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TITOLO TESI

Evaluation of clinical implications correlate to genetic polymorphisms and pharmacokinetic of transdermal fentanyl

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BACKGROUND

Cancer pain

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage and represents a vital symptom and a physical signal of alarm and defense.

It always contains an objective and a subjective component, determined by the affective and cognitive dimension, by previous experiences, by socio-cultural factors and by the psychic component. , chemical coping that are able to modify the intensity of pain in a non-linear way to tissue damage.[1]

Cancer pain can be related to causes directly related to cancer or secondary to cancer treatment or to a concomitant acute or chronic disease.

About 70-75% of patients have cancer-related pain, 20-25% secondary to cancer, and 5-10% unrelated to underlying disease.[2]

Cancer pain can be present in various stages of neoplastic disease as a factor aggravating performance status. The clinical care contexts can be different, and cancer pain can represent:

• The first manifestation of an unrecognized neoplasm;

• The expression of a known neoplastic disease, of which it represents a symptom of recovery / progression;

• The accompanying symptom of an advanced stage disease;

• The expression of iatrogenic damage (post-surgical, post-actinic, post-chemotherapy , hormone-therapy, targeted therapy, other drugs..) [3]

There are two clinical manifestations of painful symptoms:

- Acute pain (understood in its two clinical manifestations of acute pain in the strict sense, and acute exacerbation of chronic pain symptoms under treatment, or Breakthrough cancer pain);

- Chronic pain, as an expression of ongoing neoplastic disease or treatment outcome (surgical mutilation, outcome of chemo-radiant treatment).

A "didactic" subdivision into three classes can be used for an overview of the processes underlying cancer pain:

- From mass effect;
- As a paraneoplastic syndrome;
- Iatrogen [4]

The first step is represented by the evaluation of pain defining its location and characteristics (somatic, visceral, neuropathic) with its classification. Secondly, the frequency is defined (chronic, breackthrough pain or BTcP, idiopathic pain, accident or end of dose).

The third step is to define its intensity using some scales:

1) the VAS scale (subjective): one-dimensional tool that quantifies what the patient perceives as pain through a 10 cm line with two ends that correspond to "no pain" = 0 and the maximum possible = 10. to mark a point between the two lines which is then measured by the clinician.

2) the NRS scale (subjective): tool based on a numerical scale consisting of 10 degrees from 0 to 10, where 0 corresponds to the total absence of pain and 10 represents the worst pain imaginable by the patient.

3) the VDS scale (subjective): one-dimensional tool in which the patient is asked to indicate an adjective that best characterizes the pain among those proposed, for example no pain, very mild, mild, moderate strong, very strong.

4) the PAINAD scale (objects) instrument that allows the evaluation of pain in the patient unable to communicate.

5) the WONG Baker scale: an evaluation tool that is used in children or in patients with expressive difficulties

6) BPI (brief pain inventory-subjective): assessing the impact and intensity of pain in cancer patients From a structural point of view, it is made up of two sections: the first, made up of 4 items, investigates the intensity of pain in different situations (current pain, worst pain, minor and average value); the second section investigates the interference of pain in daily life through 7 items (work, recreational activities, walking, quality of sleep, mood, relationship with others, quality of life). Each item is evaluated using an 11-point Likert scale, the range of which goes from 0, which indicates "no pain/no interference" to 10, "maximum pain/maximum interference" Subsequently, in the assessment of pain, it is necessary to identify the factors that can modify or intensify symptoms, perform a thorough medical history which aims to deepen the onset of pain, the personality of the patient, the psychological state, etc. Last but not least it is essential to perform a careful physical examination, observe the signs of non-verbal pain, question family members or care givers.

Pain treatment

For clinicians to safely and effectively manage cancer-related pain with opioids, it is important that they understand the basic opioid pharmacology, are able to titrate an immediate-release or long-acting opioid, and can anticipate and treat the expected side effects of opioid therapy.

The therapeutic strategy that still remains a milestone in the treatment of cancer pain is the one proposed in 1986 by the World Health Organization, the so-called Analgesic Ladder [7], and to which the other guidelines produced by various Scientific Agencies and Societies still refer. It consists in the use of Non Steroidal Anti-Inflammatory Drugs (NSAIDs) and Paracetamol in mild pain (drugs of the first step), of "opioids for mild-moderate pain" or "weak opioids" for mild-moderate pain (drugs

of the second step), associated or not with drugs of the first step, and "opioids for moderate-severe pain" or "strong opioids" for moderate-severe pain (drugs of the third step), whether or not associated with drugs of the first. Drugs must be administered according to some simple and shared rules:

- Administration around the clock, which must necessarily be associated with a forecast of the need for administration as needed;
- By mouth: this second indication can be understood in the literal sense, or in the meaning "in the least invasive and most acceptable way for the patient"; in this sense, transdermal formulations would find space, which in the light of a recent literature review would be advantageous over the oral route in terms of some side effects and preference for the patient;
- Individualized (target doses based on patient characteristics up to the minimum effective dose);
- With attention to detail (detailing doses, schedules, side effects).

Over the years some critical observations have been made on the WHO scale, and the WHO itself has recently identified areas that may be subject to further scientific verification [8]. In the first place, its schematically "progressive" use has been stigmatized: according to this critical perspective, a patient presenting with severe pain does not necessarily have to "go" sequentially through all three steps, but it has been suggested that drugs of the second and third step from the onset, at appropriate dosages, based on the intensity of pain [9–10]. An even more radical attack brought to the strategy as a whole is represented by the accusation of being constructed only on the basis of the intensity of the pain, and not on the pathogenetic mechanisms by which it is caused[11]. It must be stated, however, that this criticism does not appear entirely founded, as the scale provides, in each step of the same, the possible use of adjuvant drugs, precisely in function of the underlying pain mechanism. Adjuvant

drugs are defined as "drugs that are not specifically analgesic but which, in the context of cancer pain, can contribute to obtaining a reduction in pain" (examples: anticonvulsants, antidepressants, corticosteroids): they can be associated with the drugs of all and three steps on the analgesic ladder. Modernly, we tend to complete the scale with two further steps,:

-one relating to the change of opioid

-change route of administration (transdermal, subcutaneous, intravenous) [12], related to invasive analgesic approaches, through neurolesion or neuro modulation interventions. The update of the EAPC Guidelines on the use of opioids in cancer pain was recently published. The basic strategy remains that relating to the WHO, but with a series of insights dictated by the most recent clinical evidence. [13–14]

Opioid Pharmacology

Appropriate opioid management needs knowledge about basic opioid pharmacology. There are three primary opioid receptors in the body: mu, kappa and delta receptors; genetic variation in receptors is a contributing factor to variation in opioid response between individuals.. Helping patients understand how much pain relief they should expect with each opioid dose and preparing them for the time when the analgesic effect peaks can set appropriate expectations for pain management outcomes and also teach patients to strategically use the pain relievers.

Fentanyl

Fentanyl, was introduced in 1960 to replace morphine and other opioids due to its higher potency (approximately 75- to 100-fold compared to morphine) [15].

Fentanyl is now used frequently for patients with either acute or chronic pain syndromes. Delivery of fentanyl for acute pain may occur by intravenous (IV), transmucosal, buccal, epidural, intrathecal, or inhalational routes [16–17]. The pharmacokinetics and clinical effects of fentanyl by these routes in the medical

setting are predictable and the drug is considered safe when used by appropriately trained clinicians. However, for practical and pharmacokinetic reasons, fentanyl is indicated for transdermal administration only for the treatment of chronic pain . Millions of patients have used a transdermal fentanyl device, also known as the fentanyl patch, since approval in 1990. The transdermal fentanyl device allows opioid analgesia to be provided in a discreet, convenient, noninvasive, and generally safe manner [18–19] .Transdermal delivery of fentanyl was initially in the postoperative setting [20], where its safety and efficacy could be evaluated under controlled clinical conditions. demonstrated that transdermal fentanyl was safe and efficacious for the outpatient treatment of chronic cancer pain [21–22].

Pharmacology

Fentanyl is a pure mu-opioid receptor agonist that demonstrates approximately 75–100 times the potency of morphine.Fentanyl possesses many of the physicochemical properties essential for transdermal use [23]. The molecular weight of fentanyl base is 337 Da within the maximum molecular weight considered suitable for skin permeation (< 1000 Da). Fentanyl, is highly potent, and produces desired clinical effects following the systemic absorption of a fraction of a milligram in nontolerant individuals with a route that is limited to drugs that are effective at doses of 50% difference in the permeability of fentanyl [24]. Skin surface areas with similar typically possess similar diffusion rates within an individual, explaining why the chest, extremities, and abdomen are acceptable sites for transdermal device application without the need for any dosage changes [25].

Following application of a transdermal fentanyl device to broken skin, blood fentanyl concentrations can rise 5-fold [26].

Skin temperature elevation enhances the absorption of transdermally-applied fentanyl, perhaps either as a result of cutaneous vasodilation or of enhanced solubility of fentanyl [27–28]: for example an increase in skin temperature to 40°C leads to a

gradual 10- to 15-fold increase in cutaneous blood flow [29].-[30]. Application of an overlay to hold in place a nonsticking transdermal device may be associated with altered fentanyl absorption, and raises the potential for toxicity [31]. Intravenously administered fentanyl has a half-life of 2–4 hours but a short duration of action of approximately 15 minutes.Extensive first-pass hepatic metabolism limits its oral bioavailability [32]., so bypassing the liver explains why the bioavailability of transdermal fentanyl is excellent (~92%)[26]. Once absorbed, fentanyl, like other lipophilic compounds, achieves a large volume of distribution (6 L/kg) [33].
Its high lipophilicity allows it to readily cross the blood-brain barrier to produce analgesia and sedation. Metabolism occurs primarily via oxidative dealkylation by hepatic CYP 3A4 to norfentanyl and other less active or inactive metabolites through an oxidative N-dealkylation process. The concomitant use of fentanyl with cytochrome CYP 3A4 inhibitors (e.g., ketoconazole, ritonavir, nefazodone) may result in an increase in both plasma fentanyl concentrations and the risk of adverse drug effects [34–35]. A small amount (8%) of fentanyl is eliminated unchanged in the urine.

Transdermal Delivery Systems

There are two general types of transdermal delivery systems currently in clinical use. The original transdermal therapeutic system (TTS), also called the reservoir transdermal device consists of four functional layers and a protective peel strip [33]. Each of these layers provides important qualities to facilitate consistent and continuous diffusion of fentanyl over a 72-hour period while minimizing the likelihood toxicity. The first layer is a polyester film backing that prevents leakage of transdermal device contents onto the surrounding skin. The second layer consists of a drug reservoir, which contains fentanyl and ethanol combined with a hydroxycellulose gel. Ethanol contained within this gel acts as an organic solvent to approximately double the rate of diffusion of fentanyl [36]. The quantity of fentanyl contained in this reservoir is appropriate to provide a sufficient concentration gradient for transdermal absorption throughout a 3-day cutaneous application . This reservoir

accounts for much of the abuse potential of this transdermal device and for the possibility of dangerous leakage onto nearby skin, both discussed below. The third layer, an ethylene vinyl acetate copolymer rate-controlling membrane, regulates the rate of delivery of fentanylethanol mixture to the skin surface. This reduces the variations in dermal transport and effectively slows diffusion and subsequent absorption by about 50%, an effect most important for those in the population who possess faster-than-average transdermal absorption [24–33]. The silicone skin adhesive represents the last layer of the transdermal fentanyl device, providing a nonirritating and secure surface area of skin contact. By containing fentanyl itself, the adhesive facilitates the development and maintenance of therapeutic fentanyl concentrations following initial transdermal device application and each subsequent change, respectively. The diffusion that occurs from this silicone adhesive layer also demonstrates that transdermal fentanyl absorption occurs in the absence of an ethanol copolymer [37]. More recently introduced to the market, the fentanyl transdermal system, commonly called the matrix patch, consists of two functional layers and a protective peel strip. The two functional layers are a backing layer of polyolefin film and a fentanyl-containing silicone adhesive layer. The major difference from the TTS is the absence of a fluid fentanyl reservoir and therefore the ethanol coadsorbant. The pharmacokinetics and clinical effects of the matrix transdermal device are similar to the original transdermal device despite the absence of a reservoir and a rate-controlling membrane [38]. This suggests that skin contact is a consequential variable in determining the absorption pharmacokinetics of fentanyl. Another transdermal delivery system consisting of fentanyl dissolved in dipropylene glycol within a silicone matrix has similar pharmacokinetic qualities as the reservoir transdermal device [39].

Fentanyl becomes detectable in the serum within 1–2 hours of application of a transdermal fentanyl device. However, therapeutic serum fentanyl concentrations are not achieved until approximately 12–16 hours after transdermal device application [40–41]. The mean time to maximal serum concentrations (Cmax) averages about 36

hours, regardless of the transdermal device strength, but there is substantial intersubject variability (17–48 hours) [42]. The Cmax achieved, which depends on the "strength" of the transdermal device, ranges from 0.3 ng/mL for a 12.5 μg/hour transdermal device to 2.6 ng/mL for a 100 μg/hour transdermal device [42]. The apparent half-life of fentanyl delivered by a transdermal device (following its removal) approaches 17 hours (16–22 hours) [40–42]. One of the advantages of this form of fentanyl delivery is exemplified by the relatively smooth pharmacokinetic curve of blood fentanyl particularly when compared to intermittent dosing by virtually any other route. [43–44]The mean curve of serum fentanyl concentration is relatively flat over the 3-day period following reaching steady state, without the peaks and troughs typical of intermittent dosing. There is a somewhat wide range between the minimum and maximum serum concentrations attained, highlighting the importance of close observation during the initiation of this therapy.The effects of cachexia, muscle wasting, or other debilitating diseases on fentanyl pharmacokinetics are not well studied.

Clinical Effects

The clinical effects of fentanyl, regardless of route of administration, are similar to those of other opioids, and are similarly dependent on both the dose and the degree of patient tolerance. Gastrointestinal effects, dyspnea, and pruritis can be discomforting [45]. The rigid chest syndrome associated with fentanyl infusion is not well described with the transdermal fentanyl device. Mydriasis, vomiting and diarrhea, may be used to identify opioid withdrawal. [46]

Therapeutic Indications

The maintenance of a relatively steady serum concentration with transdermal fentanyl, results in reduced side effects and improved efficacy, improving therapeutic compliance, However, in patients with chronic pain, the substitution of transdermal fentanyl for other opioids is often considered as much for convenience as

for any specific analgesic benefit. Several approaches have been developed for initiating transdermal fentanyl therapy, but central to all is the presupposition of preexistent opioid tolerance [33]. The transdermal fentanyl device should only prescribed for patients who are already receiving long-term therapy with strong oral or parenteral opioids . Conversion tables exist for calculating an expected transdermal fentanyl device dose requirement for patients on prior chronic oral opioid therapy [33]. Dose finding is often necessary, and short-acting opioid adjuncts will often be necessary to control pain until therapeutic serum fentanyl concentrations are achieved. Several recent literature reports suggest that certain patients who are not tolerant to strong opioids, and even opioid-naïve patients, may safely receive a low-dose transdermal fentanyl device [47]. This approach requires specialized knowledge and a highly-selected patient population.

Fentanyl CYP3A4/5 Polymorphisms

Two studies have analyzed the role of CYP3A5*3 on treatment outcomes with fentanyl in chronic cancer pain patients. In a group of Japanese the absorption rate of fentanyl was found to be significantly higher in homozygous carriers as opposed to heterozygotes or wild-type . In terms of toxicity, there was a greater incidence of central adverse effects in homozygotes,. These results suggest that CYP3A5 polymorphisms may be used in cancer patients to predict transdermal fentanyl response and toxicity [48]. Barratt et al. analyzed both the CYP3A5*3 and CYP3A4*22 genetic variants in 620 cancer pain patients from the EPOS study on transdermal fentanyl (12.5–700 mg/h) to define any differences in serum fentanyl and norfentanyl concentration and the metabolic concentration ratio (MR).[50] The results demonstrated a high variation in the delivery rate–adjusted serum fentanyl concentrations and MRs, with overall 43% of the serum fentanyl concentration variability being attributed to delivery rate. Both the CYP3A5 and CYP3A4 polymorphisms influenced the norfentanyl:fentanyl MR and serum norfentanyl concentrations, but their effect on variability was less than 2% . [49]

ABCB1 Polymorphisms

Takashina et al looked at the influence of the ABCB1 polymorphisms on the response and toxicity to transdermal fentanyl in Japanese chronic malignant pain patients. In terms of efficacy, homozygous were significantly associated with reduced breakthrough rescue medication requirements. No other significant associations were identified between any of the genotypes and response to fentanyl or fentanyl induced adverse effects [48]

Barrat [50]explored whether genetic variability in immune activation and inflammatory signaling pathway:sSerum fentanyl concentrations were not associated with any of the aforementioned outcomes. This study also confirmed previous literature that the stat6 rs167769 variant, a cytokine- and growth factor–responsive transcription activator, is able to predict the required fentanyl dose [51], but that dose alone cannot determine the interpatient variability in pain intensity [50].

OPRM1 Polymorphisms

Numerous studies have explored the association between OPRM1 polymorphisms and response to morphine in chronic cancer pain patients. One of the earlier studies analyzed OPRM1 polymorphisms in a Caucasian population and concluded that there were no significant differences between polymorphisms and required morphine dose, serum concentration of morphine, or its metabolites . This was supported by a later study , which also found no relationship between OPRM1 genotypes concluding that are similar in morphine responders and nonresponders [52] Interestingly, in the earlier study the authors showed [53] that homozygous required higher doses of morphine to achieve adequate pain control but the authors were unable to explain this difference by examining other factors such as duration of morphine treatment, time since diagnosis, and adverse symptoms [54] The OPRM1 A80G polymorphism was analyzed

in 45 cancer pain patients of Italian descent taking oral morphine, homozygotes for the wild-type allele experienced greater pain relief as compared with the homozygous genotype with no significant difference in the heterozygotes. Overall OPRM1 is an independent and strong predictor of analgesic response to morphine . Two studies have looked at the OPRM1 polymorphism in Japanese cancer pain patients taking oral morphine, one reported that OPRM1 was not associated with any morphine-related adverse effects , and the other concluded that there was no correlation between response to morphine [55]

COMT Polymorphisms

The COMT polymorphism has been shown to result in a three- to four-fold variation in enzymatic activity in fact several studies have examined the association between this variant on morphine dosing requirements, response, and adverse effects in cancer pain patient. In terms of morphine response, one study analyzed the polymorphism in a cohort of Norwegian cancer pain patients and concluded that carriers of the homozygous had higher morphine 24-hour dose requirements. The difference in dosing requirements could not be explained by other factors [55–57]. A more recent study in a 41 Japanese cancer pain cohort determined that the homozygous COMT genotype correlated with both a statistically significant lower plasma concentration of morphine and requirements for a lower morphine dose[55]. However, the relationships between pharmacogenomics and parameters to opioids genotype and morphine daily dose and plasma concentration were not significant after 1 week [55].

UGT Polymorphisms

Numerous studies have reviewed the impact of UGT2B7 polymorphisms on morphine glucuronidation and plasma concentration of morphine to its metabolites[58] A Norvegian study analyzed the UGT2B7 polymorphism in 70 cancer pain patients taking slow release morphine. There was large variation in the metabolite-to-morphine concentration ratios among individuals, and no statistically significant

differences were observed between the UGT2B7 genotypes Furthermore, no significant differences were observed in metabolite-to-morphine concentration ratios when comparing UGT1A1homozygotes with either allele heterozygotes or homozygous wild-type. This highlights the minor clinical significance of the UGT1A1 allele in influencing the rate of metabolism of morphine [59–60].
Polymorphisms in UGT2B7 in both the coding and regulatory region were further studied in a cohort of 175 Norwegian cancer patients on long-term morphine therapy. The study identified 12 but there was no evidence that these polymorphisms affected UGT2B7 activity. Overall, no association was found between both UGT2B7 genotype or haplotype and the ratio of serum morphine to morphine glucuronides..
This study concluded that the variability in morphine and its metabolite concentrations is mainly attributable to other factors [60] Additional UGT2B7 polymorphisms were analyzed both in vitro and in vivo in the Norwegian, it was concluded that variation in UGT2B7 has a clinically insignificant effect on morphine metabolism in cancer pain patients [62].
A further study on 162 Caucasian cancer patients established that the genotypes are not significantly associated with patients who respond to morphine as opposed to non-responders and that there was no relationship between genotype and the serum concentrations of morphine or its glucuronide metabolites [58]. A more recent analysis on a larger cohort of 759 Caucasian cancer pain patients from the EPOS study [51]identified two haplotypes that had weak associations with lower morphine glucuronide to morphine ratios after oral administration of morphine By contrast, polymorphisms in UGT2B7 have been significantly associated with morphine-induced adverse drug events in a group of Japanese cancer patients taking oral controlled-release morphine [63].

CYP2D6 Polymorphisms

Two studies have assessed the correlation between CYP2D6 genotype and the serum concentration of oxycodone and its metabolites (oxymorphone, noroxycodone,

noroxymorphone), and response and adverse effects in cancer pain patients. [64–65]One study investigated the CYP2D6 polymorphisms in a Japanese cohort taking extended-release oxycodone and found that there was no significant association between genotype and oxycodone trough plasma concentrations..

A cross-sectional study of 450 Caucasian patients from the EPOS study [51]measured the CYP2D6 phenotype frequency within the study population :results support that a reduction in CYP2D6 enzyme activity reduces the conversion of oxycodone to its CYP2D6-specific metabolite oxymorphone. Overall, there were no significant differences between each phenotypes and the dose required for analgesia, pain scores, or side effects of nausea, tiredness, or negative impacts on cognitive function [64].

METHODS

This is a biological interventional prospective, single-center study.

Patients were enrolled on IRCCS Istituto per la cura dei Tumori Dino Amadori from september 2018 to September 2021.The primary objective of the study was to evaluate the pharmacokinetic and pharmacogenetic of transdermal fentanyl in relation to the patient's clinical response (defined as reduction in NRS equal or greater to 2 points after 72 hours of treatment).

Secondary objective were:

• Individualization of fentanyl prescription in relation to individual genetic polymorphisms.

• Evaluation of the clinical response in terms of adverse effects related to the presence of genetic polymorphisms

• Evaluation of compatibility with concurrent inducers or inhibitors of CYP3A4

• Evaluation of possible pharmacological interactions and their effect on kinetics and clinical response.

• Identification of good responders and poor responders in relation to fentanyl kinetic and patient genetic

Study Population

Our study comprised 49 patients with solid or haematologic cancer with chronic oncologic pain who received treatment with any type of transdermal fentanyl at any dose available in the department, treated according to clinical practice.[65] . The number of patients was initially estimated at 100, however due to difficulties in enrolling it was decided to reduce it to 49.

All subject included in the study signed a written informed consent form that allowed both clinical trial and pharmacogenetic and kinetic studies.
They were free to withdraw from the study at any time.
The protocol fulfilled Italian law and biomedical research and was approved by our Ethical committee.
Inclusion criteria were as follows: age > 18 yrs with solid tumors or hematological diagnosis using transdermal fentanyl for chronic pain free from any psychiatric conditions with adequate hepatic function, able to communicate their feelings regarding the modification of pain over time.
Exclusion Criteria were as follows: pregnant or breastfeeding women, individuals with history of substance abuse or potus, allergy to study drugs or use of concomitant drugs with more interactions with fentanyl.

Study Design And Procedures

We analysed the data of 49 pts admitted to our ward in IRCCS Istituto per la cura dei Tumori Dino Amadori, using transdermal fentanyl. The analysis of fentanyl kinetics in blood were performed at the department of Life Quality studies, Rimini Campus, University of Bologna.
Blood samples (3 ml lilac sample tubes – 2 tubes only the first time) were collected from the patients included in the protocol at fixed times:
- T0 = corresponding to the period of changing patch

- T1 = 6 h from the application of FT
- T2 = 18 h from the application of FT
- T3 = 48 h from the application of FT
- T4 = 72 h from the application of FT.

At T0 and T4 was evaluate Numeric rating scale and at each blood sample collection was administrated the Brief Pain Inventory (BPI) questionnaire, to evaluate the characteristics and evolution of pain. All clinical data that was collected for this study was retrieved from the patient's medical records and from the Brief Pain Inventory questionnaires, and was treated with complete respect for confidentiality and privacy conditions, according to the current rules in terms of respect for privacy.

Clinical data were collected in our Case Report Forms and contained patient demographic data, type of primary tumor and therapy, BMI (Body Mass Index), performance Status (evaluated with Eastern Cooperative Oncology Group) , presence of hydration , hyperpyrexia , drugs, dosage and start of treatment with fentanyl, adverse events, use of opioid for breakthrough cancer pain.

Pharmacokinetic analyses

A fast and efficient blood sample preparation and a GC-MS (Gas chromatography–mass spectrometry)analytical procedure carried out in SIM (Single Ion Monitoring mode using fentanyl-D5 (FD5) as an internal standard, were developed and validated for the quantitative determination of fentanyl (F) in the whole blood samples of cancer patients.

A fast fentanyl liquid-liquid extraction (LLE) procedure was optimized as an efficient blood sample preparation procedure. The developed GC-MS method was validated in terms of selectivity, linearity, sensitivity, accuracy and recovery. Then, the validated method was applied to the analysis of cancer patient blood samples to determine the pharmacokinetic parameters (AUC, Cmax and Tmax).

The area under the concentration versus time curve, from administration time to the last blood draw time, was calculated using the linear trapezoidal rule up to Cmax and subsequently the trapezoidal rule for the remainder of the curve.

Gas Chromatography-Mass Spectrometry (GC-MS) Method

The chromatographic method was optimized with an Agilent Gas-Chromatograph coupled to a single quadrupole selective mass detector (Agilent 7820A GC System, Agilent 5977E MSD) in electron ionization (EI) mode (70 eV) under a temperature gradient elution using a HP5MSUI (5%-phenyl)-methylpolysiloxane (30 m × 0.25 mm × 0.25 μm, 19091S-433UI) Agilent column. The gas carrier was helium with a flow rate of 1.5 mL min-1. An aliquot of 1 μL of the pre-treated sample was injected in splittless mode. The MS source temperature was set at 250 °C, the MS quad temperature was adjusted to 150 °C, the AUX 1 temperature was fixed at 250 °C and the Front Inlet temperature at 250 °C. The GC oven temperature program started at 150 °C with hold time of 1 min. The temperature was increased to 240 °C by a linear gradient rate of 50 °C min-1, then to 285 °C by a rate of 10 °C min-1 and hold for 2 min, finally it was increased to 300 °C by a rate of 10 °C min-1 and it was hold for 3 min. The analyses were carried out in SIM mode. According to the signal intensity, the 245 m/Q and 250 m/Q ions were selected for fentanyl and fentanyl-D5 monitoring, respectively. The total run time was of 13.58 min and the retention time of fentanyl was found to be in the range 7.65 - 7.75 min. The method showed a good selectivity since the absence of any coeluting interference was proved by injecting solutions obtained after LLE of both blank and fentanyl and fentanyl-D5 spiked blood. Data were acquired with MassHunter GC/MS Acquisition B.07.00, 2013 and processed with MassHunter Workstation Software Qualitative Analysis B.06.00, 2012.

The analytical method was validated in terms of specificity, linearity, sensitivity, precision, accuracy and recovery.

The method specificity was determined by using three human blank blood samples and comparing the chromatograms obtained after injecting the non-spiked and spiked samples respectively. Moreover, each sample analysis was followed by a double solvent injection. The absence of any signal at fentanyl retention time has demonstrated that there was no carry-over effect.

A preliminary calibration curve, y= (1.77E-02±5.7735E-05)x + (2.83E-03±8.50E-04), was determined by analyzing ten fentanyl standard solutions diluted in methanol at the concentration range 2 to 55 ng mL-1, each containing a fixed concentration of fentanyl-D5 of 50 ng mL-1. Linearity with good correlation coefficient (R2=0.9998 ± 1.15E-04) was obtained. Linearity was also determined by analysing five blood samples spiked with fentanyl and fentanyl-D5 standard solutions (concentrations range 2 to 75 ng mL-1) and 50 ng mL-1, respectively. The enriched samples were subjected to the LLE procedure and subsequently analysed by GC-MS, (y=1.78E-02 ± 1.43E-04)x (2E-16±1.10E-17), R2= 0.9999 ± 0.001).

The limit of detection (LoD=3*SE/m) and limit of quantitation (LoQ=10*SE/m) values, were obtained by a statistical evaluation, considering the standard signal deviations. In particular, LoD was calculated multiplying the standard error (SE) of the calibration curve, of spiked blood sample solutions, for a factor of three divided for the slope of the curve (LoD= 3*SE/m). The SE was obtained from a regression analysis of the calibration curve. LoQ was calculated multiplying the standard error (SE) of the same calibration curve for a factor of ten divided for the slope of the curve (LoQ= 10*SE/m). LoD and LoQ were found to be 5.6E-02 ± 3.5E-02ng mL-1 and 1.86E-01 ± 1.18E-01 ng mL-1 respectively.

Recovery determination was carried out on blank blood samples from two different volunteers spiked with three incremental concentrations of fentanyl (10, 25, 50 ng mL-1) and a fixed concentration of fentanyl-D5 (50 ng mL-1). The recovery values were obtained by the following formula:

Eq. 1 % Recovery = [(Peak Area ratio of F/FD5 pre-spiked blank blood sample solution) / (Peak Area ratio F/FD5 of standard solution)] X 100

The mean recovery value, determined at three fentanyl spiked concentrations level (10, 25, 50 ng mL-1 of fentanyl and of a fixed concentration of 50 ng mL-1 of fentanyl D5), resulted to be 99.02 ± 9.39E-01 %, confirming the higher efficiency of this extractive method.

The intra- and inter-day precision was evaluated by analysing spiked blood sample at low (8 ng mL-1) medium (30 ng mL-1) and high (50 ng mL-1) fentanyl concentrations, each containing fentanyl-D5 at a fixed concentration of 50 ng mL-1. Spiked blood samples were extracted twice daily. Each final solution was injected into the GC-MS five times. The same GC-MS analysis were carried out on different days (n = 10).

The determination of accuracy and the intra-day and inter-day precision of the method were carried out on the same samples. The variation coefficient for intra- and inter-day assays demonstrated an average value of 1.95 ± 7.97E-01% and 1.20 ± 6.32E-01% respectively.

Accuracy, found to be more than 99%, was determined at three different fentanyl concentration levels by calculating the percentage of the deviation between the experimental concentrations of fentanyl obtained from blood analysis and the nominal ones.

The developed chromatographic method was applied to the determination of fentanyl in human blood.

The unknown fentanyl concentration was calculated by fentanyl spiked blank blood samples calibration curve.

Sample preparation

Since the blood samples were provided frozen, the optimal condition to achieve the highest fentanyl recovery was found to be LLE extraction from the whole blood. The

general procedure consisted in adding 500 µL of 0.5 M carbonate buffer (pH = 11.00) and 5.0 µL of fentanyl-D5 to 500 µL of the defrosted blood sample. The final concentration of the internal standard (IS) was equal to 50 ng mL-1. 2.5 mL of diethyl ether were added to the solution. The sample was vortexed three times at 5 second intervals and centrifuged (Thermo Scientific CL10 centrifuge) at 1500 rpm for 5 minutes at 4 °C. The solution was frozen at -80 °C for 2 hours. The supernatant was collected and evaporated under a nitrogen stream. Finally, an extracted product was dried and dissolved in 100 µL of methanol. The solution was injected and analysed by GC-MS. The selected conditions allowed to obtain an average recovery value of 99.02 ± 9.39E-01 %. Two independent LLE were performed on each blood sample of cancer patients collected at the described increasing times after the application of the transdermal patch. Fentanyl concentration in blood samples was calculated by interpolating F/F-D5 peak area ratio in fentanyl calibration curve obtained with spiked blood samples.

Data analysis

Pharmacocynetics

Data were analysed in terms of fentanyl concentration for each blood sample, then the approximate area under the curve (AUC) of each patient was calculated following the trapezoidal rule considering the formula reported below (Equation 2).

Eq. 2 AUC= [(concT0+concT1)* $(\Delta \boldsymbol{t1})/\boldsymbol{2}$]+ [(concT1+concT2)* $(\Delta \boldsymbol{t2})/\boldsymbol{2}$]+ [(concT2+concT3)* $(\Delta \boldsymbol{t3})/\boldsymbol{2}$]+ [(concT3+concT4)* $(\Delta \boldsymbol{t4})/\boldsymbol{2}$]

Eq. 2 concT is the concentration at a determined collection time; Δt is the time difference in hours between two subsequent collection times.

The maximum concentration level of fentanyl (Cmax) and its interval (Tmax) were obtained.

Genotyping

Genomic DNA was extracted from peripheral blood samples using the Maxwell® RSC Whole Blood DNA Kit and Maxwell MDx Instrument (Promega). Sample genotyping was carried out by means of Axiom PharmacoFocus Assay (Thermo Fisher Scientific), which provides information on 2000 variants in 150 genes and variants in regions of high homology to pseudo-genes (such as cytochrome P450 (CYP) genes), according to the manufacturer's recommendations. Data were analyzed by Axiom Analysis Suite software (Thermo Fisher Scientific). Normalizers are:

- for most genes: UM,RM,NM,IM,PM (ultrarapid,rapid,normal,intermediate,poor metabolizer) NM
- for transporters and MTRNR1: IF,NF,DF,PF (increased,normal,decreased,poor function) NF
- for CACNA1S and RYR1: Indeterminate,MHS (Malignant Hyperthermia susceptibility)
- for G6PD: Indeterminate,Deficient (higher risk for hemolysis)
- for IFNL3: FavorableResponseGenotype,UnfavorableResponseGenotype (for PEG-IFN-alpha containing regimens to treat hepatitis C virus)
- for NAT family: RA,IA,SA,Off (rapid,intermediate,slow,off acetylator) IA
- for VKORC1 and CYP2CRS12777823: Resistant++,Resistant+,Normal,Sensitive-,Sensitive—NORMAL

Statistical analysis To evaluate the relationship between pharmacokinetics, pharmacogenetics and clinical features with clinical response we performed association tests.

Chi-squared test or Fisher's exact were used to test the association between categorical variables, while Mann-Whitney U test or Kruskal-Wallis test were used to test the association between categorical and continuous variables. The same association tests, as appropriate, were used to correlate variables related with clinical response in order to further investigate the independent association of pharmacokinetics, pharmacogenetics and clinical features with clinical response. Multivariate analysis (principal component analysis, PCA) was performed with the effort of the SIMCA17, 17.0.2.34594, Sartorius Stedim Data Analytics AB in ordert to visualize cluster of patients.To explore how genetics influence fentanyl prescription we performed association tests comparing dosage and polymorphisms. To evaluate the relationship between genetics and side effects and between side effects and clinical response we again performed association tests.

The risk-ratio (RR) and corresponding 95% CI (bootstrap method) were reported to compare categories in case of enough evidence of association. Multiple post-hoc test were performed with exploratory purpose.

All statistical analyses were performed using STATA 15.0 statistical software (StataCorp, College Station, TX, USA).

RESULTS

Demographic characteristics

Our study population comprised 49 pts (26 male and 23 female), median age of 65 yrs old, with 98% of stage IV tumour .Performance status was respectively 1 in 22% of pts, 2 in 41% and 3 in 37%. Median weight was 65 kg and 63% exhibited a BMI values in normal range, 16% was overweight,6 % was underweight and 8 % was obese.

Type of tumour for the majority were gastrointestinal cancer (30%) , multiple myeloma(10%) and lung tumour (10%).

Fentanyl patch was administered at various dosage, 16% of cases at 12 mcg/h, 31% at 25 mcg/h, 29% at 50 mcg/h, 12% at 75%, and 12% >100 mcg/h.

The majority of participants to the study (65%) didn't experienced side effects from fentanyl administration,a little part of them experienced constipation and drowsiness.

Demographic characteristics are shown in Table 1.

Table 1. Demographic characteristics

	Overall population (n=49)
Sex- no. (%)	
Female	23 (47%)
Male	26 (53%)
Age at first treatment — yr	
Median	65
Range	(32-83)
Weight – Kg	
Median	65
Range	(36-120)
BMI– no. (%)	
Underweight (<18.5)	6 (12%)
Normal range (18.5-)	31 (63%)
Overweight	8 (16%)
Obese	4 (8%)
ECOG performace status – no. (%)	
1	11 (22%)
2	20 (41%)
3	18 (37%)
Stage– no. (%)	
III	1 (2%)
IV	48 (98%)
Tumor – no. (%)	1 (2%)
Anal	3 (6%)
Colon	2 (4%)
Esophageal	1 (2%)
Liver	1 (2%)
Tongue	1 (2%)
Hodgkin lymphoma	2 (4%)
Breast	2 (4%)
Myeloma	6 (12%)
Multiple myeloma	1 (2%)
Oropharyngeal	2 (4%)
Ovarian	5 (10%)
Pancreas	5 (10%)
Lung	1 (2%)
Prostate	2 (4%)
Kidney	2 (4%)
Nasopharyngeal	1 (2%)
Sarcoma	3 (6%)
Stomach	2 (4%)
Testicular	1 (2%)
Tonsil	1 (2%)

Urothelial	1 (2%)
Womb	1 (2%)
Bladder	2 (4%)
Bile duct	
Fentanyl dosage µg/h– no. (%)	
12	8 (16%)
25	15 (31%)
50	14 (29%)
75	6 (12%)
100	4 (8%)
150	2 (4%)
Side effects – no %	
Yes	16 (35%)
No	30 (65%)
Missing	3

Patients are classified responders if NRS T4- NRS T0≥2 or NRS T4 =0, otherwise they are classified non-responders. Among the demographic and clinical variables, sex was the only variable with enough evidence of different distribution between responders and non-responders (p=0.05). In particular males had greater chance of being responders rather than females (RR=1.36, 95% CI 1.002-2.01) as showed in Table 2.

Table 2. Demographic characteristics and responders

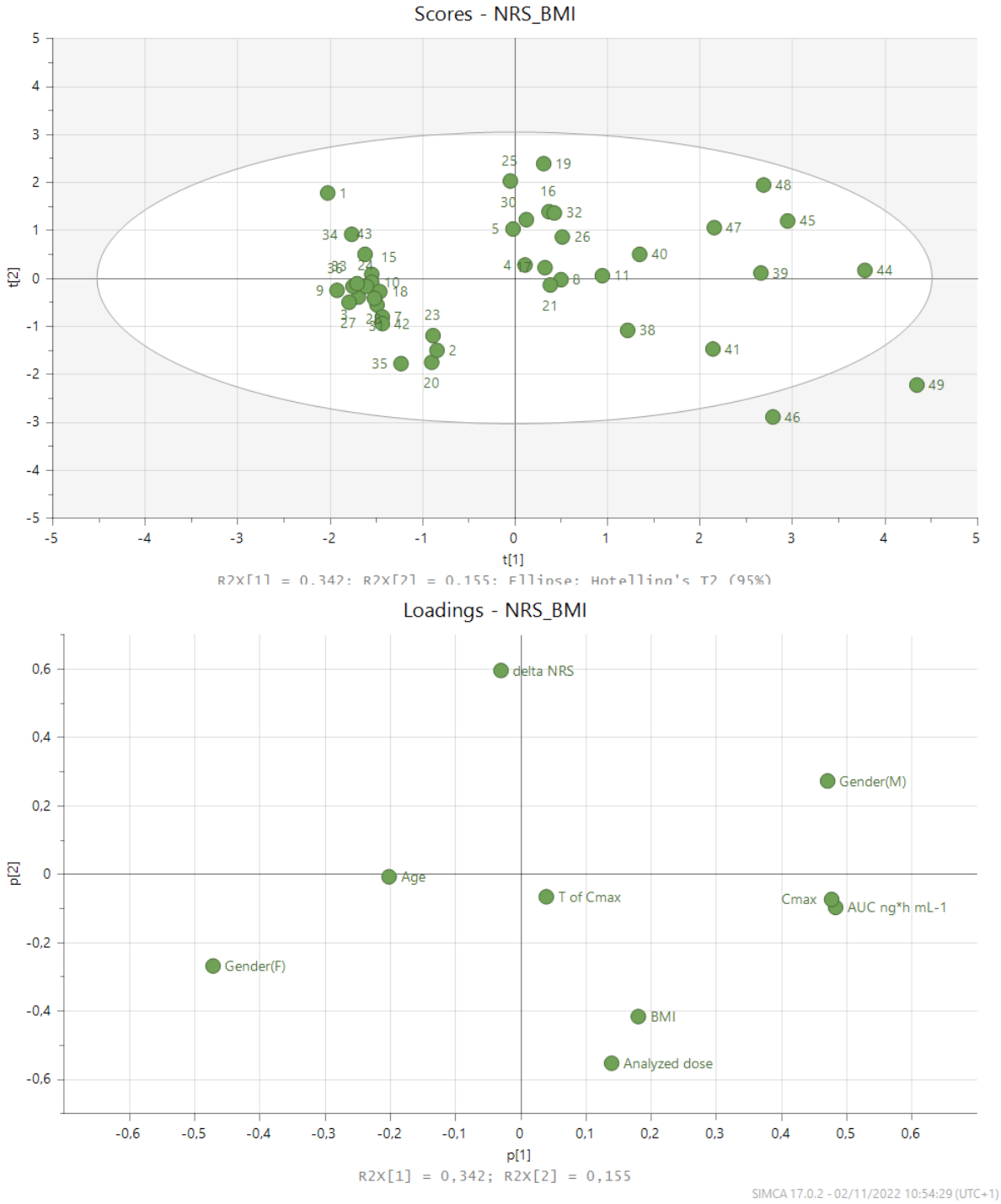
	Responders (n=38)	**Non responders (n=11)**	***p-value***
Sex- no. (%)			
Female	15 (39%)	8 (74%)	
Male	23 (61%)	3 (26%)	**0.05**
Age at first treatment — yr			
Median	64	67	
Range	(32-83)	(57-82)	0.32
BMI– no. (%)			
Underweight (<18.5)	3 (8%)	3 (26%)	
Normal range (18.5-24.9)	25 (66%)	6 (56%)	
Overweight (25-29.9)	6 (16%)	2 (18%)	
Obese (≥30)	4 (10%)	0	0.26
ECOG performace status – no. (%)			
1	9 (24%)	2 (18%)	
2	15 (39%)	5 (45%)	
3	14 (37%)	4 (36%)	0.91
Fentanyl dosage µg/h– no. (%)			
12	5 (13%)	3 (26%)	
25	13 (34%)	2 (18%)	
50	10 (26%)	4 (36%)	
75	5 (13%)	1 (10%)	
100	3 (8%)	1 (10%)	
150	2 (6%)	0	0.74
Side effects – no (%)			
Yes	12 (34%)	4 (36%)	
No	23 (66%)	7 (64%)	
Missing	3	0	0.90

Pharmacokinetic analysis

Subsequently w will describe correlation between clinical response and pharmacokinetic analysis and biometric characteristicside and concomitant treatment with inducer/inhibitor of CYP3A4. We used traditional principal component analysis (PCA) to describe this correlations.

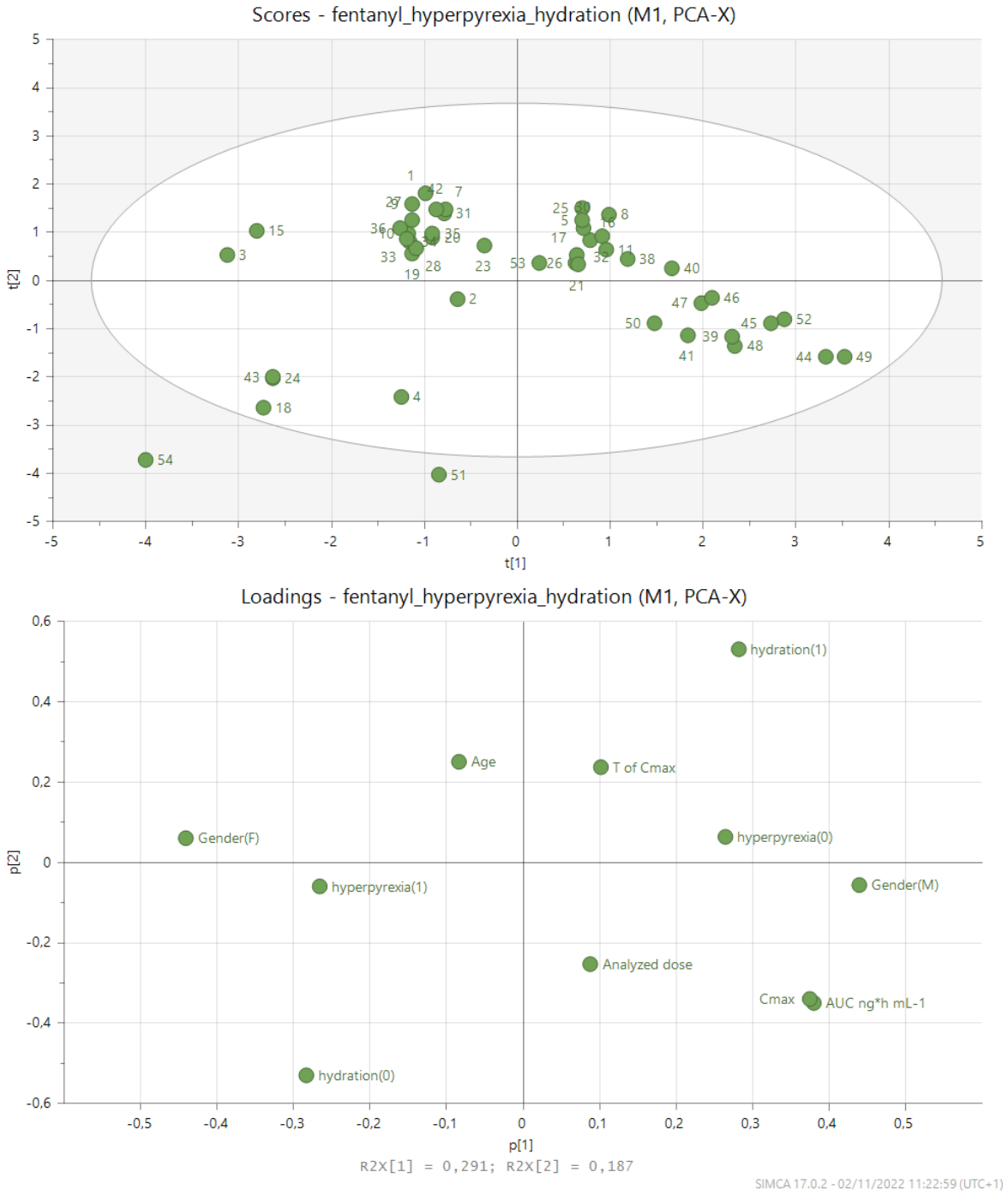
In Fig. 1 we correlated pts in base of their biometric characteristics (age, gender, BMI), dose of the patch, and NRS variation from T0 to T4 and pharmacokinetic. Delta NRS was calculated with the following formula: delta NRS= NRS T4 - NRS T0. Data obtained do not reveal detectable correlations intra-patients. Among the eight major contributors to discrimination, it is not possible to notice correlations between the clinical response (delta NRS) and Cmax, AUC, and T of Cmax. Meanwhile, a positive correlation exists between male gender and Cmax and AUC and a negative one between clinical response (delta NRS) and the drug dose.

Fig.1 PCA biometric characteristic-dose-NRS -kinetic



The traditional principal component analysis (PCA) in Fig. 2 correlates patients in base of biometric characteristic (age, gender), patch dose, hyperpyrexia,administered hydration and pharmacokinetic results collected. Data obtained do not show correlations intra-patients. A positive correlation is detected between hydration and T of C_{max}, and a negative one between hyperpyrexia and T of C_{max}. Moreover, no correlation is appreciable between hydration, hyperpyrexia and the other pharmacokinetic parameters (AUC and C_{max}).

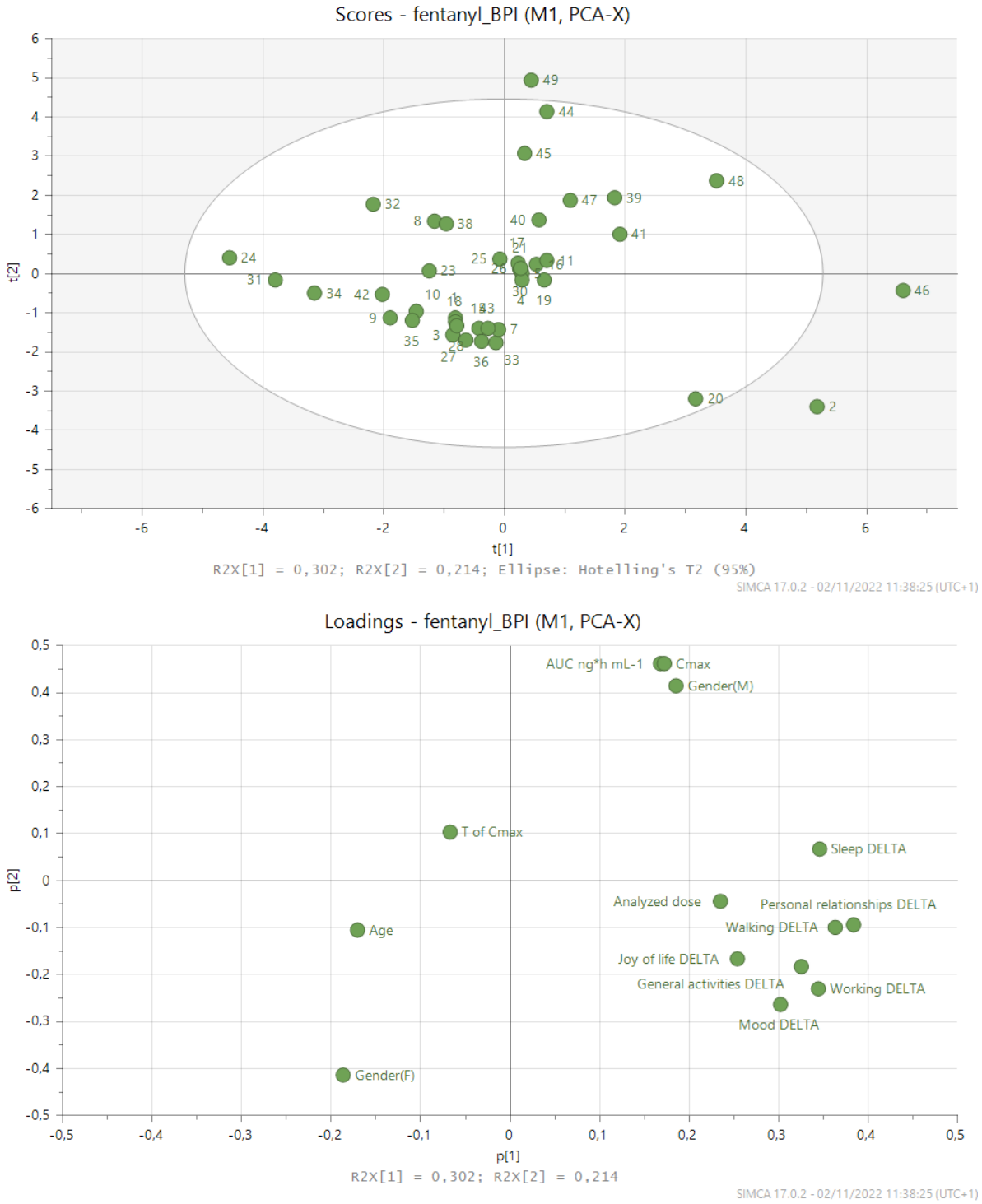
Fig.2 PCA biometric characteristic-dose-hyperpyrexiaa-hydration-kinetic



Correlation between biometric characteristics (age, gender), patch dose, variation in BPI data from T0 to T4 (calculated as: delta BPI= T4 - T0) and pharmacokinetic results in Fig.3 revealed no detectable correlations intra-patients. Among the thirteen

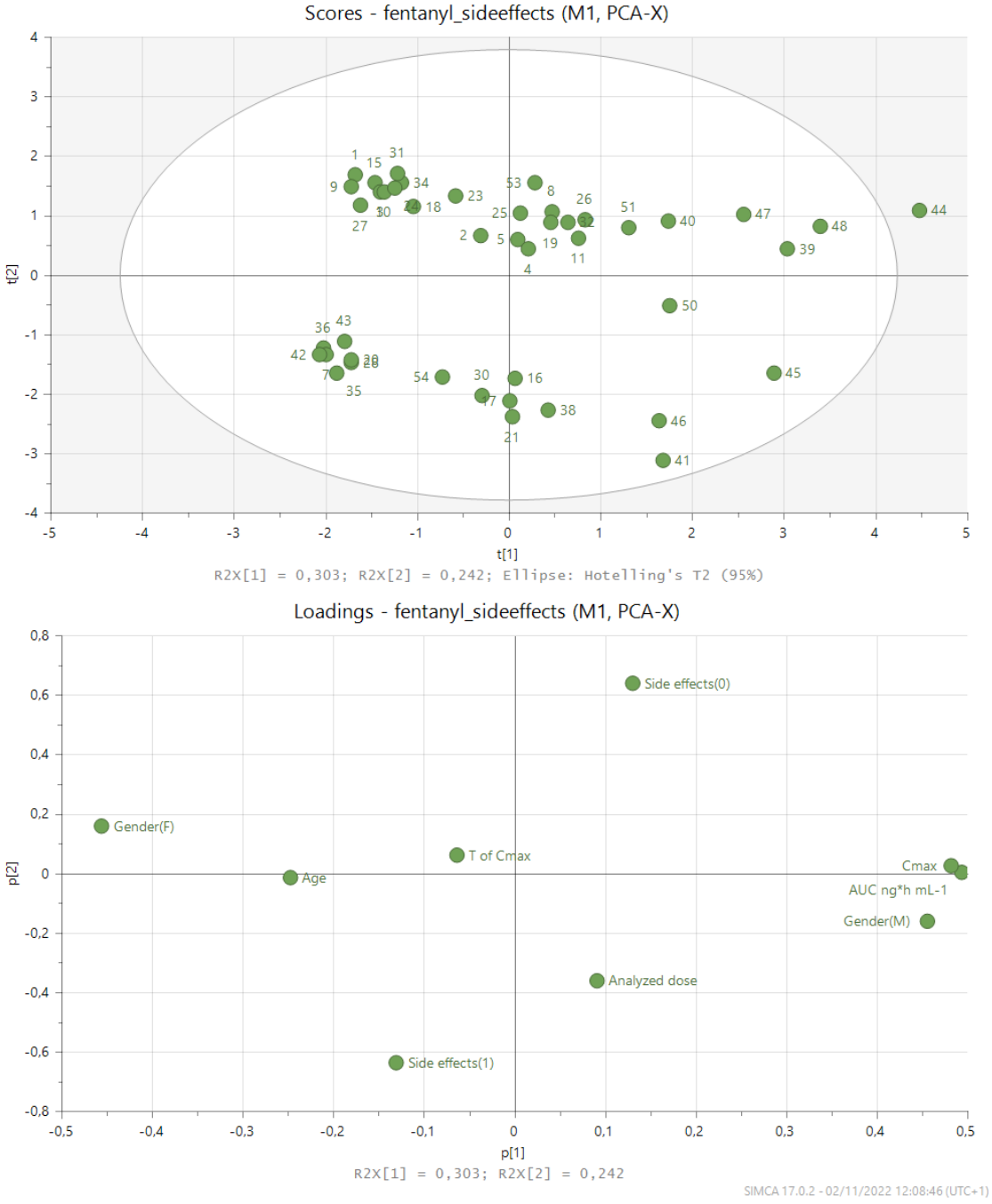
major contributors to discrimination, a positive correlation seems be determined between variation in BPI and the patch dose, and a negative correlation with T of C_{max}. Moreover, no correlation is appreciable with the other pharmacokinetic parameters (AUC and C_{max}), and gender.

Fig.3 PCA biometric characteristic-dose-BPI



In Fig. 4 we correlates patients in base of biometric characteristic (age, gender), patch dose, side effects and pharmacokinetic results collected. Data obtained do not show detectable correlations intra-patients. Among the seven major contributors to discrimination, any correlations are not present between side effects and AUC and Cmax and T of C_{max}.

Fig.4 PCA biometric characteristic- dose- side effects- pharmacokinetic



PCA in Fig. 5 correlates patients in base of biometric characteristic (age, gender), patch dose, variation in delta BPI and in delta NRS data from T0 to T4, and pharmacokinetic results collected. Data obtained do not show detectable correlations intra-patients. Among the fourteen major contributors to discrimination, a positive correlation between variation in delta BPI and in delta NRS and dose of the patch and a respectively a negative one of all these variables with T of $C_{max,}$ can be determined. No correlation exists between the clinical response and AUC and C_{max}.

Fig.5 PCA biometric characteristic- dose- delta BPI- delta NRS- pharmacokinetic

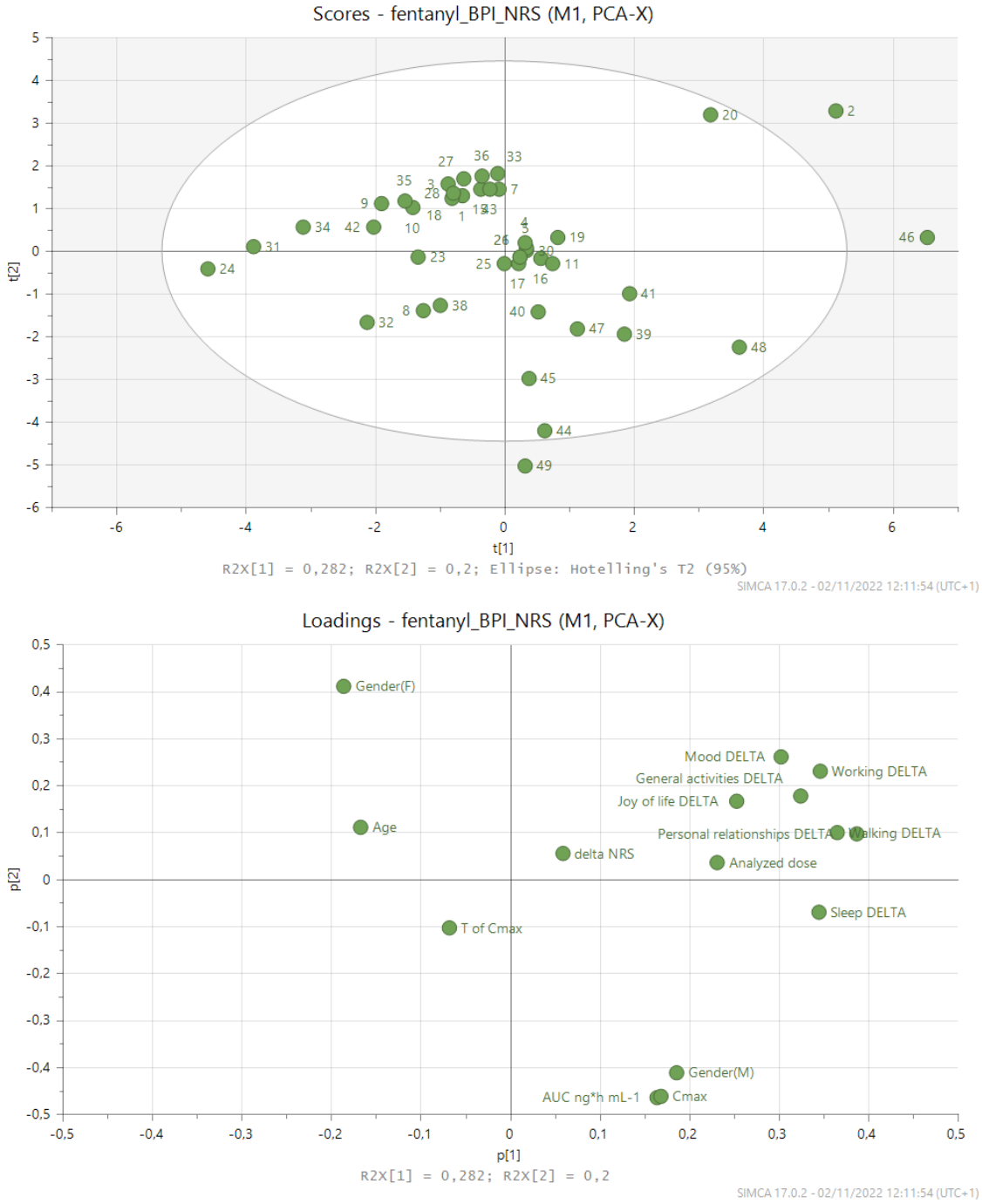
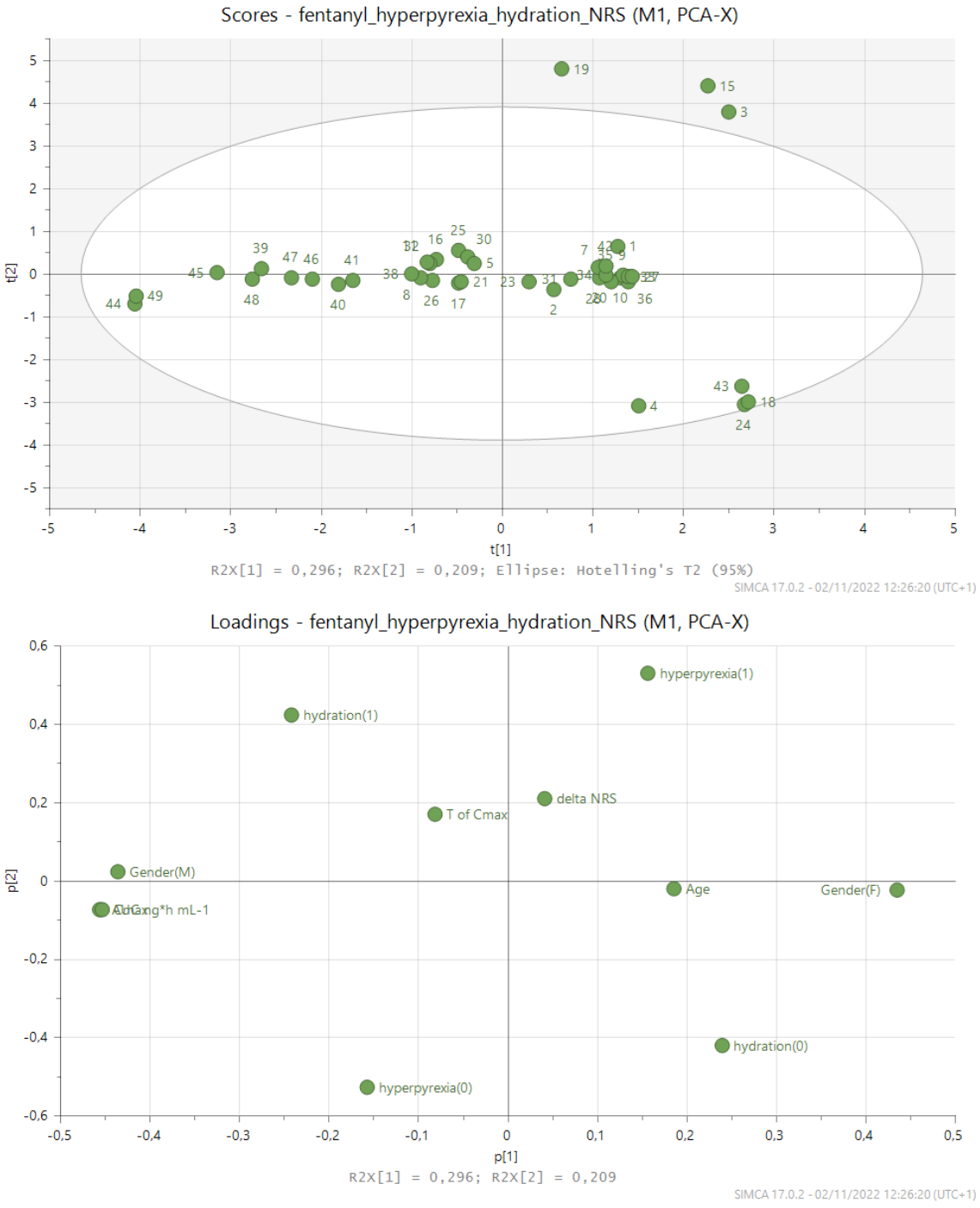


Fig. 6 correlates patients in base of biometric characteristic (age, gender), variation in delta NRS data from T0 to T4, hyperpyrexia, hydration and pharmacokinetic results collected. Data obtained do not allow to determine correlations intra-patients. Among

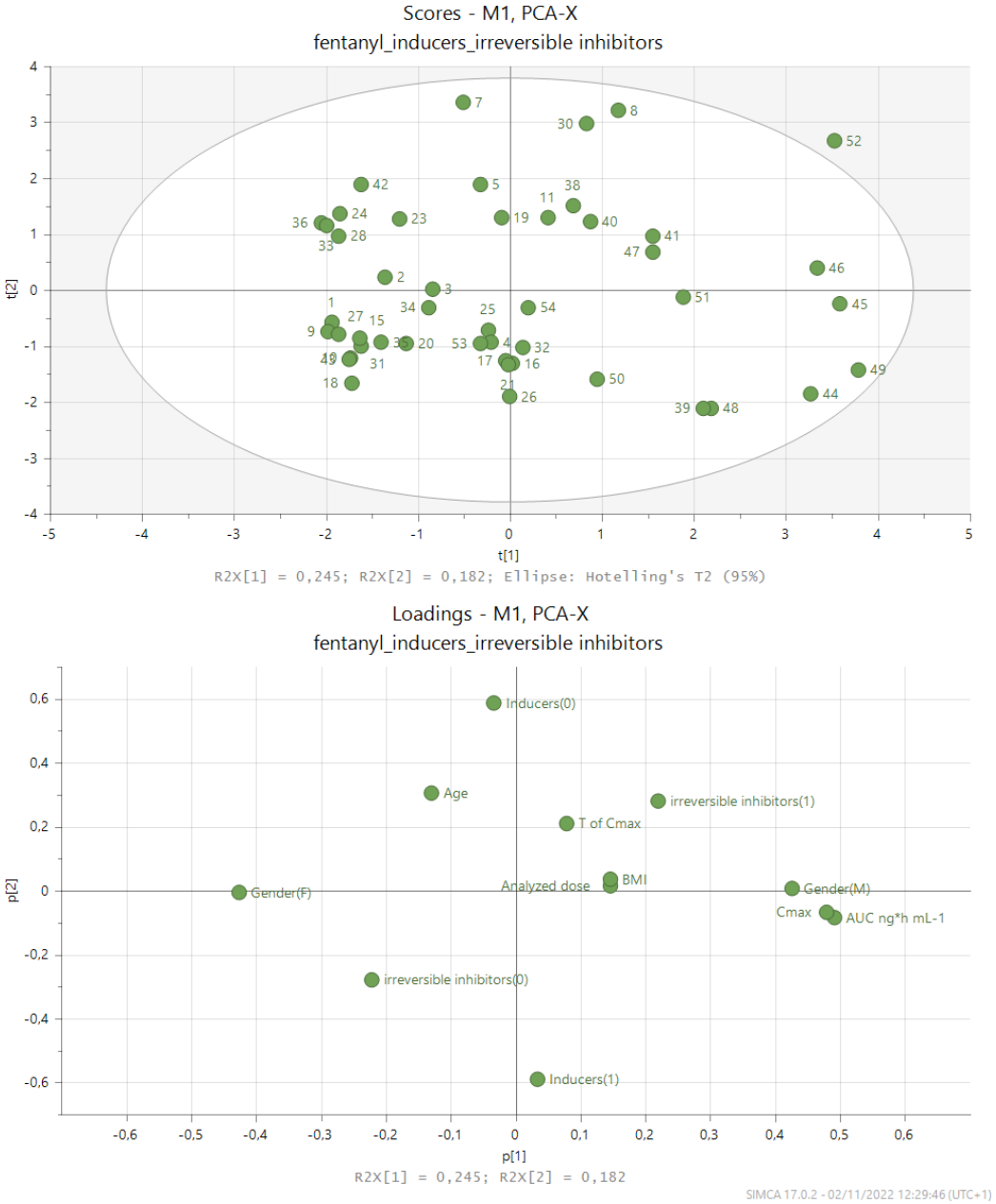
the eight major contributors to discrimination, variation of delta NRS correlate positively with hydration, T of C_{max} and hyperpyrexia, negatively with AUC and C_{max} parameters.

Fig.6 PCA biometric characteristic- delta NRS- pharmacokinetic-hyperpyrexia-hydration-kinetic



The traditional principal component analysis (PCA) in Fig. 7 correlates patients in base of biometric characteristic (age, gender, BMI), patch dose, inducer drugs and inhibitor drugs taken, and finally pharmacokinetic results collected. Data obtained do not allow to determine correlations intra-patients. Among the nine major contributors to discrimination, T of C_{max} correlates positively with BMI, patch dose, inducer drugs and male gender.

Fig.7 PCA biometric characteristic- dose- inducer drugs- inhibitor drugs-kinetic



Correlation between dosage and polymorphism analyses

As shown in Table 3, polymorphisms of NAT2 and CYP4F2 show some evidence of association with dosage (p=0.06 for both). In particular, rapid acetylator phenotype of NAT2 seems to have greater chance of receiving high dosage of fentanyl (dosage > 50 µg/h) rather than intermediate acetylator phenotype (RR 5.25, 95% CI 1.4-NA), while rapid metabolizer phenotypes of CYP4F2 shows greater chance of receiving high dosage of fentanyl rather than normal phenotype (RR 4.5, 95% CI 2.25-18).

Table 3. Association between polymorphism and dosage

Gene		Low dosage (n=11)	High dosage (n=38)	p-value
CYP4F2	IM	16	6	
	NM	14	4	
	PM	7	0	
	RM	0	2	**0.06**
NAT2	IA	12	2	
	RA	1	3	
	SA	15	4	**0.06**

Correlation between pharmacokinetics and polymorphism analyses

Metabolic phenotypes driven by *NAT2* or *UGT2B7* alleles were associated with AUC and C_{max} kynetics parameters (*NAT2*: p=0.02 and p=0.01, respectively; *UGT2B7* p=0.008 and p=0.005, respectively). Ultra-rapid metabolizer phenotypes according to *UGT2B7* genetic variation showed greater AUC and C_{max} compared with those carrying allelic variants associated with normal and rapid phenotypes (median AUC µg·h/ml= 925.32 vs 78.18 and 164.78 , respectively; median C_{max} µg/h=15.25 vs 1.51 and 4.23, respectively). Rapid metabolizer phenotypes according to *NAT2* polymorphisms showed greater AUC and C_{max} compared with those carrying allelic variants associated with intermediate and slow metabolizer phenotypes (median AUC µg·h/ml= 1083.64 vs 210.14 and 59.14 , respectively; median C_{max} µg/h=26.34 vs 4.80 and 1.43, respectively). No significant association was observed between T_{max} and genetic polymorphisms. Table 4-5 and Fig 8

Table 4.Correlation between polymorphism and AUC

Gene		AUC [range]	p-value
NAT2	Unknown		
	IA	210.14 [11.94-2578.79]	
	RA	1083.64 [540.24-1828.462]	
	SA	59.14 [9.11-1083.95]	**0.02**
UGT2B7	Unknown		
	NM	78.18 [9.11-1475.54]	
	RM	164.78 [17.39-2377.13]	
	UM	925.33 [199.88-2587.79]	**0.008**

Fig.8 AUC

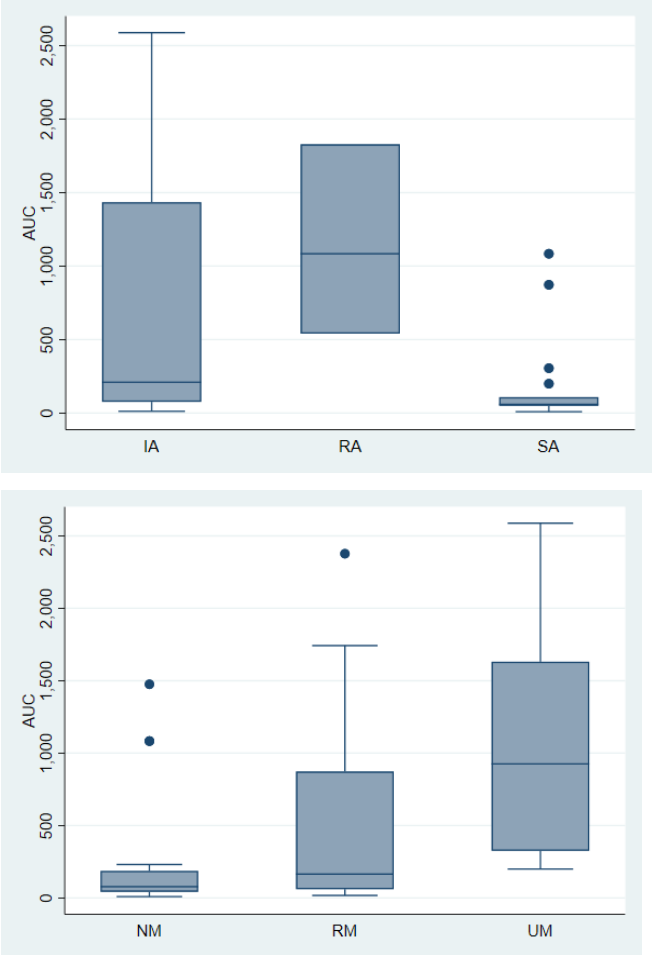


Table 5. Correlation between Cmax and polymorphism

Gene		Cmax [range]	p-value
NAT2	Unknown		
	IA	4.80 [0.78-41.55]	
	RA	26.34 [10.21-39.47]	
	SA	1.43 [0.51-17.12]	**0.01**
UGT2B7	Unknown		
	NM	1.51 [0.51-27.27]	
	RM	4.23 [0.79-40.98]	
	UM	15.25 [4.23-41.55]	**0.005**

Correlation between pharmacogenetics, pharmacocynetics and responders

Genetic data indicated a strong association between polymorphisms of the thiopurine S-methyltransferase (TPMT) gene and clinical response (p=0.009). In particular, none of the patients carrying allelic variants associated with intermediate and poor-metabolizer is a responder.

No statistically significant differences were observed in AUC, Cmax and Tmax between responder and non-responders (p=0.74, p=0.91, p=0.91, respectively). Tab.6

Table 6. Polymorphism and responders

Gene		Non responder (n=11)	Responder (n=38)	p-value
CFTR	DF	0	2	
	NF	11	36	1
COMT	Unknown	0	1	
	IM	6	26	
	NM	4	10	
	PM	1	1	0.38
CYP1A2	IM	0	2	
	NM	11	36	1
CYP2A6	Unknown	1	2	
	IM	2	8	
	NM	8	28	1
CYP2B6	Unknown	0	4	
	IM	4	13	

	NM	5	17	
	PM	0	3	
	RM	2	1	0.38
CYP2C8	Unknown	1	2	
	IM	1	13	
	NM	9	23	0.14
CYP2C9	Unknown	0	1	
	IM	2	13	
	NM	9	23	
	PM	0	1	0.59
CYP2CRS12777823	Normal	10	26	
	Sensitive	1	12	0.25
CYP2C19	Unknown	0	1	
	IM	1	11	
	NM	4	15	
	RM	6	9	
	UM	0	2	0.25
CYP2D6	Unknown	0	3	
	IM	2	11	
	NM	8	21	
	PM	1	3	0.76
CYP2E1	Unknown	0	1	
	IM	1	10	
	NM	10	27	0.42
CYP3A4	IM	1	5	
	NM	10	33	1
CYP3A5	IM	0	3	
	NM	0	1	
	PM	11	34	1
CYP3A7	NM	9	29	
	RM	1	7	
	UM	1	2	0.70
CYP4F2	IM	5	17	
	NM	3	15	
	PM	3	4	
	RM	0	2	0.49
DPDY	IM	0	1	
	NM	11	37	1
GSTM1	IM	3	17	
	NM	0	6	
	PM	8	15	0.14
GSTP1	IM	7	23	
	NM	4	15	1
IFNL3	FavorableResponseGe..	7	20	
	UnfavorableResponse..	4	18	0.73

NAT1	IA	0	2	
	RA	11	34	
	SA	0	2	1
NAT2	Unknown	5	3	
	IA	3	11	
	RA	0	4	
	SA	3	16	1
NUDT17	IM	1	1	
	NM	10	37	0.40
SLC01B1	Unknown	1	2	
	DF	4	9	
	IF	2	12	
	NF	4	15	0.60
TPMT	IM	2	0	
	IM,PM	1	0	
	NM	8	38	**0.009**
UGT1A1	IM	4	15	
	NM	5	19	
	PM	2	4	0.79
UGT2B7	Unknown	1	3	
	NM	4	13	
	RM	5	15	
	UM	1	7	0.90
VKORC1	Unknown	2	17	
	Normal	1	2	
	Resistant	2	9	
	Sensitive	6	10	0.62

Table 7. Allelic variants of the TMPT gene associated with an altered phenotype in our patients' cohort.

Patient ID	Allele(s)	Amino acid change	Nucleotide change	Genome Position	dbSNP Reference SNP ID	Metabolic phenotype
B066-007	*16	R163H	488G>A	Ch6:18138969	rs144041067	NM/IM
B066-	*3A,*3B,*3	A154T	460G>A	Ch6:1813899	rs1800460	IM,PM

023	D			7		
B066-023	*3A,*3C,*3D	Y240C	719A>G	Ch6:18130687	rs1142345	IM,PM
B066-040	*8	R215H	644G>A	Ch6:18130762	rs56161402	NM/IM

IM: intermediate metabolizer; NM: normal metabolizer; PM: poor metabolizer

When we analyzed patients' side effects in relationship with their genetic features, we observed a significant association with CYP2B6 genetic variants (p=0.009). More side effects were reported in patients carrying allelic variants associated with rapid metabolizer phenotypes rather than normal phenotypes (RR 2.40, 95% CI 1.06-5.29).

Correlation of side effects and inhibitors/inductors of CYP3A4

No correlation between side effects and use of inhibitors or inductors of CYP3A4 was found.

Table 8.

	Side effects (n=16)	No side effects (n=30)	p-value
Use of inhibitors/inductors of CYP3A4	11	20	
No use of inhibitors/inductors of CYP3A4	5	10	1

Correlation of clinical response and inhibitors/inductors of CYP3A4

no correlation between clinical response and use of inhibitors or inductors of CYP3A4 was found.

Table 9.

	Non responder (n=11)	Responder (n=38)	p-value
Use of inhibitors/inductors of CYP3A4	8	25	
No use of inhibitors/inductors of CYP3A4	3	13	1

Correlation of pharmacokinetics and inhibitors/inductors of cyp3a4

No correlation between fentanyl pharmacokinetics and use of inhibitors or inductors of CYP3A4 was found (p=0.82, p=0.78, respectively).

Correlation between change in NRS and change in BPI between T0 and T4 for each category

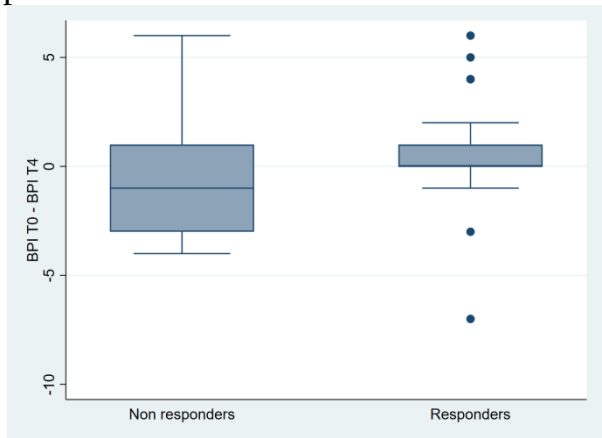
Spearman rank correlation

- Attività in generale
  Spearman's rho = 0.1550, p=0.29
- Umore
  Spearman's rho = 0.0798, p=0.59
- Camminare
  Spearman's rho = 0.1318, p=0.37
- Lavoro
  Spearman's rho = 0.2647, p=0.07
- Relazioni
  Spearman's rho = 0.4509, p=0.001
- Sonno
  Spearman's rho = 0.0771, p=0.60
- Gusto di vivere
  Spearman's rho = 0.1412, p=0.34

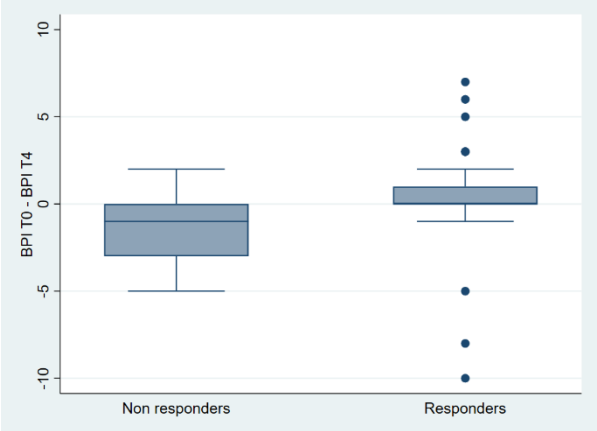
There is evidence of correlation between change in NRS and change in pain interference with relationship (p=0.001), although the correlation is not strong (rho = 0.45).

Difference in BPI change between responders and non responders

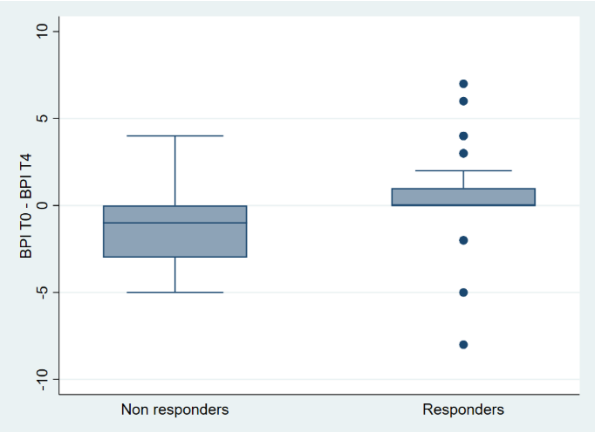
- Attività in generale
  p=0.06



- Umore
  p=0.32
- Camminare
  p=0.21
- Lavoro
  p=0.008



- Relazioni
  p=0.009



- Sonno
  p=0.38
- Gusto di vivere
  p=0.22

There is some evidence of difference between responders and non –responders in BPI change concerning general activity (p=0.06). In particular, responders show a slight improvement in pain interference with general activity rather than non responders (median BPI T4- BPI T0 = 0 , IQR = 1 vs median BPI T4- BPI T0 = -1 , IQR = 4, respectively).

There is strong evidence of difference between responders and non-responders in BPI change concerning job. In particular, responders show a slight improvement in pain interference with job rather than non responders (median BPI T4- BPI T0 = 0 , IQR = 1 vs median BPI T4- BPI T0 = -1 , IQR = 3, respectively).

There is strong evidence of difference between responders and non-responders in BPI change concerning relationship. In particular, responders show a slight improvement in pain interference with relationship rather than non responders (median BPI T4- BPI T0 = 0 , IQR = 1 vs median BPI T4- BPI T0 = -1 , IQR = 3, respectively).

Discussion

Fentanyl is widely used for cancer related pain, as many patients and health care professionals prefer a patch for drug delivery for reasons of convenience. The patch is specially appropriate for specific patients populations like pts with swallowing disorders, bowel obstruction and pts at the end of life.

However typical problems in these specific populations are cachexia and dehydration. The influence of these factors on fentanyl uptake and clearance is still largely unclear.

The effect of gender on fentanyl PK has been studied only in pts using TD patches. Gender may influence fentanyl pharmacokinetics by higher CYP3A4 activity in women compared to men and by differences in body composition between man and women.

Several factors influencing fentanyl pharmacokinetics are described in the literature. However, we still cannot completely explain the wide intra -and inter-individual variability.

A clear relationship between fentanyl and the incidence and severity of fentanyl induced side effects has not yet been demonstrated.

In our experience sex was the only variable with enough evidence of different distribution between responders and non-responders ($p=0.05$). In particular males had greater chance of being responders rather than females (RR=1.36, 95% CI 1.0022.01). Meanwhile, a positive correlation exists between male gender and Cmax and AUC and a negative one between clinical response (delta NRS) and the drug dose . A plausible justification could be differences in cancer patients,but the size of population studied does not allow us to obtained conclusive results nd justify a dose adjustment according to sex.

Concerning biometric characteristics (age, gender), patch dose, variation in BPI data from T0 to T4 and pharmacokinetic data revealed no detectable correlations intra-patients: a positive correlation seems be determined between variation in BPI and the

patch dose, and a negative correlation with T of C_{max}. Moreover, no correlation is appreciable with the other pharmacokinetic parameters (AUC and C_{max}), and gender. Concerning the influence of genotypes on fentanyl pharmacokinetics, *NAT2* and *UGT2B7* polymorphisms were associated with AUC and Cmax cynetics parameters.The *UGT2B7* gene encodes for a UDP-glucuronosyltransferase that is involved in the elimination and detoxification of drugs, xenobiotics and endogenous compounds. NAT2 enzymatic activity plays a role in the activation and deactivation of arylamine and hydrazine drugs and carcinogens. While no association has been previously reported between NAT2 and fentanyl, some metabolic products of the drug are metabolized by glucuronate conjugation. The rs7439366 polymorphysm of the *UGT2B7* gene, detected in our cohort, has been previously associated with Fentanyl sensitivity for cold pressor-induced pain in patients undergoing painful orthognathic surgery and in gynecologic patients. Regarding side effects we found correlation with *CYP2B6* genetic variants in particular in IM and NM. CYP2B6 is a cytochrome P450 monooxygenase involved in the oxidative metabolism of xenobiotics, including plant lipids and drugs. Its polymorphysms have never been associated with Fentanyl activity.

Concerning variation in BPI we found evidence of correlation between change in NRS and change in pain interference with relationship (p=0.001), although the correlation is not strong (rho = 0.45). There is some evidence of difference between responders and non –responders in BPI change concerning general activity (p=0.06) and strong evidence of difference between responders and non-responders in BPI change concerning job.

Another strong evidence of difference between responders and non-responders resulted in BPI change concerning relationship.

Furthermore, the small sample size of the studied population and the low number of pts with adverse events due to opioid treatment make difficult do find some genotype correlation.

We did not find evidence about the influence of individuals carrying the CYP3A4 enzyme activity, also regarding ABCB1 controverted in literature concerning clinical implications.
Larger studies are needed to increase knowledge about genetic, kinetic and clinical response to opioid treatment in cancer patients to better individualized pain treatment.

Study limitation

The study was perfomed in a single center study, reason that limited the number of enrolled patients.Study population was treated with transdermal fentanyl and pharmacokinetic and tolerability might vary in patients receiving chronic treatment.It is of importance that these results are interpreted with caution given the small sample size.Larger study are needed to increase the statistical power of these results in similar settings and by pts receiving chronic fentanyl treatment.

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