providers and research users such as authorities, industry and the general public. To meet these contractual obligations, CASCADE has developed a set of overarching objectives.

The overarching objectives of CASCADE are thus to (according to the original DOW):

- To provide a durable, structured and organised network of experts for integration with other activities in the field. This NoE is guided by the focus on nuclear receptors and their role as targets for food components and contaminants as well as implications for disease development. Therefore integration and collaboration with other European and international actors will be pursued.

- To increase the awareness of the need for multiple aspects of scientific information in quantitative risk assessment among scientists. Awareness of dose-effect and dose-response relationships in the low-dose ranges, complexity of the cellular network of genetic control in response to chemical exposure, cross-species variation in sensitivity, as well as variations in exposure situations are important examples of aspects that need to be considered. This is only feasible with an interdisciplinary approach, such as the current NoE.

- To improve interdisciplinary competence and thinking amongst scientists, in particular those who are at an early stage of their careers, in areas related to human health effects caused by chemical contaminants in the food chain. In addition, this is an attempt to harmonise European methodology.

- To provide education and information to consumer organisations and authorities, with particular attention towards the Candidate Countries, so that they can make informed decisions about the risks of exposure, put in relation to the potential benefits associated with the use of various chemicals.

- To provide novel scientific information on the mechanism of action of chemical residues and contaminants in food and to make this information useful in the development of mechanism- and disease-based *in vivo*, *in vitro* and *in silico* test methods, and in the risk assessment and benefit analysis.

2. RESULTS OF CASCADE

2.1 Background

The major goal for CASCADE is to support consumers to choose safer food. In order to achieve this goal, several objectives were instigated. First, the network would provide new scientific information about endocrine disruptive chemicals. This information would be used to develop new *in vivo*, *in vitro* and *in silico* test methods for fast and reliable recognition of Endocrine Disrupting Chemicals (EDCs) using an interdisciplinary approach. Second, to develop and improve quantitative risk assessment by integrating, all aspects of the scientific information achieved regarding EDC properties. Last, to provide European authorities and consumers with reliable information on the risks associated with EDC residues in food.
In order to meet these scientific objectives, CASCADE has established an extensive network where all joining partners' expertise and research activities are coordinated in a framework with a mutual scientific goal. The joint scientific program of CASCADE network is focused on the impact of EDCs

on intracellular signaling through members of the nuclear hormone receptor family. The scientific activities are divided into 10 different work packages:

- WP10 *Risk Assessment Integration*,
- WP11 *Risk Assessment Development*,
- WP12 *Analytical Methods for Selected Chemicals in Food Samples*,
- WP13 *In Silico Screening*,
- WP14 *Functional Screening*,
- WP15 *Nutrigenomics*,
- WP16 *Cross-species Comparisons*,
- WP17 *Mechanisms of Disease Development*,
- WP18 *Metabolism.*

During later stages of the project, a new and highly integrated WP was initiated by CASCADE where all partners and their methodologies were merged into the complex task of testing whole food items. This innovative WP (WP19, *Chemical Contaminants in Food*) introduced a novel concept in the field of food testing and food safety where molecular biology techniques, transgenic animals, metabolism, chemical detection, *in silico* techniques are merged into an integrated testing system (Fig.1).

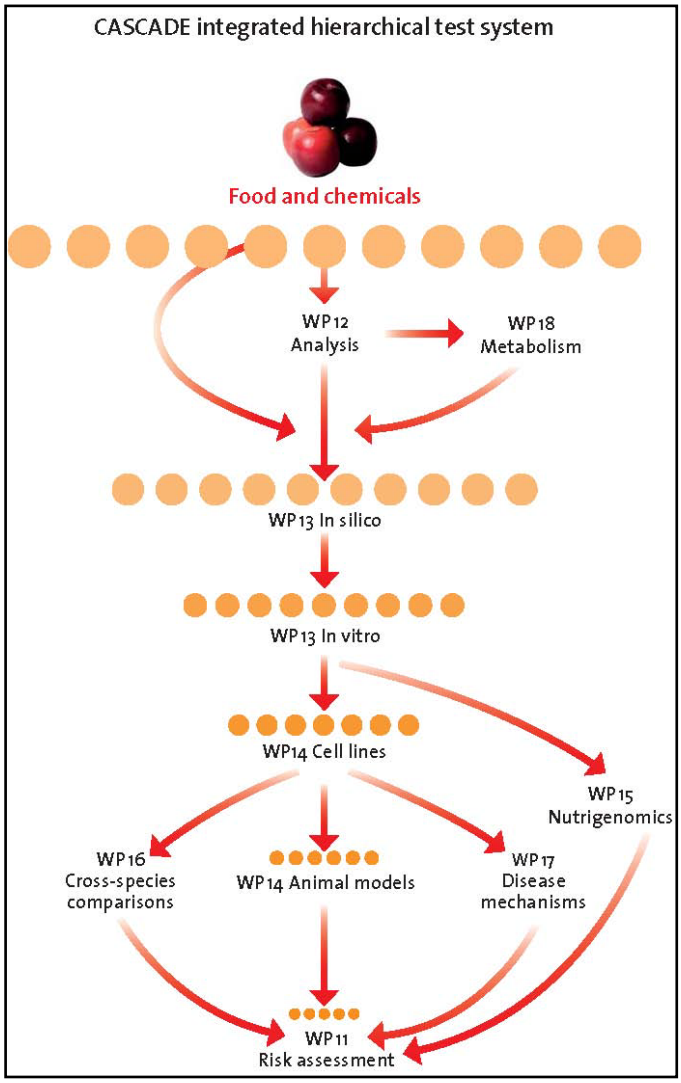


Figure 1: CASCADE Integrated hierarchal test system

2.2 Nuclear Receptors - focal points for CASCADE scientific activities

A changing environment is constantly surrounding all types of biological organisms from bacteria to eukaryotic cells. To survive, the cells need to be able to identify changes in their surroundings and react and adapt their internal cellular functions to challenges imposed on them by the environment. These challenges include changes in nutrient composition and availability, exposure to harmful or even toxic compounds or changes in temperature, to mention a few. A key factor in this adaptation process, however, is to correctly assess the conditions outside of the body and transmit this information to the inside of the cell. Cells adapt to a changing environment by altering the activity of enzymes. This alteration can be achieved either by changing the efficiency of the intracellular pool of proteins, so called allosteric control of enzymatic activity or, alternatively, by changing the total intracellular amounts of specific proteins. This latter form of adaptation is achieved mainly through regulation of gene expression, also known as transcriptional regulation. Throughout evolution, biological organisms have developed numerous different systems to identify changing environment situations and subsequently to adapt to changes. One such system of recognition is through intracellular receptors.

Receptors are small proteins present in all cells throughout the body. Their function is to bind signaling molecules that cross the outer cellular membrane and then diffuse inside the cell. As these signals enter the cell, they bind specifically to receptor proteins and form a unit. Examples of these signaling substances include steroid hormones, like estrogens, androgens and vitamins.

The nuclear receptors (NRs) represent a family of structurally related transcription factors. In mammals, 48 NRs have been identified which are involved in virtually all vital functions in the body, for example development of the fetus, reproduction, metabolism, and response to xenobiotic substances. The NRs include the family of steroid receptors, like the estrogen receptors (ERs), androgen receptor (AR), progesterone receptor (PR), glucocorticoid receptor (GR), and mineralocorticoid receptor, as well as the thyroid hormone receptors (TRs). Most environmental pollutants are able to mimic the function of the signaling hormones that trigger this group of receptors as they possess the ability to enter the cells and bind to these. The compounds form a signaling unit through binding to the receptor proteins and this unit can then influence the activity in the cell nucleus by binding to specific DNA sequences on target genes. By mimicking the actions of endogenous hormones, these compounds trigger a cellular hormone signaling pathway at a time when this pathway should not be active and thereby interfere with the normal function of the cell. This outcome is commonly referred to as endocrine disruption.

Chemicals are part of contemporary life for all members of society. A recent European survey reported that the average consumer is exposed to up to 10 000 different chemicals on a daily basis. However, the health implications of this exposure are not characterized for the vast majority of chemicals and there is a lack of knowledge of whether different chemicals are able to cause different disease states.

In mammals, three different transcription factor superfamilies are involved in the biological response to xenobiotic exposure, namely basic-helix-loop-helix/Per-ARNT-Sim (bHLH-PAS) proteins, NRs and basic leucine zipper (bZIP) proteins. In particular, selected members of the bHLH-PAS family of transcription factors are critical mediators of the biological effects exerted by environmental pollutants like, for example, dioxin. The bHLH-PAS protein arylhydrocarbon receptor (AhR), which is a ligand-dependent transcription activator, has been clearly implicated with the biological response to polycyclic aromatic hydrocarbons (PAH), like 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD, also known as Seveso dioxin).

2.3 CASCADE model compounds

The focus of CASCADE scientific activities is to characterize the health implications of exposure to chemicals acting through nuclear receptors. In addition, to synchronize and further integrate the scientific activities of the network, CASCADE partners decided to choose a limited set of model chemicals, namely:

2.3.1 Dioxin (TCDD)

TCDD and other dioxin-family members are formed as by-products in various industrial combustion processes and are widely distributed in the environment. It is well-established that humans are exposed to dioxin already from birth through breast-feeding and given the extended half-life of dioxin in humans; one has to assume that health-hazardous effects of dioxin can result from a continuous accumulation, even when overall exposure levels are considered relatively low. The dioxin family compounds are also known to affect reproduction, both on the maternal and paternal sides, to be potent cancer promoters and, at high doses, to cause the dermal condition called chloracne.

2.3.2 Bisphenol A (BPA)

BPA is a common component in plastics such as polycarbonate plastic. BPA has been around for more than 50 years. Epoxy resins containing BPA are used as coatings on the inside of almost all food and beverage cans. Because the polymerization of BPA leaves some monomers unbound, BPA molecules can be released from beverage and food containers into drink and food over time. Bisphenol A has been shown to cause early onset of puberty in girls and increases in genital abnormality in boys. Recently, BPA has been linked to diseases, like heart attack and diabetes.

2.3.3 Vinclozolin

Vinclozolin is a fungicide extensively used on fruit and vegetable farms. It is recognized as a human diet contaminant, acting as an androgen receptor (AR)-binding antagonist. Vinclozolin is interfering with lipid metabolism and/or storage and inducing reduced sperm count, decreased prostate weight and delayed puberty in test animals. Thus, it is highly plausible that vinclozolin can induce such anomalies of the reproductive tract in humans.

2.3.4 Genistein

The phyto-estrogen genistein is one of several known isoflavones found in leguminous plants. It is particularly abundant in diets containing soya or soya-derived products. Genistein binds directly to the estrogen receptors and exerts both estrogenic and anti-estrogenic effects. Genistein has attracted a lot of attention from public and medical communities because of the possible beneficial role in prevention and treatment of many disorders, such as cardiovascular diseases, osteoporosis, diabetes, menopausal symptoms, renal diseases and various cancers.

2.4 Risk Assessment Development

One objective for the risk assessment area within CASCADE was to provide reliable information on health risks associated with exposure to chemical residues in food. This has resulted in establishment of risk assessment pages on the CASCADE website. These pages contain:

1. General information about risk assessment and risk assessment methodology.
2. Drafts of summary documents describing health risk assessment status of the CASCADE model compounds TCDD, bisphenol A and vinclozolin.
3. “Fact sheets” – compact health risk assessment information about 10 selected chemicals/groups of chemicals with relevance for nuclear receptor signaling.
4. Information about MSTnet.
5. Information about RA-COURSES.

To further develop and improve exposure assessment methodology, a joint project consisting of individual national exposure data sets selected for probabilistic exposure modeling was initiated and has progressed successfully. Availability and suitability of data within the sets was investigated, probabilistic exposure analysis and modeling applied and important data gaps identified. This resulted in the start of a new breast-milk exposure project in Hungary involving a joint CASCADE PhD student, aiming at filling an identified data gap, i.e. milk consumption data on individual basis to be used in the probabilistic exposure analysis and risk assessment.

Also, a milk collection strategy was prepared between CASCADE, SAFEFOODS, the Swedish National Food Administration and subcontractor Charles University Prague (SC CUP) to be used in the Hungarian milk exposure project.

In order to develop and improve mathematical modeling of experimental data, CASCADE has contributed to the development and acceptance of the benchmark dose (BMD) method and its use in exposure- and hazard assessment. The BMD method was applied to a number of toxicological data sets on persistent organic pollutants, such as dioxins, PCBs, brominated flame-retardants and chemical mixtures.

Another objective for CASCADE was to further develop and refine methodological concepts and assumptions in risk assessment of chemicals, which act via and/or interfere with nuclear receptor signaling. CASCADE risk assessment partners has contributed to improvements of the WHO/IPCS toxic equivalency factor (TEF)-concept for dioxins and dioxin-like compounds by being part of steering/expert groups and by participation in revision of the TEF-scheme and subsequent scientific reporting. An interactive learning program on dioxin risk assessment and the TEF concept was developed and made available for CASCADE. Further, a project concerning differential tolerable daily intake (TDI) values for dioxin-like compounds in different consumer groups, as a base for risk-benefit analyses, has been completed.

2.5 Analytical Methods for Selected Chemicals in Food Samples

CASCADE also identified the need for development of innovative analytical methods based on chromatographic/mass spectrometric techniques for the four selected EDCs; dioxin, bisphenol A, vinclozolin, genistein in food and other samples, such as environmental, biota and humans. In particular, methods for the determination of

- genistein
- bisphenol A and chlorinated derivatives
- benzophenone, alkylphenols, phthalates, natural ovarian estrogens and androgens
- dioxins and polychlorinated biphenyls

have been developed for food, biological samples and environmental samples like in milk, soy products, rice prepared dishes, infant formula and babyfood, as well as analysis of other phytoestrogens derivatives (like enterolactone and enterodiol) in cow milk.

In addition, other methods have been developed, such as bioassays based on Reflectometric Interference Spectroscopy (RIfS), a label free method suitable for biosensor development. During the first years of the project, the experiments have been concentrated on development of a suitable surface chemistry for the system using two different formats: sensor surfaces based on hydrogel (aminodextrane modified transducers) for high capacity sensors and surfaces modified with polyethyleneglycol (PEG) for low non-specific binding.

The RIfS device was subsequently optimized and used to measure interactions between estrogen receptor and single, pure substances (identified by the *in silico* screening) and to perform first measurements in crude cell culture media of cell lines treated with putative EDCs. An assay for dioxin-like ligands based on RifS has also been developed using the DNA binding motifs as surface recognition elements.

2.6 In silico Screening

In silico research in medicine has the potential to speed up the rate of discovery while reducing the need for expensive lab work and clinical trials. The final goal is to reduce the need for animal testing to a minimum, by replacing them with alternative approaches. *In silico* (or virtual) screening is capable to search for an incredible amount of possibly hazardous chemicals in considerably little time. But since this screening is plainly virtual, it needs experimental data to be set up and the obtained results also have to be verified by other techniques.

In order to achieve this objective, CASCADE identified three major tasks:

1. Development of new analytical methods;
2. Combination of new analytical methods with newly developed *in silico* methods;
3. Tailoring of emerging new methods to the needs of CASCADE partners and providing these to the consortium.

To improve the knowledge about interactions between chemicals and nuclear receptors, nuclear receptor-binding assays were developed using the two isoforms of the estrogen receptors, the thyroid receptor and the arylhydrocarbon receptor as models. Further, assays based on detection of new ligands for ERalpha has been developed.

A gap has always existed between *in silico* and experimental methodologies. Typically, *in silico* models have been developed from experimental data already published but without the chances for revision. CASCADE identified two major problems dealing with obtained results from both a critical and a theoretical point of view:

1. The quality of the data used for creating the model. Normally, it is difficult to assess the quality of data obtained from bibliographical sources. There are some statistical trials, derived from data mining that provides results, but the presence of outliers in nearly every model is still a flaw in theoretical *in silico* models;
2. Difficulties to define the applicability domain of the models. This will result in uncertainties about whether screenings are real or a mathematical artefact derived from the modeling steps.

The objective was to create new analytical methods in combination with *in silico* screening methods to provide powerful and effective tools for evaluation and detection of hazardous chemicals. These methods have been developed, optimised and applied within the project to detect the presence of chemicals and to evaluate their potential to interfere with several nuclear receptors. Since the chemicals of concern are not only present in laboratory conditions, the analytical methods have been applied to samples like crude cell culture media, cell lysates, soil samples and extracts from food.

Contemporaneously with development of new experimental methods, several *in silico* methods have been developed and made available to the consortium. The most important databases of EDCs have been investigated for the possibility of building suitable prediction models for nuclear receptors. Among the nuclear receptors, models of the androgen receptor, estrogen receptor, glucocorticoid receptor, mineralcorticoid receptor, progesterone receptor and thyroid receptor have been built up. Even where no structural data was available - which is crucial for the most common *in silico* screening approaches – models have been set up. This was, for example, done for the arylhydrocarbon receptor by using the 3D-quantitative structure-activity relationship (QSAR). CASCADE further identified the need to screen for metabolites of known EDCs that can be created during detoxification processes or by microbiota within the human body. Therefore, focus was set on screening for food contaminants with unknown affinities virtually against a panel of nuclear receptors and scoring the top compounds to confirm their binding. These validation experiments demonstrated that *in silico* screening is a useful method to prioritize potential EDCs for further characterization in experimental receptor binding assays, reporter cell lines and animal models of endocrine disruption.

2.7 Functional Screening

CASCADE generated an array of model systems ranging from cells to frogs and mice where integration of reporter systems allowed studies of the spatio-temporal effects of EDC. This work was subdivided in 3 major tasks:

1. Generation of reporter cells to test EDC activity aiming to apply a systematic approach to generate a large number of cell systems representing different tissues and expressing a reporter responsive to EDCs. These systems were generated to have a reproducible and comparable way to study EDC in food extracts;
2. Develop innovative methods for generation of reporter animals and novel reporter mice to fill out the technological gap and for rapid production of reporter mice with the ubiquitous expression of the EDC-modulated reporter;

3. Construct tools for generation of reporter frogs and validate these systems for the screening of EDCs.

To meet these knowledge gaps CASCADE generated an array of stably transfected cell lines expressing NR-regulated reporters or specific hormone receptors. The reporter cell lines currently available are:

- Mammary cell ERalpha,
- Mammary cell ERbeta,
- HepG2-ERalpha,
- HepG2-ERbeta,
- ARE-luc prostate cells,
- Hela-TRbeta
- Hela-AhR
- HC11-XRE
- HC11-ERE

These cell lines have been tested with different EDC and protocols for their use have been generated. These protocols have been successfully applied to study the activity of EDCs present in food, such as cadmium, soy (genistein) and other. In addition, in collaboration with several other European programs, a major effort has been made to study ER activity in physiological settings (from embryo life to aging in both male and females) and this knowledge has provided a general vision of ER physiology, opening way for evaluation of the potential toxic effects of EDC contaminated food.

The results obtained are very important because they demonstrate the applicability of reporter animals for studies of EDCs as food contaminants. In addition, the analysis of ER activity in living animals has demonstrated that ER activity in different tissues is modulated in an oscillatory fashion. This finding may open way for better understanding of the mechanism behind tissue-specific activity of estrogens and their receptors and will be of major importance for identification of novel treatments for control of fertility and menopause. These studies will be continued in other research programs.

2.8 Nutrigenomics

CASCADE aimed to decipher the complexity in response to modulation of the activity of selected nuclear receptors, i.e. arylhydrocarbon receptor (AhR), thyroid hormone receptor (TR), estrogen receptors (ER) and androgen receptor (AR), with a specific focus on the complexity of cross-talk between these systems and discrimination between gene activation and repression by nuclear receptor modulators. This information is a requirement for designing appropriate strategies for screening of compounds and complex mixtures for selective modulation of nuclear receptor activity, as well as understanding the role of nuclear receptors in disease development.

Mainly, three organisms were subjected to analysis for the following reasons:
(i) Human cells, due to their relevance for health and disease development.
(ii) Mouse (and rat), model organisms with a broad repertoire in genetic technologies.
(iii) Zebrafish, for their importance as model organisms for human diseases.

(i) One line of investigation was based on the “cross-talk” between the Ah receptor (AhR) and the estrogen receptor (ER) system. As a first level of “cross-talk”, CASCADE could show that AhR and ER both need the obligatory dimerization partner for AhR, the ARNT protein, as a co-factor to enable proper binding to their respective DNA recognition motifs.
Evidence for a second level of “cross-talk” was established by showing that an aromatic hydrocarbon (3-methylcholanthrene, 3-MC) ligand for AhR could be metabolically converted to an estrogenic compound in liver-derived cells.

These analyses were followed by a systematic survey of alterations in gene expression profiles in liver-derived HepG2 cells upon stimulation with a presumably pure AhR activator, TCDD, a pure activator of ER, DES, and the “mixed activity” compound 3-MC.

To better understand the mechanisms by which 3-MC regulates gene expression through ERα, a search was performed for possible ERE elements that may regulate ERα-dependent gene expression in the presence of 3-MC. The MatInspector software was used collaboration with the SME Genomatix GMBH, which had developed this software. Interestingly, several 3-MC regulated genes were shown to possess possible ERE sequences that may drive ERα-dependent transcriptional activation.

Term	ID	Total	Observed	Expected	ZScore
carboxylic acid transport	GO:0046942	79	5	0.38	7.51
amino acid transport	GO:0006865	56	4	0.27	7.2
amine transport	GO:0015837	71	4	0.34	6.27
G-protein signaling, coupled to cyclic nucleotide second messenger	GO:0007187	88	4	0.43	5.51
cyclic-nucleotide-mediated signaling	GO:0019935	95	4	0.46	5.25
positive regulation of biological process	GO:0048518	1111	16	5.37	4.76
positive regulation of cellular process	GO:0048522	1008	14	4.87	4.28
negative regulation of transcription from RNA polymerase II promoter	GO:0000122	130	4	0.63	4.28
sensory perception of sound	GO:0007605	134	4	0.65	4.19
anatomical structure development	GO:0048856	1792	20	8.66	4.09
system development	GO:0048731	1555	18	7.51	4.03
nervous system development	GO:0007399	673	10	3.25	3.83

Table 1: Gene pathways affected by TCDD exposure in HepG2 cells

The complexity of responses to TCDD was investigated in a second experimental system, i.e. the AhR-dependent induction of N-myristoyltransferase 2. N-myristoyltransferase (NMT) 2 covalently attaches myristic acid residues to the aminoterminal glycine residue of specific proteins after the initiating methionin has been removed. TCDD exposure of hepatoma cells and mouse liver was established as the first system, in which the levels of NMTs are regulated in response to extracellular stimuli. Most importantly, labelling of proteins with radioactive myristic acid in cells, 2D protein separation and protein mass spectroscopy allowed the identification of NMT target proteins, whose degree of myristoylation is induced by TCDD-treatment. These proteins were the G-protein subunit GNAI2, the calcium binding protein CHP, and the 19S proteasomal subunit S4.

(i)(ii) Work in this field aimed at defining genes, whose alterations in expression could indicate exposure to hormonally active compounds, i.e. thyroid-, estrogen- and dioxin-like activity. This work was based on sophisticated cell culture models and validation studies *in vivo*.

Cultures of mouse cerebellar neurons were used for gene expression analysis as a model system that was expected to be very close to the *in vivo* situation. Microarray data obtained after T3-exposure were confirmed by Q-RT-PCR. This experiment generated an extended list of T3-responsive genes, both positively and negatively regulated, which can be used as endogenous markers of T3 signaling in cerebellum. A selection of genes (Hr, Plp1, Pfkb3, Cdh8, Klf9, Gbp4) was verified as targets in animals when T3 levels or response is changed by genetic means. This regulation does not only occur in cerebellum granular neurons, as it could be reproduced for most of these genes (except for Plp1) in a cell line (C17.2) of neural stem cells transfected with T3 receptor (TR□1). This cell system appears as very promising as it is easier to handle than primary neurons or whole animals to screen for T3-signaling disruptors and to identify the unknown mechanism of T3-mediated gene repression.

HDAC2-deficient mice have been within the scope of CASCADE work and funding in the early phase of the NoE. Our experiments demonstrate that the impact of HDAC2-deficiency as a delayed post-natal cerebellar development could depend on impaired HDAC2-dependent gene repression through the TH/TR system and suggests that chemicals that target this model may effect cerebellar development.

(ii) CASCADE has also identified additional cellular pathways affected by chemical contaminants such as TCDD. We show using protein expression analysis that the S4 non-myristoylated protein form is substantially decreased in TCDD-treated mice. These data indicate that indeed the degree of myristoylation of the S4 protein can be increased. In liver, the non-myristoylated form of S4 is

relatively high and, vice versa, the basal degree of myristoylation appears to be relatively low. Thus, the induced myristoylation can lead to a substantial reduction by about 30% in the non-myristoylated form.

Furthermore, the level of basal TCDD-independent myristoylation in different organs of the mice was analyzed. The striking result is that the degree of S4 myristoylation varies tremendously between different organs with liver and kidney being the tissues with highest levels of the non-myristoylated form and brain and colon mucosa being those with the lowest levels. Conversely, liver and kidney are expected to have low levels of myristoylated S4 protein whereas the degree of myristoylation should be high in other organs, such as brain and colon mucosa.
In summary, these data indicate that the response analysis of the endocrine disrupting compound TCDD has lead to the discovery of a regulation system that may not only be relevant for determining the cellular responses to EDCs but also to be able to differentiate regulation between different organs and possibly disease states in a non-TCDD-exposed organism.

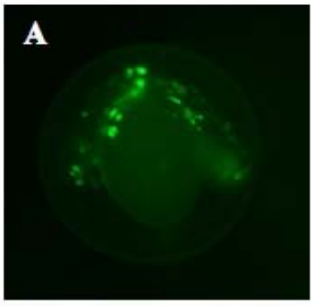
(iii) Based on the well-known estrogenic activity of bisphenol A (BPA), we tested whether BPA-induced effects on the otic vesicle could be explained through interference with ER signaling. However, the lack of any effect of E2 or tamoxifen suggests that the BPA effect on otic vesicle development is independent of the estrogenic activity. Therefore, the postulated thyroid hormone receptor repressive activity of BPA was also considered as a potential mode of BPA action on otic vesicle development. However, the treatment of zebrafish embryos with T3 did not show any indication of THR-induced impact on otic development. Co-treatment with BPA and T3 does neither show enhancement nor attenuation of the BPA effects by T3. Thus, the BPA effect on otic vesicle development indicates a novel pathway by which this CASCADE compound affects a specific developmental process in zebrafish, which is most likely independent of the well-characterised estrogenic and anti-thyroid activities of BPA.

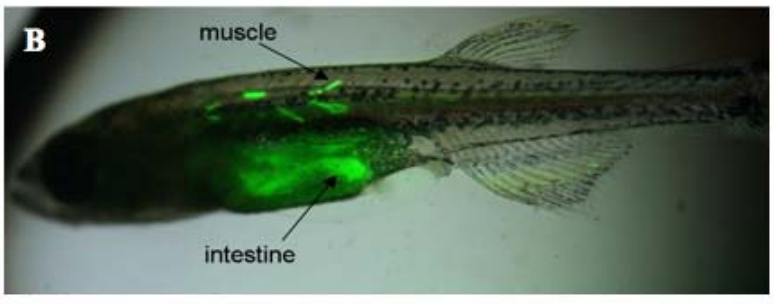
2.9 Cross-Species Comparisons

A large body of evidence suggests that chemicals can affect large numbers of species ranging from human to agronomic species, fish stock or wildlife. It is known that a given compound can have different effects in different species and this may be related to different mechanisms of action, differences in metabolism and/or differences of specificities at receptor level. In an attempt to unravel these differences, tools to study the species-specific differences and to discriminate effects based on CASCADE model compounds were developed.

Numerous different model species, fish, frog, small mammals and even reptiles, such as alligators, have been traditionally used to analyze the effects of EDCs. These various model species are used to monitor the biological effects following low dose exposure of chemicals and to understand their mode of action at both molecular and physiological levels. Given that the precise specificity or affinity of a receptor for a given compound may vary from one species to another, it is important to compare the various model systems that are used for their ability to be disrupted by specific contaminants.

The overall objectives were to develop complex approaches to study ligand/receptor interactions *in vitro* and EDC-induced effects in whole animals in terms of physiological dysfunctions by using model species. Four candidate receptors were chosen (TR, ER, AR, GR/MR), together with the four CASCADE model compounds on three different model organisms (mouse, Xenopus, zebrafish).





This task was divided into three complementary sub projects:

Sub project 1: Screening using transgenic models (frog, zebrafish, mouse).
This project involved development of similar reporter systems in the three model species, in order to compare interference between a given molecule and a NR signaling pathway in the different species. It is often difficult to compare the response obtained using several transgenic reporter systems given the variety of constructs used and the fact that insertion locus often influence the response. Our aim was, thus, to investigate the possibility of using a new technology that allows insertion of different transgenes into the same locus. In lower vertebrates, we have used a system based on the CreLox technology, which allows election of a good insertion locus that can be used, in a second step, to insert the relevant reporter construct using Recombination Mediated Target Exchange. We have validated the principle of this method and we are currently developing the relevant transgenic lines. In parallel, we have developed a transgenic zebrafish line that is used to detect and study TR endocrine disruptors.

Sub project 2: *In vitro* analysis of ligand/receptor interaction
In a second approach, we carried out an *in vitro* analysis of ligand/receptor interaction. The main goal of this sub-project was to investigate whether there are any species specificities in the interaction between EDCs and the various NRs. The methods used constituted an array of *in vitro* assays, such as transient transfection experiments, proteolysis assays and receptor/ligand- binding assays. We found that, in contrast to human, the phytoestrogen genistein does not have an ER β selectivity but instead binds and activates all zebrafish ERs in a comparable manner.

Our data suggest that the lack of selectivity in genistein activation of zebrafish ER may be explained by specific amino acid substitutions and slight changes in the relative positioning of certain residues in the zebrafish ER. In parallel, we have developed a number of cell lines allowing screening for compounds activating ERα, ERβ, AR, PR, GR, MR, TRα and AhR receptors. In addition, human PPARalpha, PPARbeta and PPARgamma cell lines were developed to characterize the impact of phthalates, flame-retardants and organotins on PPAR and RXRalpha transcriptional activity. In this study, we showed that the flame retardant tetrabromobisphenol A is a full agonist with low affinity for PPAR gamma.

Sub project 3: *In vivo* and/or *in vitro* analysis of dysfunctions observed as a consequence of exposure.
We further focused on the *in vivo* effects of two CASCADE model compounds, genistein and BPA, since these two compounds were shown to elicit novel, previously undescribed developmental effects.

We observed that genistein exposure of zebrafish embryos induces apoptosis, mainly in the hindbrain and the anterior spinal cord. By characterizing this effect, we observed that genistein acts through at least two different pathways in zebrafish and xenopus embryos:

- Genistein induces apoptosis in an ER-independent manner, and
- Genistein regulates aromatase-B expression in the brain in an ER-dependent manner.

These results, thus, highlight the multiplicity of possible actions of phytoestrogens, such as genistein.
We also investigated BPA effects during embryonic development using zebrafish and xenopus models. We observed that in zebrafish and Xenopus embryos, exposure to BPA during the first developmental day resulted in dose-dependent defects in otolith formation. The data suggest that the spectrum of BPA action is wider than previously expected and argue for a systematic survey of the developmental effects of this endocrine disruptor. These results obtained on BPA and genistein suggest that the use of standardized endpoints to study the effects of a given compound, even when this compound has well known targets, may carry the risk of overlooking interesting effects of this compound.

2.10 Mechanisms of Disease Development

The main goal of this work package is to gain further understanding of the causal roles of NRs, as well as their target genes, in human disease development. Focus is on finding novel biomarkers for aberrant NR signaling relevant to disease processes and novel *in vivo* disease models. Potential health effects of

the selected EDCs and food-derived components were integrated into model systems that would allow the assessment of their functional relevance.

Appropriate genetically modified model organisms available in the different laboratories of the consortium were utilized and developed further in joint research activities and the models were made available for the whole consortium. These models include the gonadectomised rat model, ERα- and ERβ-deficient (ERKO) and (BERKO) mice, aromatase overexpressing (AROM+) mice, aromatase deficient (ArKO) mice, mouse lines with altered expression of 17βHSD enzymes, 3xERE-Luc reporter mice, ARE-Luc reporter mice and "Alzheimer mice".

These models are used to assess causal links between NR function and phenotypic consequences for the model organism and to recognize novel critical mechanisms for disease development, likely to be targets of diet-derived NR-interacting compounds. This work block has been divided into four subsections, which initially have focused on the selected target systems, i.e., the reproductive system and the central nervous system. With regard to the reproductive system, we have continued to focus on target organs that, based on results obtained earlier, appear the most relevant, i.e. testis, prostate and mammary gland. As initially, major emphasis is on the NRs that are the likely targets of the four model compounds, namely

- The estrogen receptors ERα, ERβ,
- AR and
- AhR.

Based on work with the AROM+ mouse model, CASCADE showed that deteriorated male reproductive health has been connected to overexposure to compounds with estrogenic activity or to imbalanced androgen-estrogen ratio. In the present study, the AROM+ mice were shown to have severe abnormalities in the structure and function of Leydig cells before the appearance of spermatogenic failure.

Results from the studies with AROM+ mice crossed with ERα or ERβ knock-out mice allowed the conclusion that the structural and functional disorders caused by estrogen exposure were mediated via ERα, whereas ERβ was not involved. Furthermore, we observed that in addition to abnormal cholesterol biosynthesis, AROM+ mice showed marked accumulation of some phytosterols in testis, indicating that persistent hyper-estrogenism may disturb the metabolism of diet-derived sterols. Such link has not been described before, but may be of significance, considering the increasing use of phytosterol-enriched health foods.

It is usually assumed that isoflavonoids, such as genistein, would mainly target ER-mediated functions. However, it should be kept in mind that whenever ER signaling is disturbed, there may be indirect effects on AR signaling (e.g. through changes in CNS-gonadal axis function), and that, in addition to ERs, plant phenolics have multiple other targets, such as steroid biosynthesizing enzymes. Using ARE-Luc reporter mouse, we show that in intact males, genistein inhibited Luc expression in prostate, brain and testis but not in skeletal muscle. Furthermore, in the castrated males, genistein induced an increase in Luc expression in prostate and brain but not in skeletal muscle. The effect of genistein in the intact prostate is very interesting, as reduced AR activation could be of importance in the development of prostate cancer.

Further, genome-wide approaches were made to identify genetic networks and regulated genes – together with their response elements – controlled by estrogen, androgen and a selected endocrine disruptor (EDC) in cell types of reproductive origin. The long-term goal is to delineate genetic networks and regulatory mechanisms that govern estrogen and androgen action and to identify novel targets genes employed by EDCs to perturb with the action of natural female and/or male sex steroids. Extensive characterization of androgen receptor (AR)-binding sites has already been conducted by using both ChIP-on-Chip and ChIP-seq approaches combined with gene expression profiling in a prostate-derived cell line.

The results reveal a complex pattern of distribution of AR binding sites across the genome. Most of the AR-binding sites (ARBs) (60%) mapped to distal regions, followed by 30% in intronic regions. The proximal promoter region contained only 1% of the sites, 5% were located in exons while the rest were in the 5'/3'-untranslated regions. To understand the relationship between receptor binding and hormone-regulated gene expression, prostate-derived cells were exposed to androgen and gene expression profiles were compared to genome-wide receptor binding results. Some 500 genes were up-regulated by androgen. Most of the regulated genes possessed receptor binding sites within 200 kb upstream of transcription start sites. By contrast, genes that were down-regulated by androgen (ca. 500 genes) did not contain ARBs above that on the genomic background. Detailed analyses of the arrangement of binding elements are under way.

Genome-wide analysis of ER-binding sites (ERBs) with the receptor occupied either by the physiological ligand estradiol or the selected EDC enterolactone in a mammary cancer-derived cell line (MCF-7 cells) has also been conducted by using the ChIP-seq approach and the results are being analyzed and related to the data on gene expression profiling in the same cells.

One of the CASCADE model compounds, TCDD, is known to have impact on several aspects of intermediary metabolism. For example, when exposed to high amounts of TCDD, individuals rapidly develop wasting syndrome. In an attempt to study TCDD-induced effects on one of the key regulators in metabolism, the liver X receptor (LXR), we showed that liganded AhR/ARNT complex acts as a potent co-activator of LXR-driven transcription. As LXR target genes are mostly involved in regulation of metabolic pathways, including lipogenesis, cholesterol clearance and glucose metabolism, the consequences of increased LXR signaling could be significant.

In our society, we see a major increase of metabolic syndrome and related diseases, which is partially due to a change towards a less active lifestyle and increased food consumption, but it is also likely that constant exposure to chemical contaminants in our environment contributes to these problems. Furthermore, the chemical exposure during fetal and postnatal development could very well be a contributing factor for initiating a disease that will manifest itself in later stages of life.

Investigations of how LXR affects stromal growth and epithelial cell proliferation in the prostate showed that LXRα is strongly expressed in the luminal and basal cells of prostatic epithelium. The ventral prostates (VP) of LXRα knockout mice are characterized by the presence of smooth-muscle actin positive stromal overgrowth around the prostatic ducts and by numerous fibrous nodules pushing into the ducts and causing obstruction, so that most of the ducts were extremely dilated. We conclude that in rodents, LXRα seems to control VP stromal growth and that the LXRα knock out mouse may be a useful model to study prostatic stromal hyperplasia.

2.11 Metabolism

To gain further understanding of the role of metabolism in the endocrine disrupting effects of food contaminants interacting with NRs, a characterisation of genistein, vinclozolin and bisphenol A metabolites was performed *in vivo* and *in vitro*. Twelve metabolites were identified for genistein and more than 10 different biotransformation compounds were identified for BPA. These metabolites were analysed by radio-HPLC and the structure was confirmed by mass spectrometry (LC-MS). A urinary metabolic profile was obtained from rats treated with ^{14}C-vinclozolin, and compared with the pattern of metabolites obtained in rat precision-cut liver slices. Vinclozolin *in vitro* metabolic profiles indicate that [2-[[(3,5dichlorophenyl)carbamoyl]oxy]-2-methyl-3butanoic acid (M1) and 3,5-dichloro-2-hydroxy-2-methylbutyl-3-enanilide (M2) are the main breakdown products originating spontaneously from the parental compound in appropriate incubation conditions. The biotransformation of bisphenol A and related compounds has also been investigated for the first time in tadpoles and zebrafish showing specific metabolic pathways in these species.

The biotransformation capabilities of human HepG2 hepatoma cells, MCF7 breast cancer cells and mouse HC11 mammary gland cells were also investigated toward radiolabelled ethoxycoumarin, bisphenol A, genistein and vinclozolin. The measurements of the metabolic rates were as follows:

HepG2 > MCF7 >> HC11 (the latter showing almost no metabolic activity, even after 48h incubation periods). Differences observed between the cell lines were not only quantitative but also qualitative.

Some of the produced metabolites, especially those from vinclozolin (M1 and M2), were tested using reporter cell lines bearing a luciferase gene under the control of wild type or chimeric Gal4-fusion AR, PR, GR, MR or ER. Vinclozolin metabolites were found to be antagonists of AR but the M2 metabolite was also a PR, GR and MR antagonist and an AR partial agonist. It was also observed that vinclozolin and M1 and M2 metabolites were agonists for both ERs with a lower affinity for ERβ. The results obtained with 3-MC suggest that at least two fractions collected from the radio-HPLC metabolic profile resulting from HepG2 cell-lines incubated with 3-MC contain metabolites that can act as ER ligands, although, identification of these compound or compounds was not successful.
The biological activity of TBBPA metabolites and degradation products has been tested using reporter cell lines bearing luciferase gene under the control of wild type or chimeric Gal4-fusion ERs, AhR, PXR or PPARs. It was observed that TBBPA, tribromo-BPA, and to a lesser extent dibromo-BPA and a metabolite corresponding to a TBPA dimer were active on PPARγ.

To identify and characterize bioactive metabolites of genistein, immobilized recombinant ERα affinity columns were used. This tool followed by LC-MS analyses was successfully used in CASCADE for isolation of genistein metabolites able to bind ERα, namely orobol and, to a lesser extent, 6-hydroxy-genistein. Similarly, the use of a PPARγ-based affinity column permitted the retention of TBBPA metabolites on the basis of their affinity for PPARγ. In contrast, attempts to determine ER binding of 3-methylcholanthrene metabolites by this system were unsuccessful.

Since metabolism can result in formation of metabolites, which are biologically more active than the parental compound towards cellular targets, CASCADEs main purpose was to gain further understanding of the role for metabolism in the endocrine disrupting effects of food contaminants interacting with NRs. This issue required comprehensive information about the fate of the selected food contaminants in various *in vivo*/*in vitro* systems, but also a strong experience in the models expressing NRs. More precisely, the scientific objectives were:

1. To determine metabolic pathways of the chemicals investigated within CASCADE.
2. To understand whether metabolites and/or parental compounds are the active agents interacting with the different types of NRs.
3. To evaluate the biotransformation capabilities of the various biological systems and model organisms used within CASCADE for the analysis of the effects of EDCs.
4. To develop a metabolomic approach allowing the identification of subtle metabolic changes induced by single chemicals (or mixtures) in living systems selected in the project.

During the first three years, the major part of our efforts was focused on the 3 model compounds selected by the consortium, namely BPA, vinclozolin and genistein. More recently, we extended the metabolic studies to emerging food contaminants suspected to be endocrine disrupters, such as bisphenol F, tetrabromobisphenol A (TBBA) and tetrachlorobisphenol A (TCBA), but also to other model compounds such as 3-MC.

The *in vivo* and *in vitro* biotransformation pathways of vinclozolin, genistein and BPA were investigated using rodent models and other biological models. Additional studies were conducted on the biological systems with other bisphenols, such as tetrabromo-BPA, a flame retardant known to affect the thyroid function, or bisphenol F, a bisphenol A analog. In the case of genistein for instance, metabolic rates and pathways were investigated with rats and humans hepatocytes and microsomes and in various cell lines used for screening EDCs. The identified metabolites were compared with those predicted by MetabolExpert software.

The metabolomic approach has permitted to demonstrate that exposure of mice to an EDC resulted in significant metabolic pattern changes in urine, even at the lowest dose tested. We observed that the concentrations of ketonic bodies (acetone, acetoacetate and 3-HB) and polyamines (ornithine) were decreased by DEHP treatment. In addition, these analyses have revealed that an exposition to DEHP

induces perturbations of bile salts and amino acid metabolism. These results confirm that energetic and amino acids metabolisms were significantly affected by DEHP treatment.

The experiment regarding BPA was carried in CD1 mice exposed *in utero* (continuous sub-cutaneous delivery) during the second half of gestation to low doses of BPA. After birth, pups were grown up under standard conditions in separate lots up to the age of 3 month. At 3 month, these young mice exposed *in utero* were euthanized and their livers were immediately frozen until analysis. Analyses of the data from the liver by linear discriminant analysis showed, based on 8 variables (among which nicotinic acid and glutathione), discrimination between the doses. Therefore, the metabolomic approach confirms the potency of this EDC at very low doses as compared with intermediate levels and suggests that the liver is a potential target for this chemical.

2.12 Chemical Contaminants in Food

The ultimate goal for CASCADE is to offer European consumers state of the art research, reliable knowledge and risk assessment on chemicals in food and their effect on the human body. The final outcome was to deliver a new food testing platform based on the fact that NRs can act as sensors for environmental stimuli. Two food items were selected; bread and baby food.

2.12.1 Baby food

The main objective of the baby food project was to analyze the nutritional quality of commercial infant food and to provide information on the impact of potential EDCs. The results have been used to develop new recommendations for risks on children's health.

In summary, the baby food categories were:
• "Starting" and "follow on" milk-(M), soy-(S) and hypoallergenic-(HA) based formulae for the European or National baskets.
• "Follow on" M-, S- and HA-based formulae corresponding to the European baskets complemented with 5^{th} and 9^{th} month solid food weaning.
• Solid food used as weaning for the 5^{th}, 6^{th}, 7^{th}, 8^{th}, and 9^{th} month of age of the baby

The infant based formulae and the solid food were analyzed by a battery of *in vitro* and *in vivo* tests and complemented with analytical determinations. In addition, elemental analysis was carried out in order to control the homogenization of the pooled samples in the European and National baskets. Ten essential and non-essential elements were selected, among them cadmium (Cd) and zink (Zn), common targets in the two food categories analyzed. These two elements have also the finality to control for Zn, the recommended desired levels for essential elements and for Cd permissible levels for toxic elements in baby nourish.

The goal of this joint exercise was to obtain the "Proof of Principle", i.e. to demonstrate that the integrated test battery built in CASCADE is, indeed, capable to detect NR-modulatory activities exerted by foodstuffs. In order to reach this goal, the main objective was to start testing whole food items with the methods available in CASCADE. A Work Management Group meeting chose bread and baby food as the food items to be studied.

In the babyfood project, several joint activities have taken place and significant progress has been made towards the objectives. Chemical target elemental analysis of three different formulas at EU level was performed for ten essential and non-essential elements, such as cadmium (Cd), iron (Fe), led (Pb), selenium (Se), mercury (Hb), copper (Cu), nickel (Ni), zinc (Zn), calcium (Ca) and manganese (Mn). The highest concentration levels regarding all investigated essential metals and Cd and Pb, belonged to the soy-based formulas. Additional twelve milk-based (Mf), soy-based formulas (Sf) and hypoallergenic-based formulas (HAf) samples from the "national basket" were analysed for Cd and Zn content. It was concluded that the HAf from Slovakia shows the highest Cd content of all HA formulas. The Zn values were in agreement with those given by baby food suppliers.

CACADE also compared supplier labeled energy requirements for infant formula products with recommended standard energy values. Only 3 products of the 35 studied were in agreement with recommended daily energy intake by WHO, 2006; FAO, 2004.

The baby food analysis was further extended from infant formula to solid baby food. A tendency to higher concentrations of BPA and their derivatives was found in industrial food. Also, genistein, vinclozolin, procymidon and iprodione in infant formulae and solid food were determined. The genistein content of the Sf and followSf was 9.7 and 3.6 μg/g (dry weight), respectively. Fungicides were found neither in infant based formulae nor in solid food.

The baby food analysis was broadened in an effort to determine the EDC impact on Leydig cells capacity to produce testosterone. It was observed that fHAf significantly suppressed testosterone production. To monitor NR-mediated effects of baby food samples on the neuronal system, the murine clonal neuronal cell line N2A was used. A decreased cell survival rate was noted, even in the moderate 1:100 dilution, using the hypoallergenic formulas whereas all of the other formulas showed no detectable proliferation effects.

On the other hand, the levels of PCDD/F and PCB in solid food from the 5^{th} to the 9^{th} month were analyzed and these values were low or negligible, in accordance to the already determined values in infant formula.

2.12.2 Bread

Bread was selected for testing as it is a staple food and a highly relevant food item for all European consumers, and, furthermore, it is a significant dietary source of compounds of interest (i.e., putative NR modulators). Two types of bread having different profiles of compounds that potentially alter NR function were selected for testing:

1) Wheat toast (general type of bread consumed in all European countries);

2) Wheat toast supplemented with flaxseed (also commonly used in baking).

These bread types are likely to contain at least two different classes of NR-modulatory compounds: cadmium and plant phenols. The work-flow of the Bread Project is shown in the figure below.

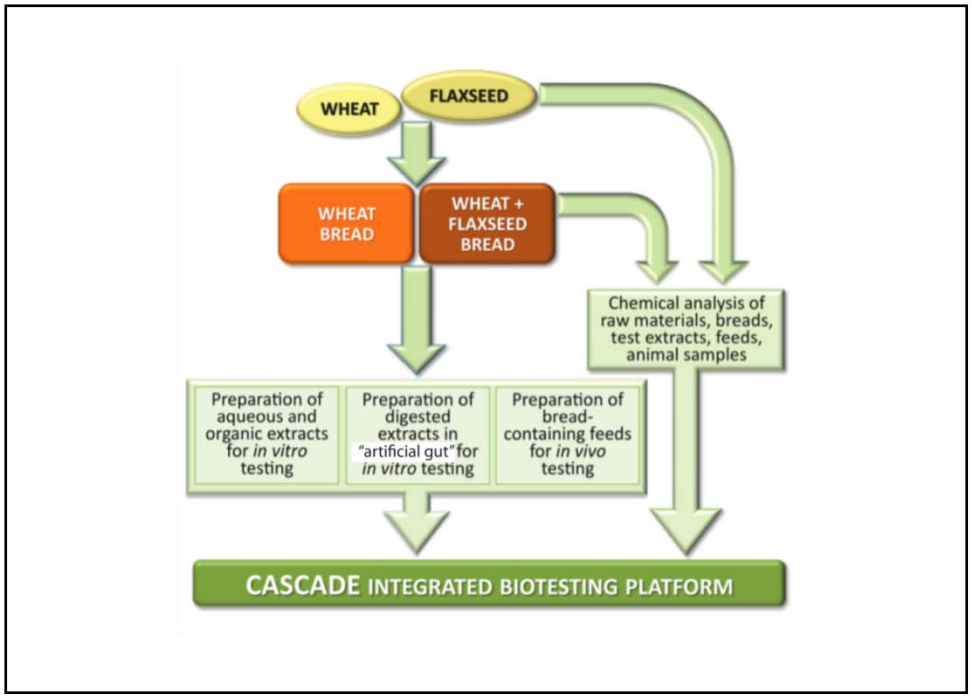


Figure 3: CASCADE Integrated Biotesting Platform

Main findings using chemical analyses:

The test breads and bread diets differ from each other with regards to the concentrations of the putative NR modulators, cadmium and plant lignans. As expected, wheat+flaxseed bread has higher cadmium and lignan concentration, compared to wheat bread. Microbial fermentation (in the "artificial gut") increases the concentration of the lignan metabolite, enterolactone, in all samples. Concentrations of mycotoxins were low in all samples.

Main findings using *in vitro* analyses:

First, CASCADE *in vitro* assays were proven suitable for testing complex materials, such as bread extracts. Bread-derived compounds were found to modulate ER, AR and AhR signalling in selected cell types, whereas TR signalling was not affected by bread extracts. Interestingly, bread-derived compounds were found influence the production of the endogenous high affinity NR ligands in steroidogenic cells. Furthermore, fermentation and the presence of flaxseed appear to change the biological activity of bread extracts.

Main findings using *in vivo* analyses:

First, CASCADE *in vivo* assays, in particular the reporter animal models, were found to be suitable for testing complex food items, such as bread diets. Modulation of ER and AR signalling was observed in estrogen and androgen reporter mouse models, whereas no effects in were seen in a traditional rat model, where estrogen and androgen targets were investigated. In line with the *in vitro* results, no effect on TR signalling was seen *in vivo*.

First, we demonstrated that complex food matrices, such as bread, could be successfully tested for their endocrine-modulatory properties in CASCADE integrated biotesting platform (state-of-the-art *in vitro, ex vivo* and *in vivo* models for NR function). Thus, the "***proof of principle***" was achieved.

Regarding *in vitro* studies, it became apparent that the preparation of the test materials that would adequately reflect the complex nature of the food item is challenging, and several different extracts need to be tested. Furthermore, in order to account for the role of gut microbiota in formation of biologically active metabolites requires *in vitro* fermentation models. With respect to *in vivo* testing, the design of custom-made diets containing complex food stuffs of interest is possible but challenging, and

special attention must be paid to the selection of the basal diet and balancing the energy and nutrient content, as well as ensuring that the basal diet is free of EDCs or phytoestrogens.

It appears that responses may be observed only in certain cell types, animal species, organs or life stages, emphasizing that multiple test systems are needed, in order to identify the likely targets of action. The main targets of bread, or bread-derived compounds, are ERs, AhR, AR and steroid metabolism, implicating a number of highly relevant and interesting health endpoints. However, it must be kept in mind that, based on these studies, demonstrating the endocrine-modulatory properties of bread, it is not yet possible to determine the nature of the effect (adverse or beneficial).

In order to make conclusions on the putative adverse or beneficial effects related to altered NR function, a wide array of test models need to be used. Demonstration of endocrine modulation by a food item *per se* does not yet allow conclusions on possible adverse or beneficial effects – these findings serve as the road signs pointing to the next level of investigation.

3. CONTRACTORS

CASCADE has integrated 25 research groups from 18 European universities, a range of research institutes and one SME located in eight countries. CASCADE mobilises several hundred scientists from various research fields. In addition, CASCADE has created a number of collaborative alliances, such as CommNet and Alliance, to strengthen the mission of the network.

4. INTEGRATION

The aim of CASCADE is to improve integration and reduce fragmentation. All CASCADE activity areas have thus been focused to achieve integration and reduce duplication of research efforts. In addition, CASCADE has played a major role in integrating research activities in the field of chemical contaminants in food in a societal setting and thus to involve not only the research community but also additional stakeholders and the general public.

Scientific integration has been achieved by the involvement of CASCADE partners in different scientific collaborations within CASCADE and also with other projects and international research actors such as the US EPA and Japan NIEHS. CASCADE will continue working towards better integration in the future, for instance with the establishment of the global post-doctoral programme on EDC research which will be run in the context of CASCADE ACERT AISBL. This legal entity is further supported by the collaboration agreement, signed by all Universities that constitute the CASCADE NoE.

Additional areas of integration will be described in more detail below.

4.1 Risk Assessment Integration to support policy development

One of the objectives for CASCADE was to integrate existing academic efforts and structures in the risk assessment area in Europe. During the course of CASCADE this task resulted in further integration both between CASCADE risk assessment partners and other risk assessment partners, as well as with other partners within CASCADE. This has been achieved through meetings, workshops, lab-visits, joint CASCADE projects (incl. PhD projects) and cooperation regarding courses within the CASCADE-initiated course programs:

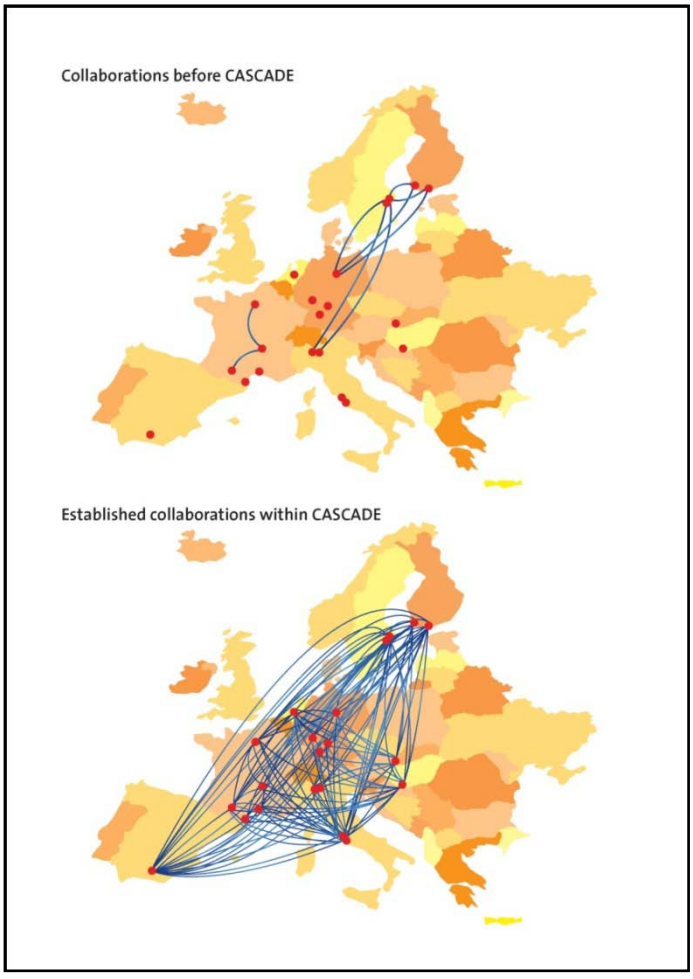
- “Advanced international training courses in health risk assessment”;
- “RA-COURSES”;
- the program for training/certification of European risk assessors - “TRISK”;
- the CASCADE Science-Risk Assessment Expertise Inventory and
- the Mathematical and Statistical Tools - “MSTnet”.

The inventory of the Science-Risk Assessment Expertise within CASCADE was carried out as personal interviews/discussions with persons from almost all CASCADE partners and was based on interview

forms. The MSTnet group was created for personnel from CASCADE partners, other universities and research institutes, authorities and governmental/intergovernmental institutions, industry and consultant companies as a discussion forum. Topics are both day-to-day communication, issues in exposure assessment and mathematical modeling of toxicological effect data. This also included a web-based journal club run from CASCADE internal website. The participants were selected based on the response to an email-based survey, where 92% of the respondents (79 persons) expressed an interest to participate in activities related to dietary exposure modeling. Contacts in the field of Risk Assessment have also been established during CASCADE with other EU-projects, such as

- SAFEFOODS,
- FIRE,
- BIOCOP,
- ECNIS,
- ESBIO,
- ATHON,
- RA-COURSES,
- TRISK.

The contacts include collaboration in the area of educational courses, exposure data modeling, benchmark dose (BMD) modeling of toxicological effect data and MSTnet participation. Contacts have also been established with stakeholders, such as EFSA, ECHA, EUROTOX, USEPA and DG SANCO, with regard to e.g. the CASCADE course program (in collaboration with RA-COURSES and TRISK) where representatives have been involved in the planning of the program as well participation as lecturers and MSTnet.



4.2 Researcher exchanges

A key integrating goal has been to establish strong links between the different CASCADE partner groups. These links should be of a durable character, to be able to create a significant impact in terms of integration. To meet these objectives, CASCADE established a combination of approaches to support integration among partners. From the start CASCADE partners committed to perform the scientific activities funded by CASCADE NoE in close collaboration with the other partners. These activities where always performed jointly and took advantage of the complementarity within the multidisciplinary CASCADE partner structure. Furthermore, exchanges were built into the collaborative projects to ensure extensive researcher exchanges.

In total CASCADE partners exchanged 250 researchers throughout the lifetime of the project, and these exchanges are still ongoing even after Commission funding has ceased. In addition, we expect that CASCADE exchanges will continue to take place. In fact, with the establishment of the CASCADE-FELLOWS post-doctoral programme, researcher exchanges will accelerate and remain a major integrating activity within the expanding CASCADE network.

4.3 Databases to support joint research

NureXbase

CASCADE has organized a database containing protein and DNA sequences, reviewed protein alignments and phylogenies, taxonomy and annotations (Duarte et al., 2002; Ruau et al., 2004). This sequence information is clustered into nested 'groups', corresponding to levels of the NR nomenclature (Nomenclature, 1999). We also integrated information from functional genomics, such as alternative transcripts and expression data based on EST repositories (Figure 4). In parallel, structural information on nuclear receptors has also been gathered to cover data on 3D structures including dimer interfaces, crystallographic contacts and all the crystallization steps. A specific emphasis has been put on NR ligands and their cognate ligand-binding pockets.
This common framework has been used as a platform to develop an "EDC-module", thus providing an integrated system allowing flexible queries on data ranging from genomic to structural features. The ultimate goal of this new EDC-module is to cluster relevant information on each EDC known to interfere with a given NR. The complete association between the DNA-oriented database, the 3D structure information and the EDC module is forming NureXBase.

Identification and acquisition of EDC data:
In a first step, we focused our efforts on EDCs binding to estrogen receptors (ERs), since these compounds represent the majority of known EDCs. We started by constructing a list of chemical contaminants that behave as ligands and/or activators of NRs. This list is largely based upon information extracted from several reports and databases available on the Internet. We have identified listings or databases containing information on established or potential EDCs that have been produced by governmental, non-governmental or industrial bodies. Efforts were made to verify the information derived from these sources using publications records. All this information has been collected and transferred to a consolidated list by eliminating redundancy. In this way we obtained a combined dataset with 169 compounds, including naturally occurring products, environmental pollutants, industrial chemicals, agro-chemicals and pharmaceutical compounds, with data about their relative binding affinity for ERs. Figure 5 summarizes the step followed to collect the relevant information.

The detail of the information gathered for each compound is as follows : Name, Formula, Molecular Weight, Image, Chemical Nature, UC (Unique code = INChI code), Smile Code, CAS Code, ChemName_IUPAC, Kow LogP, ER RBA. Specific efforts were made to verify the accuracy of the information derived from these sources through cross-referencing against on-line databases and by further searches on the Internet. To ascertain that the database contains only accurate and consensual data, all the extracted information was reviewed independently by partner 12A. Only the information recognized as accurate by the internal reviewers was conserved in the database.

Retrieve and analyze the data: the web interface
The web application gives access to the five major types of data (expressions patterns, sequences, alignments, phylogeny, 3D complexes with protein structure and ligands; Figure 4) through specialized menus (Figure 5).

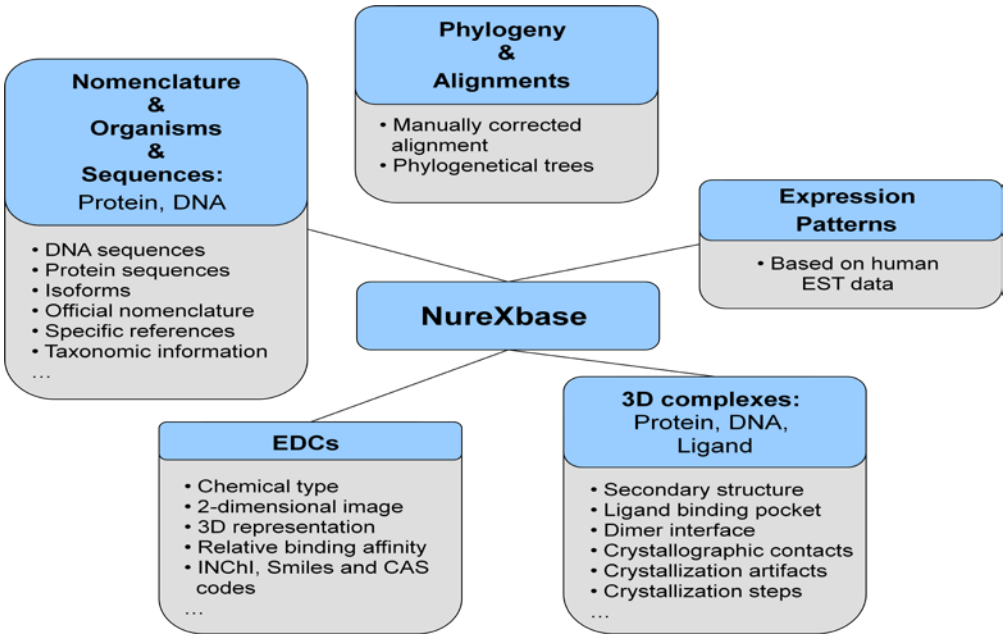


Figure 4: Schematic organization of NureXBase.
NureXBase corresponds to a fusion between two differentially focused databases, Nurebase and NRBase. NureXBase can be decomposed in 5 major domains from left to right: (i) expression patterns; (ii) alignments and phylogeny; (iii) Nomenclature, organisms and sequences (protein, DNA); (iv) 3D complexes (protein, DNA, ligands); (v) EDCs. For each domain the main properties available are indicated.

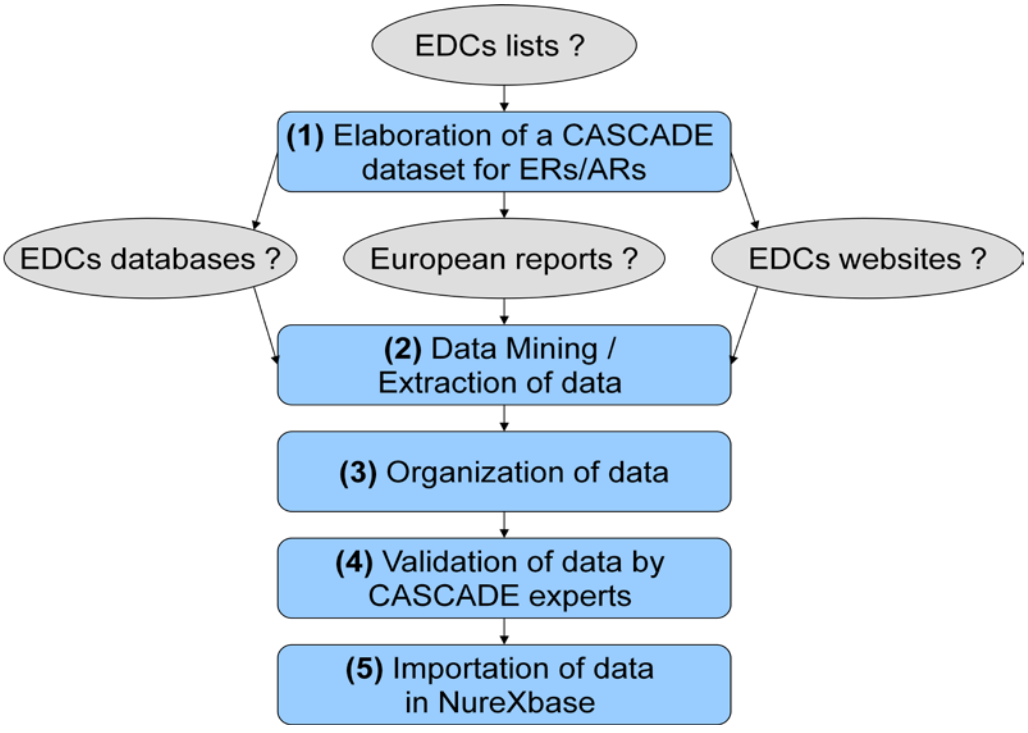


Figure 5: Flow-chart for EDCs selection.
(1) In a first step a detailed list of EDCs has been set up, based on data available on web sites from specific resources (see Table 2); (2) Existing databases were then queried to collect pertinent data on these substances. A strategy base on keywords (for example the name or CAS Number) was used; (3) The EDCs data were organized into a standardized table with relevant information for each EDC (Table 3); (4) Cascade partner experts reviewed the data; (5) Integration in the database.

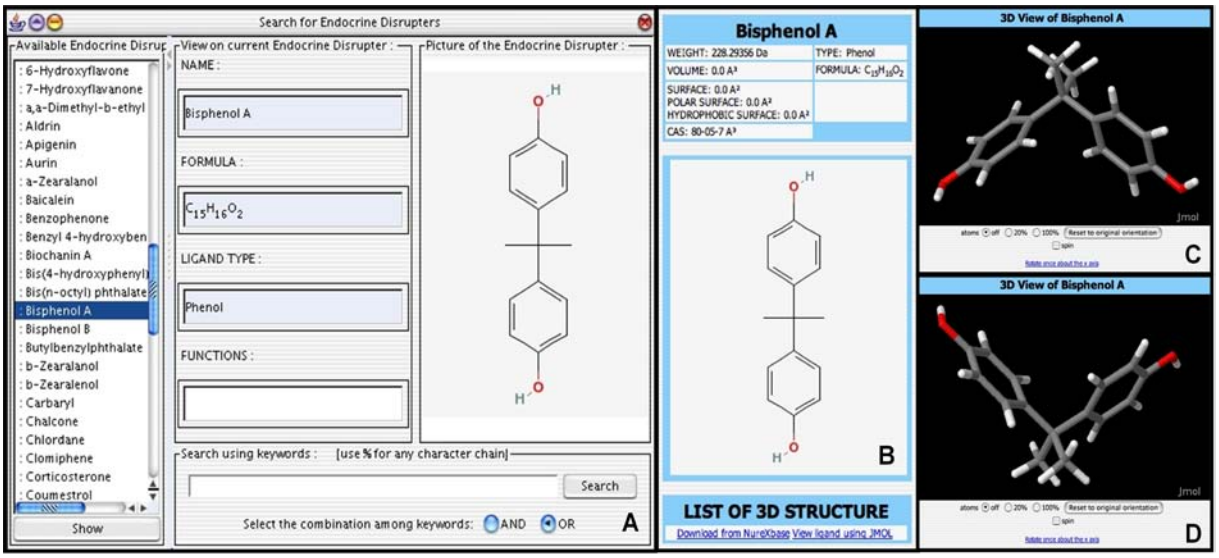


Table 2: EDC user interface for simple queries, illustrated with Bisphenol A data.
The menu to browse through the list of EDCs is shown. A few properties are displayed (name, formula, 2D picture). (B) Additional information is available in a second step using the "Show" button. (C and D) A 3D visualization is proposed using Jmol (http://www.jmol.org).

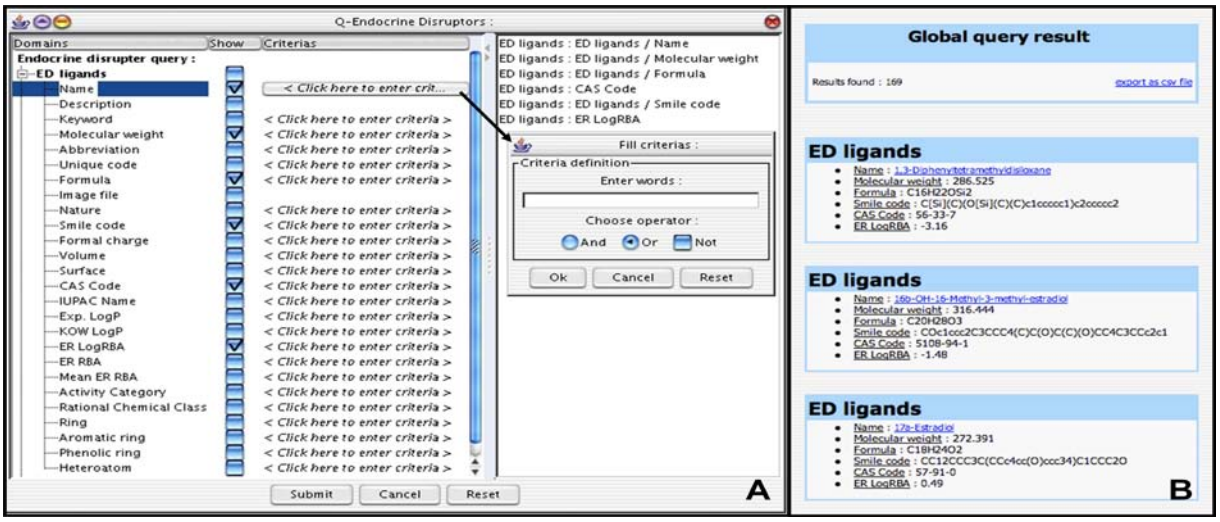


Table 3: EDC user interface for complex queries illustrated with Bisphenol A data.
(A) This interface contains a query editor with the various properties organized as a tree. Every property can be selected (check boxes in the middle) during the query. The query can be limited to a set of values, by editing each field to define a range of legitimate values. Once submitted, the SQL query is generated automatically and sent to the database and (B) the data is retrieved.

4.4 Other integrating activities

To secure the achievements of CASCADE, the network members have established an independent legal entity. This organization, called CASCADE-ACERT AISBL, is a non-profit association under Belgian jurisdiction. CASCADE ACERT will serve as an integrating structure and continue the activities of the CASCADE NoE. CASCADE ACERT AISBL was established 2009-10-09 and is currently in the process of organizing its activities.
CASCADE-ACERT will thus continue the work that was initiated by the CASCADE NoE. Currently, the initial set of activities is being set up and includes the following components:

- Run the CASCADE-FELLOWS post-doctoral programme
- Establish a research administration course programme

- Launch a set of e-learning modules aimed towards industry and general public

We expect that these activities will be launched in the fall of 2010. In addition, to support the long term development of CASCADE-ACERT, we have submitted an application (SSA) which is currently under evaluation. It is however important to stress that CASCADE-ACERT is not solely dependent on the outcome of this application.

5. EDUCATION

CASCADE has organised a large number of successful European training activities in food safety and health risk assessment. More than 1300 PhD students, post docs, senior scientists as well as professionals from industry and regulatory authorities have attended the 34 different training courses, summer schools and workshops: 10 on endocrine disrupting chemicals, nuclear receptors and food safety, 13 on risk assessment, 8 on methods, 3 on research communication. CASCADE has thus gotten established as a platform for restructuring education at the European level in food safety and risk assessment. CASCADE is actively engaged in developing a European structure for training of accredited European risk assessors. CASCADE universities are dedicated to develop joint education for Master's and PhD students and promote exchange of students and teachers between the universities. CASCADE has collaborated with DG SANCO, EUROTOX, EPAA, EU funded projects CRESCENDO, RA-COURSES, DIMI, ECNIS, BIOCOP, SAFEFOODS on training activities.

In addition, twenty PhD students/post docs involved in joint intra-network projects have been funded. 86 PhD students and post doc have participated in Junior Scientists Sessions and 10 PhD students and post docs have had personal mentors. Also, these and other CASCADE junior scientists have benefited from targeted "Junior Scientists Sessions" at CASCADE Annual Meetings.

The Junior Scientists Sessions aimed at building a strong network between the PhD students and post docs within CASCADE, allowing possibility for informal discussions on research projects and personal experience of PhD/post doc training and providing training in complementary skills. In total have 86 PhD students and post docs attended the meetings. The topics of the sessions have been workshops in research ethics, oral presentation skills, poster presentation and databases in CASCADE as well as discussions on own research projects and PhD/post doc training in different countries in Europe. Career planning and mentoring activities that are part of CASCADE Mentorship Programme have also taken place at the Junior Scientists Sessions as described in more detail below.

These sessions included the "CASCADE Mentorship Programme" aimed to support junior scientists in creating a successful career. The programme was open for PhD students and post docs in CASCADE. The programme was divided into three steps: Increased career awareness, group mentoring and one-to-one mentoring. The first step included a workshop on career planning and one-to-one career planning appointments with a career consultant. In the second step on group mentoring, junior scientists discussed in groups with senior CASCADE researchers on questions and tips on building a successful career as well as how to combine personal and professional life. In the third step, one-to-one mentoring, ten PhD students/post docs were given personal one-to-one mentoring with a senior CASCADE scientist. The one-to-one mentorship programme was launched by workshop with an expert on mentorship programmes, introducing the aims and rules of the programme. Thereafter the mentees and mentors had contact regularly during one year by face-to-face meetings and by contact through e-mail and Skype.

5.1 Agreement on collaboration on education

To support durable integration of at the institutional level, the rectors and directors from CASCADE universities and research institutes decided at the CASCADE Governing Council 2006 to collaborate on training for Master's and PhD students.

5.2 CASCADE Schools

The overall objective of the teaching activities in CASCADE was to provide a strong European training programme in the field of endocrine disruptors, food safety and health risk assessment. In addition, the teaching activities contributed to strengthen the scientific structure of the network and support scientific contacts outside the network. The teaching activities were directed towards PhD students, post docs, senior scientists and other professionals from industry and regulatory authorities and were open for participants from outside the network. The teaching activities have been developed in WP4 "CASCADE Teaching" and the individual courses and schools given in WP5 "CASCADE Schools". CASCADE course programme "Advanced international training courses in health risk assessment" was developed in WP10 "Risk Assessment Integration".

The following courses, summer schools and workshops have been organised:

Endocrine disrupting chemicals, nuclear receptors and food safety:

1. Nuclear receptors, endocrine disruptors and metabolic effects, 2004
2. Nuclear hormone receptors, 2004
3. Nuclear receptors, and their ligands in metabolism and disease, 2005
4. Nuclear receptor biology, 2005
5. Molecular nutrition in relation to cancer research, 2006
6. Nuclear receptors in health and disease, 2006
7. Nuclear receptors in health and disease, 2007
8. Nuclear receptor signalling: from molecular mechanisms to integrative physiology, 2007
9. Nuclear receptors in health and disease, 2008
10. Nuclear receptor signalling: from molecular mechanisms to integrative physiology, 2009

Health risk assessment:

1. Food safety and environment. Health risk assessment, 2005
2. Health risk assessment: principles and applications, 2006
3. Philosophy of risk in practical risk assessment, 2006
4. Contaminants in food: metabolic fate and analytical approaches. State-of-the-art and future trends, 2007
5. Health risk assessment: focus on cancer, developmental neurotoxicity and endocrine disruption as critical effects, 2007
6. Receptor-mediated toxicity, 2007
7. Statistics in risk assessment focusing on dose response, 2007
8. Health risk assessment: principles and applications, 2008
9. Regulatory toxicology, 2008

10. Reproductive and developmental toxicology, 2008
11. Child health and the environment, 2009
12. Philosophy of risk in health risk assessment, 2009
13. Exposure assessment i: chemical exposure assessment analysis and modelling, 2009

Methods:
1. Molecular imaging in drug discovery, 2006
2. Phenotype characterisation of genetically modified mouse models, 2006
3. Measurement of biomolecular interaction, 2006
4. Molecular imaging in drug discovery, 2007
5. Phenotype characterisation of genetically modified mouse models, 2007
6. Molecular imaging in drug discovery, 2008
7. Analysis of estrogenic endocrine disruptors in food, 2008
8. Phenotype characterisation of genetically modified mouse models, 2008

Research communication:
1. Communicating science and risk, 2005
2. Media training, 2006
3. Communicating research to the public and to media, 2008

6. DISSEMINATION

The main objective of the CASCADE Network of Excellence is to disseminate the research results generated by the network. The ultimate outcome of our joint efforts is to bring back these results to the European consumers. A successful communication operates in the context of external interests, such as stakeholder involvement, media interest, public sentiment and events in society. CASCADE has been active to establish CASCADE as an internationally well known and respected brand that reflects quality and excellence. CASCADE partners have actively participated in the public debate regarding chemical contaminants in food. CASCADE partners and network representatives have been contacted by authorities, industry, NGOs and individual European consumers to provide advice and support in the area of chemical contaminants and their effects on health. From the very beginning, CASCADE has had a plan for how to disseminate the results and the goals for this plan have successfully been achieved in accordance with the following activities:

6.1 Meetings

One of the most important goal for CASCADE has been to promote integration among European scientists and thus to promote interdisciplinary collaboration. To facilitate this process CASCADE has successfully established an extensive meeting agenda that enables the network participants to meet, interact and establish fruitful collaborations. These meetings are a forum for scientific exchange and also a tool to monitor progress of different activities such as integration, research and dissemination.

Meetings have also a central role to disseminate the objectives of the network and this has mainly been accomplished by informing the scientific community about this. Furthermore, meetings are excellent opportunities for members of the network to meet, interact, exchange scientific ideas as well as an

important tool to further strengthen integration and extend dissemination of CASCADE with the content adapted to target groups.

During the years the work of CASCADE partners have been present in more than 1100 meetings, conferences and workshops at regional, national and international levels. In total CASCADE partners have given more than 182 abstracts for oral presentations at meetings, shown hundreds of poster and made 351 abstracts for poster presentations. To achieve best results, CASCADE meeting activities have been adapted in such a way that it suits the audience and thus divided into different categories:

- Scientific meetings;
- Dissemination meetings;
- Meetings to support durability and strengthening of the network.

Following some highlights and total achievements in respective category:

6.1.1 Scientific meetings:

Annual meetings
The CASCADE Annual meetings have focused on the major achievements and plans for CASCADE future activities. Scientific results from all CASCADE partners have been presented and the results of CASCADE activities in other areas (risk assessment, integration and dissemination) have been discussed. In addition, these meetings have included interactive sessions to discuss new, innovative approaches that need to be undertaken to establish a vibrant collaboration across different scientific disciplines. All CASCADE partners have been represented at the Annual Meetings and external speakers have often been invited to present ideas and foster collaborations.

The following Annual Meetings have been held:

- CASCADE 1st Annual Meeting in Orvieto, Italy, January 20-23, 2005
- CASCADE 2nd Annual Meeting in St Malo, France, March 28-31, 2006
- CASCADE 3rd Annual Meeting in Helsinki, Finland, April 17-19, 2007
- CASCADE 4th Annual Meeting in Brussels, Belgium, May 5-7, 2008

Partner Meetings
CASCADE Partner Meetings have included meetings for the different CASCADE Task Forces, Work Packages (WPs), the Work Management Group and the Partner Steering Committee. Focus of these meetings has shifted from mainly administrative issues to primarily discussing research, integration and dissemination. At each Partner Meeting scientific presentations are made by the responsible WP leader on ongoing research. In total, CASCADE has organized 10 Partner Meetings. Several additional WP-meetings and joint WP-meetings have been arranged by WP-leaders throughout the years.

6.1.2. Dissemination meetings

CASCADE Open Forums
CASCADE spreads results to policy makers, consumer organisations and industry by arranging annual Open Forums. These meetings are more specialised events where the focus is directed towards research users in contrast to the conventional meeting structure, which is directed towards research-providers. The themes for these meetings have been “Chemicals in food”, “REACH in reality”, “The future of Networks of Excellence - will the billion Euro of public investment be lost?”, “Endocrine Disruption – Present health threats and future research needs” and “European Food Science Day – Bringing back the results to the consumers”, meetings that all have generated great media coverage and visibility for CASCADE.

The CASCADE Open Forums have been visited by representatives from the European Commission, European Parliament, industry organisations (e.g. CIAA), consumer organisations (e.g. BEUC), regulatory authorities (e.g. EFSA) and NGOs such as HEAL. In general, these meetings attract some hundreds of participants: the last one well above 120, and the reach out is even greater. Thousands of

stakeholders have been contacted and informed in these important issues, and afterwards the outcome has been broad casted TV-programs, video interviews on internet ("CASCADE at YouTube"), press items in printed media, opinion papers, debate articles and information on websites and in e-newsletters.

The high interest in CASCADE open forums has shown that there is a need for this kind of platform at a European level.

CommNet meetings
CASCADE has also initiated CommNet; an informal network of science communicators from EU funded food projects. Since the start in 2005, CommNet participants have met more than ten times, which has enhanced the communication quality in all involved projects.

6.1.3. Meetings to support durability and strengthening of the network

Via lobbying campaigns, position papers and opinion papers, CASCADE has tried to influence the decision makers in several urgent and important questions. The uncertain future of the Networks of Excellence was highlighted during the lobbying "Support research NoEs", which concluded in the signing of an opinion paper by over 60 Networks of Excellence, an Open Forum in Brussels 2007 and a lunch debate hosted by MEP Prof Jerzy Buzek in Brussels 2008. At the time for voting on REACH regulation, CASCADE published an article about REACH implementation in Financial Times. WWF and CASCADE jointly made a "Detox-campaign", testing 27 different foodstuffs around Europe. CASCADE's collaborations also involve successful alliances with US-EPA and OECD. The latest opinion paper, "Challenges in future European endocrine disruption research – outlining identified research needs and opportunities", was an outcome from the latest CASCADE Open Forum. Over 800 researchers from 20 different European countries jointly state the actions required to fill the knowledge gaps in ED research.

CASCADE has also had extensively collaborations with other EU-funded projects, industry and regulatory authorities during these six years. As an example, CASCADE has collaborated with the United States Environment Protection Agency (US EPA) to support the world's largest screening effort, namely the ToXCast program.

Moreover, CASCADE has participated at EuroScience Open Forum two years in a row. ESOF is the largest scientific event and public exhibition in Europe. There, CASCADE representatives have interacted with hundreds of consumers interested in information on food contaminants.

6.2 The CASCADE website and interactions with media

6.2.1 CASCADE website

CASCADE has a popular and regularly updated web site, www.cascadenet.org. It serves as a meeting portal for researchers, students and journalists interested in the area of endocrine disruption and chemicals in food. The web site offers an appreciated "Ask an expert" section, where the public can ask scientists and get immediate answer via email. There is also a section with information about state-of-the-art risk assessment on endocrine disrupting chemicals aiming to clarify the role of CASCADE as provider of scientific advice and support to authorities.

Figure 6: CASCADE website

6.2.2 Contact database

CASCADE has compiled a contact database with more than 1000 names and contact details to journalists, industry representatives, policy makers and others interested in the field of hormone disrupting chemicals. This gives CASCADE a unique opportunity to easily and quickly contact a large number of people e.g. with the Newsletter, information about Open Forum, course information and the latest research news.

6.2.3 Use of old and new media

CASCADE has used both the old and new media to spread results: researchers within CASCADE have been interviewed and quoted about CASCADE's research uncountable times in national and international media. CASCADE has also participated in press conferences on food and health and has distributed 16 press releases. Reports and information about CASCADE can also be found at several other web portals like CORDIS, EurActiv, EC DG Research, HEAL or the websites of CASCADE's 20 partner universities. CASCADE is where the consumers are. CASCADE are visible at social networking sites such as Wikipedia, Facebook, ResearchGATE and AthenaWeb.

6.3 Information material

6.3.1 Publications and consumer friendly information

CASCADE has put an effort in producing consumer-friendly information CASCADE has produced and distributed nine editions of CASCADE newsletters to hundreds of stakeholders. Several kinds of brochures and info-sheets have also been produced and distributed at conferences all over the world.

The CASCADE magazine – an attractive and easily available presentation of CASCADE for the public – was launched in 2008. The magazine has today been distributed to more than 3000 journalists, policy makers and industry representatives all over Europe.

Figure 7: Some examples of result of CASCADE dissemination activities

6.3.2 Public presentations and regional spread

CASCADE has been dedicated to disseminate our results on all levels; international, European union, regional and local. For example, CASCADE partners have during the years participated widely in local media, both written and broad casted, visited and given presentations on CASCADE research results at regional and national meetings. CASCADE has monthly sent out "Internal updates" to all CASCADE partners and hundreds of other stakeholders. CASCADE has also compiled a contact database with all regional info offices connected to the CASCADE research groups.

The website includes language derived sites to ensure spread of results and information in all member countries. During the years of CASCADE, the results of the network have been presented at more than 1100 meetings, conferences and workshops at regional, national and international levels. CASCADE has participated at EuroScience Open Forum (ESOF) two years in a row. ESOF is the largest scientific event and public exhibition in Europe and CASCADE interacted with hundreds of consumers interested in information on food contaminants.

6.4 Influence and networking activities

6.4.1 Outreach campaigns

Via lobbying campaigns, position papers and opinion papers, CASCADE has tried to influence the decision makers in several urgent and important questions. The uncertain future of the Networks of Excellence was highlighted during the lobbying campaign "Support research NoEs", which concluded in the signing of an opinion paper by over 60 Networks of Excellence, an Open Forum in Brussels 2007 and a lunch debate hosted by MEP Prof Jerzy Buzek in Brussels 2008.

At the time for voting on REACH regulation, CASCADE published an article about REACH implementation in Financial Times. WWF and CASCADE jointly made a "Detox-campaign", testing 27 different foodstuffs around Europe. CASCADE's collaborations also involve successful alliances with US-EPA and OECD.

The latest opinion paper, "Challenges in future European endocrine disruption research – outlining identified research needs and opportunities", was an outcome from the latest CASCADE Open Forum. Over 800 researchers from 20 different European countries jointly state the actions required to fill the knowledge gaps in ED research.

6.4.2 CommNet- A CASCADE initiative to improve dissemination and communication

In 2005, CASCADE took the initiative to a network of Communication managers in FP6 projects. Since then, CASCADE has chaired CommNet, which today consists of more than 20 projects and has enhanced the communication quality in all the involved projects. CASCADE arranges Communication courses, to improve our researchers' skills in communication with media and public, and also participates in ESConet's (European Science Communication Network) education of Communication educators.

The objectives of the network's communication goals have successfully been achieved in accordance with the above-mentioned activities. In addition, the following activities have been carried out:

- Popular, updated website with 140 000 hits/year and 5000 unique visitors ranked at number two on Google search
- CASCADE visibility at YouTube, Wikipedia YouTube, Wikipedia, Facebook, Cordis, AlphaGalileo, EurActive, EC DG Research, Research*eu and at least 20 other web sites
- 3000 copies of CASCADE magazines spread to European journalists and consumer organisations
- 19 arranged CASCADE meetings and over 1100 CASCADE joined meetings
- Good media coverage, 16 press releases and hundreds of reports
- Production of a multitude of information materials, including nine editions of newsletter, press invitations, position and opinion papers and monthly Internal Updates;

- More than 200 scientific publications in peer-reviewed journals, 151 manuscripts, 182 abstracts for oral presentations at meetings and 351 abstracts for poster presentations at meetings, 46 theses, 21 reports, 25 book chapters and hundreds of posters.

In total, the dissemination effects of CASCADE have developed very successfully way during the six years of activities, shown in Figure 8.

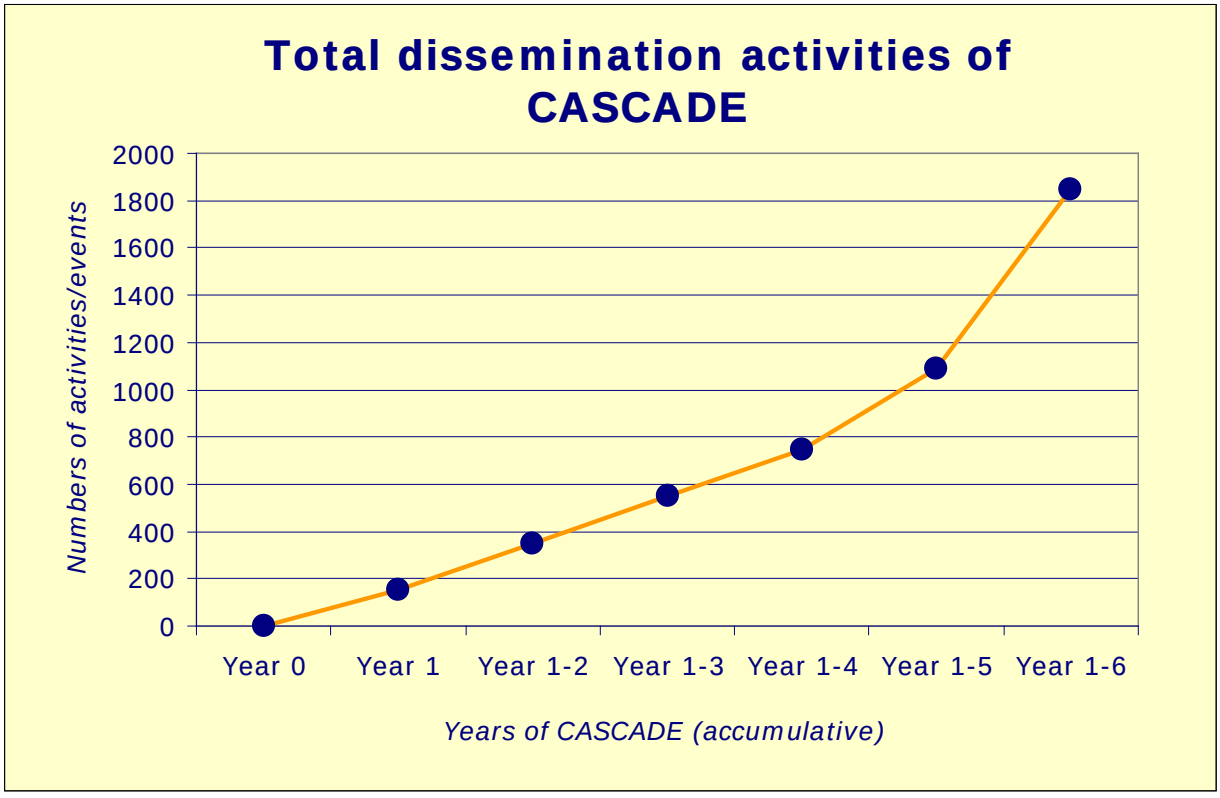


Figure 8: Total dissemination activities of CASCADE