UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

EFEITOS BIOLÓGICOS DO MATERIAL PARTICULADO GERADO EM SISTEMAS DE MINERAÇÃO DE CARVÃO A CÉU ABERTO EM POPULAÇÕES HUMANAS COM EXPOSIÇÃO AMBIENTAL

MSc. Lyda Espitia-Pérez

Programa de Pós-Graduação em Biologia Celular e Molecular – UFRGS

Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular da UFRGS como requisito parcial para a obtenção do grau de Doutor.

Orientador

Prof. Dr. João Antonio Pêgas Henriques

Porto Alegre, novembro de 2016

Indígena Wayúu tejiendo una hamaca en Media Luna, Guajira

À Jesus, o amigo que nunca falha

À meus pais, meus cúmplices eternos

Aos meus irmãos, minha fonte de energia

À minha família, tios, primos e afilhados, por ser sempre minha rede de apoio

Aos meus amigos, que mais do que isso, são parceiros e parte vital da minha vida

A ciência será sempre uma busca, jamais uma descoberta.

É uma viagem, nunca uma chegada.

Karl Popper

INSTITUIÇÕES E FONTES FINANCIADORAS

AGRADECIMENTOS

Ao Professor Henriques, por acreditar em mim, por apoiar o meu sonho de estudar no Brasil e por se arriscar em me orientar ainda sem me conhecer. Pelo exemplo, a compreensão e o conhecimento compartilhado, muito obrigada.

À Professora Juliana da Silva, quem sempre esteve disposta a me ajudar em tudo, por ter sempre palavras de encorajamento e otimismo, pela valiosa amizade e pela valiosa ajuda no desenvolvimento deste trabalho.

À Vilma e Juan, meus pais, meus eternos cúmplices e o maior suporte da minha vida, pelos sacrifícios e pelas palavras de encorajamento. Obrigada por ir junto neste sonho. Minhas conquistas, mais do que minhas, são de vocês.

Aos meus irmãos, Pedro e Juan David, os melhores amigos e parceiros que alguém possa pedir!!

À Jhonji, meu melhor cúmplice e parceiro, agradeço a Deus tua presença na minha vida cada dia.

Aos Professores membros da minha Comissão de Acompanhamento: Prof. Dr. Guido Lenz e Profa. Jennifer Saffi, pelo apoio e a ajuda constante.

Às minhas comadres Cata, Luchy e Dina, pela amizade sincera e o apoio em cada passo. Sem vocês loucas as coisas não seriam iguais.

Ao meu grupo de trabalho: Shirley Salcedo, Karina Pastor, Pedro Espitia, Claudia Galeano, Ana Toscano, Daniela Salcedo e Miguel Gómez, pela ajuda durante a fase experimental, as madrugadas em espera das amostras, as batalhas nas coletas do material particulado e a amizade e bons momentos que sempre prevaleceram ainda nos momentos difíceis.

Ao Hugo e à Marcia, pela amizade, a paciência e as excelentes contribuições ao trabalho.

À Victoria, pela amizade, a ajuda e apoio constante e as palavras com efeito Bon Ice nos momentos mais difíceis!!

Ao professor Milton, por ser o primeiro em infundir em mim os desejos de pesquisar e estudar sem importar as barreiras, pelas palavras de alento nos momentos de dúvida ou tristeza, pela luta conjunta, pela amizade... Obrigada maestro.

Ao CORPOGUAJIRA, especialmente ao Engenheiro Javier Calderón, pela colaboração na análise dos resultados e o interesse em apoiar as atividades em campo.

À Dayana Agudelo, pela amizade e as excelentes contribuições na análise dos resultados do trabalho.

Ao Sindicato de Trabalhadores do Cerrejón, pela ajuda na realização do estudo e pela boa disposição.

Às comunidades e líderes indígenas e Afro-colombianos pela confiança e disposição para participar no estudo.

À todo o corpo de professores do Programa de Pós-graduação em Biologia Celular e Molecular, pelos conhecimentos compartilhados e os valiosos ensinamentos.

INDICE GERAL

LISTA DE FIGURAS

LISTA DE TABELAS

ABREVIATURAS

ATM Ataxia-Telangiectasia Mutated ou proteína mutada na ataxia telangectasia

ATR Ataxia Telangiectasia and Rad3-related protein ou Cinase ataxia telangiectasia e RAD3 relacionada

BMNCyt Buccal Mucosa Cytome Assay ou Teste de MN em Mucosa Oral

BN Células binucleadas

CBMN-cyt Cytokinesis-block micronucleus cytome assay ou Ensaio do micronúcleo citoma com bloqueio da citocinese.

CC Células com cromatina condensada

CREST Calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia ou Calcinose, Fenômeno de Raynaud, Dismotilidade Esofágica Esclerodactilia Telangiectasia

CWP Coal workers' pneumoconiosis ou Pneumoconiose dos Trabalhadores do Carvão

ENDO III Endonuclease III

EOM Extracted organic matter ou matéria orgânica extraída

EPA U.S. Environmental Protection Agency ou Agência de Proteção Ambiental do Estados Unidos

FPG Formamidopirimidina DNA glicosilase

IARC Agência Internacional de Pesquisa do Câncer

KHC Células cariorréticas

KYL Células cariolíticas

MN Micronúcleos

MNBN Micronúcleo em células binucleadas

MNMONO Micronúcleo em células mononucleadas

NBUDS Nuclear buds ou Brotos nucleares

PAH / HAP Polycyclic Aromatic Hydrocarbons ou Hidrocarbonetos Aromáticos Policíclicos

PIXE Particle-induced X-ray emission ou Emissão de raios-X induzidas por partículas

PM Particulate matter ou material particulado

p.p.m Partes por milhão

PTFE Politetrafluoretileno ou teflon

PYK   Células picnóticas

ROS / ERO   Reactive Oxygen Species ou Espécies Reativas de Oxigênio

%Tail DNA   Percentagem de DNA na cauda

RESUMO

A Colômbia tem uma das maiores reservas de carvão do mundo, sendo o quinto exportador mundial do carvão de tipo térmico. Nos processos de extração de carvão a céu aberto, uma grande quantidade de material particulado (PM), constituído por partículas de pó, hidrocarbonetos e metais pesados, é liberada à atmosfera, onde pode formar misturas complexas e constituir um risco significativo para a saúde, tanto dos indivíduos com exposição ocupacional quanto dos que habitam em proximidade das áreas de mineração. No entanto, em populações humanas, a grande maioria dos dados sobre os efeitos da exposição ao carvão tem sido gerada a partir de estudos em populações com exposição ocupacional, o que faz com que os possíveis efeitos sobre as populações com exposição ambiental sejam desconhecidos. Considerando a pouca informação sobre o tipo de efeitos gerados pela exposição ambiental à resíduos de mineração de carvão, e com a finalidade de melhorar o conhecimento sobre o possível mecanismo do dano em diferentes tecidos, este trabalho teve como objetivo avaliar os efeitos genotóxicos e citotóxicos em populações de indivíduos com exposição ambiental à misturas complexas geradas nos sistemas de mineração de carvão a céu aberto do Departamento de Guajira, Colômbia. Para isso, foram coletados sangue e mucosa oral de 98 indivíduos residentes nas proximidades de uma mina de carvão a céu aberto e de 41 indivíduos não expostos ao carvão. O teste de CBMN-Cyt em linfócitos e BMNCyt em mucosa oral, o ensaio cometa alcalino convencional e modificado com endonucleases (FPG, ENDO III), imunocoração com anticorpos anti-centrômero (CREST), bem como o conteúdo de elementos traço e metais pesados foram avaliados nas amostras. Adicionalmente, as concentrações de PM_{10} e $PM_{2.5}$ nas áreas de coleta nas proximidades da mina foram estabelecidas e avaliadas para a presença de metais e matéria orgânica. A análise dos biomarcadores do CBMN-Cyt em linfócitos evidenciou um aumento significativo na frequência de micronúcleos em células binucleadas (MNBN) e células mononucleadas (MNMONO) dos indivíduos com proximidade residencial às áreas de exploração de carvão a céu aberto. As análises sobre a origem dos MN demonstraram um aumento significativo de 45.27% na frequência de CREST+ MN nos indivíduos expostos, sugerindo a exposição à substâncias com potencial aneugênico. Particularmente, as frequências de MNBN e a indução de CREST+ MN em residentes expostos demonstraram uma correlação altamente significativa com os níveis de $PM_{2.5}$, mas não com as concentrações de PM_{10}. A análise geoespacial demonstrou que esta correlação é proporcional à distância entre as populações e as zonas de mineração e que o relevo e a velocidade do vento estão envolvidos na distribuição do PM e o nível de efeito observado em algumas áreas avaliadas. A composição química do $PM_{2.5}$, avaliada pela técnica de PIXE, evidenciou a presença de elementos traço altamente enriquecidos como o S, e metais pesados moderadamente enriquecidos como Cr, Cu e Zn. O $PM_{2.5}$ das regiões de mineração também apresentou altas concentrações de matéria orgânica (EOM) com características apolares. Da mesma forma que nos linfócitos, a mucosa oral também mostrou um aumento significativo na frequência de todos os parâmetros do BMNCyt, enquanto que a %Tail DNA, avaliadas no ensaio Cometa convencional e modificado com o uso de endonucleases (FPG e ENDO III), também mostrou um aumento significativo em indivíduos com proximidade residencial às minas de exploração de carvão à céu aberto. Entre os indivíduos expostos, foram detectadas elevadas concentrações sanguíneas de Cr, Ni, Mn e Br quando comparados com os indivíduos não expostos. Os valores de %Tail DNA no ensaio Cometa convencional foram altamente correlacionados com as concentrações de Al, Mn e Br no sangue, enquanto que o %Tail DNA no ensaio Cometa modificado com FPG foi relacionado com as concentrações de Mn. Assim, o dano ao DNA observado nos indivíduos com exposição ambiental à resíduos de mineração de carvão pode ser consequência do dano oxidativo resultante da exposição aos resíduos do carvão, especialmente metais e matéria orgânica contidos na fração fina do material particulado.

ABSTRACT

Colombia has one of the world's largest coal reserves being the fifth biggest thermal coal exporter world-wide. In open-cast coal mining extraction, large amounts of particulate matter (PM) constituted by dust particles, hydrocarbons and heavy metals are released into the atmosphere, where they can form complex mixtures, representing a significant health risks to both, occupationally exposed workers and populations living in proximity to mining areas. However, in human populations, most of the data on the effects of exposure to coal residues have been obtained from studies involving occupationally exposed populations, causing a scarcity of data examining the impact of these industrial operations in populations with environmental exposure.

Considering the lack of information in regard to the effects of environmental exposure to coal mining residues, and in order to improve our knowledge on the possible damaging mechanism, the aim of this study was to evaluate the genotoxic and cytotoxic effects caused by environmental exposure to complex mixtures generated in open-cast coal mining systems located in the Department of Guajira-Colombia. We collected blood and oral mucosa cells from 98 individuals residents in proximity to an open-pit coal mine and 41 unexposed individuals. We assessed the CBMN-Cyt parameters in lymphocytes and BMNCyt parameters in oral mucosa, the alkaline and endonucleases modified comet assay (FPG, ENDO III), the immunofluorescent antikinetochore staining (CREST) and trace and heavy metals content in samples from exposed individuals. Additionally, PM_{10} and $PM_{2.5}$ concentrations were established in sampling areas around the mining zone and metals and organic matter content determined.

Analysis of CBMN-Cyt parameters revealed a significant increase in micronuclei frequency in binucleated (MNBN) and mononucleated (MNMONO) cells of individuals with residential proximity to open-pit coal mines compared to residents from non-mining areas. Results on the mechanism of micronucleus formation, showed a statistically significant increase in CREST+ MN (45.27%) in exposed individuals, suggesting the exposure to aneuploidy-inducing substances. Particularly, MNBN frequency and CREST+ MN induction were highly correlated to $PM_{2.5}$ levels but not to PM_{10}. Spatial interpolation analysis showed that this correlation is proportional to the distance between populations and mining areas. Local wind speed and topography were identified as major contributors to PM dispersion and damage distribution in some areas.

Chemical composition of $PM_{2.5}$ by PIXE demonstrated the presence of highly enriched elements like S and moderate enrichment of heavy metals like Cr, Cu and Zn. Mining regions had also higher concentrations of extractable organic matter (EOM) related to nonpolar and polar compounds.

Like in lymphocytes, oral mucosa cells also showed a significant increase of all BMNCyt parameters, while %TailDNA in conventional and endonuclease modified comet assay (FPG and ENDO III) also showed a significant increase in individuals living in proximity to open-pit coal mining areas.

Exposed individuals showed higher concentrations of Cr, Ni, Mn and Br in blood compared to unexposed controls. %Tail DNA in conventional Comet assay was highly correlated with Al, Mn and Br concentrations, while %Tail DNA in the FPG Comet assay was correlated to Mn concentrations.

These results suggest that DNA damage detected in environmentally exposed individuals may be caused by oxidative damage caused by exposure to coal mining residues, particularly metals and organic matter contented in the fine fraction of the particulate matter.

ESTRUTURA DO TRABALHO

O presente trabalho está estruturado em Introdução, Objetivos (gerais e específicos), três capítulos escritos na forma de artigos científicos, Discussão Geral, Conclusões, Perspectivas, Referências e Anexos.

A Introdução contém informações gerais sobre o carvão, classificação, uso, produção mundial, produção na Colômbia, métodos de exploração, mineração a céu aberto, resíduos gerados durante a exploração, efeitos sobre a saúde do material particulado, hidrocarbonetos aromáticos policíclicos e metais pesados, e mecanismos celulares envolvidos no dano relacionado com a exposição.

O *Capítulo I* apresenta um trabalho manuscrito que abordou os efeitos da exposição sobre o DNA utilizando o ensaio CBMNCyt e a imunotinção com CREST para a detecção de efeitos aneugênicos e clastogênicos. O capítulo também analisou a influência das concentrações do material particulado nas áreas de mineração e alguns aspectos da constituição química na geração do dano.

O *Capítulo II* discute os efeitos da exposição em células do epitélio oral e a indução de dano primário e oxidativo em linfócitos, utilizando o teste BMNCyt e o ensaio Cometa alcalino convencional e modificado com o uso de endonucleases (FPG e ENDO III). Como complemento, a indução do dano foi avaliada em função do conteúdo de metais no sangue dos indivíduos.

O *Capítulo III* explora o uso de métodos de SIG e IDW para avaliar a relação entre exposição ao material particulado e distribuição do dano ao redor das áreas de mineração. Adicionalmente, o capitulo analisa correlações geográficas entre o enriquecimento de determinados metais presentes no material particulado e a geração de dano.

A seguir, é apresentada uma discussão geral que integra os resultados dos três capítulos descritos, as possíveis hipóteses que poderiam explicar os resultados obtidos e sua contribuição científica no campo da toxicologia ambiental.

Por fim, são apresentadas as Conclusões**,** Perspectivas e Anexos do presente trabalho, que reúnem as considerações finais e sugerem possibilidades de aprofundamento posterior.

Nos Anexos 1 e 2 encontram-se as publicações desenvolvidas durante o doutorado e que foram utilizados na discussão deste, e nos Anexos 3 e 4 estão o consentimento livre esclarecido para a coleta de amostras de sangue e o questionário utilizado para a coleta de informação demográfica das populações.

I. INTRODUÇÃO
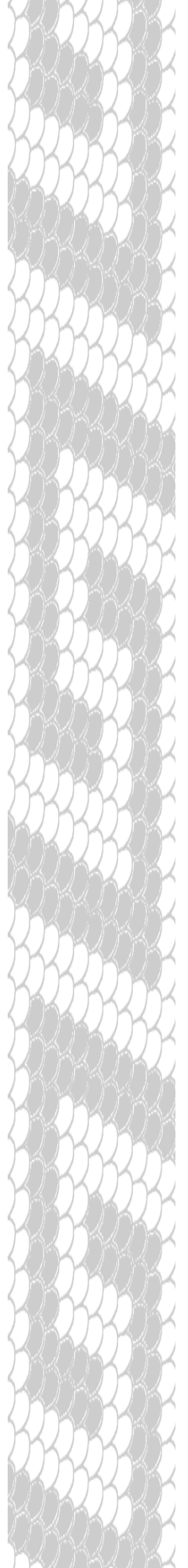
I. INTRODUÇÃO

1. Generalidades sobre o carvão

O carvão, assim como todos os combustíveis fósseis, é uma complexa e variada mistura de componentes orgânicos sólidos, fossilizados ao longo de milhões de anos [1, 2]. Como mostrado na Fig. 1, o carvão possui em sua composição átomos de carbono, oxigênio, nitrogênio e enxofre, associados a outros elementos rochosos (como arenito, siltito, folhelhos e diamictitos) e minerais (como a pirita), sendo uma das maiores fontes naturais de hidrocarbonetos. Sua qualidade é determinada pelo conteúdo de carbono e varia de acordo com o tipo e o estágio dos componentes orgânicos [3].
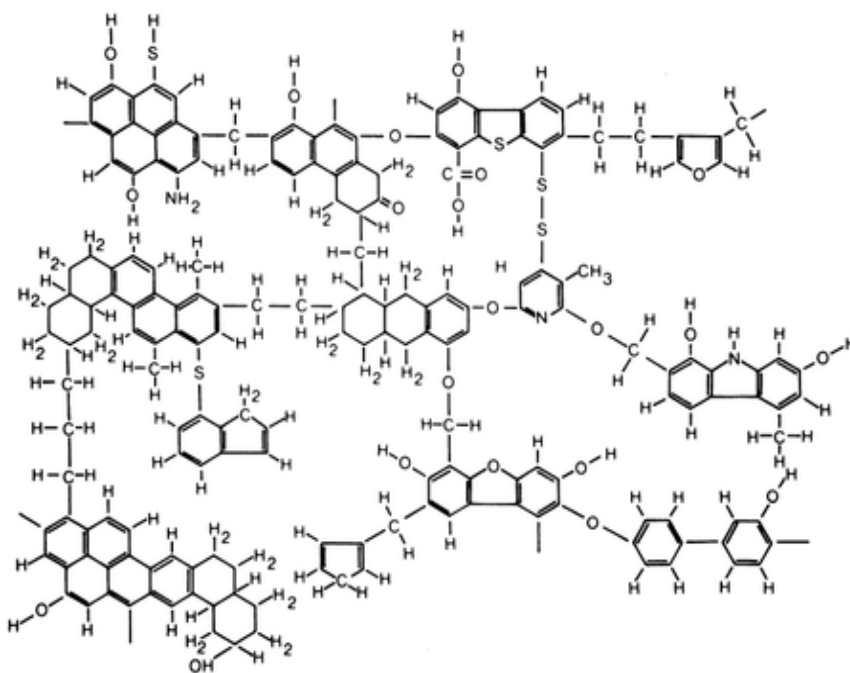
Figura 1. Estrutura química de um carvão sub-betuminoso.
Fonte: Extraído de Hill & Lyon [4].

1.1 Classificação e uso

Uma das classificações mais aceitas para o carvão corresponde à realizada pela Sociedade Americana de Testes e Materiais (ASTM D - 388-777) (Tabela 1). Conforme essa classificação, o carvão é dividido em quatro classes, levando em consideração a composição das plantas e as condições de pressão e temperatura (o grau de metamorfismo) às quais foram submetidos durante a sua formação [5]:

-**Antracite:** ou hulha, com um teor de carbono elevado (86% a 98%), um baixo teor de matéria volátil e um poder calorífico de mais de 32,6 MJ/kg (14000 BTU/lb). O antracite é usado como combustível na geração de calor ou vapor nas indústrias de aço e ferro térmico. Este carvão é também utilizado na fabricação de borracha sintética, corantes e purificação de água para consumo humano (filtros).

-**Betuminoso:** este tipo de carvão possui um teor de carbono inferior e um poder calorífico inferior aos carvões de tipo antracite. Devido ao modo como são utilizados, são conhecidos como carvão de coque, sendo empregados no processo de obtenção de aço, e carvões térmicos usados na produção de vapor para gerar energia elétrica.

-Sub-betuminoso: com um valor calórico inferior ao de outros carvões betuminosos, o seu teor de carbono situa-se entre 35% e 45%. Essa classe possui um alto teor de matéria volátil, e alguns têm poderes de coque. Ele é utilizado na geração de energia elétrica e de processos industriais.

Lignite e turfa: estes carvões apresentam alta umidade e alto teor de cinzas e materiais voláteis, possuindo, portanto, um valor calorífico baixo. Eles são usados para gerar calor (sistemas de aquecimento), energia elétrica, em alguns processos industriais para gerar vapor e, mais recentemente, foram produzidos briquetes com lenhite e turfa para serem queimados em fornos [5].

Tabela 1. Classificação dos diferentes tipos de carvão.

Tipo*	Carbono fixo (%)	Material volátil (%)	Conteúdo de umidade (%)	Valor calorífico (MJ/Kg)**	Valor calorífico (Kcal/kg)**
Antracite	86 - 98	1	< 15	> 32,6	> 7.780
Betuminoso	45 - 86	32	15 - 20	24,5 - 32,6	5.800 - 7.780
Sub-betuminoso	35 - 45	50	20 - 30	18,2 - 24,5	4.300 - 7.780
Lignite e Turfa	25 - 35	96	> 30	9,3 - 18,2	2.200 - 4.300

*Fonte: American Society for Testing and Materials (ASTMD-388-777) ; ****Cálculos**: (MJ/kg y kcal/kg) UPME (2005).

A geração de eletricidade representou 44% do consumo mundial de carvão em 2012, e é esperado que se mantenha próximo a essa percentagem até 2040 (Fig. 2). O setor industrial foi responsável por 26% do consumo total de carvão em 2012 [6]. O uso do carvão nos setores residencial e comercial foi responsável por um aumento de 4% do consumo total mundial em 2012 e a previsão é que será responsável por 3% do total em 2040 [7].
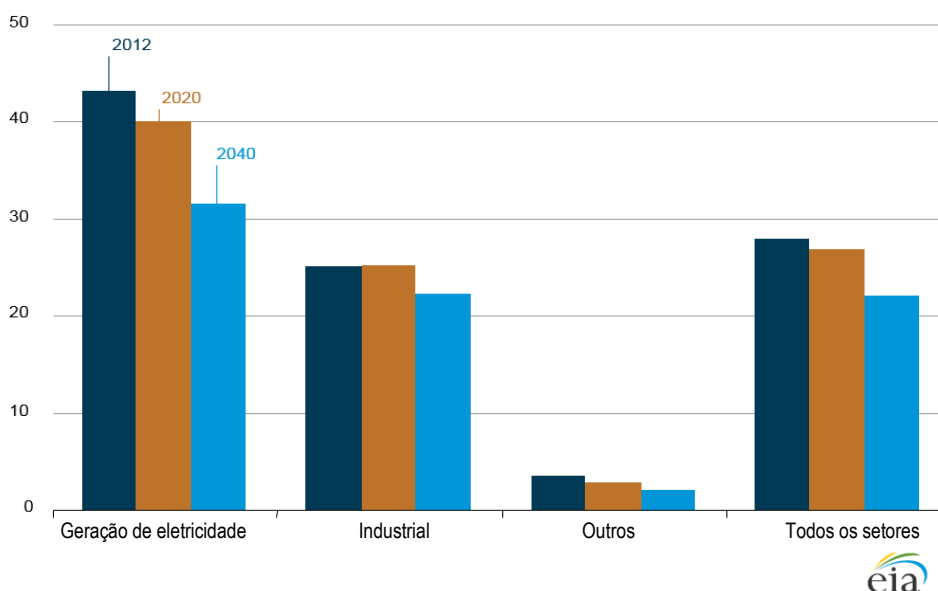
Figura 2. Consumo mundial de carvão, 2012, 2020 e 2040. **Fonte:** EIA. International Energy Outlook [6].

Conforme o *International Energy Outlook* 2016 (IEO-2016), até 2030 o carvão continuará sendo a segunda maior fonte de energia em todo o mundo, atrás somente do petróleo e outros combustíveis. De 2030 até 2040, estima-se que será a terceira maior fonte de energia, atrás de combustíveis líquidos e do gás natural. O consumo de carvão no

mundo aumentará a partir de 2012 até o 2040 à uma taxa média de 0,6%/ano, a partir de 153 quatrilhões de BTUs (*British thermal units*) em 2012 para 169 quatrilhões de BTUs em 2020, e para 180 quatrilhões de BTUs em 2040 [6].

1.2 Produção mundial

O carvão é o combustível fóssil mais abundante e está distribuído globalmente em uma ampla variedade de minas (Fig. 3). As reservas de carvão são bem distribuídas pelos continentes, com maior presença no hemisfério norte, sendo encontradas em quantidades expressivas em 75 países. No entanto, Estados Unidos (28,6%), Rússia (18,5%) e China (13,5%) concentram mais de 60% do volume total de carvão do mundo.

As reservas de carvão totalizam 847,5 bilhões de toneladas, quantidade suficiente para atender o consumo atual por 130 anos. Além disso, diferente do que acontece com petróleo e gás natural, a sua presença não está concentrada em poucas regiões.

O volume de carvão extraído e produzido não é relacionado somente à disponibilidade dos recursos naturais. Fatores estratégicos, como a existência de fontes primárias na região e, em consequência, à maior ou menor dependência da importação de combustíveis possuem influência considerável neste aspecto.

Atualmente, o maior produtor mundial de carvão é a China, que, estimulada pelo ciclo de acentuado desenvolvimento econômico, tornou-se a maior consumidora do minério. Em 2007, o país produziu 1.289,6 milhões de toneladas equivalentes de petróleo (Mtep) enquanto consumiu 1.311,4 Mtep.

1.3 Mineração de carvão a céu aberto

A mineração de carvão a céu aberto é empregada quando as camadas de carvão são aproximadamente horizontais e ocorrem em profundidades pequenas e reduzida espessura de cobertura de estéril [8]. A extração é uma operação sequencial iniciada com a limpeza da superfície e a retirada cuidadosa da capa vegetal até que as jazidas de carvão sejam expostas. Cada área de exploração é chamada *PIT,* e geralmente compreende uma área de depósito de rejeitos chamada *ÁREA DE ELIMINAÇÃO.* O solo e os resíduos adicionais de rocha são cuidadosamente recolhidos e armazenados. A mineração subsequente e o processo de transporte ("cadeia do carvão") envolve as atividades sequenciais ilustrada na Fig. 4 e resumida a seguir:

- perfuração e detonação da rocha e do carvão para quebrá-lo e facilitar a remoção;
- extração, carregamento e transporte de rochas e resíduos de carvão usando pás de alta capacidade e uma frota de transporte como caminhões que transportam o material para as áreas de eliminação ou manipulação do carvão;
- preparação de carvão, o que inclui a mistura, moagem e lavado de uma pequena percentagem do produto para a diminuição do teor de cinzas;
- carregamento de vagões ferroviários e transporte até o trem;
- armazenamento do carvão e carregamento dos navios em Puerto Bolívar.

Produção global 2015

7.942 Mt

Europa Central
O Carvão tipo lignite é extraído em grandes quantidades. No 2016 Alemanha, Republica Checa e Polônia produziram 292 Mt

Rússia
Presenta a maior extensão de vias férreas desde uma mina até o porto de embarque (4.500 – 6.000 km)

RUSSIA/ KAZAKHSTAN
485 Mt

CHINA
3.660 Mt

USA
935 Mt

Estados Unidos
Tem as maiores reservas de carvão do mundo sendo o segundo produtor mundial

INDIA
595 Mt

396 Mt

China
O Maior produtor, consumidor e importador de carvão do mundo. China é responsável pelo 46% do consumo de carvão do mundo

Índia
80% do carvão do pais é explorado por uma única companhia – carvões da Índia

Colômbia
O Mercado da mineração do carvão é dominado por companhias estrangeiras. 92% do carvão produzido é exportado

COLOMBIA
89 Mt

EXPORT 82 Mt

INDONESIA
443 Mt   383 Mt

Indonésia
Apresenta o setor mineiro com maior crescimento. A produção de carvão tem incrementado 460% desde o 2002

AUSTRALIA
421 Mt

Austrália
O Segundo exportador de carvão do mundo. Para o 2020 as emissões causadas pelas exportações de carvão da Austrália poderiam ser tão altas quanto as emissões totais da Alemanha em toda sua história

ÁFRICA DO SUL
264 Mt   79 Mt

África do Sul
94% da sua eletricidade é gerada pela queima de carvão. A mineração de carvão tem diminuído as reservas de água do país

● Produção

PRINCIPAIS IMPORTADORES DE CARVÃO

China	+ 289 Mt
Japão	+ 184 Mt
Índia	+ 160 Mt
Korean do Sul	+ 125 Mt
Taiwan	+ 64 Mt
Alemanha	+ 45 Mt
Reino Unido	+ 45 Mt

MT = Milhões de toneladas

Figura 3. Identificação geográfica dos principais produtores de carvão do mundo. O tamanho do círculo é proporcional à produção
Fonte: Adaptado de BankTrack.org [9]
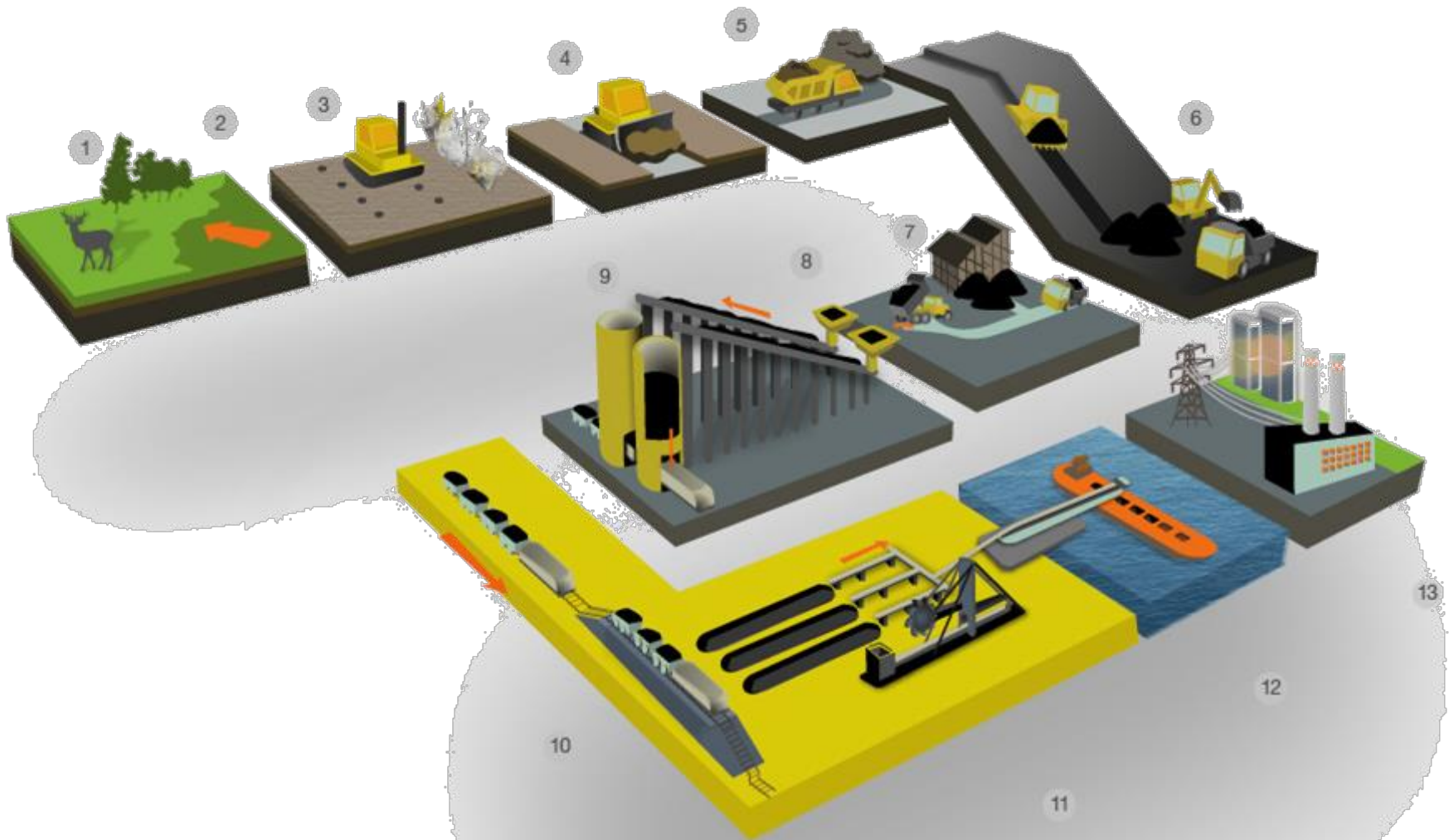
Figura 4. Processo de mineração do carvão: 1) afastamento de fauna; 2) limpeza de terrenos; 3) remoção de solo superficial, perfuração e detonação; 4) remoção de rejeitos; 5) despejo de rejeitos; 6) mineração de carvão; 7) preparação do carvão e lavagem; 8) carregamento de silos de armazenagem usando esteiras; 9) carregamento do trem; 10) transporte do carvão em trem; 11) armazenamento de carvão em áreas de embarque; 12) embarque do carvão; 13) uso final do carvão em termelétricas para a geração de energia. **Fonte:** Extraído de Cerrejón [1].

1.4 O carvão na Colômbia

A Colômbia possui as maiores reservas de carvão da América Latina. Assim, a Colômbia faz parte do grupo de exportadores mais jovens no mercado mundial. Os potenciais de recursos são de aproximadamente 16.992 milhões de toneladas (Mt), o que a torna o quinto exportador mundial do carvão, com uma participação de 6,3%, equivalente a 50 Mt de carvão anuais [10]. A produção de carvão concentra 47% da atividade de mineração do país e representa 1% do produto interno bruto colombiano, gerando cerca de 1.133 milhões de dólares. Nos últimos anos, tem se consolidado como o segundo maior produto de exportação, após o petróleo [11].

A maior parte das reservas está localizada na Costa Atlântica, e compreende 90% do carvão térmico que, por sua vez, corresponde a 98% do carvão nacional. 95% das reservas estão localizadas nos Departamentos de Guajira, Cesar, Córdoba, Norte de Santander, Cundinamarca, Boyacá, Antioquia, Valle del Cauca e Cauca (Fig. 5).

1.4.1 Costa Atlântica e Cerrejón

As jazidas de carvão da Costa Atlântica estão caracterizadas por sistemas de mineração a céu aberto, e concentram a maioria dos 25 mil trabalhadores do setor minero do país. Ao redor das áreas de mineração, assentam-se cerca de 60 mil habitantes, que podem estar sujeitos aos gases gerados pela combustão espontâdo carvão e enormes quantidades de pó e material particulado (PM) [12]. A mina de "El Cerrejón", a maior mina de carvão a céu aberto do mundo, está localizada no nordeste da Colômbia, na parte semiárida ao sul do Departamento de "La Guajira". A jazida conta com recursos estimados em 2.193 milhões de toneladas de carvão, estendendo-se por 69 mil hectares, dentro dos quais existem cinco áreas: Zona Norte, Patilla, Oreganal, Zona Central e Zona Sul (Fig. 6). Cerrejón opera atualmente quatro grandes áreas de exploração: Patilla, NAM (formada pelas áreas de Tabaco e La Puente), Oreganal e Comunidad.

59,7%

A mina

do PIB da Guajira é gerado pela extração mineira. Cerrejón é o maior produtor e exportador do departamento e da Colômbia

Cerrejon vende o 5% do carvao do mundo
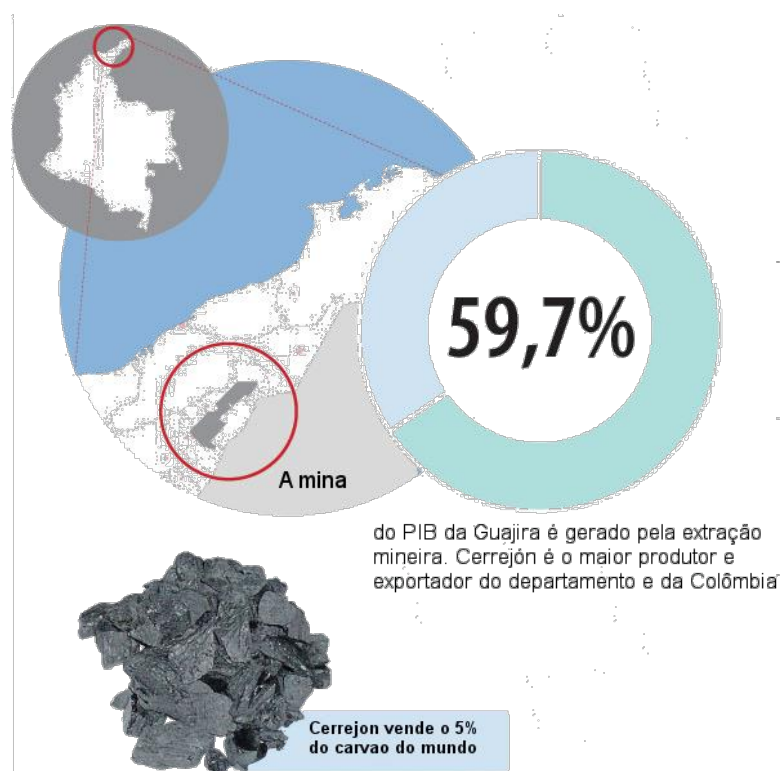
Figura 5. Produção de carvão no Cerrejón.
Fonte: Extraído de Cerrejón [1].
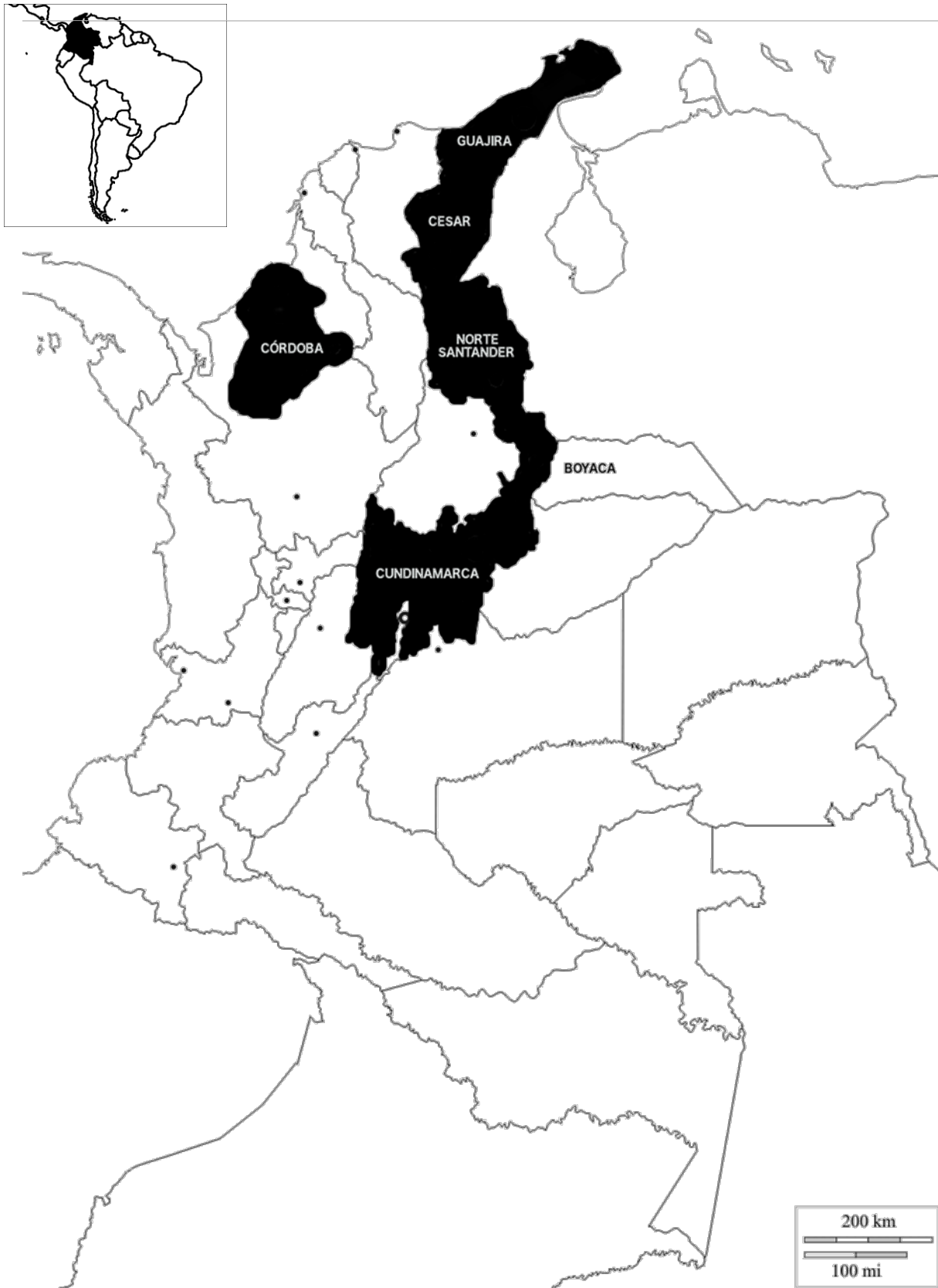
Figura 6. Distribuição geográfica das reservas de carvão da Colômbia.
Fonte: Adaptado de Jähnig et al., [10].

O carvão de Cerrejón é do tipo *betuminoso*, com um alto poder calorífico, baixo teor de cinzas e baixa concentração de elementos-traço. Algumas características físicas descritas para o carvão extraído na mina são apresentadas no ANEXO 1. Análises empregando Microscopia Eletrônica de Varredura Controlada por Computador (CCSEM) indicam que mais de 80% do peso do material mineral do carvão do Cerrejón é composto por de argila e quartzo (silicato de alumínio - Alsil), silicato de alumínio e sílica - Si-Alsil e sílica - Si) [13]. O estudo do produto da combustão revela que as cinzas são formadas principalmente de silicatos de alumínio, óxido de ferro e partículas de quartzo [14, 15]. A produção deste tipo de carvão com altos teores de sílica tem sido relacionada com altos níveis de poluição da água potável das comunidades perto da mina, algumas doenças e sintomas como dores de cabeça, erupções cutâneas e doenças do pulmão [16-18].

2. Resíduos da mineração de carvão a céu aberto: efeitos sobre a saúde, genotoxicidade e carcinogenicidade

As principais substâncias liberadas durante a mineração do carvão e envolvidas na geração de efeitos biológicos em populações com exposição ocupacional ou ambiental incluem:

- material particulado
- hidrocarbonetos aromáticos policíclicos
- metais traça e pesados

Vários estudos têm demonstrado que existe uma associação direta entre os resíduos gerados durante a mineração de carvão e várias doenças. Alguns destes são resumidos na Tabela 2.

Tabela 2. Principais resultados sobre a exposição a resíduos de mineração de carvão a céu aberto

Área de estudo	Principais conclusões	Autor (ano)
Espanha	As operações de mineração podem liberar substâncias tóxicas que podem causar problemas de saúde nas populações. Detecção de excesso de mortalidade por câncer colorretal e câncer de pulmão, especialmente relacionados com a mineração de carvão a céu aberto	Fernandez - Navarro et al. [19]
Appalachia	A exposição ambiental à PM ou agentes tóxicos presentes no carvão e que são liberados na sua mineração/processamento podem estar envolvidos na alta mortalidade por doença cardíaca, respiratória e renal em áreas de mineração de carvão	Hendryx [20]
West Virginia	Altos níveis de produção de carvão estão direta e proporcionalmente correlacionados com altas taxas de doenças cardiovasculares, hipertensão, doenças pulmonares, doenças renais, etc.	Hendryx and Ahern [21]
China	A pneumoconiose dos mineiros de carvão, caracterizada por lesões induzidas pelo pó de carvão nas regiões de troca gasosa do pulmão, está associada à mineração de carvão, principalmente pela inalação de PM.	Finkelman et al. [22]
Inglaterra	Crianças residentes em comunidades nas proximidades de minas de carvão a céu aberto estão expostas a uma significativa quantidade de PM_{10} adicional. Também foram encontradas evidências da associação entre habitar nas proximidades destas minas e o incremento na frequência de doenças respiratórias, como casos graves de asma.	Pless-Mulloli et al. [23]
	Identificaram as evidências de que a pneumoconiose e outras doenças respiratórias estão associadas com a exposição a material particulado respirável contendo quartzo.	Love et al. [24]

Fonte: Extraído de Gautam et al., [25].

2.1 Material particulado (PM) gerado em sistemas de mineração de carvão a céu aberto

O Programa das Nações Unidas para o Ambiente (UNEP/WHO) define o termo PM como um conjunto de partículas sólidas ou líquidas suspensas e dispersas no ar [27]. As propriedades dessas partículas variam em composição química, morfologia (tamanho/forma), parâmetros ópticos (cor/espalhamento da luz) e características elétricas (carga/resistência). Algumas dessas partículas podem ser grandes, escuras e, portanto, visíveis, tais como a fumaça e a fuligem. Entretanto, outras são tão pequenas que somente podem ser vistas com a utilização de um microscópio [28]. A sua fonte de origem pode ser natural ou artificial. Dentre as fontes naturais podem ser citadas aquelas que se evaporam do mar sob a forma de spray, pólens, poeiras e vulcões ou outras erupções geotérmicas. Como fontes artificiais têm-se, por exemplo, motores de veículos, caldeiras industriais e fumaça do cigarro. Dependendo dos tipos de fonte existentes e de suas interações com outros componentes presentes na atmosfera, a composição químicas dos poluentes, assim como os impactos causados à saúde humana, podem ser diferentes. As queimadas também são fontes importantes de poluição do ar, podendo causar problemas respiratórios sérios à população [29]. Baseado em sua origem, e de modo semelhante aos principais poluentes atmosféricos, o PM pode ser dividido em dois grupos: *primário e secundário.* As partículas *primárias* são produzidas por processos químicos ou físicos diretamente das fontes de poluição, enquanto que as partículas *secundárias* são formadas na atmosfera como resultado de reações químicas envolvendo gases preexistentes. Partículas primárias de PM podem tanto ser geradas por emissões naturais, tais como erupções vulcânicas e ressuspensão do solo em áreas de deserto, ou por emissões antropogênicas, como as originadas das atividades industriais e combustão de combustíveis fósseis.

2.1.1. $PM_{2.5}$ e PM_{10}

O PM pode ser dividido, basicamente, em duas modas definidas por intervalos de tamanho onde se tem maior concentração de partículas: a moda das partículas finas, menores que 2.5 μm de diâmetro aerodinâmico e a moda de partículas grossas, maiores que 2.5 μm (Fig. 7). Essa divisão é bastante conveniente, uma vez que frações de diâmetros aerodinâmicos diferentes possuem propriedades físicas e químicas distintas [29].
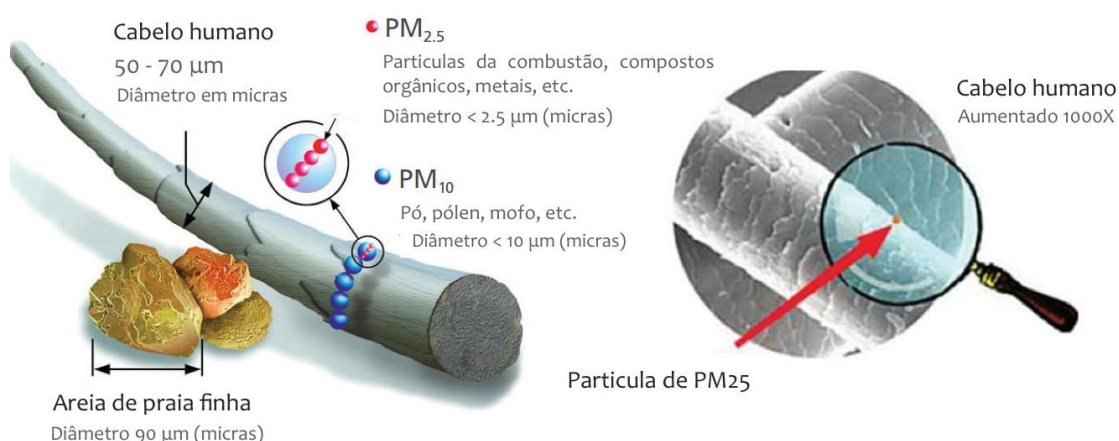
Figura 7. Comparações do tamanho do PM.
Fonte: Modificado de EPA.gov [30]

A moda grossa é geralmente constituída por partículas primárias, formadas a partir de processos mecânicos, como ressuspensão de poeira de solo por ventos, sal marinho, cinzas de combustão e emissões

biogênicas naturais [31]. A moda fina contém partículas primárias geradas por processos de combustão por indústrias, veículos e partículas secundárias, provenientes da formação de partículas na atmosfera a partir de gases, como por exemplo, a formação de sulfatos a partir de SO_2. Estas, por sua vez, possuem um tempo de permanência de dias a semanas na atmosfera e podem ser transportadas para longas distâncias por correntes de ar favoráveis, interferindo na química e na física da atmosfera, não somente em escala local, mas também em escalas regional e global.

O PM inalável (conjunto que engloba as partículas das modas fina e grossa menores que 10 μm) é constituído por sulfatos, nitratos, amônia, aerossol carbonáceo, sais marinhos (NaCl), elementos de solo (Al, Ca, Fe, Si, Ti), metais (Cd, Cr, Cu, Ni, Pb, V, Zn e outros) e água [32]. O $PM_{2.5}$ é dominado por produtos de combustão secundário e aerossóis [30].
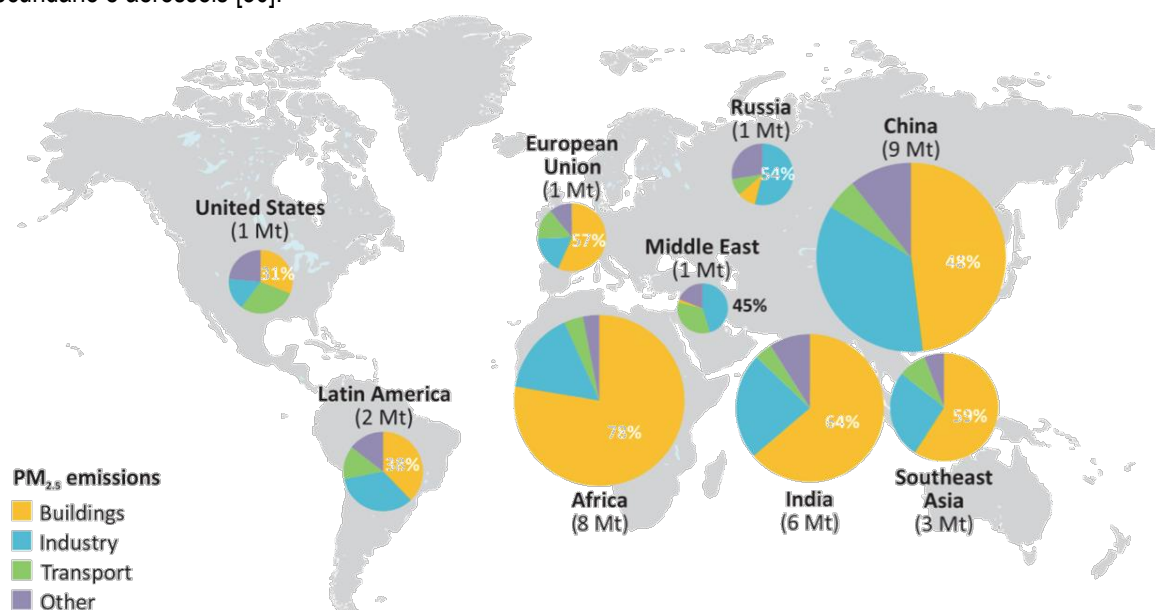
Figura 8. Emissões de $PM_{2.5}$ relacionados com a geração de energia por região e setor de 2015.
Fonte: Extraído da EPA.gov: Energy and Air pollution [1].

Durante o ano 2015, as principais fontes de $PM_{2.5}$ no mundo, foram os setores doméstico e industrial. No setor doméstico, o uso de lenha e carvão para aquecimento, preparação de alimentos e iluminação dos lares gerou 38% do $PM_{2.5}$ na América Latina e, no caso da indústria, a combustão de carvão em termelétricas para a geração de energia contribuiu com 35% (Fig. 8). O carvão também é responsável por 60% das emissões de dióxido de enxofre relacionadas com a combustão global, sendo uma das causas de doenças respiratórias e um precursor da chuva ácida [1].

2.1.2 Composição química

O PM gerado em minas de carvão a céu aberto é uma mistura complexa de partículas que variam não só em tamanho e morfologia, mas também nas suas características químicas e físicas que, por sua vez, dependem da composição do carvão [14, 15]. As atividades de mineração de carvão que geram estas partículas são perfuração, detonação, carregamento e descarga do carvão, e transporte rodoviário em estradas não pavimentadas [16]. Além disso, em minas de carvão a céu aberto, o carvão extraído é armazenado sob a luz do sol em altas temperaturas, onde a combustão incompleta e espontânea do carvão pode levar a formação de

Hidrocarbonetos Aromáticos Policíclicos (HAP) [17]. Em instalações de mineração a céu aberto, particularmente, estas substâncias tóxicas são liberadas para a atmosfera, onde podem formar misturas complexas [18], representando um dos mais importantes riscos para a saúde e de segurança das populações expostas devido aos efeitos sinérgicos potenciais das combinações resultantes [19].

A Tabela 1 do ANEXO 1 descreve os principais poluentes ambientais e substâncias químicas detectadas no carvão, cinzas e processos de combustão em torno de sistemas de mineração de carvão. Os poucos estudos sobre a caracterização química do $PM_{2.5}$ e PM_{10} no entorno de minas de carvão a céu aberto têm demonstrado a presença principalmente de metais e HAP no material particulado das duas frações.

Outros compostos que são gerados dentro do PM de minas carvão incluem emissões como óxidos de enxofre (SO_x), os óxidos de nitrogênio (NO_x) e o monóxido de carbono (CO) [1], descritos a seguir.

- **Óxidos de enxofre (SO_x), em particular o dióxido de enxofre (SO_2):** os combustíveis fósseis como o carvão e óleo contém enxofre em diferentes teores, o que pode causar a liberação para a atmosfera de SO_X durante a combustão na geração de energia ou nos processos industriais. Essas emissões podem ser precursoras na formação de partículas secundárias e estão associadas a efeitos adversos para a saúde e o ambiente.
- **Óxidos de nitrogênio (NO_x), óxido de nitrogênio (NO) e dióxido de nitrogênio (NO_2):** NO_X são gerados a partir da combustão de alta temperatura, principalmente no transporte e geração de energia, ou a partir da oxidação de NO a NO_2 na atmosfera. NO_2 é um gás tóxico e pode levar à formação de partículas e de ozônio.
- **Monóxido de carbono (CO):** o CO é um gás tóxico incolor e inodoro, gerado a partir da combustão incompleta do carvão ou da madeira.
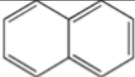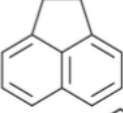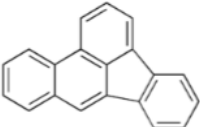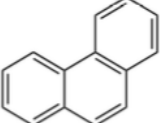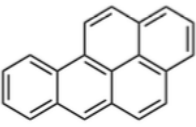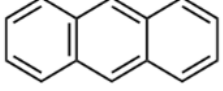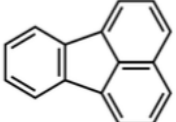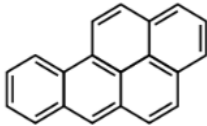
2.1.3 Genotoxicidade e Carcinogenicidade

A pneumoconiose simples dos trabalhadores de carvão (PSTC) e a fibrose massiva progressiva (FMP) são as principais doenças ocupacionais causadas pela exposição a partículas respiráveis geradas durante várias atividades de mineração de carvão a céu aberto. Donoghue [33] descreveu que o pó e carvão constituem um dos maiores riscos para a saúde dos trabalhadores e uma das principais causas da CWP e da doença pulmonar obstrutiva crônica (DPOC) nos mineiros de carvão. Diversos estudos sugerem que os efeitos sobre a saúde nestas populações expostas não estão restritos apenas a episódios ocasionais quando os poluentes estão em níveis elevados, mas que também podem acontecer quando os níveis das partículas estão abaixo dos níveis permitidos pelos padrões de qualidade do ar estabelecidos pelas agencias mundiais [27]. A agência de proteção do meio ambiente dos Estados Unidos (USEPA) tem demonstrado que a poluição com PM possui uma relação direta com as mudanças na função pulmonar e as doenças respiratórias das populações expostas. Este tipo de poluição também se encontra relacionada com um aumento na mortalidade no curto prazo. A taxa de mortalidade induzida pela poluição varia entre 2 a 8% por cada 50 µg/m^{-3} de PM [34]. Efeitos do PM sobre o meio ambiente incluem a geração de alterações climáticas que ocorrem quando as pequenas partículas de PM na atmosfera absorvem e refletem a radiação solar [35].

2.2 Hidrocarbonetos Aromáticos Policíclicos (HAPs)

Os HAPs são substâncias orgânicas constituídas por átomos de carbono e hidrogênio agrupados em pelo menos duas estruturas de anéis aromáticos condensados ou fundidos [36]. Eles podem ser divididos em duas categorias: os compostos de baixo peso molecular, formados com menos de cinco anéis, ou compostos de elevado peso molecular, formados por cinco ou mais anéis (Fig. 9). As características lipofílicas dos HAPs permitem a sua fácil penetração nas membranas celulares [37].

BAIXO PESO MOLECULAR			ALTO PESO MOLECULAR		
No de anéis	Nome	Estrutura	No de anéis	Nome	Estrutura
2	Naftaleno		5	Benzo (e) pireno	
3	Acenafteno			Benzo (b) fluorantreno	
	Fenantreno			Benzo (a) pireno	
	Antraceno				
4	Fluorantreno		6	Benzo (g,h,i) perileno	
	Pireno			Indeno (1, 2 ,3) pireno	

Figura 9. Principais HAPs, estrutura química e grau de complexidade.
Fonte: Modificado de EPA.gov [30].

Embora os HAPs com menor peso molecular sejam considerados como menos tóxicos, são capazes de reagir com outros poluentes, tais como o ozônio, óxidos de enxofre e dióxido de enxofre, para formar dionas, nitro e dinitro-HAPs, e ácidos sulfúrico, respectivamente, com uma toxicidade que pode ser mais significativa [38]. HAPs com quatro ou mais anéis apresentam um índice de vaporização insignificante em todas as condições ambientais [39].

A presença de HAPs no ambiente é devido principalmente às emissões provenientes da combustão incompleta de combustíveis que contêm carbono a partir de fontes naturais, industriais, comerciais, veiculares e residenciais. Como mostrado na Fig. 10, a presença destes processos de combustão espontânea de carvão tem sido detectada nas áreas de mineração de El Cerrejón [40].

As principais vias de exposição aos HAPs na população em geral são inalação (interior e exterior) de ar, consumo de alimentos contaminados, hábitos como o tabagismo, ou pela respiração de fumaça de lareiras [41]. Vários HAPs contidos no fumo do tabaco são possíveis cancerígenos humanos [42]. Entre os não-fumantes, a

principal via de exposição é pelos alimentos. O processamento e cozimento dos alimentos a altas temperaturas (grelhar, assar e fritar) são as principais fontes de HAPs [43]. Algumas culturas (como trigo, centeio e lentilhas) podem sintetizar HAPs ou absorvê-los através da água, ar, ou, do solo [43]. A ingestão de HAP pode ocorrer por via cutânea e pela inalação de vapores de HAPs [44]. A meia vida dos HAPs no ar é da ordem de dias mas, quando associados às PM do ambiente, pode ser mais prolongada [45].

A exposição ocupacional a HAPs pode ocorrer em trabalhadores pela respiração de gases de escape (como mecânicos, vendedores ambulantes, ou condutores de veículos motorizados) e nas pessoas envolvidas na mineração, metalurgia, ou refino do petróleo [46, 47]. Em ambos os ambientes, ocupacionais e não ocupacionais, as vias de exposição incluem ingestão, inalação e contato dérmico [48]. Algumas exposições podem envolver várias vias de exposição simultâneas, como dérmica e inalação de ar contaminado, o que afeta a dose total de absorção.

Os efeitos da *exposição aguda* aos HAPs na saúde humana dependem principalmente do grau da exposição (por exemplo, período de tempo), a concentração de HAPs, a toxicidade do HAP, e a via de contato (inalação, ingestão, ou contato com a pele). Fatores como condições de saúde pré-existentes e idade também influenciam. A exposição a curto prazo aos HAPs também tem sido relacionada com a redução da função pulmonar em asmáticos e efeitos trombóticos em pessoas afetadas por uma doença cardíaca coronariana [41]. No entanto, não se sabe quais os componentes da mistura foram responsáveis por estes efeitos. Ainda não há uma plena compreensão da capacidade de HAPs em concentrações ambientais para induzir efeitos na saúde humana a curto prazo.

Em contraste, níveis elevados de misturas de poluentes que contêm HAP na exposição ocupacional são conhecidos por resultar em sintomas como irritação nos olhos, náuseas, vómitos, diarreia, etc. [49]. Para os trabalhadores expostos a misturas de HAP e outros produtos químicos no local de trabalho, têm sido relatados uma série de problemas de saúde, tais como aumento do risco de câncer de pele, pulmão, bexiga e gastrointestinal [50-52].

A *exposição crônica* a baixos níveis de alguns HAP (por exemplo, pireno e Benzo(a)pireno) tem sido relacionada com a ocorrência de câncer em animais de laboratório [53]. Outros efeitos sobre a saúde decorrentes da exposição crônica para HAPs podem incluir diminuição da função imunológica, catarata, danos no rim e fígado (por exemplo, icterícia) [54], problemas respiratórios, sintomas semelhantes à asma e alterações da função pulmonar [55]. No caso dos HAPs, os efeitos nocivos dependem, em grande parte, da maneira pela qual o indivíduo foi exposto [56]. A Fig. 11 representa um fluxograma resumindo os efeitos na saúde da exposição de curto e longo prazo.

2.2.2 Genotoxicidade e Carcinogenicidade

Os efeitos genotóxicos de alguns HAPs têm sido demonstrados em roedores e em testes *in vitro* utilizando linhagens celulares de mamífero. A maior parte dos HAPs não são genotóxicos por si, mas quando metabolizados para diol epóxidos reagem com o DNA induzindo danos na molécula. A genotoxicidade dos HAPs desempenha um papel importante no processo de carcinogênese e possivelmente na toxicidade dessas substâncias [56].

Figura 10. Distribuição global dos incêndios em minas de carvão (nota: a figura não indica a densidade de fogos de carvão nas regiões afetadas sendo provável que a distribuição nos países em desenvolvimento seja subestimada). **Fonte:** Adaptado de Stracher et al., [40].
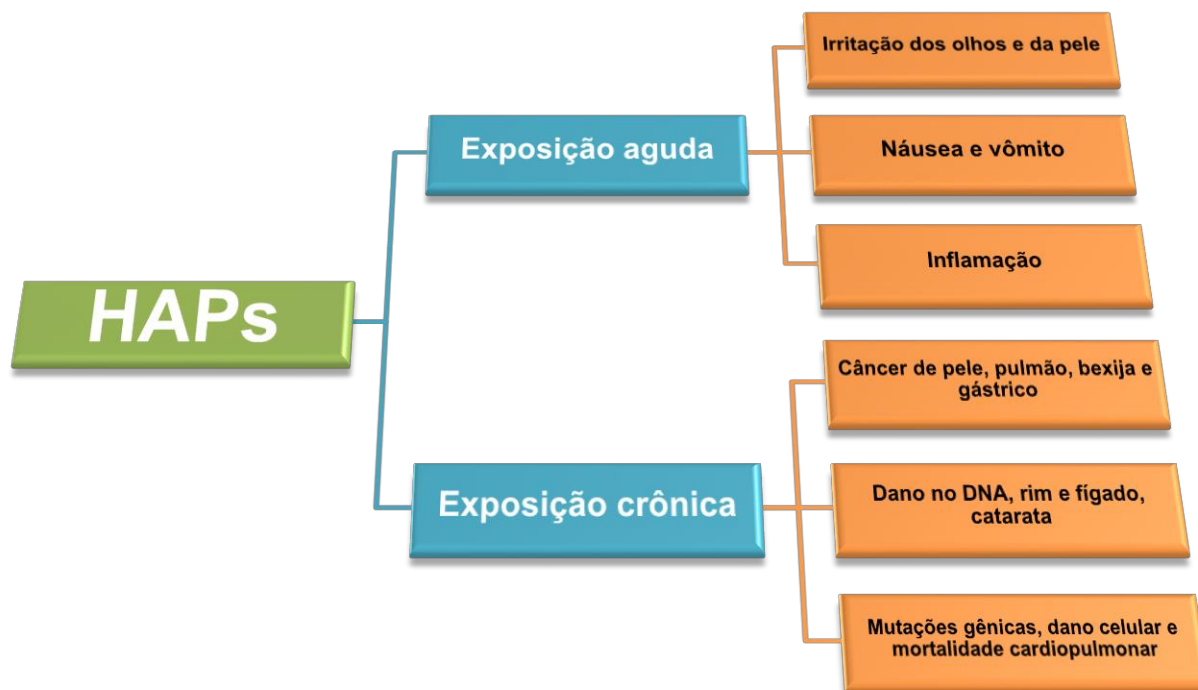
Figura 11. Efeitos na saúde causados pela exposição de curto e longo prazo à HAPs.
Fonte: Adaptado de Rengarajan, T. et al., [56].

Na maioria dos casos, a oxidação de HAPs pelo citocromo *P450 (CYP)* é um passo inicial do processo de ativação para produzir espécies eletrofílicas bioquimicamente reativas capazes de interagir com macromoléculas celulares, em particular os ácidos nucleicos e proteínas [58]. A Fig.12 mostra como a exposição ao benzo[a]pireno *(B[a]P)* depois da sua ativação ao metabólito reativo, *B[a]P diol epóxido*, pode levar à geração de lesões oxidativas no DNA, bem como a formação de adutos *B[a]P*.
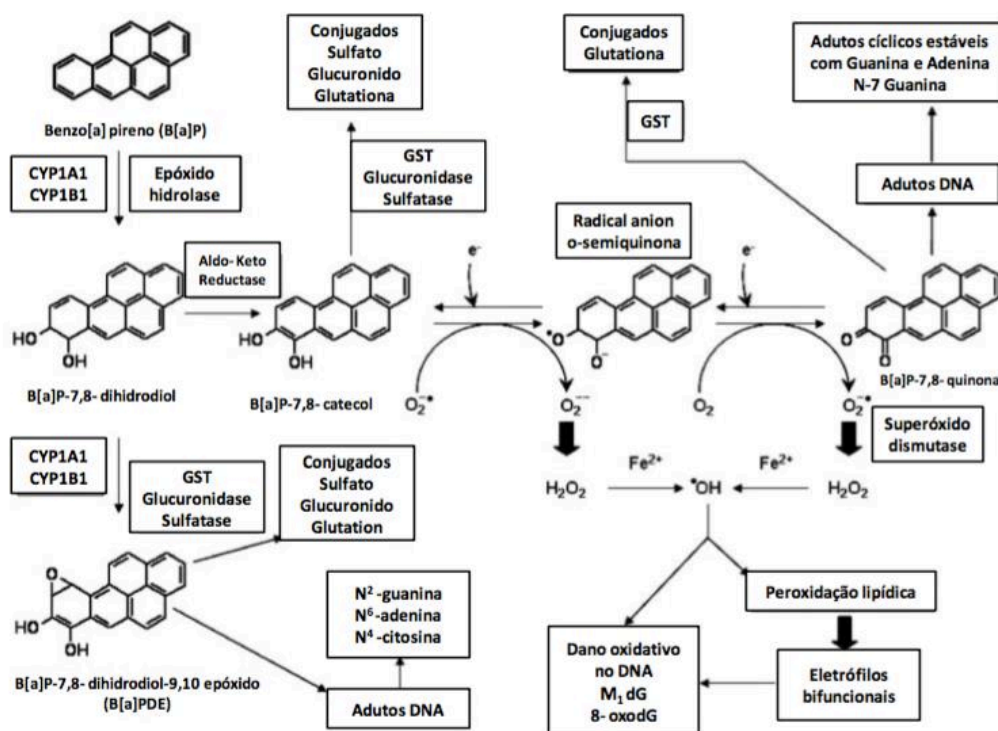
Figura 12. Geração de lesões oxidativas e formação de adutos *B[a]P* no DNA.
Fontes: Adaptado de Rengarajan et al., [56]; Singh et al.,[59].

Após sua absorção, os HAPs são distribuídos pela corrente sanguínea até as células do pulmão e/ou tecidos, onde podem ser transformados biologicamente. A ativação metabólica dos HAPs ocorre através de duas classes de enzimas: as proteínas da fase I *(CYP1A1)* e da fase II (conjugação). Nos pulmões humanos, os HAPs são metabolizados pelo *CYP1A1* membro do *CYP superfamília* [60]. Após bioativação e a reação com o DNA, os metabólitos de HAPs podem induzir mutações que podem ativar oncogenes ou inativar genes supressores de tumores, como parte do seu mecanismo carcinogênico. Este processo pode ser exacerbado nas misturas complexas de HAPs como as presentes nas misturas complexas geradas pela mineração de carvão a céu aberto, já que podem conter compostos que por si têm potencial carcinogênico fraco, mas que, em combinação com alguns iniciadores, podem atuar de forma aditiva [61]. No caso das mutações ocorrerem em genes supressores tumorais, isso pode levar a descontrole do ciclo celular, a acumulação de danos no DNA e, finalmente, à carcinogênese (Fig. 13). Por exemplo, o gene supressor de tumores p53 é mutado em muitos tipos de câncer em humanos [62] e a exposição a HAPs foi previamente associada com mutações nesse gene em câncer de pulmão e mama em humanos [63].

As exposições alimentícias e ambientais aos HAPs podem resultar na formação de adutos simultaneamente em muitos tecidos do corpo. Segundo o estudo de Pratt et al. [64], os adutos DNA/HAPs em tecidos humanos estão concentrados em tipos de células específicos dentro de determinados órgãos como o esôfago, placenta e próstata.

As evidências a partir de estudos ocupacionais em trabalhadores expostos a misturas de HAPs indicam que as misturas de HAPs podem ser mais cancerígenas para os seres humanos do que a exposição a HAPs isolados [64].
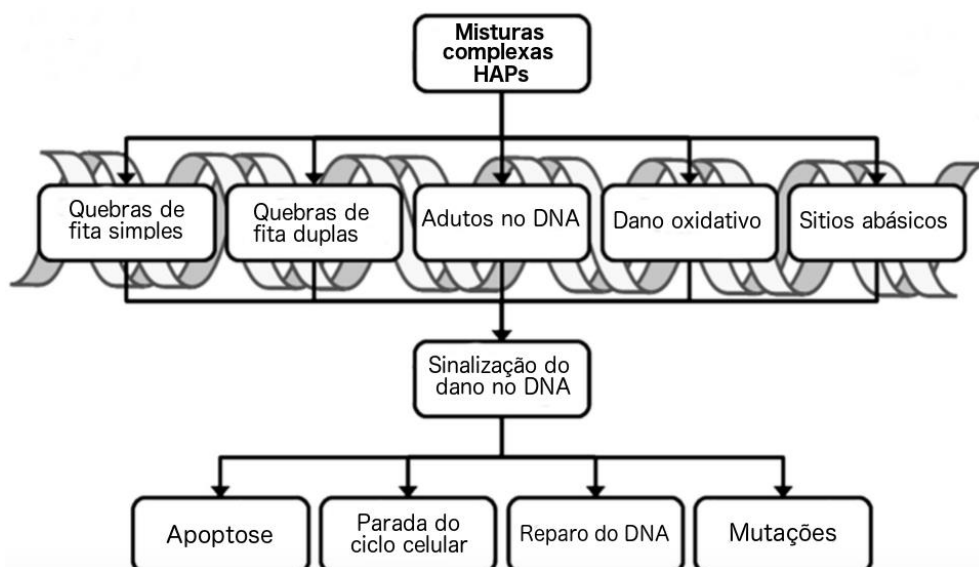
Figura 13. A sinalização dos danos no DNA coordena os danos genotóxicos causados pelas misturas complexas de HAPs. Uma ampla variedade de danos no DNA, incluindo quebras na cadeia, formação de adutos, danos oxidativos e formação de sítios abásicos resultantes de danos com a perda de purinas, podem ocorrer após a exposição a misturas complexas de HAPs. No centro da resposta ao dano no DNA, encontra-se a sinalização de danos no DNA. Um nível tolerável de dano no DNA pode gerar a parada do ciclo celular e o reparo do DNA, enquanto o dano excessivo ou irreparável no DNA pode levar à apoptose. Ambos os cenários são considerados respostas celulares positivas que impedem o processo carcinogênico. As mutações podem ocorrer como resultado de danos no reparados no DNA ou indevidamente reparados, ou erros na transdução do sinal, e são um resultado negativo que podem promover ou acelerar o processo de formação do câncer. O papel principal que a sinalização do dano no DNA desempenha na coordenação da resposta aos danos no DNA torna-o um ponto biológico adequado para a avaliação de misturas complexas.
Fonte: Adaptado de Jarvis et al., [65].

2.3 Metais pesados

Apesar dos metais pesados serem elementos que ocorrem naturalmente e são encontrados em toda a crosta terrestre, a maioria da contaminação ambiental com metais é resultado das atividades antrópicas, como as operações de mineração e fundição, produção industrial e o uso doméstico e agrícola de compostos contendo metais [66, 67]. Estudos sobre os impactos ambientais da mineração de carvão têm mostrado que a acidez do solo, a liberação de metais tóxicos [68] e os danos à vegetação [69] são os impactos negativos predominantes das atividades de exploração. Entretanto, esses elementos traço em altas concentrações no meio ambiente constituem um grave problema devido os seus efeitos nocivos no organismo, como alta toxicidade, capacidade de bioacumulação e capacidade de induzir danos ao material genético [70] e câncer, uma vez que estes efeitos têm sido atribuídos aos metais pesados em humanos [71] e/ou animais [72, 73].

Alguns dos elementos gerados durante as atividades de extração de carvão e que aparecem associados com problemas de toxicidade incluem alguns metais pesados, os quais ocorrem em altas concentrações no carvão, como o Cobre (Cu), Chumbo (Pb), Cadmio (Cd), Níquel (Ni), Vanádio (V), Zinco (Zn) e Enxofre (S), também presentes na combustão deste mineral [74].

2.3.1 Genotoxicidade e Carcinogenicidade

Além das reações características de alguns metais, três mecanismos predominantes parecem ser comuns nos processos genotóxicos e carcinogênicos da maioria dos metais:

(1) indução de estresse oxidativo - relacionado com a capacidade dos íons dos metais carcinogênicos de gerar reações *redox* nos sistemas biológicos (Fig. 14). Essas reações geralmente envolvem a produção de radicais hidroxila através das reações de tipo Fenton e Haber–Weiss. Os radicais por sua vez podem causar danos oxidativos em lipídios, proteínas e DNA [75].

(2) Modulação dos mecanismos de reparo do DNA - em baixas concentrações muitos metais têm sido identificados como inibidores da reparação de danos no DNA causada por outros xenobióticos ou por fatores endógenos [76]. Os mecanismos de reparo do DNA são alvos frequentes de interferência causada por metais tóxicos. A inibição da reparação de danos no DNA resulta em instabilidade genômica e acúmulo de mutações críticas (Tabela 3)**.**

(3) Desregulação da proliferação celular - os compostos metálicos podem alterar o crescimento celular através de vários mecanismos distintos, afetando a expressão de fatores de crescimento ou inativando mecanismos de controle do crescimento tais como os genes supressores de tumor (Fig. 15).

Sob o ponto de vista da toxicidade e abundância no meio-ambiente, os metais podem ser classificados sob três criterios [77] :
- Não críticos como Na, K, Ca, Mg, Fe e Al;
- Tóxicos mas muito raros ou insolúveis como W, Zr, Ba e Ti;
- Muito tóxicos e relativamente acessíveis como Ni, Cu, Zn, As, Cd, Hg e Pb.

A toxicidade de metais e de seus compostos depende largamente da sua biodisponibilidade, ou seja, dos mecanismos de captação através de membranas celulares, distribuição intracelular e ligações a macromoléculas

celulares. Além disso, alguns compostos metálicos ainda sofrem transformações metabólicas, tais como redução a estados oxidativos menores ou alquilação [75].

Tabela 3. Inibição dos diferentes tipos de sistemas de reparo de DNA por metais cancerígenos

Metal	Sistema de reparo	Tipo de dano no DNA	Etapa afetada	Mecanismo	Menor concentração efetiva (μM)
Níquel (II)	NER	Foto-produtos no DNA induzidos por UV-C	Reconhecimento do dano no DNA	Competição com Mg^2	50
	BER	Modificação oxidativa de bases do DNA (incluindo 8OHdG)	Excisão de base danificada	?	50
Cádmio (II)	NER	Foto-produtos no DNA induzidos por UV-C	Reconhecimento do dano no DNA	Competição com Zn^2	0,5
	BER	Modificação oxidativa de bases do DNA (incluindo 8OHdG)	Excisão de base danificada	?	0,5
Arsênico (III)	NER	Foto-produtos no DNA induzidos por UV-C	Incisão	?	2.5
Cobalto (II)	NER	Foto-produtos no DNA induzidos por UV-C	Incisão, polimerização	Competição com Mg^2	50

UV – C: Luz ultravioleta C.
Fonte: Adaptado de Hartwig, [78].

A capacidade que os metais possuem de interferir no processo de formação de radicais livres pode estar relacionado com a indução da carcinogênese. Tem sido demonstrado que metais, tais como Fe, Cu, Cd, Cr, Ni, entre outros, possuem a capacidade de produzir EROs. Quando essas espécies são formadas intracelularmente, elas podem induzir peroxidação lipídica, dano ao DNA, depleção de grupamentos tióis, alterar vias de transdução de sinais e a homeostase do cálcio [79].

Os metais podem provocar dano ao DNA de forma direta ou indiretamente pela formação das EROs. A geração das EROs pode ser afetada pelos metais por muitas vias. Por exemplo, através da reação de Fenton, da indução do processo inflamatório, ou através da formação intermediária de tioradicais. A maior parte do dano ao DNA é reparada pelo eficiente mecanismo celular de reparo, enquanto que uma pequena parte do dano resulta em mutação. Mudanças nos níveis das EROs também influenciam no equilíbrio redox intracelular, afetando fortemente as vias de transdução de sinais, as quais ativam ou inativam vários fatores de transcrição. Tanto a mutação quanto os fatores de transcrição podem modular a expressão de uma variedade relevante de genes para a transformação celular, conduzindo finalmente ao desenvolvimento do câncer.

O grupo de ERO inclui radicais aniónicos superóxido (O2 • -), peróxido de hidrogénio (H2O2) e moléculas de radicais hidroxilo (OH) que são gerados pela redução intracelular do oxigênio molecular. O2 • - é gerado principalmente como um produto secundário da respiração mitocondrial, quando os electrões são transferidos pela ubiquinona ou semi-ubiquinona directamente para o oxigênio em vez de sucessores na cadeia de transferência de electrões respiratória [80].
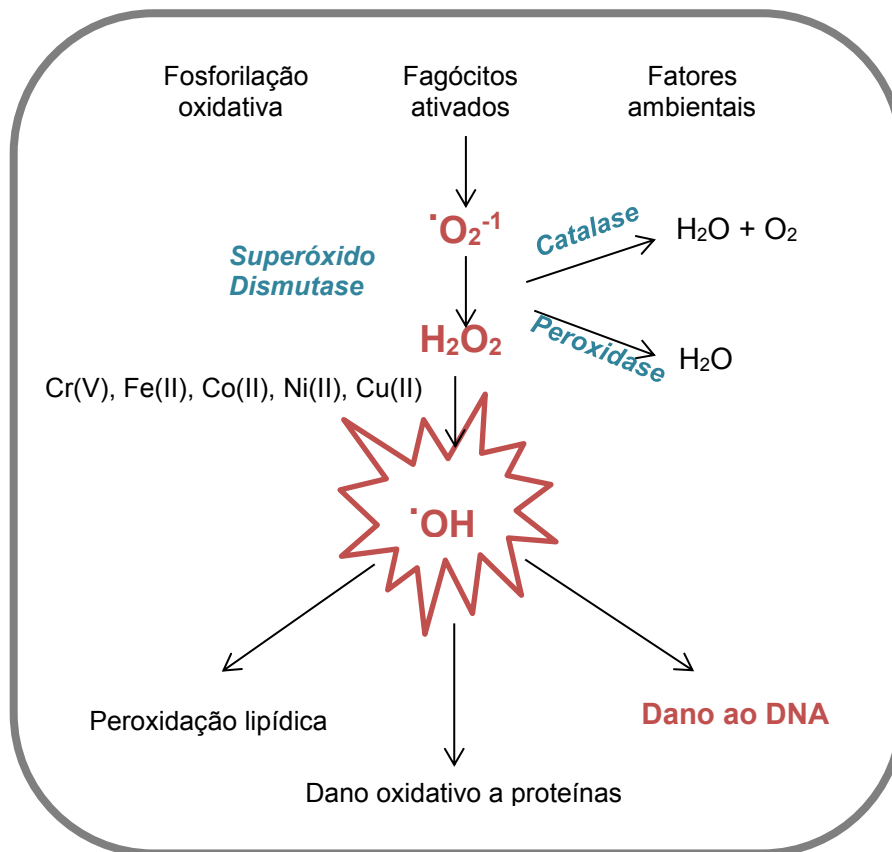
Figura 14. Íons metálicos e possíveis mecanismos na indução de estresse oxidativo.
Fontes: Adaptado de Hartwig [76].
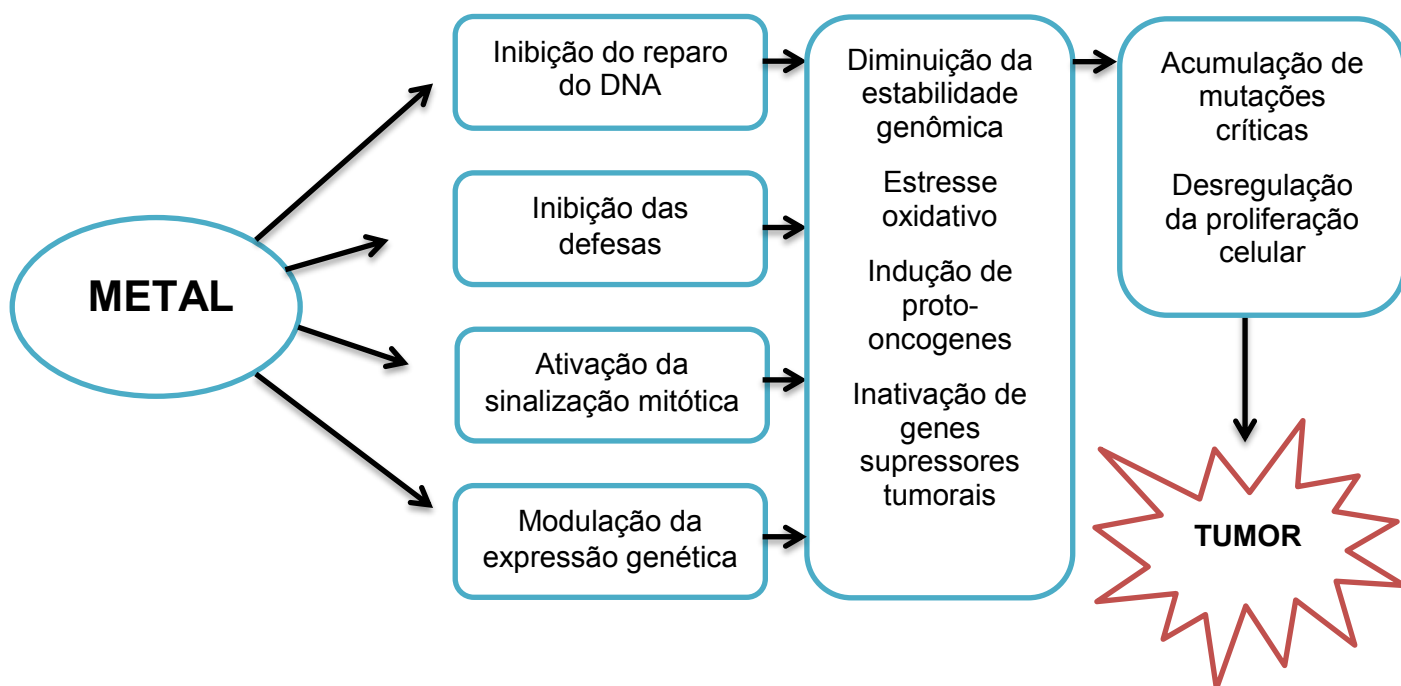
Figura 15. Principais mecanismos da indução de carcinogênese por metais. Não são mostrados alguns mecanismos únicos de alguns metais como a formação de adutos DNA - cromo, a interferência do cádmio com a adesão célula-célula ou a inibição do vanádio das proteínas fosfatases.
Fontes: Adaptado de Beyersman e Hartwig, [75].

3. Mecanismos envolvidos no dano relacionado com a exposição a carvão

3.1 Geração de EROs

O tamanho é um fator importante que influencia como o PM se deposita no trato respiratório e afeta a saúde humana (Fig.16). As partículas grandes são geralmente filtradas no nariz e na garganta, e não necessariamente causam problemas. A fração $PM_{2.5}$ ultrapassa a região pulmonar alveolar, onde ocorre a troca de sangue e, por esta razão, representa a fração respirável de maior risco [81, 82].

Uma vez na corrente sanguínea, os metais e partículas na superfície do PM podem gerar danos oxidativos (Fig.17) e genotoxicidade primaria ou secundária (Fig. 18).

O grau da deposição de $PM_{2.5}$ no pulmão é determinada pela concentração inalada, a estrutura do tecido, e a capacidade de compensação de cílios das vias aéreas. O dano resultante para os cílios das vias aéreas e a reduzida capacidade de executar limpeza das vias aéreas impede a eliminação oportuna de $PM_{2.5}$ das vias aéreas e dos pulmões [83]. Em um ambiente poluído, cada alvéolo entra em contato com uma média de 1500 moléculas de partículas num período de 24 horas. Aproximadamente 50% dos depósitos de PM ocorrem no alvéolo, 96% dos quais são constituídos por $PM_{2.5}$ [84].
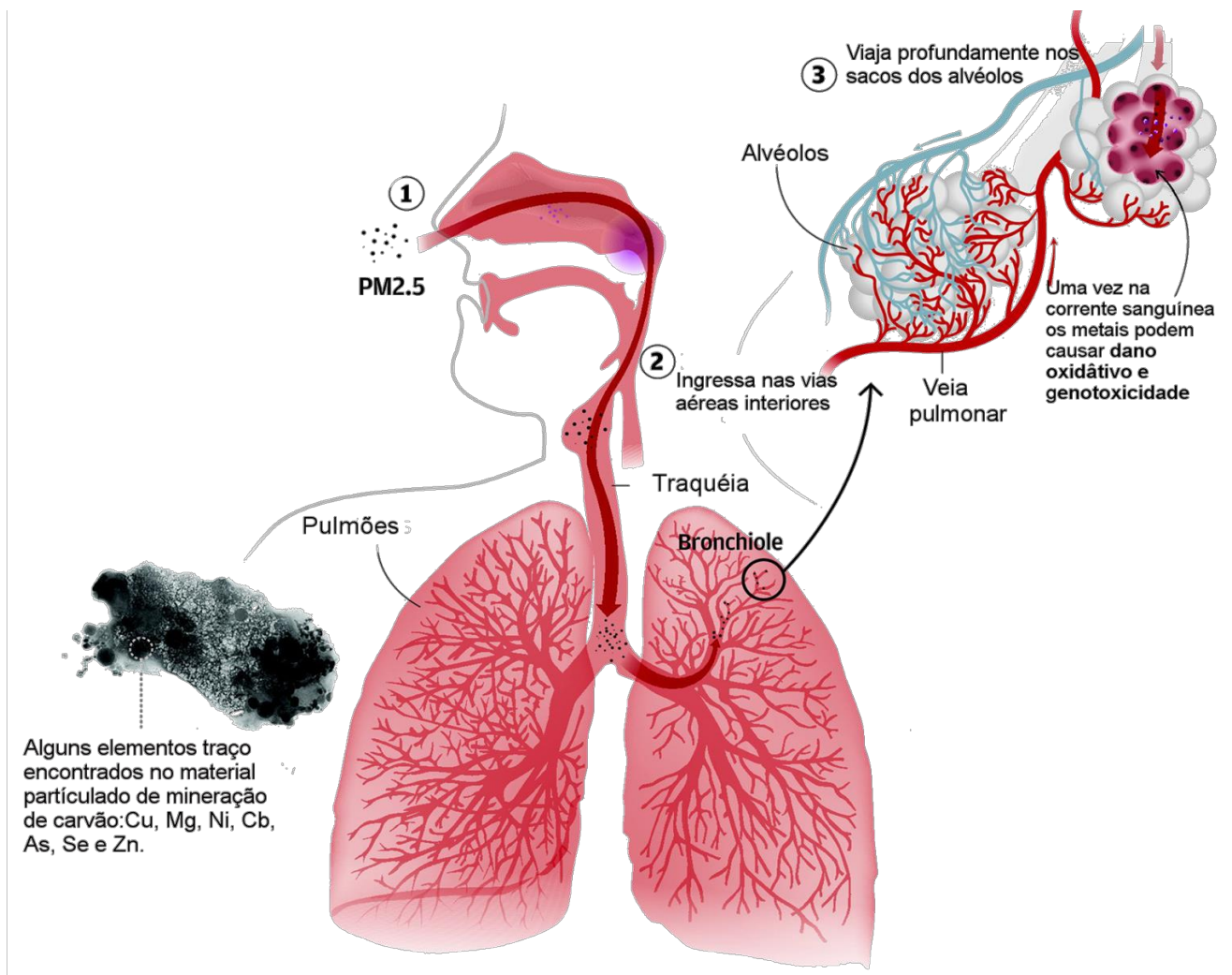
Figura 16. Deposição do $PM_{2.5}$ nas vias aéreas.
Fonte: Adaptado de EPA.gov [30] e Okuda et al., [57].

A Agência Internacional de Investigação do Câncer (IARC) classificou a exposição a PM do ar como um carcinógeno humano. Esta classificação é sustentada em provas convincentes de estudos epidemiológicos e por fortes evidências de estudos mecanicistas [85]. A crescente evidência vincula a exposição a longo prazo a $PM_{2.5}$ com o aumento do risco de mortalidade cardiovascular [86, 87] e câncer de pulmão [88, 89]. Ao redor das minas de carvão e outras instalações industriais, a exposição a $PM_{2.5}$ e PM_{10} também tem sido associada à morte prematura, aumento da morbidade das doenças respiratórias e cardiovasculares [90-92], câncer de pulmão [93-95] e doenças cardiopulmonares [21]. A elevada mortalidade ao redor das áreas de mineração de carvão ocorre em ambos os sexos masculino e feminino, sugerindo que a causa não é a exposição ocupacional mas a exposição crônica à poluição do ar e da água causada por atividades de mineração de carvão e afins [96]
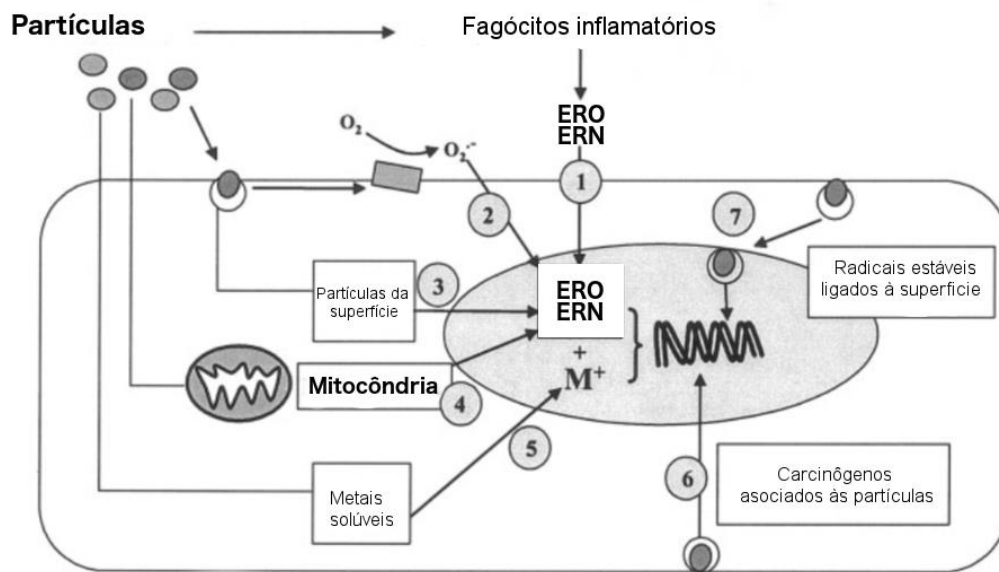
Figura 17. Possíveis mecanismos de dano ao DNA induzido por PM. As partículas podem gerar dano oxidativo através da inflamação fagocítica (genotoxicidade secundária) (1); Alternativamente, as partículas também pode gerar diretamente espécies reativas de oxigênio/nitrogênio ERO/ERN em células alvo (2-5). Por exemplo, através da ativação de enzimas tipo NAD(P)-H (2), por meio de partículas reativas de superfície (3) e através da ativação mitocondrial (4). Os metais de transição associados às partículas podem aumentar a produção de ERO através das reações de Haber-Weiss (5). Os compostos genotóxicos (por exemplo, HAPs) podem danificar diretamente o DNA (6). Finalmente, a translocação de partículas para o núcleo pode gerar dano através dos radicais ligados à superfície (7). **Fonte:** Adaptado de Knaapen et al., [97].
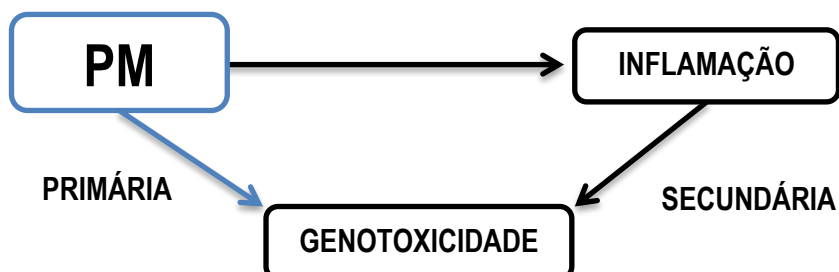
Figura 18. Genotoxicidade primária e secundária: a genotoxicidade primária do PM tem sido demonstrada em testes *in vitro* (ex., amianto), enquanto que os estudos da inalação crônica de PM de baixa toxicidade em ratos, poeiras pouco solúveis tais como o carbono negro e o dióxido de titânio levaram à identificação de genotoxicidade secundária. A formação excessiva e persistente de ERO a partir de células inflamatórias (neutrófilos, macrófagos) durante a inflamação pulmonar induzida por PM é considerada como a característica mais importante da genotoxicidade secundária. **Fonte:** Modificado de Schins,[98].

O PM gerado a partir de fontes de combustão contém um determinado número de componentes que geram ERO por uma variedade de reações. Os mais importantes são os HAPs, metais de transição com propriedades redox, radicais livres persistentes e compostos orgânicos voláteis (COVs), que podem ser metabolicamente ativados para ERO, reagindo para gerar quebras ou adutos com o DNA [99].

3.2 Alterações no ciclo celular e na integridade do fuso mitótico

A composição do PM presente no ar também tem um papel primordial na promoção dos efeitos biológicos adicionais à geração de dano celular direto ou à produção de danos oxidativos. Estudos *in vitro* têm demonstrado que o PM pode inibir o crescimento celular, reduzindo a proliferação e/ou causando a morte das células [100, 101]. A diminuição na proliferação tem sido associada a uma parada em várias etapas do ciclo celular [102, 103].

Estudos sobre as alterações no ciclo celular pela exposição a $PM_{2.5}$ revelaram um aumento no número de células (parada transiente) em *G2,* associado com um incremento da fosforilação de *Chk2*. O aumento do número de células em G2 foi seguido por uma parada transiente no ponto de transição metáfase/anáfase, associado por sua vez com a presença de aberrações do fuso mitótico [104]. Outros estudos em células V79 (fibroblastos de pulmão de hamster chinês) demonstraram que a exposição a extratos orgânicos de material particulado induziram a formação de fusos mitóticos incompletos ou multipolares [105].

A progressão do ciclo celular pode ser bloqueada e/ou retardada como resposta às lesões genotóxicas, mas também à distúrbios estruturais de várias proteínas (Fig. 19).

As paradas de avaliação da integridade do DNA G1/S, G2/M e a transição metáfase-anáfase (M/A) podem determinar atrasos no ciclo celular [106, 107]. As proteína-cinases ATM (Ataxia-telangiectasia mutada) e ATR (ATM e Rad3 relacionadas) contribuem na resposta aos danos no DNA e ativam as proteínas cinases do checkpoint Chk1/2, o que pode resultar na parada do ciclo celular por uma via dependente ou independente de p53 [108]. Ambas as vias regulam as atividades dos promotores de transição G1/S ou G2/M dependentes de ciclinas (CDK)/ciclina, como por exemplo Cdk1/ciclinaB1, que conduz a progressão de G2 para a fase mitótica [109]. Na via dependente de p53, Chk1/2 fosforila a p53 (no resíduo Ser15), que por sua vez, através da ativação transcricional de mediadores p21 e 14-3-3, inibe a Cdk1/ciclinaB1 [109]. Nos estudos sobre os efeitos do PM sobre o ciclo celular, a parada na progressão da mitose foi caracterizada por um desequilíbrio entre o número de células em cada fase mitótica (maior incidência de células pró e metafásicas em comparação a anafásicas e telofásicas), sugerindo a presença de uma possível disfunção estrutural nos microtúbulos (MT) e na montagem do fuso mitótico. Além disso, as células mitóticas apresentaram diferentes tipos de aberrações do aparelho mitótico, incluindo fusos tripolares, multipolares e incompletos [110].

Aberrações semelhantes no fuso mitótico tinham sido relatados em células V79 após exposição a PM_{10} [111] e em células do epitélio bronquial humano (BEAS-2B) expostas a $PM_{2.5}$ [104]. Estes resultados indicam que o PM pode atuar como um veneno do fuso, perturbando diretamente a dinâmica dos microtúbulos, e sugerem que a ativação da *parada do fuso (SAC)* é o principal mecanismo envolvido na parada da transição M/A. De fato, a amplificação dos centrossomas e aumento no número de polos do fuso são conhecidos por causar um atraso no início da anáfase através da ativação do SAC [112].
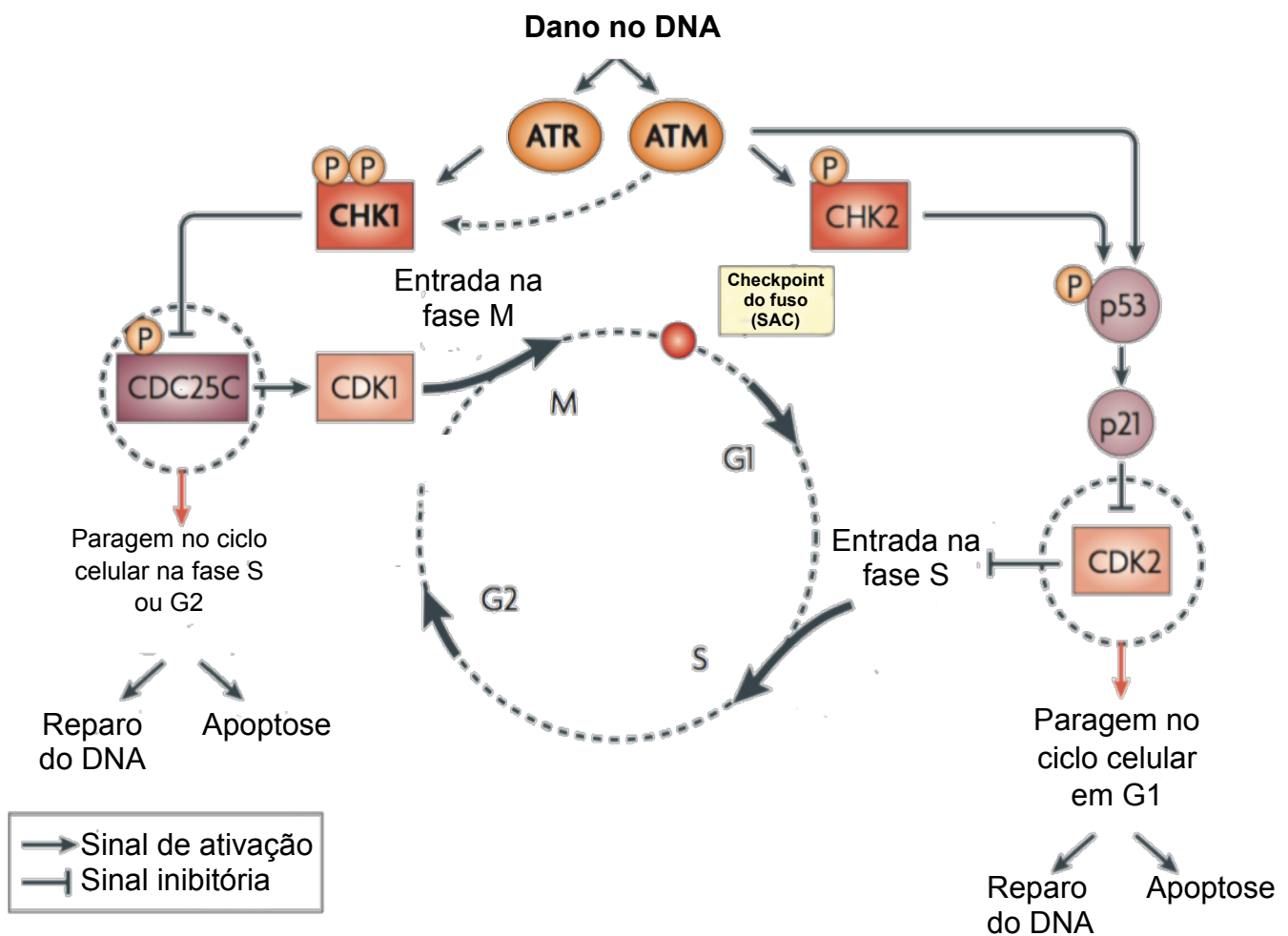
Figura 19. A cascata ATM-ATR é ativada poucos minutos depois de um alarme de dano no DNA. ATM e ATR podem fosforilar e ativar o fator de transcrição p53, diretamente ou por meio da ativação prévia das cinases do check-point 2 (CHK2). Entre os genes induzidos por p53 encontram-se o inibidor de p21, a cinase dependente de ciclinas 2 (CDK2) - também conhecido como CDKN1A e CIP1. A atividade de CDK2 impede que as células danificadas entrem na fase de síntese de DNA (S). Adicionalmente, o passo das células danificadas que já passaram a transição entre (G1) para a fase S pode ser interrompida através da ativação de CHK1, a qual fosforila a fosfatase CDC25C de dupla especificidade, fornecendo um sinal que induz seu sequestro no citoplasma. Devido ao fato de que CDC25C é responsável pela remoção de dois fosfatos inibidores de CDK1, a sua inativação impede que a célula ingresse à fase de mitose (M). A detenção do ciclo celular na fase G1, S ou em G2 é mantida até que a integridade do DNA seja restaurada. Se as lesões são irreparáveis, a morte celular é induzida pela via de sinalização ATM-ATR. **Fontes:** Adaptado de Lapenna e Giordano, [110].

3.3 Alterações cromossômicas

Estudos realizados *in vitro* [113, 114] ou *ex vivo* em populações de animais [115, 116] ou humanas que sofrem exposição ocupacional aos resíduos de mineração do carvão [17, 117] têm estabelecido que os resíduos presentes nas misturas complexas geradas a partir das atividades de exploração do carvão possuem a capacidade de gerar alterações celulares relacionadas com instabilidade genômica. Alguns dos danos mais reconhecidos incluem: micronúcleos (MN), pontes nucleoplásmicas (NPB) e brotos nucleares (NBUDS) em linfócitos, assim como em células da mucosa oral. Estas lessões podem ser detectadas em várias linhagens celulares. Em estudos *in vivo,* são principalmente avaliados em linfócitos e células exfoliadas da mucosa oral.

Os MN em células binucleadas (MNBN) originam-se principalmente a partir de cromossomos inteiros ou fragmentos de cromossomos, enquanto as NPB são causadas por cromossomos dicêntricos ou cromátides não separadas durante a anáfase/telófase. Quando uma NPB é quebrada, pode levar à formação de um MNBN ou de um NBUD [3,26,27]. Os MN em células mononucleadas (MNMONO) são formados a partir de células incapazes de se

dividir *ex vivo* devido a danos no DNA que causam a ativação de paradas (*checkpoints*) do ciclo celular, induzindo a não progressão da divisão da célula. Os MNMONO tambem podem ter sua origem em células que concluíram a replicação do DNA, mas que são incapazes de se dividir devido à saída da mitose, ou a partir de micronúcleos provenientes de NBUDS produzidos durante a fase S pela eliminação do DNA amplificado ou complexos de reparação de DNA [37, 38].

Existem vários mecanismos pelos quais os produtos químicos genotóxicos podem causar lesões no DNA que conduzem a expressão de lessões celulares *in vivo* ou *ex vivo* (Fig. 20), como segue:

(I) Produtos químicos que geram, direta ou indiretamente ERO ou ERN capazes de quebrar o esqueleto fosfodiéster do DNA, levando a quebras de DNA (por exemplo, peróxido de hidrogênio, bleomicina, ésteres de forbol).

(II) Produtos químicos que inibem a resposta a danos no DNA (DDR), interferindo com o sítio catalítico ou deslocando importantes co-fatores de enzimas de síntese ou reparacao do DNA (3-aminobenzamida, citosina-arabinósido, níquel, cádmio). Esses inibidores podem resultar em erros na reparação ou de replicação das quebras simples no DNA ou na persistência dos danos no DNA, tais como quebras ou adutos.

(III) Produtos químicos que induzem pequenos adutos no DNA como 8-hidroxi-2'-desoxiguanosina, N7-metilguanina ou adutos do benzo[a]pireno diolepoxide.

(IV) Produtos químicos que induzem cross-links entre as fitas do DNA tais como mitomicina-C ou *crosslinks* DNA/proteína DNA como o formaldeído.

(V) Produtos químicos que causam anomalias cromossômicas numéricas por inibição da polimerização de proteínas como tubulina e actina, necessárias para a formação de estruturas do citoesqueleto como os microtúbulos e microfilamentos, que são essenciais para o processo da mitose, ou causar danos aos cinetócoros ou coesinas, necessários para a segregação das cromátides durante anáfase. Os exemplos incluem o taxol (inibidor da polimerização da tubulina), tryprostatin-A (inibidor da formação de filamentos), Sepin-1 (inibidor da separase).

(VI) Químicos que interferem com mecanismos epigenéticos, como inibidores da metilação do DNA, incluindo o metabolismo doador de metilo ou a inibição direta das metiltransferases do DNA ou modificações das histonas de heterocromatina centromérica. Exemplos incluem 5-azacitidina (inibidor da DNA-metiltransferase) e o Vorinostat (inibidor das histona-desacetilases) [118]. Adicionalmente, as substâncias químicas que podem causar segregações cromossômicas incorretas e aberrações cromossômicas numéricas perturbam a formação do fuso mitótico, causando danos nos centrômeros e cinetocoros ou desativando os processos pelos quais as cromátides se alinham no eixo ou são separadas durante a anáfase [119].

O encapsulamento de um cromossoma inteiro segregado incorretamente dentro de um MNBN pode levar a um enorme número de quebras dentro do cromossoma por causa da síntese de incompleta do DNA e da união ineficaz dos fragmentos de Okazaki devido à (i) falta de sincronia entre o ciclo de replicação do DNA no núcleo principal e o MNBN e/ou (ii) captação disfuncional de enzimas de replicação do DNA e seus cofatores no MNBN, os quais podem ser afetados pela extensão da expressão de lamin A e lamin B na membrana do MNBN [120].
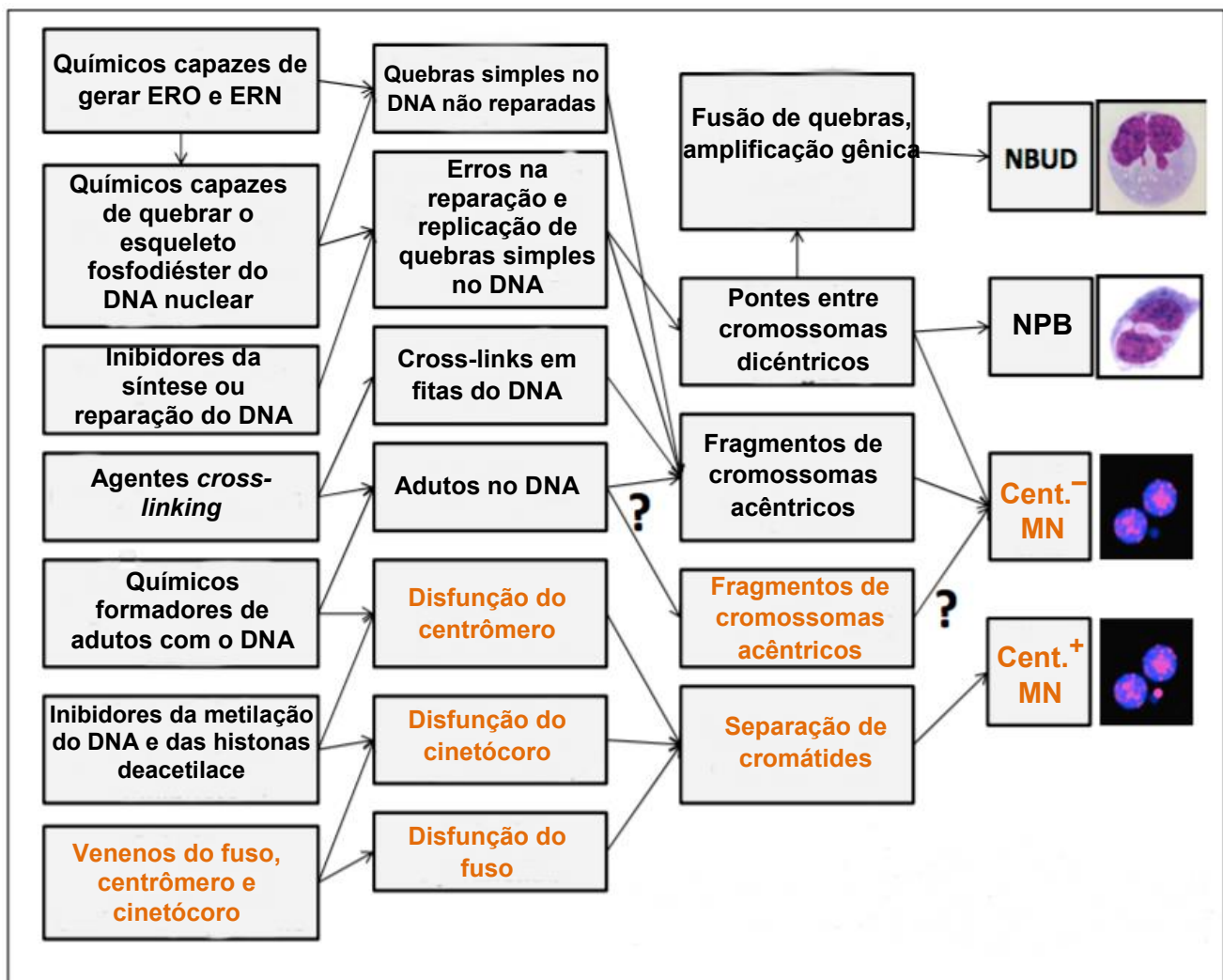
Figura 20. Mecanismos pelos quais compostos genotóxicos podem causar micronúcleos (MN), pontes nucleoplásmicos (NPB) e brotos nucleares (NBUDS). MN que contem cromossomas inteiros são centrômero positivos (Cent.+) como se detecta com o uso de provas moleculares para o DNA centromérico ou anticorpos anti-cinetócoro. MN com fragmentos cromossômicos são centrômero negativos (Cent.−). ERO: espécies reativas de oxigênio; ERN: espécies reativas de nitrogênio. ?: possível mas sem dados suficientes para afirmar.
Fontes: original de Kirsch-Volders et al., [124], adaptação de Fenech et al., [118].

Estas evidências indicam que os MNBN que contém cromossomos inteiros, que já estão presentes dentro de linfócitos *in vivo,* podem levar a novas aberrações cromossômicas e cromatídicas quando a célula se divide *ex vivo.* Esses fenômenos, conhecidos como *cromoanageneses e cromotripsis,* têm sido identificados como os mecanismos mutagênicos mais rápidos e devastadores dentro de cromossomas individuais [121, 122].

As alterações cromossômicas citadas acima podem ser estudadas na mucosa bucal (BM), um tecido de fácil acesso para a amostragem minimamente invasiva. A escolha das células da mucosa oral é considerada vantajosa, pois apresenta alto grau de divisões celulares e dispensa a realização de cultura celular *in vitro* [123]. De modo semelhante aos linfócitos, na análise da BM também se considera a formação de brotos nucleares, células binucleadas (relacionadas a defeito citocinético) e anormalidades nucleares que representam morte celular, reconhecidas pela presença de cromatina condensada, células cariorréticas e picnóticas (Fig. 21).

Diferente de outros tipos celulares, o epitélio é formado por várias camadas de células que vão sendo esfoliadas à medida que alcançam a superfície. Por esse motivo, o dano genético detectado ocorre nas camadas

basais, local onde as células se dividiram. A rápida renovação dos tecidos epiteliais faz que o máximo índice de formação de MN apareça entre 1 e 3 semanas depois da exposição ao agente genotóxico [124], que é o tempo necessário para que as células migrem das camadas basais do epitélio até a superfície. A frequência basal média dos MN nas células de esfoliação epitelial da mucosa oral tende a ser inferior à observada nas células sanguíneas [123].
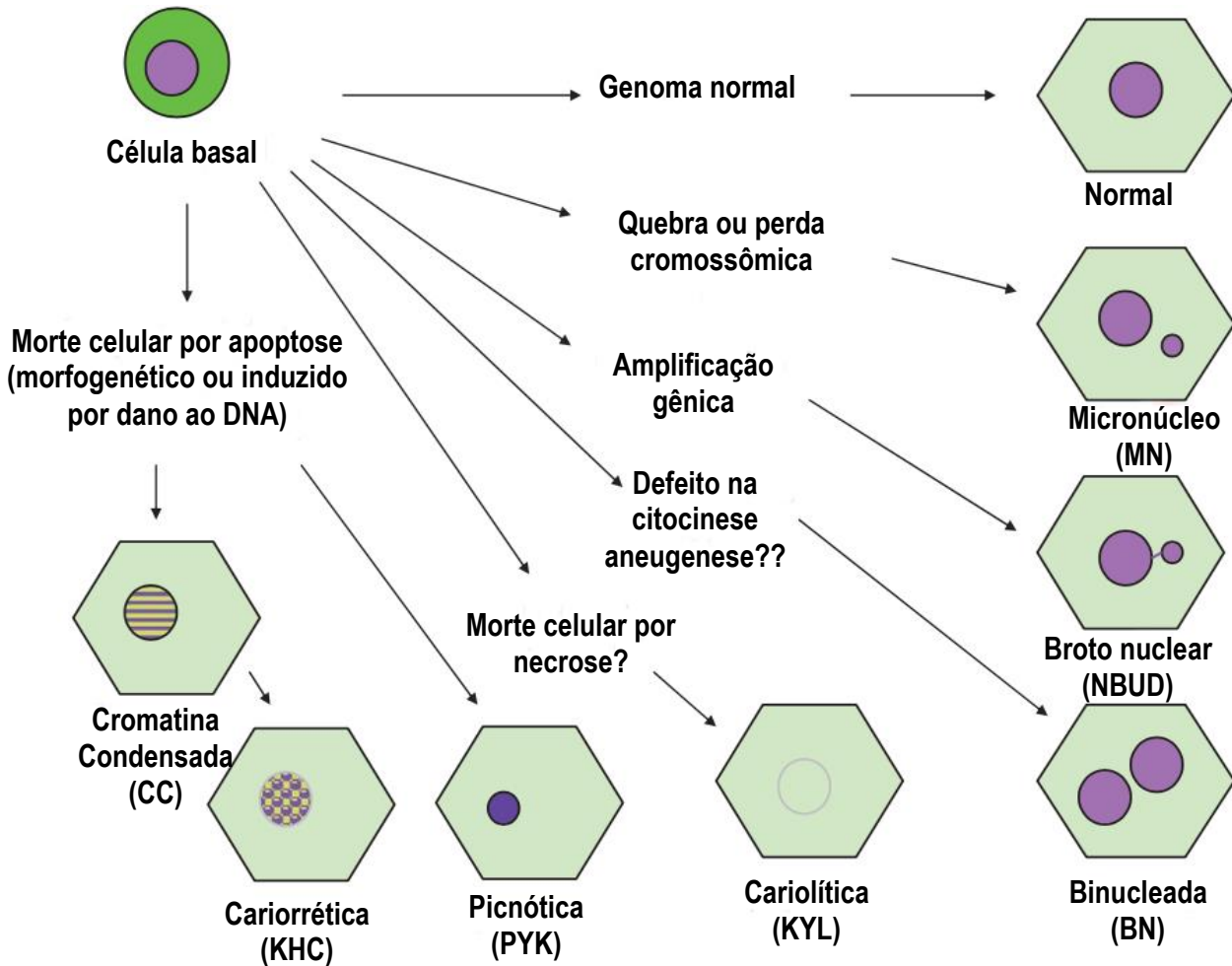
Figura 21. Representação esquemática dos tipos de células avaliados no ensaio BMNCyt. As análises de vários tipos de células são feitas na base do esquema proposto por Tolbert et al.,[125].
Fonte: Adaptação de Thomas et al., [126].

II. OBJETIVOS
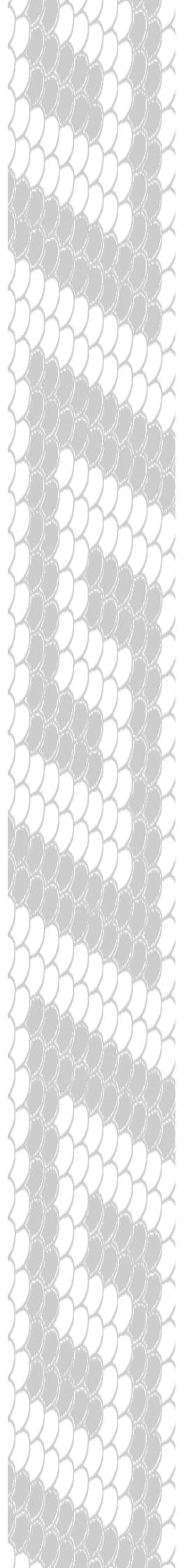
OBJETIVO GERAL

Esta Tese de Doutorado tem como objetivo principal avaliar os efeitos genotóxicos e citotóxicos em populações de indivíduos com exposição ambiental às misturas complexas geradas nos sistemas de mineração de carvão a céu aberto no Departamento de Guajira, Colômbia.

OBJETIVOS ESPECÍFICOS

- Avaliar os efeitos mutagênicos induzidos pela exposição residencial a PM dentro da área de influência direta de uma mina de carvão a céu aberto mediante a avaliação da frequência dos parâmetros do ensaio CBMNCyt em populações humanas.

- Avaliar a frequência de alterações citogenéticas em células da mucosa oral de indivíduos com exposição residencial a PM dentro da área de influência direta de uma mina de carvão a céu aberto mediante o uso do ensaio BMNCyt.

- Avaliar a presença de lesões oxidativas no DNA de populações humanas com exposição residencial a PM dentro da área de influência direta de uma mina de carvão a céu aberto mediante o uso do ensaio de Cometa modificado com enzimas - DNA Formamidopirimidina glicosilase (FPG) e Endonuclease III (Endo III).

- Avaliar o possível mecanismo de origem (aneugênico ou clastogênico) dos micronúcleos (MN) induzidos pela exposição residencial a PM dentro da área de influência direta de uma mina de carvão a céu aberto mediante o uso da imunotinção CREST.

- Determinar as concentrações de $PM_{2.5}$ e PM_{10} em populações dentro da área de influência de uma mina de carvão a céu aberto mediante o uso de amostradores de ar de alto volume (HiVol).

- Avaliar o conteúdo de elementos inorgânicos nas frações de $PM_{2.5}$ e PM_{10} de áreas de mineração de carvão a céu aberto e áreas controle sem mineração de carvão utilizando o método PIXE.

- Avaliar os níveis de metais no sangue de indivíduos com exposição residencial a PM dentro da área de influência direta de uma mina de carvão a céu aberto utilizando o método PIXE.

- Estabelecer a possível correlação entre as concentrações de $PM_{2.5}$ e PM_{10} dentro da área de influência de uma mina de carvão a céu aberto e a frequência de alterações citogenéticas e oxidativas de dano no DNA em indivíduos com exposição residencial.

- Avaliar o uso de ferramentas de informação geográfica para o estabelecimento de correlações espaciais entre a distribuição das frequências de MN e de outros fatores ambientais como as concentrações de PM e metais no ar.

III. CAPÍTULO I

CYTOGENETIC INSTABILITY IN POPULATIONS WITH RESIDENTIAL PROXIMITY TO OPEN-PIT COAL MINE IN NORTHERN COLOMBIA IN RELATION TO PM$_{10}$ AND PM$_{2.5}$ LEVELS
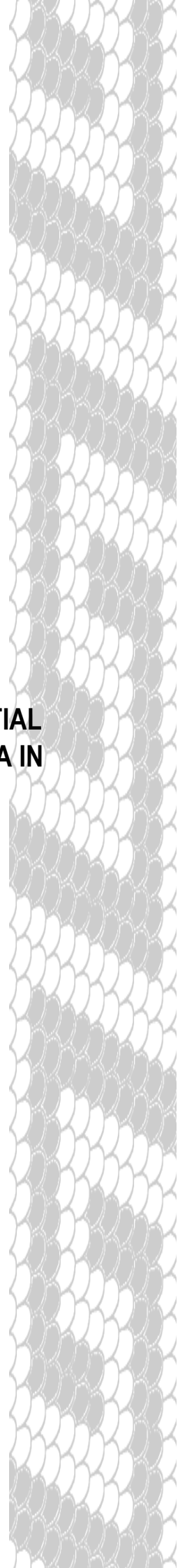
CYTOGENETIC INSTABILITY IN POPULATIONS WITH RESIDENTIAL PROXIMITY TO OPEN-PIT COAL MINE IN NORTHERN COLOMBIA IN RELATION TO PM$_{10}$ AND PM$_{2.5}$ LEVELS

Lyda Espitia-Pérez [a,b,c*], Juliana da Silva [d], Pedro Espitia - Pérez [a], Hugo Brango [e], Shirley Salcedo – Arteaga [a] Luz Stella Hoyos-Giraldo [f], Claudia T. de Souza [g], Johnny F. Dias [g], Dayana Agudelo-Castañeda [h], Ana Valdés Toscano [a], Miguel Gómez-Pérez [a], João A. P. Henriques [c**]

[a] Facultad de Ciencias de la Salud, Laboratorio de Investigación Biomédica y Biología Molecular, Universidad del Sinú, Montería, Córdoba, Colombia.

[b] Departamento de Ciencias Básicas - Facultad de Ciencias e Ingenierías, Universidad del Sinú, Montería, Córdoba, Colombia.

[c] Departamento de Biofísica, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

[d] Laboratório de Genética Toxicológica, Universidade Luterana do Brasil, ULBRA, Canoas, RS, Brazil.

[e] Instituto de Matemática e Estatística, Universidade de São Paulo, São Paulo, Brazil

[f] Department of Biology, Research Group Genetic Toxicology and Cytogenetics, Faculty of Natural Sciences and Education, Universidad del Cauca, Popayán, Cauca, Colombia.

[g] Laboratório de Implantação Iônica, Instituto de Física, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brasil.

[h] Research group in Environmental Management and Sustainability. Faculty of Environmental Sciences. Universidad De La Costa. Barranquilla, Atlántico, Colombia.

For correspondence with authors:
*E-mail address: espitiaperez@gmail.com (L. Espitia– Pérez)
**E-mail address: pegas@cbiot.ufrgs.br (JAP. Henriques)

ABSTRACT

Epidemiological studies indicate that living in proximity to coal mines is correlated with numerous diseases including cancer, and that exposure to PM_{10} and $PM_{2.5}$ components could be associated to this phenomenon. However, the understanding of the mechanisms by which PM exerts its adverse effects is still incomplete and comes mainly of studies in occupationally exposed populations. The aims of this study were to: (1) evaluate DNA damage in lymphocytes assessing the cytokinesis-block micronucleus cytome assay (CBMN-cyt) parameters; (2) identify aneugenic or clastogenic effects in lymphocytes of exposed populations using CREST immunostaining for micronuclei; (3) evaluate multielemental composition of atmospheric particulate matter; and (4) verify relation between the DNA damage and $PM_{2.5}$ and PM_{10} levels around the mining area. Analysis revealed a significant increase in micronuclei frequency in binucleated (MNBN) and mononucleated (MNMONO) cells of individuals with residential proximity to open-pit coal mines compared to residents from non-mining areas. Correlation analysis demonstrated a highly significant association between $PM_{2.5}$ levels, MNBN frequencies and CREST+ micronuclei induction in exposed residents. These results suggest that $PM_{2.5}$ fraction generated in coal mining activities may induce whole chromosome loss (aneuploidy) preferentially, although there are also chromosome breaks. Chemical composition of $PM_{2.5}$ by PIXE demonstrated the presence of highly enriched elements like S and moderate enrichment of Cr, Cu and Zn. Mining regions had also higher concentrations of extractable organic matter (EOM) related to nonpolar and polar compounds. Our results demonstrate that $PM_{2.5}$ fraction represents the most important health risk for residents living near open-pit mines, underscoring the need for incorporation of ambient air standards based on $PM_{2.5}$ measures in coal mining areas.

1. INTRODUCTION

Around coal mines and other industrial facilities, exposure to particle matters (PM) has been associated with premature death, increased morbidity from respiratory diseases [1], lung cancer [2] and cardiopulmonary diseases [3]. The biological mechanisms behind these associations are not fully understood, but the results of *in vitro* toxicological research have shown that PM induces several types of adverse cellular effects, including genotoxicity [4], mutagenicity, DNA damage and stimulation of pro-inflammatory cytokine production [5].

PM size is an important factor that influences how it is deposited in the respiratory tract and affects human health. Large particles are generally filtered in the nose and throat and do not necessarily cause problems. An important fraction of PM is referred to as PM_{10}, composed of particles ≤ 10 µm. PM_{10} is generally subdivided into a fraction of finer particles ≤ 2.5 µm ($PM_{2.5}$), and a coarser fraction of particles > 2.5 and <10 µm ($PM_{2.5-10}$). $PM_{2.5}$ is dominated by products of combustion and secondary aerosols, while $PM_{2.5-10}$ consists mainly of crustal, biological, and fine particle fraction components [6]. However, PM with a mass median aerodynamic diameter ≤ 10 µm, has been shown to be potentially hazardous to health, due to the complex mixture of compounds and the fact that it can settle in the bronchi and lungs [7]. $PM_{2.5}$ fraction overtakes the alveolar lung region where blood exchange takes place, and for this reason it represents the high-risk breathable fraction of the inhalable fraction.

Airborne PM generated in open-pit coal mines is a complex mixture of particles that vary not only in size and morphology, but also in their chemical, physical, and biological characteristics depending on coal composition [8]. PM contains high concentrations of toxic elements, such as chromium (Cr), cadmium (Cd), titanium (Ti), manganese (Mn), nickel (Ni), lead (Pb), arsenic (As), zinc (Zn), etc. [9]. These toxic elements incorporated with atmospheric $PM_{2.5}$ and PM_{10} may enter the body through inhalation [10]. Additionally, in open pit mines extracted coal is stored in the sunlight at high ambient temperatures, where spontaneous and incomplete coal combustion may lead to Polycyclic Aromatic Hydrocarbons (PAHs) emission [11, 12]. Particularly in open–cast mining facilities these toxic substances are released into the atmosphere where they can form complex mixtures [13]. Such mixtures represent one of the most important health and safety hazards to exposed populations due to potential synergistic effects of the resulting combinations [14].

Unlike inorganic elements that can be presents in both $PM_{2.5}$ and $PM_{10,}$ fractions [15], PAHs shows a strong association with $PM_{2.5}$ fraction. Several studies have reported that 87-95% of PAHs can be found in fine particles (2.5 µm) [16]. This correlation seems to be stronger for the heavier and more carcinogenic 5-6-ring PAHs [17].

While there are studies showing increased micronuclei (MN), sister chromatid exchanges (SCEs) and chromosomal aberrations (CAs) frequencies due to occupational exposure to coal residues, there is a scarcity of data examining the impact of these industrial operations to cytogenetic endpoints frequency and cancer rates of potentially exposed surrounding populations (non-occupationally exposed). In a recent review on the effect of complex mixture exposition on MN frequencies, Da Silva [18] described seven studies using the CBMN assay as endpoint of mutagenic effect of human exposition to coal, including coal dust and products from coal burning; all studies assessed occupational exposures in mines, power plants and coal fly ash processing industry.

In previous studies about the occurrence of genetic damage in human populations exposed to coal mining residues in La Guajira peninsula, we evaluated 100 employees with occupational exposure. Using the Comet assay (SCGE) and micronucleus (CBMN) frequency in lymphocytes and oral mucosa cells, we demonstrated a statistically significantly increase for all variables in the exposed group compared to the non-exposed control group [19]. The exposed group also showed higher values of silicon (Si) and aluminum (Al) in whole blood samples [13]. Similar results, also in occupational exposed individuals were obtained in workers from the open-pit region of Candiota, Brasil [20].

In the CBMN-cyt, DNA damage events are scored specifically in once-divided binucleated (BN) cells and include (a) MNBN, a biomarker of chromosome breakage and/or whole chromosome loss, (b) MNMONO a biomarker of chromosomal damage induced and expressed *in vivo* before the start of the CBMN assay culture, (c) NPBs, a biomarker of DNA misrepair and/or telomere end-fusions, and (d) NBUDs, a biomarker of elimination of amplified DNA and/or DNA repair complexes [21]. MN frequencies in MONO cells may give an estimation of the genome instability accumulated over many years in stem cells and circulating T lymphocytes, thus before the blood was sampled, whereas MN frequencies in BN cells additionally provide a measure of the lesions that have accumulated in the DNA or in key proteins [22].

This study aimed to 1) evaluate DNA damage in lymphocytes assessing the cytokinesis-block micronucleus cytome assay (CBMN-cyt) parameters; (2) identify aneugenic or clastogenic effects in lymphocytes of exposed populations using CREST immunostaining for micronuclei; (3) evaluate multielemental composition of atmospheric particulate matter; and (4) verify relation between the DNA damage and $PM_{2.5}$ and PM_{10} levels around the mining area.

2. METHODOLOGY

2.1 Study area and sampling site location

Considering previous results of mining activities related to increased DNA damage parameters in occupational exposed populations [19] and in order to differentiate substances possibly related to specific activities in the coal production chain, five sampling areas were defined, around or in proximity to different mining operations activities (Table 1).

The Barrancas municipality included three sampling areas, two denominated "resguardos" or indigenous Wayúu settlements with political status and organization: Provincial and San Francisco and a small population of Afro-Colombians named Chancleta. The latter in process of resettlement due to the high levels of air pollution. The Hatonuevo municipality included one sampling area, a resguardo named El Cerro de Hatonuevo. The last area was located in the port facility (Puerto Bolívar) in a Wayúu settlement or "ranchería" named Media Luna. The unexposed (Control) area was located in Mayapo municipality, also constituted of indigenous Wayúu settlements and Afro-Colombian populations, situated on the Caribbean sea coast without any influence of coal mining operations and located 57.12 kilometers from the city of Riohacha.

Table 1. Description of sampling sites in relation to proximity to coal mining areas and principal activities possibly related to emission of atmospheric suspended particles.

Municipality	Area	Principal activities	Rancheria/Resguardos
Mayapo	Reference	Area without coal mining activity.	Mayapo
Barrancas	Open Pit	Drilling, blasting, overburden loading and unloading, coal transport through unpaved roads.	Provincial
		The coal may be temporarily stored.	San Francisco
	Dump Site / Open Pit	Topsoil handling, coal transport through unpaved roads, overburden loading and unloading.	Chancletas
Hatonuevo	Open Pit	Drilling, blasting, overburden loading and unloading, coal transport through unpaved roads. The coal may be temporarily stored.	El Cerro de Hatonuevo
Uribia	Port Facility (Puerto Bolívar)	Rail car unloading, storage, and transport to a ship loader. The coal may be temporarily stored or sent directly from the unloading station to the cargo hold.	Media Luna

The open pit coal-mining region object of the present study is located in the semiarid southern part of the Department of La Guajira in northeast Colombia between the municipalities of Albania, Barrancas, and Hatonuevo. It is composed of indigenous Wayúu settlements, a small Afro-Colombian population and a number of campesino (rural peasant) communities. Other areas influenced by the coal mining process include those around

a 150 km railroad and areas around the port facility in the municipality of Uribia on the Caribbean Sea coast, where the coal is exported (Figure 1). Sampling sites were located inside the area of direct influence of the mining operations and around the port facility. The topography of the area is characterized by a large flat area bordered by a region of high mountains. The study focuses on villages located in the flat area where high levels of PM_{10} have been repeatedly reported by the local air quality network of the regional environmental agency (CORPOGUAJIRA).

2.2 Human biomonitoring

The Committee on Research Ethics of each institution approved the study. A meeting with the governments of each resguardo, representatives of the communities and municipal authorities was held to present the study proposal. Samples of individuals with residential proximity to open-pit coal mining area (exposed) and from non-mining area (control) were collected and transported simultaneously to prevent any interference caused by differences in sampling conditions.

Before sample collection, a written informed consent was obtained from each individual. Collection was performed on 139 healthy individuals: 98 with permanent residential proximity to open – pit coal mining area and 41 permanent residents of a control area without any proximity to open-pit coal mining facilities. Exposed and unexposed control individuals were matched by age (± 5 years), sex, similar social-economic status and ethnicity. Participants were instructed to respond to a detailed, standard questionnaire that included data on health status (use of prescription medicines), cancer history, other chronic diseases, lifestyle, nutrition, smoking habits, frequency of alcohol consumption and previous exposure to medical X-rays. Considering that domestic use of wood fuel is a common practice between Wayúu and Afro-Colombians communities in this area, all individuals in this study (exposed residents and unexposed controls) were required to cook using artisanal wood stoves to eliminate confounding factors. The exposed group was selected according to the following inclusion approaches: voluntary acceptance, been healthy and with permanent residence in proximity to coal mining area. Exclusion criteria for exposed and non-exposed groups were age over 65 years or less than 18 years, smoking (current and ex-smoking habits), actual medical treatment or up to 3 months or X-ray up to 1 year before sampling and therapeutic drugs intake, known to be mutagenic. Alcohol consumers were classified as low, moderate or heavy drinkers according to NIAAA (National Institute of Alcohol Abuse and Alcoholism) parameters. According to this, low drinking is no more than 3 drinks on any single day and no more than 7 drinks per week. For men, it is defined as no more than 4 drinks on any single day and no more than 14 drinks per week. Moderate drinking is up to 1 drink per day for women and up to 2 drinks per day for men and heavy drinking as 5 or more drinks on the same occasion on each of 5 or more days in the past 30 days. Most of exposed and unexposed individuals declared to be non-alcohol consumers, while 10.2% of exposed and 12% of unexposed defined themselves as moderate drinkers. All data was organized and recorded in databases. No major differences regarding social-economic status or dietary habits between exposed and unexposed were identified.

2.3. Blood samples collection

After informed consent was obtained from each individual, peripheral blood samples from all 139 individuals were collected by venipuncture. 8 mL of blood were drawn into EDTA and Heparin tubes (Becton
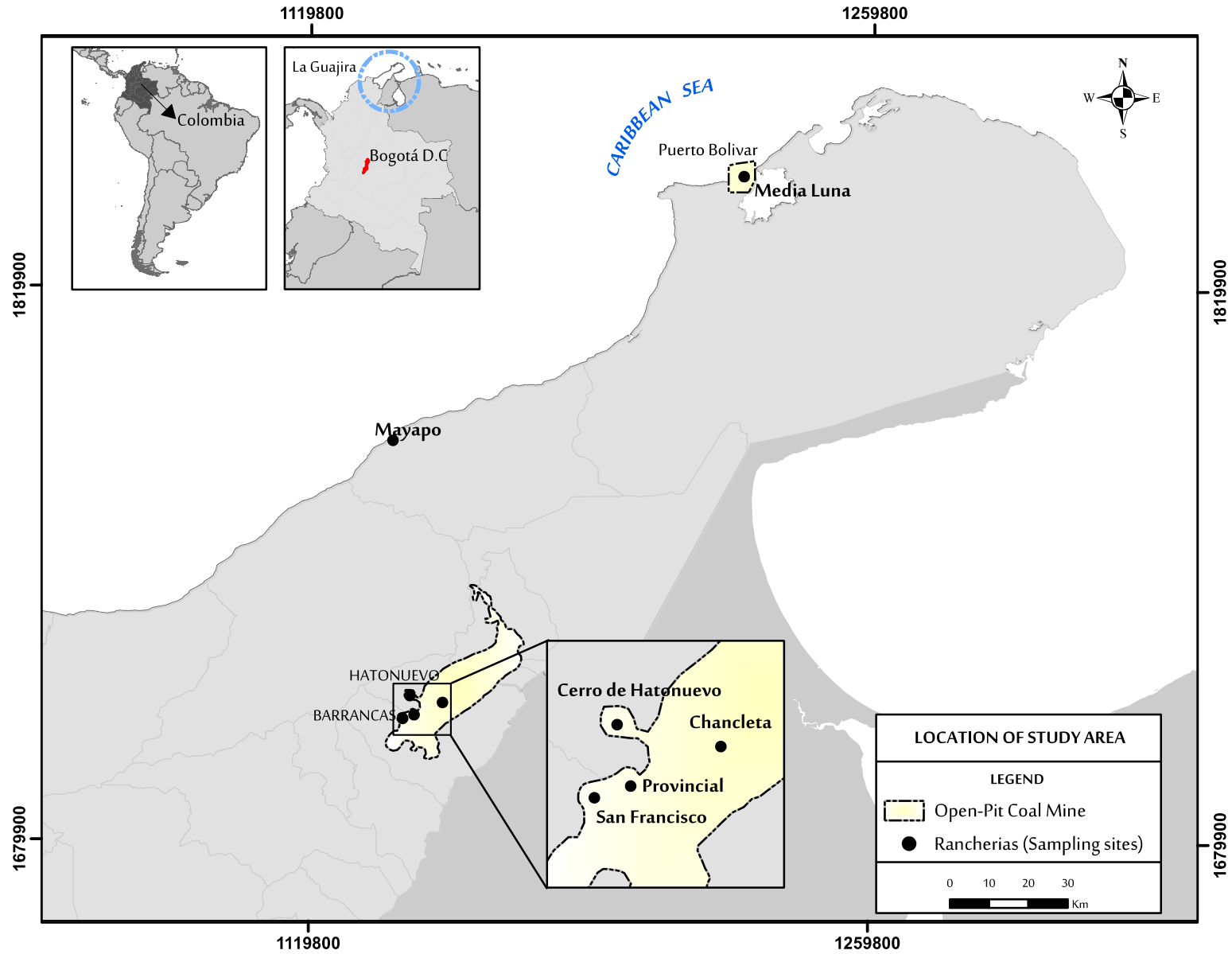
Figure 1. Sampling sites locations in Colombia

Dickenson, vacutainer) for CREST staining micronucleus test and for CBMN-cyt assay respectively. All blood sample tubes were coded, kept at 4 ∘C, and transported to the laboratory for processing within 24h of collection. Concomitantly with collection of the blood samples of exposed and unexposed individuals, additional samples of whole blood from the research staff were collected, transported and processed under the same conditions. These samples were used as internal standards (Internal controls) for the detection of potential confounding factors that may have been caused by sample handling or transportation to the laboratory.

2.4. Cytokinesis-block micronucleus cytome (CBMN-cyt) assay

CBMN assay was carried out according to methodology previously described by Fenech, 2007 [21]. Briefly, heparinized whole blood (0.5 mL) was added to 4.5 mL of RPMI 1640 medium (Sigma R8758, USA) supplemented with 2 mM l-glutamine (Sigma A5955, USA), 10% fetal bovine serum (Gibco/Invitrogen 15000-044, Brazil), 100 µL/mL antibiotic–antimycotic (Sigma A5955, USA) and 2% phytohemaglutinin (Sigma L8754, USA). Cultures were incubated at 37 °C in the dark for 44 h, under 5% CO_2. 6 µg/mL of cytocalasin B (Sigma, C6762) was added at the 44th h of incubation.

After incubation, lymphocytes were harvested via centrifugation at 1200 rpm for 8 min, re-centrifuged, fixed in 25:1 (v/v) methanol/acetic acid, placed on a clean microscope and stained with Diff-Quik stain (Lab-Aids; LP64851). For each blood sample, 2000 binucleated cells (BN) (i.e., 1000 from each of the two slides prepared from the duplicate cultures) were scored for the presence of MN (MNBN), nucleoplasmic bridges (NPB) and nuclear buds (NBUD) using bright-field optical microscopy at a magnification of 200–1000×. Additionally, MN frequency in 1.000 mononucleated cells was evaluated to determine MNMONO frequency. Based on the ratio of mononucleated (MONO), BN and multinucleated (MULT) cells, cytochalasin B proliferation index (CBPI) was also calculated. All slides were coded for blind analysis according to criteria proposed by Fenech et al [23].

2.4.1 CREST immunostaining

Since the quality of cytological preparations using whole blood cultures is not satisfactory when CREST staining is performed [24], lymphocytes were separated using a histopaque density gradient. Isolated lymphocytes (0.5 mL) were added to 4.5 mL of RPMI 1640 medium (Sigma R8758, USA) supplemented with 2 mM l-glutamine (Sigma A5955, USA), 10% fetal bovine serum (Gibco/Invitrogen 15000-044, Brazil), 100 µL/mL antibiotic–antimycotic (Sigma A5955, USA) and 2% phytohemaglutinin (Sigma L8754, USA). Cultures were incubated at 37 °C in the dark for 44 h, under 5% CO_2. 6 µg/mL of cytocalasin B (Sigma, C6762) was added at the 44th h of incubation.

Lymphocyte cell cultures were harvested using a modification of the technique described by Fenech and Morley [25]. Cells were pelleted and transferred directly from the 96-well microplate through a frosted slide using air blowing to assure the equal extension of the cell film and air-dried for 5 minutes. The cells were then fixed with methanol-acetic acid (25:1) pre-chilled at -20°C for 10 minutes, and air-dried again for maximum 10 minutes. The slides were stored in a desiccated sealed box at -20°C for further analysis.

CREST immunostaining was performed using a slight modification of the procedures previously described by Eastmond and Tucker [26] and Olivero et al. [27]. Briefly, slides were rinsed with 0,05% Tween 20-PBS three times for 5 minutes each inside a dark customized staining jar using orbital motion to allow background

elimination. The slides were directly permeabilized using 0,1% Triton X-100-PBS for 5 minutes and blocked with 1% bovine serum albumin in 0,1% Tween 20-PBS for 1 hour at room temperature. After blocking, each slide was treated directly with 40 µL of FITC conjugated IgG derived from anti-centromere positive serum (Antibodies Incorporated, Davis, CA) at a 1:40 dilution in blocking solution using a coverslip and were allowed to incubate at 4°C overnight inside a humidified chamber. Next day, slides were washed to retrieve unbound antibodies using the procedure described earlier and counterstained using 50 µL of 4´,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma D9542) at 1 µg/ml.

For mounting, each slide was treated with 50 µL of Vectashield Hard Set™ Mounting Medium (Vector Laboratories, Burlingame, CA) at a 1:1 dilution in PBS, coverslipped and sealed using clear nail polish. The slides were stored at 4°C overnight prior to observation.

Antibody application, counterstaining and slide observations were conducted under red light areas to avoid fluorochrome fading. Scoring of 1000 cells per treatment allowed the identification of micronuclei in mononucleated and binucleated cells [28], using DAPI as counterstaining, and registration of CREST+ green signals from the direct FITC conjugated antibody. Stained cells were observed using an Olympus BX51 fluorescence microscope (Olympus, Japan) equipped with a Zeiss Plan Apo 100x/1.4 oil objective. Fluorochrome observations were performed employing a set of Olympus U-MNU2 and U-MNB2 mirror units. Images were captured using an Olympus DP71 camera and DP Controller software. This probe was previously tested on metaphase chromosome for centromere-specific labeling.

2.5 Sampling of atmospheric particulate matter

Samples of atmospheric particulate matter were collected from each site concomitantly with the human biomonitoring. For all sampling sites, twenty-five PM samples were collected during a continuous period of 24h between August and December of 2015, beginning at 09:00 a.m. A BGI PQ200 FRM sampler with $PM_{2.5}$ inlet and WINS impactor (according to CFR 40 part 50, appendix L) was used with an air flow rate of 16.7 L/min, and was calibrated before its use, using a flow calibrator MesaLabs DryCal Definer 220 (Brandt Instruments, Prairieville, LA, USA). On-site calibrations included volumetric flow verification through the samplers via comparison of the calibrated orifice results to the volumetric flow controller tables. PTFE filters of 46.2 mm diameter (Tisch Environmental Inc., Cincinnati, OH, USA) were used for sampling, equilibrated in a desiccator under controlled ambient conditions (T=26.5±1 °C, RH=30.4±5 %), and weighed until reaching constant values before and after sampling. PM samples were coded, stored and transferred at 4°C for further analysis. Filters were weighed using an electronic microbalance (Radwag MYA11-3Y) with a resolution of 10^{-6} g. PM_{10} data from monitoring sites around the coal mining area were kindly provided by CORPOGUAJIRA. 24 h mean values were obtained for the same sampling period. In addition, data of air temperature, relative humidity, solar radiation, wind speed and direction were obtained from the meteorological stations located near the sampling sites.

2.6 Multielemental analysis of atmospheric particulate matter

2.6.1 Particle-Induced X-ray Emission (PIXE) assay

The elemental composition (Na, Mg, Al, Si, P, S, Cl, K, Ti, Cr, Mn, Cu and Zn) of the aerosol samples in $PM_{2.5}$ filters (blank-corrected), was measured by the conventional in vacuum Particle-Induced X-ray Emission

(PIXE) assay [29] using the accelerator facility at the Ion Implantation Laboratory of the Physics Institute, Federal University of Rio Grande do Sul. For the analysis, 1/4 of each PTFE filter was cut and use to elemental analysis.

A 3 MV Tandetron accelerator provided 2.0 MeV proton beams with an average current of 5 nA at the target. The X-rays induced by the beam in the samples were detected by a Si (Li) detector with an energy resolution of about 155 eV at 5.9 keV. The spectra were analyzed with the GUPIXWIN software package [30] and the final results are expressed in parts per million (ug/g^{-1}).

2.6.2 Enrichment factor (EF) analysis

Enrichment factor *(EF)* is widely used to identify the anthropogenic source of chemical elements [31]. For calculation of enrichment factor of each element was applied the following equation:

$$EF = \frac{\left(\frac{C_x}{C_R}\right)_{PM}}{\left(\frac{C_x}{C_R}\right)_{Crust}}$$

In this, EF is the enrichment factor of specie *x* that represents the chemical element of interest. C_x represents the concentration of the element of interest in the aerosol sample, C_R is the concentration of a reference element in the earth's crust, commonly used as Al, Si, Ti and Fe, etc. *(C_x/C_R) PM* is the concentration ratio of element *x* to R element in aerosol sample and *(C_x/C_R) Crust* is the concentration ratio of X to R element in the crustal material. In this study, Fe was selected as reference element as previously described in similar analysis [32]. For analysis, elements with EF<10 were considered as non-enriched, 10<EF<100 as moderately enriched and EF >100 as highly enriched [33]. Concentrations of elements in the upper continental crust were taken from Wedepohl [34].

2.6.3 Extractable organic matter (EOM) determination

For determination of the organic fraction, 3/4 of the PTFE filters were extracted using a Soxhlet apparatus according to standard method 3540C (USEPA Method 3540C, 1996) for 24 h (8h cycles per solvent) at 59°C in the following sequential procedure: 60 mL with cyclohexane (CH) (Merck), 60 mL dichloromethane (DCM) (Honeywell) and 60 mL acetone (ACE) (Merck). Extracted solution was concentrated to 1,5 ml using a rotary vacuum evaporator (Heidolph Instruments, Schwabach, GE). The extractable organic matter (EOM) concentration in µg/m^3 was determined from the total EOM quantity per filter divided by the total sampled air volume. The concentration of EOM in % represents the percentage of EOM in the PM$_{2.5}$.

2.7. Statistical analysis

Statistical analyses were carried out using software R version 3.3.0 (R Foundation for Statistical Computing, Vienna, Austria). A Poisson regression analysis was used in order to determine the effect of confounding factors (sex and alcohol intake) in CBMN-cyt parameters using FR data. Mean and median comparisons were performed for obtained CBMN-cyt parameters frequencies in exposed and control individuals. Correlation between CBMN-cyt parameters and PM$_{2.5}$ and PM$_{10}$ concentrations was determined using the non – parametric Spearman correlation analysis.

3. RESULTS

Demographic data of sampled populations are described in Table 2. The matching ratio between exposed and unexposed control individuals was 2.4. Considering the settlement characteristics of exposed populations, where lands and houses are of ancestral nature and generational occupation, exposure time to coal mining residues was estimated by the age of each individual. All participants have lived their whole in the exposed area life and several generations have lived in proximity of coal mining areas. The mean age of the exposed group was 35.20±13.34 years (range, 18–62 years), and of the non-exposed control group was 30.69 ± 11.56 years (range, 18–57 years). No significant difference in average age was detected between the non-exposed and exposed groups.

Table 2. Main demographic characteristics of studied population: exposed residents and unexposed individuals

Demographic characteristics	Group	
	Unexposed controls (Mean ± SD)	Exposed residents (Mean ± SD)
Number of individuals	41	98
Individuals by area		
Mayapo (Reference area / unexposed)	41	-
Provincial	-	20
San Francisco	-	26
Chancletas	-	21
Cerro de Hatonuevo	-	16
Media Luna	-	15
Gender n (%)		
Women	30 (73.17%)	68 (69.38%)
Men	11 (26.82%)	30 (30.61%)
Age (mean ±S.D)**	30.69±11.56	35.20±13.34
Alcohol consumption (%)		
Non-alcohol consumers	34 (82.92%)	84 (85.71%)
Alcohol consumers*		
Low	0	4 (4.08%)
Moderate	7 (17.07%)	10 (10.20%)

* According to NIAAA (National Institute of Alcohol Abuse and Alcoholism) parameters.
** Considered also as time of exposure in the exposed residents populations
S.D: Standard deviation.

Because of population structure and ethnic characteristics related to women role in Wayúu and Afro-Colombian communities, most participants in exposed and unexposed groups were women. However, no significant differences were observed in exposed and unexposed control groups and within groups for the proportion of male and female participants. Concerning alcohol consumption, 82.92% of unexposed individuals and of exposed residents declared to be non-drinkers. Only a small percentage of unexposed individuals were considered as moderate drinkers. There was no statistically influence of alcohol consumption in any of the parameters evaluated (Data not shown). Analysis of CBMN-cyt assay parameters in total population (Table 3)

Table 3. Poisson regression analysis of CBMN-cyt assay parameters: effect of gender in individuals with residential proximity to the open pit coal mine and unexposed controls.

Parameters	Unexposed controls			Exposed residents				
	n	Mean ± SD	Median (25th–75th percentile)	n	Mean ± SD[a]	Median (25th–75th percentile)	FR	P-value
MNBN								
Women	30	4.86 ± 3.80	5.0 (2.0 - 7.0)	68	7.53 ± 8.25	5.0 (1.2 - 10.7)	1.69	<0.01
Men	11	4.45 ± 3.67	3.0 (1.2 - 7.2)	30	6.30 ± 6.23	4.5 (2.0 - 9.0)	1.41	<0.05
Total population	41	4.75 ± 3.72	3.5 (2.0 - 7.0)	98	**7.15 ± 7.67**	**5.0 (2.0 - 10.0)**	**1.50**	**<0.01**
MNMONO								
Women	30	0.30 ± 0.70 [a]	0.0 (0.0 - 0.0)	68	**0.96 ± 1.95 [a]**	**0.0 (0.0 - 1.0)**	**3.86**	**<0.05**
Men	11	0.00 ± 0.00	0.0 (0.0 - 0.0)	30	0.33 ± 0.71	0.0 (0.0 - 0.2)	1.57	<0.05
Total population	41	0.21 ± 0.61	0.0 (0.0 - 0.0)	98	**0.76 ± 1.69**	**0.0 (0.0 - 1.0)**	**3.48**	**<0.01**
NBUD								
Women	30	0.40 ± 1.02	0.0 (0.0 - 0.2)	68	0.68 ± 1.11	0.0 (0.0 - 1.0)	0.93	NS
Men	11	0.67 ± 0.78	0.5 (0.0 - 1.0)	30	0.43 ± 0.94	0.0 (0.0 - 0.2)	0.59	NS
Total population	41	0.48 ± 0.95	0.0 (0.0 - 1.0)	98	0.60 ± 1.06	0.0 (0.0 - 1.0)	1.23	NS
NPB								
Women	30	0.20 ± 0.56	0.0 (0.0 - 0.0)	68	0.28 ± 0.62	0.0 (0.0 - 0.0)	1.78	NS
Men	11	0.09 ± 0.30	0.0 (0.0 - 0.0)	30	0.10 ± 0.31	0.0 (0.0 - 0.0)	1.10	NS
Total population	41	0.17 ± 0.49	0.0 (0.0 - 0.0)	98	0.22 ± 0.54	0.0 (0.0 - 0.0)	1.31	NS
CBPI								
Women	30	1.69 ± 0.20	1.7 (1.6 - 1.8)	30	1.61 ± 0.28	1.6 (1.4 - 1.8)	0.93	NS
Men	11	1.68 ± 0.18	1.7 (1.5 - 1.8)	68	1.63 ± 0.28	1.6 (1.4 - 1.8)	0.94	NS
Total population	41	1.70 ± 0.19	1.7 (1.5 - 1.8)	98	1.62 ± 0.28	1.6 (1.4 - 1.8)	0.98	NS

Bold for statistically significant effect in relation to unexposed controls.
[a] Significant effect in relation to men with the same exposure status; N.S: Not statistically significant

revealed a significant increase in MNBN frequency in individuals with residential proximity to the open pit coal mine compared to residents of the control area without proximity to coal mining facilities. The Relative frequency (FR) for increased MNBN frequency in exposed individuals was 1.50 representing a higher risk in relation to individuals without residential proximity to the mining corridor. Median MNMONO frequencies were also significantly higher in exposed individuals than in controls. The FR for increased MNMONO frequency in exposed individuals was 3.48. None of the other CBMN-cyt assay parameters related to asymmetrical chromosome rearrangement (NPB) and gene amplification (NBUD) were significantly influenced by residential proximity to open pit mines in general population. Even when median CBPI values in exposed individuals (1.62 ± 0.28) were lower than in unexposed participants (1.70 ± 0.19) the estimates were not statistically significant.

Considering gender and age as the major contributors to differences in CBMN-cyt assay parameters, we also assessed the influence of both confounding factors and exposure status as predictors of these parameters frequencies. MNBN frequencies were not significantly influenced by gender in exposed residents and unexposed controls populations, suggesting a similar response to exposure among women and men. Only MNMONO frequencies seemed to be influenced by gender, thus exposed women showed higher frequencies of MNMONO compared to men (p-value <0.05). In exposed individuals MNBN frequency was significantly influenced by age (Figure 2B); MNBN frequency increased in a frequency ratio of 0.14 for every year. Considering that particularly in this exposed population, exposure time was estimated by the age of each participant, we can also conclude that for every year of exposure, MNBN frequency could increase in a frequency ratio of 0.14 (p-value < 0.001). Despite the fact that MNMONO frequency may indicate a cumulative effect over a long period, our results were not significantly influenced by age/time of exposure, however seems to be inversely associated with individual age in exposed population (Figure 2D). Neither gender nor age has a statistically significant influence on other parameters as NPB, NBUDS and CBPI in exposed and unexposed populations.

Analysis of CBMN-cyt assay parameters discriminated by sampling areas (communities and "resguardos") are shown in Figure 3. Analysis of average MNBN frequencies between sampling areas showed statistically significant differences between Chancletas, Media Luna and Cerro de Hatonuevo (Figure 3A). The highest frequency was obtained for Chancletas, an Afro-Colombian community, followed by the indigenous "resguardos" of Provincial, San Francisco, the "ranchería" Media Luna and the resguardo of El Cerro de Hatonuevo. Interestingly, baseline frequency for MNBN obtained in reference area showed slightly higher MNBN frequencies than El Cerro de Hatonuevo. Internal control values showed that transportation conditions were optimal and did not adversely influence any of the results obtained. Provincial and San Francisco showed very similar MNBN frequencies that may be attributed to their close spatial location and similar exposure conditions. Frequencies of other parameters as MNMONO, NPB, NBUDS and CBPI did not differ significantly between sampling areas.

To elucidate the mechanism of micronucleus formation, an anti-kinetochore antibody (CREST staining) was used to distinguish CREST+ MN from those CREST-. A statistically significant increase in CREST+ MN (45.27%) was detected in exposed individuals (66.18%) compared to unexposed population (20.91%), suggesting a potential human hazard associated with the exposure to aneuploidy-inducing substances generated during

(A)

R$_S$= 0.0414
P= 0.7946

(B)

R$_S$= 0.3301
P= 0.0009

(C)

R$_S$= 0.3301
P= 0.1638

(D)

R$_S$= - 0.0686
P= 0.66
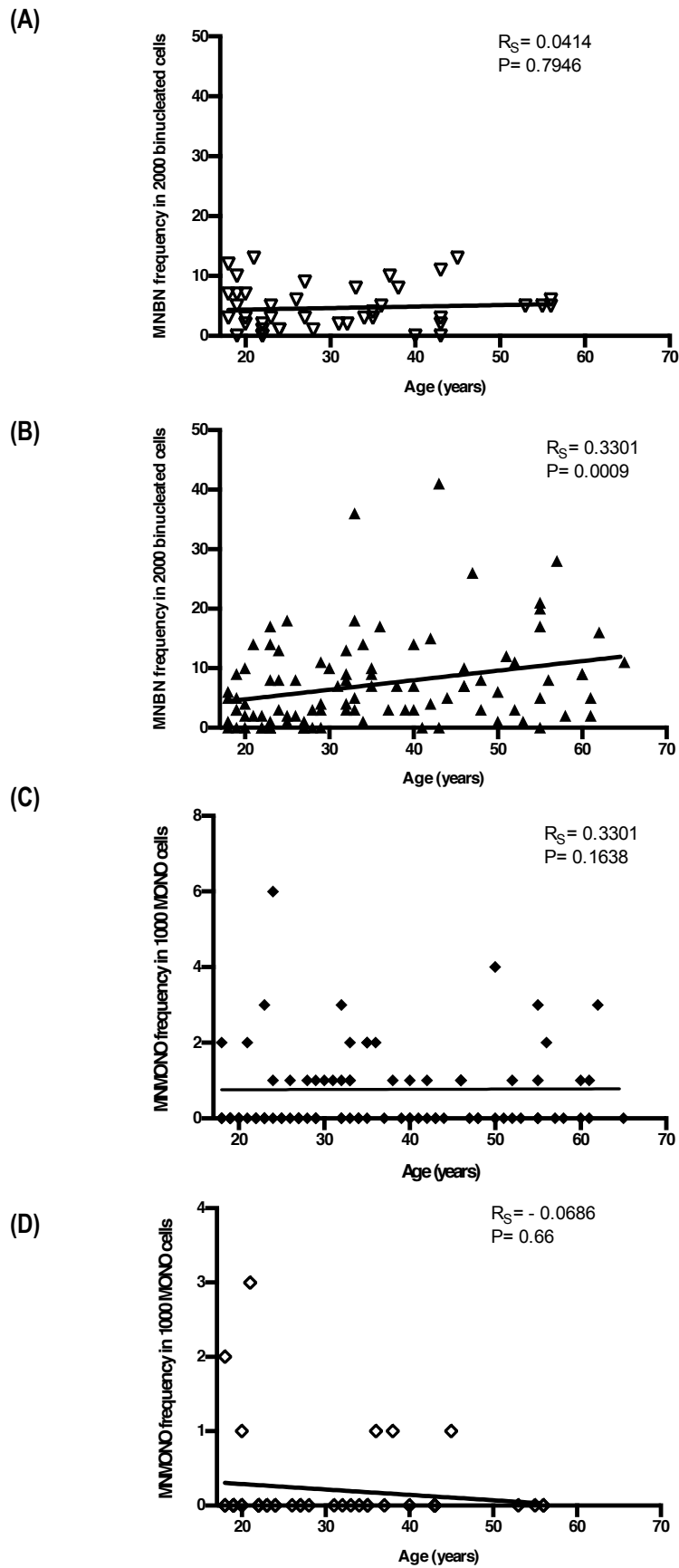
Figure 2. Nonparametric Spearman correlation analysis between MN frequency in binucleated and MONO lymphocytes and age in A-C) control and B-D) exposed individuals
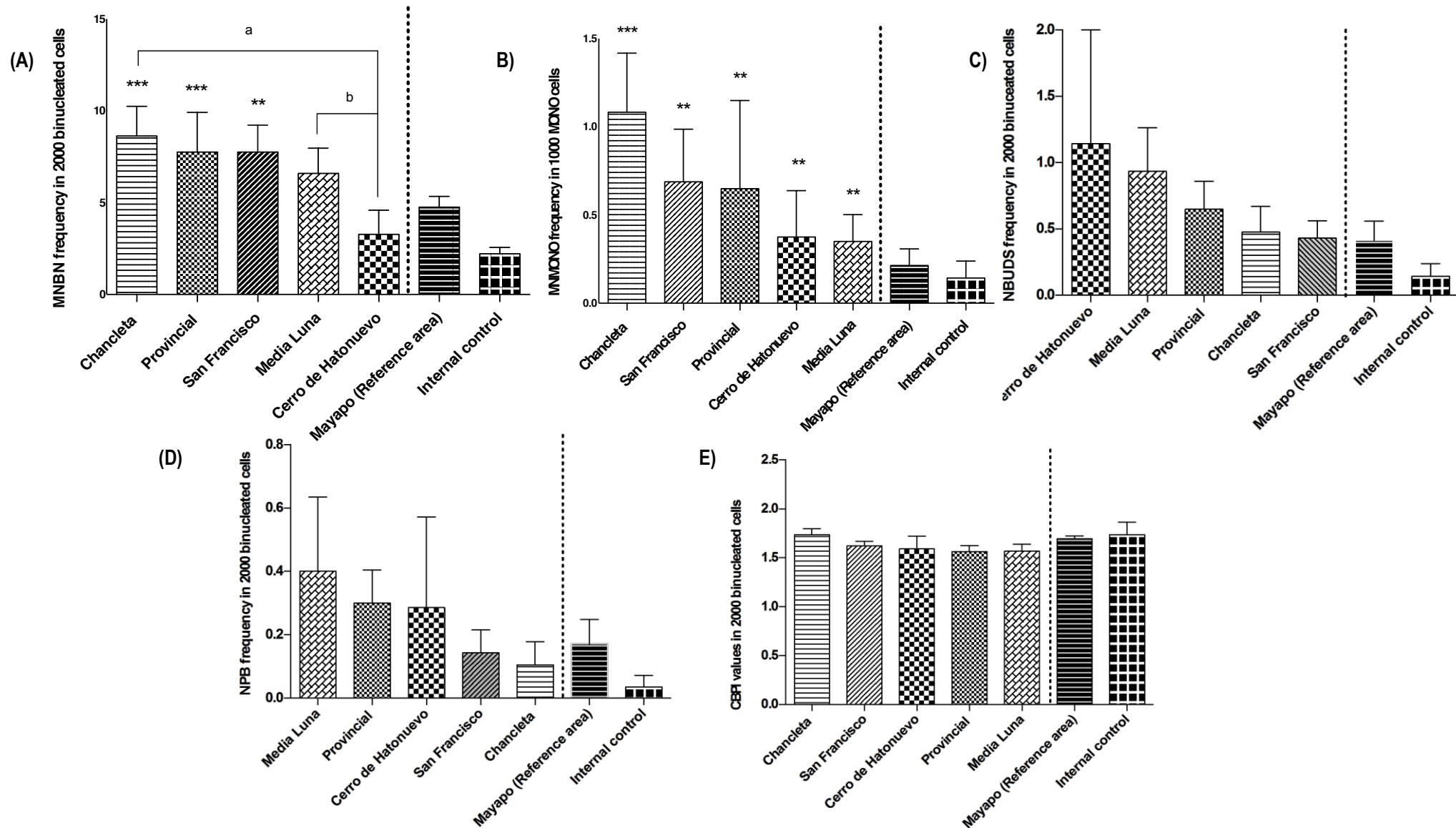
Figure 3. CBMN-cyt assay parameters frequency by sampling areas, exposed residents, unexposed and internal controls. A) MNBN frequency; B) MNMONO frequency; C) NBUDS frequency; D) NPB frequency; E) CBPI frequency. ** Significance difference in relation to Mayapo (Reference area); $P \leq 0.01$; *** Significance difference in relation to Mayapo (Reference area); $P \leq 0.001$; [a] Significance difference in relation to Cerro de Hatonuevo; $P \leq 0.001$; [b] Significance difference in relation to Cerro de Hatonuevo; $P \leq 0.05$.

45

open-pit coal mining operations (Table 4). The difference between the proportions of CREST+ and CREST- MN in unexposed and exposed residents was statistically significant (p-value< 0.001).

Even when the aneugenic effect was not related to either age/time of exposure or sex, a higher percentage (67.14 – 69.81%) was found in older individuals. Analysis of percentages of CREST+/CREST- MN discriminated by sampling areas showed statistically significant differences that could be related to geographical localization of the sampling areas and/or differentiated nature, characteristics and concentrations of the coal mining residues generated around each area (Figure 4A). Once more the highest frequency of CREST+ MN was obtained for Chancletas followed by San Francisco, Provincial, Media Luna and El Cerro de Hatonuevo. Baseline frequency for CREST- MN obtained in reference area (79.1%) was higher than that for any other region assessed.

(C).
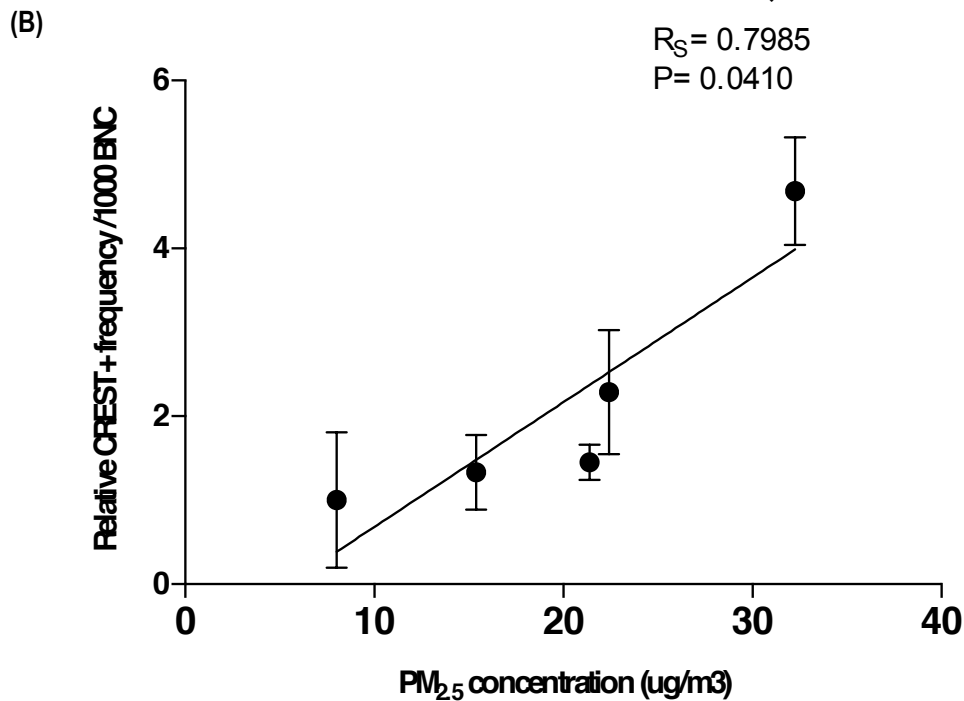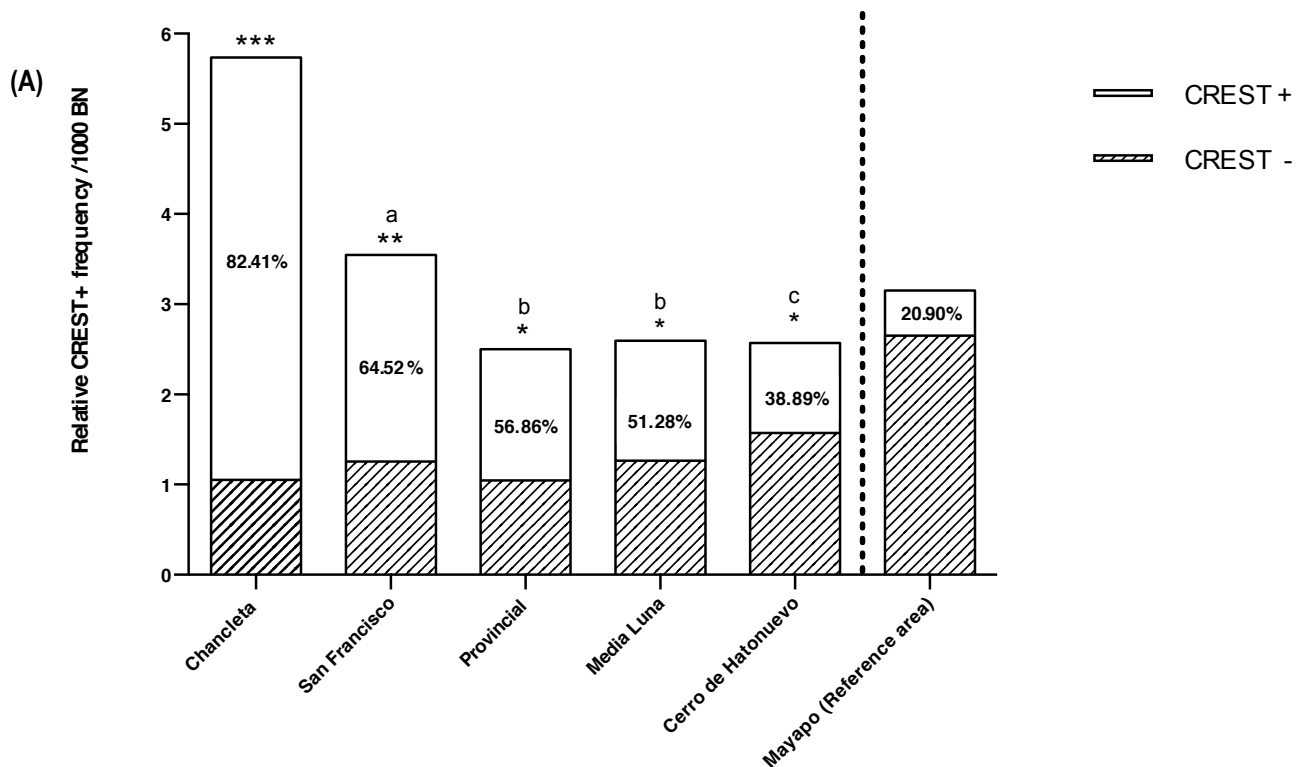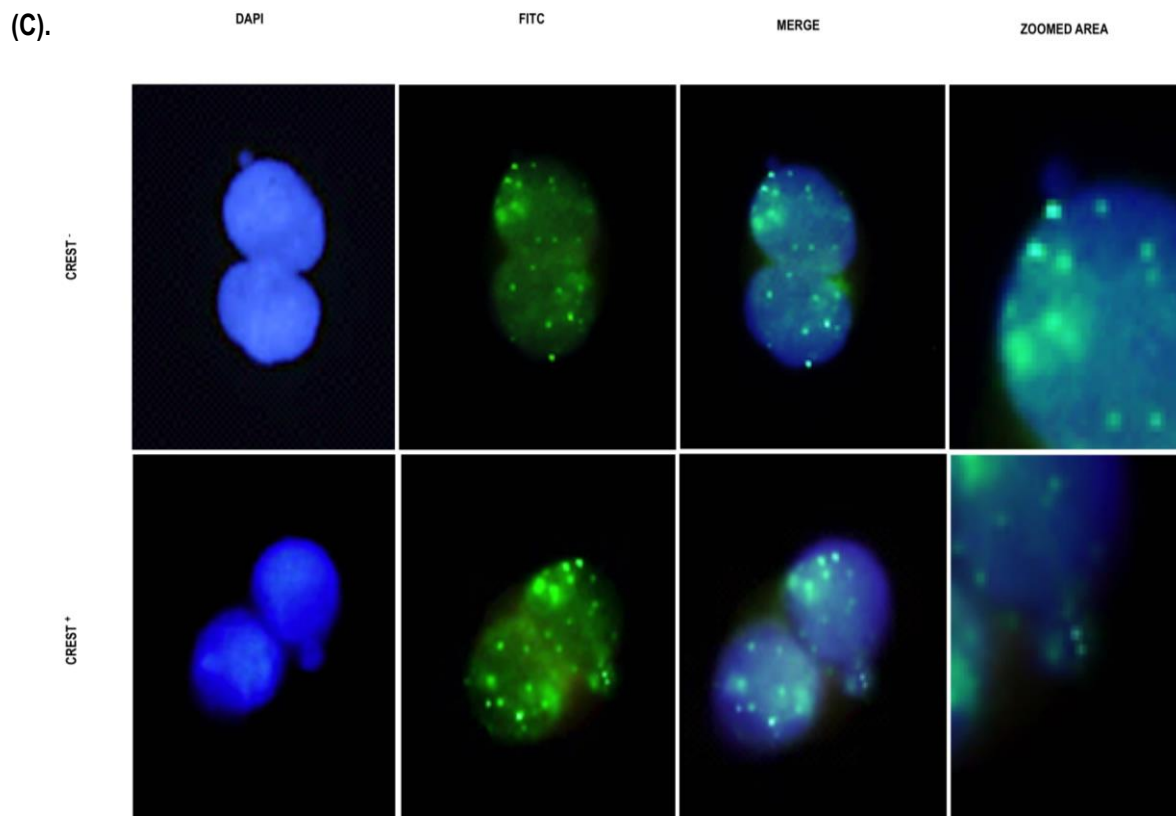
Figure 4. (A) Percentages of CREST+/CREST- micronuclei discriminated by sampling areas. * Significance difference in relation to Mayapo (Reference area) CREST+ frequency; P ≤ 0.05; ** Significance difference in relation to Mayapo (Reference area) CREST+ frequency P ≤ 0.01; * Significance difference in relation to Mayapo (Reference area) CREST+ frequency; P ≤ 0.001; [a] Significance difference in relation to Chancleta CREST+ frequency; P ≤ 0.05; [b] Significance difference in relation to Chancleta CREST+ frequency; P ≤ 0.01; [c] Significance difference in relation to Chancleta CREST+ frequency; P ≤0.001; B) Pearson correlation analysis between $PM_{2.5}$ and CREST+ micronuclei. C). Chromosomal loss evidenced by centromere positive (CREST+) micronuclei (several signals).**

Our correlation analysis showed an important and significant correlation between $PM_{2.5}$ levels and an increased induction of CREST+MN (Figure 4B). The opposite effect was observed for CREST- frequencies, while PM_{10} levels were not related to CREST+ MN induction. These results suggest that only the $PM_{2.5}$ fraction generated in the mining corridor possesses the ability to induce preferentially whole chromosome loss (aneuploidy) (Figure 4C), although there are also chromosome breaks.

Table 4. Immunofluorescent detection of kinetochores (DAPI and CREST staining): effect of gender and age in individuals with residential proximity to the open pit coal mine and unexposed controls in the aneugenic effect.

CREST (RF/1000 BN)	n	Total No. MN/1000	Mean ± S.D	CREST+/1000 BN				n	Total No. MN/1000	Mean ± S.D	CREST+/1000 BN			
				No	Mean ± S.D	Median (25th - 75th)	%				No	Mean ± S.D	Median (25th - 75th)	%
		Unexposed controls							Exposed residents					
Total population	41	107	2.61 ± 3.69	23	**0.53 ± 0.89**	**0.0 (0.0 - 1.0)**	**20.91**	98	340	3.57 ± 4.67	225	**2.34 ± 3.61**	**1.0 (0.0 - 2.0)**	**66.18***
Gender														
Women	30	96	3.20 ± 4.04	21	0.70 ± 0.98	0.0 (0.0 - 1.2)	21.87	68	238	3.50 ± 5.03	158	2.32 ± 4.02	1.0 (0.0 - 4.0)	66.39
Men	11	11	1.16 ± 1.80	2	0.16 ± 0.38	0.0 (0.0 - 0.0)	18.18	30	102	3.64 ± 3.91	67	2.39 ± 2.43	2.0 (0.0 - 4.0)	65.59
Age														
18-25	19	49	2.72 ± 3.73	10	0.52 ± 0.90	0.0 (0.0 - 1.0)	20.41	32	113	3.46 ± 4.02	61	2.03 ± 2.39	1.0 (0.0 - 4.2)	61.94
26-33	6	12	2.14 ± 3.33	3	0.57 ± 0.78	0.0 (0.0 - 1.2)	25.00	23	101	4.39 ± 7.63	69	3.00 ± 6.12	1.0 (0.0 - 4.0)	68.32
34-41	7	20	2.85 ± 4.48	3	0.42 ± 0.78	0.0 (0.0 - 0.2)	15.00	13	43	4.07 ± 2.49	37	2.84 ± 2.11	4.0 (0.5 - 4.5)	69.05
≥42	9	26	2.88 ± 3.72	7	0.62 ± 1.18	0.0 (0.0 - 1.5)	23.08	30	83	2.50 ± 2.83	47	1.67 ± 2.31	0.0 (0.0 - 3.0)	69.88

Bold* for statistically significant effect in relation to unexposed controls $P \leq 0.001$
RF – Relative frequency

Mean atmospheric $PM_{2.5}$ levels (Figure 5A) was significantly different between sampling areas (p-value 0.043); during sampling period, $PM_{2.5}$ around the coal mining corridor showed a mean value of 22.80 ± 10.74 $\mu g/m^3$ with concentrations ranged from 7.02 to 39.39 $\mu g/m^3$. Highest values of $PM_{2.5}$ were also obtained in Chancletas, followed by Provincial Media Luna and El Cerro de Hatonuevo. $PM_{2.5}$ levels in San Francisco could not be determined due to incomplete sampling procedures. Considering previous results and close spatial location we used data obtained for Provincial as informative values.

Atmospheric PM_{10} levels did not differ significantly between sampling areas. PM_{10} particles ranged from 16.70 to 77.60 $\mu g/m^3$ and showed a mean value of $43.09 \pm 11.01 \mu g/m^3$, well bellow national air quality standards (70 $\mu g/m^3$). PM_{10} values showed a different distribution between sampling areas (Figure 5B); highest values of PM_{10} were observed in El Cerro de Hatonuevo, San Francisco and Provincial, Chancletas and Media Luna (36.85 ± 11.75).

As shown in Figure 5C, the number concentration of $PM_{2.5}$ accounts for more than 50% of the observational range of the PM_{10} around the mining area. Therefore, $PM_{2.5}$ contributes an important proportion of the aerosol number concentration, especially around areas with the highest levels of DNA damage. For Chancletas and Provincial, the obtained $PM_{2.5}/PM_{10}$ ratio was 0.8 and 0.5, respectively, thus, indicating that mostly all inhalable particles are in the fine fraction (<2.5 μm), mainly for these sites. Fine particles may be originated from anthropogenic sources in the study area, such as the carbon extraction processes and transport carried out near the sampling sites. For Cerro de Hatonuevo site, $PM_{2.5}/PM_{10}$ obtained ratio were 0.2, indicating more natural sources or mechanical processes occurred that increased the PM_{10} concentration in the coarse fraction.

Next, we performed a Pearson correlation analysis to explore a possible association between $PM_{2.5}$ and PM_{10} levels in each sampling area and CBMN-cyt parameters (Table 5). In exposed individuals, MNMONO and MNBN frequencies were positively correlated. MNBN frequencies were highly positively correlated with $PM_{2.5}$ concentrations, while PM_{10} induced a slight effect although not significant in MNBN frequencies, which may be due to the presence of $PM_{2.5}$ in this fraction. None of the others CBMN-cyt parameters was correlated to $PM_{2.5}$ or PM_{10} values.

Considering these results, we proceed to evaluate chemical elemental composition of aerosol samples of $PM_{2.5}$ by PIXE. In this fraction, 13 elements were routinely found above the limit of detection: Na, Mg, Al, Si, P, S, Cl, K, Ti, Cr, Mn, Cu, Zn. Therefore, these species contribute the most to the $PM_{2.5}$ mass fractions (Table 6). Interestingly, except for S, Cr and Zn, element concentrations from reference area were found higher than those registered around the coal mining corridor. Only Si, S, K and Cr concentrations varied significantly between coal mining and control area.
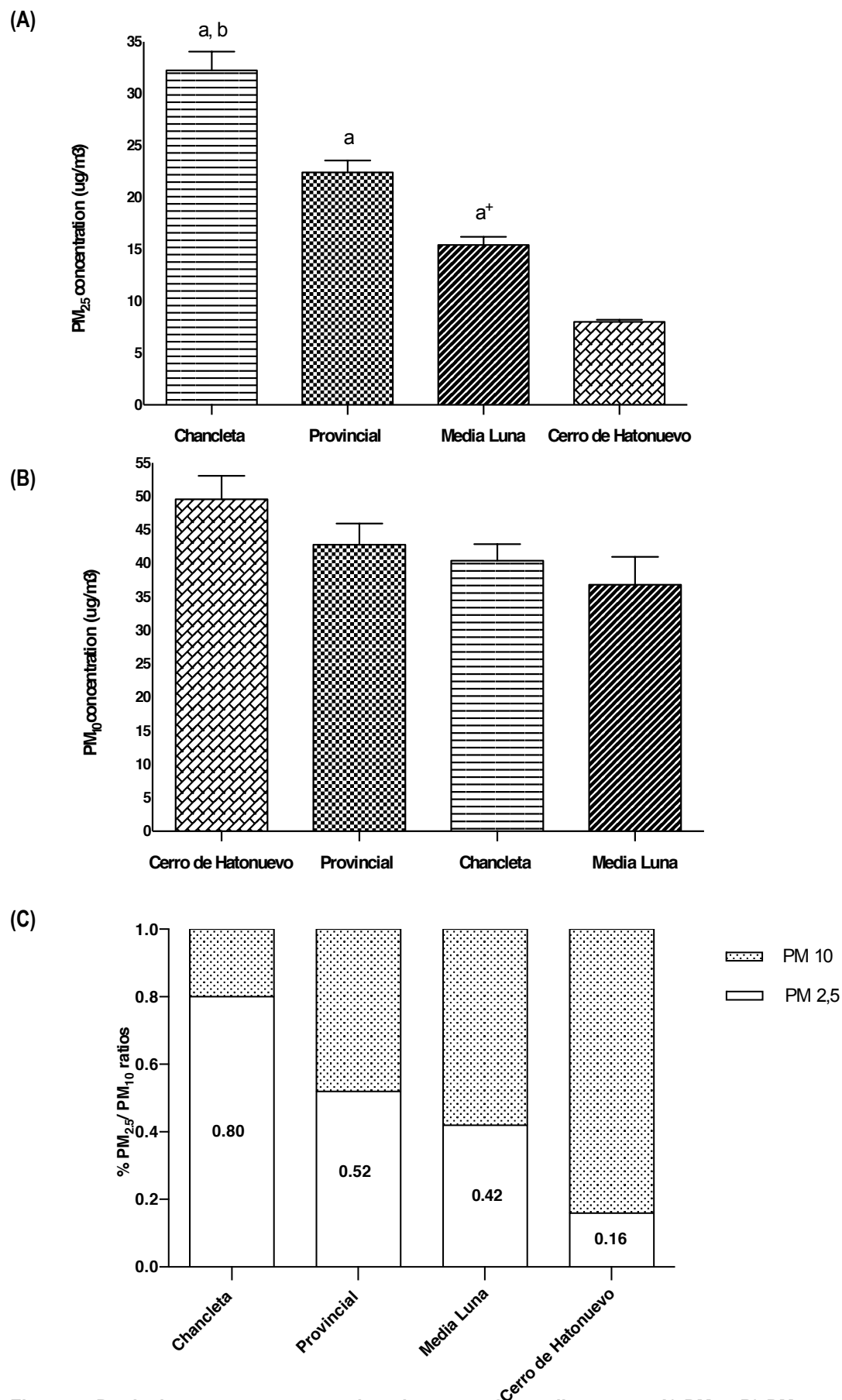
Figure 5. Particulate matter concentrations in exposed sampling areas: A) PM$_{2.5}$, B) PM$_{10}$ and C) PM$_{10}$/PM$_{2.5}$ ratio. [a] Significance difference in relation to Cerro de Hatonuevo; P $\leq$ 0.001; [a*] Significance difference in relation to Cerro de Hatonuevo; P $\leq$ 0.05; [b] Significance difference in relation to Media Luna; P $\leq$ 0.001.

Table 5. Results of the multivariate Pearson regression analysis for exposed populations and $PM_{2.5}$ and PM_{10} levels.

	Variable	$PM_{2.5}$*	PM_{10}**	MNBN	MNMONO	NPB	NBUDS	CBPI
$PM_{2.5}$*	Pearson correlation	1	- 0.52	**0.94**	0.78	- 0.70	- 0.99	0.71
	Significance		0.23	**0.03**	0.08	0.14	0.07	0.14
PM_{10}**	Pearson correlation		1	0.72	- 0.52	- 0.12	0.48	- 0.14
	Significance			0.13	0.23	0.43	0.25	0.42
MNBN	Pearson correlation			1	**0.12**	- 0.42	- 0.94	0.46
	Significance				**0.04**	0.29	0.20	0.26
MNMONO	Pearson correlation				1	0.84	0.17	- 0.76
	Significance					0.07	0.41	0.12
NPB	Pearson correlation					1	0.67	- 0.91
	Significance						0.16	0.41
NBUDS	Pearson correlation						1	- 0.63
	Significance							0.18
CBPI	Pearson correlation							1
	Significance							

Bold for statistically significant effect
$PM_{2.5}$*: $PM_{2.5}$ concentration
PM_{10} **: PM_{10} concentration

Sulfur was the most common element in the $PM_{2.5}$ fraction from the coal mining area. S concentration once more showed to be higher in Chancleta, followed by Provincial, Cerro de Hatonuevo and Media Luna. Even when Si and K values were significantly different between mining and control area, analysis of EF values showed that both concentrations in $PM_{2.5}$ filters would have mainly a crustal origin (1<EF<10) due to re–suspension of soil dust.

On the other hand, S and Cr concentrations were found enriched in $PM_{2.5}$ samples from coal mining but also in control areas, suggesting a origin from anthropogenic sources in both regions; however EF for S concentrations in coal mining (258.09 p.p.m) and control areas (90.13 p.p.m) were significantly different. Thus, S was highly enriched in coal mining area and moderately in control region. EF value for Cr also showed a 2.52 fold increase around the coal mining area (78.54 p.p.m) compared to control region (31.11 p.p.m).

Other enriched elements in coal mining and control areas included Cu and Cl. While EF value for Cu showed a 1.60 fold increase in coal mining area, Cl values in both areas were highly comparable with a slight increase in reference area (Figure 6A). EF analysis also showed enrichment of Zn only in the coal mining area suggesting an origin from anthropogenic sources. EF values for Cl, Cr, Cu and Zn did not differ significantly between sampling areas (Figure 6B). Only EF value for S was found significantly different between Chancleta (382.07 p.p.m), Provincial (349.96 p.p.m), Cerro de Hatonuevo (22.30 p.p.m) and Media Luna (35.07 p.p.m).

The Pearson correlation for inorganic elements levels in aerosol samples of $PM_{2.5}$ around coal mining areas showed an important and significant correlation between Al, Si, Ti, Fe, Mg, Na suggesting a common emission source. Sulfur showed to be positively correlated to Na (0.738) and K (0.631) and negatively to Cl (- 0.615). Other enrichment elements as Cr, Cu and Zn did not show significant association with any other element (Table 7).

51

Table 6. Chemical elements concentrations of PM$_{2.5}$ fraction in sampling areas determined by PIXE (p.p.m)

Elements	Mayapo (Reference area)	Coal mining area				
		General	Discriminated by sampling areas			
			Provincial	Cerro de Hatonuevo	Chancleta	Media Luna
Na	628.6 ± 242.80	359.48 ± 241.40	478.80 ± 147.50	500.90 ± 206.70	427.73 ±122.9	401.30 ± 78.41
Mg	305.7 ± 188.70	150.90 ± 140.20	166.00 ± 78.02	245.20 ± 231.53	159.40 ± 91.23	138.70 ± 22.39
Al	1757.33 ±1334.56	746.14 ± 752.67	651.40 ± 351.40	1197.28 ±1256.90	1111.10 ± 399.20	80.06 ± 32.87
Si	**5952.50 ± 3959.99**	**2292.70 ± 1985.40**	2117.09 ± 1215.20	3442.25 ± 3231.67	3222 ± 997.90	429.62 ± 139.40
P	411.5 ± 259.50	292.7 ± 187.80	410.48 ± 109.50	212.3 ± 129.90	466.34 ± 189.70	214.90 ± 131.45
S	**4673 ± 646.52**	**7391 ± 462.45***	10022.05 ± 2027.07	**6939.25 ± 1536.09** [b,c]	10942.09 ± 5003.00	**1064.57 ± 197.3** [a,c]
Cl	1087.8 ± 668.30	245.50 ± 396.60	84.41 ± 26.72	423.3 ± 98.99	139.05 ± 21.63	720.09 ± 592.54
K	**825.43 ± 357.10**	**417.09 ± 183.54***	466.70 ± 74.28	492.23 ± 240.30	494.43 ± 106.89	257.89 ± 121.30
Ti	113.40 ± 79.05	51.48 ± 36.69	45.84 ± 29.16	72.77 ±60.92	64.39±20.21	24.39±8.11
Cr	**58.83 ± 14.01**	**82.61 ± 19.57***	77.84 ± 16.56	82.56 ±19.21	72.31±14.51	98.92 ± 23.71
Mn	33.86 ± 33.88	18.76 ± 15.67	20.17 ± 9.20	25.58 ± 10.67	32.81 ± 0.39	20.25 ± 0.44
Cu	70.34 ± 13.20	67.07 ± 17.24	65.35 ± 3.52	69.62 ± 22.69	75.34 ± 23.23	58.45 ± 17.61
Zn	26.11 ± 4.34	28.90 ± 11.85	35.82 ± 10.36	21.69 ±12.27	38.92 ± 2.48	21.33 ± 11.15

Bold for statistically significant effect
* Significance difference in relation to Reference area $P \leq 0.05$
** Significance difference in relation to Reference area $P \leq 0.01$
[a] Significance difference in relation to Provincial; $P \leq 0.001$
[b] Significance difference in relation to Provincial; $P \leq 0.01$
[c] Significance difference in relation to Chancleta; $P \leq 0.001$

Table 7. Pearson correlation coefficient matrix for chemical elements concentrations in aerosol samples of PM$_{2.5}$ around coal mining areas

	Na	Mg	Al	Si	P	S	Cl	K	Ca	Ti	Cr	Mn	Fe	Cu	Zn
Na	1														
Mg	**0.752**	1													
Al	**0.773**	**0.909**	1												
Si	**0.750**	**0.896**	**0.989**	1											
P	0.134	-0.177	-0.069	-0.044	1										
S	**0.738**	0.369	0.444	0.396	0.337	1									
Cl	**0.518**	-0.134	-0.260	-0.244	-0.412	**-0.615**	1								
K	**0.820**	**0.815**	**0.821**	**0.820**	0.152	**0.631**	-0.238	1							
Ca	0.387	0.417	0.382	0.425	0.021	0.190	-0.095	0.417	1						
Ti	**0.675**	0.847	**0.956**	**0.955**	-0.132	0.360	-0.122	**0.718**	0.378	1					
Cr	-0.380	-0.221	-0.183	-0.235	-0.265	-0.175	0.172	-0.378	0.059	-0.105	1				
Mn	**0.702**	**0.802**	**0.811**	**0.796**	-0.129	0.335	-0.393	**0.598**	0.438	**0.780**	-0.143	1			
Fe	**0.626**	**0.889**	**0.958**	**0.974**	-0.066	0.266	-0.079	**0.782**	0.436	**0.949**	-0.197	**0.761**	1		
Cu	0.107	0.210	0.199	0.244	-0.047	0.192	-0.155	0.244	0.285	0.209	-0.243	0.384	0.262	1	
Zn	0.375	0.423	0.283	0.285	0.357	0.274	-0.412	0.341	0.366	0.235	-0.053	0.354	0.273	0.130	1

Bold for statistically significant correlation; $P \leq 0.05$
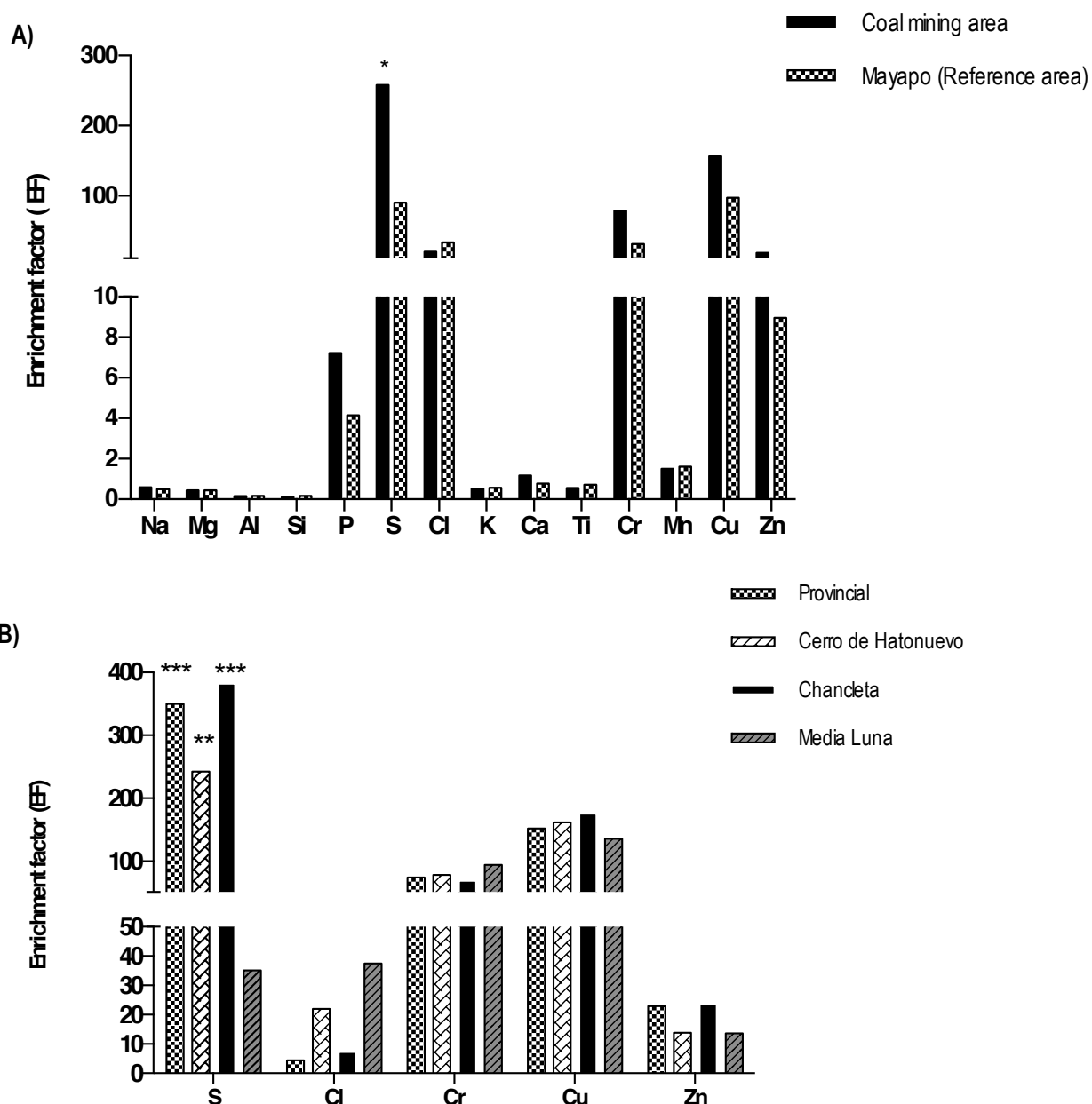
Figure 6. Enrichment factors of elements in PM$_{2.5}$ in A) coal mining and control areas and B) discriminated by coal mining areas.* Significance difference in relation to Mayapo (Reference area), P ≤ 0.05; ** Significance difference in relation to Media Luna, P ≤ 0.05; * Significance difference in relation to Media Luna, P ≤ 0.01.**

Finally, considering the documented main role of organic matter content in some of the biological PM effects, concentration of extractable organic matter (EOM) in the aerosol samples of PM$_{2.5}$ around coal mining areas was determined. Results showed that coal mining areas had higher EOM content related to substances with nonpolar characteristics extracted with CH and also the largest quantity of polar substances extracted with ACE compared to reference area (Figure 7). Concentrations of substances with moderately polar characteristics extracted with DCM were very similar between areas.
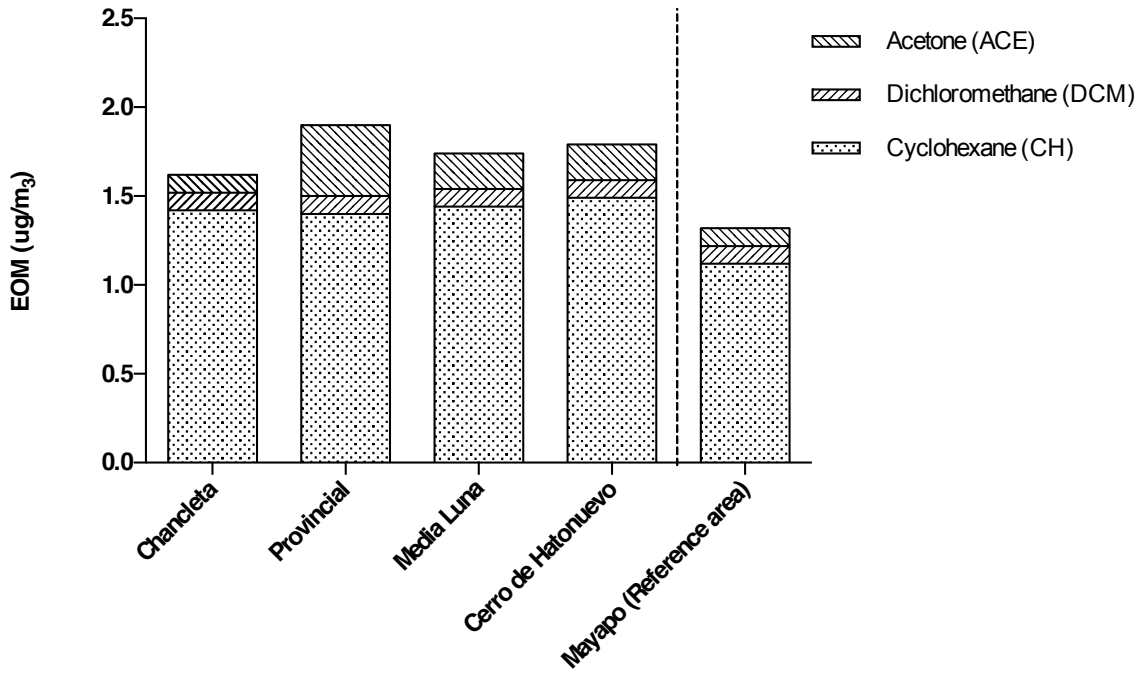
Figure 7. Concentration of extractable organic matter (EOM) in PM$_{2.5}$ from exposed sampling areas.

4. DISCUSSION

The International Agency for Research on Cancer (IARC) has classified exposure to PM in air pollution as a human carcinogen [35]. Epidemiological studies indicate that living in proximity to coal mines is correlated with higher rates of cancer [36] and that exposure to complex mixtures of chemical substances contained in coal dust particles released as fugitive PM, could be associated to this phenomenon [37]. However, most coal mining residues like coal dust remains classified as non-carcinogen for human by the International Agency for Research on Cancer [38]. The understanding of the mechanisms by which PM generated in coal mining areas exerts its various adverse effects is still incomplete and detailed *in vitro* and *in vivo* studies are highly needed.

The present study investigated the correlation between $PM_{2.5}$ and PM_{10} levels and chromosomal damage and genetic instability (CBMN-cyt) in lymphocytes of individuals with residential proximity to the coal mining corridor of Northern Colombia. To further extend the study, an anti-centromere protein antibody (CREST staining) was used to distinguish CREST+ MN from those CREST-.

Unique to the current report is the evaluation of MNMONO frequencies and the aneugenic or clastogenic effects of environmental exposure to coal mining residues in relation to PM_{10}, $PM_{2.5}$ levels, inorganic elements and EOM content, whereas a weakness of the study was the limited data about chemical characterization of organic content of the PM.

Analysis of CBMN-cyt assay parameters in total population revealed a significant increase in MNBN frequency in individuals with residential proximity to the open pit coal mine compared to residents of the control area without proximity to coal mining facilities. The Relative frequency (FR) for MNBN frequency in exposed individuals was 1.50, representing a higher risk in relation to individuals without residential proximity to the mining corridor. Data was consistent with our previous reports for occupationally exposed workers in the same mining region [19]. Similar results were obtained by Rohr et al. [39], also in occupational exposed workers from open coal mine compared to control, and have also consistently been described in amphibians [40] and rodents [41]

Since MNBN frequency in lymphocytes is considered as a predictive biomarker of cancer risk [42], our current report provides important evidence suggesting that populations living in proximity to open-pit coal mining facilities also show higher frequencies of genetic and chromosomal damage that could be associated to initial steps in cancer development.

This study demonstrated a MNBN frequency increase in exposed residents associated with age in a ratio of 0.14 for every year. The age effect was not found within the unexposed group in MNBN baseline frequencies, thereby demonstrating that time of exposure to mining residues while living in proximity to open-pit mines, is the main cause of genomic instability found in exposed residents.

A meta-analysis study coordinated by the HUMN Project confirmed the statistical significance and consistency of sex and age as modulators of MNBN frequencies [43]. Although age is the most well-established factor impacting on MNBN frequencies, a gender difference in the background incidence of MNBN is also well documented, with the frequency being consistently higher in females [44]; in our report we did not detect a significant effect of gender in MNBN values. Neither sex nor age has a statistically significant influence on others CBMN-cyt assay parameters as NPB, NBUDS and CBPI values in exposed and unexposed populations.

The original methodology of the CBMN test focused exclusively on binucleated cells, therefore MNMONO frequencies have been rarely taken into account in biomonitoring studies [45]. As far as we know, we are the first to screen for MNMONO induction by residential exposure to coal mining residues.

In our study, median MNMONO frequencies were also significantly higher in exposed individuals than in controls. Even when MNMONO frequencies were lower than MNBN frequencies in exposed populations, the FR for increased MNMONO frequency in exposed individuals was 3.48, a higher coefficient when compared to FR for MNBN. This result could be indicative of a significantly higher level of *in vivo* genetic damage induction in exposed residents compared to *in vitro* levels, which may also reflect an increase in the number of damaged cells that failed to divide. Previously reported data on MNMONO values mainly from cord blood lymphocytes are consistent with our findings [62].

Interestingly, in our results only MNMONO frequencies seemed to be influenced by sex, thus exposed women showed higher frequencies of MNMONO compared to men (p-value <0.05). The same result was obtained for general population (data not shown). Contrarily to MNBN frequencies, where higher frequencies in females are an unequivocally documented fact, there is a relative dearth in the literature exploring this phenomenon on MNMONO, especially in populations exposed to environmental genotoxins. In one of the few reports on MNMONO frequencies, El-Zein et al. [46] also reported that women had slightly higher levels of MNMONO than men in both cases and controls. Whether MNMONO frequencies could also be influenced by gender as MNBN is not known at this time, however it is possible that increase in MNMONO frequency in females can also be accounted for by the greater tendency of the X chromosome to be lost as an MN relative to other chromosomes, and to the fact that females have two copies of the chromosome compared to only one in males [47].

In exposed individuals, MNBN and MNMONO frequencies were positively correlated (Table 5). Results from studies in children exposed to radioactive isotopes from the Chernobyl disaster showed a good correlation between MNBN and MNMONO in the CBMN-cyt assay [48]. Similarly, the same concordance was described for individuals exposed to asbestos [49]. These results adds support to the evidence that MN scored in lymphocytes in the *ex vivo* CBMN assay do in fact represent DNA damage induced in vivo.

Although MNMONO frequency may indicate a cumulative effect over a long period, in our results it was not significantly influenced by age/time of exposure. However, similarly to other results [50] seems to be inversely associated with individual age.

Analysis of CREST+ MN in lymphocytes from exposed residents allowed us to identify aneuploidy-inducing activities in specific areas around the mining corridor. The highest CREST+ MN induction was found in areas near open pits and dump sites suggesting that substances or complex mixtures producing loss of chromosomes at mitosis may be characteristic of such anthropogenic environments. It is now well established that CREST+ MN originate from whole chromosomes that fail to be included in the daughter nuclei at the completion of telophase during mitosis because they did not attach properly to the spindle during the segregation process in anaphase [22]. Malsegregation of whole chromosomes may be induced if the chemical either causes centromere or kinetochore malfunction or disrupts the mitotic spindle, microtubule assembly or centrosome [51].

The proportion of centromere-positive MN in human lymphocytes increases with age, which primarily

reflects an age-dependent micronucleation of the X and Y-chromosomes. Some authors estimated that 80% of the effect of age on MN frequencies is due to CREST+ MN; women also had higher frequencies of CREST+ MN than men [52, 53]. In our results no significant effect of age or sex was observed on CREST+MN frequency in exposed and unexposed populations, but a higher percentage was found in older individuals.

The aneugenic effect observed in the exposed population would also be supported by other results reported here. Considering that several studies have indicated that aneugens, but not clastogens, also induce MNMONO [54, 55], median MNMONO frequencies also significantly higher in exposed individuals than in controls seems to confirm these observations. This data would also add to the increasing evidence that MNMONO may be useful to distinguish clastogens from aneugens and increase the sensitivity of the test.

Our correlation analysis demonstrated a highly significant association between variations of $PM_{2.5}$ levels, increased MNBN frequencies and CREST+ MN induction in exposed residents. To further study which $PM_{2.5}$ components could be responsible for the observed effects, chemical composition of aerosol samples was determined by PIXE analysis and assessment of EOM concentrations. PIXE analysis showed that most chemical elements concentrations from reference area were higher than those registered around the coal mining corridor except by anthropogenic elements such as S, Cr and Zn. Besides the influence of anthropogenic activities, geographical location of reference area, high ocean contribution, coastal erosion and highly enriched sources of inorganic elements like beach sand, could be considered as major causes of these phenomena [56].

Direct comparison of inorganic elements concentration in environmental sources is an inappropriate way to assess the level of chemical pollution because inorganic elements originate from both crustal and anthropogenic sources and accumulate in the same manner [57]. A better geochemical approaches such as EF calculation have been widely used to evaluate the impact of chemical elements in PM of polluted areas [58].

Analysis of EF for $PM_{2.5}$ samples from coal mining and control zones showed that S, Cr, Cu and Cl were enriched in both areas; however, only S concentrations inside the coal mining area were found highly enriched.

As previously evidenced, S is usually produced by the combustion of sulfur-containing fossil fuels [59] like the produced during coal mining through active mine fires [60]. Considering the absence of information on chemical speciation of inorganic elements in PIXE, it is generally assumed that many of the elements of anthropogenic origin (especially from combustion sources) are present in the atmosphere as oxides. Thus, main sulfur oxides probably present in the coal mining ambient would include SO_2 and SO_4^{2-} from coal fires [61].

Biomass burning may be also a confounding factor that would influence concentration of some combustion related elements like S in the exposed areas, especially considering that domestic use of wood fuel is a common practice between Wayúu and Afro-Colombians communities. However, other elements considered as main constituents of biomass like K and Na were not enriched in exposed areas [62]. This observation would indicate that S might originate exclusively from coal and coal combustion sources. Cr, Cu and Zn are typically found in coal mining processes [60] particularly associated with the fine fraction of particulate matter [63].

This exposure to chemical elements is of great environmental concern due to its potential long-term effects on human health [64], especially when some of the enriched elements like S [65], Cr and Cu [66] can cause DNA damage, genomic instability and have been linked to carcinogenic processes.

Main mechanism proposed for these effects includes the generation of oxidative damage through

generation of reactive oxygen species (ROS) [67].

It is worth noting that higher EF and S concentrations were found around the coal mining areas with highest levels of DNA damage and CREST+ MN. Free radicals from SO_2 metabolism, such as SO_3^{--}, SO_4^{--}, SO_5^{--} may also induce DNA strand breaks [68] and recent studies have confirmed that the SO_2 derivatives (bisulfite and sulfite) induce CA, SCE, mitotic delays and MN in cultured human blood lymphocytes *in vitro*, and that these increases occur in a dose-dependent manner [69].

Similarly, genotoxic effects of Cr are predominantly represented by formation of oxidative adducts and apurinic/apyrimidinic lesions, eventually resulting in DNA breaks [70]. Additionally, Cr (VI) has been shown by to be aneugenic as measured by both chromosome assays and centromere positive micronuclei assays [71].

Cu can induce oxidative stress by two mechanisms. First, it can directly catalyze the formation of ROS via a Fenton-like reaction [72]. Second, exposure to elevated levels of Cu significantly decreases glutathione levels [73]. Since Cu is known to promote oxidative stress and inflammation, it is likely that under non-physiological conditions of increased Cu levels, it could play a role in the development of various cancers [74].

Compared to above ions with similar chemical properties, Zn is relatively harmless. As a redox inert element does not participate in oxidation - reduction reactions [75]. Instead, some authors have also suggested that Zn could play an important role in inducing spindle assembly checkpoint bypass or centrosome amplification capacity of some compounds like Zn chromate [76].

Our results seem to confirm the possible role of oxidative stress in DNA damage generated by exposure to coal mining residues like inorganic elements, and suggest a possible new role in the induction of aneugenic effects observed in exposed populations. Possible models that connect DNA aneuploidy with ROS and oxidative stress have been proposed. However, the role of ROS in DNA aneuploidy remains controversial. Some evidence suggests a possible relationship between ploidy and ROS levels. Antioxidant agents can inhibit aneuploidy progression [77], and overexpression of the antioxidant enzyme, manganese superoxide dismutase, inhibits chromosomal instability [78]. More recently, it has been demonstrated that oxidative stress is capable of overriding the spindle checkpoint [79], inducing microtubule depolymerization [80] and alterations in the spindle structure [81].

Besides chemical elements, some authors have also suggested that exposure to organic compounds also detected in PM stimulate the continuous production of ROS, overwhelm of antioxidant defenses of the cell and thus resulting in oxidative stress [82]. Considering this, concentration of EOM in the aerosol samples of $PM_{2.5}$ around coal mining areas was determined using a three-solvent sequential extraction with increasingly polar solvents CH, DCM and ACE. CH extracts the non-polar compounds in PM, i.e., aliphatic hydrocarbons, polycyclic aromatic hydrocarbons and probably some nonpolar oxidized hydrocarbons. DCM was chosen as the second solvent because it has been shown to be about 26% more efficient than benzene in removing oxidized hydrocarbons [83]. ACE would be expected to be particularly effective in removing significant amounts of inorganic materials, including nitrate and chemical elements, as well as organic compounds.

Coal mining areas had higher EOM content related to substances with nonpolar characteristics extracted with CH and also the largest quantity of polar substances extracted with ACE compared to reference area. Since DNA damage found in reference area was well below that registered inside the coal mining area, chemical

composition of $PM_{2.5}$ in this area may not be constituted of substances with mutagenic potential. As previously discussed, marine aerosol can contain a considerable fraction of organic matter (mainly water insoluble) and sea salts dominated by carbohydrate-like material, nitrates and ammonium [84].

This observation is in line with previous results showing that organic components of $PM_{2.5}$ - particularly PAHs concentration are very important for the effects on the cell cycle and DNA damage [85]. Even when no chemical characterization of the EOM extracts was performed, about 97% of $PM_{2.5}$ around coal mining facilities is produced in the combustion of coal [86], which makes the presence of PAHs and other halogenated species in this fraction very plausible, as previously demonstrated by Pone et al. [87] and Ghose et al. [88].

Besides chemical constitution of the $PM_{2.5,}$ geographical location of sampled areas and the associated relief also constituted a very important determinant in damage distribution. Analysis of average MNBN frequencies between sampling areas showed statistically significant differences between Chancletas, Media Luna and the resguardo of El Cerro de Hatonuevo, while MNBN frequencies between Chancletas, Provincial and San Francisco were similar and comparable. The three latter zones are located downwind of pits and disposal sites in flat areas where drilling, blasting, crushing and temporary coal storage under in the sun could the highest concentrations of dust containing inorganic elements and PAHs from coal partial combustion.

Analyzing the $PM_{2.5}/PM_{10}$ ratios we could conclude that areas with higher DNA damage presented events with ratios $PM_{2.5}/PM_{10}$ above or equal to 0.5, indicating that the fine fraction of PM dominates areas with lower DNA damage showed events with ratios below 0.5 where the coarse fraction of PM is predominant. Similar results in coal mines areas have been reported [89].

It has been found that PM from no combustion sources is by far the main pollutant generated in an open pit mine [90] and transport on unpaved roads is the mining activity that generates most of the emissions. However, our results would suggest that $PM_{2.5}$ mainly from combustion processes [86] would represents the most important health risk factor for residents in proximity to open-pit mines.

Although replacement of PM_{10} by $PM_{2.5}$ measurements in ambient air quality monitoring standards is a likely trend for the future, our results also highlight the need for incorporation of ambient air standards based on $PM_{2.5}$ measures in coal mining areas of Colombia, especially those located around densely populated areas with vulnerable Indigenous and Afro-Colombian populations. This implementation process should be made gradually, under the guidance of the Colombian environmental authorities and the continuous cooperation of coal mining companies.

Characterization of chemical constitution of $PM_{2.5}$ fraction should also be included in air quality monitoring programs, considering that our results suggest that main biological effects related to genetic instability in residential exposed individuals could be related to specific combinations of inorganic elements like sulfur, chromium, copper and zinc and high concentrations of organic matter (i.e. PAH). Emerging reports have identified that more-than-additive mortality is common in inorganic elements−PAH mixtures. Individual aspects of PAH toxicity suggest they may alter the accumulation of inorganic elements and enhance element-derived reactive ROS. Redox-active elements (e.g., Cu and Ni) are also capable of enhancing the redox cycling of PAHs [91]. Several reports have implicated inorganic elements as modifiers of P450 function and regulation, which implies that such elements could alter P450 - mediated PAH mutagenicity and carcinogenicity [92].

CONCLUSIONS

Based on our data we can conclude that $PM_{2.5}$ fraction in open-pit coal mining areas of Northern Colombia contains high concentrations of enriched toxic elements (S, Cl, Cr, Cu and Zn) and organic matter related to substances with nonpolar characteristics. Correlation analysis between elements would indicate that some elements like S, Cr, Cu and Zn might originate mainly from coal and coal combustion sources. Environmental exposure to this particular complex mixture could induce an increased frequency of MNBN characterized by whole chromosome loss (aneuploidy). This aneugenic effect may be associated to an oxidative stress status inside the cell, potentially capable of causing mitotic arrest (MNMONO frequency), centromere damage, kinetochore malfunction or disruption of the mitotic spindle. Indigenous and Afro-Colombians populations with residential proximity to the Guajira coal mining corridor could present an elevated health risk of manifesting coal mining-related diseases and cancer, when compared to unexposed populations with same socio-demographic and ethnic characteristics.

ACKNOWLEDGMENTS

REFERENCES

1.   Weichenthal, S.A., K. Godri-Pollitt, and P.J. Villeneuve, *PM2.5, oxidant defence and cardiorespiratory health: a review.* Environmental Health, 2013. **12**(1): p. 1-8.

2.   Kurth, L., et al., *Atmospheric particulate matter in proximity to mountaintop coal mines: sources and potential environmental and human health impacts.* Environ Geochem Health, 2015. **37**(3): p. 529-44.

3.   Hendryx, M. and M.M. Ahern, *Relations Between Health Indicators and Residential Proximity to Coal Mining in West Virginia.* American Journal of Public Health, 2008. **98**(4): p. 669-671.

4.   Billet, S., et al., *Genotoxic potential of Polycyclic Aromatic Hydrocarbons-coated onto airborne Particulate Matter (PM 2.5) in human lung epithelial A549 cells.* Cancer Lett, 2008. **270**(1): p. 144-55.

5.   Schins, R.P. and P.J. Borm, *Mechanisms and mediators in coal dust induced toxicity: a review.* Ann Occup Hyg, 1999. **43**(1): p. 7-33.

6.   EPA, U., *Air Quality Criteria for Particulate Matter, Vols. I–II.* Washington, DC: US Environmental Protection Agency. EPA, 2004. **600/P-99/002aF-bF**.

7.   Kaonga, B. and N.A. Kgabi, *Investigation into presence of atmospheric particulate matter in Marikana, mining area in Rustenburg Town, South Africa.* Environ Monit Assess, 2011. **178**(1-4): p. 213-20.

8.   Huertas, J.I., D.A. Camacho, and M.E. Huertas, *Standardized emissions inventory methodology for open-pit mining areas.* Environ Sci Pollut Res Int, 2011. **19**(7): p. 2784-94.

9.   Srimuruganandam, B. and S.M. Shiva Nagendra, *Source characterization of PM10 and PM2.5 mass using a chemical mass balance model at urban roadside.* Sci Total Environ, 2012. **433**: p. 8-19.

10.   Li, Q., et al., *The preferential accumulation of heavy metals in different tissues following frequent respiratory exposure to PM2.5 in rats.* Sci Rep, 2015. **5**: p. 16936.

11.   Liu, G., et al., *Polycyclic aromatic hydrocarbons (PAHs) from coal combustion: emissions, analysis, and toxicology.* Rev Environ Contam Toxicol., 2008. **192**(1-28).

12.   Espitia-Pérez, L., et al., *Polymorphisms in metabolism and repair genes affects DNA damage caused by open-cast coal mining exposure.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2016. **808**: p. 38-51.

13.   Leon-Mejia, G., et al., *Genetic damage in coal miners evaluated by buccal micronucleus cytome assay.* Ecotoxicol Environ Saf, 2014. **107**: p. 133-9.

14.   White, P.A., *The genotoxicity of priority polycyclic aromatic hydrocarbons in complex mixtures.* Mutat Res Rev Mutat Res, 2002. **515**(1-2): p. 85-98.

15.   Wang, X., et al., *Chemical Composition and Sources of PM10 and PM2.5 Aerosols in Guangzhou, China.* Environmental Monitoring and Assessment, 2006. **119**(1): p. 425-439.

16.   Kawanaka, Y., et al., *Size distributions of mutagenic compounds and mutagenicity in atmospheric particulate matter collected with a low-pressure cascade impactor.* Atmospheric Environment, 2004. **38**(14): p. 2125-2132.

17.   Naser, T., et al., *Chemical composition of PM 2.5 and PM 10 and associated polycyclic aromatic hydrocarbons at a roadside and an urban background area in Saitama, Japan.* Asian Journal of Atmospheric Environment, 2008. **2**(2): p. 90-101.

18.   Da Silva, J., *DNA damage induced by occupational and environmental exposure to miscellaneous chemicals.* Mutation Research/Reviews in Mutation Research, 2016.

19.   Leon-Mejia, G., et al., *Assessment of DNA damage in coal open-cast mining workers using the cytokinesis-blocked micronucleus test and the comet assay.* Sci Total Environ, 2011. **409**(4): p. 686-91.

20.   Rohr, P., et al., *Genetic and oxidative damage of peripheral blood lymphocytes in workers with occupational exposure to coal.* Mutat Res, 2013. **758**(1-2): p. 23-8.

21.   Fenech, M., *Cytokinesis-block micronucleus cytome assay.* Nat. Protocols, 2007. **2**(5): p. 1084-1104.

22.   Kirsch-Volders, M., et al., *Commentary: critical questions, misconceptions and a road map for improving the use of the lymphocyte cytokinesis-block micronucleus assay for in vivo biomonitoring of human exposure to genotoxic chemicals-a HUMN project perspective.* Mutat Res Rev Mutat Res, 2014. **759**: p. 49-58.

23.   Fenech, M., et al., *HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures.* Mutat. Res., Genet. Toxicol. Environ. Mutagen., 2003. **534**(1-2): p. 65-75.

24.   Bonatti, S., et al., *Genotoxic effects of the carbamate insecticide methomyl. I. In vitro studies with pure compound and the technical formulation "Lannate 25".* Environmental and Molecular Mutagenesis, 1994. **23**(4): p. 306-311.

25.   Fenech, M. and A.A. Morley, *Kinetochore detection in micronuclei: an alternative method for measuring chromosome loss.* Mutagenesis, 1989. **4**(2): p. 98-104.

26.   Eastmond, D.A. and J.D. Tucker, *Identification of aneuploidy-inducing agents using cytokinesis-blocked human lymphocytes and an antikinetochore antibody.* Environ Mol Mutagen, 1989. **13**(1): p. 34-43.

27.   Olivero, O.A., et al., *Perinatal Exposure of Patas Monkeys to Antiretroviral Nucleoside Reverse-Transcriptase Inhibitors Induces Genotoxicity Persistent for up to 3 Years of Age.* Journal of Infectious Diseases, 2013.

28.   Degrassi, F. and C. Tanzarella, *Immunofluorescent staining of kinetochores in micronuclei: a new assay for the detection of aneuploidy.* Mutat Res, 1988. **203**(5): p. 339-45.

29.   Saitoh, K., et al., *PIXE analysis of spot samples on New York type of PTFE ultra-membrane filter - tape mounted in an automated beta - ray absorption mass monitor.* International Journal of PIXE, 2006. **16**(01n02): p. 95-101.

30.   Campbell, J.L., et al., *The Guelph PIXE software package IV.* Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms, 2010. **268**(20): p. 3356-3363.

31.   Cesari, D., et al., *Analysis of raw soils and their re-suspended PM10 fractions: Characterisation of source profiles and enrichment factors.* Applied Geochemistry, 2012. **27**(6): p. 1238-1246.

32.   Kothai, P., et al., *Characterization of atmospheric particulate matter using pixe technique.* Int. J. Environ. Sci. Eng, 2009. **1**(1): p. 27-30.

33.   Taner, S., B. Pekey, and H. Pekey, *Fine particulate matter in the indoor air of barbeque restaurants: Elemental compositions, sources and health risks.* Science of The Total Environment, 2013. **454–455**: p. 79-87.

34.   Hans Wedepohl, K., *The composition of the continental crust.* Geochimica et Cosmochimica Acta, 1995. **59**(7): p. 1217-1232.

35.   Loomis, D., et al., *The carcinogenicity of outdoor air pollution.* Lancet Oncol, 2013. **14**(13): p. 1262-3.

36.   Ahern, M. and M. Hendryx, *Cancer Mortality Rates in Appalachian Mountaintop Coal Mining Areas.* J Environ Occup Sci, 2012. **1**(2): p. 63-70.

37.   Aneja, V.P., A. Isherwood, and P. Morgan, *Characterization of particulate matter (PM10) related to surface coal mining operations in Appalachia.* Atmospheric Environment, 2012. **54**: p. 496-501.

38.   IARC, I.A.f.R.o.C., *Agents Classified by the IARC Monographs.* 1997. **Volumes 1–100. Cas N° 000050-41-9**: p. 68.

39.   Rohr, P., et al., *Genetic and oxidative damage of peripheral blood lymphocytes in workers with occupational exposure to coal.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2013. **758**(1–2): p. 23-28.

40.   Zocche, J.J., et al., *Heavy-Metal Content and Oxidative Damage in Hypsiboas faber: The Impact of Coal-Mining Pollutants on Amphibians.* Archives of Environmental Contamination and Toxicology, 2013. **66**(1): p. 69-77.

41.   Leon, G., et al., *Genotoxic effects in wild rodents (Rattus rattus and Mus musculus) in an open coal mining area.* Mutat Res, 2007. **630**(1-2): p. 42-9.

42.   Bonassi, S., et al., *Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies.* Mutagenesis, 2011. **26**(1): p. 93-100.

43.   Fenech, M. and S. Bonassi, *The effect of age, gender, diet and lifestyle on DNA damage measured using micronucleus frequency in human peripheral blood lymphocytes.* Mutagenesis, 2011. **26**(1): p. 43-9.

44.   Fenech, M., *Chromosomal biomarkers of genomic instability relevant to cancer.* Drug Discov Today, 2002. **7**(22): p. 1128-37.

45.   Godderis, L., et al., *Dose-dependent influence of genetic polymorphisms on DNA damage induced by styrene oxide, ethylene oxide and gamma-radiation.* Toxicology, 2006. **219**(1-3): p. 220-9.

46.   El-Zein, R.A., et al., *Cytokinesis-Blocked Micronucleus Cytome Assay Biomarkers Identify Lung Cancer Cases Amongst Smokers.* Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology, 2008. **17**(5): p. 1111-1119.

47.   Norppa, H. and G.C.-M. Falck, *What do human micronuclei contain?* Mutagenesis, 2003. **18**(3): p. 221-233.

48.   Fenech, M., G. Perepetskaya, and L. Mikhalevich, *A more comprehensive application of the micronucleus technique for biomonitoring of genetic damage rates in human populations--experiences from the Chernobyl catastrophe.* Environ Mol Mutagen, 1997. **30**(2): p. 112-8.

49.   Marini, V., et al., *Exposure to asbestos: Correlation between blood levels of mesothelin and frequency of micronuclei in peripheral blood lymphocytes.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2011. **721**(1): p. 114-117.

50. Vande Loock, K., et al., *Maternal and gestational factors and micronucleus frequencies in umbilical blood: the NewGeneris Rhea cohort in Crete.* Environ Health Perspect, 2011. **119**(10): p. 1460-5.

51. Luzhna, L., P. Kathiria, and O. Kovalchuk, *Micronuclei in genotoxicity assessment: from genetics to epigenetics and beyond.* Frontiers in Genetics, 2013. **4**: p. 131.

52. Thierens, H., et al., *Cytogenetic monitoring of hospital workers occupationally exposed to ionizing radiation using the micronucleus centromere assay.* Mutagenesis, 2000. **15**(3): p. 245-249.

53. Catalan, J., et al., *Age-associated micronuclei containing centromeres and the X chromosome in lymphocytes of women.* Cytogenet Cell Genet, 1995. **68**(1-2): p. 11-6.

54. Rosefort, C., E. Fauth, and H. Zankl, *Micronuclei induced by aneugens and clastogens in mononucleate and binucleate cells using the cytokinesis block assay.* Mutagenesis, 2004. **19**(4): p. 277-284.

55. Elhajouji, A., M. Cunha, and M. Kirsch-Volders, *Spindle poisons can induce polyploidy by mitotic slippage and micronucleate mononucleates in the cytokinesis-block assay.* Mutagenesis, 1998. **13**(2): p. 193-8.

56. Chance, R., T.D. Jickells, and A.R. Baker, *Atmospheric trace metal concentrations, solubility and deposition fluxes in remote marine air over the south-east Atlantic.* Marine Chemistry, 2015. **177, Part 1**: p. 45-56.

57. Pan, Y., et al., *Trace elements in particulate matter from metropolitan regions of Northern China: Sources, concentrations and size distributions.* Science of The Total Environment, 2015. **537**: p. 9-22.

58. Petaloti, C., et al., *Trace elements in atmospheric particulate matter over a coal burning power production area of western Macedonia, Greece.* Chemosphere, 2006. **65**(11): p. 2233-43.

59. Zhang, R., et al., *Chemical characterization and source apportionment of PM$_{2.5}$ in Beijing: seasonal perspective.* Atmos. Chem. Phys., 2013. **13**(14): p. 7053-7074.

60. Pandey, B., M. Agrawal, and S. Singh, *Assessment of air pollution around coal mining area: Emphasizing on spatial distributions, seasonal variations and heavy metals, using cluster and principal component analysis.* Atmospheric Pollution Research, 2014. **5**(1): p. 79-86.

61. Schroeder, W.H., et al., *Toxic Trace Elements Associated with Airborne Particulate Matter: A Review.* JAPCA, 1987. **37**(11): p. 1267-1285.

62. Afroz, R., M.N. Hassan, and N.A. Ibrahim, *Review of air pollution and health impacts in Malaysia.* Environmental Research, 2003. **92**(2): p. 71-77.

63. Kothai, P., et al., *Characterization of Atmospheric Particulate Matter using PIXE Technique.* International Journal of Environmental, Chemical, Ecological, Geological and Geophysical Engineering, 2009. **3**: p. 38-42.

64. Annangi, B., et al., *Biomonitoring of humans exposed to arsenic, chromium, nickel, vanadium, and complex mixtures of metals by using the micronucleus test in lymphocytes.* Mutation Research/Reviews in Mutation Research.

65. Lestari, F., et al., *In vitro cytotoxicity of selected chemicals commonly produced during fire combustion using human cell lines.* Toxicology in Vitro, 2005. **19**(5): p. 653-663.

66.   Alimba, C.G., et al., *Genotoxicity and cytotoxicity of chromium, copper, manganese and lead, and their mixture in WIL2-NS human B lymphoblastoid cells is enhanced by folate depletion.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2016. **798–799**: p. 35-47.

67.   Valko, M., et al., *Free radicals, metals and antioxidants in oxidative stress-induced cancer.* Chem Biol Interact, 2006. **160**(1): p. 1-40.

68.   Meng, Z., G. Qin, and B. Zhang, *DNA damage in mice treated with sulfur dioxide by inhalation.* Environ Mol Mutagen, 2005. **46**(3): p. 150-5.

69.   Uren, N., S. Yuksel, and Y. Onal, *Genotoxic effects of sulfur dioxide in human lymphocytes.* Toxicology and Industrial Health, 2014. **30**(4): p. 311-315.

70.   Vasylkiv, O.Y., et al., *Cytotoxicity of chromium ions may be connected with induction of oxidative stress.* Chemosphere, 2010. **80**(9): p. 1044-1049.

71.   Wise, S.S. and J.P. Wise, *Aneuploidy as an early mechanistic event in metal carcinogenesis.* Biochem Soc Trans, 2010. **38**(6): p. 1650-4.

72.   Prousek, J., *Fenton chemistry in biology and medicine.* Pure Appl. Chem. , 2007. **79**: p. 2325–2338.

73.   Speisky, H., et al., *Generation of superoxide radicals by copper–glutathione complexes: Redox-consequences associated with their interaction with reduced glutathione.* Bioorganic & Medicinal Chemistry, 2009. **17**(5): p. 1803-1810.

74.   Gupte, A. and R.J. Mumper, *Elevated copper and oxidative stress in cancer cells as a target for cancer treatment.* Cancer Treat Rev, 2009. **35**(1): p. 32-46.

75.   Bray, T.M. and W.J. Bettger, *The physiological role of zinc as an antioxidant.* Free Radic Biol Med, 1990. **8**(3): p. 281-91.

76.   Holmes, A.L., et al., *Chronic Exposure to Zinc Chromate Induces Centrosome Amplification and Spindle Assembly Checkpoint Bypass in Human Lung Fibroblasts.* Chemical Research in Toxicology, 2010. **23**(2): p. 386-395.

77.   Kraniak, J.M., et al., *Antioxidant agents transiently inhibit aneuploidy progression in Li-Fraumeni cell strains.* Mol Carcinog, 2006. **45**(3): p. 141-56.

78.   Van de Wetering, C.I., et al., *Manganese Superoxide Dismutase gene dosage affects chromosomal instability and tumor onset in a mouse model of T cell lymphoma.* Free radical biology & medicine, 2008. **44**(8): p. 1677-1686.

79.   D'Angiolella, V., C. Santarpia, and D. Grieco, *Oxidative stress overrides the spindle checkpoint.* Cell Cycle, 2007. **6**(5): p. 576-9.

80.   Parker, A.L., M. Kavallaris, and J.A. McCarroll, *Microtubules and Their Role in Cellular Stress in Cancer.* Frontiers in Oncology, 2014. **4**: p. 153.

81.   Choi, W.J., et al., *Oxidative stress and tumor necrosis factor-alpha-induced alterations in metaphase II mouse oocyte spindle structure.* Fertil Steril, 2007. **88**(4 Suppl): p. 1220-31.

82.   Zeidler, P., et al., *Role of inducible nitric oxide synthase-derived nitric oxide in silica-induced pulmonary inflammation and fibrosis.* J Toxicol Environ Health A, 2004. **67**(13): p. 1001-26.

83. Daisey, J.M., Leyko, M. A., Kleinman, M. T. and Hoffman, E. , *The nature of the organic fraction of the New York city summer aerosol.* Annals of the New York Academy of Sciences, 1979. **322**: p. 125–141.

84. Rinaldi, M., et al., *Primary and Secondary Organic Marine Aerosol and Oceanic Biological Activity: Recent Results and New Perspectives for Future Studies.* Advances in Meteorology, 2010. **2010**.

85. Longhin, E., et al., *Cell cycle alterations induced by urban PM2.5 in bronchial epithelial cells: characterization of the process and possible mechanisms involved.* Particle and Fibre Toxicology, 2013. **10**(1): p. 1-19.

86. Cao Guo Liang, Z.X., Gong SunLing, An Xin Qin & Wang Ya Qiang, *Emission inventories of primary particles and pollutant gases for China.* Atmospheric Science, 2011. **56**(8): p. 781-788.

87. Pone, J.D.N., et al., *The spontaneous combustion of coal and its by-products in the Witbank and Sasolburg coalfields of South Africa.* International Journal of Coal Geology, 2007. **72**(2): p. 124-140.

88. Ghose, M.K. and S.R. Majee, *Characteristics of hazardous airborne dust around an Indian surface coal mining area.* Environ Monit Assess, 2007. **130**(1-3): p. 17-25.

89. Tecer, L.H., et al., *Effect of meteorological parameters on fine and coarse particulate matter mass concentration in a coal-mining area in Zonguldak, Turkey.* J Air Waste Manag Assoc, 2008. **58**(4): p. 543-52.

90. Ghose, M.K. and S.R. Majee, *Assessment of Dust Generation Due to Opencast Coal Mining– An Indian Case Study.* Environmental Monitoring and Assessment. **61**(2): p. 257-265.

91. Gauthier, P.T., et al., *Metal–Polycyclic Aromatic Hydrocarbon Mixture Toxicity in Hyalella azteca. 2. Metal Accumulation and Oxidative Stress as Interactive Co-toxic Mechanisms.* Environmental Science & Technology, 2015. **49**(19): p. 11780-11788.

92. Peng, C., et al., *Micronucleus formation by single and mixed heavy metals/loids and PAH compounds in HepG2 cells.* Mutagenesis, 2015.

IV. CAPÍTULO II

GENETIC DAMAGE IN ENVIRONMENTALLY EXPOSED POPULATIONS TO OPEN-PIT COAL MINING RESIDUES: ANALYSIS OF BUCCAL MICRONUCLEUS CYTOME (BMN-cyt) ASSAY AND ALKALINE, ENDO III AND FPG HIGH-THROUGHPUT COMET ASSAY

GENETIC DAMAGE IN ENVIRONMENTALLY EXPOSED POPULATIONS TO OPEN-PIT COAL MINING RESIDUES: ANALYSIS OF BUCCAL MICRONUCLEUS CYTOME (BMN-cyt) ASSAY AND ALKALINE, ENDO III AND FPG HIGH-THROUGHPUT COMET ASSAY

Lyda Espitia-Pérez [a,b,c,d*], Juliana da Silva [e**], Hugo Brango [f], Pedro Espitia – Pérez [a], Karina Pastor-Sierra [a,b], Shirley Salcedo – Arteaga [a], Claudia T. de Souza [g], Johnny F. Dias [g], Luz Stella Hoyos-Giraldo [h], Miguel Gómez–Pérez [a], Daniela Salcedo-Restrepo [a], João A. P. Henriques [c,i]

[a] Facultad de Ciencias de la Salud, Laboratorio de Investigación Biomédica y Biología Molecular, Universidad del Sinú, Montería, Córdoba, Colombia.

[b] Departamento de Ciencias Básicas, Universidad del Sinú, Montería, Córdoba, Colombia.

[c] Programa de Pós-Graduação em Biologia Celular e Molecular (PPGBCM), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre- Brazil.

[d] Departamento de Biofísica, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

[e] Laboratório de Genética Toxicológica, Universidade Luterana do Brasil, ULBRA, Canoas, RS, Brazil.

[f] Instituto de Matemática e Estatística, Universidade de São Paulo, São Paulo, Brazil

[g] Laboratório de Implantação Iônica, Instituto de Física, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brasil

[h] Department of Biology, Research Group Genetic Toxicology and Cytogenetics, Faculty of Natural Sciences and Education, Universidad del Cauca, Popayán, Colombia

[i] Instituto de Biotecnologia, Universidade de Caxias do Sul (UCS), Caxias do Sul, RS, Brazil.

For correspondence with authors:

*E-mail address: espitiaperez@gmail.com (L. Espitia– Pérez)

**E-mail address: juliana.silva@ulbra.br (Juliana Da Silva)

ABSTRACT

DNA and chromosomal damage in individuals occupationally exposed to coal mining residues have been repeatedly reported in lymphocytes and epithelial cells, suggesting a systemic exposure response where generation of oxidative damage may play a major role. Nevertheless, understanding of this mechanism is still incomplete particularly in environmental exposures. The aims of this study were to evaluate DNA damage using the cytome assay (BMN-cyt) in buccal cells and its relation with primary and oxidative DNA damage in lymphocytes, assessed by the high-throughput alkaline and modified (FPG-ENDO III) Comet assay in individuals with environmental exposure to coal mining residues. Considering metals from coal mining activities as main source of reactive oxygen species (ROS) generation, inorganic elements content in blood samples was also assessed. Analysis revealed that frequencies of BMN-cyt parameters related to DNA damage (micronucleus), cytokinesis damage (binucleated cells) and cell death (condensed chromatin, karyorrhexis, pyknosis and karyolysis) were significantly higher in individuals environmentally exposed to coal compared to the unexposed group. The level of % Tail DNA in the alkaline and modified Comet assay was 4.0 and 4.3 times higher among exposed individuals than in unexposed controls respectively. Increased MN frequencies in buccal cells were correlated to increased %Tail DNA in alkaline and FPG Comet assay. Additionally, exposed individuals showed higher concentrations of Cr, Ni, Mn and Br in blood compared to unexposed controls. %Tail DNA in alkaline Comet assay was highly correlated with Al, Mn and Br concentrations, while %Tail DNA in the FPG Comet assay was correlated to Mn concentrations. These results suggest that oxidative damage, particularly purines oxidation, may play an important role in the DNA damage observed in individuals exposed to coal residues and that some inorganic elements are related to this effect.

1. NTRODUCTION

Over the last several years, our research group have demonstrated that occupational exposure to open-cast coal mining residues (dust particles, heavy metals and Polycyclic Aromatic Hydrocarbons - PAHs) may cause a wide range of DNA damage and genomic instability that could be associated to initial steps in cancer development and other work-related diseases [1-3] and that susceptibility to the hazardous action of this chemicals may derive from genetic or acquired characteristics of the individual [4]. However, paucity of data examining the impact of these industrial operations to cytogenetic endpoints frequency in general population has caused that the understanding of the mechanism that leads to DNA damage in environmental exposures is still incomplete.

Studies of coal dust exposure also report increased risk of lung cancer [5]. As coal may contain high amounts of carcinogens it is plausible to consider a link between coal dust inhalation into the lungs and subsequent cancer [6]. A characterization of the products generated in spontaneous combustion of coal in open-pit facilities detected thirty-two aliphatic compounds as well as halogenated compounds including bromomethane, iodomethane and trichloromethane in low concentrations, and dichloromethane and chloromethane in high concentrations [7]. Besides HAPs, metals are an important and emergent class of carcinogens [8] also related to open-pit coal mining activities [9]. They are being introduced in the atmosphere at a much higher rate than from their natural sources due to anthropogenic activities in which metals are involved. One of the major sources of anthropogenic trace elements inputs in the atmosphere is coal combustion [10]. Elemental analysis of particulate matter (PM) around coal mines showed S as the most abundant element, followed by Fe, Zn, and Pb. The examined PM also contained moderate amounts of Cd, Cr, Cu, and Ni [11], most of which exhibit well-known mutagenic and carcinogenic activity [12]. In recent years, there is a growing concern for the potential contribution of ingested dust to metal toxicity in humans. Some trace metals (such as Cu and Zn) at small amounts are harmless, but some (mainly Pb, As, Hg and Cd) even at extremely low concentrations are toxic and are potential cofactors, initiators or promoters in many diseases and cancer [13].

Inhalation of particles is considered as the main route of exposure to potentially hazardous coal residues in occupational and environmental exposed populations [14]. Therefore, buccal cells are the first barrier for the inhalation or ingestion route of these residues [15] and are considered as a suitable target site for monitoring human exposure to occupational and environmental genotoxins. Mucosal cells are not only in the direct route of exposure to ingested pollutants but also capable of metabolizing chemical agents to reactive species, for example PAH via Cytochrome P450 (CYP) [16]. Metabolic conversion of these substances can generate reactive electrophilic intermediates and reactive oxygen species (ROS) capable of interact and damage various cellular macromolecules such as proteins, lipids and DNA [17]. Micronuclei in buccal exfoliated cells emerge during mitosis of the basal layers of the epithelium and their absolute frequency could reflect the real situation in target cells [18]. The micronucleus cytome assay applied in buccal exfoliated cells (BMN-cyt) provides a complementary method for measuring DNA damage and cytotoxic effects in an easily accessible tissue not requiring *in vitro* culture [19]. The BMN-cyt assay has been used to measure biomarkers of DNA damage (MN), cytokinetic defects (binucleated cells - BN) and proliferative potential (basal cell frequency) and/or cell death (condensed chromatin - CC, karyorrhexis - KHC, pyknotic – PYK and karyolytic cells - KYL) [20].

Primary DNA damage is considered to be a key initial event in carcinogenesis. The Comet (single cell gel electrophoresis) assay has become the preferred test for qualitatively and quantitatively assessing DNA damage in small numbers of cells in vitro and human biomonitoring studies [21]. Oxidative stress may also be implicated in carcinogenesis,

and 8-hydroxy-20 -deoxyguanosine (8-OHdG) is the most frequently measured biomarker of oxidative stress [22]. Formamidopyrimidine-DNA glycosylase (FPG) and endonuclease-III (ENDO III) are most often used in the endonuclease Comet assay (called modified comet assay) for the detection of oxidative DNA damage and the presence of oxidized purine and pyrimidine bases [23]. According to Zhao et al. [24] changes in FPG-Comet assay are correlated to the cellular ROS induced by genetic toxicants. For biomonitoring purposes, enzymatic detection of DNA oxidized products by Comet assay is an easier and more feasible approach [25].

Thus, in the present study we evaluate the extent of DNA damage in individuals with environmental exposure to open-pit coal mining area using buccal exfoliated cells and Comet assay parameter (%Tail DNA) in lymphocytes. Considering that increased MN frequencies in occupational exposed populations have been attributed to oxidative stress generated by the exposure to inorganic elements and other related coal mining residues [1], we also evaluate the extend of oxidative DNA damage using the FPG-ENDO III modified comet assay. Complementary, inorganic elements content in blood samples of environmentally exposed individuals was determined and correlated with BMN-cyt and alkaline and modified comet assay parameters.

2. METHODOLOGY

2.1 Study population and sample collection

The study population was comprised of inhabitants living in the area under the direct influence of the mining operation, which comprised the municipalities of Albania, Barrancas, and Hatonuevo, constituted by indigenous Wayúu settlements, a small population of Afro-Colombians and a number of campesino (rural peasant) communities. Other areas influenced by the coal mining process include those around a 150 km railroad and areas around the port facility (Puerto Bolívar) in the municipality of Uribia on the Caribbean Sea coast, where the coal is exported. Unexposed (Control) area was located in Mayapo municipality, also constituted by indigenous Wayúu settlements and Afro-Colombians populations, situated on the Caribbean Sea coast without any influence of coal mining operations and located 57.12 kilometers from Riohacha city (La Guajira).

The Committee on Research Ethics of each institution approved the study and before sample collection, a written informed consent was obtained from each individual. Collection was performed on 139 healthy individuals: 98 with permanent residential proximity to open – pit coal mining area and 41 permanent residents of a control area without any proximity to open-pit coal mining facilities. Exposed and unexposed control individuals were matched by age (± 5 years), sex, similar social-economic status and ethnicity. Only no smokers were considered in this study. Samples of individuals with residential proximity to open-pit coal mining area (exposed) and from unexposed area (control) were collected and transported simultaneously to prevent any interference caused by differences in sampling conditions.

2.2 Blood samples collection

After informed consent was obtained, peripheral blood samples were collected by venipuncture from all 139 participants. For the application of Comet Assay, 4 mL of blood were drawn into EDTA coated tubes (Becton Dickenson, vacutainer), stored at 4°C and transported to the laboratory for processing within 24h of collection. A total of 4 mL of blood were drawn into heparin tubes (Becton Dickenson, vacutainer) for the particle-induced X-ray emission (PIXE) analysis. Concomitantly with collection of the blood samples of exposed and unexposed individuals, additional samples from the research staff were also collected, transported and processed under the same conditions. As previously described, these samples were used as internal standards in order to eliminate potential confounding factors originated during sample handling, processing or transportation to the laboratory [26]. Blood sampling was conducted by personnel with medical training, accordingly to The Committee on Research Ethics of each institution enrolled in the study.

2.3 Buccal micronucleus cytome assay (BMN-cyt) and Feulgen staining

Buccal mucosa samples were collected as previously described in Carbajal-López et al. [27]. Briefly, subjects were asked to rinse their mouth with water before sampling. The exfoliated buccal mucosa cells were collected using a cytobrush to scrape the mucosa of the inner lining of both cheeks. All buccal sample tubes were coded and kept in upright position, stored at 4°C and transported to the laboratory for processing. The cells were washed three times in 0.9 percent phosphate saline buffer; the smears were made from the pellet and fixed in methanol 80%.

Feulgen method was used for micronucleus staining. Briefly, fixed slides were treated for 1 min each in 50% and 20% ethanol, rinsed for 2 min in Milli-Q® water and immersed in 5M hydrochloric acid for 30 min. After acid treatment, slides were gently washed using running tap water for 3 min, carefully dried with fiber-free cloth, and transported into a

staining jar filled with Schiff 's reagent (Sigma 3259016). Treatment were made at room temperature in the dark for 60 min. Following this procedure, slides were washed in running tap water for 5 min and rinsed well in Milli-Q® water (1 min). For counterstaining, slides were treated with 0.2% light green (Sigma L-1886) during 30 s. Finally, slides were rinsed in Milli-Q® water for 2 min, air-dried and coverslipped for evaluation. With Feulgen method nuclei and MNs are stained magenta, while the cytoplasm appears green. Slides were scored using an Olympus BX51 microscope (Olympus, Japan) at ×1000 magnification. The frequency of MN and other nuclear abnormalities such as nuclear buds (NBUDS), binucleated (BNC), karyolytic (KYL) karyorrhectic (KHC), pyknotic (PYK) and Condensed chromatin cells (CC) were determined in 2000 cells for each person following recommendations of Thomas et al. [20]. One reader blinded to the exposure status of the individuals scored all slides.

2.4 High-Throughput Comet Assay

The alkaline Comet assay was performed as previously described by Singh et al. [28] with several modifications for a high-throughput version, which allows the processing of multiple samples. The high-throughput "96-minigel format" is an 8x12 multi-array on GelBond® film (Lonza, Rockland Inc. ME, USA) [29]. Detailed experimental protocol has been described in detail elsewhere [30].

Briefly, lymphocytes were isolated from previously collected whole blood samples (EDTA-stored) by density gradient centrifugation using Histopaque 1077 (Sigma). Cells were gently resuspended, transferred to a 96-well microplate and mixed in the ratio of 1:10 with previously prepared 0.5% low melting point agarose (LMA-Invitrogen) in PBS (pH 7.4) without calcium and magnesium. Aliquots of 5 µL of the LMA-cell suspension were added onto the hydrophilic side of pre-cutted GelBond® sheets positioned on an ice-cold metal plate. One minigel of the 8×12 array was prepared from the lymphocyte suspension harvested from each well. Minigels were immersed overnight in lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0–10.5, 1% with freshly added 1% Triton X-100 and 10% DMSO) at 4 °C in dark and rinsed the next day using cold PBS. For DNA unwinding, films were immersed in cold electrophoresis solution (300 mM NaOH and 1 mM EDTA, pH≥13) for 40 min. The alkaline electrophoresis was carried out at 0.8-1.0 V/cm for 40 min [31]. Following this procedure, the gels were neutralized with cold Tris buffer (0.4M Tris, pH 7.5) for 3×5 min, fixed with 96% ethanol for 1.5 h and kept in the dark inside a dust-free area. Finally, GelBond® films were cutted into arrays containing 3x4 minigels, stained with 10 mL of ethidium bromide (1 µg/mL), rehydrated with cold deionized water and coverslipped. Preparations were stored in a dark, moist chamber at 4°C until scoring the same or the next day [30]. For semi-automated scoring, stained cells were observed using an Olympus BX51 fluorescence microscope (Olympus, Japan) and examined at 40X magnification under green filter of 540 nm. Direct light exposure of the samples was avoided during the whole process. For each individual we analyzed 100 randomly selected Comets (50 cells from each of two replicate slides). To avoid selection bias, overlapping comets were excluded [30]. % Tail DNA was scored using the Comet Assay IV® software (Perceptive Instruments, Haverhill, England).

The alkaline comet assay using lesion-specific enzymes (ENDO III and FPG modified Comet Assay) was used to detect oxidized purines and pyrimidines [32]. Protocol was performed as previously described by other studies [33] with minor modifications for the high-throughput comet assay. Briefly, minigels were washed (3 times at room temperature) with the enzyme buffer (40 mM HEPES, 100 mM KCl, 0.5 mM EDTA and 0.2 mg/ml bovine serum albumin) immediately after lysis and were incubated at 37 °C for 30 min with (i) ENDO III (1:1000, 30 min), (ii) FPG (1:1000, 45 min) and (iii) with

enzyme buffer (control). ENDO III recognizes oxidized pyrimidines while FPG recognizes oxidized purines, specifically 8-oxo-guanine [23]. DNA unwinding, electrophoresis, neutralization, staining and scoring procedures were made according to the same high-throughput comet assay protocol, as described above. Significant differences between 7-11% in registered values of DNA in the tail were identified for each treatment (FPG, ENDO III) over control (buffer without enzyme). The net contribution of the 8-hydroxy-2'-deoxyguanosine (8-oxoG) and oxidized pyrimidines to final DNA damage evaluated by FPG—ENDO III modified comet assay respectively, was calculated by subtraction of the mean %TailDNA values obtained from slides exposed to buffer only from the mean %TailDNA values obtained from the slides exposed to ENDO III and FPG enzymes [21].

2.5 Analysis of inorganic elements content in blood samples by Particle-induced X-ray emission (PIXE)

The elemental composition (Na, Mg, Al, Si, P, S, Cl, K, Ca, Ti, Cr, Mn, Fe, Ni, Cu, Zn and Br) of the blood samples was measured by Particle-Induced X-ray Emission (PIXE), using the accelerator facility at the Ion Implantation Laboratory of the Physics Institute, Federal University of Rio Grande do Sul. Blood samples collected in heparin-coated tubes were dried at 40 °C for 72 h, then macerated using a mortar and the remaining solid were transfer into plastic containers to avoid metal interference. After drying, samples were mixed in the ratio 1:1 with high-purity cellulose and pressed into pellets using a hydraulic press (1.5 MPa). Obtained pellets were positioned on the reaction chamber at reduced pressure (10-5 mbar) [3]. Samples were irradiated with a 2.0 MeV proton beam (average current of 5 nA) generated from a 3.0 MV Tandetron accelerator. The X-rays produced by the samples were detected using a Si(Li) detector with an energy resolution of approximately 150 eV at 5.9 keV. Obtained spectra were analyzed with the GUPIXWIN software package and the final results are expressed in parts per million (mg/kg) [34]. Rutherford Backscattering Spectrometry (RBS) technique was used in order to analyze organic components of blood samples. As previously described, the organic matrix used to elemental analysis of blood was 72.50% carbon, 7.50% oxygen, 13.50% nitrogen and 6.50% fluorine.

2.6 Statistical analysis

Statistical analyses were carried out using software R version 3.3.0 (R Foundation for Statistical Computing, Vienna, Austria). The model established to confounding factors analysis included sex, alcohol intake exposure, and time of exposure. All possible two-way interactions among BMN-cyt parameters frequencies and %Tail DNA values, alcohol intake, age/time of exposure were tested. In the presence of significant interaction terms, main effects were kept in the model even when not significant. All analyses were first done in the total population and thereafter stratified by exposure status. Mean and median comparisons were performed for obtained BMN-cyt parameters frequencies (MN, NBUDS, BNC, KYL, KHC, PYK and CC) and % Tail DNA values (corrected for ENDO III and FPG enzymes) in control and exposed individuals. Correlation between BMN-cyt parameters, %TailDNA and blood inorganic elements content was determined using the non – parametric Spearman correlation analysis. For each comparison a significance level of 5% was assumed.

3. RESULTS

Detailed demographic data of sampled populations are described in Espitia – Pérez et al. [35]. All participants have lived their whole in the exposed area life and several generations have lived in proximity of coal mining areas. The mean age of the exposed group was 35.20±13.34 years (range, 18–62 years), and of the non-exposed control group was 30.69 ± 11.56 years (range, 18–57 years). No significant difference in average age was detected between the non-exposed and exposed groups. Considering the settlement characteristics of exposed populations, where lands and houses are of ancestral nature and generational occupation, exposure time to coal mining residues was estimated by the age of each individual. All participants have lived their whole in the exposed area life and several generations have lived in proximity of coal mining areas.

Frequencies of all BMN-cyt assay parameters presented in Table 1 were significantly higher in environmentally exposed individuals compared to the unexposed group. Because of their low frequencies, analysis of nuclear buds (NBUDS) in exposed and control populations was not included. Considering sex and age as possible major contributors to differences in BMN-cyt assay parameters, we also assessed the influence of both confounding factors and exposure status as predictors of these parameters frequencies. No significant correlation was found between sex or age/exposure time in relation to the parameters tested by the BMN-cyt assay (Figure 1).

Table 2 presents the cross-correlation analysis between the biomarkers of the BMN-cyt assay for all individuals in this study. MN frequency showed a positive correlation with BN cells and CC cells. BN cells were also correlated to PYK and CC cells, while KHC cells had a positive correlation with KYL cells and PYK cells. At the same time, PYK cells were correlated positively with CC cells.

Frequencies of BMN-cyt assay parameters did not differ significantly between sampling areas (Figure 2). Internal control values showed that transportation conditions were optimal and did not influence any of the results obtained. Provincial, San Francisco and Chancleta showed very similar frequencies for most of the parameters assessed, which may be attributed to their close spatial location and possible similar exposure conditions.

Our results also showed an increased level of DNA damage assessed by the % Tail DNA parameter in the alkaline Comet assay in residentially exposed individuals compared with unexposed individuals. Additionally, application of lesion-specific endonucleases showed increased levels of oxidized purines (FPG) and pyrimidines (ENDO III) in lymphocytes of exposed residents. The level of % Tail DNA in the FPG-ENDO III Comet assay was 4.0 and 4.3 times higher in exposed individuals than that of the unexposed control individuals. Similar proportions were obtained for Net FPG and ENDO III sensitive sites.

These significant differences were maintained after adjustment for sex (Table 3). Nevertheless no significant correlation was found between sex or age/exposure time in relation to % Tail DNA tested by the Comet assay.

The FPG and ENDO III treatment showed a slightly, although not significant increase level of oxidized purines compared to oxidized pyrimidines. The same tendency was observed for the FPG and ENDO III sensitive sites. No correlation was found between age/exposure times in relation to DNA damage %Tail DNA in the Comet assay (P-value > 0.05). Similarly to BMN-cyt parameters, %Tail DNA values of alkaline and modified Comet assay neither differ significantly between sampling areas (Figure 3).

Table 1. Poisson regression analysis of BMN-cyt assay parameters for cells collected from the unexposed controls and exposed residents.

Parameters	Unexposed controls			Exposed individuals			P-value
	n	Mean ± SD	Median (25th - 75th)	n	Mean ± SD	Median (25th - 75th)	
DNA damage							
MN frequency							
Women	30	1.36 ± 1.60	1.0 (0.0 - 2.0)	68	4.37 ± 3.91	3.0 (2.0 - 5.5)	NS
Men	11	1.44 ± 1.42	2.0 (0.0 - 3.0)	30	12.83 ± 39.52	4.0 (2.0 - 6.0)	NS
Total population	41	1.38 ± 1.54	1.0 (0.0 - 2.0)	98	**4.79 ± 5.30**	**3.0 (2.0 - 5.2)**	**< 0.001**
Cytokinetic damage							
BN cell No.							
Women	30	0.20 ± 0.66	0.0 (0.0 - 0.0)	68	1.70 ± 2.21	1.0 (0.0 - 3.0)	NS
Men	11	1.22 ± 3.66	0.0 (0.0 - 3.0)	30	1.72 ± 2.08	1.0 (0.0 - 3.0)	NS
Total population	41	0.43 ±1.83	0.0 (0.0 - 0.0)	98	**2.19 ± 3.29**	**1.0 (1.0 - 3.0)**	**< 0.001**
Cell death							
CC cell No.							
Women	30	3.03 ± 8.17	0.0 (0.0 - 3.5)	68	4.45 ± 10.21	0.0 (0.0 - 4.5)	NS
Men	11	0.33 ± 1.00	0.0 (0.0 - 0.0)	30	6.65 ± 11.80	1.0 (0.0 - 11.5)	NS
Total population	41	2.39 ± 7.22	0.0 (0.0 - 3.0)	**98**	**5.23 ± 10.78**	**0.5 (0.0 - 5.0)**	**<0.05**
KHC cell No.							
Women	30	35.47 ± 49.00	21.0 (9.75 - 48.0)	68	56.25 ± 37.82	51.0 (22.0 - 93.50)	NS
Men	11	33.67 ± 30.66	18.0 (16.0 - 59.5)	30	64.72 ± 41.66	71.0 (22.0 - 93.0)	NS
Total population	41	35.05 ± 45.06	21.0 (12.0 - 51.0)	**98**	**59.24 ± 39.18**	**53.5 (21.5 - 93.0)**	**<0.01**
PYK cell No.							
Women	30	11.27 ± 13.25	8.0 (3.0 - 13.0)	68	15.60 ± 16.92	12.0 (4.5 - 21.0)	NS
Men	11	18.22 ± 17.00	12.0 (3.5 - 37.0)	30	32.76 ± 62.34	14.0 (4.5 - 23.0)	NS
Total population	41	12.87 ± 14.27	11.0 (3.0 - 13.0)	**98**	**21.67 ± 39.94**	**12.0 (4.2 - 22.0)**	**<0.05**
KYL cell No.							
Women	30	211.30 ± 145.40	188.5 (123.0 - 241.0)	68	367.50 ± 176.60	366.0 (212.5 - 467.5)	NS
Men	11	176.70 ± 95.85	148.0 (101.0 - 262.0)	30	456.50 ± 281.90	410.0 (255.0 - 622.5)	NS
Total population	41	203.30 ±135.20	174.0 (108.0 - 236.0)	98	**399.00 ± 222.10**	**382.0 (243.0 - 517.3)**	**< 0.001**

Bold for statistical significant difference.
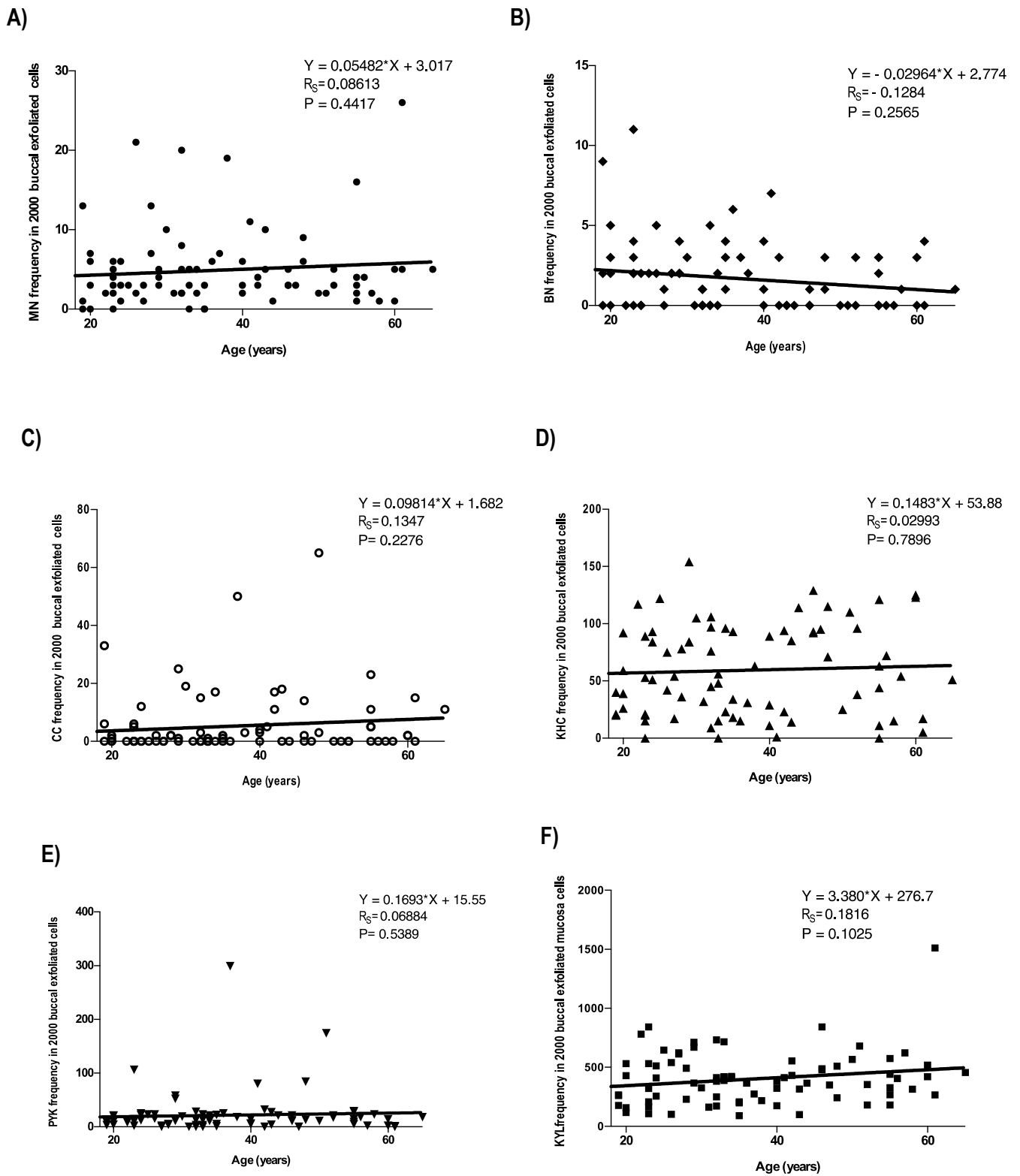
Figure 1. Non-parametric Spearman correlation analysis between BMN-cyt parameters and age in exposed individuals. A) MN frequency; B) BN frequency; C) CC frequency; D) KHC frequency; E) PYK frequency; F) KYL frequency.

Table 2. Matrix correlation between BMN-cyt Assay parameters for exposed individuals.

	Variable	MN	BN	CC	KHC	PYK	KYL
MN	Spearman correlation	1	0.419	0.384	0.042	0.137	0.051
	Significance		**<0.05**	**<0.05**	0.56	0.21	0.64
BN	Spearman correlation		1	0.441	0.072	0.265	0.234
	Significance			**<0.05**	0.52	**<0.05**	0.07
CC	Spearman correlation			1	0.037	0.221	0.024
	Significance				0.73	**<0.05**	0.82
KHC	Spearman correlation				1	0.316	0.267
	Significance					**<0.05**	**<0.05**
PYK	Spearman correlation					1	0.072
	Significance						0.52
KYL	Spearman correlation						1
	Significance						

Bold for statistical significant difference
MN: micronuclei; **BN:** binucleated cells; **CC:** condensed chromatin; **KHC:** karyorrhectic; **PYK:** pyknotic; **KYL:** karyolytic cells
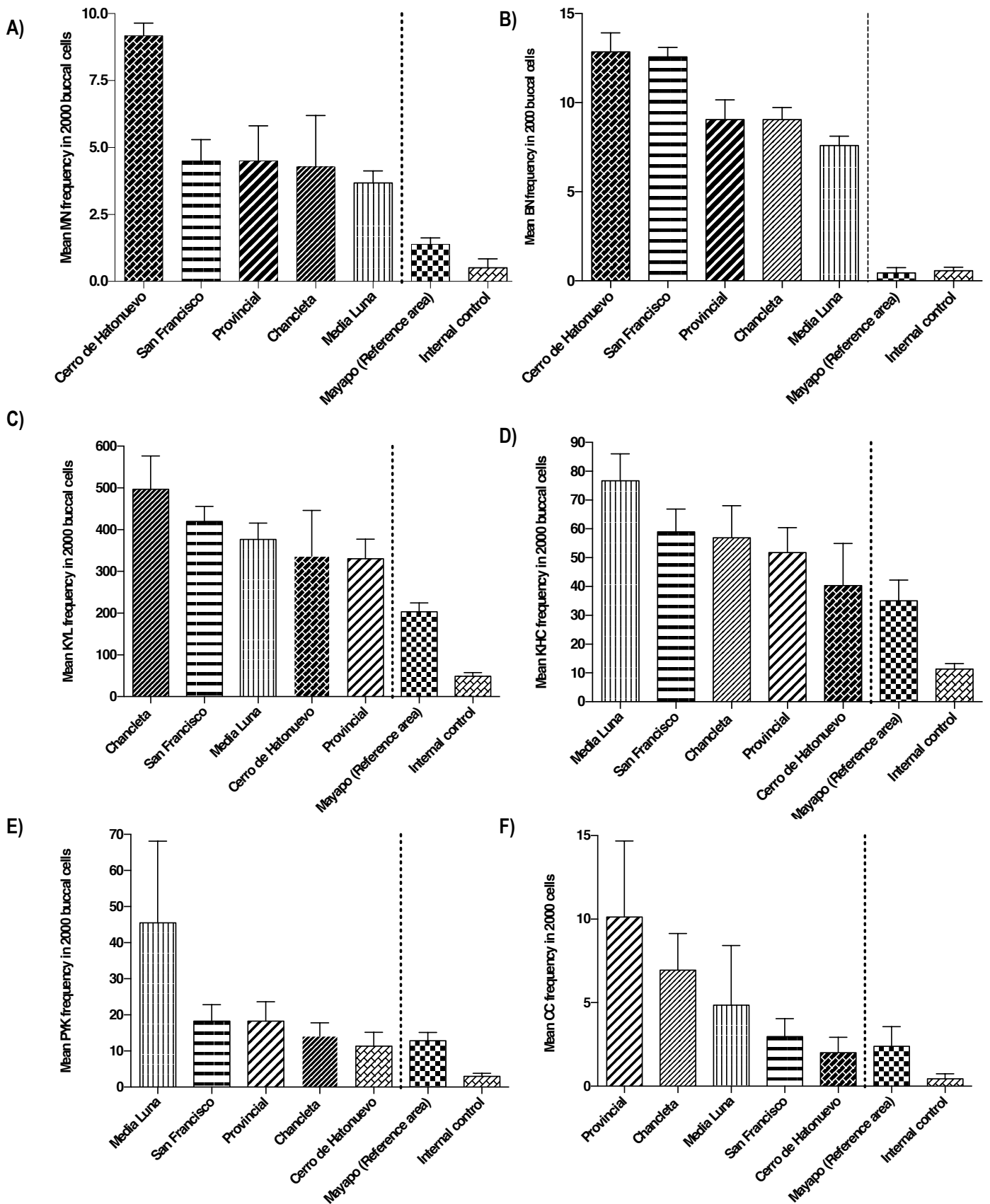
Figure 2. BMN-cyt assay parameters frequency by sampling areas in exposed residents, unexposed and internal controls. A) MN frequency; B) BN frequency; C) KYL frequency; D) KHC frequency; E) PYK frequency; F) CC frequency

Table 3. % Tail DNA values for alkaline, ENDO III and FPG modified comet assay in unexposed controls and exposed residents

Parameter	n	Mean ± SD	Median (25th - 75th)	n	Mean ± SD	Median (25th - 75th)	P-value
		Unexposed controls			Exposed controls		
Alkaline comet Assay							
% Tail DNA							
Women	30	27.54 ± 33.83	11.9 (6.9 – 27.7)	68	52.05 ± 16.95	48.1 (36.5 – 69.2)	**< 0.001**
Men	11	30.39 ± 37.55	10.4 (5.4 – 84.6)	30	47.89 ± 17.19	42.7 (38.1 – 60.7)	**< 0.001**
Total population	41	28.31 ± 34.41	11.6 (6.9 - 38.5)	98	**50.81 ± 17.04**	**46.5 (37.3 - 68.5)**	**< 0.001**
FPG							
% Tail DNA							
Women	30	12.99 ± 0.83	12.8 (12.3 - 13.6)	68	55.87 ± 17.50	56.6 (46.9 - 70.3)	**< 0.001**
Men	11	16.34 ± 5.15	17.23 (10.8 - 21.0)	30	57.87 ± 13.15	59.0 (51.9 - 70.4)	**< 0.001**
Total population	41	14.10 ± 3.14	13.6 (12.2 - 15.8)	98	**56.48 ± 16.25**	**58.1 (47.9 -70.3)**	**< 0.001**
ENDO III							
% Tail DNA							
Women	30	12.22 ± 1.53	12.2 (10.7 - 13.5)	68	52.82 ± 14.09	52.7 (41.5 - 64.0)	**< 0.001**
Men	11	13.28 ± 1.11	13.13 (12.2 - 14.4)	30	59.67 ± 14.22	57.5 (49.3 - 70.4)	**< 0.001**
Total population	41	12.57 ± 1.43	12.6 (11.4 - 13.8)	98	**54.90 ± 14.39**	**54.6 (43.8 - 65.8)**	**< 0.001**
Oxidative damage							
Net FPG sensitive sites	41	6.36 ± 2.69	6.5 (8.8 - 3.7)	98	**24.13 ± 20.97**	**24.4 (9.2 -42.38)**	**< 0.001**
Net ENDOIII sensitive sites		4.83 ± 3.39	5.0 (7.0 - 1.9)		**22.55 ± 20.31**	**22.7 (8.9 - 37.2)**	**< 0.001**

Bold for statistical significant difference.
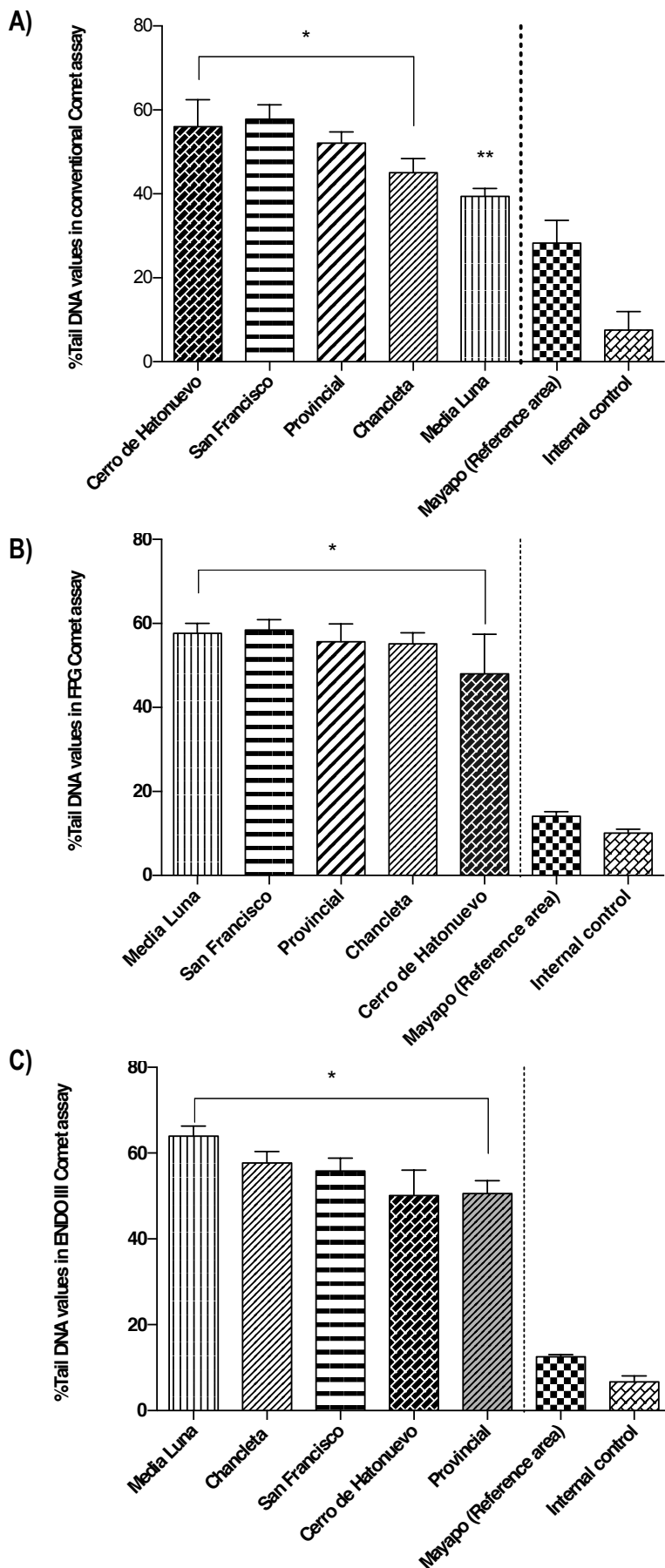
Figure 3. %Tail DNA values by sampling areas in exposed residents, unexposed and internal controls. A) Alkaline comet assay; B) FPG modified comet assay and C) ENDO III modified comet assay. *Significance difference in relation to Mayapo (Reference area); P ≤ 0.01; **Significance difference in relation to Mayapo (Reference area); P ≤ 0.05

MN frequency in buccal cells was also correlated to DNA and oxidative damage (%Tail DNA) in lymphocytes determined with the alkaline (Figure 4A) and FPG modified Comet assay (Figure 4B) respectively. Values of %Tail DNA in modified ENDO III Comet assay were not correlated to MN frequency in buccal cells (Figure 4C).
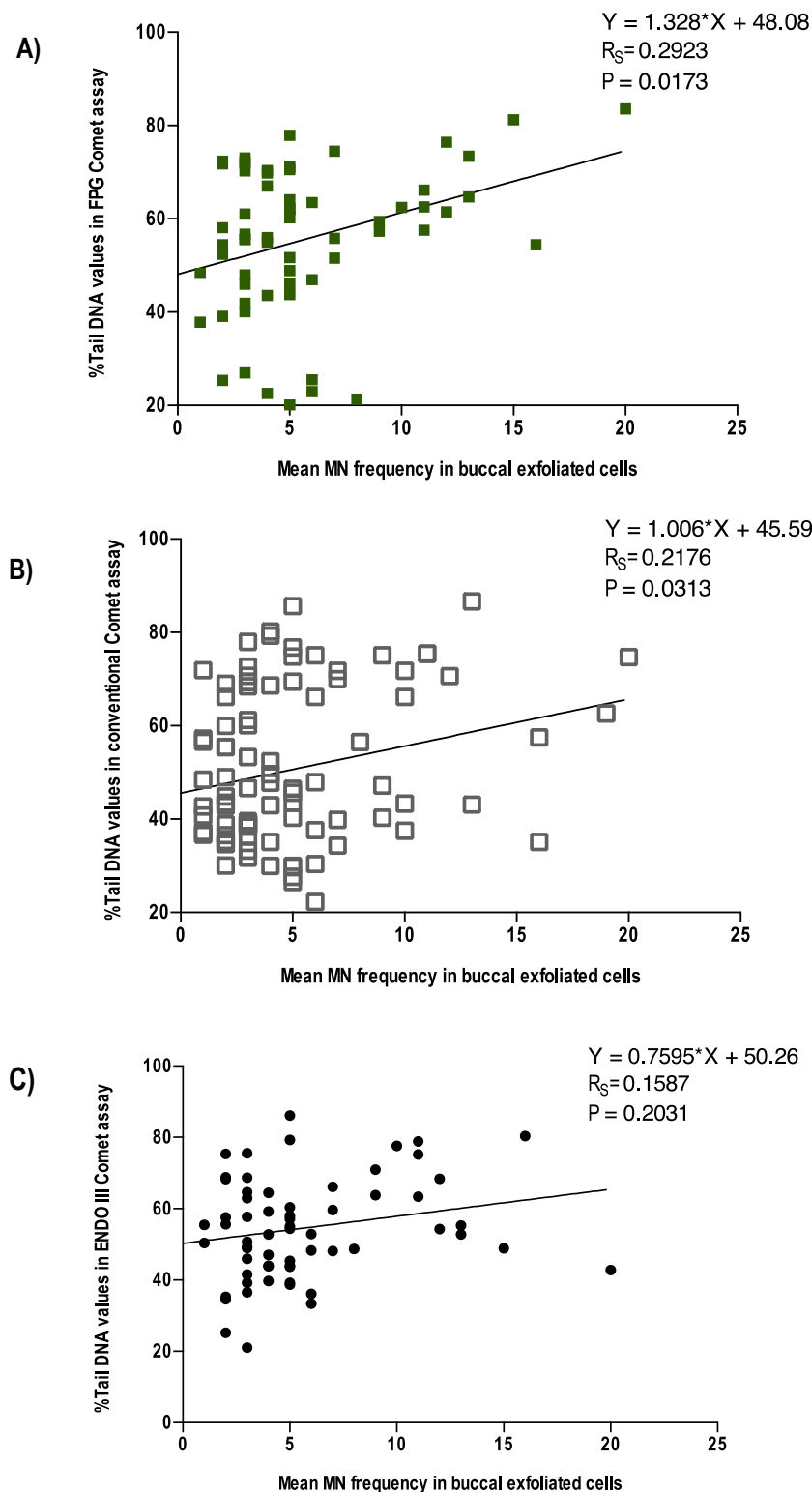
Figure 4. Nonparametric Spearman correlation analysis between MN buccal cells and %Tail DNA in A) alkaline, B) FPG and C) ENDO III modified Comet assay of exposed individuals.

In order to characterize the possible role of inorganic elements content in blood and DNA oxidative damage, chemical analyses of whole blood samples were performed via the PIXE technique (Table 4). Among *trace elements,* the most representative between exposed individuals were chlorine (Cl), sulfur (S), silicon (Si) and potassium (K). Nevertheless, none of the trace elements in blood showed a statistically significant difference between unexposed controls and residentially exposed individuals (P-value > 0.05). No correlation was found between age/exposure time and elements concentrations in blood of exposed individuals (P-value > 0.05).

Other elements like chromium (Cr), nickel (Ni), manganese (Mn) and bromine (Br) showed a statistically significant increase in blood cells of exposed individuals (P-value ≤ 0.05) compared to unexposed controls. These elements concentrations in exposed populations were neither correlated to age/time of exposure (P-value > 0.05) and highly comparable between exposed areas (P-value > 0.05) and exposed men and women (Figure 5).

While elements content in blood samples was not related to any of the BMN-cyt parameters (P ≤ 0.05), %Tail DNA in lymphocytes for alkaline Comet assay was highly correlated with Al, Mn and Br concentrations (Table 5). %Tail DNA measured by the FPG modified Comet assay was also correlated to Mn concentrations, revealing a potential role of this metal in the oxidative damage and free radical generation in blood of individuals exposed to highly enriched PM.

Table 4. Elemental composition of blood samples by PIXE analysis (Mean ± S.D).

Elements	Unexposed individuals n= 41	Total population n= 98	Residentially exposed individuals				
			Elements content by exposed areas				
			Provincial n= 20	San Francisco n= 26	Chancletas n= 21	Cerro de Hatonuevo n= 16	Media Luna n= 15
Na	5949.37 ± 2136.57	5136.67 ± 1988.75	5089.45 ± 2629.65	5927.27 ± 2860.88	4683.69 ± 737.13	5319.40 ± 1129.40	4522.33 ± 802.08
Mg	815.93 ± 787.06	1020.04 ± 978.56	1542.26 ± 1686.69	1295.90 ± 1063.78	519.63 ± 137.44	916.75 ± 605.64	294.45 ± 18.45
Al	601.58 ± 396.99	985.83 ± 274.36	742.80 ± 338.53	1044.53 ± 563.08	937.31 ± 161.25	797.55 ± 3.46	713.90 ± 1.20
Si	6268.64 ± 3910.40	8664.04 ± 16133.17	12595.08 ± 25629.64	14781.07 ± 20474.78	3496.08 ± 3850.28	10205.87 ± 11391.25	2405.46 ± 1868.87
P	2534.50 ± 295.58	2741.15 ± 395.29	2593.18 ± 360.52	2794.54 ± 620.90	2832.31 ± 244.31	2643.20 ± 325.23	2765.00 ± 227.32
S	11418.13 ± 1631.82	11170.98 ± 1636.43	11294.55 ± 174.76	10568.18 ± 2613.76	11258.13 ± 1421.23	11821.00 ± 864.29	11394.33 ± 394.57
Cl	16185.87 ± 1905.38	15045.85 ± 2905.35	14770.18 ± 1633.26	15018.72 ± 4766.04	14691.56 ± 2509.95	16803.80 ± 1510.80	15115.66 ± 1427.34
K	8164.13 ± 474.77	8258.20 ± 1269.08	8394.45 ± 839.07	7811.72 ± 2002.69	8298.07 ± 1117.02	8513.20 ± 578.43	8758.33 ± 942.14
Ti	20.63 ± 15.22	16.04 ± 7.74	20.08 ± 19.46	17.42 ± 6.30	14.72 ± 3.28	10.87 ± 5.12	17.16 ± 1.02
Cr	**3.66 ± 1.27**	**15.36 ± 3.53 ***	17.34 ± 0.88	18.32 ± 3.10	17.00 ± 2.24	16.19 ± 0.43	16.50 ± 1.47
Mn	**8.99 ± 0.90**	**14.91 ± 9.68 ***	15.32 ± 1.31	22.97 ± 20.62	10.08 ± 3.42	12.50 ± 1.06	11.00 ± 2.60
Fe	2542.25 ± 253.25	2632.11 ± 667.54	2587.18 ± 63.54	2624.08 ± 1074.94	2556.80 ± 679.49	2585.60 ± 139.20	2632.00 ± 310.78
Ni	**0.51 ± 0.73**	**14.82 ± 8.09 ***	19.24 ± 3.37	17.70 ± 4.55	19.19 ± 1.43	15.80 ± 1.00	11.78 ± 1.89
Cu	11.42 ± 3.92	17.45 ± 9.85	19.48 ± 9.16	22.48 ± 11.51	13.33 ± 10.81	11.20 ± 0.59	14.42 ± 0.65
Zn	29.30 ± 8.06	34.79 ± 26.11	32.05 ± 3.88	34.38 ± 10.86	29.34 ± 8.79	31.21 ± 4.63	28.54 ± 8.12
Br	**29.00 ± 4.23**	**39.96 ± 12.75 ***	58.11 ± 11.66	42.55 ± 13.03	30.40 ± 5.36	33.63 ± 2.73	35.57 ± 0.52

Results in parts per million (ppm)
Bold* for statistical significant difference; $P \leq 0.05$
SD: standard deviation

85
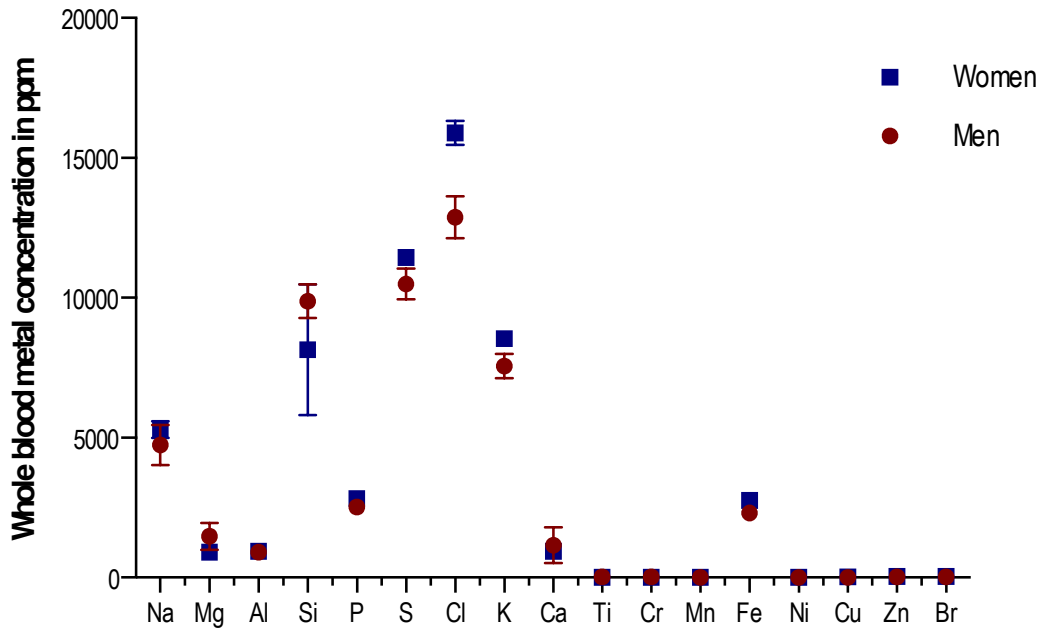
Figure 5. Elemental composition of blood samples of exposed men and women.

Table 5. Spearman correlation between blood elemental content in residentially exposed individuals, MN frequencies in buccal cells and % Tail DNA values in alkaline and FPG – ENDO III modified Comet assay.

Elements	Variable	MN	Alkaline % Tail DNA	FPG % Tail DNA	ENDO III % Tail DNA
Na	Spearman correlation	0.34	0.14	-0.37	-0.65
	Significance	0.66	0.28	0.37	0.12
Mg	Spearman correlation	0.52	0.65	0.14	-0.31
	Significance	0.09	0.67	0.21	0.34
Al	Spearman correlation	0.75	0.82	0.65	0.37
	Significance	0.55	**<0.05**	0.10	0.22
Si	Spearman correlation	0.37	0.77	0.25	-0.20
	Significance	0.34	0.51	0.34	0.78
P	Spearman correlation	0.31	0.37	0.54	0.71
	Significance	0.22	0.33	0.35	0.21
S	Spearman correlation	0.46	-0.37	-0.71	-0.60
	Significance	0.11	0.07	0.09	0.06
Cl	Spearman correlation	0.69	-0.08	-0.42	-0.42
	Significance	0.54	0.55	0.70	0.12
K	Spearman correlation	0.02	0.02	-0.02	0.20
	Significance	0.60	0.77	0.23	0.55
Ca	Spearman correlation	0.20	0.08	-0.25	-0.60
	Significance	0.21	0.29	0.21	0.20
Ti	Spearman correlation	0.23	-0.37	-0.02	-0.25
	Significance	0.76	0.41	0.11	0.54
Cr	Spearman correlation	0.23	0.54	0.71	0.77
	Significance	0.12	0.78	0.66	0.30
Mn	Spearman correlation	0.41	0.90	0.90	0.70
	Significance	0.34	**<0.05**	**<0.05**	0.21
Fe	Spearman correlation	0.46	0.37	0.71	0.54
	Significance	0.20	0.12	0.44	0.42
Ni	Spearman correlation	0.66	0.34	0.34	0.02
	Significance	0.66	0.67	0.23	0.89
Cu	Spearman correlation	0.52	0.42	0.77	0.54
	Significance	0.98	0.45	0.09	0.31
Zn	Spearman correlation	0.75	0.77	0.25	-0.14
	Significance	0.55	0.22	0.65	0.55
Br	Spearman correlation	0.34	0.88	0.37	0.14
	Significance	0.32	**<0.05**	0.33	0.46

Bold for statistical significant difference.

4. DISCUSION

In the past decades, many studies highlighted consistently that exposure to coal mining residues are related to increased DNA damage in lymphocytes and buccal cells, however most results arise from occupationally exposed populations where exposure concentrations are generally considered higher than environmental concentrations. This particular trend has caused a scarcity of data examining the impact of these industrial operations to cytogenetic endpoints frequency and cancer rates of potentially exposed surrounding populations (non-occupationally exposed) [36]. Accordingly, the present study investigated the extent of DNA damage in individuals with environmental exposure to open – pit coal mining area using the BMN-cyt assay in exfoliated buccal cells and Comet assay parameter (%Tail DNA) in lymphocytes. We also evaluated oxidative DNA damage using the FPG- ENDO III modified comet assay.

In our results, frequencies of all BMN-cyt assay parameters and DNA damage assessed by the % Tail DNA parameter in the alkaline Comet assay were significantly higher in environmentally exposed individuals compared to the unexposed group. This observation is in agreement with several earlier studies that reported a statistically significant elevation of these parameters in occupationally exposed individuals inside coal mining areas [3, 37].

Most chemical compounds found in complex mixtures generated in open cast mines enter the body through inhalation and ingestion [3]. Thus, epithelial cells located in upper aerodigestive tract are the first barrier for the inhalation or ingestion route; these cells are highly proliferative [38] and are capable of metabolizing proximate carcinogens to reactive products [39] like ROS, capable of binding to cellular macromolecules and DNA [40].

As sex and age are considered the most important demographic variables affecting the MN index, we also assessed the influence of both variables as confounding factors and exposure status as predictors of BMN-cyt parameters frequencies. Similarly to occupationally exposed populations previously studied in the same area [3], no correlation was found between sex or age/exposure time in relation to the parameters tested by the BMN-cyt assay. Contrarily to MN frequencies in lymphocytes, where increased MN frequencies with age and sex are an unequivocally documented fact [41] in BMN-cyt parameters and particularly in MN frequencies the situation is less clear. According to Holland et al. [38], although many studies report the age and sex of the study subjects, only a fraction of these studies have been able to establish a statistically significant effect by gender or by age.

In buccal cells, alcohol intake may be also a risk factor for increased frequency of oral anomalies and a causal association has been established between alcohol consumption and cancers of the oral cavity, pharynx and larynx [42]. In our study there was no statistically influence of alcohol consumption in any of the parameters evaluated. Considering that most of exposed and unexposed individuals declared to be non-alcohol consumers and only 10.20% were moderate consumers [35], this result could be expectable. .

Results from the cross-correlation analysis between the biomarkers of the BMN-cyt assay for exposed individuals showed a positive correlation between MN frequency with BN cells and CC cells. Rohr et al. [43] evaluated individuals occupationally exposed to coal and observed similar correlations. Considering models of possible inter-relationships between the various cell types observed in the BMN-cyt assay [44], our results may suggest that MN and BN derived directly from basal cells as a results of a genotoxic insult in the basal cells, while CC cells may originate secondarily neither from BN or MN cells as part of the elimination process of damaged cells via the apoptotic process. In line with

these observations, BN cells were also correlated to PYK and CC cells. Tolbert *et al.* [45] suggested that apoptosis is a surveillance mechanism, which eliminates buccal epithelial cells with genetic damage.

Even when BN cells were initially related to failed cytokinesis following the last nuclear division [20], more recently studies suggest that formation of BN cell seems to be related to cytokinesis failure either due to defects in microfilament ring formation or cell cycle arrest due to aneuploidy or telomere dysfunction [46]. According to Shi and King [47] chromosomal non-disjunction occurs with a higher frequency in binucleated cells that fail to complete cytokinesis, rather than in cells that have completed cytokinesis; thus chromosome nondisjunction would be tightly coupled to regulation of cytokinesis. These data would suggest that cytokinesis might be inhibited in cells that spontaneously mis-segregate chromosomes during the preceding mitosis. This mechanism is thought to be a cytokinesis checkpoint for aneuploid binucleated cells [47]. Accordingly, observed higher frequency of BN cells in buccal mucosa could also be indicative that complex mixtures generated during mining operations may have major aneugenic effects. This assumption is supported by previous results [35] on a possible predominant aneugenic effect detected in lymphocytes of the same environmentally exposed populations; however, further evidence (chromosome specific centromeric probes) would be required to verify the accuracy of the model and also the aneugenic origin of MN in buccal cells of these populations.

On the other hand, KHC cells had a positive correlation with KYL cells and PYK cells. These biomarkers of cell death have been reported to be positively correlated in previous reports [48]. Positive correlation between condensed chromatin and karyorrhectic cells, suggests that the latter are either derived directly from basal cells, or may originate secondarily from condensed chromatin cells. Similar results have been described in previous reports [49]. Because of their low frequencies, analysis of NBUDS in exposed and control populations was not included. These results differ from earlier findings in occupational exposed populations were NBUDS frequencies were significantly higher in exposed individuals compared to control unexposed and showed a major average rate during cells analysis [3, 48].

ROS are believed to play a major role in primary DNA damage caused by occupational or environmental exposure to coal mining residues [50]. Our results confirm that DNA damage observed in individuals living in proximity of coal mines is also caused mainly by oxidative damage of DNA bases with a slight increase of oxidized purines compared to oxidized pyrimidines. Using a different approach, Rohr et al. [2], identified an increased superoxide dismutase (SOD) activity in individuals occupationally exposed to coal, while other authors showed an increased formation of 8-OHdG in peripheral blood lymphocytes and altered antioxidant capacities in the serum and red blood cells of coal miners [51].

Additionally to primary DNA damage as a consequence of ROS generation in blood, other cells as well as interstitial components may be affected due to translocation of particles, mediators and ROS. Several cells types may act as targets for these mediators including fibroblasts, epithelial cells and endothelial cells [52].

In accordance with this observation, our results showed that MN frequency in buccal cells was correlated to %Tail DNA in the alkaline and FPG modified Comet assay. This correlation suggests that in fact, systemic genotoxic effects within the blood stream may also impact on and be detectable in other cell types like buccal cells; oxidative damage, particularly purines oxidation may play an important role in the DNA damage observed in the buccal epithelium. Similar results were obtained by Katarkar et al. [53] in oral cancer patients. Considering that MN in buccal cells are expressed in the dividing basal cells which are in the deepest layers of the buccal mucosa is most likely that they are influenced to similar extents by the toxins and nutrients in the blood stream [20]. Thus, MN induction in buccal cells resulting from systemic exposure to coal mining residues throughout bloodstream should be considered. MN in buccal exfoliated cells

was also correlated to MN frequencies in lymphocytes previously analyzed from the same sample groups (Figure 6). A linear correlation between MN in lymphocytes and buccal exfoliated cells was also described by Ceppi et al. [54] as well as by Rohr et al. [43] and León-Mejía et al. [3] for individuals occupationally exposed to coal. In brief, these results indicate that genetic factors and exposure affecting MN frequency and primary DNA damage in lymphocytes, may possibly also apply to some degree to buccal cells, including the association of MN with cancer risk [53].

$$Y = 0.5866*X + 4.214$$
$$R_S = 0.2003$$
$$P = 0.0198$$
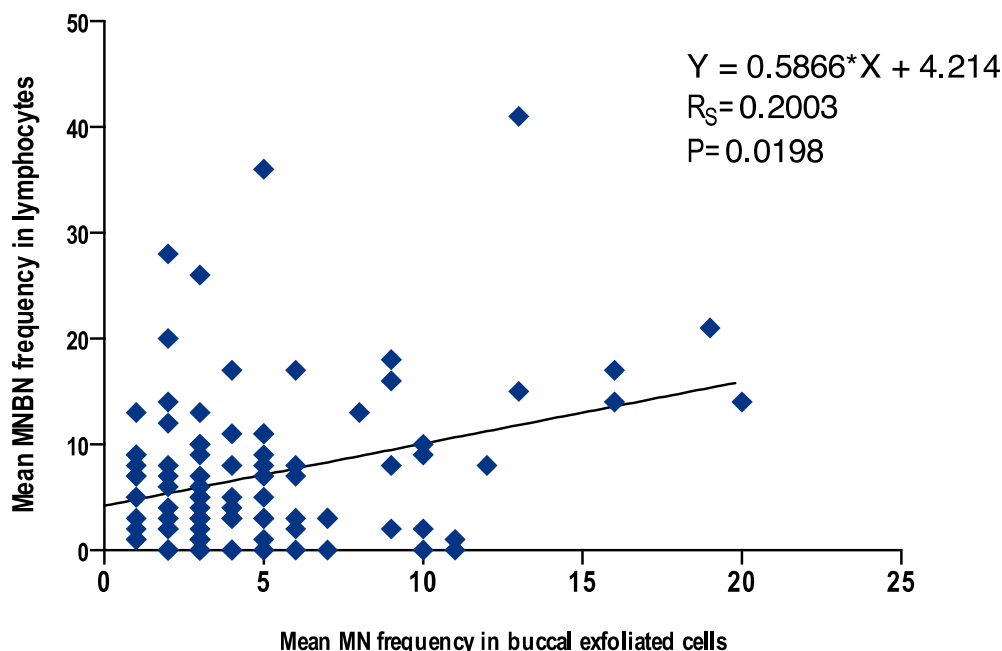
Figure 6. Nonparametric Spearman correlation analysis between MN in buccal cells and MN frequency in lymphocytes.

Populations around coal mine areas are usually exposed to complex mixtures of organic and inorganic compounds [55]. Metal exposure in particular is potentially capable of generate oxidative damage through generation of ROS [56]. Inorganic elements enter the human body either by ingestion or inhalation and are subsequently transferred to the vascular compartment (blood) [57]. These elements derived from the circulation get deposited in the tissues by various mechanisms [58] and can be related to DNA damage detected. Consequently, we decided to characterize inorganic elements content in blood samples of exposed and unexposed individuals in order to established a possible relation with oxidative damage, BMN-cyt and Comet assay parameters.

Among *trace* elements, the most representative in blood samples of exposed individuals were Cl, S, Si, Fe, Zn, Br and Ti. Only Cr, Ni, Mn and Br showed a statistically significant increase (P-value ≤ 0.05) compared to unexposed controls. Most of these elements had been found in blood samples in previous studies in occupationally exposed populations [3] and wild mammals [59, 60] of coal mining areas of Colombia and Brazil. In a previous report [35], some of these were found enriched around the coal mining area indicating that area release to atmosphere from anthropogenic sources.

It has been reported that elements such as Cr, Mn, Ni and Br are essential nutrients that are required for various biochemical and physiological functions, however is also known that levels of these elements in blood also rise following

90

exposure [61]. Most of these are usually present in coal mining ambience and consequently might be important source for metal - induced diseases or cancer risk related to this exposure [62]. The carcinogenic potency of Cr and Ni is well known. Hexavalent chromium and divalent nickel show positive effects in several genotoxic tests [63]. Hexavalent chromium can easily pass the cell membranes, and it is reduced inside the cells to its trivalent form. Trivalent chromium, intermediates like Cr (V) and Cr (IV) and radicals are suspected to react with DNA and cause DNA damage [64]. It is also known that divalent nickel ions are able to induce DNA damage in various cell systems [65]. The nickel-induced DNA damage is probably induced by nickel catalyzed oxygen radicals and not by direct involvement of nickel compounds [66].

On the other hand, Br is one of the most ubiquitous elements in the biosphere [67]. Only limited evidence supports the essentiality of Br to man, and the chemical forms in which bromine exists in human tissues are not well known [68]. Moreover, some compounds that contain Br in their chemical structure like potassium bromate are considered as possibly carcinogen for human [69]. In particular, exposure of mammalian cells to bromate (BrO_3^-) generates oxidative DNA modifications, in particular 8-oxoG. The damaging mechanism is quite unique, since glutathione, which is protective against most oxidants and alkylating agents, mediates a metabolic activation, while BrO_3^- itself does not react directly with DNA [70]. Nevertheless, none of these elements were correlated to BMN-cyt parameters. Similar results were obtained for Benedetti et al. [71] when analyzed the influence of metals presents in the oral mucosa of pesticides exposed populations. We could assume that after inhalation inorganic elements are transferred to blood [57] where are involved in ROS generation via mechanisms such as Haber-Weiss and Fenton-type reactions [72]. It is possible that other compounds present on complex mixtures from coal mining activities, like PHA may play a major role in DNA damage in oral mucosa. Especially inside the oral cavity, some genes related to PAH metabolism like *CYP1A1* are highly expressed, suggesting an *in situ* activation of this xenobiotic compounds [73]. Several studies have described a significant association of *CYP1A1* variants with genetic damage in buccal mucosa cells suggesting that these polymorphisms could modulate the effects of PAH exposure in this epithelial tissues [74]. However, researches about intracorporal metabolic distribution of some metals in the major organs are still insufficient [75].

Additionally, considering that environmental exposition to coal mining residues consists of exposure to complex mixtures of inorganic elements, is difficult to assign the observed effects to one particular compound [76]. Synergism or antagonism would occur between all kinds of elements in different organisms. A specific element can affect the absorption of another or change its distribution in the body. Studies *in vitro or in vivo* have shown that Pb, Cd, Cr and Ni even in low concentrations from $PM_{2.5}$ *in vivo* or *in vitro* can exhibit genetic toxicity through producing primary DNA or chromosomal damage [77]. In line with this assumption, primary DNA damage determined by alkaline Comet assay in lymphocytes was highly correlated with Al, Mn and Br concentrations, while FPG oxidative damage was correlated only to Mn concentrations in blood samples of exposed individuals. Manganese is a common component of coal ashes after combustion processes [78] and constitutes an important contaminant of subterranean and superficial waters due to its leaching from coal fly ash [79].

Al and Mn play important biological roles as essential trace elements in enzymes and are usually present inside the body [68], however higher concentrations and inorganic elements imbalance can be related to several diseases [80]. Possible mechanisms through which Al can exert its genotoxic and carcinogenic properties have not yet been clearly established. The fraction of Al that reaches the systemic circulation and is not excreted in the urine rapidly, accumulates in peripheral tissues, where it is strongly bound [81]. The intracellular distribution of Al among organelles varies but

lysosomes, mitochondria and nuclei are reported to be the major sites of binding [82]. Exposure to Al is responsible for the promotion of iron-induced generation of ROS and lipid peroxidation [83, 84]. There is evidence that Al also induces chromosomal aberrations, micronuclei and sister-chromatid exchanges in human lymphocytes [85]. Lankoff et al. [86] using the alkaline Comet assay, demonstrated that Al induces DNA damage in a dose-dependent manner. Cells treated with Al also showed a decreased repair capacity indicating that Al would also inhibit DNA repair. Similarly, relatively high doses of Mn can disrupt DNA integrity and DNA replication [87]. Previous studies have correlated Mn concentrations in blood with 8OHdG levels in urine of workers exposed to $PM_{2.5}$ [88]. Evidence suggest that Mn alone disturbs mitochondrial respiration and inhibits the antioxidant system [89], thus consequently straining cell ability to combat oxidative stress.

CONCLUSIONS

Our results confirm that DNA damage observed in individuals exposed to coal mines residues is caused mainly by oxidative damage of DNA bases with a slight increase of oxidized purines compared to oxidized pyrimidines. This primary DNA damage could be related to high concentration of some elements like Cr, Mn, Ni and Br in blood samples of exposed individuals and particularly to Mn concentrations, significantly correlated to purine oxidative DNA damage. MN frequency and other parameters of cell death and cytokinetic damage in buccal cells were also increased in exposed populations. MN frequencies were correlated to %Tail DNA in the alkaline and FPG modified Comet assay, suggesting that oxidative damage, particularly purine oxidation may play an important role in the DNA damage. Considering that MN in buccal cells are correlated with MN in lymphocytes for individual exposed in the environment contaminated with coal, MN in buccal cells resulting from systemic exposure to coal mining residues throughout bloodstream could be considered in environmental biomonitoring.

REFERENCES

1. Leon-Mejia, G., et al., *Assessment of DNA damage in coal open-cast mining workers using the cytokinesis-blocked micronucleus test and the comet assay.* Sci Total Environ, 2011. **409**(4): p. 686-91.

2. Rohr, P., et al., *Genetic and oxidative damage of peripheral blood lymphocytes in workers with occupational exposure to coal.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2013. **758**(1–2): p. 23-28.

3. León-Mejía, G., et al., *Genetic damage in coal miners evaluated by buccal micronucleus cytome assay.* Ecotoxicology and Environmental Safety, 2014. **107**: p. 133-139.

4. Espitia-Pérez, L., et al., *Polymorphisms in metabolism and repair genes affects DNA damage caused by open-cast coal mining exposure.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2016. **808**: p. 38-51.

5. Morabia, A., et al., *Lung cancer and occupation: results of a multicentre case-control study.* British journal of industrial medicine, 1992. **49**(10): p. 721-727.

6. Jenkins, W.D., et al., *Population Cancer Risks Associated with Coal Mining: A Systematic Review.* PLoS ONE, 2013. **8**(8): p. e71312.

7. Pone, J.D.N., et al., *The spontaneous combustion of coal and its by-products in the Witbank and Sasolburg coalfields of South Africa.* International Journal of Coal Geology, 2007. **72**(2): p. 124-140.

8. Waalkes, M.P., T.P. Coogan, and R.A. Barter, *Toxicological principles of metal carcinogenesis with special emphasis on cadmium.* Crit Rev Toxicol, 1992. **22**(3-4): p. 175-201.

9. Bhuiyan, M.A.H., et al., *Heavy metal pollution of coal mine-affected agricultural soils in the northern part of Bangladesh.* Journal of Hazardous Materials, 2010. **173**(1–3): p. 384-392.

10. Zereini, F., et al., *Concentration and distribution of heavy metals in urban airborne particulate matter in Frankfurt am Main, Germany.* Environ Sci Technol, 2005. **39**(9): p. 2983-9.

11. Sun, L., et al., *Contribution of heavy metals to toxicity of coal combustion related fine particulate matter (PM2.5) in Caenorhabditis elegans with wild-type or susceptible genetic background.* Chemosphere, 2016. **144**: p. 2392-2400.

12. Celik, M., et al., *Cytogenetic damage in workers from a coal-fired power plant.* Mutat. Res., Genet. Toxicol. Environ. Mutagen., 2007. **627**(2): p. 158-163.

13. El-Sergany, M.M. and M.F. El-Sharkawy, *Heavy Metal Contamination of Airborne Dust in the Environment of Two Main Cities in the Eastern Province of Saudi Arabia.* Journal of King Abdulaziz University, 2011. **22**(1): p. 135.

14. Pless-Mulloli, T., et al., *Living near opencast coal mining sites and children's respiratory health.* Occupational and Environmental Medicine, 2000. **57**(3): p. 145-151.

15. Shashikala, R., et al., *Role of micronucleus in oral exfoliative cytology.* Journal of Pharmacy & Bioallied Sciences, 2015. **7**(Suppl 2): p. S409-S413.

16. Janot, F., et al., *Principal xenobiotic-metabolizing enzyme systems in human head and neck squamous cell carcinoma.* Carcinogenesis, 1993. **14**(7): p. 1279-83.

17. Avezov, K., A.Z. Reznick, and D. Aizenbud, *Oxidative stress in the oral cavity: sources and pathological outcomes.* Respir Physiol Neurobiol, 2015. **209**: p. 91-4.

18.   Bolognesi, C., et al., *Buccal micronucleus cytome assay: results of an intra- and inter-laboratory scoring comparison.* Mutagenesis, 2015.

19.   Bolognesi, C. and M. Fenech, *Micronucleus assay in human cells: lymphocytes and buccal cells.* Methods Mol Biol, 2013. **1044**: p. 191-207.

20.   Thomas, P., et al., *Buccal micronucleus cytome assay.* Nat Protoc, 2009. **4**(6): p. 825-37.

21.   Collins, A.R., *The comet assay for DNA damage and repair.* Molecular Biotechnology, 2004. **26**(3): p. 249-261.

22.   Valavanidis, A., T. Vlachogianni, and C. Fiotakis, *8-hydroxy-2'-deoxyguanosine (8-OHdG): a critical biomarker of oxidative stress and carcinogenesis.* Journal of environmental science and health Part C, 2009. **27**(2): p. 120-139.

23.   Kushwaha, S., et al., *Alkaline, Endo III and FPG modified comet assay as biomarkers for the detection of oxidative DNA damage in rats with experimentally induced diabetes.* Mutat Res, 2011. **726**(2): p. 242-50.

24.   Zhao, J., et al., *[Endonuclease modified comet assay for oxidative DNA damage induced by detection of genetic toxicants].* Zhonghua Yu Fang Yi Xue Za Zhi, 2014. **48**(3): p. 208-12.

25.   Gedik, C.M. and A. Collins, *Establishing the background level of base oxidation in human lymphocyte DNA: results of an interlaboratory validation study.* The FASEB Journal, 2004.

26.   Leon, G., et al., *Genotoxic effects in wild rodents (Rattus rattus and Mus musculus) in an open coal mining area.* Mutat Res, 2007. **630**(1-2): p. 42-9.

27.   Carbajal-López, Y., et al., *Biomonitoring of agricultural workers exposed to pesticide mixtures in Guerrero state, Mexico, with comet assay and micronucleus test.* Environmental Science and Pollution Research, 2016. **23**(3): p. 2513-2520.

28.   Singh, N.P., et al., *A simple technique for quantitation of low levels of DNA damage in individual cells.* Experimental Cell Research, 1988. **175**(1): p. 184-191.

29.   Azqueta, A., et al., *A comparative performance test of standard, medium- and high-throughput comet assays.* Toxicology in Vitro, 2013. **27**(2): p. 768-773.

30.   Gutzkow, K.B., et al., *High-throughput comet assay using 96 minigels.* Mutagenesis, 2013. **28**(3): p. 333-40.

31.   Speit, G. and A. Hartmann, *The Comet Assay*, in *DNA Repair Protocols: Mammalian Systems*, D.S. Henderson, Editor. 2006, Humana Press: Totowa, NJ. p. 275-286.

32.   Collins, A., M. Dusinská, and A. Horská, *Detection of alkylation damage in human lymphocyte DNA with the comet assay.* Acta Biochim. Pol, 2001. **48**: p. 611-614.

33.   Collins, A.R., *Investigating oxidative DNA damage and its repair using the comet assay.* Mutation Research/Reviews in Mutation Research, 2009. **681**(1): p. 24-32.

34.   Campbell, J.L., et al., *The Guelph PIXE software package IV.* Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms, 2010. **268**(20): p. 3356-3363.

35.   Espitia-Pérez, L., et al., *Cytogenettic instability in populations with residential proximity to Open-pit coal mine in Northern Colombia in relation to PM10 and PM2.5 levels (Unpublished work),* 2016. p. 40.

36.   Nersesyan, A., et al., *Use of the lymphocyte cytokinesis-block micronucleus assay in occupational biomonitoring of genome damage caused by in vivo exposure to chemical genotoxins: Past, present and future.* Mutation Research/Reviews in Mutation Research, 2016.

37.   Klimkina, I., *Assessment of mutagenic health effects in coal mine workers.* Genome, 2013. **18**: p. 31.

38.   Holland, N., et al., *The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: the HUMN project perspective on current status and knowledge gaps.* Mutat Res, 2008. **659**(1-2): p. 93-108.

39.   Tanaka, T. and R. Ishigamori, *Understanding carcinogenesis for fighting oral cancer.* J Oncol, 2011. **2011**: p. 603740.

40.   Sram, R.J., et al., *Chromosomal aberrations in environmentally exposed population in relation to metabolic and DNA repair genes polymorphisms.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2007. **620**(1-2): p. 22-33.

41.   Bonassi, S., et al., *HUman MicroNucleus project: international database comparison for results with the cytokinesis-block micronucleus assay in human lymphocytes: I. Effect of laboratory protocol, scoring criteria, and host factors on the frequency of micronuclei.* Environ Mol Mutagen, 2001. **37**(1): p. 31-45.

42.   Boffetta, P. and M. Hashibe, *Alcohol and cancer.* The Lancet Oncology. **7**(2): p. 149-156.

43.   Rohr, P., et al., *Evaluation of genetic damage in open-cast coal mine workers using the buccal micronucleus cytome assay.* Environ Mol Mutagen, 2013. **54**(1): p. 65-71.

44.   Thomas, P., et al., *The buccal cytome and micronucleus frequency is substantially altered in Down's syndrome and normal ageing compared to young healthy controls.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2008. **638**(1): p. 37-47.

45.   Tolbert, P.E., C.M. Shy, and J.W. Allen, *Micronuclei and other nuclear anomalies in buccal smears: methods development.* Mutat Res, 1992. **271**(1): p. 69-77.

46.   Bonassi, S., et al., *The HUman MicroNucleus project on eXfoLiated buccal cells (HUMN XL): the role of life-style, host factors, occupational exposures, health status, and assay protocol.* Mutation Research/Reviews in Mutation Research, 2011. **728**(3): p. 88-97.

47.   Shi, Q. and R.W. King, *Chromosome nondisjunction yields tetraploid rather than aneuploid cells in human cell lines.* Nature, 2005. **437**(7061): p. 1038-42.

48.   Rohr, P., et al., *Genetic and oxidative damage of peripheral blood lymphocytes in workers with occupational exposure to coal.* Mutat Res, 2013. **758**(1-2): p. 23-8.

49.   Yadav AS, J.S., *Buccal Micronucleus Cytome Assay- A Biomarker of Genotoxicity.* J Mol Biomark Diagn **6**(236).

50.   Melody, S.M. and F.H. Johnston, *Coal mine fires and human health: What do we know?* International Journal of Coal Geology, 2015. **152, Part B**: p. 1-14.

51.   Schins, R.P., P.A. Schilderman, and P.J. Borm, *Oxidative DNA damage in peripheral blood lymphocytes of coal workers.* International archives of occupational and environmental health, 1995. **67**(3): p. 153-157.

52.   Schins, R.P. and P.J. Borm, *Mechanisms and mediators in coal dust induced toxicity: a review.* Annals of Occupational Hygiene, 1999. **43**(1): p. 7-33.

53.   Katarkar, A., et al., *Comparative evaluation of genotoxicity by micronucleus assay in the buccal mucosa over comet assay in peripheral blood in oral precancer and cancer patients.* Mutagenesis, 2014. **29**(5): p. 325-334.

54.   Ceppi, M., et al., *Human population studies with the exfoliated buccal micronucleus assay: statistical and epidemiological issues.* Mutat Res, 2010. **705**(1): p. 11-9.

55. Gautam, S., et al., *Characterization of PM2.5 generated from opencast coal mining operations: A case study of Sonepur Bazari Opencast Project of India.* Environmental Technology & Innovation, 2016. **6**: p. 1-10.

56. Valko, M., et al., *Free radicals, metals and antioxidants in oxidative stress-induced cancer.* Chem Biol Interact, 2006. **160**(1): p. 1-40.

57. Schweinsberg, F. and L. Von Karsa, *Heavy metal concentrations in humans.* Comparative Biochemistry and Physiology Part C: Comparative Pharmacology, 1990. **95**(2): p. 117-123.

58. Bridges, C.C. and R.K. Zalups, *Molecular and ionic mimicry and the transport of toxic metals.* Toxicology and applied pharmacology, 2005. **204**(3): p. 274-308.

59. José Zocche, J., et al., *Heavy metals and DNA damage in blood cells of insectivore bats in coal mining areas of Catarinense coal basin, Brazil.* Environmental Research, 2010. **110**(7): p. 684-691.

60. Guerrero-Castilla, A., J. Olivero-Verbel, and J. Marrugo-Negrete, *Heavy metals in wild house mice from coal-mining areas of Colombia and expression of genes related to oxidative stress, DNA damage and exposure to metals.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2014. **762**: p. 24-29.

61. WHO/FAO/IAEA, *Trace elements in human nutrition and health.* World Health Organization, Geneva, 1996.

62. Avila Junior, S., et al., *Occupational airborne contamination in south Brazil: 1. Oxidative stress detected in the blood of coal miners.* Ecotoxicology, 2009. **18**(8): p. 1150-7.

63. IARC, *IARC Monographs on the Valuation of the Carcinogenic Risks of Chemicals to Humans: Chromium, Nickel and Welding.* IARC, Lyon, 1990. **49**: p. 447–525.

64. Fang, Z., et al., *Genotoxicity of Tri- and Hexavalent Chromium Compounds <italic>In Vivo</italic> and Their Modes of Action on DNA Damage <italic>In Vitro</italic>.* PLoS ONE, 2014. **9**(8): p. e103194.

65. Barceloux, D.G. and D. Barceloux, *Nickel.* Journal of Toxicology: Clinical Toxicology, 1999. **37**(2): p. 239-258.

66. Dally, H. and A. Hartwig, *Induction and repair inhibition of oxidative DNA damage by nickel (II) and cadmium (II) in mammalian cells.* Carcinogenesis, 1997. **18**(5): p. 1021-1026.

67. Milman, N., et al., *Elements in normal and cirrhotic human liver tissue II. Potassium, sulphur, chlorine and bromine measured by X- ray fluorescence spectrometry.* Liver, 1987. **7**(4): p. 206-210.

68. Bogden, J.D. and L.M. Klevay, *Clinical nutrition of the essential trace elements and minerals: the guide for health professionals.* 2000: Humana Press.

69. Hayes, A.W., *Principles and Methods of Toxicology, Fifth Edition.* 2007: Taylor & Francis.

70. Ballmaier, D. and B. Epe, *DNA damage by bromate: Mechanism and consequences.* Toxicology, 2006. **221**(2–3): p. 166-171.

71. Benedetti, D., et al., *Genetic damage in soybean workers exposed to pesticides: Evaluation with the comet and buccal micronucleus cytome assays.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2013. **752**(1–2): p. 28-33.

72. Knaapen, A.M., et al., *Inhaled particles and lung cancer. Part A: Mechanisms.* International Journal of Cancer, 2004. **109**(6): p. 799-809.

73. Sam, S.S., et al., *Gene-environment interactions associated with CYP1A1 MspI and GST polymorphisms and the risk of upper aerodigestive tract cancers in an Indian population.* J Cancer Res Clin Oncol, 2010. **136**(6): p. 945-51.

74. Kvitko, et al., *Cytochrome P4501A1 polymorphisms in South American Indians.* Hum Biol, 2000. **72**(6): p. 1039-43.

75. Li, Q., et al., *The preferential accumulation of heavy metals in different tissues following frequent respiratory exposure to PM(2.5) in rats.* Scientific Reports, 2015. **5**: p. 16936.

76. Annangi, B., et al., *Biomonitoring of humans exposed to arsenic, chromium, nickel, vanadium, and complex mixtures of metals by using the micronucleus test in lymphocytes.* Mutation Research/Reviews in Mutation Research.

77. de Kok, T.M., et al., *Genotoxicity and physicochemical characteristics of traffic-related ambient particulate matter.* Environ Mol Mutagen, 2005. **46**(2): p. 71-80.

78. Prasad, B. and K.K. Mondal, *Environmental impact of manganese due to its leaching from coal fly ash.* J Environ Sci Eng, 2009. **51**(1): p. 27-32.

79. Chakraborty, R. and A. Mukherjee, *Mutagenicity and genotoxicity of coal fly ash water leachate.* Ecotoxicol Environ Saf, 2009. **72**(3): p. 838-42.

80. Oubrahim, H., P.B. Chock, and E.R. Stadtman, *Manganese(II) Induces Apoptotic Cell Death in NIH3T3 Cells via a Caspase-12-dependent Pathway.* Journal of Biological Chemistry, 2002. **277**(23): p. 20135-20138.

81. Ward, R.J., Y. Zhang, and R.R. Crichton, *Aluminium toxicity and iron homeostasis.* Journal of inorganic biochemistry, 2001. **87**(1): p. 9-14.

82. Dobson, C.B., et al., *Location of aluminium and gallium in human neuroblastoma cells treated with metal-chelating agent complexes.* Toxicology and applied pharmacology, 1998. **152**(1): p. 145-152.

83. Zatta, P., et al., *Aluminium (III) as a promoter of cellular oxidation.* Coordination Chemistry Reviews, 2002. **228**(2): p. 271-284.

84. Yousef, M.I., *Aluminium-induced changes in hemato-biochemical parameters, lipid peroxidation and enzyme activities of male rabbits: protective role of ascorbic acid.* Toxicology, 2004. **199**(1): p. 47-57.

85. Banasik, A., et al., *Aluminum-induced micronuclei and apoptosis in human peripheral-blood lymphocytes treated during different phases of the cell cycle.* Environmental toxicology, 2005. **20**(4): p. 402-406.

86. Lankoff, A., et al., *A comet assay study reveals that aluminium induces DNA damage and inhibits the repair of radiation-induced lesions in human peripheral blood lymphocytes.* Toxicol Lett, 2006. **161**(1): p. 27-36.

87. Beckman, R.A., A.S. Mildvan, and L.A. Loeb, *On the fidelity of DNA replication: manganese mutagenesis in vitro.* Biochemistry, 1985. **24**(21): p. 5810-5817.

88. Kim, J.Y., et al., *Urinary 8-hydroxy-2'-deoxyguanosine as a biomarker of oxidative DNA damage in workers exposed to fine particulates.* Environ Health Perspect, 2004. **112**(6): p. 666-71.

89. Brouillet, E.P., et al., *Manganese injection into the rat striatum produces excitotoxic lesions by impairing energy metabolism.* Exp Neurol, 1993. **120**(1): p. 89-94.

V. CAPÍTULO III

GEOSPATIAL ANALYSIS OF RESIDENTIAL PROXIMITY TO OPEN-PIT COAL MINES IN RELATION TO PARTICULATE MATTER CONCENTRATION, ELEMENTAL ENRICHMENT FACTORS AND MICRONUCLEUS FREQUENCIES
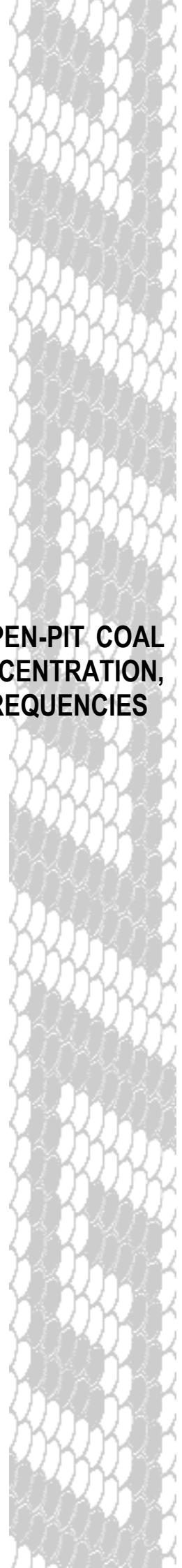
GEOSPATIAL ANALYSIS OF RESIDENTIAL PROXIMITY TO OPEN-PIT COAL MINES IN RELATION TO PARTICULATE MATTER CONCENTRATION, ELEMENTAL ENRICHMENT FACTORS AND MICRONUCLEUS FREQUENCIES

Lyda Espitia-Pérez [a,b], Marcia Arteaga [a,c], José Salvador Soto [a,c], Pedro Espitia-Pérez [a], Hugo Brango [d], Shirley Salcedo-Arteaga [a], Juliana da Silva [e], João A. P. Henriques [b]

[a] Facultad de Ciencias de la Salud, Laboratorio de Investigación Biomédica y Biología Molecular, Universidad del Sinú, Montería, Córdoba, Colombia.

[b] Departamento de Biofísica, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

[c] Departamento de Geografía y Medio Ambiente, Universidad de Córdoba

[d] Instituto de Matemática e Estatística, Universidade de São Paulo, São Paulo, Brazil

[e] Laboratório de Genética Toxicológica, Universidade Luterana do Brasil, ULBRA, Canoas, RS, Brazil.

For correspondence with authors:
*E-mail address: espitiaperez@gmail.com (L. Espitia– Pérez)
**E-mail address: pegas@cbiot.ufrgs.br (JAP. Henriques)

ABSTRACT

Occupational exposure to coal mining residues has been associated with increase of DNA damage, but there is a relative dearth in the literature exploring environmental exposition. We used a Geographic Information Systems (GIS) approach and Inverse Distance Weighting (IDW) methods to perform spatial and statistical analyses to explore whether exposure to $PM_{2.5}$ and PM_{10} pollution, and additional factors, including inorganic elements enrichment of particulate matter (PM), contribute to cytogenetic damage in residents living in proximity to open-pit coal mining areas. Our results showed a spatial relationship between exposure to elevated concentrations of $PM_{2.5}$ and PM_{10} and micronucleus frequencies in binucleated (MNBN) and mononucleated (MNMONO) cells. Active pits, disposal and storage areas were identified as the main emission sources of combustion elements associated with spontaneous coal seams fires. Mining activities were also correlated to increased concentrations of highly enriched elements like S, Cu and Cr in the atmosphere, corroborating its role in the inorganic elements pollution of areas in proximity to coal mines. Elements enriched in the $PM_{2.5}$ fraction contributed partially to the increase of MNBN, but seems to be more related to increased MNMONO frequencies and DNA damage accumulated *in vivo.* The combined use of GIS and IDW methods could represent an important tool for monitoring potential cancer risk associated to dynamically distributed variables like PM. Future studies are needed to further elucidate the precision of the interpolation models obtained around coal mining areas using individual-level exposures and cancer outcomes.

1. INTRODUCTION

In coal mines, after coal is removed from the ground, it is typically transported off-site via large trucks that travel on public roads generating large amounts of particulate matter (PM). These coal trucks frequently travel through communities located in steeply sided valleys, or hollows, where homes are situated very close to the narrow roads. Some communities experience up to hundreds of truck trips a day being exposed to high concentrations of PM [1].

PM generated during coal mining is described as PM_{10} (particles with aerodynamic diameters smaller than 10 μm) and $PM_{2.5}$ (particles with diameters smaller than 2.5 μm). PM_{10} often referred to as the coarse fraction, is mostly produced by mechanical processes. By contrast, $PM_{2.5}$ is mostly derived from combustion sources, such as automobiles, trucks, and other vehicle exhaust, as well as from stationary combustion sources [2]. These toxic elements incorporated with atmospheric $PM_{2.5}$ and PM_{10} may enter the body through inhalation [3]. PM_{10} is potentially hazardous to health, due to the complex composition of this fraction and easily mediated deposition in bronchi and lungs [4]. $PM_{2.5}$ fraction represents the highly-inhalable fraction of PM directly affecting the alveolar lung region [5]. Besides the size component, PM can contains high concentrations of toxic trace elements, such as Cr, Cd, Ti, Mn, Ni, Pb, As and Zn [6].

Health related studies indicate a strong association of airborne PM generated around coal mines with adverse impacts such as increased cardiovascular disease, pneumoconiosis, cancer, and neurotoxic effects [7]. Evidence suggests that PM exerts its genotoxic and carcinogenic effects through generation of DNA damage and chromosomal instability [8, 9]. A powerful tool for measurement of chromosomal abnormalities is the cytokinesis-block micronucleus cytome (CBMNCyt) assay. Micronuclei (MN) frequency in peripheral blood lymphocytes can be predictive of cancer risk, and an increased MN formation is associated with early events in carcinogenesis [18, 19]. Several studies have used MN frequencies to assess the potential risk of populations exposed to coal mining residues at workplace [8, 10, 11]. However, there is a scarcity of data examining the impact of these industrial operations in potentially exposed surrounding populations.

In a recent study, our research group demonstrated elevated MNBN and MNMONO frequencies in populations living in proximity to coal mining operations of northern Colombia [12]. Statistical correlation analysis suggested a strong association between MN frequencies, PM concentrations and elemental composition (particularly with some enriched elements like Cr, Cu, Zn and S). Accurate ambient PM concentration measurements are critical for epidemiological studies on chronic human exposure, but remain a challenge for large geographical areas [13]. The open pit coal-mining region of northern Colombia is located in the semiarid southern part of the Department of La Guajira between the municipalities of Albania, Barrancas, and Hatonuevo. The extension of the mining area covers an area of 69.000 hectares (ha) and includes a 150 Km railroad and a port facility in the Atlantic ocean [14]. Air quality systems established to monitor PM levels around the coal mining area, are located in zones under direct influence of prevailing winds and are limited to the south of open-pits, roads, industrial activities and disposal sites within the mining concession [15]. Thus, most inhabitants around the mining zone may live in unmonitored areas or monitor-sparse locations. PM distribution may be related to geomorphological characteristics and wind conditions in sampled areas, however these factors are usually not considered in common statistical analysis applied to exposure risk studies in human populations. Therefore, in this study we decided to explore the spatial relation between $PM_{2.5}$, PM_{10} concentrations, MNBN, MNMONO frequencies and enrichment factors (EF) values for chemical elements presents in the PM of areas around the open pit coal mining operations. This approach

allows the evaluation of the proximity effect of coal mining on MN frequencies and the identification of potential distribution patterns that can reveal a potential risk in unmonitored populations. To accomplish this task we used several spatial interpolation and geostatistics methodologies. Spatial interpolation is a method or mathematical function that estimates the values at locations where no measured values are available. Spatial interpolation assumes the attribute data are continuous over space and spatially dependent, indicating that values closer together are more likely to be similar than the values farther apart. The goal of spatial interpolation is to create a surface that is intended to best represent empirical reality thus the method selected must be assessed for accuracy [16]. Inverse Distance Weighting (IDW), Ordinary Kriging (OK) and Radial Basis Functions (RBF) are three well-known spatial interpolation techniques commonly used for characterizing the spatial variability and interpolating between sampled points and generating the prediction maps [17]. IDW and its modifications are the most often applied deterministic interpolation method [18]. This technique is based on the assumption that nearby values contribute more to the interpolated values than distant observations. In other words, for this method the influence of a known data point is inversely related to the distance from the unknown location that is being estimated. According to several authors, IDW method can be used as the first choice to predict PM concentrations in regions where not enough measurement data are available [19, 20].

2. METHODOLOGY

2.1 Study area and sampling collection

As previously described in Espitia-Pérez et al. [12], the open pit coal-mining region object of the present study is located in the semiarid southern part of the Department of La Guajira in northeast Colombia between the municipalities of Albania, Barrancas, and Hatonuevo. Sampling sites were located inside the area of direct influence of the mining operations and around the port facility (Media Luna). The study focus in villages located at the flat area around the coal mining corridor where high levels of PM_{10} have been repeatedly reported by local air quality network of the regional environmental agency (CORPOGUAJIRA). [10]. The unexposed (Control) area was located in Mayapo municipality, also constituted of indigenous Wayúu settlements and Afro-Colombian populations, situated on the Caribbean sea coast without any influence of coal mining operations and located 57.12 kilometers from the city of Riohacha. A total number of 139 healthy individuals were enrolled to this study: 98 with permanent residential proximity to open – pit coal mining operations and 41 permanent residents of the control area. Detailed demographic characteristics of the studied population can be also found in a previous study in Espitia – Pérez et al. [12]

2.2 Cytokinesis-block micronucleus (CBMN) assay.

CBMN assay was carried out according to previously described methodology [12]. For each blood sample, 2000 binucleated cells (BN) (i.e., 1000 from each of the two slides prepared from the duplicate cultures) were scored for the presence of MN (MNBN). Additionally, MN frequency in 1.000 mononucleated cells (MONO) was evaluated to determine MNMONO frequency.[21].

2.3 Sampling of atmospheric particulate matter

For $PM_{2.5}$ collection, a total of twenty-five aerosol samples was collected using 46.2 mm PTFE filters (Tisch Environmental Inc., Cincinnati, OH, USA). . Samplings were performed during a 5-month period using a PQ200 FRM air sampler (BGI MesaLabs, Butler, NJ, USA) equipped with $PM_{2.5}$ inlet and WINS impactor (according to CFR 40 part 50, appendix L). Meteorological data and PM_{10} samples were supplied by Hi-Vol Air Quality Measurement Systems of CORPOGUAJIRA (Riohacha, La Guajira, COL).

2.4 Elemental composition and Enrichment factor (EF) analysis

Using data from element concentrations previously obtained by PIXE analysis [12], we performed an Enrichment Factor (EF) analysis in order to identify those elements with anthropogenic source [22]. Iron (Fe) was employed as reference [23, 24] and concentrations of elements in the upper continental crust were taken from Wedepohl [25].

2.5 Spatial analysis

To explore the relationship between the variables described above *(PM2.5, PM10, MNBN, MNMONO and EF values)*, the statistical analysis was performed by the software package SPSS version 16.0. to make the calculated data visualization spatially, geo-statistical method was employed by using ArcGIS Software Version 10.3.1 (ESRI, Redlands, CA, USA) and previous data obtained during the sampling process for each variable [12].

Direct influence area of mining project, open-pits and disposal sites were georeferenced from previous reports [26]. Sampling sites (Rancherias) and surrounding coal mining areas were digitalized as reference points and used as a

base layer for variable processing, analysis and spatial representation during the whole study. Digitalized map shows currently active open-pits, which include: Comuneros Pit, Oreganal Pit and NAM (New Areas of Mining) Pits (actually open-pits composed by La Puente and Tabaco Pits) (Figure 1).

Geographic information system (GIS) was used to spatially analyze the distribution, enrichment level, and induced MNBN and MNMONO frequencies of $PM_{2.5}$ and PM_{10} around the coal mining area. Interpolation layers of each of the variables were obtained by IDW. The IDW method was applied to map the spatial characteristics of pollutants based on the ArcGIS 10.3.1 software.

IDW employs a specific number of nearest points that are then weighted according to their distance from the point being interpolated applying the algorithm:

$$z_{est}^{j} = \frac{\Sigma\left(\dfrac{Z_i}{(h_{ij}+s)^2}\right)}{\Sigma\left(\dfrac{1}{(h_{ij}+s)^2}\right)}$$

Where z_{est}^{j}: estimated value for location

j, Z_i: measured sample values at point i

h_{ij}: distance between z_{est}^{j} and Z_i

S: smoothing factor (0)

IDW interpolation method was selected considering sampling sites density and topography conditions of the studied areas [27] where coastal zones and areas with proximity to open-cast pits share almost exclusively a flat geomorphology, except south, when it becomes slightly undulated in the vicinity of Cerro de Hatonuevo, with the presence of small hill tops (Figure 2) [28].

2.5.1 Overlay analysis

Spatial relationships between the different data sets were studied creating new layers from the joint analysis of MN frequencies, $PM_{2.5}$, PM_{10} and EF values. To enable the quantitative comparison of the different maps, they were all produced at the same spatial scale and on the same grid. Contour intervals obtained using the IDW method were used to established a three-rank categorization for each variable (high, medium and low) represented using a color scale, where lower values were represented by light colors and higher values using red. Using weighted overlay tools, output raster overlay maps were obtained for MN frequencies/$PM_{2.5}$ – PM_{10} and MN frequencies/EF values input rasters.
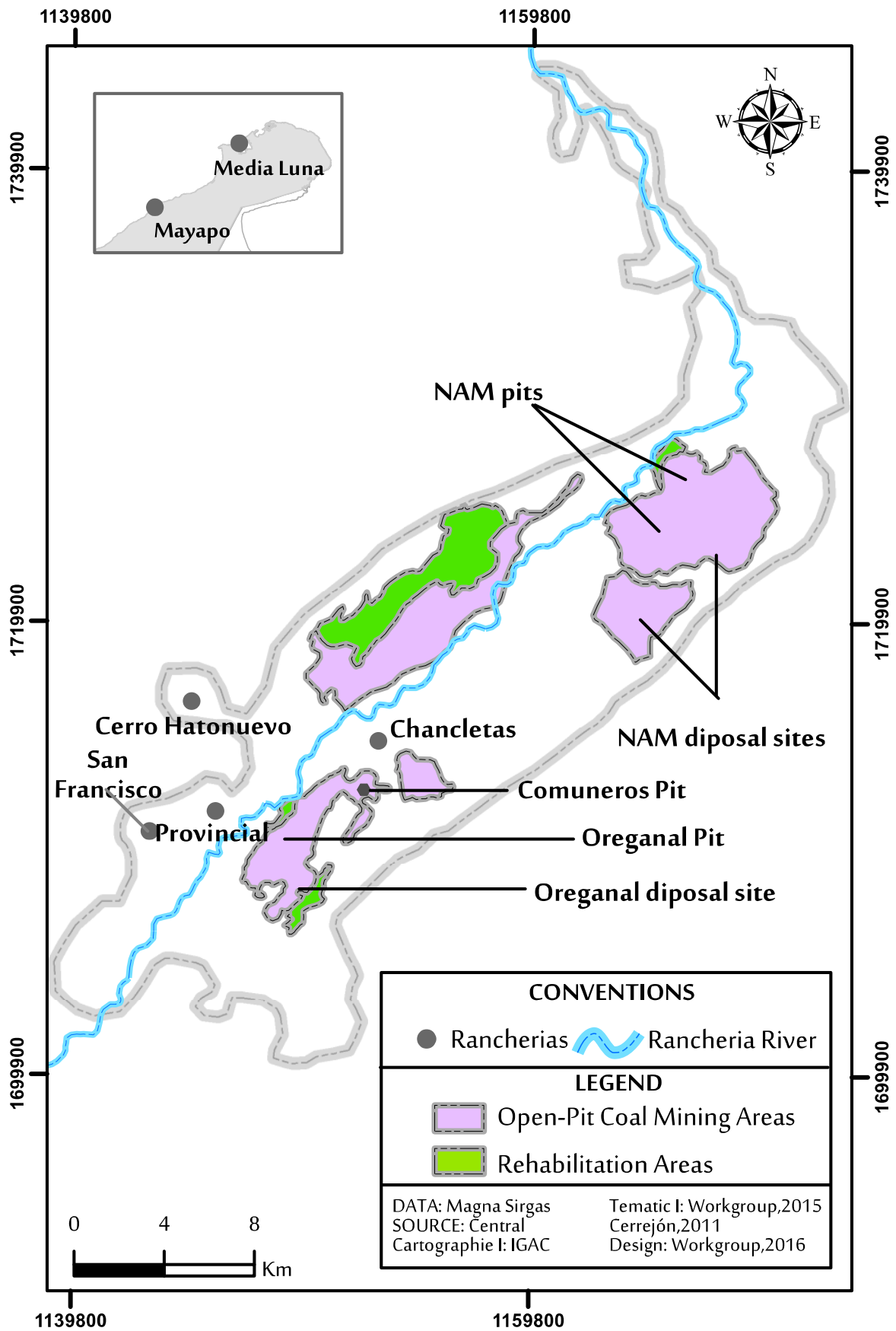
Figure 1. Sampling site locations, open-pits, disposal sites and recovery areas in opencast coal mine.

3. RESULTS

To identify possible emission sources in the sampling sites, all maps show spatial location of pits, disposal and storing areas inside the mining areas. Coal storage piles (A1, A2 and A3) were identified from a previous report [29].

Spatial analysis of wind characteristics (Speed and direction) and geomorphology conditions in sampling areas in proximity of the open pit coal mining operations is showed in Figure 2.

Figure 3 represents the spatial relation between MNBN frequencies and $PM_{2.5}$ concentrations around the coal mining area. Geographical distribution of both variables confirmed previous observations about the high correlation between MNBN and $PM_{2.5}$ concentrations. Higher spatial correlations between these variables were established for Chancletas, Provincial and San Francisco, located between disposal areas and active pits. A moderate correlation was observed around the north and south of the mining area.

Interestingly, even when in previous reports of MNMONO frequencies showed a negative correlation with PM_{10} concentrations, the geographical distribution showed a high positive correlation of both variables at the south of the mining area (Figure 4). A moderate low correlation was observed around rehabilitated, pits and disposal sites located north of the mining area, were lower values of both variables were registered.

Based on the EF values of studied elements and IDW interpolation method, their spatial distribution maps are shown in Figure 5. Elements typically found around coal mining areas like S, Cu and Zn showed highly enriched patterns around areas with proximity to pits and disposal sites, with a decrease of enrichment factor with distance from the mining operations.

Sulfur was highly enriched around the whole mining area, showing higher values around Chancletas, Provincial and San Francisco, while Cerro de Hatonuevo and Media Luna showed lower levels of S but higher concentrations of Cl and Cr. Analysis of the enrichment pattern for Cr also showed higher values at north and south of the mining region, particularly around pits and disposal sites. Similarly, Cl showed a poor enrichment pattern around the coal mining area and higher values at north of the mining area.

Spatial correlation between MNBN and MNMONO frequencies and enriched elements in mining areas are show in Figure 5 and Figure 6 respectively. Compared to MNBN frequencies, MNMONO were more correlated to enrichment factor values of S, Cr and Cl. Instead, MNBN/MONO showed no spatial correlation with Zn enrichment values. Areas with enriched values of Cu were proportionately correlated with MNBN and MNMONO frequencies.
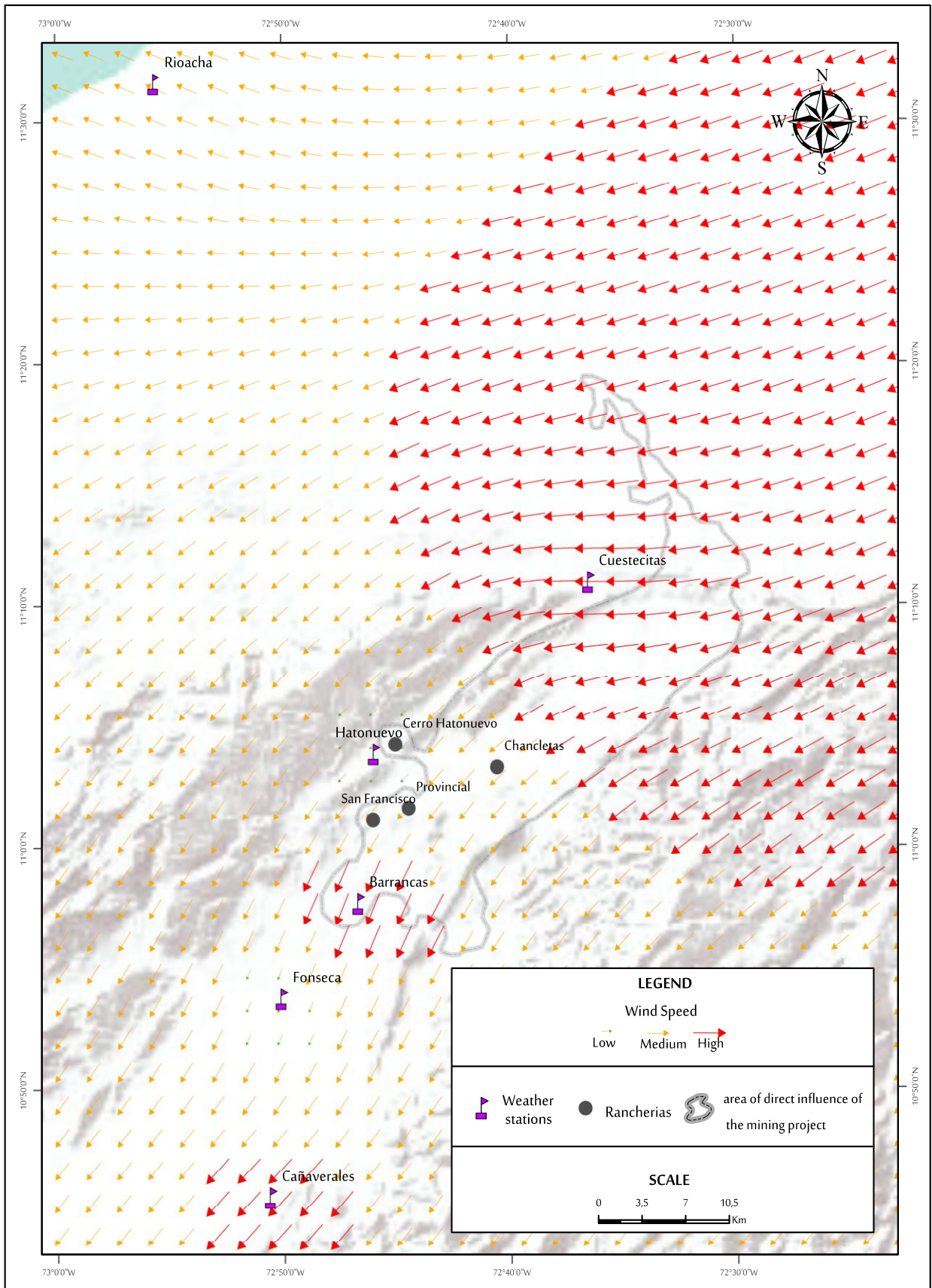
Figure 2. Spatial analysis of wind characteristics (Speed and direction) and geomorphology conditions in sampling areas in proximity of the open pit coal mining operations
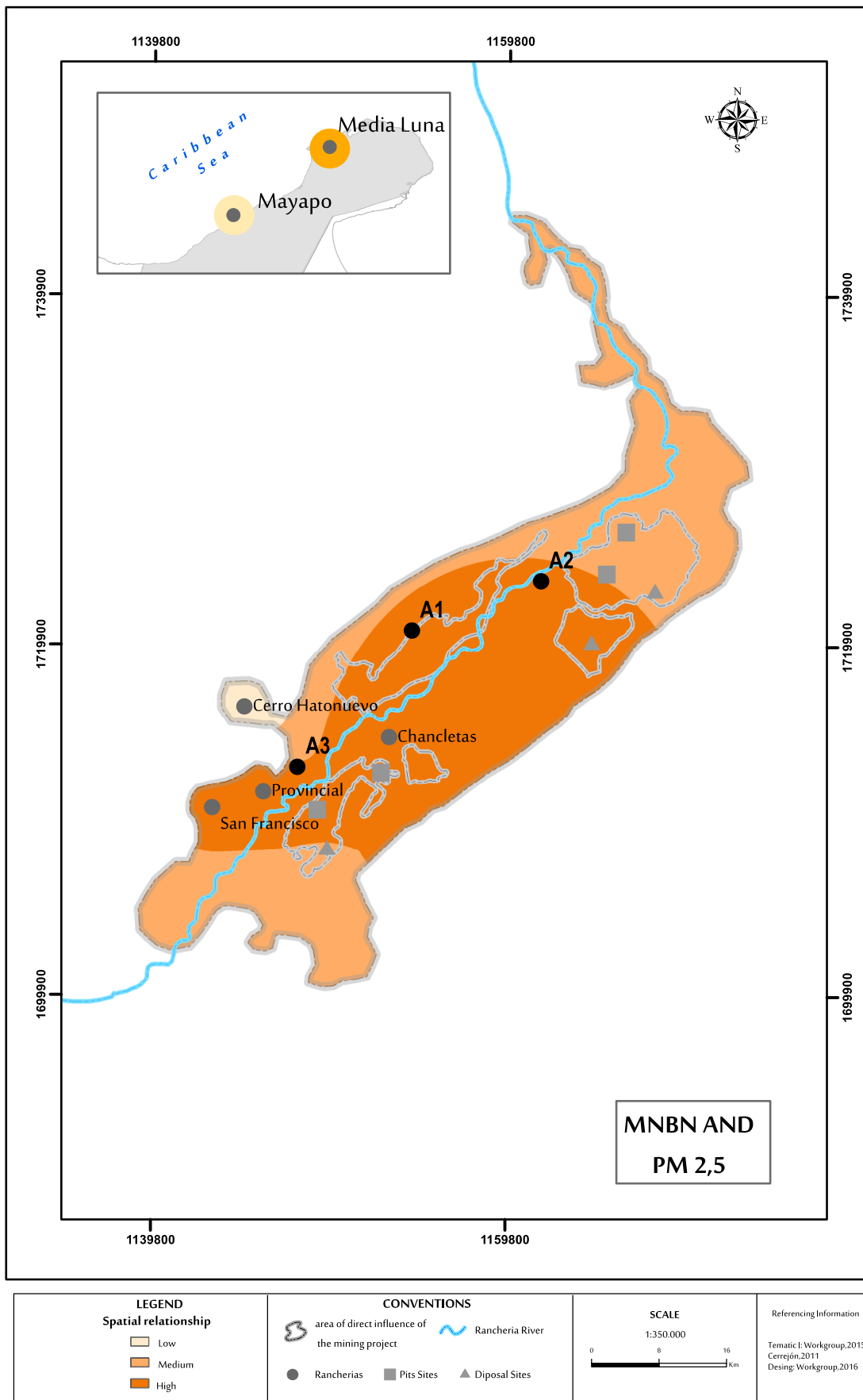
Figure 3. Overlay analysis of MNBN frequency and PM$_{2,5}$ concentrations in areas located near the direct influence area of the open pit coal mining operations
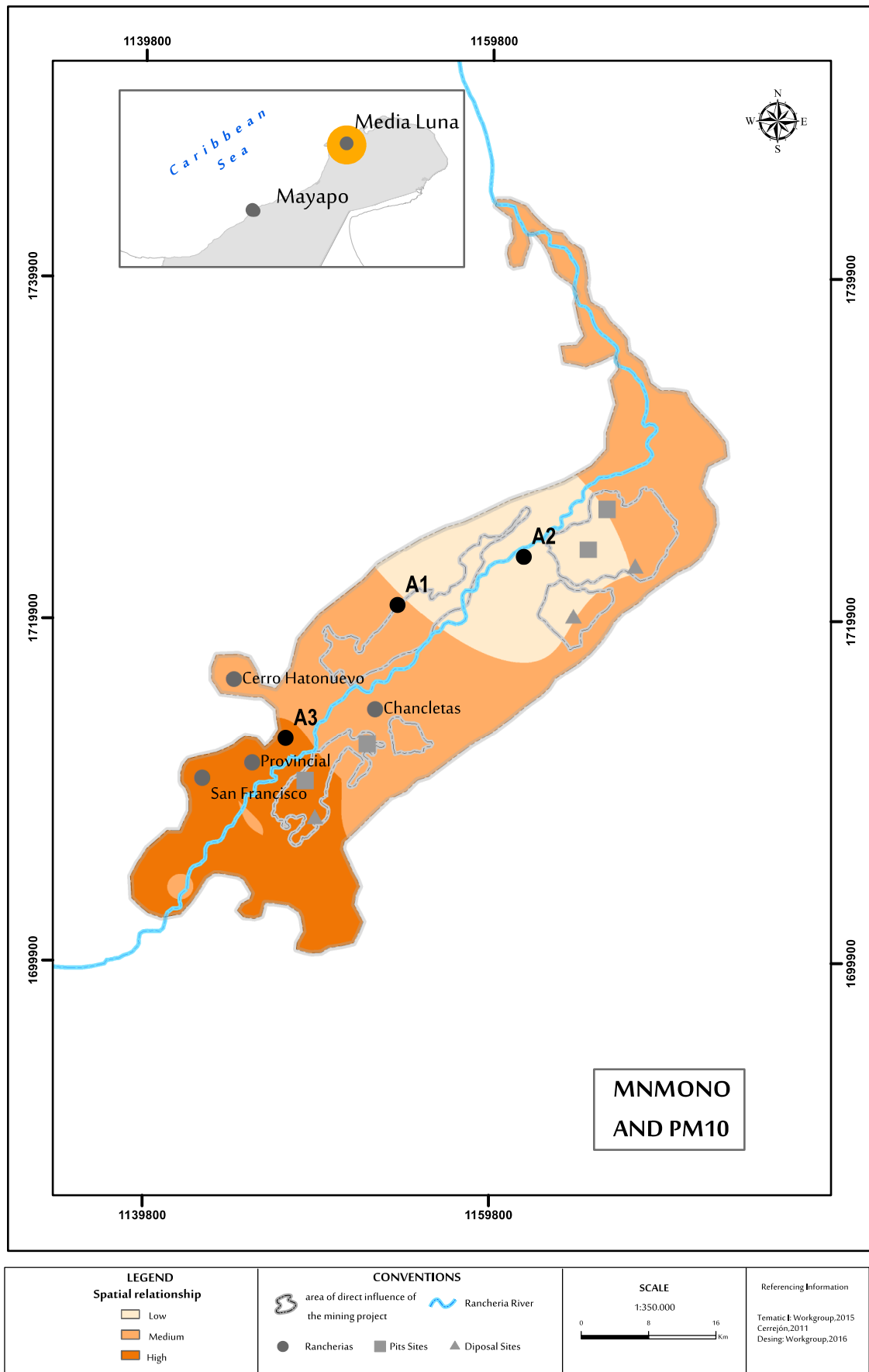
Figure 4. Overlay analysis of MNMONO frequency and PM$_{10}$ concentrations in areas located near the direct influence area of the open pit coal mining operations
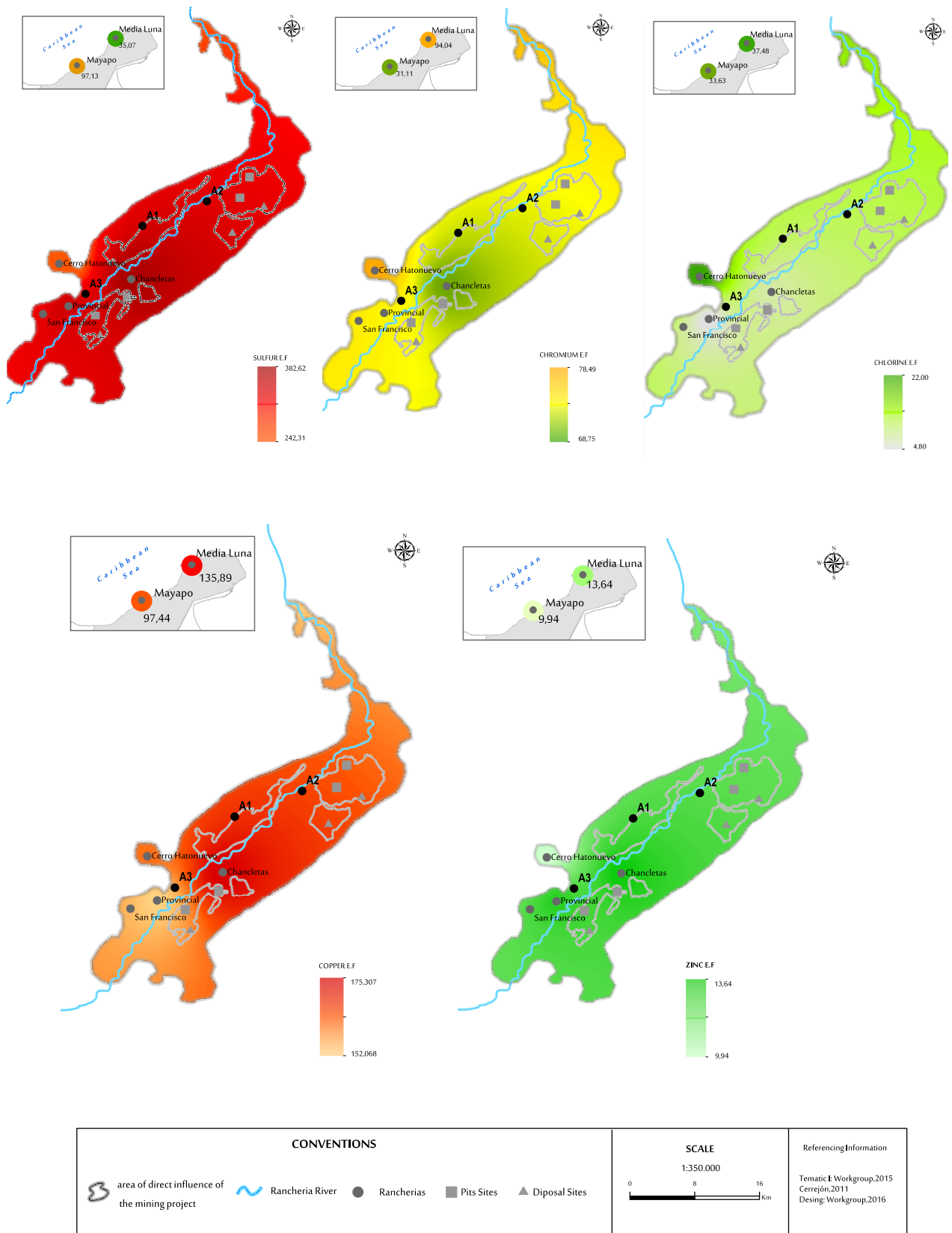
Figure 5. Spatial EF distribution of S, Cr, Cl, Cu and Zn in sampling areas in proximity of the open pit coal mining operations

Figure 6. Overlay analysis of interpolated MNBN frequencies and EF values of enriched elements in sampling areas in proximity of the open pit coal mining operations.

Figure 7. Overlay analysis of interpolated MNMONO frequencies and enriched elements concentrations in sampling areas in proximity of the open pit coal mining operations.

4. DISCUSSION

An initial assessment of current exposure to mean atmospheric concentrations for $PM_{2.5}$ and PM_{10} was performed for each sampled area. It has to be noted that the Colombian legislation stipulated national standards only for PM_{10}, thus $PM_{2.5}$ levels are not regularly monitored. As previously described, during sampling period, $PM_{2.5}$ around the coal mining corridor showed a mean value of 22.80 ± 10.74 $\mu g/m^3$ with concentrations ranged from 7.02 to 39.39 $\mu g/m^3$, while PM_{10} levels showed a mean value of $43.09 \pm 11.01 \mu g/m^3$ below national air quality standards (50 $\mu g/m^3$). Compared to previously reported values of PM_{10} in proximity to open pit coal mines of England and Czech Republic, our measures were significantly higher (Table 1). Interestingly, our values also surpassed previous reports for PM_{10} around the same coal mining area [30]. As shown in Table 1 up to now, $PM_{2.5}$ assessment has been rarely taken into account in biomonitoring studies. The few previously reported data on $PM_{2.5}$ concentrations are higher than our findings, however in our report $PM_{2.5}$ levels in areas like Chancletas and Provincial exceeded the 24-hour fine particle standard established by the WHO (25 $\mu g/m^3$). Nevertheless, because our objective was to record actual exposure levels experienced by local residents and do not intend to evaluate regional air quality, this information was mainly used for informative means. It is worth to mention that, $PM_{2.5}$ concentrations described in this study are the first reported for areas in proximity to open pit coal areas from La Guajira, Colombia.

Results from the spatial analysis performed to MNBN frequencies and $PM_{2.5}$ concentrations using the IDW interpolation confirmed our previous observations about the high correlation between MNBN and $PM_{2.5}$. Higher spatial correlations between these variables were established for Chancletas, Provincial and San Francisco, located between disposal areas and active pits, where spontaneous coal seams fires are more common [31]. These spontaneous fires could be the main source of $PM_{2.5}$ around these areas. As shown in Figure 3, exposure to $PM_{2.5}$ as well as MN frequency tends to decrease as the distance from the emission source increases, as evidenced in distant and elevated areas like Cerro de Hatonuevo. Despite being located in proximity of the coal mining operations and inside the mine direct influence area, Cerro de Hatonuevo showed a low spatial correlation between MNBN frequencies and $PM_{2.5}$ values. As previously discussed [12], this result could be a direct consequence of Cerro de Hatonuevo geographic location on a hill-top up above the main coal mining areas. Pollutant dispersion mechanisms, depending both on the site topography and on the meteorological conditions strongly influence the concentrations of particles. It has been widely observed that for most airborne particulate and gas metrics, concentration reduces as wind speed increases, although in some cases, an increase in the concentration may be observed at the highest wind speeds [32]. In consequence, as shown in Figure 2, wind direction and speed around mining areas could disperse chemical substances from pits, storing and disposal sites and concentrate them around the flat areas of Chancletas, Provincial and San Francisco where predominant wind speed is reduced. This effect would be greatly enhanced by the mountainous area that borders these regions increasing the exposure. Besides low wind speed registered in Cerro de Hatonuevo, elevated topography in the area would also influence particles concentrations, increasing the deposition of PM_{10} and decreasing $PM_{2.5}$ levels. These results also demonstrate that geographical location of sampled areas and the associated relief also

Table 1. Particulate matter values from different regions of the world in or near an opencast coal mine. Literature review is done based on the table described by Aneja et al., 2012 and recent reported studies.

Region	Site location	Sampling	PM$_{2.5}$ (µg/m^3)	PM$_{10}$ (µg/m^3)	Reference
North East England	Proximal to mine	Average	_	22.1	Pless-Mulloni et al., 2000 [33]
Czech Republic	Proximal to mine	Heating period	_	37	Hykysova and Brejcha, 2009 [34]
		Non heating period	_	26	
		Transition period	_	33	
		Annual mean	_	33.5	
Turkey	Inside mine	Drilling	_	3080	Onder and Yigit, 2009 [35]
		Coal handling plant	_	1840	
		Stock yard	_	1670	
		Overburden loading	_	1350	
		Coal loading	_	1300	
Zonguldak City, Turkey	Proximal to mine	Winter	34.17	63.59	Tecer et al., 2008 [36]
		Spring	29.84	59.16	
		Summer	25.03	41.83	
		Autumm	23.03	39.66	
Jharia, India	Inside mine	Average (6 stations)	_	_	Ghose and Majee, 2002 [37]
Dhanbad, India	Inside mine	Average (31 stations)	_	194±32 (range 59 to 339)	Dubey and Pal, 2012 [38]
Central Appalachian Region,U.S.A	Proximal to mine	Campbell Site	_	250.2±135.0 (range 31.9 to 469.7)	Aneja et al., 2012 [1]
		Willis Site	_	144.8±60.0 (range 19.2 to 218.5)	
Jharia, India	Inside mine	Winter	217	534	Roy and Singh, 2014 [39]
		Summer	232	549	
		Post-Monsoon	205	509	
Jharia, India	Inside mine	Winter	212±20	549±54	Roy et al., 2015 [40]
		Summer	177±29	509±47	
		Post-Monsoon	185±54	533±48	
Sonepur Bazari Opencast Project, India	FCR (pit top)	Average	156.77±3.78	_	Gautam et al., 2016 [41]
	CHP (near pit boundary)	Average	152.02±2.94	_	
	CTR (inside mine)	Average	141.82±3.22	_	
Czech Republic[1]	Proximal to mine	Annual mean	1.5	30	Máca and Melichar, 2016 [42]
La Guajira, Colombia	Proximal to mine	Average	_	32.90	Rojano et al., 2015 [30]
La Guajira, Colombia	Proximal to mine	Average	22.80±10.74 (range 7.02 to 39.39)	43.09±11.01 (range 16.70 to 77.60)	Present study

SPM suspended particulate matter, RPM respirable particulate matter
FCR field control room, CHP coal handling plant, CTR coal transport road
Brown coal min

constitute a very important determinant in damage distribution and that must be considered in the analysis of health impact caused by dynamically distributed variables like PM.

In previous reports we did not find a statistically significant association between PM_{10} levels and MNMONO frequency in exposed populations [12]. However, the spatial analysis showed a clustered relation between these variables at south of the area of direct influence of the mining operations, as depicted in Figure 4. Due to the fact that exposed areas possess flat geomorphology, obtained results suggests that wind direction may be also related with spatial behavior rather than the localization of extraction zones. Prevailing wind would determine to a large extent the direction and deposition of a large portion of PM in the southern exposed areas, increasing the risk of elevated MNMONO frequencies in these populations. Even when spatial relation was determined between MNMONO and PM_{10}, previous results about the $PM_{10}/PM_{2.5}$ ratio suggest that a high proportion of PM_{10} is constituted by $PM_{2.5}$ in these areas; thus, the influence of $PM_{2.5}$ on MNMONO frequency must also be considered. On the other hand, considering that high deposition of PM_{10} occurs in areas near the emission source [24, 43], it is also possible that some activities inside the Oreganal pit and disposal sites could be producing high quantities of PM_{10}, also capable of induce DNA damage [44, 45]. These results also demonstrate that geographical analyses of variables like PM and MN frequencies are capable of establishing correlations that commonly used statistical analysis may not.

Further analysis of other components of the PM also described interesting spatial correlations. Using inorganic elements concentrations previously obtained by PIXE analysis [12], and consequently EF values calculated for each element found in the $PM_{2.5}$ fraction, we established the presence of highly (S) and moderate enriched elements (Cr, Cu, Cl and Zn) in coal mining areas. We employed EF values rather than element concentrations in order to evaluate the effects of anthropogenic sources in DNA damage, excluding possible interferences due to crustal elements contribution. We also wanted to corroborate if anthropogenic emission sources possessed any influence in the detected elemental EF, and whether EF values and location of open-pits presented any spatial behavior. To locate the emission source responsible to high EF values in the sampling sites, we decided to perform an identification of pits, disposal and storing areas inside the mining areas. In a previous report by Rojano et al. [29], were identified three areas for coal storage (A1, A2 and A3). A1 and A2 are located in the northern of the mining zone. A1 consisted in 11 storing piles at north of the main area and A2 is formed by 1 storing pile. A3 was located in the southern area of the mine, with a total number or 2 auxiliary piles for storing. We only considered A1 and A3, as they were located near our sampling sites. However, residues from A2 may be also transported to sampled areas by predominant wind. Figure 5 shows the spatial distribution of EF values for S, Cr, Cu, Cl and Zn and possible emission sources around the coal mining area. Elements typically found around coal mining areas like S, Cu and Zn showed highly enriched patterns around pits and disposal sites, with a decrease of EF values with distance from the mining operations.

Sulfur was highly enriched around storing piles A1 and A3, which indicates that emissions are produced during coal mining and through active mine fires [46]. For S, emission sources may include S from common sulfide minerals contained in the coal, such as pyrite [47], thus, punctual emission sources of sulfur can be related as A1 in the north, A3 in the south and the Comuneros and Oreganal open-pits. These combustion residues could be dispersed by the wind along the whole mining area as shown in the interpolation results. Presence of S found in sampled areas is a matter of concern, since contributes to acid rain due to its responsibility in production of sulfuric acid, responsible

for the solubilization of metals in aerosol [48] and especially in other ambient matrices, such as soil and water. Sulfate contamination of surface and ground water from mining and processing operations is well recognized and commonly monitored as a primary indicator of coal mining impact to surface water [49]. Due to the fact that several of the Wayúu and Afro-Colombians communities from the exposed areas use artesian wells in water supply, more future studies, focusing in analytical determination of S deposition in water samples are highly recommended around the coal mining influence zone.

Analysis of the enrichment pattern for Cr showed higher values at north and south of the mining region, particularly around pits and disposal sites. Enrichment of Cr is a well-documented indication of their anthropogenic origin from traffic emissions [50], specially caused by transporting operation of coal and coal mining sub-products around some areas inside the mining corridor. In line with this observation, slightly elevated enrichment values of Cr were obtained around pits sites characterized by large-scale coal transportation through unpaved roads, as well as coal material overburden loading and unloading. Particularly in Media Luna, the presence of a coal railroad transportation may be also related with the EF found for Cr, commonly found in areas of increased trains activities [51]. Differential pattern obtained for the Cr enrichment values would suggest that dispersion of Cr from emission source might not be related to wind.

Other element related to high vehicle traffic is Cu, however in our results the enrichment pattern was different from other traffic related elements like Cr. Other possible sources of Cu enrichment in coal mines is related to coal fly ashes [52], that could be released by coal combustion in proximity to A3 and A1 storing piles. Similar results were obtained in an abandoned coal mine in Turkey [53], in which Cu showed higher concentrations within the coal storage area and dump sites. Similarly, high EF values for Zn may be also correlated with coal and coal bottom fly ash [54]. Corroborating with these results, similar distribution patterns observed for Cu and Zn seem to confirm a similar origin and distribution.

On the other hand, enrichment distribution pattern for Cl is possibly related to the presence of the water-soluble ion Cl from marine aerosol [55]. In accordance with this assumption, EF values were lower around the coal mining area and higher in Mayapo and Media Luna both located near the coastal area. Higher concentrations of Cl around of Cerro de Hatonuevo coincide with the presence of clay soil with high salinity levels and brackish underground water zones.

Spatial correlation between MNBN and MNMONO frequencies and enriched elements distribution in mining areas are show in Figure 5 and Figure 6 respectively. Moderate (medium) and low correlations between MNBN frequencies and S, Cr and Cu EF values around mining areas, would indicate that exposure to this enriched elements typically produced in coal mining activities is partially related to increased MNBN frequencies. These observations would confirm previous observations [12] showing that organic components of $PM_{2.5}$ - particularly PAHs concentration are very important for the effects on the cell cycle and DNA damage [56]. Even when no chemical characterization of the organic extracts of $PM_{2.5}$ was performed, about 97% of $PM_{2.5}$ around coal mining facilities is produced in the combustion of coal [57], which makes the presence of PAHs and other halogenated species in this fraction very plausible. MNBN/MONO showed no spatial correlation with Cl and Zn enrichment values.

Interestingly, areas with higher EF values for S showed also higher frequencies of MNMONO cells, while

116

other elements like Cr and Cu showed moderate (medium) and low correlations. With exception of Cu, Cl and Zn, that seems to have similar correlations patterns, MNMONO frequencies apparently were more susceptible to enrichment of S and Cr than MNBN. These results would indicate that enrichment of some elements like S and Cr around coal mining areas are potentially more related to accumulated *in vivo* genetic damage induction in exposed residents, which may also reflect an increase in the number of damaged cells that failed to divide. Some of these elements presents in the $PM_{2.5}$ fraction are involved in generation of oxidative damage through reactive oxygen species (ROS) production [58]. As previously discussed, oxidative stress status inside the cell is potentially capable of causing mitotic arrest (increasing MNMONO frequency), centromere damage, kinetochore malfunction [59] or disruption of the mitotic spindle [60] that may be associated to aneugenic effects [61].

CONCLUSION

Results showed a spatial relationship between exposure to higher concentrations of $PM_{2.5}$ and PM_{10} and micronucleus in individuals with residential proximity to coal mining areas. Our study is one of the first attempts to perform a spatial analysis on the relationship between PM generation, proximity to coal mining areas and predictive cancer risk biomarkers in open-pit systems. Active pits, disposal and storage areas were identified as the main sources of combustion elements associated with spontaneous coal seams fires. Wind speed and topography around the coal mining areas were identified as major contributors to PM dispersion and damage distribution. Inorganic element enrichment of the $PM_{2.5}$ fraction contributes partially to the increase of MNBN frequencies of exposed populations and corroborates the main role of the organic components of the PM in the biological effects and DNA damage; however seems to be more related to increase MNMONO frequencies and accumulated *in vivo* DNA damage. The present study would pose a useful tool to assess the human health risk associated with residential proximity to open-pit coal mining areas and could help to supply detailed and hierarchical information to the public or government about detailed priority pollutants/ regions of spatially concern.

REFERENCES

1.   Aneja, V.P., A. Isherwood, and P. Morgan, *Characterization of particulate matter (PM10) related to surface coal mining operations in Appalachia.* Atmospheric Environment, 2012. **54**: p. 496-501.

2.   (EPA), U.S.E.P.A., *Executive summary, in Air Quality Criteria for Particulate Matter.* National Center for Environmental Assessment, Research Triangle Park, 1996. **I**(Publication No. EPA/600/P-95/001/aF): p. 1–21.

3.   Li, Q., et al., *The preferential accumulation of heavy metals in different tissues following frequent respiratory exposure to PM2.5 in rats.* Sci Rep, 2015. **5**: p. 16936.

4.   Kaonga, B. and N.A. Kgabi, *Investigation into presence of atmospheric particulate matter in Marikana, mining area in Rustenburg Town, South Africa.* Environmental monitoring and assessment, 2011. **178**(1-4): p. 213-220.

5.   Barja, B., et al., *Atmospheric particulate matter levels, chemical composition and optical absorbing properties in Camagüey, Cuba.* Environmental Science: Processes & Impacts, 2013. **15**(2): p. 440-453.

6.   Srimuruganandam, B. and S.M. Shiva Nagendra, *Source characterization of PM10 and PM2.5 mass using a chemical mass balance model at urban roadside.* Sci Total Environ, 2012. **433**: p. 8-19.

7.   Patra, A.K., S. Gautam, and P. Kumar, *Emissions and human health impact of particulate matter from surface mining operation—A review.* Environmental Technology & Innovation, 2016. **5**: p. 233-249.

8.   Rohr, P., et al., *Genetic and oxidative damage of peripheral blood lymphocytes in workers with occupational exposure to coal.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2013. **758**(1–2): p. 23-28.

9.   León-Mejía, G., et al., *Genetic damage in coal miners evaluated by buccal micronucleus cytome assay.* Ecotoxicology and Environmental Safety, 2014. **107**: p. 133-139.

10.   Leon-Mejia, G., et al., *Assessment of DNA damage in coal open-cast mining workers using the cytokinesis-blocked micronucleus test and the comet assay.* Sci Total Environ, 2011. **409**(4): p. 686-91.

11.   Klimkina, I., *Assessment of mutagenic health effects in coal mine workers.* Genome, 2013. **18**: p. 31.

12.   Espitia-Pérez, L., et al., *Cytogenettic instability in populations with residential proximity to Open-pit coal mine in Northern Colombia in relation to PM10 and PM2.5 levels (Unpublished work)*, 2016. p. 40.

13.   Zou, B., et al., *Spatially differentiated and source-specific population exposure to ambient urban air pollution.* Atmospheric Environment, 2009. **43**(26): p. 3981-3988.

14.   S.A, C., *Summary of the Iiwo´uyaa Expansion Project for Stakeholders*, 2011. p. 70.

15. Calderon, J. and J. Nieves, *Informe calidad del aire corredor minero*, in *Corporación Autónoma*, C.A.r.d.l. Guajira, Editor 2010, Corporación Autónoma regional de la Guajira.

16. Azpurua, M.A. and K.D. Ramos, *A comparison of spatial interpolation methods for estimation of average electromagnetic field magnitude.* Progress In Electromagnetics Research M, 2010. **14**: p. 135-145.

17. Zandi, S., A. Ghobakhlou, and P. Sallis, *Evaluation of spatial interpolation techniques for mapping soil pH.* 2011.

18. Nalder, I.A. and R.W. Wein, *Spatial interpolation of climatic normals: test of a new method in the Canadian boreal forest.* Agricultural and forest meteorology, 1998. **92**(4): p. 211-225.

19. Wu, J., A. M Winer, and R. J Delfino, *Exposure assessment of particulate matter air pollution before, during, and after the 2003 Southern California wildfires.* Atmospheric Environment, 2006. **40**(18): p. 3333-3348.

20. Jha, D.K., et al., *Evaluation of interpolation technique for air quality parameters in Port Blair, India.* Universal Journal of Environmental Research and Technology, 2011. **1**(3): p. 301-310.

21. Kirsch-Volders, M. and M. Fenech, *Inclusion of micronuclei in non-divided mononuclear lymphocytes and necrosis/apoptosis may provide a more comprehensive cytokinesis block micronucleus assay for biomonitoring purposes.* Mutagenesis, 2001. **16**(1): p. 51-58.

22. Taner, S., B. Pekey, and H. Pekey, *Fine particulate matter in the indoor air of barbeque restaurants: Elemental compositions, sources and health risks.* Science of The Total Environment, 2013. **454–455**: p. 79-87.

23. Kothai, P., et al., *Characterization of atmospheric particulate matter using pixe technique.* Int. J. Environ. Sci. Eng, 2009. **1**(1): p. 27-30.

24. Rojano, R.E., L.C. Angulo, and G. Restrepo, *Niveles de Partículas Suspendidas Totales (PST), PM10 y PM2.5 y su Relación en Lugares Públicos de la Ciudad Riohacha, Caribe Colombiano.* Información tecnológica, 2013. **24**: p. 37-46.

25. Hans Wedepohl, K., *The composition of the continental crust.* Geochimica et Cosmochimica Acta, 1995. **59**(7): p. 1217-1232.

26. S.A., C., *Summary of the Iiwo'uyaa Expansion Project for Stakeholders*. Vol. 1. 2011. 70.

27. Wong, D.W., L. Yuan, and S.A. Perlin, *Comparison of spatial interpolation methods for the estimation of air quality data.* J Expo Anal Environ Epidemiol, 2004. **14**(5): p. 404-15.

28. Hermelin, M., *Landscapes and Landforms of Colombia*. 2015: Springer International Publishing.

29. Rojano, R., et al., *PM10 emissions due to storage in coal piles in a mining industrial area.* WIT Transactions on Ecology and The Environment, 2016. **207**: p. 87-97.

30.   Rojano, R., L. Angulo, and R. G., *Concentración y relación de PST, PM10 y PM2.5 en poblaciones cercanas a minería a cielo abierto: caso Cerrejón Colombia.* Revista de la Facultad de Ingeniería U.C.V, 2015. **30**(1): p. 1-10.

31.   Colaizzi, G.J., *Prevention, control and/or extinguishment of coal seam fires using cellular grout.* International Journal of Coal Geology, 2004. **59**(1–2): p. 75-81.

32.   Jones, A.M., R.M. Harrison, and J. Baker, *The wind speed dependence of the concentrations of airborne particulate matter and NOx.* Atmospheric Environment, 2010. **44**(13): p. 1682-1690.

33.   Pless-Mulloli, T., et al., *Living near opencast coal mining sites and children's respiratory health.* Occup Environ Med, 2000. **57**(3): p. 145-51.

34.   Hykysova, S. and J. Brejcha, *Monitoring of PM10 air pollution in small settlements close to opencast mines in the North-Bohemian Brown Coal Basin.* WIT Transactions on Ecology and the Environment, 2009. **123**: p. 387-398.

35.   Onder, M. and E. Yigit, *Assessment of respirable dust exposures in an opencast coal mine.* Environ Monit Assess, 2009. **152**(1-4): p. 393-401.

36.   Tecer, L.H., et al., *Effect of meteorological parameters on fine and coarse particulate matter mass concentration in a coal-mining area in Zonguldak, Turkey.* J Air Waste Manag Assoc, 2008. **58**(4): p. 543-52.

37.   Ghose, M.K. and S.R. Majee, *Assessment of the Status of Work Zone Air Environment Due to Opencast Coal Mining.* Environmental Monitoring and Assessment, 2002. **77**(1): p. 51-60.

38.   Dubey, B., A.K. Pal, and G. Singh, *Trace metal composition of airborne particulate matter in the coal mining and non–mining areas of Dhanbad Region, Jharkhand, India.* Atmospheric Pollution Research, 2012. **3**(2): p. 238-246.

39.   Roy, D. and G. Singh, *Chemical Characterization and Particulate Distribution of PM10 and PM2.5 at Critically Polluted Area of Dhanbad/Jharia Coalfield.* International Journal of Engineering Research and Applications, 2014. **4**(3): p. 625-634.

40.   Roy, D., G. Singh, and N. Gosai, *Identification of possible sources of atmospheric PM10 using particle size, SEM-EDS and XRD analysis, Jharia Coalfield Dhanbad, India.* Environ Monit Assess, 2015. **187**(11): p. 680.

41.   Gautam, S., et al., *Characterization of PM2.5 generated from opencast coal mining operations: A case study of Sonepur Bazari Opencast Project of India.* Environmental Technology & Innovation, 2016. **6**: p. 1-10.

42.   Máca, V. and J. Melichar, *The Health Costs of Revised Coal Mining Limits in Northern Bohemia.* Energies, 2016. **9**(2): p. 81.

43.   Huertas, J.I., et al., *Assessment of the natural sources of particulate matter on the opencast mines air quality.* Science of The Total Environment, 2014. **493**: p. 1047-1055.

44.   Roubicek, D.A., et al., *Micronuclei induced by airborne particulate matter from Mexico City.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2007. **631**(1): p. 9-15.

45.   Jung, M.H., et al., *Genotoxic effects and oxidative stress induced by organic extracts of particulate matter (PM10) collected from a subway tunnel in Seoul, Korea.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2012. **749**(1–2): p. 39-47.

46.   Pandey, B., M. Agrawal, and S. Singh, *Assessment of air pollution around coal mining area: Emphasizing on spatial distributions, seasonal variations and heavy metals, using cluster and principal component analysis.* Atmospheric Pollution Research, 2014. **5**(1): p. 79-86.

47.   Ward, I.C.L.a.C.R., *Composition and mode of occurrence of mineral matter in some Colombian coals.* Int. J. Coal Geol, 2008. **73**(1): p. 3–18.

48.   Medunic, G., et al., *Toxic airborne S, PAH, and trace element legacy of the superhigh-organic-sulphur Rasa coal combustion: Cytotoxicity and genotoxicity assessment of soil and ash.* Sci Total Environ, 2016. **566-567**: p. 306-19.

49.   Simonton, D.S. and S. King, *Hydrogen Sulfide Formation and Potential Health Consequences in Coal Mining Regions.* Water Quality, Exposure and Health, 2013. **5**(2): p. 85-92.

50.   Kothai, P., et al., *Characterization of Atmospheric Particulate Matter using PIXE Technique.* International Journal of Environmental, Chemical, Ecological, Geological and Geophysical Engineering, 2009. **3**: p. 38-42.

51.   Cartledge, B.T. and B.J. Majestic, *Metal concentrations and soluble iron speciation in fine particulate matter from light rail activity in the Denver-Metropolitan area.* Atmospheric Pollution Research, 2015. **6**(3): p. 495-502.

52.   Petaloti, C., et al., *Trace elements in atmospheric particulate matter over a coal burning power production area of western Macedonia, Greece.* Chemosphere, 2006. **65**(11): p. 2233-2243.

53.   Yenilmez, F., et al., *Evaluation of pollution levels at an abandoned coal mine site in Turkey with the aid of GIS.* International Journal of Coal Geology, 2011. **86**(1): p. 12-19.

54.   (ATSDR), A.f.T.S.a.D.R., *Toxicological profile for Zinc.* 2005, Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

55.   Dao, X., et al., *Chemical Characteristics of Water-Soluble Ions in Particulate Matter in Three Metropolitan Areas in the North China Plain.* PLoS ONE, 2014. **9**(12): p. e113831.

56.   Longhin, E., et al., *Cell cycle alterations induced by urban PM2.5 in bronchial epithelial cells: characterization of the process and possible mechanisms involved.* Particle and Fibre Toxicology, 2013. **10**(1): p. 1-19.

57.   Cao Guo Liang, Z.X., Gong SunLing, An Xin Qin & Wang Ya Qiang, *Emission inventories of primary particles and pollutant gases for China.* Atmospheric Science, 2011. **56**(8): p. 781-788.

58.   Valko, M., et al., *Free radicals, metals and antioxidants in oxidative stress-induced cancer.* Chem Biol Interact, 2006. **160**(1): p. 1-40.

59.   Parker, A.L., M. Kavallaris, and J.A. McCarroll, *Microtubules and Their Role in Cellular Stress in Cancer.* Frontiers in Oncology, 2014. **4**: p. 153.

60.   Choi, W.J., et al., *Oxidative stress and tumor necrosis factor-alpha-induced alterations in metaphase II mouse oocyte spindle structure.* Fertil Steril, 2007. **88**(4 Suppl): p. 1220-31.

61.   Kraniak, J.M., et al., *Antioxidant agents transiently inhibit aneuploidy progression in Li-Fraumeni cell strains.* Mol Carcinog, 2006. **45**(3): p. 141-56.

VI. DISCUSSÃO GERAL
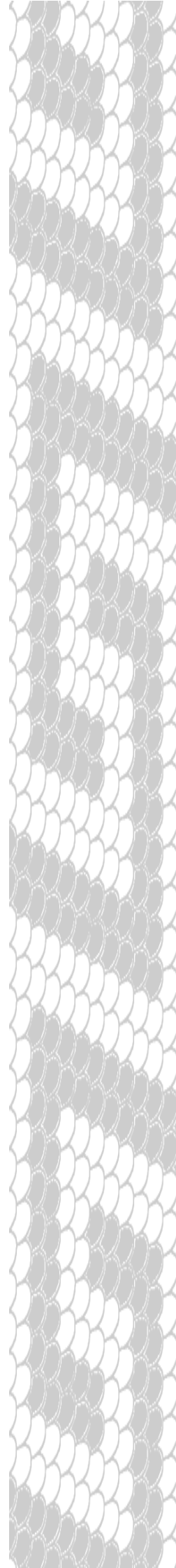
VI. DISCUSSÃO GERAL

Durante os últimos anos, a poluição atmosférica causada pela liberação de material particulado (PM) em torno de minas de carvão tem recebido especial atenção em todo mundo [127]. Estudos epidemiológicos sugerem que residir nas proximidades de minas de carvão está correlacionado com uma alta incidência de vários tipos de câncer [128], doenças respiratórias [129] e cardiovasculares [130], e que a exposição a misturas complexas de substâncias químicas contidas no material de escape gerado durante as atividades de mineração pode estar associado a este fenômeno [131].

O tamanho do MP é um fator importante na sua deposição no trato respiratório e consequentemente na forma como afeta a saúde humana. As frações maiores do MP referidas como PM_{10} são compostas por partículas com um diâmetro $\leq$ 10µm e são geralmente filtradas pelo nariz e pela garganta, sendo menos lesivas para a saúde. Adicionalmente, o PM_{10} é subdividido numa fracção de partículas mais finas $\leq$ 2,5 µm ($PM_{2,5}$), e uma fracção grosseira de partículas > 2,5 e <10 µm ($PM_{2,5-10}$) [132]. O $PM_{2,5}$ contém partículas primárias geradas por processos de combustão por indústrias e veículos, e partículas secundárias provenientes da formação de partículas na atmosfera a partir de gases, como por exemplo, a formação de sulfatos a partir de SO_2 [133]. O $PM_{2,5}$ é capaz de ultrapassar a região pulmonar alveolar, onde ocorre a troca de sangue e por esta razão representa a fração respirável de maior risco.

Os mecanismos biológicos pelos quais o MP exerce os seus efeitos adversos não são completamente compreendidos, mas os resultados dos ensaios *in vitro* têm mostrado que o MP induz vários tipos de efeitos celulares nocivos, incluindo genotoxicidade [134, 135], mutagenicidade, danos no DNA e a estimulação da produção de citosinas pró-inflamatórias [136]. Vários estudos têm relacionado a geração de espécies reativas de oxigênio (ERO) no interior da célula como o principal mecanismo pelo qual o MP pode gerar dano no DNA [137-139]

Além dos estudos com abordagem epidemiológica, o uso de outras ferramentas como marcadores de dano no DNA e dano citogenético ou cromossômico têm sido amplamente utilizadas na determinação do risco das populações expostas a resíduos de mineração de carvão [140-142]. Alguns dos mais usados, como o teste de micronúcleos em linfócitos (MN) ou em células epiteliais (BMN), têm sido relacionados com um aumento no risco de câncer [143, 144].

Em populações humanas, a grande maioria dos dados sobre os efeitos da exposição ao carvão têm sido gerados a partir de estudos em populações com exposição ocupacional [20]. Consequentemente, os possíveis efeitos sobre populações com exposição residencial são ainda desconhecidos.

Com a finalidade de avaliar os possíveis efeitos causados pela exposição ambiental a resíduos de mineração de carvão, este trabalho estudou populações assentadas ao redor da maior mina de carvão a céu aberto do mundo, localizada na região da Guajira ao Norte da Colômbia, e avaliou a possível relação entre o dano observado e as concentrações de PM_{10} e $PM_{2,5}$. Considerando que a exposição ao MP depende de fatores como o vento predominante e o relevo das áreas de residência dos indivíduos, também foi explorado o uso de ferramentas do sistema de informação geográfica (GIS) e modelos de interpolação de distância inversa (IDW) para avaliar a influência da proximidade das diferentes áreas de mineração sobre o dano genético e o enriquecimento de alguns elementos inorgânicos considerados de risco para as populações expostas.

No **CAPÍTULO I,** o dano genético foi avaliado utilizando o ensaio de citoma de micronúcleos (CBMN-cyt) em linfócitos. Para determinar o mecanismo de origem dos micronúcleos (clastogênico ou aneugénico) foi utilizada a imunotinção de MN com anticorpos anti-centrômero utilizando a técnica CREST. A população exposta foi comparada com uma população não exposta com características demográficas similares, mas residente em uma área sem mineração de carvão. Finalmente, considerando o papel do MP na geração do dano, durante o biomonitoramento populacional, as concentrações de PM_{10} e $PM_{2,5}$ foram estabelecidas nas áreas residenciais e foi determinado o conteúdo de elementos inorgânicos e orgânicos.

É importante ressaltar que, embora a análise das frequências de MN em linfócitos e outros tecidos tem sido usada em inúmeros estudos para avaliar a exposição populacional a agentes genotóxicos [145-148], a inclusão de outros parâmetros de dano celular como o a frequência de MNMONO para a avaliação do dano *in vivo* é relativamente recente. Dessa forma, os resultados obtidos no CAPÍTULO I constituem o primeiro relato de frequências de MNMONO em populações com exposição ambiental a resíduos de mineração de carvão.

As análises de correlação demostraram uma associação altamente significativa entre as concentrações de $PM_{2,5}$, as frequências de MNBN e a indução de micronúcleos CREST+ nos residentes expostos. As concentrações de PM_{10} apresentaram uma correlação menor, mas esta não foi significativa em relação às frequências de MNBN e micronúcleos CREST+. Estes resultados sugerem que a fração $PM_{2,5}$ gerada durante os processos de mineração de carvão tem a capacidade de induzir preferencialmente a perda de cromossomos inteiros (aneuploidia) embora a quebra de cromossomos também foi evidenciada.

Considerando a influência da fração $PM_{2,5}$ no dano, decidimos analisar sua composição química elementar, demonstrando a presença, nas áreas de mineração, de elementos altamente enriquecidos como o S e moderadamente enriquecidos como Cr, Cu, Cl e Zn (Fig. 7A e 7B; CAPÍTULO I). As concentrações da maior parte dos elementos inorgânicos foram comparáveis entre áreas controle e expostas, sendo maiores em alguns casos dentro das áreas de referência, especialmente para os elementos de origem edáfico (Tabela 6; CAPÍTULO I). Além das atividades antropogênicas da área controle, a alta concentração de elementos químicos presentes na areia do mar, as correntes de vento que favorecem a ressuspensão do solo e a erosão da costa marinha podem ser consideradas como as principais causas deste fenômeno [149]. A presença de elementos inorgânicos no MP atmosférico é comum, principalmente a partir da ressuspensão do solo. No entanto, durante os últimos anos, os metais têm sido introduzidos na atmosfera em grandes quantidades a partir de fontes antropogênicas, principalmente da combustão do carvão [150].

Vários estudos têm confirmado que o S é normalmente produzido pela combustão de combustíveis fósseis [24] como a que pode acontecer durante a mineração de carvão quando acontecem os incêndios das minas ativas [25]. Considerando que o método PIXE não oferece informação sobre a especiação química dos elementos inorgânicos, geralmente assume-se que muitos dos elementos estão presentes na atmosfera, como óxidos [26]. Assim, os principais óxidos de S provavelmente presentes ao redor das minas de carvão incluam SO_2 e SO_4^{2-} [27]. Outra possível fonte de S na área é constituída pela utilização, previamente descrita, de fogões à lenha pelas comunidades indígenas e Afro. No entanto, outros elementos considerados como principais constituintes da queima de biomassa como K e Na não foram encontrados enriquecidos nas áreas de mineração [151]. Esta observação indicaria que o S é enriquecido quase exclusivamente a partir das fontes de combustão do carvão. Uma análise

detalhada das fontes de enriquecimento potenciais e da distribuição espacial destes elementos é realizada no CAPÍTULO III e apoia essa hipótese.

O Cr, Cu e Zn estão particularmente associados com a fração fina do MP [152]. O enriquecimento desses elementos na atmosfera das áreas de mineração são motivo de preocupação devido a seus possíveis efeitos a longo prazo sobre a saúde humana [153], especialmente quando alguns dos elementos enriquecidos como S [154], Cr [155] e Cu [156] têm sido relacionados com a geração de danos no DNA, instabilidade genômica e processos cancerígenos. O principal mecanismo proposto para esses efeitos inclui a geração de danos oxidativos através da geração de ERO [157].

Os radicais livres gerados durante o metabolismo do SO_2, como $SO_3^{\cdot-}$, $SO_4^{\cdot-}$, $SO_5^{\cdot-}$, também podem induzir quebras na cadeia de DNA [158] e estudos recentes têm confirmado que os derivados de SO_2 (bissulfeto e sulfito) são capazes de induzir alterações cromossômicas (CA), troca de cromátides irmãs (SCE), paradas do ciclo celular e MN em linfócitos de sangue humano *in vitro* de uma maneira dependente da dose [159]. Da mesma forma, os efeitos genotóxicos do Cr são predominantemente produzidos pela formação de adutos e lesões oxidativas de tipo apurínicos/apirimidínicos que podem resultar em quebras no DNA [160]. Recentemente, outros estudos utilizando o CBMN-cyt e a tinção do centrómero demonstraram que o Cr (VI) apresenta características aneugênicas [38].

Estudos prévios sobre a capacidade oxidativa do Cu comprovaram que este pode catalisar diretamente a formação de ERO pela reação de Fenton [161] e diminuir significativamente os níveis de glutationa [162]. Este mecanismo é particularmente interessante considerando que a depleção da glutationa durante o dano oxidativo extremo é uma das possíveis causas pelas quais indivíduos portadores dos polimorfismos selvagens para as glutationa θ e μ (*GSTT1/GSTM1(+)*) e com exposição ocupacional a carvão apresentam uma frequência elevada de MN comparados com os indivíduos com os polimorfismos nulos [163]. Diferentemente dos outros elementos enriquecidos, o Zn não participa em reações redox [164]. Em vez disso, alguns autores têm sugerido que o Zn pode desempenhar um papel importante na capacidade de alguns compostos como o cromato de zinco de induzir erros nas paradas do ciclo celular relacionadas com a formação do fuso mitótico [43].

Além dos elementos inorgânicos, alguns autores sugerem que a exposição a compostos orgânicos também presentes no MP pode estimular a produção contínua de ERO, resultando em estresse oxidativo [165]. Por isso, a concentração de matéria orgânica extraída (EOM) nas amostras de aerossóis de PM$_{2,5}$ em torno das áreas de mineração de carvão foi determinada utilizando uma extração sequencial de três solventes com polaridade crescente: ciclohexano (CH), diclorometano (DCM) e acetona (ACE) [166].

Quando comparadas com a área controle, as áreas de mineração de carvão apresentaram uma maior concentração de EOM com características apolares (extraídos com CH) e de substâncias polares (extraídas com ACE). Levando em consideração os resultados obtidos para os elementos inorgânicos, podemos concluir que a diferença mais importante entre as PM$_{2,5}$ das áreas expostas e controle é causada pelas diferenças no conteúdo de matéria orgânica.

Considerando que o DNA encontrado na área controle foi muito menor do que o registrado dentro da área de mineração de carvão, é possível assumir que a composição química das partículas PM$_{2,5}$ nesta área não inclui substâncias com potencial mutagênico. Como discutido previamente, o aerossol marinho da área controle pode conter uma fração considerável de matéria orgânica (sobretudo insolúvel em água) e sais marinhos dominados por material

de natureza similar aos hidratos de carbono, nitratos e amónio [167]. Uma área similar à controle, mas dentro da área de exploração de carvão como Media Luna, apresentou valores superiores de EOM e maior frequência de dano, que poderiam ser correlacionadas com a proximidade a zonas de armazenamento e transporte de carvão.

Os nossos resultados concordam com resultados prévios que mostram que os componentes orgânicos do $PM_{2,5}$ - em particular os hidrocarbonetos aromáticos policíclicos (HAPs) - são os mais importantes para os efeitos sobre o ciclo celular e a geração de danos no DNA [168]. Embora a análise química dos extratos da EOM não tenha sido efetuada, a evidência sugere que aproximadamente 97% do $PM_{2,5}$ em torno das instalações de extração de carvão é produzido pela combustão do carvão [169], o que torna a presença de HAPs e outras espécies halogenadas nesta fracção muito plausível [170, 171]. É importante salientar que, durante a análise dos resultados, além da influência da concentração e das características químicas do MP na geração do dano, também foi evidenciada a presença de outro componente de natureza geográfica. A localização das áreas, o relevo e a velocidade do vento pareceram estar envolvidos nas diferenças de efeito observadas entre algumas áreas avaliadas. Este componente em particular foi avaliado extensivamente no **CAPÍTULO III.**

Quando foram analisadas as proporções de $PM_{2,5}/PM_{10}$, foi evidenciado que a fração fina do MP é dominante nas áreas com maior frequência de danos no DNA, com valores entre 0,80 e 0,52. Estes resultados sugerem que, contrário ao relatado em estudos prévios [172], o MP gerado em sistemas de mineração a céu aberto é constituído principalmente por $PM_{2,5}$. Esta fração constitui o maior risco para a saúde dos residentes em áreas próximas a minas de exploração de carvão, e por isso os resultados destacam a necessidade da incorporação de sistemas de monitoramento da qualidade do ar baseados na medição do $PM_{2,5}$. Também é fortemente sugerida a inclusão da caracterização química desta fração, tendo em conta que os nossos resultados sugerem que os principais efeitos biológicos relacionados à instabilidade genética poderiam estar relacionados com combinações específicas de elementos inorgânicos como o S, Cr, Cu e Zn e altas concentrações de material orgânico (isto é, HAP).

De fato, um número crescente de trabalhos sugerem que existe um comportamento aditivo nas misturas complexas de elementos inorgânicos e HAPs, onde os HAPs poderiam alterar a acumulação dos elementos e aumentar a geração de ERO intermediários [173]. Por sua vez, os elementos inorgânicos poderiam atuar como modificadores da função e regulação do citocromo P450, alterando o metabolismo dos HAPs e sua mutagenicidade e carcinogenicidade associada [174] .

Como foi discutido previamente, os efeitos da exposição a resíduos da mineração de carvão sobre o DNA têm sido relatados em numerosos estudos avaliando linfócitos e células epiteliais, sugerindo a presença de uma resposta sistémica que poderia envolver a produção de ERO na geração do dano e de outros intermediários [175, 176]. Contudo, o mecanismo do dano não é muito claro, especialmente em comunidades com exposição não ocupacional.

Assim, levando em conta que a inalação das partículas é a principal rota de exposição a resíduos de mineração de carvão em populações de residentes e em trabalhadores [177], o **CAPÍTULO II** avaliou o dano no DNA em células esfoliadas da mucosa oral (BMNCyt) e o dano primário e oxidativo no DNA em linfócitos, utilizando o ensaio Cometa convencional e modificado com endonucleases específicas: FPG e ENDO III.

Os resultados do CAPÍTULO I sugeriram que junto a outros compostos, a presença de alguns elementos inorgânicos enriquecidos diferencialmente nas áreas de mineração poderiam constituir a principal fonte de ERO nos

indivíduos expostos. Assim, o conteúdo de elementos inorgânicos no sangue dos indivíduos também foi determinado e correlacionado com as frequências de dano em células da mucosa e linfócitos.

Os resultados obtidos na mucosa oral evidenciaram um aumento significativo na frequência de micronúcleos (MN), células binucleadas (BN) e células representativas de processos de morte celular como células cariorréticas (KHC), carioliticas (KYL), picnóticas (PYK) e com cromatina condensada (CC) nas populações expostas.

Da mesma forma que em linfócitos, os MN na mucosa oral são formados a partir de fragmentos e de cromossomo inteiros, que permanecem excluídos do núcleo principal após a mitose [178], o que pode indicar, portanto, um risco aumentado para o desenvolvimento de câncer [179]. As células bucais estão em contato constante com o ambiente, razão pela qual o epitélio oral é um importante alvo para tóxicos inalados, sendo razoável esperar, em virtude da exposição crônica ao MP gerado nas minas de carvão, que a mucosa oral dos residentes em áreas próximas as minas apresentem sinais de maior genotoxicidade em relação aos grupos de indivíduos não expostos.

A mucosa oral é um tecido altamente ativo e com uma alta taxa de divisão celular [180]. Os modelos sobre a dinâmica nas células bucais têm demonstrado que existe uma forte correlação entre alguns tipos celulares, sendo que a proporção entre um e outro reflete o estado do tecido em geral [181].

Os resultados da análise de correlação entre os diferentes tipos celulares na mucosa evidenciaram uma forte associação positiva entre as células com MN, células BN e células CC. Com base nos modelos sobre as interações celulares no tecido bucal, os nossos resultados sugerem que as células com MN e as células BN derivam diretamente das células basais como resultado do dano genotóxico gerado pela exposição, enquanto que as células CC podem ser originadas de forma secundária a partir de células BN ou com MN, como parte do processo de eliminação de células danificadas pela via da apoptose. Em concordância com estas observações, as células BN também foram correlacionadas com outros parâmetros de morte celular como as células PYK e CC. Segundo Tolbert et al. (1992) [182], a apoptose no tecido bucal pode ser considerado como um mecanismo de vigilância, que elimina as células epiteliais danificadas.

Embora a origem das células BN tinham sido inicialmente relacionadas com falhas na citocinese na última divisão nuclear [181], estudos recentes sugerem que a formação de células BN também poderia estar relacionada com a parada do ciclo celular devido a uma disfunção do telômero ou à presença de efeitos aneuploides no interior da célula. Os resultados reportados por Shi e King (2005) [183] demonstram que os erros na disjunção cromossômica ocorrem com maior frequência em células BN incapazes de completar o processo citocinético do que em células que têm completado a citocinese. Assim, a não-disjunção cromossômica estaria estreitamente relacionada com a regulação da citocinese. Os autores indicam que a citocinese seria inibida em células que espontaneamente não segregam seus cromossomos durante a mitose [183].

Sendo assim, é provável que a elevada frequência de células BN encontrada nos indivíduos expostos seja também indicativa do potencial aneugénico das misturas complexas geradas durante a mineração de carvão. Esta hipótese é suportada pelos resultados obtidos no CAPÍTULO I onde o mesmo efeito aneugénico é descrito em linfócitos do mesmo grupo de indivíduos. No entanto, outras evidências (sondas centroméricas específicas) seriam necessária para verificar a precisão do modelo e também a origem aneugênica dos MN em células bucais dessas populações.

O dano primário no DNA (%Tail DNA) e o dano oxidativo a purinas (FPG) e pirimidinas (ENDO III) também foram significativamente maiores nos indivíduos expostos, corroborando com a informação de que o dano no DNA observado nos indivíduos expostos a resíduos de mineração de carvão é gerado a partir de lesões oxidativas. Estudos prévios em populações com exposição ocupacional relataram resultados similares na mucosa oral e no dano ao DNA avaliado pelo ensaio Cometa convencional [142, 184]. Os nossos resultados são os primeiros a utilizar enzimas de reparo em populações com exposição ambiental para avaliar o reparo de lesões oxidativas no DNA. Outros estudos têm demonstrado o mesmo efeito em populações com exposição ocupacional utilizando a medição da atividade enzimática da superóxido dismutase (SOD) e das concentrações de 8OHdG na urina [185].

Além do dano primário no DNA como consequência da geração de ERO no sangue, outras células, assim como o componente intersticial, podem ser afetadas pela translocação de partículas, mediadores e ERO através do sangue. Vários tipos celulares podem atuar como alvos desses mediadores incluindo fibroblastos, células epiteliais e endoteliais [175].

Em concordância com esta observação, a frequência de MN em células bucais foi correlacionada com a %TailDNA no ensaio Cometa convencional e modificado com FPG (Fig. 4; CAPÍTULO II). Esta correlação sugere que, de fato, os efeitos genotóxicos sistêmicos dentro da corrente sanguínea também podem afetar e ser detectados em outros tipos de células como as células bucais. Da mesma forma, os danos oxidativos no DNA, particularmente a oxidação da guanina a 8-oxoguanina (8-OHdG) detectada pela FPG, podem desempenhar um papel importante no dano do DNA observado no epitélio bucal. Resultados semelhantes foram obtidos por Katarkar et al. [179] em pacientes com câncer oral.

Considerando que, em células bucais, os MN são expressos nas células basais que estão em divisão nas camadas mais profundas da mucosa bucal, é mais provável que estas células sejam influenciadas em graus semelhantes pelos compostos absorvidos e distribuídos pela corrente sanguínea [9]. Assim, a indução de MN em células bucais pode ser o resultado de uma resposta sistêmica à exposição a resíduos de mineração do carvão induzida pela geração de dano oxidativo na corrente sanguínea.

As frequências de MN em células bucais também foram correlacionadas com as frequências de MN em linfócitos previamente analisadas a partir nos mesmos grupos de amostras (Tabela 3, CAPÍTULO II). Uma correlação linear entre MN em linfócitos e MN em células bucais também foi descrita por Ceppi et al. [186].

Em resumo, os resultados indicam que os fatores genéticos e de exposição que afetam a frequência de MN e de dano no DNA em linfócitos podem eventualmente ser compartilhados em algum grau pelas células bucais, incluindo a associação dos MN com o risco de câncer [179].

Populações de residentes em proximidade de áreas de minas de carvão são normalmente expostos a misturas complexas de compostos orgânicos e inorgânicos [187]. Em particular, a exposição a elementos inorgânicos é potencialmente capaz de gerar dano oxidativo pela produção de ERO [157]. Os elementos inorgânicos ingressam no corpo humano por ingestão ou inalação e são, subsequentemente, transferidas para o sangue, sendo espalhados por todo o organismo [188] e depositando-se nos tecidos por meio de vários mecanismos [189]. Por conseguinte, decidimos caracterizar a presença de elementos inorgânicos nas amostras de sangue dos indivíduos expostos e não expostos com a finalidade de estabelecer uma possível relação entre as concentrações do elemento inorgânico e o dano em células da mucosa e linfócitos.

Os elementos traço mais representativos em amostras de sangue dos indivíduos expostos foram Cl, S, Si, K, Fe, Zn, Br e Ti (Tabela 4; CAPÍTULO II).

Apenas os elementos Cr, Ni, Mn e Br mostraram um aumento estatisticamente significativo em relação aos indivíduos não expostos. A maioria destes elementos já tinha sido detectada em amostras de sangue de populações com exposição ocupacional [142] e em mamíferos selvagens [190, 191] de áreas de mineração de carvão da Colômbia e do Brasil. No CAPÍTULO I, alguns destes elementos inorgânicos foram encontrados em altas concentrações no MP das zonas estudadas e, particularmente, Cr, Zn e Cu foram encontrados enriquecidos nas áreas próximas às zonas mineração de carvão, indicando que são liberados para a atmosfera a partir de fontes antropogênicas.

Vários estudos têm demonstrado que elementos como Cr, Ni, Mn e Br são nutrientes essenciais necessários para várias funções bioquímicas e fisiológicas. No entanto, também é conhecido que os níveis destes elementos no sangue podem aumentar significativamente após a exposição [192]. Adicionalmente, a maioria destes elementos estão normalmente presentes no ambiente da mineração de carvão e, consequentemente, podem ser um fator de risco importante na aparição de doenças ou câncer relacionados com esta exposição [193].

Como foi discutido acima, o potencial cancerígeno do Cr é bem conhecido. Sabe-se também que os íons bivalentes de Ni são capazes de induzir danos no DNA em vários tipos de células [194]. O dano ao DNA induzido pelo Ni é provavelmente gerado a partir de ERO intermediários e não pelo envolvimento direto dos compostos de níquel [195]. Por outro lado, o Br é um dos elementos mais ubíquos na biosfera [196] e a evidência sobre se é essencial ou não para o homem é pouca [197]. Alguns compostos que contêm Br na sua estrutura química, como o brometo de potássio, são considerados como possíveis carcinógenos em humanos [198]. Em particular, a exposição de células de mamífero a brometo (BrO_3^-) pode gerar modificações oxidativas, em particular 8-oxoG. No mecanismo de toxicidade descrito , o BrO_3^- é ativado pela glutationa a um metabólito reativo que interage com o DNA [199].

Apesar disso, nenhum destes elementos foram correlacionados com o incremento nos parâmetros do BMNCyt. Resultados semelhantes foram obtidos por Benedetti et al. [200], quando analisaram a influência dos elementos inorgânicos presentes na mucosa oral de indivíduos expostos a pesticidas. A partir dos resultados, podemos inferir que, depois de inalados, os elementos inorgânicos são transferidos para o sangue [188], onde estão envolvidos na geração de ERO por meio de mecanismos como as reações de Fenton e Haber-Weiss [201]. É possível que outros compostos presentes nas misturas complexas das atividades de mineração de carvão, como os HAPs desempenhem um rol mais significativo nos danos no DNA da mucosa oral. No interior da cavidade oral, especialmente, alguns genes relacionados com o metabolismo dos HAPs, como o *CYP1A1*, são altamente expressos, o que sugere uma ativação *in situ* deste tipo de compostos xenobióticos [202]. Vários estudos têm descrito uma associação significativa entre algumas variações no gene *CYP1A1* e a presença de danos genéticos nas células da mucosa oral, sugerindo que esse polimorfismo pode modular os efeitos da exposição a HAPs neste tecido epitelial [203].

No mesmo sentido, %TailDNA avaliado com o ensaio Cometa convencional foi altamente correlacionado com as concentrações de Al, Mn e Br, enquanto o dano oxidativo avaliado com o Cometa modificado com FPG foi correlacionado com as concentrações de Mn. Al e Mn desempenham papéis biológicos importantes como oligoelementos essenciais para as atividades de algumas enzimas do organismo humano [197]. No entanto,

concentrações mais elevadas podem ocasionar o desequilíbrio metálico e estar relacionadas a várias doenças [204]. Os possíveis mecanismos através dos quais o Al pode exercer suas propriedades genotóxicas e cancerígenas ainda não foram claramente estabelecidas. A fração do Al que atinge a circulação sistêmica não é excretada na urina rapidamente e se acumula nos tecidos periféricos [205]. Há evidências de que o Al é capaz de induzir aberrações cromossômicas (CA), MN e trocas de cromátides irmãs em linfócitos humanos [206]. Utilizando o ensaio cometa alcalino, Lankoff et al. [207] demonstraram que o Al induz danos no DNA de um modo dependente da dose. As células tratadas com Al também mostraram uma diminuição na capacidade de reparação do DNA, indicando que o mesmo também pode inibir a reparação do DNA. De modo semelhante, doses relativamente elevadas de Mn podem causar dano no DNA e afetar o processo de replicação [208]. Estudos anteriores têm correlacionado elevadas concentrações de Mn no sangue com altos níveis de 8OHdG na urina de trabalhadores expostos a PM$_{2,5}$ [209]. As evidências sugerem que o Mn perturba a respiração mitocondrial e inibe o sistema antioxidante celular, diminuindo a capacidade das células para combater o estresse oxidativo [210].

Finalmente, considerando que a exposição ambiental a resíduos de mineração de carvão envolve a presença de misturas complexas de elementos inorgânicos e outras substâncias, é difícil atribuir os efeitos observados a um composto em particular [153]. Durante a exposição, podem acontecer fenômenos como o sinergismo ou antagonismo, onde um elemento inorgânico pode afetar a absorção de outro ou alterar a sua distribuição no corpo. Vários estudos têm mostrado que Pb, Cd, Cr e Ni em baixas concentrações no PM$_{2,5}$ podem apresentar toxicidade *in vivo* ou *in vitro* pela produção de dano primário ou cromossómico no DNA [135].

A análise dos resultados obtido nos **CAPÍTULOS I** e **II** demonstrou que a localização geográfica das áreas de amostragem, o relevo e a velocidade do vento poderiam estar envolvidos nas diferenças de efeito observadas entre algumas áreas avaliadas. Para explorar a possível influência dos fatores geográficos na distribuição do dano, o **CAPÍTULO III** utilizou sistemas de informação geográfica (SIG) e métodos de interpolação pela ponderação do inverso da distância (IDW) para explorar a relação entre a exposição a PM$_{2,5}$ e PM$_{10}$ e a indução de dano citogenético e risco de câncer nas populações que residem em proximidade das áreas de mineração de carvão. Adicionalmente, o capítulo avaliou a influência de outros fatores como o enriquecimento de alguns elementos inorgânicos no PM$_{2,5}$ na geração do dano e sua relação com a proximidade às áreas de exploração.

Os resultados da análise espacial confirmaram as conclusões dos capítulos anteriores sobre a alta correlação entre os níveis de PM$_{2,5}$ e elevadas frequências de MNBN. As áreas de maior correlação foram estabelecidas para Chancletas, Provincial e San Francisco, localizadas entre áreas de armazenamento de rejeitos e áreas de mineração, onde a presença de incêndios espontâneos de carvão é mais alta [211]. Como foi mostrado na Fig. 4 do **CAPÍTULO III,** a exposição a PM$_{2,5}$ assim como a frequência de MNBN, são inversamente proporcionais à proximidade da mina, corroborado a relação entre a proximidade às áreas de combustão e o dano no DNA.

Embora os resultados do **CAPÍTULO I** não estabeleçam uma correlação significativa entre as frequências de MNMONO e a exposição a PM$_{10}$, a análise espacial estabeleceu uma forte associação entre as variáveis, especialmente ao norte das áreas de mineração. Considerando a velocidade e direção do vento no local (**CAPITULO III,** Fig. 2), é possível que as regiões localizadas no sul das áreas de mineração recebam uma alta concentração de MP que provoca o aumento das frequências de MNMONO nessas populações. Outra possível explicação considera que o fenômeno seja, na realidade, devido às concentrações de PM$_{2,5}$ nas regiões do sul, onde as proporções

PM$_{10}$/PM$_{2,5}$ são elevadas. Esses resultados também demonstraram que as análises espaciais de variáveis que, como o MP, variam em função de condições ambientais como o relevo e o vento, permitem estabelecer correlações que não podem ser determinadas pelos métodos estatísticos comumente utilizados.

Considerando que a exposição a elementos químicos inorgânicos constitui uma importante fonte na geração de ERO e estresse oxidativo, para complementar as análises, verificamos a distribuição espacial dos fatores de enriquecimento (EF) calculados para o S, Cr, Cu, Cl e Zn e sua relação com a presença de altas frequências de MNBN e MNMONO nas áreas de mineração de carvão. O uso dos valores do EF para cada elemento permitiu avaliar os efeitos das fontes antropogênicas sobre as frequências de MNBN e MNMONO sem a presença de possíveis fatores de interferência como a contribuição da ressuspensão do solo.

Para localizar as fontes de emissão responsáveis pelos altos valores de EF nos locais de amostragem, decidimos realizar a identificação dos pits e das áreas de armazenamento de carvão e rejeitos dentro das áreas de mineração. Em um estúdio prévio, Rojano et al. [212] identificaram três áreas para armazenamento de carvão (A1, A2 e A3). A1 é formada por 11 pilhas de armazenamento localizadas ao norte da área principal de mineração, enquanto que A3 contem 2 pilhas de armazenamento auxiliares localizadas na zona sul da mina. A análise da distribuição espacial das possíveis fontes de emissão de elementos inorgânicos demonstrou que elementos como S, Cu e Zn apresentaram valores de enriquecimento superiores em torno das áreas com proximidade a pits e áreas de armazenamento de carvão. O fator de enriquecimento foi inversamente correlacionado com a distância das operações de mineração. O S apareceu altamente enriquecido em torno das pilhas de armazenamento A1 e A3, o que indicaria que as emissões de S nas proximidades das minas são produzidas através de incêndios espontâneos dentro das áreas de mineração [213]. A análise também demonstrou que os resíduos de S parecem ser gerados ao longo de toda a área de mineração e que também podem ser dispersos pelo vento, como mostrado nos resultados de interpolação.

A presença do S nas áreas próximas às zonas de mineração é um motivo de preocupação devido principalmente a sua contribuição para a chuva ácida [214]. A contaminação das águas superficiais e subterrâneas com sulfato gerado nas operações de mineração é reconhecida e comumente monitorada como um indicador primário do impacto da mineração de carvão na superfície da água [215]. Devido ao fato de que várias das comunidades Wayúu e de Afro-colombianos das áreas expostas usam poços artesianos para o abastecimento de água, é altamente recomendado a realização de estudos em amostras de água focados na determinação analítica da deposição do S.

A distribuição do padrão de enriquecimento do Cr apresentou valores mais elevados ao norte e sul da região de mineração, particularmente em torno de pits e áreas de armazenamento de rejeitos. O enriquecimento do Cr é uma indicação bem documentada de sua origem antropogênica a partir de emissões do tráfego [152], especialmente causados pelo transporte do carvão, material de rejeito e subprodutos da mineração em torno de algumas áreas no interior das zonas expostas. Particularmente em Media Luna, a presença de trens para o transporte do carvão também pode ser relacionada com o EF encontrado para o Cr, comumente enriquecido em áreas com atividades ferroviárias [216]. Em nossos resultados, outro elemento comumente relacionado com o tráfego de veículos como o Cu mostrou um padrão de enriquecimento completamente diferente ao padrão do Cr. Outras possíveis fontes de enriquecimento do Cu em minas de carvão incluem as cinzas voláteis [217], que poderiam ser liberadas pela combustão de carvão em proximidade das pilhas de armazenamento A3 e A1. Da mesma forma, para o Zn, valores

elevados no EF podem estar correlacionados com a presença de cinzas de carvão [218]. Os padrões de distribuição semelhantes observados para Cu e Zn parecem confirmar uma origem comum **(**Fig. 5; **CAPITULO III).**

Finalmente, a distribuição dos valores do EF para o Cl podem ser possivelmente relacionados com a presença de íons de Cl provenientes do aerossol marinho [219]. Esta hipótese foi suportada pelas análises de distribuição que mostraram que os maiores valores do EF para Cl foram localizados em Mayapo e Media Luna, ambos localizados na área costeira.

Quando foi analisada a possível correlação entre a distribuição dos EF nas áreas de mineração e a frequências de MNBN e MNMONO, foi observada uma correlação de moderada a baixa entre as frequências de MNBN e os valores de enriquecimento do S, Cr e Cu. Esses resultados podem sugerir que a exposição a elementos inorgânicos (tipicamente enriquecidos em atividades de mineração de carvão) se encontra apenas parcialmente relacionada com o aumento da frequência de MNBN obtida para indivíduos expostos. Estas observações apoiam algumas conclusões prévias **(CAPÍTULO II)** que mostraram que os componentes orgânicos do $PM_{2,5}$ constituem os fatores mais importantes para os efeitos sobre o ciclo celular e a geração do dano no DNA [168].

Curiosamente, as áreas com os valores mais elevados de enriquecimento para o S mostraram também as frequências mais elevadas de células MNMONO. Com exceção do Cu, Cl e Zn, que parecem ter padrões de correlação semelhantes, as frequências de MNMONO foram, aparentemente, mais suscetíveis ao enriquecimento de S e Cr do que MNBN. Estes resultados indicam que o enriquecimento de alguns elementos como S e Cr em torno de áreas de mineração de carvão poderia estar potencialmente mais relacionado com a indução do dano genético cumulativo em residentes expostos e, consequentemente, com o aumento no número de células com dano que não conseguiram se dividir. Alguns destes elementos presentes na fracção de $PM_{2,5}$ estão envolvidos na geração de danos oxidativos pela geração de ERO [157]. Como discutido durante todo o trabalho, o estresse oxidativo dentro da célula é potencialmente capaz de causar a parada do ciclo celular/mitose (aumentando a frequência de MNMONO) e gerar danos no centrômero ou no fuso mitótico, o que também poderia estar associado à indução de efeitos aneugênicos durante a divisão celular.

A partir dos principais achados dos **CAPITULOS I, II e III,** podemos sugerir um **modelo de exposição e dano** descrito na **Fig. 23 (deste capítulo):** Neste modelo, as $PM_{2,5}$ e PM_{10} gerados principalmente a partir de fontes de combustão e atividades mecânicas ingressam no organismo dos indivíduos expostos pela inalação das partículas. Outras frações menores como a $PM_{<1,0}$ também seriam gerados durante as atividades de mineração de carvão. O $PM_{2,5}$ transportaria elementos orgânicos altamente enriquecidos como o S presumivelmente em forma de óxidos (SO_2; SO_4^{-2}) e outros medianamente enriquecidos como o Cr, Cu, Zn e Cl. Junto com o conteúdo de elementos inorgânicos, uma grande proporção do $PM_{2,5}$ estaria constituída por matéria orgânica de natureza apolar (muito provavelmente HAPs e outras espécies halogenadas) e outra proporção menor de elementos polares como nitratos e sulfatos. Devido ao tamanho do $PM_{2,5}$, os resíduos transportados pela fração seriam rapidamente absorvidos pelos pulmões e bronquíolos onde seriam assimilados por macrófagos e outras células epiteliais, iniciando a produção de ERO. Outra porção do $PM_{2,5}$ seria difundido na corrente sanguínea, onde ocorreria a translocação até outros tecidos. No sangue e no interior de células como os linfócitos, a geração de ERO seria a causa do dano oxidativo do DNA, gerando,

RESUMO
COMO OS RESÍDUOS DA MINERAÇÃO DO CARVÃO PODEM AFETAR A INSTABILIDADE GENÉTICA NA POPULAÇÃO GERAL?

Al   Si   Cr

HAPs
Outras espécies halogenadas

Cu

Cl   S
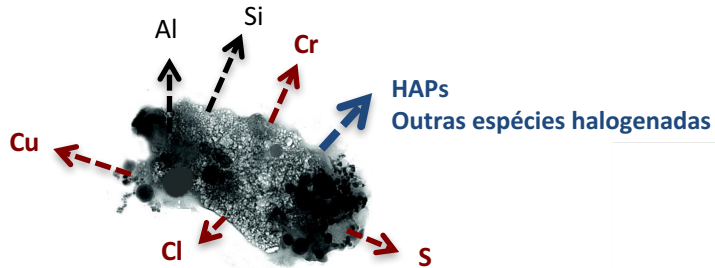
Transporta elementos inorgânicos não enriquecidos como Si, K e Al
Elementos inorgânicos enriquecidos como **S, Cr, Cu** e **Cl**
Altas concentrações de matéria orgânica **não – polar**

1. Inalação/Ingestão

Poderia ter uma maior influência sobre o dano observado na mucosa oral ???

$PM_{2.5}$
$PM_{<1.0}$
(nanopartículas)

PM_{10}

Combustão espontânea

Atividades mecânicas

Atividades de mineração de carvão a céu aberto

2. É provável que alguns elementos presentes em maior proporção no $PM_{2.5}$ **(HAPs de 5-6 anéis)**, estejam mais envolvidos nos níveis de dano observados na mucosa oral.

**DANO NO DNA
(MN, BN)**

**Morte celular
(CC, KHC, PYK, KYL)**

7. Em contato direto com o sangue, as células basais da mucosa entram em contato com os intermediários genotóxicos aumentado a frequência de MN, BN e células apoptóticas/necróticas na cavidade oral.
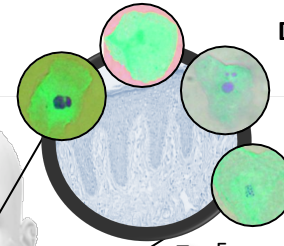
Mucosa oral

Bronquíolos

ERO

Brônquios

3. As partículas contidas no $PM_{2.5}$, seriam rapidamente absorvidas pelos alvéolos onde seriam assimiladas pelos macrófagos e outras células epiteliais, iniciando a produção de **ERO.** As partículas de PM_{10}, seriam absorvido pelos brônquios e bronquíolos pelo que também estão envolvidas na geração de **ERO.**

ERO

4. Na corrente sanguínea, a geração de **ERO** pode estar relacionada à presença de altas concentrações de elementos inorgânicos como *Cr, Ni, Mn, Al e Br*. Estes metais podem causar dano no DNA, particularmente pela da oxidação das guaninas.

**Ensaio cometa; FPG, ENDO III
(Tail%DNA)**

Linfócitos da corrrente sanguinea

6. A corrente sanguínea pode transportar **ERO** e outros intermediários até outros tecidos em um processo de translocação sistêmica.

5. A produção crônica de **ERO** e efeitos sinérgicos e aditivos entre produtos presentes nas misturas complexas **(HAPs e elementos inorgânicos)** podem gerar o acúmulo de danos no DNA, que, por sua vez, produz a parada do ciclo celular e o dano em estruturas como o fuso mitótico.

**MNBN; MNMONO
CREST+MN**
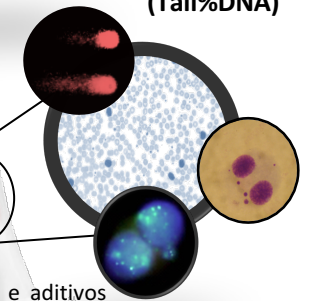
Fig. 23. Modelo de exposição e dano proposto a partir dos principais achados do estudo

especialmente, um aumento nas concentrações de purinas oxidadas. A produção crônica de ERO e os possíveis efeitos aditivos ou sinérgicos de alguns produtos presentes na mistura complexa (HAPs e elementos inorgânicos) poderiam causar o acúmulo de danos no DNA, causando paradas no ciclo celular e/ou danificando estruturas como o fuso mitótico, incrementando a frequência de células MNBN e MNMONO, assim como de CREST+MN.

O papel dos ERO na aparição da aneuploidia permanece controverso, mas algumas evidências sugerem uma possível relação entre ploidia e os níveis de ERO. Agentes antioxidantes podem inibir a progressão da aneuploidia [220] e a super-expressão da enzima antioxidante superóxido-dismutase dependente de manganês, inibe a instabilidade cromossómica [221]. Mais recentemente, demonstrou-se que o estresse oxidativo é capaz de contornar o ponto de checagem (checkpoint) do controle do fuso mitótico [222], induzindo a despolimerização dos microtúbulos [223] e alterações na estrutura do fuso [224]

Como consequência da geração de ERO no sangue, outras células como as células da mucosa oral poderiam ser afetadas pela translocação de partículas secundárias, mediadores e ERO. Em contato direto com o sangue, as células basais da mucosa poderiam entrar em contato com intermediários genotóxicos capazes de incrementar a frequência de MN e células BN em células da mucosa oral. O aumento das células BN poderia constituir uma evidência de um efeito aneugênico similar ao descrito nos linfócitos, mas na mucosa oral. Outros elementos como os HAPs poderiam representar um maior impacto nos níveis de dano observados neste tecido. Considerando o tamanho das partículas e as características de deposição da fração PM_{10}, seria interessante determinar se alguns dos componentes diferencialmente presentes nesta fração grosseira apresentam uma maior influência no dano observado na mucosa oral.

Finalmente, é importante considerar que o acúmulo dos elementos inorgânicos e outras substâncias como os HAPs em matrizes não avaliadas neste estudo (água, solo ou alimentos) também podem constituir rotas de exposição dos indivíduos e influenciar a distribuição do dano. Uma análise futura dos principais poluentes detectados nos sistemas de mineração de carvão deverá incluir a avaliação de outras matrizes. O estudo também demonstrou que a fração $PM_{2,5}$ representa maior risco para a saúde dos residentes das proximidades de minas de carvão a céu aberto, e revelou a necessidade de incorporar o monitoramento desta fração nos programas de avaliação da qualidade do ar em áreas de exploração de carvão. Além de confirmar o papel do estresse oxidativo no dano no DNA observado nas populações expostas a resíduos de mineração de carvão, os nossos resultados sugerem um possível novo papel de alguns resíduos gerados durante a mineração de carvão na indução de efeitos aneugênicos em populações expostas.

VII. CONCLUSÕES
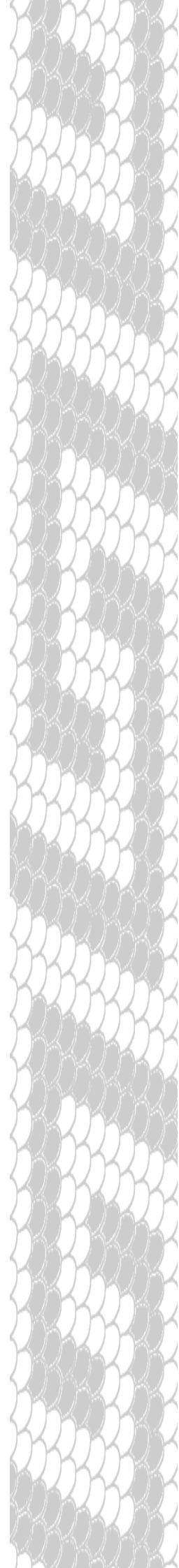
VII. CONCLUSÕES

CONCLUSÃO GERAL

O conjunto de resultados desta tese permite concluir que a exposição ambiental a resíduos de mineração de carvão constitui um fator de risco, possibilitando a geração de dano no DNA e a indução de instabilidade cromossômica em indivíduos que residem nas proximidades de zonas de mineração a céu aberto. O dano no DNA está relacionado com a indução de um estado de estresse oxidativo em células da corrente sanguínea, estando este relacionado com a indução de ERO e a presença de elementos inorgânicos no sangue. A instabilidade cromossômica, evidenciada em linfócitos e células epiteliais, encontra-se relacionada com a perda de cromossomos inteiros e fragmentos de cromossomos, revelando que misturas complexas de compostos gerados durante a mineração de carvão apresentam atividade tanto aneugénica quanto clastogênica, sendo a perda de cromossomos inteiros relativamente mais frequente.

Adicionalmente, foi possível estabelecer que estes efeitos sobre a integridade do material genético estão estreitamente correlacionados com as características físicas e químicas da fração fina do material particulado; a composição química desta fração é constituída principalmente por grandes quantidades de elementos inorgânicos e material orgânico de natureza apolar. Similar ao descrito em outros estudos, o componente orgânico da fração fina parece constituir o elemento mais importante na resposta biológica observada.

CONCLUSÕES ESPECÍFICAS

- A análise dos biomarcadores do CBMN-cyt em linfócitos evidenciou um aumento significativo na frequência de micronúcleos em células binucleadas (MNBN) e células mononucleadas (MNMONO) dos indivíduos que habitam regiões próximas ás minas de exploração de carvão a céu aberto.

- Os indivíduos expostos apresentaram um aumento significativo de 45,27% na frequência de CREST+ MN quando comparados com indivíduos não expostos, indicando a exposição a substâncias indutoras de aneuploidia nas áreas de mineração.

- As análises de correlação demonstraram uma relação altamente significativa entre os níveis de $PM_{2,5}$, as frequências de MNBN e a indução de CREST+ MN em residentes expostos.

- A determinação da composição química do $PM_{2,5}$ pela técnica de PIXE demonstrou a presença de elementos traço altamente enriquecidos, como o S, e moderadamente enriquecidos como Cr, Cu e Zn.

- A análise do dano na mucosa oral, revelou um aumento significativo na frequência de todos os parâmetros do BMNCyt.

- Os resultados do ensaio Cometa convencional e modificado com o uso de endonucleases (FPG e ENDO III) também demonstraram um aumento significativo nos valores de % Tail DNA nos indivíduos que habitam próximos à região de minas de exploração de carvão a céu aberto.

- Os indivíduos expostos apresentaram elevadas concentrações sanguíneas de Cr, Ni, Mn e Br quando comparados com os indivíduos não expostos.

- Os valores de %Tail DNA no ensaio Cometa convencional apresentaram alta correlação com as concentrações de Al, Mn e Br no sangue, enquanto

- O aumento no %Tail DNA no ensaio Cometa modificado com FPG apresentou uma correlação com altas concentrações de Mn.

- Existe uma relação espacial entre a proximidade a minas de carvão a céu aberto e um incremento na frequência de marcadores de risco de câncer em populações com exposição ambiental.

- A localização geográfica das áreas de amostragem, o relevo e a velocidade do vento estão envolvidos na distribuição de $PM_{2,5}$ e PM_{10} nas áreas próximas às zonas de mineração, e, por sua vez, afetam a distribuição do dano observado em algumas áreas avaliadas.

- As zonas de pits, as áreas de armazenamento de carvão e de materiais de rejeito constituem os principais pontos de emissão de $PM_{2,5}$ para a atmosfera e de elementos associados à combustão do carvão como S, Cu e Cr.

- O incremento na frequência de MNBN em indivíduos expostos relaciona-se parcialmente com o enriquecimento de elementos orgânicos no $PM_{2,5}$ dentro das áreas próximas à zonas de mineração de carvão.

- Em proximidade a minas de carvão, o enriquecimento do $PM_{2,5}$ com elementos inorgânicos como o S está fortemente relacionado com o incremento na frequência de MNMONO nos indivíduos expostos.

VIII. PERSPECTIVAS
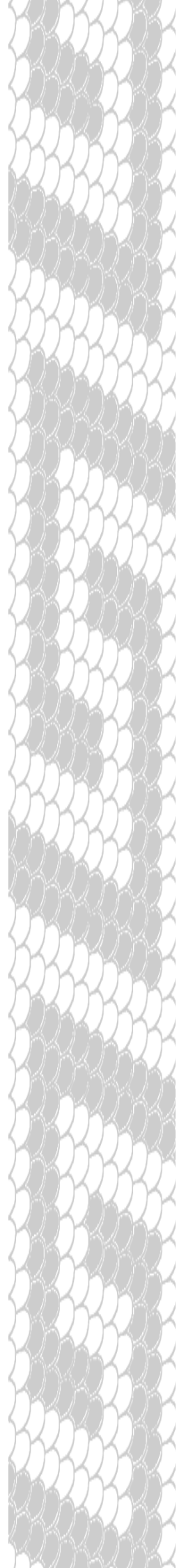
VIII. PERSPECTIVAS

Para aprofundar os conhecimentos sobre os efeitos da exposição ambiental a resíduos da mineração do carvão, poderiam ser implementadas as avaliações dos aspectos relacionados a seguir.

- Caracterizar a constituição química dos extratos orgânicos da fração $PM_{2,5}$.
- Determinar e comparar a constituição química da fração PM_{10} com seus efeitos biológicos, especialmente na mucosa oral, e os efeitos observados na fração $PM_{2,5}$.
- Avaliar a exposição à HAPs nos indivíduos com exposição ambiental através da determinação de metabólitos característicos como o 1-hidroxipireno urinário.
- Analisar a contribuição de outras matrizes ambientais como solo, água e alimentos para a presença de elementos inorgânicos no sangue dos indivíduos expostos.
- Estudar a capacidade dos extratos orgânicos da fração $PM_{2,5}$ (CH, DCM, ACE) de induzir a formação de CREST+MN *in vitro.*
- Avaliar o ajuste e capacidade de interpolação dos modelos de IDW estabelecidos no estudo pela avaliação dos parâmetros em outras localidades ao redor da mina.
- Confirmar as concentrações de elementos inorgânicos utilizando técnicas mais sensíveis como o ICP-MS (espectrometria de massas com plasma de acoplamento indutivo).
- Determinar a presença de dano em outros tecidos das vias respiratórias superiores como a mucosa nasal.
- Avaliar as concentrações de HAPs no sangue dos indivíduos expostos pelo uso da cromatografia líquida com detecção de fluorescência de alto desempenho (HPLC-FD) e sua possível correlação com o dano.
- Realizar a caracterização química dos compostos presentes no $PM_{2,5}$ utilizando difração de raios X (XRD) e a caracterização das partículas mediante microscopia eletrônica.
- Avaliar a presença de óxidos de silício nas frações de PM_{10} e $PM_{2,5}$ mediante o uso de XRD.
- Aprofundar nas características dos íons solúveis em água presentes na fração de $PM_{2,5}$ mediante o uso de cromatografia iônica (IC).
- Avaliar outros parâmetros indicativos de dano oxidativo como a atividade da Superóxido dismutase (SOD)

REFERENCES

1.   (EIA), U.S.E.I.A., *Energy and Air Pollution*, I.E.O.S. Report, Editor 2016.

2.   S.A, C., *Summary of the Iiwo´uyaa Expansion Project for Stakeholders*, 2011. p. 70.

3.   Chadwick, M.J., N.H. Highton, and N. Lindman, *Environmental Impacts of Coal Mining & Utilization: A Complete Revision of Environmental Implications of Expanded Coal Utilization*. 2013: Elsevier Science.

4.   Hill, G.B. and L.B. Lyon, *A NEW CHEMICAL STRUCTURE FOR COAL.* Industrial & Engineering Chemistry, 1962. **54**(6): p. 36-41.

5.   (UPME), U.d.P.M.E., *La Cadena del Carbón en Colombia: fuente de energía para el mundo*. 1995.

6.   (EIA), U.S.E.I.A., *International Energy Outlook*, in *International Energy Outlook*2016.

7.   (EIA), U.S.E.I.A., *Analysis of the Impacts of the Clean Power Plants* 2015.

8.   Aguiar, L.A.A., G. H.; Almeida, J. R.; Soares, P. S.; Possa, M. V., *Análise e Avaliação de Risco Ambiental como Instrumento de Gestão em Instalações de Mineração. .* In: Carvão Brasileiro: Tecnologia e Meio Ambiente. Rio de Janeiro, CETEM/MCT, 2008: p. 213-235.

9.   Ryan Brightwell, B.C., Ashish Fernandes, Katrin Ganswindt,, Y.L. Kuba Gogolewski, Calvin Quek, Sebastian Rötters, Jan Rovensky, Alex Scrivener,, and A.S. Vladimir Slivyak, Klara Sutlovicova, Hanna Yang, *Banking on coal* 2013: BanckTrack.

10.   Jähnig, A., *Coal Deposits of Colombia.* 2013.

11.   Energética., U.U.d.P.n.M., *Mercado nacional e internacional del carbón colombiano.* 2007.

12.   Bahamón, J.E.S., *El Cerrejon y sus efectos: Una perspectiva socioeconómica y ambiental.* Banco de la República, 2004.

13.   Irons, R.M.A., Quick, W., , *The Fate of Trace Elements in Pulverised Fuel (PF) Combustion Systems.* PowerGen UK plc, CRE Group Ltd, University of Nottingham 2000: p. p. R193.

14.   Valentim, B., Guedes, A., Flores, D., Ward, C.R., Hower, J.C., , *Variations in fly ash composition with sampling location: Case study from a Portuguese power plant. .* Coal Combustion and Gasification Products, 2009. **1** p. 14 - 24.

15.   López, I.C. and C.R. Ward, *Composition and mode of occurrence of mineral matter in some Colombian coals.* International Journal of Coal Geology, 2008. **73**(1): p. 3-18.

16.   Leon-Mejia, G., et al., *Genetic damage in coal miners evaluated by buccal micronucleus cytome assay.* Ecotoxicol Environ Saf, 2014. **107**: p. 133-9.

17.   Leon-Mejia, G., et al., *Assessment of DNA damage in coal open-cast mining workers using the cytokinesis-blocked micronucleus test and the comet assay.* Sci Total Environ, 2011. **409**(4): p. 686-91.

18.   Pulido, A., *Coal and its Effects: Case Study: Cerrejón Zonal Norte.* in Chomsky et. al. The People Behind Colombian Coal - Mining, Multinationals and Human Rights 2007.

19.   Fernandez-Navarro, P., et al., *Proximity to mining industry and cancer mortality.* Sci Total Environ, 2012. **435-436**: p. 66-73.

20.   Hendryx, M., *Mortality from heart, respiratory, and kidney disease in coal mining areas of Appalachia.* Int Arch Occup Environ Health, 2009. **82**(2): p. 243-9.

21. Hendryx, M. and M.M. Ahern, *Relations Between Health Indicators and Residential Proximity to Coal Mining in West Virginia.* American Journal of Public Health, 2008. **98**(4): p. 669-671.

22. Finkelman, R.B., et al., *Health impacts of coal and coal use: possible solutions.* International Journal of Coal Geology, 2002. **50**(1–4): p. 425-443.

23. Pless-Mulloli, T., et al., *Living near opencast coal mining sites and children's respiratory health.* Occupational and Environmental Medicine, 2000. **57**(3): p. 145-151.

24. Love, R.G., et al., *Respiratory health effects of opencast coalmining: a cross sectional study of current workers.* Occupational and Environmental Medicine, 1997. **54**(6): p. 416-423.

25. Gautam, S., et al., *Characterization of PM2.5 generated from opencast coal mining operations: A case study of Sonepur Bazari Opencast Project of India.* Environmental Technology & Innovation, 2016. **6**: p. 1-10.

26. Gautam, S., et al., *Particulate matter pollution in opencast coal mining areas: a threat to human health and environment.* International Journal of Mining, Reclamation and Environment, 2016: p. 1-18.

27. ORGANIZATION, U.N.E.P.W.H., *AIR metodology review handbook series.* Geneva, WHO, 2004. **3: Measurement of suspended particulate matter in ambient air.**

28. Alves, C., *Aerossóis atmosféricos: perspectiva histórica, fontes, processos químicos de formação e composição orgânica.* Química Nova, 2005. **28**: p. 859-870.

29. Queiroz, P.G.M., V.M.F. Jacomino, and M.Â.d.B.C. Menezes, *Composição elementar do material particulado presente no aerossol atmosférico do município de Sete Lagoas, Minas Gerais.* Química Nova, 2007. **30**: p. 1233-1239.

30. EPA, U., *Air Quality Criteria for Particulate Matter, Vols. I–II.* Washington, DC: US Environmental Protection Agency. EPA, 2004. **600/P-99/002aF-bF**.

31. Kouimtzis, T., et al., *Airborne Particulate Matter*. 2013: Springer Berlin Heidelberg.

32. Yanagi, Y., J.V.d. Assunção, and L.V. Barrozo, *The impact of atmospheric particulate matter on cancer incidence and mortality in the city of São Paulo, Brazil.* Cadernos de Saúde Pública, 2012. **28**: p. 1737-1748.

33. Donoghue, A.M., *Occupational health hazards in mining: an overview.* Occupational Medicine, 2004. **54**(5): p. 283-289.

34. Roy, G.A.a.T.S., *Development of Emission Factors for Quantification of Blasting Dust at Surface.* Journal of Environmental Protection, 2010. **1**(4): p. 346-361.

35. (EPA), U.S.E.P.A., *Executive summary, in Air Quality Criteria for Particulate Matter.* National Center for Environmental Assessment, Research Triangle Park, 1996. **I**(Publication No. EPA/600/P-95/001/aF): p. 1–21.

36. CCME, *Canadian soil quality guidelines for potentially carcinogenic and other PAHs: scientific criteria document*, 2010, Canadian Council of Ministers of the Environment Winnipeg: CCME.

37. Yu, M.-H., Tsunoda, H., Tsunoda, M., , *Environmental Toxicology: Biological and Health Effects of Pollutants, Third Edition. CRC Press.* 2011.

38. Park JS, W.T., Sweet S. , *Atmospheric distribution of polycyclic aromatic hydrocarbons and deposition to Galveston Bay, Texas, USA. .* Atmos Environ 2001. **35**: p. 3241–9.

39. T., K., *Atmospheric chemistry of polycyclic aromatic hydrocarbons and related compounds. .* J Health Sci 2011. **57**(6): p. 504-11.

40. Stracher, G.B., A. Prakash, and E.V. Sokol, *Coal and Peat Fires: A Global Perspective: Volume 1: Coal - Geology and Combustion*. 2010: Elsevier Science.

41. Hygienists), A.A.C.o.G.I., *Polycyclic aromatic hydrocarbons (PAHs) biologic exposure indices (BEI) Cincinnati. OH: American Conference of Governmental Industrial Hygienists.* 2005.

42. Lannerö, E., et al., *Exposure to environmental tobacco smoke and sensitisation in children.* Thorax, 2008. **63**(2): p. 172-176.

43. Chen, B.H. and Y.C. Chen, *Formation of polycyclic aromatic hydrocarbons in the smoke from heated model lipids and food lipids.* J Agric Food Chem, 2001. **49**(11): p. 5238-43.

44. Warneck P, W.J., *Chapter 5. The atmospheric aerosol. Atmospheric chemist's companion*, Springer, Editor. 2012. p. 127–87.

45. Mumtaz, M.M., et al., *ATSDR evaluation of health effects of chemicals. IV. Polycyclic aromatic hydrocarbons (PAHs): understanding a complex problem.* Toxicol Ind Health, 1996. **12**(6): p. 742-971.

46. Armstrong, B., et al., *Lung Cancer Risk after Exposure to Polycyclic Aromatic Hydrocarbons: A Review and Meta-Analysis.* Environmental Health Perspectives, 2004. **112**(9): p. 970-978.

47. Wei See, S., S. Karthikeyan, and R. Balasubramanian, *Health risk assessment of occupational exposure to particulate-phase polycyclic aromatic hydrocarbons associated with Chinese, Malay and Indian cooking.* J Environ Monit, 2006. **8**(3): p. 369-76.

48. Ravindra, K., R. Sokhi, and R. Van Grieken, *Atmospheric polycyclic aromatic hydrocarbons: Source attribution, emission factors and regulation.* Atmospheric Environment, 2008. **42**(13): p. 2895-2921.

49. Unwin, J., et al., *An assessment of occupational exposure to polycyclic aromatic hydrocarbons in the UK.* Ann Occup Hyg, 2006. **50**(4): p. 395-403.

50. Bach, P.B., et al., *Screening for lung cancer: a review of the current literature.* Chest, 2003. **123**(1 Suppl): p. 72S-82S.

51. Boffetta, P., N. Jourenkova, and P. Gustavsson, *Cancer risk from occupational and environmental exposure to polycyclic aromatic hydrocarbons.* Cancer Causes Control, 1997. **8**(3): p. 444-72.

52. Olsson, A.C., et al., *Occupational exposure to polycyclic aromatic hydrocarbons and lung cancer risk: a multicenter study in Europe.* Occup Environ Med, 2010. **67**(2): p. 98-103.

53. Diggs, D.L., et al., *Tumor microsomal metabolism of the food toxicant, benzo(a)pyrene, in Apc(Min) mouse model of colon cancer.* Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine, 2012. **33**(4): p. 1255-1260.

54. ATSDR (Agency for Toxic Substances, D.R., *Toxicological profile for polycy- clic aromatic hydrocarbons.* , U.D.o.H.a.H. Services, Editor 1995, US Government Printing Office. p. 298.

55. Srogi, K., *Monitoring of environmental exposure to polycyclic aromatic hydrocarbons: a review.* Environmental Chemistry Letters, 2007. **5**(4): p. 169-195.

56. Rengarajan, T., et al., *Exposure to polycyclic aromatic hydrocarbons with special focus on cancer.* Asian Pacific Journal of Tropical Biomedicine, 2015. **5**(3): p. 182-189.

57. Okuda, T., et al., *Trends in hazardous trace metal concentrations in aerosols collected in Beijing, China from 2001 to 2006.* Chemosphere, 2008. **72**(6): p. 917-924.

58.   Nebert, D.W., et al., *Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer.* J Biol Chem, 2004. **279**(23): p. 23847-50.

59.   Singh, R., et al., *The relationship between biomarkers of oxidative DNA damage, polycyclic aromatic hydrocarbon DNA adducts, antioxidant status and genetic susceptibility following exposure to environmental air pollution in humans.* Mutat Res, 2007. **620**(1-2): p. 83-92.

60.   Schwartz, A.G., et al., *The molecular epidemiology of lung cancer.* Carcinogenesis, 2007. **28**(3): p. 507-18.

61.   USEPA, *Development of a Relative Potency Factor (RPF) Approach for Polycyclic Aromatic Hydrocarbon (PAH) Mixtures (External Review Draft, EPA/635/R-08/012A)*, 2010, U.S. Environmental Protection Agency: Washington, DC.

62.   Hainaut, P., et al., *IARC Database of p53 gene mutations in human tumors and cell lines: updated compilation, revised formats and new visualisation tools.* Nucleic Acids Res, 1998. **26**(1): p. 205-13.

63.   Mordukhovich, I., et al., *Associations between Polycyclic Aromatic Hydrocarbon–Related Exposures and p53 Mutations in Breast Tumors.* Environmental Health Perspectives, 2010. **118**(4): p. 511-518.

64.   Pratt, M.M., et al., *Polycyclic Aromatic Hydrocarbon (PAH) Exposure and DNA Adduct Semi-Quantitation in Archived Human Tissues.* International Journal of Environmental Research and Public Health, 2011. **8**(7): p. 2675-2691.

65.   Jarvis, I.W., et al., *Interactions between polycyclic aromatic hydrocarbons in complex mixtures and implications for cancer risk assessment.* Toxicology, 2014. **321**: p. 27-39.

66.   Herawati, N., et al., *Cadmium, copper, and zinc levels in rice and soil of Japan, Indonesia, and China by soil type.* Bull Environ Contam Toxicol, 2000. **64**(1): p. 33-9.

67.   He, Z.L., X.E. Yang, and P.J. Stoffella, *Trace elements in agroecosystems and impacts on the environment.* J Trace Elem Med Biol, 2005. **19**(2-3): p. 125-40.

68.   Adriano, D.C., *Trace Elements in Terrestrial Environments*. 2 ed. 2001: Springer-Verlag New York.

69.   Madejón, P., et al., *Bioaccumulation of As, Cd, Cu, Fe and Pb in wild grasses affected by the Aznalcóllar mine spill (SW Spain).* Science of The Total Environment, 2002. **290**(1–3): p. 105-120.

70.   Sunjog, K., et al., *Assessment of status of three water bodies in Serbia based on tissue metal and metalloid concentration (ICP-OES) and genotoxicity (comet assay).* Environ Pollut, 2016. **213**: p. 600-7.

71.   Moran-Martinez, J., et al., *Genotoxicity in oral epithelial cells in children caused by nickel in metal crowns.* Genet Mol Res, 2013. **12**(3): p. 3178-85.

72.   Lanctot, C., et al., *Effects of coal mine wastewater on locomotor and non-locomotor activities of empire gudgeons (Hypseleotris compressa).* Ecotoxicol Environ Saf, 2016. **127**: p. 36-42.

73.   Lourenco, J., et al., *Metal bioaccumulation, genotoxicity and gene expression in the European wood mouse (Apodemus sylvaticus) inhabiting an abandoned uranium mining area.* Sci Total Environ, 2013. **443**: p. 673-80.

74.   Tchounwou, P.B., et al., *Heavy Metals Toxicity and the Environment.* EXS, 2012. **101**: p. 133-164.

75.   Beyersmann, D. and A. Hartwig, *Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms.* Arch Toxicol, 2008. **82**(8): p. 493-512.

76.   A, H., *Kanzerogene Metallverbindungen. Aktuelle Aspekte zu Wirkungsmechanismen und Risikobewertung. Oesterreichisches Forum Arbeitsmedizin* 2007. **01/07**: p. 5-10.

77.   Förstner, U. and G.T.W. Wittmann, *Metal pollution in the aquatic environment*. 1981: Springer-Verlag.

78.   Hartwig, A., *Carcinogenicity of metal compounds: possible role of DNA repair inhibition.* Toxicology Letters, 1998. **102–103**: p. 235-239.

79.   Stohs, S.J. and D. Bagchi, *Oxidative mechanisms in the toxicity of metal ions.* Free Radic Biol Med, 1995. **18**(2): p. 321-36.

80.   Cadenas, E. and K.J. Davies, *Mitochondrial free radical generation, oxidative stress, and aging.* Free Radic Biol Med, 2000. **29**(3-4): p. 222-30.

81.   Kaonga, B. and N.A. Kgabi, *Investigation into presence of atmospheric particulate matter in Marikana, mining area in Rustenburg Town, South Africa.* Environ Monit Assess, 2011. **178**(1-4): p. 213-20.

82.   Barja, B., et al., *Atmospheric particulate matter levels, chemical composition and optical absorbing properties in Camaguey, Cuba.* Environ Sci Process Impacts, 2013. **15**(2): p. 440-53.

83.   Ling, S.H. and S.F. van Eeden, *Particulate matter air pollution exposure: role in the development and exacerbation of chronic obstructive pulmonary disease.* Int J Chron Obstruct Pulmon Dis, 2009. **4**: p. 233-43.

84.   Valavanidis, A., K. Fiotakis, and T. Vlachogianni, *Airborne particulate matter and human health: toxicological assessment and importance of size and composition of particles for oxidative damage and carcinogenic mechanisms.* J Environ Sci Health C Environ Carcinog Ecotoxicol Rev, 2008. **26**(4): p. 339-62.

85.   Loomis, D., et al., *The carcinogenicity of outdoor air pollution.* Lancet Oncol, 2013. **14**(13): p. 1262-3.

86.   Pope, C.A. and D.W. Dockery, *Health Effects of Fine Particulate Air Pollution: Lines that Connect.* Journal of the Air & Waste Management Association, 2006. **56**(6): p. 709-742.

87.   Brook, R.D., et al., *Particulate matter air pollution and cardiovascular disease: An update to the scientific statement from the American Heart Association.* Circulation, 2010. **121**(21): p. 2331-78.

88.   Pope, C.A., 3rd, et al., *Lung cancer, cardiopulmonary mortality, and long-term exposure to fine particulate air pollution.* Jama, 2002. **287**(9): p. 1132-41.

89.   Pope, C.A., 3rd, et al., *Lung cancer and cardiovascular disease mortality associated with ambient air pollution and cigarette smoke: shape of the exposure-response relationships.* Environ Health Perspect, 2011. **119**(11): p. 1616-21.

90.   Pope, C.A., 3rd and D.W. Dockery, *Health effects of fine particulate air pollution: lines that connect.* J Air Waste Manag Assoc, 2006. **56**(6): p. 709-42.

91.   Weichenthal, S.A., K. Godri-Pollitt, and P.J. Villeneuve, *PM2.5, oxidant defence and cardiorespiratory health: a review.* Environmental Health, 2013. **12**(1): p. 1-8.

92.   Liberda, E.N. and L.C. Chen, *An evaluation of the toxicological aspects and potential doses from the inhalation of coal combustion products.* Journal of the Air & Waste Management Association, 2013. **63**(6): p. 671-680.

93.   Vinikoor-Imler, L.C., J.A. Davis, and T.J. Luben, *An Ecologic Analysis of County-Level PM2.5 Concentrations and Lung Cancer Incidence and Mortality.* International Journal of Environmental Research and Public Health, 2011. **8**(6): p. 1865.

94.   Kurth, L., et al., *Atmospheric particulate matter in proximity to mountaintop coal mines: sources and potential environmental and human health impacts.* Environ Geochem Health, 2015. **37**(3): p. 529-44.

95.   Kurth, L.M., et al., *Atmospheric particulate matter size distribution and concentration in West Virginia coal mining and non-mining areas.* J Expo Sci Environ Epidemiol, 2014. **24**(4): p. 405-11.

96.   Hendryx, M. and M.M. Ahern, *Mortality in Appalachian coal mining regions: the value of statistical life lost.* Public Health Rep, 2009. **124**(4): p. 541-50.

97.   Knaapen, A.M., et al., *Inhaled particles and lung cancer. Part A: Mechanisms.* Int J Cancer, 2004. **109**(6): p. 799-809.

98.   Schins, R.P., *Mechanisms of genotoxicity of particles and fibers.* Inhal Toxicol, 2002. **14**(1): p. 57-78.

99.   Risom, L., P. Moller, and S. Loft, *Oxidative stress-induced DNA damage by particulate air pollution.* Mutat Res, 2005. **592**(1-2): p. 119-37.

100.   Poma, A., et al., *Genotoxicity induced by fine urban air particulate matter in the macrophages cell line RAW 264.7.* Toxicol In Vitro, 2006. **20**(6): p. 1023-9.

101.   Schwarze, P.E., et al., *Particulate matter properties and health effects: consistency of epidemiological and toxicological studies.* Hum Exp Toxicol, 2006. **25**(10): p. 559-79.

102.   Gualtieri, M., et al., *Airborne urban particles (Milan winter-PM2.5) cause mitotic arrest and cell death: Effects on DNA, mitochondria, AhR binding and spindle organization.* Mutat Res, 2011. **713**(1-2): p. 18-31.

103.   Deng, F., et al., *Effects of dust storm PM2.5 on cell proliferation and cell cycle in human lung fibroblasts.* Toxicol In Vitro, 2007. **21**(4): p. 632-8.

104.   Longhin, E., et al., *Cell cycle alterations induced by urban PM2.5 in bronchial epithelial cells: characterization of the process and possible mechanisms involved.* Particle and Fibre Toxicology, 2013. **10**(1): p. 1-19.

105.   Glowala, M., et al., *HSP70 overexpression increases resistance of V79 cells to cytotoxicity of airborne pollutants, but does not protect the mitotic spindle against damage caused by airborne toxins.* Toxicology, 2002. **170**(3): p. 211-219.

106.   Rieder, C.L., *Mitosis in vertebrates: the G2/M and M/A transitions and their associated checkpoints.* Chromosome Res, 2011. **19**(3): p. 291-306.

107.   Okada, H. and T.W. Mak, *Pathways of apoptotic and non-apoptotic death in tumour cells.* Nat Rev Cancer, 2004. **4**(8): p. 592-603.

108.   Pearce, A.K. and T.C. Humphrey, *Integrating stress-response and cell-cycle checkpoint pathways.* Trends Cell Biol, 2001. **11**(10): p. 426-33.

109.   Branzei, D. and M. Foiani, *Regulation of DNA repair throughout the cell cycle.* Nat Rev Mol Cell Biol, 2008. **9**(4): p. 297-308.

110.   Lapenna, S. and A. Giordano, *Cell cycle kinases as therapeutic targets for cancer.* Nat Rev Drug Discov, 2009. **8**(7): p. 547-66.

111.   Glowala, M., et al., *HSP70 overexpression increases resistance of V79 cells to cytotoxicity of airborne pollutants, but does not protect the mitotic spindle against damage caused by airborne toxins.* Toxicology, 2001. **170**.

112.   Acilan, C. and W.S. Saunders, *A tale of too many centrosomes.* Cell, 2008. **134**(4): p. 572-5.

113.   Zhai, Q., et al., *Genetic damage induced by organic extract of coke oven emissions on human bronchial epithelial cells.* Toxicol In Vitro, 2012. **26**(5): p. 752-8.

114.   Morris, D.L., et al., *Genotoxic effects of fly ash in bacteria, mammalian cells and animals.* Teratog Carcinog Mutagen, 1989. **9**(5): p. 297-314.

115.   da Silva, J., et al., *Genotoxicity biomonitoring in coal regions using wild rodent Ctenomys torquatus by Comet assay and micronucleus test.* Environ Mol Mutagen, 2000. **35**(4): p. 270-8.

116.   Cabarcas-Montalvo, M., J. Olivero-Verbel, and H. Corrales-Aldana, *Genotoxic effects in blood cells of Mus musculus and Iguana iguana living near coal mining areas in Colombia.* Sci Total Environ, 2012. **416**: p. 208-14.

117.   Rohr, P., et al., *Genetic and oxidative damage of peripheral blood lymphocytes in workers with occupational exposure to coal.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2013. **758**(1–2): p. 23-28.

118.   Fenech, M., et al., *Molecular mechanisms by which in vivo exposure to exogenous chemical genotoxic agents can lead to micronucleus formation in lymphocytes in vivo and ex vivo in humans.* Mutation Research/Reviews in Mutation Research, 2016.

119.   Kirsch-Volders, M., et al., *The in vitro MN assay in 2011: origin and fate, biological significance, protocols, high throughput methodologies and toxicological relevance.* Arch Toxicol, 2011. **85**(8): p. 873-99.

120.   Okamoto, A., K. Utani, and N. Shimizu, *DNA replication occurs in all lamina positive micronuclei, but never in lamina negative micronuclei.* Mutagenesis, 2012. **27**(3): p. 323-7.

121.   Zhang, C.-Z., et al., *CHROMOTHRIPSIS FROM DNA DAMAGE IN MICRONUCLEI.* Nature, 2015. **522**(7555): p. 179-184.

122.   Holland, A.J. and D.W. Cleveland, *Chromoanagenesis and cancer: mechanisms and consequences of localized, complex chromosomal rearrangements.* Nat Med, 2012. **18**(11): p. 1630-8.

123.   Thomas, P., et al., *The buccal cytome and micronucleus frequency is substantially altered in Down's syndrome and normal ageing compared to young healthy controls.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2008. **638**(1): p. 37-47.

124.   Holland, N., et al., *The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: the HUMN project perspective on current status and knowledge gaps.* Mutat Res, 2008. **659**(1-2): p. 93-108.

125.   Tolbert, P.E., C.M. Shy, and J.W. Allen, *Micronuclei and Other Nuclear Anomalies in Buccal Smears: A Field Test in Snuff Users.* American Journal of Epidemiology, 1991. **134**(8): p. 840-850.

126.   Thomas, P., et al., *Buccal micronucleus cytome assay.* Nat Protoc, 2009. **4**(6): p. 825-37.

127.   Melody, S.M. and F.H. Johnston, *Coal mine fires and human health: What do we know?* International Journal of Coal Geology, 2015. **152, Part B**: p. 1-14.

128.   Ahern, M. and M. Hendryx, *Cancer Mortality Rates in Appalachian Mountaintop Coal Mining Areas.* J Environ Occup Sci, 2012. **1**(2): p. 63-70.

129.   Hendryx, M., *Mortality from heart, respiratory, and kidney disease in coal mining areas of Appalachia.* Int Arch Occup Environ Health, 2009. **82**(2): p. 243-9.

130.   Weichenthal, S.A., K. Godri-Pollitt, and P.J. Villeneuve, *PM2.5, oxidant defence and cardiorespiratory health: a review.* Environmental Health, 2013. **12**(1): p. 1-8.

131.   Aneja, V.P., A. Isherwood, and P. Morgan, *Characterization of particulate matter (PM10) related to surface coal mining operations in Appalachia.* Atmospheric Environment, 2012. **54**: p. 496-501.

132. (EPA), U.S.E.P.A., *Executive summary, in Air Quality Criteria for Particulate Matter.* National Center for Environmental Assessment, Research Triangle Park, 1996. **I**(Publication No. EPA/600/P-95/001/aF): p. 1–21.

133. Kouimtzis, T., et al., *Airborne Particulate Matter*. 2013: Springer Berlin Heidelberg.

134. Billet, S., et al., *Genotoxic potential of Polycyclic Aromatic Hydrocarbons-coated onto airborne Particulate Matter (PM 2.5) in human lung epithelial A549 cells.* Cancer Lett, 2008. **270**(1): p. 144-55.

135. de Kok, T.M., et al., *Genotoxicity and physicochemical characteristics of traffic-related ambient particulate matter.* Environ Mol Mutagen, 2005. **46**(2): p. 71-80.

136. Schins, R.P. and P.J. Borm, *Mechanisms and mediators in coal dust induced toxicity: a review.* Ann Occup Hyg, 1999. **43**(1): p. 7-33.

137. Jomova, K. and M. Valko, *Advances in metal-induced oxidative stress and human disease.* Toxicology, 2011. **283**(2-3): p. 65-87.

138. Valavanidis, A., K. Fiotakis, and T. Vlachogianni, *Airborne particulate matter and human health: toxicological assessment and importance of size and composition of particles for oxidative damage and carcinogenic mechanisms.* J Environ Sci Health C Environ Carcinog Ecotoxicol Rev, 2008. **26**(4): p. 339-62.

139. Rohr, P., et al., *Genetic and oxidative damage of peripheral blood lymphocytes in workers with occupational exposure to coal.* Mutat Res, 2013. **758**(1-2): p. 23-8.

140. Leon-Mejia, G., et al., *Assessment of DNA damage in coal open-cast mining workers using the cytokinesis-blocked micronucleus test and the comet assay.* Sci Total Environ, 2011. **409**(4): p. 686-91.

141. Rohr, P., et al., *Genetic and oxidative damage of peripheral blood lymphocytes in workers with occupational exposure to coal.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2013. **758**(1–2): p. 23-28.

142. León-Mejía, G., et al., *Genetic damage in coal miners evaluated by buccal micronucleus cytome assay.* Ecotoxicology and Environmental Safety, 2014. **107**: p. 133-139.

143. Bonassi, S., et al., *Micronuclei frequency in peripheral blood lymphocytes and cancer risk: evidence from human studies.* Mutagenesis, 2011. **26**(1): p. 93-100.

144. Bonassi, S., et al., *An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans.* Carcinogenesis, 2007. **28**(3): p. 625-31.

145. Çelik, A., et al., *Bio-monitoring for the genotoxic assessment in road construction workers as determined by the buccal micronucleus cytome assay.* Ecotoxicology and Environmental Safety, 2013. **92**: p. 265-270.

146. Thierens, H., et al., *Cytogenetic monitoring of hospital workers occupationally exposed to ionizing radiation using the micronucleus centromere assay.* Mutagenesis, 2000. **15**(3): p. 245-249.

147. Roy, P., A. Mukherjee, and S. Giri, *Evaluation of genetic damage in tobacco and arsenic exposed population of Southern Assam, India using buccal cytome assay and comet assay.* Ecotoxicology and Environmental Safety, 2016. **124**: p. 169-176.

148. Bonassi, S., M. Milić, and M. Neri, *Frequency of micronuclei and other biomarkers of DNA damage in populations exposed to dusts, asbestos and other fibers. A systematic review.* Mutation Research/Reviews in Mutation Research.

149. Chance, R., T.D. Jickells, and A.R. Baker, *Atmospheric trace metal concentrations, solubility and deposition fluxes in remote marine air over the south-east Atlantic.* Marine Chemistry, 2015. **177, Part 1**: p. 45-56.

150. Zereini, F., et al., *Concentration and distribution of heavy metals in urban airborne particulate matter in Frankfurt am Main, Germany.* Environ Sci Technol, 2005. **39**(9): p. 2983-9.

151. Afroz, R., M.N. Hassan, and N.A. Ibrahim, *Review of air pollution and health impacts in Malaysia.* Environmental Research, 2003. **92**(2): p. 71-77.

152. Kothai, P., et al., *Characterization of Atmospheric Particulate Matter using PIXE Technique.* International Journal of Environmental, Chemical, Ecological, Geological and Geophysical Engineering, 2009. **3**: p. 38-42.

153. Annangi, B., et al., *Biomonitoring of humans exposed to arsenic, chromium, nickel, vanadium, and complex mixtures of metals by using the micronucleus test in lymphocytes.* Mutation Research/Reviews in Mutation Research.

154. Lestari, F., et al., *In vitro cytotoxicity of selected chemicals commonly produced during fire combustion using human cell lines.* Toxicology in Vitro, 2005. **19**(5): p. 653-663.

155. Nickens, K.P., S.R. Patierno, and S. Ceryak, *Chromium genotoxicity: A double-edged sword.* Chemico-Biological Interactions, 2010. **188**(2): p. 276-288.

156. Alimba, C.G., et al., *Genotoxicity and cytotoxicity of chromium, copper, manganese and lead, and their mixture in WIL2-NS human B lymphoblastoid cells is enhanced by folate depletion.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2016. **798–799**: p. 35-47.

157. Valko, M., et al., *Free radicals, metals and antioxidants in oxidative stress-induced cancer.* Chem Biol Interact, 2006. **160**(1): p. 1-40.

158. Meng, Z., G. Qin, and B. Zhang, *DNA damage in mice treated with sulfur dioxide by inhalation.* Environ Mol Mutagen, 2005. **46**(3): p. 150-5.

159. Uren, N., S. Yuksel, and Y. Onal, *Genotoxic effects of sulfur dioxide in human lymphocytes.* Toxicology and Industrial Health, 2014. **30**(4): p. 311-315.

160. Vasylkiv, O.Y., et al., *Cytotoxicity of chromium ions may be connected with induction of oxidative stress.* Chemosphere, 2010. **80**(9): p. 1044-1049.

161. Prousek, J., *Fenton chemistry in biology and medicine.* Pure Appl. Chem. , 2007. **79**: p. 2325–2338.

162. Speisky, H., et al., *Generation of superoxide radicals by copper–glutathione complexes: Redox-consequences associated with their interaction with reduced glutathione.* Bioorganic & Medicinal Chemistry, 2009. **17**(5): p. 1803-1810.

163. Espitia-Pérez, L., et al., *Polymorphisms in metabolism and repair genes affects DNA damage caused by open-cast coal mining exposure.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2016. **808**: p. 38-51.

164. Bray, T.M. and W.J. Bettger, *The physiological role of zinc as an antioxidant.* Free Radic Biol Med, 1990. **8**(3): p. 281-91.

165. Zeidler, P., et al., *Role of inducible nitric oxide synthase-derived nitric oxide in silica-induced pulmonary inflammation and fibrosis.* J Toxicol Environ Health A, 2004. **67**(13): p. 1001-26.

166. Alfaro-Moreno, E., et al., *Biologic effects induced in vitro by PM10 from three different zones of Mexico City.* Environmental Health Perspectives, 2002. **110**(7): p. 715-720.

167. Rinaldi, M., et al., *Primary and Secondary Organic Marine Aerosol and Oceanic Biological Activity: Recent Results and New Perspectives for Future Studies.* Advances in Meteorology, 2010. **2010**.

168. Longhin, E., et al., *Cell cycle alterations induced by urban PM2.5 in bronchial epithelial cells: characterization of the process and possible mechanisms involved.* Particle and Fibre Toxicology, 2013. **10**(1): p. 1-19.

169. Cao Guo Liang, Z.X., Gong SunLing, An Xin Qin & Wang Ya Qiang, *Emission inventories of primary particles and pollutant gases for China.* Atmospheric Science, 2011. **56**(8): p. 781-788.

170. Pone, J.D.N., et al., *The spontaneous combustion of coal and its by-products in the Witbank and Sasolburg coalfields of South Africa.* International Journal of Coal Geology, 2007. **72**(2): p. 124-140.

171. Ghose, M.K. and S.R. Majee, *Characteristics of hazardous airborne dust around an Indian surface coal mining area.* Environ Monit Assess, 2007. **130**(1-3): p. 17-25.

172. Ghose, M.K. and S.R. Majee, *Assessment of Dust Generation Due to Opencast Coal Mining– An Indian Case Study.* Environmental Monitoring and Assessment. **61**(2): p. 257-265.

173. Gauthier, P.T., et al., *Metal–Polycyclic Aromatic Hydrocarbon Mixture Toxicity in Hyalella azteca. 2. Metal Accumulation and Oxidative Stress as Interactive Co-toxic Mechanisms.* Environmental Science & Technology, 2015. **49**(19): p. 11780-11788.

174. Peng, C., et al., *Micronucleus formation by single and mixed heavy metals/loids and PAH compounds in HepG2 cells.* Mutagenesis, 2015.

175. Schins, R.P. and P.J. Borm, *Mechanisms and mediators in coal dust induced toxicity: a review.* Annals of Occupational Hygiene, 1999. **43**(1): p. 7-33.

176. Schins, R.P., *Mechanisms of genotoxicity of particles and fibers.* Inhal Toxicol, 2002. **14**(1): p. 57-78.

177. Pless-Mulloli, T., et al., *Living near opencast coal mining sites and children's respiratory health.* Occupational and Environmental Medicine, 2000. **57**(3): p. 145-151.

178. Bolognesi, C., et al., *Clinical application of micronucleus test in exfoliated buccal cells: A systematic review and metanalysis.* Mutat Res Rev Mutat Res, 2015. **766**: p. 20-31.

179. Katarkar, A., et al., *Comparative evaluation of genotoxicity by micronucleus assay in the buccal mucosa over comet assay in peripheral blood in oral precancer and cancer patients.* Mutagenesis, 2014. **29**(5): p. 325-334.

180. Thomas, P., et al., *The buccal cytome and micronucleus frequency is substantially altered in Down's syndrome and normal ageing compared to young healthy controls.* Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 2008. **638**(1): p. 37-47.

181. Thomas, P., et al., *Buccal micronucleus cytome assay.* Nat Protoc, 2009. **4**(6): p. 825-37.

182. Tolbert, P.E., C.M. Shy, and J.W. Allen, *Micronuclei and other nuclear anomalies in buccal smears: methods development.* Mutat Res, 1992. **271**(1): p. 69-77.

183. Shi, Q. and R.W. King, *Chromosome nondisjunction yields tetraploid rather than aneuploid cells in human cell lines.* Nature, 2005. **437**(7061): p. 1038-42.

184. Klimkina, I., *Assessment of mutagenic health effects in coal mine workers.* Genome, 2013. **18**: p. 31.

185.   Schins, R.P., P.A. Schilderman, and P.J. Borm, *Oxidative DNA damage in peripheral blood lymphocytes of coal workers.* International archives of occupational and environmental health, 1995. **67**(3): p. 153-157.

186.   Ceppi, M., et al., *Human population studies with the exfoliated buccal micronucleus assay: statistical and epidemiological issues.* Mutat Res, 2010. **705**(1): p. 11-9.

187.   Gautam, S., et al., *Characterization of PM2.5 generated from opencast coal mining operations: A case study of Sonepur Bazari Opencast Project of India.* Environmental Technology & Innovation, 2016. **6**: p. 1-10.

188.   Schweinsberg, F. and L. Von Karsa, *Heavy metal concentrations in humans.* Comparative Biochemistry and Physiology Part C: Comparative Pharmacology, 1990. **95**(2): p. 117-123.

189.   Bridges, C.C. and R.K. Zalups, *Molecular and ionic mimicry and the transport of toxic metals.* Toxicology and applied pharmacology, 2005. **204**(3): p. 274-308.

190.   José Zocche, J., et al., *Heavy metals and DNA damage in blood cells of insectivore bats in coal mining areas of Catarinense coal basin, Brazil.* Environmental Research, 2010. **110**(7): p. 684-691.

191.   Guerrero-Castilla, A., J. Olivero-Verbel, and J. Marrugo-Negrete, *Heavy metals in wild house mice from coal-mining areas of Colombia and expression of genes related to oxidative stress, DNA damage and exposure to metals.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2014. **762**: p. 24-29.

192.   WHO/FAO/IAEA, *Trace elements in human nutrition and health.* World Health Organization, Geneva, 1996.

193.   Avila Junior, S., et al., *Occupational airborne contamination in south Brazil: 1. Oxidative stress detected in the blood of coal miners.* Ecotoxicology, 2009. **18**(8): p. 1150-7.

194.   Barceloux, D.G. and D. Barceloux, *Nickel.* Journal of Toxicology: Clinical Toxicology, 1999. **37**(2): p. 239-258.

195.   Dally, H. and A. Hartwig, *Induction and repair inhibition of oxidative DNA damage by nickel (II) and cadmium (II) in mammalian cells.* Carcinogenesis, 1997. **18**(5): p. 1021-1026.

196.   Milman, N., et al., *Elements in normal and cirrhotic human liver tissue II. Potassium, sulphur, chlorine and bromine measured by X‐ray fluorescence spectrometry.* Liver, 1987. **7**(4): p. 206-210.

197.   Bogden, J.D. and L.M. Klevay, *Clinical nutrition of the essential trace elements and minerals: the guide for health professionals*. 2000: Humana Press.

198.   Hayes, A.W., *Principles and Methods of Toxicology, Fifth Edition*. 2007: Taylor & Francis.

199.   Ballmaier, D. and B. Epe, *DNA damage by bromate: Mechanism and consequences.* Toxicology, 2006. **221**(2–3): p. 166-171.

200.   Benedetti, D., et al., *Genetic damage in soybean workers exposed to pesticides: Evaluation with the comet and buccal micronucleus cytome assays.* Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2013. **752**(1–2): p. 28-33.

201.   Knaapen, A.M., et al., *Inhaled particles and lung cancer. Part A: Mechanisms.* International Journal of Cancer, 2004. **109**(6): p. 799-809.

202.   Sam, S.S., et al., *Gene-environment interactions associated with CYP1A1 MspI and GST polymorphisms and the risk of upper aerodigestive tract cancers in an Indian population.* J Cancer Res Clin Oncol, 2010. **136**(6): p. 945-51.

203.   Kvitko, et al., *Cytochrome P4501A1 polymorphisms in South American Indians.* Hum Biol, 2000. **72**(6): p. 1039-43.

204. Oubrahim, H., P.B. Chock, and E.R. Stadtman, *Manganese(II) Induces Apoptotic Cell Death in NIH3T3 Cells via a Caspase-12-dependent Pathway.* Journal of Biological Chemistry, 2002. **277**(23): p. 20135-20138.

205. Ward, R.J., Y. Zhang, and R.R. Crichton, *Aluminium toxicity and iron homeostasis.* Journal of inorganic biochemistry, 2001. **87**(1): p. 9-14.

206. Banasik, A., et al., *Aluminum-induced micronuclei and apoptosis in human peripheral-blood lymphocytes treated during different phases of the cell cycle.* Environmental toxicology, 2005. **20**(4): p. 402-406.

207. Lankoff, A., et al., *A comet assay study reveals that aluminium induces DNA damage and inhibits the repair of radiation-induced lesions in human peripheral blood lymphocytes.* Toxicol Lett, 2006. **161**(1): p. 27-36.

208. Beckman, R.A., A.S. Mildvan, and L.A. Loeb, *On the fidelity of DNA replication: manganese mutagenesis in vitro.* Biochemistry, 1985. **24**(21): p. 5810-5817.

209. Kim, J.Y., et al., *Urinary 8-hydroxy-2'-deoxyguanosine as a biomarker of oxidative DNA damage in workers exposed to fine particulates.* Environ Health Perspect, 2004. **112**(6): p. 666-71.

210. Brouillet, E.P., et al., *Manganese injection into the rat striatum produces excitotoxic lesions by impairing energy metabolism.* Exp Neurol, 1993. **120**(1): p. 89-94.

211. Colaizzi, G.J., *Prevention, control and/or extinguishment of coal seam fires using cellular grout.* International Journal of Coal Geology, 2004. **59**(1–2): p. 75-81.

212. Rojano, R., et al., *PM10 emissions due to storage in coal piles in a mining industrial area.* WIT Transactions on Ecology and The Environment, 2016. **207**: p. 87-97.

213. Pandey, B., M. Agrawal, and S. Singh, *Assessment of air pollution around coal mining area: Emphasizing on spatial distributions, seasonal variations and heavy metals, using cluster and principal component analysis.* Atmospheric Pollution Research, 2014. **5**(1): p. 79-86.

214. Medunic, G., et al., *Toxic airborne S, PAH, and trace element legacy of the superhigh-organic-sulphur Rasa coal combustion: Cytotoxicity and genotoxicity assessment of soil and ash.* Sci Total Environ, 2016. **566-567**: p. 306-19.

215. Simonton, D.S. and S. King, *Hydrogen Sulfide Formation and Potential Health Consequences in Coal Mining Regions.* Water Quality, Exposure and Health, 2013. **5**(2): p. 85-92.

216. Cartledge, B.T. and B.J. Majestic, *Metal concentrations and soluble iron speciation in fine particulate matter from light rail activity in the Denver-Metropolitan area.* Atmospheric Pollution Research, 2015. **6**(3): p. 495-502.

217. Petaloti, C., et al., *Trace elements in atmospheric particulate matter over a coal burning power production area of western Macedonia, Greece.* Chemosphere, 2006. **65**(11): p. 2233-2243.

218. (ATSDR), A.f.T.S.a.D.R., *Toxicological profile for Zinc.* 2005, Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service.

219. Dao, X., et al., *Chemical Characteristics of Water-Soluble Ions in Particulate Matter in Three Metropolitan Areas in the North China Plain.* PLoS ONE, 2014. **9**(12): p. e113831.

220. Kraniak, J.M., et al., *Antioxidant agents transiently inhibit aneuploidy progression in Li-Fraumeni cell strains.* Mol Carcinog, 2006. **45**(3): p. 141-56.

221. Van de Wetering, C.I., et al., *Manganese Superoxide Dismutase gene dosage affects chromosomal instability and tumor onset in a mouse model of T cell lymphoma.* Free radical biology & medicine, 2008. **44**(8): p. 1677-1686.

222. D'Angiolella, V., C. Santarpia, and D. Grieco, *Oxidative stress overrides the spindle checkpoint.* Cell Cycle, 2007. **6**(5): p. 576-9.

223. Parker, A.L., M. Kavallaris, and J.A. McCarroll, *Microtubules and Their Role in Cellular Stress in Cancer.* Frontiers in Oncology, 2014. **4**: p. 153.

224. Choi, W.J., et al., *Oxidative stress and tumor necrosis factor-alpha-induced alterations in metaphase II mouse oocyte spindle structure.* Fertil Steril, 2007. **88**(4 Suppl): p. 1220-31.
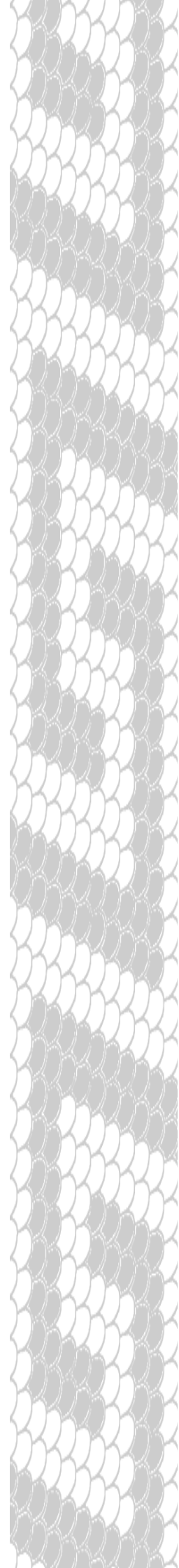
ANEXOS
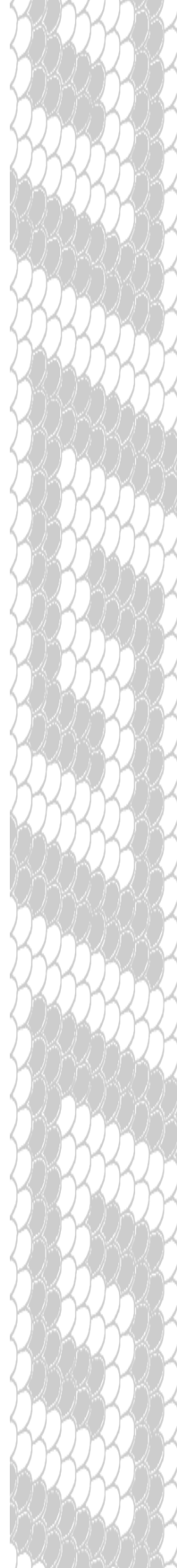
ANEXO I

Polymorphisms in metabolism and repair genes affects DNA damage caused by open-cast coal mining exposure

Polymorphisms in metabolism and repair genes affects DNA damage caused by open-cast coal mining exposure

Lyda Espitia-Pérez [a,b,c,*], Milton Quintana Sosa [c,1], Shirley Salcedo-Arteaga [c], Grethel León-Mejía [a,b,1], Luz Stella Hoyos-Giraldo [d], Hugo Brango [e], Katia Kvitko [f], Juliana da Silva [g], João A.P. Henriques [a,b,**]

[a] Programa de Pós Graduação em Biologia Celular e Molecular (PPGBCM), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Brazil
[b] Departamento de Biofísica, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[c] Laboratorio de Investigación Biomédica y Biología Molecular, Universidad del Sinú, Montería, Córdoba, Colombia
[d] Department of Biology, Research Group Genetic Toxicology and Cytogenetics, Faculty of Natural Sciences and Education, Universidad del Cauca, Popayán, Cauca, Colombia
[e] Instituto de Matemática e Estatística, Universidade de São Paulo, São Paulo, Brazil
[f] Programa de Pós-Graduação em Genética e Biologia Molecular (PPGGBM), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil
[g] Laboratório de Genética Toxicológica, Universidade Luterana do Brasil, ULBRA, Canoas, RS, Brazil

A R T I C L E   I N F O

A B S T R A C T

Increasing evidence suggest that occupational exposure to open-cast coal mining residues like dust particles, heavy metals and Polycyclic Aromatic Hydrocarbons (PAHs) may cause a wide range of DNA damage and genomic instability that could be associated to initial steps in cancer development and other work-related diseases. The aim of our study was to evaluate if key polymorphisms in metabolism genes $CYP1A1_{Msp1}$, $GSTM1_{Null}$, $GSTT1_{Null}$ and DNA repair genes $XRCC1_{Arg194Trp}$ and $hOGG1_{Ser326Cys}$ could modify individual susceptibility to adverse coal exposure effects, considering the DNA damage (Comet assay) and micronucleus formation in lymphocytes (CBMN) and buccal mucosa cells (BMNCyt) as endpoints for genotoxicity. The study population is comprised of 200 healthy male subjects, 100 open-cast coal-mining workers from "El Cerrejón" (world's largest open-cast coal mine located in Guajira – Colombia) and 100 non-exposed referents from general population. The data revealed a significant increase of CBMN frequency in peripheral lymphocytes of occupationally exposed workers carrying the wild-type variant of $GSTT1_{(+)}$ gene. Exposed subjects carrying $GSTT1_{null}$ polymorphism showed a lower micronucleus frequency compared with their positive counterparts (FR: 0.83; P = 0.04), while BMNCyt, frequency and Comet assay parameters in lymphocytes: Damage Index (DI) and percentage of DNA in the tail (Tail % DNA) were significantly higher in exposed workers with the $GSTM1_{Null}$ polymorphism. Other exfoliated buccal mucosa abnormalities related to cell death (Karyorrhexis and Karyolysis) were increased in $GSTT/M1_{Null}$ carriers. Nuclear buds were significantly higher in workers carrying the $CYP1A1_{Msp1}$ (m1/m2, m2/m2) allele. Moreover, BMNCyt frequency and Comet assay parameters were significantly lower in exposed carriers of $XRCC1_{Arg194Trp}$ (Arg/Trp, Trp/Trp) and $hOGG1_{Ser326Cys}$ (Ser/Cys, Cys/Cys), thereby providing new data to the increasing evidence about the protective role of these polymorphisms. This modulation may involve specific and differentiated pathways in different tissues that also may cause a differential sensitivity related to differential induction of some enzymes.

* Corresponding author at: Calle 38 Carrera 1W, Barrio Juan XXIII – Laboratorio de Investigación Biomédica y Biología Molecular, Universidad del Sinú – Elías Bechara Zainúm, Montería, Córdoba, Colombia.
** Corresponding author at: Departamento de Biofísica, Prédio 43422, Laboratório 210, Campus do Vale, Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves 9500, Bairro Agronomia, CEP 91501-970, Porto Alegre, RS, Brazil.
E-mail addresses: espitiaperez00@gmail.com (L. Espitia-Pérez), pegas@cbiot.ufrgs.br (J.A.P. Henriques).
[1] Present address: Unidad de Investigación, Desarrollo e Innovación en Genética y Biología Molecular, Universidad Simón Bolívar, Barranquilla, Colombia.

1. Introduction

The coal-mining region in northern Colombia is one of the largest open pit mining regions of the world. In 2009, there were eight mining companies in operation with an approximate coal production of 70 Mt/year. In 2010, more than 33,372 workers were employed full time in coal mines in the country and more than 25,000 individuals worked in open-cast systems [1,2]. In this particular extraction method, large amounts of dust particles are released into the atmosphere as fugitive particulate matter [3]. In addition to coal, which is the main component, this mixture can also contain oxygen, nitrogen, hydrogen, trace elements, and several inorganic minerals. The trace elements may include silica, copper, aluminum, nickel, cadmium, boron, antimony, iron, lead, and zinc [3]. Table 1 shows some of the main environmental pollutants and chemical substances detected in coal, ashes and combustion processes in coal mining systems around the world.

Excess occupational exposure to metals, particularly in mining is considered to be major cause of metal-related cancer [4]. Additionally, in open-cast mines from north Colombia extracted coal is stored under the sunlight at high ambient temperatures, where spontaneous and incomplete coal combustion may lead in Polycyclic Aromatic Hydrocarbons (PAHs) emission [5], most of which exhibit well-known mutagenic and carcinogenic activity [6]. Particularly, in open-cast mining facilities these toxic substances are released in the atmosphere where they can form complex mixtures [7]. Such mixtures represent one of the most important health and safety hazards to this industry's workers due to potential synergistic effects of the resulting combinations [8]. For most chemical compounds found in complex mixtures generated in open cast mines, metabolic activation is required for the formation of electrophilic intermediates capable of binding to cellular macromolecules and DNA [9,10]. As consequence of cellular metabolism, some of these intermediates and some heavy metals found in blood samples from exposed individuals could be involved in the generation of oxidatively damaged DNA and proteins [11,12]. In this order, susceptibility to the hazardous action of this chemicals may derive from genetic or acquired characteristics of the individual, and may be associated with variations in genes encoding for carcinogen or xenobiotic-metabolizing enzymes, such as Cytochrome P-450 *(CYP)* in Phase I and Glutathione *S*-transferases *(GST)* in Phase II [13] and also in DNA repair genes majorly involve in oxidative damage repair such as X-ray repair complementing defective in Chinese hamster 1 *(XRCC1)* and 8-oxo guanine-DNA glycosylase 1 *(hOGG1)*.

CYP and *GST* genes play an important role in the detoxification of a wide range of human carcinogens, including several residues as PAHs, and particulate matter from coal mining activities [14]. In general, carcinogenic PAHs, such as benzo (a) pyrene, need to be activated by the phase I enzymes *(*e.g., Cytochrome P450 1A1*)* to form ultimate carcinogens, such as B (a) P diol epoxide (BPDE), whereas the phase II enzymes (e.g., glutathione S transferases) generally mediate the conjugation of water-soluble moieties, such as glutathione, which are responsible for detoxification of these reactive metabolites [15]. Hence, the coordinated expression and regulation of phase-I and −II enzymes determines the outcome of carcinogen exposure. The capacity to repair DNA damage induced by activated carcinogens is also a host factor that may influence the risk of cytogenetic instability. Human 8-oxoguanine DNA glycosylase 1 *(hOGG1)* and X-ray repair cross complementing group 1 *(XRCC1)* are BER enzymes involved in the core processes of single-strand break repair. *hOGG1,* a glycosylase, helps in the excision of oxidized guanine, while *XRCC1* stimulates endonuclease action and acts as a scaffold protein in the subsequent restoration of the site.

Cytogenetic endpoints have been extensively employed in surveillance of human genotoxic exposure and increased chromosomal damage has been shown to be predictive of elevated cancer risk [16]. The micronucleus (MN) assay in target as well as non-target cells is used as an indicator of chromosomal damage in interphase cells and is associated with early events in carcinogenesis. Micronuclei in exfoliated cells emerge during mitosis of the basal layers of the epithelium and their absolute quantities could reflect the real situation in target cells [17]. The micronucleus cytome assay applied in buccal exfoliated cells (BMNCyt) provides a complementary method for measuring DNA damage and cytotoxic effects in an easily accessible tissue not requiring in vitro culture [18]. The comet assay is a rapid, sensitive and relatively simple method for measuring DNA damage and has been widely adopted in as a biomarker assay in human biomonitoring studies, in 'biological effect dosing' of occupational and environmental exposures [19,20].

The mining region of "El Cerrejón" includes the world's largest open-cast coal mine located in the northwest Colombian state of Guajira and concentrates most of the country's mining sector workers. In this scenario, the purpose of the present cross-sectional study is to evaluate if the $CYP1A1_{Msp1}$ *(rs.4646903)*, $GSTM1_{null}$, $GSTT1_{null}$, $XRCC1_{Arg194Trp}$ *(rs.1799782)* and $hOGG1_{Ser326Cys}$ *(rs. 1052133)* polymorphisms could have an influence on the individual susceptibility to DNA damage caused by coal residues exposure, as previously demonstrated by primary DNA damage in lymphocytes and MN formation in lymphocytes [21] and oral mucosa of exposed workers [11]. Our results will contribute to identify metabolic and DNA-repair polymorphisms involved with the modulation of DNA damage in populations occupationally exposed to open-cast coal mining residues.

2. Methods

2.1. Study population and sample collection

The Committee on Research Ethics of each institution approved this study and a written informed consent was obtained from each individual before sample collection. The study population comprised a total of 200 healthy males (in total). To calculate the size of the sample was considered the minimum necessary to be detected at least 1% of the genetic polymorphism less frequent in the studied population. Within the study population 100 were exposed workers from "El Cerrejón" open-cast mine engaged in surface activities inside several areas of the mining complex showed in Fig. 1, who were exposed to coal dust for at least 5 years. Main chemical and mineralogical properties of coal and coal fly ashes from sampling areas ares described at Table 2. The mean time of service ± standard deviation (SD) of the exposed group was 17.7 ± 6.9 years (range, 5–30 years). The non-exposed reference group consisted of 100 males with no known exposure to genotoxic agents such as coal dust, radiation, chemicals, cigarette, etc., and was selected from the general local population. Exposed workers were matched to non-exposed referents by age (± 2 years) and similar social-economic status. The mean age of exposed group was 44.0 ± 7.5 years (range, 24–60 years), and non-exposed reference group was 43.7 ± 7.8 years (range, 26–60 years).

Confounding and exclusion factors were collected from all participants who responded to an interviewer-administered, detailed and standard questionnaire which included data on lifestyle, health status, cancer history, other chronic diseases, nutrition and smoking habits, alcohol and medication intake, occupational and time exposure, adoption of protective measurements, and previous exposure to medical X-rays or treatment with known carcinogens. Exclusion criteria for exposed and non-exposed reference groups were age over 60 years or less than 18 years, current and previous smoking habits, medical treatment for up to 3 months or X-ray up to 1 year before sampling, as well as therapeutic drugs intake,

Table 1
Main environmental pollutants and chemical substances detected in coal, ashes and combustion processes around coal mining systems.

Compounds	Sample	Methods	Country of origin	References
Volatile Organic Compounds (VOC)				
Benzene	Coal-fire gas mineral; gas samples	XRD	South Africa	Pone et al. [43]
m/p-Xylene	Coal-fire gas mineral; gas samples	XRD	South Africa	Pone et al. [43]
Toluene	Coal gases	GC–MS	United States	Puettmann et al. [82]
Ethylbenzene	Coal-fire gas mineral; gas samples	XRD	South Africa	Pone et al. [43]
Phenanthrene	PM_{10}, $PM_{2.5}$ filters	LVI- GC/MS	Greece	Evagelopoulos et al. [83]
Anthracene	Burning coal waste samples	GC–MS	Portugal	Ribeiro et al. [84]
Pyrene	PM_{10}, $PM_{2.5}$ filters	LVI- GC/MS	Greece	Evagelopoulos et al. [83]
Benzo[a]anthracene	PM_{10}, $PM_{2.5}$ filters	LVI- GC/MS	Greece	Evagelopoulos et al. [83], Voutsa et al. [85]
Benzo[k]fluoranthene	Burning coal waste samples, TSP samples	GC–MS; HRGC/HRMS	Portugal, Greece	Ribeiro et al. [84], Voutsa et al. [85]
Benzo[b]fluoranthene	TSP samples	HRGC/HRMS	Greece	Voutsa et al. [85]
Fluorene	Burning coal waste samples, TSP samples	GC–MS; HRGC/HRMS	Portugal, Greece	Ribeiro et al. [84], Voutsa et al. [85]
1,2,4-Trimethylbenzene	TSP samples	HRGC/HRMS	Greece	Voutsa et al. [85]
Aliphatic hydrocarbon				
Ethane	Coal-fire gas mineral; gas samples	XRD, TPO	South Africa, China	Pone et al. [43], Yu-guo et al. [86]
Ethene	Coal-fire gas mineral; gas samples	XRD	South Africa	Pone et al. [43]
1-Butene	Coal-fire gas mineral; gas samples	XRD	South Africa	Pone et al. [43]
Cis-2-pentene	Coal-fire gas mineral; gas samples	XRD	South Africa	Pone et al. [43]
n-Heptane	Coal-fire gas mineral; gas samples	XRD	South Africa	Pone et al. [43]
Cyclopentane	Coal-fire gas mineral; gas samples	XRD	South Africa	Pone et al. [43]
n-Octane	Coal-fire gas mineral; gas samples	XRD	South Africa	Pone et al. [43]
Halogenated hydrocarbon				
Trichloroethylene	Coal-fire gas mineral; gas samples	XRD	South Africa	Pone et al. [43]
Methyl iodide	Coal-fire gas mineral; gas samples	XRD	South Africa	Pone et al. [43]
Greenhouse gases and others				
Methane	Coal gases	FTIR, TPO	United States, China	Kirchgessner et al. [87], Yu-guo et al. [86]
Carbon monoxide	Coal gases	FTIR, TPO	United States; China	Kirchgessner et al. [87], Yu-guo et al. [86]
Carbon dioxide	Coal-fire gas mineral; gas samples	XRD	South Africa; China	Pone et al. [43]
Oxides				
K_2O	Coal ashes	XRD	China	Xing et al. [89]
Ti_2O	Coal-fire gas mineral; gas samples	XRD	South Africa	Pone et al. [43]
CaO	Coal ashes	XRD	China	Xing et al. [89]
Na_2O	Coal-fire gas mineral; gas samples	XRD	South Africa	Pone et al. [43]
Fe_2O_3	Solid waste samples, combused ashes	XRF	China	Zhao et al. [90]
SO_3	Coal-fire gas mineral; gas samples	XRD	South Africa	Pone et al. [43]
Others elements				
C	PM_{10} filters	XPS	Colombia	Huertas et al. [91], Querol et al. [92]
O	PM_{10} filters	XPS	Colombia	Huertas et al. [91]
Sb	Coal ashes	SEM-EDS	United States, Serbia	Damle et al. [93], Cvetkovicí et al. [94]
Ca	Coal samples	SEM-EDX	United States	Damle et al. [93], Jones et al. [95]
Na	Coal ashes	GFAAS	Poland	Kaupplnen and Pakkanen [96]
Cd	TSP samples	XRF	Greece	Petaloti et al. [97]
S	Coal and coal clays	SEM, EXMA	United States	Finkelman [98], Jones et al. [95]
Al	TSP samples	XRF	Greece	Petaloti et al. [97]; Jones et al. [95]
As	Coal gangue and products from gas vents	XRD; SEM	Czech Republic, China	Tichý [88], Querol et al. [92]
Hg	Coal ashes	GFAAS	Poland	Kaupplnen and Pakkanen [96], Gustin et al. [99]
Be	Solid waste samples, combused ashes, PM_{10} filters	XRF; ICP-MS;	China	Zhao et al. [90], Aneja et al. [100]
Si, Se	Solid waste samples, combused ashes	XRF; ICP-MS	China	Zhao et al. [90], Querol et al. [92]
Pb	Solid waste samples, combused ashes	XRF; ICP-MS	China	Zhao et al. [90], Querol et al. [92]
Zn	TSP samples	XRF	Greece	Petaloti et al. [97]
Ba	Coal gangue and products from gas vents	XRD; SEM	China	Querol et al. [92]

XRD: X-ray diffraction; XPS: X-ray photoelectron spectroscopy; ICP-MS: Inductively coupled plasma-mass spectrometer; XPS: X-ray photoelectron spectroscopy; XRF: X-ray fluorescence spectrometry; ICP-MS: Inductively coupled plasma-mass spectrometer; SEM: Scanning Electron Microscope; FTIR: Fourier transform infrared spectroscopy; LVI–GC/MS: Large Volume–Gas Chromatography/Mass Spectrometry; GC–MS: Gas chromatography–mass spectrometry; HRGC/HRMS: High Resolution Gas Chromatography/High Resolution Mass Spectrometry; GFAAS: furnace atomic absorption spectrometer; TPO: temperature programmed oxidation (TPO); EDS: energy-dispersive spectrometer,; EXMA: electron probe X-ray microanalysis.
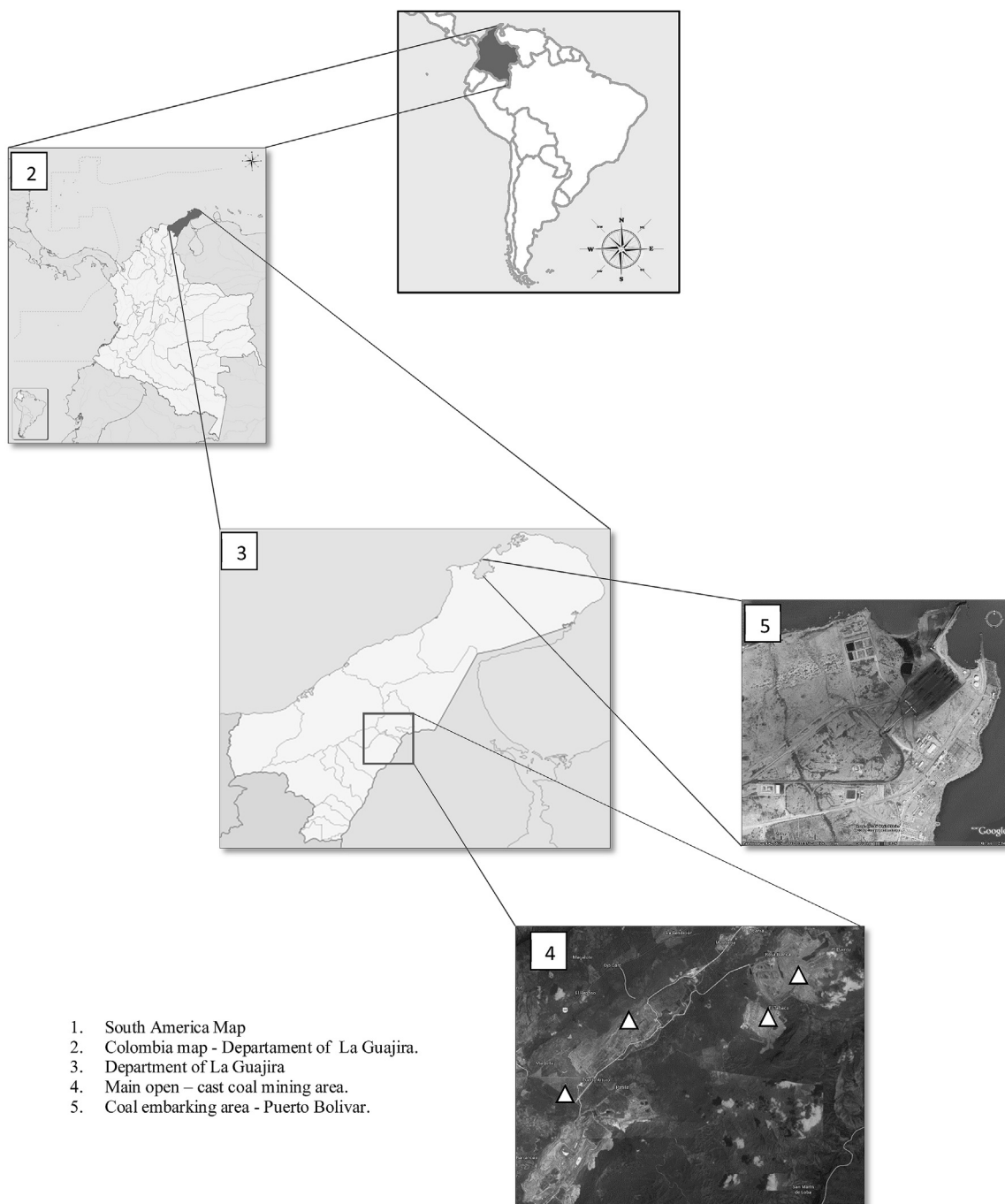
1. South America Map
2. Colombia map - Departament of La Guajira.
3. Department of La Guajira
4. Main open – cast coal mining area.
5. Coal embarking area - Puerto Bolivar.

Fig. 1. Geographic localization of sampling areas in Guajira's coal deposits.

known to be genotoxic, mutagenic or carcinogenetic. All data were structured and maintained in databases. No major differences in social-economic status and dietary habits were recorded. Detailed demographic characteristics of the studied population can be found in a previous study in León-Mejía et al. [21].

2.2. Blood samples collection

Peripheral blood samples were collected by venipuncture from all 200 participants: 20 mL of blood in heparin tubes (Becton Dickenson, vaccutainer), 10 mL for the MN assay and 10 mL for the Comet Assay, and 10 mL of blood were collected in EDTA coated tubes for the DNA isolation and genotyping. Blood sampling was conducted by personnel with medical training at the end of a working period, consisting in a 15-days period of 8 h inside the mine. As previously described [22], concomitantly with collection of the blood samples of exposed and non-exposed individuals, additional samples of whole blood from the research staff were collected, transported and processed under the same conditions. These samples were used as internal standards for the detection of potential confounding factors that may have been caused by sample handling, processing or transportation to the laboratory. All blood samples tubes were coded and kept upright at room temperature in the dark during the transportation (overnight) to the laboratory, where the samples were processed immediately upon arrival. Blood sampling was carried out from June 2009 to January 2010.

Table 2
Main chemical and mineralogical properties of coal and coal fly ashes from sampling areas.

General properties (wt%)			
Moisture	5.40	Ashes	12
Elemental analysis (wt%)[b]			
C	70.10	N	4.30
H	5.50	S	0.90
Coal trace elements (ppm)[a]			
Si	0.69	Mn	52.40
Al	0.67	Ni	9.90
Cr	14.50	Zn	24.80
Cu	9.80	Pb	1.39
Coal ash major oxides (wt%)[b]			
SiO_2	73.00	CaO	1.97
Al_2O_3	8.20	MgO	2.22
K_2O	0.57	Na_2O	2.10
Ti_2O	0.89	MnO	0.06
P_2O_5	0.18	Fe_2O_3	11.90
SO_3	9.77	K_2O	0.93
Mineralogy of LTA[c] residues (wt.%)[a]			
Quartz	54.20	Pyrite	12.90
Kaolinite	16.90	Illite	9.40

[a] Valentim et al. [101].
[b] Carmona-Ward [102].
[c] LTA: Low-temperature ash.

2.3. Micronucleus assay (CBMN assay)

CBMN assay was carried out according to previous methodology described in León-Mejía et al [21]. Briefly, heparinized whole blood (0.5 mL) was added to 4.5 mL of RPMI 1640 medium (Sigma R8758, USA) supplemented with 2 mM l-glutamine (Sigma A5955, USA), 10% fetal bovine serum (Gibco/Invitrogen 15000-044, Brazil), 100 μL/mL antibiotic–antimycotic (Sigma A5955, USA) and 2% phytohemaglutinin (Sigma L8754, USA). Cultures were incubated at 37 °C in the dark for 44 h, under 5% CO_2. 6 μg/mL of cytocalasin B (Sigma, C6762) was added at the 44th h of incubation. The scoring criteria followed those proposed by Fenech et al. [23].

2.4. Buccal micronucleus cytome assay (BMCyt assay)

Buccal mucosa samples were collected as previously described in León-Mejía et al. [21]. Briefly, subjects were asked to rinse their mouth with water before sampling. The exfoliated buccal mucosa cells were collected using a cytobrush to gently scrape the mucosa of the inner lining of both cheeks. All buccal sample tubes were coded and kept in upright position at room temperature. The cells were washed three times in 0.9% phosphate saline buffer, the smears were made from the pellet and fixed in methanol: acetic acid (3:1). For microscopic analysis, the slides were incubated at 37 °C overnight and then stained with Giemsa [24]. The frequency of MN and other nuclear abnormalities such as karyorrhectic and karyolytic cells (different forms of cell death), and nuclear buds (indicative of gene amplification) were determined in 2000 cells for each person following recommendations of Thomas et al. [25]. All slides were scored by one reader blinded to the exposure status of the individuals.

2.5. Single cell gel electrophoresis (SCGE – comet assay test)

The alkaline Comet assay was performed as described by León-Mejía et al. [21]. Briefly, 30 μL of isolated lymphocytes by Histopaque 1077, were mixed with 270 μL 0.5% of low melting point (LMA-Invitrogen) at 37 °C. This mixture was placed into a slide

previously coated with 1.5% of normal melting point agarose (NMA-Cambrex Bioscience Rockland) processed at 60 °C. The agarose layers were covered with a cover slip and after gel solidifying the cover slips were removed. The slides were immersed overnight in lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0–10.5, 1% with freshly added 1% Triton X-100 and 10% DMSO) at 4 °C in dark. Afterwards, the slides were placed for 30 min in alkaline buffer at 4 °C (300 mM NaOH and 1 mM EDTA, pHN13) to unwind the DNA. The alkaline electrophoresis was carried out for 30 min at 25 V and 300 mA. This standard alkaline procedure allows single-strand DNA breaks to be detected and alkali labile lesions (i.e., apurinic/apirimidinic sites) are converted to strand breaks under these conditions as well. The gels were neutralized with 0.4 M Tris (pH 7.5) with 3 washes of 5 min each. Finally, the slides were stained with 50 μL ethidium bromide (2 μL/mL) and examined at 40 × magnification under a fluorescence microscope equipped with a green filter of 540 nm. Direct light exposure of the samples was avoided during the whole process. For each individual we analyzed 100 randomly selected Comets (50 cells from each of two replicate slides). Two parameters were evaluated: *(i)* damage index (DI), in which each cell was designated to one of five classes (from no damage = 0 to maximum damage = 4) according to tail size and shape and *(ii)* percentage of DNA in the tail (% tail DNA) [26]. % tail DNA was scored using the Comet Assay IV® software (Perceptive Instruments, Haverhill, England).

2.6. DNA isolation and genetic polymorphisms of xenobiotic-metabolizing enzymes: CYP1A1$_{Msp1}$ (rs.4646903), GSTM1$_{null}$, GSTT1$_{null}$ and DNA repair enzymes XRCC1$_{Arg194Trp}$ (rs.1799782) and hOGG1$_{Ser326Cys}$ (rs. 1052133)

10 mL of blood sample were collected in EDTA coated tube; buffy coat containing peripheral blood mononuclear cells (PBMC) was isolated according to the Ficoll–Histopaque method. PBMC were digested with proteinase-K. Genomic DNA was isolated from PBMC by means of commercially available DNeasy Blood and Tissue® extraction kit (Qiagen, USA), according to the manufacturer's instructions. Each DNA sample was stored at −20 °C until analysis. The *CYP1A1*, *XRCC1* and *hOGG1* genotypes were determined by Polymerase chain reaction (PCR) and Restriction Fragment Length Analysis (RFLP) techniques according to Lum and Le Marchand [27] for *CYP1A1* and De Ruyck et al. [28] for *XRCC1* and *hOGG1*. The *CYP1A1* RFLP was carried out using the *MspI* endonuclease, whose restriction site polymorphism resulted in three genotypes: a homozygous *m1* allele without *MspI* site (unique 340 bp fragment), the heterozygote (340, 200 and 140 bp fragments) and a rare homozygous *m2* allele with the *MspI* site (200 and 140 bp fragments). The *XRCC1*$_{194Trp}$ alleles were detected after digestion with *PvuII* enzyme. For *hOGG1*$_{Ser326Cys}$ alleles, an aliquot of the PCR product was digested with *Fnu4HI*. Genotypes were resolved using a 2% agarose gel stained with ethidium bromide. The genotyping of *GSTM1* and *GSTT1*, which detects homozygous deleted genes that results in deficiency of the *GSTM1/T1* activity, was carried out by allele-specific multiplex PCR as described earlier by Abdel-Rahman et al. [29], using *CYP1A1* gene amplification as an internal control. Both PCRs, *CYP1A1* and *GSTM1* and *T1*, were carried out using appropriate primers and approximately 50 ng of DNA. The *GSTM1* and *GSTT1* genotypes were classified as null or positive (at least one undeleted allele). All the resulting bands were separated in agarose gels by electrophoresis and visualized with ethidium bromide staining (10 mg/mL) and ultraviolet transillumination. The presence or absence of *GSTM1* and *GSTT1* genes was detected by the presence or absence of a band at 480 bp (corresponding to *GSTT1*) and a band at 215 bp (corresponding to *GSTM1*). A band at 312 bp (corresponding to *CYP1A1* gene) was always present. Genotypes were confirmed by randomly re-genotyping 10% of the samples.

There were no discrepancies between the genotypes determined in duplicate.

2.7. Statistical analysis

Statistical analyses were carried out using software R version 3.3.0. The agreement of genotypic frequencies with Hardy–Weinberg expectations for $CYP1A1_{MspI}$, $GSTM1_{null}$, $GSTT1_{null}$, $XRCC1_{Arg194Trp}$ and $hOGG1_{Ser326Cys}$ was performed using a χ^2 test with $1°$ of freedom. The influence of genotype, age, alcohol intake, exposure and time of exposure on micronucleus formation in lymphocytes (CBMN) and buccal mucosa cells (BMNCyt) was determined using Poisson regression analysis. A scale variable was introduced into the model to account for overdispersion. Normalization of data was not necessary. Link function used for continuous variables followed a Gaussian distribution.

The model included genotype, alcohol intake (drinker or not drinker) exposure (occupationally exposed or nonoccupationally exposed), and time of exposure as fixed factors and age as a continuous covariate.

All possible two-way interactions among genotypes, alcohol intake, age and time of exposure were tested. In the presence of significant interaction terms, main effects were kept in the model even when not significant. All analyses were first done in the total population and thereafter stratified by occupational exposure. Frequency ratio (FR) and the corresponding P-values were estimated. For categorical variables, the FR indicates the proportional increase of the micronucleus frequency in the study group; for example, a FR of 1.81 for exposed versus non-occupationally exposed means a 81% increase of karyorrhexis frequency in exposed individuals. For continuous variables, the FR represents the proportional increase of micronucleus frequency due to the increase of one unit of the variable evaluated; for example, a FR for time of exposure of 1.12 means a 12% increase of BMNCyt frequency per year of exposure.

3. Results

The allele/genotype frequencies for *CYP1A1, GSTM1, GSTT1, XRCC1* and *hOGG1* studied for the exposed and reference non-exposed groups are shown in Table 3. The allele/genotype frequencies of *CYP1A1, GSTM1, GSTT1* were according to the Hardy–Weinberg equilibrium making selection bias less likely (p >0.05), however frequencies for *XRCC1* and h*OGG1* were not agreed with Hardy-Weinberg expectations. This deviation from Hardy-Weinberg equilibrium may be due to a moderate effect of genetic drift previously described for sampled population [30] or incomplete panmixia, strongly associated to assortative mating aspects of indigenous populations that constitutes the main racial component in this region from northern Colombia. All variant alleles were in agreement with literature values for Colombian [31], Brazilian [32] and Caucasian populations [33].

In total population, the FR estimated by Poisson regression analysis, including age, alcohol intake, exposure and time of exposure as potential confounding factors (Table 4) showed a significant increase of micronuclei only for the variable exposure (P < 0.001) for all parameters assessed.

Micronucleus frequencies, BMNCyt parameters and DNA damage were not significantly influenced by age, alcohol intake or time of exposure in non-exposed and exposed populations (Tables 4, 6 and 8).

The results of the Poisson logistic regression, taking into account the effect of *CYP1A1, GSTM1, GSTT1, XRCC1* and *hOGG1* polymorphisms on CBMN frequencies, are summarized in Table 5. Exposed $GSTT1_{null}$ subjects showed a lower micronucleus frequency compared with their positive counterparts (FR: 0.83; P = 0.04).

Interestingly, this protective effect of the $GSTT1_{null}$ genotype was observed only in occupationally exposed individuals. In non-exposed population the opposite effect was observed: CBMN frequencies were significantly increased in $GSTT1_{null}$ carriers (FR: 1.4; P = 0.03). Although not statistically significant, the $GSTT1_{null}$ genotype was shown to influence micronucleus frequencies in an age/time of exposure-dependent way (FR: 1.00; P = 0.99; FR: 1.01; P = 0.76).

In total and non-exposed populations, $CYP1A1_{Msp1}$ (m1/m2, m2/m2) carriers displayed a statistically significant increase in micronuclei frequencies (FR: 1.34, P = 0.01; FR: 1.29, P = 0.02), while $GSTM1_{null}$ individuals showed significant lower CBMN frequencies (FR: 0.72, P = 0.01; FR: 0.71; P = 0.03). These modulation effects were not observed in exposed individuals. Although not statistically significant, $CYP1A1_{Msp1}$ (m1/m2, m2/m2) and $GSTM1_{null}$ effects were reversed in exposed carriers, indicating a possible modulation effect of exposure status on these polymorphisms effects.

Internal control values showed that handling, processing and transportation conditions were optimal along all sampling period and did not adversely influence any of the results obtained.

Other polymorphisms studied, $hOGG1_{Ser326Cys}$ and $XRCC1_{Arg194Trp}$ had no significant impact on CBMN frequencies.

As shown in Table 7, BMNCyt frequency and other exfoliated buccal mucosa parameters related to cell death (karyorrhexis and karyolysis) and DNA damage (nuclear buds) were significantly modulated by metabolism and DNA repair polymorphisms: BMNCyt and nuclear buds were significantly higher in exposed workers with $GSTM1_{Null}$ polymorphisms (FR: 1.28, P = 0.03; FR: 1.50, P = 0.02). Karyolysis frequency was significantly increased in exposed carriers of $GSTM1_{Null}$ and $GSTT1_{Null}$ alleles, while karyorrhexis frequencies in exposed populations were also found increased in individuals with $GSTT1_{Null}$ polymorphism. Carriers of $XRCC1_{Arg194Trp}$ (Arg/Trp, Trp/Trp) also showed lower levels of karyolitic in total, non-exposed and occupational exposed populations. Nuclear buds were also significantly augmented in exposed carriers of the $CYP1A1_{Msp1}$ (m1/m2, m2/m2) gene (FR: 1.07, P = 0.02).

Together with $XRCC1_{Arg194Trp}$ (Arg/Trp, Trp/Trp), $hOGG1_{Ser326Cys}$ (Ser/Cys, Cys/Cys) polymorphism was significantly associated with lower levels of BMNCyt and Comet assay parameters like % tail DNA and DI in exposed carriers, thereby demonstrating a possible protective effect of both polymorphisms in exposed population (Tables 7 and 9). $hOGG1_{Ser326Cys}$ (Ser/Cys, Cys/Cys) protective effect was also observed for total and non-exposed populations. Comet assay parameters like DI and % tail DNA also seemed to be significantly influenced by glutathione polymorphisms; DI and % DNA in tail were significantly higher in exposed individuals with the null variant of *GSTM1*.

4. Discussion

Biotransformation plays an important role in the carcinogenic activity of environmental carcinogens. Large inter-individual variation in the biotransformation has been reported, and genetic polymorphisms in some xenobiotic – metabolizing and DNA repair enzymes can in part explain some of these differences. Thus, the aim of our study was to evaluate if key polymorphisms in metabolisms genes $CYP1A1_{Msp1}$, $GSTM1_{Null}$, $GSTT1_{Null}$ and DNA repair genes $XRCC1_{Arg194Trp}$ and $hOGG1_{Ser326Cys}$ could modify individual susceptibility to adverse coal exposure effects, considering the DNA damage (SCGE/Comet assay) and micronucleus formation in lymphocytes (CBMN) and buccal mucosa cells (BMNCyt) as endpoints for genotoxicity. Given the importance of *GST* in the detoxification the null genotypes of both genes have become the object of molecular epidemiology studies because homozygous deletions are expected to result in an impaired ability to detoxify carcinogenic

Table 3
Distribution of *CYP1A1, GSTM1, GSTT1, XRCC1* and *OGG1* genotypes and variant alleles frequencies in non-exposed and exposed groups.

Gene	Genotype	Exposure		p[*]	Total frequency observed	Equilibrium frequency	p[**]	Allele/Genotype Frequency
		Non-exposed group	Exposed group					
		n (%)	n (%)		n (%)	n (%)		
XRCC1 Arg194Trp[b]	*Arg/Arg*	94 (94)	86 (86)		180 (90)	174 (87)		*Arg: 0.93*
	Arg/Trp	4 (4)	9 (9)	0.17	13 (6.5)	25 (12.5)	<0.01	
	Trp/Trp	2 (2)	5 (5)		7 (3.5)	1 (0.5)		*Trp: 0.07*
OGG1 Ser326Cys[b]	*Ser/Ser*	56 (56)	63 (63)		119 (60)	108 (54)		*Ser: 0.73*
	Ser/Cys	25 (25)	21 (21)	0.6	46 (23)	78 (39)	<0.01	
	Cys/Cys	19 (19)	16 (16)		35 (18)	14 (7)		*Cys: 0.26*
CYP1A1$_{MspI}$	*m1/m1*	49 (49)	38 (38)		87 (43.5)	84 (42.5)		*m1: 0.65*
	m1/m2	40 (40)	47 (47)	0.17	87 (43.5)	91 (45.5)	0.739	
	m2/m2	11 (11)	15 (15)		26 (13)	24 (12)		*m2: 0.35*
GSTM1	*Positive*	57 (57)	59 (59)	0.77	116 (58)	116 (58)	>0.75	*Positive: 0.35*[a]
	Null	43 (43)	41 (41)		84 (42)	84 (42)		*Null: 0.65*[a]
GSTT1	*Positive*	80 (80)	80 (80)	1.0	160 (80)	160 (80)	1.0	*Positive: 0.55*[a]
	Null	20 (20)	20 (20)		40 (20)	40 (20)		*Null: 0.45*[a]

[a] Allelic frequencies determined assuming genetic equilibrium in general population.
[b] Didn't meet with Hardy–Weinberg equilibrium.
[*] Statistical significance of genotypic differences between exposed and non-exposed groups determined by χ2 test.
[**] Statistical significance of genotypic differences between expected and observed values.

Table 4
Lymphocyte micronucleus frequency (CBMN): Poisson regression analysis of the total, non-occupationally exposed, and occupationally exposed populations without taking into account genetic polymorphisms.

Population	Variable	FR	CI (95%)	P
Total population (n = 200)	Age	1.00	0.18–5.45	0.43
	Alcohol intake[a]	1.12	0.25–5.01	0.28
	Exposure (Exposed)[a]	**3.04**	**1.65–5.59**	**0.00**[*]
	Exposure (Exposed/drinker)[**,b]	0.92	0.30–2.79	0.15
	Exposure (Age)[**]	0.98	0.33–2.84	0.12
Non-occupationally exposed (n = 100)	Age	0.98	0.52–1.84	0.17
	Alcohol intake	1.11	0.46–2.65	0.26
Exposed (n = 100)	Age	1.02	0.65–1.59	0.10
	Alcohol intake	1.15	0.64–2.04	0.23
	Time of exposure	0.99	0.52–1.85	0.22

CI: Confident intervals.
[*] Bold: For statistically significant level.
[**] Interaction term.
[a] Reference category: non-occupationally exposed.
[b] Reference category: non drinkers individuals.

compounds and therefore an increasing risk for genetic damage. However, in our study we could not detect any significant effect of *GSTM1, CYP1A1, OGG1* or *XRCC1* in CBMN frequency of exposed individuals. Exposed subjects carrying *GSTT1*$_{null}$ polymorphism showed a lower micronucleus frequency compared with their positive counterparts (FR: 0.83; P = 0.04).

While many previous studies have found the *GSTT1*$_{Null}$ variant to be associated with increased DNA damage and adduct levels [34,35], there is a growing body of evidence on the protective effect of *GSTT1* deletion in both disease and intermediary end-points related to environmental carcinogenesis. A pooled analysis have described that *GSTT1*$_{Null}$ subjects have lower micronucleus frequencies than their *GSTT1*$_{(+)}$ counterparts, counteracted only by the effect of age and gender [36]. However, results of the Poisson logistic regression, considering the genetic factor and the effect of age, alcohol intake, exposure and time of exposure as potential confounding factors in CBMN frequency, confirmed the lack of influence of these confounding factors in micronucleus frequency.

Other studies have also described a similar results for CBMN frequencies [37], chromosome alterations in human lymphocytes [31] and oxidative damaged DNA induced by PAH exposure [38]. *GSTT1*$_{Null}$ genotype have been also linked with decreased risk of breast cancer among premenopausal nurses [39] and as a protective factor against several types of cancer [40,41]. A possible explanation for this protective effect has focused on the possibility that the products of *GSTT1* conjugating reactions could produce in certain instances more carcinogenic metabolites. *GSTT1*$_{(+)}$ phenotype could catalyze the glutathione conjugation of dichloromethane, a metabolic pathway that has been shown to be mutagenic in *Salmonella typhimurium* strains and is believed to be responsible for the carcinogenicity of dichloromethane in the mouse [42]. These facts indicate that *GSTT1*$_{(+)}$ carriers might be more prone to the genotoxic action of halogenated compounds by way of the *GSTT1* pathway, and *GSTT1*$_{Null}$ individuals might be less susceptible [40]. Some of our findings about the protective effect of the *GSTT1*$_{null}$ genotype only in occupationally exposed individuals seem to confirm this hypothesis. In non-exposed population the opposite effect

Table 5
Lymphocyte micronucleus frequency (CBMN): Poisson regression analysis of the total, non-occupationally exposed, and occupationally exposed populations taking into account genetic polymorphisms.

Population	Variable	FR	CI (95%)	P
Total population (n = 200)	*XRCC1Arg/Trp;Trp/Trp*[a]	1.13	0.71–1.77	0.2
	OGG1Ser/Cys; Cys/Cys[b]	0.99	0.66–1.46	0.52
	CYP1A1m1/m2;m2/m2[c]	**1.34**	**1.10–1.63**	**0.01**[*]
	*GSTM1*_{Null}[d]	**0.72**	**0.62–0.84**	**0.01**[*]
	*GSTT1*_{Null}[e]	1.00	0.67–1.47	0.87
Non-occupationally exposed (n = 100)	*XRCC1Arg/Trp;Trp/Trp*[a]	0.98	0.66–1.45	0.43
	OGG1Ser/Cys; Cys/Cys[b]	0.99	0.59–1.64	0.29
	CYP1A1m1/m2;m2/m2[c]	**1.29**	**1.14–1.45**	**0.02**[*]
	*GSTM1*_{Null}[d]	**0.71**	**0.58–0.86**	**0.03**[*]
	*GSTT1*_{Null}[e]	**1.40**	**1.19–1.63**	**0.03**[*]
Exposed (n = 100)	*XRCC1Arg/Trp;Trp/Trp*[a]	0.95	0.64–1.40	0.81
	OGG1Ser/Cys; Cys/Cys[b]	0.99	0.54–1.78	0.86
	CYP1A1m1/m2;m2/m2[c]	0.97	0.57–1.64	0.18
	*GSTM1*_{Null}[d]	1.05	0.66–1.64	0.23
	*GSTT1*_{Null}[e]	**0.83**	**0.70–0.97**	**0.04**[*]

CI: Confident intervals.

[*] Bold: For statistically significant level.

[a] Reference genotype *XRCC1*_{Arg/Arg} individuals.

[b] Reference genotype: *OGG1*_{Ser/Ser} individuals.

[c] Reference genotype: *CYP1A1*_{m1/m1} individuals.

[d] Reference genotype: *GSTM1*₍₊₎ individuals.

[e] Reference genotype: *GSTT1*₍₊₎ individuals.

was observed: CBMN frequencies were significantly increased in *GSTT1*_{null} carriers (FR: 1.4; P = 0.03).

In open-cast coal mining facilities, exposition to PAH could occur in several activities during coal extraction. A characterization of the products generated in spontaneous combustion of coal in open pit detected thirty-two aliphatic compounds as well as halogenated compounds including bromomethane, iodomethane and trichloromethane in low concentrations, and dichloromethane and chloromethane in high concentrations [43]. We propose that, in open cast mines occupationally exposed workers with the *GSTT1*₍₊₎ polymorphism could produce in certain instances more carcinogenic metabolites, such as di-halomethanes and other chlorinated hydrocarbons [44,45], which are known to be bioactivated by *GSTT1*. Garte et al. [38], determined that the frequency of the *GSTT1* deletion was significantly higher in individuals resistant to the DNA damaging effects of PAH exposure than in people who were the most sensitive. According to authors the mechanism for this effect might be related to specific PAH substrate specificities, or could be related to other functions of *GSTT1* gene in oxidative stress that may induced damage pathways. Data from the EXPAH project (population EXposure to PAH) also concluded that variations on the protective effect of *GSTT1* deletion for DNA oxidation could depend on the mix of PAHs presents in occupational settings that could have different specificity for the *GSTT1* enzyme. The same data also suggest that *GSTM1* alone does play a small role in detoxification of PAHs [46].

Other hypothesis suggested that combined conjugation activities of wild genotypes, for all glutathione-*S*-transferases, may lead to glutathione activity depletion and thereby become counterproductive [47]. Interestingly our results report a protective influence by *GSTT1*_{Null} genotype over CBMN frequencies increase without any combined effect of other glutathione-*S*-transferases polymorphism. Possible explanations could be the effects of substrate specificity for *GSTT1* (which is clearly different from that of *GSTM1),* the presence of other detoxification systems that may render the loss of one system like *GSTT1* or the fact that *GSTT1* shows important differences in the catalytic activity compared with the other GSTs [48].

In total and non-exposed populations, *CYP1A1*_{Msp1} *(m1/m2, m2/m2)* carriers displayed a statistically significant increase in micronuclei frequencies (FR:1.34, P = 0.01; FR:1.29, P = 0.02), while *GSTM1*_{null} individuals showed significant lower CBMN frequencies (FR:0.72, P = 0.01; FR:0.71; P = 0.03). These modulation effects were not observed in exposed individuals.

Together with *CYP1A1,* other polymorphisms considered in this study, *OGG1* and *XRCC1* had no significant impact on CBMN frequencies in lymphocytes. Possible explanations for this lack of modulation effect could arise from the nature of the exposure itself. The results described so far in the literature demonstrate that the inhibition of DNA repair, essentially base-excision repair (BER) and nucleotide excision repair (NER), is a common mechanism in metal-induced genotoxicity [49].

The abundance of different mineral elements in Cerrejón coal determined by Scanning Electron Microscopy Computer-Controlled shows that more than 80% of weight of the mineral material is composed of clay and quartz minerals (aluminum silicate, aluminum silicate, and silica). Analysis of the product reveals that the combustion ashes are formed mainly of aluminum silicates, iron oxide and quartz particles [50].

In a previous study, with same individuals, we detected a significant content of inorganic elements as Aluminum (Al) and Silicon (Si) in peripheral blood of the same coal miner's population [11]. Several studies have demonstrated that Al ions inhibit proteins with zinc finger domains [51] and DNA repair in lymphocytes [52]. Such domains have been identified in several DNA repair enzyme like *OGG1* [53] and the members of the polyADP-ribose polymerase (PARP) family [54] that interact with *XRCC1* in BER pathway [55].

On the other hand, Marston et al. [56] demonstrated in mouse skin that some PAH present in complex mixtures can alter the carcinogenic activity of other PAH present in the mixture by inhibit the activation of carcinogenic PAH by the induced of *CYP* enzymes. Thus, depending on the PAHs presents in the mixture, an inhibitory effect could be strong enough to reduce *CYP1A1* induction and PAH-DNA adducts formation. Therefore, the main factor involved in carcinogenic of complex mixtures could be the potential of PAH presents in the mixture to induced or not CYPs enzymes expression in lymphocytes [5]. Other findings also suggest that coal dust inhalation could be involved in modulation of *CYP1A1* expression [57]. A growing number of studies have also shown that moderate oxidative stress specifically down-regulates the expression of var-

Table 6

Buccal micronucleus cytome (BMNCyt) parameters: poisson regression analysis of the total, non-occupationally exposed, and occupationally exposed populations without considering genetic polymorphisms.

Groups and variables Total population (n = 200)	FR	CI (95%)	P
BMNCyt			
Age	0.99	0.40–3.49	0.37
Alcohol intake[a]	1.02	0.28–3.34	0.92
Exposure (Exposed)[b]	**8.10**	**2.13–12.99**	**0.00**[*]
Exposure (Exposed/drinker)[**]	1.09	0.89–6.39	0.12
Exposure (Age)[**]	1.04	0.57–4.18	0.09
Nuclear buds			
Age	0.98	0.73–4.86	0.34
Alcohol intake[a]	1.00	0.71–4.82	0.99
Exposure (Exposed)[b]	**4.80**	**2.04–8.52**	**0.00**[*]
Exposure (Exposed/drinker)[**]	1.05	0.69–4.82	0.06
Exposure (Age)[**]	1.03	0.67–4.67	0.09
Karyolysis			
Age	0.98	0.22–3.29	0.23
Alcohol intake[a]	1.03	0.42–3.61	0.15
Exposure (Exposed)[b]	**1.30**	**1.13–9.96**	**0.03**[*]
Exposure (Exposed/drinker)[**]	0.96	0.17–3.36	0.39
Exposure (Age)[**]	1.03	0.34–3.42	0.42
Karyorrhexis			
Age	0.98	0.33–3.32	0.23
Alcohol intake[a]	0.97	0.25–3.25	0.56
Exposure (Exposed)[b]	**1.81**	**1.19–12.94**	**0.03**[*]
Exposure (Exposed/drinker)[**]	1.10	0.58–4.34	0.18
Exposure (Age)[**]	1.01	0.51–3.86	0.47
Non-occupationally exposed (n = 100)			
BMNCyt			
Age	0.98	0.32–3.31	0.12
Alcohol intake[a]	1.09	0.55–4.19	0.34
Nuclear buds			
Age	0.95	0.79–5.19	0.08
Alcohol intake[a]	0.97	0.71–4.74	0.23
Karyolysis			
Age	0.98	0.27–3.27	0.14
Alcohol intake[a]	1.05	0.64–4.56	0.38
Karyorrhexis			
Age	0.97	0.20–3.29	0.12
Alcohol intake[a]	0.96	0.81–5.38	0.34
Exposed (n = 100)			
BMNCyt			
Age	0.99	0.85–5.74	0.19
Alcohol intake[a]	1.02	0.93–6.49	0.10
Time of exposure	1.12	0.94–6.95	0.08
Nuclear buds			
Age	1.02	0.69–4.72	0.18
Alcohol intake[a]	0.98	0.61– 4.25	0.19
Time of exposure	1.09	0.75–5.30	0.20
Karyolysis			
Age	1.01	0.66–4.55	0.59
Alcohol intake[a]	1.01	0.41–3.55	0.62
Time of exposure	0.99	0.32–3.33	0.93
Karyorrhexis			
Age	0.97	0.85–5.69	0.09
Alcohol intake[a]	1.02	0.84–5.73	0.11
Time of exposure	1.19	1.09–8.92	0.08

CI: Confident intervals.

[*] Bold: For statistically significant level.

[**] Interaction term.

[a] Reference category: non drinkers individuals.

[b] Reference category: non-occupationally exposed.

ious genes. Interestingly, several *CYP* isoforms have been shown to release ROS during their catalytic cycles, especially with uncoupled substrates [58]. This ROS production could contribute to repress the expression of oxidative-stress-sensitive genes. A possible consequence could be a negative-feedback mechanism controlling *CYP* gene expression. Indeed, high *CYP1A1* activity within the cell represses the promoter of its own gene [59]. This negative auto-regulation could limit the intracellular production of ROS by *CYP1A1* (and subsequent damage, such as DNA alterations), as well as the

activation of particular *CYP1A1* substrates into carcinogenic compounds [60]. Thus, the cellular adaptive response to an oxidant insult could comprises both the induction of antioxidant defenses and the repression of endogenous ROS-generating systems or physiological pathways that indirectly increase the risk of ROS generation, such as the action of *CYP1A1* [60,61]. A reduced induction of *CYPs* enzymes and therefore of reactive metabolites from phase-I metabolic pathways could also explain the lack of modulation in other enzymes from phase II like *GSTM* or from DNA damage repair

Table 7

Buccal micronucleus cytome (BMNCyt) parameters: poisson regression analysis of the total, non-occupationally exposed, and occupationally exposed populations considering genetic polymorphisms.

Total population (n = 200)				Non-occupationally exposed (n = 100)				Exposed (n = 100)			
BMNCyt[**]	FR	CI (95%)	P	BMNCyt[**]	FR	CI (95%)	P	BMNCyt[**]	FR	CI (95%)	P
XRCC1Arg/Trp;Trp/Trp[a]	1.05	0.66–1.64	0.92	*XRCC1Arg/Trp;Trp/Trp*[a]	0.00	0.00–4.58	1.00	*XRCC1Arg/Trp;Trp/Trp*[a]	**0.79**	**0.62–0.99**	**0.04**[*]
OGG1Ser/Cys; Cys/Cys[b]	**0.78**	**0.68–0.89**	**0.03**[*]	*OGG1Ser/Cys; Cys/Cys*[b]	**0.77**	**0.60–0.97**	**0.04**[*]	*OGG1Ser/Cys; Cys/Cys*[b]	**0.92**	**0.80–0.99**	**0.04**[*]
CYP1A1m1/m2;m2/m2[c]	1.24	0.83–1.83	0.79	*CYP1A1m1/m2;m2/m2*[c]	1.05	0.70–1.55	0.35	*CYP1A1m1/m2;m2/m2*[c]	1.02	0.80–1.50	0.65
GSTM1Null[d]	1.14	0.97–1.33	0.52	*GSTM1Null*[d]	1.15	0.98–1.34	0.06	*GSTM1Null*[d]	**1.28**	**1.05–1.55**	**0.03**[*]
GSTT1 Null[e]	**0.91**	**0.84–0.98**	**0.04**[*]	*GSTT1 Null*[e]	**0.10**	**0.07–0.12**	**0.01**[*]	*GSTT1 Null*[e]	0.98	0.60–1.59	0.54
Nuclear buds[**]				**Nuclear buds**[**]				**Nuclear buds**[**]			
XRCC1Arg/Trp;Trp/Trp[a]	**1.87**	**1.12–3.11**	**0.02**[*]	*XRCC1Arg/Trp;Trp/Trp*[a]	0.87	0.52–1.44	0.79	*XRCC1Arg/Trp;Trp/Trp*[a]	**1.70**	**1.54–1.87**	**0.03**[*]
OGG1Ser/Cys; Cys/Cys[b]	0.93	0.83–1.03	0.06	*OGG1Ser/Cys; Cys/Cys*[b]	1.14	0.49–2.64	0.51	*OGG1Ser/Cys; Cys/Cys*[b]	0.89	0.71–1.11	0.10
CYP1A1m1/m2;m2/m2[c]	1.17	0.96–1.42	0.09	*CYP1A1m1/m2;m2/m2*[c]	1.11	0.87–1.40	0.70	*CYP1A1m1/m2;m2/m2*[c]	**1.07**	**0.87–1.30**	**0.02**[*]
GSTM1Null[d]	**1.35**	**1.16–1.56**	**0.04**[*]	*GSTM1Null*[d]	0.88	0.52–1.46	0.43	*GSTM1Null*[d]	**1.50**	**1.41–1.59**	**0.02**[*]
GSTT1 Null[e]	0.94	0.63–1.39	0.10	*GSTT1 Null*[e]	**0.67**	**0.58–0.76**	**0.03**[*]	*GSTT1 Null*[e]	0.93	0.62–1.37	0.10
Karyolysis[**]				**Karyolysis**[**]				**Karyolysis**[**]			
XRCC1Arg/Trp;Trp/Trp[a]	**0.62**	**0.50- 0.75**	**0.03**[*]	*XRCC1Arg/Trp;Trp/Trp*[a]	**0.70**	**0.58–0.83**	**0.03**[*]	*XRCC1Arg/Trp;Trp/Trp*[a]	**0.69**	**0.54–0.87**	**0.02**[*]
OGG1Ser/Cys; Cys/Cys[b]	0.92	0.56–1.50	0.28	*OGG1Ser/Cys; Cys/Cys*[b]	0.91	0.55–1.48	0.10	*OGG1Ser/Cys; Cys/Cys*[b]	0.96	0.58–1.56	0.32
CYP1A1m1/m2;m2/m2[c]	0.96	0.68–1.33	0.34	*CYP1A1m1/m2;m2/m2*[c]	**0.73**	**0.55–0.96**	**0.04**[*]	*CYP1A1m1/m2;m2/m2*[c]	1.08	0.77–1.50	0.18
GSTM1Null[d]	1.13	0.62–2.03	0.11	*GSTM1Null*[d]	1.03	0.56–1.89	0.75	*GSTM1Null*[d]	**1.24**	**1.12–1.36**	**0.03**[*]
GSTT1 Null[e]	**1.32**	**1.22–1.42**	**0.02**[*]	*GSTT1 Null*[e]	**0.72**	**0.55–0.92**	**0.04**[*]	*GSTT1 Null*[e]	**1.71**	**1.58–1.84**	**0.02**[*]
Karyorrhexis[**]				**Karyorrhexis**[**]				**Karyorrhexis**[**]			
XRCC1Arg/Trp;Trp/Trp[a]	**0.86**	**0.77–0.94**	**0.04**[*]	*XRCC1Arg/Trp;Trp/Trp*[a]	0.87	0.68–1.10	0.11	*XRCC1Arg/Trp;Trp/Trp*[a]	1.14	0.64–2.01	0.12
OGG1Ser/Cys; Cys/Cys[b]	0.95	0.55–1.61	0.30	*OGG1Ser/Cys; Cys/Cys*[b]	0.92	0.54–1.56	0.39	*OGG1Ser/Cys; Cys/Cys*[b]	1.04	0.61–1.76	0.38
CYP1A1m1/m2;m2/m2[c]	1.04	0.87–1.24	0.34	*CYP1A1m1/m2;m2/m2*[c]	0.95	0.79–1.13	0.51	*CYP1A1m1/m2;m2/m2*[c]	1.02	0.85–1.21	0.65
GSTM1Null[d]	0.94	0.53–1.65	0.28	*GSTM1Null*[d]	0.83	0.60–1.13	0.07	*GSTM1Null*[d]	1.10	0.62–1.94	0.29
GSTT1 Null[e]	1.08	0.58–1.98	0.21	*GSTT1 Null*[e]	0.91	0.53–1.54	0.48	*GSTT1 Null*[e]	**1.28**	**1.03–1.58**	**0.04**[*]

CI: Confident intervals.

[*] Bold: For statistically significant level.

[**] Poisson analysis model for adjusted covariates.

[a] Reference genotype *XRCC1Arg/Arg* individuals.

[b] Reference genotype: *OGG1Ser/Ser* individuals.

[c] Reference genotype: *CYP1A1m1/m1* individuals.

[d] Reference genotype: *GSTM1(+)* individuals.

[e] Reference genotype: *GSTT1(+)* individuals.

as *XRCC1* and *OGG1*. Our results could also suggest that detoxification by *CYP1A1* and *GSTM1* can represent a minor metabolic pathway for xenobiotic compounds from coal mining exposure in lymphocytes. Enzymatic pathways related to oxidative damage response like SOD (superoxide dismutase) [12], catalase [62], glutathione peroxidase (GPx) and systemic inflammation enzymes like TNF (tumor necrosis factor alpha) [63] could represent a major pathway for detoxification of coal mining compounds in blood [64,65]. However, we cannot exclude the possibility that these findings may be due to chance considering the small sample size formed by a group of exposed workers and multiple comparisons, so that a firm conclusion would be premature pending more extensive investigations.

Interestingly, results obtain for gene modulation of DNA damage parameters in oral mucosa (BMNCyt frequency and nuclear buds) were quite different from those obtain in CBMN frequencies in lymphocytes. For these biomarkers, significantly higher values were observed in exposed workers with the *GSTM1Null* polymorphism and *CYP1A1 (m1/m2, m2/m2)* allele. Because of their direct exposure to ambient air, cell from the oral cavity may experience enhanced oxidant stress by environmental irritants and pollutants including particulate matter, metals and PAH from complex mixtures presents in coal mining settings. Similar results were obtained by Rohr et al., [66] from a population of coal workers from Candiota – Brasil, where *GSTM1Null* carriers showed a significant increase in the frequency of nuclear buds related to individuals with the *GSTT1(+)* genotype.

On the other hand, *CYP1A1* is expressed in many epithelial tissues especially in buccal mucosa, suggesting an in situ activation of xenobiotic compounds [67]. Several studies have described a sig-

nificant association of *CYP1A1 m1* and *m2* variants with genetic damage in buccal mucosa cells suggesting that these polymorphisms could modulate the effects of PAH exposure in occupational settings [68]. The *CYP1A1* variant genotype has also been found to be associated with higher DNA adducts levels in coke oven workers [69] and increased Comet assay tail inertias in pot-room workers [37]. Giri et al. [15], described that synergistically *GSTM1* and *CYP1A1 (m1/m2, m2/m2)* genotypes were significantly associated with DNA damage as compared to *GSTM1(+)* and *CYP1A1 (m1/m1)* genotypes among coal tar workers. *GSTM1* enzyme had been reported to be present in oral tissue [70], thus in our set of data, excess formation of toxins in *CYP1A1Msp1 (m1/m2, m2/m2)* mutants and reduced detoxification of the toxins in *GSTM1Null,* genotypes among coal mining workers may result in a larger amount of toxic compounds that might have a role in the increased frequency in BMNCyt and other buccal abnormalities. This hypothesis seems to be supported by other results obtained in this study in relation to buccal parameters related to cell death. Karyolysis and karyorrhexis frequencies were also significantly increased in exposed carriers of *GSTM1Null* and *GSTT1Null* alleles, suggesting a role of gluthathione genes in initial response to coal residues. The combined effect of both genes is similar to the results reported in previous studies [67,71].

Genetic polymorphisms in base-excision repair pathway (BER) genes like *XRCC1Arg194Trp (Arg/Trp, Trp/Tpr)* and *hOGG1Ser326Cys (Ser/Cys, Cys/Cys)* were significantly associated with lower levels of BMNCyt and Comet assay parameters: DI and % tailDNA in referents and exposed carriers, demonstrating a possible protective effect of both polymorphisms in exposed population. In pesticide exposed workers, Rohr et al. [72] described the same protective

Table 8
Comet assay parameters: Poisson regression analysis of the total, non-occupationally exposed, and occupationally exposed populations without considering genetic polymorphisms.

Groups and variables	FR	CI (95%)	P
Total population (n = 200)			
Tail % DNA			
Age	1.00	0.53–3.92	0.90
Alcohol intake[a]	0.89	0.58–3.91	0.11
Exposure (Exposed)[b]	**4.10**	**1.53–33.64**	**0.00**[*]
Exposure (Exposed/drinker)[**]	0.91	0.41–3.36	0.59
Exposure (Age)[**]	1.00	0.25–3.31	0.97
DI			
Age	1.00	0.82–5.53	0.08
Alcohol intake[a]	0.92	0.51–3.67	0.10
Exposure (Exposed)[b]	**4.80**	**1.21–21.88**	**0.00**[*]
Exposure (Exposed/drinker)[**]	1.17	0.87–6.41	0.09
Exposure (Age)[**]	1.01	0.72–4.89	0.47
Non-occupationally exposed (n = 100)			
Tail%DNA			
Age	1.00	0.20–3.35	0.91
Alcohol intake[a]	0.96	0.30–3.26	0.78
DI			
Age	0.99	0.29–3.31	0.85
Alcohol intake[a]	1.09	0.18–3.55	0.53
Exposed (n = 100)			
Tail%DNA			
Age	1.00	0.24–3.31	0.87
Alcohol intake[a]	0.84	0.57–3.76	0.06
Time of exposure	0.99	0.27–3.29	0.78
DI			
Age	1.00	0.27–3.31	0.72
Alcohol intake[a]	0.92	0.47–3.54	0.21
Time of exposure	0.98	0.21–3.30	0.35

CI: Confident intervals.
[*] Bold: For statistically significant level.
[**] Interaction term.
[a] Reference category: non drinkers individuals.
[b] Reference category: non-occupationally exposed.

Table 9
Comet assay parameters: Poisson regression analysis of the total, non-occupationally exposed, and occupationally exposed populations considering genetic polymorphisms.

Total population (n = 200)				Non-occupationally exposed (n = 100)				Exposed (n = 100)			
% tail DNA[**]	FR	CI (95%)	P	% tail DNA[**]	FR	CI (95%)	P	% tail DNA[**]	FR	CI (95%)	P
XRCC1Arg/Trp;Trp/Trp[a]	0.96	0.47–1.94	0.67	*XRCC1Arg/Trp;Trp/Trp*[a]	1.03	0.53–1.96	0.89	*XRCC1Arg/Trp;Trp/Trp*[a]	**0.72**	**0.54–0.94**	**0.04**[*]
OGG1Ser/Cys; Cys/Cys[b]	0.88	0.36–2.12	0.54	*OGG1Ser/Cys; Cys/Cys*[b]	0.94	0.42–2.05	0.84	*OGG1Ser/Cys; Cys/Cys*[b]	**0.94**	**0.52–0.98**	**0.03**[*]
CYP1A1m1/m2;m2/m2[c]	1.18	0.93–1.49	0.07	*CYP1A1m1/m2;m2/m2*[c]	1.00	0.27–3.57	0.99	*CYP1A1m1/m2;m2/m2*[c]	1.05	0.43–2.53	0.45
GSTM1Null[d]	1.04	0.87–1.24	0.24	*GSTM1Null*[d]	1.04	0.76–1.42	0.88	*GSTM1Null*[d]	**1.08**	**0.68–1.69**	**0.02**[*]
GSTT1 Null[e]	0.97	0.39–2.38	0.79	*GSTT1 Null*[e]	0.94	0.38–2.31	0.90	*GSTT1 Null*[e]	0.95	0.60–1.49	0.33
DI[**]				**DI**[**]				**DI**[**]			
XRCC1Arg/Trp;Trp/Trp[a]	1.03	0.52–2.00	0.65	*XRCC1Arg/Trp;Trp/Trp*[a]	1.16	0.48–2.80	0.56	*XRCC1Arg/Trp;Trp/Trp*[a]	**0.75**	**0.62–0.89**	**0.03**[*]
OGG1Ser/Cys; Cys/Cys[b]	0.85	0.55–1.30	0.12	*OGG1Ser/Cys; Cys/Cys*[b]	1.03	0.61–1.71	0.78	*OGG1Ser/Cys; Cys/Cys*[b]	**0.93**	**0.55–0.98**	**0.04**[*]
CYP1A1m1/m2;m2/m2[c]	1.18	0.82–1.67	0.10	*CYP1A1m1/m2;m2/m2*[c]	0.97	0.49–1.88	0.87	*CYP1A1m1/m2;m2/m2*[c]	1.01	0.51–1.96	0.71
GSTM1Null[d]	1.04	0.57–1.87	0.54	*GSTM1Null*[d]	1.05	0.45–2.43	0.82	*GSTM1Null*[d]	**1.06**	**0.54–1.34**	**0.03**[*]
GSTT1 Null[e]	0.95	0.55–1.61	0.38	*GSTT1 Null*[e]	1.13	0.61–2.07	0.67	*GSTT1 Null*[e]	0.94	0.51–1.72	0.56

CI: Confident intervals.
[*] Bold: For statistically significant level.
[**] Poisson analysis model for adjusted covariates.
[a] Reference genotype *XRCC1_{Arg/Arg}* individuals.
[b] Reference genotype: *OGG1_{Ser/Ser}* individuals.
[c] Reference genotype: *CYP1A1_{m1/m1}* individuals.
[d] Reference genotype: *GSTM1_{(+)}* individuals.
[e] Reference genotype: *GSTT1_{(+)}* individuals.

effect for CBMN frequencies, while Kvitko et al. [73] found no effect of *XRCC1_{Arg194Trp}* on BMNCyt in workers exposed to coal mining residues. The protective effect observed for *XRCC1_{Arg194Trp}* could be linked to a diminish repair capacity in BER pathway in relation to *(Arg/Trp, Trp/Tpr)* alleles. According to Bretton et al. [55], *XRCC1* is known to interact with several enzymes in the BER pathway and, since the *Arg194Trp* polymorphism resides in the linker region sep-

arating the POL β domain from the poly (ADP-ribose) polymerase (PARP)-interacting domain, the polymorphism could affect *XRCC1's* ability to bind to either *POL β* or poly (ADP-ribose) polymerase. Thus a decreased DNA repair ability could lead to increased accumulation of DNA damage that enhances cell cycle arrest and apoptosis [74,75]. In addition, several studies also have demonstrated that environmental exposure may down-regulate expression of some

DNA repair genes including *XRCC1*. Carriers of *XRCC1*$_{Arg194Trp}$ also showed lower levels of karyolitic cells in total, non-exposed and occupational exposed populations. This effect might be linked to the dynamic nature of the cell population on the oral mucosa or a protective role of *XRCC1*. Rohr et al., [66] described a similar result but only in non-exposed groups. Individuals with the *XRCC1*$_{Arg194Trp}$ (*Arg/Trp*) genotype had lower levels of karyorrhectic and pyknotic cells when compared with individuals with *XRCC1*$_{Arg194Trp}$ (*Arg/Arg*) genotype. On the other hand, evidence about protective effect of *hOGG1*$_{Ser326Cys}$ (*Ser/Cys, Cys/Cys*) still contradictory. According with some theories, individuals with the *hOGG1*$_{Ser326Cys}$ (*Cys/Cys*) variant genotype would repair oxidatively damaged DNA less efficiently and could be predisposed to cancer. Several studies have linked this polymorphism to a reduced DNA repair capacity [76] and different types of cancer [77,78]. In contrast, protective effects of *Cys* allele have also been reported for breast [79], lung [80] and colorectal cancer [81]. How a defective repair of oxidatively damaged DNA could lead to lower levels of BMNCyt and primary genetic DNA damage needs further investigation.

5. Conclusions

The present study suggests that DNA damage caused by occupational exposure to coal residues is modulated by the combination of different polymorphisms in key genes involved in xenobiotic – metabolism and DNA repair (BER pathway). These unfavorable genotypes are individual susceptibility factors that have a functional impact and influence on potential cancer risk in coal mining residues exposed workers. This modulation may involve specific and differentiated pathways in different tissues that also may cause a differential sensitivity related to differential induction of some enzymes. Understanding the complexity of the relationships between exposure, metabolism, DNA repair, CBMN frequencies and DNA damage will require larger scale studies and complementary biomarkers.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

References

[1] MINMINAS, in: M.d.M.y.E.D.d. Minas (Ed.), Política Nacional de Seguridad Minera, Política Nacional de Seguridad Minera, Gobierno de Colombia, 2011, p. 54.

[2] M. Ahern, M. Hendryx, Cancer mortality rates in appalachian mountaintop coal mining areas, J. Environ. Occup. Sci. 1 (2012) 63–70.

[3] J.I. Huertas, M.E. Huertas, S. Izquierdo, E.D. Gonzalez, Air quality impact assessment of multiple open pit coal mines in northern Colombia, J. Environ. Econ. Manage. 93 (2012) 121–129.

[4] E.J. Gloscow, New Research on Genomic Instability, Nova Science Publishers Inc., New York, 2007.

[5] G. Liu, Z. Niu, D. Van Niekerk, J. Xue, L. Zheng, Polycyclic aromatic hydrocarbons (PAHs) from coal combustion: emissions analysis, and toxicology, Rev. Environ. Contam. Toxicol. 192 (2008).

[6] M. Celik, L. Donbak, F. Unal, D. Yüzbasĺoglu, H. Aksoy, S. Yilmaz, Cytogenetic damage in workers from a coal-fired power plant, Mutat. Res. Genet. Toxicol. Environ. Mutagen. 627 (2007) 158–163.

[7] J. Gibson, Coal—an introduction to its formation and propierties, in: G.J. Pitt, G.R. Millward (Eds.), Coal and Modern Coal Processing: An Introduction, Academic Press, 1979, pp. 1–25.

[8] P.A. White, The genotoxicity of priority polycyclic aromatic hydrocarbons in complex mixtures, Mutat. Res. Rev. Mutat. Res. 515 (2002) 85–98.

[9] R.J. Sram, O. Beskid, B. Binkova, I. Chvatalova, Z. Lnenickova, A. Milcova, I. Solansky, E. Tulupova, H. Bavorova, D. Ocadlikova, P.B. Farmer, Chromosomal aberrations in environmentally exposed population in relation to metabolic and DNA repair genes polymorphisms, Mutat. Res. 620 (2007) 22–33.

[10] J. Klinchid, B. Chewaskulyoung, S. Saeteng, N. Lertprasertsuke, W. Kasinrerk, R. Cressey, Effect of combined genetic polymorphisms on lung cancer risk in northern Thai women, Cancer Genet. Cytogenet. 195 (2009) 143–149.

[11] G. Leon-Mejia, M. Quintana, R. Debastiani, J. Dias, L. Espitia-Perez, A. Hartmann, J.A. Henriques, J. Da Silva, Genetic damage in coal miners evaluated by buccal micronucleus cytome assay, Ecotoxicol. Environ. Saf. 107 (2014) 133–139.

[12] P. Rohr, K. Kvitko, F.R. da Silva, A.P.S. Menezes, C. Porto, M. Sarmento, N. Decker, J.M. Reyes, M.D. Allgayer, T.C. Furtado, M. Salvador, C. Branco, J. da Silva, Genetic and oxidative damage of peripheral blood lymphocytes in workers with occupational exposure to coal, Mutat. Res. Genet. Toxicol. Environ. Mutagen. 758 (2013) 23–28.

[13] S. Garte, E. Taioli, S. Raimondi, V. Paracchini, B. Binkova, R.J. Sram, I. Kalina, T.A. Popov, R. Singh, P.B. Farmer, Effects of metabolic genotypes on intermediary biomarkers in subjects exposed to PAHS: Results from the EXPAH study, Mutat. Res. Fundam. Mol. Mech. Mutagen. 620 (2007) 7–15.

[14] H.D. Hosgood, S.I. Berndt, Q. Lan, GST genotypes and lung cancer susceptibility in Asian populations with indoor air pollution exposures: a meta-analysis, Mutat. Res. 636 (2007) 134–143.

[15] S.K. Giri, A. Yadav, A. Kumar, K. Dev, S. Gulati, R. Gupta, N. Aggarwa, S.K. Gautam, Synergistic effect of GSTM1 and CYP1A1-m1 (Msp1) polymorphisms on DNA damage in coal-tar workers, Int. J. Adv. Pharm. Anal. 2 (2012) 51–57.

[16] S. Bonassi, A. Znaor, M. Ceppi, C. Lando, W.P. Chang, N. Holland, M. Kirsch-Volders, E. Zeiger, S. Ban, R. Barale, M.P. Bigatti, C. Bolognesi, A. Cebulska-Wasilewska, E. Fabianova, A. Fucic, L. Hagmar, G. Joksic, A. Martelli, L. Migliore, E. Mirkova, M.R. Scarfi, A. Zijno, H. Norppa, M. Fenech, An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans, Carcinogenesis 28 (2007) 625–631.

[17] C. Bolognesi, P. Roggieri, M. Ropolo, P. Thomas, M. Hor, M. Fenech, A. Nersesyan, S. Knasmueller, Buccal micronucleus cytome assay: results of an intra- and inter-laboratory scoring comparison, Mutagenesis 4 (2015) 545–555.

[18] C. Bolognesi, M. Fenech, Micronucleus assay in human cells: lymphocytes and buccal cells, Methods Mol. Biol. (Clifton, N.J.) 1044 (2013) 191–207.

[19] M. Neri, D. Milazzo, D. Ugolini, M. Milic, A. Campolongo, P. Pasqualetti, S. Bonassi, Worldwide interest in the comet assay: a bibliometric study, Mutagenesis 30 (2015) 155–163.

[20] A. Collins, G. Koppen, V. Valdiglesias, M. Dusinska, M. Kruszewski, P. Møller, E. Rojas, A. Dhawan, I. Benzie, E. Coskun, M. Moretti, G. Speit, S. Bonassi, The comet assay as a tool for human biomonitoring studies: the ComNet Project, Mutat. Res. 759 (2014) 27–39.

[21] G. León-Mejía, L. Espitia-Pérez, L.S. Hoyos-Giraldo, J. Da Silva, A. Hartmann, J.A.P. Henriques, M. Quintana, Assessment of DNA damage in coal open-cast mining workers using the cytokinesis-blocked micronucleus test and the comet assay, Sci. Total Environ. 409 (2011) 686–691.

[22] G. Leon, L.E. Perez, J.C. Linares, A. Hartmann, M. Quintana, Genotoxic effects in wild rodents (Rattus rattus and Mus musculus) in an open coal mining area, Mutat. Res. 630 (2007) 42–49.

[23] M. Fenech, W.P. Chang, M. Kirsch-Volders, N. Holland, S. Bonassi, E. Zeiger, HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures, Mutat. Res. Genet. Toxicol. Environ. Mutagen. 534 (2003) 65–75.

[24] H. Acar, U. Caliskan, S. Demirel, D.A. Largaespada, Micronucleus incidence and their chromosomal origin related to therapy in acute lymphoblastic leukemia (ALL) patients: detection by micronucleus and FISH techniques, Teratog. Carcinog. Mutagen. 21 (2001) 341–347.

[25] P. Thomas, N. Holland, C. Bolognesi, M. Kirsch-Volders, S. Bonassi, E. Zeiger, S. Knasmueller, M. Fenech, Buccal micronucleus cytome assay, Nat. Protoc. 4 (2009) 825–837.

[26] A. Hartmann, E. Agurell, C. Beevers, S. Brendler-Schwaab, B. Burlinson, P. Clay, A. Collins, A. Smith, G. Speit, V. Thybaud, R.R. Tice, Recommendations for conducting the in vivo alkaline Comet assay, Mutagenesis 18 (2003) 45–51.

[27] A. Lum, L. Le Marchand, A simple mouthwash method for obtaining genomic DNA in molecular epidemiological studies, Cancer Epidemiol. Biomarkers Prev. 7 (1998) 719–724.

[28] K. De Ruyck, M. Van Eijkeren, K. Claes, R. Morthier, A. De Paepe, A. Vral, L. De Ridder, H. Thierens, Radiation-induced damage to normal tissues after

radiotherapy in patients treated for gynecologic tumors: association with single nucleotide polymorphisms in XRCC1 XRCC3, and OGG1 genes and in vitro chromosomal radiosensitivity in lymphocytes, Int. J. Radiat. Oncol. Biol. Phys. 62 (2005) 1140–1149.

[29] S.Z. Abdel-Rahman, R.A. El-Zein, W.A. Anwar, W.W. Au, A multiplex PCR procedure for polymorphic analysis of GSTM1 and GSTT1 genes in population studies, Cancer Lett. 107 (1996) 229–233.

[30] M.Y. Rojas, L.A. Alonso, V.A. Sarmiento, L.Y. Eljach, W. Usaquén, Structure analysis of the La Guajira-Colombia population: a genetic, demographic and genealogical overview, Ann. Hum. Biol. 40 (2013) 119–131.

[31] L.S. Hoyos-Giraldo, S. Carvajal, N. Cajas-Salazar, M. Ruiz, A. Sanchez-Gomez, Chromosome aberrations in workers exposed to organic solvents: influence of polymorphisms in xenobiotic-metabolism and DNA repair genes, Mutat. Res. 666 (2009) 8–15.

[32] M.C. Duarte, J. Colombo, A.R.B. Rossit, A.E. Silva, Polymorphisms of the DNA repair genes XRCC1 and XRCC3 in a Brazilian population, Genet. Mol. Biol. 28 (2005) 397–401.

[33] S. Garte, Metabolic susceptibility genes as cancer risk factors time for a reassessment? AACR (2001) 1233–1237.

[34] A. Santovito, P. Cervella, M. Delpero, Evaluation of genomic damage in peripheral lymphocytes from occupationally exposed anesthetists: assessment of the effects of age, sex, and GSTT1 gene polymorphism, J. Biochem. Mol. Toxicol. (2015).

[35] S. Karaca, M. Karaca, T. Cesuroglu, S. Erge, R. Polimanti, GSTM1, GSTP1, and GSTT1 genetic variability in Turkish and worldwide populations, Am. J. Hum. Biol. 27 (2015) 310–316.

[36] M. Kirsch-Volders, R.A. Mateuca, M. Roelants, A. Tremp, E. Zeiger, S. Bonassi, N. Holland, W.P. Chang, P.V. Aka, M. Deboeck, L. Godderis, V. Haufroid, H. Ishikawa, B. Laffon, R. Marcos, L. Migliore, H. Norppa, J.P. Teixeira, A. Zijno, M. Fenech, The effects of GSTM1 and GSTT1 polymorphisms on micronucleus frequencies in human lymphocytes in vivo, Cancer Epidemiol. Biomarkers Prev. 15 (2006) 1038–1042.

[37] L. Godderis, P. Aka, R. Mateuca, M. Kirsch-Volders, D. Lison, H. Veulemans, Dose-dependent influence of genetic polymorphisms on DNA damage induced by styrene oxide, ethylene oxide and gamma-radiation, Toxicology 219 (2006) 220–229.

[38] S. Garte, E. Taioli, T. Popov, I. Kalina, R. Sram, P. Farmer, Role of GSTT1 deletion in DNA oxidative damage by exposure to polycyclic aromatic hydrocarbons in humans, Int. J. Cancer 120 (2007) 2499–2503.

[39] M. García-Closas, K.T. Kelsey, S.E. Hankinson, D. Spiegelman, K. Springer, W.C. Willett, F.E. Speizer, D.J. Hunter, Glutathione S-transferase mu and theta polymorphisms and breast cancer susceptibility, J. Natl. Cancer Inst. 91 (1999) 1960–1964.

[40] W.J. Kim, H. Kim, C.H. Kim, M.S. Lee, B.R. Oh, H.M. Lee, T. Katoh, GSTT1-null genotype is a protective factor against bladder cancer, Urology 60 (2002) 913–918.

[41] A. Risch, H. Wikman, S. Thiel, P. Schmezer, L. Edler, P. Drings, H. Dienemann, K. Kayser, V. Schulz, B. Spiegelhalder, H. Bartsch, Glutathione-S-transferase M1, M3 T1 and P1 polymorphisms and susceptibility to non-small-cell lung cancer subtypes and hamartomas, Pharmacogenetics 11 (2001) 757–764.

[42] S. Landi, Mammalian class theta GST and differential susceptibility to carcinogens: a review, Mutat. Res. 463 (2000) 247–283.

[43] J.D.N. Pone, K.A.A. Hein, G.B. Stracher, H.J. Annegarn, R.B. Finkleman, D.R. Blake, J.K. McCormack, P. Schroeder, The spontaneous combustion of coal and its by-products in the Witbank and Sasolburg coalfields of South Africa, Int. J. Coal Geol. 72 (2007) 124–140.

[44] E. Hallier, K. Schröder, K. Asmuth, A. Dommermuth, B. Aust, H. Goergens, Metabolism of dichloromethane (methylene chloride) to formaldehyde in human erythrocytes: influence of polymorphism of glutathione transferase Theta (GST T1-1), Arch. Toxicol. 68 (1994) 423–427.

[45] S.J. London, J.M. Yuan, F.L. Chung, Y.T. Gao, G.A. Coetzee, R.K. Ross, M.C. Yu, Isothiocyanates, glutathione S-transferase M1 and T1 polymorphisms, and lung-cancer risk: a prospective study of men in Shanghai, China, Lancet 356 (2000) 724–729.

[46] S. Garte, E. Taioli, S. Raimondi, V. Paracchini, B. Binkova, R.J. Sram, I. Kalina, T.A. Popov, R. Singh, P.B. Farmer, Effects of metabolic genotypes on intermediary biomarkers in subjects exposed to PAHS: results from the EXPAH study, Mutat. Res. 620 (2007) 7–15.

[47] F.F. Parl, Glutathione S-transferase genotypes and cancer risk, Cancer Lett. 221 (2005) 123–129.

[48] P. Jemth, B. Mannervik, Kinetic characterization of recombinant human glutathione transferase T1-1, a polymorphic detoxication enzyme, Arch. Biochem. Biophys. 348 (1997) 247–254.

[49] A. Hartwig, T. Schwerdtle, Interactions by carcinogenic metal compounds with DNA repair processes: toxicological implications, Toxicol. Lett. 127 (2002) 47–54.

[50] G.B.C.C.T. Programme, The Fate of Trace Elements in Pulverised Fuel (PF) Combustion Systems, Great Britain, Department of Trade and Industry, Cleaner Coal Technology Programme.

[51] J.S. Hanas, C.G. Gunn, Inhibition of transcription factor IIIA-DNA interactions by xenobiotic metal ions, Nucleic Acids Res. 24 (1996) 924–930.

[52] A. Lankoff, A. Banasik, A. Duma, E. Ochniak, H. Lisowska, T. Kuszewski, S. Góźdź, A. Wojcik, A comet assay study reveals that aluminium induces DNA damage and inhibits the repair of radiation-induced lesions in human peripheral blood lymphocytes, Toxicol. Lett. 161 (2006) 27–36.

[53] K. Shinmura, J. Yokota, The OGG1 gene encodes a repair enzyme for oxidatively damaged DNA and is involved in human carcinogenesis, Antioxid. Redox Signal. 3 (2001) 597–609.

[54] J.P. Mackay, M. Crossley, Zinc fingers are sticking together, Trends Biochem. Sci. 23 (1998) 1–4.

[55] C.V. Breton, W. Zhou, M.L. Kile, E.A. Houseman, Q. Quamruzzaman, M. Rahman, G. Mahiuddin, D.C. Christiani, Susceptibility to arsenic-induced skin lesions from polymorphisms in base excision repair genes, Carcinogenesis 28 (2007) 1520–1525.

[56] C.P. Marston, C. Pereira, J. Ferguson, K. Fischer, O. Hedstrom, W.M. Dashwood, W.M. Baird, Effect of a complex environmental mixture from coal tar containing polycyclic aromatic hydrocarbons (PAH) on the tumor initiation, PAH–DNA binding and metabolic activation of carcinogenic PAH in mouse epidermis, Carcinogenesis 22 (2001) 1077–1086.

[57] M. Ghanem, M. Hubbs-A.F. Kashon, D. Weissman, D. Porter, V. Batelli-L.A. Vallyathan, J. Nath, Cytochrome P4501A1 (CYP1A1) induction is suppressed by coal dust exposure in the ovine lung, Environ. Mol. Mutagen. 44 (2004) 200.

[58] T.A. Richardson, E.T. Morgan, Hepatic cytochrome P450 gene regulation during endotoxin-induced inflammation in nuclear receptor knockout mice, J. Pharmacol. Exp. Ther. 314 (2005) 703–709.

[59] H.R. Andersen, A.-M. Andersson, S.F. Arnold, H. Autrup, M. Barfoed, N.A. Beresford, P. Bjerregaard, L.B. Christiansen, B. Gissel, R. Hummel, E.B. Jørgensen, B. Korsgaard, R. Le Guevel, H. Leffers, J. McLachlan, A. Møller, J. Bo Nielsen, N. Olea, A. Oles-Karasko, F. Pakdel, K.L. Pedersen, P. Perez, N.E. Skakkebœk, C. Sonnenschein, A.M. Soto, J.P. Sumpter, S.M. Thorpe, P. Grandjean, Comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals, Environ. Health Perspect. 107 (1999) 89–108.

[60] R. Barouki, Y. Morel, Repression of cytochrome P450 1A1 gene expression by oxidative stress: mechanisms and biological implications, Biochem. Pharmacol. 61 (2001) 511–516.

[61] Y. Morel, R. Barouki, Repression of gene expression by oxidative stress, Biochem. J. 342 (Pt. 3) (1999) 481–496.

[62] R. Perrin-Nadif, G. Auburtin, M. Dusch, J.M. Porcher, J.M. Mur, Blood antioxidant enzymes as markers of exposure or effect in coal miners, Occup. Environ. Med. 53 (1996) 41–45.

[63] R. Nadif, A. Jedlicka, M. Mintz, J.P. Bertrand, S. Kleeberger, F. Kauffmann, Effect of TNF and LTA polymorphisms on biological markers of response to oxidative stimuli in coal miners: a model of gene-environment interaction. Tumour necrosis factor and lymphotoxin alpha, J. Med. Genet. 40 (2003) 96–103.

[64] J. Gasparotto, N. Somensi, F.F. Caregnato, T.K. Rabelo, K. DaBoit, M.L.S. Oliveira, J.C.F. Moreira, D.P. Gelain, Coal and tire burning mixtures containing ultrafine and nanoparticulate materials induce oxidative stress and inflammatory activation in macrophages, Sci. Total Environ. 463–464 (2013) 743–753.

[65] E.L. Petsonk, C. Rose, R. Cohen, Coal mine dust lung disease. new lessons from an old exposure, Am. J. Respir. Crit. Care Med. 187 (2013) 1178–1185.

[66] P. Rohr, Avaliação do risco ocupacional em trabalhadores de minas de carvão de Candiota (Rio Grande do Sul), Instituto de Biociências. Programa de Pós-Graduação em Genética e Biologia Molecular Universidade Federal do Rio Grande do Sul, 2012.

[67] S.S. Sam, V. Thomas, K.S. Reddy, G. Surianarayanan, A. Chandrasekaran, Gene-environment interactions associated with CYP1A1 MspI and GST polymorphisms and the risk of upper aerodigestive tract cancers in an Indian population, J. Cancer Res. Clin. Oncol. 136 (2010) 945–951.

[68] K. Kvitko, J.C. Nunes, T.A. Weimer, F.M. Salzano, M.H. Hutz, Cytochrome P4501A1 polymorphisms in South American Indians, Hum. Biol. 72 (2000) 1039–1043.

[69] T. Kuljukka-Rabb, L. Nylund, R. Vaaranrinta, K. Savela, P. Mutanen, T. Veidebaum, M. Sorsa, A. Rannug, K. Peltonen, The effect of relevant genotypes on PAH exposure-related biomarkers, J. Expo. Anal. Environ. Epidemiol. 12 (2002) 81–91.

[70] F. Janot, L. Massaad, V. Ribrag, I. de Waziers, P.H. Beaune, B. Luboinski, O. Parise, A. Gouyette, G.G. Chabot, Principal xenobiotic-metabolizing enzyme systems in human head and neck squamous cell carcinoma, Carcinogenesis 14 (1993) 1279–1283.

[71] J. Smart, A.K. Daly, Variation in induced CYP1A1 levels: relationship to CYP1A1 Ah receptor and GSTM1 polymorphisms, Pharmacogenetics 10 (2000) 11–24.

[72] P. Rohr, J. da Silva, B. Erdtmann, J. Saffi, T.N. Guecheva, J. Antonio Pegas Henriques, K. Kvitko, BER gene polymorphisms (OGG1 Ser326Cys and XRCC1 Arg194Trp) and modulation of DNA damage due to pesticides exposure, Environ. Mol. Mutagen. 52 (2011) 20–27.

[73] K. Kvitko, E. Bandinelli, J.A. Henriques, V.D. Heuser, P. Rohr, F.R. da Silva, N.B. Schneider, S. Fernandes, C. Ancines, J. da Silva, Susceptibility to DNA damage in workers occupationally exposed to pesticides, to tannery chemicals and to coal dust during mining, Genet. Mol. Biol. 35 (2012) 1060–1068.

[74] H.H. Nelson, K.T. Kelsey, L.A. Mott, M.R. Karagas, The XRCC1 Arg399Gln polymorphism, sunburn, and non-melanoma skin cancer: evidence of gene-environment interaction, Cancer Res. 62 (2002) 152–155.

[75] G. Frosina, Commentary: DNA base excision repair defects in human pathologies, Free Radic. Res. 38 (2004) 1037–1054.

[76] N.J.H.A.J. Lee, J.K. Chipman, Interindividual variability in response to sodium dichromate-induced oxidative DNA damage: role of the Ser(326)Cys

polymorphism in the DNA-repair protein of 8-oxo-7,8-dihydro-2-deoxyguanosine DNA glycosylase-1, Cancer Epidemiol. Biomarkers Prev. 14 (2005) 497–505.

[77] A. Yamane, T. Kohno, K. Ito, N. Sunaga, K. Aoki, K. Yoshimura, H. Murakami, Y. Nojima, J. Yokota, Differential ability of polymorphic OGG1 proteins to suppress mutagenesis induced by 8-hydroxyguanine in human cell in vivo, Carcinogenesis 25 (2004) 1689–1694.

[78] W. Wei, X.-F. He, J.-B. Qin, J. Su, S.-X. Li, Y. Liu, Y. Zhang, W. Wang, Association between the OGG1 Ser326Cys and APEX1 Asp148Glu polymorphisms and lung cancer risk: a meta-analysis, Mol. Biol. Rep. 39 (2012) 11249–11262.

[79] H. Luo, Z. Li, Y. Qing, S.H. Zhang, Y. Peng, Q. Li, D. Wang, Single nucleotide polymorphisms of DNA base-excision repair genes (APE1, OGG1 and XRCC1) associated with breast cancer risk in a Chinese population, Asian Pac. J. Cancer Prev.: APJCP 15 (2014) 1133–1140.

[80] K. De Ruyck, M. Szaumkessel, I. De Rudder, A. Dehoorne, A. Vral, K. Claes, A. Velghe, J. Van Meerbeeck, H. Thierens, Polymorphisms in base-excision repair and nucleotide-excision repair genes in relation to lung cancer risk, Mutat. Res. 631 (2007) 101–110.

[81] R. Hansen, M. Sæbø, C.F. Skjelbred, B.A. Nexø, P.C. Hagen, G. Bock, I.M. Bowitz Lothe, E. Johnson, S. Aase, I.-L. Hansteen, U. Vogel, E.H. Kure, GPX Pro198Leu and OGG1 Ser326Cys polymorphisms and risk of development of colorectal adenomas and colorectal cancer, Cancer Lett. 229 (2005) 85–91.

[82] W. Puettmann, K. Steffens, W. Gossel, Analysis of Aromatic Hydrocarbons in Overburden from Coal Mines: Assessment of the Environmental Impact, TechBooks, United States, 1991.

[83] V.A. Evagelopoulos, T.A. Albanis, A. Asvesta, S. Zoras, Polycyclic Aromatic Hydrocarbons (PAHs) in fine and coarse particles, Global NEST J. 12 (2010) 63–70.

[84] J. Ribeiro, E.F. da Silva, D. Flores, Burning of coal waste piles from Douro Coalfield (Portugal): petrological, geochemical and mineralogical characterization, Int. J. Coal Geol. 81 (2010) 359–372.

[85] D. Voutsa, H. Terzi, L. Muller, C. Samara, T. Kouimtzis, Profile analysis of organic micropollutants in the environment of a coal burning area, NW Greece, Chemosphere 55 (2004) 595–604.

[86] W.U. Yu-guo, W.U. Jian-ming, Experimental study on significant gases of coal spontaneous combustion by temperature programmed (TP), Procedia Eng. 26 (2011) 120–125.

[87] D.A. Kirchgessner, S.D. Piccot, S.S. Masemore, An improved inventory of methane emissions from coal mining in the United States, J. Air Waste Manag. Assoc. 50 (2000) 1904–1919.

[88] R. Tichý, V. Mejstřík, Heavy metal contamination from open-pit coal mining in Europe's Black Triangle and possible remediation, Environ. Rev. 4 (1996) 321–341.

[89] P. Xing, L.I. Darvell, J.M. Jones, L. Ma, M. Pourkashanian, A. Williams, Experimental and theoretical methods for evaluating ash properties of pine and El Cerrejon coal used in co-firing, Fuel 183 (2016) 39–54.

[90] Y. Zhao, J. Zhang, C.-L. Chou, Y. Li, Z. Wang, Y. Ge, C. Zheng, Trace element emissions from spontaneous combustion of gob piles in coal mines, Shanxi, China, Int. J. Coal Geol. 73 (2008) 52–62.

[91] J.I. Huertas, M.E. Huertas, D.A. Solis, Characterization of airborne particles in an open pit mining region, Sci. Total Environ. 423 (2012) 39–46.

[92] X. Querol, M. Izquierdo, E. Monfort, E. Alvarez, O. Font, T. Moreno, A. Alastuey, X. Zhuang, W. Lu, Y. Wang, Environmental characterization of burnt coal gangue banks at Yangquan, Shanxi Province, China, Int. J. Coal Geol. 75 (2008) 93–104.

[93] A.S. Damle, D.S. Ensor, M.B. Ranade, Coal combustion aerosol formation mechanisms: a review, Aerosol Sci. Technol. 1 (1981) 119–133.

[94] Ž. Cvetković, M. Logar, A. Rosić, Mineralogy and characterization of deposited particles of the aero sediments collected in the vicinity of power plants and the open pit coal mine: Kolubara (Serbia), Environ. Sci. Pollut. Res. 20 (2013) 3034–3049.

[95] T. Jones, P. Blackmore, M. Leach, K. BéruBé, K. Sexton, R. Richards, Characterisation of airborne particles collected within and proximal to an opencast coalmine: South Wales, U.K, Environ. Monit. Assess. 75 (2002) 293–312.

[96] E.I. Kauppinen, T.A. Pakkanen, Coal combustion aerosols: a field study, Environ. Sci. Technol. 24 (1990) 1811–1818.

[97] C. Petaloti, A. Triantafyllou, T. Kouimtzis, C. Samara, Trace elements in atmospheric particulate matter over a coal burning power production area of western Macedonia, Greece, Chemosphere 65 (2006) 2233–2243.

[98] R.B. Finkelman, Trace elements in coal: environmental and health significance, Biol. Trace Elem. Res. 67 (1999) 197–204.

[99] M.S. Gustin, K. Ladwig, An assessment of the significance of mercury release from coal fly ash, J. Air Waste. Manage. Assoc. (1995) 54 (2004) 320–330.

[100] V.P. Aneja, A. Isherwood, P. Morgan, Characterization of particulate matter (PM10) related to surface coal mining operations in Appalachia, Atmos. Environ. 54 (2012) 496–501.

[101] B. Valentim, A. Guedes, D. Flores, C.R. Ward, J.C. Hower, Variations in fly ash composition with sampling location: case study from a Portuguese power plant, CCGP 1 (2009) 14–24.

[102] I.C. López, C.R. Ward, Composition and mode of occurrence of mineral matter in some Colombian coals, Int. J. Coal Geol. 73 (2008) 3–18.

ANEXO II

Genetic damage in coal miners evaluated by buccal micronucleus cytome assay

CrossMark
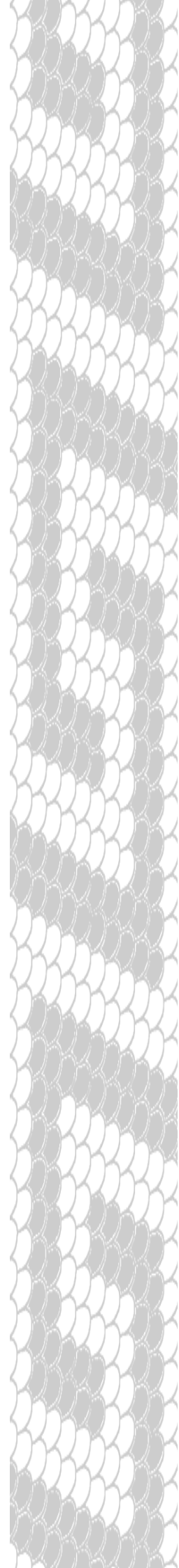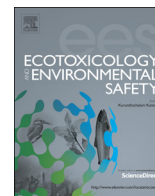
Genetic damage in coal miners evaluated by buccal micronucleus cytome assay

Grethel León-Mejía [a,*], Milton Quintana [b], Rafaela Debastiani [c], Johnny Dias [c],
Lyda Espitia-Pérez [d], Andreas Hartmann [e], João Antônio Pêgas Henriques [a,f,*],
Juliana Da Silva [g]

[a] Departamento de Biofísica, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brasil
[b] Unidad de Investigación, Desarrollo e Innovación en Genética y Biología Molecular, Universidad Simón Bolívar, Barranquilla, Colombia
[c] Laboratório de Implantação Iônica, Instituto de Física, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brasil
[d] Laboratorio de Investigación Biomédica y Biología Molecular, Universidad del Sinú, Montería, Colombia
[e] Novartis Pharma AG, Basel, Switzerland
[f] Instituto de Biotecnologia, Universidade de Caxias do Sul (UCS), Caxias do Sul, RS, Brasil
[g] Laboratório de Genética Toxicológica, Universidade Luterana do Brasil (ULBRA), Canoas-RS, Brasil

ARTICLE INFO

ABSTRACT

During coal mining activities, large quantities of coal dust, ashes, polycyclic aromatic hydrocarbons and metals are released into the environment. This complex mixture presents one of the most important occupational hazards for health of workers. The aim of the present study was to evaluate the genetic damage together with the presence of inorganic elements, in an exposed workers population to coal mining residues of Guajira-Colombia. Thus, 100 exposed workers and 100 non-exposed control individuals were included in this study. To determine genetic damage we assessed the micronucleus (MN) frequencies and nuclear buds in buccal mucosa samples (BMCyt) assay, which were significantly higher in the exposed group than non-exposed control group. In addition, karyorrhectic and karyolytic cells were also significantly higher in the exposed group (cell death). No significant difference was observed between the exposed groups engaged in different mining activities. No correlation between age, alcohol consumption, time of service and MN assay data were found in this study. However, the content of inorganic elements in blood samples analyzed by a Particle-induced X-ray emission technique (PIXE) showed higher values of silicon (Si) and aluminum (Al) in the exposed group. In this study we discuss the possibility of DNA damage observed in the mine workers cells be a consequence of oxidative damage.

1. Introduction

It is known that coal mining activities are a major source of environmental contamination. Mining activities release large amounts of substances that can form complex mixtures containing CO_x, NO_x, SO_x, aluminum silicon crystals, quartz, metals (arsenic, boron, cadmium, chromium, lead, copper, selenium, iron, and zinc), and polycyclic aromatic hydrocarbons (PAH) into the environment (Zhou et al., 2005).

The main route of coal mining exposure to these potentially hazardous residues is by inhalation of coal dust particles from the extraction and manipulation activities. Currently, it is known that chronic inhalation of coal dust particles can result in lung disorders including simple pneumoconiosis, progressive massive fibrosis, bronchitis, lung function loss, emphysema and cancer. Studies were able to establish that some of these disorders could have their origin in genetic damage generated by the inhalation of mineral particles. In particular interaction of particles with macrophages, epithelial cells and other cells could lead to generation of reactive oxygen species (ROS) (Schins and Borm, 1999; Cooke et al., 2003).

The effects of coal exposure have been studied using bacteria (Nakajima et al., 2008), bats (Zocche et al., 2010), rodents (Da Silva et al., 2000, León et al., 2007) and human cells (Celik et al., 2007; Rohr et al., 2013a, 2013b). Some studies in workers exposed to coal mining residues assessed by chromosomal aberrations (Santa Maria et al., 2007), sister chromatid exchange, and micronuclei

* Corresponding authors at: Departamento de Biofísica, Prédio 43422, Laboratório 210, Campus do Vale, Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves 9500, Bairro Agronomia, CEP 91501-970, Porto Alegre, RS, Brasil. Fax: +55 5133167003.

E-mail addresses: grethelleon@gmail.com, grethy40@hotmail.com (G. León-Mejía), pegas@cbiot.ufrgs.br (J.A.P. Henriques).

(MN) in peripheral blood lymphocytes (Donbak et al., 2005; León-Mejía et al., 2011) demonstrated that occupational exposure to coal dust can lead to a significant induction of cytogenetic damage. In a previous study, we found elevated DNA damage in coal mining workers from Guajira-Colombia, assessed by the Comet assay and MN test in lymphocytes (León-Mejía et al., 2011). Despite these findings, coal dust remains classified as "not classifiable as to its carcinogenicity to humans" (Group 3) by the International Agency for Research on Cancer (IARC, 1997).

The fact that a very high percentage of cancers have an epithelial origin suggests that micronuclei in epithelial cells are an important biomarker that can be used for epidemiological studies. Micronuclei that are detected in exfoliated buccal cells reflect genotoxic events that occurred in basal cells, and these events can be observed in exfoliated cells over an approximately three week period (Holland et al., 2008). The buccal micronucleus cytome assay (BMCyt assay) is considered a fast and simple method for *in situ* biomonitoring of human populations exposed to environmental genotoxicans (Majer et al., 2001; Bonassi et al., 2011).

The aim of the present study was to evaluate the genotoxic effects in exfoliated buccal cells and concentrations of inorganic elements in a population exposed to coal residues in the open-cast mine "El Cerrejón" in Guajira-Colombia using the buccal micronucleus cytome assay (exfoliated buccal cells; BMCyt assay) and the particle-induced X-ray emission (PIXE) in blood samples. The MN data in buccal were compared to MN data in lymphocytes from our previous study (León-Mejía et al., 2011) to assess whether buccal cells can be used as a non-invasive source to investigate biomarkers of genetic damage in exposed individuals.

2. Materials and methods

2.1. Individuals and sampling

This study was approved by the Committee on Research Ethics at University of Sinú Ethic and details of the study through the informed consent were obtained from each individual before the research began.

This study involved a total of 200 individuals, who live in the same region in order to ensure a comparable genetic background and life habits. The exposed group were 100 workers occupationally exposed to coal with a minimum time of service of 5 years in "El Cerrejón" open-cast coal mine, in the Guajira Department in the north coast of Colombia, South America. The non-exposed control group consisted of 100 individuals with no known exposure to genotoxic agents including coal, radiation, chemicals or cigarettes. Both study populations (exposed and non-exposed groups) lived in the same region; it was considered that the two populations should have presented the same genetic background and the same life habits.

The workers were involved in different activities in the mine: (i) *transport of extracted coal* ($n=50$), in which the workers are involved in coal transport up to arrival in the storing centers; (ii) *equipment field maintenance* ($n=18$), these workers drive trucks to spread water onto the roads where large quantities of coal dust are generated, and also maintain the coal extraction equipment; (iii) *coal stripping* ($n=17$), these workers are engaged in coal stripping activities and the accumulation of the material for the transport in trucks, they also extinguish fires generated by spontaneous combustion of coal; (iv) *coal embarking* ($n=15$), these workers are involved in shipping of coal in containers to be exported to other countries. All workers were exposed to large quantity of coal dust, but was perceived that the coal stripping group was the most exposed to coal mining residues.

All individuals in the study were required to answer a questionnaire and participate in a face-to-face interview, which included determination of standard demographic data and questions concerning medical issues (exposure to X-rays, vaccinations, medication, *etc.*), life style (smoking, alcohol consumption, diet, *etc.*), cancer history, other chronic diseases and occupation (number of working hours per day, protective measures adopted). All individuals included in the study were non-smokers and have time of service ≥ 5 years. Buccal cell and blood samples were obtained from all individuals.

2.2. Buccal micronucleus cytome assay (BMCyt assay)

After informed consent was obtained from each individual, buccal mucosa samples from all 200 individuals were collected. The subjects were asked to rinse their mouth with water before sampling. The exfoliated buccal mucosa cells were

collected using a cytobrush to gently scrape the mucosa of the inner lining of both cheeks. All buccal sample tubes were coded and kept in upright position at room temperature.

The cells were washed three times in 0.9 percent phosphate saline buffer, the smears were made from the pellet and fixed in methanol:acetic acid (3:1). For microscopic analysis, the slides were incubated at 37 °C overnight and then stained with Giemsa (Stich and Rosin, 1984; Acar et al., 2001). The frequency of MN was determined in 2000 cells for each person following recommendations of Thomas et al. (2009). All slides were scored by one reader blinded to the exposure status of the individuals.

MN and other nuclear abnormalities were classified according to Tolbert et al. (1992) and Thomas et al. (2009). Nuclear anomalies, such as karyorrhectic and karyolytic cells (different forms of cell death), and nuclear buds (indicative of gene amplification) were assessed in 2000 cells/individual and recorded separately.

2.3. Particle-induced X-ray emission (PIXE)

Peripheral blood samples from all 200 individuals were collected by venipuncture. Thus, 5 mL of blood were drawn into heparin tubes (Becton Dickenson, vacutainer) for the particle-induced X-ray emission (PIXE) analysis. All blood samples tubes were coded and kept at room temperature. Blood samples were analyzed for the total content of metals by the particle induced X-ray emission (PIXE) technique (He et al., 1993; Johansson et al., 1995). This technique has been successfully employed to detect trace elements in plants and animals because of its multielemental character, high sensitivity, simplicity and high sample throughput (Mireles et al., 2004).

For the analyses, the blood samples were dried at 40 °C for 72 h, then macerated using a mortar, and finally pressed into pellets which were positioned on the target of the reaction chamber. A 3 MV Tandetron accelerator provided 2.0 MeV proton beams with an average current of 5 nA at the target. The X-rays induced by the beam in the samples were detected by a Si(Li) detector with an energy resolution of about 155 eV at 5.9 keV. The spectra were analyzed with the GUPIXWIN software package (Maxwell et al., 1995; Campbell, 2000) and the final results are expressed in parts per million (ug g^{-1}). The chemical elements analyzed in the samples by the PIXE method were: sodium (Na), magnesium (Mg), aluminum (Al), silicon (Si), phosphorus (P), sulfur (S), chlorine (Cl), potassium (K), calcium (Ca), iron (Fe), copper (Cu), zinc (Zn), bromine (Br) and rubidium (Rb). The organic matrix of the blood (the organic composition of the sample) was determined by the Rutherford Backscattering Spectrometry (RBS) technique.

2.4. Statistical analysis

The normality of the variables was evaluated using the Kolmogorov–Smirnov test; χ^2 and t-tests were used to compare the demographic characteristics of study populations and chemical elements analyzed by PIXE. The statistical analysis of differences in MN frequency between the exposed and control group were carried out using the non-parametric Mann–Whitney U-test, and statistical differences between the five groups (non-exposed control, extracted coal transport, equipment field maintenance, coal stripping, and coal embarking) were analyzed using the non-parametric two-tailed Kruskal–Wallis test with the Dunn correction. Correlations between MN frequency in lymphocytes obtained in our previous study (León-Mejía et al., 2011) and MN frequencies in buccal cells of the present study in control and exposed individuals were determined by Spearman rank correlation test. The critical level for rejection of the null hypothesis was considered to be $P < 0.05$. All analyses were performed with the PRISMA 5.0 statistical software package.

3. Results

The mean age and standard deviation of exposed group was 44.0 ± 7.5 years (range, 24–60 years), and non-exposed control group was 43.7 ± 7.8 years (range, 27–60 years). The mean time of service of the exposed group was 17.7 ± 6.9 years (range, 5–30 years). The percentage of alcohol consumption for non-exposed group was 45 percent and for exposed group was 55 percent, considering as alcohol consumer to drink alcohol in excess of once/week.

Table 1 summarizes the values of the MN frequencies for both study groups, exposed and control groups, with exposed group differentiated by the mining area activities. There was no statistically significant difference between the different mining area activities ($P > 0.05$; Kruskal–Wallis test), however the micronuclei frequencies observed to each individual subgroup exposed to coal mining were significantly increased compared to control group values ($P < 0.05$; Kruskal–Wallis test).

Table 1
Parameters of genetic damage (micronuclei), gene amplification (nuclear bud) and cell death (karyorrhexis and karyolitic cells) observed in exfoliated buccal of the control and exposed group divided by mining area activities (mean ± standard deviation).

Groups	Micronucleus test parameters			
	Micronucleus	Nuclear bud	Karyorrhexis	Karyolytic
Control (n=100)	1.0 ± 2.2	1.0 ± 1.1	35.8 ± 30.5	25.3 ± 17.3
Exposed (n=100)	8.8 ± 12.8[a]	2.7 ± 4.2[b]	64.8 ± 54.9[b]	36.1 ± 40.1[b]
Exposed per mining area				
Transport of extracted coal (n=50)	8.9 ± 11.7[c]	2.3 ± 2.9[c]	63.1 ± 49.0[c]	30.3 ± 38.4
Equipment field maintenance(n=18)	8.7 ± 14.9[c]	1.6 ± 2.3	64.4 ± 56.0[c]	36.3 ± 37.6[c]
Coal stripping (n=17)	11.3 ± 17.7[c]	4.1 ± 6.5[c]	85.7 ± 78.3[c]	38.2 ± 68.8[c]
Coal embarking (n=15)	5.7 ± 6.1[c]	3.0 ± 4.3[c]	52.0 ± 33.7[c]	29.0 ± 16.1

[a] $P < 0.001$, Mann–Whitney U-test.
[b] Significant difference compared to the control group at $P < 0.05$.
[c] Significant difference compared to the control group at $P < 0.05$, Kruskal Wallis test.
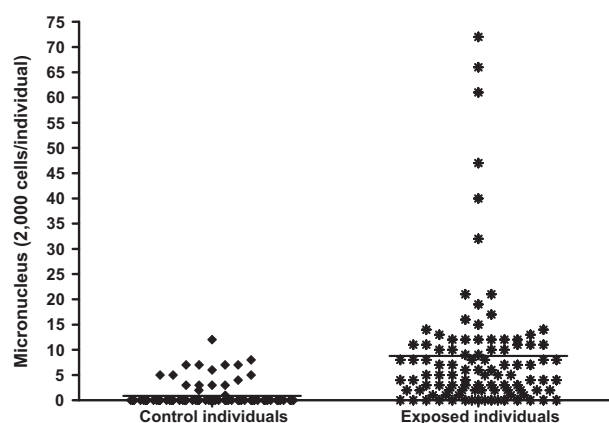
Fig. 1. Scatterplot comparing MN frequencies (BMCyt assay) of control individuals and exposed individuals. Horizontal lines represent the group mean MN frequencies.

The results obtained for MN frequency in exfoliated buccal cells showed that the values in the exposed group to coal mine residues are significantly higher compared with the control group, which were evaluated by Mann–Whitney U-test ($P < 0.001$). In addition, Table 1 lists additional markers of nuclear abnormalities in exfoliated buccal cells: nuclear buds, karyorrhectic and karyolytic cells. The mean values of the exposed group of these biomarkers were statistically significant compared to control group ($P < 0.05$).

The Spearman correlation coefficient of MN frequency with respect to age for the exposed ($P=0.7159$; $r = -0.03685$) versus control group ($P=0.1475$; $r=0.5272$) were not significant ($P > 0.05$). A high inter-individual variation between MN frequencies was only observed in the exposed group and ranged from 0 percent to 3.6 percent (0–72 MN/2000 cells) (Fig. 1). Fig. 2 shows the scatterplot comparing MN frequencies of the control group and exposed group divided by mining area activities. However, there were no significant correlations between the MN frequency and the service time for the different exposed groups ($P > 0.05$): extracted coal transport ($P=0.9116$; $r = -0.0500$); equipment field maintenance ($P=0.1618$; $r=0.5126$); coal stripping ($P=0.9359$; $r = -0.02112$); and coal embarking ($P=0.0760$; $r = -0.6360$). The MN data in buccal cells obtained in this study were compared with MN data in lymphocytes obtained in our previous study (León-Mejía et al., 2011). Fig. 3 shows a significant and positive correlation between MN frequency in lymphocytes and buccal cells of control and exposed individuals ($P < 0.001$; $r=0.573$).

The chemical elements present in the samples determinate by the PIXE method are presented in Table 2. The organic matrix of the blood (the organic composition of the sample) was 72.50 percent carbon, 7.50 percent oxygen, 13.50 percent nitrogen and 6.50 percent fluorine. There was no individual differences in the ppm concentrations of metals in the blood of workers measured by PIXE, in relation to the function performed in coal mining area as assessed by Kruskal–Wallis ANOVA and Dunn's post-test. Thus, for the evaluation of different correlations with regards to chemical present in blood samples, the whole study population of the exposed individuals was considered as the "exposed group". In the analysis of the difference between exposed and control group, the exposed group showed significantly higher ppm levels of aluminum (Al) and silicon (Si) using t-Student test with Welch correction ($P < 0.05$). The ppm amounts of metals showed no correlation with age or exposure time (Spearman correlation).

4. Discussion

Coal mining is an activity with a high potential for environmental pollution. In the case of exposure to coal mining residues, the studies that used biomarkers of biological effects, susceptibility and exposure as epidemiological tools are still scarce and most studies assessed underground mining activities (Agostini et al., 1996; Moriske et al., 1996; Santa Maria et al., 2007; Donbak et al., 2005). However, potential genotoxic effects caused by coal open-cast mining activities on human health remain poorly explored.

In the present study, MN formation in exfoliated buccal cells of workers exposed to open coal mining was used as a biomarker for genotoxic exposure. This study did not show any effect of alcohol consumption, age and time of service on the MN frequency of the populations investigated. In concordance, Holland et al. (2008) cite that most occupational studies conducted with the buccal micronucleus cytome assay do not find a statistically significant influence of age and lifestyles in the MN frequency of study populations.

When we compared the MN frequencies in the group exposed to coal mining residues we observed a significantly higher frequency compared to the matched control group. No significant difference was observed in the extent of MN formation among the four different mining activities (transport of extracted coal, equipment field maintenance, coal stripping, and coal embarking). This observation indicates that the workers did show a genotoxic response to a complex mixture independent of the working area. Several individuals in the exposed group showed a higher MN frequency and high inter-individual variability. There was no clear difference in the exposed subgroups of the different working areas; therefore it can be assumed that there was no specific factor that would induce a particular high MN frequency. In a recent study using lymphocytes from coal mine workers from
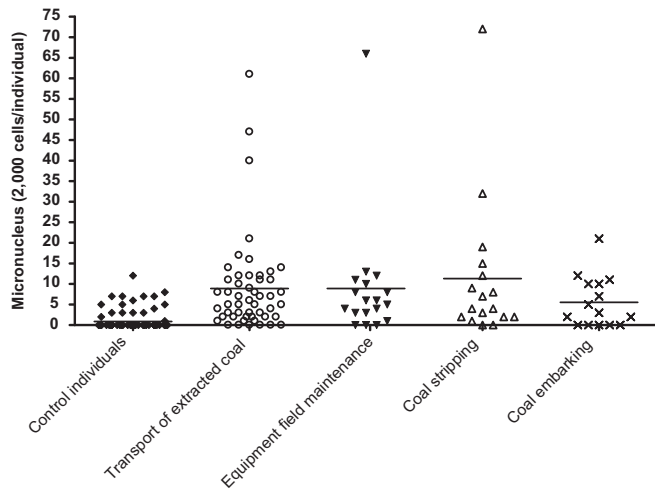
Fig. 2. Scatterplot comparing MN frequencies (BMCyt assay) of the control group and exposed group divided by mining area activities. Horizontal lines represent the group mean MN frequencies.
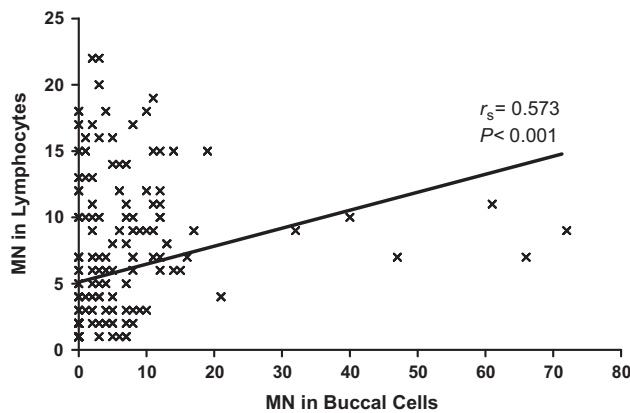

Fig. 3. Nonparametric Spearman correlation analysis between MN frequency in lymphocytes and buccal cells of control and exposed individuals ($n=200$).

Guajira-Colombia, we found comparable results with regards to occupational hazard effects using the Comet assay and MN test in lymphocytes (León-Mejía et al., 2011). These previous results demonstrated that the group exposed to coal mining residuals exhibited a significantly higher extent of DNA damage in peripheral lymphocytes in the Comet assay. In the exposed group, MN frequency was 2.9-fold and DNA damage index was 6.6-fold higher compared to the control group (León-Mejía et al., 2011), in this study we observed that MN frequency in buccal cells was 8.8-fold higher than control group. While the comet assay detects primary DNA damage with high sensitivity (Collins et al., 2008), the assessment of the MN frequency in isolated lymphocytes has become a reliable biomarker of chromosome breakage and/or whole chromosome loss (Fenech et al., 2003; Fenech, 2006). It has been demonstrated that high frequencies of MN in peripheral blood lymphocytes are predictive of cancer risk and that high levels of MN formation are associated with early events in carcinogenesis (Bonassi et al., 2007; Kirsch-Volders et al., 2014).

In our previous study (León-Mejía et al., 2011), MN frequencies were analyzed in lymphocytes from the same sample groups as those used in the current study, and these data were compared with data on MN in buccal cells from the current study. There was a significant and positive correlation between MN frequencies in the lymphocytes and buccal cells of the control and exposed individuals ($P < 0.001$; $r=0.573$; Fig. 3). Similarly Ceppi et al.

Table 2
Concentration of inorganic elements in the blood samples (ppm) of the control group and exposed group divided by mining area activities by PIXE method (mean ± standard deviation).

Groups	Inorganic elements (ppm)													
	Na	Mg	Al	Si	P	S	Cl	K	Ca	Fe	Cu	Zn	Br	Rb
Control (n=100)	8942 ± 1657	209 ± 81	109 ± 50	44 ± 30	1629 ± 284	5717 ± 892	14,927 ± 2623	9285 ± 1481	312 ± 116	2945 ± 431	5 ± 2	39 ± 10	18 ± 9	17 ± 9
Exposed (n=100)	8049 ± 2019	210 ± 170	127 ± 60*	81 ± 95*	1529 ± 422	5396 ± 1051	13,572 ± 3366	8731 ± 2109	292 ± 106	2777 ± 538	5 ± 2	37 ± 10	17 ± 8	19 ± 11
Exposed per mining area														
Transport of extracted coal (**n=50**)	8042 ± 1900	219 ± 202	117 ± 63	71 ± 91	1525 ± 467	5401 ± 878	13,531 ± 2794	8675 ± 1786	291 ± 120	2783 ± 482	5 ± 2	37 ± 9	17 ± 8	18 ± 11
Equipment field maintenance (**n=18**)	8450 ± 2235	172 ± 89	150 ± 61	97 ± 123	1517 ± 360	5354 ± 1491	14,473 ± 3834	9001 ± 2315	298 ± 103	2854 ± 704	5 ± 2	37 ± 13	17 ± 7	18 ± 12
Coal stripping (**n=17**)	7855 ± 2398	205 ± 121	127 ± 46	91 ± 65	1526 ± 474	5388 ± 1234	13,003 ± 3916	8524 ± 2544	276 ± 81	2775 ± 635	4 ± 2	34 ± 11	15 ± 7	19 ± 9
Coal embarking (**n=15**)	7788 ± 1784	232 ± 183	133 ± 63	73 ± 101	1565 ± 290	5443 ± 779	13,216 ± 3973	8811 ± 2499	306 ± 91	2658 ± 363	5 ± 2	38 ± 8	18 ± 6	21 ± 10

* Significant increase in relation to the control group at $P < 0.05$; Student's *t* test (Welch correction).

(2010) prepared a compilation of 19 studies which measured the MN frequency in buccal cells and lymphocytes showed a high correlation between both tissues, revealing that the MN evaluation in buccal cells has a similar potential to demonstrate the effects of exposure to genotoxic agents. The formation of micronuclei in both lymphocytes and epithelial cells has been proposed as a useful biomarker to assess the cytogenetic damage in biomonitoring studies (Diler and Celik, 2011; Rohr et al., 2013b; Kirsch-Volders et al., 2014). The formation of micronuclei in both lymphocytes and epithelial cells has been proposed as a useful biomarker to assess the damage cytogenetic in biomonitoring studies (Diler and Celik, 2011).

Our results further support that the mechanism of MN formation in buccal exfoliated cells is consistent with the model proposed for lymphocytes (recently reviewed by Ceppi et al. (2010)).

Besides the formation of MN, cell death indicators (karyorrhectic and karyolytic cells) and other nuclear anomalies such as nuclear buds (indicative of gene amplification) (Diler and Celik, 2011) were also evaluated in buccal cells. The results showed that the number of karyorrhectic and karyolytic cells were significantly higher in the exposed group compared with the control group. These markers of genetic damage found in our study suggest that these events could be a consequence of exposure to some genotoxic agents related to coal mining residues forming a complex mixture of agents present at low concentrations can interact additively or synergistically (Kalantzi et al., 2004), similar to what had been demonstrated by Rohr et al. (2013a, 2013b).

The main route of exposure of coal mine workers to potentially hazardous coal residues is by inhalation of particles. Today it is known that chronic inhalation of this cocktail (which may contain a mixture of substances such as inorganic elements and PAH) can produce pulmonary disorders (Schins and Borm, 1999; Beckman and Ames, 1997; Cooke et al., 2003). Some characteristics of coal from "El Cerrejon" are moisture ($\sim$10 percent), volatile ($\sim$30 percent), ash ($\sim$8 percent), sulfur ($\sim$1 percent), carbon (70 percent), hydrogen ($\sim$6 percent), oxygen ($\sim$5 percent), nitrogen ($\sim$1 percent) and different metals (ETSU and Department of Trade and Industry, 2000). In our study we included the assessment of several elements (Na, Mg, Al, Si, P, S, Cl, K, Ca, Fe, Cu, Zn, Br and Rb). The elements assessed in peripheral blood samples showed no difference when comparing the four mining area activities and no correlation with age or time of service was observed. In the analysis of the levels of the different elements in the study population we observed significantly higher amounts of silicon (Si) and aluminum (Al) in the exposed compared to the control group. In the composition of the Cerrejón-Guajira coal these elements are found in substantial quantities in the form of oxides (ETSU and Department of Trade and Industry, 2000), and presence of Al and Si in coal fly ash is recognized in coal fly ash (Prahalad et al., 2000). The abundance of different mineral elements in Cerrejón coal determined by Scanning Electron Microscopy Computer-Controlled shows that more than 80 percent of weight of the mineral material is composed of clay and quartz minerals (aluminum silicate, aluminum silicate, and silica). Analysis of the product reveals that the combustion ashes are formed mainly of aluminum silicates, iron oxide and quartz particles (Irons and Quick, 2000). It is known that inhalation of particulate material typically contains high levels of Al. Experimental studies indicate that the presence of excessive Al is associated with inflammatory processes in the lung which can trigger respiratory diseases (Clarke et al., 2000; Wagner et al., 2007) and carcinogenic processes (Spinelli et al., 2006; Exley et al., 2007; Neumann et al., 2011). The interaction of inorganic elements with living matter is complex, but it is possible that common mechanisms for the majority of inorganic compounds include oxidative stress,

DNA repair modulation and disturbance of signal transduction pathway (Beyersmann and Hartwig, 2008).

PAH are also associated with the generation of oxidative stress. The spontaneous combustion of coal is very common in centers of open mining storage systems, and is a major cause of the production of PAH. Many PAH have mutagenic and carcinogenic effects (Cherng et al., 1996; IARC, 1997; Da Silva et al., 2000). Exposure to PAH has been associated with increased DNA damage by cytokinesis-block micronucleus cytome and oxidative stress in occupational exposed populations (Duan et al., 2009, Guo et al., 2014). Several studies have showed buccal MN induction in exposed populations (Giri et al., 2012, Karahalil et al., 1999) and in vitro studies suggest that PAH-quinones induce genotoxic effects by modulating the metabolic machinery inside the cells by a combined effect of oxidative stress (Gurbani et al., 2013, Ekstrand-Hammarstrom et al., 2013). Mixtures of DNA-reactive procarcinogens compounds such as PAH at environmentally relevant low-dose concentrations give rise to markedly elevated DNA damage (Hewitt et al., 2007). One of the proposed mechanisms of generation of DNA damage by exposure to PAH is associated with oxidation–reduction processes occurring during the metabolism of these compounds, which result in the formation of quinones. These quinones can undergo redox cycling and produce reactive oxygen species (ROS) (Singh et al., 2007). Another way of ROS generation by exposure to coal mine residues is related to the inhalation of coal dust particles which triggers an inflammatory cell response in macrophages and lung epithelial cells producing large amounts of ROS and cytokines. There is evidence of oxidative damage in coal mining workers, as higher levels of SOD (superoxide dismutase) in individuals exposed to coal (due to an enzymatic response) comparing with non-exposed individuals (Rohr et al., 2013a, 2013b). ROS may also be generated independently of the cellular pathway due to the intrinsic chemical properties of coal dust such as iron content and the radicals on the surface (Schins and Borm, 1999). It is known that ROS are capable of causing oxidative damage to DNA such as single strand breaks and base and nucleotide modifications, particularly in guanosine. The oxidative modifications induce a broad response in the repair characterized by excision of modified bases and nucleotides (Bennett, 2001; Klaunig et al., 2011).

5. Conclusions

In summary, increased levels of micronuclei in the BMCyt assay were observed in coal mining workers. The data of the present study are in agreement with the results of a previous study assessing DNA damage in lymphocytes using the comet assay and MN assay (León-Mejía et al., 2011). The increased MN frequencies observed in the mine workers may be a consequence of oxidative damage resulting from their exposure to coal residues mixtures, including inorganic elements, as Al and Si. However, there are several additional compounds that are released during the processes of exploration and extraction of coal and therefore, due to the complex mixture, it is difficult to relate the genotoxic effects found to the actions of a single compound. Therefore, our study demonstrates that buccal cells present a suitable and non-invasive source to investigate biomarkers of genetic damage in exposed individuals.

References

Acar, H., Caliskan, U., Demirel, S., Largaespada, D.A., 2001. Micronucleus incidence and their chromosomal origin related to therapy in acute lymphoblastic leukemia (ALL) patients: detection by micronucleus and FISH techniques. Teratog. Carcinog. Mutagen. 21, 341–347.

Agostini, J., Otto, P., Wajntal, A., 1996. Chromosome damage in underground coal miners: detection by conventional cytogenetic techniques and by submitting lymphocytes of unexposed individuals to plasma from at-risk groups. Braz. J. Genet. 19, 641–646.

Beckman, K.B., Ames, B.N., 1997. Oxidative decay of DNA. J. Biol. Chem. 272, 19633–19636.

Bennett, M.R., 2001. Reactive oxygen species and death: oxidative DNA damage in atherosclerosis. Circ. Res. 88 (7), 648–650.

Beyersmann, D., Hartwig, A., 2008. Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. Arch. Toxicol. 82, 493–512.

Bonassi, S., Znaor, A., Ceppi, M., Lando, C., Chang, W.P., Holland, N., Kirsch-Volders, M., Zeiger, E., Ban, S., Barale, R., Bigatti, M.P., Bolognesi, C., Cebulska-Wasilewska, A., Fabianova, E., Fucic, A., Hagmar, L., Joksic, G., Martelli, A., Migliore, L., Mirkova, E., Scarfi, M.R., Zijno, A., Norppa, H., Fenech, M., 2007. An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. Carcinogenesis 28, 625–631.

Bonassi, S., Coskun, E., Ceppi, M., Lando, C., Bolognesi, C., Burgaz, S., Holland, N., Kirsh-Volders, M., Knasmueller, S., Zeiger, E., Carnesoltas, D., Cavallo, D., da Silva, J., de Andrade, V.M., Demircigil, G.C., Dominguez, Odio A., Donmez-Altuntas, H., Gattas, G., Giri, A., Giri, S., Gomez-Meda, B., Gomez-Arroyo, S., Hadjidekova, V., Haveric, A., Kamboj, M., Kurteshi, K., Martino-Roth, M.G., Montero Montoya, R., Nersesyan, A., Pastor-Benito, S., Favero Salvadori, D.M., Shaposhnikova, A., Stopper, H., Thomas, P., Torres-Bugarin, O., Yadav, A.S., Zuniga Gonzalez, G., Fenech, M., 2011. The HUman MicroNucleus project on eXfoLiated buccal cells (HUMN(XL)): the role of life-style, host factors, occupational exposures, health status, and assay protocol. Mutat. Res. 728, 88–97.

Campbell, J.L., 2000. The Guelph PIXE software package III. Nucl. Instrum. Methods Phys. Res. B 170, 193.

Celik, M., Donbak, L., Unal, F., Yuzbasioglu, D., Aksoy, H., Yilmaz, S., 2007. Cytogenetic damage in workers from a coal-fired power plant. Mutat. Res. 627, 158–163.

Ceppi, M., Biasotti, B., Fenech, M., Bonassi, S., 2010. Human population studies with the exfoliated buccal micronucleus assay: statistical and epidemiological issues. Mutat. Res. 705 (1), 11–19.

Cherng, S.H., Lin, S.T., Lee, H., 1996. Modulatory effects of polycyclic aromatic hydrocarbons on the mutagenicity of 1-nitropyrene: a structure-activity relationship study. Mutat. Res. 367 (4), 177–185.

Clarke, R.W., Coull, B., Reinisch, U., Catalano, P., Killingsworth, C.R., Koutrakis, P., Kavouras, I., Murthy, G.G., Lawrence, J., Lovett, E., Wolfson, J.M., Verrier, R.L., Godleski, J.J., 2000. Inhaled concentrated ambient particles are associated with hematologic and bronchoalveolar lavage changes in canines. Environ. Health Perspect. 108 (12), 179–187.

Collins, A.R., Oscoz, A.A., Brunborg, G., Gaivão, I., Giovannelli, L., Kruszewski, M., Smith, C.C., Stetina, R., 2008. The comet assay: topical issues. Mutagenesis 23, 143–151.

Cooke, M.S., Evans, M.D., Dizdaroglu, M., Lunec, J., 2003. Oxidative DNA damage: mechanisms, mutation, and disease. FASEB J. 17, 1195–1214.

Da Silva, J., De Freitas, T.R.O., Heuser, V., Marinho, J., Erdtmann, B., 2000. Genotoxicity biomonitoring in coal regions using wild rodent *Ctenomys torquatus* by Comet assay and micronucleus test. Environ. Mol. Mutagen. 35, 270–278.

Diler, B.S., Celik, A., 2011. Cytogenetic biomonitoring of carpet fabric workers using micronucleus frequency, nuclear changes, and the calculation of risk assessment by repair index in exfoliated mucosa cells. DNA Cell Biol., 821–827.

Donbak, L., Rencuzogullari, E., Yavuz, A., Topaktas, M., 2005. The genotoxic risk of underground coal miners from Turkey. Mutat. Res. 588, 82–87.

Duan, H., Leng, S., Pan, Z., Dai, Y., Niu, Y., Huang, C., Bin, P., Wang, Y., Liu, Q., Chen, W., Zheng, Y., 2009. Biomarkers measured by cytokinesis-block micronucleus cytome assay for evaluating genetic damages induced by polycyclic aromatic hydrocarbons. Mutat. Res. 677 (1–2), 93–99.

Ekstrand-Hammarstrom, B., Magnusson, R., Osterlund, C., Andersson, B.M., Bucht, A., Wingfors, H., 2013. Oxidative stress and cytokine expression in respiratory epithelial cells exposed to well-characterized aerosols from Kabul, Afghanistan. Toxicol. In Vitro 27 (2), 825–833.

ETSU and Department of Trade and Industry, 2000. The Fate of Trace Elements in PF Combustion Systems. Report No. COAL R193 – DTI/Pub URN 00/947, ⟨http://webarchive.ationalarchives.gov.uk/tna/+/⟩⟨http://www.dti.gov.uk/files/file18569.pdf/⟩.

Exley, C., Charles, L.M., Barr, L., Martin, C., Polwart, A., Darbre, P.D., 2007. Aluminium in human breast tissue. J. Inorg. Biochem. 101 (9), 1344–1346.

Fenech, M., Chang, W.P., Kirsch-Volders, M., Holland, N., Bonassi, S., Zeiger, E., 2003. Human MicronNucleus project, HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutat. Res. 534, 65–75.

Fenech, M., 2006. Cytokinesis-block micronucleus assay evolves into a cytome assay of chromosomal instability, mitotic dysfunction and cell death. Mutat. Res. 600, 58–66.

Giri, S.K., Yadav, A., Kumar, A., Dev, K., Gulati, S., Gupta, R., Aggarwa, N., Gautam, S.K., 2012. CYP1A1 gene polymorphisms: modulator of genetic damage in coal-tar workers. Asian Pac. J. Cancer Prev. 13 (7), 3409–3416.

Guo, H., Huang, K., Zhang, X., Zhang, W., Guan, L., Kuang, D., Deng, Q., Deng, H., Zhang, X., He, M., Christiani, D., Wu, T., 2014. Women are more susceptible than men to oxidative stress and chromosome damage caused by polycyclic aromatic hydrocarbons exposure. Environ. Mol. Mutagen, 10.1002/em.21866, in press.

Gurbani, D., Bharti, S.K., Kumar, A., Pandey, A.K., Ana, G.R., Verma, A., Khan, A.H., Patel, D.K., Mudiam, M.K., Jain, S.K., Roy, R., Dhawan, A., 2013. Polycyclic aromatic hydrocarbons and their quinones modulate the metabolic profile and induce DNA damage in human alveolar and bronchiolar cells. Int. J. Hyg. Environ. Health 216 (5), 553–565.

He, F., Buoso, M.C., Burattini, E., Fazinic, S., Galassini, S., Haque, A.M.I., Jaksic, M., Moschini, G., 1993. Target preparation for trace element determination of biological materials using techniques. Nucl. Instrum. Meth. Phys. Res. Sect. A 334, 238–245.

Hewitt, R., Forero, A., Luncsford, P.J., Martin, F.L., 2007. Enhanced micronucleus formation and modulation of BCL-2:BAX in MCF-7 cells after exposure to binary mixtures. Environ. Health Perspect. 115 (Suppl. 1), S129–S136.

Holland, N., Bolognesi, C., Kirsch-Volders, M., Bonassi, S., Zeiger, E., Knasmueller, S., Fenech, M., 2008. The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: the HUMN project perspective on current status and knowledge gaps. Mutat. Res. 659 (1–2), 93–108.

IARC, International Agency for Research on Cancer, 1997. Agents Classified by the IARC Monographs, vols. 1–105, Cas N 000050-41-9, p. 68.

Irons, R.M.A., Quick, W., 2000. The Fate of Trace Elements in Pulverised Fuel (PF) Combustion Systems. PowerGen UK plc, CRE Group Ltd, University of Nottingham p. R193.

Johansson, S.A.E., Campbell, J.L., Malmqvist, K.G., 1995. Paticle-induced X-ray Emission Spectrometry (PIXE). John Wiley & Sons, INC..

Kalantzi, O.I., Hewitt, R., Ford, K.J., Alcock, R.E., Thomas, G.O., Morris, J.A., 2004. Inter-individual differences in the ability of human milk-fat extracts to enhance the genotoxic potential of the procarcinogen benzo[a]pyrene in MCF-7 breast cells. Environ. Sci. Technol. 38, 3614–3622.

Karahalil, B., Karakaya, A.E., Burgaz, S., 1999. The micronucleus assay in exfoliated buccal cells: application to occupational exposure to polycyclic aromatic hydrocarbons. Mutat. Res. 442 (1), 29–35.

Klaunig, J.E., Wang, Z., Pu, X., Zhou, S., 2011. Oxidative stress and oxidative damage in chemical carcinogenesis. Toxicol. Appl. Pharmacol., 86–99.

Kirsch-Volders, M., Bonassi, S., Knasmueller, S., Holland, N., Bolognesi, C., Fenech, M.F., 2014. Commentary: critical questions, misconceptions and a road map for improving the use of the lymphocyte cytokinesis-block micronucleus assay for in vivo biomonitoring of human exposure to genotoxic chemicals–A HUMN project perspective. Mutat. Res. 759, 49–58.

León, G., Perez, L.E., Linares, J.C., Hartmann, A., Quintana, M., 2007. Genotoxic effects in wild rodents (*Rattus rattus* and *Mus musculus*) in an open coal mining area. Mutat. Res. 630, 42–49.

León-Mejía, G., Espitia-Pérez, L., Hoyos-Giraldo, L.S., Da Silva, J., Hartmann, A., Henriques, J.A., Quintana, M., 2011. Assessment of DNA damage in coal open-cast mining workers using the cytokinesis-blocked micronucleus test and the comet assay. Sci. Total Environ. 409 (4), 686–691.

Majer, B.J., Laky, B., Knasmüller, S., Kassie, F., 2001. Use of the micronucleus assay with exfoliated epithelial cells as a biomarker for monitoring individuals at elevated risk of genetic damage and in chemoprevention trials. Mutat. Res. 489, 147–172.

Maxwell, J.A., Teesdale, W.J., Campbel, J.L., 1995. The Guelph PIXE software package II. Nucl. Instrum. Methods Phys. Res. Sect. 95, 407–421.

Mireles, A., Solís, C., Andrade, E., Lagunas-Solar, M., Pina, C., Flocchini, R.G., 2004. Heavy metal accumulation in plants and soil irrigated with wastewater from Mexico city. Nucl. Instrum. Methods Phys. Res. Sect. 220, 187–190.

Moriske, H.J., Drews, M., Ebert, G., Menk, G., Scheller, C., Schondube, M., Konieczny, L., 1996. Indoor air pollution by different heating systems: coal burning, open fireplace and central heating. Toxicol. Lett. 88, 349–354.

Nakajima, T., Hasegawa, H., Nakamata, S., Takanashi, H., Ohki, A., 2008. Mutagenicity of eluent by hot water extraction of various coals: effect of chlorination. Fuel 87, 3132–3136.

Neumann, V., Schulz, F., Theile, A., Loseke, S., Püschel, K., Tannapfel, A., 2011. Case report of a rare occupational disease: a during life non-recognised occupational disease – talcosis. Pneumologie 65 (8), 471–476.

Prahalad, A.K., Inmon, J., Ghio, A.J., Gallagher, J.E., 2000. Enhancement of 2′-deoxyguanosine hydroxylation and DNA damage by coal and oil fly ash in relation to particulate metal content and availability. Chem. Res. Toxicol. 13 (10), 1011–1019.

Rohr, P., da Silva, J., da Silva, F.R., Sarmento, M., Porto, C., Debastiani, R., dos Santos, C.E.I., Dias, J.F., Kvitko, K., 2013a. Evaluation of genetic damage in open-cast coal

mine workers using the buccal micronucleus cytome assay. Environ. Mol. Mutagen. 54 (1), 65–71.

Rohr, P., Kvitko, K., da Silva, F.R., Menezes, A.P., Porto, C., Sarmento, M., Decker, N., Reyes, J.M., Allgayer, Mda C., Furtado, T.C., Salvador, M., Branco, C., da Silva, J., 2013b. Genetic and oxidative damage of peripheral blood lymphocytes in workers with occupational exposure to coal. Mutat. Res. 758 (1–2), 23–28.

Santa Maria, S.R., Arana, M., Ramirez, O., 2007. Chromosomal aberrations in peripheral lymphocytes from male native miners working in the Peruvian Andes. Genet. Mol. Biol. 30, 1135–1138.

Schins, R., Borm, P., 1999. Mechanisms and mediators in coal dust induced toxicity: a review. Ann. Occup. Hyg. 32 (1), 7–33.

Singh, R., Sram, R.J., Binkova, B., Kalina, I., Popov, T.A., Georgieva, T., Garte, S., Taioli, E., Farmer, P.B., 2007. The relationship between biomarkers of oxidative DNA damage, polycyclic aromatic hydrocarbon DNA adducts, antioxidant status and genetic susceptibility following exposure to environmental air pollution in humans. Mutat. Res./Fundam. Mol. Mech. Mutagen. 620, 83–92.

Spinelli, J.J., Demers, P.A., Le, N.D., Friesen, M.D., Lorenzi, M.F., Fang, R., Gallagher, R.P., 2006. Cancer risk in aluminum reduction plant workers (Canada). Cancer Causes Control 17 (7), 939–948.

Stich, H.F., Rosin, M.P., 1984. Micronuclei in exfoliated human cells as a tool for studies in cancer risk and cancer intervention. Cancer Lett. 22, 241–253.

Thomas, P., Holland, N., Bolognesi, C., Kirsch-Volders, M., Bonassi, S., Zeiger, E., Knasmueller, S., Fenech, M., 2009. Buccal micronucleus cytome assay. Nat. Protoc. 4 (6), 825–837.

Tolbert, P.E., Shy, C.M., Allen, J.W., 1992. Micronuclei and other nuclear anomalies in buccal smears: methods development. Mutat. Res. 271, 69–77.

Wagner, A.J., Bleckmann, C.A., Murdock, R.C., Schrand, A.M., Schlager, J.J., Hussain, S.M., 2007. Cellular interaction of different forms of aluminum nanoparticles in rat alveolar macrophages. J. Phys. Chem. B. 111 (25), 7353–7359.

Zhou, H.C., Zhong, Z.P., Jin, B.S., Huang, Y.J., Xiao, R., 2005. Experimental study on the removal of PAHs using in-duct activated carbon injection. Chemosphere 59 (6), 861–869.

Zocche, J.J., Leffa, D.D., Damiani, A.P., Carvalho, F., Mendonca, R.A., Iochims, dos Santos, C.E., Boufleur, L.A., Dias, J.F., de Andrade, V.M., 2010. Heavy metals and DNA damage in blood cells of insectivore bats in coal mining areas of Catarinense coal basin, Brazil. Environ. Res. 110, 684–691.

ANEXO III

CONSENTIMENTO LIVRE ESCLARECIDO
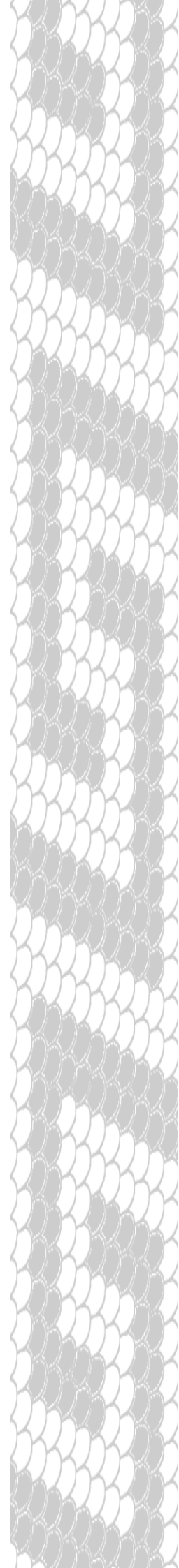
ANEXO III

CONSENTIMIENTO INFORMADO
EVALUACIÓN Y CARACTERIZACIÓN DE MEZCLAS COMPLEJAS GENERADAS EN UNA MINA DE CARBÓN A CIELO ABIERTO Y DE SUS EFECTOS BIOLÓGICOS EN LINFOCITOS HUMANOS POLIMORFICOS

Yo _____, mayor de edad, he sido informado que el GRUPO DE INVESTIGACIÓN BIOMÉDICA Y BIOLOGÍA MOLECULAR de la Universidad del Sinú (GIBM) en cooperación con el GRUPO DE INVESTIGACIÓN EN TOXICOLOGÍA GENÉTICA Y CITOGENÉTICA de la Universidad del Cauca (UNICAUCA) y el LABORATORIO DE BIOFÍSICA de la Universidade Federal do Rio Grande do Sul en Brasil (UFRGS) realizan el estudio "EVALUACIÓN Y CARACTERIZACIÓN DE MEZCLAS COMPLEJAS GENERADAS EN UNA MINA DE CARBÓN A CIELO ABIERTO Y DE SUS EFECTOS BIOLÓGICOS EN LINFOCITOS HUMANOS POLIMORFICOS" en poblaciones localizadas alrededor de las zonas de producción de carbón del Departamento de La Guajira. El estudio comprende la ejecución de dos componentes: 1) Componente medio ambiental y 2) Componente en salud de las comunidades.

Se me ha solicitado participar voluntariamente como sujeto de estudio del Componente 2): salud de las comunidades.

OBJETIVO Y PROPÓSITO DEL ESTUDIO
Evaluar los efectos citotóxicos, genómicos, mutagénicos, y genotóxicos inducidos por la exposición in vitro de cultivos de linfocitos humanos polimórficos a las mezclas complejas generadas en el interior de una mina de carbón a cielo abierto localizada en el Departamento de la Guajira, con la finalidad de la aplicación de nuevas tecnologías y fortalecimiento científico en salud, ya que la investigación ofrecerá nuevos conocimientos para incorporar a los programas de diagnóstico y vigilancia epidemiológica, que motiven la prevención de problemas en salud y una mejor calidad de vida de las poblaciones que viven en cercanías a proyectos de minería a cielo abierto, teniendo en cuenta las diferencias individuales (susceptibilidad) y los factores de riesgo genético relacionado con los efectos en la salud.

YO HE SIDO INFORMADO SOBRE LOS OBJETIVOS, PROPÓSITO, JUSTIFICACIÓN, METODOLOGÍA, RIESGOS, Y BENEFICIOS DEL ESTUDIO Y DE SU COMPONENTE EN SALUD DE LAS COMUNIDADES. En este estudio serán seleccionados 400 individuos, 200 pobladores expuestos a residuos de minería carbón y 200 personas no expuestas como grupo control, con el fin de diferenciar al momento del análisis de los resultados, que individuos son más susceptibles a la exposición a residuos de minería carbón. El propósito de la investigación tiene relevancia social y científica y obedece a una problemática de salud pública.

Sobre la competencia, formación integral y calidad de los investigadores será responsable cada institución vinculada al proyecto.

Los resultados del estudio son confidenciales y serán informados y explicados de manera personal y confidencial al grupo objeto de estudio en forma anónima por parte de la profesora LYDA ESPITIA-PÉREZ, investigador principal. Los datos no serán utilizados con otra finalidad distinta a la descrita en esta investigación.

REQUERIMIENTOS. Yo, en pleno uso de mis facultades mentales, libre y consciente, estoy de acuerdo en participar en este estudio y entiendo que éste requiere de mi lo siguiente: Contestar un cuestionario de aproximadamente 20 minutos, para suministrar información personal referente a mi edad, estado de salud, estilo de vida, historia ocupacional y familiar. Si soy seleccionado para el estudio debo donar 20 ml. de sangre tomada de la vena del brazo, de los cuales se tomarán 5 ml para ser procesada en el laboratorio Investigación Biomédica y Biología Molecular de la Universidad del Sinu para la prueba in vitro de Micronúcleos y para la prueba del Ensayo Cometa. La sangre restante se utilizará en la UFRGS para la detección de metales en sangre. También me será solicitada una muestra de mucosa del interior de las mejillas derecha e izquierda que serán colectados mediante un frotis en el área con un cepillo citológico. Estas muestras serán usadas para la prueba de Micronucleos en mucosa oral.

RIESGOS DE PARTICIPACIÓN. Los riesgos potenciales de participación en el estudio son sangrados en el sitio de la toma de la muestra de sangre, los cuales serán controlados por un profesional experto en la toma de muestras de sangre del brazo y el empleo de técnicas médicamente aceptadas y uso de jeringas y/o tubos y agujas estériles nuevas.

Para garantizar la confiabilidad de la información suministrada, los resultados de las pruebas serán codificados y se darán a conocer en forma grupal más no individual en un seminario, con el propósito de hacer una autorreflexión, luego de haber recibido una serie de conferencias.

Tengo claro que no se me proveerá con ninguna compensación económica.

BENEFICIOS PARA EL PARTICIPANTE: Atender a las capacitaciones sobre los diferentes efectos en la salud de la exposición a corto y largo plazo de la exposición a residuos de minería a cielo abierto.

Reflexión y motivación hacia el cambio de actitud para la prevención de riesgos a la salud por exposición a residuos de minería. Conocer los resultados grupales del estudio

YO ENTIENDO QUE

Mi participación es completamente voluntaria y que puedo rehusarme a responder cualquier pregunta si así lo deseo o puedo tomar libremente la decisión de finalizar mi participación en este monitoreo en cualquier momento, sin que ello represente perjuicios de índole legal con mi trabajo.

Esta investigación fue evaluada y aprobada por el comité ético de la Universidad del Sinú. La información recolectada será tratada de manera confidencial y mis respuestas serán reunidas con las de otros participantes para obtener resultados grupales.

La Universidad del Sinú se compromete a vigilar que las muestras de sangre sean tomadas por un profesional experto y autorizado y en forma aséptica para evitar complicaciones.

Puedo preguntar cualquier interrogante o duda que tenga antes, durante o después del estudio, al investigador principal LYDA ESPITIA – PÉREZ de la Universidad del Sinú responsable del estudio, en el Laboratorio de INVESTIGACIÓN BIOMÉDICA Y BIOLOGÍA MOLECULAR ubicado en la Calle 38 Cra 1W – Barrio Juan XXIII de la ciudad de Montería, en los teléfonos 7840340 Ext. 402.

La firma del documento del consentimiento informado es requerida para todas las personas participantes en un estudio como éste.

Los procedimientos alternativos principales incluyendo procedimientos experimentales en este estudio, me han sido explicados en un lenguaje claro que yo he podido entender.

Los riesgos y molestias que pueden presentarse me han sido explicados claramente.

También entiendo que como mi nombre no será vinculado con los resultados del estudio, el investigador principal y sus coinvestigadores no estarán en la posibilidad de informar a ninguna otra persona sobre los resultados míos de la pruebas.

Los resultados de este estudio podrán ser divulgados y/o publicados en revistas científicas en forma grupal sin que se de a conocer mi nombre.

He leído este consentimiento, he entendido en que consiste este estudio y también me fueron aclaradas las dudas al respecto, en consecuencia voluntariamente acepto participar como sujeto de estudio en el monitoreo biológico "**EVALUACIÓN Y CARACTERIZACIÓN DE MEZCLAS COMPLEJAS GENERADAS EN UNA MINA DE CARBÓN A CIELO ABIERTO Y DE US EFECTOS BIOLÓGICOS EN LINFOCITOS HUMANOS POLIMORFICOS**".

_____   _____
Nombre del Participante   Firma del Participante

_____   _____
Nombre del Testigo   Firma del Testigo
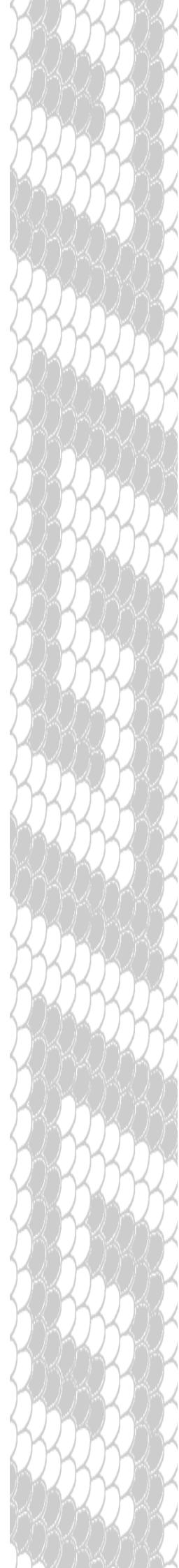
_____
LYDA ESPITIA - PÉREZ.
Coordinador del proyecto

ANEXO IV

QUESTIONÁRIO POPULACIONAL

EVALUACIÓN Y CARACTERIZACIÓN DE MEZCLAS COMPLEJAS GENERADAS EN UNA MINA DE CARBÓN A CIELO ABIERTO Y DE SUS EFECTOS BIOLÓGICOS EN LINFOCITOS HUMANOS POLIMÓRFICOS

INSTITUCIONES PROPONENTES: Universidad del Sinú (Unisinu – Elías Bechara Zainúm), Grupo de investigación Biomédica y Biología Molecular (GIBM), Centro de Biotecnologia CBiot , Universidade Federal do Rio Grande do Sul(UFRGS), GENOTOX ROYAL.

ENCUESTA PARA LA COLECTA DE INFORMACION SOCIODEMOGRAFICA
Lea y responda cuidadosamente las siguientes preguntas. La información que entregue no será asociada con su nombre, y sólo será conocida por los investigadores responsables del estudio. Las respuestas de este cuestionario podrán tener influencia directa en la interpretación de nuestros resultados.

1. CÓDIGO: _____
2. Nombre:_____
3. Coordenadas:_____
4. Población: _____
5. Tiempo de residencia: Años (   ), Meses (   ), Semanas (   ), Días (   ).
6. Teléfono de contacto: _____
7. Edad: _____ en años
8. Fecha de nacimiento: ____/____/_____
9. Sexo: (   ) Masculino (   ) Femenino   *FUM*: ___/___/___*
10. Grupo étnico: (   ) blanco; (   ) mestizo; (   ) negro; (   ) amarillo; (   ) indígena
11. Matrimonio consanguíneo: (   ) Si   (   ) No / Si es así, ¿qué grado de relación tiene con su conyugue?_____
12. ¿Cuál es su trabajo actual? _____
13. ¿Cuánto tiempo lleva ejerciendo esta función?_____
14. Cuantas horas al día trabaja? _____

En su trabajo actual a estado expuesto a alguna de estas sustancias? (Diga el tiempo de exposición en horas / semana):
(   ) Derivados de petróleo _____
(   ) Tintas/colorantes _____
(   ) Solventes _____
(   ) Pesticidas/herbicidas _____
(   ) Mercurio/vapores de otros metales pesados - cual? _____
(   ) Otras sustancias químicas – cual? _____

15. Utiliza/va en su trabajo actual o pasado equipo de seguridad personal?
( ) Si   (   ) No (   ) / (   ) Siempre   (   ) Casi siempre   (   ) Pocas veces
Cual? _____
16. Alguna vez fumó? ( ) Si ( ) No - Si no, pase a la pregunta 24
17. ¿Por cuánto tiempo lo ha hecho? _____ (en años)
18. Cuantos cigarros fumaba por día?
(   ) Menos de la mitad de un paquete (   )medio paquete
(   ) 1 a 2 paquetes   (   ) más de 2 paquetes
Todavía fuma? ( ) Si ( ) No - Si no, hace cuánto tiempo dejó de fumar? _____ (en años)
19. En caso afirmativo, ¿cuántos cigarrillos fuma al día?
(   ) Menos de la mitad de un paquete (   ) medio paquete
     (   ) 1 a 2 paquetes   (   ) más de 2 paquetes

* FUM: Fecha Última Menstruación

181

20. Fuma también: ( ) tabaco; ( ) Pipa - ¿Cuántas veces al día? _____

21. Vive con personas que fuman? Si ( ), No ( ). Hace cuanto tiempo_____

22. Medicamentos utilizados habitualmente (especificar horas de frecuencia / día):

( ) Hormonas _____

( ) Vitaminas y / o suplementos _____

( ) Pastillas para la presión _____

( ) Antibióticos _____

( ) Insulina _____

( ) Tranquilizantes _____

( ) Relajantes musculares _____

( ) Otros – cual? _____

23. La ingesta de estos medicamentos obedece a algún tratamiento? ( ) Si ( ) No

24. Cual? _____

25. Consume ó ha consumido algunas de las siguientes sustancias psicoactivas

LSD ( )

Cocaína ( )

Marihuana ( )

Alucinógenos ( )

Hoja de coca ( )

Otra cual? _____

26. Se hizo alguna radiografía en el último año? ( ) Sí   ( ) No

27. Con que frecuencia usted consume bebidas alcohólicas? _____

Cuales?_____

28. Consume frutas y verduras? / En caso afirmativo, indique la frecuencia (días / semanas)

( ) Si   ( ) No

 ( ) 1 a 2   ( ) 3 a 4   ( ) 5 a 6   ( ) todos os días

29. ¿Ha tenido / tiene cualquiera de estas enfermedades?

( ) Cáncer   ( ) VIH/SIDA   ( ) Diabetes   ( ) Anemia

( ) Enfermedades cardiacas   ( ) Otras – cuales? _____

30. ¿ Tiene conocimiento de cualquier defecto congénito o una enfermedad hereditaria que afecta a sus padres, hermanos, hermanas o esposa? ( ) No ( ) Si

Cual? _____

31. De donde proviene El agua que usted consume _____

32. El agua es hervida? ( ) Si   ( ) No

Qué   tipo   de   tratamiento   le   hace   al   agua   antes   de   beberla   o   usarla?

_____

_____

_____

33. Cocina con leña? ( ) No ( )Si

Cuantas veces al día? _____

34. Usted y su cónyuge han tenido dificultades para concebir ( ) o ha sido diagnosticado como infértiles? ( ) No   ( ) Si

35. Ha sufrido abortos involuntarios?

 ( ) No   ( ) Si – Cuantas veces? _____

36. Historia bebé con defectos?

 ( ) No   ( ) Si – cual? _____

37. Tiene hermano gemelo? ( )No   ( ) Si

38. Tiene parientes cercanos en otros cabildos o rancherias ubicados lejos de la mina de carbón? ( ) No   ( ) Si

Dónde?_____

Nombre:_____

Telefono:_____

39. Casos de cáncer en la familia: (   ) No   (   ) Si   –   Grado de parentesco_____

¿Qué tipos de cáncer?

( ) Piel   ( ) mama   ( ) leucemia   ( ) esófago

( ) Otros: _____

Doy fe que la información proporcionada arriba es verídica

Fecha _____

Firma del Voluntario: _____

Huella (índice derecho)

Firma del investigador responsable: _____