UNIVERSITY OF SIENA
DOCTORAL SCHOOL OF ONCOLOGY AND GENETICS

HEPATOBILIOPANCREATIC DISEASES AND MULTITUMORAL SYNDROMES SECTION

PhD student, XXI cycle

Role of the diet and gender in the onset and/or protection in tumors

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1. INTRODUCTION

Individual susceptibility to cancer may result from several host factors including differences in metabolism, DNA repair, altered expression of protooncogenes, tumor suppressor genes, and nutritional status. Since most carcinogens require metabolic activation before binding to DNA, variations in an individual's metabolic phenotype that have been detected in enzymes involved in activation and detoxification should play an essential role in the development of environmental cancer. This phenotypic metabolic variation has been related to genetic polymorphisms.

The mechanisms of chemical food and PM-induced health effects are believed to involve chronic inflammation and oxidative stress. The oxidative stress mediated by PM may arise from direct generation of reactive oxygen species (ROS) from the surface of particles, soluble compounds such as transition metal or organic compounds, altered function of mitochondria or NADPH-oxidase, and activation of inflammatory cells capable of generating ROS and reactive nitrogen species. Carcinogens require metabolic activation, and it was proposed that genetic control of activation or elimination might account for genetically mediated variation in PM and diet-related cancer susceptibility. A broader appreciation of human carcinogenesis suggests categories of genes that go beyond metabolic activation/detoxification. These include genes that influence DNA repair, chromosome stability, the activity of oncogenes or tumor suppressor genes, cell cycle control or signal transduction, hormonal or vitamin metabolism pathways, immune function and receptor or neurotransmitter action. While epidemiological studies indicate that susceptibility to cancer is to a large extent determined by environment, certain individuals are more susceptible than others in a similar environment, suggesting that genetically determined factors also play a role. In some cases this is associated with genetically determined increased or decreased activity of critical enzymes involved in xenobiotic metabolism. Amongst the cytochrome P450s, responsible for the bulk of phase I xenobiotic metabolism, genetic polymorphisms have been found in CYP 1A1, 2A6, 2C9, 2C19, 2D6, 2E1, and 3A5. Polymorphisms have also been identified in four families of the human glutathione *S*-transferase (GST) supergene family, the best characterized being in GST M1, M3, T1 and P1. Since isoenzymes encoded by these polymorphic genes catalyze the activation and detoxification of a variety of carcinogenic compounds, it follows that altered expression resulting from polymorphisms could have deleterious

consequences in terms of disease susceptibility. Since activity of theses enzymes implicated in the metabolism of carcinogenens presents a great variability between individuals due to the existence of a polymorphism in gene coding for these enzymes, individual susceptibility to develop cancer depend not only on exposure to enviromental carcinogens (PM, diet, etc), but also on genetic capacity to activate or inactivate these carcinogens.

Asbestos is the principal etiological factor of Malignant Mesothelioma (MM) an aggressive and highly lethal tumor originating from the epithelium of serosal cavities. The mechanisms of asbestos-induced genotoxicity explaining carcinogenicity of asbestos fibers is linked to pleural inflammation. In fact, asbestos fibers could cause prolonged cycles of damage, repair, and local inflammation and induce DNA damage and strand breaks. The effect of the oxidative stress, caused by free radicals and reactive oxygen species (ROS), is contrasted by molecules which have an antioxidant action, such as the glutathione S-transferase family, which includes several isozymes, many of them are polymorphic in humans. The final outcome seems to be less dependent on the toxic potency of the pollutants or the exposure dose and more on individual susceptibility of the host. Our initial working hypothesis was to perform genome wide analysis in a cohort of people with long lasting occupational exposure to asbestos in the same working place, in order to detect genetic differences which could be responsible for the different incidence of mesothelioma.

Comparative genomic hybridization showed that each study group, a first couple including an indirectly exposed affected female and her exposed healthy husband, and a second couple including an exposed affected male and his healthy wife differed for a panel of 7 and 4 different polymorphisms respectively.

The presence of a peculiar polymorphisms in some common genes, among "predisposed" subjects, i.e. developing mesothelioma; and "non susceptible" subjects, i.e. not developing mesothelioma, despite being exposed to the same dose of pollutants or asbestos, strongly suggests that these genes could be involved in, or at least be partially responsible, for the congenital or inherited (or even acquired) predisposition. In subjects which were exposed to the same or similar doses of asbestos for similar time lags, the predisposition facilitates the occurrence of mesothelioma – or the absence of mesothelioma.

This approach should explain the absence of health effects in the husband of one of the couple with chronic occupational exposure to asbestos but the occurrence of mesothelioma in the wife with minor indirect exposure from her husband.

1.1 Reactive Oxygen Species (ROS) are main cellular stressors generated by PM exposure

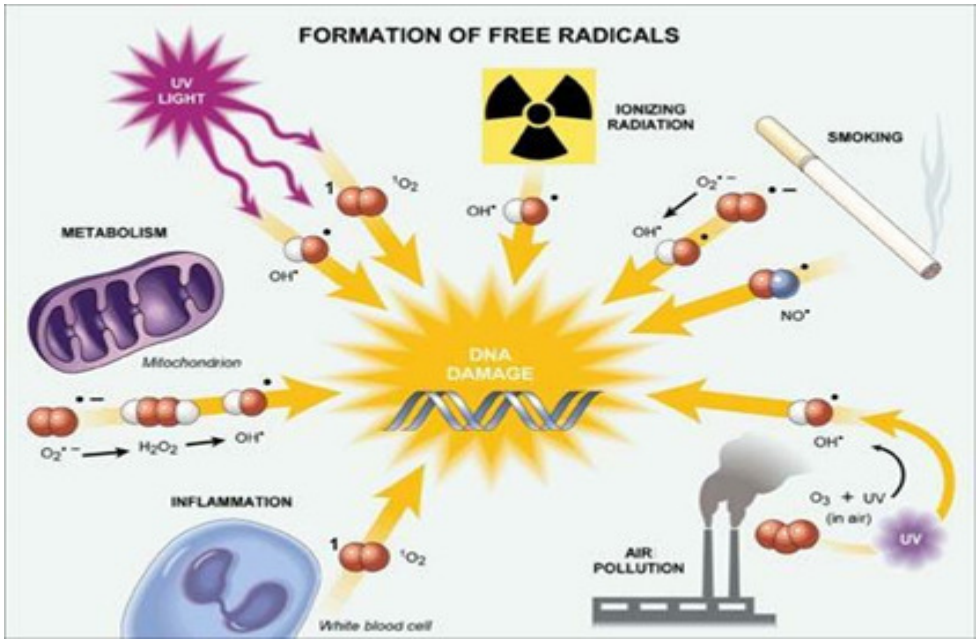
Reactive oxygen species (ROS) are a variety of molecules and free radicals (chemical species with one unpaired electron) derived from the metabolism of molecular oxygen. Molecular oxygen in the ground state is a bi-radical, containing two unpaired electrons in the outer shell (also known as a triplet state). Since the two single electrons have the same spin, oxygen can only react with one electron at a time and therefore it is not very reactive with the two electrons in a chemical bond. On the other hand, if one of the two unpaired electrons is excited and changes its spin, the resulting species (known as singlet oxygen) becomes a powerful oxidant because the two electrons with opposing spins can quickly react with other pairs of electrons, especially pairs with double bonds.

The reduction of oxygen by one electron at a time produces relatively stable intermediates. Superoxide anion ($O2^{-}\bullet$), the product of a one-electron reduction of oxygen, is the precursor of most ROS and a mediator in oxidative chain reactions. Dismutation of $O2^{-}\bullet$, either spontaneously or through a reaction catalysed by superoxide dismutases (SOD), produces hydrogen peroxide (H2O2), which in turn may be fully reduced to water or partially reduced to hydroxyl radical ($OH\bullet$), one of the strongest oxidants in nature. The formation of $OH\bullet$ is catalyzed by reduced transition metals, which in turn may be re-reduced by $O2^{-}\bullet$, repeating this process. In addition, $O2^{-}\bullet$ may react with other radicals including nitric oxide ($NO\bullet$), controlled by the rate of diffusion of both radicals. The product, peroxynitrite, is also a very powerful oxidant. The oxidants derived from $NO\bullet$ are called reactive nitrogen species (RNS).

ROS normally exist in all aerobic cells in balance with biochemical antioxidants. Oxidative stress occurs in cells or tissues when this critical balance is disrupted in favor of pro-oxidants and in disfavor of antioxidants, potentially leading to cellular macromolecule damage: such as in DNA, lipids, and proteins. To counteract the oxidant effects and to restore redox balance, cells must reset important homeostatic parameters. Oxidative stress is generated by a large variety of mechanisms, including mitochondrial respiration, ischemia/reperfusion, inflammation, and metabolism of foreign compounds. ROS can be produced either endogenously or exogenously. Endogenous oxidative stress can be the result of normal cellular metabolism and oxidative phosphorylation. The

metabolism of substances by the P450 enzyme system generates oxygen free radicals through normal or futile cycling mechanism (1). Exogenous sources of ROS can also impact on the overall oxidative status of a cell. Drugs, hormones, and other xenobiotic chemicals can produce ROS, via either direct or indirect mechanisms (2, 3). Alternatively, oxidative stress can also occur when there is a decrease in the antioxidant capacity of a cell. Non-enzymatic antioxidant levels (vitamin E, vitamin C, glutathione, etc) and enzymatic antioxidant levels (superoxide dismutase, glutathione peroxidase, and catalase) in the cell can be decreased through modification in gene expression, decreased in their uptake in the diet, or can be overloaded in ROS production, which creates a net increase in the amount of oxygen free radicals present in the cell (4, 5).

Figure 1: Oxidative DNA damage may participate in ROS-induced carcinogenesis



The production of ROS seems to play an important role in the primary citotoxic effects of respirable diesel exhaust particles (DEP) and urban street Particulate Material (PM). Air pollution consists of tiny ambient particles measuring < 10–15 microns (PM10) that arise from dust, smoke, or aerosol liquids produced by vehicles, factories, or burning wood. Air pollution can include residual oil fly ash (ROFA), an organic and inorganic

mixture of silicates and metal salts containing vanadium, zinc, iron, and nickel, released during the combustion of low-grade oil.

PM have several mechanisms of adverse cellular effects, such as citotoxicity through oxidative stress mechanisms, oxygen-free radical-generating activity, DNA oxidative damage, mutagenicity, and stimulation of pro-inflammatory factors.

The size of the airborne particles and their surface area determine the potential to elicit inflammatory injury, oxidative damage, and other biological effects. These effects are stronger for fine and ultrafine particles because they can penetrate deeper into the airways of the respiratory tract, reach the alveoli and go into the circulation, whereas larger sized particles deposit mainly in the upper airways and can be cleared by the mucociliary system and swallowed.

The chemical composition of PM varies greatly and depends on many factors, such as combustion sources, climate, seasons, and type of urban or industrial pollution. The major components of PM are organic compounds adsorbed on to particles. These compounds can be volatile or semivolatile organic species (e.g., PAHs, nitro-PAHs, quinones), transition metals (iron, nickel, vanadium, copper, etc.), ions (sulfate, nitrate, acidity), reactive gases (ozone, peroxides, aldehydes), particle core of carbonaceous material (mainly from combustion processes and vehicular exhaust particles), materials of biologic origin (endotoxins, bacteria, viruses, animal and plant debris), and minerals (quartz, asbestos, soil dust). The composition of coarse particles consists mainly of insoluble crust-derived minerals, sea salt, material of biologic origin, and so on. By contrast, the fine and ultrafine particles are mainly carbonaceous aggregates with metals and organic species adsorbed on their surface cavities (7).

Associations between chemical compositions and particle toxicity tend to be stronger for the fine and ultrafine PM size fractions. Diesel exhaust particles (DEP) are the most predominant particles in small-sized airborne PM air pollution in urban areas. The ultrafine particles are generated directly by combustion and photochemical activity. The particles exist briefly in an unstable form, and then aggregate to form larger particles. Ultrafine and fine particles are very high in numbers, have greater total surface area than larger particles and because of their porous surface, can adsorb and retain toxic substances.

The oxidative stress mediated by PM may arise from mixed sources, including: 1) direct generation of ROS from the surface of particles; 2) soluble compounds such as transition

metals or organic compounds (PAHs); 3) altered function of mitochondria or NADPH-oxidase; and 4) activation of inflammatory cells capable of generating ROS and Reactive nitrogen species (RNS).

Direct particles generation of ROS can occur through the presence of free radicals and oxidants on the particle surface (8). Although this mechanism can explain the generation of DNA damage in cell experiments, the generation of particles-induced oxidative damage in intact cells, can only be explained assuming that: i) particles traverse to the nucleus; ii) extracellular ROS initiate free radical chain reactions which ultimately reach the nucleus and damage DNA.

Transition metals are thought to be very important in PM cellular toxicity. On their surface, particles may contain soluble transition metals such as iron, copper, chromium and vanadium that can generate ROS through Fenton type reaction and act as catalyst by Harber-Weiss reactions:

$$^{\bullet}O_2 + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^- + O_2$$

Ferrous iron (Fe^{2+}) reduces hydrogen peroxide (H_2O_2) with the formation on hydroxyl radical and oxidation of ferrous iron to ferric iron (Fe^{3+}). This reaction can recycle via reductants, such as superoxide anions, glutathione and ascorbic acid by reducing Fe^{3+} to Fe^{2+}.

The hydroxyl radical ($^{\bullet}OH$) is extremely reactive, which implicates that it attacks any biological molecule at diffusion distance (9). Several studies have shown that iron and other transition metals leaching from particles or that are present on particle surface play a role in the generation of ROS in biological system (10, 11). It was recently suggested that DEP contain surface functional groups with the capacity to complex host iron, whereby iron accumulates and oxidative stress is induced (10,11). This is in accordance with in vitro studies demonstrating that DEPs generate superoxide anions (12, 13), which can lead to hydrogen peroxide and hydroxyl radicals without any biochemical or biological activation.

Diesel exhaust is composed of both particulate and gaseous phases. DEPs have a mass medium diameter of 0.05 to 1 μm (mean = 0.2 μm), a size that renders them easily breathable and capable of depositing in the airways and in alveoli (14, 15). The particles consist of a carbonaceous core with a large surface area to which various hydrocarbons are absorbed. The gaseous phase contains various combustion products, including hydrocarbons. Once emitted, diesel exhaust components undergo atmospheric

transformation, either through heterogeneous processes involving particle-associated compounds or homogeneous gas phase reactions (16). DEPs contain more than 400 chemicals, among which the following components have been identified: polycyclic aromatic hydrocarbons (PAHs), nitroderivatives of PAH, oxygenated derivatives of PAH (ketones, quinones, and diones), heterocyclic compounds, aldehydes, and aliphatic hydrocarbons (17-20). PAHs, including oxyderivatives in DEP, exert pro-inflammatory and tissue-damaging effects through generation of reactive oxygen species (ROS) (21). Metabolic activation of PAHs by cytochrome P450 enzymes (CYP1A1) and peroxidases leads to oxidized derivatives, such as quinones that generate ROS (22, 23). In addition, some PAH species are chemically oxidized to quinones by the fuel combustion process (17). Some quinones undergo one electron (1e-) reductions by NADPH-dependent reductase to yield semiquinone radicals (24-26). These semiquinones reduce oxygen to superoxide radicals (O_2^-), which can be re-oxidized to the original quinone (24-26). This leads to a futile redox cycle during which cytotoxic amounts of ROS accumulate. Through this mechanism, quinones contribute to cellular activation and toxicity of DEP chemicals.

Moreover PAHs and volatile organic compounds (e.g. benzo[*a*]pyrene) may be metabolically activated to reactive species that form bulky adducts on the DNA.

The principal pathways of metabolic activation of PAHs are: 1) generation of diol epoxides catalyzed by cytochrome P450, leading to DNA adduct formation, considered to be essential to PAHs mechanism of carcinogenesis; 2) formation of radical cations catalyzed by cytochrome P450 peroxidases; and 3) formation of redox-active quinones catalyzed by dihydrodiol dehydrogenases, contributing to PAHs carcinogenesis and tumor promotion (27-30).

Organic compounds of DEP undergo metabolic activation, causing induced expression of cytochrome P450 enzymes (CYP1A1), and generate ROS and reactive PAH-quinones. PM initiate inflammatory damage and upregulation of pro-inflammatory mediators (cytokines and chemokines), endotoxin effects, stimulation of capsaicin/irritant receptors, procoagulant effects, and modification of cellular components, cellular mutagenicity, and DNA damage (31).

All aerobic organisms contain elaborate antioxidant defenses (enzymatic and non-enzymatic) for cellular redox homeostasis and avoidance of oxidative damage to important biological macromolecules (proteins, carbohydrates, membrane lipids, and

mitochondrial and cellular DNA). Endogenous metabolic factors can be responsible for excessive ROS production or the weakening of antioxidant defenses but are regulated to a great extent by intracellular enzymatic mechanisms. High levels of ROS from exogenous factors (air pollution, dietary factors, cigarette smoke, etc.) can change the redox status of the cell, thereby triggering a cascade of events associated with inflammation (32, 33). Many recent observations showed that diesel exhaust particles (DEP), because of their fine and ultrafine composition, play an important role of oxidative cellular damage through the generation of oxygen-free radicals (e.g., hydroxyl, HO•, and superoxide anion, $O•_2^-$) and ROS (e.g., H_2O_2), which take part in a series of mechanisms that cause membrane lipid peroxidation and oxidative DNA damage (34-36).

In vitro studies have shown that exposure to diesel soot and other PM10 particles activate pro-inflammatory genes in a process mediated by free radical/oxidative stress mechanisms. These, in turn, induce pro-inflammatory transcription factors, such as nuclear factors-κB (NF-κB) and activator protein 1 (AP-1), which promote increased histone acetyl transferase activity, histone acetylation, the release of interleukin-8 (IL-8), a marker of inflammation, and the expression of inflammatory genes. ROFA exposure stimulates a similar cascade of events. Samet et al. [48] showed that the vanadium component of ROFA can inhibit tyrosine phosphatases, causing phosphorylation of NF-κB and other pro-inflammatory transcription factors, including activating transcription factor 2 and c-Jun. Again, this leads to the expression of inflammatory genes, chronic inflammation, and, in some cases, cancer development.

Oxidative stress caused by activation of the inflammatory system encompasses alveolar macrophage and neutrophils in multicellular organisms that are important DEPs targets, which contribute to the proinflammatory effects.

Ultrafine particles cause inflammation by surface-mediated effects after inhalation (transition metals, PAHs, organic and volatile compounds), while large, coarse particles cause inflammation through endotoxins.

Activated alveolar macrophages (AM) are able to release pro-inflammatory mediators (e.g. cytokines) and toxic oxygen radical, which produce reactive oxygen species (ROS) (38, 39). ROS are known to be involved in the regulation of transcription factors, which have an important function in the cytokine network (40-43). Transcription factors initiate the transcription of specific genes by binding to their promoter region. Nuclear factor-κB (NF-κB) is an important transcription factor activated by ROS-dependent mechanisms.

NF-κB activation has been linked to the carcinogenesis process because of its roles in inflammation, differentiation, and cell growth. NF-κB regulates several genes involved in cell transformation, proliferation, and angiogenesis (44). Carcinogens and tumor promoters including UV radiation, phorbol esters, NNK, asbestos, alcohol, and benzo (a) pyrene are among the external stimuli that activate NF-κB (45, 46).

Reactive oxygen species have been implicated as second messengers involved in activation of NF-κB via tumor necrosis factor (TNF) and interleukin-1 (47). Suppression of TNF and interleukin-1 leads to downregulate the expression of active NF-κB and inhibit proliferation of lymphoma and myelogenous leukemia cells (48). Of note is the fact that protein kinases are also involved in cell response mediated by the TNF superfamily. In fact, the binding of TNF to its receptor is associated with H_2O_2 generation and protein-protein disulphide bond formation (49, 50). Oxidative changes may amplify the TNF receptor-mediated signal, and can function either to activate protein kinases [e.g., stress-activated protein kinase (SAPK), extracellular signal regulated kinase (ERK), and p38] or inhibit transcription factors, such as AP-1 and NF-κB (51). Therefore, the decision to commit to cell death or cell survival will in part depend on the strength and duration of oxidant exposure and on the cell type involved. The importance of reactive oxygen species on NF-κB activation is further supported by studies demonstrating that activation of NF-κB by nearly all stimuli can be blocked by antioxidants, including L-cysteine, NAC, thiols, green tea polyphenols, and vitamin E (52, 53). That NF-κB activation appears to be selectively mediated by peroxides as activation was observed only following exposure to H_2O_2 or butylperoxide, and not superoxide or hydroxyl radicals (54). Likewise, NF-κB activity was increased in cells that overexpressed superoxide dismutase and decreased in cells overexpressing catalase (55). NF-κB activation signals the transcription of genes such as manganese containing SOD and γ-GCS. Collectively these findings support the linkage of NF-κB activation by reactive oxygen species with the carcinogenesis process. Activation of transcription factors is clearly stimulated by signal transduction pathways that are activated by H_2O_2 and other cellular oxidants. Through the ability to stimulate cell proliferation and either positive or negative regulation of apoptosis, transcription factors can mediate many of the documented effects of both physiological and pathological exposure to H_2O_2, or chemicals that induce reactive oxygen species and/or other conditions that favor increased cellular oxidants. Through regulation of gene transcription factors, and disruption of

signal transduction pathways, reactive oxygen species are intimately involved in the maintenance of concerted networks of gene expression that may interrelate with neoplastic development.

However, individuals are usually co-exposed to a broad range of pollutants that usually exert their dangerous effects by similar mechanisms or pathways, mainly involving the generation of reactive oxygen species (ROS). The effects of co-exposure to multiple pollutants are not only accumulative but may also enhance their individual effects. Accordingly, epithelial cell alterations are greatly enhanced by co-exposure to particulate material (PM), gaseous pollutants such as NOx, SOx, ozone, and/or biogenic substances such as pollen, aeroallergens, and bacterial endotoxins.

In particular, the various components of PM seem to have different specific biological effects. For example, PM10 is more effective in "priming" cells to the subsequent activity of PM2, 5, which is then able to produce DNA adducts. While the ultrafine component of PM0,001-1 is responsible for damage to cell and mitochondrial membranes.

In summary, it can be argued that Particulate Material (PM), especially traffic-related airborne particles, contains a large number of genotoxic/mutagenic chemical substances, which can cause DNA damage and promote malignant neoplasms. Most studies focused their observations on the genotoxicity of extractable organic compounds and mixtures but also on the water-soluble substances (such as metals) and volatile organic compounds (56-58). Studies showed that the mutagenicity of airborne PM is due to at least 500 identified organic compounds from varying chemical classes. Mutagenicity was associated with moderately polar and highly polar classes of substances that tend to contain nitroaromatics (nitro-PAHs), aromatic amines, and aromatic ketones. These compounds are produced in the atmosphere when organic compounds (even non-mutagenic) are exposed to NOx and sunlight. Combustion emissions were associated with much of the mutagenicity and carcinogenicity of urban PM (59).

These studies suggest that the mechanisms of genotoxicity of PM are the result of adduct-forming compounds (through cell-particulate interactions) and oxidizing DNA damage (45, 60, and 61).

Apart from organic-soluble fractions of PM, other studies focused on the water soluble fractions (mainly transition metals) and compared their DNA damage potential. Results of these studies showed that the constituents of the water-soluble PM extracts are more

likely to induce oxidative DNA damage than the organic compounds (44, 62). A series of studies in the past decade suggest that after inhalation and deposition of PM in the lung, alveoli are able to stimulate the formation of reactive oxygen species (ROS), especially hydroxyl (HO•) and superoxide anion radicals ($O_2^{\bullet -}$). These ROS, which can be generated by transition metals and/or quinoid redox cycling, initiate a cascade of reactions and can play an important role in oxidative damage to cellular membrane lipids, proteins-enzymes, and DNA. In addition, ROS can initiate pulmonary inflammation and, through complex mechanisms, might contribute to the impairment of excision repair mechanisms of DNA and activation of oncogenes (46, 63, and 64).

1.2 Alteration of DNA methylation of the promoter is a common finding in environmental - related chronic or cancerous diseases

Previous in vitro experiments have shown that repetitive element hypomethylation and transcription occur in response to biological processes, such as cellular stress and inflammation (65), which are also induced by particulate pollution in exposed subjects (66, 67, 68). Air particles are known to increase the production of reactive oxygen species, perhaps in a catalytic fashion via redox cycling (68). Oxidative DNA damage can interfere with the ability of methyltransferases to interact with DNA (69), thus resulting in hypomethylation of cytosine residues at CpG sites. In addition, reactive oxygen species have been recently shown to alter the expression of genes belonging to DNA methylation machinery (70). Changes in DNA methylation through generation of reactive oxygen species may be induced by components of airborne particulate matter, such as transition metals (71).

Methylation status of cellular DNA is considered an epigenetic mechanism that influences gene expression (72). Altered methylation does not involve a change or miscoding of DNA base-coding sequence, but rather leads to aberrant gene expression, in part, by affecting the ability of methylated DNA-binding proteins to interact with cis elements (73). Post synthetic DNA methylation of the 5 position on cytosine [5-methylcytosine (5mC)] is a naturally occurring modification to DNA in higher eukaryotes. Under normal conditions, DNA is methylated symmetrically on both strands. Immediately following DNA replication, the newly synthesized double-stranded DNA contains hemimethylated sites that signal for DNA maintenance methylases to transfer methyl groups from S-adenosylmethionine to cytosine residues on the new DNA strand (74). If a cell is signaled to undergo DNA synthesis prior to maintenance methylation, then double-stranded DNA with hypomethylated regions will be propagated in subsequent cell division cycles, giving rise to potentially heritable genetic changes. 5mC in DNA is known to affect gene expression and alteration of cellular processes such as development and differentiation, and appears to be an important mechanism in carcinogenesis (75-77).

During the carcinogenesis process, DNA methylation may be such that both hypomethylation and hypermethylation occur (75, 76). The degree of methylation within a gene inversely correlates with the expression of that gene. Hypermethylation of genes

may inhibit transcription of tumor suppressor genes (78) and is associated with decreased gene expression or gene silencing. Important to the cancer process, tumor suppressor genes are known to be hypermethylated and subsequently inactivated (75-77). Progressive increases in methylation of CpG islands have been observed in bladder cancer and specific tumor suppressor genes have been reported to be methylated in tumors, e.g., the retinoblastoma gene, p16ink4a, and p14ARF (79-82). Inactivation of p16ink4a by hypermethylation of the promoter region appears to be an early event in lung cancer (83). Regional hypermethylation may impart molecular changes associated with genetic instability and may participate in the progression of neoplasia. Conversely, hypomethylation is considered an early and frequent event in the carcinogenesis process (84). A hypomethylated gene is considered to possess an increased potential for expression as compared to a hypermethylated gene (85). In addition, hypomethylation has been associated with increased mutation rates. Most metastatic neoplasms in humans have significantly lower 5 MeC than normal tissue (86). Oncogenes can become hypomethylated and their expression amplified (75, 83).

Dietary constituents containing choline and methionine provide the methyl groups used in methylation reactions. Exposure of rats to a choline/methioninedeficient diet results in hepatocellular proliferation and neoplasia (87, 88). The induction of cell proliferation by a methyl-deficient diet appears to function through decreased hepatic levels of S-adenosyl-methionine and, thus, promotes hypomethylation and subsequent expression of oncogenes. Prolonged administration of a diet deficient in choline or methyl donor groups resulted in hypomethylation of c-myc, c-fos, and c-H-ras protooncogenes andwas associated with the induction of hepatocarcinogenesis in rodents (88, 89). Also consistent with the role of methylation of DNA in the promotion stage of the carcinogenesis process, the induction of hepatocarcinogenesis by methyl-deficient diets was shown to be reversible by the administration of S-adenosyl-methionine (90, 91).

Among the agents and situations that can alter methylation status, reactive oxygen species can modify DNA methylation patterns. In particular, oxidative DNA damage (92) can result in decreased DNA methylation. Several chemical carcinogens modify DNA methylation, methyltransferase activity, and chromosomal structure. Of particular importance, the formation of oxidative DNA lesions has been linked to changes in DNA methylation profiles and the carcinogenesis process. Oxidative DNA damage can interfere with the ability of methyltransferases to interact with DNA, thus resulting in a

generalized hypomethylation of cytosine residues at CpG sites. The formation of OH8dG in DNA by reaction of the hydroxyl radical or singlet oxygen with DNA can lead to hypomethylation of DNA since the presence of OH8dG in CpCpGpGp sequences inhibits the methylation of adjacent C residues. Additionally, OH8dG formation can interfere with the normal function of DNA methyltransferase and alter DNA methylation status (93). Thus, oxidative DNA damage may be an important contributor to the carcinogenesis process brought about by the loss of DNA methylation, allowing the expression of normally quiescent genes. Also, the abnormal methylation pattern observed in cells transformed by chemical oxidants may contribute to an overall aberrant gene expression and promote the tumor process. Hypomethylation of repetitive DNA sequences is expected to lead to the transcriptional activation of those repetitive sequences that still contain active promoters, potentially resulting in disruption of transcription factor balance, sense or antisense transcriptional interference, and production of transcripts complementary to endogenous transcripts or to alterations in genomic organization and stability. The decrease in methylation may be part of the systemic events consequent to alveolar inflammation and the release of inflammatory mediators resulting from particle inhalation.

1.3 ROS increase expression and activity of inducible nitric oxide synthase (*iNOS*)

Inhaled particulate pollutants have been shown to produce systemic changes in gene expression due to their ability to generate chronic inflammatory processes which induce oxidative/nitrosative stress, thereby generating excess of reactive oxygen species (ROS), reactive nitrogen species (RNS) and DNA damage. Initial observations of *in vitro* and animal models have shown that air particles, or air particle components, such as toxic metals, can induce changes in DNA methylation (94, 95). Genomic DNA hypomethylation is likely to result from demethylation in transposable repetitive elements, which plays a crucial role in gene regulation and genomic stability.

Gene expression of human genes is controlled by DNA methylation, which in mammals, involves the post replication addition of methyl groups to the 5´ position of cytosine ring within the context of CpG dinucleotides to form 5-methylcytosine (5mC).

Specific studies on *iNOS* have shown that lower DNA methylation in the gene promoter is associated with increased expression (96) and activity of this enzyme in the presence of ROS (97).

iNOS is induced, at least partially, via activation of NF-κB and by cytokines. *iNOS* catalyzes the production of nitric oxide ($NO^{\bullet}$) from L-arginine and its expression was consistently reported in human cancer at a variety of sites. Various RNS can be formed from $NO^{\bullet}$. $NO^{\bullet}$ reacts with oxygen to yield the strong nitrosating agent N_2O_3, which deaminates DNA bases and reacts with secondary amines to form carcinogenic N-nitrosamines. They were detected in experimental animals and in humans with infections and inflammation (98). The reaction of $NO^{\bullet}$ with O_2 forms a highly reactive nitrating and oxidizing species, the peroxynitrite anion ($ONOO^-$). Peroxynitrite can also induce DNA single-strand breakage. In addition to DNA damage, caused by $HO^{\bullet}$ and $NO^{\bullet}$, overproduction under inflammatory conditions, the peroxynitrite-induced stress also leads to secondary LPO-derived DNA modifications.

1.4 Chronic exposure to toxic or carcinogenic environmental substances does not elicit the same results in all individuals: Individual Susceptibility of the host

Recent epidemiological and experimental studies have shown that in the occurrence of pollution related health effects, in addition to intrinsic toxicity of pollutants, a major role is played by individual susceptibility i.e. host predisposition to generate- because of host-particle interaction- pathological outcomes, which are partly independent of the triggering event. Individual susceptibility includes inherited and acquired susceptibility. The former is inherited and mainly depends on genetic polymorphisms, which globally account for the diverse immunologic and metabolic response that is typical of each individual. The latter depends on environmental stimuli and on the capability of activating immunologic or epigenetic pathways because of external factors.

The adverse health effects of air pollution are difficult to dissect since the atmosphere contains about 18,000 different substances, each of which is present at very low concentrations. Despite the well-known in vitro toxicity, mutagenicity, and carcinogenicity of many pollutants documented by experiments in animal models, it must be stressed that in most of these in vitro studies, the exposure level to each pollutant, e.g. polycyclic aromatic hydrocarbons (PAHs) and TCDD (dioxin), is higher than that occurring under actual conditions, in which PAHs are present at 10 parts per million (ppm), ozone at ppb (parts per billion), and TCDD at ppt (parts per trillion). Therefore the health damage caused by a single pollutant, even after long-term exposure, is likely to be very low.

In particular, airborne pollutants are currently considered weak pollutants. These are responsible for health effects which occur as a no-threshold phenomenon, namely that is no threshold above which all humans are affected and no threshold below which no effect is observable (99-101). In other words, even very low concentrations of airborne pollutants (PM) can be responsible for health effects in particularly susceptible individuals (99-101).

It is suggested that, from one hand, individual susceptibility plays a role greater than expected vs intrinsic toxicity of pollutants in the occurrence of pollution related diseases; on the other hand, the list of PM related diseases is going to expand, also including diseases affecting districts distant from the site of pollutant entrance. Finally, even if host factors, namely individual susceptibility, are of major importance, exposure of newborns

to high PM concentration is able to dramatically affect “acquired susceptibility” during the first months of life, determining an increased prevalence of “susceptible individuals” in the general population of future generations. This susceptibility to future damage, measurable only decades after exposure, should always be added to the burden of diseases concomitantly detected.

Carcinogenic risk from exposure to exogenous chemical carcinogens depend not only on intrinsic nature and dose of each chemical, but also may depend on inter-individual variability in sensitivity to the carcinogens.

Individual susceptibility to cancer may result from several host factors including differences in metabolism, DNA repair, altered expression of protooncogenes and tumor suppressor genes, and nutritional status. Since most carcinogens require metabolic activation before binding to DNA, individual features of carcinogen metabolism play an essential role in the development of environmental cancer. Variations in an individual's metabolic phenotype, i.e., phenotypic polymorphism, have been detected in a variety of enzymes involved in activation and detoxification of chemical carcinogens. This phenotypic metabolic variation has now been related to genetic polymorphisms. A growing number of genes encoding carcinogen-metabolizing enzymes have been identified and cloned. Consequently, there is increasing knowledge of the allelic variants or genetic defects that give rise to the observed variation. Many of the polymorphic genes of carcinogen metabolism show considerable ethnic differences in gene structure and allelic distribution (e.g., rare alleles, gene amplifications, and pseudogenes).

Enzymes coded by different variants of the same gene can differ in their catalytic activities. Up to the present time, most information on the effect of genetic polymorphism on the individual's ability to activate or deactivate environmental carcinogenic xenobiotics, and the associated risk of cancer, has been collected from studies of cytochromes P-450 belonging to gene families CYP1, CYP2 and CYP3, and of glutathione S-transferases and N-acetyltransferases.

Polymorphisms of the genes enconding for enzymes involved in the metabolism of these hazardous compounds may be related to inter-individual differences in cancer susceptibility. Thus, for each of those genes, a number of protein products, i.e. enzymes with different catalytic activity, can be found in the cells (102). This refers both to the enzymes participating in carcinogen biotransformation phase I and to those participating in phase II (103-105).The genetic principles of the polymorphisms of enzymes

participating in the metabolism of environmental carcinogens have already been quite well explained at the DNA level, and genetic population studies have revealed numerous allelic forms present in the gene pool of the human genome (106, 107).

Numerous carcinogens contain various functional groups in their chemical structures and may serve as the substrate for more than one enzyme. Those carcinogens may be metabolised at different rates and specificities. Thus, one carcinogen molecule may be transformed into several metabolites with different carcinogenic potentials. Cancer development may be associated only with some of those metabolites (108, 109). Although many other (non-metabolic) factors may also affect the manifestation of the carcinogenic activity of a chemical, the genetic variability of biotransformation process enzymes constitutes a major factor in the increased risk of cancer development (110). Most of the information collected up to now on the effect of genetic polymorphism on the individual's ability to activate and deactivate environmental carcinogenic xenobiotics, and the associated cancer risk related to cytochromes P-450, glutathione S-transferases and N-acetyltransferases.

Most xenobiotics, including dietary components, are metabolically processed by hepatic and extrahepatic xenobiotic-metabolizing enzymes in two broad steps: activation via Phase I enzymes mediated by cytochrome p450s (CYPs) and detoxification by conjugation via Phase II enzymes catalyzed by glutathione S-transferases (GSTs), N-acetyltransferases (NATs). Phase I reactions expose functional groups of the substrates and therefore yield highly reactive intermediates. These intermediates form the substrates for phase II reactions that involve their conjugation with endogenous molecules such as glutathione (GSH) and thus facilitate their elimination. Hence, the coordinated expression and regulation of phase I and II enzymes determines the outcome of carcinogen exposure. Sequence variants or polymorphisms in these genes can alter the expression, function and/or activity of these enzymes and, in turn, cancer risk (111). The CYP1A1 encodes an aromatic hydrocarbon hydroxylase enzyme that catalyzes the oxidation of PAHs to their phenolic metabolite or diol epoxide [e.g. Benzo [a] pyrene (B (a) P) to Benzo [a] pyrene-Diol-Epoxide (BPDE)] (8). A transition from T to C in the 3’ noncoding region results in the introduction of an MspI restriction site and is associated with increase in enzyme activity and hence cancer risk (112, 113).

GSTs are a superfamily of ubiquitous, multifunctional enzymes that facilitate detoxification, in particular, taking part in the conversion of many reactive electrophilic

intermediates, especially epoxides, to less reactive and more easily excreted glutathione (GSH) conjugates, thus protecting cells from oxidative stress. Four families of soluble GSTs have so far been identified in humans, referred to as α, μ, π and θ (114). To date, 4 of the GST genes, i.e., GSTM1, GSTM3, GSTP1 and GSTT1, have been found to be polymorphic.

One of the μ class genes, GSTM1, is expressed in only about 50% of Caucasians. The lack of GSTM1 activity is due to a homozygous deletion (null genotype) of the GSTM1 gene. Because GSTM1 catalyzes conjugation of GSH with epoxide metabolites of polycyclic aromatic hydrocarbons (PAHs) (114), individuals lacking the GSTM1 activity show an increased risk of cancers.

Another polymorphic GST, GSTP1, is known to metabolize many carcinogenic compounds, among them benzo (a) pyrene diolepoxide (BPDE), which is one of the most important carcinogenic metabolites derived from tobacco smoke (114). Given that GSTP1 is the most abundant GST isoform in the lungs (115), it is anticipated to be of particular importance in the detoxification of inhaled carcinogens.

A deletion polymorphism similar to that observed for GSTM1 has also been observed for the GSTT1 gene (122). The prevalence of GSTT1 null individuals shows a wide variation between ethnically different populations; in Caucasians the prevalence is 10–20% (116). Thus, a structural deletion in these genes represents a null genotype potentially leading to an increase of cancer risk.

Activity of some enzymes implicated in the metabolism of carcinogens presents a great variability between individuals due to the existence of a polymorphism in gene coding for these enzymes. Individual susceptibility to develop cancer depends not only on exposure to environmental pollution (including their intrinsic toxicity), but also on genetic capacity of the host to activate or inactivate these carcinogens.

Increased exposure to traffic-related air pollution in densely populated metropolitan areas and to a wide variety of genotoxic xenobiotics introduced either by diet or by inhalation, together with spontaneous mutations related to aging are likely responsible not only for the observed incidence of chronic inflammatory diseases but also of malignant tumors.

The phenotypic manifestations of the same germline mutation of a tumor suppressor gene are highly variable, even when patients belong to the same kindred. This is mainly due to superimposed epigenetic factors, which could be sex-based or environmentally related (117). Likewise, health damage from occupational exposure to known carcinogens such

as PAHs or even asbestos greatly varies among individuals with the same exposure level and/or belonging to the same family because of individual susceptibility (118-125). This includes not only inherited predisposition due to ethnic or individual differences in genetic polymorphisms for the genes encoding enzymes involved in xenobiotic metabolism, but also in acquired predisposition, related to the effects of aging, concomitant chronic or metabolic disease, such as infections, immunodepression or diabetes, and variable exposure to environmental agents, beginning from fetal development and/or the first weeks of life.

1.5 Malignant Mesothelioma

Malignant Mesothelioma (MM) is an aggressive and highly lethal tumor originating from the epithelium of serosal cavities, that has been strictly related the asbestos exposure. The incidence of this neoplasm, in Western countries is dramatically increasing – reflecting past exposure to asbestos – and the trend is not expected to level off until the second decade of the century (126). The aggressiveness of this tumor, and its ability to put up with all current therapies, is responsible for the limited efficacy of treatments and the poor survival. Although many alternative exposures have been suggested (127-130), including biological agents such as the simian virus 40 (SV40) (131,132), asbestos is universally considered as the principal etiological factor of MM, and most estimates attribute to this exposure a proportion ranging around 80% of all observed cases (133). Another mineral fiber, erionite, has been indicated as the cause of a MM epidemic in a cluster of isolated Turkish villages (134, 135). The most circumstantiated mechanism explaining carcinogenicity of asbestos fibers is linked to pleural inflammation. In fact, asbestos fibers could directly penetrate the lung, engrave the mesothelial surface and cause prolonged cycles of damage, repair, and local inflammation either directly (136) or indirectly, generating iron-related oxygen free radicals (137) that could induce DNA damage and strand breaks (138). The effect of the oxidative stress, caused by free radicals and reactive oxygen species (ROS), is contrasted by molecules which have an antioxidant action, such as the glutathione (GSH) (138). The redox system of GSH consists of primary and secondary antioxidants, such as the glutathione S-transferase family, which includes several isozymes, many of them (e.g., GSTM1, GSTP1, and GSTT1) are polymorphic in humans resulting in individual differences in cancer risk. Mesothelioma is a rare tumor originating from mesothelium. Therefore, it has been reported in all sites covered by a mesothelial layer: pleura, peritoneum, pericardium and tonaca vaginalis testis. In particular, pleural mesothelioma is an aggressive tumor, with invariably dismal prognosis and a median survival of 10-12 months after clinical diagnosis. In particular, exposure to crocidolite, an asbestos variant characterized by long and thin fibers (length to width ratio > 3:1) is associated with an increased incidence of pleural mesothelioma. Erionite, another fibrous substance similar to asbestos, has been considered responsible for a striking prevalence of pleural and peritoneal mesothelioma in Cappadocian villages

of Tuzkoy and Kazan, with up to 40% of people living in the village affected by mesothelioma.

Exposure to asbestos fibers is a major risk factor for malignant mesothelioma, lung cancer and other non-neoplastic conditions, such as asbestosis and pleural plaques. However, in the last decade many studies have shown that polymorphism in the genes involved in xenobiotic and oxidative metabolism or in DNA repair processes may play an important role in the etiology and pathogenesis of these diseases (139).

In the last decade, the role of genetic polymorphisms in the pathogenesis of mesothelioma and other diseases has been the object of intensive research. Many studies have focused on polymorphic metabolic genes that encode enzymes involved in conjugation and detoxification of environmental toxicants (140).

Previous studies have shown that both malignant and non malignant asbestos-related diseases develop more frequently in asbestos-exposed subjects carrying homozygous deletion (null genotype) of GSTM1 gene (139, 141, 142). In highly exposed Finnish and Italian workers, the GSTM1 deletion in combination with the NAT2 slow acetylator genotype was associated with a remarkably increased risk of mesothelioma (140-143). Therefore, it was hypothesized that, in addition to exposure to the pathogenic xenobiotic (erionite or asbestos), inherited factors, responsible for different individual susceptibility, should also be considered. Actually, epidemiologic studies in selected cohorts of subjects with long term occupational exposure to asbestos have shown that mesothelioma occurs in less than 5 % of exposed individuals. In particular, long-term follow-up of subjects working for 30 years or more in ship-building (where asbestos has been used as sealing and insulating material), has shown that only 2-3% of subjects belonging to these subgroups with occupational exposure actually developed mesothelioma. Mesothelioma occurs long time after initial asbestos exposure and then a later mesothelioma occurrence should be expected in some individuals belonging to these populations. However, there is enough evidence that up to 95% of severely exposed people never develop mesothelioma. This observation could underline a common exposure to the same xenobiotic, with peculiar intrinsic toxicity, but could also points to common, genetically determined predisposing factors.

2. MATERIAL AND METHODS

2.1 Patients and demographics

The present study reports on a consecutive series of patients with mesothelioma (MM), who entered from January 2000 to December 2007 the same Department of Pathology for final histological diagnosis.

Most of these patients also underwent radical or palliative removal of primary tumors in the same University Hospital (n=30). Some of them, had only biopsies (n=45) and various types of palliative treatment (Chemo or Radioterapy).

There were 75 subjects (51 males and 22 female) with a mean age of 65 years. Fourteen patients had uncertain diagnosis or were lacking of basic information and were discharged.

Sixty-one patients had complete informations.

In particular, there were 49 pleural mesotheliomas, (20 with radical surgery), 7 peritoneal MM (4 malignant, 3 cystic or benigns cell with remove; 1 cystic mesothelioma of the round ligament, 3 mesothelioma of the pericardium and 1 mesothelioma of the tunica vaginalis testis. Therefore, 48% had surgical remove of evident lesions. For all patients, personal history was recorded, with particular reference to asbestos exposure, either concerning the affected subject or other family members. In particular, there were two family cluster cases. In one case, there were two brothers, who had both occupational exposure to asbestos, and who were affected by pleural mesothelioma, at age of 50 and 56, respectively. In another there was the husband, who suffered from asbestos (more than 30 years) whereas the unexposed wife developed pleural mesothelioma at age 72 and another case where the husband occupationally exposed to asbestos had tumor at age 63.

2.2 Molecular analysis

2.2.1 DNA extraction

Genomic DNA was extracted from peripheral blood using a QIAamp DNA Blood Maxi kit according to the manufacturer protocol (Qiagen, www.qiagen.com). The OD260/280 method on a photometer was employed to determine the appropriate DNA concentration.

DNA samples were sonicated to produce a homogeneous smear DNA extending from approximately 600 bp to 2 kb. DNA samples were then purified using the DNA Clean and Concentrator kit (Zymo Research, Orange, CA). Ten micrograms of genomic DNA was used for array CGH analysis. In a first experiment the DNA of an indirectly exposed affected female was used as DNA test and the DNA of her exposed healthy husband was used as control (see Clinical case 1 in the result). In a second experiment, the DNA of an exposed affected male was used as DNA test and the DNA of his healthy wife used as control (see clinical case 2 in the result)

2.2.2 Array CGH analysis

Array based CGH analysis was performed using commercially available oligonucleotide microarrays containing 99,000 60-mer probes with an estimate average resolution of 50-65 kb (Human Genome CGH Microarray 105A Kit, Agilent Technologies). Physical positions of the probes correspond to the UCSC genome browser - NCBI build 36, March 2006. (http://genome.ucsc.edu). DNA labelling was executed essentially according to the Agilent protocol (Oligonucleotide Array-Based CGH for Genomic DNA Analysis 2.0v) using the Bioprime DNA labelling system (Invitrogen). Genomic DNA (2 µg) was mixed with 20 µl of 2.5X Random primer solution (Invitrogen) and MilliQ water to a total volume of 41 µl. The mix was denaturated at 95° C for 7 minutes and then incubated in ice/water for 5 minutes. Each sample was added with 5 µl of 10X dUTP nucleotide mix (1.2 mM dATP, dGTP, dCTP, 0.6 mM dTTP in 10 mM Tris pH 8 and 1 mM EDTA), 2.5 µl of Cy5-dUTP (test sample) or 2.5 µl of Cy3-dUTP (reference sample) and with 1.5 µl of Exo-Klenow (40 U/µl, Invitrogen). Labeled samples were subsequently purified using CyScribe GFX Purification kit (Amersham Biosciences) according to the manufacturer protocol. Test and reference DNA were pooled and mixed with 50 µg of Human Cot I DNA (Invitrogen), 50 µl of Blocking buffer (Agilent Technologies) and 250 µl of Hybridization buffer (Agilent Technologies). Before hybridization to the array the mix was denatured at 95° C for 7 minutes and then pre-associated at 37°C for 30 minutes. Probes were applied to the slide using an Agilent microarray hybridization station. Hybridization was carried out for 40 hrs at 65° in a rotating oven (20 rpm). The array was disassembled and washed according to the manufacturer protocol with wash buffers

supplied with the Agilent 44B kit. The slides were dried and scanned using an Agilent G2565BA DNA microarray scanner. Image analysis was performed using the CGH Analytics software v. 3.4.40 with default settings. The software automatically determines the fluorescence intensities of the spots for both fluorochromes performing 35 background subtraction and data normalization, and compiles the data into a spreadsheet that links the fluorescent signal of every oligo on the array to the oligo name, its position on the array and its position in the genome. The linear order of the oligos is reconstituted in the ratio plots consistent with an ideogram. The ratio plot is arbitrarily assigned such that gains and losses in DNA copy number at a particular locus are observed as a deviation of the ratio plot from a modal value of 1.0.

3. RESULTS

3.1 Searching for the susceptibility genes of mesothelioma

The following cases were selected for being of particular interest:

1) A couple, husband and wife: the husband was occupationally exposed to asbestos for 30 years, but didn't have either mesothelioma or asbestosis; the wife, who was not occupationally exposed (only indirectly exposed through her husband clothes), developed pleural mesothelioma at age 72. (Clinical Case 1)

2) Two brothers, both living and working in the same environment: the former developed mesothelioma of the tunica vaginalis testis; the latter, even if living in the same house and doing the same work, didn't develop mesothelioma.

3) Three sibligs, all male brothers, all doing the same work: two of them developed pleural mesothelioma (the former died after 12 months and the latter after 4 years). The third brother didn't develop mesothelioma.

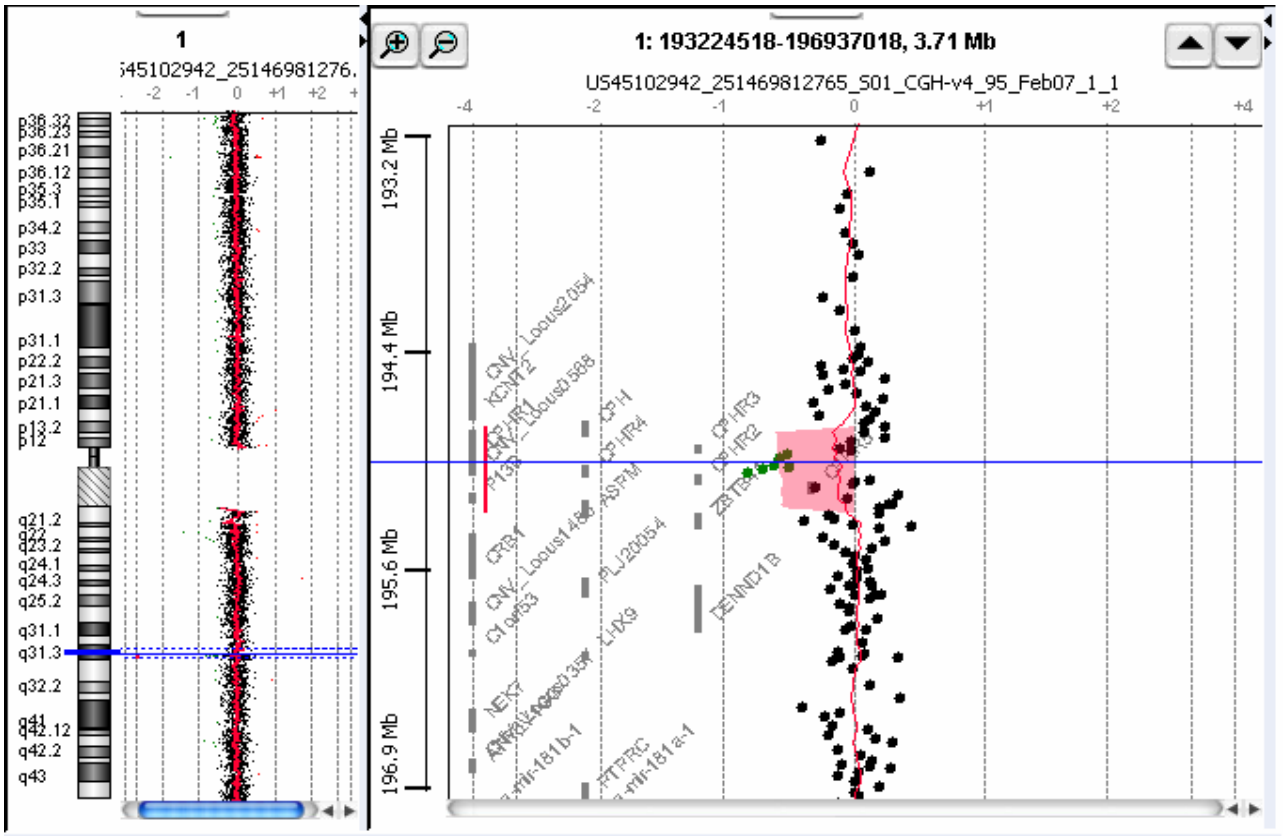
4) A couple, husband and wife: the husband was occupationally exposed to asbestos and developed tunica vaginalis testis mesothelioma; the wife was healthy. (Clinical Case 2)

Biological samples (DNA extracted from blood) were available only from the first and the last couple (Clinical Case 1 and Clinical Case 2).
By using array –CGH (comparative Genomic Hybridization) 105K (Agilent) 9 different genetic polymorphisms were identified in the affected wife that were not present in the healthy exposed husband. Seven polymorphisms involved genes while the remaining 2 do not contain genes (Table 2, Figure 2 and 3).
By using the same method 4 different genetic polymorphisms were identified in the husband of the second couple (Table 3).
All rearrangements reported above, are already presented, as polymorphisms, in known Databases.

Table 2: Results of Clinical Case 1: Nine different genetic polymorphisms present in the affected indirectly exposed wife

	MUTATION	POSITION	GENE	PROTEIN	FUNCTION
1	**Del 1q31.3**	193513-193614 kB	CFHR1 CFHR4	Complement Factor H-related 1/4	Immunological response and lipid metabolism
2	**Del 3q26.1**	163997-164101 kB			
3	**Dup 4q13.2**	69203-69311 kB	UGT 2B17	UDP-glucuronosyl-transferase (UGTs)	Enzymes that catalyze the transfer of glucuronic acid to a variety of substrates, including steroid hormones. Implicate in the conjugation and subsequent elimination of potentially toxic xenobiotics.
4	**Dup 4p15.33**	763-873 kB	ZTH HC11	Zinc-finger DHHC domain 11	
5	**Del 6p21.32**	32595-32660 kB	HLA-DRB5 HLA-DRB1	Major Histocompatibility complex class II DR	Immune system regulators play a central role in the immune system by presenting peptides derived from extracellular proteins and are expressed in the B-lymphocytes, dentritic cells, macrophages.
6	**Dup 8p11.22**	39341-39449 kB	BCO 67864	ADAM5 protein	These proteins are membrane-anchored glycoproteins, named from two of structural domains they contain: a disintegrin domain, and a metalloprotease domain. These two domains possess both proteolytic and adhesive functions as endopeptidases and/or adhesion proteins.
7	**Del 10q21.1**	56779-57249 kB			
8	**Del 15q12**	19869-19988 kB	OR4M2 OR4N4	Olfactory receptor 4M2 protein	They codify for olfactory receptors, which interact with odorant molecules in the nose, to initiate a neuronal responses, that triggers the perception of smell. They are members of a large family of G-protein coupled receptors (GPCL), that is the largest in the genoma;
9	**Del 15q14**	32482-32751 KB	CR749361	Golgi autoantigen golgin-67	Probably involved in Golgi apparatus maintenance.

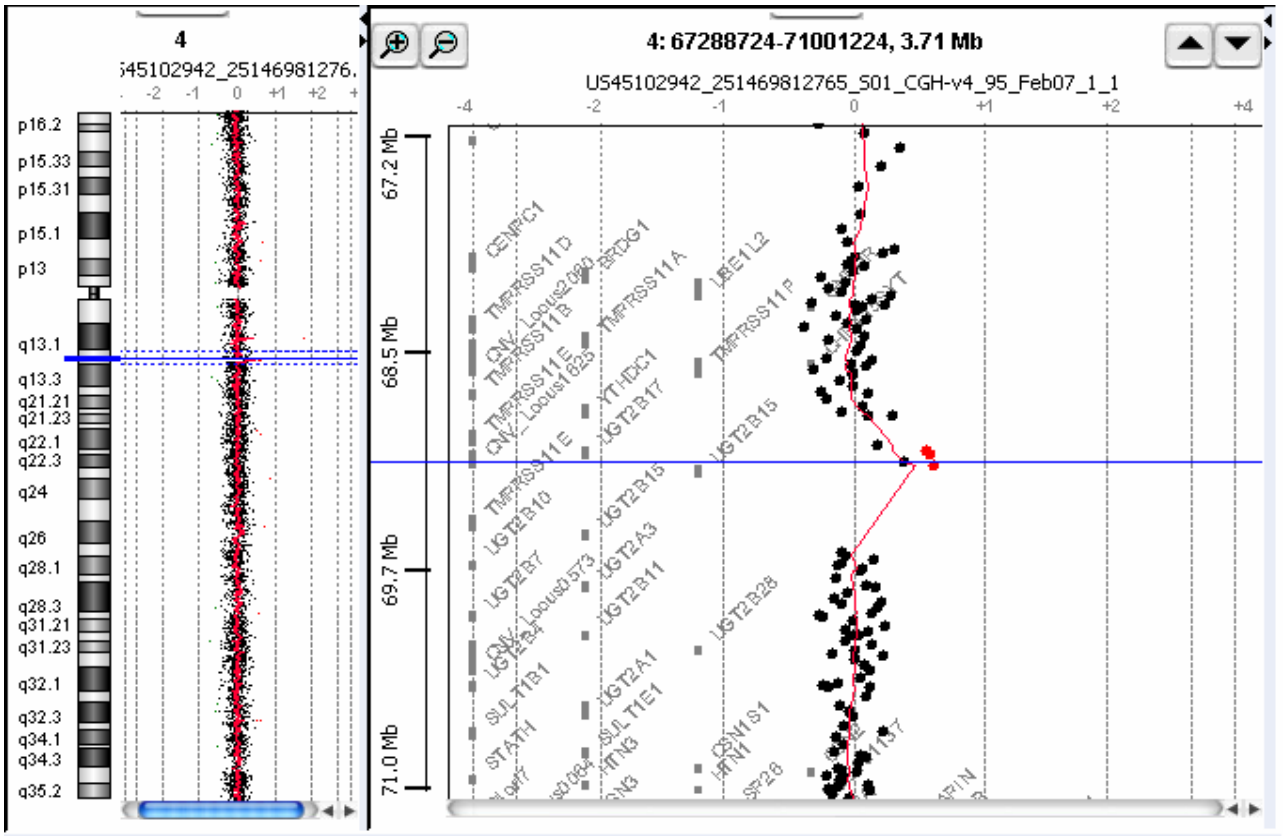
Figure 2: Clinical Case 1- Array-CGH 105K



Del 1q31.3 (193513-193614 kB)

Genes involved: CFHR1 e CFHR4 (complement factor H-related1 e 4)

Figure 3: Clinical Case 1- Array-CGH 105K



Dup 4q13.2 (69203-69311 kB)

Genes involved: UGT 2B17 (UDP-glucuronosyl-transferase (UGTs))

Table 3: Results of Clinical Case 2: Four different genetic polymorphisms present in the affected exposed husband

	MUTATION	POSITION	GENE	PROTEIN	FUNCTION
1	**Dup 1q31.3**	195011344-195065867 kB	CFHR1 CFHR3	Complement Factor H-related 1/3	Immunological response and lipid metabolism
2	**Dup 7q11.23**	73301068-73662251 kB	RFC2	Replication factor C subunit 2	RFC is an accessory protein implicates in the elongation of primed DNA templates by DNA polymerase. It, in the presence of ATP, assembles proliferating-cell nuclear antigen and DNA polymerase-delta (174761) or polymerase-epsilon (174762) on primed DNA templates.
			CLIP2	CAP-GLY domain containing linker protein 2	The protein belongs to the family of cytoplasmic linker proteins, which have been proposed to mediate the interaction between specific membranous organelles and microtubules.
			GTF2IRD1	GTF2I repeat domain containing 1	The protein contains five GTF2I-like repeats and each repeat possesses a potential helix-loop-helix (HLH) motif. It may have the ability to interact with other HLH-proteins and function as a transcription factor or as a positive transcriptional regulator under the control of Retinoblastoma protein. May be a transcription regulator involved in cell-cycle progression and skeletal muscle differentiation.
3	**Dup 16p11.2**	31958973-33875313 kB	HERC2P4	Hect domain and RLD 2 pseudogene 4	
			TP53TG3		May play a significant role in p53/TP53-mediating signaling pathway
			SLC6A10P		Solute carrier family 6 (neurotransmitter transporter, creatine), member 10 (pseudogene)
4	**Dup 22q11.23**	22676549-22706765 KB	GSTTP1	glutathione S-transferase theta pseudogene 1	Conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles.
			GSTT1	Glutathione S-transferase (GST) theta 1 (GSTT1)	Member of a superfamily of proteins that catalyze the conjugation of reduced glutathione to a variety of electrophilic and hydrophobic compounds. The theta class includes GSTT1 and GSTT2. The GSTT1 and GSTT2 have an important role in human carcinogenesis

3.3 Role of the gender in the onset of the diseases and its biology

The immune system is a complex and highly developed system, yet its mission is simple: to seek and kill invaders. If a person is born with a severely defective immune system, death from infection by a virus, bacterium, fungus or parasite will occur.
In severe combined immuno deficiency, lack of an enzyme means that toxic waste builds up inside immune system cells, killing them and thus devastating the immune system.
Most of the immune disorders result from either an excessive immune response or an "autoimmune attack". Asthma, familial Mediterranean fever and Crohn's disease (inflammatory bowel disease) all result from an over-reaction of the immune system, while autoimmune polyglandular syndrome and some facets of diabetes are due to the immune system attacking "self cells" and molecules.
A key part of the immune system's role is to differentiate between invaders and the body's own cells - when it fails to make this distinction, a reaction against "self cells" and molecules causes autoimmune disease. It is notable that women have higher absolute numbers of CD4+ lymphocytes relative to men (143), which likely contributes to their increased responses. Direct comparisons of cytokine production under conditions of immunization have shown higher production of Th1cytokines in females (143). Cytokine secretion is generally enhanced in vitro in the presence of estrogen—observed most prominently with interferon-γ (IFN-γ), interleukin 1 (IL-1) and IL-10—and decreased in the presence of androgens (IFN-γ, IL-4 and IL-5).The increased prevalence of autoimmune disease in women, the sexual dimorphism of the immune response and the modulatory effects of sex steroids on immune function in vitro have focused attention on the role of these hormones—mainly estrogen, progesterone and testosterone—as primary mediators of the sex differences. Sex hormones appear to influence the immune system, with estrogens as enhancers at least of the humoral immunity and androgens and progesterone (and glucocorticoids) as natural immune suppressors.
This results in a sexual dimorphism in the immune response in humans. In females both the humoral and cell mediated immune response is more active than in males. A large amount of information supports the view that hormones of the endocrine system are intimately involved in this immunological dimorphism.

Such hormones include the gonadal steroids, the adrenal glucocorticoids, growth hormone (GH) and prolactin (Prl) from the pituitary, thymic hormones, and substances generated by activated lymphocytes. The most important of these hormonal interactions leading to immunological dimorphism are the effects elicited by estrogen (E) elaborated at elevated levels from the female ovary after puberty.

Elevated E leads to basal GH secretion, increased Prl, and increased thymosin release, all of which are hypothesized to effect lymphocyte development and stimulate adult T- and B-cell function in females. Interactions of hormonal regulatory axes involving the hypothalamus, pituitary, gonads, adrenals, and thymus are also thought to be involved. Factors elaborated by activated immune cells including IL-1 and IL-2 may also play a role in down regulation of these responses.

Finally, genetic components are also considered pertinent especially under conditions of pathological disequilibrium leading to autoimmune disease. Since sex hormones are intimately involved in immunological regulation it is quite possible that the increased immune response in females allows them to compensate for the increased physiological stress which accompanies reproduction.

Several physiological, pathological, and therapeutic conditions may change the serum estrogen milieu and/or peripheral conversion rate, including the menstrual cycle, pregnancy, postpartum period, menopause, being elderly, chronic stress, altered circadian rhythms, inflammatory cytokines, and use of corticosteroids, oral contraceptives, and steroid hormonal replacements, inducing altered androgen/estrogen ratios and related effects.

In particular, cortisol and melatonin circadian rhythms are altered, at least in rheumatoid arthritis (RA), and partially involve sex hormone circadian synthesis and levels as well. Abnormal regulation of aromatase activity (i.e., increased activity) by inflammatory cytokine production (i.e., TNFα, IL-1, and IL-6) may partially explain the abnormalities of peripheral estrogen synthesis in RA (i.e., increased availability of 17-beta estradiol and possible metabolites in synovial fluids) and in systemic lupus erythematosus, as well as the altered serum sex-hormone levels and ratio (i.e., decreased androgens). In the synovial fluids of RA patients, the increased estrogen concentration is observed in both sexes and is more specifically characterized by the hydroxylated forms, in particular 16alpha-hydroxyestrone, which is a mitogenic and cell proliferative endogenous hormone. Local effects of sex hormones in autoimmune rheumatic diseases seem to consist mainly in

modulation of cell proliferation and cytokine production (i.e., TNFα, IL-1, IL-12). In this respect, it is interesting that male patients with RA seem to profit more from anti-TNFα strategies than do female patients. Altered serum hydroxylated estrogens have been found also in serum of systemic lupus erythematosus (SLE) patients. Recent studies indicate that 17-b estradiol (E_2) clearly enhanced the expression of markers of cell growth and proliferation, whereas testosterone (T) induced an increase of markers indicating DNA damage and apoptosis. In particular, the enhancing role of estrogens on immune/inflammatory response is exerted by activating the NF-κB complex pathway. Locally increased estrogens (i.e., synovial tissue in RA or skin in SLE) might exert activating effects on cell proliferation, including macrophages and fibroblasts, suggesting new roles for estrogens in autoimmunity.

Perhaps the most striking evidence comes from pregnancy, in which estrogen and progesterone increase greatly during the third trimester. In both MS and RA, disease activity decreases throughout pregnancy, but most profoundly during the third trimester, when estrogen and progesterone concentrations are highest (144, 145).

This is often followed by a flare of disease activity during the post-partum period, when estrogen and progesterone concentrations fall. These observations are in contrast to SLE, which appears to either worsen or remain unchanged during pregnancy (146-148). This fluctuation of disease activity during and after pregnancy has been explained by a hormonal environment during pregnancy that favors a Th2 response. In MS and RA, this environment may suppress the ongoing Th1 responses to central nervous system and joint antigens, whereas in SLE, a Th2 environment would enhance antibody production and possibly exacerbate disease progression. Interestingly, men with RA have significantly lowered testosterone concentrations (149).

The modulatory effects of estrogen seem to be quite different between normal immune responses measured in vitro and autoimmune responses observed in vivo, with enhancement of the former and apparent suppression of autoimmunity. This dilemma was partly resolved with the realization that estrogen shows biphasic dose effects: lower doses facilitate immune responses and higher doses, as occur in pregnancy, suppress such responses (150).Sex steroids may act directly on the immune system, modulating aspects of antigen presentation, lymphocyte activation, cytokine gene expression and/or homing of immune cells.

The identification of estrogen and androgen receptors on immune cells provided a means for direct communication. Sex steroids also have indirect effects that must be considered. Sex hormones modulate the hypothalamic-pituitary adrenal (HPA) axis and, thus, modulate the stress response. Females of many species, including humans, have higher corticosterone-cortisolconcentrations than males do (151, 152); in addition, glucocorticoids suppress the production of sex hormones and the action of these hormones in tissues.

The sharp spike of corticotropin-releasing hormone (CRH) and cortisol at parturition undoubtedly participates in the decline of estrogen postpartum.

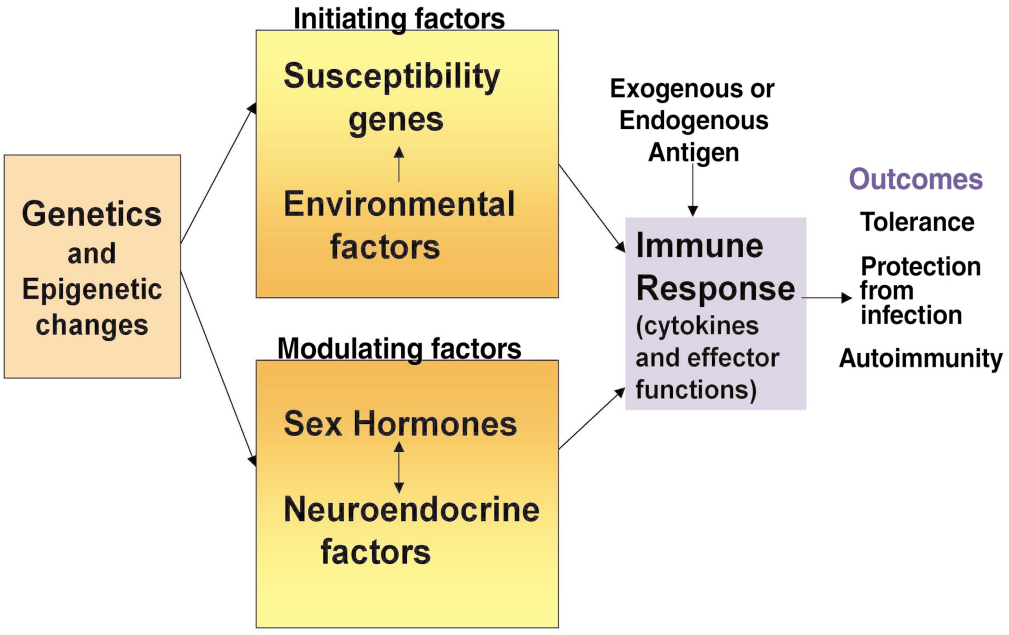
The discovery of an estrogen-response element in the promoter region of the gene encoding CRH indicated that these two hormone systems are inter-regulated.

Genetics plays a key role in defining autoimmune disease susceptibility and determining the expression of sex hormones and neuro endocrine factors. In an individual with a susceptible genotype, exposure to environmental factors (such as sunlight, diet, allergens, infectious agents or environmental toxins) can act to initiate an autoimmune process.

All these factors together affect the immune response to self and foreign antigens through modulation of cytokine production and effector cell function. The nature of the antigen and the character of the immune response—that is, Th1 orTh2—dictate the outcome of the immune response.

Therefore, the interactions between the sex hormones, HPA axis and immune system are complex: all these factors must be considered when studying the sex differences in autoimmunity (Fig. 4).

Figure 4: A model for the multifactorial nature of autoimmune disease.



Sex hormones represent an important modulatory factor in the immune and autoimmune response. Sex hormones include the gonadal sex steroids as well as other hormones that indicate differences between men and women.

Autoimmunity designates the presence of lymphocytes and/or antibodies that are reactive with self-antigens. Autoimmune disease occurs when such autoimmune responses cause tissue damage or dysfunction (153) and are conventionally divided into systemic autoimmune diseases, classically SLE, where the target antigen is widely distributed, as for all cell nuclei, and organ specific autoimmune diseases where the target antigen is confined to a specific tissue or organ. However, many autoimmune diseases, exemplified by scleroderma, Sjögren's syndrome and primary biliary cirrhosis (PBC), have elements of both systemic and organ-specific disease, with some bearing a strong resemblance to graft-versus-host disease. Most organ-specific autoimmune diseases have a strong inflammatory component, notably rheumatoid arthritis (RA) or multiple sclerosis (MS). Some, like myasthenia gravis (MG), are caused by antibody-mediated dysfunction and are noninflammatory. The nervous system is a very rich field for different types of autoimmune disorder including antibody- mediated autoimmune diseases of the central

and peripheral nervous system that arise as part of the immune response to malignancy (the paraneoplastic syndromes) (154). Paraneoplastic autoimmune diseases can also affect other tissues such as the eye (155) and the muscles (156). Some of the more common autoimmune diseases are listed in Table 4.

Table 4: Sex differences in Human Autoimmune Diseases

Disease	Female : Male Ratio	References
Systemic		
SLE	13.1 : 1 (African American), 8.7 : 1 (white)	156
Sjögren's syndrome	4 : 1 (Israel) ; 8.7 : 1 (Denmark)	157,158
Endocrine		
Type 1 diabetes	0.5 : 1 (Sweden) ; 0.8 : 1 (Denmark)	157,159
Grave's disease	3.5 : 1	160
Hashimoto's thyroiditis	5.2 : 1	160
Gastro-intestinal and liver		
Ulcerative colitis	1 : 1	161
Crohn's disease	1.3 : 1	161
Primary biliary cirrhosis	9 : 1	162
Coeliac disease	1.8 : 1	163
Rheumatological		
Ankylosing spondylitis	1 : 3	164
Rheumatoid arthritis	2.7 : 1	165
Psoriatic arthritis	1 : 1	166
Neurological		
Multiple sclerosis	1.9 : 1 (Canada); 2.4 : 1 (Japan); 4.3 : 1 (Wales)	167,168,169
Myasthenia gravis	2 : 1	170
Guillain Barré	0.9 : 1	171
CIDP	0.6 : 1	172
Skin		
Psoriasis	0.8 : 1 (Italy) ; 1.1 : 1	157,173
Scleroderma	4 : 1	157,174

The underlying cause of autoimmune disease is a complex combination of elements that cause disruption of natural immune tolerance, including genetic, environmental and immunologic influences and, to an unknown degree, stochastic components. Current thinking emphasizes a loss of tolerance such as that provided either by anomalies of deletion of self-reactive lymphocytes in primary lymphoid organs, or faulty development of regulatory T cells. At least one-third of the risk of developing an autoimmune disease

is attributed to heritable factors and the remainder is thought to be associated with non-inherited events. In some families affected by autoimmune diabetes there is what is described as “extreme genetic risk” (175) of transmission of disease. Among families, members can express different autoimmune diseases and, rarely, the development of autoimmune disease is inherited in an autosomal dominant fashion, (176, 177). In the case of MS, however, there has been a recent contrary view that there is no association (clustering) with other autoimmune disorders (178). The most potent genetic contributor to the development of autoimmune disease is gender and after this the major histocompatibility complex (MHC) comprising multiple alleles at two particular loci (MHC class I and MHC class II) that encode molecules important for immunological function. Indeed a link to MHC has become one of the defining criteria for a diagnosis of autoimmune disease (179). However, allelic variations in many other genes as well have been linked to the occurrence of autoimmune diseases (180, 181). In isolation these genes have relatively small genetic effects when compared to susceptibility variants located in the MHC region and hence the inheritance of many of such “autoimmunity genes” is seen as necessary for the development of autoimmune disease. Genes recently identified as predisposing to autoimmunity overall include several cytokine receptors. For example, autoimmune Type I diabetes (T1D) (182), autoimmune thyroid disease (183) and MS (184, 185) are associated with polymorphisms in IL2 receptor (R) alpha chains, and polymorphisms in IL7R alpha chain are linked to MS (186-188). Variation in the IL23R has been linked to susceptibility to ankylosing spondylitis (189). It is interesting to note that these

polymorphisms predisposing to disease result in variation in the amount of circulating soluble receptor, which suggests that the predisposition to autoimmunity may be related to the blocking effects of soluble receptor.

Environmental factors participating in susceptibility to the development of autoimmune disease include exposure to certain chemicals, xenobiotics or toxins (e.g. drug-induced lupus, toxic oil syndrome, polyvinyl chloride-associated scleroderma, and the eosinophilia-myalgia syndrome), or stress (190). Levels of non-concordance in monozygotic twins, the observed increases in the incidence of several autoimmune diseases including Type I diabetes and MS, at a rate too rapid to be due to changes in genetic risk, and a role for season of birth in determining risk of disease, provide strong evidence for the predisposing role of environmental factors in autoimmunity. These

environmental factors include exposure to infectious agents and to dietary constituents (191, 192). In some acutely developing autoimmune diseases, the influence of the preceding infection is very clear, as in human Guillain Barre Syndrome (GBS) of peripheral neuropathy (193). In other cases, the presumed environmental provocation could have acted so remotely in time as to be unascertainable. After infections, autoimmunity might be initiated by the process of molecular (epitope) mimicry (194, 195) entailing activation of the innate immune system (196). There is also the possibility of molecular mimicry involving the adaptive immune system. However, although mechanistically molecular mimicry is an appealing idea to explain the association of autoimmunity with infections, another school of thought is that antigen spillage after any form of cellular injury, together with inherited liability of natural self-tolerance, is of more importance. A middle view is that infections cause more immune stimulation than simple tissue damage, because molecules found in infectious agents are potent activators of the innate immune system, and so are more likely to trigger an immune response.

3.4 Role of inflammation and immune response/autoimmunity in the onset of the diseases and their biology

The main function of the mammalian immune system is to monitor tissue homeostasis, to protect against invading or infectious pathogens and to eliminate damaged cells. Therefore, it is surprising that cancer occurs with such a high frequency in humans. Some molecular mechanisms underlie harmful, excessive stimulation of immune-cell responses. Genetic predisposition underlies some disorders, such as pancreatitis, ulcerative colitis and some rheumatoid diseases. Others are associated with infectious pathogens that are able to evade natural tissue immune clearance mechanisms (197). For example, Helicobacter pylori, a gram-negative bacterium, causes chronic gastritis in infected hosts, whereas infection with hepatitis B or hepatitis C virus (HBV and HCV, respectively) is linked to chronic hepatitis (197, 198). Unresolved inflammation also results from exposure to toxic factors such as PM, dietary, asbestos or smoke, as well as from ongoing chemical or physical irritation, such as acid-reflux disease or exposure to ultraviolet (UV) light. Mutations and/or genetic polymorphisms in crucial genes that regulate cytokine function, metabolism and leukocyte survival have also been implicated as aetiological factors in chronic inflammation (199). During acute inflammation, innate immune cells form the first line of immune defence and regulate activation of adaptive immune responses. By contrast, during chronic inflammation, these roles can be reversed — adaptive immune responses can cause ongoing and excessive activation of innate immune cells (200). In arthritis, for example, activation of T and B lymphocytes results in antibody deposition into affected joints, prompting recruitment of innate immune cells into tissue (201). Once within the tissue, activation and/or degranulation of mast cells, granulocytes and macrophages, in combination with humoral immune responses, leads to joint destruction (201). By contrast, whereas acutely activated innate immune cells contribute to efficient T-cell activation, chronically activated innate immune cells can cause T-cell dysfunction through the production of reactive oxygen (201). Regardless of the underlying initiating cause, if an infectious or assaulting agent is inadequately cleared and persists in tissue, or a tissue is subjected to ongoing insult and damage that fails to heal in a timely manner, host inflammatory responses can persist and exacerbate chronic tissue damage, which can cause primary organ dysfunction and systemic complications.

Cancer is an insidious disease that originates from mutant DNA sequences that reroute crucial pathways regulating tissue homeostasis, cell survival and/or cell death. Cancers are composed of multiple cell types, such as fibroblasts and epithelial cells, innate and adaptive immune cells, and cells that form blood and lymphatic vasculature, as well as specialized mesenchymal cell types that are unique to each tissue microenvironment. Whereas tissue homeostasis is maintained by collaborative interactions between these diverse cell types, cancer development is enhanced when mutant cells harness these collaborative capabilities to favour their own survival.

Cancer development largely depends on the ability of mutant cells to hijack and exploit the normal physiological processes of the host. Each stage of cancer development is exquisitely susceptible to regulation by immune cells (Table 5).

Table 5: Mechanisms by which immune cells regulate cancer development

Mechanisms by which innate immune cells contribute to cancer
Direct mechanisms • Induction of DNA damage by the generation of free radicals • Paracrine regulation of intracellular pathways (through nuclear factor κB)
Indirect mechanisms • Promotion of angiogenesis and tissue remodelling by the production of growth factors, cytokines, chemokines and matrix metalloproteinases • cyclooxygenase-2 upregulation • Suppression of antitumour adaptive immune responses
Mechanisms by which adaptive immune cells modulate cancer
Direct mechanisms • Inhibition of tumour growth by antitumour cytotoxic-T-cell activity • Inhibition of tumour growth by cytokine-mediated lysis of tumour cells
Indirect mechanisms • Promotion of tumour growth by regulatory T cells that suppress antitumour T-cell responses • Promotion of tumour development by humoral immune responses that increase chronic inflammation in the tumour microenvironment

Whereas full activation of adaptive immune cells in response to the tumour might result in eradication of malignant cells, chronic activation of various types of innate immune cells in or around pre-malignant tissues might actually promote tumour development. The mammalian immune system is composed of many cell types and mediators that interact with non-immune cells and each other in complex and dynamic networks to

ensure protection against foreign pathogens, while simultaneously maintaining tolerance towards self-antigens.

Based on antigen specificity and timing of activation, the immune system is composed of two distinct compartments — adaptive and innate. Whereas the cellular composition and antigen specificity of these are distinct, they have each evolved sophisticated communication networks that enable rapid responses to tissue injury. Innate immune cells, such as dendritic cells (DCs), natural killer (NK) cells, macrophages, neutrophils, basophils, eosinophils and mast cells, are the first line of defence against foreign pathogens. DCs, macrophages and mast cells serve as sentinel cells that are pre-stationed in tissues and continuously monitor their microenvironment for signs of distress. When tissue homeostasis is perturbed, by the activating of the inflammation system, sentinel macrophages and mast cells immediately release soluble mediators, such as cytokines, chemokines, matrix remodelling proteases and reactive oxygen species (ROS), and bioactive mediators such as histamine, that induce mobilization and infiltration of additional leukocytes into damaged tissue. Macrophages and mast cells can also activate vascular and fibroblast responses in order to orchestrate the elimination of invading organisms and initiate local tissue repair. DCs, on the other hand, take up foreign antigens and migrate to lymphoid organs where they present their antigens to adaptive immune cells. They are, therefore, key players in the interface between innate and adaptive immunity. NK cells also participate in cellular crosstalk between innate and adaptive immune cells through their ability to interact bidirectionally with DCs; certain NK-cell subsets eliminate immature DCs, whereas others promote DC maturation, which can then also reciprocally regulate activation of NK cells1–3. The unique characteristic of innate immune cells — their inherent ability to rapidly respond when tissue injury occurs, without memory of previous assaults or antigen specificity — is a defining feature that sets them apart from adaptive immune cells. Acute activation of innate immunity sets the stage for activation of the more sophisticated adaptive immune system. Induction of efficient primary adaptive immune responses requires direct interactions with mature antigen-presenting cells and a proinflammatory milieu. Adaptive immune cells, such as B lymphocytes, CD4+ helper T lymphocytes and CD8+ cytotoxic T lymphocytes (CTLs), distinguish themselves from innate leukocytes by expression of somatically generated, diverse antigen-specific receptors, which are formed as a consequence of random gene rearrangements and allow a flexible and broader repertoire of responses than innate

immune cells, which express germline-encoded receptors. As individual B and T lymphocytes are antigenically committed to a specific unique antigen, clonal expansion upon recognition of foreign antigens is required to obtain sufficient antigen-specific B and/or T lymphocytes to counteract infection. Therefore, the kinetics of primary adaptive responses are slower than innate responses. However, during primary responses a subset of lymphocytes differentiate into long-lived memory cells, resulting in larger responses upon subsequent exposure to the same antigen. Together, acute activation of these distinct immune-response pathways efficiently removes or eliminates invading pathogens, damaged cells and extracellular matrix (ECM). In addition, once assaulting agents are eliminated, immune cells are crucially involved in normalizing cell-proliferation and cell death pathways to enable re-epithelialization and new ECM synthesis. Once wound healing is complete, inflammation resolves and tissue homeostasis returns. Because of their enormous plasticity, immune cells exert multiple effector functions that are continually fine-tuned as tissue microenvironments are altered. Therefore, the immune system is integrally involved in maintaining tissue homeostasis as well as being implicated in the pathogenesis of many chronic diseases, such as arthritis, heart disease, Alzheimer disease and cancer.

When tissue homeostasis is chronically perturbed, interactions between innate and adaptive immune cells can be disturbed. Although duration and resolution are defining features of chronic versus acute inflammation, the cellular profiles, soluble mediators and downstream tissue-responsive pathways of the two states are also distinct. The destructive cycles that are initiated within tissues by failure to appropriately engage and/or disengage either arm of the immune system can result in excessive tissue remodelling, loss of tissue architecture due to tissue destruction, protein and DNA alterations due to oxidative stress, and, under some circumstances, increased risk of cancer development.

Population-based studies reveal that individuals who are prone to chronic inflammatory diseases have an increased risk of cancer development (202). In addition, over 15% of all human cancers are believed to be caused by infectious conditions (215), of which some — for example, chronic infection with cag+ strains of Helicobacter pylori or with hepatitis viruses — indirectly promote carcinogenesis through induction of chronic inflammatory states. One prediction that can be made from these population-based and experimental studies is that mutations or polymorphisms in genes that encode immune modifiers exist in individuals with chronic inflammatory disorders who have an increased

risk of cancer. This is in fact the case — genetic polymorphisms in genes that encode crucial cytokines, proteases and signal-transduction proteins have been identified as aetiological factors in several chronic inflammatory disorders (203). Perhaps the most compelling clinical evidence for a causative link between chronic inflammation and cancer development comes from epidemiological studies reporting that inhibiting chronic inflammation in patients with pre-malignant disease, or who are predisposed to cancer development, has chemopreventative potential. These studies revealed that long-term usage of anti-inflammatory drugs, such as aspirin and selectivecyclooxygenase-2 (COX2) inhibitors, significantly reduces cancer risk (204), indicating that COX2 or other key molecules that are involved in prostaglandin biosynthesis might be effective anticancer targets. Thus, even if, the immune system is designed to eradicate 'damaged' cells or tissues, inflammation potentiates cancer development rather than protect against it. One plausible explanation for why tumour cells escape immune-surveillance mechanisms is that neoplastic microenvironments favour polarized chronic pro-tumorigenic inflammatory states rather than ones that represent acute anti tumour immune responses (205-209). Clinical data indicate that the 'immune status' of healthy individuals is distinct from that of those who harbour malignant tumours; in the latter, T lymphocytes are functionally impaired. In addition, accumulations of chronically activated myeloid suppressor cells and regulatory T cells are found in the circulation, lymphoid organs and neoplastic tissues (210, 211). Together, immune states such as these can disable tumour-killing CD8+CTL responses and enable states of immune privilege that foster escape from anti tumour immunity while simultaneously exploiting activated immune cells that, as we are beginning to appreciate, enhance cancer development.

Diverse agents trigger the inflammation associated with human cancer. These links have been confirmed in a number of murine models, especially in terms of gastric (H. pylori infection) (121), liver (cholangitis) (213) and colon (colitis) (214) cancers. In these and other animal cancer models (215), the cells and mediators of chronic inflammation act as tumor promoters at distinct phases of malignant progression (Table 6).

Table 6: Inflammatory conditions that predispose to cancer

Malignancy	Inflammatory stimulus
Bladder cancer	Schistosomiasis
Gastric cancer	H. pylori-induced gastritis
MALT lymphoma	H. pylori
Hepatocellular carcinoma	Hepatitis virus (B and C)
Kaposi.s sarcoma	HHV8
Bronchial carcinoma	Silica
Mesothelioma	Asbestos
Bronchial carcinoma	Asbestos
Ovarian cancer	Salpingitis/talc/ovulation/endometriosis
Colorectal cancer	Inflammatory bowel disease
Oesophageal cancer	Barrett.s metaplasia
Papillary thyroid carcinoma	Thyroiditis
Prostate cancer	Prostatitis

4. DISCUSSION

Genome wide array-CGH analysis is a powerful research tool, which is going to change present knowledge. In particular, the possibility of detecting copy number polymorphisms in matched couples with a different phenotype immediately suggests genes of interest. If further confirmed these preliminary data, which should contribute to highlight the basic host-differences in the response to asbestos exposure, instead of suggesting the possible alteration of a single susceptibility gene, tend to outline a panel of genes involved either in the immunoresponse or in the perception process or in the metabolism of xenobiotics. Array-CGH showed that each couple (husband and wife) differed for a panel of genes. Copy Number Variants (CNVs) involved genes belonging to complement/HLA-complex and genes implicated in the perception of olfactory stimuli. It is also noteworthy that one CNV on 4q13.2 contains *UDP*, a gene involved in the conjugation and elimination of potential xenobiotics, and another CNV on 22q11.23 includes *GSTT1*, a gene that catalyzes the conjugation of reduced glutathione which plays a basic role in the response to oxidative stress induced by environmental pollutants.

One limiting factor to genome-wide array-CGH analysis for the present purpose of detecting susceptibility genes is the rarity of informative cases. In fact, our initial working hypothesis was to perform array-CGH analysis in a cohort of subjects with long lasting occupational exposure to asbestos in the same working place in order to detect genetic differences which could be responsible for the different incidence of mesothelioma. However, this study is time consuming, and difficult to accomplish nowadays after banning of asbestos from working sites. In addition, it should be taken into account that positive informative cases usually live less than one year after clinical diagnosis and therefore the possibility of "to enrol a significant number of individuals is low. Anyway, considering all these limitations, for the first time in a Western country initial evidence is presented that "individual susceptibility" to mesothelioma occurrence after asbestos exposure also includes, among other factors, a panel of different copy number polymorphisms including a series of interesting candidate genes. Even if in some cases no obvious candidate genes are emerged, these genes may have a possible relevance in the future with improvement of present knowledge.

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We have previously outlined that in environment related health-effects, when trying to define in more details host-particle interactions, the relative "power" of increased concentration and intrinsic toxicity of the pollutant and that of individual susceptibility of each subject is far from being established (117-119). Clinically evident diseases usually occur irrespective of a threshold able to distinguish dangerous from non dangerous concentrations of pollutants. In particular, we have recently stressed that individual susceptibility could play, in the occurrence of clinically evident diseases (and not in the simple occurrence of local or systemic oxidative stress because of particulate or fibrous material), a greater role than intrinsic toxicity of the various pollutants. In particular, after focusing on pollutant concentration (μg/m3) and pollutant type, surface reactivity, new parameters such as inflammatory potency are increasingly taken into accounts. "Inflammatory potency" is not simply related to intrinsic toxicity, but also to host reactivity. In addition, individual susceptibility includes both inherited susceptibility, due to genetic predisposition, but also "previous patient history", i.e. age, previous and concomitant diseases and functional alterations.

In conclusion present data, even if preliminary and far from being conclusive, because of the small number of informative subjects, suggest that:

1) There is not a single gene or a single genetic variation which confers predisposition/susceptibility to develop mesothelioma after asbestos exposure;

2) t is likely that there is a panel of genes, which are all together responsible for a different susceptibility or resistance to inhalated xenobiotics (and that could show a peculiar susceptibility for asbestos fibers). This higher susceptibility or resistance, mediated by diverse pathophysiologic mechanisms and pathways, may determine in the presence of a similar exposure to a toxic agent with the same intrinsic toxic potential the occurrence of different phenotypic manifestations, ranging from invasive and rapidly lethal pleural mesothelioma, to less severe mesothelioma of the tunica vaginalis testis and even to no mesothelioma development at all.

Obviously, further studies and a larger number of informative samples (subjects who have different phenotypic outcomes, despite living in the same environmental conditions) is required for a better knowledge of the relative role of the intrinsic toxicity of the pathogenic agent (asbestos, crysotile) and of individual susceptibility in the occurrence of mesothelioma. However, it is clear from the present study that even long-lasting and high-grade exposure to asbestos is not able, by itself, to determine the occurrence of mesothelioma.

5. CONCLUSION

In conclusion, the present study has basic limitations due to the small number of cases. However, for the first time evidence is presented suggesting that a panel of genes involved in the immune response and in the uptake and metabolism of xenobiotic may partly explain individual susceptibility,responsible for the the different occurrence of mesothelioma in subjects with similar asbestos exposure ..

Our approach i.e. genome wide array-CGH analysis in informative casesdeserves further studies that may be able to define more strictly the panel of interesting genes .

Informative cases should include families with occupationally exposed but not affected husbands and not occupationally exposed wive and siblingsCohorts including subjects with long term-occupational exposure in the same working site could also be of interest for recruiting a greater number of possible informative subjects. Rarity of informative subjects is the main drawback and limitation of these kind of studiesin rare diseases such as asbestos related mesothelioma.

Multicentric cooperation and in particular international cooperation, also including cohorts belonging to different racial and /or ethic groups, could better refine present preliminary data, defining more precisely which different regions/genes in affected vs non affected individuals recur more frequently.

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7. APPENDIX

Cetta F., Dhamo A., Malagnino G., Galeazzi M.

Linking environmental parti culate matter with genetic alterations.

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