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Biological markers as a tool in cancer risk assessment in Upper Silesia, Poland

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ABSTRACT

Upper Silesia is a densely populated and most polluted, industrial region of southern Poland. The major health hazard for humans comes from mining and/or processing of black coal, which generates a variety of compounds including polycyclic aromatic hydrocarbons (PAH). Ambient pollution with PAH plays an important role in risk of cancer. Between 1982 and 1997 Upper Silesia served as a study model for multi-center collaborative cancer risk assessment research, which utilized a variety of biological markers (biomarkers). This research started from testing genotoxic activity of air pollutants (using seven *in vitro* and *in vivo* tests) and was continued as the molecular epidemiology studies, which utilized eleven biomarkers of internal and biological doses of mutagen, early biologic effects as well as susceptibility markers. An ambient air genotoxicity studies were performed on benzene extracts of suspended matter collected in Upper Silesia while the molecular epidemiologic research involved human volunteers – residents of Upper Silesia and controls from a north-eastern, rural part of Poland. This review summarizes the results, presenting biomarkers as a valuable tool in the assessment of human gene damage. The preliminary results of the follow-up research on cancer incidence or mortality performed, over 10 years later, in the previously studied populations are also presented.

INTRODUCTION

Application of biomarkers is a modern way for assessing genetic damage in humans and other organisms in the environment. While environmental studies performed on fauna and flora living in contaminated water and soils give a chance to protect both the wild-life and humans from hazardous substances, utilization of biomarkers directly in the human body allows estimating risks of genetic-based diseases, including cancer. Considering future generations and the quality of life, both approaches are of great importance. This paper summarizes research on risk of human gene damage caused by polluted city air and the occupational environment in Upper Silesia, Poland.

Upper Silesia is a heavily industrialized region of Poland where major environmental contamination comes from black coal. Coal mining and/or processing (metallurgical works, smelters, coke ovens, chemical factories) generates a variety of hazardous substances, including polycyclic aromatic hydrocarbons (PAHs). At the end of the last century (1982-1997), the level of PM10 averaged between 80 and 314$\mu g \cdot m^{-3}$ of ambient air and non-occupational chronic exposure to a known PAH carcinogen – benzo[*a*]pyrene (B[*a*]P) varied from 1 to 125$ng \cdot m^{-3}$ of air with the maximum levels, permitted in Poland, 22$\mu g \cdot m^{-3}$ and 10$ng \cdot m^{-3}$ of air, respectively. The above data were drawn from the results of continuous monitoring of air quality, which was performed by the District Sanitary-Epidemiology Station in Katowice in cooperation with the Institute of Oncology in Gliwice (District Sanitary-Epidemiology Station in Katowice, 1980-1997; Chorąży et al. 1994; Motykiewicz et al. 1990a, 1998). In 1982 Upper Silesia (district of Katowice) encompassed 6500 square kilometres and was inhabited by about 4 million people.

The biomarker studies were started in Gliwice in 1982 to evaluate potential risk of genetic damage among residents of the District of Katowice. Over time and through development of new methods, these studies evolved from testing mutagenic and clastogenic activity of air-borne pollutants to molecular epidemiology research. This research utilized biomarkers in human tissues (blood, urine and buccal mucosa cells) to

indicate potential damage to genetic material. In general, there were three major steps in the research:

- determination of genotoxic activity of extracts of air pollutants in Upper Silesia,
- molecular epidemiology studies performed on residents of Upper Silesia and a reference region in north-eastern Poland,
- follow-up studies on the incidence and mortality from cancer in the previously studied populations.

I. GENOTOXIC ACTIVITY OF AIR-BORNE POLLUTANTS IN UPPER SILESIA

These studies were performed on crude benzene extracts or the sequential elution solvent chromatography (SESC) fractions of air-borne particulate matter collected at the District of Katowice. Samples were collected 6x24h·month^{-1} over a period of 6 months at 24 measuring points situated mainly in the center of the towns and, after extraction, combined for genotoxicity testing. The details on sampling, extraction and the SESC method were published previously (Motykiewicz et al. 1985, 1988).

Considering the assessment of potential carcinogenic activity, samples were tested in a battery of short-term assays according to the scheme proposed by Ashby (1986). In this scheme, genotoxic and potential carcinogenic activity is estimated in a combination of prokaryotic and eukaryotic tests performed *in vitro* and *in vivo*. There were seven different methods applied for testing activity of samples collected in Upper Silesia. The data are summarized in Table 1. Among the *in vitro* tests, applied methods ranged from the simplest bacterial mutagenicity assay performed on *Salmonella* Typhimurium mutants (strains TA100 and TA98), through cytogenetic methods using human lymphocytes and hamster fibroblasts (V79 cell line), to a transformation assay performed on a primary hamster kidney cell line. The only *in vivo* method, the micronucleus assay, was performed on BALB/c mouse bone marrow. As the by-product upon testing SCE, we have also investigated an epigenetic activity of the studied samples. This effect was measured by the mitotic arrest method based on mitotic division profile studies (Hadnagy and Seemayer 1988; Motykiewicz et al. 1991). As shown in Table 1, apart from one negative result obtained for fraction 3 of the crude benzene extract (polar aromatics and N, S, O nonbasic heterocyclics), tested by the mitotic arrest method, we observed positive responses in all tests. The results were dose-dependent. The details on the methods and the dose-response curves were previously published (Motykiewicz et al. 1985, 1988, 1989, 1990b, 1991).

II. MOLECULAR EPIDEMIOLOGY STUDIES ON RESIDENTS OF UPPER SILESIA AND REFERENCE REGION OF POLAND

Molecular epidemiology is a relatively new discipline which merges highly sophisticated molecular biology techniques with epidemiologic methods in order to use biomarkers in human tissues as indicators of potential risk of genetic-based

Table 1. Genotoxic activity of air-borne pollutants from Upper Silesia, Poland.

Test		Pollutant			
		CE	F2	F3	F4
Salmonella Typhimurium (-S9)	TA 100	+++	++	++	+++
	TA 98	++	NT	NT	NT
Salmonella Typhimurium (+S9)	TA 100	++	+++	++	++
	TA 98	+++	NT	NT	NT
SCE (human lymphocytes, hamster fibroblasts)		++	++	+	+
CA (hamster fibroblasts)		NT	NT	+	++
Micronucleus test (mouse bone marrow)		+	+	+	+
Transformation test (primary hamster kidney cells)		++	+++	+	++
Mitotic arrest test (hamster fibroblasts)		+++	+	-	++

CE - crude benzene extract; F2 - fraction 2 containing parent PAH compounds; F3 - fraction 3 enriched in polar derivatives of PAH; F4 - monophenol fraction; NT - not tested; (+) – positive response; (-) – negative result.

diseases, including cancer. According to the scheme proposed by Perera and Santella (1993), biomarkers include internal and molecular dosimeters of mutagen exposure (internal dose and biological dose, respectively), alterations in genes and chromosomes (early biologic effects), and genetic susceptibility factors controlling individual mutagen metabolism and DNA damage repair (individual susceptibly factors). As compared to classical (descriptive) cancer epidemiology, the use of "molecular" biomarkers allows for better understanding of environmental causes of diseases, cancer risk assessment, an early diagnosis as well as disease prevention. Biomarkers are also useful as predictive measures of the development of cancer and during the treatment of this disease.

The term "molecular epidemiology" was created by Summers and first published by Klein (1975). An important impact to this scientific approach was made by Lower (Lower 1982; Lower et al. 1979) and Perera and Weinstein (1982). While the works of Lower showed the connection between metabolic phenotype of N-acetyltransferase and carcinogen exposure (aromatic amines), Perera and Weinstein made use of the biomarkers as a very sensitive and accurate measure of human internal level (biological dose of mutagen) of a known carcinogenic metabolite of B[*a*]P, benzo[*a*]pyrene diol epoxide (BPDE) adducted to DNA. This was possible due to the development of antibodies against BPDE-DNA adducts (Poirier 1980; Santella et al. 1984).

Between 1989 and 1997 four independent molecular epidemiology projects were run in the Institute of Oncology in Gliwice, Poland, in cooperation with Columbia University in the City of New York (USA) and six top European laboratories from Finland, The Netherlands, Germany, Great Britain, Norway and Sweden. These studies were focused on the estimation of genetic damage caused by polluted ambient air or occupational hazards to residents of the region of Upper Silesia. Since the major carcinogenic components polluting the studied region were PAHs, most of the biomarkers were directed to determine PAH exposure. The same biomarkers were studied in residents of a control region of north-eastern Poland where environmental pollution with PAH was 10-times lower than that in Upper Silesia.

Cytogenetic studies in men exposed to different levels of air pollutants

Having experience with cytogenetic methods performed *in vitro*, we started our molecular epidemiology research studying SCE and CA in human lymphocyte cultures. Blood samples were collected from three groups of volunteers with different levels of exposure to air-borne pollutants. The donors included extremely highly exposed coke-factory workers (ambient air B[*a*]P level from 0.1 to 89.6$\mu g \cdot m^{-3}$), residents of Upper Silesia chronically exposed to lower doses of PAH (without working exposure to coal processing products) and residents of a north-eastern rural part of Poland (Biała Podlaska). Studies were performed on lymphocytes collected in summer and winter seasons. Since tobacco smoke contains a large amount of PAHs, we paid special attention to smoking habit. Before sampling, detailed questionnaires were used to collect information from all volunteers enrolled in the studies. The donors were healthy and, apart from smoking, they were questioned about age, occupation, exposure to genotoxic substances at home, diet (including alcohol consumption), family history of cancer, etc. As a result of these studies, we have demonstrated significant damage to human chromosomes among residents of Upper Silesia as compared to controls. We have also shown an additive effect of smoking and influence of season on biomarkers levels, with higher results in winter, as well as a dose-response effects (Motykiewicz et al. 1992; Pendzich et al. 1997; Perera et al. 1992). An example of the above results is the graph (Figure 1) presenting percentage of high frequency cells (HFC, cells exceeding 95th percentile of the SCE distribution) in three groups of men with different levels of exposure to PAH.

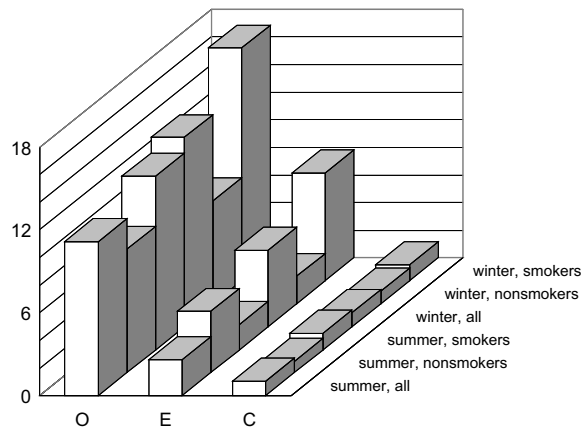

Figure 1. Comparison of HFC levels (%) in three groups of men (O, E, C) with different levels of exposure to PAH with respect to the seasons (summer, winter) and smoking habit (nonsmokers, smokers). O-occupational exposure, E-environmental exposure, C-control. According to Pendzich et al. (1997).

Comparison of cytogenetic methods with biomarkers of internal and biological doses in male and female populations

At the same time when lymphocyte cultures for the SCE and CA analyses were set up, DNA was isolated from a portion of collected blood. These samples were used for the measurements of PAH-DNA adducts determined by the enzyme-linked immunosorbent assay (ELISA) and aromatic adducts by ^{32}P-postlabelling. Measurements were performed at Columbia University (USA) and CNT Karolinska Institute

(Sweden), respectively. Serum samples were also saved and used for p21 protein (ras oncogene product) analyses. The results were previously published (Perera et al. 1992) and indicated that exposure to environmental pollution is associated with the significant increase in carcinogen-DNA adducts (both methods), SCE including HFC, CA as well as a doubling in the frequency of detection of p21 protein. In the early 1990-ties, this study was new and probably the most comprehensive evaluation of biomarkers in population exposed to environmental pollution (Garner 1992).

The molecular epidemiologic studies were continued in the Institute of Oncology in Gliwice till 1997. Over these years, we focused on the influence of seasons on the level of biomarkers, investigated the dose-response by including a highly exposed group of coke-factory workers as well as we extended studies to a female population not exposed occupationally to PAH compounds. We also included additional markers of internal dose of mutagen, which were determined in urine samples (urine mutagenicity by the Ames test and urinary level of 1-hydroxypyrene (1-OH-P)), the biological dose (BPDE-DNA adducts by immunohistochemistry in buccal mucosa cells) as well as susceptibility markers (bleomycin sensitivity assay, *GSTM1* and *CYP1A1* genotypes). All markers, which were used in this research, are summarized in Table 2.

Molecular epidemiologic study in women from Upper Silesia and the reference region of Poland

In this study, we paid special attention to already known and possible confounding factors in the molecular epidemiology research. Therefore, we focused on better study design including a narrow age range, smoking status and similar occupation without working exposure to PAH compounds. Both, our "study" (donors from Upper Silesia) and "control" (volunteers from north-eastern Poland) populations were composed of healthy 35-46 year old female, non-smoking city hall clerks. Samples of blood, urine and buccal mucosa cells were collected in summer 1994 and winter 1995. Our control region (city of Białystok) was also carefully selected with respect to the level of pollution and the type of urbanization. Although located in relatively unpolluted north-eastern Poland, similar to the Upper Silesian towns, Białystok is a large city (the capital of the province) and the population density was comparable in both studied regions (3076 people·km^{-2} and 3041 people·km^{-2}, respectively). At the time of sampling biologic materials, the medium ambient air level of B[*a*]P was about six times higher in Upper Silesia than in Białystok in both winter and summer seasons (43.4ng·m^{-3} versus 7.2ng·m^{-3} and 3.7ng·m^{-3} versus 0.6ng·m^{-3} in winter and summer, respectively). There were eight biomarkers applied in the study and control populations. They included urinary mutagenicity (*Salmonella* Typhimurium strains TA98 and YG1024), urinary level of 1-hydroxypyrene, PAH-DNA adducts in oral mucosa, SCE, HFC, CA and sensitivity to bleomycin in lymphocytes as well as glutathione s-transferase (GSTM1)/cytochrome P4501A1 (CYP1A1) genotypes in DNA isolated from blood samples. Biomarkers of internal and biological doses of mutagen and their biologic effects showed statistically significant increases in the study group of women as compared to control. No difference between groups was found for susceptibility markers. This negative result can be explained by very restrictive study design:

Table 2. Biomarkers used in molecular epidemiology research performed on residents of Upper Silesia and control region between 1989 and 1997.

Type of biomarker	Method	References
Internal dose of mutagen	Urinary 1-hydroxypyrene	Motykiewcz et al. 1998
	Ames urinary mutagenicity test (TA 98 and YG 1024 strains)	
Biological dose of mutagen	PAH-DNA adducts by ELISA	Perera et al. 1992
	Aromatic adducts by ^{32}P-postlabelling	Grzybowska et al. 1993; Hemminki et al. 1990; Perera et al. 1992
	Immunohistochemical detection of BPDE-DNA adducts	Motykiewcz et al. 1995, 1998
Early biologic effects	SCE in lymphocytes	Michalska et al. 1999; Motykiewicz et al. 1992;
	CA in lymphocytes	Pendzich et al. 1997; Perera et al. 1992
	ras p21 protein in plasma	Perera et al. 1992
Individual susceptibility	bleomycin sensitivity test in lymphocytes	Michalska et al. 1998; Motykiewicz et al. 1998
	GSTM1 and *CYP1A1* genotypes	Butkiewicz et al. 1998, 2000; Grzybowska et al. 2000; Motykiewicz et al. 1998
	Serum retinol level	Perera et al. 1992

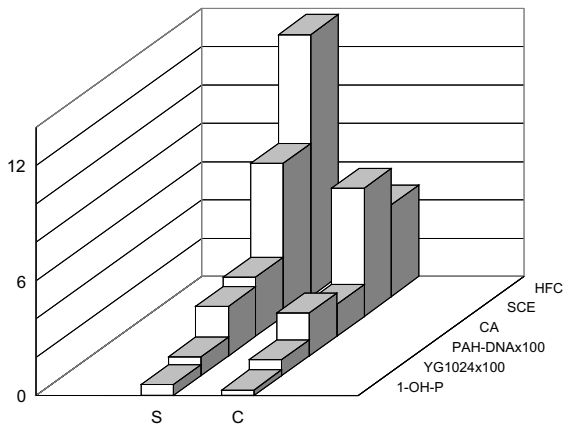

Figure 2. The mean levels of six biomarkers studied in Upper Silesian (S) and control (C) groups; 1-OH-P, urinary 1-hydroxypyrene (μmol·mol^{-1} creatinine); YG1024, Ames urinary mutagenicity (number of revertants·24ml^{-1} of urine); PAH-DNA adducts detected in buccal mucosa cells by immunohistochemistry (OBAD units); CA, structural chromosomal aberrations in lymphocytes, including gaps (number of aberrations·100^{-1} cells); SCE, sister chromatid exchanges in lymphocytes (number of exchanges·cell^{-1}); HFC, high frequency cells (percentage of cells with >12 SCE·cell^{-1}). According to Motykiewicz et al. (1998).

genetically homogenous non-smoking healthy population at the same age range without working exposure to PAHs. We realize that in the genotype studies, a positive result is very much dependent on the study size, which in referred research was small (67 and 72 donors, for the study group and controls, respectively). On the other hand, with a similarly small groups of male-donors (30 occupationally exposed workers, 38 Silesian citizens, and 35 rural inhabitants) significantly higher number of breaks per cell (b/c) generated by bleomycin in lymphocytes collected from donors living in Upper Silesia, as compared to controls, was reported in our previous studies (Michalska et al. 1998). Using the same method we have not found significantly higher level of b/c among women from Upper Silesia as compared to controls. The bleomycin sensitivity test has been well recognized as relatively sensitive, phenotype, cancer susceptibility marker (Chang et al. 2002; Hsu et al. 1989; Spitz and Bondy 1993).

The results obtained for six markers showing significantly higher results in Upper Silesian group (S) as compared to controls (C) are summarized in Figure 2. The details on study design and the original data were published previously (Grzybowska et al. 2000; Michalska et al. 1999; Motykiewicz et al. 1998).

III. THE FOLLOW-UP STUDIES IN UPPER SILESIA AND REFERENCE REGION

As shown above, there were molecular epidemiology studies on residents of Upper Silesia and a control region of Poland performed between 1989 and 1997. This research, which built on previous studies on genotoxicity of air-borne pollutants, showed significant damage to the human genome among residents of Upper Silesia as compared to controls from Biała Podlaska and Białystok. Having in mind a possible predictive value of biomarkers in cancer disease, our last goal for molecular epidemiology research in Poland was to study cancer incidence and mortality among donors previously

Table 3. Cancer incidence or mortality in previously studied populations.

Study groups			No. of subjects	No. of cancer cases
Upper Silesia	males	residents	86	3
		workers	170	16
	females		68	1
Control	males		132	2
	females		71	2
Total	males		388	21
	females		139	3

enrolled into the projects. For this purpose, we compared our database with cancer registries of the relevant regions, the Regional Silesia Cancer Registry and the Białystok Cancer Registry, respectively. Since we started searching cancer registries in 2004 and molecular epidemiology studies began in 1989, we were able to deal with the latency period, which is known to be an important factor in the development of most cancer diseases.

To date we identified 527 donors, who served in our previous biomarker studies. They include 388 males and 139 females. Table 3 gives the details on study groups and the results of the follow-up research. Among enrolled subjects, cancer registries identify 21 cancer cases in males (out of 388 subjects, 5.4%) and 3 cancer cases in females (out of 139 subjects, 2.1%). Among women, given the small group studied and the low overall cancer incidence, there was no difference between Upper Silesia and control regions (1 case out of 68 and 2 cases out of 71 subjects, respectively). Therefore, we eliminated this group from further examination. Instead, we focused on the data obtained for the male group. There were 19 cases (out of 256 subjects, 7.4%) and 2 cases (out of 132 subjects, 1.5%) found in the Upper Silesian and control groups, respectively. Considering occupational exposure to carcinogens as a possible modifier of cancer incidence, we have further divided the Upper Silesian group into "residents" not connected with coal mining and/or processing and to donors employed in relevant factories ("workers"). There were only 3 cases (out of 86 subjects, 3.5%) and 16 cases (out of 170, 9.4%) found for residents and workers from Upper Silesia, respectively. All workers were current smokers and half of them suffered from respiratory tract cancers.

We realize that these preliminary results are limited by the small sample size and need extended statistical evaluation. But even now, the observed high number of cancer cases among men from Upper Silesia (especially workers occupationally exposed to coal products), as compared to controls, suggests a causative relation between chemical exposure and cancer development. It was recently found that, among other factors (emphysema, age of smoking cessation, and individual efficiency of DNA damage repair), exposure to dust is a predictor for lung cancer (Spitz et al. 2008).

DISCUSSION AND CONCLUSIONS

Presented research is an example of utilizing biomarkers in the assessment of risk of one of the most damaging human diseases – cancer. At the same time, the above research shows an evolution in the development of biomarkers, which mirrors the progress in molecular biology, cell biology, genetics, biochemistry, etc. It also shows a better understanding how to use biomarkers to limit confounding factors and the value of proper study design in cancer epidemiology research.

Recent results building on previous hospital-based molecular epidemiology research have shown the usefulness of incorporating biomarkers of exposure, biological effect and susceptibility for predicting lung cancer (Reid et al. 2008; Schwartz et al. 2007; Spitz et al. 2008). Similar approach might be used for environmental exposures by linking previous biomarkers' results with already existing cancer registries. Although the presented follow-up research is substantially limited by the small number of subjects, and suggested conclusion on the link between occupational exposures to PAH and the development of cancer should be taken with care, other results from a pooled cohort studies have demonstrated the CA assay predict risk of cancer (Bonassi et al. 2008).

In the mid 1980-ties, estimating environmentally related risk of cancer based on testing samples collected from polluted air, water, soil and other sources was well accepted. Using this approach, the impact of environmental factors on risk of cancer was estimated in the USA at 2% (Doll and Peto 1981). Collected samples were tested in a battery of bioassays (Ashby 1986) and the only rational conclusion to be drawn was to accept or reject the thesis of a "possible carcinogenic activity of tested samples". Now we understand that testing the level of pollutants in a contaminated environment gives us only information on external dose of mutagens. Considering individual differences in mutagen absorption, metabolism and excretion, this measure is not accurate in assessing human exposure. A good example is an external level (e.g. ambient air level) of PAH compounds. It is well recognized, that PAHs are promutagens, which in their basic form are biologically not active. They need to be converted, through metabolic pathways, to become mutagenic (Alberts et al. 2002; Ledemsa et al. 2000; Scicchitano 2005). Therefore, instead of testing air level of B[*a*]P a much better measure of real human exposure to PAH is testing urinary level of 1-OH-P, a metabolite of one of PAH compound – pyrene. Similarly, instead of testing mutagenic activity of air-borne extracts by the Ames test (mostly strains TA98 and TA100), which provides only limited measure of human exposure to mutagens; much more informative is testing (with the same method) the mutagenicity of urine collected from the donors. Obviously, this change was possible by the development and utilization of more sensitive *Salmonella* Typhimurium mutants (e.g. YG1024, *O*-acethyltransferase derivative of strain TA98). Both approaches were used in the presented research and are examples of the evolution in the way of thinking about risk assessment. This change has happened during the last two decades of the last century. Over this time, the research evolved from testing genotoxic activity of samples collected from the environment to the molecular epidemiology in humans. As shown on Figure 3, in both approaches similar or even the same methods are utilized (e.g. Ames test, SCE, CA) in a battery of assays. The major difference lays in the origin of tested material.

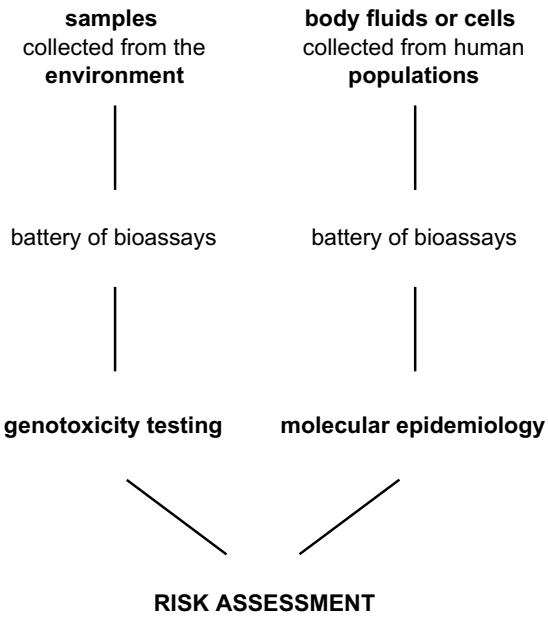

Figure 3. Evolution in cancer risk assessment. According to Motykiewicz et al. (1998).

Another important issue of biomarker studies is sample collection, preservation and long-term storing without loss of their biological value. At this point, researchers encounter many problems of both a scientific and logistic nature. It appears that the best solution is creation of professional Biobanks and linking them in the networks that would serve laboratories (in connection with hospitals) worldwide or, at least on the same continent. Till now, the best example of how to realize this idea is the Scandinavian net of Biobanks, which was created in the 1960-ties. Apart from Scandinavian countries, Biobanks exist for at least 30 years in the USA. However, the idea of how to build up and maintain Biobanks might be a subject for another review article.

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