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# **Biomarkers of evaluation of complex environmental exposure : exposure assessment of sewage workers to genotoxics**

Hamzeh Alzabadi

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par **Hamzeh ALZABADI**

**Le 25 Mars 2010**

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## I Know Why the Caged Bird Sings

A free bird leaps on the back of the wind  
and floats downstream till the current ends  
and dips his wing in the orange sun's rays and dares to claim the sky.

But a bird that stalks down his narrow cage  
can seldom see through his bars of rage  
his wings are clipped and his feet are tied so he opens his throat to sing.

The caged bird sings with a fearful trill  
of things unknown but longed for still  
and his tune is heard on the distant hill  
for the caged bird sings of freedom.

The free bird thinks of another breeze  
and the trade winds soft through the sighing trees  
and the fat worms waiting on a dawn bright lawn and he names the sky his own.

But a caged bird stands on the grave of dreams  
his shadow shouts on a nightmare scream  
his wings are clipped and his feet are tied so he opens his throat to sing.

Maya Angelou (1928-Present)



## **Dedication**

*To my dear mother and father,*

*To my sisters and brothers,*

*To my wife “Reham”,*

*To my son “Tejan”,*

*You are always in my heart,*

**Hamzeh ALZABADI**



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## Abbreviations

AChE:	Acetylcholinesterase
[B(a)P]eq:	Benzo[a]pyrene equivalent concentration
B[a]P:	Benzo[a]pyrene
BNed:	Binucleated cells
CBMN:	Cytokinesis Block Micronucleus assay
95%CI:	95% Confidence Interval
EIA:	Enzymatic immunoassay
h:	Hour
HPLC:	High Performance Liquid Chromatography
INSERM:	Institut National de la Santé Et de la Recherche Médicale
min:	Minute
MN:	Micronucleus assay
MNi:	Micronuclei
NDI:	Nuclear Division Index
8-oxodG:	8-oxo-2'-deoxyguanosine
PAHs:	Polycyclic Aromatic Hydrocarbons
PCBs:	Poly Chlorinated Biphenyls
ppm:	Part Per Million
pmole:	Picomole
RR:	Relative Risk
SD:	Standard Deviation
SEM:	Standard Error of the Mean
SMR:	Standard Mortality Ratio
SUVIMAX2:	SUplémentation en VItamines et Minéraux Anti-oXydants
TEFs:	Toxicity equivalent factors
USA:	United States of America
UV:	Ultraviolet
v:v:	Volume by Volume
VOCs:	Volatile Organic Compounds
w:v:	Weight by Volume



# 1. Introduction

## 1.1. Background

The sewage system is very important for the disposal of wastewaters and other materials derived from life of society. It is used to transport the wastewater effluents of the cities, and associated liquids, to the surrounding areas where, after appropriate treatment, they to be used for fertilization or just to be dumped. Sewage workers who clean and repair this system are exposed to human excreta and domestic wastewater. However, with increasing emphasis on industrialization and urbanization, this system has been increased in complexity. This resulted in new exposures including multiple and complex chemicals used in homes, commercial facilities and in small industries [1,2], yielding an increased risk of chemical exposure to sewage workers. The main pollutant input sources to sewers are domestic and industrial wastes, rainfall and runoff waters and also, street sweeping waters that collect atmospheric dry deposits, dusts from asphalt erosion, residues from petroleum products and various trash, as well as discharge from hospitals and research institutions that use emit a large array of drugs, such as anti-neoplastic drugs or experimental genotoxics [3]. Although in recent years this exposure has decreased due to regulations on waste recycling, hundreds of chemicals have been shown to exist in sewage and wastewater systems [4-6].

Studies showed that sewage systems contain generally; (1) chemicals and microorganisms from normal/abnormal industrial discharges and any products arising from bio or chemical conversion of these materials; (2) chemicals from domestic premises, including those in human excrement; (3) microorganisms in human excreta; and (4) materials in the runoff from pipes, road surfaces, gardens and others. This results in the release of a wide variety of chemicals including methane, hydrogen sulphide, carbon dioxide, heavy metals, chlorinated organic solvents like chloroform, dichloroethane, perchloroethanol, other solvents (benzene, toluene), aldehydes, nitrosamines, pesticides, dyes, polychlorobiphenyls, and polycyclic aromatic hydrocarbons (PAHs) [6-8]. Thus, in the sewage system, occupational exposure to hazardous agents occurs definitively. It is very difficult to characterize this exposure qualitatively and quantitatively, due to the multitude of chemicals and the large range of their concentrations [9]. However, many of the compounds to which sewage workers are exposed are known or suspected to be genotoxics and/or carcinogens [10-12] and as a result of their contact with the wastes, sewage workers exposed to this complex mixture might be subject to elevated risk of cancer.

One of the great difficulties in evaluating this risk has been the inability to identify the many possible substances to which they are potentially exposed. Furthermore, this composition of chemicals varies along time and/or by location. Therefore, it is inappropriate to associate the adverse outcomes with a single chemical. Studies showed that many contaminants that are released into surface water may have no detectable, acute effect on exposed organisms, but may reduce their survival via long term, chronic effects [13]. Such effects, which can occur at doses that are often lower than those required for acute effects, may take the form of excessive tissue damage, genetic damage of somatic and germ cells, accumulation of persistent contaminants, and disorders that require long latency periods before becoming clinically visible, e.g., cancer [13].

Sewage workers are usually not aware of this potential complex exposure in their workplace, and thus they might be at higher risk than other industrial workers with well defined exposures [14-16]. Thus in view of this multiple exposure, sewage workers represent a unique population for the study of related health effects. There has been limited and conflicting research on the health effects of exposure to mixtures of chemicals, even though most workplace environments involve sequential or simultaneous exposure to multiple chemicals.

It is well known that in such complex exposure situations, which are very often encountered in workplaces, different agents may interact, either enhancing or inhibiting the effects of the individual compounds. And since individuals differ in their genetic constitution and capacity to adapt to this stress, they exhibit a great variability in their response. Thus, it may be reasonable to look for early biological effects directly in the exposed individuals or groups rather than try to predict hazards of complex exposure patterns from data pertaining to single compounds. In this setting, using biomarkers to assess changes associated with this exposure situation is described as a relevant approach [17]. Human biomonitoring uses samples of body fluids or other easily obtainable biological material for the measurement of exposure to specific or non-specific substances and/or their metabolites or for the measurement of the biological effects of this exposure [17,18]. Biological monitoring allows estimating total individual exposure through different exposure pathways (lungs, skin, and gastrointestinal tract) and different sources of exposure (air, diet, lifestyle or occupation) [17].



## **1.2. Health hazards in the workplace of sewage workers**

### **1.2.1. Biological and physical hazards**

In addition to chemicals, sewage workers are also exposed to biological and physical agents. Various pathogenic bacteria and viruses as well as protozoa and helminthes have been reported in sewage system [1]. Accordingly, infections and associated health effects have been the focus of the majority of research in sewage exposed workers in the past [1,2]. A summary of a brief review of literature among sewage workers showed that enteric viruses, bacteria, parasites and pathogenic fungi have been found in sewage and aerosols from wastewater treatment plants, suggesting that sewage workers are at increased risk of infection [19-22]. In France, hepatitis A virus seroprevalence in Parisian sewage workers was found increased with age, reaching 80% for workers older than 40 years [23]. Giardiasis and entamoeba histiolytica have been also reported among sewage workers in France and other countries [24,25].

Sewage workers are also exposed to a variety of physical hazards putting them at risk for significant injuries. Physical contact with the effluent is possible through cleaning up work, contact with spray during decontamination processes, and while touching contaminated surfaces, clothes, shoes, etc [7]. A study found that in 255 sewage workers, the 12 month prevalence symptoms rates were 52.4% neck symptoms, 54.8% upper back and 72.8% low back symptoms [26].

### **1.2.2. Chemical hazard**

Sewer workers, especially those working under-ground for maintenance and repair tasks, are working in confined spaces and are exposed to many chemicals including volatile organic solvents (halogenated solvents) disposed and discarded into the sewer from industrial and commercial facilities [27,28].

During the sewage treatment process, a number of gases are produced [29]. They are classified according to their toxic action and include irritants (e.g. ammonia), simple asphyxiant (methane and nitrogen), or chemical asphyxiant (carbon monoxide and hydrogen sulphide) [29]. Carbon monoxide, which could cause carboxyhemoglobin and progressing to collapse, coma and death, may be introduced into the confined air sewage system from combustion of fossil fuels (e.g., fires, diesel engines) [29]. Methane, which is a flammable by-product gas of natural breakdown and anaerobic digestion of sewage is lighter than air and collects at the top of enclosed spaces. Thus it is a recognizable hazard for sewer workers [29].

Carbon dioxide is heavier than air and can collect at the bottom of enclosed spaces, possibly causing narcosis after a few minutes of breathing [29]. Ammonia a strong respiratory tract irritant [29], is produced by the natural breakdown of sewage and collects in the upper areas of enclosed spaces. In high concentration, it can cause respiratory distress by affecting the lower respiratory system at levels of 100 ppm [29]. Hydrogen sulphide, a dense gas concentrating at the bottom of confined spaces, is a potent mucous membrane and respiratory tract irritant [29]. It may cause eye irritation, and at high concentration (100 ppm) it inhibits the respiratory center, causing death. The characteristic “rotten egg” odor of the gas is detectable at low levels, but at high concentrations, olfactory paralysis prevents the exposed person from detecting the presence of the gas which could lead to a sudden loss of consciousness (“knockdown”) [29].

Workers might come in contact with industrial waste from accidental or illegal release. Studies have shown contamination of sewage with chlorinated hydrocarbons (chloroform, dichloroethane, and perchloroethanol), polychlorinated biphenyls (PCBs) [15,28], heavy metals, other solvents (benzene, toluene), formaldehydes, pesticides, dyes, PAHs and others [28]. Fluorinated hydrocarbons may be also present in sewage system. Freon-11 (trichloromono-fluoromethane) has also been detected in sewage system and found to be responsible for the death of two sewer workers at Crymlyn Burrows, South Wales [30]. Chlorine, used as a disinfectant, could be found in sewage system; it is extremely irritating to the mucous membrane, eyes, and the skin. At high levels it could cause adult pulmonary distress syndrome [29]. Exposures may also occur with alkaline cleaners, paints, lubricants, hydraulic fluids, acrylamide and 2-nitropropane, acids. Nitrosamines and a broad spectrum of toxic elements were also detected in sewage system [31]. Asbestos and dioxins have been identified in the sewage sludges [32]. In a study in the USA, 15 sludges samples were analyzed and detected the carcinogen N-nitrosodimethylamine in 14, and various other N-nitrosamines in 12 [33]. Further, hexachlorocyclopentadiene and hexachlorobicycloheptadiene in urine samples of sewage treatment plant receiving wastes from a pesticide manufacturer have been found [15].

### **1.3. Characterization of exposure among sewage workers**

Workers involved with chemically contaminated wastewater are exposed through inhalation, dermal contact and/or ingestion. Because of the time and location variability of the sewage make-up [34,35], it is difficult to characterize this mixture or to quantify the exposure dose. Exposures are variable, often intermittent, occasionally acute, but generally of a low and

chronic nature. Adverse health effects, which can occur at doses that are often far lower than those required for acute effects, can manifest as tissue damage that might lead to disorders that require long latency periods, e.g., cancer [13]. Indeed, many studies have detected an increase in the incidence of cancer among sewage workers [28,36-38].

On the other hand, elevated exposure may occur for brief periods such as during a periodic dumping in sewer lines or while working in confined spaces, e.g. during the repair of drainage pipes. The employees have a variety of job activities, some which involve work in confined spaces and unventilated areas (e.g., sewers, tunnels, pipes, wet wells, manholes, pits, pumping stations, and sludge digestors) where there is potential for exposures to high concentrations. In these confined spaces, there is little or no ventilation, little room for movement, and few exits or entrances. Such settings can be especially dangerous because gases can rapidly accumulate at the bottom of these areas or in isolated pockets. Although the job description of these sewage workers differed from those of sewage workers employed in sewage treatment plants, data from treatment plants suggest that both groups of workers are, to some extent, exposed to similar agents and develop similar symptoms [4]. Within a sewage treatment plant, workers with the same job title may have different job responsibilities, however, that lead to different and varying levels of exposure.

### **1.3.1. Sewage drains indoor air pollution**

Urban surfaces receive deposits of PAHs and volatile organic compounds (VOCs) from different sources such as car traffic, industries, waste incinerators, and domestic heating, via both atmospheric transport and local activity [39]. Industrial wastewater treatment plants might as also be a source for these hazardous substances with known health effects [41]. No study has measured the PAHs or VOCs in the air of the sewage system workplace. However, many have measured these substances in the wastewater treatment plants, in municipal solid waste, and in the sewage sludge, although rarely in the air of these plants. In Paris, a study assessed concentrations of certain pollutants in wastewater during dry and wet periods across different sampling sites and sewer networks. For each sample, a total of 66 elements, including PAHs, VOCs were analysed. A broad range of pollutants was observed during dry as well as wet weather periods. Of the 66 elements investigated, 33 and 40 substances were observed in raw sewage and wet weather effluent, respectively. The majority of organic pollutants were identified within the  $\mu\text{g.L}^{-1}$  range [42]. In the sewage sludge and wastewater samples, PAHs and VOCs have also been reported in other studies [43,44].

### **1.3.1.1. Polycyclic aromatic hydrocarbons (PAHs)**

We found no study that assessed the PAHs in the air of the under-ground sewage workplaces. However, in Italy, possible and probable carcinogens PAHs, such as benzo(a)anthracene, benzo(a)pyrene, benzo(a)fluoranthene, and benzo(k)fluoranthene, were found in the aerosol collected from the aeration tanks of the sewage treatment plant in Prato, Italy [45].

In Korea, a study reviewed the characteristics of PAH found in sewage sludge. The study was performed on 16 PAHs. It showed that concentrations of the PAHs on the inlet and on the outlet of the air measurement devices to be ranged from 3.926-925.748  $\mu\text{g}/\text{m}^3$  and from 1.153-189.449  $\mu\text{g}/\text{m}^3$ , respectively [46]. In Greece, higher concentrations of benzo[a]pyrene (B[a]P), and total PAHs were found in the urban sewage sludge than in the industrial sludge [47]. Pham et al. reported an increase of PAHs concentration in Montreal wastewaters during the winter period [48]. Blanchard et al. [6] found the concentration of PAHs in raw wastewater entering the Seine Aval treatment plant in Paris to be (as  $\text{ng L}^{-1}$ ) 0.2 to 400 for fluoranthene (mean, 77.4), 2 to 104 (mean, 21.4) for benzo(a)pyrene, and 0.3 to 63 (mean, 12.2) for benzo(b)fluoranthene. These results are consistent with that of Pham and Proulx (1997) in the Montreal wastewater treatment plant (83 to 216, 20 to 77, and 42 to 168  $\text{ngL}^{-1}$  for fluoranthene, benzo(a)pyrene, and benzo(b)fluoranthene; respectively) [49]. Similar toxicants were found by other authors who analyzed the wastewater of municipal sewage treatment plants [28,29,50]. Over a period of two years (2000-2001), sediment samples were extracted from 40 silt traps spread through the combined sewer system of Paris. All samples were analysed for 16 PAHs. The results show that there are some important (between- and within-site) variations in hydrocarbon contents. PAHs contamination levels (50th percentile) in the Parisian sewer sediment were estimated at 18  $\mu\text{g g}^{-1}$  [51]. In USA, study in the wastewater, some PAHs, like fluoranthrene, pyrene, chrysene, phenanthrene, benzo[a]anthracene, and naphthalene have been revealed [52].

### **1.3.1.2. Volatile organic compounds (VOCs)**

We found no study that evaluated VOCs concentrations in the confined air of the under-ground sewage workplaces. However, VOCs have been identified in the air of the municipal solid waste treatment plants [53]. Benzene, toluene and organic solvents have been reported in the air of the sewage treatment plants receiving industrial sewage [9,27]. In a recent study on sewage management plants, concentrations of seven volatile organic sulfur compounds samples were determined. A wide range of concentrations was observed.

Dimethyl sulfide, carbon disulfide and dimethyl disulfide were the most abundant compounds, the highest concentrations being  $608.5\mu\text{g m}^{-3}$ ,  $658.5\mu\text{g m}^{-3}$  and  $857.8\mu\text{g m}^{-3}$ , respectively. The results were strongly influenced by the characteristics of the sampling point, e.g. whether the sample was taken at a confined site or in the open air [54]. The emissions of different VOCs from a wastewater treatment plant in Turkey has been studied; 1,3-dichlorobenzene, tetrachloroethylene were the most abundant with a total hourly emission rate of  $0.041\text{ kg h}^{-1}$  [55]. VOCs were monitored in the different sections of a wastewater treatment plant in Taiwan samples over several air sampling points. In the drainage and effluent system in each season, acetone, isopropanol and dimethyl sulfide were the major species and maximum concentrations were 400.4, 22.8 and 641.2 ppbv (part per billion by volume), respectively [56]. In Greece, 41 VOCs was analysed in wastewater samples collected seasonally in four wastewater treatment plants: 31 VOCs was observed [57]. The total organic vapour concentrations were measured in the influents of two wastewater treatment plants in a Midwestern city in USA and identified chlorobenzene, tetrachloroethylene, trichlorobenzene, 1, 1, 1- trichloroethane, trichloroethylene, toluene, xylene, benzene, methylethylketone, methylisobutylketone, and aliphatic naphtha [58,59].

### **1.3.2. Other exposures in sewage system (skin contact and ingestion)**

Sewage workers might also come in contact with industrial waste from accidental or illegal release, putting them at risk from extremely irritants to the mucous membrane, eyes, and skin [38,29]. An outbreak of airborne irritant contact dermatitis developed among incinerator workers employed in a sewage treatment facility. Contamination of the workplace and workers' clothing by sludge from the interstices of an incinerator exhaust fan proved to be the cause of the problem [60]. Exposure to wastewater in Mexico revealed no significant risk from ingestion or dermal contact except from nitrate exposure [61]. Dermal contact to acrylamide in sewage and wastewater treatment plants have been suggested [62].

### **1.4. Health effects among sewage workers**

The demonstration of toxic chemicals in sewage system, in the sludge and in the air in specific sites in sewage system, has raised suspensions regarding their possible health effects on sewage workers [50,63,64]. A variety of health hazard relating to work in sewage treatment plants and sewage system in general have been suggested caused by infectious agents and toxic materials which might be present in the wastewater.

Acute and sub-acute effects among sewage workers are well described [28,34,65-68]. Long term effect evidence is more scant and less consistent. Studies on long term effects have indicated an increase in total mortality and in the incidence of cancer [28,37,38].

#### **1.4.1. Morbidity studies**

Over the years, there have been several reports about work related symptoms among sewage workers. Of these, eye and nose irritation, lower airway symptoms, fatigue, skin symptoms, dizziness, and flu like symptoms [29,69]. An increased risk and/or prevalence of asthma and chronic bronchitis among sewage workers as well as decreased lung function values have been reported compared to water treatment plants [70,71]. Similar results were found for gastrointestinal symptoms [70,72], prevalence of hepatitis A antibodies [66,73] or leptospirosis [65]. A national wide study in Sweden among sewage workers [74] reported significantly increased risks for airway symptoms, chronic bronchitis, and toxic pneumonitis, as well as symptoms of the central nervous system including headache, unusual tiredness, and concentration difficulties in sewage workers compared to controls. Moreover, it reported an increased risk for non-specific gastrointestinal symptoms and for joint pains. A study of pregnancy outcomes in 101 wives of workers employed in a waste water treatment plant (WWTP), suggested an increased relative risk of fetal loss when paternal exposure to the WWTP occurred within 4 months prior to conception compared to exposure any time prior the conception [75].

#### **1.4.2. Mortality and cancer studies**

In association with the many substances in the sewage system there are carcinogens and mutagens [15,50,64]. Elevated levels of mutagen activity in the urine of sewage workers were found [50]. A Swedish study, however, found no difference in the level of DNA damage between sewage workers and municipal construction workers using comet assay on peripheral lymphocytes as a markers of genotoxic exposure [76]. When compared with workers from drinking water plants, waste workers at 14 treatment plants in the New York State were 12.9 times more likely to have urinary mutagens determined with Ames test, both with and without *in vitro* metabolic activation, after controlling for smoking [50].

Providing evidence of increased exposure to mutagenic substances among sewage workers does not mean that the long term health effects of this exposure are well characterized [50]. In Denmark, it was demonstrated that the wastewater workers have a mortality significantly higher than a population of water suppliers (RR = 1.27; 95%CI, 0.97-1.67) or than a

non-exposed population (RR = 1.88; 95%CI, 1.15-3.09) [28]. A significant increase in mortality from gastric cancer in workers in garbage handling, waste incineration and sewage treatment was reported in Italy (standard mortality ratio, SMR=3; 95% CI 1.2-6.4) [77]. A case-control British study of urothelial tumours and occupation showed an increased risk among sewage workers [78]. In Buffalo, USA, a retrospective cohort mortality study showed a significant increased mortality from liver and larynx cancer among sewage workers while the total mortality in cancer was not increased [37]. Standardized incidence ratios of some cancers were increased in a cohort of Swedish sewage workers: for prostate it was 1.2, 95% CI= 1-2.5, and was also significantly increased for cancer of the nose and the nasal sinuses (Standardized incidence ratio and 95%CI; 12, 1.5-44); incidence of stomach cancer was also increased but not significantly [38].

In Paris sewage workers showed an excess in mortality (SMR=1.25, 95% CI; 1.15-1.36), in particular, mortality for cancer (SMR= 1.37). This excess was to a large extent due to cancers of the digestive tract, in particular cancer of the oesophagus and the liver. Cancer of pleura and brain were also noted to be in excess. The study concluded that both malignant and non malignant liver diseases was probably due to over alcohol consumption, but they might be also partially related to occupational exposure to chemical or infectious agents [36]. However, this study did not use any objective measurement for either personal or ambient monitoring of exposure and used only information gathered by questionnaire or through the computerized register of the employees of the city of Paris. Unfortunately, there was an absence of any precise job codes, which might be lead to inclusion of other workers who were only administratively attached to sewage and thus possibly yielding under-estimation of the possible occupational risk.

To summarize, some studies have indicated an increase in the incidence of cancer among sewage workers [28,36-38]. Analyses on specific cancer sites have reported excess numbers of laryngeal, primary liver cancer [28,37], cancer of the prostate gland, nose and nasal sinuses cancers, stomach [38], central nervous system [79], and bladder cancers [50]. However, these data exhibit conflicting results. Furthermore, the use of employment records and the lack of direct biomonitoring or real measurement of exposure accompanying the biological assessment make such types of studies less accurate. Using national rates of cancer to compare the incidence in sewage workers calculations of cancer development might also be misleading in many aspects (e.g., geographically) [37,38].

## **1.5. Biomarkers of exposure to genotoxicants**

Occupational exposure may contribute to the development of serious diseases and impact on health through mechanisms that involve DNA damage. Thus, continuous efforts have been made to identify exposure to genotoxicants and to monitor populations that are exposed to harmful agents [80]. There are many methods available for the scoring of DNA damage and repair. The most commonly used are the bacterial Ames test [81], the chromosomal aberrations [82], micronucleus assay [83], sister chromatid exchanges [84], unscheduled DNA synthesis assay [85] and comet assay [86].

### **1.5.1. *In vitro* comet assay**

For its sensitivity to detect low levels of DNA damage in individual cells, ease of application, and the requirements of small amounts of a test substance, the *in vitro* alkaline comet assay developed by Singh et al. [86] represents a basic tool in research areas ranging from human and environmental biomonitoring to DNA repair processes and genetic toxicology [87].

#### **1.5.1.1. History and definition**

Comet test or “Single Cell Gel Electrophoresis” (SCGE) assay in an alkaline condition is defined as a rapid, simple, visual and sensitive electrophoretic technique that permits the evaluation, *in vitro* and *in vivo*, single and double DNA strand’s breaks, alkali-labile sites in individual mammalian cells [88].

The test is most frequently used as a genotoxicity screening test and a biomarker of exposure [89]. The test is easy to apply, require small amounts of the substance to test and can detect low levels of DNA damage and repair in individual cells.

Rydberg and Johanson in 1978 [90] were the first that quantified the DNA damage in individual cells (Chinese Hamster, CHO) embedded in an agarose gel. Then after, Östling and Johanson in 1984 [91] were the first who described comet assay by introducing an electrophoresis step in a neutral conditions to the previous procedure described Rydberg and Johanson in 1978. In this version of the technique, cells embedded in agarose on microscope slides were lysed and subjected to electrophoresis under neutral conditions, enabling the detection of DNA double-strand breaks. A later modification, in 1988, Singh et al. [86] introduced an electrophoresis at pH >13. In these alkaline conditions, the test permitted the detection of single DNA strand’s break and alkali-labile sites in individual cells. Singh called this comet test “Single Cell Gel Electrophoresis” (SCGE). Nowadays, this technique can



detect further forms of DNA damage and repair (e.g., oxidative DNA base damage, and DNA-DNA/DNA-protein/DNA-Drug crosslinking) in virtually any eukaryotic cell [89].

### 1.5.1.2. General principle of the assay

After exposure to a test substance, cells are embedded in agarose on a microscope slide, lysed with detergent and after a denaturation step during electrophoresis at high pH >13, DNA fragments as outcome of single-stranded and double-stranded breaks migrate faster in the electric field than intact DNA. The DNA is stained with ethidium bromide after neutralization. Cells with damaged DNA form a comet, consisting of a head (nuclear matrix) and a tail formed by DNA fragments which are visible under fluorescence microscopy conditions. This is due to the migration of (negatively charged) DNA fragments or DNA loops towards the positive pole. The likely basis for this is that loops containing a break lose their supercoiling and become free to extend toward the anode. Under these conditions, cellular cores fluoresce in red-orange. The relative intensity of fluorescence in the tail is a function of the frequency of DNA breaks; it can be assessed either visually or using densitometry and computer-based image analysis systems which make it possible to evaluate the DNA damage quantitatively [86,92].

Figure 1, shows general images of comet assay on Hep G2 cells according to the level of DNA damage.

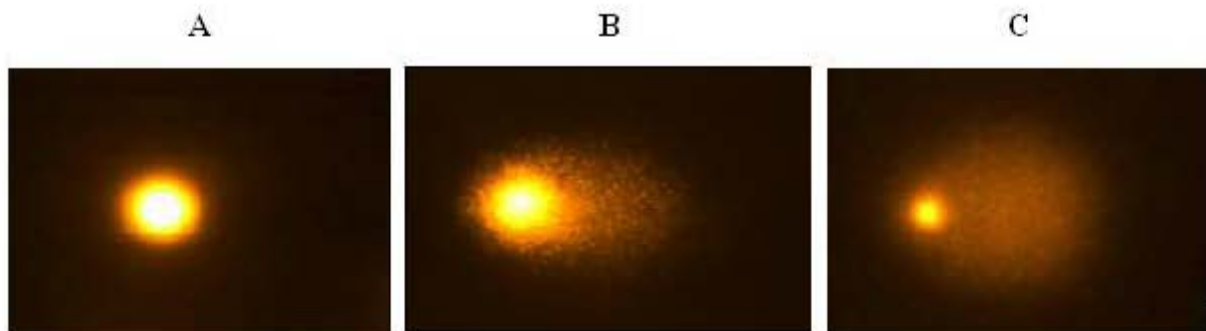


Figure 1: General images in comet assay on Hep G2 cells according to the level of DNA damage. A) Cell without DNA fragments. B) Cell with DNA fragments. C) Cell with major DNA fragments

### 1.5.1.3. Fields of application

One of the main advantages of the comet assay is that it can be successfully applied to almost every cell type (culture cell lines, isolated tissue, human, animal and plant cells). All eukaryotic cells can be employed to realise the test provided that a homogenous single cell preparation with an acceptable viability of > 60% are available [85]. Over the past two decades, the comet assay, has become one of the standard methods for assessing DNA damage, with applications

include but not limited to; (1) genotoxicity fields (*in vivo and in vitro*) where pharmaceutical, novel chemicals and/or toxic testing could be applied for example [89,93]; (2) ecogenotoxicological monitoring, and environmental biomonitoring where environmental samples and/or studies in different species living in the particular environments could be carried out [94,95]; (3) human biomonitoring ranging from aging and clinical investigations to genetic toxicology and molecular epidemiology, examples of this application include but not limited to monitoring occupational exposure to genotoxic chemicals or radiation [96-99], assessment of oxidative stress associated with various human diseases [100], and detection of DNA damage associated with smoking for example [101], as well as fundamental research in background DNA damage and repair capacity both cellular and *in vitro* [102-104]; (4) some human diagnosis application also could be followed by comet assay like [105]; (5) investigating nutrient or micronutrient effects at the level of DNA damage in humans as lipid content could lead to changes in oxidative DNA damage in lymphocytes [106], the test also could be applied to study the protective effects of anti-oxidants [107].

### **1.5.2. *In vitro* micronucleus assay**

It is becoming increasingly evident that an increased rate of DNA damage and chromosome breakage or loss is an important risk factor for elevated risk for cancer [108]. Human biomonitoring of early genetic effects requires accurate, sensitive and, if possible, easy and not too time-consuming methodologies to assess mutations. One of the most promising methodologies at the present time for assessing DNA damage is the cytokinesis block micronucleus (CBMN) assay, which detects both chromosome and genome mutations in binucleated (BNed) cells. Micronucleus (MN) assay could be considered as biomarker of effect that is relevant for risk assessment of cancer [83].

The MN assay is a mutagenicity test that reflects chromosomal damage and aneuploidy, whereas other currently used human biomonitoring methods such as DNA-adducts, and comet assay reflect DNA damage that might be repaired and not give rise to heritable DNA alterations. Positive results in the comet assay do not always correspond to positive results in the MN tests, especially when the exposure to genotoxic agents is small. The comet assay usually detects more defects than the MN test [109]. The positive results in the comet and MN tests are due to different mechanisms; the MN test detects injuries that survive at least one mitotic cycle, while the comet assay identifies reparable injuries or alkali-label sites [109,110]. Consequently, Goethem, 1997 [109] suggests the use of both MN and comet tests.

Thus, MN assay is suitable to monitor specific human health risk arising from various kinds of exposure to carcinogenic hazards [111].

### **1.5.2.1. History and definition**

As the name implies, a “micro”-nucleus is a small nucleus. Normally, during the process of cell division, the genetic material replicates and is then divided equally between the two daughter cells. In the case of a chromosome break however, the distribution of DNA between the two daughter cells may be affected. In this case, the fragment may fail to be included in either of the two daughter nuclei, and the genetic material left behind forms a “micro” nucleus.

A micronucleus arise from a whole lagging chromosome (aneugenic event leading to chromosome loss) or an acentric chromosome fragment detaching from a chromosome after breakage (clastogenic event) which do not integrate in the daughter nuclei [112,113] during the metaphase/anaphase transition of mitosis (cell division) and thus can only be expressed once a cell has completed nuclear division. These events may be induced by oxidative stress, exposure to clastogens or aneugens, genetic defects in cell cycle checkpoint and/or DNA repair genes, as well as deficiencies in nutrients required as co-factors in DNA metabolism and chromosome segregation machinery [114-117].

Slide reading is easy, more objective and quick. It has been also shown to be a robust test which can be applied to any type of primary cells or cell lines [118]. Finally, it has greater accuracy and statistical power as thousands of cells can be scored compared to a few hundred in the *in vitro* chromosomal aberration test. A limitation of the *in vitro* MN assay is that the assay does not provide information about the types of structural chromosome aberrations.

The first study of importance on the *in vitro* MN assay was performed in 1976 by Countryman and Heddle on human lymphocytes exposed to X-rays [119]. The suitability of the MN test for human biomonitoring studies was first described in 1982 by Stich et al [120] who used exfoliated cells of the bucal mucosa.

Currently, two methods are used to perform the assay. In the original method proposed by Countryman and Heddle, (1976) micronuclei (MNi) are scored in a dividing cell population without discriminating between cells that have completed nuclear division and those that have not following exposure to the genotoxic agent [119]. It has been shown that this approach in human lymphocyte cultures tends to underestimate MN frequency [121,122]. To improve the *in vitro* MN assay, Fenech and Morley (1986) proposed that MN should only be scored in cells that had completed one nuclear division, both to obtain an accurate estimate of

spontaneous MNi frequency as well as a reliable estimate of MNi induced by radiation or chemicals [121]. This was particularly important for chemicals (and UV radiation) as genotoxic doses of several chemicals also tend to inhibit nuclear division. Several methods were proposed and developed to score MNi only in cells that complete nuclear division but of these only the CBMN assay could efficiently and reliably identify such cells [123]. The CBMN assay was subsequently adopted by numerous laboratories leading to an unabated interest in its use and application for human biomonitoring, radio-sensitivity testing and *in vitro* genotoxicity studies [83,124-126].

In general, MN assay has a high sensitivity, but not necessarily a high specificity (it is likely that the positive results indeed reflect exposure to carcinogenic factors, whereas it is unclear if the lack of MN induction indicates the lack of a cancer risk) [111].

### **1.5.2.2. General principle of the assay**

Recently, Fenech described the general methodology for performing the MN assay. This protocol that has been published in nature protocols" journal [83] and includes; at time (t) = 0: a cellular suspension is prepared and a fraction of this suspension is deposited on a microscopic slides. At t+24h: the medium is then discarded and replaced by freshly diluted test medium contain the tested substance. At t+44h: the test medium is discarded and replaced by freshly diluted culture medium contain cytochalasin-B. At t+72h: the slides are removed from the dishes, rinsing in a KCL solution, and then dipped into another fresh KCL solution for 10 min. They were then shortly-dried at room temperature and then dipped into CARNOY solution (acetic acid: methanol (1: 3 v:v) for nearly 10 seconds before being dried in the open air. Before reading and analysis, the slides are dipped 10 min in an Acridine orange solution then dipped into a phosphate buffer solution for 10 min. After the initial screening; well-prepared slides are scored using a high power magnification (400-1000 folds) with both bright field and phase-contrast microscopy or a microscope with a filter for propidium iodide (fluorescent in situ hybridization; FISH technique). Under these conditions, the cellular cytoplasm appear in red and the DNA in green. At least 1000 binucleated cells non-apoptotic, non-necrotic are counted per slide. Approximately 500 more cells are counted to allow calculation of the nuclear division index according to the criteria described by Fenech, 2007 [83]. Cells of different lines could be used as well as lymphocytes.

Statistical significance should not be the sole factor for determining positive results in MN assay but serve as an aid in evaluating the test results. Biological meaning and relevance of the results should be considered taking into account the historical control range and

cytotoxicity. Only individual MN frequencies based on the scoring of at least 1000 BNed cells are considered for statistical analysis. All frequencies, averages, and fold-increase calculations could be taken into account. For example, a significant induction of MN  $\geq$  2-fold mean increase over concurrent negative control might be considered.

### **1.5.2.3. Fields of application**

The MN test is among the most-well validated intermediate endpoints. It represents a change that is biologically relevant to the process of carcinogenesis and its frequency change in response to carcinogen exposures. The high reliability, simplicity, low cost and the non-invasively running of this technique, have contributed to the worldwide success and adoption of this biomarker for *in vitro* and *in vivo* studies of genome damage [128]. This assay has been also successfully applied to identify dietary and genetic factors that have a significant impact on genome stability [115]. Measurement of micronucleus frequency in peripheral blood lymphocytes is extensively used in molecular epidemiology and cytogenetics to evaluate the presence and the extent of chromosomal damage in human populations exposed to genotoxic agents or bearing a susceptible genetic profile [129].

The MN assay in human biomonitoring studies is mainly applied to peripheral blood lymphocytes and to a lesser extent in epithelial cells [126,130]. This assay is being also used to: (i) compare genetic damage rate between populations exposed to different environmental, occupational and lifestyle factors [129]; (ii) assess differences in radio-sensitivity between individuals at risk for cancer both as a predictor of cancer risk as well as for optimization of radiotherapy [125]; (iii) assess the genotoxic potential of new chemicals produced by the agrochemical and pharmaceutical industries [118].

Furthermore, an association between MN induction and cancer development had been established. This association is supported by numerous observations. The most substantiated include: (i) the high frequency of this biomarker in untreated cancer patients and in subjects affected by cancer-prone congenital diseases, e.g. Bloom syndrome or ataxia telangiectasia [129,131]; (ii) the correlation existing between genotoxic MN-inducing agents and carcinogenicity, e.g. ionizing or ultraviolet radiation [132]; and (iii) the inverse correlation between MN frequency and the blood concentration and/or dietary intake of certain micronutrients associated with reduced cancer risk, such as folate, calcium, vitamin E and nicotinic acid [117]. Moreover, the assessment of MN in BNed cells remains an important and sensitive method for biomonitoring of exposure to clastogenic chemicals (eg., complex mixtures of PAHs) [133].

The wide-spread use of the MN assay in the monitoring of environmental and occupational exposure to genotoxicants, its responsiveness to the effects of micronutrients and diet and its ability to identify high-risk groups of susceptible individuals, provides a further possibility for the use of the MN assay in the planning, implementation and validation of cancer surveillance and prevention policies [134]. It is now evident that the *ex vivo/in vitro* MN assay has evolved into a strong test as a DNA damage biomarker with application in nutrition, radiation sensitivity both for cancer risk assessment and optimization of radiotherapy, ecotoxicology, biomonitoring of human populations, and testing of new pharmaceuticals and other chemicals.

### **1.5.3. Comet and micronucleus assays *in vitro* human occupational studies**

The comet and MN assays have been suggested suitable for monitoring occupational exposure [96,98,129,139]. To our knowledge, no study has specifically investigated the use of comet and micronucleus tests among the urine extracts of sewage workers or other occupationally or environmentally exposed workers. Only one study investigated the level of DNA damage among sewage workers using the comet test but on blood lymphocytes and it did not find significant differences between the sewage workers and the control group [76]. In this study, the sewage workers were classified into three different groups of exposure, based on self-reported levels of exposure, and the group with the highest exposure was defined as those workers who had worked in sewage-contaminated environments for at least 8 h in the preceding 2 weeks. This may indicate that the sewage workers were not exposed to an extent that causes DNA damage in lymphocytes. However, the assessment of exposure may not accurately discriminate between different exposure circumstances. It would have been of great value if some sort of definition of the DNA damaging agents and measurement of exposure had been included in the study. However, these tests have been evaluated in various occupational studies in using other body or tissue samples, mainly blood lymphocytes or oral mucosa. Analysis of leukocytes from workers exposed to environmental pollutants at a waste disposal site exhibited significant DNA damage as presented by comet assay in exposed group [135].

Regarding other settings of occupational exposures to complex mixture of chemicals and low doses of carcinogens, comet and micronucleus tests have been widely applied in *ex vivo* epidemiological biomonitoring studies to evaluate genotoxic effects and DNA damage.

In Italy, paving workers exposed to complex mixture including several PAHs contained in asphalt fumes during road paving, were evaluated for early genotoxic and oxidative anomalies

on lymphocytes. Using the comet assay, they found a significant increase in DNA damage in exposed group than the control [136]. In Turkey, a significant increase in MN frequency among asphalt workers compared to control on peripheral lymphocytes was reported [137], while a lack of MN induction was reported for road pavers in Sweden [138]. Another work evaluated the occupational genotoxicity risk through the comet and MN test applied to exfoliated cells of the buccal mucous among storage battery renovation workers and car painters in Brazil. Highly significant effects of occupational exposure were found in both tests [139].

Among workers in a 1,3-butadiene production plant. Comet and MNi assays on lymphocytes did not indicate that the exposed workers had more DNA damage than unexposed [140]. Other studies have investigated occupational exposure to mixtures of organic solvents and hydrocarbons on lymphocytes and exfoliated buccal cells. The exposed groups included airport personnel [141], gasoline station attendants [142], and shoe factory workers [143]. Both the airport personnel and the gasoline attendants were exposed to variety of DNA-damaging agents such as motor vehicle exhausts and gasoline vapors, including benzene and its metabolites. The gasoline station attendants and the airport workers had more DNA damage than the control groups as evaluated by comet assay. The airport workers had no significant MN frequency than the control. The study of shoe factory workers included employees from two shoe factories. In both factories, acetone, gasoline, and toluene were detected in the air of the workplaces. There was no difference in the level of DNA damage (comet assay) between exposed workers and controls. Footwear manufacturing industry workers occupationally exposed to solvent based adhesive and solutions containing organic solvents, mainly toluene were evaluated comet assay in blood cells and micronucleus frequencies in lymphocytes and epithelial buccal cells. Comet assay showed statistical increased values among footwear-workers relative to controls ( $P < 0.001$ ) but no difference was observed in frequencies of MNi [144]. Coke oven and graphite-electrode producing plant workers exposed to PAHs were evaluated for comet test in blood lymphocytes. The mean tail extent moment was higher in both coke oven workers (1.38 times,  $P > 0.05$ ) and graphite-electrode producing plant workers (3.13 times,  $P < 0.05$ ) when compared with controls (mean $\pm$ SD, 2.54 $\pm$ 0.68) [145]. In another recent study, coke oven workers showed a significant increase in MNi and comet tail moment than controls ( $P < 0.05$ ) [146].

A study aimed to determine the level of environmental exposure to VOCs and the possible DNA damage induced on the population involved in the different cleaning tasks related to VOCs exposure, reported an increase in MN and comet assays [147].

On exfoliated oral mucosa cells, the group of Surralles et al. [148] used MN test for benzene exposed workers in a petrochemical industry and found no genotoxic effects at concentration of 1 ppm TWA (part per million time weighted average). However, in engine exhaust and used engine oils workers in engine repair workshops and traffic, tobacco industry workers, tunnel construction workers (lymphocytes), and gasoline station attendants and traffic enforcers, positive results were reported by MN test [149-152]. While rubbers factory workers, showed negative results in MN test on oral mucosal cells [153], positive results as measured by comet assay on lymphocytes were reported [154].

Occupational exposure to gasoline, diesel, butane gas, styrene, benzene, and chloroform in petroleum hydrocarbons workers showed that exposed individuals presented a greater amount of damage at the DNA level (comet assay) as well as at the chromosomal level (MN assay) than the individuals from the control populations [155]. The results of a Polish study showed that workers employed in a wooden furniture factory had more DNA damage in lymphocytes than a control group as measured by comet assay [156].

## **1.6. Biomarkers of early effects of genotoxicants**

### **1.6.1. The 24h urinary 8-oxodG**

Oxidative stress-induced damage to DNA includes a multitude of lesions, many of which are mutagenic and have multiple roles in cancer. The oxidative damage biomarkers occur early in the exposure-to-disease pathway. Many of these early damage biomarkers have been shown to be associated with increased risk of developing diseases as cancer. They are therefore considered as important tools for investigating the mechanisms behind exposure induced adverse health effects. In relation to cancer, biomarkers of oxidative damage DNA are considered most important in this context [157-160]. One of the major products of hydroxyl radical attack of DNA is 8-oxo-2'-deoxyguanosine (8-oxodG) [161]. The 8-oxodG (a DNA base lesion) is highly mutagenic and thus may be important in carcinogenesis process [161,162]. The cell perceives it as a threatening lesion that has to be removed rapidly [161]. Its measurement is among the most widely used biomarkers for assessing oxidative DNA damage [163]. It can be detected in urine, DNA and tissues [164]. The 8-oxodG level in cells or tissues reflects the balance between damage and repair [164], while its level in urine is used to assess the whole-body oxidative DNA damage [165,166].

In situation implied the exposure to genotoxic chemicals, the measurement of 8-oxodG in blood cells DNA and urine has become very popular. However, there is still a lack of



well-established dose-response relations between occupational or environmental exposures and the induction of 8-oxodG [167]. In the meanwhile, experimental and population-based studies indicate that many environmental factors, including particulate air pollution, cause oxidative damage to DNA, whereas diets rich in fruit and vegetables or antioxidant supplements may reduce the levels and enhance repair [168,169].

To our knowledge, there is no study that has evaluated the 8-oxodG among sewage workers neither in the urine nor in the DNA of blood cells. However, it has been assessed in other similar occupational settings that involved exposure to complex chemical mixtures. Workers who smoked and were exposed to hard metal dusts had elevated 8-oxodG in urine [170]. In urine of occupationally exposed workers, 8-oxodG was assessed in 57 non-smoking bus drivers in Denmark. The excretion of 8-oxodG was significantly higher in bus drivers from the central areas of Copenhagen compared with suburban/rural areas [171].

Urinary levels of 8-oxodG range between 2.7-13 ng/mg creatinine (see StressMarq Biosciences Inc., Victoria, BC Canada reference). The European Standards Committee on Oxidative DNA Damage has concluded that the true levels of the most widely studied lesion, 8-oxodG, in cellular DNA is between 0.5 and 5 lesions per  $10^6$  dG bases [102]. Few studies have assessed 8-oxodG in the DNA of blood cells. Population exposed to environmental factors (e.g., air pollution, smoking) [172], have shown markedly higher than normal amounts of oxidative DNA damage in terms of the 8-oxodG concentration in circulating leukocytes. Occupationally exposed workers (boat builders) to styrene, a genotoxic compound and a potential carcinogenic hazard that is present in sewage system, showed elevated levels of 8-oxodG in white blood cells. The 8-oxodG / $10^5$  dG ratio from 17 styrene-exposed workers showed significant increases (mean  $\pm$  SD,  $2.23 \pm 0.54$ , median 2.35,  $P < 0.001$ ) in comparison to the controls ( $1.52 \pm 0.45$ , median 1.50) [173].

### **1.6.2. DNA-adducts**

Of the complex mixtures to which sewage workers are exposed, are PAHs and other genotoxic chemicals that are metabolized by and induce the expression of cytochrome P450 enzymes (e.g. CYP1A2) [174-176]. The catalyzed metabolism by CYP1A2 can generate reactive oxygen species (ROS) which might lead to oxidative DNA damage [163,174,177]. This damage has been associated with an increased risk of cancer generally ascribed to DNA adducts [174,178]. PAHs related DNA-adducts measured by  $^{32}\text{P}$ - postlabeling technique is frequently described as a biomarker of choice for early biological effects of these genotoxicants [179-181].

In this context of an exposure to complex mixtures, the technique of  $^{32}\text{P}$ - postlabeling for the measurement of the adducts of the DNA has several advantages: (1) it allows the detection of adducts of varied structures and their quantification; (2) it has a very good sensitivity, which makes it useful even when the levels of exposure remain moderate; (3) its application is possible for the biomonitoring of human populations since the use of radioelement take place only after DNA extraction from the tissue samples; (4) in addition, the necessary amount of DNA is rather small, about 5 to 10  $\mu\text{g}$  by analysis, requiring only small quantities of tissue samples [179-181].

### **1.6.3. Metabolism of caffeine and CYP1A2 activity**

The cytochromes P450 (CYTP450) are a super family of genes containing currently approximately 4000 members in species going from bacteria to plants and animals. The P450 cytochromes belong to the reactions of phase I of the metabolism. They catalyse the oxidative transformation of a great number of different chemical compounds, as xenobiotics (e.g. drugs and pollutants) and endogenous compounds (e.g. steroids, fatty acids, and biliary acids). There is a great inter-individual variability of these P450 cytochromes. The main reason is their regulation by various chemical compounds, resulting either from the environment, food or medical drugs intake [182]. Environmental pollutants (e.g., PAHs, the solvents, and heavy metals) are usually described as likely to modulate the expression of some P450s, and thus their activities [182]. All these products are found in the environment and are found in worn water. They are likely to change the expression of P450 in a population of sewage workers.

The exposure to PAHs is easily evaluated by a measurement of the activity of the CYTP1A1 in animals or *in vitro* (rats, fish) [183,184]. *In vivo*, this technique is invasive and requires the sacrifice of the animals.

For human being, the CYP1A2 was shown to be responsible for the 3-demethylation of caffeine, which is the initial major step in the biotransformation of caffeine in the human body [185]. Urinary metabolites of dietary caffeine are the most non-invasively-used method in the assessment of CYP1A2 activity [186-188]. Caffeine test is a simple and non-invasive test which makes it possible to explore the activity of the CYP1A2 induced by PAHs. It was used successfully to carry out measurements of exposure to the PCBs. The rise in the metabolism of caffeine, and more particularly of the ratio [1,7-dimethyluric acid (17U)+ 1,7-dimethylxanthine (17X)/ 1,3,7-trimethylxanthine (137X)] is the mark of an exposure to molecules of the type PAHs [189,190]. A correlation between the metabolism of caffeine and the presence of mutagen in urine was previously highlighted [191]. However, recent studies

have demonstrated that the polymorphism of CYP1A2 could be critical in investigating the induction of the enzyme [192]. The -163C>A (allele CYP1A2\*F) polymorphism has been associated with higher enzyme inducibility by smoking [192]. Even if the clinical relevance of this polymorphism remains controversial [192], it is necessary to assess it for a good interpretation of the caffeine metabolism data.

### **1.7. Description of the Parisian sewage system**

The Parisian sewage system is a large network of underground canals whose network duplicates nearly the street network. The sewage system usually collects domestic and industrial effluents and runoff waters from the Paris urban area. It drains the waste water towards the treatment plants and it consists of three main lines; (A) small or primary lines (1.2 to 2 m in diameter) that connect buildings to the system. The sewage workers intervene directly with handheld tools when these lines need to be repaired; (B) secondary and main collectors or canals that re-group the output of the primary lines. These are larger and have small sidewalks that allow the workers to walk along these canals. The clearing of these canals is done by wagons or small boats that allow the regulation of the flow of wastewater; (C) more or less large sedimentation basins slow down the wastewater flow to get rid of the sediments that are taken by aspiration from the streets. Inspection of the lines is the job of the sewage workers that enable the detection of any perturbation of the wastewater flow and other possible defaults like cracks or leaks of the duct, it enables also the curing of the wastewater lines and the extraction of the sediments. This final job has been subcontracted in the recent years. A number of maintenance tasks like carpentry and masonry are usually done by specialists [36].

### **1.8. Rationale and justification of the study**

Occupational exposure may contribute to the development of pernicious illnesses, often through mechanisms that involve genotoxicity and chromosomal changes. Genotoxicity is a pathogenetic process for many neoplasias and according to the multi-step hypothesis of cancer development; the formation of initiated cells by genotoxic compounds is causally related to cancer [193]. Continuous efforts have been made to identify genotoxic agents, to determine conditions of harmful exposition and to monitor populations that are excessively exposed [80].

Studies showed that sewage workers may be exposed to various toxic agents present in the atmosphere or in wastewater of the sewage system [29,50,52]. Obviously, exposure of those

workers implies contact with multiple compounds at varying levels (by concentration, time and/or location) and routes of exposure (by inhalation, dermal and/or ingestion) [38]. These multiple agents, to which sewage workers are exposed to, might interact together resulting in an immeasurable amount of different chemicals. Also, changes in the composition of the sewage system over-time may affect the level and character of worker's exposure. Rather than trying to describe this immense array of exposures or pursuing the goal to relate the biological health outcomes to one specific compound, it might be more reasonable to look for unspecific early effects. Moreover, assessment of genotoxicants at only one point in time may not represent long term occurrence of these substances in the body [194].

Sewage workers of the city of Paris show characteristics of morbidity higher than the general working population but few data are available concerning the exposure of this category of workers. Many of the compounds previously identified in sewage system are known or suspected carcinogens and/or mutagens. Sewage workers exposed to this mixture of toxicants might be subject to elevated risk of cancer [10-12]. It is in this population, whose exposure to carcinogenic agents is certain, that we will assess the relevance of the various biomarkers proposed earlier. The usage of biomarkers to study the association between exposure and early biological effects seems even more relevant in this setting of complex exposure [11]. Consequently, workplace environment sampling, at various locations over-time when the biological specimens are taken might be more representative of actual exposure and would further support the link between exposure to unknown chemical mixtures and the occurrence of genotoxicants in urine and blood. We believe that the possible carcinogenic effects of occupational exposure in sewage workers should be further explored by quantitative exposure estimates.

In this thesis we will assess occupational exposure of the city of Paris sewage workers to a complex environment using in parallel environmental and biological measurements. A selected number of biomarkers of exposure and of early biological effects in surrogate human tissue will be evaluated. Also, occupational air sampling for 13 PAHs and 12 VOCs on a weekly basis will be assessed. The validation of these biomarkers in terms of their ability to detect alterations related to this exposure would allow a more convenient and, most important, earlier evaluation of the risk to populations exposed to cocktails of toxicants than would the traditional epidemiological approach.

## 1.9. Aim of the study

The study is interested in an association of simple, early and non-invasive biomarkers intended to highlight exposure to cocktails of undefined toxic substances having genotoxic properties. The traditional epidemiological studies evaluate the impact of exposure in terms of well established incident pathology. Little research has been done regarding associations between a poorly understood and very complex exposure and changes in biomarkers as a means to evaluate risks associated with this exposure. This study aims to provide useful tools in this context.

This thesis explore certain integrated biomarkers of exposure and of early effects among sewage workers in order to better assess their carcinogenic risk with a focus on urinary genotoxicity and oxidative stress biomarkers.

The biomarkers selected for this study are the *in vitro* comet and micronucleus tests, which seek the presence of genotoxics in urine. A second group of biomarkers highlights early effects of these substances: the caffeine test, relevant in the event of exposure to PAHs [195]; urinary 8-oxodG in 24h urine samples, a biomarker of early effect, corresponding to DNA oxidative stress [196] and DNA-adducts in the peripheral blood cells, a biomarker of early effect indicating the exposure to genotoxics (future work).

Hence, the specific objectives of the thesis are the following:

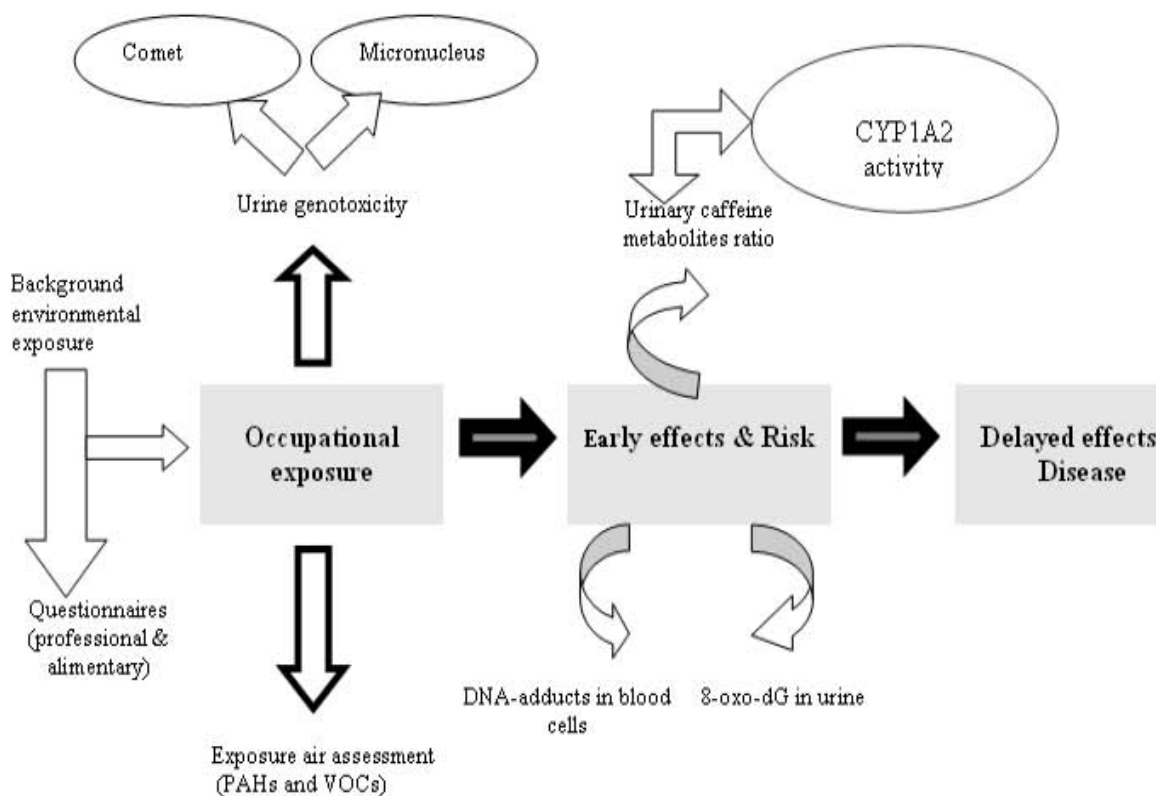
- To measure personal exposures to 13 PAHs and 12 VOCs in the workplaces air environment.
- To evaluate the urine genotoxicity and DNA damage through *in vitro* comet and micronucleus assays (biomarkers of exposure) applied on the urinary organic extracts of sewage and office workers, using human-derived culture cell line (Hep G2).
- To assess the oxidative stress through urinary 8-oxodG in the 24h urine samples (biomarker of early biological effect) retrieved from those workers.
- To analyze the urinary caffeine metabolites ratio in view to evaluate the activity of cytochrome P450 1A2 enzyme (biomarker of exposure to PAHs).
- To analyze peripheral blood cells for DNA-adducts through the <sup>32</sup>P-post labelling technique (biomarker of early biological effects). Due to time shortage, this part of the study will be dedicated to further work.

## **1.10. Study hypothesis**

The study hypothesis is that exposure of the sewage workers to multiple genotoxics leads to an increase in certain biomarkers of exposure and other biomarkers of early biological effects. Figure 2 presents the theoretical-overview of development from exposure to disease and the study assessment biomarkers. It also shortly presents the study tools and assessment biomarkers. They range from background environmental exposure and assessment of the health status of the study population by questionnaires, to assessment of early biological effects through biological assays with blood and urine.

The results of this thesis aim to improve understanding of the biomarkers of exposure and of early biological effects in a way that might help overcome the limitations of environmental exposure assessment in very complex occupational or environmental settings. By using a biomarker approach, this thesis will increase our knowledge regarding total exposure to complex mixtures of toxic chemicals along different pathways (lung, skin, and gastro-intestinal tract), and different sources (air, diet, lifestyle and occupation).

The conclusion will be important for the better understanding of human biomonitoring approaches in complex occupational settings where accumulative risk may occur. The conclusion will also highlight areas for future research.



**Figure 2: Schematic diagram of progression from exposure to disease showing the study assessment tools and biomarkers. 8-oxodG: 8-oxo-2'-deoxyguanosine; PAHs, polycyclic aromatic hydrocarbons; VOCs, volatile organic compounds.**

## 1.11. Thesis overview

This thesis is composed of five chapters. Chapter 1, “Introduction”, presents the background and details the aspects that are explored in this work including the relevant literature; it also provides background information for readers unfamiliar with this particular area of research.

Chapter 2, “Materials and methods”, describes the experimental techniques and measurements used for data collection and analysis. This includes population recruitment procedures, occupational air sampling for 13 PAHs and 12 VOCs, and the analysis of various biomarkers of exposure and of early effects related to genotoxicity and oxidative DNA damage. It includes also the published study protocol article (1<sup>st</sup> article) and the under submission comet article (2<sup>nd</sup> article).

This is followed in Chapter 3, “Results”, by presentation of the research results. These are the findings concerning the outcomes related to occupational exposure of sewage workers. Emphasis has been directed toward (i) occupational air sampling findings, (ii) organic urine genotoxicity assays (*in vitro* comet and micronucleus), as biomarkers of exposure that explore the exposure and presence of genotoxics in urine, (iii) oxidative DNA damage using urinary

8-oxodG in 24h urine samples as surrogate biomarker of early biological effects. Points (i and iii) were presented in the exposure and genotoxicity article (3<sup>rd</sup> article). Other detailed findings were presented in the results chapter separately.

Findings that were not discussed in the 3<sup>rd</sup> article are reviewed and discussed in Chapter 4, “General discussion”, which allows evaluation of our findings, along with data and analysis techniques validation. Chapter 5, “Conclusion and research perspectives”, completes this study by gathering learnings from all the results and presents suggestions for areas of future work.

Finally, several appendices at the end of the thesis provide summaries about the work which was conducted as part of this thesis. They also provide some details of and information about various aspects followed along this thesis (e.g., ethical and administrative procedures).



## **2. Materials and methods**

### **2.1. Protocol development**

#### **2.1.1. Study protocol article (1<sup>st</sup> article)**

The protocol article first introduces the study and its significance. It focuses on the study objectives and the biomarkers dedicated for the study. Then the materials and method section describes the study design, population and setting. It also presents the sampling chronology of the study and the ethical consideration. Later, the experimental protocol and technique for the assessment of the study biomarkers are explained. The discussion section highlights the importance of the biomonitoring approach of the biomarkers of exposure and of early biological effects in complex occupational and environmental exposures of multiple pollutants.

The protocol of this study has been previously published in BMC cancer journal (AL Zabadi et al., 2008). It is presented in the following pages as part of this thesis.



# **Biomonitoring of complex occupational exposures to carcinogens: the case of sewage workers in Paris\***

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\* AL Zabadi H, Ferrari L, Laurent AM, Tiberguent A, Paris C, Zmirou-Navier D. BMC Cancer 2008; 8:67.

## **Abstract**

### **Background**

Sewage workers provide an essential service in the protection of public and environmental health. However, they are exposed to varied mixtures of chemicals; some are known or suspected to be genotoxics or carcinogens. Thus, trying to relate adverse outcomes to single toxicant is inappropriate. We aim to investigate if sewage workers are at increased carcinogenic risk as evaluated by biomarkers of exposure and early biological effects.

### **Methods/design**

This cross sectional study will compare exposed sewage workers to non-exposed office workers. Both are voluntaries from Paris municipality, males, aged (20–60) years, non-smokers since at least six months, with no history of chronic or recent illness, and have similar socioeconomic status. After at least 3 days of consecutive work, blood sample and a 24-hour urine will be collected. A caffeine test will be performed, by administering coffee and collecting urines three hours after. Subjects will fill in self-administered questionnaires; one covering the professional and lifestyle habits while the second one is alimentary. The blood sample will be used to assess DNA adducts in peripheral lymphocytes. The 24-hour urine to assess urinary 8-oxo-7, 8-dihydro-2'-deoxy-Guanosine (8-oxodG), and the *in vitro* genotoxicity tests (comet and micronucleus) using HeLa S3 or HepG2 cells. In parallel, occupational air sampling will be conducted for some Polycyclic Aromatic Hydrocarbons and Volatile Organic Compounds. A weekly sampling chronology at the offices of occupational medicine in Paris city during the regular medical visits will be followed. This protocol has been accepted by the French Est III Ethical Committee with the number 2007-A00685-48.

### **Discussion**

Biomarkers of exposure and of early biological effects may help overcome the limitations of environmental exposure assessment in very complex occupational or environmental settings.

## Background

Sewage workers provide an essential service in the protection of public and environmental health. In large cities, sewage is composed of organic residues but also incorporate a wide variety of chemicals produced by roadways scrubbing by rain, water from office and industrial facilities, domestic activities (remainders of painting, drugs, pesticides used indoor, etc). As a result of their contact with wastes, sewage workers are exposed to complex mixtures of toxicants including pathogens, heavy metals, chlorinated organic solvents like chloroform, dichloroethane, perchloroethanol, other solvents (benzene, toluene), aldehydes, nitrosamines, pesticides, dyes, polychlorobiphenyls, and polycyclic aromatic hydrocarbons (PAH) [1-3]. Many of these compounds are known or suspected to be genotoxics and/or carcinogens [4-6], which suggests that those workers may be subject to elevated risk of cancer.

Previous studies have indicated an increase in the incidence of cancer among sewage workers [7-10]. Analyses on specific cancer sites have reported excess numbers of laryngeal, primary liver cancer [8,10], cancer of the prostate gland, nose and nasal sinuses cancers, stomach [9], central nervous system [11], and bladder cancers [12]. However, these data exhibit conflicting results [8,9]. A more recent mortality study among the sewage workers of Paris published in 2006 assessed their cause-specific mortality from 1970 until 1999 [7]. A slight but significant excess in mortality was found (SMR = 1.25, 95% CI; 1.15–1.36) in particular from cancer mortality (SMR = 1.37, 95% CI; 1.20–1.56), with a suggested excess for oesophagus, liver, pleura and the brain cancers albeit not significant. However, this study didn't measure personal or workplace exposures; it used only qualitative information gathered by a questionnaire and the computerized register of the employees.

As exposure of sewage workers implies contact with multiple potent genotoxics at varying levels (by concentration, time and location) and routes of exposure (by inhalation, dermal and ingestion) [9], characterizing and quantifying it are extremely difficult, and trying to relate adverse outcomes to single toxicant is inappropriate. However, usage of biomarkers to study the association between exposure and early biological genotoxics effects seems more relevant in this setting [5]. These findings may explain that previous studies among sewage workers exhibited conflicting results; some were biased by many confounding factors; others relied on qualitative and/or a questionnaire data; while others used urine or blood samples to evaluate the exposure without workplace measurements. However, sewage workers might be exposed to many agents that may interact with one another resulting in an immeasurable amount of

different chemicals. Rather than trying to describe this immense array of exposures or pursuing the goal to relate the biological health outcomes to specific compound, it might be more reasonable to look for unspecific early effects. Further, changes in the composition of the sewage system over-time may affect the level and character of worker's exposure longitudinally. Thus, assessment of genotoxics at only one point in time may not represent long term occurrence of these substances in the body [13]. However, workplace environment sampling at various locations over-time when the biological specimens were taken might be more representable and would further support the link between occupational sewage exposure and the appearance of genotoxics in both sample types (urine and blood).

In order to precisely assess such exposures to genotoxics/carcinogens compounds, urine genotoxicity has been widely used as a noninvasive method to evaluate recent exposure among populations exposed to environmental and/or workplace-related complex mixtures of chemicals [14-16]. *In Vitro* comet [14] and micronucleus [17] assays are among the most widely-used biomarkers of urine genotoxicity for monitoring the risk of DNA damage that stems from occupational and environmental exposures to genotoxics. Comet assay is a sensitive technique, can detect DNA damage in terms of double and single-strand breaks, and alkaline-labile sites [18,19]. Micronucleus test is a reliable biomarker of irregularity of genetic material due to non-specific genotoxic exposure [20,21].

Of the complex mixtures to which sewage workers are exposed, are PAHs and other genotoxic chemicals that are metabolized by and induce the expression of cytochrome P450 enzymes (e.g. CYP1A2) [22-24]. The CYP1A2 enzyme is involved in the metabolic activation of a wide range of chemicals and carcinogens like PAHs and aromatic amines [23,24]. Its activity has been shown to be increased by smoking, ingestion of charbroiled meat, cruciferous vegetables, PAHs and PCBs exposures [23,25-28]. The catalyzed metabolism by CYP1A2 can generate ROS which might lead to oxidative DNA damage [22,29,30]. This damage has been associated with an increased risk of cancer generally ascribed to DNA adducts [22,31]. Thus, measurement of CYP1A2 activity *in vivo* may be an important tool to assess the exposure to chemical carcinogens and cancer risk. PAHs related DNA-adducts measured by <sup>32</sup>P- postlabeling technique is frequently described as the biomarker of choice [32-34]. Oxidative DNA damage may be also important in carcinogenesis since the DNA base lesions, such as 8-oxodG, are abundant and highly mutagenic [35,36]. However, DNA repair via nucleotide and base excision processes leads to elimination and excretion of 8-oxodG in urine quantitatively without metabolism [37-40]. Urinary excretion of 8-oxodG is the most widely used noninvasive urinary biomarker of

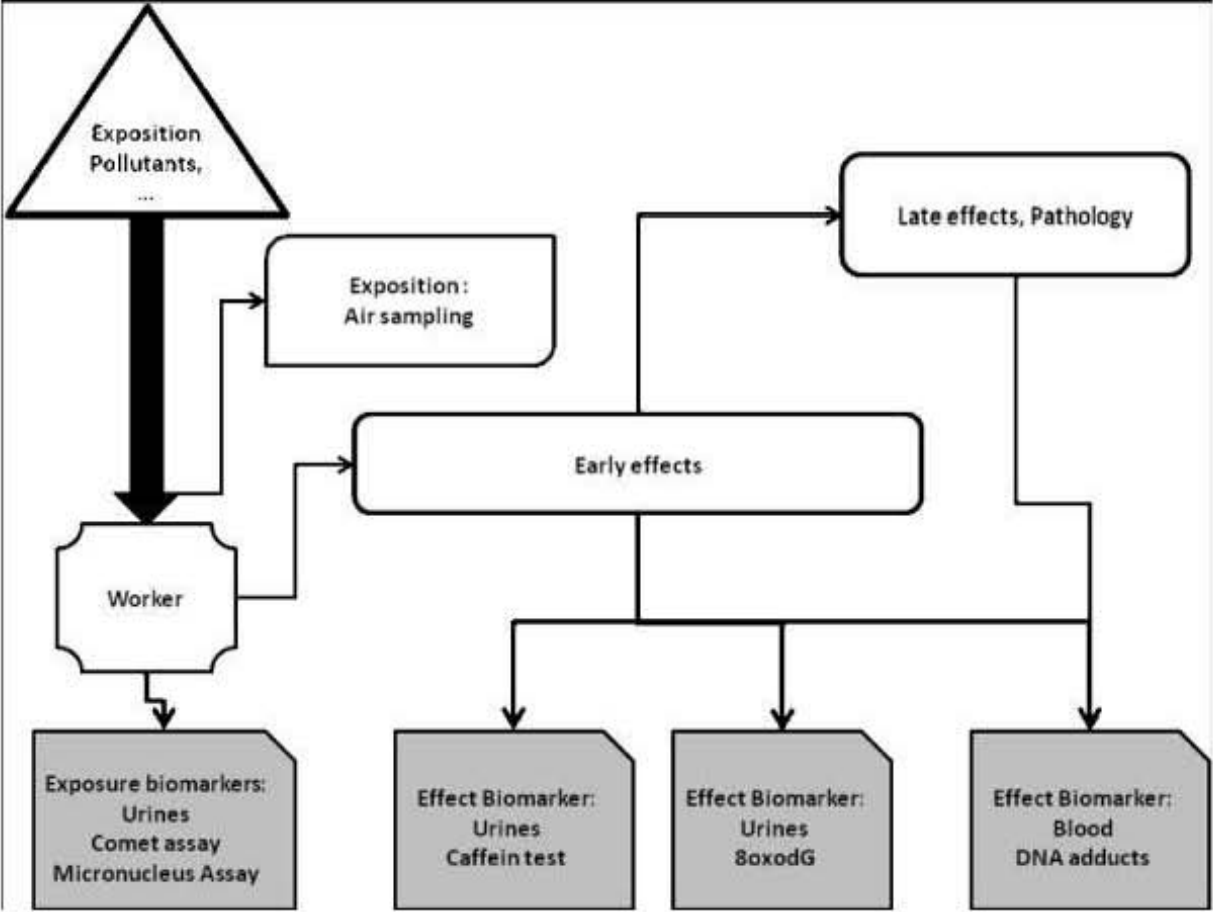
oxidative stress and its measurement in urine has been proposed to assess whole-body oxidative DNA damage [41,42].

The CYP1A2 was shown to be responsible for the 3-demethylation of caffeine, which is the initial major step in the biotransformation of caffeine in human's body [43]. Urinary metabolites of dietary caffeine are the most noninvasively-used method in the assessment of CYP1A2 activity [44-46]. Recent studies have demonstrated that the polymorphism of CYP1A2 could be critical in investigating the induction of the enzyme [47]. The -163C>A (allele CYP1A2\*F) polymorphism has been associated with higher enzyme inducibility by smoking [48]. Even if the clinical relevance of this polymorphism remains controversial [47], it is necessary to assess it for a good interpretation of the caffeine metabolism data.

This project is interested in an association of simple, early and non-invasive biomarkers intended to highlight exposure to cocktails of undefined toxic substances having genotoxic properties. We propose to carry out a cross sectional study comparing a particularly exposed category of workers to multiple professional pollutants (Parisians sewage workers) with a non-exposed professional category workers (municipality office workers) by using biomarkers of exposure and early biological effects. The biomarkers dedicated for this study are; the comet and micronucleus tests, which seek the presence of genotoxics in the urine. A second group of biomarkers highlights early effects of these substances; the caffeine test, relevant in the event of exposure to PAHs [49], DNA-adducts in the lymphocytes, a biomarker of early effect indicating the exposure to genotoxics, and urinary 8-oxodG, a biomarker of early effect, corresponding to DNA oxidative stress [50]. Our primary objective is to study if the exposed present an increased risk of genotoxic lesions, compared to the nonexposed. The secondary objective is to evaluate the early effects of an exposure to complex genotoxic agents. To achieve these objectives we will; (1) analyze the urine for DNA damage and genotoxicity (using in vitro comet and micronucleus assays and analysis of oxidative stress through 24 h urinary 8-oxodG), (2) analyze peripheral blood lymphocytes for DNA-adducts by <sup>32</sup>P-post labelling technique, (3) assess personal exposure to PAHs and VOCs in the workplace environment, and (4) evaluate the PAHs exposure through assessment of CYP1A2 activity by urinary metabolites of dietary caffeine.

The study hypothesis is that exposure of the sewage workers to multiple genotoxics leads to an increase in certain biomarkers of exposure and other biomarkers of early biological effects. The validation of our hypothesis through these biomarkers, would allow the estimation of the total personal exposure to complex mixture of toxic chemicals from different exposure pathways (lungs, skin, and GIT), and different sources (air, diet, lifestyle or occupation),

whereas the traditional epidemiological studies don't. Figure 1 presents the theoretical-overview of development from exposure to disease and the study assessment biomarkers.



**Figure 1:** Schematic diagram of progression from exposure to disease and the study assessment tools and biomarkers.

**Methods/Design**

**Study design, population and setting**

This cross sectional study will compare an exposed population (under-ground sewage workers) to a control group (office workers). Both groups are from Paris municipality workers and selected among occupational categories with similar socio-economic status. Participation will be voluntary. Subjects will be current nonsmokers since at least 6 months, aged (20–60) years old, being employed during at least the same period, have no history of chronic or recent illness (diabetes, influenza for example) and are not taking any medication (omeprazole for instance) that could interfere with the study results. As sewage workers are mostly males, the study population will be only of males. The study will be conducted in the framework of



regular occupational medical visits. All interviews and primary procedures will be taken place at the offices of occupational medicine in Paris city.

### **Ethical consideration**

The study protocol was approved by the local ethical committees (CPP, N°2007-A00685-48). All participants will be given an explanation of the nature of the study, and a signed informed consent will be obtained.

### **Sampling chronology**

Table 1 shows the weekly sampling chronology of the study participants. Briefly, controls will be frequency-matched for age with sewage workers with a 1 to 1 ratio. After at least three consecutive days of work a 24h urinary sample will be collected from all participants (starting at 9:00 a.m). Subjects will receive a urine collecting bottle and written/oral information describing urine collection. After given their urine samples at 9:00 a.m of the next day (Friday), the exposed will undergo medical examination by occupational health physicians. Blood samples will then be taken by nurses. Thereafter, they will receive a cup of decaffeinated coffee added with 110 mg of caffeine. Three hours later, a urine sample will be taken, from which three aliquots (200 µl each) will be collected to assess the urinary caffeine metabolites and the corresponding CYP1A2 activity. During these 3 hours, subjects will fill in two self-administered questionnaires under the supervision of study researchers. A professional one covering socio-demographic factors, non-occupationally exposures (especially PAHs-related: commuting means, area of residence and indoor sources), medical history, lifestyle (smoking history including passive smoking exposure, alcohol and medications) and other confounders. The other is an alimentary questionnaire collecting detailed-data on diet habits [51]. For the control group, the sequence will be the same but start at 13:30 p.m.

Blood and urine samples will be processed on the same day as described further. For the 48 h before and during the 3 hours of the caffeine test, subjects will be asked to avoid diets or cooking procedures known to increase CYP1A2 activity or elicit urinary mutagenicity (e.g., cruciferous vegetables; charcoal-broiled or grilled meat) or inhibit CYP1A2 (e.g., grapefruit). They will also be asked to refrain from consuming alcoholic drinks and beverages containing methylxanthines and to avoid massive physical activity as it could increase DNA damage [52,53]. In table 1 we present the morning sampling chronology for the exposed participants.

### **Occupational atmospheric sampling**

During the first part of the week, before coming to the medical examination, the air of the working places will be collected to be assessed for their COV or HAP content.

**Table 1:** Morning sampling chronology of study participants.

*9h00	9h30	10h00-12h00	12h30	13h00	13h30
24h urine collection					
Pre-treatment of 24h urine samples					
	Cup of Coffee				
Medical visit					
Blood sample					
Isolation and pre-treatment of lymphocytes					
Biological tests (urea, creatinine)					
Questionnaires					
			3h urine collection		
				Pre-treatment of 3h urine samples	
			Thanks	Departure	

\*Friday of each week. In each study week, 16 participants will be sampled (8 exposed in the morning and 8 non-exposed in the corresponding afternoon).

### Sample size

Sample size was calculated for a type 2 error ( $\alpha$ ) of 5% and power expectation of 80%. In a non-exposed population, urines are not mutagenic in theory, and both genotoxicity tests should be negative. Thus, if the expected prevalence in the control is 1%, a number of 75 subjects in each group are sufficient to highlight a prevalence of the anomalies of 17% in the exposed. The number of subjects necessary for DNA-adducts study is similar. For urinary 8-oxodG, the expected value in reference (control) population is nearly  $10.78 \pm 6.6$  (Mean  $\pm$  SD) nmole/24h, [54]. Thus 75 subjects in each group are sufficient to detect 18% modification of this value. For CYP1A2 activity, the urinary “molar concentration ratio of 1, 7-dimethylurate plus paraxanthine over caffeine” measured in a reference population is  $5.6 \pm 1.5$  (Mean  $\pm$  SD) [55], so 75 subjects in each group allow to detect a modification of 13% of this ratio.

As describe above, 16 subjects (8 exposed and 8 non-exposed) will be sampled each week. This will result in 10 weeks of sampling procedures to complete data collection according to study sample size of 75 subjects in each group. Each corresponding afternoon, 8 non-exposed participants will follow a similar sampling procedure.

The Parisians sewage workers are nearly 400 individuals, recruitment will be comfortable, even after exclusion of smokers (approximately 45%) [7].

## **Experimental protocol and technique**

### ***Isolation of lymphocytes***

This will be carried out using Ficoll gradient centrifugation method of Bøyum [56] with few modifications according to the study conditions. Briefly, 25 ml of freshly obtained venous blood will be collected on anticoagulant (EDTA) and diluted with an equal volume of standard balanced salt solution and layered carefully over Ficoll-Paque Plus density gradient medium, without intermixing, in a centrifuge tube. After centrifuging at room temperature (400 g for 30–40 min), drawing of the upper layer by a clean Pasteur pipette will be done leaving the lymphocytes layer undisturbed at the interface. The upper layer which contains the plasma will be saved for later usual clinical chemistry tests. Using a clean Pasteur pipette the lymphocytes layer will be harvested from the interface and transferred to a clean centrifuge tube. Then it will be centrifuged twice (60–100 g for 10 min at 18–20°C) in a balanced salt solution to wash the lymphocytes and remove any remnants of platelets. The lymphocytes will be suspended with 10% DMSO, coded, and frozen at -80°C until extraction of DNA.

### ***Extraction of DNA***

Frozen lymphocytes suspensions will be thawed in a 37°C water bath with gentle agitation. DNA extraction will be carried out using a standard phenol-chloroform method including treatment with RNAses as described elsewhere [57]. DNA purity will be checked by determination of UV spectra between 228 and 300 nm (associated with ratio values:  $1.8 < A_{260}/A_{280} < 1.95$  and  $A_{260}/A_{230} > 2.3$ ) and the DNA concentration will be deduced from the  $A_{260}$ , as described [58]. DNA solutions will be divided into three portions and frozen at -80°C in glass vials.

### ***Polymerase chain reaction “PCR” analysis of the CYP1A2***

The polymorphism of CYP1A2 will be assessed by real-time polymerase chain reaction and melting curve analysis, as described by Casley and LeBlanc-Westwood [59]. Reactions will be carried out in 20 µL volumes containing 3.5 mM MgCl<sub>2</sub> and 50 pg genomic DNA, using Fast Start DNA master mix for hybridization probes from Roche Diagnostics. All conditions will be adapted from Casley and LeBlanc-Westwood [59].

### ***Analysis of DNA-adducts***

DNA-adducts will be analyzed by <sup>32</sup>P-postlabelling assay as described [60,61], using Nuclease P1 for enrichment, with modifications from le Goff [57]. Briefly, 5 µg of DNA will be digested, then µCi γ-<sup>32</sup>P-ATP. Separation will be achieved on thin layer chromatography. Autoradiograms will be obtained after exposure of Kodak Biomax film to the TLC-plates. Each sample will be analyzed two times and in at least two different experiments. The

detection limit will be fixed at  $0.02 \times 10^{-10}$ , i.e. half of the lowest quantifiable Relative Adduct Level (RAL) value. For qualitative analysis, the mean number of adducts per individual will be calculated.

#### ***Pre-treatment of urine samples***

The volume of the 24h urine collected in sterile plastic urine collection bottles will be measured immediately and expressed per subject and body weight. Then, three 10 ml aliquots will be coded and frozen at  $-20^{\circ}\text{C}$  for 8-oxodG analysis. Another up to 100 ml aliquots will be coded and frozen at  $-20^{\circ}\text{C}$  for organic extraction and genotoxicity tests. Both samples will be transferred to the laboratory of analysis (within the same day). The concentration of 8-oxodG in urine stored at  $-20^{\circ}\text{C}$  was shown to be constant for at least 3 years [62].

#### ***Measurement of 8-oxodG concentration in 24h urine***

This will be done as described [63]. Briefly, frozen urine samples will be thawed at  $37^{\circ}\text{C}$  for 25 min, mixed and cooled to room temperature. HPLC separation will be performed on a C18 HPLC column ( $150 \times 2$  mm,  $5 \mu$ ) protected by a C18 guard column ( $10 \times 2$  mm,  $5 \mu$ ). The mobile phase for urine samples will be 10 mM ammonium formate, adjusted to pH 3.75 with formic acid and 2% acetonitrile. Electrospray will be performed in the positive ion mode. A stable isotopically marked internal standard of 8-oxodG will be used ( $[^{15}\text{N}_5]$  8-oxodG) (for details, see reference 63).

#### ***Urine organic extracts***

This will be carried on Sep-Pak C18 cartridges (Waters Associates, Inc) adsorption chromatography as described [64] with some modifications. Briefly, frozen urine samples will be thawed at room temperature and filtered through Whatman filter paper No. 1. Then it will be adjusted to pH 7 using 0.1 M NaOH. The cartridge will firstly be washed 3 times with 3 ml of absolute methanol and 3 ml of ultra-pure water successively before preparation of the columns. Then, the cartridge will be loaded with urine using a glass powder funnel on the column to facilitate the loading process. All operations will be at room temperature. The column will then be washed 3 times with 10 ml distilled water in order to eliminate the residual urine and histidine. The adsorbed components will then be eluted with methanol (5 ml/100 ml urine) into glass test tube. The eluate will be dried at  $40^{\circ}\text{C}$  under a nitrogen stream until complete dryness. Then, the residue will be dissolved in DMSO (0.4 ml/100 ml urine) and stored at  $-20^{\circ}\text{C}$  until analysis of genotoxicity tests.

### ***Cell culture***

For the two tests (comet and micronucleus) two cell lines will be used. HeLa S3 cellular line cells will be used (ECACC, catalog number 87110901, adherent cells of human cervical carcinoma). Hep G2 is a perpetual adherent cell line which was derived from the liver tissue of a 15 year old Caucasian male with a well differentiated hepatocellular carcinoma (ATCC, catalog number HB-8065).

### ***Comet assay “Single Cell Gel Electrophoresis” (SCGE)***

The urine extracts kept at  $-20^{\circ}\text{C}$  will be thawed and warmed to room temperature shortly before the assay. Comet assay will be performed basically according to Sing et al. 1988 [65], with modifications according to Muller et al. 2000 [66]. Briefly, the cells will be incubated with the organic extract of urine (200  $\mu\text{l}$ ) during 24h (typical division duration of these cells). Viability of cells will be determined by trypan blue test. Microscopic slides will be precoated with 100  $\mu\text{l}$  of agarose (1%). The slides will be gently immersed in ice-cold freshly lysis solution and will be covered with fresh electrophoresis buffer for 20 min and placed in a horizontal electrophoresis unit tank filled with new fresh electrophoresis buffer. After electrophoresis, they will be washed with a freshly made neutralizing buffer and stained with 50  $\mu\text{l}$  ethidium bromide solution. They will then be examined for analysis of DNA migration under a fluorescence microscope (Olympus BX-40, Olympus, Japan) using a computerized image analysis system (Komet 5, Kinetic Imaging). Two slides will be analyzed for each sample with fifty cells scored in each slide. Olive tail moment will be used for analysis of results [67].

### ***Micronucleus assay***

It will be performed according to the standard protocol of the International Workshops on Genotoxicity Test Procedures [17,68,69]. Briefly, after the initial screening; well-prepared slides will be scored using a high power magnification (400–1000 folds) with both bright field and phase-contrast microscope. Frequency of micronucleated cells will be evaluated by the number of cells containing one or more micronuclei (but less than 5). The induction factor will be calculated by dividing treated values by the control ones. Chi-square will be used for the comparisons and when P value is  $< 0.05$  the concentration will be considered positive.

### ***Assessment of CYP1A2 activity by urinary caffeine metabolites***

Subjects will be instructed to empty their bladder. Then they will receive a cup of decaffeinated coffee added with 110 mg caffeine. Three hours later, a urine sample will be collected and transferred to tube with 1 ml HCl, pH 3.5. Samples will be coded and frozen at  $-20^{\circ}\text{C}$  and then transferred to the laboratory of analysis (within the same day), where it will be

stored at -20°C until HPLC analysis. Caffeine and its metabolites will be extracted as described [55]. Briefly, the concentrated residue will be dissolved in 800 µl of 0.05% acetic acid and filtered through a 0.45-µm filter. Here, 100 µl of the filtrate will be injected into HPLC column. Caffeine and its metabolites will be analyzed using an HPLC system as described elsewhere [70]. The metabolites will be identified and quantified by UV detector with a computerized photodiode array detector as compared with definite standards [1,7-dimethylurate (17 U), 1,7-dimethylxantine (17X), and 1,3,7-trimethylxanthine (137X)]. To assess CYP1A2 activity, urinary molar concentration ratio index [17U+ 17X/137X] will be used as it reflects caffeine 3-demethylation activity in this phenotyping procedure [71,72].

### ***Occupational air sampling***

The targeted indicators will be VOCs and PAHs. They have been selected because they are present in the confined environments of the sewers while also emitted by automobile traffic, hence present in ambient and indoor atmospheres, and because they are of health significance. Since the sewage system is deprived of electricity, air sampling will be carried out using battery-powered devices or passive samplers. The sampling procedure will strive at evaluating exposure near the breathing zone. However, not to disturb the sewage workers, measurements will be done by a companion worker (or a study personnel) who will accompany each studied team and carry the sampling equipment in a back bag. For the reference population (office workers) the same type of sampling materials will be placed in a bag located in the working area, for example on a desk.

### ***Measurement of Volatile Organic Compounds (VOCs)***

Collection of VOCs will be carried out on thermal desorption sorbent tubes exposed during the sewage workers worktime from Monday to Thursday and the sampler is recapped after every exposure. Analysis will be carried out by coupling gas chromatography and mass spectrometry. As work in sewage system takes place in a wet environment, a sorbent tube not very sensitive to moisture will be chosen. The list of the selected indicators will be at least the substances measured inside residences within the framework of the national inventory carried out by the Observatory of Indoor Air Quality (OIAQ) in more than 560 French residences. These data were published in November 2006 [73] and can be used as reference values. It is probable that most of compounds found in the residences are also present in the air of the offices. The basic list is as follows:

- Alkanes: decane, undecane
- Monocyclic aromatic hydrocarbons: benzene, toluene, meta and para-xylenes, orthoxylene, 1, 2, 4-trimethylbenzene, styrene

- Chlorinated hydrocarbons: trichloroethylene, tetrachloroethylene, 1–4-dichlorobenzene.

The tubes used for sampling will be analyzed at the Paris city hygiene laboratory on a chain including a thermal desorption module on line with a chromatograph in gas phase equipped with a capillary column and coupled to a mass spectrometer (Quadripole). Quantitative analysis will be carried out on the basis of ion extracts and a range of calibration prepared by doping a lot of sorbent tubes with various quantities of a mixture of the selected VOCs. The first samples (2 to 3) will be devoted to the qualitative analysis (screening) of the chromatographic profiles with the aim to adjust the list of targeted compounds.

### ***Measurement of Polycyclic Aromatic Hydrocarbons***

They will be collected with personal air samplers allowing the simultaneously trapping of the volatile and the particulate phases (in case of heavy loss of charge due to high charged XAD2 resin, it will be necessary to carry out two distinct samples, one collecting only gaseous PAHs, the second one for particulate PAHs). The head of the samplers will consist of a cassette containing a filter, to collect particles coupled to a marketed tube filled with XAD2 resin or polyurethane foam. Air will be drawn using a constant flow sampling pump at a calibrated flow-rate of 2 L/min.

Duration of sampling will be at least equal to the daily worktime. However, four consecutive days of cumulative sampling may be necessary because concentrations are expected to be low. In this case, filter will be preserved in an aluminum sheet to avoid photochemical transformations. A first series of measurements will allow determination of the minimal duration of exposure for an acceptable quantification limit.

Thirteen PAHs will be measured: phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(j)fluoranthene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, benzo(g,h,i) perylene, indeno-pyrene. PAHs will be extracted at the laboratory by a solvent in a pressurized cell, so the extracts will be concentrated in an automatic evaporator. They will be analyzed by high HPLC associated with fluorimetric detection. A binary elution gradient consisting of water and acetonitrile will be used to separate the different PAHs. Several couples of wavelengths of excitation and emission will be selected to optimize the sensitivity of the response of the compounds and to limit the chromatographic interferences. The quantitative analysis will be carried out according to the response of standard solutions that are prepared from a marketed mixture of the selected PAHs.

## **Statistical analysis**

For each parameter, data will be compared between the exposed and non-exposed groups. Data will be tested for homogeneity of variance and normality after variable transformation if appropriate. Two-tailed Student's t-test will be used for group and/or sample comparisons relative to DNA-adducts level. Fisher's exact test will also be used for comparison of DNA-adducts pattern distributions between groups. Linear regression analysis will be used for quantitative variables, adjusting for parameters that reflect exposures in the questionnaires. For other parameters, ANOVA will be performed. Correlation analysis of DNA-adducts levels and urine genotoxicity with qualitative parameters will be evaluated by Spearman tests. Potential confounding factors, like age, socioeconomic or passive smoking will be studied, mainly by evaluating their distribution in both groups and by looking for possible association with DNA-adducts levels, urine genotoxicity or caffeine metabolism tests. The influence of confounding factors will be determined by multiple logistic regressions after a check of normality (Kolmogorov Smirnov's test). The analysis will be processed by the statistical software SAS (SAS Inc., Version 8.02).

## **Discussion**

This study aims to investigate the carcinogenic risk associated with occupational exposure of sewage workers to complex chemical mixtures. While the comet assay can detect DNA repairable lesions or alkali-labile sites, micronucleus can detect fixed mutations that persist at least one mitotic cycle [74]. Positive results in the comet don't necessarily correspond to positive results in the micronucleus, especially when genotoxic exposure is small. Thus, the combination of both assays might be more accurate and reasonable. Urinary excretion of 8-oxodG is a repair product of oxidative DNA damage and under the usual steady conditions it reflects the general average risk of a promutagenic oxidative stress in DNA of all tissues and organs [75]. Further, DNA-adducts in peripheral lymphocytes is considered as a good biomarker when studying the early effect of genotoxic exposures in humans [76,77].

This study is limited by its cross sectional design where systematic differences between exposed and non-exposed could cause under or overestimation of the risk, as exposed subjects may be more motivated to participate than non-exposed. However, genotoxicity tests (urine and lymphocytes) are not likely to be affected by the subjects' interest to the study. Moreover, choosing office workers as a control group may alleviate sources of strong bias such as "healthy worker effect" and social class differences, as both groups belong to the same socioeconomic class. Further, urine genotoxicity is a short-term measure that reflects



exposure 24 to 72h before collection [78] and our blood samples will be taken at rest. Airborne assessment will assess exposure by inhalation only, thus possibly misclassifying exposure both quantitatively and qualitatively. Using biomarkers of exposure and of early effects aims to overcome this shortcoming in view to assess the risk. Some difficulties might stem from the tiny amounts of promutagens in urine and the presence of urinary histidine that leads to false positive results. Filtration and concentration of urine might help to solve these problems [64]. Finally, day to day variability in laboratory procedures will be calculated and estimated by analysis.

To summarize, sewage workers are exposed to multiple chemicals from multiple pathways resulting in a complicated matrix of exposure to chemicals and concentrations. In this complex chemical exposure setting, this study combines biological sampling, both in blood and urine, to assess biomarkers of exposure and of early biological effects. These biological indicators will be scaled with results of workplace environment air sampling that will be conducted in parallel. Such biomarkers of exposure and of early biological effects may help overcome the severe limitations of environmental exposure assessment in very complex occupational or environmental settings. If shown discriminating in the framework of this study population, these non-specific biomarkers might be used to assess the genotoxic risk in other populations also experiencing complex exposures.

**Abbreviations**

CI–Confidence interval, CYP1A2–Cytochrome P1A2, DNA–Deoxyribo-nucleic Acid, EDTA–Ethylene-diamine-tetra-acetic acid, HCl–Hydrochloric acid, HPLC–High performance liquid chromatography, MgCL2–Magnesium chloride, MN–Micronucleus, PAHs–Polycyclic aromatic hydrocarbons, SMR–Standardized mortality rate, VOCs–Volatile organic compounds, 8-oxodG–8-Oxo-7, 8-Dihydro-2'-deoxy-Guanosine.

**Competing interests**

The author(s) declare that they have no competing interests.

**Authors' contributions**

HA drafted the manuscript. LF, DZN and CP participated in the design and coordination of the study protocol and helped to draft the manuscript. Other authors reviewed the manuscript, provided further contributions and suggestions. All authors read and approved the final manuscript.

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### 2.1.2. Comet article (2<sup>nd</sup> article)

As part of this thesis, our choice of the cellular line is of great importance in studying the *in vitro* genotoxicity assays. In this comet article, we aimed to compare the response of two model genotoxic compounds (B[a]P and H<sub>2</sub>O<sub>2</sub>) in two widely used human derived cell lines (Hep G2 and HeLa S3), in order to assess whether the choice of the cell line depends upon the mechanism of action of the toxicants under study. Further aim of this study was to choose the cell line that could better represent our investigations on the organic urine extracts of the studied subjects.

Hep G2 is a differentiated hepatoma cells that have retained certain activities of various phase I and phase II enzymes, while HeLa S3 is unspecialized epithelial cells that are believed to lack many metabolic enzymes. We found that Hep G2 cell line could be a more convenient human *in vitro* model to study genotoxicity in comet assay for promutagens (e.g. B[a]P) than HeLa S3, which could be more convenient model for directly-acting genotoxicants (e.g. H<sub>2</sub>O<sub>2</sub>). The comet article is submitted and it is presented in the following page as part of this thesis.



## **Arguments for a choice between HepG2 or HeLa S3 human-derived cell lines for a comet assay\***

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## **Abstract**

Comet assay represents a basic tool in assessing the genotoxicity of a compound. Among the quality criteria for the test and its interpretation, the choice of the cell line is of particular importance. In this study we compare two different cell lines, HepG2 and HeLa S3. HepG2 is a metabolically active cell line, while HeLa S3 has a very low capacity to transform xenobiotics. One hypothesis is that a metabolically competent cell line is interesting to detect a pro-mutagenic compound, while it is of less interest for a direct genotoxic agent. In this work we provide data for the choice of the cell line, according to the compound tested, here Benzo[a]Pyrene (B[a]P) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Concentration ranges (5-60 μM) B[a]P and (5-20 μM) H<sub>2</sub>O<sub>2</sub> were investigated on HepG2 and HeLa S3. The DNA migration was examined by evaluating the percent of DNA tail. Both cell lines showed statistically significant concentration-dependent increase in DNA damage after B[a]P and H<sub>2</sub>O<sub>2</sub> treatments compared to negative controls DMSO 1% (Dimethyl sulphoxide) for B[a]P and MEM (Minimum essential medium) for H<sub>2</sub>O<sub>2</sub>. HepG2 exhibits greater response and sensitivity towards B[a]P induced DNA damage than HeLa S3. However, HeLa S3 was more susceptible to oxidative DNA damage by H<sub>2</sub>O<sub>2</sub> than the more differentiated cell line Hep-G2. HepG2 cell line could be a more convenient human *in vitro* model to study genotoxicity in comet assay for promutagens than HeLa S3, which could be more convenient for directly acting genotoxicants. Thus, the most suitable cell line should be chosen according to the toxic mode of action, to better design and interpret the results of a comet assay.

**Key words:** Benzo[a]pyrene; Comet assay; HeLa S3; HepG2; Hydrogen peroxide.

## 1. Introduction

Comet assay or single-cell gel electrophoresis (SCGE) is a frequently used genotoxicity screening test [Tice et al., 2000]. The test is rapid, easy to apply, requires small amounts of the tested substance, sensitive and can detect low levels of DNA damage in individual cells. Depending on the experimental conditions, this technique allows the detection of single and double DNA strand's breaks, oxidative DNA damage, alkali-labile sites and DNA cross-links. This assay developed by Singh et al. [Singh et al., 1988 ] represents also a basic tool in research areas ranging from human and environmental biomonitoring to DNA repair processes and genetic toxicology [Kassie et al., 2000].

The international workgroup on genotoxicity testing pointed the need to develop adequate historical controls by conducting appropriately-designed multi-laboratory validation studies, in order to demonstrate the stability of the negative/positive control data [Burlinson et al., 2007]. To date, many different protocols have been used and the variations encompass use of different cell types with different model compounds, exposure regimes, use of DNA repair inhibitors, lysis, and electrophoresis techniques and scoring criteria [Burlinson et al., 2007]. In addition, different parameters of measurement and statistical analysis with various techniques were reported. Among these discrepancies, the choice of the appropriate cell type that must be used is of great importance.

In this framework, the present work provides background data for the choice of the cell line in comet assay in the response of two widely used human derived cell lines (HepG2 and HeLa S3). Here, data from B[a]P and H<sub>2</sub>O<sub>2</sub> are presented. These two compounds are commonly used as positive controls in genotoxicity studies. These two cell lines exhibit different levels of enzyme activities, DNA repair capacity, and degrees of specialization. HepG2 was isolated from a hepatoblastoma of an 11-year old Argentinean boy [Aden, et al., 1979]. It is a differentiated hepatoma cells that have retained certain activities of various phase I and phase II enzymes [Knasmüller et al., 2004; Rueff et al., 1996] that play a key role in the activation and detoxification of various promutagens/procarcinogens [Park et al., 2006; Uhl et al., 2000]. This cell line is among one of the preferred human *in vitro* models to study genotoxicity and DNA damage [Wilkening et al., 2003]. HeLa S3 is unspecialized epithelial cells that are believed to lack many metabolic enzymes [Duthie and Collins, 1997].

Two model compounds, B[a]P and H<sub>2</sub>O<sub>2</sub>, were tested. They cause genotoxicity and produce different spectra of DNA lesions by different mechanisms. B[a]P is a promutagen that needs metabolizing enzymes for its bioactivation. It is the best known and characterized genotoxicant among the polycyclic aromatic hydrocarbons (PAHs) family, and a

widely-spread environmental pollutant as a consequence of its formation during incomplete combustion processes of organic materials [ATSDR, 2009]. Its carcinogenic and mutagenic effects have been extensively investigated in mammalian and other animal cell systems [Pei et al., 1999]. It can be absorbed by human body and metabolized via Cytochrome P4501A1, producing the ultimate carcinogen B[a]P-diol-epoxide that binds to DNA, forming bulky-adducts that induce GC to TA transversions [Moody et al., 1995; Sims et al., 1974]. On the other hand, it is believed that during the metabolic activation process, B[a]P produces reactive oxygen species (ROS). These metabolites and ROS can cause oxidative DNA damage, starting the mutagenic chain of events responsible for cancer initiation [Burczynski and Penning, 2000].

In turn, H<sub>2</sub>O<sub>2</sub> is an oxidizing agent that represents a highly reactive and genotoxic form of oxygen and it might be involved in several human diseases such as aging and various degenerative conditions including cancer [Ames, 1989]. In living cells, it can oxidize critical target molecules generating DNA damage by producing single/double strand breaks, and/or alkali label sites [Joenje, 1989]. It is believed that H<sub>2</sub>O<sub>2</sub> causes DNA strand breaks mainly after conversion to hydroxyl radical via the Fenton reaction [Halliwell and Aruoma, 1991]. The reactive oxygen species generated from H<sub>2</sub>O<sub>2</sub> can react with most cellular components, they are also genotoxic because they react with both the deoxyribose and bases in DNA, and so generate base lesions and strand breaks.

The aim of this work is to compare the response of these two genotoxic agents in HepG2 and HeLa S3 cells, in order to assess whether the choice of the cell line depends upon the mechanism of action of the toxicants under study.

## **2. Materials and methods**

### *2.1. Chemicals and media*

Unless otherwise specified, all chemicals and culture medium used for cells cultivation and B[a]P (CAS No. B-1760), were purchased from Sigma-Aldrich Chimie S.A.R.L (L'Isle d'Abeau Chesnes, France). H<sub>2</sub>O<sub>2</sub> 3% (v/v) solution was obtained from Gilbert laboratories (H rouville, France).

### *2.2. Cells culture*

HepG2 and HeLa S3 cells were routinely cultured in the laboratory in monolayers. HepG2 were maintained in EMEM (Eagle's minimum essential medium, 9.6g/L) supplemented with 10% FBS (Fetal bovine serum) and 1% antibiotics solution (penicillin 10000U/ml; streptomycin 10mg/ml), sodium carbonate (2.2g/L), Hepes (5.96g/L), and sodium pyruvate (0.11g/L). HeLa S3 cells were maintained in MEM Eagle supplemented as in HepG2 but with



5% FBS and without sodium pyruvate. The pH of cultured mediums was maintained between (7.2-7.4). Cells were grown at 37°C in a humidified 5% CO<sub>2</sub> and 95% air atmosphere.

### *2.3. Choice of positive and negative controls*

Stock solutions of B[a]P (10mM in DMSO) and H<sub>2</sub>O<sub>2</sub> (4.4mM in distilled water) were prepared and kept at -20°C. Immediately before use, B[a]P was diluted in DMSO with a final concentration adjusted to 1%. Five final concentrations of B[a]P were tested (5, 10, 20, 40, and 60 µM). DMSO 1% was used as negative control. For H<sub>2</sub>O<sub>2</sub>, three final concentrations were tested (5, 10, and 20 µM). MEM was used as negative control. The dose range for each compound was chosen based on prior trial experiments. Each concentration of each compound was assessed in triplicate on each cell line, i.e. three identical independent experiments per cell line and compound concentration.

### *2.4. Treatment*

HeLa S3 lacks metabolic activation enzymes and had shown a more rapid DNA repair capacity half life in comet assay than HepG2 [Duthie and Collins, 1997; Collins et al., 1995]. Consequently, the sensitivity of the test might be poor [Kassie et al., 2000, Gutiérrez et al., 1998]. In preliminary experiments, testing HeLa S3 without a DNA repair inhibitor (aphidicoline), detected no genotoxicity and the data were inconclusive (not shown). To further enhance the sensitivity, aphidicoline was added to HeLa S3 cell cultures [Gedik et al., 1992]. HepG2 cells have low repair capacity and 24h exposure period was found to be optimal for comet formation regardless of the tested toxicant [Uhl et al., 1999; Sanyal et al., 1997].

Before the test compound treatment, subcultures for the experiments were prepared. HepG2 and HeLa S3 were passaged at confluence using trypsin-EDTA solution (0.5% trypsin, 0.2% EDTA; w:v) for HepG2, and EDTA 0.02% for HeLa S3 to 25cm<sup>2</sup> flasks (Greiner, A 12.078.019) at a density of 3x10<sup>6</sup> (HepG2) and 1x10<sup>6</sup> (HeLa S3) cell/flask. Attached cells were allowed to grow in 5ml complete medium until 80% confluence. The medium was then discarded and replaced by 5ml of freshly-diluted test medium. The medium included aphidicoline, 5µg/ml (CAS No.A-0781) for HeLa S3. Cells were incubated for 24h, then washed twice with PBS buffer (Phosphate buffered saline, pH 7.4) and harvested immediately by rinsing for 5 min with EDTA, for HeLa S3, and (5-7) min with trypsin-EDTA solution for HepG2. After inactivation of trypsin and EDTA by the culture medium, cells were centrifuged at 500x g for 5 min. Subsequently, the cell pellet was suspended in 3ml culture medium, counted, and then diluted to reach a solution of 5x10<sup>5</sup>cell/ml. Trypan blue exclusion test was used to assess toxicity, and cell viability was always ≥ 95%.

### *2.5. Slides preparation and comet assay*

Comet assay was performed essentially as in Singh et al. [Singh et al., 1988], with some modifications. Conventional microscope slides were dipped into a solution of 1% hot NMP agarose (Normal melting point agarose) and dried for 24h at ambient temperature in dark. In the test day, a second layer of 100 $\mu$ l NMP agarose was sprayed on the slides, covered with a coverslip and maintained at 4°C for 5 min. Then, 100 $\mu$ l of cell suspension was dissolved in 600 $\mu$ l LMP agarose (Low melting point agarose). From this, 100 $\mu$ l were rapidly layered onto the slides and covered with coverslip, maintained at 4°C for 5 min and then the coverslip was removed. Cells were lysed at 4°C in the dark for at least 1 h in a freshly made solution of 2.5 M NaCl, 100mM Na<sub>2</sub>EDTA, 10mM Tris and 1% Triton X-100 and 10% DMSO, pH 10. The slides were then immersed into an electrophoresis buffer (300mM NaOH, 1 mM Na<sub>2</sub>EDTA, pH 13) for 20 min to allow the unwinding of DNA and the expression of alkaline labile sites. Afterwards, the slides were placed side by side in a horizontal electrophoresis gel system (EC340, Maxicell<sup>®</sup> Primo, Holbrook, New York) filled with a fresh electrophoresis buffer and the electrophoresis was conducted for 20 min, 20 V, and 300 mA at room temperature. Each experimental replicate was done in one electrophoretic run. The slides were then gently washed for 5 min in a cold neutralizing buffer (0.4 M Tris, pH 7.5) in order to neutralize the alkali and were then stained with 40 $\mu$ l ethidium bromide (2 $\mu$ g/ml in Milli-Q water), covered with cover slips, dried and stored at 4C° in dark and low humidity environment until analysis in the next day. For each cell line and each tested compound, the independent experiments were separated by one week time interval. This procedure enabled us to compare the baseline DNA damage for each experiment.

### *2.6. Selection of comets and image analysis parameter used*

Slides were examined for DNA migration using an Olympus BX fluorescence microscope (Olympus, Tokyo, Japan) at a 40-fold magnification. Two slides per concentration and 25 cells per slide were randomly scored (total count is 50 cells). Quantitative analysis of DNA damage in individual cells was evaluated by a computerized image analysis system, Komet 4.02 software (Kinetic Imaging, UK). The gel was scanned in a systematic way and the comets represented the whole gel. Edges and areas around air bubbles were avoided. DNA damage was assessed by % DNA tail parameter (the fraction of DNA in the tail divided by the amount of DNA in the cell, multiplied by 100). This parameter has been shown to be the most linearly related to dose, the easiest to understand, and is relatively unaffected by the threshold settings [Tice et al., 2000; Burlinson et al., 2007; Kumaravel and Jha, 2006; Kirkland et al., 2007a]. It also gives a clear indication of what the comets actually look like [Collins, 2004].

Tail length tends to reach a maximum at a low level of damage, and tail moment (despite its wide usage) tells nothing about the appearance of the comets and is the least informative parameter [Collins, 2004; Olive et al., 1990]. Olive tail moment or extent tail moment may also be calculated differently by Image Analysis systems [Burlinson et al., 2007].

### 2.7. Statistical analysis

Data were analyzed by the non-parametric Kruskal-Wallis test to compare the three experiments, followed by a non-parametric multiple comparison test (Tukey test) if a significant difference was found. Subsequently, Mann-Whitney U test was used to analyze for differences between the control group and each treatment dose. It was also used to analyze the response of each cell line at the same concentration of the toxicants. Results are presented as means $\pm$ SEM; standard error of the mean. Three sets of 50 cells per concentration were evaluated. In order to facilitate comparison between the cells response, the mean response of the negative controls (DMSO or M.E.M) were considered as 100% and the other treatments means were normalized in respect to negative controls. Statistical significance was set at  $p < 0.05$ . All analyses were carried out with the SPSS software (Statistical Package for Social Science) version 12 [SPSS Inc, 2003].

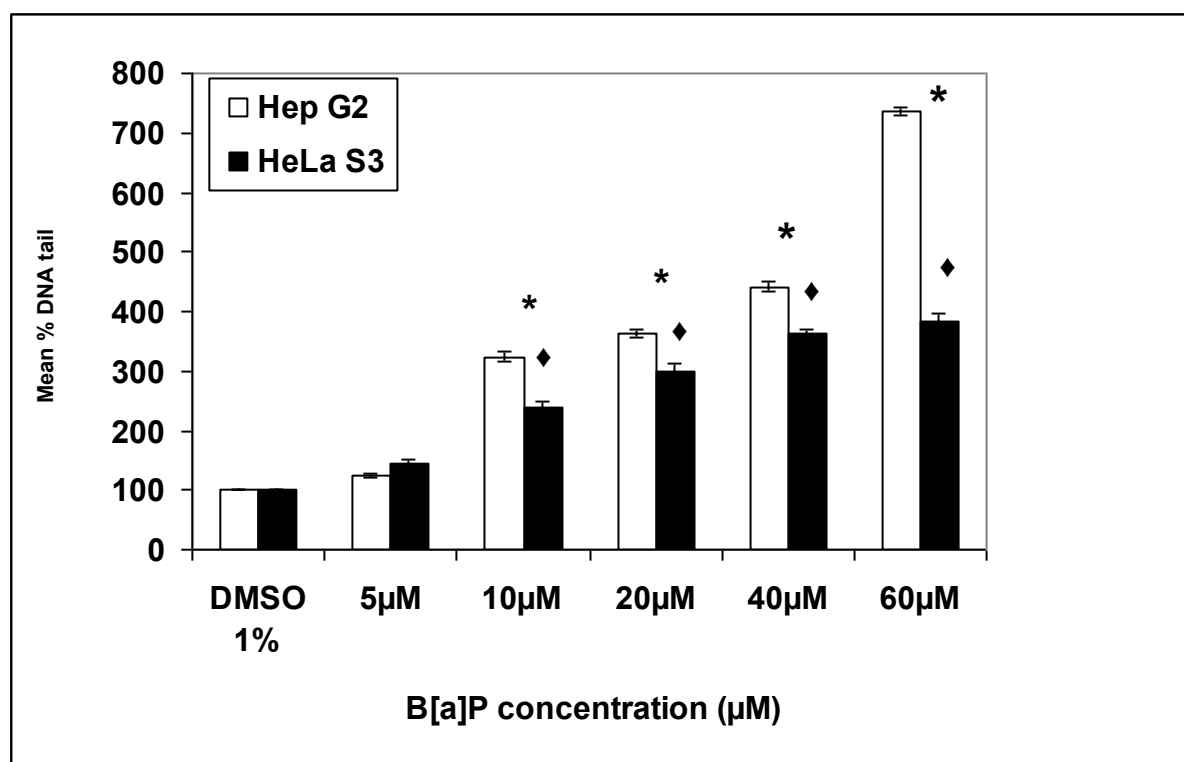
## 3. Results

In both HepG2 and HeLa S3, B[a]P and H<sub>2</sub>O<sub>2</sub> were tested during 24h exposure period in a non-toxic dose range of 5 to 60 $\mu$ M compared to 1% DMSO for B[a]P, and 5 to 20 $\mu$ M compared to MEM for H<sub>2</sub>O<sub>2</sub>. Three independent experiments for each compound were conducted. Cell viability was always  $\geq 95\%$  and % DNA tail parameter was evaluated. There was no statistical significant difference between any of the three identical experiments (data not shown).

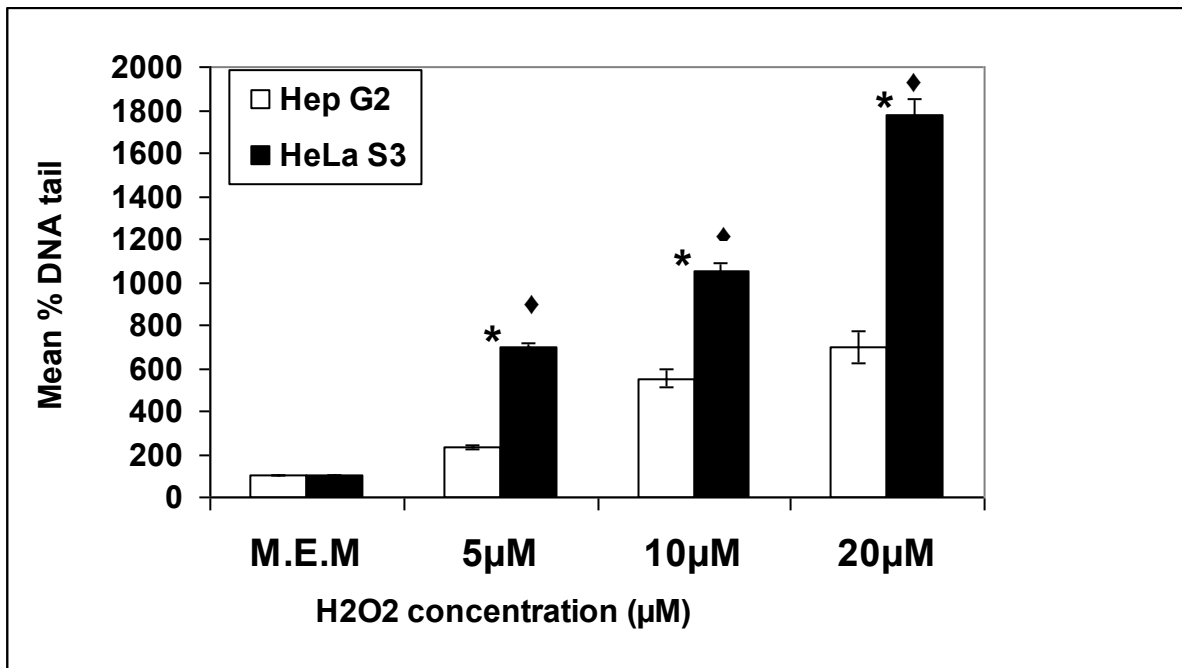
The average (mean $\pm$ SEM) % DNA tail parameters of each experimental point for each cell line are illustrated in Figs. 1 and 2, for B[a]P and H<sub>2</sub>O<sub>2</sub>; respectively. Both cell lines showed concentration-dependent increase in DNA damage after B[a]P and H<sub>2</sub>O<sub>2</sub> treatment. Statistically significant dose-response effects by all concentrations of B[a]P and H<sub>2</sub>O<sub>2</sub> were observed compared to negative controls (1% DMSO and M.E.M; respectively;  $p$  values  $< 0.05$ , U-test).

As observed from Figs. 1 and 2, once the % DNA tail response is normalized after DMSO and MEM negative controls (set to 100%) in both cell lines, the maximal means values obtained by the 60 $\mu$ M B[a]P were 736% and 384% in HepG2 and HeLa S3 respectively. The corresponding figures for 20 $\mu$ M H<sub>2</sub>O<sub>2</sub> were 697% and 1780% in HepG2 and HeLa S3, respectively. Clearly, the maximal mean values upon B[a]P maximum treatment were nearly

up to 7-folds higher than the control values (DMSO) in HepG2, and 4-folds higher for HeLa S3 cells. The contrasts for H<sub>2</sub>O<sub>2</sub> were up to 7-fold for HepG2 and 18-fold for HeLa S3 compared to control values (MEM). The DNA damage across HepG2 compare to HeLa S3 cells associated with H<sub>2</sub>O<sub>2</sub> was significantly differed ( $p < 0.001$ ) at each concentration. This observation held true for B[a]P ( $p < 0.001$ ) for all concentrations except for 5 $\mu$ M ( $p = 0.53$ ). These results demonstrate that HepG2 cells exhibit a greater response and sensitivity to B[a]P-induced DNA damage in the comet assay than HeLa S3 cells. Regarding H<sub>2</sub>O<sub>2</sub>, HeLa S3 was more susceptible to oxidative DNA damage than the more differentiated HepG2 cells. In the present study, cells viability after exposure was always  $\geq 95\%$ . The comet tail formation in HepG2 and HeLa S3 treated with B[a]P and H<sub>2</sub>O<sub>2</sub>, expressed different shapes and ranges. Short but dense tails were usually seen, with a few long and sparse tails. These differences between the DNA damage levels on different concentrations, and sometimes within the same tested dose, can be explained by the great variability and diversity in response from one cell to another which is a normal feature of comet assay [Collins, 2004].



**Figure 1:** DNA damage in HepG2 and HeLa S3 cells by B[a]P. Each bar indicates the mean $\pm$ SEM of each experimental point in three experiments (50 cells, N=150 in total). The % DNA tail in DMSO was 3.07 in HepG2 and 2.26 in HeLa S3, both were normalized to 100%. \*Significantly different from DMSO,  $p < 0.001$  for both cell lines. For 5 $\mu$ M,  $P = 0.011$  and  $0.002$  in HepG2 and HeLa S3; respectively. ♦ HeLa S3 Significantly different from HepG2,  $p < 0.001$ .



**Figure 2:** DNA damage in HepG2 and HeLa S3 cells by H<sub>2</sub>O<sub>2</sub>. Each value indicates the mean±SEM of each experimental point in three experiments (50 cells, N=150 in total). The % DNA tail in MEM was 1.71 in HepG2 and 1.09 in HeLa S3, both were normalized to 100%. \* Significantly different from MEM,  $p < 0.001$  for both cell lines. ♦ HeLa S3 Significantly different from HepG2,  $p < 0.001$ .

#### 4. Discussion

In the present study, the effect of various concentrations of B[a]P and H<sub>2</sub>O<sub>2</sub> were investigated in a metabolically competent cell line (HepG2) and a metabolically incompetent cell line HeLa S3, using *in vitro* comet assay. The main study finding is that, concentrations range of 5-60 µM of B[a]P and 5-20 µM of H<sub>2</sub>O<sub>2</sub> caused significant dose-dependent effects in both cell lines. The maximal response differences were nearly 2-fold higher in HepG2 than in HeLa S3 cells for B[a]P, and, conversely, 2.5-fold higher in HeLa S3 than in HepG2 cells for H<sub>2</sub>O<sub>2</sub>. These results showed that HepG2 cells are more susceptible towards B[a]P-induced oxidative DNA damage than HeLa S3, while the reverse is seen for H<sub>2</sub>O<sub>2</sub>.

B[a]P is a promutagen which needs further bioactivation before exhibiting its DNA genotoxicity [Tice et al., 2000]. H<sub>2</sub>O<sub>2</sub> is a direct genotoxicant that does not need bioactivation to exert its genotoxicity [Joenje, 1989]. B[a]P has induced DNA damage in the two cell lines after a 24h incubation, which suggests that this damage is due to B[a]P metabolites and that these cells have to a certain degree some metabolic activity. Our results show that the metabolic activity of HepG2 cells is sufficient to activate B[a]P into DNA reactive metabolites. In contrast, the weak response seen in HeLa S3 cells is likely due to its

low metabolic activity that is not sufficient to catalyze B[a]P into a large amount of reactive metabolites [Duthie and Collins, 1997].

H<sub>2</sub>O<sub>2</sub> is more genotoxic in HeLa S3 than in HepG2. This suggests that cellular defence against oxidative DNA damage is higher in HepG2 than HeLa S3. Antioxidant enzymes and other metabolizing enzymes decrease the concentrations of ROS and therefore the level of damage inflicted to DNA. Although weak, the in between cells" variability and permeability to H<sub>2</sub>O<sub>2</sub> and B[a]P cannot be ruled out as another source of heterogeneity in cellular response [Fairbairn et al., 1995].

In our study, a concentration range between 5 to 60µM for B[a]P and 5 to 20 µM for H<sub>2</sub>O<sub>2</sub> showed significant differences in comet formation compared with controls in both cells without addition of metabolic activation. Studies had shown that significant differences were seen using 10, 50, and 100µM of B[a]P compared to controls (0.1% DMSO) in HepG2 [Wilkening et al., 2003]. Heterocyclic aromatic amines such as 2-amino-3-methyl-imidazo[4,5-*f*]quinoline, that known to need metabolic activation to exert their mutagenicity, were shown to have positive results in HepG2 (doses  $\geq$  20 µM) and HeLa S3 (doses  $\geq$  60 µM) without addition of metabolically active enzymes [IARC, 1993; Knasmüller et al., 1999]. However, on HepG2 cells, Gábelová et al. [Gábelová et al., 2007] found that 7.5µM B[a]P for incubated with cells for 24h was sufficient to detect a significant difference from DMSO 0.5%. In the other hand, Uhl et al. [Uhl et al., 2000; Uhl et al., 1999] treated HepG2 with 150µM B[a]P for 24h showed about 5-fold increase in DNA migration compared to control and the lowest effective dose was 25µM (dose range 25-150µM). Yusuf et al. [Yusuf et al., 2000] reported a significant differences in a dose range of 1-100 µM of B[a]P on HepG2.

Although shorter periods (30 min) of exposure were examined by another study that compared the DNA damage between the two cellular lines in response to H<sub>2</sub>O<sub>2</sub> with concentrations range (0-100 µM), our results are in accordance with this study [Duthie and Collins, 1997]. Another study on HepG2, during a one hour exposure period, with various concentrations of H<sub>2</sub>O<sub>2</sub> (from 4.4 to 17.6 µM), found significant dose-response relationships compared to a negative control in comet assay [Mba Gachou et al., 1999]. Others reported that exposure of HepG2 to 50µM H<sub>2</sub>O<sub>2</sub> during 30 min at 37 C° resulted in a significant DNA damage [Aherne and O'Brien, 1999].

As HeLa S3 show a rapid DNA repair capacity, 50µM H<sub>2</sub>O<sub>2</sub> concentration exhibited a significant DNA repair response during the first 12h of cell culture [Ramana et al., 1998]. Another study carried out on human lymphocytes showed almost complete repair after

25-100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  during the first 16h of culture. However, DNA damage was shown to persist to a large extent when cells were cultured in the presence of repair inhibitor [Andreoli et al., 1999]. The study also showed that DNA damage was almost undetectable after 16h or 48h incubation periods without DNA repair inhibitor. On the other hand,  $\text{H}_2\text{O}_2$  produced a significantly extensive DNA damage in treated cells analysed immediately after treatment, when the highest response was observed [Andreoli et al., 1999]. Thus, the onset of repair processes with HeLa S3 cells will attenuate comet formation as a function of exposure time unless a DNA repair suppressor like aphidicoline is added, as it was the case in our study. In HepG2 cells, however, the enzymes are continuously active, thus the possibility to get false results is low [Knasmüller et al., 2004; Mersch-Sundermann et al., 2004; Kirkland et al., 2007b].

In summary, to our knowledge, no study had yet compared the metabolically competent HepG2 and incompetent HeLa S3 cells using the promutagen B[a]P and the directly genotoxicant  $\text{H}_2\text{O}_2$ . Our results show that HepG2 cell line could be a more convenient human *in vitro* model to study genotoxicity in comet assay for promutagens than HeLa S3. The high metabolic capacity of this cell line, however, might restrain the range of response, due to the anti-oxidant action of the xenobiotic metabolising system. When no activation is required, a cell line with a low metabolic capacity might be more appropriate. In both cases, the DNA repair system should be inhibited. While these results need to be extended to other compounds, they point out the fact that suitability of the cell lines should be carefully assessed to better design and interpret the results of comet assay.

### **Abbreviations**

B[a]P, benzo[a]pyrene; E.M.E.M, eagle's minimum essential medium; FBS, fetal bovine serum;  $\text{H}_2\text{O}_2$ , hydrogen peroxide; LMP agarose, low melting point agarose; M.E.M, minimum essential medium; NMP agarose, normal melting point agarose; PAHs, polycyclic aromatic hydrocarbons; PBS, phosphate buffered saline; ROS, reactive oxygen species; SCGE, single cell gel electrophoresis; SEM, standard error of the mean.

### **Conflict of interest statement**

The author (s) declare that they have no competing interests.

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### 2.1.3. Validation of urine extraction protocol on comet assay

Before the analysis of the study samples, 50ml urine from a voluntary non-smoker person was taken and added with 30µM B[a]P final concentration, then after extraction, comet assay was applied as previously described. DMSO 1% (50µl) and urine extract (50µl) each in 5ml of culture medium and B[a]P 40µM (with a final DMSO concentration of 1%) as positive control were compared. As shown in table 1, statistical significant differences in changes between the DMSO negative control and the urine extract was seen ( $< 10^{-3}$ ).

**Table 1:** DNA damage in Hep G2 cells as expressed in % DNA tail by DMSO, urine extracts and B[a]P 40µM.

Treatment	% DNA tail		Differences between groups (M-WUT)*	
	Means±SEM	Median (range)	P value	Z-Score
DMSO 1% (negative control)	0.91±0.10	0.82 (0.05-2.63)	-----	
Urine extract (50µl)	2.37±0.20	1.88 (0.51-6.24)	$< 10^{-3}$	-6.1
B[a]P 40µM (positive control)	7.28±0.48	6.38 (1.56-15.15)	$< 10^{-3}$	-8.4

\* Mann-Whitney U Test (M-WUT) was used to analyse differences in changes between the DMSO 1% control group and the other groups (50 cells were analysed in each).

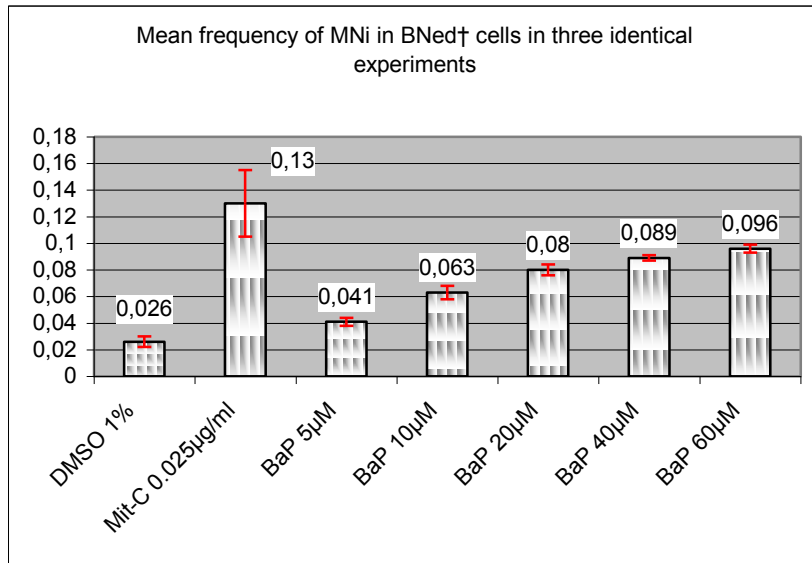
### 2.1.4. Validation of micronucleus assay on B[a]P using Hep G2 cells

B[a]P could serve as a potential biomarker for PAHs biomonitoring and risk assessment among occupationally exposed workers to such genotoxicants [197]. As we are applying the micronucleus assay on the urine organic extracts of the sewage workers; i.e. who are according to our hypothesis are exposed to PAHs, using B[a]P and its solvent DMSO as positive and negative controls respectively would be more appropriate than other chemicals as B[a]P is closely related to PAHs in its chemistry and mechanism of action.

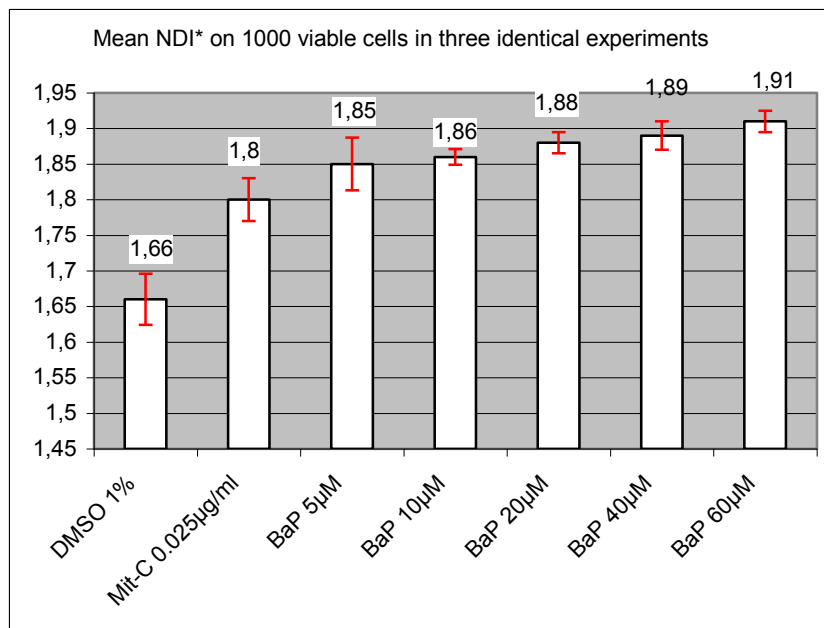
In consequence, to find out a validated data on the proper use of the positive and negative solvent control when applying the MN assay on Hep G2 cells, and to test if B[a]P could serve as a positive control and its solvent DMSO as negative one, three identical experiments were carried out using different range doses. Figures 3 and 4 represent the mean values of MNi (micronuclei) and NDI (nuclear division index); respectively, in three preliminary identical

experiments of the micronucleus assay using Hep G2 cells on Mitomycin 0.025 $\mu$ g/ml and 5-60  $\mu$ M range concentrations of B[a]P. In each experimental point, the total number of cells counted was 1000 cells with a range number of BNed cells between 500-728 cells among different treatments. As we could see from figures 3 and 4, DMSO 1% and B[a]P 40 $\mu$ M were judged to be proper negative and positive controls; respectively, as they showed no toxicity levels and permit a wide interpretations of the data. Furthermore, the same concentrations have been found to be proper and in accordance with the results we obtained from the comet assay (see comet article section).

As shown from figure 4, the average mean value of the NDI for the maximum dose of B[a]P did not differ more than 25% from that for the negative solvent control (DMSO 1%), which means no cytotoxicity has been reached. These data demonstrate that B[a]P 40  $\mu$ M and DMSO 1% could be used as positive and negative controls respectively when applying MN assay on Hep G2 human derived cell lines. Consequently, these measured data of controls were applied in our study. Detailed description of the data in each preliminary experiment could be found below in table 2.



**Figure 3:** Presentation of the mean frequency values of MNi in BNed cells in three identical experiments of micronucleus assay on Hep G2. Each bar represents Mean±SD of each point. †Frequency of MNi in BNed cells was calculated in a minimum of 500 cells.



**Figure 4:** Presentation of the mean NDI values on 1000 viable cells in three identical experiments of micronucleus assay on Hep G2. Each bar represents Mean±SD of each point. \*NDI =  $(M1 + 2M2 + 3M3 + 4M4) / N$ , where M1-M4 represent the number of cells with 1-4 nuclei and N is the total number of viable cells scored (excluding necrotic and apoptotic cells).

**Table 2: Three preliminary identical experiments of micronucleus test on Hep G2 cells with DMSO 1%, Mitomycin 0.025µg/ml and 5-60 µM range concentrations of B[a]P.**

Treatment	DMSO 1%	Mitomycin-C 0.025 µg /ml	B[a]P 5µM	B[a]P 10µM	B[a]P 20µM	B[a]P 40µM	B[a]P 60 µM
<b>First preliminary experiment</b>							
*M1+ 2M2+ 3M3+ 4M4	1672	1812	1836	1852	1876	1892	1900
BNed cells	536	568	728	648	696	648	692
MNi in BNed	12	92	32	44	56	60	68
*NDI on 1000 viable cells	1.67	1.81	1.83	1.85	1.87	1.89	1.90
Frequency of MNi in BNed	0.022	0.16	0.044	0.068	0.08	0.092	0.098
<b>Second preliminary experiment</b>							
*M1+ 2M2+ 3M3+ 4M4	1697	1770	1932	1879	1883	1884	1935
BNed cells	545	550	690	660	715	635	702
MNi in BNed	15	75	29	44	61	55	70
*NDI on 1000 viable cells	1.69	1.77	1.90	1.87	1.88	1.88	1.93
Frequency of MNi in BNed	0.027	0.13	0.042	0.066	0.085	0.087	0.099
<b>Third preliminary experiment</b>							
*M1+ 2M2+ 3M3+ 4M4	1624	1832	1843	1872	1902	1921	1924
BNed cells	526	539	562	623	687	726	684
MNi in BNed	16	60	21	36	53	64	63
*NDI on 1000 viable cells	1.62	1.83	1.84	1.87	1.90	1.92	1.92
Frequency of MNi in BNed	0.03	0.11	0.037	0.057	0.077	0.088	0.092

\*NDI = (M1+ 2M2+ 3M3+ 4M4) /N, where M1-M4 represent the number of cells with 1-4 nuclei and N is the total number of viable cells scored and = 1000 here (excluding necrotic and apoptotic cells). MNi, microneuclei; BNed, binucleated; NDI, nuclear division index.

### 2.1.5. Urinary caffeine metabolites and assessment of CYP1A2 activity

After extraction line (solid phase extraction (SPE)) of the analytes from the urine matrix, the different metabolites were separated and quantified by HPLC coupled with UV detection. Quantification was achieved using calibration curves made by extracting mixtures of metabolites control targets. An internal standard (acetaminophen) was introduced for all samples and controls used before extraction.

#### ❖ Equipments used:

- Pump: Spectra system P1000 XR (Thermo Separation Products).
- Extractor/injector: Aspeca XL (Gilson).
- Detector: Spectra series UV100 (Thermo Separation Products).
- HPLC-Columns: Column monolithic Onyx finish first (Phenomenex).

➤ Two columns in series were used (100 \* 3mm) with a guard cartridge of the same type.

➤ Extraction cartridges: Cartridge Oasis<sup>®</sup> HLB 1cc (30mg) (Waters).

❖ **Chromatographic conditions:**

➤ A mobile phase consisting of 0.1% acetic acid, methanol and ethanol in the following proportions: 96/2/2 (v / v / v).

➤ A loop-injection of 50µL

➤ Wavelength detection 280nm.

➤ A gradient-flow described as follows: 1ml/min during the first 4.5 minutes, 2.5 ml/min up to 16min, and a return of 1ml/min until the end of the chromatogram (20min).

❖ **Samples:**

Urine samples were acidified before extraction with hydrochloric acid and stored in the freezer. They were thawed immediately before use and were added with 16µL of internal standard (0.5 mM in 0.1% acetic acid) to 400µL samples.

❖ **Control solutions:**

The controls were made as follows: 200µL of original solution concentration chosen for each metabolite (40 - 400µM) + 200µL of acetic acid 0.1% + 16µL of paracetamol. They were then extracted identically to the samples and the standard ranges were integrated with the sets of analysis. Each line contains three points: a low point at 20µM, a moderate point at 100µM and a peak high point at 200µM.

❖ **Extraction:**

Samples were extracted before injection to avoid the impurities present in the urine matrix from damaging the column and disrupting the detection of the interested compounds. Most of the reported extractions were liquid/liquid extraction that require more time, non-automated work and products such as chloroform that laboratory use should be restricted to a minimum. Therefore we chose a method of solid phase extraction on oasis cartridges HLB. This extraction is effective and allows rapid automated analysis.

❖ **The extraction conditions were as follows:**

➤ Conditioning the cartridge with 2ml of methanol followed by 2ml of water at a rate of 3ml/min.

➤ Loading of the cartridge with 0.5ml of sample at a rate of 1ml/min then pushed by 1ml of air.

➤ Rinse the cartridge with 1ml of water pushed by 1ml of air.

➤ Elution with 0.5 ml of methanol.

- Dilution in 2ml of acetic acid.
- Vacuum/dispersion successive mixing and then injection into the loop of 50 $\mu$ L.

❖ **Sequence of injection:**

The samples were analyzed twice in a completely independent manner to allow statistical interpretation of results (results reported in terms of mean and standard deviation). Each sample was thus extracted and quantified in duplicate.

The extraction and separation were connected in series and the extractor injects the sample directly into the chromatographic column and then starts the extraction of the next sample during the passage of the first column in order to optimize to the maximum the time of analysis.

This procedure allows launching night sequences up to 33 injections (33x20min = 11 hours).

A sequence type is given below:

12 samples performed in duplicate (= 24 injections) + three range of three-point of concentration (beginning, middle and an end range) =33 injections.

The activity of CYP1A2 enzyme was determined by a formula that used the urinary concentrations of 3 compounds: caffeine (1,3,7- trimethylxanthine; 137X) and 2 of its metabolites; 1,7- dimethyluric acid (17U) and 1,7- dimethylxanthine (17X). The ratio selected for CYP1A2 activity is= (17U+17X)/137X.

The calibration lines were constructed for each compound from the standard lines of each injection sequence. Each sample was then determined by comparison with calibration lines that are passed in the same series of it.

An average calibration curve was constructed for each sequence taking into account of all the standard injections (for an example, see figure 5). A chromatogram type for a control 100 $\mu$ M and a sample is given in Figure 6 and 7; respectively.



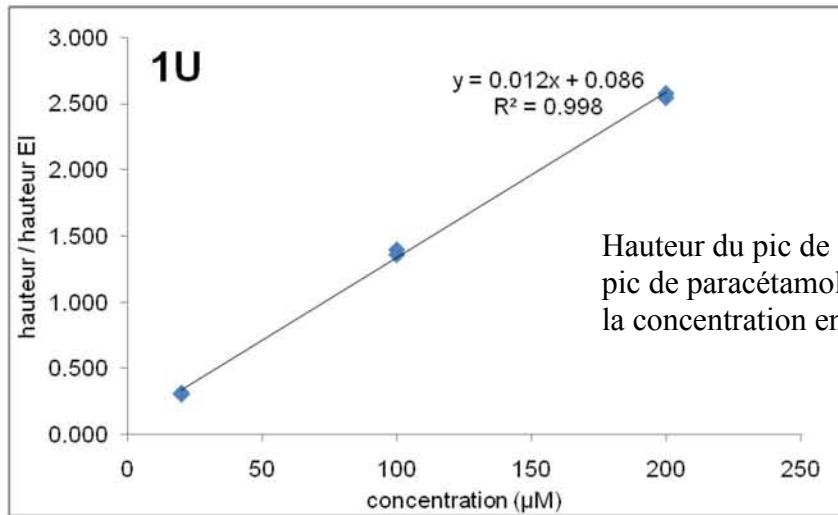


Figure 5: Example of a calibration curve for the compound (1-methyluric acid; IU).

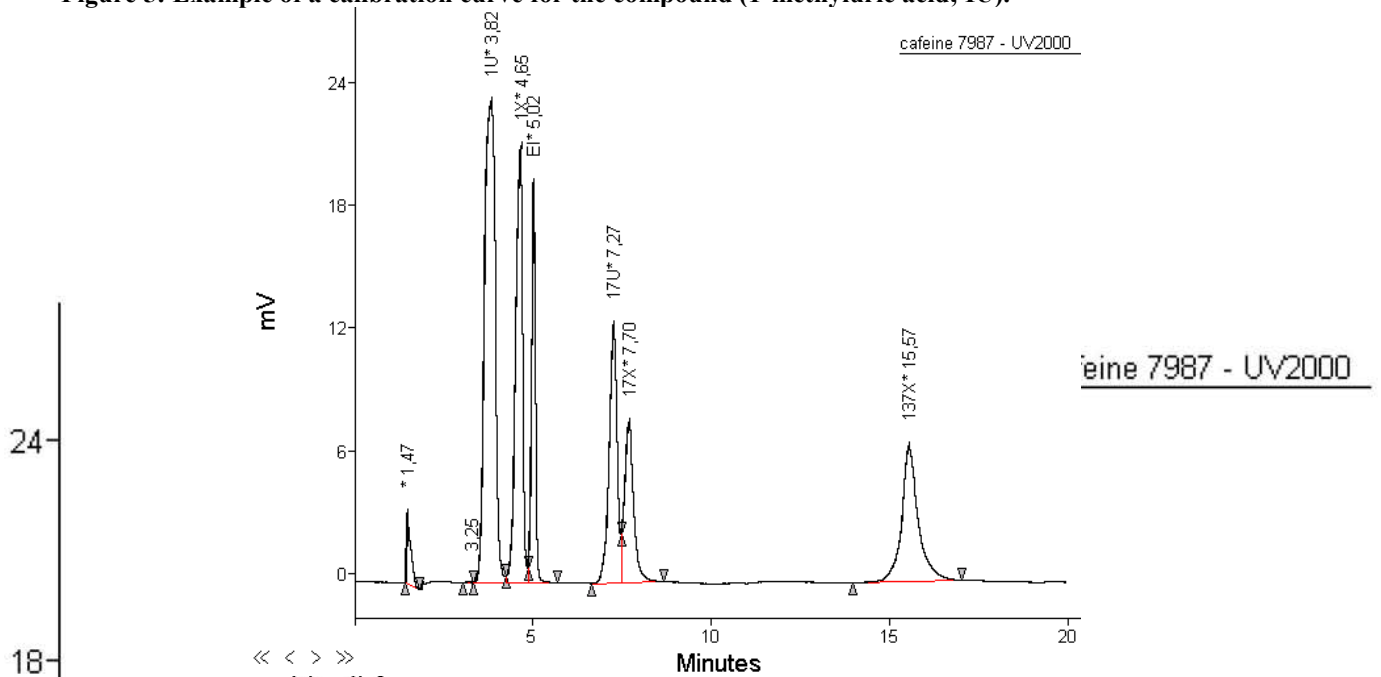
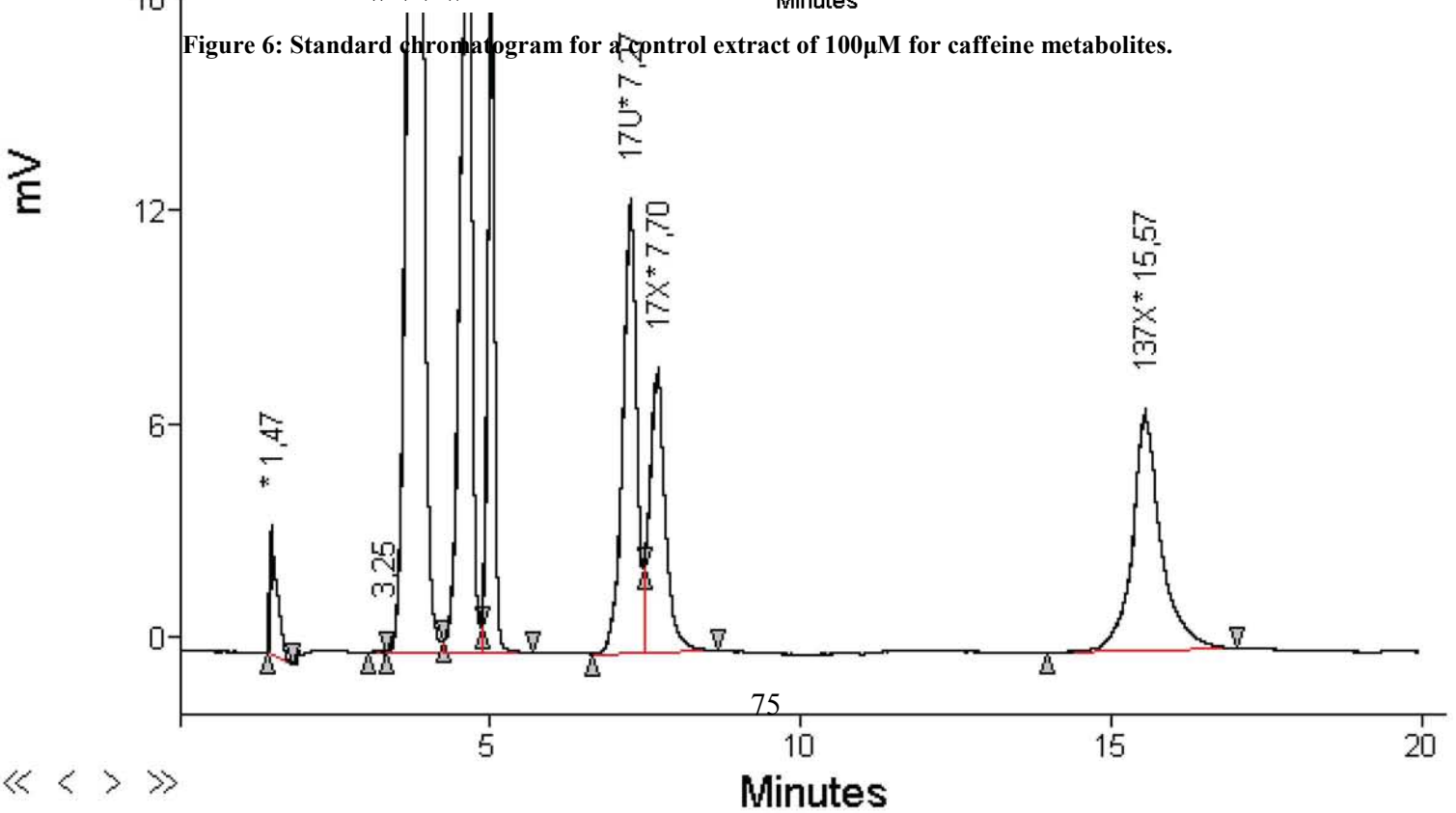


Figure 6: Standard chromatogram for a control extract of 100 $\mu\text{M}$  for caffeine metabolites.



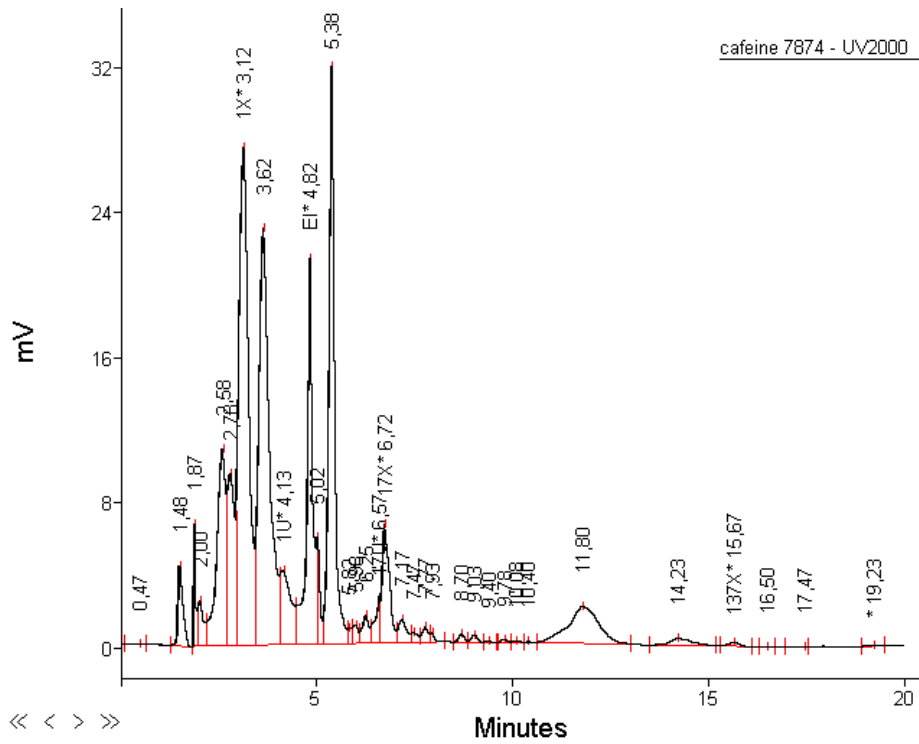
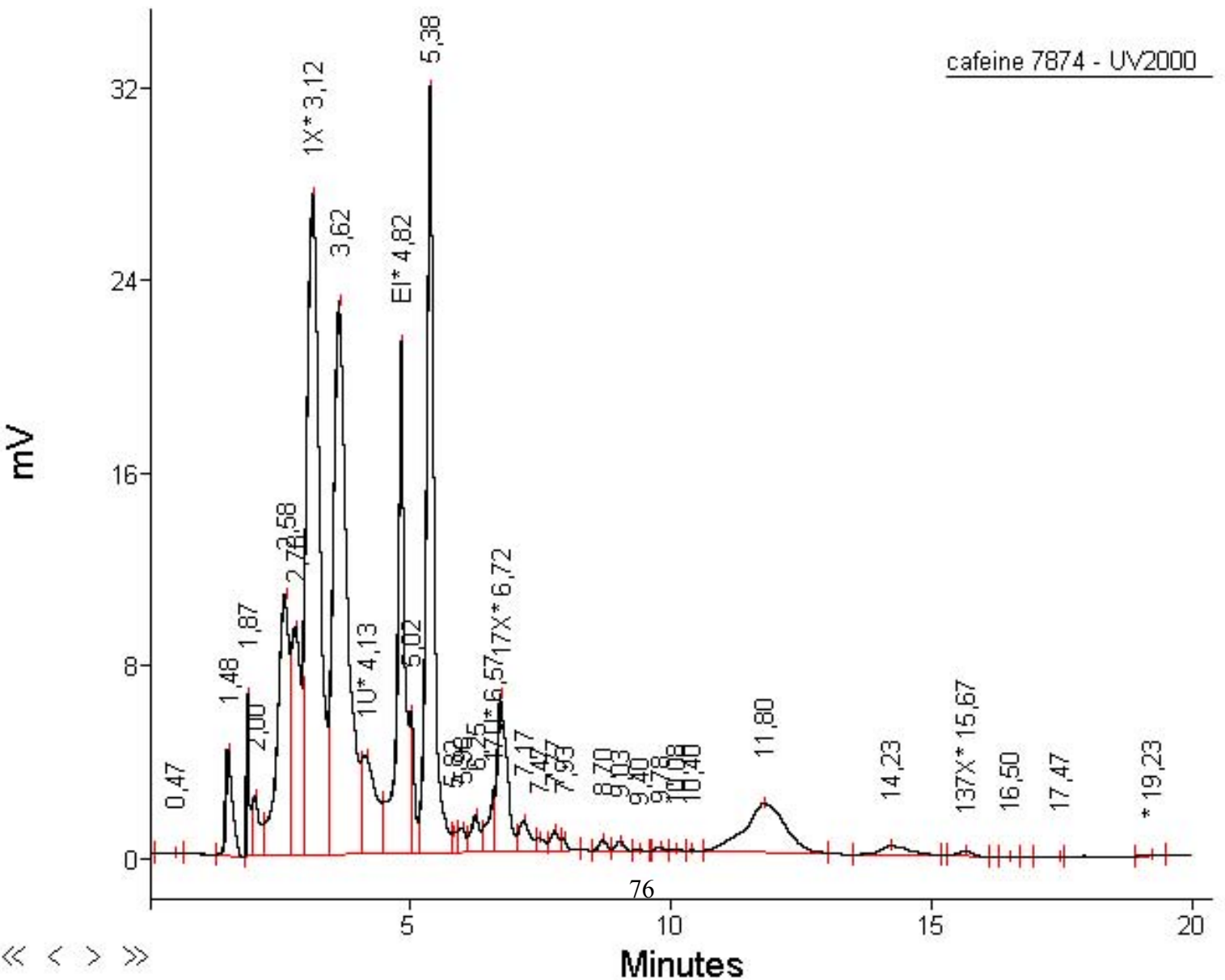


Figure 7: Standard chromatogram for a sample of urine analyzed for caffeine metabolites.



## **2.2. Epidemiological study**

### **2.2.1. Details out of protocol article**

Sample size is the main topic that encountered modifications of the study protocol after the article has been published. While the sample size was anticipated to be 75 participants in each group, we were only able during the study period of nearly 10 months (June 2008 to April 2009), to recruit 34 exposed sewage workers and 30 non-exposed administrative office workers. It should be kept in mind that the Parisian sewage workers are nearly 400 individuals. We excluded the smokers that represent approximately 45% of them [36].

Further other details concerning the study protocol that were not mentioned in the published protocol article are found in the following sections of this chapter.

#### **2.2.1.1. Study setting, administrative and ethical consideration**

All interviews and primary procedures of this study were taken place at the offices of occupational medicine in Paris city municipality (44 rue Charles Moureu, 75013 Paris). The study was conducted in the framework of regular occupational medical visits.

This project has been included in the framework of the normal activity of the occupational medicine department of the Paris municipality; it does not induce any particular risk for the participants. The protocol of this study respect the French regulation, especially regarding the applications relative to the biomedical research code of public health, article L 1121-1, and follow the law of bioethical, the law of informatics, Fichiers et Libertés of CNIL-Commission Nationale de l'Informatique et des Libertés (and specially the methodology of reference MR-001 relative to the biomedical research, as well as the Helsinki declaration.

The “promoter” of this project is the INSERM-department of clinical and therapeutic research (DRCT) under the number C07-20. It subscribed for the whole period of the project a contract of assurance for the civil responsibility under the number 907882007007 with the accordance of the French legal applications and regulations on the biomedical researches. A copy of the assurance certificate is found in appendix 1.

The promoter submitted the whole study protocol, information and consent form (Appendix 2), observation sheet (Appendix 3), tracking sheet (Appendix 4), transport sheet (Appendix 5), the questionnaires and all other documents related to the project to the Direction Générale de la Santé (DGS) and to local ethical committee (CPP-Comité de Protection des Personnes de Nancy Est III). The DGS authorized this biomedical research under the number

DGS2007-0433 (Appendix 6). The local ethical committee approved the study protocol and all its related documents under the number 2007-A00685-48 (Appendix 7).

This project has also been authorized by the Committee Hygiene and Safety and Management of Cleanliness and Environment of the Town of Paris (Comité Hygiène et Sécurité et de la Direction de la Propreté et de l'Environnement de la ville de Paris) where the exposed population recruitment has been occurred (Appendix 8), and the services of Occupational and Preventive Medicine of the town of Paris (les services de médecine professionnelle et préventive de la ville de Paris) where the non-exposed population recruitment has taken place (Appendix 8). The laboratory of hygiene of the Town of Paris (LHVP) gave its agreement to participate in the study and carried out the air sampling for measurement of PAHs and VOCs in the workplaces.

Oral and written explanatory information, as well as a consent form, appendix 2, (for sewage workers “exposed”, and office workers “non-exposed”) that explain the study aim, importance, confidentiality and anonymity of the information with optional participation, were given to all participants. Three similar signed informed consent forms have been obtained from those who agreed to participate in the study. One was kept by the participant and the other two by the study team. No one was given access to completed questionnaires, signed informed consents, biological samples and/or any other documents except the research team which also kept record of all processing steps of the samples to ensure the privacy, security, confidentiality and accuracy. Declaration to the study promoter (INSERM) of any undesirable event which might have occurred to any of the study participants was also taken into account by the research team (Appendix 9).

#### **2.2.1.2. Sample size**

In a non-exposed population, urines are not mutagenic in theory, and both genotoxicity tests should be negative. Thus, if the expected prevalence in the controls is 1%, our sample size (at least 30 subjects in each group) is sufficient to highlight a prevalence of anomalies of nearly 25% in the exposed group. For urinary 8-oxodG, the expected value in reference (control) population is  $10.78 \pm 6.6$  (Mean  $\pm$  SD) nmole/24h [171]. With our sample size, we could detect nearly 33% modification of this value. For CYP1A2 activity, the urinary “molar concentration ratio of 1,7- dimethyluric acid (17U) puls 1,7- dimethylxanthine (17X) over caffeine (1,3,7- trimethylxanthine; 137X)” measured in a reference population is  $5.6 \pm 1.5$  (Mean $\pm$ SD) [190], this allows us to detect a modification of nearly 18% of that ratio.

These estimates were based on a type I error ( $\alpha$ ) of 5% and a power expectation of 80%.

### **2.2.1.3. Recruitment procedure and data collection**

Weekly-meetings were scheduled to present the study in the Committee Hygiene and Safety and Management of Cleanliness and Environment of the Town of Paris (Comité Hygiène et Sécurité et de la Direction de la Propreté et de l'Environnement de la ville de Paris) for the Parisian's sewage workers where their recruitment has been occurred, and in the services of Occupational and Preventive Medicine of the town of Paris (les services de médecine professionnelle et préventive de la ville de Paris) for the office workers where their recruitment has been occurred. The persons that met the study inclusion and exclusion criteria and gave their consent to participate voluntary in the study were selected.

Then, the recruitment procedure and sampling chronology for the study populations were done during their regular annual occupational medical visits at the offices of occupational medicine in Paris city municipality and had the following sequence: after at least three consecutive days of work, a 24h (work shift included) urinary sample was collected from all participants (starting at 9:00 a.m for exposed and 13:30 p.m for non-exposed) to carry out the urine genotoxicity tests (comet and micronucleus). Subjects received a sterile plastic urine-collecting bottle and written/oral information (Appendix 10) describing how to collect urine. After given their urine samples on the next day at 9:00 a.m (Friday for the exposed group) or 13:30 p.m (mostly Thursday and sometimes Friday for the non-exposed group) participants had undergone a general medical examination by occupational health physicians, followed by an electrocardiogram (ECG) test. These were done to insure that they have no history of chronic or recent illness (e.g. diabetes, hepatic, cardiac, renal, rheumatoid arthritis and/or influenza) that could lead to interference with the quality of the biological samples and/or could potentially expose the study subjects to risk from caffeine intake during the study.

Then after, 20ml of blood samples were taken by nurses in 5 sterile glass tubes of 4ml each with EDTA (K3) added (VENOSAFE™, VF-054STK). Thereafter, they received a cup of decaffeinated coffee (Maxwell House, Qualité filtre décaféiné, Kraft foods, France) added with 200 mg of caffeine (Sigma, C-7731, CAS N° 58-08-2). Three hours later, 1ml of urine sample was taken to assess the urinary caffeine metabolites and the corresponding CYP1A2 activity. During these 3 hours, subjects filled in two self-administered questionnaires under the supervision of study researchers. A professional one covering socio-demographic factors, non-occupationally exposures (especially PAHs-related: commuting means, area of residence and indoor sources), medical history, lifestyle (smoking history including passive smoking

exposure, alcohol and medications) and other confounders (Appendix 11). The other is an alimentary questionnaire collecting detailed-data on diet habits specially the 8-oxodG, which is a biomarker sensitive to alimentation specially vitamins of B-group and anti-oxidants that could influence with the test results [168,169]. That was the SUVIMAX2 alimentary questionnaire (Appendix 12) which had been previously validated (Pr S Hercberg, UMR U557 INSERM/U1125 Inra/EA3200 Cnam/Université Paris 13) [198]. Blood and urine samples were pre-treated on the same day as described further in this chapter. In Table 3, we present the sequence of this weekly-sampling procedure of the study participants.

In parallel and during at least three consecutive days of working shifts, the workplace indoor air measurements were made for 13 PAHs and 12 VOCs using passive and active samplers, where the participants had been given oral/written information on how to use them (Appendix 13). Measurements were done on the first part of the week, before coming to the medical examination. PAHs and VOCs are present in the confined environments of the sewers but also emitted by automobile traffic, hence they were selected. They are both present in ambient and indoor atmospheres and are of health significance. The protocol of air sampling is described in details further in this chapter.

For at least 48h before taking the biological samples, subjects were asked to avoid diets or cooking procedures known to increase CYP1A2 activity or elicit urinary mutagenicity (e.g., cruciferous vegetables; charcoal-broiled or grilled meat) or inhibit CYP1A2 (e.g., grapefruit). They were also asked to refrain from consuming alcoholic drinks and beverages containing methylxanthines and asked to avoid massive physical activity as it could increase DNA damage [199].

During the study period of 10 months, 34 sewage workers and 30 office workers were recruited. There was no regular number of participants each week or month. Some weeks we were not be able to recruit any subject, while on some other weeks nearly up to five subjects on each group were recruited. Hence, the dominant number of participants each week was 3-4 participants.

**Table 3: Sequence of the sampling procedure of the study participants in the offices of occupational medicine in Paris city municipality<sup>†</sup>.**

<b>A) Collection of 24h urine sample</b>	code	Time (hh:mm) h	Volume (ml)		
Mix thoroughly and then	150 ml for urine extraction <b>(1)</b>	1ml for 8-oxodG <b>(2)</b>	1ml for creatinine <b>(3)</b>	Tickets and congelation (-20°C).	Tickets and Congelation and then to Nancy city.
<b>B) Medical visit &amp; ECG</b>	Blood samples (4ml glass tubes in 5 times=20ml)	Centrifugation 4000 t/ 5-10 min	Plasma by a pipet to other 5 glass tubes <b>(5-9)</b>	Cells in the same original tubes <b>(10-14)</b>	Tickets and Congelation and then to Nancy city.
<b>C) 200 mg caffeine</b>	A cup of decaffeinated coffee with 200mg added caffeine after the medical visit and a normal ECG			Time (hh:mm) h	<b><u>From C to E :</u></b> <b><u>3hrs</u></b>  Tickets and Congelation and then to Nancy city.
<b>D) Questionnaires</b>	Professional	Suvimax2 (food)			
<b>E) Urine sample (caffeine analysis)</b>	Time (hh:mm) h	1ml + 50µL HCL 1N to get (3-4) PH <b>(4)</b>			

**1:** urine extraction tube of 150 ml for urine genotoxicity tests (comet and micronucleus); **2:** 8-oxodG in 24h urine; **3:** creatinine in 24 h urine; **4:** caffeine test metabolism after 3hrs; **5-9:** plasma cells (upper layer); **10-14:** blood layer (lower layer). 8-oxodG: 8-oxo-2'-deoxyguanosine; ECG, Electrocardiogram. The congelations in Paris were done at -20°C and at the end of sampling the samples were transferred to Nancy city in a cooler and when arrived, they were congelated at -20°C.

<sup>†</sup> Friday morning of each study week at 9:00 a.m for the exposed group. For the control group, the sequence was the same but started at 13:30 p.m mostly on Thursday and sometimes on Friday.





## **2.2.2. Details on the tests out of the article**

### **2.2.2.1. Blood samples**

The four glass tubes of the 20ml of the blood samples were pre-processed in Paris. They were centrifuged at 4000t/min for 5-10 min. The plasma upper layer was saved in other 4 glass tubes for later usual clinical chemistry tests. The lower cells layer was remained in the same glass tubes for the DNA extraction to further measurement of DNA-adducts. All tubes were coded and frozen at -20C° until they were delivered to the laboratory in Nancy city in a cooler where they were kept in -20C°. The DNA-adducts from these blood samples will be dedicated to another future work.

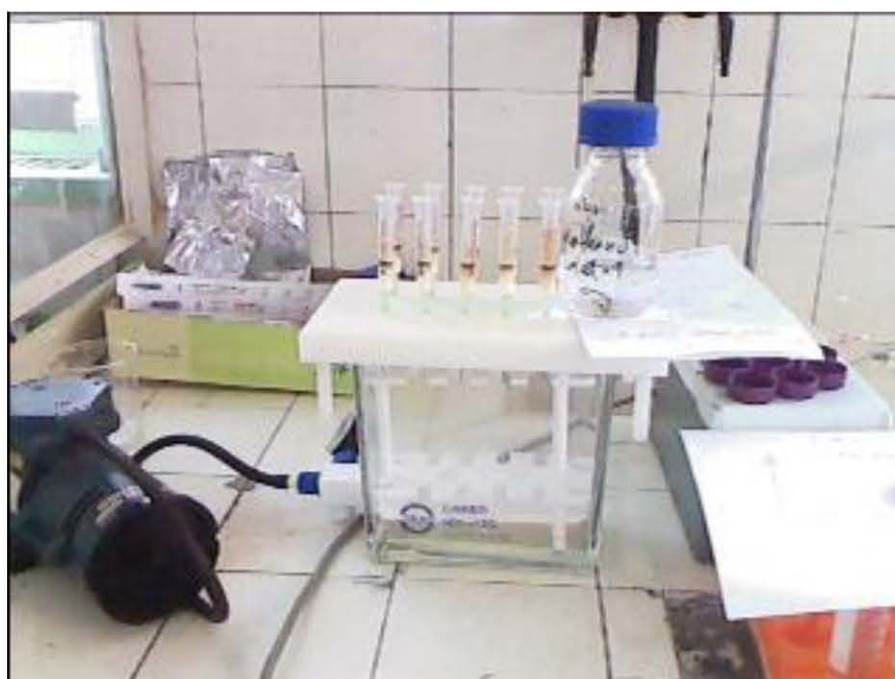
### **2.2.2.2. Treatment of urine samples**

The volume of the 24h urine collected in sterile plastic urine-collecting bottles was measured immediately for each participant. Then after, about 150 ml was dedicated for organic urine extraction and the urine *in vitro* genotoxicity tests (comet and micronucleus) and 1ml for further creatinine analysis. In addition, 1ml of urine sample was taken after 3h of coffee consumption and was added with 50µl of 1M HCL for the assessment of CYP1A2 activity by urinary caffeine metabolites. All urine samples where coded and frozen at -20C°, and then transferred to the laboratory of analysis (within the same day) in Nancy city in a cooler where they kept in -20C° until use.

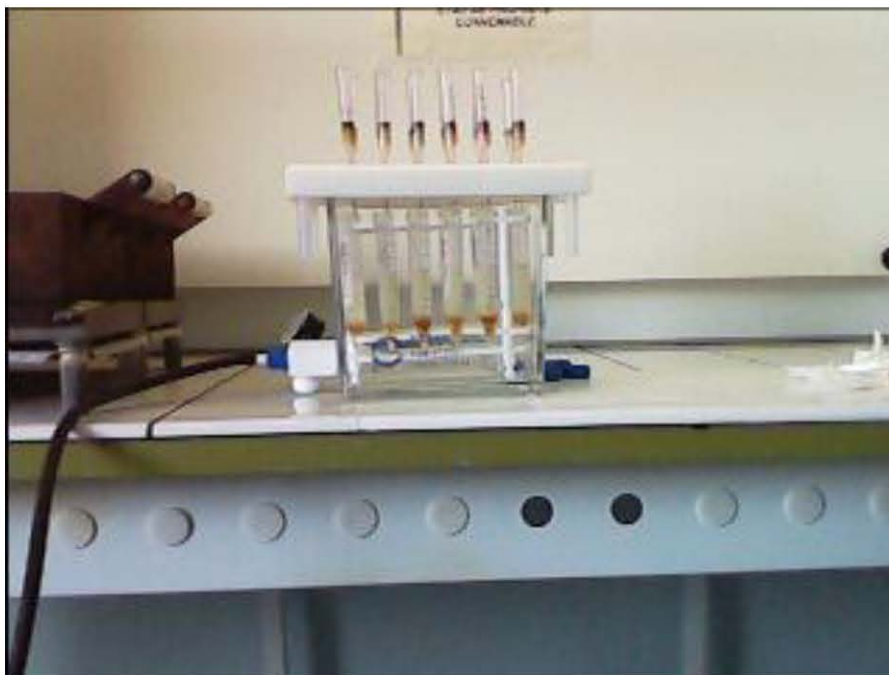
### **2.2.2.3. Extraction of organic fraction from urine samples**

Nearly up to 150ml aliquot of urine sample collected from the total volume of the 24h urinary sample at the day of sampling procedure had been coded and frozen at -20°C to be used for the organic extraction and then after conduction of *in vitro* genotoxicity tests (comet and micronucleus). The organic urine extracts have been carried out as follows; frozen urine samples were thawed at room temperature and 50ml were added in a sterilized corning tube, centrifuged at 3000 t/min for 5 min. The remaining 100ml were frozen again to be kept for further uses. Then, 40ml of the supernatant was collected in a new sterilized corning tube. The separation of the urine extracts was done using Column Sep Pak<sup>®</sup> Vac C18 Cartridges (Waters, WAT020805, Associates, Inc) using an aspirated tray (J.T. Baker spe -12G) with vacuum pump (GAST<sup>®</sup>, CORP. BENTON HARBOR. MCH USA, AC 1PH, model: MOA-V12-CD, S/N 0182). The cartridges were firstly washed with 3ml of absolute methanol (Sigma, M-1775, EC N° 200-659-6) , then three times by 3ml of ultra-pure water followed by

3ml of absolute methanol and three times of 3ml of ultra-pure water successively before preparation of the columns. Then, the cartridge was loaded with the 40ml of the urine (Figure 8). The column was then washed 2 times with 3ml of ultra-pure water in order to eliminate the residual urine and non-organic contaminants. The adsorbed components were then eluted 3 times by 3ml of absolute acetone (CARLO ER BA REAGENTI, CAS N° 67-64-1) into a sterilized test tube (Figure 9). The eluate was evaporated at 45°C under a nitrogen stream (AirLiquid, France) until complete dryness. Then, the residue (extract) was re-suspended in 500µl DMSO and then stored at -20°C until analysis of genotoxicity tests. All operations were done at room temperature.



**Figure 8: The loading of the 40ml of urine showing the adsorbed organic components into the cartridges.**



**Figure 9:** The elution of the adsorbed organic components of urine by acetone into sterilized test tubes.

#### **2.2.2.4. Cellular line used (Hep G2)**

The cellular cell line used is Hep G2; which is a perpetual adherent cell line derived from the liver tissue of a 15 year old Caucasian male with a well-differentiated hepatocellular carcinoma (ATCC, catalog number HB-8065). It has retained certain activities of various phase I and phase II enzymes [200,201] that play a key role in the activation and detoxification of various promutagens/procarcinogens [202,203]. This cell line is among one of the preferred human *in vitro* models used to study genotoxicity and DNA damage for promutagens and compounds that need bioactivation before they exert their genotoxic effects [204]. Hep G2 cells were also suggested to have a metabolic capacity for PAHs similar to human hepatocytes and therefore represent a good *in vitro* model for investigating the genotoxic potential of complex mixtures containing PAHs [205]. Our previous work also demonstrates the benefits and suitability of these cell lines (see comet article section for the comparison between HeLa S3 and Hep G2 cells in comet assay).

#### **2.2.2.5. Chemicals and media for cell culture**

Unless otherwise specified, all chemicals, solutions used and culture medium used for cell cultivation and treatment were purchased from Sigma-Aldrich Chimie S.A.R.L (L'Isle D'Abeau Chesnes, France).

The composition of culture medium (E.M.E.M. Eagle, 1L) for Hep G2 includes (pH, 7.2-7.4, and conserved at 4C°):

- 9.6 g of Eagle's Minimum Essential Medium (E.M.E.M, Sigma M-0643).
- 5.96 g Hepes (Sigma H-4034).
- 2.2 g Sodium bicarbonate (Sigma S-6297).
- 0.11 g Sodium pyruvate (Acros- 132150250).
- 100 ml Fetal Bovine Serum (FBS, Sigma F-7524).
- 10 ml Antibiotic solution (penicillin 10000U/ml, streptomycin 10mg/ml; Sigma P- 4333).
- 890 ml Milli-Q water.

The solutions used for the passage of cell culture from one flask to another, conserved at 4C°:

- 9.6 g Dulbecco's Phosphate Buffered Saline (D.P.B.S, pH 7.4, Sigma D- 5652) in 1L Milli-Q water.
- Trypsin-EDTA solution (0.5% trypsin, 0.2% EDTA; w:v).
- Complete culture medium (E.M.E.M. Eagle).

The solutions of positive and negative controls (comet and micronucleus assays):

- Positive control: B[a]P 40  $\mu$ M (Sigma B-1760). Stock solutions of B[a]P (10mM in DMSO) were prepared and kept at -20°C. Immediately before use, B[a]P was diluted in DMSO and adjusted to reach 40 $\mu$ M B[a]P with 1% DMSO final concentrations in the culture medium.
- Negative control: DMSO 1% final concentration in the culture medium.

By choosing DMSO to be 1% final concentrations in all medium (test medium, positive and negative mediums) this procedure enabled us to attenuate the effect of DMSO in the results.

### **2.2.3. Comet assay**

#### **2.2.3.1. The solutions used**

The solutions used for comet assay are:

- Agarose routine 1%: 500 mg of agarose (first layer, Sigma A-5093) in 50 ml Milli-Q water.
- Agarose N 1% (normal melting point agarose, NMP): 110 mg of agarose type I-B Low EEO (Sigma A- 0576) in 10 ml D.P.B.S.
- Agarose L 1% (low melting point agarose, LMP): 110 mg of agarose type VII Low Gelling (Sigma A- 4018) in 10 ml D.P.B.S.

- Ethidium bromide (Sigma E-8751): original solution of 20µg/ml in Milli-Q water diluted ten times to reach 2µg/ml in Milli-Q water.
- Electrophoresis buffer solution pH, 13: 300mM NaOH (Sigma S- 0899) and 1 mM Na<sub>2</sub>EDTA (Sigma E- 5134) in 2L Milli-Q water, conservation at 4C°.
- Lysis solution pH, 10: 2.5 M NaCl (Sigma S- 9625), 100mM Na<sub>2</sub>EDTA, 10mM Tris (Sigma T-1378) in 356 ml Milli-Q water, conservation at 4C°. In the test day and two hours before utilization, 1% Triton X-100 (Prolabo 28.817.295) and 10% dimethyl sulphoxide (DMSO, Sigma D-5879) were added.
- Tris neutralizing solution 0.4M, pH, 7.5: 48.56 g of Tris in 1L Milli-Q water, conservation at 4C°.
- Trypan blue 0.4% (CAS 72-57-1) exclusion test to assess toxicity.

### **2.2.3.2. Culture and treatment of cellular line used (Hep G2)**

As described previously in comet article section, Hep G2 cells were routinely cultured in the laboratory in 5ml complete culture medium until 80% confluence. The medium was then discarded and exactly replaced by 5ml of freshly diluted test medium contain 50µl of organic extract of urine that were thawed and warmed to room temperature shortly before use. Cells were then incubated for 24h, then washed twice with PBS buffer (pH 7.4) and harvested immediately by rinsing for 5-7 min with trypsin-EDTA solution. After inactivation of trypsin and EDTA by the culture medium, cells were centrifuged at 1000 t/min during 5 min. Subsequently, the cell pellet was suspended in 3ml culture medium, homogenated, counted, diluted to reach a solution of 5x10<sup>5</sup>cell/ml cell suspension. Trypan blue exclusion test was used to assess toxicity, and cell viability was always ≥ 95%.

### **2.2.3.3. Methodology**

Comet assay was performed essentially as in Singh et al. [86], with some modifications as in Muller-Pillet et al. [206] and followed the recommendations of the International Workshop on Genotoxicity Test Procedures [89]. Methodology detail was previously described in comet article section.

### **2.2.3.4. Selection of comets and image analysis parameter used**

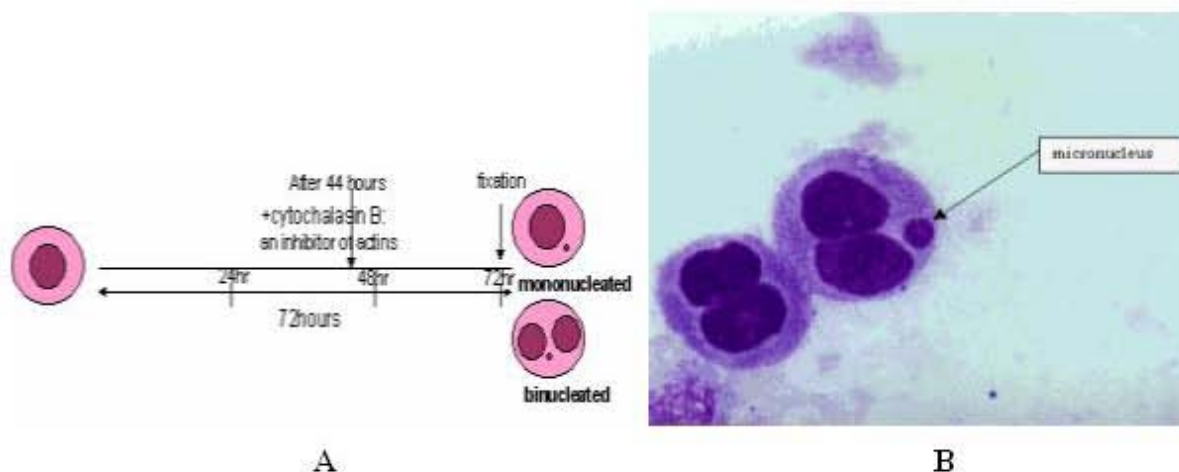
This has been previously described in comet article section. Briefly, slides were examined for DNA migration using an Olympus BX40 fluorescence microscope (Olympus, Tokyo, Japan). For each organic urine sample, two slides were analyzed and 25 cells per slide were randomly

scored. Quantitative analysis of DNA damage was performed by a computerized image analysis system (Komet 4.02 software, Kinetic Imaging, UK). DNA damage was assessed by % DNA tail parameter, which is the fraction of DNA in the tail divided by the amount of DNA in the cell, multiplied by 100. This parameter has been shown to be the most linearly related to dose, the easiest to understand [89,207,208]. It also gives a clear indication of what the comets actually look like [209]. Tail length tends to reach a maximum at a low level of damage, and tail moment (despite its wide usage) tells nothing about the appearance of the comets and is the least informative parameter [209,210]. Olive tail moment and extent tail moment may be also calculated differently by Image Analysis systems [89].

#### **2.2.4. Micronucleus assay**

There is evidence that performing the MN assay in a manner that does not account for inhibition of nuclear division can lead to an underestimate of micronuclei (MNi) induction [83]. Cytochalasin-B in a range of (1–10 µg/ml) is added during the first cell cycle following the start of the treatment and the cells should be harvested prior to the second mitosis [83]. Cell proliferation and assessment of toxicity are performed by calculating the nuclear division index (NDI) defined as:  $NDI = (M1 + 2M2 + 3M3 + 4M4) / N$ , where M1-M4 represent the number of cells with 1-4 nuclei and N is the total number of viable cells scored (excluding necrotic and apoptotic cells) [83,112].

Cytochalasin-B an inhibitor of actins allows distinguishing easily between mononucleated cells which did not divide and binucleated cells (BNed) which completed nuclear division during *in vitro* culture (Figure 10). Indeed, in these conditions the frequencies of mononucleated cells provide an indication of the background level of chromosome/genome mutations accumulated *in vivo* and the frequencies of binucleated cells with MNi a measure of the damage accumulated before cultivation plus mutations expressed during the first *in vitro* mitosis. Thus, in the absence of cytochalasin B, mononucleated cells are analyzed for the presence of micronuclei. However, with cytochalasin-B, the scoring of micronuclei in mononucleated cells is optional [127].



**Figure 10: A) CBMN assay with cytochalasin-B addition 44 hours after the start of cultivation to distinguish between mono- and BNed cells. B) A MNi in BNed cells. Adapted from [http://we.vub.ac.be/~cege/volders/ENG/tests/MN.htm]. Last accessed 30 November 2009.**

#### 2.2.4.1. The solutions used

The solutions used for micronucleus assay are:

- Acridine orange (Sigma A- 6014): original solution of 4 mg/ml in Milli-Q water conserved in the dark at ambient temperature. The utilized solution was 40µg/ml and conserved in the dark.
- CARNOY: methanol (Carlo Erba 525.102): acetic acid (Prolabo 20.104.298), 3:1 v:v.
- Cytochalasin-B (Sigma C-6762): original solution of 2 mg/ml of DMSO and conserved in fractions at -18C°. The utilized solution was 3µg/ml of E.M.E.M. Eagle.
- Dulbecco's Phosphate Buffered Saline (D.P.B.S, pH 7.4, Sigma D- 5652): 9.6 g in 1L Milli-Q water.
- KCL 0.075 M (Prolabo 26.764.298): 559 mg KCL in 100 ml Milli-Q water.

#### 2.2.4.2. Methodology

Slides preparation and methodology for micronucleus assay were carried out essentially as in Fenech. 2007 protocol which was published recently in nature protocols" journal [83]:

- At time (t) = 0: a cellular suspension of  $1 \times 10^4$  cell/ml was prepared and 1 ml of this suspension were deposited on a conditional microscopic slide that was autoclaved and placed in a Petri dish (L.E.S Sayag BP50). Then, the volume of the culture medium was adjusted to 10ml.
- At t+24h: the medium was then discarded and exactly replaced by 10ml of freshly diluted test medium contain 100µl of organic extract of urine that were thawed and warmed to room temperature shortly before use.

- At t+44h: the test medium was discarded and replaced by 10ml of freshly diluted culture medium contain cytochalasin-B at 3µg/ml. The addition of cytochalasin-B was realised at the total volume of culture medium used at that time in order to obtain an identical concentration in every dish.
- At t+72h: the cells were harvested in a way that the slides were removed from the dishes by a clamp, rinsing in a KCL solution of 0.075M, and then dipped into another fresh KCL solution of 0.075M for 10 min. They were then shortly-dried at room temperature and then dipped into CARNOY solution for nearly 10 seconds. After that, they were let to dry in the room.
- Before reading and analysis under the microscope, the slides were dipped 10 min in an Acridine orange solution of 40µg/ml in the dark, rinsed shortly in Milli-Q water and then dipped into D.P.B.S buffer solution for 10 min in the dark. They were then read under an Olympus BX fluorescence microscope at a 40-fold magnification (Olympus, Tokyo, Japan) that is equipped with UMN-B-II cube: excitation filter 470-490nm, dichroic mirror 500nm and d'arrêt 515nm,. Under these prescribed conditions, the cellular cytoplasm appear in red and the DNA in green.
- The slides were manipulated under laminar flow hood until the time t+48h.

#### **2.2.4.3. Selection and calculation of the parameters**

On a score sheet for MN assay, the following information was included:

- Name of the person scoring the slides.
- Code number of each slide.
- Number of BNed cells scored.
- Frequency of MNi in 1000 BNed cells.
- Frequency of BNed cells containing MNi in 1000 BNed cells.
- Frequency of viable mono-, bi-, tri- and tetranucleated cells in a total of 150 cells.
- Nuclear division index (NDI).

So, 1000 BNed cells were scored in each slide. Among these 1000 BNed cells, the total number of MNi was determined. The MNi with the following criteria were scored [83]:

- Morphologically identical to but smaller than main nuclei.
- The diameter of MNi should be less than one-third of the mean diameter of the main nuclei.
- MNi not linked or connected to the main nuclei.



- MNi can be readily distinguished and may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.
- MNi have the same staining intensity but occasionally staining may be more intense than the main nuclei.

The Nuclear Division Index (NDI) was also calculated. It provides a measure of the proliferative status of the viable cell fraction. It is therefore an indicator of cytostatic effects (cytostasis of cell death and cytotoxicity). NDI was calculated according to Fenech, 2000 [211] which was performed on 150 viable cells to determine the frequency of cells with 1, 2, 3 or 4 nuclei, and calculate the NDI following the formula:

$NDI = (M1 + 2M2 + 3M3 + 4M4) / N$ , where M1-M4 represent the number of cells with 1-4 nuclei and N is the total number of viable cells scored (excluding necrotic and apoptotic cells). The NDI is a useful parameter for comparing the cytostatic effects of agents examined in the assay. If the mean index in the exposed sample was 25% below the mean index of the control sample, this led to the rejection of the sample. This limit of rejection is based on the desire to test the genotoxicity outside the cytotoxicity [127].

The lowest NDI value possible is 1 which occurs if all of the viable cells have failed to divide during the cytokinesis-block period and are therefore all mononucleated. If all viable cells completed one nuclear division and are therefore all binucleated, the NDI is 2. An NDI value can only be greater than 2 if a substantial proportion of viable cells have completed more than one nuclear division during the cytokinesis-block phase and therefore contain more than two nuclei. For example, if 50% of viable cells are BNed, 10% trinucleated and 10% quadrinucleated, the NDI value is 2.2.

### **2.2.5. Sewage system's indoor air sampling protocol**

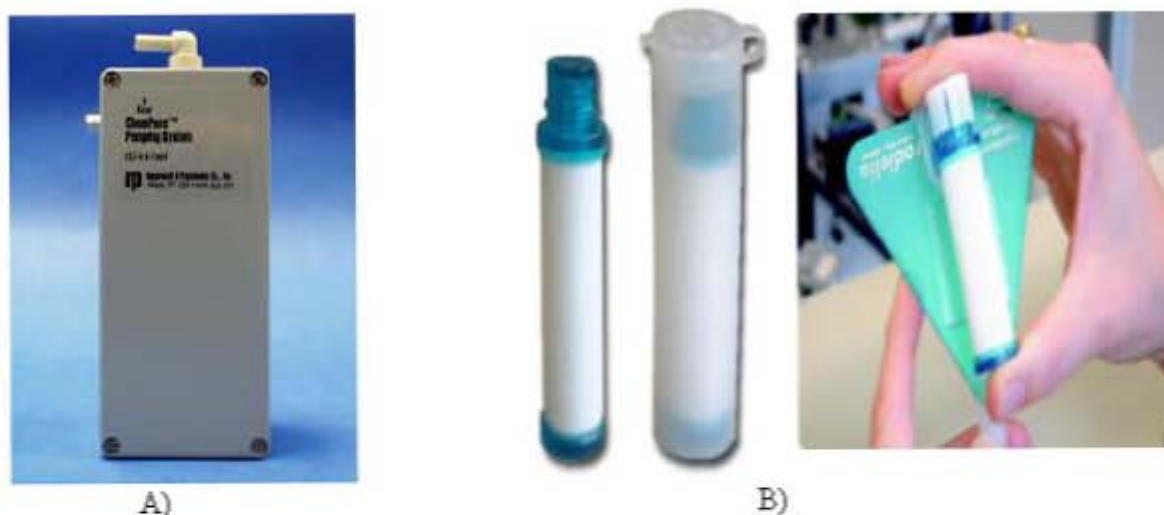
The equipments for atmospheric sampling were prepared in the laboratory of hygiene of the City of Paris (LHVP). Since the sewage system is deprived of electricity, air sampling was carried out using battery-powered devices (active sampling) and passive samplers. The sampling procedure strived at evaluating the exposure near the breathing zone. The sampling equipments were placed in a backpack and handed to each participant with a sheet of instructions (Appendix 13). This material was provided for collecting PAHs and VOCs.

#### **2.2.5.1. Sampling and collection of PAHs and VOCs**

The PAHs compounds were sampled by active sampling. Measurements were performed using a ChemPass pump (Rupprecht and Patastnick Co., Inc. NY, USA) that sampling air at a

calibrated flow rate of 4 L/min. The sampling support apparatus consisted of a quartz filter to which the PAHs presented in the air in a particulates form were collected (quartz filter, Supelco 21038, 32 mm of diameter) and a cartridge containing polyurethane foam (PUF) that retaining the PAHs presented in the air a gaseous form (Supelco ORBO 2-0600, previously cleaned) (Figure 11A). The flow was measured before and after sampling. A gap of 10% was allowed between these two measures. The pumps used were equipped with a timer and the sampling time chosen was the time of pumping shown on the pump. However, it should be noted that in some cases, a discrepancy was noted between the periods specified on the sampling sheet and the pump timer.

The VOCs compounds were sampled by passive sampling method on thermal desorption sorbent tubes. The gaseous pollutants migrate by molecular diffusion to the surface of a cartridge containing an adsorbent on which they were retained. VOCs were collected on passive sensors to radial diffusion. These tubes composed of a cartridge containing an adsorbent type Carbograph 4 (charcoal graphitization) - thermally preconditioned (cartridge Radiello code 145, Sigma-Aldrich), and a diffusive body of porous polyethylene membrane (Radiello code 120-2, Sigma-Aldrich) placed on a triangular support plate (code 121) (Figure 11B). The supplier indicated the molecular diffusion rates. The sampling time was calculated from the time shown on the sampling sheet filled by the participant.



**Figure 11: A) ChemPass pumping system. B) Cartridge Radiello code 145 adsorbent.**

Before descending into the underground, a sewer worker equipped so as to collect the PAHs and VOCs compounds in the air of the workplace. He carried the backpack containing the pump and fixed the head of the PAHs sampling to a carabineer of his working clothes. The participant did the same for the passive sampling of VOCs. He noted on the sampling sheet,

the date and time of installation and removal of the equipment. These operations were carried out for four days (Monday through Thursday) during one working week. Filter was preserved in an aluminium sheet to avoid photochemical transformations.

The same equipments were made available to the volunteers working in an office who constitute the reference population (non-exposed). In this case, the equipments were placed in a fixed site of the workplace, near the office of the participant. Nearly, each office worker made the samples during the working hours and for three consecutive days (Monday to Wednesday) of one working week. There was an exception in one case where two office workers sampled together as a team. For the sewage workers (exposed), two workers were sampled together as a team insofar as they were carrying the same task. However, in some few cases, each worker made the samples alone and in only one case, 4 workers were sampled together as a team. The same exposure values were assigned to each subject within a team.

The duration of sampling corresponded to the time passed by the participants in the sewage system for the exposed population, and in the office excluding the break of the lunchtime (from 12h00 to 14h00) for the administrative non-exposed workers.

During the analysis of PAHs and VOCs, a number of certain values obtained were higher than the high value of the calibration range (i.e., quantification limit). This limit was variable since the sample volume, in relation to the sampling time was different from one sample to another. For the comparison, the Paris air quality monitoring network (Surveillance de la qualité de l'air en Ile-de-France-AIRPARIF), provided the ambient (traffic and urban) concentrations values of the selected PAHs and of 6 out of the 12 VOCs under study. Values corresponded to the nearest sampled sites and dates of the study were compared.

#### **2.2.5.2. Extraction and analysis of PAHs**

The filter and the foam were subjected to extraction by Accelerated Solvent (ASE) with a mixture of hexane-acetone (50/50). The concentration was carried out under nitrogen. Firstly in a water bath at 35 ° C, using the automatic evaporator Turbo Vap II Zymark until obtaining 10 ml and then under nitrogen flow with the evaporator N-evap until obtaining an oily drop of the extract. This was taken by 1ml of acetonitrile (solvent compatible with the high performance liquid chromatography (HPLC, Sigma-Aldrich). The analysis of the extract was done by HPLC in a polarity of reverse phase with a detector by fluorescence emission (HPLC/Fluo).

The apparatus of the chromatographic system consisted of:

- ❖ A thermostat automatic injector Agilent 1200 G1329A and G1330B.

- ❖ A quaternary pump Agilent 1200 G1311A.
- ❖ A furnace to maintain the column and the pre-column at  $40^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$  (manufacturer data) Agilent 1200 G1316A.
- ❖ A fluorimetric detector Waters 2475.
- ❖ A column filled with C18 phase: Restek, Pinnacle II PAH, L = 20 cm, diameter = 4.6 mm, dp = 5 $\mu\text{m}$ .
- ❖ A pre-column filled with C18 phase: Restek Pinnacle II PAH, L = 1 cm, diameter = 4.6 mm, pore size = 5 $\mu\text{m}$ .

The quantification analysis was carried out according to the calibrated response of standard solutions that were prepared from a marked mixture of the selected PAHs. The concentrations were expressed in  $\text{ng}/\text{m}^3$  of the compound in the air. The quantification limits expressed in  $\text{ng}/\text{m}^3$  were differed from one compound to another because the volume of sampled air was different from one sample to another (Table 4).

**Table 4: The quantification limits (QLs) expressed in ( $\text{ng}/\text{m}^3$ ) calibrated for the 13 PAHs on the HPLC.**

<b>PAHs</b>	Phenant.	Anthrac.	Fluorant.	Pyrene	B(a)A	Chrysene	B(j)F
<b>QL</b>	52.0	0.70	14.0	7.3	1.7	7.0	1.4
<b>PAHs</b>	B(b)F	B(k)F	B(a)P	DiB(ah)A	B(ghi)P	IP	
<b>QL</b>	1.0	0.8	1.7	0.2	1.2	1.0	

Phenant, Phenanthrene; Anthrac, anthracene; Fluorant, Fluoranthene; B(a)A, Benzo(a)anthracene; B(j)F, Benzo(j)fluoranthene; B(b)F, Benzo(b)fluoranthene; B(k)F, Benzo(k)fluoranthene; B(a)P, Benzo(a)pyrene; DiB(ah)A, Dibenzo(a,h)anthracene; B(ghi)P, Benzo(g,h,i) perylene; IP, Indeno(1,2,3,-c,d)pyrene.

13 PAHs priority pollutants listed by US EPA were quantified:

- ❖ 9 compounds in particulate form: chrysene, benzo(b)fluoranthene [B(b)F], benzo(a)anthracene [B(a)A], benzo(k)fluoranthene [B(k)F], benzo(a)pyrene [B(a)P], dibenzo(a,h)anthracene [DiB(ah)A], benzo(g,h,i) perylene [B(ghi)P], indeno(1,2,3,-c,d)pyrene [IP] and benzo(j)fluoranthene [B(j)F].
- ❖ 4 compounds mainly in gaseous form: phenanthrene, anthracene, fluoranthene and pyrene.

### 2.2.5.3. Extraction and analysis of VOCs

The VOCs were thermally desorbed and analyzed by coupling gas phase-chromatography and mass-spectrometry (TD GC-MS) technique. The gaseous compounds, recovered from the adsorbent, were thermally desorbed (primary desorption), then further concentrated on a cryogenic trap. By a thermal flash, they were desorbed from the trap (secondary desorption)

and transported via an inert gas carrier through a line of transfer to a gas chromatography equipped with a capillary column and coupled to a mass spectrometry detector (Quadripole). The compounds were desorbed from the tube at 370 ° C for 15 min.

The apparatus consisted of:

- ❖ Thermal Désorbeur Turbomatrix, Perkin Elmer (PMDEST003) equipped with a cryogenic trap (ref. M041-3627) containing a volume of nearly 0.2 ml of carbotrap C 20/40 carbotrap B 20/40 and carbosieve SIII 60/80 in voisines quantities. The pneumatic system was powered by nitrogen or by the air from a zero air generator.
- ❖ Gas phase chromatography Autosystems XL Perkin Elmer, equipped with a capillary column RTX-1 100% dimethyl polysiloxane RESTEK, L= 60 m, diameter = 0.32 mm, pore size= 3µm, powered by helium, and a mass spectrometer Turbomass Gold, Perkin Elmer.

The qualitative analysis of VOCs was obtained from the current profile of total ion by comparing the mass spectra of pollutants in the sample with those in a library of spectra. The qualitative analysis of the chromatographic profile obtained for VOCs revealed that the compounds identified are mainly saturated (n-alkanes) and unsaturated (alkenes) hydrocarbons, branched hydrocarbons and aromatic hydrocarbons. Some oxygenated compounds (alcohols, aldehydes) and chlorinated were also identified. It was noted that the observed profiles were quite comparable from one analysis to another. Figure 12 below shows a chromatographic profile for the exposed population.

The quantitative analysis was realised according to the response of ions extracts from a pre-determined list of 12 compounds. The concentrations were expressed in µg/m<sup>3</sup> of the compound in the air. Calibration was achieved by doping liquid using standard solutions. For the 12 compounds of VOCs, the quantitative analysis was made from a straight line of calibration included:

- ❖ Monocyclic aromatic hydrocarbons: benzene, ethylbenzene, toluene, m + p-xylenes, o-xylene, 1,2,4-trimethylbenzene and styrene.
- ❖ Alkanes: decane and undecane.
- ❖ Chlorinated compounds: trichloroethylene, tetrachloroethylene and 1,4-dichlorobenzene.

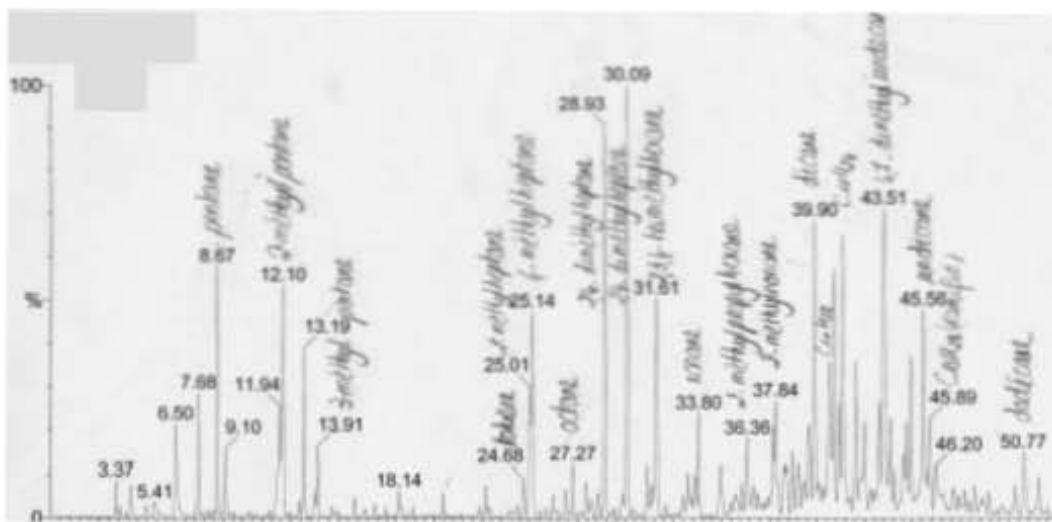


Figure 12: A chromatographic profile for the exposed population to some VOCs.

### 2.2.6. Measurement of 24h urinary 8-oxodG

The 8-oxodG was measured with a competitive enzymatic immunoassay (EIA) kit named 8-oxodG EIA kit (StressMarq Biosciences Inc., Victoria, BC Canada). This assay utilizes a specific 8-oxodG monoclonal anti-body, 8-oxodG-Acetylcholinesterase (AChE) conjugate and an anti-mouse IgG-coated plate. The assay is based on a competition between 8-oxodG and the 8-oxodG AChE conjugate for 8-oxodG monoclonal anti-body binding. The 8-oxodG-anti-body complex binds to goat polyclonal anti-mouse IgG that is attached to the well.

We followed the protocol provided by the manufacturer in the analysis [212]. Briefly, all the materials in EIA kit and the urine samples were kept at  $-20^{\circ}\text{C}$  until the day of analysis. After thawing at room temperature, the 8-oxodG standard (300ng/ml) was diluted by ultra pure water into bulk standard (30ng/ml). This was then serially diluted by EIA buffer into 8 concentrations ranged from 10.3-3000 pg/ml. Then, we diluted the 8-oxodG-AChE conjugate and its dye as well as the 8-oxodG monoclonal anti-body and its anti-serum dye by the EIA buffer. The 96-well plates that were supplied ready to use contained two blanks, two non-specific binding (NSB) wells, two maximum binding ( $B_0$ ), and 8 point standard in duplicate.

After a wash step with EIA buffer to remove any unbound reagents, Ellman's reagent containing AChE substrate (acetylthiocholine) and [5,5'-dithio-bis-(2-nitrobenzoic acid)] was added to the well. The AChE-substrate reaction produced thiocholine that reacted with 5,5'-dithio-bis-(2-nitrobenzoic acid) resulting in 5-thio-2-nitrobenzoic acid. This final product has a yellow color that absorbed strongly at 412 nm. Spectrophotometrically determined, the

intensity of this color is proportional to the bound concentration of 8-oxodG AChE conjugate binding to the 8-oxodG monoclonal anti-body in the well, while inversely proportional to the concentration of free 8-oxodG in the test sample. To insure the accuracy and reproducibility of results, each sample was assayed at two dilutions (1/300 and 1/400) and each at duplicates. For the analysis of results, we plotted the data as % B/B<sub>0</sub> (% bound/maximum bound; ratio of the absorbance of the standard or sample well to that of the maximum binding well) versus log concentration of standard 8-oxodG. A calibration curve of the standard concentrations of 8-oxodG (S1-S8) was plotted with a detection limit of 33 pg/ml (80% B/B<sub>0</sub>). Quantification of the samples concentration was determined using the calibration curve after calculating their %B/B<sub>0</sub> and taking into account the samples dilution factor prior to the assay.

### **2.2.7. Statistical analysis**

Chi square and Fisher exact tests were used to analyse the differences between categorical variables. Comparisons between different treatment groups were done by the non-parametric Kruskal-Wallis test, followed by the Mann-Whitney U test for differences between the control and treated groups. ANOVA analysis was also carried out to compare the mean differences of quantitative variables between the sewage and the office groups and to evaluate the influence of some confounding factors (e.g. age, smoking and alcohol consumption). Multiple linear regressions were conducted and confounders were retained if  $p < 0.1$  in univariate analyses. ANOVA multi-way was also used to screen for possible interaction effects. P-values  $< 0.05$  were regarded as significant. All the statistical analysis was performed using SPSS 16 software (Statistical Package for Social Science) [213].

To assess and characterize the carcinogenic potency of PAH mixtures, toxicity equivalent factors (TEFs) were used where B(a)P is chosen as the reference compound and assigned a TEF value of 1. Cancer risk by inhalation for PAH mixtures was calculated by multiplying the concentration of each PAH by its assigned TEF and then summed to obtain the total B(a)P equivalent concentration ([B(a)P]<sub>eq</sub>). This final concentration was multiplied by the B(a)P inhalation risk cancer unit value. Regarding the VOCs, only benzene has an inhalation cancer unit risk. We used this unit risk to calculate the cancer risk by inhalation for benzene.





### **3. Results**

#### **3.1. Exposure and genotoxicity article (3<sup>rd</sup> article)**

This article describes the characteristics of the study population, and details the concentrations of the PAHs and VOCs measured in the different study workplaces and sectors (sewage and office occupational measurements and traffics and urban sectors). From these results, we estimated the lifetime cancer risks from PAHs and benzene inhalation exposure using the TEFs and the inhalation unit risks for these compounds. It then includes the results from the *in vitro* genotoxicity assays (comet and CBMN) on Hep G2 cells with the organic urine extracts from the workers. We further present the results from the 24hours urinary 8-oxo-2'-deoxyguanosine (8-oxodG), the chosen early effect biomarker of DNA oxidative stress. We hypothesized that exposure of sewage workers to higher levels of certain chemicals, like PAHs and VOCs, could generate an excess in reactive oxygen species during their metabolic activation in human body that could lead to oxidative DNA damage. This damage represented partially by the urinary excretion of the most widely formed DNA oxidized lesion named "8-oxodG", could have an important role in the development of many diseases including cancer risk. We compare the results obtained from the analysis of the 24 hours urinary 8-oxodG by an enzymatic immunoassay between sewage and office workers. These biomarkers have also been related to the air sampling of the various PAHs and VOCs in multiple linear regression models. The results were then discussed under the discussion section of this article and a conclusion has been derived.

This exposure and genotoxicity article is presented in the following page (submitted).



# **Integrated Exposure Assessment of Sewage Workers to Genotoxics: an Urinary Biomarker Approach and Oxidative stress Evaluation<sup>†</sup>**

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<sup>†</sup> Submitted.

**Running title:** Urinary genotoxicants and 8-oxodG in sewage workers.

**Key words:** 8-oxo-2'-deoxyguanosine (8-oxodG); Comet assay; Micronucleus assay; Oxidative stress; Sewage workers; urine genotoxicity; Workplace air sampling.

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**Competing Interests:** None of the authors has any competing financial interest.

#### **List of abbreviations**

8-oxodG: 8-oxo-2'-deoxyguanosine.

B(a)P: Benzo(a)pyrene

[B(a)P]eq: Benzo(a)pyrene equivalent concentration

BNed: Binucleated cells

CBMN: Cytokinesis block micronucleus assay

MNi: Micronuclei

NDI: Nuclear division index

PAHs: Polycyclic aromatic hydrocarbons

TEFs: Toxicity equivalent factors

U.S. EPA: United States Environmental Protection Agency

VOCs: Volatile organic compounds

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## **Abstract**

**BACKGROUND:** Sewage workers are exposed to multiple chemicals among which many are suspected genotoxicants. Therefore, they might incur DNA damage and oxidative stress.

**OBJECTIVES:** To explore integrated biomarkers of exposure and early effects, respectively, among sewage workers, assessing the overall urine genotoxicity by *in vitro* comet and micronucleus assays and measuring urinary 8-oxo-2'-deoxyguanosine (8-oxodG).

**METHODS:** During three consecutive working days, polycyclic aromatic hydrocarbons (PAHs) and volatile organic compounds (VOCs) were sampled in workplace air of 34 sewage and 30 office workers, as indicators of airborne exposure. The last day, subjects collected their 24hr urine. Genotoxicity of urinary extracts was assessed by comet and micronucleus assays on a HepG2 cell line. Using competitive enzymatic immunoassay we evaluated the 24hr urinary 8-oxodG excretion. Benzo(a)pyrene [B(a)P] toxicity equivalent factors and inhalation unit risk for B(a)P and benzene were used to characterize cancer risks.

**RESULTS:** The urinary extracts of sewage workers showed higher genotoxicity ( $P < 0.001$ ) than office workers. Although not statistically greater than office workers, the 24hr urinary 8-oxodG mean levels among sewage workers were associated ( $P = 0.04$ ) with years of work in the sewage system. Workplace air concentrations of PAHs (e.g. 23.7 [range 2.4-104.6]  $\text{ng.m}^{-3}$  for fluoranthene) and VOCs (e.g.  $19.1 \pm 2.9$  [standard error]  $\mu\text{.m}^{-3}$  for benzene) were elevated in sewage compared to office workplaces ( $P < 0.01$ ) and resulted in an increased lifetime cancer risk.

**CONCLUSIONS:** The integrated and non-specific urinary biomarkers showed that sewage workers experience exposure to mixtures of genotoxicants in the workplace, and suggest a DNA oxidative stress that probably increases with work seniority.

## **Background**

Sewage workers provide an essential service that contributes to the protection of public health. Their role is to maintain the sewage system through which wastewaters and hazardous agents produced by our urbanized society are disposed of. Sewage system receives deposits of polycyclic aromatic hydrocarbons (PAHs) and solubilised volatile organic compounds (VOCs) from different sources such as traffic exhausts, industries, waste incinerators, and domestic heating via both atmospheric deposition and activities of the society (Bris et al. 1999; Manoli and Samara 1999). Many other chemicals could also be found in the sewage workplace environment, such as fluorinated hydrocarbons, heavy metals, pesticides, dyes, nitrosamines and polychlorinated biphenyls (Hansen et al. 2003; Mulloy 2001; Lyons et al. 2000). As a result, sewage workers might be subjected to exposure to a wide and complex variety of chemicals; many are known or suspected genotoxicants and/or carcinogens (Krishnamurthi et al. 2003; Sorensen et al. 2003). Indeed, although scant and not completely consistent, many studies showed an increase in the incidence of cancer and total mortality (Friis et al. 1999; Hansen et al. 2003; Wild et al. 2006) among sewage workers.

This complex exposure varies along time, locations, concentration levels and pathways. It is often intermittent, occasionally acute, over a chronic background. These exposure fluctuations cannot be easily captured by measuring a single or limited number of pollutants at a given time and place or by exploring one route of exposure (Callahan and Sexton 2007). An attractive alternative approach is the use of biomarkers. This may be achieved by collecting samples from easily obtainable biological material in order to assess the total individual exposure to non-specific substances with which subjects come in contact through different pathways (lung, skin and gastrointestinal tract) and sources (air, diet, lifestyle or occupation) (Ryan et al. 2007). In addition, the use of non-specific biomarkers of exposure and of early effects in exposed workers, together with careful assessment of workplace at various locations and over time, could be a tool to gain insight into the hazardous potency of such complex occupational settings. It might also support the link between occupational exposure and the risk of adverse health effects (Callahan and Sexton 2007).

In the human body, toxicants like PAHs and VOCs may appear as intact compounds or metabolites, in particular in the urine, within a few hours or days following exposure (Ma et al. 1992). In contrast to measurements of workplace air concentrations of specific compounds, assessment of the internal dose of these compounds or of their metabolites, offers the advantage to represent the effective body uptake through different routes of exposure, to

account for personal metabolism activities and the usage of protective devices by the workers. However, specific biomarkers fall short to express a complex exposure to a variety of compounds, a situation that sewage workers experience, among other occupations. Many compounds encountered in the sewage system are genotoxicants (Hansen et al. 2003). Urine genotoxicity assessment might thus be an appropriate approach to integrate the exposure to an array of genotoxic compounds that eventually results in a variety of urinary excreted metabolites which are too many to be individually quantified. Hence, the genotoxic potency of urine might be used as a biomarker of exposure to genotoxicants.

When the genotoxicants are incorporated by the human body, their metabolism may generate reactive oxygen species. The latter might interact with cell nucleus DNA, leading to oxidative DNA damage (Olinski et al. 2006). The 8-oxo-2'-deoxyguanosine (8-oxodG) is a biomarker of guanine oxidation in DNA and one of the most easily-formed DNA lesions. The DNA base excision repair pathway of oxidant-induced bases recognizes 8-oxodG as a threatening lesion resulting in its excretion in human urine without further metabolism (Cooke et al. 2005). Urinary assessment of this biomarker of early effect is an increasingly used non-invasive biomonitoring approach to estimate the overall DNA oxidative stress produced in the body (Olinski et al. 2006). The DNA damage represented by 8-oxodG is important in the pathogenesis of many diseases, including cancer (Cooke et al. 2005).

There is no information on the levels of DNA oxidized bases, mainly 8-oxodG, among underground sewage workers. However, male workers exposed to fly ash at solid waste incinerators, showed a significant increase in the mean levels of urinary 8-oxodG with duration and level of exposure (Yoshida et al. 2003). Data on personal exposure to PAHs and VOCs in the workplace air of underground sewage workers are not available. However, many studies have found these chemicals in wastewater treatment plants (Walker et al. 1999; Atasoy et al. 2004; Gasperi et al. 2008), in the air of municipal solid waste (Pierucci et al. 2005) and in sewage sludge (Mantis et al. 2005).

As part of a biomarker study to assess exposure of sewage workers to complex chemical mixtures (AL Zabadi et al. 2008), the aims of the present study were: (1) to evaluate the overall genotoxicity of urinary extracts of Parisian underground sewage workers, as a biomarker of exposure, and compare it with urines from office workers by comet and micronucleus assays, (2) to explore early effects through the assessment of DNA oxidative stress measured as the urinary excretion of 8-oxodG. In addition, we compared workplace air concentrations of PAHs and VOCs, used as indicators of airborne exposure in these two occupations.



## **Materials and Methods**

***Study population, setting and design.*** The study protocol has been described in detail elsewhere (AL Zabadi et al. 2008). Briefly, after organising periodical meetings with the sewage and office workers on a weekly-basis and over a 10 months period (July 2008-April 2009), 34 underground sewage workers and a control group of 30 office workers from the city of Paris were recruited. All were male volunteers, current non-smokers since at least 6 months, aged 20-60 years, employed at the same function for at least six months and having no history of chronic or recent illness. Interviews and biological sampling were conducted in the framework of regular occupational medical visits at the offices of occupational and preventive medicine in the Paris municipality. The study was approved by the local ethical committees and has been conducted according to Common Rule and in accordance with the principles for human experience as defined by the Helsinki Declaration. A signed informed consent was obtained from each participant.

During three consecutive days of work shifts prior to the medical visit, workplaces indoor air concentrations for 13 PAHs and 12 VOCs were assessed using personal active and passive samplers. Each subject collected 24hr urine in a sterile plastic collecting bottle in the last day of air sampling. Then, during the medical visit that took place on Thursdays or Fridays, subjects filled in two self-administered questionnaires. One for socio-demographic factors, non-occupational exposures (especially possible PAHs and VOCs exposures related to commuting, to area of residence and indoor sources), medical history, lifestyle (smoking history, including passive smoking, alcohol consumption and medications), and other confounders. The second for diet habits including daily intake of fruits and vegetables.

***Chemicals, media and reagents.*** Unless otherwise specified, all chemicals and culture media used were purchased from Sigma-Aldrich Chimie S.A.R.L (L'Isle d'Abeau Chesnes, France). For 8-oxodG analysis, the 96-well kits were purchased from CliniSciences SA, Montrouge, France (origin: StressMarq Biosciences Inc., Victoria, BC Canada).

***Workplaces air sampling and analysis.*** The sampling equipments were placed in a backpack and handed to one subject per work team (a team being generally composed of 2 to 3 subjects) with oral and written instructions. The backpack contained a personal active sampling ChemPass pump (Rupprecht and Patastnick Co., Inc. NY, USA) set with a calibrated flow rate of 4 L/min, to measure PAHs. It was carried during the sewage activities or placed on a desk nearby the office workers. A VOCs passive sampling badge (Radiello

code 145, Sigma-Aldrich, France) was also provided and attached to workers' clothes near the breathing zone.

PAHs in particulates and gaseous forms were collected on a quartz filter (Supelco 21038, 32 mm of diameter) and a cartridge containing polyurethane foam (Supelco ORBO 2-0600); respectively. The pump was equipped with a timer and subjects had to turn it on during work shifts and off when finished. The flow rate was measured before and after sampling and the sample was rejected if the difference was greater than 10%. For the analysis, the filter and the foam were subjected to extraction by accelerated solvent (hexane-acetone; 50/50, v/v). The extract was concentrated in a water bath at 35°C using automatic evaporator (Turbo Vap II Zymark), then under nitrogen flow evaporator (N-Evap, MA, USA) until obtaining an oily drop. The drop was taken by 1ml of acetonitrile solvent compatible high performance liquid chromatography by which the extracts were analyzed using a fluorescence emission detector (Waters 2475). The quantification analysis was carried out according to the calibrated response of standard solutions of marked-mixture for the selected PAHs.

VOCs were captured on sorbent tubes composed of a thermally-preconditioned cartridge containing an adsorbent (Type Carbograph 4, charcoal graphitization), and a diffusive body of porous polyethylene membrane (Radiello code 120-2, Sigma-Aldrich, France) placed on a triangular support plate (code 121). The supplier indicated the molecular diffusion rates. The sampling time was informed by the participants who filled a sampling sheet. The filters were immediately preserved in an aluminium sheet to avoid photochemical transformations. The filters and sorbent tubes were stored at 4°C until analysis. Analysis was carried out by coupling gas chromatography and mass-spectrometry (TD GC-MS). VOCs were thermally-desorbed from the tube at 370°C for 15 min on a thermal adsorbent (Turbomatrix, Perkin Elmer, PMDEST003) and concentrated on a cryogenic trap (M041-3627). Then desorbed from the trap (secondary desorption) by a thermal flash and transported via an inert gas carrier to a gas chromatography (Autosystems XL, Perkin Elmer) coupled to a mass spectrometry quadripole detector (Turbomass Gold, Perkin Elmer). The gas chromatography was equipped with a capillary column (RTX-1 100% dimethyl polysiloxane, RESTEK) powered by helium. The quantitative analysis was realised according to the response of ions extracts of a pre-determined list. Calibration was achieved by doping liquid using standard solutions.

The workplace collection of the PAHs and VOCs were performed from Monday to Thursday for sewage workers and from Monday to Wednesday for office workers. For comparison with ambient air pollution, results from the Paris air quality monitoring network (AIRPARIF) were

retrieved. It assesses the same PAHs and 6 out of the 12 VOCs selected for this study in different background monitoring stations throughout Paris. The average concentrations at the same or at the nearest days of the study period, were obtained from the closest monitoring stations relative to the sampled underground workplaces, with each time two types of monitors: one measuring background air quality (away from specific traffic or industrial emission sources) and the other close to traffic sources.

Thirteen PAHs were quantified [twelve listed as priority pollutants by the United States Environmental Protection Agency (U.S. EPA) plus benzo(j)fluoranthene classified as probable carcinogen-B2 by the U.S EPA]. Of these, nine in particulate form (chrysene, benzo(b)fluoranthene, benzo(a)anthracene, benzo(k)fluoranthene, benzo(a)pyrene (B(a)P), dibenzo(a,h)anthracene, benzo(g,h,i)perylene, indeno(1,2,3,-c,d)pyrene and benzo(j)fluoranthene), and 4 in gaseous form (phenanthrene, anthracene, fluoranthene and pyrene). Twelve VOCs were also quantified: 7 monocyclic aromatic hydrocarbons (benzene, ethylbenzene, toluene, m + p-xylenes, o-xylene, 1,2,4-trimethylbenzene and styrene), two alkanes (decane and undecane) and 3 chlorinated compounds (trichloroethylene, tetrachloroethylene and 1,4-dichlorobenzene).

***Extraction of urinary organic fraction.*** The volume of the 24hr urine was measured and a sample of 150 ml was immediately coded and frozen at -20°C for each subject. For the assays, samples were then thawed and 50ml were centrifuged at 3000 t/min for 5 min. The supernatant (40ml) was collected in a sterile tube. The organic fraction was extracted on a Column Sep Pak<sup>®</sup> Vac C18 Cartridges (Waters, WAT020805, Associates, Inc) on an aspirated tray (J.T. Baker spe -12G). The cartridge was washed 2x 3 ml of absolute methanol, then 3x 3ml of ultra-pure water. It was then loaded with the 40ml urine. The column was washed 2x 3ml of ultra-pure water and the adsorbed organics were eluted with 3x 3ml of absolute acetone (Carlo, CAS 67-64-1). The eluate was evaporated at 45°C under nitrogen stream (Tech Lab Faster-Chemfree, France) until complete dryness. The residue was suspended in 500µl DMSO and stored at -20°C. All operations were done at room temperature.

***Comet assay.*** Comet assay was performed essentially as in Singh et al. (1988), with some modifications as in Muller-Pillet et al. (2000). Briefly, HepG2 cells (ATCC, catalog number HB-8065) were cultivated for 24hr in 5ml complete culture medium supplemented with 50µl of organic urine extract. After harvesting and centrifugation, the cell pellet was diluted by the culture medium to  $5 \times 10^5$  cell/ml. Cell viability determined by trypan blue exclusion technique was constantly found to be over 95%. After layering the cells on conventional

microscopic slides previously sprayed by normal melting point agarose, they were lysed at 4°C in the dark for at least 1hr (2.5 M NaCl, 100mM Na<sub>2</sub>EDTA, 10mM Tris and 1% Triton X-100, Prolabo 28.817.295 and 10% DMSO, pH= 10). The electrophoresis was conducted (20V, 0.62 V/cm, 300 mA, 20 min) in an electrophoresis gel system (EC340, Maxicell<sup>®</sup> Primo, Holbrook, New York) filled with electrophoresis buffer (300mM NaOH, 1 mM Na<sub>2</sub>EDTA, pH 13). B(a)P 40µM and DMSO 1% final concentrations (positive and negative controls; respectively) were added with each electrophoretic run. After neutralizing the slides in 0.4M Tris buffer, they were stained with 40µl ethidium bromide (2µg/ml in ultra-pure water). 50 cells (two slides/subject and 25 cells/slide) were examined for DNA migration using an Olympus BX40 fluorescence microscope (Olympus, Tokyo, Japan). We quantified DNA damage by a computerized image analysis system (Komet 4.02 software, Kinetic Imaging, UK) in evaluating the % DNA tail parameter.

***Cytokinesis block micronucleus assay.*** We adapted the assay described by Fenech (2007). Briefly, at time=0, HepG2 cells were grown in 10 ml complete medium for 24hr on autoclaved conventional microscopic slides placed in Petri dishes (L.E.S Sayag BP50). The culture medium was then replaced by 10ml of freshly diluted medium supplemented with 100µl of organic urine extract (time=24hr). Subsequently, at time=44hr, the cells were cultivated in a fresh complete culture medium supplemented with 3µg/ml cytochalasin-B final concentration. At time=72hr, cells were rinsed in cold hypotonic KCL solution (0.075M, Prolabo 26.764.298) and then fixed in CARNOY solution (methanol, Carlo Erba 525.102: acetic acid, Prolabo 20.104.298, 3:1 v:v). The air-dried slides were stained with 40µg/ml Acridine orange solution in dark. The slides were examined at 200-fold magnification with an Olympus BX fluorescence microscope (Olympus, Tokyo, Japan). We evaluated the frequency of the micronuclei (MNi) formation in 1000 binucleated cells (BNed)/slide for each subject. We respected Fenech (2007) criteria in scoring the MNi. Cell proliferation and cytotoxicity were assessed by calculating the nuclear division index (NDI) on 150 viable cells according to Fenech (2007) formula:  $NDI = (M1 + 2M2 + 3M3 + 4M4) / N$ , where M1-M4 represent the number of cells with 1-4 nuclei and N is the total number of viable cells scored (excluding necrotic and apoptotic cells). The NDI mean difference between exposed and non-exposed and between positive and negative controls (B(a)P 40µM and DMSO 1% final concentrations; respectively) was always acceptable and below 25% (Kirsch-Volders et al. 2000).

***Analysis of 24hr urinary 8-oxodG.*** The urine aliquots (1ml each) were kept at -20°C. They were thawed at room temperature immediately before analysis. The 8-oxodG was measured with a competitive enzymatic immunoassay (EIA) kit named 8-oxodG EIA kit. This assay

utilizes a specific 8-oxodG monoclonal anti-body (Catalog# SKC-120A), 8-oxodG-acetylcholinesterase (AChE) conjugate and an anti-mouse IgG-coated plate. We followed the protocol provided by the manufacturer in the analysis (StressMarq Biosciences Inc. 2008). To insure the accuracy and reproducibility of results, each sample was assayed at two dilutions (1/300 and 1/400) and each at duplicates. The 24hr 8-oxodG excretion was related to body weight (Loft et al. 1999; Sørensen et al. 2005).

**Urinary creatinine analysis.** Creatinine in 24hr urine was determined photometrically as picrate, according to Jaffé method (Tausky, 1954).

**Statistical analysis.** PAHs values were highly dispersed; their concentrations are presented as mean (range) while VOCs are exhibited as mean±SE . For comparisons between workplace and ambient air concentrations, the number of measurement data (26 in workplaces and 23 in ambient air) were used. For analyses of associations between biomarkers and exposure, the workplace air concentrations were assigned to each subject within a team as his exposure level. The *in vitro* genotoxicity assays results are expressed as mean±SD. Chi square and Fisher exact tests were used to analyse the differences between categorical variables. ANOVA was used to compare the mean differences of quantitative variables between the exposed and control groups, and to evaluate the effect of some socio-demographic and putative confounding factors (e.g., smoking and alcohol consumption) on comet and micronucleus results. Multiple linear regression was conducted to evaluate the level of 8-oxodG among the two study groups while adjusting for possible confounding factors. Multiple linear regression were also implemented to assess the association between exposure to occupational agents and the two genotoxicity assays while adjusting for confounding variables. Only pollutants significantly associated ( $P<0.05$ ) in univariate analysis were tested, while confounders were retained if  $P<0.1$  in univariate analyses. Effect modification was tested, in particular by age whose distribution was partitioned by the median value into two categories ( $\leq 39$  and  $<39$ years). All statistical analyses were performed using SPSS 16 software (Statistical Package for Social Science) (SPSS, Inc., Chicago, USA).

To assess and characterize the carcinogenic potency of PAH mixtures, toxicity equivalent factors (TEFs) were used to convert PAH exposure into an estimated B(a)P equivalent. We used the Nisbet and Lagoy (1992) TEFs with the exception for benzo(j)fluoranthene where the Collins et al. (1998) proposed TEF has been used. Cancer risk due to inhalation of PAH mixtures was then calculated using the B(a)P inhalation risk cancer unit value of  $1.1 \times 10^{-6} (\text{ng}/\text{m}^3)^{-1}$  proposed by the U.S. EPA (U.S. EPA 2009). We also used the benzene

unit risk range of  $2.2 \times 10^{-6}$  to  $7.8 \times 10^{-6} (\mu\text{g}/\text{m}^3)^{-1}$  (U.S. EPA 2007) to assess the cancer risk by inhalation of benzene.

## **Results**

***Characteristics of the study population.*** General characteristics of the study population are summarized in Table 1. All participants were males, with a mean age of 35.9 (SD, 7.5) and 43.3 (SD, 8.2) years in sewage (n=34) and office (n= 30) workers respectively ( $P<0.001$ ). The mean $\pm$ SD number of working years in sewage was  $7.05\pm 6.9$  years. Never smokers were more frequent in sewage than in office workers ( $P=0.03$ ). However, sewage workers were more likely to drink alcohol regularly than the control group ( $P=0.01$ ), and less likely to eat vegetables than the office workers ( $P=0.01$ ). The 24hr urinary creatinine differed between the two groups ( $P=0.003$ ) but was within the normal human male values for both groups. The level of education also distinguished the study groups ( $P=0.04$ ). No other difference was seen regarding factors that might influence study-relevant exposures: environmental tobacco smoke ( $P=0.87$ ), type of heating system used at home (individual or collective,  $P=0.52$ ), declared proximity of homes to industrial installations ( $P=0.82$ ) or consumption of barbecue grilled food.

**Table 1.** Population characteristics and exposure factors.

<b>Characteristics and exposure factors</b>	<b>Sewage workers (N=34)</b>	<b>Office workers (N=30)</b>	<b>Total Sample (N=64)</b>	<b>P-value</b>
<b>Age (year)</b>	35.85±7.54	43.30±8.15	39.3±8.6	<b>0.001*</b>
<b>Weight (Kg)</b>	77.8±12.2	76.9±10.6	77.4±11.4	0.75
<b>BMI (Kg/m<sup>2</sup>)</b>	25.5±3.3	25.3±4.2	25.4±3.7	0.82
<b>24hr urinary volume (ml)</b>	1500±691.5	1750 ±655.8	1617.2±681.4	0.14
<b>24hr Urinary creatinine (g/l)</b>	1.3±0.55	0.91±0.43	1.2±0.54	<b>0.003*</b>
<b>Place of residence</b>				
-Urban	8 (23.5)	9 (30)	17 (26.6)	0.47
-Suburbs	22 (64.7)	15 (50)	37 (57.8)	
-Rural	4 (11.8)	6 (20)	10 (15.6)	
<b>Marital status</b>				
-Married	17 (50)	10 (33.3)	27 (42.2)	0.20
-Not married	17 (50)	20 (66.6)	37 (57.8)	
<b>Level of education</b>				
-< 12 years of schooling	2 (5.9)	8 (26.7)	10 (15.6)	<b>0.04*</b>
-> 12 years of schooling	32 (94.1)	22 (73.3)	54 (84.4)	
<b>Smoking</b>				
-Never smokers	29 (85.3)	18 (60)	47 (73.4)	<b>0.03*</b>
-Ex-smokers	5 (14.7)	12 (40)	17 (26.6)	
<b>Exposure to passive smoking</b>				
-Yes	20 (58.8)	17 (56.7)	37 (57.8)	0.87
-No	14 (41.2)	13 (43.3)	27 (42.2)	
<b>Alcohol consumption</b>				
-Regularly	23 (67.6)	10 (33.3)	33 (51.6)	<b>0.01*</b>
-Occasionally	11 (32.4)	20 (66.7)	31 (48.4)	
<b>Use of barbecue last week</b>				
-Yes	3 (8.8)	0 (0)	3 (4.7)	0.20
-No	31 (91.2)	30 (100)	61 (95.3)	
<b>Massive physical activity (last two days)</b>				
-Yes	6 (17.6)	4 (13.3)	10 (15.6)	0.74
-No	28 (82.4)	26 (86.7)	54 (84.4)	
<b>Fruits intake</b>				
-Usually	21 (61.8)	23 (76.7)	44 (68.8)	0.29
-Sometimes	13 (26.5)	7 (16.7)	20 (21.9)	
<b>Vegetables intake</b>				
-Usually	19 (55.9)	26 (86.7)	45 (70.3)	<b>0.01*</b>
-Sometimes	15 (44.1)	4 (13.3)	19 (29.7)	
<b>Intake of vitamins/minerals</b>				
-Yes	6 (17.6)	2 (6.7)	8 (12.5)	0.27
-No	28 (82.4)	28 (93.3)	56 (87.5)	

Data are frequencies (percentage) or mean±SD. BMI, body mass index (the square of the height over the body weight). \*Statistically significant (p <0.05).

**Concentrations of PAHs and cancer risk characterization.** The mean workplace exposure levels of each PAH compound presented in Table 2 were significantly higher among sewage workers compared to office workers and to ambient air concentrations ( $P<0.01$ ). In general, phenanthrene, fluoranthene and pyrene contribute the largest portion of the total PAHs exposure (e.g. among sewage workers they ranged from 43%, 15%, and 12%; respectively). The other PAHs amount to less than 5%, except anthracene after the traffic monitor ambient

air measurements (11.5%). The highest B(a)P concentration was found among sewage workers with a range of 0.5 to 62.1 ng/m<sup>3</sup> and a mean value of 7 ng/m<sup>3</sup>.

Based on these data, single PAHs concentrations were converted into total B(a)P equivalent concentration ([B(a)P]eq) using the TEFs (see statistical analysis) and translated into lifetime cancer risk estimates. The average sewage workers' total [B(a)P]eq exposure value (13.66 ng/m<sup>3</sup>) is more than 10 times greater than those encountered for office workers, traffic and urban ambient air levels (respectively 1.15 ng/m<sup>3</sup>, 1.08 ng/m<sup>3</sup>, and 0.73 ng/m<sup>3</sup>). Table 2 also shows the associated lifetime cancer risks. The PAHs cancer risk level for sewage workers is 1.5 x 10<sup>-5</sup> (1.3 x 10<sup>-6</sup> for office workers).

**Table 2.** The mean (range) (ng.m<sup>-3</sup>) concentration at the workplaces and the corresponding nearest ambient air measurements of PAHs, and the derived total [B(a)P]eq and cancer risk estimates.

PAHs	Sewage workplace (n=26)	Office workplace (n=26)	Traffic (n=23)	Urban (n=23)
Benzo(a)pyrene	6 (0.5-62.1)	0.4 (0.2-2.4)	0.7 (0.1-1.6)	0.5 (0.2-5.8)
Anthracene	6.7 (0.5-32.1)	0.9 (0.1-1.8)	3.2 (0.3-14.5)	0.2 (0.02-0.8)
Benz(a)anthracene	4.6 (0.3-31.8)	0.5 (0.2-2.4)	0.5(0.2-1.2)	0.2 (0.02-0.6)
Benzo(b)fluoranthene	4.3 (0.5-30.1)	0.4 (0.1-2.4)	0.6 (0.2-1.6)	0.3 (0.05-1.1)
Benzo(g,h,i)perylene	4.4 (0.4-24)	0.5 (0.1-3.8)	0.8 (0.2-1.7)	0.3 (0.06-1)
Benzo(k)fluoranthene	2.0 (0.2-15.2)	0.2 (0.08-1.2)	0.2 (0.07-0.6)	0.2 (0.01-2.5)
Chrysene	7.7 (1-30)	2.2 (1.1-6.8)	0.7 (0.3-0.2)	0.3 (0.1-0.8)
Dibenz(a,h)anthracene	0.9 (0.01-5.4)	0.1 (0.01-0.5)	0.02 (0.01-0.06)	0.02 (0.01-0.06)
Fluoranthene	23.7 (2.4-104.6)	4.3 (2.5-8.1)	3.8 (2-5)	1.6 (0.8-4.3)
Indeno(1,2,3-cd)pyrene	3 (0.3-15.9)	0.3 (0.08-2.4)	0.4 (0.07-1)	0.2 (0.02-0.7)
Phenanthrene	71.2 (12.5-220)	22.3 (9-43.9)	11.9 (5.2-18.1)	5.2 (2.3-14.2)
Pyrene	19.3 (2.3-78.4)	5.5 (1.2-21.7)	4.8 (2.8-6.3)	1.3 (0.6-3.4)
Benzo(j)fluoranthene	3.9 (0.3-21.6)	0.4 (0.1-3.3)	0.4 (0.03-1.2)	0.2 (0.03-0.8)
Total [B(a)P]eq (ng m <sup>-3</sup> )	13.66	1.15	1.08	0.73
B(a)P equivalent lifetime cancer risk	15.02 x 10 <sup>-6</sup>	1.26 x 10 <sup>-6</sup>	1.19 x 10 <sup>-6</sup>	0.80 x 10 <sup>-6</sup>

a. B(a)P TEFs according to Nisbet and LaGoy (1992), for benzo(j)fluoranthene Collins et al. (1998) value was applied.

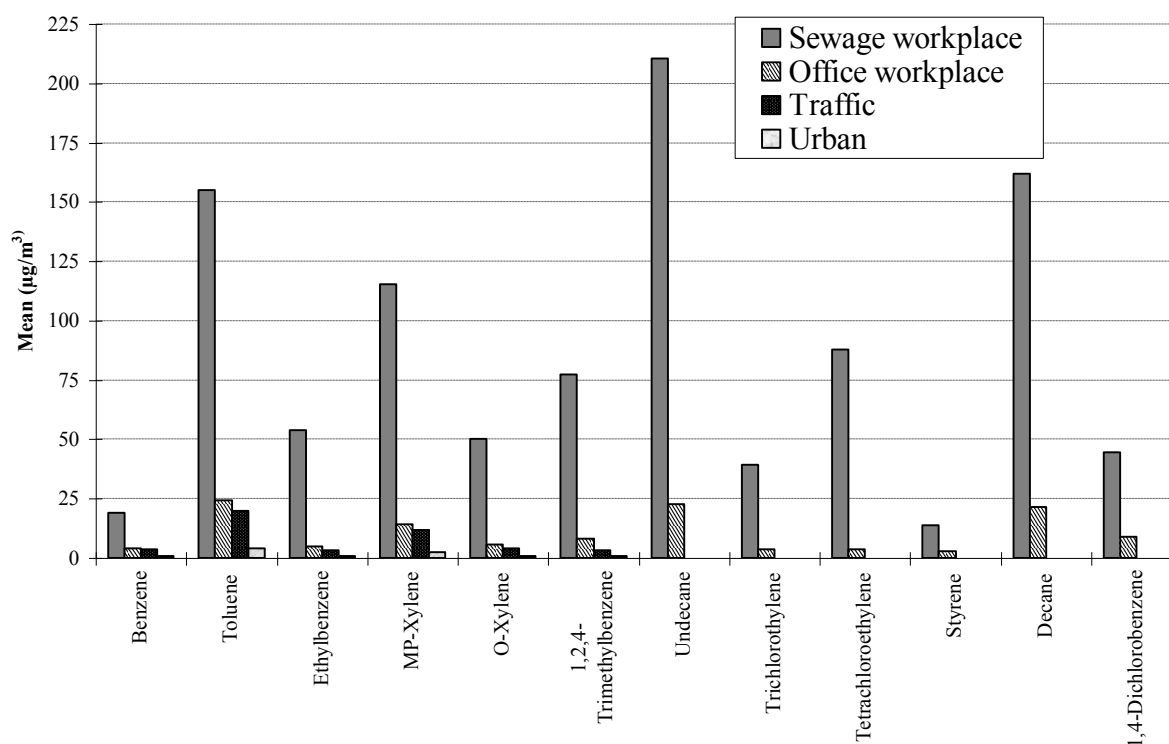
b. The U.S. EPA inhalation unit cancer risk of B(a)P = 1.1 x 10<sup>-6</sup> (ng/m<sup>3</sup>)<sup>-1</sup> was used (U.S. EPA 2009). Total [B(a)p]eq, total benzo(a)pyrene equivalent concentration.

**Concentrations of VOCs and related cancer risk characterization.** Figure 1 presents the mean of each VOC concentrations in the workplace air of sewage and office workers, and in



the air of urban and traffic environments nearest to the study locations. A high heterogeneity was observed between the different locations with the highest values found in the sewage workplace ( $P<0.01$ ), followed by indoor air of office workers, traffic and urban background ambient air, respectively. Comparison of workplace and ambient air concentrations is incomplete because some VOCs measured in the workplaces are not measured by traffic or background urban monitors (undecane, trichloroethylene, tetrachloroethylene, styrene, decane and 1,4-dichlorobenzene).

Benzene (mean $\pm$ SE) concentrations were  $19.1\pm 2.9$  and  $4.1\pm 0.53$   $\mu\text{g}/\text{m}^3$  among sewage and office workers, respectively; corresponding values were  $3.7\pm 0.13$  and  $1.0\pm 0.09$   $\mu\text{g}/\text{m}^3$  in traffic and urban background, respectively. Using the Integrated Risk Information System (IRIS) unit risk estimate range (U.S. EPA 2007), the benzene associated lifetime excess cancer risk for sewage workers ranged between  $4.2 \times 10^{-5}$  to  $14.9 \times 10^{-5}$  ( $9 \times 10^{-6}$  to  $3.2 \times 10^{-5}$  for office workers).

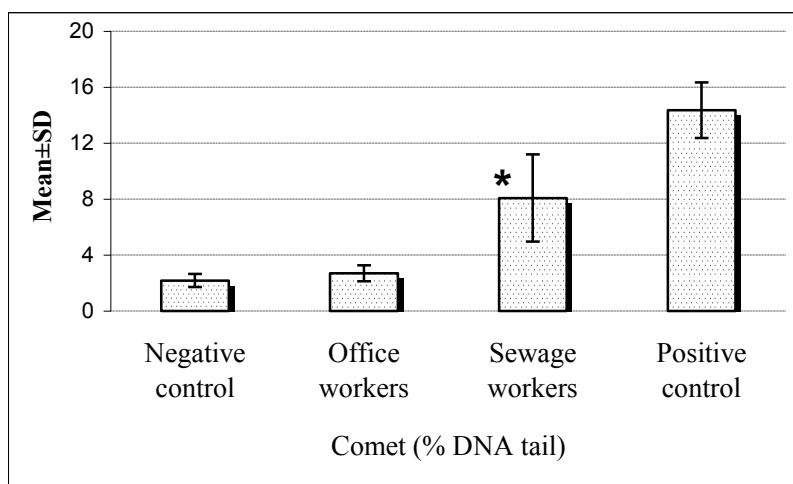


**Figure 1.** Volatile organic compounds (VOCs) concentration patterns in the workplaces air and from the nearest outdoor monitoring stations.

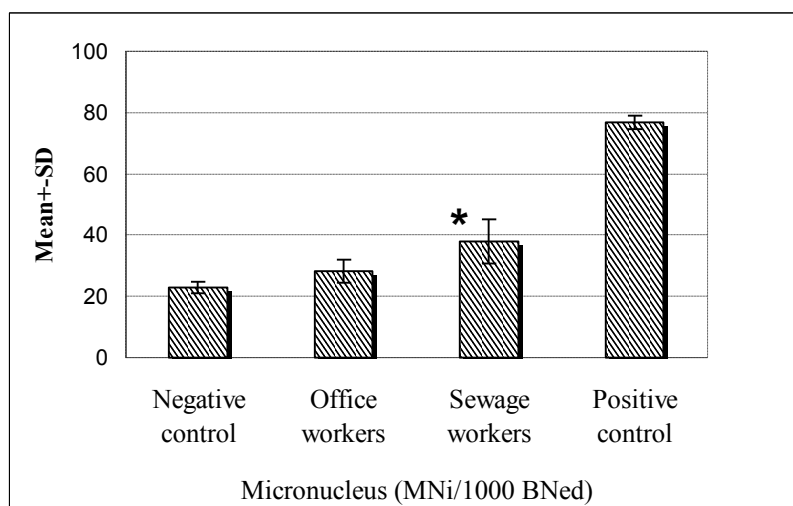
Statistically significant higher means concentrations ( $P< 0.01$ ) among sewage workers compared to all other groups for all substances. Empty data were not measured by Paris air quality monitoring network (AIRPARIF).

***In vitro genotoxicity assays on urinary extracts.*** The mean % DNA tail and MNi/1000 BNed among sewage workers was statistically higher than in office workers [mean $\pm$ SD of % DNA

tail=  $8.07 \pm 3.12$  and  $2.70 \pm 0.58$ , and mean $\pm$ SD of MNi/1000 BNed=  $38.02 \pm 7.16$  and  $28.30 \pm 3.74$ ; respectively in the two populations [ $P < 0.001$  in both tests] (Figure 2A and B). In multivariate linear regression models, we tested the differences in % DNA tail and MNi/1000 BNed between the two study occupation groups while adjusting for possible confounders including 24hr urinary creatinine. The differences between sewage and office worker are, respectively (point estimate and [95% confidence interval]), 5.01 [3.01-7.00] for % DNA tail and 9.41 and [4.47-14.36] for MNi/1000 BNed. No interaction was observed according to subjects' characteristics.



**Figure 2A**



**Figure 2B**

**Figure 2.** The means $\pm$ SD of urine genotoxicity biomarkers of exposure as performed with the subjects organic urine extracts on HepG2 cells.

A) Mean $\pm$ SD of %DNA tail of comet; B) Mean $\pm$ SD of MNi/1000 BNed.

For comet assay, 9 pairs of positive and negative controls were performed. For micronucleus assay, 5 pairs of positive and negative controls were performed. \*Statistically significant ( $P < 0.001$ ) compared to office workers. BNed, Binucleated cells; MNi, micronuclei.

***In vitro genotoxicity assays and workplace air concentrations.*** The %DNA tail was positively associated with age and educational level with a borderline positive effect of alcohol consumption ( $P=0.09$ ) and a protective effect of vegetable intake ( $P=0.05$ ). MNi/1000 BNed was positively associated with age and alcohol consumption while inversely with vegetable intake. Table 3 also presents the results of the association of % DNA tail and MNi/1000 BNed with each individual VOC and PAH.

**Table 3:** Univariate analysis for factors associated with genotoxicity assays (%DNA tail and MNi/1000 BNed) performed with urine extracts on HepG2 cells: statistical significance of associations (N=64).

Independent variables	P values	
	%DNA tail	MNi/1000 BNed
<b>Socio-demographic characteristics</b>		
Marital status (married/not married)	0.12	0.94
Smoking (ex-smokers/never smokers)	0.16	0.18
Educational level; years (>12/≤12)	0.04	0.23
Age; years (≤39/>39)	0.01	0.02
Alcohol consumption (regularly/occasionally)	0.09	0.05
Vegetable intake (usually/sometimes)	0.05	0.02
<b>VOCs</b>		
Benzene	0.01	0.001
Toluene	0.14	0.004
Ethylbenzene	0.01	0.001
M+P-Xylene	0.002	<0.001
O-Xylene	0.003	<0.001
1, 2,4 Trimethylebenzene	0.02	<0.001
Undecane	0.05	<0.001
Trichloroethylene	0.07	0.05
Tetrachloroethylene	0.01	0.19
Styrene	0.09	0.002
Decane	0.001	<0.001
1,4 Dichlorobenzene	0.06	0.02
<b>PAHs</b>		
Benzo(a)pyrene	0.40	0.35
Anthracene	0.02	0.17
Benzo(a)anthracene	0.20	0.20
Benzo(b)fluoranthene	0.16	0.22
Benzo(g,h,i)perylene	0.09	0.22
Benzo(k)fluoranthene	0.21	0.24
Chrysene	0.01	0.03
Dibenz(a,h)anthracene	0.02	0.51
Fluoranthene	0.04	0.06
Indeno(1,2,3-cd)pyrene	0.08	0.20
Phenanthrene	0.01	0.03
Pyrene	0.03	0.11
Benzo(j)fluoranthene	0.06	0.26
Total [B(a)P]eq	0.15	0.34

Total [B(a)p]eq, total Benzo(a)pyrene equivalent concentration; MNi, micronuclei; BNed; binucleated cells.

Results of the multiple linear regression models are presented in Table 4 that exhibits the association between each of the *in vitro* genotoxicity assays and the assigned personal atmospheric exposure variables, controlling for covariates. Effect modification is also accounted for, with age being the sole factor influencing this association. A significantly positive association with the comet test response was seen among older workers only (>39 ears; n=34) for nine PAHs (the 4 gaseous PAHs and 5 out of the 9 particulate PAHs). This association with PAHs was not found for the MNi/1000 BNed. All VOCs were significantly associated with MNi/1000 BNed among older workers, while % DNA tail was only influenced by exposure to benzene, ethylbenzene, m+p-xylene, o-xylene, decane, tri and tetra-chloroethylene. Noteworthy is that no difference in 24hr urinary volume or creatinine levels between the two age groups was observed ( $P=0.91$  and  $0.20$ ; respectively).

**Table 4:** Association between exposures to workplace toxicants and genotoxicity assays (% DNA tail and MNi/1000 BNed) performed with urine extracts on HepG2 cells: results of multiple linear regression models (N=64)\*.

Independent variable	%DNA tail			MNi/1000 BNed		
	B	P value	95%CI for B	B	P value	95%CI for B
<b>VOCs</b>						
Benzene						
❖ ≤ 39 yrs	0.03	0.61	-0.08-0.14	0.03	0.78	-0.17-0.23
❖ > 39 yrs	0.08	0.03	0.01-0.16	0.41	0.00	0.23-0.56
Ethylbenzene						
❖ ≤ 39 yrs	0.02	0.24	-0.01-0.05	-0.01	0.80	-0.06-0.05
❖ > 39 yrs	0.02	0.05	0.00-0.03	0.10	0.00	0.07-0.13
M+P-Xylene						
❖ ≤ 39 yrs	0.01	0.09	-0.002-0.02	0.002	0.9	-0.02-0.03
❖ > 39 yrs	0.01	0.03	0.001-0.02	0.05	0.00	0.03-0.06
O-Xylene						
❖ ≤ 39 yrs	0.03	0.12	-0.01-0.06	0.01	0.66	-0.05-0.07
❖ > 39 yrs	0.02	0.04	0.001-0.03	0.11	0.00	0.07-0.14
Decane						
❖ ≤ 39 yrs	0.01	0.07	-0.001-0.02	0.003	0.76	-0.02-0.02
❖ > 39 yrs	0.01	0.03	0.001-0.01	0.04	0.00	0.03-0.05
Tetrachloroethylene				NT	NT	NT
❖ ≤ 39 yrs	0.02	0.31	-0.02-0.05			
❖ > 39 yrs	0.01	0.002	0.004-0.02			
Trichloroethylene						
❖ ≤ 39 yrs	0.003	0.84	-0.03-0.04	-0.03	0.27	-0.10-0.03
❖ > 39 yrs	0.02	0.02	0.003-0.03	0.06	0.002	0.03-0.10
Toluene	NT	NT	NT			
❖ ≤ 39 yrs				-0.004	0.57	-0.02-0.01
❖ > 39 yrs				0.02	0.00	0.01-0.03
1,4 Dichlorobenzene <sup>§</sup>	0.01	0.39	-0.01-0.03			
❖ ≤ 39 yrs				-0.03	0.38	-0.10-0.04
❖ > 39 yrs				0.11	0.00	0.06-0.16
1, 2,4Trimethylebenzene <sup>§</sup>	0.01	0.12	-0.002-0.02			
❖ ≤ 39 yrs				0.01	0.81	-0.03-0.04
❖ > 39 yrs				0.07	0.00	0.05-0.09

Table 4 (continued) :

Undecane <sup>§</sup>	0.003	0.23	-0.002-0.01			
❖ ≤ 39 yrs				0.01	0.14	-0.004-0.03
❖ > 39 yrs				0.03	0.00	0.02-0.03
Styrene <sup>§</sup>	0.03	0.36	-0.03-0.09			
❖ ≤ 39 yrs				-0.07	0.52	-0.29-0.15
❖ > 39 yrs				0.34	0.00	0.23-0.44
<b>PAHs</b>						
Chrysene <sup>‡</sup>				0.10	0.50	-0.19-0.38
❖ ≤ 39 yrs	0.07	0.57	-0.18-0.32			
❖ > 39 yrs	0.20	0.04	0.01-0.39			
Fluranthene <sup>‡</sup>				0.02	0.68	-0.07-0.10
❖ ≤ 39 yrs	-0.002	0.96	-0.07-0.07			
❖ > 39 yrs	0.09	0.02	0.02-0.16			
Phenanthrene <sup>‡</sup>				0.01	0.51	-0.02-0.05
❖ ≤ 39 yrs	0.01	0.67	-0.02-0.04			
❖ > 39 yrs	0.04	0.01	0.01-0.07			
Pyrene				NT	NT	NT
❖ ≤ 39 yrs	0.004	0.92	-0.09-0.10			
❖ > 39 yrs	0.08	0.03	0.01-0.15			
Anthracene				NT	NT	NT
❖ ≤ 39 yrs	0.02	0.88	-0.24-0.27			
❖ > 39 yrs	0.25	0.01	0.06-0.44			
Benzo(g,h,i)perylene				NT	NT	NT
❖ ≤ 39 yrs	-0.01	0.96	-0.33-0.31			
❖ > 39 yrs	0.20	0.04	0.01-0.39			
Indeno(1,2,3-cd)pyrene				NT	NT	NT
❖ ≤ 39 yrs	-0.01	0.98	-0.49-0.48			
❖ > 39 yrs	0.34	0.03	0.03-0.65			
Benzo(j)fluoranthene				NT	NT	NT
❖ ≤ 39 yrs	-0.01	0.98	-0.37-0.36			
❖ > 39 yrs	0.26	0.04	0.02-0.51			
Dibenz(a,h)anthracene <sup>§</sup>	0.85	0.02	0.13-10.57	NT	NT	NT

\* Variables in the final models. (i) MNi/1000 BNed models: toxicants (one each time), age (≤ 39/>39 years), toxicant-age interaction, alcohol consumption (regularly/occasionally), vegetable intake (usually/sometimes). (ii) % DNA tail models: same as MNi/1000 BNed plus level of education (>12/≤12years).

<sup>§</sup> or <sup>‡</sup> Not significant interaction between toxicant and age for comet<sup>§</sup> or micronuclei<sup>‡</sup> assays. Toxicant effect adjusted for the other models variables.

NT: Not Tested because  $p > 0.1$  in univariate association between toxicant and biomarker (table 2).

Notes: Variables entered in the models are those with  $P < 0.1$  in univariate analysis. B, regression coefficient; CI, confidence interval; yrs, years; MNi, micronuclei; BNed; binucleated cells. Enter method was used.

**24hr urinary 8-oxodG.** Figure 3A shows the box and whisker plots of 24hr urinary 8-oxodG in sewage and office workers. There was a slightly (but not significantly) higher mean level in sewage compared to office workers (mean±SD, 8.26±4.26 pmole/kg 24h and 7.22±3.32 pmole/kg 24h respectively,  $P=0.28$ ). Interestingly, as shown by the scatterplot and the linear regression line in Figure 3B, 8-oxodG was positively associated with the number of working years in the sewage system (office workers were assigned a zero value;  $P=0.04$ ). Controlling for 24hr urinary volume and creatinine did not alter the association with working years in sewage ( $P=0.06$ ). There was no significant difference in 8-oxodG level regarding other exposure factors mentioned in Table 1. No clear association could be found between the

workplace concentrations of the measured pollutants or of total [B(a)P]eq and the level of 24hr urinary 8-oxodG (data not shown).

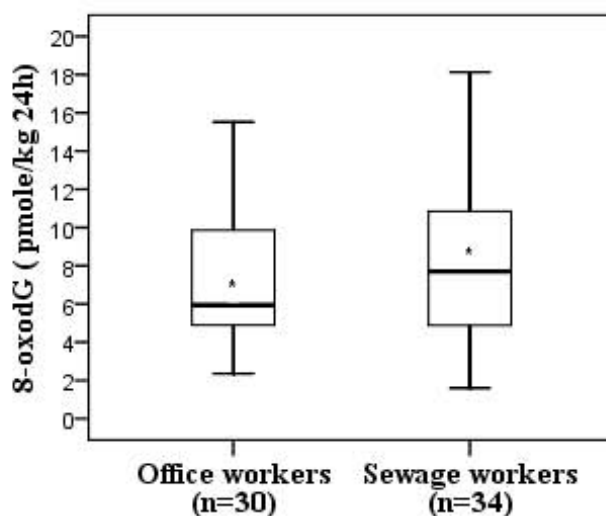


Figure 3A

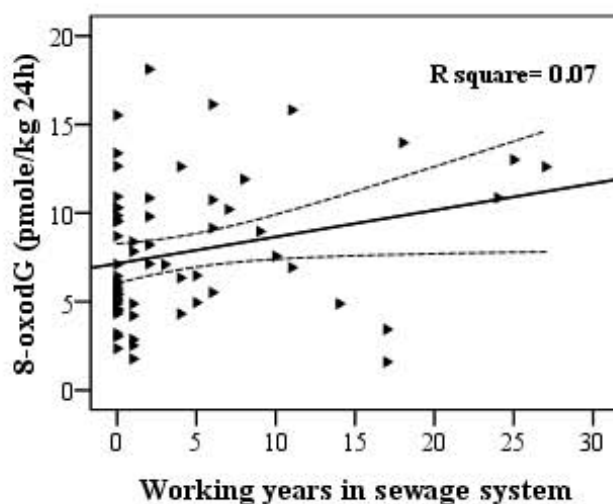


Figure 3B

**Figure 3.** The 24hr urinary 8-oxodG level (pmole/kg 24h). A) Box and whisker plot in sewage and office workers,  $P=0.28$  for the mean differences, \* Mean; B) Relationship with the number of working years in sewage system; assigned zero value to the office workers ( $P=0.04$ ). Scatterplot and regression line with 95% confidence intervals of the mean are shown. 8-oxodG, 8-oxo-2'-deoxyguanosine.

## Discussion

This is, to our knowledge, the first work that (i) applied the HepG2 cells comet and CBMN assays with urine organic extracts (as urinary biomarkers of exposure), (ii) assessed the 24hr urinary 8-oxodG excretion (biomarker of early effect), and (iii) measured workplace air concentrations of PAHs and VOCs (indicators of external exposure) among underground

sewage workers. The urinary extracts of sewage workers induced significantly greater DNA and chromosomal damage than office workers ones (Figure 2A and B), suggesting that sewage workers are exposed to substances that could lead to genotoxic effects. In contrast to many published biological monitoring studies that measure specific urinary metabolites, our novel *in vitro* approach measures the overall genotoxicity of urine as a mean to assess in a more global way the exposure of sewage workers to complex mixture of chemicals encountered in their work environment. Those integrated biomarkers reflect exposure from hours to a few days prior to urine sampling. In addition to encompassing the diversity and time variable levels of exposure to genotoxic agents, it allows to account for their multiple portals of entry into the human body, not only by inhalation.

In accordance with our results, mutagens in the urine of sewage workers with the Ames test have been detected (Scarlett-Kranz et al. 1986). We found no other reference in the literature that bears on this population. Although results of this study cannot be directly compared with *ex-vivo* assays, other authors have assessed the hazardous potency of this occupational environment. Friis et al. (1997) investigated the level of DNA damage on lymphocytes of sewage workers by comet assay, and found no difference compared with construction workers. Comet and micronucleus assays have also been performed in other occupational settings. Significant increase in MNi frequency on lymphocytes was reported among asphalt workers (Karaman and Pirim 2009), while elevated lymphocytes DNA damage by comet assay was shown in workers of a petroleum hydrocarbons facility (Paz-y-Miño et al. 2008).

In our study, the 24hr urinary 8-oxodG was evaluated and failed to show a statistically significant difference (due to our small sample size) between sewage and office workers, yet average value were slightly greater among the former (Figure 3A). Similarly, a study among workers exposed to diesel particles could not have shown increased levels after a working week (Harri et al. 2005). However, several studies had shown an increased level of 24hr urinary 8-oxodG among workers exposed to different sources of genotoxicants like coke oven emissions and ambient air pollutants (Loft et al. 1999; Liu et al. 2006). Interestingly, we found that 24hr urinary 8-oxodG was positively, although weakly, associated with the number of years subjects had been working in the sewage system, i.e. were exposed to the suspected genotoxicants (Figure 3B). This is in agreement with Yoshida et al. (2003) who found that male workers exposed to fly ash at municipal solid waste incinerators showed a significant increase in the mean levels of urinary 8-oxodG with duration of exposure.

We attempted to interpret our data in terms of cancer risk in this population based on specific PAHs and benzene workplace air levels that account only for a part of this complex exposure.

We found that the Paris city sewage workers experience a substantial lifetime cancer risk via inhalation ranging from  $1.5 \times 10^{-5}$  to  $14.9 \times 10^{-5}$ , which is over the acceptable cancer risk ranges defined by American regulatory agencies (OEHHA 1994; U.S. EPA 2007 and 2009). Our results might partially explain the excess in cancer incidence in sewage workers found by studies that used national records or follow-up designs (Wild et al. 2006; Friis et al. 1999).

The workplace air concentrations of specific pollutants were significantly higher in the sewage workplace than in office and than ambient air concentrations measured by urban background monitors and even in traffic areas (Table 2 and Figure 1). The present data show associations between the urinary biomarkers of exposure and the short-term (3 consecutive days) measurements of workplace PAHs and VOCs levels (Table 3). The multivariate analysis revealed significant associations between several VOCs and both urinary biomarkers of exposure while PAHs were significantly associated with %DNA tail only. These associations were detected among older but not the younger workers (Table 4). Keeping in mind that these associations should not be causally ascribed to specific compounds; all very much correlated, several hypotheses might be advanced to explain these differences. Of them is that the two *in vitro* assays have different mechanisms, and positive results in comet does not necessarily yield positive ones in CBMN. Indeed, comet assay reflects repairable DNA damage and breaks (Tice et al. 2000), while CBMN assay reflects chromosomal damage (Fenech 2007). Further, benzene is the only confirmed human carcinogen in our study (IARC 2004; U.S. EPA 2009), a hazardous potency that could be reflected by the observable responses on both assays to VOCs exposure levels. It seems worthy, however, to indicate that the average VOCs concentrations in this study were all below the French and American recommended occupational permissible exposure limits (INRS 2007; OSHA 2009). In absence of a substance-specific standard for workplace air exposure to PAHs in France, we can compare the exposure levels measured for B(a)P to the provisional limit value proposed by INRS, the occupational security institute in France ( $150 \text{ ng/m}^3$ , 8-hr time-weighted average): the observed concentrations are an order of magnitude lower. Hence, higher PAHs exposure levels might be needed to elicit a significant observable damage at the chromosomal level on HepG2 cells using CBMN assay, that may be when assessed *in vitro* an exposure biomarker less sensitive than repairable DNA damage and breaks biomarker (Aouadene et al. 2008). PAHs exposures were also lower than those detected in the workplace air of coke-oven and graphite-electrode producing workers (Marczynski et al. 2002). The studied PAHs and VOCs families differ in their chemical, physical and toxicokinetic properties. Furthermore, they represent only part of the many chemicals to which sewage workers are exposed in the



workplace. Clearly, the true exposure in this complex environment is unknown and the possible observed integrated effects could relate to the overall synergistic and/or antagonistic interactions (ATSDR 2009).

We have no firm explanation for the difference observed across the two age categories. It cannot be explained by a lower capacity of DNA repair mechanisms in older subjects since these results are based on *in vitro* assays (Faragher et al. 2009); nor should it be due to differences in kidney function and excretion rates, as we found no significant difference between younger and older subjects in the 24hr urinary creatinine or 24hr urinary volume levels (mean±SD, 1.2±0.60 g/l and 1.0±0.45 g/l,  $P=0.20$ ; and 1.63±0.71 l and 1.60±0.66 l,  $P=0.91$ ; respectively). We offer for discussion tentative hypotheses. Many xenobiotics in the workplace (e.g., PAHs and VOCs) are lipophilic, and therefore stored in fatty tissue (ATSDR 2009). Aged volunteers may have more saturated fatty tissues, a feature that could explain higher release and excretion of such substances in urine (Jandacek and Tso 2001). The diversity of tasks of sewage workers according to age might also play a role if more experienced subjects are called for activities that incur greater exposure (Wild et al. 2006). Now, contrasts in exposure levels cannot be assessed in our study using the workplace concentrations data stratified according to age, because a work team is usually composed of subjects of all ages and measurements represent exposure levels for all the team members. Differences concerning wearing protections devices are also factors influencing the degree of true individual exposure. In our study, older sewage workers were less likely to wear protective devices than younger ones ( $P=0.01$ ).

No association was detected between indicators of external exposure and the 24hr urinary 8-oxodG; biomarker of early effect. One reason might be related to differences in the time dynamics of the two types of measures. While measurements of external exposure represent the last work shift or up to 3 days before, our biomarker of oxidative-stress effect encompasses a much longer period of exposure (Ma et al. 1992; Olinski et al. 2006). More important in our view is the fact that the workplace's PAHs and VOCs concentrations are poor proxies of the occupational complex exposure that sewage workers experience, so that exposure misclassification may be large. This, in our opinion, gives weight to the integrated exposure approach we propose. The two urinary biomarkers of exposure account for the whole body burden and the effective biological uptake through the assessment of the overall genotoxicity of urinary metabolites. They both show elevated levels among sewage compared to office workers, reflecting exposure to mixtures of genotoxicants in the sewage workplace. Similar studies in other complex occupational settings should be conducted to assess the

generalizability of these results and how sensitive these biomarkers of exposure might be to a variety of mixtures and differences in exposure levels. Once the performance of this approach evaluated, it might be extended to environmental settings such as coastal or soil petroleum pollution and/or contaminated industrial sites.

## **Conclusions**

Sewage workers are exposed through different pathways to a variety of toxicants. We propose an integrated approach to assess exposure to a blend of genotoxicants that could lead to genotoxic effects, using noninvasive urinary biomarkers. In view of the small sample size of our population, the possibility of lack of adjustment for unknown confounders, and/or because of the multifactorial nature in the production of 8-oxodG, which represents only a fraction of the repair mechanism to 8-oxo-guanine, it is not possible to simply link the increased urinary excretion of 8-oxodG and its relation with duration of exposure trend with an increased cancer risk, even more so, with the observations of cancer cases observed and described among sewage workers.

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### 3.2. Multivariate analysis of *in vitro* assays with the study groups

This section and the following ones include findings that were not shown or presented in the main article (exposure and genotoxicity article, 3<sup>rd</sup> article).

As mentioned but not presented in the main article, we tested the differences in % DNA tail and MNi/1000 BNed between the two study groups while adjusting for possible confounders. The multivariate linear regression results are shown in Table 5 and 6 for % DNA tail and MNi/1000 BNed respectively. No significant association with any of these confounders was found. However, the positive association between the % DNA tail and MNi/1000 BNed with the sewage group was still remained strongly associated.

**Table 5: Multivariate linear regression model for comet (%DNA tail) assay among the study groups (sewage/office workers)\* [N=64].**

Comet (%DNA tail)					
Independent variables	B	SE	Beta	P value	95%CI for B
Occupation (sewage workers/office workers)	5.01	0.99	0.71	<0.001	3.01-7.00
Occupation * age Interaction	0.98	1.11	0.14	0.38	-1.23-3.21
Age (year)	0.001	0.05	0.002	0.99	-0.10-0.10
24hr Urinary creatinine (g/l)	-0.33	0.65	-0.05	0.61	-1.64-0.97
Educational level (>12 / ≤12 years)	0.33	0.91	0.034	0.72	-1.51-2.17
Smoking habits (ex-smokers/never smokers)	0.28	0.74	0.035	0.71	-1.21-1.77
Alcohol consumption (regularly/occasionally)	-0.42	0.67	-0.06	0.53	-1.76-0.92
Vegetable intake (usually/sometimes)	0.08	0.70	0.01	0.91	-1.34-1.49

\*The model is adjusted for variables of  $P < 0.1$  in univariate analysis that are showed in the model.

Note: The same model was also tested without the occupation \* age Interaction variable and the  $P$  values of the confounders were always not-significant at  $P < 0.1$ , while it still remained  $< 0.001$  for the occupation variable. SE, standard error; B, regression coefficient Enter regression method was used.

**Table 6: Multivariate linear regression model for CBMN (MNi/1000 BNed) assay among the study groups (sewage/office workers)\* [N=64].**

MNi/1000 BNed					
Independent variables	B	SE	Beta	P value	95%CI for B
Occupation (sewage workers/office workers)	9.41	2.47	0.63	<0.001	4.47-14.36
Occupation * age Interaction	1.006	2.75	0.06	0.72	-4.49-6.51
Age (year)	-0.06	0.12	-0.07	0.63	-0.31-0.19
24hr Urinary creatinine (g/l)	-2.41	1.61	-0.17	0.14	-5.65-0.82
Educational level (>12 / ≤12 years)	-1.40	2.27	-0.06	0.54	-5.96-3.16
Smoking habits (ex-smokers/never smokers)	-0.24	1.84	-0.01	0.89	-3.93-3.46
Alcohol consumption (regularly/occasionally)	-0.10	1.66	-0.007	0.95	-3.43-3.23
Vegetable intake (usually/sometimes)	-1.27	1.76	-0.08	0.47	-4.79-2.25

\*The model is adjusted for variables of  $P < 0.1$  in univariate analysis that are showed in the model.

Note: The same model was also tested without the occupation \* age Interaction variable and the  $P$  values of the confounders were always not-significant at  $P < 0.1$ , while it still remained  $< 0.001$  for the occupation variable. MNi: Micronuclei; BNed: Binucleated; SE, standard error; B, regression coefficient. Enter regression method was used.

### 3.3. Population characteristics factors and 8-oxodG associations

Table 7 shows the simple association between the 24hr 8-oxodG (biomarker of early effect) and the populations characteristics. It also presents the simple associations between this biomarker of early effect and the urinary biomarkers of exposure (*in vitro* comet and and micronucleus assays). As shown in the table, 8-oxodG was only found to be significantly associated with the 24hr urinary volume and urinary creatinine, as well as with the number of working years in the sewage system ( $P$  values  $<0.05$ ). No association was found between this biomarker of early effect and the urinary biomarkers of exposure. However, a positive trend with age was observed, with older subjects exhibiting greater 8-oxodG values ( $P=0.14$ ).

**Table 7: The simple associations between oxidative stress biomarkers (24hr urinary 8-oxodG; pmole/kg 24h) and the population characteristics.**

Independent variables	8-oxodG (pmole/kg 24h)			
	B	SE	Beta	P-value
Weight (Kg)	-0.04	0.04	-0.11	0.39
BMI (Kg/m <sup>2</sup> )	-0.06	0.13	-0.05	0.65
Age (years)	0.08	0.05	0.19	0.14
24hr Urinary volume (ml)	-0.003	0.001	-0.51	<0.001*
24hr Urinary creatinine (g/l)	2.86	0.84	0.39	0.001*
Number of working years in the sewage	0.15	0.07	0.26	0.04*
Educational level (>12/≤12 years)	-1.31	1.33	-0.12	0.33
Marital status (married/non-married)	1.06	0.97	0.14	0.28
Smoking habits (ex-smokers/non-smokers)	-1.22	1.09	-0.14	0.27
Alcohol consumption (regularly/occasionally)	-0.06	0.97	-0.007	0.95
Fruits intake (usually/sometimes)	-0.84	1.04	-0.10	0.42
Vegetable intake (usually/sometimes)	-0.26	1.06	-0.03	0.80
Intake of vitamins/minerals (yes/no)	-0.92	1.47	-0.08	0.53
Use of barbecue in the last week (yes/no)	-1.26	2.29	-0.07	0.59
Exposure to passive smoking (yes/no)	-0.35	0.98	-0.05	0.72
Massive physical activity in last two days (yes/no)	-0.87	1.33	-0.08	0.52
Type of heating system (collective/individual)	0.44	1.003	0.06	0.66
Resident proximity to industrial installations (yes/no)	1.32	1.55	0.11	0.39
Place of residence (urban/suburban/rural)	-	-	-	0.70
% DNA tail (comet assay)	0.12	0.14	0.11	0.37
MNi/1000 BNed (micronucleus assay)	-0.08	0.06	-0.15	0.24

BMI, body mass index (the square of the height over the body weight); pmole, picomole; 8-oxodG, 8-oxo-2'-deoxyguanosine; SE, standard error; B, regression coefficient. \* Statistically significant ( $p < 0.05$ ). Simple multiple regression was used except for place of residence variable where ANOVA was applied.

### 3.4. Multivariate analysis of 8-oxodG with the study groups

Table 8 below, shows the multivariate linear regression analysis of 24hr urinary 8-oxodG with the two study groups while adjusting for possible confounders. A significant positive association with age and a significant negative association with the 24hr urinary volume were observed. The 24hr urinary creatinine did not remain significantly associated in this 8-oxodG multivariate model.

**Table 8: Multivariate linear regression model for the association of 24 hour urinary 8-oxodG (pmole/kg 24h) with the two exposure groups under study [N=64]\*.**

Independent variables	8-oxodG (pmole/kg 24h)			
	B	SE	Beta	P-value
Occupation (sewage workers/office workers)	0.31	1.20	0.04	0.80
Age (years)	0.15	0.07	0.32	0.03*
Occupation x Age Interaction	1.80	1.55	0.23	0.25
24hr urinary volume (ml)	-0.003	0.001	-0.52	0.005*
24hr Urinary creatinine (g/l)	-0.39	1.36	-0.05	0.78

\*Variables entered in the model are those with a *P*-value <0.15 in univariate analysis.

Note: Testing the associations with age as only a confounder yielded also a margin significant association with age ( $p=0.06$ ) and a positive association with the 24 hr urinary volume ( $P=0.009$ ). pmole, picomole; 8-oxodG, 8-oxo-2'-deoxyguanosine; SE, standard error; B, regression coefficient. \*Statistically significant ( $p < 0.05$ ). Enter regression method was used.

### 3.5. Multivariate analysis of 8-oxodG with exposure duration

As mentioned in the exposure and genotoxicity article, the 24hr 8-oxodG was significantly associated with the number of working years in the sewage system ( $P=0.04$ ) assigned zero value to office workers. The adjustment for possible confounders (24hr urinary volume and creatinine) in multivariate regression model did not change the association ( $P=0.06$ , Table 9).

**Table 9: Multivariate linear regression model for the association of 24 hour urinary 8-oxodG (pmole/kg 24h) with the working years in sewage system [N=64]\*.**

Independent variables	8-oxodG (pmole/kg 24h)			
	B	SE	Beta	P-value
Number of working years in the sewage system	0.12	0.06	0.21	0.06*
24hr urinary volume (ml)	-0.003	0.001	-0.49	0.004*
24hr Urinary creatinine (g/l)	-0.03	1.19	-0.003	0.98

\*Variables entered in the model are those with a *P*-value <0.1 in univariate analysis. The office workers were assigned zero value in the variable “number of working years in the sewage system”. pmole, picomole; 8-oxodG, 8-oxo-2'-deoxyguanosine; SE, standard error; B, regression coefficient. \*Statistically significant ( $p < 0.05$ ). Enter regression method was used.

### 3.6. The associations between 8-oxodG and workplace air pollutants

As shown in Table 10, we tested the association between the 24hr urinary 8-oxodG and the workplace air pollutants (indicators of the external exposure; PAHs and VOCs). No significant association has been detected between 8-oxodG and any of these indicators, as well as with the total [B(a)P]eq.

**Table 10: Crude associations between oxidative stress biomarkers (24hr urinary 8-oxodG; pmole/kg 24h) and concentrations of workplace air pollutants (indicators of the external exposure; PAHs and VOCs).**

Pollutants (indicators of external exposure)	8-oxodG (pmole/kg 24h)			
	B	SE	Beta	P-value
<b>VOCs</b>				
Benzene	0.03	0.04	0.10	0.42
Toluene	0.002	0.002	0.11	0.42
Ethylbenzene	0.01	0.01	0.12	0.37
M+P-Xylene	0.002	0.004	0.06	0.64
O-Xylene	0.01	0.01	0.07	0.59
1, 2,4Trimethylebenzene	0.01	0.01	0.18	0.16
Undecane	0.003	0.002	0.16	0.20
Trichloroethylene	0.01	0.01	0.15	0.26
Tetrachloroethylene	0.01	0.01	0.12	0.36
Styrene	0.03	0.03	0.12	0.39
Decane	0.004	0.003	0.14	0.29
1,4 Dichlorobenzene	0.01	0.01	0.13	0.33
<b>PAHs</b>				
Benzo(a)pyrene	-0.03	0.04	-0.10	0.46
Anthracene	-0.06	0.07	-0.10	0.45
Benz(a)anthracene	-0.05	0.08	-0.09	0.52
Benzo(b)fluoranthene	-0.06	0.09	-0.09	0.50
Benzo(g,h,i)perylene	-0.02	0.09	-0.03	0.83
Benzo(k)fluornathene	-0.11	0.17	-0.09	0.51
Chrysene	-0.03	0.08	-0.05	0.71
Dibenz(a,h)anthracene	0.07	0.42	0.02	0.87
Fluoranthene	-0.01	0.02	-0.08	0.53
Indeno(1,2,3-cd)pyrene	-0.03	0.14	-0.03	0.82
Phenanthrene	-0.004	0.01	-0.06	0.66
Pyrene	-0.01	0.03	-0.07	0.61
Benzo(j)fluoranthene	-0.06	0.11	-0.08	0.57
Total [B(a)P]eq	-0.01	0.03	-0.06	0.63

pmole, picomole; 8-oxodG, 8-oxo-2'-deoxyguanosine; SE, standard error; B, regression coefficient; VOCs, volatile organic compounds; PAHs, polycyclic aromatic hydrocarbons. \*Statistically significant ( $p < 0.05$ ). Simple multiple regression was used.

### 3.7. The association between the two biomarkers of exposure

Figure 13 shows a positive linear relationship between the *in vitro* genotoxicity assays (integrated biomarkers of exposure) with a  $P$  value of 0.002. This association only remained marginally significant, however, after adjustment for age, alcohol consumption and vegetable intake ( $P$  value =0.07). No association between the two *in vitro* assays and the 24hr urinary 8-oxodG levels was observed ( $P$  values are respectively 0.37 and 0.24 for % DNA tail and MNi/1000 BNed).

To get an idea about the homogeneity and the distribution of the biomarkers results, data were plotted as normal probability plots and scatterplots (Appendix 14). Although comet assay results were shown to be slightly deviated from the normal distribution curve being skewed right with a peaked distribution (mainly resulted from the sewage population as seen in Fig. I B and C), general trends toward normal distribution were observed among the other two

biomarkers results. Noteworthy also is that 8-oxodG values among the office workers were found to be a little skewed to the right with a flat distribution.

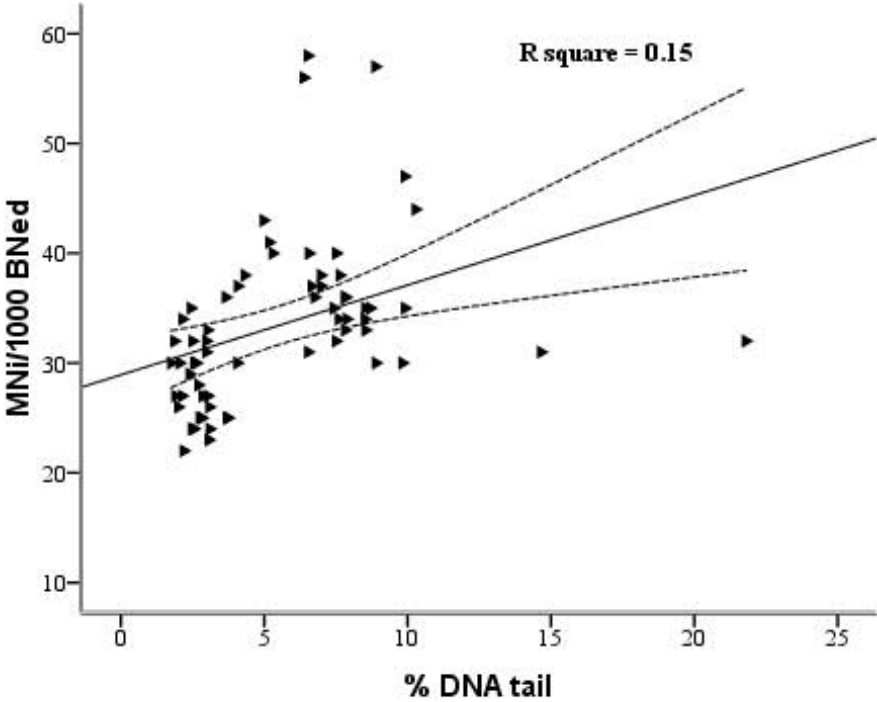


Figure 13: Relationship between %DNA tail and MNI/1000 BNed (urine genotoxicity biomarkers of exposure) as assessed with the subjects organic urine extracts on HepG2 cells. Scatterplot and regression line with 95% confidence intervals of the mean are shown. BNed, Binucleated cells; MNI, micronuclei.



## 4. General discussion

This thesis explored the association between genotoxicity and oxidative stress biomarkers with occupation in the underground sewage system of the city of Paris in a view to assess effects of exposure to cocktails of chemicals at work following various pathways and sources. We selected these factors to explore integrated biomarkers of exposure and of early biological effects, that are easy to sample, with a focus on urine genotoxicity (biomarkers of exposure) and oxidative stress (biomarker of early effect). The underground sewage workers were compared to office workers of Paris city.

This is the first study to directly measure personal exposure of sewage workers to such complex occupational mixtures of chemicals in the workplace air, mainly PAHs and VOCs. Neither had been used any objective measurement, nor had been characterized the workplace air quality of those underground workers [36]. Most of the published studies focused on the wastewater treatment workers in municipal solid waste and in the sewage sludges [6,42,47,214]. However, the two situations and environments might greatly differ, both in workspaces and in exposure characterization. As a consequence, we have no other source to compare our results with.

### 4.1. The study's main results

In the 3<sup>rd</sup> article (exposure and genotoxicity article), we found that the workplaces air sampling for several PAHs and VOCs among sewage workers showed significantly higher levels compared with office workers, urban and traffic background monitors. We also carried out two *in vitro* genotoxicity assays (comet and CBMN) on the organic urine extracts of sewage and office workers performed on Hep G2 cellular line, showing significantly greater DNA and chromosomal damage by comet and CBMN, respectively, among sewage compared to office workers. Using the TEFs for the PAHs and inhalation unit risk of cancer calculations for PAHs and benzene [215-218], sewage workers showed a substantial elevated lifetime estimated cancer risk ( $1.5 \times 10^{-5}$ ) to PAHs mixtures and to benzene ( $4.2 \times 10^{-5}$  to  $14.9 \times 10^{-5}$ ) in reference with office workers. These cancer estimates are higher than the acceptable cancer risk ranges defined by American regulatory agencies [217,218]. Concentrations of several VOCs (indicators of the external exposure) in the workplace air were found to be significantly associated with the *in vitro* comet and micronucleus assays. By contrast, PAHs were found associated only with the *in vitro* micronucleus assay. The exposure and genotoxicity article also presents the main results from the biomarker of early effect, the 8-oxodG (oxidative

stress effect), with higher levels among sewage than among office workers; the differences failed to be statistically significant, however. The biomarker of early effect was also positively associated with the number of working years in the sewage system.

In the second article (comet article), we evaluated the choice between HeLa S3 and Hep G2 cellular lines in performing the comet assay. This aimed to choose which cell line to use in our study. The main results showed that Hep G2 cellular line is a more convenient *in vitro* model than HeLa S3 to study the promutagens and compounds that need to be metabolically activated before exerting their effects. According to our results and in accordance with the literature data [205], we decided to use the Hep G2 cellular line.

These main results have been previously discussed in the 3 published or submitted papers. The following sections will discuss further points related to this work that have not been presented or discussed yet.

Our approach is an integrated and non-specific one that assesses the overall urine genotoxicity. In such a complex, varied and multiple occupational exposure setting, measurement of a single and specific urinary metabolite like 1-hydroxypyrene as an indicator of PAHs exposure would not reflect the multiple mixtures that may include other toxic chemical groups, including but not limited to VOCs, PCBs and pesticides for examples. Specific biomarkers also fall short to express a complex exposure to a variety of compounds, a situation that sewage workers experience. Urine genotoxicity assessment might thus be an appropriate approach to integrate the exposure to an array of genotoxic compounds that eventually results in a variety of urinary excreted metabolites which are too many to be individually quantified. These compounds are usually excreted intact or as metabolites in human urine.

There are many other methods available for scoring of DNA damage and repair. The MNi formation in *ex-vivo* associated with fluorescence in situ hybridization (FISH) technique on peripheral blood lymphocytes is a technique that highlights the clastogenic or aneugenic mechanism in the formation of the micronuclei and is considered to be biomarker of early effect [219]. Its application would have allowed us the later comparison and validation of our *in vitro* urinary MN assay as biomarker of exposure. Also, the bacterial Ames test carried out on several strains of the bacterium *Salmonella typhimurium* is another assay. The classical version of this assay has some disadvantages: (i) it is time-consuming (a 2 day incubation period is needed as the end-point is the growth of revertants on minimal medium); (ii) the test sample or soluble components of the metabolising system may diffuse into the bottom-agar, thus varying the final concentration over time; (iii) it is labour-intensive; and (iv) it does not



provide direct toxicity measurements (toxicity is determined by subjective criteria such as background clearing or a reduction in the number of revertants on treated plates compared with the solvent control); (v) as *Salmonella* is a prokaryote, it is not a perfect model for humans although an adapted *in vitro* model has been made for eukaryotic cells, for example yeast structure; (vi) Ames test at certain strain was shown not to be effective at monitoring occupational exposure to PAHs [220]; (vii) finally, substances that contain the nitrate fraction sometimes come back positive for Ames test when they are indeed safe. Nitroglycerin is an example that gives a positive Ames yet is still used in treatment today. The conditions of the Ames test are dosed at very high concentrations and with nitrate compounds that can potentially generate nitric oxide (NO), an important molecule signal, will give false positive results [221]. Despite these shortcomings, further work is now in planning to apply our urine samples on Ames assay for comparison and validation purposes.

#### **4.2. The results of urinary caffeine metabolites and CYP1A2 activity**

We have reported that sewage workers are exposed to PAHs and VOCs. In humans, the cytochrome P450 isoform CYP1A2 is induced by and involved in the metabolic activation of some procarcinogens like PAHs, some VOCs, aromatic amines and other resembling chemicals, thus probably modifying the toxicity of these chemicals [175]. Indeed, the metabolic activation and detoxification by CYP1A2 can generate ROS which might lead to oxidative DNA damage [175,177]. The activity of CYP1A2, may be considerably increased by exposure to aromatic compounds (e.g., PAHs), and have effects on DNA oxidative damage. When the expression levels of this enzyme increase, the metabolism of some procarcinogens into DNA-reactive metabolites is enhanced, probably leading to elevated carcinogenic risk [175]. The measurement of CYP1A2 activity in human would thus be a useful tool to assess the association between exposure to chemicals and cancer risk or to show changes in the enzyme activity after exposure to inducers [175]. CYP1A2 is responsible for the 3-demethylation of caffeine in human's body [185]. The determination of caffeine metabolic ratio in urine after dietary caffeine intake is an increasingly-used simple and non-invasive biomonitoring approach that makes it possible to explore the activity of the CYP1A2 induced by PAHs and similar compounds [187,188]. Particularly, the urinary caffeine metabolic ratio [1,7-dimethyluric acid (17U)+ 1,7-dimethylxanthine (17X)/1,3,7-trimethylxanthine (137X or caffeine)] is widely used as it reflects the caffeine 3-demethylation [190].

In sewage workers, CYP1A2 activity has never been assessed. Very few studies have evaluated the CYP1A2 activity by caffeine metabolic ratio in occupational settings that involved exposure to PAHs or VOCs. However, among the non-smokers bus drivers, it was increased with high exposure to air pollution and significantly correlated with the urinary 8-oxodG [171]. Further, tobacco smoke was shown to increase CYP1A2 activity [222] and a significant positive correlation between the CYP1A2 activity and the presence of mutagen in urine of smokers was established [223].

We intended to assess the CYP1A2 activity and the samples have been collected from participants, as explained in the methods and materials section. We analyzed these samples by HPLC and standard calibration curves have been established (see section 2.1.5. for details). Unfortunately, at the experimental stage of testing the subjects' urine samples, it was found that the chromatograms were too different from those obtained during the validation of the method. We have no clear explanation for this point. The large number of peaks was a major problem to identify correctly the peaks for each caffeine metabolite and consequently we could not produce data with enough accuracy and security of interpretation. It is possible that the composition of the participants' urine contain various interfering and unidentified contaminants that prevented accurate analysis of their data, so that it was not possible to avoid overlapping peaks in the chromatography. These data could have helped us in studying the link between the exposure and biological modifications that could happen in subject's body as a result of this exposure. Due to time shortage, further verifications and analysis were not possible. They will be, however, subjected to future verifications, and, hopefully, explanations.

### **4.3. Cancer risk estimates and workplace sampling**

The cancer risk estimates in our study (presented in the exposure and genotoxicity article) should be interpreted with caution. The reasons are that some of the studied PAHs compounds have no sufficient data to assess their carcinogenicity to human and their true risk could be null [217]. Further, all cancer unit risks used are based on linear extrapolation. Thus, if the dose response relationships for some of these substances are nonlinear, this might result in a significant over or under-estimation of the risk. These unit risk estimates were based on inhalation of B(a)P or benzene exposure which might be differed from sewage or office workplaces air exposure, both in composition and properties [215-218]. Workers protection by wearing personal protective equipments such as filters and masks would further decrease

the biological uptake of these substances by the workers body and therefore reduce their true cancer risk.

In environmental cancer, it should be noted that *nitro*-PAHs compounds (e.g., 1-nitropyrene and 1,8-dinitropyrenes) are major environmental pollutants. They could be found in the air of the sewage and office workplaces. These substances are suspected animal and human carcinogens. Mutations may occur when inhaled nitropyrenes are metabolized to active derivatives that combine with DNA leading to DNA-adducts which could result in mutations that may ultimately affect a gene's function and initiate carcinogenesis. If nitropyrene adducts were measured in humans, it might provide information on the level of the deposited material in the lungs and, perhaps, permit estimates of resulting risk [224].

It would have been interesting to identify and measure these derivatives in our study as they would add to our understanding of the complex genotoxic mixtures in the sewage system, these compounds being thought as being among the most toxic in the PAHs family. *Nitro-PAHs* are thought to be emitted through atmospheric combustion processes or composed from parent PAHs by hydroxyl radical addition followed by the reaction with nitrogen dioxide and by nitrate radical addition to the PAHs. They therefore could be accompanied by exposure to PAHs [225,226]. Unfortunately, the study did not collect the data to assess the association between all PAHs families. This would merit further investigation of the potential contribution of *nitro*-PAHs to the overall occupational health risk associated with airborne PAHs among sewage workers.

#### **4.4. The urinary biomarkers of exposure associations**

As previously explained (but not presented) in the exposure and genotoxicity article (3<sup>rd</sup> article), we implemented multivariate linear regression models to study the differences in the comet and CBMN assays between the two occupational groups while adjusting for potential confounders. As shown in tables 5 and 6, none of the suspected confounders remained significant, nor was observed an occupation x age interaction. On the other side, the differences in both the *in vitro* genotoxicity assays remained strongly significant between sewage and office workers.

These results indicate that the observed higher genotoxic effects on Hep G2 cells produced by the organic urine extracts from the sewage workers could not be due to confounders (at least those taken into account in this study). However, they are due to the presence of genotoxicants in the subjects' urine extracts that are linked to occupational exposure. In the multivariate models of comet and CBMN assays with single PAHs and VOCs, it was not

possible to include the occupation groups because, as expected, this variable was strongly associated with exposure levels (see exposure and genotoxicity article).

The comet and MN were carried out *in vitro*. Here, they are considered as biomarkers of exposure. At this stage of the new approach presented in this work, these observed effects on Hep G2 could not be directly extrapolated to a possible development of pathology due to genotoxic effects in the subjects. Furthermore, it must be remembered that assessment of genotoxicants at only one point in time may not represent long term occurrence of these substances in the urine [194]. Changes in the composition of the sewage system over time (months and years) may affect longitudinally the level and characters of exposure and adverse effects [194]. A follow-up of these chemicals on a weekly basis, and at various locations, with simultaneous evaluation of urine genotoxicity would be of relevance to back the view that sewage workers incur a chronic occupational exposure to genotoxicants.

The two biomarkers of exposure to genotoxicants (comet and CBMN) showed significant higher levels among sewage workers and were positively associated, as shown in figure 13. However, with an  $R^2$  of about 15% this association is weak. Indeed, positive results in the comet assay do not always correspond to positive results in the CBMN assay, especially when exposure to genotoxic agents is low and time variable [109]. Thus, the poor relationship observed in our study between the two assays is not surprising. Moreover, the positive results in these two assays are also due to different mechanisms. The CBMN assay reflects chromosomal damage, aneuploidy and detects injuries that survive at least one mitotic cycle. Whereas, the comet assay reflect DNA damage and lesions that might be repaired (such as alkali-label sites) and do not give rise to fixed DNA alterations [109,110]. Therefore, in such complex occupational setting of multiple and time variable genotoxicants exposure, it is not easy to predict the final effect as it depends on the overall synergistic and/or antagonistic interaction of the different families of chemicals. Consequently, we suggest that the combination of both assays might be more accurate, suitable and reasonable in such complex setting to monitor the overall urine genotoxicity arising from non-specific, multiple and various kinds of exposure to carcinogenic hazards [111,227].

#### **4.5. The early effect biomarker (8-oxodG) associations**

As shown in table 8, the multivariate linear regression model of 8-oxodG adjusted for occupation (sewage/office workers), age, 24hr urinary volume and creatinine resulted in a significant positive association with age and a negative association with the 24hr urinary volume (the same was observed when adjusting for duration of exposure to the sewage

system, table 9 model). The significant positive association of 24hr urinary creatinine observed in univariate analysis (table 7) did not remain when adjusting for age, occupation, and other showed factors in the models.

The older workers could be more sensitive to oxidative DNA damage resulting from occupational exposure to genotoxicants than younger ones [228]. Indeed, production of oxidative stress represented here by urinary 8-oxodG is the consequence of genotoxicants' metabolism and of the generation of reactive oxygen species [166]. Therefore, 8-oxodG might be influenced by the many anti-defence mechanisms in the human body that is hypothesized to decrease with age [172, 229]. Higher levels of urinary 8-oxodG with age were also found among workers exposed to PAHs in silicon production plants [230]. However, this result was not found among garage workers exposed to diesel particulate exhaust [231].

The increase in the urinary volume could result in greater dilution and therefore could abate the concentrations of the urinary excreted substances such as 8-oxodG. In our study, the 24hr urinary creatinine was not associated with 24hr urinary 8-oxodG when adjusting for occupation, age and the 24hr urinary volume. In a normal kidney function, creatinine has a constant rate of excretion and is often measured in urine as a marker of the effect of hydration on other analysts. Here, the 24hr urinary creatinine was within the normal human range while the 24hr urinary volume did not differ between the two study groups ( $P=0.14$ ). As a consequence, analysis of 8-oxodG was not standardized for creatinine in the statistical analysis. Adjustment for creatinine should be done when spot (but not 24hr) urine samples are taken and/or when the 24hr urinary volume differs between groups, to take into account the urine dilution.

As presented in table 7, there was no significant association between the *in vitro* genotoxicity assays (biomarkers of exposure) and the 24hr urinary 8-oxodG (biomarker of early effect). Absence of such association could be due to the fact that these urinary biomarkers of exposure and the biomarker of early effect are parameters with different targets, mechanisms, compartment and/or time periods [145]. While the urinary biomarkers of exposure represent exposure to genotoxicants that appeared intact or as metabolites in the urine within the sampling period (the last work shift or a few days before), the biomarker of early effect represented a much longer period of exposure and the body metabolism and interaction (e.g., production of reactive oxygen species) with genotoxicants [166]. The 8-oxodG translates an action in the human body that corresponds to DNA damage repair [171]. Therefore, the 8-oxodG (but not our used *in vitro* urinary biomarkers of exposure) is usually

influenced by the numerous repair capacities and anti-defense mechanisms in the human body [172]. When the mean values of exposure are not subject to large daily fluctuations, comparison between the short-term biomarkers of exposure and the long-term biomarker of early effect may be appropriate [145]; this is not the case in our study. Furthermore, as indicated by our measured indicators of external exposure (PAHs and VOCs), it seems that exposure is under the permissible exposure limits, thus, higher levels of exposure might have been needed to result in clear associations.

Another comment on 8-oxodG results is that the free radical theory proposes that ageing is the cumulative result of oxidative damage to the cells and tissues. The hypothesis claims that: (a) variation in species life span is correlated with metabolic rate and protective anti-oxidant activity; (b) enhanced expression of anti-oxidative enzymes in experimental animals can produce a significant increase in longevity; (c) cellular levels of free radical damage increases with age; and (c) reduced calorie intake leads to a decline in the production of reactive oxygen species and an increase in life span. Lipid peroxidation, age pigments, DNA damage and decline of mitochondrial function that develop with ageing can also be explained by the free radical theory. However, ageing is a multi-factorial process. Thus, despite its positive features, the evidence for the free radical theory is either correlative or inconclusive [232]. This oxidative stress hypothesis of aging (or free radical hypothesis) is currently one of the most popular explanations for how aging occurs at the biochemical level and was shown in several human and animal studies that found higher levels of 8-oxodG levels in older subjects or animals [232,233].

The level of oxidative-stress biomarker (8-oxodG) and the period of working years in the sewage were significantly associated in our study. This could further raise the possibility of the radical inflammatory reaction responses produced by the repeated exposure to low levels of harmful chemicals and biological agents in the workplace, but also might be associated with cellular aging. The reactive oxygen and nitrogen species generated from these agents can react with most cellular components; they are also genotoxic because they react with, both the deoxyribose and bases in DNA, and so generate base lesions like 8-oxodG and strand breaks. Hence, these inflammatory responses might differ across individuals due to variations in sensitivity to oxidative stress and/or due to the consumption of protective anti-oxidants like vegetables [234,235]. Although we performed *in vitro* assays, higher comet results among older subjects in this study might be cautiously explained by this cellular aging and decrease in the anti-oxidant capacity with age, assuming that the underlying mechanism is the free radical one [234].

A final comment on the results of the 24hr urinary 8-oxodG, is the discrepancy observed between chromatography and immunoassay methods in the determination of baseline levels of urinary 8-oxodG in healthy individuals, with immunoassays argued to overestimate the levels of 8-oxodG [236]. However, some studies have found a significant correlation with immunoassays using HPLC-GC/MS (gas chromatography/mass spectrometry) and Electrochemical Detection (ECD) [237]. Furthermore, the anti-body used in our study is highly specific so that its application to urine samples seems completely appropriate [212]. The use of anti-mouse IgG-coated plate and a 8-oxodG enzyme conjugate has a low variability and increase sensitivity compared to other assays that use an antigen-coated plate. The enzyme used is stable and capable of high turnover for the hydrolysis of acetylthiocholine. Over other enzymes that are used in immunoassays, it does not self-inactivate during turnover, highly stable under the assay conditions, has wide pH range (5-10), and is not inhibited by common buffer salts or preservatives. Moreover, the enzymatic immunoassay is easy to use, rapid and no expensive equipment is required, and it could be applied to numerous extracellular matrices including urine and plasma [238].

#### **4.6. Choice of the cellular line (Hep G2)**

To date, several protocols have been used to perform genotoxicity assays and the variations encompass use of different cell types. These cell lines exhibit different levels of enzyme activities, DNA repair capacity, and degrees of specialization. Of them is the Hep G2 cellular line that was used in our study. It is a well-differentiated hepatocellular carcinoma. Several studies had shown that these cells retained certain activities of various phase I and phase II enzymes [200,201] that play a key role in the activation and detoxification of various promutagens/procarcinogens [202,203]. This cell line is one of the *in vitro* models suggested to study genotoxicity and DNA damage for promutagens and compounds that need bioactivation before they exert their genotoxic effects [204]. Hep G2 cells were also suggested to have a metabolic capacity for PAHs similar to human hepatocytes and therefore represent a good *in vitro* model for investigating the genotoxic potential of complex mixtures containing PAHs [205]. Our previous work also demonstrates the benefits and suitability of this cell line (see comet article section).

On the other side, some studies suggested that Hep G2 cellular line lack several specific enzymes that account for their inability to process some promutagens. A broad range of genes code (cytochrome P450 and some phase II enzymes) was quantified in both Hep G2 and primary human hepatocytes. The expression level of these genes in human hepatocytes was

similar to those previously reported for human liver samples. Expression levels in Hep G2 differed significantly from that in human. Activity and expression, especially of phase I enzymes, were demonstrated to be low in Hep G2 cells. However, up-regulation of specific genes by test substances [e.g., B(a)P] was found to be similar in both cell types [239]. In our study, the high formation of MNi in the negative control cultures might suggest that Hep G2 could also be affected by a genetic instability where they might unpredictably acquire a mutated phenotype. Further, their shortage in some of phase I enzymes might limit their use in the prediction of some promutagens genotoxicity.

Clearly, human hepatocytes cells are the preferred model for biotransformation in human liver and are more sensitive toward the promutagens. They express phase I enzymes at significantly higher levels than Hep G2 cells and thereby more closely reflect metabolism in human liver. However, they are currently available at a cost almost prohibitive but indeed might represent the most promising alternative cellular line due to their high metabolic capacity.

Despite its shortcomings, Hep G2 cellular line has been shown by several groups to possess the enzymes necessary for the activation of many chemicals. For instance, benzo-[a] pyrene [240], 7,12-dimethylbenz-[a]anthracene, dibenz[a,h]anthracene, 7-methyldibenz[a,h]anthracene, 7,12-dimethyldibenz[a,h]anthracene, 3-methylcholanthrene, 3,6-dimethylcholanthrene, 1-methylbenzo[e] pyrene, 7,12-dimethylbenz[a]anthracene, and 1-,4-, or 10-fluoro-7,12-dimethylbenz[a]anthracene [241], several N-nitroso compounds [242], benzidine, acetylbenzidine, 2-aminofluorene and 2-aminoanthracene [243].

A more recent study [244] investigated the oxidative damage of selected individual carcinogenic PAHs (c-PAHs: benzo[a]pyrene and dibenzo[a,l]pyrene), an artificial mixture of c-PAHs (c-PAHs mix) and extractable organic matter (EOM) from urban air particulate matter. Two cell lines (Hep G2 and human diploid lung fibroblasts, HEL) were treated for 24 and 48h with various concentrations of compounds and mixtures. A panel of oxidative stress markers included 8-oxodG was investigated. The results showed the ability of extractable organic matter to induce oxidative damage to DNA and lipids after 24h of treatment, and to proteins after 48h, in Hep G2 cells, while the effect of c-PAHs was substantially less. The induction of oxidative stress by c-PAHs and EOM in HEL cells was weak compared to HepG2.

Another study investigated the genotoxic effects of some heterocyclic aromatic amines (e.g., 2-amino-3-methyl-imidazo[4,5-f]quinoline) in Hep G2 cells compared to Chinese hamster ovary (CHO) cells [245]. The study indicated that the MNi formation in Hep G2 might reflect



the mutagenic effects of heterocyclic aromatic amines more adequately than other *in vitro* mammalian cell systems due to the presence of enzymes involved in the metabolic conversion of the amines. The results of enzyme measurements indicate that the N-acetyltransferase activity in Hep G2 cells is ~ 40-fold higher than in CHO cells and similar to that found in primary human hepatocytes. Unless associated with S9 mix, it is likely that the negative results seen with quinolines and quinoxalines in CHO cells are due to their low acetyltransferase activity [246]. In contrast, the acetyltransferase activity in Hep G2 cells is apparently sufficiently high to catalyze the generation of DNA-reactive metabolites from the heterocyclic aromatic amines. On the other hand, the Chinese hamster ovary (CHO-K1) cells associated with S9 mix were shown to be reliable cells to detect clastogenic or aneugenic potential of genotoxic substances as well as chromosomal aberrations [247,248,249].

In our opinion, the ability to detect a significant difference in comet and MNi formation applied on Hep G2 cells would further suggest a good sensitivity of these two tests to detect DNA and chromosomal damage and that this cellular line is to some extent metabolically competent (despite some reports of low levels of various phase I and phase II enzymes from certain laboratories).

Hep G2 cells have low repair capacity (unlike HeLa S3 for example) and 24h exposure period was found to be optimal for comet formation regardless of the tested toxicant [250,251], therefore, it was unlikely in our study to add DNA repair inhibitor. Indeed, in preliminary experiments (as argued in the comet article section), testing HeLa S3 without a DNA repair inhibitor (Aphidicoline), detected no genotoxicity and the data were inconclusive. We thus further enhance the sensitivity and Aphidicoline was added to HeLa S3 cell cultures in comet assay. Cells that are metabolically incompetent (e.g., HeLa S3) will attenuate comet formation as a function of exposure time unless a DNA repair suppressor is added. In Hep G2, however, the enzymes are likely to be continuously active, thus the possibility to get false results is low [200,252].

To date, however, the human hepatocytes are the most promising *in vitro* model for biotransformation in human liver and are of great importance for toxicological and pharmaceutical studies [253]. Their disadvantage is the shortage of available human liver material and poses the additional problem of interindividual variations. Additionally, primary hepatocytes do not proliferate and lose their metabolic activity after some weeks although this may be overcome by the generation of differentiated hepatocytes from adult or embryonic stem cells or immortalization of differentiated hepatocytes. The Hep G2 cell line is easy to

handle and provides a reproducible human system although could be less suitable to predict metabolism in adult human liver cells.

#### **4.7. The study sample size**

The sample size of this study (34 sewage workers and 30 office workers) is small. Therefore, generalization of our results to the general population of sewage workers should be done with caution. Furthermore, the small sample size might have restricted the statistical analysis power, especially when developing the multivariate regression models and studying the modification effects of age for example.

The Parisian sewage workers are approximately 400 workers and smokers represent about 45% of them [36]. We anticipated recruiting 75 subjects in each group [227]. However, our criteria of inclusion required that volunteers be current non smokers and have no history of chronic or recent illness, especially inflammatory ones (e.g., diabetics, hepatic and renal disorders) since at least six months, as these conditions could interfere with the study outcomes. Consequently, the recruitment and inclusion procedures were not easy despite the long study period (10 months) and the several meetings to include as many subjects as possible. Moreover, it was difficult and long to obtain all the authorizations from the committees involved in health and occupational security in Paris. Two different departments were involved; the Committee Hygiene and Safety and Management of Cleanliness and Environment of the Town of Paris for sewage workers, and the services of Occupational and Preventive Medicine of the town of Paris for office workers. Nearly one year was needed to pass the all necessary steps.

Despite these shortcomings, our sample size included a group of sewage workers from different job categories performing several tasks in the sewage system. It included workers carrying tasks of maintenance, intervention, surveillance, sampling and collections. This wide difference in duties function might have enabled us to examine an informative group of sewage workers that represent different levels of exposure and job types. In section 2.2.1.2 we explained in details the differences between the two study groups that our sample size could have enabled us to detect.

#### **4.8. Study questionnaires**

Two self-administered questionnaires have been used in this study. The first was the SUVIMAX2 food questionnaire (Appendix 12) that collected detailed data on diet habits. This questionnaire has been previously validated by Pr S. Hercberg (UMR U557

INSERM/U1125 Inra/EA3200 Cnam/Université Paris 13). The second was the professional one. It covered the sociodemographic factors, non-occupational exposures and other confounders (Appendix 11). This questionnaire has been previously standardized by the services of the occupational and preventive medicine in Paris municipality. We have integrated and developed (with the agreement and accord of occupational and preventive medicine services) several questions to meet the objectives of our study. These were mainly related to non-occupational PAHs exposures, area of residence and indoor sources of exposures, medical history and medications intake.

Answering the questionnaires depends on recall, thus under or over estimation of questionnaire-dependant factors could have occurred (e.g., vegetable and fruit intake). However, they were administered to the study subjects under the supervision of the study team in a standardized manner and enough time has been given to fill them (3 hours). Further, several studies in France used the same food questionnaire and concluded that it is a reliable tool [198]. In the meanwhile, the professional questionnaire is routinely administered as an annual survey means for the population of sewage workers in Paris city and was shown to be well adapted (personal communication, Dr Aziz TIBERGUENT, head of the occupational and preventive medicine office of the City of Paris). Data entry was performed by optical and intelligent character recognition using computerized DataScan software (Neoptec-Data-Scan automatic forms-processing software, Montpellier, France, 2007), thus data entry errors that are usually encountered are likely to have been reduced to the minimum.

#### **4.9. Bias of the study**

This was a cross-sectional study. Although we tried to control biases to the maximum, this type of study is prone to different types of bias. We cannot exclude the probability that some might have affected the associations under study. Selection bias could have occurred. Example of this is the “healthy worker effect” that is mainly likely to appear in sewage worker group whose job is more difficult and demanding. The study subjects were all volunteers, thus motivation to participate was important. In order to minimize this source of selection bias, we encouraged the workers and performed several meeting to explain the study objectives and confidentiality procedures. Oral and written information was always provided to the study populations. It is well established that this study design provides limited information about the sequence of events in causation and/or prognosis. However, it provided us with a useful baseline assessment and we were able to study multiple outcomes and

exposures. In this study design, faster, cheaper, and easier procedures were applied. Measurement bias and inter- and intra-observer variations are of concern. To control this source of bias, the same researcher carried out the comet and CBMN *in vitro* assays. PAHs and VOCs as well as the 8-oxodG were also analyzed by the same research team. Previously validated and tested questionnaires under the supervision of the study team were used. Data entry for the professional questionnaire was done through a computerized optical reading, as discussed earlier. Other sources of bias in such studies are the recall and over or under-reporting (mainly from questionnaires).

Confounders and/or effect modifiers (interaction factors) were assessed and accommodated through adjustment and stratification in multivariate models (e.g., PAHs and VOCs with age and occupation with age when testing for genotoxicity assays associations). Other unknown or, hence, uncontrolled confounders (e.g., risk-taking behaviour) may interfere.

Another source of uncertainty in this work is that sewage system exposure may typically fluctuate over time, thus increasing inter individual differences or mis-classifying exposure. Using biomarkers that integrate exposure over time or serial sampling (on weekly-basis and over 10 months period) would have been indicated to reduce such variability and exposure mis-classification. The interindividual variability (e.g., in metabolizing enzyme activity) could not be excluded in this study. Further discussion on the study biases and limitations may be found in the study protocol article (1<sup>st</sup> article) under the discussion section.

## 5. Conclusion and research perspectives

This work provides essential information on integrated exposure assessment of underground sewage workers to complex, time variable and nonspecific exposure to genotoxicants. As indicators of the external exposure, air sampling at the workplace revealed elevated levels of PAHs and VOCs in sewage workers' workplaces compared to workplaces of office workers, and to ambient air concentration at traffic and background monitoring sites. The integrated biomarkers of exposure approach showed that the urinary extracts of those workers are capable to produce *in vitro* genotoxic effects on a Hep G2 cellular line. This suggests an occupational exposure to multiple genotoxicants that could not have been captured by a single specific metabolite or biomarker, let alone single air pollutants.

However, providing evidence of increased exposure to mutagenic substances among sewage workers does not mean that the long term health effects of this exposure are well characterized. I agree with this point. In this study we merely found a positive relationship between the duration of exposure and rates of 24h urinary 8-oxodG. Caution is called upon to interpret these results regarding the role of inflammatory response to the prolonged cellular oxidative stress. Indeed, in view of the small sample size of our population, the possibility of lack of adjustment for unknown confounders, and/or because of the multifactorial nature in the production of 8-oxodG, which represents only a fraction of the repair mechanism to 8-oxo-guanine [235], it is not possible to simply link this trend with an increased cancer risk and, even more so, with the observations of cancer cases observed and described among sewage workers.

The main goal of this study was to demonstrate that it was possible to assess the exposure of a group of worker to an "unknown" complex mixture containing genotoxic compounds. We succeed to point out this by the use of genotoxic tests applied on the urine extracts of those workers. To validate the fact that they are exposed, we also checked the presence of known genotoxics in the air of the occupational setting of sewer workers. This allows us to affirm that sewer workers are more exposed to genotoxic substances than administrative workers.

Therefore, the overall picture from this work shows that sewage workers experience exposure in confined spaces to many chemicals that have genotoxic potentials. When entering the human body, these agents may interact with one another, either enhancing or inhibiting the effects of single compounds, a phenomenon that results in a fact that specific effects become immeasurable. This work highlights the importance of occupational biomonitoring approaches in such setting. Using urinary biomarkers of exposure and of early biological

effects to assess changes associated with such complex environment is a relevant approach, since urine reflects the overall body uptake [194] and removal of the oxidative stress products, among which the 8-oxodG [165,166]. Because it is difficult to quantify the exposure dose to relevant single compounds, our biomarkers approach allowed estimation and integration of the total exposure through multiple sources and portals of entry.

In 1954, the German physician “Anders” reported his retrospective results after examination of 449 male sewage workers in West Berlin. He concluded that the biological and chemical composition of the sewage system did not present an undue risk to health [254]. Now, using more sophisticated tools, we can say that the lifestyles have changed and that the sewage system could be a source of hazard to the sewage workers’ health. The recent increased demand for urban transportation, and its associated air pollution, and the wide usage of chemicals in industries, shops and in our homes have increased the complexity of the sewage system where most of this chemical production of liquid waste eventually ends. This results in exposure of those workers involved in the maintenance of the system. Therefore, strict and new environmental regulations should be implemented to control this exposure and abate it to the minimum.

This work emphasized a small but an important population in our daily life, “the sewage workers”. Effective steps towards risk prevention should be taken. This includes (1) communication of this research results to those involved in risk management both locally and through occupational health dedicated agencies, (2) intervention of decision makers to reduce or prevent the occupational risk to the sewage workers, (3) enhancing awareness of the workers and educating them on how to avoid adverse health effects of exposures encountered in their workplaces through practicing the hygiene and protective processes.

I hope the findings of this thesis will increase the attention among the researchers and policy makers, and contribute to the discussion on how to develop appropriate solutions to manage the potential risks to sewage system.

The perspectives of this research are numerous. At this stage, the new integrated approach and the observed effects on Hep G2 could not be directly extrapolated to a possible development of pathology due to genotoxic effects in the subjects. The scheme of “Exposure-Disease development” is not yet completed from this work. Future work and research studies are still required. Further biomarkers of early biological effects are needed to better characterize the risk and draw consistent conclusions regarding adverse effects to workers. We collected blood samples from the participants to conduct the DNA-adducts. Due to time shortage, we were not

able to undergo the DNA-adducts analysis. Results from the DNA-adducts (biomarker of early biological effect) will better help to clarify, strengthen and support risk characterization. However, it would have been of great value if this work had assessed the MNi formation in *ex-vivo* associated with fluorescence in situ hybridization (FISH) technique on peripheral blood lymphocytes. This technique could have highlighted the clastogenic or aneugenic mechanism in the formation of the micronuclei, and allowed later comparison; it might also have validated our *in vitro* urinary MNi as biomarker of exposure with the MNi in *ex-vivo* as biomarker of early effect. Obviously, this work was primarily designed to establish exposure among those workers. Now, we have collected blood samples from those workers, therefore, this idea should be taken into account in further data analyses to enhance the risk evaluation. DNA-adducts is now under analysis. In the meanwhile, further work is now in planning to apply our urine samples on Ames assay for comparison and validation purposes.

Furthermore, the human population is under persistent exposure to a wide variety of toxic and carcinogenic  $\alpha$ ,  $\beta$ -unsaturated aldehydes derived from the metabolism of natural cellular constituents and of xenobiotics, including from the environment. In the human body, genotoxic  $\alpha$ ,  $\beta$ -unsaturated aldehydes (e.g., 4-Hydroxyalkenals) are also produced as a result of free radical initiated the lipid peroxidation [255]. Thus, exposure to genotoxic substances may give rise to generation of free radicals and thus lipid peroxidation according to the free radical hypothesis. Therefore, future analysis and measurement of urinary thiobarbituric-acid-reacting substances (TBARS), that represent  $\alpha$ ,  $\beta$ -unsaturated aldehydes resulting from lipid peroxidation by free radical species, would be essential in quantifying the lipid peroxidation as an index of the whole body oxidative stress in our population [255]. We have collected sufficient urine samples from each participant (150 ml). Therefore, this study perspective may help better understand the hypothesis of radical mechanism.

In the present study, a relatively small number of subjects were involved. Larger and preferably prospective studies should be implemented to verify the observed associations and outcomes stemming from this study. As part of this work, we collected detailed information by questionnaire regarding diet intake. This is still to be analyzed and correlated with other clinical investigations. Further, interindividual differences, in particular in metabolizing enzymes activity may also contribute to the explanation and understanding of the biological modifications resulting from this occupational exposure. Monitoring and functional follow-up at specific time intervals and close medical surveillance for those workers are also essential.

The cooperation between different services throughout large cities in Europe could be undertaken to further characterize the risk we have shown.

The classical epidemiological studies assess the impact of exposure on an established pathology. This project proposes the simultaneous evaluation of biomarkers of exposure and of early effects to genotoxicants. It assesses the risk prior to the detection of the pathology. This work permits, by biomarkers of exposure and of early effect, a better understanding and perception of cancer risk in a population for whom the characterization of exposure remains unclear, complex and varied. This research thus improves the biological characterization of a complex exposure to occupational pollutants. This will also provide appropriate tools to define the degree of exposure of a given population by toxic cocktails while traditional metrology allows only the identification and quantification of a limited number of these substances in the environment. Another work is now started and the data are being collected from the garbage workers of the city of Paris as another occupational setting that could experience exposure to complex chemical mixtures. We are planning to apply our biomarkers approach described in this thesis on those workers. Once the biomonitoring approach applied in this research is evaluated in other occupational environments that also experience complex occupational exposure to genotoxicants, we believe that it could be extended to other environmental settings such as land petroleum pollution or contaminated industrial sites.



## **Sommaire (Français)**

### **Biomarqueurs d'évaluation d'une exposition environmental complexe : Évaluation de l'exposition des égoutiers aux substances génotoxiques**

#### **1. Justification de l'étude**

L'exposition aux polluants atmosphériques concerne une large fraction de la population française et plus particulièrement les citadins. L'urbanisation et le chauffage résidentiel et tertiaire, la croissance du trafic automobile et parfois la proximité de sources industrielles provoquent localement des conditions d'exposition à une large gamme de polluants. Aux premiers rangs des pathologies associées, dans la littérature, à cette exposition figurent les cancers, deuxième cause de mortalité en France, et dont la part attribuable à des facteurs environnementaux et professionnels est estimée entre 8 et 20 %. Selon le Plan National Santé-Environnement (PNSE), la biosurveillance a le mérite de fournir une estimation intégrée de l'exposition des individus. Si elle est plus développée en milieu de travail qu'en population générale, elle demeure cependant très insuffisamment utilisée. Etant donné le caractère souvent différé des pathologies étudiées (cancers notamment), l'apport de méthodes d'évaluation précoce de l'exposition est essentiel. De plus, la métrologie analytique est confrontée à la grande variabilité temporelle et spatiale de l'exposition, rendant souvent illusoire la mesure d'indicateurs spécifiques ; de plus, elle ne prend pas en compte fidèlement l'interaction avec l'organisme. Il est donc fondé de recourir à des marqueurs biologiques pour chercher à mieux définir l'exposition des individus. Ces approches ne permettront pas une évaluation produit par produit, mais par familles de substances, voire par mélanges indéterminés ayant des effets cocktails.

L'étude s'intéresse à une association de biomarqueurs simples, précoces et non invasifs destinés à mettre en évidence l'exposition à des cocktails de substances toxiques indéfinis ayant des propriétés génotoxiques. Pour étudier la pertinence de ces biomarqueurs, nous avons identifié une population de personnes particulièrement exposées professionnellement à un tel environnement complexe : les égoutiers parisiens. Un rapport récent de l'INRS met en évidence un excès de mortalité (SMR=1,25), par cancers notamment (SMR=1,37), chez les égoutiers de la ville de Paris, en regard de populations actives de mêmes catégories sociales (Wild et al., 2006). Quelques études, plutôt anciennes, ont mis en évidence une augmentation de certains marqueurs pouvant suggérer une exposition à des molécules potentiellement

génotoxiques (Kuusimaki et al., 2002). Dans cette catégorie professionnelle, l'évaluation de biomarqueurs d'exposition et d'effets précoces semble d'autant plus intéressante que l'étiologie des risques est mal définie.

Les biomarqueurs retenus pour cette étude sont des marqueurs généralement utilisés pour mettre en évidence une exposition à des génotoxiques. Ils sont révélés par les tests du micronoyau et des comètes, qui recherchent la présence de substances toxiques dans les urines. Un second groupe de biomarqueurs met en évidence des effets précoces de ces substances, sans préjuger du devenir des événements ainsi mis en exergue. Différentes méthodes sont utilisées : le test à la caféine, pertinent en cas d'exposition aux hydrocarbures aromatiques polycycliques (HAPs) conduisant à l'expression des cytochromes P450. (Faber et al., 2005), l'évaluation de la 8-oxo-déoxyguanosine urinaire (8-oxodG), marqueur d'effet précoce, correspondant à un stress oxydant de l'ADN (Wu et al., 2004), et la recherche d'adduits à l'ADN dans les lymphocytes, marqueur d'effet précoce indiquant l'exposition à des génotoxiques actifs (Le Goff et al., 2005).

L'association des biomarqueurs d'exposition à des génotoxiques et des biomarqueurs d'effets précoces permettrait de compléter la métrologie et la démarche analytique classique, en s'affranchissant du besoin de connaissance *a priori* sur les conditions et la composition spécifique de l'exposition à des polluants de proximité. Il s'agit d'étudier, grâce à une enquête transversale personnels exposés/personnels non exposés portant sur des biomarqueurs d'exposition, si les populations ciblées présentent un risque accru d'exposition à des génotoxiques par rapport à une population de référence.

## **2. Hypothèse et objectifs**

L'hypothèse de cette étude est que l'exposition des égoutiers aux génotoxiques conduit à une augmentation de certains marqueurs d'exposition associée à une augmentation de certains marqueurs d'effets précoces.

L'objectif principal de cette étude transversal était d'explorer des biomarqueurs intégrés d'exposition chez les égoutiers afin de mieux apprécier leur risque cancérigène. L'objectif secondaire est d'évaluer les effets précoces d'une exposition à des agents génotoxiques chez les égoutiers.

La validation de l'association entre les marqueurs d'exposition et marqueurs d'effets précoces chez les égoutiers permettra d'améliorer la caractérisation biologique d'une exposition complexe à des polluants professionnels. La validation de notre hypothèse permettra de disposer d'outils appropriés pour définir le degré d'exposition d'une population donnée à une

pollution par des cocktails de substances toxiques alors que la métrologie traditionnelle ne permet que l'identification et la quantification d'un nombre limité de ces substances dans l'environnement. Ces biomarqueurs non spécifiques seront alors disponibles pour évaluer le risque génotoxique dans une population dont l'exposition à la pollution n'est pas définie de façon précise et spécifique.

### **3. Considération éthique**

Cette étude est réalisée conformément aux articles L1121-1 et suivants du Code de la Santé Publique, relatifs à la protection et confidentialité de données personnelles des personnes qui se prêtent à des recherches biomédicales et conformément aux Bonnes Pratiques Cliniques Internationales. L'Inserm-Département Recherche Clinique et Thérapeutique, promoteur de cette recherche, a contracté une police d'assurance garantissant sa responsabilité civile et celle de tout intervenant (Annexe 1). Cette étude a reçu l'avis favorable du Comité de Protection des Personnes de Nancy – Est III (Annexe 7), et a été autorisée par la direction générale de la santé (Annexe 6). L'information a été donnée oralement et par écrit dans la première partie du formulaire d'information et de consentement (Annexe 2). Le consentement éclairé écrit et signé de toutes les personnes qui se prêtent a été obtenu.

### **4. Résultats**

#### **4.1. La population d'étude**

Tous les participants étaient de sexe masculin, avec un âge moyen de 35,9 (écart-type, 7.5) et 43.3 (écart-type, 8,2) ans dans les égoutiers et les employés de bureau, respectivement ( $P < 0,001$ ). Les égoutiers étaient plus susceptibles d'être n'ayant jamais fumé ( $P = 0,03$ ) comparativement aux travailleurs de bureau. D'autres caractéristiques de la population de l'étude sont présentées au tableau 1 de l'exposition et genotox article. Aucune différence n'a été vu concernant les autres facteurs qui pourraient influencer sur l'étude d'exposition pertinentes: l'exposition à la fumée de tabac ambiante ( $P = 0,87$ ), le type de système de chauffage utilisé à la maison (individuelle ou collective,  $P = 0,52$ ), a déclaré la proximité des résidences pour les installations industrielles ( $P = 0,82$ ) ou la consommation de barbecue.

#### **4.2. Concentrations d'HAPs et la caractérisation du risque de cancer**

Les niveaux d'exposition moyenne de chaque composé HAP présentés au tableau 2 de l'exposition et genotox article, étaient significativement plus élevés chez les égoutiers par

rapport aux travailleurs de bureau et de concentrations dans l'air ambiant ( $P < 0,01$ ). En général, fluoranthène, le phénanthrène et pyrène contribuent à la plus grande partie de la somme des HAPs (par exemple parmi les égoutiers, ils s'échelonnent de 43 %, 15 % et 12 % respectivement). Les HAPs d'autres encore contribuent à moins de 5 %, sauf pour l'anthracène dans le trafic de surveiller les mesures de l'air ambiant, où il s'élève à 11,5 %. Le plus haut B(a)P concentrations a été constaté chez les égoutiers avec une gamme de 0,5 à 62,1  $\text{ng}/\text{m}^3$  et une valeur moyenne de 7  $\text{ng}/\text{m}^3$ .

Sur la base de ces données, les concentrations des HAPs ont été converties en totale [B(a)P] équivalent en utilisant les facteurs d'équivalence de toxicité et traduit en estimations de la durée de vie risque de cancer. Chez les égoutiers, la moyenne [B (a)P] équivalent valeur d'exposition (13,66  $\text{ng}/\text{m}^3$ ) est supérieur à 10 fois à celles trouvé pour les employés de bureau, le trafic urbain et l'air ambiant (respectivement 1,15  $\text{ng}/\text{m}^3$ , 1,08  $\text{ng}/\text{m}^3$ , et de 0,73  $\text{ng}/\text{m}^3$ ). Le tableau 2 dans l'exposition et genotox article présent également les risques de cancer unitaire associés à la vie. Le niveau de risque pour les égoutiers est de  $1,5 \times 10^{-5}$  ( $1,3 \times 10^{-6}$  pour les travailleurs de bureau).

#### **4.3. Concentration de COVs et la caractérisation du risque de cancer**

Figure 1 dans l'exposition et genotox article présente la moyenne de chaque concentration de COV dans l'air en milieu de travail des égoutiers et les employés de bureau, et dans l'air des zones urbaines et les environnements de circulation le plus proche aux lieux d'étude. Une hétérogénéité élevée a été observée entre les différents groupes avec les valeurs plus élevés dans le milieu de travail des égoutiers ( $P < 0,01$ ), suivi par l'air ambiant des employés de bureau, le trafic et l'air ambiant de fond urbaines, respectivement. Certains de ces COVs mesurées dans les lieux de travail n'ont pas été mesurés par le trafic ni par l'air ambiant de fond urbaines (undécane, le trichloroéthylène, le tétrachloroéthylène, le styrène, le décane et le 1,4-dichlorobenzène).

Benzène moyenne  $\pm$  erreur standard variaient entre  $19,1 \pm 2,9$  et  $4,1 \pm 0,53$   $\mu\text{g}/\text{m}^3$  entre les égoutiers et les employés de bureau, avec des concentrations de  $3,7 \pm 0,13$  et  $1,0 \pm 0,09$   $\mu\text{g}/\text{m}^3$  dans le trafic et les zones urbanisées, respectivement. Utilisation de l'estimation de l'Integrated Risk Information System (IRIS) pour le risque unitaire (US EPA, 2007), le benzène associé à vie de cancer excès de risque pour les égoutiers variait entre  $4,2 \times 10^{-5}$  à  $14,9 \times 10^{-5}$ . La gamme correspondante pour les employés de bureau de  $9 \times 10^{-6}$  à  $3,2 \times 10^{-5}$ .

#### 4.4. Tests de génotoxicité sur des extraits d'urine

Le moyene de la queue % d'ADN et MNi/1000 BNed chez les égoutiers a été statistiquement plus élevés que chez les employés de bureau [moyenne  $\pm$  écart-type de la queue % d'ADN =  $8,07 \pm 3,12$  et  $2,70 \pm 0,58$ , et la moyenne  $\pm$  écart-type de MNi/1000 BNed =  $38,02 \pm 7,16$  et  $28,30 \pm 3,74$ , respectivement dans les deux populations,  $p < 0,001$  dans les deux essais] (figure 2 A et B de l'exposition et genotox article). Adjuster pour les facteurs de confusion de la valeur  $p < 0,1$  en analyse univariée, les différences étaient toujours restées élevées ( $P < 0,001$ ) entre les deux groupes d'étude.

#### 4.5. Tests de génotoxicité et l'association d'exposition

La queue% d'ADN a été associée positivement avec l'âge et le niveau d'éducation avec un borderline effet positif de la consommation d'alcool ( $p = 0,09$ ) et un effet protecteur des légumes ( $p = 0,05$ ). MNi/1000 BNed a été positivement associée avec l'âge et la consommation d'alcool et l'inverse avec la consommation de légumes. Tableau 3 de l'exposition et genotox article présente également les résultats de l'analyse de régression de la queue % d'ADN et MNi/1000 BNed avec chaque individu de COV et de HAP.

Résultats des modèles de régression linéaire multiple sont présentés dans le tableau 4 de l'exposition et genotox article qui présente l'association entre les marqueurs de dommages aux cellules et les variables d'exposition, avec le contrôle de covariables. Modification de l'effet est également représentée, avec l'âge étant le seul facteur influençant cette association. Une association significativement positive avec la réponse à l'essai comète observée chez les travailleurs âgés seulement ( $> 39$  ans) pour neuf HAP (les 4 HAPs gazeux et 5 sur les 9 particules HAPs). Cette association avec les HAPs n'a pas été trouvée pour le test de micronoyaux. Tous les COVs étaient significativement associés à MNi/1000 BNed parmi les travailleurs âgés, cependant, la queue % d'ADN a été seulement influencée par l'exposition au benzène, éthylbenzène, m + p-xylène, le o-xylène, le décane, tri et tétra-chloroéthylène.

#### 4.6. Le 8-oxodG urinaire

La figure 3A dans l'exposition et genotox article montre le 24hr 8-oxodG urinaire dans les égoutiers et des travailleurs de bureau. La moyenne chez les égoutiers était plus élevée que dans les travailleurs de bureau (mais non significativement) (moyenne  $\pm$  écart-type,  $8,26 \pm 4,26$  pmole / kg 24h et  $7,22 \pm 3,32$  pmole / kg 24h respectivement,  $p = 0,28$ ). Comme le montre la figure 3B, 8-oxodG était associée positivement avec le nombre d'années de

travail dans les égouts (attribué une valeur zéro pour les travailleurs de bureau,  $p = 0,04$ ). Le contrôle pour 24hr volume urinaire et de la créatinine ne modifie pas l'association avec les années de travail dans les égoutiers ( $P = 0,06$ ). Il n'y avait aucune différence significative dans 8-oxodG au niveau d'autres facteurs d'exposition mentionnés dans le tableau 1 („exposition et genotox article). Aucun lien clair n'a pu être trouvé entre les concentrations des polluants en milieu de travail mesurés ou du total [B (a) P] eq et le 24hr 8-oxodG urinaire.

## 5. Discussion and conclusion

C'est le premier travail qui a appliqué *in vitro* teste de comète et de micronoyau (biomarqueurs d'exposition) sur les extraits organiques d'urine, qui a évalué la 24hr 8-oxodG urinaire (biomarqueurs d'effet précoce) et mesuré les HAP et COV (indicateurs d'exposition externe) dans l'air du milieu de travail parmi les égoutiers. Les extraits urinaires des égoutiers ont montré significativement plus des dommages d'ADN et de chromosomes comparé aux personnels administratifs sur les cellules Hep G2, suggérant que les égoutiers sont exposés à des substances capables de produire des effets génotoxiques. Ces biomarqueurs intégrées d'exposition également liée aux concentrations de polluants de l'air en milieu de travail qui sont sensiblement plus élevés dans les environnements des égoutiers que chez les administratifs et les concentrations dans l'air ambiant mesurées par les moniteurs de urbaine et même par les moniteurs trafic.

Contrairement à de nombreuses études publiés de surveillance biologique qui mesurent sanguin ou des métabolites urinaires spécifiques, nôtre approche nouvelle de la génotoxicité *in vitro* mesure d'ensemble de l'urine comme un moyen d'évaluer d'une manière plus globale l'exposition des égoutiers au mélange complexe de substances chimiques rencontrées dans leur environnement de travail. Ces biomarqueurs intégrées d'exposition reflètent l'exposition heures à quelques jours avant le prélèvement urinaire. En plus elle prend en compte la diversité et les variabilités d'exposition aux agents génotoxiques, il permet de rendre compte de leurs multiples portails d'entrée dans le corps humain, non seulement par inhalation. Conformément à nos résultats, des mutagènes dans l'urine des travailleurs des eaux usées avec le test d'Ames ont été détectés (Scarlett-Kranz et al. 1986). Nous n'avons trouvé aucune autre référence dans la littérature qui porte sur cette population.

Dans notre étude, le 24hr 8-oxodG urinaire a été évalué et a échoué à montrer une différence statistiquement significative entre les égoutiers et les administratifs, mais les valeurs moyennes étaient plus élevées chez les premiers. Plusieurs études ont montré une

augmentation du niveau de 24hr 8-oxodG urinaire chez les travailleurs exposés à différentes sources de substances génotoxiques provenant de coke-oven émissions et des polluants atmosphériques (Loft et al. 1999 ; Liu et al. 2006). Ce biomarqueur d'effet génotoxique pourrait être appropriée pour des périodes d'exposition longues (Loft et al. 1999 ; Harri et al. 2005). Dans notre étude, les niveaux d'exposition ou la période d'échantillonnage ont été trop courts pour tout changement à se produire, ou alternativement, la taille de l'échantillon et puissance statistique de notre étude était trop faible. Une étude chez les travailleurs exposés à des particules diesel n'a pas montré des niveaux plus élevés de ce biomarqueur, après une semaine de travail (Harri et al. 2005). Fait intéressant, nous avons constaté que 24hr 8-oxodG urinaire était significativement associée à une augmentation du nombre d'années avaient été les sujets travaillent dans le système d'égouts. Ceci est en accord avec Yoshida et al. (2003) qui révèle que les travailleurs de sexe masculin exposés aux fly-ash dans les incinérateurs municipaux de déchets solides ont montré une augmentation significative de la moyenne des niveaux 8-oxodG urinaire avec la durée d'exposition. Dans une tentative d'interpréter nos données du risque de cancer unitaire dans cette population, nous avons constaté que, sur la base des HAP spécifiques et des niveaux de benzène en milieu de travail aérien qui ne représentent qu'une partie de ce complexe d'exposition, les égoutiers de la ville de Paris ont un cancer unitaire importante ( $1,5 \times 10^{-5}$ ) par inhalation qui est plus que le risque de cancer acceptable définies par les organismes américains de réglementation (US EPA, 2007; US EPA, 2009 ). Nos résultats pourraient expliquer en partie l'excédent de l'incidence du cancer chez les égoutiers trouvée dans les études ayant utilisé des registres nationaux (Wild et al. 2006).

Les données actuelles montrent des associations entre l'*in vitro* biomarqueurs d'exposition et les indicateurs d'exposition externe (HAP et COV). L'analyse multivariée a révélé des associations significatives entre plusieurs COV et les deux biomarqueurs d'exposition alors que les HAP étaient significativement associées au teste de comète seulement. Ces associations ont été détectées parmi les plus âgés. En gardant à l'esprit que ces associations ne devraient pas être causalement attribuées pour spécifiques composés, tous très associées, plusieurs hypothèses peuvent être avancées pour expliquer ces différences. Bien que les dommages de l'ADN détectée par test des comètes reflète lésions réparables (Tice et al. 2000), le test de micronoyaux reflète les dommages chromosomiques (Fenech 2007). En plus, les concentrations moyennes des COV dans cette étude étaient toutes inférieures aux valeurs limites d'exposition professionnelle en France et aux Etats-Unis (INRS 2007; OSHA 2009). Par conséquent, la hausse des niveaux d'exposition HAP aurait été nécessaire pour obtenir des

dommages observables au niveau des chromosomes sur les cellules Hep G2. Les HAP ont également été inférieurs à ceux détectés dans l'air en milieu de travail de coke-oven émissions et du graphite-électrode (Marczynski et al. 2002). En outre, les deux familles de composés que nous avons mesurées diffèrent dans leurs propriétés chimiques, physiques et toxicologiques, et d'autres nombreux composés n'ont pas été mesurés pourraient aussi exprimer dans les biomarqueurs d'exposition urinaires que nous avons utilisé. Alors que l'exposition vraie dans cet environnement complexe est inconnue et son effet final sur la santé pourrait être lié à la synergie globale et/ou des interactions antagonistes.

Nous n'avons pas d'explication ferme pour la différence observée entre les deux catégories d'âge. Il ne devrait pas être dû à des différences de fonction rénale et les taux d'excrétion, comme nous n'avons trouvé aucune différence significative entre les sujets jeunes et âgés dans la 24hr créatinine urinaire ou 24hr de volume urinaire. Nous proposons à la discussion une autre hypothèse provisoire. De nombreux xénobiotiques en milieu de travail sont lipophiles, tels que les HAP et les COV, et sont donc stockés dans le tissu adipeux (ATSDR 2009). Les personnes âgées pourraient avoir saturé la charge corporelle du tissu adipeux à des xénobiotiques. Cela pourrait sans doute augmenter le taux d'élimination urinaire et l'excrétion de ces substances de l'organisme (Jandacek et Tso 2001). La diversité des tâches des égoutiers en fonction de l'âge pourrait jouer un rôle si des sujets avec plus d'expériences sont appelés pour des activités qui engagent une plus grande exposition. Les différences concernant le port des dispositifs de protection sont également des facteurs influençant le degré d'exposition individuelle. Dans notre étude, les travailleurs âgés ont été moins susceptibles de porter des dispositifs de protection que les plus jeunes ( $P=0,01$ ).

Aucune association n'a été décelée entre les indicateurs de l'exposition externe et de la 24hr 8-oxodG urinaire (biomarqueur d'effet précoce). Une des raisons pourrait avoir trait à des différences dans les dynamiques temporelles des deux types de mesures. Bien que les indicateurs de l'exposition externe représentent l'exposition dans les quelques dernières jours, notre biomarqueur d'effet englobe une période beaucoup plus longue de l'exposition (Ma et al. 1992 ; Olinski et al. 2006). Plus important aussi est le fait que les HAP et les COV sont des indicateurs faibles de l'exposition complexe dans les égouts, que les erreurs de classification de l'exposition sont certainement importantes. Cela, à notre avis, donne une importance à l'approche intégrée de l'exposition que nous proposons. Les deux biomarqueurs de l'exposition prennent en compte l'efficace absorption biologique par l'évaluation de la génotoxicité globale de métabolites urinaires. Les deux montrent des niveaux élevés chez les égoutiers par rapport aux administratifs, ce qui reflète l'exposition à un mélange de substances



génomiques dans l'environnement des égoutiers. Des études similaires dans d'autres environnements complexes du travail devraient être entreprises pour évaluer la possibilité de généraliser cette déclaration et la sensibilité de ces biomarqueurs de l'exposition d'une variété de mélanges et des différences de niveaux d'exposition. Une fois que l'évaluation de cette approche aura lieu, il pourrait être étendu à des paramètres environnementaux tels que la pollution pétrolière ou les sites industriels contaminés.

En conclusion, les égoutiers sont exposés à une variété de substances toxiques, par des voies différentes. Nous proposons une approche non invasive intégrée pour évaluer l'exposition à un mélange de substances génotoxiques capables de produire des effets génotoxiques en utilisant des biomarqueurs urinaires. Dans cette étude on retrouve une relation positive entre la durée de l'exposition et les taux urinaires de 8-oxodG ce qui mérite d'être approfondi notamment quant au rôle de la réaction inflammatoire prolongée sur le stress oxydant cellulaire. Mais compte tenu de la faible population étudiée, la possibilité de manque d'ajustement pour les facteurs confondants inconnu et/ou en raison de la nature multifactoriel de la production de ce métabolite qui ne représente qu'une fraction de la réparation de la 8-oxo-guanine, les résultats ne préjugent en rien du niveau des lésions d'oxydation de l'ADN initiales et donc il n'est pas possible de relier cette tendance avec un accroissement du risque cancérigène et à fortiori avec les observations de cancers décrites chez les égoutiers.

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## **Partnerships of the study**

- Yvon Le MOULLEC, Laboratoire d'Hygiène de la Ville de Paris, 11, rue George Eastman, 75013 PARIS : Atmospheric sampling and analysis.
- Aziz TIBERGENT, Service de Médecine du Travail de la Ville de Paris, 44 rue Charles Moureu, 75013 Paris : Clinical examinations.
- Pierre LEROY, Laboratoire de Chimie Physique et Microbiologie pour l'Environnement, UMR 7564 CNRS - Université Henri Poincaré, Nancy-Université : Caffeine metabolism.
- Véronique ANDRE, EA 1772, GRECAN, Centre Baclesse, Avenue général Harris, 14076 Caen cedex : DNA adducts.


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# Appendices

## Appendix 1: A copy of the assurance certificate from the study promoter (INSERM).

 **Biomedicinsure**  
Société de courtage d'Assurances  
SAS au capital de 48.000 €  
RCS VANNES B 347 531 089 - APE 672Z  
PARC D'INNOVATION BRETAGNE SUD  
C.P. 142 - 56038 VANNES CEDEX  
Tel 33 2 97 69 19 19 - Fax 33 2 97 69 11 11  
E-mail : biomail@biomedic-insure.com

 **GERLING FRANCE**

**ATTESTATION D'ASSURANCE**  
**RESPONSABILITE CIVILE**  
**PROMOTEUR DE RECHERCHE BIOMEDICALE**

ADHESION n° 907882007007

Nous, soussignés GERLING FRANCE - 111, rue de Longchamp 75116 PARIS, agissant en qualité d'assureur, attestons par la présente que :  
INSERM  
101 rue de Tolbiac  
75654 PARIS CEDEX 13

a souscrit un contrat de Responsabilité Civile sous le n° (1680) 90788

Conforme aux dispositions légales et réglementaires Françaises sur les recherches biomédicales et notamment aux dispositions de la loi 88.1138 du 20/12/1988, modifiée par les textes subséquents: loi 90.86 du 23/01/1990, décret 91-440 du 14/05/1991, loi 94.630 du 25/07/1994, décret 97-888 du 01/10/1997, décret 2002-722 du 03/05/2002, loi 2004.806 du 09/08/2004, décret 2006-477 du 26/04/2006.

**Description précise de la recherche assurée :**


Biomarqueurs d'Evaluation d'une Exposition Complexe : exposition à des polluants professionnels.  
Protocole n° C07-20

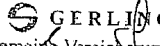
La garantie est conforme à l'obligation d'assurance instituée par les textes de la loi précitée, article L 1121-10 du Code de la Santé Publique, à la charge du promoteur, tant pour sa responsabilité que pour celle des intervenants.

La garantie prévue au contrat restera acquise à l'Assuré en cas de modification affectant la prise d'effet du protocole.

La présente attestation est valable pour la durée de la recherche concernée et sa présentation vaut présomption de garantie à la charge de l'assureur.

Fait , le 5 juillet 2007

**Le Courtier**  
 **Biomedicinsure**  
Société de courtage d'Assurances  
SAS au capital de 48.000 €  
RCS VANNES B 347 531 089 - APE 672Z  
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**L'Assureur**  
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304/2007

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Siège social : Gerling-Konzern  
Allgemeine Versicherungs-AG,  
Von-Werth-Strasse, 4-14, D - 50670 Köln  
Téléphone : 00 49 221 144-1  
Téléfax : 00 49 221 144-33 19

**Appendix 2: The explanatory information and consent form of the study for the exposed sewage workers and the non-exposed office workers.**



Département de la  
Recherche Clinique et Thérapeutique

Institut national  
de la santé et de la recherche médicale

*N° DE DOSSIER (RESERVE A L'ADMINISTRATION) : C07-20*

## **Notice d'Information et de Consentement Personnel Exposé/Non exposé**

### **Titre de l'essai**

**«Biomarqueurs d'Evaluation d'une Exposition Complexe :  
exposition à des polluants professionnels «**

**«Etude BEEC «**

**Investigateur coordonnateur principal:**  
Dr Christophe PARIS

**Responsable scientifique :**  
Dr Luc FERRARI

**Fonction :** Professeur de Médecine de travail  
**Appartenance administrative :** Unité Inserm ERI  
11 - Faculté de Médecine de Nancy

**Fonction :** Maître de Conférences en Toxicologie  
**Appartenance administrative :** Unité Inserm ERI  
11 - Faculté de Pharmacie de Nancy

**Adresse :** Inserm ERI 11 - Faculté de Médecine –  
BP.184, 9 boulevard de la Forêt de Haye, 54505  
Vandœuvre-lès-Nancy

**Tel :** 03 83 68 37 01

**Fax :** 03 83 68 39 19

**Email :** Christophe.Paris@nancy.inserm.fr

**Adresse :** Inserm ERI 11 - Faculté de Médecine –  
BP.184, 9 boulevard de la Forêt de Haye, 54505  
Vandoeuvre-les-Nancy

**Tel :** 03 83 68 37 02

**Fax :** 03 83 68 39 19

**Email :** Luc.Ferrari@pharma.uhp-nancy.fr



## Notice d'information – Personnel Exposé/Non exposé

Le Docteur.....du Service de Médecine du Travail de la ville de Paris, médecin investigateur, m'a proposé de participer à la recherche biomédicale intitulée :

### **«Biomarqueurs d'Evaluation d'une Exposition Complexe : exposition à des polluants professionnels».**

Le médecin m'a précisé que j'étais libre d'accepter ou de refuser de participer à cette recherche.

Afin d'éclairer ma décision, j'ai reçu et bien compris les informations suivantes :

#### **1. Cadre général de cette étude**

L'exposition aux polluants atmosphériques concerne une large fraction de la population française et plus particulièrement les citadins.

Cette étude fait suite à un travail de l'INRS (Institut National de Recherche et de Sécurité) qui avait mis en évidence une espérance de vie plus faible dans certaines catégories professionnelles particulièrement exposées à des polluants.

Selon le PNSE (Plan National Santé-Environnement), la surveillance biologique permet de fournir une estimation intégrée de l'exposition des individus qui demeure cependant très insuffisamment utilisée. Etant donné le caractère souvent différé des pathologies étudiées (cancers notamment), l'apport de méthodes d'évaluation précoce utilisant des marqueurs biologiques de l'exposition aux polluants est essentiel et permet de mieux définir l'exposition des individus.

L'association des marqueurs biologiques d'exposition et d'effets précoces à des substances toxiques permettrait également de compléter les méthodes de mesure classiques, en s'affranchissant du besoin de connaissance *a priori* sur les conditions et la composition spécifique de l'exposition à des polluants de proximité.

#### **2. Objectif de la recherche**

Nous proposons de réaliser une enquête comparant une catégorie de personnels exposée à de multiples polluants professionnels (égoutiers et éboueurs) à une catégorie professionnelle non exposée (personnels de bureaux) en recherchant des marqueurs biologiques d'éventuelle exposition à des substances toxiques présentes dans l'environnement de travail.

L'objectif principal de ce projet est d'étudier si la population exposée présente un risque accru de lésions génotoxiques (modifications de l'ADN induites par des substances toxiques) évocatrices d'un risque de cancer, par rapport à la population non exposée.

L'objectif secondaire est d'évaluer les effets précoces d'une exposition à des agents génotoxiques.

#### **3. Méthode et durée de participation**

Afin d'évaluer votre niveau d'exposition à des polluants professionnels et ses conséquences, vous devrez répondre à des questionnaires spécifiques et faire des prélèvements d'échantillons biologiques que nous allons vous détailler ci-dessous. *Des prélèvements aériens seront réalisés sur votre lieu de travail, du lundi au jeudi précédant le vendredi de la Visite Médicale. Ils seront réalisés, pour les égoutiers par des personnes sentinelles qui porteront les appareillages de prélèvements, pour les éboueurs en plaçant les appareils de prélèvements dans un sac derrière le camion. La durée des prélèvements aériens sera de 4 fois 7 heures, pour respecter les durées de travail.*

Un entretien préalable d'inclusion avec un médecin investigateur est effectué afin de déterminer si aucune pathologie cardiaque ou rénale n'affecte votre santé. Lors de cet entretien, l'investigateur vous remettra un récipient afin de collecter vos urines durant les 24 heures précédant la visite annuelle de

médecine du travail (recherche de substances toxiques dans les urines et de marqueurs d'une atteinte toxique). *Le recueil des urines par les participants se fera dans un flacon fourni par les responsables de l'étude, du jeudi matin au lever, veille de la visite au Centre de Médecine du Travail de la Ville de Paris, au vendredi matin au lever, jour de la visite, y compris sur le lieu de travail, soit une durée totale de 24 heures.*

Notice d'information et de consentement  
Paraphe investigateur :

Version 5 du 19/09/2007  
Paraphe participant :

Au cours de la visite annuelle de médecine du travail:

- vous devrez répondre à deux questionnaires : un questionnaire professionnel et de mode de vie ; un questionnaire d'habitudes alimentaires;

- *Le jour de la visite médicale* vous devrez faire un recueil d'urine : en plus du recueil des urines de 24h précédant cette visite, un second recueil d'urine sera fait 3 heures après la prise d'une quantité définie de café (test qui permet d'évaluer la réponse à une exposition à des hydrocarbures aromatiques polycycliques) ;

- *Le jour de la visite médicale* vous devrez faire des prélèvements sanguins : 2 fois 10 millilitres de sang vous seront prélevés pour réaliser un test d'atteinte du noyau des cellules du sang ; et 5 millilitres pour extraire du matériel génétique à partir de ces mêmes cellules, en vue de valider les résultats du test à la caféine et de faire des analyses génétiques de marqueurs biologiques. Le plasma obtenu lors de ces prélèvements sera congelé pour permettre une mesure des vitamines et de l'homocystéine (indicateur d'apports alimentaires). Ces prélèvements ne présentent aucun risque et ne seront qu'en supplément de ceux qui vous seront faits dans le cadre normal des examens biologiques relevant de la visite médicale annuelle en médecine du travail.

La participation des agents s'étale sur une semaine.

#### **4. Risques prévisibles**

Il n'y a pas de risques prévisibles pour les personnes participant à ce protocole dans les conditions d'inclusion de l'étude.

#### **5. Période d'exclusion**

*Existence d'une période d'exclusion : Vous vous engagez à ne participer à aucune autre recherche biomédicale pendant votre participation à la présente étude (c'est-à-dire pendant 7 jours à partir de la signature du consentement, correspondant à la date d'inclusion).*

#### **6. Modalités de prise en charge médicale**

L'investigation est réalisée dans le cadre de la visite annuelle de médecine du travail. Tout examen approprié sera effectué par le médecin du travail quelle que soit la participation de la personne à l'étude ou sa sortie de l'étude (à la fin de l'étude, par exclusion par l'investigateur ou sur sa décision propre).

#### **7. Droit d'information concernant la recherche et la santé du patient**

Vous serez aussi tenu informé(e) de toute modification ou nouvelle donnée importante concernant l'étude (par exemple, examen nouveau ou visite supplémentaire) à laquelle vous participez. Dans ce cas, la confirmation de votre consentement de participation vous sera redemandée.

Les informations concernant la santé générale du participant lui seront communiquées par le médecin du travail de la ville de Paris, d'une part lors de la visite médicale annuelle relevant de la législation du travail, d'autre part si besoin et sur sa demande personnelle, sur rendez-vous.

#### **8. Droit d'information des résultats globaux de la recherche**

A l'issue de la recherche et après analyse de toutes les données liées à cette étude (c'est-à-dire pour tous les patients), vous pourrez demander à être informé des résultats globaux de l'étude par l'intermédiaire du médecin qui vous suit dans le cadre de cette recherche. Les résultats de cette étude peuvent être présentés à des congrès ou dans des publications scientifiques. Cependant, vos données personnelles ne seront aucunement identifiables et votre anonymat sera entièrement respecté.

Dans cette étude, il est prévu que les résultats seront communiqués à tous les participants, par l'envoi d'un document présentant ces résultats, et lors d'une communication générale à tous les participants réunis par le service de médecine du travail de la ville de Paris en assemblée de restitution des résultats.

#### **9. Données informatisées**

Dans le cadre de la recherche biomédicale à laquelle l'Inserm vous propose de participer, un traitement de vos données personnelles va être mis en œuvre pour permettre d'analyser les résultats de la recherche au regard de son objectif.

A cette fin, les données médicales vous concernant et les données relatives à vos habitudes de vie seront transmises à Luc FERRARI (responsable scientifique de l'étude) ou aux personnes agissant pour le compte de l'Inserm (promoteur), en France. Ces données seront identifiées par un numéro de code alphanumérique (jour, mois, initiale prénom, deux premières lettres nom). Ces données pourront également, dans des conditions assurant leur confidentialité, être transmises aux autorités de Santé françaises. Conformément aux dispositions de la loi relative à l'informatique, aux fichiers et aux libertés (n°78-17 du 6 janvier 1978 modifiée par les lois n°94-

548 du 1<sup>er</sup> juillet 1994, n°2002-303 du 4 mars 2002 et n°2004-801 du 6 août 2004), vous disposez d'un droit d'accès et de rectification de ces données. Vous disposez également d'un droit d'opposition à la transmission des données couvertes par le secret professionnel susceptibles d'être utilisées dans le cadre de cette recherche et d'être traitées.

A la fin de l'étude, l'ensemble des échantillons sera détruit.

Vous pouvez également accéder directement ou par l'intermédiaire d'un médecin de votre choix, à l'ensemble de vos données médicales en application des dispositions de l'article L. 1111-7 du Code de la Santé Publique.

Ces droits s'exercent auprès du médecin qui vous suit dans le cadre de la recherche et qui connaît votre identité.

#### **10. Inscription sur le fichier national des personnes qui se prêtent à des recherches biomédicales**

Nous vous informons que vous serez inscrit dans le fichier national des personnes qui se prêtent à des recherches biomédicales prévu à l'article L. 1121-16 du code de la Santé Publique du fait de la période d'exclusion d'une semaine prévue dans le protocole. Vous avez la possibilité de vérifier auprès du ministre chargé de la santé l'exactitude des données vous concernant présentes dans ce fichier et la destruction de ces données au terme du délai prévu par le Code de la Santé Publique.

#### **11. Indemnités :**

Aucune indemnité n'est prévue pour la participation à cette recherche et aucun éventuel avantage financier ne peut en être tiré.

#### **12. Cadre législatif de cette étude**

Conformément à la Loi, l'Inserm, promoteur de cette recherche, dont l'adresse est Inserm-Département Recherche Clinique et Thérapeutique, 101 rue de Tolbiac, 75654 Paris Cedex 13, a contracté une police d'assurance garantissant sa responsabilité civile et celle de tout intervenant auprès de la compagnie Gerling, France, dont l'adresse est 111 rue de Longchamp, 75116 Paris, sous le numéro 907882007007.

Cette étude est réalisée conformément aux articles L1121-1 et suivants du Code de la Santé Publique, relatifs à la protection des personnes qui se prêtent à des recherches biomédicales et conformément aux Bonnes Pratiques Cliniques Internationales. Des moniteurs de Recherche Clinique désignés par le promoteur et des auditeurs mandatés par le promoteur, ainsi que les inspecteurs des autorités de santé peuvent demander l'accès aux données cliniques de votre dossier médical aux seules fins de vérification des données présentées dans le cahier de recueil de données. Ils sont également soumis à une clause de confidentialité concernant vos données personnelles.

Cette étude a reçu l'avis favorable du Comité de Protection des Personnes de Nancy – Est III, le 05/10/2007 et a été autorisée par la DGS, le 24/08/2007.

## Formulaire de consentement – Personnel exposé/ Non exposé

Je soussigné Nom, prénom : .....  
Adresse : .....

accepte par la présente, conformément aux titres 2 et 3 du Livre 1 du Code de la Santé Publique, de participer à la Recherche Biomédicale intitulée : « **Biomarqueurs d'Evaluation d'une Exposition Complexe : exposition à des polluants professionnels** ».

Avant de participer à cette recherche, j'ai bénéficié d'un examen médical dont les résultats m'ont été communiqués.

Je certifie sur l'honneur être affilié à un régime français de sécurité sociale. Je m'engage à ne participer à aucune autre recherche biomédicale pendant ma participation à la présente étude sur une durée d'une semaine.

J'ai été avisé qu'aucune indemnité n'est prévue pour la participation à cette recherche.

J'accepte le prélèvement, la conservation et l'utilisation des échantillons biologiques humains de sang, d'urine, de sérum, de plasma et d'ADN des cellules de sang afin de procéder à des analyses génétiques dans le cadre de la recherche portant sur l'évaluation de l'exposition aux toxiques. Ces échantillons seront traités, stockés et conservés sous un code non nominatif à Nancy (INSERM ERI11). A la fin de l'étude l'ensemble des échantillons sera détruit. En cas de retrait de mon consentement de participation à la recherche, les échantillons me concernant seront détruits.

En application de la loi «Informatique et Liberté» du 6 janvier 1978, modifiée par les lois n° 94-548 du 1er juillet 1994, n° 2002-303 du 4 mars 2002 et n° 2004-801 du 6 août 2004, j'accepte que les données enregistrées à l'occasion de cette étude et comportant des données génétiques puissent faire l'objet d'un traitement informatisé par le promoteur ou pour son compte. J'ai bien noté que le droit d'accès (article 39) et de rectification (article 40), que m'ouvrent les textes susvisés, pourra s'exercer à tout moment auprès du médecin investigateur et que les données me concernant pourront m'être communiquées directement ou par l'intermédiaire d'un médecin de mon choix.

Je peux à tout moment demander des informations complémentaires au Docteur Christophe Paris (☎ 03 83 68 37 01), INSERM ERI 11, Nancy.

J'ai bien noté que j'ai le droit d'être informé des résultats globaux de cette recherche selon les modalités qui ont été précisées dans la note d'information conformément à l'article L. 1122-1 du Code de la Santé Publique

Après avoir discuté et obtenu les réponses à mes questions, j'accepte librement et volontairement de participer à la recherche décrite ci-dessus. Je suis parfaitement conscient(e) que je peux retirer à tout moment mon consentement à ma participation à cette recherche et cela quelles que soient mes raisons et sans encourir aucune responsabilité ni aucun préjudice. Le fait de ne plus participer à cette recherche ne portera pas atteinte à mes relations avec le médecin investigateur et avec mon médecin traitant.

Mon consentement ne décharge en rien l'investigateur et le promoteur de l'ensemble de leurs responsabilités et je conserve tous mes droits garantis par la loi.

J'ai reçu un exemplaire signé de ce consentement.

Signature de l'investigateur :

Signature du patient :

Date :

Date :

**Toutes les pages de ce document doivent être paraphées par l'investigateur et la personne sollicitée, excepté la dernière page qui doit comporter leurs signatures et doit être daté de la main de la personne qui a consenti dans l'emplacement prévu et qui lui est réservé.**

Ce document est établi en trois exemplaires originaux : un exemplaire est remis à la personne, un exemplaire est conservé par l'investigateur, le troisième est conservé à l'attention du promoteur sous enveloppe sécurisée afin de préserver la confidentialité de l'identité de la personne.

Notice d'information et de consentement  
Paraphe investigateur :

Version 5 du 19/09/2007  
Paraphe participant :

**Appendix 3: Observation sheet of the study.**

INSERM U954

Protocole BEEC

**Feuille d'observations**

Date d'ouverture (jj/mm/aaaa) :

Nom :  (spécifier les deux premières lettres du nom)

Prénom :  (initiale)

Code :

Initiales du Médecin investigateur :

Initiales de l'Investigateur ER111 :

**Examen médical**

Respect des critères d'inclusion : OUI  NON

Respect des critères de non-exclusion : OUI  NON

ECG : test caféine possible OUI  NON

Remarques spécifiques utiles à l'étude :

Heure de la prise de caféine (hh:mm) :  :

Heure du recueil d'urines (hh:mm) :  :

Temps entre la prise de caféine et le recueil d'urines (mm) :

**Biologie clinique**

*Paramètres des urines de 24 heures*

	Valeur	Date de la mesure	Initiales de l'investigateur
Volume des urines de 24 h			

*Paramètres sériques*

	Valeur	Date de la mesure	Initiales de l'investigateur
Créatinine			
Urée			

**Questionnaires**

Questionnaire professionnel rempli :

Questionnaire alimentaire rempli :

Feuille remplie totalement

Date clôture feuille :

**Feuille de suivi BEEC**

**Numéro d'anonymat :**

--	--	--	--	--	--	--	--

	Date de réalisation	Données transmises à Nancy	Date de numérisation	Initiales de l'investigateur
Recueils atmosphériques (LHVP)				
Dosages COVs (LHVP)				
Dosages HAPs (LHVP)				
Réception des urines de 24h				
Traitement des urines de 24 h				
Recueil des urines caféine				
Traitement des urines caféine				
Codage des tubes urines				
Prélèvement sanguin 20 ml				
Isolement des culots				
Aliquotage du sérum				
Codage des tubes culots				
Codage des tubes sérum				
Dosages biologiques (MdT)				
Mise des échantillons sur la glace				
Transports des échantillons à Nancy				
Dosages urée/créatinine urinaire				
Extraction de l'urine des 24h (ERI 11)				
Test des comètes (ERI 11)				
Test des micronoyaux (ERI 11)				
Transmission de l'ADN au GRECAN				
Adduits ADN (GRECAN)				
Transmission d'échantillons à l'U724				
8-oxodG (U724)				
Paramètres sériques (U724)				
Génotypage (ERI 11, U724)				
Transmission d'échantillons au LCPME				
Métabolites de la caféine (LCPME)				

Feuille totalement remplie

Date de clôture de la Feuille

**«Biomarqueurs d'Evaluation d'Expositions Complexes»**

**BEEC**

**Fiche de traçabilité**

**Transport des Echantillons**

Numéro d'anonymat :

Date de prélèvement :

Date du départ de Paris :

Nombre de tubes :

Nom du transporteur :

Date d'arrivée à Nancy :

Nombres de tubes :

Je soussigné ..... Atteste avoir réceptionné à Nancy les

tubes correspondant au numéro d'anonymat .....

Fait à Nancy le

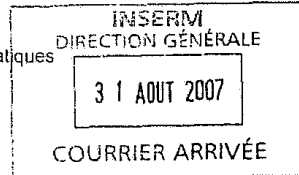
Signature

**Appendix 6: The Direction Générale de la Santé (DGS) authorization of the study project.**



Ministère de la Santé, de la Jeunesse et des Sports

Direction générale de la santé  
Sous-Direction de la politique des pratiques  
et des produits de santé  
Bureau de la qualité des pratiques  
et des recherches biomédicales  
DGS / PP1 / RBM 07.856



Paris, le 24 AOUT 2007

Personne chargée du dossier :  
Mme le Dr. Sabine KENOUCHE  
Tél. : 01 40 56 57 33  
Fax : 01 40 56 56 55  
E-mail : sabine.kenouch@sante.gouv.fr

INSERM  
Monsieur le Directeur Général  
101, rue de Tolbiac  
75654 PARIS CEDEX 13

A l'attention de Madame Héléne POLLARD

N° d'enregistrement : 2007-A00685-48

Madame,

Par votre courrier du 09/08/2007, reçu le 13/08/2007, vous m'avez adressé, en qualité de promoteur ou de représentant du promoteur, une demande d'autorisation de mise en œuvre de la recherche biomédicale suivante :

**Biomarqueurs d'évaluation d'une exposition complexe : exposition à des polluants professionnels. Etude BEEC.**

Investigateur coordonnateur : Christophe PARIS

Je vous informe que l'autorisation de mise en œuvre de cette recherche biomédicale vous est accordée sous le numéro : DGS2007-0433

En outre, je vous rappelle qu'en application de l'article L. 1123-6 du code de la santé publique, cette recherche ne pourra commencer qu'après qu'un Comité de protection des personnes aura émis un avis favorable sur le projet.

Pour toute demande de modification substantielle, toute déclaration d'effets indésirables graves inattendus, toute déclaration de fin de recherche ou toute autre correspondance relative à cette recherche, il conviendra de rappeler le numéro d'enregistrement mentionné en haut de la présente lettre.

Veuillez agréer, Madame, l'expression de ma considération distinguée.

Le Directeur Général de la Santé,

Pr Didier HOUSSIN



**Appendix 7: The local ethical committee de Nancy Est III -CPP (Comité de Protection des Personnes)  
approval of the study protocol and its documents.**

**COMITÉ de PROTECTION des PERSONNES EST-III**

Hôpital de Brabois. Rue du Morvan - 54511 VANDŒUVRE LES NANCY Cedex  
Téléphone : 03 83 15 43 24 - Télécopie : 03 83 15 43 05 - Courriel : cppetl3@chu-nancy.fr

Projet de recherche enregistré  
sous les références  
N° : 2007-A00685-48  
N° DGS :  
N° CPP : 07.09.02

Le Comité a été saisi le 16 août 2007 par Madame Hélène POLLARD, représentant  
l'Inserm, promoteur, pour l'examen d'un protocole intitulé :

**Biomarqueurs d'évaluation d'une exposition complexe : exposition à des polluants professionnels.  
Protocole BEEC**

dont l'investigateur est M. le Professeur PARIS (Inserm ERI 11 à la Faculté de Médecine de Nancy).

Le Comité a examiné cette étude lors de sa séance du 5 septembre 2007. Ont participé aux  
délibérations :

- les membres du Collège n° 1 : M. le Docteur Beau (Titulaire, cat. 1), M. le Docteur Lamaze (Suppléant, cat. 1), M. le Docteur Robert (Suppléant, qualifié en raison de sa compétence en matière de biostatistique), M. le Docteur Peton (Titulaire, cat. 1), Mme Picaut (Titulaire, cat. 3), Mme Laumesfeld (Suppléante, cat. 4) ;
- les membres du Collège n° 2 : Mme Blondelet (Titulaire, cat. 2), Mme Batt (Titulaire, cat. 3), M. Dory (Titulaire, cat. 4), Mme Claudot (Suppléante, cat. 4), Mme Boutet (Suppléante, cat. 5), M. Vidal (Titulaire, cat. 5).

Le 28 septembre 2007, le Comité a reçu les documents modifiés demandés et les a  
examinés lors de sa séance du 3 octobre 2007. Ont participé aux délibérations :

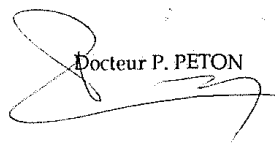
- les membres du Collège n° 1 : M. le Docteur Lamaze (Suppléant, cat. 1), Mme le Docteur Luporsi (Titulaire, qualifiée en raison de sa compétence en matière de biostatistique), M. le Docteur Peton (Titulaire, cat. 1), M. le Docteur Fenot (Titulaire, cat. 2), Mlle Raffy (Suppléante, cat. 3), Mme Léonard (Titulaire, cat. 4) ;
- les membres du Collège n° 2 : M. Hoffman (Titulaire, cat. 1), Mme Blondelet (Titulaire, cat. 2), Mme Batt (Titulaire, cat. 3), M. Dory (Titulaire, cat. 4), Mme Boutet (Suppléante, cat. 5), M. Vidal (Titulaire, cat. 5).

Le Comité a adopté la délibération suivante : **AVIS FAVORABLE** pour

- le protocole (version 6 du 19/09/2007)
- la notice d'information et de consentement destinée au personnel exposé (version n° 5 du 19.09.2007)
- la notice d'information et de consentement destinée au personnel non exposé (version n° 5 du 19.09.2007).

Nancy, le vendredi 5 octobre 2007

Le Président

  
Docteur P. PETON

**Appendix 8: The project authorization by the Committee Hygiene and Safety and Management of Cleanliness and Environment and by the services of Occupational and Preventive Medicine of the town of Paris).**

**Inserm**

Institut national  
de la santé et de la recherche médicale

**PARTICIPEZ A UNE ETUDE EPIDEMIOLOGIQUE**

Suite aux résultats de l'enquête réalisée par l'INRS les années passées, le Service de Médecine du Travail de la Ville de Paris (Dr Aziz Tiberguent), en collaboration avec l'INSERM, ERI11 met en place une étude épidémiologique

L'Unité Inserm ERI11 du Pr Denis Zmirou-Navier (Faculté de Médecine de NANCY), recherche des volontaires pour participer à cette étude épidémiologique coordonnée par le Pr Christophe Paris.

Cette étude a pour but d'évaluer l'impact d'une exposition à des polluants professionnels sur la santé des personnels de la Ville de Paris. Pour cela, nous recherchons des égoutiers, des éboueurs et des personnels non-égoutiers/non-éboueurs appartenant aux mêmes directions techniques.

Cette étude s'inscrit dans le cadre normal de votre visite annuelle de médecine du travail.

La participation des agents s'étale sur une semaine.

**Si vous souhaitez participer et aider la recherche, ou recevoir plus d'informations :**

**Dr Aziz Tiberguent**

**Service de Médecine du Travail de la Ville de Paris**

**01 44 97 86 40**



Département de la  
Recherche clinique et thérapeutique

Institut national  
de la santé et de la recherche médicale

## MODALITES DE DECLARATION D'UN EVENEMENT INDESIRABLE GRAVE (EIG)

dans le cadre d'un projet de recherche biomédicale

**Rappel :** Tout événement ayant pu contribuer à la survenue d'un décès, provoquer ou prolonger une hospitalisation ou entraîner des séquelles organiques ou fonctionnelles durables et susceptibles d'être dues à la recherche doit être déclaré à l'autorité administrative compétente par le promoteur (Article L.1123-8 du Code de la Santé).

POINTS A SUIVRE	MODALITES
<b>Information du promoteur</b>	- tout événement indésirable grave doit être déclaré <b>immédiatement</b> au promoteur : <b>Béatrice Barraud</b> Mission Réglementation et Qualité en Recherche Clinique Inserm Siège Département de la Recherche Clinique et Thérapeutique 101, rue de Tolbiac 75654 Paris Cedex 13 Téléphone : 01 44 23 67 29 / 60 55 Télécopie : 01 44 23 67 10 E-mail : <a href="mailto:beatrice.barraud@tolbiac.inserm.fr">beatrice.barraud@tolbiac.inserm.fr</a>
<b>Comment déclarer</b>	- à l'aide du formulaire de déclaration des EIG disponible sur le site Internet, à l'adresse suivante : <a href="http://www.inserm.fr">www.inserm.fr</a>  - l'investigateur doit adresser la fiche par télécopie au <b>01 44 23 67 10</b> puis par courrier.  - en complément du formulaire, toute information concernant l'événement indésirable, compte rendu d'hospitalisation, les mesures prises et les conséquences envisagées pour la poursuite de la recherche, doivent être signalées au promoteur.
<b>Visite sur site</b>	- selon la nature de l'événement, le promoteur peut demander à l'investigateur des informations complémentaires et envisager le cas échéant une visite sur site.
<b>Poursuite de la recherche</b>	- la décision de poursuivre la recherche est prise conjointement par l'investigateur et par le promoteur - le promoteur en informe l'autorité administrative compétente.

# Inserm

Département de la  
Recherche clinique et thérapeutique

Institut national  
de la santé et de la recherche médicale

## DECLARATION D'UN EVENEMENT INDESIRABLE GRAVE

Ce document est à adresser :

- par télécopie au **01 44 23 67 10**
- puis par courrier à l'**Inserm**:

Département de la Recherche clinique et thérapeutique  
Mission Réglementation et Qualité en Recherche Clinique  
101, rue de Tolbiac,  
75654 Paris Cedex 13

- Déclaration Initiale  
 Déclaration complémentaire

<b>1. Identification de l'étude</b>			
Titre :		Inserm n°	
		IDRCB n°	
<b>2. Identification du participant</b>			
Identifiant Participant : <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/>		Age : <input type="text"/> ans	Sexe : M <input type="checkbox"/> F <input type="checkbox"/>
Centre      Patient      Code Anonyme			
Date d'inclusion : <input type="text"/> <input type="text"/> <input type="text"/> / <input type="text"/> <input type="text"/> / <input type="text"/> <input type="text"/>		Poids : <input type="text"/> <input type="text"/> <input type="text"/> <input type="text"/> kg	Taille : <input type="text"/> <input type="text"/> <input type="text"/> cm
<b>3. Evénement indésirable grave (EIG)</b>			
<input type="checkbox"/> Décès, date <input type="text"/> <input type="text"/> <input type="text"/> / <input type="text"/> <input type="text"/> <input type="text"/>		<input type="checkbox"/> Anomalie/malformation congénitale	
Cause probable : .....		<input type="checkbox"/> Evénement potentiellement grave selon l'investigateur	
<input type="checkbox"/> Mise en jeu du pronostic vital		<input type="checkbox"/> Autre, préciser : .....	
<input type="checkbox"/> Invalidité ou incapacité			
<input type="checkbox"/> Hospitalisation ou prolongation d'hospitalisation			
<b>4. Description de l'EIG</b>			
Date de survenue : <input type="text"/> <input type="text"/> <input type="text"/> / <input type="text"/> <input type="text"/> / <input type="text"/> <input type="text"/>		Prise de connaissance par l'investigateur : <input type="text"/> <input type="text"/> <input type="text"/> / <input type="text"/> <input type="text"/> / <input type="text"/> <input type="text"/>	
Description (symptômes, intensité, chronologie):   			
(joindre les copies et comptes rendus d'hospitalisation des résultats relatifs à l'EIG)			
<b>5. Informations sur le traitement au moment de l'EIG</b>			
Médicament : .....		Posologie : .....	Voie d'administration : .....
Autres : .....			
Antécédents pertinents (médicaux, médicamenteux, sociaux...)   			
<b>6. Diagnostic de l'EIG</b>			
Identifié : <input type="checkbox"/> Oui, si oui, préciser : .....			
<input type="checkbox"/> Non			
Examens complémentaires réalisés :   			

**7. Dispositions prises vis à vis du participant suite à l'EIG**

Aucune mesure thérapeutique

Diminution de la posologie du traitement évalué, préciser : .....

Arrêt momentané du traitement    Date : UU/UU/UU  
Reprise du traitement (si connue) : UU/UU/UU

Arrêt définitif du traitement    Date : UU/UU/UU

Hospitalisation    Date d'entrée : UU/UU/UU  
Date de sortie : UU/UU/UU  
 En cours

Administration d'un traitement spécifique, préciser : .....

**8. Evolution de l'EIG hors décès**

Guérison sans séquelles     Guérison avec séquelles, préciser : .....

Date de guérison : UU/UU/UU

Patient non encore rétabli :  évolution inconnue\*  
 amélioration\*  
 aggravation\*  
 stable\*

(\* dans ces situations une fiche de déclaration complémentaire d'EIG devra être remplie)

**9. Selon l'investigateur, l'EIG semble plutôt lié**

Au(x) traitement(s) étudié(s)     Aux procédures de l'essai

Au(x) médicament(s) associé(s)     A la progression de la maladie

A une maladie intercurrente     Autre, préciser : .....

**10. Informations concernant l'investigateur notificateur de l'EIG**

Nom : .....

Adresse : .....

Tel : ..... Fax : ..... E-mail : .....

**11. Commentaires de l'investigateur sur la relation possible avec le(s) traitement(s) de l'étude**

.....

Signature de l'investigateur :

Date de déclaration : UU/UU/UU

**Appendix 10: Urine collection sheet with written information to study participants explaining how to collect their urine in a sterile plastic collecting bottle.**

**Début du prélèvement le Jeudi**

Numéro d'anonymat :

Avant le début du recueil :

- Uriner normalement aux toilettes. (heure théorique : jeudi, 9h00)
- Noter l'heure et la date. Ce sont les dates et heures de début du recueil des urines de 24 heures.
- heures.

Date de début de recueil :

Heure de début de recueil :

Tout le temps du recueil, à chaque fois qu'il y a un besoin d'uriner, il faut uriner dans le bidon de recueil des urines de 24 heures.

A la fin de la période de recueil, environ 24 heures après le début (soit vendredi 9h00)

- Uriner une dernière fois dans le bidon
- Noter date et heure

Date de fin de recueil :

Heure de fin de recueil :

Volume estimé :

## Début du prélèvement le Mercredi

Numéro d'anonymat :

Avant le début du recueil :

- Uriner normalement aux toilettes. (heure théorique : mercredi, 13h00)
- Noter l'heure et la date. Ce sont les dates et heures de début du recueil des urines de 24 heures.

Date de début de recueil :

Heure de début de recueil :

Tout le temps du recueil, à chaque fois qu'il y a un besoin d'uriner, il faut uriner dans le bidon de recueil des urines de 24 heures. Le sachet est prévu pour permettre un transport facile et discret durant les 24 heures du recueil.

A la fin de la période de recueil, environ 24 heures après le début (soit jeudi 13h00)

- Uriner une dernière fois dans le bidon
- Noter date et heure

Date de fin de recueil :

Heure de fin de recueil :

Volume estimé :

- divorcé(e)
- veuf(ve)
- séparé(e)

**SURVEILLANCE MEDICALE RENFORCEE DES AGENTS  
TRAVAILLANT EN EGOUT**

Population de référence : 400 agents travaillant en égout pour le suivi médical renforcé et la surveillance épidémiologique longitudinale (2007-2012 Version du 25 /10/2007 mise en forme Data Scan 05/06/2008)

**DPE - SAP**  
2007 - 2012

Dossier                      Codification

1. Dossier

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La réponse doit être comprise entre 1 et 1000.  
La réponse est obligatoire.

3. Nom Prénom

2. Numéro d'agent

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4. Votre date de naissance

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La réponse doit être comprise entre 02/01/2007 00:00:00 et 28/09/2012 00:00:00.

CARACTERISTIQUES DE L'AGENT

5. Situation matrimoniale

- Célibataire
- Marié(e)
- divorcé(e)
- veuf(ve)
- séparé(e)
- Pacsé(e)
- Concubinage

6. Masculin/Féminin

- Homme
- Femme

7. Date de naissance

						1	9		
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La réponse doit être comprise entre 02/01/1900 et 31/12/1999.

8. Taille

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La réponse doit être comprise entre 100 et 200.



02/01/

100 et



# 14. Quel est votre poste de travail actuel ?

Egoutier

## 9. Poids

La réponse doit être comprise entre 40 et 150.

## 10. Formation/Etude

- Ecole primaire
- 1er cycle secondaire
- 2ème cycle secondaire
- Enseignement technique (BEP, CAP)
- Etudes supérieures
- Jamais scolarisé
- Autre : précisez :

## 11. Type d'habitat

- Centre ville
- Banlieue ou périphérie de ville
- Rural (village, habitat dispersé, bourg)

## POSTE DE TRAVAIL ET STATUT ACTUEL

### 12. Quel est votre statut au travail ?

- Catégorie C
- Catégorie B
- Catégorie A
- Autre

### 13. Date d'embauche à la Ville de Paris ?

La réponse doit être comprise entre 01/01/1945 et 01/01/2012.

### 14. Quel est votre poste de travail actuel ?

- Egoutier
- Egoutier principal
- Chef d'équipe égoutier
- Chef égoutier
- Agent de maîtrise égoutier
- Maître ouvrier professionnel
- Maître ouvrier principal
- Maître ouvrier
- Ouvrier professionnel
- Agent de maîtrise maintenance
- Agent supérieur d'exploitation
- Chef de service
- Eboueur
- Autre :



ANO1



2



V D P S L B E E C



ANO1

Travaux de soudage, perçage...

Travaux avec produits chimiques (sol

15. Quelles sont vos tâches au poste de travail actuel ?

- Curage petites lignes
- Curage Collecteurs
- Collecte
- Prélèvement
- Musée
- Permanence
- Maintenance DGF
- Sécurité
- Autre : précisez:

Vous pouvez cocher plusieurs cases (8 au maximum).

16. A votre poste de travail vous effectuez les tâches suivantes?

- Curage manuel petites lignes
- Curage manuel collecteurs
- Curage camion mixte (Haute Pression)
- Nettoyage de galeries techniques
- Pose de barrages
- Collecte
- Surveillance et inspection des équipements
- Accompagnement des personnes extérieures
- Traçage chemins des câbles par pulvérisation de peinture (à l'aide de bombes aérosols)
- Enquêtes et visites (branchements des particuliers)
- Vidange de cuves des immeubles et habitation
- Garde orifice
- Intervention de maintenance sur les équipements (vannes, pompes, dégriffeur et autres installations...)
- Intervention et Maintenance dans les usines
- Intervention et Maintenance électriques des locaux techniques
- Travaux de maçonnerie
- Travaux de soudage, perçage...
- Travaux avec produits chimiques (solvants, résines)
- Travaux en atelier de mécanique général
- Travaux en atelier de menuiserie
- Autres :

Vous pouvez cocher plusieurs cases.

17. Depuis combien d'années travaillez-vous en égout?

La réponse doit être comprise entre 0 et 50.

18. Quelle est le nombre d'heures moyen que vous passez par jour en égout?

La réponse doit être comprise entre 0 et 8.



Les EPI sont-ils désinfectés  Oui,

Si oui:  UV,  Bain de détergent

19. En fin de travail, quelles sont les conditions et les mesures d'hygiène? Disposez-vous de :

- Douches avec eau chaude
- WC
- Lavabos avec eau chaude
- Robinet classique
- Robinet commande à pédale
- Vestiaires avec double compartiment (vêtement de ville et vêtement de travail)
- Cantine
- Salle de pause
- Les moyens mis à disposition sont-ils suffisants
- Les moyens mis à disposition présentent-ils une bonne hygiène : /Oui/,/Non/
- Les vêtements de travail sont-ils lavés par le service :  Oui/,  Non/
- Existe-t-il un circuit propre et un circuit sale:  Oui/,  Non/
- Autres :

Vous pouvez cocher plusieurs cases (10 au maximum).

20. Au cours de votre activité professionnelle à la Ville de Paris et en dehors du travail en égout, avez-vous porté des équipements de protection individuelle (EPI)

- Casque avec jugulaire
- Lunettes
- Vêtements de travail
- Combinaison jetable
- Chaussures de sécurité et/ou bottes
- Gants
- Harnais
- Casquette et gilet de sécurité
- Détecteur de gaz
- Coquilles anti-bruit
- Bouchons d'oreille
- Les EPI sont-ils désinfectés  Oui,  Non
- Si oui:  UV,  Bain de détergent

Vous pouvez cocher plusieurs cases (12 au maximum).

#### ANTECEDENTS ET EXPOSITIONS PROFESSIONNELS A LA VILLE DE PARIS

21. Avez-vous occupé d'autres postes de travail à la Ville de Paris avant de travailler au poste actuel ?

- Aucun poste
- Ouvrier
- Agent administratif
- Agent de service, Employé travaillant en égout
- Agent de maîtrise
- Autre:

22. Avant de travailler au poste actuel, avez-vous occupé des postes de travail de ? Indiquez le nombre d'année pour le ou les postes que vous avez occupé(s) (dans la liste énumérée).

- Menuiserie pendant   ans





# Ouvrier désamiantage pendant

- Chauffagiste pendant 

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 ans
- Plombier pendant 

--	--

 ans
- Maçon pendant 

--	--

 ans
- Carreleur 

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 ans
- Paveur-granitier 

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 ans
- Soudeur pendant 

--	--

 ans
- Métalier pendant 

--	--

 ans
- Mécanique générale pendant 

--	--

 ans
- Mécanicien Auto pendant 

--	--

 ans
- Peintre pendant 

--	--

 ans
- Electricien pendant 

--	--

 ans
- Eboueur pendant 

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 ans
- Couvreur pendant 

--	--

 ans
- Ouvrier dans usine de matières plastiques pendant 

--	--

 ans
- Manoeuvre BTP pendant 

--	--

 ans
- Agent d'entretien BTP pendant 

--	--

 ans
- Ouvrier BTP (démolition, ravalement) pendant 

--	--

 ans
- Ouvrier granitier-paveur pendant 

--	--

 ans
- Ouvrier désamiantage pendant 

--	--

 ans
- Ouvrier granitier-paveur 

--	--

 ans
- Ouvrier de chantier naval pendant 

--	--

 ans
- Agent de salubrité, Dératiseur-Désinfecteur pendant 

--	--

 ans
- Jardinier pendant 

--	--

 ans
- Applicateur de pesticides pendant 

--	--

 ans
- Pompiers pendant 

--	--

 ans
- Pompiste (station d'essence) pendant 

--	--

 ans
- Garagiste pendant 

--	--

 ans
- Agriculteur (utilisation de pesticides) pendant 

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 ans
- Maître-nageur, Agent de service ou ouvrier dans les piscines pendant 

--	--

 ans



idant

r-Désin

idant

pendant

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ice ou c

Vestiaires avec double compartiment  
 Cantine

- Ouvrier fondeur, Ouvrier métallurgiste pendant   ans
- Chaudronnier pendant   ans
- Ouvrier de traitement de surface (chimique ou mécanique) pendant   ans
- Ouvrier de maintenance des installations techniques pendant   ans
- Autres : \_\_\_\_\_ pendant   ans

Vous pouvez cocher plusieurs cases (30 au maximum).

23. Au cours de votre activité professionnelle hors Ville de Paris, avez-vous porté des équipements de protection individuelle (EPI)

- Casque avec jugulaire
- Lunettes
- Vêtements de travail
- Combinaison jetable
- Chaussures de sécurité et/ou bottes
- Gants
- Harnais
- Casquette et gilet de sécurité
- Détecteur de gaz
- Coquilles anti-bruit
- Bouchons d'oreille
- Autres :

24. En fin de travail, quelles sont les conditions et les mesures d'hygiène? Disposez-vous de :

- Douches avec eau chaude
- WC
- Lavabos avec eau chaude
- Vestiaires avec double compartiment (vêtement de ville et vêtement de travail)
- Cantine
- Salle de pause
- Les moyens mis à disposition étaient-ils suffisants
- Les moyens mis à disposition présentaient-ils une bonne hygiène :  Oui,  Non
- Les vêtements de travail étaient-ils lavés par le service :  Oui,  Non
- Existait-il un circuit propre et un circuit sale:  Oui,  Non
- Autres :

Vous pouvez cocher plusieurs cases (10 au maximum).

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# Mécanicien Auto pendant

## ANTECEDENTS ET EXPOSITIONS PROFESSIONNELS EN DEHORS DE LA VILLE DE PARIS

25. Avez-vous occupé d'autres postes de travail avant votre embauche à la Ville de Paris?

- Aucun poste
- Ouvrier
- Agent administratif
- Agent de service, Employé travaillant en égout
- Agent de maîtrise
- Autre:

26. Pendant combien d'années avez-vous travaillé dans des entreprises ou sociétés hors de la Ville de Paris?

La réponse doit être comprise entre 0 et 50.

27. Hors de la Ville de Paris, avez-vous occupé des postes de travail de ? Indiquez le nombre d'année pour le ou les postes que vous avez occupés.

- Menuiserie pendant   ans
- Chauffagiste pendant   ans
- Plombier pendant   ans
- Maçon pendant   ans
- Carreleur   ans
- Paveur-granitier   ans
- Soudeur pendant   ans
- Métalier pendant   ans
- Mécanique générale pendant   ans
- Mécanicien Auto pendant   ans
- Peintre pendant   ans
- Electricien pendant   ans
- Eboueur pendant   ans
- Couvreur pendant   ans
- Ouvrier dans usine de matières plastiques pendant   ans
- Manoeuvre BTP pendant   ans
- Agent d'entretien BTP pendant   ans
- Ouvrier BTP (démolition, ravalement pendant   ans
- Ouvrier granitier-paveur pendant   ans
- Ouvrier désamiantage pendant   ans





Vêtements de travail



Combinaison jetable

ottes

- Ouvrier granitier-paveur pendant   ans
  - Ouvrier de chantier naval pendant   ans
  - Agent de salubrité, Dératiseur-Désinfecteur pendant   ans
  - Jardinier pendant   ans
  - Applicateur de pesticides pendant   ans
  - Pompiers pendant   ans
  - Pompiste (station d'essence) pendant   ans
  - Garagiste pendant   ans
  - Agriculteur (utilisation de pesticides) pendant   ans
  - Maître-nageur, Agent de service ou ouvrier dans les piscines pendant   ans
  - Ouvrier fondeur, Ouvrier métallurgiste pendant   ans
  - Chaudronnier pendant   ans
  - Ouvrier de traitement de surface (chimique ou mécanique) pendant   ans
  - Ouvrier de maintenance des installations techniques pendant   ans
  - Autres : \_\_\_\_\_ pendant   ans
- Vous pouvez cocher plusieurs cases (30 au maximum).

28. Au cours de votre activité professionnelle hors ville de Paris aviez-vous porté des équipements de protection individuelle (EPI)

- Casque avec jugulaire
- Lunettes
- Vêtements de travail
- Combinaison jetable
- Chaussures de sécurité et/ou bottes
- Gants
- Harnais
- Casquette et gilet de sécurité
- Détecteur de gaz
- Coquilles anti-bruit
- Bouchons d'oreille
- Autres :

Vous pouvez cocher plusieurs cases.





# Fluides de lubrification et de refroidissement

exposition faible , exposition moyenne , exposition forte

29. En fin de travail, quelles étaient les conditions et les mesures d'hygiène? Disposiez-vous de :

- Douches avec eau chaude
- WC
- Lavabos avec eau chaude
- Vestiaires avec double compartiment (vêtement de ville et vêtement de travail)
- Cantine
- Salle de pause
- Les moyens mis à disposition étaient-ils suffisants
- Les moyens mis à disposition présentaient-ils une bonne hygiène :  Oui,  Non
- Les vêtements de travail étaient-ils lavés par le service :  Oui,  Non
- Existait-t-il un circuit propre et un circuit sale:  Oui,  Non
- Autres :

Vous pouvez cocher plusieurs cases (10 au maximum).

## EXPOSITION AUX AGENTS CHIMIQUES

30. Avez-vous été exposé au cours de votre carrière professionnelle aux produits chimiques suivants?

- Acides :  
exposition faible , exposition moyenne , exposition forte
- Solvants:  
exposition faible , exposition moyenne , exposition forte
- Essences, fuel, gazole:  
exposition faible , exposition moyenne , exposition forte
- Fibres d'amiante, céramique, verre, roche:  
exposition faible , exposition moyenne , exposition forte
- Gaz et fumée:  
exposition faible , exposition moyenne , exposition forte
- COV :  
exposition faible , exposition moyenne , exposition forte
- Fluides de lubrification et de refroidissement:  
exposition faible , exposition moyenne , exposition forte
- Matières plastiques:  
exposition faible , exposition moyenne , exposition forte
- Métaux:  
exposition faible , exposition moyenne , exposition forte
- Poussières de bois:  
exposition faible , exposition moyenne , exposition forte
- Aérosols biologiques ou poussières animales:  
exposition faible , exposition moyenne , exposition forte
- Produits phytosanitaires:  
exposition faible , exposition moyenne , exposition forte
- Fumées et poussières de goudrons, de bitumes:  
exposition faible , exposition moyenne , exposition forte

Vous pouvez cocher plusieurs cases (2 au maximum).





Radiateurs

Panneaux radiants

**EXPOSITION ENVIRONNEMENTALE**

31. Quelle durée passez-vous en moyenne par jour dans un véhicule à moteur (moto, voiture, camion, bus, ...) en nombre d'heures par jour

- Trajet domicile-travail:   h/jour  
 Durant l'activité professionnelle   h/jour  
 Durant vos loisirs   h/jour

Vous pouvez cocher plusieurs cases.

32. Etes-vous ou avez-vous été pompier volontaire au moins 3 mois dans les 15 dernières années ?

- Oui  
 Non

33. Au cours de la dernière semaine, combien de fois avez-vous utilisé le barbecue?

- Jamais  
 Nombre de fois

34. Avez-vous utilisé le barbecue au cours des 2 derniers jours?

- Oui  
 Non

35. Utilisez-vous une cuisinière à bois ?

- Oui  
 Non

36. Quels types d'appareils utilisez-vous à votre domicile?

- Chauffe-bain  
 Chauffe-eau  
 Chaudière  
 Radiateurs  
 Panneaux radiants  
 Poêle  
 Foyer ouvert (cheminée sans récupération de chaleur)  
 Foyer ouvert "cheminée" avec récupération de chaleur  
 Foyer fermé ou insert sans récupération de chaleur  
 Foyer fermé ou insert avec récupération de chaleur  
 Rien, pas de chauffage  
 Autres :



# MODE DE VIE : TABAC, BRICOLAGE

37. Indiquez le type de combustibles utilisés ?

- Bois
- Charbon
- Fuel/mazout
- Gaz de réseau urbain
- Gaz bouteille/réservoirs
- Pétrole
- Autres :

Vous pouvez cocher plusieurs cases.

38. Quel chauffage utilisez-vous ?

- Chauffage individuel
- Chauffage collectif

39. Résidez-vous ou avez-vous résidé à proximité d'une ou des installations industrielles suivantes ?

- Production de charbon de bois
- Usine d'incinération d'ordures ménagères, hospitalières, industrielle
- Industrie métallurgique, fonderie
- Industrie de production de pâte à papier
- Industrie pétrolière, raffinerie, pétrochimie
- Production de pesticides chlorés
- Centrale thermique
- Cimenterie

Vous pouvez cocher plusieurs cases.

40. Avez-vous été victime ou exposé à un incendie à votre domicile ou sur votre lieu de travail dans les 5 dernières années?

- Oui
- Non

## MODE DE VIE : TABAC, BRICOLAGE, SPORT

41. Fumez-vous ?

- Jamais
- Fume régulièrement   cig/jour depuis   ans
- J'ai arrêté de fumer depuis   ans

42. Si vous n'êtes pas fumeur, avez-vous été exposé au tabagisme passif de la part de votre entourage (à domicile ou au travail)?

- Non
- Oui, nombre d'heures   par jour et pendant   ans

43. Faites-vous de l'exercice physique ou une activité sportive?

- Non
- Oui, nombre   heures/jour et depuis   ans



44. Avez-vous fait une activité physique massive au cours des 2 derniers jours ?

- Oui
- Non

45. Faites-vous régulièrement des activités de bricolage ou de loisirs?

- Travaux de réfection de son domicile : maçonnerie, peinture, plomberie
- Jardinage
- Pêche
- Chasse
- Autre :

46. Buvez-vous régulièrement des boissons alcoolisées (vin, bière, alcool fort, liqueurs)?

- Très souvent
- Souvent
- Rarement
- Jamais

Vous pouvez cocher plusieurs cases.

Si c'est souvent ou très souvent:

nombre de verres de vin par jour

--	--

Nombre de canettes de bière par jour

--	--

Nombre de verres d'alcool fort par jour

--	--

**DES QUESTIONS CONCERNANT VOTRE SANTE?**



51. Au cours des 12 derniers mois, combien

3 crises

2 crises

**TOUX, CRACHATS, SIFFLEMENTS ET ESSOUFLEMENTS**

47. Au cours des 12 derniers mois, avez-vous toussé?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Seulement pendant une infection respiratoire
- Pas du tout

48. Au cours des 12 derniers mois, avez-vous craché?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Seulement pendant une infection respiratoire
- Pas du tout

49. Au cours des 12 derniers mois, avez-vous été essoufflé?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Seulement pendant une infection respiratoire
- Pas du tout

50. Au cours des 12 derniers mois, avez-vous des sifflements dans la poitrine?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Seulement pendant une infection respiratoire
- Pas du tout

51. Au cours des 12 derniers mois, combien de fois avez-vous eu de crises graves de sifflements dans la poitrine?

- 3 crises
- 2 crises
- 1 crise
- Aucune crise

52. Au cours des 12 derniers mois, combien de temps a duré la crise la plus pénible?

- Une semaine ou plus
- 3 jours ou plus
- 1 à 2 jours
- moins d'une journée

53. Quand vous avez des sifflements, c'est pire?

- Le matin
- Le soir
- Le matin et le soir



combien

s, c'est

## 58. Que pensez-vous de vos problèmes resp

Mon travail est la cause de mes problè

54. Ma toux ou ma respiration perturbe mon sommeil?

- Oui
- Non

55. Je suis essoufflé?

- Au repos Oui
- Au repos Non
- Marcher sur un terrain plat Oui
- Marcher sur un terrain plat Non
- Monter un étage Oui
- Monter un étage Non
- Monter une côte Oui
- Monter une côte Non
- Le travail manuel ou activité physique Oui
- Le travail manuel ou activité physique Non

56. Au cours des 12 derniers mois, combien de fois avez-vous eu de la bronchite aiguë (avec toux, crachats, fièvre)?

- 3 fois
- 2 fois
- 1 fois
- Pas du tout

57. Que pensez-vous de votre état respiratoire?

- C'est mon plus gros problème
- Cela me pose pas mal de problèmes
- Cela me pose quelques problèmes
- Cela ne me pose aucun problème

58. Que pensez-vous de vos problèmes respiratoires et de de votre travail?

- Mon travail est la cause de mes problèmes respiratoires
- Mon travail n'est pas la cause de mes problèmes respiratoires
- Mon travail a aggravé mes problèmes respiratoires
- Mon travail n'a pas aggravé mes problèmes respiratoires
- Mon travail déclenche souvent mes problèmes respiratoires
- Mon travail déclenche rarement mes problèmes respratoires

Vous pouvez cocher plusieurs cases (3 au maximum).

### IRRITATIONS ALLERGIES: NEZ, SINUS, GORGE, ORL, YEUX

59. Au cours des 12 derniers mois, avez-vous eu le nez qui coule?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Seulement pendant une infection respiratoire ou nasale
- Pas du tout

ANO1

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ANO1

**64. Au cours des 12 derniers mois, avez-vous**

60. Au cours des 12 derniers mois, avez-vous eu mal au crâne (sinusite)?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Seulement pendant une infection respiratoire ou nasale
- Pas du tout

61. Au cours des 12 derniers mois, avez-vous eu mal à la gorge?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Seulement pendant une infection respiratoire ou de la gorge
- Pas du tout

62. Au cours des 12 derniers mois, avez-vous eu mal aux yeux (irritations et démangeaisons des yeux)?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Seulement pendant une infection des yeux
- Pas du tout

63. Au cours des 12 derniers mois, avez-vous eu mal aux oreilles (irritations et démangeaisons aux oreilles)?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Seulement pendant une infection des oreilles
- Pas du tout

**PEAU ET ALLERGIES CUTANÉES**

64. Au cours des 12 derniers mois, avez-vous eu des irritations ou des démangeaisons sur tout le corps?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Seulement pendant une infection de la peau
- Pas du tout

65. Au cours des 12 derniers mois, avez-vous eu des irritations ou des démangeaisons de la peau sur les parties découvertes (mains, bras, visage, crâne)?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Seulement pendant une infection de la peau
- Pas du tout



Presque tous les jours de la semaine

Plusieurs jours par semaine (2-4 jours)

66. Si les irritations et les démangeaisons sont localisées sur les mains, pensez-vous que ce soit lié au port de gants ?

Non

Oui

Si oui :

à quel type de gants

--	--	--	--	--	--	--	--	--	--

67. Au cours des 12 derniers mois, avez-vous eu des irritations ou des démangeaisons dans les pieds, entre les orteils ?

Presque tous les jours de la semaine

Plusieurs jours par semaine (2-4 jours)

Quelques jours par mois

Seulement pendant une infection des pieds

Pas du tout

68. Si les irritations et les démangeaisons sont localisées sur les pieds, pensez-vous que ce soit lié au port de chaussures ou de bottes ?

Non

Oui

Si oui :

à quel type de chaussures ou bottes

--	--	--	--	--	--	--	--	--	--

69. Au cours des 12 derniers mois, avez-vous eu sur la peau des plaques avec des croûtes et qui vous démangent (exemple eczéma) ?

Presque tous les jours de la semaine

Plusieurs jours par semaine (2-4 jours)

Quelques jours par mois

Seulement pendant une infection de la peau

Pas du tout

70. Au cours des 12 derniers mois, avez-vous eu une infection de la peau (exemple : abcès, furoncle, plaie infectée, acné surinfectée, eczéma surinfectée ...) ?

Presque tous les jours de la semaine

Plusieurs jours par semaine (2-4 jours)

Quelques jours par mois

Pas du tout

Vous pouvez cocher plusieurs cases.

#### COEUR ET VAISSEAUX

71. Au cours des 12 derniers mois, avez-vous eu une douleur thoracique comme un étai ou une compression sur la poitrine (angine de poitrine) ?

Presque tous les jours de la semaine

Plusieurs jours par semaine (2-4 jours)

Quelques jours par mois

Pas du tout



ivez-vo

naine

4 jours

72. Au cours des 12 derniers mois, avez-vous eu des maux de tête intenses ou des migraines ?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Pas du tout

73. Au cours des 12 derniers mois, avez-vous eu des douleurs ou des crampes aux jambes vous obligeant de marcher lentement ou de vous arrêter ?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Pas du tout

74. Au cours des 12 derniers mois, avez-vous eu les chevilles ou les jambes qui gonflent ?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Pas du tout

75. Au cours des 12 derniers mois avez-vous eu des étourdissements?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Pas du tout

76. Au cours des 12 derniers mois, avez-vous eu un malaise (exemple pâleur, fatigue intense, sans perte de conscience)?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Pas du tout

#### INFECTIONS, FOIE ET TROUBLES DIGESTIFS

77. Au cours des 12 derniers mois, avez-vous eu des diarrhées?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Seulement pendant une infection digestive (gastro-entérite)
- Pas du tout

78. Au cours des 12 derniers mois, avez-vous eu des vomissements?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Seulement pendant une infection digestive (gastro-entérite)
- Pas du tout



ANO1



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ANO1



Presque toutes les fois quand j'ai les tr

Souvent quand j'ai les troubles digesti

es diges

79. Au cours des 12 derniers mois, avez-vous eu des nausées?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Seulement pendant une infection digestive (gastro-entérite)
- Pas du tout

80. Au cours des 12 derniers mois, avez-vous eu des brûlures gastriques ?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Seulement pendant une infection digestive (gastro-entérite)
- Pas du tout

81. Au cours des 12 derniers mois, avez-vous eu des crampes au ventre?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Seulement pendant une infection digestive (gastro-entérite)
- Pas du tout

82. Au cours des 12 derniers mois, avez-vous eu des constipations?

- Presque tous les jours de la semaine
- Plusieurs jours par semaine (2-4 jours)
- Quelques jours par mois
- Seulement pendant une infection digestive (gastro-entérite)
- Pas du tout

83. Au cours des 12 derniers mois, avez-vous eu de la fièvre au cours des troubles digestifs: diarrhée, nausées, vomissements, crampes au ventre, constipations ?

- Presque toutes les fois quand j'ai les troubles digestifs
- Souvent quand j'ai les troubles digestifs
- Rarement quand j'ai les troubles digestifs
- Pas du tout

#### LES DOULEURS MUSCULO-SQUELETTIQUES

84. Avez-vous eu durant les 12 derniers mois des problèmes (courbatures, douleur, inconfort) au niveau du cou ?

- oui
- non

Une seule réponse.



## 89. En moyenne, quelle est l'intensité de ce

85. Quelle a été la fréquence de ce problème ?

- presque jamais
- rarement (tous les 2 à 3 mois)
- parfois (tous les mois)
- fréquemment (toutes les semaines)
- presque toujours (tous les jours)
- non concerné

Une réponse.

86. En moyenne, quelle est l'intensité de ce problème ?

- Faible
- Modérée
- Forte
- Insupportable
- Non concerné

Une seule réponse.

87. Avez-vous eu durant les 12 derniers mois des problèmes (courbatures, douleurs, inconfort) au niveau du haut du dos ?

- Oui
- Non

Une seule réponse.

88. Quelle a été la fréquence de ce problème ?

- presque jamais
- rarement
- parfois
- fréquemment
- presque toujours
- non concerné

Une seule réponse.

89. En moyenne, quelle est l'intensité de ce problème ?

- Faible
- Modérée
- Forte
- Insupportable
- Non concerné

Une seule réponse.

90. Avez-vous eu durant les 12 derniers mois des problèmes (lumbago, lombalgies) au niveau du bas du dos ?

- Oui
- Non

Une seule réponse.



## 95. En moyenne, quelle est l'intensité de ce

91. Quelle a été la fréquence de ce problème ?

- presque jamais
- rarement
- parfois
- fréquemment
- presque toujours
- non concerné

Une seule réponse.

92. En moyenne, quelle est l'intensité de ce problème ?

- Faible
- Modérée
- Forte
- Insupportable
- Non concerné

Une seule réponse.

93. Avez-vous eu durant les 12 derniers mois des problèmes (courbatures, douleur, inconfort) au niveau de l'épaule droite ?

- Oui
- Non

Une seule réponse.

94. Quelle a été la fréquence de ce problème ?

- presque jamais
- rarement
- parfois
- fréquemment
- presque toujours
- non concerné

Une seule réponse.

95. En moyenne, quelle est l'intensité de ce problème ?

- Faible
- Modérée
- Forte
- Insupportable
- Non concerné

Une seule réponse.

96. Avez-vous eu durant les 12 derniers mois des problèmes (courbatures, douleur, inconfort) au niveau de l'épaule gauche ?

- oui
- non

Une seule réponse.

97. Quelle a été la fréquence de ce problème ?

- presque jamais
- rarement
- parfois



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roblèm

Forte

Insupportable

- fréquemment
  - presque toujours
  - non concerné
- Une seule réponse.

98. En moyenne, quelle est l'intensité de ce problème ?

- Faible
  - Modérée
  - Forte
  - Insupportable
  - Non concerné
- Une seule réponse.

99. Avez-vous eu durant les 12 derniers mois des problèmes (courbatures, douleur, inconfort) au niveau du coude droit?

- Oui
  - Non
- Une seule réponse.

100. Quelle a été la fréquence de ce problème ?

- presque jamais
  - rarement
  - parfois
  - fréquemment
  - presque toujours
  - non concerné
- Une seule réponse.

101. En moyenne, quelle est l'intensité de ce problème ?

- Faible
  - Modérée
  - Forte
  - Insupportable
  - Non concerné
- Une seule réponse.

102. Avez-vous eu durant les 12 derniers mois des problèmes (courbatures, douleur, inconfort) au niveau du coude gauche?

- Oui
  - Non
- Une seule réponse.

103. Quelle a été la fréquence de ce problème ?

- presque jamais
  - rarement
  - parfois
  - fréquemment
  - presque toujours
  - non concerné
- Une seule réponse.



miers n

e problè

# 108. Avez-vous eu durant les 12 derniers m

104. En moyenne, quelle est l'intensité de ce problème ?

- Faible
- Modérée
- Forte
- Insupportable
- Non concerné

Une seule réponse.

105. Avez-vous eu durant les 12 derniers mois des problèmes (courbatures, douleur, inconfort) au niveau du poignet-main droit ?

- Oui
- Non

Une seule réponse.

106. Quelle a été la fréquence de ce problème ?

- presque jamais
- rarement
- parfois
- fréquemment
- presque toujours
- non concerné

Une seule réponse.

107. En moyenne, quelle est l'intensité de ce problème ?

- Faible
- Modérée
- Forte
- Insupportable
- Non concerné

Une seule réponse.

108. Avez-vous eu durant les 12 derniers mois des problèmes (courbatures, douleur, inconfort) au niveau du poignet-main gauche ?

- Oui
- Non

Une seule réponse.

109. Quelle a été la fréquence de ce problème ?

- presque jamais
- rarement
- parfois
- fréquemment
- presque toujours
- non concerné

Une seule réponse.



problè

Non  
Une seule réponse.

re trava

110. En moyenne, quelle est l'intensité de ce problème ?

- Faible
- Modérée
- Forte
- Insupportable
- Non concerné

Une seule réponse.

111. Votre travail vous oblige-t-il à travailler très vite ?

- rarement
- parfois
- assez souvent
- très souvent

Une seule réponse.

112. Votre travail vous oblige-t-il à être très productif ?

- rarement
- parfois
- assez souvent
- très souvent

Une seule réponse.

113. Généralement, est ce que vous avez beaucoup de chose à faire ?

- rarement
- parfois
- assez souvent
- très souvent

Une seule réponse.

114. En ce moment, est ce que vous avez beaucoup de choses à faire ?

- Oui
- Non

Une seule réponse.

115. Etes-vous débordé(e) dans votre travail ?

- jamais
- de temps en temps
- souvent
- toujours

Une seule réponse.

116. Dans quelle mesure êtes-vous confronté(e) aux conditions suivantes dans votre propre travail : travail sous délai en général ?

- pas du tout
- un peu
- assez
- beaucoup

Une seule réponse.



confron

aucun

un peu

117. Est-ce le cas en ce moment ?

Oui

Non

Une seule réponse.

118. Avez-vous du retard dans le travail (en général)

pas du tout

un peu

assez

beaucoup

Une seule réponse.

119. Avez-vous du retard dans votre travail en ce moment?

Oui

Non

Une seule réponse.

120. Avez-vous des objectifs de rendement ?

pas du tout

un peu

assez

beaucoup

Une seule réponse.

121. Votre travail nécessite-t-il votre pleine attention ?

jamais

de temps en temps

souvent

toujours

Une seule réponse.

122. Quel est le risque d'erreur dans votre travail si vous arrêtez de vous concentrer pendant un moment ?

aucun

un peu

assez

beaucoup

Une réponse.

123. Pouvez-vous choisir l'ordre dans lequel vous accomplissez vos tâches ?

très fortement

beaucoup

modérément

un peu

très peu

Une seule réponse.



ANO1



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ANO1

## 128. Participez-vous à l'organisation de vo

124. Pouvez-vous décider quelle quantité de travail vous allez effectuer ?

- très fortement
- beaucoup
- modérément
- un peu
- très peu

Une seule réponse.

125. Pouvez-vous travailler à la vitesse ou au rythme que vous souhaitez ?

- très fortement
- beaucoup
- modérément
- un peu
- très peu

Une seule réponse.

126. Pouvez-vous prendre de l'avance dans votre travail ?

- souvent
- parfois
- rarement
- jamais
- non concerné

Une seule réponse.

127. Etes-vous partie prenante dans les décisions qui concernent votre travail ?

- énormément
- beaucoup
- moyennement
- un peu
- presque pas

Une seule réponse.

128. Participez-vous à l'organisation de votre travail ?

- énormément
- beaucoup
- moyennement
- un peu
- presque pas

Une seule réponse.

129. Quelle influence avez-vous sur la qualité du travail qui vous est confié ?

- très grande
- beaucoup
- modérée
- un peu
- très peu

Une seule réponse.



A N O 1



2 5



V D P S L B E E C



A N O 1

r la qua



Beaucoup

assez

130. Décidez-vous quelle partie du travail vous allez effectuer ?

- énormément
- beaucoup
- moyennement
- un peu
- presque pas

Une seule réponse.

131. Est-ce que votre supérieur hiérarchique immédiat délaisse momentanément son travail pour vous aider dans le votre ?

- Beaucoup
- assez
- un peu
- pas du tout

Une seule réponse.

132. Est-ce que vos collègues délaissent momentanément leur travail pour vous aider dans le votre ?

- Beaucoup
- assez
- un peu
- pas du tout

Une seule réponse.

133. Est-il facile de discuter avec votre supérieur hiérarchique immédiat ?

- Beaucoup
- assez
- un peu
- pas du tout

Une seule réponse.

134. Est-il facile de discuter avec vos collègues ?

- Beaucoup
- assez
- un peu
- pas du tout

Une seule réponse.

135. Pouvez-vous compter sur votre supérieur hiérarchique immédiat en cas de difficultés dans le travail ?

- Beaucoup
- assez
- un peu
- pas du tout

Une seule réponse.

136. Pouvez-vous compter sur vos collègues en cas de difficultés dans le travail ?

- Beaucoup
- assez
- un peu
- pas du tout

Une seule réponse.



: supérieur

collègue

# 141. Quel intérêt portez-vous à votre travail ?

Donnez une note de 0 à 10 : 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10

137. Est-ce que votre supérieur hiérarchique immédiat est disponible pour écouter vos problèmes personnels ?

- Beaucoup
- assez
- un peu
- pas du tout
- non concerné

Une seule réponse.

138. Est-ce que vos collègues sont disponibles pour écouter vos problèmes personnels ?

- Beaucoup
- assez
- un peu
- pas du tout
- non concerné

Une seule réponse.

139. Selon vous, quelles sont les possibilités de voir votre emploi supprimé dans les prochaines années ?

- Nulle
- Moyenne
- Probable
- Certaine

Une seule réponse.

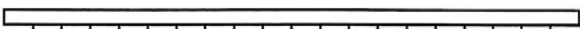
140. Selon vous, quelles sont les possibilités que dans les prochaines années votre emploi soit automatisé ?

- Nulle
- Moyenne
- Probable
- Certaine

Une seule réponse.

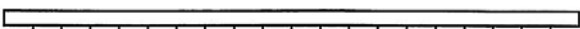
141. Quel intérêt portez-vous à votre travail ?

Donnez une note de 0 à 10 : 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 : de très peu d'intérêt à très grand intérêt.

0  10

142. Comment jugez-vous la complexité de votre travail ?

Donnez une note sur une échelle de 0 à 10 : 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 : de travail très peu complexe à travail très complexe.

0  10

143. Travail manuel : S'agit-il d'un travail à la chaîne ?

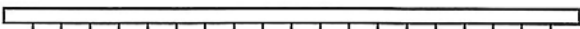
- Oui
- Non

Une seule réponse.

144. Selon vous la contrainte (ou pression) de temps lors du travail est-elle faible ou très forte ?

Donnez une note sur une échelle de 0 à 10 : 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10.

De très faible 0 à très forte 10.

0  10



# 151. Au travail, avez-vous déjà éprouvé la

Non

145. Les gestes de travail sont-ils répétitifs ?

Donnez une note sur une échelle de 0 à 10: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10.

De très peu répétitif (0) à très répétitif (10).

0  10

146. La cadence (ou rythme de travail) est-elle rapide ou non ?

Donnez une note sur une échelle de 0 à 10: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10.

Cadence très faible (0) à cadence très forte (10).

0  10

147. La force musculaire requise est-elle forte ou non ?

Donnez une note sur une échelle de 0 à 10: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10.

Force musculaire requise très faible (0) à force musculaire requise très forte (10).

0  10

148. Le travail nécessite-t-il des mouvements précis et très fins ?

Donnez une note sur une échelle de 0 à 10 : 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10.

Mouvements peu précis et rarement fins (0) à mouvements précis et très fins (10).

0  10

149. Après votre travail, la fatigue musculaire dans les membres supérieurs est-elle intense ou non ?

Donnez une note sur une échelle de 0 à 10 : 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10.

Pas de fatigue (0) à fatigue très intense (10).

0  10

150. Utilisez-vous plus souvent une main que l'autre ?

non

main droite

main gauche

Une seule réponse.

151. Au travail, avez-vous déjà éprouvé la sensation de froid (<10°C)

Oui

Non

Une seule réponse.

152. Au travail, avez-vous déjà éprouvé la sensation d'humidité ?

Oui

Non

Une seule réponse.

153. Au travail, avez-vous déjà éprouvé la sensation de bruit ?

Oui

Non

Une seule réponse.

154. Au travail, avez-vous déjà éprouvé la sensation de chaleur ?

Oui

Non

Une seule réponse.



un peu

beaucoup

155. Au travail, avez-vous déjà éprouvé la sensation d'empoussièrément ?

- Oui
- Non

Une seule réponse.

156. Quels sont les trois outils les plus pénibles que vous utilisez ?


157. Selon vous, au cours de votre travail, la température de vos mains est-elle ?

- ni chaude ni froide
- plutôt chaude
- plutôt froide

Une seule réponse.

158. Selon vous, l'un ou plusieurs des outils que vous utilisez vibrent-ils ?

- pas du tout
- un peu
- beaucoup
- non concerné

Une seule réponse.

159. Quels sont les outils vibrants ?

--	--	--	--	--	--	--	--	--	--

160. Cette vibration vous gêne-t-elle ?

- pas du tout
- un peu
- beaucoup
- non concerné

Une seule réponse.

161. Portez-vous des gants ?

- Oui
- Non

Une seule réponse.

162. La pression que vous exercez sur l'objet travaillé est-elle:

- très faible
- faible
- moyenne
- forte
- très forte

Une seule réponse.



ANO1



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ANO1

sur l'obj

# Une seule réponse.

163. Durant les 12 derniers mois, avez-vous

163. Travail informatisé : Quelle est votre tâche principale ?

- Saisie de données
- acquisition de données
- traitement de texte
- dialogue
- tâche créative

Une seule réponse.

164. Quelle est en moyenne la durée journalière de cette tâche ?

En heure par jour.

165. Faites-vous toujours la même tâche ?

- oui
- non

Une seule réponse.

## STRESS AU TRAVAIL

166. Depuis que vous travaillez ici, vous sentez-vous stressé(e)?

- pas du tout
- un peu
- beaucoup
- énormément

Une seule réponse.

167. Durant les 12 derniers mois, avez-vous ressenti des palpitations ?

- jamais ou rarement
- quelquefois
- assez souvent
- très souvent ou constamment

Une seule réponse.

168. Durant les 12 derniers mois, avez-vous ressenti des douleurs au niveau du coeur (gêne précordiale) ?

- jamais ou rarement
- quelquefois
- assez souvent
- très souvent ou constamment

Une seule réponse.

169. Durant les 12 derniers mois, avez-vous ressenti des sueurs en l'absence d'effort physique ?

- jamais ou rarement
- quelquefois
- assez souvent
- très souvent ou constamment

Une seule réponse.





assez souvent



très souvent ou constamment

170. Durant les 12 derniers mois, avez-vous ressenti une nervosité ou des tremblements ?

- jamais ou rarement
- quelquefois
- assez souvent
- très souvent ou constamment

Une seule réponse.

171. Durant les 12 derniers mois, avez-vous ressenti des étourdissements ou des vertiges ?

- jamais ou rarement
- quelquefois
- assez souvent
- très souvent ou constamment

Une seule réponse.

172. Durant les 12 derniers mois, avez-vous ressenti la bouche sèche ?

- jamais ou rarement
- quelquefois
- assez souvent
- très souvent ou constamment

Une seule réponse.

173. Durant les 12 derniers mois, avez-vous ressenti des brûlures d'estomac ?

- jamais ou rarement
- quelquefois
- assez souvent
- très souvent ou constamment

Une seule réponse.

174. Durant les 12 derniers mois, avez-vous ressenti des ballonnements, des gaz ?

- jamais ou rarement
- quelquefois
- assez souvent
- très souvent ou constamment

Une seule réponse.

175. Durant les 12 derniers mois, avez-vous ressenti une digestion difficile ?

- jamais ou rarement
- quelquefois
- assez souvent
- très souvent ou constamment

Une seule réponse.

176. Durant les 12 derniers mois, avez-vous ressenti des constipations ou des diarrhées ?

- jamais ou rarement
- quelquefois
- assez souvent
- très souvent ou constamment

Une seule réponse.



ANO1



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ANO1

vez-vous

vez-vous



assez souvent



très souvent ou constamment

177. Durant les 12 derniers mois, avez-vous ressenti la sensation d'avoir l'estomac noué ?

- jamais ou rarement
- quelquefois
- assez souvent
- très souvent ou constamment

Une seule réponse.

178. Durant les 12 derniers mois, avez-vous ressenti la sensation de tension, de crispation ?

- jamais ou rarement
- quelquefois
- assez souvent
- très souvent ou constamment

Une seule réponse.

179. Durant les 12 derniers mois, avez-vous ressenti de l'anxiété ?

- jamais ou rarement
- quelquefois
- assez souvent
- très souvent ou constamment

Une seule réponse.

180. Durant les 12 derniers mois, avez-vous ressenti de l'irritabilité ?

- jamais ou rarement
- quelquefois
- assez souvent
- très souvent ou constamment

Une seule réponse.

181. Durant les 12 derniers mois, avez-vous ressenti des états dépressifs ?

- jamais ou rarement
- quelquefois
- assez souvent
- très souvent ou constamment

Une seule réponse.

182. Durant les 12 derniers mois, avez-vous ressenti des difficultés d'endormissement ?

- jamais ou rarement
- quelquefois
- assez souvent
- très souvent ou constamment

Une seule réponse.

183. Durant les 12 derniers mois, avez-vous ressenti des insomnies ?

- jamais ou rarement
- quelquefois
- assez souvent
- très souvent ou constamment

Une seule réponse.



ANO1



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ANO1

avez-vous

avez-vous

D'accord

Tout à fait d'accord

184. Durant les 12 derniers mois, avez-vous ressenti des périodes de fatigue intense ou d'épuisement ?

- jamais ou rarement
- quelquefois
- assez souvent
- très souvent ou constamment

Une seule réponse.

185. Durant les 12 derniers mois avez-vous eu des soucis ?

- non
- professionnels
- familiaux

Plusieurs réponses.

#### VOTRE OPINION SUR VOTRE SITUATION DE TRAVAIL

186. Dans mon travail, je dois apprendre des choses nouvelles?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

187. Dans mon travail, j'effectue des tâches répétitives?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

188. Mon travail me demande d'être créatif?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

189. Mon travail me permet souvent de prendre des décisions moi-même?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

190. Mon travail demande un haut niveau de compétence

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord



ANO1



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ANO1



Pas du tout d'accord

191. Dans ma tâche, j'ai très peu de liberté pour décider comment je fais mon travail?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

192. Dans mon travail, j'ai des activités variées?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

193. J'ai la possibilité d'influencer le déroulement de mon travail?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

194. J'ai l'occasion de développer mes compétences professionnelles?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

195. Mon travail demande de travailler très vite?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

196. Mon travail demande de travailler intensément?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

197. On me demande d'effectuer une quantité de travail excessive?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

198. Je dispose du temps nécessaire pour exécuter correctement mon travail?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord



e quanti

pour ex

Pas du tout d'accord

199. Je reçois des ordres contradictoires de la part d'autres personnes?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

200. Mon travail nécessite de longues périodes de concentration intense?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

201. Mes tâches sont souvent interrompues avant d'être achevées, nécessitant de les reprendre plus tard?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

202. Mon travail est très "bousculé" ?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

203. Attendre le travail de collègues ou d'autres services ralentit souvent mon propre travail?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

204. Mon supérieur se sent concerné par le bien-être de ses subordonnés?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

205. Mon supérieur prête attention à ce que je dis?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

206. Mon supérieur m'aide à mener ma tâche à bien?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord



A N O 1



3 5



V D P S L B E E C



A N O 1

# 212. Dans l'ensemble, comment estimez-vous Placez-vous sur cette échelle de 1 (=

207. Mon supérieur réussit facilement à faire collaborer ses subordonnés?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

208. Les collègues avec qui je travaille sont des gens professionnellement compétents?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

209. Les collègues avec qui je travaille me manifestent de l'intérêt?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

210. Les collègues avec qui je travaille sont amicaux?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

211. Les collègues avec qui je travaille m'aident à mener les tâches à bien?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord

212. Dans l'ensemble, comment estimez-vous votre état de santé?

Placez-vous sur cette échelle de 1 (= très mauvais) à 10 (= très bon)



213. Pensez-vous que votre travail influence votre santé?

- Non, mon travail n'influence pas ma santé
- Oui, mon travail est plutôt bon pour ma santé
- Oui, mon travail est plutôt mauvais pour ma santé

214. Dans l'ensemble, je suis satisfait de mon travail?

- Pas du tout d'accord
- Pas d'accord
- D'accord
- Tout à fait d'accord



Non, mais j'ai essayé

Non

Oui

215. Il m'arrive de ne pas dormir parce que je pense à mon travail?

- Jamais
- Rarement
- Parfois
- Souvent

216. Dans l'ensemble, estimez-vous que votre travail est fatigant?

Placez-vous sur cette échelle de 1, pas du tout fatigant à 10, extrêmement fatigant

1  10

217. Dans l'ensemble, estimez-vous que votre travail est stressant?

Placez-vous sur cette échelle de 1, pas du tout stressant à 10, extrêmement stressant

1  10

218. Quand vous êtes très fatigué(e) ou stressé(e) par votre travail, que faites vous de préférence?

- Je prends des médicaments
- Je vais voir mon médecin
- Je m'absente de mon travail
- Je regarde la télé
- Je dors davantage
- Je fais du sport
- Je lis ou j'écoute de la musique
- Je consomme davantage de tabac ou d'alcool
- Je mange
- Autre: .....

3 réponses maximum

219. Au cours des 12 derniers mois, avez-vous changé de poste ou de travail en raison de votre santé?

- Oui
- Non, mais j'ai essayé
- Non
- Non, pas de problème de santé

220. Au cours des 12 derniers mois, combien avez-vous eu d'arrêts maladie? (Hors accidents de travail ou de trajet)

- Nombre d'arrêt maladie
- Nombre de jours d'arrêt maladie   jours
- Aucun arrêt

Vous pouvez cocher plusieurs cases (2 au maximum).



Oui actuellement:

Vous empêche de vous exprimer:

221. Au cours des 12 derniers mois, avez-vous eu des accidents de travail (Hors accident de trajet)

- Pas d'accident
- 1 accident
- 2 accidents
- 3 accidents
- 4 accidents et plus

Nombre de jours d'arrêt de travail pour des accidents :   jours  
Vous pouvez cocher plusieurs cases (2 au maximum).

222. Souhaitez-vous changer de poste ou de travail ?

- Non
- Oui, plus tard
- Oui, rapidement
- Quelle que soit votre réponse, indiquez pour quelles raisons :
- Le contenu du travail
- Les risques du travail
- L'organisation du travail
- L'ambiance de travail
- Les conditions de travail et salariales
- Votre âge
- La sécurité de l'emploi
- Autre :.....

Indiquez 3 réponses maximum

223. Vous arrive-t-il de vivre au travail les situations difficiles décrites ci-dessous?

Une personne ou plusieurs personnes se comporte(ent) systématiquement avec vous de la façon suivante :

- Vous ignore, fait comme si vous n'étiez pas là:  
 Oui actuellement,  Oui, dans le passé,  Non
- Tient sur vous des propos désobligeants  
 Oui actuellement:  Oui, dans le passé,  Non
- Vous empêche de vous exprimer:  
 Oui actuellement,  Oui, dans le passé,  Non
- Vous ridiculise en public:  
 Oui actuellement,  Oui, dans le passé,  Non
- Critique injustement votre travail:  
 Oui actuellement:  Oui, dans le passé,  Non
- Vous charge de tâches inutiles ou dégradantes:  
 Oui actuellement:  Oui, dans le passé,  Non
- Sabote votre travail, vous empêche de travailler correctement :  
 Oui actuellement:  Oui, dans le passé,  Non
- Laisse entendre que vous êtes mentalement dérangé(e):  
 Oui actuellement:  Oui, dans le passé,  Non
- Vous dit des choses obscènes ou dégradantes:  
 Oui actuellement:  Oui, dans le passé,  Non
- Vous fait des propositions à caractère sexuel de façon insistante :  
 Oui actuellement:  Oui, dans le passé,  Non

Vous pouvez cocher plusieurs cases.

ANO1

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ANO1

224. Au cours des 12 derniers mois, avez-vous été victime, de la part du public dans le cadre de votre travail :

- Agression verbale :
  - Non,  Oui:   fois
- Agression physique:
  - Non,  Oui:   fois
- Agression sexuelle:
  - Non,  Oui:   fois
- Agression à caractère raciste:
  - Non,  Oui:   fois

Vous pouvez cocher plusieurs cases.



## Questionnaire des troubles du sommeil de l'Hôtel Dieu

Test effectué le :         (jour/mois/année)

Consultez un proche si nécessaire pour répondre attentivement aux questions suivantes.

1/ Avez-vous des problèmes de sommeil ? Parmi les propositions suivantes, avez-vous ?

<i>1 seule réponse par ligne</i>	OUI	NON
Des difficultés à vous endormir	<input type="checkbox"/>	<input type="checkbox"/>
Des réveils nocturnes fréquents	<input type="checkbox"/>	<input type="checkbox"/>
Un réveil trop précoce	<input type="checkbox"/>	<input type="checkbox"/>
Un sommeil de mauvaise qualité	<input type="checkbox"/>	<input type="checkbox"/>
Une sensation de manque de sommeil	<input type="checkbox"/>	<input type="checkbox"/>

*Si vous avez répondu au moins 1 OUI à la question 1/ passez à la question 2/, sinon passez à la question 3/*

2/ Quand vos problèmes de sommeil ont-ils commencé ?

Depuis   mois ou   années

3/ Avez-vous déjà des troubles de sommeil dans l'enfance ou lors de l'adolescence ?

OUI.....  → *Passez à la question 4/*  
 NON.....  → *Passez à la question 5/*

4/ Quels types de troubles ?

*Pour les questions qui suivent, nous vous remercions de bien vouloir compléter les espaces vides dans les phrases qui vous sont proposées. Pour les questions 5/ à 10/, complétez chacune des phrases.*

5/ Pendant la semaine :

“Je me couche à   h  ”

“Je me lève à   h  ”

Pendant le week-end :

“Je me couche à   h  ”

“Je me lève à   h  ”

6/ Quelle est en moyenne votre durée de sommeil (par nuit) ?

“Pendant la semaine, je dors   h   par nuit”

“Pendant le week-end, je dors   h   par nuit”



Jamais.....

# 13/ Après un sommeil normal de nuit, j

*1 seule*

7/ Si vous étiez absolument libre de vous coucher à l'heure que vous voulez, à quelle heure vous coucheriez-vous ?

"Je me coucherai à   h

8/ Si vous étiez absolument libre de vous lever à l'heure que vous voulez, à quelle heure vous leveriez-vous ?

"Je me lèverai à   h

9/ Combien de minutes vous faut-il pour vous endormir ?

"Il me faut habituellement   minutes pour m'endormir"

10/ Combien de fois par nuit vous réveillez-vous ?

"Je me réveille en général   fois par nuit"

Si vous vous réveillez au moins 1 fois par nuit, passez à la question 11/, sinon passez à la question 13/

11/ Pour quelle(s) raison(s) ?

12/ Je n'arrive pas à m'endormir, une fois réveillé(e) :

*1 seule réponse*

- Chaque nuit.....
- Chaque semaine.....
- Rarement.....
- Jamais.....

13/ Après un sommeil normal de nuit, je me sens :

*1 seule réponse*

- Rafraîchi(e).....
- Bien reposé(e).....
- Un peu fatigué(e).....
- Très fatigué(e).....

14/ Faites-vous quelques fois la sieste ?

- OUI.....1  → Passez à la question 15/
- NON.....2  → Passez à la question 17/

15/ Combien de fois par semaine ?

fois par semaine

16/ Après une sieste, je me sens :

*1 seule réponse*

- Rafraîchi(e).....
- Bien reposé(e).....
- Un peu fatigué(e).....
- Très fatigué(e).....

17/ "Je fume en moyenne   cigarettes par jour"

"Ma consommation habituelle de café est de l'ordre de   tasses par jour"

"Ma consommation habituelle de thé est de l'ordre de   tasses par jour"

"Ma consommation habituelle de Coca-cola (ou Pepsi-cola) est de l'ordre de   verres par jour"

"Ma consommation habituelle d'alcool est de l'ordre de   verres par jour"





NON.....



→ Passez à

18/ Prenez-vous habituellement des médicaments pour dormir ?

OUI.....  → Passez à la question 19/

NON.....  → Passez à la question 20/

19/ De quel(s) médicament(s) s'agit-il ?

1<sup>er</sup> médicament.....  
2<sup>ème</sup> .....  
3<sup>ème</sup> .....  
4<sup>ème</sup> .....

20/ Quelles méthodes utilisez-vous pour dormir ?

21/ Vous a-t-on fait remarquer que vous ronflez  
bruyamment ?

OUI.....  → Passez à la question 22/

NON.....  → Passez à la question 24/

22/ Depuis quel âge (ronflez-vous) ?

“Depuis que j’ai   ans”

23/ Cela vous arrive-t-il (de ronfler) ?

Rarement.....

Souvent.....

Presque tous les jours.....

24/ Vous a-t-on fait remarquer qu’il vous arrivait  
d’arrêter de respirer plusieurs secondes  
pendant votre sommeil ?

OUI.....  → Passez à la question 25/

NON.....  → Passez à la question 27/

25/ Qui vous l’a fait remarquer ?

.....  
.....

26/ Si vous vous réveillez à ce moment-là, avez-vous :

<i>1 seule réponse par ligne</i>	OUI	NON
Le cœur battant ?	<input type="checkbox"/>	<input type="checkbox"/>
Des crampes dans les jambes ?	<input type="checkbox"/>	<input type="checkbox"/>
Une sensation de gêne respiratoire ?	<input type="checkbox"/>	<input type="checkbox"/>

27/ Dans la journée, avez-vous des problèmes de nez, ou des difficultés à respirer par le nez ?

OUI.....  → Passez à la question 28/

NON.....  → Passez à la question 29/

28/ Expliquez de quels problèmes il s’agit ?



ANO1



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ANO1

# 30/ Faites vous de l'exercice (du sport)

OUI

→ *Passer à*  
*Passer à*

29/ Pour chacune des phrases suivantes, entourez le code correspondant à votre situation.

<i>1 seule réponse par ligne</i>	Chaque nuit	Chaque semaine	Rarement	Jamais
Je me réveille en ayant mal à la tête	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
On m'a dit que je m'agite dans mon sommeil et que je ne cesse de me tourner et de me retourner toute la nuit	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Pendant mon sommeil, il semble que je donne des coups de pied et que je sursaute	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Je ressens ou j'entends des choses qui ne sont pas réelles quand je m'allonge dans mon lit, alors que je suis encore réveillé(e)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Immédiatement après m'être endormi(e), je rêve	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Après m'être allongé(e), avant de m'endormir, j'ai la sensation de ne plus pouvoir bouger	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Je suis somnambule	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Je suis dérangé(e) par des cauchemars	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Je parle pendant le sommeil	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Je grince des dents quand je dors	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Je me réveille en toussant	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Je me réveille avec des brûlures gastriques	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Une dépression ou une anxiété m'empêchent de dormir	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Je m'endors pendant la journée, pendant le travail, en écoutant la radio ou de la musique, dans les transports, devant la télé	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Lors d'une colère, une déprime, un fou-rire ou une émotion, je ressens une faiblesse dans les genoux, le cou, les mâchoires ou les bras	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

30/ Faites vous de l'exercice (du sport) ?

OUI.....  → *Passer à la question 31/*

NON.....  → *Passer à la question 34/*

31/ Quel type d'exercice (de sport) ?

32/ A quel moment de la journée ?

33/ Combien de fois par semaine ?

fois par semaine

34/ Vous semble-t-il que votre humeur ait changé récemment ?

OUI.....

NON.....



NON.....



35/ Vous semble-t-il que votre mémoire ait baissé soudainement ?

OUI.....

NON.....

36/ Avez-vous noté une diminution de l'intérêt ou de la fonction sexuelle (réponse facultative) ?

OUI.....

NON.....

37/ Avez-vous dans votre famille des antécédents de troubles du sommeil ?

OUI.....  → Passez à la question 38/

NON.....  → Passez à la question 40/

38/ Quel type de troubles ?

39/ Chez qui ces troubles étaient-ils présents ?

40/ Si vous avez des problèmes de sommeil, est-ce que ces problèmes sont plus importants :

<i>1 seule réponse par ligne</i>	OUI	NON
En hiver	<input type="checkbox"/>	<input type="checkbox"/>
En automne	<input type="checkbox"/>	<input type="checkbox"/>
En été	<input type="checkbox"/>	<input type="checkbox"/>
Au printemps	<input type="checkbox"/>	<input type="checkbox"/>

41/ Avez-vous déjà consulté un médecin sur vos problèmes de sommeil ?

OUI.....

NON.....

42/ Quel est votre poids ?

Kilos

43/ Quelle est votre taille ?

m  cm



# Comme passager(e) d'une voiture (ou d'un train) sans arrêt pendant une heure

## Echelle de somnolence d'Epworth

Quelle chance avez-vous de somnoler ou de vous endormir, pas simplement de vous sentir fatigué(e) dans les situations suivantes ?

Cette question concerne votre mode de vie habituel au cours des derniers mois. Au cas où une de ces situations ne s'est pas encore produite récemment, essayez d'imaginer de qui se passerait.

Pour répondre, utilisez l'échelle suivante en cochant la case à côté du chiffre le plus approprié pour chaque situation :

- 0 = aucune chance de s'endormir
- 1 = faible chance de s'endormir
- 2 = chance modérée de s'endormir
- 3 = forte chance de s'endormir

Situation	Probabilité de s'endormir			
	0	1	2	3
Assis(e) en train de lire	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
En train de regarder la télévision	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Assis(e), inactif(e) dans un lieu public (théâtre, cinéma, réunion, ...)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Comme passager(e) d'une voiture (ou d'un transport en commun) roulant sans arrêt pendant une heure	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Allongé(e) l'après midi pour vous reposer, lorsque les circonstances le permettent	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Etant assis(e) en train de parler avec quelqu'un	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Assis(e) au calme après un repas sans alcool	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Dans une voiture immobilisée depuis quelques minutes	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Score =





## Questionnaire Alimentaire

Numéro de volontaire : | \_ | \_ | \_ | \_ | \_ | \_ |

Numéri

Code à 4 lettres : | \_ | \_ | \_ | \_ |

Code à

Date: .....

Date:

**U 557 Inserm (UMR Inserm/INRA/CNAM/Paris 13)**

# INSTRUCTIONS

## A lire attentivement avant de remplir le questionnaire

### A lire

Ce questionnaire a pour but d'estimer vos apports alimentaires.

Il est important de le remplir vous-même. Vous pouvez toutefois vous faire aider de quelqu'un qui connaît bien vos habitudes alimentaires.

Ce question

En suivant les instructions vous verrez qu'il est simple à remplir puisqu'il traduit vos habitudes alimentaires.

Il est importai

quelqu'un qui

En suivant les

habitudes alim

### I- Evaluer la fréquence de consommation des aliments

Ceci est un questionnaire dit "de fréquence" : il porte sur la fréquence de consommation de chaque aliment. Il est composé de tableaux représentant chacun une grande famille d'aliments. Ces tableaux comportent 1 aliment ou groupe d'aliments par ligne. Pour chaque ligne du questionnaire, vous devez répondre à la question suivante : "**Combien de fois avez-vous mangé cet aliment au cours des 12 derniers mois, par jour, par semaine, par mois ou par an ?**"

Ceci est un

consommation

une grande f

d'aliments par

question suiva

derniers mois

Il faut donc y indiquer ce que vous avez mangé en moyenne **au cours des 12 derniers mois**, que ce soit au cours des repas (à domicile ou à l'extérieur) ou entre les repas.

Tous les jours et toutes les occasions sont à prendre en compte (jours de la semaine, week-end et jours de fête).

Il faut donc

derniers mois

repas.

Les colonnes des tableaux vous permettent d'indiquer la fréquence par jour, par semaine, par mois ou par an. *Si, par exemple, vous mangez du pain chaque jour, il est plus facile d'utiliser la colonne 'par jour', par contre si vous mangez des frites plusieurs fois par mois, il sera plus facile d'utiliser la colonne 'par mois'.*

Tous les j

semaine, weel

Les colonne

semaine, par

est plus facile

plusieurs fois

Pour chaque aliment, vous devez mettre un chiffre dans l'une des quatre colonnes ou cocher la case 'jamais' de la cinquième colonne. Il faut donc donner une réponse pour chaque ligne (remplir une case par ligne).

**Attention il est indispensable de mettre un chiffre lorsqu'il y a une consommation, la croix est réservée à la case 'jamais'.**

### Exemple :

Pour chaqu

ou cocher la c

pour chaque li

La réponse ci-dessous correspond à une personne qui mange 4 tranches de pain complet par jour, 2 biscottes par semaine, 3 fois par mois une viennoiserie et 6 brioches individuelles au cours des 12 derniers mois. Le pain blanc et les céréales ne sont jamais consommés.

### Attention

**consommation, la croix est réservée à la case 'jamais'.**

### Exemple :

La réponse ci-dessous correspond à une personne qui mange 4 tranches de pain complet par jour, 2 biscottes par semaine, 3 fois par mois une viennoiserie et 6 brioches individuelles au cours des 12 derniers mois. Le pain blanc et les céréales ne sont jamais consommés.

	PAIN ET CEREALES	FREQUENCE				
		par jour	par semaine	par mois	par an	jamais
	Un quart de baguette, une tranche de pain de mie (y compris dans les sandwiches)					<input checked="" type="checkbox"/>
	Une tranche de pain complet ou aux céréales	4				<input type="checkbox"/>
Un quart de (y compris c	Une biscotte ou cracotte		2			<input type="checkbox"/>
Une tranche	Une portion de céréales de type petit déjeuner (corn-flakes, cheerios, au chocolat, muesli, etc.)					<input checked="" type="checkbox"/>
Une biscotte	Une viennoiserie (croissant, pain au chocolat, etc.)			3		<input type="checkbox"/>
Une portion (corn-flakes	Une brioche (individuelle ou en tranche)				6	<input type="checkbox"/>

Vérifiez à la fin de chaque tableau que vous avez rempli toutes les lignes.

#### Cas particuliers :

Les produits saisonniers (notamment les fruits) : comment exprimer dans le tableau la consommation d'un produit pendant une période limitée dans le temps ?

→ Evaluer la durée de la saison (ex : 4 mois)

→ Evaluer la quantité d'aliment consommée pendant cette période

(ex : 3 tranches de melon par semaine)

→ Exprimer la fréquence par an.


Cela revient à  $(3 \times 4 \text{ semaines}) \times 4 \text{ mois} = 48$  à reporter dans la colonne 'par an'.


**Remarque** : Il est possible d'utiliser les décimales. Par exemple, si vous consommez un produit entre deux et trois fois, notez 2,5.

#### II- Evaluer la quantité mangée à chaque fois

Pour la plupart des aliments, la quantité d'aliment considérée comme une portion unitaire est inscrite dans le tableau. Ainsi, le jambon est compté par tranche : on demande " combien de tranches de jambon avez-vous mangé en moyenne au cours des 12 derniers mois ? ", donc si vous mangez du jambon une fois par semaine et que vous en mangez trois tranches à la fois, vous devez mettre '3' dans la colonne 'par semaine'.

#### II- Evaluer

Pour la plupart des aliments, la quantité d'aliment considérée comme une portion unitaire est prédéfinie, mais un symbole  . Ce symbole renvoie aux photos d'aliments situées sous le tableau concerné. Ces photos ont pour but de vous aider à évaluer la portion des 12 derniers mois ? ", donc si vous mangez du jambon une fois par semaine et que vous en mangez trois tranches à la fois, vous devez mettre '3' dans la colonne 'par semaine'.

**Mais attention** : Pour certains aliments, il n'y a pas de quantité unitaire prédéfinie, mais un symbole  . Ce symbole renvoie aux photos d'aliments situées sous le tableau concerné. Ces photos ont pour but de vous aider à évaluer la portion



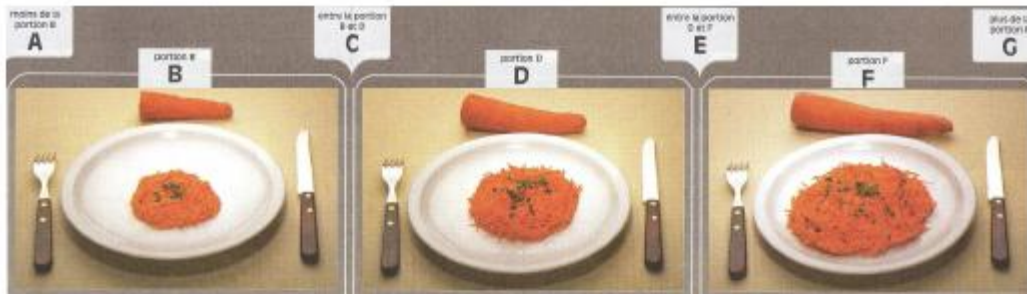
moyenne que vous consommez habituellement. Cochez uniquement la lettre qui correspond le mieux à cette quantité.

**Attention !** Cette photo ne représente qu'un exemple pour les aliments de la famille concernée.

La photographie ci-dessous, par exemple, est destinée à vous aider à évaluer la portion moyenne de légumes que vous mangez en général, quel que soit le légume. La carotte n'est qu'un exemple. Il faut répondre à la question : " En général, lorsque vous mangez des légumes, quelle quantité en mangez-vous ? " en cochant une case : les lettres B, D, F correspondent aux photos, les lettres A, C, E, G aux portions se situant entre les portions B, D, F.

**Regardez la photo ci-dessous : quand vous mangez des légumes en général, quelle quantité moyenne en mangez-vous ? Cochez la lettre qui correspond à la portion de légumes que vous mangez en moyenne (cochez une seule case) :**

A      B      C      D      E      F      G  
                       



**III- Indiquer les produits que vous mangez**

Pour certains aliments, il vous est demandé de noter les deux produits que vous mangez le plus fréquemment : notez bien la marque et les noms du produit.

Exemple : pour les céréales, quelqu'un qui mange la plupart du temps des Spécial K de Kellog's ou des Golden Grahams de Nestlé remplira le tableau ainsi:

	Marque	Nom du produit
Produit 1	KELLOG'S	SPECIAL K
Produit 2	NESTLE	GOLDEN GRAHAMS

Cette information est indispensable, dans la mesure du possible, pour prendre en considération les différentes compositions nutritionnelles d'un même produit.

Merci de votre participation,  
 Bon courage !

	Marque	Nom du produit
Produit 1	KELLOG'S	SPECIAL K
Produit 2	NESTLE	GOLDEN GRAHAMS

**III- Indique**

Pour certai

mangez le

Exemple :

de Kellog's

Cette information est indispensable, dans la mesure du possible, pour prendre en considération les différentes compositions nutritionnelles d'un même produit.

Merci de votre participation,  
 Bon courage !



## A - QUESTIONNAIRE ALIMENTAIRE

A1 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :

A - QUEST

A1 - Au co

¼ de baguette

compris a

¼ de baguette

tranche d

les sandw

1 biscotte

1 part de

cheerios,

1 viennois

1 brioche

A2. Si vous

que vous c

A3 - Au co

1 cuilleré

1 cuilleré

1 morceai

(dans le cc

A4 - Au co

1 tasse de

1 tasse de

1 tasse de

1 tasse de

1 tasse de

1 tasse de

PAIN ET CEREALES	FREQUENCE				
	par jour	par semaine	par mois	par an	Jamais
¼ de baguette de pain blanc, une tranche de pain de mie (y compris dans les sandwiches)					<input type="checkbox"/>
¼ de baguette de pain complet ou aux céréales, une tranche de pain complet ou aux céréales (y compris dans les sandwiches)					<input type="checkbox"/>
1 biscotte, petit-grillé ou cracotte					<input type="checkbox"/>
1 part de céréales de type petit déjeuner (corn-flakes, cheerios, au chocolat, muesli, etc.)					<input type="checkbox"/>
1 viennoiserie (croissant, pain au chocolat, etc.)					<input type="checkbox"/>
1 brioche (individuelle ou en tranche)					<input type="checkbox"/>

A2. Si vous mangez des céréales, citez la ou les marque(s) et dénomination(s) exacte(s) que vous consommez le plus fréquemment :

Marque

Nom du produit

Produit 1

Produit 2

A3 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :

CONFITURE, SUCRE, MIEL	FREQUENCE MOYENNE				
	par jour	par semaine	par mois	par an	Jamais
1 cuillerée à café de miel, confiture ou marmelade					<input type="checkbox"/>
1 cuillerée à café de Nutella					<input type="checkbox"/>
1 morceau ou cuillerée à café de sucre (hors édulcorant) (dans le café, le thé ou dans les yaourts, etc.)					<input type="checkbox"/>

A4 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :

BOISSONS CHAUDES	FREQUENCE MOYENNE				
	par jour	par semaine	par mois	par an	Jamais
1 tasse de café au lait					<input type="checkbox"/>
1 tasse de café noir					<input type="checkbox"/>
1 tasse de chicorée au lait					<input type="checkbox"/>
1 tasse de chicorée nature					<input type="checkbox"/>
1 tasse de chocolat chaud					<input type="checkbox"/>
1 tasse de thé nature ou au citron					<input type="checkbox"/>
1 tasse d'infusion nature ou au citron					<input type="checkbox"/>
1 tasse de thé au lait					<input type="checkbox"/>

1 tasse de chicorée au lait

1 tasse de chicorée nature

1 tasse de chocolat chaud

1 tasse de thé nature ou au citron

1 tasse d'infusion nature ou au citron

1 tasse de thé au lait

A5. Si vous buvez du chocolat, citez la ou les marque(s) et dénomination(s) exacte(s) que vous consommez le plus fréquemment :

	Marque	Nom du produit
A5. Si vous buvez du chocolat, citez la ou les marque(s) et dénomination(s) exacte(s) que vous consommez le plus fréquemment :	Produit 1 _____	_____
	Produit 2 _____	_____

A6 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :

L	LAIT (hors café, thé, chocolat, chicorée)	FREQUENCE MOYENNE				
		par jour	par semaine	par mois	par an	Jamais
1 bol de lait entier						<input type="checkbox"/>
1 bol de lait demi-écrémé						<input type="checkbox"/>
1 bol de lait écrémé						<input type="checkbox"/>
1 bol de lait fermenté						<input type="checkbox"/>
1 bol de lait de soja						<input type="checkbox"/>

A7. Si vous buvez du lait, citez la ou les marque(s) et dénomination(s) exacte(s) que vous consommez le plus fréquemment :

	Marque	Nom du produit
1 bol de lait	Produit 1 _____	_____
1 bol de lait	Produit 2 _____	_____

A8 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :

L	YAOURTS	FREQUENCE MOYENNE				
		par jour	par semaine	par mois	par an	Jamais
1 yaourt au lait entier nature						<input type="checkbox"/>
1 yaourt au lait entier aux fruits, aromatisé, sucré						<input type="checkbox"/>
1 yaourt au lait ½ écrémé nature						<input type="checkbox"/>
1 yaourt au lait ½ écrémé aux fruits, aromatisé, sucré						<input type="checkbox"/>
1 yaourt à 0% de matière grasse nature						<input type="checkbox"/>
1 yaourt à 0% de matière grasse aux fruits, aromatisé, sucré						<input type="checkbox"/>
1 yaourt à 0% de matière grasse à l'aspartame et aux fruits (type Taillefine, Panier de Yoplait 0%)						<input type="checkbox"/>
1 yaourt au bifidus (type Bio)						<input type="checkbox"/>
1 yaourt au soja						<input type="checkbox"/>

1 yaourt au lait ½ écrémé nature						<input type="checkbox"/>
1 yaourt au lait ½ écrémé aux fruits, aromatisé, sucré						<input type="checkbox"/>
1 yaourt à 0% de matière grasse nature						<input type="checkbox"/>
1 yaourt à 0% de matière grasse aux fruits, aromatisé, sucré						<input type="checkbox"/>
1 yaourt à 0% de matière grasse à l'aspartame et aux fruits (type Taillefine, Panier de Yoplait 0%)						<input type="checkbox"/>
1 yaourt au bifidus (type Bio)						<input type="checkbox"/>
1 yaourt au soja						<input type="checkbox"/>

**A9 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :**

FROMAGE BLANC	FREQUENCE MOYENNE				
	par jour	par semaine	par mois	par an	jamais
½ bol de fromage blanc à 0% de matière grasse nature					<input type="checkbox"/>
½ bol de fromage blanc à 0% de matière grasse aux fruits, aromatisé, sucré					<input type="checkbox"/>
½ bol de fromage blanc à 0% de matière grasse aux fruits, à l'aspartame (type <i>Taillefine</i> )					<input type="checkbox"/>
½ bol de fromage blanc à 20% de matière grasse nature					<input type="checkbox"/>
½ bol de fromage blanc à 20% de matière grasse aux fruits, aromatisé, sucré					<input type="checkbox"/>
½ bol de fromage blanc à 40% de matière grasse nature					<input type="checkbox"/>
½ bol de fromage blanc à 40% de matière grasse aux fruits, aromatisé, sucré					<input type="checkbox"/>
1 Petit suisse (nature, <i>Petits filous</i> , <i>Petits musclés</i> , etc.)					<input type="checkbox"/>

**A9 - Au cou**

½ bol de fi

½ bol de fi aromatisé,

½ bol de fi l'aspartame

½ bol de fi

½ bol de fi aromatisé,

½ bol de fi

½ bol de fi aromatisé,

1 Petit sui:

**A10. Si vous mangez des yaourts ou petits suisses, citez la ou les marque(s) et dénomination(s) exacte(s) que vous consommez le plus fréquemment :**

	Marque	Nom du produit
Produit 1	_____	_____
Produit 2	_____	_____

**A10. Si vous dénominati**

**A11 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :**

CREMES ET ENTREMETS	FREQUENCE MOYENNE				
	par jour	par semaine	par mois	par an	jamais
1 cuillère à soupe de crème fraîche entière (dans une tarte, dans un plat, etc.)					<input type="checkbox"/>
1 cuillère à soupe de crème fraîche allégée (dans une tarte, dans un plat, etc.)					<input type="checkbox"/>
1 cuillère à soupe de crème chantilly					<input type="checkbox"/>
1 coupe d'entremet (y compris maison) (crème dessert type <i>Danette</i> , <i>Liégeois</i> , <i>mousse</i> , etc.)					<input type="checkbox"/>
1 coupe de crème caramel, crème brûlée, crème anglaise					<input type="checkbox"/>

**A11 - Au co**

1 cuillère à soupe de crème fraîche entière (dans une tarte, dans un plat, etc.)


1 cuillère à soupe de crème fraîche allégée (dans une tarte, dans un plat, etc.)


1 cuillère à soupe de crème chantilly

1 coupe d'entremet (y compris maison) (crème dessert type *Danette*, *Liégeois*, *mousse*, etc.)

1 coupe de crème caramel, crème brûlée, crème anglaise

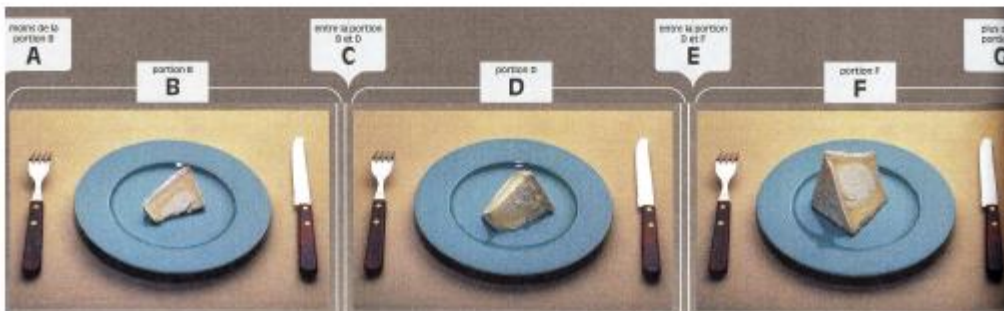
**A12 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :**

FROMAGE (lors d'un repas ou dans un sandwich) 	FREQUENCE MOYENNE				
	par jour	par semaine	par mois	par an	jamais
fromage fondu ( <i>La vache-qui-rit, Apéricubes, etc.</i> )					<input type="checkbox"/>
Bleu, Roquefort, Gorgonzola					<input type="checkbox"/>
Brie, Camembert, Munster, Pont-l'Évêque, fromage type Caprice des Dieux					<input type="checkbox"/>
fromage de chèvre					<input type="checkbox"/>
Gouda, Emmental, Gruyère, Comté, Beaufort, Parmesan, Bonbel, Babybel, Port-Salut, Saint-Paulin ( <i>en morceau ou râpé</i> )					<input type="checkbox"/>
Edam, Mimolette					<input type="checkbox"/>
Mozzarella, Feta, Mascarpone					<input type="checkbox"/>
fromage allégé					<input type="checkbox"/>
fromage frais ( <i>Tartare, Kiri, Boursin, St. Môret, etc.</i> )					<input type="checkbox"/>


 **Regardez la photo ci-dessous : quand vous mangez du fromage en général, quelle quantité moyenne en mangez vous ? Cochez la lettre qui correspond à la portion de fromage que vous mangez en moyenne (cochez une seule case) :**

fromage fondu  
Bleu, Roquefort, Gorgonzola  
Brie, Camembert, Munster, Pont-l'Évêque, fromage type Caprice des Dieux  
fromage de chèvre  
Gouda, Emmental, Gruyère, Comté, Beaufort, Parmesan, Bonbel, Babybel, Port-Salut, Saint-Paulin (*en morceau ou râpé*)  
Edam, Mimolette

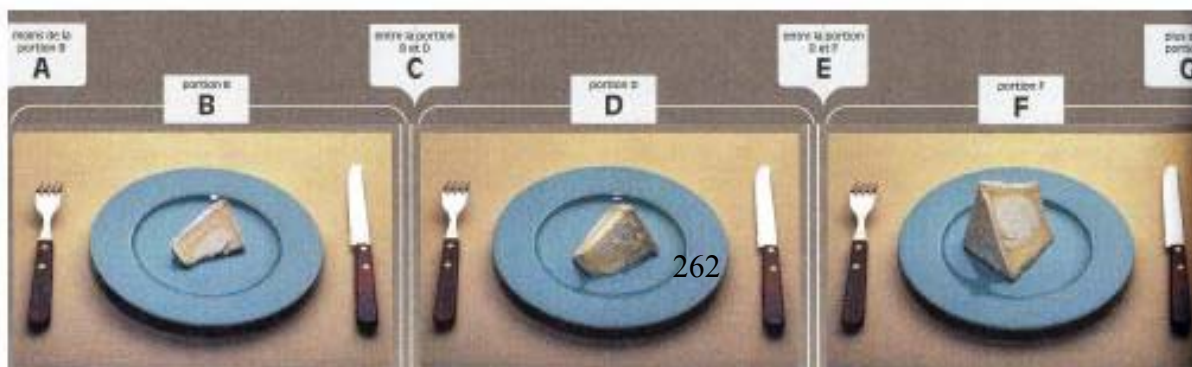
A B C D E F G



Mozzarella, Feta, Mascarpone							<input type="checkbox"/>
fromage allégé							<input type="checkbox"/>
fromage frais ( <i>Tartare, Kiri, Boursin, St. Môret, etc.</i> )							<input type="checkbox"/>

 **Regardez la photo ci-dessous : quand vous mangez du fromage en général, quelle quantité moyenne en mangez vous ? Cochez la lettre qui correspond à la portion de fromage que vous mangez en moyenne (cochez une seule case) :**

A B C D E F G

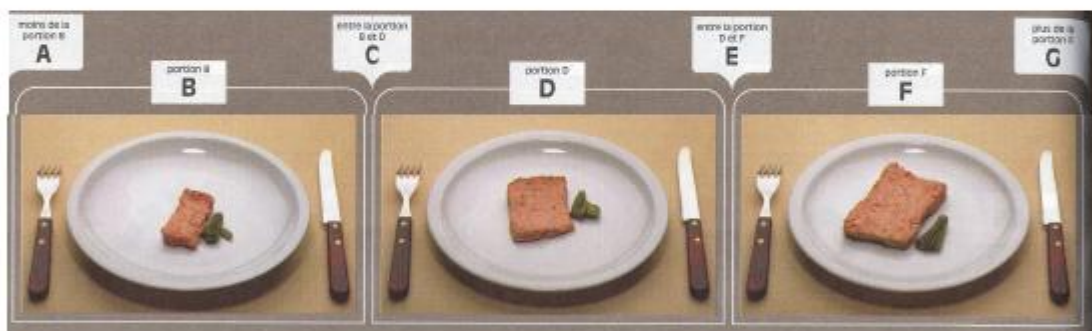




A13 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :

A13 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :	CHARCUTERIE (lors d'un repas ou dans un sandwich)	FREQUENCE MOYENNE						
		par jour	par semaine	par mois	par an	Jamais		
	1 tranche de jambon blanc					<input type="checkbox"/>		
	1 tranche de jambon cru ou de bacon					<input type="checkbox"/>		
	1 tranche de saucisson sec					<input type="checkbox"/>		
	1 tranche de cervelas					<input type="checkbox"/>		
	1 tranche de mortadelle					<input type="checkbox"/>		
	1 tranche de pâté					<input type="checkbox"/>		
	1 tranche de rillettes					<input type="checkbox"/>		
	<b>Si vous avez coché pâté ou rillettes :</b>							
	<b>Regardez la photo ci-dessous : quand vous mangez du pâté ou des rillettes en général, quelle quantité moyenne en mangez vous ? Cochez la lettre qui correspond à la portion de pâté ou de rillettes que vous mangez (cochez une seule case) :</b>							
		A	B	C	D	E	F	G
		<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

**Si vous**  
**Regardez la**  
**quelle quan**  
**de pâté ou**



A14 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :

A14 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :	OEUFS	FREQUENCE MOYENNE				
		par jour	par semaine	par mois	par an	Jamais
	1 oeuf à la coque, dur ou poché					<input type="checkbox"/>
	1 oeuf sur le plat ou en omelette					<input type="checkbox"/>

A14 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :

A15. Si vous mangez des œufs enrichis, citez la marque ou la dénomination exacte du produit :

A15. Si vous mangez des œufs enrichis, citez la marque ou la dénomination exacte du produit :	Marque ou dénomination du produit					
	Produit	par jour	par semaine	par mois	par an	Jamais
	1 oeuf à la coque, dur ou poché					<input type="checkbox"/>
	1 oeuf sur le plat ou en omelette					<input type="checkbox"/>

A15. Si vous mangez des œufs enrichis, citez la marque ou la dénomination exacte du produit :

Marque ou dénomination du produit

Produit \_\_\_\_\_

**A16 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :**

**A16 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :**

1 crêpe salée

1 part de quiche ou de tarte salée

1 crêpe salée

1 part de quiche ou de tarte salée

1 croque-monsieur

1 part de pizza

1 croque-monsieur

1 assiette de raviolis ou de lasagnes

1 sandwich grec/turc

1 plat chinois/vietnamien

1 hamburger

1 panini

1 assiette de choucroute garnie

1 hamburger

1 assiette de cassoulet

1 panini

1 assiette de choucroute garnie

1 assiette de choucroute garnie

PLATS GARNIS, COMPOSES, FOURRES (du commerce ou maison)	FREQUENCE MOYENNE				
	par jour	par semaine	par mois	par an	jamais
1 crêpe salée garnie					<input type="checkbox"/>
1 part de quiche ou de tarte salée					<input type="checkbox"/>
1 croque-monsieur					<input type="checkbox"/>
1 part de pizza					<input type="checkbox"/>
1 assiette de raviolis ou de lasagnes					<input type="checkbox"/>
1 sandwich grec/turc					<input type="checkbox"/>
1 plat chinois/vietnamien					<input type="checkbox"/>
1 hamburger					<input type="checkbox"/>
1 panini					<input type="checkbox"/>
1 assiette de choucroute garnie					<input type="checkbox"/>
1 assiette de cassoulet					<input type="checkbox"/>

**A17 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :**

**A17 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :**

1 assiette de fruits de mer (coquillages et crustacés)

1 portion de 3 bâtonnets de poisson pané

1 portion de poisson 'gras' (maquereau, hareng, anguille, sardine, saumon)

1 assiette de poisson 'mi-gras' (anchois, bar, carpe, espadon, flétan, rouget, roussette, thon, mullet, truite, turbot)

1 portion de poisson 'maigre' (les autres espèces, comme cabillaud, colin, merlan, sole)

(maquereau)

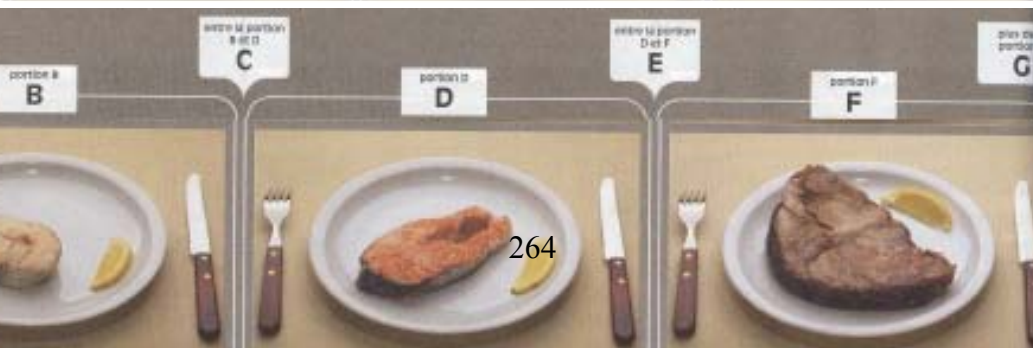
1 assiette de poisson 'mi-gras' (anchois, bar, carpe, espadon, flétan, rouget, roussette, thon, mullet, truite, turbot)

1 portion de poisson 'maigre' (les autres espèces, comme cabillaud, colin, merlan, sole)

POISSON	FREQUENCE MOYENNE				
	par jour	par semaine	par mois	par an	jamais
1 assiette de fruits de mer (coquillages et crustacés)					<input type="checkbox"/>
1 portion de 3 bâtonnets de poisson pané					<input type="checkbox"/>
1 portion de poisson 'gras' (maquereau, hareng, anguille, sardine, saumon)					<input type="checkbox"/>
1 assiette de poisson 'mi-gras' (anchois, bar, carpe, espadon, flétan, rouget, roussette, thon, mullet, truite, turbot)					<input type="checkbox"/>
1 portion de poisson 'maigre' (les autres espèces, comme cabillaud, colin, merlan, sole)					<input type="checkbox"/>

Regardez la photo ci-dessous : quand vous mangez du poisson en général, quelle quantité moyenne en mangez-vous ? Cochez la lettre qui correspond à la portion de poisson que vous mangez en moyenne (cochez une seule case) :

A B C D E F G



Regardez la photo ci-dessous : quand vous mangez du poisson en général, quelle quantité moyenne en mangez-vous ?



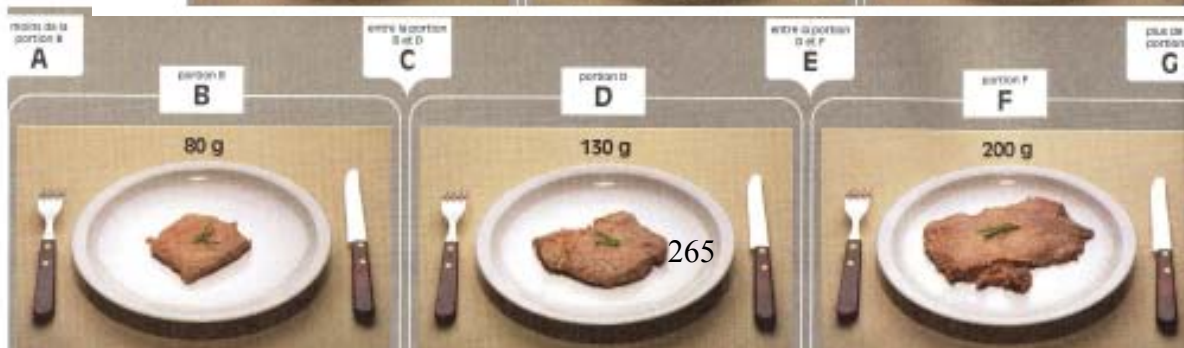
A18 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :

A18 - Au co

VIANDE OU CHARCUTERIE CHAUDE	FREQUENCE MOYENNE				
	par jour	par semaine	par mois	par an	Jamais
lapin ou volaille sans la peau ( <i>dinde, poulet, canard, etc.</i> )					<input type="checkbox"/>
volaille avec la peau ( <i>dinde, poulet, canard, etc.</i> )					<input type="checkbox"/>
steak haché					<input type="checkbox"/>
rôti ou steak de bœuf					<input type="checkbox"/>
côte ou entrecôte de bœuf					<input type="checkbox"/>
bœuf à la bourguignonne ( <i>ou braisé</i> )					<input type="checkbox"/>
pot-au-feu					<input type="checkbox"/>
escalope de veau					<input type="checkbox"/>
rôti de veau					<input type="checkbox"/>
côte de veau					<input type="checkbox"/>
sauté de veau, blanquette, osso-bucco					<input type="checkbox"/>
rôti de veau					<input type="checkbox"/>
côte de veau					<input type="checkbox"/>
sauté de veau					<input type="checkbox"/>
côte ou grillade de porc					<input type="checkbox"/>
rôti de porc					<input type="checkbox"/>
échine ou travers de porc					<input type="checkbox"/>
filet mignon de porc					<input type="checkbox"/>
lardons ( <i>dans une salade ou dans un plat</i> )					<input type="checkbox"/>
saucisse ( <i>merguez, chipolatas</i> )					<input type="checkbox"/>
foie ( <i>génisse, veau, volaille, etc.</i> )					<input type="checkbox"/>
autres abats ( <i>rognons, tripes, boudin, andouillette, etc.</i> )					<input type="checkbox"/>
viande panée ( <i>cordon bleu, escalope panée</i> )					<input type="checkbox"/>

Regardez la photo ci-dessous : quand vous mangez de la viande en général, quelle quantité moyenne en mangez-vous ? Cochez la lettre qui correspond à la portion de viande que vous mangez en moyenne (cochez une seule case) :

A B C D E F G



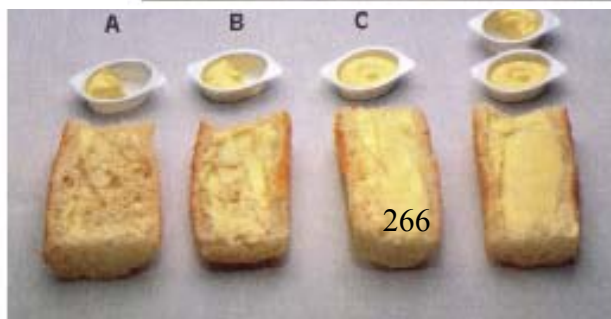
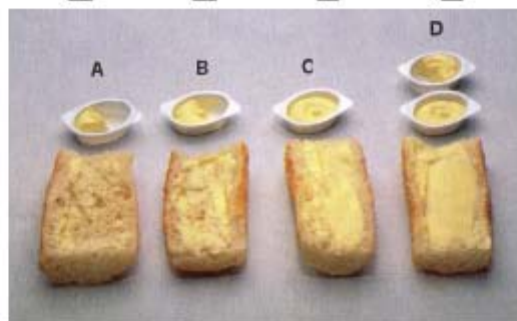
Regardez la quantité moyenne de viande que vous mangez en moyenne

**A19 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :**

	SAUCES ET MATIERES GRASSES	FREQUENCE MOYENNE				
		par jour	par semaine	par mois	par an	Jamais
	1 cuillerée à café de mayonnaise (du commerce ou maison)					<input type="checkbox"/>
1 cuillerée	1 cuillerée à café de moutarde					<input type="checkbox"/>
1 cuillerée	1 cuillerée à café de ketchup					<input type="checkbox"/>
1 cuillerée	1 cuillerée à café de sauce froide du commerce (type tartare, béarnaise, américaine)					<input type="checkbox"/>
1 cuillerée	1 cuillerée à soupe de sauce béchamel					<input type="checkbox"/>
1 cuillerée	1 portion de 2 cuillerées à soupe de sauce pour pâtes (tomates, bolognaise, carbonara, etc.)					<input type="checkbox"/>
1 portion d	1 portion de 5 cuillerées à soupe de sauce chaude accompagnant la viande ou le poisson (commerce ou maison)					<input type="checkbox"/>
(tomates, b	1 cuillerée à soupe de jus de cuisson de la viande					<input type="checkbox"/>
1 portion d	1 noisette de beurre ou margarine dans les préparations (viandes, pâtes, légumes, salades, etc.) ou pour la cuisson					<input type="checkbox"/>
accompagn	1 cuillerée à soupe de sauce vinaigrette du commerce					<input type="checkbox"/>
1 cuillerée	<b>Dans les préparations, pour la cuisson ou pour la vinaigrette maison :</b>					
1 noisette	1 cuillerée à soupe d'huile de tournesol					<input type="checkbox"/>
(viandes, p	1 cuillerée à soupe d'huile d'olive					<input type="checkbox"/>
1 cuillerée	1 cuillerée à soupe d'huile d'arachide					<input type="checkbox"/>
<b>Dans les pi</b>	1 cuillerée à soupe d'huile de colza					<input type="checkbox"/>
1 cuillerée	1 cuillerée à soupe d'huile de maïs					<input type="checkbox"/>
1 cuillerée	1 cuillerée à soupe d'huile de soja					<input type="checkbox"/>
1 cuillerée	1 cuillerée à soupe d'huile mélangée (type Isio 4)					<input type="checkbox"/>
1 cuillerée	1 cuillerée à soupe d'huile Primevère					<input type="checkbox"/>
1 cuillerée	1 cuillerée à soupe d'une autre huile					<input type="checkbox"/>
1 cuillerée	préciser : _____					<input type="checkbox"/>
1 cuillerée	beurre ou margarine sur le pain					<input type="checkbox"/>

**Si vous avez coché « beurre ou margarine sur le pain » :**  
 Regardez la photo ci-dessous : quand vous étalez du beurre ou de la margarine sur du pain, quelle quantité en mettez-vous, en moyenne ? Cochez la lettre qui correspond à la quantité de beurre ou margarine que vous mangez en moyenne sur le pain (cochez une seule case) :

A      B      C      D  
        





**A20 a- Cochez le type de beurre, margarine ou autre type de matière grasse que vous consommez le plus fréquemment dans les préparations ou pour la cuisson (une seule réponse) :**

margarine ordinaire à environ 80% de matière grasse	<input type="radio"/>
margarine à environ 70% matière grasse (type Astra)	<input type="radio"/>
margarine à environ 80% de matière grasse au tournesol	<input type="radio"/>
margarine à environ 70% de matière grasse (type Fruit d'Or)	<input type="radio"/>
margarine allégée à environ 60% de matière grasse (type Plantafin, Le Fleurier)	<input type="radio"/>
margarine allégée à environ 60% de matière grasse à l'huile d'olive (type Olivio)	<input type="radio"/>
margarine allégée à environ 60% de matière grasse au tournesol	<input type="radio"/>
margarine allégée à environ 60% de matière grasse (type Fruit d'Or)	<input type="radio"/>
margarine allégée à environ 60% de matière grasse (type Primevère)	<input type="radio"/>
margarine allégée à environ 40% de matière grasse nature (type Effi ou St. Hubert 41)	<input type="radio"/>
margarine allégée à environ 25% de matière grasse nature (type Bridelight)	<input type="radio"/>
Fruit d'Or "pro-activ"	<input type="radio"/>
beurre nature ou demi-sel	<input type="radio"/>
beurre allégé	<input type="radio"/>
Végétaline	<input type="radio"/>
suif, saindoux, autre graisse animale	<input type="radio"/>

**A20 a- Cochez le type de matière grasse que vous consommez le plus fréquemment dans les préparations ou pour la cuisson (une seule réponse) :**

margarine à environ 80% de matière grasse	<input type="radio"/>
margarine à environ 70% matière grasse (type Astra)	<input type="radio"/>
margarine à environ 80% de matière grasse au tournesol	<input type="radio"/>
margarine à environ 70% de matière grasse (type Fruit d'Or)	<input type="radio"/>
margarine allégée à environ 60% de matière grasse (type Plantafin, Le Fleurier)	<input type="radio"/>
margarine allégée à environ 60% de matière grasse à l'huile d'olive (type Olivio)	<input type="radio"/>
margarine allégée à environ 60% de matière grasse au tournesol	<input type="radio"/>
margarine allégée à environ 60% de matière grasse (type Fruit d'Or)	<input type="radio"/>
margarine allégée à environ 60% de matière grasse (type Primevère)	<input type="radio"/>
margarine allégée à environ 40% de matière grasse nature (type Effi ou St. Hubert 41)	<input type="radio"/>
margarine allégée à environ 25% de matière grasse nature (type Bridelight)	<input type="radio"/>
Fruit d'Or "pro-activ"	<input type="radio"/>
beurre nature ou demi-sel	<input type="radio"/>
beurre allégé	<input type="radio"/>
Végétaline	<input type="radio"/>
suif, saindoux, autre graisse animale	<input type="radio"/>

**Si le type de matière grasse que vous utilisez le plus fréquemment pour la cuisson n'est pas listé ci-dessus, citez la marque ou la dénomination exacte du produit :**

Marque ou dénomination du produit

Produit

margarine allégée à environ 40% de matière grasse nature (type Effi ou St. Hubert 41)	<input type="radio"/>
margarine allégée à environ 25% de matière grasse nature (type Bridelight)	<input type="radio"/>
Fruit d'Or "pro-activ"	<input type="radio"/>
beurre nature ou demi-sel	<input type="radio"/>
beurre allégé	<input type="radio"/>
Végétaline	<input type="radio"/>
suif, saindoux, autre graisse animale	<input type="radio"/>

**Si le type de matière grasse que vous utilisez le plus fréquemment pour la cuisson n'est pas listé ci-dessus, citez la marque ou la dénomination exacte du produit :**

Marque ou dénomination du produit

Produit

**A20 b- Cochez le type de beurre, margarine ou autre type de matière grasse que vous utilisez le plus fréquemment pour tartiner (une seule réponse) :**

**A20 b- Cochez le type de matière grasse que vous utilisez le plus fréquemment pour tartiner**

margarine ordinaire à environ 80% de matière grasse	<input type="radio"/>
margarine à environ 70% matière grasse (type Astra)	<input type="radio"/>
margarine à environ 80% de matière grasse au tournesol	<input type="radio"/>
margarine à environ 70% de matière grasse (type Fruit d'Or)	<input type="radio"/>
margarine allégée à environ 60% de matière grasse (type Plantafin, Le Fleurier)	<input type="radio"/>
margarine allégée à environ 60% de matière grasse à l'huile d'olive (type Olivio)	<input type="radio"/>
margarine allégée à environ 60% de matière grasse au tournesol	<input type="radio"/>
margarine allégée à environ 60% de matière grasse (type Fruit d'Or)	<input type="radio"/>
margarine allégée à environ 60% de matière grasse (type Primevère)	<input type="radio"/>
margarine allégée à environ 40% de matière grasse nature (type Effi ou St. Hubert 41)	<input type="radio"/>
margarine allégée à environ 25% de matière grasse nature (type Bridelight)	<input type="radio"/>
Fruit d'Or "pro-activ"	<input type="radio"/>
beurre nature ou demi-sel	<input type="radio"/>
beurre allégé	<input type="radio"/>
Végétaline	<input type="radio"/>
suif, saindoux, autre graisse animale	<input type="radio"/>

**Si le type de matière grasse que vous utilisez le plus fréquemment pour tartiner ou assaisonner n'est pas listé ci-dessus, citez la marque ou la dénomination exacte du produit :**

	Marque ou dénomination du produit
Fruit d'Or "	
beurre nature	
beurre allégé	
Végétaline	
suif, saindoux	


**A21 - A propos de votre consommation de sel :**

Si le type de produit :	OUI	NON
Mettez-vous systématiquement la salière sur la table ?	<input type="checkbox"/>	<input type="checkbox"/>
Re-salez vous toujours ou presque toujours les aliments dans votre assiette ?	<input type="checkbox"/>	<input type="checkbox"/>
Proc Mettez-vous toujours ou presque toujours du sel lors de la préparation des plats ?	<input type="checkbox"/>	<input type="checkbox"/>

**A21 - A propos de votre consommation de sel :**

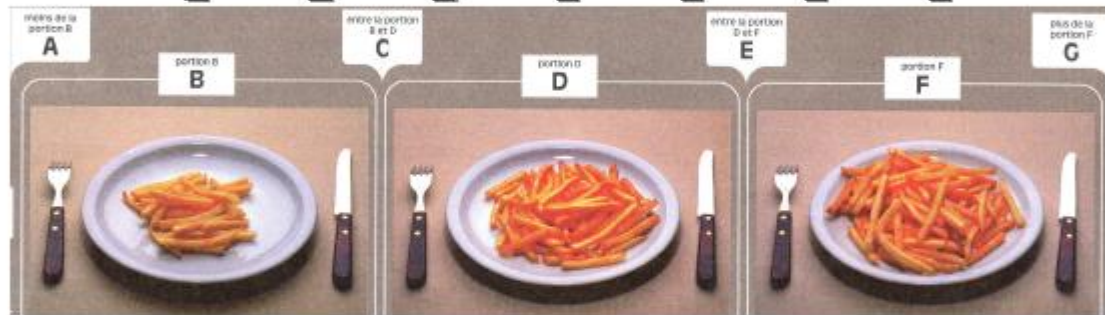
	OUI	NON
Mettez-vous systématiquement la salière sur la table ?	<input type="checkbox"/>	<input type="checkbox"/>
Re-salez vous toujours ou presque toujours les aliments dans votre assiette ?	<input type="checkbox"/>	<input type="checkbox"/>
Mettez-vous toujours ou presque toujours du sel lors de la préparation des plats ?	<input type="checkbox"/>	<input type="checkbox"/>

**A22 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :**


POMMES DE TERRE 	FREQUENCE MOYENNE				
	par jour	par semaine	par mois	par an	jamais
salade de pommes de terre					<input type="checkbox"/>
pommes de terre à l'eau ou au four					<input type="checkbox"/>
salade de pommes de terre sautées à l'huile ou au beurre					<input type="checkbox"/>
Purée (du commerce ou maison)					<input type="checkbox"/>
frites					<input type="checkbox"/>

**Regardez la photo ci-dessous : quand vous mangez des pommes de terre, de la purée ou des frites en général, quelle quantité moyenne en mangez vous ? Cochez la lettre qui correspond à la portion que vous mangez en moyenne (cochez une seule case) :**

A  B  C  D  E  F  G



**A23 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :**

PATES, RIZ, SEMOULE 	FREQUENCE MOYENNE				
	par jour	par semaine	par mois	par an	jamais
pâtes					<input type="checkbox"/>
riz					<input type="checkbox"/>
semoule (y compris couscous), blé concassé, ebly					<input type="checkbox"/>

**Regardez la photo ci-dessous : quand vous mangez des pâtes, du riz ou de la semoule en général, quelle quantité moyenne en mangez vous ? Cochez la lettre qui correspond à la portion que vous mangez en moyenne (cochez une seule case) :**

A  B  C  D  E  F  G




**Regardez en général, la portion que**



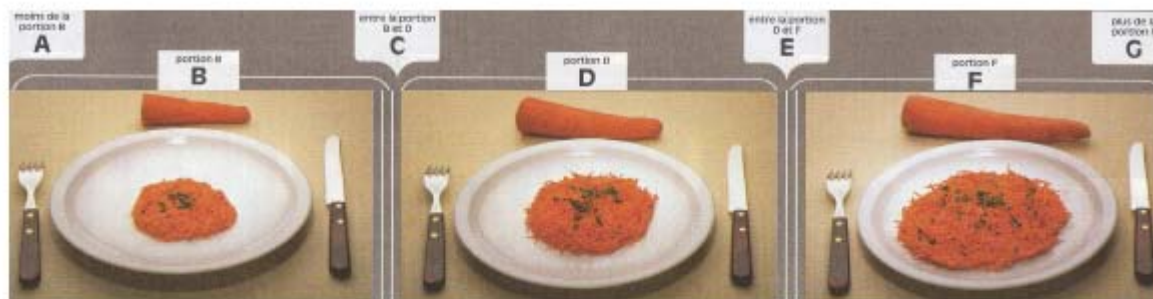
**A24 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :**

LEGUMES (frais, conserve, surgelé)	FREQUENCE MOYENNE				
	par jour	par semaine	par mois	par an	Jamais
½ avocat					<input type="checkbox"/>
1 artichaut moyen					<input type="checkbox"/>
oignons (dans une sauce, une tarte, une ratatouille, etc.)					<input type="checkbox"/>
ail (dans un plat)					<input type="checkbox"/>
petit pois, légumes secs (lentilles, haricots blancs, fèves, pois cassés, pois chiches) 🍲					<input type="checkbox"/>
1 portion de champignons					<input type="checkbox"/>
½ avocat					<input type="checkbox"/>
1 artichaut					<input type="checkbox"/>
oignons (c					<input type="checkbox"/>
ail (dans t					<input type="checkbox"/>
petit pois, pois cassé					<input type="checkbox"/>
1 portion					<input type="checkbox"/>
soupe de l maison)					<input type="checkbox"/>
salade ver					<input type="checkbox"/>
carottes (					<input type="checkbox"/>
céleri 🍲					<input type="checkbox"/>
tomate 🍲					<input type="checkbox"/>
betterave 🍲					<input type="checkbox"/>
chou roug					<input type="checkbox"/>
chou blan					<input type="checkbox"/>
chou vert					<input type="checkbox"/>
choux de					<input type="checkbox"/>
chou-fleur					<input type="checkbox"/>
brocolis					<input type="checkbox"/>
haricots verts 🍲					<input type="checkbox"/>
endives (en salade ou cuites) 🍲					<input type="checkbox"/>
épinards 🍲					<input type="checkbox"/>
concombre, courgettes ou aubergines 🍲					<input type="checkbox"/>
poivrons rouge/ vert/ jaune 🍲					<input type="checkbox"/>
poireaux 🍲					<input type="checkbox"/>
fenouil 🍲					<input type="checkbox"/>
potiron 🍲					<input type="checkbox"/>
navets, radis 🍲					<input type="checkbox"/>
maïs 🍲					<input type="checkbox"/>

haricots verts 🍲					<input type="checkbox"/>
endives (en salade ou cuites) 🍲					<input type="checkbox"/>
épinards 🍲					<input type="checkbox"/>
concombre, courgettes ou aubergines 🍲					<input type="checkbox"/>
poivrons rouge/ vert/ jaune 🍲					<input type="checkbox"/>
poireaux 🍲					<input type="checkbox"/>
fenouil 🍲					<input type="checkbox"/>
potiron 🍲					<input type="checkbox"/>
navets, radis 🍲					<input type="checkbox"/>
maïs 🍲					<input type="checkbox"/>

 Regardez la photo ci-dessous : quand vous mangez des légumes en général, quelle quantité moyenne en mangez-vous ? Cochez la lettre qui correspond à la portion de légumes que vous mangez en moyenne (cochez une seule case) :

A      B      C      D      E      F      G  
                       



✓ **Rappel** : calcul de la consommation des fruits saisonniers.

→ Evaluer la durée de la saison (ex : 4 mois)

→ Evaluer la quantité d'aliment consommée pendant cette période (ex : 3 tranches de melon par semaine)

→ Exprimer la fréquence par an (soit : 3 x 4 semaines x 4 mois = 48 à reporter dans la colonne 'par an')

**A25 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :**

	FRUITS (entier ou sous forme de jus de fruits pressés maison)	FREQUENCE MOYENNE				
		par jour	par semaine	par mois	par an	Jamais
	1 pomme					<input type="checkbox"/>
	1 poire					<input type="checkbox"/>
(entier ou	1 agrume (orange, mandarine, pamplemousse, citron)					<input type="checkbox"/>
	1 banane					<input type="checkbox"/>
	1 pêche, nectarine, brugnion ou abricot					<input type="checkbox"/>
	1 morceau de melon					<input type="checkbox"/>
	1 pomme	1 bol de cerises				<input type="checkbox"/>
1 poire	1 bol de fraises, framboises ou d'autres fruits rouges				<input type="checkbox"/>	
1 agrume (o	1 prune				<input type="checkbox"/>	
1 banane	1 kiwi				<input type="checkbox"/>	
1 pêche, ne	1 bol de raisins (blanc ou noir)				<input type="checkbox"/>	
1 morceau c	1 morceau d'ananas				<input type="checkbox"/>	
1 bol de cer	1 mangue				<input type="checkbox"/>	
1 bol de frai	1 portion de litchis frais				<input type="checkbox"/>	
1 prune	1 poignée de fruits secs (pruneaux, abricots, raisins, etc.)				<input type="checkbox"/>	
	1 coupe de fruits en compote ou au sirop				<input type="checkbox"/>	

1 kiwi					<input type="checkbox"/>
1 bol de raisins (blanc ou noir)					<input type="checkbox"/>
1 morceau d'ananas					<input type="checkbox"/>
1 mangue					<input type="checkbox"/>
1 portion de litchis frais					<input type="checkbox"/>
1 poignée de fruits secs (pruneaux, abricots, raisins, etc.)					<input type="checkbox"/>
1 coupe de fruits en compote ou au sirop					<input type="checkbox"/>

**A26 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :**

BISCUITS, GATEAUX, SUCRERIES (du commerce ou maison)	FREQUENCE MOYENNE				
	par jour	par semaine	par mois	par an	Jamais
1 carré de chocolat ( <i>noir, au lait, aux noisettes, etc.</i> )					<input type="checkbox"/>
1 bonbon					<input type="checkbox"/>
1 part de tarte aux fruits					<input type="checkbox"/>
1 part de flan					<input type="checkbox"/>
1 tranche de cake ou de quatre-quarts, madeleine					<input type="checkbox"/>
1 biscuit sec au chocolat					<input type="checkbox"/>
1 biscuit sec nature ( <i>petit beurre, galette</i> )					<input type="checkbox"/>
1 petit gâteau ( <i>barquettes, figolu, etc.</i> )					<input type="checkbox"/>
1 brownie, 1 part de gâteau au chocolat					<input type="checkbox"/>
1 pâtisserie à la crème					<input type="checkbox"/>
1 barre chocolatée ou aux céréales ( <i>Mars, Twix, Granny, etc.</i> )					<input type="checkbox"/>
1 crêpe sucrée ou 1 gaufre					<input type="checkbox"/>
1 boule de sorbet					<input type="checkbox"/>
1 boule de glace					<input type="checkbox"/>

**A26 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :**

1 carré de c

1 bonbon

1 part de ta

1 part de fl

1 tranche d

1 biscuit se

1 biscuit se

**A27. Si vous mangez des biscuits, citez la ou les marque(s) et dénomination(s) exacte(s) que vous consommez le plus fréquemment ?**

Marque

Nom du produit

Produit 1 \_\_\_\_\_

Produit 2 \_\_\_\_\_

1 petit gâteau (*barquettes, figolu, etc.*)

1 brownie, 1 part de gâteau au chocolat

1 pâtisserie à la crème

1 barre chocolatée ou aux céréales (*Mars, Twix, Granny, etc.*)

1 crêpe sucrée ou 1 gaufre

1 boule de sorbet

1 boule de glace

**A27. Si vous mangez des biscuits, citez la ou les marque(s) et dénomination(s) exacte(s) que vous consommez le plus fréquemment ?**


Marque


Nom du produit

Produit 1 \_\_\_\_\_ 272 \_\_\_\_\_

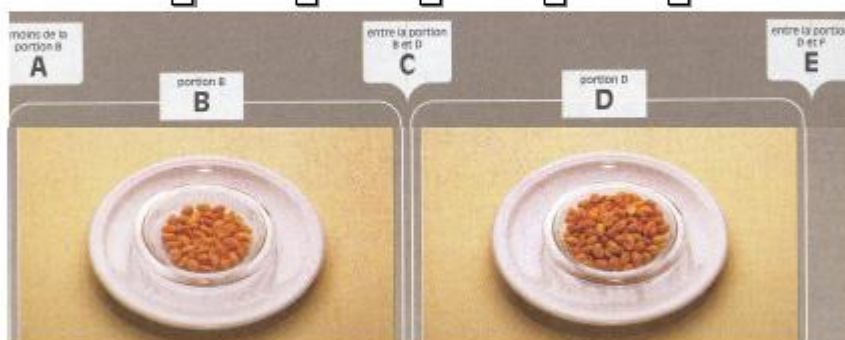
Produit 2 \_\_\_\_\_ \_\_\_\_\_

**A28 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :**

SNACKS SALES	FREQUENCE MOYENNE				
	par jour	par semaine	par mois	par an	Jamais
1 poignée de gâteaux apéritifs salés (hors mélange de fruits secs)					<input type="checkbox"/>
1 portion de chips					<input type="checkbox"/>
1 cornet ou un sachet de pop-corn					<input type="checkbox"/>
1 poignée de fruits oléagineux salés (cacahuètes, amandes, pistaches, noisettes, noix, etc.) 					<input type="checkbox"/>
1 portion de fruits oléagineux non salés (cacahuètes, amandes, pistaches, noisettes, noix, etc.) 					<input type="checkbox"/>

 **Regardez la photo ci-dessous : cochez la lettre qui correspond à la portion de fruits oléagineux que vous mangez en moyenne (cochez une seule case) :**

A  B  C  D  E



**A29 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :**

BOISSONS FROIDES NON ALCOOLISEES	FREQUENCE MOYENNE				
	par jour	par semaine	par mois	par an	Jamais
1 verre de jus de fruits du commerce					<input type="checkbox"/>
1 verre de sirop à l'eau					<input type="checkbox"/>
1 verre de soda (Coca-cola, Sprite, Fanta, Orangina, Ice Tea, etc.)					<input type="checkbox"/>
1 verre de soda light					<input type="checkbox"/>
1 verre d'eau minérale: remplissez la case et précisez la marque : _____					<input type="checkbox"/>
1 verre d'eau du robinet					<input type="checkbox"/>
1 verre de bière sans alcool					<input type="checkbox"/>

1 verre de sirop à l'eau					<input type="checkbox"/>
1 verre de soda (Coca-cola, Sprite, Fanta, Orangina, Ice Tea, etc.)					<input type="checkbox"/>
1 verre de soda light					<input type="checkbox"/>
1 verre d'eau minérale: remplissez la case et précisez la marque : _____					<input type="checkbox"/>
1 verre d'eau du robinet					<input type="checkbox"/>
1 verre de bière sans alcool					<input type="checkbox"/>

A30. Si vous buvez du jus de fruits du commerce, citez la ou les marque(s) et dénomination(s) exacte(s) que vous consommez le plus fréquemment ?

	Marque	Nom du produit
A30. Si vous	Produit 1 _____	_____
dénominati	Produit 2 _____	_____

Pro A31 - Au cours des 12 derniers mois, à quelle fréquence avez-vous consommé :

Pro	BOISSONS ALCOOLISEES	FREQUENCE MOYENNE				
		par jour	par semaine	par mois	par an	Jamais
A31 - Au co	1 verre de cidre					<input type="checkbox"/>
	1 verre de bière blonde ou brune					<input type="checkbox"/>
	1 verre de vin blanc, rosé ou de champagne					<input type="checkbox"/>
	1 verre de vin rouge					<input type="checkbox"/>
	1 verre de	1 verre d'alcool anisé (Ricard, Pastis)				<input type="checkbox"/>
	1 verre de	1 verre d'apéritif (Cherry, Porto, Martini, etc.)				<input type="checkbox"/>
	1 verre de	1 verre d'alcool fort (whisky, gin, vodka, etc.)				<input type="checkbox"/>
	1 verre de	1 verre de liqueur (Amaretto, Cointreau, etc.)				<input type="checkbox"/>
	1 verre d'a	1 verre de digestif (cognac, calvados, rhum, etc.)				<input type="checkbox"/>
	1 verre d'i					

A32- Si des aliments ou boissons que vous consommez habituellement (au moins une fois par semaine) ne sont pas mentionnés dans ce questionnaire, merci de les noter ci-dessous :

	NOM DE L'ALIMENT OU DE LA BOISSON	UNITE (verre, tranche, cuillère etc.)	NOMBRE DE FOIS PAR SEMAINE
1 verre de			
1 verre de			
A32- Si des			
par semaine			
ci-dessous :			
NOM DE			



A33- Si vous consommez habituellement (au moins une fois par semaine) des aliments enrichis (jus de fruits, céréales pour petit-déjeuner, pâtes, lait etc.) merci de les noter ci-dessous :

	NOM DE L'ALIMENT OU DE LA BOISSON	UNITE (verre, tranche, cuillère etc.)	NOMBRE DE FOIS PAR SEMAINE
A33- Si vous consommez habituellement (au moins une fois par semaine) des aliments enrichis (jus de fruits, céréales pour petit-déjeuner, pâtes, lait etc.) merci de les noter ci-dessous :			

**B - REGIME ALIMENTAIRE**

B1. Actuellement, suivez-vous un régime alimentaire particulier ?

- Oui  si oui, passez à la question suivante  
 Non  si non, passez à la page suivante

B2. Pour quelle raison suivez-vous un régime ? (plusieurs réponses possibles)

Pour diminuer le taux de cholestérol	<input type="checkbox"/>
Pour diminuer la pression artérielle	<input type="checkbox"/>
Pour le diabète	<input type="checkbox"/>
Pour maigrir	<input type="checkbox"/>
Pour rester en forme	<input type="checkbox"/>
Pour éviter de maigrir	<input type="checkbox"/>
Autre raison précisez laquelle :	<input type="checkbox"/>
.....	
.....	

B2. Pour quelle raison suivez-vous un régime : (plusieurs réponses possibles)

Pour diminuer le taux de cholestérol	<input type="checkbox"/>
Pour diminuer la pression artérielle	<input type="checkbox"/>
Pour le diabète	<input type="checkbox"/>
Pour maigrir	<input type="checkbox"/>
Pour rester en forme	<input type="checkbox"/>
Pour éviter de maigrir	<input type="checkbox"/>
Autre raison précisez laquelle :	<input type="checkbox"/>
.....	
.....	

**B3. Quelle sont les caractéristiques de votre régime ? (plusieurs réponses possibles)**

pauvre en graisses	<input type="checkbox"/>
pauvre en glucides	<input type="checkbox"/>
pauvre en sel	<input type="checkbox"/>
pauvre en alcool	<input type="checkbox"/>
riche en graisses	<input type="checkbox"/>
riche en glucides	<input type="checkbox"/>
riche en (huile de) poisson	<input type="checkbox"/>
riche en produits laitiers	<input type="checkbox"/>
Autres caractéristiques précisez laquelle : .....	<input type="checkbox"/>

**B4. Votre régime est :**

<b>B3. Quelle s</b>	Préscrit par un médecin, généraliste, spécialiste ou diététicien	<input type="checkbox"/>
	Lu / trouvé / entendu dans un magazine, un livre, sur Internet, à la radio ou à la télévision	<input type="checkbox"/>
pauvre en gr	Défini par vous-même ou par un proche	<input type="checkbox"/>
pauvre en gl	Autre (précisez) : .....	<input type="checkbox"/>
pauvre en se		

**C - LES COMPLEMENTS ALIMENTAIRES**

pauvre en al	<b>C1. Prenez-vous actuellement des vitamines ou des minéraux sous forme de compléments alimentaires c'est-à-dire sous forme de comprimés, gélules, sachets de poudre, sirop ?</b>
riche en grai	(une seule réponse possible)
riche en gluc	Oui <input type="checkbox"/>
riche en (hu	Non <input type="checkbox"/>
riche en pro	
Autres carac	

**B4. Votre régime est :**

Préscrit par un médecin, généraliste, spécialiste ou diététicien	<input type="checkbox"/>
Lu / trouvé / entendu dans un magazine, un livre, sur Internet, à la radio ou à la télévision	<input type="checkbox"/>
Défini par vous-même ou par un proche	<input type="checkbox"/>
Autre (précisez) : .....	<input type="checkbox"/>

**C - LES COMPLEMENTS ALIMENTAIRES**

<b>C1. Prenez-vous actuellement des vitamines ou des minéraux sous forme de compléments alimentaires c'est-à-dire sous forme de comprimés, gélules, sachets de poudre, sirop ?</b>
(une seule réponse possible)

Oui

Non

**C2. Si oui, quelles sont les marques et dénomination exactes de ce(s) complément(s) alimentaire(s) ?** Cette question portent uniquement sur les 3 compléments alimentaires que vous consommez le plus souvent. Si vous ne consommez qu'un complément alimentaire, remplissez uniquement la ligne Produit 1. Si vous ne consommez que 2 compléments alimentaires, remplissez uniquement les lignes Produit 1 et Produit 2. Pour répondre aux questions munissez-vous, si possible, des emballages.

	Laboratoire	Nom du produit	Fréquence
Produit 1	_____	_____	_____
Produit 2	_____	_____	_____
Produit 3	_____	_____	_____

**D- Remplissage du questionnaire**

**C2. Si oui, D1- Avez-vous rempli ce questionnaire :**

seul(e)	<input type="checkbox"/>
avec l'aide de votre conjoint(e)	<input type="checkbox"/>
avec l'aide d'une autre personne : précisez laquelle.....	<input type="checkbox"/>

vous consommez  
remplissez  
alimentaire  
questions r

**Vous voici arrivé à la fin du questionnaire: vérifiez bien que vous avez rempli chaque ligne de chaque tableau d'aliments.**

**MERCI BEAUCOUP D'AVOIR PRIS LE TEMPS DE REMPLIR CE QUESTIONNAIRE !**

Produit 1	_____	_____	_____
Produit 2	_____	_____	_____
Produit 3	_____	_____	_____

**D- Remplissage du questionnaire**

**D1- Avez-vous rempli ce questionnaire :**

seul(e)	<input type="checkbox"/>
avec l'aide de votre conjoint(e)	<input type="checkbox"/>
avec l'aide d'une autre personne : précisez laquelle.....	<input type="checkbox"/>

**Vous voici arrivé à la fin du questionnaire: vérifiez bien que vous avez rempli chaque ligne de chaque tableau d'aliments.**

**MERCI BEAUCOUP D'AVOIR PRIS LE TEMPS DE REMPLIR CE QUESTIONNAIRE !**

## Appendix 13: Active and passive samplers' information sheet for PAHs and VOCs sampling.

LHVP

Affaire 2007/323/PPCE - 0739

### Instructions à suivre pour prélèvements d'air lors de travaux du service de propreté

Rappel : un agent de l'équipe en charge des prélèvements, porte le matériel, la semaine prévue, du lundi au jeudi.

#### 1) Matériels fournis dans le sac à dos :

- Une pompe équipée d'une batterie externe et qui est reliée par un tuyau au dispositif de prélèvement protégé par un sac en plastique
- 1 boîte en plastique qui contient un triangle bleu sur lequel est fixé un cylindre blanc et un triangle bleu sur lequel est fixé un cylindre jaune. Chaque support de prélèvement est protégé de la pluie par un plastique.
- 1 pochette plastique contenant les instructions à suivre
- 1 pochette plastique contenant la feuille de prélèvement à remplir chaque jour

#### 2) Au début de chaque matinée de travail et après chaque pause à l'atelier :

- Ouvrir la boîte en plastique
- Fixer chaque triangle bleu sur le sac à dos au moyen de l'épingle à nourrice
- Fermer la boîte
- Noter la date et l'heure de début de prélèvement sur la fiche placée dans le sac
- Enlever le sac en plastique protégeant le dispositif de prélèvement et le papier d'aluminium
- Mettre en marche la pompe en positionnant le petit bouton sur le corps de pompe sur «ON»
- Fixer le dispositif de prélèvement bien verticalement (la tête de prélèvement vers le, bas) sur le sac au moyen de l'épingle à nourrice
- Porter le sac à dos

#### 3) A chaque pause à votre atelier et à la fin de la journée :

- Arrêter la pompe en positionnant le petit bouton sur le corps de pompe sur «OFF»
- Ranger la pompe dans le sac après avoir recouvert le dispositif au bout du tuyau par un petit sac en plastique
- Placer chaque triangle bleu dans la boîte
- Bien fermer la boîte et la ranger dans le sac à dos
- Noter sur la fiche l'heure de fin de prélèvement

Au retour à l'atelier, l'agent en charge des prélèvements range le sac à dos dans son casier.

Recommencer les opérations 2 et 3, du lundi au jeudi.

Lors de la convocation le vendredi au centre médical, rapporter le sac à dos avec l'ensemble du matériel et la fiche de prélèvement

Si nécessaire, contacter le Laboratoire d'Hygiène au 01.44.97.87.87  
Demander Madame LAURENT – Madame KERAUTRET ou Monsieur LEMOULLEC

Appendix 14: Presentations of biomarkers variables results as normal probability plots (by the whole study population [C] but also by population categories: office [A] and sewage workers [B]). Diagram [D] shows the dispersion of the biomarkers for the two categories.

Fig. I: Normal probability-plots and diagram of dispersion for comet %DNA tail variable.

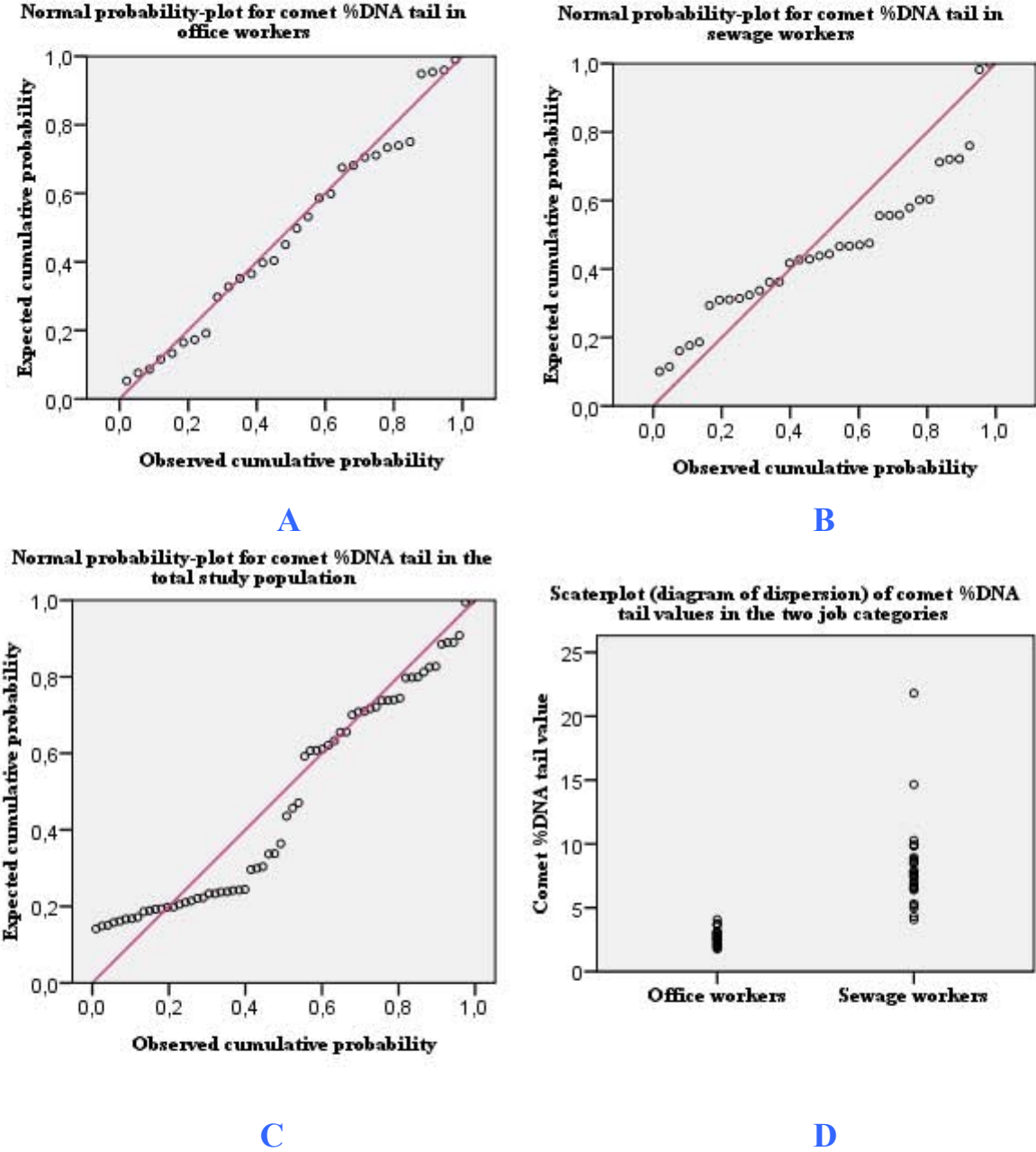
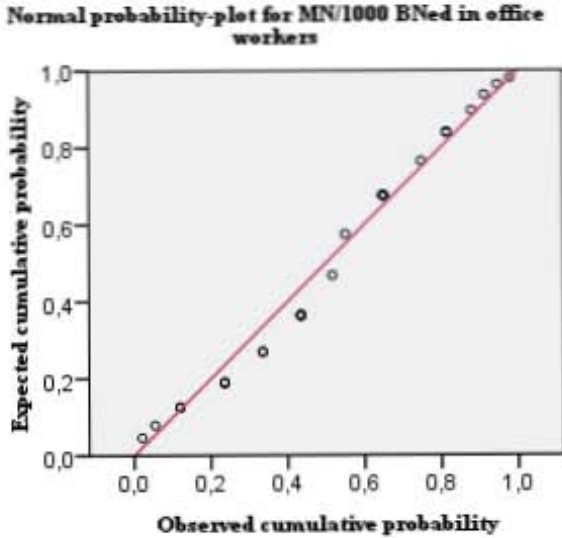
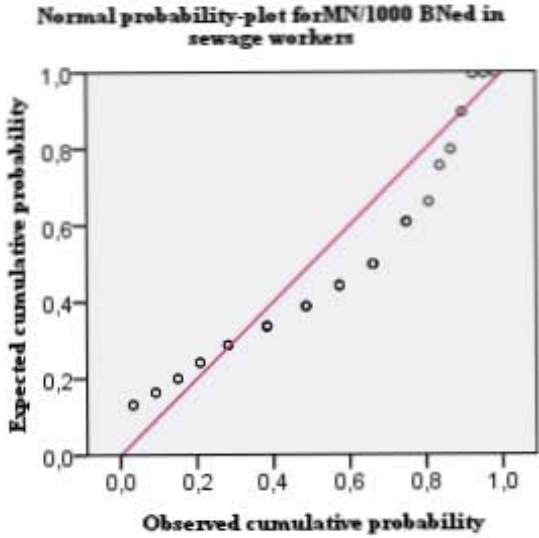


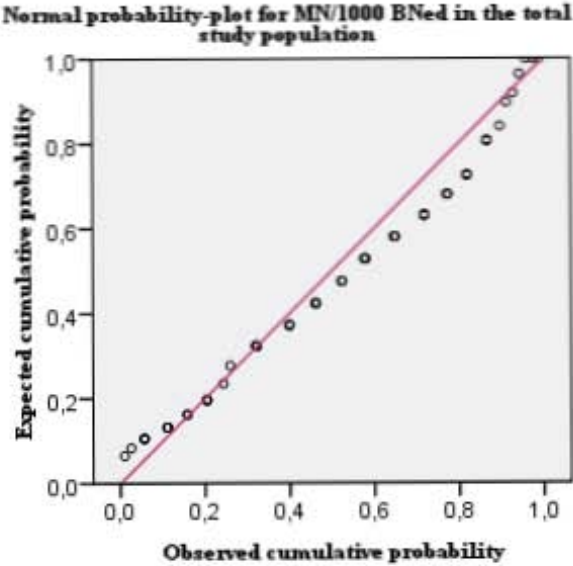
Fig. II: Normal probability-plots and diagram of dispersion for MN/1000 BNed variable.



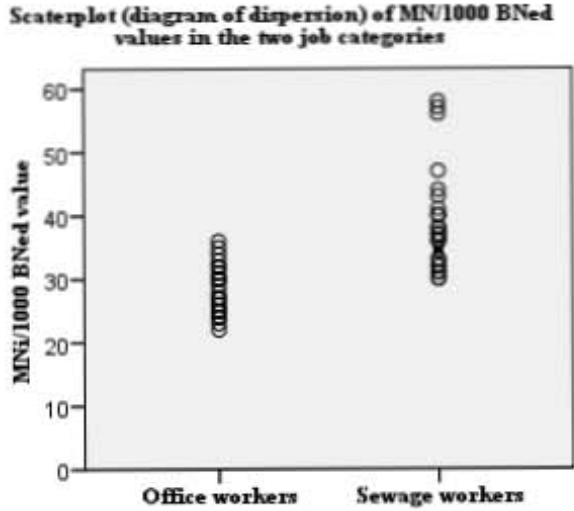
A



B



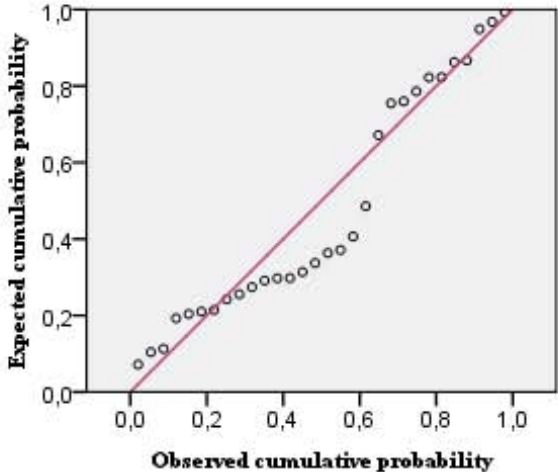
C



D

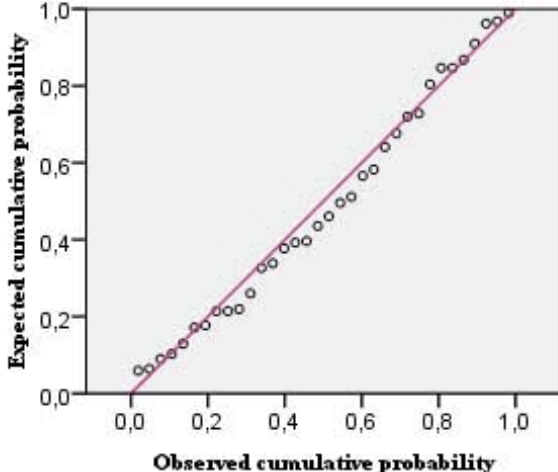
**Fig. III:** Normal probability-plots and diagram of dispersion for 8-oxodG (pmole/kg 24h) variable.

**Normal probability-plots for 8-oxodG (pmole/kg 24h) in office workers**



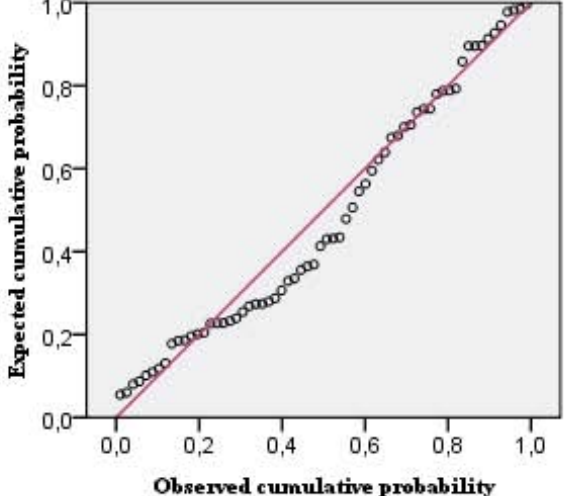
A

**Normal probability-plots for 8-oxodG (pmole/kg 24h) in sewage workers**



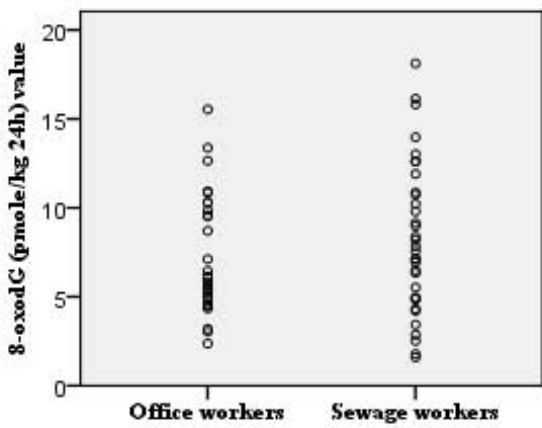
B

**Normal probability-plots for 8-oxodG (pmole/kg 24h) in the total study population**



C

**Scatterplot (diagram of dispersion) of 8-oxodG (pmole/kg 24h) values in the two job categories**



D





**Appendix 15: Poster communication presented in the 36<sup>ème</sup> Forum of Young Researchers (Biological Interaction of the molecule in the cell) and published in its textbook of the French Society of Biochemistry and Molecular Biology, France-Nancy 25-27 August 2009.**

«Étude BEEC»

**Biomarqueurs d'Évaluation d'une Exposition Complexe**

**\*Biomonitoring of Complex Occupational Exposures to Carcinogens: The Case of Sewage Workers in Paris**

Hanzeh Al Zabadi<sup>1</sup>, Luc Ferrari<sup>1,2</sup>, Anne-Marie Laurent<sup>3</sup>, Aziz Tiberquient<sup>4</sup>, Christophe Paris<sup>1</sup> and Denis Zmirou-Navier<sup>1</sup>

<sup>1</sup>INSERM-ERI 11, Nancy University Medical School, 9 av de la Forêt de Haye, BP 184, 54505 Vandœuvre-les-Nancy Cedex, France.

<sup>2</sup>Faculty of Pharmacy of Nancy, 5 rue Albert Lebrun, 54000 Nancy, France.

<sup>3</sup>Hygiene Laboratory of the City of Paris, 11, rue George Eastman, 75013 Paris, France.

<sup>4</sup>Department of Occupational Medicine, Municipality of Paris, 44 rue Charles Maureu, 75013 Paris, France.

**Background:** Sewage workers are exposed to complex mixtures of chemicals suspected to be genotoxins and/or carcinogens. We aim to investigate if those workers are at increased carcinogenic risk as evaluated by biomarkers of exposure and of early biological effects.

**Methods/design:** A cross sectional study of Parisian sewage workers and non-exposed office workers (75/75). Approved by French Est III Ethical Committee. Each week 5 exposed and 5 non-exposed will be sampled. After three days of consecutive work....

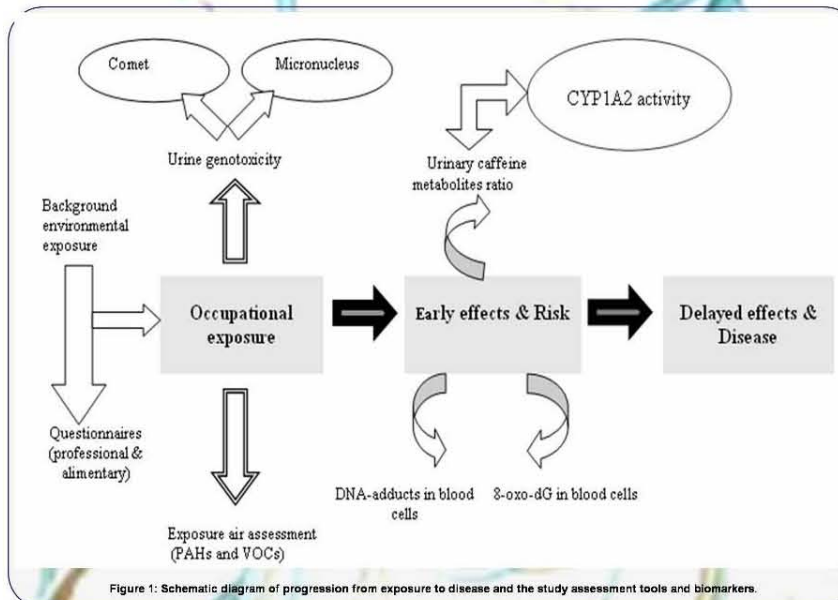


Figure 1: Schematic diagram of progression from exposure to disease and the study assessment tools and biomarkers.

Table: Morning sampling chronology of study participants.

	9h00	9h30	10h00-12h00	12h30	13h00	13h30
24h urine collection						
Pre-treatment of 24h urine samples						
Cup of Coffee						
Medical visit						
Blood sample						
Isolation and pre-treatment of lymphocytes						
Biological tests (urea, creatinine)						
Questionnaires						
3h urine collection						
Pre-treatment of 3h urine samples						
Thanks						
Departure						

Friday of each week. In each study week, 10 participants will be sampled (5 exposed in the morning and 5 non-exposed in the corresponding afternoon).

**Discussion:** Biomarkers of exposure and of early biological effects might help to overcome the extreme limitations in the exposure assessment of a very complex occupational or environmental setting.

Al Zabadi et al., *BMC Cancer*, 8: 67, 2008

**Appendix 16: Poster communication presented during the INSERM evaluation committee of two units of the INSERM (ERI 11 and U724) to integrate them in one unit (now U-954), 2008.**

**Biomonitoring of Complex Occupational Exposures to Carcinogens and Nutrition Status (OCM)  
"BEEC : Biomarqueurs d'Évaluation d'une Exposition Complexe"**

Hamzeh Al Zabadi<sup>1,2</sup>, Luc Ferrari<sup>1,3</sup>, Christophe Paris<sup>1,2</sup> and Denis Zmirou-Navier<sup>1,2</sup>

1. INSERM-ERI 11, School of Medicine, 9 avenue de la forêt de Hôye ; 54505 Vandœuvre-lès-Nancy Cedex France - 2. Nancy Universities, School of Medicine 3 Nancy Universities, Faculty of Pharmacy.

**Aim of the study**

Put in Evidence genotoxic effect of exposure to complex mixtures ?  
Is there a role of nutritional status on this response, if exists ?  
Biomonitoring early events reflecting the exposure to toxics.

**Methods/Study design**

A cross sectional study of Parisian sewage workers and non-exposed office workers (75/75). Each week 5 exposed and 5 non-exposed will be sampled.  
After four days of consecutive work, workers will be submitted to a clinical examination, sampling of 24h urines, blood (clinical chemistry and DNA), they will answer to questionnaires and they will perform a caffeine test.  
Approved by French Est III Ethical Committee.

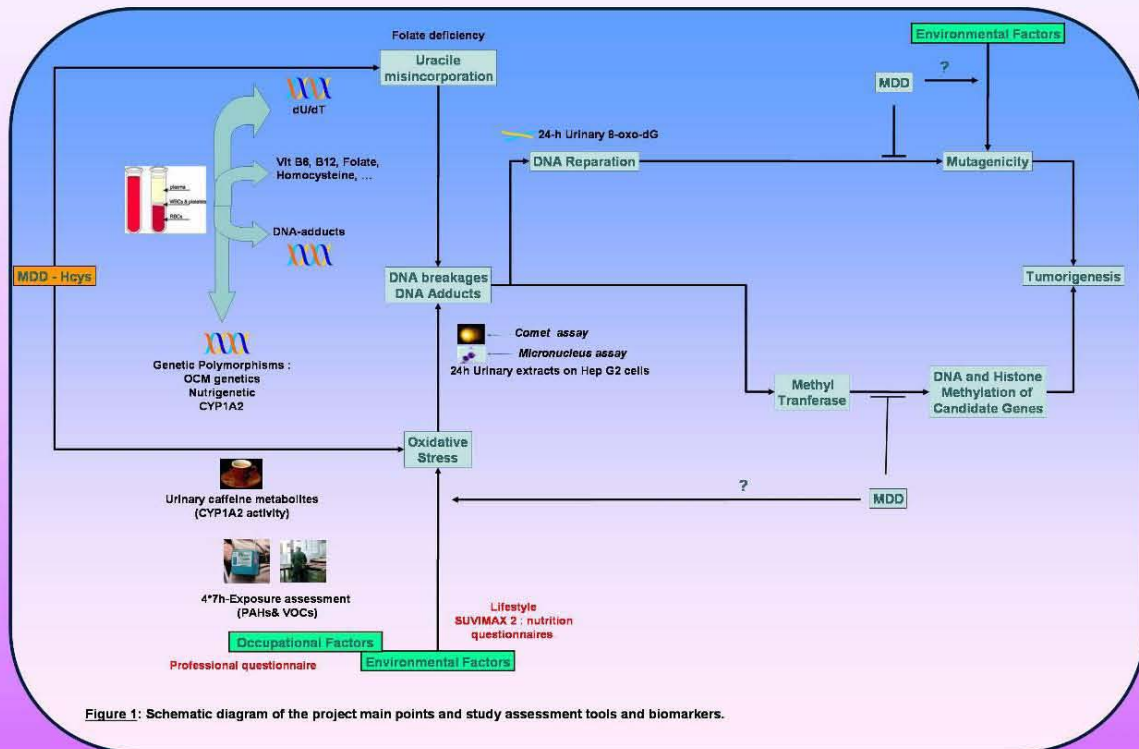


Figure 1: Schematic diagram of the project main points and study assessment tools and biomarkers.

**Work Packages**

ERI 11 : Study management, Questionnaires, Sampling, Comet and Micronucleus assays, caffeine test, Data analysis  
U724 : OCM clinical chemistry, dU/dT ratio, 8-oxo-dG, polymorphism studies

**Interpretation of the results**

Biomarkers of exposure and of early biological effects might help to overcome the extreme limitations in the exposure assessment of a very complex occupational or environmental setting.  
Nutrition status (methyl donor) evaluated through SUVIMAX questionnaire, clinical chemistry data and genetic polymorphisms will be discussed according to the genotoxicity results.

A follow up of both population for 5 years with the inclusion of nearly 400 subject is planned

Al Zabadi et al., BMC Cancer, 8: 67, 2008.

**Collaborations**

- Véronique ANDRE, EA 1772, GRECAN, Centre Baclesse, Avenue général Harris, 14076 Coen cedex : DNA adducts,
- Yvon Le MOULLEC, Laboratoire d'Hygiène de la Ville de Paris, 11, rue George Eastman, 75013 PARIS : Atmospheric sampling and analysis,
- Aziz TIBERGENT, Service de Médecine du Travail de la Ville de Paris, 44 rue Charles Moureu, 75013 Paris : Clinical examinations,
- Pierre LEROY, Laboratoire de Chimie Physique et Microbiologie pour l'Environnement, UMR 7564 CNRS - Université Henri Poincaré, Nancy-Université : Caffeine metabolism.



## Résumé :

### Évaluation de l'Exposition des Égoutiers aux Génotoxiques

Les égoutiers sont exposés à une grande variété de mélanges de produits chimiques beaucoup ce sont des substances génotoxiques ou cancérigènes. Le but de cette étude transversale était d'explorer des biomarqueurs intégrés d'exposition et des effets précoces chez les égoutiers afin de mieux apprécier leur risque cancérigène (article I-protocole). L'article II expose l'étape de validation préalable effectuée sur la lignée cellulaire pour nos objectifs.

Avec un recueil en 10 mois et sur une base hebdomadaire, nous avons, a) évalué l'exposition personnelle des égoutiers (n=34) et administratifs (n=30) (groupe témoin) à des hydrocarbures aromatiques polycycliques (HAP) et composés organiques volatils (COV) dans l'air des lieux de travail, b) évalué la génotoxicité de leurs extraits organiques urinaire par des tests *in vitro* : tests des comète et des micronoyaux sur les cellules Hep G2, c) évalué la réponse à un stress oxydatif de l'ADN par la mesure du 8-oxo-2'-deoxyguanosine urinaire (8-oxodG) dans les urines de 24h (Article III).

Les tests ont montré la présence de plus de génotoxiques dans les urines des égoutiers ( $P < 0,001$ ). La moyenne du 8-oxodG urinaire chez les égoutiers était plus élevée (non significatif,  $P = 0,28$ ) que les administratifs et ses valeurs moyennes ont été associées ( $P = 0,04$ ) avec les années de travail dans le système des égouts. Les concentrations des HAP et COV dans l'atmosphère professionnelle des égoutiers étaient élevées par rapport à celles des administratifs ( $P < 0,01$ ) et il a été montré une augmentation de risque du cancer en utilisant les facteurs d'équivalence de toxicité et de risque unitaire de cancer. En conclusion, les biomarqueurs intégrés urinaire d'exposition et non spécifiques montrent que les égoutiers sont professionnellement exposés aux mélanges des substances génotoxiques. Il n'est pas possible de relier nos résultats avec l'accroissement du risque cancérigène chez les égoutiers.

**Mots-clés:** 8-oxodG; biomarqueurs ; teste de comète et de micronoyau ; ADN stress oxydatif; génotoxicité; HAP, les égoutiers; COV.

## Abstract:

### Exposure Assessment of Sewage Workers to Genotoxicants

Sewage workers are exposed to a wide-variety of mixtures of chemicals many were shown to be genotoxicants or carcinogens. The aim of this cross sectional study was to explore integrated biomarkers of exposure and of early effects among sewage workers in order to better assess their carcinogenic risk (Paper I-protocol). Paper II, describes the validation step performed prior to choose the cellular line that meet our objectives.

Over 10 months and on weekly-basis, we, a) assessed the personal exposure of sewage (n=34) and office (n=30) workers (control group) to polycyclic aromatic hydrocarbons (PAHs) and volatile organic compounds (VOCs) in the air of workplaces, b) evaluated the genotoxicity of organic urine extracts by *in vitro* comet and micronucleus assays on Hep G2 cells, c) assessed the DNA oxidative stress by the 24h urinary 8-oxo-2'-deoxyguanosine (8-oxodG) (Paper III).

The tests showed greater genotoxicity in the urine extracts of sewage workers ( $P < 0.001$ ). The 24hr urinary 8-oxodG mean level in sewage workers was higher (nonsignificant,  $P = 0.28$ ) than office workers and its mean values were associated ( $P = 0.04$ ) with working years in sewage system. Workplace air concentrations of PAHs and VOCs were elevated in sewage compared to office workplaces ( $P < 0.01$ ) and resulted in an increased lifetime cancer risk using toxicity equivalent factors and inhalation unit risk of cancer. In conclusion, the applied integrated and non-specific urinary biomarkers show that sewage workers experience exposure to multiple genotoxicants at the workplace. It is not possible to simply link our results with the increased cancer risk in sewage workers.

**Key-words:** 8-oxodG; biomarkers; comet and micronucleus assays; DNA oxidative stress; genotoxicity; PAHs; sewage workers; VOCs.