Urinary antihypertensive drug metabolite screening using molecular networking coupled to high-resolution mass spectrometry fragmentation

Chae-Myeong Ha, Kaysaw Tuy, Liliana Baptista

Justin J. J. van der Hooft• Sandosh Padmanabhan • Karl E. V. Burgess • Michael P. Barrett (2016). *Metabolomics*. DOI 12:125 DOI 10.1007/s11306-016-1064-z

Introduction

- Mass Spectrometry is a powerful analysis of drug metabolism but the complexity of the data offers significant analytical challenges.

- Few metabolomics studies have reported on the use of molecular networking combined with high-resolution metabolomics data to aid in analysis of the large amount of spectral information resulting from data-dependent fragmentation.

AIM: To detect and visualize antihypertensive drug metabolites in untargeted metabolomics experiments based on the spectral similarity of their fragmentation spectra

Methods

Participants

Cohort of 26 patients diagnosed with hypertension and on antihypertensive therapy

Age range- 42 to 87 years

15 male, 11 female;

4 were smokers; 5 have diabetes;

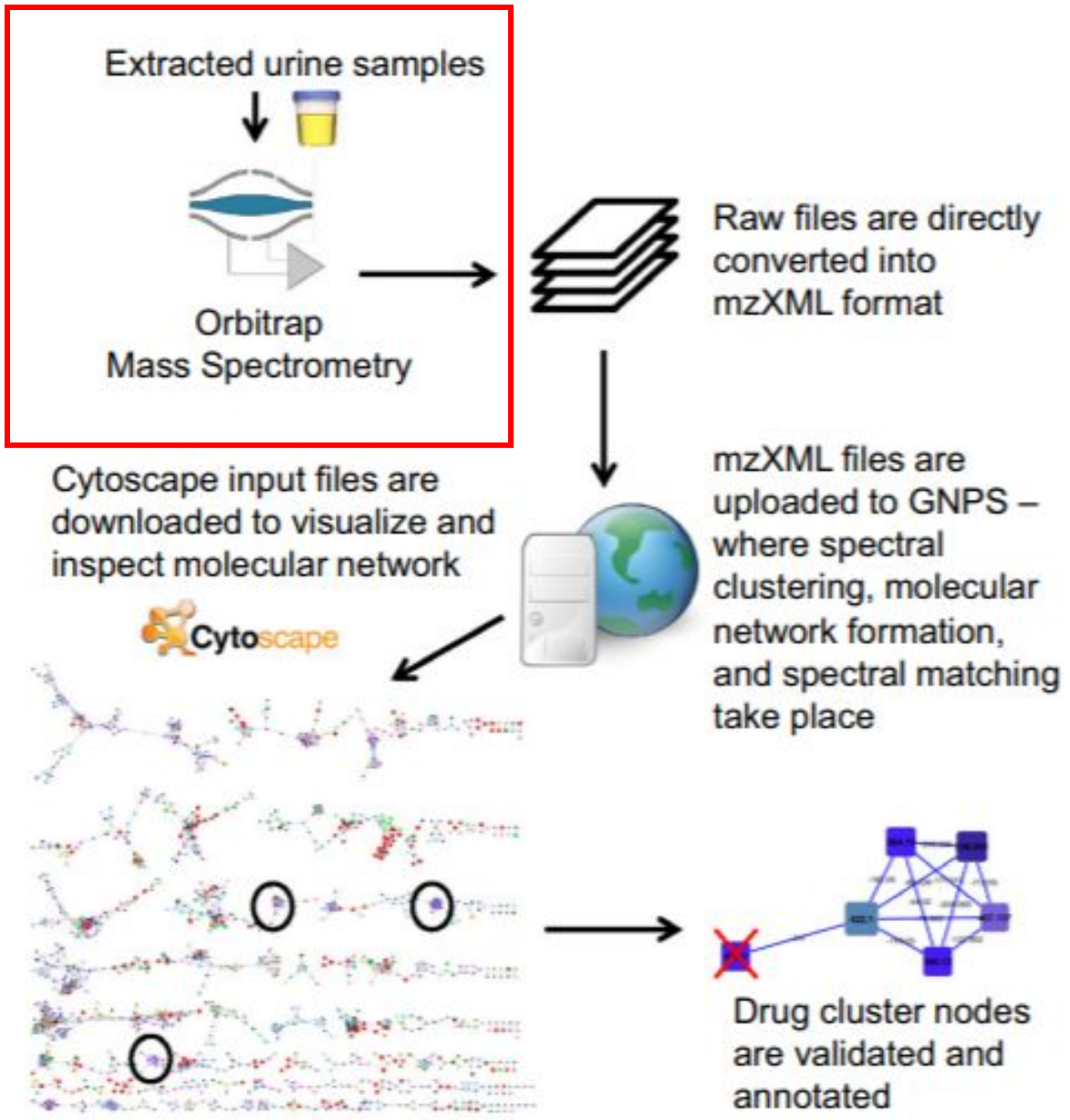
Medication- 2 to 7 different classes of antihypertensive drugs

ACE I	angiotensin converting enzyme inhibitors (drugs ending on -pril)
ARB	angiotensin type II receptor blockers (drugs ending on -sartan)
Diuretic	promoting production of urine (drugs often ending on -zide)
Statin	low-density lipoprotein blood level lowering drugs (drugs ending on -statin)
β-blocker	beta-adrenergic blocking agent (drugs often ending on -olol)
α-blocker	adrenergic inhibitors
	Ca Antag Calcium-channel blockers (two types - dihydropyridines (drugs ending on -ipine) and non-dihydropyridines)
NSAID	non-steroidal anti-inflammatory drugs (i.e., iboprufen)
Nitrate	Anti-anginal drugs

Methods

- Urine Samples
- 5 µL urine was extracted in 200 µL chloroform/methanol/water (1:3:1) at 4ºC;
- centrifuged for 3 min (13,000 g) at 4ºC. Supernatant was stored at -80ºC until analysis
- Pooled urine sample prior to LC-MS

- Analytical approach
- A **Thermo Scientific Ultimate 3000 RSLC nano liquid chromatography** system was coupled to a **Thermo Scientific Q-Exactive Orbitrap mass spectrometer**. Thermo **Xcalibur Tune software** (version 2.5) was used for instrument control and data acquisition.



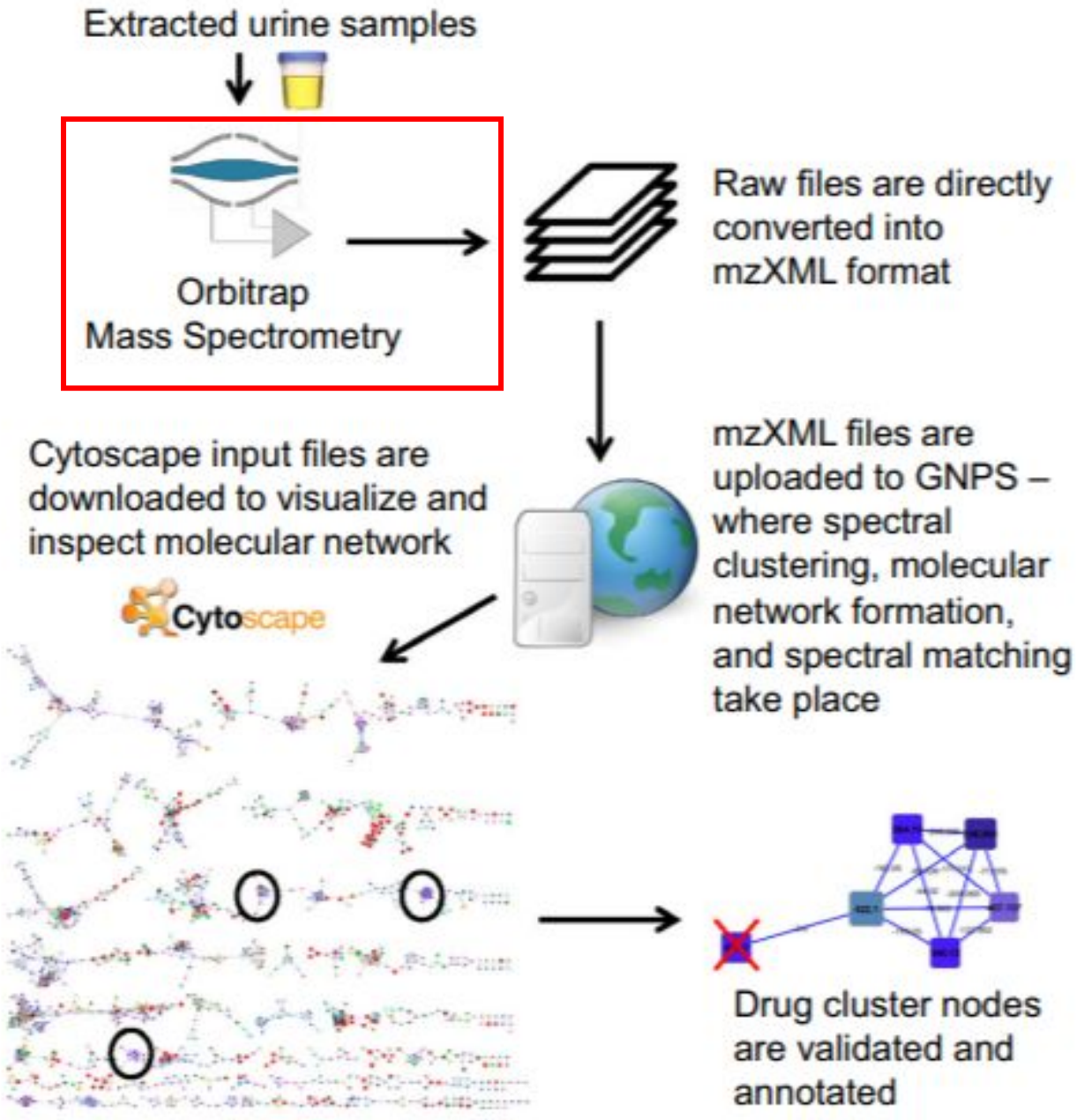
Methods

LC Settings

- **Hydrophilic interaction chromatograph(HILIC) seperation:** A linear biphasic LC gradient was conducted from 80 % B to 20 % B over 15 min, followed by a 2 min wash with 5 % B, and 7 min re-equilibration with 80 % B, where solvent B is acetonitrile and solvent A is 20 mM ammonium carbonate in water.
- Flow rate: 300 µL/ min,
- Column temperature: 25ºC,
- Injection volume: 10 µL

MS and MS/MS settings

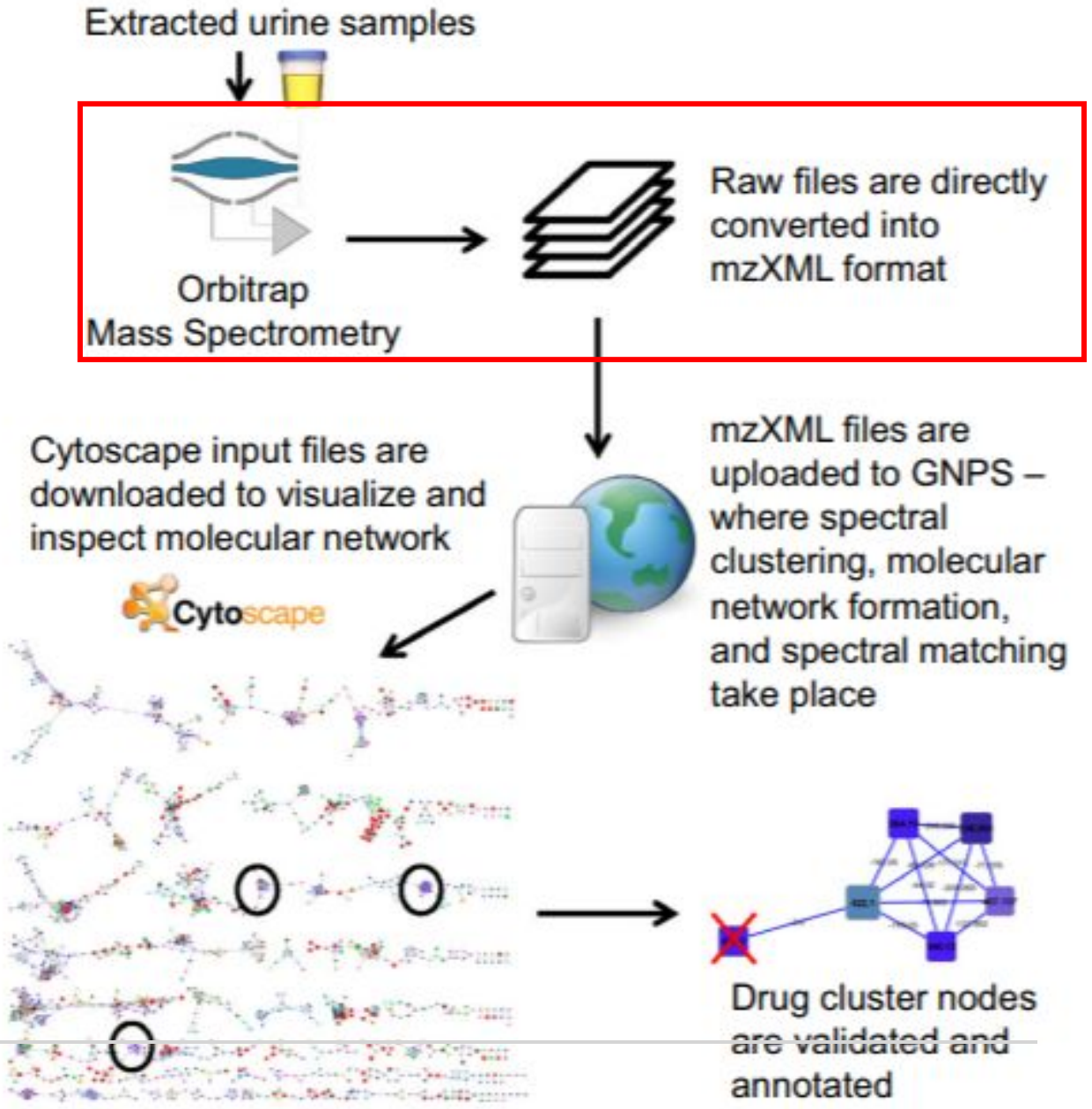
- Positive/Negative ionization combined fragmentation mode
- 2 scans positive mode and then 2 scans in the negative-10 most abundant ion
- **Lock Mass**: m/z 74.0964 (+) (ACN cluster), 88.07569 (contaminant), and m/z 112.98563 (-) (Formic Acid cluster)
- **MS1**: both ionization modes in profile mode at 35,000 resolution (at m/z 200) using 1 microscan, 10^6 AGC target, spray voltages +3.8 and -3.0 kV, capillary temperature 320ºC, full scan mass window of 70–1050 m/z
- **MS2:** 35000 resolution 1 microscan, 10^5 AGC target, max injection time 120ms, isolation window 1 Da (offset 0 Da),



Methods

Data acquisition

- Stability/quality of samples monitor by running pooled samples every 6th randomize sample that was run
- After acquisition, all files were converted to mzXML, two seperate mzXML files for positive and negative ionization spectra
- Accurate mass accuracy of standard within 3 ppm
- All 26 urine samples ran in combined fragmentation mode
  - 12 underwent separate fragmentation mode
  - 6 ran in combined full scan mode with triplicate injection



Methods

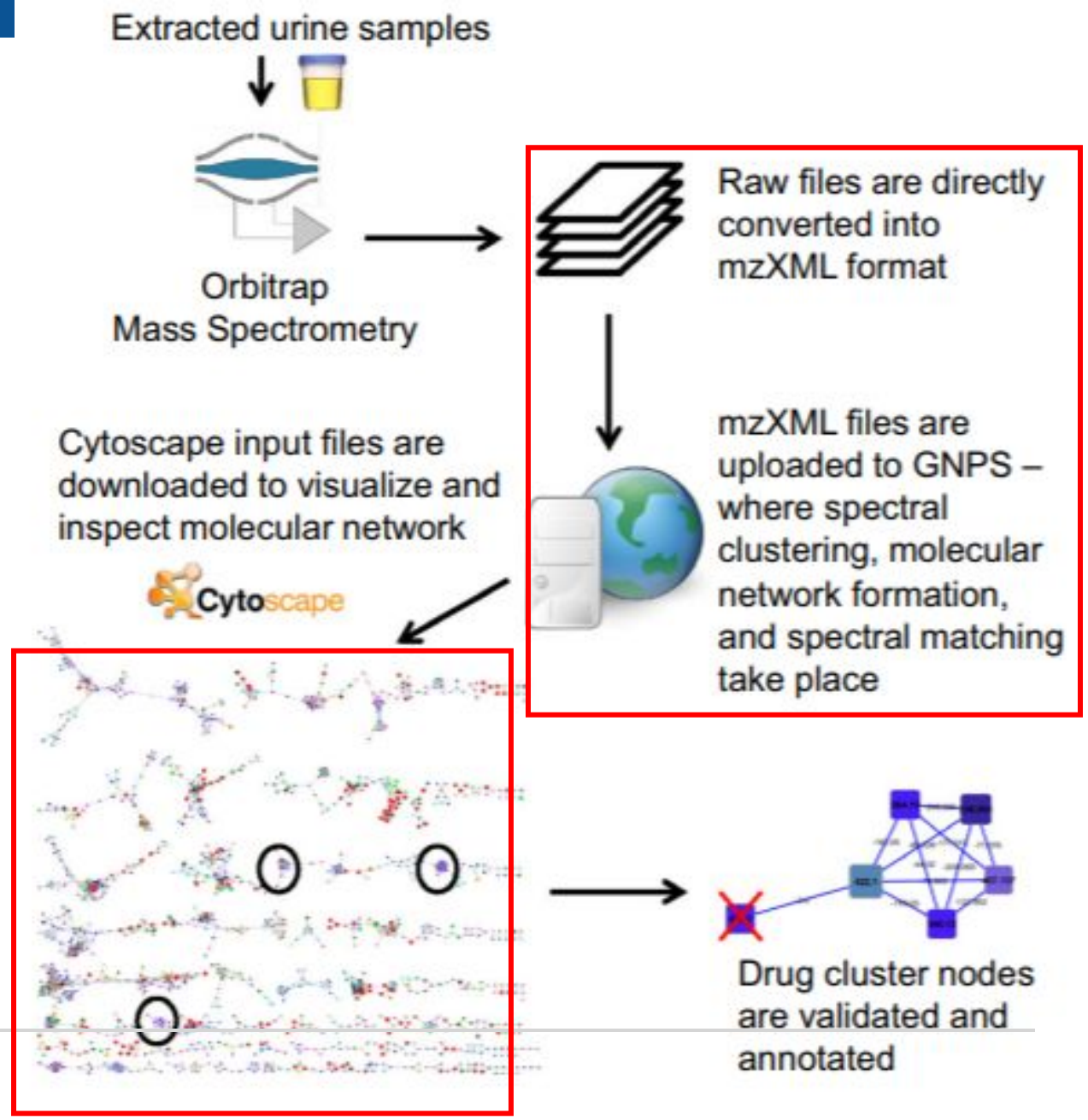
Data processing

- The mzXML files uploaded to Global Natural Products Social Molecular Networking (GNPS) environment

- Consensus spectra created:
  - Parent mass tolerance = 0.25 Dd
  - MS/MS fragment ion tolerance = 0.00 Da
  - Discarded spectras with less than 2 spectras
- A network was created for Cosine scores above 0.55 and 2+ matched peaks
  - Distant nodes kept if they were in 10 top most similar node of respective spectra

- Network ran in GNPS spectral libraries
  - Matches with Cosine score above 0.6 and 4+ peak matches

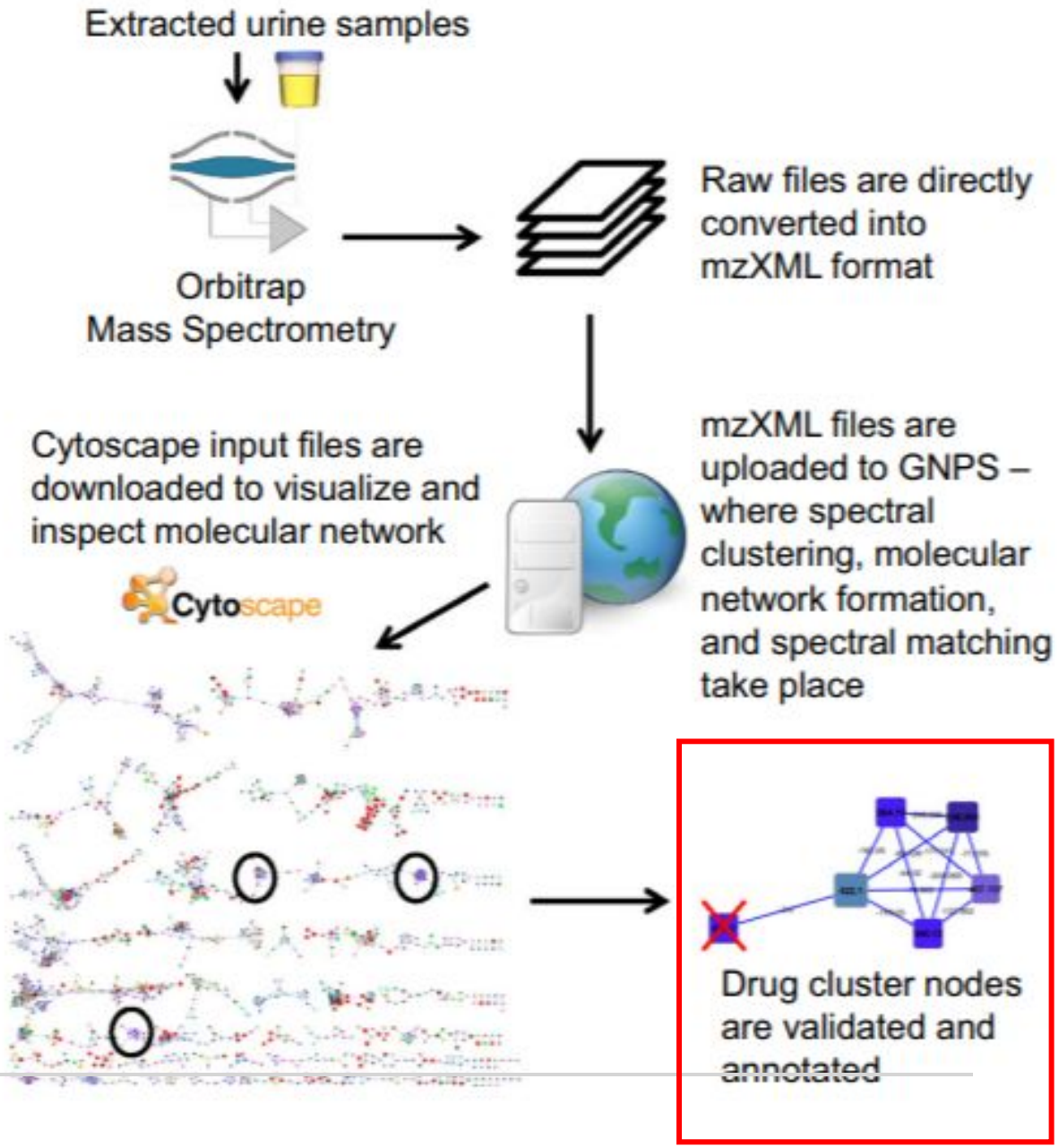
- Cytoscape used for data visualization



Methods

Data analysis

- Drug related clusters were identified based on parent compound
  - GNPS and MassBank
  - MAGMa used for potential matches when there was no spectra match
- Nodes comprised of isomers or related compounds
  - isotopes, in-source fragments of adducts on “real metabolites”
- Nodes were validated by checking number of metabolites within cluster
  - most likely elemental/theoretical mass was assigned
- Drug metabolite annotation based of MzCloud and MassBank North America libraries and were assigned based on the following in order:
- (1) unambiguously identified,
- (2) spectral or literature match,
- (3) metabolite classification,
- (4) metabolites characterized via retention time, mass, and fragmentation spectra



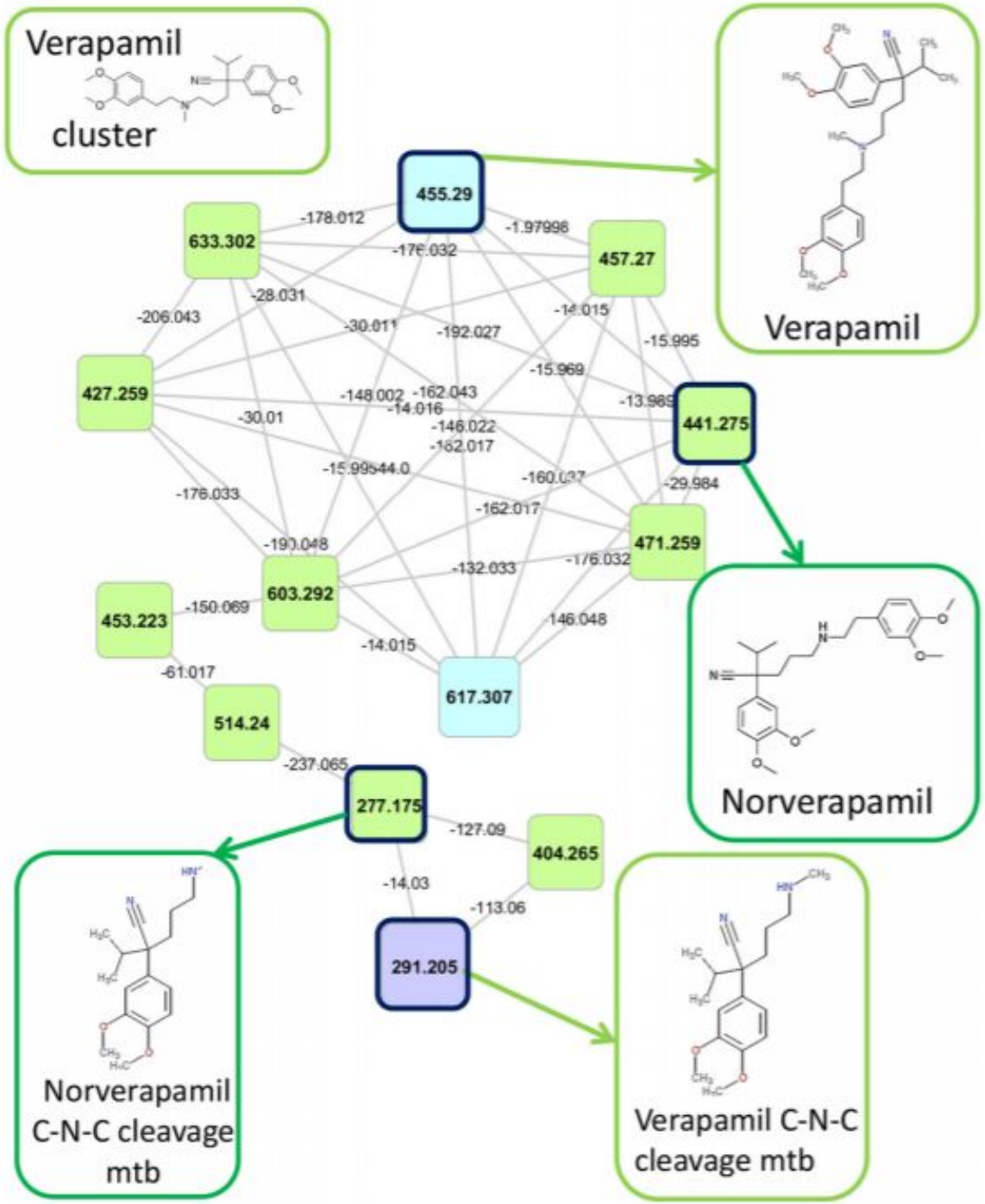
Results

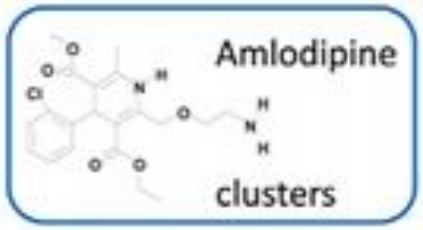
Drug clustering analysis identified 4 correctly annotated matches

Parent drug	Total annotated nodes	Correctly annotated	Related compound
Clodipogrel	1	1	0
Irbesartan/losartan	3	1	2
Verapamil	3	1	2
Atenolol/bisoprolol	2	1	1
Ranitidine	3	0	3
Metformin	3	0	3
Paracetamol	1	0	1
Total	16	4	12

Results

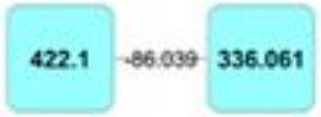
Verapamil Drug-Clustering Indicates Extensive Biotransformation



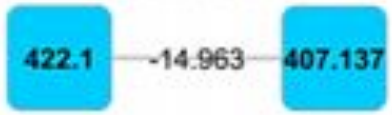


Urine 19

Urine 67

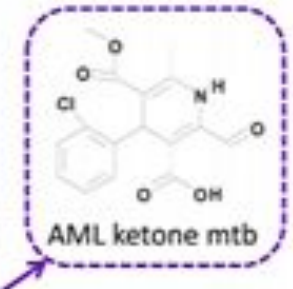


Urine 76

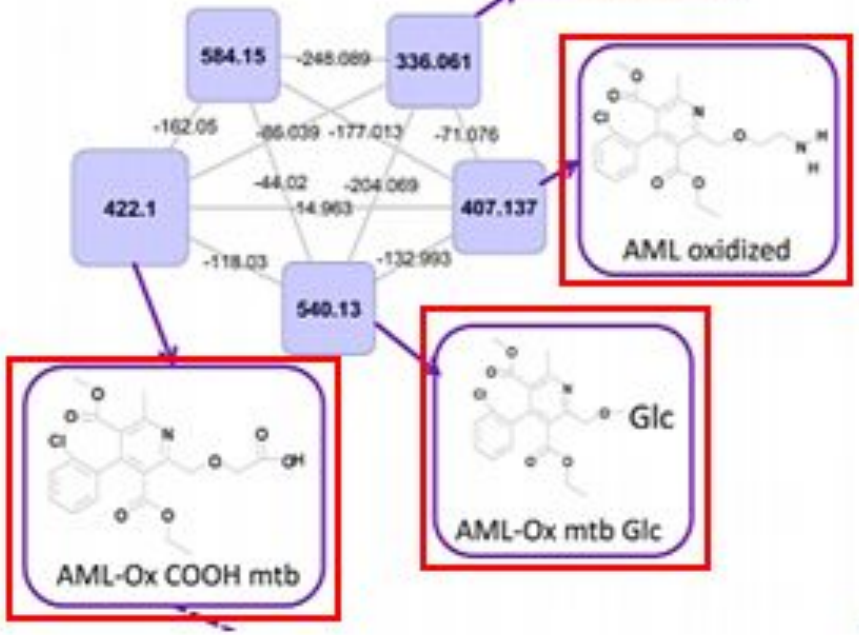


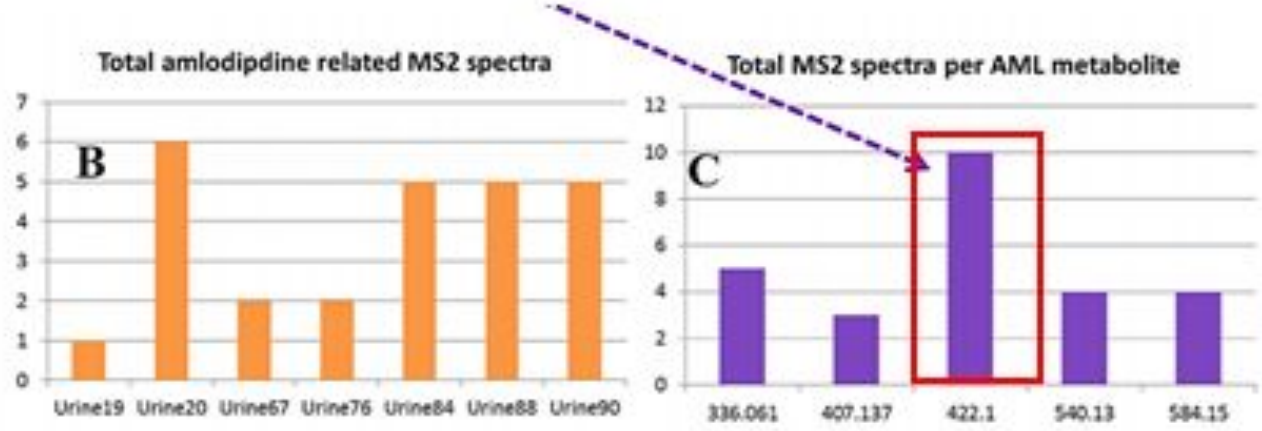
A

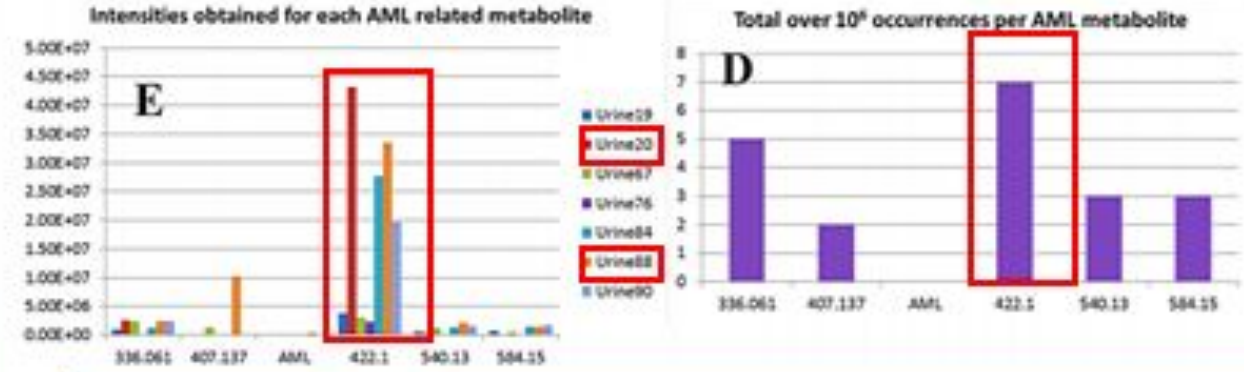
Urines 84 and 88



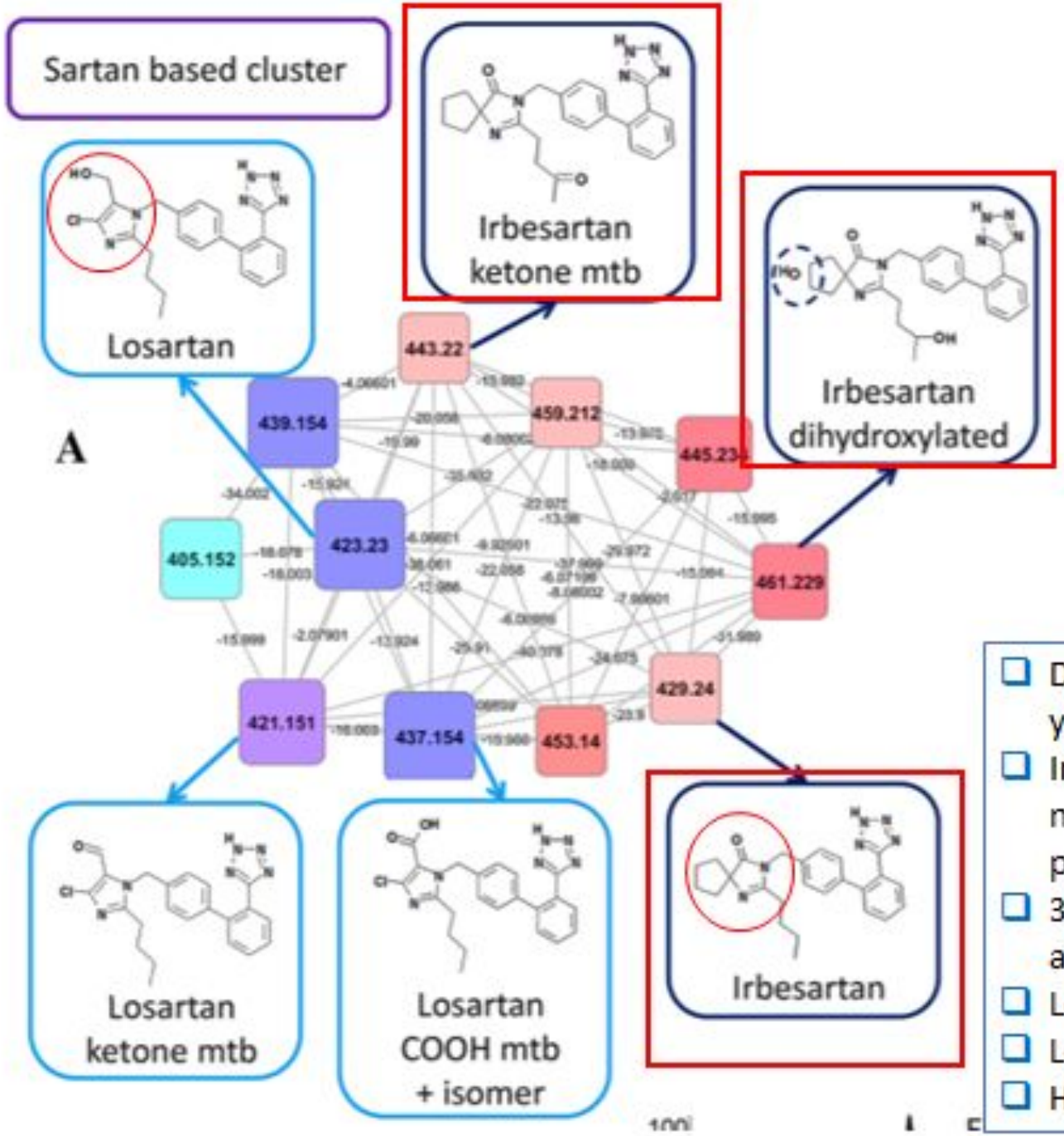
Urines 20 and 90

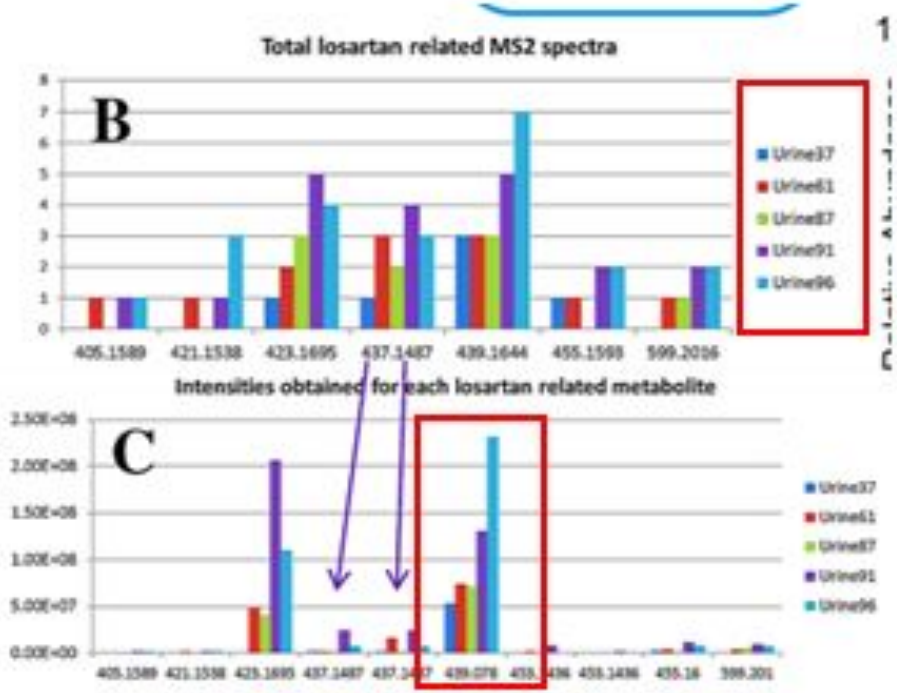






- Amlodipine- related cluster networking showed 3 metabolites that matched with literature search and 1 that was matched due to mass similarity
- From MS2- Amlodipine metabolites were found in 7 samples
- Higher abundance was of Amlodipine-OX-COOH metabolite
- Amlodipine-OX-COOH- abundant in urine samples 20, 88, 84
- Amlodipine was not found, only traces
- Amlodipine-OX-COOH- could be a better marker for amlodipine intake than amlodipine parent drug





- Distinguish Irbesartan from losartan- (1Htetrazol-5-yl)biphenyl-4-yl) vs chloride atom backbone
- Irbesartan- related cluster networking showed 2 metabolites MSI level 2 that matched with literature search plus the metabolite itself
- 3 other metabolites were found but the spectral match was ambiguous- further analysis
- Losartan was found in 5 urine samples
- Losartan COOH+ isomer- intensity MS and retention time
- Higher abundance of Losartan hidroxylation

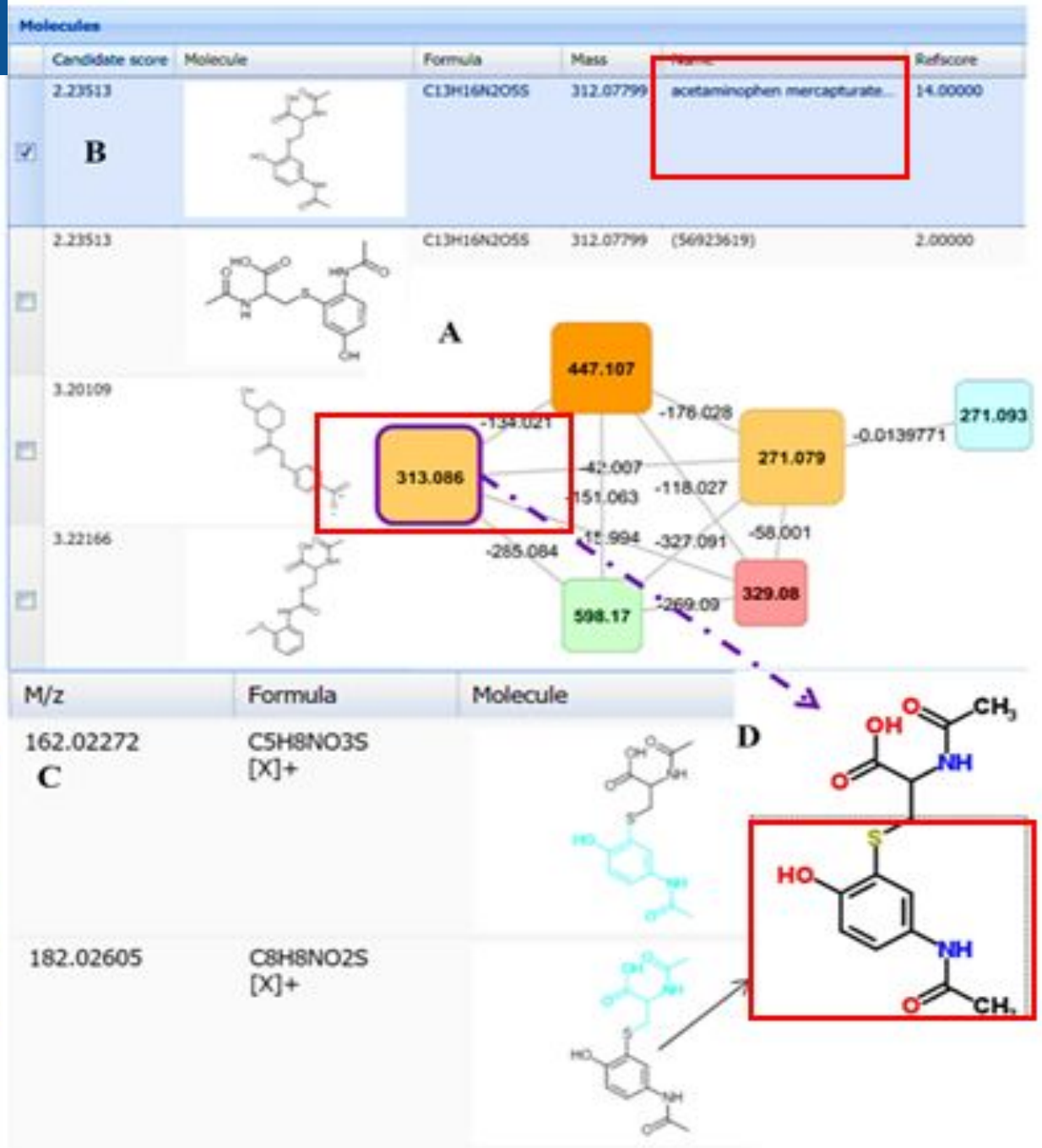
Other drug metabolites

- Paracetamol mercapturate and N-acetylcysteine)- m/z= 313.086 and 271.079, respectively
- Contained paracetamol-O-sulphate and paracetamol-O-glucuronide

- Type 2 diabetes- Metformin- 3 Pt had diabetes but not reported on 1 Pt
- Metformin- sulfonylurea metabolite

- Histamine H2-receptor antagonist- ranitidine- 20 metabolites in 1 subject

Metabolites
165 drug metabolites
17 with annotated MS (MSI MI level 2)
122 clustered fragmentation (MSI MI level 3)
13 different drugs



Other endogenous metabolites

Compound class	Nodes in cluster combined fragmentation mode — POS	Nodes in cluster separate fragmentation mode — POS	MaxUniqueFileCount No. unique urine files (# nodes)
Carnitine based	52	52	26 (5), 25 (6)
Glutamine based	18	18	26 (3), 25 (2)
Trigonelline based	12	11	26 (1), 11(1)
Betaine based	–	11	12 (4), 8 (1) [POS only]
Steriod skeleton	2 + 2	12	11 (1), 8 (2)
Pyrriline-CO based	16	9	15 (1), 9 (3)
Pipecolic acid based	20	12	26 (4), 25 (2)
Lysine based	9	7	24 (1), 19 (1)
N containing oxygen rich substructure	10	10	2 (4), 1 (6)
Total	137	142	N/A

- Carnitine, glutamine-related metabolites, and trigonelline- plant base Vitamin B- diet or supplement?
- Most were found in the combined fragmentation mode but betaine cluster only found using the separate fragmentation mode
- Four compound classes- at least one associated metabolite- present in all 26 urine extracts

Conclusion/Implications

- Molecular **networking** approach clearly offers a means to derive important information from **large and complex datasets.**
- Coupling HILIC-based liquid chromatography to Orbitrap high resolution spectrometry allows for the simultaneous detection of a wide range of polar urinary compounds in both positive and negative ionization modes
- Approach- study **drug metabolism** with relative ease.
- Typical mass differences of 176.032 (glucuronidation), 14.015 (methylation), and 16.000 Da (hydroxylation) that are commonly associated to drug (or xenobiotic) metabolism
- Spectral clustering and matching **enhances metabolite annotation and classification**; however, extensive manual interpretation and validation remain essential for confident assessment of metabolite structures
- It is a way to assess drug adherence, drug physiology, drug-drug interactions, drug- endogenous metabolites interactions

Limitations

- Subjects **age range and sex**- samples had a wide age range- influence drugs pharmacokinetics and pharmacodynamics (older organisms have lower metabolization rates than younger/ males have better metabolization than female/ body composition
- **Drugs have different half life**- Amlodipine half-line between 30-50 hours; Losartan 6-9 hours; Paracetamol 1-4 hours; Drug extended release- Time of urine collection **may dictate different metabolite abundance** that may not correspond to patient reality.

Concerns

- **Ethical- clinical data**
  - patient 61 was on losartan and bisoprolol- second stage hypertension.
  - patient 66 was on enalapril, bisoprolol, metformin, and likely gliclazide (a sulfonylurea class drug), and also took paracetamol- Obese patient with hypertension and a second stage Type 2 diabetes
  - patient 91 was on losartan, perindopril, and atenolol, and also took paracetamol- Resistant Hypertension
- **Drugs are prescribed for other conditions**
  - Metformin- 1 patient did not report diabetes (it could be taken for hair loss)