

COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

Toxicogenomics: A selective update of the literature since 2004

At the 2006 horizon scanning exercise, the Committee reviewed a small number of papers which had been published in the previous year on toxicogenomics. It decided that it would be useful to have an update in 2 areas: whether toxicogenomics was useful in distinguishing between genotoxic and non-genotoxic chemicals, and whether it is useful in testing the hypothesis for a mode of action or of help in proposing a mode of action for a chemical. It was suggested that this could be a joint project with the COM and COT.

As a first step, the Department of Health Toxicology Unit at Imperial College London has identified a sample of papers from the literature which address these areas, for the committee to consider. These include papers on the mechanism of arsenic-induced transplacental hepatotoxicity in mice; on the mechanism of induction of renal tumours by ochratoxin A; models of hepatocellular carcinoma development; and on differences in gene expression between genotoxic and non-genotoxic carcinogens. However, it is concluded that, without an intimate knowledge of the methodology and approaches used, it is difficult to comment on the quality of the studies reviewed. Moreover, the use of different microarrays by different authors, and selection of different genes for study, makes comparisons between studies difficult.

The committee is asked to consider the attached paper and to address the following questions:

- Based on the sample of studies presented here, how effective do members consider these methods currently to be in predicting carcinogenicity and in proposing or confirming a mechanism of action?
- Members are asked whether the existing statement on toxicogenomics requires revision based on the information presented. Are there any further considerations which require attention, such as input into global initiatives, or whether further work needs to be undertaken in this area.

Secretariat

June 2007

TOXICOGENOMICS: A SELECTIVE UPDATE OF THE LITERATURE SINCE 2004

1. The Committee considered the topic of Toxicogenomics during its horizon scanning exercise in November 2006 and it decided that a further update was warranted but recommended this should be restricted to considering the role of toxicogenomics in predicting carcinogenicity and in confirming or proposing a mechanism of action. The previous update was presented and discussed in 2004 and a COT/COC/COM joint statement was issued. The following overall conclusions are detailed in the appended statement (Appendix A).
2. A large number of papers were retrieved during this review, covering a wide variety of investigative aims. It was not possible to describe them all in the current review, and this paper summarises a representative selection attempting to cover the use of different models (*in vivo* and *in vitro*), carcinogen category identification, dose response determinations and mechanistic investigations. Appendix B contains full copies of a selection of these papers. The use of statistical methods and bioinformatics in toxicogenomics has not been fully addressed as it warrants a more detailed review which is under discussion with Dr David Lovell.
3. There are currently a number of international initiatives addressing the usefulness and robustness of toxicogenomics approaches in chemical risk assessment ongoing. A recent OECD progress report and the FDA MAQC project are briefly summarised. The carcinoGENOMICS project is being considered separately.

Investigation of Arsenic induced carcinogenesis

4. In the last review a paper which described aberrant gene expression in liver tumours of mice, particularly males, exposed to inorganic arsenic (As) during gestation was presented (Liu et al 2004). Exposure to inorganic arsenic *in utero* in C3H mice produces hepatocellular carcinoma in male offspring when they reach adulthood. Since then, a number of studies originating from the same group have been published which extend the investigations reported therein and further attempt to elucidate the mechanisms involved in the observed transplacental hepatocellular carcinogenesis .

Chen et al 2004

5. As prior work implicates the role of DNA hypomethylation as a causative factor in As-induced tumourigenicity, this study was designed to assess alterations in methylation status and associated aberrant gene expression. Although this study did not use the offspring of treated dams, it contributes to the overall understanding of As-induced carcinogenic mechanisms.

6. Method: 129/SvJ mice were given sodium arsenite in the drinking water at 45ppm for 48 weeks. The livers were taken and global DNA methylation determined by 'methyl acceptance assay' and HPLC of deoxyribonucleosides gene specific methylation analysis. Microarray was conducted using pooled RNA (4/group)- tRNA converted to ³²P-labelled cDNA probes which were purified using Clontech Atlas mouse cDNA syntheses primer mix. Microarray membrane was prehybridised with Expresshyb (Clontech). RT-PCR was also conducted on selected genes.

7. Of the 588 genes on the array, 30 were aberrantly expressed. These included genes involved in cell cycle regulation (eg cyclins D 1,2 and 3), growth factors (eg EGFR, ER α , IGF BP-1), apoptosis (caspases) and stress related genes (NF κ β , p65, p105). Expression of ER α and cyclin D1 genes were considered to be of particular significance from a mechanistic perspective and induction of these was confirmed by RT-PCR at the expression level. The methylation status of these genes was also assessed; a decrease in methylation by up to 90% was noted, considered to be responsible for the gene overexpression. Surprisingly, the authors did not appear to study genes specifically involved in methylation.

Liu et al 2006a

8. C3H mice were given As in drinking water at 85ppm from day 8 to 18 of gestation. Samples of hepatocellular carcinoma (HCC) and surrounding tissues from off-spring and liver from untreated control animals were taken and RNA processed using

microarray Agilent mouse 22K oligo assay. The software system Rosetta Resolver¹ was used to generate intensity plots – signature genes were those with $p < 0.001$.

9. Ingenuity Pathway Analysis² used Fischers exact test, with a 1.5-fold cut-off and $p < 0.001$, to identify differentially expressed genes. Interactions based on ‘focus genes’ provided a score for each network and biological functions assigned.

10. Under the Rosetta criteria, 2010 genes were altered in As exposed normal tissue compared to control tissue and 2540 in As exposed HCC tissue. Clustering revealed similar patterns as regards up and down regulated genes in normal and HCC tissues from treated off-spring, although qualitatively more pronounced in tumour tissue. However the similarities in gene expression changes between tumour and non tumour tissue implies that the pre-natal exposure induced changes persist throughout the tissue, some of which are likely associated with the carcinogenic process.

11. The following principle gene clusters were identified: oncogenes (*c-myc*, *c-met*, *k-ras* Npn3), cell cycle regulators (including IGF, cyclins D1 & E, PCNA), stress response genes (GST's EGR-1, SOD-1), metabolism (feminisation pattern of CYP, ER- α) and cell communication (E-cadherin, β -catenin). A number of HCC biomarkers were also increased, including AFP, PAI-1. The consequences of genetic reprogramming during critical development stages leading to carcinogenesis in adults are discussed.

Liu et al 2007

12. This follow up study was designed to investigate the involvement of estrogenic mechanisms and ‘feminisation’ as discussed in the previous papers.

13. Pregnant C3H mice were given drinking water containing 85ppm arsenic from days 8-18 of gestation and foetuses were then removed. Male liver RNA was analysed using Agilent 22K chip microarray, statistical analysis utilised Rosetta resolver (genes considered to be signature genes if $p < 0.001$) and clustering analysis was performed (method not stated).

14. Using Rosetta Resolver, 187 of 22,000 genes were significantly altered by As exposure. Clustering revealed induction of genes related to oestrogen signalling, including marked increases in *Agr2* (134-fold), *Spr2a* (125-fold) *Tff1* (31-fold), *CRP-ductin*, *ghrelin* (*Ghrl*) *cytokeratin Krt-1*, *Xist* and *cholesterol binding protein* (*Cbg*). Genes related to steroid metabolism showed marked expression changes, this included increased *HSD17 β 7* and decreased *HSD11 β 1* and *Akr1c18*. Genes involved in methyl metabolism decreased and P450 enzyme expression were also reduced. Due to the way in which the results were reported (i.e focus on oestrogen and steroid related genes only) it was not possible to compare directly genes involved in other processes or those

¹ A commercially available customised gene expression data management and analysis system.

² A commercially available programme which identifies and maps biological pathways, molecular mechanisms and process from gene expression data.

discussed in other papers. However, there is sufficient evidence for the authors to conclude that steroid metabolism and oestrogen signalling pathways are involved in genetic reprogramming *in utero* which may lead to carcinogenesis in later life.

Liu et al 2006b

15. Another follow up study was carried out in which C3H mice given As in drinking water at 85ppm as before were additionally treated with 12-O-tetradecanoyl phorbol-13-acetate (TPA) for 21 weeks following weaning (2µg/twice a week to shaved skin). This dosing protocol produces HCC in female off-spring as well as males, and therefore was considered to provide an opportunity to compare molecular events and gene changes with those when As acts as a complete carcinogen in males.

16. Livers and HCC tissue of off-spring from TPA-treated animals, with or without prior As exposure, were removed and RNA assessed using a custom designed Microarray (Clontech : 588 genes). Pooled samples were prepared in triplicate. Images were analysed using Atlas image software and expression levels quantified using real time RT-PCR. Clustering was applied but the methodology was not detailed.

17. Using a combined criteria of 2-fold increases and $p < 0.05$ for significance, 70 genes were significantly altered by TPA/As treatment. Clustering revealed that non-tumour and tumour tissue from TPA/As males and females had largely similar gene alteration patterns. Gene expression changes of note included: basal expression of PAI-1 and cycD1 significantly higher in females than in males, and As/TPA induced a further increase. Cdk2na was increased in both male and female HCC. There were no apparent changes in c-myc expression. A feminisation of metabolic enzymes was apparent in male livers and tumour tissue with significant increases in CYP2A4, 2B9. In females, lower expression of male predominant CYP2F2 and 7B1, was further decreased after As/TPA treatment. Expression of IGFII was increased in male and female tumour tissue. Basal expression of IGF-binding proteins (1 and 3) was higher in females, and As/TPA exposure increased this in both females and males. Other changes of note were increased expression of GST's and EGR1 in both male and female livers.

18. In summary:

Together, these papers provide an example of how toxicogenomics methodologies are being used to elucidate carcinogenic mechanisms. As follow up studies to the original general observation of altered gene expression changes following *in utero* exposure to As, the authors have addressed the hypotheses of altered methylation and oestrogen signalling as potential modes of action of As-induced transplacental carcinogenesis. However, even within the same laboratory, different arrays and statistical methods have been used in different studies which, although all apparently robust, must diminish the opportunity to directly compare results from study to study.

Papers revealing mechanistic insights of ochratoxin A carcinogenicity

Marin-Kuan et al 2006 (Appendix B):

19. With a view to investigating the potential mechanism of ochratoxin A (OTA)-induced renal carcinomas, groups of F344 rats (n=4) were administered OTA in the diet (calculated to average 300µg/kg/day) for 7 or 21 days or 4, 7, or 12 months. Concurrent controls were included for each group. Kidney and liver cRNA was hybridised to GeneChip cartridge and analysed using a Affymetrix RG-U34A platform containing 8,799 probe sets. Differences in gene expression were assessed using ANOVA (Global Error Assessment method) based on log-transformed signal values comparing treatment, time, and interaction between treatment and time. Gene clustering was obtained using Spotfire software following UPGMA clustering and Tanimoto similarity measures. Genes significant at $p < 0.001$ at at least two time-points were included in dendrograms. The variability in gene modulation by OTA over time was assessed using a PCA technique, the singular value decomposition (SVD) method³. Selected genes were subjected to RT-PCR and correlation between mRNA and protein expression confirmed by Western Blotting.

20. A total of 470 genes in kidney and 233 in the liver were differentially modulated ($p < 0.001$ at at least two time-points), although generally less than 2-fold, with 45 genes common to both organs. Clustering revealed that greatest similarities were, interestingly, the 7 day and 12 month time-points with no distinct correlation by time (e.g in kidney, next similar was the 7 month point followed by the 21 days and 4 months). Gene down regulation was more common than up regulation, with 60 and 56% of altered genes decreased in kidney and liver respectively. SVD analysis showed eight clusters which exhibited time-dependent expression profiles. The two main clusters were either continuously up regulated (31 and 25% in kidney and liver respectively) or down regulated (47% kidney, 27% liver).

21. Gene grouping according to biological functions indicated a wide diversity of alterations. In the kidney, genes involved in xenobiotic metabolism and oxidative stress responses were generally down-regulated. It is noted that many of these have a common promoter targeted by hepatocyte nuclear factor α (HNF4 α) and the mRNA specific for HNF4 α was itself down regulated. Other notable gene expression changes were transporter-related genes (a number of solute carriers, Slc 3, 21, 22) and some genes recognised as markers of kidney injury, regeneration and oncogenesis were up regulated (eg KIM-1, c-myc, Akt-1, Cdkn1a). Regucalcin (REG), known as senescence marker protein 30, was down regulated by 10-fold. Only small changes were noted in the expression of genes involved in DNA synthesis and repair and similarly, little effects on the expression of apoptosis-related genes.

³ This method provides a low-dimensional projection of the original data set which can be interpreted as the relative frequencies with which a given gene is modulated at every one of the original single time points which, when combined, form the temporal modes of gene modulation.

22. The authors discuss their findings in relation to identifying an epigenetic or a genotoxic mode of action for OTA. Firstly, distinct patterns were observed in the liver and kidney reflecting the organ specificity of OTA's carcinogenicity and its known active transport into the kidney. KIM-1, highly expressed in early stage renal tubule damage and regeneration, exhibited maximal expression at 21 days, although there was no histological evidence of proliferation at the dose level used. Inhibition of protein synthesis is implicated in OTA toxicity; however only a few genes were altered, namely, prostaglandin F2 receptor negative regulator, and eukaryotic translation initiation factor 4E binding protein (negative regulation of protein synthesis). The notable down regulation of regucalcin is considered to be compatible with the effects of OTA on Ca²⁺ homeostasis. Transport proteins Oat1, Oat-k1 and Oat-p1 are known to transport OTA; here they were down regulated suggesting an impact on toxicokinetics. Genes implicated in genotoxicity were generally not altered.

23. Considered of importance is the involvement of the transcription factor HNF4 α although the biological significance is unclear. Also, a number of stress related genes sharing the antioxidant response element (ARE) recognised by Nrf2 family of transcription factors which are involved in redox status and cellular defence against oxidative damage. The authors discuss a number of possible ways in which this could be of significance in addressing OTA mode of action.

Arbillaga et al 2007: (Appendix B)

24. This study investigated changes in OTA induced gene expression changes *in vitro* and provides an opportunity to compare with the data obtained *in vivo* by Marin Kuan (2006) with regard to mode of action investigations.

25. HK-2 cells (human renal proximal tubule cells) were cultured and treated with OTA (50 μ m, based on induction slight cytotoxicity) for 6 or 24 hours. Cell RNA was hybridised to Human Genome U133 A 2.0 Gene Chip which contains 22,277 probe sets. Expression data were analysed using BRB-ArrayTools (3.4). Hierarchical clustering (method not stated) and differentially expressed genes using t-test on normalised log₂-transformed values (p<0.0001) were applied for analyses. Annotation was done using DAVID and pathway analysis using GenMAPP and MAPPfinder. A concomitant comet assay was conducted.

26. After 6 hours treatment, 179 genes were modulated by treatment with OTA, 18 up-regulated and 161 down-regulated; after 24 hours this had increased to 2083 (697 up-regulated, 1386 down-regulated). Pathways affected included down regulation of genes associated with MAPK signalling and Wnt signalling at both time-points and apoptosis, cell cycle control, p38 MAP signalling and mRNA processing at 24 hours. Up-regulated pathways included mitochondrial electron transport chain at 6 and 24 hours and oxidative stress, inflammatory response and calcium regulation at 24 hours.

27. Particular genes highlighted as being of significance include: up-regulated mitochondrial genes encoding complexes I, II, III, IV and V and antioxidant genes CYBA, GPX1, GPX2, TXN2, SOD3. In addition, the absence of significant effects on genes considered characteristic of a genotoxic response was discussed; indeed GADD45, MYC, p21 and several caspases were slightly down regulated.

28. The authors conclude that the changes observed support a non-genotoxic mechanism of action of OTA. However, when comparing these data with work specifically aiming to investigate non-genotoxic and genotoxic mechanisms, it is not apparent which specific genes in these processes have been considered.

In summary:

29. Here an *in vitro* and an *in vivo* study have reached the same conclusion, that OTA does not significantly modulate the expression of genes typically thought to be affected by genotoxic carcinogens. Furthermore, both have discussed oxidative stress and calcium homeostasis as potential mechanisms. However, there appear to be many differences in gene expression changes. For example, some of the genes and processes highlighted by Marin-Kuan (2006), such as HNF4 α , were not apparently affected by OTA *in vitro*, although it is not clear whether these were included for assessment or whether any particular attention was drawn to them. Furthermore, as different microarrays and statistical analyses were used it is not possible to understand whether these differences are a function of *in vitro* vs *in vivo*, other biological variables or the methodologies employed.

Models of Hepatocellular carcinoma development

Osada et al 2006

30. This study aimed to assess the gene expression profiles of Glutathione-S-transferase placental form (GST-P) positive foci induced using the Solt-Farber protocol (single ip injection of DEN at 200mg/kg, followed two weeks later by dietary administration of 0.02% 2-acetylaminofluorene, followed one week later by partial hepatectomy). Livers were removed 8 weeks after the start of treatment. RNA was prepared from GST-P positive foci and from control rat livers (3/group). Biotinylated and fragmented RNA was hybridized onto GeneChip Rat Expression Array 230A. Statistics of differentially expressed genes was estimated using the linear modelling feature of the limma library (p-values of t-statistics by empirical Bayes shrinkage of the standard error).

31. Scatter plots show that gene expression profiles were reproducible across subjects (average correlation coefficient 0.93 ± 0.035 for controls, 0.95 ± 0.0068 for GST-P foci). Of the 15,923 probes on the chip, 375 were up-regulated and 199 down-regulated. Overexpression of metabolic enzyme associated genes including Gstp1/2, aldehyde dehydrogenase, aflatoxin B1 aldehyde reductase, NADPH dehydrogenase and glutathione peroxidase were noted. Also highlighted were changes in a number of

transcriptional regulatory factors . The authors focus on the significance of Pwrr upregulation and its association with WT-1 (which interacts with Wilms tumour) a tumour suppressor gene. Additionally, Srebp1 (as discussed earlier) was down regulated in positive foci together with the expression of some genes associated with fatty acid metabolism. High mobility group box 2 (Hmgb2) was up-regulated; the protein family are non-histone nuclear proteins which play a role in the assembly of nucleoprotein complexes. It is suggested that genes involved in these processes may provide a candidate link to early stages of hepatocarcinogenesis.

Coulouarn et al 2006

32. Although this study did not examine chemically induced tumours, it provides insight into the multi-stage nature of tumour progression and a further example of the use of microarray data. Three transgenic mouse models of liver cancer which over express specific genes implicated in liver cancer (*c-Myc*, *E2f1* or both) were used. The over expression of these genes has been demonstrated to be sufficient to promote tumour growth and thus it is suggested that these models are useful investigative tools.

33. RNA was isolated from livers taken from 3 month old mice (dysplastic stage) and 15 month old mice (tumour stage; hepatocellularcarcinoma and non tumour tissue). Microarray utilised a genome wide set of longmer mouse oligonucleotides based on annotated sequence information from the Mouse Exonic Evidence Based Oligonucleotide consortium. Differentially affected genes were identified using a univariate 2-sample t test $P < 0.001$. Data mining was performed using GeneOntology annotations and gene networks were confirmed using Ingenuity Pathway Analysis.

34. The expression of 5,331 genes was significantly altered compared to wild-type mice (2,541 in early dysplastic livers, 2,520 in non-tumour liver, 2,570 in HCC). Three major clusters were identified. Wild type mice profiles clustered with early dysplastic livers and non-tumour tissue. Whilst tumour tissues clustered, those from *c-Myc* and *E2f1* transgenic mice were clearly separated. Tumours from *E2f1/c-Myc* livers displayed variability, resembling *c-Myc* or *E2f1* specific gene expression.

35. The analysis also attempted to identify oncogene specific signatures. In HCC, genes were up or down regulated similarly in different strains of mice, whereas the dysplastic livers showed distinct expression patterns in the different lines. In dysplastic livers 68% of the 2541 genes were differentially expressed compared to HCC.

36. Due to over expression of transgenes, it was not possible to draw conclusions on the functional classification of genes in tumours from the different mice strains. However, in early dysplastic livers, cell cycle and cell death associated genes were identified in all three mice strains correlating with increased mitosis and apoptosis compared to wild-type controls. Down regulation of immune response genes were also common to all. Functional categories differentially expressed in different strains included: In *c-Myc* mice, increases in genes involved in protein synthesis catabolism were suggested to be

as a consequence of c-Myc's translational role. In E2f1 mice, induction of genes associated with lipogenesis and cholesterologenesis was noted. This is considered to be associated with sterol regulatory element binding factor1 (Srebp1), a transcription factor that relies on E2f1 and this was up-regulated in these mice. Concomitant overexpression of both E2f1 and c-Myc was associated with mitochondrial metabolism, specifically increased in the double transgenic mice. Overall conclusions suggest that the oncogenes E2f1 and c-Myc modulate specific metabolic functions which contributes to the development of HCC.

37. In summary:

Although significantly different from one another, these studies indicate the potential utility of gene expression analyses as investigative tools for the assessment of genetic changes of significance during the stages of tumour progression.

Genotoxic and non-genotoxic mechanisms and fingerprints

38. The largest number of papers retrieved during this review had the aim of assessing differences between, and profiles of, genotoxic and non-genotoxic carcinogens or attempting to establish gene 'fingerprints' for types of carcinogen. Some assessed detailed gene profile changes induced by matched carcinogens and some applied complex analytical tools to evaluate more general changes induced by larger numbers of chemicals. A representative selection are presented, which includes *in vivo* and *in vitro* models.

Ellinger Ziegelbauer et al 2005 (Appendix B)

39. This substantial study was designed with the aim of discriminating non-genotoxic carcinogens from genotoxic carcinogens. Groups of male rats were dosed orally with four non-genotoxic carcinogens, methapyrilene (MPY), diethylstilbesterol (DES), Wy14643 and piperonylbutoxide (PPB), and livers were taken after 1, 3, 7 and 14 days. Histopathology was performed and gene expression assessed using Affymetrix GeneChip RG-U34A arrays. Expression profiles were compared with those for 2NF, DMN, NNK and aflatoxin B.

40. Significantly deregulated genes were identified using a two-sample t-test ($p < 0.001$) and genes differentiating between treated and control by Welch test ($p < 0.0001$). Cut off was set at 1.7-2.0 fold.

41. Altered gene expressions were assigned to toxicological categories which can be related to elements of carcinogenic processes such as oxidative stress (DNA and protein damage responses), apoptosis and cell cycle progression. However, the methods used to do this are not detailed.

42. Some notable alterations discussed include:

- More pronounced induction of p53 target genes by genotoxic compounds compared to non-genotoxins, including p21, BAX, cyclin G1, CGR11, CGR19, MDM2, suggestive of a direct response to DNA damage.
- APEX1 DNA repair enzymes were induced only by non-genotoxins – these are activated by oxidative stress via oxidation of cysteine residues in terminal redox regulation domain. O⁶ methyl transferase was up-regulated only by genotoxins.
- Genes encoding DNA replication and cell cycle progression (PCNA, cyclin B1, CDC2, tubulin components) were upregulated by non-genotoxins and DMN. These increases can account for the increased mitosis observed. A time course correlation was noted for their expression.
- Up-regulation of heat shock response proteins eg HSP105 ETC, mainly by non-genotoxins but also 2NF
- Increased expression of ribosomal proteins (interpreted as a regenerative response) principally by non genotoxins , MPY and DES in particular. However RPS27 appears to be specific to genotoxins and also MPY.
- Down regulation of antiproliferative genes RB1 and MKP3 by both genotoxins and non-genotoxins.
- GADD45a – induction generally greater for genotoxicants. Induction of p53 specific genes appears to be a consequence of direct DNA damage...

43. MPY showed the strongest responses of the non-genotoxins examined although it is pointed out that this is positive in some genotoxicity assays. It is possible that these effects may contribute to enhanced responses.

44. The overall conclusions were that individual pathways or genes could not reliably distinguish between non-genotoxins and genotoxins.

Nakayama et al 2006

45. This study aimed to assess differences in gene expression profiles of carcinogenic and non-carcinogenic analogues of hepatocarcinogens. The pairs of compounds were administered orally for 1, 3 , 7, 14 or 28 days to groups of rats (n=4) as detailed below.

Compound	Dose mg/kg
2-acetylaminofluorene (2AAF)	6
4-acetylaminofluorene (4AAF)	40
2,4-diaminotoluene (2,4-DAT)	10
2,6-diaminotoluene (2,6-DAT)	10
2-nitropropane (2NP)	40
1-nitropropane (1NP)	80
2-nitro-p-phenylenediamine (2NpP)	100
4-nitro-p-phenylenediamine (4NpP)	250

46. RNA was hybridised to a prepared oligo-microarray, NEDO-ToxArray III which consisted of 6709 genes. Images were analyzed using GenePix Pro 4.0 and differentially expressed genes were selected based on ratios of levels in treated vs concurrent controls and Welch's approximate t-test (p value not stated). Ratios greater than 2 were considered to be up-regulation or 0.5 for down-regulation. Sets of isomers were assessed together, with and without inclusion of 2-NpP which was considered to have only limited carcinogenicity. Finally, 54 genes significantly altered at 28 days were clustered using GeneSpring in a Pearson correlation.

47. Carcinogenic analogues clustered together with the exception of 2NpP which clustered with 4-NoP and 4-AAF. There was no apparent time course of up or down regulation when assessing the numbers with altered ratios, but statistical analysis showed that the numbers were greatly increased after 28 days administration. *Pgy1* (ABC family of membrane transporters) was greatly up-regulated by carcinogenic but not non-carcinogenic analogues (up to 65-fold). Others highlighted were *Aldh1a1*, and cell cycle, repair and apoptosis genes (*Ccng1*, *Mgmt*, *Bax*, and *Btg2*). The authors concluded that the clustering of identified genes is a useful method for determining the hepatocarcinogenic potential of chemicals.

Iida et al 2005

48. This study investigated the differences in gene expression changes in mouse liver following dietary administration of a number of carcinogens (oxazepam, o-nitrotoluene, methyleugenol, all non-genotoxic or weakly genotoxic) and non-carcinogens (p-nitrotoluene, eugenol and acetaminophen) for 2 weeks. Dose levels were selected based upon NTP 2-year bioassay information and 4 animals/group were used. RNA was processed using Agilent Mouse Oligo arrays (20,000 genes including selected markers from NIEHS Toxicogenomics research consortium). Data analysis was conducted by the Rosetta Resolver; genes were considered to be signature genes if $p < 0.05$. Gene Spring 6.2 was used to analyse replicate arrays and clustering was performed with Condition tree algorithms. Ingenuity Pathway Analysis was used to identify networks of interacting genes and functional groups.

49. Genes identified in a previous study as potential biomarkers of mouse liver carcinogenesis, namely *Cyp2b10*, *Gadd 45b*, *Tsc-22*, *Lapser1*, *Bad*, *Maff*, *Igf-I*, *Igf-II*, *Igfbp1*, *Igfbp5* and *EST 2* (Iida et al 2003), were studied using QRT-PCR. In this study, however, most of these genes were not altered including following treatment with the carcinogens o-nitrotoluene and methyleugenol. Clustering using the 421 signature genes identified showed that non-carcinogenic o-nitrotoluene and low dose p-nitrotoluene, and methyleugenol and eugenol clustered together suggesting there are patterns of early gene expression which distinguish different carcinogens from one another as well as carcinogenic vs non-carcinogenic analogues. Metabolism related genes appeared to be up-regulated only in oxazepam treated animals.

50. Analysis of the paired carcinogens/non-carcinogens showed that there were 76 changes with o-nitrotoluene and 33 with p-nitrotoluene, with 17 of these common to both. Interestingly, o-nitrotoluene has different carcinogenic potency in male and female differences and of the 74-76 gene changes in males & females only 20 were common. Forty seven genes were altered in methyleugenol treated livers and 22 following eugenol treatment, 14 of these were the same, although expression changes were less pronounced in eugenol group. Notable differences in expression of *Fhit* and *Wwox* were indicated, with decreased expression following methyleugenol treatment but not eugenol. However, it was suggested that eugenol may have same effect as low dose methyleugenol.

51. Cell cycle associated genes cyclin G1 and p21 were strongly up-regulated with o-nitrotoluene and methyleugenol. However, *Egr1* was down regulated with oxazepam and up-regulated with p-nitrotoluene and methyleugenol. *Gadd45b* was highly up-regulated by oxazepam.

52. The authors highlight reduced expression of *Tsc-22*, *Fhit* and *Wwox* in carcinogen treated livers, suggesting that they are involved in apoptosis. However statistical assessment of the fold changes do not show this to be as clear cut as they conclude. Also, cell cycle genes were more significantly affected by carcinogens although individual genes differed amongst the carcinogens. The authors conclude that here are gene expression changes after 2 –weeks which can differentiate between carcinogenic and non-carcinogenic analogues.

Nie et al 2006.(Appendix B)

53. This is an apparently robust study which has assessed similarities and differences in gene expression changes between a large number of non-genotoxic carcinogens and non carcinogens, in rat liver. Data from over 100 different chemicals were examined and thus the aim was to establish more generalised patterns of change rather than to evaluate the significance of individual genes.

54. High single doses (30-50% of MTD) were given to male rats and livers removed 24h later. Specifically constructed microarrays were used (1471). Differentially expressed genes were identified using Ingenuity Pathways Knowledge Base, $p < 0.001$ cut off. A stepwise approach was used to identify signature genes. Fifty appeared in the final selection, and the best 6 selected by an exhaustive selection method. Of these 6, 3 were induced by non-genotoxins but not genotoxins. They were *NUTF2*, *Pgrmc1* and *UDPGTr2*. Three were repressed by non-genotoxins; *MT1A*, *Sel1h* and *Mat1a*. Overall expression changes were not large. With this procedure 88.5% of non-genotoxins were accurately predicted not to be genotoxic.

55. 125 genes were significantly up or down-regulated by non-genotoxins, although providing only 77% prediction of class. Of these 125, 71 were found in Ingenuity Pathways Knowledge, 16 associated with cancer, 19 to growth and proliferation, 7 to cell

cycle and 10 to cell death, 11 to immune and lymphatic system development. Of these 71, 62 were built into Ingenuity network building tools. c-myc was not differentially expressed but showed interaction with 4 of the 5 networks established. Genes associated with myc include up regulation of PGK1, iron responsive element binding protein (IREB2) and LEF-1 and down regulation of MTA1, fibronectin 1 (FN1) and EGR1.

56. Of genes singled out for discussion, induction of UDPGT (phenobarbitone inducible UDP glucuronyl transferase) is a fairly common mechanism in non-genotoxic carcinogenesis in rat. Methionine adenosyltransferase 1 alpha is hepatic specific involved in methylating (SAM) Mat2a leading to its downregulation. High levels of SAM also inactivates NFκB which protects against apoptosis and has a possible role in cell proliferation. Mat1a repression was seen which suggests that hypomethylation is important in non-genotoxic carcinogenesis. Metallothionein (MT1a) was also identified as a signature gene; this has a role in protecting against metal induced toxicity but probably also have a role in protecting against oxidative stress.

57. The authors concluded the system employed predicted non-genotoxic carcinogens by establishing gene signatures and provided useful information on involved pathways. However, it was considered that the high doses used in these investigations may overestimate the non-genotoxic potential of some chemicals.

Hu et al 2004

58. This study aimed to compare direct and indirect acting genotoxicants using TK L5178Y cells. The chemicals examined were: direct acting, DNA x-linking agents MMC and cisplatin; an alkylating agent MMS; and indirect acting agents hydroxyurea (ribonucleotide reductase inhibitor), taxol (microtubule inhibitor) and etoposide (topo II inhibitor). Three independent studies for each chemical were conducted in which cells were exposed to chemicals for 4 hours and harvested 4 or 20 hours post dosing. Cytotoxicity and micronuclei (MN) were also assessed as direct comparitors.

59. For gene expression profiling, Affimetrix microarray was used with a total of 9977 probe sets. Data analysis utilised ANOVA for general treatment effects on log-transformed signal values and nonparametric test for trends (Jonckheere-Terpestra). Analyses assessed: 1) treatment effects for genes regulated by all 6 chemicals 2) genes differentially expressed between direct and indirect acting chemicals. Hierarchical clustering analysis was also used with Euclidean distance measure of fold change for genes $p < 0.001$ to discriminate between indirect and direct genotoxins at both timepoints.

60. At 4 hours MMS and HU altered the expression of 281 and 148 genes respectively. The other chemicals only altered small numbers of genes (20-38). At 24 hours, all chemicals induced large numbers of changes (151-643). At 4 hours 38 genes were affected by all chemicals, most of which were involved with processes expected to be involved in DNA damage such as cell cycle, transcription regulation and chromosome

organization. At 24 hours, there were 1046 genes with $p < 0.0001$; 39 were singled out as particularly significant and included those involved in signal transduction, immune/stress response, transcription regulation and protein synthesis.

61. Discrimination of direct-acting vs indirect-acting chemicals was shown by hierarchical clustering. At 4 hours, it was demonstrated that 43 genes were differentially expressed in cells treated with direct compared to indirect genotoxins. Analysis of the biological pathways showed that there is suppression of cellular survival pathways by direct acting chemicals and moderate activation of apoptosis pathways by indirect acting chemicals. At 24 hours, a set of 58 genes discriminated the two classes of chemicals. It is considered that fingerprints can be generated to classify chemicals of unknown mechanism of action.

Kim et al 2005

62. L5178Y Tk⁺ cells were treated with 1,2-dibromomethane, glycidol (genotoxic carcinogens), 8-hydroxyquinolone, emodin (genotoxic non-carcinogens), methylcarbamate, o-nitrotoluene (non-genotox carcinogens), d-mannitol and 1,2-dichlorobenzene (non-genotoxic non-carcinogens). Dose levels used were those that induced maximum mutation frequencies in the absence of excessive toxicity for the positive chemicals and 90% viability or 5000 $\mu\text{g}/\text{mL}$ for the others. TwinChip Mouse 7.4K microarray used (7,400 genes); gene expression ratios were normalized using LOWESS regression. Data analyses included q-value computing, SAM and hierarchical clustering (Eisen Lab Web). Genes were considered differentially expressed when log ratios in 4 independent hybridisations were more than 1 or less than -1 (ie 2-fold difference in expression, q values $< 5\%$).

63. One gene consistently unregulated by genotoxins was *Trp63* (although not identified using real time PCR), which is required for p53 dependent apoptosis. In total there were 10 genes consistently altered by the genotoxins and 7 by the carcinogens.

64. Clustering categories included signal transduction, cell cycle control, cell growth and death, response to stress, transcription and apoptosis. >2 fold changes only considered. Differences in expression profiles for non-genotoxins vs genotoxins were noted for cell cycle control, response to stress and immune response related genes. No gene clusters were found in any category that distinguished carcinogenic from noncarcinogenic chemicals. Genes altered by nongenotoxic noncarcinogens were excluded.

65. It was concluded that although gene expression analyses are potentially useful in the evaluation of genotoxicity, the diversity of mechanisms of carcinogenesis means there are inherent limitations in the application of the technique.

Islaih et al 2005(Appendix B)

66. This study aimed to explore the relationship between mutagenesis, cell cycle and gene expression in TK6 cells (human lymphoblastoid) exposed to genotoxins with diverse mechanisms of action. MMC (crosslinking agent), MMS (alkylating agent), H₂O₂ (reactive oxygen species), Bleomycin (reactive oxygen generator), etoposide and doxorubicin (topo II inhibitors) were incubated with cells for 4 hours and harvested after 24 hours. Cytotoxicity and mutant frequency data were generated for all and cell cycle analysis was performed using flow-cytometry. RNA from treated cells was hybridised to AffimetrixGeneChipHuGeneFL Arrays (5600 human genes) and data analysed using Microarray Suite software. Data mining tool used criteria of <1.5 fold change consistent between duplicate hybridizations. Gene annotations were obtained from NetAffix and analysed using Pathway Assist software.

67. There was an inverse relationship between mutation frequency and magnitude of effect on global gene expression. MMS induced the strongest mutation frequency response but altered the least number of genes; these included DNA repair genes (GADD45 α , BTG1 &2), cell cycle regulation (p21, CCNG1) and 2 apoptosis associated genes. H₂O₂ induced changes in the largest number of genes (150, all but 3 up-regulated) including 3 DNA repair genes, 5 cell cycle regulators and 3 pro-apoptosis genes. Common to all chemicals was the induction of p53 and TNF pathways. Relationships were seen between cell cycle G2 arrest, MF and p53 activation.

68. In summary:

These studies indicate the usefulness of clustering techniques to discriminate between genotoxic, non-genotoxic and non-carcinogens. Some authors suggest that gene fingerprints could potentially be used for the identification of mechanisms. However, other studies indicate the limitations. Furthermore, it was not possible to compare expression changes of the same chemicals examined in different test systems.

No effect level and dose responses determinations

Seidel et al 2006

69. Dose responses in gene expression were examined following administration of 2-AAF and PB to rats for 28 days. Doses ranged across carcinogenic and non-carcinogenic levels (AAF 0, 0.1, 0.3, 1 and 3 mg/kg/day, PB 10, 30, 60 and 100mg/kg/day). Atlas Rat Toxicology II (Clontech) microarrays were used and analysed using GeneSpring. Genes >2 background were considered.

70. Fifteen altered genes were identified for AAF and 18 for PB. Many expression changes, particularly for 2-AAF exhibited U-shaped dose responses, with changes returning to control levels at higher doses. Greatest expression changes following AAF administration were MGMT and osteopontin (inducible by p53 as a direct result of DNA damage). Many of the genes altered by PB were predictable, GST, CYP genes. Four genes were altered by administration of both compounds.

71. It is postulated that the unusual shaped dose response curves are a consequence of an adaptive responses at high dose levels and of liver remodeling following a toxic insult.

Assessment of analysis systems:

Van delft 2005 (Appendix B)

72. This paper examined four different supervised clustering methodologies to discriminate between genotoxin and non-genotoxins. They were: pearson correlation analysis, nearest shrunken centroids analysis, k-nearest neighbour analysis, and weighted voting. All methods used steps to discriminate between the two classes and then expression profiles of classifiers to predict class of toxicity.

73. Microarray data was taken from a previously published study (van Delft et al 2004) in which HepG2 cells treated with a 11 genotoxic carcinogens and 9 non-genotoxic carcinogens for 24hrs. Secondly, chemicals which had at least 6 responding genes were assessed. Cross validation and training sets of chemicals were employed. Assessments for each method were annotated '1' as correct or '0' as incorrect.

74. Overall, treatments tetrachlorethylene and reserpine were wrongly classified consistently by all clustering methods. However, there were no consistent misclassifications using the >6 genes approach.

75. Involved genes and pathways - the number of genes selected for each method varied from 14 to 31. The genes selected at least three times by the different methods and approaches numbered 27. BAX was selected in 10/10 cases (induced by genotoxins and suppressed by non-genotoxins), ZFP36 in 9/10 and PAHR, MT1E and TTR in 8/10 cases. In general the effects elicited by non-genotoxic carcinogens were more profound than those by genotoxic carcinogens. Gene annotations indicated that apoptosis associated genes figured most highly. Also CDKN1A was picked out as a marker. Interestingly no genes involved in DNA repair were identified as altered although present on the microarrays.

76. Overall, it was concluded that the NSC, KNN and WV methods performed more robustly than the Pearson method.

Currie et al 2005 (Appendix B)

77. This report aimed to assess Gene Ontology (GO) and pathway mapping tools with the goal to understand molecular processes associated with gene expressions changes and their relation to conventional toxicological endpoints. DEHP was used as model non-genotoxic compound.

78. Groups of B6C3F1 mice were given 3 daily doses of DEHP at 1150mg/kg for 3 days and killed 1, 2, 4, 8, 24, 48 and 72h after first dose (3/group). BrDU was used to assess DNA synthesis

79. Gene expression profiling was conducted using Affimetrix. Significant changes were determined using 2-way ANOVA $p < 0.05$. This generated three probe lists; time (1863), treatment (899) and time/treatment (76). A final probe set of 1786 whose expression was significantly altered by DEHP was identified. DEHP responsive genes were mapped to Gene Ontology and genMAPPs. Classical peroxisome proliferator-responsive genes such as the CYP4a family were assessed.

80. Gene ontology clustering approach: the largest group of genes were associated with 'metabolism'; 'organismal/cell physiological processes' and 'cell communication' were the next largest. This is considered to be a simplified approach which assigns and reflects annotations. Given representation indices (R_i) and associated GO terms identified specific processes eg fatty acid β oxidation, lipid and fatty acid transport, bile acid metabolism. There are several tools to perform the overrepresentation analysis of GO, with an apparent good degree of correlation. The usefulness of this approach was confirmed.

81. Genes associated with epigenetic status: 'ontology of 'regulation of gene expression, epigenetic' was not reliably overrepresented in these analyses. However CpG binding domain protein (Mbd1) was identified as an early DEHP responsive gene, and also genes involved in 1C metabolism, eg Mat2a, Shmt1 and 2.

82. The authors conclude that the use of GO and mapping procedures are a powerful approach to evaluate mechanisms of action.

Toxicogenomics initiatives

OECD

83. The OECD/IPCS Advisory group on Toxicogenomics is spearheading a project '*Molecular Screening for Characterizing Individual Chemicals and Chemical Categories Project*' which will select a number of chemicals for screening with the aim of establishing a strategy for prioritizing chemicals for further evaluation based on molecular properties linked to toxicology.

84. The project proposal invites participation in collaborative efforts such as (i) data generation through additional assays and/or additional chemicals to the U.S. EPA's ToxCast Program, (ii) data sharing and/or (iii) analysis of gathered data. It was also suggested that all data should be made publicly available.

85. The following issues for implementing OECD-wide collaboration in the Molecular Screening Project are considered:

- Endpoints of concern (e.g., cancer, reprotox, devtox, neurotox and immunotox)

- Chemicals of concern (e.g., pesticides, HPVs, PBTs, water contaminants, etc.)
- Modes of action of concern (e.g., genotoxic and non-genotoxic carcinogenicity; nuclear receptor mediated xenobiotic response)
- Screening data generation (e.g., new assays for common chemicals; new chemicals for common assays)
- Data management, analysis and sharing

86. Participants in the meeting generally recognized that the Molecular Screening Project would be a very useful initial opportunity to explore regulatory application of toxicogenomic methods and HTS/HTC methods in chemical assessment. It was considered that the project should keep regulatory focuses and pay attention to quality assurance and quality control (QA/QC) issues.

87. The aims are to select a fairly large number of chemicals which have been well examined using traditional mammalian toxicity testing methodologies, and hence have known properties representative of a number of differing structural classes and phenotypic outcomes (e.g., carcinogens, developmental toxicants, reproductive toxicants, and neurotoxicants). These chemicals would then be evaluated in a series of molecular screening assays.

FDA Microarray Quality Control (MAQC) Programme

88. The executive summary for this project is appended (Appendix C). This is a comprehensive project which analysed reference RNA samples at multiple test sites using different arrays with view to establishing the best performance of the microarray technology so that end users can judge the quality of their data. It is anticipated that it will also help in the development of best experimental practises.

Conclusions

89. General observations of the papers reviewed include:

- There are substantial differences in the way in which the studies are reported. This includes variation in the level of detail of experimental methodologies (including details of animal numbers or microarray replicates), statistical and other analytical approaches. Without intimate knowledge of the approaches it is difficult to ascertain the quality and any bias of the work. Hopefully initiatives such as the MAQC project will help to address these.
- A large number of different microarrays are utilised which appear to contain substantially different numbers of genes. Furthermore, the ways in which individual genes are singled out for discussion is highly variable. This makes it difficult to compare the results of studies examining the same chemicals or processes.

- Studies are very data rich. Many authors, particularly those investigating specific mechanisms, include a lot of detail on specific gene changes and these assessments frequently appear to be subjective. For example, it is not always those that are most significantly altered that are discussed
- Clustering is widely used to examine gene expression patterns induced by classes of chemicals such as genotoxins vs non-genotoxins. There is a moderate degree of success with these approaches.
- The ability of the programme used to identify pathways involved in carcinogenic processes clearly has utility for mechanism investigations. As well as anticipated processes such as cell cycle control and apoptosis modulation, a number of papers have revealed new insights, particularly when assessing individual chemicals.

Questions for the Committee:

90. i) Based on the sample of studies presented here, how effective do members consider these methods currently to be in predicting carcinogenicity and in proposing or confirming a mechanism of action?

ii) Members are asked whether the existing statement on toxicogenomics requires revision based on the information presented. Are there any further considerations which require attention, such as input into global initiatives, or whether further work needs to be undertaken in this area.

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**2004 JOINT STATEMENT ON THE USE OF TOXICOGENOMICS IN
TOXICOLOGY**

**COC/04/S8
December 2004**

**This statement can be found on the COC website at
<http://www.advisorybodies.doh.gov.uk/cotnonfood/toxicogenomics.htm>**

Appended papers:

Arbillaga, L., Azqueta, A., van Delft, J.H.M. et al (2007). In vitro gene expression data supporting a DNA non-reactive genotoxic mechanism for ochratoxin A. *Toxicol. Appl. Pharmacol.* **220** 216-224

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