Identification and assessment of new psychoactive substances: a European network **(NPS-EURONET)**

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Plenary Meeting No. 2

Chair: Ettore Zuccato **Rapporteur: Lubertus Bijlsma**

Venue: Faculty of Pharmacy of the University of Lisbon, Lisbon (Portugal) **Date: 28/09/2016**

Attendees: Ettore Zuccato (**EZ**), Emma Gracia Lor (**EGL**), Mario Dias (**MD**), João Franco (**JF**), Maria Bronze (**MB**), Cristina Sampayo (**CS**), Alexandre Quintas (**AQ**) Felix Hernandez (**FH**), Suzana Simões (**SS**), Lubertus Bijlsma (**LB**), Dora Brites (**DB**), Alvaro Lopes (**AL**), Nuno Silva (**NS**)

The meeting started at 09:00 on Wednesday September 28th

1. Welcome

Attendees were welcomed to the FFUL by **MD**

2. Opening of the meeting and information (EZ)

All 4 partners, 3 associated partners and the project officer were invited to attend the meeting.

Apologies were received from the Norwegian associated partners and the project officer.

- *As announced in the first plenary meeting in Milan, the Norwegian members are now associate partners and don't receive budget for traveling and other activities. Therefore, they have applied for finance in order to be able to work, but without success*.

Project website:

- A project website has been developed by MN: http://www.npseuronet.eu/
- It consist of a *public area* and a *private area* (access by password, for partners and associated partners)

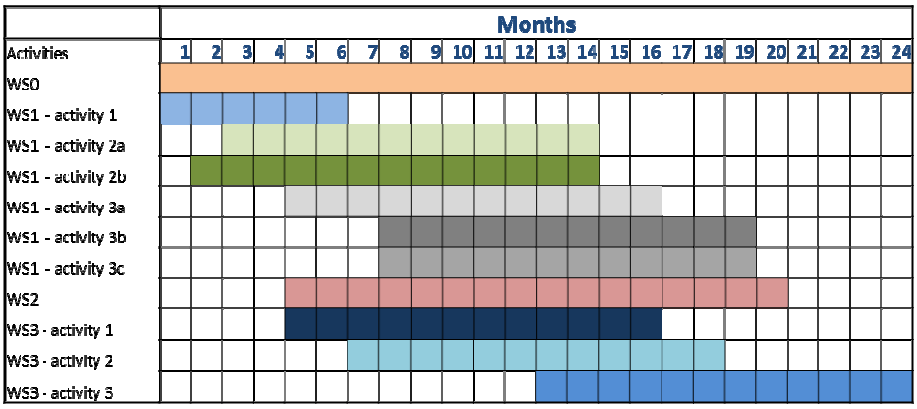
- *Seafile*: is a server (platform) which allow to exchange large files between Partners - one/two persons per partner could apply for a password. These persons in charge will receive a program to be installed and a password for access.

➢ *All Partners have been asked to report errors, modifications and/or amendments of the website*

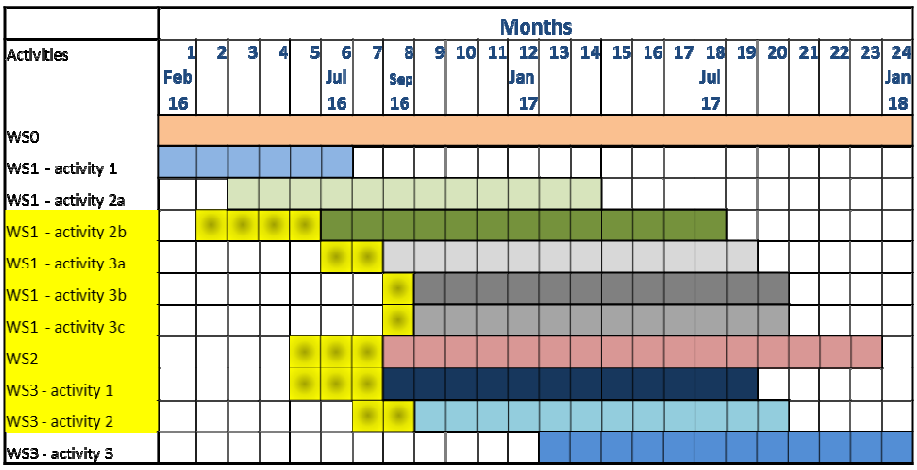
Gantt modification:

- There is a delay of activity 2b, due to sampling of the music festivals in Norway. This took place in August instead of March. Furthermore there were some laboratory issues at UJI and MN. There is therefore a proposal of modification of the Gantt. The proposal was accepted by the Partners.

Original



Revised



The Norwegian members: Although no financial support is received, the Norwegian members do support the project. We have decided to assist them in sampling the music festivals and two persons were sent 1) Francesco Riva of MN, 2) Alberto Celma Tirado of UJI in august 2016.

Communication with EMCDDA and UNODC:

- EMCDDA: Paul Griffiths – Liesbeth Van Dam (Lisbon Office)
- UNODC: Angela Me – Thomas Pietschmann (Vienna Office - Research and Trend Analysis Branch)

- Discussion topics proposal (EZ)
  - NPS list revision
  - NPS selection for RA studies
  - Definition of tasks and timing (Gantt revision)
  - Next project meeting
  - 1 year report
  - Committee activities (steering committee for publication; progress monitoring committee , coordinating groups for the WS)

3. Presentations of partners and associated partners

Each partner gave a short presentation (10-15 min/group): explaining their activities of the last months within the different WS.

➢ *The presentation will be made available for all project partners (in the restricted private area of the website of the project). In addition a short summary is provided below.*

- Instituto Mario Negri (Milan, Italy) by **EGL**:

  Mario Negri prepared the list of priority NPS (200 compounds), based on information provided by EMCDDA, National Focal Point and UNODC. Screening of these priority

compounds was performed in pooled wastewater samples, which were received from 19 European cities, and 3 pooled urine samples collected in a rock music festival in Oslo. The methodology used for the analysis of both types of samples was presented: SPE extraction, analysis by QExactive-Orbitrap and data processing using two Thermo softwares. Some preliminary results were presented but more analysis are needed for the identification and confirmation of the "suspected" compounds. This work will be done in the coming weeks.

- Instituto Nacional de Medicina Legal e Ciencias Forenses (Lisbon, Portugal) by **SS**:

  Wastewater and Urine Drug Analysis by LC-MS/MS – Our Experience. In the year of 2012, the problem related with the NPS abuse all over the world, became also a problem in Portugal. In beginning of 2013; a new law was published to regulate the commercialization of these products. Following these events, an SPE-UPLC-MS/MS method that included eight of the most common synthetic cannabinoids at the time, some of the main metabolites, and the classic cannabinoids (THC, 11OHTHC and THCCOOH) was developed and validated to analyse urine samples. This method has been applied in the laboratory routine and in a partnership between a Central Hospital of Lisbon and our institute, where 72 urine samples were collected in the emergency department. Five samples were positive for synthetic cannabinoids, three of them in association with THC and/or THCCOOH. The synthetic cannabinoids detected were, mainly, metabolites of JWH018, the N-hydroxypentyl and the N-pentanoic acid metabolites, and the N-5-hydroxypentyl metabolite of JWH122. Regarding the wastewater analysis, since 2014, our laboratory collaborates with the University of Pharmacy of Lisbon, in the SCORE project. An SPE-UPLC-MS/MS analytical method was developed and validated according to the international guidelines. The project included an inter-laboratory calibration protocol, and the analysis of wastewater samples collected from sewage treatment plants from two different cities of the Lisbon region (Alcântara and Almada) in seven consecutive days.

- Universitat Jaume I (Castellón, Spain) by **FH**:

  UJI collected and received individual and pooled urine and wastewater samples, ~600 and 10 samples, respectively. Furthermore, 5 pooled urine samples were collected from Norwegian festivals (Oslo, Trondheim). A target and post-target methodology has been developed for screening of NPS in both type of samples. Although some preliminary results were presented, there is a considerable delay in data processing, due to the change in software and the adoption to the new workflow. In the coming weeks extra effort will be made to perform screening on the priority list of NPS. Finally, outreach and dissemination has been presented.

- Faculdade de Farmacia Universidade de Lisboa (Lisbon, Portugal) by **MB**:

  The presentation was focused on the work the group from FFULisboa did, concerning WS3/1: Synthesis and Analysis of NPS and their metabolites: the synthesis and NMR and MS characterization of buphedrone hydrochloride (cathinone chemical class) was performed. In order to start the synthesis of metabolites defined by the in silico studies it was mentioned that it was mandatory to define which drugs are going to be studied under the scope of the project

- Egas Moniz - Cooperativa de Ensino Superior (Monte Caparica, Portugal) by **AQ** and **AL**:

  AQ: *Development of a high throughput methodology to screen drugs toxicological impact.* The yeast Saccharomyces cerevisiae is the most studied eukaryotic cell model being the first organism which genome was fully sequenced in 1996. Yeast models have been paramount for the current understanding of conserved cellular mechanism such as cell division, DNA replication, metabolism, protein folding and intracellular transport. This high degree of cellular pathways conservation makes S. cerevisiae an exceptional tool to study human diseases pharmaceutical drugs, environmental pollutants impact and toxic substances. The study of the growth of yeast cultures is a basic method to screen substances. The study of the growth of yeast cultures is the basic method to screen substances. For this purpose investigators do a time course of absorbance at 600 nm. The absorbance reading is due to light scattering associated to cell suspension. This means that absorbance increasing at 600 nm reflects optical density increase associated to cell growth. The plot of optical density at 600 nm versus time gives information such as the lag phase, acceleration phase, exponential phase, retardation phase and stationary phase. In here we have coupled large throughput of yeast growth cultures with different amounts of diclofenac with a non-linear regression analysis of the data to obtain rate constant, duplication time, lag time and final biomass. Yeast growth at different concentrations of diclofenac has shown statistically significant differences.

  AL: NPS's State of the Art: The author presented a selection of recent NPS's reports/data/events (2nd quarter of 2016) which might be of interest for the NPS-Euronet project partners/associate partners.
  Focus was put on four main topics: The Euro-DEN (European Drug Emergencies Network) report, the amending on the EU's Early Warning System and risk assessment, the IV International Conference on NPS's in Budapest and the EMCDDA-Europol 2015 Annual Report.
  The August 2016 report of the Euro-DEN Plus centers was briefly presented showing hospital emergency data from 20 sentinel sites in 14 countries collected over a two-year period (October 2013 to September 2015). There was a total of 10956 presentations and in what concerns NPS's they were much less reported (11%) being

cathinones, in particular mephedrone the most frequent reported. NPS's (mephedrone) were involved in nine deaths (fatality rate=1.1%). Only a minority of cases had a laboratory toxicology confirmation which in the author's opinion and also briefly mentioned in the end of the report, corresponds to a strong limitation in the study based mainly in consumer's information. It was underlined the best value that the current NPS-Euronet project will be able to bring in the future bypassing this limited type of approach when analytical data will come out from all the hospital samples after being processed.

Also, in the month of August an update has been made by the Commission to the EU's Early Warning System and Risk Assessment follow up's, briefly creating quicker and more expedite forms of evaluating new cases of NPS's with an emphasis on risk assessment procedures and information, among others, on pharmacological and toxicological properties, abuse liability and behavior effects. To the author's opinion the NPS-Euronet project, mainly through its Workstream 2 is in perfect tune with these aims.

The third topic was about the IV International Conference on NPS's held in Budapest in 30-31 May. The author has participated as an online stream viewer of the main sessions and as a later access viewer of most of the presentations. A brief selection has been made with the purpose of possible interest for the project. The EMCDDA described the ongoing rules of the signal management system and prioritization for risk assessment on new cases. Serious adverse events are the very important signals. The rising of new opioid derivatives was also mentioned as the case study of U-47700 with a kilogram seizure in Spain which might suggest a search on the project samples mainly those from Spain. Ocfentanil and mainly acetylfentanyl has been involved recently in 32 deaths and 8 acute intoxications in 4 member states. Also in the meeting it was pointed out the rise of the prevalence of injected substances (NPS's) which is a growing concern on health risks. The last selected topic from this meeting was about the so called "Pharma-terrorism" with the widespread use of Captagon (fenetylline) in middle east countries and among Jihadists for their terrorism actions namely those that occurred in Paris attacks. It could be relevant to consider this drug on the project search but the metabolism into some other common drugs (amphetamine and theophylline) will pose obstacles to this aim. Several million tablets have been seized in 2016 (Egypt). This drug could appear in Europe as well.

The fourth topic was about the EMCDDA-Europol 2015 Annual Report with the presentation of the 100 NPS's notified during the year of 2015 and the EWS alerts. The author showed that an "old" NPS (PMMA) has been notified multiple times and involved in several fatal cases all across Europe giving a glimpse of recurrent appearance into the drug scene after a decade. The author suggested that this 2015 (recent) notified NPS's list could also be a good starting point for project samples search.

In the end, diagrams of the ongoing Workstream 1 (W1) and starting Workstream 2 (W2) tasks were shown with an emphasis on the forthcoming W2 multiple protocols

involved with an "urgent" need for clarification of the NPS's to be assessed in order to avoid penalizing delays

- Faculdade de Farmacia Universidade de Lisboa (Lisbon, Portugal) by **DB**:

  Risk assessment of NPS: neurotoxic and neuroinflammatory effects
  Our group "Neuron Glia Biology in Health and Disease at the Research Institute for Medicines, Faculty of Pharmacy, University of Lisbon, are interested in dissecting how neurotoxic compounds, such as NPS, activate neuropathological pathways that lead to neuroinflammation and neurodegeneration with the final aim to define targets and develop novel preventive actions and target-driven therapies. Not much is known about the dangerous properties of NPS as proinflammatory mediators and inducers of neuronal dysfunction. Therefore, further investigation is needed to clarify these issues, which are necessary to better understand the acute and lasting consequences of their administration. Our preliminary results with methedrone indicate that in concentrations above 10 µg/mL decrease neuronal viability and lead to the release of alarmins, such as high mobility group box protein 1 (HMGB1). Levels above 150 µg/mL lead to a reduction on neurite length, compromising neuronal network, and trigger the death of microglia, the innate immune cells of the central nervous system. As soon as we will have the identification of the most used drugs with potential neurotoxicity, to be selected by the consortium members, we will pursuit our studies on their comparative neurotoxic and neuroinflammatory properties.

- Faculdade de Farmacia Universidade de Lisboa (Lisbon, Portugal) by **CS** and **AL**:

  CS: Since 1999 the EMCDDA has conducted the risk assessment (RA) for near 20 substances among which 9 are new psychoactive substances (NPS) (1). As the number of NPS keeps growing, biomedical data, based on systematic pharmacological and toxicological studies, will rarely be available for the RA of these NPS. Behavior does represent the integration and the integrity of the nervous system (function and structure). Thus, it is generally considered a sensitive indicator of nervous system compromised structure and function as a result of chemical exposures. The observational battery of behaviors (FOB) data, published so far on these NPS, showed that in addition to typical stimulant effect, similar to those produced by classic stimulants, at higher doses some cathinones produced ataxia, convulsions and increased exploration and bizarre behaviors (2-4). Contrariwise to cathinones behavior effects, 6 synthetic cannabinoids belonging to the JWH family, in addition to the typical depressor effect of THC, unlike THC, they also produce signs of CNS excitability, sensor motor reactivity as well as autonomic effects (5). Therefore, when selecting substances to study in WS2, which should happen the sooner as possible, it is of relevance to take

into account the substances that have been already submitted to a RA and those that have been deeply studied.

1. EMCDDA site, visited on Sept 2016
2. Marusich et al., Neuro Toxicol, 2012;33:1305
3. Marusich et al., Neuropharmacol, 2014;87:206
4. Marusich et al., Neuropharmacol, 2016;101:68
5. Wiley et al., Neuropharmacol, 2016;110:143

- Faculdade de Farmacia Universidade de Lisboa (Lisbon, Portugal) by **NS**:

  The objectives of activity 3C to be performed by FFUL (Nuno Silva and Paulo Paixão) include the investigation of NPS oral absorption potential, biodistribution potential patterns, as well as metabolism and excretion patterns. The understanding of what the body does to a selected NPS will enable to predict plasma pharmacokinetic profiles and dose dependence, as well as specific organs biodistribution.
  For this purpose, specific QSAR, artificial neuronal network (ANN) and physiologically based in silico models will be used.
  Chemical properties of selected NPS will obtain from different in silico sources through SMILES notation, using specific software (ALOGPS 2.1, E-Dragon, CORINA, Chemaxon). Molecular descriptors will be obtain for ionization, lipophilicity, size, compactness, and solubility profiles.
  An ANN will be used with 12 molecular descriptors to estimate NPS apparent permeability in caco-2 model and then the human effective permeability
  An ANN and other algorithms will be used to estimate tissue-to-blood partition coefficients for several tissues and the apparent volume of distribution in individual organs. Additionally, NPS plasma protein binding and red blood cells binding will also be predicted.
  An ANN with 21 molecular descriptors will be used to estimate in vitro intrinsic clearance and therefore estimate total clearance.
  Apart from ANN methodology, a 'whole body' physiologically based model will be used to reach activity objectives, through the professional software (PK-Sim - Bayer Technology Services). This software has incorporated algorithms for the estimation of human effective permeability and organ/plasma partition coefficients (Rodgers & Rowland, Schmitt, Poulin & Theil and Berezhkovskiy). Elimination characteristics will be based in ANN estimates.
  With the use of this software it will be possible to estimate pharmacokinetic patterns for different population cohorts (stratification by race, gender, age groups) and for different administration protocols (iv, oral, fasted, fed).

4. Discussion and conclusions

- A priority list of NPS (*WS 1, activity 1*) has been compiled by MN and UJI. This list consist of approximately 200 compounds and is available for all partners at the private area of the website. Within this list there is a so called "yellow list", consisting of 18 NPS which has been most frequently reported to National Focal Points and EMCDDA. **EZ** will revise this list and include some synthetic cannabinoids according to frequency of reporting (before 10th of October). It was also suggested (FH) to include fenetheylline, also known as Captagon or "the terrorist" drug.

The "Yellow list"

25B-NBOMe
25C-NBOMe (2C-C-NBOMe)
25I-NBOMe
6-APDB
PMA
PMMA
Methoxethamine (MXE / 3-MeO-2'-Oxo-PCE)
3-methylmethcathinone (3-MMC)
3,4-dimethylmethcathinone (3,4-DMMC)
4-MEC (4-Methylethcathinone)
bk-MDMA (3,4-methylenedioxy-N-methylcathinone/ Methylone)
MDPV (3,4-Methylenedioxypyrovalerone)
Mephedrone (4-MMC)
Pentedrone (α-methylamino-valerophenone/ β-ethyl-methcathinone)
α-PVP (α-Pyrrolidinopentiophenone / α-pyrrolidinovalerophenone)
Acetylfentanyl
MT-45
Para methyl-4-methylaminorex (4-4'-DMAR)

- MN (**EGL and Sara Castiglioni**) and UJI (**LB**) will focus on the pooled urine samples collected i.e. Oslo festivals, Lisbon (INMLFC), German (USAAR). First screening for NPS included in the yellow list in a later stage this will be expanded by other lists. The analytical groups will closely collaborate in order to try to confirm suspects. (*WS1, activity 2a*)
- Criteria for positive identification of the compounds is of great importance. An action group (**LB**, **EGL**, **SS**, **MB and Sara Castiglioni**) is in charge of writing a proposal regarding the criteria to be applied using low- and high-resolution mass spectrometry for identification of NPS in urine and wastewater samples.

- The yellow list is the priority list for in vitro experiments (*WS1, activity 3a*). In theory there is no limitation to the number of compounds to perform these studies, but reference standards should be available. EMCES (**AQ**) and FFUL (**NS**) will evaluate the yellow list and start performing experiments. The two most promising compounds will be synthesized by **MB** (*WS3, activity 1*) and studied by **DB** and **CS** (*WS2, activity 1 and 2a)*.
- FFUL (**DB, MB** and **CS**) will meet with the aim to select two compounds of the yellow list for further investigation (*see above)*
- A candidate to be studied is methylone (bk-MDMA) as this compounds has been found by the UJI (**LB** and **FH**) in wastewater from three treatment plants (Bristol, Copenhagen and Utrecht)

5. Next meeting: when and where

University Jaume I (Castellon, Spain), agrees in hosting the next plenary meeting (1 day), it will probably be planned the last week of March 2017 or the first week of April 2017. A doodle will be made to concrete the final date.

6. Closing

The meeting was closed by EZ at 18:00 September 28th.