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DEFINITION OF THE PHARMACOLOGICAL PROFILE OF CONVENTIONAL CHEMOTHERAPEUTIC DRUGS BASED ON PHARMACOGENETICS DETERMINANT IN CRC PATIENTS

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Riassunto

Pazienti oncologici, con lo stesso tipo di tumore, non sempre rispondono in modo uguale al medesimo trattamento farmacologico: la somministrazione della stessa dose di un dato farmaco antiblastico in una popolazione di pazienti implica spesso la manifestazione di un vasto range di tossicità, che in alcuni casi può risultare addirittura mortale.

La variabilità intersoggettiva che si osserva nell'efficacia e nella tossicità dei farmaci antiblastici impiegati nella chemioterapia può pertanto essere determinata da interazioni complesse tra le componenti fisiologiche, ambientali e fattori genetici individuali.

L'attività svolta in ambito del progetto di dottorato ha puntato l'attenzione su come i fattori genetici possano influenzare l'esito di un trattamento farmacologico, individuando dei possibili biomarcatori genetici prognostici e predittivi.

Per valutare la correlazione tra genotipo e fenotipo del paziente siamo partiti da uno studio di farmacogenetica che ha definito la relazione esistente tra un determinato polimorfismo (UGT1A1*28) e l'alterazione dell'effetto del farmaco che ne consegue.

Successivamente, ci si è indirizzati verso il trasferimento e l'applicazione nella pratica clinica attraverso uno studo clinico di fase Ib *("Studio di fase I guidato dal genotipo dell'irinotecano in combinazione con 5-fluorouracile/leucovorina (FOLFIRI) e bevacizumab in pazienti con carcinoma colonrettale")*, condotto presso il Centro di Riferimento Oncologico di Aviano (PN).

Lo scopo di tale studio è stato quello di modulare il dosaggio dell'irinotecano (CPT-11) in presenza dell'inibitore angiogenetico bevacizumab (BV), non essendo ancora nota l'interazione tra i due farmaci, in base al genotipo di UGT1A1, al fine di ottenere un miglioramento dell'indice terapeutico per ogni singolo paziente.

Per fare ciò, si è ritenuto necessario sviluppare un metodo di analisi di farmacocinetica specifico e si è pensato di procedere nel seguente modo:

- Sviluppo di un metodo quantitativo per l'analisi del farmaco d'interesse
- Validazione del metodo in accordo con le linee guida dell'FDA
- Misurazione delle concentrazioni plasmatiche del farmaco
- Determinazione della farmacocinetica del farmaco
- Correlazione dei dati di farmacocinetica e farmacogenetica

Sono stati arruolati pazienti con diagnosi istologica di adenocarcinoma colorettale (CRC) metastatico, non pretrattati con chemioterapia o trattati con terapia adiuvante (escluso irinotecano), e rispondenti ai criteri di eleggibilità/esclusione previsti dal protocollo.

I pazienti sono stati assegnati al loro gruppo di trattamento in base al genotipo (*1/*1 o *1/*28) fino al completamento del reclutamento per ogni livello di dose in ogni gruppo di pazienti. I pazienti con genotipo *28/*28 sono stati esclusi perché ad alto rischio di tossicità. La dose iniziale di irinotecano somministrata nei pazienti portatori dell'allele *1 (wild type ed eterozigoti) è di 260 mg/m^2. La dose di BV è di 5 mg/kg, somministrata anch'essa in infusione ogni due settimane.

Il dosaggio dell'irinotecano è stato incrementato a 310 e 370 mg/m^2 qualora nel gruppo di trattamento con dosaggio più basso non vi sia stata tossicità.

La valutazione delle interazioni farmacocinetiche e farmacodinamiche tra bevacizumab (BV) ed irinotecano (CPT-11) è stata condotta descrivendo il profilo farmacocinetico del CPT-11 (e dei suoi metaboliti) in assenza ed in presenza di BV nello stesso paziente.

Da una prima analisi dei parametri farmacocinetici sembra che si possa escludere un effetto del BV sulla farmacocinetica dell'irinotecano. La sovrapponibilità del dato farmacocinetico con o senza BV, è valida per entrambi i dosaggi di irinotecano considerati. Tuttavia, sono stati riscontrati dei valori di Dose Massima Tollerata (MTD) inferiori rispetto a quelli determinati da lavori precedenti, e ciò potrebbe suggerire che l'aggiunta del BV comporti una variazione nella manifestazione di tossicità in regimi ad alto dosaggio.

Una percentuale non trascurabile di pazienti ha ottenuto una riduzione del numero e delle dimensioni delle lesioni secondarie epatiche tanto da renderle aggredibili chirurgicamente o tramite termoablazione, facendo pertanto concludere che il regime terapeutico si configuri quindi come una *conversion therapy*.

Poiché i farmaci che sono stati studiati in questo protocollo sono presenti nella pratica clinica da diversi anni, la notevole variabilità riscontrata nella risposta e nello sviluppo di tossicità rende indispensabile trovare dei criteri utili alla personalizzazione del trattamento.

Criteri fondamentali per la personalizzazione della terapia sono i parametri clinici, principalmente il genere e l'età dei pazienti. Molti dei soggetti anziani, infatti, ricevono dei trattamenti ridotti poiché tollerano meno le terapie dei protocolli clinici standard, con una notevole riduzione del dosaggio di farmaco o del numero di cicli di trattamento. Proprio per le loro caratteristiche fisiologiche, i pazienti oncologici anziani riportano maggiori effetti di tossicità associati al trattamento rispetto ai soggetti definiti giovani. Analogamente, anche le donne sono spesso escluse da alcuni protocolli soprattutto per la variabilità ormonale che caratterizza l'età fertile e il periodo della menopausa.

A tale proposito, in una seconda parte del progetto di dottorato, abbiamo considerato una casistica molto ampia di pazienti affetti da CRC trattati con fluoropirimidine associate ad altri farmaci (FOLFIRI o FOLFOX) presso il Centro di Riferimento Oncologico di Aviano (PN) e altri centri aderenti al programma.

Lo scopo è stato quello di individuare dei possibili determinanti farmacogenetici (PG) che condizionano gli effetti del farmaco (tossicità e risposta) in maniera differente tra soggetti giovani e anziani, e tra i due generi (maschio e femmina), nonché dei possibili marcatori di rischio tumorale specifici.

Sono stati analizzati diversi polimorfismi di geni codificanti per proteine coinvolte nei meccanismi di attivazione, metabolizzazione ed eliminazione dei farmaci principalmente usati nelle terapie contro il CRC e altri polimorfismi di geni che

regolano il ciclo cellulare in quanto coinvolti nel processo di carcinogenesi e di progressione tumorale.

Molte delle variazioni alleliche analizzate confermano un'associazione con il rischio d'insorgenza del tumore, altre con la sopravvivenza dei pazienti.

In particolare, la variazione del *copy number* dei geni codificanti per l'enzima Glutatione S-trasferasi, GSTT1 e GSTM1, ha messo in evidenza una correlazione con l'età di diagnosi dei pazienti, nonché con il genere. L'aumento delle copie di GSTM1 o la riduzione del GSTT1 sono associati a una maggiore sopravvivenza nei soggetti con età superiore a 70 anni e di sesso maschile (P_{GSTM1}=0.047, HR=3.937, 95% CI=0.31–0.89; P_{GSTT1}=0.039, HR=4.246). Abbiamo ulteriormente riscontrato una specifica associazione del genotipo GST*null* con un aumentato rischio d'insorgenza tumorale nei soggetti giovani, ma non nei soggetti anziani. Si può quindi ipotizzare che vi sia una riduzione dell'attività enzimatica della Glutatione-S Transferasi con conseguente deficit ai meccanismi di detossificazione cellulare che comportano un incremento dei danni a carico del DNA.

Gli ulteriori dati ottenuti, ci permettono di concludere che ci possono essere delle differenze associate alle caratteristiche genotipiche relative sia al genere che all'età dei pazienti: ci sono polimorfismi predittivi di rischio prevalentemente associati ad uno dei due generi (MTHFR 1298A>C nelle giovani donne); altri polimorfismi localizzati nei geni del riparo del DNA sono prevalentemente associati ad un rischio maggiore e ad una sopravvivenza minore nella popolazione anziana.

È pertanto fondamentale che, grazie alle nuove conoscenze nell'ambito della medicina molecolare e alle nuove tecnologie, si arrivi alla personalizzazione della terapia, al fine di massimizzare la risposta e ridurre gli effetti tossici in ciascun paziente.

Abstract

Cancer patients do not always respond in the same way to the same drug treatment: the administration of the same dose of an antiblastic drug in a population of patients induces the manifestation of a wide range of toxicity, which sometimes can even be deadly.

The intersubject variation, that is observed in efficacy and toxicity of anticancer drugs, can be determined by complex interactions among components of the physiological, environmental and genetic factors.

The activities carried out in the framework of the PhD project have focused the attention on the influence of genetic factors on the outcome of drug treatment, identifying possible prognostic and predictive genetic biomarkers.

To evaluate the correlation between genotype and phenotype of the patient, we considered a pharmacogenetics study which defined the relationship between a specific polymorphism (UGT1A1 * 28) and the effects of the drug. A phase Ib clinical trial was conducted at the CRO, Aviano (PN). The purpose of this study was to modulate the dosage of CPT-11 in the presence of the inhibitor angiogenetic BV for each individual patient, based on genotype of UGT1A1, in order to obtain an improvement of the therapeutic index .

To do this, it was considered necessary to develop a specific method of pharmacokinetic analysis and we proceeded as follows :

- Development of a quantitative method for the analysis of the drug of interest
- Validation of the method in accordance with FDA guidelines
- Measurement of plasma concentrations of the drug
- Determination of the pharmacokinetics of the drug
- Correlation of pharmacokinetics and pharmacogenetics

We enrolled patients with a histological diagnosis of metastatic colorectal adenocarcinoma, naïve or treated with adjuvant chemotherapy (excluding irinotecan), and corresponding to the criteria for eligibility/exclusion described in the protocol.
The patients were assigned to their treatment group based on genotype (*1/*1 or *1/*28) until the completion of recruitment for each dose level in each group of patients. Patients with genotype *28/*28 were excluded because of high risk of toxicity. The starting dose of irinotecan administered in patients carrying the *1 (wild-type and heterozygous) was 260mg/m^2. The dose of BV was 5mg/kg and it was administered in infusion after two weeks. In the treatment group with lower dose, the irinotecan dose was increased to 310 and 370mg/m^2 in case of lack of toxicity.
The evaluation of pharmacokinetic and pharmacodynamic interactions between bevacizumab (BV) and irinotecan (CPT-11) was conducted describing the pharmacokinetic profile of the CPT -11 (and its metabolites) in the absence and in the presence of BV in the same patient.
The obteined pharmacokinetic parameters exclude an effect of BV on the pharmacokinetics of CPT-11. However, the registered values of MTD were lower than those determined by previous works with FOLFIRI alone, and that might suggest that the addition of BV results in changes in the manifestation of toxicity in the high-dose regimens.

The drugs that have been investigated in this protocol have been present in the clinical practice since several years, and the high variability of the response and toxicity makes it essential the definition of useful criteria for treatment personalization. Basic criteria for personalization of therapy are clinical parameters, like gender and age of the patients. Many of the elderly subjects, in fact, receive reduced treatments comparing standard clinical protocols, with a considerable reduction of the dosage of the drug or of the number of therapy cycles. Due to their physiological characteristics, the older cancer patients reported greater toxicity effects associated with the treatment.

In clinical trials, elderly patients are under-represented because they don't satisfy all the inclusion criteria, like comorbid conditions and baseline functional status of the patient. Similarly, women are often excluded from some protocols especially for the variability that characterizes the hormonal status.

In a second part of the PhD project, we considered a very large series of patients with CRC treated with other medications associated with fluoropyrimidine (FOLFOX or FOLFIRI) at the CRO, Aviano (PN), and other centers participating in the program. We performed a pharmacogenetic study to see if there are genetic biomarkers useful to define a personalization of therapy for subjects considered elderly (age at diagnosis >70 years).

In particular, the aim was to identify possible pharmacogenetic (PG) determinants which influence drug effects (toxicity and response) in a different way between young and elderly subjects, and between the two genders (male and female).

We analyzed several polymorphisms of genes coding for proteins involved in the mechanisms of activation, metabolism and elimination of drugs mainly used in therapies against CRC and other polymorphisms in genes that regulate the cell cycle and involved in the process of carcinogenesis and tumor progression. Many allelic variations confirm an association with the risk of developing cancer risk, while others with the survival of patients. In particular, the variation in the copy number of GSTM1 and GSTT1 genes, showed a correlation with the age of diagnosis of the patients, as well as with the gender. The increase in copies of GSTM1 or GSTT1 reduction are associated with greater survival in male patients older than 70 years (P_{GSTM1}=0.047, HR=3.937, 95% CI=0.89-0:31; P_{GSTT1}=0.039, HR=4.246). Moreover, we further found a specific association of genotype GSTnull with an increased risk of tumor in young subjects, but not in the elderly. It can therefore be assumed that there is a deficiency in the enzymatic activity of glutathione S-transferase deficiency resulting in reduced cell detoxification mechanisms that involve an increase in damage to the DNA damnage.

Additional data allowed us to conclude that genetic characteristics may be associated

with both gender and age of the patients: there are polymorphisms predictive of risk related to one of the two parameters (MTHFR 1298A>C in young women); polymorphisms localized in repair genes are mainly associated with a greater risk and a lower survival in the elderly population.

Translational research is a fundamental step for the application of experimental research to clinical practice. One of the most important aspects in this area is the customization of the therapy that should be considerate important to get treatment optimization especially in the field of oncology.

INTRODUCTION

1.1 Pharmacogenetics as Innovative Approach for Phase I Clinical Studies

The development of anticancer drugs is expensive due to the high rate of failure of evaluated agents and the duration of this process. Only 1 out of 20 cancer drugs entering clinical trials gains regulatory approval: inadequate therapeutic activity and toxicity are the major causes for failure. Drug development is commonly described in "phases". Phase I trials provide information about safety and aim to define toxicity and maximum tolerated dose (MTD) in patients. While these trials are conventionally conducted in healthy volunteers and include ascending doses, antineoplastic drugs phase I trials involve cancer patients with advanced-stage disease, and not suitable for conventional treatment. This is due to of the low therapeutic index of antineoplastic drugs (i.e. the ratio between the dose efficacy for the antitumor effect and the dose causing severe toxicity).

Pharmacokinetic (PK) and pharmacodynamic (PD) assessments are used to evaluate optimal dose and schedule in phase I trials. Objective response rates within these trials in cancer patients remain low and in some instances do not justify the risk of severe toxicity (earlier analysis of tumor responses in unselected patients recruited to phase I trials indicate a response rate of 3.8%, with a risk of toxic death of 0.54%) [1, 2]. Improvement of phase I clinical trial design, hence, represents a scientific, ethical and financial imperative.

The classical design for phase I study does not require genotyping. This procedure is eventually performed during or after the trial in order to investigate genetic association with toxicity. An innovative approach is based on stratification of patients on an existing hypothesis. Regarding this, a genetic profile related with high risk for toxic adverse event, could improve the outcome of phase I studies.

An early discovery of clinically important genomic differences is expected to drive the early development of drugs in the future. In November 2003 FDA realized the first

Draft Guidance for Industry Clinical Pharmacogenomics: premarketing evaluation in early phase clinical studies.

Several pharmaceutical and biotechnology companies have submitted comments to the FDA regarding the voluntary submission process and the procedure for validating exploratory biomarkers. But how forthcoming the firms will be with genomics data still remain to be seen.

Genetic differences among individuals can affect response to drug treatment. In particular, PK (adsorption, distribution, metabolism and excretion-ADME) is deeply influenced by some genes. Genetic differences concerning PK have been well described for antineoplastic drugs including 6-mercaptopurine and azathioprine with thiopurine methyltransferase (TPMT) [3]; irinotecan with uridyne difosfoglucuronosyl transferase (UGT) [4]; and for several other drugs including warfarin, with CYP2C9 and VKORC1 [5], and abacavir with HLA-B*5701 [6]. These drugs required dose adjustments in 'high risk' patients with a specific genetic profile. Also genetic differences concerning drug target can explain differences in response or toxicity among individuals: for example, the number of cytosine/adenine repeats in the intron 1 of epidermal growth factor receptor (EGFR), can affect the receptor activity and could potentially interfere with the activity of EGFR inhibitors as cetuximab [7].

In most cases, PG suggestions derive from data from postmarketing experience and are performed relatively late in the drug development process.

1.2 Colorectal Cancer Therapy

Colorectal cancer (CRC) is the third most prevalent cause of cancer-related death. Approximately 40% of all patients develop metastatic disease. Consequently, chemotherapy which provides an increase of survival in metastatic CRC is quite useful. Until recently, 5-fluoruracil (5-FU), which has been available for over 40 years,

and folinate calcium [leucovorin (LV)] have been the standard therapy. However, LV/5-FU showed no major impact on survival. Chemotherapy against advanced or metastatic CRC has steadily improved with the introduction in the recent years of several new cytotoxic and biologic agents in the therapeutic arnamentarium. These agents include irinotecan and newer monoclonal antibodies targeting the vascular endothelial growth factor (bevacizumab, BV).

Irinotecan {7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin} is a topoisomerase I inhibitor, approved world wide for the treatment of metastatic CRC also in association with oxalilatin or antiangiogenetic (i.e. BV) or EGFR inhibitors (i.e. cetuximab) [8] [9]. It is a semisynthetic derivative of the natural alkaloid camptothecin that acts as prodrug generating *in vivo* the active SN-38 metabolites through carboxylesterases [10]. The response rate for single agent irinotecan is 32% in CRC untreated patients and 13% in patients with prior 5-FU therapy, with a 9-month median duration response [11] [12] [13].

A randomized trial compared a 125mg/m^2 weekly, for 4 out of 6 weeks, schedule with a 300mg/m^2 once every 3 weeks schedule, demonstrating no difference in response rate, survival or time to progression [14]. The type of serious toxicity differed between the two schedules: grade III (G3) or grade IV (G4) diarrhea was observed in 36% of patients treated weekly and in 19% of patients receiving the therapy every three weeks, while G3-G4 neutropenia occurred in 29% of weekly and 34% of tri-weekly patients. Several studies demonstrated that neutropenic fever is unusual, approximately 3% [12] [13]. Nausea or acute colinergic symptoms are more common with a tri-weekly schedule [14]. Diarrhea can generally be well controlled with the aggressive use of loperamide or atropine.

Randomized trials have shown improvements in clinical efficacy as related to overall response rates, time to tumor progression, and median overall survival when irinotecan has been added to either infusional (FOLFIRI) [15] or bolus [16] 5-FU and leucovorin (LV) in the initial treatment of patients with metastatic CRC. These two studies demonstrated, in terms of overall response and survival, the superiority of

irinotecan combination with 5-FU/LV compared to 5-FU/LV [15] or irinotecan alone [16].

Conventional infusional 5-FU regimens (FOLFIRI) resulted more efficacious and less toxic than bolus regimens] [16] that have been associated to toxic death due to cardiovascular and gastrointestinal toxicity [18]. The life-threatening toxicity of FOLFIRI became evident in randomized trials for both metastatic disease and in adjuvant setting [15]. However, a more accurate revision of the data suggested that toxicity could be related to the 5-FU bolus more than irinotecan and 5-FU association [14].

FDA approved the bevacizumab (Avastin™) in combination with fluoropyrimidine-based chemotherapy for first-line treatment of patients with metastatic carcinoma of the colon or rectum. It is a recombinant humanized monoclonal antibody that binds and neutralizes effects induced by human vascular endothelial growth factor (VEGF) [19] in cell proliferation and new blood vessel formation (angiogenesis process). Preclinical studies have suggested that combining BV with chemotherapy and radiotherapy may results in synergistic antitumor activity. The addition of BV (5mg/kg every 2 weeks) as an intravenous infusion in combination with irinotecan 5-FU and LV (FOLFIRI) has been found to increase the response rates from 34.8% to 44.8% and extend median overall survival from 15.6 months to 20.3 months and prolonged the duration of response from a median of 6.2 months to 10.6 months as compared to FOLFIRI alone [20].

Although the improvements offered by the introduction of BV, a great inter-patient variability in both response and toxicity associated to irinotecan treatment still remain the major concern. This could be related to differential plasma levels of the active metabolite SN-38 [10] among patients. Several factors can affect SN-38 plasma levels, in particular activation of irinotecan to SN-38 by carboxylesterase enzymes [9] or glucuronidation of SN-38 to the inactive SN-38 glucuronide (SN-38G) by uridine diphosphate glucuronosyltransferase (UGT1A1), the enzyme that conjugates bilirubin [21]. Other metabolic or transport pathways can affect irinotecan and SN-38

disposition. In particular cytochrome p450 isoform 3A4 (CYP3A4) and 3A5 (CYP3A5) [22] converted irinotecan into inactive metabolites and ATP-binding Cassette B1 (ABCB1) and C2 (ABCC2) transporters are involved in intracellular concentration and excretion of irinotecan and metabolite [21].

Presently an impaired glucuronidation activity of the UGT1A1 enzyme, possibly due to the genetic polymorphism of the UGT1A1 gene, can explain toxicity variability. More than 50 genetic variations in the promoter and coding regions of the gene are currently known [23]. In particular, UGT1A1*28 (alias TA indel) polymorphism, characterized by an extra TA repeat in the promoter region of the gene [$A(TA)_7TAA$] is thought to be involved in irinotecan toxicity and response. This polymorphism is thought to be associated with a reduced glucuronidation of SN-38 compared with wild-type UGT1A1 [$A(TA)_6TAA$], leading to variability in pharmacokinetics of SN-38 [4] [24] [21]. UGT1A1*28 is associated with Gilbert's syndrome (mildly unconjugated hyperbilirubinemia), and irinotecan-induced severe toxicity has been reported in patients with Gilbert's syndrome [77].

In a prospective study [25], we analyzed the effect of UGT1A1*28 polymorphism on irinotecan pharmacodynamics and pharmacokinetics in 250 Caucasian subjects affected by metastatic CRC and homogeneously treated as first-line with FOLFIRI regimen. The patients with *28/*28 and *1/*28 genotype exhibited a 8.63 (95% CI 1.31-56.55) and 3.47 (95% CI 0.69-17.34) increased risk of developing G3-G4 hematological toxicity after the first cycle of chemotherapy compared to wild type patients respectively. However, conventional strategies to manage toxicity after the first cycle of chemotherapy was sufficient to prevent severe toxicity in the subsequent course of chemotherapy. On this ground no differences in the toxicity was observed in the entire course of chemotherapy among patients carrying the UGT1A1*28 allele comparing to the wild type (UGT1A1) allele. No association was also found between non-hematological toxicity (i.e. diarrhea), and UGT1A1*28 genotype either after the 1st or the 6th cycle of chemotherapy. Conversely, the response to treatment seemed to be affected from UGT1A1*28 polymorphism. The *28/*28 mutation of the UGT1A1

gene appeared to have a beneficial effect on disease progression. Homozygous *28/*28 patients have an increased response rate (CR+PR) of about 5 fold compared with the wild-type genotype (*1/*1), and a significantly lower risk of disease progression after 4 cycles of chemotherapy respect to wild type subjects (OR=0.32; 95% CI=0.12-0.86). In addition, tumor response was inversely correlated with glucuronidation ratio (SN-38G/SN-38) and directly correlated to biliary index.

These results suggest that patients carrying the UGT1A*28 variant have a better response in terms of stable disease or partial response, and longer median survival compared to wild type patients (11 months and 8 months respectively). Based on that, dose reduction in UGT1A*28 homozygous patients could be questionable. Since the optimal dose of irinotecan for tumor response appears to be the standard dose (180mg/m^2) only in homozygous *28/*28 patients whereas patients with the wild type allele could be under dosage for optimal response.

We conduced a phase I study in patients with CRC treated with FOLFIRI as first line to define the MTD of irinotecan used in the FOLFIRI regimen (fixed doses of 5-FU 400mg/m^2 bolus followed by FU 600mg/m^2 continuous infusion and LV 200mg/m^2) in heterozygous *1/*28 patients and in homozygous *1/*1 patients [59]. The conclusions of this pharmacogenetic study indicated that the MTD in *1/*28 patients and in *1/*1, was 310mg/m^2 and 370mg/m^2, respectively. Even if tumor response is not the main end-point of the phase I studies, we observed an increased response rate by increasing the irinotecan dose with minimal increases in adverse drug events. Moreover, although tumor response was not the primary endpoint of the phase I study, it was observed an improved response rate with higher dose of irinotecan dose with minimal increases in adverse drug events, suggestive of major benefit of administering irinotecan at higher doses [25].

The addition of BV in the FOLFIRI regimen is effective [20], and generally well tolerated however its use is associated with some unique toxicities [26]. The more common side effects are: hemorrhage, blood hypertension and proteinuria. Nonetheless, rare, yet serious side effects and sometimes fatal have been observed

with BV administration including arterial thrombotic events, gastrointestinal perforation, risk of bowel perforation, risk of reversible posterior leukoencephalopathy syndrome and wound healing complications were infrequent, but were potentially serious events and occasionally fatal [26]. No formal drug interaction studies with anti-neoplastic agents and BV have been conducted. In early registration studies, where FOLFIRI was given with BV, the irinotecan plasma concentrations were found similar to those observed in patients receiving FOLFIRI alone however the concentrations of active metabolite SN-38 were 33% higher in patients receiving FOLFIRI plus BV as compared with FOLFIRI alone. This can in part explain the higher incidence of Grade 3-4 diarrhea and neutropenia observed in the group of patients receiving FOLFIRI plus BV [26]. This observation imposes caution to irinotecan dose increment when administrated in combination with BV.

In this contest we have proposed a Phase I study to assess the recommended dose of irinotecan according to UGT1A1 genotype for FOLFIRI plus BV regimen in patients with mCRC with the intent of increasing the overall efficiency of the treatment.

Impaired glucuronidation activity of the UGT1A1 enzyme is a predisposing factor to severe irinotecan toxicity, due to a genetic polymorphism of the UGT1A1 gene. UGT1A1*28 is a TA in-del polymorphism characterized by an extra TA repeated in the promoter region of the gene [$A(TA)_7TAA$]. This polymorphism is thought to be associated with reduced glucuronidation of SN-38, the active metabolite of irinotecan, compared with wild-type UGT1A1 [$A(TA)_6TAA$], leading to variability in the PK of SN-38 [4]. Several studies have shown a clear correlation between UGT1A1*28 and severe toxicity of neutropenia [24] [25]. The product label for irinotecan in the US has been revised to include UGT1A1*28 as a risk factor of severe neutropenia.

We hypothesize that patients without the UGT1A1*28/*28 (*28/ *28) genotype are less sensitive to the toxic effects of the standard dose of irinotecan, and that higher doses of irinotecan in the FOLFIRI regimen would be tolerated by patients without the risk genotype. Hence, we performed a dose-finding study in patients with the

UGT1A1*1/*1 (*1/*1) and UGT1A1*1/*28 (*1/*28) genotypes treated with escalated doses of irinotecan. This increase is almost double compared to the irinotecan dose typically used in FOLFIRI (180mg/m^2).

Most of genetic determinants currently considered for PG study potentially affect PK. Therefore it is important to define the real impact of PK on PD. It must be considered that several observable phenotypes of drug response in human result from the interactions of multiple factors or covariances, including demographic and environmental factors. On this ground genetic differences affecting PK could be easy to detect, but genetic differences affecting PD would be more difficult to recognize. Despite of these limitations, for the phase I studies based on genetic profile it becomes fundamental to determine the relationship among doses, defined by expected blood levels in individuals rather than by administered doses and response (toxicity) and how specific genetic characteristics affect drug doses. Finally, new ethical issues derive from phase I studies designed on genetic profile of patients. Prospective DNA sample collection from patients requires a formal consent from all participants in phase I clinical trials and for retaining DNA in the event that new genomic issues arise after the completion of the studies.

Pharmacogenomic markers are now increasingly available, but remain poorly utilized. It is hoped that in future subject selection by genotype during prescreening can be used to ensure adequate enrollment of subjects to create a balanced homogeneous subgroup of population for PK and PD effect of the drug under investigation in phase I studies.

1.3 Elderly Pharmacogenetics

It is important that cancer clinical trials enroll a representative sample of patients to ensure that the obtained results are applicable to all those with cancer. Federal laws

require that cancer trials enroll representative samples of women and members of minority groups [27] [28] [29] [30].

These laws may have had some success; several studies indicate that women and minorities are proportionately enrolled onto National Cancer Institute (NCI)-sponsored, cooperative group treatment trials [31] [32] [33]. Despite that, research indicates that the elderly are underrepresented in cancer clinical trials [34] [35] [36] [2]. A study of Southwest Oncology Group about clinical trials active between 1993 and 1996 found that, although 63% of United States cancer patients were over 65 years old, the elderly comprised only 25% of trial participants [2]. However, this study evaluated elderly participation using data from only one cooperative group. Furthermore, the investigators did not evaluate whether elderly participation differed by phase of the trial or stage of disease, or investigate the reasons for the underrepresentation among the elderly. Recent federal efforts have focused on expanded Medicare coverage for clinical trials. To assess the likely impact of improved insurance coverage, it is important to determine the numerous factors that may affect the representation of elderly persons in cancer clinical trials.

The incidence of CRC increases as age progresses [37]. At present, elderly patients have received substandard cancer treatment not supported by “evidence”. Geriatric assessment should be performed preoperatively and selected elderly patients must be offered standard surgical treatment receiving the same complementary therapies as a younger patient. It should be stressed that elderly patients should not be deprived of their decision-making role. In our experience, more than 43% of patients with CRC patients are ≥70 years of age, and we believe that they should receive the same type of follow-up. This would allow for the detection and removal of polyps, treatment of malignant tumors, and psychological support similarly to younger patients. Significantly, in our experience, the incidence of reoperation for neoplastic disease is similar in the two patient populations. Women and men aged 70 years have a life expectancy above or slightly below 15 years. This is a life span long enough to plan and make important decisions, like those involving possible curative cancer

treatments, which, if not started, could unequivocally compromise both life expectancy and quality of life. [38].

Another indisputable issue is that the incidence of CRC increases as age progresses, [38], thus becoming a disease proper to old age [39]: it is in fact the most common tumor in more than 70 years old patients [40] [41].

At present, elderly patients have not had the same oncologic "privileges" as younger patients [41], and 50% of cases, or even more, have received substandard cancer treatment [38] [40]. Often this is not related to the anatomopathologic features of the tumor, to the type of medical procedure, or to the little clear evidence in the literature, but rather to the clinical prejudices associated with the mythical, but not sufficient, "*surgeon's gut feeling*" [40] or to the fatalistic approach of the elderly population. Paradigmatically, in Europe, the age factor alone influences the percentage of overall surgical resections, and, despite the many reports, about 50% of elderly patients treated for stage III CRC, both in Europe and the United States, does not receive any adjuvant chemotherapy treatment [39] [42] [43].

We cannot deny that not all elderly people have a biologic age younger than their chronologic age. More frequent comorbidities [41] [44], poor functional reserves, precarious physical conditions, doubtful mental health, poor rehabilitative potential, and poor social support [45] are all factors that can have a catastrophic impact on optimal treatment.

Therefore, the goal of the physician must be to select elderly patients who are suitable for a standard oncologic treatment. Using scores, such as Charlson's comorbidity score, Comprehensive Geriatric Assessment, Physiologic and Operative Severity Score, or Acute Physiology and Chronic Health Evaluation (APACHE) [42] [46] [40], which identify "fit" patients who can receive the same treatment as that offered to younger patients, also identify "vulnerable" patients who need a tailored treatment, and finally "frail" patients, who will probably not tolerate a radical therapy proper to the disease [47].

Another certainty is that at present, these patients are diagnosed later because of the longer interval between the onset of the first symptoms and the diagnosis [48] [49]. It is also true that elderly patients often delay medical consultation [41], and often the doctor's or family's attention is different for a person with a “limited” life expectancy. Therefore, more urgent operations are required, with a negative impact on postoperative mortality, morbidity and prognosis [38] [49] [41], the staging is worse, and there is also the reluctance of the surgeon to offer to an elderly patient with an advanced neoplasia an optimal curative operation [42] [41] [45] despite the fact that often the tumors in these patients are more amenable to treatment (right colon) or less undifferentiated [38] [48]. Therefore, there is no reason for the “selected” elderly patient with a “not advanced” cancer stage to be treated surgically, or even laparoscopically, in a manner different from that of a younger counterpart.

Even more controversial is the use of complementary therapies. Emblematically, Köhne *et al,* [39] in a well-selected group of elderly patients treated for CRC, report an increase in 5-year survival rate due to a decrease in postoperative mortality and an increase in curative resection, but not with the use of adjuvant therapy, which is still underused. Both adjuvant chemotherapy and radiochemotherapy are “drastically” less used than in younger patients [42] [48] [49] [41]. This is not because of the “evidence” in the literature or the refusal of the patient, who often is willing to receive even a “strong” chemotherapy. If a higher noncancer-related mortality is reported in elderly patients receiving adjuvant chemotherapy, it must be stressed that compared to younger patients, the treatment is not less effective, and toxicity only increases due to some minor complications with a similar survival increase [39] [42]. Actually, the SIOG9 recommends the use of preoperative radiotherapy for the resectable rectal cancer in the elderly patients, and in patients with stage III, the use of adjuvant 5-FU-based chemotherapy in continous infusion, capecitabine, with regard to renal function, and oxaliplatin. With regard to chemotherapy, some investigators report that elderly patients accept the toxicity and the discomforts associated with the treatment, as they are seen as a surrogate of efficacy, which does not affect their quality of life,

but rather improves it. They have lower expectations, pragmatically more than younger patients, they need to fight every day against cancer.

Nevertheless, it is difficult to recommend chemotherapy, as a rule to all elderly patients, especially if “very old” or “non-fit” who should require a rationally tailored treatment [50]. The decision must be made by mutual consent between the physician and the patient taking into consideration comorbidities, performance status, and, last but not least, their own preference.

The majority of elderly patients survive for 5 years or more after colorectal resection and, along with other investigators, they reported no significant difference in cancer-specific long-term survival between elderly and younger patients with the same cancer stage [38] [48] [46] [49] [41] [51]. The age factor may have a negative impact only on the short-term survival [45]. The elderly patients who survive the first year have a prognosis similar to younger patients.

In conclusion, a standard surgical treatment must be offered to “selected” elderly patients with CRC and they must receive the same complementary therapies of a younger patient. In addition, patients more than 70 years of age should receive the same follow-up as their younger counterparts, and not a surrogate that would not produce the same favorable results. [52].

Significantly, “specific” follow-up protocols for the elderly were not even recommended by the 2005 update of the American Society of Clinical Oncology practice guidelines [53] and more recently by Cooper *et al*, [54] who reviewed the international guidelines on colorectal cancer follow-up.

At present, “frail” patients remain the real challenge and it is unclear what is the most appropriate approach, which, as Ugolini *et al* [40] said recently, is “a key point in the everyday life of a surgeon”.

AIMS OF THE STUDY

The intersubject variation that is observed in efficacy and toxicity of anticancer drugs can be determined by complex interactions among components of the physiological, environmental and genetic factors.

To evaluate the correlation between genotype and phenotype of the oncological patients, we considered a pharmacogenetics study which defined the relationship between a specific polymorphism (UGT1A1*28) and the effects of the drugs used for the treatment, irinotecan (CPT-11) and bevacizumab (BV).

A phase Ib clinical trial was conducted at the CRO, Aviano (PN). The purpose of this study was to modulate the dosage of CPT-11 in the presence of the angiogenic inhibitor BV, being yet unknown the interaction between the two drugs, for each individual patient based on genotype of UGT1A1, in order to obtain an improvement of the therapeutic index .

We enrolled patients with a histological diagnosis of metastatic colorectal adenocarcinoma, naïve or treated with adjuvant chemotherapy (excluding irinotecan), and meeting the criteria for eligibility/exclusion of the protocol.

The patients were assigned to their treatment group based on genotype (*1/*1 or *1/*28) until the completion of recruitment for each dose level in each group of patients. Patients with genotype *28/*28 were excluded because of high risk of toxicity. The starting dose of irinotecan in patients carrying the *1 (wild-type and heterozygous) was 260mg/m^2. The dose of BV was 5mg/kg, also administered in infusion every two weeks. In case of lack of toxicity in the lower dosage treatment, the CPT-11 dose was increased to 310 and 370mg/m^2.

The evaluation of pharmacokinetic and pharmacodynamic interactions between BV and CPT-11 was conducted describing the pharmacokinetic profile of the CPT-11 (and its metabolites) in the absence and in the presence of BV in the same patient.

The high variability of the response and toxicity makes essential the definition of useful criteria to personalize the treatment.

Basic criteria for personalization of therapy are clinical parameters, mainly the gender and the age. In a second part of the PhD project, we considered a large series of patients with CRC treated with different therapies with fluoropyrimidine (FOLFOX or FOLFIRI) at the CRO, Aviano (PN), and other centers participating in the program. We performed a pharmacogenetic study to see if there are genetic biomarkers useful to define a personalization of therapy for subjects considered elderly (age at diagnosis >70 years).

In particular, the aim was to identify possible pharmacogenetic (PG) determinants which influence the effects of the drug (toxicity and response) in a different way between young and elderly subjects, and between the two genders (male and female).

In this study we examined associations of different genotypes and clinical factors (age, gender, stage, localization of the tumor) with risk and we assessed the effect of genetic polymorphisms on survival in CRC patients treated with adjuvant/palliative chemotherapy.

MATERIALS AND METHODS

3.1 Pharmacokinetics Analysis

The study was conduced in patients with metastatic adenocarcinoma of the colon and rectum, enrolled at the National Cancer Institute CRO (Aviano, PN). All patients were registered and all signed the consent to the processing of personal data and informed consent for genetic analysis approved by the local Ethics Committee and by the Institute of Medicine.

3.1.1 Patients Enrollment

Eligibility criteria are: histologically or cytologically confirmed diagnosis of metastatic colorectal adenocarcinoma, beyond a curative option for surgery to be defined**,** no prior chemotherapy for metastatic disease. Age ≥18 or ≤75 years; Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, life expectancy >3months; measurable disease (defined as >1cm on spiral computed tomography scan); adequate organ function, including bone marrow (absolute neutrophil count (ANC) ≥1,500/μL, haemoglobin ≥9.0g/dL, platelets ≥100,000/μL); hepatic (total bilirubin <1.6mg/dL, international normalized ratio or ≤2x for Gilbert's Syndrome, aspartate aminotransferase/alanine aminotransferase <2.5x upper limit of normal for patients without liver metastases, <5x upper limit of normal for patients with liver metastases); and kidneys (serum creatinine ≤1.5x upper limit of normal) function.

Elegible patients were genotyped for the UGT1A1*28 polymorphism and stratified in two groups based on the presence of *1/ *1 or *1/ *28 genotype. Patients with both variant alleles *28/*28 were excluded. Signed informed consent and local IRB approval was requested.

The exclusion criteria are: prior irinotecan and bevacizumab treatment; chronic enteric diseases (Crohn disease, ulcerous colitis), unresolved diarrhea and bowel obstruction; documented cerebral metastasis; serious active infectious disease; serious functional alteration of visceral and metabolic disease; pregnancy status. Radiotherapy or major surgery within 4 weeks. All patients in fertile age must have

been under contraceptive treatment; presence of previous or concomitant neoplasm with exclusion of in situ cervical cancer; and patients who could not attend periodic clinical check-ups.

3.1.2 Drug Administration and Dose Escalation

Patients were be treated with the FOLFIRI regimen plus BV, where irinotecan was administered at doses higher than the standard dose in patients with the UGT1A1 *1/*1 and *1/TA_7 genotypes, while the dose of infusional 5-FU and leucovorin remained unchanged.

The initial dose of irinotecan for the two groups of patients (the UGT1A1 *1/*1 and *1/*28) was 260mg/m^2 administered as a 120min intravenous infusion every 2 weeks. The dosage of irinotecan was be increased to 310, 370, and 420 mg/m^2, and further irinotecan doses were increased of 14%; 5-FU was administered as 400mg/m^2 bolus right after the end of the irinotecan infusion, followed by 2400mg/m^2 over a 46h continuous infusion plus leucovorin 200mg/m^2 every two weeks. Bevacizumab will be administered at a dose of 5mg/kg by 90min IV on day 3 and 15 during the first cycle of treatment. No dose modification will be performed for 5-FU, LV and bevacizumab. One cycle lasts 28 days. Before starting irinotecan, patients will be pre-treated with atropine 0.5mg, dexamethasone 8mg, granisetron 3mg or ondansetron 8mg. Diarrhea will be treated promptly with loperamide 4mg at the onset, and then with 2mg every 2h, until the patient will be diarrhea-free for at least 12h. Growth factors (i.e., G-CSF) will be allowed only in patients who had grade 3-4 neutropenia at previous cycles.

DLT is defined as hematological grade 4 toxicity or non hematological grade 3-4 toxicity recorded at cycle 1 that developed or persisted despite supportive measures (i.e. anti-diarrheas or anti-emetics). Toxicity is classified and graded according to the United States NCI's common toxicity criteria.

Three patients will be enrolled at any dose level, will be treated with irinotecan at 260mg/m^2 and if DLT is observed in <1/3 of them, dose level will be escalated and 3

patients will be treated at the next dose level (310mg/m^2). If DLT was observed in 1/3 of the patients, 3 additional patients were enrolled at the same dose level and the escalation to the next dose level (310mg/m^2) continued if DLT occurred in ≤1/6 of the 6 patients treated at 260mg/m^2. If DLT was observed in ≥1/3 or >1/6 patients treated at any given dose level, the dose escalation was stopped, and 10 patients total were then enrolled at one dose level below to assess the safety and the inter-patient pharmacokinetic variability. If DLT is observed in <1/3 of patients enrolled at this dose level experience DLT, this dose level was declared as the MTD.

The MTD recommended for phase II studies will be defined as the dose level immediately below that at which ≥1/3 of patients out of three patients or ≥1/6 out of six patients experienced DLT. Therefore at the MTD, ≤1/3 out of at least 10 patients experienced DLT. No intra-patient dose escalation is allowed.

At present, 9 patients with *1*1 genotype have been treated at the 260mg/m^2 dose level and nowadays just one exhibited DLT. Therefore, we propose to re-escalate the dose in the *1/*1 cohort to 310mg/m^2. Thus far, 3 patients with *1/*1 genotype have been treated at the 310mg/m^2 dose level, and 2 of these 3 had a DLT. After re-escalating the dose, we would plan to expand up to 10 patients in the *1/*1 cohort at 310mg/m^2 and proceed as follows:

- If > 3 of 10 patients have a DLT, then 260mg/m^2 would be declared the MTD
- If exactly 3 patients have a DLT, then 310mg/m^2 would be declared the MTD
- If < 3 of 10 patients have a DLT, then the dose would be escalated further to 370mg/m^2.

Patients can continue receiving the same dose of irinotecan in absence of major toxicity according to the following criteria: before re-treatment, full recovery from any non haematological toxicity, an absolute neutrophil count ≥1.5*10^3/μL and platelet count ≥1000*10^3/μL, are required. Chemotherapy is discontinued on evidence of disease progression, or the appearance of new lesions on serial magnetic resonance or CT scans. Patients experiencing a major toxicity during the first or successive cycles of therapy are allowed to receive additional treatment with a 25% reduction in the dose

of irinotecan. The cumulative haematological and non haematological toxicity as well as the number of dose reductions and a delay in starting the next cycle of treatment will be used as secondary indicators to differentiate the two genotype cohorts of patients.

3.1.3 Measurement of Effect

Although response is not the primary endpoint of this trial, patients with measurable disease will be assessed by standard criteria. For the purposes of this study, patients should be re-evaluated every 2 cycles.

Response and progression will be evaluated in this study using the international criteria proposed by the Response Evaluation Criteria in Solid Tumors (RECIST) Committee [55]. Changes in only the largest diameter (unidimensional measurement) of the tumor lesions are used in the RECIST criteria.

To do that, the following response criteria are considered:

A)Evaluation of Target Lesions

Complete Response (CR): Disappearance of all target lesions.

Partial Response (PR): At least a 30% decrease in the sum of the longest diameter (LD) of target lesions, taking as reference the baseline sum LD.

Progressive Disease (PD): At least a 20% increase in the sum of the LD of target lesions, taking as reference the smallest sum LD recorded since the treatment started or the appearance of one or more new lesions.

Stable Disease (SD): Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum LD since the treatment started.

B) Evaluation of Non-target Lesions

Complete Response (CR): Disappearance of all non-target lesions and normalization of tumor markers' level.

Incomplete Response/Stable Disease (SD): Persistence of one or more non-target lesion(s) and/or maintenance of tumor marker level above the normal limits.

Progressive Disease (PD): Appearance of one or more new lesions and/or unequivocal progression of existing non-target lesions.

Although a clear progression of "non-target" lesions only is exceptional, in such circumstances, the opinion of the treating physician should prevail, and the progression status should be confirmed at a later time by the review panel (or study chair).

C) Evaluation of Best Overall Response

The best overall response is the best response recorded from the start of the treatment until disease progression/recurrence (taking as reference for progressive disease the smallest measurements recorded since the treatment started). The patient's best response assignment will depend on the achievement of both measurement and confirmation criteria.

Target Lesions	Non-target Lesions	New Lesions	Overall Response
CR	CR	No	CR
CR	Incomplete response/SD	No	PR
PR	Non-PD	No	PR
SD	Non-PD	No	SD
PD	Any	Yes or No	PD
Any	PD	Yes or No	PD
Any	Any	Yes	PD

Patients with a global deterioration of health status requiring discontinuation of treatment without objective evidence of disease progression at that time should be classified as having "symptomatic deterioration". Every effort should be made to document the objective progression, even after discontinuation of treatment.

3.1.4 Drugs Assay and Pharmacokinetics

Serial blood samples were collected into heparinized tubes before drug administration, and at 1.0, 2.0, 2.25, 2.50, 3.0, 4.0, 6.0, 8.0, 10.0, 14.0, 26.0, 50.0 h following the start of the irinotecan infusion. Plasma was obtained immediately by centrifugation of the blood samples at 3,000g for 15min at 4°C, and stored at -80°C.

The total plasma concentration of irinotecan (lactone plus carboxylate) and its metabolites SN-38 and SN-38G, were determined by using a new LC-MS/MS method that was specifically developed.

Non-compartmental analysis was used for pharmacokinetics analysis. A linear-log trapezoidal numerical integration method was used to calculate the area under the irinotecan, SN-38 and SN-38G plasma concentration-time curve ($AUC_{0\text{-last}}$) from time 0 to the last sampling time. The glucuronidation ratio (GR) measured the extent of glucuronidation of SN-38 to SN-38G in plasma (SN-38G AUC divided by the SN-38 AUC). The biliary index (BI) was defined as the product of the irinotecan AUC and the ratio of the SN-38 AUC over the SN-38G AUC.

3.1.5 Development of the Analytical Method

In order to describe the pharmacokinetics in patient's plasma, it has been developed a HPLC-MS for the dosage of the irinotecan (CPT-11), its main metabolites (SN-38, SN-38G, APC and NPC) and the Internal Standard (SI). As an IS was selected a compound having the chemical-physical properties as similar as possible to those of the analytes of interest: camptothecin (CPT). The IS is added to all the samples analyzed and each calibration solution.

The solutions used in this development phase of the method are prepared by progressive dilution of each standard. A mixture of equal parts of the mobile phases used for the chromatography (A and B) is used as solvent. The final concentration used for each standard was 50ng/mL; this amount is sufficient to generate in the mass spectrometer a good signal for the optimization of the parameters (in the order of $1x10^6$cps).

We used milliQ water and acetonitrile as mobile phases for the chromatographic run; both are commonly used for the analyzed compounds and were added with 0.1% acetic acid to allow the ionization of the compounds.

The development of the method is divided into stages of optimization of the analysis conditions, which chronologically follow one another in the opposite way of the path

that will accomplish by the sample during the analysis: it starts from the determination of the parameters of the analyzer, the mass spectrometer, passing to the chromatographic component, to then define the method of processing the biological sample.

The instuments we used were:

- HPLC shimatzu Prominence, made of controller CBM-20A, solvent dispensation unit LC-20A, refrigerate autosampler SIL-20A, column support termostatate CTO-20A.
- Chromatographic column (Gemini C18, 100x2mm, 3µm, Phenomenex) with a pre-column (C18, 4 x 3mm, Phenomenex).
- Spectrometer triple quadrupole (Applied Biosystems API 4000) with an elettrospray source (ESI), used in positive mode, and connected to a computer with the software Analyst^{R}.

The reagents we used were:

- Acetic acid glacial (99,9%), acetonitrile and methanol (HPLC-grade, Carlo Erba, Milan).
- milliQ water made by Milli Ro 60 Water System, Millipore (Milford, MA, USA).

3.1.5.1 LC-MS/MS Method

The *first* step is the optimization of the compound dependent parameters, that are variables depending on the analyte and therefore they must be optimized individually for each compound. The solution containing the compound was infused directly to the source of the spectrometer via infusion pump; in this case it has been used a constant flow of 10mL/min. The variations step by step for the development were performed through AnalystR, the management software of the mass spectrometer. We proceed with the "Manual tuning", starting from the initial baseline values for the parameters of the source: TEM=0, CUR=10, CAD=10, GS1=11, GS2=0, IS=5500.

We determined the monoisotopic mass of the analyte in the range of decimal in a scan mode Q1MS, and without applying energy to the collision cell. It starts by performing

a scan in a range of values of m/z in which to perform the reading. During the first analysis we used a wider range to detect all the possible adducts of the test compound with the mobile phase used. For example using acetic acid, it will be possible to obtain the formation of adducts with the acetate, highlighting the presence of a signal having m/z of 59amu.

Another aspect to consider is the time to scan: in this mode the spectrometer operates a scan of all the values of m/z belonging to the selected range, so if the dwell time (the time at which the analyzer collect events for each step of mass) is not proportionate to the range of m/z, inaccurate readings are obtained.

After obtaining the stabilization of the signal, we mesured it in "MCA" (Multiple Count Acquisition) mode. In this way the TIC (Total Ion Count) will be the sum total of all the recorded events in the interval of time and mass selected; then we obtained the m/z value detected by the instrument.

Then it's possible to proceed with the optimization of the parameters DP and EP (compound dependent) in Q1MI mode selecting a specific value of m/z that is the one obtained by the above analysis . For example, in the case of SN-38, was used the value of 393.3 . It's important to test again the stability of the ion signal selected (eg 393.3) and then, using the " RAMP" mode, it's possible to scan the voltage values of DP between (0 and 400V). The signal starts from the minimum and increases to a maximum value, then decreased again and return to minimum values. The value of DP that will be used for the analysis will be the one which will present the maximum intensity. At the same way, we determined the optimal value of EP, having first carefully insert the value determined for DP .

We proceed with the analysis of the fragmentation pattern using again the command "RAMP". In this way it was possible to evaluate the formation of several fragments by applying different collision energies (CE) activating the "MCA" option in Product ions (M2) mode. Once we had selected the main fragments, we proceed with the determination of the optimal value of EC for each one, using the MRM mode, with a dwell time of 200ms, varying the value of the EC. The result was a graph that shows

the intensity of the signal for each fragment and can we identified the most representative fragments and the relative EC value suitable to obtain the maximum signal intensity. For each fragment was determined the optimal value of the CXP (Collision Exit Potential) parameter, using the MRM mode again and the m/z values of each fragment with the corresponding value of CE.

Once obtained the values of DP, EP, m/z of the fragments and their CE and CXP, we used the method in MRM mode. In the method Multiple Reaction Monitoring (MRM), Q1 functions as a filter and allows only the passage of the analyte of interest (Precursor ion), Q2 fragments it, and Q3 filters the mass of one or more of its fragments (Product ion). The precursor iongoes into the collision cell in which it is fragmented into the so called “product ions” or “ions children”. Finally Q3 filters the mass of one or more of the fragments thus generated.

The MRM acquisition mode is best suited for quantitative experiments as it maximizes the signal/noise ratio of the analyte of interest and, monitoring the m/z of the precursor ion and of its fragments, appears to be highly specific.
Using Flow Injection Analysis (FIA), it is possible to define the source dependent parameters. These are optimized to obtain the maximum signal intensity of the analyte of main interest (in this thesis the SN-38).

With the FIA method we performed the optimization of source dependent parameters mimicking what will be the actual conditions in the analysis of biological samples. The solution of the analyte of interest was injected every 30 seconds in a constant flow of the mobile phase by autosampler. During the optimization phase by FIA, the chromatographic column is not mounted. In this way, the analyte reached the source in a time dependent only on the length of capillary tubes which separate the injection site from the entrance into the source of mass. We then guided the optimization of each parameter of the source by the management by the software of the mass spectrometer going to increase as much as possible the signal intensity of the transition selected as the main quantifier of the analyte of interest.

In that way the determination of the temperature conditions, the flow gas and the elettrospray potential that will be used for the analysis were possible. The flow and the mobile phases used in this phase of optimization are obviously those that will be used for the analysis of plasma of patients enrolled in the clinical study.

The *second step* of the development of the method provides for the determination of the best chromatographic conditions for the analysis. This allows the separation by chromatography of the analytes. To determine the best chromatographic conditions, different chromatographic parameters were verified:

- Chromatographic column
- Initial percentage of organic phase ($\%B_{min}$)
- Maximum percentage of organic phase ($\%B_{max}$)
- Type of gradient
- Flow velocity
- Temperature of the column
- Injection volume

A chromatographic method is described by the variation of the percentage of mobile phases in time. There are precise phases that occour to develop a chromatographic method: firstly it is important to determin the initial percentage of the organic phase ($\%B_{min}$), which increases to a maximum value ($\%B_{max}$), mantained for a certain time, up to a rapid return to $\%B_{min}$. The high percentage of organic phase is needed to prolong the usage time of the column, avoiding possible occlusion generated by residues of the organic matrix accumulated during subsequent chromatographic runs. The final stage, called reconditioning, restores the initial conditions before the next race. Failure reconditioning may lead to alterations in retention times and therefore the failure reproducibility of repeated runs. In fact, if the analytes at the time of injection are in a different condition compared to T0, it should be possible to have different behaviors from those we expected. Before to start a chromatographic run is necessary that the column is completely conditioned. The duration of the gradient should be established so that all the substances elute before the reconditioning

process. The initial conditions of the chromatographic run determine the conditions encountered by the sample when it is injected into the column. In the case of a reverse phase chromatography, where the analytes are eluted in order of increasing affinity for the organic phase, if the percentage is too high in the initial stages it may interfere with the interaction between the analytes and the functional groups of the particulate of the column. This can lead for example to the formation of two chromatographic peaks for a single analyte (peak-split), in which the first peak comes out together with the solvent front .

The parameters that must be considered in the selection of the best method are:

- the best separation of the different substances;
- the shape of the chromatographic peaks, especially in relation to the compound in the queue of the race, which is the one most affected by the change of $\%B_{max}$ and from the slope of the increase in %B;
- reproducibility and absence of carry-over;
- minimum dual time analysis obtained by retention times reduced.

Generally a good chromatographic method allows the distinction of each substance as a single chromatographic peak. When two analytes co-elute, it is necessary to demonstrate that there is signal interference between the two. The possible interference between the two compounds occurs by injection of each analyte. If there is no interfering signal in the chromatogram trace of each of the fragments of the injected analyte, the chromatographic method should be considered acceptable. (Figures 1a, 1b, 1c).

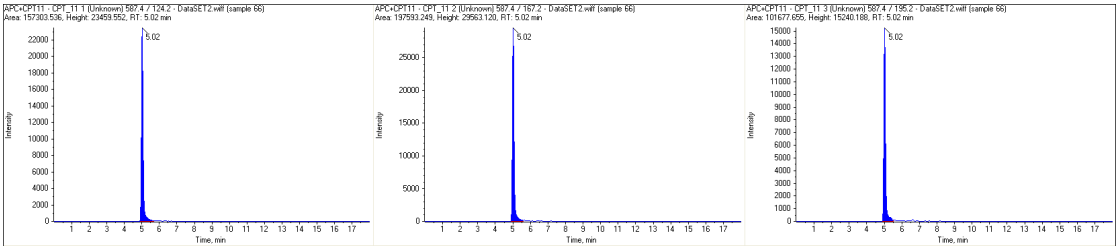


Figure 1a

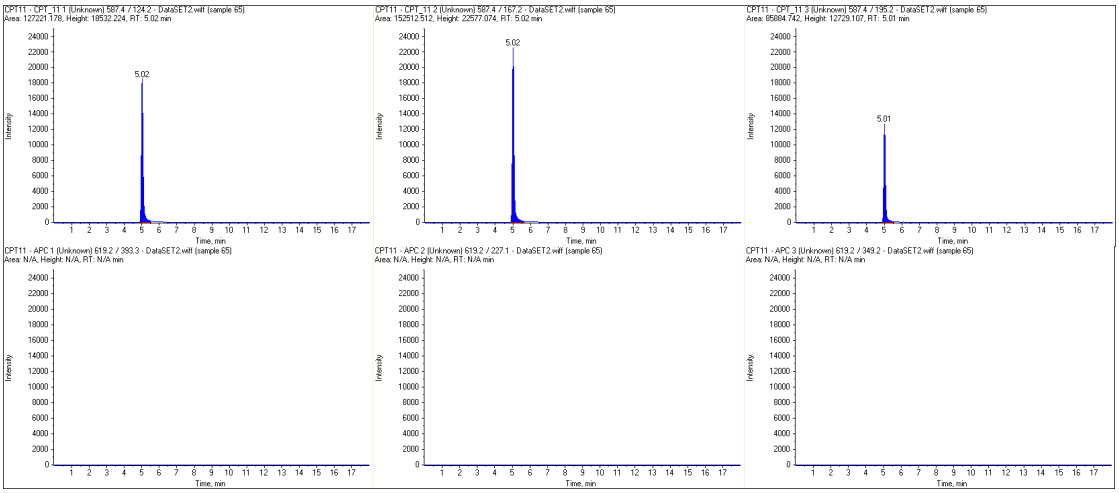


Figure 1b

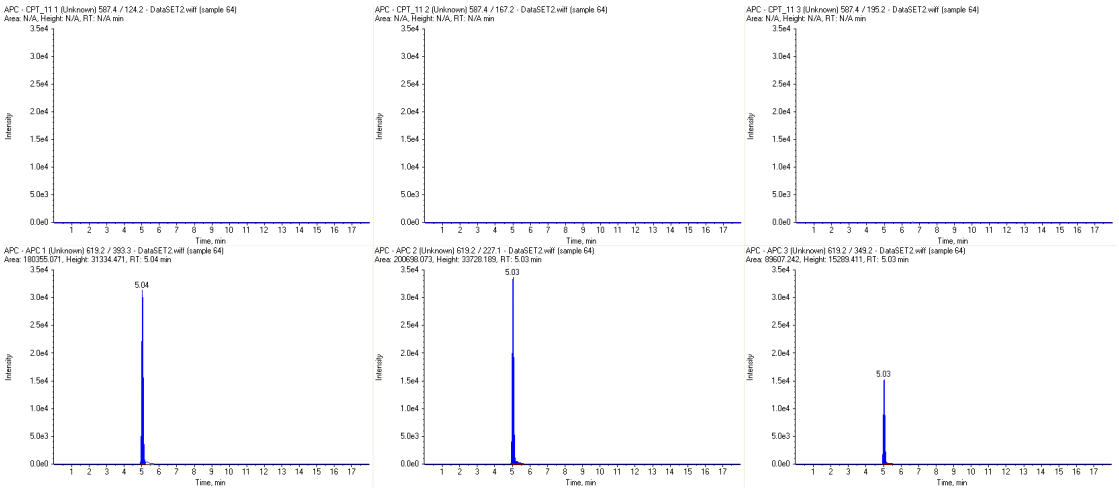


Figure 1c

Figure 1. Check of interference between two co-eluting analytes. The three groups of graphs (a), (b), (c), respectively, are related to the solutions composed of: CPT-11 and APC together, CPT-11 alone, APC alone. The tracks of the transitions considered for each,

are listed on the same line in the same order, respectively, of CPT-11 and APC. When are injected separately, there is no signal for relative to the transitions of the other compound as shown by the graphs (b) and (c).

3.1.5.2 Matrix Effect Evaluation

To assess the matrix effect on the ionization of the analytes, it may proceed with the infusion of each test compound during the chromatographic run of a sample extracted by the matrix. For this purpose, we used a three-way entrance, connecting simultaneously the capillary tube of the HPLC to the source of the mass spectrometer and the capillary tube of the infusion syringe to the spectrometer (Figure. 18). The flow of the syringe is operated by an infusion pump with constant flow adjustable. The infusion pump must be regulated with a flow able to counterbalance the one coming by HPLC: in the case of a flow of 300µL/min will be needed the infusion of the flow coming from the syringe at a rate of 99µL/min.

Distinct solutions were prepared for each analyte, and eachone was infused simultaneously to a chromatographic matrix. It was observed then the variation of the compound signal infusing a concentration capable to generate a signal of the order of $7x10^5$- $1x10^6$cps.

If the matrix does not generate any effect on the ability of ionization of the analyte, a stable signal would be observed over time, without any change. In the case of a matrix effect, we should be able to observe a chromatogram trace on the transmission of the analyte selected as quantifier which presents peaks and valleys (Figure 2). In the latter case, we should have to check the stability of the signal in the neighborhood of the retention time of the analyte. In the event that the signal does not prove stable, we should have to proceed with the change of gradient chromatography and re-evaluation of the matrix effect.

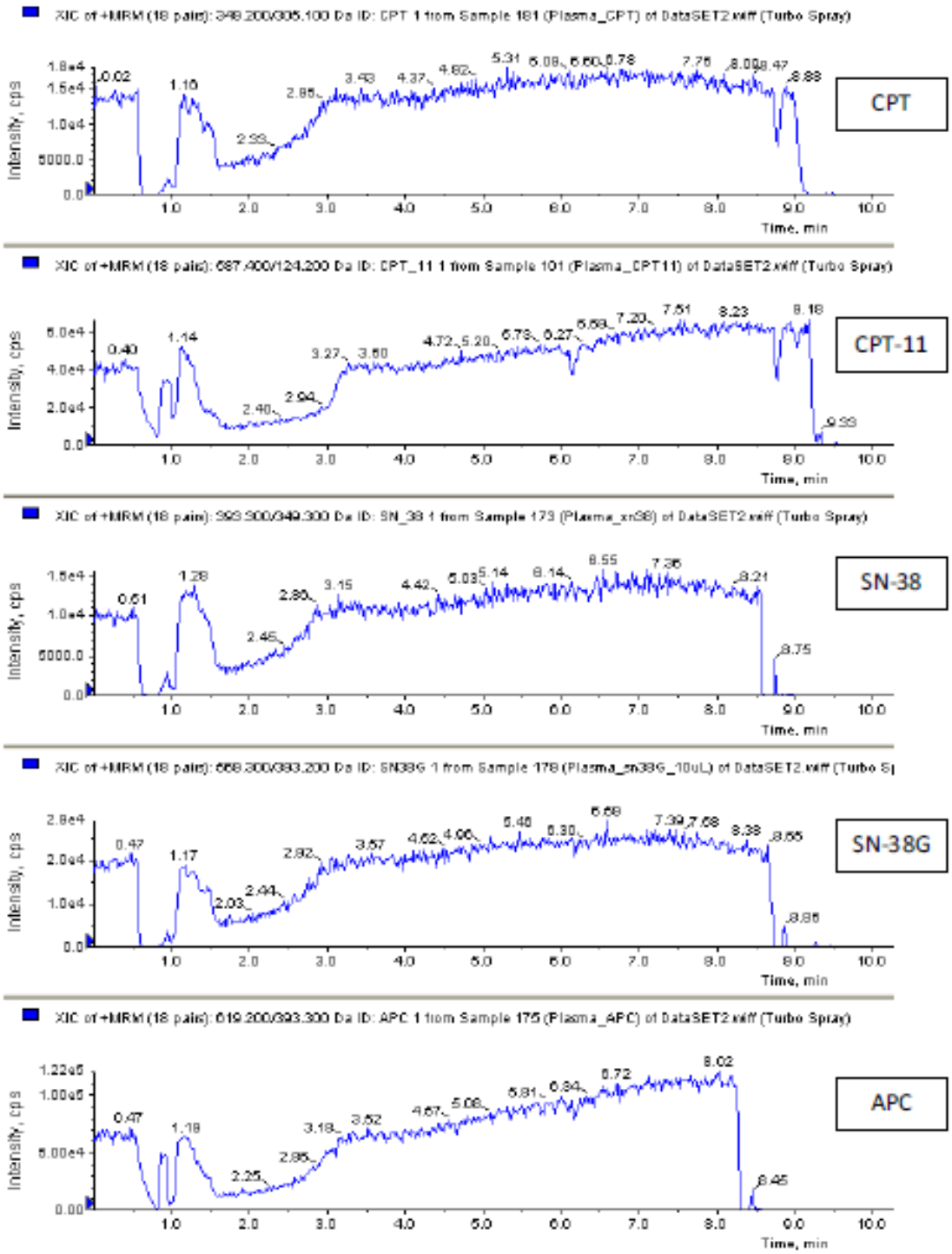


Figure 2. Evaluation of the matrix effect for the analyzed compounds.

3.1.6 Biological Samples Preparation

The plasma samples are made to thaw at room temperature, vortexed and centrifuged for 10min at 3000rmp in a thermostated centrifuge (4°C). Then the subsequent steps are performed on a small aliquot (100µL), which is transferred into a clean tube.
The extraction method has been established by comparison with the methods used in the literature. In a first phase is provided the use of acetonitrile added to 0.1% of acetic acid. However, the use of methanol instead of acetonitrile has improved the shape and the definition of the chromatographic peaks.
5µL of CPT 0.5g/mL were added to each sample and then we proceed with the extraction:

- 300µL of precipitant solution were added
- the sample was mixed by vortexing for 10sec at maximum speed
- the sample was centrifuged for 10min at 13000rpm at 4°C
- 150µL of supernatant were transferred to a vial for analysis

3.1.7 Analytical method validation

3.1.7.1 Preparation Of Standard Solutions And QC

The solutions of standards were prepared in volumetric flasks, using DMSO as a solvent, which resulted the only one able to guarantee the complete dissolution of the compounds to be used. The solution of CPT was instead prepared in methanol. The concentrations of the stock solutions (in DMSO) are shown in table:

Compound	*Mg/mL*
CPT-11	*5006*
APC	*1000*
SN-38	*99.8*
SN38-G	*99.6*
CPT	*0.5*

From these solutions ''stock'' were obtained solutions of the standard curve and of the QCs, preparing dilute solutions in methanol, containing all the compounds (to the exclusion of the SI), always in volumetric flasks, in order to obtain standard solutions and QCs. These were then transferred into appropriate tubes and stored at -80°C. For each test performed in plasma, were used 95µL of control plasma from a healthy donor pool, to which were added 5µL of the solution of each of the standards (prepared at a concentration 20x compared to the value reported in Table 1). They were then added to 5µL of the solution of CPT and was finally performed the extraction process developed.

(ng/mL)	**A**	**B**	**C**	**D**	**E**	**F**	**QCH**	**QCM**	**QCL**
CPT-11	10000	8000	5000	1000	100	10	9000	6000	25
APC	5000	2500	1000	100	10	1	4000	2000	2
SN-38	500	250	100	25	5	1	400	150	2
SN-38G	500	250	100	25	5	1	400	150	2

*Table 1. Concentrations used for the points of the straight line (A, B, C, D, E, F) and for the QC*H, M, L. The values refer to the final concentration in the sample before processing and the addition of IS.*

3.1.7.2 Recovery value

The recovery value was calculated as the average of the ratio of the signal detected for each compound when added to the matrix and then processed, compared to the addition of the same compounds to the matrix already processed. The experiment was repeated in five replicates with three different concentrations (QCL, QCM, QCH). The table 6 shows the average values obtained.

3.1.7.3 Linearity of the calibration curve

The linearity of the calibration curve was validated by comparison of the straight lines of five different days, determining the values of the intercept and the slope. These values were obtained by weighted regression (weighted least-squares regression), with $1/x^2$ as ''weight''. The intercept (q) and the slope (m) of the equation of the line

y=m*x+b were then calculated by the software MultiquantR elaborating the values of y (area analyte/area of IS) and x (nominal concentration) obtained from the points of the calibration curve. The goodness of the values obtained was evaluated by determining the Pearson coefficient (r) and the reproducibility was determined by the average of the values of m, q and r, with the relative standard deviation (SD) and coefficient of variation (CV%). In the graph (Figure 3) there is an example of the straight lines obtained for the different compounds in one of the days of the validation.

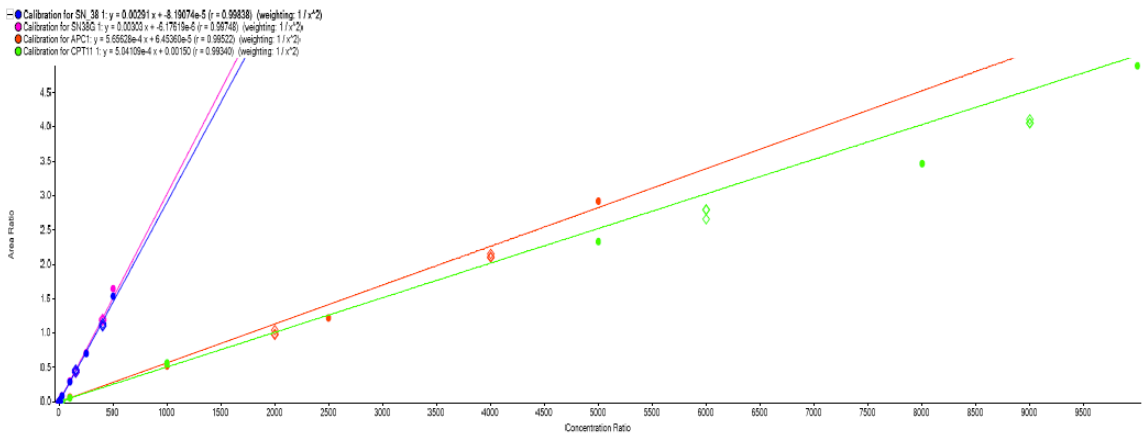


Figure 3. Example of a summary chart of the calibration lines used during a day of validation.

3.1.7.4 Lower Limit of Quantitation

The lower limit of quantification (Lower Limit of Quantitation, LLOQ) was defined on the basis of lower concentration of each analyte detected with precision and accuracy by 20% and the concentration that would guarantee a signal/noise ratio less than or equal to 10. The value dell'LLoQ has been established to be the lowest concentration of the calibration curve. As a reference, this is also the graph of the signal on a control sample obtained by the extraction method of a control plasma pool of healthy donors (Figure 4). The figure shows the graphs and the corresponding signal / noise ratio for the LLOQ for the different compounds (Figure 5).

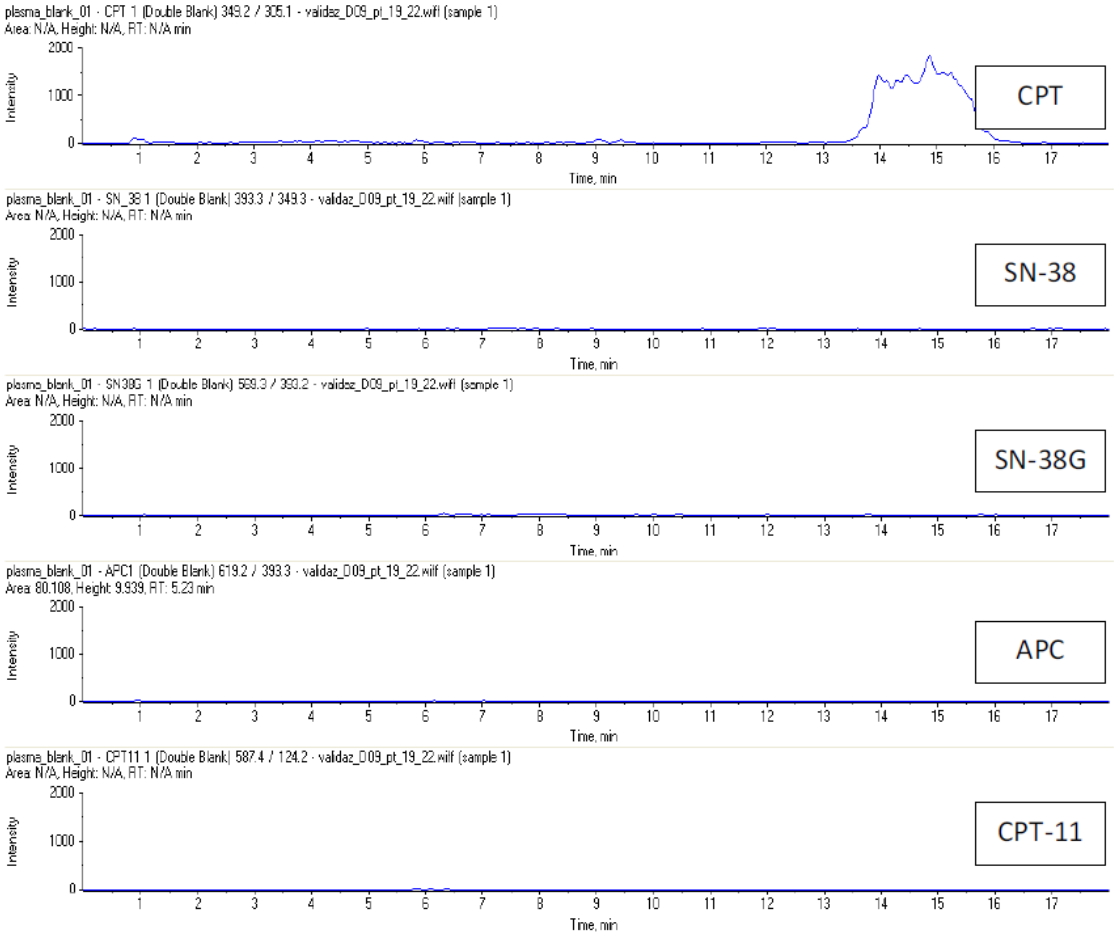


Figure 4. Signal relative to the main transitions of each compound for a control sample. It can be noted how there are no signals related to the presence of the analytes, but only background noise. In the transition of the CPT (the internal standard) there is a high amount of noise towards the end of the chromatographic run, when all compounds therefore have already been recognized, and therefore does not affect the measurement.

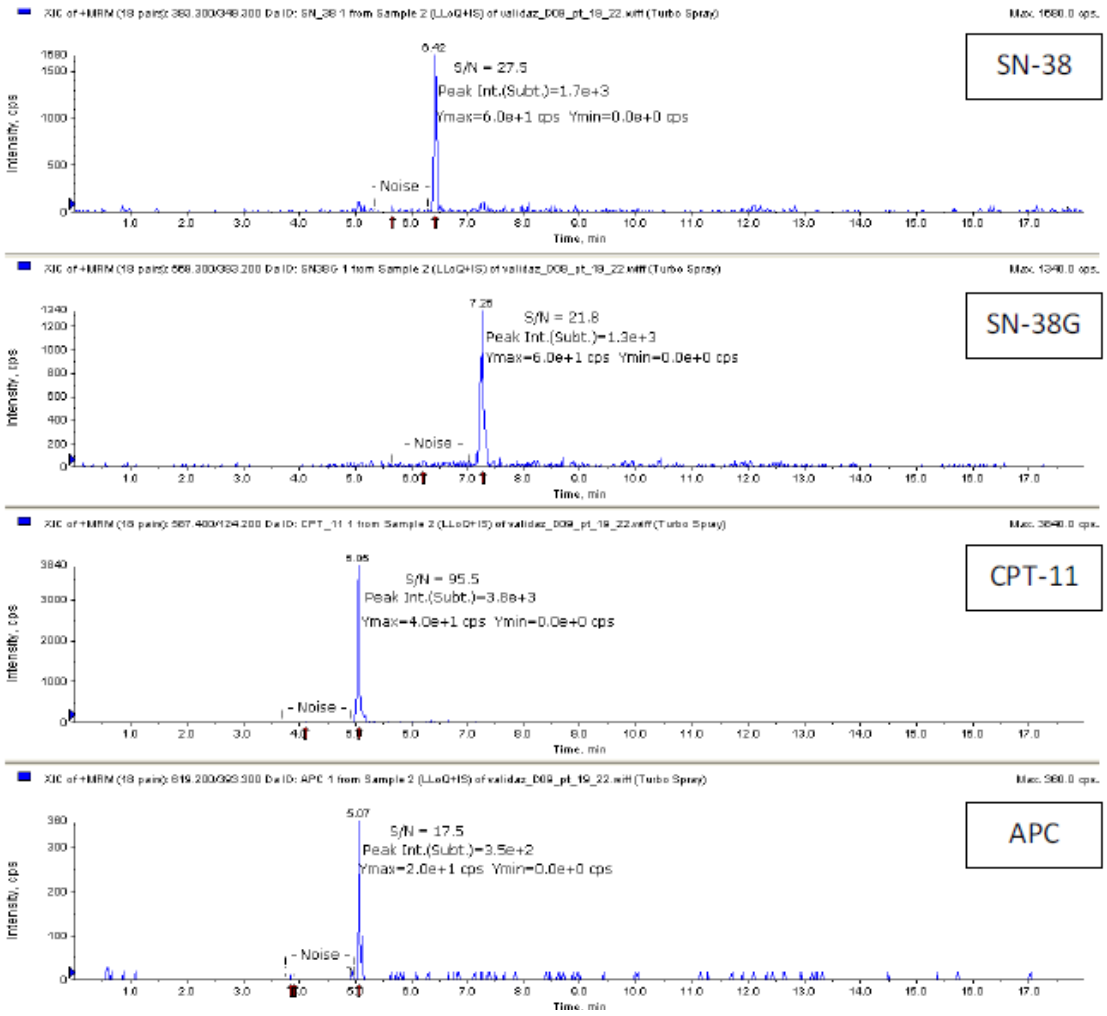


Figure 5. Chromatographic tracings for the transition used as a quantifier of the four analytes, illustrating the relative signal/noise ratios (S/N). As can be seen from the image, the values S/N are related to the ratio between the signal of the peak and the noise signal marked as ''Noise'' and for each compound is greater than 10.

3.1.7.5 Stability

The stability of each analite was evaluated using the matrix (plasma control) in three different concentrations (QCL, QCM, QCH). There were compared the different values obtained by the following conditions:

- Ambient Temperature at 4h, 3h, 2h, 1h;
- On ice at 4h;
- After two cycles of frozen/melting (FTC);

- In the autosampler (4°C) at 72h and 96h.

3.1.8 Statistical Analysis

Differences between the irinotecan pharmacokinetics parameters when administrated alone and in association to bevacizumab were investigated by the non parametric Wilcoxon signed ranks matched pairs test while the statistical significance of correlation between pharmacokinetics parameters was investigated by Spearman non parametric Spearman Rank Correlation Test. Two-tailed p values of <0.05 were considered to be statistically significant.

Exploratory analyses were conducted and a P value less than 0.05 was considered of nominal statistical significance. The effect of irinotecan dose and UGT1A1*28 genotype on TTP was estimated using the Kaplan-Meier method, and differences were tested using the log-rank test. The effect of irinotecan dose and UGT1A1*28 genotype on response rate was evaluated using multivariate logistic regression modeling, adjusting for age, sex, and adjuvant chemotherapy. The correlation between irinotecan dose and pharmacokinetic parameters was tested by Spearman's rank correlation test. The Mann-Whitney test was used for two-group comparisons (UGT1A1*1/*1 and *1/*28 genotypes and dose-normalized AUCs).

3.2 Molecular Analysis

3.2.1 Candidate Gene And Pathway-Based Approaches

In the candidate gene approach, a panel of genes of interest was selected according to their involvments in the drug's mechanism of action. This included genes encoding for its target for the drug-metabolizing enzymes and the membrane transporters involved in drug's absorption, distribution, metabolism and elimination. In this study we analyzed 49 variations in 29 genes coding for protein involved in different regulatory

pathways (drug transport, DNA repair, phase I metabolism, phase II metabolism, folate cycle, catabolism and cell cycle regulation) as shown in the following table.

REGULATORY PATHWAYs		INVESTIGATED GENES	INVESTIGATED VARIATIONS	AA VARIATIONS
DNA Repair Enzymes	**BER**	hOGG1	hOGG1 1245C>G	Ser326Cys
		HEXO1	HEXO1 Ex12+49C>T	Thr439Met
		XRCC1	XRCC1 28512G>A	Arg399Gln
			XRCC1 Ex9 +16G>A	Arg280His
			XRCC1 26304C>T	Arg194Trp
			XRCC1 1449delGGCC	NA
		PARP	PARP Ex17+8T>C	Val762Ala
		APE1	APE1 2197T>G	Asp148Glu
	NER	ERCC1	ERCC1 8092C>A	NA
			ERCC1 19007C>T	Asn118Asn
		XPD	XPD 23591G>A	Asp312Asn
			XPD 3591A>C	Lys751Gly
		XPG	XPG 3507G>C	NA
	MMR	hMLH1	hMLH1 676A>G	Ile219Val
		hMSH2	hMSH2 IVS12 -6C>T	NA
		hMSH6	hMSH6 556G>T	NA
	DSBR	XRCC3	XRCC3 17893A>G	NA
			XRCC3 18067C>T	Thr241Met
			XRCC3 4541A>G	NA
		MGMT	MGMT Ex5-25C>T	NA
Phase I metabolism		SOD2	SOD2 16C>T	Ala16Val
		SOD3	SOD3 231C>T	Arg231Gly
		CYP3	CYP32D6	NA
			CYP3A4	NA
			CYP3A5	NA
		CYP17	CYP17	NA
Phase II metabolism		GSTA1	GSTA1*B	NA
		GSTT1	GSTT1 pos/neg	NA
			GSTT1 COPY NUMBER	NA
		GSTM1	GSTM1 pos/neg	NA
			GSTM1 COPY NUMBER	NA
		GSTP1	GST P1 313A>G	Ile105Val
			GSTP1 5C>T	NA
		UGT1A1	UGT1A1 *28	NA
			UGT1A1 *60	NA
			UGT1A1 *93	NA
		UGT1A9	UGT1A9 *22	NA
		UGT1A7	UGT1A7 *2	NA
			UGT1A7 *4	NA
			UGT1A7 *3	NA
Folate cycle		TS	TSER	NA
			TSUR	NA
		MTHFR	MTHFR 1298A>C	Glu429Ala

		MTHFR 677C>T	Ala222Val
Catabolism	DPD		
Cell Cycle	TP53	TP53 ex4+119C>G	Arg72Pro
		TP53 IVS2+38C>G	NA
		TP53 IVS3+16bp (PIN3)	NA
	MDM2	MDM2 309T>G	NA

Table 2. GENEs under investigation.

3.2.2 Sample Storage

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.

3.2.3 Genomic DNA Extraction

The extraction of genomic DNA from whole blood was performed with the automated extractor BioRobot EZ1 (Qiagen SPA, Milano, Italy). The Card "EZ1 DNA Blood", in association with the Kit "EZ1 DNA Blood Kit 200μL", was used for the extraction of genomic DNA from 200μl of whole blood obtaining 200μl as final volume, corresponding approximately 4-8ng 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 extraction is performed by lysis. Once the white blood cells are lysed, the DNA strand released in solution 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. The extracted DNA is maintained at 2-8°C.

3.2.4 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 (Figure 6).

Figure 6: Thermal Cyclers.

In the reaction tube, two primers are added, one to allowing the synthesis of the sense strand (sense or forward primer) and the other one for the synthesis of the antisense strand (antisense or reverse primer). The two primers define the target region to be amplified (Figure 7).

PCR requires several reagents and reaction conditions that vary with the time. In particular, the samples undergo 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, able 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 and 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 neo-strands of undefined length. In subsequent cycles only fragments of the desired length are formed and they contain the specific region you want to amplify.

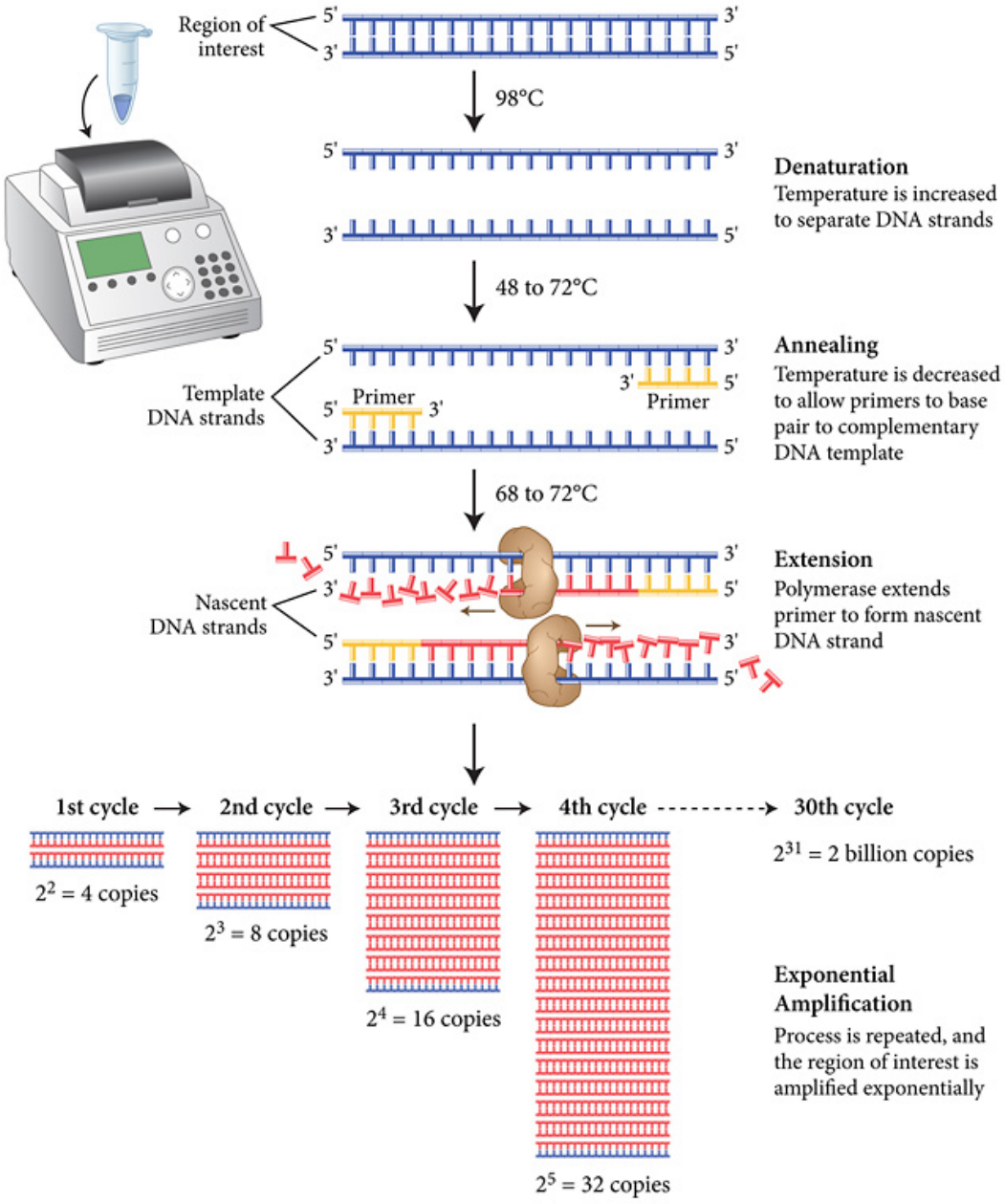


Figure 7: Polymerase Chain Reaction (PCR).

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:

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 1.2-1.3mM. The concentration of free magnesium is, however, influenced by the concentration of nucleotides as there is a link between equimolar magnesium and dNTPs. Magnesium concentrations higher than those indicated above can be also used, 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 50-200μM. 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: the DNA polymerase used is named Taq Polymerase due to its derivation from *Thermophilus bacterium acquaticus*. It 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.

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.

3.2.5 Optimization of the Polymerase Chain Reaction (PCR) Conditions

In order to obtain good results in the PCR process there are three key parameters to consider: efficiency (or yield), reaction specificity and accuracy. To perform a PCR with high efficiency, the larger number of products with the least number of cycles as possible has to be obtained. 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: the number of cycles 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 should guarantee 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, because the Tm is variously affected by the buffer components, primers 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%.

3.2.6 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).

3.2.6.1 Pyrosequencing

Pyrosequencing is a nanotechnology of recent development for SNP identification consisting of a real time pyrophosphate detection method [56].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, which determines and provides directly the genotype at the level of the analyzed SNP.

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.

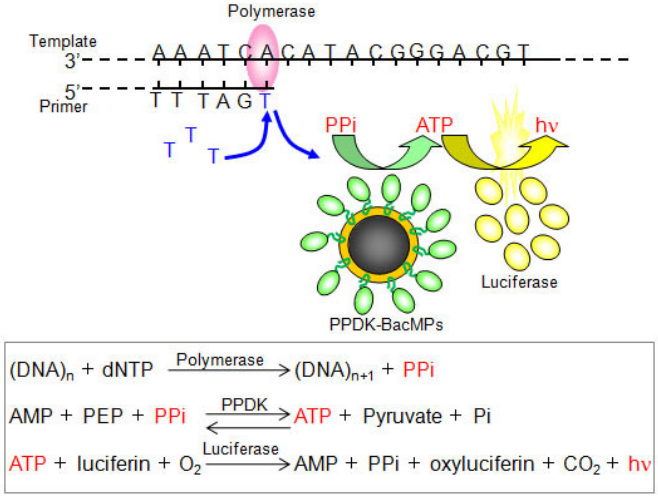


Figure 8: PCR schematic representation of Pyrosequencing method. Schematic diagram of the principle of pyrosequencing using PPDK-BacMPs. dNTP, deoxynucleoside triphosphate; PPi, pyrophosphate; AMP, adenosine monophosphate; Pi, phosphate.

PCR reaction product is mixed with streptavidin coated High Performance Sepharose beads (Amersham Biosciences, Uppsala, Sweden) in the presence of a binding buffer (Tris 10mM, Sodium Chloride 2M, EDTA 1mM 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 into a 96-well filter plate and vacuum (vacuum manifold for 96 well filter plate, Millipore) is applied to remove all the liquid. Denaturation solution (Sodium Hydroxide 0.2M) 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 10mM, pH 7.6) in the presence of the vacuum. The beads with the immobilized template are resuspended by adding 45µl of annealing buffer (Tris 20mM, Magnesium Acetate Tetra-Hydrate 2mM, pH 7.6) and sequencing primer (2µM) 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 15bp. 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).

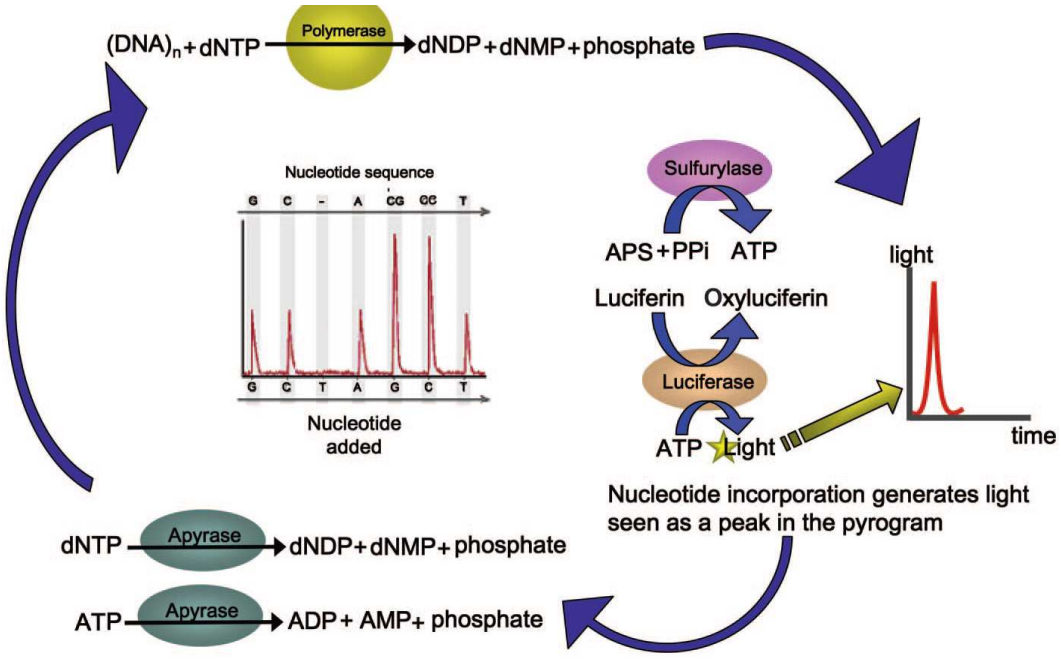


Figure 9: Pyrosequencing chemistry: biochemical reactions and enzymes involved in the generation of light signals by DNA pyrosequencing. Each peak in the pyrograms represents a pulse of light detected in the instrument. dNTP, deoxynucleoside triphosphate; dNDP, deoxynucleoside diphosphate; dNMP, deoxynucleoside monophosphate; PPi, pyrophosphate; APS, adenosine 5-phosphosulfate.

The plate is incubated for 5 minutes at 60°C to allow the complete annealing of sequencing primer 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 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 nascent 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 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 (Figure 8).

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 by one. 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 (Figure 9).

"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 the 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 100mM;
- 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 in solution;
    - dCTP in solution;
    - dGTP in solution;
    - dTTP in solution.
- PSQTM 96 Sample Preparation Kit (Biotage AB, Uppsala, Sweden) constituted by:
    - Binding Buffer (10mM Tris-HCl, 2M NaCl, 1mM EDTA, 0,1% Tween 20; pH=7,6);
    - Denaturation Solution (0,2M NaOH);
    - Washing Buffer (10mM Tris-acetate; pH=7,6);
    - Annealing Buffer (20mM Tris-acetate, 2mM 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).

3.2.6.2 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 employed by the Taq polymerase for the elongation.

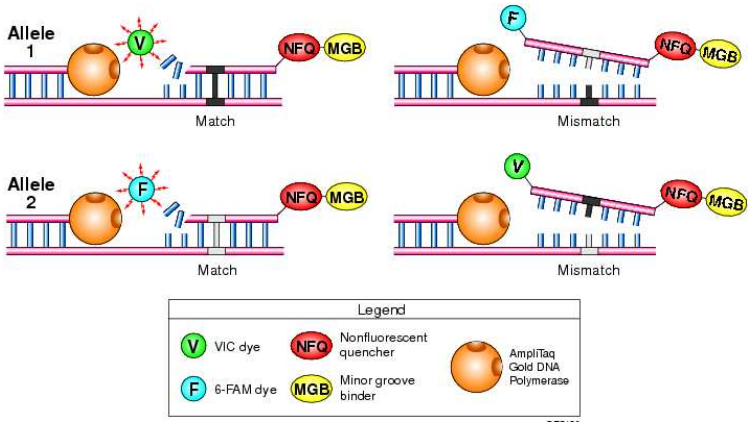


Figure 10: Schematic representation of TaqMan® technology.

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 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 figure 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 20-30bp 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.

Samples are analyzed using the Applied Biosystems 7500 Real-Time PCR System instrument. 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 "TaqMan® SNP Genotyping Assay" is employed: it is available on-line in the catalog of Applied Biosystems. 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 (20µl) are determined, and the markers 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).

3.2.6.3 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 (Figure11).

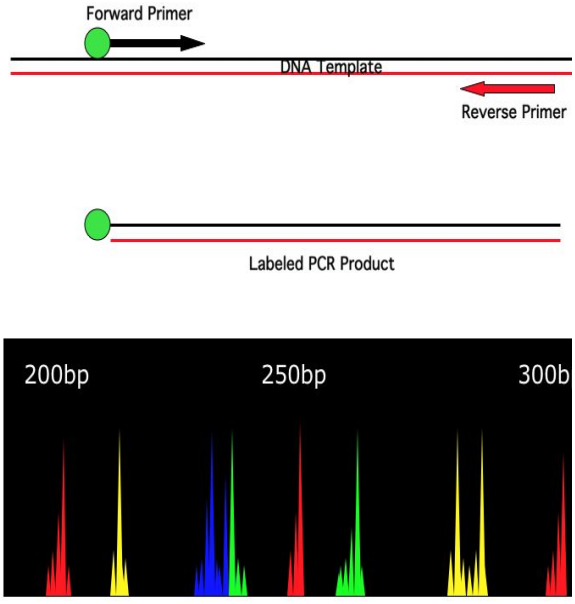


Figure 11: Scheme of the separation of different sized DNA fragments labeled with different fluorophores by capillary gel electrophoresis. (http://www.agctsequencing.com)

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.

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). Gene Scan analysis software (Applied Biosystems) allows data extraction and elaboration.

The process is very simple. The first phase consists in the amplification of the gene fragment containing the polymorphism of interest by 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 14.5µl of deionized and purified formamide and 0.5µl 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 1µl of the diluted sample for a total of 16µl 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 (2min 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 (Figure 12).

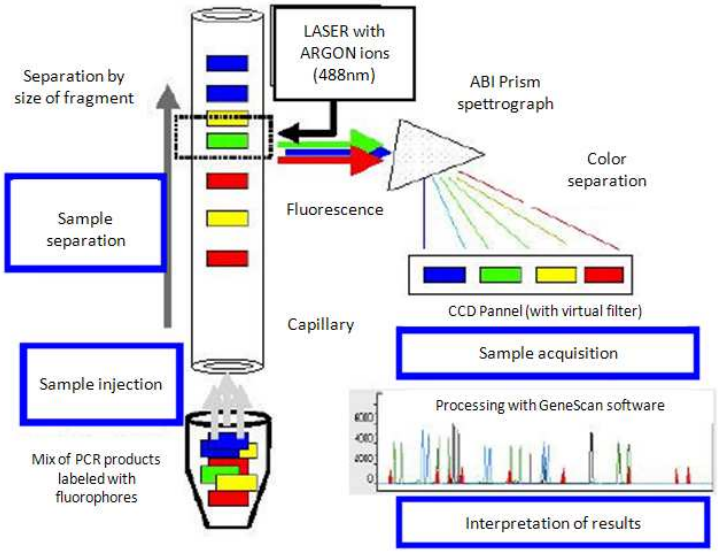


Figure 12: Scheme of the processing of DNA fragments within the Genetic Analyzer ABI PRISM 3100 instrument. The involved steps are: injection of the samples, separation of the fragments by size using electrophoresis, determination of fluorescence with CCD camera, interpretation of the results with the 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 the DNA diffusion. 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.

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 [57]:

- 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 the physical gels can be used, 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 ROXTM DYE (5-carbossi-X-rodamina, succinil estere) (Gene ScanTM 400HD [ROX] Size Standard, Applied Biosystems, Foster City, CA).

3.2.7 Statistical Analysis

The study was prospectively designed to test the association between genetic polymorphisms and the risk of CRC as first end point. 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 K^2-test was reported. All P values were two-sided. 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.

Information on progression or recurrence was obtained through an active follow-up on a periodical verification of the recurrence status of the patients. It was computed by Kaplan-Meier method, and log-rank test was 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 $p<0.05$, 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) 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) 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 statistically test their difference. The determination of the relative risk of death or progression in patients with different genotype 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.

RESULTS

4.1 Pharmacokinetics (PK)

4.1.1 Patients Enrollment

Elegible patients for the study were 51, 35 males and 16 females, aged between 32 and 76 years (mean 58yrs) and all with a confirmed diagnosis of metastatic adenocarcinoma of the colon and rectum. Following the signing of informed consent to the exclusive genetic investigation and approved by the ethics committee of each of the three institutions and from the ISS, patients were genotyped for the UGT1A1 * 28 polymorphism in each center. As described above, only the patients with genotype * 1 / * 1 and * 1 / * 28 were enrolled. (Table 2).

Center	PATIENTS				
	Total	Not enrolled	Enrolled		
		*28/*28	*1/*1	*1/*28	Total
[1] CRO, Aviano	34	14	7	13	20
[2] UC, Chicago (USA)	14	2	5	7	12
[3] S. Pietro FBF, Roma	3	0	3	0	3
Total	51	16	15	20	35

*Table 2. Patients genotyped for the polymorphism UGT1A1*28 and patients enrolled by each participant center.*

[1]Centro di Riferimento Oncologico, Aviano; [2]University of Chicago Medical Center,IL (USA); [3]S. Pietro Fate Bene Fratelli, Roma.

4.1.2. Analytical Method Optimization

After the optimization of the mass and chromatographic methods, each analytical conditions was determined (Table 3) and used for samples' analysis. The stability tests were positive in all the condions of samples mantainance: two cycles of freeze-thaw, maintainance in the autosampler for 96 hours and 4 hours on ice. Thus ensured the possibility to conduct the analysis on all of the patient samples.

Analytical Parameters **(Scan MRM (SRM))**			
Parent		Fragment	
Compound		Q3 CE CXP Time (amu) (volts) (volts) (msec)	
Q1 DP EP (amu) (volts) (volts)			
CPT (IS)	349,2 75 10	305,1 33 15	50
		248,9 43 16	
		220,1 48 13	
CPT-11	587,4 125 11	124,2 51 6	50
		195,2 44 13	
SN-38	393,3 103 13	349,3 35 8	50
		249,1 68 15	
		293,2 47 13	
SN-38G	569,3 113 11	393,2 40 8	50
		349,2 60 7	
		249,2 104 16	
APC	619,2 115 12	393,3 45 9	50
		227,1 36 14	
		349,2 62 7	

Table 3a. Analytical conditions determined for each compound.

Source Parameters	
Curtain gas (CUR)	20 psi
Ionspray voltage	5500V
Temperatura (TEM)	650°C
Nebulizer gas (GS1)	30 psi
Turbo gas (GS2)	65 psi

Table 3b. Source parameters optimized.

Column	**C18, 100x2mm, 3µm**	**Analite**	**Retention Time**
Flux	300 µL/min	CPT	6.49 min
Column temperature	25°C	CPT-11	5.02 min
Autosampler temperature	4°C	SN-38	6.80 min
Injection volume	5µL	SN-38G	7.40 min
Run Time	18 min	APC	5.02 min
Reconditioning Time	7min		
$\%B_{min}$	5%		
$\%B_{max}$	70%		

Table 3c. Chromatographic parameters determined for each compound.

4.1.3 Preliminary Results in the Ongoing Study

The study, still in progress, expected to enroll a maximum number of 5 patients in group 1, and 3 patients in group 2 (Figure 13). Patients who experienced DLTs at the first administration left the protocol prior to the completion of the full course of the planned treatment. Therefore, their samples were not available for the second administration and it was not possible to compare plasma levels of CPT-11 administered after BV.

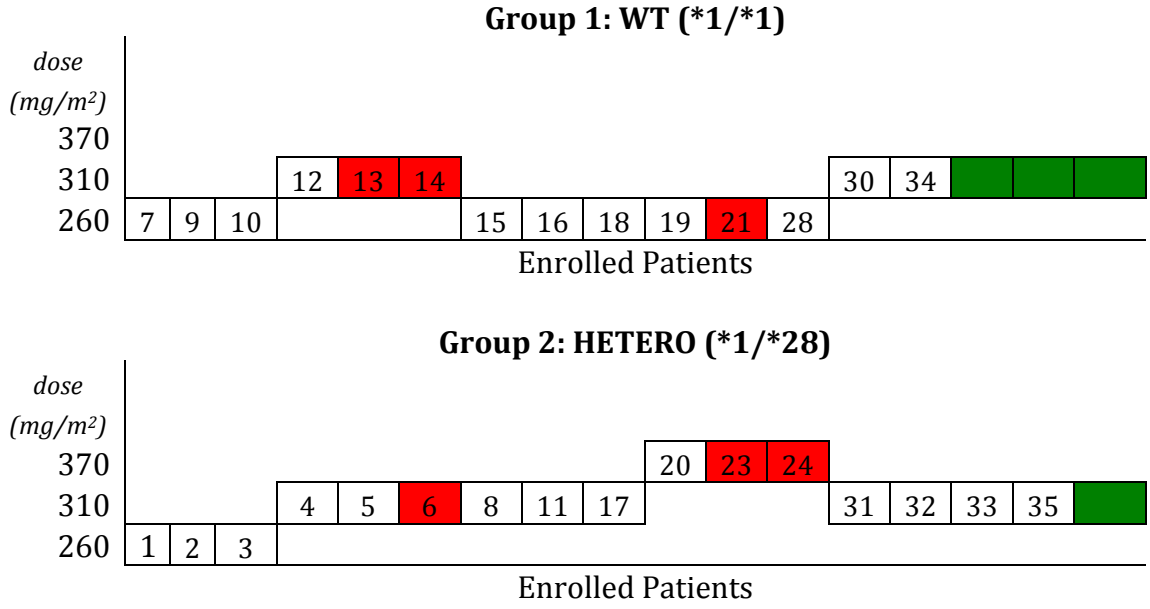


Figure 13. Scheme enrollment stage. The numbers inside the boxes indicate the identification number of each enrolled patient.

The obseved DLTs and the reported adverse events' type are summarized in Table 4:

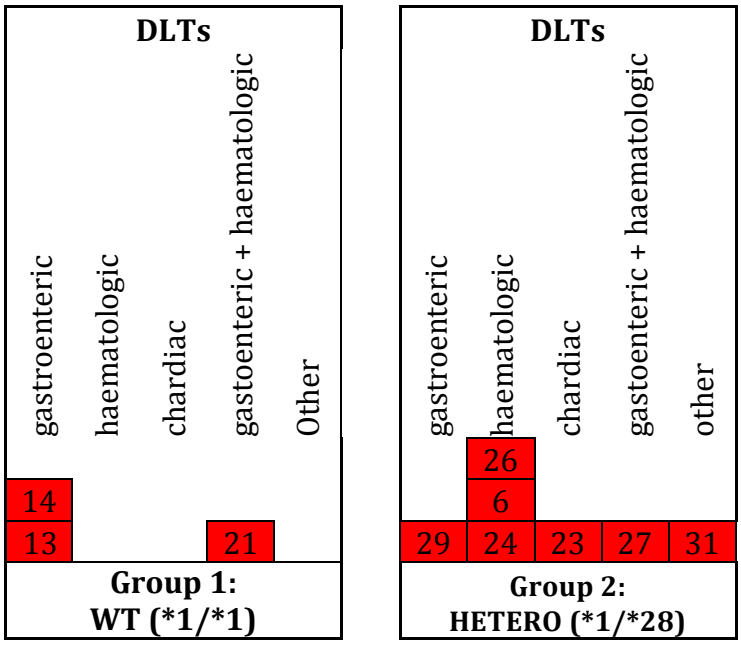


*Table 4. List of the observed DLTs, subdivided by the UGT1A1*28 genotype. The numbers correspond to the number of enrollment of patients.*

The quantification of irinotecan and its metabolites was done on 5 samples of patients enrolled in the CRO of Aviano (Pz_19, Pz_20, Pz_21, Pz_22, Pz_23) and it was performed by the analytical method which was developed and validated by our group (Figure 14).

As described by the schema related to the enrollment, patients number 19, 21 and 22 were treated with a dose of 260mg/m^2, they were characterized by the wt genotype. The patient 21 experienced the reported DLTs. On the other hand, patients 20 and 23 belong to a cohort of heterozygous genotype treated at 370mg/m^2, and the patient 23 showed DLTs.

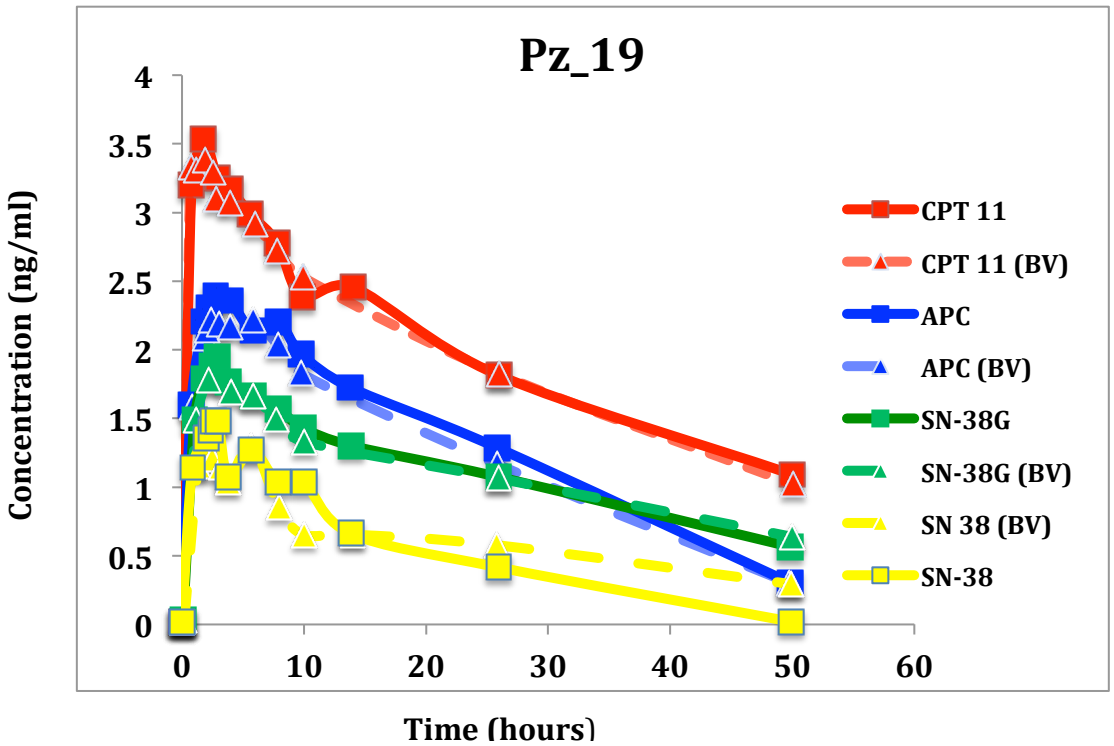


Figure 14a

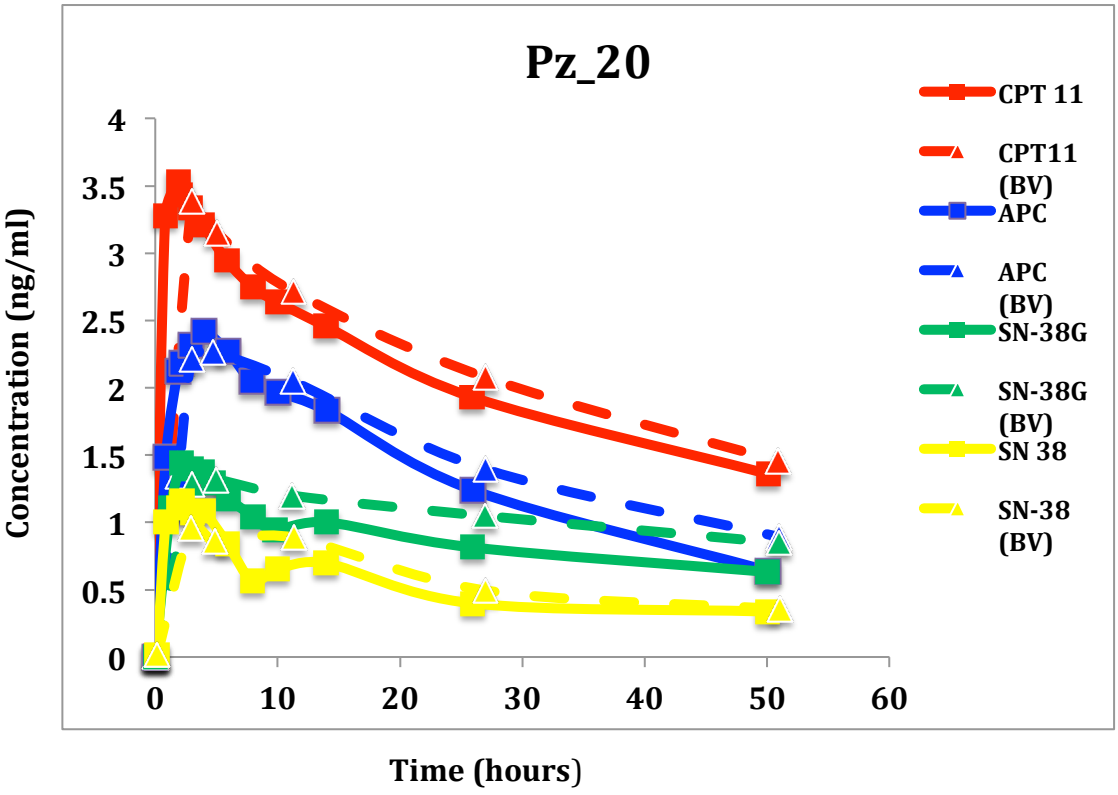


Figure 14b

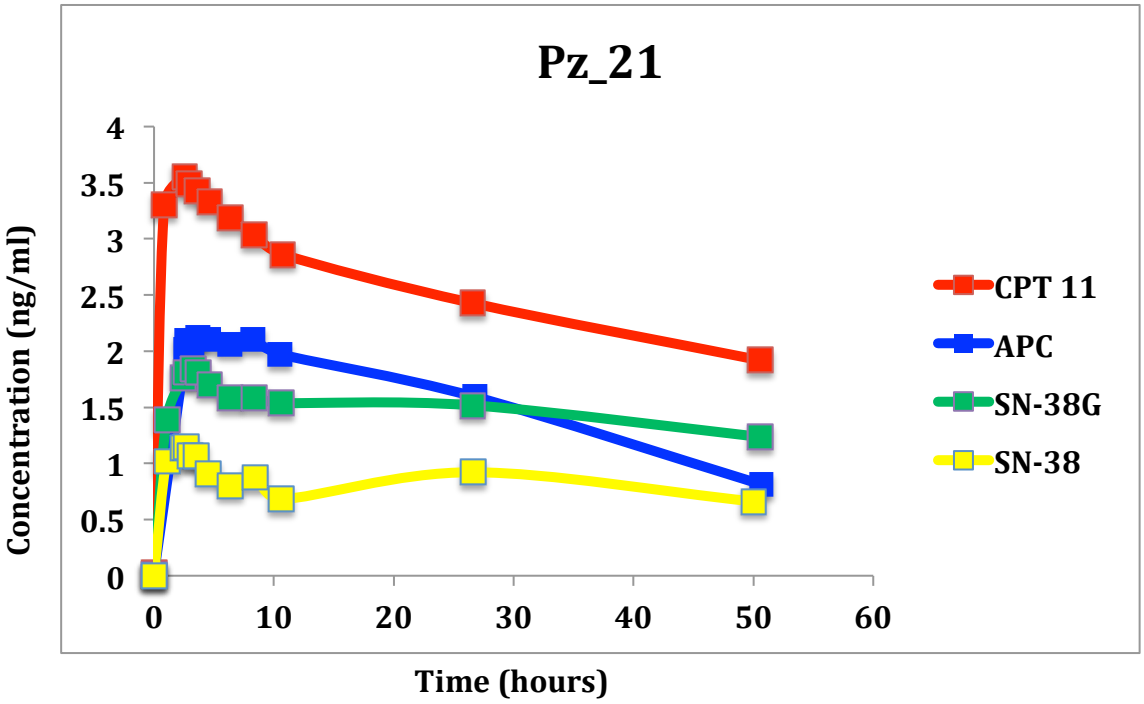


Figure 14c

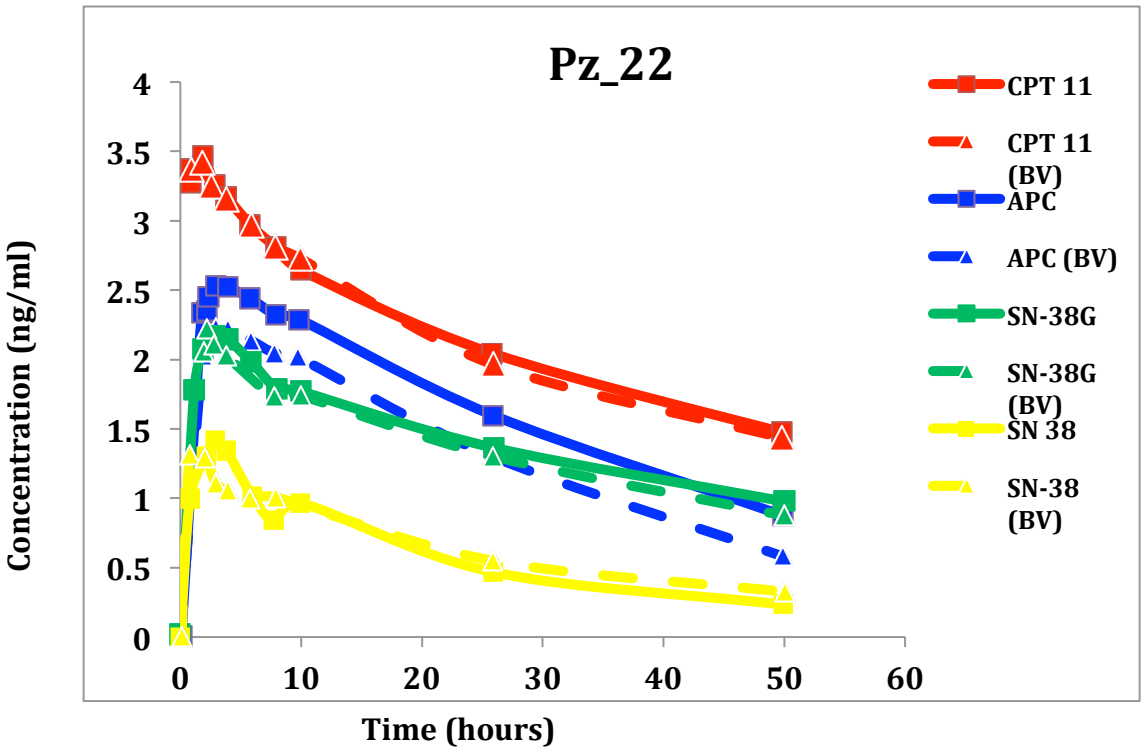


Figure 14d

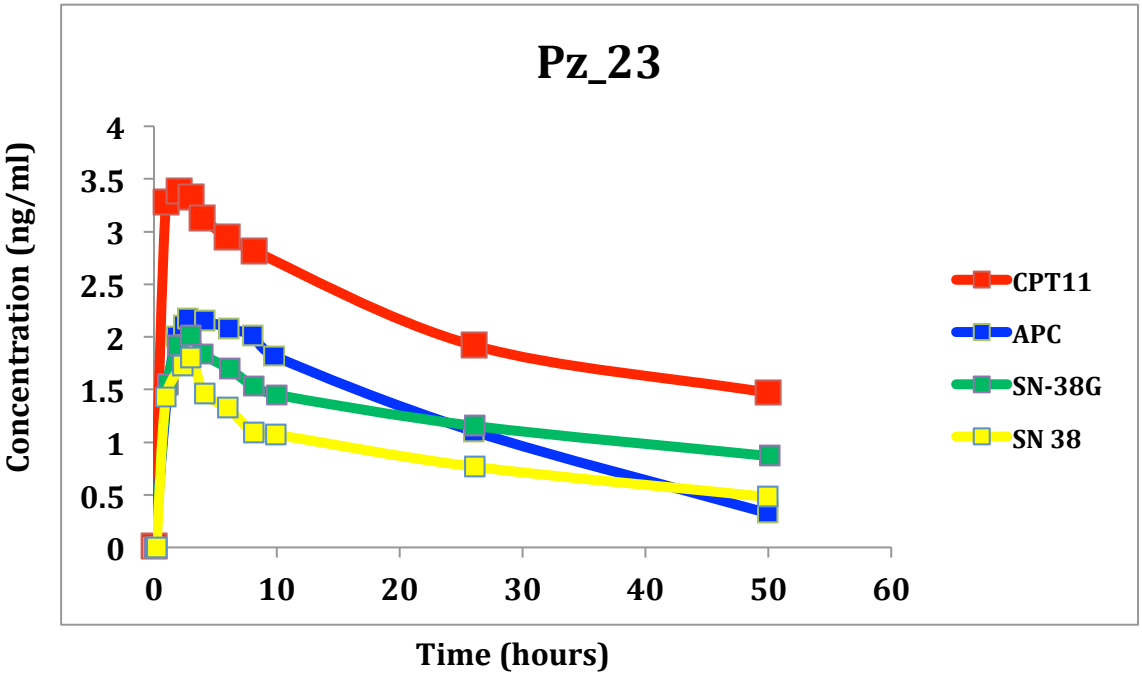


Figure 14e

Figure 14. Graphs of the plasma concentrations of CPT-11 and its major metabolites in the plasma of patients. In each graph the plasma levels between the first and second administration are compared (indicated by a dashed line and in the legend from "BV"). Patients 21 (c) and 23 (e) experienced DLTs after the first administration, so we reported only the figures for the 1-3 days of treatment.

Then pharmacokinetic analysis were performed with the software *WinNonlin Pro Node 4.1 (Pharsight Co., Mountain View, Ca, USA)* to determine the following parameters:

C_{max} = maximum plasma concentration, expressed in mg/mL

t_{max} = time to reach the Cmax, expressed in hours (h)

AUC_{last} = area under the concentration curve from time 0 to the last point detected in the samples, expressed as h*mg/mL

AUC_{inf} = AUC extrapolated to infinity, mathematically expressed as h*mg/mL

$t_{1/2}$ = half-life in the terminal phase, expressed in hours (h)

CL = plasma clearance, expressed in $L/h/m^2$

V_z = volume of distribution, expressed in L/m^2

The results for each patient are shown in the following tables.

- At the first somministration:

	CPT-11							SN-38				
ID pz	**AUC_{last}**	**AUC_{inf}**	**C_{max}**	**$t_{1/2}$**	**t_{max}**	***Vz***	***C_L***	**AUC_{last}**	**AUC_{inf}**	**C_{max}**	**$t_{1/2}$**	**t_{max}**
19	15.51	15.65	3.31	8.3	2.0	*199.4*	*16.6*	0.30	0.31	29.4	10.7	3.0
21	31.53	32.96	3.54	12.5	2.7	*142.3*	*7.9*	0.35	0.54	14.3	28.8	2.9
22	17.92	18.34	2.85	10.3	2.0	*209.8*	*14.2*	0.28	0.32	25.2	17.4	3.0
20	17.75	18.04	3.34	9.4	2.0	*278.3*	*20.5*	0.20	0.29	14.0	32.1	2.3
23	17.76	18.19	2.37	10.3	2.0	*301.2*	*20.3*	0.52	0.61	66.0	21.0	3.0

	SN-38G					APC				
ID pz	**AUC_{last}**	**AUC_{inf}**	**C_{max}**	**$t_{1/2}$**	**t_{max}**	**AUC_{last}**	**AUC_{inf}**	**C_{max}**	**$t_{1/2}$**	**t_{max}**
19	0.95	1.02	87.5	12.5	3.0	2.63	2.65	241.6	6.5	3.0
21	1.59	2.53	67.0	12.5	3.2	2.70	2.80	135.8	10.3	3.7
22	1.9	2.1	2.85	156.6	3.0	4.55	4.64	331.7	8.7	3.0
20	0.42	0.6	3.34	26.6	2.3	2.50	2.55	252.2	9.3	4.0
23	1.13	1.33	2.37	100.7	3.0	1.82	1.84	155.2	8.1	3.0

- At the second somministration:

	CPT-11							SN-38				
ID pz	**AUC_{last}**	**AUC_{inf}**	**C_{max}**	**$t_{1/2}$**	**t_{max}**	***Vz***	***C_L***	**AUC_{last}**	**AUC_{inf}**	**C_{max}**	**$t_{1/2}$**	**t_{max}**
19	15.04	15.16	2.38	8.0	2.0	*198.1*	*17.2*	0.24	0.33	19.1	31.3	6.0
21	*Not available*							*Not available*				
22	18.22	18.56	2.45	9.4	2.0	*190.0*	*14.0*	0.27	0.33	20.6	21.0	1.0
20	19.97	20.33	2.38	9.4	3.0	*247.3*	*18.2*	0.22	0.29	9.2	23.1	2.0
23	*Not available*							*Not available*				

	SN-38G					APC				
ID pz	**AUC_{last}**	**AUC_{inf}**	**C_{max}**	**$t_{1/2}$**	**t_{max}**	**AUC_{last}**	**AUC_{inf}**	**C_{max}**	**$t_{1/2}$**	**t_{max}**
19	0.86	0.96	60.3	16.6	2.5	2.00	2.02	167.7	7.8	6.0
21	*Not available*					*Not available*				
22	1.70	1.85	153.1	14.3	2.5	3.38	2.43	162.8	8.5	4.0
20	0.59	0.89	19.9	31.7	4.0	2.82	2.92	179.5	10.4	4.0
23	*Not available*					*Not available*				

All the patients referred to the CRO (Aviano, PN) and S.PIETRO FBF (Rome) were genotypized for other UGT polimorphisms (Table 5).

For the same patients, polymorphism IVS14 +1 G>A in the gene of the DPYD*2A was also analyzed, to rule out any toxicity due to 5-FU, in this treatment regimen FOLFIRI. All patients were found to carry the wt genotype for this polymorphism.

NUMBER OF THE PATIENT ENROLLED	UGT1A1 *28	UGT1A1 *60	UGT1A1 *93	UGT1A1 *22	UGT1A1 *2	UGT1A1 *4	UGT1A1 *3
not enrolled	*28/*28	mut	hetero	wt	mut	mut	*3/*3
not enrolled	*28/*28	wt	wt	mut	mut	hetero	*3/*2
not enrolled	*28/*28	hetero	hetero	wt	wt	wt	*3/*1
2	*1/*28	hetero	hetero	mut	mut	hetero	*3/*2
not enrolled	*28/*28	mut	wt	wt	hetero	hetero	*3/*1
not enrolled	*28/*28	wt	wt	hetero	hetero	hetero	*3/*1
4	*1/*28	hetero	hetero	-	hetero	hetero	*3/*1
5	*1/*28	hetero	hetero	hetero	hetero	hetero	*3/*1
7	*1/*1	wt	wt	hetero	wt	wt	*1/*1
8	*1/*28	hetero	hetero	hetero	mut	hetero	*3/*2
10	*1/*1	wt	wt	hetero	hetero	hetero	*3/*1
9	*1/*28	wt	wt	mut	wt	wt	*1/*1
11	*1/*28	hetero	wt	hetero	hetero	hetero	*3/*1
13	*1/*1	hetero	wt	wt	mut	wt	*2/*2
12	*1/*1	hetero	wt	hetero	hetero	wt	*2/*2
not enrolled	*28/*28	mut	hetero	wt	mut	hetero	*3/*2
15	*1/*1	wt	wt	hetero	hetero	wt	*2/*1
not enrolled	*28/*28	mut	mut	wt	mut	mut	*3/*3
17	*1/*28	hetero	hetero	hetero	hetero	hetero	*3/*1
not enrolled	*28/*28	hetero	hetero	wt	mut	hetero	*3/*2
not enrolled	*28/*28	hetero	hetero	hetero	mut	hetero	*3/*2
19	*1/*1	wt	wt	-	wt	wt	*1/*1
20	*1/*28	hetero	hetero	wt	mut	hetero	*3/*2
22	*1/*1	wt	wt	mut	wt	wt	*1/*1
23	*1/*28	mut	hetero	wt	mut	hetero	*3/*2
21	*1/*1	hetero	wt	mut	wt	wt	*1/*1
not enrolled	*28/*28	mut	mut	wt	mut	hetero	*3/*2
25	*1/*28	hetero	hetero	wt	mut	mut	*3/*3
26	*1/*28	hetero	hetero	-	mut	mut	*3/*3
not enrolled	*28/*28	wt	wt	mut	-	-	-
28	*1/*1	wt	wt	mut	wt	wt	*1/*1
not enrolled	*28/*28	hetero	hetero	wt	-	-	-
not enrolled	*28/*28	hetero	hetero	hetero	-	-	-
31	*1/*28	hetero	hetero	hetero	hetero	hetero	*3/*1
33	*1/*28	hetero	hetero	hetero	hetero	hetero	*3/*1
not enrolled	*28/*28	hetero	hetero	wt	-	-	-
35	*1/*28	mut	mut	wt	mut	hetero	*3/*2

Table 5. Results of genotyping for polymorphisms of the UGT family genes in patients enrolled at CRO Aviano and St. Peter FBF in Rome.

4.2 PHARMACOGENETICS (PG)

4.2.1 Patient's Characteristics and Treatment

This study, sponsored by the CRO-National Cancer Institute of Aviano, Italy, includes 812 CRC patients. All of them were Caucasians and have been enrolled in centers located in Northern and Central Italy. The subjects were treated with 5-FU in adjuvant chemotherapy (FOLFOX or FILFIRI regimen).

Patients with histologically confirmed CRC, 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 Institutional Review Board of each participating institution approved the study protocol, and all patients signed a written informed consent before entering the study.

1307 blood donors were enrolled as controls They were older than 18 years and without cancer diagnosis.

The characteristics of the studied populations are reported in the following table (Table 6a, 6b, 6c).

	CASES (N=812)		CONTROLS (N=1307)	
media	62ys		49ys	
median	64ys		44ys	
range	20ys – 85ys		18ys – 97ys	
	Male (N=510)	**Female (N=302)**	**Male (N=862)**	**Female (N=475)**
media	63ys	60ys	47ys	53ys
median	65ys	62ys	35ys	48ys
range	20ys -84ys	24ys – 85ys	18ys – 92ys	18ys – 97ys

Table 6a. Characteristics of the studied population.

	CASE		CONTROL	
	M	F	M	F
11ys - 15ys	0	0	0	0
16ys - 20ys	1	0	14	16
21ys - 25ys	0	2	40	34
26ys - 30ys	1	5	83	47
31ys - 35ys	4	4	130	45
36ys - 40ys	12	8	129	47
41ys - 45ys	11	18	95	29
46ys - 50ys	33	32	73	30
51ys - 55ys	52	33	69	31
56ys - 60ys	60	38	47	10
61ys - 65ys	108	52	21	3
66ys - 70ys	92	40	14	13
71ys - 75ys	84	38	53	49
76ys - 80ys	46	25	52	50
81ys - 85ys	6	7	27	45
86ys - 90ys	0	0	13	20
91ys - 95ys	0	0	2	4
96ys - 100ys	0	0	0	2
tot	510	302	862	475

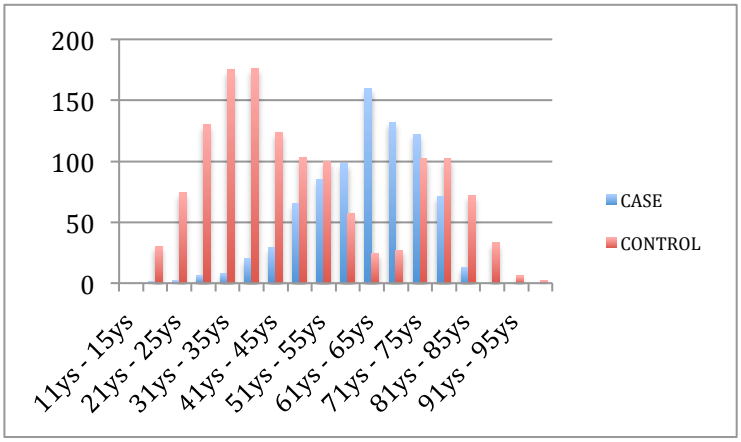


Table 6b. Age distribution of cases and controls stratified in 5 years.

As we can see, the distribution of age is different between case and control because the enrollment of elderly donors was performed requesting subjects ≥70 years old (Geriatric Unit), so there is a gap in the 60s caused by the low quantity of blood donor volunteers.

4.2.2 CRC-Risk Analysis

As first end point, we tested the association between genetic polymorphisms and the risk of CRC. 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 K^2-test was reported.

4.2.2.1 Case-Control Risk Analysis

The first analysis was conducted on the entire population matching cases and controls. In the following table the risk associations with a significance ($P<0.05$) were reported only. Mostly of them are variations on genes involved in the DNA repair mechanisms.

	Model		Controlli		Casi		OR	95% CI	*p-value**	*p-value***
			No	(%)	No	(%)				
XRCC1 28152G>A (Arg399Gln) (n= 2182)	Genotype	GG	568	(44,8)	377	(41,2)	1	-	0,069	0,026
		GA	555	(43,8)	407	(44,5)	1,105	0,921 - 1,326	0,284	
		AA	144	(11,4)	131	(14,3)	1,370	1,046 - 1,795	0,022	
	Recessive	GG+GA/AA	1123/144	(88,6)/(11,4)	784/131	(85,7)/(14,3)	1,304	1,011 - 1,678	0,040	
	Dominant	GG/GA+AA	568/699	(44,8)/(55,2)	377/538	(41,2)/(58,8)	1,160	0,977 - 1,377	0,091	
XRCC1 Ex9+16G>A (Arg280His) (n= 2163)	Genotype	GG	1101	(87,1)	767	(85,3)	1	-	0,047	0,093
		GA	157	(12,4)	119	(13,2)	1,088	0,842 - 1,404	0,517	
		AA	6	(0,5)	13	(1,4)	3,106	1,176 - 8,197	0,022	
	Recessive	GG+GA/AA	1258/6	(99,5)/(0,5)	886/13	(98,6)/(1,4)	3,077	1,166 - 8,13	0,017	
	Dominant	GG/GA+AA	1101/163	(87,1)/(12,9)	767/132	(85,3)/(14,7)	1,163	0,907 - 1,488	0,233	
XPD 23591G>A (Asp312Asn) (n= 2184)	Genotype	GG	496	(39,1)	362	(39,6)	1	-	0,079	0,19
		GA	561	(44,2)	430	(47,0)	1,050	0,873 - 1,263	0,603	
		AA	213	(16,8)	122	(13,3)	0,785	0,605 - 1,018	0,068	
	Recessive	GG+GA/AA	1057/213	(83,2)/(16,8)	792/122	(86,7)/(13,3)	0,765	0,601 - 0,973	0,029	
	Dominant	GG/GA+AA	496/774	(39,1)/(60,9)	362/552	(39,6)/(60,4)	0,978	0,821 - 1,163	0,795	
ERCC1 8092C>A 3'UTR (n= 2182)	Genotype	CC	662	(52,3)	452	(49,3)	1	-	0,004	0,011
		CA	499	(39,4)	348	(38,0)	1,021	0,852 - 1,225	0,819	
		AA	105	(8,3)	116	(12,7)	1,618	1,211 - 2,16	0,001	
	Recessive	CC+CA/AA	1161/105	(91,7)/(8,3)	800/116	(87,3)/(12,7)	1,603	1,214 - 2,119	0,001	
	Dominant	CC/CA+AA	662/604	(52,3)/(47,7)	452/464	(49,3)/(50,7)	1,125	0,949 - 1,333	0,174	
hMSH2 (GIVS12-6T>C) (n= 2024)	Genotype	TT	1033	(85,9)	690	(83,9)	1	-	0,091	0,105
		TC	163	(13,6)	121	(14,7)	1,111	0,862 - 1,433	0,416	
		CC	6	(0,5)	11	(1,3)	2,747	1,01 - 7,463	0,048	
	Recessive	TT+TC/CC	1196/6	(99,5)/(0,5)	811/11	(98,7)/(1,3)	2,703	0,996 - 7,353	0,042	
	Dominant	TT/TC+CC	1033/169	(85,9)/(14,1)	690/132	(83,9)/(16,1)	1,170	0,913 - 1,497	0,215	
hMSH6-556G>T rs3136228 (n= 2134)	Genotype	GG	477	(37,8)	309	(35,4)	1	-	0,006	0,014
		GT	606	(48,0)	394	(45,2)	1,004	0,829 - 1,215	0,97	
		TT	179	(14,2)	169	(19,4)	1,458	1,13 - 1,88	0,004	
	Recessive	GG+GT/TT	1083/179	(85,8)/(14,2)	703/169	(80,6)/(19,4)	1,453	1,155 - 1,832	0,001	
	Dominant	GG/GT+TT	477/785	(37,8)/(62,2)	309/563	(35,4)/(64,6)	1,107	0,925 - 1,325	0,266	
XRCC3 17893A>G (int) (n= 2163)	Genotype	AA	629	(49,6)	497	(55,5)	1	-	0,017	0,047
		AG	505	(39,9)	305	(34,0)	0,765	0,636 - 0,919	0,004	
		GG	133	(10,5)	94	(10,5)	0,894	0,67 - 1,195	0,45	
	Recessive	AA+AG/GG	1134/133	(89,5)/(10,5)	802/94	(89,5)/(10,5)	0,999	0,756 - 1,321	0,996	
	Dominant	AA/AG+GG	629/638	(49,6)/(50,4)	497/399	(55,5)/(44,5)	0,792	0,667 - 0,94	0,008	
GST T1 pos/ neg (n= 1701)	Genotype	pos	608	(85,5)	801	(80,9)	1	-	0.013	
		neg	103	(14,5)	189	(19,1)	1,393	1,007 - 1,818		
GST M1 COPY NUMBER (n= 1648)	Genotype	0	479	(50,4)	419	(60,1)	1	-	0,000	0,001
		1	401	(42,2)	233	(33,4)	0,664	0,54 - 0,818	0,000	
		2	71	(7,5)	45	(6,5)	0,725	0 - 1,55	0,111	
	Recessive	0 + 1 / 2	880/71	(92,5)/(7,5)	652/45	(93,5)/(6,5)	0,855	0,001 - 1,814	0,429	
	Dominant	0 / 1 + 2	479/472	(50,4)/(49,6)	419/278	(60,1)/(39,9)	0,673	0,552 - 0,82	0,000	
MTHFR 1298A>C (Glu429Ala) (n= 2297)	Genotype	AA	556	(44,2)	521	(50,1)	1	-	0,016	0,005
		AC	557	(44,3)	420	(40,4)	0,805	0,676 - 0,958	0,014	
		CC	144	(11,5)	99	(9,5)	0,734	0,553 - 0,973	0,032	
	Recessive	AA+AC/CC	1113/144	(88,5)/(11,5)	941/99	(90,5)/(9,5)	0,813	0,621 - 1,065	0,133	
	Dominant	AA/AC+CC	556/701	(44,2)/(55,8)	521/519	(50,1)/(49,9)	0,790	0,67 - 0,932	0,005	

*Pearson Chi-Square or Likelihood Ratio
** Armitage trend test

Table 7. Associations between polymorphisms and healthy subjects (control) vs CRC patients (case).

4.2.2.2 Case-Control Risk Analysis Gender Related: Male Cases

We stratified the population by gender to underline the possible differences of the risk associations in male or female for the different polymorphic gene variations. Firstly we reported the analysis of possible relationships between polymorphisms and case-controls for the only males (*p-value* del $\chi^2 < 0,05$).

Table 8a. Summary of the significant associations between polymorphism and case-control, only in males						
Genotype						
GST T1 pos/neg		Case	Control	*p-value*	OR	95% CI
	0	446	404	*0,014*	ref	-
	1	112	67		0,660	0,474 - 0,919
Total	1029	558	471			
GST M1 COPY NUMBER		Case	Control	*p-value*	OR	95% CI
	0	255	315	*0,005*	ref	-
	1	150	283	*0,001*	1,527	1,180 - 1,976
	2	28	45	*0,302*	1,301	0,789 - 2,145
Total	1076	433	643			
TP53 ex4+119C>G (arg72pro)		Case	Control	*p-value*	OR	95% CI
	CC	311	462	*0,042*	ref	-
	CG	200	298	*0,980*	1,003	0,797 - 1,262
	GG	45	38	*0,015*	0,568	0,361 - 0,896
Total	1354	556	798			

Table 8a. Associations between polymorphisms and male healthy subjects (control) vs male CRC patients (case).

4.2.2.3 Case-Control Risk Analysis Gender Related: Female Cases

Than we reported the analysis of possible relationships between polymorphisms and case- controls for the only females (*p-value* del $\chi^2 < 0,05$).

Table 8b. Summary of the significant associations between polymorphism and case-control, only in females						
Genotype						
XRCC1 Ex9+16G>A (Arg280His)		Case	Control	*p-value*	OR	95% CI
	GG	269	398	*0,011*	ref	-
	GA	40	60	*0,950*	1,014	0,660 - 1,557
	AA	10	2	*0,010*	0,135	0,029 - 0,622
Total	779	319	460			
ERCC1 8092C>A 3'UTR		Case	Control	*p-value*	OR	95% CI
	CC	168	233	*0,044*	ref	-
	CA	123	186	*0,575*	1,090	0,806 - 1,475
	AA	50	42	*0,031*	0,606	0,384 - 0,955
Total	802	341	461			
hMSH6-556G>T rs3136228		Case	Control	*p-value*	OR	95% CI
	GG	96	178	*0,007*	ref	-
	GT	141	213	*0,219*	0,815	0,588 - 1,130
	TT	70	67	*0,002*	0,516	0,340 - 0,783
Total	765	307	458			
TP53 ex4+119C>G (arg72pro)		Case	Control	*p-value*	OR	95% CI
	CC	199	242	*0,042*	ref	-
	CG	120	170	*0,318*	1,165	0,863 1,572
	GG	17	43	*0,015*	2,080	1,151 - 3,760
Total	791	336	455			
TP53 IVS2+38C>G		Case	Control	*p-value*	OR	95% CI
	CC	189	236	*0,04*	ref	-
	CG	121	179	*0,268*	1,185	0,878 - 1,599
	GG	18	46 (10%)	*0,015*	2,047	1,149 - 3,646
Total	789	328	461			
MTHFR 1298A>C (Glu429Ala)		Case	Control	*p-value*	OR	95% CI
	AA	177	201	*0,009*	ref	-
	AC	144	203	*0,149*	1,241	0,925 - 1,666
	CC	22	56	*0,003*	2,242	1,315 - 3,819
Total	803	343	460			

Table 8b. Associations between polymorphisms and female healthy subjects (control) vs female CRC patients (case).

4.2.2.4 Gender Related Risk Analysis

Analysis was then performed considering all the patients divided by gender to demonstrate the possible relationships between expression of polymorphisms and gender, in all cases (*p-value* of χ^2<0,05).

Table 8c. Summary of the significant associations between polymorphism and gender

Genotype						
XRCC1 Ex9+16G>A (Arg280His)		Female	Male	*p-value*	OR	95% CI
	GG	269	456	*0,011*	ref	-
	GA	40	74	*0,678*	1,091	0,722 - 1,65
	AA	10	3	*0,009*	0,177	0,048 - 0,649
Recessive	GG+GA/AA	309/10	530/3	*0.006*	0.175	0.48 – 0.640
Total	852	319	533			
XPG 3507G>C		Female	Male	*p-value*	OR	95% CI
	GG	174	260	*0,027*	ref	-
	GC	97	198	*0,048*	1,366	1,002 - 1,862
	CC	17	15	*0,152*	0,590	0,287 - 1,214
Total	761	288	473			
MTHFR 1298A>C (Glu429Ala)		Female	Male	*p-value*	OR	95% CI
	AA	177	278	*0,041*	ref	-
	AC	144	222	*0,897*	0,982	0,740 - 1,301
	CC	22	65	*0,017*	1,881	1,119 - 3,161
Recessive	AA+AC/CC	321/22	500/65	*0.012*	1.897	1.147 – 3.138
Total	908	343	565			

Table 8c. Associations between polymorphisms and female CRC patients vs female colorectal cancer patients.

4.2.2.5 Risk Analysis Age Related

To evaluate the influence of age, we performed the same analysis in young and old population separately. Three of the gene variations maintain a significant value related to the CRC risk, but they characterized only one group (not in both): as we can see in the following tables (Table 9a, 9b), XRCC1 Ex9+16AA and MTHFR 1298CC can be considered risk factors in young patients respectively in female and male; XPG 3507CC is predictive only in old female patients.

Genotype			< 70 years n (%)						
	Model		Female		Male		OR	95% CI	*p-value**
XRCC1 Ex9+16G>A (Arg280His) (n= 578)	Genotype	GG	191	(85,7)	310	(87,3)	rif	-	**0,014**
		GA	25	(11,2)	44	(12,4)	1,084	0,643 - 1,829	0,761
		AA	7	(3,1)	1	(0,3)	0,88	0,011 - 0,721	0,024
	Recessive	GG+GA/AA	216/7	(96,9)/(3,1)	354/1	(99,7)/(0,3)	**0,087**	0,011 - 0,713	**0,006**
	Dominant	GG/GA+AA	191/32	(85,7)/(14,3)	310/45	(87,3)/(12,7)	0,866	0,532 - 1,412	0,564
XPG 3507G>C (n= 507)	Genotype	GG	120	(63,2)	176	(55,5)	rif	-	0,240
		GC	64	(33,7)	129	(40,7)	1,374	0,941 - 2,008	0,100
		CC	6	(3,2)	12	(3,8)	1,364	0,498 - 3,733	0,546
	Recessive	GG+GC/CC	184/6	(96,8)/(3,2)	305/12	(96,2)/(3,8)	1,207	0,445 - 3,277	0,712
	Dominant	GG/GC+CC	120/70	(63,2)/(36,8)	176/141	(55,5)/(44,5)	1,373	0,949 - 1,986	0,091
MTHFR 1298A>C (Glu429Ala) (n= 584)	Genotype	AA	114	(51,1)	170	(47,1)	rif	-	**0,018**
		AC	98	(43,9)	148	(41,0)	1,013	0,715 - 1,435	0,943
		CC	11	(4,9)	43	(11,9)	2,621	1,297 - 5,297	0,007
	Recessive	AA+AC/CC	212/11	(95,1)/(4,9)	318/43	(88,1)/(11,9)	**2,606**	1,314 - 5,168	**0,005**
	Dominant	AA/AC+CC	114/109	(51,1)/(48,9)	170/191	(47,1)/(52,9)	1,175	0,841 - 1,641	0,344

*Pearson Chi-Square or Fisher's Exact Test

Table 9a: Comparison of each polymorphic variations and gender, in young patients (<70yrs).

Genotype			≥ 70 years n (%)						
	Model		Female		Male		OR	95% CI	*p-value**
XRCC1 Ex9+16G>A (Arg280His) (n= 268)	Genotype	GG	78	(83,9)	143	(81,7)	rif	-	0,354
		GA	12	(12,9)	30	(17,1)	1,364	0,661 - 2,813	0,401
		AA	3	(3,2)	2	(1,1)	0,364	0,059 - 2,223	0,273
	Recessive	GG+GA/AA	90/3	(96,8)/(3,2)	173/2	(98,9)/(1,1)	0,347	0,056 - 2,113	0,345
	Dominant	GG/GA+AA	78/15	(83,9)/(16,1)	143/32	(81,7)/(18,3)	1,164	0,594 - 2,28	0,659
XPG 3507G>C (n= 249)	Genotype	GG	52	(54,2)	83	(54,2)	rif	-	**0,005**
		GC	33	(34,4)	67	(43,8)	1,272	0,740 - 2,187	0,384
		CC	11	(11,5)	3	(2,0)	0,171	0,046 - 0,641	0,009
	Recessive	GG+GC/CC	85/11	(88,5)/(11,5)	150/3	(98,0)/(2,0)	0,155	0,042 - 0,570	**0,002**
	Dominant	GG/GC+CC	52/44	(54,2)/(45,8)	83/70	(54,2)/(45,8)	0,997	0,597 - 1,663	0,99
MTHFR 1298A>C (Glu429Ala) (n= 316)	Genotype	AA	63	(54,8)	105	(52,2)	rif	-	0,663
		AC	43	(37,4)	74	(36,8)	1,033	0,633 - 1,683	0,898
		CC	9	(7,8)	22	(10,9)	1,467	0,636 - 3,384	0,369
	Recessive	AA+AC/CC	106/9	(92,2)/(7,8)	179/22	(89,1)/(10,9)	1,447	0,643 - 3,260	0,37
	Dominant	AA/AC+CC	63/52	(54,8)/(45,2)	105/96	(52,2)/(47,8)	1,107	0,699 - 1,754	0,663

*Pearson Chi-Square or Fisher's Exact Test

Table 9b: Comparison of each polymorphic variations and gender, in elderly patients (≥70yrs).

4.2.2.6 Cart Analisy

To complete the risk association analysis a test was made to allowed us to combine all possible stratifications and associations with the polymorphic variations. It has been used a model of classification and regression trees, the CART analysis, which evaluates the behavior of the polymorphisms associated with the gender in the two groups separate by age (<70ys vs ≥70yrs), in order to be able to locate subjects in low or high risk of onset of CRC (Table 10).

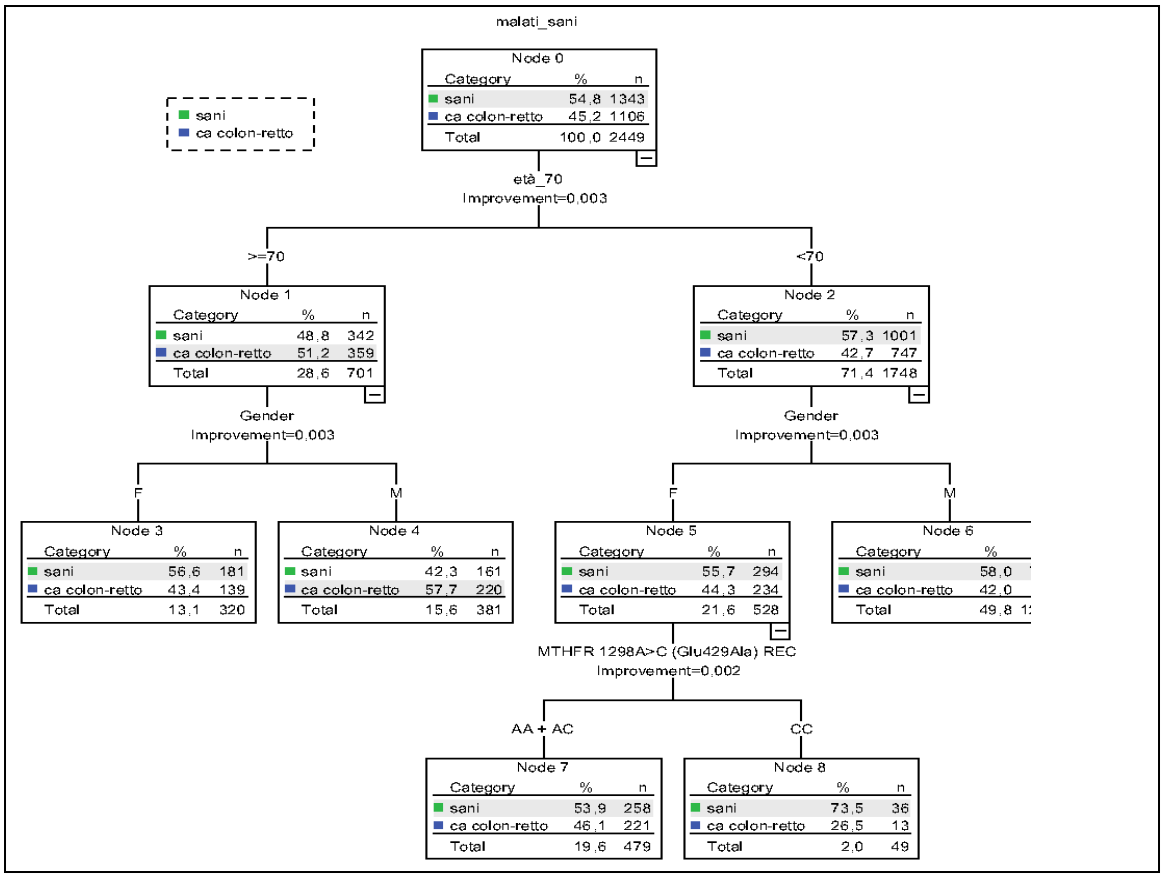


Table 10: CART analysis – MTHFR 1298A>C

This analysis suggestsed that young women carryng the mutant genotype of MTHFR 1298A>C had a lower probability to develop CRC, compared with the control group.

4.2.3 Overall Survival Analysis

Evaluations on overall survival were performed to associate clinical parameters to the genetic characteristics. In particular data were collected for each patient in the 120 months of the date of diagnosis of the illness. Information was available for 548 patients: 301 (55%) patients had died and 247 (45%) were still alive. The median survival, calculated as patients died, is 62.7 months (range 51.1-74.45).

4.2.3.1 Clinicopathologic Features – Univariate Analysis

For this analysis we considered gender (male/female), age (<70ys vs ≥70yrs), surgery (yes/no), TNM grade (T_1_2_3/T_4), and localization - IVO (colon dx, colon sx, rectum).

In the following table have been reported the results of the Log rank test for each of the clinical parameters that hitherto have been considered (Table 11).

We reported in Table 15 the variables that resulted statistically significant. In particular the overall survival of patients was found to be directly associated with the stage of the tumor at the time of diagnosis, as well as to the making the surgical intervention associated with the therapy or therapy alone.

Overall Survival		***Median***	***Sdt. Error***	***IC 95%***		***Log Rank Test***
		62.73	5.98	51.02	74.45	-
N pts	548					
N events	301 (55%)					
N censored	247 (45%)					
Gender	Female	75.93	7.94	60.38	91.49	0.393
	Male	58.87	6.52	46.08	71.66	
Age	<70yrs	64.33	7.42	49.78	78.88	0.98
	≥70yrs	58.87	9.48	40.29	77.45	
Localization	Colon DX	26.13	4.39	17.53	34.74	0.000
	Colon SX	45.17	6.77	31.90	58.43	
	Rectum	116.50	-	-	-	
TNM	T_1_2_3	116.5	-	-	-	0.000
	T_4	18.1	1.4	15.3	20.9	
Surgery	Yes	13.70	0.62	12.49	14.91	0.000
	No	76.57	7.00	62.85	90.28	

Table 11. Log Rank Test for clinical parameters.

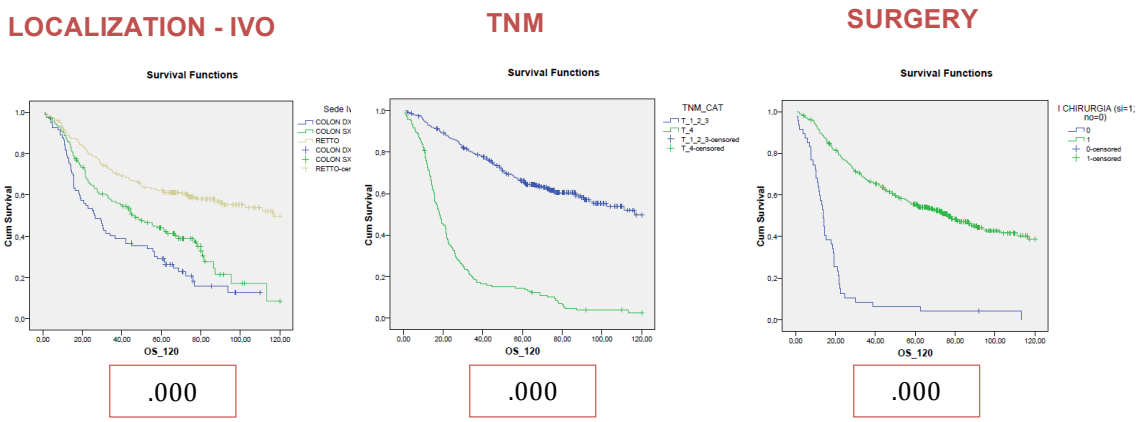


Figure 15. Survival Curves related to clinical parameters (p-value >0.05).

4.2.3.2 Polymorphic Covariates – Univariate Analysis

For this analysis we analyzed 49 polymorphisms in 29 genes described before. The significative associations between the polymorphic variables considered and survival are shown in the following table:

		Median	*Std. Error*	*IC 95%*		*Log Rank Test*
XRCC3 18067C>T	CC	61.1	10.8	39.9	82.3	0.116
	CT	55.7	6.7	42.7	68.8	
	TT	87.3	11.6	64.5	110.0	
XRCC3 18067C>T	CC+CT	57.3	6.6	44.4	70.1	0.042
	TT	87.3	11.6	64.5	110.0	
XRCC3 4541A>G	AA	72.2	8.1	56.3	88.1	0.145
	AG	51.6	7.5	36.9	66.3	
	GG	61.4	17.3	27.4	95.4	
XRCC3 4541A>G	AA	72.2	8.1	56.3	88.1	0.055
	AG+GG	51.6	7.1	37.6	65.6	
GSTT1	0	45.6	7.2	31.6	59.6	0.017
COPY NUMBER	1	49.6	7.4	35.1	64.1	
	2	87.3	-	-	-	
GSTT1	0+1	48.5	4.99	38.8	58.3	0.004
COPY NUMBER	2	87.3	-	-	-	
GSTM1	0	77.9	10.1	58.2	97.6	0.005
COPY NUMBER	1	42.2	6.8	28.8	55.5	
	2	33.3	11.3	11.2	55.3	
GSTM1	0	77.9	10.1	58.2	97.6	0.001
COPY NUMBER	1+2	41.8	6.1	29.8	53.8	
MDM2 2309T>G	TT	61.1	6.6	48.2	74.0	0.042
	TG	76.0	9.4	57.6	94.3	
	GG	30.5	3.9	22.9	38.1	

MDM2 2309T>G	TT+TG	67.2	6.3	54.9	79.5	0.020
	GG	30.5	3.9	22.9	38.1	
TSER	WT	36.7	8.9	19.2	54.1	0.012
	HET	78.0	8.6	61.2	94.9	
	MUT	58.9	8.9	41.5	76.2	
TSER	WT	36.7	8.9	19.2	54.1	0.010
	HET+MUT	72.2	6.9	58.8	85.7	

Table 12. Log Rank Test for the polymorphic gene variations.

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.

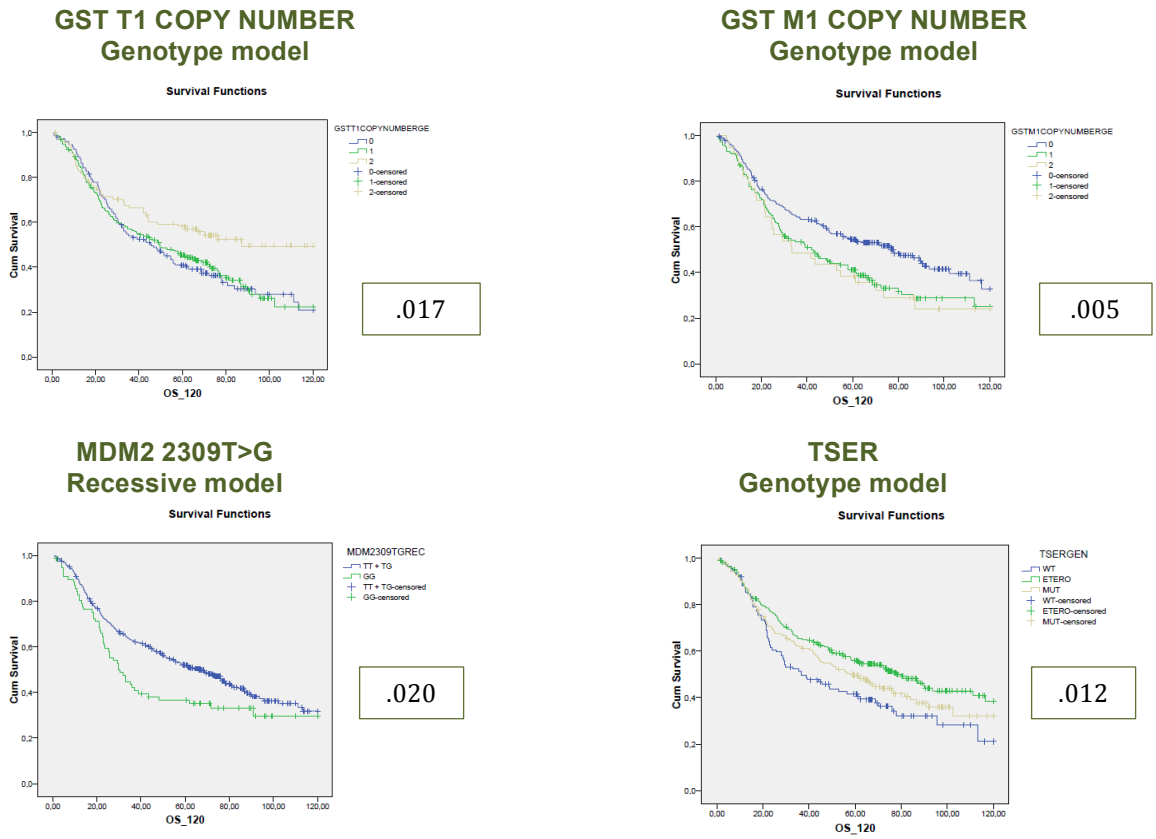


Figure 16. Survival Curves related to genetic polymorphisms (p-value >0.05).

4.2.3.3 Polymorphic Covariates – Gender Related Univariate Analysis

Also to evaluate the survival, we stratified the data by gender and age and the analysis were conducted for each polymorphism according to the previously described models. Only significant survival curves obtained with Kaplan Meyer Method are reported.

The first analyses performed were related to gender. Two genetic markers were significant in describing the overall survival: male patients with the variated polymorphisms GSTT1 and TSER are both characterized by significant longer median survival.

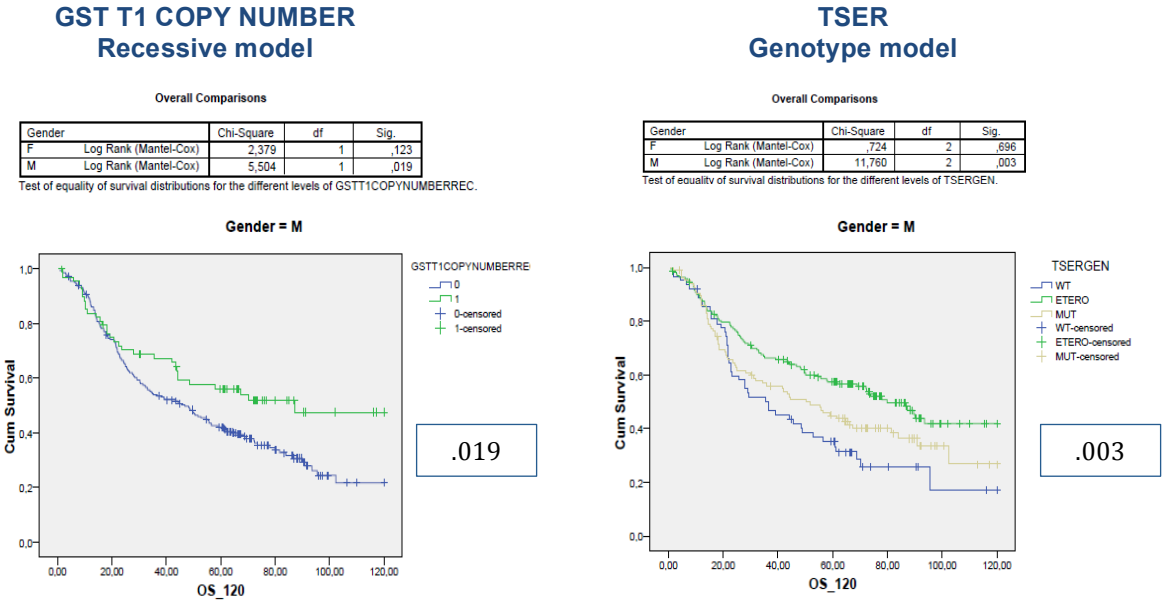


Figure 17. Survival Curves related to genetic polymorphisms (p-value >0.05) in male cases.

For the female analysis, four genetic markers were significant in describing the overall survival, the polymorphisms GSTM1 pos/neg and COPY NUMBER, SOD2 16G>A and MDM2 309T>G. Female patients with wild type genotype for GSTM1, SOD2 and MDM2 wild type showed a significantly longer median survival than patients with mutated genotype.

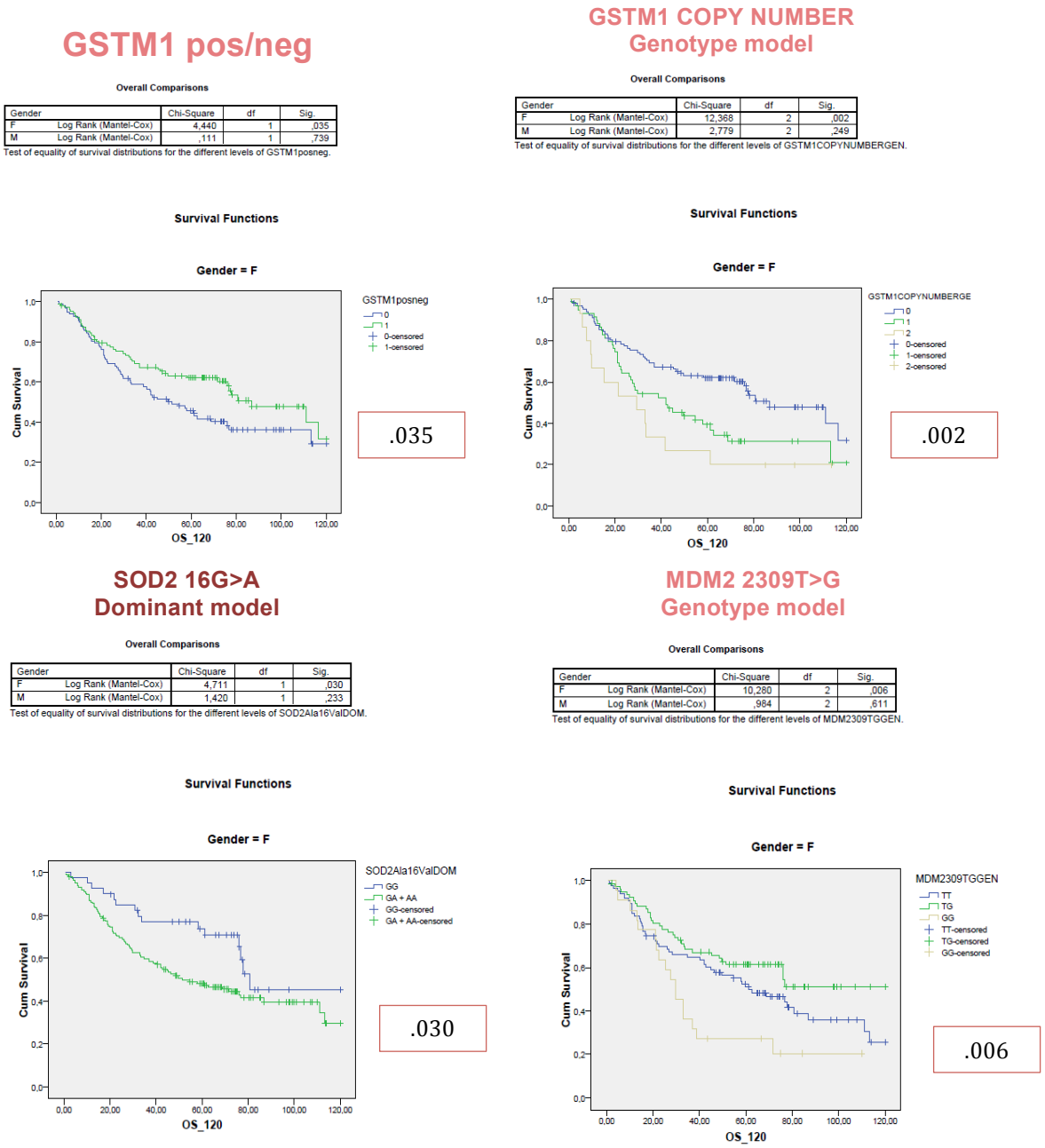


Figure 18. Survival Curves related to genetic polymorphisms (p-value >0.05) in female cases.

4.2.3.3 Polymorphic Covariates – Gender Related Univariate Analysis

The second analyses performed were related to age. Three genetic markers were significant in describing the overall survival in young patients, and five in old patients.

Young patients with homozygote genotype for XRCC3 17893A>G polymorphism showed a significantly longer median survival than patients with heterozygous genotype. The high copy number of GSTT1 and the low copy number of GSTM1 are both related to a longer survival time.

XRCC3 17893A>G
Genotype model

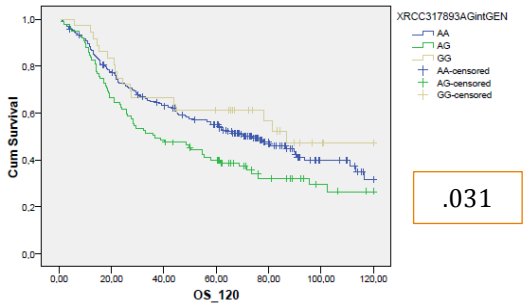
Overall Comparisons

Età_70		Chi-Square	df	Sig.
< 70	Log Rank (Mantel-Cox)	6,969	2	,031
>= 70	Log Rank (Mantel-Cox)	,358	2	,836

Test of equality of survival distributions for the different levels of XRCC317893AGintGEN.

Survival Functions

Età_70 = < 70



.031

GSTT1 COPY NUMBER
Recessive model

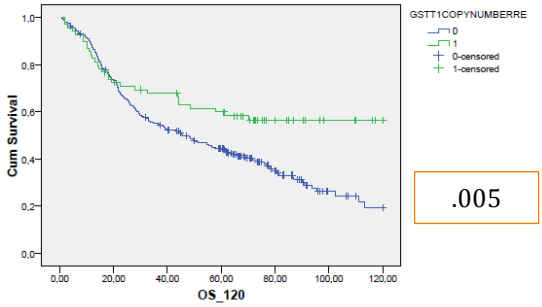
Overall Comparisons

Età_70		Chi-Square	df	Sig.
< 70	Log Rank (Mantel-Cox)	8,030	1	,005
>= 70	Log Rank (Mantel-Cox)	,770	1	,380

Test of equality of survival distributions for the different levels of GSTT1COPYNUMBERREC.

Survival Functions

Età_70 = < 70



.005

GSTM1 COPY NUMBER
Genotype model

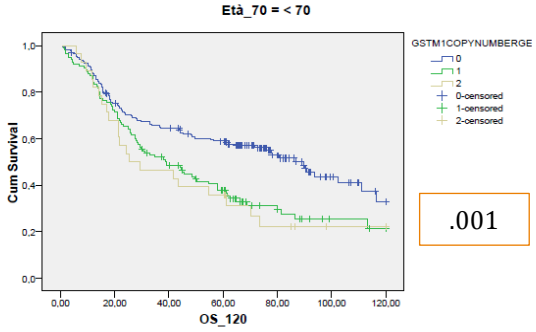
Overall Comparisons

Età_70		Chi-Square	df	Sig.
< 70	Log Rank (Mantel-Cox)	13,481	2	,001
>= 70	Log Rank (Mantel-Cox)	,182	2	,913

Test of equality of survival distributions for the different levels of GSTM1COPYNUMBERGEN.

Survival Functions

Età_70 = < 70



.001

Figure 19. Survival Curves related to genetic polymorphisms (p-value >0.05) in young patients.

Old patients survival seemed to be related mostly to the polymorphic variations on genes involved on the DNA damage repair. Patients with the variant allele on the polymorphisms XPD 23591G>A and hMLH1 A>G showed a shorter median survival than patients with wild type genotype. On the contrary, the deletion in position 1449 on the XRCC1 gene characterized a better survival curve.

The hOGG1 1245C>G genotype showed two different curves for patients with or without the variation but the frequency of the mutated events were not enough strong to describe a real prognostic value about that polymorphism.

As in the overall survival study performed on female cases, MDM2 wild type showed a significantly longer median survival than patients with mutated genotype.

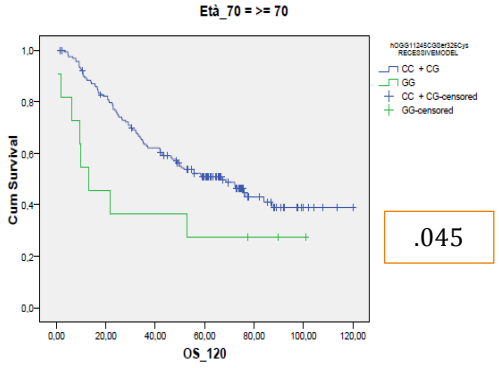
hOGG1 1245C>G
Genotype model

Overall Comparisons

Età_70		Chi-Square	df	Sig.
< 70	Log Rank (Mantel-Cox)	1,237	1	,266
>= 70	Log Rank (Mantel-Cox)	4,030	1	,045

Test of equality of survival distributions for the different levels of hOGG11245CGSer326CysRECESSIVEMODEL.

Survival Functions



.045

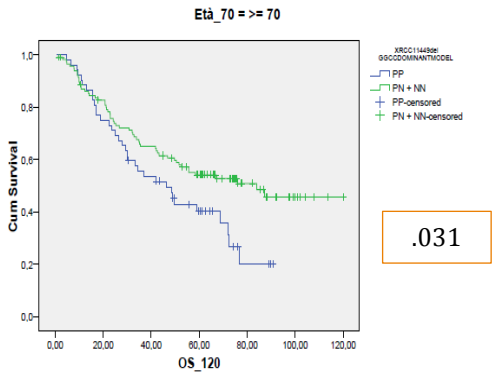
XRCC1 1449del
Dominant model

Overall Comparisons

Età_70		Chi-Square	df	Sig.
< 70	Log Rank (Mantel-Cox)	,061	1	,806
>= 70	Log Rank (Mantel-Cox)	4,672	1	,031

Test of equality of survival distributions for the different levels of XRCC11449delGGCCDOMINANTMODEL.

Survival Functions



.031

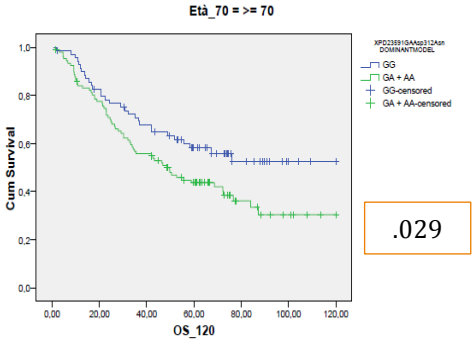
XPD 23591G>A
Dominant model

Overall Comparisons

Età_70		Chi-Square	df	Sig.
< 70	Log Rank (Mantel-Cox)	,566	1	,452
>= 70	Log Rank (Mantel-Cox)	4,738	1	,029

Test of equality of survival distributions for the different levels of XPD23591GAAsp312AsnDOMINANTMODEL.

Survival Functions



.029

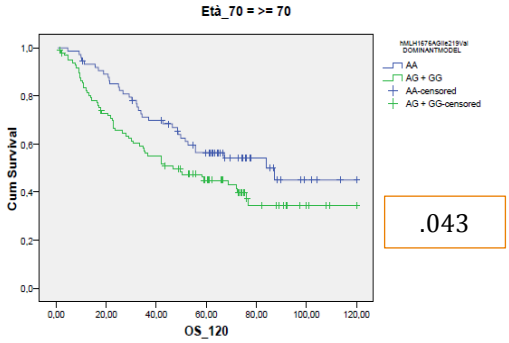
hMLH1 676A>G
Dominant model

Overall Comparisons

Età_70		Chi-Square	df	Sig.
< 70	Log Rank (Mantel-Cox)	,200	1	,655
>= 70	Log Rank (Mantel-Cox)	4,081	1	,043

Test of equality of survival distributions for the different levels of hMLH1676AGlle219ValDOMINANTMODEL.

Survival Functions



.043

Figure 20. Survival Curves related to genetic polymorphisms (p-value >0.05) in old patients.

4.2.3.3 Multivariate Cox Analysis

In the multivariate Cox proportional hazard model we have adjusted the clinicopathologic features that were significant in the previous multivariate analysis (age, gender, TNM, surgery, IVO) with the polymorphisms that were significant in the univariate analysis (XRCC1 1449del, XRCC3 17893A>G, XRCCE 18067C>T, XRCC3 4541A>G, hMLH1 676A>G, hOGG11245C>G, XPD 23591G>A, GSTT1 pos/neg, GSTT1 COPY NUMBER, GSTM1 pos/neg, GSTM1 COPY NUMBER, MDM2 2309T>G). After adjustment with the clinicopathologic covariates only two polymorphisms remained significant (Table 13).

Variables in the Equation

TSER	rif	B	SE	Wald	df	Sig.	Exp(B)	95,0% CI for Exp(B) Lower
TSER	WT			6,498	2	**0,039**		
TSER (ETERO)		-0,816	0,328	6,211	1	**0,013**	0,442	0,233
TSER (MUTATO)		-0,436	0,331	1,738	1	0,187	0,646	0,338
TNM (T1_2_3)		-1,617	0,134	146,234	1	**0,000**	0,199	0,153
SEDE				8,210	2	0,016		
SEDE (Colon DX)		0,477	0,166	8,207	1	**0,004**	1,610	1,162
SEDE (Colon SX)		0,215	0,146	2,163	1	0,141	1,240	0,931
Età <70		-0,423	0,299	1,992	1	0,158	0,655	0,364
Gender (F)		-0,245	0,253	0,938	1	0,333	0,783	0,477
TSER*Età <70	WT, >= 70			1,811	2	0,404		
TSER (ETERO)*Età <70		0,463	0,357	1,677	1	0,195	1,589	0,789
TSER (MUTATO)*Età <70		0,426	0,371	1,319	1	0,251	1,532	0,740
TSER*Gender	WT, M			2,139	2	0,343		
TSER (ETERO)*Gender (F)		0,415	0,314	1,748	1	0,186	1,515	0,818
TSER (MUTATO)*Gender (F)		0,100	0,337	0,088	1	0,766	1,105	0,571

Method = Enter

MDM2	rif	B	SE	Wald	df	Sig.	Exp(B)	95,0% CI for Exp(B)	
								Lower	Upper
MDM2309	TT			9,907	2	0,007			
MDM2309 (TG)		0,184	0,261	0,495	1	0,482	1,202	0,720	2,004
MDM2309 (GG)		1,030	0,333	9,546	1	**0,002**	2,801	1,457	5,383
TNM (T1_2_3)	T 4	-1,662	0,138	145,789	1	0,000	0,190	0,145	0,248
SEDE	Retto			8,571	2	0,014			
SEDE (Colon DX)		0,491	0,169	8,486	1	0,004	1,634	1,174	2,275
SEDE (Colon SX)		0,255	0,149	2,946	1	0,086	1,291	0,964	1,728
Età (<70)	>= 70	0,225	0,204	1,215	1	0,270	1,253	0,839	1,870
Gender (F)	M	-0,030	0,187	0,026	1	0,871	0,970	0,673	1,399
MDM2309*Età_70	TT >= 70			7,748	2	**0,021**			
MDM2309 (TT)*Età <70		-0,362	0,289	1,575	1	0,209	0,696	0,395	1,226
MDM2309 (GG)*Età <70		-1,040	0,375	7,697	1	**0,006**	0,353	0,169	0,737
MDM2309*Gender	TT, M			0,141	2	0,932			
MDM2309 (TT)*Gender (F)		-0,086	0,286	0,091	1	0,763	0,918	0,524	1,606
MDM2309 (TT)*Gender (F)		0,040	0,360	0,012	1	0,912	1,041	0,514	2,109

Method = Enter

Table 13: Association between TSER and MDM2 2309T>G polymorphisms and clinicopathologic features among CRC patients.

DISCUSSION

5.1 Pharmacokinetics Study

Therapies based on the use of chemotherapy are characterized by a narrow therapeutic index and it is evident that it should be necessary to identify markers useful for tailoring therapy. Being the anti-tumor drugs' toxicity characterized by severe life-threatening reactions, the identification of markers correlated to the onset of toxicity would help in preventing the clinical manifestation in the patient.

In the treatment with FOLFIRI, the most frequent toxicities are caused by the irinotecan. It is a prodrug, whose active metabolite is SN-38. The major route of elimination of SN-38 is the glucuronidation, with the formation of SN-38G. Variations in the efficiency of the reaction of glucuronidation may lead to a lower detoxification of the active metabolite, resulting that it should be candidate as a marker of toxicity associated to the irinotecan treatment.

Several polymorphisms in the UGT gene, which encodes for proteins responsible for the process of glucuronidation, have been linked to a decrease in the levels of glucuronidated metabolites in vitro and in patients with the hyperbilirubinemia syndromes. The enzyme mainly involved in SN-38 glucuronidation is the UGT1A1. A variant of this gene (UGT1A1*28) is associated with lower levels of UGT glucuronidation.

In several studies, the plasma levels of SN-38 and SN-38G were analyzed in patients treated with FOLFIRI, carriers of the variant UGT1A1*28 and it was found a correlation between the occurrence of haematological toxicity type and plasma levels of SN-38 [58].

The work previously carried out by our laboratory [59] have shown that patients wild type and heterozygous for the polymorphism could tolerate higher doses of irinotecan compared to homozygous mutant. This has led to a redefinition of the MTD for irinotecan in groups of patients stratified according to genotype for the UGT1A1*28 polymorphism [25].

The use of BV in combination with FOLFIRI in polychemotherapy regimen was approved in the clinical practice and it is generally well tolerated [26]. However,

preliminary studies reported that the addition of BV induced an increase of SN-38 AUC of approximately 33% in patients treated with regimens BV+FOLFIRI versus FOLFIRI alone.

Therefore, given the correlation between AUC SN-38 and the onset of toxicity, the determination of the possible role of BV in the plasma levels of CPT-11 or its metabolites would therefore be of clinical interest, particularly in schemes in which irinotecan was administered at doses higher than expected from the standard treatment regimen FOLFIRI. The study of the interaction between irinotecan and BV can be evaluated through the analysis of the relationship between the pharmacokinetic properties of CPT-11 and its metabolites, with particular reference to the SN-38, and the toxicity manifested by patients. Even in the study object of this thesis, dose increments in this protocol were established on the basis of the stratification of patients according to the UGT1A1*28 polymorphism. The study examined 35 patients till now and is still ongoing. To define the pharmacokinetic properties of irinotecan, a method for the quantification of CPT-11, SN-38, SN-38G and APC was developed and validated. It was decided to start the analysis with the dosage of the samples collected from patients experiencing DLTs in order to assess the possible correlation between the known toxicity with pharmacokinetics of the analyzed compounds and pharmacogenetic aspects of treated patients. Moreover, we analyzed three patients characterized as wt for the polymorphism UGT1A1*28 and two heterozygous for the same polymorphism, treated at a dose of 260 and 370 mg/m^2, respectively.

The range for the parameters observed in patients (AUC, C_{max}, $t_{1/2}$, t_{max}, volume of distribution, clearance) related to the first administration (withou bevacizumab, BV-), is in line with the values previously reported in the literature for all compounds, thus demonstrating the validity of the method [60].

Two patients (patient 21 and patient 23) showed hematologic and gastrointestinal DLTs, which led to their exit from the study.

Patient 21 presented an AUC of CPT-11 of 31 h*mg/mL and therefore much higher than the range observed in the other analyzed patients (15.5-17.9 h*mg/mL). Another pharmacokinetic parameter different from that of the other patients is the

clearance, which is equal to 7.9, compared to the observed range of 14.2-20.5. However, the observed toxicity did not appear to be ascribed to the SN-38 because the value of AUC is comparable with those calculated for the other analyzed patients.

Patient 23 presented an extremely high value for AUC of SN-38 that is about twice compared to that of the other ones. This may be linked to the observed DLTs correlations described also in other works [59] [60].

One of the purposes of the protocol is to analyze from the point of view of a pharmacokinetic interaction CPT-11 and BV. To assess the presence of an effect on the pharmacokinetic properties of the BV, irinotecan were compared to all the parameters calculated from the plasma concentrations of CPT-11 and its metabolites, in the 2 treatments provided for the first cycle of therapy. In fact, in the first treatment (days 1-3) the BV can not show any effect on the irinotecan PK because it is administered only during the third day, and then at the end of the sampling performed for this first treatment.

First analysis of the pharmacokinetic parameters seem to exclude an effect of BV on the pharmacokinetics of irinotecan. Performing an intra-patient comparison between the first and the second administration, it is evident that all the parameters of CPT-11, SN-38 and SN-38G are comparable. The curves related to the plasma concentrations are practically the same between the first and second cycle.

It is necessary to underline that data obtained so far are preliminary and it's important to note that a small intra-variability should be very high in interindividual comparisons, with values fluctuation by more than 50% for some patients even if the same dosage.

In addition, we observed an overlap of pharmacokinetic data with or without BV. Although the study is still in progress, the values of MTD determined by genotype analysis should be lower than previously determined for FOLFIRI regimen [25]. This seems to suggest that the addition of BV in some way is able to contribute to the onset of toxicity in high-dose regimens of irinotecan, but this difference does not seem to be correlated to plasma concentrations of CPT-11 or its metabolites.

We deeped the role of some genetic biomarkers in patients carryng the wild type or heterozygous for the UGT1A1*28 polymorphism who have developed severe toxicity (DLT). In particular, we focused our attention on genetic determinants already described in literature. Previous studies [61] have shown the impact of UGT1A7*3 polymorphism on the development of severe haematological toxicity after treatment with FOLFIRI. This polymorphism is included in an haplotype with UGT1A9*22 and UGT1A1*28 polymorphisms and the linkage disequilibrium was associated with an increased risk of toxicity to treatment with FOLFIRI. Patients with DLT were characterized by all of these polymorphisms. Because the patients were selected for the UGT1A1*28 polymorphism, it has not been possible to identify any patient with two alleles at high risk of toxicity. However, patient 29, who presented severe neutropenia, is characterized by the genotype UGT1A7*3/*3, which may have contributed to the development of severe toxicity, even in the absence of genotype UGT1A1*28/*28.

With regard to the clinical response of the treatment provided by the protocol, a preliminary analysis was performed only in patients enrolled in CRO, Aviano (PN) and having completed at least 2 cycles of therapy (protocol stipulates the assessment of the asset after every 2 cycles of therapy by CT with contrast medium). In the 18 evaluable patients, eight (44%) had partial remission of the disease, nine (50%) had stable disease and one patient (6%) had tumor progression. After administration of a variable number of cycles (from six to eight), four patients (22%) became subjected to surgery or thermoablation excision of the liver and that has made them free of disease for a period of time not yet assessable.

Although the study is still ongoing and we have been assessed only patients enrolled in the CRO, the first conclusion that we can drawn from this preliminary data analysis of clinical activity is that the combination of the chemotherapy regimen FOLFIRI and BV administered in patients with metastatic CRC as first-line treatment, can stabilize the disease in 50% of patients. Moreover, in a significant percentage of patients there was a reduction in the number and size of lesions in liver; this is noteworthy because it makes them surgically resectable or removable

by thermal ablation. The combined therapy allows the conversion of a disease initially judged as unresectable, in an operable disease in selected cases.

5.2 Pharmacogenetics Study

The therapeutic protocols used for the treatment of CRC have a high degree of variation in toxicity and antitumor activity, making it difficult to select the optimal treatment for each individual patient. In addition, the cost of new drugs is prohibiting the ability for many patients to have access to therapy.

The prevalence of therapeutic failures (TFs) and adverse drug reactions (ADRs) markedly increase in older subjects. However, both TFs and ADRs did not always appear related to the presence of multiple pharmacologic treatments, a common status in subjects aged 70 and over. They seem instead more related to variations in the genes encoding for metabolizing and transporting drugs protein. The genetics of drug metabolizing enzymes (DMEs) and drug transporters (DTs) is a very active area of multidisciplinary research, overlapping the fields of medicine, biology, pharmacology, and genetics. These proteins are virtually responsible for metabolism and disposition, and thus for the efficacy of a number of drugs currently used in clinical practice.

Older patients have been long under-represented in cancer clinical trials. This age discrepancy in trial enrollment was largely driven by the lack of enrollment of patients older than 70 years, since the proportions of trial subjects and population cancer cases aged 65–69 years were nearly identical [2].

This part of the study was designed to analyze the age and gender-related associations between polymorphisms of candidate genes with risk and overall survival (OS) in patients with colorectal cancer (CRC) treated with 5-fluoruracil (5-FU)-based adjuvant chemotherapy.

The greatest burden from CRC falls on the elderly, with nearly 70% of cases diagnosed in those older than age 65 and 40% diagnosed in those over 75 years of age [62]. As a result, approximately 75% of CRC deaths occur in people older than

65 years of age [63] Recently, evidence that supports the use of chemotherapy in older CRC patients has begun to emerge; however, these investigations have largely focused on the outcomes in the most fit older patients.

The knowledge of what we know and what we need to know is needed to promote the application of pharmacogenetics biomarkers in clinical practice, in order to introduce personalized treatments for elderly people.

We enrolled 812 patients with stage II and III CRC treated with adjuvant 5-FU-based chemotherapy. Genotypes for 49 variations in 29 genes were determined by different genotyping methods (see M&M). 510 males and 302 females were included in the study.

Risk analysis evidenced an important role of most of the polymorphic variation on genes involved in the mechanisms of DNA repair. We stratified the population by age and gender to underline the correlation between these two clinical parameters to the genetics. After the stratification by gender, the risk associations with the variations in the DNA repair genes were maintained only in female. In particular, female with the variations on XRCC1 Ex9+16G>A (Arg280His), ERCC1 8092C>A 3'UTR and hMSH6-556G>T have higher risk to develop CRC (*p: 0.010*, OR: 0,135, CI95%: 0,029 – 0,622; *p: 0.031,* OR: 0,606, CI95%: 0,384 – 0,955; *p: 0.002,* OR: 0,516, CI95%: 0,340 – 0,783). It is mostly confirmed by a lot of studies that the mutated genotype of these SNPs are associated with an increase of cancer risk because of the reduction of DNA damages repair.

The TP53 ex4+119C>G variation is the only one which is associated to the higher cancer risk both in male and female (*p: 0.015,* OR: 0,568, CI95%: 0,361 - 0,896; *p: 0.015,* OR: 2,08 CI95%: 1,151 - 3,760). The polymorphism in the p53 72nd codon involves a proline to arginine substitution, leading to changes in gene transcription activity, interaction with other proteins and modulation of apoptosis. Studies evaluating the association between this polymorphism and CRC have shown an association with increased cancer risk [64] [65].

A second stratification was made by age. We use the cut-off of 70yrs because it is the age used in the clinical practice to define a patient as a geriatric case. The double stratificated analysis lost the significance of most of the genes' variations previously

associated to the risk, except for XPG 3507G>C (related only to elderly patients), and for XRCC1 Ex9+16G>A and MTHFR 1298A>C (characterizing only young patients). The CART analysis was made to combine all the clinical paramiters considered to each gene variations. MTHFR 1298A>C, a polymorphism altering the function of the encoded protein, has been strongly associated with a lower risk to develope CRC in young females. Alterations in the kinetics of MTHFR due to the presence of polymorphisms in this gene have been associated with the risk of CRC [66]. In fact, previously studies demostrated that plasma folate level is influenced by MTHFR genotypes: in particular, MTHFR 1298AC+CC genotypes had a lower plasma folate concentration than those with the MTHFR 1298AA genotype with a reduced risk do develop cancer [67].

However there are no confirmed association with age and gender.

Glutathione S-transferases (GSTs) catalyse reactions between glutathione and lipophilic compounds with electrophilic centres, leading to neutralisation of toxic compounds, xenobiotics and products of oxidative stress. The role of GST polymorphisms (GSTM1 null/present genotype, GSTT1 null/present genotype, GSTP1 Ile105Val and GSTA1 *A/*B) as CRC risk factors is still not clearly defined [68]. GSTT1 null genotype was associated with a small but significant increase in risk ($p = 0.013$, OR = 1.393, 95% CI = 1.007–1.818). At contrary, copy-number variant of GSTM1 was associated with a reduction of risk ($p_{\text{Dominant model}} < 0.001$, OR = 0.673, 95% CI = 0.552–0.820). The same associations were founded in male cases after gender stratification and the frequencies observed in our study are in accordance with those from other European Caucasian populations [69].

There is a clear increase in the number of chronic comorbid conditions with age [70], and comorbidity is repeatedly cited as a reason behind the decision not to treat an older patient [71] [72] [73]. However, the influence that an older patient's comorbidities should have on treatment decision making is not clear. Certainly, comorbidity likely places patients at a higher risk of suffering adverse outcomes from treatment, and increases the risk that competing causes of mortality will shorten an older patient's life to such an extent that chemotherapy benefit is not realized.

Our analysis about the associations between genotypes and overall survival were assessed using Kaplan–Meier curves and Cox proportional hazards regression. Our results underline the pivotal role of the detoxification mechanisms played by the Glutathione-S-transferase. GSTM1 null and GSTT1 copy number variations were associated with low survival rates in younger patients (p_{GSTM1} = 0.047, HR = 3.937, 95% CI = 0.31– 0.89; p_{GSTT1} = 0.039, HR = 4.246). However, survival increase is observed in young patients with the GSTM1 copy number variant ($p_{Dominant\ model}$ < 0.001, HR = 13.246).

There were no significant associations between GSTT1 and GSTM1 genotypes with other clinical factors (localization, stage and tumor node metastasis classification) in the total case group. Following stratification by age (<70 vs ≥70 years at diagnosis), GSTT1 null was associated with an increased cancer risk in young patients (p < 0.001, OR = 1.942, 95% CI = 1.523–3.440). This study confirms the association with the risk and the effect of GSTT1 and GSTM1 polymorphisms on survival in CRC patients who received chemotherapy. We also suggest that the specific risk association with GST null genotype in younger patients, particularly in those with presentation of tumor under the age of 70 years, could be related to an improved immune response in younger patients, but less detoxification and increased rates of DNA damage in older patients] [74].

The incorporation of pharmacogenomics in everyday clinical practice will bring with it many basic ethical and legal issues. Furthermore, the weight of the published literature including information with conflicting data, coupled with genetic admixtures and changing population structures, seems to challenge the clinical applicability and practicality of the information. Thus, molecular diagnostics based on individual biomarkers capable of a more precise prognosis is imperative to the successful clinical adaptation of the field of pharmacogenetics.

The inclusion of pharmacogenetic biomarkers in the paradigm of CRC therapy will enable the determination of patients who are most likely to benefit from therapeutic interventions tailored specifically for them. Use of predictive biomarkers should be made an integral part of current clinical practice and be used as an aid to clinical

experience and expertise in making patient therapy decisions. However, in our study most of the predictive biomarkers were not confirmed as prognostic, underlining the necessity to identify different panels of gene variations to be analyzed in the clinical practice.

Our analysis suggests an important role of the variation of the gene coding the protein MDM2. The Mouse Double Minute 2 (MDM2) gene encodes a phosphoprotein that interacts with P53 and negatively regulates its activity. The polymorphism (309T>G) in the promoter of MDM2 gene has been reported to be associated with enhanced MDM2 expression and tumor development. Individuals carrying the GG genotype of the MDM2 309T>G polymorphism were found to have higher MDM2 levels, which led to attenuation of the TP53 pathway and acceleration of tumor formation in humans [75]. Studies investigating the association between MDM2 309T>G polymorphism and CRC risk reported conflicting results. We did not found any association with risk too, but the GG genotype was related to a reduced overall survival in all cases. These data were confirmed after age stratification, both in the young and elderly populations. Only female cases showed an association between the GG genotype and a reduced overall survival. COX analysis confirmed the independence of the genotype, and all the other clinical parameters. All the patients received the same therapy and the role of the MDM2 protein is not related to the pharmacology of the drug used for the treatment, so we can suppose that the modification of the protein, which increases the tumor progression, reduces the possibility to survive.

Conclusions

To conclude, the aim of pharmacogenetics is the establishment of connections between pharmacology and genetics, and in particular between a pharmacologic phenotype and a genotype, to predict different individual response to treatments [76]. This connection was not a statistical association of a given pharmacologic phenotype with a given genotype in a number of individuals, but rather the

identification in a single individual of a unique genotype univocally responsible for the observed pharmacologic phenotype. This was pivotal for the utility of pharmacogenetics in setting up an individualized therapy, and the starting point leading to a personalized medicine in clinical practice. To this end, several points must be underlined. First of all, understanding the relationships between pharmacologic phenotypes and genotypes is not simple and requires their unequivocal identification. The metabolic phenotype may be quite different from the pharmacologic phenotype observed in clinical practice, because it results from the interaction of metabolic phenotypes with a number of factors, including environmental factors.

Thus, the combination of studies investigating clinical drug interactions and pharmacogenetics is a pivotal step, and its integration in drug development may constitute an integrated approach that potentially increases the clinical safety of drugs.

We recommend that older patients and their physicians earnestly discuss risks and benefits of treatment using the current best evidence. Research in geriatric oncology is in an early stage but expanding. Our hope for the near future is to have improved methods for determining which older patients are likely to experience a benefit from therapy, and which older patients are likely to be harmed by therapy.

REFERENCES

[1] Roberts TG, Jr., Goulart BH, Squitieri L, Stallings SC, Halpern EF, Chabner BA, et al. Trends in the risks and benefits to patients with cancer participating in phase 1 clinical trials. JAMA. 2004 Nov 3; 292(17): 2130-40.

[2] Hutchins LF, Unger JM, Crowley JJ, Coltman CA, Jr., Albain KS. Underrepresentation of patients 65 years of age or older in cancer-treatment trials. N Engl J Med. 1999 Dec 30; 341(27): 2061-7.

[3] Relling MV, Gardner EE, Sandborn WJ, Schmiegelow K, Pui CH, Yee SW, et al. Clinical Pharmacogenetics Implementation Consortium guidelines for thiopurine methyltransferase genotype and thiopurine dosing. Clin Pharmacol Ther. 2011 Mar; 89(3): 387-91.

[4] Ando Y, Ueoka H, Sugiyama T, Ichiki M, Shimokata K, Hasegawa Y. Polymorphisms of UDP-glucuronosyltransferase and pharmacokinetics of irinotecan. Ther Drug Monit. 2002 Feb; 24(1): 111-6.

[5] Dickinson GL, Rezaee S, Proctor NJ, Lennard MS, Tucker GT, Rostami-Hodjegan A. Incorporating in vitro information on drug metabolism into clinical trial simulations to assess the effect of CYP2D6 polymorphism on pharmacokinetics and pharmacodynamics: dextromethorphan as a model application. J Clin Pharmacol. 2007 Feb; 47(2).

[6] Mallal S, Nolan D, Witt C, Masel G, Martin AM, Moore C, et al. Association between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse-transcriptase inhibitor abacavir. Lancet. 2002 Mar 2; 359(9308): 727-32.

[7] Costa BM, Viana-Pereira M, Fernandes R, Costa S, Linhares P, Vaz R, et al. Impact of EGFR genetic variants on glioma risk and patient outcome. Cancer Epidemiol Biomarkers Prev. 2011; 20(12): 2610-7.

[8] Hegde SR, Sun W, Lynch JP. Systemic and targeted therapy for advanced colon cancer. Expert Rev Gastroenterol Hepatol. 2008 Feb; 2(1): 135-49.

[9] Slatter JG, Schaaf LJ, Sams JP, Feenstra KL, Johnson MG, Bombardt PA, et al. Pharmacokinetics, metabolism, and excretion of irinotecan (CPT-11) following I.V. infusion of [(14)C]CPT-11 in cancer patients. Drug Metab Dispos. 2000 Apr; 28(4): 423-33.

[10] Mathijssen RH, van Alphen RJ, Verweij J, Loos WJ, Nooter K, Stoter G, et al. Clinical pharmacokinetics and metabolism of irinotecan (CPT-11). Clin Cancer Res. 2001 Aug; 7(8): 2182-94.

[11] Benson AB, 3rd, Goldberg RM. Optimal use of the combination of irinotecan and 5-fluorouracil. Semin Oncol. 2003 Jun; 30(3 Suppl 6): 68-77.

[12] Pitot HC, Wender DB, O'Connell MJ, Schroeder G, Goldberg RM, Rubin J, et al. Phase II trial of irinotecan in patients with metastatic colorectal carcinoma. J Clin Oncol. 1997 Aug; 15(8): 2910-9.

[13] Rougier P, Bugat R. CPT-11 in the treatment of colorectal cancer: clinical efficacy and safety profile. Semin Oncol. 1996 Feb; 23(1 Suppl 3): 34-41.

[14] Rothenberg ML, Berlin JD. Therapeutic strategies for metastatic colorectal cancer: simultaneous, sequential, or specific? J Clin Oncol. 2003 Oct 15; 21(20): 3716-7.

[15] Douillard JY, Cunningham D, Roth AD, Navarro M, James RD, Karasek P, et al. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. Lancet. 2000 Mar 25; 355(9209): 1041-7.

[16] Saltz LB, Cox JV, Blanke C, Rosen LS, Fehrenbacher L, Moore MJ, et al. Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer. Irinotecan Study Group. N Engl J Med. 2000 Sep 28; 343(13): 905-14.

[17] de Gramont A, Vignoud J, Tournigand C, Louvet C, Andre T, Varette C, et al. Oxaliplatin with high-dose leucovorin and 5-fluorouracil 48-hour continuous infusion in pretreated metastatic colorectal cancer. Eur J Cancer. 1997 Feb; 33(2): 214-9.

[18] Sargent DJ, Goldberg RM, Jacobson SD, Macdonald JS, Labianca R, Haller DG, et al. A pooled analysis of adjuvant chemotherapy for resected colon cancer in elderly patients. N Engl J Med. 2001 Oct 11; 345(15): 1091-7.

[19] Presta LG, Chen H, O'Connor SJ, Chisholm V, Meng YG, Krummen L, et al. Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. Cancer Res. 1997 Oct 15; 57(20): 4593-9.

[20] Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med. 2004 Jun 3; 350(23): 2335-42.

[21] Iyer L, Ratain MJ. Pharmacogenetics and cancer chemotherapy. Eur J Cancer. 1998 Sep; 34(10): 1493-9.

[22] Haaz MC, Rivory L, Riche C, Vernillet L, Robert J. Metabolism of irinotecan (CPT-11) by human hepatic microsomes: participation of cytochrome P-450 3A and drug interactions. Cancer Res. 1998 Feb 1; 58(3): 468-72.

[23] Kadakol A, Ghosh SS, Sappal BS, Sharma G, Chowdhury JR, Chowdhury NR. Genetic lesions of bilirubin uridine-diphosphoglucuronate glucuronosyltransferase (UGT1A1) causing Crigler-Najjar and Gilbert syndromes: correlation of genotype to phenotype. Hum Mutat. 2000 Oct; 16(4): 297-306.

[24] Innocenti F, Undevia SD, Iyer L, Chen PX, Das S, Kocherginsky M, et al. Genetic variants in the UDP-glucuronosyltransferase 1A1 gene predict the risk of severe neutropenia of irinotecan. J Clin Oncol. 2004 Apr 15; 22(8): 1382-8.

[25] Toffoli G, Cecchin E, Gasparini G, D'Andrea M, Azzarello G, Basso U, et al. Genotype-driven phase I study of irinotecan administered in combination with fluorouracil/leucovorin in patients with metastatic colorectal cancer. J Clin Oncol. 2006 Feb 10; 28(5): 866-71.

[26] McCormack PL, Keam SJ. Bevacizumab: a review of its use in metastatic colorectal cancer. Drugs. 2008; 68(4): 487-506.

[27] Schmucker DL, Vesell ES. Underrepresentation of women in clinical drug trials. Clin Pharmacol Ther. 1993 Jul; 54(1): 11-5.

[28] Pinn VW. Commentary: women, research, and the National Institutes of Health. Am J Prev Med. 1992 Sep-Oct; 8(5): 324-7.

[29] Kessler DA. Food and Drug Administration: Guideline for the study and evaluation of gender differences in the clinical evaluation of drugs: Notice. . Fed Regist 1993; 58: 39406-16.

[30] Cotton P. FDA lifts ban on women in early drug tests, will require companies to look for gender differences. JAMA. 1993 Apr 28; 269(16): 2067.

[31] Tejeda HA, Green SB, Trimble EL, Ford L, High JL, Ungerleider RS, et al. Representation of African-Americans, Hispanics, and whites in National Cancer Institute cancer treatment trials. J Natl Cancer Inst. 1996 Jun 19; 88(12): 812-6.

[32] Klabunde CN, Springer BC, Butler B, White MS, Atkins J. Factors influencing enrollment in clinical trials for cancer treatment. South Med J. 1999 Dec; 92(12): 1189-93.

[33] Chamberlain RM, Winter KA, Vijayakumar S, Porter AT, Roach M, 3rd, Streeter O, et al. Sociodemographic analysis of patients in radiation therapy oncology group clinical trials. Int J Radiat Oncol Biol Phys. 1998 Jan 1; 40(1): 9-15.

[34] Sateren WB, Trimble EL, Abrams J, Brawley O, Breen N, Ford L, et al. How sociodemographics, presence of oncology specialists, and hospital cancer programs affect accrual to cancer treatment trials. J Clin Oncol. 2002 Apr 15; 20(8): 2109-17.

[35] Trimble EL, Carter CL, Cain D, Freidlin B, Ungerleider RS, Friedman MA. Representation of older patients in cancer treatment trials. Cancer. 1994 Oct 1; 74(7 Suppl): 2208-14.

[36] Goodwin JS, Hunt WC, Humble CG, Key CR, Samet JM. Cancer treatment protocols. Who gets chosen? Arch Intern Med. 1988 Oct; 148(10): 2258-60.

[37] Lewis JH, Kilgore ML, Goldman DP, Trimble EL, Kaplan R, Montello MJ, et al. Participation of patients 65 years of age or older in cancer clinical trials. J Clin Oncol. 2003 Apr 1; 21(7): 1383-9.

[38] Smith JJ, Lee J, Burke C, Contractor KB, Dawson PM. Major colorectal cancer resection should not be denied to the elderly. Eur J Surg Oncol. 2002 Sep; 28(6): 661-6.

[39] Kohne CH, Folprecht G, Goldberg RM, Mitry E, Rougier P. Chemotherapy in elderly patients with colorectal cancer. Oncologist. 2008 Apr; 13(4): 390-402.

[40] Ugolini G, Rosati G, Montroni I, Zanotti S, Manaresi A, Giampaolo L, et al. Can elderly patients with colorectal cancer tolerate planned surgical treatment? A practical approach to a common dilemma. Colorectal Dis. 2009 Sep; 11(7): 750-5.

[41] Papamichael D, Audisio R, Horiot JC, Glimelius B, Sastre J, Mitry E, et al. Treatment of the elderly colorectal cancer patient: SIOG expert recommendations. Ann Oncol. 2009 Jan; 20(1): 5-16.

[42] Pasetto LM, Rossi E, Jirillo A, Monfardini S. Colorectal cancer adjuvant treatment in elderly patients. Crit Rev Oncol Hematol. 2005 Sep; 55(3): 201-6.

[43] Bouvier AM, Jooste V, Bonnetain F, Cottet V, Bizollon MH, Bernard MP, et al. Adjuvant treatments do not alter the quality of life in elderly patients with colorectal cancer: a population-based study. Cancer. 2008 Aug 15; 113(4): 879-86.

[44] Schiffmann L, Ozcan S, Schwarz F, Lange J, Prall F, Klar E. Colorectal cancer in the elderly: surgical treatment and long-term survival. Int J Colorectal Dis. 2008 Jun; 23(6): 601-10.

[45] Iversen LH, Pedersen L, Riis A, Friis S, Laurberg S, Sorensen HT. Age and colorectal cancer with focus on the elderly: trends in relative survival and initial treatment from a Danish population-based study. Dis Colon Rectum. 2005 Sep; 48(9): 1755-63.

[46] Heriot AG, Tekkis PP, Smith JJ, Cohen CR, Montgomery A, Audisio RA, et al. Prediction of postoperative mortality in elderly patients with colorectal cancer. Dis Colon Rectum. 2006 Jun; 49(6): 816-24.

[47] Wedding U, Honecker F, Bokemeyer C, Pientka L, Hoffken K. Tolerance to chemotherapy in elderly patients with cancer. Cancer Control. 2007 Jan; 14(1): 44-56.

[48] Basili G, Lorenzetti L, Biondi G, Preziuso E, Angrisano C, Carnesecchi P, et al. Colorectal cancer in the elderly. Is there a role for safe and curative surgery? ANZ J Surg. 2008 Jun; 78(6): 466-70.

[49] Ong ES, Alassas M, Dunn KB, Rajput A. Colorectal cancer surgery in the elderly: acceptable morbidity? Am J Surg. 2008 Mar; 195(3): 344-8; discussion 8.

[50] Aschele C, Bergamo F, Lonardi S. Chemotherapy for operable and advanced colorectal cancer. Cancer Treat Rev. 2009 Oct; 35(6): 509-16.

[51] Devon KM, Vergara-Fernandez O, Victor JC, McLeod RS. Colorectal cancer surgery in elderly patients: presentation, treatment, and outcomes. Dis Colon Rectum. 2009 Jul; 52(7): 1272-7.

[52] Yu X, McBean AM, Virnig BA. Physician visits, patient comorbidities, and mammography use among elderly colorectal cancer survivors. J Cancer Surviv. 2007 Dec; 1(4): 275-82.

[53] Desch CE, Benson AB, 3rd, Somerfield MR, Flynn PJ, Krause C, Loprinzi CL, et al. Colorectal cancer surveillance: 2005 update of an American Society of Clinical Oncology practice guideline. J Clin Oncol. 2005 Nov 20; 23(33): 8512-9.

[54] Cooper GS, Kou TD, Reynolds HL, Jr. Receipt of guideline-recommended follow-up in older colorectal cancer survivors : a population-based analysis. Cancer. 2008 Oct 15; 113(8): 2029-37.

[55] Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst. 2000 Feb 2; 92(3): 205-16.

[56] Fakhrai-Rad H, Pourmand N, Ronaghi M. Pyrosequencing: an accurate detection platform for single nucleotide polymorphisms. Hum Mutat. 2002 May; 19(5): 479-85.

[57] Butler JM, Buel E, Crivellente F, McCord BR. Forensic DNA typing by capillary electrophoresis using the ABI Prism 310 and 3100 genetic analyzers for STR analysis. Electrophoresis. 2004 Jun; 25(10-11): 1397-412.

[58] Innocenti F, Kroetz DL, Schuetz E, Dolan ME, Ramirez J, Relling M, et al. Comprehensive pharmacogenetic analysis of irinotecan neutropenia and pharmacokinetics. J Clin Oncol. 2009 Jun 1; 27(16): 2604-14.

[59] Toffoli G, Cecchin E, Corona G, Russo A, Buonadonna A, D'Andrea M, et al. The role of UGT1A1*28 polymorphism in the pharmacodynamics and pharmacokinetics of irinotecan in patients with metastatic colorectal cancer. J Clin Oncol. 2006 Jul 1; 24(19): 3061-8.

[60] Poujol S, Bressolle F, Duffour J, Abderrahim AG, Astre C, Ychou M, et al. Pharmacokinetics and pharmacodynamics of irinotecan and its metabolites from plasma and saliva data in patients with metastatic digestive cancer receiving Folfiri regimen. Cancer Chemother Pharmacol. 2006 Sep; 58(3): 292-305.

[61] Cecchin E, Innocenti F, D'Andrea M, Corona G, De Mattia E, Biason P, et al. Predictive role of the UGT1A1, UGT1A7, and UGT1A9 genetic variants and their

haplotypes on the outcome of metastatic colorectal cancer patients treated with fluorouracil, leucovorin, and irinotecan. J Clin Oncol. 2009 May 20; 27(15): 2457-65.

[62] Edwards BK, Howe HL, Ries LA, Thun MJ, Rosenberg HM, Yancik R, et al. Annual report to the nation on the status of cancer, 1973-1999, featuring implications of age and aging on U.S. cancer burden. Cancer. 2002 May 15; 94(10): 2766-92.

[63] Yancik R, Ries LA. Cancer in older persons. Magnitude of the problem--how do we apply what we know? Cancer. 1994 Oct 1; 74(7 Suppl): 1995-2003.

[64] Song HR, Kweon SS, Kim HN, Piao JM, Yun WJ, Choi JS, et al. p53 codon 72 polymorphism in patients with gastric and colorectal cancer in a Korean population. Gastric Cancer. Aug; 14(3): 242-8.

[65] Dastjerdi MN. TP53 codon 72 polymorphism and P53 protein expression in colorectal cancer specimens in Isfahan. Acta Med Iran. 49(2): 71-7.

[66] Reeves SG, Meldrum C, Groombridge C, Spigelman AD, Suchy J, Kurzawski G, et al. MTHFR 677 C>T and 1298 A>C polymorphisms and the age of onset of colorectal cancer in hereditary nonpolyposis colorectal cancer. Eur J Hum Genet. 2009 May; 17(5): 629-35.

[67] Li H, Xu WL, Shen HL, Chen QY, Hui LL, Long LL, et al. Methylenetetrahydrofolate reductase genotypes and haplotypes associated with susceptibility to colorectal cancer in an eastern Chinese Han population. Genet Mol Res. 10(4): 3738-46.

[68] Economopoulos KP, Sergentanis TN. GSTM1, GSTT1, GSTP1, GSTA1 and colorectal cancer risk: a comprehensive meta-analysis. Eur J Cancer. Jun; 46(9): 1617-31.

[69] Hezova R, Bienertova-Vasku J, Sachlova M, Brezkova V, Vasku A, Svoboda M, et al. Common polymorphisms in GSTM1, GSTT1, GSTP1, GSTA1 and susceptibility to colorectal cancer in the Central European population. Eur J Med Res. 17: 17.

[70] Yancik R, Wesley MN, Ries LA, Havlik RJ, Long S, Edwards BK, et al. Comorbidity and age as predictors of risk for early mortality of male and female colon carcinoma patients: a population-based study. Cancer. 1998 Jun 1; 82(11): 2123-34.

[71] Potosky AL, Harlan LC, Kaplan RS, Johnson KA, Lynch CF. Age, sex, and racial differences in the use of standard adjuvant therapy for colorectal cancer. J Clin Oncol. 2002 Mar 1; 20(5): 1192-202.

[72] Mahoney T, Kuo YH, Topilow A, Davis JM. Stage III colon cancers: why adjuvant chemotherapy is not offered to elderly patients. Arch Surg. 2000 Feb; 135(2): 182-5.

[73] Lemmens VE, van Halteren AH, Janssen-Heijnen ML, Vreugdenhil G, Repelaer van Driel OJ, Coebergh JW. Adjuvant treatment for elderly patients with stage III colon cancer in the southern Netherlands is affected by socioeconomic status, gender, and comorbidity. Ann Oncol. 2005 May; 16(5): 767-72.

[74] Rudolph A, Hein R, Hoffmeister M, Forsti A, Hemminki K, Risch A, et al. Copy number variations of GSTT1 and GSTM1, colorectal cancer risk and possible effect modification of cigarette smoking and menopausal hormone therapy. Int J Cancer. Sep 1; 131(5): E841-8.

[75] Bond GL, Hu W, Bond EE, Robins H, Lutzker SG, Arva NC, et al. A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. Cell. 2004 Nov 24; 119(5): 591-602.

[76] Al-Ghoul M, Valdes R, Jr. Fundamentals of pharmacology and applications in pharmacogenetics. Clin Lab Med. 2008 Dec; 28(4): 485-97.

[77] Carrer A, Giacca Mas, Giacca Mau, Chapter 4: Molecular Parameters for Prognostic and Predictive Assesment in Colorectal Cance, Rectal Cancer - Strategy and Surgical Techniques. Springer. 2013.