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# NANOTECHNOLOGIES IN ONCOLOGY: PHARMACOGENETICS AND PHARMACOGENOMICS TO OPTIMIZE ANTITUMOR THERAPIES

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*Alla mia famiglia*

*Questo lavoro di tesi è stato svolto presso la Struttura Operativa Complessa di Farmacologia Sperimentale e Clinica del Centro di Riferimento Oncologico di Aviano (Istituto di ricerca e cura a carattere scientifico), diretta dal Dott. Giuseppe Toffoli.*

# CONTENTS

# RIASSUNTO

La *nanomedicina* è l'applicazione di nanotecnologie nella prevenzione e trattamento delle malattie nel corpo umano. La ricerca farmacologica in campo oncologico si rivolge sia alla scoperta di nuove molecole ad azione antitumorale che soprattutto a migliorare l'uso dei farmaci già presenti in terapia. Infatti, la risposta ad un agente chemioterapico è molto variabile sia sotto il profilo dell'efficacia che della tollerabilità e questo può dipendere sia da differenze tra individuo e individuo che da caratteristiche molecolari della neoplasia. La farmacogenetica, insieme alla radiogenetica, si occupano di indagare come le caratteristiche genetiche di ciascun individuo possano influenzare l'effetto terapeutico del farmaco o delle radiazioni e la predisposizione alla comparsa di effetti indesiderati. In particolare si focalizzano sulle alterazioni costitutive del patrimonio genetico di ciascun individuo, i cosiddetti polimorfismi, in grado di influenzare il trasporto, il metabolismo o l'interazione con il target di un farmaco o trattamento.

L'oxaliplatino è un chemioterapico usato in terapia da molti anni per il trattamento di numerosi tumori solidi, tra cui carcinoma ovarico, colon rettale, gastrico, della mammella, del polmone, e mesotelioma. Nonostante l'ampia diffusione dovuta all'efficacia dimostrata in numerosi studi clinici, l'impiego dell'oxaliplatino è talvolta associato a fenomeni di neurotossicità, tossicità ematologica e non ematologica talvolta molto gravi e ad oggi rimane impossibile prevedere nel singolo paziente quale sarà la risposta al trattamento sia in termini di efficacia che di tollerabilità.

Inoltre, l'impiego della radioterapia nel carcinoma prostatico può causare dei danni sulla doppia elica del DNA. La radioterapia, infatti, esplica il suo effetto antineoplastico sia direttamente, su macromolecole cellulari quali il DNA, sia indirettamente generando specie reattive dell'ossigeno e relativi sottoprodotti. Di conseguenza si focalizza l'attenzione su geni coinvolti nei meccanismi di riparo dei danni sul DNA indotti da radiazioni e dello stress ossidativo.

In questo studio l'obiettivo principale è quello di analizzare l'effetto delle variazioni geniche (polimorfismi) sull'outcome clinico dei pazienti con carcinoma colon rettale trattati con oxaliplatino e di coloro che sono affetti da carcinoma prostatico e che sono stati trattati con la radioterapia, con lo scopo di individuare quei parametri farmacogenetici in grado di fornire delle indicazioni su una possibile "personalizzazione" della terapia.

Sono stati arruolati 154 pazienti affetti da tumore colonrettale in trattamento con oxaliplatino (regime FOLFOX4) in terapia adiuvante, e 924 pazienti con carcinoma prostatico trattati con radioterapia, e/o chirurgia e/o ormonoterapia.

I pazienti sono stati sottoposti ad un prelievo ematico all'inizio della terapia. Dall'aliquota di sangue è stato estratto il DNA, e poi amplificato mediante Polymerase Chain Reaction (PCR). Per la genotipizzazione sono state impiegate le seguenti tecniche: Pyrosequencing, una nanotecnologia per l'identificazione di mutazioni puntiformi (SNP), metodo Taqman, per la discriminazione allelica, e l'analisi dei frammenti automatizzata.

Il primo obiettivo di questo studio è il seguente:

- ✓ Studiare il ruolo di alcuni polimorfismi precedentemente validati nel cancro metastatico come possibili markers di neurotossicità in pazienti affetti da carcinoma colonrettale e trattati con regime FOLFOX4;
- ✓ Valutare il ruolo di alcuni polimorfismi precedentemente validati nel cancro metastatico come possibili markers di tossicità ematologica e non-ematologica in pazienti affetti da carcinoma colonrettale e trattati con regime FOLFOX4.

*Neurotossicità di grado ≥ 2*: la frequenza degli alleli minori dei polimorfismi rs2074087 e rs35587 nel gene ABCC1 e la frequenza degli alleli minori dei polimorfismi rs2273697 nel gene ABCC2 risultano associati ad una riduzione del rischio di sviluppare neurotossicità.

*Tossicità ematologica di grado ≥ 3*: solo il polimorfismo rs3136228 nel gene hMSH6 risulta associato ad un aumento del rischio di questa tossicità (neutropenia).

*Tossicità non-ematologica di grado ≥ 3*: solo il polimorfismo rs1799794 nel gene XRCC3 risulta associato ad un aumento del rischio di questa tossicità.

Il secondo obiettivo di questo studio è il seguente:

- ✓ Identificare dei markers radiogenetici di risposta in termini di rischio di recidiva biochimica del PSA nel cancro prostatico;
- ✓ Identificare dei markers radiogenetici di risposta in termini di overall survival (OS) nel cancro prostatico.

*Rischio di recidiva biochimica del PSA*: i polimorfismi XRCC1 (rs1799782) e TP53 (rs1042522) risultano correlati con un aumento del rischio di recidiva biochimica del PSA; mentre i polimorfismi ERCC2 (rs1799793) e VEGF (rs1570360) risultati associati ad una diminuzione del rischio di recidiva biochimica del PSA.

*Overall survival (OS)*: i polimorfismi XRCC1 (rs1799782), RAD51 (rs1801320), e NOS2A (rs9282801) sembrano influenzare la sopravvivenza (overall survival) dei pazienti affetti da carcinoma prostatico trattati con radioterapia.

In conclusione, il lavoro di questa tesi ha permesso di definire nuovi markers molecolari che potrebbero avere un valore prognostico nel trattamento del carcinoma colonrettale con regime FOLFOX4 e di quello prostatico con radioterapia. L'applicazione di questi biomarcatori nella pratica clinica potrebbe essere utile per progettare una personalizzazione del trattamento basato su specifiche caratteristiche genetiche del paziente con carcinoma colonrettale e prostatico.

## ABSTRACT

Nanomedicine is the application of nanotechnology in biomedical field to the prevention and treatment of diseases in the human body. Pharmacological research in oncology is aimed at the discovery of new antitumor molecules and especially to improve the use of drugs already present in therapy. In fact, the response to a chemotherapeutic agent is highly variable both in terms of efficacy and tolerability and this may be due both to differences between individuals and molecular characteristics of the tumor. Pharmacogenetics, along with radiogenetics, are concerned to investigate how the genetic characteristics of each individual can influence the therapeutic effect of the drug or radiation and susceptibility to side effects. In particular, they focus on how the constitutive alterations of genetic asset of each individual, so-called polymorphisms, can affect transport, metabolism or the interaction with the target of a drug or treatment.

Oxaliplatin is a chemotherapy used in therapy for many years for the treatment of many solid tumors, including ovarian, colorectal, gastric, breast, lung cancer, and mesothelioma. Despite the prevalence due to the effectiveness demonstrated in numerous clinical trials, the use of oxaliplatin is sometimes associated with phenomena of neurotoxicity, haematological and non-haematological toxicity at times very serious and today it remains impossible to predict in the individual patient what will be response to the treatment both in terms of efficacy and tolerability.

Furthermore, the use of radiation therapy in prostate cancer may lead to damage on the DNA double helix. Radiotherapy, in fact, exerts its antineoplastic effect both directly, on cellular macromolecules such as DNA, or indirectly by generating reactive oxygen species and their by-products. As a result, attention is focused on genes involved in the repair mechanisms of DNA damage induced by radiation and oxidative stress.

In this study, the main objective was to analyze the effect of genetic variations (polymorphisms) on clinical outcome of colorectal cancer patients treated with oxaliplatin and those with prostate cancer treated with radiotherapy, with the aim to identify those pharmacogenetic parameters able to provide an indication of a possible "personalization" of therapy.

We enrolled 154 patients with colorectal cancer treated with oxaliplatin (FOLFOX4 regimen) in adjuvant therapy, and 924 patients with prostate cancer treated with radiotherapy and/or surgery and/or hormone therapy.

The patients were subjected to blood sampling at the beginning of therapy. DNA was extracted from blood, and then amplified by Polymerase Chain Reaction (PCR). For the genotyping were employed the following techniques: Pyrosequencing, a nanotechnology for the identification of punctiform mutations (SNP), Taqman method for allelic discrimination, and the automated fragments analysis.

The first objective of this study is as follows:

- ✓ Investigate the role of genetic polymorphisms previously identified in the metastatic disease as predictive markers of neurotoxicity from oxaliplatin in patients treated with adjuvant FOLFOX4 regimen;
- ✓ Investigate the role of genetic polymorphisms previously identified in the metastatic disease as predictive markers of hematological and non-hematological toxicities in patients treated with adjuvant FOLFOX4 regimen.

*Neurotoxicity of grade ≥ 2*: the minor frequency alleles of rs2074087 and rs35587 polymorphisms in ABCC1 gene and the minor frequency alleles of rs2273697 in ABCC2 were associated with a reduced risk of neurotoxicity; while the minor frequency alleles of rs3740066, rs1885301, rs4148396, and rs717620 polymorphisms in ABCC2 gene and the minor frequency alleles of rs2622604 in ABCG2 were associated with an increased risk of neurotoxicity.

*Hematological toxicity of grade ≥ 3*: only the polymorphism rs3136228 in hMSH6 gene resulted associated with an increased risk of this toxicity (neutropenia).

*Non-hematological toxicity of grade ≥ 3*: only the polymorphism rs1799794 in XRCC3 gene resulted associated with an increased risk of this toxicity.

The second objective of this study is as follows:

- ✓ Identification of radiogenetic markers of response in terms of risk of biochemical PSA recurrence in prostate cancer;
- ✓ Identification of radiogenetic markers of response in terms of overall survival (OS) in prostate cancer.

*Risk of biochemical PSA recurrence*: XRCC1 (rs1799782) and TP53 (rs1042522) are significant factors predicting for higher risk of biochemical PSA recurrence; while ERCC2 (rs1799793) and VEGF (rs1570360) polymorphisms are significant factors predicting lower risk of biochemical PSA recurrence.
*Overall survival (OS)*: XRCC1 (rs1799782), RAD51 (rs1801320), and NOS2A (rs9282801) seem to influence the overall survival of prostate cancer patients treated with radiation therapy.

In conclusion, the work of this thesis allowed defining new molecular markers that may have prognostic value in the treatment of colorectal cancer with FOLFOX4 regimen and in prostate cancer with radiotherapy. The application of these biomarkers in clinical practice may be useful for designing a personalization of treatment based on specific genetic characteristics of the patient with colorectal cancer and prostate cancer.

# *INTRODUCTION*

# 1. *Nanotechnologies in medicine*

Nanoscience and nanotechnology are the study and application of extremely small things and can be used across all the other science fields, such as chemistry, biology, physics, materials science, and engineering. Nanotechnology is not just a new field of science and engineering, but a new way of looking at and studying at the nanoscale, which ranges from 1 to 100 nanometres.

The ideas and concepts behind nanoscience and nanotechnology started with a talk entitled "There's Plenty of Room at the Bottom" by physicist Richard Feynman at American Physical Society meeting at the California Institute of Technology (CalTech) on December 29, 1959, long before the term "nanotechnology" was used. In his talk, Feynman described a process in which scientists would be able to manipulate and control individual atoms and molecules.

Nanotechnology is being used to refine discovery of biomarkers, molecular diagnostics, and drug discovery and drug delivery, which could be applicable to management of patients. Nanotechnology strives to develop and combine new materials by precisely engineering atoms and molecules to yield new molecular on the scale of individual cells, organelles or even smaller components, providing a personalized medicine [1,2].

Over the past twenty-five years nanotechnologies have assumed a growing importance in, insomuch that in 2000 the National Institute of Health (USA) coined the term "nanomedicine", to include all applications of nanotechnology in medical-biological field.

The early genesis of the concept of nanomedicine sprang from the visionary idea that tiny nanorobots and related machines could be designed, manufactured, and introduced into the human body to perform cellular repair at the molecular level. Nanomedicine today has branched out in hundreds of different directions, each of them embodying the key insight that the ability to structure materials and devices at the molecular scale can bring enormous immediate benefits in the research and practice of medicine [3].

Nanomedicine is the process of diagnosing, treating, and preventing disease and traumatic injury, of relieving pain, and of preserving and improving human health, using molecular tools and molecular knowledge of the human body (Fig. 1). Currently nanomedicine is being utilized in wide ranging areas, and these applications have a potential to revolutionize the way we detect and treat damage to the human body and diseases in the future [4].

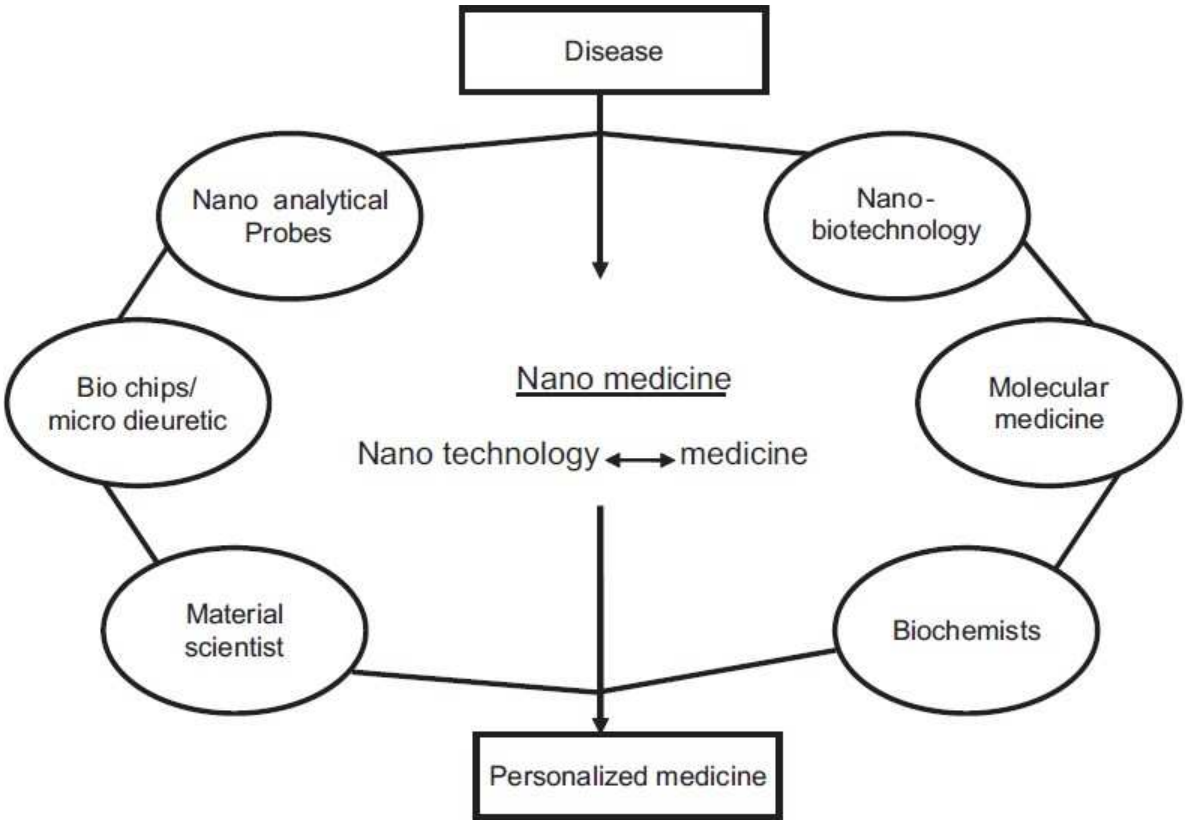


**Figure 1:** Technologies involved in the field of medicine

So nanotechnology lends itself to the application in the medical field, designing a potentially endless series of nanosystems, which can be used to deliver therapeutic or diagnostic agents through biological barriers, to promote access to molecules. These properties are incorporated in new nanosystems, some of which have already been approved by the Food and Drug Administration (FDA), others of them are undergoing clinical trials, and relate to drug delivery, gene delivery, contrast agents, diagnostic devices and tissue engineering.

The main applications of nanotechnology in the field of medicine are to date the following, although are still in part on an experimental basis:

1) Nanocarriers for the transport of drugs: they include nanotransporters, nanoparticles for targeted delivery of therapeutic substances. Drug delivery has several purposes including reducing toxicity and improving the penetration of the drug.
2) Nanogene therapy to improve cancer diagnosis. It is a new technology able to read the fingerprint of proteins, detect tumour markers and control the toxicity of drugs will be developed by building nanodevices. Actually nanomedical sensors will detect the

presence of tumour markers from a drop of blood and monitor the drug concentration in the tissues in real time.

3) Nanoprobes of DNA in molecular biology to better characterize cancers and create targeted treatments.
4) Nanoparticles to identify pathogens and improve tumour diagnosis: a nanoparticle is defined as the smallest unit ($10^{-9}$ metres) that can still behave as a whole entity in terms of properties and transport to improve drug bioavailability, abrogate treatment-induced drug resistance, and reduce nonspecific toxicity in the field of medicine [5].

Other nanotechnology platforms like fullerenes, nanotubes, quantum dots, nanopores, dendrimers, liposomes, magnetic nanoprobes and radio-controlled nanoparticles are being developed [8].

One of the most important challenges undertaken by nanomedicine is the fight against cancer, so it is true that the National Cancer Institute (USA) established as ambitious goal victory against cancer in 2015. This complex disease is caused by genetic instability and the sum of multiple molecular alterations. Until now, the medical knowledge was not sufficient to diagnose the cancer effectively, let alone predict the occurrence, in addition to being proved insufficient to make predictions on the outcome of an effective therapy. To be resolved the current difficulty of care requires:

1) advanced technology to observe and detect early disease;
2) new methods to obtain accurate diagnoses and prognoses;
3) strategies to overcome the toxicity and side-effects of chemotherapy drugs;
4) new treatments for aggressive and hit particularly lethal diseases.

The progress on these points is also of paramount importance for the new frontiers of medicine, "custom", with which the detection, diagnosis and treatment of cancer are linked to tumour molecular profile of each individual, and "predictive" which is based on the detection of molecular and genetic markers for predicting the appearance of any diseases [4].

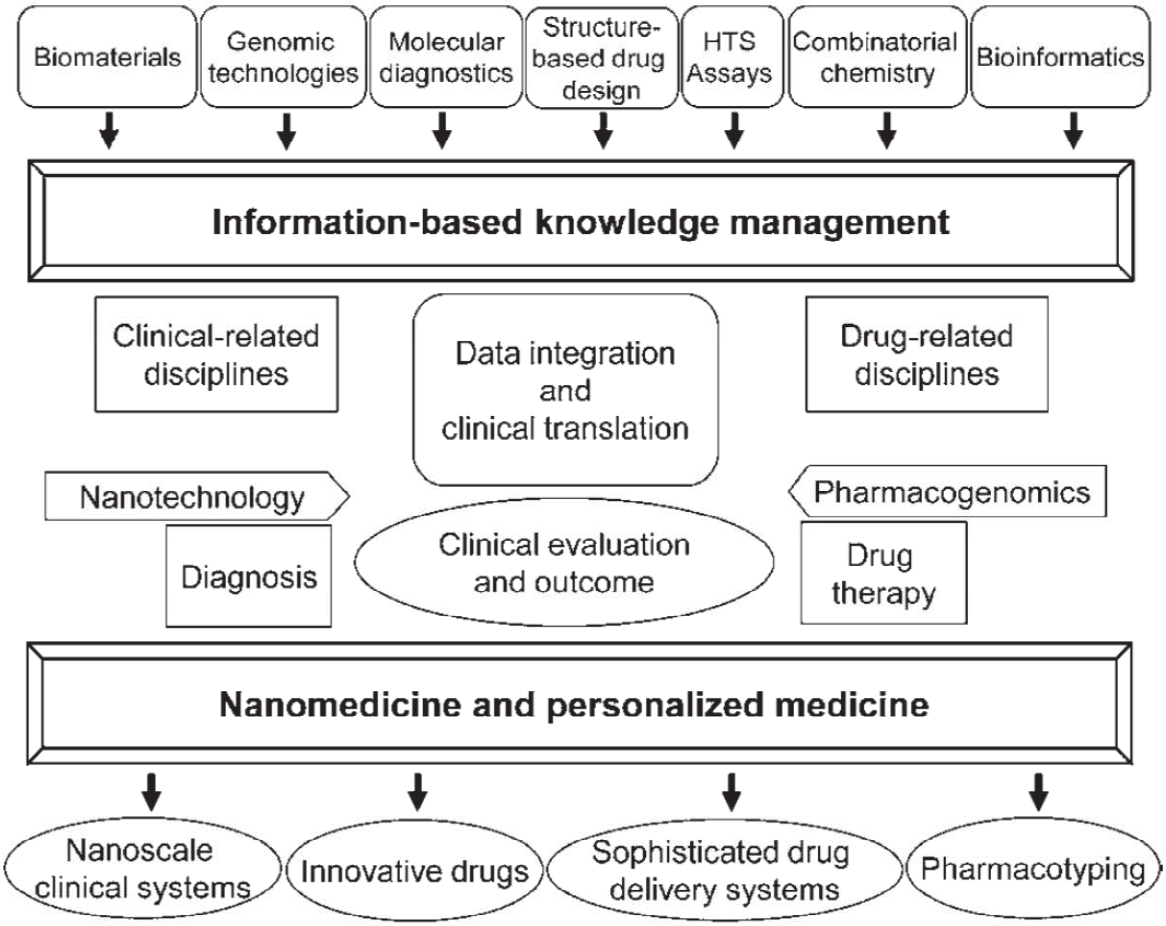


**Figure 2.** Diagrammatic outline of the multidisciplinary environment proposed for nanomedicine and personalized medicine to ensure major benefits for disease diagnosis and drug development and delivery to improve clinical outcomes.

Nanotechnology will play an important role in this area and in particular nanobiotechnology (Fig. 2) is being used to refine discovery of biomarkers, molecular diagnostics, drug discovery and drug delivery, which are important basic components of personalized medicine and are applicable to management of cancer as well [7]. Examples are given by the application of quantum dots, gold nanoparticles, and molecular imaging in diagnostics and combination with therapeutics, that constitute another important feature of personalized medicine. Personalized management is usually based on pharmacogenetic, pharmacogenomic, pharmacoproteomic and pharmacometabolic information, but other individual variations in patients and environmental factors are also taken into consideration [2]. Nanobiotechnology through personalized management of cancer is expected to enable early detection of cancer, more effective and less toxic treatment increasing the changes of cure.

# 2. Pharmacogenetics and Pharmacogenomics

The term "pharmacogenetics" was coined by the German Pharmacologist Friedrich Vogel in 1959 two years after Arno Motulsky [9] wrote his seminal paper on how "[...] drug reactions [...] may be considered pertinent models for demonstrating the interaction of heredity and environment in the pathogenesis of disease" (Fig. 3) [8]. Pharmacogenetics can be defined as the study of the variability in drug response because of heredity. In 1997, Marshall introduced the term "pharmacogenomics" [10]. Both terms are used interchangeably; however, the latter one, pharmacogenomics, signifies that we have the knowledge and technology to evaluate the whole genome and we have the ability to interrogate multiple genes on drug response, rather than having to concentrate on a single gene at a time [11,12].

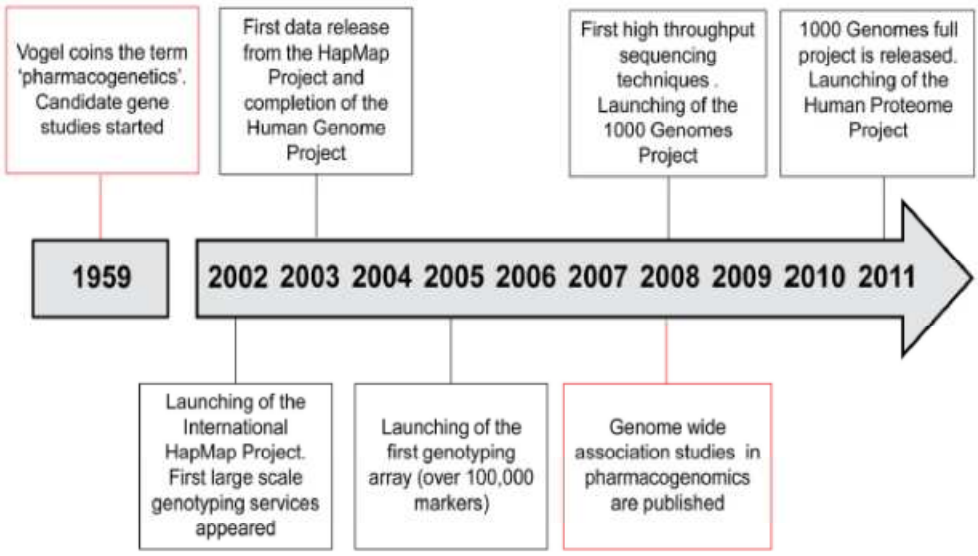


**Figure 3:** Current pipeline used in pharmacogenomics.

Pharmacogenetics, the study of the role of inheritance in interindividual variability in drug response, has the potential to contribute to the development of more rational pharmacological therapies for various type of cancers. There is wide interpatient variability in the dose-effect relationship of chemotherapeutics drugs; some patients respond well to treatment and others do not, and the nature and severity of adverse events also show wide variations among patients. Several host-related factors have evolved over time as determinants affecting anticancer drug treatment outcome such as age, gender, renal and

liver function, concomitant medication leading to drug-drug interactions, (co-)morbidity, compliance, environment, and lifestyle (Fig. 4).

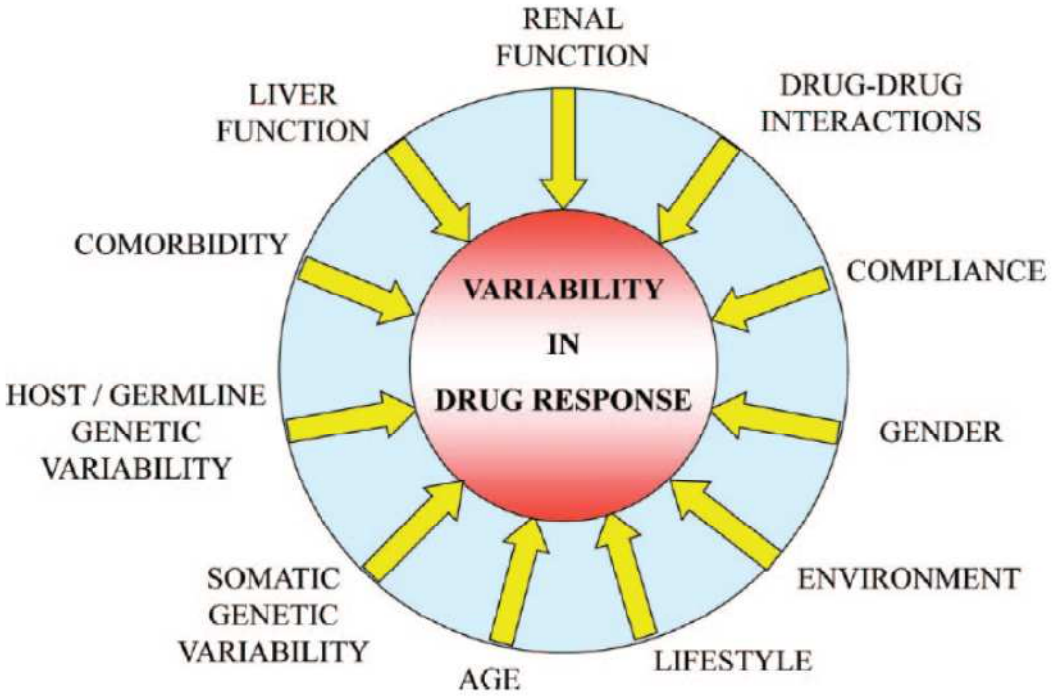


**Figure 4: Interindividual variability in drug response:** beside genetic polymorphism, various additional nongenetic factors may contribute to interindividual differences in drug response.

Other sources of interpatient variability in drug response are interindividual differences in pharmacokinetics (PK), that is, drug absorption, distribution, metabolism, and elimination, and in pharmacodynamics (PD), i.e., effects on drug receptors and other drug targets. Variations in the genetic constitution of genes that encode proteins involved in the PK and PD of a drug thereby significantly contribute to individual differences in drug response. Among the various biological mechanisms for genetic variability there are differences in transcription factor activity, gene expression, gene silencing (epigenetics), and genetic polymorphism [13].

Genetic polymorphisms are DNA sequence alterations consisting of single-nucleotide polymorphisms (SNPs), mutations, deletions, insertions, and gene copy number variations and they are present in >1% of the population. The single-nucleotide polymorphism (SNP) which involves a single nucleotide, leads to the alteration of a single base in DNA sequence; in the human genome this event occurs approximately every 100 basis. Other common types of polymorphisms are minisatellites which are characterized by a variable number of tandem

repeats of multiple copies of DNA sequences (0,1-10kb), and microsatellites in which a sequence of up to four nucleotides is repeated many times.

Finally, among polymorphic variations we can find insertions or deletions that are formed by the addition or loss of a nucleotide portion of variable size in the genomic sequence. Polymorphisms are defined: "Nonsense" when substitutions of single basis lead to the appearance of a stop codon and to the early termination of protein synthesis; "Missense" or "Non-synonymous" when, instead, substitutions create the incorporation of an inappropriate amino acid in the protein; and finally "Frameshift" when they establish a shift in the reading frame during protein translation (Fig.5).

| | |
|---|---|
| Wild-type sequence | DNA: CTC CGA GAA AAC<br>*Protein: Leu - Arg - Glu - Asn* |
| Nonsynonymous SNP (missense) | DNA: CTC CCA GAA AAC<br>*Protein: Leu - Pro - Glu - Asn* |
| Nonsynonymous SNP (nonsense) | DNA: CTC CGA TAA AAC<br>*Protein: Leu - Arg* stop |
| Synonymous SNP | DNA: CTC CGA GAA AAT<br>*Protein: Leu - Arg - Glu - Asn* |
| Insertion | DNA: CTC CAG AGA AAA C<br>*Protein: Leu - Leu - Arg - Phe* |

**Figure 5: Effect of genetic polymorphism on the encoded protein.** Depending on its type and physical location, a genetic polymorphism may elicit changes the primary amino acid sequence of a protein in various ways.

From a phenotypic point of view, these genetic variations may alter or totally silence the normal gene or protein product activity. In particular, when the mutagenic event involves the coding region of the gene we have a structural aberration in the produced protein with consequent alteration of its functions; if genetic variations are located in the regulating regions as the gene promoter or in not translated areas into 5' (5'UTR) and 3' (3'UTR), or modify the normal processes of transcription, translation and RNA splicing we have a variation in the amount of protein produced. There are three different approaches to analyze the genetic variations described above (Fig.6):

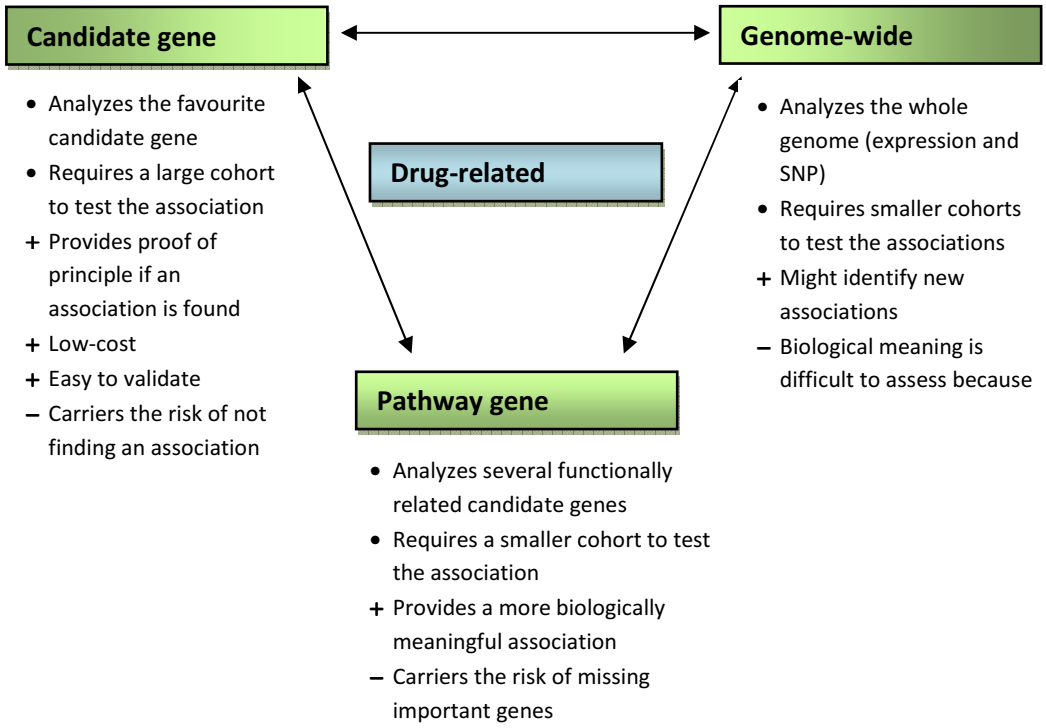


**Figure 6: Pharmacogenetic approaches:** differences among candidate-gene, pathway gene and genome wide approaches: main characteristics (•), advantages (+), and disadvantages (-).

1) A ***candidate gene approach***: treatment response can be altered by polymorphisms of single genes encoding enzymes that metabolize chemotherapeutic drugs; the clear disadvantage in considering a single gene approach is the possibility of losing significant association.
2) A ***pathway gene approach***: combines the advantages of the single-gene approach and the genome wide approach. A characteristic of the pathway gene method is that a set of SNPs is selected based on a description of pathways regarding the mechanism of action and pharmacokinetics of the drug under study [14]. The main advantages of the gene candidate pathway approach are to combine information of several genes that are common in a metabolic pathway and to minimize the "noise" of a non-targeted genome wide approach, although it probably excludes some genes of importance.
3) A ***genome wide approach***: in contrast to the candidate gene approach, in which only a limited number of polymorphisms are tested, the genome-wide approach analyzes multiple polymorphisms (mostly SNPs) across the entire human genome. Therefore, it is independent of whether or not a gene is a priori expected to be involved in the

pharmacological pathway of a drug. This approach requires high-throughput genotyping technologies that are able to analyze multiple SNPs simultaneously [13]. The number of SNPs on the array may range from a few hundred to even hundreds of thousands. The main disadvantage of this method is the expression signals of irrelevant genes (defined as "noise") that could increase the number of false positive.

The ultimate goal of a pharmacogenetic analysis is to be able to forecast, through the preliminary analysis of the genetic characteristics of the individual patient, the therapeutic effect and the toxicity of the used drug, allowing optimization and customization of therapy. In oncology, the personalization of chemotherapy is certainly a very important goal (Fig. 7).

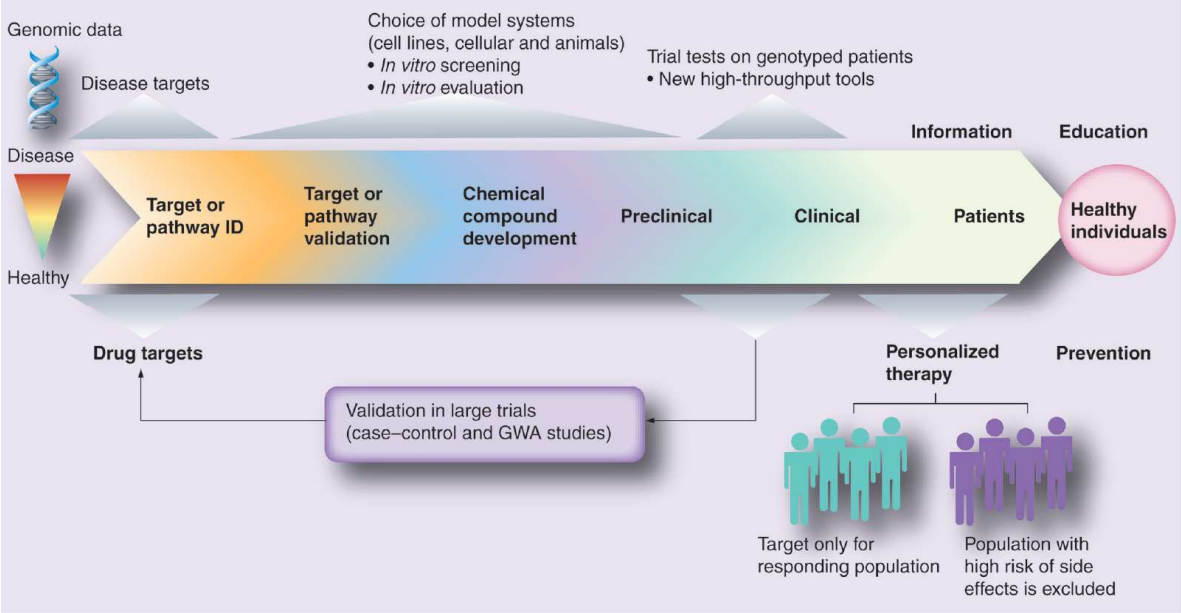


**Figure 7:** Pharmacogenomic and drug development steps for personalized therapy.

Several studies have shown, for example, that the administration of the same dose of a given antineoplastic drug in a population of patients often shows a huge range of toxicities which, in some cases, can result even lethal. Moreover these drugs have a low therapeutic index, i.e. the ratio between the minimum effective dose and the maximum tolerated dose (MTD); this obviously carries a high risk of developing adverse effects for the subpopulation genetically predisposed to a changed drug metabolism.

Currently the dose of most anticancer drugs is generally based on the individual's body weight or body surface area, but this is not clearly enough to overcome the inter-individual differences observed in the outcome of treatment [15,16]. Therefore it becomes of primary

importance to have a genetic test that can predict the answer to the drug and that allows to identify the effective and non-toxic dose for each individual. The rationale for the introduction of genetic testing in clinical practice is that on one hand the most effective therapy permits to avoid both the waste of time connected to the choice of therapeutic unfit regimens, and the psychological negative relapses on patient due to prescribed therapy inappropriateness; on the other hand it is possible to assess a priori the risk of adverse events and to avoid to administer drugs or too high doses that could damage the patient. However, pharmacogenetics has currently several limitations, among them; the most obvious is the lack of clinical validation of obtained data due to the heterogeneity and sometimes to the conflicts that emerges when the results reported in several studies are compared. This discrepancy may be due to several factors [15,17,18]: differences in study design (e.g. retrospective/prospective, low statistical power), survey unsuitable sample to evaluate the effect of low penetrance polymorphisms or to assess the importance of a haplotypic approach with the risk of generating false positives/negatives, differences in the clinical setting and treatment plan (e.g. dose and methods of administration of the drug, co-administration of other chemotherapeutic agents), heterogeneity in tumor pathology (e.g. stage of disease, tumor site, pre-treatment) and in clinical and demographic characteristics of patients (e.g. concomitant diseases, performance status, age, sex), inability to control environmental confusing factors (alcohol consumption, smoking, diet) and, finally, differences in experimental techniques employed for the determination of polymorphisms and in parameters and methods of measurement of clinical outcome. To identify efficient genetic markers of a specific chemotherapy treatment used in a particular clinical pathology, it is important, therefore, to manage multicenter, methodologically well defined prospective on pharmacogenetic studies using a population of patients adequately large and as uniform as possible from clinical, demographic and behavioural point of view. The integration of results from pharmacogenetic studies so structured is certainly an efficient strategy for reaching finally towards of tumor therapy and for getting from each treatment maximum effectiveness and minimal toxicity, choosing the most suitable drug and the optimal dose for the individual.

# 3. Colorectal cancer

Colorectal cancer (CRC) is the fourth most frequent cancer in the world and the second leading cause of cancer death in the United States [19]. In 2001, 135,400 new patients were diagnosed and 56,700 (of them) died of colorectal cancer. In particular, 50% of patients die during the metastatic phase of the disease. This neoplasia has a multifactorial etiology in which environmental, dietary and genetic factors contribute together to the development of the disease.

The majority of colorectal cancers, regardless of etiology, arise from adenomatous polyps and the patient's prognosis strongly depends on the degree of penetration of the tumor in the intestinal wall, on the involvement of regional lymph nodes or less and the presence of distant metastases [20]. For the majority of patients with metastatic colorectal cancer, the main therapy consists of the surgical removal of the tumor. Other treatments for this type of cancer include radiotherapy and chemotherapy [21].

The colorectal cancer presents a significant chemo-resistance: in particular, some platinum derivatives such as, cisplatin and carboplatin, have not been shown to be active in this tumor type, on the contrary, oxaliplatin has proved effectiveness in the treatment of such cancer and has been included in the current treatment regimens [22,23]. In the past, in chemotherapy of colon carcinoma was used 5-fluorouracil (5-FU) which, however, in monotherapy treatment, showed a very low percentage of response [24]. It is sought, therefore, to develop new drugs that could be used either alone or in combination with 5-FU so as to enhance the effects.

Today irinotecan and oxaliplatin [25,19] are used for the treatment of advanced-stage colorectal cancer, in addition to 5-FU, usually with folic acid (leucovorin, LV) [26], as a biochemical modulator. In IFL (Irinotecan, 5-FU and LV) regimen used in the United States, 5-FU is administered as a bolus [27], while in European regimen (FOLFIRI) by continuous infusion [28].

Moreover, many studies have shown, in terms of response and survival, the superiority of the combination of irinotecan with 5-FU/LV compared to 5-FU, leucovorin or irinotecan alone [29,30]. Oxaliplatin, when used as a single agent, has only a modest activity against colon cancer, while when it is used in combination with 5-FU/LV, has a higher antitumor efficacy, evidently for a synergy of cytotoxic effects. Oxaliplatin has thus become one of the most

important therapeutic agents in the treatment of colorectal cancer and it is today officially approved by the U.S. Food and Drug Admistration (FDA) for the treatment of this type of tumor, in combination with 5-FU and LV.

## 3.1 Oxaliplatin and neurotoxicity

### Pharmacokinetics of oxaliplatin

Oxaliplatin (Eloxatin®) is an antineoplastic agent currently used in association with 5-fluorouracil and leucovorin (FOLFOX) for the treatment of advanced-stage colorectal cancer [31]. It is a third-generation organoplatinum compound that was introduced in the European Union in 1999 and in the United States since 2002. The oxaliplatin molecular structure consists of a central platinum atom (Pt), surrounded by a 1,2-diaminocyclohexane group (DACH) and a bidentate oxalate ligand (Fig. 8).

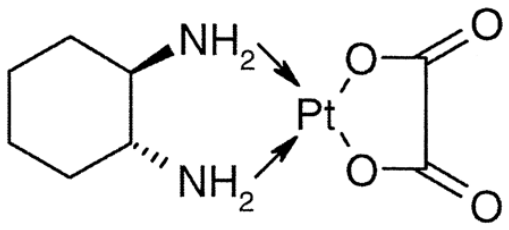


**Figure 8:** Molecular structure of oxaliplatin.

First, the pro-drug oxaliplatin is activated by conversion to monochloro, dichloro and diaquo compounds by non-enzymatic hydrolysis and displacement of the oxalate group (Fig. 9). The kinetics of hydrolysis differs among platinum compounds, being slower for oxaliplatin than for cisplatin [32].

The highly reactive monochloro, dichloro and diaquo intermediates react with sulphur- and amino groups in proteins, RNA and DNA. Its anti-tumor effects are thought to be related to the formation of Pt-DNA adducts.

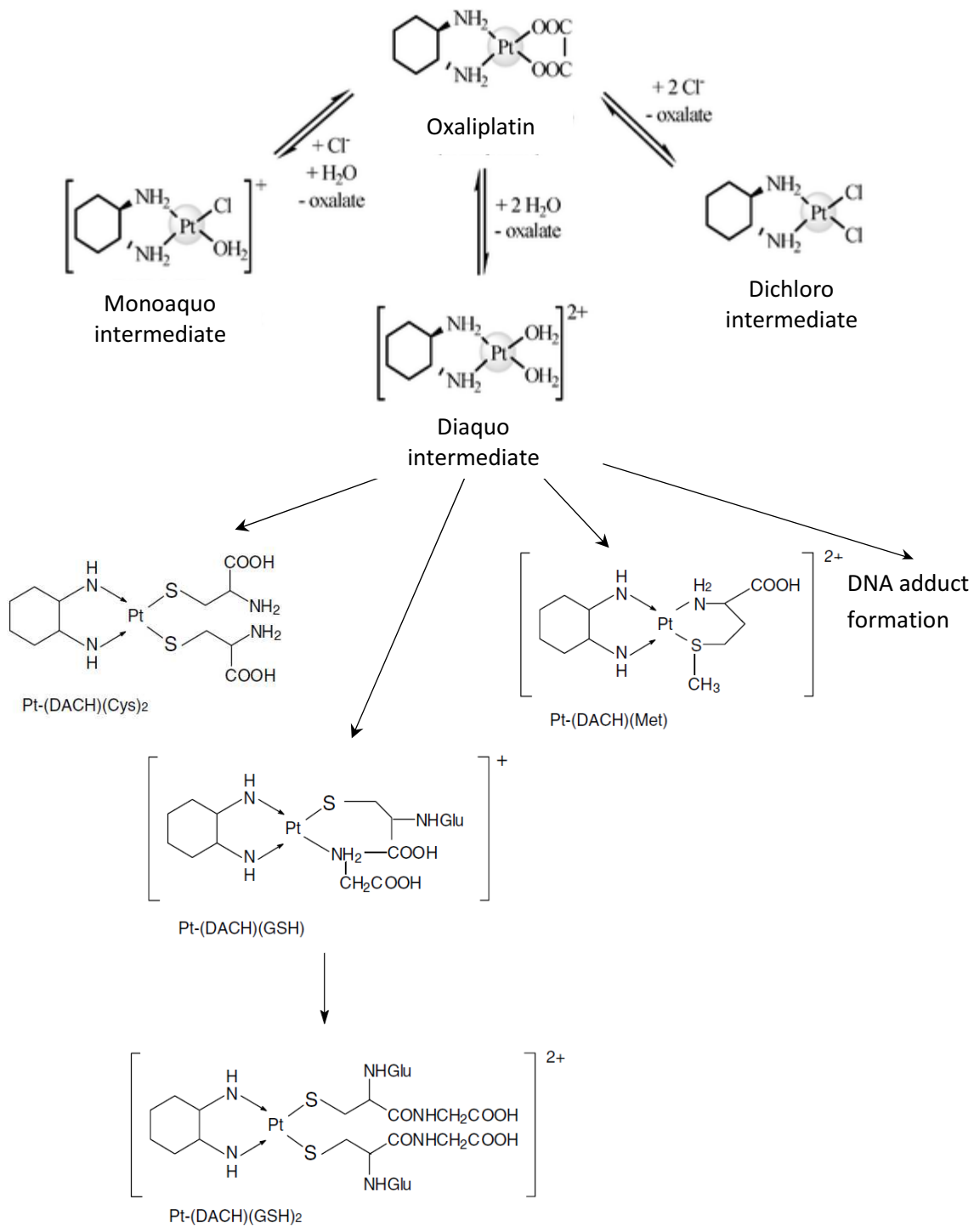


**Figure 9: Non-enzymatic hydrolysis reactions of oxaliplatin *in vivo* and *in vitro*.** The aquated derivates of oxaliplatin are considered to be the biologically active species, capable of adduct formation with various sulphide- and amino groups. Such groups are abundant in cellular DNA and biomolecules. Cellular detoxification processes competing with DNA-adduct formation include conjugation of the aquated compound to glutathione (GSH), methionine (Met) and cysteine (Cys). The conjugation products are subsequently excreted from the cell and eliminated from the body.

Other reactions include irreversible binding to biomolecules such as albumin, cysteine (Cys), methionine (Met) and reduced glutathione (GSH), which are in fact the first steps of *in vivo* biotransformation and cellular detoxification [33].

Oxaliplatin species are widely distributed among various tissue sites and free platinum is eliminated from the body, mainly by renal clearance. Unbound platinum fraction is eliminated from circulation by irreversible bonds with plasma/blood constituents and then is excreted through urine. The platinum irreversibly bound to plasma proteins and erythrocytes is generally considered pharmacologically active.

## *Pharmacodynamic of oxaliplatin*

The cytotoxic activity of oxaliplatin is initiated by formation of a DNA adduct between the aquated oxaliplatin derivative and a DNA base. The adducts are preferentially formed with N-7 positions of guanine and adenine and in most cases these reactions result in intrastrand crosslink's. In general, the cytotoxic efficacy of platinum compounds in cancer cells can be related to inhibition of DNA synthesis or to saturation of the cellular capacity to repair Pt-DNA adducts. Platinum atoms modify the three-dimensional DNA structure, which inhibits the normal DNA synthesis and –repair processes. Platinum *trans* adducts are 30 times less toxic than *cis* adducts due to more effective repair [34]. Despite the fact that oxaliplatin forms only *trans* adducts, it induces more efficient or different damage since a 10-fold lower adduct ratio was found relative to cisplatin at an equitoxic dose of oxaliplatin [32]. In addition, more DNA strand breaks are formed with oxaliplatin compared to cisplatin.

Compared to the amino ligands of both cisplatin and carboplatin, the DACH ligand of oxaliplatin is remarkably bulkier and more hydrophobic. In contrast to other DACH complexes however, oxaliplatin shows relatively good water solubility. Together, these properties result in a greater deformation of tumor DNA by steric hindrance of adduct formation and this may explain the more effective inhibition of DNA synthesis by oxaliplatin as compared to, e.g., cisplatin.

## *Neurotoxicity*

At clinically recommended doses, oxaliplatin is reported to be less toxic than cisplatin and carboplatin to auditory, hematological and renal systems. However, one of the most common adverse effects associated with oxaliplatin administration is neurotoxicity. The latter is of two types: acute and chronic. It has been suggested that the acute neurological symptoms reflect a state of peripheral nerve hyperexcitability that likely represents a transient oxaliplatin-induced impairment of ion channels, while the chronic treatment induces an axonal neuropathy similar to the other platinum-based drugs [35].

*Acute neuropathy:* it is related to the amount of the individual dose and is more likely to occur with higher dose. It is not a dose-limiting toxicity and is usually triggered by cold exposure [19]. It is an acute transient syndrome that may begin during the infusion, within minutes to hours, or within 1-2 days of administration but is usually self-limiting, often disappearing within days [36,37]. Signs and symptoms include paraesthesia, hypoaesthesia, and synaesthesia, usually beginning in the hands or feet, but sometimes occurring around the mouth or in the throat. The neuropathy is at times associated with shortness of breath or difficulty in swallowing, but without bronchospasm, laryngospasm, wheezing, or stridor. Patients have also experienced an unusual sensation in the tongue, jaw spasms, eye pain, and muscle spasms or cramps, which are sometimes described as stiffness in the hands or feet or the inability to release the grip. Acute neuropathy may be triggered by exposure to cold temperatures and often returns on retreatment [37].

*Chronic neuropathy:* the signs and symptoms of chronic oxaliplatin neurotoxicity are cumulative, and are usually similar to the acute signs and symptoms, and usually consist of paraesthesia, hypoaesthesia and dysaesthesia, in addition to changes in proprioception that do not resolve between cycles, and can affect fine motor coordination, such as writing, holding objects, picking up coins, buttoning shirts and walking [31]. It is a dose-limiting toxicity [38] but does not seem to be schedule-dependent [37]. A review of several trials with patients treated with oxaliplatin revealed that neuropathy associated with functional impairment occurred in approximately 10% of patients who receive a median cumulative dose of 780 $mg/m^2$ and in 50% of those who receive a median cumulative dose of 1170 $mg/m^2$ of the drug [39]. Rare central neuropathy, characterized by Lhermitte's sign (an electric sensation experienced with flexing of the neck), proprioception deficiencies, and urinary retention,

have also been reported [40]. Chronic neuropathy gradually resolves over several months after therapy is discontinued [40,41]. This type of neuropathy has been shown to be exacerbated immediately after surgery in about 58% of treated patients [19,37].

## *Mechanism of oxaliplatin neurotoxicity*

The exact mechanism of oxaliplatin-induced neurotoxicity is unknown. Adelsberger et al. [42] demonstrated that oxaliplatin causes a prolonged opening of sodium channels in some sensory nerves resulting in a hyperexcitable state. The disruption of ion channels is sometimes referred to acute channelopathy and has been suggested as the cause of acute oxaliplatin-induced neuropathy [35]. Grolleau et al. [43] concluded that oxaliplatin was capable of altering the voltage-gated sodium channels through a pathway involving calcium ions probably immobilized by its metabolite oxalate. Finally, Gamelin et al. [44] established that $Ca^{2+}/Mg^{2+}$ infusions seem to reduce the incidence and the intensity of acute oxaliplatin-induced symptoms and might delay cumulative neuropathy, especially in 85 $mg/m^2$ oxaliplatin dosage. Patients treated with oxaliplatin excrete oxalate and cations after drug infusion, accompanied by increased excretion of four amino acids (glycine, serine, alanine and taurine) that are linked to oxalate metabolism. Glycoxylate aminotransferase genotyping was suggested to be employed before the start of therapy to predict patients who may be more susceptible to neurotoxicity [45]: it was demonstrated indeed that the minor haplotype of the enzyme glycoxylate aminotransferase is significantly related with both acute and chronic neurotoxicity.

The biochemical mechanism of “channelopathy” has been associated both with acute and chronic neurotoxicity. Instead chronic neurotoxicity was selectively associated with morphological damage linked to reduced speed of conduction and changed sensory potential. The drug seems to exert its damaging action on peripheral neurons through its accumulation in distal ganglia of nervous system with consequent morphological and functional alteration or loss of the constitutive cells of the same nervous ganglia [46].

## Clinical management of oxaliplatin-induced neurotoxicity

Various strategies have been proposed to prevent or treat oxaliplatin-induced neurotoxicity:

1. *Stop-and-go* strategy: "Stop" after either an appearance pre-defined cumulative oxaliplatin or a development of a critical sensory neurotoxicity of a certain grade; and "Go" when either sensory neurotoxicity has regressed or when oxaliplatin therapy is required to stop tumour progression [38]. This strategy uses the predictability and reversibility of neurologic symptoms to allow patients to stay on an oxaliplatin-containing first-line therapy for a prolonged period [47].
2. *Neuromodulatory agents*: these include agents such as $Ca^{2+}$ and $Mg^{2+}$ infusion; antiepileptic drugs like carbamazepine, gabapentin, and venlafaxine; amifostine; $\alpha$-lipoic acid, and glutathione. $Ca^{2+}$ and $Mg^{2+}$ solutions may be a good first choice to prevent and treat acute oxaliplatin neuropathy. They offer clinical activity, availability, and the convenience of requiring administration only on the treatment day. Gabapentin is a second choice. It offers the dual advantage of treatment and prevention of the signs and symptoms of neuropathy, but requires daily administration until oxaliplatin therapy is completed. A third choice is subcutaneous administration of amifostine. It offers the advantages of administration on treatment days only and greater safety than intravenous route, but does not appear to be as effective as gabapentin [31].

The predictability of neurotoxicity associated with oxaliplatin-based therapy should allow patients and physicians to develop strategies to manage this side effect in view of the individual patient's clinical situation.

## Pharmacogenetics of oxaliplatin

Several mechanisms, that confer decreased sensitivity or resistance to oxaliplatin, including diminished cellular drug accumulation, increased intracellular drug detoxification and increased Pt-DNA adduct repair, are described.

The cellular uptake of platinum is not completely understood but there is evidence that decreased accumulation is the most common mechanism of resistance to cisplatin. The uptake of platinum by cells is an energy requiring process, but it is not saturable and possibly involves transport by a yet unidentified efflux pump [48]. Once inside the cell, conjugation to

glutathione (catalyzed by the enzyme glutathione-S-transferase, GST) effectively inactivates platinum compounds before DNA damage is induced. This conjugation reaction is followed by cellular excretion and is therefore related to cellular drug resistance as well.

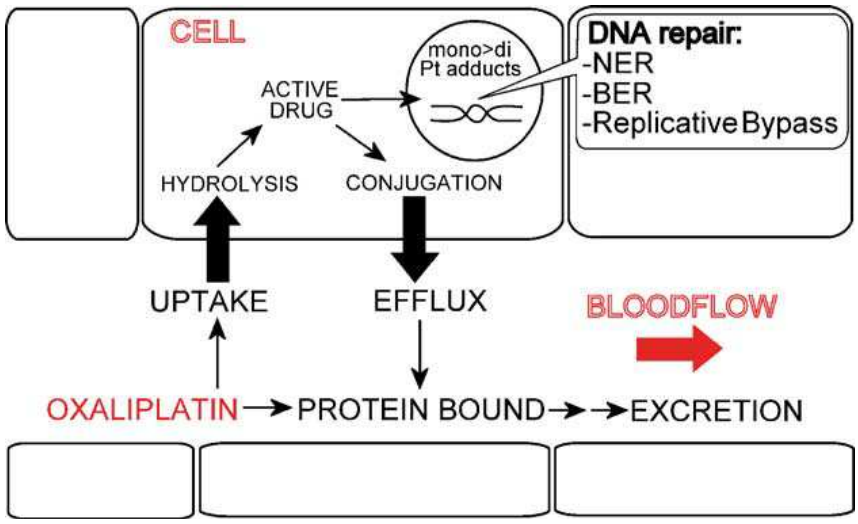


**Figure 10: Schematic view of cellular defence mechanisms involved in oxaliplatin resistance.** Cellular uptake and efflux determine the level of oxaliplatin in intracellular fluid. In plasma, extracellular conjugation of oxaliplatin to plasma proteins (mainly albumin) results in renal excretion of inactive drug species. Once inside the cell, the oxaliplatin prodrug is hydrolyzed to monochloro-, dichloro and diaquo active species which form DNA adducts. Intracellular conjugation to glutathione effectively inactivates these highly reactive oxaliplatin-species before DNA damage occurs, followed by cellular excretion into plasma. DNA damage is repaired by nucleotide excision repair (NER), base excision repair (BER) and replicative bypass.

Platinum-induced DNA adducts can be repaired by several enzyme systems: base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR). Induction of the enzymes involved in these systems results in increased DNA repair activity, more efficient adduct removal and hence decreased sensitivity to platinum drugs (Fig. 10).

There is growing evidence that common gene variants (polymorphisms) affect the activity of cellular DNA repair and platinum conjugation. Such polymorphisms in genes coding for enzymes involved in oxaliplatin accumulation, detoxification and Pt-DNA repair may influence cellular response to oxaliplatin [49]. The effects of polymorphisms in DNA repair and inactivation of oxaliplatin through conjugation with glutathione are described below.

# 4. Prostate cancer

Prostate cancer is now the most common cancer in humans. Worldwide over 660000 new cases are diagnosed each year, 42800 of which are diagnosed in Italy. Prostate cancer is predominantly an old age disease and is rare before the age of 40-50 years. Autopsy studies worldwide have shown that histological disease increases with age and that roughly three-quarters of men older than 80 years will have some evidence of latent disease [50]. Prostate cancer includes, several forms from slow-growing ones also known as “indolent”, which cause no significant problems during the whole life of the patient to other more aggressive forms which, by contrast, grow rapidly beyond the limits of the gland and they can spread through the blood and lymphatic system to other parts of the body, thus giving rise to the spread of cancer in other areas, i.e. to metastasis. The real causes of prostate cancer are unknown, however some risk factors and some conditions that increase the probability of occurrence were identified. The “certain” risk factors are familiarità and the age. Another significant risk factor is membership of the African-American ethnicity: in this population the incidence is higher. Moreover, it seems that the probability of getting sick can increase in the presence of certain conditions such as inflammation of the prostate (mainly chronic and recurrent prostatitis), chronic or recurrent, the high values of male hormones, exposure to environmental pollutants, smoking, obesity and a diet rich in animal fats (particularly dairy products and red meat) and low consumption of fruits and vegetables.

In the 1980s the PSA test has been introduced in the treatment of prostate cancer as diagnostic factor. The PSA is a glycoprotein of callicreine produced by prostate cells and periurethral glands. PSA (prostate-specific antigen) in the bloodstream binds to protease inhibitors, such as antichymotrypsin (ACT) and $\alpha$2-macroglobulina (MG) and in a negligible part at $\alpha$1-antitrypsin and inter-$\alpha$-trypsin. Plasma concentration of PSA is about 1,000 times smaller than that of the seminal fluid and is found mainly in complexed or free inactive form. PSA values less than 4 ng/ml are considered normal.

Once the presence of the tumor has been confirmed, some clinical pathological indicators are used to analyze the aggressiveness of the tumor. The characteristics of cancer cells under the microscope are evaluated through the "grading", describing their degree of differentiation and is quantified by the Gleason's score (which ranges from 2 to 10). The

lower Gleason score, the less aggressive is the cancer. Staging allows you to assess the extent of the tumor. The used method used is the TNM system that indicates the extent of the tumor (T), the lymph node involvement (N), and any distant metastases (M). If the cancer is found only in the prostate gland, is defined as "localized" (T1/T2); if it has passed the prostatic capsule or invaded the seminal vesicles is defined as "locally advanced"; if there is tumor metastasis to lymph nodes or organs or bones, it is called "Advanced" or "metastatic". In relation to the different characteristics of the tumor and the PSA value it is possible to define the "hazard classes", on the basis of which focus therapeutic strategies.
Although several clinicopathologic indicators, such as prostate-specific antigen level (PSA), Gleason score, pathologic stage, and surgical margin status, are currently used to predict therapy outcome, there is a need to find new biomarkers to improve the prediction of disease recurrence such as DNA-based genetic biomarkers that will the primary object of study of this thesis.

## *4.1 Treatments of prostate cancer*

### *Radiotherapy*

The radiation treatment may be either radical or adjuvant.

- Radical: if it is the definitive treatment; it will start a radical radiotherapy program. The planned dose: 76 Gy to 80 Gy in 38 to 40 fractions.
- Adjuvant: the treatment will begin no earlier than 3 months after surgery (EORTC), the dosage will be provided from 66 Gy to 70 Gy in 33 to 35 fractions.

Radiotherapy in prostate cancer can cause many side effects:

- adverse effects on the rectum with an increase in intestinal peristalsis: sometimes the anal region may be irritated and sore;
- cystitis: urination may become more frequent with a burning sensation. This effect usually disappears gradually within a few weeks after the end of treatment. In some cases, to avoid difficult urination, catheterization is required.
- fatigue: can also be highly disabling, depending on the intensity of the irradiation dose and duration of treatment.

Most of side effects of radiotherapy gradually disappear at the end of the course of therapy, although some may last for several months and others may be permanent.

Radiotherapy may also cause long term side effects:

- sexual impotence;
- permanent damage to the bowel or bladder: the blood vessels supplying the bowel and bladder may become weakened after radiation treatment, with the appearance of traces of blood in the urine or faeces;
- Urinary incontinence.

## *Surgery*

There are three types of surgical treatment for prostate cancer:

- radical prostatectomy;
- transurethral resection (TUR), to relieve symptoms;
- orchiectomy (removal of the testicles)

Patients enrolled in this study are only subjected to radical prostatectomy. It is one of the therapeutic options for prostate cancer treatment in early stage. This procedure is usually done only in cases where the cancer has not spread to other organs and in patients younger than 70 years. Sometimes surgery can cause sexual impotence, the inability to have an erection, and urinary incontinence, although modern surgical techniques have reduced the frequency of such complications. Impotence is caused by reduced blood flow to the penis as a result of impairment of the arteries or nerves. The risk of impotence after radical prostatectomy is 50% in patients aged less than 60 years, and reaches 70% or more in those over 70 years. The problems of incontinence after radical prostatectomy are less frequent. An year after surgery about 25% of subjects undergoing radical prostatectomy will have occasional loss of urine, and less than 1% will have assistance such as a catheter.

The most important side effects of radical prostatectomy are urinary incontinence and difficulty or absence of erection.

### *Hormone therapy*

In the hormone treatment analogues alone and anti-androgens alone or combined are accepted. The hormonal treatment can be used as neoadjuvant, concomitant or adjuvant therapy. Hormone therapy will be continued for two years after radiotherapy in very high risk patients. In this therapy the following drugs are used:

a. Analogs and antagonists of GnRH (Gonadotropin Releasing Hormone), the hormone that stimulates the gonadotropins: these drugs block the action of hormones that stimulate the production of testosterone by the testes. They are administered by intramuscular or sottodermica injection monthly or quarterly. The analogues most used are: Goserelin (Zoladex®), Leuprorelin (ENANTONE®, Eligard®), and Triptorelin (Decapeptyl®); the antagonist is Degarelix (Firmagon®).
b. Antiandrogens: these drugs bind to proteins or receptors present inside the cancer cells, thereby preventing the stimulating action of testosterone. They are often administered in tablets to be taken daily, also in combination with GnRH analogues. The most common are: Bicalutamide (Casodex®) and Flutamide (Drogenil®, Eulexin®).

Possible side effects during hormonal therapy are: hot flushes, excessive sweating, fatigue, decreased muscle mass, weight gain, mood disorders, anemia, osteoporosis, loss of libido and erectile dysfunction, increased fatigue, both physical and mental.

## *4.2 Radiogenetics*

Since the discovery of radiation at the end of the 19$^{th}$ century, radiotherapy has become one of the most important modalities for cancer treatment. When radiation interacts with tissue energy, deposition along a track causes ionizations with a clustering of ionizations at radiation track ends. Ionizations produce free radicals, which are highly reactive and can break chemical bonds. The most harmful effect to cells is damage to DNA, like single-strand breaks, double-strand breaks (DSBs) and DNA crosslinks (Fig. 11). DBSs are the most important lesions as they are harder to repair than other DNA lesions because the two DNA ends can separate, and accompanying base damage hampers DSB ligation [52]. There are two main pathways for repairing DSBs: non-homologous end joining (NHEJ) and homologous

recombination (HR) [53]. Radiation causes other types of damage. Formation of reactive oxygen species (ROS) and reactive nitric oxide species (RNOS) induce stress responses, inflammation, and release of cytokines, growth factors and chemokines [54,55]. The finding that ATM is also involved in protecting cells from ROS accumulation by stimulating NADPH production and promoting the synthesis of nucleotides for DSB repair highlights the complex interplay between multiple molecular radiation responses [56]. When radiation interacts with tissue, ionizations produce free radicals that damage DNA. Cells respond by undergoing cell cycle arrest to allow DNA damage repair. Most damage is repaired, but unrepaired damage causes cell death via necrosis or apoptosis.

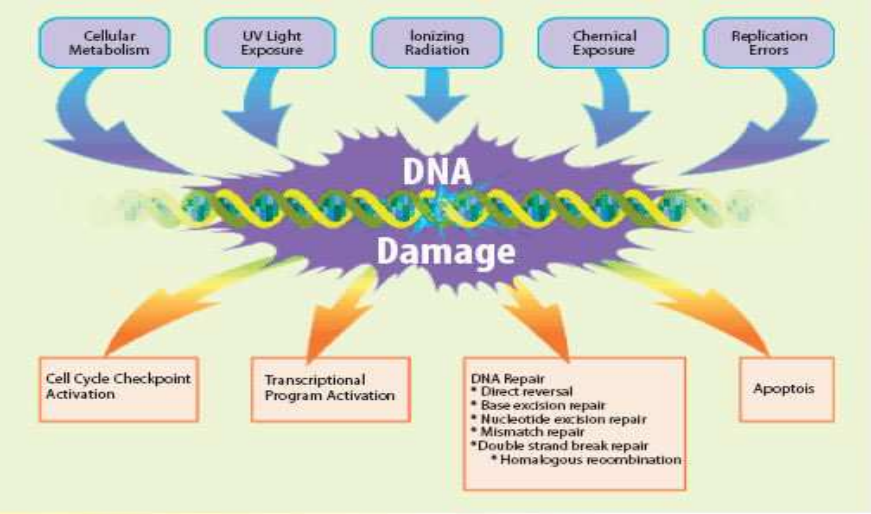


**Figure 11: DNA damage.** Different pathways that result in changes in DNA and the response of the body to damage.

There is evidence that, in addition to patient-related factors such as age and life-style factors, these patient-to-patient differences are attributable to a genetic basis. The genome of some people is more unstable than others and individuals with cancer-prone conditions associated with genomic instability, such as ataxia telangiectasia, tend to be radiosensitive. It was therefore hypothesized that common genetic variants with modest functional effects (low penetrance variants) cause the bulk of the unexplained inter-individual variability. Radiosensitivity is thus suggested to be a complex, polygenic trait which results from the interaction of a number of genes in different cellular pathways [57,27]. These pathways include genes related to DNA damage induction and repair, apoptosis, pro-fibrotic and inflammatory

cytokines, endogenous antioxidant enzymes, as well as to general metabolism and homeostasis (Fig. 12).

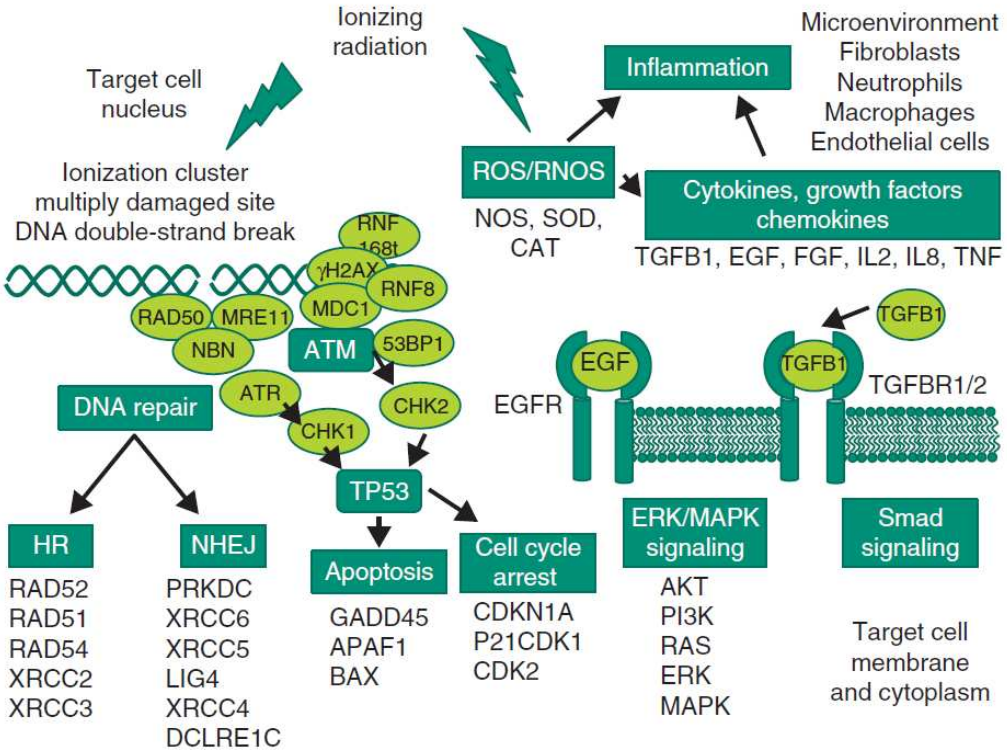


**Figure 12: Summary of the pathways and mechanism involved in cell and tissue response to radiotherapy.** The interaction of ionizing radiation with tissue leads to multiple types of DNA damage (i.e. base damage, single-strand breaks, double-strand breaks). Double-strand breaks are harder to repair and are the most important DNA lesion induced by radiation. Radiation also produces reaction oxygen (ROS) and nitrogen (RNOS) species that simulate cytokine, growth factor and chemokine responses. There are multiple interconnected signaling networks that respond to radiation damage that can lead to cell death, cell senescence, genomic instability, mutations and inflammatory response.

A major challenge is to identify the combination of multiple low-penetrance genetic variants (single nucleotide polymorphisms, SNPs) which affect the complex cellular and clinical phenotype and may therefore be useful as potential biomarkers to predict normal tissue response after radiotherapy [58,59].

# 5. Genetic polymorphisms

## DNA repair

Since the primary anti-tumor mechanism of oxaliplatin is the formation of Pt-DNA adducts ultimately leading to cell cycle arrest and apoptosis), polymorphisms in genes involving the repair mechanisms of these adducts, such as *base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), and double-strand break repair (DSBR)* are investigated in radiogenetics studies (Fig. 13).

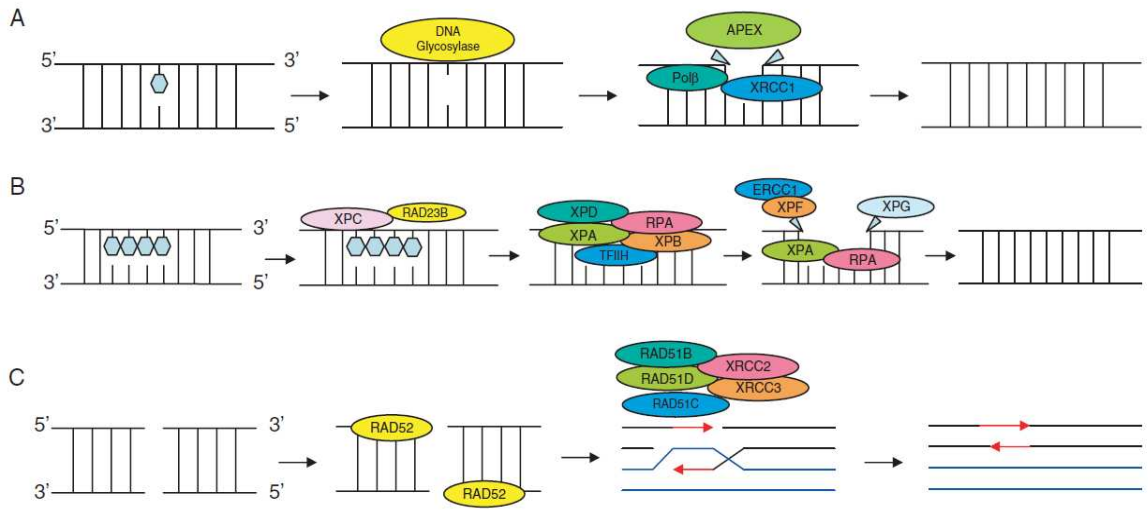


**Figure 13: A) BER (base excision repair)** pathway targets DNA damaged during replication or by environmental agents. The single damaged base in DNA caused by endogenous metabolism or environmental oxidizing agents results in DNA adducts. BER involves removing the mutated base out of the DNA and repairing the base alone. **B) NER (nucleotide excision repair)** is associated with the repair of bulky adducts induced by several suspected environmental prostate cancer carcinogens. The NER pathway is a complex biochemical process that requires at least four steps: 1) damage recognition by a complex of bound proteins including XPC, XPA and RPA; 2) unwinding of the DNA by TFIIH complex that includes XPD (ERCC2); 3) removal of the damaged single stranded fragment (usually about 27-30bp) by molecules including an ERCC1 and XPF complex and XPG; and 4) synthesis by DNA polymerases. **C) DSBR (double strand break repair)** is induced by replication failure or by DNA-damaging agents. Two repair pathways exist to repair double strand breaks. The homologous recombination repair relies on DNA sequence complementarity between the intact chromatid and the damaged chromatid as the bases of stand exchange and repair.

- *Base excision repair (BER)*: it repaired single-strand breaks resulting from exposure to endogenously produced active oxygen, ionizing radiation or alkylating agents.

**hOGG1** *(oxoguanine glycosylase 1)* is the glycolase involved in the excision of 7,8-dihydro-8-oxoguanine, a common oxidized form of guanine [52]. It has various polymorphisms, among which the most studied is 1245 C>G which leads to amino acid substitution of a serine with a cysteine at codon 326 (Ser326Cys). The allele frequency of this polymorphism is 22-45% [53] according to the population considered, in particular, in Caucasians rate is 22%. This polymorphism is responsible for decreased activity of hOGG1.

**XRCC1** *(X-ray repair cross-complementing group 1)* contains a domain which functions as a protein-protein interface that interacts with poly(ADP-ribose)polymerase (PARP). PARP is a zinc finger-containing enzyme that detects strand breaks and subsequently removes proteins from the DNA helix, which in turn becomes more accessible for repair enzyme [54]. The interaction between XRCC1 and PARP allows not only the recognition and repair of DNA damage but also an investigation of its entities and therefore the opportunity to activate repair mechanisms, or, in case the damage is particularly extensive, of activate apoptotic mechanisms [55]. For the XRCC1 gene have been described three polymorphic variants: 28 152 G> A in exon 10 leading to amino acid substitution of an arginine with a glutamine at codon 399 (Arg399Gln), 26304 C>T in exon 6 which leads to the replacement amino acid of an arginine with a tryptophan at codon 194 (Arg194Trp) and 27466 G>A in exon 9 which leads to the amino acid substitution of an arginine with a histidine at codon 280 (Arg280His) [56]. The polymorphisms Arg194Trp and Arg280Hys have an allele frequency respectively of 6-9% and 5% in the Caucasian population [53]. The Arg399Gln polymorphism, instead, presents an allele frequency of 32-36% in the Caucasian population.

This polymorphism plays a role in tumor response to platinum-based chemotherapy: *Stoehlmacher et al.* [55] have studied a population of 61 patients with advanced colorectal cancer and treated with 5-FU and oxaliplatin. They found a significantly higher proportion of responders among the homozygous wild-type genotype patients (8/25) than among the other patients (3/36). Patients with at least one of the mutant alleles have a more than fivefold risk of combined oxaliplatin/5-FU chemotherapy failure compared to patients with two wild-type alleles. It would therefore appear that this polymorphism is associated with a resistance to the platinum derivatives although, in theory, an inefficient repair of platinum-DNA adducts should on the contrary increase the cytotoxic power of the same drugs. The apparent contradiction may be explained by assuming that a sub-

optimal level in tumor DNA repair leading to an increased accumulation of mutations and accordingly render the same more aggressive tumor. Recently, associations between genetic variants in XRCC1 and the risk of developing cancer, cancer progression, or adverse reactions after radiotherapy have been reported [27,58]. A clinical study conducted by Popanda et al, aimed at defining the impact of the variant XRCC1 Arg399Gln in the radiotherapeutic treatment modulation, shows that heterozygous individuals had a higher risk of toxicity compared to Gln carriers [59]. In another study conducted by Gao R et al [60], patients possessing at least one variant allele A of Arg399Gln and wild-type CC of Arg194Trp had significantly longer survival time after radiotherapy, while patients having at least one wild-type allele G of Arg399Gln and the heterozygous genotype CT of Arg194Trp had shorter survival time.

**APE1** *(apurinic/apyrimidinic endonuclease 1)* is an important enzyme in the BER pathway which is responsible for the repair of DNA damaged by oxidation/alkylation; it protects therefore cells against the effect of endogenous and exogenous agents. A total of 18 polymorphisms have been reported, but the most studied is APE1 2197 T>G (Asp148Glu) [61].

- *Nucleotide excision repair (NER)*: is a pathway involved in the recognition and repair of damaged or inappropriate nucleotides. A wide variety of DNA damages is repaired by NER, including UV-induced photoproducts, helix-distorting monoaducts, cross-links and endogenous oxidative damage [62].

  **XPD** *(Xeroderma Pigmentosum Group D)* encodes for an helicase that allows the opening of the double-stranded DNA and facilitates the accessibility for the endonucleases in charge of the excision. Several polymorphisms have been described: 23591 G>A in exon 10 (Asp312Asn), and 35931 A>C in exon 23 (Lys751Gln). The variants Asp312Asn and Lys751Gln have a frequency of allelic variant of 33-44% and 6-42% respectively by ethnicity; in the Caucasian population the two variants have an allelic frequency of around 30% [63]. In a study involving 73 patients receiving combination chemotherapy, with Oxaliplatin and 5FU, for metastatic colorectal cancer, the effect of the XPD polymorphism on response and survival was examined. Only 10% of patients with the lysine/glutamine (A/C) or glutamine/glutamine (C/C) genotypes achieved an objective response compared to 24% of patients with the lysine/lysine (A/A) genotype. Median survival in the A/A group was 17.4 months compared to 12.8 months for the A/C heterozygous group and 3.3

months for the C/C homozygous group [64]. The lysine variant in the XPD gene has been seen to cause decreased DNA repair capacity in some studies and increased capacity in others [58]. Regarding the polymorphism Asp312Asn, in the study of Gurubhagavatula [59], it has been observed that patients with 312 Asp/Asp genotype have a longer survival as compared those with the allelic variant 312Asn.

**ERCC1** *(Excision repair cross-complementation group 1)* consists of 297 amino acids and appears to harbor a nuclear localization signal and a domain characteristic of a DNA-binding protein. In normal individuals, a common polymorphism at exon 4 can be found at codon 118 (19007 C>T), changing AAC into the less used AAT codon. This is a conservative nucleotide change, as both codons result in the same amino acid (Asn) being incorporated into the protein. The allelic variant 118T has a frequency allelic particularly high both in the healthy population (up to 61%) than in the pathological (55-60%) [61]. This polymorphism, even if classified as silent, was found to be associated with decreased ERCC1 expression in ovarian cancer cells, but, contrasting, with increased intratumor levels of ERCC1 in 32 patients with metastatic colorectal cancer treated with 5-FU/oxaliplatin chemotherapy [63]. Shirota et al. [64] reported that increased expression of ERCC1 is associated with a lower survival and vice versa. In another study of 91 patients with metastatic colon rectal cancer treated with 5-FU/oxaliplatin, it was found that individuals with 118TT genotype have a higher responsiveness to therapy [65]. A further polymorphism of ERCC1 is 8092 A> C in the 3'-untranslated region of the gene: it presents an allele frequency of 27% and assumes ports to an alteration of mRNA stability and, therefore, the expression of ERCC1. Even this polymorphism, therefore, can have a good predictive value on the outcome of a therapy with platinum derivatives.

- *Mismatch repair (MMR)*: is a pathway that corrects base impairs and small strand loops that occur during replication.

  **hMSH2** (human MutS homolog 2) presents several polymorphisms among which GIVS12-6T>C in position -6 of the intronic site in the 3' splice acceptor of exon 13 with a allelic variant frequency in the Caucasian population of approximately 5%. This polymorphism has been associated with an uncreated sensitivity to DNA damaging agents.

  **hMLH1** (human mutL homolog 1) presents various polymorphisms among which the most studied is 676 A>G in exon 8 (Ile219Val) with a frequency of variation of approximately 31% in Caucasians: this is a highly conserved residue and the amino acid variation due to a

change of polarity of the same residue [66]. This polymorphism has been noted to lead to both topoisomerase inhibition and alkylating agent resistance [67].

- *Double strand break repair (DSBR)*: DNA double-strand break (DSB) is one of the most serious forms of damage induced by ionizing irradiation. Genome stability in response to DSBs is maintained principally through the two evolutionarily conserved DNA repair pathways, non-homologous DNA end joining (NHEJ) and homologous recombination (HR) [68]. HR is known to play a crucial role in repairing one-ended DSBs arising in S-phase when the replication fork encounters single-stranded breaks or base damages.

  **XRCC3** *(X-ray repair cross-complementing group 3)*: a polymorphic variant 18067 C>T in exon 7 (THR241Met), associated with reduced repair activity of the enzyme, has been described for the XRCC3 encoding gene [69]. The phenotypic effect of this polymorphism on the enzyme is related to *in vitro* increased cellular radiosensitivity [70]. Another variant, XRCC3 4541 A>G, was described. An increased clinical late rectal toxicity after radiotherapy in patients carrying 4541G variant has been highlighted in patients with prostate cancer.

  **RAD51** is likely that polymorphic variants of the encoding gene may have an effect on the outcome of radiotherapeutic treatment. In fact, several studies *in vitro* and *in vivo* have shown that altered levels of RAD51 expressions may influence cell susceptibility to ionizing radiation, with a possible consequent impact on radiotherapy clinical outcome [71]. The polymorphic variant considered for this gene is 135 G>C in the 5' untranslated region of the gene. Although the biological effect of this polymorphism is still unclear, this is hypothesized to affect mRNA stability and/or the efficiency of the translation process, thus impacting the expression level of the protein and possibly modulating the effect of radiation therapy [72].

## *Ataxia telangiectasia mutated*

The Ataxia telangiectasia mutated **(ATM)** contributes to DNA damage signaling and cell cycle control and has been historically related to radiosensitivity. Individuals with alterations, such as deletions or stop codons in the ATM gene, are highly sensitive to radiation. In prostate cancer patients, the presence of ATM sequence alterations was found to be associated with

a radiotherapy-related decline of erectile function, in a pilot study with 37 patients affected by prostate cancer [73]. This association is confirmed in another study with 35 prostate cancer patients [74].

## *Glutathione S-transferase*

Glutathione S-Transferase (GST) is a family of enzymes that are involved in detoxification of many xenobiotics, including anticancer drugs (oxaliplatin), and the polymorphisms encountered in the various enzymes may be both associated with a modification in cancer risk and with the anticancer treatment efficacy (Fig. 14) [75]. These enzymes catalyse the conjugation of a glutathione moiety with electrophilic, hydrophobic compounds, in order to increase their water solubility and facilitate their excretion. They also appear able to detoxify the free radicals formed by the action of certain drugs as well as ionising radiations. Any individual variation in enzymes or factors that affect the level of reactive oxygen species after radiation are thus likely to be significant in the response to RT and may influence the patient's prognosis after treatment, as well as the level of long-term side effects [76].

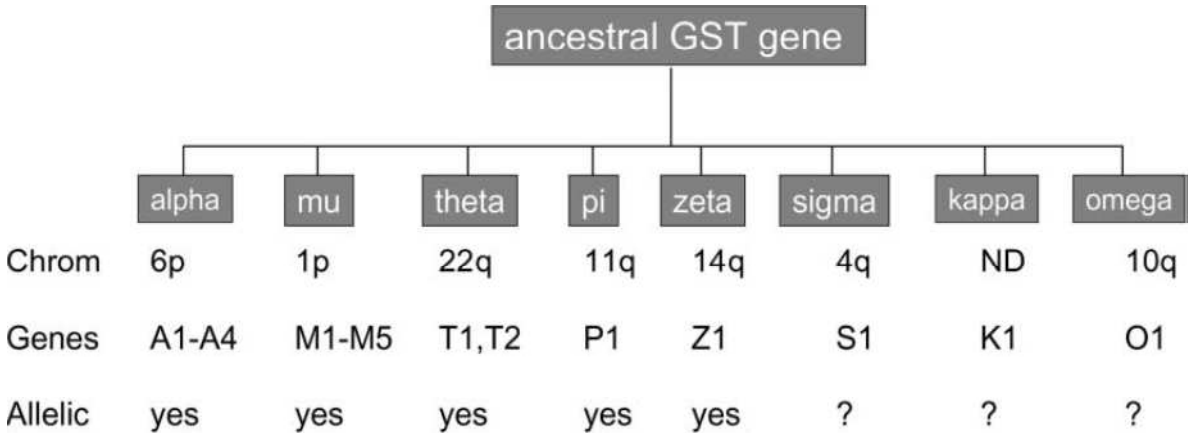


**Figure 14:** The glutathione-*S*-transferase supergene family.

Several isoforms have been identified, with different substrate specificities, and are mainly distributed between alpha (α), mu (μ), pi (π), theta (θ) and zeta (ζ) classes.

- **GSTM1** *(glutathione S-transferase mu)* and **GSTT1** *(glutathione S-transferase theta)* are characterized by a polymorphism resulting in the deletion of the gene, with consequent loss of the enzyme activity that is no longer expressed. Subjects, who are homozygous

variants in both genes, have reported an increased risk of developing various forms of cancer including that colon rectum. GSTM1 and GSTT1 polymorphisms are characterised by the complete loss of enzyme activity in 50% and 20% of Caucasians, respectively [77,78].

- **GSTP1** *(glutathione S-transferase pi)* presents at least two functional polymorphisms; the first one, 313 A>G (Ile105Val), with a high frequency (30%), results in a substantial decrease in enzyme activity for several classes of substrates [79]. The second one, 341 C>T (Ala114Val), is much less frequent (10%). Stoehlmacher et al. have shown that the one mutant allele (Ile/Val) is less potent in detoxification of carcinogens and individuals with two mutant alleles (Val/Val) have shown a significant survival benefit from combined oxaliplatin/5-FU treatment with a median survival of 24.9 months compared to only 7.9 months for metastatic colorectal cancer patients with two wild-type alleles [80].
- **GSTA1** *(glutathione S-transferase alpha)* consists in several combined SNPs in the proximal promoter region of the gene and determines the existence of two alleles, *A and *B, with allele frequencies of 60% and 40% respectively.
- **GSTM3** *(glutathione S-transferase mu)* in particular the allele GSTM3*B presents a deletion of 3bp in intron 6 which is related with the formation of a new binding site for Yin Yang 1 (YY1) transcription factor.

## *Superoxide dismutase*

Superoxide dismutase are a class of proteins associated to oxidative stress. They may have a role in neurotoxicity by oxaliplatin as are the basis of several species of neuropathies also of iatrogenic type. It is an antioxidant enzyme that is a major defense system against reactive oxygen species (ROS), particularly superoxide anion-radical. Of this enzyme three isoforms (SOD1, SOD2 and SOD3) are known.

- **SOD2** *(superoxide dismutase 2)*: the polymorphism most studied is 47 C> T (Ala16Val) and has a frequency of 41-55% of the allele variant. It falls into the signal sequence of the protein and produces a conformational change of the helical structure which is thought to lead to a decrease of efficiency of transport of SOD2 in the mitochondria, the seat of the enzyme activity [81]. This polymorphism seems to have a role in the development of radiation injury resulting from radiotherapy for prostate cancer: according to Burri et al. [82]

patients who have the SOD2 C/T genotype exhibited a significant increase in grade 2 late rectal bleeding compared to patients who had either the C/C or T/T genotype for this SNP.

- **SOD3** *(superoxide dismutase 3)*: the polymorphism most studied is 760 C> G (Arg213Gly) and presents a variant allele frequency of 4-6%. This polymorphism falls in the carboxy-terminal region at the center of a group of positively charged amino acids which represent the binding domain of the protein with heparin and heparan sulfate, two negatively charged molecules present in the interstitial tissue. Furthermore, this amino acid variation is to alter the binding of the enzyme SOD3 with these molecules thus decreasing the presence and activity of the same enzyme at a tissue level.
  In conclusion two polymorphisms described above are both associated with an altered ability to control oxidative stress and may therefore affect the individual predisposition to the development of neurotoxicity.

## *Cytochromes*

The cytochromes P450 enzymes (CYP) play an important role in the metabolism of a wide variety of endogenous and foreign compounds, including anticancer drugs and steroid hormones [83]. The localization of most cytochromes P450 is manly hepatic, and the liver is the organ most heavily involved in xenobiotic metabolism.

- **CYP2B6** is involved in the metabolism of several therapeutically significant drugs and environmental toxicants. In addition, CYP2B6 hydroxylates testosterone in the liver, deactivating testosterone's hormonal function, and also inactivates dihydrotestosterone (DHT) in the prostate [84]. The polymorphism CYP2B6*6 (Lys262Arg and Gln172His) is the most studied and its frequency in the Caucasian population is 16%. If the decrease in protein expression levels of the CYP2B6 caused by the genetic variants of CYP2B6 were to hinder the hydroxylation of testosterone, this process could affect the development and progression of prostate cancer.
- **CYP3A4** is the most abundant cytochrome P450 in human liver. It is responsible for the metabolism of over 50% of all clinically used drugs, and also metabolizes several toxins and endogenous compounds [85]. The most common variation is -392 A>G (CYP3A4*1B) and it has been shown to induce increased transcriptional activity *in vitro*. Ethnic and

geographic differences reflecting the rates of prostate cancer in different populations (highest among the African-Americans, intermediate in Caucasian and lowest in Asians) have been reported for this alteration and association with higher clinical stage and grade has been reported although also contradictory reports exist [86].

- **CYP3A5** catalyzes the 6-hydroxylation of testosterone, producing a metabolite that is less biologically active and more readily eliminated, thus inhibiting testosterone metabolism to more biologically active androgen forms [87]. It is expressed in only 10-20% of adult livers. The CYP3A4*1B was suggested to show linkage disequilibrium with CYP3A5*1 and to be associated with a risk of developing more aggressive prostate cancer. In following study an aggressive CYP3A4*1B/CYP3A5*3 (6986 A>G, rs776746) haplotype has been reported by Plummer et al., 2003 [86]. Several SNPs have also been reported for CYP3A5 gene which impact enzyme function. CYP3A5*3 and CYP3A5*6 variant alleles has been reported to cause alternative splicing and to block the production of proteins, resulting in either a reduction or absence of CYP3A5 enzyme activity. Individuals homozygous for the CYP3A5*3 (6986 A>G in intron 3) allele appear to not express a functional version of the CYP3A5 enzyme due to a cryptic splice site that results in the incorporation of intronic sequence in the mature mRNA and the production of a truncated protein due to a premature appearance of termination codon. This is important in anticancer therapies as CYP3A is involved in the metabolism of many anticancer drugs, including oxaliplatin and docetaxel.

## *Glyoxylate metabolism*

Oxalate synthesis and outcome are closely linked to those of glyoxylate, it self produced from serine, glycine, and alanine metabolisms, used by the liver to produce glucose.

Glyoxylate is detoxified by alanine glyoxylate transferase (**AGT**), exclusively in peroxisomes, through its transformation in glycine in a reaction coupled to alanine conversion in pyruvate.

In the cytosol, glyoxylate is metabolized into glycolate by glyoxylate reductase-hydroxypyruvate reductase (Fig. 15). The main role of this enzyme is to prevent the accumulation of glyoxylate in the cytosol, converting it into glycolate. The last catabolic pathways of glyoxylate are its transformation in oxalate by lactate dehydrogenase in cytosol.

It usually remains minor because AGT detoxifies most of glyoxylate, consequently considerably limiting its oxidation in oxalate.

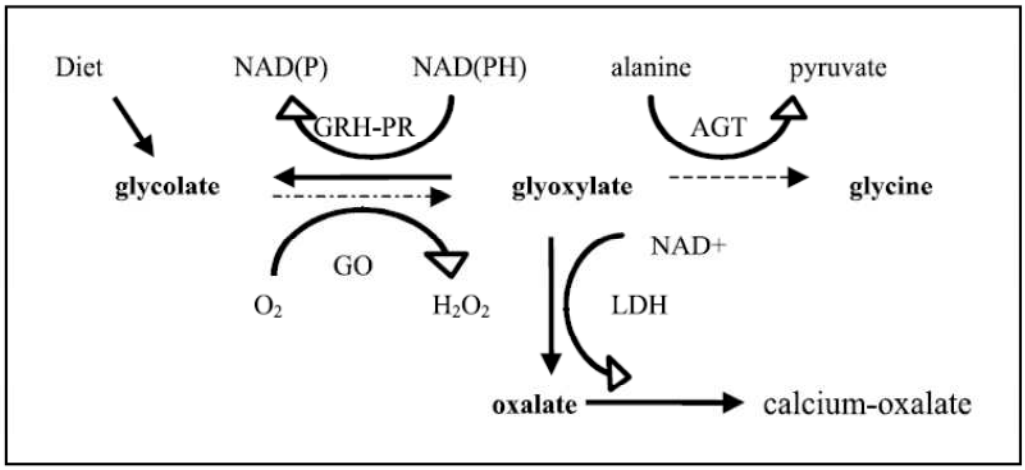


**Figure 15. The metabolism of oxalate and glyoxylate.** The implications of enzymes and links with certain amino acids. GO, glycolate oxydase; LDH, lactate dehydrogenase. Solid arrows, cytosol; dashed arrows, peroxisome.

A deficiency in one of these two enzymes, AGT and GRHPR, known as genetic disease, can lead to an abnormal accumulation of oxalate called hyperoxaluria. AGXT gene variants are very rare, but besides the genetic disease, two variants are frequent, the wild-type known as a major allele and less frequent minor allele, found in 20% of Caucasian patients, a haplotype characterized by three mutations: two substitutions Pro11Leu and Ile1142Met and a duplication of74 bp. Only Pro11Leu single nucleotide polymorphism has a phenotypic effect. It reduces AGT catalytic activity by 3 fold in homozygote patients (i.e., 4% of Caucasian patients). GRHPR variants, leading to the formation of a truncated protein or to the loss of catalytic enzyme activity (965T>G, 103 delG), are very rare. Because glyoxylate is an intermediate step of the two metabolic pathways of oxalate, we hypothesized that patients with a partial deficiency of one of these enzymes could be at high risk of neurotoxicity after oxaliplatin infusion because they would not be able to manage repeated acute and high levels of oxalate production [88].

## *Transporter genes*

ABCC1 and ABCC2 belong to ATP-binding cassette transporter superfamily, containing several family members that mediate the cellular trafficking of drugs, their metabolites and endogenous factors (Fig. 16) [89].

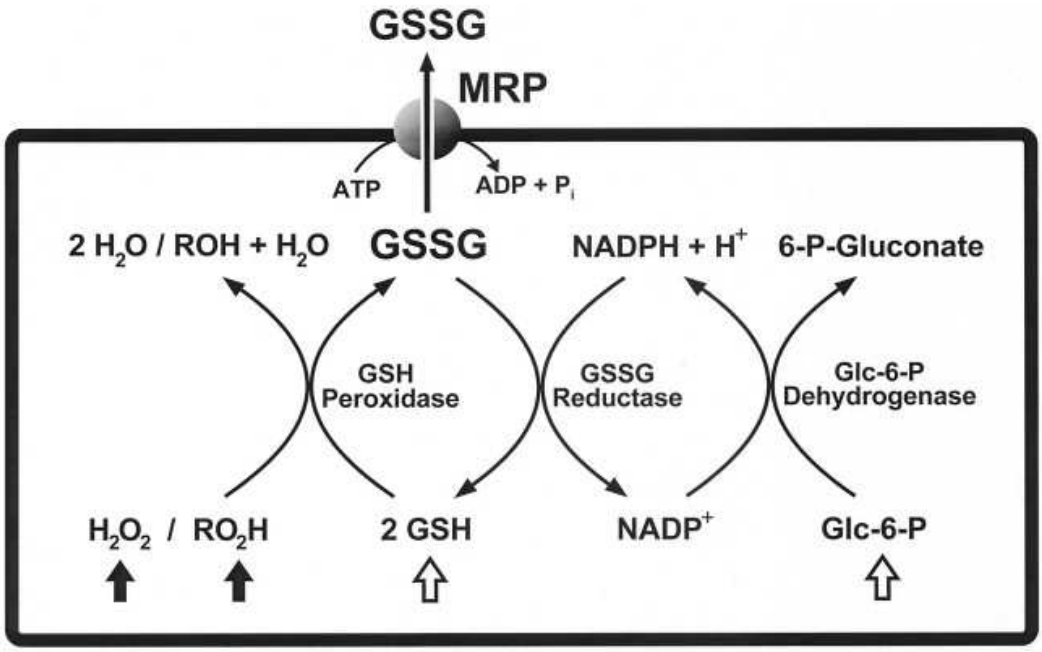


**Figure 16: MRP-mediated export of glutathione disulfide.** Filled arrows indicate enhanced formation of hydroperoxides; open arrows indicate supply of metabolites counteracting oxidative stress.

ABCC1 and ABCC2 may act in a synergistic way modulating the effect of oxaliplatin and 5-fluorouracil at the cellular level [90]. High-affinity substrates for these genes include the glutathione S-conjugate, in particular the glutathione disulfide. Under conditions of oxidative stress, the reduction of GSSG by glutathione reductase may become rate-limiting, thus leading to an increase in the export of GSSG. Proteins of the MRP family are indispensable for transport of glutathione S-conjugates and glutathione disulfide into the extracellular space and play. Therefore, a decisive role in detoxification and defense against oxidative stress [91].

## *Toll-like receptor*

- **TLR4** *(Toll-like receptor)* it is one of the main regulators of innate immune response. So far we identified two polymorphisms in exon 4, which are in close linkage disequilibrium: 896 A>G (Asp299Gly) and 1196 C>T (Thr399Ile). It is seen that the two missense polymorphisms, situated in the extracellular domain of the receptor, go to alter the interaction of TLR4 with the ligand and lead, therefore, to less activation of the same receptor (Fig. 17) [92].

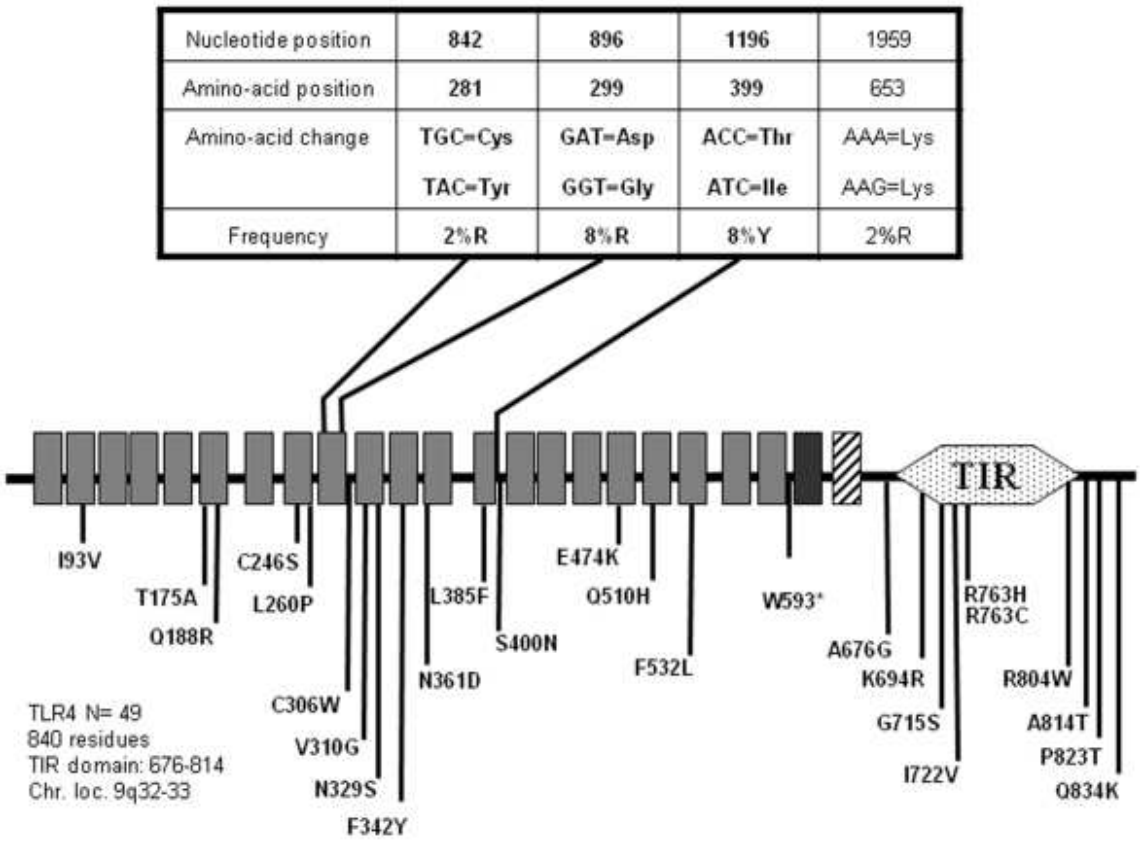


**Figure 17:** Schematic representation of TLR4 and position and frequency of Single Nucleotide Polymorphisms (SNPs).

In a study of Rudofsky [93] it is seen that two polymorphisms mentioned above have a role in the development of peripheral neuropathy in patients with type 2 diabetes. In particular, we have seen that patients with both mutated alleles have a lower prevalence of peripheral neuropathy. This is probably due to a decreased stimulation of the receptor TLR4 by endogenous molecules, such as oxLDL, and to a lesser consequent activation of

the inflammatory mechanisms which over time can go to damage the same peripheral neurons.

Although the role of these polymorphisms in the pathogenesis of diabetic neuropathy remains to be clarified, we can still assume that, in a similar manner, an altered stimulation of TLR4 in patients treated with oxaliplatin can affect on individual susceptibility to develop neurotoxicity.

## *5,10-Methylenetetrahydrofolate reductase*

- **MTHFR** (*5,10-Methylenetetrahydrofolate reductase)* catalyses the conversion of 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-methylenetetrahydrofolate (5-MTHF). MTHFR is an important enzyme in folate metabolism. This enzyme directs the folate pool toward homocysteine remethylation at the expense of DNA and RNA synthesis. This enzyme is known to have at least two polymorphisms associated with it. One of them, the 677 C>T polymorphism reduces the activity in homozygotes by 30% in comparison to heterozygotes [94]. This reduced activity leads to accumulation of 5-MTHF. The allele frequency of this polymorphism is 24-40% in Caucasians.

## *NADPH Oxidase*

NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) consists of a membrane-bound complex (glycosylated protein gp91 *phox* and non-glycosylated protein **p22 *phox***) and three cytosolic subunits (p47 *phox*, p67 *phox*, p40 *phox*). This enzyme is one of the main sources of $O_2^{\bullet -}$ and thus has an important role in maintaining the balance of ROS in the cell. On the other hand, several studies have demonstrated the role of NOX in ovarian and colon cancer, and only in PCa cell lines. Xia et al [95], Brar et al [96], and Lim et al [97] found that cells from ovarian cancer, colon cancer, and prostate carcinoma cell line DU145 present an overexpression of NOX; they concluded that ROS have an important role in the induction of angiogenesis and tumoral growth. Moreover Floriano-Sanchez et al [98] in his study suggests that NADPH oxidase in combination with other classic markers (such as PSA) could function

as a marker in post-operative monitoring of patients diagnosed with hyperplasia and others minor pathologies of the prostate.

## *Vascular endothelial growth factor*

Vascular endothelial growth factor (VEGF) is an important hypoxia-related angiogenesis stimulating factor, is a potential prognostic factor for radiotherapy outcome for prostate cancer, because its expression has been linked with poor prognosis, including adverse outcome after radiotherapy, in other tumor types (Fig. 18) [99].

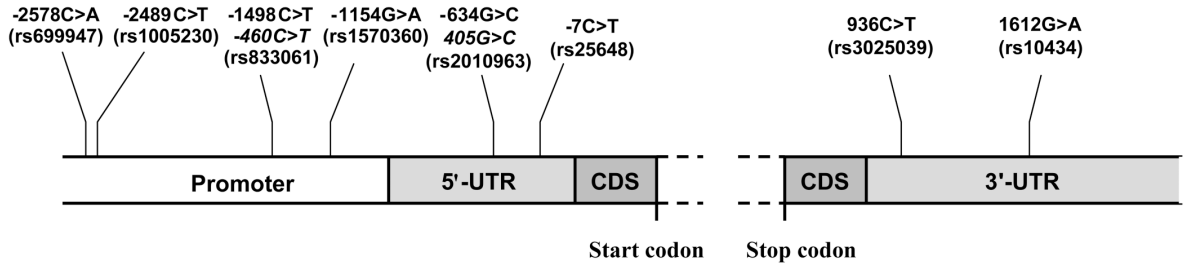


**Figure 18. Structure of VEGF gene and position of VEGF SNPs relative to translation start site.** Dashed lines indicate the region consisting of coding sequence (CDS) and seven introns. UTR: Untranslated region.

VEGF is major player in angiogenesis and one of the most potent inducers of vascular permeability. Angiogenesis involves the development of abnormal vessels that prevent the normal tissue oxygenation and transport of macromolecules, endothelial cell growth, proliferation, migration, and differentiation [94]. Preclinical studies have shown a protective effect of VEGF against endothelial damage induced by exposure to radiation.

- **VEGF-A:** -634 G>C and 1154 G>A are the most studied polymorphisms. In particular the first variant is situated in the 5'-UTR of VEGF gene and it affects the protein translation efficiency. The other polymorphism, 1154 G>A, is located in the gene promoter and seems to be associated with a lower expression of VEGF-a. This polymorphism was associated to the risk of prostate cancer development and could be a genetic marker of response to radiotherapy.

## *Nitric oxide synthase*

Nitric oxide (NO) is a multifaceted compound that may inhibit carcinogenesis through cell death or promote carcinogenesis through angiogenesis [99,100]. Nitric oxide synthase (NOS) is a family of enzymes that generate NO from L-arginine and have been classified as calcium-dependent endothelial NOS (NOS3) and neuronal NOS (NOS1) and calcium-independent inducible NOS (NOS2). The expression of inducible NOS (NOS2A) is increased in prostate carcinoma [101,102] and endothelial NOS (NOS3) is reported to protect prostate cancer cells from apoptosis [103].

# 6. Diagnostic kits for the determination of pharmacogenetic variants

A considerable problem that occurs in the management of pharmacological treatment in oncology is the significant inter-individual variability in the toxicities and in the efficacy associated with therapy. Adverse reactions to a drug are the fourth leading cause of death in Western countries. In oncology, these effects are amplified due to the narrow therapeutic index of drugs (small margin between toxic dose to normal tissues and effective dose for antitumor activity). Several studies have shown different susceptibility to the toxic effects of a given chemotherapeutic agent in patients treated with the same dose or a different sensitivity to the identical agent in clinically homogeneous patients. All these factors, in addition to determining a potential inadequate provision of care for patients, can aggravate the economic burden associated with the management of cancer patients [15,104,16].

The genetic characteristics of the patient represent one of the determining factors in intersubjective variability observed in the outcome of cancer therapy, and pharmacogenetics has the aim to validate and to use in clinical practice certain diagnostic kits, with the purpose to personalize and optimize the therapy. Currently, in oncology, there are no tools able to predict with certainty the validity of the therapy to be administered to the patient. Therefore there is a need to find new technologies, diagnostic tools predictive of toxicity and response to anticancer drugs.

The development of diagnostic kits easy to manage results, therefore, of particular utility because, allowing the preliminary analysis of the genetic characteristics of the patient, can provide to oncologists an additional tool in the choice of the most appropriate drug and the optimal dose for the individual. The diagnostic kits are innovative tools, useful to derive from a simple blood test valuable information on the activity of anticancer drugs, particularly Fluoropyrimidines, Taxanes, and Irinotecan in terms of toxicity and response.

The kits, born in oncology, are potentially useful in other contexts of drug therapy (HIV infection, blood clotting, etc..). Another added value lies in their simplicity of use and versatility of employ, factors that provide an easy diagnostic applicability.

The qualitative and functional characteristics of kits make them suitable for various applications:

- Predictive markers of pharmacological therapy toxicity;
- Predictive markers of pharmacological therapy efficacy;
- Construction of genetic risks profiles for drug susceptibility;
- Construction of genetic risks profiles for susceptibility to other substances related to medical practice;
- Monitoring of toxicity and efficacy in retrospective and prospective studies to epidemiological purposes, and of clinical monitoring;
- Genetic basis of pharmacological interactions;
- Pharmacological research;
- Determination of risk of developing tumors;
- Other parameters related to techniques of molecular biology.

The chemotherapeutic drugs are natural or synthetic substances which have a low selectivity being cytotoxic for all the cells in duplication, both tumor and normal. The genetic characteristics of the host organism may be especially important in determining the degree of toxicity of the therapy, meanwhile those of the tumor, characterized by important aberrations, may instead affect especially the effectiveness of treatment [104]. The determination of the genetic germinal variants of the patient has, therefore, primarily the purpose of guiding the therapy as a function of the potential toxic effects, characterizing the normal tissue, rather than as a function of tumor response, that is a more problematic phenotype more problematic related to the complex tumoral genetic.

The multigenic approach allows, moreover, to perform simultaneous analysis of multiple polymorphisms within the same gene or multiple genes, favoring the complexity of metabolic and cellular pathways involving the drug [15].

# *AIM*

The genetic characteristics of the patient are one of the determining factors in the inter-individual variation observed in the onset of cancer and in outcome of anti-tumor therapy. The ability to predict the pharmacological response of a patient on the basis of the individual's genetic makeup is the basic concept of pharmacogenetics, also commonly known by the term of personalized medicine. The genetic characteristics of the patient represent one of the determining factors in inter subjective variability observed in the outcome of antitumoral therapy, and pharmacogenetics has the aim to validate and to use in clinical practice certain genetic markers, with the aim to customize and optimize the therapy.

This project aims to study the genetic characteristics involved in toxicity and response to drug therapy and radiotherapy in cancer patients with the aim of improving the treatment and research of pharmaco/radiogenetics kits of possible use in clinical practice.

The main objectives of this thesis were the following:

1. *Selection of polymorphisms in the genes of interest through the research in scientific literature and the use of bioinformatics tools;*
2. *Development of analytical methods for the characterization of genetic polymorphisms;*
3. *Identification of pharmacogenetic markers of neurotoxicity, haematological and non-haematological toxicities to oxaliplatin in colorectal cancer;*
4. *Identification of radiogenetic markers of response in terms of risk of biochemical PSA recurrence in prostate cancer;*
5. *Identification of radiogenetic markers of response in terms of overall survival (OS) in prostate cancer;*
6. *Development of diagnostic kits for the determination of pharmacoradiogenetic variants.*

# *MATERIALS AND METHODS*

# 7. Patients enrolment and therapy

In this study two groups of patients were enrolled by their characteristics and their pathology:

1) *colorectal cancer* patients treated with oxaliplatin in adjuvant therapy (FOLFOX4 regimen);

2) *prostate cancer* patients treated with radiotherapy and/or surgery and/or hormone therapy.

## 7.1 Colorectal cancer patients

This study, sponsored by the CRO-National Cancer Institute of Aviano, Italy, includes one hundred and fifty-four colorectal patients. All of them were of Caucasian ethnicity and have been enrolled in centers located in Northern and Central Italy. The subjects were treated with oxaliplatin in adjuvant chemotherapy (FOLFOX4 regimen).

Patients with histologically confirmed colorectal cancer, and radiologically confirmed absence of distant metastases were eligible. Eligibility criteria were as follows: stage II-III CRC; age>18 years; performance status (WHO) 0-2; normal bone marrow, renal and liver function. Patients affected by chronic inflammatory enteric diseases, evidence of neurosensory disease or assuming neurotoxic medications were excluded from the study.

The main endpoint of this study was neurotoxicity during FOLFOX4 therapy in curatively-resected patients with stage II–III CRC. Neutropenia and any non-hematological toxicity were evaluated as secondary endpoints. The highest grade of toxicity recorded during the treatment for each toxicity endpoint (i.e., neurotoxicity, neutropenia and any non-hematological toxicity) was used for association with polymorphisms. The Institutional Review Board of each participating institution approved the study protocol, and all patients signed a written informed consent before entering the study.

Eligible patients were treated with FOLFOX4 (oxaliplatin 85 mg $m^{-2}$ (2 h infusion on day 1), leucovorin (100 mg $m^{-2}$ as 2 h infusion on day 1), 5-fluorouracil bolus (400 mg $m^{-2}$) and 22 h infusion (600 mg $m^{-2}$) on days 1 and 2 every two weeks) for 6 months (12 cycles).

Objective clinical evaluation, blood counts, hepatic and renal function tests were performed within 48 h before each cycle. Patients were questioned about nausea and vomiting, mucositis, diarrhea, asthenia (i.e., fatigue, malaise and weakness symptoms), and appetite at every cycle. Toxicity was evaluated according to NCI-CTC criteria version 2.0 (http://ctep.cancer.gov/). Neurotoxicity was evaluated according to the oxaliplatin-specific scale. Patients undergoing at least one cycle of chemotherapy were included in this study. Chemotherapy was delayed until recovery from hematological toxicities or in the case of significant, persisting, non-hematological toxicity. In the event of severe (grade 3–4) toxicity, the doses of oxaliplatin and 5-fluorouracil were reduced by 25% or 50% based on the physician's evaluation. Treatment was discontinued either in the event of anaphylactic reaction, or repeated severe toxicity in spite of dose reduction, or patient refusal.

## *7.2 Prostate cancer patients*

Nine hundred and twenty four patients with prostate cancer were enrolled at the CRO-National Cancer Institute of Aviano, Italy, between 2000 and 2008. All patients are of Caucasian ethnicity and 864 are eligible. All cases recruited in this study signed a written informed consent approved by the local Ethical Committee for research purposes before entering this study. Eligibility criteria were as follows: histological diagnosis of prostate cancer; age ≥ 18 years; performance status (ECOG) ≤ 2; adequate hematologic, hepatic and renal function, and absence of lymph node metastases.

The main endpoint was biochemical PSA recurrence after the end of radiotherapy. PSA recurrence was defined as two consecutive PSA measurements of >0.2 ng/ml at an interval of >3 months. The PSA level of >0.2 ng/ml in the first time of follow-up was considered the date of recurrence.

All patients included in this study were treated with radiotherapy. Some of them underwent surgery (161 patients) or hormone therapy (468 patients) or both treatments (155 patients).

Radiotherapy: The radiation treatment may be either radical or adjuvant. If it is radical, the planned dose is between 76 Gy to 80 Gy in 38 to 40 fractions. If it is adjuvant, it begins no earlier than 3 months after surgery (EORTC, European Organisation for Research and Treatment of Cancer) and the dosage provides from 66 Gy to 70 Gy in 33 to 35 fractions.

Surgery: The patients enrolled in this study have undergone radical prostatectomy. Sometimes surgery can cause sexual impotence, the inability to have an erection, and urinary incontinence, although modern surgical techniques have reduced the frequency of such complications.

Hormone therapy: In hormone treatment analogues alone and anti-androgens alone or combined were used. For the total androgen blockade a similar LH-RH (Enantone, Zoladex or Decapeptyl) and a peripheral anti-androgen (Casodex, Flutamide or Androcur) were administered while for the partial androgen blockade an anti-androgen alone (Casodex, Flutamide or Androcur) was used. Hormone treatment has been used as neoadjuvant or adjuvant in relation to radiotherapy. This treatment will be continued for 2 years after radiotherapy in very high risk patients.

# 8. Molecular analysis

## 8.1 Sample storing

Whole blood samples were collected from the patients and stored in freezer at -20° C. All personal and clinical data were catalogued in appropriate databases, prepared in accordance with the Privacy Policy.

## 8.2 Genomic DNA extraction

The extraction of genomic DNA from whole blood was performed with the automated extractor BioRobot EZ1 (Qiagen SPA, Milano, Italy) (Fig. 19).


**Figure 19:** BioRobot® EZ1 for automated genomic DNA extraction from whole blood.

The Card "EZ1 DNA Blood", in association with the Kit "EZ1 DNA Blood Kit 200 µl", was used for the extraction of genomic DNA from 200 µl of whole blood obtaining 200 µl as final volume, corresponding approximately 4-8 µg of DNA. Once introduced the appropriate card and start the program, the BioRobot allows to process 6 samples simultaneously, without any intervention by the operator. The DNA strand binds to the magnetic particles coated

with silica in the presence of chaotropic salt and thus the DNA is held and purified from the blood sample. DNA extracted is maintained at 2-8°C.

## 8.3 Polymerase Chain Reaction (PCR)

The technique of DNA polymerase chain reaction (PCR) was invented in 1983 by K. Mullis and allows to produce a large number of copies of a specific DNA sequence *in vitro*. It also allows to isolate and amplify any gene from any organism and then analyze the sequence, perform cloning or mutagenesis procedures, or even establish diagnostic tests that detect the presence of mutated forms of the gene. In the *in vitro* process, DNA is initially heated to temperatures close to boiling, in order to denature it and thus obtain single-stranded mold, then Taq polymerase is used to catalyze the duplication of the parental strand. To start the synthesis reaction, this enzyme requires a primer represented by a small sequence of double-stranded DNA (Fig. 20).

In the reaction tube are added two primers, one to allow the synthesis of the sense strand (sense or forward primer) and one for the synthesis of the antisense (antisense or reverse primer). The two primers define the target region to be amplified (Fig. 21).


**Figure 20:** Thermal Cyclers.

PCR requires several reagents and reaction conditions that vary with the time. In particular, the samples are subjected to a series of thermal cycles which are summarized below:

- An initial period at elevated temperature (94-95°C) that allows the DNA denaturation, in order to completely separate the template's strands that act as a mold.

- A variable number of consecutive cycles of amplification, each of which consists of three phases corresponding to three different temperatures:
    1. Complete DNA denaturation, carried out by heating at elevated temperature (94-95° C);
    2. Pairing (annealing) of sense and antisense primers with complementary sequences on the DNA template. In this phase the temperature is lowered to values which may vary from 50° C to 65° C according to the specific characteristics of the primers used;
    3. extension (elongation) of the primers and synthesis of new strands by the Taq polymerase, at a temperature of 72° C optimum for the enzyme activity.

    To obtain the amplification of the desired DNA sequence, this cycle of three steps must be repeated several times, typically 25 to 40 times.
- A final period at 72° C to complete the elongation.

This series of thermal cycles is carried out thanks to a programmable instrument, the thermal cycler, capable of changing the temperature very quickly and keep it constant for a given period of time. The result of a PCR is that, at the end of *n* cycles of amplification, the reaction mixture contains a theoretical maximum number of double-stranded DNA equal to $2^n$ (where "*n*" represents the number of amplification cycles). In the first cycle of PCR the two primers anneal with the two strands of the denatured template, thus providing the trigger for the polymerase that synthesizes complementary strands; as result of this cycle, two new strands, longer than the region to be amplified, whose end parts correspond to the sequence of the primers used to identify the target sequence, are created. In the second cycle, the primers anneal to the original template again and so it produces other neostrands of undefined length. In subsequent cycles only fragments of the desired length are formed and they contain the specific region you want to amplify.

The starting material of the PCR is the genomic DNA containing the sequence to be amplified, it is not necessary to isolate this sequence because it is directly bounded by two specific primers used in the reaction. The reagents used in a PCR are: the reaction buffer, magnesium ions supplied by the magnesium chloride ($MgCl_2$), the deoxynucleotides triphosphate (dNTPs), the primers, DNA polymerase and the template. In particular, for each sample, a reaction mixture, containing the reaction buffer, a solution of $MgCl_2$, the dNTPs, primers and DNA polymerase, is made before adding genomic DNA.

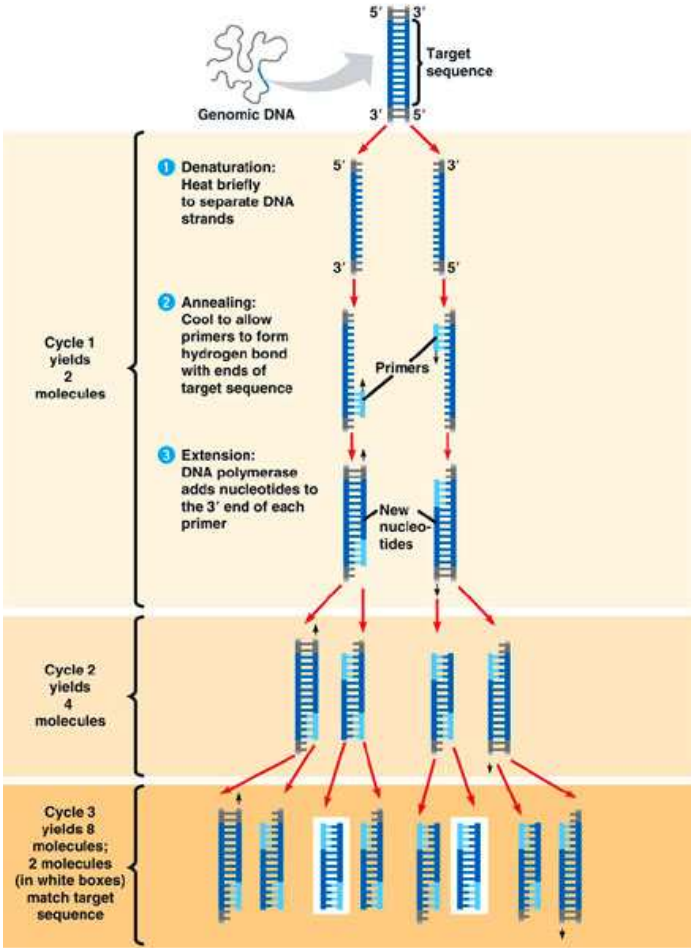


**Figure 21:** Polymerase Chain Reaction (PCR).

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1) Reaction Buffer: it is a Tris-HCl and KCl based buffer and it is necessary to reproduce the optimal conditions for the activity of the polymerase thus increasing the throughput or the number of nucleotides that the enzyme can insert in succession before separating from the template strand.
2) $Mg^{2+}$: it is essential for the activity of Taq polymerase as its bond with the enzyme stabilizes it in a three-dimensional conformation that facilitate its activities. The Taq polymerase shows its highest activity around a concentration of free magnesium equal to 1.2-1.3 mM. The concentration of free magnesium is, however, influenced by the concentration of nucleotides as there is a link between equimolar magnesium and dNTPs. You can then also use magnesium concentrations higher than those indicated above, but at higher concentrations of magnesium polymerase tends to incorporate incorrect nucleotides.

3) dNTPs: the solutions of dNTPs contain the four nitrogenous bases of DNA: dATP, dGTP, dTTP and dCTP. For a good efficiency of the PCR the four nucleotides must be present in equimolar concentrations and the optimum concentration is around 50-200 µM. A too high concentration may increase the incorrect rate of incorporation, while a too low concentration may damage the efficiency of the reaction.
4) Primers: the design of the primers can be performed manually, or more frequently through the use of some softwares that facilitate the choice such as "Primer3_www.cgi, version 0.2". The aim of primer design is to obtain a balance between two goals: efficiency and specificity of amplification. Given a target DNA sequence, primer analysis software attempts to strike a balance between these two goals by using pre-selected default values for each of the primer design available. In particular, optimal primer pairs should be closely matched in Melting Temperature and must not be able to form loops and primer dimers. Primer length (about 20-base pairs), sequence and GC contents are taken into account to select proper primers sequences.
5) DNA polymerase: it is derived from Thermophilus bacterium acquaticus, is stable at high temperatures and works with maximum efficiency between 72°-75° C. The thermal stability is a critical feature of this enzyme. Taq polymerase at 72° C has an enzymatic activity that allows the incorporation of 50-60 nucleotides per second which corresponds to approximately 3 Kb per minute. The optimal concentration of DNA polymerase Taq ranges from 0.5 to 2.5 U. A too high concentration may decrease the specificity of the reaction, while a too low concentration may not enable the conclusion of all cycles.

### *8.3.1 Optimization of the Polymerase Chain Reaction (PCR) conditions*

In order to obtain good results in the PCR process the three key parameters to consider: efficiency (or yield), reaction specificity and accuracy. To perform a PCR with high efficiency, we have to obtain a large number of products with the least number of cycles as possible. The specificity of the reaction is intended as the ability to amplify only the sequence of

interest, without obtaining nonspecific products. Finally, a high accuracy is given by the presence of a negligible number of errors introduced by DNA polymerase.

Once the primers are designed in an opportune way, there are conditions that, if modified in an appropriate manner, can improve these parameters:

- ✓ $Mg^{2+}$ concentration: the presence of divalent cations is critical, and it has been shown that magnesium ions are superior to manganese, and that calcium ions are ineffective. The optimal $Mg^{2+}$ concentration for Taq polymerase efficiency must be set up to match dNTPs and primer concentration/ sequence. dNTPs are the major source of phosphate groups in the reaction, and any change in their concentration affects the concentration of available $Mg^{2+}$.
- ✓ Number of cycles**:** to be performed for each PCR protocol, it was set up checking the accumulation of target sequence by gel electrophoresis after each amplification cycle. The correct number of cycles to be used should guarantees a sufficient balance between efficiency and specificity of amplification. Thirty–five cycles of amplification resulted correct for most of the protocol employed in this work.
- ✓ Annealing Temperature: was set up performing a temperature gradient PCR (Mastercycler Gradient Eppendorf, Hamburg, Germany). Annealing temperatures could be calculated by several methods considering the Melting Temperature (Tm) of the primer-template pairs. However, in practice, because the Tm is variously affected by the individual buffer components and the primer and template concentrations, any calculated Tm value should be considered just as a first approximation. A range of 60±5°C was tested by 1 degree increments to find the optimal reaction conditions.
- ✓ Additives: they may be added to the reaction mixture of PCR to increase the specificity of annealing of primers or the amount of amplified products. They are denaturants substances that lead to a destabilization between the bases and, consequently, also to a high destabilization of the complex primer/aspecific DNA. The substances may be used are: DMSO (dimethylsulfoxide) up to 10%, formamide up to 5% and glycerol up to 10-15%.

## 8.4 Methodologies for the analysis of polymorphisms

NCBI (National Center for Biotechnology Information) database and tools were used to select the analyzed polymorphisms. The NCBI presents a web site showing links to all kinds of important protein and nucleotide database, literature (PubMed), and search and analysis tools. Important databases included in the web site and consulted for assay design (genetic sequences, polymorphisms description, primer design), during this work of thesis, were the "GenBank sequence database", "SNP500" (Single Nucleotide Polymorphisms database), "PharmaGKB" (The Pharmacogenomics Knowledge Base), and "1000 Genomes Browser".

Subsequently, according to the type of polymorphism to be analyzed and to the specific characteristics of the nucleotide sequence, the most suitable method of genotyping has been chosen and we proceeded with its finalization.

In particular, in this PhD thesis, semi-automated, recently developed, genotyping methods have been used. These are based on the PCR reaction and allow the identification of genetic polymorphisms in a very simple and easy method: **Pyrosequencing** technology **(PSQ)**, the methodology for allelic discrimination based on **TaqMan** chemistry, and the **Analysis of Fragments (Gene Scan)**.

### 8.4.1 Pyrosequencing

Pyrosequencing is a nanotechnology of recent development for SNP identification consisting of a real time pyrophosphate detection method [105,106].

This technique is based on indirect bioluminometric assay of the pyrophosphate (Ppi) that is released from each dNTP upon DNA chain elongation. Following Klenow polymerase mediated base incorporation, Ppi is released and used as substrate, together with adenosine 5'-phosphosulfate, for ATP sulfurylase, which results in the formation of ATP. Subsequently, the ATP accomplishes the conversion of luciferin to its oxi-derivative by luciferase. The ensuing light output becomes proportional to the number of added bases, up to about four bases. To allow processivity of the method, dNTPs in excess are degraded by apyrase, which is also present in the starting reaction mixture, so that only dNTPs are added to the template during sequencing procedure. The process is fully automated and adapted to a 96-well format, which allows rapid screening of large panel of samples.

This method has a first phase of preparation of the sample and then the use of an instrument, the PSQ 96MA Pyrosequencing (Fig. 22), which determines and provides directly the genotype at the level of the analyzed SNP.

**Figure 22:** PSQ 96MA Pyrosequencing.

Pyrosequencing analysis is performed on PCR- amplified DNA. One of the PCR primers must be biotin-labeled for immobilization to streptavidin coated Sepharose beads. This allows the separation of the two DNA strands produced by PCR, since the assay must be carried out on single stranded DNA. If reverse primer is biotynilated we have the forward assay, otherwise, if the forward primer is biotin labeled, the assay is called reverse.

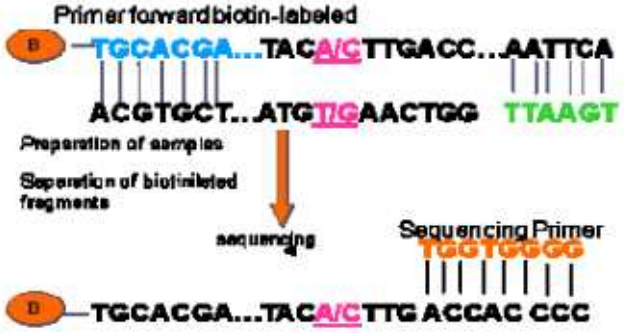


**Figure 23: PCR schematic representation of Pyrosequencing method.** SNP position is indicated by the slash (/). "B" stands for biotin.

PCR reaction product is mixed with streptavidin coated High Performance Sepharose beads (Amersham Biosciences, Uppsala, Sweden) in the presence of a binding buffer (Tris 10 mM, Sodium Chloride 2 M, EDTA 1 mM and Tween 20 0.1%, pH 7.6). The mixture is allowed to shake for 10 minutes at room temperature. The samples are subsequently transferred to a 96-well filter plate and vacuum (vacuum manifold for 96 well filter plate, Millipore) applied

to remove all liquid. Denaturation solution (Sodium Hydroxide 0.2 M) is added to denature double stranded PCR product DNA. After 1 minute incubation vacuum is applied to remove the solution and the non immobilized DNA. The beads are washed twice with a washing buffer (Tris 10 mM, pH 7.6) in the presence of the vacuum. The beads with the immobilized template are resuspended by adding 45 µl annealing buffer (Tris 20 mM, Magnesium Acetate Tetra-Hydrate 2 mM, pH 7.6) and sequencing primer (2 µM) is added to each sample. Design of sequencing primers for Pyrosequencing follows the same criteria as for the PCR primers, except that the melting temperature of this primer may, if necessary, be lowered. The sequencing primer could thus be shorter than the PCR primers, typically 15 bp. The position of the primer is flexible within 5 bases from the SNP and can be designed on both the positive (reverse assay) or on the negative (forward assay) strand. Thirty-five µl of this mixture is transferred to a Pyrosequencing 96 wells plate (PSQ 96 Plate Low).

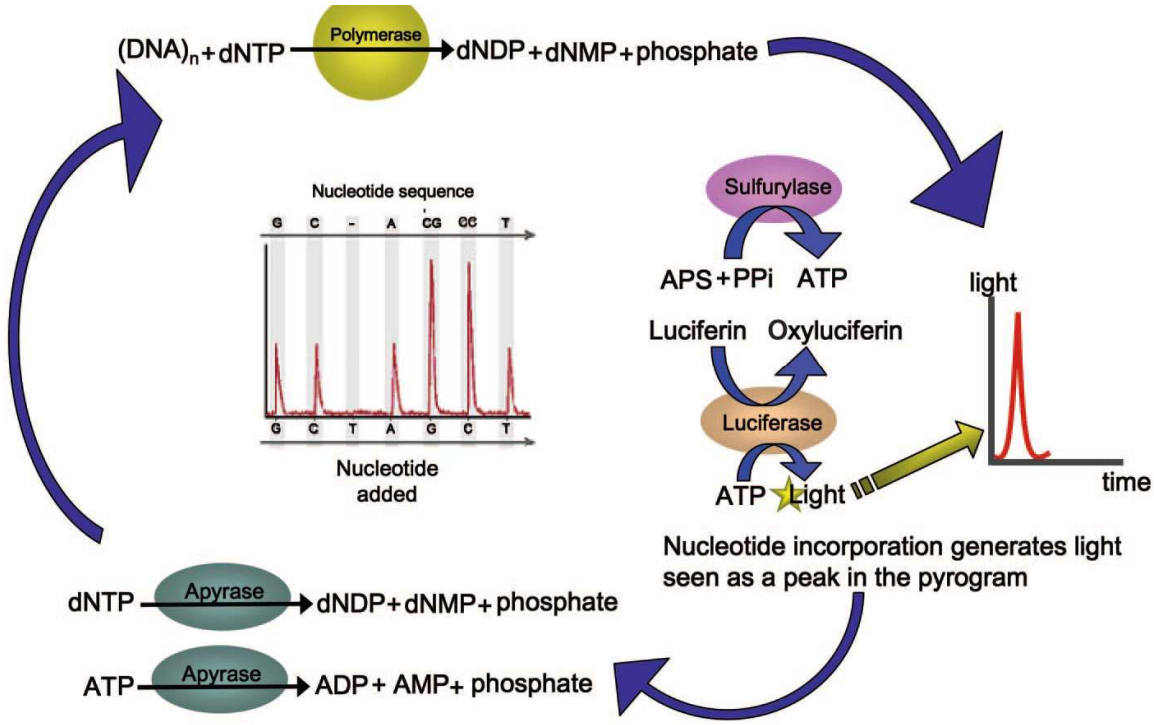


**Figure 24: Pyrosequencing chemistry:** biochemical reactions and enzymes involved in the generation of light signals by DNA pyrosequencing. Each peak in the pyrograms represents a pulse of light detected in the instrument. dNTP, deoxynucleoside triphosphate; dNDP, deoxynucleoside diphosphate; dNMP, deoxynucleoside monophosphate; PPi, pyrophosphate; APS, adenosine 5_-phosphosulfate.

The plate is incubated for 5 minutes at 60° C to allow complete sequencing primer annealing on the template DNA. After samples cooling, the plate is transferred on the Pyrosequencing

instrument. The biotin labeled DNA template, annealed to the sequencing primer, is incubated with enzymes (DNA polymerase, ATP sulfurylase, luciferase and apyrase) and the substrates (adenosine 5'phosphosulfate and luciferin). The first of four dNTPs is added to the reaction. DNA polymerase catalyzes the incorporation of the dNTP into the DNA strand, complementary to the base in the template strand. Each incorporation event is accompanied by release of Ppi in a quantity directly related with the amount of incorporated nucleotide. ATP sulfurylase converts PPi to ATP in the presence of adenosine 5'phosphosulfate. This ATP drives the luciferase mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a charge coupled device (CCD) camera and seen as peak in a pyrogram. The height of each peak (light signal) is proportional to the number of nucleotides incorporated (Fig. 23).

Apyrase, a nucleotide degrading enzyme, continuously degrades ATP and unincorporated dNTPs. This switches off the light and regenerates the reaction solution. The next dNTP is then added. Addition of dNTPs is performed one at a time. It should be noted that deoxyadenosine alfa-thio triphosphate is used as a substitute for the natural dATP since it is efficiently used by the DNA polymerase, but not recognized by the luciferase. As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peaks in the pyrogram (Fig. 24).

"PSQ Assay Design" software was used for the planning of the described assays: it allows to easily choose the set of primers (sense and antisense primers for PCR and sequencing primer for subsequent analysis at PSQ) most suitable for the study of each SNP.
The analysis of the results of an experiment at PSQ is accomplished with the "PSQTM 96 MA software" on your computer that is connected to the Pyrosequencer.

The reagents and solutions that are used in Pyrosequencing methodology are:

- Aqua B. Braun Ecotainer, sterile water for injection, (B. Braun, Melsugen AG, Germany);
- Streptavidin SepharoseTM High Performance, (Amersham Biosciences AB, Uppsala, Sweden);
- Sequencing primer provided in lyophilized form (Sigma Genosys, Cambridge, UK) and then resuspended in sterile water to obtain a final concentration of 100 μM;

- Pyro Gold Reagents Kit (Biotage AB, Uppsala, Sweden) constituted by:
  - Enzyme mixture (luciferase, DNA polymerase, apyrase, and proteins binding to single-stranded DNA) provided in lyophilized form and then resuspended in sterile water;
  - Substrate mixture (adenosina 5'fosfosulfato [APS] and luciferin) provided in lyophilized form and then resuspended in sterile water;
  - dATP$\alpha$S in solution;
  - dCTP in solution;
  - dGTP in solution;
  - dTTP in solution.
- PSQTM 96 Sample Preparation Kit (Biotage AB, Uppsala, Sweden) constituted by:
  - Binding Buffer (10 mM Tris-HCl, 2M NaCl, 1 mM EDTA, 0,1% Tween 20; pH=7,6);
  - Denaturation Solution (0,2 M NaOH);
  - Washing Buffer (10 mM Tris-acetate; pH=7,6);
  - Annealing Buffer ( 20 mM Tris-acetate, 2 mM $Mg^{2+}$-acetate; pH=7,6).

Other materials and instruments used in Pyrosequencing methodology are:

- PSQ 96 Plate Low (Biotage AB, Uppsala, Sweden);
- 96-well filter plates (Millipore, MA, USA);
- PSQTM 96 Reagent Cartridge (Biotage AB, Uppsala, Sweden);
- PSQTM 96 Sample Prep Tool Termoplate (Biotage AB, Uppsala, Sweden);
- Multichannel Pipette (Matrix Technologies Corporations, NH, USA);
- Vacuum pump (Millipore, MA, USA);
- Shaker (Analitica De Mori, MI, Italia);
- PyroMarkTM Vacuum Prep Workstation (Biotage AB, Uppsala, Sweden);
- PSQ96 MA Pyrosequencing (Biotage AB, Uppsala, Sweden), software PSQTM 96 MA;
- Pyrosequencing Assay Design Software, version 1.0.6 (Biotage, Westbrough, MA, USA).

### 8.4.2 TaqMan® assay

The allelic discrimination consists in the determination of the two variants of a single nucleic acid sequence by means of the "5 'fluorogenic nuclease assay". In particular, this technique exploits the exonuclease property in the direction 5'→3' of the Taq polymerase when it encounters, during its activity of elongation of a DNA fragment, an oligonucleotide perfectly matched with the DNA template that the Taq employs as a template for the elongation. Through this method it is possible to investigate missense polymorphic alterations.

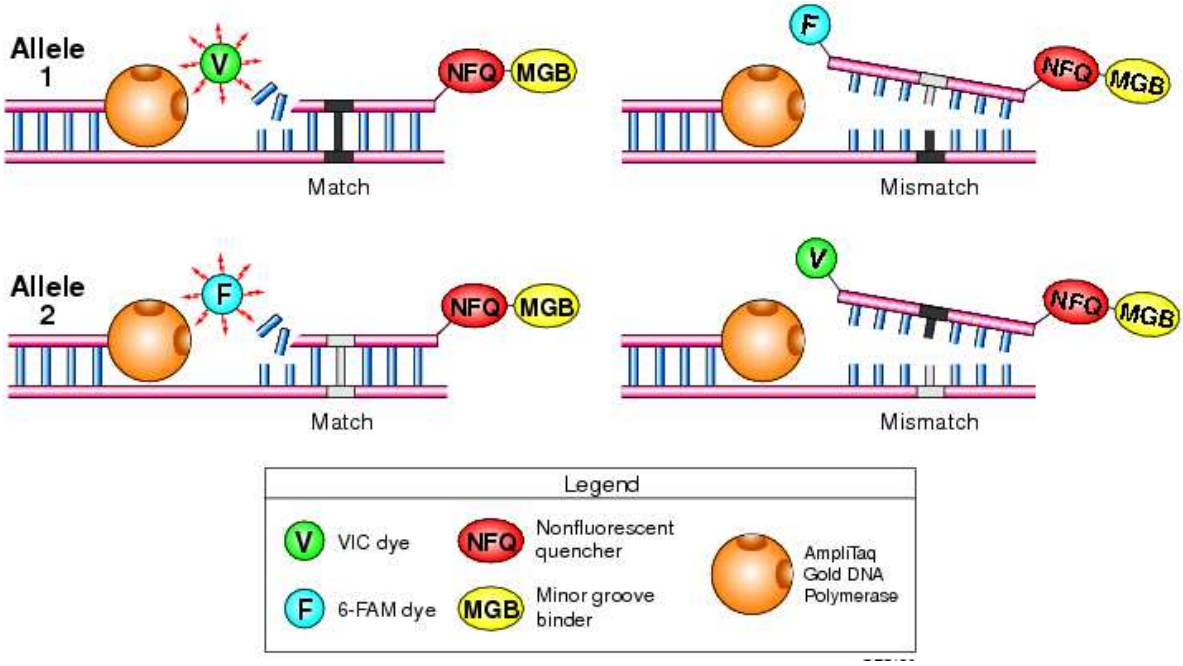


**Figure 25:** Schematic representation of TaqMan® technology.

At the base of the TaqMan® allelic discrimination is the use of a Real Time PCR (RT PCR), that in addition to the sense and antisense primers needed for the amplification of the fragment of interest containing the polymorphism, involves the use of an oligonucleotide *(probe)* that is able to base-pair to the template occupying an intermediate position between the sense and the antisense primer. The probe is marked at the two ends: in one part there is a "quencher" (fluorophore TAMRA) which acts as a silencer of fluorescence, the other one is tied to a "reporter" (fluorophore FAM or VIC). The action of silencing by the quencher occurs by transfer of energy from one fluorochrome to the other when the latter are near to each other. In the reaction two different allele-specific probes are placed: they are labeled with different fluorophores (fluorochrome FAM or VIC). One fluorescent dye detector

contains a perfect match to the wild type (allele 1) and the other fluorescent dye detector presents a perfect match to the mutation (allele 2). The allelic discrimination assay classifies unknown samples as: homozygotes (samples having only allele 1 or allele 2) and heterozygotes (samples having both allele 1 and allele 2).

TaqMan probe-based chemistry uses a fluorogenic probe to detect specific PCR product as it accumulates during PCR cycles. In Fig. 25 is reported a schematic representation of a TaqMan Assay. During the denaturation step, the reporter (R) and the quencher (Q) are attached to the 5' and 3' ends of a TaqMan probe. When both dyes are attached to the probe, reporter dye emission is quenched. During each extension cycle, the hot-start DNA polymerase system cleaves the reporter dye from the probe. After being separated from the quencher, the reporter dye emits its characteristic fluorescence.

The probes are chosen according to certain characteristics:

- The $T_m$ must be at least 5° C higher than the $T_m$ of the two primers because they must bind to the nucleotide sequence when executing the synthesis of the complementary strand;
- The oligonucleotide must have a length of about 20-30 bp and 50% of G and C;
- The extension phase must be performed at a temperature below 72° C standard, used in the PCR, in order not to cause the detachment of the probe from the template (for this reason we use high concentrations of $MgCl_2$);
- The probe must not form dimers or even pair with itself.

**Figure 26:** Applied Biosystems 7500 Real-Time PCR System instrument.

Samples are analyzed using the Applied Biosystems 7500 Real-Time PCR System instrument (Fig. 26). The allelic discrimination was performed with the SDS software 2.3 (Applied Biosystems).

The practical procedure of the TaqMan® technology is really very simple and allows to analyze quickly the genotype using only a universal mix (master mix) and a specific marker for the polymorphism of interest. The step of sample preparation involves the use of 96-well plates. The reaction mixture is prepared by combining the specific mix for the gene variation under investigation (SNP Assay 20X or 40X), containing primers (sense and antisense) and the two probes labeled with FAM or VIC, to the Master Mix (TaqMan Genotyping Master Mix 2X) universal for all genotypic analyzes, containing dNTPs, Taq Polymerase, $MgCl_2$ and salts in a suitable concentration creating an adequately buffered environment. The solution is dispensed into wells and, finally, is added to the genomic DNA (approximately 20 ng of DNA for each sample). For SNP assay a preformed assay “TaqManR SNP Genotyping Assay” is employed: it is available on-line in the catalog of Applied Biosystems (http://www3.appliedbiosystems.com/AB_Home/index.htm). As an alternative, you can use the service offered by the same company that, on sending the gene sequence containing the nucleotide variation, develops and tests specifically an assay called "Custom SNP Genotyping assay TaqMan®". Once set up the plate, this is covered with an adhesive film and centrifuged for a few minutes in order to eliminate the presence of any air bubbles at the bottom of the wells. Then the plate is loaded into the ABI PRISM 7900HT machine, at this stage RT-PCR conditions (temperature, duration and cycles), the test volumes (20 µl) are determined, and above marker (FAM and VIC) are assigned to polymorphism’s alleles. The amplification is carried out with a thermal cycler integrated into the instrument using the following thermal profile:

- 50° C for 2 minutes;
- 95° C for 10 minutes;
- 40 cycles for (92° C for 15 seconds; 60° C for 1 minute)

At the end of the PCR reaction an end point scanning of the 96-well plate containing the samples is carried out, in order to detect the fluorescence signal produced in each well by the two fluorophores (FAM and VIC) used for marking the allele-specific probes. Finally, thanks to the processing of obtained data by software SDS 2.3, the assignment of the genotype corresponding to each sample analyzed occurs.

For the analysis with TaqMan® technology were used the following reagents:

- 2X TaqMan Genotyping Master Mix, (Applied Biosystems,CA,USA);
- 20X or 40X "TaqMan® SNP Genotyping Assay" or "CustomTaqMan® SNP Genotyping Assay" (Applied Biosystems,CA,USA);
- MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems,CA,USA);
- Optical Adhesive Covers (Applied Biosystems,CA,USA);
- Real-Time ABI PRISM 7900HT instrument (Applied Biosystems,CA,USA);
- SDS 2.3 software (Applied Biosystems,CA,USA).

### *8.4.3 Automated fragment analysis*

Automated fragment analysis is performed to detect small variation in the length of a DNA fragment. It is based on the technique of capillary electrophoresis coupled with fluorescence detection. It occurs when an electric field is applied to an electrolyte (DNA fragment) solution within a capillary, causing ions migration. DNA fragments, having a negative charge, move toward the anode (+) and are separated by size (Fig. 27).

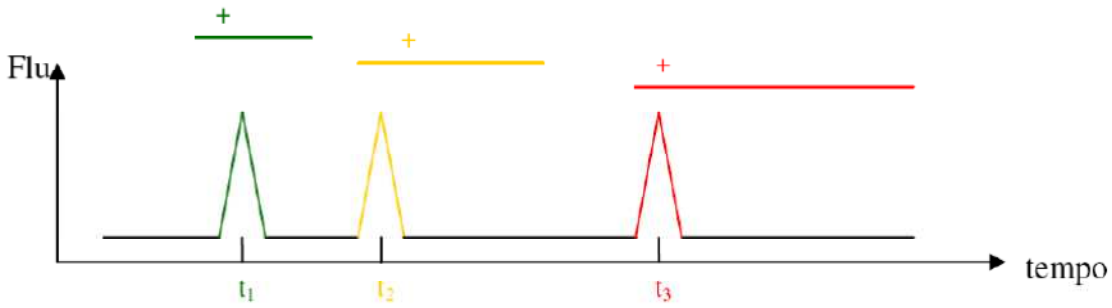


**Figure 27:** Scheme of the separation of different sized DNA fragments labeled with different fluorophores (ROX=red, JOE=green, LIZ=yellow) by capillary gel electrophoresis.

Polyacrylamide gels are used as the electrolyte solution to provide the sieving medium for the separations. DNA fragments are produced by PCR employing one 5' fluorescence labeled primer with the HEX (isomer-free succinimidyl ester of 6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein, excitation and emission maxima of 535 and 556 nm) fluorophore. In the analysis is also used a marker of DNA molecular weight labeled with a different fluorophore, the ROX which serves as internal standard (Fig. 28).

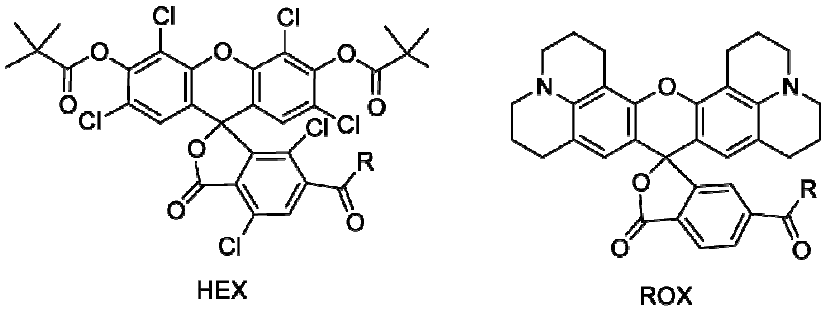


**Figure 28:** Molecular structure of the two fluorophore used to characterize the PCR sense primer (HEX) and the molecular marker (ROX) for automated fragment analysis.

These dye-labeled fragments are detected by fluorescence and in turn rendered into a sequence or sized fragment. The pherogram analyzed by the software presents on the abscissa the separated molecular weight fragments, while on the ordinate the intensity of the fluorescence peak. The samples are analyzed in the Genetic Analyzer ABI Prism 3100 instrument (Applied Biosystems) (Fig. 29). Gene Scan analysis software (Applied Biosystems) allows data extraction and elaboration.

**Figure 29:** Genetic Analyzer ABI Prism 3100.

The process is very simple. The first phase consists in the amplification of the gene fragment containing the polymorphism of interest by means of a PCR that presents one of the two primers labeled with the fluorophore HEX covalently linked in 5' (not reactive extremity).
Since this method is very sensitive, it is sufficient a small concentration of amplified fragment to conduct the analysis. Consequently, the samples, after being analyzed by electrophoresis on agarose gel, are suitably diluted. The mix needed to perform the analysis

consists of 14.5 µl of deionized and purified formamide and 0.5 µl of Internal Lane Size Standard [ROX] for each sample. Once prepared the mix, this is aliquoted into a 96-well plate and, subsequently, is added 1 µl of the diluted sample for a total of 16 µl per well. The formamide is a strong denaturant and is sufficient the contact with the DNA to exert its effect. The plate is covered, to prevent evaporation of the solution, and then it is placed in the thermal cycler for denaturation (2 min at 95° C). Immediately after denaturation, the plate is placed in ice in order to avoid the rewinding of DNA strands and it is loaded into the Genetic Analyzer ABI PRISM 3100 instrument (Fig. 30).

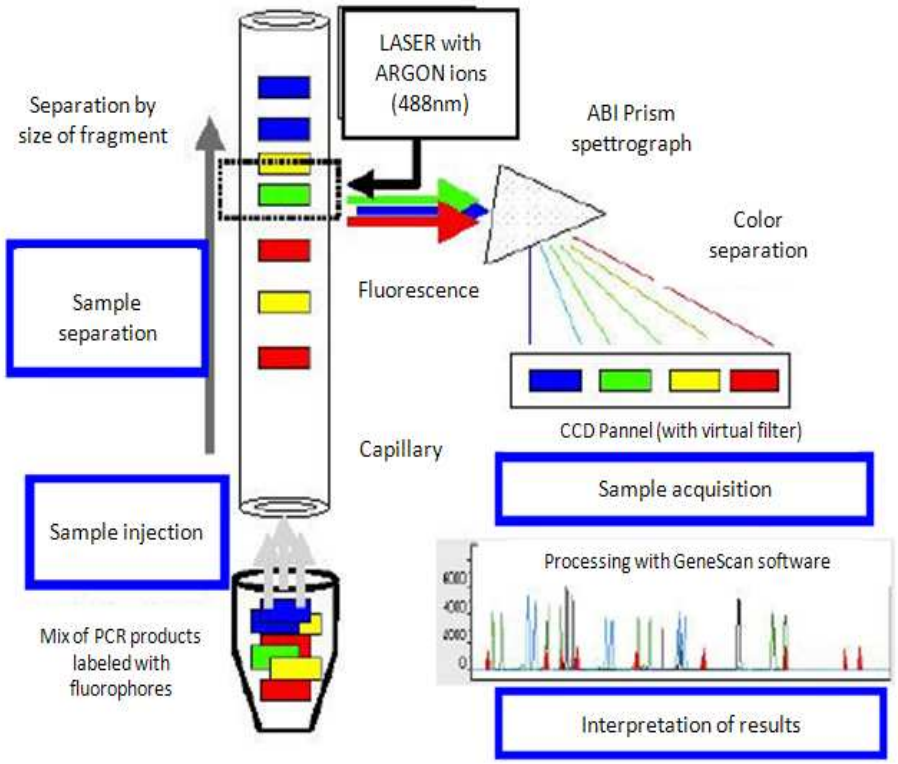


**Figure 30:** Scheme of the processing of DNA fragments within the Genetic Analyzer ABI PRISM 3100 instrument. The involved steps are: injection of the samples, separation of the fragments by size using electrophoresis, determination of fluorescence with CCD camera, interpretation of the results with the Gene Scan software.

Once started the instrument, the 16 capillaries penetrate in the plate and take samples. The loading of the samples takes place through electrokinetic injection, i.e. through the application of a potential of 15 KV for about 5 seconds which moves all the charged molecules within the capillary. There are activities of competition by charged molecules or ions, present in the sample, which can interfere with this delicate phase of the process. The

sample dilution in sterile water and purified formamide is also useful to reduce these interference phenomena. To guarantee the correct injection of the samples in the capillaries is also the phenomenon of stacking, which allows to the fragments and the mix to be loaded into a restricted and compact zone of the capillary, avoiding DNA diffusion phenomena. Stacking permits to produce an area of low conductivity, and this is made possible from immersion of the capillary in water before loading the samples. After the first phase of injection, the samples are separated by an electrophoretic run and, at the exit of the capillary, they are bombarded by a laser that excites all fluorophores emitting fluorescence in different regions of the spectrum. An analyzer of multiple wavelengths (CCD camera, charged-coupled device) identifies the emissions of each fragment passing through the detector. The analysis of fluorescence occurs both for unknowns fragments and for the internal standard's fragments (Fig.31).

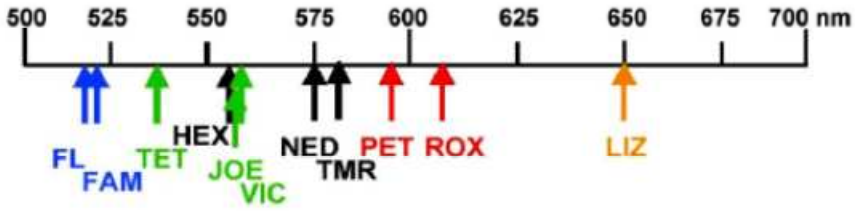


**Figure 31:** Range of the visible spectrum captured in CCD camera

The ABI 3100 data collection software allows to control the conditions of electrophoresis, directs which light wavelength will be analyzed by the CCD camera (through the use of virtual filter) and manages the creation of files of samples and lists of injections. The extraction and processing of data are managed by the Gene Scan analysis software that allows the conversion of the data into appropriate colored peaks which have assigned values of fragment length, based on the time of output and the type of emission. The instrument Genetic Analyzer ABI PRISM 3100 (Applied Biosystems, Foster City, CA) was also employed, managed by the Gene Scan analysis software (Applied Biosystems, Foster City, CA).

The success of an electrophoretic run depends on several factors [107]:

- The capillary: in capillary electrophoresis the separation is performed using a tube with an internal diameter of 50-100 micron. This thinness allows the application of a high electric field and therefore reduces the times of escalation without overheating problems associated with the high voltage used.

- The polymer: There are many different types of means of sieving used in electrophoretic separations that depend on their physical characteristics. For example you can use the physical gels, products from agarose, or the common chemical gels, such as polyacrylamide used in electrophoretic denaturants gel plates, which are cross-linked rigid materials in which the porous structure is linked through covalent bonds. These two materials are however problematic to be used inside a capillary for the formation, for example, of air bubbles, both during the coating of the lumen and in the phase of gel contraction, due to the polymerization. The third type of sieving materials are tangled polymers also characterized by intermolecular interactions. These (for example the linear polyacrylamide that is not cross-linked) have replaced the other two types of gels as they are less problematic.
- The formamide: is very important to use high-quality formamide with low conductivity. Such substance in fact generates ionic products from its decomposition, including formic acid, that is negatively charged at neutral pH and would compete with DNA in the run on the capillary. This can also cause problems of both sensitivity and resolution.
- The buffer: the solution used to dissolve the polymers is important to: stabilize and solubilize the DNA, provide charge carriers to the electrophoretic current and to increase the injection. If the concentration and the concomitant conductivity of the buffer are too high, the column will overheat and as a result will lose resolution.
- The temperature: to maintain the DNA denaturation, promoted by formamide and by the rapid heating-cooling, the temperature of the column must be greater than room temperature. Furthermore the requested internal standard is sensitive to temperature variations and, therefore, can be also used as an indicator of a stable and well calibrated system.

The reagents and solutions used in the sequencer methodology are:

- Aqua B. Braun Ecotainer, sterile water for injection, (B. Braun, Melsugen AG, Germany);
- Hi-DiTM Formamide (Applied Biosystems, Foster City, CA);
- Fluorophore ROX™ DYE (5-carbossi-X-rodamina, succinil estere) (Gene Scan™ 400HD [ROX] Size Standard, Applied Biosystems, Foster City, CA).

## 8.5 Analyzed polymorphisms and their related methods of analysis

### 8.5.1 Pyrosequencing

The polymorphisms (SNPs) that have been analyzed with the Pyrosequencing technology (PSQ) are reported in Table 1:

| GENE | SNP | FUNCTION | AA CHANGE | SEQUENCE |
|---|---|---|---|---|
| ABCB1 | rs1045642 | cds-synon | Ile1145Ile | CCGGGTGGTGTCACAGGAAGAGAT[C/T]GTGAGGGCAGCAAAGGAGGCCAACATA |
| ABCB1 | rs1128503 | cds-synon | Gly412Gly | CACTCGTCCTGGTAGATCTTGAAGGG[C/T]CTGAACCTGAAGGTGCAGAGTGGGC |
| ABCC1 | rs2074087 | intron | NA | GCTAGGCAGTCTCACACATGTGCACT[C/G]ACGTGGCCGGGTGTCCCCTTTGCCC |
| ABCC1 | rs35529209 | missense | Ala989Thr | TCCTTTTCATGTGTAACCATGTGTCC[A/G]CGCTGGCTTCCAACTATTGGCTCAG |
| ABCC1 | rs35605 | cds-synon | Leu562Leu | ACTGGGCGTTCTGCTTGCAGGTGGCC[C/T]TGTGCACATTTGCCGTCTACGTGAC |
| ABCC1 | rs2230671 | cds-synon | Ser1334Ser | GTGGGGCGGACGGGAGCTGGGAAGTC[G/A]TCCCTGACCCTGGGCTTATTTCGGAT |
| ABCC1 | rs28364006 | missense | Thr1337Ala | GGACGGGAGCTGGGAAGTCGTCCCTG[A/G]CCCTGGGCTTATTTCGGATCAACGA |
| ABCC1 | rs212088 | intron | NA | GGCAAACTCCCAAAGCCTAGAGGCCA[C/T]TGTGCCAGTGGCATCACCTTGTGTA |
| ABCC2 | rs2273697 | missense | Val417Ile | CCAACTTGGCCAGGAAGGAGTACACC[A/G]TTGGAGAAACAGTGAACCTGATGT |
| ABCC2 | rs717620 | UTR-5 | NA | ACACAATCATATTAATAGAAGAGTCTT[C/T]GTTCCAGACGCAGTCCAGGAATCATG |
| ABCC2 | rs2804402 | neargene-5 | NA | CTCAAACTCCAGGCTTCAACAATCCT[C/T]CTGCCTTGGCCTCCCAAAGGGCTGG |
| ABCC2 | rs7910642 | neargene-5 | NA | CAATCCCAGCCCTTTGGGAGGCCAAG[A/G]CAGAAGGATTGTTGAAGCCTGGAGT |
| ABCC2 | rs4148396 | intron | NA | TGTTTATATACTGAGGATCTTTCTGA[C/T]AGGGAGGAATTATTATGTCCCCCTA |
| ABCG2 | rs2046134 | intron | NA | CAACCTGAGAGAGAAGGGAAAATGCA[A/G]CAAAATGGGTACTTAGACAGAATCA |
| AGXT | rs34116584 | missense | Pro11Leu | TCTCACAAGCTGCTGGTGACCCCCC[C/T]CAAGGCCCTGCTCAAGCCCCTCTCC |
| CYP2B6 | rs3745274 | missense | Gln172His | CTCATGGACCCCACCTTCCTCTTCCA[G/T]TCCATTACCGCCAACATCATCTGCT |
| CYP3A4 | rs2740574 | neargene-5 | *1B | TGAGGACAGCCATAGAGACAAGGGCA[A/G]GAGAGAGGCGATTTAATAGATTTTA |
| CYP3A5 | rs776746 | splice-3 | *3 | CTCTTTAAAGAGCTCTTTTGTCTTTCA[A/G]TATCTCTTCCCTGTTTGGACCACATTAC |
| ERCC2 | rs1799793 | missense | Asp312Asn | CCCACCTGGCCAACCCCGTGCTGCCC[A/G]ACGAAGTGCTGCAGGGTGAGCCCC |
| ERCC2 | rs13181 | missense | Lys751Gln | CTGCTGAGCAATCTGCTCTATCCTCT[G/T]CAGCGTCTCCTCTGATTCTAGCTGC |
| GSTA1 | rs3957357 | neargene-5 | *B | CAATAGTTCTCTCCCACTGAAAGAAG[A/G]GTCAAGTTAGGGAAAAGCCACTCCC |
| hMLH1 | rs1799977 | missense | Ile219Val | CCTCAACCGTGGACAATATTCGCTCC[A/G]TCTTTGGAAATGCTGTTAGTCGGTA |
| hMSH2 | rs2303428 | intron | NA | AATCTTGCTTTCTGATATAATTTGTT[C/T]TGTAGGCCCCAATATGGGAGGTAAA |
| hOGG1 | rs1052133 | missense | Ser326Cys | GCTGTTCAGTGCCGACCTGCGCCAAT[C/G]CCGCCATGCTCAGGAGCCACCAGCA |
| IL6 | rs1800795 | neargene-5 | NA | CACTTTTCCCCCTAGTTGTGTCTTGC[C/G]ATGCTAAAGGACGTCACATTGCACA |

| | | | | |
|---|---|---|---|---|
| MDM2 | rs2279744 | intron | NA | CGGGGGCCGGGGGCTGCGGGGCCGCT[G/T]CGGCGCGGGAGGTCCGGATGATCGC |
| RAD51 | rs1801320 | UTR-5 | NA | CGAGTAGAGAAGTGGAGCGTAAGCCA[C/G]GGGCGTTGGGGGCCGTGCGGGTCGG |
| SCN2A | rs17183814 | missense | Arg19Lys | ACCTGACAGCTTCCGCTTCTTTACCA[A/G]GGAATCCCTTGCTGCTATTGAACAA |
| SOD3 | rs1799895 | missense | Arg231Gly | CGCGGGAGCACTCAGAGCGCAAGAAG[C/G]GGCGGCGCGAGAGCGAGTGCAAGGC |
| TLR4 | rs4986790 | missense | Asp299Gly | AGCATACTTAGACTACTACCTCGATG[A/G]TATTATTGACTTATTTAATTGTTTG |
| TLR4 | rs4986791 | missense | Thr399Ile | CTGTTCTCAAAGTGATTTTGGGACAA[C/T]CAGCCTAAAGTATTTAGATCTGAGC |
| XPG | rs17655 | missense | Asp1104His | ATTCATTAAAGATGAACTTTCAGCAT[C/G]TTCACTTGAAGATCCATCAGATGAT |
| XRCC1 | rs1799782 | missense | Arg194Trp | TGAGGCCGGGGGCTCTCTTCTTCAGC[C/T]GGATCAACAAGACATCCCCAGGTGA |
| XRCC1 | rs3213239 | UTR-5 | NA | CCCTGTTTTCTCACCTTCCCACGGCC[-/GGCC] CATTGTTGTTTGTTTGTTTGCTTGA |
| XRCC3 | rs861539 | missense | Thr241Met | CAGGCATCTGCAGTCCCTGGGGGCCA[C/T]GCTGCGTGAGCTGAGCAGTGCCTTC |

**Table 1:** SNPs analyzed by Pyrosequencing technology.

## *8.5.2 TaqMan® assay*

The polymorphisms (SNPs) that have been analyzed with the Taqman® method are reported in Table 2:

| GENE | SNP | FUNCTION | AA CHANGE | SEQUENCE |
|---|---|---|---|---|
| ABCC1 | rs35588 | intron | NA | TGGGGCTGTCTATCGGAAGGTAGGGG[A/G]CGCTGTGCCATTGGCATGTGGCCC |
| ABCC1 | rs504348 | neargene-5 | NA | ACACACCCTGCGACCACTTTTCAAAT[C/G]CTGTTTAAGGACAGTATCCGTCACC |
| ABCC1 | rs3765129 | intron | NA | AATAGCTGGTGATGTTGAGTGATGGG[C/T]TGATCCCAGGGTCGCCCCAGATGT |
| ABCC1 | rs35587 | cds-synon | Asn354Asn | TGCAGGTTGCTCATCAAGTTCGTGAA[C/T]GACACGAAGGCCCCAGACTGGCAG |
| ABCC2 | rs3740066 | cds-synon | Ile1324Ile | ACTCCACCTACCTTCTCCATGCTACC[A/G]ATGTCACAAGTGATCCCTCTGAGGA |
| ABCC2 | rs8187710 | missense | Cys1515Tyr | CCTAGACAACGGGAAGATTATAGAGT[A/G]CGGCAGCCCTGAAGAACTGCTACA |
| ABCC2 | rs1885301 | neargene-5 | NA | TGAGTTGTATGAGTTCCTTATAGTAT[A/G]TTGTGGATATTAACTCTTCATCAGT |
| ABCC2 | rs2002042 | intron | NA | GAGTTTCTGGATTCTGTTATAAACCA[C/T]TTCTCCTTTGAAGAATCCTGTCTTT |
| ABCG2 | rs2622604 | intron | NA | AAAACATCTCTTCCTTACTACTCTGG[C/T]GTATTTATTTATGACTAGCTGACAT |
| AGXT | rs4426527 | missense | Ile340Met | TGGAGAGACATCGTCAGCTACGTCAT[A/G]GACCACTTCGACATTGAGATCATG |
| APE1 | rs1130409 | missense | Asp148Glu | TAATTCTGTTTCATTTCTATAGGCGA[G/T]GAGGAGCATGATCAGGAAGGCCGG |
| ATM40 | rs1800054 | missense | Ser49Cys | TAAACATCTAGATCGGCATTCAGATT[C/G]CAAACAAGGAAAATATTTGAATTG |
| ATM61 | rs1801516 | missense | Asp1853Asn | ACTTGATTCATGATATTTTACTCCAA[A/G]ATACAAATGAATCATGGAGAAATCT |
| CYP17A1 | rs743572 | UTR-5 | NA | GGGTGCCGGCAGGCAAGATAGACAGC[A/G]GTGGAGTAGAAGAGCTGTGGCA |
| ERCC1 | rs3212986 | UTR-3 | NA | AGACTACACAGGCTGCTGCTGCTGCT[G/T]CTTCCGCTTCTTGTCCCGGCCTGTG |

| | | | | |
|---|---|---|---|---|
| **ERCC1** | **rs11615** | **cds-synon** | **Asn118Asn** | AATCCCGTACTGAAGTTCGTGCGCAA[C/T]GTGCCCTGGGAATTTGGCGACGTAA |
| **GSTM1** | **NA** | **del** | **null** | NA |
| **GSTP1** | **rs1695** | **missense** | **Ile105Val** | GCGTGGAGGACCTCCGCTGCAAATAC[A/G]TCTCCCTCATCTACACCAACTATGT |
| **GSTP1** | **rs1138272** | **missense** | **Ala114Val** | GATACATGGTGGTGTCTGGCAGGAGG[C/T]GGGCAAGGATGACTATGTGAAGG |
| **GSTT1** | **NA** | **del 54251 bp in chr 22** | **null** | NA |
| **hEXO1** | **rs4149963** | **missense** | **Thr439Met** | GTTGAGTCAGTATTCTCTTTCATTTA[C/T]GAAGAAGACCAAGAAAAATAGCTCT |
| **hMSH6** | **rs3136228** | **neargene-5** | **NA** | ACTAAAGTTTGGTCCCTTTCGCTCCG[G/T]CTCCTTGCGAAAATGCTCTAACGGC |
| **MGMT** | **rs12917** | **missense** | **Leu115Phe** | CTATCGAAGAGTTCCCCGTGCCGGCT[C/T]TTCACCATCCCGTTTTCCAGCAAGG |
| **MTHFR** | **rs1801133** | **missense** | **Ala222Val** | CTTGAAGGAGAAGGTGTCTGCGGGAG[C/T]CGATTTCATCATCACGCAGCTTTTC |
| **MTHFR** | **rs1801131** | **missense** | **Glu429Ala** | GTGGGGGGAGGAGCTGACCAGTGAAG[A/C]AAGTGTCTTTGAAGTCTTCGTTCT |
| **NOS2A** | **rs9282801** | **intron** | **NA** | GGGGATGGAACTGGGTGTGCACAGGG[G/T]GTCTTCTCAGAACCGTGCAGGCA |
| **NOS3** | **rs2070744** | **neargene-5** | **NA** | ACCAGGGCATCAAGCTCTTCCCTGGC[C/T]GGCTGACCCTGCCTCAGCCCTAGTC |
| **P21** | **rs1801270** | **missense** | **Ser31Arg** | GGCCCAGTGGACAGCGAGCAGCTGAG[A/C]CGCGACTGTGATGCGCTAATGGC |
| **P22** | **rs4673** | **missense** | **Tyr72His** | TTCCTCCCTCCCCCAGGGGACAGAAG[C/T]ACATGACCGCCGTGGTGAAGCTGTT |
| **PARP1** | **rs1136410** | **missense** | **Val762Ala** | CTTTCTTTTGCTCCTCCAGGCCAAGG[C/T]GGAAATGCTTGACAACCTGCTGGAC |
| **SOD2** | **rs4880** | **missense** | **Ala16Val** | CAGCACCAGCAGGCAGCTGGCTCCGG[C/T]TTTGGGGTATCTGGGCTCCAGGCAG |
| **TP53** | **rs1642785** | **UTR-5** | **NA** | CATTGGAAGGGCAGGCCCACCACCCC[C/G]ACCCCAACCCCAGCCCCCTAGCAGA |
| **TP53** | **rs1042522** | **missense** | **Pro72Arg** | TCCCAGAATGCCAGAGGCTGCTCCCC[C/G]CGTGGCCCCTGCACCAGCAGCTCCT |
| **VEGFA** | **rs2010963** | **UTR-5** | **NA** | GCGCGCGGGCGTGCGAGCAGCGAAAG[C/G]GACAGGGGCAAAGTGAGTGACC |
| **VEGFA** | **rs1570360** | **neargene-5** | **NA** | GAGCCCGGGCCCGAGCCGCGTGTGGA[A/G]GGGCTGAGGCTCGCCTGTCCCCGC |
| **XRCC1** | **rs25487** | **missense** | **Gln399Arg** | CCGCATGCGTCGGCGGCTGCCCTCCC[A/G]GAGGTAAGGCCTCACACGCCAACCC |
| **XRCC1** | **rs25489** | **missense** | **Arg280His** | GTCTTCTCCAGTGCCAGCTCCAACTC[A/G]TACCCCAGCCACAGCCCCAGTCCCT |
| **XRCC3** | **rs1799796** | **intron** | **NA** | ACTTGCTGACCAGCATAGACAATGAC[A/G]GCTGTCCCCACAGGACACCTTGTTG |
| **XRCC3** | **rs1799794** | **UTR-5** | **NA** | GCCTGTTAAACCAAGTTCTCAGCAGG[A/G]TGTGCACAGAGGGCCCAGGAGGA |

**Table 2:** SNPs analyzed by Taqman® method.

### 8.5.3 Automated fragment analysis

The polymorphisms (SNPs) that have been analyzed with the automated fragment analysis are reported in Table 3:

| GENE | SNP | FUNCTION | AA CHANGE | SEQUENCE |
|---|---|---|---|---|
| ABCG2 | rs3219191 | neargene-5 | NA | GTAGATGTTGGGATGGCTACACTCA [-/CTCA]CAAAGCCTGATGGCCCGTTTCCT |
| GSTM3 | rs1799735 | intron | *B | AAGGTAGGAAGAAGGGAAAAGAAG[-AGG]ATACTTCTCTATCTCTGCAGGCTA |

**Table 3:** SNPs analyzed by automated fragment analysis.

### 8.5.4 Gel electrophoresis

The polymorphisms (SNPs) that have been analyzed with the gel electrophoresis are reported in Table 4:

| GENE | SNP | FUNCTION | AA CHANGE | SEQUENCE |
|---|---|---|---|---|
| AGXT | NA | intron | 74bp_dup | NA |
| TP53 | rs17878362 | intron | NA | CTGGGGACCTGGAGGGCTGGGG[-/ACCTGGAGGGCTGGGG]GGCTGGGGGG |

**Table 4:** SNPs analyzed by gel electrophoresis.

## 8.6 Linkage disequilibrium

In population genetics, linkage disequilibrium (LD) is the non-random association of alleles at two or more loci, not necessarily on the same chromosome. Linkage disequilibrium describes a situation in which some combinations of alleles or genetic markers occur more or less frequently in a population than would be expected from a random formation of haplotypes from alleles based on their frequencies. Non-random associations between polymorphisms at different loci are measured by the degree of linkage disequilibrium. The level of linkage disequilibrium is influenced by a number of factors including genetic linkage, the rate of recombination, the rate of mutation, random drift, non-random mating, and population structure.

The deviation of the observed frequency of a haplotype from the expected is a quantity commonly denoted by a capital D': its value could be included between 0 and 1 where 0 indicates a linkage equilibrium and 1 indicates a linkage disequilibrium. Another measure of LD which is an alternative to D' is the correlation coefficient between pairs of loci, denoted as $r^2$. This is also adjusted to the loci having different allele frequencies.

A perfect LD was defined when the allele frequencies are identical and thus only two haplotype could exist (D'=1, $r^2$=1); a complete LD was present when the allele frequencies are not identical and thus almost three haplotype exist (D'=1, $r^2$<1) [108].

The definition of linkage disequilibrium among the studied polymorphisms could be useful to define the haplotypes (the combination of several SNPs on a single chromosome) that could be more informative than the single polymorphisms. In this work of thesis, analyses of linkage disequilibrium among SNPs were performed.

## 8.7 Response and toxicity evaluation

### 8.7.1 Colorectal cancer patients

#### ✓ Toxicity evaluation

Objective clinical evaluation, blood counts, hepatic and renal function tests were performed within 48h before each cycle. Patients were questioned about nausea and vomiting, mucositis, diarrhea, asthenia (i.e., fatigue, malaise and weakness symptoms), and appetite at every cycle. Toxicity was evaluated according to NCI-CTC criteria version 2.0 (http://ctep.cancer.gov/). Neurotoxicity was evaluated according to the oxaliplatin-specific scale [109]. Patients undergoing at least one cycle of chemotherapy were included in this study. Chemotherapy was delayed until recovery from haematological toxicities or in the case of significant, persisting, non-hematological toxicity. In the event of severe (grade 3-4) toxicity, the doses of oxaliplatin and 5-fluorouracil were reduced by 25% or 50% based on the physician's evaluation. Treatment was discontinued either in the event of anaphylactic reaction, or repeated severe toxicity in spite of dose reduction, or patient refusal.

### 8.7.2 Prostate cancer patients

#### ✓ Response evaluation

- Biochemical response: partial response is defined as a PSA reduction of 50% for two consecutive tests performed at least 3 weeks; complete biochemical response is defined as a PSA value <0.1 ng/ml after chemotherapy, surgery, radiotherapy.

  a) duration of clinical response: it is defined as the time elapsing from the date of the first post-treatment PSA (3 months after the end of radiotherapy) to the time of biochemical progression or death from any cause.

- Clinical response: absence of injury and/or nodules identified at clinical or instrumental examination.

  b) duration of clinical response: it is defined as the time elapsing from the date of the end of radiotherapy to the time of clinical or instrumental progression or death from any cause.

## 8.8 Statistical analysis

For colorectal cancer patients, the study was prospectively designed to test the association between genetic polymorphisms and oxaliplatin neurological toxicity (grade ≥ 2), as the primary endpoint. Severe neutropenia and any severe non-hematological toxicity were evaluated as secondary endpoints. For each polymorphism, deviation from hardy-Weinberg equilibrium was tested by Fisher's exact test and no deviation was found ($P > 0.05$). Odds ratio and 95% confidence interval were estimated by unconditional logistic regression. We investigated three genetic models (that is, dominant, recessive and additive) for the association, and the most statistically significant by Wald $\chi^2$-test was reported. All *P* values were two-sided. Logistic models adjusting for age and sex of the patients, neo-adjuvant treatment (yes/no) and cumulative oxaliplatin dose normalized by body surface area ($mg/m^2$), was also tested through a sensitivity analysis by excluding patients who underwent pre-operative radio- or chemotherapy. The SAS software (version 9.2) (SAS Institute Inc., Milan, Italy) was used for all the analyses. To control for multiple testing *q*-value (a false discovery rate (FDR)-adjusted *P* value, FDR 0.1) was calculated for each SNP implemented in the R-package [110].

Pairwise linkage disequilibrium (LD) analysis for ABCC1 and ABCC2 polymorphisms was performed by HaploView 3.32 [111]. Haplotypes and diplotypes were inferred by Phase version 2.1.1 [112,113].

For prostate cancer patients, information on risk to biochemical PSA recurrence will be obtained through an active follow-up on a periodical verification of the recurrence status of the patients. Biochemical PSA recurrence was defined when the PSA level is >0.2 ng/ml in the PSA measurement during the follow-up after radiotherapy. It will be computed by Kaplan-Meier method, and log-rank test will be used to test the differences between subgroups. Differences between subgroups will be subjected to univariate analysis using the Cox proportional hazards model to compute the hazard ratio (HR) and corresponding 95% confidence interval (CI). First the clinical and pathological covariates that are significant in the univariate analysis will be tested in the multivariate model. Then the clinical and pathological covariates significant in multivariate analysis will be adjusted for other covariates as genotypes. In all cases, statistical significance will be claimed for $p \leq 0.05$, indicating a lack of agreement with Hardy–Weinberg equilibrium evaluated by a permutation

procedure based on an exact test. The SAS software (version 9.2) (SAS Institute Inc., Milan, Italy) was used for all analyses.

To investigate the role of polymorphisms in influencing the overall survival (OS) and the disease-free survival (DFS) we have used the Kaplan-Meier product-limit method and the log-rank test statistic. The first method was used to trace the curve showing the overall survival (OS) or the disease-free survival (DFS) and to calculate the median time to progression or survival; the log-rank test, however, has been used to compare the curves obtained for each genotype, and to test their difference statistically. The determination of the relative risk of death or progression in patients with different genotype asset was performed by univariate analysis. The overall survival was evaluated as the months elapsed from the date of diagnosis until the date of death or last control, and the time to progression was calculated as the months elapsed from the date of the first PSA dosage after the end of radiotherapy (about 3 months) until the date of the determination of recurrence or last control.

# *RESULTS*

# 9. *Colorectal cancer patients*

## 9.1 Patient's characteristics and treatment

In this multi-institutional study, sponsored by the CRO-National Cancer Institute of Aviano, were enrolled 154 colorectal cancer patients, all of Caucasian ethnicity. The median age of patients was 59 years (range 25 to 82 years). Ten of the 154 patients enrolled into the study were considered ineligible for this pharmacogenomic analysis according to the study criteria (1 was stage I; 2 were completely resected stage IV; 1 received 5-fluorouracil alone; 1 received FOLFOX plus bevacizumab; 5 were lost during follow-up), thus leaving 144 eligible patients (Table 5).

| Characteristic | N | % |
|---|---|---|
| **Total** | 144 | |
| ***SEX*** | | |
| Male | 82 | 56.9 |
| Female | 62 | 43.0 |
| ***AGE*** | | |
| Mean, range | 59 (25-82) | |
| ***PRIMARY TUMOR SITE*** | | |
| Colon | 111 | 77.1 |
| *Right* | 39 | 35.1 |
| *Left* | 68 | 61.3 |
| *Transverse* | 3 | 2.7 |
| *Sigma* | 1 | 0.9 |
| Rectum | 33 | 22.9 |
| ***STAGE OF DISEASE AT DIAGNOSIS*** *(TNM scale)* | | |
| II | 21 | 14.6 |
| III | 123 | 85.4 |
| ***NEO-ADJUVANT RADIOTHERAPY*** *(only for rectum)* | | |
| Yes | 19 | 57.6 |
| No | 14 | 42.4 |
| ***NEO-ADJUVANT RADIOTHERAPY*** *(only for rectum)* | | |
| 5-FU | 10 | 30.3 |
| 5-FU–FA and oxaliplatin | 3 | 9.1 |
| **NUMBER OF CYCLES** | | |
| Mean, range | 10.4 (1-12) | |
| **PATIENTS WHO RECEIVED ALL 12 PLANNED CYCLES** | 81 | 56.3 |
| **TOTAL (MG) OXALIPLATIN DOSE** | | |
| Median (range) | 1460 (145-2166) | |
| Dose per $m^2$ ($mg/m^2$): median (range) | 886 (85-1020) | |
| Dose per $m^2$ per week: median (range) | 35 (17-54) | |

**Table 5:** Patient's characteristics.

## 9.2 Polymorphisms and genotyping assay

| Cellular pathway | Gene | rs | Function | AA change | Previously investigated for FOLFOX4 in metastatic CRC | Association with toxicity in at least one study | Analysis method |
|---|---|---|---|---|---|---|---|
| Cellular transport | ABCB1 | rs1045642 | cds-synon | Ile1145Ile | Yes [114] | | Pyrosequencing |
| | | rs1128503 | cds-synon | Gly412Gly | Yes [114] | | Pyrosequencing |
| | | rs35588 | intronic | N/A | | | TaqMan |
| | ABCC1 | rs2230671 | cds-synon | Ser1334Ser | Yes [114] | | Pyrosequencing |
| | | rs212088 | intronic | N/A | | | Pyrosequencing |
| | | rs2074087 | intronic | N/A | Yes [114] | | Pyrosequencing |
| | | rs504348 | 5'UTR | N/A | | | TaqMan |
| | | rs3765129 | intronic | N/A | | | TaqMan |
| | | rs35587 | cds-synon | Asn354Asn | | | TaqMan |
| | ABCC2 | rs717620 | 5'UTR | N/A | Yes [88, 114] | | Pyrosequencing |
| | | rs2273697 | cds-synon | Val417Ile | Yes [88, 114] | | Pyrosequencing |
| | | rs7910642 | 5'UTR | N/A | | | Pyrosequencing |
| | | rs4148396 | intronic | N/A | | | Pyrosequencing |
| | | rs3740066 | cds-synon | Ile1324Ile | Yes [114] | | TaqMan |
| | | rs8187710 | cds-synon | Cys1515Tyr | Yes [114] | | TaqMan |
| | | rs1885301 | 5'UTR | N/A | | | TaqMan |
| | | rs2002042 | intronic | N/A | | | TaqMan |
| | ABCG2 | rs2046134 | intronic | N/A | | | Pyrosequencing |
| | | rs2622604 | intronic | N/A | | | TaqMan |
| | | rs3219191 | 5'UTR | N/A | | | Fragment analysis |

| Cellular pathway | Gene | rs | Function | AA change | Previously investigated for FOLFOX4 in metastatic CRC | Association with toxicity in at least one study | Analysis method |
|---|---|---|---|---|---|---|---|
| DNA repair | RAD51 | rs1801320 | 5'UTR | N/A | | | Pyrosequencing |
| | hMLH1 | rs1799977 | cds-synon | Ile219Val | | | Pyrosequencing |
| | hMSH2 | rs2303428 | intronic | N/A | | | Pyrosequencing |
| | hOGG1 | rs1052133 | cds-synon | Ser326Cys | | | Pyrosequencing |
| | XRCC1 | rs1799782 | cds-synon | Arg194Trp | Yes [115] | Yes [(54)] | Pyrosequencing |
| | | rs3213239 | intronic | N/A | | | Pyrosequencing |
| | | rs25487 | cds-synon | Gln399Arg | Yes [116, 114, 115] | | TaqMan |
| | | rs25489 | cds-synon | Arg280His | Yes [114] | | TaqMan |
| | XRCC3 | rs861539 | cds-synon | Thr241Met | Yes [116] | | Pyrosequencing |
| | | rs1799794 | 5'UTR | N/A | | | TaqMan |
| | | rs1799796 | intronic | N/A | | | TaqMan |
| | ERCC1 | rs3212986 | 3'UTR | N/A | Yes [114] | | TaqMan |
| | | rs11615 | cds-synon | Asn118Asn | Yes [116, 117, 114, 115] | Yes [119, 120] | TaqMan |
| | ERCC2 | rs13181 | cds-synon | Lys751Gln | Yes [116, 117, 114, 115, 58] | Yes [120, 58] | Pyrosequencing |
| | | rs1799793 | cds-synon | Asp312Asn | Yes [118] | | Pyrosequencing |
| | APE1 | rs1130409 | cds-synon | Asp148Glu | | | TaqMan |
| | hMSH6 | rs3136228 | 5'UTR | N/A | | | TaqMan |
| | ATM | rs1801516 | cds-synon | Asp1853Asn | | | TaqMan |
| | XPG | rs17655 | cds-synon | Asp1104His | | | Pyrosequencing |
| | MDM2 | rs2279744 | Intronic | N/A | | | Pyrosequencing |
| | MGMT | rs12917 | cds-synon | Leu115Phe | | | TaqMan |
| | PARP1 | rs1136410 | cds-synon | Val762Ala | | | Taqman |
| | hEXO1 | rs4149963 | cds-synon | Thr439Met | | | TaqMan |

| Cellular pathway | Gene | rs | Function | AA change | Previously investigated for FOLFOX4 in metastatic CRC | Association with toxicity in at least one study | Analysis method |
|---|---|---|---|---|---|---|---|
| Oxidative damage | GSTP1 | rs1695 | 5'UTR | Ile105Val | Yes [118, 116, 117, 114, 115] | §Yes [118, 116, 117, 114, 120, 121] | TaqMan |
| | | rs1138272 | cds-synon | Ala114Val | Yes [118, 88, 114] | §Yes [114] | TaqMan |
| | GSTA1 | rs3957357 | intronic | N/A | | | Pyrosequencing |
| | GSTT1 | N/A | cds-synon | null | Yes [118, 116] | | TaqMan *CNV |
| | GSTM1 | N/A | cds-synon | null | Yes [118, 116, 114] | ^Yes [114] | TaqMan *CNV |
| | GSTM3 | rs1799735 | intronic | N/A | | | Fragment analysis |
| | SOD2 | rs4880 | cds-synon | Ala16Val | | | TaqMan |
| Glyioxylate metabolism | AGXT | rs4426527 | cds-synon | Ile340Met | Yes [88] | §Yes [88] | TaqMan |
| | | rs34116584 | cds-synon | Pro11Leu | Yes [88] | §Yes [88] | Pyrosequencing |
| | | N/A | 5'UTR | del-74 bp | Yes [88] | §Yes [88] | Gel electrophoresis |
| Apoptosis | TP53 | rs1642785 | intronic | N/A | | | TaqMan |
| | | rs1042522 | 3'UTR | Arg72Pro | | | TaqMan |
| | | rs17878362 | cds-synon | N/A | | | Gel electrophoresis |
| Sodium channel | SCN2A | rs17183814 | cds-synon | Arg19Lys | | | Pyrosequencing |

Abbreviations: N/A, not applicable.
* CNV: Copy Number Variation assay
§ neurotoxicity
^ neutropenia

**Table 6**: Polymorphisms and genotyping assay.

## 9.3 Treatment tolerance

The majority of patients (56%) completed all the 12 planned cycles of chemotherapy (mean of 10 cycles, range 1-12). Nineteen patients (13%) underwent neo-adjuvant radiotherapy with or without systemic chemotherapy. Grade 3-4 toxicity of any type was experienced by 48% of patients (69/144): 18% (26/144) developed non-hematological grade 3-4 toxicity and 39% (56/144) developed haematological grade 3-4 toxicity. Neutropenia (38%) was the most common severe haematological toxicity, whereas diarrhea (11%) and neurotoxicity (7%) were the most frequent non-hematological severe toxicities. Neurotoxicity was also the most common adverse event of any grade developed during the treatment (83%). A detailed description of patient toxicity is in Table 7.

| **Toxicity** | **Patients** | | | |
|---|---|---|---|---|
| | Any grade N° of patients | % | Grade 3-4[a] N° of patients | % |
| ***NON-HEMATOLOGICAL TOXIC EFFECTS*** | | | | |
| Diarrhea | 53 | 36.8 | 16 | 11.1 |
| Nausea | 68 | 47.2 | 3 | 2.1 |
| Vomiting | 30 | 20.8 | 4 | 2.8 |
| Asthenia | 44 | 30.5 | 0 | 0 |
| Alopecia | 19 | 13.2 | 0 | 0 |
| Mucositis | 32 | 22.2 | 2 | 1.4 |
| Hepatic (hyperbilirubinemia) | 6 | 4.2 | 1 | 0.7 |
| Infection without severe neutropenia | 9 | 6.2 | 0 | 0 |
| Neurotoxicity[b] | 120 | 83.3 | 10 | 6.9 |
| ***HEMATOLOGICAL TOXIC EFFECTS*** | | | | |
| Anemia | 64 | 44.4 | 0 | 0 |
| Neutropenia | 91 | 63.2 | 54 | 37.5 |
| Leukopenia | 54 | 37.5 | 8 | 5.5 |
| Fever with severe neutropenia | 4 | 2.8 | 2 | 1.4 |
| Thrombocytopenia | 59 | 41.0 | 1 | 0.7 |

[a]According to the NCI-CTC version 2.
[b]According to the oxaliplatin-specific scale [(108)]

**Table 7:** Common toxicities in patients treated with FOLFOX4.

### 9.3.1. Validation of polymorphisms of the adjuvant study in a metastatic setting

There is a need to investigate the role of genetic polymorphisms previously identified in the metastatic setting and discover new putative biomarkers for additional investigation. We analyzed 23 genetic polymorphisms previously investigated in metastatic CRC patients treated with FOLFOX4 as significant markers of toxicity. Six of them were associated with severe toxicity (either neurotoxicity or neutropenia) in at least one study. For neurological toxicity we found that *GSTP1 Ile105Val (rs947894)* and *Ala114Val (rs1138272), AGXT Ile340Met (rs4426527), Pro11Leu (rs34116584),* and *del-74 bp (N/A)* were associated. Whereas for severe neutropenia only *GSTM1-null (N/A)* resulted associated. We investigated three genetic models (i.e., dominant, recessive and additive) for the association, and the most statistically significant by Wald $\chi^2$-test was reported in Table 8. All *P*-values were adjusted for sex, age, neo-adjuvant therapy and oxaliplatin cumulative dose normalized by BSA (for neurological toxicity). None of the associations reported in previous studies in the metastatic setting were replicated in the present study.

| SNP | GENE | BASE CHANGE | MAF | | MOST SIGNIFICANT GENETIC MODEL | | |
|---|---|---|---|---|---|---|---|
| | | | Grade ≥2 toxicity | Grade 0-1 toxicity | Model | OR (95% CI) | *p-value* |
| **Neurological toxicity** | | | | | | | |
| rs947894 | GSTP1 | A>G | 0.321 | 0.301 | Dominant | 1.16 (0.59-2.30) | 0.6647 |
| rs1138272 | GSTP1 | C>T | 0.071 | 0.080 | Additive | 0.91 (0.35-2.38) | 0.8433 |
| rs4426527 | AGXT | A>G | 0.241 | 0.247 | Additive | 0.93 (0.52-1.65) | 0.7946 |
| rs34116584 | AGXT | C>T | 0.277 | 0.227 | Additive | 1.29 (0.73-2.27) | 0.3863 |
| N/A | AGXT | del-74bp | 0.232 | 0.276 | Additive | 0.73 (0.40-1.32) | 0.3029 |
| **SNP** | **GENE** | **BASE CHANGE** | **MAF** | | **MOST SIGNIFICANT GENETIC MODEL** | | |
| | | | Grade ≥3 toxicity | Grade 0-2 toxicity | Model | OR (95% CI) | *p-value* |
| **Neutropenia** | | | | | | | |
| N/A | GSTM1 | null | 0.192 | 0.237 | Additive | 0.73 (0.41-1.32) | 0.2995 |

*Abbreviations: CI=confidence interval; MAF=minor allele frequency; OR=odds ratio; SNP=single-nucleotide polymorphisms.*

**Table 8:** Association between genetic polymorphisms previously related to toxicity in FOLFOX4 metastatic CRC.

## 9.3.2. Polymorphisms and neurological toxicity

Oxaliplatin-based chemotherapy is limited by the occurrence of severe toxicities in a considerable number of patients. Among those side effects, peripheral neurotoxicity is one of the most common and disabling. In particular neurotoxicity of grade ≥ 2 was the primary endpoint for this analysis, because of its prevalence (39%) and clinical relevance (grade 2 indicates moderate motor symptoms and sensory symptoms extended to ankle and wrist, and grade 3 indicates motor symptoms requiring help/assistance and sensory symptoms extended to knee and elbow).

In this study for toxicity analysis we have genotyped 144 patients for 57 genetic polymorphisms in 29 candidate genes. We have found that the minor frequency alleles of *ABCC1 N/A (rs2074087)* and *Asn354Asn (rs35587)* resulted associated with a reduced risk of neurotoxicity. By contrast, the minor frequency alleles of *ABCC2 Ile1324Ile (rs3740066), N/A (rs1885301), N/A (rs4148396),* and *N/A (rs717620)* were associated with an increased risk of neurotoxicity, whereas the minor frequency allele of *Val417Ile (rs2273697)* was associated with a reduced risk of neurotoxicity. Finally for *ABCG2* we have found that the minor frequency allele of *N/A (rs2622604)* was associated with an increased risk of neurotoxicity.

We investigated three genetic models (i.e., dominant, recessive and additive) for the association, and the most statistically significant by Wald $\chi^2$-test was reported in Table 9. All *P*-values were adjusted for sex, age, neo-adjuvant therapy and oxaliplatin cumulative dose normalized by BSA (for neurological toxicity). FDR (false discovery rate) analysis pointed out that five out of eight predictive markers have a *q*-value < 0.1.

| SNP | GENE | BASE CHANGE | MAF | | MOST SIGNIFICANT GENETIC MODEL | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Grade ≥2 toxicity | Grade 0-1 toxicity | Model | OR (95% CI) | *p-value* | *q-value*** |
| **Neurological toxicity** | | | | | | | | |
| rs2074087 | ABCC1 | G>C | 0.0750 | 0.2529 | Additive | 0.43 (0.22-0.86) | 0.0170 | **0.0881** |
| rs35587 | ABCC1 | T>C | 0.2455 | 0.3000 | Dominant | 0.47 (0.23-0.96) | 0.0375 | 0.1049 |
| rs1885301 | ABCC2 | G>A | 0.5982 | 0.4419 | Recessive | 3.06 (1.35-6.92) | 0.0072 | **0.0747** |
| rs717620 | ABCC2 | C>T | 0.2455 | 0.1782 | Recessive | 14.39 (1.63-127.02) | 0.0164 | **0.0881** |
| rs2273697 | ABCC2 | G>A | 0.1340 | 0.2300 | Dominant | 0.44 (0.20-0.98) | 0.0434 | 0.1049 |
| rs4148396 | ABCC2 | C>T | 0.4629 | 0.3512 | Recessive | 4.69 (1.60-13.74) | 0.0048 | **0.0747** |
| rs3740066 | ABCC2 | C>T | 0.4545 | 0.3353 | Recessive | 2.99 (1.16-7.70) | 0.0231 | **0.0958** |
| rs2622604 | ABCG2 | C>T | 0.3092 | 0.2118 | Recessive | 3.61 (1.01-12.88) | 0.0478 | 0.1049 |

*Abbreviations: CI=confidence interval; MAF=minor allele frequency; OR=odds ratio; SNP=single-nucleotide polymorphisms.*
***FDR-adjusted P-value*

**Table 9:** Significant associations between genetic polymorphisms and grade ≥2 neurological toxicity. Associations with q <0.10 are in bold.

### 9.3.3. Polymorphisms and severe (grade ≥3) hematological and non-hematological toxicity

The most common side effects of oxaliplatin in combination with 5-fluorouracil and leucovorin are neurological, as described in the previous paragraph, hematological (anemia, neutropenia, leukopenia, fever with severe neutropenia, thrombocytopenia) and non-hematological (diarrhea, nausea, vomiting, asthenia, alopecia, mucositis, hepatic, infection without severe neutropenia).

Neutropenia (grade 3-4: 38%) was the most common severe hematological toxicity, whereas diarrhea (grade 3-4: 11%) was the most frequent non-hematological severe toxicity.

For grade ≥ 3 neutropenia we have found three significant genetic polymorphisms: *hMSH6 N/A (rs3136228), ABCC1 Asn354Asn (rs35587)* and *ABCC2 N/A (rs717620)*. One of them, *hMSH6 N/A (rs3136228),* was below the FDR threshold.

For non-hematological toxicity we have found six genetic polymorphisms: XRCC3 N/A (rs1799794), Thr241Met (rs861539), XRCC1 N/A (rs3213239), APE1 Asp148Glu (rs1130409) PARP1 Val762Ala (rs1136410), and GSTT1-null (N/A). Only one of them XRCC3 N/A (rs1799794) passed the FDR cutoff and was associated with an increased risk of toxicity.

We investigated three genetic models (i.e., dominant, recessive and additive) for the association, and the most statistically significant by Wald $\chi^2$-test was reported in Table 10. All *P*-values were adjusted for sex, age, neo-adjuvant therapy and oxaliplatin cumulative dose normalized by BSA (for neurological toxicity). FDR (false discovery rate) analysis pointed out that five out of eight predictive markers have a *q*-value < 0.1.

| SNP | GENE | BASE CHANGE | MAF | | MOST SIGNIFICANT GENETIC MODEL | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Grade ≥3 toxicity | Grade 0-2 toxicity | Model | OR (95% CI) | *p-value* | *q-value*** |
| **Neutropenia** | | | | | | | | |
| rs3136228 | hMSH6 | T>G | 0.509 | 0.410 | Recessive | 3.23 (1.38-7.57) | 0.0071 | **0.0937** |
| rs35587 | ABCC1 | T>C | 0.204 | 0.325 | Additive | 0.54 (0.31-0.96) | 0.0368 | 0.1273 |
| rs717620 | ABCC2 | C>T | 0.269 | 0.165 | Additive | 1.81 (1.01-3.26) | 0.0466 | 0.1273 |
| **Any non-hematological toxicity** | | | | | | | | |
| rs1799794 | XRCC3 | A>G | 0.365 | 0.225 | Recessive | 8.90 (2.48-31.97) | 0.0008 | **0.0150** |
| rs861539 | XRCC3 | C>T | 0.2917 | 0.4435 | Additive | 0.49 (0.24-1.00) | 0.0495 | 0.1326 |
| rs3213239 | XRCC1 | In/Del | 0.212 | 0.406 | Additive | 0.39 (0.19-0.82) | 0.0130 | 0.1217 |
| rs1130409 | APE 1 | T>G | 0.6042 | 0.4396 | Additive | 1.99 (1.05-3.74) | 0.0339 | 0.1326 |
| rs1136410 | PARP | T>C | 0.2500 | 0.1710 | Dominant | 2.77 (1.07-7.21) | 0.0366 | 0.1326 |
| N/A | GSTT1 | null | 0.500 | 0.364 | Recessive | 2.82 (1.02-7.83) | 0.0467 | 0.1326 |

*Abbreviations: CI=confidence interval; MAF=minor allele frequency; OR=odds ratio; SNP=single-nucleotide polymorphisms.*
***FDR-adjusted P-value*

**Table 10:** Significant associations between genetic polymorphisms and grade 3-4 toxicity. Associations with q <0.10 are in bold.

## 9.4 Linkage disequilibrium and haplotypes

For ABCC2 (rs1885301; rs717620; rs2273697; rs4148396; rs3740066) were studied the possible relationships of linkage disequilibrium (LD), described by the coefficient of Lewontin's (D') and the coefficient of correlation (r2), and we have then determined statistically the most common haplotypes. In Figure 32 the relationships of linkage among the various markers and the values of D' and $r^2$ are represented graphically; the intensity of the color (black-white) as well as the values of the coefficients indicate the degree of statistical significance of the association.

A strong linkage disequilibrium was found between: rs1885301 and rs717620 (D'=0.94; $r^2$=0.22); rs1885301 and rs4148396 (D'=0.91; $r^2$=0.52); rs717620 and rs4148396 (D'=0.92; $r^2$=0.34); rs717620 and rs3740066 (D'=0.93; $r^2$=0.37); rs4148396 and rs3740066 (D'=0.88; $r^2$=0.71).

Then we performed a haplotype analysis on ABCC2 variants to test whether haplotypes are more predictive than single variants. Haplotypes I (16%, all the protective alleles) and II (19%, all the risk alleles) were selected but no significant association with grade ≥ 2 neurotoxicity was found (Table 11).

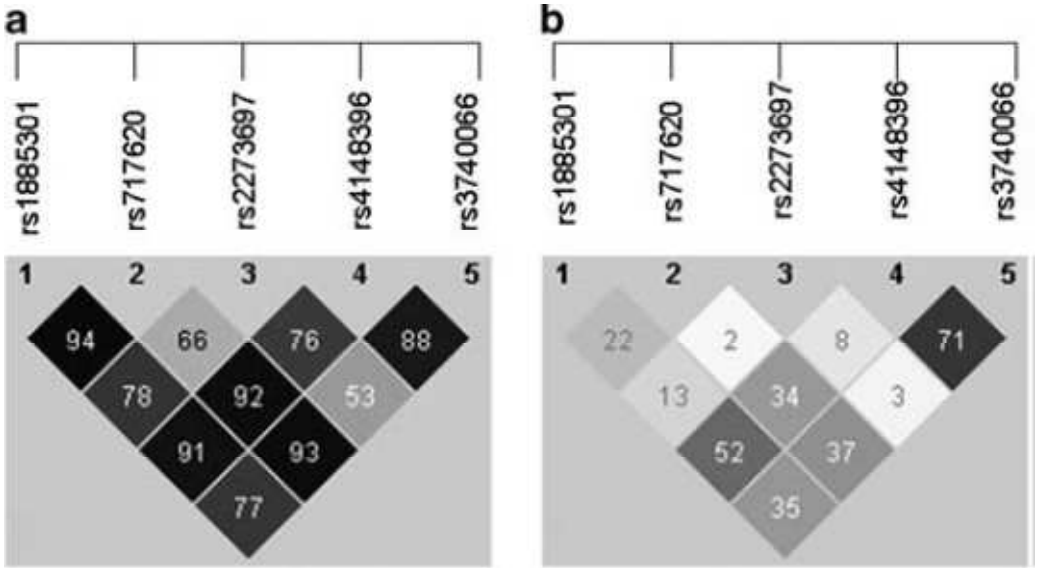


**Figure 32:** Pairwise linkage disequilibrium analysis among ABCC2 polymorphisms associated to neurotoxicity grade ≥ 2. The value within each diamond represents the pairwise correlation between polymorphisms (measured as D' (a) or $r^2$ (b)). The black to white gradient reflects higher to lower values. The plots were generated by HaploView 3.32.

| ABCC2 haplotype | rs1885301 | rs717620 | rs2273697 | rs4148396 | rs3740066 | Model | OR (95% CI) | *p-value* | *q-value*** |
|---|---|---|---|---|---|---|---|---|---|
| I (protective) | G | C | A | C | C | Additive | 0.60 (0.30-1.16) | 0.1296 | - |
| II (risk) | A | T | G | T | T | Additive | 1.72 (0.95-3.09) | 0.0722 | - |

*Abbreviations: CI=confidence interval; OR=odds ratio.*
***FDR-adjusted P-value*

**Table 11:** ABCC2 haplotypes and their association with grade ≥ 2 neurotoxicity.

# 10. *Prostate cancer patients*

## 10.1 Patient's characteristics and treatment

In this study 924 prostate cancer patients were enrolled from CRO-National Cancer Institute of Aviano. 60 of these patients were considered ineligible and were excluded from the analysis due to lack of clinical data. Thus 864 prostate cancer patients were eligible and included in the study. The median age of patients was 67 years (range 44 to 83 years) and all were of Caucasian ethnicity.

To date, 82% of patients are alive with a median follow-up of 73 months (range 1 to 246 months).

All patients included in the study were treated with radiotherapy (adjuvant or radical), some of them have received surgery, others, hormone therapy and the rest (of them) either surgery or hormone therapy. 24% of the total patients found to have a PSA biochemical recurrence.

Demographic and clinical characteristics of the patients are shown in Table 12: in particular Gleason score, TNM grade, stage and PSA at diagnosis were considered as factors of diagnosis.

| Characteristic | N | % |
|---|---|---|
| **Total** | 924 | |
| Eligible | 864 | |
| Ineligible* | 60 | |
| ***AGE*** | | |
| Mean, range | 67 (44-83) | |
| ***FOLLOW-UP** (months)* | | |
| Mean, range | 73 (1-246) | |
| ***STATUS*** | | |
| Alive | 710 | 82.2 |
| Dead | 154 | 17.8 |
| ***GLEASON*** | | |
| ≤ 7 | 634 | 73.4 |
| > 8 | 227 | 26.3 |
| N/A | 3 | 0.3 |
| ***TNM GRADE*** | | |
| 0-2 | 506 | 58.6 |
| *3-4* | 357 | 41.3 |
| *N/A* | 1 | 0.1 |
| ***STAGE*** | | |
| A-B | 288 | 33.3 |
| C-D | 576 | 66.7 |
| ***BIOCHEMICAL PSA RECURRENCE*** | | |
| Yes | 207 | 24.0 |
| No | 657 | 76.0 |
| ***PSA AT DIAGNOSIS (mg/ml)*** | | |
| < 7 | 297 | 7.3 |
| 7-12.9 | 301 | 44.9 |
| ≥ 13 | 260 | 42.9 |
| N/A | 6 | 4.9 |
| **ineligible for lack of clinical data* | | |

**Table 12:** Patient's characteristics.

## 10.2 Polymorphisms and genotyping assay

| Cellular pathway | Gene | rs | Function | AA change | Previously investigated for radiotherapy in PCA | Association with toxicity in at least one study | Analysis method |
|---|---|---|---|---|---|---|---|
| Enzymes of Phase I | CYP3A4 | rs2740574 | neargene-5 | *1B | | | Pyrosequencing |
| | CYP3A5 | rs776746 | splice-3 | *3 | Yes [122] | Yes [122] | Pyrosequencing |
| | CYP2B6 | rs3745274 | missense | Gln172His | | | Pyrosequencing |
| | CYP17A1 | rs743572 | 5'UTR | NA | | | TaqMan |
| | SOD2 | rs4880 | cds-synon | Ala16Val | Yes [82] | | TaqMan |
| | SOD3 | rs1799895 | missense | Arg231Gly | | | Pyrosequencing |
| Oxidative damage | GSTP1 | rs1695 | 5'UTR | Ile105Val | | | TaqMan |
| | | rs1138272 | cds-synon | Ala114Val | | | TaqMan |
| | GSTA1 | rs3957357 | intronic | N/A | | | Pyrosequencing |
| | GSTT1 | N/A | cds-synon | null | | | TaqMan *CNV |
| | GSTM1 | N/A | cds-synon | null | | | TaqMan *CNV |
| | GSTM3 | rs1799735 | intronic | N/A | | | Fragment analysis |
| Apoptosis | TP53 | rs1042522 | 3'UTR | Arg72Pro | Yes [123, 124] | | TaqMan |
| | MDM2 | rs2279744 | Intronic | N/A | Yes [123] | | Pyrosequencing |
| Cell cycle | P21 | rs1801270 | missense | Ser31Arg | Yes [123] | | TaqMan |
| | P22 | rs4673 | missense | Tyr72His | | | TaqMan |
| Folate cycle | MTHFR | rs1801133 | missense | Ala222Val | | | TaqMan |
| | | rs1801131 | missense | Glu429Ala | | | TaqMan |

| Cellular pathway | Gene | rs | Function | AA change | Previously investigated for radiotherapy in PCA | Association with toxicity in at least one study | Analysis method |
|---|---|---|---|---|---|---|---|
| DNA repair | RAD51 | rs1801320 | 5'UTR | N/A | Yes [122] | | Pyrosequencing |
| | hMLH1 | rs1799977 | cds-synon | Ile219Val | Yes [122] | Yes [122] | Pyrosequencing |
| | hMSH2 | rs2303428 | intronic | N/A | | | Pyrosequencing |
| | hOGG1 | rs1052133 | cds-synon | Ser326Cys | Yes [123, 124] | | Pyrosequencing |
| | XRCC1 | rs1799782 | cds-synon | Arg194Trp | Yes [123, 60, 122, 124] | Yes [123] | Pyrosequencing |
| | | rs3213239 | intronic | N/A | Yes [123, 124] | Yes [123] | Pyrosequencing |
| | | rs25487 | cds-synon | Gln399Arg | Yes [123, 60, 124] | Yes [123] | TaqMan |
| | | rs25489 | cds-synon | Arg280His | Yes [123, 82, 122, 124] | Yes [123, 82, 122] | TaqMan |
| | XRCC3 | rs861539 | cds-synon | Thr241Met | Yes [123, 82, 124] | | Pyrosequencing |
| | | rs1799794 | 5'UTR | N/A | Yes [122, 124] | Yes [122] | TaqMan |
| | | rs1799796 | intronic | N/A | Yes [122, 124] | | TaqMan |
| | ERCC1 | rs3212986 | 3'UTR | N/A | Yes [123] | | TaqMan |
| | | rs11615 | cds-synon | Asn118Asn | Yes [123, 60] | | TaqMan |
| | ERCC2 | rs13181 | cds-synon | Lys751Gln | Yes [60, 122, 124] | Yes [122] | Pyrosequencing |
| | | rs1799793 | cds-synon | Asp312Asn | | | Pyrosequencing |
| | APE1 | rs1130409 | cds-synon | Asp148Glu | Yes [123, 124] | | TaqMan |
| | hMSH6 | rs3136228 | 5'UTR | N/A | | | TaqMan |
| | ATM61 | rs1801516 | missense | Asp1853Asn | Yes [123, 125, 122, 74, 124, 126] | Yes [123, 125, 122, 74, 126] | TaqMan |
| | ATM40 | rs1800054 | missense | Ser49Cys | | | TaqMan |
| | MGMT | rs12917 | cds-synon | Leu115Phe | | | TaqMan |
| | PARP1 | rs1136410 | cds-synon | Val762Ala | Yes [60] | | Taqman |
| | hEXO1 | rs4149963 | cds-synon | Thr439Met | | | TaqMan |

| Cellular pathway | Gene | rs | Function | AA change | Previously investigated for radiotherapy in PCA | Association with toxicity in at least one study | Analysis method |
|---|---|---|---|---|---|---|---|
| Angiogenesis | VEGF | rs2010963 | UTR-5 | N/A | Yes [127, 128] | Yes [127, 128] | TaqMan |
| | | rs1570360 | neargene-5 | N/A | Yes [127, 128] | Yes [127, 128] | TaqMan |
| Nitric Oxyde synthase | NOS2A | rs9282801 | intron | N/A | | | TaqMan |
| | NOS3 | rs2070744 | neargene-5 | N/A | | | TaqMan |
| Immune system | IL6 | rs1800795 | neargene-5 | N/A | | | Pyrosequencing |
| | TLR4 | rs4986790 | missense | Asp299Gly | | | Pyrosequencing |
| | | rs4986791 | missense | Thr399Ile | | | Pyrosequencing |
| Cellular transport | ABCB1 | rs1045642 | cds-synon | Ile1145Ile | | | Pyrosequencing |
| | | rs1128503 | cds-synon | Gly412Gly | | | Pyrosequencing |
| | ABCC2 | rs717620 | 5'UTR | N/A | | | Pyrosequencing |
| | | rs2273697 | cds-synon | Val417Ile | | | Pyrosequencing |

*Abbreviations: N/A, not applicable.*
** CNV: Copy Number Variation assay*

**Table 13:** Polymorphisms and genotyping assay.

## 10.3 Treatment and response tolerance

All patients included in the study (864) were treated with radiotherapy (adjuvant or radical), some of them have received surgery (161), others hormone therapy (468) and the rest (of them) either surgery or hormone therapy (155). In particular 208 (24.1%) patients received an adjuvant radiotherapy and 656 (75.9%) received a radical radiotherapy. For the toxicity following radiotherapy, generally patients well tolerated the treatment, with severe toxicity (grade 2) only in 9 out of the 864 patients (1.0%), and with low toxicity (grade 0-1) in 616 patients (71.3%). The most frequent toxicity was urological and/or intestinal. Furthermore for hormone therapy, 291 (33.7%) patients received a partial androgen blockade, while 330 (38.2%) patients received a total androgen blockade.

Finally all the 864 patients were evaluable for the Disease Free Survival (DFS) and Overall Survival (OS) analysis.

All treatment's characteristics of the patients are shown in Table 14.

| Treatment | N | % |
|---|---|---|
| Radiotherapy | 864 | |
| Surgery | 161 | 18.6 |
| Hormone therapy | 468 | 54.2 |
| Surgery + Hormone therapy | 155 | 17.9 |
| ***ANDROGEN BLOCKADE*** | | |
| Partial | 291 | 33.7 |
| Total | 330 | 38.2 |
| N/A | 2 | 0.2 |
| ***RADIOTHERAPY*** | | |
| Adjuvant | 208 | 24.1 |
| Radical | 656 | 75.9 |
| ***RADIOTHERAPY (dose, cycles)*** | | |
| > 7000 (> 33) | 200 | 23.1 |
| 7000 ≥ 7600 (33 ≥ 38) | 641 | 74.2 |
| > 7600 (> 38) | 23 | 2.7 |
| ***TOXICITY*** *(from RT)** | | |
| Urological | 314 | 36.3 |
| *Grade 0* | 2 | |
| *Grade 1* | 308 | |
| *Grade 2* | 4 | |
| Intestinal | 311 | 36.0 |
| *Grade 0* | 1 | |
| *Grade 1* | 305 | |
| *Grade 2* | 5 | |
| *EORTC=European Organisation for Research and Treatment of Cancer | | |

**Table 14:** Treatment's characteristics of the patients.

### 10.3.1. Polymorphisms and risk of biochemical PSA recurrence

The principal aim of this study for prostate cancer patients is to study the effects of the polymorphisms described before on the risk of biochemical PSA recurrence. In total, 864 clinically prostate cancer patients who underwent radiotherapy were included in the final analysis. Among them, 207 (24%) experienced recurrence of PSA during the 73 months (mean). We analyzed 50 polymorphisms in 37 genes. First we have done an univariate analysis of the clinicopathologic features and polymorphisms, then we have analyzed the clinicopathologic variables, that are significant in univariate analysis, in a multivariate analysis. Finally we have adjusted the polymorphisms that are significant in univariate analysis with the clinicopathologic covariates that are significant in multivariate analysis.

## A) Univariate analysis

- ✓ **Clinicopathologic features:** for this analysis we considered Gleason score (2-10), PSA at diagnosis (ng/ml) value, surgery (yes/no), hormone therapy (yes/no), age, TNM grade, stage, androgen blockade (partial/total), radiation therapy dose (Gy), and radiation therapy cycles as clinicopathologic features.

  We reported in Table 15 the variables that resulted statistically significant. In particular patients with a Gleason score higher or equal to 8, have an higher risk of PSA biochemical recurrence (HR=2.02; 95%CI=1.52-2.67; p=<0.0001). In the same way for PSA at diagnosis value, where patients that have a PSA at diagnosis higher or equal to 13 ng/ml, have an higher risk of PSA biochemical recurrence (HR=2.63; 95%CI=1.85-3.74; p=<0.0001). Age is also a very important clinicopathologic feature: patients older than 71 years have a lower risk of PSA biochemical recurrence (HR=0.52; 95%CI=0.37-0.74; p=0.0003).

  Finally for the treatment patients who received surgery or a total androgen blockade have a higher risk of PSA biochemical recurrence (HR=1.47; 95%CI=1.12-1.94; p=0.006 and HR=1.49; 95%CI=1.07-2.07; p=0.0198 respectively); while for radiation therapy dose we have an opposite result (HR=0.71; 95%CI=0.54-0.93; p=0.0124).

| | Total | N° | N relapse | % relapse | HR | 95%CI | p-value |
|---|---|---|---|---|---|---|---|
| **GLEASON SCORE** | (n=861) | | | | | | |
| 2-7 | | 634 | 128 | 62.1% | 1 [#] | | |
| ≥ 8 | | 227 | 78 | 37.9% | 2.02 | 1.52-2.67 | **<0.0001** |
| **PSA AT DIAGNOSIS (ng/ml)** | (n=858) | | | | | | |
| > 7 | | 297 | 47 | 22.8% | 1 [#] | | |
| 7-12.9 | | 301 | 65 | 31.6% | 1.35 | 0.93-1.96 | 0.1498 |
| ≥ 13 | | 260 | 94 | 45.6% | 2.63 | 1.85-3.74 | **<0.0001** |
| **SURGERY** | (n=864) | | | | | | |
| No | | 548 | 116 | 56.0% | 1 [#] | | |
| Yes | | 316 | 91 | 44.0% | 1.47 | 1.12-1.94 | **0.006** |
| **AGE (years)** | (n=864) | | | | | | |
| < 65 | | 293 | 90 | 43.5% | 1 [#] | | |
| 65-71 | | 280 | 68 | 32.9% | 0.76 | 0.56-1.04 | 0.0904 |
| ≥ 71 | | 291 | 49 | 23.6% | 0.52 | 0.37-0.74 | **0.0003** |
| **PARTIAL OR TOTAL ANDROGEN BLOCKADE** | (n=864) | | | | | | |
| No hormone therapy | | 243 | 61 | 29.5% | 1 [#] | | |
| Partial | | 291 | 58 | 28.0% | 0.99 | 0.69-1.43 | 0.9724 |
| Total | | 330 | 88 | 42.5% | 1.49 | 1.07-2.07 | **0.0198** |
| **RT DOSE (Gy)** | (n=864) | | | | | | |
| ≤ 7000 | | 346 | 104 | 50.2% | 1 [#] | | |
| > 7000 | | 518 | 103 | 49.8% | 0.71 | 0.54-0.93 | **0.0124** |

Abbreviations: RT=radiotherapy; *CI=confidence interval; HR=hazard ratio;* [#] *reference category.*

**Table 15:** Significant clinicopathologic features of patients in univariate analysis.

- ✓ **Polymorphisms covariates:** for this analysis we analyzed 50 polymorphisms in 37 genes described before. In Table 16, there are the polymorphisms that are significant in univariate analysis. We have two significant polymorphisms in two different DNA repair genes. Concerning ERCC2 (rs1799793) gene, patients with the heterozygous genotype have a lower risk of PSA biochemical recurrence than wild type genotype (HR=0.67; 95%CI=0.49-0.92; p=0.0123). For the other significant DNA repair gene, XRCC1 (rs1799782), patients with heterozygous genotype have a higher risk of biochemical PSA recurrence than wild type genotype (HR=1.57; 95%CI=1.12-2.19; p=0.0083).

  Then there is a significant enzyme of Phase I, SOD2 (rs4880) associated with an increase of PSA biochemical recurrence: in particular the heterozygous genotype determines a higher risk of recurrence (HR=1.48; 95%CI=1.01-2.17; p=0.0439).

  We also have acquired a significant data for a transporter gene, ABCC2 (rs2273697), where the homozygous variant genotype presents a higher risk of recurrence (HR=2.07; 95%CI=1.26-3.40; p=0.0039).

  Interestingly, the probability of biochemical PSA recurrence was significantly higher in patients with homozygous variant for TP53 (rs1042522) gene (HR=1.86; 95%CI=1.12-3.09; p=0.0171); while for the heterozygous genotype in MDM2 (rs2279744) gene it was lower (HR=0.74; 95%CI=0.55-1.00; p=0.0468).

  Finally we investigated the relationship between polymorphism and the risk of biochemical PSA recurrence in VEGF (rs1570360) and we obtained a significant data for homozygous genotype that results associated with a lower risk of recurrence (HR=0.45; 95%CI=0.25-0.82; p=0.0090).

| | Total | N° | N relapse | % relapse | HR | 95%CI | p-value |
|---|---|---|---|---|---|---|---|
| **XRCC1 *(rs1799782)*** | (n=858) | | | | | | |
| TT | | 724 | 161 | 78.2 | 1[#] | | |
| TC | | 128 | 44 | 21.4 | 1.57 | 1.12-2.19 | **0.0083** |
| CC | | 6 | 1 | 0.5 | 0.61 | 0.09-4.38 | 0.6249 |
| **ERCC2 *(rs1799793)*** | (n=857) | | | | | | |
| AA | | 384 | 113 | 54.9 | 1[#] | | |
| AG | | 348 | 60 | 29.1 | 0.67 | 0.49-0.92 | **0.0123** |
| GG | | 125 | 33 | 16.0 | 1.05 | 0.71-1.55 | 0.8111 |
| **SOD2 *(rs4880)*** | (n=859) | | | | | | |
| TT | | 185 | 34 | 16.5 | | | |
| TC | | 446 | 116 | 56.3 | 1.48 | 1.01-2.17 | **0.0439** |
| CC | | 228 | 56 | 27.2 | 1.45 | 0.95-2.23 | 0.0875 |
| **ABCC2 *(rs2273697)*** | (n=858) | | | | | | |
| AA | | 551 | 123 | 60.0 | 1[#] | | |
| AG | | 268 | 64 | 31.2 | 0.99 | 0.73-1.34 | 0.9318 |
| GG | | 39 | 18 | 8.8 | 2.07 | 1.26-3.40 | **0.0039** |
| **TP53 *(rs1042522)*** | (n=850) | | | | | | |
| GG | | 501 | 125 | 61.0 | 1[#] | | |
| GC | | 298 | 63 | 30.7 | 0.80 | 0.59-1.08 | 0.1464 |
| CC | | 51 | 17 | 8.3 | 1.86 | 1.12-3.09 | **0.0171** |
| **MDM2 (rs2279744)** | (n=858) | | | | | | |
| TT | | 368 | 98 | 47.8 | 1[#] | | |
| TG | | 370 | 78 | 38.1 | 0.74 | 0.55-1.00 | **0.0468** |
| GG | | 120 | 29 | 14.2 | 0.85 | 0.56-1.28 | 0.4275 |
| **VEGF *(rs1570360)*** | (n=854) | | | | | | |
| AA | | 400 | 107 | 52.5 | 1[#] | | |
| AG | | 363 | 85 | 41.7 | 0.88 | 0.66-1.17 | 0.3697 |
| GG | | 91 | 12 | 5.9 | 0.45 | 0.25-0.82 | **0.0090** |

*Abbreviations: CI=confidence interval; HR=hazard ratio; [#] reference category.*

**Table 16:** Significant polymorphisms of patients in univariate analysis.

## B) Multivariate analysis

- ✓ **Clinicopathologic features:** in multivariate analysis only Gleason score, PSA at diagnosis (ng/ml) value and age remain statistically significant in the association with the risk of biochemical PSA recurrence. For other covariates (surgery, partial or total androgen blockade and radiotherapy dose) we did not find any significant correlation with PSA recurrence risk (p=0.7232 for surgery; p=0.9673 and p=0.3544 for partial or total androgen blockade; p=0.7727 for radiotherapy dose).

  Gleason score and PSA at diagnosis are two very important clinicopathologic indicators for the diagnosis of prostate cancer and are currently used to predict therapy outcome. In particular patients with a Gleason score ≥ 8 and/or a PSA at diagnosis ≥ 13 resulted statistically associated with the disease recurrence (HR=1.66; 95%CI=1.21-2.27; p=0.0016 and HR=2.33; 95%CI=1.62-3.36; p=<0.0001 respectively). Furthermore patients older than 71 years have a lower risk of biochemical PSA recurrence compared to younger patients (HR=0.59; 95%CI=0.39-0.89; p=0.0114).

  In Table 17 are summarize the results obtained.

| | Total | N° | N relapse | % relapse | HR | 95%CI | p-value |
|---|---|---|---|---|---|---|---|
| **GLEASON SCORE** | (n=861) | | | | | | |
| 1-7 | | 634 | 128 | 62.1% | 1 [#] | | |
| ≥ 8 | | 227 | 78 | 37.9% | 1.66 | 1.21-2.27 | **0.0016** |
| **PSA AT DIAGNOSIS (ng/ml)** | (n=858) | | | | | | |
| > 7 | | 297 | 47 | 22.8% | 1 [#] | | |
| 7-12.9 | | 301 | 65 | 31.6% | 1.31 | 0.90-1.91 | 0.1650 |
| ≥ 13 | | 260 | 94 | 45.6% | 2.33 | 1.62-3.36 | **<0.0001** |
| **SURGERY** | (n=864) | | | | | | |
| No | | 548 | 116 | 56.0% | 1 [#] | | |
| Yes | | 316 | 91 | 44.0% | 1.10 | 0.65-1.86 | 0.7232 |
| **AGE (years)** | (n=864) | | | | | | |
| < 65 | | 293 | 90 | 43.5% | 1 [#] | | |
| 65-71 | | 280 | 68 | 32.9% | 0.85 | 0.61-1.19 | 0.3360 |
| ≥ 71 | | 291 | 49 | 23.6% | 0.59 | 0.39-0.89 | **0.0114** |
| **PARTIAL OR TOTAL ANDROGEN BLOCKADE** | (n=864) | | | | | | |
| No hormone therapy | | 243 | 61 | 29.5% | 1 [#] | | |
| Partial | | 291 | 58 | 28.0% | 1.01 | 0.68-1.49 | 0.9673 |
| Total | | 330 | 88 | 42.5% | 1.20 | 0.81-1.78 | 0.3544 |
| **RT DOSE (Gy)** | (n=864) | | | | | | |
| ≤ 7000 | | 346 | 104 | 50.2% | 1 [#] | | |
| > 7000 | | 518 | 103 | 49.8% | 0.93 | 0.57-1.52 | 0.7727 |

*Abbreviations: RT=radiotherapy; CI=confidence interval; HR=hazard ratio; [#] reference category.*

**Table 17:** Significant clinicopathologic features of patients in multivariate analysis.

## C) Adjustment of polymorphisms for clinicopathologic features

In the multivariate Cox proportional hazard model we have adjusted the clinicopathologic features that were significant in the previous multivariate analysis (Gleason score, PSA at diagnosis, age) with the polymorphisms that were significant in the univariate analysis (XRCC1 -*rs1799782*-; ERCC2 -*rs1799793*-; SOD2 -*rs4880*-; ABCC2 -*rs2273697*-; TP53 -*rs1042522*-; MDM2 -*rs2279744*-; VEGF -*rs1570360*-).

After adjustment with the clinicopathologic covariates only four polymorphisms remained significant.

✓ **XRCC1 *(rs1799782)*:**

| | Total | N° | N relapse | % relapse | HR | 95%CI | p-value |
|---|---|---|---|---|---|---|---|
| **GLEASON SCORE** | (n=861) | | | | | | |
| 1-7 | | 634 | 128 | 62.1% | 1 [#] | | |
| ≥ 8 | | 227 | 78 | 37.9% | 1.73 | 1.30-2.32 | **0.0002** |
| **PSA AT DIAGNOSIS (ng/ml)** | (n=858) | | | | | | |
| > 7 | | 297 | 47 | 22.8% | 1 [#] | | |
| 7-12.9 | | 301 | 65 | 31.6% | 1.33 | 0.91-1.95 | 0.1360 |
| ≥ 13 | | 260 | 94 | 45.6% | 2.43 | 1.70-3.47 | **<0.0001** |
| **AGE (years)** | (n=864) | | | | | | |
| < 65 | | 293 | 90 | 43.5% | 1 [#] | | |
| 65-71 | | 280 | 68 | 32.9% | 0.81 | 0.59-1.11 | 0.1942 |
| ≥ 71 | | 291 | 49 | 23.6% | 0.54 | 0.38-0.78 | **0.0008** |
| **XRCC1 *(rs1799782)*** | (n=864) | | | | | | |
| TT | | 724 | 161 | 78.2% | 1 [#] | | |
| TC+CC | | 134 | 45 | 21.8% | 1.51 | 1.08-2.10 | **0.0153** |

*Abbreviations: CI=confidence interval; HR=hazard ratio; [#] reference category.*

**Table 18:** Association between XRCC1 (rs1799782) polymorphism and clinicopathologic features among prostate cancer patients.

this polymorphism, in particular the heterozygous and the homozygous variant genotype, was associated with a higher risk of biochemical PSA recurrence (HR=1.51; 95%CI=1.08-2.10; p=0.0153). After adjusting for the clinicopathologic covariates (Gleason, PSA at diagnosis, age), the XRCC1 TC and CC genotypes remained

independent risk factors for biochemical PSA recurrence after radiotherapy (Table 18).

✓ **ERCC2 *(rs1799793)*:**

| | Total | N° | N relapse | % relapse | HR | 95%CI | p-value |
|---|---|---|---|---|---|---|---|
| **GLEASON SCORE** | (n=861) | | | | | | |
| 1-7 | | 634 | 128 | 62.1% | 1[#] | | |
| ≥ 8 | | 227 | 78 | 37.9% | 1.83 | 1.37-2.45 | **<0.0001** |
| **PSA AT DIAGNOSIS (ng/ml)** | (n=858) | | | | | | |
| > 7 | | 297 | 47 | 22.8% | 1 [#] | | |
| 7-12.9 | | 301 | 65 | 31.6% | 1.31 | 0.90-1.92 | 0.1600 |
| ≥ 13 | | 260 | 94 | 45.6% | 2.33 | 1.63-3.32 | **<0.0001** |
| **AGE (years)** | (n=864) | | | | | | |
| < 65 | | 293 | 90 | 43.5% | 1 [#] | | |
| 65-71 | | 280 | 68 | 32.9% | 0.85 | 0.61-1.17 | 0.3033 |
| ≥ 71 | | 291 | 49 | 23.6% | 0.56 | 0.39-0.80 | **0.0015** |
| **ERCC2 *(rs1799793)*** | (n=864) | | | | | | |
| AA | | 384 | 113 | 54.9% | 1 [#] | | |
| AG | | 348 | 60 | 29.1% | 0.67 | 0.49-0.93 | **0.0148** |
| GG | | 125 | 33 | 16.0% | 0.99 | 0.67-1.46 | 0.9453 |

*Abbreviations: CI=confidence interval; HR=hazard ratio; [#] reference category.*

**Table 19:** Association between ERCC2 (rs1799793) polymorphism and clinicopathologic features among prostate cancer patients.

after adjusting for clinicopathologic features, ERCC2 (rs1799793) remained a significant and independent predictor of biochemical PSA recurrence after radiation therapy. In particular patients with the heterozygous variant genotype have a lower risk of PSA failure (HR=0.67; 95%CI=0.49-0.93; p=0.0148) (Table 19).

✓ **TP53 *(rs1042522)*:**

| | Total | N° | N relapse | % relapse | HR | 95%CI | p-value |
|---|---|---|---|---|---|---|---|
| **GLEASON SCORE** | (n=861) | | | | | | |
| 1-7 | | 634 | 128 | 62.1% | 1[#] | | |
| ≥ 8 | | 227 | 78 | 37.9% | 1.76 | 1.32-2.36 | **0.0001** |
| **PSA AT DIAGNOSIS (ng/ml)** | (n=858) | | | | | | |
| > 7 | | 297 | 47 | 22.8% | 1 [#] | | |
| 7-12.9 | | 301 | 65 | 31.6% | 1.34 | 0.91-1.95 | 0.1354 |
| ≥ 13 | | 260 | 94 | 45.6% | 2.41 | 1.68-3.45 | **<0.0001** |
| **AGE (years)** | (n=864) | | | | | | |
| < 65 | | 293 | 90 | 43.5% | 1 [#] | | |
| 65-71 | | 280 | 68 | 32.9% | 0.77 | 0.56-1.07 | 0.1174 |
| ≥ 71 | | 291 | 49 | 23.6% | 0.55 | 0.39-0.79 | **0.0011** |
| **TP53 *(rs1042522)*** | (n=864) | | | | | | |
| GG | | 501 | 125 | 61.0% | 1 [#] | | |
| GC | | 298 | 63 | 30.7% | 0.88 | 0.64-1.19 | 0.4028 |
| CC | | 51 | 17 | 8.3% | 2.21 | 1.32-3.70 | **0.0027** |

*Abbreviations: CI=confidence interval; HR=hazard ratio; [#] reference category.*

**Table 20:** Association between TP53 (rs1042522) polymorphism and clinicopathologic features among prostate cancer patients.

in this analysis, TP53 (rs1042522) polymorphism was associated with post-radiotherapy biochemical PSA recurrence. In particular CC genotype was associated with a higher risk of biochemical PSA recurrence, and this variant TP53 genotype remained an independent predictive factor after adjusting for other clinicopathologic covariates (HR=2.21; 95%CI=1.32-3.70; p=0.0027) (Table 20).

✓ **VEGF *(rs1570360)*:**

| | Total | N° | N relapse | % relapse | HR | 95%CI | p-value |
|---|---|---|---|---|---|---|---|
| **GLEASON SCORE** | (n=861) | | | | | | |
| 1-7 | | 634 | 128 | 62.1% | 1[#] | | |
| ≥ 8 | | 227 | 78 | 37.9% | **1.75** | **1.31-2.33** | **0.0002** |
| **PSA AT DIAGNOSIS (ng/ml)** | (n=858) | | | | | | |
| > 7 | | 297 | 47 | 22.8% | 1[#] | | |
| 7-12.9 | | 301 | 65 | 31.6% | 1.31 | 0.90-1.91 | 0.1641 |
| ≥ 13 | | 260 | 94 | 45.6% | **2.31** | **1.61-3.29** | **<0.0001** |
| **AGE (years)** | (n=864) | | | | | | |
| < 65 | | 293 | 90 | 43.5% | 1[#] | | |
| 65-71 | | 280 | 68 | 32.9% | 0.80 | 0.58-1.10 | 0.1659 |
| ≥ 71 | | 291 | 49 | 23.6% | **0.55** | **0.38-0.78** | **0.0009** |
| **VEGF *(rs1570360)*** | (n=854) | | | | | | |
| AA | | 400 | 107 | 52.4% | 1[#] | | |
| AG | | 363 | 85 | 41.7% | 0.92 | 0.69-1.22 | 0.5520 |
| GG | | 91 | 12 | 5.9% | **0.52** | **0.29-0.95** | **0.0332** |

*Abbreviations: CI=confidence interval; HR=hazard ratio; [#] reference category.*

**Table 21:** Association between VEGF (rs1570360) polymorphism and clinicopathologic features among prostate cancer patients.

In this multivariate Cox proportional hazard model including the clinicopathologic features (Gleason, PSA at diagnosis and age), the VEGF (rs1570360) polymorphism, in particular GG genotype, remained significant factor predicting for lower risk of biochemical PSA recurrence (HR=0.52; 95%CI=0.29-0.95; p=0.0332) (Table 21).

## 10.3.2. Polymorphisms and Time to Recurrence (TTR)

In this part of the study we assessed the role of various genetic variants influencing the time to recurrence (Time to Recurrence, TTR) of each patient. The study was conducted on all eligible patients (864). 207 patients were in relapse (24.0%) and 657 non-recurrence (76.0%). The median time to recurrence was 45 months (range 0.0-166.5).

For each genotype of the various polymorphisms considered, it was obtained, according to the representation of Kaplan-Meier analysis, the curve representing the time to recurrence and it has been calculated the median time to recurrence. The various curves obtained were then compared using the log-rank statistical test. The results obtained are shown below. From this analysis, four genetic markers were significant in describing the time to recurrence, the polymorphisms XRCC1 (rs1799782) (Fig.33) (Table 22), ERCC2 (rs1799793), TP53 (rs1042522), and VEGF (rs1570360) (Fig. 36) (Table 25).

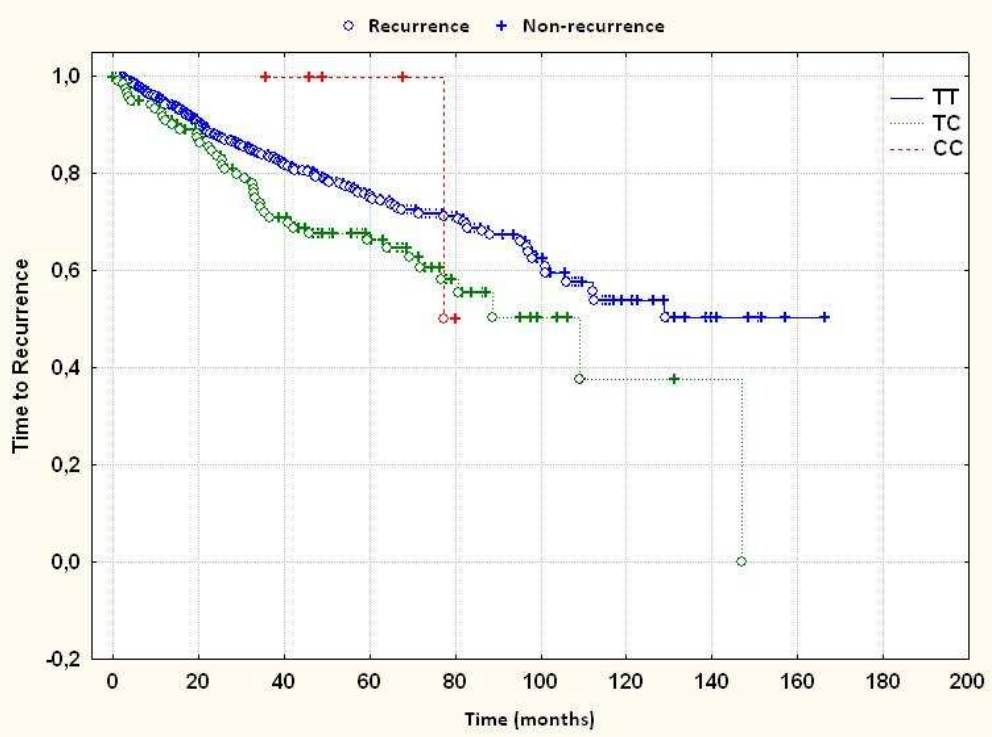


**Figure 33:** Time to Recurrence according with the genotypes of XRCC1 (rs1799782).

| | N° Total | N relapse | TTR (median) | Log rank test (p) |
|---|---|---|---|---|
| **XRCC1 *(rs1799782)*** | | | | |
| TT | 724 | 161 | N/A | |
| TC | 128 | 44 | 89.4 | **0.023** |
| CC | 6 | 1 | N/A | |

**Table 22:** XRCC1 (rs1799782) and time to recurrence (TTR).

Patients with wild type genotype for ERCC2 (rs1799793) polymorphism have a significantly lower risk of recurrence than patients with homozygous variant genotype (122.60 vs 93.4 months, p=0.039) (Fig. 34) (Table 23).

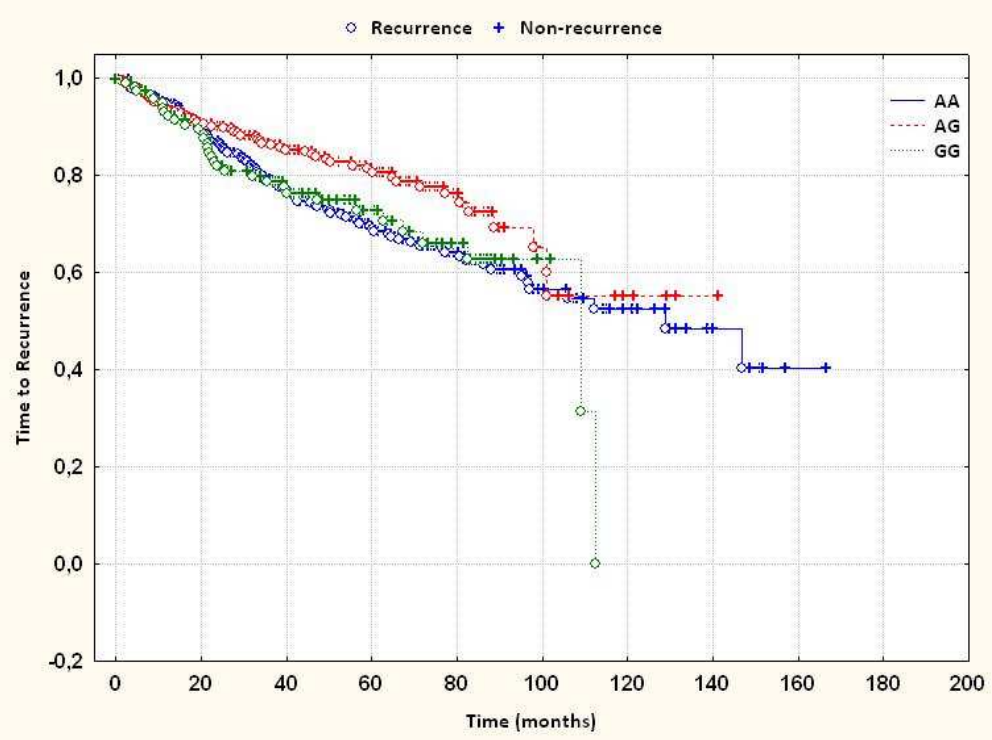


**Figure 34:** Time to Recurrence according with the genotypes of ERCC2 (rs1799793).

| | N° Total | N relapse | TTR (median) | Log rank test (p) |
|---|---|---|---|---|
| **ERCC2 *(rs1799793)*** | | | | |
| AA | 384 | 113 | 122.60 | |
| AG | 348 | 60 | N/A | **0.039** |
| GG | 125 | 33 | 93.4 | |

**Table 23:** ERCC2 (rs1799793) and time to recurrence (TTR).

By contrast, individuals with the genotype heterozygous for the TP53 (rs1042522) polymorphism showed a median time to recurrence significantly longer than those with the wild type or homozygous variant genotype (127.89 vs 112.85 or 62.78 months, p=0.022) (Fig. 35) (Table 24).

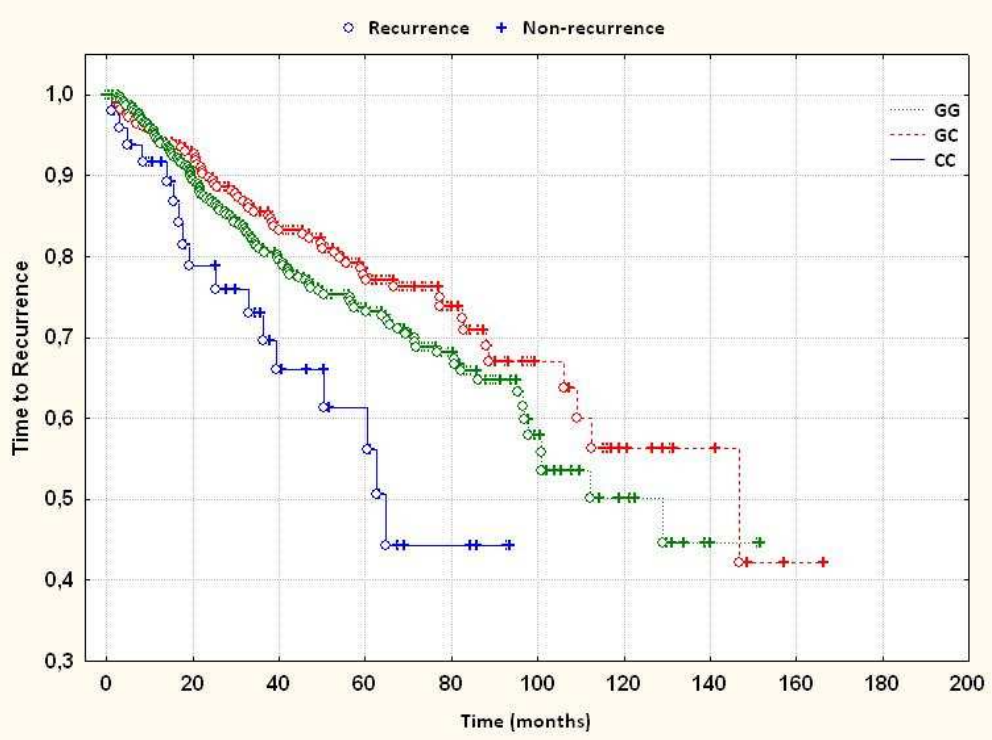


**Figure 35:** Time to Recurrence according with the genotypes of TP53 (rs1042522).

| | N° Total | N relapse | TTR (median) | Log rank test (p) |
|---|---|---|---|---|
| **TP53 *(rs1042522)*** | | | | |
| GG | 501 | 125 | 112.85 | |
| GC | 298 | 63 | 127.89 | **0.022** |
| CC | 51 | 17 | 62.78 | |

**Table 24:** TP53 (rs1042522) and time to recurrence (TTR).

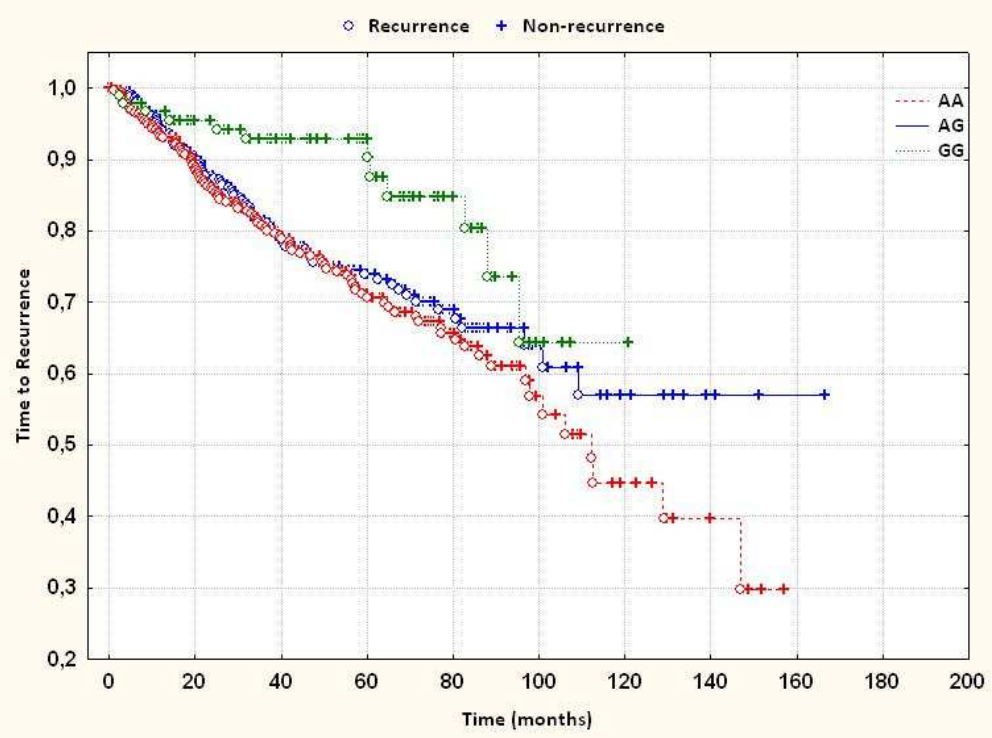


**Figure 36:** Time to Recurrence according with the genotypes of VEGF (rs1570360).

| | N° Total | N relapse | TTR (median) | Log rank test (p) |
|---|---|---|---|---|
| **VEGF *(rs1570360)*** | | | | |
| AA | 400 | 107 | 108.79 | |
| AG | 363 | 85 | N/A | **0.012** |
| GG | 91 | 12 | N/A | |

**Table 25:** VEGF (rs1570360) and time to recurrence (TTR).

### 10.3.3. Polymorphisms and Overall Survival (OS)

The informations of survival was available for all 864 patients was possible to obtain the informations of survival: at the end of the clinical trial, 154 (17.8%) patients had died and 710 (82.2%) were still alive. The median survival, calculated as patients died, is 72.1 months (range 3.4-245.6).

Analyzing one by one all the genetic markers under investigation, for each genotype the survival curve was derived according to the method of Kaplan-Meier and median survival (OS) was calculated; using the log-rank test, the difference between the same curves obtained was then evaluated statistically. Below are shown the results obtained from the analysis; in particular are reported associations that have shown a degree of significance less than 0.5. From this analysis, three genetic markers were significant in describing the overall survival, the polymorphisms XRCC1 (rs1799782), RAD51 (rs1801320), NOS2A (rs9282801).

Patients with wild type genotype for XRCC1 (rs1799782) polymorphism showed a significantly longer median survival than patients with heterozygous genotype (168.35 vs 137.17 months, p = 0.040). While patients with homozygous genotype did not reach the median survival (Fig. 37) (Table 26).

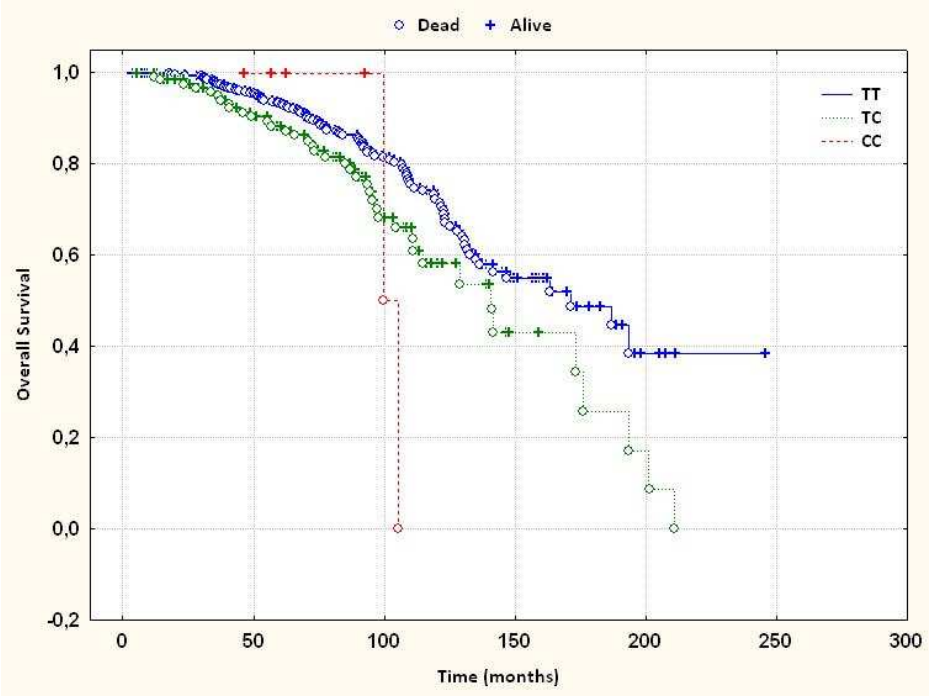


**Figure 37:** Overall Survival according with the genotypes of XRCC1 (rs1799782).

| | N° Total | N relapse | OS (median) | Log rank test (p) |
|---|---|---|---|---|
| **XRCC1 *(rs1799782)*** | | | | |
| TT | 724 | 113 | 168.35 | |
| TC | 128 | 39 | 137.17 | **0.040** |
| CC | 6 | 2 | N/A | |

**Table 26:** XRCC1 (rs1799782) and time to recurrence (TTR).

For RAD51 (rs1801320) polymorphism, the heterozygous genotype seems to have a protective role for the survival in prostate cancer: in fact the median survival for the patients with heterozygous genotype is greater than patients with wild type genotype (161.00 vs 157.44 months, p=0.023). Also in this case the median survival for the homozygous genotype has not been reached (Fig. 38) (Table 27).

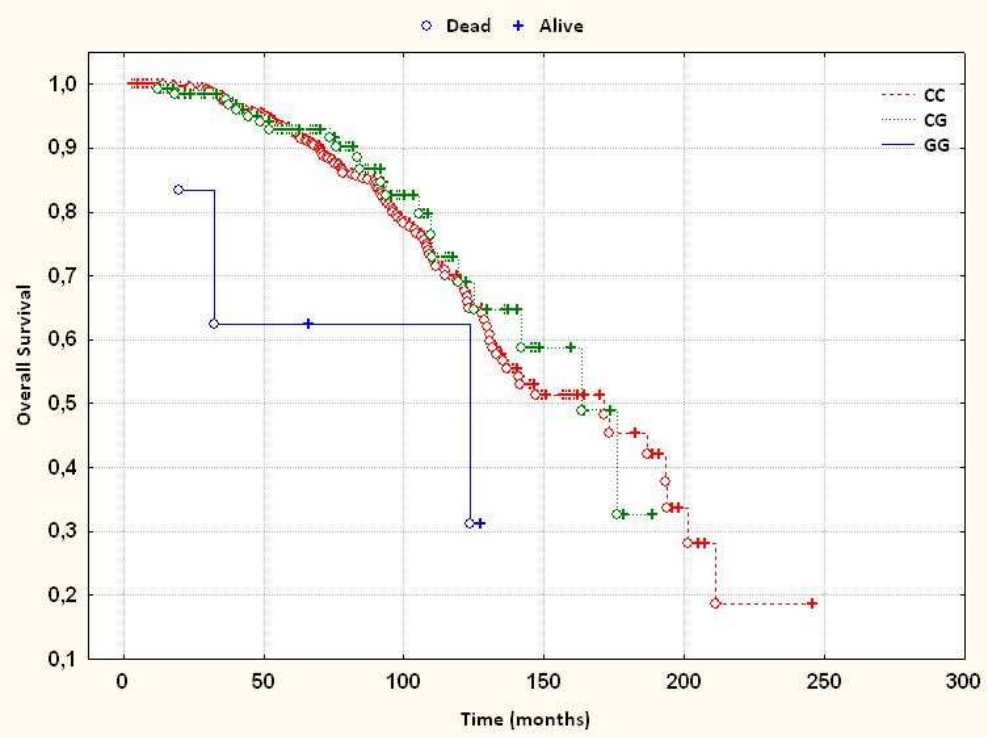


**Figure 38:** Overall Survival according with the genotypes of RAD51 (rs1801320).

| | N° Total | N relapse | OS (median) | Log rank test (p) |
|---|---|---|---|---|
| **RAD51 *(rs1801320)*** | | | | |
| CC | 715 | 129 | 157.44 | |
| CG | 140 | 22 | 161.00 | **0.023** |
| GG | 6 | 3 | N/A | |

**Table 27:** RAD51 (rs1801320) and time to recurrence (TTR).

Finally a significant association between Overall Survival and genotypes was found for the NOS2A (rs9282801) polymorphism: patients with wild type genotype survive longer than patients with heterozygous genotype (181.80 vs 135.77 months, p=0.003) (Fig. 39) (Table 28).

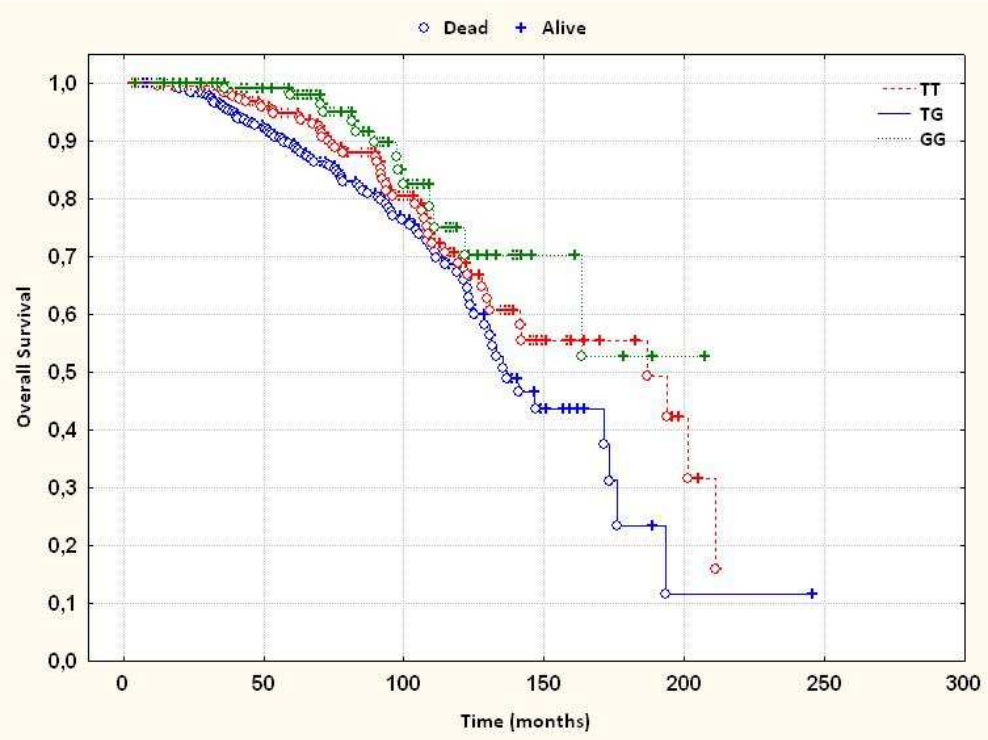


**Figure 39:** Overall Survival according with the genotypes of NOS2A (rs9282801).

| | N° Total | N relapse | OS (median) | Log rank test (p) |
|---|---|---|---|---|
| **NOS2A *(rs9282801)*** | | | | |
| TT | 312 | 50 | 181.80 | **0.003** |
| TG | 419 | 90 | 135.77 | |
| GG | 123 | 14 | N/A | |

**Table 28:** NOS2A (rs9282801) and time to recurrence (TTR).

# 11. Developments of diagnostic kits

The development of diagnostic kits easy and practical to manage results, therefore, of particular utility because, allowing the preliminary analysis of the genetic characteristics of the patient, can provide to oncologists an additional tool in the choice of the most appropriate drug and the optimal dose for the individual. The diagnostic kits are innovative tools, useful to derive from a simple blood test valuable information on the activity of anticancer drugs, particularly *Fluoropyrimidines, Taxanes,* and *Irinotecan* administered to patients in terms of toxicity and response.

## ✓ Fluoropyrimidines

The treatment with fluoropyrimidine has the thymidylate synthase (TYMS), whose expression levels can be critical in influencing the clinical outcome of drug treatment with these anti-cancer agents [129,130], as its main intracellular target. It has been described for the TYMS gene, a genetic variation that is the variability in the number of repetitions (2 or 3) of a sequence of 28 bases located in the promoter region of the gene. Several *in vitro* and *in vivo* studies have shown that the polymorphic allele 3R (characterized by 3 repetitions) is associated with an increase of expression and enzyme activity [131,132]. The allelic variant of the TYMS gene characterized by 2 repetitions (2R) was associated with a significant increase in the incidence of adverse events to a therapy based on the use of 5-fluorouracil (5-FU) in colorectal cancer patients [132,133]. The same 2R allele has been associated, in many studies, also to a better therapeutic outcome, both in terms of tumor response and survival and time to progression in patients treated with regimens containing 5-FU [132,134,135], although tumor response to capecitabine was higher in colorectal cancer patients with the genotype 2R/2R of TYMS gene [136] (Table 29).

A primary role in catabolic fluoropyrimidines inactivating and then in determining the levels of active drug available in the patient is played by the dihydropyrimidine dehydrogenase gene (DPYD). In particular, a polymorphism characterizing the gene coding for DPYD at the level of intron 14 (IV14+1G>A) is responsible for an error of splicing of mRNA associated with

the production of an nonfunctional enzyme [137,138]. This polymorphism is relatively rare in the population, with a frequency of allele variation <1% in Caucasians [139] but carriers of the allele variant IV14+1A, even in the heterozygous state, are exposed, following chemotherapy with fluoropyrimidine, to high-risk to develop toxicity of grade 3 and 4 with results sometimes lethal [140,141].

| | Genotype | Effect on toxicity risk | | *In vitro/ in vivo* | References |
|---|---|---|---|---|---|
| **TYMS** | | | | | |
| 2 or 3 repeats of 28 bp | 2R/2R | ++ | *Possible increased risk* | *In vivo* | [132, 133] |
| | 2R/3R | / | *No indication* | | |
| | 3R/3R | / | *No indication* | | |
| **DYPD** | | | | | |
| IV14+1 G>A | GG | / | *No indication* | *In vivo* | [140, 141] |
| | GA | ++ | *Possible increased risk* | | |
| | AA | **+++** | ***Strong increase in the risk of severe toxicity even lethal*** | | |
| **MTHFR** | | | | | |
| 677 C>T | CC | / | *No indication* | *In vitro* and *in vivo* | [142, 143-146] |
| | CT | + | *Possible small increased risk (possible increased efficacy)* | | |
| | TT | + | *Possible small increased risk (possible increased efficacy)* | | |
| 1298 A>C | AA | / | *No indication* | *In vivo* | [140] |

**Table 29:** Genes that are associated with toxic effects following treatment with fluoropyrimidines.

A protein encoded by a gene particularly polymorphic, which in turn plays a key role in the metabolism and homeostasis of folate is the 5-10-methylenetetrahydrofolate reductase (MTHFR) which can in turn be indirectly implicated in the mechanism of action fluoropyrimidines. The 677C>T polymorphism in MTHFR gene, which leads to an amino acid change in the codon 222 (Ala222Val) [147,148], and 1298A>C polymorphism, which leads to an amino acid variation at codon 429 (Glu429Ala) [149,150] are in reciprocal relationship of linkage disequilibrium and have a significantly different distribution in the various regions and ethnic groups [153,154]. Both polymorphisms have been associated with a reduced activity of MTHFR

enzyme resulting in increased levels of homocysteine and abnormal intracellular distribution of folate [147,151,152].

Some *in vitro* studies and in mouse models have suggested that both genetic variation 677C>T and 1298A>C may increase the chemosensitivity to 5-FU [143,145]. The effect of these variations on the outcome of therapy with fluoropyrimidine in patients has not yet been fully elucidated, however, several clinical studies conducted in patients with metastatic colorectal cancer treated with therapy including 5-FU, showed a significant increase in tumor response, time to progression and survival in patients carrying the allele variant 677T [142,146,144]. In addition, the polymorphic variant 1298C is associated with an increased toxicity after treatment with fluoropyrimidine [140]. The effect of this allelic variant on the prognosis of patients treated with fluoropyrimidine has not yet been elucidated [140,143,155,156].

## ✓ Taxanes

The oxidative metabolism of taxanes is mediated by different isoforms of cytochrome P-450, (3A4 and 3A5), which are characterized by a marked inter-individual variability which can then modify the bio-availability. Although not completely understood, the cause of this variability can be found in the presence of genetic polymorphisms. In particular, the polymorphism CYP3A4*1B (-392A>G), located at the level of the regulatory region of untranslated 5' of the gene, seems to be associated with an increase in the level of protein expression and of its oxidative metabolism efficiency [157,158]. Referring to the isoform CYP3A5, CYP3A5*3 (6986A>G) polymorphism, located in intron 3 of the gene, is associated with enzymatic expression levels. In particular, the absence of the polymorphism (variant CYP3A5*1) in at least one of the two alleles carried by the patient, is reflected in a dramatic increase in the level of expression of the cytochrome CYP3A5, which leads it to represent 50% of the total content of liver enzymes of the CYP3A subfamily. The inter-ethnic variability in the prevalence of this polymorphism may be associated, then, with differences in the degree of expression of this isoform in different ethnic groups [159]. It has been reported as such genetic variations, and the relative change of expression and activity of the corresponding enzymes, it can be translated into an an alteration of the metabolism of the

compounds and drug substrates of CYP3A4 and CYP3A5, including precisely the taxanes, with potential consequences on the toxicity produced in the patient [160-163] (Table 30).

| | Genotype | Effect on toxicity risk | | *In vitro/ in vivo* | References |
|---|---|---|---|---|---|
| **CYP3A4** | | | | | |
| -392 A>G | AA | / | *No indication* | *In vitro* | [157, 158] |
| | AG | / | *No indication* | | |
| | GG | - | *Possible decreased risk* | | |
| **CYP3A5** | | | | | |
| 6986 A>G | AA | - | *Possible decreased risk* | *In vitro* | [160-163] |
| | AG | / | *No indication* | | |
| | GG | / | *No indication* | | |
| **ABCB1** | | | | | |
| 3435 C>T | CC | / | *No indication* | *in vivo* | [163, 164] |
| | CT | + | *Possible small increased risk* | | |
| | TT | ++ | *Possible increased risk* | | |
| 1236 C>T | CC | / | *No indication* | *In vivo* | [165, 166] |
| | CT | / | *No indication* | | |
| | TT | ++ | *Possible increased risk* | | |

**Table 30:** Genes that are associated with toxic effects following treatment with taxanes.

The taxanes are also substrates for the membrane transporter P-glycoprotein (P-gp) belonging to the family of ATP binding cassette proteins and encoded by the ABCB1 gene. Genetic alterations of the ABCB1 gene, associated with changes in activity and/or expression of this transporter, may cause oscillations in intracellular drug levels with an effect on its cytotoxic power [167,168]. In particular, the 3435C>T polymorphism in exon 26 of the gene, was associated with changes in the level of expression of P-gp [169-171] and in the efficiency of intra-cellular transport of the molecules that constitute a substrate, such, for example, digoxin [17]. The 3435C>T polymorphism in ABCB1 gene, has also been implicated in the development of toxic effects associated with chemotherapy with taxanes. In particular, an analysis of 58 patients with solid tumors treated with docetaxel, patients carrying the 3435TT genotype were exposed to increased haematological toxicity [163]. In addition, a study of 26 patients with advanced solid tumors treated with paclitaxel showed that individuals carrying at least

one allele variant 3435T showed a significant increase in the risk of developing neutropenia and peripheral neuropathy associated with therapy [164]. Some authors have suggested also an effect of the 1236 C> T polymorphism in ABCB1 gene, located in exon 12, and in linkage disequilibrium with the variation 3435C> T, on the stability of the mRNA of the gene and, consequently, on its expression [172]. Two clinical studies have been recently pubished in support of an association between this polymorphism and pharmacokinetics of taxanes. In particular, the published results suggest a possible role of polymorphism in influencing the clearance of docetaxel and paclitaxel in two independent studies [165,166]. These associations support the possibility of an involvement of these polymorphisms, in gene ABCB1, in systemic exposure to the drug and therefore the possibility of developing toxic effects consequently to therapy.

## ✓ Irinotecan

Irinotecan is a prodrug that is converted *in vivo* into its active metabolite SN-38 through a hydrolysis reaction by the isoforms 1 and 2 of the human carboxylesterase. The subsequent conversion of SN-38 in its inactive derivative takes place by conjugation of the drug with a molecule of glucuronic acid operated by enzymes belonging to the family of uridino-glucuronosyltransferase 1A (UGT1A) and mainly by UGT1A1 isoform. This enzyme and in particular its polymorphism UGT1A1*28, which consists in the variability of the number of repetitions (commonly 6 or 7 in the Caucasian population) of the TA microsatellite in the promoter region of the gene, have been extensively studied for their important implications from the point of pharmacogenetic view. Such polymorphic variant that causes a decreased gene expression and, below, a decreased ability of the individual glucuronidation [173-175], was found to be associated with a significant increase in the risk of developing severe haematological and non-haematological toxicities (especially diarrhea and neutropenia) after therapy based on the administration of irinotecan. In particular, individuals carrying the allele variant characterized by seven TA repeats were more exposed to the drug's active metabolite, SN-38, and to its toxic effects [176-182]. Based on the findings in the literature on this item, in July 2005, the American Food and Drug Administration (FDA) has decided to include in the booklet of the irinotecan, an indication of pharmacogenetics, recommending

an adjustment of the dose to administer to the patient in treatment with irinotecan on the basis of its genotype in UGT1A1*28 polymorphism (Revised Irinotecan label, American Food and Drug Administration; http://www.fda.gov). Additional studies are still ongoing in order to establish conclusively the correct dosage to be used in each group of patients with different genotype for UGT1A1*28 [177,183,184] (Table 31).

| | Genotype | Effect on toxicity risk | | *In vitro/ in vivo* | References |
|---|---|---|---|---|---|
| **UGT1A1** | | | | | |
| 6 or 7 repeats of TA (UGT1A1*28) | 6/6 | / | *No indication* | *In vivo* | [176, 185] |
| | 6/7/ | + | *Possible small increased risk* | | |
| | 7/7 | ++ | *Possible increased risk* | | |
| **CYP3A4** | | | | | |
| -392 A>G | AA | / | *No indication* | *In vitro* | [186-187] |
| | AG | / | *No indication* | | |
| | GG | - | *Possible decreased risk* | | |
| **CYP3A5** | | | | | |
| 6986 A>G | AA | - | *Possible decreased risk* | *In vitro* and *in vivo* | [188-191] |
| | AG | / | *No indication* | | |
| | GG | / | *No indication* | | |
| **ABCB1** | | | | | |
| 1236 C>T | CC | / | *No indication* | *In vitro* | [190-191] |
| | CT | + | *Possible small increased risk* | | |
| | TT | + | *Possible small increased risk* | | |

**Table 31:** Genes that are associated with toxic effects following treatment with irinotecan.

once administered Irinotecan to the patient, there may be, in addition to the activation process described above, with an oxidative reaction that transforms it into derivatives not pharmacologically active and which is realized by the 3A4 and 3A5 isoforms of cytochrome P-450 (CYP3A4 and CYP3A5). The isoforms CYP3A4 and CYP3A5 are characterized by a marked inter-individual variability in terms of expression and enzymatic activities that may be reflected in a change in the bio-availability of irinotecan. Although not completely understood, the cause of this variability can be found in the presence of genetic polymorphisms. In particular, with the abbreviation CYP3A4*1B is identified a polymorphism

(-392A>G) located at the level of the regulatory region of untranslated 5' of the gene, which seems to be associated with an increase in the level of protein expression and of its oxidative metabolism efficiency [186-187]. Referring to the isoform CYP3A5, CYP3A5*3 (6986A>G) polymorphism, located in intron 3 of the gene, is associated with enzymatic expression levels. In particular, the absence of the polymorphism (variant CYP3A5*1) in at least one of the two alleles carried by the patient, is reflected in a dramatic increase in the level of expression of the cytochrome CYP3A5, which leads him to represent 50% of the total content of liver enzymes of the CYP3A subfamily. The inter-ethnic variability in the prevalence of this polymorphism may be associated, then, to differences in the degree of expression of this isoform in different ethnic groups [192]. These genetic variations studied in patients with cancer who are undergoing pharmacological treatment including irinotecan, were involved in fluctuations in plasma levels of the active metabolite SN-38 with potential consequences on the toxicity produced in the patient [188-191]. In particular, individuals bearing the allele variant CYP3A4*1B or the wild-type allele CYP3A5*1 have been found to have a more extensive metabolism of oxidation substrates, and are consequently less exposed to the drug in its active form. Although there are still supporting clinical data, it is likely that this predisposition will reduce the risk of exposure to the toxic effects of the drug. Studies *in vitro* and in animal models have also highlighted that irinotecan represents a substrate for the membrane transporter P-glycoprotein (P-gp) belonging to the family of ATP binding cassette proteins and encoded by the gene ABCB1 [193-199]. Polymorphisms in ABCB1 gene, associated with changes in activity and/or expression of this transporter, can cause changes in the process of drug efflux with important consequences on bioavailability. Some authors have suggested an effect of the silent polymorphism 1236 C>T, located in exon 12 of the gene, on the stability of the mRNA of the gene and, consequently, on its expression [200]. Some clinical studies, published in recent years, have shown an association between this polymorphism and the pharmacokinetics of irinotecan; in particular the variant 1236T was associated with a significant increase in plasma exposure to irinotecan and to its active metabolite SN-38 [190-191]. Although there are currently no confirmatory data on this subject in the scientific literature, the impact of the polymorphism on the pharmacokinetics of irinotecan and its metabolites, can potentially be reflected in an effect on the susceptibility of the patient bearing the polymorphism to develop toxic effects to the treatment.

# *DISCUSSION*

Due to the advancements of nanotechnology, nanomedicine formulations of therapeutics are now developed with proper PK/PD behavior to specifically accumulate in their site of action within the body and to achieve better pharmacotherapeutic outcomes. In fact, a balance between maximum therapeutic efficacy with lower toxicity can be attained. Nanodrugs and nanodiagnostics often lead to greater bioavailability profiles with lower doses, thus decreasing the rate of ADRs in clinical practice and increasing patients' compliance [201]. Important nanotechnological applications for personalized medicine are now considered for the development of targeted drug formulations that achieve maximum efficacy and optimal safety profiles. Furthermore, the capability to generate cheaper high-throughput DNA sequencing and other genomic technologies permits the application of personal genome analysis in clinical practice for each patient, which will facilitate the movement of pharmacogenomics and personalized medicine toward pharmacotyping in drug prescription.

Personalized medicine has been defined by the US President's Council of Advisors on Science and Technology as referring to *"-the tailoring of medical treatment to the individual characteristics of each patient; to classify individuals into subpopulations that differ in their susceptibility to a particular disease or their response to a specific treatment so that preventive or therapeutic interventions can then be concentrated on those who will benefit, sparing expense and side effects for those who will not*" [202]. To this definition, the Personalized Medicine Coalition has added, "-*the molecular methods that make personalized medicine possible include testing for variations in genes, gene expression, proteins, and metabolites, as well as new treatments that target molecular mechanisms. Test results are correlated with clinical factors – such as disease state, prediction of future disease states, drug response, and treatment prognosis – to help physicians individualize treatment for each patient*" [203].

The impact of pharmacogenetics on our ability to predict drug response is one of the most promising and fertile areas of genomic and personalized medicine [204]. Inter-individual variability in the response to similar doses of a given drug is an inherent characteristic of drug therapy. Some patients experience no treatment benefits, while in others the treatment is associated with undesirable side effects [205,206]. The interindividual variability contributes to the broad range of drug responses and the "optimal" drug that would be effective and safe for all patients does not exist. The role of genetic factors in drug

disposition and response is studied by pharmacogenetics/pharmacogenomics. In addition, environmental factors (e.g. dietary habits, smoking, co-medication, exposure to toxic substances), factors of physiological differences (age, sex, disease, pregnancy), and patient compliance contribute to variations in drug metabolism and responses. Pharmacogenetics, by analyzing the correlation between genotype and phenotype of the patient, has the aim to define the relationship between a given polymorphic variation and alteration of the effect of a specific drug that it follows, thereby identifying valid predictive genetic markers that, once validated, can predict the outcome of cancer and help determine the optimal use of new drugs are required to determine the efficacy of a specific therapy [207].

In this field of study, it also places the study of pharmacogenetics presented in this thesis, undertaken to determine the role of individual genetic characteristics influencing the clinical outcome of oxaliplatin therapy, used in combination with 5-fluorouracil and leucovorin (FOLFOX4 regimen) in colorectal cancer patients and radiation therapy used in prostate cancer patients.

## *Colorectal cancer patients*

The study was conducted on 154 colorectal cancer patients treated with oxaliplatin in combination with 5-fluoropyrimidine and leucovorin (FOLFOX4 regimen). The main objectives of this study are 1) investigate the role of genetic polymorphisms previously identified in the metastatic disease as predictive markers of toxicity (mainly neurotoxicity and neutropenia) in patients treated with adjuvant FOLFOX4 regimen; and 2) to discover associations with putative novel biomarkers.

As reported in the literature, this is the first study investigating pharmacogenomic markers of toxicity in a group of CRC patients homogeneously treated with adjuvant FOLFOX4 regimen, whereas several studies have been published in the advanced disease [115,113,111,208]. FOLFOX4 regimen has led to significant improvements in the clinical outcome of CRC patients [209,40] as has an acceptable toxicity profile, with neutropenia and sensory peripheral neuropathy as the most clinically significant and often dose-limiting toxicities. Nonetheless, the persistence of neurological toxicity after the interruption of FOLFOX4 has been recently reported in a high percentage of patients (58–83%) [210]. Therefore, there is an urgent need to

identify predictive markers of mild-to-severe toxicity in order to avoid long-term disability, especially in patients with favorable prognosis. In agreement with previously published studies [209], 39% of patients developed grade 2 or 3 toxicity in our study. This toxicity is common and clinically relevant, and deserves a thorough investigation of its potential genetic basis.

At first it was created a comprehensive panel of SNPs to include all the most relevant polymorphisms previously reported as significant markers of toxicity in FOLFOX4 in metastatic CRC patients. Contrary to what is reported in the literature, the polymorphism GSTP1-rs947894 was not predictive for the neurotoxicity in advanced CRC patients treated with FOLFOX4 [115,113,106,111]. The clinical validity of this marker has been already put into question [85,112,211]. GSTP1 is an enzyme mediating glutathione-related detoxification of oxaliplatin. This detoxification pathway might be dependent upon systemic oxidative stress, which is known to be impaired in patients with metastatic disease [106]. Findings from similar pharmacogenomic studies conducted in different settings should be interpreted with caution. The biological scenario of patients with early disease treated with adjuvant oxaliplatin differs from that of patients with metastatic disease. The molecular and metabolic changes related to tumor metastatization involve several pathways, including the glycolysis, the tricarboxylic acid cycle, the pentose phosphate pathway, fatty acid and nucleotide biosynthesis and the GSH-dependent antioxidative pathway [212]. Thus, because of the role played by these pathways in the pathophysiology of oxaliplatin neurotoxicity, [210, 213] it is plausible that genetic polymorphisms could have a different impact depending upon the clinical setting.

Furthermore as regards the second aim of this study five genetic polymorphisms have been identified in ABCC2 (rs3740066, rs1885301, rs4148396, rs717620) and ABCC1 (rs2074087) genes as predictors of grade 2–3 neurological toxicity, one polymorphism in hMSH6 (rs3136228) as predictor of grade 3-4 neutropenia, and one polymorphism in XRCC3 (rs1799794) as predictor of grade 3-4 non-hematological toxicity all with an FDR <10%, therefore considered noteworthy predictive markers. Similar associations were also obtained for severe neutropenia though they did not pass the 10% FDR cutoff.

ABCC1 and ABCC2 belong to ATP-binding cassette transporter superfamily, containing several family members that mediate the cellular trafficking of drugs, their metabolites and endogenous factors [86]. ABCC1 and ABCC2 may act in a synergistic way modulating the effect

of oxaliplatin and 5-fluorouracil at the cellular level [87]. According to the findings published by Haenisch S, et al, [214] ABCC2 (rs717620), located on the gene promoter region, has been associated to decreased protein expression *in vitro*, and in our study it was associated with a 13-fold increased risk of grade 2–3 neurological toxicity. It was also associated to a fivefold increased risk of severe neutropenia, though this association had a *q*-value higher than 0.10 (*q*-value 0.1228). This polymorphism and rs2273697 that lies in ABCC2 gene too, had a combined effect in increasing platinum related toxicity in lung cancer patients [215], similar to our results. In addition to the ABCC2 variants passing the FDR cutoff, ABCC2 (rs2273697) was associated with a protective effect for grade 2–3 neurological toxicity in our study. Although this association did not pass the FDR cutoff (*q*-value 0.1109), rs2273697 is a missense polymorphism causing a Val417Ile amino-acid substitution increasing the transporter efficiency [214]. Enhanced ABCC2 expression can lead to decreased cellular glutathione content [215]. Glutathione is needed for oxaliplatin detoxification via conjugation, and it was reported that low glutathione intra-cellular levels can cause increased oxaliplatin cytotoxicity [87]. Moreover, ABCC2 mediates the export of the oxaliplatin-glutathione conjugated form, and ABCC2 over expressing cells were resistant to platinum derivatives [216]. Taken together with our results, ABCC2 variants might change the susceptibility of patients to oxaliplatin toxicity via a glutathione mediated mechanism.

Also with regard to the association with grade 2-3 neurological toxicity only one genetic polymorphism (rs2074087) in ABCC1 was significant. Instead, the other polymorphism (rs35587) in ABCC1 that was not significantly associated with grade 2-3 neurological toxicity, was associated to grade 3-4 neutropenia. The functional effect of these variants is not known, and they are not in LD with any known ABCC1 functional variants. Overexpression of the ABCC1 protein was related to resistance to 5-fluorouracil *in vitro* [87]. This could be due to the ability of ABCC1 to extrude folates and thus depleting their intra-cellular availability for the activity of 5-fluorouracil. This might in part explain the effect of ABCC1 (rs35587) on both neutropenia and neurological toxicity, suggesting that ABCC1 (rs35587) might increase the function or expression of the ABCC1 transporter. More confirmatory studies (both at the clinical and molecular level) should be conducted to confirm the clinical associations and their mechanistic basis.

Regarding grade 3-4 neutropenia, only a polymorphism hMSH6 (rs3136228) has been resulted associated. In particular it increased the risk of severe neutropenia (*q*-value 0.0937).

hMSH6, expressed in normal marrow cells [217], deals with the DNA mis-match repair. In particular, it forms a heterodimeric complex with MSH2 able to recognize mispaired bases in DNA. A functional mis-match repair system is required for the detection of damaged DNA created by platinum derivatives [218]. The rs3136228 polymorphism, seems to modulate gene transcription causing the loss of a Sp1-binding site [219]. It is likely that the rs3136228 variant can affect mismatch repair (MMR) activity in non cancer cells modulating the toxic effects of FOLFOX.

Finally, for grade 3-4 non-hematological toxicity, we have found a significant association with XRCC3 (rs1799794) polymorphism. It is a DNA repair gene and belongs to the double-strand break repair (DSBR) pathway. Its reduced activity is associated with significantly higher levels of bulky DNA adducts [69]. Our study suggests that this SNP might confer reduced DNA repair of oxaliplatin DNA adducts, leading to more toxicity. However, the molecular function of this SNP is presently unknown, and controversial data have been reported on the possible clinical role of this SNP on the response and toxicity to DNA damaging agents [220,119].

For the novel SNPs indentified in this study, the limited number of patients and lack of an independent validation cohort make our findings preliminary, requiring further confirmation. The use of an oxaliplatin-specific scale [106] could impair the ability to replicate our findings in studies using the definitions of the NCI-CTC [221,222]. Our study did not validate existing markers previously identified in metastatic patients, and provides the basis for testing the validity of new markers in future studies in adjuvant FOLFOX4. Among those, the variants in ABCC2 have a clinical effect that is consistent with their molecular function, and should be prioritized for testing of their clinical validity in future studies.

## *Prostate cancer patients*

The study presented here investigated the possible association between 50 polymorphisms in 37 genes and clinical outcome of radiotherapy in patients with prostate cancer (in term of risk of biochemical PSA recurrence, time to recurrence (TTR), and overall survival (OS).

The clinical course of prostate cancer is difficult to predict given that men with similar tumor features can experience strikingly diverse outcomes. So, new markers are needed to more accurately predict the risk of relapse and enhance the selection of high-risk patients who may benefit from therapy and intensive follow-up. DNA-based genetic markers have advantages, such as preoperative availability, easy conductance, and objective interpretation without individual bias.

In this study, two polymorphisms in different DNA repair genes (XRCC1 (rs1799782) and ERCC2 (rs1799793)) were associated with an higher and lower biochemical PSA recurrence risk respectively, and these variants remained independent predictive factors after adjusting for other covariates. In particular for XRCC1 (rs1799782), the heterozygous and the homozygous variant genotypes (26304TC and 26304CC), were associated with a higher risk of biochemical PSA recurrence, while for ERCC2 (rs1799793), the heterozygous variant genotype (23591AG) have a lower risk of PSA failure.

DNA repair plays a key role in carcinogenesis through the removal and repair of DNA damage induced by endogenous and environmental sources. Base-excision repair is an important DNA repair pathway responsible for repair of base damage from X-rays, oxygen radicals, and alkylating agents [223,224]. The XRCC1 acts as a central scaffolding protein by binding DNA ligase III, DNA polymerase β, and poly(ADP-ribose) polymerase in base excision-repair [225,226]. In a study of Chinese hamster ovary cell lines mutations in the XRCC1, there has been found a reduced ability to repair single-strand breaks in DNA and concomitant cellular hypersensitivity to ionizing radiation and alkylation agents [227]. These findings suggest that XRCC1 plays an essential role in the removal of endogenous and exogenous DNA damage. In literature we have conflicting results: two studies (n=304) showed a clear negative effect on survival in Caucasians patients [228], another study conducted on a set of 52 patients found inconsistent results as the worst outcome was observed for heterozygous patients [229], and in another the variant allele of XRCC1 (rs1799793) showed a protective effect on radiotherapy [60].

ERCC2 protein has DNA-dependent helicase activity and is a component of transcription factor complex, TFIIH, which participates in both NER and basal transcription. Amino acid substitutions within the conserved region may alter ERCC2 function, which may in turn influence its capacity to repair damaged DNA, increasing cancer susceptibility. For ERCC2 (rs1799793) polymorphism, although all preclinical studies suggested a sub-optimal DNA repair capacity for the Asn/Asn genotype relative to Asn-containing genotypes [230], one showed surprisingly fewer unrepaired radiation-induced DNA alterations associated with Asn/Asn genotype.

TP53 (rs1042522) is another polymorphism that resulted associated with the risk of biochemical PSA recurrence in this study. In particular, the homozygous variant genotype (ex4+119CC) showed shorter survival time than patients with the wild type genotype (62.78 months comparing to 112.85 months). In addition this polymorphism remained an independent risk factor for PSA failure also after adjusting for other covariates. To our knowledge, our series might be the first to demonstrate the significant prognostic role of the TP53 (rs1042522) polymorphism on PSA recurrence in Caucasian population. Before our study, Bin Xu et al. [231] found an association between this polymorphism on PSA recurrence but in Chinese population, and by contrast Huang et al [232], Hirata et al. [233] did not find an association in this ethnicity. However, how exactly the TP53 (rs1042522) polymorphism influences prostate cancer progression requires more detailed *in vitro* and *in vivo* studies.

In this study, we investigated whether common SNPs in the VEGF gene were associated with the risk of biochemical PSA recurrence in prostate cancer patients after radiotherapy. The most notable finding was a significant association between the VEGF (rs1570360) polymorphism and PSA failure after radiation treatment. We found that this variant, in particular the homozygous variant genotype (-1154GG), determine a lower risk of biochemical PSA recurrence in prostate cancer patients. Vascular endothelial growth factor (VEGF) is an important hypoxia-related angiogenesis stimulating factor and a potential prognostic factor for radiotherapy outcome for prostate cancer, because its expression has been linked with poor prognosis, including adverse outcome after radiotherapy, in other tumour types [234-237]. Hypoxia is known to generate reactive oxygen species and to mediate the production of inflammatory and fibrogenic cytokines, leading to an increase in vascular permeability and collagen formation. It is also known to play a role in inducing VEGF gene expression [238]. It has been shown that VEGF promotes angiogenesis, tumour aggressiveness

and invasiveness in prostate cancer [239,240], and that the homozygous variant genotype of VEGF (-1154GG) is associated with a significantly higher production of VEGF that the homozygous genotype [241]. Furthermore, Mazzucchelli et al. [242]have reported higher VEGF levels in some high grade prostatic intraepithelial neoplasia lesions than in normal epithelium, suggesting that the increase in VEGF expression is related to the poor cellular differentiation and to an aggressive behaviour of tumour. This thesis is supported by another study where appears that the *VEGF* −1154 A allele reduces risk of prostate cancer and furthermore imparts protection against high-grade tumour and tumour aggressiveness.

In our study, we also have investigated the associations of selected polymorphisms with overall survival (OS) in prostate cancer patients. We demonstrated a significant association of two DNA repair genes, XRCC1 (rs1799782) and RAD51 (rs1801320), and a nitric oxide synthase gene, NOS2A (rs9282801) with overall survival. In particular for the polymorphism XRCC1 (rs1799782) we found an increased of median survival for wild type genotype than heterozygous genotype (168.35 months comparing to 137.17 months).

XRCC1 protein, having no known enzymatic activity, functions as a scaffold protein that provides a platform upon which the necessary repair complexes can form, and plays regulative and coordinative role in consecutive stages of the BER pathway. The role of XRCC1 has been mainly attributed to its interactions with a series of repair enzymes known to be involved in BER and single-strand break repair machinery. Considering the importance of these repair proteins and their interactions with XRCC1 for a complete DNA damage repair process, it is proposed reasonably that noted one non synonymous mutation occurring within related protein binding domains of XRCC1 gene, may change the protein structure, subsequently influence its activity (or affinity) to bind to other partners, which in turn lead to the alterations of its communication with other enzymes as well as altered DNA repair capacity. Based on these evidences, we speculated that this polymorphism XRCC1 (rs1799782) may play an advance role in cancer carcinogenesis and development. In agreement with our results, Zhang et al, reported that the homozygous genotype of XRCC1 (rs1799782) conferred a longer median survival than the other genotypes. On the contrary, Wang et al. [243], reported that individuals carrying one or two variant (26304TC or 26304CC) alleles exhibited significantly fewer chromosomal breaks than those with the wild type genotype (26304TT) due to the lower mutagen sensitivity of CC genotype, suggesting a protective role of this genetic mutation.

RAD51 (rs1801320) is another polymorphism that resulted associated with the overall survival: the heterozygous genotype (-135CG) presents a longer median survival than the wild type genotype (-135CC) (161.00 months comparing to 157.44 months). The RAD51 gene family consists of several proteins that show DNA-stimulated ATPase activity and plays a central role in the homologous recombination (HR) activation. The RAD51 (rs1801320) polymorphism, located in the promoter region of the gene, results in the up-regulated gene expression through an increased promoter activity by substituting G for C allele. RAD51 is essential for optimal repair of DSBs, and its expression level was a single important factor in modifying DSB repair capacity as reported in previous studies [244,245]. Based on these findings, the association of the RAD51 -135C allele with a reduced overall survival can be reasonably explained by an increased radioresistance due to the anticipated up-regulated RAD51 expression.

Finally, the last polymorphism that is associated with overall survival is NOS2A (rs9282801) polymorphism: patients with heterozygous genotype (IVS16+88TG) have a poor median survival than wild type genotype (IVS16+88TT) (135.77 months comparing to 181.80 months). Endogenously generated NO may play a role as a reactive oxygen species (ROS) scavenger protecting growing tumor-spheroids from ROS-induced apoptosis. Lower ROS levels were observed in invasive prostate cancer cells (PC3) compared with non-invasive ones (LNCaP). Thus, the effect of NOS2A IVS16+88T.G on aggressive prostate cancer among those with higher antioxidant intake level might be due to decreased ROS-induced apoptosis or increased angiogenesis in prostate cancer cells. To our knowledge, this is the first study in literature that reported the association of this polymorphism with overall survival in prostate cancer.

Our results indicate that XRCC1 (rs1799782), ERCC2 (rs1799793), TP53 (rs1042522), and VEGF (rs1570360) polymorphisms may have a significant effect on PSA failure in prostate cancer patients after radiotherapy, and XRCC1 (rs1799782), RAD51 (rs1801320), and NOS2A (rs9282801) seem to influence the overall survival of prostate cancer patients treated with radiation therapy. Our results illustrate the potential of using these polymorphic variants as a pharmacogenetic tool that can be used in clinical practice of prostate cancer.

## *Conclusions*

In conclusion, this PhD project has allowed us to identify new molecular markers of the clinical outcome of treatment carried out with FOLFOX4 regimen in colorectal cancer and the risk of biochemical PSA recurrence in prostate cancer. The analysis has identified a set of significant genetic markers about in particular the process of intra-extracellular transport, DNA repair, oxidative stress and cell cycle, along with some clinical and demographic characteristics, can predict the outcome of specific aspects of the treatment. We have developed, in particular, the significant predictive models for neurotoxicity, haematological and non-haematological toxicities in colorectal carcinoma and the effectiveness of treatment in terms of tumor response, time to progression and survival in prostate cancer. The preliminary results obtained in this thesis, once validated by further studies, may become for clinicians valid biomarkers to optimize and personalize the treatment of colorectal cancer patients treated with oxaliplatin in FOLFOX4 regimen, and the clinical outcome of prostate cancer patients treated with radiotherapy.

*Biomarkers and molecular medicine are replacing "one size fits all" medicine with individualized medicine. This paradigm shift requires that personalized oncology rapidly implement new validated biomarkers. In the next decade oncology will move from a reactive to a proactive discipline — a discipline that is predictive, personalized, preventive and participatory. In addition tools, for implementing preemptive medicine based on genetic and molecular diagnostics and interventions will emerge and these will improve prevention, early detection, and access to care. Healthcare oncology professionals need to keep themselves informed of new tumor biomarkers, their optimal clinical use, and the management of side effects* [246].

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