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DRUGS OF ABUSE ANALYSIS IN URINE AND HAIR FOR THE EVALUATION OF THE DRIVING FITNESS. AN EPIDEMIOLOGICAL STUDY

S.S.D. MED/43

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1. ABBREVIATIONS

6-MAM	6-monoacetylmorphine
A	amphetamines
BDZ	benzodiazepines
BMI	body mass index
CdS	Codice della Strada (*Road Code*)
C.M.L	Local Medical Commission
CNS	Central Nervous System
DUI	driving under influence
EDDP	2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
GC-MS	Gas Chromatography-Mass Spectrometry
GTFI	Group of Italian Forensic Toxicologists
m/z	mass-to-charge ratio
MA	methamphetamine,
MBDB	*N*-methyl-1-(1,3-benzodioxol-5yl)-2-butamine
MDA	3,4-methylenedioxyamphetamine
MDE	methylenedioxyetamphetamine
MDMA	3,4-methylenedioxymethamphetamine
MSTFA	*N*-methyl-*N*-trimethylsilyl-trifluoroacetamide
SIM	Selected Ion Monitoring
SPE	solid-phase extaction
Δ^9-THC	Δ^9-tetrahydrocannabinol
Δ^9-THC-COOH	11-nor-9-carboxy-Δ^9-tetrahydrocannabinol

2. ABSTRACT

Background and goals: According to the Italian Road Code, driving under the influence of drugs is forbidden and the violation of this rule is sanctioned with, among others, the withdrawal or the revocation of the driving license. In order to have his/her license back, the sanctioned individual is submitted to a clinical examination aimed at evaluating his/her fitness to drive, including analysis for drugs of abuse in urine and/or in hair. A positive result to these tests implies a diagnosis of non-fitness to drive for a given time period (typically 6 months to 1 year) after which the individual may reapply for driving license. This research project is aimed at providing an insight in the data collected by the toxicology laboratory of the Legal Medicine Unit of the Department of Medicine & Public Health over the years concerning urine and/or hair analysis for drugs of abuse for the evaluation of the fitness to drive with the purposes of (i), defining trends in drug abuse, and identifying specific risk factors for drug abuse and relapse in drug abuse in the observed population, (ii) selecting the most effective and efficient analytical strategy to detect drug abuse.

Methods: Analysis for drugs of abuse either in urine or in hair was carried out in the laboratory using a combined analytical approach: immunochemical screening for opiates, cocaine, amphetamines and derivatives (urine and hair) and cannabinoids, benzodiazepines and barbiturates (urine only) and GC-MS confirmation of positives. The following screening cut-offs were applied: 500 ng/ml for amphetamines and derivatives, 300 ng/ml for opiates, methadone and cocaine, 200 ng/ml for benzodiazepines and barbiturates, 50 ng/ml for cannabinoids in urine; 0.1 ng/mg for all analytes in hair.

Hair analysis was carried out on one hair sample (3-4 cm proximal segment in order to infer on the history of drug use during the 3-4 month period preceding sample collection) collected at the beginning of the sampling protocol. In the case of baldness

or when hair appeared to have been submitted to strong cosmetic treatments (*e.g*. bleaching, perming), pubic hair was collected instead. In the case of urine, different sampling protocols were applied: 3 urine samples randomly collected without notice to the subject within a 4-week period or 8 urine samples collected at pre-defined times with notice to the patient within the same period of time. The biological substrate (urine and/or hair) and the urine sampling protocol were established by the Medical Commission in charge of evaluating driving fitness on the basis of the clinical records of the subject.

Personal as well as clinical and anamnestic data on the individuals undergoing urine and/or hair testing were recorded together with test results in a database constructed using Microsoft Access (2003 version). Statistical analysis on the cases examined during 2003 and 2008 (981 cases) was carried out using Microsoft Excel (2003 version).

Results: In the period under study, the total number of subjects who underwent driving fitness diagnosis was 981. 24% (n=231) of them tested positive, 49% (n=483) were negative and 27% (n=267) didn't show up after being called upon for clinical examination and samples collection.

Cocaine (n=124, 17%), was the drug most frequently detected, followed by cannabinoids (n=86, 12%), benzodiazepines (n=42, 6%), opiates (n=22, 3%), amphetamines and derivatives (n=21, 3%), methadone (n=11, 2%) and barbiturates (n=5, 1%). Thirty percent of the subjects who tested positives, were positives for more the one drug class, and one of these was usually cocaine.

The subjects analyzed were mostly men 93% (n=907). The mean age was 31 (range 18-63) and was the same for positives and negatives. Nevertheless, different mean ages were observed among subjects testing positive for different drug classes: younger subjects, with a mean age of 23 years, seem to have a higher risk to use amphetamines while older subjects, with a mean age of 35/36 years, test more frequently positive for benzodiazepines and barbiturates.

Smokers and subjects with an anamnesis for alcohol-abuse were more represented among positives, however the difference was not statistically significant probably due to the relatively small number of cases.

Generally, an increase of BMI with age was observed among positive and negative subjects. However a different BMI trend was found out in subjects testing positive for different drug classes. In opiates and amphetamines users the increase of BMI with age was more marked then in negatives. For cocaine and benzodiazepines users, the BMI trend was approximately the same of negatives. Furthermore, a peculiar BMI trend in cannabinoids users was observed: in the first two age ranges (18-25 and 26-35 years) the trend was similar to negatives but in the last range (36-45 years) a decrease with respect to negatives was observed.

The toxicological anamnesis of the subjects appeared to be very informative. In fact, thirty percent of the subjects who reported a previous use of a specific drug, tested actually positive for that substance. In addition, the probability to find a positive subject for any substances increased when he/she declared a previous use of cocaine.

The comparison between hair and urine analysis for cocaine, opiates and amphetamines showed the complementary features of these two matrices. In fact, there were subjects testing positive in hair and negative in urine and viceversa.

Part of the research was dedicated to assessing the possible correlation between our results and the numbers of road accidents occurred on the provincial area. In general, a positive but weak correlation between the percentage of subjects testing positive for different drugs and the frequency of road accidents within the same year (no. road accidents/number of cars in the province of Verona). According to the Italian road code, after a car accident where drug use was ascertained, the subject has to undergo a clinical examination to test his fitness to drive (Art. 119), so one could argue that these positive trends may be due to the fact that if the number of accidents increases, also the clinical examinations should increase. However, during the studied period, no

positive trend between the absolute number of traffic accidents and number of clinical examination requested was observed (Art. 119).

The comparison between results of the first and second check (which is mandatory for both negative and positive subjects after a variable period of time, typically 6-12 months) has shown that only 27% of positives at the first check were still positive at the second but also that 9% of negatives at the first check were positive at the second.

Conclusion: The percentage of positives at the first check was 24%, increasing to 32% excluding subjects who didn't show up for the clinical examination.

Cocaine was the most frequently detected illicit drug and was commonly found with other illicit substances. The majority of subjects involved in the study were males (93%) and the same percentage was discovered among positives. Different mean ages were observed in users of different substances: younger age appeared to be more frequently associated to amphetamines use and older age to benzodiazepines/barbiturates use. The comparison of results from urine and hair testing confirmed the complementary features of these two biological substrates and the importance to have both data in order to increase the sensitivity in detecting illicit drug use. Moreover, this study showed that testing for driving fitness is an effective deterrent to illicit drug use, as only about one quarter of subjects testing positive at the first testing are still positive at the second testing.

3. INTRODUCTION

Traffic crashes are a common cause of death in many countries. Among the numerous risk factors (*e.g*. speed, talking on cell phones, road infrastructures), the effects of illegal drugs have an important role.

Driving a motor vehicle is a necessary daily activity for many people, and it is a complex multifunctional task involving visual search and recognition, vigilance, information processing under variable demand, decision-making, risk taking and enough sensory-motor control to carry out all these activities correctly. It is believed that an ever-increasing number of people might be driving under the influence of illegal drugs (DUI), which would mean a high-risk factor in terms of road safety **[1]**.

In the last few years the relation between illegal drugs and driving has been a subject of growing interest.

A large body of literature demonstrates that the abuse of illicit drugs and alcohol is one of the main causes of the most serious road accidents [2,3,4]. In fact, use of narcotic and psychoactive drugs may impair driving skills, in respect of their effects on the central nervous system, by affecting alertness, visual acuity, reaction time, judgment and decision making, and so on **[5,6]**.

3.1 EFFECTS ON DRIVING PERFORMANCE OF CONTROLLED DRUGS OF ABUSE

MORPHINE:

Morphine produces its major effects on the CNS primarily through μ-receptors, and also at κ-receptors and δ-receptors. μ–receptors are involved in pain modulation,

analgesia, respiratory depression, miosis, euphoria, and decreased gastrointestinal activity.

Effects on Driving:

Drug manufacturers state that morphine may impair the mental and/or physical abilities needed to perform potentially hazardous activities such as driving a car, and patients must be cautioned accordingly.

In several driving under the influence case reports, where the subject tested positive for morphine and/or 6-acetylmorphine, observations included slow driving, weaving, poor vehicle control, poor coordination, slow response to stimuli, delayed reactions, difficulty in following instructions, and falling asleep at the wheel.

Miosis might be a cause of impaired vision at the entrance of a tunnel or when passing from light to shadow, as the eye is not able to rapidly adapt to the lower amount of light by dilating the pupil.

METHADONE:

Methadone is a long acting μ opioid receptor agonist with potent central analgesic, sedative, and antitussive actions. Methadone inhibits ascending pain pathways, alters perception of and response to pain (dissociative effect), and produces generalized CNS depression.

Effects on driving:

Drug manufacturers caution that methadone may impair the mental and/or physical abilities required for the performance of potentially hazardous tasks, and that the sedative effects of the drug may be enhanced by concurrent use of other CNS depressants, including alcohol. Numerous European studies of long-term methadone maintenance patients have shown that appropriately administered methadone does not cause significant psychomotor or cognitive impairment when administered regularly and when the subject abstains from all other drugs. However, in the majority of cases, patients did not exhibit stable abstinence from drug use and had an increased

occurrence of simultaneous psychiatric/neurotic disorders or personality disturbances which, by themselves, could be a reason to doubt their driving ability.

COCAINE:

Cocaine is a strong CNS stimulant that interferes with the reabsorption process of catecholamines, particularly dopamine, a chemical messenger associated with pleasure and movement. Cocaine prevents the reuptake of dopamine by blocking the dopamine transporter which leads to increased extracellular dopamine, resulting in chronic stimulation of post-synaptic dopamine receptors. This results in the euphoric "rush". When dopamine levels subsequently fall, users experience a dysphoric "crash".

Effects on Driving:

Observed signs of impairment in driving performance have included subjects speeding, losing control of their vehicle, causing collisions, turning in front of other vehicles, high-risk behavior, inattentive driving, and poor impulse control. As the effects of cocaine wear off, subjects may suffer from fatigue, depression, sleepiness, and inattention. In epidemiology studies of driving under the influence cases, accidents, and fatally injured drivers, between 8-23% of subjects had cocaine and/or metabolites detected in their blood.

METHAMPHETAMINE AND AMPHETAMINE:

Methamphetamine increases synaptic levels of the neurotransmitters dopamine, serotonin, (5-HT) and norepinephrine, and has α and β adrenergic agonist effects. Norepinephrine is responsible for methamphetamine's alerting, anorectic, locomotor and sympathomimetic effects; dopamine stimulates locomotor effects, psychosis, and perception disturbances; and 5HT is responsible for delusions and psychosis.

Effects on Driving:

Drug manufacturers state that patients should be informed that methamphetamine and amphetamine may impair the ability to engage in potentially hazardous activities such as driving a motor vehicle. In epidemiology studies drive-off-the-road type accidents, high speed, failing to stop, diminished divided attention, inattentive driving performance would also be expected during drug withdrawal. Driving and driver behaviors included speeding, lane travel, erratic driving, accidents, nervousness, rapid and non-stop speech, unintelligible speech, disorientation, agitation, staggering and awkward movements, irrational or violent behavior, and unconsciousness. Impairment was attributed to distraction, disorientation, motor excitation, hyperactive reflexes, general cognitive impairment, or withdrawal, fatigue and hypersonnolence.

METHYLENEDIOXYMETHAMPHETAMINE (MDMA, Ecstasy):

MDMA is a phenylethylamine that has stimulant as well as psychedelic effects. MDMA is related in structure and effects to methamphetamine, however, it has significantly less CNS stimulant properties than methamphetamine. MDMA has a high affinity for 5-HT_2 receptors. The MDMA metabolite, S-(+)-MDA, destroys serotonin-producing neurons which play a direct role in regulating aggression, mood sexual activity, sleep, and sensitivity to pain.

Effects on Driving:

In an advanced driving simulator study, subjects were given a mean single dose of 56 mg MDMA. Compared to a sober state, moderate effects on vehicle control, acceptance of higher levels of risk, acute changes in cognitive performance, and impaired information processing ability were observed.

CANNABINOIDS:

Δ9-Tetrahydrocannabinol (THC) binds to cannabinoid receptors and interferes with important endogenous cannabinoid neurotransmitter systems. Receptor distribution correlates with brain areas involved in physiological, psychomotor and cognitive effects.

Effects on Driving:

Epidemiology data from road traffic arrests and fatalities indicate that after alcohol, marijuana is the most frequently detected psychoactive substance among driving populations. Marijuana decreased car handling performance, increased reaction times, impaired time and distance estimation, inability to maintain headway, lateral travel, subjective sleepiness, motor incoordination, and impaired sustained vigilance have all been reported. Mixing alcohol and marijuana may dramatically produce effects greater than either drug on its own.

BENZODIAZEPINES:

Benzodiazepines bind with high affinity to the $GABA_A$ receptor in the brain to reduce arousal and to affect emotions.

Effects on Driving:

The drug manufacturer suggests patients treated with benzodiazepines be cautioned against engaging in hazardous occupations requiring complete mental alertness such as driving a motor vehicle. Use of benzodiazepines can increase lateral deviation of lane control, reduce reaction times, reduce ability to perform multiple tasks, decrease attention, adversely effect memory and cognition, and increase the effects of fatigue. Significant impairment is further increased when benzodiazepines are combined with low concentrations of alcohol.

3.2 DIAGNOSIS OF DRIVING FITNESS WITH REFERENCE OF DRUG ABUSE ACCORDING TO THE ITALIAN ROAD CODE

The diagnosis of driving fitness in relation to the use of narcotic drugs and psychotropic substances is regulated in our country by Article 119 ("physical and psychological requirements for obtaining a driving license") of Legislative Decree No. 285 of 30 April 1992 (better known as "Nuovo Codice della Strada") **[7]** and subsequent amendments and additions, and Art. 319 and 320 of its implementing Regulation (DPR 495/1992 and subsequent amendments and additions) **[8]**.

According to the laws presently in force in Italy, the driving license must not be issued neither confirmed to anyone who abuses, is addicted to, or suffers for dependence from alcohol and illicit or psychotropic drugs which may impair her/his driving ability. The evaluation of these conditions is carried out by a specific medical commission of the Public Health Service set up in each province, on the basis of clinical and laboratory tests, whose cost is borne to the user applying for the issue or renewal of driving license. It should be clarified, in this regard, that on one hand the local medical commissions have broad freedom of action as to the criteria for diagnostic evaluation; on the other, users have the possibility of choosing the local medical commission where to undergo the diagnostic evaluation.

Among the individuals who are required to undergo this evaluation are: subjects sent by the prefect or by the office of the department responsible for land transportation, those for which the medical doctor examination requires toxicological test to assess the fitness to drive, as well as subjects tested positives for alcohol and drugs of abuse at road-side testing (Art. 186 and 187 CdS).

A recent change in Art. 119, in force since 13th august 2010, also requires preventive toxicological examinations for the first issue of the driving license and for workers assigned to duties which may endanger public safety **[9].**

As regards workers employed in duties that may imply a risk to security, safety and health of their own and third parties, the diagnosis of drug addiction or of sporadic use of drugs is achieved, in accordance with the provisions of the Conferenza Unificata Stato-Regioni e P.A. of 30 October 2007 **[10]**, by means of a clinical exam including the testing of itself of an urine specimen collected from the employee after a notification time not exceeding 24 hours **[11]**.

4. AIM OF THE STUDY

The Forensic Toxicology laboratory, Section of Forensic Medicine, Department of Public Health and Community Medicine, University of Verona, exclusively performs clinical and analytical toxicological investigations on subjects sent by the Medical Commission of the Province of Verona. In particular, each subject is submitted to a clinical examination, to an interview for the collection of anamnestic and clinical data and to the collection of biological samples (urine and/or hair) for drugs of abuse testing.

The amount of data collected so far (an average of 160 new subjects per year to which are to be added subjects who were requested to undergo subsequent examinations after the suspension/validity period of their driving license was expired) has led us to a thorough examination of the data collected in recent years (2003-2008).

Although other authors have previously ventured into the development of statistical and epidemiological case studies relating to ascertainment of fitness to drive in relation to the use of drugs **[12-15]** with the aim of evaluating the relative frequency of positivity for different classes of drugs of abuse and any trend of these frequencies, our study intended to investigate, in addition to the aspects just mentioned, also the evaluation of possible risk factors for testing positive and for relapse and the comparison of the effectiveness of different diagnostic established approaches.

5 MATERIALS AND METHODS

The survey was conducted on the requests for control of the use of narcotic and psychotropic substances sent by the Local Medical Commission (C.M.L) of Verona to our laboratory for the purpose of diagnosis of driving fitness within the period 2003-2008. Based on these request, we proceeded to summon the people for carrying out clinical examination and biological sampling. For most of the statistical and epidemiological process only the first check was taken into account with the inclusion of checks after the first only for specific purposes.

During the period under study the requests received from the C.M.L. were 981 and corresponded, in the vast majority of cases, to one of the following types:

- Drivers tested positive for driving under the influence of drugs
- Drivers to whom the driving license had been suspended/withdrawn because they were found positives to drug testing after a car accident
- Subjects dependent on public or private facilities for the care and rehabilitation of drug use
- Subjects to whom the driving license had been suspended following a report to the prefecture for use or possession of drugs

5.1 SAMPLING PROTOCOL

The type of sampling protocol (*e.g*. urine and/or hair) was established by the Medical Commission on the basis of the clinical records of the subjects.

The adopted protocol consisted in the collection of clinical and anamnestic data and urine and/or hair samples. In particular, the protocol for urine collection consisted either

in 3 urine samples randomly collected without notice to the subjects within a 4-week period or in 8 urine samples collected at pre-defined times with notice to the patient within the same period of time. The first urine protocol was adopted only in the 4% of the case, the second was more common and was carried out on the 96% of the subjects.

In the case of hair analysis, a single hair sample (3-4 cm proximal segment in order to infer on the history of drug use during the 3-4 month period preceding sample collection) was collected at the beginning of the sampling protocol. Personal as well as clinical and anamnestic data on the individuals undergoing urine and or hair analysis were recorded together with test results in a database constructed using Microsoft Access (Version 2003). The subsequent statistical elaboration was performed with Microsoft Excel (Version 2003) and software PSPP (Version 0.6.2).

5.2 CHEMICALS AND REAGENTS

Pure standards of morphine, codeine, 6-monoacetylmorphine (6-MAM), dihydrocodeine, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), cocaine, benzoylecgonine, cocaethylene, ecgonine methyl ester, amphetamines (A), methamphetamine (MA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), *N*-methyl-1-(1,3-benzodioxol-5yl)-2-butamine (MBDB), methylenedioxyetamphetamine (MDE) and 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (Δ^9-THC-COOH) were purchased from S.a.l.a.r.s. S.p.a. (Como, Italy). Benzodiazepines (bromazepam, clobazam, chlordesmethyldiazepam, diazepam, flunitrazepam, flurazepam, lorazepam, lormetazepam, medazepam, nitrazepam, nordazepam, oxazepam, temazepam) and barbiturates (fenobarbital and butalbital) were obtained from commercial drugs.

N-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) was purchased from Sigma (St Louis, Italy), β-glucuronidase was also purchased from Sigma.

TWEEN was purchased from A.C.E.F. S.p.a., (Fiorenzuola d'Arda (PC), Italy). The bidistilled water used throughout the present study was obtained from an aqua MAX-Ultra 370 Series water purification system (Young Lin Instrument, Anyang, Korea).

Solvents and reagents (HCl, NaOH, monobasic phosphate) of C. Erba, Milan, Italy were reagent grade.

Bond Elut Certify LRC cartridges (10 ml capacity, 130 mg) and C18 cartridges (6 ml capacity, 100 mg) were obtained from Agilent (Santa Clara, CA, USA). Toxi-Tube B from Varian (Harbor City, CA, USA).

5.3 IMMUNOCHEMICAL SCREENING

Urinary screening was performed with CEDIA MULTI-DRUG kits (Microgenics Corporation, Fremont, CA, USA), after sample centrifugation (3500 rpm, 10 min), for the following substances: opiates, methadone, cocaine, amphetamines and metilendioximethamphetamines, cannabinoids, benzodiazepines and barbiturates. Hair samples (100 mg) were washed with 3 % TWEEN solution in water, thoroughly rinsed with water and then were manually cut into small fragments and incubated overnight in 1 ml of HCl 0.1 M at 45°C. The resulting mixtures were neutralized with NaOH 0.1 M and screened for opiates, cocaine and amphetamines and derivates using the CEDIA kits for urine adapted.

The adopted cut-off for urine screening was 500 ng/ml for amphetamines and derivates, 300 ng/ml for opiates, methadone and benzoylecgonine, 200 ng/ml for benzodiazepines and barbiturates and 50 ng/ml for cannabinoids in agreement with those provided by the commission of the group of Italian Forensic Toxicologists (GTFI). Positives samples in the keratin matrix screening were reported when the

concentration values were above 10 ng/ml for opiates and cocaine and 25 ng/ml for amphetamines and derivates corresponding to a concentration of 0.1 ng/mg and 0.25 ng/mg in keratin matrix.

5.4 CONFIRMATION ANALYSIS

Confirmation analysis for both matrices was carried out by gas chromatography/mass spectrometry (GC-MS) after solid-phase extraction (SPE) and silyl-derivatization.

For urine, solid-phase extraction cartridges was performed with a mixed mode extraction 130 mg Bond Elut Certify for analytes predominantly alkaline and C18 cartridges of 100 mg for cannabinoids. The eluates were evaporated to dryness under air stream on hot plate at 65°C, the residues were reconstituted with 50 μl of *N*-metil-*N*-trimetilsililtrifluoroacetamide (MSTFA) and examined by GC-MS by monitoring 3-4 specific ions in Selected Ion Monitoring (SIM) mode.

Subjects screened positives for benzodiazepines and barbiturates were confirmed only if the use was not declared by the user during the clinical examination. The determination of total benzodiazepines was made through liquid-liquid extraction with butyl acetate at alkaline pH after hydrolysis with β-glucuronidase while the determination of barbiturates was made with a liquid-liquid extraction using Toxi-Tube B. Cut-off values used for confirmation analysis were 100 ng/ml for each substances detected, except for cannabinoids and amphetamines and derivates which have cut-off respectively of 250 ng/ml and 30 ng/ml.

The confirmation analysis in hair, by GC-MS, was preceded by a solid phase extraction (Bond Elut Certify cartridges 130 mg) for the three classes of substances after addition of 1 ml of 0,1 M phosphate buffer at pH 6-7 on the incubation mixtures obtained as described above. In this case a sample was considered positive when the concentration of drugs of abuse was above the cut-off of 0,1 ng/mg.

The GC-MS system used, consisted of an Agilent Model 6850 gas chromatograph equipped with a Model 5975C mass-selective detector (Agilent Technologies, Santa Clara, CA, USA). The column used (Agilent Technologies, Santa Clara, CA, USA) was a fused silica capillary column (HP-5 fused-silica capillary column, 30m x 0,25 mm I.D., 0.25 μm film thickness cross-linked 5% phenyl-methylsiloxane). Helium was used as the carrier gas at a constant flow-rate of 1 ml/min. The injector operated in the pulsed-splitless mode at 250°C and the injection volume was 1 μl. Initial oven temperature was 100°C, maintained for 1 min, increasing (30°C/min) at 200°C and followed by a ramp at a rate of 15°C/min to 290°C and maintained at this temperature for 7 min.

Identification criteria were based on the retention time ± 0.02 min with respect to the same criterion in the spiked hair sample and on the relative abundance of the three confirming ions with respect to the target. Quantitative data were obtained by selected ion monitoring for each compound and for internal standards. Monitored ions and the retention time for each compound are shown in [Table 1].

[Table 1] **Selected ions and retention times.**

Compound	Rt (min)[a]	Ion *m/z*
Morphine	10.35	**429**, 401, 414
Codeine	10.09	**371**, 313, 343
6-MAM	10.70	**399**, 340, 287
Dihydrocodeine	9.74	**373**, 236, 282
Methadone	8.41	**72**, 178, 294
EDDP	7.89	**277**, 276, 220
Cocaine	8.74	**182**, 82, 303
Benzoylecgonine	9.09	**82**, 240, 346
Cocaethylene	9.04	**82**, 196, 272
ecgonine methyl ester	5.30	**82**, 96, 240, 271
A	4.02	**116**, 91, 192
MA	4.42	**130**, 91, 206
MDA	5.46	**116**, 135, 236
MDMA	5.79	**130**, 250, 91
MBDB	6.30	**144**, 264, 135, 73
MDE	6.09	**144**, 264, 135
Δ^9-THC-COOH	5.88	**371**, 473, 488, 489

Quantifier ion in bold

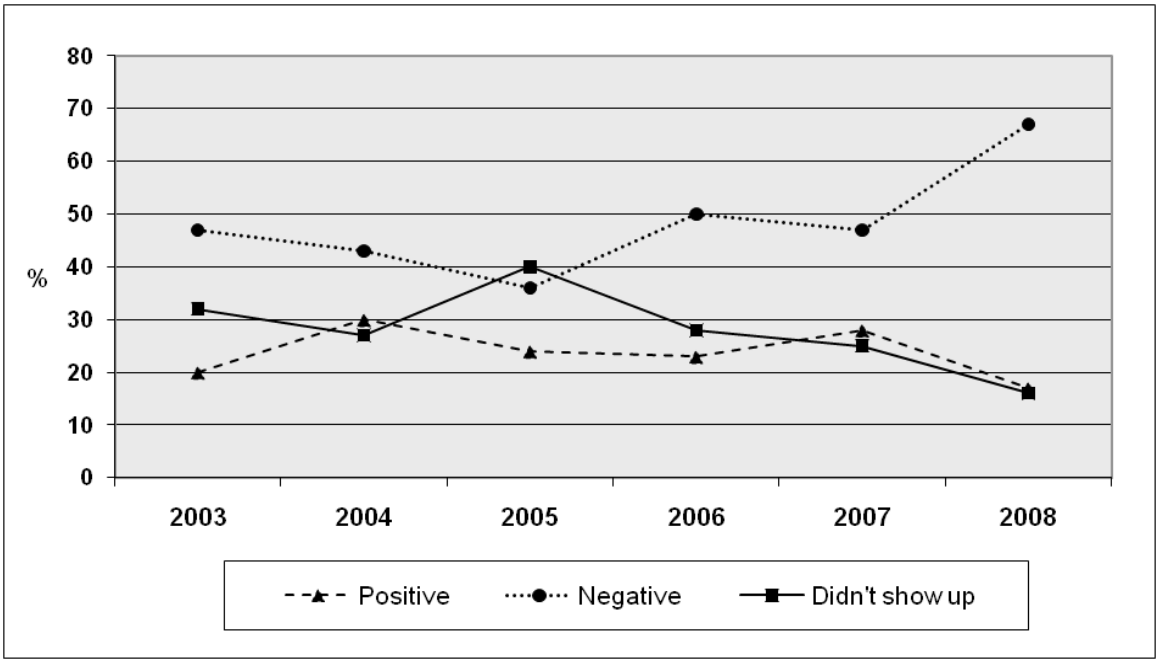
[a] Retention time ± 0.02 min

6. RESULTS AND DISCUSSION

In the period under study, from 2003 to 2008, more than a quarter of subjects didn't show up after being called upon for clinical examination and samples collection. Among the remaining, 231 subjects tested positive for one or more than one substances in urine and/or hair (24% of the total number, 32% of the subjects analyzed) and 483 subjects tested negative (49 % of the total, 68 % of the subjects analyzed).

We observed a progressive increase of request for investigations: from 152 in 2003 to 187 in 2008; with an increase of approximately 23%.

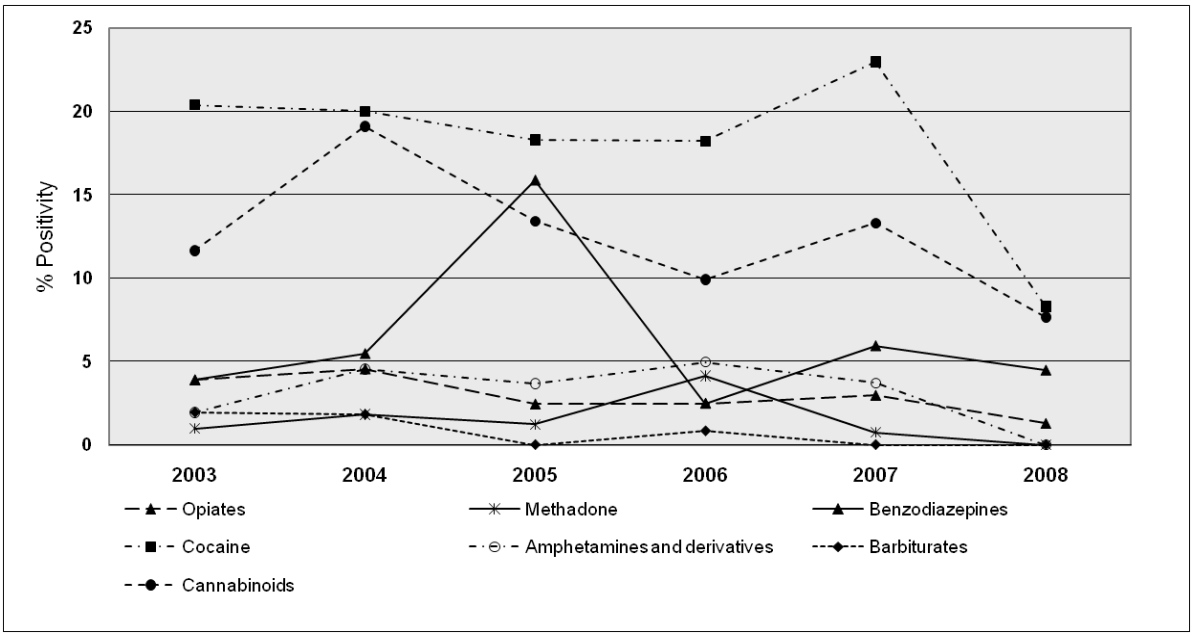
The time course of the percentage of people positive for one or more drugs of abuse respect to request for investigation was fluctuating between 20% and 30% and characterized by a modest decline in the last year considered [Fig. 1].



[Fig. 1] **Time course of the frequency of positivity for different classes of drugs of abuse.**

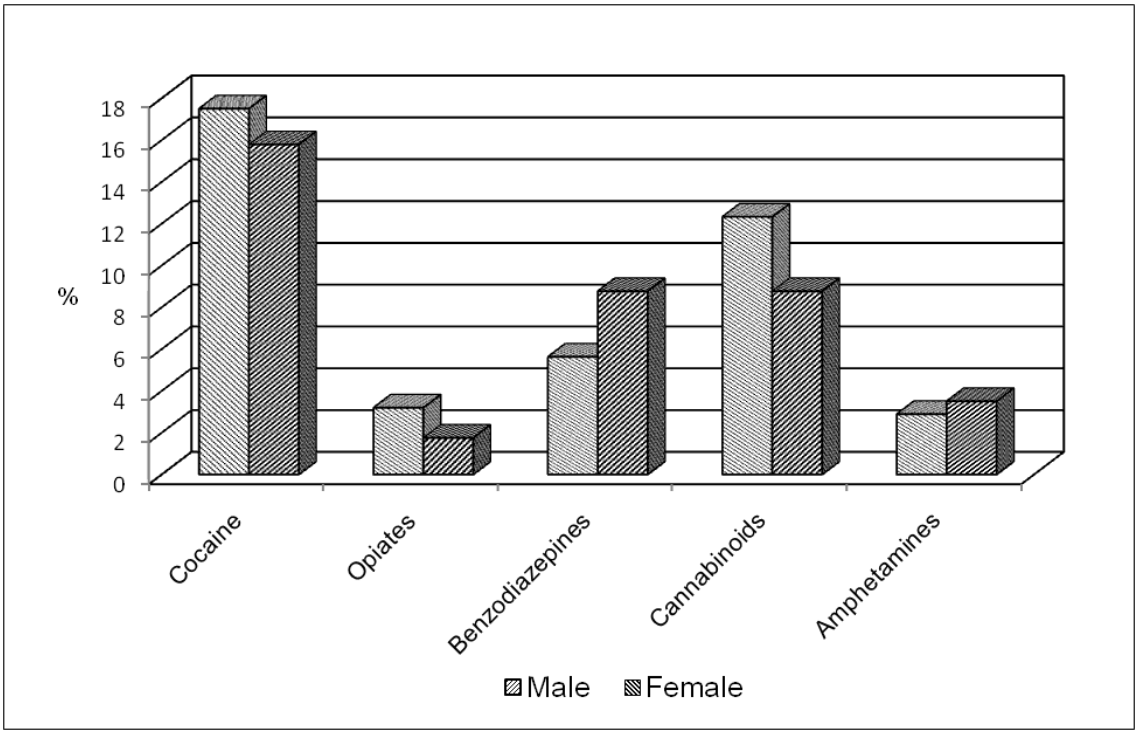
The positivity rate of subjects tested, ranged from 30% to 40% with the exception of 2008, the year in which there was a substantial decline (approximately 20% positivity). Cocaine, 17% (n=124), was the drug most frequently detected, followed by cannabinoids 12% (n=86), benzodiazepines 6% (n=42), opiates 3% (n=22), amphetamines and derivates 3% (n=21), methadone 3% (n=21) and barbiturates 1% (n=5). Thirty percent of the subjects who tested positives, were positives for more the one drug class, and one of these was usually cocaine. The most frequent combination was cocaine plus cannabinoids.

The time course of positivity for different classes of substances was relatively constant for opiates, methadone, amphetamines and barbiturates, cocaine and cannabinoids have shown a more fluctuating trend with a significant increase of positivity in 2007 but was not confirmed in the next year where there was a decline of the subjects tested positive for all the substances [Fig. 2].



[Fig. 2] **Drug classes detected over the years.**

The request for investigation were mostly to male subjects (n=907, 93%). However, over the years, was found a steady increase of request for investigation to female subjects (from 5% in 2003 to 11% in 2007) to which is also corresponded an increase of positivity. However, this trend was not confirmed in 2008 when there was a decline both of percentage of request (6%) and of percentage of female subjects tested positive (1%). No relevant difference in the distribution of drug classes detected in the two genders was observed [Fig. 3].



[Fig. 3] **Distribution of positivity for different classes of drugs between males and females.**

The mean age of the subjects analyzed was 31±8 years (median, 30 years) and was almost the same for the subjects who didn't show up for the clinical examination (32±8 years). Nevertheless, the age range was wider in the first group (18-63 and 18-52

respectively). Mean ages very similar were also observed between positive and negative subjects (31±8 years and 31±9 years respectively).

Instead different mean ages were observed among subjects testing positive for different drug classes: benzodiazepines > barbiturates > opiates > cocaine = THC >amphetamine [table 2].

[Table 2] **Age (in years) of the users tested positive the different classes of substances.**

	MEAN	SD	MEDIAN	MIN	MAX	N°of Subjects
Benzodiazepines	36	10	36	21	63	42
Barbiturates	35	6	36	28	44	5
Opiates	31	8	32	20	42	22
Cocaine	29	8	28	19	57	124
Cannabinoids	29	8	27	18	51	86
Amphetamines	23	3	23	19	30	21

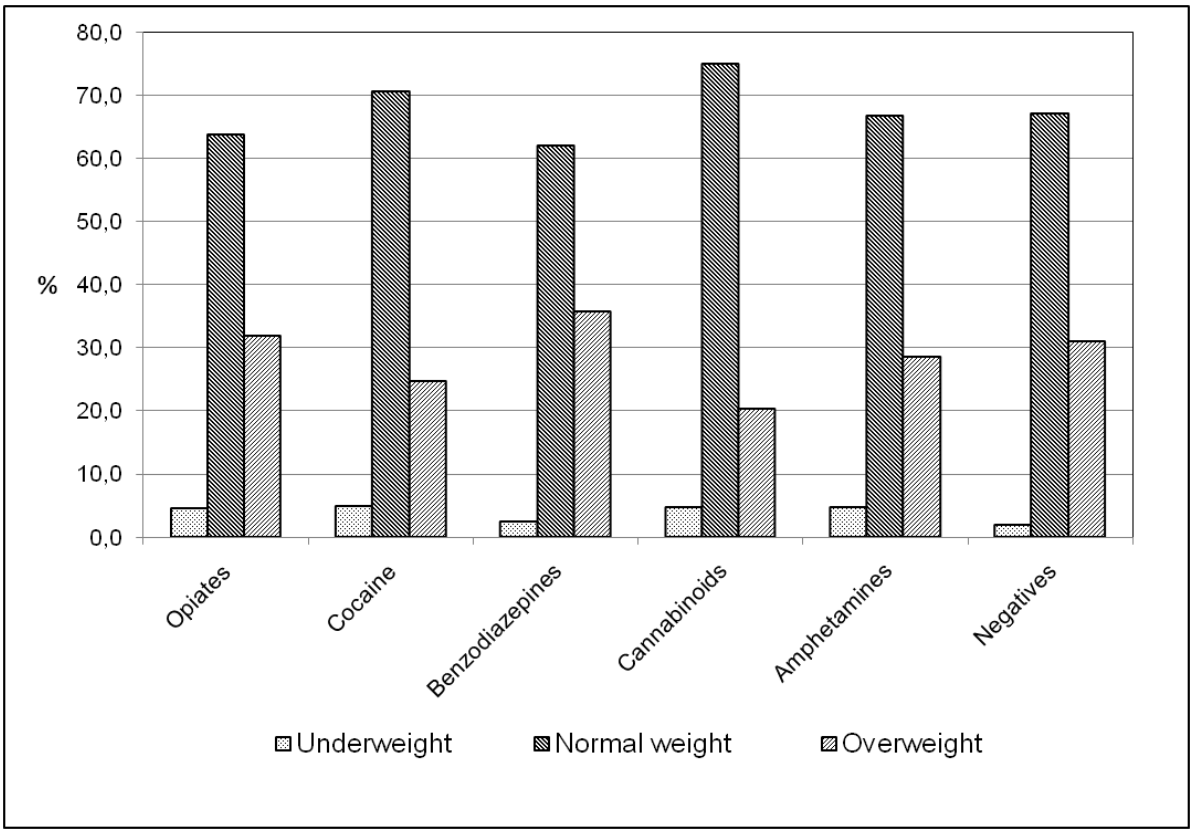
In particular, younger subjects, with a mean age of 23 years, seem to have a higher risk to use amphetamines while older subjects, with a mean age of 35/36 years, are more frequently exposed to benzodiazepines and barbiturates. This difference was also significant (P<0,001).

Nearly 10% of requests for investigation were from subjects of foreign nationality (n=94). 95% residing in the Province of Verona (n=929), one quarter of which was a resident in the centre of Verona (n=233). The comparison between positive and negative in relation to place of birth, and residence (city of Verona, Verona Province, Veneto and other regions, foreign countries) did not show considerable differences, highlighting that these factors do not significantly change the likelihood of a positive finding.

Among smokers, who represent approximately the 91% (n=543) of the subjects analyzed, and among subjects with an anamnesis for alcohol-abuse (17 %, n=121) it was possible observe a greater frequency of positive: 33% and 23% positivity in smokers and non smokers, 35% and 31% between subjects with or without a history of alcohol abuse, respectively. However, the sample size does not seem sufficient to show a possible statistical significance; only between smokers and not smokers we observed a chi square: P = 0,14.

The same analysis carried out on non drinkers and coffee drinkers highlighted that among the subjects who doesn't drink coffee (8.2%; n=36, of the subjects for which that information was available), the positivity for drugs of abuse is more common (44% versus 31% positivity among coffee drinkers). However, also in this case, the observed difference was not supported by statistical significance.

67.4% (n=475) of the subjects analyzed, has a normal body mass index (18.5<BMI<24.9), while just under one third are overweight or obese (BMI>24.9, 30.4% n=214) and 2.3% (n=16) is underweight (BMI<18.5). The BMI distribution between subjects positive and negative is essentially comparable with regard to the classes of normal weight (68% between positive, and 67.1% between negative) and overweight/obese (28.9% between positive, 31% between negative) with a more marked difference with regard to individuals underweight. The BMI distribution among subjects tested positive for different drugs classes shows that subjects overweight/obese are less represented among positives for cocaine and THC, while overweight subjects are more frequent between positives for opiates and benzodiazepines [Fig. 4].

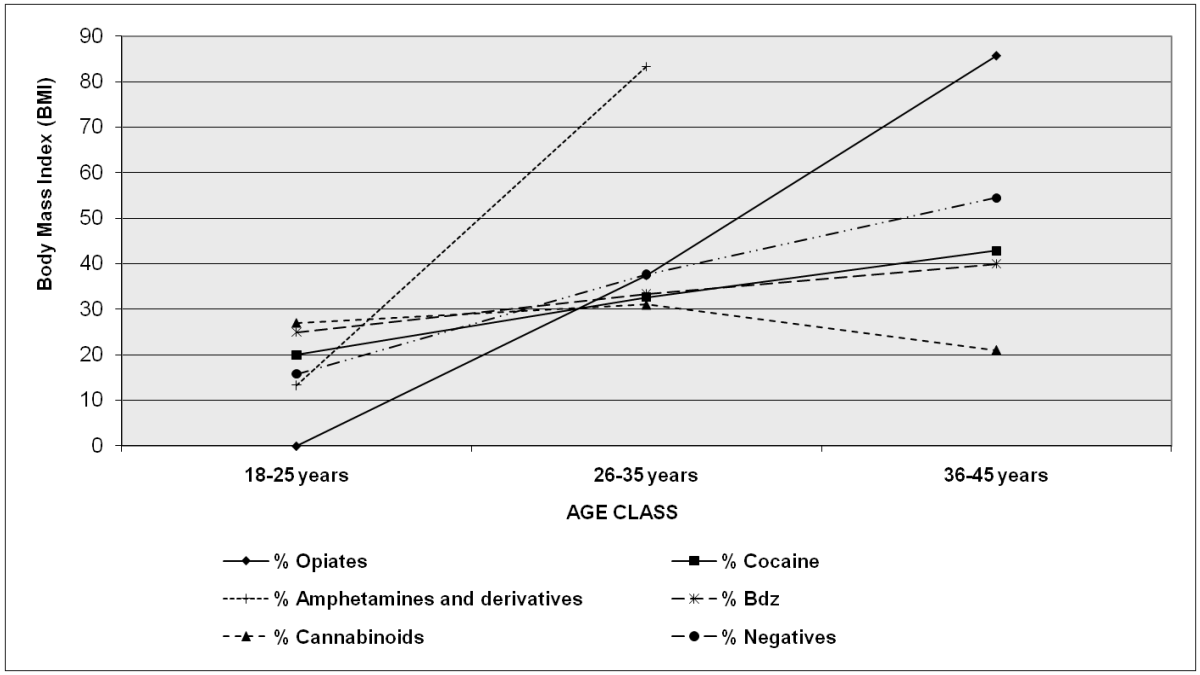


[Fig. 4] **Distribution of BMI for different drug classes.**

This observation appears to be correlated to the mean age of subjects tested positives for the different drug classes [Table 2]. In other words, the higher BMI in subjects tested positive for opiates and benzodiazepines may be justified by the increased mean age of the subjects positive for these substances and, therefore, by the physiological gain in weight that occurs with age **[16]**. Therefore, in order to isolate the effect of BMI from age, subjects tested positive for each class of substances have been further broken down into three age groups: 18-25 years, 26-35 years and >45 years [Fig. 5].

This analysis showed, as expected, an overall increase in BMI with age in both subjects tested positives and negatives, but also other interesting observation. In opiates and amphetamines users the increase of BMI with age was much more marked than in negatives. Instead, in cannabinoids users, the curve differs from that of

negative subjects in the second half, while for cocaine and benzodiazepines users, the BMI trend was approximately the same of negatives [Fig. 5].



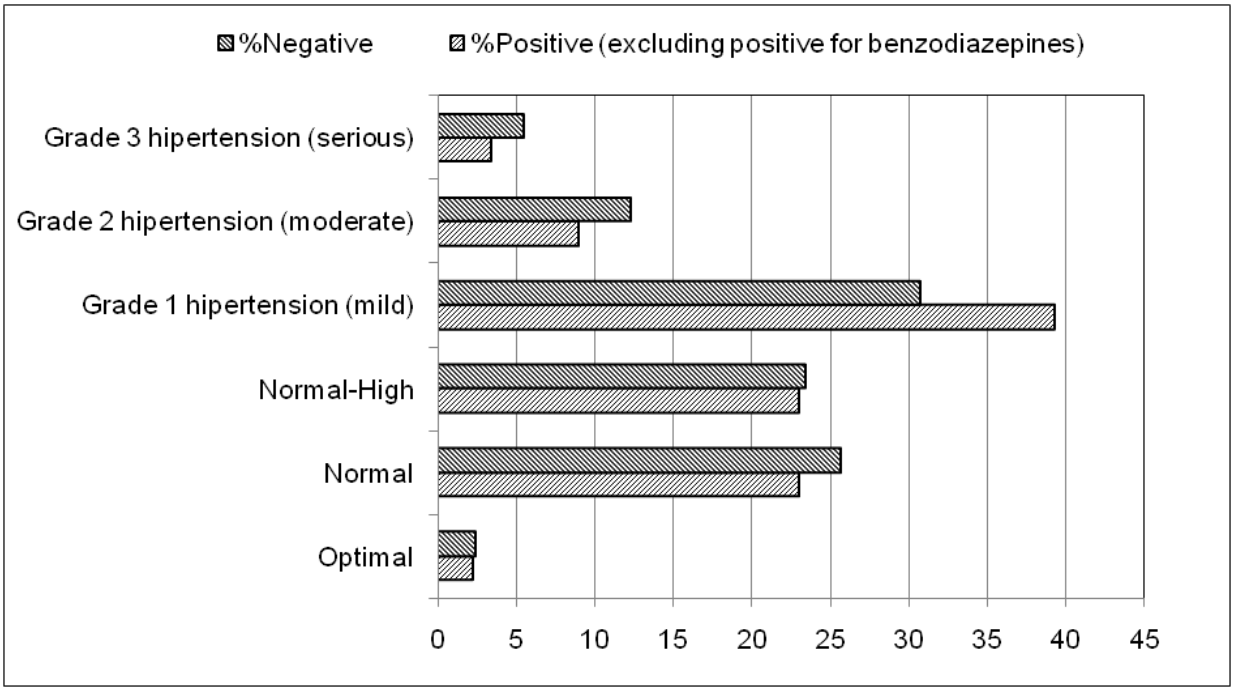
[Fig. 5] **Variation in body mass index (BMI) for different classes of drugs of abuse in relation to the age.**

The statistical comparison (Student's t-test) between positive for each class of substances and negative within each age group did not differ significantly, with the exception of cannabinoids users in the age >36 years, which are characterized by an average BMI lower than of negatives in the same age range (P=0,005).

Not unlike what is found in the general population, the female gender, of the subjects analyzed, tends to have a BMI lower (the percentage of overweight corresponds to 21% and 31% among females and males respectively). Interesting was to observe a lower BMI among females tested positive respect to negative (with a difference at the limit of the statistical significance, P=0,07) compared to a substantial overlap between the distributions of BMI in positives and negatives males. Therefore, it can be

concluded that the greater proportion of underweight subjects observed in the overall sample of positives respect to negatives is mainly due to female gender.

The data of systolic and diastolic blood pressure recorded at the time of the clinical examination were used to make a classification of blood pressure according to what is proposed by the Italian Society of Hypertension **[17]**. In particular, the systemic blood pressure was classified considering the highest class between systolic and diastolic pressure. Since clinical examination is carried out away from any acute episode (*e.g*. driving under the influence of drugs) the data of blood pressure may be related to possible chronic consequences of the assumption or simply to a condition of stress related to the investigation, particularly by those who fear a positive test results. The analysis of the data seems to support the latter hypothesis because among positives appears to be a higher rate of subjects with mild hypertension compared to negatives (P=0,11675). This difference is approaching to statistical significance by excluding positive for benzodiazepines (P=0.0659) [Fig. 6].



[Fig. 6] **Distribution of blood pressure for users, respectively, positive and negative to the toxicological investigation.**

The distribution in age groups shows that the difference is statistically significant only for the age group 18-25 years (P=0.0609, including benzodiazepines, P=0.0389 excluding benzodiazepines). Is also interesting to note that, from the breakdown by class of substances, the greater frequency of cases of mild hypertension is observed between positives to cocaine (P=0.0123), while for the other classes the difference are far from a statistical significance.

About 1 in 10 subjects analyzed declared the positivity to HBV (3%) and/or HCV (11%) and/or HIV (2%). The distribution of singularly or combined positivity in the outcome of the investigation showed a lower rate of positivity for drugs of abuse in subjects positives for one of these marker. This difference, which is approaching to statistical significance (Chi-Square, P=0.09), however, appear significantly affected by the lack of specific question in medical records related to the positivity or negativity to these markers. This allowed us to detect only positive self-declaration, but it was not possible

to distinguish between negative to these markers and missing data. The statistical evaluation could therefore show a greater tendency to hide this information from the subjects tested positive, rather than a “protective effect” due to the presence of one of these infections.

The medical record contained a question on the subject’s involvement in road accidents or work accidents, in a state of intoxication or withdrawal. Was observed a no significantly higher frequency of previous accidents among those had a positive results (15% versus 11% among negative, P=0.25). However in doubt that the answer to these question could include the road accident that led to the call for the examination, becoming an obvious confounding factor, the analysis was repeated excluding all cases in which appeared a car accident as the reason for the call. In fact, the largest proportion of cases with previous accidents among positives remains although smaller (7% vs. 5% of negatives). The breakdown of the data for classes of substances reveals a higher frequency of previous accidents among positives for benzodiazepines (12%), opiates (10%) and cocaine (7%), while for cannabinoids (3%) and amphetamines (0%) the frequency was lower than negatives.

The toxicological anamnesis (previous use of one or more than one drugs or psychotropic substances) appears in many cases useful to have ideas on the present use of drugs by the subjects [Table 3]. Even though in about one third of cases the self-declaration of previous use of drugs match with the test results (with a rate of positivity of 32% among cases with a positive anamnesis and 30% among cases with a negative anamnesis) high differences are found separating the anamnestic data into classes of substances.

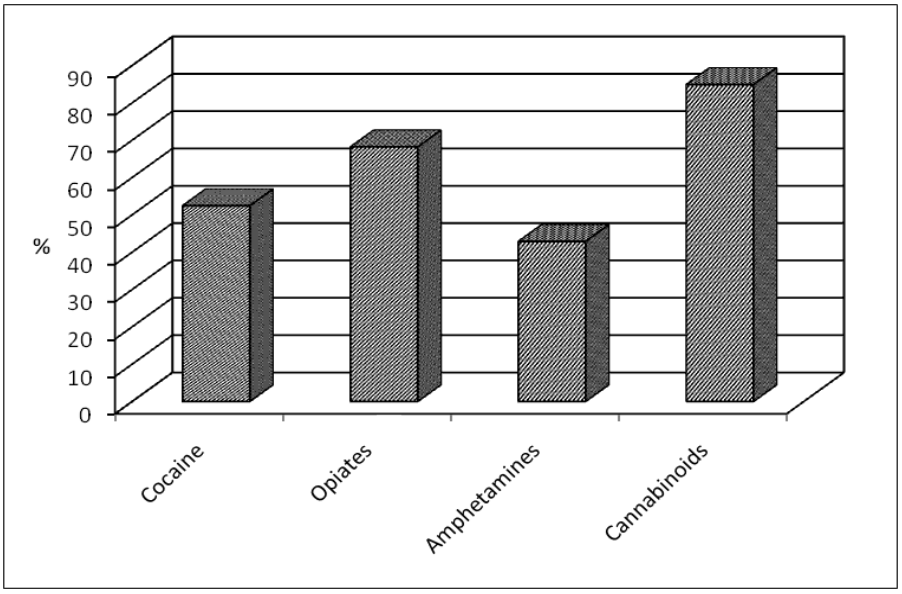
In particular, was observed that a positive anamnesis for cocaine increased significantly (Chi-square, P=0.004) the probability of a positive outcome of the investigation for any substance (the P value increases to 0.006 excluding positive for benzodiazepines). Instead the positive anamnesis for amphetamines or opiates or cannabinoids has no predictive value of the outcome of the investigation. Only in the

case of a positive anamnesis for opiates there is a nearly significantly lower rate of positive subjects for any substances (P=0.068 excluded benzodiazepines). Positive anamnesis for a class of drug of abuse appears in all cases significantly increase the probability of positivity for the same class of substances (Chi-square: cocaine, P<0,0001; opiates, P<0,0001; amphetamines, P<0,0001; cannabinoids, P=0,012; benzodiazepines, P<0,0001). In particular was found that 25% of subjects who reported a previous use of cocaine, were than actually positive for this substances, while the corresponding percentages for opiates, amphetamines and cannabinoids are respectively, 8%, 10%, 13%.

[Table 3] **Comperison between self-reported toxicological history and outcome of investigation.**

ANAMNESIS		OUTCOME INVESTIGATION											
		ALL of DRUGS		ALL-BDZ		COCAINE		OPPIATES		AMPHETAMINES		CANNABINOIDS	
		% POS	% NEG	% POS	% NEG	% POS	% NEG	% POS	% NEG	% POS	% NEG	% POS	% NEG
ALL of DRUGS	% POS	32	68										
	% NEG	30	71										
COCAINE	% POS	39	61	36	64	25	75						
	% NEG	28	72	26	74	13	87						
OPPIATES	% POS	30	70	24	76			8	92				
	% NEG	33	67	31	69			1	99				
AMPHETAMINES	% POS	31	69	30	70					10	90		
	% NEG	32	68	29	71					2	98		
CANNABINOIDS	% POS	31	69	28	72							13	87
	% NEG	37	63	33	67							6	94

Therefore, the proportion of subjects tested positive for the same class of substances which reported during the clinical examination, can be used as an indicator of the truthfulness of the statement provided by the subject. This proportion appears to be higher among positive for cannabinoids (85%) followed by positive for opiates (68%), cocaine (52%) and amphetamines (43%) [Fig. 7].



[Fig. 7] **Percentages of subjects tested positive for a class of drugs of abuse that had declared the use of the substance before the investigation.**

Part of the research was dedicated to assessing the possible correlation between the outcome of the investigation for drugs of abuse (performed, as previously reported, on subjects in almost all cases, residents in the province of Verona) and data on road accidents occurred on the provincial area **[18]** [Table 4].

[Table 4] **Comparison between self-reported toxicological analysis history and outcome of the investigation.**

Year	N. of Requests	% of positives	% of positives for BDZ	% of positives for THC	% of positives for cocaine	% of positives for opiates	% of positives for amphe.	N. Accidents Prov.VR / Cars circulating	N. Fatal accidents/ Cars circulating	N. Injured per accident/ Cars circulating
2003	152	30	4	12	20	4	2	0,0060	0,00022	0,0082
2004	150	41	5	19	20	5	5	0,0057	0,00015	0,0077
2005	135	39	16	13	18	2	4	0,0052	0,00012	0,0071
2006	167	31	2	10	18	2	5	0,0050	0,00013	0,0069
2007	183	38	6	13	23	3	4	0,0049	0,00011	0,0067
2008	187	20	4	8	8	1	0	0,0045	0,00010	0,0060

A first comparison regarded the percentage of positivity and the number of road accidents in relation to the car fleet circulating in the province. Effectively, between the two parameters, there was a positive trend: with the growth of positivity was observed a progressive increase in the rate of car accidents. However, the coefficient of linear regression is small (R^2=0.197). If we substituted the accident rate with the rate of fatal accidents, the modest correlation disappears (R^2=0.009), while it increase slightly considering the number of injured per accident in relation to the car fleet circulating (R^2=0.226). More detailed information were obtained comparing the accident rate, with the frequency of positives decomposed into different classes of substances. In particular there was no correlation with regard to amphetamines and benzodiazepines, however, there was a better correlation with regard to the frequency of positivity for cannabinoids, cocaine and in particular for opiates in relation both to the accident rate (R^2=0.343, 0.347 and 0.779, respectively) and the number of injured per accident, compared to the car fleet circulating (R^2=0.337, 0.415, and 0.786, respectively). In relation to the frequency of fatal accident, positive trends were observed for cannabinoids (R^2=0.050), cocaine (R^2=0.186) and opiates (R^2=0.489), although only in the last case, the regression coefficient is of some importance. Since the Italian Road Code (Art. 187), requires obligatory toxicological test after a car accident, and the positive individual to these test is submitted to a clinical examination aimed at evaluating his/her fitness to drive (Art. 119 CdS), it can be argued that the positive trend observed between subjects tested positive and the number of car accident are due to the fact that if increases the number of accidents, so also increases the number of controls. However, this hypothesis is contradicted by the fact that during the period under studying, there is no positive trend between the absolute number of traffic accidents and number of checks required by Art.119 (visits for fitness to drive).

Part of the research was dedicated to evaluating the effectiveness of the investigation carried out in urine or hair. To this aim were considered the subjects for which the C.M.L. has requested both matrices (n=885, 90% of the subjects; n=628, 67%

excluded the subjects who didn't show up for the examination). Since the investigation on hair was only for opiates, cocaine and metabolites and amphetamines, the comparison was limited to these substances. In the case of urine, although we used two different protocols (see materials and methods), the protocol based on 3 urine samples randomly collected without notice to the subjects was applied in a small minority of cases (4%) therefore it was decided to carry out statistical analysis without breaking down for urine protocol. A first important result, which is largely confirmed in the literature **[19]** is that a determination on keratin matrix appears with a greater epidemiological sensitivity than urine matrix, although with interesting differences between the different classes. Indeed, while the difference in sensitivity is low in the case of opiates (0.68 against 0.55) becomes significantly higher for cocaine and metabolites (0.91 against 0.25) and amphetamines (0.86 against 0.14). This observation could be at least partly related to the different time windows of detection in urine matrix (generally greater in the case of opiates) and/or a greater tendency of opiates to determine a depending resulting in frequent use **[20]**. Furthermore, the urine protocol has shown its utility, since for all classes of substances there were subjects tested positive exclusively in urine (8% for cocaine, 14% for amphetamines and 32% for opiates). This confirm the complementary nature rather than alternative of the two biological matrices.

Apart of the outcome of the investigation and the diagnosis of fitness to drive by the C.M.L. all subjects underwent a further analysis after a variable period of time, usually determined by the diagnostic evaluation of the first check (greater interval for negative subjects)

Therefore it was considered useful to undertake a valuation of the outcome of the second check in relation to the first, in order to determine whether and how the suspension of the driving license, and the consequences that this implies on social and professional level, is an effective deterrent to drug use. The answer to this question is positive, since just over a quarter (27%) of cases positivity at the first check was

followed by a positive result at the second check. Also in this case the breakdown by classes of substances reveals interesting differences [Table 5].

[Table 5] **Analysis of positivity at the II°check**

POSITIVITY I°CHECK	% OF POSITIVITY II°CHECK				
CLASS OF SUBSTANCE	for any substances	for the same substances	for a substance different from the I°Check	% of negative	% of didn't show up
COCAINE (88)	**20**	**44**	**56**	**64**	**16**
THC (56)	**34**	**68**	**32**	**48**	**18**
AMPHETAMINES (13)	**46**	**17**	**83**	**54**	**0**
OPIATES (14)	**43**	**50**	**50**	**43**	**15**
METHADONE (6)	**33**	**50**	**50**	**67**	**0**

In fact, positivity at the second check are more frequent among subjects who tested positive at the first check for opiates and amphetamines (43% and 46% respectively) followed by cannabinoids and methadone (34% and 33% respectively) and a greater distance by cocaine. Moreover within the different classes of substances there are marked differences with regard to fidelity to the substance found in the first analysis. In fact if, for example, the positivity at the first check for opiates, methadone and cocaine, is confirmed in almost one case out of two, and in two out of three for cannabinoids, in the case of amphetamines, the second check reveals a percentage of positives less than 20%. Also the time period between the first and the second check seems to influence the percentage of positives at the second. In fact, it tends to decrease with the increasing of this range (34% within 6 months, 25% between 6 and 12 months).

7. CONCLUSIONS

The analysis of cases presented offered many points of interest and reflection. First, results confirm the complementary nature of the information provided by the analysis of hair and serial urine, since each of the two analytical strategies applied individually leads to a significant reduction in the epidemiological sensitivity of the investigation. It is also interesting to note that several associations between "risk factors" and testing positive for any drug of abuse (*i.e.* BMI<18,5, smoking, not drinking coffee, mild hypertension and, of course, a previous history of drug abuse) or for one or more classes/specific substances (*e.g.* young age and amphetamines, mild hypertension and cocaine, BMI and THC). For some of these factors, the observed difference between positives and negatives, although approaching statistical significance, does not reach it and will therefore be interesting to verify their effective role on a larger amount of data.
The comparison between self-reports of illicit drug use and urine/hair testing results shows that the reliability of self-report decreases in the following order: THC>opiates>cocaine>amphetamines.
An interesting outcome that deserves a further development is the possible relation between the number of opiates positives and the rate of car crashes observed in corresponding years.
In addition, and this is probably the most relevant and reassuring outcome of our study, it is clear from the comparison between first and the second check on the same subject, that the chance of being diagnosed as unfit for driving as a consequence of testing positive for drugs of abuse exerts a substantial deterrent effect on their consumption. In other terms, it is evident that possessing a driving license plays a crucial role both professionally and socially speaking, and avoiding the risk of not getting it back is likely considered a valid return to abstaining from illicit drugs.

A final consideration refers to the large number of subjects who didn't show up for the clinical examination requested by the C.M.L., representing over one quarter of the total requests. This phenomenon might, at least partially, be explained by the chance for the subject to decide to which Medical Commission to apply for the diagnosis of driving fitness. As a consequence, the adoption of less restrictive diagnostic criteria by C.M.L. of geographic areas close to the province of Verona might be a reason for diverting from the Verona C.M.L.. For example, the cut offs adopted for drug testing in hair are generally higher in the C.M.L of Lombardy (*e.g.* 0.5 ng/mg for cocaine) than in the Veneto Region (0.1 ng/mg), providing the applicant a more than good reason for choosing a lombard C.M.L. Therefore, it will be interesting in perspective to cross-check the databases of other proximal C.M.L. to estimate to what extent this phenomenon occurs. However, the only possibility of this occurrence is a more than relevant motivation to invoke a rapid and appropriate standardization of the analytical and diagnostic criteria applied by the different C.M.L..

REFERENCES

[1] Carmen del Rio M., Alvarez Javier F., Illicit drugs and fitness to drive: assessment in Spanish Medical Driving Test Centres. Drug Alcohol Depend, 2001, 64: 19-25.

[2] Drummer O.H., Gerostamoulos J., Batziris H., Chu M., Caplehorn J.R., Robertson M.D., Swann P., The incidence of drugs in drivers killed in Australian road traffic crashes. Forensic Sci Int, 2003, 134: 154-162.

[3] Schwilke E.W., Sampaio dos Santos M.L., Logan B.K., Changing patterns of drug and alcohol use in fatally injured drivers in Waschington State. J. Forensic Sci, 2006, 51: 1191-1198.

[4] Movig K.L., Mathijssen M.P., Nagel P.H., Van Egmond T., De Gier J.J., Leufkens H.G., Egberts A.C. Psychoactive substance use and the risk of motor vehicle accidents. Accid. Anal Prev, 2004, 36: 631-636.

[5] Landauer A.A., Drugs and driving: historical perspectives, methodological principles and current problems in : J.F. O'Hanlon, J.J. de Gier (Eds.), Drug and Driving, Taylor & Francis, London, 1986, pp17-28.

[6] Augsburger M., Donzè N., Menetrey A., Brossard C., Sporkert F., Giroud C., Mangin P. Concentration of drugs in blood of suspected impaired drivers. Forensic Sci Int, 2005, 153: 11-15.

[7] D. Lgs. N. 285 del 30/04/1992. Gazzetta Ufficiale n. 114 del 18/05/1992 http://www.mit.gov.it/mit/site.php?p=normativa&o=vd&id=1&id_cat=&id_dett=0 (ultimo accesso novembre 2010).

[8] D.P.R. 16 dicembre 1992, n. 495. Gazzetta Ufficiale 28 dicembre 1992, n. 303, S.O. http://www.altalex.com/index.php?idnot=34641 (ultimo accesso novembre 2010).

[9] D. Lgs. N. 285 del 30/04/1992. Art. 119. http://www.mit.gov.it/mit/site.php?p=normativa&o=vd&id=1&id_dett=121 (ultimo accesso novembre 2010).

[10] CONFERENZA UNIFICATA STATO-REGIONI E P.A. - PROVVEDIMENTO 30 ottobre 2007, “Intesa, ai sensi dell'articolo 8, comma 6, della legge 5 giugno 2003, n. 131, in materia di accertamento di assenza di tossicodipendenza. (Repertorio atti n. 99/CU).” Gazzetta Ufficiale n. 266 del 15 novembre 2007. http://www.gtfi.it/doc/Conferenza_unificata_Stato.pdf (ultimo accesso novembre 2010).

[11] Paraluppi P, Fassina G, Ferrari G, Tronconi L. Sostanze voluttuarie e idoneità alla guida in ambito lavorativo. G Ital Med Lav Erg 2009, 31:33-36.

[12] Tagliaro F, De Battisti Z, Lubli G, Neri C, Manetto G, Marigo M. Integrated use of hair analysis to investigate the physical fitness to obtain the driving license. A casework study. Forensic Sci Int,1997, 84:129-135.

[13] Montagna M, Stramesi C, Vignali C, Groppi A, Polettini A. Simultaneous hair testing for opiates, cocaine, and metabolites by GC-MS: a survey of applicants for driving licenses with a history of drug use. Forensic Sci Int 2000, 107:157-167.

[14] Ricossa MC, Bernini M, De Ferrari F. Hair analysis for driving licence in cocaine and heroin users. An epidemiological study. Forensic Sci Int 2000, 107:301-308.

[15] Tagliaro F, Valentini R, Manetto G, Crivellente F, Carli G, Marigo M. Hair analysis by using radioimmunoassay, high-performance liquid chromatography and capillary electrophoresis to investigate chronic exposure to heroin, cocaine and/ or ecstasy in applicants for driving licences. Forensic Sci Int 2000, 107:121-128.

[16] Centro Nazionale di Epidemiologia, Sorveglianza e Promozione della Salute. Obesità.
http://www.epicentro.iss.it/problemi/obesita/epid.asp (ultimo accesso novembre 2010).

[17] Società Italiana dell'Ipertensione Arteriosa. Linee Guida 2007 per il Trattamento dell'Ipertensione Arteriosa.
http://www.siia.it/it/siia-guidelines/siia-articles/0/detail/0/155/131/linee-guida-2007-per-il-trattamento-dellipertensio.html (ultimo accesso Novembre 2010).

[18] Automobile Club d'Italia. Studi e Ricerche. Dati e Statistiche. http://www.aci.it/?id=54 (ultimo accesso Novembre 2010).

[19] Drummer OH, The Forensic Pharmacology of Drugs of Abuse, Arnold Publishers, London, 2001.

[20] Gruber SA et al. Neuropsychological consequences of opiate use. Neuropsychol Rev, 2007, 17:299-315.