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DNA Research, Genetics and Cell Biology

DNA and RNA: Properties and Modifications, Functions and Interactions, Recombination and Applications

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DNA RESEARCH, GENETICS AND CELL BIOLOGY

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PREFACE

This book presents and discusses topical research in the study of DNA, genetics and cell biology. Topics discussed include DNA damage related to exposure to oil spills; mutation detection in TP53 gene; DNA and protein sequence analysis; the role of effectors on hypoxia due to nitric oxide production in human alveolar epithelial cells and plasma cell dyscrasias.

Chapter I - One of the major challenges involved mainly in searching of point mutations for clinical relevance is the technology used; in particular for cancer research will be focused on the gains or loss-of-suppression function, v.gr. in cancer genes as *RET*, *TP53*, *RAS*, etc. *TP53* has been used as an excellent model for point mutation detection, because its more than 20,000 different mutations, being this gene the most frequently found in many human cancers.

Furthermore, there are uncommon somatic and germline mutations that might be related to specific cancers or predispositions. In particular case, the precise nature of the *TP53* mutation presents both challenges and opportunities for alternate treatment strategies in specific cancers. These highlight the clinical need to accuratelly identify often unknown inherited aberrations or infrequently represented mutations in mixed populations of DNA molecules.

Chapter II - *TP53* is the most commonly mutated gene in human cancers, and the p53 protein is a potent inhibitor of cell growth, arrestingcell cycle progression at several points and inducing apoptosisof cells undergoing uncontrolled growth. The loss of p53 function by mutation is too common in cancer. However,most natural p53 mutations occur at a late stage in tumor development,and many clinically detectable cancers have reduced p53 expression but no p53 mutations.

Since, approximately 90% of the *TP53* gene mutations are localized between domains encoding exons 5 to 8. Much research suggests that *TP53* mutations have prognostic importance and sometimes are a significant factor in clinical Oncology. The presence of specific p53 mutational hotspots in different types of cancer implicates environmental carcinogens and endogenous processes in the etiology of human cancer. Oxidative stress and the generation of reactive species may cause mutations in cancer-related genes, and affect key regulator proteins of DNA repair, cell cycle, and apoptosis.

This review gives a brief perspective of some of the landmark discoveries in mutation research. The molecular and biochemical characteristics of *TP53* and p53 are then covered,

followed by an overview of how it can be studied in the laboratory. Finally, the implications of mutational hotspotsof *TP53* gene at level of DNA damage are discussed.

Chapter III - Oil spills are one of the most frequent ecological disasters giving rise to the pollution of enormous coastal areas and affecting also the local flora and fauna. As a direct consequence of them, a large number of individuals is always involved, as they take part in the different tasks derived from the need of cleaning the spilled oil and recovering the natural environments. From a toxicological point of view, oil is a complex mixture of compounds that can penetrate into the body burden through dermal, respiratory or digestive routes. Despite the huge number of spills occurred all around the world, the international literature dealing with the harmful effects of this exposure is very scarce and restricted to the acute and psychological effects. This seems paradoxical attending to the large number of carcinogenic agents composing this mixture.

So, after the *Prestige* oil spill (November 2002, NW of Spain) and taking into account the seriousness of the catastrophe, the large number of individuals involved, the damaging character of oil components and especially the lack in the scientific literature of reports considering the consequences of this exposure from a genotoxic or carcinogenic point of view, the author developed an extensive biomonitoring study including effect and susceptibility biomarkers. Three groups of exposed individuals whose exposure during the recovery of *Prestige* oil polluted areas differed quantitatively (short and acute or more prolonged in time) and qualitatively (as a consequence of the different methods used for this purpose) were included. Environmental concentrations of volatile organic compounds were evaluated by means of passive dosimeters in each exposure group. Two types of effect biomarkers were applied: the comet assay, characterized by its high sensitivity in population studies and for reflecting also DNA repair phenomena, and two well established cytogenetic assays, micronucleus test and sister chromatid exchanges. Moreover, due to the fact that individual differences in terms of susceptibility to xenobiotics have been extensively reported and mainly attributed to some polymorphisms in genes encoding for biotransformation enzymes and DNA repair proteins with functional consequences, a complete set of the most relevant were also included in this study.

Chapter IV - In an age of molecular genetics, viable but not culturable organisms, and highly sophisticated DNA sequence analysis technologies, is there a chance for a new breakthrough? It is quite extraordinary that 99% of microorganisms not only can’t be cultured but are totally unknown to the scientific community. Many bacterial gene functions and identifications in the environment have been uncovered by recent advances in metagenomics or culture-independent genomic analysis in addition to the DNA microarray technology. Our knowledge of detecting and classifying bacterial isolates has been also improved by cutting-edge molecular tools such as biosensors and molecular subtyping as well as phage recombinant probes. Microbial communities, many to be discovered, are slowly revealed by these sharp scientific discoveries.

Chapter V - The effect of hypoxia on cell viability, proliferation and possible induced apoptosis in human alveolar epithelial cells and human hepatocytes is not clearly understood.In cultured human alveolar epithelial cells, IL-1β, TNF-α and INF-γ stimulated the nitric oxide production and resulted with inflammation manifested by hypoxia and apoptosis.In cultured hepatocytes,enhanced expression of superoxide dismutase and glutathione reductase enzymes indicated hypoxia associated with the enhanced level of *cAMP* dependent phosphodiesterase, NADPH dependentCytochrome C Oxidase enzyme activities

due to energy insufficiency in hepatocyte cultured medium.Both hypoxia and energy insufficiency reduced hepatocyte viability.The author propose that extent of inflammation leading to hypoxia initially and programmed cell death later both can enhance the MRI visible edema fluid content in lungs due to oxygen and energy insufficiency to surviving inflammatory alveolar epithelial cells.The mechanism of A549 cell damage due to nitric oxide (NO) production included: Cytokines regulated the expression of nitric oxide synthase (NOS) through NF-κB activation; Cofactor tetrahydro biopterin-catalyzed synthesis of iducible nitric oxide synthase (iNOS) enzyme.The NO production and increased level of NOS expression lead to Na^+ ion transport and cell proliferation with differentiation or apoptosis.Hypoxic human alveolar epithelial cells showed the possibility of NO production associated with the decrease of their viability and rapid increase of apoptosis. In conclusion, increased level of NO production can be a cause of hypoxia induced apoptosis and possibly MRI visible indicator of inflamed alveolar cell viability. High resolution MRI can track these sites of inflammation in lungs.

Chapter VI - Cancer is a disease resulting from the breakdown of several checkpoints and tumor-suppressing mechanisms. In cancer research, the development of new technologies, which have produced genomic tools indispensable for understanding how gene products are regulated in normal and diseased conditions on a global genome scale; one of these technologies is the DNA arrays. Although the most common use of DNA arrays is gene expression profiling and mutation detection, scientists have successfully used them for multiple applications, including genotyping, re-sequencing, DNA copy number analysis and DNA-protein interactions mainly.

This section then will be dedicated to the use of a public sequence database that can be accessed, and the design of DNA oligonucleotide probes for oligoarrays derived from sequences of special interest. Single probe and stacking hybridization are explored as possible microarray designs. Then the use of thermodynamic models and *in silico* hybridization are explored in order to access the sensitivity and specificity of the oligoarray and how its design can be improved. However, many commercial and public applications do not consider that the hybridization between target DNA's and microarray probes is a chemical reaction which is influenced by several thermodynamic parameters. One of the most important of such parameters is the thermal stability of the nucleic acid duplexes, which are formed as a result of the hybridization. According to the reaction conditions, these duplexes can be perfectly or imperfectly paired, which is of critical importance when a diagnostic kit is developed in order to assess its sensitivity and specificity.

In silico hybridization can be used to avoid undesired hybridization events (such as multiple target-probe interactions and stable mismatched hybrids). Also, the strategy can facilitate (through use of different length probes) selection of a probe set that has a narrow duplex stability allowing maximal specificity under a single hybridization condition.

Chapter VII - Plasma cell dyscrasias (PCDs) include plasmacytomas and various forms of plasma cell myeloma (multiple myeloma-MM). Diagnosis of plasmacytoma requires demonstration of a monoclonal/aberrant plasma cell (PC) population in a tissue biopsy; whereas, diagnosis of a PCD involving the bone marrow (BM) (i.e., various forms of MM) requires quantitation of the PCs in the BM in addition to the demonstration of monoclonality/aberrancy. This chapter will describe the definitions of the various forms of PCD (including the International Staging System-ISS), the morphological variants and immunophenotypic features of neoplastic PCs, the diagnostic laboratory techniques useful in

establishing a diagnosis (including immunophenotypic techniques), and the ancillary studies that may aid in diagnosis, prognostication, and therapeutic decision-making (i.e., immunophenotypic features, as well as cytogenetic and molecular findings).

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Chapter I

MUTATION DETECTION IN *TP53* GENE: ALTERNATIVES IN POINT MUTATION TECHNOLOGIES

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One of the major challenges involved mainly in searching of point mutations for clinical relevance is the technology used; in particular for cancer research will be focused on the gainsor loss-of-suppression function, v.gr. in cancer genes as *RET*, *TP53*, *RAS*, etc. *TP53* has been used as an excellent model for point mutation detection, because its more than 20,000 different mutations, being this gene the most frequently found in many human cancers.

Furthermore, there are uncommon somatic and germline mutations that might be related to specific cancers or predispositions. In particular case, the precise nature of the *TP53* mutation presents both challenges and opportunities for alternate treatment strategies in specific cancers. These highlight the clinical need to accuratelly identify often unknown inherited aberrations or infrequently represented mutations in mixed populations of DNA molecules.

Different polymorphisms or mutation detection technologies have been developed to identify known changes: these include DNA microarrays, the polymerase chain

reaction/ligase detection reaction (PCR/LDR), now used in combination with the universal DNA microarray and primer extension assays. On the other hand, the technologies used for unknown mutations: hybridization analysis using high-density oligonucleotide arrays, denaturing high-performance liquid chromatography (DHPLC), capillary electrophoresis-based single strand conformation polymorphism (CE-SSCP), denaturing gradient gel electrophoresis (DGGE) and heteroduplex analysis (HA). Finally, dideoxy-sequencing has difficulty in detecting heterozygous mutations, and is of limited utility in the analysis of solid tumors where mutant DNA may represent as little as 15% of the total. Some other methods include *in vitro* transcription/translation-based approaches, chemical and enzymatic mismatch cleavage detection (e.g. Cleavase, RNase, T4 endonuclease VII, MutS enzymes and CEL I). *In vitro* mismatch cleavage methods encounter variability in signal intensity compared with background bands.

Hybridization analysis using low-density oligonucleotide arrays for unknown mutations, certainly might have the potential for custom fabrication and the detection of all desired features and could conveniently provide reliable results and decrease production cost considerably. A variant of this kind of technique is based on tandem hybridization. Tandem hybridization attempts to compare the annealing of matched versus mismatched probes to targets ("probe" typically refers to the DNA immobilized on the surface, whereas "target" generally refers to DNA in solution) over a range of hybridization conditions. Moreover, tandem hybridization method offers several advantages over traditional oligonucleotide array configuration, mainly because a unique feature of tandem hybridization is that unlabeled target DNA is annealed with relatively long-labeled stacking oligonucleotides which bind at a unique site together with short capture probes positioned immediately adjacent to stacking oligonucleotides. In consequence, this system being a highly specific and sensitive because capture and stacking probes must be contiguous, in order to obtain a specific hybridization signal. This system has been successfully applied to *RET* oncogene and *TP53* gene. In this particular context, we have designed a small microarray directed against the hotspot mutations that are more commonly observed in exons 5, 7 and 8 of *TP53* because in this region are clustered the most frequently mutations found in clinical samples. The minimum amount of target detected, as estimated by the proportion of equimolar amount of labeled stacking oligonucleotide annealed with the sample. It is important to comment that the intensity of signals seen with the same sample, using 7-mer probes, under traditional, single tandem and double tandem hybridizations increases from 3 to 6 times in Single Tandem Hybridization (which uses only a single stacking oligonucleotide) and 6 to 12 times in the Double Tamdem Hybridization (which uses both stacking oligonucleotides) when compared to traditional hybridization. Some mutations, such as those causing G's mismatched, are very stable, and for this reason are difficult to discriminate with the traditional hybridization techniques due especially to the length of the probes that are normally used (20-25 nt or even longer). Short duplexes are considerably more destabilized by even relatively stable mismatches than longer duplexes, and for this reason short probes (7-mer) have higher discrimination power. However, short probes when used individually (without tandem hybridization) are not quite adequate because theses sequences can occur by random chance multiple times within relatively long targets, which would limit their specificity in the single-probe approach. However, this is not the case in the tandem hybridization approach because the specificity arises from the short probes plus the contiguous stacking oligonucleotides (the fact that they must be contiguous in order to see a hybridization signal is an important

requirement for this technique). In other words, only 7-mer sites located adjacent to the stacking oligonucleotides are interrogated, since the stacking hybridization allows only their detection since isolated 7-mer duplexes (not stabilized by the base stacking) are unstable under the hybridization conditions used. Finally, in order to decide whether one and other method is adequate to detect point mutations it is important previously to know the mutation frequency expected for any non-selected gene in normal human and in tumor tissue which makes the analysis less difficult.

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Chapter II

MUTATIONAL HOTSPOTS OF *TP53* GENE ASSOCIATED TO DNA DAMAGE

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ABSTRACT

TP53 is the most commonly mutated gene in human cancers, and the p53 protein is a potent inhibitor of cell growth, arrestingcell cycle progression at several points and inducing apoptosisof cells undergoing uncontrolled growth. The loss of p53 function by mutation is too common in cancer. However,most natural p53 mutations occur at a late stage in tumor development,and many clinically detectable cancers have reduced p53 expression but no p53 mutations.

Since, approximately 90% of the *TP53* gene mutations are localized between domains encoding exons 5 to 8. Much research suggests that *TP53* mutations have prognostic importance and sometimes are a significant factor in clinical Oncology. The presence of specific p53 mutational hotspots in different types of cancer implicates environmental carcinogens and endogenous processes in the etiology of human cancer. Oxidative stress and the generation of reactive species may cause mutations in cancer-related genes, and affect key regulator proteins of DNA repair, cell cycle, and apoptosis.

This review gives a brief perspective of some of the landmark discoveries in mutation research. The molecular and biochemical characteristics of *TP53* and p53 are then covered, followed by an overview of how it can be studied in the laboratory. Finally, the implications of mutational hotspotsof *TP53* gene at level of DNA damage are discussed.

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GLOSSARY

The following terms are used in the text of this chapter.

Allele — An alternative form of a gene.

Alternative splicing— A regulatory mechanism by which variations in the incorporation of a gene's exons, or coding regions, into messenger RNA lead to the production of more than one related protein, or isoform.

Conservative mutation— A change in a DNA or RNA sequence that leads to the replacement of one amino acid with a biochemically similar one.

Epigenetic — A term describing nonmutational phenomena, such as methylation and histone modification, that modify the expression of a gene.

Exon — A region of a gene that codes for a protein.

Frame-shift mutation— The addition or deletion of a number of DNA bases that is not a multiple of three, thus causing a shift in the reading frame of the gene. This shift leads to a change in the reading frame of all parts of the gene that are downstream from the mutation, often leading to a premature stop codon and ultimately, to a truncated protein.

Gain-of-function mutation— A mutation that produces a protein that takes on a new or enhanced function.

Genomics — The study of the functions and interactions of all the genes in the genome, including their interactions with environmental factors.

Genotype — A person's genetic makeup, as reflected by his or her DNA sequence.

Heterozygous — Having two different alleles at a specific autosomal (or X chromosome in a female) gene locus.

Homozygous— Having two identical alleles at a specific autosomal (or X chromosome in a female) gene locus.

Intron — A region of a gene that does not code for a protein.

Loss-of-function mutation— A mutation that decreases the production or function of a protein (or does both).

Missense mutation— A substitution of a single DNA base leads to an alternative amino acid, because of the way in which it changes the three-base sequence, or codon, that codes for an amino acid.

Motif — A DNA-sequence pattern within a gene that, because of its similarity to sequences in other known genes, suggests a possible function of the gene, its protein product, or both.

Nonconservative mutation— A change in the DNA or RNA sequence that leads to the replacement of one amino acid with a very dissimilar one.

Nonsense mutation— Is a more dramatically deleterious type of point mutation that change the codon to a "stop" codon, thus leading to the truncation of a protein.

Penetrance— The likelihood that a person carrying a particular mutant gene will have an altered phenotype.

Phenotype— The clinical presentation or expression of a specific gene or genes, environmental factors, or both.

Point mutation— The substitution of a single DNA base in the normal DNA sequence.

Regulatory mutation— A mutation in a region of the genome that does not encode a protein but affects the expression of a gene.

Repeat sequence— A stretch of DNA bases that occurs in the genome in multiple identical or closely related copies.

Silent mutation— Substitution of a single DNA base that produces no change in the amino acid sequence of the encoded protein.

Single-nucleotide polymorphism (SNP)— A common variant in the genome sequence; the human genome contains about 10 million SNPs.

Stop codon— A codon that that causes the termination of the protein instead of producing an amino acid. The three stop codons are TGA, TAA, and TAG.

Tautomeric shifts— Chemical fluctuations of Hydrogen atoms that can move from one position in a purine or pyrimidine to another position, for example, from an amino group to a ring nitrogen.

Transitions— Mutations resulting from tautomeric shifts in the bases of DNA involve the replacement of a purine in one strand of DNA with the other purine and the replacement of a pyrimidine in the complementary strand with the other pyrimidine.

Transvertions — Base-pair substitutions involving the replacement of a purine with a pyrimidine and vice versa.

INTRODUCTION

During their life, normal cells are constantly exposed to various endogenous and exogenous stresses that alter their normal behavior. Alterations in the chemical structure of the DNA occur frequently, interfere with transcription and replication, and kill the organism if allowed to accumulate. This accumulation activates the transcription of a wide range of genes involved in various activities, including cell cycle inhibition and apoptosis depending on the cellular context, the extent of damage or other unknown parameters. For all that "wearandtear" of DNA can take two forms: *mutation and DNA damage*. Genetic insults that can lead to mutations are particularly harmful, as their transmission to daughters cells can lead different alterations, including complex diseases such as cancer[1].

Cancer is a disease resulting from the breakdown of several checkpoints and tumor-suppressing mechanisms. Carcinogenesis typically involves multiple somatic mutations or, less commonly, germline mutations in caretaker and gatekeeper genes [2, 3].The caretakers are broadly defined DNA repair genes that are responsible for maintenance of genome stability. Mutations in the caretaker genes, which are considered to be typical tumor suppressors, compromise genome stability and, more specifically, increase the probability of mutation in the gatekeepers which include both tumor suppressor genes and oncogenes [4, 5].

Tumor suppressors are genes that control cell proliferation, in particular, by causing cell death in response to DNA damage; accordingly, mutational inactivation of tumor suppressors may cause transformation. In contrast, oncogenes are genes that, when mutated, acquire new functions promoting cell proliferation and, eventually, transformation [2].

Of more than 200 human cancer genes reported in the current databases, ~90% have somatic mutations, 20% have germline mutations and 10% have both, and inactivation of tumor suppressors is considered to be the main driving force of tumorigenesis[6].

In the post-genomic era, interest is beginning to focus on the differences between the genomes of individuals and on the effects of mutations. Today, the term *mutation* often is

used in a narrow sense to refer only to changes occurring within genes. The immediate effects of the mutation and its ability to produce a phenotypic change are determined by its dominance, the type of cell in which it occurs, and the time at which it takes place during the life cycle of the organism. However, changes in the genotype, and thus in the phenotype, of an organism that result from recombination events that produce new combinations of preexistings genetic variation must be carefully distinguished from changes caused by new mutations. Both events sometimes give rise to new phenotypes at very low frequencies. Mutational changes in the genotype of an organism include changes in chromosome number and structure, as well as changes in the structures of individual genes [7].

Mutation data fall largely into two classes: single nucleotide polymorphisms (SNPs) and disease-related mutations. Further sub-divisions are possible including the split between non-coding, silent, mis-sense and nonsense mutations. Mutations occur naturally in DNA, but are generally corrected through DNA repair systems. Mutations are rarely maintained and inherited by daughter cells or future generations [8]. When Watson and Crick in 1953 described the double-helix structure of DNA and proposed its semiconservative replication based on specific base-pairing to account for the accurate transmission of genetic information from generation to generation, they also proposed a mechanism to explain spontaneous mutation, by *tautomeric shifts*. Although tautomeric shifts are rare, they may be of considerable importance in DNA metabolism because some alter the pairing potential of the bases. The more stable keto forms of thymine and guanine and the amino forms of adenine and cytosine may infrequently undergo tautomeric shifts to less stable enol and imino forms, respectively. The bases would be expected to exist in their less stable tautomeric forms for only short periods of time. However, if a base existed in the rare form at the moment that it was being replicated or being incorporated into a nascent DNA chain, a mutation would result. When the bases are present in their rare imino or enol states, they can form adenine-cytosine and guanine-thymine base pair. The net effect of such an event, and the subsequent replication required to segregate the mismatched base pair, is an A:T to G:C or a G:C to A:T base-pair substitution. Mutations resulting from tautomeric shifts in the bases of DNA involve the formation of *transitions* and *transversions*. Four different transitions and eight different transversions are possible [9].

Clearly, mutations may affect organisms in very different ways; they may exhibit the complete range of phenotypes from drastic detrimental effects, through mild and completely phenotypically silent effects to minor improvements or the introduction of new function [10]. The larger the change brought about by the mutation, the more likely it is to have a drastically affected phenotype. For example, frameshifts, deletions, insertions, repetitions or nonsense codons leading to early termination of a protein are almost guaranteed to have a drastic effect, whereas single amino acid mutations may have a much more limited effect on phenotype. Because around 90% of sequence variants in humans are single DNA base changes [11], there is an increasing interest in this family of mutations [12, 13]. Formally, SNPs can be defined as alleles which exist in normal individuals in a population with the least frequent allele having an abundance of at least 1% [14]. In principle, SNPs could be bi-, tri- or tetra-allelic variations, but tri- and tetra-allelic SNPs are very rare in humans. In practice, the term SNP is often applied in a more generic context and may encompass disease-causing mutations which are recessive, or low-penetration dominant alleles, the latter generally being present at much lower frequencies [15].

The discovery of chemical mutagens with known effects on DNA led to a better understanding of mutation at the molecular level. DNA is damaged by both environmental and cellular (endogenous) sources. Many of the environmental agents that damage DNA have been demostrated to be mutagens. Epidemiologic data indicate that many of these agents are also human carcinogens. DNA damage by chemicals can be divided into two categories: (i) those that produce large bulky adducts and are repaired by the nucleotide excision pathway; and (ii) those that cause small alterations, such as alkylating agents that add methyl and ethyl groups onto nucleotides in DNA, and are repaired by the base excision repair pathway. Also, chemical mutagens can be divided into two groups: (1) those that are mutagenic to both replicating and nonreplicating DNA, such as the alkylating agents and nitrous acid; and (2) those that are mutagenic only to replicating DNA, such as base analogs—purines, and pyrimidines with structures similar to the normal bases in DNA. The base analogs must be incorporated into DNA chains in the place of normal bases during replication in order to exert their mutagenic effects. Mutations can also result from radiation, in which the portion of the electromagnetic spectrum with wavelengths shorter and of higher energy than visible light is subdivided into ionizing radiation (X rays, gamma rays, and cosmic rays) and nonionizing (ultraviolet light). Ionizing radiations are of high energy they penetrate living tissues for substantial distances. These high-energy rays collide with atoms and cause the release of electrons, leaving positively charged free radicals or ions. Ultraviolet rays, having lower energy that ionizing radiations and dissipate their energy to atoms that they encounter, raising the electrons in the outer orbitals to higher energy levels, a state referred to as excitation. The increased reactivity of atoms present in DNA molecules is responsible for the mutagenicity of ionizing radiation and ultraviolet light [16, 17].

When we consider mutations in terms of the inactivation of the gene, most genes mitin a species show more or less similar rates of mutation relative to their size. This suggest that the gene can be regarded as a target for mutation, and that damage to any part of it can abolish its function. As a result, susceptibility to mutation is roughly proportional to the size of the gene. But consider the sites of mutation within the sequence of DNA some base pairs in a gene more than susceptible to be mutated than others. So some sites will gain one, two, or three mutations, while others will not gain any. But some sites gain far more than the number of mutations expected from a random distribution; they may have 10x or even 100x more mutations than predicted by random hits. These sites are called *hotspots*. Defining such hotspot regions and natural mutants is of invaluable help in defining critical regions in an unknown protein. Spontaneous mutations may occur at hotspots; and different mutagens may have different hotspots.

Sites containing 5-methylcytosine, generated by a methylase enzyme that adds a methyl group to a small proportion of the cytosine residues—at specific sites in the DNA, provide hotspots for spontaneous point mutations. The reason for the existence of the hotspots is that 5-methylcytosine suffers spontaneous deamination at an appreciable frequency; replacement of the amino group by a keto group converts 5-methylcytosine to thymine (causing C▪G to T▪A transitions) [18, 19].

To understand the role of individual mutants in carcinogenesis and to assess the possibility of rescuing their function, it is important to know the effect of the mutation not only on the overall stability but also on the local structure. Qualitative NMR studies indicate that hotspot mutants evince characteristic local structural changes [20].

THE ROLE OF THE *TP53* GENE

The *TP53* gene is the most prominent and best studied tumor suppressor. Its a single copy gene located on the short arm of chromosome 17 (17p13.1), and is composed of 11 exons, 10 intervening introns and 393 amino acids (MIM # 191117). *TP53* was the first gene to be identified as a mutant in tumours, in 1979 [21], and it has been figuratively called the "genome guard" [22]. Thus, this gene coordinates all the essential processes of maintaining genome stability, and its one of the most important tumour suppressor genes controlling DNA transcription and cell cycle regulation. Therefore, this gene is both a caretaker (by inducing DNA repair) and a gatekeeper (by inducing apoptosis in the case of irreparable damage) [23]. And it has an unusually large number of functions in the cell, binds to many other proteins and ligands, and has a complex architecture [24, 25]. *TP53* gene encodes a transcription factor with multiple, anti-proliferative functions activated in response to several forms of cellular stress.

The *TP53* gene is mutated in nearly 60% of human tumors. The spectrum of somatic mutations in the *TP53* gene, of which ~85% are missense mutations, implicates environmental carcinogens and endogenous processes in the etiology of human cancer, and occur at over 200 codons within the central portion of the gene [26]. This somatic *TP53* gene alterations are frequent in most human cancers [27-34], and inherited *TP53* mutations predispose to a wide spectrum of early onset cancers (e.g., Li-Fraumeni Syndrome, LFS; MIM# 151623). Most *TP53* mutations result in gain of oncogenic functions including attenuating the function of the *p73* gene [35]. The types of mutations observed in the germline and sporadic cancer cases are similar, with a high prevalence of missense mutations (>75%). Thus, *TP53* differs from other large tumor suppressor genes such as *RB1* (27 exons, 928 amino acids), *APC* (15 exons, 2843 amino acids), or *BRCA1* (24 exons, 1863 amino acids), which are frequently inactivated by deletions, frameshift mutations, or nonsense mutations. *TP53* missense mutations are scattered throughout the coding sequence but 97% of them cluster in exons encoding the DNA-binding domain (DBD). In this domain, six mutation hotspots (defined by a mutation frequency superior to 2% of all mutations) have been identified at codons 175, 245, 248, 249, 273, and 282. The position and nature of the mutations vary from one cancer type to the other, and in some instances from one cohort to another ―suggesting that mutation patterns may reveal clues on the mutagenic mechanism involved in causing cancer [36].

There are a database of all published mutations which is maintained at the International Agency for Research on Cancer (IARC). The IARC *TP53* Mutation Database compiles all *TP53* gene mutations identified in human cancers and cell lines that have been reported in the peer-reviewed literature since 1989. The database has been updated with data reported in publications edited in PubMed in 2005. This R11, version release in October 2006 is the lates, contains 23544 somatic mutations, 376 germline mutations, functional data on 2314 mutant proteins and *TP53* gene status of 1569 cell-lines. (http://www-p53.iarc.fr/p53database. html[37].

THE *P53* PROTEIN

p53 is probably the most popular molecule in the field of cellular biology [38-40]. p53 is a polyfunctional protein which functions in the nucleus. The *TP53* gene is continuously transcribed and translated, but the protein is rapidly subjected to ubiquitin-dependent degradation in proteosomes [41, 42]. Therefore, the concentrations of p53 in cells of most tissues are quite low and may be at the limit of detection. The activation of p53 as a response to various stresses and damages proceeds mainly post-translationally via a decrease in its degradation rate and a change of its conformation to generate increased functional activity. Several functions and activities are attributed to p53. This ubiquitous factor is kept in a repressed state in normal cells, but is activated by post-translational modifications in response to multiple forms of stress, both genotoxic (such as irradiation, chemical carcinogens, or cytotoxic agents used in cancer therapy) or non-genotoxic (such as hypoxia, depletion of ribonucleotides, and oncogenic activation of growth signaling cascades) [43].When active, the p53 protein accumulates to high levels in the nucleus and acts as a multi-functional transcription factor to enhance or repress the expression of several sets of genes involved in cell cycle progression, apoptosis, adaptive response to stress, differentiation, and DNA repair [24].

Thus, p53 controls and coordinates anti-proliferative responses to prevent DNA replication from occurring when cells are exposed to adverse conditions. The mechanism of the p53 mediated suppression of cell cycle progression involves arrest within the G1 phase [23, 44], as a consequence of the p53 induced synthesis of p21, an inhibitor of cyclin E/cdk2 and cyclin A/cdk2 kinases. In this way, p53 gives DNA repair mechanisms time to correct damage before the genome is replicated. If damage to the cell is too severe, p53 initiates apoptosis by inducing transcription of genes encoding proapoptotic factors [45, 46] p53 also enhances or represses the expression of genes involved in the adaptive response to stress, differentiation, and the DNA repair process. These properties have led to the concept that p53 plays a central role in carcinogenesis.

As a transcription factor, p53 mediates many cellular responses to genotoxic insults and hypoxia [24, 25, 47]. Through coordination of over 50 genes, activated p53 is central to a variety of biological functions including cell cycling, apoptosis, differentiation, cellular senescence [23, 44, 48], angiogenesis [49, 50],and the removal of DNA damage [51-55]. Analysis of p53-regulated global gene expression reveals differences in strength, kinetics, and specificity that depend on the levels of p53, its posttranslational modifications, its degradation, nature of stress, cell type, and other as yet unidentified parameters [56-59]. It is likely that subsets of genes can be chosen from the complex spectrum of potentially inducible genes to mediate a specific p53 response in a given physiological situation [60].

The open reading frame of human p53 codes for 393 amino acids, The p53 molecule forms a tetrameric complex that recognizes a specific DNA sequence and stimulates transcription of several genes having an appropriate DNA element adjacent to the promoter. The DNA element with which p53 couples consists of two “semi-sites” of general structure PuPuPuC(A/T)(A/T)GPyPyPy positioned one after another at 0-13 nucleotides distance consisting of three major structural domains: an N-terminal domain which contains a strong transcription activation signal [61], a DNA-binding core domain, and C-terminal domain which mediates oligomerization. Comparisons of p53 sequences from different species

indicate five blocks – boxes I-V —of highly conserved residues which coincide with mutation clusters found in p53 in human cancers. The majority (approximately 80%) are missense mutations comprising GC to AT transitions at cytosine phosphate guanine dinucleotides and occur principally in five hotspot codons (175, 245, 248, 249, 273, and 282) in exons 5 to 8, in highly conserved areas, and in three principal structural domains of the *TP53* protein (L2, L3, and loop-sheet-helix [LSH]). The most frequent changes are missense mutations in the DNA-binding domain (DBD) of the protein. These can lead to nuclear accumulation of mutant p53 protein and loss of its normal functions, such as transcriptional activation of target genes [37, 62].

The DNA-binding core domain of the protein has been structurally characterized in complex with its cognate DNA by x-ray crystallography [63-65], and in its free formin solution by NMR [66]. It consists of a central ß-sandwich that serves as a basic scaffold for the DNA-binding surface. The DNA-binding surface is composed of two large loops (L2 and L3) that are stabilized by a zinc ion and a loop–sheet–helix motif. Together, these structural elements form an extended surface that makes specific contacts with the various p53 response elements. The six amino acid residues that are most frequently mutated in human cancer are located in or close to the DNA-binding surface (compare release R10 of the *TP53* mutation database at www-p53.iarc.fr) [37]. These residues have been classified as ‘‘contact’’ (Arg-248 and Arg-273) or ‘‘structural’’ (Arg-175, Gly-245, Arg-249, and Arg-282) residues, depending on whether they directly contact DNA or play a role in maintaining the structural integrity of the DNA-binding surface [63].

Urea denaturation studies have shown that the contact mutation R273H has no effect on the thermodynamic stability of the core domain, whereas structural mutations substantially destabilize the protein to varying degrees, ranging from 1 kcal/mol for G245S and 2 kcal/mol for R249S up to >3 kcal/mol for R282W [67]. The destabilization has severe implications for the folding state of these mutants in the cell. Because the wild-type core domain is only marginally stable and has a melting temperature only slightly above body temperature, highly destabilized mutants such as R282W are largely unfolded under physiological conditions and, hence, are no longer functional [68].

A flexible proline-rich domain is located between amino acids 63 and 97 in which the PxxP elements are found. This domain is needed for the full suppressor activity of p53. Although the mechanism of action of the proline-rich domain is not completely clear, it is supposed to take part in both transcription-dependent, and transcription-independent apoptosis [69]. A locus between amino acids 323 and 356 is responsible for tetramerization of p53 molecules. This locus has a clear *alpha*-helical structure. Loss of the ability of p53 to oligomerize occurs if the *alpha*-helical domain is damaged, and this causes functional inactivation [70].An alkaline domain essential for the regulation of p53 activity is located immediately at the C-terminus of the molecule (amino acids 363-393).This domain is a target for a series of modifying enzymes. The unmodified alkaline domain prevents formation of the complex of DNA and the central DNA-binding domain of the p53. Removal of the alkaline domain stimulates the DNA-binding activity of p53 *in vitro* [71]. The C-terminal domain is essential for regulation of p53 activity *in vivo*. Modification of this element by kinases, acetylases, glycosylases, and binding with other proteins causes a delicate transformation of the DNA-binding and transactivation ability of p53. Also, the C-terminal p53 fragment is able to bind nonspecifically with single-stranded DNA elements, unpaired bases, and DNA ends indicating its possible involvement in the process of recognition of damaged DNA [72]. The

structure supports the hypothesis that DNA binding is critical for the biological activity of p53, and provides a framework for understanding how mutations inactivate it [63].

TP53 SCREENING TECHNIQUES

Although the publication of the human genome sequence and the immense technological advancements have facilitated the analysis of cancer genomes, detection of mutations in tumor specimens may still be challenging and fraught with technical problems. Today it is generally accepted that molecular analyses in tumor samples should be performed in precisely defined homogeneous tumor cells with low or even no contamination by nontumor cells. Therefore, the success of nucleic acid diagnostic in the clinical setting depends heavily on the method used for purification of the nucleic acid target from biological samples.

Actually, exists a variety of methods for the detection of point mutations as well as small deletions or insertions, the number of methods that may be used for detection of genetic variability is growing steadily. Most methods are applicable only in situations where the location and type of variability have been defined. These include the allele-specific oligonucleotide assay [73], the oligo-ligation assay [74], minisequencing [75, 76], TaqMan assay [77], Invader assay [78], pyrosequencing [79] Real-time PCR [80], and PCR-restriction fragment length polymorphism analysis [81]. And another methods that can detect genetic variability irrespective of the location and type of aberration are less common and, in general, less reliable. These methods include heteroduplex analysis [82], single strand conformation polymorphism (SSCP) [83], desnaturing gradient gel electrophoresis [84], chemical cleavage [85], dideoxy fingerpriting [86], denaturing HPLC (dHPLC) [87], primer extension [88], and Microarray technology [89-91]. that facilitates large-scale mutation/polymorphism detection. Finally, dideoxy-sequencing has difficulty in detecting heterozygous mutations, and is of limited utility in the analysis of solid tumors where mutant DNA may represent as little as 15% of the total. Other methods include *in vitro* transcription/translation-based approaches, chemical and enzymatic mismatch cleavage detection (e.g. Cleavase, RNase, T4 endonuclease VII, MutS enzymes and CEL I) [92, 93]. *In vitro* mismatch cleavage methods encounter variability in signal intensity compared with background bands. But, for the appropriate choice of any one of these methods, several criteria must be considered, some of them are:

(1) What type of nucleic acid is analyzed (DNA or RNA)?
(2) What kind of specimen is analyzed (e.g., peripheral blood, bone marrow, tissues, secretions, excretions)?
(3) Are the mutations to be detected known before analysis?
(4) How large is the number of potential mutations to be detected?
(5) Need each of the potential mutations be detected?
(6) What is the ratio between wild-type and mutant alleles?
(7) How reliable is the method to be used, and how far can it be standardized?
(8) How does the test perform?
(9) Is the test suited for routine diagnosis?
(10) What kind of quality assessment can be achieved?

In general, PCR is either used for the generation of DNA fragments, or is part of the detection method. Screening methods for unknown mutations as well as methods for the detection of known mutations must be are included. DNA sequencing is considered the gold standard and remains the definitive procedure for the detection of mutations so far. For this reason, mutations assumed from the results of screening methods must be confirmed by DNA sequencing. Any one of the above methods is suited for the analysis of allelic differences in hereditary disease. Non-gel-based detection systems have been developed for most of the assays described, making these methods favorable for application in routine laboratories. For each technique, reaction conditions must be standardized and appropriate internal controls must be included. In cases in which a large number of different mutations or polymorphisms are to be detected, the DNA chip technology most probably will be the method of choice in the near future. At present, one technique addresses by combining the ability of termostable Endonuclease V (Endo V) enzyme to recognize and nick mismatched DNA, with the high fidelity of thermostable DNA ligase to suppress nicks at matched DNA [94]. Endo V can nick either or both strands of the mismatch. Unlike of others enzymes, Endo V nicks DNA close to the mismatched base. This allows the thermostable ligase to effectively discriminate between perfectly matched and mismatched regions of the DNA [95] and to ligate only perfectly matched nicks. This results in greatly reduced background noise. This method has very high sensitivity, and can distinguish one mutant sequence in a 20 fold excess of unaltered DNA [96].

At level for detect the protein status the adequate method its immunohistochemistry techniques, Western-blot and Flow cytometry that using different monoclonal antibodies [97, 98].

Despite the accumulation of studies on *TP53* mutations, their significance for cancer detection and prognosis remains elusive. One of the reasons for this is the functional heterogeneity of mutations. Its important therefore to develop studies aimed at better understanding the lessons that can be learned from *TP53* mutation detection. For example: in liver cancer, mutations can be found ahead of diagnosis, in free DNA fragments retrieved in the plasma, thus providing a possible mean for early cancer detection. In Western Africa, a region where chronic infection by HBV and dietary contamination by aflatoxin is widespread, the so-called « *aflatoxin-signature* » mutation at codon 249 is found in the plasma DNA of chronic HBV carriers, with seasonal variations reflecting variations of exposure to aflatoxin [99]. Thus, in this case, plasma *TP53* mutation appears to be a biomarker of exposure to a mutagen, rather than of early cancer development. Another factor of complexity is the possible impact of *deltaN isoforms of p53* family members. In keratinocytes infected by skin-type HPVs such as HPV38, expression of deltaNp73 contributes to inhibit p53 activity and to protect cells against p53-dependent apoptosis during virus-induced immortalization. Thus, expression of isoforms may, in specific contexts, provide alternative mechanisms to down-regulate p53 function during progression towards cancer [100]. Another example to be consider its that p53 is a short-lived transcription factor that has evolved to respond to a variety of stimuli to initiate cell cycle arrest or apoptotic programs, or to induce genes that participate in some forms of DNA repair. As p53 participates in life and death decisions, it is critical that its output be stringently regulated. The importance of the p53 pathway for tumor suppression is demonstrated by mutation of the p53 gene or alterations in its negative regulators in almost all cancers [101]. Besides abrogating the tumor suppressor activity of the wt p53 protein, some of the frequently encountered tumor-associated mutant p53 isoforms

often also elicit a pro-oncogenic gain of function. One of the manifestations of this gain of function is increased resistance to killing by anti-cancer agents.

Exist evidence that at least some of the anti-apoptotic gain of function of mutant p53 is due to its ability to modulate the transcription of apoptosis-related genes. One such example is CD95/Fas, a death receptor whose gene is subject to transcriptional repression by mutant p53 [102]. Another mutant p53-respressed gene is MSP (macrophage stimulating protein). While the literature suggests that MSP has anti-apoptotic effects, some study shows that, in cultured tumor cells, it actually contributes to apoptosis. Analysis of MSP gene expression in tumors supports the notion that its downregulation may benefit at least some human tumors [103]. Most notably, mutant p53 contributes to activation of NF-kB in cancer cells, resulting in enhanced induction of anti-apoptotic genes and increased resistance to apoptosis. The underlying mechanism may involve recruitment of mutant p53 protein to genomic NF-kB binding sites, which may be facilitated by a physical interaction between mutant p53 and NF-kB. NF-kB is a well-documented contributor to cancer development and therapy resistance. Its hyperactivation by mutant p53 may thus provide substantial benefits to tumors. Thus, mutant p53 may constitute an important target for future anti-cancer therapies [104].

There are five regions – boxes I-V - in p53 protein which reveal strikingly high conservation between species, suggesting they are functionally important. Box I comprises the binding site of mdm2, which supports the importance of negative regulation of p53 by mdm2. Boxes II-V, residing in DNA binding domain of p53 are the most frequent sites of mutations found in human tumours. p53 mutants with deletions of conserved boxes II-V behave like tumor derived point mutants in this region in that they fail to bind DNA and are therefore not transcriptionally active. In addition, these mutants adopt a conformation associated with tumor derived p53 mutants [70].

p53 point mutants are stable in human tumors and expressed at higher levels in tumor cells compared to wild-type p53. However, recent studies of mutant p53 knock-in mice suggest that these p53 proteins are not stable in normal tissue, indicating that a failure to activate expression of Mdm2 is not the underlying cause of mutant p53 stability in tumors. Another possibility is that the stability of mutant p53 is related to the over-expression of ARF. However, exist some studies have suggested that inhibiting ARF expression does not reduce the stability of mutant p53 in tumor cell lines [105]. Although mutant p53 has been reported to be sensitive to Mdm2-mediated degradation, previous studies be showed that mutations in the DNA binding region could render p53 resistant to degradation by HPV E6. *In vitro* assays the p53 deletion mutants are less efficiently ubiquitinated by Mdm2 compared to wild type p53. These results suggest that the degradation of mutant p53s by Mdm2 may not be dependent on efficient ubiquitination [106]. Interestingly, significantly higher levels of ubiquitination of each of the mutant p53s were found in Mdm2 null cells compared to wild type p53, suggesting that the mutants may acquire an increased sensitivity to other E3 ligases such as Cop1 or Pirh2 [107].

IMPLICATIONS OF MUTATIONAL HOTSPOTSOF *TP53* GENE AT LEVEL OF DNA DAMAGE

In general terms “Damage” to DNA consists of any change introducing a desviation from the usual double-helical structure. We can divide such changes into two general classes: *single base changes* and *structural distortions*.

Single base changes affect the sequence but not the overal structure of DNA. They do not affect transcription or replication, when the strands of the DNA duplex are separated. So these changes exert their damaging effects on future generations through the consequences of the change in DNA sequence. Such an effect is caused by the conversion of one base into another that is not properly paired with the partner base. For example deamination of cytosine (spontaneously or by chemical mutagen) creates a mismatched T▪G pair; while a replication error that inserts adenine instead of cytosine creates an A▪G pair. Similar consequences could result from covalent addition of a small group to a base that modifies its ability to base pair. These changes may result in very minor structural distortion (as in the case of T▪G pair) or quite significant change (as in the case of A▪G pair), but the common feature is that the mismatch persists only until the next replication.

*Structural distortions*may provide a physical impediment to replication or transcription, e.g., introduction of covalent links between bases on one strand of DNA or between bases on opposite strands inhibits replication and transcription. A well studied example of a structural distortion is caused by ultraviolet irradiation, which introduces covalent bonds between two adjacent thymine bases, giving the intrastrand pyrimidine dimer. Similar consequences could result from addition of a bulky adduct to a base that distorts the structure of the double helix. A single-strand nick or the removal of a base prevents a strand from serving as a proper template for synthesis of RNA or DNA. The common feature in all these changes is that the damaged adduct remains in the DNA, continuing to cause structural problems and/or induce mutations, until it is removed. Repair systems can often recognize a range of distortions in DNA as signals for action, and a cell may have several systems able to deal with DNA damage. We may divide them into several general types:

(1) *Direct repair* is rare and involves the reversal or simple removal of the damage. Photoreactivation of pyrimidine dimers, in which the offending covalent bonds are reversed by a light-dependent enzyme, is the best example.
(2) *Excision repair* is initiated by a recognition enzyme that sees an actual damaged base or a change in the spatial path of DNA. Recognition is followed by excision of a sequence that includes the damaged bases; then a new stretch of DNA is synthesized to replace the excised material. Such systems are common; some recognize general damage to DNA, while others act upon specific types of base damage (glycosylases remove specific altered bases; AP endonucleases remove residues from sites at which purine bases have been lost). There are often multiple excision repair systems in a single cell type, and they probably handle most of the damage that occurs.
(3) Mismatch repair is accomplished by scrutinizing DNA for apposed bases that do not pair properly. Mismatches that arise during replication are corrected by distinguishing between the “new” and “old” strands and preferentially correcting the sequence of the newly synthesized strand. Mismatches also occur when hybrid DNA

is created during recombination, and their correction upsets the ratio of parental alleles. Other systems deal with mismatches generated by base conversions, such as the result of deamination. The importance of these systems is emphasized by the fact that cancer is caused in human populations by mutation of genes related to those involved in mismatch repair in yeast.

(4) *Tolerance systems* cope with the difficulties that arise when normal replication is blocked at a damage site. They provide a means for a damaged template sequence to be copied, probably with a relatively high frequence of errors. They are especially important in higher eukaryotic cells.

(5) *Retrieval systems* comprise another type of tolerance system. When damage remains in a daughter molecule, and replication has been forced to bypass the site, a retrieval system uses recombination to obtain another copy of the sequence from an undamaged source. These "recombination-repair" systems are well characterized in bacteria; it is not clear how important they are elsewhere.

Most of the mutations in the *TP53* gene occur in the part encoding the DNA-binding domain (DBD). In this domain, every residue has been found to be targeted for substitutions in human cancers (with one exception, residue 123) [37]. These mutations in DBD that inactivate p53 and evidently impair or abolish the ability of p53 to bind to specific DNA sequences that are embedded in its target genes, thereby preventing the transcriptional activation of these genes. Thus, mutations in the DBD are typically recessive, loss-of-function mutations. Other types of mutations are found in the C-terminal homo-oligomerization domain (OD) portion of the polypeptide. Molecules of p53 with these type of mutations dimerize with wild-type p53 polypeptides and prevent the wild-type polypeptides from functioning as transcriptional activators. Thus, mutations in the OD have a *dominant negative* effect on p53 function [108-110]. However, some codons are more frequently mutated than others. Mutations at five major hotspots account for about 30% of all known mutations. These codons are R175, G245, R248, R249, R273 and R282. The apparent hypermutability of these sites is due to two factors. First, these codons encompass CpG sites where cytosines are often methylated, and their spontaneous deamination induces a transition mutation from C to T [111]. This type of mutation is frequent in all cancers. Second, these residues play important roles at the surface of contact between the protein and target DNA. Thus, substitution of these residues results in a protein with decreased affinity for DNA, which has lost the capacity to suppress proliferation [112]. Hotspot mutations can thus be explained by an interplay between mutagenesis, which occurs at specific sites, and selection, which gives to cells with deficient *TP53* function a selective, proliferative advantage during tumour progression. There are many mutagens that can damage DNA in specific ways, leaving fingerprints in the genome of cancer cells. However, many of the mutations found in cancers probably arise spontaneously, through endogenous mechanisms. The most common of these mechanisms are polymerase errors during DNA replication or repair and deamination of methylated cytosine in CpG motifs to form thymine. The latter is enhanced by nitric oxide (NO)— an important bioregulatory agent and signaling molecule that mediates a variety of physiological functions such as vasodilation, neurotransmission, host defense, and iron metabolism— and this type of mutations developed by accumulation nitrotyrosine in the inflamed mucosa of patients and the formation of peroxynitriteare common in tumours occurring within a chronic inflammatory context, such as colorectal or stomach cancer [113].

So far, the identification of precise fingerprints left by mutagens in *TP53* has been possible in a number of cancers where there is good, experimental and molecular demonstration that specific carcinogens play an important role. The most significant examples are liver cancer arising in a context of chronic infection by hepatitis viruses and dietary intoxication by aflatoxins, skin cancers (other than melanoma) resulting from exposure to solar radiations, and lung cancers linked with tobacco smoke. A well-documented mutagen fingerprint is found in non-melanoma skin cancers (NMSC) in relation to sunlight exposure [114]. The *TP53* mutation spectrum in NMSC shows a high frequency of C to T transitions (56% of all mutations), including tandem CC to TT transitions (6%) that are not found in other tumours. They are due to inefficient repair of a common photoproduct, cyclobutane pyrimidine dimers. In individuals with the inherited syndrome xeroderma pigmentosum, a multi-trait disease associated with hypersensitivity to UV, CC to TT transitions represent about half of all observed mutations, though with important differences depending upon the complementation group of the patient [115]. Thus, the CC to TT transitions can be taken as good evidence of the direct DNA-damage generated by exposure to UV. In lung cancers of smokers, the *TP53* mutation pattern is consistent with mutagenesis by at least some classes of tobacco carcinogens [116]. Overall, lung cancer differs from cancers unrelated to smoking by a high prevalence of G to T transversions (30% in lung cancer, compared to an average of 9% in non-tobacco related cancers such as brain, breast or colorectal cancers). These transversions are preferentially located on the non-transcribed strand of *TP53* DNA, and often occur at codons 157, 158, 245, 248 and 273. Although data on non-smokers are still limited, it is known that this type of transversion is not frequent in lung cancers of non-smokers (12%) [117]. Tobacco smoke contains many agents that can potentially induce G to T transversions, particularly oxidative stress agents, nitrosamines, aromatic amines and polycyclic aromatic hydrocarbons. However, the bases at which the G to T transversions occur in lung cancers of smokers are the same as those where benzo(*a*)pyrene preferentially forms DNA adducts *in vitro* [118]. It seems that the presence of a methylated cytosine adjacent to a guanine is an important factor for preferential adduct formation and a direct mutagenic effect of some major tobacco components in lung cancers of smokers [119].

Hepatocellular carcinoma (HCC), the major manifestation of primary liver cancer, is one of the most frequent and malignant disease worldwide and offers one of the most striking example of a mutation “fingerprint” left by a carcinogen in the human genome. The major HCC risk factors include various chemicals and viruses. Among those, chronic HBV and HCV infections attribute to the HCC development in many areas of the world. Other known risk factors, including Aflatoxin B1, cigarette smoking, or heavy alcohol consumption are capable of inducing HCC alone, but they also have synergetic effects. Aflatoxins are mycotoxins produced by species of *Aspergillus* that frequently contaminate staple foods. AFB1 is one of the most potent hepatocarcinogens known, is the predominant form of exposure in humans, *TP53* is mutated in about 30% of HCCs in low-incidence areas, e.g. Western Europe and the U.S.A., and these mutations are scattered along the DNA-binding domain of the gene, with no particular hotspot. However, in areas of high incidence resulting from aflatoxin B1 exposure and HBV infections, *TP53* is mutated in over 50% of cases, with a high proportion of a single missense mutation at codon 249, AGG to AGT, leading to the substitution of an arginine by a serine (Ser-249) [120]. This mutation represents 26% of all *TP53* mutations described to date in HCC and is rather uncommon in other cancers, with no tumour type having more than 2% of Ser-249 mutations. Ser-249 is by far the predominant

mutation in areas of high HCC incidence and high aflatoxin exposure, like Mozambique, Senegal, Qidong country in China, [121], and The Gambia [122]. In contrast, the prevalence of Ser-249 is lower in other areas of China as well as in those African and Asian countries (such as Thailand) where average levels of aflatoxin exposure are lower, and it is virtually absent in HCCs from Europe and the U.S.A., where alcohol but not aflatoxin is an important contributor to liver carcinogenesis. There is good evidence that metabolites of aflatoxin can induce this mutation *in vitro* as well as in cultured cells [123, 124]. However, this is not the only type of mutations that aflatoxin can generate in *TP53* DNA and the reasons for its almost exclusive presence in tumours of high-incidence areas are not yet elucidated. It is possible that Ser-249 has specific, functional properties conferring a capacity to enhance liver carcinogenesis. Indeed, it is quite remarkable that this mutant is rare in tumours other than HCC [125].

An increased cancer risk occurs in tissues of the body undergoing chronic inflammation [126]. Reactive oxygen and nitrogen species —hydrogen peroxide (H_2O_2), nitric oxide ($NO^{\bullet}$), and reactive intermediates such as hydroxyl radicals($OH^{\bullet}$), superoxide ($O_2^{-\bullet}$), and peroxynitrite ($ONOO^-$)— produced by inflammatory cells can interact with key genes involved in carcinogenic pathways such as *TP53*, DNA mismatch repair genes, and even DNA base excision-repair genes [127]. Free radicals, as well as hydroxyurea, can also lead to DNA replication stress involving the ATR kinase network [128, 129]. Although it appears that the onset of carcinogenesis associated with inflammation is mediated by free radical species, identification of specific free radicals and their targets remains vague.

In most researches be has hypothesized that the inflammatory microenvironment activates the p53 network and inactivates the tumor suppressor activity by mutation of the *TP53* gene by NO pathway —NO is a candidate free radical, and the p53 tumor suppressor is a candidate molecular target— and *TP53* mutations contribute to clonal cellular expansion and genomic instability through deregulation of cell cycle checkpoints, DNA repair, and apoptosis[130, 131]. However, before somatic mutations, *TP53* mediates these anticarcinogenic cellular functions through a DNA damage-response [24, 127].

To place the molecular pathogenesis of some tumors, it is first helpful to appreciate the molecular events involved in the development of neoplasia. Since arises as a result of genomic instability. The two main types of genomic instability that contribute to carcinogenesis are chromosomal instability (CIN) and microsatellite instability (MSI). Chromosomal instability results in abnormal segregation of chromosomes and abnormal DNA content (aneuploidy). As a result, loss of chromosomal material [loss of heterozygosity (LOH)] often occurs, contributing to the loss of function of key tumor suppressor genes such as *APC* and *TP53*. These genes can also be rendered nonfunctional by mutation. In either event, it is the accumulation of molecular disturbances mainly in tumor suppressor genes that drives the sporadic adenomacarcinoma progression, and therefore this pathway has sometimes been referred to as the "suppressor pathway" [132].

Epigenetic alterations also contribute to altered gene expression in carcinogenesis. A recently recognized molecular alteration is the CpG island methylator phenotype (CIMP) [133]. CpG islands are dense aggregates of cytosine-guanine dinucleotide sequences that may occur in the promoter region of genes. Extensive methylation of the cytosine bases is associated with promoter silencing and loss of gene expression. Many genes involved in cell cycle control, cell adhesion, and DNA repair can be methylated in colon cancer [134].

To reduce the level of reactive oxidants and limit their damaging effects (particularly to DNA, RNA, proteins, and lipids), several defense mechanisms have evolved. In addition to reactive oxidants, the microenvironmental changes within inflamed tissues are typically associated with hypoxic conditions, in which oxygen tensions<10 mm Hg (i.e., <1% oxygen) have been reported [135]. Thus, hypoxia is an additional cell stress mediator concurrently found during chronic inflammation [136].

CONCLUSION

Over the course of evolution, mammalian cells have acquired an intrincate network of protective mechanisms to safeguard the genomic integrity. A serious consequence from a failure in the safety networks is cancer. The fact that p53 pathway is defective in the majority of human cancers, underscores its importance in protecting the cells from genetic, biochemical, and physiological dysregulation that can contribute to tumor development. Moreover, with the development of new, sensitive and high-throughput methods for mutation detection, analysis of *TP53* hotspots mutations may become an essential aid to the identification of specific cancer risk factors in human populations. Currently, the number of types of *TP53* mutations described in the world literature increases by two to three thousand every year. It is very likely that this trend will continue in the coming years, confirming the status of *TP53* as a central piece of the puzzle in the molecular biology of human cancer.

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Chapter III

DNA DAMAGE RELATED TO EXPOSURE TO OIL SPILLS: THE *PRESTIGE* EXPERIENCE

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ABSTRACT

Oil spills are one of the most frequent ecological disasters giving rise to the pollution of enormous coastal areas and affecting also the local flora and fauna. As a direct consequence of them, a large number of individuals is always involved, as they take part in the different tasks derived from the need of cleaning the spilled oil and recovering the natural environments. From a toxicological point of view, oil is a complex mixture of compounds that can penetrate into the body burden through dermal, respiratory or digestive routes. Despite the huge number of spills occurred all around the world, the international literature dealing with the harmful effects of this exposure is very scarce and restricted to the acute and psychological effects. This seems paradoxical attending to the large number of carcinogenic agents composing this mixture.

So, after the *Prestige* oil spill (November 2002, NW of Spain) and taking into account the seriousness of the catastrophe, the large number of individuals involved, the damaging character of oil components and especially the lack in the scientific literature of reports considering the consequences of this exposure from a genotoxic or carcinogenic point of view, we developed an extensive biomonitoring study including effect and susceptibility biomarkers. Three groups of exposed individuals whose exposure during the recovery of *Prestige* oil polluted areas differed quantitatively (short and acute or more prolonged in time) and qualitatively (as a consequence of the different methods used for this purpose) were included. Environmental concentrations of volatile organic compounds were evaluated by means of passive dosimeters in each exposure group. Two types of effect biomarkers were applied: the comet assay, characterized by its high sensitivity in population studies and for reflecting also DNA repair phenomena, and

two well established cytogenetic assays, micronucleus test and sister chromatid exchanges. Moreover, due to the fact that individual differences in terms of susceptibility to xenobiotics have been extensively reported and mainly attributed to some polymorphisms in genes encoding for biotransformation enzymes and DNA repair proteins with functional consequences, a complete set of the most relevant were also included in this study.

1. INTRODUCTION

Oil is obviously one of the main sources of energy acquiring a central position for human development since 1900, when oil first entered general use. Nowadays oil serves a wide diversity of purposes, which include transportation, heating, electricity and industrial applications, and is an input into over 2,000 end products (International Labour Organization, 2002). But all these benefits entail a risk derived from need of on and off shore exploration, drilling and extraction activities besides its transportation and refining activities, all of them dangerous and affecting ecosystems and human health.

In 2003 there were approximately 40,000 oil fields in the world (Mead, 1993). The global seaborne trade increased by almost 70% from 1990 to 2004, and oil transportation grew by 30% in the same period (UNCTAD, 2005). Actually, the European Union assumes a continued increase in trade from Baltic ports until 2017, leading to a 25% higher risk of large oil spills (HELCOM, 2003).

As a consequence of the intense exploitation and maritime traffic, a large amount of oil is spilled to the oceans both from leaks during the extraction activities and especially from accidents and wrecks of oil tankers. In 2002, the US National Academy of Sciences estimated that 38,000 tons of petroleum hydrocarbons were released into the world's oceans each year during the 90s as a result of oil and gas operations (Committee on Oil in the Sea: inputs, fates and effects, 2003). Specifically, in the past two decades several major spills took place generating the pollution of huge coastal areas all over the world as it happened with the wreck of Exxon Valdez (Alaska, 1989), Braer (Shetland Islands, 1993) Sea Empress (Wales, 1996), Nakhodka (Japan, 1997) and Erika (France, 1999).

The last one, considered the biggest large-scale catastrophe of this type in Europe (Alonso-Alvarez, 2007), took place in the northwest of Spain on November 13, 2002. At 15:15h the big 26-years old single-hulled oil tanker *Prestige* sent a SOS call as a consequence of having a water leak. The tanker containing more than 77,000 tons of heavy oil remained listed between 25 and 45 degrees to starboard at 28 miles of Finisterre cape. After rescuing the crew the ship started being towed, but in the morning of Thursday 14, the spot of oil spilled already exceeded 5 miles length. The same night around 3,000 tons of oil were spilled and four days later 150 km of coastal area were polluted. On Tuesday 19, the oil tanker broke in two and sank in roughly 3,000 meters of water about 150 miles off Galicia. The more than 5,000 tons of oil spilled in that moment constituted the first one of the three black tides that reached the shore during the following months favoured by the strong weather conditions. The precise following day the first fishermen started working on the recovery tasks.

The tanker continued leaking more than 125 tons per day through the 14 cracks of its hull, and eventually more than 60,000 tons of fuel polluted around 900 km of Spanish and French coast including 675 Galician beaches and eight natural spaces. Beside this a large

number of animals, especially seabirds, were affected by the spill, actually on February 3, 2003, 130,000 birds were recorded and only 10% of them survived.

In view of such catastrophe, a huge human mobilisation took place in order to try to minimize the adverse consequences. Between November 2002 and July 2003, more than 300,000 volunteers from different places of Spain and Europe, added to the staff hired by the Galician Autonomous Government, collaborated in the cleaning tasks to recover the fauna and shoreline affected by the oil. This human collaborative response usually takes place after this kind catastrophes, and is indirectly generating an enormous population all over the world highly exposed to oil during certain periods of time and in different ways.

The United States Environmental Protection Agency (USEPA) gathers refined petroleum products into 8 classes namely gasoline, kerosene, No. 2 to 6 fuel oils, and lubricating oil, many of them classified as possible human carcinogens by the International Agency for Research on Cancer (IARC), as it is the case of the oil transported by the *Prestige* (No. 6 fuel oil). This type of oil is defined as heavyweight material difficult to dissolve and disperse, and likely to form tar balls, lumps and emulsions; its low solubility and volatility make it extremely persistent in the environment. *Prestige* oil was a complex mixture of compounds with high density (0.99 g/cm^3) and viscosity (30,000 cSt at 15 °C). The analyses of this oil made by the Spanish National Research Council revealed 50% aromatic hydrocarbons, 22% saturated hydrocarbons and 28% resins and asphaltenes (CSIC, 2003a). Moreover, high concentrations of zinc, cadmium and lead, and other heavy metals like aluminium or nickel were found in the seawater column on the shipwreck and in the emulsified oil that reached the coast, respectively (CSIC, 2003b; Prego and Cobelo-García, 2004). Three groups of compounds must be highlighted among all these with regard to their toxic properties to human health, namely volatile organic compounds (VOC), polycyclic aromatic compounds (PAH) and heavy metals. In fact, the European Communitarian Normative establishes these three as the main in which the risk evaluation must be focused (Commission Directive 93/67/EEC; Council Regulation (EEC) No. 793/93; Commission Regulation (EC) No. 1488/94).

The term VOC refers to substances containing carbon atoms whose vapour pressure at 20ºC is at least 0.13 kPa, with exception of CO, CO_2, organometallic compounds and organic acids. Among the wide range of agents integrating this group there are many well established toxic compounds, some of them presenting mutagenic and/or carcinogenic properties. Their effects are increased due to their high volatility in natural conditions, and this is a clue factor that facilitates their intake through the respiratory pathway. From a toxicological point of view, benzene and alkylbenzenes [toluene, ethylbenzene and xylenes (*orto-*,*meta-* and *para-*)] (BTEX) (Figure 1) deserve special attention, and are usually present into the light fractions of distilled petroleum. The initial concentrations of benzene and BTEX in the *Prestige* oil were 59mg/kg and 345mg/kg, respectively (CSIC, 2003c).

PAH are ubiquitous contaminants whose presence is associated with the use of fossil fuels, but they can also be generated during some industrial processes as iron industry, tar and asphalt production, etc. The precise mechanism by which they are formed is not fully understood, but it is considered as due to two phenomena: pyrolysis and pyrosynthesis. So, at high temperatures (from 400 to 1000ºC) organic compounds are easily fragmented generating free radicals, that further react and give rise to relatively stable PAH (Jägerstad y Skog, 2005). Among their effects on the organisms, their potential mutagenicity and carcinogenicity stand out again. Actually, six PAH specifically present in the *Prestige* oil were classified as

possible or probable human carcinogens by the IARC, and were included among the 16 PAH designated by the USEPA as primary contaminants: benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenz[ah]anthracene and indeno[1,2,3-cd]pyrene (Figure 2). Nowadays PAH are considered as procarcinogens and tumour inductors. The mechanisms responsible for their genotoxic properties find their origin in their high affinity by the nucleophilic centres of the great organic macromolecules (RNA, proteins and especially DNA), with which they covalently bind constituting a key process in chemical carcinogenesis(Borgert *et al.*, 1993). Beside this, their involvement in the generation of DNA strand breaks via free radical mechanisms has also been reported (Mitchelmore *et al*., 1998).

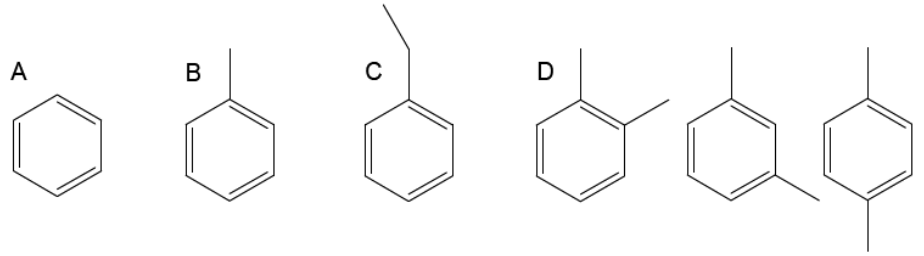


Figure 1. Chemical structures of benzene (A), toluene (B), ethylbenzene (C) and o-, m- and p-xylene (D).

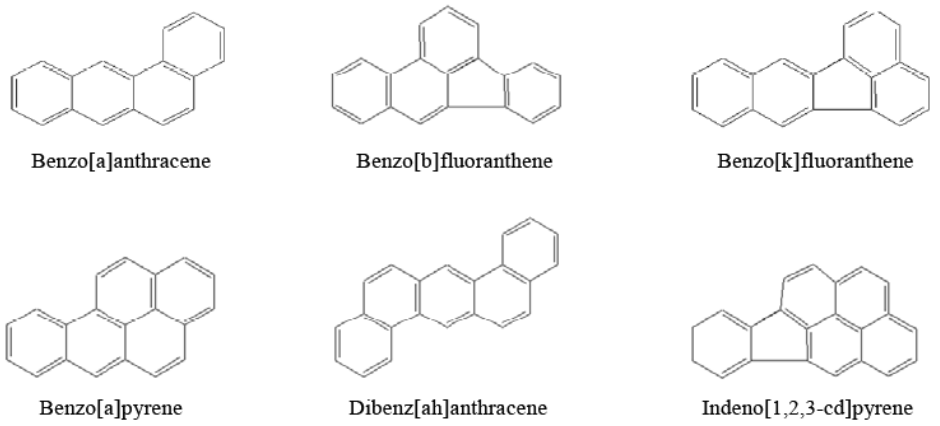


Figure 2. Chemical structures of possibly carcinogenic polycyclic aromatic hydrocarbons contained in the Prestige oil.

The third group of compounds included among the oil integrants that deserve especial attention due to its health effects is integrated by heavy metals. These are highly toxic agents both in short-term and especially in long-term, mainly due to bioaccumulation phenomena. They were classified as toxic by the USEPA and the IARC as they give rise to numerous toxicological processes essentially derived from their ability to denature macromolecules, and many of them also act by altering the fidelity of DNA transcription, generating apurinic mediators or competing with essential metals needed to macromolecule stabilisation (Léonard and Bernard, 1993).

Even though all these compounds have always been present in the environment, human being activities magnified their concentrations to limits that started being dangerous. The first references regarding to the toxicity of these compounds go back to the 16th century when Paracelsus designated the "disease of the miners" (silicosis). Subsequently, in the 18th century Hill and Pott focused their studies on the evaluation of the effects of PAH present in tobacco smoke and soot in London. Nowadays a large number of studies reported the mutagenic and carcinogenic effects of these compounds.

Nevertheless if we have a look to the international literature dealing with human health effects after oil spills, we find a hollow in this regard. Although there are several works on the environmental consequences of these catastrophes (Le Hir and Hily, 2002; Harwell and Gentile, 2006; Laffon *et al*., 2006a), very few are focused on human health and most evaluated acute toxicity indicators (Attias *et al*., 1995; Lyons *et al*., 1999; Morita *et al*., 1999; Suárez *et al*., 2005; Carrasco *et al*., 2006), or psychological symptoms (Palinkas *et al*., 1992, 1993; Lyons *et al*., 1999). Moreover, none of them consider the long-term effects derived from the exposure, excepting one that analysed the prolonged respiratory symptoms (Zock *et al*., 2007), which turns out to be surprising in view of the large number of exposed individuals all over the world and the seriousness of the mutagenic and carcinogenic properties of oil components. Genotoxicity tests are the optimal tool for this purpose due to the narrow relationship between carcinogenesis and genotoxic events. The number of existing assays is quite wide comprising some well established to evaluate the carcinogenic risk associated with an environmental or occupational exposure. Nevertheless, as the type of damage exerted on the DNA can be different, the employment of a set of tests constitutes the most accurate way to reflect what is taking place. Some of the most extensively used biomarkers of gentoxic effect are the single-cell gel electrophoresis (comet assay) micronucleus (MN) test, and sister chromatid exchanges (SCE).

On the other hand, after an exposure, organisms proceed to the biotransformation of the chemicals and the repair of the damage caused, and at this point the effectiveness of the mechanisms involved plays a crucial role. Individual variations in polymorphic genes participating in these processes have been linked with an increased risk of cancer in several case-control studies (IARC, 1999), and may be important in the estimation of the risk to humans from exposure to environmental toxicants. In fact, they are increasingly evaluated in the recent years as susceptibility biomarkers in human population toxicological studies.

The following sections of this chapter deal with the evaluation of the exposure and its genotoxic effects in a population of 240 individuals directly exposed to oil during the recovery tasks after the *Prestige* oil spill. To the best of our knowledge, this is the first study on the genotoxic damage caused in humans by oil exposure after an accidental spill.

2. Study Population

As a response to the *Prestige* serious environmental catastrophe a large number of volunteers coming from numerous points in Spain and Europe was involved in the shoreline cleaning tasks. Furthermore, the Galician Autonomous Government hired a broad staff to the same purpose. So that, individuals included in this study experienced two main types of exposure: the volunteers (V group, N=61) a short-term one (only 5 days), while the exposure

period of the hired workers was a long-term one (3-4 months). This last group was also divided qualitatively, as some of them used high-pressure water cleaning machines in the rocks (HPW group, *N*=60), and the rest collected the oil manually in the beaches as the volunteers (MW group, *N*=59). A fourth category of non-exposed individuals was included as control group (*N*=60). A detailed questionnaire guaranteed the absence of previous exposures that could mask or distort the results, as well as gave information referred to physiological and lifestyle factors. All individuals gave their informed consent for participating in the study. The characteristics of the study individuals are gathered in Table 1.

Table 1. Characteristics of the study population

	Controls	Exposed			
		Total	V	MW	HPW
Total No. of individuals	60	180	61	59	60
Sex					
female	37	88	38	20	30
male	23	92	23	39	30
Age (years)[a]	23.15±4.80	32.26±11.68	22.95±4.55	38.49±11.46	35.78±10.96
Smoking habits					
non-smokers	41	112	44	39	29
smokers	19	68	17	20	31

[a]Mean±SD.

In the exposed population 38.3% were males and 37.8% smokers (8.81±10.99 packs-year). Protective devices used included clothes (waterproof overalls) and cellulose mask, although some individuals did not wear them (18.3% for clothes and 6.7% for mask). In the control group 51.1% were males and 31.7% were smokers (3.92±4.64 packs-year).

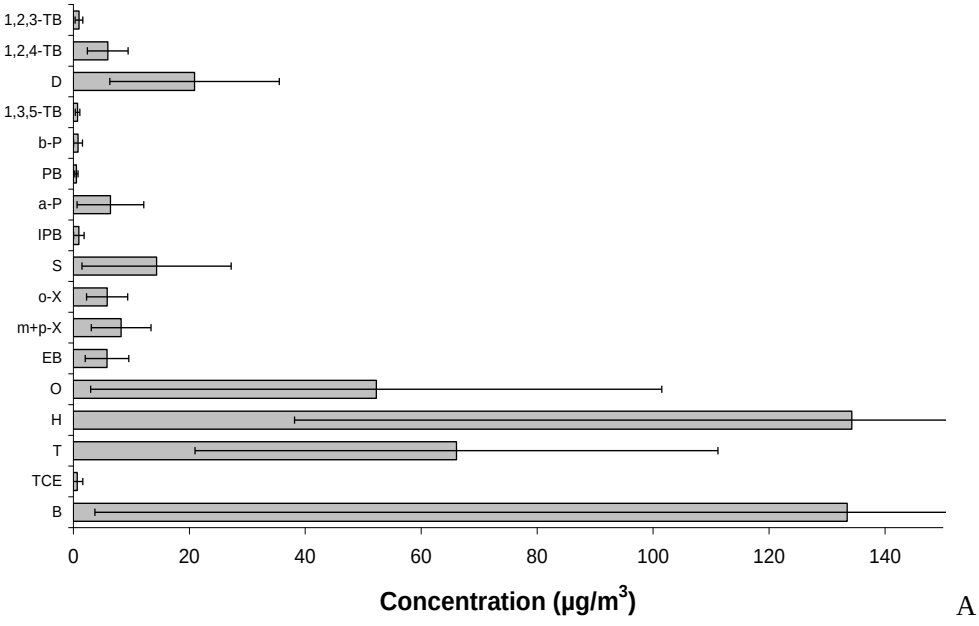
Samples were collected between March and May 2003. Peripheral blood was drawn early in the morning before the working shift by venipuncture in three types of tubes, one containing lithium heparine for SCE and MN test, one EDTA container for DNA extraction and genotyping, and a special BD Vacutainer™ CPT™ for leukocyte isolation for the comet assay. All samples were codified and refrigerated until arrive to the laboratory, where they were processed according to each established protocol.

3. EXPOSURE EVALUATION

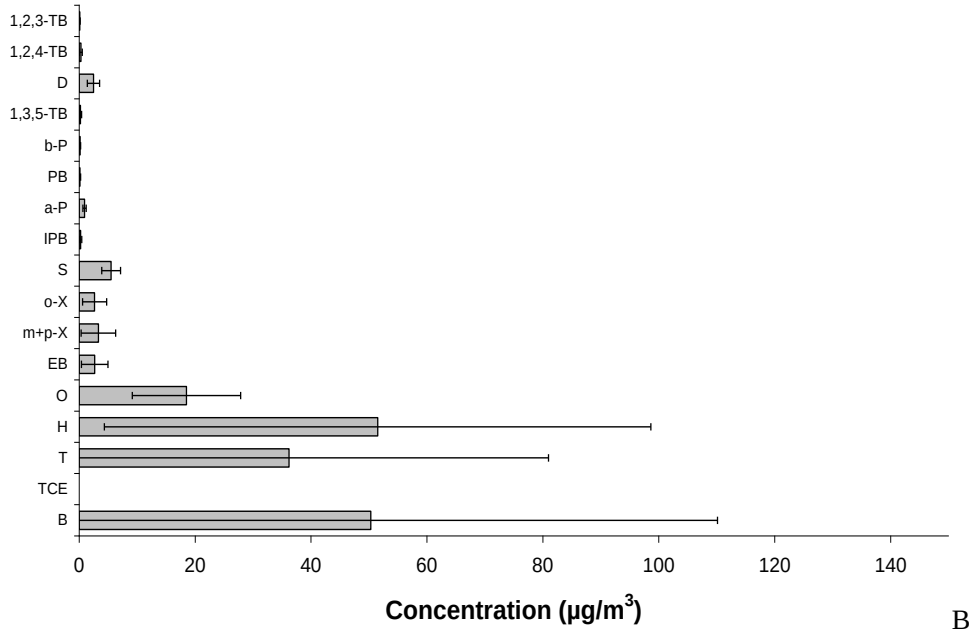
Respiratory tract constituted the main exposure route for the oil components. In order to evaluate the exposure levels, passive dosimeters were randomly distributed among individuals (28 to V, 19 to MW and 19 to HPW) who wore them during the complete working shift. Both Perkin Elmer tubes filled with 200mg of Tenax TA and Radiello® tubes were thermally desorbed and analyses were carried out by gas chromatography coupled to ion trap mass spectrometry (ITMS) (Fernández-Villarrenaga *et al*., 2004). A wide list of the most representative VOC, including also the most harmful was analysed: benzene (B), tetracloroethene (TCE), toluene (T), *n*-heptane (H), *n*-octane (O), ethylbenzene (EB), *m*+*p*-xylene (m+p-X), *o*-xylene (o-X), styrene (S), isopropylbenzene (IPB), *a*-pynene (a-P), *n*-

propylbenzene (PB), *b*-pynene (b-P), 1,3,5-trimethylbenzene (1,3,5-TB), *n*-decane (D), 1,2,4-trimethylbenzene (1,2,4-TB), and 1,2,3-trimethylbenzene (1,2,3-TB). Two commonly used indices were determined: the sum of the total quantity of the VOC determined (TVOC), and the sum of benzene, toluene, ethylbenzene and *o*-, *m*-, and *p*-xylenes (BTEX).

Figure 3 shows the profiles obtained for every VOC in each exposure group.



A



B

Figure 3. (Continued)

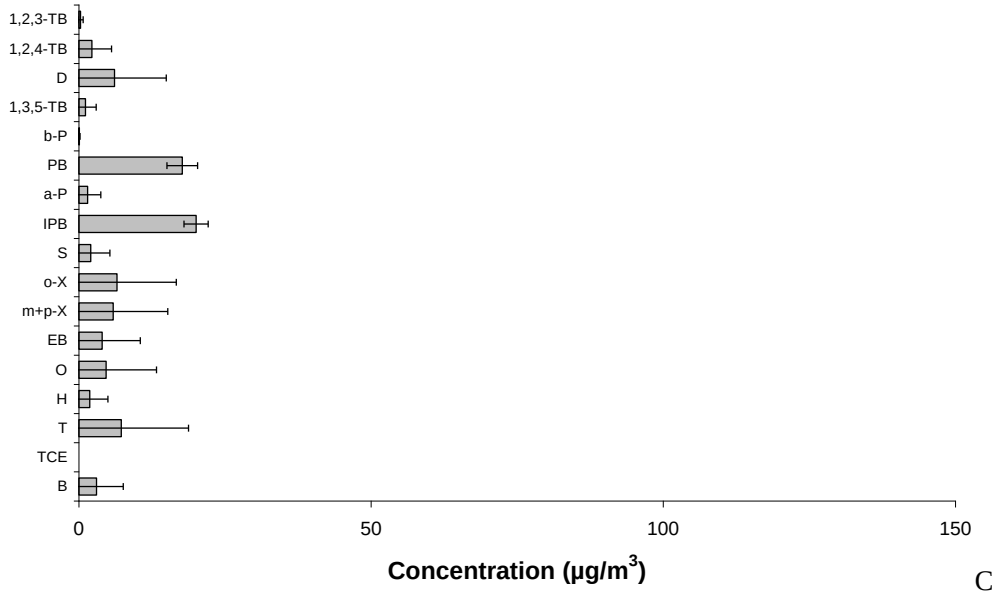


Figure 3. VOC profiles for each exposed group: V (A), MW (B), and HPW (C). Bars indicate standard deviation. Acronyms used for VOC are shown in the text.

A major presence of light hydrocarbons as benzene, *n*-heptane, toluene and *n*-octane was observed in all cases, followed by styrene and *n*-decane, and in HPW group isopropylbenzene and *n*-propylbenzene.

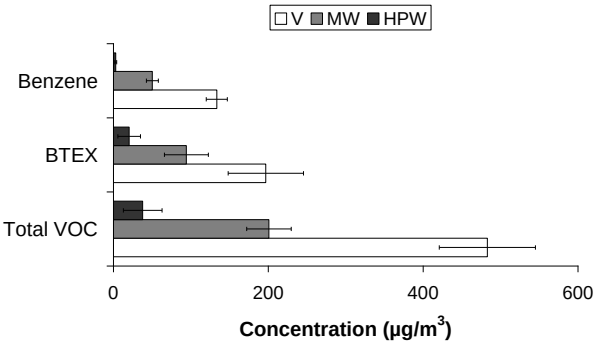


Figure 4. Mean environmental concentrations of TVOC, BTEX and benzene for each exposed group. Bars indicate mean standard error. **P<0.01, *P<0.05, significant difference with regard to the V group.

When comparing quantitatively the exposure levels among groups (Figure 4), the three evaluated parameters (TVOC, BTEX and benzene) were significantly higher for V group than for MW or HPW, the last one presenting the lower concentrations. Significant differences were not obtained between the two groups of hired workers. In general, concentrations obtained in V and MW groups were similar to those usually present in cities with very intense traffic, and higher than those expected in an open environment like a beach. Anyway, environmental concentrations observed were below the limits established by the American Conference of Governmental Industrial Hygienists (ACGIH, 2005) and the Spanish *Instituto*

Nacional de Seguridad e Higiene en el Trabajo (INSHT, 2006) referred to closed environments. VOC values obtained in HPW were lower than in the other two groups probably due to the use of high-pressure cleaning machines which may contribute to the dispersion of the compounds, or to the fact that the oil deposited on the rocks was more aged.

A similar dosimetry methodology was used by Morita *et al*. (1999) to evaluate these compounds in the environment after *Nakhodka* spill in Japan. Values obtained were close to our minimum levels, but they were considerably lower than our mean values for benzene, toluene and xylene (3.49±1.83, 15.78±11.22 and 3.41±1.99μg/m^3 *vs.* 76.36±14.05, 81.58±36.40 and 30.68±17.38μg/m^3, respectively). Both oils were fairly similar qualitatively, but *Nakhodka* spill was less substantial, wasting over 6,000 tons of oil.

4. EFFECT BIOMARKERS

4.1. Comet Assay

The comet assay is a reliable method to assess DNA damage in individual cells based on the principle of quantifying the amount of denatured DNA fragments migrating out of the cell nuclei during electrophoresis. The alkaline version allows the detection of double and single strand breaks, delay DNA excision repair sites, and alkali-labile sites. So it gives a good reflect of a very early response of the organism to the effects of genotoxic agents on DNA integrity. This is a key point in the interpretation of the results provided by this assay, as these lesions are usually repaired quite fast and thus the reflected damage is relatively recent. Furthermore, its high sensitivity provides this assay with a high potential for quantifying DNA damage in populations exposed to low levels of genotoxic agents (Sul *et al*., 2003).

Mononuclear leukocyte isolation was made using BD Vacutainer™ CPT™ Cell Preparation Tubes (Becton Dickinson)following manufacturer's instructions. Cells were resuspended in frozen medium (40% RPMI 1640, 50% foetal bovine serum and 10% DMSO) and stored at -80ºC in a Nalgene® Cryo 1ºC Freezing Container (Nalgene Nunc International) until their use. Leukocytes were thawed at 37ºC and their viability was checked with trypan blue, being always ≥85%. Alkaline comet assay was performed as described in Singh *et al*. (1988) with minor modifications (Laffon *et al*., 2002). One hundred cells per donor (50 per replicate slide) were analyzed by a blind scorer employing the QWIN Comet software (Leica Imaging Systems, Cambridge, UK). The percentage of DNA in the comet tail (%TDNA), tail length (TL) measured from the estimated centre of the cell, and tail moment (%TDNAxTL) were used as DNA damage parameters.

Results from the three variables, adjusted by sex, age and smoking habits (Figure 5), showed the same increasing tendency in all exposed groups regarding to controls. Specially, V group experienced the more noticeable variation, agreeing with its highest VOC exposure levels. On the other hand, nowadays several studies suggest the possible existence of an adaptive response to stress situations (Benner *et al*., 1992; Gourabi and Mozdarani, 1998; Rothfuss *et al*., 1998; Bonassi *et al*., 2003). Thus, a prolonged low or medium intensity exposure to an agent would minimize the damage induced by it in a subsequent exposure. Taking into account the immediate response and sensitivity characteristics of comet assay, the adaptive response seems a suitable explanation for the higher damage values obtained in V

group regarding to MW and HPW, who were exposed to oil for 4 and 3 months, respectively, prior to the sample collection.

Although the three parameters used could be adequate to reflect the DNA damage, %TDNA was selected in order to simplify subsequent analysis since it results more sensitive and has a better dose-response relationship (Kumaravel and Jha, 2006).

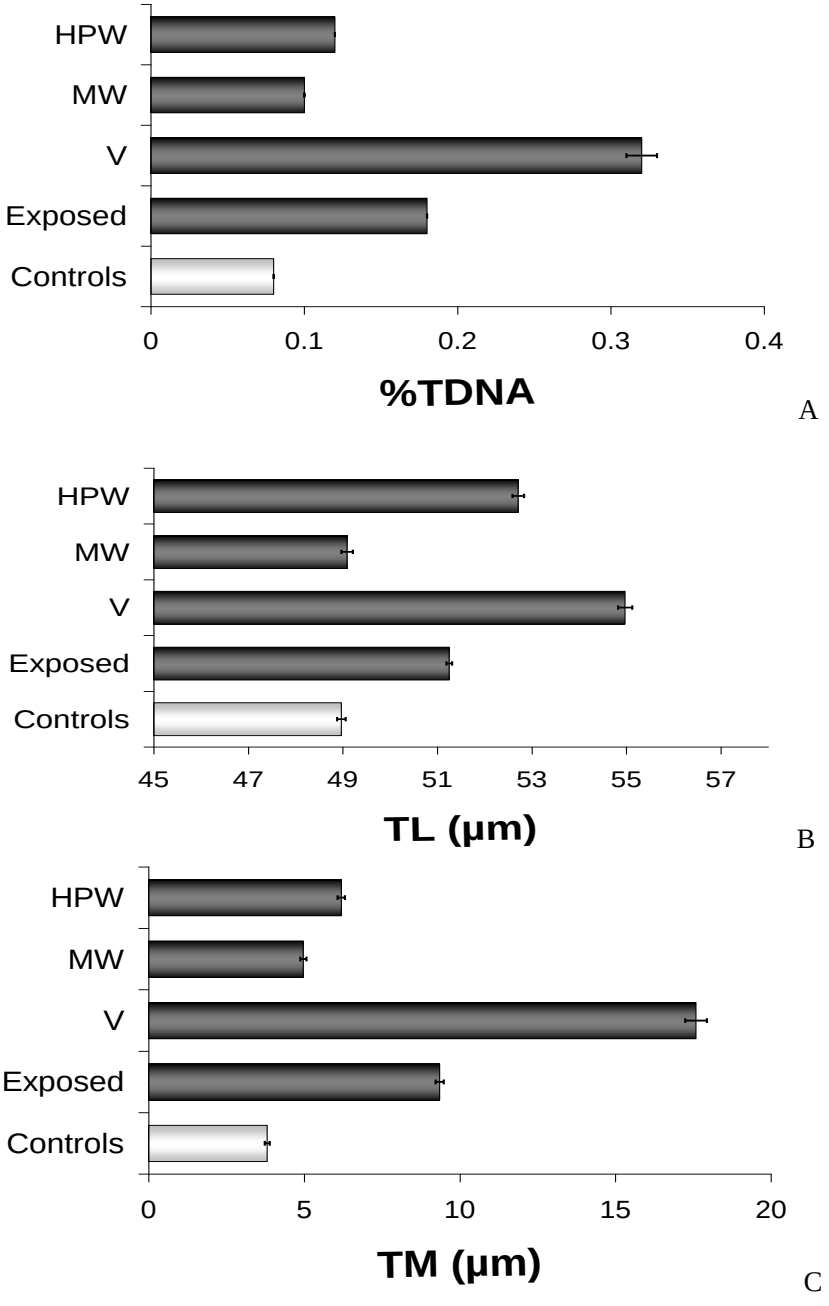


Figure 5. Effect of exposure on DNA damage expressed as %TDNA (A), TL (B) and TM (C). Results adjusted by sex, age and smoking habits. Bars indicate mean standard error. **P<0.01, significant difference with regard to the controls.

A major problem affecting the design of studies in molecular epidemiology is the uncertainty about the impact of confounding factors, as the role of some lifestyle factors that are widely considered to be influential in this kind of events. Given that we performed a specific analysis evaluating three of the most relevant: sex, age and tobacco consumption (Table 2). When considering these factors individually after adjusting by exposure, comet assay results were influenced by sex (males higher DNA damage than females) and age (increasing DNA damage with increasing age); both of them remained significant when they were mutually adjusted (partial *P* values 0.003 and <0.001, respectively, model *P* value <0.001). Furthermore, there was a significant correlation between %TDNA and age (Pearson coefficient r=0.043, *P*<0.01). These results agree with the influential role of sex on DNA damage, specially when employing short-term markers (Collins, 1999), and with the fact that age has been widely related to increases in the background damage levels, losses in the efficacy of the repair mechanisms and the subsequent damage accumulation. The decrease of the defence mechanisms against toxicity related to age was found to be associated with the generation of reactive oxygen species and the decrease of agents participating in the detoxification processes as glutathione (Rihs *et al*., 2005). Tobacco smoke is especially associated with the exposure evaluated in this work due to the great number of highly toxic component substances that share with oil (e.g. benzo[a]pyrene, benzene, cadmium, etc.). Although smoking did not influence comet assay results, smokers presented significantly higher DNA damage levels than non-smokers in V group (0.375±0.012 *vs*. 0.304±0.007, *P*<0.01) but the opposite in MW group (0.087±0.003 *vs*. 0.111±0.003, *P*<0.01).

Table 2. Influence of sex, age, smoking habits (pack-year) on DNA damage (Models adjusted by exposure)

Model	Unstandardized coefficients β	95% CI		Partial *P* value	Model *P* value
Males *vs.* females	0.015	0.009 to	0.022	0.000	0.000
Age (years)	0.002	0.002 to	0.002	0.000	0.000
Smoking habits	$-1.8x10^{-5}$	$-4.3x10^{-5}$ to	$0.7x10^{-5}$	0.161	0.000

Garaj-Vrhovac and Kopjar (2005) suggested that enhanced DNA repair mechanisms in smokers might be the reason why the effect of an additional occupational exposure was more pronounced in non-smokers. Faust *et al*. (2004) also supported this hypothesis proposing that the stimulation of DNA repair or detoxifying mechanisms induced by xenobiotics coming from occupational exposures are important in the attenuation of the effect of chemical agents from the tobacco smoke, and this could happen also in the inverse way.

Exposed individuals were provided with protective devices (clothes and cellulose mask). Influence of their employment was evaluated and increased DNA damage was associated with the absence of using clothes (partial *P* value 0.003, model *P* value <0.001), while no influence of wearing mask was observed. These data suggest that dermal exposure was suitably avoided, but inhalation exposure occurred in spite of using mask. The inappropriate characteristics of the masks employed or their inadequate utilization by the individuals are probably the reasons that underlie these unexpected results. In contrast, use of mask determined a significant decrease in DNA damage levels (comet assay) in individuals

engaged in the cleaning and autopsies of *Prestige* oil contaminated birds (Laffon *et al*., 2006b).

4.2. Micronucleus Test

MN originate from chromosome fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division. As a consequence, MN require a cell division to be expressed. The cytokinesis-block technique proposed by Fenech and Morley (1985) uses cytochalasin-B, which arrests division of cytoplasm without inhibiting nuclear division, and enables the recognition of cells that had completed one nuclear division by their binucleated appearance. On the other hand, nucleoplasmic bridges (NPB) are an important biomarker of chromosome rearrangement originated from dicentric chromosomes that are pulled to opposite poles of the cell at anaphase (Fenech, 2000), and they may be also scored in binucleated cells.

The frequency of MN in peripheral blood lymphocytes is extensively used as a biomarker of chromosomal damage and genome stability in human populations exposed to genotoxic agents (Fenech *et al*., 1999), and preliminary evidence that it is a predictive biomarker of cancer risk within a population of healthy subjects has been recently reported (Bonassi *et al*., 2007). Moreover, MN test is unlike to generate false positive results (Fenech 2006), and its key advantage is the relative ease of scoring and the statistical power obtained from scoring larger number of cells than are typically used for metaphase analysis.

Heparinised whole blood (0.5ml) was cultured in duplicate at 37ºC in 4.5ml RPMI 1640 medium supplemented with 15% heat inactivated foetal bovine serum, 1% phytohaemagglutinin, 1% L-glutamine (200mM) and 1% penicillin (5000U/ml)/streptomycin (5000μg/ml) (all from Invitrogen, Barcelona, Spain). Cultures were maintained for 44h and then 10μl of cytochalasin B were added at a final concentration of 6μg/ml. Cells were harvested by centrifugation after 64h of culture and treated with a mild hypotonic solution (0.075mM KCl at 4ºC). Then cells were centrifuged again, fixed in Carnoy (methanol-acetic acid 3:1 v/v), placed on dry slides and stained with 4,6-diamidino-2-phenylindole (DAPI). Binucleated cytokinesis-blocked cells and MN were identified on a Leica DM-RXA microscope equipped with a 100W mercury lamp, according to the criteria of Fenech *et al*. (2003). Two genotoxic parameters were evaluated by scoring 1000 binucleated cells (500 from each duplicate culture): frequency of binucleated cells with MN (BNMN) and frequency of NPB. Furthermore, a cytokinesis-block proliferation index (CBPI) was determined as an estimation of possible cytotoxic processes. Five hundred cells (250 from each replica) were examined and classified according to the number of nuclei. CBPI was calculated following the formula CBPI=(MI+2xMII+3x(MIII+MIV)/Total, where MI-MIV represent the number of cells with 1-4 nuclei.

Figure 6 represent the results obtained for each exposure group, after adjusting by age, sex and tobacco smoking. Higher levels of BNMN frequencies were obtained in both groups of workers, but not in V, although statistical significance was only reached in MW. This suggests that a relatively prolonged exposure time was required for this cytogenetic damage to become obvious. Similar data were obtained from NPB frequency parameter, although in this case the effect was much smoother and statistical significance was not reached.

MN test reflects both aneugenic and clastogenic types of damage. Thomas *et al*. (2003) proposed the relation NPB/MN as an index to determine the type of damage, in such a way that if the coefficient is close to 0 the effect would be aneugenic, while the index for clastogens would be around 0.77. The values obtained in this study are 0.03, 0.03 and 0.04 for the groups V, MW and HPW, respectively, indicating that *Prestige* oil constitute a mainly aneugenic mixture of agents.

Based on current knowledge, it could be anticipated that agents that induce genome damage are also likely to cause cell cycle delay (Fenech, 2000).

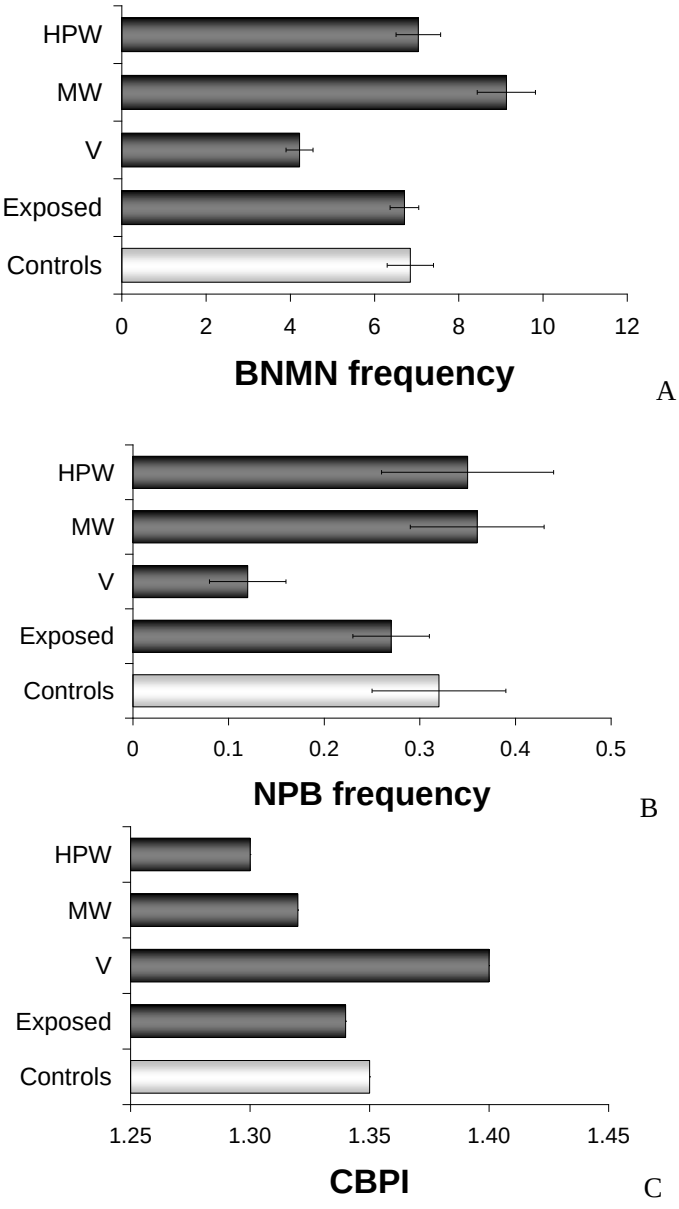


Figure 6. Results of cytokinesis-block MN test in the study population, after adjusting by age, sex and tobacco smoking: BNMN frequency (A), NPB frequency (B) and CBPI (C). Bars indicate mean standard error. **P<0.01, significant difference with regard to the controls.

This fact could be observed in the response of CBPI to exposure, bringing to light a significant decrease in the proliferation rate in lymphocytes from individuals belonging to MW and HPW, the two groups with longest exposures, pointing again to the need of a relatively long period of exposure both for the genotoxic and cytotoxic effects of oil to be detected.

Table 3 details the analysis of the effect of sex, age and tobacco consumption on the MN test genotoxic response variables. When considering these factors separately, significant influence of the first two was observed on BNMN frequency in an analysis adjusted by exposure. Among them, age has shown to exert the strongest influence, as it was the only one that remained significant in a further analysis where variables were mutually adjusted (partial *P* value 0.010, model *P* value <0.001), agreeing with previous data presented by the HUman MicroNucleus (HUMN) international project (Bonassi *et al*., 2001). The increase of cytogenetic damage with age indicated by the model can be in accordance with the reduction in the ability of the metabolic enzymes involved in biotransformation processes to detoxify compounds as a result of the ageing process (Ingelman-Sundberg, 2001). This observation, together with the absence of tobacco consumption effect on the cytogenetic damage levels, supporting previous findings of the HUMN project for soft-smokers (Bonassi *et al*., 2003), agree with the results obtained by means of the comet assay, generating a common pattern of influential factors. Significant influence of these factors on NPB frequency was not obtained, either individually or mutually adjusted.

Table 3. Influence of sex, age and smoking habits (pack-year) on BNMN and NPB frequencies (Models adjusted by exposure)

Model	Unstandardized coefficients β	95% CI		Partial *P* value	Model *P* value
1. BNMN					
Males *vs.* females	-1.120	-2.19 to	-0.05	0.041	0.000
Age (years)	0.085	0.02 to	0.15	0.011	0.000
Smoking habits	-0.014	-0.09 to	0.07	0.733	0.000
2. NPB					
Males *vs.* females	-0.038	-0.18 to	0.10	0.592	0.074
Age (years)	-0.002	-0.01 to	0.01	0.684	0.168
Smoking habits	-0.008	-0.02 to	0.00	0.142	0.026

When the possible influence of wearing protective clothes and mask was evaluated no effect was observed for any of them either on BNMN frequency or on NPB frequency. Nevertheless, in individuals who participated in cleaning and autopsies of oil contaminated birds higher frequencies were observed linked to the use of mask, although statistical significance could not be reached (Laffon *et al*., 2006b).

4.3. Sister Chromatid Exchanges

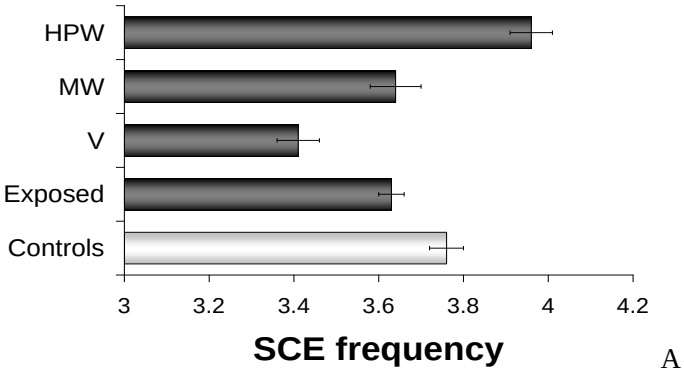
SCE are cytogenetic manifestations of DNA breaks and their join in the homologous locus in the sister chromatid of a chromosome (Lazutka *et al*., 1994). There is uncertainty

surrounding their mechanism and biological significance, although it has been suggested that they constitute the display of repair processes of DNA double strand breaks homologous recombination (Norppa, 2004) SCE represent a sensitive and reliable effect biomarker extensively used from the beginning of Genetic Toxicology due to the fact that they are induced by a broad spectrum of genotoxic agents. The SCE test system using human peripheral lymphocytes is proposed as an especially valuable tool for the cytogenetic monitoring of exposure to potential chemical mutagens.

For that purpose, duplicate lymphocyte cultures were set up by adding 0.5ml of whole blood to 4.5ml of culture medium (the same used for MN test). Cultures were incubated at 37ºC for 68h, and after 24h of culture initiation 5-bromo-2'-deoxyuridine was added at a final concentration of 7µg/ml. Colchicine (2µg/ml) was added 2h prior to harvesting to arrest the cells at metaphase. Cells were collected by centrifugation, resuspended in a warm hypotonic solution (0.075M KCl at 37ºC) for 10min, and fixed in Carnoy. Air-dried slides were prepared and stained with fluorescence plus Giemsa technique, following Perry and Wolff (1974). A total of 50 well-spread second division metaphases were examined for each donor, half from each duplicate culture, on a Nikon HFX-DX light microscope by the same scorer, to determine the number of SCE/cell. Moreover, 100 metaphases were scored for each donor to evaluate the proportion of cells that underwent 1, 2 or 3 or more divisions. Proliferative rate index (PRI) was calculated as an estimation of cytotoxicity following the formula PRI=(M1+2MII+3MIII)/Total, where MI, MII, and MIII indicate the number of metaphases in first, second, and third or subsequent divisions.

Data from this assay adjusted by sex, age and exposure are gathered in Figure 7. Effect of exposure to *Prestige* oil was only observed in the group of HPW, as a statistically significant increase in the SCE frequency. This group of exposed individuals showed the lowest level of VOC exposure, but the use of high-pressure cleaning machines in their work probably determined the existence of substantial differences in the chronic exposure experienced with regard to that experienced by MW or V. On the other hand, cytotoxicity index PRI did not indicate a significant delay in the cell cycle associated with any of the exposed groups.

The results presented in this work support the hypothesis that oil exposure induces fixed cytogenetic alterations in peripheral blood lymphocytes of individuals exposed for a relatively long period of time, detected as increases in BNMN frequency, a reliable biomarker specially recommended to assess the risk associated with benzene exposure (one of the main integrants of the oil) (Maffei *et al*., 2005) and in SCE frequency.



A

Figure 7. (Continued)

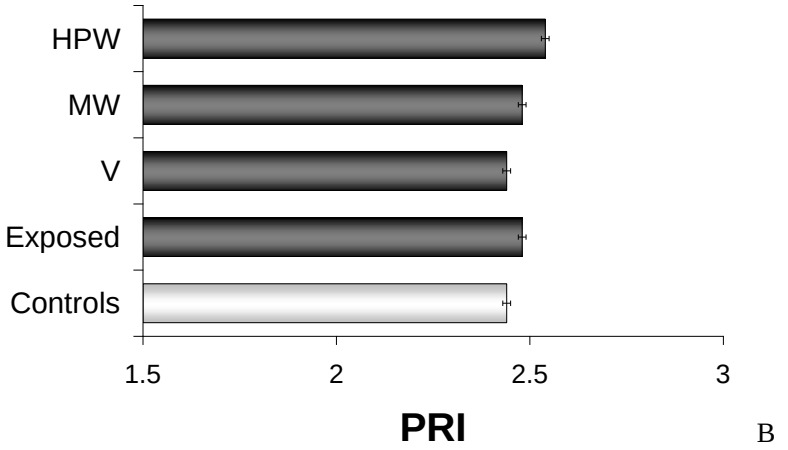


Figure 7. Results of SCE in the study population, after adjusting by age, sex and smoking: SCE frequency (A) and PRI (B). Bars indicate mean standard error. **P<0.01, significant difference with regard to the controls.

Effects of lifestyle factors on SCE frequency were studied and data are shown in Table 4. As the other cytogenetic variables evaluated, SCE frequency was influenced by sex (higher rates in females), and also by tobacco smoking, when evaluating these factors individually after adjusting by exposure.

Table 4. Influence of sex, age and smoking habits (pack-year) on SCE frequency (Models adjusted by exposure)

Model	Unstandardized coefficients β	95% CI		Partial *P* value	Model *P* value
Males *vs.* females	-0.263	-0.369 to	-0.158	0.000	0.000
Age (years)	-0.001	-0.007 to	0.006	0.883	0.000
Smoking habits	0.008	0.002 to	0.014	0.014	0.000

Moreover, both of them remained significant when they were mutually adjusted (both partial *P* values <0.001, model *P* value <0.001). These results agree with Lazutka *et al.* (1994), who evaluated the differences between genders in SCE and reported that the frequency of this parameter was 7.5% higher in females than in males. Also, the strong influence of smoking on this cytogenetic rate was extensively reported (Hirsch *et al*., 1992; Barale *et al*., 1998). On the other hand, it is well known that age affects SCE frequency (Bolognesi *et al*., 1997; Barale *et al*., 1998); the absence of influence of this parameter in the present work is probably related to the narrow range of age covered by our study population.

As occurred in the comet assay, increased SCE frequencies were observed in those individuals who did not wear protective clothes (partial *P* value <0.001, model *P* value <0.001), demonstrating their effectiveness against dermal exposure, but no influence of using mask could be detected, pointing again to unsuitable properties of the devices or inadequate utilization by the individuals.

Taken together the results brought by the three genotoxicity biomarkers evaluated, data seem to indicate that either the DNA strand breaks observed in the individuals was a short-

time damage that could be easily repaired and therefore it was not finally fixed, or it actually reflected incomplete excision repair processes. In fact, some authors believe that the comet assay is more linked to exposure and does not represent a true biomarker of effect, but preferably a biomarker of exposure, whereas MN or SCE are more consistently considered as biomarkers of early effect (Migliore *et al*., 2006). According to this idea, the higher levels of %TDNA detected in V, a group of individuals that experienced a very short and recent exposure to oil, are probably related to an adaptive response as it was explained above, and a longer term exposure such as that experienced by MW and HPW is needed for the cytogenetic and cytotoxic effects to be displayed.

On the other hand, among the possible explanations for the differences in the results obtained by means of the three genotoxicity assays employed, the overlapping influence of confounding factors, the high complexity of the oil mixture and the different types of damage reflected appear to be the most reliable, supporting and strengthening the need of applying a set of biomarkers when evaluating this kind of exposures.

5. SUSCEPTIBILITY BIOMARKERS

Nowadays it is well known that the presence of polymorphisms in several genes encoding for enzymes involved in metabolic and repair pathways are considered key in epidemiological studies due to the repercussions on the detoxification processes and the genetic damage levels. This acquires even more importance when taking into account that in some occasions the initial agent is not by itself the determinant cause of the toxicity phenomena, but the formation and elimination of intermediary metabolites that are often more harmful than the original compound. That is the reason why an important part of this work dealt with the determination and analysis of the influence of the polymorphic variants in the most relevant genes involved in the metabolism of the main toxic compounds present in the oil, as well as in the DNA repair pathways, on the effect biomarkers studied. In order to be able to differentiate the interactions of the genotypic factors with the exposure and their influence on the background levels of the biomarkers, and following the indications of Norppa (2004) we evaluated the effect of the polymorphisms in the control and exposed populations separately.

For the genotyping purposes, DNA was extracted from 300µl of whole peripheral blood samples using Puregene™ DNA isolation kit (Gentra Systems, Minneapolis, USA). Polymorphisms in metabolizing genes were evaluated by means of polymerase chain reaction-restriction fragments length polymorphisms (PCR-RFLP) methodologies following Laffon *et al*. (2003a) for CYP1A1 3'-UTR *1A>*2A, Pérez-Cadahía *et al*. (2007) for CYP1B1 codon 432 *1>*3 (amino acid substitution Leu>Val), Laffon *et al*. (2003b) for EPHX1 codon 113 Tyr>His and Laffon *et al*. (2006c) for codon 139 His>Arg, Saarikoski *et al*. (1998) for GSTP1 codon 105 Ile>Val and Laffon *et al*. (2003b) for simultaneous detection of GSTM1 and GSTT1 deletion polymorphisms. Polymorphisms in DNA repair genes were analysed using PCR-RFLP as described in Laffon *et al*. (2006b) for XRCC1 codon 194 Arg>Trp, and Hu *et al*. (2001) for XRCC3 codon 241 Thr>Met. XPD codon 751 Lys>Gln polymorphism was determined by means of melting curve analysis after a real-time PCR process using resonance energy transfer probes. After confirming that all studied polymorphisms, excepting GSTM1 and GSTT1, were in Hardy-Weinberg equilibrium,

individuals were grouped depending on the most suitable heredity model for each gene according to the results (Analysis of Variance), since no data were available in the literature, excepting for EPHX1 (Vesell, 1979).

5.1. Metabolic Genes

CYP enzymes are considered as the first defence against lipophilic toxic substances (Garte *et al*. 2001). They are mainly expressed in liver, although some isozymes are tissue-specific, and they catalyze the insertion of an oxygen atom in the substrate (Pavanello and Clonfero, 2000). Specifically, CYP1A1 and CYP1B1 catalyze the formation of mutagenic intermediaries of many PAH (Savas *et al*., 1997), so an activating role has been assigned to this two enzymes. Their expression is mediated by the aryl hydrocarbon receptor and is inducible by exposure to PAH. CYP1A1 is one of the most abundant CYP isozymes in lungs, and thus polymorphisms affecting this gene may be important in toxicity induced by inhaled chemicals. CYP1B1 is expressed in all organs excepting liver and lungs (Nishimura *et al*., 2003). McFayden and Murray (2005) identified CYP1B1 as the main CYP present in a wide range of human cancers of different histological types, and it is considered nowadays as a biomarker of neoplastic phenotype. Two of the most studied polymorphisms in these genes are located in the 3'-untranslated region (UTR) of the first one (allele change CYP1A1*1 to CYP1A1*2A) and in the codon 432 of the second one (allele change CYP1B1*1 to CYP1B3*, amino acid change Leu to Val), the resulting enzymes having a higher inducibility than the wild-type ones (Smith *et al*., 1998). Data coming from the comet assay (Figure 8) showed a statistically significant increase in the DNA damage levels associated with the presence of the CYP1A1*2A variant allele in individuals belonging to control and V groups.

On the contrary, data from the cytogenetic parameters showed general decreases in the damage rates related to the presence of the variant allele in an alelle number-dependent way, suggesting a relationship between lower damage levels and higher detoxifying activity of the routes. These differences could be due to the higher concentrations of the most reactive intermediate metabolites produced in a first moment which could increase the damage, but in a long term the higher enzyme activity would yield a higher detoxifying efficiency. Results obtained for CYP1B1 polymorphism did not show any homogeneous tendency in the comet assay, and no influence was observed in the cytogenetic tests.

Microsomal epoxide hydrolase is encoded by EPHX1 gene and has a great importance in xenobiotic detoxification. It catalyzes the addition of water molecules to very reactive and instable aromatic and aliphatic epoxides, to form much less reactive trans-dihydrodiols. Several polymorphisms have been reported in EPHX1 gene, two of them involving amino acid changes have consequences on the activity of the resulting enzyme and were established as risk factors against several exposures, e.g. to benzene (Bauer *et al*. 2003). Polymorphism located in codon 113 (Tyr to His) gives rise to a 40% decrease in enzyme activity, while the one located in codon 139 (Arg to His) increases it in 25% (Hasset *et al*. 1994). In general, their influence is usually evaluated by means of the expected activity resulting from both polymorphisms (Lee *et al*., 2002). No influence of epoxide hydrolase expected activity was observed in the MN test (Table 5). In the comet assay and SCE test it seems that in general decreases in the damage rates were related to the increase in enzyme detoxifying activity, although in some cases lower damage was obtained in individuals with low activity. Anyway,

as those differences were detected not only in exposed subjects but also in controls, they can probably be due to variations in the background level of the effect biomarkers used. Rihs *et al.* (2005) maintained that these EPHX1 variants have no appreciable effect on enzyme kinetics since amino acid changes are not close to the catalytic centre. Moreover, other polymorphic loci were identified in the promoter region of EPHX1 (Raaka *et al*., 1998) that could also modify the enzyme activity, and post-transcriptional regulation processes (Hasset *et al*., 1997) and other variations in the EPHX1 sequence (Saito *et al*., 2001) must also be taken into account.

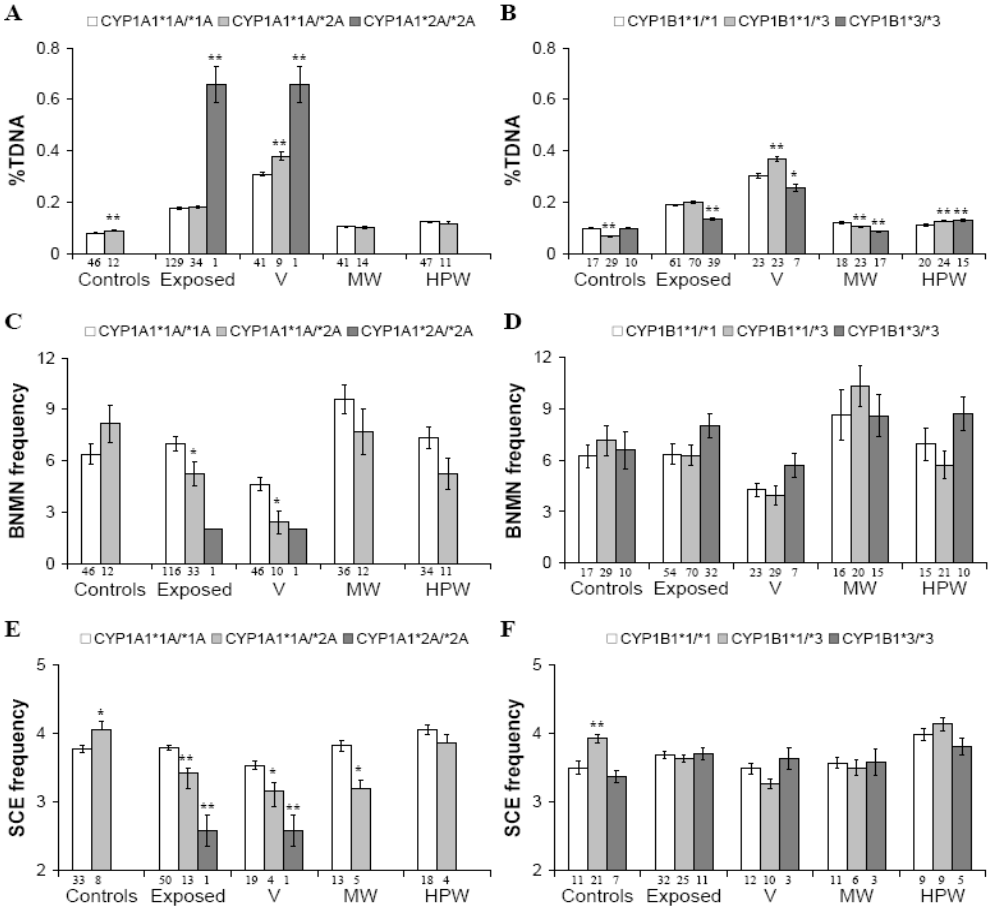


Figure 8. Influence of CYP1A1 (A, C, E) and CYP1B1 (B, D, F) studied polymorphisms on DNA damage (A, B), BNMN frequency (C, D), and SCE frequency (E, F). Bars indicate mean standard error. The number of individuals included in the groups is indicated under each bar. **P<0.01, *P<0.05, significant difference with regard to the controls.

Glutathione S-transferases (GST) are a superfamily of polymorphic enzymes involved in the conjugation of reactive chemical intermediates, and play an important role in the detoxification of endogenous and exogenous compounds. Although glutathione conjugation is a minor pathway in liver, it has a great importance in extrahepatic tissues where epoxide hydrolase activity is low (Norppa 2004). Three of the main enzymes involved in this conjugation are encoded by the genes GSTP1, GSTM1 and GSTT1, all of them present

polymorphisms with effect on the enzyme catalytic activity. It is well known that GSTP1 enzyme takes part in the pulmonary detoxification of inhaled toxicants, specifically PAH (Buschini *et al*. 2003). It has also been described that the activity of the codon 105 variant enzyme (amino acid change Ile to Val) against low size substrates is threefold lower than the wild type (Ali-Osman *et al*., 1997), but its activity rate against PAH dihydrodiol-epoxides is up to 7-fold higher (Hu *et al*., 1997). On this basis it has been proposed that variant allele homozygous carriers are more susceptible to certain mutagens but less susceptible to PAH-induced carcinogenesis (Whyatt *et al*., 2000). Results obtained in the comet assay and MN test (Table 6) generally support this hypothesis, although data from SCE indicate quite the contrary. The main reason behind this apparent controversy is likely the complex mixture of substances which individuals were exposed to.

Table 5. Influence of epoxide hydrolase expected activity on the effect biomarkers analyzed

Bio-marker	En-zyme activity	Controls	Exposed	V	MW	HPW
% TDNA	High	0.058±0.003** (*12*)	0.166±0.005** (*37*)	0.455±0.018 (*9*)	0.103±0.004 (*17*)	0.120±0.005 (*11*)
	Me-dium	0.084±0.002 (*33*)	0.187±0.004 (*85*)	0.340±0.009 (*31*)	0.107±0.003 (*25*)	0.116±0.003 (*29*)
	Low	0.085±0.003 (*15*)	0.174±0.004* *(58)*	0.284±0.001** (*21*)	0.096±0.004 (*17*)	0.129±0.004 * (*20*)
BNMN	High	7.42±0.78 (*12*)	7.00±0.47 (*32*)	4.33±0.69 (*9*)	8.43±0.77 (*14*)	7.44±0.91 (*9*)
	Me-dium	6.91±0.46 (*33*)	6.75±0.31 (*72*)	4.45±0.39 (*29*)	10.14±0.69 (*21*)	6.55±0.54 (*22*)
	Low	6.93±0.68 (*15*)	6.32±0.34 (*53*)	4.14±0.44 (*21*)	8.71±0.71 (*17*)	6.67±0.66 (*15*)
SCE	High	3.95±0.10 (*9*)	3.50±0.06* (*18*)	3.20±0.10 (*7*)	3.54±0.09 (*8*)	4.08±0.15 (*4*)
	Me-dium	3.91±0.06 (*22*)	3.73±0.05 (*29*)	3.39±0.08 (*9*)	3.69±0.13 (*6*)	3.95±0.08 (*13*)
	Low	3.45±0.08** (*11*)	3.72±0.06 (*21*)	3.60±0.09 (*9*)	3.45±0.11 (*6*)	4.11±0.10 (*6*)

N is indicated between brackets. ***P*<0.01, **P*<0.05, significant difference with regard to the medium activity.

The polymorphisms of GSTM1 and GSTT1 genes owing to gene deletions result in null alleles, and homozygous individuals for the deletion lack enzyme activity. GSTM1 is involved in PAH dihydrodiol-epoxides detoxification (Thier *et al*., 2003), and the null genotype was associated with higher PAH DNA adduct levels (Norppa, 2004). Similarly to results obtained for GSTP1, GSTM1 null individuals showed lower damage levels than the positive ones in the comet assay and MN test, but opposite results were observed in SCE (Table 7), probably due to the same cause. On the other hand, Parl *et al*. (2005) found an increased risk of breast cancer among GSTM1 positive women, and suggested that the

combination of all GST conjugation activities may cause glutathione depletion and therefore result counterproductive.

Table 6. Influence of GSTP1 codon 105 polymorphism on the effect biomarkers analyzed

	%TDNA		BNMN		SCE	
	Ala/Ala	Ala/Val+ Val/Val	Ala/Ala	Ala/Val+ Val/Val	Ala/Ala	Ala/Val+ Val/Val
Controls	0.085±0.002 (*30*)	0.079±0.002 (*25*)	7.59±0.49 (*32*)	6.58±0.52 (*24*)	3.65±0.05 (*17*)	3.95±0.08** (*14*)
Exposed	0.176±0.003 (*88*)	0.180±0.003** (*86*)	7.16±0.31 (*75*)	5.84 ±0.28** (*74*)	3.52±0.04 (*39*)	3.85±0.05** (*29*)
V	0.355±0.010 (*26*)	0.296±0.008** (*33*)	4.20±0.41 (*25*)	4.55±0.38 (*31*)	3.32±0.06 (*18*)	3.62±0.10* (*11*)
MW	0.110±0.003 (*29*)	0.094±0.003 (*26*)	10.04±0.62 (*26*)	7.19±0.58** (*21*)	3.37±0.08 (*11*)	3.75±0.10** (*7*)
HPW	0.109±0.003 (*33*)	0.138±0.004** (*27*)	7.13±0.54 (*24*)	6.36±0.54 (*22*)	3.98±0.09 (*10*)	4.04±0.08 (*13*)

N is indicated between brackets. ***P*<0.01, **P*<0.05, significant difference with regard to the homozygous wild type genotype.

Table 7. Influence of GSTM1 deletion polymorphism on the effect biomarkers analyzed

	%TDNA		BNMN		SCE	
	Positive	Null	Positive	Null	Positive	Null
Controls	0.081±0.002 (*33*)	0.076±0.003 (*27*)	8.06±0.50 (*32*)	5.82±0.45** (*28*)	3.61±0.05 (*28*)	4.17±0.08** (*14*)
Exposed	0.203±0.003 (*119*)	0.151±0.003** (*61*)	6.72±0.29 (*81*)	6.59 ±0.29 (*76*)	3.52±0.04 (*39*)	3.85±0.05** (*29*)
V	0.329±0.007 (*40*)	0.312±0.011 (*21*)	4.68±0.35 (*37*)	3.73±0.41 (*22*)	3.32±0.06 (*18*)	3.62±0.10* (*7*)
MW	0.114±0.003 (*28*)	0.093±0.003** (*31*)	9.29±0.62 (*24*)	9.14±0.57 (*28*)	3.37±0.08 (*11*)	3.75±0.10** (*9*)
HPW	0.118±0.004 (*26*)	0.123±0.003 (*34*)	7.40±0.61 (*20*)	6.27±0.49 (*26*)	3.98±0.09 (*10*)	4.04±0.08 (*13*)

N is indicated between brackets. ***P*<0.01, **P*<0.05, significant difference with regard to the positive genotype.

GSTT1 catalyzes the conjugation of several substances such as monohalomethanes and ethylene oxide (Autrup, 2000). In general, decreases in the damage rates were observed in GSTT1 null genotype individuals in the three effect biomarkers analyzed (Table 8), agreeing with studies that reported associations between lower MN frequencies and GSTT1 null genotype (Falck *et al*., 1999; Kirsch-Volders *et al*., 2006). Anyway it is well known that gene expression induction or inhibition by exogenous agents is one of the most influential factors on the detoxifying role of this kind of enzymes. In this regard, despite an inhibitor effect of benzo[a]pyrene on GSTT1 expression was described, the ability of this compound or other PAH to alter the expression balance of GST requires to be investigated in depth (Kemp *et al*.

2006). On the other hand, it is necessary to further consider the complex relationship among genetic damage, its repair, and cell transformation and cell death (Mishra *et al*. 2003).

Table 8. Influence of GSTT1 deletion polymorphism on the effect biomarkers analyzed

	%TDNA		BNMN		SCE	
	Positive	Null	Positive	Null	Positive	Null
Controls	0.078±0.002 (*58*)	0.112±0.010** (*2*)	7.10±0.35 (*59*)	2.00±1.41 (*1*)	3.82±0.05 (*40*)	3.40±0.18* (*2*)
Exposed	0.178±0.002 (*161*)	0.181±0.007 (*19*)	6.78±0.22 (*142*)	5.43 ±0.62 (*14*)	3.67±0.03 (*63*)	3.60±0.12 (*5*)
V	0.329±0.001 (*55*)	0.355±0.018 (*6*)	4.39±0.28 (*52*)	3.60±0.85 (*5*)	3.42±0.06 (*22*)	3.37±0.14 (*3*)
MW	0.114±0.000 (*55*)	0.086±0.007* (*4*)	9.39±0.44 (*49*)	6.33±1.45 (*3*)	3.55±0.06 (*20*)	(*0*)
HPW	0.124±0.003 (*51*)	0.104±0.005** (*9*)	6.80±0.41 (*40*)	6.50±1.04 (*6*)	4.02±0.06 (*21*)	3.98±0.22 (*2*)

N is indicated between brackets. ***P*<0.01, **P*<0.05, significant difference with regard to the positive genotype.

5.2. DNA Repair Genes

DNA repair systems are responsible for the maintenance of genome integrity both in germinal and somatic cells, and inherited or acquired deficiencies in such pathways may contribute to the onset of tumorigenesis (Waisberg *et al*., 2003). Three of the most relevant and widely studied DNA repair polymorphic genes were included in this study: XRCC1, XRCC3 and XPD. The possible influence of their polymorphisms in the development of several types of cancer was previously reviewed (Goode *et al*., 2002; Benhamou and Sarasin, 2002).

XRCC1 protein participates in base excision repair and recombinational repair pathways (Thompson and West, 2000). Several variants of XRCC1 have been described, including one affecting codon 194 (amino acid substitutionArg to Trp) that resides in the hinge region separating the DNA polymeraseβ from the PARP-binding domains (Mateuca *et al*., 2005). XRCC3 is involved in the homologous recombinational pathway (Brenneman *et al*., 2000) for repair of DNA double strand breaks induced either directly or indirectly following replication of closely spaced single strand breaks (O'Driscoll and Jeggo, 2006). Additionally, XRCC3 was also shown to promote correct chromosome segregation in mammalian cells (Griffin, 2002). A nucleotide substitution in codon 241 of XRCC3 results in the amino acid change Thr to Met. Lastly, the XPDgene codes for a DNA helicase involved in transcription and nucleotide excision repair (Evans *et al*., 1997). The DNA repair function of XPD is critical for the repair of genetic damage from tobacco and other carcinogens (Moncollin *et al*., 2001). Several common single base pair substitution polymorphisms in thisgene have been identified. The one located in codon 751 (amino acid change Lys to Gln) is perhaps the most relevant due to its decreasing effect on repair activity and its frequency in the general population (Pavanello, 2003).

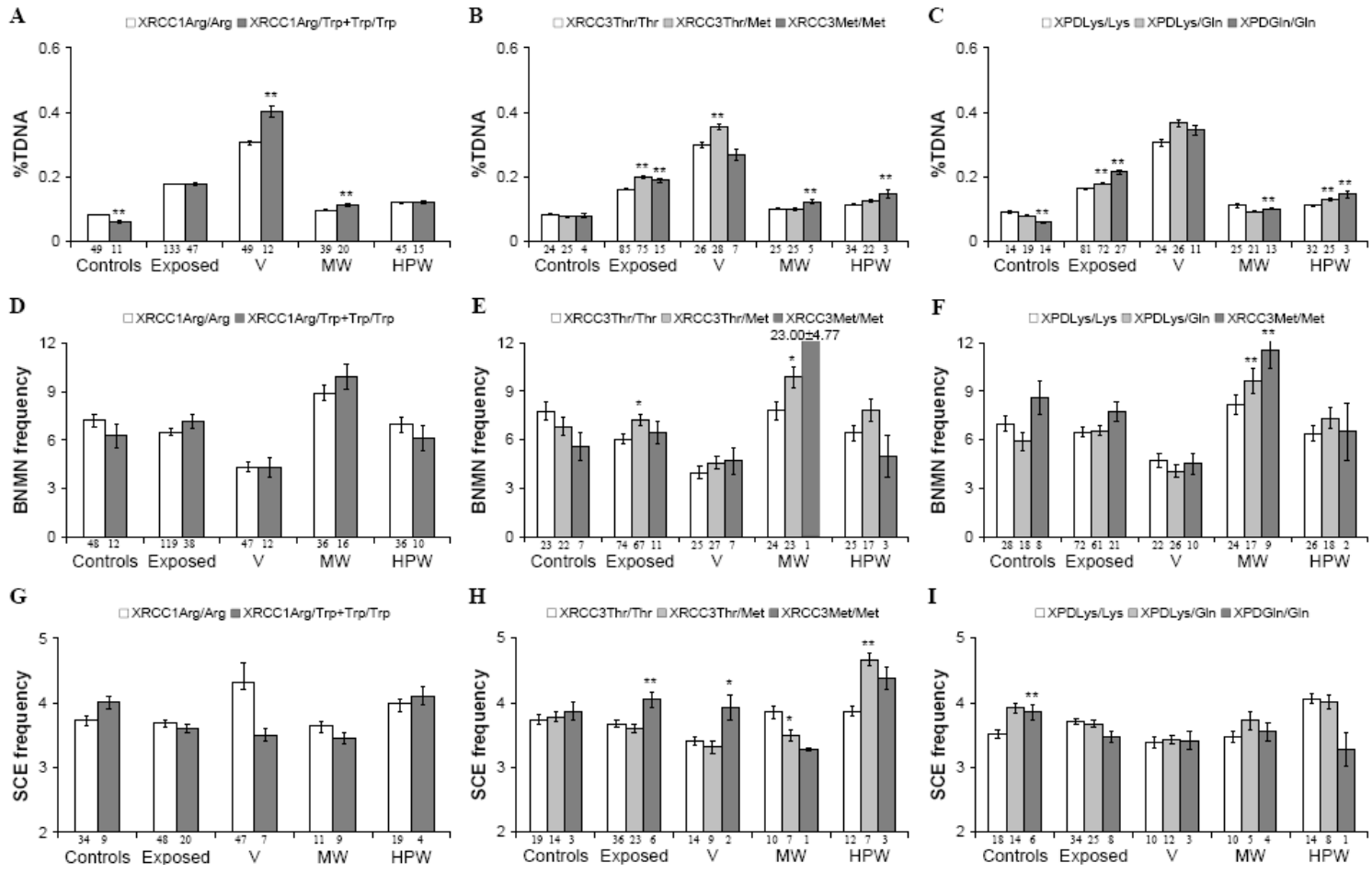


Figure 9. Influence of XRCC1 (A, D, G), XRCC3 (B, E, H), and XPD (C, F, I) studied polymorphisms on DNA damage (A, B, C), BNMN frequency (D, E, F), and SCE frequency (G, H, I). Bars indicate mean standard error. The number of individuals included in the groups is indicated under each bar. **P<0.01, *P<0.05, significant difference with regard to the controls.

General increases in DNA damage levels were observed associated with the presence of XRCC1 variant allele (Figure 9). More outstanding effects could be assigned to XRCC3 and XPD polymorphisms, since increases in not only DNA damage but also in fixed cytogenetic damage were obtained in individuals carrying the variant allele. These results agree with the proposed reduction in the repair activity for the variant alleles (Pavanello 2003). The general maintenance of genome stability for which XRCC3 appears to be required (Cui *et al*., 1999), and the removal of DNA adducts, main effect of exposure to PAH and other compounds contained in the oil (Wei *et al*., 2000), in which XPD is directly involved, are essential mechanisms in the organism response to damaging environmental exposures. The influence of these polymorphisms seems to be more emphasized in the longest exposed groups, indicating that they must be fundamentally taken into account in chronic exposures.

Numerous genetic polymorphisms may influence the same pathway and therefore the various gene-gene interactions and their modulation of genotoxic effects in their whole complexity must be considered. In this work no statistically significant interaction could be detected among metabolizing or DNA repair polymorphisms. This fact does not likely mean that interactions among enzymes were not taking place, but reflects the limitations that this kind of approaches usually find in epidemiological studies, as the more factors included in the analysis, the larger population needed to detect statistically significant differences; that is mainly the reason why they are so scarce in epidemiological studies.

CONCLUSION

Data obtained in this work showed DNA and cytogenetic damage related to exposure to *Prestige* oil, influenced by sex, age, smoking habits and some metabolizing and DNA repair polymorphisms. In view of these results, it seems essential to pay more attention to the human health effects of exposure to oil after unfortunately too frequent accidental spills, and focus new studies on such relevant but overlooked Public Health field that involve a large number of people all over the world. If similar effects supported those reported here, a major urgent topic would be to develop a more optimal international acting protocol to ensure the safety of individuals involved in the recovery and cleaning tasks.

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Chapter IV

THE BIGGEST WINNERS IN DNA AND PROTEIN SEQUENCE ANALYSIS: METAGENOMICS, DNA MICROARRAY, BIOSENSORS, MOLECULAR SUBTYPING, AND PHAGE RECOMBINANT PROBES

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ABSTRACT

In an age of molecular genetics, viable but not culturable organisms, and highly sophisticated DNA sequence analysis technologies, is there a chance for a new breakthrough? It is quite extraordinary that 99% of microorganisms not only can't be cultured but are totally unknown to the scientific community. Many bacterial gene functions and identifications in the environment have been uncovered by recent advances in metagenomics or culture-independent genomic analysis in addition to the DNA microarray technology. Our knowledge of detecting and classifying bacterial isolates has been also improved by cutting-edge molecular tools such as biosensors and molecular

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subtyping as well as phage recombinant probes. Microbial communities, many to be discovered, are slowly revealed by these sharp scientific discoveries.

METAGENOMIC STRATEGIES FOR UNCULTURED MICROBES

Many bacterial genomes sequenced lately were recovered from cultured bacteria which represent 1% of total microbial communities [4]. This limitation of exploring the DNA sequences and missing 99% of non-cultured bacteria represents a big gap in our knowledge on bacterial strains of microbial communities. Because vast amounts of bacterial strains do not live or exist in isolation in their natural environments, metagenomic approaches - culture-independent genomic analysis- of DNA sequences can help to address the real representation of full genetic and metabolic potential. Therefore, allowing the bio-discovery of gene functions associated with natural population can lead to a) establishment of gene inventories b) discovery of new natural products c) understanding microbial group dynamics in extreme environments d) discovery of new viruses (bacteriophages).

Although different microbial communities were studied using PCR analysis, PCR has shown some biases in primer binding and amplification of specific DNA regions hindering the real representation of microbial diversity [70]. Metagenomic strategies of microbial habitat, however, are more powerful tools for profiling DNA sequences from complex mixtures of uncultured bacterial cells.

Metagenomic DNA Strategies

There are many approaches that can be used in metagenomic investigations. Almost all investigated strategies require some form of cloning and sequencing of the DNA fragments. Cloning different DNA fragments from microbial communities is followed by several possible strategies including:

1. Screening clones for the phenotype. This is usually followed by determination of phylogenetic origins of cloned DNA [10].
2. Screening clones for specific phylogenetic anchors such as 16S RNA or predetermined genes. This is followed by sequencing the entire clones and trying to find other genes residing near the anchor genes [7].
3. Random sequencing of the cloned fragments, identifying the interesting genes, and finally reconstruction of the genomic map [7, 8, 11, 12].
4. Using small DNA fragments to build DNA microarray chips followed by hybridizations with known reference bacterial strains [77], Figure 1.

Metagenomic in Bacterial Communities

In the last five years, DNA sequence technologies have seen rapid improvements. Because of these improvements, it is feasible now to sequence entire metagenomes of

complex bacterial communities. The complexity of bacterial communities due to microheterogeneity makes the metagenomic DNA sequence reconstructions simple representatives of real life. Since first attempts at conducting metagenomic sequencing started in viral communities in the ocean [12, 87] and human feces [11, 87], it is now feasible to sequence vast amounts of culture independent bacterial communities from different microbial environments.

Venter et al [87] and Tyson et al [86] were first to conduct large-scale metagenomic DNA sequencing. The DNA, in one study for example [87], was isolated from environmental genomes and inserted into cosmid libraries using shotgun cloning. The cloned DNA was sequenced without the need to culture or isolate the bacterial cells. As a result, seawater samples from whole-genome shotgun sequences were collected and further analyzed revealing at least 1800 genomic species including 148 previously unknown bacterial phylotypes. More than 1.2 million genes, including 782 new rhodopsin-like photoreceptors, were identified. In addition, six plasmids larger than 100-kb were discovered as well as putative genes of arsenate, mercury, copper, and cadmium resistance sequences. Putative conjugal transfer systems and plasmid transfer genes were also found suggesting the presence of DNA transfer mechanisms between bacterial strains in the ocean. The discovery of these gene sequences exposed the presence of substantial oceanic microbial diversities. Similarly, Tyson et al [86] used random DNA shotgun sequencing from natural acidophilic biofilm. A total of 76.2 million base pairs were sequenced and around 103, 462 genes (high-quality reads) were identified.

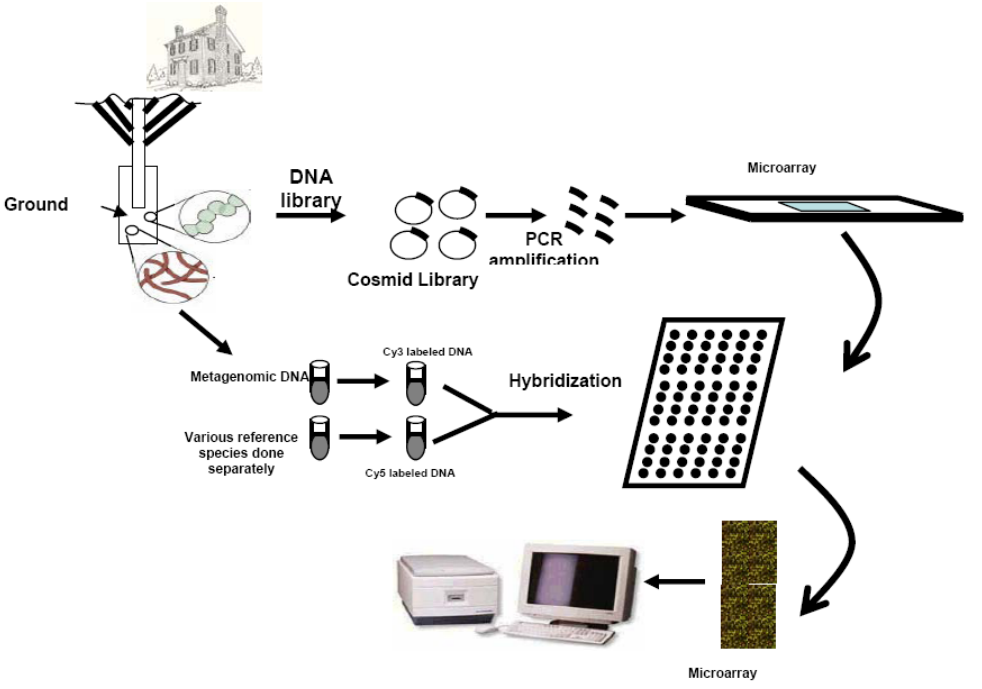

Figure 1. DNA was isolated from groundwater microbial communities to build cosmid DNA library. The DNA fragments were fabricated on the chemically treated slide to build the DNA chip. Reference known bacterial strains mixed with unknown samples were labeled and hybridized to the DNA chip revealing the DNA profiling of the bacterial water communities.

Recently, microarray platforms were used to screen 1-kb metagenomic libraries [77]. The DNA 1-kb fragments from 672 cosmid metagenomic library clones were amplified using PCR, Figure 1. The PCR fragments were printed on chemically treated slides and hybridized with labeled DNA. The DNA hybridization mixture containing a labeled Cy5-dCTP environmental DNA and labeled Cy3-d-CTP bacterial reference strains DNA created comparative genomic hybridizations (CGH). Positive results were based on the ratios which are greater than one of Cy5/Cy3. This strategy was able to profile each clone in the metagenomic library by using known reference strains.

Metagenomic in Virus Communities

Metagenomic analysis of genome is important in the discovery of viruses and bacteriophages. Bacteriophages take part in diversity and population structures of microbial communities in the human gut. There are approximately 500 human intestinal microbial species with around 40 predominant species in the total population [51, 52].

The presence of bacteriophages was documented in many environments where bacteria live in soil, the intestines of animals and humans, and sea and drinking waters. Since bacteriophages have complex diversity and many of their hosts have not been cultured and therefore not yet discovered, metagenomic approaches were great discovery genomic tools. Bacteriophages isolated from human feces using metagenomic analyses of an uncultured viral community were found, naturally, to be unrelated to any known viruses [11]. A total of 1200 viral genotypes, not including bacterial strains, were recognized revealing the novelty of these microscopic lives. Most of these bacteriophages were found to be siphophages.

Metagenomic research was instrumental in finding the virus causing honey bee colony collapse disorder (CCD) (21). In fact, CCD has caused a loss of 50-90% of colonies in beekeeping operations in the US. The devastating effect of this disorder has triggered mass fear of economic losses in agricultural farm productions. Metagenomic analysis together with different molecular techniques was used to study the CCD hives for three years revealing that CCD in bees, was caused, most likely, by Israeli acute paralysis virus (IAPV). This breakthrough conclusion highlighted the importance of metagenomic research as instruments capable of finding the cause of unknown complex diseases especially when microorganisms cannot be cultured.

Finally, it is paramount to recognize that metagenomic DNA analyses have opened the door to the discovery of many bacterial species as well as viruses. Discovering the black box with complex microbial environments, metagenoic research represents instrumental tool in the microbial investigation. Once again [1], high throughput screening technologies will be valuable in unlocking many of the unknowns' microbial reservoirs. The future will be filled with new discoveries of genes which will be used to improve our daily lives.

Characterization and Detection of DNA Hybridization by Using Metal Nanoparticles (NP) and Surface-Enhanced Vibrational Spectroscopy

The successful development of new microanalytical biosensing assays must meet several challenging requirements that include specificity of biological recognition probes and sensitivity of detection. The present discussion will be limited to the sequence-specific recognition of capture DNA, a process that is based on the hybridization of a strand of nucleic acid (probe) to a complementary target sequence. Fluorescent dyes traditionally used for the detection of DNA microarrays (gene chips) suffer from drawbacks such as broad emission peaks which limit multiplexing, quenching of fluorescence and photobleaching [27, 39]. Advances in the development of DNA-nanoparticle (NP) bioconjugates have led to their use as novel analytical research tools for the characterization and detection of DNA [17, 39, 44] in an attempt to overcome these limitations.

There has been an increased interest in applying metal, particularly gold and silver, NPs to the detection of DNA hybridization and to other assays. This is because NPs significantly enhance the sensitivity of physicochemical detection methods including those based on surface-enhanced vibrational spectroscopy, such as surface-enhanced infrared absorption (SEIRA) and surface-enhanced Raman scattering (SERS). These applications were possible due to the fact that the surface of metal NPs as well as the surface of metal island films (with nanometric asperities) could successfully be functionalized via an organic linker with stable receptors, such as oligonucleotides suitable for hybridization in aqueous buffers. In one conjugation technique, thiol or disulfide molecules with appropriate endgroups are used to form gold-bound thiolate, self-assembled monolayers (SAMs) on metal surfaces (93), thus making it possible to design substrates for tethering molecular recognition receptors such as ssDNA probes.

NPs were first reported to tether to thiol-modified DNA by self-assembly in 1996 (3, 48). These advances in NP-nucleic acid bioconjugates have since been applied to biosensing and have led to the development of many novel assays with a number of different detection methods. With respect to vibrational spectroscopy, there is a paucity of SEIRA publications on monolayer formation and/or detection of DNA hybridization. By contrast, there is a large body of SERS literature on the detection of binding reactions, including those between nucleic acid targets and probes. A few examples of recent studies are presented next as an overview of the application of NPs and surface-enhanced vibrational spectroscopy to DNA hybridization.

SERS is an emerging technology that can overcome many of the challenges associated with fluorescence detection. SERS signals occur from the combination of electromagnetic [31, 37, 75] and chemical [54, 59, 88] enhancement effects that allow for signals up to 10^{14} times larger than normal Raman scattering [38]. While many theories have been proposed to elucidate the mechanisms underlying these effects, electromagnetic enhancement is recognized to be from enhanced local electric fields experienced by the Raman active molecule and the formation of surface plasmons at the metal surface [32], while chemical enhancement is attributed to an increased polarizability of the molecule due to adsorption at the metal surface and charge-transfer [61].

Hairpin DNA structures have traditionally been used in molecular beacon systems with fluorescence detection. However, by using a plasmonic-based nanoprobe called a molecular sentinel [89], a DNA hairpin loop with a Raman-active molecule at one end and a thiol

moiety at the other linked to a metal NP can be used as a SERS-beacon in solution. The middle circular section of the hairpin consisted of the DNA sequence probe; the two straight arm sections had complementary sequences in order to form a hairpin configuration. Under normal conditions, the very close proximity of the two arms, and hence that of the NP (SERS surface) to the label (Raman-active molecule), induces a strong SERS signal upon excitation with a laser light. However, this SERS signal disappears upon hybridization with the complementary target DNA sequence. This decrease in SERS signal as the loop of the hairpin opens up upon formation of a DNA duplex occurs from the two arms physically moving away from each other thus separating the NP from the Raman label and decreasing the SERS enhancement effects [89].

Another study deals with multiplexed SERS assays carried out with Au NP probes functionalized with oligonucleotides containing Raman-active labels that exhibit distinctive spectroscopic fingerprints [17]. A microarray of 48 amine-modified capture ssDNA probes were immobilized on a commercially available glass slide coated with a hydrophilic polymer functionalized with succinimidyl ester groups. The tethered capture probes, which consisted of 15-nucleotide strands each, were incubated with a 30-nucleotide target. The overhanging region of the target sequence was subsequently hybridized to a probe consisting of 13-nm Au NPs with an adlayer of 15-nucleotide complementary strands containing Raman-active labels. To carry out measurements, the Au NPs were augmented by a Ag metal shell in order to allow for electromagnetic enhancement and achieve SERS detection of microarrayed spots. Multiplexed detection of oligonucleotide targets was possible with this SERS assay due to the unique spectral signature of the six different Raman-active labels. The potential of this assay to differentiate single nucleotide polymorphisms was demonstrated, and most importantly a detection limit of 20 femtomolar was reported [17].

Another recent advance in SERS-based DNA detection is the bio-bar-code amplification method of Mirkin and coworkers [36]. In this system, a bar-code DNA sequence that serves as a unique identifier for a target sequence is detected, rather than the target itself, thus allowing a route for PCR-less target amplification. Through a combination of NPs for SERS signal amplification and magnetic microparticles for separation of unreacted elements, this assay was reportedly able to detect an oligonucleotide associated with anthrax lethal factor in the 500 zeptomolar concentration range [36]. Other methods in DNA detection by SERS that show promise include gene diagnostics via hybridization of probes to complementary DNA-coated, silver chips [22] and the detection of tagged PCR products [49].

Another alternative to fluorescent labeling has been the use of surface-enhanced infrared reflection absorption (SEIRA) spectroscopy. In this SEIRA effect, IR radiation generates oscillating dipole moments in metal nanostructures, which in turn produce an external electromagnetic field that can interact with molecules on or near the metal surface; this interaction leads to the enhancement of IR vibrations perpendicular to the metal surface [58]. In one example, grazing-angle external-reflection Fourier-transform infrared (IR) microspectroscopy and Au NPs were used to detect hybridization [44]. NP-labeled complementary DNA targets were hybridized to thiol-modified capture single stranded DNA probes chemisorbed onto slides coated with Au island films. The surface of the slide was passivated with mercaptohexanol to reduce the number of non-covalent interactions between bases in ssDNA probes and the Au surface. Non-selective binding of cDNA targets was reported to be dependent on the size of NPs and the target concentration, and nearly complete slide surface coverage was observed with a 20% probe density, 10 nM target concentration

and 5-nm diameter NPs. IR spectra for ssDNA, dsDNA and NP-dsDNA conjugates were measured; however, only those observed for the latter exhibited characteristic spectral features indicating the necessity of NP conjugation. For instance, bands at 1126 and 1222 cm^{-1} attributed to the relatively intense symmetric and antisymmetric stretching vibrations of the phosphodiester DNA backbone were reported to suggest that the DNA duplex axis makes a significant projection along the surface normal.

Spectral enhancements for nucleic acids or their constituents were also reported in several SEIRA studies [5, 28, 35, 53, 67, 71]. In an external-reflection IR study to characterize SAMs (66), a thiol-modified polythymine deoxyribonucleotide $(dT)_{25}$ probe was immobilized on a Au slide. The data indicated that at a lower coverage, much of the $(dT)_{25}$ probe laid nearly flat on the surface, with a substantial fraction of the thymine bases chemisorbed; while at a higher surface density, the probe was attached via the thiol group and at possibly one or two bases. An observed IR band at 1714 cm^{-1} was attributed to carbonyl groups in thymine bases, while IR features between 1600 and 1550 cm^{-1} were consistent with carbonyl vibration modes in thymine bases chemisorbed on the Au slide.

In conclusion, NPs as signal amplification tags have been used in DNA biosensing applications and offer advantages over fluorescence-based detection methods due to multiplexing capabilities and signal stability. As such, the complementary techniques of SEIRA and SERS are coming to the forefront of DNA detection. Most promising are heterogeneous SERS assays, which include immobilization of capture probes on a solid substrate (chip-based) and provide high selectivity with sensitivities that are similar to those of conventional molecular biology techniques. SERS assays have also been shown to support multiplexed DNA detection, where unique Raman molecules can be used to simultaneously interrogate multiple targets of interest. Reflection and absorption mode SEIRA are increasingly being used as surface analytical techniques. For food pathogen, medical, defense, environmental and other applications, the full potential of applying NPs and surface-enhanced vibrational spectroscopy (including multispectral imaging) to gene detection in high throughput screening on a microarray platform has yet to be reached, and advances in this field are expected.

APPLICATION OF DNA MICROARRAY TECHNOLOGY

The vast multitude of sequenced genomes and the rapid increase in their discovery has increased the output of genomic DNA data. Recently, DNA microarray technology has emerged as a leader in this analysis, testing gene expression for thousands of genes in a single experiment. In a typical DNA microarray assay, a series of oligonucleotide sequences known as “oligoprobes” are selected to identify genes spotted onto a chemically modified glass slide, creating a “chip”. DNA collected from a test sample would be amplified and then fluorescently labeled for hybridization on the chip.

Along with its application to gene expression studies, DNA microarray technology has emerged as an indispensable technique for microbial identification and classification in different environments. Recent concern for accidental and intentional contamination of food has called to develop of microarray technology to quickly and selectively characterize microbes, even in cases where only a small concentration of pathogens is present. Another

important development in DNA microarray has been its clinical application, identifying and characterizing pathogenic microbes and viruses for diagnoses of infectious diseases in humans.

Gene Expression and Microbial Identification in Food Bacterial Pathogens

Gene expression of virulence and resistance factors in bacterial isolates is an area of high interest for public health and food safety. The study of certain genes provides insight into the interrelationship between virulence and fitness genes. By selecting for unique sets of genes, it is possible to characterize and serotype bacterial strains, as well. Previously, the go-to method for detecting multiple genes has been multiplex PCR combined with gel electrophoresis. Multiplex PCR utilizes more than one set of primers to simultaneously amplify multiple DNA sequences.

A multiplex PCR method was developed to test for virulence genes of *Escherichia coli* O157:H7, a major food pathogen. This method detected *slt*-I and –II toxin structural genes, the *eaeA* gene whose product mediated the adherence of *E. coli* O157:H7 to host cells, and genes involved in the biosynthesis of O157 and H7 antigens. The combination of genes selected successfully identified *E. coli* O157:H7 in bovine feces [1].

This general method has its limitations. The number of different amplifications is restricted due to primer-primer interactions. Also, nonspecific amplification causes multiple bands and problems in identifying PCR product due to DNA fragment size similarity. The application of DNA microarray technology provides a solution to these problems.

A highly sensitive and flexible microarray scheme was developed to identify and genotype*E. coli* O157:H7. Various oligonucleotide sequences were selected to represent four genes in wild-type and mutant*E. coli* O157:H7. They were: *stl*-I and –II, *eaeA*, and *hylA*. The method was successful in genotyping four pure cultures of *E. coli* O157:H7 and one isolate of O91:H2. The hybridization results were 32-fold more sensitive than the comparable multiplex PCR-gel electrophoresis assay [2, 16].

DNA microarray is not only used to test gene expression of bacterial isolates but also to serotype bacterial strains. A recent study of *Staphylococcus aureus* isolated from cattle utilized DNA microarray technology to study 144 virulence and resistance genes in a single experiment. Its efficiency also allowed 128 isolates from Germany and Switzerland to be tested much faster than any other method would allow. The majority of isolates were closely related clonal complexes 8, 25, and 97 (34.4% together) or were related to the bovine strain RF122 (48.4%) [50]. The simultaneous characterization and serotyping of *Salmonella enterica* isolates was also recently achieved using DNA microarray. Whole genomic DNA was fluorescently labeled with Cy5 and hybridized to the chip. A total of 109 oligoprobes detected genes encoding virulence factors, antibiotic resistance factors, and serotype-specific flagella and somatic antigens [47].

The ability to quickly and precisely identify bacteria is especially important in cases of food outbreaks. *Yersinia enterocolitica* is a pathogen able to grow in refrigerated and high salt conditions. It is typically transmitted through consumption of contaminated pork, water, and milk. Myers et al [55] recently developed a DNA microarray chip using four virulence genes (*virF, ail, yst, blaA*) and a positive control encoding 16S ribosomal components. The chip successfully identified five genes from adulterated pasteurized whole milk, and the detection

limit was 1,000 CFU per hybridization. The ultimate goal of DNA microarray chip is to selectively detect multiple food contaminants in a single assay with high sensitivity and low false-positive occurrence.

Possible Clinical Applications of DNA Microarray

A rather general response of any physician examining a patient with high fever in the emergency room would be to take a blood sample and send it to the bacteriology laboratory. Analysis would take two to three days to confirm an infection, to identify a pathogen, and to determine its antibiotic susceptibility profile [76]. The latter is especially important with the increasing number of multiple-drug resistant microbes.

The application of DNA microarray technology provides for highly sensitive and rapid characterization and subtyping of pathogens resistant to multiple antibiotics. Majtan et al (46) recently developed a novel *Salmonella*-specific DNA microarray chip for determining virulence factors, prophage sequences, and antimicrobial resistance genes. Preliminary screening of 24 *Salmonella* clinical isolates was done by susceptibility testing, plasmid profiling, and class 1 integron PCR. The microarray analysis characterized and localized the genetic markers, while the antimicrobial resistance genes were also detected and confirmed with sequencing. All strains revealed resistance to two or more antibiotics, some even showing resistance to up to nine drugs.

Pseudomonas aeruginosa infections, in particular, need fast and reliable methods for antibiotic susceptibility testing in order to achieve therapy improvement. Weile et al (90) have developed a DNA microarray chip and amplification protocol for genotyping multiple-drug resistant *P. aeruginosa*. DNA isolation, target gene amplification, fluorescence labeling, fragmentation, and hybridization could all be completed in less than five hours. There was an 87.8% coverage of relevant resistance genes used in therapy of critically ill patients calculated from genotype-phenotype comparisons.

Adenoviruses infect the respiratory tract of humans and cause acute respiratory disease. There is an absence of effective therapeutic or alternative prophylactic treatment for the infection. Lopez-Campos et al [42] recently developed a simple, cost-effective microarray assay for detection and serotyping of adenoviruses.

In order for these applications to be clinically significant, there must be an initial understanding of the patient's condition and at least a suspicion of the bacterial species causing the infection. If no such information can be determined, broad-range microarray techniques can be applied.

Schrenzel [76] designed a method to select for pathogens causing blood infection. These pathogens were identified from the past ten years' records at the Geneva University Hospitals, by analyzing more than 12,000 isolates. About 1000 species were considered and short regions of their ribosomal RNA genes were compared. A combination of highly conserved and highly variable oligonucleotide probes were chosen to design the microarray. Francois et al [30] have identified 21 genera and species responsible for approximately 80% of bacteremic episodes. Bacterial cultures isolation validated the microarray further by fluorescently labeling and hybridizing immediately after nucleic acid extraction.

The advantages of microarray technology are clear, yet certain limitations prevent its acceptance as a routine diagnostic technique. Broad-range microarray, in particular, is too

complex and costly to integrate as a diagnostic technique. Even with a broad range, the microarray is not cost effective like culturing [76]. The application of this technology can be integrated where it is most helpful. Zhu et al [94] have developed a base stacking hybridization method that uses microarray to rapidly identify clinical isolates without nucleic acid extraction. The technique was completed in less than two hours and matched with biochemical identification for 150 out of 152 clinical strains. Currently, it would be most effective to reserve DNA microarray technology to specific validated applications.

Looking Forward – The Future of DNA Microarray

This section has covered many of the recent applications of DNA microarray technology to bacterial detection in contaminated foods, an area especially important to counter-bioterrorism and public health. The development of this area has been the emerging trend in the literature over the past three to five years. In the future, DNA microarray technology may be used more for understanding the interrelationship between bacteria and the host environment they occupy as pathogens or normal flora.

Palmer et al [60] used DNA microarray technology to study the bacterial inhabitants of fourteen human infants' colons. They reported comprehensive analysis of bacteria residing in the human intestine. A previous study of adult intestinal microbiota showed the abundance of Gram-positive bacteria, Firmicutes and Actinobacteria, and Bacteroidetes. The recent infant study showed similar prevalence of these two categories of bacteria, but also a major appearance of Proteobacteria, a group of facultative anaerobes. It is speculated that they may be early inhabitants of the colon to clear oxygen content and allowing the anaerobes to colonize. A comparison of the infants' microbiota showed a high degree of species-level variations, but no discernable pattern of abundant species or temporal modes of acquisition. Two fraternal twins were also compared to show that environment may play a greater role than genetics in determining microbial profiles [60].

Recent Advances in the Molecular Subtyping of Bacterial Strains

Molecular genetic techniques (*i.e.*, pulsed field gel electrophoresis (PFGE) and single nucleotide polymorphism (SNP) analysis) that pinpoint subtle genotypic differences between closely related strains of a species have allowed for significant advances in strain identification and differentiation. Whether the molecular subtyping involves the tracking of nosocomial outbreaks, identifying the reservoirs of foodborne infections, or the discrimination of plant pathogenic strains through an agricultural field commodity, the association of a specific genotype with a particular outbreak of disease cluster greatly enhances our understanding of the epidemiological principles that govern the spread of a bacterium.

Molecular subtyping methods that yield unique genotypes among strains have revolutionized the way we characterize strain diversity and scrutinize pathogen outbreaks. These techniques offer numerous advantages over classical typing methods including averting the need for stable monocultures of bacterial cells, increased assay sensitivity, and significantly faster reaction times [68]. Molecular subtypes, stemming from DNA fingerprints

or polymorphic DNA sequences, can provide substantial genetic information that distinguishes closely related strains. As an example, a single hypervariable gene sequence can offer dozens of potentially polymorphic nucleotide substitution capable of discriminating among highly homogeneous pathogenic bacterial strains. As a rule, strain identification methods can be broken down into two basic conventions: (i) those that fingerprint the chromosome into polymorphic size fragments using gel electrophoretic techniques and oligonucleotide primers or restriction endonucleases (*e.g.*, PFGE or ribotyping); and (ii) those that target specific DNA sequences and discriminate based upon the individual nucleotide substitutions retained within a particular strain (e.g., multilocus sequence typing (MLST) or SNP analysis). Emanating historically from a need for the high resolution study of metazoan populations, DNA-based strain subtyping approaches, whether they be fragmentation based or sequence based, have transformed radically the way we differentiate strains and study their phylogenetic relationships [15, 41].

Molecular subtyping methodologies have been widely effective for the differentiation of many outbreak clusters and for high resolution delineation of discrete evolutionary clades (*i.e.*, the hierarchical classification of species based on evolutionary ancestry of strains). A wide spectrum of mounting epidemiological challenges, however, has begun to expose the various "Achilles' heels" of these once highly touted subtyping methodologies. Newly emerging bacterial pathogens (*e.g.*, the non-O157 strains of hemorrhagic *E. coli*) as well as several re-emerging classical pathogens (*e.g.*, the group I salmonellae) associated with the food supply are now present with unusual population structures, many of which confound conventional subtyping strategies. *Salmonella* Enteritidis, for example, has re-emerged in poultry as a highly virulent clone with little known genetic variation partitioning any two strains [72]. Such genetic homogeneity stymies current subtyping protocols designed for differentiating *Salmonella* strains.

Here, several novel and recent advances in PFGE and DNA sequence-based subtyping schemes are presented that hold promise in countering the aberrant genetic challenges forged within certain foodborne pathogen populations. Specifically, conventional *Salmonella* PFGE schemes, largely ineffective in disciminating *S.* Enteritidis and other clonal strain lineages, have been enhanced by the addition of several more informative restriction enzymes, markedly improving method resolution. Additionally, the slow substitution rates of housekeeping gene sequences have proven incapable of providing the discriminatory power among genetically uniform clades of salmonellae. Thus, other more rapidly evolving or hypervariable gene sequences are now being examined for their utility in discriminating closely related strains. The availability of full-length genome sequences of two or more strains from the same serovar have provided extensive utility in allowing the pinpointing of loci with greater numbers of sequence polymorphisms.

Enhanced PFGE-Based Subtyping Schemes for Clonal Serovars of the Salmonellae

PFGE is applied for the separation of large linear pieces of DNA up to 10 Mb in length. PFGE relies on rare-cutting restriction enzymes which macro-restrict genomic digestions for input to be loaded onto agarose gels. As such, PFGE can successfully resolve significantly larger fragments than conventional electrophoresis thereby yielding banding patterns with

fewer band classes easily discernable among strains. This unique property of the technique has made it useful as a molecular epidemiological approach for detecting outbreak clusters of bacterial pathogens based on common PFGE banding patterns or fingerprints [84].

PFGE has been reported widely as a useful approach for differentiating strains of *Salmonella* serovars often associated with foodborne illness. The technique has greatly improved the accuracy of pinpointing bacterial sources of foodborne outbreaks while significantly reducing the amount of time required to complete an investigation [83]. While PFGE remains the subtyping "gold standard" for many strains and species, however, a few serovars continue to confound PFGE-based subtyping strategies.

Conventional PFGE protocols call for the restriction enzymes *Xba*I and *Bln*I (and sometimes *Spe*I) to resolve *Salmonella* strains. Unfortunately, these particular enzymes lack the discriminatory power to amass the genotypic signatures required to partition many *S.* Enteritidis strains and certain *E. coli* O157:H7 strains–to name two examples. Regarding the former, *S.* Enteritidis comprises a limited number of highly clonal genotypes with a single electrophoretic type (ET) accounting for nearly 80% of the ETs for this serovar [72]. These data reinforce the notion that the vast majority of *S.* Enteritidis strains are derived from a single endemic clone irrelevant of host or geography.

In order to overcome this limitation, several public health and food safety laboratories have collaborated to provide enhanced dicriminatory PFGE protocols for a more effective subtyping of these particular pathogens [92].

Specifically, improvement of the discriminatory power of PFGE for highly clonal salmonellae has relied on two strategies. First, several additional macrorestriction enzymes were selected that provided a relatively even distribution and a moderately informative number of cut sites within the genomes of *S.* Typhimurium strain LT2 , *S.* Typhi CT18, *Escherichia coli* K12 and *E. coli* O157:H7 (www.rebase.com). These additional enzymes provided discriminatory PFGE fingerprints among strains that were previously unresolvable using conventional *Xba*I/*Bln*I protocols. The second strategy has been to combine individual enzyme datasets into a single supermatrix thus allowing for a combined analysis of up to six different enzymes simultaneously. This approach has had a substantial effect in differentiating highly homogeneous strains of *S.* Enteritidis. As an example, concatenated binary band class data for six enzymes derived for a clonal strain set of *S.* Enteritidis derived from poultry sources was subjected to a combined single cluster analysis. The dendrogram that resulted from this simultaneous analysis was highly resolved, yielding numerous previously unidentified branching patterns at the tips of the tree which translated into unique PFGE pattern types for nearly every strain included in the analysis (Figure 2). Additionally, a series of streamlined three-enzyme combined protocols have been developed for several notably clonal *Salmonella* serovars such as *S.* Enteritidis, Hadar, Kentucky, and Heidelberg. These strategies capture most of the six-enzyme discriminatory capability while allowing for a more technically streamlined approach. A *Sfi*I/*Pac*I/*Not*I combination, for example, was found to actually exceed corresponding values in the six-enzyme analysis for *S.* Enteritidis indicating that the diversity present in six-enzyme analyses is captured by certain three-enzyme combinations [92].

In addition to markedly improving the discriminatory potential of PFGE when subtyping closely related enteric strains, it has been observed that six enzyme combined PFGE data matrices can be used to infer fairly accurate genetic relationships among strains, an attribute of six enzyme analysis that is particularly helpful in the absence of any relevant

epidemiologic findings [24]. Deriving accurate genetic relatedness of individuals from homogeneous strain populations allows for assignment of molecular epidemiological relatedness among strains.

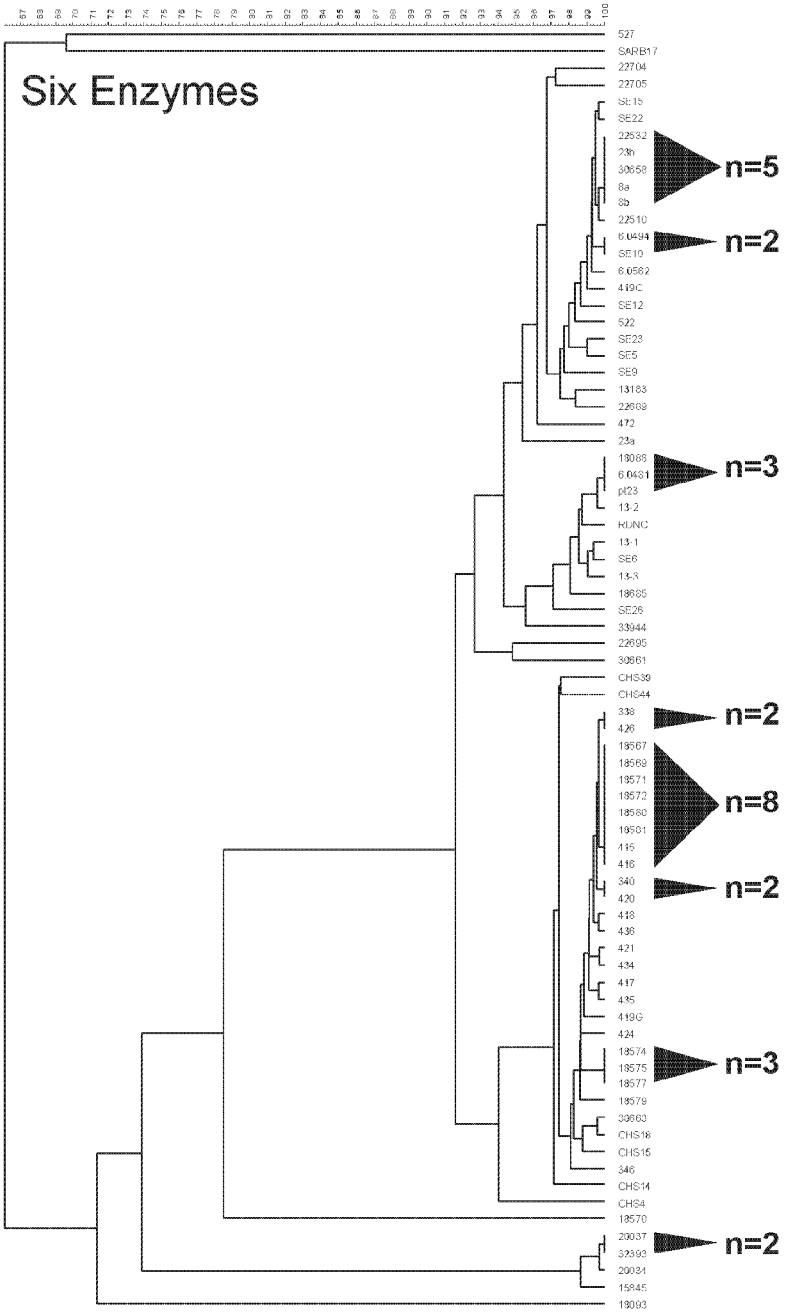

Figure 2. Combined six-enzyme dendrogram of *S*. Enteritidis strains derived from poultry. The tree shown depicts the enhanced discriminatory power resulting from the simultaneous clustering of PFGE data from six enzymes (*Xba*I, *Bln*I, *Spe*I, *Sfi*I, *Pac*I, and *Not*I). Shaded cones to the right of the terminal branches denote polytomies within each dendrogram, while adjacent *n* numbers reveal the strain totals composing their respective polytomies. A scale depicting percent divergence is presented above the dendrogram.

Studies focusing on these PFGE-based estimates of genetic similarity have shown great promise in linking potential sources of infection (*e.g.*, feedstuffs) with outbreak strains of *E. coli* O157:H7 in cattle as well as demonstrating a strong genetic correlation with geographical distribution of O157:H7 strains from common cattle herds (25, 26).

Combined three and six enzyme schemes presented here should serve as useful alternatives for surmounting the problem of extreme strain homogeneityamong pathogens. Specifically, in cases where strains are tightly linked both ecologically and temporally, potentially resulting from clonal expansion, the simultaneous multi-enzyme approach can allow for an epidemiologically useful partitioning of strains. Indeed, combined data strategies such as those noted above highlight PFGE analysis as a continued essential and informative subtyping method for the molecular epidemiological investigation of foodborne and clinical enteric pathogens.

Enhanced DNA-Sequence Based Subtyping for Clonal Serovars of the Salmonellae

Arguably, the most precise and potent method for bacterial strain discrimination remains the nucleotide sequence itself. Nucleotide sequences have been instrumental in discerning evolutionary relationships and assigning genotypes between disparate lineages of bacteria. DNA sequencing has been applied commonly for discriminating individual genotypes at the strain level [41]. Nucleotide sequence results offer several advantages over fingerprinting methods. Given the analysis of an effectively variable locus, larger numbers of polymorphic sites are usually found to be unique to a given strain. Additionally, results are portable and easily transferred between laboratories without the use of elaborate or expensive image-based databases. Finally, genotypic conclusions drawn from DNA sequence data are unambiguous and do not rely on a secondary measurements such as base pair size to make determinations.

MLST is perhaps the most well known and widely used strain subtyping method for direct sequence comparisons. MLST distinguishes strains or clusters of strains based on nucleotide sequence differences or distinct allelic types of conserved housekeeping (HK) genes [45]. Numerous loci are now available that are known to yield reliable phylogenetic clusters of strains for several enteric species (www.mlst.net). Moreover, the simultaneous analysis of multiple HK genes scattered around the chromosome often yields a snapshot of the general taxonomic or evolutionary relationships of the strains or species in question. These types of binned sequence or allele data are also amenable to cladistic analysis, a classification method in which the members of a cluster have been grouped together on the basis of a more recent common ancestor with each other than with the other members of any other group. Taken together, the advantages of MLST have made it a highly effective method, in general, for subtyping, and the method has been used to subtype populations of a variety of bacterial species including *Salmonella enterica*, *E. coli*, *Vibrio cholerae*, *Listeria monocytogenes*, and *Mycobacteriumspp*. [82].

Much like DNA fragment-based subtyping methodologies, one caveat associated with MLST analysis is the inability to differentiate highly clonal lineages of enteric bacteria such as the aforementioned serovars of *Salmonella*. This shortcoming lies in part with the choice of loci being analyzed in a conventional MLST approach. Housekeeping genes represent more evolutionarily stable and slowly evolving portions of a bacterial genome and, as such, are

prone to substantially slower rates of substitution. The subsequent lack of substantive nucleotide variation at the DNA level prevents an effective discriminatory scheme for pathogens like *Salmonella* Enteritidis derived from only a few genetic clones.

While an ever expanding arsenal of automated subtyping technologies, such as sequence tiling on DNA microarray, ESI-TOF mass spectrometry, and whole-genome pyrosequencing offer higher throughput approaches for assessing nucleotide diversity between strains, much of the success of these techniques is belied by the choice of genomic targets. With multiple whole-genome sequences now readily available in the public database for many notable enteric pathogens including *S.* Enteritidis, it has become possible to comparatively seek out those sequences that are under more relaxed selective constraints allowing them to evolve at more rapid rates between strains. This provides a more promising strategy for harvesting a greater number of nucleotide polymorphisms between any two strains. Two places in the bacterial genome where attention is being focused include (i) the relatively anonymous hypervariable ORFs that dapple the genome amongst more conserved HK gene loci and (ii) the intergenic segments of unexpressed DNA that separate many of the ORFs in the enteric genome. For some *Salmonella* serovars, strategies that target hypervariable loci are beginning to yield success. A recent publication demonstrated an effective typing scheme for *Salmonella* Typhymurium clone DT104 by simultaneously analyzing a collection of intergenic non-transcribed sequences [34]. In addition, numerous hypervariable loci have now been identified bioinformatically in *S.* Enteritidis through whole-genome comparison of syntenic ORFs between the PT4 and PT8 genomes in GenBank. Several examples of these targets and their inter-genome sequence divergence values are listed in Table 1. Taken together, these novel genome targeting strategies should facilitate more effective typing schemes capable of culling the subtle genomic differences that distinguish strains residing amongst troublesome clonal pathogen complexes.

Table 1. Examples of hypervariable loci shared between *S.* Enteritidis strains PT4 and PT8

Locus	SE PT4 ORF[a]	SE PT8 Contig[b]	Length (bp)	Putative Function	%PT4/PT8 similarity
map	00632	1419	828	methionine aminopeptidase	97.5
nfnB	01472	2007	651	dihydropteridine reductase	97.1
ybdN	01552	1972	1233	3'- phosphoadenosine	97.7
yliH	02208	2037	381	cytoplasmic protein	97.9
yliJ	02230	786	486	glutathione S-transferase	97.4
lonH	02574	1463	1758	protease	97.3
ycgB	03280	318	1035	cytoplasmic protein	97.7
ynfI	04161	412	612	anaerobic dehydrogenase	97.2
ydgP	04274	832	618	Na+ transport NADH:ubiquinone	97.9
ynhG	04426	342	999	lysM domain	97.9
celD	04581	1017	840	transcriptional repressor	97.9
csgG	05067	273	831	transcriptional regulator (curly)	97.6

[a]S. Enteritidis strain PT4 genome was available from www.sanger.ac.uk/Projects/Salmonella/

[b]S. Enteritidis strain PT8 genome was available in contig format from http://www.salmonella.org/genomics/sen.dbs.

Recombinant Phage Probes in Microbial Diagnostics

Development of systems for monitoring of the environment and food for biological threat agents is a challenge because it requires environmentally stable, long lasting, sensitive and specific diagnostic probes capable of tight selective binding of pathogens in unfavourable conditions. To respond to the challenge, large financial investments and extensive collaborative efforts of specialists in different areas of science and technology are required. In the last years, probe technology is being revolutionized by utilizing methods of combinatorial chemistry and directed molecular evolution. In particular, phage display is recently identified [64, 65] as a new technique for development of diagnostic probes which may meet the strong criteria —fastness, sensitiveness, accuracy, and inexpensiveness— for biological monitoring. Phage display is an exponentially growing research area, and numerous reviews covering different aspects of it have been published [20, 29, 79].

Phage Display Technology

Phage display technology is a powerful tool for selection of short random peptides with high affinity to target structures of interest. It is most fully developed in the Ff class of filamentous bacteriophage, which includes three wild-type strains: f1, M13, and fd. The outer coat of the phage is composed of thousands of subunits of the major coat protein pVIII, which form a tube encasing the viral DNA. At the tips of the phage are several copies of each of the minor coat proteins, pIII, pVI, pVII, and pIX. To create a phage display library, degenerate synthetic oligonucleotides are spliced in-frame into one of the coat protein genes, usually VIII or III, so that the "foreign" peptides encoded by those sequences are fused to the major coat protein pVIII or to the terminus pIII protein, respectively, and thereby displayed in several copies on the exposed surface of the virions. These foreign peptides are arranged in a repeating pattern on the phage capsid, which may thus serve as an interacting scaffold to constrain them into a particular conformation, creating a defined organic surface structure(defined "landscape" by Smith and Petrenko, [79]) that varies from one phage clone to the next. A landscape library is a huge population of such phage, encompassing billions of clones with different surface structures and biophysical properties.

Recombinant peptides specifically binding a target of interest can be selected from random peptidic libraries (usually from 8- to 15-mer), by a process of affinity selection known as "biopanning". Briefly, a simple method for biopanning involves incubating the library with the target (either a single receptor tethered to a solid support or intact cells for selection of tissue and cell targeting proteins) to allow phage displaying a complementary protein to the target to bind. Non-binding phage are then washed away and those that are bound – usually a tiny minority – are eluted in a solution that loosens target-peptide bonds without destroying phage infectivity. The eluted phages are propagated simply by infecting fresh bacterial host cells, yielding an amplified eluate that can serve as input to another round of affinity selection. Successive rounds of biopanning enrich the pool of phage with clones that specifically bind the target. Phage clones from the final eluate (typically after 2–4 rounds of selection) are propagated and characterized individually. The amino acid sequences of the peptides responsible for binding the target receptor are determined simply by ascertaining the corresponding DNA coding sequence in the phage genome.

Recombinant Phage Probes

The proteins displayed on phage surface range from short amino acid sequences to antibody fragments, enzymes, cDNA and hormones. Recently, the possibility of using landscape phage as detection probes in place of antibodies has been presented [13, 62, 64, 65]. Recombinant phage probes with highspecificity and selectivity for a wide range of targets have been selected from phage display libraries [40, 62, 63, 69, 74].

Advanced bioselective sensors may meet prerequisite for isolation, concentration of the agents and their immediate real-time detection. The majority of rapid detection biosensors described in the literature has utilized antibodies as bioreceptors [43]. However, while sensitive and selective, antibodies have numerous disadvantages for use as diagnostic biodetectors in biological monitoring, including high cost of production, low availability, great susceptibility to environmental conditions [78] and the need for laborious immobilization methods to sensor substrates [65].

An effective alternative to antibodies may be the short peptides affinity-selected from random phage-displayed peptide libraries for specific, selective binding to biological targets (80). Recombinant phage-displayed peptides can recognize and bind specific targets, such as cell surface receptors. Thus, they can act as antibody surrogates, possessing distinct advantages including durability, stability, standardization and low-cost production, while achieving equivalent specificity and sensitivity [63, 65]. A selected phage itself can be used as a probe in a detection device, without chemical synthesis of the displayed peptide or fusion to a carrier protein. For example, to be used in an automated fluorescence based sensing assay [33] or FACS [85], phage, exposing thousands of reactive amino-groups, can be conjugated with fluorescent labels and, in this format, successfully compete with antibody-derived probes. The use of antibodies as diagnostic probes outside of a laboratory may be problematic because they are often unstable in severe environmental conditions. Environmental monitoring requires stable probes, such as landscape phage, that carry thousands of foreign peptides on their surfaces, are as specific and selective as antibodies, and can operate in non-controlled conditions. Filamentous phages are probably the most stable natural nucleoproteins capable of withstanding high temperatures (up to 80°C), denaturing agents (6–8 mol/L urea), organic solvents [e.g., 50% alcohol or acetonitrile], mild acids (pH 2), and alkaline solutions. The thermostability of a landscape phage probe was recently examined in comparison with a monoclonal antibody specific for the same target [14]. They were both stable for greater than six months at room temperature, but at higher temperatures the antibody degraded more rapidly than the phage probe. Phage retained detectable binding ability for more than 6 weeks at 63°C, and 3 days at 76°C. These results confirm that phage probes are highly thermostable and can function even after exposure to high temperatures during shipping, storage and operation.

Phage-derived probes inherit the extreme robustness of the wild-type phage and, in addition, allow fabrication of bioselective materials by self-assemblage of phages or their composites on metal, mineral, or plastic surfaces [65]. The recombinant phage probes appear to be highly amenable to simple immobilization through physical adsorption directly to the sensor surface, thus providing another engineering advantage while maintaining biological functionality [18, 19, 57, 80]. This property allows phage to be used as a recognition element in biosensors by using a method to immobilize phage on the sensor surface simpler than Langmuir–Blodgett and molecular assembling technologies [56].

Applications

Numerous phage applications have been proposed, including the detection of small molecules [73], receptors [6], bacterial spores [13], and whole-cell epitopes [18, 19, 23, 57, 64, 80, 81, 91]. In particular, this technology represents a powerful tool for the selection of peptides binding to specific motifs on whole cells since it is a non-targeted strategy, which also enables the identification of surface structures that may not have been considered as targets or have not yet been identified [9].

In our studies, we used a whole-cell phage display approach to isolate phage probes specifically binding to surface of bacterial cells [18, 19].We demonstrated, along with the other published data, that phage clones show promise as probes for biosensor applications. In fact, these phage probes could be used to build micro-biosensor systems in which biological sensing element are the selected recombinant phage. Our experience shows that recombinant phage selected by phage display technology may be the most suitable for obtaining long lasting specific probes against *L. monocytogenes*, *P. aeruginosa* and other bacterial pathogens.

Recombinant phage selected by phage-display may find application as biosorbent and diagnostic probe for monitoring bacterial cellsby various devices in which antibodies have been used to date. The potential advantages of phage probes include: the simplicity of manipulation of the phage libraries, their great variability, high binding affinity, great stability and the possibility to select probes to targets of different nature, also to small molecules or toxic compounds or immunosupressants against which it is difficult to raise natural antibodies. For these properties they may be exploited for development of bioaffinity sensors, whose essential elements are probes that specifically recognize and selectively bind target structures and, as parts of the analytical platform, generate a measurable signal. For example, they may be used for separation and purification of bacteria prior to their identification with polymerase chain reaction, immunoassays, flow cytometry, or other methods. Furthermore, they may find application as bio-recognition elements of real-time biosensor devices. Recombinant phage selected by phage-display selectively recognize and specifically bind complex structures, such as bacterial cells, thus they can be used to develop rapid diagnostic arrays. In fact, traditional diagnostic systems usually involve a multi-step detection method with the use of labelled secondary antibody. Phage-displayed detection microsystems could be considered one-step, simultaneously bind and identify the target microorganism, with no need of further characterization steps. Furthermore, the nature of the bioreceptor phage holds potential utilization for development against any bacterium, virus or toxin to which a corresponding recombinant phage could be affinity-selected for. Therefore, different phage clones could be isolated specifically binding to isolated proteins, enzyme or inorganic material, as well as to different microbial species, thus, with the same microsystem different enzymes, toxins and pathogens might be detected, by performing several different parallel assays in real-time, within the same miniaturized substrate, in a single run. This could ultimately translate to a much lower cost per test.Much of the promise of these microarrays relies in their small dimensions, which reduce sample and reagent requirements and reaction times, while increasing the amount of data available from a single assay. Through the use of different labels in parallel, such as different specific phage probes, multiple tests could be simultaneously performed on the same microarray in a single step, so that standardizing data

from multiple separate experiments is unnecessary and truly meaningful comparisons can be made.

The development of highly sensitive and accurate field-usable devices for detection of multiple biological agents could have a number of applications in biomedical field as well as in monitoring of agro-food pathogens and detection of biological warfare agents.

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Chapter V

ROLE OF EFFECTORS ON HYPOXIA DUE TO NITRIC OXIDE PRODUCTION INHUMAN ALVEOLAR EPITHELIAL CELLS AND OXYGEN DEPLETION IN HUMAN HEPATOCYTES

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ABSTRACT

The effect of hypoxia on cell viability, proliferation and possible induced apoptosis in human alveolar epithelial cells and human hepatocytes is not clearly understood.In cultured human alveolar epithelial cells, IL-1β, TNF-α and INF-γ stimulated the nitric oxide production and resulted with inflammation manifested by hypoxia and apoptosis.In cultured hepatocytes,enhanced expression of superoxide dismutase and glutathione reductase enzymes indicated hypoxia associated with the enhanced level of *cAMP* dependent phosphodiesterase, NADPH dependentCytochrome C Oxidase enzyme activities due to energy insufficiency in hepatocyte cultured medium.Both hypoxia and energy insufficiency reduced hepatocyte viability.We propose that extent of inflammation leading to hypoxia initially and programmed cell death later both can enhance the MRI visible edema fluid content in lungs due to oxygen and energy insufficiency to surviving inflammatory alveolar epithelial cells.The mechanism of A549 cell damage due to nitric oxide (NO) production included: Cytokines regulated the expression of nitric oxide synthase (NOS) through NF-κB activation; Cofactor tetrahydro biopterin-catalyzed

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synthesis of iducible nitric oxide synthase (iNOS) enzyme.The NO production and increased level of NOS expression lead to Na^+ ion transport and cell proliferation with differentiation or apoptosis.Hypoxic human alveolar epithelial cells showed the possibility of NO production associated with the decrease of their viability and rapid increase of apoptosis. In conclusion, increased level of NO production can be a cause of hypoxia induced apoptosis and possibly MRI visible indicator of inflamed alveolar cell viability. High resolution MRI can track these sites of inflammation in lungs.

Key Words: cell viability; apoptosis; energy insufficiency; inflammation; MRI; oxygen insufficiency; cytokines; nitric oxide synthase; Na^+ ion transport

INTRODUCTION

The influences of inflammatory cytokine effectors and energy metabolizing enzymes on hypoxia, cell viability loss, and possible induced apoptosis in human alveolar epithelialcells and human hepatocytes are not clearly understood.The alveolar epithelial cells are very sensitive to inflammation and hypoxia while hepatocytes are very sensitive to energy insufficiency during regeneration after hypoxia.However, events of hypoxia in pathological damage of alveoli and energy starved hepatocytes were reported due to reactive oxygen species, disrupted inflammatory cells and epithelial cell injury in alveoli manifested as accumulation of fluid or edema [1].The hypoxia and hepatocyte survival indicated the interplay of nitric oxide production, chemokines with mitochondrial glutathione and oxide synthesis but poorly understood [2-5].Still Alveolar epithelial cells play key role in the development and recovery from acute lung injury [6].Recently, our report suggested the possibility of metabolic energy insufficiency and cytokine-induced nitric oxide production as major causes of hypoxia that can induce apoptosis and edema [7].The regulatory role of nitric oxide was described crucial on the redox activated *in vivo* target interactions during apoptosis [8].However, the role of energy insufficiency on hypoxia induced apoptosis in tissue injury is not well understood [9-11].We believe that cytokine stimulation and metabolic energy insufficiency both play their role side by side in hypoxia. However, these events if lead to apoptosis is not investigated [11].Recent studies suggested the mechanistic role of IL-1β, TNF-α and INF-γ molecules in iNOS synthesis as main step in hypoxia induced apoptosis [12-14]. The increased reactive oxygen radicals play active role associated with hypoxia-induced apoptosis [16].Other studies suggest that hypoxia may downregulate the sodium ion channels and affect the sodium transport in isolated alveolar epithelial cells [17, 18].Moreover, apoptosis has been reported to increase sodium transport in intact tumor cells [19].

Aim of the present study was to determine the possible mechanistic role of nitric oxide production in cell viability loss in alveolar epithelial cells and different effectors on oxygen and metabolic energy insufficiency in hepatocytes.We exposed both alveolar epithelial cells and hepatocytes to hypoxic conditions for different time periods in the presence of effectors and measured the hypoxia biomarkers. In hypoxia-induced hepatocytes, cAMPphosphodiesterase, superoxide dismutase, HMP shunt and glutathione reductase enzyme activities were measured.In alveolar epithelial cells, NO and cell viability were monitored at 24 hour interval for a total of 96 hours in the presence of cytokine mixture.The

present study indicated the active role of superoxide radical and nitric oxide production as early events in hypoxia.

MATERIALS AND METHODS

Dulbecco's modified eagle's medium (DMEM), Dulbecco's phosphate buffered saline (DPBS), Trypsin-EDTA solution, antibiotics (penicillin-streptomycin, amphotericin B), recombinant human interleukin-1β (IL-1β), recombinant human tumor necrosis factor-α (TNF-α), and recombinant human interferon-γ (IFN-γ) were obtained from Sigma Chemical (St. Louis, MO).Fetal bovine serum was purchased from Hyclone Laboratory (Logan, UT).L-glutamine was obtained from ICN Biochemicals (Cleveland, Ohio), and 48-well plates were purchased from Corning Costar (Cambridge, MA).

Cell Culture

Immortal Type II alveolar cell lines of human origin (A549) were obtained from the American Type Culture Collection (ATCC).A549 is epithelial-like in morphology and originates from a human lung carcinoma. The cells were seeded at 4 X 10^4 cells/cm^2 onto 48 well plates (Corning Costar) containing 0.5 ml of DMEM supplemented with 10 % FBS, L-glutamine (2 mM), amphoterincin B (5.6 mg/L), and penicillin-streptomycin (100 U/mL), and allowed to grow to confluence.

Cultured primary hepatocytes of human origin were cultured and seeded at 1 x 10^8cells/cm^2 onto 48 well plates (Corning Costar, NY) containing 1.0 ml of RPMI supplemented with 4 % CSF, and 1.5% penicillin-streptomycin (v/v) and allowed to grow to confluence.In other set of hepatocytes, the medium was maintained with 1 % O_2+ 5 % CO_2 and 94% nitrogen to keep hepatocytes in hypoxic conditions.Hypoxic conditions were achieved in humidified aerobic system(INVIVO O_2, Ruskinn Technologies, USA) as described elsewhere [19].

Alveolar Cells Exposed to Cytokines and Cell Viability

Following confluency, the cells were incubated in serum-free media (DMEM) for 24 hours prior to cytokine exposure.Baseline concentration of NO in the media due to constitutive NOS (cNOS) was determined initially.Monolayers were initially exposed to a combination of cytokines ranging in concentration from 1-100 ng/ml for a period of time up to 120 hours to determine the point of maximum NO production.Once this was determined, the monolayers were then exposed to a factorial combination of three cytokines (IL-1β, TNF-α, and IFN-γ) at three concentrations (0, 5, and 100 ng/mL) with or without the presence of 0.1 mM L-N-arginine methyl ester (L-NAME).L-NAME is a competitive inhibitor of NOS and can be used to determine the source of NO synthesis.Stable end-products of NO oxidation ($NO^{2-}$and NO^{3-}) were then measured using chemiluminesence (Model 280 NOA, Sievers Inc., Boulder, CO) in the media bathing the apical surface of the cells at 24 hour intervals for 96 hours following exposure.The number of viable cells at each time interval was measured by

Trypan Blue dye-exclusion.Cell mass was determined by a modification of the colorimetric Lowry assay for total cell protein, using a phenol reagent (Folin-Ciocalteau) for the development of color as described elsewhere [14, 15]. Each exposure was repeated in duplicate, and NO concentration was normalized by the cell mass in each well.

Nitric Oxide Assay

NO activity was analyzed by chemiluminescence (Model 280 NOA, Sievers Inc., Boulder, CO).NO is highly unstable in the presence of oxygen, and is rapidly converted into $NO^{2-}$and NO^{3-} in liquid media.In order to detect NO, both $NO^{2-}$and NO^{3-} were converted into NO using a reducing agent (vanadium (III) chloride). To achieve high conversion efficiency, the reduction is performed at 90 ^{o}C [19].

NADPH Oxidase

NADPH oxidase activity was determined by spectrophotometric assay by measuring the decrease in extinction at 340 nm followed at substrate concentration of 0.1 mM.The assay mixture contained 3.3 mM phosphate buffer pH 7.4, 1 mM KOH and 1.5 mM rotenone in 5 ml reaction mixture.The reaction was started by addition of 0.1 mM NADPH, Controlled reaction with equal water instead of NADPH.One unit was expressed in nanomoles of NADPH oxidized per minute per mg protein as described [20].

cAMP Dependent Calmodulin Mediated Phosphodiesterase Stimulation

Phosphodiesterase activity was measured in hepatocytes isolated after 48 hours from their cultures. Calcium dependent phosphodiesterase was measured based upon measurement of cyclic AMP before and after incubation with 3 μ moles $MgCl_2$, 0.25 %,Triton X-100(w/v), 80μmoles imidazole buffer pH 6.91, hepatocyte preparations of above supernatants and calcium ion 1.5 x 10^5 M.After incubation of 15-20 minutes at 37^{0}C in presence of ^{3}H cAMP for monitoring recovery, reaction was stopped by placing vessels in boiling water bath.The reaction mixture was suitably diluted and cyclic 5' AMP was measured. One unit was expressed as picomoles of cAMP degraded per mg. of hepatocyte cell protein per minute. For intracellular cyclic AMP levels, hepatocyte cells adhering to surface were removed and boiled $HC10_2$ was poured on these cells.After cooling and centrifugation at 3000 x g for five minutes the supernatant was isolated and cAMP was measured in 0.05 M acetate buffer pH 6.2 [21].^{3}H labeled cAMP was added 25000 dpm (disintegrations per minutes) to supernatant to determine the recovery Prostaglandins E_2 and F2α were measured by radioimmunoassay. Appropriately diluted antibody 0.1 ml was incubated overnight with 0.1 ml of respective tritium labeled prostaglandin 10000 dpm either E2 or F2α with 0.1 ml sample supernatant or standard unlabelled F2α prostaglandin.The solution was prepared in 0.1 M phosphate buffer pH 7.4 containing 0.9 % sodium chloride and 0.1 % gelatine.One ml supernatants containing prostaglandin-antiprostaglandin antibody complex were counted in a Tricarb 3380 liquid scintillation. Vials and determined in ng per mg on scintillation counter.The incubations were

performed for 30 minutes before estimating prostaglandin by radioimmunoassay using standard curve as described elsewhere [22].

Superoxide Dismutase

Superoxide dismutase activity in isolated hepatocytes was determined in a 2.0 ml reaction mixture containing 0.1M phosphate buffer pH 7.4, 25μM dianisidine in ethanol, 26 nM riboflavin and 100 -400μL hepatocyte homogenate fraction.At 25^0C photochemical reaction started for four minutes by illumination provided by pair of parallel 20 W Sylvania florescence tubes positioned in 3 inches away from assay tube. One unit of enzyme measured at 460 nm after four minutes incubation time which reduced one μmole of riboflavin as described [23].

Hexose Monophosphate Shunt Activity

Hexose monophosphate shunt activation was measured by method [24], ^{14} C glucose 1 μCi/dish was added along with particles latex and erythrocytes as mentioned above experimental and without particles as control.After one hour two ml cell free medium was transferred into wells of Warburg type reaction flasks containing *a* strip of filter paper with 100 ml of 10 % potassium hydroxide in central well. The flasks were closed and 0.2 ml sulphuric acid added from side arm. Later filter paper strip and 250 μl of water was used for washing well and transferred to scintillation vial for counting in Trisol for $^{14}C0_2$.The unit of hexose monophosphate shunt activation was expressed in $^{14}CO_2$ liberated in dpm from labelled glucose per minute per mg protein.

Glutathione Reductase

Glutathione reductase activity was estimated by using spectrophotometry method in reaction mixture 2.5 ml containing 2.0 ml phosphate buffer pH 7.4 with 0.1 ml 50 mM GSSG 0.1 ml 250 UM flavin adenine dinucleotide 0.05 ml 80 mM EDTA and 0.1 ml hepatocyte mitochondrial fraction in cuvette was incubated at 37^0C for 15 minutes following which 0.1 ml NADPH solution (4 mM) was added to reaction mixture as described elsewhere[25]. In blank cuvette, flavin adenine dinuleotide was not added. After five minutes, absorbance at 340 nm was measured.One unit of activity was expressed as number of moles of NADPH oxidised per hour per mg protein

RESULTS

Nitric Oxide Production in Human Alveolar Epithelial Cells

TNF-α-induced NO production did not exceed constitutive NO production (Figure 1). In contrast, both IL-1β (Figure 2) and IFN-γ (Figure 3) significantly increased NO production

from A549 over the basal or constitutive level.The effect of IFN-γ is only observed at the largest concentration (100 ng/ml) (Figure 3); whereas the effect of IL-1β is observed at the lowest and highest concentrations (Figure 2).

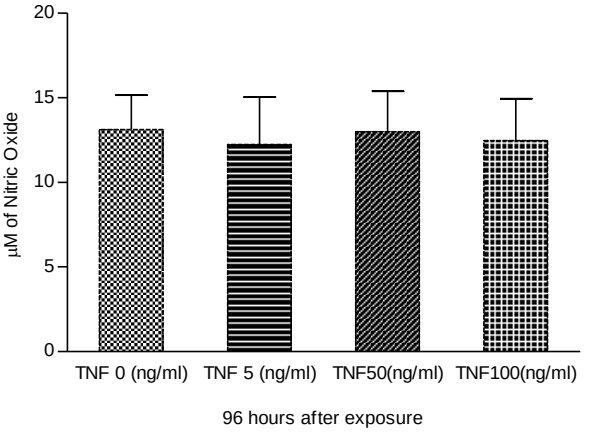

Figure 1 Effects of TNF-□ on nitric oxideproduction in A549 cells.

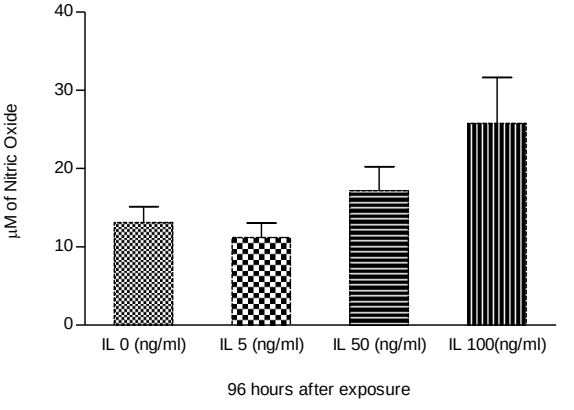

Figure 2. Effects of IL-1□ on nitric oxideproduction in A549 cells.

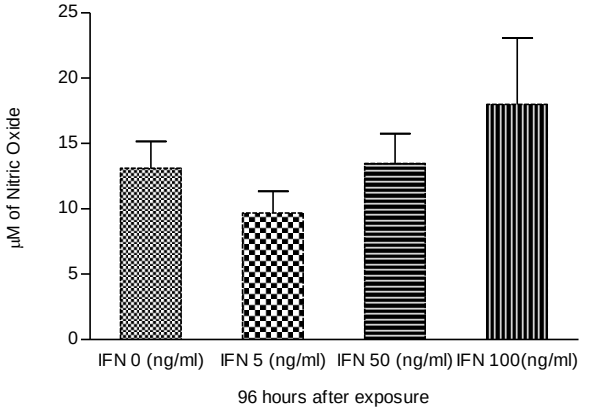

Figure 3. Effects of IFN-□ on nitric oxideproduction in A549 cells.

Cell Viability in Human Alveolar Epithelial Cells

NO concentrations (Figure 4A) and cell viability (Figure 4B) were simultaneously monitored at 24 hour intervals for 120 hours following exposure to cytokine mixtures to determine potential cytotoxicity of the exogenous cytokines or the increased concentrations of NO, $NO^{2-}$and NO^{3-}.Cell viability decreased as time and concentrations of cytokine mixtures increased.When cytokine mixtures were added to the culture media, the number of viable cells, determined by Trypan blue dye-exclusion assay, declined by more than 50 % within 5 days at the maximal cytokine concentration (Figure 4).

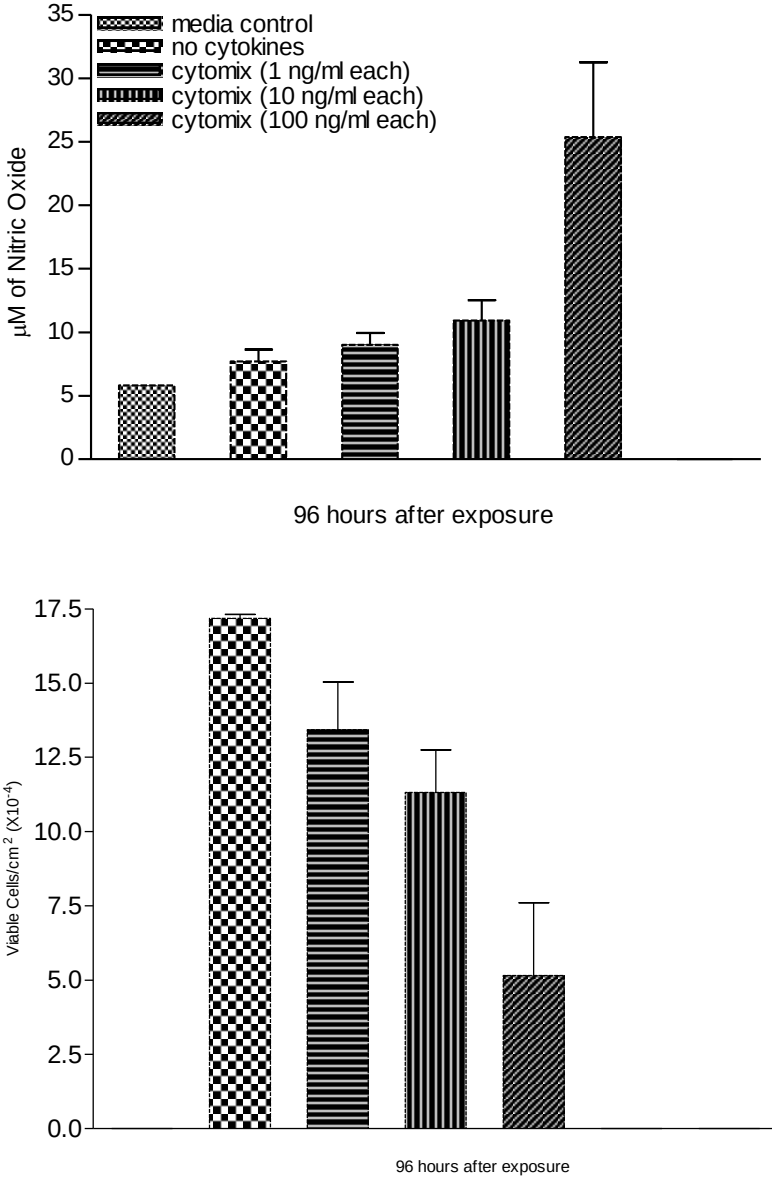

Figure 4. Time profiles of nitric oxide production (A) and cell viability (B) for A549 alveolar cells.

The viability of A549 cells was reduced more in the media containing TNF-α alone than IL-1β or IFN-γ alone (Figure 5).The decrease in cell viability may be due to oxidative damage of the higher concentration of NO induced by cytokines.However, the half-life of the NO free radical is very short (order 1 second) and the NO free radical is rapidly changed into nitrite and nitrate.To test this hypothesis, A549 cells were exposed to exogenous nitrate (NO^{3-}).As demonstrated in Figure 6, exogenous nitrate (NO^{3-}) in the absence of exogenous cytokines did not affect the cell viability and growth of A549 cells.

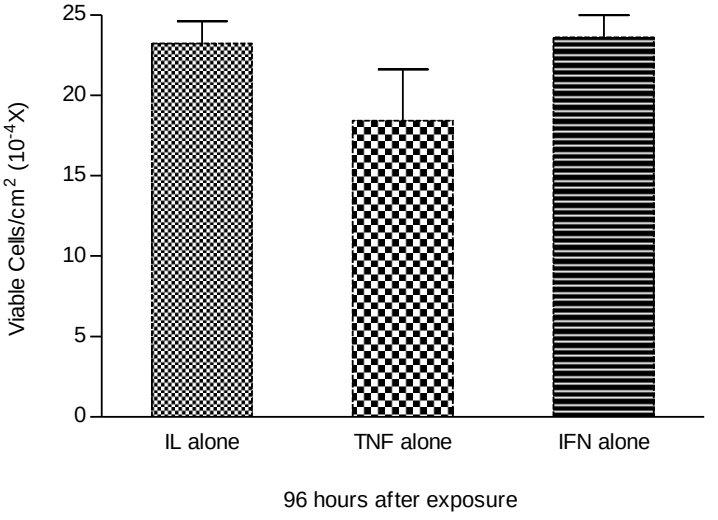

Figure 5. . Effects of IL-1□ , TNF-□ , or IFN-□ on cell viability. Viable cells were measured 96 hours after exposure to each cytokine.

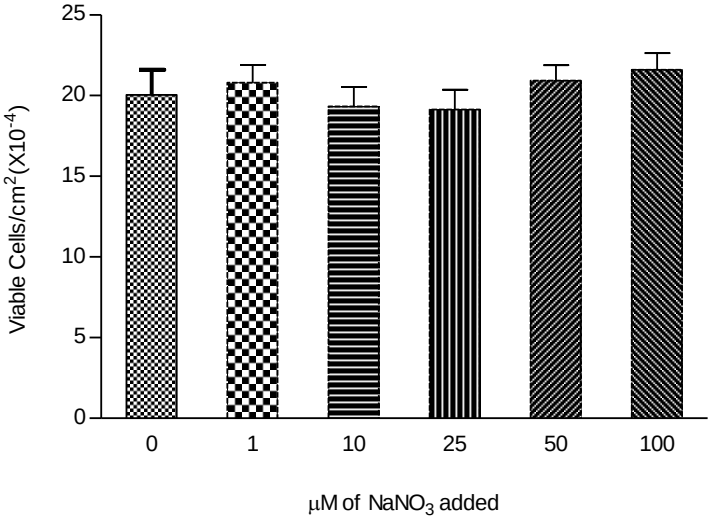

Figure 6. Effects of exogenous nitrate (NO3-) on cell growth and viability. Various concentrations (0 – 100 □ M) of sodiumnitrates were added to media on A549 monolayers to determine effects of cytokine-induced increased NO production on cell viability.

NADPH Oxidase Activity in Hypoxia

Specific activity of NADPH Oxidase enzyme in hypoxic hepatocytes was enhanced in comparison to control hepatocytes (23.7 $\pm$ 1.8 vs 16.2 $\pm$ 2.1) (P value < 0.0014).

cAMPPhosphodiesterase Activity in Hypoxia

The specific activity change of PDE was minimal and insignificant estimated as unutilized cAMP by enzyme in control and hypoxic hepatocytes. In presence of calcium ions the PDE activities in hypoxic hepatocytes were comparable and higher than control hepatocytes (P value = 0.3489; coefficient of variance 180.6 %).The activities of NADPH oxidase in control hepatocytes were measured in specific enzyme activity in I.U. The hypoxic hepatocytes showed inhibited PDE activities by addition of both prostaglandin E2 and prostaglandin F2 alpha but higher than control hepatocytes.However, inhibition by PGE2 was higher than PGF2 alpha.These results indicated the dependence of PDE enzyme activities on prostaglandins as effectors.

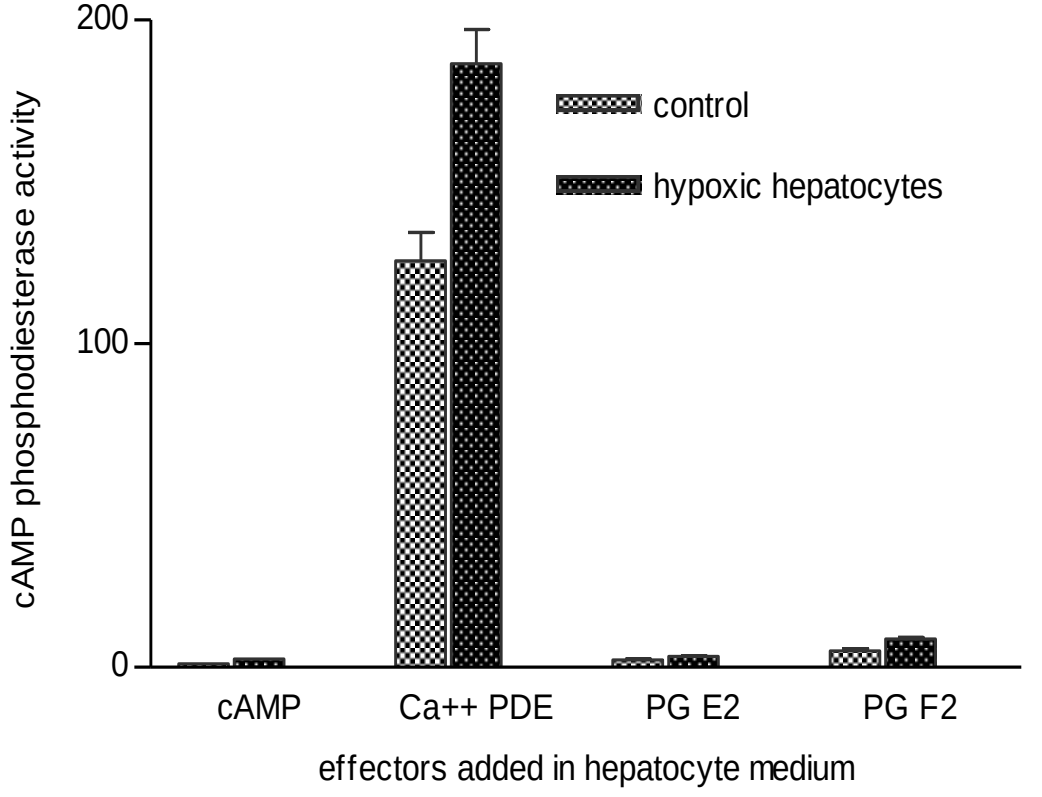

Figure 7. Effect of added prostaglandin E2 and prostaglandin F2α on cAMPPhosphodiesterase enzyme activity of hypoxic hepatocytes.

Superoxide Dismutase Activitiy in Hypoxia

Superoxide dismutase enzyme activity was comparable and showed minimal difference in both control and hypoxic hepatocytes.The presence of sodium cyanide concentration showed the elevated activities ofSOD in both control and hypoxic hepatocytes.However, the hypoxic hepatocytes showed elevated activity significantly high after adding 10 nM of sodium cyanide in the medium.

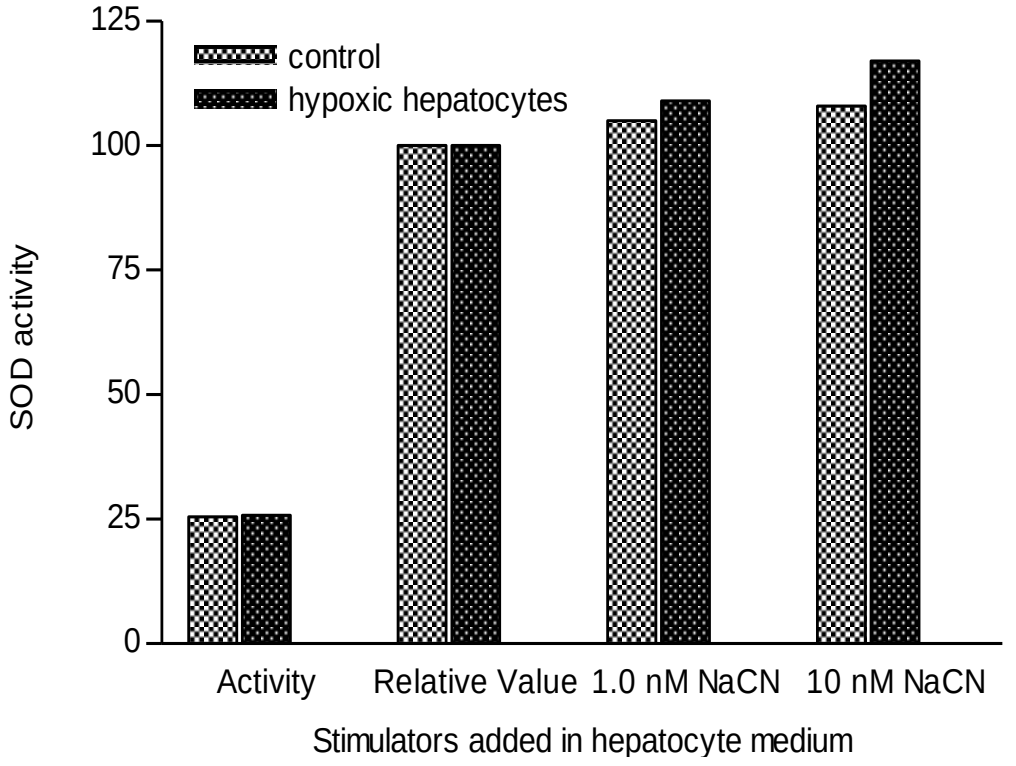

Figure 8. Effect of added sodium cyanide on superoxide dismutase activity in hypoxic hepatocytes.

Hexose Monophosphate Shunt Activity in Hypoxia

The HMP shunt activity in control and hypoxic hepatocytes was measured by specific activity of enzyme in presence of different effectors known as quenching the hexose monophosphates in glycolysis. The enzyme activity was similar with no difference (P value 0.4434) in control and hypoxic hepatocytes without any effector and latex did not alter it.The activity was significantly enhanced in hypoxic hepatocytes in presence of trophozoits (P value 0.1587) but the enhancement was lesser in presence of erythrocytes.It indicated the dependance of activity on nature of effectors. The latex was inert, erythrocytes were nonvirulent and trophozoits were virulent as a result HMP shunt activity was enhanced in the order of latex < erythrocytes < trophozoits added in the medium of hypoxic hepatocytes.

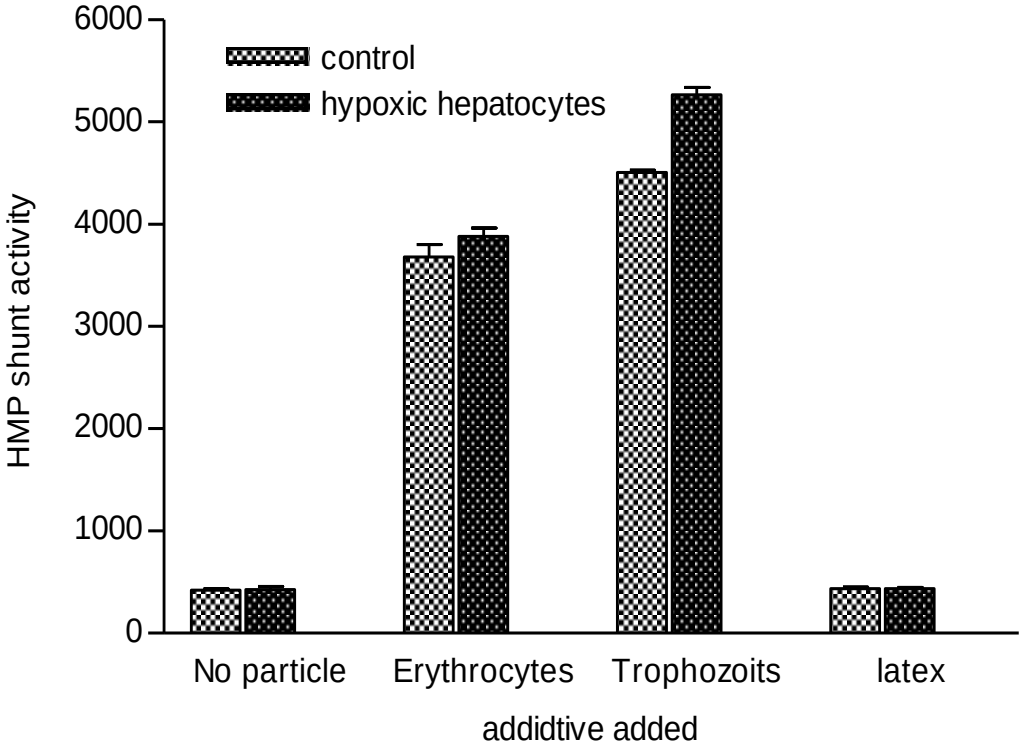

Figure 9. Effect of added particles on HMP shunt activity in hypoxic hepatocyte cells in culture.

Glutathione Reductase (GSR) Enzyme Activity in Hypoxia

The GSR specific activity was measured in hypoxic hepatocytes in presence of different GSR enzyme inhibitors such as DEM, antimycin, BSO as effectors.To demonstrate the dependence of enzyme activity on exposure time of effectors, the hypoxic hepatocytes were incubated in presence of effectors and enzyme activity was mesured in medium. At 0 hour, the enzyme specific activity was same in hepatocytes in all groups.After 24 hours, the activity was decreased in hepatocytes maximum by actinomycin while DEM and BSO inhibited the enzyme at similar extent and hypoxic hepatocytes exhibited no difference in enzyme activities ($P < 0.05$, 0 vs 24 hr, 24 vs 48 hr, and 24 vs 72 hr; $P < 0.001$, 24 vs 96 hrs).After 48 hours, the GSR enzyme activity was decreased maximum by BSO and minimum by antimycin ($P > 0.05$, 48 vs 72 hr; $P < 0.05$, 48 vs 96 hr).However, the behavior pattern of enzyme inhibition by DEM and BSO was similar.After 72 hours, the inhibition pattern of GSR enzyme activitity by inhibitors was same as after 48 hours, but it changed after 96 hours($P > 0.05$, 72 vs 96 hr). After 96 hours, all inhibitors showed maximal inhibition and hypoxic hepatocytes showed same specific activities in all groups.Antimycin inhibitor exhibited lesser inhibition activity of hypoxic GSR enzyme initially during 24-48 hours.

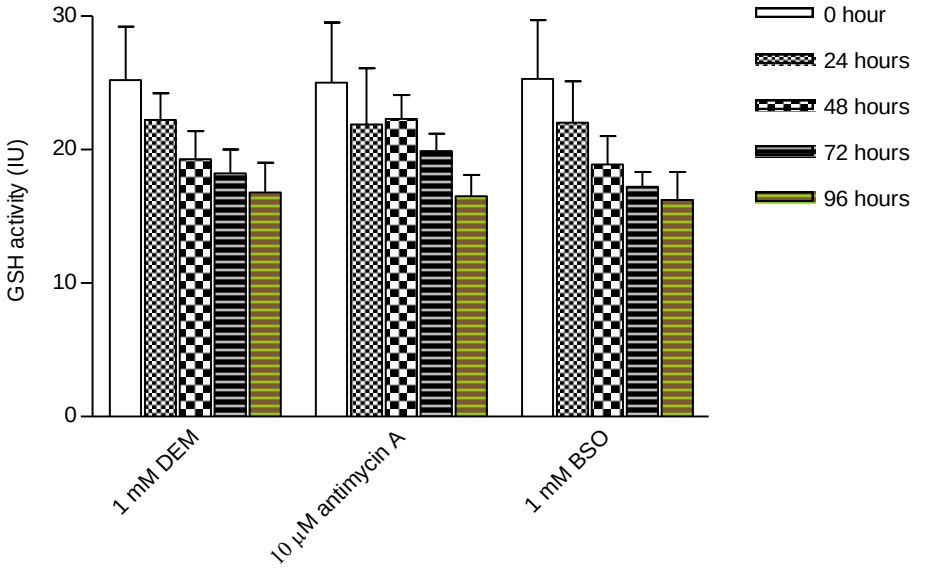

Figure 10. Effect of added stimulators on Glutathione reductase enzyme activity in hypoxic hepatocytes in culture.

DISCUSSION

The hypoxia model of alveolar epithelial cells was chosen due to their cell viability as indicator of hypoxia and sensitivity to cytokines to explain their mechanistic role.The hepatocytes served the purpose of demonstrating the role of intra-hepatocellular enzymes as indicator of energy depletion during low oxygen hypoxic state possibly associated with. nitric oxide production and inflammation. The present study indicates that three cytokines (IL-1β, TNF-α, and IFN-γ) exhibit differently induced responses to human alveolar epithelial cells such as hypoxia and induced cell viability loss.However, cytokines may cause NO production and lead to cytotoxicity. The reduction of cell viability may also be caused by cytokine itself.

The decrease in cell viability observed following exposure to the cytokines may be due to oxidative damage of NO induced by cytokines.It was confirmed, when A549 cells were exposed to exogenous NO^{3-} the cell viability and growth was not affected (Figure 6).We observed the active role of reactive oxygen species and superoxide dismutase activity in cultured hepatocyte damage during hypoxia (Figure 11).We also observed that NO free radical could actually protect against cellular damage and cytotoxicity from reactive oxygen species by reacting with them (i.e., superoxide anion) and acting as a scavengeras suggested earlier [30].However, short half-life of NO suggests another possibility of decrease in cell viability.The decrease may be due to the toxicity of cytokines, specifically TNF-α which demonstrated the greatest cytotoxicity.These observations suggested that the growth and proliferation of the A549 cell line is sensitive to TNF-α.Recent studies demonstrated that TNF-α had selective cytoxicity for tumor cells and transformed cell lines [26, 27]. An anti-proliferate response of TNF-α was evident in 16 out of 34 tumor cell lines, while the growth of normal human cell lines from the colon, fetal skin, and lung was not inhibited [28].

Recently TNF-α from inflammatory epithelial cells was reported important mediator in acute lung injury with its possible role in reversing hypoxia and induced apoptosis [19, 29].Recently, a report suggested the oxygen insufficiency induced hypoxia can contribute in decreased protein and lipid production, edema fluid, destruction from neutrophil proteases and oxygen reactive species [1].However, the mechanism is not understood involved in these events of cell damage.In present study, we consider hypoxia may be induced by cytokine mediated nitric oxide production as associated with the event of cell viability loss. The important suggestion here is TNF-α and NO free radical production cause hypoxia and cell viability loss. Other studies also support these speculations [31].

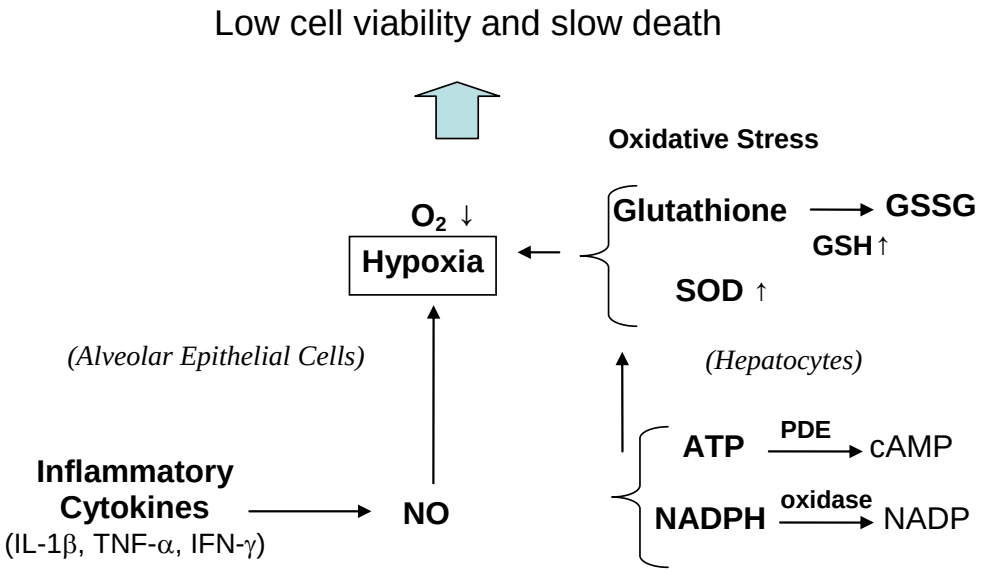

Figure 11. A schematic sketch of hypoxia events represent the role of cytokines on NO production and possible role of reduced NADPH and ATP on SOD and oxidative stress leading to energy and oxygen insufficiency. Abbreviation: NO; nitric oxide, GSH; , GSSG; ,SOD; superoxide dismutase, NADPH; reduced nicotine amide adenine dinucleotide phosphate, PDE; phosphodiesterase.

We observed the decreased hepatocyte cell viability associated with enhanced high NADPH oxidase activity and NADPH consumption with altered cAMPphosphodiesterase enzyme activity.It may be attributed that available cAMP supply in cell is inhibited by 'feed back inhibition' in oxygen deficient cells resulting with higher phosphodiesterase demand and its high activity.Other reports suggest the similar explanation during [32].However, the oxygen supply to cell gets interrupted by less available oxygen and formation of free superoxide radical.In attempt to scavenge the superoxide radical, hepatocyte cells utilize superoxide dismutase enzyme to stimulate the energy supply in form of NADH and ATP.Superoxide dismutase removes the active oxygen species to make available oxygen to cells.Our results suggest the hyperactive state of hypoxic hepatocytes and high SOD activity.However, its action depends on several other factors [33]. During this response, mitochondrial glutathione and HMP shunt pathway play significant role as a result of hypoxia [34]. The antioxidant enzyme SOD and other antioxidants provide protection to the cells against oxidative damage of hypoxia.

Our study suggested that the oxygen insufficiency may possibly be a consequence of energy depletion due to less NADPH available and increased oxygen reactive species manifested by enhanced activity of SOD.Other observation was that prostaglandin effectors affect the activities of cAMPphosphodiesterase and indicate the quick depleted ATP in hepatocytes and it can be attributed for alveolar epithelial cells as vasodilation sensitivity.Recent report supports this view leading to ATP depletion in hepatocytes and alveolar epithelial cells [35].The reactive oxygen species production appears as another cause of hypoxia as suggested by the nature of super oxide dismutase as cyanide sensitive.Previous reports indicated the possibility of superoxide dismutase as oxygen reactive scavenger [36].We observed here the good reason of association between superoxide anion scavenge and hypoxia as first cause of cell viability loss leading further to cell damage as illustrated in Figure 11.In this direction, ample evidence supports the possibility of burst or slowing down of HMP shunt activity due to depleted ATP and NADPH in the hepatocytes [35, 37].However, our experiments suggest the specific roles of effectors on hypoxia biomarkers.The prostaglandins and calcium are known to show antagonistic behavior on cAMPphosphodiesterase activity.Superxide anion plays a significant role in hypoxia. NO acts as a scavenger of superoxide. We observed the SOD sensitivity to cyanide anion. The increased sodium cyanide concentration showed stimulation of superoxide dismutase activity in Hepatocytes.It indicates the role of superoxide in hypoxia leading to respiratory burst. The other effectors as live erythrocytes and trophozoits showed HMP shunt stimulation while inert latex particles showed no effect on HMP shunt activity suggesting the association of effector nature as determinant of the HMP shunt activity.The glutathione plays a critical role in surviving hepatocytes during hypoxia and indicated the stimulated reactive oxygen species [3].All these hypoxia biomarker activities showed dependence on the nature of effectors in corroboration with earlier reports [3, 38, 39, 40].The hexose monophosphate (HMP) shunt enzymes generate NADPH required for the activity of GSR which may affect the glutathione (GSH) content in the hepatocytes. The GSH content and the decreased activity of GSR in presence of DEM, antimycin and BSO suggested the cause for increased LPO and suggested the production of NADPH by the HMP shunt to reduce the oxidative damage. The observation suggests the active role of antioxidant system with energy conservation.

In present study, important finding was the observation of energy insufficiency and oxygen depletion as distinct causes of hypoxia lead to cell viability loss.However, association of this depletion and hypoxia is not conclusive and needs further investigation.Our recent study suggested that hypoxia may induce cell cycle G1 phase arrest, limiting cell proliferation and increased apoptosis of breast tumor cells in first 24 hours [41].These observations are consistent with other recent reports [19, 42].

The present study has some notable limitations to predict the role of oxygen radical and energy insufficiency in cytokine induced nitric oxide production as initiator of hypoxia induced cell damage. The cytokine effectors are not sole cause of nitric oxide synthase stimulation. Hypoxia can be induced by several other factors of mitochondrial glutathione and membrane integrity.Our study is limited to the role of nitric oxide production in alveolar epithelial cells and possibility of energy and oxygen depletion in hepatocytes, but presents a partial explanation of pre-hypoxia events in hepatocytes and cell viability loss in alveolar epithelial cells.

Our recent report on intracellular sodium MRI as indicator of apoptosis and decreased cell proliferation with differentiation supported the possibility of MRI as potential technique in alveolar imaging of inflammation [1, 7].

In conclusion, our results show that hypoxia is induced by nitric oxide production mediated by cytokines in alveolar cells and hypoxia is also induced by oxygen and energy depletion in hepatocytes. These results indicate the active role of effectors on hypoxic alveolar cells and hepatocytes during cell injury.

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Chapter VI

METHODS, STRATEGIES AND FACTS FOR IMPROVING THE ANALYSIS OF GENETIC VARIATIONS USING DNA ARRAYS

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ABSTRACT

Cancer is a disease resulting from the breakdown of several checkpoints and tumor-suppressing mechanisms. In cancer research, the development of new technologies, which have produced genomic tools indispensable for understanding how gene products are regulated in normal and diseased conditions on a global genome scale; one of these technologies is the DNA arrays. Although the most common use of DNA arrays is gene expression profiling and mutation detection, scientists have successfully used them for multiple applications, including genotyping, re-sequencing, DNA copy number analysis and DNA-protein interactions mainly.

This section then will be dedicated to the use of a public sequence database that can be accessed, and the design of DNA oligonucleotide probes for oligoarrays derived from

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sequences of special interest. Single probe and stacking hybridization are explored as possible microarray designs. Then the use of thermodynamic models and *in silico* hybridization are explored in order to access the sensitivity and specificity of the oligoarray and how its design can be improved. However, many commercial and public applications do not consider that the hybridization between target DNA's and microarray probes is a chemical reaction which is influenced by several thermodynamic parameters. One of the most important of such parameters is the thermal stability of the nucleic acid duplexes, which are formed as a result of the hybridization. According to the reaction conditions, these duplexes can be perfectly or imperfectly paired, which is of critical importance when a diagnostic kit is developed in order to assess its sensitivity and specificity.

In silico hybridization can be used to avoid undesired hybridization events (such as multiple target-probe interactions and stable mismatched hybrids). Also, the strategy can facilitate (through use of different length probes) selection of a probe set that has a narrow duplex stability allowing maximal specificity under a single hybridization condition.

INTRODUCTION

Cancer is a genetic disorder arising from the breakdown of several checkpoints and tumor-suppressing mechanisms or by accumulation of somatic mutations in genes involved in critical cellular pathways. These mutations typically result in proteins which exhibit their oncogenic effect by altering signaling through vital transduction networks, or in haploinsufficiency of critical tumor suppressor proteins. The detection of these variations in DNA is very important for the early prediction of cancer, diagnostics of disease and clinical prognosis.

In cancer, the development of new technologies has changed the way we do research in biology and medicine, one of these technologies is the DNA arrays. Array-based technologies are revolutionizing genomics, especially the analysis of DNA variation. Array technologies are not without limitations, however, and one major drawback is the poor flexibility of typical array formats. It is cumbersome to create one's own tailored arrays by spotting DNA. Commercially available microarrays, on the other hand, either contain a fixed and usually broadly applicable content or are expensive to purchase with customized features.

This review gives a brief perspective of some of the landmark discoveries in array-based technology related to analysis of DNA variation. The fundamental concepts behind the technology and some characteristics of DNA oligoarrays are then covered, followed by an overview of how those can be studied in the laboratory. Finally, the use of public sequence database that can be accessed, and the design of DNA oligonucleotide probes for oligoarrays arising from sequences of special interest as possible microarray designs are discussed.

MICROARRAY-DEFINITION

The term microarray is both descriptive and somewhat ambiguous as it is commonly used to describe a collection of microscopic spots arranged in an array or grid-like format and attached to a solid support, typically a glass, filter, or silicon wafer, hence the term. These

spots are typically referred as “probes” and are designed such that each probe binds a specific nucleic acid sequence (“target”) corresponding to a particular gene or other sequence through a process termed hybridization. The sequence bound to a probe, often referred to as the target, is labeled with some kind of detectable molecule or dye such as a fluorophore. The level of binding between a probe and its target is quantified by measuring the fluorescence or signal emitted by the labeling dye when scanned. This signal, in turn, provides a measure of the expression of the specific gene containing the target sequence [1]. This term is commonly used to describe also a variety of platforms including protein microarrays and tissue microarrays [2].

MICROARRAY- NOMENCLATURE

Oligonucleotide arrays are also known as “DNA chips”, “gene chips”, or “genesensor chips” [3]. Currently there are in the literature at least two nomenclature systems for referring to hybridization partners. Both use common terms: “probes” and “targets”. With symmetry akin to the hybridization reaction itself, each system mirrors the other. What one describes as “probes”, the other describes as “targets”. With respect to the nucleic acids whose entwining represents the hybridization reactions, the identity of one is defined—it tends to be tethered to the solid phase, making up the microarray itself. The identity of the other is revealed by hybridization. The strategy of the “standard” microarray therefore parallels that of a reverse dot-blot, in which the probe is immobilized. For this reason, here we described the tethered nucleic acid as “probe”, and the free nucleic acid as “target” [3].

MICROARRAY- TECHNOLOGY

The latest microarray technology is a result of rapid development in the 1990s and early 2000s. Although the foundation for microarray technology was established much earlier in 1975, when the first hybridization between nucleic acids was performed [4], it took researchers another 20 years to develop microarrays, due to underdevelopment computer, robotic and DNA technologies. DNA microarray technologies initially were designed to measure the transcriptional levels of RNA transcripts derived from thousands of genes within a genome in a single experiment. Today's arrays are vastly superior to their predecessors in terms of quality, probe density, and structural layout [1-3].

This technology has made it possible to relate physiological cell states to gene expression patterns for studying tumors, disease progression, cellular response to stimuli, and drug target identification. At present, DNA microarrays have become a vital component in genomic research for high-throughput gene expression analysis [5, 6], mutation detection[7, 8], gene discovery and genetic mapping studies [9] and protein–DNA interaction analysis [10-13].

More recent uses of DNA microarrays in biomedical research are not limited to gene expression. DNA microarrays are being used to detect single nucleotide polymorphisms (SNPs) of our genome (Hap Map project) [14], aberrations in methylation patterns [15], alterations in gene copy number [16], alternative RNA splicing[17], and pathogen detection[18, 19].

Several platforms for fabricating DNA arrays have been described [20]. All platforms use hybridization between probe nucleic acids bound to solid support (microarray) and labeled target nucleic acids. It is well known that complementary single-stranded sequences of nucleic acids form double-stranded hybrids. This property is the basis of the very powerful molecular biology tools such as Southern and Northern blots, in situ hybridization, and Polymerase Chain Reaction (PCR). In these, specific single-stranded DNA sequences are used to probe for its complementary sequence (DNA or RNA) forming hybrids. This same idea also is used in DNA microarray technologies.

The aim, however, is not only to detect but also to measure the expression levels of not a few but rather thousands of genes in the same experiment. For this purpose, thousands of single-stranded sequences that are complementary to target sequences are bound, synthesized, or spotted to a glass support whose size is similar to a typical microscope slide.

Platforms can be grouped according to the type of fabrication and type of probe. There are mainly two types of DNA arrays, depending on the type of spotted probes. One uses small single-stranded oligonucleotides (~22 nt) synthesized in situ whose leading provider is Affymetrix (Santa Clara, CA, USA) [21]. The other type of array uses complementary DNA (cDNA) obtained by reverse transcription of the genes' messenger RNAs (mRNA), completion of the second strand, cloning of the double-stranded DNAs, and typically PCR amplification of their open reading frames (ORF), which become the bound probes. One of the limitations of using large ORF or cDNA sequences is an uneven optimal melting temperature caused by differences in their sizes and the content of GC-paired nucleotides. A second problem is cross-hybridization of closely related sequences, overlapped genes, and splicing variants. In oligo-based DNA arrays, the targeted nucleic acid specie is redundantly detected by designing several complementary oligonucleotides spanning each entire target sequence by segments. The oligonucleotides are designed in such a way to avoid the cDNA probe drawbacks and to maximize the specificity for the target gene [22, 23].

See some links for more detailed information about the microarray technology[24-26].

SOME LIMITATIONS OF OLIGONUCLEOTIDE ARRAY TECHNOLOGY

Several technical challenges remain to be solved, however, before oligonucleotide arrays can reach their full potential and be implemented in a robust fashion. Hairpin structures are a common feature of single-stranded DNA and RNA molecules. In relation to arrays oligonucleotides, one limitation of the method is that stable secondary structures in the target nucleic acid can make the target sequence inaccessible to intermolecular Watson-Crick base pairing [27-29]. Several techniques have been explored to alleviate this problem, but none appears to be totally successful. Fragmenting the nucleic acid sequence, preferably to a size close to that of the oligonucleotides on the array, by heating the DNA or RNA in the presence of Mg^{2+}[30] or by the use of uracil-*N-glycosylase*[31] can reduce the secondary structure effect. However, the extent of fragmentation is difficult to control. Oligonucleotide analogues such as peptide nucleic acid (PNA) exhibit PNA-DNA heteroduplexes with a high thermal stability at low salt concentration. The authors suggest that under these conditions the DNA strands are less likely to fold to form a stable secondary structure and therefore would be

accessible to the oligonucleotide probes [32]. However, there is an increasing tendency for non specific DNA binding [33]. It's important to point out that these stable structures probably explain why some regions of targets are not accessible for heteroduplex formation with complementary oligonucleotides. Heteroduplex formation between a hairpin structure and a complementary oligonucleotide probe implies two main steps: opening of the structure to allow formation of one or a few base pairs in a transient nucleation complex followed by further base pairing between the two complementary strands to form a heteroduplex which is more stable than the starting structures. The secondary structure of nucleic acid targets can be minimized by using a hybridization temperature above the melting temperature of intramolecular structure or low salt concentration solutions. However, these conditions are not appropriated for hybridization of nucleic acid molecules with short oligodeoxynucleotides.

The Mirzabekov laboratory previously described a contiguous stacking hybridization strategy for potential use in *de novo* sequencing by hybridization [34, 35], and more lately reported the successful use of contiguous stacking hybridization to investigate point mutations [36]. In the latter study, the hybridization was carried out in two steps: The target DNA was first hybridized to decanucleotide probes (10 mer) covalently attached within a thin polyacrylamide gel matrix, to place the mutant site adjacent to the 10-mer duplex, then fluorescently labeled contiguous stacking pentanucleotide probes (5 mers) were applied to gel matrix to detect point mutations within the target sequence immediately adjacent to 10-mer duplex.

TANDEM HYBRIDIZATION ON OLIGONUCLEOTIDE MICROARRAYS

Tandem hybridization (TH) or coaxial hybridization as is known also is a novel technology with high sensitivity and specificity which is especially useful for the detection of point mutation.

This strategy is designed to minimize or eliminate some difficulties in simultaneous hybridization of DNA sample to numerous oligonucleotide probes attached to a solid support material. Some of these challenges are as follows:

1. The need to generate single-stranded target nucleic acids in order to achieve optimal hybridization signals.
2. Spontaneous formation of secondary structure in the single-stranded target nucleic acid, causing certain stretches of target sequences to be poorly accessible to hybridization.
3. Imperfect specificity of hybridization, making it difficult or impossible to distinguish between certain sequences variations.
4. The strong influence of base composition on the stability of short duplex structures, making it difficult to use an extensive array of oligonucleotides (differing in base composition) to analyze a nucleic acid sample under a single hybridization condition.

5. Multiple occurrences of sequences complementary to other oligonucleotide probes with the nucleic acid sample, limiting the genetic complexity of a nucleic acid sample than can be analyzed by arrays of short oligonucleotide probes.
6. The need to label each nucleic acid analyte prior to hybridization to DNA probe array, a significant factor in the overall time and cost of analysis.

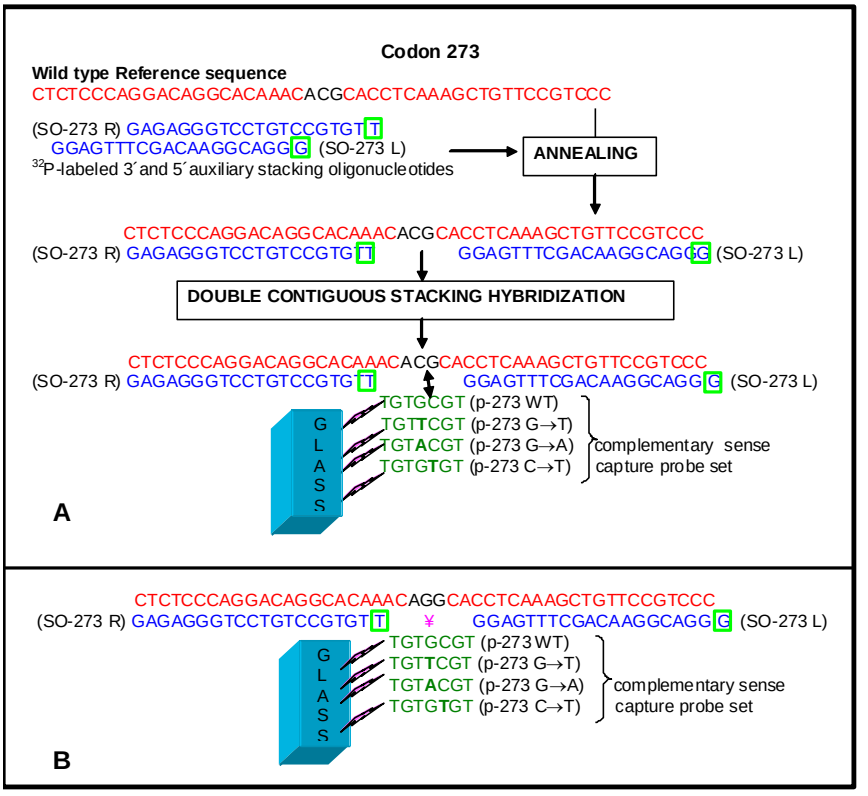

Figure 1 – Tandem hybridization strategy on design of *TP53* Low density DNA microarray. (A) An example of the alignment of stacking oligonucleotides and probes to their respective synthetic wild type target sequence in codon 273 of the *TP53* gene. As shown, a nucleic acid target is first annealed with a molar excess of labeled auxiliary oligonucleotide (the green square marks the 32P-labeled end of the oligonucleotide). The labeled auxiliary oligonucleotide serves as a reagent to introduce the detection tag into a single specific site within the nucleic acid target. The partially duplex labeled target is then applied to the array of 7-mer capture probes, end-tethered to a glass slide or chip. Each capture probe is designed to hybridize in the gap formed on the target strand, in tandem with both labeled stacking probes preannealed to the target. Base stacking interactions between the short capture probe and the long stacking probe stabilize the binding of partially duplex labeled target to the glass-tethered capture probe. Hybridization is carried out at an (elevated) temperature at which the short capture probe by itself does not form a stable duplex structure with the target; capture of label to the glass occurs only when the capture probe hybridizes in tandem with the labeled stacking probes. As depicted in (B), a single base mismatch between the short capture probe and the target sequence will disrupt the short duplex, preventing binding of label to the glass.

The principle of TH is: unlabeled, single-stranded target DNA is preannealed to one or more labeled "auxiliary" oligonucleotides which can be designed to disrupt interfering secondary or higher-order structures, or to cover up alternative hybridization sites that any of surface tethered probes may have within the target. TH method archives sequence specificity

by using short, glass-tethered capture probe along with one or two longer, contiguously stacking oligonucleotides that are preannealed to the target. Good discrimination of point mutations is achieved within the short capture probe, while the longer stacking probe(s) contribute the label and stabilize the binding of the target via base-stacking interactions. The stacking probe enables hybridization to be conducted at an elevated temperature at which the capture probe alone cannot bind to the target, and directs the analysis to a single site within the target, even within a complex nucleic acid analyte (see **Figure 1**). This preannealing strategy may facilitate more reliable applications of genesensor technology, and avoids the inconvenient and costly labeling of numerous DNA samples [37].

The TH approach and double tandem hybridization strategy (DTH), wherein 7-mer capture probes are hybridized with duplex polymerase chain reaction products that have been heat-denatured and preannealed with two longer stacking probes to form a 7-nucleotide gap at the site of capture-probe binding, were successfully tested in several experimental models. Between the experimental models tested are: the ΔF508 mutation and other sequence changes responsible for cystic fibrosis (a genetical disease) [38], the identification of mutations in the *RET* gene associated to medullary thyroid carcinoma[39], the identification of *TP53* mutations hotspots in lung cancer samples [40] and, the identification of the Asiatico-American (AA) and European (E) HPV 16 variants [41].

SOME RELEVANT GUIDELINES FOR THE DESIGN OF OLIGONUCLEOTIDE PROBES

As it has been previously mentioned the detection of genetic variation with microarray approaches can in theory be capable of diagnoses thousands of genetic variations due to the parallel nature of this technology. However this technology still suffers of important limitations in detecting known single polymorphism. In particular there is a high rate of false positives when specificity in determined by the hybridization, is difficult to distinguish all the polymorphisms, is difficult to detect low level of polymorphism [42].

An important and key issue for the multiple possible applications of microarrays for the study of genetic variations is the design of probes. Typically the design of these probes is a process involving three stages:

1. Selecting the appropriated (desired) targets.
2. Calculating the sequence of the probe.
3. Validating the specificity of the probe

Therefore the process of selecting probes looks similar to that commonly used for genetic expression. However some important precautions are necessary for this type the design. We will explain some guidelines which can be applied for selection of probes for the analysis of genetic variations.

SELECTING THE APPROPRIATED (DESIRED) TARGETS

There are two different models for exploring for genetic variations on the genes of interest:

1. Exploring the whole sequence of the gene of interest or
2. Designing a set of probes for detection of the most common or important genetic variations on a gene of interest.

In the first case the microarray must be integrated by a high number of probes in order that the whole gene can be scanned for searching potential mutations. This approach is then similar to the concept of Sequencing by Hybridization [42, 43]. However this approach has some important limitations:

1. If the array includes only the probes that allows to screen the "reference" sequence of the target, then this microarray would be able to detect only the presence of the mutation but not its identity.
2. If additional probes are included for detection of the all possible variations (in the case of single base variations), then the number of require probes could be excessive.
3. Alternatively the microarray can include the whole set of combinations of probes of a defined length as the microarrays for sequencing by hybridization or resequencing. However, this design can be problematic as the variation of stability is very broad and then it will be difficult for distinguishing ambiguous hybridizations.
4. Most part of the potential sequence variations will no be observed in any sequence, then most part of the data provided by the microarray will not be informative.

Table 1: Some databases on genetic variations related with cancer

Database name	URL	Brief description
IARC TP53 Database	http://www-p53.iarc.fr/index.html	Most frequent somatic mutations in the tumor suppressor gene TP53 associated with most human cancers and germline TP53 mutations.
Tumor Associated Gene database	http://www.binfo.ncku.edu.tw/TAG/GeneDoc.php	Provides information as gene content and mutation, from well-characterized oncogenes and tumor suppressor genes to facilitate cancer research.
SNP500Cancer	http://snp500cancer.nci.nih.gov/	Re-sequenced SNPs from 102 reference samples
Cosmic	http://www.sanger.ac.uk/genetics/CGP/cosmic/	Catalogue of somatic mutations in cancer
HGMD	http://www.hgmd.cf.ac.uk/ac/index.php	Human Gene Mutation Database at the Institute of Medical Genetics
Human Genome Variation Society HGVS	http://www.hgvs.org/dblist/dblist.html	Formerly the HUGO Mutation Database Initiative. Includes Variation Databases and Related Sites
OMIM	http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim	A catalog of human genes and genetic disorders for the World Wide Web by NCBI
dbSNP	http://www.ncbi.nlm.nih.gov/SNP/	Database of genome variation of the NCBI which provides a comprehensive catalogs of common genomic variations in humans and other organisms
OncoDB.HCC	http://www.oxfordjournals.org/nar/database/summary/1026	Oncogenomic database of hepatocellular carcinoma (HCC).
Tumor Gene Family Databases (TGDBs)	http://www.tumor-gene.org/tgdf.html	Contain a broad range of data about the genes involved in cancer (e.g. proto-oncogenes and tumor supressor genes).

A more efficient microarray can be designed for the second case, as the number of required probes will be significantly reduced. In such case the sets of probes can be created for detection of all the potential variations and approaches as the tandem hybridization can be used in order to allow a more sensitive detection. However the main limitation of this technique is that some genetic variations will be skipped. Fortunately most part of the genetic variations observed in diseases show important "hot spots" or variations which are especially frequent on human populations. For example the *TP53* database of the International Agency for Research on Cancer (see **Table 1**) shows six especially abundant somatic mutations at codons 175, 245, 248, 249, 273 and 282. These mutations represent almost the 30% of the genetic variations observed for this gene [44]. Therefore, although some information is missed this type of microarray still can be important for the diagnosis of variations commonly observed or associated with some diseases.

For a convenient selection of the targets for designing of the probes probe there are several databases available up to date [45]. **Table 1** resumes some important databases that can be used for such purpose. It is important to mention that some of this databases show genetic variations observed in some important genes, but sometimes there is a lack of statistical evidence that can definitively associate the variation with the disease as in the case of the SNPs database. For such cases the information should be carefully interpreted. Sometimes, additional annotation of the genes can provides information about the relatedness of the observed variations with the disease of interest.

DERIVING THE SEQUENCES OF THE PROBES

When probes are directed against a sequence variation, there are few choices about the sequence zone on the targets that can be used to derive the sequence of the probe. Most important is to define the length of the probe and the possible placement of the recognized area when the duplex is formed. However as the probes are usually required for detection of small sequence variations, the thermal stability of the duplex formed during the hybridization reaction becomes of critical importance. In this context it is important to mention that the stability of duplex formation between probes and targets, in the presence of small sequence variations, is strongly dependent on the sequential context [46].

Typically thermodynamic nearest-neighbor models, based on experimental data, have been developed for predicting the thermal stability of short DNA duplexes [46-51]. According with these models, the main contribution of duplex stability are the stacking interactions between neighboring bases. For perfect matches there are 10 different types of such interactions (see **Table 2**). Moreover, the nearest-neighbor model is appropriated for calculating the stability of single mismatched duplexes and it can be expanded to include the contribution of other components of the secondary structure [46, 52, 53]. Some stability values are affected by nucleic acid concentrations and by the ionic strength. But the relative values between them are conserved on a wide range of ionic conditions [54]. Most part of the thermodynamic parameters used for this model have been derived from experiments in solution. It can be possible to find significant differences when the model is applied to solid-liquid system as that used in the microarrays, where one of the strands is fixed to a solid

support. However, it has been observed that the relative stabilities are conserved also in these systems [55].

Table 2. Unified set of DNA parameters for the nearest-neighbor model for predicting stability of DNA/DNA duplexes. [1)]

NN	ΔH° (kcal/mol) [2)]	ΔS° (cal/mol K) [2)]	ΔG° (kcal/mol) [2)]
AA/TT	-7.9	-22.2	-1.00
AT/TA	-7.2	-20.4	-0.58
TA/AT	-7.2	-21.3	-0.58
CA/GT	-8.5	-22.7	-1.45
GT/CA	-8.4	-22.4	-1.44
CT/GA	-7.8	-21.0	-1.28
GA/CT	-8.2	-22.2	-1.30
CG/GC	-10.6	-27.2	-2.17
GC/CG	-9.8	-24.4	-2.24
GG/CC	-8.0	-19.9	-1.84
Initiation with GC	0.1	-2.8	0.98
Initiation with AT	2.3	4.1	1.03
Symmetry correction	0.0	-1.4	0.40

1) Source data from SantaLucia, 1998.
2) ΔH^O enthalpy, ΔS^o entropy, ΔG^o free energy

Probe length can be adjusted in order to have a similar stability for the probes in the microarray. Additionally, the probe length is an important factor which determines the ability of the probes for the detection of small sequence variations. The use of the nearest-neihgbor model allows the prediction of the effect of the probe length on the discrimination power of single sequence variations. Figure 2 illustrates that the presence of a CT mismatch produces a higher variation of Tm in the hybridization with a short probe (about 3oC for a 19mer probe) than a longer probe (about 1oC for a 50mer probe). However the identity of the mismatch is very important as is showed in Table 3, where it can be seen than AC and CT mismatches are highly destabilizing, whereas GA, GT and GG mismatches are highly stable in some sequential contexts. Therefore short probes are better suitable for the detection of single variations as SNPs. However the special stability of some single mismatches represents an important limitation for the application of the "classic" microarray technology for detecting single variation where single probes are used for detection of individual mutations. In such cases even the use of short probes will produce intense signals (ambiguous hybridization) in the presence of highly stable mismatches. Moreover, if the hybridization conditions are not rigorously controlled for detection of these mutations, ambiguous hybridization is produced. This issue becomes critical when a high number of probes are hybridized simultaneously where is not possible to control rigorously the hybridization conditions for all them. A possible solution for such problem could the use of probes of different length which have similar stability.

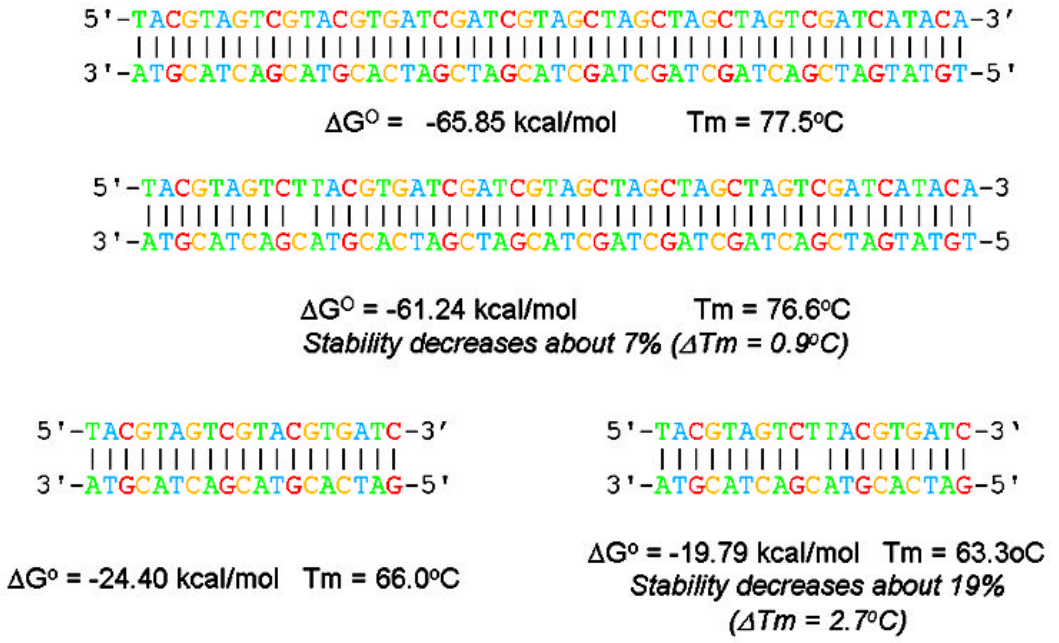

Figure 2. Effect of the probe length on the discrimination power for single mismatches with microarray DNA probes. Thermal stability of the probes was calculated with the Nearest-Neighbor (NN) model which considers that the main source of duplex stability is the stacking between neighboring bases. It can be seen that the discrimination power (measures as the difference of thermal stabilities between the perfect and the mismatched target-probe duplex) increases as the probe length reduces. Thermodynamic values calculated with NN data from SantaLucia and Allawi et al. setting [Na+] = 50 mM and [oligonucleotide] = 100 µM. The Tm estimates are intended only to describe the relative stability of different oligonucleotides in a given hybridization reaction, since the absolute values will depend on variables of DNA and ionic concentrations.

Typically the presence of mutations with classic microarrays is carried up by comparing the signal intensity of the mismatched probe against the perfectly complementary duplex. Moreover several probes are designed to screen the entire sequential context around the mismatch. If the loss of the signal intensity is the observed within several probes this will constitute a confirmation of the mutation presence. However, even with this approach, the detection of mutations that produce especially stable mismatches would be difficult when the Tm variation is less of 0.5°C. Even with the use of several probes for screening the same mutation will produce similar sequential contexts, and then the detection of such special mutations remains difficult. Some developing technologies are promising for solving several of these problems. The method termed DASH (dynamic allele-specific hybridization), which entails dynamic tracking of probe (oligonucleotide) to target (PCR product) hybridization as reaction temperature is steadily increased allows for an more accurate genotype assignment [56]. Current failures on this methodology are assumed to be caused by secondary structures in the target molecule, which could be reliably predicted from thermodynamic theory. Electric chips use probes coupled to electric contacts sending a current to denature target-probe hybrids, allow a more strict control of the hybridization-denaturing conditions [57].

Table 3: Tm Differences for single internal mismatches

Mismatch = G-T				
Mismatch	3' match	^{0}C	5' match	^{0}C
AGA/TTT	AGA/TCT	9.4	AAA/TTT	7.3
AGC/GTT	AGC/GCT	9.2	AAC/GTT	5.7
AGG/CTT	AGG/CCT	8.7	AAG/CTT	5.6
AGT/ATT	AGT/ACT	9.1	AAT/ATT	6.3
CGA/TTG	CGA/TCG	7.9	CAA/TTG	5.1
CGC/GTG	CGC/GCG	7.9	CAC/GTG	3.8
CGG/CTG	CGG/CCG	7.5	CAG/CTG	3.8
CGT/ATG	CGT/ACG	7.7	CAT/ATG	4.2
GGA/TTC	GGA/TCC	7.5	GAA/TTC	4.4
GGC/GTC	GGC/GCC	7.2	GAC/GTC	2.8
GGG/CTC	GGG/CCC	6.7	GAG/CTC	2.6
GGT/ATC	GGT/ACC	7.3	GAT/ATC	3.5
TGA/TTA	TGA/TCA	8.8	TAA/TTA	5.0
TGC/GTA	TGC/GCA	8.7	TAC/GTA	3.5
TGG/CTA	TGG/CCA	8.2	TAG/CTA	3.4
TGT/ATA	TGT/ACA	8.6	TAT/ATA	4.0
G:T Median	discriminatory values	8.15		4.40
Mismatch = C-T				
Mismatch	3' match	^{0}C	5' match	^{0}C
ACA/TTT	ACA/TGT	11.1	AAA/TTT	8.2
ACC/GTT	ACC/GGT	12.2	AAC/GTT	9.2
ACG/CTT	ACG/CGT	11.6	AAG/CTT	7.9
ACT/ATT	ACT/AGT	11.2	AAT/ATT	8.4
CCA/TTG	CCA/TGG	12.4	CAA/TTG	9.4
CCC/GTG	CCC/GGG	13.4	CAC/GTG	10.2
CCG/CTG	CCG/CGG	13.0	CAG/CTG	9.3
CCT/ATG	CCT/AGG	12.6	CAT/ATG	9.6
GCA/TTC	GCA/TGC	12.7	GAA/TTC	8.4
GCC/GTC	GCC/GGC	13.6	GAC/GTC	9.1
GCG/CTC	GCG/CGC	13.0	GAG/CTC	8.1
GCT/ATC	GCT/AGC	12.9	GAT/ATC	8.6
TCA/TTA	TCA/TGA	11.9	TAA/TTA	8.1
TCC/GTA	TCC/GGA	13.1	TAC/GTA	9.1
TCG/CTA	TCG/CGA	12.3	TAG/CTA	7.8
TCT/ATA	TCT/AGA	12.0	TAT/ATA	8.3
C:T Median	discriminatory values	12.43		8.73

Tandem mismatches are other kind of sequence variations that can be present in some special cases. From the thermodynamic point of view, internal tandem mismatches can not be considered as the combination of two consecutive single mismatches [58]. Tandem mismatches follow a special behavior, some tandem mismatches are exceptionally stable with a stability even similar to the AT base pairs [59]. Therefore tandem mismatches are especially problematic for their detection with this technology. The deletion of one or few consecutive bases will produce bulges in the bimolecular secondary structure of the target-probe duplexes. Some one base bulges are also relatively stable [60]. Moreover, when short probes are used, one-base bulges can be found easier by chance, allowing the formation of non-desired cross reactions, but it has been reported an efficient detection of several one-deletions with microarrays [61].

The placement of the mismatch in the duplex is also important. In general the higher discrimination power for mismatches is reached when they are located at the middle of the probe. However mismatches located at the ends or close to them (especially at the penultimate positions) are highly stable in general [33, 62].

All these facts indicate that the classical microarray technology is not well suitable for the detection of all single base variations with this technology. In particular there is a high rate of false positives for distinguishing all the polymorphisms and it is difficult to detect low level of polymorphism[42]. However an interesting alternative is the use of tandem or coaxial hybridization (TH) as it has been previously described. In general the use of coaxial stacking highly increases the ability for discriminating single mismatches .

The formation of a tandem probe-auxiliary oligonucleotide duplex with the target is accompanied by the appearance of the stacking interaction between the terminal base pairs of the contiguous duplexes and then it contributes to the stabilization of the complex. Some studies have showed that such stabilization reach the maximum when there are not gaps between the duplexes and indicate that such stabilization can produce an increase of 1 to 4^{o}C of the Tm of the probe [63-66]. This discrimination power reaches a maximum when both flanking sequences to binding site of the probe are hybridized with auxiliary (stacking) oligonucleotides [40]. Moreover the presence of additional auxiliary oligonucleotides, which must be pre-aligned with the samples, helps to eliminate the any interfering secondary structure of the targets. They also allow the correct location of the site where the probe must be hybridized.

When the probe produces mismatches with the target, the duplex stability is highly disturbed. Although this stabilization is dependent on the sequence context as in the case of the simple hybridization, the effect is higher with the tandem hybridization approach even for highly stable mismatches. Tandem hybridization has been successfully tested in the laboratory and it has been demonstrated that it can reach highly sensitivity levels for single mutation detection. However the hybridization with this approach requires the presence of additional auxiliary oligonucleotides, which must be pre-aligned with the targets. This pre-annealing must be carried up in a multiplex way and then special precautions must be taken for the design of the auxiliary oligonucleotides to avoid the formation of stable dimers between them.

VALIDATING THE SPECIFICITY OF THE PROBE

There are several software applications both commercial and free that can be used for microarray probe design [67-71]. Many of them use thermodynamic models to predict the stability of the duplexes formed by the probes when they hybridize against their targets as well as tools for ensuring the specificity of the probes. However most part of these software applications are aimed to the design microarrays for differential expression analysis. In general these applications could not be appropriated for designing of probes for monitoring single genetic variations for the reasons exposed next.

Small sequence variations are not of special interest when monitoring the gene-expression. As long probes are used for such experiments the formation of ambiguous mismatched duplexes is quite possible within highly similar sequences. No validation of such variations is considered for designing probes for gene expression. However this aspect is critical when designing probes for evaluation of genetic variations. The validation of the specificity of the probes is commonly carried up with software tools that evaluate the

similarity of the sequence recognized by the probe. If multiple sequences similar to the targeted site are detected then they can produce cross reactions.

However software tools like BLAST or FASTA are not optimized for searching of similarities with short nucleotide sequences. Moreover even when similar sequences the identity of the mismatches is important the correct identification of the binding sites.

Our team has proposed than alternative way for testing microarrays aimed for testing genetic variation is to check their performance*in silico*. The hybridization between the probes in the microarrays and the potential targets can be virtually simulated and this simulation can calculates the hybridization patterns where the differences between spot intensities in the presence of single mismatches can be visualized and therefore it can be possible to anticipate if the microarray could produce the desired results. This approach is called Virtual Hybridization and it has been successfully tested to analyze hybridization results and for improving the microarray design [72].

Search of specificity of the probes in the microarray yields more satisfactory results as in this case all potential sites where ambiguous hybridization can occur are searched, and then thermodynamic models are used to calculate the stability of the potential duplex. Therefore this represents an improvement compared with the tools commonly used for assessing specificity based in the sequence similarity/complementary only.

Moreover the concept of *in silico* hybridization can be used to avoid undesired hybridization events (such as multiple target-probe interactions and stable mismatched hybrids). The strategy can facilitate (through the use of different length probes) selection of a probe set having a narrow duplex stability allowing maximal specificity under a single hybridization condition.

Applications Developed for the Study of Organisms on DNA Arrays

Microarrays have been successfully used for several important purposes—gene expression, *SNP* analysis, detection of genetic variations, organism identification, sequencing, resequencing and barcode identification. The size of the probes can be critical for some of these applications. In the case of gene expression, organism identification and for detection of some barcode sequences long sizes probes are preferred, since this capture probes are able to detect the gene or the expected sequence even when small sequence variations are present in the sample. However, when single base changes are searched, as happens in the *SNP* analysis or resequencing, the use of relatively smaller probes is obligated to get better discrimination of single base variations. Numerous improvements in the performance of microarrays have been developed through studies for gene expression. The array quality has been estimated by measuring the amount of fluorescence (Cy5-UTP) incorporated by terminal deoxynucleotidyl transferase (TdT) at the probes 3´-end. In another procedure the 3´-end ligation is used. The TdT approach detects the probes 3´-end while the ligation is also detecting the performance of the hybridization. Therefore, both array characteristics can be assessed by the combination of the two assays [73].

The preparation of the target is another important issue. Zhang *et al.*[74] have successfully tested genomic DNA isolation, for use in microarray hybridization, by a

combination of sonication with a heat treatment. This procedure is fast, cheap and easily adaptable for high throughput comparative genomics. In another study, Stangegaard *et al.*[75] demonstrated that the yield of cDNA is doubled by using 15-mer random primers instead of 6-mer random hexamers. 11-fold more genes were detected in the whole transcriptome DNA microarray tested with this preparative protocol. The homogeneity of the hybridization signal can be critical for quantitative analysis. In some cases the formation of doughnut shaped spots is observed. Doughnut appears as a consequence of evaporation of droplets. However doughnuts can also be formed during the hybridization process by diffusion-limited conditions (the situation in which the actual hybridization rate is slower than that expected on the basis of the intrinsic reaction kinetics). This situation occurs with short hybridization targets which reach the spot from the sides and not from above the spot. It is expected that appropriated microfluidics, such as that obtained in the MAUI hybridization chamber, will solve this problem. Pappaert *et al.*[76]. Since a 3-fold augment in positive signals was obtained in the MAUI hybridization system while negative controls show lower hybridization. Schaupp *et al.*[77]. Feature characteristics, such as probe sequence, can cloud the relationship between observed intensity and actual expression. Although this "probe effect" is large, it is also very consistent across hybridizations, which implies that relative measures of expression are substantially more useful than absolute ones. By this reason generally differential expression it is used instead than expressed or unexpressed. However this work uses the consistency in the intensity of each probe target pair as a way to determine if a given gene is or not expressed [78].

One of the common ways to reveal differential gene expression is the use of scatterplot (two-dimensional, 2-D, plot), which is created with the intensity of the hybridization signals. This plots obscure useful information when a large gene count caused saturation across areas of the graph. Park *et al.*[79] found that topographical plots are useful for quick qualitative analysis that reveals significant information, especially with respect to regions of low differential gene expression not visible in standard analysis.

Two key studies aimed to identify the sources of error and data variability in gene expression analysis were performed by the Toxicogenomics Research Group. In one of them they Izarry *et al*[80] demonstrated that standardization of protocols (Sample preparation, microarray processing, hybridization conditions, data acquisition and treatment) good reproducibility was obtained. This work suggests that microarray results, for gene expression, can be comparable across multiple laboratories, especially when a common platform and a set of procedures are used. In this study three microarray platforms (Affymetrix GeneChip, Spotted cDNA arrays and Long oligonucleotide arrays) were compared in ten laboratories using identical RNA samples. In general was found that the lab had a larger effect, on for example, precision than did the platform, and that the results from the best performing lab agreed rather well. In another, well controlled study [81], two platforms were compared (The Affymetrix Mouse Genome 430 2.0 GeneChip and a spotted cDNA array). The results, validated by qRT-PCR, suggest that biological treatment had a far greater impact on measured expression than did platform for more than 90% of genes. In the cases of inconsistency between platforms only one of eleven genes tested by qRT-PCR supported one platform over the other. One of the factors could be the difference in probe design due to splice variants. The qRT-PCR primer was based on the TIGR tentative consensus sequence, which was derived from the assembly of multiple expressed sequence tags (ESTs). Due to imperfections in genome annotation the Affymetrix probe set may target one or more

variants, the cDNA probes on the spotted array can test one or more different variants, and the qRT-PCR probe yet others. Another possibility is that some gene family member can cross-hybridize with cDNA or Affymetrix probe sets, testing different combinations of these family members while the qRT-PCR probably tests only a single member. By comparing the Affymetrix probe sequences against the corresponding TIGR EST sequences. For genes that had similar expression patterns across platforms full identity or almost identical sequences were found. However, some of the eleven tentative consensus sequences that disagreed across all three platforms had Affymetrix probes that mapped to the corresponding TIGR EST, supporting the hypothesis that the two platforms were interrogating different sequences for the genes that disagreed across platforms. The alignment of multiple EST sequences to the genome in the region containing the array probes generally suggested multiple splice variants in regions where there was generally a single annotated gene structure in the EnsEMBL database. Therefore the improvement in the annotation of the multiple splice variants will improve the design of oligonucleotide probes for gene expression.

Another important issue in gene expression is the normalization of data. One of the factors is the use of appropriated reference genes. These genes should show minimal expression variability among replicates, tissues and individuals. However, it is impossible to predict how different experimental conditions may affect the expression of putative normalization genes. Therefore it should be imperative to select the best reference genes and use the same normalization strategy in order to reduce the experimental variables [82]. A variation in expression of some housekeeping genes was observed during cancer. Therefore especial quantitation of the expression of these genes should be done previously to use them for normalization [83]. The quantitative expression of genes commonly is validated by real-time PCR and this relies on housekeeping genes. However, no single housekeeping gene can be used for all studies. Abruzzo *et al*[84] have developed a statistical approach that automatically identifies those genes whose expression remains unchanged in the samples. Another statistical method is able to show unusual expression patterns or the variability between slides [79]. Both procedures can be used for normalization.

Between the recent advances in differential gene expression arrays there is the hMitChip3, which contains probes to search nuclear and mitochondrial genes[85]. Recently a human exon array has been also developed (AffymetrixGeneChipR Human exon 1.0 ST array). This array has been made to test 1 million known exons. It contains 5.5 million probes. This chip should be able to perform differential exon expression in various tissues under normal or other conditions, revealing more precisely the targets for prevention or the treatment of genetic diseases [86].

BARCODE

Another increasingly relevant application of arrays is the detection of barcode sequences. A molecular barcode composed of 384 array features, Affymetrix GeneChip, was tested using defined template quantities of mixed PCR templates. The individual concentration response of each feature to each sample was highly reproducible [87]. Barcode has also been applied to predict tissue type [78]. Besides, plant species can be identified by using a two-locus (The rbcL gene and the non-coding region tmH-psbA spacer region) global DNA barcode [88]. In

yet another study a universal barcode array, made with unique 20-base-pair DNA tags, was used in parallel for the analysis of thousands of biological samples [89]. Recently nucleotide barcode was combined with pyrosequencing to reach successful large-scale sample multiplexing analysis [90].

ORGANISM IDENTIFICATION

A set of 60-mer probes to search approximately 100 CNS pathogens: The probes were designed on open reading frames from highly conserved and heterogenic regions within viral families. Additionally viral genes reflecting different stages of pathogen infection were also included to potentially define the stage of the viral infection. This array is aimed to found evidence of viral infection in postmortem tissue from psiquiatric patients [18]. In another study an array of approximately 39 000 probes was done to identify influenza virus subtypes. The array was designed based in the comparison of approximately 5000 viral sequences. Each probe was designed to be exclusive for a given subtype [91].

METAGENOMICS

Metagenomics can also be done with arrays of probes designed to detect specific sequences. Sequence reads can be then assembled to reach gene prediction, functional annotation and metagenomics [92].

Resequencing

Resequencing is an oligonucleotide array based technology aimed to search for sequence changes in a given gene or sequence of interest. The DNA region in study is tested by a set of 25-mer probes covering, base by base, the complete wild type sequence. The chip also contains additional probes looking for the presence of mutations in the sample. Commonly the mutations tested are single base substitutions and single base eliminations or insertions. Other types of mutations, such as double base insertions, can also be included in the probe array. The sequence changes are placed in the middle of the probes and the target is transcribed to RNA with the purpose to increase the sensibility in the detection of point mutations. One of the main advantages of resequencing is that genetic variations, such as point mutations, are more confidently identified by a combination of the loss of signal in the group of probes corresponding to the wild type sequence with a gain of signal in the complementary mutant capture probe. Besides, the analysis is performed in both target DNA strands which increases the discrimination of single base changes, especially in the cases where in one target strand the mutant/wild type, target/probe, forms a relatively stable single mismatched duplex when compared with the perfectly paired wild type target/probe duplex. In these cases the mismatch produced by the base present in the other target strand at the site of the mutation with the probe to capture the wild type sequence produces a big loss in

stability in the target/probe duplex formed compared with its respective, wild type target/probe duplex [93].

Successful resequencing of point mutations has been demonstrated since 1996 [94]. Only one, high quality, reference sequence is required to search for variations to that sequence. Resequencing was applied first to search for sequence changes in genes frequently associated to cancer such as *TP53* and *BRCA-1*. It also has been used to search variations in microorganisms between them to detectgroup A streptococci and their associated antibiotic resistancemarkers[95], severe acute respiratory syndrome(SARS) virus [96, 97] and smallpox virusgenomes [98].

Resequencing can be also be efficiently done by arrayed primer extension (APEX). In this case the 3´-end of each primer used as a capture probe is extended after hybridization to interrogate the template base in the target [99].

Recently, a novel resequencing application has been tested by Affymetrix. 14 125 688 probes were used for whole genome analysis (excluding repetitive sequences but including two plasmids) in *Francisella tularensis*. The main purpose was to perform genotyping and polymorphism discovery. Some of the current limitations in the interpretation of results are the presence of deletions in the sample relative to the reference sequence, since in this case a mixture of no-calls and false positive SNP calls is observed, besides the large populationof target DNA fragments in a whole-genome sample may containsequences capable of high-efficiency hybridization with morethan one of the probe pairs, resulting in a false-positive SNPcall (the alternate homology effect) and the local destabilizingeffect of genuine SNPs in the sample leads to false-positivecalls at adjacent genome locations, which is known as the footprint effect. It is expected that in the near future some improvements, such as the use of smaller-sized probes and hybridization under more astringent conditions will improve the resequencing technology [100].

High throughput resequencing can be done by universal PCR amplification of DNA fragments, with an average of 300 bp, with adaptors ligated to TdT created 3´-adenine overhangs and captured and eluted from a custom high-density microarray consisting of complementary sequences identified from a reference genome sequence SNPs [101].

However in some cases the results were not as expected. This happened when testing SNPs forgenotype-calling of individuals and in allele frequency determination of DNA pools from patients with lung cancer. Affymetrix GenChipR Mapping 100K Array-set, which includes 116,204 SNAps with a median and mean intermarker distance of 8.5 and 23.6 kb, respectively. In this study not reliable hybridization data was obtained in a quite large number of SNPs [102].

All these works shows the enormous and highly informative applications developed for the study of organisms on DNA arrays. The methodologies in some cases, such as differential expression, are able to detect either relatively long sequences even when some minor and unknown sequence variations can be present. In other cases, such as organism identification or barcoding, predefined sequences are tested with capture probes. In yet another case, such as SNPs or resequencing, point sequence variations are identified. However, there is not an array-based method able to precisely identify the sequence of unknown complex samples. There are many challenges to reach this goal. Between them, the quality of the array, the preparation of the sample for hybridization, the conditions of hybridization and the capture and interpretation of the results.

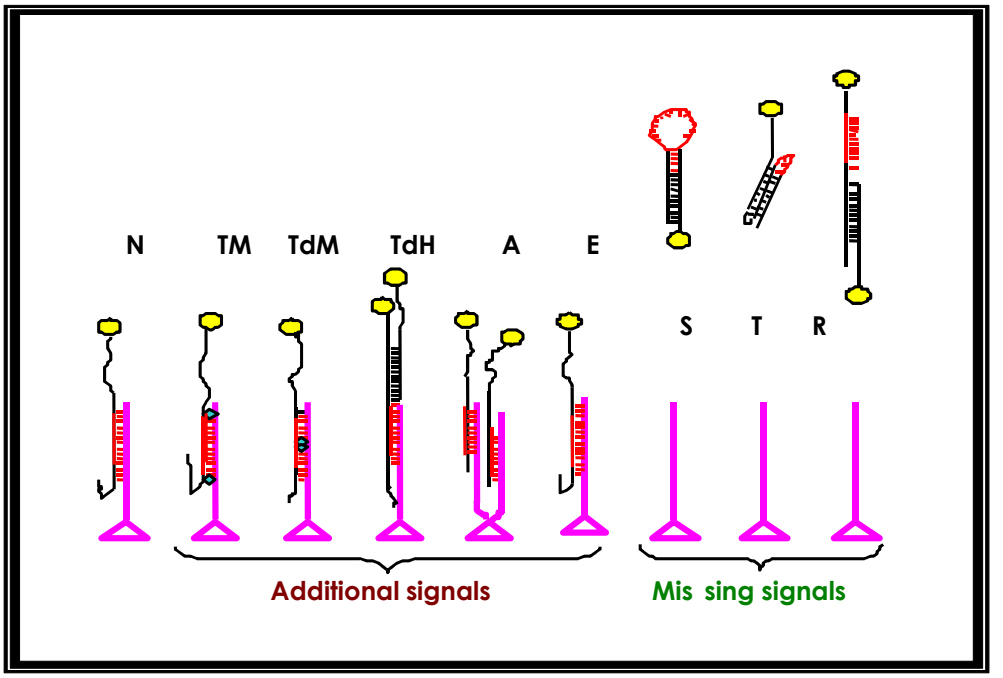

Figure 3. **Factors affecting the pattern of hybridization in complex target samples. N**, normal hybridization. *Additional hybridization signals* due to: **TM**, terminal mismatches; **TdM**, tandem mismatches; **TdH**, tandem hybridization; **A**, additive hybridization; **E**, extrachromosomal DNA. *Missing hybridization signals* due to: **S**, Secondary structure; **T**, Tertiary structure and **R**, repeats. Yellow circles, fluorescent label; blue circles, mismatches; red portions, target sequences; violet lines, probes.

The array of immobilized probes is covered with a solution containing hundreds of thousands of single stranded DNA fragments. Many of the fragments have repeated, inverse repeats or in tandem repetitive sequences, therefore many duplexes can be formed in the solution, besides stable secondary structures can be acquired in the hybridization conditions. Non repeated sequences located in the vicinity of repeated sequences can then become unavailable for hybridization. All these interactions have more chances to occur before interacting with the tethered probes. The remaining exposed target sequences can then produce normal and many unusual interactions with the probes. The following figure (**Figure 3**) illustrates some of the possible interactions. The effect on the final pattern of hybridization due to these interactions is discussed.

CONCLUSION

Microarrays are a powerful genomic tool that can be applied for biomedical and clinical research. The strength of the technology rests on several factors including: ease of use, availability of platforms and lower cost relative to other exploratory methods. Biological interpretation however requires the integration of several sources of information. In this context, about guidelines for designing, there some useful databases describing genetic

variations which can be accessed in order to obtain sequences for deriving the probes for the microarrays.The data should be carefully examinated because in several cases there is not enough statistical evidence on the degree of relatedness of some variations with the disease. As many microarrays will be directed for the detection of small genetic variations as single nucleotide polymorphisms special attention should be taken about the high stability of some mismatch sequence contexts.

Thermodynamic data based in the nearest-neighbor model are useful for predicting the stability of heteroduplexes as they include data for calculating the stability of duplexes in the presence of single mismatches and some other important components of the secondary structure which can arise when ambiguous hybridization is observed. The length of the probes is critical as short probes show a higher sensitivity for detection of especially stable mismatches. However, short probes are prone to problems of interfering secondary structure and specificity for recognizing the target site. An interesting alternative is the use of coaxial or tandem hybridization (TH), where the use of single or double auxiliary oligonucleotides increases the sensitivity for detecting stable mismatches, reduces interfering secondary structure of the targets and improves the specificity of the probes.

In silico validation of the performance of designed microarray through virtual hybridization is a useful technique that can be used to avoid undesired hybridization events (such as multiple target-probe interactions and stable mismatched hybrids). Also, this strategy can facilitate (through use of different length probes) selection of a probe set that has a narrow duplex stability allowing maximal specificity under a single hybridization condition.

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Chapter VII

PLASMA CELL DYSCRASIAS

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INTRODUCTION

Plasma cell dyscrasias (PCDs) include plasmacytomas and various forms of plasma cell myeloma (multiple myeloma-MM). Diagnosis of plasmacytoma requires demonstration of a monoclonal/aberrant plasma cell (PC) population in a tissue biopsy; whereas, diagnosis of a PCD involving the bone marrow (BM) (i.e., various forms of MM) requires quantitation of the PCs in the BM in addition to the demonstration of monoclonality/aberrancy. This chapter will describe the definitions of the various forms of PCD (including the International Staging System-ISS), the morphological variants and immunophenotypic features of neoplastic PCs, the diagnostic laboratory techniques useful in establishing a diagnosis (including immunophenotypic techniques), and the ancillary studies that may aid in diagnosis, prognostication, and therapeutic decision-making (i.e., immunophenotypic features, as well as cytogenetic and molecular findings).

DEFINITIONS OF VARIOUS FORMS OF PLASMA CELL DYSCRASIA (SEE TABLE 1)

Plasmacytoma

Solitary plasmacytoma of bone and extramedullary plasmacytoma and multiple solitary plasmacytomas are defined as distinct entities. If a solitary lytic lesion of PCs is found on skeletal survey in an otherwise asymptomatic patient, and a BM examination from an

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uninvolved site contains less than 5% to 10% PCs, the patient has an isolated plasmacytoma of bone. Likewise, patients with isolated PC tumors of soft tissues, most commonly occurring in the tonsils, nasopharynx, or paranasal sinuses, should have skeletal x-rays and a BM biopsy. [1-3] If these tests are negative, the patient has extramedullary plasmacytoma.

Table 1. Definitions in Multiple Myeloma

Solitary Plasmacytoma
All four criteria must be met:
Biopsy-proven solitary lesion of bone or soft tissue with evidence of clonal plasma cells
Normal bone marrow with no evidence of clonal plasma cells
Normal skeletal survey and MRI of spine and pelvis (except for the primary solitary lesion)
Absence of end-organ damage such as CRAB (see below)
Clinical features of end-organ damage: hypercalcemia, renal insufficiency, anemia, or bone lesions (CRAB)
Calcium levels increased: 0.5 mg/dL above the upper limit of normal or 10.5 mg/dL
Renal insufficiency: creatinine>2 mg/dL
Anemia: hemoglobin 2 g/dL below the lower limit of normal or hemoglobin <10 g/dL
Bone lesions: lytic lesions or osteoporosis with compression fractures (MRI or CT may clarify)
Other: symptomatic hyperviscosity, amyloidosis, recurrent bacterial infections (>2 episodes in 12 months)
Monoclonal gammopathy of undetermined significance (MGUS)
All three criteria must be met:
Serum monoclonal protein <3g/dl
Clonal BM plasma cells<10% and
Absence of end-organ damage such as hypercalcemia, *r*enal insufficiency, *a*nemia and bonelesions (CRAB) that can be attributed to the plasma cell proliferative disorder
No evidence of other B-cell lymphoproliferative disorder
AL amyloid and the IgM paraprotien-related neurological syndromes would be instance of "MG associated with..."
Asymptomatic myeloma (smoldering myeloma)
Both criteria must be met:
M protein in serum>3 g/dL and/or bone marrow clonal plasma cells>10%
No CRAB
Symptomatic multiple myeloma
All three criteria must be met (except as noted):
Clonal bone marrow plasma cells>/=10% or plasmacytoma
Presence of serum and/or urinary monoclonal protein (except in patients with true non-secretory multiple myeloma) and
Evidence of end-organ damage that can be attributed to the underlying plasma cell proliferative disorder, specifically:
-Hypercalcemia: serum calcium>/=11.5 mg/100 ml or
-Renal insufficiency: serum creatinine>1.73 mmol/l)
-Anemia: normochromic, normocytic with a hemoglobin value of >2 g/100 ml below the lower limit of normal or a hemoglobin value <10 g/100 ml
-Bone lesions: lytic lesions, severe osteopenia, or pathologic fractures
+CRAB (Some patients may have no symptoms, but have CRAB.)

Plasma Cell Leukemia
Circulating plasma cells exceed 2,000/ul or 20% of the peripheral white blood cells
MRI=magnetic resonance imaging

Monoclonal Gammopathy of Undetermined Significance (MGUS)

Monoclonal gammopathy of undetermined significance (MGUS) is characterized by a serum monoclonal protein <30g/l, <10% PCs in the BM, and the absence of end-organ damage (i.e., CRAB–hyper<u>c</u>alcemia, <u>r</u>enal insufficiency, <u>a</u>nemia, or <u>b</u>one lesions).

Smoldering Multiple Myeloma

Smoldering (asymptomatic) MM is characterized by having a serum IgG or IgA monoclonal protein of 30g/l or higher and/or 10% or more PCs in the BM but no evidence of end-organ damage.

Symptomatic Multiple Myeloma

Symptomatic MM requires evidence of related organ or tissue impairment (ROTI).The diagnostic criteria for symptomatic MM include the following:

- • Monoclonal PCs in the BM (≥ 10%)
- • Monoclonal protein in serum and/or urine by electrophoresis
- • One or more of the CRAB features of organ damage (elevatedcalcium (> 10.5 mg/dl); renal dysfunction (creatinine> 2 mg/dl); anemia (Hgb < 10 g/dl or 2 g below normal); and bonedisease (lytic lesions or osteoporosis).

Non-Secretory Multiple Myeloma

Non-secretory multiple myeloma is characterized by the absence of an M-protein in the serum and urine, but with BM plasmacytosis and ROTI.

Plasma Cell Leukemia

Plasma cell leukemia is defined as circulating PCs exceeding 2,000/ul or exceeding 20% of the peripheral white blood cells.

Multiple Myeloma International Staging System

The MM ISS consists of the following stages:stage I, serum ß$_2$ microglobulin (Sß$_2$M) less than 3.5 mg/L plus serum albumin≥3.5 g/dL (median survival, 62 months); stage II, neither stageI nor III (median survival, 44 months); and stage III, Sß$_2$M≥5.5 mg/L (median survival, 29 months).[5]

MORPHOLOGIC VARIANTS OF NEOPLASTIC PLASMA CELLS (SEE TABLE 2)

Neoplastic PCs may be so well-differentiated cytologically as to be indistinguishable from normal/reactive PCs. This mature appearance is known as the Marshalko type morphology.Russell bodies (large intracytoplasmic globules of proteins) and Dutcher bodies (pale intranuclear inclusions) are relatively common findings in neoplastic PCs. However, in addition, various unusual cytological appearances of neoplastic PCs seen have been well described in the literature.[6]These include the following:

- "Flame cells" (deep magenta to pink staining in the peripheral part of the cytoplasm)
- "Mott, morular, or grape cells" (cells with multiple small globular cytoplasmic inclusions similar to multiple small Russell bodies)
- "Thesaurocytes" (plasma cells with abundant reticulated cytoplasm and/or cytoplasm trabeculated by eosinophilic bands): The appearance of flame cells and thesaurocytes is due to the accumulation of flocculent or granular material in the endoplasmic reticulum, as observed ultrastructurally.
- Cells intermediate in appearance between flame cells and thesaurocytes may be seen, possibly representing stages in the development of a shared subcellular composition
- Plasma cells with the presence of cytoplasmic spindle-shaped crystalline deposits (single or multiple)
- Cleaved, Multilobated, and Monocytoid Cells/Polymorphous Variant: These cytological variants may be confused with poorly differentiated carcinoma, lymphoma, and myeloid or monocytic leukemia. The multilobated cells may resemble neutrophils or histiocytes.
- Histiocytic variant: Neoplastic PCsmay have abundant homogeneous eosinophilic cytoplasm and dense nuclei, mimicking epithelioid histiocytes or histiocytes of a storage disorder. The neoplastic PCs in such cases contain paranuclear cytoplasmic inclusions,representing clonal light chain.
- Plasma cell dyscrasia mimicking erythrophagocytosis: Recently Pillai et al.[7] have documented a case of plasmacytoma in which the neoplastic PCs mimicked erythrocyte-laden macrophages. This was the result of accumulation of waxy, pale eosinophilic material in the cytoplasm. Rarely, true erythrophagocytosis may be seen in myelomas.
- Clear cell variant: The cells contain abundant clear cytoplasm. Signet-ring cells (see next variant description) may also be present in this variant. Occasionally multiple

clear vacuoles are present within the cytoplasm and may mimic a germ cell tumor or clear cell carcinoma.

- Signet-ring cell variant: Signet-ring cell MM is an extremely rare neoplasm. This variant may be easily confused with metastatic carcinoma. Signet-ring cell morphology has also been described in lymphomas. The cells in this variant contain peripheral crescentic nuclei and empty cytoplasmic vacuoles. Immunostains for light and heavy chains show a delicate ring of positivity around the vacuoles.
- Pleomorphic variant: Rarely, neoplastic PCs exhibit marked cellular pleomorphism and multinuclearity. These neoplasms simulate an anaplastic carcinoma or anaplastic large cell lymphoma.
- Blastic variant: This variant is composed of either plasmablasts (large PCs with hyperchromatic nuclei and prominent nucleoli) or cells closely resembling large-cell non-Hodgkin lymphoma. The cells in the latter type contain centrally placed large hyperchromatic or vesicular nuclei with one or more prominent nucleoli and scanty cytoplasm. Pleomorphic cells may also be seen in the blastic variant.
- Small cell (lymphoid) variant: The small-cell type of MM in the marrowmay mimic a small cell lymphocytic leukemia/lymphoma or an acute leukemic infiltrate. This is a particular problem since acute leukemiamay coexist with MM in the marrow. It may also be confused with other types of small-cell malignancies. The small cells in these neoplasms contain dense, round, angulated or notched nuclei and scanty cytoplasm.
- Spindle cell variant: The neoplastic PCsmay assume a spindle cell morphology or may be associated with a fibroblastic stromal response, closely resembling a spindle cell mesenchymal tumor.
- Oncocytic variant: This is an extremely rare variant of non-secretory MM.
- Thus, neoplastic PCs may histologically mimic carcinomas of various types (including anaplastic carcinoma), lymphomas (including anaplastic lymphoma), leukemias, germ-cell tumours, and very rarely sarcomas and histiocytic lesions. The clue to the correct diagnosis is finding PCs with typical morphology. However, such typical PCs may not always be readily identified, and thus recognition of these variants of neoplastic cells ultimately relies on their immunophenotypic features, described in the next section.

Table 2. Morphologic Features of Neoplastic Cells

Morphologic Variants of Neoplastic Cells	**Inclusions Within or Structures Associated With Neoplastic Plasma Cells**
Marshalko (Mature plasma cell morphology)	Russell Bodies
Flame	Dutcher Bodies
Mott	Intracytoplasmic Crystals
Thesaurocyte	
Histiocytic variant	
Mimicking erythrophagocytosis	
Clear cell variant	
Signet-ring cell variant	
Pleomorphic variant	
Blastic variant	

Table 2. (Continued)

Morphologic Variants of Neoplastic Cells	Inclusions Within or Structures Associated With Neoplastic Plasma Cells
Small cell (lymphoid) variant	
Spindle cell variant	
Oncocytic variant	

IMMUNOPHENOTYPIC FEATURES OF NEOPLASTIC PLASMA CELLS

The single best marker for identifying neoplastic PCs is syndecan-1 (i.e., CD138) combined with demonstration of monoclonal cytoplasmic light chain expression. Neoplastic PCs may display a heterogeneous phenotype consistent with the fact that the neoplastic clone is able to undergo a certain degree of differentiation. Although neoplastic PCs usually lack surface expression of B-cell associated antigens (i.e., CD19 and CD20), weak expression of CD20 may be seen in less than 1/3 of cases, and strong expression of CD20 may be seen in the majority of cellsin <10% of cases. Likewise, CD19 may be expressed by neoplastic PCs. CD79a is typically, though not invariably, positive. Although normal PCs and most neoplastic PCs lack surface light chain expression, they do demonstrate cytoplasmic light chain expression (restricted in neoplastic PCs). In addition, restricted surface light chain expression may be demonstrated in some neoplastic PCs. Likewise, although leukocyte common antigen (CD45) is not expressed by normal PCs and most neoplastic PCs, it may be expressed in some neoplastic PCs.

Of note, neoplastic PCs also frequently display reactivity for markers which are not restricted to the B-cell lineage, such as the neural cell adhesion molecule-CD56, CD31, [8]CD43, UCHL1 (CD45RO),[9] CD10,[10] and myelomonocytic markers (i.e., CD13 and CD33),[11]as well as cyclin D1,[12]cytokeratin,[11] and epithelial membrane antigen (EMA). Thus, cytokeratin and EMA positivity, with LCA negativity, could lead to an erroneous diagnosis of carcinoma.

Likewise, the small cell, 'lymphocyte-like' variant of MMmay be a particular diagnostic challenge, being confused morphologically and immunophenotypically with other CD20+ mature B-cells neoplasms. Furthermore, this variant frequently demonstrates over-expression of cyclin D1, asseen in mantle cell lymphoma (MCL). Immunophenotyping using a panel of antibodies, including CD3, CD5, CD20, CD23, CD103, CD11c, surface and cytoplasmic light chain expression, and CD138 aid in distinguishing the small cell variant of MM from MCL, chronic lymphocytic leukemia, lymphoplasmacytic lymphoma, and marginal zone lymphoma.

Of the immunophenotypic markers described above, some have prognostic and/or clinicopathologic significance. The presence of CD56 expression by plasma cells has been shown to indicate clonality, since they have been identified in plasma cell myeloma and lymphoplasmacytic lymphoma, but not in any cases of reactive plasmacytosis.[13] CD56 has also been associated with the presence of lytic bone lesions. In addition, it has been demonstrated that CD56 expression is inversely and significantly linked to the degree of BM involvement as well as peripheral blood and central nervous system involvement.[14-16]As

mentioned previously, cyclin D1 expression has been associated with the small cell variant of MM. The prognostic significance of cyclin D1 expression in MM is controversial. Cyclin D1 protein expression has been associated with a significantly unfavorable overall and progression-free survival.[17] However, other studies using conventional chemotherapy or high dose chemotherapy with autologous stem cell transplantation have generally shown either no effect or a slightly favorable effect on prognosis.[18-20]

Expression of CD10 and/or myelomonocytic markers (i.e., CD13 and CD33) by neoplastic PCs has likewise been associated with a worst prognosis.

Not only are CD138 and the demonstration of monoclonal cytoplasmic light chain expression important in recognizing neoplastic PCs, particularly in the morphologic variant cases, but the evaluation of the percentage and clonality of PCs in BM specimens is essential in establishing a diagnosis of a MM.Unfortunately, PCs tend to strip in BMA smear preparations. Based on this stripping artifact, as well as the occurrence of atypical morphological forms, PCs may be difficult to recognize and thus, tend to be underestimated in BMA differential counts. However, immunohistochemical (IHC) analysis of CD138 has been shown to be useful in quantitating PCs in paraffin sections of BM clots and cores and is superior to BMA differential counts in evaluating an accurate percentage of PCs in the BM, and preferentially in the BM core biopsy sections than in the BM clot sections.[13]

Immunophenotypic Techniques in Diagnosing Plasma Cell Dyscrasias

The following markers may be detected by both flow cytometric (FC) and IHC or in-situ hybridization (ISH) techniques: CD138, CD56, cytoplasmic and surface light chains, CD45, CD10, CD13, CD33, CD19, CD20, CD3, CD5, CD23, CD103, CD11c, and CD43. the ISH technique is much superior to IHC technique in evaluating light chain expression, since it better detects surface light chain expression and has much less background staining. The following markers are detected by IHC techniques, but not by FC techniques: CD31, UCHL-1, cytokeratin, EMA, and cyclin D1.

Immunohistochemistry is the preferred technique in quantifying PCs, since the cellular processing involved in performing flow cytometry appears to reduce PC percentages in many cases.[21]In addition, CD56 may be particularly helpful in determining the presence of neoplastic PCs, particularly in cases of PCD with “biclonal” populations and in the non-secretory variant.

Clinically Significant Molecular Abnormalities In Plasma Cell Myeloma (See Table 3)

Chromosome 13 Abnormalities

Monosomy of chromosome 13 or deletions of chromosome 13q represent one of the most frequent abnormalities in MM, being detectable by FISH in approximately 50% of

cases.[19,22,23]In cases with interstitial 13q deletions, the deleted segment appears to be centered about 13q14. However, in the great majority of cases, chromosome 13 abnormalities consist of monosomy 13.[24]In cases with chromosome 13 abnormalities detected by FISH, the percent of PCnuclei carrying the deletion varies from approximately 25% to >95%, suggesting this abnormality represents a secondary change in the pathogenesis of MM. However, chromosome 13 abnormalities are also detectable in 25-50% of cases of MGUS, indicating this abnormality appears to occur relatively early in the evolution of plasma cell neoplasms.[19,22]

In patients treated with either standard chemotherapy or high dose chemotherapy and autologous bone marrow transplantation (BMT), the presence of a chromosome 13 abnormality is associated with an adverse prognosis.[25-29]Chromosome 13 abnormalities are also highly associated with other molecular abnormalities with an adverse prognosis, such as t(4;14), discussed later. Of interest, the prognostic impact of chromosome 13 abnormalities is greater when the abnormality is detected by metaphase cytogenetics rather than by FISH, likely reflecting the combined influence of chromosome 13 abnormalities and the PC proliferative rate.[30,31]

Table 3. Correlation of Cytogenetic and Molecular Abnormalities with Prognosis in Multiple Myeloma

Cytogenetic/Molecular Abnormality	Prognostic Impact/Stage of Disease
Chromosome 13 abnormalities	Adverse
17p13 deletion/p53 mutation	Adverse
t(11;14)	No effect or slightly favorable
t(4;14)	Adverse
t(14:16)	Adverse
MYC translocation	Advanced Disease
Chromosome 1p21 deletion	Adverse
Hypodiploidy	Adverse
K-ras mutation	Adverse

Del(17p) and P53 Abnormalities

Somatic point mutations or hemizygous deletions of the tumor suppressor gene p53, located at locus 17p13, are observed in many malignant neoplasms.[32]As detected by FISH, deletions of the 17p13 locus are found in 5-10% of cases of MM. 17p deletions have been shown to be more common in patients with advanced MM and in more than 60% of human myeloma cell lines, suggesting that p53 mutations and deletions develop as secondary abnormalities during the course of disease progression.[19,22]In patients treated with conventional chemotherapy or high dose chemotherapy with autologous BMT, the FISH detectionof 17p13 deletionsis associated with short survival.[20,33-35]

Although most of the data in the literature regarding the clinical and prognostic significance of p53 abnormalities comes from studies employing FISH, other techniques, such as metaphase cytogenetics, may also be of clinical utility. Furthermore, deletions of the 17p13 locus may represent a marker for functional abnormalities of the p53 tumor suppressor pathway, rather than being of intrinsic biologic significance. In general, the hemizygous loss

of one p53 locus is thought to be associated with acquired mutations in the remaining locus that lead to abnormal function. Sequencing based studies have suggested that point mutations in p53 are less common than 17p deletions.[36]Furthermore, because p53 mutations lead to an abnormally long half-life of the protein, the presence of p53 mutations is associated with increased nuclear p53 expression as detected by IHC techniques. The presence of nuclear p53 staining by IHC techniques has been shown to be associated with deletions of 17p13 by FISH, and with poor survival.[37] IHC studies, which are much more widely available in routine pathology practice than FISH studies, may be useful to examine for p53 abnormalities in MM. It is currently unclear which of these methodologies (FISH, sequencing, or IHC) provides the most clinically relevant assessment of the p53 pathway in MM.

IMMUNOGLOBULIN HEAVY CHAIN (14Q32) TRANSLOCATIONS

General

Translocations involving the immunoglobulin heavy chain locus (*IGH*) at chromosome 14q32 are identified in approximately 50-60% of cases of MM, and are associated with non-hyperdiploid karyotypes.[19] The incidence of *IGH* translocations has also been shown to be inversely correlated with patient age.[38]Unlike several B-cell non-Hodgkin lymphomas that are associated with specific translocations involving the *IGH* locus and one partner gene (e.g. *IGH/BCL2* in follicular lymphoma), numerous *IGH* translocation partner genes have been described in myeloma. The major recurring partner genes include *CCND1* (11q13), *MMSET* (4p16), and *CMAF* (14q62). These recurring translocations can be detected in cases of MGUS and are therefore thought to arise early in the pathogenesis of MM.[19,39]Other *IGH* translocations, however, such as the *IGH/CMYC* translocation, are thought to represent secondary abnormalities acquired during disease progression.[40]The major recurring translocations, discussed below, are associated with specific pathologic and clinical features.

t(11;14)(Q13;Q32)*IGH/CCND1*

The t(11;14)(q13;q32) is found in approximately 15-20% of cases of MM using FISH techniques.[19,22,41] This translocation is also found in essentially all cases of mantle cell lymphoma. The precise translocation breakpoints, however, differ between MM and MCL, consistent with differing molecular pathogenesis (i.e. translocations arising during VDJ recombination for mantle cell lymphoma versus during class switching for MM).[19,42,43]

Cases of MM containing the *IGH/CCND1* translocation are associated with distinct clinicopathologic features including a small cell (i.e., lymphoid) morphology and CD20 expression, as previously discussed. Oligosecretory/non-secretory myeloma and IgM myeloma are also associated with the presence of the *IGH/CCND1*.[18,44,45]The influence of *IGH/CCND1* on prognosis has been controversial. As discussed previously, studies using conventional chemotherapy or high dose chemotherapy with autologous stem cell

transplantation have generally shown either no effect or a slightly favorable effect on prognosis.[18-20]

Several techniques have been used for the detection of *IGH/CCND1* in routine practice. When informative karyotypes are available, the translocation is typically readily identified by metaphase cytogenetics. Many *IGH/CCND1* positive cases, however, may yield non-informative results by standard karyotyping. Using modern, sensitive antibodies to Cyclin D1 protein, the vast majority of cases containing the *IGH/CCND1* show strong, uniform positivity for Cyclin D1 protein by IHC analysis (as discussed previously). One should keep in mind that approximately 30% of MM also show weak, partial expression of Cyclin D1 in the absence of the t(11;14) through other mechanisms of dysregulation of the *CCND1* locus.[46] The detection of Cyclin D1 mRNA by RT-PCR has also been shown to be of prognostic significance; however, this technique did not discriminate between *IGH/CCND1* and other forms of *CCND1* dysregulation.[47]FISH studies offer the most sensitive and specific method for detection of this translocation, especially when using simultaneous phenotyping (such as cIg-FISH or CD138 gating) or purification of plasma cells prior to FISH analysis.

t(4;14)(P16;Q32) *MMSET/IGH*

The t(4;14)(p16;q32) is found by FISH studies in approximately 15-20% of cases of MM.[18,48-50]The translocation leads to the production of an *IGH/MMSET* fusion gene. The normal function of the *MMSET* gene and the role of the dysregulated *IGH/MMSET* fusion gene in MM pathogenesisare largely unknown. In addition, the t(4;14) also leads to dysregulation of the *FGFR3* gene at chromosome 4p16 in about 80% of cases containing this translocation. Dysregulation of the FGFR3 protein is also thought to contribute to the molecular pathogenesis of t(4;14) positive MM.[19,22,48]

In MM treated with either conventional chemotherapy or high dose chemotherapy with autologous BMT, the presence of the t(4;14) has been associated with an adverse prognosis.[18,33,48] The prognosis appears to be poor in cases with the t(4;14), regardless of whether or not *FGFR3* expression is detectable.[51]This latter observation suggests that the *IGH/MMSET* fusion gene contributes directly to the adverse prognosis in these patients. Cases with the t(4;14) also show a strong association with chromosome 13q abnormalities that may also contribute to the adverse prognosis. [19,22,50]MM cases containing the t(4;14) are also associated with specific clinicopathologic features, including a blastoid morphology, IgA paraproteins, and preferential restriction of lambda light chains.[19,22,52]Novel small molecule inhibitors targeting the function of FGFR3 and cytotoxic anti-FGFR3 antibodies are currently in development for treatment of t(4;14) positive MM.[53-55]

The t(4;14)(p16;q32) is cryptic by routine banded karyotyping. The *IGH-MMSET* fusion transcript may be detected by RT-PCR or interphase FISH studies.[19,22]More recently, studies have shown that IHC detection of FGFR3 protein may also be used to screen for the presence of t(4;14).[56,57]However, since FGFR3 protein expression is found in only 75-80% of cases containing the t(4;14), FISH studies remain the gold standard for detection of this abnormality.

t(14;16)(Q32;Q23) *IGH/CMAF*

A t(14;16)(q32;q23) involving the *IGH* locus and the *CMAF* oncogene is identified in 2-10% of MMcases by FISH.[19,22]Due to the rarity of this translocation, there is limited data regarding the prognosis of cases carrying this abnormality. However, the available findings suggest the abnormality is associated with an adverse prognosis. [33]The *IGH/CMAF* translocation is generally cryptic by metaphase cytogenetics, and is best detected by interphaseFISH(dual fusion FISH probes specific for this abnormality are commercially available). Rare MM cases contain a variant t(14;20)(q32;q12) involving *IGH* and another member of the maf family, *MAFB*.[58]Although only a small number of cases with this abnormality have been described, it also appears to be associated with short survival.

Other *IGH* Translocations

Several other *IGH* translocation partner genes have also been identified in MM. Translocations involving *MYC* are frequent in MM cell lines, but are found in only a small percentage of MM patient samples.[59,60]The *IGH/CMYC* translocations are generally found in patients with advanced disease and are thought to generally represent secondary, acquired abnormalities. A t(6;14)(p21;q32) involving *IGH* and the Cyclin D3 gene (*CCND3*) is found in <5% of cases. A t(6;14)(p25;q32) involving the *MUM1/IRF4* locus has also been described in several patients.[19,22,61,62]The clinical significance of CCND3 and MUM1/IRF4 translocations are not presently well characterized.

Other Chromosomal Abnormalities

Abnormalities of chromosome 1 are frequently found in MM. Duplication or amplification of the *CKS1B* gene on chromosome 1q21 is found in approximately 30% of cases of MM using FISH.[63,64] The *CKS1B* protein regulates the degradation of p27, thereby promoting cell cycle progression. Amplification of the CKS1B locus is associated with the presence of other prognostically significant abnormalities, including 17p13 deletions and chromosome 13 abnormalities, and the presence of CKS1B amplification conveys poor survival. Another chromosome 1 abnormality, deletion of chromosome 1p21, has been reported in 20% of MM cases.[65]The deleted locus contains the cyclin dependant kinase phosphatase, *CDC14A*. Deletion of 1p21 is also associated with other chromosomal abnormalities, including chromosome 13 abnormalities and the t(4;14)(p16;q32). Cases with the 1p21 deletion display an adverse prognosis in cases treated with high dose chemotherapy and autologous BMT.

In addition, the presence of hypodiploidy predicts a significantly reduced survival.[66]

Other Genetic Abnormalities

Activating *Ras* mutations have been noted in 35–50% ofMM patients.The prevalence of the mutations may increase somewhat with advancingstages of the disease. Most mutationsinvolve K- and N-*ras* at codons 12, 13, and 61. MM cases with t(4;14) (p16.3;q32) can have activating mutations of *FGFR3* or *Ras*, but these eventsappear to be mutually exclusive, suggesting that activatingmutations of *Ras* or *FGFR3* have a similar effect. *Ras*mutations appear to be rare in MGUS, suggesting that this isa molecular marker if not causative in the progression fromMGUS to MM for some tumors. Mutations of K-*ras*, but not of N-*ras*, have been associated withshorter survival. Treatment strategies addressing theconstitutive activation of *ras* are being explored.[19]

A Practical Approach to Molecular Evaluation of MM

To allow for identification of these abnormalities in routine practice, metaphase cytogenetic studies and FISH studies for IGH translocations, chromosome 13 abnormalities, and p53 are suggested in all cases at initial diagnosis. The p53 pathway may also be assessed by IHC methods. Although other recurring abnormalities may also be of prognostic significance, it is currently unclear how such information should be incorporated into routine practice.

Current Therapeutic Guidelines for MM

In the past decade, there have been major advances in the treatmentof myeloma. Thalidomide,[67]bortezomib,[68,69]and lenalidomide [70,71]have emerged as highly active agents in the treatment of myeloma.There is no evidence that early treatment of patients with smoldering MM prolongs survival. However, clinicaltrials are ongoing to determine whether newer agents can delayprogression.

The treatment of symptomatic MM depends oneligibility for stem-cell transplantation and risk assessment.Current therapy for MM relies on the ISS as well as important prognostic factors that stratify patients into highrisk and standard risk, including deletion 13 or hypodiploidy on conventionalkaryotyping, deletion 17p– or immunoglobulin heavy chaintranslocations [t(4;14) or t(14;16)] on molecular genetic studies,and plasma cell labeling index of 3% or higher.[72]The presenceof any one or more of these high-risk factors classifies a patientas having high-risk myeloma. The median survival of patientswith high-risk features is only 2 to 3 years, even with tandemstem-cell transplantation, compared with 5 or more years inpatients with standard-risk disease.Figure 1 outlines the current approach to the treatment of newly diagnosed MM.

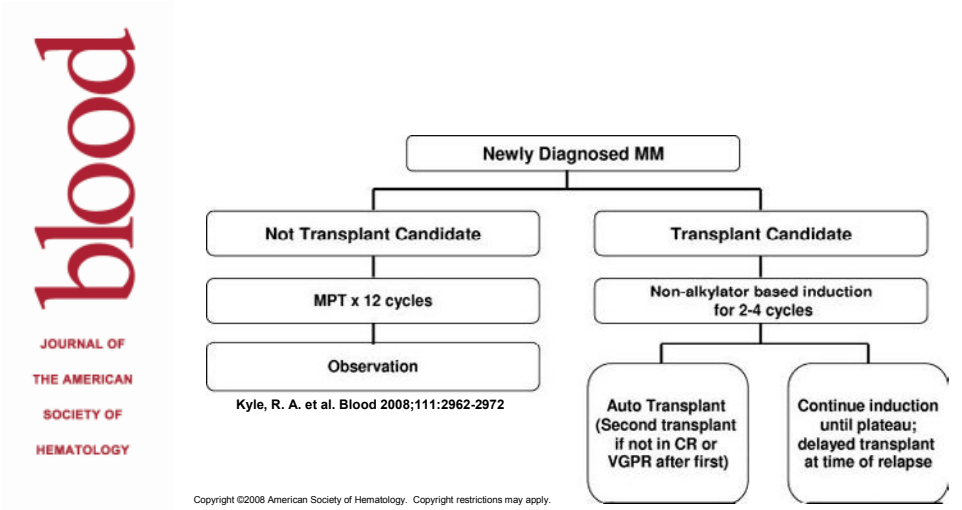

Figure 1. Algorithm outlining the current approach to the treatment of newly diagnosed myeloma. CR indicates complete response; MPT, melphalan, prednisone, thalidomide; and VGPR, very good partial response. Reprinted with permission.

Initial Therapy in Patients Eligible for Transplantation

The most commoninduction regimens used today are thalidomide–dexamethasone (Thal/Dex), bortezomib-based regimens, and lenalidomide–dexamethasone(Rev/Dex).

Initial Therapy in Patients not Eligible for Transplantation

Patients who are not transplant candidates are treated withstandard alkylating agent therapy. The 3 most commonly usedregimens are *Melphalan, prednisone, thalidomide (MPT); Melphalan, prednisone, bortezomib (MPV; and Melphalan, prednisone, lenalidomide (MPR).*

Hematopoietic Stem-Cell Transplantation

Autologous Stem Cell Transplant (ASCT)

Although not curative, *ASCT* improves complete response ratesand prolongs median overall survival in MM by approximately12 months.[73,74]The mortality rate is 1% to 2%. Melphalan, 200mg/m^2, is the most widely used conditioning regimen.Randomized trials have shown similar survival ratesin patients in which*ASCT*is done early (immediately after 4 cycles of induction therapy)or later (at the time of relapse as salvage therapy).[75,76] It has also been shown that patients responding to inductiontherapy had similar overall and progression-free survival witheither *ASCT* or 8 additional courses of chemotherapy, questioning the benefit of *ASCT* in patients respondingto induction chemotherapy.[77]The need for early *ASCT* in this eraof new promising drugs is the present real therapeutic question in MM.

Tandem Transplantation

With tandem (double) *ASCT*, patients receive a second planned*ASCT* after recovery from the first *ASCT*. This approach has been restrictedto patients failing to achieve a complete response or significantpartial response with the first *ASCT*. [78,79]

Allogeneic Transplantation

Only a limited number of MM patients are candidates forallogeneic transplantation due to age, availability of anHLA-matched sibling donor, and adequate performance status. Thehigh treatment-related mortality, mainly related to graft-versus-hostdisease, has made conventional allogeneic transplants unacceptablefor most MM patients. Initial trials employing anapproach of an *ASCT* followed by a planned reduced intensity (RIC) allogeneic*SCT* have shown promise.[80]However, additionalconfirmation is needed.

Maintenance Therapy

Observation is still the standard following initial therapyas described (Figure 1). Interferon does not appear to providesignificant clinical benefit. [76]A recent trial (IFM 99-02) randomized597 patients (age < 65 years) after tandem ASCT to no maintenance, pamidronate, or pamidronate plus thalidomide. [81] The 4-yearoverall survival rate was superior with thalidomide (77%, 74%,and 87%, respectively, $P < .04$), but these results need further confirmation. Clinical trials are currently evaluating thalidomide, lenalidomide, bortezomib, and other novel approaches as maintenancetherapy.

Treatment of High-Risk Myeloma

As mentioned previously, patients with high-risk MM tend to do poorly with medianoverall survival of approximately 2 years even with tandem *ASCT*. [82]Incorporation of new agents early in the disease course is amajor option for treatment. [72]*ASCT* followed by RIC-allogeneicSCT may also be an option in selected patients. A third optionis to incorporate routine maintenance therapy after treatmentas outlined earlier for patients with standard-risk disease.

Supportive Care

Numerous improvements in supportive care have greatly improvedthe outcome of MM patients. Some of the most important advancesare the advent of bisphosphonates to treat hypercalcemia andto prevent MM bone disease, the use of vertebroplasty andkyphoplasty to treat vertebral fractures, and judicious useof prophylactic antibiotics in selected patients.

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