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MUT/2015/06

COMMITTEE ON MUTAGENICITY OF CHEMCIALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

HORIZON SCANNING 2015

Introduction

1. The Committee on Mutagenicity (COM) undertakes an annual horizon scanning exercise which provides an opportunity for Members and assessors from Government Departments/Regulatory agencies to discuss and suggest topics for further work.

2. A formal Horizon scanning exercise was not carried out in 2014. At the meeting in March 2015 Members considered paper MUT/2015/05, which provided a brief outline of some topics recently reviewed (cell transformation assays; mutation spectra) and those for consideration (gene expression profiling; integration of *in vivo* genotoxicity assays in repeat dose toxicity testing; quantification of genotoxic response; epigenetics and epimutations; and 3D tissue models). Members were also asked to suggest other topics that they would like the Committee to review.

3. With regards to suggested topics:
   - A presentation on 3D tissue models is scheduled for this meeting.
   - A Member informed the secretariat that a journal special issue on quantitative approaches to genotoxicity would be published later in the year. It was noted that this will be useful for the Committee to be aware of and consider the topic then.
   - Gene expression profiling /toxicogenomics in genetic toxicology had been considered relatively recently (in 2013 - MUT-2013/09) and it was decided that this was presently of very low priority.

4. The other topics will be discussed in brief here together with new topics suggested by Members (mitochondrial DNA mutations, age-related mutations in man and disease). Members are invited to consider the subjects presented, to provide guidance and to advise on priorities.

Integration of in vivo genotoxicity assays into repeat dose toxicity testing

5. Members initially considered this topic in Horizon scanning in 2013. More recently Members noted that integrating *in vivo* genotoxicity assays into repeat dose toxicity tests has become routine practice when testing human pharmaceuticals. Whilst the use of the combined assays in other regulatory areas is not yet routine, EFSA encourage protocols to reduce the use of animals, and discuss the feasibility of incorporating micronucleus assay into repeat dose toxicity studies (EFSA 2012 section 6.1).

6. ICH S2(R) (2012) describes the requirements for acceptability of the top dose when genotoxicity assays are integrated into a routine toxicology study (Multiple Administrations 4.3.2). They are detailed as a) maximum feasible dose based on physiocochemical properties, b) limit does of 1000mg/kg/ for studies of 14days or longer if tolerated. c) a plateau of exposure demonstrated or evidence of accumulation in toxicokinetic measurements and d) the top dose is > 50% of top dose of an acute administration study. Sampling of blood or bone marrow can be undertaken the day after the final administration. The ICH guideline is based on studies conducted for human pharmaceutical development during which toxicokinetic measurements are routinely incorporated into the study design.

- What recommendations can be made for studies in which TK studies are not routinely incorporated?

7. There are a number of publications reporting on integrating genotoxicity assays into 28 days studies (Pfuhler et al 2009; Kushwaha et al 2010; Rothfuss et al 2011) – ECVAM workshop during which genotoxicity experts discussed ways in which animal number can be reduced in genotoxicity testing emphasised that reduction in animal number should not compromise study quality and thus interpretation, and that poorly designed in vivo studies are a waste of animals. Sampling flow-cytometric scoring of peripheral blood micronuclei will improve the scoring.

- It is possible and/or worthwhile for COM to collect and collate data obtained from using the combined assay?
- Do Members feel that COM should provide guidance on this topic?

Epigenetics and epimutations

8. Members first suggested the topic of epigenetics and epimutations during the Horizon scanning exercise in 2013. Epigenetics is defined as modifications in gene expression or cell phenotype caused by mechanisms other than a direct change in the DNA sequence. Epigenetic mechanisms are known to be involved with many regulatory processes in the cell, including cell growth and transformation. The topic of epigenetics was considered by COC in May 2013 when an introductory overview paper and a paper looking at some specific examples (e.g. benzene, arsenic, endocrine disruption) were presented. It was concluded that it was possible that epigenetic changes contribute to carcinogenicity for arsenic and benzene but much more work would be required for this to be proven. It was agreed that epigenetic changes could be considered on a case-by-case basis when undertaking a carcinogen risk assessment, though it was unclear when epigenetic changes were causal for tumour development or for an effect of tumour development. The introductory paper is appended (Annex 1).

9. Epimutation is defined as abnormal transcriptional repression of active genes and/or abnormal activation of usually repressed genes caused by errors in epigenetic gene repression (Hesson et al 2010). Epimutation can arise in somatic and germ cells. Epimutations have been identified in several tumour suppressor genes including MLH1, MSH2, *retinoblastoma* (RB), *cyclin-dependent kinase*

inhibitor (CDKN2A) and *breast cancer susceptibility gene* (BRACA1) (Dobrovic et al 2009, Banno et al 2012). Constitutional epimutations, occur at early stages of embryonic development and thus are present in all tissues. Controversy exists whether this arises from the mother or father or whether it arises de novo. However there is limited information associating epimutations with chemical exposures. Search terms such as 'epimutations AND benzene OR PAH OR arsenic' yielded no reports.

- Would Members like to review this topic?
- If so, given that there are limited reports on the role of chemicals in epimutations, what aspects are of greatest importance?
- What can be added to the review undertaken by COC?

Mitochondrial DNA mutations

10. Mitochrondrial DNA mutation was initially suggested as a topic for consideration at the COM Horizon scanning exercise in 2013. Mitochondrial DNA (mtDNA) is a double stranded circular DNA of 15000-17000 base pairs coding for 37 genes (13 proteins involved in oxidative phosphorylation, 22 for tRNA's, 2 for rRNA's) and has only approximately 600 non-coding nucleotides (Taylor and Turnbull 2005). The spontaneous mutation rate is much higher in mtDNA than in nuclear DNA (by up to 100-fold), and therefore mtDNA has a higher level of heterogeneity. It is speculated that the high mutation rate is a function of lower DNA polymerase gamma fidelity in mitochondria and due to a lack of histones which may have a protective effect. However it is noted that this is a hypothesis only and lacks empirical evidence (Alexeeyev et al 2013). Alexeyev et al (2013) list five types of mtDNA damage:

- alkylation damage (exogenous and endogenous sources);
- hydrolytic damage; formation of adducts (endogenous and exogenous sources);
- mismatched bases (due to replication errors or base modification);
- DNA strand breaks;
- oxidative damage.

11. Oxidative damage is the most widely studied type of damage as mitochondria themselves are the principal generator of ROS in the cell. H_2O_2 induced strand breaks are considered to be the predominate lesion in mtDNA (Shokolenko et al 2009). There are some claims that mtDNA is damaged preferentially to nuclear DNA although evidence is controversial and inconsistent for different types of damage (e.g. oxidative vs alkylation). Glutathione is thought to play an important role in the maintenance of the mitochondrial genome (Ayer et al 2010)

12. There are substantive reports considering mtDNA mutation repair (Copeland and Longley 2014, Muftuoglu et al 2014, Scheibye-Knudsen et al 2015). Initially it was believed that mtDNA lacked or had reduced repair capacity due to their inability to repair UV-induced pyrimidine dimers. However more recently there is evidence for most DNA repair pathways such as DNA mismatch repair (MMR), DNA single-strand break repair (SSBR), double-strand break repair, base excision repair (BER;

short and long patch), but with the exception of nucleotide excision repair (NER) (Alexeyev et al 2015).

13. Homoplasmy and heteroplasmy There are multiple mitochondrial genomes per cell; it is possible to have a heterogeneous population of mitochondrial genomes in one cell or individual—a condition known as heteroplasmy (Taylor and Turnbull 2005)

14. The clinical impact of mtDNA mutations is well documented and is believed to contribute to a wide variety of disorders (Schon et al 2012; Copeland and Longley 2014). Primary mtDNA related diseases arising directly from a mutation associated with mitochondrial oxidative phosphorylation (OXPHOS) respiratory chain deficiency and thus failure to generate energy in the cell – this is classical mitochondrial DNA disease affecting tissues with high metabolic demand (CNS, skeletal muscle, heart) Conditions such as Pearsons syndrome, Kearns Sayre syndrome (Greaves et al 2012). Most mtDNA deletions are sporadic and not transmitted to offspring. However point mutations can be maternally inherited.

15. There are hypotheses that mitochondrial dysfunction contributes to neurodegeneration susceptibility and late onset neurodegenerative diseases, though the link to mtDNA variants and mutations is not definitively proven. There a number of diseases linked to mitochondrial DNA dysfunction or mutation – these include Parkinsons disease, Alzheimer's disease, amylotrophic lateral sclerosis (Schon et al 2012 , Subramanian and Chesselet 2013), epilepsy, atherosclerosis, diabetes (Taylor and Turnbull 2005). mtDNA mutations are considered to have a role in the aging process as they disrupt balance of ROS production and scavenging (Greaves et al 2012). It has been suggested that mtDNA mutations also modify the risk of developing other diseases in later life (Hudson et al 2014).

16. There are only limited associations between environmental chemical exposures and disease and mitochondrial mutation; for example an association between atherosclerosis and polyaromatic hydrocarbons (Pulliero et al 2015). However conclusive evidence for an understanding of the impact of oxidative damage caused by these chemicals and metals and role in mtDNA damage has not been found.

- Would Members like to review this topic?
- If so, given that there are limited reports on the role of chemicals in mtDNA mutations, what aspects are of greatest importance?

Human mutation rates and disease

17. COM considered the development of tests to investigate germ cell mutagenicity in 2013 (MUT/2013/05). Members concluded that further validation work was needed before germ cell assays could be incorporated into general genotoxicity testing and that there were a number of methodological difficulties such as DNA extraction. Good study design was considered to be crucial. DeMarini (2012) recently suggested that there is a need to investigate and advise on the existence and implications of human germ cell mutagens, in a manner similar to that

undertaken by IARC for human carcinogens. This would lead to a greater understanding of germ cell mutations and potential disease in off-spring.

18. The advent of high throughput, next generation sequencing methodologies has enabled substantially more detailed analyses of human genome mutations, incidence and patterns, including those that occur from generation to generation (Veltman and Brunner 2012). There is considerable interest in the contribution that *de novo* mutations in germ cells make to human illness and disease in off-spring (McGrath et al 2014). An investigation in the Icelandic population which sequenced the entire genomes of 78 parent offspring trios provided information on the importance of father's age on *de novo* mutation rates (Gudbjartsson et al 2015; Kong et al 2012). The frequency and diversity of the single nucleotide polymorphisms (SNPs) increased with increasing age, and it has been estimated that 36 year old man will pass on twice as many mutations to his offspring as a 20 year old man (Callaway 2012).

19. This increase in mutation rate has been associated with some diseases in children, particular focus on neurodevelopmental disorders, for example autism and schizophrenia (Goriely et al 2013; Malaspina et al 2015 Pedersen et al 2014). There is mounting evidence that *de novo* copy number variants (CNVs) are more abundant in children with autism spectrum disorders than in controls (Ronemus 2014).

20. To date no reports have been retrieved which have addressed whether this increased risk with age is a consequence of intrinsic aging effects or a consequence of the cumulative effects of environmental exposures.

- Do Members think this is a topic that should be considered?
- If so, how can we go about addressing this?

Development of the Pig-A assay in human erythrocytes

21. COM evaluated the PIG-A assay in 2010 (MUT/2010/13) and 2013 (MUT/2013/03) and agreed that the assay was robust but that it was difficult to see how it would provide additional information to the comet and micronucleus assays in rodents though it could contribute to weight of evidence decisions. At the time it was noted that an assay using this endpoint in human cells would be useful for estimating genotoxicity in human biomonitoring studies and to provide information on background and spontaneous mutation rates in man.

22. A recent publication from Dertinger et al (2014), the group who have been the principle protagonists for the assay, describes the further development of a method for evaluating pig-A mutations in human erythrocytes. The proof-of principle study which assessed the utility of the $CD59^9$ protocol for detecting mutations in human erythrocytes from cancer patients undergoing chemotherapy was reviewed in 2013 (Dobrolvsky et al 2011). Minutes of the meeting state: "there was only one published study in humans but [Members] were advised that a clinical trial using 50 compounds was underway and the unpublished clinical data were good and would be reported in due course". The Dertinger study has looked at anti- CD59 and CD55

in 52 subjects to determine pig-A mutant frequencies in red blood cells (RBC) and reticulocytes (RET) and it is surmised that this is the work referred to. They report the applicability of the rat blood cell assay to human cells and the effective use of 100 µL blood samples. Reproducibility was demonstrated across three samples. Inter-subject variability – the mean mutant frequency values given are 6.0 x 10^{-6} and 2.9 x 10^{-6} for RET and RBC respectively. Standard deviations are not given but inter-subject variability was high. The ranges are 0.4-11.3 x 10^{-6} for RBC and 0-29.4 x 10^{-6} for RET. A particularly low or high MF in one cell type in a subject was sometimes but not always reflected in the second cell type, e.g. the subject with the highest RBC MF also had the highest RET MF. However it is evident that the variability in human subjects is substantially greater than in rats.

23. The authors performed some preliminary assessments of variability with sex and age. A positive correlation with increasing age was noted though it was concluded that the result should be considered preliminary given the relatively small sample size. The study did not assess the impact of any chemical exposures.

- What are Members opinion of the development of the Pig-A assay using human cells?
- Do Members consider it potentially useful in monitoring background mutation rates in man

PHE Toxicology Unit
June 2015

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Annex 1 to MUT/2015/06

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

HORIZON SCANNING 2015

COC discussion paper CC/2013/05 – Epigenetics and carcinogenesis

Available from here:
http://webarchive.nationalarchives.gov.uk/20140722183324/https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/315923/9_May_2013_minutes_and_discussion_papers.zip

Secretariat
May 2015

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CC/2013/5

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

Epigenetics and carcinogenesis

1. The role of epigenetics in carcinogenesis was considered by the Committee at the Horizon scanning exercise in November 2010. It was concluded that it would be useful to review information on any demonstrable role of epigenetics in experimental carcinogenesis. Transgenerational epigenetics has been discussed previously by the COM in 2006 (MUT/06/15) and was the topic of a workshop organised by the COT in February 2008 (http://cot.food.gov.uk/pdfs/cotstatementtransepi200803.pdf).

2. Evaluation of the literature revealed a very large amount of information and it was realised that a review of the entire topic was not feasible. Therefore, initially, Dr Tim Gant has kindly agreed to make a presentation to the Committee on epigenetics and its association with carcinogenesis. The two papers presented aim to provide an introduction to the subject. This first paper provides an outline of general principles and discussion of lifestyle and other factors known to impact on epigenetic modification. A second paper provides information on epigenetic changes induced by two human carcinogens, arsenic and benzene, for which there are data from *in vitro* models, and from exposed humans. Neither of the papers is a detailed systematic review, but are intended for general information in the first instance.

3. Members are asked to review the information and consider the questions given at the end of each paper.

Secretariat

April 2013

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Epigenetics: an introductory overview

Introduction

1. Epigenetics is defined as modification in gene expression or cell phenotype caused by mechanisms other than a direct change in the DNA sequence. Epigenetic mechanisms are known to be involved with many regulatory processes in the cell, including cell growth and transformation. The principal mechanisms commonly described as epigenetic are DNA methylation changes and histone modification (Hamilton, 2011; Tammen et al., 2012). The modulation of small non-coding RNAs, such as micro RNA (miRNA), which can alter the translation of mRNAs, can also be considered to be an epigenetic mechanism. MiRNAs have been addressed in a previous COC paper (CC/2011/06) and will not be discussed in further detail here. Maintenance of, or changes to, the epigenome are recognised to play important roles in the regulation of gene expression during normal cell growth, foetal development and in the manifestation of diseases (Bernal and Jirtle, 2010; Calvanese et al., 2009). This includes the developmental origins of diseases following *in utero* exposures and also some evidence for their role in transgenerational effects, when acquired changes are passed on to offspring. These aspects of epigenetics were the topics of a one-day workshop held by the COT in 2008 (http://cot.food.gov.uk/pdfs/cotstatementtransepi200803.pdf). The overall conclusions were that there is reasonable evidence that epigenetic changes associated with environmental exposures during development can result in adverse effects in subsequent generations, although it is not clear whether transmission of acquired epigenetic changes occurs across generations in humans. The workshop did not address in detail epigenetic alterations in carcinogenesis. Epigenetics, and how it could be incorporated into safety assessments, was also the topic of an ILSI/HESI workshop in 2009 (Goodman et al., 2010) (Appendix 1).

2. The importance of epigenetic regulation of gene expression in susceptibility to, and development of, cancer is increasingly being recognized. Alterations in DNA methylation (global hyper-methylation and promoter specific hypo- methylation) can result in changed expression of tumour suppressor genes or induce loss of imprinting and there are a large number of examples of aberrant methylation patterns in a wide variety of tumours (Esteller, 2011; Jones and Baylin, 2002; Sharma et al., 2010). Histone modification is also considered to have a role in tumour progression via post transcriptional modification by histone deacetylases (HDACs) which can alter the regulation of DNA repair or replication (Sawan and Herceg, 2010). It is now widely accepted that epigenetic mechanisms are as important in the development of cancer as genetic mechanisms, and that exploration of this area will yield insight into tumour development, an understanding of the origins of tumours and also cancer therapies. Some overviews of the role of epigenetic mechanisms in the development and causation of cancer are attached - Appendix 1 (Herceg, 2007; Weidman et al., 2007).

3. There is substantial research interest in the impact of environmental chemicals on the epigenome and efforts to understand and quantify these effects on health outcomes (Baccarelli and Bollati, 2009; Christensen and Marsit, 2011; Hou et al., 2012). It is well documented that the epigenome is susceptible to modulation and that there are a wide variety of factors, such as diet and

other lifestyle factors, which impact on its integrity (Alegria-Torres et al., 2011; Poirier, 2002). Whilst not directly relevant to the current evaluation of chemical exposures, carcinogenesis and epigenetics, it is likely that there will be significant interplay between different factors and accordingly, the analysis and interpretation of chemical-induced changes, particularly in epidemiological studies, will be difficult. Moreover, there are bodies of work investigating epigenetic changes in animal models which provide 'proof-of-principle' models from which to generate hypotheses (for example, transgenerational studies using Agouti mice or investigating environmental exposures). The similarities and differences between epigenetic modification in animals and man are not well defined and, therefore, it is not known whether findings in animals can be accurately extrapolated to man. However, there are significant areas of research which investigate the modulation of epigenetic changes in humans, for example, the after effects of famine. It is possible that examining the human epidemiological consequences of epigenetic modulation by all factors will provide insight into how chemical exposures impact on carcinogenesis via epigenetic mechanisms.

4. This paper aims to serve as an introduction to some basic concepts of epigenetics and to examine some factors which can modulate epigenetics, with view to understanding their possible relationship with carcinogenesis. Some examples of specific carcinogens and their impact on epigenetic changes *in vitro*, in animal models and in humans are provided in the accompanying paper (CC/13/24).

Epigenetic mechanisms

5. DNA methylation: Covalent addition of a methyl group at the 5' carbon of cytosine generates 5-methylcytosine which is present in approximately 4% of genomic DNA, principally (90%) in cytosine-guanosine dinucleotides (CpG sites). However, there are clusters of non-methylated CpG rich regions, referred to as CpG islands, which are often located at or near promoter regions where transcriptional regulation occurs (Hamilton, 2011; Santos et al., 2005). These regions are particularly susceptible to chemical-induced alterations in methylation status.

6. DNA methylation is regulated by a family of enzymes, DNA methyltransferases (DNMTs) which catalyse the transference of a methyl group from S-adenosylmethionine (SAM) (Santos et al 2005). Different isoforms are responsible for *de novo* methylation (DNMT 3a, 3b) and for the maintenance of established DNA methylation patterns (DNMT1, (Baylin, 2005)). Modulation of these enzymes or the availability of SAM can result in epigenetic changes.

7. A cancer epigenome is characterised by global hypomethylation and site specific CpG island promoter hypermethylation, which can cause the silencing of tumour suppressor genes such as p53, p16, BRCA1, Rb promoter (Jones and Baylin, 2002). Hypomethylation can play a significant role in the carcinogenic process by increasing genomic instability (Sharma et al., 2010) However, it is difficult to distinguish whether methylation changes are cause or effect, particularly in relation to cancer, as significant changes in gene expression are taking place and the cell is undergoing deregulation of a large number of complex cellular processes.

8. Histone modification: Chromatin, the DNA nucleoprotein complex, varies considerably between different cell types. Chromatin packaging can be mediated by the modification of histones and other proteins which results in a relaxation of chromatin packaging and a more transcriptionally

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active state. Histone modification can occur by acetylation, phosphorylation and methylation or by modification of the enzymes responsible for histone maintenance (Histone deacytylases -HDAC) (Rakyan et al 2001). Perturbation of these processes represents other plausible epigenetic mechanisms of carcinogenesis. Histone modifications described in cancer progression include the global loss of acetylated H4-lysine 16 (H4K16ac) and H4-lysine 20 trimethylation (H4K20me3). These losses may result in gene repression. HDACs have been found to be over expressed in some tumours (Jones and Baylin, 2002; Sharma et al., 2010). Histone methylation patterns are also associated with aberrant gene expression, for example gene silencing of tumour suppressors such as EZH2 over expression in breast and prostate cancers, or G9a in liver cancer when it is implicated in the development of a malignant phenotype by the modulation of chromatin structure (Esteller, 2011)

9. Gene Imprinting: Genomic imprinting is an epigenetic modification of DNA which is parental origin specific and /or the preferential expression of a parental specific allele in offspring (Feinberg et al 2002). Thus imprinting is a phenomenon whereby the expression of a gene is dependent on whether it resided in the male or female of the previous generation (Jirtle, 1999). It is most commonly studied in the context of gene silencing but the general term refers to the heritable modification. The most commonly observed alterations (and most commonly investigated) are methylation changes.

10. Loss of imprinting (LOI) is when normal genomic imprinting is disrupted by mechanisms such as altered methylation. The consequences are that either a normally silenced gene is activated or an expressed allele is silenced. This phenomenon is observed quite frequently in a wide variety of different human tumours, particularly tumours embryonic in origin (Feinberg et al., 2006). To date the most commonly observed affected gene is the insulin-like growth factor II (IGF2) gene, normally expressed only from the paternal allele but which is capable of undergoing activation of the normally silenced maternal allele.

Primary concepts:

11. Agouti mouse model: A preliminary understanding of the impact of epigenetic changes on gene expression arose from the use of the Agouti mouse model. The mouse has an A^{VY} allele upstream transposable element which is variably methylated, resulting in differential gene expression. The Agouti protein directs follicular melanocytes to produce yellow pigment instead of black melanin. Hypomethylation causes the gene to be expressed, producing yellow mice; hypermethylation silences the gene and produces black mice. These markers are established early in development. The methylation status is affected by dietary changes especially folate, B12, choline, some chemical exposures (for example, arsenic) and also the phytoestrogen genistein (Dolinoy, 2008; Ruemmele and Garnier-Lengline, 2012)

12. Epigenetic effects in human twins: Epigenetic changes are thought to be responsible for the phenotypic differences in monozygotic twins (i.e. genetically identical but with disease and developmental differences). The peri/postnatal epigenetic twins study (PETS) is an Australian initiative which has examined epigenetics in twins from birth (250 pairs), with the aim of understanding the similarities or differences in epigenetic markers throughout life-spans and any potential effects on the developmental origins of health and disease (Loke et al 2012). To date, studies have shown that there are discordances in DNA methylation between twins at birth and suggested that subtle differences in the uterine environment contribute to this epigenetic variation

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and to gene expression differences . Furthermore, one third of the epigenome has altered within the first 18 months of life. In a review of studies examining the heritable DNA methylation effects and differential methylation in twins in relation to disease, correlations between disease-discordance in twins and differentially methylated regions was evident for a number of diseases including schizophrenia and diabetes and psoriasis (Bell and Spector, 2012). Differences in DNA methylation have been identified between twins discordant for breast cancer (Heyn et al., 2013).

13. ***Epigenetic changes during famine:*** A series of studies have examined the impact of the human starvation endured during the Dutch famine of 1944 on nutritional deficiency during pregnancy. Schizophrenia, obesity and cardiovascular diseases in later life were linked to under-nutrition at certain stages of pregnancy, and the hypothesis is that these conditions can be attributed to alterations in epigenetic programming. (Ruemmele and Garnier-Lengline, 2012) (Roseboom et al., 2006). It was evident that the period of famine was critical to these observations. A possible link between prenatal exposure to famine and breast cancer has been suggested (Painter et al., 2006).

Lifestyle factors

14. It is considered that numerous lifestyle factors, including diet, physical activity, tobacco smoking, alcohol consumption, psychological stress and working on night shifts, might modify epigenetic patterns.

15. Nutrition: The link between nutritional status and methylation has been shown in the famine studies and in the Agouti mouse model. Specifically, the impact of dietary micronutrients such as folate and vitamin B12 is of great importance in the normal functioning of processes which regulate methyl donors, one-carbon metabolism and DNA methylation generally. Folate has a significant impact on methylation as it acts as a methyl donor (via the production of dihydrofolate and tetrahydrofolate) for use in the activity of SAM. There are epidemiological studies which examine the correlation between folate status, global methylation and a number of different cancers (Herceg, 2007; Williams, 2012).

16. Vitamins B12 and B6 act as co-enzymes in the generation of methylation via methionine synthase and are also known to impact on global methylation status. Other dietary factors lead to changes in the enzymes which control de novo DNA methylation such as DNMT3A and DNMT3B. These principles are described by Anderson (Anderson et al., 2012) and this paper is appended (Appendix 1). It has also been shown that histones are modified by dietary exposures.

***17. Alcohol*:** The potential of alcohol to cause epigenetic alterations has been recognised as it interferes with folate absorption and thus the one-carbon metabolism mechanism as with nutritional deficiency. It has also been demonstrated that it impacts on LINE-1 hypomethylation a marker of global methylation and that the effects are partially abrogated by intake of folate (Schernhammer et al, 2010). Epidemiological studies have shown that alcohol consumption is associated with epigenetic changes in human colon cancers including hypermethylation of some gene promoters including CDKN2D, MGMT and RASSF1A (Herceg, 2007). Hepatocellular carcinoma (HCC), associated with chronic liver injury which can be attributed to excess alcohol use, exhibits global hypomethylation, promoter hypermethylation and overexpression of the DNA methylating enzymes (DNMT1, 3A, 3B) (Ozen et al 2013). It has also been noted that aberrant methylation

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distinguishes alcoholic induced HCC from those attributable to HCV/HBV infection (Lambert et al 2011). Analysis of tumours of the upper aerodigestive tract, such as oral and oesophageal cancers, indicates a high frequency of aberrant hypermethylation of some genes thought to be associated with carcinogenic progression (MYOD1, MTHFR) (Mani et al 2012). Whilst the correlation is not direct – the associations are noteworthy.

18. Smoking : A link between aberrant promoter methylation and smoking has been established (Belinsky et al, 2002;(Lim and Song, 2012). Epigenome wide studies have shown a strong correlation between tobacco usage and methylation identified in blood and lung tissues and associations with cancer risks were also drawn (Shenker et al., 2013). Maternal tobacco use is also correlated with changes in genome wide placental methylation CpG sites and alterations in gene expression (Suter et al., 2011). Histone modifications and chromatin remodelling have also been reported.

19. Physical activity and stress: The effect of physical activity on global methylation (LINE-1) in women was assessed and it was concluded that higher levels of physical activity over three time periods was associated with increased global DNA methylation. The link between exercise and decreased cancer risk was also noted (White et al., 2013). A study which examined DNA methylation in tai-chi practitioners showed that the reduction in methylation associated with aging was less evident in practitioners compared to matched controls (Ren et al., 2012). In a large study examining DNA methylation in over 14,000 genes of the PBLs of 93 human subjects, early-life poverty and adult stress (perceived and cortisol levels) were correlated with a decrease in methylation (Lam et al., 2012). However the changes were small and were not associated with changes in gene expression.

Discussion

20. Epigenetics has emerged as a critical factor in the regulation of gene expression and the onset and pathogenesis of diseases including cancer. Studies investigating the mechanisms which underpin its maintenance and modification indicate a substantial complexity which is impacted upon by numerous lifestyle factors. This includes the capacity to be altered *in utero,* leading to changes which can manifest in disease in adult life

21. With regard to disease (cancer), it is also clear that epigenetic modifications change over time and therefore assigning causality is likely to be problematic as the disease may influence the epigenetic patterns rather than the epigenetic pattern changes underpinning the disease. Timings of exposures to chemicals and the resulting alterations will need to be clearly understood (Hou et al., 2012). It seems reasonable to suggest that an understanding of these background characteristics is important before an accurate evaluation of the impact of chemical exposures on cancer risk can be undertaken.

Questions for the Committee:

- What are Members' opinions of the proposed role of epigenetics in the development of tumours?
- Should a more detailed review of the impact of background factors on epigenetics and cancer be undertaken? If so, given the extensive data in this area, which aspects are of greatest importance?

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