carcinoGENOMICS

a Project of the European Union

- Operational since 1/11/2006
- Total budget amount: 11.988.801 €
- Project contribution by the EC: 10.440.000 €

carcinoGENOMICS consortium with 19 participating groups

- 6 groups represent industry
- 11 groups represent universities or research institutes
- 2 groups represent organizations, i.e. EURL-ECVAM and *ecopa*

This integrated project (IP) supports the 3R-based research in Europe, consisting of Refinement, Reduction and Replacement of animal experimentation.

Info

For more information:
Project Coordinator: Jos Kleinjans
Project Manager: Rene Reijnders
r.reijnders@maastrichtuniversity.nl
www.maastrichtuniversity.nl
Secretariat of **carcino**GENOMICS
Maastricht University, PO Box 616, 6200 MD Maastricht
Universiteitssingel 50, 6229 ER Maastricht
The Netherlands
Tel.: +31(0) 43-388 18 45, Fax: +31(0) 43-388 41 46

www.carcinoGENOMICS.eu

June 2012 • R.E.: carcinoGENOMICS, Vrije Universiteit Brussel/*ecopa*, Laarbeeklaan 103, B-1090 Brussel • Design © www.nickvanhee.be

SIXTH FRAMEWORK PROGRAMME

An integrated Project financially supported by a grant from the European Commission PL 037712

carcinoGENOMICS

a Project of the European Union

Development of a high throughput genomics-based test for assessing genotoxic and carcinogenic properties of chemical compounds *in vitro*

carcinoGENOMICS
a Project of the European Union

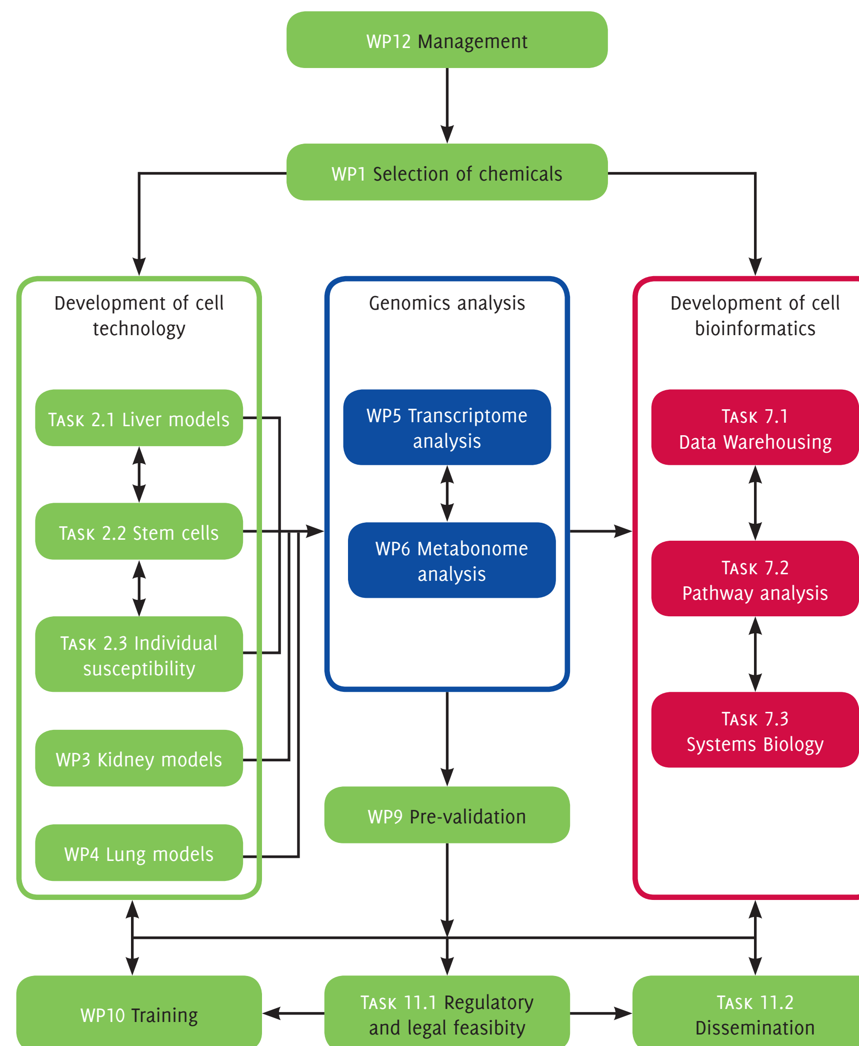
Goals of the carcinoGENOMICS Project

The major goal of **carcinoGENOMICS** was to develop and select appropriate omics-based *in vitro* methods for assessing the carcinogenic potential of compounds, as a potential alternative to the 2-year rodent carcinogenicity bioassay. The idea was to design a battery of mechanism-based *in vitro* tests covering major target organs for carcinogenic action e.g. liver, lung, and kidney. "Omic" responses (i.e. genome-wide transcriptomics as well as metabonomics) were generated following exposure to a well-defined set of model compounds, namely genotoxic (GTX) carcinogens, non-genotoxic (NGTX) carcinogens and non- carcinogens (NC). Through extensive biostatistics, literature mining, and analysis of molecular-expression datasets, differential genetic pathways were searched capable of predicting GTX or NGTX mechanisms of chemical carcinogenesis. Finally, integration of the generated transcriptomic and metabonomic data into a holistic understanding of systems biology was pursued to build an iterative *in silico* model of chemical carcinogenesis.

Structure of the carcinoGENOMICS Project

To achieve the goal of being an effective integrated, co-ordinated project, the management of the project was based upon optimisation of the natural and direct interactions between the different workpackages (WPs) and individual participants (research partners). The multidisciplinary approach required to reach the objectives, set by **carcinoGENOMICS**, involved different methodologies ranging from cell technology, microarray technology to metabonomics and bioinformatics.

Therefore, the project was subdivided in 3 strong interdisciplinary fields being the development of cell technology, genomics analysis and the development of cell bioinformatics (Fig. 1). For that reason the **carcinoGENOMICS** partners were composed of university groups, governmental research institutes, large enterprises and Small and Medium Enterprises (SMEs). Extensive areas of interaction were clearly present between the WPs. Their interconnections are illustrated in the Pert diagram on the right.



WP8 : High Throughput analysis is not operational anymore and has been closed in Year 3 of the project. WP9 has taken over the tasks.

Fig. 1. Pert diagram showing the interconnections between the different interdisciplinary research fields.

SELECTION OF CHEMICALS

A crucial step at the start of **carcino**GENOMICS was the selection of chemicals to be tested throughout the project. In fact, 2 sets of compounds were necessary, namely for the first and the second phase of the **carcino**GENOMICS project. The first series of chemicals was established with the goal of generating a vast collection of data that could serve as a platform to predict the carcinogenic potential of chemicals in lung, liver and kidney. Three classes of chemicals were defined, namely GTX carcinogens, NGTX carcinogens and NC. At least 5 compounds per class and per organ were selected by screening relevant scientific literature and public electronic databases. A number of clear-cut criteria were used for selection purposes, including the availability of toxicological data and gene expression profiles, diversity and selectivity of the compounds, biochemical and biophysical properties, and legal aspects and safety measures. For the second phase of the project, yet an additional criterion was considered while selecting chemicals. Indeed, compounds with human toxicity data were prioritised with the aim of increasing the scope and applicability of the developed *in vitro* systems.

Overall, a wealth of "omics" data has been produced by testing these compounds in **carcino**GENOMICS. This not only resulted in a substantial number of high-quality research papers, but it is also expected to provide a solid basis for a follow-up project that could bring the results obtained into the regular framework of safety assessment of pharmaceuticals, cosmetics and chemicals.

DEVELOPMENT OF CELL TECHNOLOGY

Liver Models – a successful tool for mechanism-based *in vitro* detection of genotoxicants

Partners Involved: Biopredic International (BPI), Cellartis (CELL), University Hospital La Fe (HULAFE), Vrije Universiteit Brussel (VUB), Maastricht University (UM).

Selected Compounds: Genotoxic (GTX) carcinogens, non-genotoxic (NGTX) carcinogens, non-carcinogens (NC)

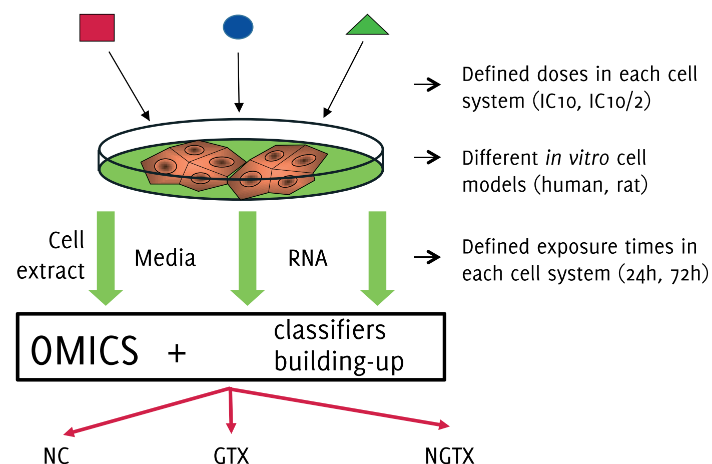


Fig. 2. Schematic representation of the in vitro research on liver carried out in the **carcino**GENOMICS *project.*

Among the different *in vitro* liver models used in the **carcino**GENOMICS project, the human HepaRG cell line generated the most robust gene classifier that discriminated the GTX from the NGTX carcinogens and NC. This model could now be taken foreward to develop a novel *in vitro* test to detect genotoxic compounds.

The main task of the liver workpackage was to provide the best possible liver *in vitro* model for the **carcino**GENOMICS project. The partners provided six liver-based *in vitro* models including models of rat and human origin as well as fully-differentiated and progenitor-based cell systems.
The models chosen were: (i) human hepatoma-derived cell lines including HepaRG, HepG2 and transcription factor-transfected HepG2 cells, (ii) human embryonic stem cell-derived hepatocyte-like cells (hES-Heps) and (iii) conventional and Trichostatin-A (TSA) stabilized primary rat hepatocytes.
In a first phase, in order to establish the most appropriate exposure time and doses, all *in vitro* models were challenged with 3 GTX and 3 NGTX carcinogens and with 3 NC. Two different time-points (24h and 72h) and 2 different low-cytotoxic concentrations (IC10 and IC10/2) were set. Each cell model was exposed to all individual compounds and RNA, cell extracts and media were collected for further analysis (transcriptomics, metabonomics)(Fig. 2).

As from these results, exposure time seemed to affect the results more than the concentrations used, thus in the second phase of the project, the next set of 6 prototypical compounds (2 of each group) was tested for both time points (24h and 72h) but only at one dose (IC10). For full characterization of all experimental systems, several approaches of data analysis were applied. Although each approach was specific and oriented towards a different methodology, they all came to the same conclusion namely that the HepaRG cell line generated the most reliable gene classifier able to discriminate the GTX carcinogens from the NGTX carcinogens and NC (Table 1 and Fig. 3). All other *in vitro* models also succeeded to yield cancer-relevant characteristic results for the GTX exposure gene groups, but they were less performing than HepaRG cells as some genes were also deregulated by NGTX carcinogens and NC. Irrespective of the tested *in vitro* model, it was very clear that the most uniformly expressed pathway following GTX exposure of the cells is the p53 pathway and its subsequently-induced networks.

% of correctly classified experiments

Model	Categories	All exp	All exp - controls	IC 10/2 24h	IC 10 24h	IC 10/2 72h	IC 10 72h	IC10/2 24h - controls	IC 10 24h - controls	IC 10/2 72h - controls	IC 10 72h - controls
Primary rat hepatocytes TSA treated	only GC-RMA	80	94	44	47	46	48	61	60	76	72
	GC-RMA / half-z	76	81	70	75	64	76	71	80	80	74
		188/52	144/48	49	67	49	67	27	45	27	45
Primary rat hepatocytes TSA non-treated	only GC-RMA	82	93	46	50	51	61	65	61	69	69
	GC-RMA / half-z	80	89	70	83	76	68	84	74	83	78
	# of experiments/groups	192/52	144/48	49	67	53	71	27	45	27	45
HepG2-up	only GC-RMA	76	82	42	44	45	49	46	51	54	52
	GC-RMA / half-z	59	71	54	48	52	47	49	60	54	51
HepG2	only GC-RMA	75	81	47	39	57	58	56	47	68	62
	GC-RMA / half-z	60	71	45	39	42	56	50	49	42	63
	# of experiments/groups	179/52	146/48	44	62	44	62	28	46	27	45
hESC-Hep	only GC-RMA	92	94	40	71	58	74	49	77	63	76
	GC-RMA / half-z	92	93	65	90	69	82	73	89	63	86
	# of experiments/groups	183/52	151/48	44	63	45	63	28	47	29	47
HepaRG	only GC-RMA	90	97	60	72	69	70	73	82	84	84
	GC-RMA / half-z	88	98	72	74	77	80	85	90	92	90
	# of experiments/groups	186/56	156/52	45	63	45	63	30	48	30	48

Table 1. Classification analysis for the different liver-based in vitro models used during the carcinoGENOMICS project.

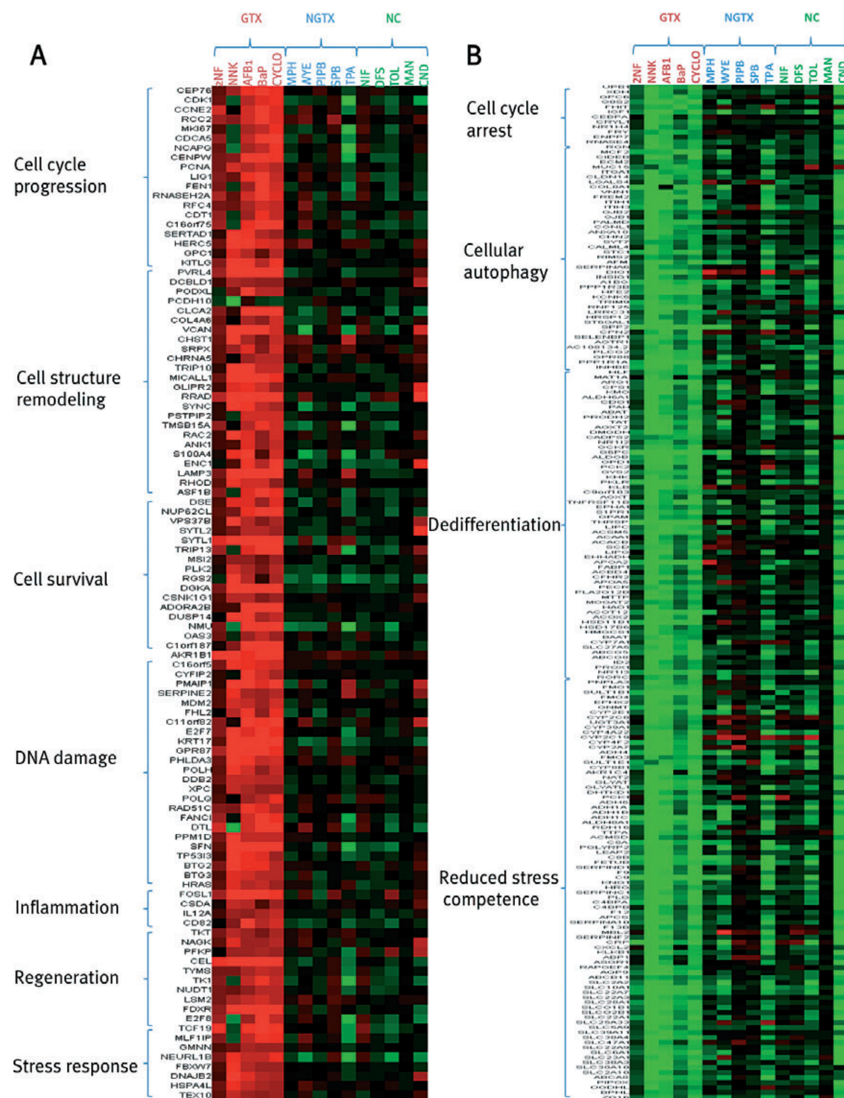


Fig. 3. Heatmap of the upregulated (A) and downregulated (B) genes, selectively affected following exposure to GTX carcinogens in the HepaRG cell model. The genes were separated into different toxicological categories.

(GTX, genotoxic; NGTX, non-genotoxic; NC, non-carcinogens; HepsC, conventional cultures of primary rat hepatocytes; HepsT, TSA-stabilized cultures of primary rat hepatocytes; hES-Hep, human embryonic stem cell-derived hepatocyte-like cells; 2NF, 2-nitrofluorene; AFB1, aflatoxin B1; BaP, Benzo(a)pyrene; CND, clonidine; CYCLO, Cyclophosphamide; MPH, methapyrilene hydrochloride; NIF, nifedipine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; MAN, D-mannitol; PIPB, piperonyl butoxide; SDF, diclofenac sodium; SPB, phenobarbital sodium; TOL, tolbutamide; TPA, tetradecanoyl phorbolacetate; WYE, Wy-14643)

Based on these results, the HepaRG cell line was chosen as the best performing human liver-based *in vitro* model and was further used in the second phase of the project, in which 15 additional compounds (5 of each group) were tested. These experiments were performed by the lead laboratory VUB. Once again, samples were taken following exposure to all compounds at IC10 doses for 24h and 72h. Very convincingly, the different data analysis approaches again led to a similar outcome, namely that GTX carcinogens could be separated at the gene and pathway level from NGTX carcinogens and NC (Fig. 4 and Fig. 5).

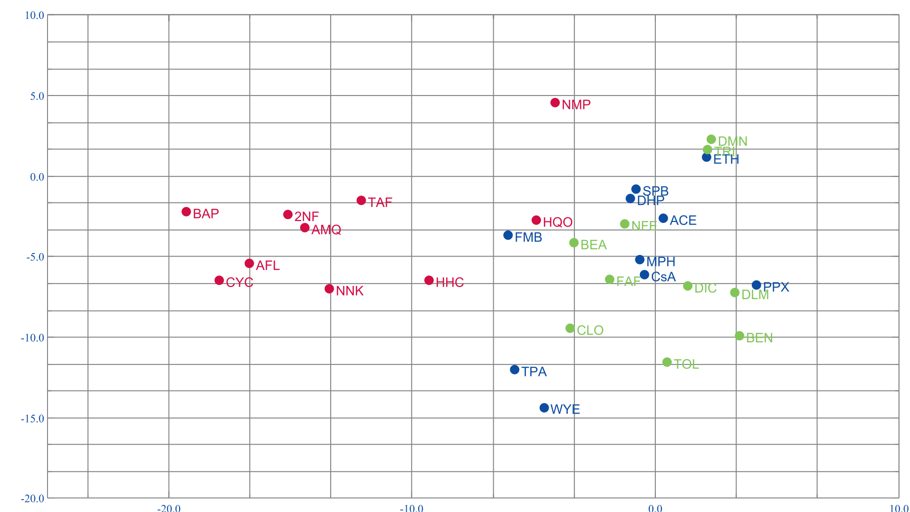


Fig. 4. Principle component analysis (PCA) based on pathways preselected after performing an ANOVA test of the results obtained for the compounds used in the first and second phase of the carcino*GENOMICS project in the HepaRG cells.*

(2NF, 2-nitrofluorene; ACE, Acetamide; AFL, aflatoxin B1; AMQ, IQ (2-amino-3-methylimidazo(4,5-f)quinoline); BaP, Benzo(a)pyrene; BEA, Benzyl alcohol; BEN, Benzoin; CLO, clonidine; CsA, Cyclosporine A; CYC, Cyclophosphamide; DIC, diclofenac sodium; DHP, Diethylhexylphthalate; DLM, D,L –Menthol; DMN, D-mannitol; ETH, Ethanol; FAF, 4-acetylaminofluorene; FMB, Fumonisin B1; HHC, Hydrazine dihydrochloride; HQO, Hydroquinone; MPH, methapyrilene hydrochloride; NFE, nifedipine; NMP, N-Nitrosomorpholine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PPX, piperonyl butoxide; SPB, phenobarbital sodium; TAF, 2-acetylaminofluorene; TOL, tolbutamide; TPA, tetradecanoyl phorbolacetate; TRI, Triclosan; WYE, Wy-14643)

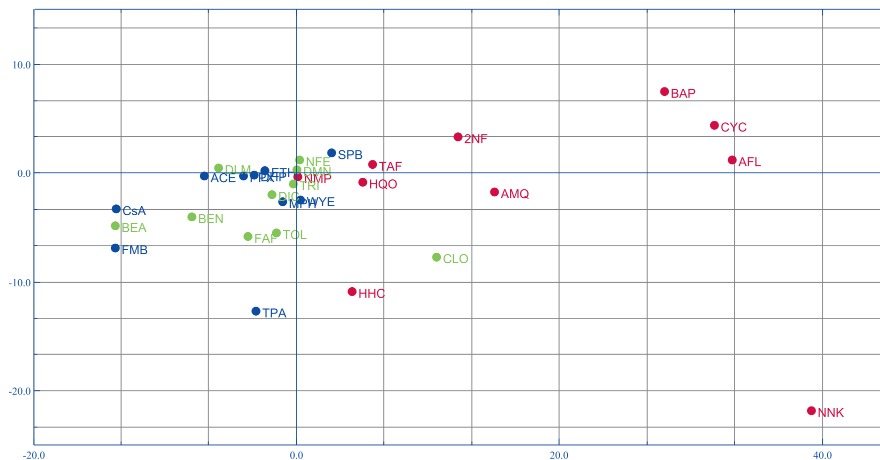


Fig. 5. Principle component analysis (PCA) based on genes preselected after performing an ANOVA test of the results obtained for the compounds used in the first and second phase of the* carcino**GENOMICS** *project in the HepaRG cells. *(2NF, 2-nitrofluorene; ACE, Acetamide; AFL, aflatoxin B1; AMQ, IQ (2-amino-3-methylimidazo(4,5-f)quinoline); BaP, Benzo(a)pyrene; BEA, Benzyl alcohol; BEN, Benzoin; CLO, clonidine; CsA, Cyclosporine A; CYC, Cyclophosphamide; DIC, diclofenac sodium; DHP, Diethylhexylphthalate; DLM, D,L –Menthol; DMN, D-mannitol; ETH, Ethanol; FAF, 4-acetylaminofluorene; FMB, Fumonisin B1; HHC, Hydrazine dihydrochloride; HQO, Hydroquinone; MPH, methapyrilene hydrochloride; NFE, nifedipine; NMP, N-Nitrosomorpholine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PPX, piperonyl butoxide; SPB, phenobarbital sodium; TAF, 2-acetylaminofluorene; TOL, tolbutamide; TPA, tetradecanoyl phorbolacetate; TRI, Triclosan; WYE, Wy-14643)*

This model can now be taken foreward in a proof of principle study to evaluate the usefulness of the **carcino**GENOMICS-generated reference gene biomarker set as a mechanistic follow-up of false positive findings in the EU standard battery of mutagenicity/genotoxicity tests of pharmaceuticals, cosmetics and chemicals in general.

In addition, it is worthwhile mentioning that CELL has - within the five years of **carcino**GENOMICS - developed a homogenous, reproducible, feeder-free monolayer culture of human embryonic stem cell-derived hepatocyte-like cells (hES-HEP). The hES-HEP display a morphology resembling primary hepatocytes and they express important hepatic markers and liver-related proteins.

The **carcino**GENOMICS project has shown that the hES-HEP can be produced with a robustness and reproducibility to allow repeated toxicity testing. The results indicate the potential for using human embryonic stem cell-derived hepatocyte-like cells as an *in vitro* model for hazard assessment of chemical carcinogenesis.

Another aspect of the project was the development of an *in vitro* system to potentially assess individual idiosyncratic reactions of chemicals. In standard *in vivo* risk assessment procedures for NGT compounds, extrapolation from animal to man takes a factor of 100 into account which covers inter- and intra-species kinetics and dynamics. In order to gain a better insight in the relevance of this default assumption when *in vitro* tests are involved, transcriptomic and metabonomic responses were investigated in a liver model made more susceptible to carcinogenicity by genetical manipulation of the enzymes playing a role in the bioactivation of procarcinogens.

Finally, an interlaboratory study was carried out in order to test the reproducibility/transferability of the HepaRG-based methodology, an important point in the validation process of an *in vitro* method.

Kidney Models – A Success Story

Partners involved: University College Dublin (UCD), Innsbruck Medical University (IMU), Liverpool John Moores University (LJMU).

The kidney cell model has been a real success story in the carcinoGENOMICS project. The model is ready to be taken forward to the next steps in developing a novel *in vitro* assay to detect carcinogens.

Initially, in the early stages of the project, the strategy of the 3 participating laboratories was to focus on the biology of the renal cells and provide a model reflecting the *in vivo* characteristics of the kidney. The cells were chosen to reflect the renal proximal tubule as this is the main site of renal tumours in humans. Three human renal cells were investigated, namely primary human renal cells, the HK-2 human renal cell line and the RPTEC/TERT1 a novel human cell line. The novel RPTEC/TERT1 cell was found to maintain excellent characteristics of the proximal tubule including transport capabilities and maintenance of a primary cilium. It also showed normal chromosomes and nuclear stability. It was therefore chosen as the human renal cell model. The NRK-52E cell line was chosen as the rat model in order to incorporate interspecies comparison and *in vitro* – *in vivo* comparison.

Both cell lines were challenged in phase 1 with 15 compounds including 5 GTX, 5 NGTX carcinogens and 5 NC and gene expression analysis and selected metabonomics were examined following 6, 24 and 72 hour exposure to the compounds at the IC10 concentrations for cell viability reduction at 72 hour. Following RNA extraction in the participating laboratories, the samples were forwarded for transcriptomics analysis. Both the NRK-52E rat cells and the human RPTEC/TERT1 performed very well in these assays in terms of being able to classify the 15 compounds into the respective classes of GTX, NGTX carcinogens and NC. However, in terms of pathway analysis, the RPTEC/TERT1 cells demonstrated more pathways which seemed to be intuitively more relevant to cancer development. Pathway analysis was able to more clearly separate the 15 compound into the respective 3 classes compared to gene based analysis. Also an initial interlaboratory study between UCD and IMU demonstrated excellent comparability of the results in the RPTEC/TERT1 cells. It was therefore decided to carry the RPTEC/TERT1 cells into phase 2 of the project, where the cells were challenged with an additional 15 compounds – 5 GTX, 5 NGTX carcinogens and 5 NC in the lead laboratory in UCD. Once again, RNA was extracted following with the 72 hour IC10 viability at the 6, 24 and 72 hour. The 30 compounds (total from phase 1 and phase 2) into the respective classes of GTX, NGTX and NC could be separated. While this could be done to some extent on gene-based PCA, the correct classification was more successful with a pathways-based PCA (Fig. 6 and Fig. 7).

An EURL-ECVAM supervised interlaboratory initial prevalidation study was carried out with 3 coded compounds and blinded to the 3 participating laboratories lead by UCD and also including IMU and LJMU. A training workshop was carried out

Fig. 6. Principle component analysis (PCA) based on pathways preselected after performing an ANOVA test of the results obtained for the compounds used in the first and second phase of the carcinoGENOMICS project in the RPTEC/TERT1 cells. *(2NF, 2-Nitrofluorene; ADA, 1-Amino-2,4-dibromoanthra-quinone; ARA, Aristolochic acid; BAP, Benzo[a]pyrene; BDM, Bromodichloromethane; BEA, Benzylalcohol; BEN, Benzoin; CIT, Citrinin ; CLO, Clonidine hydrochloride; CTN, Chlorothalonil; DIC, Sodium Diclofenac; DLM, D, L-Menthol; DMA, D-mannitol; EHN, N-Ethyl-N-(2-hydroxyethyl) nitrosamine; FAF, 4-Acetylaminofluorene; FMB, Fumonisin B1; GAL, Gallium arsenide; HQO, Hydroquinone; LAT, Lead (II) acetatetri hydrate; MON, Monuron; NFE, Nifedipine; NMP, N-Nitrosomorpholine; NT A, Nitrilotriacetic acid; OTA, Ochratoxin A; PBR, Potassium bromate; SDC, S-(1.2-dichlorovinyl)-L-cysteine; STZ, Streptoxotocin; TDP, Tris(2,3-dibromopropyl)phosphate; TOL, Tolbutamide; TRI, Triclosan)*

and SOPs were developed for all stages in the laboratory studies.
Additional experiments carried out in UCD showed that loss of the primary cilium from the RPTEC/TERT1 cells by carcinogens may be an additional useful assay to detect carcinogens in these cells. Work in UCD also demonstrated that many of the gene changes resulting from carcinogen exposure in the RPTEC/TERT1 cells are common to gene changes detected in human renal cancers.

Overall the results with the kidney cell culture model are optimal and the next steps are to bring this model assay further along the pathway towards development of a validated novel human cell assay to detect carcinogens.

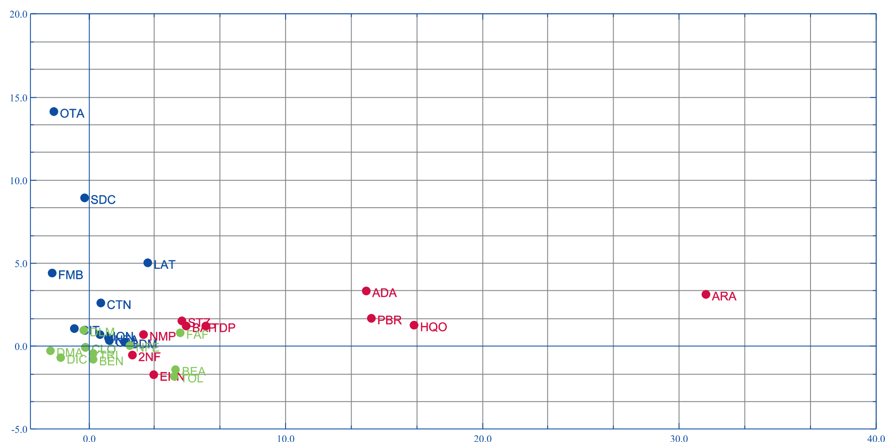


Fig. 7. Principle component analysis (PCA) based on genes preselected after performing an ANOVA test of the results obtained for the compounds used in the first and second phase of the carcinoGENOMICS project in the RPTEC/TERT1 cells. *(2NF, 2-Nitrofluorene; ADA, 1-Amino-2,4-dibromoanthra-quinone; ARA, Aristolochic acid; BAP, Benzo[a]pyrene; BDM, Bromodichloromethane; BEA, Benzylalcohol; BEN, Benzoin; CIT, Citrinin ; CLO, Clonidine hydrochloride; CTN, Chlorothalonil; DIC, Sodium Diclofenac; DLM, D, L-Menthol; DMA, D-mannitol; EHN, N-Ethyl-N-(2-hydroxyethyl) nitrosamine; FAF, 4-Acetylaminofluorene; FMB, Fumonisin B1; GAL, Gallium arsenide; HQO, Hydroquinone; LAT, Lead (II) acetatetri hydrate; MON, Monuron; NFE, Nifedipine; NMP, N-Nitrosomorpholine; NT A, Nitrilotriacetic acid; OTA, Ochratoxin A; PBR, Potassium bromate; SDC, S-(1.2-dichlorovinyl)-L-cysteine; STZ, Streptoxotocin; TDP, Tris(2,3-dibromopropyl)phosphate; TOL, Tolbutamide; TRI, Triclosan)*

% correctly classified experiments							
		RPTEC/TERT1			NRK-52E		
Categories	+- controls	# of experiments/groups	GC-RMA condensed	GC-RMA condensed & half-z normalized	# of experiments/groups	GC-RMA condensed	GC-RMA condensed & half-z normalized
All experiments							
tox class	+	189/48	79	75	192/52	88	81
	-	153/42	88	88	144/46	100	97
tox class & time point	+	189/48	68	59	192/52	84	78
	-	153/42	82	67	144/46	99	89
6h experiments							
tox class	+	63	70	58	60	75	74
	-	51	84	81	45	100	94
24h experiments							
tox class	+	63	68	56	60	90	93
	-	51	73	71	45	99	99
72h experiments							
tox class	+	63	86	73	72	92	90
	-	51	83	88	54	100	100

Table 2. Classification analysis for the different kidney-based in vitro models used during the carcinoGENOMICS project.

Lung Model - A lung epithelial cell-based test system for discriminating genotoxic and non-genotoxic carcinogens.

Partner involved: Novozymes (NZ), Leiden University Medical Center (LUMC).

During the course of the project, an immortal cell line maintaining its differentiation capacity was generated. This cell line could also maintain the same classifying capacity as observed with *in vitro* reconstructed lung tissue cultures (n=18, > 95%) without having the many variables which could disturb the transcriptomics analysis. These immortal lung tissue cultures are ready now for further validation.

In a first instance, a human bronchial model with relevant physiology was developed. While adapting to the cell culture conditions and establishing a 3-dimensional reconstituted tissue, the transcriptional changes induced in these primary cells were investigated. At the microscopic level, the cells were found to establish a physiologically relevant tight barrier with an *in vivo*-relevant Trans-Epithelial Electrical Resistance (TEER), functional tight junctions, beating cilia, mucus-production and xenobiotic-metabolizing enzyme activity.
The physiology of this *in vitro* tissue correlated well with that of human bronchial tissue *in vivo*. The number of passages, however, could not exceed 4.

Dose-finding experiments were performed and TEER was selected as the endpoint of choice.

The cell cultures were exposed baso-laterally for 24 and 72 hrs to concentrations corresponding to IC10 and IC10/2.

A number of exposure experiments with a potent carcinogen was carried out and it was found that a number of factors could disturb their outcome. These were the variability between the independent studies themselves, cell batches and cell donors. The exposure period did not add to the test performance variability.

Other variables were the water solubility of the compounds to be tested and alternatively the properties of the organic solvent to be used.

Eighteen solid compounds (6 GTX and 6 NGTX carcinogens and 6 NC) were tested. These were correctly classified (> 95%) in both repeats, by all 4 donors for both treatment times (Table 3), although the transcriptional responses were rather weak.

Classification and Cross Validation

Without controls > 95% correct

Failure controls due to DMSO

Confusion matrix and AUC	True NGTX	True GTX	True NC	True DMSO	True MED	Sum	Correct [%]		Tox class	AUC
LOO sampling without controls										
Predicted NGTX	111	2	1	-	-	114	97.37		NGTX	0.9982
Predicted GTX	1	107	3	-	-	111	96.40		GTX	0.9974
Predicted NC	0	3	108	-	-	111	97.30		NC	0.9969
Sum	112	112	112	-	-	-	-			
Correct [%]	99.11	95.54	96.43	-	-	-	-			
K-fold sampling without controls										
Predicted NGTX	1087	21	8	-	-	1116	97.40		NGTX	0.9977
Predicted GTX	16	1069	24	-	-	1109	96.39		GTX	0.9962
Predicted NC	17	30	1088	-	-	1135	95.86		NC	0.9964
Sum	1120	1120	1120	-	-	-	-			
Correct [%]	97.05	95.45	97.14	-	-	-	-			
LOO sampling with controls										
Predicted NGTX	111	4	1	2	0	118	94.07		NGTX	0.9977
Predicted GTX	1	102	2	1	1	107	95.33		GTX	0.9864
Predicted NC	0	4	108	4	0	116	93.10		NC	0.9925
Predicted DMSO	0	1	0	17	0	18	94.44		DMSO	0.9892
Predicted MED	0	1	1	0	23	25	92.00		MED	0.9983
Sum	112	112	112	24	24	-	-			
Correct [%]	99.11	91.07	96.43	70.83	95.83	-	-			
K-fold sampling with controls										
Predicted NGTX	1107	38	8	14	0	1167	94.86		NGTX	0.9974
Predicted GTX	10	1027	18	12	15	1082	94.92		GTX	0.9832
Predicted NC	3	35	1075	49	9	1171	91.80		NC	0.9906
Predicted DMSO	0	9	7	165	0	181	91.16		DMSO	0.9874
Predicted MED	0	11	12	0	216	239	90.38		MED	0.9978
Sum	1120	1120	1120	240	240	-	-			
Correct [%]	98.84	91.70	95,98	68.75	90.00	-	-			

Table 3. Classification analysis for the primary lung tissue model used in the* carcinoGENOMICS *project. DMSO: Dimethylsulfoxide treated controls; LOO: Leave One Out; MED: Untreated Control (medium); AUC: Area Under the Curve.

In order to eliminate the issue of variability and to assure the availability of a standardized easily accessible test system, efforts were undertaken to establish an immortalized cell line.

The selected strategy comprised the introduction of the human Tert gene in combination with suppression of the p16 gene in human lung cells.

The effect of immortalization was studied by comparing the transcription profiles of primary cells and immortalized cell lines. As expected, significant differences were observed between primary cells and immortalized cells.

The immortalized cells were then differentiated into air-exposed reconstituted lung tissue with functional characteristics that were similar to the properties of the corresponding tissue derived from primary cells. Exposure to IC10 concentrations of NC, NGTX and GTX carcinogens was carried out and gene profiles discriminating the 3 different compound classes could be identified, suggesting that immortalization did not significantly affected the differentiating and classifying capacity of the test model.

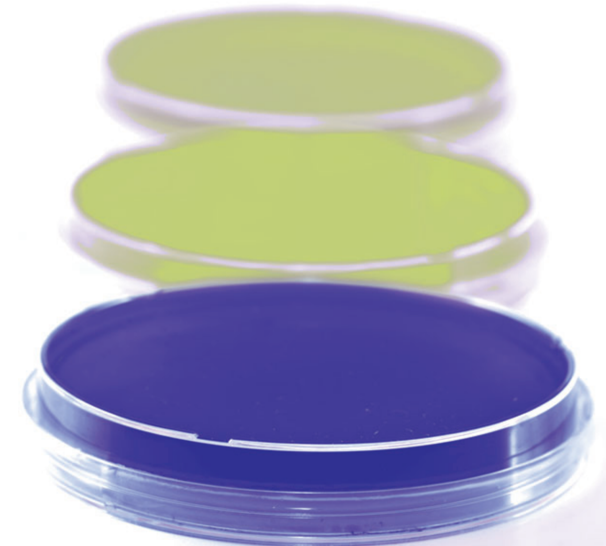
Interestingly, the most accurate identification was obtained for NGTX carcinogens, a chemical class that is difficult to assess with the currently available test models.

The immortalized tissue model is actually under study to allow the assessment of airborne substances (e.g. gasses and volatile substances).

Transcriptomics

Partner involved: Maastricht University (UM).

In order to minimize the bias between the different laboratories, the generation of the RNA samples was carried out according to standardized protocols. Following RNA extraction, the complementary targets were further prepared and hybridized at the Maastricht University. All procedures were performed according to the manufacturer's instructions on high-density oligonucleotide microarrays (i.e. Affymetrix RAT 230 2.0 GeneChip or Affymetrix U133 Plus 2.0 GeneChip). The bioinformatics analyses were further carried out by other partners of the **carcino**GENOMICS project, including Genedata (Basel), Max Plank Institute for Molecular Genetics (Berlin) and TNO (Zeist).



Metabonomics

Partners involved: Imperial College London (IC), Max Planck Institute for Molecular Genetics (MPIMG).

Metabolic characterisation of the *in vitro* cell systems used by the **carcino**GENOMICS project was another aspect of the project. The aim was to contribute to the overall understanding of the biochemical mechanisms involved, to integrate the metabolic data with other assays, such as transcriptomic profiling and to obtain a system wide overview of the cell systems and their response to chemical treatment.

Initially, detailed protocols were developed for obtaining reliable metabolic profiles of *in vitro* cell systems including culture media and intracellular metabolites. Nuclear Magnetic Resonance (NMR) metabolic profiles were generated from four liver and two kidney cell models and made available to the consortium. One of the main scientific achievements was the definition of the intra- and extracellular NMR metabonome of the RPTEC/TERT1 cell line and its response to model toxicants. Another significant advance was the demonstration of bile acid production in human embryonic stem cell-derived hepatocyte-like cells, indicating that the novel cell line also exhibits this critical aspect of the hepatic phenotype. Additionally, protocols and criteria for assessment of reproducibility of *in vitro* toxico-metabonomics experiments have been defined.

DEVELOPMENT OF CELL BIOINFORMATICS

Bioinformatics and Systems Biology

Partners involved: Nederlandse Organisatie voor toegepast-natuurwetenschappelijk onderzoek (TNO), Max Planck Institute for Molecular Genetics (MPIMG), Genedata, European Bioinformatics Institute (EBI).

As part of the development of suitable *in vitro* assays for carcinogenicity screening of chemicals for liver, kidney and lung as target organs, bioinformatics support needed to be provided to the consortium. This primarily concerned analysis of gene expression (transcriptome) data for the first phase of the project involving the selection of *in vitro* models for each target organ and for the second phase of the project, in which additional chemicals were tested with selected *in vitro* models. One of the first bioinformatics activities consisted of the collection and storage of metadata (descriptive experiment data in ISATAB format). This step allowed for tracking of the raw data, accurate normalization of the data towards corresponding solvent controls, proper annotation of toxicity classes as well as correction for possible confounding factors in downstream bioinformatics analyses. Several bioinformatics analyses where subsequently applied to the microarray data. After quality control and condensing of microarray raw data, partner Genedata employed extensive support vector machine-based classification and cross-validation approaches to several datasets from the consortium using its Expressionist® platform. Further, the effect on the classification outcome of different normalization approaches was investigated. Moreover, MPIMG applied ANOVA modeling to discover gene lists differentially expressed between the different toxicological classes. In addition, the MPIMG ConsensusPathDB interaction resource was used to characterize the response genes at the level of biological networks. TNO also contributed to the selection of the cell models, using ToxProfiler in which the overrepresentation of relevant gene sets in microarray data was analysed. MPIMG, in collaboration with Imperial College London, developed a novel technique and tool Integrated Molecular Pathway Level Analysis (IMPaLA) for integrative pathway analysis of multiple omics data sets and demonstrated its practical applicability by predicting the sensitivity of cancer cell lines for chemical toxicity. The work is a proof-of-concept study illustrating that metabolomic data provides crucial additional information beyond that provided by transcriptomics.

Optimisation - prevalidation

Partners involved: Imperial College London (IC), Maastricht Univerity (UM), University College Dublin (UCD), European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM), Biopredic International (BPI), Liverpool John Moores University (LJMU), Innsbruck Medical University (IMU).

Objectives

The second phase of the project focused on the optimisation of the most promising organ-specific omics-based assays.

During the last decade the field of toxicogenomics has expanded rapidly. However, to date there is still limited experience with the validation of toxicogenomics data from *in vitro* systems, especially with regard to the evaluation of their reproducibility. Moreover, a method to assess the reproducibility of *in vitro* metabonomics-based tests is also lacking. The **carcino**GENOMICS project offered an excellent platform for the investigation of the reproducibility of omics-based tests in general and for the assessment of various bioinformatics approaches. In collaboration with the bioinformatics workpackage, several approaches were identified to judge data reproducibility, ranging from evaluation of response gene lists over correlation analyses to multivariate statistical methods such as support vector machine classification and analysis of variance. For more information on the bioinformatics methodologies used, read the section on bioinformatics.

Based on transcriptome data and other established criteria, the most promising test methods used in phase 1 were selected. As such, 2 test methods were identified as having the highest potential for distinction of GTX and NGTX carcinogens and NC controls: the HepaRG model for the liver and the RPTEC/TERT1 model for the kidney. Both models are based on human cell lines. No preferred model for the lung was identified, as it was considered that the lung models needed further development. The objectives were to 1) assess test method transferability and between-laboratory reproducibility by using 3 coded chemicals in 3 laboratories for each test model, applying the same SOPs and controlled conditions, and 2) develop

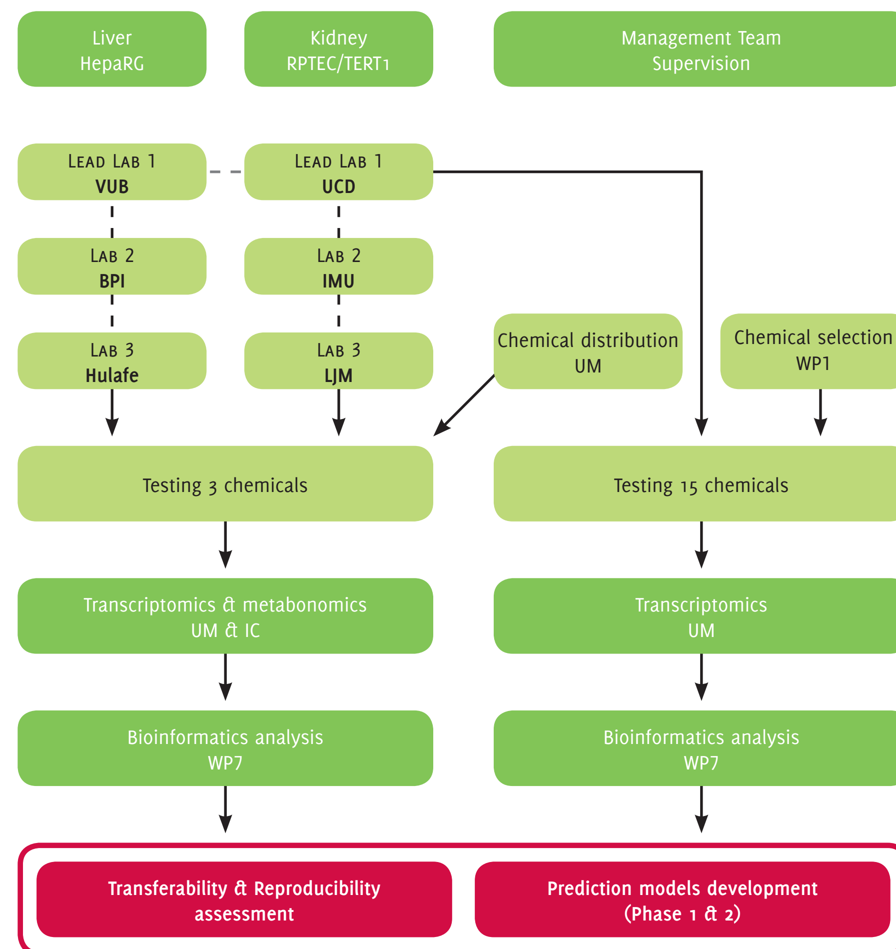


Fig. 8. Overview of WP9 study design
The liver HepaRG and the kidney RPTEC/TERT1 models were assessed for transferability and reproducibility, as well as for further development of the respective prediction models. For the analysis of inter-laboratory reproducibility, 3 laboratories tested three coded chemicals in each cell model, while the lead laboratories tested additional 15 chemicals to further build the prediction models. Transcriptomics and subsequent bioinformatics analyses were centralised and carried out by WP5 and WP7, respectively. Metabonomics analysis was conducted by IC for reproducibility assessment. The work was coordinated by EURL-ECVAM and supervised by a management team.

dedicated bioinformatics tools to serve as a basis for future validations of omics-based tests. The read-outs for the transferability and reproducibility assessments were transcriptomics and metabonomics (Fig. 8).

Preparatory work

The choice of the participating laboratories was based on expression of interest and experience with the test models. VUB and UCD were chosen as the lead laboratory for the liver and for the kidney model respectively.

As part of the transferability phase, the preparatory work included trainings of the participating laboratories on the respective test methods and agreement on and finalisation of the SOPs.

A Management Team (MT) comprising some of the project partners was nominated to supervise the inter-laboratory study. One of the first tasks of the MT was the selection of the chemicals for the transferability and between-laboratory reproducibility studies. The MT also monitored the work conducted in the laboratories to ensure that it was performed according to the agreed controlled conditions.

Experimental design

To assess transferability and reproducibility 3 coded chemicals were tested by 3 laboratories in each test model. The lead laboratories also tested an additional 15 chemicals (5 GTX and 5 NGTX carcinogens, 5 NC). For the HepaRG liver model, the experimental design was: one dose (IC10 at 72h), 2 time points (24h, 72h), 3 replicates. For the RPTEC/TERT1 kidney model, the experimental design was: one testing dose (IC10 at 72h), 3 time points (6h, 24h, 72h), 3 replicates.

The transcriptomics analysis was conducted at UM while the metabonomics analysis was carried out by IC. The purchase, coding and distribution of the test chemicals to the participating laboratories were under the responsibility of UM. An overview of the overall experimental design of the prevalidation work is given in Fig. 8.

Inter-laboratory transferability and reproducibility assessment

IC10 assessment

In both models the IC10 dose was established independently by each laboratory according to the agreed SOPs. However, before proceeding to the main experiments, the cytotoxicity results were compared across the laboratories and discussed to identify potential outliers, based on criteria defined by the lead laboratories. The calculation of IC10 appeared to be the most challenging step of the experimental phase of the study and was especially difficult for weak cytotoxic substances. During these experiments it was realised that the criteria for the evaluation of cytotoxicity required some refinement and better definition, which led to the amendment of the SOPs. Overall, the laboratories generated acceptable and comparable IC10 values for each of the compounds and could then proceed to the main experiments. Thus, each laboratory used its own IC10 values to carry out the main experiments and generate the samples for the transcriptomics and the metabolomics analyses.

Further development of prediction models

The lead laboratories tested 15 new chemicals in addition to those tested in phase 1 of **carcino**GENOMICS in order to further optimise the prediction model. New bioinformatic models to predict the 3 toxicity classes were developed, including all 30 compounds evaluated during Phase 1 and Phase 2. Subsequently, the same data set was used to classify the coded chemicals tested during the inter-laboratory study and assess the concordance of the predictions. More details about the classification models, their development and validation can be found in the section on Bioinformatics.

Reproducibility of omics data

Independent of the bioinformatics approaches applied, the HepaRG model generated reproducible transcriptomics results, with the exception of a single experiment in one laboratory. In this cell model the GTX carcinogen was classified correctly and reproducible in all laboratories. Although the overall results were

reproducible, the NGTX carcinogen could not be discriminated from the NC in all laboratories. After proper training and agreeing on a unified standard operating procedure, 3 coded compounds (belonging to the set of GTX previously analysed and with the following conditions : IC10, 72h exposure time) were blindly tested by 3 laboratories, namely VUB, HULAFE and BPI. As the results of the 3 laboratories were nicely reproducible, it can be concluded that the HepaRG cell line seems to be a robust and promising *in vitro* model especially when GTX carcinogens need to be identified.

Regarding the RPTEC/TERT1 model, 2 laboratories showed highly reproducible results, while one laboratory generated non-reproducible results. This outcome was in line with the experimental observations of much slower cell growth in comparison to the other laboratories. Interestingly, despite these results the 3 coded chemicals were classified in the correct classes by all laboratories, indicating that the prediction model is quite robust. Metabonomics analysis in the kidney model also showed a reproducible response for some metabolite patterns. IC10 determinations at 72 hours for the 3 coded compounds were carried out in each of the laboratories. The cells were then exposed to the respective IC10 values in each laboratory and RNA and supernatant/cell extracts for metabonomics collected at the 6, 24 and 72 hour time points. Metabonomics data was determined in Imperial College, London. During the period of the cell culture experiments, it became clear that there were some problems with the cell culture in LJMU as the cells were growing and reaching confluency and full differentiation at a much slower rate in LJMU. The metabonomics results also indicated some issues with the LJMU results. The bioinformatics results on the interlaboratory comparisons of gene changes also indicated that while the results from UCD and IMU were comparable, the results for gene changes in LJMU were not comparable with UCD and IMU. However despite this, a striking finding was that two different bioinformatics approaches showed that the 3 laboratories UCD, IMU and LJMU were able to correctly classify the 3 coded compounds into GTX carcinogens, NGTX carcinogens and NC. This finding is encouraging and suggests that the 'carcinogenic signal' in the RPTEC/TERT1 cells is robust and able to successfully classify carcinogens despite some noise in the system from a laboratory displaying some cell culture problems.

Overall, these results present a proof of concept that such *in vitro* models can be used for transcriptomics analysis.

Bioinfomatics approaches for reproducibility assessment

Finally, the **carcino**GENOMICS project seized the opportunity of this exercise to value the considerable amount of work achieved by the bioinformatics workpackage to develop and optimise dedicated bioinformatics tools to interpret -omics data. A Workshop on bioinformatics approaches for the evaluation of omics-based tests was held in Angera (Italy) in January 2012 to discuss and review the data produced and the possible approaches to be used in the validation (i.e. reproducibility assessment) of transcriptomics- as well as metabonomics-based tests. Overall, the various approaches used independently to analyse the inter-laboratory reproducibility led to consistent results. The demonstration that the different bioinformatics tools are not a source of result variability is reassuring, especially in view of future regulatory use of transcriptomics data. The outcome of this work will contribute to the drafting of a best practice document on bioinformatics approaches, which will represent a guide for future users. This document will also set the basis for the (pre)validation of such high-content test methods.

DISSEMINATION

Partners involved: European Consensus Platform for 3R-Alternatives (ecopa), Vrije Universiteit Brussel (VUB).

Besides of organizing the dissemination part of the project for the partners and the general public, as well as taking care of prevalidation and intellectual property issues, a main topic was the direct interaction with regulatory authorities from the very beginning to the very end of the project.

At the start of the project, it was tried to get feedback from regulators via questionnaires and interviews, but it became soon apparent that direct interaction with the respective experts of agencies, committees and working groups was highly necessary. This was done through several workshops (2008, 2009, 2010), one of them in an international setting at Venice (2009). With the final results becoming available in April 2012, the final dialogue-event with participation of shareholders, partners and agency representatives was realized/ organized in Maastricht (17th of April 2012).

All in all, 15 representatives of authorities and international bodies including the European Medicines Agency (EMA), the European Chemicals Agency (ECHA), the Organisation for Economic Co-operation and Development (OECD), the Scientific Committee on Consumer Safety (SCCS) and the Medical Products Agency (MPA) were involved. A detailed commentary publication addressed the international aspects and the way forward (Fig. 9).

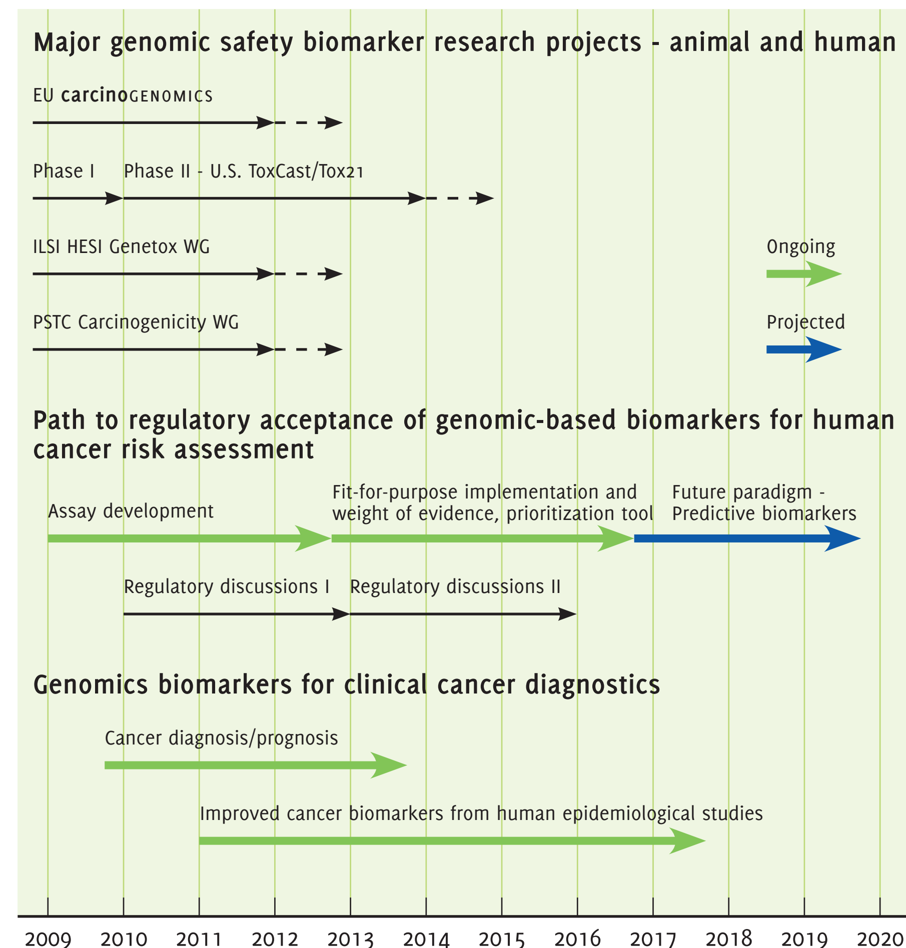


Fig. 9. Road map for human cancer risk assessment. (Paules et al, "Moving forward in human cancer risk assessment" Environmental Health Perspectives, 119 (6), 2011, 739-742).

CONCLUSIONS

The major aim of **carcino**GENOMICS was the development of *in vitro* methods for assessing the carcinogenomic potential of compounds, serving all kinds of purposes, i.e. the safety of chemical, pharmaceutical, consumer products etc.
Therefore, the discriminative power between GTX and NGTX carcinogens of a battery of mechanism-based *in vitro* tests covering the most important target organs, i.e. liver, lung and the kidney, was evaluated.

Although the degree of applicability and information depth of the potential tests identified, is different for the different organs, the overall task was achieved to a major extent. Thereby, a basis is laid for a number of follow-up activities, e.g. addressing testing of consumer goods such as cosmetics in much more detail, where animal testing for risk assessment is banned in the EU anyhow.
Of major importance was the very positive interaction with regulators of European registration and licensing authorities, including the competent agencies of Member States, early on in the project. Similarly, the worldwide context was brought in by organizing an international conference with participation of regulators of the US and others- a satellite to the world congress for alternatives in Rome.
These activities resulted in an intense dialogue between developers, potential users in industrial settings and regulators of authorities, who finally have to accept the applicability of novel alternative procedures for risk assessment in carcinogenesis, e.g. when faced by the submission documents from the enterprises in life sciences. This dialogue has to be pursued.

When highlighting major achievements, and the general conclusions to be drawn from these, the following has to be stated:

- a crucial step in this as in all FP projects regarding alternative methodology, is the identification of “reference” compounds, a series which can be used by others now,
- the mass of data out of the genomics analyses of all organ cell systems were collated and screened in new techniques and tools, e.g. an integrative pathway analysis of multiple –omics data sets, something that will assist analysis in future
- the *in vitro*-based assays for carcinogenicity screening of the diverse partners for liver, kidney and lung , analysed across the project finally enabled a prevalidation - on this basis, future activities can be easily conceived, and must be pursued in order to use the outstanding value of the project work and results
- the experimental expertise and procedural knowledge in cell systems and result analysing accumulated in this project and its partner groups, especially for kidney and liver, should be taken up in future R&D programme projects of the EU.

More specifically, the **carcino**GENOMICS consortium concludes that the toxico-genomics-based RPTEC/TERT1 kidney model is a promising novel tool for predicting renal genotoxicity/carcinogenicity *in vivo*. Where it adequately discriminates between GTX carcinogens, NGTX carcinogens and NC at fairly high accuracy, and has performed well in the course of the inter-laboratory reproducibility comparisons, it is ready for use by academic toxicologists and during early R&D developments within the chemical industry, e.g. for prioritizing interesting new chemicals. This assay is also ready to be subjected to relevant procedures to be ultimately accepted as an official guideline for testing for kidney genotoxicity/carcinogenicity.

Furthermore, the HepaRG liver model is able of outperforming classical *in vitro* genotoxicy assays, because of its capability of generating fewer miss-classifications and this model performed rather well during the inter-laboratory comparisons. In this sense, the HepaRG model is ready for use by academic toxicologists and during early R&D developments within the chemical industry, for predicting liver genotoxicity/carcinogenicity. However, its capacity for segregating NGTX liver carcinogens from NC is quite low; improving this will require follow-up studies.

The consortium furthermore believes that the human embryonic stem cell-derived hepatocyte model is promising and may bring added value for predicting liver carcinogenesis. This should be further explored.
The consortium also believes that the toxicogenomics-based human primary bronchial model for predicting lung genotoxicity/carcinogenicity is quite promising, certainly upon immortalization which will bypass the problem of considerable donor variability. Due to its unavoidably late development in the course of the project, this assay could be subjected to only a limited set of model compounds, so challenging it by larger number of test compounds is definitely warranted.

Lastly, in one of the first efforts ever, assessments of inter-laboratory repro-ducibility of toxico-genomics-based assays have been successfully protocoled and applied. For this, an integrated bioinformatics approach has been set in place, from which the toxico-genomics research community may considerably benefit in the near future.

Publication list carcinoGENOMICS April 2012

- Benet M., Lahoz A., Guzmán C., Castell J.V., Jover R., 2010, **CCAAT/enhancer-binding protein alpha (C/EBPalpha) and hepatocyte nuclear factor 4alpha (HNF4alpha) synergistically cooperate with constitutive androstane receptor to transactivate the human cytochrome P450 2B6 (CYP2B6) gene: application to the development of a metabolically competent human hepatic cell model.** *J Biol Chem.* 2010 Sep 10;285(37):28457-71.
- Bonora-Centelles A., Jover R., Mirabet V., Lahoz A., Carbonell F., Castell J.V., Gómez-Lechón M.J., 2009, **Sequential hepatogenic transdifferentiation of adipose tissue-derived stem cells: relevance of different extracellular signaling molecules, transcription factors involved, and expression of new key marker genes.** *Cell Transplant.* 18(12):1319-40.
- Cavill R., Keun H.C., Holmes E., Lindon J.C., Nicholson J.K., Ebbels T.M. **Genetic algorithms for simultaneous variable and sample selection in metabonomics.** *Bioinformatics.* 2009 Jan 1;25(1):112-8.
- Cavill R., Sidhu J.K., Kilarski W., Javerzat S., Hagedorn M., Ebbels T.M., Bikfalvi A., Keun H.C. **A combined metabonomic and transcriptomic approach to investigate metabolism during development in the chick chorioallantoic membrane.** *J Proteome Res. 2010* Jun 4;9(6):3126-34.
- Cavill R., Kamburov A., Ellis J.K., Athersuch T.J., Blagrove M.S., Herwig R., Ebbels T.M., Keun H.C. **Consensus-phenotype integration of transcriptomic and metabolomic data implies a role for metabolism in the chemosensitivity of tumour cells.** PLoS Comput Biol. 2011 Mar;7(3):e1001113.
- Ceelen L., Haesebrouck F., Vanhaecke T., Rogiers V., Vinken M., 2011, **Modulation of connexin signalling by bacterial pathogens and their toxins.** *Cellular and Molecular Life Sciences* 68: 3047-3064.
- Chan E.C., Koh P.K., Mal M., Cheah P.Y., Eu K.W., Backshall A., Cavill R., Nicholson J.K. and Keun H.C. **Metabolic profiling of human colorectal cancer using high-resolution magic angle spinning nuclear magnetic resonance (HR-MAS NMR) spectroscopy and gas chromatography mass spectrometry (GC/MS).** *J Proteome* Res 81 (2009): 352-61.
- Doktorova T., Vanhaecke T., Rogiers V., Vinken M., 2010, **Epigenetically stabilized primary hepatocyte cultures: a potential sensitive screening tool for non-genotoxic carcinogenicity.** *Methods in Bioengineering: Alternative Technologies to Animal Testing*, eds: Maguire T., Novik E., published by: Artech House, 133–145.
- Doktorova T., Doctoral thesis in Pharmaceutical Sciences entitled: **Evaluation of carcinogen-modified global gene expression profiles in liver-based *in vitro* models with focus on primary hepatocyte cultures?** Defended on 16th of March 2012 at the Vrije Universiteit Brussels - Belgium, Promotors: Prof. Rogiers V., Prof. Vanhaecke T. and Prof. Vinken M.
- Doktorova T., Pauwels M., Vinken M., Vanhaecke T., Rogiers V., 2012, **Opportunities for an alternative integrating testing strategy for carcinogen hazard assessment?** *Critical reviews in Toxicology*, 42: 91-106.
- Doktorova T., Ellinger-Ziegelbauer H., Vinken M., Vanhaecke T., Van Delft J., Kleinjans J., Ahr H.-J., Rogiers V., 2012, **Comparison of hepatocarcinogen-induced gene expression profiles in conventional primary rat hepatocytes with *in vivo* rat liver. Archives of Toxicology**, April 8.
- Donato M.T., Hallifax D., Picazo L., Castell J.V., Houston J.B., Gomez-Lechón M.J., Lahoz A., 2010, **Metabolite formation kinetics and intrinsic clearance of phenacetin, tolbutamide, alprazolam, and midazolam in adenoviral cytochrome P450-transfected HepG2 cells and comparison with hepatocytes and *in vivo*.** *Drug Metab Dispos.*:1449-55.
- Ebbels T.M., Lindon J.C., & Coen M., 2011, **Processing and modeling of nuclear magnetic resonance (NMR) metabolic profiles.** *Methods in Molecular Biology*, 708, 365-388.
- Ebbels T.M.D. & Cavill R., 2009, **Bioinformatic methods in NMR-based metabolic profiling.** *Progress in Nuclear Magnetic Resonance Spectroscopy*, 55, 361-374.
- Ellis J.K., Chan P.H., Doktorova T., Athersuch T.J., Cavill R., Vanhaecke T., Rogiers V., Vinken M., Nicholson J.K., M.D. Ebbels T., Keun H.C, **Effect of the histone deacetylase inhibitor trichostatin a on the metabolome of cultured primary hepatocytes.** *J Proteome Res.* 2010 Jan;9(1):413-9.
- Ellis J.K., Athersuch T.J., Cavill R., Radford R., Slattery C., Jennings P., McMorrow T., Ryan M.P., Ebbels T.M.D., and Keun H.C., 2011, **Metabolic response to low-level toxicant exposure in a novel renal tubule epithelial cell system.** *Molecular Biosystems 7*, 247-257.
- Gómez-Lechón M.J., Castell J.V., Donato M.T., 2010, **The use of hepatocytes to investigate drug toxicity.** *Methods Mol Biol.* 2010;640:389-415.
- Hector C. Keun & Toby Athersuch, **Nuclear Magnetic Resonance (NMR)-based metabolomics.** *From Metabolic Profiling: Methods & Protocols.* Ed. Thomas O. Metz. Humana Press, 2011.
- Jennings P., Aydin S., Bennett J., McBride R., Weiland C., Tuite N., Gruber L.N., Perco P., Gaora P.O., Ellinger-Ziegelbauer H., *et al.*, 2009, **Inter-laboratory comparison of human renal proximal tubule (HK-2) transcriptome alterations due to Cyclosporine A exposure and medium exhaustion.** *Toxicology in vitro 23*, 486-499.
- Jennings P., Weiland C., Limonciel A., Bloch K., Radford R., Aschauer L., McMorrow T., Pfaller W., Ahr H.J., Slattery C., *et al.*, 2011, **Transcriptomic alterations induced by Ochratoxin A in rat and human renal proximal tubular *in vitro* models and comparison to a rat *in vivo* model.** *Arch Toxicol.* 2012 Apr; 86(4):571-89.

- Kamburov A., Pentchev K., Galicka H., Wierling C.K., Lehrach H., Herwig R., 2010, **ConsensusPathDB - towards a more complete picture of cell biology.** *Nucleic Acids Res* 39(1):712-717.
- Kamburov A., Cavill R., Ebbels T.M.D., Herwig R., and Keun H.C., 2011, **Integrated pathway-level analysis of transcriptomics and metabolomics data with IMPaLA.** *Bioinformatics* 27(20):2917-2918.
- Keun H.C., Athersuch T.J., Beckonert O., Wang Y., Saric J., Shockcor J.P., Lindon J.C., Wilson I.D., Holmes E., Nicholson J.K., **Heteronuclear 19F-1H statistical total correlation spectroscopy as a tool in drug metabolism: study of flucloxacillin biotransformation.** *Anal Chem. 2008* Feb 15;80(4):1073-9.
- Klipp E., Liebermeister W., Wierling C., Kowald A., Lehrach H., Herwig R., 2009, **Systems biology – a textbook**. Wiley-Blackwell Weinheim, 569 p., ISBN: 978-3-527-31874-2.
- Limonciel A., Aschauer L., Wilmes A., Prajczer S., Leonard M.O., Pfaller W., and Jennings P., 2011, **Lactate is an ideal non-invasive marker for evaluating temporal alterations in cell stress and toxicity in repeat dose testing regimes**. *Toxicoloy In vitro*. 2011 Dec;25(8):1855-62.
- Marfil V., Moya M., Pierreux C.E., Castell J.V., Lemaigre F.P., Real F.X., Bort R., 2010, **Interaction between Hhex and SOX13 modulates Wnt/TCF activity.** *J Biol Chem*. 2010 Feb 19;285(8):5726-37.
- Parry J.D., Pointon A.V., Lutz U., Teichert F., Charlwood J.K., Chan P.H., Athersuch T.J., Taylor E.L., Singh R., Luo J., Phillips K.M., Vetillard A., Lyon J.J., Keun H.C., Lutz W.K., Gant T.W. **Pivotal role for two electron reduction in 2,3-dimethoxy-1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone metabolism and kinetics *in vivo* that prevents liver redox stress.** *Chem Res Toxicol. 2009* Apr;22(4):717-25.
- Pascual M., Gómez-Lechón M.J., Castell J.V., Jover R., 2008, **ATF5 is a highly abundant liver-enriched transcription factor that cooperates with constitutive androstane receptor in the transactivation of CYP2B6: implications in hepatic stress responses**. *Drug Metab Dispos*. 36(6):1063-72.
- Radford R., Slattery C., Jennings P., Blaque O., Pfaller W., Gmuender H., Van Delft J., Ryan M.P., McMorrow T. **Carcinogens induce loss of the primary cilium in proximal tubular epithelial cells independent of effects on cell cycle**. *Am J Physiol Renal Physiol*. 2012 Apr;302(8):F905-16.
- Rasche A., Al-Hasani H., Herwig R., 2008, **Meta-analysis approach identifies candidate genes and associated molecular networks for type-2 diabetes mellitus.** *BMC Genomics* 9:310.
- Rasche A., Yildirimman R. and Herwig R., 2010, **Integrative Analysis of microarray data - a path for systems toxicology**. *In Handbook of Systems Toxicology* (Eds. D. A. Casciano and S. C. Sahu). John Wiley & Sons Ltd., ISBN-13: 978-0-470-68401-6.

- Sansone S.A., Rocca-Serra P., Field D., Maguire E., Taylor C., Hofmann O., Fang H., Neumann S., Tong W., Amaral-Zettler L., Begley K., Booth T., Bougueleret L., Burns G., Chapman B., Clark T., Coleman L.A., Copeland J., Das S., de Daruvar A., de Matos P., Dix I., Edmunds S., Evelo C.T., Forster M.J., Gaudet P., Gilbert J., Goble C., Griffin J.L., Jacob D., Kleinjans J., Harland L., Haug K., Hermjakob H., Ho Sui S.J., Laederach A., Liang S., Marshall S., McGrath A., Merrill E., Reilly D., Roux M., Shamu C.E., Shang C.A., Steinbeck C., Trefethen A., Williams-Jones B., Wolstencroft K., Xenarios I., Hide W. **Toward interoperable bioscience data.** *Nat Genet.* 2012 Jan 27;44(2):121-6.
- Teahan O., Bevan C.L., Waxman J., Keun H.C. **Metabolic signatures of malignant progression in prostate epithelial cells.** *Int J Biochem Cell Biol. 2011* Jul;43(7):1002-9.
- Vinken M., Doktorova T., Ellinger-Ziegelbauer H., Ahr H.J., Lock E., Carmichael P., Roggen E., van Delft J., Kleinjans J., Castell J., *et al.*, 2008. **The carcinoGENOMICS project: critical selection of model compounds for the development of omics-based *in vitro* carcinogenicity screening assays.** *Mutat Res* 659, 202-210.
- Vinken M., De Rop E., Decrock E., De Vuyst E., Leybaert L., Vanhaecke T., Rogiers V., 2009, **Epigenetic regulation of gap junctional intercellular communication: more than a way to keep cells quiet?** *Biochimica et Biophysica Acta Reviews on Cancer* 1795: 53-61.
- Vinken M., Decrock E., De Vuyst E., Leybaert L., Vanhaecke T., Rogiers V., 2009, **Biochemical characterization of an *in vitro* model of hepatocellular apoptotic cell death.** *Alternatives to Laboratory Animals* 37: 209-218.
- Vinken M., Doktorova T., Decrock E., Leybaert L., Vanhaecke T., Rogiers V., 2009, **Gap junctional intercellular communication as a target for liver toxicity and carcinogenicity.** *Critical Reviews in Biochemistry and Molecular Biology* 44: 201-222.
- Vinken M., Decrock E., Leybaert L., Vanhaecke T., Rogiers V., 2010, **Proteolytic cleavage of adherens junction components during Fas-dependent cell death in primary cultures of rat hepatocytes.** *ALTEX* 27: 151-157.
- Vinken M., Ceelen L., Vanhaecke T., Rogiers V., 2010, **Inhibition of gap junctional intercellular communication by toxic metals.** *Chemical Research in Toxicology* 23: 1862-1867.
- Vinken M., Decrock E., De Vuyst E., Ponsaerts R., D'hondt C., Bultynck G., Ceelen L., Vanhaecke T., Leybaert L., Rogiers V., 2011, **Connexins: sensors and regulators of cell cycling.** *Biochimica et Biophysica Acta Reviews on Cancer* 1815: 13-25.
- Vinken M., Decrock E., Bultynck G., Vanhaecke T., Leybaert L., Rogiers V., 2012, **Connexin43 signalling contributes to spontaneous apoptosis in cultures of primary hepatocytes.** *Toxicological Sciences* 125: 175-186.
- Vinken M., Vanhaecke T., Rogiers V., 2012, **Primary hepatocyte cultures as tools in drug development: quo vadis?** *Toxicology in Vitro* 26: 541-544.

- Vinken M., Pauwels M., Ates G., Vivier M., Vanhaecke T., Rogiers V., 2012, **Screening of repeated dose toxicity data present in SCC(NF)P/SCCS safety evaluations of cosmetic ingredients.** *Archives of Toxicology* 86: 405-412.
- Vinken M., Buyl K., Vanhaecke T., Rogiers V., 2012, **Effects of RNA interference-mediated suppression of connexin43 production on the expression of differentiation markers in cultures of adult primary rat hepatocytes.** *ALTEX Proceedings* 1: 155-164.
- Wierling, C., Herwig, R., and Lehrach, H., 2007, **Resources, Standards and Tools for Systems Biology.** *Brief Funct Genom Proteom* 6(3):240-51.
- Wierling C., Kühn A., Hache H., Daskalaki A., Maschke-Dutz E., Mareva S., Li J., Herwig R., and Lehrach H., 2012, **Prediction in the face of uncertainty: A Monte Carlo-based approach for systems biology of cancer treatment.** *Mutat Res.* 2012 Aug 15;746(2):163-70.
- Wieser M., Stadler G., Jennings P., Streubel B., Pfaller W., Ambros P., Riedl C., Katinger H., Grillari J., and Grillari-Voglauer R., 2008, **hTERT alone immortalizes epithelial cells of renal proximal tubules without changing their functional characteristics.** *Am J Physiol Renal Physiol 295*, F1365-1375.
- Wilmes A., Crean D., Aydin S., Pfaller W., Jennings P., and Leonard M.O., 2011, **Identification and dissection of the Nrf2 mediated oxidative stress pathway in human renal proximal tubule toxicity.** *Toxicology in vitro 25*, 613-622.
- Yildirimman R., Brolén G., Vilardell M., Eriksson G., Synnergren J., Gmuender H., Kamburov A., Ingelman-Sundberg M., Castell J., Lahoz A., Kleinjans J., van Delft J., Björquist P., Herwig R., 2011, **Human embryonic stem cell derived hepatocyte-like cells as a tool for *in vitro* hazard assessment of chemical carcinogenicity.** *Toxicological Sciences* 124(2): 278-290.