UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE VETERINARIA

TESIS DOCTORAL

Gamma-H2AX como nuevo criterio de valoración para detectar daños en el ADN en modelos *in vitro*:
Aplicación para la evaluación del humo de los cigarrillos y constituyentes

Gamma H2AX as a new endpoint of DNA damage in *in vitro* models:
application to tobacco smoke and constituents

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

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Gamma–H2AX AS A NEW ENDPOINT OF DNA DAMAGE IN *IN VITRO* MODELS: APPLICATION TO TOBACCO SMOKE AND CONSTITUENTS

Memoria presentada para optar al Grado de Doctor por la Universidad Complutense de Madrid

Departamento de Toxicología y Farmacología

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CERTIFICAN que **Dña. CAROLINA GARCÍA CANTÓN**

Ha realizado bajo nuestra dirección su Tesis Doctoral titulada **"gamma–H2AX as a new endpoint of DNA damage in *in vitro* models: application to tobacco smoke and constituents"** en el Departamento de Toxicología y Farmacología de la Facultad de Veterinaria de la Universidad Complutense de Madrid y en el Grupo Investigación y Desarrollo de British American Tobacco Ltd.

Y para que conste a los efectos oportunos, se expide el presente certificado en Madrid, a cinco de Octubre del dos mil trece.

Prof. Dr. Arturo Anadón Navarro Dr. Clive Meredith

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CONTENTS

ESTRUCTURA DE LA TESIS

Esta Tesis Doctoral se presenta en formato publicaciones donde todos los capítulos de la Tesis Doctoral, que se enumeran a continuación, han sido enviados a revistas indexadas en el Journal Citation Reports (JCR). La Tesis Doctoral comienza con un resumen que incluye una breve introducción a la misma junto con los objetivos, a continuación se describen los materiales y métodos de forma abreviada, seguida por una sección de resultados y por una discusión final con las conclusiones.

CAPÍTULO I – γH2AX como nuevo criterio de valoración para detectar daños en el ADN: aplicaciones para la evaluación de la genotoxicidad *in vitro* del humo de los cigarrillos. Este capítulo introductorio contiene una revisión detallada de la bibliografía especializada relativa al uso de la γH2AX como biomarcador de las roturas de la doble cadena de ADN, además de incluir una descripción general de los criterios actuales de valoración que se emplean en la evaluación *in vitro* de la toxicología genética.

CAPÍTULO II – Caracterización metabólica de los sistemas celulares empleados en los experimentos toxicológicos *in vitro*: línea celular pulmonar BEAS-2B como ejemplo. Este capítulo está centrado en comprender la competencia metabólica del sistema celular BEAS-2B empleado durante esta Tesis Doctoral. También subraya la importancia de la actividad metabólica en los sistemas *in vitro* en el contexto de la bioactivación de tóxicos.

CAPÍTULO III – Evaluación del ensayo *in vitro* γH2AX mediante "High Content Screening" (HCS) como nuevo test de genotoxicidad. Este capítulo describe el desarrollo del ensayo *in vitro* de la γH2AX mediante HCS en células BEAS-2B. El sistema de análisis automatizado contribuye al alto rendimiento del ensayo y elimina la subjetividad del análisis manual. En este capítulo se describe una evaluación preliminar para calcular la sensibilidad y

la especificidad del ensayo analizando la respuesta de un grupo de controles positivos y negativos.

CAPÍTULO IV – Evaluación de la genotoxicidad de tóxicos específicos del humo de los cigarrillos empleando el ensayo *in vitro* de la γH2AX mediante "High Content Screening" (HCS). Este capítulo se centra en la aplicación del nuevo ensayo *in vitro* sobre la γH2AX mediante HCS en la evaluación de las sustancias tóxicas del humo de los cigarrillos de la fase de partículas, tanto a nivel individual como en mezcla binaria.

CAPÍTULO V – Caracterización de un sistema de exposición a aerosoles para la evaluación de la genotoxicidad del humo de los cigarrillos usando el ensayo *in vitro* de la γH2AX mediante "High Content Screening" (HCS). Este capítulo describe la optimización del nuevo ensayo para evaluar aerosoles, seguida de la aplicación del ensayo *in vitro* optimizado de la γH2AX mediante HCS en la evaluación íntegra del humo de los cigarrillos.

1.- RESUMEN DE LA TESIS DOCTORAL

Hay multitud de formas en las que el ADN puede sufrir daños; una de las lesiones más graves son las roturas de la doble cadena, puesto que no hay ninguna cadena complementaria que quede intacta para actuar como plantilla durante la reparación del ADN. Esta lesión puede desembocar en la muerte celular si falla mecanismo de reparación; ahora bien, si se repara de forma incorrecta, la información del ADN puede quedar comprometida, lo que puede producir una mutación y, potencialmente, la conservación de la información de ADN que puede ser incorrecta (Jeggo y Lobrich 2007).

La fosforilación de la histona 2AX denominada γH2AX se produce como respuesta temprana a las roturas de la doble cadena de ADN, y fue descrita por primera vez en 1998 (Rogakou *et al*., 1998). La fosforilación de la H2AX es un biomarcador de daño en el ADN conservado en las células eucariotas y, por tanto, está presente en células humanas, así como en animales y plantas (Redon *et al*., 2011). Desde su descubrimiento, este biomarcador de daño en el ADN ha sido empleado en diferentes ámbitos científicos, como estudios clínicos, investigación sobre la integridad del genoma y desarrollo de medicamentos. En el siglo XXI, el campo preclínico de la toxicología genética está experimentando una revolución, en la que se están utilizando tecnologías nuevas y emergentes para desarrollar y mejorar los métodos de análisis tanto como para los nuevos como para los ya existentes dentro de la toxicología genética (Lynch *et al*., 2011). En esta Tesis Doctoral, la γH2AX ha sido estudiada como un nuevo criterio de evaluación del daño sobre el ADN, en particular de las posibles aplicaciones en la disciplina de la toxicología genética *in vitro*. Se ha sugerido que la medición de la γH2AX se utilice como una herramienta complementaria a la batería de ensayos toxicológicos existente, y de forma más específica como ensayo de pre-evaluación basado en el alto rendimiento generado por sistemas de análisis como son: la citometría de flujo, la

inmunoelectrotransferencia *In Cell Western* y el HCS (Smart *et al*., 2011; Audebert *et al*., 2010; Garcia-Canton *et al*., 2012). El capítulo I de esta Tesis Doctoral revisa los mecanismos de fosforilación de la H2AX y su relevancia como nuevo ensayo *in vitro* de genotoxicidad en el contexto de las pruebas *in vitro* de genotoxicidad actuales, tanto reguladas como no reguladas.

El diseño experimental para medir la γH2AX *in vitro* requiere la selección de un sistema celular, teniendo en cuenta la ruta de exposición y los requisitos metabólicos del compuesto a analizar. En esta Tesis Doctoral se ha previsto que el ensayo *in vitro* de la γH2AX mediante HCS sea aplicado a la evaluación del humo de los cigarrillos, que constituye de forma irrefutable una de las principales causas del cáncer de pulmón y contribuye a otras enfermedades, entre las que se incluyen trastornos cardiovasculares y respiratorios. Algunos componentes del humo de los cigarrillos, como el benzo[a]pireno (B[a]P), no son genotóxicos *per se*, pero pueden ser metabolizados por enzimas específicas de un tejido, como los citocromos P450 (CYP), y transformarse en especies reactivas. Estos compuestos reciben el nombre de protóxicos. Por este motivo, la capacidad metabólica del sistema celular seleccionado tiene que caracterizarse para garantizar que los compuestos protóxicos sean correctamente evaluados.

Se seleccionó como sistema celular la línea celular humana BEAS-2B derivada del pulmón. Esta línea celular fue establecida por Reddel y colaboradores a partir de células epiteliales bronquiales con fenotipo normal, inmortalizadas por el adenovirus 12-SV40/híbrido sin capacidad de replicación (Reddel *et al*., 1988). Cuando se mide la γH2AX, las líneas celulares derivadas de los tejidos normales, como las células BEAS-2B, se consideran más fiables que aquellas procedentes de tejidos premalignos o tumorales. Por ejemplo, se ha observado que algunas líneas celulares tumorigénicas pueden ser defectuosas en algunas

funciones fundamentales, como mecanismos de reparación de daño en el ADN o que no tienen una regulación normal del ciclo celular (Hanahan y Weinberg, 2000).

La transformación maligna celular podría afectar a la respuesta de la γH2AX; por ejemplo, existen trabajos que demuestran una importante cantidad de γH2AX en ausencia de roturas de la doble cadena de ADN, posiblemente como respuesta al "estres oncogénico" (Svetlova *et al*., 2010). Además, el órgano de origen de esta línea celular es de especial interés en la evaluación de compuestos, puesto que el objetivo principal es el pulmón. Este es el caso del humo de los cigarrillos, los contaminantes y los nanomateriales, entre otros (Ansteinsson *et al*., 2011; Veljkovic *et al*., 2011). No obstante, era necesario caracterizar de manera adicional las capacidades metabólicas de la línea celular BEAS-2B para respaldar un diseño experimental sólido de cara a la bioactivación de protóxicos. En el capítulo II de esta tesis se detalla la caracterización metabólica de la línea celular BEAS-2B.

Tras nuestra evaluación usando células BEAS-2B, se puede llegar a la conclusión de que en estas células se detectaba una actividad limitada de las enzimas del citocromo P450 en comparación con las células del tejido pulmonar normal. Para evitar una evaluación incorrecta de los compuestos protóxicos, se incluyó en diseño experimental durante el desarrollo del ensayo *in vitro* de la γH2AX una fuente externa de activación metabólica, tal y como se recomienda en las guías destinadas al asesoramiento de ensayos de genotoxicidad de medicamentos de uso humano (ICH, 2011). El desarrollo del ensayo incluyo un sistema de análisis automatizado basado en microscopía denominado HCS (Bickle, 2010). Existen otros sistemas automatizados alternativos empleados en otros estudios para medir la γH2AX, como la citometría de flujo (Smart *et al*., 2011) y el sistema *In Cell Western*, método de inmuno-electrotransferencia en el que se emplea una placa con múltiples pocillos (Audebert *et al*., 2010). En esta Tesis Doctoral se seleccionó un panel de 22 compuestos positivos y negativos

empleados en evaluaciones anteriores de ensayos de genotoxicidad (Hastwell *et al*., 2006) y recomendados por el Centro Europeo para la Validación de Métodos Alternativos. Se calcularon la sensibilidad, especificidad y precisión del ensayo mediante HCS siguiendo las recomendaciones publicadas por un grupo de expertos (Kirkland *et al*., 2008). El panel de compuestos seleccionado incluía compuestos con diferentes mecanismos de acción conocidos, como protóxicos, agentes aneugénicos y compuestos no genotóxicos. En el capítulo III de esta tesis se presenta el desarrollo del ensayo *in vitro* de la γH2AX mediante HCS y la evaluación preliminar de su precisión.

Tras finalizar la evaluación del rendimiento del ensayo H2AX con sustancias tóxicas de referencia, aplicamos el ensayo H2AX a las sustancias tóxicas del humo de los cigarrillos y a la exposición al humo. El humo de los cigarrillos es una mezcla compleja, que consta de más de 5600 compuestos identificados (Perfetti y Rodgman, 2011). Los cálculos sugieren que la mezcla íntegra contiene más de 150 sustancias tóxicas conocidas (Cunningham *et al*., 2011), incluyendo compuestos clasificados por la IARC como "carcinógenos para los humanos" (grupo 1), por ejemplo B[a]P o 4-(metilnitrosamino)-1-(3-piridil)-1-butanona (NNK) (IARC, 2012a; AIRC, 2012b). Los resultados experimentales obtenidos de una mezcla compleja podrían ser complicados de interpretar, puesto que las interacciones entre los componentes separados podrían influir sobre el resultado final. El fraccionamiento de la mezcla compleja en componentes individuales y mezclas sencillas podría ayudar a determinar los elementos tóxicos fundamentales presentes en el humo de los cigarrillos. La regulación de los productos derivados del tabaco por parte de diferentes organizaciones, como el Centro de Productos de Tabaco de la Administración de Alimentos y Medicamentos o el Convenio Marco de la Organización Mundial de la Salud para el Control del Tabaco, requiere herramientas de evaluación de riesgos sensibles y rápidas, que sirvan para complementar el conocimiento ya existente basado principalmente en datos *in vivo* (WHO 2008; FDA, 2012). La tarea más

exigente del análisis previo del potencial de genotoxicidad *in vitro* de un gran número de compuestos tanto por separado como en mezcla simple requiere un ensayo de alto rendimiento con un diseño experimental simplificado. Este enfoque también sirve para respaldar la reducción en el uso de modelos animales, tal y como recomienda el enfoque de las 3R (Schechtman, 2002). En esta tesis, el nuevo desarrollo del ensayo *in vitro* de la γH2AX mediante HCS se aplicó a la evaluación del potencial genotóxico de algunos elementos tóxicos del humo de los cigarrillos (que se describen en el capítulo IV) y al humo de los cigarrillos (descrito en el capítulo V).

En el capítulo IV de esta Tesis Doctoral procedimos a seleccionar una serie de sustancias tóxicas del humo de los cigarrillos de la fase de partículas para ser evaluadas en el nuevo ensayo *in vitro* de la γH2AX mediante HCS. El hidrocarburo aromático policíclico B[a]P, las nitrosaminas específicas del tabaco 4-(metilnitrosamino)-1-(3-piridil)-1-butanona (NNK) y N-nitrosonornicotina (NNN) y el cadmio (metal pesado) fueron evaluados individualmente, mientras que las nitrosaminas especifícas del tabaco, NNN y NNK, también fueron evaluados en mezcla binaria. Las tres primeras sustancias han sido recomendadas para una reducción obligatoria de su nivel en los productos derivados del tabaco, mientras que para el cadmio se recomienda su monitorización por parte de la Organización Mundial de la Salud para el Control del Tabaco (WHO, 2008).

El ensayo *in vitro* de la γH2AX mediante HCS también se optimizó en el capítulo V para la evaluación de aerosoles en la fase aire-líquido. Esta metodología se considera fisiológicamente más relevante para la evaluación íntegra del humo directo de los cigarrillos, puesto que las células están expuestas directamente a la mezcla compleja de la combustión de productos derivados del tabaco (Phillips *et al.*, 2005).

En resumen, esta Tesis Doctoral describe el desarrollo, la evaluación preliminar y la aplicación de un nuevo ensayo para la detección de daño en el ADN mediante el uso de nuevas tecnologías para automatizar la medida de la respuesta. Además, el nuevo ensayo ha demostrado su potencial para optimizarse y aplicarse en el campo reglamentario del tabaco como herramienta para efectuar un análisis inicial de un gran número de compuestos y mezclas, incluyendo aerosoles.

SUMMARY

There are multiple forms of DNA damage, one of the most deleterious lesions are double-strand breaks (DSBs) since no complementary strand is left intact to act as a template during DNA repair. This lesion can lead to cell death if the repair mechanism fails, however, if repaired incorrectly, the DNA information can be compromised leading to mutation and potentially the perpetuating incorrect DNA information (Jeggo and Lobrich 2007).

Phosphorylation of the histone 2AX resulting in γH2AX occurs as an early response to DNA DSBs and was first described in 1998 (Rogakou *et al*., 1998). The phosphorylation of H2AX is a conserved biomarker of DNA damage across eukaryotes and is therefore present in humans as well as animals and plants cells (Redon *et al*., 2011). Since its discovery, this biomarker of DNA damage has been used in different disciplines of scientific research such as clinical studies, genome integrity research and drug development. The field of pre-clinical genetic toxicology testing in the 21st century is experiencing a revolution where new and emerging technologies are being used to develop and improve new and existing testing methods in genetic toxicology (Lynch *et al*., 2011). In this PhD dissertation, γH2AX has been studied as a novel endpoint of DNA damage focusing on the potential applications in the discipline of *in vitro* genetic toxicology. The measurement of γH2AX has been suggested as a complementary tool to the existing battery of genetic toxicology tests, more specifically

as a pre-screening assay based on the high throughput given by scoring systems such as flow cytometry, *in cell western* immunoblotting and High Content Screening (HCS) (Smart *et al*., 2011; Audebert *et al*., 2010; Garcia-Canton *et al*., 2012). Chapter I of this PhD dissertation reviews the mechanisms of H2AX phosphorylation and the relevance of γH2AX as a novel *in vitro* genotoxicity assay in the context of current *in vitro* genotoxicity tests both regulatory and non-regulatory.

The experimental design for measuring γH2AX *in vitro* requires the selection of a cell system taking into consideration route of exposure and metabolic requirements. In this PhD dissertation, the *in vitro* γH2AX assay by HCS is planned to be applied to the evaluation of cigarette smoke which is known to be a major cause of lung cancer and contributes to other diseases including cardiovascular and respiratory related diseases. Some cigarette smoke constituents such as Benzo[a]pyrene (B[a]P) are not genotoxic per se, but can be metabolized by tissue specific enzymes such as cytochromes P450 (CYPs) into reactive species. These compounds are known as pro-toxicants. Therefore, the metabolic capability of the selected cell system needs to be characterised to ensure that pro-toxicant compounds are correctly evaluated.

Here, the human lung-derived BEAS-2B cell line was selected as the cell system. This cell line was established by Reddel and colleagues from normal bronchial epithelial cells that were immortalised by a replication-defective 12-SV40/adenovirus hybrid (Reddel *et al*., 1988). When measuring γH2AX, cell lines derived from normal tissues, such as BEAS-2B cells, are considered to be more reliable than those from pre-malignant and cancer tissues. For instance, it has been reported that some tumorigenic cell lines can be defective in key functions including DNA damage repair mechanisms and lack normal cell cycle regulation (Hanahan and Weinberg 2000). Malignant cell transformation could affect the γH2AX

response, for instance, there have been reports showing significant amount of γH2AX in the absence of DNA DSBs, possibly as a response to "oncogenic stress" (Svetlova *et al*., 2010). Moreover, the organ of origin of this cell line is of special interest in the evaluation of compounds since the primary target is the lung. This is the case of cigarette smoke, pollutants and nanomaterials among others (Ansteinsson *et al*., 2011; Veljkovic *et al*., 2011). However, the metabolic capabilities of the BEAS-2B cell line needed to be further characterised to support robust experimental design for pro-toxicant bioactivation. The metabolic characterisation of the BEAS-2B cell line is detailed in Chapter II of this PhD dissertation.

Following our assessment of the BEAS-2B cells, we concluded that limited cytochrome P450 enzyme activity could be detected in BEAS-2B cells compared to normal lung-tissue. To avoid an incorrect evaluation of pro-toxicants, a standardised external source of metabolic activation was incorporated in the experimental design of the *in vitro* γH2AX assay development as recommended in testing guidelines (ICH, 2011). The assay development included an automated microscopy-based scoring system referred to as High Content Screening (Bickle 2010). Other alternative automated screening systems previously used to measure γH2AX include flow cytometry (Smart *et al*., 2011) and *In Cell Western* a multi-well plate based immunoblotting (Audebert *et al*., 2010). A panel of 22 positive and negative compounds used in previous genotoxicity assay assessments (Hastwell *et al*., 2006) and recommended by the European Centre for the Validation of Alternative Methods (ECVAM) were selected. The sensitivity, specificity and accuracy of the assay by HCS were calculated following recommendations published by an expert group (Kirkland *et al*., 2008). The selected panel included compounds with different known mechanisms of action, such as pro-toxicants, aneugens and non-genotoxic compounds. The *in vitro* γH2AX assay by HCS development and preliminary assessment is presented in Chapter III of this PhD dissertation.

Following the completion of the H2AX assay performance evaluation with reference toxicants, we applied the H2AX assay to cigarette smoke toxicants and whole smoke exposure. Cigarette smoke is a complex mixture consisting of more than 5,600 identified compounds (Perfetti and Rodgman 2011). Estimations suggest that the whole mixture contains over 150 known toxicants (Cunningham *et al*., 2011), including compounds classified by IARC as "carcinogenic to humans" (Group 1) such as B[a]P and NNK (IARC, 2012a; IARC, 2012b). Experimental results obtained from complex mixture testing could be difficult to interpret as interactions between single components could affect the final outcome. Fractionation of the complex mixture into single components and simple mixtures could assist with the determination of key toxic drivers present in the cigarette smoke. The regulation of tobacco products from different organisations such as the Food and Drug Administration Center for Tobacco Products (FDA CTP) and the World Health Organisation Framework Convention on Tobacco Control (WHO FCTC) require sensitive and fast risk assessment tools that complement existing knowledge based mainly on *in vivo* data (WHO 2008; FDA, 2012). The challenging task of pre-screening the *in vitro* genotoxicity potential of a large number of compounds individually and in simple mixture requires a high throughput assay with a simplified experimental design. This approach also supports the reduction in the use of animal models as recommended by the 3Rs approach (Schechtman 2002). In this PhD dissertation, the newly developed *in vitro* γH2AX assay by HCS was applied to the evaluation of the genotoxic potential of cigarette smoke toxicants (described in Chapter IV) and whole mainstream cigarette smoke (WMCS) (described in Chapter V).

In Chapter IV of this PhD dissertation, we selected a number of cigarette smoke toxicants from the particulate phase to be tested in the novel *in vitro* γH2AX assay by HCS. The polycyclic aromatic hydrocarbon B[a]P, the tobacco-specific nitrosamines (TSNAs) 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosonornicotine (NNN) and

the heavy metal cadmium were all tested individually whereas the TSNAs were also tested in binary mixture. The first three toxicants are recommended for mandated lowering while the last one is recommended for monitoring by the WHO FCTC (WHO 2008).

The *in vitro* γH2AX assay by HCS was also optimised in Chapter V for the evaluation of aerosols at the air-liquid interface. This methodology is considered more physiologically relevant for the evaluation of WMCS as cells are directly exposed to the complex mixture of combusted tobacco (Phillips *et al.*, 2005).

In summary, this PhD dissertation describes the development, preliminary assessment and application of a novel assay for the detection of DNA damage using new technologies to automate the measurement of the response. Furthermore, the novel assay has demonstrated the potential to be further optimised and applied in the area of tobacco regulation as a tool to pre-screen large numbers of compounds and mixtures including aerosols.

2.- OBJETIVOS

- Caracterizar las capacidades metabólicas de la línea celular BEAS-2B procedente del pulmón para su uso *in vitro* en experimentos toxicológicos.
- Determinar si la respuesta de la γH2AX puede medirse en las células BEAS-2B mediante un sistema automatizado basado en microscopía y que es conocido como HCS.
- Evaluar la sensibilidad, la especificidad y la precisión del ensayo *in vitro* de la γH2AX mediante HCS con un conjunto preliminar de compuestos de referencia.
- Determinar si el ensayo *in vitro* de la γH2AX mediante HCS puede aplicarse a la evaluación de sustancias tóxicas procedentes del humo de los cigarrillos, tanto de forma separada como en mezcla binaria.
- Investigar si el ensayo *in vitro* de la γH2AX mediante HCS puede desarrollarse y adaptarse a la exposición de aerosoles.

3.- MATERIALES Y MÉTODOS

3.1.- Líneas celulares

La línea celular epitelial bronquial humana (BEAS-2B) fue adquirida a ATCC (Estados Unidos). Las células fueron conservadas en un medio de crecimiento epitelial bronquial (Bronchial Epithelial Growth Medium, BEGM®) a 37°C y con CO_2 al 5% en un incubador humidificado (Lonza Group Ltd., Bélgica).

El tipo II alveolar humano procedente de un adenocarcinoma (A549) fue adquirido a ATCC (Estados Unidos). Las células fueron conservadas en un medio Eagle modificado por Dulbecco, complementado con suero fetal bovino (10% v/v), L-glutamina (2 mM), penicilina (50 U/mL) y estreptomicina (50 µg/mL) (DMEM completo) a 37°C y con CO_2 al 5% en un incubador humidificado.

La línea celular procedente del hepatocarcinoma (HepG2) fue adquirida a ATCC (Estados Unidos). Las células fueron conservadas en medio Eagle modificado por Dulbecco, complementado con suero fetal bovino (10% v/v), L-glutamina (2 mM), penicilina (50 U/mL) y estreptomicina (50 µg/mL) (DMEM completo) a 37°C y con un 5% de CO_2 en un incubador humidificado.

La línea celular humana derivada de un hepatocarcinoma (HepaRG) fue adquirida ya diferenciada como una capa confluente a Biopredic International (Francia). Las células fueron conservadas en un medio basal, complementado con una mezcla de recuperación durante 24 horas seguido de un medio basal complementado con una mezcla de mantenimiento/metabolismo. Los medios y los complementos fueron suministrados por el fabricante (Biopredic, Francia).

Las células BEAS-2B, A549 y HepG2 fueron cultivadas y expandidas en el laboratorio. Los experimentos se llevaron a cabo únicamente entre los pasos 3 y 12. Todos los cultivos fueron analizados para descartar la presencia de contaminación por micoplasma, y todos resultaron ser negativos. Además, las células fueron autentificadas mediante el análisis de repeticiones cortas en tándem para confirmar la naturaleza de los cultivos celulares (LGC Standards, Reino Unido) (Nims *et al*., 2010).

3.2.- Metodologías

3.2.1.- Caracterización metabólica de las células BEAS-2B

Expresión génica: Se examinó un panel de 47 genes con codificación de enzimas de fase I y fase II del metabolismo a través del uso de qPCR en las células BEAS-2B, A549 y HepG2, tanto en presencia como en ausencia del inductor 2,3,7,8-tetraclorodibenzo-p-dioxina (TCDD). La línea celular HepaRG fue analizado en ausencia de inductor.

Ensayos de actividad enzimática: se generaron perfiles de actividad para cuatro enzimas del citocromo P450 implicadas en el metabolismo de algunas sustancias tóxicas del humo de los cigarrillos. La actividad de CYP1A1/1B1 se evaluó mediante el ensayo P450-Glo™ después de su inducción por TCDD. CYP2A6/CYP2A13, CYP2E1 y CYP1A2 se evaluaron mediante UPLC-MS/MS con los sustratos específicos de la enzima: cumarina, clorzoxazona y etoxiresorufina. Estos métodos fueron empleados en el capítulo II.

3.2.2.- Detección y cuantificación de γH2AX en células BEAS-2B mediante inmunotinción

Se identificó la γH2AX como marcador de la rotura de las dobles cadenas de ADN mediante inmunotinción, utilizando un anticuerpo primario específico de la propia γH2AX seguido de un anticuerpo secundario acoplado con fluoróforo.

La intensidad de la fluorescencia fue detectada y medida a través de la plataforma Cellomics Arrayscan® VTi (Thermo Scientific, EE. UU.), y posteriormente analizada con el software Target Activation Bioapplication V.6.6.1.4.

Una vez comprobados los compuestos protóxicos, se empleó la fuente externa de activación metabólica el sobrenadante homogeneizado inducido por aroclor-1254 procedente del hígado de rata con cofactores (mezcla S9) (Moltox, EE. UU.). La mezcla S9 es uno de los sistemas estándar empleados como fuente externa de activación metabólica en los actuales y regulados ensayos *in vitro*.

Los criterios de aceptación para la citometría de flujo descritos para el ensayo *in vitro* de la γH2AX (Smart *et al.*, 2011) fueron aplicados a los resultados del ensayo *in vitro* de la γH2AX mediante HCS para evaluar la relevancia de utilizar el mismo enfoque (Tabla 1).

Tabla 1: Criterios de evaluación de la genotoxicidad para el ensayo *in vitro* de la γH2AX, adaptados (Smart *et al.*, 2011).

Respuesta γH2AX	**Clasificación**
> 1,5 veces γH2AX x RCC > 25%	Genotóxico (+)
< 1,5 veces γH2AX x RCC 100-0%	No genotóxico (–)
> 1,5 veces γH2AX x RCC < 25%	"Falso" positivo; genotoxicidad (C) provocada por citotóxico
= 1,5 veces γH2AX x RCC ≥ 25%	Equívoco (±)

Esta metodología se aplicó en los capítulos III, IV y V.

3.2.3.- Optimización para la exposición a aerosoles

Para la optimización por la exposición a los aerosoles se empleó la máquina fumadora "Borgwaldt RM20S®" (RM20S) para generar el humo directo de los cigarrillos. Las células cultivadas en soportes con membrana porosa Transwell™ se mantuvieron expuestas durante 3 horas a una serie de diluciones del humo directo de los cigarrillos en aire procedentes de dos cigarrillos de referencia. El cigarrillo de referencia 3R4F es una mezcla de tabaco "estilo EE.UU." que genera 9,4 mg de alquitrán y 0,7 mg de nicotina cuando se fuma siguiendo las condiciones experimentales detalladas por la Organización Internacional de Normalización (ISO) sobre el consumo de cigarrillos (ISO 3308:2012). El cigarrillo de referencia M4A es un cigarrillo con tabaco curado al aire caliente que genera 10 mg de alquitrán y 1,0 mg de nicotina cuando se fuma siguiendo las condiciones experimentales detalladas por la ISO (ISO 3308:2012). Esta optimización se describe en el capítulo V.

4.- RESULTADOS

4.1.- Capítulo II – Caracterización metabólica de los sistemas celulares *in vitro* empleados en los experimentos toxicológicos: línea celular pulmonar BEAS-2B

En la introducción, subrayamos la importancia de la actividad metabólica en la bioactivación de algunas sustancias tóxicas, aunque no necesariamente en todas. Por este motivo, describimos la expresión génica por qPCR de un panel de genes que codifican enzimas relacionadas con el metabolismo y la actividad de un grupo seleccionado de enzimas del citocromo P450 en células BEAS-2B.

Expresión génica: el análisis qPCR indicó una expresión génica limitada en las células BEAS-2B, con la excepción de CYP1A1, CYP1B1 y CYP1A2, que mostraron una importante regulación al alza (25, 5 y 4 veces más, respectivamente) tras una incubación previa con TCDD, inductor de algunas enzimas del citocromo P450.

Actividad enzimática: las células BEAS-2B inducidas por TCDD mostraron una actividad CYP1A1/1B1 de 0,2 RLU/mg/min, cantidad baja comparada con otras células metabólicamente competentes. En cambio, no se observó ninguna actividad enzimática en las células BEAS-2B para CYP1A2, CYP2A6/2A13 y CYP2E1.

4.2.- Capítulo III – Evaluación del ensayo *in vitro* γH2AX mediante *High Content Screening* (HCS) como nuevo test de genotoxicidad

Se utilizó un panel de 22 compuestos de referencia, que tenían diferentes mecanismos de acción conocidos para llevar a cabo una evaluación preliminar de la sensibilidad y la especificidad del ensayo *in vitro* de la γH2AX mediante HCS. También como se había previsto en la evaluación se determinaron si los criterios de evaluación de la genotoxicidad

descritos por Smart y colaboradores (2011) para el sistema de citometría de flujo podrían emplearse con el sistema HCS.

La evaluación preliminar indica que el ensayo *in vitro* de la γH2AX mediante HCS tiene una alta precisión (86%) como consecuencia de su alta sensibilidad y especificidad (86-92% y 80-88% respectivamente). Los resultados también confirmaron que los criterios empleados para el sistema de análisis por citometría de flujo (Tabla 1) también pueden aplicarse al sistema HCS.

4.3.- Capítulo IV – Evaluación de la genotoxicidad de sustancias tóxicas específicas del humo de los cigarrillos empleando el ensayo *in vitro* de la γH2AX mediante High Content Screening (HCS)

A continuación, se aplicó el ensayo de la γH2AX mediante HCS sobre las sustancias tóxicas conocidas del tabaco, incluyendo hidrocarburos policíclicos aromáticos, nitrosaminas y metales pesados, con la finalidad de evaluar el ensayo en el contexto de la toxicología del tabaco. El compuesto B[a]P no indujo ningún incremento en la frecuencia de la γH2AX por encima de los controles tratados con el vehículo (control negativo) después de 3 horas de tratamiento en cultivos previamente inducidos por TCDD o no inducidos. Cuando se evaluó el B[a]P durante 24 horas, se comprobó que se había producido un incremento significativo de la frecuencia de la γH2AX en los cultivos tanto inducidos por TCDD como no inducidos, en concentraciones superiores a 0,5 μM.

No obstante, la frecuencia de la γH2AX en cultivos no inducidos para concentraciones superiores a 4 μM después de un tratamiento de 24 horas, volvió a los niveles tratados con vehículo (inducción < 1,5 veces).

El cloruro de cadmio produjo una respuesta positiva después de un tratamiento de tres horas en concentraciones superiores a 250 μM. Después de las 24 horas de tratamiento, las concentraciones por encima de 16 μM mostraban una genotoxicidad generada por efecto citotóxico.

En el caso de las nitrosaminas especificas del tabaco, la NNK y la NNN no mostraron incremento estadísticamente significativo alguno en la frecuencia de la γH2AX en comparación con los controles tratados con vehículo para ningúno del los intervalos de tiempo en presencia de mezcla S9. Cuando se evalúan los precursores reactivos NNK-acetato y NNN-acetato, ambos produjeron un incremento en la frecuencia de la γH2AX en comparación con el control tratado con vehículo después de un tratamiento de 3 horas en concentraciones por encima de las 16 μM y las 500 μM respectivamente. Después de un tratamiento de 3 horas seguido de un período de recuperación de 24 horas, el NNK-acetato provocó un incremento significativo en concentraciones superiores a los 125 μM; aunque el NNN-acetato provocó un incremento dosis-dependiente en la frecuencia de la γH2AX, aunque los resultados no alcanzaron el umbral de respuesta positiva.

Para evaluar el efecto de las mezclas binarias, se llevaron a cabo tratamientos con 5 permutaciones de mezclas de TSNA con diferentes proporciones de los precursores reactivos de NNK y NNN (NNK-acetato y NNN-acetato, respectivamente) durante 3 horas, en concentraciones entre 7,8 μM y 1000 μM. El tratamiento con NNK-acetato al 100% provocó un incremento significativo en la frecuencia de γH2AX en concentraciones por encima de los 31,25 μM, mientras que NNN-acetato al 100% generó un incremento importante únicamente a la dosis más alta evaluada (1000 μM). Todas las demás combinaciones de mezcla comprobadas generaron incrementos importantes en los niveles de γH2AX en concentraciones por encima de 62,5 μM. La respuesta inducida por diferentes proporciones

de nitrosaminas específicas del tabaco sugiere que el NNK-acetato es el principal responsable de la respuesta genotóxica cuando se encuentra presente en una mezcla con NNN-acetato. En este estudio no se observaron efectos sinérgicos ni antagonistas al utilizar precursores reactivos NNK y NNN independientes del citocromo P450 en mezcla binaria.

4.4.- Capítulo V – Caracterización de un sistema de exposición a aerosoles para la evaluación de la genotoxicidad del humo de los cigarrillos usando el ensayo *in vitro* de la γH2AX mediante High Content Screening (HCS)

Algunos productos nuevos derivados del tabaco, como son los cigarrillos "heat not burn" (que no necesitan combustión), están apareciendo en el mercado con la ventaja de un potencial de una menor exposición a sustancias tóxicas y sugieren por ello un menor riesgo. En el contexto regulador del tabaco en constante evolución, las sugerencias relativas a la menor exposición y a un menor riesgo tienen o tendrán que corroborarse de acuerdo a las legislaciones nacionales específicas. Por este motivo, es interesante desarrollar ensayos genotóxicos, como el ensayo de la γH2AX sobre la exposición a los aerosoles en contraposición a las sustancias tóxicas separadas o en solución. En este capítulo comprobamos la respuesta de la γH2AX tras la exposición íntegra al humo de dos cigarrillos de referencia con una serie de diluciones del propio humo.

Los cigarrillos de referencia 3R4F y M4A generaron un incremento importante en la frecuencia de la γH2AX (incremento superior a 1,5 veces) en comparación con el control tratado con aire en todas las diluciones comprobadas (de 1:500 a 1:16.000). Mientras que la exposición íntegra al humo directo de los cigarrillos del 3R4F produjo una respuesta dosis-dependiente, no se observó el mismo efecto con el humo directo de los cigarrillos del M4A. Los trabajos que se lleven a cabo en un futuro requerirán una caracterización en profundidad

del efecto de las variaciones del producto en la inducción de la γH2AX para comprender las diferencias en la respuesta.

5.- DISCUSIÓN

Con el paso de los años, el paradigma de la evaluación de riesgos de la toxicología genética ha pasado de los métodos de ensayo en animales (*in vivo*) a los ensayos *in vitro* (Anadón *et al.*, 2014). Por ejemplo, en la industria cosmética, las pruebas en animales están prohibidas por la 7.ª modificación de la Directiva de la UE (European Commission, 2003). No obstante, en muchos casos, los ensayos *in vitro* actuales producen un elevado número de "falsos positivos", una respuesta *in vitro* positiva que no se confirma *in vivo*. La frecuencia actual de estos "falsos positivos" incrementa la necesidad de continuar con los ensayos *in vivo*, lo cual demora mucho el desarrollo de nuevos productos. En consecuencia, durante la última década se han realizado multitud de esfuerzos en lo que respecta al desarrollo y la validación de nuevos métodos de prueba *in vitro* por parte de organizaciones como el Centro Europeo para la Validación de Métodos Alternativos o el Centro Nacional para el Reemplazo, la Refinación y Reducción de Animales de Investigación (Schechtman, 2002).

En el proceso de desarrollo de nuevos ensayos, tenemos que tomar en consideración todas las características relevantes que pueden hacer que el nuevo método de ensayo pueda ser utilizado. Lo ideal es que un ensayo sea:

i. Fisiológicamente relevante: el sistema celular seleccionado para el ensayo tiene que ser relevante para la vía de exposición, de origen humano y tiene que tener en cuenta las necesidades metabólicas del tejido diana.
ii. La evaluación de los compuestos debe ser precisa: la sensibilidad (o capacidad de detectar compuestos genotóxicos) y la especificidad (o capacidad para discriminar compuestos no genotóxicos) tienen que ofrecer una mejora sobre los métodos de ensayo existentes.

iii. Alto rendimiento: el ensayo tiene que utilizar nuevas técnicas y tecnologías con el fin de suministrar un método rápido y en consecuencia barato, que sea fiable y reproducible con el paso del tiempo. La automatización o el potencial de automatización de la metodología representarían una ventaja, especialmente en lo que respecta a la pre-selección.

iv. Objetivo: la medida de la respuesta será mucho más eficaz si puede llevarse a cabo mediante un sistema automático validado en vez de por un operador humano. Asimismo, la medida de la respuesta por parte del operador humano se limita a los términos cualitativos (sí/no) más que a los cuantitativos (un valor medido).

v. Aplicable: el ensayo nuevo tiene que poder aplicarse a la investigación actual.

Se ha utilizado ampliamente la medida de la γH2AX como biomarcador de la rotura de las dobles cadenas de ADN desde su desde su descubrimiento (Rogakou *et al*., 1998). En el campo de la toxicología genética *in vitro*, la medida de la γH2AX se ha apuntado como un posible ensayo complementario a la batería de ensayos existente, especialmente como herramienta de pre-evaluación o pre-selección. A pesar de ello, la medida de la γH2AX se sigue utilizando mucho en la investigación, y son pocos los estudios que se han centrado en el desarrollo completo del ensayo investigando las características mencionadas anteriormente. Los diferentes capítulos de esta Tesis Doctoral han analizado las características necesarias para el desarrollo del ensayo *in vitro* de la γH2AX mediante HCS.

La línea celular humana BEAS-2B procedente del pulmón fue seleccionada por su naturaleza humana y su tejido de origen. No obstante, los resultados de la caracterización metabólica mostraron que había ausencia de actividad en la mayoría de las enzimas del citocromo P450 evaluadas en comparación con el tejido pulmonar normal de una persona. La ausencia de competencia metabólica podría mitigarse añadiendo una fuente externa de activación

metabólica. En los ensayos reguladores rutinarios de genotoxicidad, se utiliza el sobrenadante homogeneizado inducido por aroclor-1254 procedente del hígado de rata con cofactores (mezcla S9) como fuente externa de activación metabólica. Las mezclas S9 derivadas de animales no son representativas de la especie de la línea celular ni del órgano diana, pero hasta la fecha son la opción disponible más normalizada. Las mezclas S9 procedentes de humanos están disponibles en el mercado, aunque las variaciones fenotípicas y los diferentes factores medioambientales hacen que estas fracciones no resulten fiables para ensayos rutinarios. En consecuencia, se selecciona el S9 procedente del hígado de rata inducido por aroclor-1254 como la fuente externa de activación metabólica para la evaluación de los compuestos protóxicos durante el desarrollo del ensayo *in vitro* de la γH2AX mediante HCS.

El ensayo *in vitro* de la γH2AX mediante evaluación preliminar del HCS mostró una alta precisión (86%) en la detección de compuestos genotóxicos y en la discriminación de compuestos no genotóxicos; estos resultados coinciden con las evaluaciones llevadas a cabo previamente con otras metodologías de análisis (Smart *et al.*, 2011; Tsamou *et al.*, 2012). No obstante, se observaron algunas limitaciones durante la fase de desarrollo. La cantidad de mezcla S9 empleada para la comprobación de los compuestos protóxicos no alcanzó el mínimo recomendado de un 1% v/v como consecuencia de la toxicidad intrínseca observada y, por tanto, solo se utilizó un 0,5% v/v. Esta reducción en la cantidad de mezcla S9 utilizada podría haber provocado la falta de respuesta positiva del 2-acetilaminofluoreno, uno de los cuatro compuestos protóxicos comprobados en esta Tesis Doctoral. Las optimizaciones adicionales de ensayos podrían incluir una línea celular humana metabólicamente competente para realizar una mejor evaluación de los compuestos protóxicos. En términos generales, las líneas celulares hepáticas son conocidas por mantener un cierto nivel de metabolismo, como es el caso de HepG2 y HepaRG (Jennen *et al.*, 2010). No obstante, estas líneas celulares sacrificarán la relevancia en la vía de exposición en investigaciones donde el órgano diana

sea el pulmón. Lo ideal es que se desarrolle una línea celular pulmonar metabólicamente competente, con ayuda de la nueva metodología de células madre pluripotentes inducidas se puede a largo plazo conseguir un avance en este área.

Se observó una segunda limitación cuando se comprobaron los compuestos aneugénicos. El tratamiento con agentes aneugénicos produjo cambios morfológicos en las células, puesto que detiene su ciclo celular. Las células se redondean y la adhesión celular se reduce, lo que produce una elevada pérdida de células durante el proceso de inmunotinción. El diseño experimental tendrá que ajustarse para comprobar esta familia de compuestos, con el fin de asegurar que la adhesión celular quede restablecida antes de que comience el proceso de inmunotinción.

Tras el desarrollo y la optimización adicional del ensayo *in vitro* de la γH2AX mediante HCS, se aplica el nuevo ensayo para evaluar el humo de los cigarrillos y una serie de componentes presentes en el humo. Los resultados mostraron que el ensayo *in vitro* de la γH2AX mediante HCS puede detectar el efecto de la genotoxicidad del humo de dos cigarrillos de referencia, con incrementos en la respuesta de hasta 7 veces por encima de los controles tratados con aire. El nuevo ensayo también puede detectar y cuantificar el efecto genotóxico de las sustancias tóxicas del humo de los cigarrillos, tanto en forma separada como en forma de mezcla binaria, lo que muestra el potencial como herramienta para investigar la aportación relativa de cada sustancia tóxica a la totalidad de la toxicidad del tabaco asociada al humo en este criterio de valoración específico de los daños sobre el ADN.

6.- CONCLUSIONES

- La línea celular BEAS-2B parece no tener actividades en las enzimas del citocromo P450 relevantes para la bioactivación de algunos compuestos protóxicos.
- La γH2AX puede detectarse y cuantificarse en las células BEAS-2B mediante un sistema automatizado basado en microscopía, conocido como High Content Screening.
- El ensayo *in vitro* de la γH2AX mediante HCS mostró una alta precisión (86%), producida por una alta sensibilidad (86-92%) y una alta especificidad (80-88%).
- El ensayo *in vitro* de la γH2AX mediante HCS puede aplicarse a la evaluación de sustancias tóxicas procedentes del humo del tabaco en fase de partículas, tanto separadamente como en mezcla primaria.
- El ensayo *in vitro* de la γH2AX mediante HCS fue optimizado de forma satisfactoria para determinar la evaluación de la genotoxicidad de los aerosoles.

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DISSERTATION ORGANISATION

This PhD dissertation is presented by publications where all the chapters have been submitted to peer-reviewed journals included in the Journal Citation Reports (JCR) and are listed below. The PhD dissertation starts with a summary that includes a brief introduction to the PhD topic together with the objectives, simplified materials and methods followed by the result section and a final discussion with conclusions.

CHAPTER I - γH2AX as a novel endpoint to detect DNA damage: Applications for the assessment of the *in vitro* genotoxicity of cigarette smoke: This introduction chapter contains a detailed literature review on the γH2AX as a biomarker of DNA double strand breaks including an overall description of the current endpoints used in *in vitro* genetic toxicology evaluation.

CHAPTER II - Metabolic characterization of cell systems used in *in vitro* toxicology testing: Lung cell system BEAS-2B as a working example: This chapter is focused on understanding the metabolic competency of the cell system BEAS-2B used during this PhD project. It also highlights the importance of metabolic activity in *in vitro* systems in the context of toxicant bioactivation.

CHAPTER III - Assessment of the *in vitro* γH2AX assay by High Content Screening as a novel genotoxicity test: This chapter describes the development of the *in vitro* γH2AX assay by High Content Screening in BEAS-2B cells. The automated scoring system contributes to the high throughput of the assay and eliminates the subjectivity from the manual scoring. A preliminary assessment to calculate the sensitivity and specificity of the assay by testing a panel of positive and negative controls is described in this chapter.

CHAPTER IV - Genotoxicity evaluation of individual cigarette smoke toxicants using the *in vitro* γH2AX assay by High Content Screening: This chapter focuses on the application of the novel *in vitro* γH2AX assay by High Content Screening in the evaluation of cigarette smoke toxicants from the particulate phase individually and in binary mixture.

CHAPTER V - Characterisation of an aerosol exposure system to evaluate the genotoxicity of whole mainstream cigarette smoke using the *in vitro* γH2AX assay by High Content Screening: This chapter describes the optimisation of the novel assay to evaluate aerosols, followed by the application of the optimised *in vitro* γH2AX assay by High Content Screening in the evaluation of whole mainstream cigarette smoke.

1.- SUMMARY INTRODUCTION

There are multiple forms of DNA damage, one of the most deleterious lesions are double-strand breaks (DSBs) since no complementary strand is left intact to act as a template during DNA repair. This lesion can lead to cell death if the repair mechanism fails, however, if repaired incorrectly, the DNA information can be compromised leading to mutation and potentially the perpetuating incorrect DNA information (Jeggo and Lobrich 2007).

Phosphorylation of the histone 2AX resulting in γH2AX occurs as an early response to DNA DSBs and was first described in 1998 (Rogakou *et al*., 1998). The phosphorylation of H2AX is a conserved biomarker of DNA damage across eukaryotes and is therefore present in humans as well as animals and plants cells (Redon *et al*., 2011). Since its discovery, this biomarker of DNA damage has been used in different disciplines of scientific research such as clinical studies, genome integrity research and drug development. The field of pre-clinical genetic toxicology testing in the 21st century is experiencing a revolution where new and emerging technologies are being used to develop and improve new and existing testing methods in genetic toxicology (Lynch *et al*., 2011). In this PhD dissertation, γH2AX has been studied as a novel endpoint of DNA damage focusing on the potential applications in the discipline of *in vitro* genetic toxicology. The measurement of γH2AX has been suggested as a complementary tool to the existing battery of genetic toxicology tests, more specifically as a pre-screening assay based on the high throughput given by scoring systems such as flow cytometry, *in cell western* immunoblotting and High Content Screening (HCS) (Smart *et al*., 2011; Audebert *et al*., 2010; Garcia-Canton *et al*., 2012). Chapter I of this PhD dissertation reviews the mechanisms of H2AX phosphorylation and the relevance of γH2AX as a novel *in vitro* genotoxicity assay in the context of current *in vitro* genotoxicity tests both regulatory and non-regulatory.

The experimental design for measuring γH2AX *in vitro* requires the selection of a cell system taking into consideration route of exposure and metabolic requirements. In this PhD dissertation, the *in vitro* γH2AX assay by HCS is planned to be applied to the evaluation of cigarette smoke which is known to be a major cause of lung cancer and contributes to other diseases including cardiovascular and respiratory related diseases. Some cigarette smoke constituents such as Benzo[a]pyrene (B[a]P) are not genotoxic per se, but can be metabolized by tissue specific enzymes such as cytochromes P450 (CYPs) into reactive species. These compounds are known as pro-toxicants. Therefore, the metabolic capability of the selected cell system needs to be characterised to ensure that pro-toxicant compounds are correctly evaluated.

Here, the human lung-derived BEAS-2B cell line was selected as the cell system. This cell line was established by Reddel and colleagues from normal bronchial epithelial cells that were immortalised by a replication-defective 12-SV40/adenovirus hybrid (Reddel *et al.*, 1988). When measuring γH2AX, cell lines derived from normal tissues, such as BEAS-2B cells, are considered to be more reliable than those from pre-malignant and cancer tissues. For instance, it has been reported that some tumorigenic cell lines can be defective in key functions including DNA damage repair mechanisms and lack normal cell cycle regulation (Hanahan and Weinberg 2000). Malignant cell transformation could affect the γH2AX response, for instance, there have been reports showing significant amount of γH2AX in the absence of DNA DSBs, possibly as a response to "oncogenic stress" (Svetlova *et al.*, 2010). Moreover, the organ of origin of this cell line is of special interest in the evaluation of compounds since the primary target is the lung. This is the case of cigarette smoke, pollutants and nanomaterials among others (Ansteinsson *et al.*, 2011; Veljkovic *et al.*, 2011). However, the metabolic capabilities of the BEAS-2B cell line needed to be further characterised to

support robust experimental design for pro-toxicant bioactivation. The metabolic characterisation of the BEAS-2B cell line is detailed in Chapter II of this PhD dissertation.

Following our assessment of the BEAS-2B cells, we concluded that limited cytochrome P450 enzyme activity could be detected in BEAS-2B cells compared to normal lung-tissue. To avoid an incorrect evaluation of pro-toxicants, a standardised external source of metabolic activation was incorporated in the experimental design of the *in vitro* γH2AX assay development as recommended in testing guidelines (ICH, 2011). The assay development included an automated microscopy-based scoring system referred to as High Content Screening (Bickle 2010). Other alternative automated screening systems previously used to measure γH2AX include flow cytometry (Smart *et al.*, 2011) and *In Cell Western* a multi-well plate based immunoblotting (Audebert *et al.*, 2010). A panel of 22 positive and negative compounds used in previous genotoxicity assay assessments (Hastwell *et al.*, 2006) and recommended by the European Centre for the Validation of Alternative Methods (ECVAM) were selected. The sensitivity, specificity and accuracy of the assay by HCS were calculated following recommendations published by an expert group (Kirkland *et al.*, 2008). The selected panel included compounds with different known mechanisms of action, such as pro-toxicants, aneugens and non-genotoxic compounds. The *in vitro* γH2AX assay by HCS development and preliminary assessment is presented in Chapter III of this PhD dissertation.

Following the completion of the H2AX assay performance evaluation with reference toxicants, we applied the H2AX assay to cigarette smoke toxicants and whole smoke exposure. Cigarette smoke is a complex mixture consisting of more than 5,600 identified compounds (Perfetti and Rodgman 2011). Estimations suggest that the whole mixture contains over 150 known toxicants (Cunningham *et al.*, 2011), including compounds classified by IARC as “carcinogenic to humans” (Group 1) such as B[a]P and NNK (IARC,

2012a; IARC, 2012b). Experimental results obtained from complex mixture testing could be difficult to interpret as interactions between single components could affect the final outcome. Fractionation of the complex mixture into single components and simple mixtures could assist with the determination of key toxic drivers present in the cigarette smoke. The regulation of tobacco products from different organisations such as the Food and Drug Administration Center for Tobacco Products (FDA CTP) and the World Health Organisation Framework Convention on Tobacco Control (WHO FCTC) require sensitive and fast risk assessment tools that complement existing knowledge based mainly on *in vivo* data (WHO 2008; FDA, 2012). The challenging task of pre-screening the *in vitro* genotoxicity potential of a large number of compounds individually and in simple mixture requires a high throughput assay with a simplified experimental design. This approach also supports the reduction in the use of animal models as recommended by the 3Rs approach (Schechtman 2002). In this PhD dissertation, the newly developed *in vitro* γH2AX assay by HCS was applied to the evaluation of the genotoxic potential of cigarette smoke toxicants (described in Chapter IV) and whole mainstream cigarette smoke (WMCS) (described in Chapter V).

In Chapter IV of this PhD dissertation, we selected a number of cigarette smoke toxicants from the particulate phase to be tested in the novel *in vitro* γH2AX assay by HCS. The polycyclic aromatic hydrocarbon B[a]P, the tobacco-specific nitrosamines (TSNAs) 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosonornicotine (NNN) and the heavy metal cadmium were all tested individually whereas the TSNAs were also tested in binary mixture. The first three toxicants are recommended for mandated lowering while the last one is recommended for monitoring by the WHO FCTC (WHO 2008).

The *in vitro* γH2AX assay by HCS was also optimised in Chapter V for the evaluation of aerosols at the air-liquid interface. This methodology is considered more physiologically

relevant for the evaluation of WMCS as cells are directly exposed to the complex mixture of combusted tobacco (Phillips *et al*., 2005).

In summary, this PhD dissertation describes the development, preliminary assessment and application of a novel assay for the detection of DNA damage using new technologies to automate the measurement of the response. Furthermore, the novel assay has demonstrated the potential to be further optimised and applied in the area of tobacco regulation as a tool to pre-screen large numbers of compounds and mixtures including aerosols.

2.- OBJECTIVES

- To characterise the metabolic capabilities of the lung-derived cell line BEAS-2B for its use in *in vitro* toxicology testing.
- To determine whether the γH2AX response can be measured in BEAS-2B cells by an automated microscopy-based system known as High Content Screening.
- To assess the sensitivity, specificity and accuracy of the *in vitro* γH2AX assay by HCS with a preliminary set of control compounds.
- To determine whether the *in vitro* γH2AX assay by HCS can be applied to the evaluation of cigarette smoke toxicants individually and in binary mixture.
- To investigate whether the *in vitro* γH2AX assay by HCS can be further developed and adapted to aerosol exposure.

3.- MATERIALS AND METHODS

3.1.- Cell Lines

The human bronchial epithelial cell line (BEAS-2B), was purchased from ATCC (United States). Cells were maintained in Bronchial Epithelial Growth Medium (BEGM®) at 37°C and 5% CO_2 in a humidified incubator (Lonza Group Ltd., Belgium).

The human type II alveolar adenocarcinoma-derived (A549), was purchased from ATCC (United States). Cells were maintained in Dulbecco's modified Eagle medium supplemented with fetal bovine serum (10% v/v), L-glutamine (2 mM), penicillin (50 U/mL) and streptomycin (50 µg/mL) (complete DMEM) at 37°C and 5% CO_2 in a humidified incubator.

The hepatocarcinoma-derived (HepG2) cell line was purchased from ATCC (United States). Cells were maintained in Dulbecco's modified Eagle medium supplemented with fetal bovine serum (10% v/v), L-glutamine (2 mM), penicillin (50 U/mL) and streptomycin (50 µg/mL) (complete DMEM) at 37°C and 5% CO_2 in a humidified incubator.

The human hepatocarcinoma-derived cell line (HepaRG) was purchased as a differentiated confluent monolayer from Biopredic International (France). Cells were maintained in basal medium supplemented with recovery mix for 24 hours followed by basal medium supplemented with maintenance/metabolism mix. Media and supplements were provided by the manufacturer (Biopredic, France).

BEAS-2B, A549 and HepG2 cells were cultured and expanded in-house. Experiments were performed between passages 3 and 12 only. All cultures were negative for mycoplasma. Additionally, the cells were authenticated using the short tandem repeat profiling to confirm the nature of the cell cultures (LGC Standards, United Kingdom) (Nims *et al.*, 2010).

3.2.- Methodologies

3.2.1.- Metabolic characterization of BEAS-2B cells

Gene expression: A panel of 47 phase I and phase II enzyme-encoding genes was examined using qPCR in BEAS-2B, A549 and HepG2 cells in the presence and absence of the inducer 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD). HepaRG was examined in the absence of inducer.

Enzyme activity assays: Activity profiles were generated for four cytochrome P450 enzymes involved in the metabolism of some cigarette smoke toxicants. CYP1A1/1B1 activity was assessed with the P450-Glo™ assay after induction with TCDD. CYP2A6/CYP2A13, CYP2E1, and CYP1A2 were assessed by UPLC-MS/MS with the enzyme-specific substrates; coumarin, chlorzoxazone, and ethoxyresorufin respectively. These methods were used in Chapter II.

3.2.2.- Detection and quantification of γH2AX in BEAS-2B cells by immunostaining

γH2AX as a marker of DNA DSBs was identified by immunostaining using a fluorophore-coupled γH2AX-specific antibody. Fluorescence intensity was detected and measured with the Cellomics Arrayscan® VTI platform (Thermo Scientific, USA), then analysed with the Target Activation Bioapplication software V.6.6.1.4.

When testing pro-toxicants, the external source of metabolic activation used was Aroclor-1254 induced rat liver homogenate supernatant with cofactors (S9 mix) (Moltox, USA). S9 mix is one of the standard systems employed as an external source of metabolic activation in current regulatory *in vitro* assays.

The acceptance criteria described for the *in vitro* γH2AX assay by flow (Smart *et al*., 2011) were applied to the results from the *in vitro* γH2AX assay by HCS to evaluate the relevance of using the same approach (Table 1).

Table 1: Genotoxicity evaluation criteria for the *in vitro* γH2AX assay, adapted from (Smart *et al*., 2011).

γH2AX response	**Classification**
> 1.5-fold γH2AX @ RCC > 25%	Genotoxic (+)
< 1.5-fold γH2AX @ RCC 100-0%	Non-genotoxic (–)
> 1.5-fold γH2AX @ RCC < 25%	"False" positive; Cytotoxic-driven genotoxicity (C)
= 1.5-fold γH2AX @ RCC ≥ 25%	Equivocal (±)

This methodology was applied in Chapters III, IV and V.

3.2.3.- Aerosol exposure optimisation

The aerosol exposure optimisation used the Borgwaldt RM20S® smoking machine (RM20S) as the engine to generate WMCS. Cells grown on porous Transwell™ inserts were exposed for 3 hours to a range of WMCS dilutions in air from two reference cigarettes. Reference cigarette 3R4F is a "US style" blended cigarette that delivers 9.4 mg tar and 0.7 mg nicotine under the International Standard Organisation (ISO) conditions for cigarette smoking (ISO 3308:2012). Reference cigarette M4A is a flue cured cigarette that delivers 10 mg of tar and 1.0 mg nicotine under ISO smoking conditions (ISO 3308:2012). This optimisation is discussed in Chapter V.

4.- RESULTS

4.1.- Chapter II - Metabolic characterization of cell systems used in *in vitro* toxicology testing: Lung cell system BEAS-2B as a working example

We highlighted in the introduction the importance of metabolic activity in the bioactivation of some but not all toxicants. Therefore we profiled the expression of a panel of metabolic genes by qPCR and activity of selected cytochromes P450 in BEAS-2B cells.

Gene Expression: qPCR analysis indicated limited gene expression in BEAS-2B cells, with the exception of CYP1A1, CYP1B1 and CYP1A2 which showed a significant up-regulation (25-, 5- and 4-fold, respectively) following pre-incubation with the CYP-inducer TCDD.

Enzyme activity: TCDD-induced BEAS-2B cells showed a CYP1A1/1B1 activity of 0.2 RLU/mg/min which is low compared to other metabolically competent cells. Conversely, no enzyme activity was observed in BEAS-2B cells for CYP1A2, CYP2A6/2A13 and CYP2E1.

4.2.- Chapter III - Assessment of the *in vitro* γH2AX assay by High Content Screening as a novel genotoxicity test

A panel of 22 compounds with different known mechanisms of action were used to perform a preliminary assessment of the sensitivity and specificity of the *in vitro* γH2AX assay by HCS. The assessment was also intended to determine if the genotoxicity evaluation criteria suggested by Smart and colleagues for the flow cytometry system could be employed with the HCS system.

The preliminary assessment indicates that the *in vitro* γH2AX assay by HCS has a high accuracy (86%) as a result of high sensitivity and specificity (86-92% and 80-88%

respectively). The results also confirmed that the criteria employed for the flow cytometry scoring system (Table 1) can also be applied to the HCS system.

4.3.- Chapter IV - Genotoxicity evaluation of individual cigarette smoke toxicants using the *in vitro* γH2AX assay by High Content Screening

We next applied the γH2AX HCS assay to known tobacco toxicants, including aromatic hydrocarbons, nitrosamines, and heavy metals, in order to evaluate the assay in the context of tobacco toxicology. B[a]P did not induce any increase in γH2AX frequency above the vehicle-treated controls after 3 hour treatment in TCDD pre-induced or non-induced cultures. When B[a]P was tested for 24 hour, there was a significant increase in γH2AX frequency in both TCDD-induced and non-induced cultures at concentrations above 0.5 μM. However, the γH2AX frequency in non-induced cultures at concentrations above 4 μM after 24 hour treatment returned to vehicle-treated levels (< 1.5-fold induction).

Cadmium chloride produced a positive response after 3 hour treatment at concentrations above 250 μM. After 24 hour treatment, concentrations above 16 μM showed cytotoxic-driven genotoxicity.

Both TSNAs, NNK and NNN, failed to show any statistically significant increase in γH2AX compared to the vehicle-treated controls at all timepoints in the presence and absence of S9 mix. When the reactive precursors NNK-acetate and NNN-acetate were tested, both produced an increase in γH2AX frequency compared to the vehicle-treated control after 3 hour treatment, at concentrations above 16 μM and 500 μM respectively. After a 3 hour treatment followed by 24 hour recovery period NNK-acetate induced a significant increase at concentrations above 125 μM, although NNN-acetate induced a dose-related increase in γH2AX frequency, the results failed to reach the positive response threshold.

Treatments with 5 TSNA mixture permutations containing different ratios of the reactive precursors of NNK and NNN (NNK-acetate and NNN-acetate respectively) were performed for 3 hour at concentrations from 7.8 μM to 1000 μM. Treatment with 100% NNK-acetate produced a significant increase in γH2AX at concentrations above 31.25 μM while 100% NNN-acetate produced a significant increase only at the highest tested dose, 1000 μM. All other mixture combinations tested produced significant increases in γH2AX levels at concentrations above 62.5 μM. The response induced by different ratios of tobacco specific nitrosamines suggests that NNK-acetate is the primary driver of the genotoxic response when present in a mixture with NNN-acetate. Neither synergistic nor antagonistic effects were observed in this study using CYP-independent NNK and NNN reactive precursors.

4.4.- Chapter V - Characterisation of an aerosol exposure system to evaluate the genotoxicity of whole mainstream cigarette smoke using the *in vitro* γH2AX assay by High Content Screening

Novel tobacco products such as “heat not burn” cigarettes are emerging on the market with potential reduced toxicant exposure and risk claims. In the evolving tobacco regulatory context, claims regarding reduced exposure and risk have or will have to be substantiated according to specific national legislations. Therefore it is of interest to develop genotoxic assays such as the γH2AX assay for aerosol exposure as opposed to single toxicants or toxicants in solution. Here, we tested the γH2AX response following whole smoke exposure to two reference cigarettes with a range of smoke dilutions.

Both reference cigarettes 3R4F and M4A produced a significant increase in γH2AX frequency (above 1.5-fold increase) compared to the air-treated control in all the dilutions tested (1:500 to 1:16,000). While exposure to 3R4F WMCS produced a slight dose-related response, the same effect was not observed with M4A WMCS. Future work will require an

in-depth characterisation on the effect of product variations in γH2AX induction to understand the differences in response.

5.- DISCUSSION

Over the years, the genetic toxicology risk assessment paradigm has been moving away from animal based test methods (*in vivo*) towards *in vitro* test methods. For instance, in the cosmetic industry, the use of animal testing is banned by the EU 7th Amendment Directive (European Commission 2003). However, in many cases the current *in vitro* assays produce an elevated number of "false positive" results, a positive response *in vitro* that is not confirmed *in vivo*. The current frequency of "false positive" increases the need of follow up *in vivo* tests delaying the development of new products. As a consequence, over the last decade multiple efforts have been placed on the development and validation of new *in vitro* test methods by organisations such as the European Centre for Validation of Alternative Models (ECVAM) and the National Centre for the Reduction, Refinement and Reduction of Animals in Research (NC3Rs).

In the process of new assay development we have to consider all the relevant characteristics that can make the novel test method worthy of use. Ideally, an assay has to be:

i. Physiologically relevant: The cell system selected for the assay has to be relevant to the route of exposure, of human origin and needs to take into account the metabolic needs of the target tissue.
ii. Accurate in the evaluation of compounds: The sensitivity (or ability to detect genotoxic compounds) as well as the specificity (or ability to discriminate non-genotoxic compounds) have to provide an improvement over existing testing methods.
iii. High Throughput: the assay has to utilise new techniques and technologies in order to provide a fast, inexpensive method that is reliable and reproducible over time. The

automation or potential for automation of the methodology would be an advantage, especially towards pre-screening.

iv. Objective: the measure of the response will be more efficient if it can be carried out by a validated system rather than human operator. Also, the measure of the response by human operator is limited to qualitative (yes/no) rather than quantitative (a measured value).

v. Applicable: the novel assay needs to have applicability in current research.

Measuring γH2AX has been extensively used as a biomarker of DNA DSBs since its discovery in 1998 (Rogakou *et al*., 1998). In the field of *in vitro* genetic toxicology, measuring γH2AX has been suggested as a potential complementary assay to the existing battery, especially as a pre-screening tool. Even though, measuring γH2AX is currently used extensively in research, limited studies have focused on the full assay development investigating the characteristics afore mentioned. The different chapters of this PhD dissertation have explored the characteristics required for the development of the *in vitro* γH2AX assay by HCS.

The human lung-derived BEAS-2B cell line was selected for its human source and tissue of origin. However, the results from the metabolic characterisation showed that there was a lack of activity in the majority of the cytochrome P450 enzymes evaluated compared to human normal lung tissue. The lack of metabolic competency could be mitigated by adding an external source of metabolic activation. In routine regulatory genotoxicity testing, Aroclor-1254 induced rat liver homogenate supernatant with cofactors (S9 mix) is used as an external source of metabolic activation. Animal-derived S9 mixes are neither representative of the cell line species nor the target organ, but to date they are the most standardised option available. Human-derived S9 mixes are commercially available, yet, the phenotypic variations and

different environmental factors made these fractions unreliable for routine testing. As a result, Aroclor-1254 induced rat liver S9 was selected as the external source of metabolic activation for the testing of pro-toxicant compounds during the *in vitro* γH2AX assay by HCS assay assessment.

The *in vitro* γH2AX assay by HCS preliminary assessment showed a high accuracy (86%) in detecting genotoxic compounds and discriminating non-genotoxic compounds, these results concur with previous evaluations using other scoring methodologies (Smart *et al.*, 2011; Tsamou *et al.*, 2012). Nevertheless, some limitations were observed during the developmental phase. The amount of S9 mix used for testing pro-toxicants could not reach the minimum recommended amount of 1% v/v due to intrinsic toxicity and therefore only 0.5% v/v was employed. This reduction in the amount of S9 mix used could have caused the lack of positive response from 2-acetylaminoflurene, one of the four pro-toxicants tested in this study. Further assay optimisations could include a metabolically competent human-derived cell line to better assess pro-toxicants. Generally, liver-derived cell lines are known to maintain a degree of metabolism, this is the case for HepG2 and HepaRG (Jennen *et al.*, 2010), however, these cell lines will sacrifice the route of exposure relevance in research where the target organ is the lung. Ideally, a metabolically competent lung-derived cell line needs to be developed, and the novel induced pluripotent stem cell (iPS) methodology may help with this issue in the long term.

A second limitation was observed when aneugenic compounds were tested. Treatment with aneugens produced morphological changes in the cells as their cell cycle is arrested. The cells become more round and cell adhesion decreases producing an elevated loss of cells during the immunostaining process. The experimental design will need to be adjusted for the testing

of this family of compounds to ensure the cell adhesion is restored before the immunostaining process starts.

Following the development and further optimisation of the *in vitro* γH2AX assay by HCS, the novel assay was applied to the evaluation of cigarette smoke and a number of cigarette smoke constituents. The results showed that the *in vitro* γH2AX assay by HCS can detect the genotoxicity effect of WMCS from two reference cigarettes with increments in the response up to 7-fold increases over the air-treated controls. The novel assay can also detect and quantify the genotoxicity effect of cigarette smoke toxicants both individually and in binary mixture showing potential as a tool to investigate the relative contribution of each toxicant to the whole tobacco smoke-related toxicity in this particular end-point of DNA damage.

6. - CONCLUSIONS

- The BEAS-2B cell line appears to lack cytochrome P450 enzyme activities relevant to the bioactivation of pro-toxicants.
- γH2AX can be detected and quantified in BEAS-2B cells by an automated microscopy-based system known as High Content Screeening.
- The *in vitro* γH2AX assay by HCS showed a high accuracy (86%) which results from a high sensitivity (86-92%) and a high specificity (80-88%).
- The *in vitro* γH2AX assay by HCS can be applied to the evaluation of cigarette smoke toxicants from the particulate phase both individually and in primary mixture.
- The *in vitro* γH2AX assay by HCS was successfully optimised for the genotoxicity evaluation of aerosols.

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Chapter I

γH2AX as a novel endpoint to detect DNA damage: Applications for the assessment of the *in vitro* genotoxicity of cigarette smoke

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Review

γH2AX as a novel endpoint to detect DNA damage: Applications for the assessment of the *in vitro* genotoxicity of cigarette smoke

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ABSTRACT

Histone H2AX is rapidly phosphorylated to become γH2AX after exposure to DNA-damaging agents that cause double-strand DNA breaks (DSBs). γH2AX can be detected and quantified by numerous methods, giving a direct correlation with the number of DSBs. This relationship has made γH2AX an increasingly utilised endpoint in multiple scientific fields since its discovery in 1998. Applications include its use in pre-clinical drug assessment, as a biomarker of DNA damage and in *in vitro* mechanistic studies.

Here, we review current *in vitro* regulatory and non-regulatory genotoxicity assays proposing the γH2AX assay as a potential complement to the current test battery.

Additionally, we evaluate the use of the γH2AX assay to measure DSBs *in vitro* in tobacco product testing.

1.- INTRODUCTION

DNA damage can be caused by products from internal metabolism such as reactive oxygen species but also by a range of exogenous agents, from energetic radiations such as UV light to chemicals. There are multiple forms of DNA damage; DNA single-strand breaks (SSBs), DNA-DNA crosslinks or DNA–protein crosslinks or covalent binding to DNA bases, nucleotide substitution, DNA frameshifts, double-strand breaks (DSBs) etc. DSBs are one of the most deleterious lesions since they affect both strands of the DNA helix. This lesion can lead to cell death by triggering apoptosis but if the lesion fails to repair or it is repaired incorrectly, DNA information can be compromised leading to mutation and ultimately cancer and/or heritable damage (Jeggo and Lobrich, 2007).

Histones are highly conserved proteins which play a role not only in DNA packing but also in DNA repair and gene regulation. There are 5 families of histones: 1, 2A, 2B, 3 and 4. Histone 2AX (H2AX) from the histone 2A family becomes rapidly phosphorylated (γH2AX) at serine-139 in response to DSBs (Rogakou *et al.*, 1998). There are mechanisms that get activated after DNA damage has occurred to avoid genomic instability; they are known as DNA damage response (DDR). One of the earliest DDRs is the activation of γH2AX as a result of a DSB. This response occurs within minutes of the damage, thus making it a useful marker of DNA damage. The description of events involved in this activation in mammalian cells leading to γH2AX and beyond is a complex process that has been described in detail in previous reviews (Riches *et al.*, 2008; Paull *et al.*, 2000; Fernandez-Capetillo *et al.*, 2004; Cann and Dellaire, 2011; Bekker-Jensen and Mailand, 2010; Srivastava *et al.*, 2009; Svetlova *et al.*, 2010).

Briefly, the earliest responding proteins are those of the phosphatidylinositol 3-kinase-like family of kinases (PIKK) including ataxia telangiectasia-mutated (ATM), ATM- and Rad3-

related (ATR) and the catalytic subunit of DNA-dependent protein kinase (DNA-PKc). The proteins are activated by DNA damage and are rapidly recruited to the site of damaged chromatin. Once there, they phosphorylate the histone 2AX at serine residue 139 located at the C-terminal tail resulting in the formation of γH2AX. However, to date it is still not fully understood how DNA damage is detected by the cellular machinery. Cann *et al*. suggested two models. The first postulates that changes in the chromatin structure following a DSB release topological constraints on the DNA helix that ultimately activate ATM. The second model, however, postulates that the MRE11-RAD50-NBS1 (MRN) complex in its task of keeping both ends of the broken DNA together is the critical DSB sensor but also the initial repair force, recruiting ATM to the site where it becomes activated (Cann and Dellaire, 2011).

Some investigations with cell lines deficient in DNA-PK and ATM showed a limited increase in H2AX phosphorylation after DSB damage (Paull *et al*., 2000). The roles played by the PI3K enzymes are thought to be different depending on toxic stimulus or cell type (Yan *et al*., 2011; Riches *et al*., 2008).

Either way, after the initial γH2AX activation, a positive feedback loop is created between γH2AX and the PIKKs for further DDR. The signal amplification acts as a repair signal calling for the repair systems to move to the location of the damage (Nakamura *et al*., 2010). Within minutes of the damage occurring, γH2AX can be detected in high quantities in the areas surrounding the DSB (Rogakou *et al*., 1999). These areas are known as nuclear foci and could extend several megabases of chromatin around the site of damage (Riches *et al*., 2008). Multiple studies (Cann and Dellaire, 2011; Xu and Price, 2011) suggest that γH2AX foci formation is mainly limited to euchromatin considered transcriptionally active and moderately compacted. Heterochromatin representing the transcriptionally inactive and highly compacted chromatin could be inaccessible to phosphorylation or more resistant to DNA damage. One

could also hypothesise that DNA damage in the heterochromatin does not lead to genomic instability as there is no active transcription. Therefore, repair resources are not invested.

The formation of nuclear foci in response to DNA DSBs differs from the formation of the "apoptotic γH2AX ring" (Solier and Pommier, 2009). They demonstrated that γH2AX ring staining is an early apoptosis indicator that precedes a global nuclear staining or pan-nuclear staining and apoptotic body formation. The main driver of this particular phosphorylation is DNA-PK in contrast to ATM and ATR associated with γH2AX nuclear focus formation. This morphology variation could potentially be used to discriminate DNA DSBs from other forms of DNA damage.

γH2AX could also act as a cell cycle checkpoint (Downey and Durocher, 2006). H2AX could become phosphorylated at any point during the cell cycle, including during mitosis while other DDR proteins are limited to interphase cells (Nakamura *et al.*, 2010). It has been suggested that DSB repair mechanisms may be suspended during mitosis. However, γH2AX foci continue to form during mitosis. The foci act as indicators to activate the repair mechanisms as soon as the cell has finished the division process. If the DNA DSB occurs in G1, the cell cycle would stop to prevent the cell moving into S-phase with damaged DNA. Likewise, DNA replication could be slowed if the DNA DSB has occurred in S-phase, so that the repair mechanisms could act before the DNA polymerase reaches the damaged section. Finally, when the damage occurs in G2-phase, the cell is prevented from moving into mitosis, avoiding the fracture of chromosomes during anaphase and cytokinesis (Jackson, 2002).

1.1.- Kinetics of phosphorylation

Following the induction of DSBs, phosphorylation of the serine 139 residue starts within minutes, reaching a plateau at around 30 minutes after damage occurs (Paull *et al.*, 2000). The phosphorylation then decreases over a period of hours (Rogakou *et al.*, 1998).

The mechanism of γH2AX elimination has not been fully unravelled. There are multiple phosphatases involved in γH2AX dephosphorylation. Dephosphorylation could occur directly on the chromatin or could happen after the histone has been displaced from the nucleosomes (Chowdhury *et al.*, 2005; Redon *et al.*, 2011a). Both mechanisms could potentially occur simultaneously, independent of the location of the γH2AX in the foci. Other mechanisms mentioned by Bao involve histone chaperone proteins in the process of γH2AX elimination (Bao, 2011). Experiments carried out by Keogh and colleagues suggest that the loss of γH2AX could be triggered not only by DSB repair but also by the activation of steps that precede DSB repair (Keogh *et al.*, 2006). However, some of their results seem to indicate that γH2AX loss is not mediated by single-stranded DNA resection, one of the cellular responses to DSBs.

1.2.- Rationale for measuring γH2AX

There are several reasons why γH2AX is used to detect DSBs. The formation of γH2AX is proportional to the amount of DSBs, giving a direct 1:1 correlation to existing damage (Sedelnikova *et al.*, 2002). This correlation indicates that for every DSB one nuclear focus would be created. Moreover, H2AX is distributed throughout the mammalian chromatin and when it becomes phosphorylated it covers a large area of the chromatin producing the easily detectable nuclear foci (Rogakou *et al.*, 1999). The foci can be measured by different techniques in what is known as the γH2AX assay to give an account of the DSBs. In addition,

this marker is conserved across eukaryotic evolution, giving the γH2AX assay potential use not only in human studies but also in other organisms including plants (Redon *et al.*, 2011b).

The standard battery of genotoxicity tests measure fixed DNA damage as their endpoint e.g. mutations in the Ames test (OECD, 1997a) or chromosome damage in the *in vitro* micronucleus test (OECD, 2010). However, measuring total DNA damage could provide a complement to the current tests. In general, DNA damage could produce genome instability or cell death. Mis-repaired DNA damage could lead to mutation and unrepaired DNA damage to chromosome breaks. Moreover, repeat DNA damage could saturate the cell repair system leading to accumulation of unrepaired lesions. The γH2AX assay can provide an indication of DNA damage which can be used as a pre-screening tool or as a complement to the standard battery of genotoxicity tests (Watters *et al.*, 2009).

2.- CURRENT GENOTOXICITY TESTS

2.1.- Regulatory Assays

From the total number of assays described to measure genotoxicity *in vitro*, only a small number are accepted for regulatory purposes. These are deemed acceptable for estimating the genotoxic risks posed by compounds commercially employed for human use and thus are required by regulatory authorities. This group includes the Ames test, mouse lymphoma assay (MLA), the micronucleus and chromosomal aberration tests. These assays have been extensively validated and are accompanied by an Organisation for Economic Co-operation and Development (OECD) guideline describing the proper conduct of these tests. There is a wealth of literature available on each of these genotoxicity assays. Therefore, this section will only briefly describe each assay, its application and limitations.

2.1.1.- Ames Test

The Ames test is a bacterial gene mutation assay widely used for its simplicity, accuracy and low cost (OECD, 1997a). The assay measures the number of colonies formed after exposure to the test chemical. If the bacteria have suffered mutations, the frequency of colonies would be significantly higher than the frequency of colonies in the negative control cultures. This assay detects most tested genotoxic carcinogens with a high sensitivity. However, the Ames test sometimes fails to detect genotoxic compounds, primarily those that cause large DNA deletions or compounds that are non-DNA reactive (aneugens and carcinogens that have a non-genotoxic mechanisms). Other carcinogenic compounds that have a specific target in mammalian cells such as the cell division spindle apparatus or DNA polymerases and topoisomerases can also be mislabelled by the Ames test. Moreover, compounds such as antibiotics or bacteriocides cannot be tested adequately in the Ames test as they are toxic to

bacteria per se. Additionally, false positives (i.e. non-carcinogens detected as mutagens) do occur in the Ames test. There are a small number of compounds that are Ames positive mutagens due to their bacterium-specific metabolism e.g. sodium azide and some nitro-group containing compounds (Prival, 1983).

The strains of *Salmonella typhimurium* used in the Ames test contain different mutations in various histidine synthesis genes (Table 1). The mutations carried by the specific strains prevent the bacteria from growing in media without histidine. However, if the test chemical mutates the defective mutation back to functional status (revert initial mutation), the bacteria will acquire the ability to grow in histidine-free media and form colonies. These colonies are thus known as revertants (Ames *et al.*, 1975).

Table 1: *Salmonella typhimurium* strains with mutation gene

Strain	**Mutated gene (allele)**	**uvrB**	**pKM101**	**Mutation**
TA97	hisD6610	No	Yes	Frameshift
TA1537	hisC3076	No	No	Frameshift
TA98	hisD3052	No	Yes	Frameshift
TA1538		No	No	
TA100	hisG46	No	Yes	Base substitution
TA1535		No	No	
TA102	hisG428	Yes	Yes	Base substitution
TA104		No	Yes	

All strains except TA102 are missing the uvrB DNA repair gene, thus removing the main error-free DNA excision repair pathway, compared to wild-type cells. This will amplify the mutations as DNA repair, in the absence of excision repair, occurs by error-prone pathways. TA102 bacteria strain maintains the excision repair system to be able to detect DNA cross-

linking agents such as mitomycin C. Otherwise compounds with DNA cross-link mechanism of action will not be detected, as unrepaired cross-links are lethal to the cell.

In addition, all strains have the mutation known as deep rough or rfa genotype. This is an alteration of the phenotype, where the polysaccharide capsule surrounding the cell is no longer present. Therefore, larger compounds are able to enter through the cell membrane reaching the bacterial DNA.

Various strains possess the plasmid pKM101 which contains the operon muc. Enzymes encoded by this operon allow the damaged DNA to continue its synthesis. The effect of this operon is to amplify the translation of DNA damage to mutations. The plasmid also contains a gene coding for resistance to the antibiotic ampicillin. This ampicillin-resistant property permits the selection of mutants containing the plasmid.

Alternatively, some *Escherichia coli* strains can be used to screen for mutagens. These strains have base change mutations in one of the tryptophan synthesis operon genes (trpE) instead of the histidine operon genes. Strains with and without the uvrA mutation are available as are strains with and without the plasmid pKM101. *E. coli* WP2 strains are equivalent to TA102 in terms of types of mutagen detected (including oxidative mutagens). However, if a cross-linking effect is to be detected, then the *E. coli* strain must have an intact excision repair system. The rfa mutation is not required as *E. coli* cells are naturally permeable to larger molecules.

Each strain of bacteria used in the Ames test detects a different spectrum of mutagens. Compounds are usually tested in multiple bacterial strains to understand the nature of their mutagenicity and also to broaden the range of mutagens detected (Table 1).

There are two types of mutations, base substitution or frameshift point mutations. A base substitution is a type of mutation where one nucleotide is replaced by another. As a consequence, a codon that will not code for any amino acid could be produced. This is also referred to as a nonsense mutation creating a stop codon which results in a truncated, incomplete or non-functional protein, when the relevant mRNA is translated. If the substitution leads to a codon that codes for a different amino acid then it is referred to as a missense mutation. Missense mutations do not always lead to marked protein changes but can give rise to non-functional proteins. Frameshift mutations are typically caused by loss or gain of a number of nucleotides that are not evenly divisible by three. As a result, the whole sequence will be modified from the point of mutation as the reading frame or sequence of codons will be changed. This in turn leads to a completely different translation.

The bacterial mutation assays are normally carried out in the presence and absence of a surrogate for human liver activity such as rat liver S9 fraction. Liver S9 is obtained from animals treated with inducers of P450 enzymes required for phase I metabolism. Thus, compounds that are innocuous but which have DNA reactive metabolites can be detected.

2.1.2.- Mouse Lymphoma Assay

The mouse lymphoma LY5178Y TK assay (MLA) is a gene mutation assay used to assess the mutagenicity of chemicals (OECD, 1997c). The principle of this assay is very similar to the Ames test, although in this case forward mutations are induced rather than reverse mutations. The selected mutation will cause the cell to be resistant to a toxic chemical. Thymidine kinase-competent ($TK^{+/+}$ or $TK^{+/-}$) mouse lymphoma cells are treated with test chemicals, then the cells are transferred to selective media containing lethal analogues such as trifluorothymidine. Only cells that have mutated to $TK^{-/-}$ survive and form colonies. The loss of this specific enzyme does not cause any other deleterious effect to the cell. However, if the

mutation results from an extensive deletion causing the loss of essential genes, the cell will die and no colonies will form. There are also genes close to the TK gene that are involved in cell growth, thus a deletion that removes these genes will result in a slow growing colony. This contrasts with point mutations within the TK gene, where a large mutant colony will be formed. By measuring the numbers of small mutant colonies that are induced after exposure to a test chemical, an assessment of clastogenicity can be obtained, as chromosome damage could result in deletions. By measuring the number of large mutant colonies, an estimate of induced point mutations can be obtained.

In addition to the MLA, rodent cell lines such as Chinese hamster V79 cells, and Chinese hamster ovary (CHO) cells have also been used for mammalian cell mutation assays, measuring mutations in the hypoxanthine-guanine phosphoribosyl transferase (hprt) gene. Moreover, mutation of the TK gene can also be measured in the human lymphoblastoid cell line TK6. However, the MLA test is most commonly used as it detects both aneugens (non-direct effect on DNA) and clastogens (direct effect on DNA).

The current methodology includes the use of either a plate assay in soft agar or a liquid exposure in a 96-well microplate increasing the throughput. However, the scoring of the colonies has to be done manually by an operator, adding subjectivity to the process.

Initially, the MLA assay was conducted using short treatments of 3 to 6 hours. However, the genotoxicity testing guideline from the International Conference on Harmonisation (ICH) for the registration of pharmaceuticals recommended a continuous treatment (24 hours) when there is a negative response in the short treatments in the absence of S9 (ICH, 2008). The longer treatments allow the cells to go through 1.5 to 2 normal cell cycles, ensuring that weak positive chemicals are readily detectable. Additionally, some evidence suggests that aneugenic compounds can also be detected with this longer treatment time (Moore *et al*.,

2002). However, Fellows *et al*. have recently advised against the use of MLA as a routine test to detect aneugens, as some of their tested compounds did not generate a positive response, while others only produced positive results at toxic concentrations (Fellows *et al*., 2011).

As with the Ames test, the MLA assay can be conducted in the presence of S9. However, S9 can only be used in the short treatments as it is toxic per se when the cells are exposed for more than 3 hours.

2.1.3.- Chromosomal Aberration Test

The *in vitro* chromosomal aberration test is a cytogenetic assay that has traditionally been used to evaluate chromosome abnormalities and stability after chemical treatment (OECD, 1997b). The assay evaluates the karyotype in the first metaphase after a short (3-6 hours) and long (24 hours) treatment with test compounds.

This assay is laborious and requires observational skills to score the different chromosome aberrations. These include chromosome and chromatid gaps and breaks and more complex rearrangements including chromosome fusions to produce dicentric chromosomes and exchange figures. Although many of these lesions are lethal to the cell, they are surrogates for stable chromosomal exchanges and translocations which are compatible with cell survival and are important in activation of oncogenes and, in some cases, deletion of tumour suppressor genes. The development of fluorescence *in situ* hybridization (FISH) has facilitated the identification of chromosome abnormalities.

The *in vitro* chromosomal aberration test focuses primarily on structural aberrations. For this reason, carcinogens with a non-genotoxic potential will not be identified. Several cell types have been used routinely for these studies including human peripheral lymphocytes as well as various established Chinese hamster cell lines such as V79, CHO and CHL cells.

This assay can include an exogenous source of metabolic activation such as S9 for use with the shorter exposures.

This test is not easily automated and the throughput is limited. However, there is imaging technology available to find and sort well spread metaphases for scoring, which significantly decreases the time needed to score experiments. Although an option in the international guidelines for genotoxicity testing, in general, this assay is beginning to be superceded by the *in vitro* micronucleus test, which has the advantage of detecting aneugens as well as clastogens more easily (Lynch and Parry, 1993).

2.1.4.- *In vitro* Micronucleus Assay

The *in vitro* micronucleus assay is a cytogenetic test that measures genetic damage using the formation of micronuclei as an endpoint (OECD, 2010). Micronuclei are small membrane-bound structures that contain chromosome fragments or sometimes whole chromosomes that are not incorporated into either daughter nucleus. The majority of micronuclei contain DNA fragments giving a measure of chromosomal damage or clastogeniticy. The content of the micronuclei can be identified by adding an extra step in the standard method: Centromere immunostaining gives this assay the ability to identify aneuploidy when the micronucleus contains a whole chromosome (Lynch and Parry, 1993).

The micronuclei should be present in cells that have undergone at least one mitosis. Segregating the populations that have experienced mitosis was initially a challenge. This led to the development by Fenech of the cytokinesis-blocked micronucleus assay (CBMN) which uses cytochalasin B to inhibit membrane division after mitosis (karyokinesis) (Fenech, 2007). This allows the scorer to identify which cells have undergone mitosis by counting the micronuclei present in binucleated cells (cells containing both daughter nuclei).

The micronucleus test shows fixed DNA damage in the form of chromosomal breaks or chromosomal loss but does not give an indication of total damage as some of the initial damage can be repaired or the cell can undergo apoptosis. The micronucleus test does not detect point mutations. For this reason, a mutation assay is always needed as a complementary test in genotoxicity test batteries.

This assay can be performed in the presence of S9 to detect promutagens. However, S9 is only employed for the short treatments as it is toxic per se to mammalian cells in culture.

The technique involved in this assay is much simpler than the chromosomal aberration test where the analysts require greater skills to prepare the metaphases and score the aberrations. However, a degree of subjectivity is associated with the manual scoring which also limits the throughput. Over the past few years, some automation methods for scoring micronuclei have gained acceptance, in particular flow cytometry (Lynch *et al* 2011).

In a recent review, Dearfield *et al*. compiled a list of all available genotoxicity assays and organised them into 4 categories based on their validation status, strengths and weaknesses (Dearfield *et al*., 2011). Table 2 below details the assays contained in their "category one" also known as regulatory assays comprising of assays that are well-characterised and have an issued OECD guideline and are currently used for regulatory purposes.

Table 2: *In vitro* genotoxicity regulatory assay. Adapted from (Dearfield *et al.*, 2011).

Assay	Endpoint	Strengths	Limitations	Opportunities
Ames bacterial reverse mutation assay	Gene mutations (point mutations including base pair substitutions and frameshift mutations) in bacterial cells.	Commonly used, well validated, inexpensive, recognized guideline, ability to differentiate frameshift and point mutations (different bacterial strains). Can be conducted both with and without metabolic activation.	Prokaryotic (bacterial specific); does not detect clastogens.	Use as high throughput screening test. (Jacobson-Kram and Contrera, 2007)
In vitro mouse lymphoma L5178Y $tk^{+/-}$ assay	Gene mutations (point mutations including base pair substitutions and frameshift mutations) in mammalian cells. Can also detect various sizes of chromosome deletions, mitotic recombination, chromosome rearrangements, and some aneugens	Can detect point mutation inducers, clastogens and some aneugens. Can be conducted both with and without metabolic activation.	Use of positive controls for colony sizing essential for quality control. Colony sizing required to distinguish between point mutation or clastogen endpoints. Evaluation and interpretation changed over the years. Recent protocol updates recommended. Cytotoxicity (necrosis and apoptosis) needs to be controlled to avoid false positive results	Recent protocol updates recommended (adjusted Relative Total Growth and Global Evaluation Factor), might improve data interpretation. (Moore *et al.*, 2002; Moore *et al.*, 2007)

In vitro gene mutation assay in mammalian cells (excluding mouse lymphoma assay)	Gene mutations (point mutations including base pair substitutions and frameshift mutations) in mammalian cells (e.g. hprt). Can also detect various sizes of chromosome deletions when autosomal gene (e.g., in AS52 cells) is used; hprt locus assay can detect some deletions, though not very efficiently.	Detects gene mutation in mammalian cells without confounding chromosome damage when non-autosomal genes are used Can be conducted both with and without metabolic activation.	Currently infrequently used but may be used more in future (e.g. REACH, interest in *in vitro* alternatives to *in vivo* testing), Chinese hamster cells considered by some to be insufficiently sensitive. Cytotoxicity (necrosis and apoptosis) needs to be controlled to avoid false positive results mammalian cells.	For AS52 cell lines, can be used as alternative to metaphase analysis.
In vitro chromosome aberration assay	Structural and numerical chromosome damage in mammalian cells (i.e., clastogenicity and polyploidy).	Detects clastogens and polyploidy inducers, including some aneugens. Some information on aneugenicity can be obtained with extended culture times. Applicable to different cell types, able to proliferate in culture. Provides information on the type of chromosome aberrations. Analysis made on individual cells. Can be conducted both with and without metabolic activation.	Resource intensive and time consuming scoring of aberrations. Requires skilled scientists. Polyploidy provides suboptimal predictivity of aneugenicity. Limited number of cells evaluated. Cytotoxicity (necrosis and apoptosis) needs to be controlled to avoid false positive results.	FISH/chromosome painting can provide additional mechanistic information.
In vitro micronucleus	Structural and numerical chromosome damage in	Can detect both aneugens and clastogens. Rapid and easy to conduct.	Resource intensive and time consuming scoring of micronuclei.	Mechanistic studies: can be used with and without

assay	mammalian cells (i.e., clastogenicity and aneuploidy).	Applicable to different cell types that can proliferate in culture. Analysis made on individual cells. Can be conducted both with and without metabolic activation.	Does not distinguish complex rearrangements and chromosome breaks. Cytotoxicity (necrosis and apoptosis) needs to be controlled to avoid false positive results.	FISH analysis of centromeres to differentiate clastogens from aneugens. Can be used for screening assay. Potential for automation, e.g. flow cytometry, image analysis. (Kirsch-Volders *et al.*, 2011)

The Ames test is considered to have high specificity, with a low frequency of false positive results with non-carcinogens. However, the sensitivity is limited because some carcinogens only show activity with eukaryotic cells. Additionally, compounds such as antibiotics or bacteriocides cannot be tested adequately in the Ames test as they are toxic to bacteria per se. False positives (i.e. non-carcinogens detected as mutagens) do occur in the Ames test. Those include compounds with bacterial-specific metabolism (e.g. sodium azide) and some nitro-group containing compounds which will not produce a harmful effect in mammalian cells. Therefore, *in vitro* mammalian assays are required to generate a complete safety assessment of genotoxicity potential (Kirkland *et al*., 2007a).

Unfortunately, the established *in vitro* mammalian cell tests produce an unacceptable rate of false positives (Kirkland *et al*., 2007b). For this reason they are defined as low specificity assays, and several causes are thought to be responsible for this lack of specificity. Many of the cell systems used for these assays are deficient in DNA repair mechanisms. In addition, genetic drift occurring during repeated subculturing can make them artificially prone to genetic damage. The high rates of false positives are also increased by the current guidelines requiring very high test concentrations of up to 10 mM or 5000 µg/mL. Furthermore, guidelines require top concentrations to elicit high levels of cytotoxicity of 50% or even higher (90% for the MLA). These conditions can result in the appearance of genetic damage that is unrelated to the inherent genotoxicity of the test compounds themselves. Moreover, the use of different cytotoxicity measures such as relative cell counts (RCC), relative population doubling (RPD), and mitotic index (MI) among others, could lead to different cytotoxicity results (Kirkland *et al*., 2007b; Greenwood *et al*., 2004). Kirkland showed that, by using different cytotoxicity measures, the same compound could give a positive or negative response at the maximum level of toxicity (50%) in the *in vitro* micronucleus test (Kirkland, 2010).

Finally, the *in vitro* assays only have the inherent ability to detect mutagens and carcinogens but they cannot detect the metabolites produced by hepatic metabolism from compounds known as promutagens or procarcinogens. To cover this deficiency, the majority of the assays require an exogenous metabolic source, such as rat liver S9 fraction from animals treated with inducers of P450 enzymes. However, S9 is deficient in detoxification phase II enzymes (and no co-factors for these enzymes are included in the S9 mix) giving rise to a high level of metabolites which may be irrelevant to *in vivo* systems. Alternatively, some mammalian cell tests can use genetically modified cell lines with some degree of metabolic capability like engineered Chinese hamster V79 cells or can be co-cultured with primary hepatocytes (Kirkland *et al*., 2007b; Pfuhler *et al*., 2011; Dearfield *et al*., 2011).

2.2.- Non-regulatory Assays

The current mammalian *in vitro* genotoxicity assays have a high rate of positive results that do not translate into positive rodent carcinogenicity results. This raises the concern that these *in vitro* assays are overly sensitive and therefore generate false positives (Dearfield *et al*., 2011; Kirkland *et al*., 2007b). Some companies use non-regulatory assays as early screening tools (Jacobson-Kram and Contrera, 2007).

Recently, Lynch *et al*. have reviewed the status of new and emerging technologies, comparing them with the current battery of genotoxicity tests (Lynch *et al*., 2011). These tests do not yet have an accompanying OECD guideline, or not enough data has been collected to fully establish them (trials, validations). This group of assays includes, for example, the comet assay, GreenScreen assay and the γH2AX detection assay. These assays are classified as replacements or improvements of the traditional genotoxicity assays, forming a new approach to replace traditional assays or providing mechanistic understanding complementary to the

traditional assays. Subcategories to classify these assays have been defined by experts in the field and are described as mature, maturing and emerging (Lynch *et al*., 2011).

Mature refers to methods or technologies that have been in the field for a relatively long time and are amongst those tests that are likely to become accepted in the foreseeable future. However, these are still not yet fully accepted by regulatory bodies. One reason for this lack of acceptance is the need for generating more data by comprehensive validation exercises. This category includes, for example, the comet assay, and *in silico* technologies for genotoxicity prediction based on chemical structure-activity relationships (SARs) etc.

Maturing refers to those methods or technologies that have proved to add value to the existing methods but have not yet gone through an extensive validation exercise. Maturing assays are the novel GreenScreen assay and yeast DEL assay. Additionally, this category also encompasses the automation of existing methods such as, for example, the development of flow cytometry to score *in vitro* micronucleus samples.

Emerging refers to new technologies that are currently in development, i.e. they show interesting capabilities but require further testing/development. While the standard battery of genotoxicity assays looks at gene mutation or chromosomal damage and variation in chromosome numbers (aneugenicity), there are a number of promising new genotoxicity endpoints of interest related to DNA repair-related protein modification as a response to DNA damage, such as the histone phosphorylation to form γH2AX, subject of this paper. Other genotoxicity approaches classified as emerging technologies are toxicogenomics, the use of humanised cell lines and Pig-a gene mutation assay among others (Lynch *et al*., 2011).

2.2.1.- *In vitro* Comet Assay

The *in vitro* comet assay or single cell gel electrophoresis assay is currently considered as a mature technology (Lynch *et al*., 2011). The assay detects DNA damage in individual cells. The methodology employs a microgel electrophoresis technique at alkaline pH (pH>13). The measurements of the comet tails (DNA migration) after the cells are lysed gives an indication of the amount of DNA damage present in the cells (Tice *et al*., 2000; Kumaravel and Jha, 2006). It is a very sensitive assay. However, in the past the comet assay has shown a high variability caused mainly by physical factors such as temperature, and materials that generate variation not only in inter-laboratory but also in intra-laboratory comparisons. At this point, the method is still not fully optimised or validated, however, further research is still ongoing (Zainol *et al*., 2009).

The comet assay can take advantage of existing software to score the comets. However, its throughput is limited. This assay does not require cell division. Therefore, a parallel assessment of the compound cytotoxicity would be needed to ensure the DNA damage is not caused by high toxicity (Dearfield *et al*., 2011).

This assay can use any eukaryotic cell or tissue and it has the versatility to be used *in vitro* and *in vivo* where it may be included in tests being carried out for other purposes such as a repeat dose general toxicology study.

The addition of an external source of metabolic activation in the *in vitro* comet assay is possible if the selected cell system is not metabolically competent.

2.2.2.- GreenScreen

The GreenScreen assay, considered to be a maturing assay, is a completely new approach to genotoxicity evaluation. It uses the transcriptional response of the human GADD45a gene as a

marker of genotoxic stress. The gene for green fluorescent protein (GFP) is fused to the GADD45a promoter allowing a fluorescent signal to be generated when the GADD45a gene is induced following exposure to genotoxins. The host cell line is the human lymphoblastoid line TK6, which has the advantage of being p53-competent. This competency allows the cells to maintain genomic stability after genotoxic stress reducing the rate of false positives (Kirkland *et al*., 2007b; Lynch *et al*., 2011).

This assay has initially been developed without the use of rat liver S9 in a multi-well microplate format, which allowed for a reasonable throughput in use (Hastwell *et al*., 2006). After the initial development, it was further modified to include the use of S9 with flow cytometry scoring (Jagger *et al*., 2009), although this resulted in a lower throughput.

2.2.3.- Yeast DEL assay

The Yeast DEL assay is another new approach to genotoxicity evaluation and is also classified as a maturing assay. The assay uses homologous recombination scoring for DNA deletion in the yeast *Saccharomyces cerevisiae*. The assay can detect carcinogens that act directly on the DNA (clastogens) (Kirpnick *et al*., 2005). The methodology has been modified to support microwell plate use thereby increasing throughput (Hafer *et al*., 2010). However, there are still concerns about the cell wall permeability of the yeast and the perceived relevance of the cell system (Lynch *et al*., 2011).

3.- γH2AX AS A NEW GENOTOXICITY ASSAY

There are 2 major limitations to the current *in vitro* mammalian genotoxicity assays:

- Low throughput: This is mainly linked to the manual scoring that limits large scale screening in terms of time. In the last few years, some technologies have been developed to increase the throughput. For example, automated flow cytometric analysis is used to score *in vitro* micronucleus samples (Bryce *et al.*, 2007). This methodology could potentially be used as a pre-screening tool while awaiting further validation, as detailed in a recent review (Avlasevich *et al.*, 2011).

- High frequency of false positives: A large proportion of non-carcinogenic compounds produced positive results in at least one of the *in vitro* assays of the standard battery of genotoxicity tests (Kirkland *et al.*, 2007b). The relevance of those *in vitro* positives was not confirmed in the follow-up in vivo genotoxicity tests. This contributes to delays in drug development and an increase in animal use. Moreover, for the cosmetic industry, where animal testing is banned by the EU 7th Amendment Directive (European Commission, 2003), the effect of a false positive has serious consequences in potential loss of useful compounds.

3.1.- Methods

The γH2AX assay could be of potential use in overcoming the 2 major limitations mentioned above. There are several methods for detecting γH2AX and these have evolved to become simpler, quicker and more automated.

Initially, γH2AX detection employed acetic acid-urea-triton and acid-urea-cetyltrimethylammonium bromide polyacrylamide gel electrophoresis (aut-aucPAGE), a two-dimensional gel analysis to detect the level of phosphorylated H2AX. Gels from untreated mammalian cell cultures were compared to gels generated using radiated cultures. The gels from the treated cells showed an additional shadowed area identified as a region containing the γH2AX protein which migrates through the gel differently than non-phosphorylated H2AX (Rogakou *et al*., 1998).

However, after the initial development of this approach, immunocytochemical detection as described by Rogakou *et al*. became the primary method of detection, as it is several orders of magnitude more sensitive and has the potential for quantitation (Rogakou *et al*., 1999), (Sedelnikova *et al*., 2002). This method is based on the use of a γH2AX-specific monoclonal fluorophore-coupled antibody. Once γH2AX presence has been detected by the antibody based method, the results can be quantified using various methods. These approaches have been discussed extensively in a previous review (Bonner *et al*., 2008) and are summarised briefly below.

3.1.1.- Immunofluorescence analysis

A phosphospecific antibody is used to detect γH2AX, the antibody does not bind to any non-phosphorylated H2AX. This antibody can either be directly labelled with a fluorophore reporter or detected by addition of a secondary, fluorophore-labelled antibody. The stained γH2AX can then be analysed by manual or automated scoring.

Manual scoring: The stained cells are evaluated by eye using a fluorescence microscope. This method will only be able to give qualitative results, i.e. presence or absence of fluorescence.

Additionally, the number of foci per cell could be counted. This scoring method is considered very time consuming and has a high level of subjectivity (Sedelnikova *et al*., 2002).

Automated detection using a cell enzyme-linked immunosorbent assay (cell-ELISA): A peroxidase-conjugated secondary antibody substitutes the standard fluorophore-conjugated secondary antibody used in immunocytochemistry. The enzymatic reaction carried out by the peroxidase generates an amount of product that can be measured by absorbance. This absorbance measure would be directly proportional to the amount of γH2AX present in the cells. This methodology has the advantage of high throughput technology. However, no measure of focus number per cell or distribution would be available (Matsuzaki *et al*., 2010). A variation of this method consists of reading the infrared fluorescence dye from the secondary antibody directly on the microwell plate (Audebert *et al*., 2010).

- Automated scoring using flow cytometry: The flow cytometer uses a laser to detect the fluorescence per nucleus. This method will generate qualitative results based on measures of γH2AX antibody present in the sample. However, the cells are destroyed in the process and no measure of focus number per cell or distribution is available (Banath and Olive, 2003).

- Automated scoring using high content screening (HCS): This qualitative method uses automatic microscopy to acquire cell images and image analysis software to measure different endpoints such as brightness, focus number per cell, distribution etc. This method generates and stores the images for future re-scan or quality control check (Kim *et al*., 2011; Hou *et al*., 2009).

3.1.2.- Immunoblotting analysis

Here absolute amounts of γH2AX protein are measured and compared to the total H2AX and H2A content. However, different cell types have different γH2AX/H2AX and H2AX/H2A ratios yielding as a result different absolute amounts of γH2AX for the same number of DSBs (Rogakou *et al*., 1998).

Overall, microscopic analysis of γH2AX is considered to be more sensitive than other methods such as flow cytometry (Kim *et al*., 2011). Initial microscopy developments in this area were limited to manual scoring of the samples which is restrictive in terms of sample generation (slide vs microwell plate), operator time and subjectivity.

New developments in the area of automated microscopy and image analysis software have increased the sensitivity of the results obtained by HCS. Additionally, the use of microplates and robotic systems has promoted the development of high throughput assays. Moreover, the use of software analysis allows objective quantitative scoring, avoiding operator subjectivity. The potential for multiplexing or evaluating various endpoints simultaneously is an attractive option as there would be a reduction in experimental time and resources. Therefore, from the current methods described above, HCS is considered a strong candidate for routine testing of γH2AX.

4.- ASSESSMENT OF γH2AX AS A GENOTOX ASSAY

In the last decade, the use of γH2AX to assess DNA damage has grown exponentially as demonstrated by the number of publications (Figure 1A). This growth comes as a consequence of the diversification of scientific fields where H2AX is used (Figure 1B). Initial studies were carried out in the field of radiation research, but once the relation between the phosphorylation of H2AX and DSBs was demonstrated (Rogakou *et al*., 1998), the use of γH2AX soon expanded to other areas.

The initial methodologies supported experimentation focused on DNA damage and repair mechanisms (Mukherjee *et al*., 2006; Marti *et al*., 2006; Celeste *et al*., 2003; Bassing *et al*., 2003) to mention some. Other studies were orientated to assess the DNA damage potential of drugs, potency of chemotherapy agents and other medical materials (Tanaka *et al*., 2006; Ansteinsson *et al*., 2011; Olive and Banath, 2009).

Further optimisations in γH2AX detection allowed the use of this indicator of DSBs as a biomarker (Muslimovic *et al*., 2008; Cornelissen *et al*., 2011). For example, Muslimovic *et al*. used non-fixed blood cells from irradiation patients to develop a biomarker that could potentially lead to modulation of radiological treatment (Muslimovic *et al*., 2008; Johansson *et al*., 2011). The clinical use of γH2AX as a biomarker has been reviewed recently (Redon *et al*., 2010).

In the field of genetic toxicology, Albino *et al*. proposed the use of γH2AX as a novel genotoxicity assay using flow cytometry (Albino *et al*., 2004) and was soon followed by Gallmeier *et al*. recommending immunocytochemistry (Gallmeier *et al*., 2005). Since then, multiple compounds have been tested in the γH2AX assay to assess its utility as a genotoxicity assay (Table 3). Overall, γH2AX is considered as a good marker of genotoxic

damage. Moreover, the large number of compounds tested by Smart *et al.* has shown the γH2AX assay to be a sensitive and specific assay for the assessment of genotoxicity (Smart *et al.*, 2011).

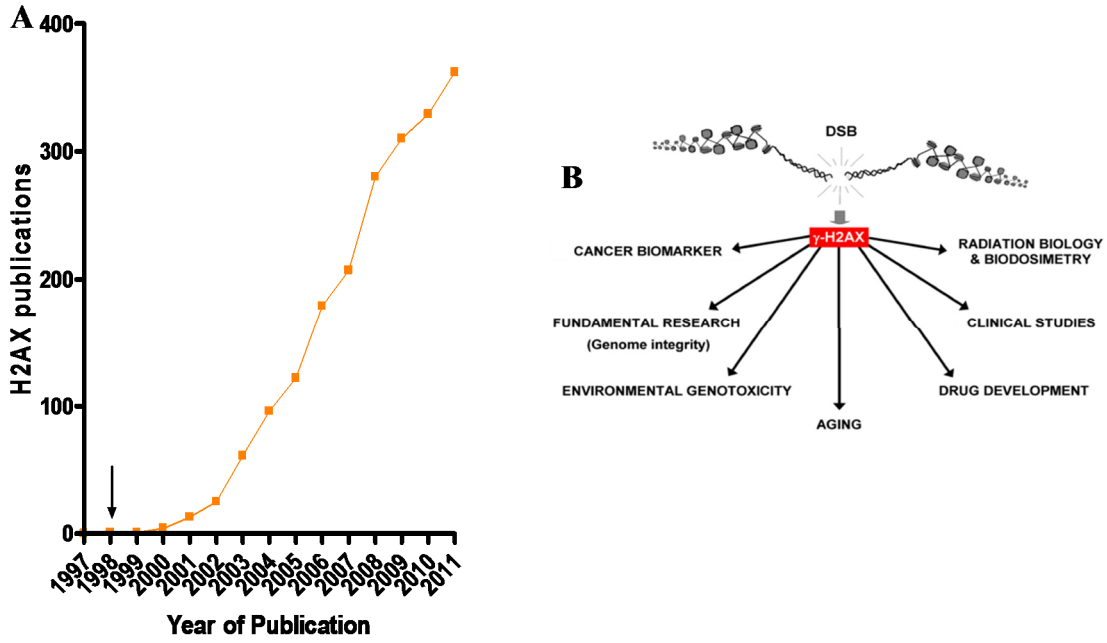

Figure 1: [A] Since its discovery as a DNA DSB marker in 1998 (arrow), the number of publications containing H2AX in the title/abstract has grown exponentially. Adapted and updated from (Dickey *et al.*, 2009). [B] Areas in which γH2AX is studied as a marker of DNA damage. Taken from (Redon *et al.*, 2011b).

Table 3: Studies using well-characterised compounds to assess γH2AX as a marker of DNA DSB damage.

Year	Detection Method	Compounds	Reference
2005	Immunoblotting and Microscopy	Norethindrone	(Gallmeier *et al*., 2005)
2006	Microscopy	Methyl methanesulfonate, *N*-ethyl-*N*-nitrosourea, benzo[a]pyrene, 2-acetyl-aminofluorene, azathioprine, cyclosporine A.	(Zhou *et al*., 2006)
2008	Microscopy	Bleomycin	(Rakiman *et al*., 2008)
2008	Flow cytometry	Etoposide and mitoxantrone	(Smart *et al*., 2008)
2009	Flow cytometry	Etoposide, methyl methanesulfonate, bleomycin, ampicillin, sodium chloride	(Watters *et al*., 2009)
2010	Microscopy and Immunoblotting*	Benzo[a]pyrene, fluoranthene, 3-methylcholanthrene	(Audebert *et al*., 2010)
2010	Cell-ELISA	Methyl methanesulfonate, *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine, mitomycin C, cis-diamminedichloroplatinum II, irinotecan hydrochloride hydrate, etoposide, methotrexate hydrate, 5-fluorouracil, colcemid, vincristine sulfate salt, paclitaxel, griseofulvin, 17-allylaminogeldanamycin, sodium dodecyl sulfate,	(Matsuzaki *et al*., 2010)

		sodium chloride	
2011	Microscopy	Doxorubicin, cisplatin, etoposide, camptothecin, bleomycin	(Kim *et al*., 2011)
2011	Flow cytometry	Cyclophosphamide, ethyl methane sulphonate, methyl methane sulphonate, n-butyl chloride, trimethyl ammonium chloride,2-acetylaminofluorene, 2-aminoanthracene, 5-chloro-o-toluidine, 2,4-diaminotoluene, p-chloroaniline, 4-nitroquinolineoxide, p-nitroaniline, mitomycin C, glycidol, 1,2-propylene oxide, styrene oxide, benzo[a]pyrene, hydrogen peroxide, endrin, methyl nitrosourea, ethyl nitrosourea, dimethylbenz(a)anthracene, hydroquinone, phenanthrene, isobutyraldehyde, ethyl acrylate, D-mannitol, cyclohexanone, methyl carbamate, hexachloroethane, phthalic anhydride, resorcinol, propyl gallate, eugenol	(Smart *et al*., 2011)

* Score in multi-well plate, no gel required.

Some cell systems used in *in vitro* toxicology testing are reported to have different deficiencies in their metabolism leading to incorrect evaluation of test compounds (Kirkland *et al.*, 2007a). These limitations could also affect the predictivity of the γH2AX assay. To prevent this, study designs need to incorporate a metabolically competent cell system or, alternatively, an exogenous source of metabolic activation to detect pro-toxicants. These are compounds that have to be metabolically activated before their toxic form is active, a prime example being benzo[a]pyrene known as B[a]P (Figure 2).

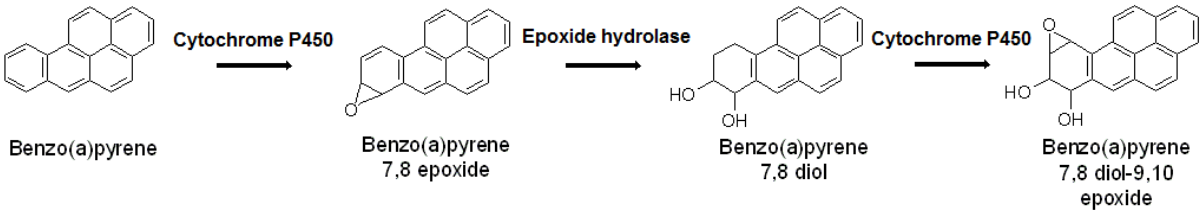

Figure 2: Activation of Benzo[a]pyrene (B[a]P) to Benzo[a]pyrene diol-epoxide (BPDE) by a Phase I enzyme from the cytochrome P450. BPDE binds to the DNA molecule creating DNA-adducts.

Audebert *et al*. tested various polycyclic aromatic hydrocarbons (PAHs), such as B[a]P, in three different cell lines. They demonstrated that in HepG2, B[a]P can be oxidised and conjugated (Audebert *et al.*, 2010), however, the metabolic competency of HepG2 has some limitations as discussed previously (Jennen *et al.*, 2010). The use of cell lines with metabolic capabilities has been previously recommended to improve the specificity without compromising the sensitivity of the method (Rueff *et al.*, 1996; Kirkland *et al*., 2007b).

An alternative approach to the use of cell lines with full or limited metabolic competency, is the introduction of an exogenous source of metabolism during the experimentation. The most commonly used is the hepatic S9 fraction or S9, liver microsomes from rats pre-stimulated with Aroclor1254 or phenobarbital/β-naphthoflavone. This methodology is currently applied to the entire battery of regulatory tests, where S9 is added for short treatments (3 hours) due to

its toxicity (OECD, 2010; OECD, 1997c). The same approach was followed by Smart *et al.* where mouse lymphoma L5178Y cells were used to assess γH2AX induction after exposure to a panel of pro-toxicants in the presence of S9 (Smart *et al.*, 2011). Alternatively, other sources of metabolic activation could be employed. Hepatic human microsomes could be used for a human-specific metabolism or a lung subcellular fraction for a more organ-specific metabolism. However, incorporating human material could increase the variability compared to the S9 from laboratory animals.

The use of metabolically competent cell systems like HepaRG or human stem cells has also been discussed as an option to reduce the false positives produced by the higher activation capacity of the rat S9 fraction (Kirkland *et al.*, 2007b).

5.- APPLICATION OF γH2AX AS A GENOTOXICITY ASSAY IN THE EVALUATION OF CIGARETTE SMOKE

Cigarette smoke is a complex mixture consisting of a particulate phase and a vapour phase. It is estimated that the whole mixture contains approximately 5600 compounds (Perfetti and Rodgman, 2011). Over 150 known toxicants are present in tobacco smoke (Cunningham *et al*., 2011). Some of those compounds are known carcinogens, such as B[a]P, a PAH and 4-(N-methylnitrosamino)-1-(3-pyridinyl)-1-butanone (NNK), a tobacco-specific *N*-nitrosamine. Both compounds are classified by IARC as "carcinogenic to humans" (Group 1) (IARC, 2012a; IARC, 2012b).

Testing of complex mixtures is problematic as a small number of particular components can mask the effects of others, especially if they elicit high cytotoxicities. In addition, components can also act synergistically or act competitively, so the testing of mixtures can only give a global picture obscured by these factors. Fractionation of the different smoke components can assist determining of what the key toxic drivers in smoke may be. A global picture can also be used to compare smoke from different tobaccos, which may have different toxicities.

Table 4: Physical forms of cigarette smoke used in *in vitro* testing. Taken from (Breheny *et al.*, 2011).

Name	Description
Cigarette smoke condensate (CSC)	Comprises the particulate phase along with some vapour phase components. Generated by cold-trapping and condensation of smoke at extremely low temperatures. The condensed 'tar' is then typically extracted and diluted using acetone.
Cigarette smoke total particulate matter (TPM)	Comprises the particulate phase only. Particulates are collected by passing cigarette smoke through a Cambridge filter pad and are subsequently eluted using a solvent such as dimethylsulphoxide.
Cigarette smoke extract (CSE)	Comprises the particulate phase along with some vapour phase components. Generated by bubbling smoke in a liquid (e.g. phosphate-buffered saline or cell culture medium).
Whole mainstream cigarette smoke (WMCS)	Cells are directly exposed to smoke at the air-liquid interface. This is the most representative of human exposure conditions, as cells are exposed to the gas and vapour phase components in an aerosol (Phillips *et al.*, 2005).

There are different mechanisms by which cigarette smoke carcinogens interact with DNA (Hecht, 1999). DNA adduct formation and oxidative DNA damage are mechanisms known to generate DSBs by acting directly on the DNA. Cigarette smoke has also shown to have aneugenic activity (Van *et al.*, 2008), an indirect-acting mechanism of genotoxicity. However, no single compound present in cigarette smoke has been classified as an aneugenic compound.

Currently, the genotoxic potential of cigarette smoke is measured mostly using methods focusing on fixed DNA damage after acute exposures to different forms of cigarette smoke (DeMarini *et al.*, 2008; Schramke *et al.*, 2006; Van *et al.*, 2008; Wolz *et al.*, 2002; Nakayama *et al.*, 1985; Johnson *et al.*, 2009). There are also multiple clinical studies focusing on the genotoxic effects of cigarette smoke in humans (Hruba *et al.*, 2010; Laubenthal *et al.*, 2011;

Chapman *et al*., 2011; Choudhury *et al*., 2008; Mondal *et al*., 2010). However, these clinical studies fall out of the scope of this review.

Recent reviews described the different physical forms of cigarette smoke used in *in vitro* testing (Table 4) and the history of the collection of tobacco smoke for toxicology testing (Breheny *et al*., 2011; Johnson *et al*., 2009).

De Marini conducted a detailed review of the genotoxicity of tobacco smoke and tobacco smoke condensate (DeMarini, 2004). Overall, cigarette smoke condensate (CSC) and cigarette smoke total particulate matter (TPM) have been the main testing forms of cigarette smoke *in vitro*. The use of CSC or TPM for *in vitro* genotoxicity testing has the advantage that test material can be prepared as a concentrated stock solution in a compatible solvent (usually DMSO) and applied at a relatively high top concentration in a range of *in vitro* test systems, thus maximizing the potential to detect and quantify a genotoxic effect. Resultant data can be normalized on a per milligram tar, per cigarette or per milligram nicotine basis, facilitating product comparisons (DeMarini *et al*., 2008). However, it is also clear that such assays cannot assess the contribution that the vapour phase of cigarette smoke may make toward toxicity. Moreover, these exposure agents are not fully representative of human exposure as cells are not fully exposed to both the particulate and vapour phase components of the cigarette smoke. A number of whole smoke exposure systems are being developed to address these problems, but have only recently entered a phase where dosimetric comparisons can be made and have not yet been validated. Whole smoke exerts significant cytotoxicity and therefore precise exposure conditions need to be defined in order to detect specific genotoxic effects. Of course the real key to definition of appropriate smoke exposure systems for toxicity testing is to understand the contribution of individual tobacco smoke constituents to the genotoxic effects (both singly and in combination) and to estimate their

concentration in tobacco smoke particulate and vapour phase fractions. This understanding then facilitates the design of appropriate tobacco smoke exposure systems, focusing on key drivers of genotoxicity, facilitating product comparisons and providing a scientific rationale for any observed differences in genotoxic potential.

To date, there are a limited number of studies using whole mainstream cigarette smoke (WMCS) in *in vitro* genotoxicity assays. WMCS was first used as a smoke exposure system in the *in vitro* micronucleus assay (Massey *et al*., 1998; Okuwa *et al*., 2010). In addition, Aufderheide *et al*. developed a WMCS method to evaluate the mutagenicity of cigarette smoke in various bacterial strains in the Ames test (Aufderheide and Gressmann, 2007; Aufderheide and Gressmann, 2008). To date, there is no published information of this exposure system in the MLA assay.

In the field of non-regulatory assays, WMCS was used by Thorne *et al*. to measure oxidative DNA damage in the *in vitro* comet assay (Thorne *et al*., 2009). Studies to measure the activation of H2AX in response to DNA damage *in vitro* after cigarette smoke exposure have also used CSC, TPM or cigarette smoke extract (CSE) as a smoke exposure system (Albino *et al*., 2004; Albino *et al*., 2006; Tanaka *et al*., 2007b; Tanaka *et al*., 2007a; Luo *et al*., 2004; Albino *et al*., 2009; Zhao *et al*., 2009; Jorgensen *et al*., 2010; Darzynkiewicz *et al*., 2011).

The *in vitro* γH2AX assay was originally used to measure DSBs following cigarette smoke exposure (Albino *et al*., 2004). Human A549 pulmonary adenocarcinoma cells were exposed to cigarette smoke and normal human bronchial epithelial (NHBE) cells to CSC. Both cell systems showed a dose-related response in γH2AX activation. Once the relationship between smoke exposure and γH2AX activation was confirmed, Albino *et al*. used the assay to evaluate cigarettes with different tar deliveries. The results indicated that the increment in

γH2AX intensity was proportional to the estimated tar delivery rather than the cigarette type or smoking behaviour (Albino *et al*., 2009).

Interestingly, when Kato *et al.* evaluated the response of CSC in the commonly used CHO cell system without metabolic activation, they obtained a negative result (Kato *et al*., 2007). However, Kato *et al.* synchronised the cultures to evaluate DSBs only in G1 phase. Direct-acting genotoxic compounds in CSC may require metabolic activation in order to generate DSBs. Other indirectly acting genotoxic compounds in CSC would need the cell to progress through cell division to generate DSBs as these compounds interfere with cell division mechanisms. Further experiments would be needed to elucidate if the negative result was caused by the lack of metabolic activation, the synchronisation or both.

Cigarette sidestream smoke (CSS) or environmental cigarette smoke has also been reported to generate a dose- and time-related γH2AX induction in A549 cells (Toyooka and Ibuki, 2009).

Additionally, a recent publication reported the induction of γH2AX in A549 cells after exposure to smoke of tobacco- and nicotine-free cigarettes (T&N-free cigarettes) and a commercially available control cigarette (2R4F) (Jorgensen *et al*., 2010). The results showed that T&N-free cigarettes produce a consistently higher induction of γH2AX compared to 2R4F. The results indicated that the driver for the γH2AX increase is the tar as T&N-free cigarettes produced an average of 30.9 mg of TPM per cigarette while 2R4F generate around 8.9 mg TPM per cigarette. This result concurs with the conclusions reported by Albino *et al*. that the γH2AX intensity was proportional to the estimated tar delivery (Albino *et al*., 2009).

6.- CONCLUSIONS

Since its discovery in 1998, the phosphorylation of H2AX to γH2AX has been used as a tool in multiple scientific fields, from the *in vitro* assessment of new drugs to a clinical biomarker. However, the main focus of this review is to collect the efforts of the last decade to demonstrate that the γH2AX assay could be a potential complement to the current battery of *in vitro* genotoxicity tests.

Furthermore, we have reviewed the applications of the γH2AX assay in the *in vitro* evaluation of cigarette smoke, showing that the γH2AX assay could unravel some of the DNA damaging effects of this complex mixture.

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Chapter II

Metabolic characterization of cell systems used in *in vitro* toxicology testing: Lung cell system BEAS-2B as a working example

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Metabolic characterization of cell systems used in *in vitro* toxicology testing: Lung cell system BEAS-2B as a working example ☆

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ABSTRACT

The bioactivation of pro-toxicants is the biological process through which some chemicals are metabolized into reactive metabolites. Therefore, *in vitro* toxicological evaluation should ideally be conducted in cell systems retaining adequate metabolic competency and relevant to the route of exposure. The respiratory tract is the primary route of exposure to inhaled pro-toxicants and lung-derived BEAS-2B cell line has been considered as a potentially suitable model for *in vitro* toxicology testing. However, its metabolic activity has not been characterized.

We performed a gene expression analysis for 41 metabolism-related genes and compared the profile with liver- and lung-derived cell lines (HepaRG, HepG2 and A549). To confirm that mRNA expression was associated with the corresponding enzyme activity, we used a series of metabolic substrates of CYPs (CYP1A1/1B1, CYP1A2, CYP2A6/2A13 and CYP2E1) known to bioactivate inhaled pro-toxicants. CYP activities were compared between BEAS-2B, HepaRG, HepG2, and A549 cells and published literature on primary bronchial epithelium cells (HBEC).

We found that in contrast to HBEC, BEAS-2B and A549 have limited CYP activity which was in agreement with their CYP gene expression profile. Control cell lines such as HepG2 and HepaRG were metabolically active for the tested CYPs. We recommend that similar strategies can be used to select suitable cell systems in the context of pro-toxicant assessment.

1.- INTRODUCTION

The need for *in vitro* cell systems as alternatives to animal models for toxicological testing is increasing in response to new regulations, such as the EU 7th Amendment Directive (European Commission 2003), and to ethical considerations like the 3Rs principle (Schechtman 2002). Due to their relative homogeneity and ability to be maintained in culture indefinitely, established cell lines have been one of the preferred cell systems employed in the development and validation of *in vitro* toxicology assays. Most continuous cell lines, however, have been derived from malignant or transformed tissue and fail to replicate the physiology and morphology of normal cells. Historically, hepatic cell lines have been thoroughly characterized, as they are the prime systems used for drug metabolism and toxicity testing in pre-clinical development (Guguen-Guillouzo and Guillouzo 2010; Brandon *et al*., 2003). For instance, the hepatoma cell line HepG2 lacks normal metabolic activity and has been engineered to express hepatic cytochrome P450 (CYP) enzymes (CYP3A4, CYP2E1) to study *in vitro* drug hepatotoxicity caused by compounds such as paracetamol (Yoshitomi *et al*., 2001). CYP3A4 and CYP2E1 catalyze the transformation of paracetamol into a highly reactive metabolite responsible for the tissue specific toxicity of the drug. This process of metabolic transformation of a chemical (pro-toxicant) into a toxic species is defined as bioactivation and is a key element in many toxicity studies. In contrast, many other cell lines used in toxicology, and in particular non-hepatic cells, have not been extensively characterized for their metabolic competency. The deficiencies in the metabolic capabilities of cell lines could lead to inaccurate evaluation of test compounds (Kirkland *et al*., 2007). This is the case for benzo[a]pyrene (B[a]P), a well-known tobacco smoke chemical that is ultimately metabolized to a diol-epoxide carcinogen by the inducible lung CYPs, CYP1A1/1B1. The formation of B[a]P DNA adducts has been reported *in vitro* using lung carcinoma-derived A549 cells (Feldman *et al*., 1978) but the role of CYP1A1/1B1 in the

formation of such adducts in A549 was not demonstrated at the time. In 2000, Hukkanen and colleagues reported the expression and inducibility of CYP1A1/1B1 in the A549 cell line but activity was not verified. In 2008, Quinn established that CYP1A1 was not required in A549 for the oxidation of B[a]P to its reactive form and that this reaction could be catalyzed by AKR1B10 (Quinn *et al.*, 2008). However CYP1A1 activity was reported the same year by EROD assay after A549 induction (Billet *et al.*, 2008). In contrast, in a comparison between CYP1A1/1B1 activity in A549 and HBECS Newland *et al.* showed that the CYP1A1 activity in A549 was limited when compared to a culture of human primary lung epithelial cells when incubated with a luminogenic probe substrate. Thus the mechanism of adduct formation in A549 can potentially follow multiple metabolic routes different than what would be expected in a normal lung epithelium. CYP2B6 activity has also been reported in A549 together with mRNA expression of CYP2D6, 2E1, 3A5, and CYP3A7, the latest is not expected to be present in normal adult tissue. Other key lung epithelium CYPs such as CYP2A6, CYP2A13, and CYP2F1 involved in the bioactivation of toxicants such as nitrosamines were not detected in this cell line. This example highlights the importance of characterizing the metabolic enzyme profile in cell lines used for toxicological evaluation, with the possibility to restrict such study to enzymes relevant to the metabolic pathway of specific toxicants. However, to date, there is no standard approach to metabolic characterization. Where some researchers focus on gene expression only (Jennen *et al.*, 2010) others may combine gene expression with enzyme activity (Westerink and Schoonen 2007).

The aim of our investigation was to describe an experimental strategy combining quantitative real time PCR (qPCR) and functional enzymatic assays applied to the lung-derived BEAS-2B cell line. Initially, we profiled the gene expression of a panel of oxidative and conjugative metabolism-related genes involved in xenobiotics metabolism, more specifically related to the toxicity of cigarette smoke to human lung (Hecht 2006). Next, we used a series of

metabolic substrates and inhibitors to test the enzyme activity of key CYP enzymes. The selection was made taking into account the relevance of the target organ and the nature of the test article. The human lung-derived BEAS-2B cell line was first described in 1988, when normal bronchial epithelial cells obtained from autopsy of non-cancerous individuals were isolated, then infected with a replication-defective 12-SV40/adenovirus hybrid and cloned to create an immortalized phenotype (Reddel *et al*., 1988). The non-cancerous phenotype of BEAS-2B cells is an advantage in the investigation of carcinogenic processes such as DNA damage and cell transformation (Sun *et al*., 2011). Therefore, BEAS-2B cells have been considered as a relevant cell line for *in vitro* toxicology testing in the field of pollutants, tobacco products and nanomaterials (Persoz *et al*., 2012; Veljkovic *et al*., 2011; Haniu *et al*., 2011). Although, several studies have employed BEAS-2B cells to evaluate the effect of some pro-toxicants such as B[a]P and 4-(N-methylnitrosamino)-1-(3-pyridinyl)-1-butanone (NNK), the metabolic capacity of this particular cell line has not been fully characterized (Ovrevik *et al*., 2010; Proulx *et al*., 2005).

Here, BEAS-2B cells were tested and compared to the lung-derived A549 cells broadly used as pulmonary *in vitro* system but with no cytochrome P450 expression reported for CYP1A2 and the CYP2A family, and inducibility documented for CYP1A1/1B1 genes *(*Hukkanen *et al*., 2002; Castell *et al*., 2005). Cells derived from hepatocarcinomas considered to have a more extensive cytochrome P450 enzyme activity (HepG2 and HepaRG) were used as a more comprehensive control for our CYP assays (Jennen *et al*., 2010). Moreover, the results were contrasted to those reported in primary human bronchial epithelium culture (Newland *et al*., 2011; Courcot *et al*., 2012). The results of this study are considered to be useful for *in vitro* toxicological testing using the cell line BEAS-2B as cell system. Furthermore, we propose that the outlined strategy can be incorporated in the characterization of cell systems used in *in vitro* testing.

2.- MATERIALS AND METHODS

2.1.- Cell culture

The human bronchial epithelial cell line (BEAS-2B), purchased from ATCC (United States), was seeded into culture vessels that had been pre-coated with 0.03 mg/mL PureCol® bovine collagen solution (Nutacon, The Netherlands). Cells were maintained in Bronchial Epithelial Growth Medium (BEGM®) at 37°C and 5% CO_2 in a humidified incubator. BEGM® was prepared by supplementing Bronchial Epithelial Basal Medium with growth supplements provided in the manufacturer's BEGM® SingleQuot® kit (Lonza Group Ltd., Belgium) containing: bovine pituitary extract, hydrocortisone, human epidermal growth factor, epinephrine, insulin, triiodothyronine, transferrin, gentamicin /amphotericin-B and retinoic acid.

The human type II alveolar adenocarcinoma-derived (A549) and hepatocarcinoma-derived (HepG2) cell lines purchased from the Global Bioresource Centre (ATCC, USA), were maintained in Dulbecco's modified Eagle medium supplemented with fetal bovine serum (10% v/v), L-glutamine (2 mM), penicillin (50 U/mL) and streptomycin (50 µg/mL) (complete DMEM) at 37°C and 5% CO_2 in a humidified incubator.

The human hepatocarcinoma-derived cell line (HepaRG) was purchased as a differentiated confluent monolayer from Biopredic International (France). After shipment, the cells were maintained in basal medium supplemented with recovery mix for 24 hours followed by basal medium supplemented with maintenance/metabolism mix. Media and supplements were provided by the manufacturer (Biopredic, France).

BEAS-2B, A549 and HepG2 cells were cultured and expanded in-house. Experiments were performed between passages 3 and 12 only. All cultures were negative for mycoplasma.

Additionally, the cells were authenticated using the short tandem repeat profiling to confirm the nature of the cell cultures (LGC Standards, United Kingdom) (Nims *et al.*, 2010).

2.2.- Gene expression assay

BEAS-2B, A549 and HepG2 cells were plated in 12-well tissue culture plates, at 60% confluency. A total of 6 wells per plate were treated for 48 hours with 10nM of the CYP1A1/1B1 inducer 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD). HepaRG cells were not used as positive control cell line for CYP1A1/1B1, therefore, they were not pre-induced with TCDD.

After 96 hours from seeding, total RNA was isolated from the cells using the RNeasy mini kit (Quiagen, United Kingdom). The RNA quantity was measured by using the NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, USA) and the quality assessed with the Agilent 2100 Bioanalyzer (Agilent, United Kingdom).

The RNA was converted to cDNA using the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems, United Kingdom).

qPCR was carried out using custom TaqMan® array 96-well plates and TaqMan® Fast Universal Master mix (Applied Biosystem, United Kingdom). Each plate contained two assays with the probes of 1 manufacturing control, 5 endogenous controls and 41 metabolism-related genes from both phase I and phase II (Table 1). qPCR amplification mixtures (20 µL) contained 2 µL of cDNA and 18 µL of fast master mix and were amplified using the fast PCR 7500 (Applied Biosystems, United Kingdom). The cycle conditions comprised 2 min at 50°C, 20 seconds at 95°C, then 40 cycles of 3 seconds at 95°C and 30 seconds at 60°C.

Threshold cycle (Ct) values for the genes were normalised to RPLP0, and relative expression levels were calculated using the ΔΔCt method (Livak and Schmittgen 2001).

Table 1: Panel of genes classified by phase of metabolism and function. Standard gene nomenclature taken from (HGNC, 2012).

Controls		**Phase I**			
Manufacturing Control	**Endogenous control**	**Cytochrome P450**		**Aldo-keto reductases**	**Others**
RN18S1	GAPDH	CYP1A1	CYP3A4	AKR1B10	EPHX1
	HPRT1	CYP1B1	CYP3A5	AKR1C1	CBR1
	GUSB	CYP1A2	CYP2C9	AKR1C2	DCXR
	B2M	CYP2A6	CYP2D6	AKR1C3	AOX1
	RPLP0	CYP2A13	CYP2E1	AKR7A3	FMO3
		CYP2B6	CYP2F1		HSD11B1
		CYP2B7P1			NQO1

Phase II

glutathione S-transferases	**UDP-glucuronosyltransferase**	**Others**
GSTA1	UGT1A4	SULT1A1
GSTM1	UGT1A6	NAT1
GSTP1	UGT1A7	COMT
GSTT1	UGT1A1	
	UGT1A8	
	UGT1A9	
	UGT1A10	
	UGT2B7	
	UGT2B10	

2.3.- Cytochrome P450 enzyme activity assays

Range finder experiments were initially carried out to select optimal concentrations for substrates, inducer and inhibitors where maximal activity, induction or inhibition were obtained without cytotoxicity.

For the enzyme activity profiling, phenol free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 0.5 mM L-Glutamine was used as a basal experimental medium.

CYP1A1/1B1 activity was measured using the specific P450-Glo™ kit (Promega, United Kingdom). The probe used was Luciferin 6' chloroethyl ether (luciferin-CEE) (50 µM) added directly to the basal medium (0 and 4 hour incubation) of 3 wells per condition. For induced conditions, cultures were pre-incubated for 48 hours with 10nM of TCDD. For inhibited conditions, α-naphthoflavone (10 µM) was added to the basal medium 30 minutes prior to the probe. After the incubation, the luminescence was measured using a LMaxII® luminometer (Molecular Devices, United States). HepG2 cells were used as *'positive control'*.

The measurement of CYP2A6/2A13 activity was based on the methodology described previously (Newland *et al*., 2011). The same methodology was adapted, including probes and inhibitors, for the measurement of CYP1A2 and CYP2E1 activities. A549 cells were used as *'negative control'* for CYP2A6/2A13 and CYP1A2, the status of CYP2E1 activity is unknown in A549. HepaRG cells were used as a positive control for CYP1A2 and CYP2A6/2A13. HepG2 cells were used as *'positive control'* for CYP2E1.

For the CYP1A2 activity assay, 7-ethoxyresorufin (20 µM) was used as a probe and fluvoxamine (100 µM) was used as inhibitor. The metabolite quantified was resorufin.

In the case of the CYP2A6/2A13 activity assay, coumarin (200 μM) was used as a probe and 8-methoxypsoralen (8-MOP) (100 μM) as inhibitor. The metabolite measured was 7-hydroxycoumarin.

Finally, the CYP2E1 activity assay used chlorzoxazone (100 μM) as probe and disulfiram (20 μM) as inhibitor. 6-hydroxychlorzoxazone was the metabolite quantified.

After the probe incubations, 250 μL of basal medium was adjusted to pH 5.0 with hydrochloric acid and treated with 2.5 μL of β-glucuronidase from *Helix pomatia* for 18 hours at 37°C while shaking. Once the glucuronidase treatment finished, 250 μL of methanol and the internal standard 4-methylumbelliferon (5 μM) was added to the solution. The metabolites where then quantified using an UPLC- AB SCIEX/API 4000 Q-Trap® mass spectrometer using the column Phenomenex Kinetex 2.6μm PFP, 100Å (Applied Biosystems, United States).

Once all basal medium was removed, cells were lysed using Mammalian Protein Extraction Reagent (M-PER) lysate buffer (Thermo Fisher Scientific Inc., United Kingdom) and protein content was measured employing the bicinchoninic acid protein assay (BSA) together with a Multiskan Ascent® spectrophotometer (Thermo Fisher Scientific Inc., United Kingdom).

Lactate dehydrogenase (LDH) release was used as a measure of cytotoxicy during the enzyme activity assays. The CytoTox-ONE® homogeneous membrane integrity assay (Promega, United Kingdom) was used following manufacture recommendations and analysed with a Fluoroskan Ascent® fluorometer (Thermo Fisher Scientific Inc., United Kingdom). The percentage LDH release is inversely proportional to the cell viability which was >85% for all treatments and timepoints.

2.4.- Gene expression data analysis

After completion of the qPCR, the threshold cycle (Ct) values were visually inspected using the fast PCR 7500 software v.2.0.5. When required, the threshold setting default (0.2) was manually adjusted to ensure optimal sensitivity was maintained. All Ct > 36, indicative of the plateau phase of qPCR, were considered non-expressed genes.

The Ct values were then normalized against the selected endogenous control gene to generate ΔCt values ($Ct_{gene\ of\ interest} - Ct_{endogenous\ control\ gene}$).

2.5.- Statistical analysis

All the experiments were repeated three times containing three replicates per condition and timepoint.

GeneSpring^{TM} GX11.5.1 (Agilent, United Kingdom) was used to perform the gene expression graphical and statistical analysis. Principal Component Analysis (PCA) and hierarchical clustering were selected for graphical representations. For the hierarchical clustering algorithm, Euclidean distance measured with average linkage was selected for interpretation of the normalized gene expression data (ΔCt). One-way ANOVA was used to analyse the effect of the TCDD induction on the expression of each gene.

The enzyme activity data are represented by the arithmetic mean of three experiments + standard deviation (SD). Minitab v.16 was used to perform Student's t-test. Difference was significant when $p<0.05$.

3.- RESULTS

3.1.- Gene expression

The first stage in the metabolic characterization was to quantify the mRNAs of a panel of enzyme-encoding genes involved in oxidative (phase I) and conjugative (phase II) metabolism.

The endogenous control gene RPLP0 showed the most stable expression across the different samples and treatments (data not shown). Furthermore, RPLP0 has been reported as being highly conserved across tissues and species (Akamine *et al.*, 2007). Therefore, RPLP0 was chosen for normalisation of data, generating ΔCt values ($Ct_{gene\ of\ interest} - Ct_{RPLP0}$).

PCA was used to visualise the dataset in a 3D scatter plot graph shown in Figure 1. This analysis demonstrated the segregation of cell lines based on their gene expression profile. The graph shows a clear separation of the different cell lines (represented by colours) indicating that the expression profile differs from cell line to cell line. In addition, only HepG2 cells show a variation between the induced (triangle shaped icon) and non-induced samples (rectangle shaped icon), while there is no apparent separation of the induced from the non-induced BEAS-2B and A549 samples. HepaRG cells were not induced so Figure 1 only represents the basal gene expression levels.

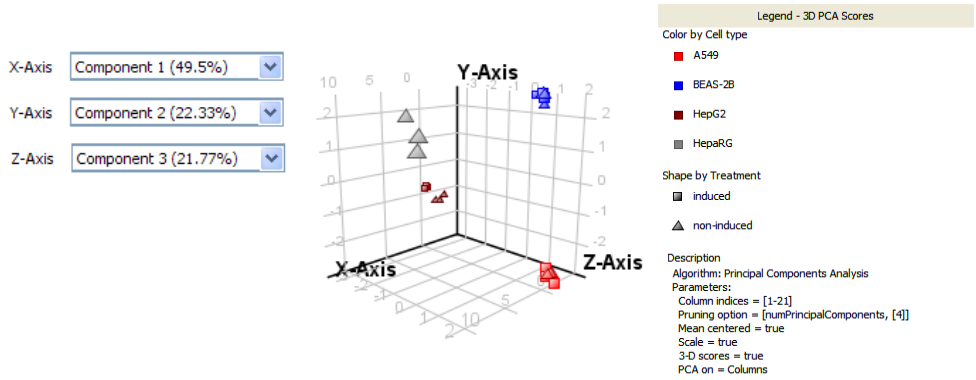

Figure 1: 3D scatter plot representing principal component analysis (PCA) of qPCR data. Cell type is represented by colour - A549 (red), BEAS-2B (blue), HepG2 (maroon) and HepaRG (grey). Shaped icons represent induction status, triangle (▲) represents induced cultures and square (■) represents non-induced cultures. (Note: HepaRG gene expression was only measured in non-induced cultures).

The hierarchical cluster shown in Figure 2 was generated to visualise the gene expression and induction profiles of each individual cell line. This graphical representation contained the expression value for each individual gene normalised (ΔCt values); red, blue and yellow indicate increased (positive ΔCt), reduced (negative ΔCt) and undetectable (ΔCt close to or 0), respectively. The details contained in the hierarchical cluster allowed a gene by gene comparison between induced and non-induced treatments but also, between different cell lines. This cluster analysis confirms the observations made above by PCA. Gene expression profiles in BEAS-2B and A549 cells do not show a significant difference between induced and non-induced samples. In contrast, the HepG2 profile shows some changes between induced and non-induced samples. However, there are many genes that are not differentially expressed. HepaRG cells show a high expression in the majority of the tested genes.

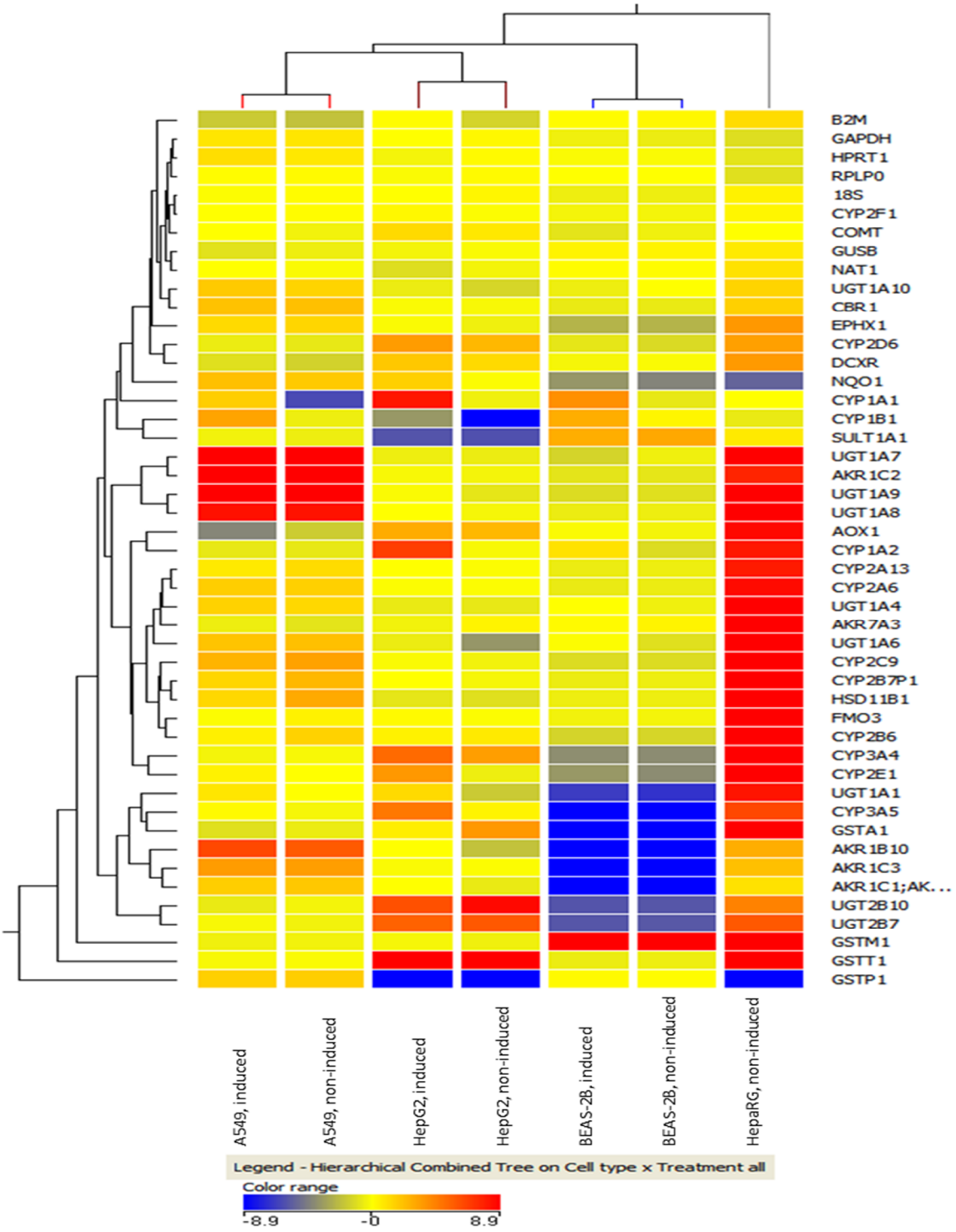

Figure 2: Hierarchical cluster representing the gene expression profiles of all four cell lines dependent on treatment (induced or non-induced). (Note: HepaRG gene expression was only measured in non-induced cultures). Columns represent individual samples and rows represent genes. Red, blue and yellow indicate high signal intensity, low signal intensity or no signal in normalised gene expression data (ΔCt), respectively.

To allow fine observations between TCDD-induced and non-induced samples, ΔΔCt data representing fold-changes in gene expression for BEAS-2B, A549 and HepG2 are detailed in Table 2.

As expected, CYP1A1/1B1 were inducible across the three cell lines. In BEAS-2B cells, CYP1A2 also showed a degree of inducibility. However, no other gene studied in BEAS-2B cells shows a relevant up- or down-regulation.

Table 2: Gene expression data represented as fold change comparing TCDD- induced with non-induced cells. In red: genes with at least 2-fold increase in gene expression after induction (up-regulated). In blue: genes with at least 2-fold decrease in gene expression after induction (down-regulated).

	Gene ID	BEAS-2B	A549	HepG2
Phase I	CYP1A1	25.35 ↑	256.99 ↑	389.97 ↑
	CYP1B1	5.79 ↑	13.69 ↑	154.52 ↑
	CYP1A2	4.47 ↑	1.02 ↑	124.64 ↑
	CYP2A6	-1.06 ↓	1.05 ↑	-1.02 ↓
	CYP2A13	-1.05 ↓	-1.42 ↓	1.02 ↑
	CYP2B6	-1.05 ↓	-2.11 ↓	-1.22 ↓
	CYP2B7P1	-1.05 ↓	-2.11 ↓	1.18 ↑
	CYP3A4	1.02 ↑	-1.11 ↓	3.60 ↑
	CYP3A5	-1.62 ↓	1.36 ↑	20.43 ↑
	CYP2C9	1.02 ↑	-1.52 ↓	1.23 ↑
	CYP2D6	1.28 ↑	1.14 ↑	1.96 ↑
	CYP2E1	1.37 ↑	1.32 ↑	18.18 ↑
	CYP2F1	-1.05 ↓	-1.06 ↓	1.18 ↑
	AKR1B10	1.25 ↑	1.55 ↑	4.33 ↑
	AKR1C1;AKR1C2	1.21 ↑	-1.14 ↓	1.67 ↑
	AKR1C2	-1.05 ↓	1.44 ↑	1.10 ↑
	AKR1C3	1.63 ↑	1.04 ↑	-1.04 ↓
	AKR7A3	-1.15 ↓	1.28 ↑	-1.72 ↓

	EPHX1	-1.01 ↓	-1.10 ↓	1.29 ↑
	CBR1	-1.02 ↓	-1.05 ↓	1.017 ↑
	DCXR	-1.11 ↓	1.43 ↑	1.54 ↑
	AOX1	1.18 ↑	-5.51 ↓	1.34 ↑
	FMO3	-1.05 ↓	-1.35 ↓	1.18 ↑
	HSD11B1	1.02 ↑	-3.08 ↓	1.18 ↑
	NQO1	1.54 ↑	1.30 ↑	3.30 ↑
Phase II	GSTA1	-1.05 ↓	-1.35 ↓	-8.38 ↓
	GSTM1	-1.09 ↓	-1.06 ↓	1.18 ↑
	GSTP1	1.03 ↑	-1.02 ↓	87.66 ↑
	GSTT1	-1.05 ↓	-1.06 ↓	1.71 ↑
	UGT1A4	1.41 ↑	1.13 ↑	1.05 ↑
	UGT1A6	1.94 ↑	-1.15 ↓	8.17 ↑
	UGT1A7	-1.93 ↓	-1.34 ↓	1.05 ↑
	UGT1A1	1.29 ↑	1.82 ↑	8.51 ↑
	UGT1A8	-1.05 ↓	1.04 ↑	1.18 ↑
	UGT1A9	-1.12 ↓	-1.03 ↓	1.62 ↑
	UGT1A10	-1.47 ↓	1.31 ↑	1.72 ↑
	UGT2B7	-1.01 ↓	1.16 ↑	-1.26 ↓
	UGT2B10	-1.05 ↓	-1.28 ↓	-5.73 ↓
	SULT1A1	-1.17 ↓	1.09 ↑	1.13 ↑
	NAT1	-1.02 ↓	1.10 ↑	-1.72 ↓
	COMT	-1.28 ↓	1.39 ↑	1.40 ↑

3.2.- Enzyme activity

The enzymatic activities of four cytochrome P450s enzymes involved in the oxidative metabolism of smoke toxicants were further evaluated in BEAS-2B, HepG2, HepaRG, and A549 cells to complement the gene expression data.

Data represent the rate of metabolite formation in pmol/mg protein/minute, normalised to soluble protein, except for CYP1A1/1B1 where the metabolite is represented as a measure of

luminescence (RLU). Each experiment included data for the cell line intended for characterization (BEAS-2B), A549 and the *'positive control'* cell line (Hep-G2 or HepaRG).

Results in Figure 3A represent CYP1A1/1B1 enzyme activity. In the absence of TCDD, only background activity was detected for BEAS-2B (0.0470 RLU/mg/min±0.0082). In TCDD-induced BEAS-2B, the activity levels increased 3.7-fold compared to non-induced cells (0.1740 RLU/mg/min±0.0317) and were inhibited in the presence of the CYP1A1/1B1 inhibitor α-naphthoflavone. The activity increase in TCDD-treated cells was statistically significant with a p value <0.0001 and was consistent with the CYP1A1/1B1 mRNA induction observed in our gene expression data.

HepG2 cells gave a high level of enzyme activity as expected from the positive control cell line following induction with TCDD. In contrast, A549 cells produced only background activity both in the presence and absence of the inducer TCDD (0.0284 and 0.0121 RLU/mg/min respectively).

The results observed for CYP2E1 enzyme activity (Figure 3B) showed no statistically significant difference in the levels of enzyme activity between BEAS-2B or A549 cultures treated in the absence or presence of inhibitor disulfiram (p=0.793 and p=0.222 respectively). The positive control cell line (HepG2), on the other hand, showed a significant reduction of enzyme activity in the presence of inhibitor (p=0.022).

CYP2A6/2A13 oxidises coumarin to 7-hydroxycoumarin. The results presented in Figure 3C showed no statistically significant difference (p=0.741) in BEAS-2B CYP2A6/2A13 activity in the presence and absence of inhibitor 8-MOP. A similar profile was observed for A549 cells. These results are in agreement with the lack of CYP2A6/2A13 mRNA expression (Ct>36). HepaRG cells (positive control cell line) showed a reduction in enzyme activity in the presence of inhibitor (p<0.0001).

Figure 3D represents the enzyme activity levels measured for CYP1A2. The results showed that the levels of activity detected in BEAS-2B cells were equivalent to those observed when the CYP1A2 inhibitor fluvoxamine was present in the cultures. These results concurred with the results obtained for CYP1A2 gene expression that indicate that without induction there is no expression of the CYP1A2 gene in BEAS-2B cells based on a Ct>36. HepaRG cells (positive control cell line) showed a reduction in enzyme activity (1.6-fold) in the presence of inhibitor, however, this reduction was not statistically significant ($p=0.127$).

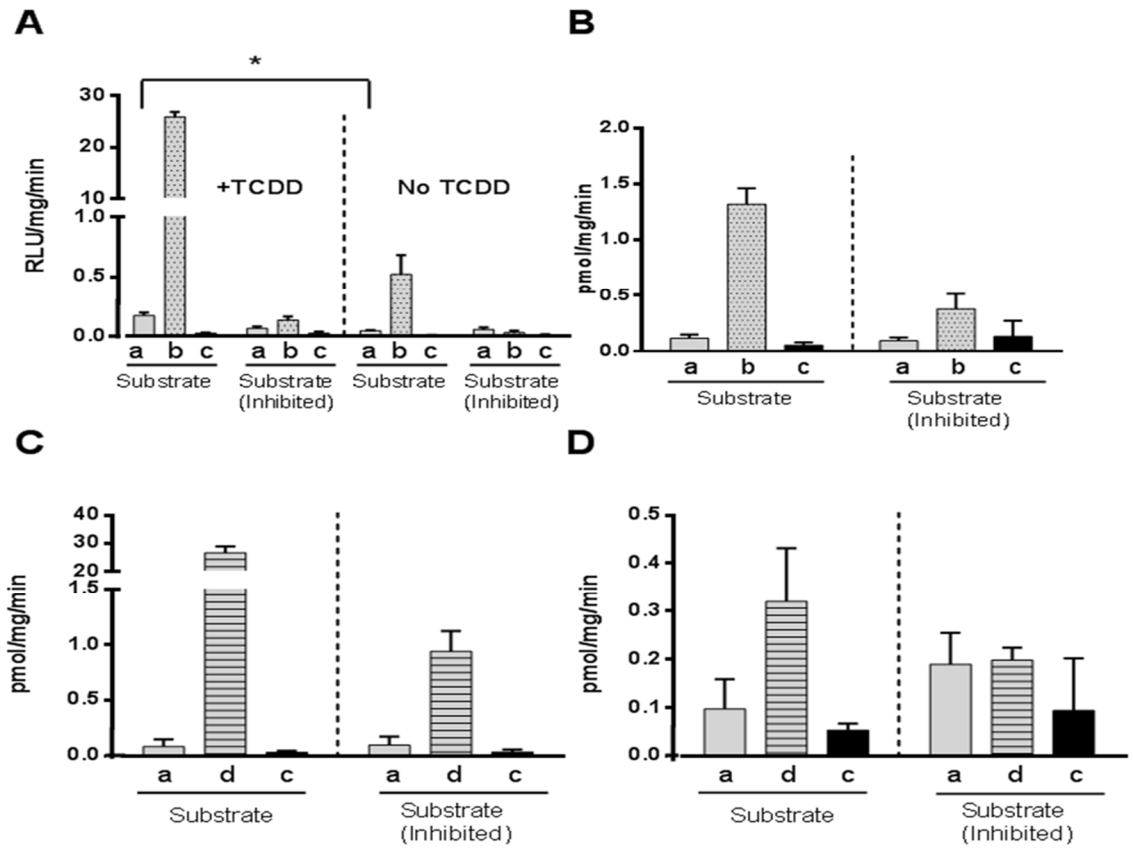

Figure 3: Enzyme activity represented by metabolite formation. **[A]** Luciferin formation measured in BEAS-2B (a), A549 (c) and Hep-G2 (b) with and without induction with TCDD in the presence and absence of a specific inhibitor (α-naphthoflavone). Luciferin rate of formation is indicative of CYP1A1/1B1 activity when luciferin-CEE was used as a substrate. **[B]** 6-hydroxychlorzoxazone formation measured in BEAS-2B (a), A549 (c) and Hep-G2 (b) in the presence and absence of a specific inhibitor (disulfiram). 6-hydroxychlorzoxazone rate of formation is indicative of CYP2E1 activity when chlorzoxazone was used as a substrate. **[C]** 7-hydroxycoumarin formation measured in BEAS-2B (a), A549 (c) and HepaRG (d) in the presence and absence of a specific inhibitor (8-MOP). 7-hydroxycoumarin rate of formation is indicative of CYP2A6/2A13 activity when coumarin was used as a substrate. **[D]** Resorufin formation measured in BEAS-2B (a), A549 (c) and HepaRG (d) in the presence and absence of a specific inhibitor (fluvoxamine). Resorufin ratio of formation is indicative of CYP1A2 activity when 7-ethoxyresorufin was used as a substrate. CYP activities are expressed as the mean + SD of three independent experiments with triplicate samples. The asterisk (*) indicates a $p<0.001$.

4.- DISCUSSION

The lung-derived cell line BEAS-2B has been identified as a cell line of interest in the *in vitro* toxicological testing of inhaled toxicants (Veljkovic *et al.*, 2011; Ansteinsson *et al.*, 2011). However, to date the metabolic capabilities of this cell line have not been thoroughly investigated. In this study, we employed high throughput technology to provide a rapid screening for gene expression and inducibility for a panel of 41 metabolism-related genes, producing a profile of gene expression and gene inducibility. Then, four key CYP enzymes involved in the bioactivation of some smoke pro-toxicants were selected for functional enzyme activity assay. The data obtained from both analysis would confirm if enzyme activity was consistent with gene expression. The scientific approach used in this study is a working example of our proposed strategy for the metabolic characterization of cell systems used in the context of *in vitro* toxicology testing.

Our gene expression results show that non-induced BEAS-2B cells have high and moderate mRNA expression for, GSTM1 and SULT1A1 respectively, both related to conjugative reactions which mainly act as detoxification mechanisms (Castell *et al.*, 2005). As expected, when cultures were pre-induced with TCDD, CYP1A1, CYP1B1 and CYP1A2 genes showed an up-regulation of 25-fold, 6-fold and 4-fold respectively compared with non-induced cultures. The up-regulation of CYP1A1 and CYP1B1 genes after pre-incubation with TCDD has also been reported in normal human primary bronchial epithelium (NHBE) cells (Newland *et al.*, 2011). Surprisingly, in a recent publication Courcot and colleagues report high levels of CYP1B1 gene expression in non-induced cultures of BEAS-2B cells and high levels of CYP1A1/1B1 gene expression in non-induced cultures of human primary bronchial epithelium cells (HBEC), among other lung cell systems (Courcot *et al.*, 2012). This contrasts with our gene expression results and other published results (Newland *et al.*, 2011; Castell *et*

al., 2005). It is possible that the high levels of CYP1A1 and CYP1B1 reported in HBEC by Courcot and colleagues could be as a result of the smoking habit of the donor; cigarette smoke is known to activate these enzymes in the lung (Nishikawa *et al*., 2004; Anttila *et al*., 2011). However, this information is not disclosed in their methodology. In order to confirm the relevance of the gene expression, activity assessment is generally recommended as a follow up experiment.

The gene expression results we obtained were compared with the enzyme activity data obtained for the tested CYPs (CYP1A1/1B1, CYP1A2, CYP2A6/2A13 and CYP2E1). When BEAS-2B cells were pre-incubated with TCDD, CYP1A1/1B1 activity showed a statistically significant increase compared to non-treated cultures (Figure 3A). This concurs with the gene up-regulation described earlier. TCDD-induced BEAS-2B cells showed an activity of 0.2 RLU/mg/min while HBEC cultures have been reported to show an enzyme activity level between 4.3 and 7.3 RLU/mg protein/min (Newland *et al*., 2011). No activity was observed in BEAS-2B cells for the other three CYPs analysed (CYP2E1, CYP2A6/2A13 and CYP1A2) which confirms the findings from our gene expression analysis. Previous studies have also reported no detectable CYP1A2 activity in BEAS-2B cells and lung microsomes (Van Vleet *et al*., 2002; Shimada *et al*., 1992), however, CYP1A2 activity could be induced by environmental factors and specific CYP1A2 gene polymorphisms increasing lung cancer risk as recently reviewed (Pavanello *et al*., 2012). The activity related to CYP2A and CYP2E1 has not been previously reported in BEAS-2B cells, but has been detected in human lung (Hukkanen *et al*., 2002). Newland *et al.* also reported that HBEC cultures from three different donors showed a CYP2A6/2A13 activity between 0.15 and 1.33 pmol/mg/min (Newland *et al*., 2011) a similar study by Runge and colleagues showed that CYP2E1 activity in HBEC (0.6 pmol/mg/min), however substantial inter-individual variability was reported as only two out of the four donors showed CYP2E1 activity (Runge *et al*., 2001).

Overall, the relative enzyme activity level in BEAS-2B cells appears limited compared with normal tissue. For instance, immunobloting of human lung microsomes have been used to detect CYP1A1, 1B1, 2A6, 2B6, 2C9, 2D6, 2E1, 2F1 and 3A4/5 in normal airway tissue (Hukkanen *et al*., 2002; Bernauer *et al*., 2006). In HBEC, these CYPs have been reported to show both gene expression and enzyme activity, however, high interindividual variability between different donors was also noted (Runge *et al*., 2001; Newland *et al*., 2011; Anttila *et al*., 2011; Castell *et al*., 2005). The lack of gene expression for the majority of metabolizing enzyme-encoding genes tested, with or without induction by TCDD, and the lack of activity for three out of the four selected P450 enzymes indicates that BEAS-2B cells might not be suitable to study the toxicity of some inhaled pro-toxicants without an external source of metabolic activation (S9 fractions, microsomes, co-cultures or *in vitro* liver-like cell lines amongst others) (Brandon *et al*., 2003). Alternatively to an external source of metabolic activation, Macé and colleagues developed a series of cell lines, derived from BEAS-2B cells, expressing one single human cytochrome P450 cDNA, CYP1A2, 2A6, 2B6, 2C9, 2D6, 2E1, 2F1, 3A4 and 3A5 (Mace *et al*., 1997a; Mace *et al*., 1997b). These cell lines have been mainly used for the toxicological assessment of single compounds (Mace *et al*., 1994; Van Vleet *et al*., 2002; Nichols *et al*., 2003). Although useful for the toxicity evaluation of single compounds, genetically engineered cell lines have toxicity testing limitations with complex mixtures and compounds with unknown metabolic pathway. The complex mixture could contain various pro-toxicants bioactivated by multiple CYPs. Nevertheless, pro-toxicants which are metabolised by CYP1A1/1B1 enzymes such as PAHs could be bioactivated in pre-induced BEAS-2B cultures. In this study CYP1A1/1B1 gene expression and enzyme activity were induced using TCDD, however, other xenobiotics such as B[a]P have been used previously to induce these isoforms (Nebert *et al*., 1993; Tsuji and Walle 2006).

It is important to consider that the BEAS-2B cell line has a wider application for biological endpoint assessment such as DNA damage and repair mechanisms *in vitro*. The non-cancerous phenotype and wild-type p53 status of the BEAS-2B cell line makes them an ideal cell system in cell transformation research (Reddel *et al*., 1988; Petitjean *et al*., 2007; IARC-TP53 2013). Moreover, the "oncogenic stress" exhibited by pre-malignant and cancer tissues could affect the measure of certain biomarkers of DNA damage such as the γH2AX (Svetlova *et al*., 2010). The BEAS-2B cell line has also been selected as a cell system in the study of nanomaterials cellular transport and intracellular response (Gilbert *et al*., 2012; Ekstrand-Hammarstroem *et al*., 2012).

During this study a number of well-characterised cell lines were used in parallel with the same treatment conditions. The A549 cell line was selected as a lung carcinoma-derived cell system for comparison purpose while the HepG2 and HepaRG cell lines were used as *'positive control'* with a more extensive cytochrome P450 enzyme activity. A549 cells showed a small number of up-regulated genes in basal cultures such as AKR1B10 and AKR1C2 known to be associated with the cell line's tumorigenic origin (Quinn *et al*., 2008). As expected, in pre-induced cultures CYP1A1 and CYP1B1 genes were up-regulated (260-fold and 14-fold increase respectively). Interestingly, in our study the up-regulation of these genes was not translated into enzyme activity. The lack of CYP1A1/1B1 enzyme activity has been observed previously (Newland *et al*., 2011). With respect to the results obtained for HepG2 and HepaRG cells, we observed that HepaRG express more genes involved in phase I and phase II metabolism than HepG2. Our results concur with data published previously (Gerets *et al*., 2012; Jennen *et al*., 2010).

Our data on BEAS-2B have shown a different profile to the data published recently by Courcot *et al*. They recommended BEAS-2B cells as a surrogate for metabolism and

bioactivation of toxicants in the lung, based on gene expression similarities between these cells and primary cultured cells (Courcot *et al.*, 2012). Although, our functional experiments include a subset of metabolic enzymes, our results suggest that BEAS-2B cells do not have significant phase I metabolism capabilities. The different results could be explained by variations in the culture conditions and cell origin. These protocol variations have been reported as causes of differences in phase I and phase II activities (Hewitt and Hewitt 2004). Nevertheless, while qPCR is a sensitive method to measure gene expression, not all mRNAs are translated into active proteins. There are multiple processes that could interfere with the translation and activation of proteins from mRNA one example is the emerging field of microRNA research which has shown the ability to modify the regulation of both gene expression and translation (Lee and Vasudevan 2013). Thus, mRNA level is not always correlated with protein or activity. For instance, Halladay and colleagues studied the induction of various hepatic cytochrome P450 at the mRNA, protein and activity level from different donors. For CYP1A2, the inducer rifampicin did not increased mRNA and protein levels (1.00 and 1.03-fold induction respectively), however, the activity was induced by an average of 2.55-fold (one donor's activity reaching above 4-fold induction). On the contrary, CYP3A4/5 inducer ritonavir (5 µM) increased mRNA expression by 2.5-fold but protein and activity levels were not induced (<0.3-fold induction) (Halladay *et al.*, 2012). In our study, we observed that the HepG2 cell line showed enzyme activity for both CYP1A1/1B1 and CYP2E1 (Figure 3**A** and 3**B**) but a low mRNA expression was detected in un-induced HepG2 cultures (Figure 2). The lack of correlation between activity and mRNA could be caused by post-transcriptional factors and is also a function of the protein stability. Also, it is worth noting that the mRNA expression of both CYP1A1/1B1 and CYP2E1 was upregulated in induced HepG2 cultures, the substrates used during the enzyme activity assays could have had an inducibility effect. For these reasons, key enzymatic activities should be included in

any metabolic characterization to confirm the gene expression results prior the use of the cell line for further *in vitro* toxicological testing.

In summary, we would like to outline an experimental strategy that benefits from the high throughput of qPCR but includes key functional assays (i.e. enzymatic activity).

i. Define an experimental design considering the nature of the test article, route of exposure and metabolic pathways. In our study, the experimental design was orientated towards toxicological studies on cigarette smoke toxicants.
ii. Obtain the cell systems from an established supplier such as the European Collection of Cell Cultures (ECACC) to avoid the propagation of misidentified cultures (Lacroix 2008). Furthermore, whenever the cell line is expanded in-house, simple authentication methods will confirm the authenticity of the cells (Nims *et al.*, 2010).
iii. Select a panel of pathway-relevant metabolism genes to carry out expression analysis.
iv. Assess corresponding enzyme activity.

The metabolic characterization of the cell line BEAS-2B carried out in this study will support future experimental designs, taking into account the cell system limitations. Moreover, we propose that this strategy can be applied to unravel the metabolic capabilities of other cell systems considered for *in vitro* toxicology testing.

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Chapter III

Assessment of the *in vitro* γH2AX assay by High Content Screening as a novel genotoxicity test

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Assessment of the *in vitro* γH2AX assay by High Content Screening as a novel genotoxicity test☆

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ABSTRACT

The γH2AX assay is widely used as a marker of DNA damage in multiple scientific fields such as cancer biomarker, clinical studies and radiation biology. In particular, the *in vitro* γH2AX assay has been suggested as a novel *in vitro* genotoxicity test with potential as a pre-screening tool. However, to date, limited assessments have been carried out to evaluate the sensitivity, specificity and accuracy of the *in vitro* γH2AX assay.

In this study, the microscopy-based system combining automated cellular image acquisition with software quantification for High Content Screening (HCS) has been used for the first time to evaluate the *in vitro* γH2AX assay. A panel of well-characterised genotoxic and non-genotoxic compounds was selected to assess the performance of the *in vitro* γH2AX assay in the human bronchial epithelial cell line BEAS-2B.

The results obtained during this preliminary assessment indicate that the *in vitro* γH2AX assay has a high accuracy (86%) as a result of high sensitivity and specificity (86-92% and 80-88% respectively). Our data highlight the potential for γH2AX detection in HCS as a complement to the current regulatory genotoxicity battery of *in vitro* assays. We therefore recommend more comprehensive assessments to confirm the performance of the *in vitro* γH2AX assay by HCS with a more extensive set of compounds.

1.- INTRODUCTION

The field of genetic toxicology testing in the 21st century faces two challenges recently as described by (Mahadevan *et al*., 2011); *"The first challenge is to take full advantage of new technologies to improve our ability to assess the impacts of chemically induced genetic damage. The second is to use these technologies to reliably assess new and existing chemicals for genetic toxicity potential more efficiently, cost-effectively and with less reliance on animal models"*.

In particular, current advisory bodies such as the Committee of Mutagenicity (COM) and the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), recommend two options for pre-clinical genotoxicity testing (COM, 2011; ICH, 2011) :

Option 1

i. A test for gene mutation in bacteria
ii. A cytogenetic test for mammalian chromosomal damage (*in vitro* metaphase chromosome aberration test or *in vitro* micronucleus) or an *in vitro* mouse lymphoma Tk gene mutation assay
iii. An *in vivo* test for genotoxicity, generally a test for chromosomal damage using rodent hematopoietic cells.

Option 2

i. A test for gene mutation in bacteria
ii. An *in vivo* test for genotoxicity assessment with two different tissues.

The recommended *in vitro* mammalian tests are very laborious, especially due to their manual scoring requirements. Additionally, they have the limitation of reporting a large number of "false positives" (Kirkland *et al*., 2007b). For pharmaceuticals, both options require the addition of an *in vivo* test. However, this generates an increase in animal use and might lead to delays in product development. Moreover, in the cosmetic industry, the use of animal testing is banned by the EU 7th Amendment Directive (European Commission 2003). In order to avoid unnecessary *in vivo* testing, alternative *in vitro* assays could be used as part of the weight of evidence to support non-relevant *in vitro* positives that could be negative *in vivo*, also known as "false positive" (Kirkland *et al*., 2007a). Alternative *in vitro* assays could also become part of a pre-screening high throughput filter tool to discard potential genotoxic compounds early on during development. Some of these alternative or "non-core" *in vitro* assays have been reviewed and classified into mature, maturing or emerging technologies (Lynch *et al*., 2011). A number of emerging technologies are related to the understanding of the mechanisms of DNA damage. One novel endpoint of DNA damage is the phosphorylation of the histone 2AX at serine-139 named γH2AX, in response to double-strand breaks (DSB). This early event in the DNA damage response (DDR) mechanism is triggered when DNA damage occurs to avoid genome instability and was first described by (Rogakou *et al*., 1998). Since then, the measurement of γH2AX has increasingly been used as a biomarker of DNA damage in multiple scientific fields such as *in vitro* mechanistic studies and drug development, *in vivo* DNA damage and repair studies and clinical trials (Dickey *et al*., 2009; Redon *et al*., 2011). In recent years, the automation of microscopy in cellular-based assays and the potential for higher throughput have increased its applicability as a complement to the existing battery of *in vitro* genotoxicity assays (Bickle 2010). However, the main efforts have been focused on measuring γH2AX by flow cytometry (Smart *et al*., 2011; Tsamou *et al*., 2012), even though such methodologies have been described to be less

sensitive than microscopic analysis (Banath and Olive 2003). The sensitivity of the microscopy-based method is based on the possibility to identify single foci within the cell's nucleus (Sedelnikova *et al.*, 2002). Other technical benefits include the ability to score attached cells directly from the micro-well plate instead of adding an extra step in the methodology to generate a cell suspension. Moreover, the cell sample is not destroyed during scoring, allowing cell distribution, morphology and other quality control checks to be carried out post-scoring.

In this study, microscopy has been selected as an alternative to flow cytometry for the detection of γH2AX *in vitro*. The Cellomics ArrayScan® VTI platform (ThermoScientific, USA) is an automated microscopy system that combines cellular image acquisition from multi-well microplates with software quantification analysis for High Content Screening (HCS) (Taylor 2010). The sensitivity and specificity of the *in vitro* γH2AX assay to detect genotoxic potential was assessed by testing a panel of 22 compounds that had been recommended by the European Centre for the Validation of Alternative Methods (ECVAM) and used in previous assessments of *in vitro* genotoxicity tests (Kirkland *et al.*, 2008; Hastwell *et al.*, 2006). The compounds selected represent different mechanism of action that could lead to the formation of DNA damage in the form of DSB directly or indirectly as previously described (Bonner *et al.*, 2008). Bleomycin sulphate, for example, was used as reactive oxygen species generator known to directly produce DSBs in the DNA. Etoposide, on the other hand, is a topoisomerase II inhibitor that increases the number of topoisomerase-DNA complexes causing an increase in collisions between the complexes and the replication forks. The collisions result in DSB by an indirect mechanism.

Selecting an appropriate cell line could also affect the sensitivity of the assay. Recently published recommendations on cell line selection suggest the use of human p-53 competent

cell lines to reduce the incidence of "false positives" (Fowler *et al*., 2012). To develop the assay, we selected the normal phenotype-derived BEAS-2B cells obtained from non-cancerous lung epithelium cells and immortalised by (Reddel *et al*., 1988). When measuring γH2AX, cell lines derived from normal tissues, such as BEAS-2B cells, are considered to be more reliable than those from pre-malignant and cancer tissues. Tumorigenic cell lines have an impairment to functions such as the DNA damage repair mechanisms and cell cycle regulation (Hanahan and Weinberg 2000). The phosphorylation of H2AX is an early event in DNA damage response and is also involve in cell cycle progression (Fernandez-Capetillo *et al*., 2004; Downey and Durocher 2006), therefore, malignant transformation could affect the γH2AX response. For instance, there have been reports showing significant amount of γH2AX in the absence of DSB, possibly as a response to "oncogenic stress" (Svetlova *et al*., 2010). BEAS-2B cells, however, have been reported to have limited metabolic capabilities reducing its ability to detect compounds that require metabolic activation i.e. pro-toxicants (Garcia-Canton *et al*., 2013). Therefore, this study included the addition of a standard rat-derived subcellular fraction as external source of metabolic activation.

The preliminary results obtained were used to define the sensitivity and specificity of the assay using previously recommended criteria (Kirkland *et al*., 2008). The results were also compared with existing *in vitro* γH2AX relevant data published in the scientific literature. Overall, the *in vitro* γH2AX assay by HCS has shown potential as an *in vitro* genotoxicity assay that could be included as a complementary tool to the existing battery of *in vitro* regulatory genotoxicity assays. Moreover, the high throughput technology and reproducibility of the *in vitro* γH2AX assay by HCS could be a very useful tool in pre-screening for *in vitro* genotoxicity.

2.- MATERIALS AND METHODS

2.1.- Chemicals

All compounds were of the highest purity available from Sigma-Aldrich (United Kingdom) except MNNG (Toronto Research Chemicals, Canada). They were dissolved in an appropriate vehicle and further dilute to a final concentration of 1% v/v vehicle in cell culture medium. Selection of doses and treatment times followed current ICH regulatory guidelines (ICH, 2011). Therefore, the maximum concentration tested was 1mM unless precipitation was observed.

2.2.- Cell culture

The human bronchial epithelial cell line BEAS-2B, purchased from ATCC (USA), was seeded into culture vessels that had been pre-coated with 0.03 mg/mL PureCol® bovine collagen solution (Nutacon, The Netherlands). Cells were maintained in Bronchial Epithelial Growth Medium (BEGM®) at 37°C and 5% CO_2 in a humidified incubator. BEGM® was prepared by supplementing Bronchial Epithelial Basal Medium with growth supplements provided in the manufacturer's BEGM® SingleQuot® kit (Lonza Group Ltd., Belgium) containing: bovine pituitary extract, hydrocortisone, human epidermal growth factor, epinephrine, insulin, triiodothyronine, transferrin, gentamicin /amphotericin-B and retinoic acid. BEAS-2B cells were cultured and expanded in-house. All cultures were negative for mycoplasma. Additionally, the cells were authenticated using the short tandem repeat profiling to confirm the nature of the cell cultures (LGC Standards, United Kingdom) (Nims *et al*., 2010).

2.3.- Treatments

The day before the treatment, BEAS-2B cells were seeded into 96-well, black, clear-bottom microplates (PerkinElmer, United Kingdom) at a concentration of $8x10^4$ cells/mL.

Cells were maintained at 37°C in an atmosphere of 5% CO_2 in air and tested with compounds for either 3 hour or 24 hour. For compounds that required metabolic activation, the treatment was for 3 hour with and without the metabolic activation system. Since some of these compounds are known to bind DNA, a 24 hour recovery period treatment was also included in the experimental design to quantify potentially delayed DNA damage (3+24 hour).

The external source of metabolic activation used in this study was rat hepatic S9 (Aroclor 1254-induced animals) (Moltox, USA), used as the standard system for metabolic activation in regulatory *in vitro* assays.

The preparation of the S9 mix (0.5% v/v final concentration in medium) was carried out in accordance with the suppliers' instructions. The concentration of subcellular fraction used was below the typically used range (1-10% v/v) due to an intrinsic cytotoxicity observed in BEAS-2B cells. Briefly, Reagent A (Moltox, USA) a mixture of phosphate-buffered salt solution and glucose-6-phosphate and Reagent B (Moltox, USA) containing NADP were mixed at a proportion of 1:3.4 creating a clear solution named co-factor mix. The co-factor mix was then used to dilute the rat S9 to a 10% v/v intermediate solution (S9 mix). The intermediate solution was further diluted in BEGM® media to prepare the treatment solution containing the test compound.

2.4.- Immunostaining

Following treatment, the compound or recovery media was aspirated from the cells and the plates were processed for γH2AX immunostaining following manufacturer's recommendations (ThermoScientific, USA). Briefly, cells were fixed with 4% paraformaldehyde (100 µL/well) and incubated for 15 minutes at room temperature. After fixation, plates were washed twice with 100 µL/well phosphate buffered saline (PBS) (0.14 M sodium chloride, 0.003 M potassium chloride, 0.002 M potassium phosphate and 0.01 M sodium phosphate). The cells were then permeabilised for 15 minutes by incubating them at room temperature with 100 µL/well of permeabilisation buffer (PBS with 1% Triton X-100). After permeabilisation, the cells were washed twice with 100 µL/well of PBS. Blocking buffer (ThermoScientific proprietary formulation supplemented with 2% fetal bovine serum) (100µL/well) was then added for 15 minutes at room temperature to avoid unspecific antibody binding. The blocking buffer was aspirated and 50 µL primary antibody solution (0.1% γH2AX primary antibody in PBS) was added to each well and plates were incubated for 1 hour at room temperature. After the incubation, the primary antibody solution was aspirated and plates washed twice with PBS II (PBS with 1% Tween-20) followed by one wash with PBS. Then, 50 µL secondary antibody (DyLight^{TM} 549 conjugated goat anti-mouse IgG) containing Hoechst dye DNA staining solution (0.2% secondary antibody and 0.01% Hoechst in PBS) was added to each well and plates were incubated for 45 minutes at room temperature in the dark. After the incubation, the wells were washed twice with PBS II followed by one wash with PBS. Finally, the wells were filled with 200 µL of PBS and sealed for scanning. Plates were stored at 4°C after the scan or while awaiting the scan.

2.5.- Imaging analysis

Images from the wells at 20x magnification were taken using the Cellomics Arrayscan® VTI platform (Thermo Scientific, USA) and analysed with the Target Activation Bioapplication software V.6.6.1.4.

The algorithm parameters for object selection was set to count a minimum of 250 cells per well, giving a minimum of 1500 cells per concentration tested. Two different nuclear stains were measured. Nuclear DNA fluorescence intensity (Hoechst dye) was assessed in channel 1 to identify viable cells. Micrographies were obtained with a fixed exposure time of 0.04 seconds. Object area and shape parameters were defined during the algorithm set up to fit the specific nuclei morphology of the cell line, generating the criteria to define valid nuclei. The valid nuclei from channel 1 showed a green overlay that served as the target to measure the fluorescence intensity of channel 2. Rejected nuclei from channel 1 showed an orange overlay and no further measures are taken from them. No cell rejection in channel 1 was based on fluorescence intensity.

Channel 2 measures whole nuclei fluorescence intensity of the secondary antibody (DyLight^{TM} 549) in valid nuclei. Micrographies were obtained with a fixed exposure time of 0.06 seconds. All intensities are automatically calculated by the software per pixel in the identified target area of every valid object.

With all the micrographies parameters quantified, the software generated a number of measurements to report, in this study we selected two for their relevance to the γH2AX endpoint:

“Selected Object Count per Valid Field” is the average number of objects (viable cells) selected for analysis per valid field in the well. These data were obtained from the identified

nuclei in channel 1. Untreated wells would have a higher number of nuclei per field while toxic doses would have lower number of nuclei per field. These data were converted into cell viability data. The vehicle treated counts were defined as 100% cell viability. The cell counts in the compound treated wells were then compared to those in the vehicle-treated wells, and the percentage cell viability was calculated. This comparison against the vehicle control data is referred to as Relative Cell Counts (RCC) and expressed as percentage.

"Mean average intensity in channel 2" is the average intensity in channel 2 of all pixels within the overlay mask identified in valid nuclei in channel 1. The mean average intensity is measured in intensity units and was reported as γH2AX frequency (intensity units) in this study. This measurement relates to the number of DSBs present in the cells.

2.6.- Data analysis and criteria

All experiments were repeated at least 3 independent times. The results for both γH2AX intensity and RCC for all tested compounds were graphically represented using GraphPad Prism software v.6

The evaluation criteria for the *in vitro* γH2AX assay selected in this study is described in Table 1 and was previously presented by Smart *et al.* (Smart *et al.*, 2011).

Table 1: Genotoxicity evaluation criteria for the *in vitro* γH2AX assay, adapted from (Smart *et al*., 2011).

γH2AX response	**Classification**
> 1.5-fold γH2AX @ RCC > 25%	Genotoxic (+)
< 1.5-fold γH2AX @ RCC 100-0%	Non-genotoxic (–)
> 1.5-fold γH2AX @ RCC < 25%	"False" positive; Cytotoxic-driven genotoxicity (C)
1.5-fold γH2AX @ RCC ≥ 25%	Equivocal (±)

The *in vitro* to *in vivo* predictivity of the assay was defined by the calculation of the "Sensitivity", "Specificity" and "Accuracy" defined previously by Kirkland *et al.* (Kirkland *et al.*, 2005). The sensitivity is defined as the ability of the assay to detect *in vitro* genotoxic compounds. These compounds should give a positive response in *in vitro* mammalian cell genotoxicity tests. They are also *in vivo* genotoxic compounds and are often classified as carcinogenic compounds (Kirkland *et al*., 2008).

Specificity, on the other hand, is defined as the ability of the assay to discriminate non-genotoxic compounds. These compounds should give a negative response in *in vitro* mammalian cell tests. When there is data available in *in vivo* genotoxicity testing, the results are usually negative. They are often non-carcinogenic or if carcinogenicity is observed *in vivo*, the mode of action assumed is non-mutagenic (Kirkland *et al*., 2008).

Some of the compounds tested are classified as artifactual *in vitro* genotoxic positives (false positive). These compounds should give a negative response in *in vitro* mammalian cell tests but often have been reported to produced positive genotoxicity, especially at high cytotoxic concentrations. They are commonly negative in *in vivo* gentotoxicity tests (Kirkland *et al*., 2008).

Sensitivity = A/B

Specificity = C/D

Accuracy = (A+C)/(B+D)

where A is the number of *in vivo* genotoxic compounds that tested positive in the *in vitro* γH2AX assay evaluated in this study, B is the total number of *in vivo* genotoxic compounds tested. C is the number of *in vivo* non-genotoxic compounds that tested negative in the *in vitro* γH2AX assay, and D is the total number of *in vivo* non-genotoxic compounds tested.

3.- RESULTS

The assessment of the *in vitro* γH2AX assay by HCS was performed by treating BEAS-2B cells with different control compounds. The increase of histone 2AX phosphorylation (γH2AX) labelled by immunostaining and detected by automated fluorescence microscopy was used as an indication of DNA double strand breaks. An illustration γH2AX detection is given in Figure 1 after 3 hour treatment of the cells with etoposide.

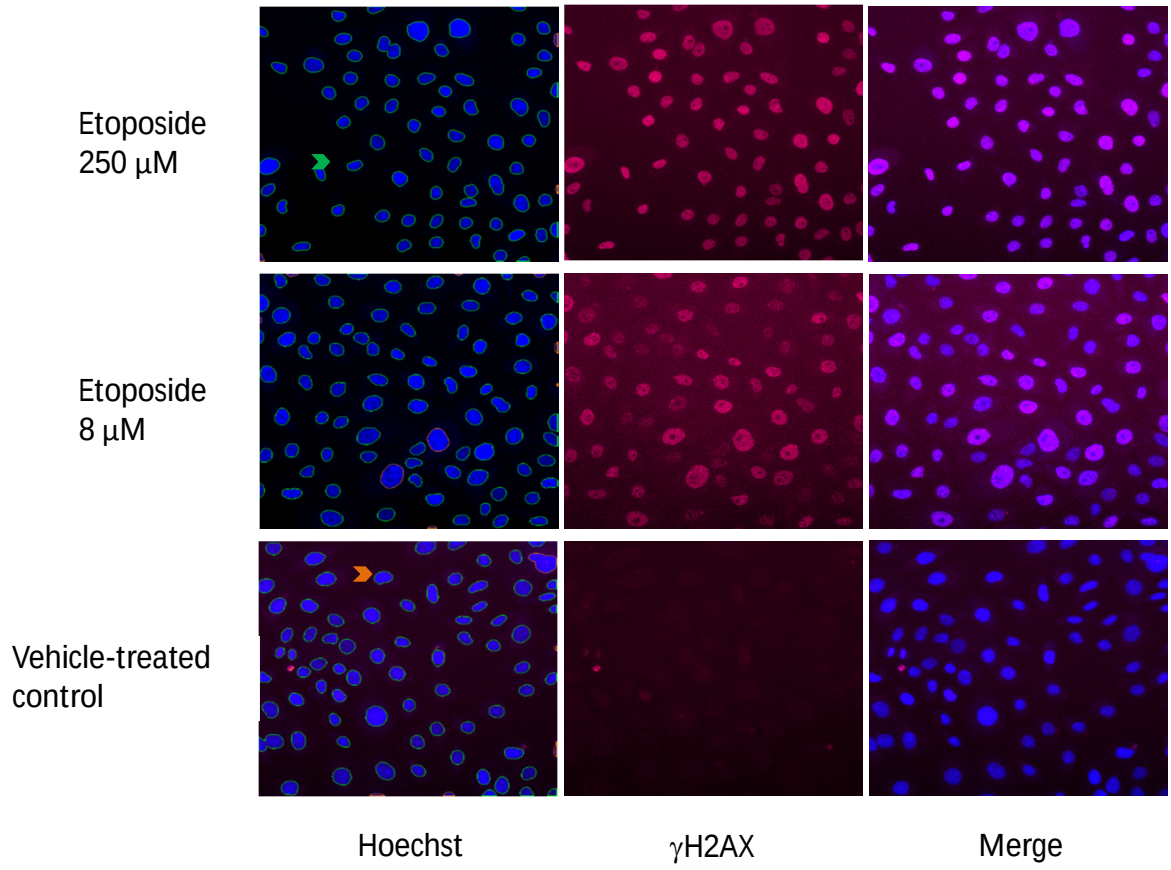

Figure 1: Example of γH2AX detection after 3 hour treatment with one of the genotoxic compounds, etoposide. The nuclei of the cells were stained with Hoechst and γH2AX with a combination of primary anti-γH2AX and secondary fluorophore-conjugated antibody. Hoechst micrographs indicate with a green overlay the scored nuclei (green arrow) and orange overlay the rejected nuclei (orange arrow).

For this study, we selected a series of genotoxic compounds with different mechanisms of action in order to have a comprehensive selection of DNA-damaging agents. Direct DNA-damaging compounds interact directly with the DNA causing different degree of DNA damage ranging from point mutations to crosslinks. Ethyl methanesulfonate, mitomycin C and methylnitronitrosoguanidine (MNNG) were used in this study as they interact directly with the DNA. Similarly, reactive oxygen species (ROS) generators such as bleomycin sulphate, also used here, generate ROS molecules that have the potential to oxidise DNA. Some direct DNA-damaging compounds have to be metabolically activated before their toxic form is active, these compounds are known as pro-toxicants. The limitations of some cell systems used in *in vitro* toxicological testing to activate these compounds implies that external sources of metabolic activation would be required to carry out subsequent endpoint analysis. Benzo(a)Pyrene, cyclophosphamide, 2-acetylaminofluorene and aflatoxin B1 were used as pro-toxicant models during this study.

Indirect DNA-damaging compounds interfere with cellular functions involved in DNA repair, synthesis or mitosis. Damage in these fundamental functions could ultimately translate into DNA damage. In this assessment, amsacrine and etoposide were used as topoisomerase inhibitors while aphidicolin and zidovudine were used as DNA synthesis inhibitors.

Aneugenic compounds affect mitosis mechanisms such as microtubules synthesis and cytoeskeleton morphology. These changes could lead to aneuploidy in daughter cells (abnormal chromosomal number), cell cycle deregulation and so on, rather than actual DNA damage. These compounds are included here in the non-genotoxic carcinogenic class. In this study, colchicine and nocodazole were used as model compounds.

Artifactual *in vitro* compounds typically show a positive response in some *in vitro* genotoxicity assays but are not genotoxic *in vivo*. False positive responses are commonly

produced when toxic concentrations are tested (Kirkland *et al*., 2007b; Kirkland *et al*., 2008). Here, 2,4-dichlorophenol and D,L-menthol were used as examples. Negative *in vitro* genotoxic compounds selected have been extensively tested, consistently giving negative results for genotoxicity or carcinogenicity both *in vitro* and *in vivo* (Kirkland *et al*., 2008; Hastwell *et al*., 2006). Acetonitrile, 3-amino-1,2,4-triazole, dimethyl formamide, ethylene glycol, D-mannitol and sodium chloride were chosen as model negative compounds for this work.

The sensitivity and specificity of the *in vitro* γH2AX assay has been calculated by the number of *in vitro* genotoxic and non-genotoxic compounds that the assay could discriminate during this assessment as previously defined (Kirkland *et al*., 2008; Kirkland *et al*., 2005).

3.1.- *In vitro* γH2AX assay assessment

Table 2 summarised the results obtained during the assessment of the *in vitro* γH2AX assay by HCS performed in this study on BEAS-2B cells using Smart *et al.* evaluation criteria (Table 1) (Smart *et al*., 2011). For comparison purposes we have included published data available on regulatory *in vitro* genotoxicity tests as well as other γH2AX studies where these control compounds have been used.

Table 2: Control compounds tested in the *in vitro* γH2AX assay by High Content Screening in 96-well plates, and data from published data on regulatory *in vitro* genotoxicity assays and other γH2AX studies. Positive results (+), negative (–), equivocal (±), cytotoxic (C).

Compound (CAS number)	**IARC Group**	**Regulatory *in vitro* tests (refs)***		**Other γH2AX**	**γH2AX by HCS**
		Ames	***In vitro* mammalian test**		
Direct DNA-damaging compounds					
Ethyl methanesulfonate (62-50-0)	2B	+	+	+ (Smart *et al*., 2011)	+ (500 µM)
Mitomycin C (50-07-7)	2B	±	+	+ (Smart *et al*., 2011)	+ (0.47 µM)
MNNG (70-25-7)	2A	+	+	+ (Staszewski *et al*., 2008)	+ (0.49 µM)
Pro-toxicant compounds					
Benzo[a]pyrene (50-32-8)	1	+	+	+ (Smart *et al*., 2011)	+ (7.8 µM)
Cyclophosphamide (6055-19-2)	1	+	+	+ (Smart *et al*., 2011)	+ (1000 µM)
2-Acetylaminofluorene (53-96-3)	N/A	+	+	+ (Smart *et al*., 2011)	–
Aflatoxin B1 (1162-65-8)	1	+	+	+ (Gursoy-Yuzugullu *et al*., 2011)	+ (4 µM)
Reactive Oxygen Species Generator					
Bleomycin 162ulphate (9041-93-4)	2B	+	+	+ (Kim *et al*., 2011)	+ (0.019 µM)
Topoisomerase inhibitors					
Amsacrine (m-AMSA) (54301-15-4)	2B	+	+	N/A	+ (0.2 µM)
Etoposide (33419-42-0)	1	–	+	+ (Matsuzaki *et al*., 2010)	+ (0.24 µM)
Nucleotide/DNA synthesis inhibitors					
Aphidicolin (38966-21-1)	N/A	–	+	+ (Tanaka *et al*., 2007)	+ (1.56 µM)
Zidovudine (30516-87-1)	2B	+	+	N/A	+ (125 µM)
Aneugen compounds					
Colchicine (64-86-8)	N/A	–	±	N/A	+ (7.8 µM)
Nocodazole (31430-18-9)	N/A		+	+ (Dalton *et al*., 2010)	C
Artifactual *in vitro* genotoxic positives (false positive)					
2,4-Dichlorophenol (120-83-2)	N/A	±	±	N/A	+ (1000 µM)
D,L-menthol (15356-70-4)	N/A	–	±	N/A	–

In vitro genotoxic negatives					
Acetonitrile (75-05-8)	N/A	–	±	N/A	–
3-Amino-1,2,4-triazole (61-82-5)	3	–	–	N/A	–
Dimethyl formamide (68-12-2)	3	–	–	N/A	–
Ethylene glycol (107-21-1)	N/A	–	–	N/A	–
D-Mannitol (69-65-8)	N/A	–	–	– (Smart *et al*., 2011)	–
Sodium chloride (7647-14-5)	N/A	–	–	– (Matsuzaki *et al*., 2010)	–

* (United State National Library of Medicine, 2013; Department of Health and Human Services, 2013; IARC, 2000; IARC, 1987)

Figure 2 illustrates a summary of the graphical results obtained during the assessment of the *in vitro* γH2AX assay. The data represented is expressed as mean fluorescence (γH2AX frequency) ± Standard Deviation (SD) of three separate experiments to show the reproducibility between experiments. The RCC is expressed as a percentage of the vehicle-treated control calculated for the three separate experiments. The X-axis represents the compound concentration (μM) in logarithmic scale, the left Y-axis represents the γH2AX frequency in absolute units (intensity units) and the right Y-axis represents the cell viability as RCC in percentage relative to vehicle-treated control. In those selected examples, the direct-DNA damaging compound, Ethyl methanesulfonate (Figure 2A) produced a statistically significant increase in the γH2AX frequency compared to vehicle-treated control at concentrations above 500 μM after treatment for 24 hour without a reduction in cell viability below 80%. Ethyl methanesulfonate is typically used in DNA damage and repair studies for its mutagenic activity (Munroe and Schimenti 2009). In contrast, the treatment with the pro-toxicant B[a]P in the presence of aroclor-induced rat S9 mix (Figure 2B) produced a significant increase in γH2AX frequency at concentrations above 8 μM at 3 hour (RCC > 80%) and concentrations above 16 μM at 3 hour followed by 24 hour recovery

period (RCC > 60%). In the absence of S9 mix B[a]P did not produce any increase in γH2AX frequency above vehicle-treated controls at either timepoint.

B[a]P is a polycyclic aromatic hydrocarbon present in cigarette smoke which is oxidised by cytochrome P450 enzymes, primarily CYP1A1 but also CYP1A2 and CYP1B1.

Treatment for 3 hour with ROS generator, bleomycin sulphate (Figure 2C), produced a statistically significant increase in γH2AX frequency at concentrations above 0.3 μM without a reduction in cell viability (RCC > 95%). However, treatment for 24 hour produced an increase in γH2AX frequency above 1.5-fold at concentrations above 0.02 μM with a dose-related decrease in cell viability (RCC from 65% to 33%).

Both, topoisomerase and DNA synthesis inhibitors, etoposide and zidovudine, produced a gentoxic response in the *in vitro* γH2AX assay. Etoposide treatment (Figure 2D) produced a positive response after 3 hour at concentrations above 7.8 μM without a reduction in cell viability below 80%. The range of concentrations used for the 24 hour treatments was lower as high cytotoxicity was observed in the initial range finder (data not shown). Treatment with etoposide for 24 hour produced a positive response at concentrations above 0.24 μM with a cell viability of 75%. A dose-related decrease in cell viability was observed after 24 hour treatment when etoposide concentration increased (supplementary data). Contrarily, only treatment for 24 hour with zidovudine (Figure 2E) produced a significant increase in γH2AX frequency at concentrations above 125 μM with a cell viability above 65%.

The aneugenic compound, colchicine (Figure 2F), produced a significant increase in γH2AX frequency compared to vehicle-treated controls after 24 hour continuous treatment at concentrations above 7.8 μM (RCC > 25%). However, as the concentration increased, there

was a reduction in γH2AX frequency and at concentrations above 250 μM the γH2AX frequency was equivalent to background response (< 1.5-fold).

The artifactual genotoxic compound 2,4-dichlorophenol generated a significant increase in γH2AX frequency at the highest dose tested (1000 μM) after both 3 and 24 hour treatments (Figure 2G). There was no significant reduction in cell viability after the 3 hour treatment (RCC > 95%), however, after 24 hour treatment, there was a dose-related reduction in cell viability from 100% to 40% RCC.

Treatments with the non-genotoxic D-mannitol (Figure 2H) did not produce any significant increase in γH2AX frequency compared to vehicle-treated controls at any timepoint. The cell viability was also maintained at around 100% RCC at all timepoints.

Overall, the majority of tested compounds, both *in vitro* genotoxic and non-genotoxic, produced the expected increment in γH2AX frequency (above and below 1.5-fold increase respectively). All the results are graphically represented in the supplementary data and discussed in more detail in the *in vitro* γH2AX assay performance section.

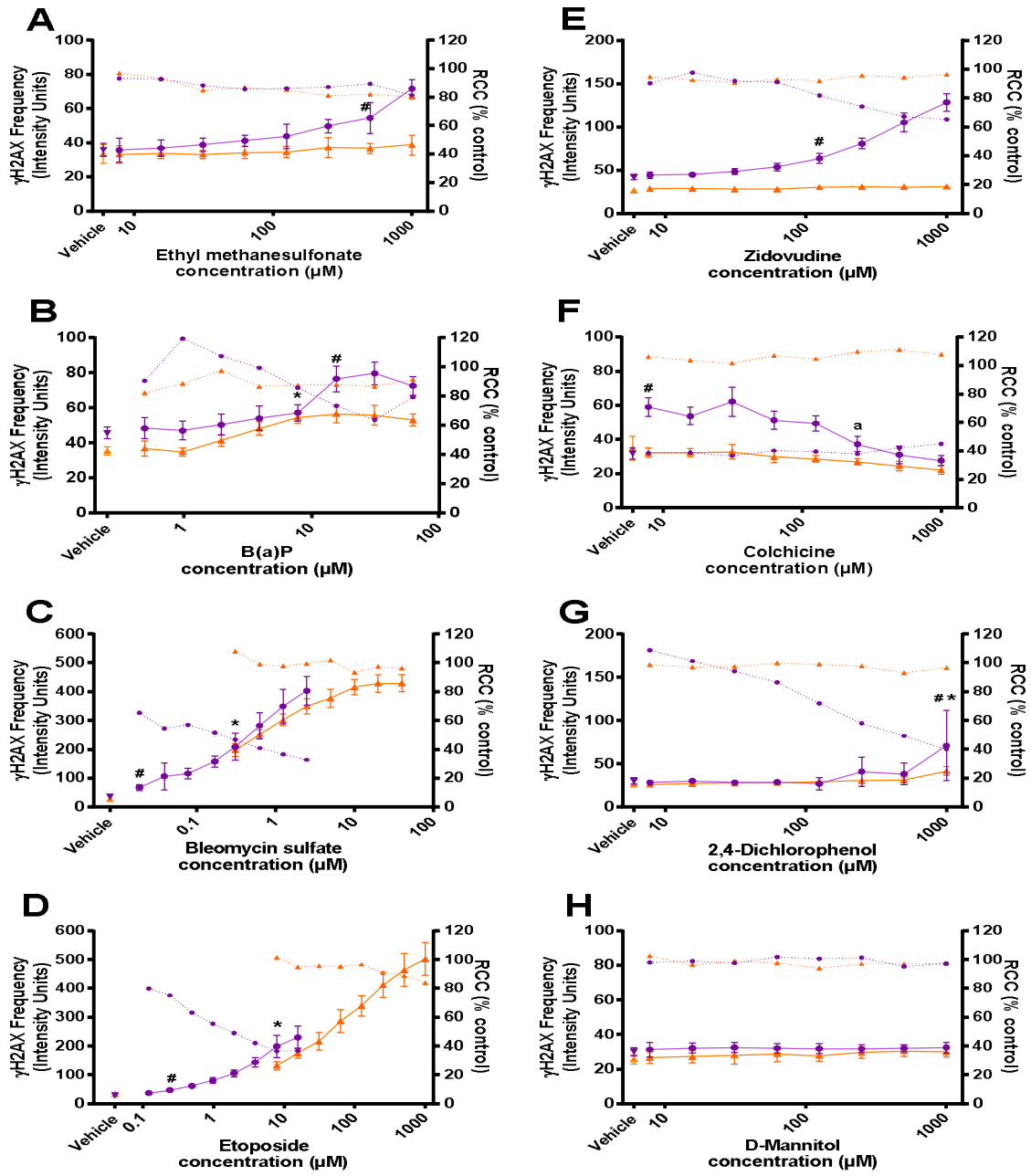

Figure 2: Example data from the assessment of the *in vitro* γH2AX assay. Left Y-axis represents γH2AX frequency (continuous line) and right Y-axis represents RCC (% control) (dotted line). Compounds: [A] Ethylmethane sulfonate, [B] B[a]P, [C] bleomycin sulphate, [D] etoposide, [E] zidovudine, [F] colchicine, [G] 2,4-dichlorophenol and [H] D-mannitol. Triangle (-▲-) represents short treatment and circle (-●-) represents long treatment. Asterisk (*) indicates the minimum concentration that shows genotoxicity compared to the vehicle treated control after short treatment. Hash (#) indicates the minimum concentration that shows genotoxicity compared to the vehicle treated control after long treatment.

3.2.- *In vitro* γH2AX assay performance

The results obtained in the *in vitro* γH2AX assay assessment were used to calculate the sensitivity, specificity and accuracy of this single assay to predict *in vivo* genotoxicity (Kirkland *et al.*, 2005).

The sensitivity (or ability to detect genotoxic compounds) calculated for this preliminary assessment showed that 91.6% of genotoxic compounds could be detected if aneugenic compounds are considered non-genotoxic. When aneugenic compounds were considered as indirect-acting genotoxic compounds, the sensitivity fell to 85.7%. For specificity (or ability to discriminate non-genotoxic compounds), this preliminary assessment indicated an 80% specificity if aneugenic compounds are considered non-genotoxic or 87.5% if aneugenic compounds are considered to have genotoxic potential.

The accuracy of the *in vitro* γH2AX assay by HCS in this preliminary assessment is therefore, calculated to be 86.4%, independent of the aneugenic compound classification.

To put our performance results in context with existing *in vitro* regulatory genotoxicity tests, we have evaluated previously published data for the 22 compounds tested in this study (Table 2) to calculated the sensitivity, specificity and accuracy of the current regulatory *in vitro* tests. The results in Table 3 have been calculated considering aneugens as both genotoxic and non-genotoxic compounds.

Table 3: Performance results based on the 22 compounds tested in this study.

Results	Ames test	*In vitro* Mammalian test	γH2AX by HCS
Aneugens considered genotoxic compounds			
No. of genotoxic compounds clearly positive (A)	9	13	12
No. of genotoxic compounds tested (B)	14	14	14
Sensitivity (%)	**64%**	**93%**	**86%**
No. of non-genotoxic compounds clearly negative (C)	7	5	7
No. of non-genotoxic compounds tested (D)	8	8	8
Specificity (%)	**88%**	**63%**	**88%**
Accuracy (%)	**73%**	**82%**	**86%**
Aneugens considered non-genotoxic			
No. of genotoxic compounds clearly positive (A)	9	12	11
No. of genotoxic compounds tested (B)	12	12	12
Sensitivity (%)	**75%**	**100%**	**92%**
No. of non-genotoxic compounds clearly negative (C)	8	5	8
No. of non-genotoxic compounds tested (D)	10	10	10
Specificity (%)	**80%**	**50%**	**80%**
Accuracy	**77%**	**77%**	**86%**

4.- DISCUSSION AND CONCLUSIONS

The *in vitro* γH2AX assay has been previously proposed as a potential complement to the existing battery of *in vitro* genotoxicity assays (Garcia-Canton *et al.*, 2012). Although, measuring γH2AX as a biomarker of DNA damage is commonly used in current scientific research, there is a limited number of studies focused on assessing the sensitivity and specificity of the *in vitro* γH2AX assay (Matsuzaki *et al.*, 2010; Smart *et al.*, 2011; Tsamou *et al.*, 2012). More data is needed to support its move from emerging to maturing technology in genetic toxicology testing as described previously (Lynch *et al.*, 2011). To date, the main system employed to measure γH2AX has been flow cytometry (Smart *et al.*, 2011; Tsamou *et al.*, 2012). Here, we present the first study where automated microscopy-based HCS in multi-well microplates has been used to assess the sensitivity, specificity and accuracy of the *in vitro* γH2AX assay.

Although flow cytometry incorporates high throughput qualitative scoring, the sensitivity of detection for γH2AX has been described to be lower than in microscopy (Banath and Olive 2003). Additionally, flow cytometry scoring requires cells to be in suspension; however, the majority of cell lines used in *in vitro* toxicology testing are adherent cells. The requirement for a cell suspension increases the time and complexity of the methodology as a trypsin-mediated detachment step will be required. Moreover, the use of trypsin have been shown to affect the cell viability and can cause DNA damage during detachment (Wischermann *et al.*, 2007). In contrast, HCS applies the sensitivity of microscopy with an automated high-throughput system for acquiring cellular images and software to aid with the qualitative scoring. Other benefits include the scoring of samples *in situ* in multi-well plates, elimination of the detachment step and storage of images as raw data for quality control purposes. HCS methods could also incorporate cell cycle information if required by measuring DNA content

or multiplexing nuclei dyes (Lyman *et al.*, 2011; Chan *et al.*, 2013). An overview of the technology with current capabilities, limitations and potential developments has been published by Bickle (Bickle 2010).

The genotoxicity assessment of the *in vitro* γH2AX assay performed in this study involved the treatment of the phenotypically-normal cell line BEAS-2B to a panel of genotoxic and non-genotoxic compounds following ICH guidelines (ICH, 2011). One of the major limitations of current *in vitro* assays is the rate of "false" or misleading positives, non-carcinogenic compounds that produce a positive response *in vitro* (Kirkland *et al.*, 2007b). In this study, all the well-established *in vitro* genotoxic negative compounds tested produced a negative response at both treatment timepoints (3 and 24 hour). Of the two previously reported artifactual *in vitro* genotoxic positive compounds, D,L-menthol produced a clear negative result. By contrast, 2,4-dichlorophenol produced a positive response at the highest concentration tested after both 3 and 24 hour treatments, however, this statistically significant increase in γH2AX frequency was only achieved in one experiment. The same inconsistent response has been reported for 2,4-dichlorophenol in other *in vitro* genotoxicity studies and summarised recently (Fowler *et al.*, 2012). Although there is no evidence for carcinogenicity of 2,4-dichlorophenol *in vivo*, there have been some studies indicating endocrine disruption in the zebra fish model (Ma *et al.*, 2012). The hormonal imbalance caused by 2,4-dichlorophenol could potentially elevate the levels of estrogens, an endogenous genotoxic agent. These events suggest that 2,4-dichlorophenol have an indirect DNA-damage effect caused by hormonal imbalance (Cavalieri *et al.*, 2000; Amer and Aly 2001).

The acceptance criteria described for the γH2AX assay by flow cytometry (Smart *et al.*, 2011) were applied to our data to evaluate the relevance of using the same approach for the γH2AX assay by HCS. Our data showed that all increments in γH2AX frequencies over the vehicle-

treated control observed were also identified by the 1.5-fold increase “cut off” criterion defined by Smart and colleagues. The RCC levels selected as a measure of cytotoxicity was the second criterion employed to monitor that increases in γH2AX frequencies were not caused solely by a toxic effect.

The performance of the *in vitro* γH2AX assay by HCS to detect direct-acting genotoxic compounds showed a high sensitivity. Only the pro-toxicant 2-acetylaminofluorene (2AAF) failed to show a positive response. 2AAF is an aromatic amine not classified by IARC regarding its carcinogenicity. However, the IARC monograph describing some aromatic amines refers to its mechanism of action as formation of DNA-adducts by different metabolites (IARC, 2010). 2AAF is mainly activated by CYP1A2 present in the standard rat S9 mix. Although, in this study 2AAF show a dose-related increase in γH2AX frequency, the response was never greater than 1.5-fold. In a previous study, Zhou and colleagues reported that the ability of 2AAF to induce DSBs depends on the cell type tested (Zhou *et al*., 2006). They obtained a positive response at 90 µM in Chinese hamster CHL cells after short treatments only, while no positive response was observed in human ambion FL cells at any timepoint. Similarly, Tsamou *et al*. report a positive response at 50 µM after 24 hour treatment in HepG2 cells, known to have a degree of metabolic competency (Tsamou *et al*., 2012). Smart *et al*. report a positive response at a concentration above 125 µM after 3 hour treatment in the presence of 1% v/v of rat S9 mix (Smart *et al*., 2011). In our experiments, we tested 2AAF up to a concentration of only 125 µM as precipitation was observed at higher doses. However, in our experimental design for the testing of pro-toxicants, the limiting factor was the inherent toxicity produced by the rat S9 to the cells. At the minimum concentration of 1% v/v S9, typically used *in vitro*, the cell viability fell below 55%, which is not compatible with robust testing for DNA damage. The reduction in concentration to 0.5%

v/v S9 maintained the cell viability above 80% but could have compromised the metabolic activation of 2AAF.

In the case of indirect-acting genotoxic compounds, the *in vitro* γH2AX assay performance was also highly sensitive. The cellular targets affected by these compounds are essential for the DNA synthesis and repair, both functions are intrinsic of the cell cycle. All the indirect-acting genotoxic compounds tested in this study produced a positive response in the *in vitro* γH2AX assay.

During the *in vitro* γH2AX assay assessment, we also tested two aneugenic compounds. We define these as non-genotoxic carcinogens as they can cause aneuploidy (Aardema *et al*., 1998). Aneugenic compounds do not damage DNA directly, instead, they affect cellular mechanisms involve in the cell cycle process to the point of forcing the cell to programmed death i.e. apoptosis (Yamada and Gorbsky 2006). In this assessment, nocodazole produced a genotoxic response at highly cytotoxic concentrations (RCC < 25%) after 24 hour treatment. This effect is considered an artifactual genotoxic positive result (Scott *et al*., 1991). Colchicine, on the other hand, produced a genotoxic response after 24 hour treatment with cell viability around 40%. These contrasting results could be influence by the different levels of cytotoxicity exerted by the compounds. In general, treatments with aneugenic compounds could lead to DNA damage by products liberated to the culture media during cell death and other mechanisms of toxicity (Scott *et al*., 1991). Therefore, a small degree of clastogenicity could be observed after treatment with aneugenic compounds. This effect could lead to an increase in γH2AX frequency above the vehicle-treated control when aneugens are tested, and potentially an additional mechanism of indirect DNA damage. Increases in γH2AX have been associated to other mechanisms such as apoptosis (Mukherjee *et al*., 2006; Darzynkiewicz *et al*., 2012). Briefly, during a late-stage event of apoptosis, fragmentation of

cellular DNA could produce an increase in the frequency of γH2AX followed by a reduction of both γH2AX levels and cell viability as a result of the programmed cell death. Thus, it is considered important to report both γH2AX frequency and cell viability data.

The performances of other *in vitro* regulatory assays calculated from the panel of compounds tested in this study (Table 3), showed that the Ames test is better at discriminating negative compounds (specificity). Other *in vitro* mammalian tests (i.e. Micronucleus test, mouse lymphoma test etc.) are better at detecting positive compounds (sensitivity). Our observations concur with more extensive assessments such as the evaluation carried out by Kirkland *et al.* with over 800 compounds (Kirkland *et al*., 2005). The performance of the *in vitro* γH2AX assay by HCS obtained in this preliminary assessment shows a consistently high level of both sensitivity and specificity giving as a result a greater accuracy than the other *in vitro* tests. It is as yet not possible to compare the sensitivity and specificity of the *in vitro* γH2AX assay by HCS with previously reported methods such as flow cytometry. Only a limited subset of the reference compounds have been used across all studies (Table 2). However, it would be interesting for future studies to perform a parallel comparison between HCS and flow cytometry using the same experimental conditions e.g. cell lines.

Overall, the *in vitro* γH2AX assay by HCS results obtained during this preliminary assessment are in line with previously published evaluations (Smart *et al*., 2011; Tsamou *et al*., 2012; Audebert *et al*., 2010). However, we believe that the use of HCS for the scoring of micro-well plates and detecting γH2AX may offer advantages in terms of higher throughput and sensitivity. Initially, the reproducibility and accuracy of the data gathered supports the use of the *in vitro* γH2AX assay as a complementary tool to the existing battery of genotoxicity assays, principally as a pre-screening assay. Nevertheless, a more comprehensive assessment including a more extensive set of compounds would be required

to confirm the performance of the *in vitro* γH2AX assay by HCS and to provide further support for future validation trials.

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Chapter IV

Genotoxicity evaluation of individual cigarette smoke toxicants using the *in vitro* γH2AX assay by High Content Screening

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Genotoxicity evaluation of individual cigarette smoke toxicants using the *in vitro* γH2AX assay by High Content Screening☆

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ABSTRACT

Cigarette smoke is a complex mixture consisting of more than 5600 identified chemical constituents of which approximately 150 have been identified so far as "tobacco smoke toxicants". Proposals made by the World Health Organisation Framework Convention on Tobacco Control mandate the lowering of nine tobacco smoke priority toxicants, including 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosonornicotine (NNN), and benzo[a]pyrene (B[a]P) and monitoring the levels of a further nine including cadmium.

Here, we evaluated the genotoxic potential in human bronchial epithelial BEAS-2B cells of four cigarette smoke toxicants; NNK, NNN, B[a]P and cadmium using the novel *in vitro* γH2AX assay by High Content Screening (HCS). We also examined the genotoxicity of binary mixtures of NNK and NNN reporting their relative contribution to the genotoxic end-point.

The results of this preliminary assessment showed that the *in vitro* γH2AX assay by HCS could be used as a pre-screening tool to detect and quantify the genotoxicity effect of cigarette smoke toxicants individually and in binary mixture. Moreover, the data produced could contribute to the prioritisation of toxicant reduction research in modified tobacco products.

1.- INTRODUCTION

Cigarette smoke is a complex mixture consisting of more than 5,600 identified compounds (Perfetti and Rodgman 2011), of which over 150 are known toxicants (Cunningham *et al*., 2011). In 2008, the World Health Organisation Framework Convention on Tobacco Control (WHO FCTC) published "The scientific basis of tobacco product regulation" a report containing recommendations on tobacco product regulation from a group of international experts (TobReg) (WHO 2008). From the initial report, Burns and colleagues summarised the proposal in a paper where a list of 18 priority toxicants was shortlisted based on evidence of their direct toxicity and their hazard indices as described by Fowles and Dybing (Burns *et al*., 2008; Fowles and Dybing 2003). Of these 18 toxicants, 9 were proposed for mandated lowering, including NNK, NNN, and B[a]P and 9 were proposed for monitoring (Table 1). Similarly, the Food and Drug Administration (FDA) Center for Tobacco products (CTP) has recently drafted a guideline for reporting Harmful and Potentially Harmful Constituents (HPHCs) in tobacco products. From the list of 93 established HPHCs (FDA, 2012a), 20 are initially shortlisted for reporting in the draft guideline (FDA, 2012b). The WHO FCTC priority toxicants list and the abbreviated HPHC list contain 14 toxicants in common as detailed in Table 1.

Burns and colleagues further discussed the limitation of the prioritisation model used which was established based on individual toxicant exposure, as opposed to complex mixture, and is mostly based on animal data. In this context, it is of interest to characterise the toxicity of smoke toxicants and mixtures using relevant human target tissue to prioritise toxicant reduction. However, the level of characterisation proposed along with ethical considerations like the 3Rs principle (Schechtman 2002) requires a high throughput *in vitro* method to overcome lengthy and expensive testing as well as being an alternative to animal models.

Table 1: Cigarette smoke toxicants. Cigarette smoke is an aerosol composed of condensed-phase particle in a gaseous medium. Compounds with low volatility have been found to be exclusively in the particulate phase, in the gas phase or partitioned between both phases (vapour).

Priority toxicants (Burns *et al.*, 2008)	Abbreviated HPHCs (FDA, 2012a)	Chemical Family	Smoke Phase	Recommended for lowering by FCTC
Acetaldehyde		Carbonyls	Vapour	Yes
Acrolein		Carbonyls	Vapour	Yes
Acrylonitrile		Organic Compounds	Vapour	No
4-Aminobiphenhyl		Aromatic Amines	Particulate	No
-	1-Aminonaphthalene	Aromatic Amines	Particulate	No
2-Aminonaphthalene		Aromatic Amines	Particulate	No
-	Ammonia	Inorganic Compound	Gas	No
-	Arsenic	Metal/Metalloid	Particulate	No
Benzene		Volatile Hydrocarbons	Vapour	Yes
Benzo[a]pyrene - B[a]P		Polyaromatic hydrocarbons	Particulate	Yes
1,3-Butadiene		Volatile Hydrocarbons	Vapour	Yes
Cadmium		Heavy Metal	Particulate	No
Carbon Monoxide		Organic Compounds	Gas	Yes
Catechol	-	Phenol	Particulate	No
Crotonaldehyde		Carbonyls	Vapour	No
Formaldehyde		Carbonyls	Vapour	Yes
-	Isoprene	Volatile Hydrocarbons	Vapour	No
-	Nicotine	Aza-arenes	Particulate	No
Hydrogen cyanide	-	Organic Compounds	Vapour	No
Hydroquinone	-	Phenol	Particulate	No
Nitrogen Oxides – various	-	Nitrogen Oxides	Gas	No
NNK *		Tobacco Specific Nitosamines	Particulate	Yes
NNN **		Tobacco Specific Nitrosamines	Particulate	Yes
-	Toluene	Volatile Hydrocarbons	Vapour	No

* 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone
** *N´*-nitrosonornicotine

The aim of this study was to utilise the recently developed *in vitro* γH2AX assay by High Content Screening (HCS) to evaluate the genotoxicity effect, in human lung-derived cells, of a group of cigarette smoke toxicants shown to cause genotoxicity *in vitro* in the battery of assays described in regulatory guidelines, such as International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Humans (ICH) (ICH, 2011; IARC, 2012a; IARC, 2012b; IARC, 2012c). The high throughput scoring system employed was the Cellomics ArrayScan® VTI platform (ThermoScientific, USA), an automated microscopy system that combines cellular image acquisition from multi-well microplates with software quantification analysis (Taylor 2010). HCS could provide simplified experimental design that allows a larger number of toxicants and mixtures to be pre-screened for genotoxicity effects compared to established regulatory *in vitro* tests.

The novel *in* vitro γH2AX assay HCS in human lung-derived BEAS-2B cells has been recently described (Garcia-Canton *et al*., 2013b). Briefly, the phosphorylation of the histone 2AX named γH2AX has been demonstrated to be a sensitive marker of DNA damage in the form of double strand breaks in different scientific fields since its discovery in 1998 (Rogakou *et al*., 1998). In the field of regulatory genotoxicity, the *in vitro* γH2AX assay has been suggested as a complementary tool to the existing battery of *in vitro* assays based on its sensitivity, specificity and automated scoring (Garcia-Canton *et al*., 2012; Watters *et al*., 2009; Smart *et al*., 2011). Moreover, the use of the human-derived BEAS-2B cells as the *in vitro* cell system could provide a more physiologically relevant testing system than existing assays where rodent-derived cell lines are used routinely. The BEAS-2B cell line was first described by Reddel and colleagues in 1988 (Reddel *et al*., 1988). BEAS-2B cells have a non-cancerous phenotype and wild-type p53 status, both of which are important advantages in the measurement of DNA damage response such as γH2AX (Petitjean *et al*., 2007; Svetlova *et al*., 2010; IARC, 2013). However, the cell line BEAS-2B lacks normal metabolic

activity for the majority of the cytochrome P450 family (Garcia-Canton *et al*., 2013a). The lack of metabolic activation could cause some pro-toxicants to produce a "false negative" response *in vitro* (Kirkland *et al*., 2007). To solve the BEAS-2B metabolic competency limitation, the standardised Aroclor-1254 induced rat S9 mix was included in our experimental design as an external source of metabolic bioactivation.

In this study, we have selected 3 chemicals recommended for mandated lowering by the FCTC, namely B[a]P, NNK and NNN. We also tested cadmium, a toxicant recommended for monitoring by the FCTC. The toxicants belong to polyaromatic hydrocarbons (PAH), Tobacco-Specific Nitrosamines (TSNAs), and heavy metals, respectively. B[a]P, NNK, NNN and cadmium can be found in the particulate phase of cigarette smoke and are all classified by the international agency for research on cancer (IARC) as *Group 1 – carcinogenic to humans* (IARC, 2012a; IARC, 2012b; IARC, 2012c).

The PAH B[a]P is found in tobacco smoke but also in barbequed foods, polluted air (both outdoor and indoor) and coal tar-based pharmaceuticals. The toxic effects associated with B[a]P exposure are caused by the diol-epoxide metabolite (BPDE) formed as a result of the CYP1A and CYP1B1 metabolism. This metabolism-mediated toxicity designates B[a]P as a pro-toxicant. The principal mechanism of action is the formation of DNA-adducts that could ultimately lead to mutation and DNA breaks (Alexandrov *et al*., 2010). B[a]P was previously evaluated in the presence of S9 mix during the assessment of the *in vitro* γH2AX assay by HCS (Garcia-Canton *et al*., 2013b). In this study B[a]P was tested in BEAS-2B cultures where CYP1A1/1B1 were pre-induced using 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) as previously described (Garcia-Canton *et al*., 2013a).

The heavy metal Cadmium is absorbed from soil by the tobacco plant which can accumulate relatively high amounts in its leaves (Lugon-Moulin *et al*., 2006). Different *in vivo* and *in*

vitro studies defined cadmium as a weak mutagenic compound with various mechanisms of action such as causing DNA strand breaks, inducing oxidative stress and acting as a co-mutagen that increases the mutagenicity of other compounds by inhibition of DNA-repair mechanisms (Waisberg *et al.*, 2003; Hartwig 2010). Cadmium also has the potential to be a spindle poison *in vivo* i.e. an aneugenic compound (Watanabe *et al.*, 1979). However, *in vitro* studies have shown conflicting results that could be caused by the use of different cell lines and differing ranges of tested concentrations (Seoane and Dulout 2001; Lynch and Parry 1993). In this study, the soluble form of cadmium tested was cadmium chloride as it has been widely used both *in vivo* and *in vitro* (IARC, 2012c; Fowler *et al.*, 2010; Forti *et al.*, 2010).

The TSNAs NNK and NNN are present in all tobacco products as a result of the curing process. The toxicity of NNK and NNN is mainly caused by their metabolites, formed as a result of the CYP2A6/2A13 metabolism (Hecht and Tricker 1999). Their mechanism of action is to bind covalently to DNA producing mutations and strand breaks (Liu *et al.*, 1990; Hecht 1999). Here we tested both TSNAs in the presence and absence of S9 mix. We have also tested NNK-acetate and NNN-acetate as CYP-independent reactive forms of NNK and NNN respectively. The acetate forms are stable intermediates that do not require cytochrome P450 activity to become activated, instead they are activated by esterase enzyme activity which are ubiquitous in mammalian cells (Peterson *et al.*, 2001).

Here, we report and discuss the results obtained from the *in vitro* γH2AX assay by HCS applied to the selected cigarette smoke toxicants tested individually or for the TSNAs as a binary mixture. The results obtained support the use of the *in vitro* γH2AX assay by HCS as a high throughput tool to investigate the genotoxicity effect of cigarette smoke toxicants.

2.- MATERIALS AND METHODS

2.1.- Chemicals

All compounds were of the highest available grade. Etoposide (positive control) (CAS 33419-42-0), B[a]P (CAS 50-32-8) and cadmium chloride (CAS 10108-64-2) were purchased from Sigma-Aldrich (United Kingdom). NNK (CAS 64091-91-4), NNK-acetate (CAS 127686-49-1), NNN (CAS 80508-23-2) and NNN-acetate (CAS 68743-65-7) were purchased from Toronto Research Chemicals (Canada). Dimethyl sulfoxide (DMSO) was used as a vehicle at a final concentration of 1% v/v in cell culture medium. Current guidelines for the genotoxicity testing of pharmaceuticals were followed to define doses and treatment times (ICH, 2011). As a result, the maximum concentration tested was 1 mM except for B[a]P where the maximum concentration was 15 μM due to poor solubility and precipitation after treatment.

NNK-acetate and NNN-acetate mixture assessment followed simple binary mixture experimental design. Five different permutations containing a different ratio of each TSNA were prepared and each permutation contained 8 separate concentrations.

1.- 100% NNK-acetate

2.- 25% NNK-acetate and 75% NNN-acetate

3.- 100% NNN-acetate

4.- 50% NNK-acetate and 50% NNN-acetate

5.- 75% NNK-acetate and 25% NNN-acetate

For example, to prepare 1 mL final volume of the binary mixture 25% NNK-acetate and 75% NNN-acetate at a concentration of 1000 μM, 0.25 mL of NNK-acetate solution at a concentration of 1000 μM was mixed with 0.75 mL of NNN-acetate solution at a concentration of 1000 μM. The same approach was followed for the rest of the binary mixtures.

2.2.- Cell culture

The human bronchial epithelial cell line BEAS-2B, purchased from ATCC (United States), was seeded into culture vessels that had been pre-coated with 0.03 mg/mL PureCol® bovine collagen solution (Nutacon, The Netherlands). Cells were maintained in Bronchial Epithelial Growth Medium (BEGM®) at 37°C and 5% CO_2 in a humidified incubator. BEGM® was prepared by supplementing Bronchial Epithelial Basal Medium with growth supplements provided in the manufacturer's BEGM® SingleQuot® kit (Lonza Group Ltd., Belgium) containing: bovine pituitary extract, hydrocortisone, human epidermal growth factor, epinephrine, insulin, triiodothyronine, transferrin, gentamicin /amphotericin-B and retinoic acid. BEAS-2B cells were cultured and expanded in-house. All cultures were negative for mycoplasma. Additionally, the cells were authenticated using the short tandem repeat profiling to confirm the nature of the cell cultures (LGC Standards, United Kingdom) (Nims *et al*., 2010).

2.3.- Treatment, immunostaining and imaging analysis

The methodology to detect and quantify γH2AX by HCS has been previously described (Garcia-Canton *et al.*, 2013b). Here we introduced a minor variation for the testing of B[a]P where 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) was used as CYP1A1/1B1 inducer. Briefly, 72 hours before B[a]P treatment, cells were seeded into collagen pre-coated 96-well, black,

clear bottom microplates (PerkinElmer, United Kingdom) at a concentration of $6x10^4$ cells/mL and maintained overnight at 37°C in an atmosphere of 5% CO_2 in air. Then, TCDD was added to the culture media (10nM final) for a further 48 hour. Then, the media containing TCDD was removed and cells washed with pre-warmed phosphate buffered salt solution prior to treatment with B[a]P.

Aroclor-induced rat S9 and NADPH cofactor (Moltox United States) (0.5% v/v final concentration in medium) were used as the exogenous source of metabolic activation for the pro-toxicants NNK and NNN. Preparation of the S9 mix was carried out following suppliers' instructions and as detailed in (Garcia-Canton *et al.*, 2013b).

In this study compounds were tested for either 3 hour or 24 hour. For compounds that required an external source of metabolic activation compounds were tested with and without metabolic activation system for 3 hour and 3 hour followed by a 24 hour recovery period.

2.4.- Data analysis and criteria

All experiments were repeated at least 3 times and results were graphically represented using GraphPad Prism software v.6

γH2AX frequency (response) is reported as fluorescence intensity units. Our methodology scores whole nuclei fluorescence instead of foci numbers; Counting foci numbers will decrease the throughput of the assay reducing its potential application as a pre-screening tool. In addition, the length of our treatments, i.e. minimum of 3 hour, would have an effect in the heterogeneity of foci size (overlapping of foci) compromising the sensitivity of the results.

Relative Cell Counts (RCC) as a percentage of the vehicle-treated control are used as the measure of cell viability. The vehicle treated cell counts were defined as 100% cell viability. The cell counts in the compound treated wells were then compared to those in the vehicle-

treated wells, and the percentage cell viability was calculated. This comparison against the vehicle control data is referred to as Relative Cell Counts (RCC).

The evaluation criteria for this study is described in Table 2, it was initially presented by Smart *et al.* for the analysis of γH2AX by flow cytometry (Smart *et al.*, 2011) and recently employed by Garcia-Canton *et al.* for the analysis of γH2AX by HCS (Garcia-Canton *et al.*, 2013b).

Table 2: Genotoxicity evaluation criteria for the *in vitro* γH2AX assay, adapted from (Smart *et al.*, 2011)

γH2AX response	**Classification**
> 1.5-fold γH2AX @ RCC > 25%	Genotoxic (+)
< 1.5-fold γH2AX @ RCC 100-0%	Non-genotoxic (–)
> 1.5-fold γH2AX @ RCC < 25%	"False" positive; Cytotoxic-driven genotoxicity
=1.5-fold γH2AX @ RCC ≥ 25%	Equivocal (±)

3.- RESULTS

The novel *in vitro* γH2AX assay by HCS was used to evaluate the genotoxicity effect of four cigarette smoke toxicants and the binary mixture of two TSNAs. Automated fluorescence microscopy was selected as the scoring system to detect and quantify the specific γH2AX immunostaining, used as a marker of DNA double strand breaks. The graphical representations below show the γH2AX response obtained after BEAS-2B cells were treated with the positive control etoposide (Figure 1), different cigarette smoke toxicants (Figures 2 to 5) and the TSNAs binary mixture (Figure 6). The X-axis represents the compound concentration (µM) on a logarithmic scale while the Y-axis represents the γH2AX frequency in absolute units (intensity units). RCC representing cell viability are graphically represented in the supplementary data for clarity.

A range of etoposide concentrations up to 1mM was used in this study as a positive control in all experiments conducted. A sample dose response is graphically represented in Figure 1.

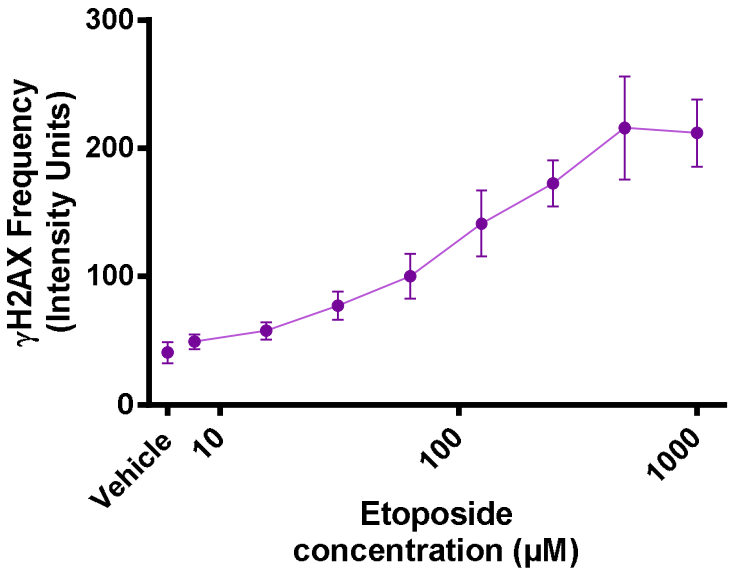

Figure 1: Etoposide positive control dose response example after 3 hour treatment.

B[a]P was tested in both TCDD-induced and non-induced cultures for 3 hour and 24 hour. Initial range finder experiments testing up to 1mM showed that B[a]P precipitated at high concentrations (data not shown). Therefore, the maximum concentration tested in this study was 15 µM. B[a]P did not produce any increase in γH2AX frequency above the vehicle-treated controls after 3 hour treatment in the presence and absence of the inducer TCDD (Figures 2A and 2B). When B[a]P was tested for 24 hour, there was a significant increase in γH2AX frequency in both TCDD-induced and non-induced cultures at concentrations above 0.5 µM without any significant reduction in cell viability (RCC > 80%). However, the γH2AX frequency in non-induced cultures at concentrations above 4 µM after 24 hour treatment returned to vehicle-treated levels (< 1.5-fold induction) as illustrated in Figure 2B.

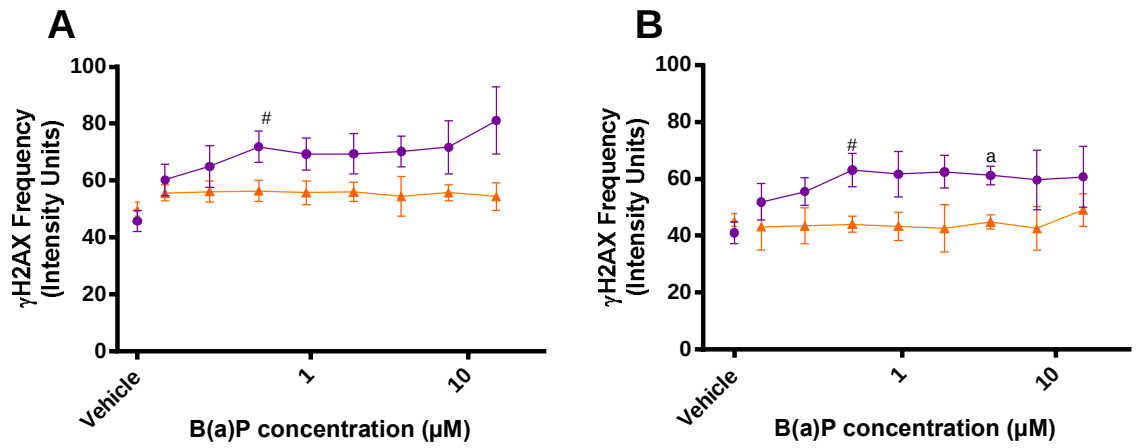

Figure 2: γH2AX frequency after treatment with B[a]P. [A] B[a]P treatment in TCDD-induced cultures [B] B[a]P treatment in non-induced cultures. Triangle (-▲-) represents short treatment (3 hour) and circle (-●-) represents long treatment (24 hour). Hash (#) indicates the minimum concentration that shows genotoxicity (1.5 fold increase) compared to the vehicle treated control after long treatment. (a) indicates the minimum concentration where γH2AX frequency reverts to non-genotoxic range (< 1.5 fold-induction).

Both tobacco-specific nitrosamines NNK and NNN failed to show any statistically significant increase in γH2AX compared to the vehicle-treated controls at all timepoints in the presence and absence of aroclor-induced rat S9 mix. Figure 3 illustrates the results obtained for NNK in the presence of S9 mix, the same profile was obtained for NNN in the presence and absence of S9 mix (data not shown).

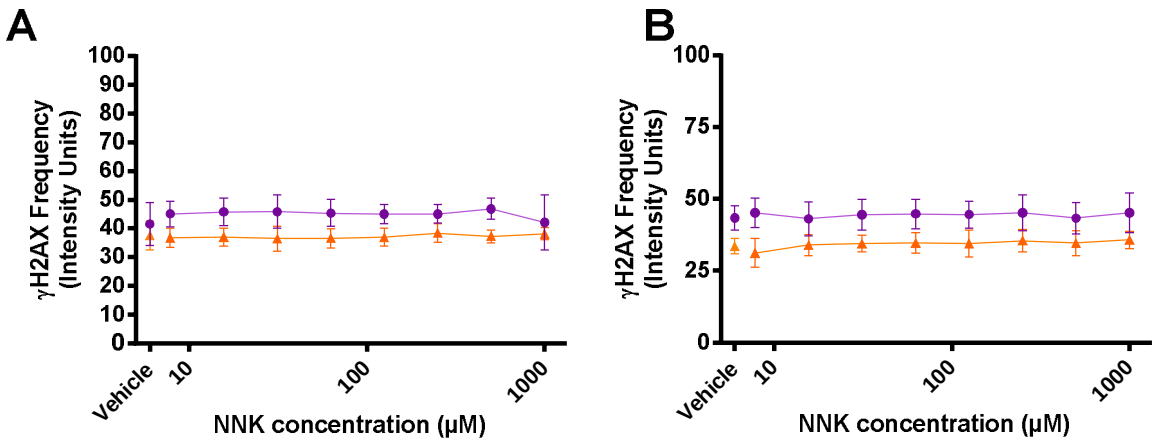

Figure 3: γH2AX frequency after treatment with NNK. [A] NNK in the presence of S9 mix and [B] NNK in the absence of S9 mix. Triangle (-▲-) represents short treatment (3 hour) and circle (-●-) represents short treatment (3 hour) followed by a recovery period (24 hour).

The results represented in Figure 4 show that both NNK-acetate and NNN-acetate produced DNA damage in the form of DSBs. NNK-acetate (Figure 4A) produced a significant increase in γH2AX frequency compared to the vehicle-treated control at concentrations above 16 µM after a 3 hour treatment, without reduction in cell viability (RCC > 90%), at 3 hour followed by 24 hour recovery period concentrations above 125 µM showed an increase in γH2AX above 1.5-fold with a high reduction in cell viability (RCC ~ 40%). The highest concentration tested (1000 µM) produced almost total cell death after a 3 hour treatment followed by 24 hour recovery period, this could explain the high variability observed in the data.

When NNN-acetate was tested at 3h there was a positive response at concentrations above 500 µM without a reduction in cell viability below 90%. NNN-acetate did not produce a positive response after 3 hour treatments followed by 24 hour recovery time, although, a dose-related increase in γH2AX frequency and reduction in cell viability were observed (Figure 4B).

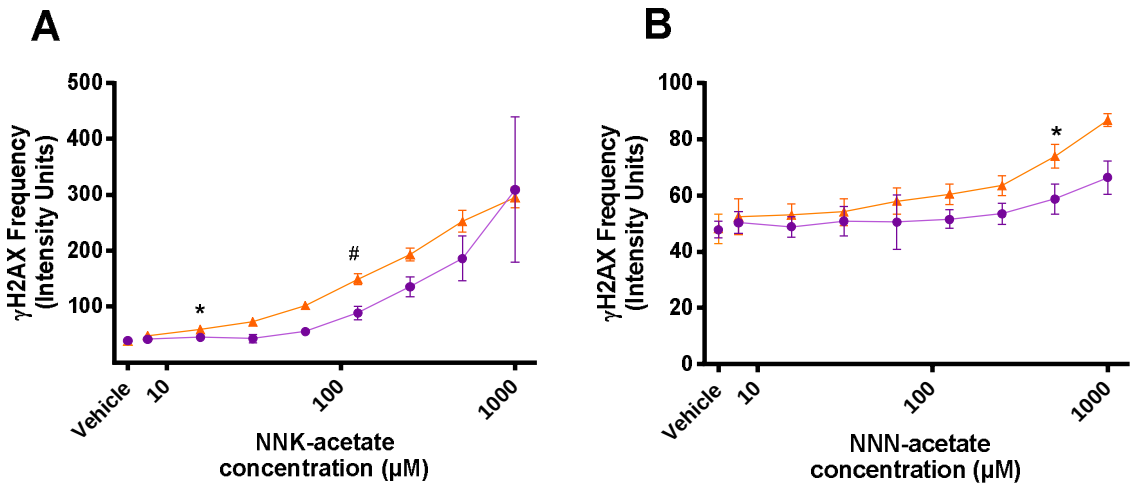

Figure 4: γH2AX frequency after treatment with the active form of NNK and NNN. [A] NNK-acetate treatment, [B] NNN-acetate treatment. Triangle (-▲-) represents short treatment (3 hour) and circle (-●-) represents short treatment (3 hour) followed by a recovery period (24 hour). Asterisk (*) indicates the minimum concentration that shows genotoxicity compared to the vehicle treated control after short treatment. Hash (#) indicates the minimum concentration that shows genotoxicity compared to the vehicle treated control after long treatment.

Figure 5 illustrates the results obtained after BEAS-2B cells were treated for 3 hour and 24 hour with cadmium chloride. A positive response was observed after 3 hour treatment at concentrations above 250 µM (RCC > 40%). After 24 hour treatment, concentrations above 16 µM showed cytotoxic-driven genotoxicity with RCC < 25%.

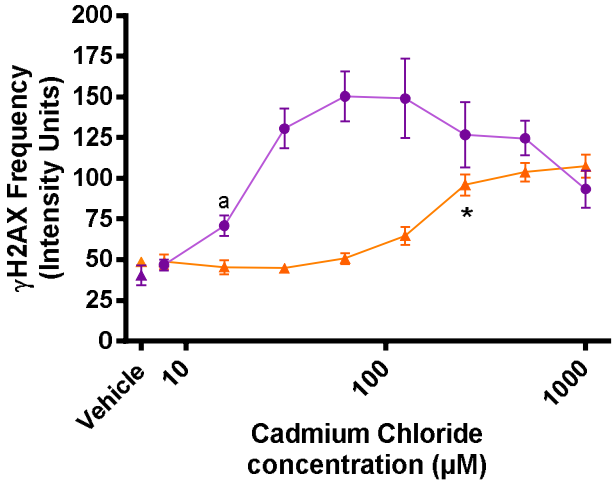

Figure 5: γH2AX frequency after treatment with Cadmium chloride. Triangle (-▲-) represents short treatment (3 hour) and circle (-●-) represents long treatment (24 hour). Asterisk (*) indicates the minimum concentration that shows genotoxicity compared to the vehicle treated control after short treatment. (a) Indicates the minimum concentration that shows cytotoxic-driven genotoxicity after long treatment (RCC < 25%).

The results obtained after BEAS-2B cells were treated for 3 hour with binary mixtures of NNK-acetate and NNN-acetate are graphically represented in Figure 6. The binary mixture involved the testing of 5 different permutations containing different percentages of both NNK-acetate and NNN-acetate. All permutations tested showed a significant increase in the γH2AX frequency compared to the vehicle-treated control without a reduction in cell viability to levels below 60%. NNK-acetate (100%) produced a more potent genotoxic response than NNN-acetate (100%) as the positive response with NNK-acetate is observed at lower concentrations than NNN-acetate. The mixtures tested do not suggest any compound-to-compound interaction such as synergistic or antagonistic effects.

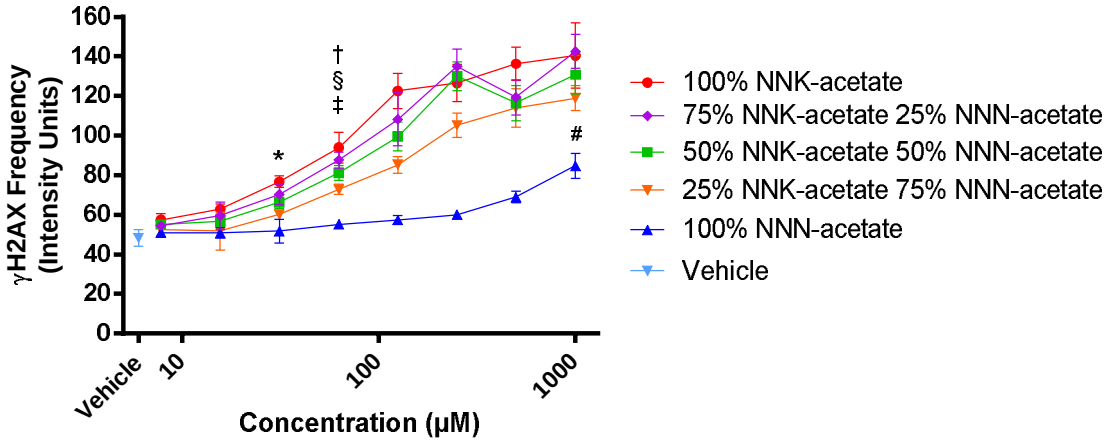

Figure 6: γH2AX frequency after 3 hour treatments with different ratios of TSNA mixtures. Circle (-●-) represents 100% NNK-acetate, rhomb (-◆-) represents 75% NNK-acetate and 25% NNN-acetate, square (-■-) represents 50% NNK-acetate and 50% NNN-acetate, down-pointing triangle (-▼-) represents 25% NNK-acetate and 75% NNN-acetate and up-pointing triangle (-▲-) represents 100% NNN-acetate. Asterisk (*) indicates the minimum concentration that shows genotoxicity compared to the vehicle treated control after treatment with 100% NNK-acetate. Dagger (†), section sign (§) and double dagger (‡) indicate the minimum concentration that shown genotoxicity compared to the vehicle treated control after treatment with 75% NNK-acetate and 25% NNN-acetate, 50% NNK-acetate and 50% NNN-acetate and 25% NNK-acetate and 75% NNN-acetate respectively. Hash (#) indicates the minimum concentration that shows genotoxicity compared to the vehicle treated control after treatment with 100% NNN-acetate.

4.- DISCUSSION

The objective of this study was to evaluate the applicability of the novel *in vitro* γH2AX assay by HCS for the *in vitro* genotoxicity assessment of cigarette smoke toxicants in a high-throughput approach. Additionally, we assessed the genotoxicity effect of a mixture of two TSNAs (Acetylated NNK and NNN reactive precursors) developing further understanding of their relative contribution to the genotoxic response.

Traditionally, the *in vitro* genotoxic effect of tobacco cigarette smoke has been widely evaluated by testing the particulate matter collected on Cambridge filter pads in animal-derived cell lines such as CHO or V79. A thorough review on the evaluation of *in vitro* assays used for assessing cigarette smoke was published by Johnson and colleagues in 2009 (Johnson *et al*., 2009). Although these methods are useful when comparing products, it is not possible to determine which compounds in the mixture are causing the response or "driving" the toxicity. Fractionation of the different components present in tobacco cigarette smoke has supported the segregation of the toxicants from other compounds present in the mixture as described by (Burns *et al*., 2008) and the FDA CTP (FDA, 2012a). Nonetheless, mixture testing is still required to understand potential toxicant interactions and relative contributions. The information obtained from single toxicant and mixture testing would be essential to prioritise the reduction of cigarette smoke toxicants in modified tobacco products as recommended by the WHO FCTC (WHO 2008). To date, the information existing on cigarette smoke toxicants are mainly derived from animal models (*in vivo*) or current regulatory *in vitro* genotoxicity assays such as Ames, micronucleus or mouse lymphoma assay (MLA). Existing regulatory *in vitro* genotoxicity assays have two main limitations, low throughput and high frequency of false positives, for these reasons newer technologies and end-points are currently taken into consideration in the genetic toxicology field (Lynch *et al*.,

2011). The *in vitro* γH2AX assay has been described as a potential complementary tool to the current battery of genotoxicity assays, especially as a pre-screening assay, using scoring systems such as manual microscopy, flow cytometry and *in cell western* (Watters *et al.*, 2009; Smart *et al.*, 2011; Audebert *et al.*, 2010). Automated microscopy in the form of High Content Screening (HCS) has recently been proposed as an alternative to flow cytometry in the *in vitro* γH2AX assay and offer the advantage of a high accuracy (86%), which results from a high sensitivity (86-92%) and a high specificity (80-88%) (Garcia-Canton *et al.*, 2013b). The use of the *in vitro* γH2AX assay by HCS would facilitate the analysis of large number of compounds and of mixtures.

Here, our results indicate that the *in vitro* γH2AX assay by HCS has the ability to detect the genotoxicity response from cigarette smoke toxicants in different chemical families such as PAHs (B[a]P), heavy metals (Cadmium) and CYP-independent forms of TSNAs (NNK-acetate and NNN-acetate). The *in vitro* γH2AX assay by HCS has also shown the potential to detect and quantify the genotoxicity effect of binary mixtures supporting the assessment of complex chemical to chemical interactions and the relative contribution to the toxicological end-point. In some cases, an elevated response in γH2AX levels was observed at concentrations with low cell viability. This effect has been associated to mechanisms such as apoptosis as a result of the fragmentation of cellular DNA that is part of the programmed cell death sequence (Mukherjee *et al.*, 2006; Darzynkiewicz *et al.*, 2012).

During this study some limitations were observed when aroclor-induced rat S9 mix was used as an external source of metabolic activation for TSNAs. Both NNK and NNN produced a negative response in the presence of the S9 mix but a positive response was later obtained when the active intermediates were tested. The S9 mix is a standardised hepatic product used routinely in *in vitro* testing. However, not all the relevant cytochrome P450s are present in

this external source of metabolic activation as described by Easterbrook *et al* (Easterbrook *et al*., 2001). They performed a comparison between aroclor-induced rat S9 and human liver microsomes finding that the rat S9 lacks 7-hydroxylation activity (equivalent to human CYP2A6). In addition, CYP2A13 enzyme activity is mostly restricted to the respiratory tissue and therefore not expected in liver S9 mix (Su *et al*., 2000; Zhang *et al*., 2007). Both CYP2A6 and CYP2A13 enzymes are required for the activation of NNK and NNN (Hecht 1999), explaining why our experiments did not show any response for these TSNAs in the presence of S9 mix. Engineered BEAS-2B cells expressing human cytochrome P450 (Mace *et al*., 1997; Grosse *et al*., 1997), human- and animal-derived lung fractions or purified CYPs could also be employed as alternatives to standard rat S9. The pros and cons of some of these methodologies *in vitro* have been thoroughly reviewed by Brandon and colleagues (Brandon *et al*., 2003). Cell lines with a more comprehensive expression of CYP enzymes such as the human hepatoma-derived HepaRG cells could be considered as cell systems (Antherieu *et al*., 2010). However, these "metabolically competent" cell lines are derived from the liver which in this case is not an appropriate surrogate for the target organ of exposure.

In summary, the *in vitro* γH2AX assay by HCS can be used as a pre-screening tool for the genotoxicity evaluation of cigarette smoke toxicants both individually and in mixtures supporting the ranking of priority toxicants, defined by the WHO FCTC, and the HPHCs, defined by the FDA CTP (WHO 2008; FDA, 2012a). The data produced could scientifically support the prioritisation of toxicant reduction in modified tobacco products. Furthermore, the *in vitro* γH2AX assay by HCS can be further developed to be adapted to aerosol exposure and this could become a more physiologically relevant model in the evaluation of toxicants in the form of gases and vapours as well as whole cigarette smoke.

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Chapter V

Characterisation of an aerosol exposure system to evaluate the genotoxicity of whole mainstream cigarette smoke using the *in vitro* γH2AX assay by High Content Screening

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ABSTRACT

The genotoxic effect of cigarette smoke is routinely measured by treating cells with cigarette Particulate Matter (PM) at different dose levels in submerged cell cultures. However, PM exposure cannot be considered as a complete exposure as it does not contain the gas phase component of the cigarette smoke. The *in vitro* γH2AX assay by High Content Screening (HCS) has been suggested as a complementary tool to the standard battery of genotoxicity assays as it detects DNA double strand breaks in a high-throughput fashion. The aim of this study was to further optimise the *in vitro* γH2AX assay by HCS to enable aerosol exposure of human bronchial epithelial BEAS-2B cells at the air-liquid interface (ALI). Whole mainstream cigarette smoke (WMCS) from two reference cigarettes (3R4F and M4A) were assessed for their genotoxic potential. During the study, a further characterisation of the Borgwaldt RM20S® aerosol exposure system to include single dilution assessment with a reference gas was also carried out.

The results of the optimisation showed that both reference cigarettes produced a positive genotoxic response at all dilutions tested. However, the correlation between dose and response was low for both 3R4F and M4A (Pearson coefficient, r = -0.53 and -0.44 respectively). During the additional characterisation of the exposure system, it was observed that several pre-programmed dilutions did not perform as expected.

Overall, the *in vitro* γH2AX assay by HCS could be used to evaluate WMCS in cell cultures at the ALI. Additionally, the extended characterisation of the exposure system indicates that assessing the performance of the dilutions could improve the existing routine QC checks.

1.- INTRODUCTION

Cigarette smoke is a complex aerosol mixture consisting of more than 6,000 identified compounds that can be divided between the particulate phase, accounting for 4.5% of the total aerosol mixture mass, and the gas phase, accounting for 95.5% of the total aerosol mixture mass (Rodgman and Perfetti 2013).

Testing and understanding the toxicity of cigarette smoke *in vitro* is a key step in the characterisation of modified tobacco products with potentially reduced harm. Adopting such strategies are in line with recommendations published by the Institute of Medicine "Clearing the Smoke" (Stratton *et al.*, 2001) and the World Health Organisation Framework convention on Tobacco Control (WHO FCTC) "The scientific basis of tobacco product regulation" (WHO 2008).

Johnson and colleagues published a thorough review on the *in vitro* systems used to evaluate the toxicity of cigarette smoke (Johnson *et al.*, 2009). In this review, the authors highlighted that the majority of tobacco-related *in vitro* toxicology studies are carried out in non-human cell models which are poorly validated for tobacco product comparison. They also concluded that better methods are needed, especially in relation to regulation and health claims. In the field of *in vitro* genotoxicity, the authors described that the evaluation of cigarette smoke has been carried out using mainly cigarette smoke condensate (CSC). However, CSC contains primarily particulate phase components compared to whole mainstream cigarette smoke (WMCS) which contains both particulate and gas phase components. We consider WMCS a more comprehensive exposure system to study toxicological effects *in vitro* (Table 1). Moreover, the *in vitro* genotoxicity data has been mainly obtained from animal-derived cell systems which are functionally very different from human-derived cells.

Table 1: Physical forms of cigarette smoke used in *in vitro* testing. Adapted from (Breheny *et al.*, 2011).

Name	Description
Cigarette smoke condensate (CSC)	Comprises the particulate phase along with some vapour phase components. Generated by cold-trapping and condensation of smoke at extremely low temperatures. The condensed 'tar' is then typically extracted and diluted using acetone.
Cigarette smoke particulate matter (PM)	Comprises the particulate phase only. Particulates are typically collected by passing cigarette smoke through a Cambridge filter pad and are subsequently eluted from the filter pad using a solvent such as dimethylsulphoxide.
Cigarette smoke extract (CSE)	Comprises the particulate phase along with some vapour phase components. Generated by bubbling smoke through a liquid (e.g. phosphate-buffered saline or cell culture medium).
Whole mainstream cigarette smoke (WMCS)	Cells are directly exposed to smoke at the air-liquid interface. This is more representative of human exposure conditions, as cells are exposed to the gas and vapour phase components in an aerosol (Phillips *et al.*, 2005).

There are different *in vitro* genotoxicity assays that have been widely used in the assessment of tobacco products (Johnson *et al.*, 2009). Some of the assays described such as the micronucleus or the mouse lymphoma assay focus on fixed DNA damage like chromosomal damage and mutations, their strengths and limitations have been previously summarised (Garcia-Canton *et al.*, 2012). The comet assay is the only assay described by Johnson and colleagues that specifically detects DNA strand breaks. Although the assay is widely accepted and considered a mature method (Lynch *et al.*, 2011), it does not discriminate between single or double strand breaks and has shown high inter- and intra-experimental variation (Zainol *et al.*, 2009). The *in vitro* γH2AX assay, on the other hand, is an emerging method for DNA damage detection. The phosphorylation of H2AX (named γH2AX) in response to DNA double strand breaks (DSB) was first described in 1998 (Rogakou *et al.*,

1998) and has since been extensively investigated (Fernandez-Capetillo *et al.*, 2004). Some applications in which γH2AX has been used as a biomarker of DNA damage are pre-clinical drug development and clinical studies (Dickey *et al.*, 2009). More recently, γH2AX has been suggested as a potential complement to the current battery of *in vitro* genotoxicity assays with applications in the assessment of cigarette smoke (Smart *et al.*, 2011; Garcia-Canton *et al.*, 2012).

The aim of this study was to optimise the novel *in vitro* γH2AX assay by High Content Screening (HCS) that we had previously developed (Garcia-Canton *et al.*, 2013a), in order to adapt it for the assessment of aerosols and to evaluate the genotoxic effect of two reference cigarettes in human lung-derived BEAS-2B cells at the air-liquid interface (ALI). The optimisation employs the Borgwaldt RM20S® smoking machine (RM20S®) as part of the exposure system that delivers WMCS to cells at the ALI (Phillips *et al.*, 2005).

The *in vitro* γH2AX assay has been previously used in the assessment of cigarette smoke using mainly CSC and indirect exposure to WMCS i.e. cell cultures that had a layer of media covering the cells continuously or intermittently during smoke exposure and therefore not considered true ALI exposure (Albino *et al.*, 2004; Albino *et al.*, 2006; Albino *et al.*, 2009; Tanaka *et al.*, 2007; Zhao *et al.*, 2009). In general, flow cytometry has been the main method for γH2AX detection and analysis. In this study, we selected a microscopy-based automated scoring system known as HCS to acquire and quantify the γH2AX response after WMCS exposure to BEAS-2B cells at the ALI. WMCS was tested from two different cigarettes, 3R4F a reference cigarette from the University of Kentucky (University of Kentucky, 2013) and M4A a historical control used as internal reference in genotoxicity studies by British American Tobacco (Combes *et al.*, 2012).

Overall, the results show that the *in vitro* γH2AX by HCS can be used as a high throughput tool to assess the genotoxic effect of WMCS in cultures exposed at the ALI. The results can be used to compare the genotoxic responses of different tobacco products. Furthermore, the optimised *in vitro* γH2AX assay for aerosol exposure could be a useful high content screening tool to assess the genotoxic potential of toxicants in gaseous form.

2.- MATERIALS AND METHODS

2.1.- Cell culture

The human bronchial epithelial cell line BEAS-2B was purchased from ATCC (United States). Normal bronchial epithelium cells obtained from autopsy of non-cancerous individuals had been isolated, then infected with a replication-defective 12-SV40/adenovirus hybrid (Ad12SV40) and cloned to create an immortalised phenotype (Reddel *et al.*, 1988). Cells were seeded into culture vessels that had been pre-coated with 0.03 mg/mL PureCol® bovine collagen solution (Nutacon, The Netherlands). Cells were then maintained in Bronchial Epithelial Growth Medium (BEGM®) at 37°C and 5% CO_2 in a humidified incubator. BEGM® was prepared by supplementing Bronchial Epithelial Basal Medium with growth supplements provided in the manufacturer's BEGM® SingleQuot® kit (Lonza Group Ltd., Belgium) containing: bovine pituitary extract, hydrocortisone, human epidermal growth factor, epinephrine, insulin, triiodothyronine, transferrin, gentamicin /amphotericin-B and retinoic acid. BEAS-2B cells were cultured and expanded in-house, the cells were used between passages 3 and 12 only. All cultures were negative for mycoplasma. Additionally, the cells were authenticated using the short tandem repeat profiling to confirm the nature of the cell cultures (LGC Standards, United Kingdom) (Nims *et al.*, 2010).

2.2.- Smoking system

The selection of the RM20S® 8-syringe smoking machine as the WMCS exposure system was based on previous *in vitro* studies (Massey *et al.*, 1998; Phillips *et al.*, 2005) and thorough evaluations of precision, accuracy, repeatability and reproducibility (Kaur *et al.*, 2010; Adamson *et al.*, 2011). The smoking exposure system is schematically represented in Figure 1. The RM20S® employs a dilution system that mixes WMCS with different proportions of air to generate a dilution ratio represented as 1 : X (smoke volume : air

volume). Cigarettes are automatically loaded into cigarette holders (Figure 1A) where WMCS is drawn directly into the glass syringe and diluted with air taken from the laboratory environment (Figure 1B) following a multi-step process operated by a plunger (Figure 1C). The diluted WMCS is then delivered to an exposure chamber (Figure 1D) containing four Transwell® inserts with BEAS-2B cells seeded on top of the insert's membrane (Figure 1E). At the time of the exposure the cells are directly exposed to WMCS at the air-liquid interface (ALI).

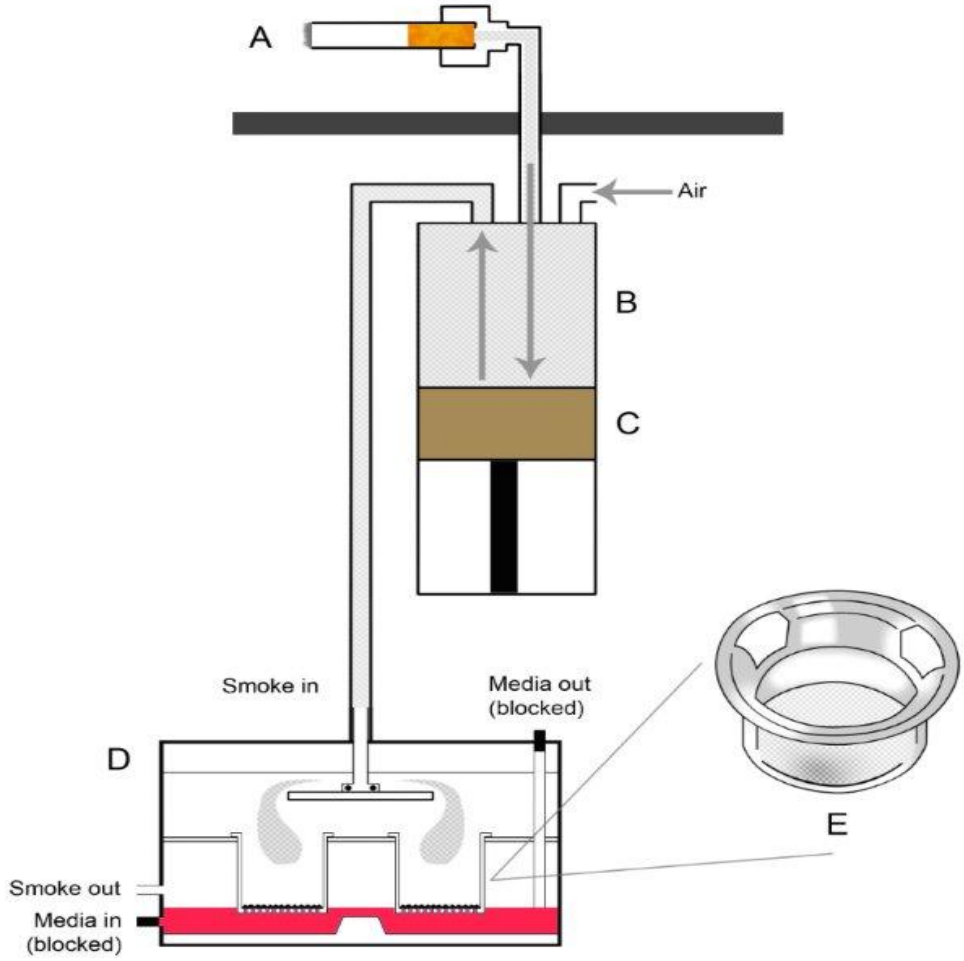

Figure 1: Schematic representation of a single RM20S® syringe combined with British American Tobacco's exposure chamber (UK patent publication WO 03/100417/ A1) (not to scale). The RM20S® can smoke up to eight cigarettes simultaneously. [A] Cigarette holder with cigarette in place; [B] 150mL glass syringe where cigarette smoke dilution in air is prepared; [C] Plunger; [D] Exposure chamber containing porous membrane inserts with cells seeded on top at the ALI [E] Transwell® insert representation. Figure adapted from (Adamson *et al*., 2011).

2.3.- Dilution performance evaluation

A range of dilutions were selected for this study from 1:25 to 1:20000 (smoke volume : air volume) (Table 2). The methodology employed by Kaur and colleagues used methane (CH_4) as a reference gas standard with known parts per million (PPM) to compare syringe performance and has been adapted here to assess dilution performance (Kaur *et al*., 2010). For our experiments, three different methane reference standards in nitrogen were purchased from Air Products PLC (United Kingdom), 10% containing 100,000 PPM methane, 50% containing 500,000 PPM methane and 99.95% containing 1,000,000 PPM methane. The relevant reference gas was loaded into a sealed bag and connected directly to the smoking machine cigarette holder (Figure 1A). The dilution to be tested was then programmed into the RM20S® and then gas diluted following International Standard Organization (ISO) 3308:2012 puffing profile consisting of 35 mL puff volume, 2 sec puff duration, and 60 sec puff interval (ISO, 2012). A second empty sealed bag was connected to the exhaust position in the place of the exposure chamber to collect the diluted gas (Figure 1D). Quantification of methane in PPM was performed with a 3010 MINIFID portable heated flame ionization detector total hydrocarbon analyser (Signal Group Ltd, United Kingdom) as per manufacturer's instructions. Table 2 summarises details about dilutions, reference gas standard used per dilution and expected PPM. The laboratory environment was conditioned at 22±2°C and 60±5% Relative Humidity (RH).

Table 2: Range of dilutions, details of methane reference gas and expected PPM.

Dilutions	Expected PPM with 10% methane	Expected PPM with 50% methane	Expected PPM with 100% methane
1:25	4000		
1:50	2000		
1:100	1000		
1:250	400		
1:500	200		
1:1000	100		
1:1500	67		
1:2000		250	
1:3000		167	
1:4000		125	
1:6000		83	
1:8000		63	
1:16000			63
1:20000			50

2.4.- Smoke exposure

Cigarettes were conditioned for a minimum of 48 hours prior to use (60±3% relative humidity, 22±1°C according to ISO 3402:1999) (ISO, 2010) and smoked continuously throughout the exposure on a RM20S® smoking machine (Borgwaldt KC, Germany) using a 35 ml puff volume drawn over 2 seconds, once every minute according to ISO 3308:2012 (ISO, 2012). The smoking environment was conditioned at 22±2°C and 60±5% RH.

In this study two reference cigarettes were used to test whether the *in vitro* γH2AX assay by HCS could discriminate between products. The reference cigarette 3R4F supplied by the

University of Kentucky, is a “US style” blended cigarette that delivers 9.4 mg tar and 0.7 mg nicotine under ISO conditions for cigarette smoking (ISO 3308:2012) (ISO, 2012). Internal reference cigarette M4A is a flue cured cigarette that delivers 10 mg of tar and 1.0 mg nicotine under ISO conditions for cigarette smoking (ISO 3308:2012) (ISO, 2012).

2.5.- Controls

Etoposide (1mM final) was used as a positive control during the experimentation (Sigma-Aldrich, United Kingdom). Etoposide is a well-known DNA-damaging compound and has previously been used in the *in vitro* γH2AX assay by HCS as a reference compound and positive control respectively (Garcia-Canton *et al*., 2013a; Garcia-Canton *et al*., 2013b). Two different negative controls were used in this study; air control and incubator control. The air control was generated by the smoking machine to evaluate the quality of the air used to dilute the WMCS and mimic the exposure conditions. The incubator control evaluated the incubation conditions used to generate the positive controls.

2.6.- WMCS treatment, γH2AX immunostaining and imaging analysis

The methodology used during this study to detect and quantify γH2AX by HCS was previously described (Garcia-Canton *et al.*, 2013a) with variations for the ALI exposure. In this study, cells were seeded on top of the membrane of collagen pre-coated 24-Transwell® plate (Corning Incorporated Life Sciences, Unites States) at a concentration of $1.2x10^5$ cells/mL and 500 µL of BEGM® were added underneath to keep the cells hydrated. The plates were then incubated overnight at 37°C in an atmosphere of 5% CO_2 in air. At the time of treatment, the culture media was removed from the Transwell® membrane so the cells could be exposed directly at the ALI. Then, four inserts were transferred to each exposure chamber containing 25 mL of Dulbecco's Modified Eagle Medium supplemented with 1% L-

Glutamine and 0.5% penicillin/streptomycin (10000 IU/mL – 10000uG/mL). The exposure chambers were then placed in an incubator at 37°C and connected with plastic tubing to the smoking machine as represented in Figure 1D (smoke in/smoke out connectors). The smoking machine pre-programmed with the appropriate dilutions was set for a 3 hour exposure. We selected a 3 hour exposure as it is the minimum recommended in the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH guidelines) (ICH, 2011). Following exposure, the inserts were placed in clean pre-labelled 24-well plates where the cells were fixed with 4% paraformaldehyde (100 µL/insert) and incubated for 15 minutes at room temperature. The fixed samples were processed for γH2AX immunostaining following manufacturer's recommendation (ThermoScientific, United States).

Image acquisition was performed using the Cellomics ArrayScan® VTI platform (ThermoScientific, USA). Image analysis used the Target Activation Bioapplication software v.6.6.1.4. The protocol was set to count a minimum of 500 cells per insert, giving a minimum of 2000 cells per concentration tested. Nuclear DNA staining (Hoechst dye) was used to identify viable cells nuclei. These nuclei were used as the target areas for the measurement of γH2AX specific fluorescence intensity represented as absolute intensity units. Viable cell counts from negative controls were defined as 100% cell viability. The viable cell counts in the WMCS and etoposide treated samples were then compared to those in the negative control, and the percentage cell viability was calculated and referred to as Relative Cell Counts (RCC).

2.7.- Data analysis and criteria

- Dilution performance evaluation

1-sample t-test was used to compare the results obtained in PPM for each dilution with the expected PPM. A variability of ±10% over the expected PPM was included afterwards as accepted measurement variation (Supplier Quality Requirements Taskforce 2002). Repeatability and Reproducibility statistics were computed for all data points according to ISO 5725 -2:1994 (ISO, 2013). Experiments were replicated 3 times, with 6 repeats per dilution per experiment. Data analysis and graphical representations were performed with Minitab software v.16.

- WMCS genotoxicity evaluation

The evaluation criteria used in this study (Table 3) was first described by Smart *et al.* for the analysis of γH2AX by flow cytometry (Smart *et al.*, 2011) and recently applied by Garcia-Canton *et al.* for the analysis of γH2AX by HCS (Garcia-Canton *et al.*, 2013a). Experiments were replicated at least three times and graphical representation was performed using GraphPad Prism software v.6.01.

Table 3: Genotoxicity evaluation criteria for the *in vitro* γH2AX assay by HCS, adapted from (Smart *et al.*, 2011).

γH2AX response	**Classification**
> 1.5-fold γH2AX @ RCC > 25%	Genotoxic (+)
< 1.5-fold γH2AX @ RCC 100-0%	Non-genotoxic (–)
> 1.5-fold γH2AX @ RCC < 25%	"False" positive; Cytotoxic-driven genotoxicity (C)
1.5-fold γH2AX @ RCC ≥ 25%	Equivocal (±)

3.- RESULTS

The Borgwaldt RM20S® smoking machine combined with British American Tobacco's chamber were used as an exposure system during the optimisation of the novel *in vitro* γH2AX assay by HCS for the evaluation of aerosols.

The initial steps in this optimisation included extending the QC checks of the RM20S® to include 14 dilution performance evaluations (Table 2). From the 10 dilutions generating accurate deliveries, 6 smoke dilutions were selected for further experiments based on range finder experiments (data not shown). The tested smoke dilutions covered a wide range of WMCS dilutions to assess the genotoxicity effect of two reference cigarettes (3R4F and M4A).

3.1.- Dilution performance evaluation

An initial range of 14 dilutions from 1:25 to 1:20,000 were selected to evaluate the actual dilution delivery in PPM units using reference methane gases (Table 2). The data in Figure 2 graphically represents the results from the statistical 1-sample t-test analysis performed comparing PPM obtained per dilution (box plot) against the expected PPM (red dot), the analysis did not incorporate the ±10% tolerance accepted for machinery measurement variation and was, therefore, added to the expected PPM value afterwards (Supplier Quality Requirements Taskforce 2002). Results indicate that in the majority of the cases (10 out of 14 dilutions) the dilution delivery was as expected when the ±10% tolerance was included in the analysis. There were four dilutions where the statistical analysis (1-sample t-test) showed a statistically significant difference between measured and expected PPM (including ±10% measurement variation), those dilutions are identified in Figure 2 with a hash (#) (1:1,000,

1:6,000, 1:8,000 and 1:20,000) and were not taken into consideration for the assessment of WMCS in the *in vitro* γH2AX assay.

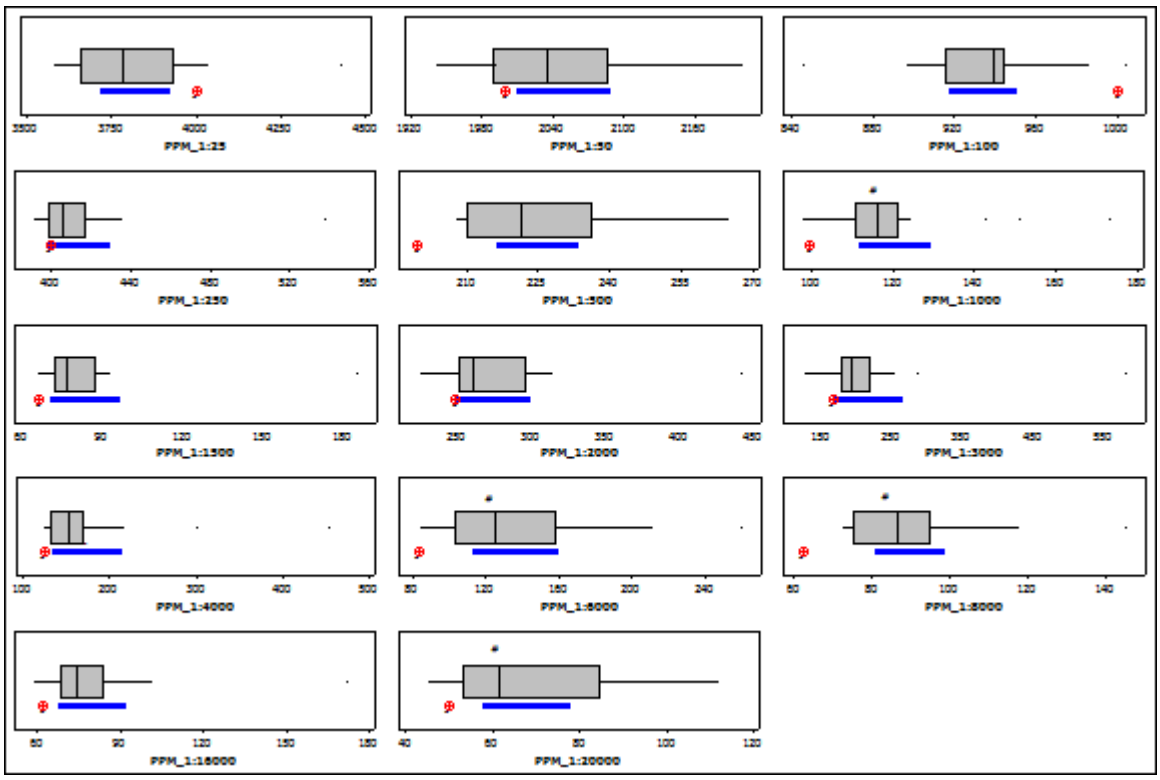

Figure 2: Test dilutions t-test boxplots. Expected PPM (red dot), 95% confidence interval from PPM results (blue line). The asterisk (*) indicates outliers while hash (#) indicates dilutions producing a significant different PPM than expected (confidence interval includes the 10% machinery measurement variability).

Figure 3 represents the repeatability and reproducibility results indicating the precision of the smoking machine dilution performance within the same experiment and in different experiments respectively. The repeatability and reproducibility increased linearly with concentration as expected.

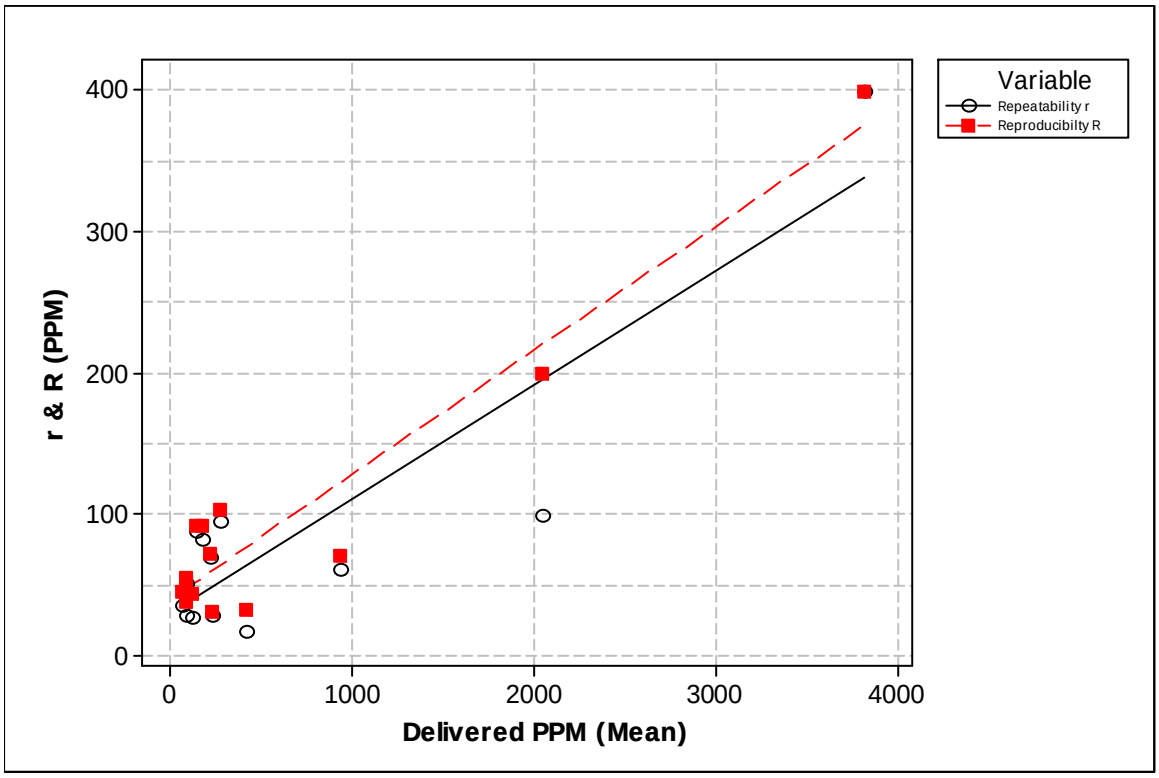

Figure 3: Scatterplot of repeatability (r) (-○-) and reproducibility (R) (-■-).

3.2.- WMCS genotoxicity assessment

Initial range finder experiments showed that 3 hour exposures to WMCS from 3R4F cigarettes at dilutions more concentrated than 1:500 produced tar depositions, this effect was considered equivalent to precipitation. Only dilutions greater than 1:500 were included in further experiments. Both reference cigarettes 3R4F and M4A produced a significant increase in γH2AX frequency (above 1.5-fold increase) compared to the air-treated control in all the dilutions tested (Figure 4). In all experiments the positive control etoposide produced an increase greater than 1.5-fold compared to the air-treated control (Figure 4A and 4B). Relative Cell Counts (RCC) for all results presented were above the acceptance limit of toxicity (RCC > 25%) and therefore no cytotoxic-driven genotoxicity was observed (Table 3).

Figure 4A illustrates the response produced after 3 hour exposure to 3R4F WMCS. A variation in the response can be observed between the most concentrated WMCS (1:500) and

the most diluted WMCS dilution (1:16,000). However, the linear regression model indicates a low correlation between the dose and the response (Pearson coefficient, r = -0.53). Figure 4B showed the results obtained after 3 hour exposure to M4A WMCS. In this case, a variation in the response can only be observed at the most diluted WMCS dilution tested (1:16,000). The linear regression model produced a low correlation between the dose and the response (Pearson coefficient r = -0.44). Figure 4C graphically represents the fold-induction results from both reference cigarettes. In general, 3R4F WMCS exposure seems to have a more potent genotoxic effect compared to M4A WMCS exposure, especially at the most concentrated dilution 1:500.

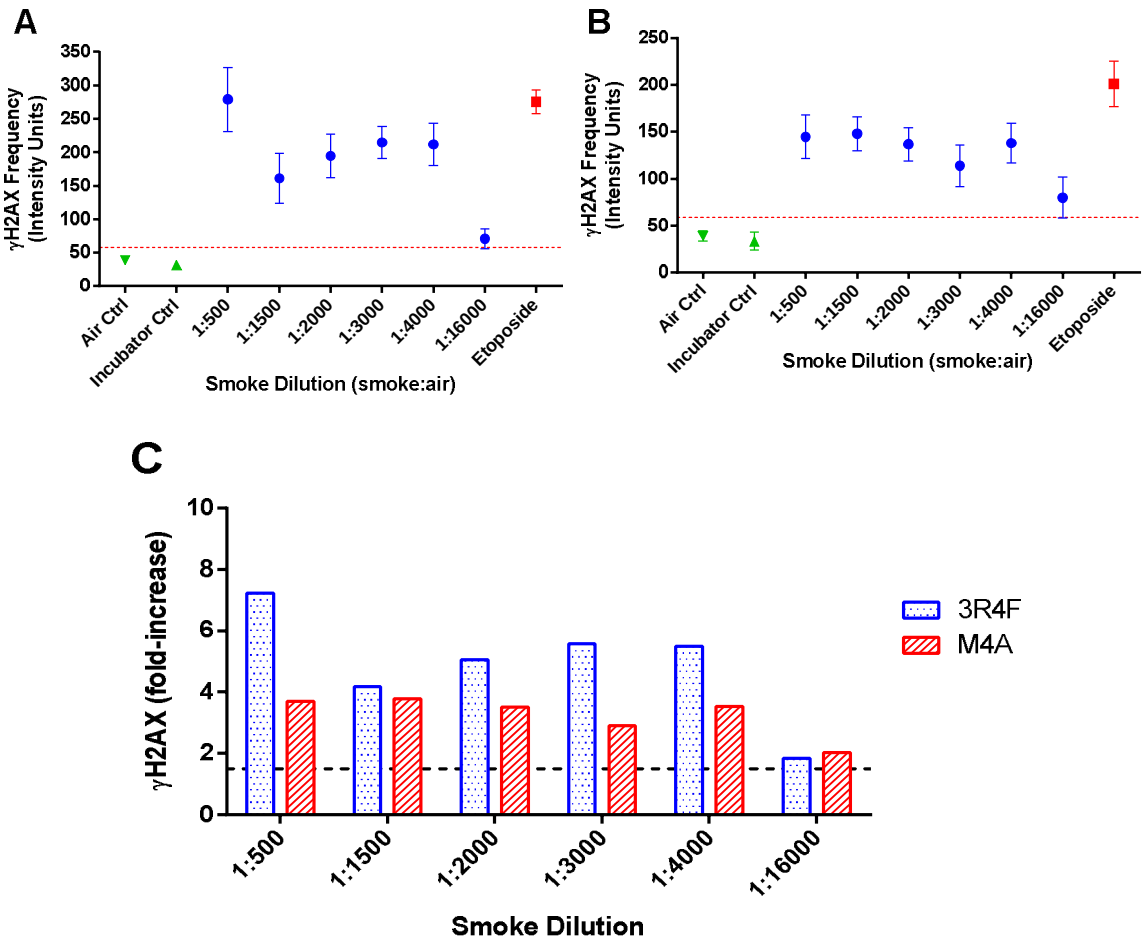

Figure 4: γH2AX frequency mean ±SD after 3 hour exposure to WMCS from reference cigarette [A] 3R4F, [B] M4A. Circle (-●-) represents WMCS results, square (-■-) represents positive control etoposide (1mM final), triangles (-▼- and -▲-) represents negative controls, air and incubator controls respectively and dotted red line represents the 1.5-fold increase over the air control indicating the threshold of genotoxic response. [C] γH2AX fold-induction for both reference cigarettes 3R4F (blue) and M4A (red), dotted line indicates genotoxic level (>1.5-fold γH2AX response).

4.- DISCUSSION

The main objective of this study was to optimise the novel *in vitro* γH2AX by HCS for the genotoxicity assessment of aerosols. During the optimisation, the genotoxic potential in the form of γH2AX induction from various dilutions of WMCS of two reference cigarettes were tested and differences in the response evaluated.

The cell system selected was the BEAS-2B cell line, a human-derived cell line from the lung, the first target tissue of inhaled aerosols. The non-tumorigenic human-derived BEAS-2B cell line was isolated from normal human epithelium and immortalised by virus infection (Reddel *et al*., 1988). The normal phenotype and wild-type p53 status support the use of this cell line in DNA damage studies (Svetlova *et al*., 2010; Petitjean *et al*., 2007; IARC, 2013). BEAS-2B cells, however, lack normal metabolic activity for the majority of cytochrome P450 family, an essential factor for the phase I bioactivation of some cigarette smoke toxicants such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Garcia-Canton *et al*., 2013c). The limitation in the metabolic capability of the cell line would need to be considered in future experimental designs i.e. including an external source of metabolic activation in part of the experiments to have a more comprehensive genotoxicity evaluation of the WMCS.

The Borgwaldt RM20S® smoking machine has been extensively used for the *in vitro* evaluation of WMCS (Massey *et al*., 1998; Phillips *et al*., 2005; Thorne *et al*., 2009). Although, some QC analyses have been reported for the accurate performance of the syringes (Kaur *et al*., 2010; Adamson *et al*., 2011) further QC tests for the accurate performance of the programmed dilutions have proved necessary. Our results in this study indicate that not all of the programmed dilutions deliver the expected amount of reference gas in PPM (Figure 2). We have observed that higher dilutions seem to produce less accurate deliveries; this effect could be caused by the smoking machine dilution programming. The smoking machine

performs a multistep process to dilute WMCS with laboratory conditioned air, the process requires the programming of more dilution steps for more higher dilutions, hence, the potential for more variation. The discrepancy between expected and delivered aerosol could affect the exposure to the cell cultures and ultimately the outcome of the assay. The same approach could be applied in the future to the particulate phase expected in the different dilutions employing Quartz Crystal Microbalances (QCM) previously described for this aerosol exposure system (Adamson *et al.*, 2012). Nevertheless, the smoking machine performance has shown an overall good reproducibility and repeatability from dilutions delivering 50 PPM or above as can be seen in Figure 3. The performance of syringes and dilutions can be carried out using the same methodology and apparatus already in place for the standard QC checks. Moreover, the extended QC check could easily be incorporated into the routine service of the Borgwaldt RM20S® smoking machine.

The γH2AX results obtained during the assessment of two reference cigarettes seem to indicate that the *in vitro* γH2AX assay by HCS was able to detect the genotoxic potential of WMCS, however the correlation between the dose and the response was low for both reference cigarettes evaluated in this study across all the tested dilutions (Figure 4). Nevertheless, the γH2AX response obtained after BEAS-2B cells were exposed to a range of 3R4F WMCS dilutions for 3 hours was in general more potent than the response obtained for M4A WMCS, and can be visually observed in Figure 4C. If the genotoxicity response was primarily associated to the gas phase we would have expected a better γH2AX dose-response correlation with the different dilutions tested. Therefore, we have considered that the particulate phase may have a significant effect in driving the genotoxic potential. This could be further investigated by characterising the level of particulates deposited at different dilutions with tools such as the QCM balance mentioned earlier in this discussion.

It is important to notice that 3 hour continuous exposure as recommended by ICH guidelines (ICH, 2011) could be the longest exposure time a submerged monolayer culture might be exposed at the ALI. In our experiments, the cell cultures were immediately fixed after the exposure to evaluate the DNA damage in the form of γH2AX. Pilot experiments were conducted where the cell cultures were left to recover for a further 24 hours submerged in media to evaluate potential DNA repair after the acute 3 hour exposure. The proliferation of the BEAS-2B cells was greatly affected in WMCS and air control samples. Interestingly, the same effect was not observed in incubator control cultures where the humidity is maintained at a higher level (data not shown). We concluded that for *in vitro* assays using submerged cultures as cell systems, 3 hour exposure at the current conditions of ALI exposure system would cause irreversible damage due to drying as opposed to aerosol exposure.

Following the optimisation described in this study, further investigations employing different exposure times, a larger number of products and an external source of metabolic activation would be necessary to support the applicability of the *in vitro* γH2AX assay for the evaluation of tobacco products in aerosol exposure. Future work could also carry out an in-depth characterisation on the effect that product variations such as different tobacco blends have in γH2AX induction to understand the differences in response.

Nevertheless, the optimisation performed here could also be applied to the genotoxicity evaluation of other aerosols such as aerosolised drugs, pollutants and cigarette smoke toxicants present in the gas phase (e.g. benzene).

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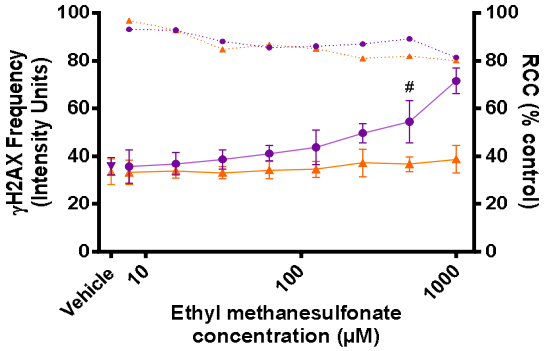
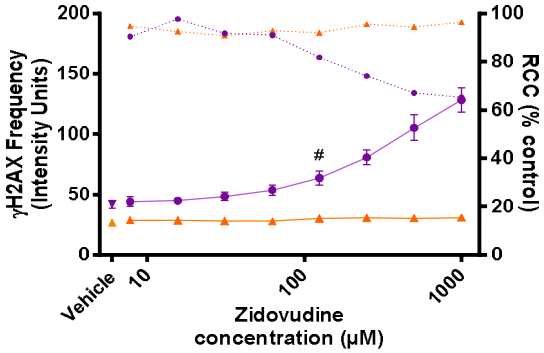
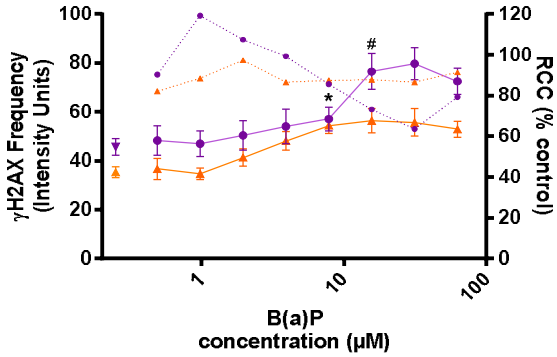
APPENDICES

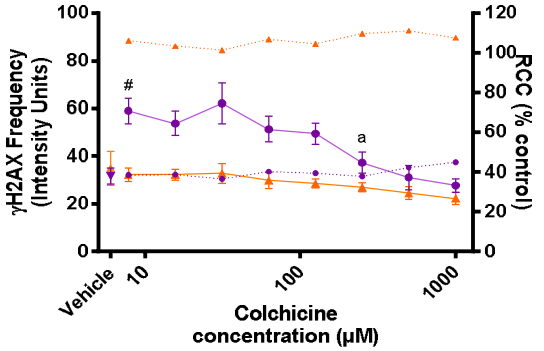
APPENDIX 1 – SUPPLEMENTARY DATA

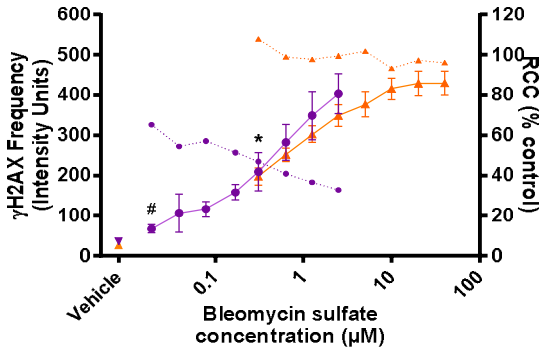
CHAPTER III

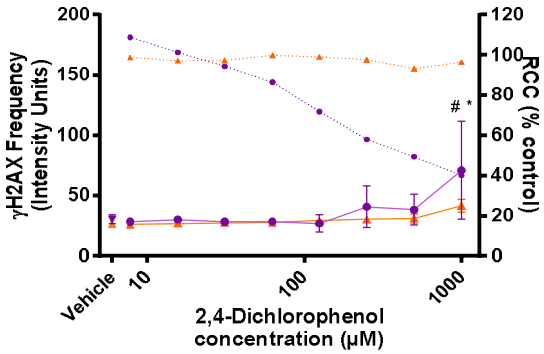


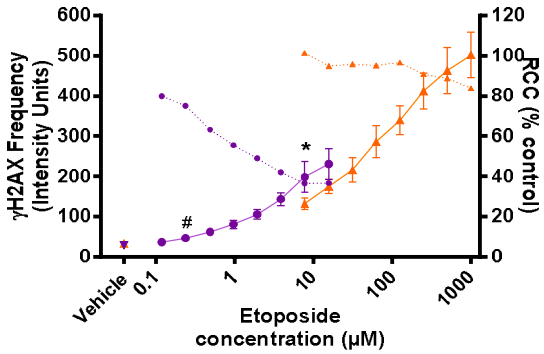


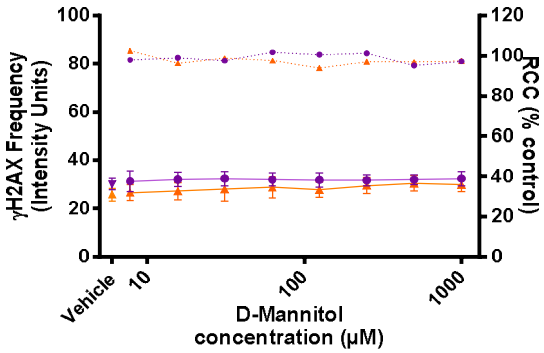


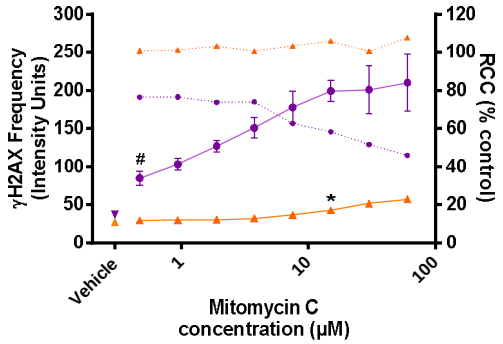


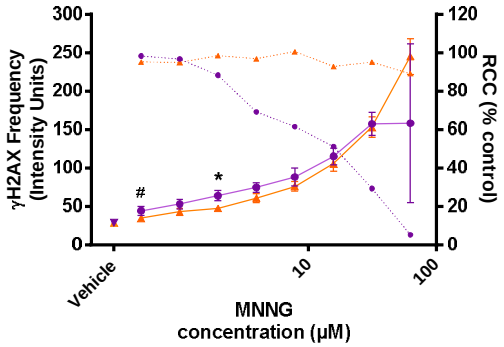


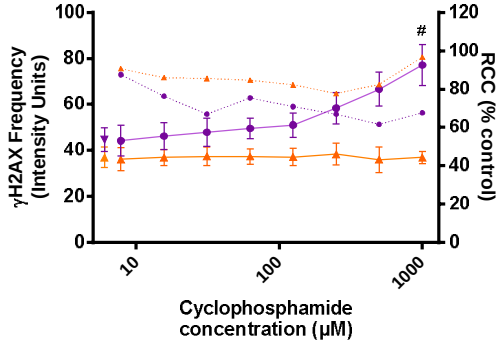


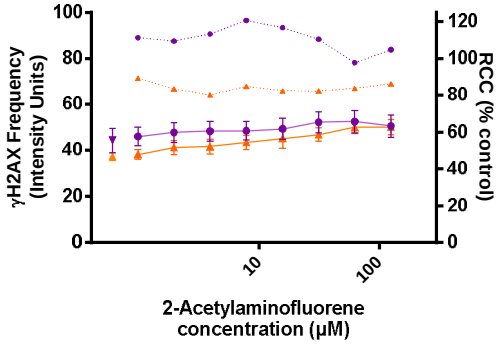


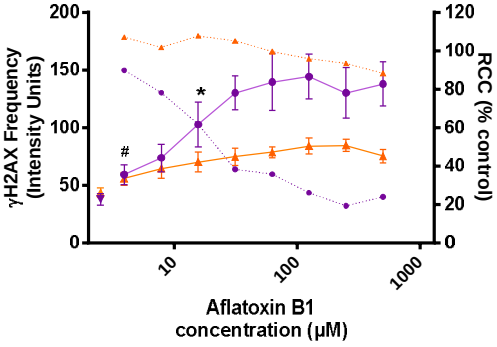


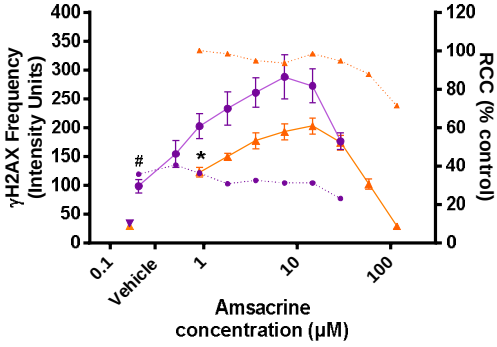

γH2AX Frequency (Intensity Units)
RCC (% control)
Vehicle
1
10
100
Mitomycin C concentration (μM)

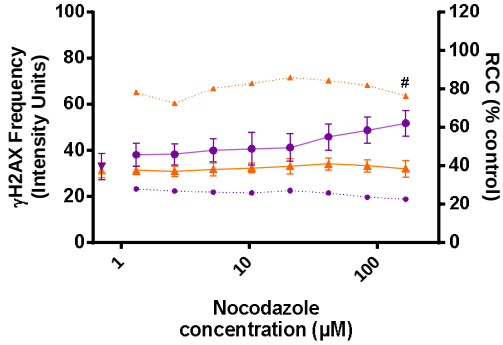

γH2AX Frequency (Intensity Units)
RCC (% control)
Vehicle
10
100
MNNG concentration (μM)

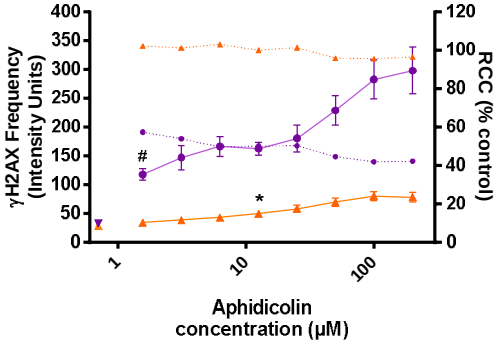

γH2AX Frequency (Intensity Units)
RCC (% control)
10
100
1000
Cyclophosphamide concentration (μM)

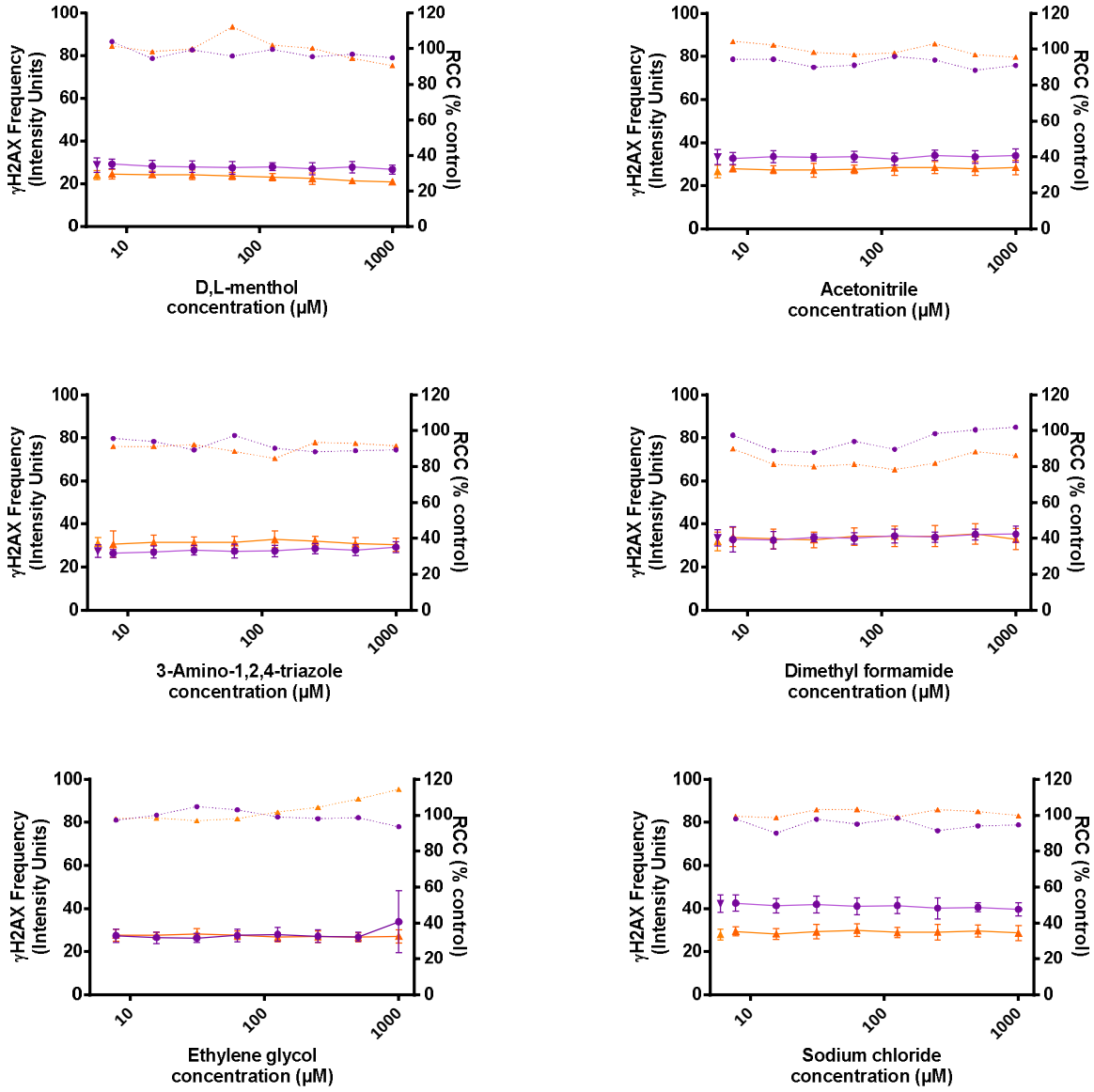

γH2AX Frequency (Intensity Units)
RCC (% control)
10
100
2-Acetylaminofluorene concentration (μM)


γH2AX Frequency (Intensity Units)
RCC (% control)
10
100
1000
Aflatoxin B1 concentration (μM)

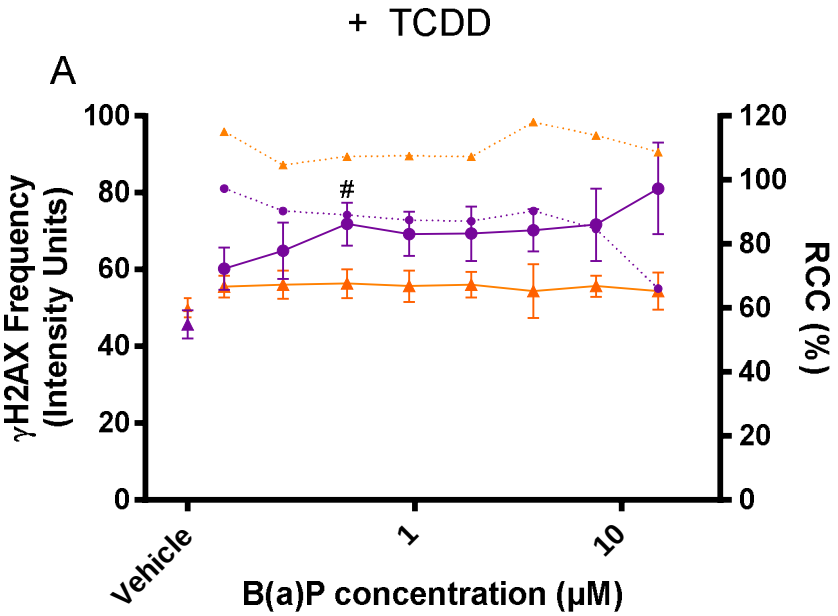

γH2AX Frequency (Intensity Units)
RCC (% control)
0.1
Vehicle
1
10
100
Amsacrine concentration (μM)

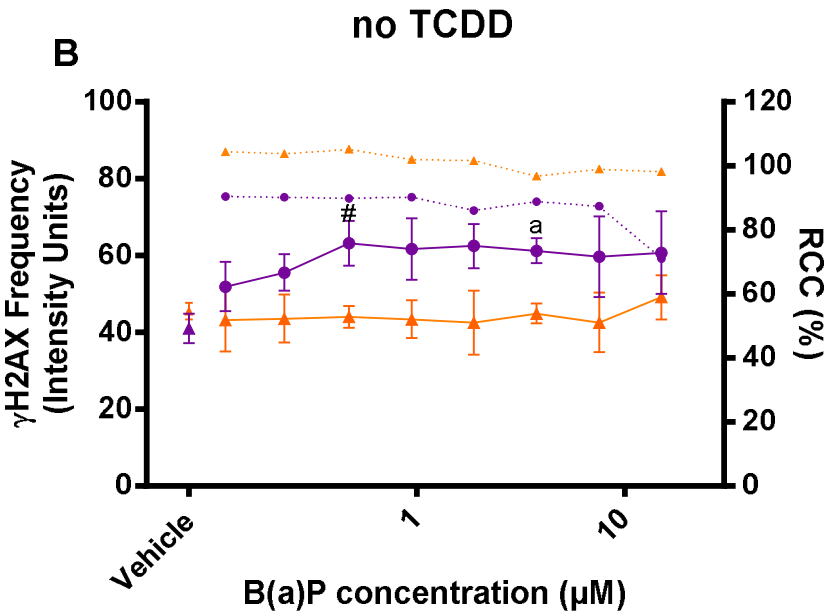

γH2AX Frequency (Intensity Units)
RCC (% control)
1
10
100
Nocodazole concentration (μM)

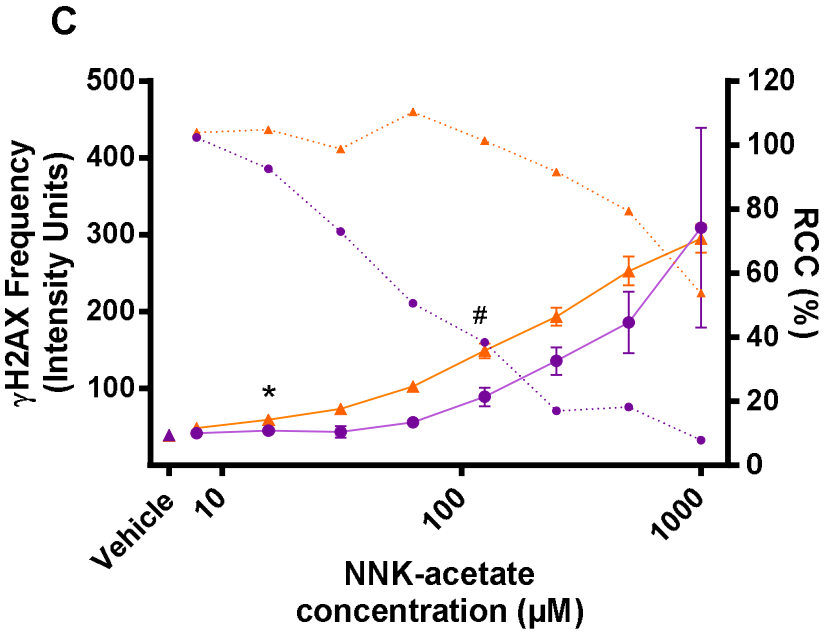

γH2AX Frequency (Intensity Units)
RCC (% control)
1
10
100
Aphidicolin concentration (μM)

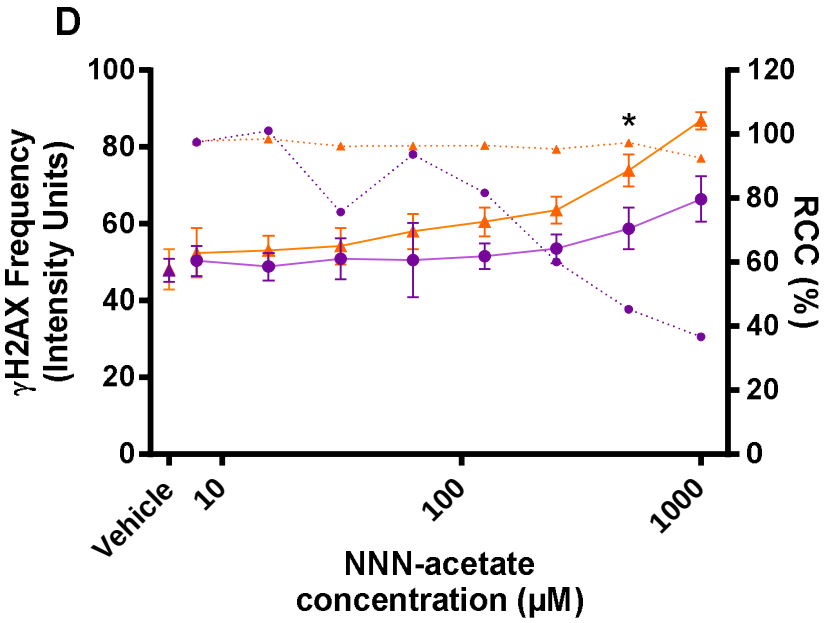


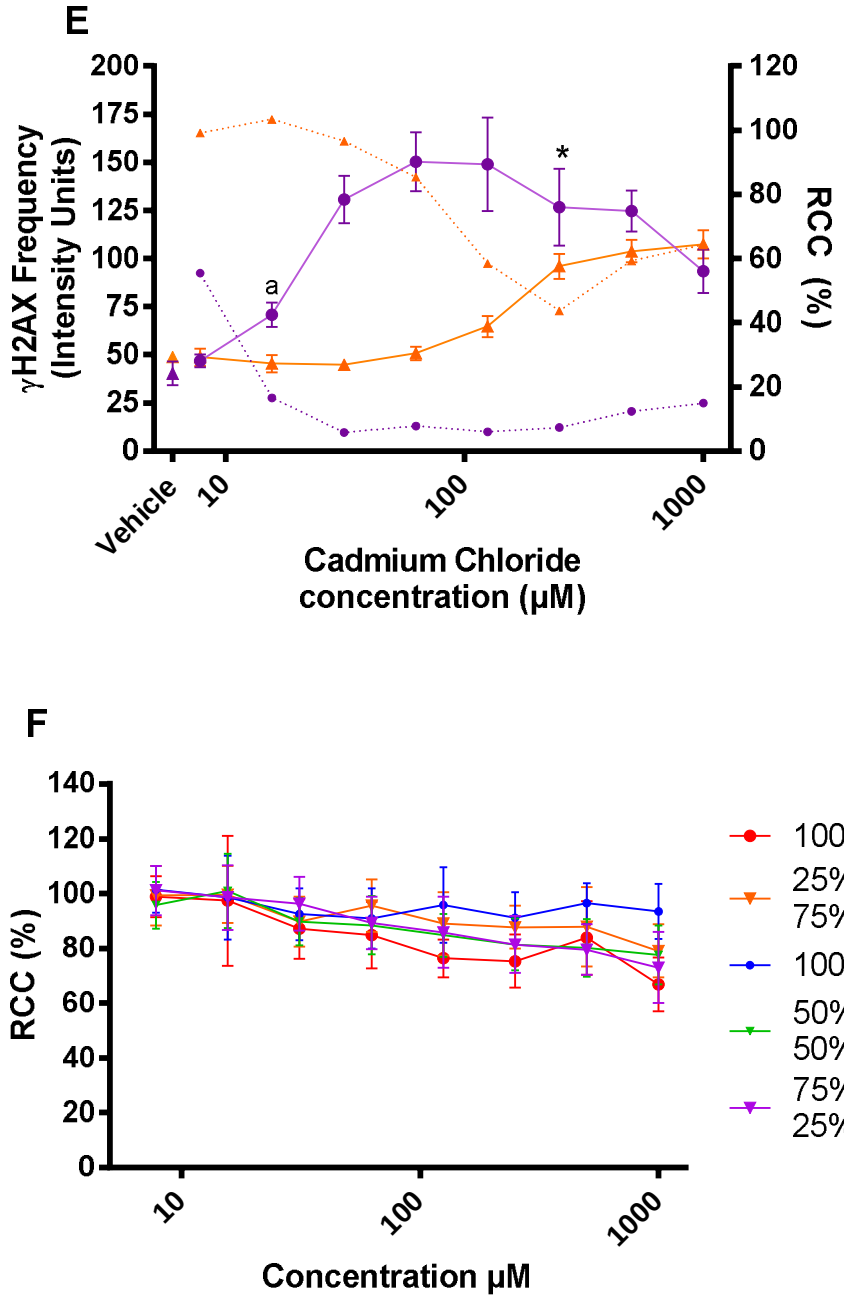
Supplementary graphs: graphical representation from the 22 compounds used during the assessment of the *in vitro* γH2AX assay. Left Y-axis represents γH2AX frequency (continuous line) and right Y-axis represents cell viability (%) (dotted line). Triangle (-▲-) represents short treatment and circle (-●-) represents long treatment. Asterisk (*) indicates the minimum concentration that shows genotoxicity compared to the vehicle treated control after short treatment. Hash (#) indicates the minimum concentration that shows genotoxicity compared to the vehicle treated control after long treatment.


+ TCDD
A
γH2AX Frequency (Intensity Units)
RCC (%)
100
80
60
40
20
0
120
100
80
60
40
20
0
#
Vehicle
1
10
B(a)P concentration (μM)


no TCDD
B
γH2AX Frequency (Intensity Units)
RCC (%)
100
80
60
40
20
0
120
100
80
60
40
20
0
#
a
Vehicle
1
10
B(a)P concentration (μM)


C
γH2AX Frequency (Intensity Units)
RCC (%)
500
400
300
200
100
120
100
80
60
40
20
0
*
#
Vehicle
10
100
1000
NNK-acetate concentration (μM)


D
γH2AX Frequency (Intensity Units)
RCC (%)
100
80
60
40
20
0
120
100
80
60
40
20
0
*
Vehicle
10
100
1000
NNN-acetate concentration (μM)



Supplementary graphs: graphical representation from the 4 cigarette smoke toxicants tested in the *in vitro* γH2AX assay. [A to E] Left Y-axis represents γH2AX frequency (continuous line) and right Y-axis represents cell viability (% RCC) (dotted line). Triangle (-▲-) represents short treatment and circle (-●-) represents long treatment. Asterisk (*) indicates the minimum concentration that shows genotoxicity compared to the vehicle treated control after short treatment. Hash (#) indicates the minimum concentration that shows genotoxicity compared to the vehicle treated control after long treatment. [F] Graphical representation of the mixture permutation cell viability (% RCC).

APPENDIX 2 – ACRONYMS

2AAF – 2-Acetylaminofluorene

8-MOP – 8-Methoxypsoralen

ALI – Air Liquid Interfase

ATM – Ataxia Telangiectasia-Mutated

ATR – ATM- and Rad3-Related

aut-aucPAGE – acid-urea-cetyltrimethylammonium bromide Polyacrylamide Gel Electrophoresis

B[a]P – Benzo[a]pyrene

BEGM – Bronchial Epithelial Growth Medium

BPDE – Benzo[a]pyrene Diol-Epoxide

BSA – Bicinchoninic Acid protein Assay

CBMN – Cytokinesis-Blocked Micronucleus

cDNA – complementary DNA

CHO – Chine Hamster Ovary

CSC – Cigarette Smoke Condensate

CSE – Cigarette Smoke Extract

CSS – Cigarette Sidestream Smoke

Ct – threshold Cycle

COM – Committee Of Mutagenicity

CYP – Cytochrome P450

DDR – DNA Damage Response

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimetyl Sulfoxide

DNA/ADN - Deoxyribonucleic Acid / Ácido Desoxirribonucleico

DNA-PKc – DNA-dependent Protein Kinase

DSBs – Double Strand Breaks

ECACC – European Collection of Cell Cultures

ECVAM – European Centre for the Validation of Alternative Methods

ELISA – Enzyme-Linked Immunosorbent Assay

FDA – Food and Drug Administration

FDA CTP – Food and Drug Administration Center for Tobacco Products

FISH – Fluorescence *In Situ* Hybridization

GFP – Green Fluorescent Protein

HCS – High Content Screening

HPHCs – Harmful and Potentially Harmful Constituents

Hprt – Hypoxanthine-guanine phosphoribosyl transferase

IARC – International Agency for Research on Cancer

ICH – International Conference on Harmonisation

iPS – induced Pluripotent Stem cell

ISO – International Standard Organization

JRC – Journal Citation Reports

LDH – Lactate Dehydrogenase

Luciferin-CEE – Luciferin 6' Chloroethyl Ether

MI – Mitotic Index

MLA – Mouse Lymphoma Assay

MN – Micronucleus

MNNG – Methylnitronitrosoguanidine

MoA – Mode of Action

M-PER – Mammalian Protein Extraction Reagent

MRN – MRE11-RAD50-NBS1 complex

mRNA – messenger RNA

NC3Rs – National Centre for the Reduction, Refinement and Reduction of Animals in Research

NHBE – Normal Human primary Bronchial Epithelium

NNK – 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone

NNN – *N´*-nitrosonornicotine

OECD – Organisation for Economic Co-operation and Development

PAH – Polycyclic Aromatic Hydrocarbons

PBS – Phosphate Buffered Saline

PCA – Principal Component Analysis

PM – Particulate Matter

PIKK – Phosphatidylinositol 3-Kinase-like family of Kinases

PPM – Parts Per Million

QCM – Quartz Crystal Microbalances

qPCR – quantitative Polymerase Chain Reaction

RCC – Relative Cell Count

RH – Relative Humidity

RLU – Relative Luminescence Units

RNA – Ribonucleic Acid

ROS – Reactive Oxygen Species

RPD – Relative Population Doubling

SARs – Structure-Activity Relationships

SD – Standard Deviation

SSB – Single Strand Break

TCDD – 2,3,7,8-tetraclorodibenzo-p-dioxina

TK – Thymidine Kinase

T&N-free – Tobacco and Nicotine free

TPM – Total Particulate Matter

trpE – tryptophan synthesis operon

TSNAs – Tobacco Specific Nitrosamines

WHO – World Health Organisation

WHO FCTC – World Health Organisation Framework Convention on Tobacco Control

WMCS – Whole Mainstream Cigarette Smoke

WoE – Weight of Evidence