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Amatoxin poisoning treatment decision-making: Pharmaco-therapeutic clinical strategy assessment using multidimensional multivariate statistic analysis

Patrick Poucheret [a,*], Françoise Fons [b], Jean Christophe Doré [c], Didier Michelot [c,1], Sylvie Rapior [b]

[a] Laboratoire de Pharmacologie et Physiopathologie Expérimentale, UMR 95 Qualisud, Faculté de Pharmacie, Université Montpellier I, 15 Avenue Charles Flahault, F-34093 Montpellier Cedex 5, France
[b] Laboratoire de Botanique, Phytochimie et Mycologie, UMR 5175 Centre d'Ecologie Fonctionnelle et Evolutive, Faculté de Pharmacie, Université Montpellier I, 15 Avenue Charles Flahault, F-34093 Montpellier Cedex 5, France
[c] Muséum National d'Histoire Naturelle, Département Régulations, Développement et Diversité Moléculaire, CBAF: Chimie bio-organique des associations fongiques, FRE 3206 CNRS, USM 502 MNHN, Case postale 54, 57, rue Cuvier, 75005 Paris, France

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ABSTRACT

Ninety percent of fatal higher fungus poisoning is due to amatoxin-containing mushroom species. In addition to absence of antidote, no chemotherapeutic consensus was reported. The aim of the present study is to perform a retrospective multidimensional multivariate statistic analysis of 2110 amatoxin poisoning clinical cases, in order to optimize therapeutic decision-making. Our results allowed to classify drugs as a function of their influence on one major parameter: patient survival. Active principles were classified as first intention, second intention, adjuvant or controversial pharmaco-therapeutic clinical intervention. We conclude that (1) retrospective multidimensional multivariate statistic analysis of complex clinical dataset might help future therapeutic decision-making and (2) drugs such as silybin, N-acetylcystein and putatively ceftazidime are clearly associated, in amatoxin poisoning context, with higher level of patient survival.

1. Introduction

The total number of fungi species on earth is estimated to be 1.5 million including about 1000 either pathogenic or hazardous species (Bresinsky and Besl, 1990; Kavanagh, 2007). In western countries, i.e., North American and Europe as well as in Asian countries, i.e., China and Japan, higher fungi have been collected and cultivated for hundred years as medicine, "functional foods" and food (Cheung, 2008). Poisonings caused by "mushroom" toxins, i.e., inner toxic secondary metabolites, result from the confusional consumption of fruiting bodies from edible and poisonous related species (Himmelmann et al., 2001). Many case reports demonstrate that number of "mushroom" fatal poisonings increases worldwide (Barceloux, 2008; Diaz, 2005; Enjalbert et al., 2002). Among toadstools, several dozen of species representing three main genera, i.e., *Amanita*, *Lepiota* and *Galerina*, induce toxic reaction and even lethal outcome in man (Enjalbert et al., 2002; Karlson-Stiber and Persson, 2003). Of great interest is the worldwide distribution of increased number of

* Corresponding author. Tel.: +33 1 4 67 66 81 06; fax: +33 1 4 67 54 80 39.
E-mail address: patrick.poucheret@univ-montp1.fr (P. Poucheret).
[1] In memoriam.

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fatalities among cases of poisonings by amanitas, i.e., *Amanita phalloides* (Vaill.:Fr.) Lamarck (Death Cap), *Amanita verna* (Bull.:Fr.) Lamarck (White Deadly Amanita) and *Amanita virosa* (Lamarck) Bertillon (Destroying Angel), for health care and consequently management of mushroom poisoning treatment (Barceloux, 2008; Berger and Guss, 2005). In the United States and Europe, poisonous *Amanita* species are responsible for 90–95% of the fatalities occurring after mushroom ingestion (Enjalbert et al., 2002; Karlson-Stiber and Persson, 2003; Wieland, 1984). The prominent, toxic constituents of the green death cap *A. phalloides* and its relatives have been identified. Cyclic octapeptides named amatoxins, i.e., mainly α, β, and γ-amanitins, are responsible for severe liver and secondary kidney damages (Enjalbert et al., 2002; Faulstich, 1979; Karlson-Stiber and Persson, 2003; Michelot et al., 1985); α-amanitin accounts for ca. 40% of the amatoxins. Amatoxins belong to the most violent poisons from higher fungi: only one medium-size amatoxin-containing specimen contains from 10 to 12 mg amatoxins, a deleterious dose (Lethal Dose: LD_{50} of 0.1–0.5 mg kg^{-1} body weight) for human adults (Wieland, 1984).

Amanitins are cyclic octapeptides with specific properties: heat stability, water solubility to various extents and resistance to enzyme degradation. On a pharmacokinetic point of view the toxins are readily absorbed, 90–120 min post-ingestion. It is not bound to blood proteins and distribute readily in target tissues (Faulstich et al., 1985). It follows the enterohepatic cycle inducing an increased half-life that worsens the syndrome. In this context, the octapeptides would accumulate in the liver upon uptake via an organic anion-transporting octapeptide (OATP) located in the sinusoidal membrane of hepatocytes (Letschert et al., 2006). Blood detection is possible after 36–48 h (Karlson-Stiber and Persson, 2003). Excretion is mainly urinary (Jaeger et al., 1993). On the pharmacological point of view, amanitins might act through two mechanisms of action. Firstly, the toxins bind non-covalently to and inhibit RNA polymerase II mostly in liver. Shortly after, about 1 h *in vivo*, protein synthesis decreases. An elegant hypothesis relies on the amanitins behaving as suicide substrates on its target (Michelot and Labia, 1988). Since liver is an organ with high rate of protein synthesis and cell turnover, it is not surprising that it is majorly injured during amanitin poisoning (Burkhart, 1995). Secondly amanitins would act synergistically with cytokines, such as Tumor Necrosis Factor (TNF), to induce apoptosis (Karlson-Stiber and Persson, 2003). Free radical intermediates generated are associated with increased Reactive Oxygen Species (ROS) production. Subsequent intense oxidative stress would then lead to hepatocyte peroxidation and death, contributing to severe hepatotoxicity with massive centro-lobular necrosis (Zheleva et al., 2007).

Amanitin poisoning clinical scheme includes three major stages: (1) an asymptomatic latency period, (2) a gastrointestinal phase and (3) the hepatic-kidney final stage.

The asymptomatic period starts at ingestion time and lasts for 6–10 h rarely reaching 24–36 h in extreme cases (Michelot and Labia, 1988; Vetter, 1998). The gastrointestinal phase lasts for 24–48 h. Initial symptoms include vomiting, abdominal pain and choleriform aqueous diarrhea that lead to severe dehydration and hydroelectrolytic unbalance (Jaeger et al., 1993; Karlson-Stiber and Persson, 2003). Simultaneously amatoxins impair endocrine pancreatic β-cells functions inducing insulin release and hyperinsulinemia responsible for a drastic hypoglycemia. This happens before liver failure and hepatic glycogen depletion (De Carlo et al., 2003). These combined disorders can induce a hypovolemic shock. The hepatic-kidney final stage starts 48–72 h post-ingestion and lasts for 6–16 days. Liver function impairment is objectified by progressive increase of hepatic enzymes transaminases as well as higher lactate dehydrogenase (LDH) and bilirubin blood levels associated with liver enlargement and discrete jaundice. Then acute hepatic failure (centro-lobular necrosis and vacuolar degeneration) takes place with hyperbilirubinemia, liver glycogen depletion, hypoglycemia, metabolic acidosis, coagulation disorders, hemorrhage, disseminated intravascular coagulation and vascular shock (Enjalbert et al., 2002). Kidney damages, indicated by elevated urea and creatinine, occur following various offenses from all previous phases, i.e., amatoxins direct deleterious cell effects, hypovolemia and vascular shock. Ultimately, without intense and proper medical care, encephalopathy and coma happen frequently leading to patient death 6–16 days post-ingestion of amatoxins (Vetter, 1998).

As previously cited, molecular basis of amatoxin mechanism of action on their main physiological targets starts to be understood. Unfortunately, so far, no consensual antidote was discovered to counter this mushroom poisoning. In addition no appropriate therapeutic arsenal is available for the clinicians to manage the amatoxin poisoning with consistent efficacy. Therefore physician use various combinations of non-chemotherapeutic and chemotherapeutic strategies to increase survival rate of patients and minimize sequels. Waiting for a putative antidote and in order to provide an objective analysis of therapeutic schemes efficacy, two options can be chosen that are synergistic: (1) *in vivo* studies on animal models or (2) retrospective analyses of human clinical cases. Elegant investigations on animal models are performed by several research teams (Tong et al., 2007; Zheleva et al., 2007). They provide valuable insights on the interface between the physiopathological process of amanitin poisoning and the pharmacological effects of different treatments whether administered alone or in combination. In parallel to these explorations, retrospective clinical case analyses provide major information on human responsiveness to the same type of treatments (Enjalbert et al., 2002; Gonmori and Yoshioka, 2003; Giannini et al., 2007) as well as to more complex therapeutic protocols. All these investigations have one fundamental common point: to save lives.

The present statistical pharmaco-epidemiologic study originates in an amatoxin poisoning survey based on literature review of 2110 detailed clinical cases from North America and Europe; 2108 from Enjalbert et al. (2002) report and 2 complementary cases from the original database.

An integrative retrospective highly multifactorial/multidimensional statistic analysis of these properly

documented human clinical amatoxin poisoning cases was carried out in order to explore further in depth treatment schemes. Results are discussed on the base of current knowledge about amatoxin toxicology and drugs pharmacology. Prominent drug candidates are then proposed to help and optimize therapeutic decision-making in amatoxin poisoning.

2. Material and methods

2.1. Bibliographical source

Our study used a database build up from Enjalbert et al. (2002) report regarding a 20-year retrospective analysis on clinical treatment of amatoxin poisoning cases.

Chemotherapies used to treat these patients are as numerous and diverse as patients' profiles, countries, date and supportive care protocols. In consequence, traditional statistic approaches (Fischerian) or too selective multidimensional data reduction analysis such as PCA type multivariate analysis (PCA standing for Principal Component Analysis) are both inappropriate in such case of complexity. Indeed for high complexity dataset, elevated information diversity and multiple interactions prevent the analyst from researching and identifying the major elements of causality. In this situation, use of inappropriate statistic method would lead to two major drawbacks, either the impossibility to reach significant conclusion or worse to reach erroneous conclusion which is not acceptable. Therefore we used a correspondence factorial analysis.

2.2. Statistic method

A multidimensional multivariate statistic analysis was performed. In order to realize proper and refined dataset analysis, our procedure followed several steps.

We computerized all data to allow binary to n-ary correlation analyses to move from Fischerian logic (correlation coefficient on measurable variable) to Pearsonian logic (causality search using Chi-2). We prepared a logic matrix (full disjunctive table) relating, on a binary mode (1/0), patients and all continuous and discontinuous modalities encountered. Then a Burt table was constituted to systematically relate all modalities in the considered population. This gave a contingency/frequency table as reference integrating all parameters classified by type and associated with clinical case reports. This contingency table between all variables taken two by two privileging survival rate, allowed to support further analysis. On this base, we subjected these data to a multidimensional synthesis using convergent approaches: an automatic classification procedure, the ascendant hierarchical classification and a Correspondence Factor Analysis (CFA) as previously described (Doré and Jaubert, 1984; Doré et al., 1996; Hans et al., 2000). This method acts as a filter bringing to light the most legitimate correlation between variables and therefore the hidden fundamental organization of the analyzed problem. It led to determination of a therapeutic model based on analysis of drugs associations incidence on patients survival rate. This was graphically represented under a factorial map format. Position of the treatment groups on the factorial map is a function of their pharmacological and therapeutic impact on patient survival rate.

2.3. Factorial map

Let's consider the case of various drugs interacting with different patient profiles for defining a survival rate. A data matrix of the (i) molecules ($\Sigma i = n$) (rows) by the (j) survival rate ($\Sigma j = p$) (columns) is constituted. The data table is subjected to an appropriate transformation procedure to allow representation of both molecules and survival rates on single display factorial maps. The j survival rates are thus projected into the multidimensional space made up of the i molecules R^i and vice versa the i molecules are projected into the j dimensional space of the survival rate R^j. The position of each point representing a single survival rate result for a given molecule in the R^i space is given by the probability test (j) has an amplitude of f_{ij} for molecule (i) and is defined by the ratio:

$$f_{ij}/f_{\cdot j}$$

where

$$f_{ij} = k_{ij} / \sum_{ij} k_{ij}$$

and where

$$f_{\cdot j} = \sum_{j} f_{ij}$$

A symmetrical calculation defines the position of each molecule for each survival rate ($f_{ij}/f_{i\cdot}$) in the R^j space. This dual procedure yields comparable normalized profiles for the rows and columns, thus enabling their comparison by a technique that can be considered as a form of pattern recognition.

To represent these two sets of points, principal projection axes are established as in PCA by determining eigenvalues (λ) and eigenvectors (V_x). A symmetrical matrix is constituted of the distances $S_{jj'}$ between pairs of molecules (χ^2-distance) as follows:

$$R = \left\{S'_{ij}\right\}\left[{}^{n}\sum_{i=1}\left(1/f'_{ij}\right)\cdot\left(f_{ij}f'_{ij}/\sqrt{\left(f_{\cdot j}f'_{\cdot j}\right)}\right)\right]$$

The calculation is performed by solving the equations of type $[R] - \lambda\,[x] = 0$ and $[R][V_x]$ (diagonalization of the symmetric matrix). This procedure is simpler in CFA than in PCA because one of the sets of points is given by the matrix $[R]=[M]\cdot[M']$. The permutation of the indices is, thus, equivalent to transposing the matrix onto the other set of points $[M]\cdot[M']$ with the same eigenvalues as those of R. The coordinates φ_j of the tests for factorial axis α are calculated by using the formula:

$$\varphi_{\alpha j} = \lambda_{\alpha}^{1/2} V_{\alpha j} / f_{\cdot j}^{1/2}$$

Where $\lambda_{\alpha}^{1/2}$ is the square root of the non-trivial eigenvalue la, $V_{\alpha j}$ the corresponding eigenvector, and $f_j^{1/2}$ the square root of the marginal relative frequency of test (j) for the (i) molecules. The correspondence between the

molecules and tests is given by the transition formula:For the molecules

$$\varphi_{\alpha i} = \left(1/\lambda_{\alpha}^{1/2}\right)^{p} \sum_{j=1} (fij/f_{j\cdot})\varphi_{\alpha j}$$

For the tests

$$\varphi_{\alpha j} = \left(1/\lambda_{\alpha}^{1/2}\right)^{n} \sum_{i=1} (fij/f_{\cdot j})\varphi_{\alpha i}$$

The factorial axes φ_{α} are ranked by their order of importance in accounting for the total variance of the system (φ_1, φ_2, φ_3, …, φ_{n-1},). Factorial maps are then drawn by plotting any two of these orthogonal axes and displaying the projection of points.

If a large part of the total variance is not accounted for by the two principal factorial axes φ_1 and φ_2, i.e., the true points are not close to their projections onto the $\varphi_1\varphi_2$ map, it is necessary to refer to the absolute contribution (AC) and relative contribution (RC) of each variable to all factorial axes, in order to assess how properly a particular axis represents the variance of the system (ACs of the variables) and how a variable is dispersed across all the axes (RCs of the variables). For test j,

$$AC\alpha(j) = f \cdot \varphi_{\alpha i}^2/\lambda_{\alpha} 100\left(\sum ACs = 100\%\ for\ any\ axis\ \alpha\right)$$

$$RC\alpha(i) = \varphi_{\alpha i}^2/d_p^2(i,G)100 \times \left(\sum RCs\ of\ each\ variable\ to\ all\ axes = 1\right)$$

Where G is the distance from the center of gravity of the points. RC is in fact the square of the cosine of the test j for axis α.

3. Results

Results were obtained after analysis of data from a retrospective analysis of reported amatoxin poisoning clinical cases (Enjalbert et al., 2002). On 2110 patients receiving medical care, 1866 patients survived and 244 died defining an average mortality rate (MR) of 11.58%. From the same set of 2110 subjects, 1632 (77.34%) were treated with various types of chemotherapy. Among these 1632 treated patients 1458 survived and 174 deceased. Overall, mortality rate under chemical treatment was 10.66%. Thirteen different active principles were reported whether used alone as monochemotherapy or in combination as bi, tri or polychemotherapy.

3.1. Basic analysis

Statistic comparisons between groups MR were performed before further exploration. This analysis highlighted significant differences between the chemotherapy used. Based on these data, 6 pooled mortality/survival ratios (from A to F) were made up (Fig. 1). As shown in Fig. 1, chemotherapies with the highest average survival rates (97.2%)/lowest MR (2.8%) were the silybin/ceftazidime (group F) and NAC (N-acetylcystein) (group E) with an average MR of 6.8%. Within group D (MR of 13.32%) no statistic difference could be observed between its composing active principles: benzylpenicillin, vitamin C (ascorbic acid), cimetidine and antiseptic agents. Group D demonstrated a significantly higher MR when compared to groups E/F. No statistic difference between treatments within group C (insulin/glucagon and insulin/growth hormone and corticosteroids) was shown. Finally, group B (antibiotics and thioctic acid) and group A (vitamin E) presented the lowest survival rates. Therefore groups A, B and C demonstrated the highest MR ranging from 15.96 to 40% (Fig. 1).

A Burt matrix was then realized in order to perform advanced analysis. This matrix integrated all parameters from reported clinical cases such as chemotherapy type, non-chemotherapeutic clinical care, dates, country, mushroom types and patient age. Chemotherapy part of this large matrix is presented as Table 1.

3.2. Advanced analysis

Results were then further analyzed and plotted as a two axes factorial map (Fig. 2). This map indicates the impact of the treatment factor on patient survival. The following

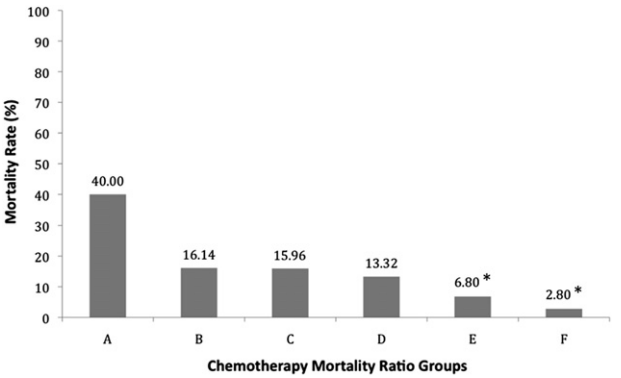

Fig. 1. Pooled mortality rate associated with statistically stratified chemotherapy groups. Group A (vitamin E), group B (antibiotics and thioctic acid), group C (insulin/glucagon, insulin/growth hormone and corticosteroids), group D (benzylpenicillin, vitamin C, cimetidine and antiseptic agents), group E (N-acetylcystein), group F (silybin/ceftazidime). (*stands for statistic significance).

Table 1
Burt matrix excerpt: chemotherapy part of the contingency/frequency table. ATB (antibiotics: gentamycin, neomycin, streptomycin, vancomycin, clindamycin), B-Pen. (benzylpenicillin), Cimet. (cimetidine), NAC (N-acetylcystein), Thioct. A. (thioctic acid), ATS (antiseptics), Vit. C (vitamin C) Vit. E (vitamin E), Ins. (insulin), Gluc. (glucagon), hGH (human growth hormone), Silyb. (silybin), Ster. (steroids), Cefta. (ceftazidime).

	ATB	B-Pen.	Cimet.	NAC	Thioct. A.	ATS	Vit. C	Vit. E	Ins./Gluc.	Ins./hGH	Silyb.	Ster.	Cefta.
ATB	63	48	0	1	27	8	16	0	0	0	34	36	0
B-Pen.	48	1411	18	102	429	9	43	25	128	69	530	443	0
Cimet.	0	18	21	3	0	0	1	0	0	0	4	4	0
NAC	1	102	3	192	0	0	6	0	0	0	1	1	0
Thioct. A.	27	429	0	0	450	0	12	0	0	0	110	157	0
ATS	8	9	0	0	0	19	18	0	0	0	6	5	0
Vit. C	16	43	1	6	12	18	60	0	0	0	10	15	0
Vit. E	0	25	0	0	0	0	0	25	0	0	0	25	0
Ins./Gluc.	0	128	0	0	0	0	0	0	128	69	0	128	0
Ins./hGH	0	69	0	0	0	0	0	0	69	69	0	69	0
Silyb.	34	530	4	1	110	6	10	0	0	0	624	67	12
Ster.	36	443	4	1	157	5	15	25	128	69	67	459	0
Cefta.	0	0	0	0	0	0	0	0	0	0	12	0	12

groups were defined: group A (vitamin E), group B (antibiotics and thioctic acid), group C (insulin/glucagon, insulin/growth hormone and corticosteroids), group D (benzylpenicillin, vitamin C, cimetidine and antiseptics), group E (N-acetylcystein) and group F (silybin). Four major points can be made.

First, two active principles, located in the left middle and upper side of the factorial map, clearly showed positive effect on patient survival: silybin and NAC. Silybin, whether administered alone or in combination with other treatment, brought MR down to 5.6%, meaning half the average mortality (10.66%). This result was consolidated by the elevated number of case report for this active principle (624 patients treated with silybin out of the 2110 clinical cases, Table 1) when compared to other drugs. NAC, with 192 treated patients reduced MR to 6.8%, also close to half the average. Therefore these two active principles stood apart from all others. In addition, in the apparent highest range of survival we found ceftazidime. Two specific remarks should be made regarding its result reaching 0% mortality: (1) ceftazidime was always associated to silybin and (2) the number of cases was limited to 12. Obviously further investigations should be carried out on ceftazidime in order to confirm or not this observation with a statistically reliable result.

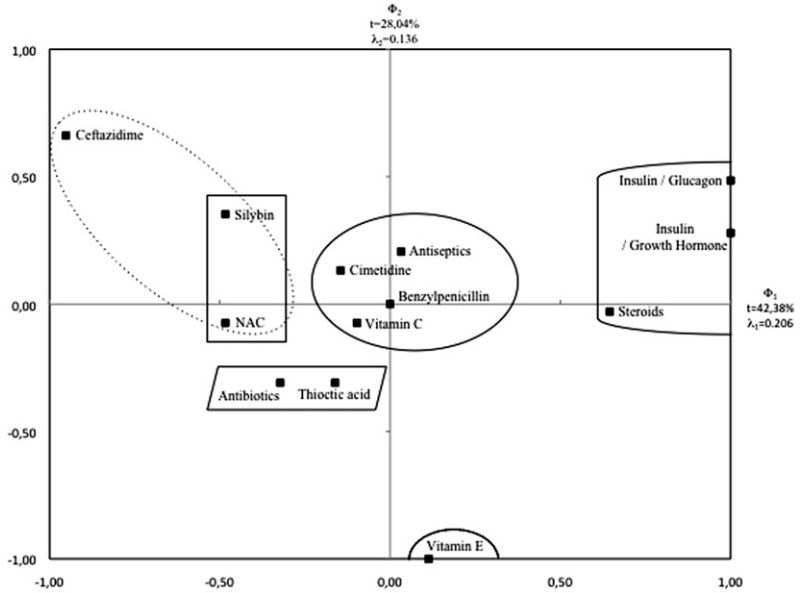

Fig. 2. Factorial map of chemotherapy classes associated incidence on patient survival rate after multidimensional statistic analysis. Therapies with statistically positive impact on amatoxin poisoning are located in the left superior quarter, neutral therapies are located in the center of the map, treatments with no or inappropriate effects are respectively located in the lowest part and the far right center of the factorial map. Number of clinical cases per therapy: Group A (vitamin E: 25 patients), group B (antibiotics: 63 patients and thioctic acid: 450 patients), group C (Insulin/Glucagon: 128 patients, Insulin/Growth hormone: 69 patients and corticosteroids: 459 patients), group D (benzylpenicillin: 1411 patients, vitamin C: 60 patients, cimetidine: 21 patients and antiseptic agents: 19 patients), group E (N-acetylcystein: 192 patients), group F (silybin: 624 patients/ceftazidime: 12 patients).

Second, a central group on the map is group D. In this cluster of treatments, benzylpenicillin represented the most frequently used drug with 1411 patients treated (Table 1). In spite of its classical use in amatoxin poisoning medical care, MR was 10.68% still close to average. Regarding vitamin C (60 cases) and cimetidine (21 cases), they presented above from average MR of 19.05% and 14.28%, respectively. In the same group, antiseptics (19 cases) seemed to be advantageous (9.1% MR) but, as for ceftazidime (12 cases), limited number of reports decreases the confidence that one could grant in the impact of this therapy. More data would be necessary to sort out this issue. Overall, in group D, the multidimensional analysis indicated that no significant tendency toward either favorable or unfavorable incidence on survival could be detected. These treatments appeared somehow as neutral in term of pharmacological effect when considering the course of poisoning syndrome.

Group B included antibiotics (63 cases) and thioctic acid (450 cases) with respective MR of 20.27% and 12% therefore above or close to average. These compounds, located in the lowest left part of the factorial map (Fig. 2), tended not to have a significant influence on survival and were associated to poor drug action in the amatoxin poisoning context.

Third, moreover, group A with vitamin E (25 cases), as seen in the lowest middle zone of the map (Fig. 2), showed pharmacological and therapeutic inefficacy. However, in spite of its 40% MR (Fig. 1), vitamin E did not seem to jeopardize patients survival.

Fourth, oppositely, the most unfavorable group of molecules for patient survival, located at the far right of the factorial map (Fig. 2) is composed of steroids (459 cases) on one side and of combinations of insulin with either growth hormone (69 cases) or glucagon (128 cases) on the other side. These treatments with pharmacological intrinsic efficacy appeared to have a deleterious incidence on survival rate suggesting an increased risk for survival prognostic.

4. Discussion

The present study was designed to analyze the benefit/risk balance of various anti-amatoxin chemotherapy used among the 2110 amatoxin poisoning cases reported in a survey realized by Enjalbert et al. (2002). The aim of this analysis was dual as (1) to demonstrate the interest of complex data multidimensional statistic analysis for pharmacological and therapeutic identification of safe and effective chemotherapy and (2) to valorize this large reported dataset through optimization of chemotherapeutic decision-making in order to save human lives.

In order to identify a clue of optimality among treatments, multidimensional statistic analysis was applied to literature clinical reports. Focusing on one discriminant factor (MR $<$ 10%), treatment classes were ranked as first intention, second intention, adjuvant and recovery treatments. Obviously, drugs identified as potentially harmful would be excluded from amatoxin poisoning therapeutic arsenal.

In this respect, our data indicate that treatments based on hormones (insulin, growth hormone, glucagon) and steroids tend to have a negative impact on patient survival (Figs. 1 and 2). The benefit/risk balance seems to be oriented toward higher risk when compared to any other treatment. The intrinsic pharmacological properties of these compounds are elevated. Their proper action belongs to the most powerful ligands as they are themselves mediators or structurally close to endogenous mediators. Therefore their lack of clinical benefit is not associated to an absence of action but rather to an inappropriate influence on poisoning generated metabolism imbalance and glucose homeostasis disturbance (De Carlo et al., 2003).

Drugs such as vitamin C, vitamin E, cimetidine or thioctic acid have direct and indirect antioxidant properties with no particular tissue tropism. Cell exposure to high level of xenobiotics, such as amatoxins, is correlated with high level of ROS production (Reactive Oxygen Species) (Zheleva et al., 2000, 2007). In the amatoxin poisoning case, ROS but also very reactive RSS (Reactive Sulfur Species) are produced, both leading to major oxidative structural and functional hepatic cell injury, i.e., lipoperoxidation of cell membranes and oxidation of proteins, nucleic acids as well as all cell components (Zheleva et al., 2007). Absence of significant action of cited drugs on patient survival might be due to a free radical scavenging effect that appears not to be focused enough on the major site of acute oxidative stress in amatoxin poisoning: the liver. In addition, it should be noted that cimetidine inhibits cytochrome P450. Cimetidine influence on putative amatoxin detoxication pathway might influence native toxin and metabolites levels in hepatocytes (Burkhart, 1995). Regarding thioctic acid, also called lipoic acid, this molecule is well-known for its potentially deleterious side effects such as hypoglycemia that may increase amatoxins induced glucose homeostasis disturbance. Beside this compound stimulates COX (cyclooxygenase) activity and associated prostaglandin production. This action could partially antagonize potent drugs such as silybin if associated in therapeutic schemes. All these elements taken together may explain why thioctic acid is a controversial molecule in the context of amatoxin poisoning treatment. It could also explain the reason why it is frequently considered as potentially able to jeopardize patient recovery and even survival (Marshall et al., 1982). Globally considering their non significant positive impact or inefficacy on poisoning outcome, all non-specific antioxidants still have to demonstrate their own interest if any.

Close to the central cluster of drugs previously mentioned (Fig. 2), are located antibiotics (ATBs). Most ATBs are now considered not to be used anymore. Our analysis argues for such a trend, since ATBs do not improve survival rate and demonstrate poor therapeutic effect in the poisoning context.

However, one particular molecule, benzylpenicillin (B-Pen), has to be studied specifically. This drug is historically the most widely used by clinicians for its hepatoprotective potential (Giannini et al., 2007). On a pharmacological point of view, if inhibition of amatoxins hepatocyte uptake by B-Pen was ruled out in rat hepatocytes (Kröncke et al., 1986) this might not be the case in human. Indeed, in human the OATP transporter is markedly different from rat in its amino acid sequence leading to difference in affinity for substrates and/or inhibitors. Indeed benzylpenicillin

was demonstrated to be a substrate for OATP and to inhibit amanitin uptake (Letschert et al., 2006). However this effect might only be obtained for very high dose and blood concentration. B-Pen mechanism of action at cell entry and intracellular level remain to be fully elucidated. In any case it should be noted that β-lactams (including B-Pen), administered at high dose, would inhibit DNA polymerase alpha, eukaryote cell proliferation and decrease γ-aminobutyric acid production, thereby limiting hepatic injury (Daoudal et al., 1989). To obtain this effect it should be mentioned that high doses are required, narrowing the therapeutic/safety margin. Therefore these elements explain why, our statistic results position B-Pen in a neutral zone of the factorial map (Fig. 2) in term of survival rate and therapeutic potency. These results and conclusion are further consolidated by the highest number of reported cases with B-Pen when compared to any other compound (Table 1). Consequently, in order to avoid risk of collateral damages to amatoxin poisoning therapy, B-Pen should only be used when other more efficient treatment is not available as previously suggested (Karlson-Stiber and Persson, 2003).

Another antibiotic with hepatoprotective properties was suggested to be administered in amatoxin poisoning care: ceftazidime (CFZ). This compound belongs to the third generation of cephalosporins class which demonstrate the same effects than β-lactam described in the previous paragraph. However regarding cephalosporins and more specifically CFZ two major differences should be pointed out (Cottagnoud and Neftel, 1986): (1) it is more powerful than penicillin and (2) it produces much less adverse effects. Our statistic result, as well as literature (Daoudal et al., 1989), tends to confirm these observations. On our factorial map (Fig. 2), CFZ is positioned in the highest left corner suggesting major positive impact on patient survival correlated with elevated therapeutic potency. Nonetheless this result should be moderated. Indeed, in our study the number of amatoxin poisoning cases treated by CFZ was limited (12 cases out of 2110). In addition CFZ was always associated to a very potent drug, namely silybin. So, even if strong arguments support CFZ as a potentially good therapeutic candidate for amatoxin intoxication care, further investigations and/or case report analysis are clearly needed to validate this observation for larger clinical application.

Among drugs statistically demonstrating significant therapeutic interest for amatoxin poisoning treatment from our factorial mapping (Fig. 2), the antioxidant N-acetylcystein (NAC) steps out (192 cases out of 2110). However, our results (previous paragraphs) suggest that antioxidant compounds with non-specific/non-targeted action do not demonstrate significant therapeutic interest. Therefore high positive impact of NAC on patient care indicates a singularity in the action profile of this compound. NAC potency could differentiate from other antioxidant molecules because it would combine classical non-specific free radical scavenging effects with additional specific effects. During poisoning course, amatoxin generates, among other consequences, an important production of free radical species (ROS and RSS) (Zheleva et al., 2007). It promotes hepatocyte death and liver glutathione depletion (Michelot and Labia, 1988; Kawaji et al., 1990). In this context, our hypothesis is that NAC would act at two levels. First NAC would scavenge free radicals as a non-specific effect. Second NAC more specific effect could rely on liver glutathione repletion since N-acetylcystein is a recognized glutathione precursor. In addition NAC might alter amatoxin intramolecular tryptathione bridge whose integrity is known to be essential for toxicity (Wieland, 1984; Zheleva et al., 2000). This hypothesis needs further investigation.

In the left superior corner of the factorial map (Fig. 2), is located the bioactive compound named silybin. Silybin is a flavolignan isolated from *Silybum marianum*, a Mediterranean milk thistle (Flora et al., 1998). Based on our multivariate analysis of 2110 amatoxin poisoning clinical cases (Fig. 2), both active principles, silybin and NAC (respectively group F and group E on Fig. 1), seemed to demonstrate more pronounced clinical benefit in term of patient survival.

Silybin was the second most frequently used chemotherapy (624 cases out of 2110), after B-Pen but generated the highest survival rate. Beneficial effect of silybin in amatoxin poisoning starts to be recognized and our statistic retrospective clinical analysis supports these clinical observation and trend. Several facts might explain this therapeutic potency. Silybin would act at two main levels: (1) pharmacodynamic level, and (2) pharmacokinetic level. At the pharmacodynamic level, silybin shows protection properties and restoration abilities. The first protective effect is based on silybin being an antioxidant, inhibiting production and propagation of oxidative stress. This molecule behaves as a free radical scavenger on ROS, RSS, lipoperoxides, superoxides and prevents liver glutathione depletion. It also inhibits production of these highly reactive molecular species and their potential derivatives such as AGE (Advanced Glycation Endproducts) (Zheleva et al., 2000, 2007). Propagation of membrane oxidation is limited because of silybin promoting membrane stabilization and fluidity, thereby avoiding peroxidative chain reaction and ultimately cell death, i.e., hepatocyte death. The second protective effect is anti-inflammatory. Silybin inhibits lipoxygenase and decrease leucotrien production (Saller et al., 2001). Additionally, the third protective effect, anti-fibrotic, allows liver to architecture preservation thereby avoiding organ remodeling and associated hepatic insufficiency. Silybin also demonstrates tissue and metabolic restoration abilities coming from DNA-dependant-RNA polymerase I stimulation. This molecular activation, happening only in injured hepatocytes, would promote liver regeneration (Pradhan and Girish, 2006). All pharmacological effects cited above tend to protect liver against both molecular and tissue lesions but also promote regeneration of organ damaged territories. At the kinetic level, silybin would be active at two levels: (1) amatoxins input and (2) amatoxins output from hepatocytes. Indeed first silybin can compete with amatoxins for cells entry. It would lock amatoxins binding site thereby reducing amatoxins liver uptake (Faulstich et al., 1985; Kröncke et al., 1986). In addition, silybin inhibits P-glycoprotein induced cellular efflux therefore reducing xenobiotic (toxin) rejection by affected hepatocytes. These two kinetic effects would therefore reduce amatoxins enterohepatic

recirculation and consequently its half-life, deleterious effects and prolonged hepatocyte exposure. Finally, silybin efficacy is correlated with reduction of lag time between intoxication and treatment initiation. Combining such converging pharmacological and pharmacokinetic particularities associated with intrinsic hepatoprotective action and clinical safety, currently makes silybin an active principle of major interest in amatoxin poisoning chemotherapy (Pradhan and Girish, 2006).

5. Conclusion

In conclusion, our investigation provides the first rational scientific trend for optimizing chemotherapeutic decision-making in amatoxin poisoning clinical care strategy.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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