UNIVERSITY OF SÃO PAULO
School of Pharmaceutical Sciences
Department of Clinical and Toxicological Analysis
Graduate Program in Pharmacy (Physiopathology and Toxicology)

Investigation on the relationship between violent death, cocaine abuse and single nucleotide polymorphisms

Ana Miguel Fonseca Pêgo

Thesis for the attainment of the title of DOCTOR

Supervisor:
Prof. Dr. Mauricio Yonamine

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Ficha Catalográfica elaborada eletronicamente pelo autor, utilizando o programa desenvolvido pela Seção Técnica de Informática do ICMC/USP e adaptado para a Divisão de Biblioteca e Documentação do Conjunto das Químicas da USP

Bibliotecária responsável pela orientação de catalogação da publicação:
Marlene Aparecida Vieira - CRB - 8/5562

Pego, Ana Miguel Fonseca
P376i Investigation on the relationship between violent death, cocaine abuse and single nucleotide polymorphisms / Ana Miguel Fonseca Pego. - São Paulo, 2018.
208 p.

Tese (doutorado) - Faculdade de Ciências Farmacêuticas da Universidade de São Paulo. Departamento de Análises Clínicas e Toxicológicas.
Orientador: Yonamine, Mauricio

1. Toxicologia Forense. 2. Morte. 3. Drogas de abuso. 4. Genética. I. T. II. Yonamine, Mauricio, orientador.

Ana Miguel Fonseca Pêgo

Investigation on the relationship between violent death, cocaine abuse and single nucleotide polymorphisms

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São Paulo, August 2018

Financial Support

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Projeto CAPES Pró-Forenses AUXPE 3419/2014.

To my Auntie Helena

Para a Tia Helena

Acknowledgements

To my adviser, Mauricio Yonamine, thank you for being, during these years, much more than a "Prof", but a scientific father and a friend. You have taught me so much beyond toxicology. Thank you for the knowledge you have given me, for the talks and the laughter! I'll take all these lessons with me. Thank you for trusting me!

To my parents, Isabel and Pedro, I owe them everything! Thank you for being my homeport, my inspiration. Thank you for letting me fly and even more, for flying with me! Thank you for believing in me, thank you for learning to be the most present parents even from afar. I do not simply miss you; you are my one and only longing. Dad, thank you for making me dream up to heaven and mum, thank you for helping me keep my feet on the ground. You are the perfect match and my best friends!

To boo, thank you for always being by my side all this time through thick and thin. Thank you for staying until dawn with me in the lab while I validated methods, thank you for believing in me when I stopped believing in myself. Thank you for making me smile without any effort. Thank you for so many times having taught me to be my best version and of course, thank you for being the IT boyfriend who knows the most about Forensic Toxicology! I admire you and grow along with you!

My family. Thank you auntie Céu and Mimi for taking care of grandma and grandpa! Thank you uncle "Zéntonio" and uncle Zé. Thank you to my 4 grandparents for still being here with me, especially my grandmother Maria Conceição who is the most incredible woman the World has ever known. Thanks to Tati and Tomtom, without you I would never be here and my story would definitely not be this one! It was for you that everything started...

To ToxiGirls. During these years, those 5 girls were everything to me! They were my friends, my colleagues, my sisters ... Thank you Gabys for being the first to show me everything in the lab and for helping me to this day, untiringly - I do not know if I'll ever be able to thank you for your friendship! Thank you Fuf for having validated a method with me, for having been my right arm, for being this incredible person who taught me so much - I'm your #1 fan! Thank you Idy for all your love, your affection, your laughter, I admire your authenticity and humility so much - you teach me every day. Thank you Maita, only you know how special you are to me! When we're apart, we're both like an "airplane without a wing," it does not fly! And with you by my side, everything is better - I admire you so much and without you nothing is amusing!

Thank you Sarinha for being my adoptive mother, for helping me endless days and nights, for guiding me, for advising me, for being an example of a researcher for me – nothing will ever be enough to thank you! Words will never do justice for how much I admire you and how much you mean to me!

To Anax! I will not have enough words in a doctoral thesis to thank you. You are a unique friend and more than that, you are part of my family, part of me. Thank you for teaching me so much, especially for making me chuckle till it hurts! Thank you for taking me to your home, thank you for sharing your life with me - it was and it will always be a pleasure!

To Lucas, you were my first friend in SP, without even knowing each other! You showed me this city like no one else! Thanks for all the stories, all the walks, all the St. Patricks, all your friendship and partnership! A lifetime would not be enough to thank you.

To Tiago, my adoptive dad! Thank you for helping me so many times! Thank you for our wine conversations and for representing your role very well!

To chuchu, our friendship was never very common! I feel like I've known you forever! We are sisters without knowing how! Thank you for your friendship without burdens or labels!

To Wanessinha and Bruno, you are incredibly special to me, your friendship is a gift that I will keep for life! And nothing is by chance. Thank you for letting me be a part of your families!

To Ju, thank you for all the coffees, all the trips to IML, the family Easter, all the conversations and gossip. Our friendship definitely did not start in a common way but for us this was never a problem! We knew how to make our time in São Paulo the best of them all! Thanks friend!

To boo's family, Neuza, Gih, Dimas, Claudio, Tica, thank you for opening your houses for me! You have shown me how to have family Sundays so far from home. To Luquinhas, thank you for being this child so full of life and so happy. Thank you for teaching me how to play video games and to fight like a ninja!

To Samantha, thank you for your patience with me since before I even arrived in Brazil! Thank you for everything!

To Bia, thank you for having so much patience to teach me everything there is, for all our conversations both in English and Portuguese! For never denying a request for help!

To Katia, thank you for always listening to me and having some advice ready to give me! Thank you for your friendship and fellowship!

To Professor Tania, thank you for all the things you've taught me, our conversations and our laughter! You are an incredible woman for whom I have the greatest admiration!

To Professor Beth, thank you for the conversations, dinners and endless advice! "How chic you are!".

To Professor Felipe Lourenço, thank you for all your help in the statistical part of my project and all the patience! Thank you!

To Jânio and Du. Thank you for making my IML collections fun! I learned a lot from you! To all IML-Sul staff for opening their doors to me and for trusting my project, thank you very much!

Ju and Paulo from Incor, thank you for having done the genetic analyses of this project and therefore taught me during this process. Thank you Paulo for all the patience with me and my questions and thanks Ju for having spent so much time until dawn analysing the statistics with me - you are awesome!

To Professor Paulo Caleb, thank you so much for the opportunity of working with you and for all the help and support!

To Angelo, thank you for always having a joke to tell me the corridors! We had a good laugh together!

To the Gaúcho, Juliano, thank you for your company, your help with everything! You are an incredible person and a friend that I will not forget!

To the Professor Mauricio's students: Grillo, Menck, Jeff, Ale, Pine, Ana Luiza and Marcelo, thank you for having helped me in so many different ways during this journey! And thank you for the company in and out of the lab!

To Professor Vilma Leyton and to Professor Ivan Miziara, thank you for the collaboration, help and all the support during the progress of the project. To Professor Mario Hirata and Raul Bortolin, thank you for all your help and contribution to this project.

To CAPES for the scholarship granted me so that I could carry out this project during those four years.

Agradecimentos

Ao meu orientador, Mauricio Yonamine, muito obrigada por ter sido durante esses anos muito mais do que um “Prof”, mas um Pai científico e um amigo. Com você aprendi muito além da toxicologia. Obrigada pelo conhecimento que me passou, pelas conversas e as risadas! Levo todo esse aprendizado comigo na bagagem. Obrigada por ter confiado em mim!

Aos meus pais, Isabel e Pedro, a eles eu devo tudo! Obrigada por serem o meu porto de abrigo, a minha inspiração. Obrigada por me deixarem voar e mais ainda por voarem comigo! Obrigada por acreditarem em mim, obrigada por terem aprendido a ser os Pais mais presentes mesmo de longe. Por vocês eu não sinto só saudade, vocês SÃO a minha única saudade. Pai, obrigada por me fazeres sonhar até ao céu e Mãe, obrigada por me ajudares a manter os pés assentes na terra. Vocês são a combinação perfeita e os meus melhores amigos!

Ao boo, obrigada por ter estado sempre do meu lado todo esse tempo, faça chuva ou faça sol. Obrigada por ter ficado até de madrugada comigo no laboratório enquanto eu validava métodos, obrigada por acreditar em mim quando eu mesma deixei de acreditar. Obrigada por me fazer sorrir sem qualquer esforço. Obrigada por tantas vezes me ter ensinado a ser a minha melhor versão e claro, obrigada por ser o namorado de T.I. que mais sabe de toxicologia forense! Eu te admiro e cresço junto com você!

À minha família. Obrigada tia Céu e Mimi por cuidarem da avó e do avô! Obrigada tio “Zéntonio” e tio Zé. Obrigada aos meus 4 avós por ainda estarem aqui comigo, especialmente a minha avó Conceição que é a mulher mais incrível que o mundo já conheceu. Obrigada à Tati e ao Tomtom, sem eles eu jamais estaria aqui e a minha história não seria esta! Foi por eles que tudo começou...

Às ToxiGirls. Durante esses anos, essas 5 meninas foram tudo para mim! Elas foram amigas, colegas, irmãs... Obrigada à Gabys por ter sido a primeira a me mostrar tudo no laboratório e me ajudar até hoje incansavelmente – não sei se um dia vou conseguir agradecer pela sua amizade! Obrigada Fuf por ter validado um método comigo, por ter sido o meu braço direito, por ser essa pessoa incrível que tanto me ensinou – sou sua fã nº1! Obrigada Idy por todo o seu amor, o seu carinho, a sua risada, eu admiro demais a sua autenticidade e humildade – você me ensina

todos os dias. Obrigada Maita, só você sabe o quanto você é especial para mim! Quando estamos separadas, nós duas somos como um “avião sem asa”, ele não voa! E com você do meu lado, tudo é melhor – eu te admiro demais e sem você nada tem graça! Obrigada Sarinha por ser a minha mãe adotiva, por ter me ajudado dias e noites sem fim, por me guiar, por me aconselhar, por ser um exemplo de pesquisadora para mim – nada é suficiente para te agradecer! Palavras nunca farão justiça ao quanto eu te admiro e ao tanto que você significa para mim!

Ao Anax! Não terei palavras suficientes em uma tese de doutorado para te agradecer. Você é um amigo sem igual e mais do que isso, você é parte da minha família, é parte de mim. Obrigada por ter me ensinado tanto, especialmente a dar risada de doer a bochecha! Obrigada por me levar a sua casa, obrigada por partilhar a sua vida comigo – foi e sempre será um prazer!

Ao Lucas, você foi o meu primeiro amigo em SP, sem nem sequer nos conhecermos ao vivo! Você me mostrou essa cidade como ninguém! Obrigada por todas as histórias, todos os passeios, todos os St. Patricks, toda a sua amizade e parceria! Toda uma vida não seria suficiente para te agradecer.

Ao Tiago, meu Pai adotivo! Obrigada por ter me ajudado tantas e tantas vezes! Obrigada pelas conversas regadas a vinho e por ter representado muito bem a sua função!

À chuchu, a nossa amizade nunca foi muito comum! Sinto que te conheço desde sempre! Somos irmãs sem saber como! Obrigada pela sua amizade sem cobranças nem rótulos!

À Wanessinha e ao Bruno, vocês são incrivelmente especiais para mim, a vossa amizade é um presente que eu vou guardar para toda a vida! E nada é por acaso. Obrigada por terem me deixado fazer parte das vossas famílias!

À Ju por todos os cafés, todas as idas ao IML, a Páscoa em família, todas as conversas e fofocas. A nossa amizade definitivamente não começou de uma forma comum mas para nós isso nunca foi um problema! Soubemos fazer desse nosso tempo em SP o melhor de todos! Obrigada amiga!

Ao Professor Paulo Caleb, muito obrigada pela oportunidade que me concedeu de trabalhar com você, toda a sua ajuda e apoio!

À família do boo, Neuza, Gih, Dimas, Claudio, Tica, obrigada por terem aberto as vossas casas para mim! Os Domingos em família passaram a existir para mim, tão

longe de casa. Ao Luquinhas, obrigada por ser essa criança tão cheia de vida e tão feliz. Obrigada por me ensinar a jogar videogame e a lutar como um ninja!

À Samantha por toda a sua paciência que tem comigo desde antes mesmo de eu chegar ao Brasil! Muito obrigada por tudo!

À Bia, obrigada por ter tanta paciência para me ensinar tudo e mais alguma coisa, por todas as nossas conversas em inglês e em português! Por nunca ter negado um pedido de ajuda!

À Katia, obrigada por sempre me ouvir e ter um conselho pronto para me dar! Obrigada pela sua amizade e companheirismo!

À Professora Tânia, obrigada por todos os ensinamentos, conversas e risadas! Você é uma mulher incrível pela qual eu tenho a maior admiração!

À Professora Beth, obrigada pelas conversas, jantares e conselhos sem fim! “How chic you are!”.

Ao Professor de Felipe Lourenço por toda a sua ajuda na parte estatística do meu projeto e toda a paciência! Muito obrigada!

Ao Jânio e ao Du. Obrigada por terem tornado as minhas coletas no IML em algo divertido! Aprendi muito com vocês! A todo o staff do IML-Sul por terem aberto as portas para mim e terem confiado no meu projeto, muito obrigada!

À Ju e ao Paulo do Incor por terem feito as análises genéticas deste projeto e por tanto me ensinarem durante esse processo. Obrigada Paulo por toda a paciência comigo e as minhas perguntas e obrigada Ju por ter passado tanto tempo até de madrugada analisando a estatística comigo – você é demais!

Ao Ângelo, obrigada por sempre ter uma piada para me contar pelos corredores! Demos boas risadas juntos!

Ao Gaúcho, Juliano, obrigada pela sua companhia, sua ajuda com tudo! Você é uma pessoa incrível e um amigo que não irei esquecer!

Aos alunos do Professor Mauricio: Grillo, Menck, Jeff, Ale, Pine, Ana Luiza e Marcelo, obrigada por terem me ajudado de tantas diversas formas durante essa jornada! E obrigada pela companhia dentro e fora do laboratório!

À Professora Vilma Leyton e ao Professor Ivan Miziara, obrigada pela colaboração, ajuda e todo o apoio durante o andamento do projeto. Ao Professor Mario Hirata e Raul Bortolin por toda a sua ajuda e contribuição para este projeto.

À CAPES pela bolsa que me foi concedida para que pudesse realizar esse projeto durante esses 4 anos.

“To dream is the worst of all cocaines, because it is the most natural of all.”

Fernando Pessoa, in “*Livro do Desassossego*”

“O sonho é a pior das cocaínas, porque é a mais natural de todas.”

Fernando Pessoa, em “*Livro do Desassossego*”

Abstract

PEGO, A.M.F. **Investigation on the relationship between violent death, cocaine abuse and single nucleotide polymorphisms.** 2018. 208p. Thesis (PhD) – School of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil.

Violence is a dreadful phenomenon spread throughout the world, resulting in unfortunate events that can ultimately cause death. It is known that some countries play a much worrying role in this scenario than others. Brazil is one of them. The present study has focused on identifying the use of cocaine within 105 *postmortem* cases arriving at the Institute of Legal Medicine of São Paulo (IML-SP) through analytic toxicological methods and latter applying genetic testing to see whether the presence of certain single nucleotide polymorphisms (SNPs) is more predominant within users rather than non-users, which would help to better understand one's susceptibility to abuse the drug. Both blood and hair samples have been analysed through ultra-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (UPLC-ESI-MS/MS) in order to distinguish between recent or chronic cocaine use among violent individuals whose violence has ultimately leaded to their death. Two dilute-and-shoot methods have been validated and used for this purpose, and the final residue was analysed through the UPLC-ESI-MS/MS system. From the 105 *postmortem* cases, a rather high proportion of cocaine and its metabolites was found. A chronic use of the drug was denoted in 53% of the cases, which were positive for cocaine and benzoylecgonine, followed by 43% for norcocaine, 40% for cocaethylene and 13% for anhydroecgonine methyl ester, in hair. As for blood, reflecting the use of cocaine prior to death, 51% of the cases have shown to be positive for benzoylecgonine, followed by 41% for cocaine, 23% for cocaethylene and 20% for norcocaine. These findings suggest a probable association between the use of the drug and risky/violent behaviours. Genetic wise, a significant difference has been observed for SNP rs4263329 from the *BCHE* gene in its dominant model, with higher frequencies of the genotypes A/G and G/G seen in cocaine users rather than non-users (OR=8.91; 95%CI=1.58–50.21; p=0.01). Likewise, also SNP rs6280 from the *DRD3* gene presented a significant association in both its additive and dominant model, suggesting that the C allele may be playing a role in cocaine use as both genotypes T/C and C/C were significantly more frequent in users than non-users. This association was not lost when adjusted for co-variants using logistic regression (OR=4.96; 95%CI=1.07; p=0.04). Finally, a statistically significant association (p = 0.003) was also encountered among individuals with both A/G and G/G genotypes within SNP rs4263329 and the use of cocaine HCl (f(A/G+G/G)=44.7%) versus *crack*-cocaine (f(A/G+G/G)=7.7%) and non-users (f(A/G+G/G)=16.2%). In conclusion, this study has found significant associations within two SNPs related to cocaine use, however, due to several inherent limitations, these must be confirmed by further studies with a higher number of subjects and within a more controlled setting. Definite assumptions may not be made at this point and future researches are to be conducted.

Keywords: Cocaine; *postmortem*; violence; toxicogenetics; SNPs

Resumo

PEGO, A.M.F. **Estudo da relação entre morte violenta, uso de cocaína e polimorfismos de nucleotídeo único.** 2018. 208f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, Brasil.

A violência é um fenômeno aterrador espalhado por todo o mundo, resultando em eventos que podem, em última instância, causar a morte. Sabe-se que, em alguns países esse cenário é mais preocupante que em outros. O Brasil é um deles. O presente estudo teve como objetivo identificar o uso de cocaína em 105 casos *postmortem* provenientes do Instituto de Medicina Legal de São Paulo (IML-SP) por meio de métodos toxicológicos analíticos e posterior aplicação de testes genéticos para verificar se a presença de determinados polimorfismos de nucleotídeo único (SNPs) é mais predominante dentro dos usuários do que dos não usuários, o que explicaria uma possível suscetibilidade de um indivíduo ao abuso da droga. Amostras de sangue e cabelo foram analisadas através de cromatografia líquida de ultra-eficiência acoplada a espectrometria de massas e ionização por electrospray (UPLC-ESI-MS/MS) para distinguir entre uso recente ou crônico de cocaína entre indivíduos violentos cuja violência levou à sua morte. Para tal, dois métodos de extração baseados na técnica de "*dilute-and-shoot*" foram validados e utilizados para esse fim, e o resíduo final foi analisado através de um sistema UPLC-ESI-MS/MS. Dos 105 casos *postmortem*, foi encontrada uma proporção significativa de cocaína e seus produtos de biotransformação. O uso crônico da droga foi denotado em 53% dos casos, sendo estes positivos para cocaína e benzoilecgonina, seguidos de 43% para norcocaína, 40% para cocaetileno e 13% para anidroecgonina metil éster, no cabelo. Quanto ao sangue, refletindo o uso de cocaína antes da morte, 51% dos casos mostraram-se positivos para benzoilecgonina, seguido de 41% para cocaína, 23% para cocaetileno e 20% para norcocaína. Esses dados corroboram a hipótese provável da relação entre o uso da droga e comportamentos de risco/violentos. Quanto à genética, uma diferença significativa foi observada para o SNP rs4263329 do gene *BCHE* em seu modelo dominante, com maiores frequências dos genótipos A/G e G/G vistos em usuários de cocaína ao contrário de não usuários (OR=8,91; 95%IC=1,58-50,21; p=0,01). Da mesma forma, também o SNP rs6280 do gene *DRD3* apresentou uma associação significativa tanto no seu modelo aditivo quanto dominante, sugerindo que o alelo C pode estar desempenhando um papel no uso de cocaína, pois ambos os genótipos T/C e C/C foram significativamente mais frequentes nos usuários do que não usuários. Essa associação não foi perdida quando ajustada para co-variáveis usando regressão logística (OR=4,96; 95%IC=1,07; p=0,04). Finalmente, uma associação estatisticamente significativa (p=0,003) também foi encontrada entre indivíduos com ambos os genótipos A/G e G/G dentro do SNP rs4263329 e o uso de cocaína HCl (f(A/G + G/G)=44,7%) versus *crack* (f(A/G + G/G)=7,7%) e não usuários (f(A/G + G/G)=16,2%). Em conclusão, este estudo encontrou associações significativas em dois SNPs relacionados ao uso de cocaína, no entanto, devido a várias limitações inerentes, estas devem ser confirmadas por mais estudos com um maior número de indivíduos e dentro de um cenário mais controlado. Hipóteses definitivas não poderão ser feitas neste momento e futuras pesquisas devem ser conduzidas.

Palavras-chave: Cocaína; *postmortem*; violência; toxicogenética; SNPs

List of Abbreviations

ACN	Acetonitrile
ANOVA	Analysis of variance
AEME	Anhydroecgonine methyl ester
BChE	Butyrylcholinesterase enzyme
BCHE	Butyrylcholinesterase gene
BZE	Benzoylecgonine
CE	Cocaethylene
CG	Candidate-gene
CI	Confidence interval
CLE	Collision energy
COC	Cocaine
COMT	Catechol-*O*-methyltransferase gene
CNS	Central Nervous System
CV	Coefficient of variation
DAT 1	Dopamine transporter gene
DNA	Deoxyribonucleic acid
DRD2	Dopamine D2 receptor gene
DRD3	Dopamine D3 receptor gene
EDTA	Ethylenediaminetetra-acetic acid
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EME	Ecgonine methyl ester
FAB	Femoral arterial blood
FVB	Femoral venous blood
g	Gravitacional force

GWAS	Genome-wide association study
HCl	Hydrochloric acid
HQC	High quality control
HWE	Hardy-Weinberg equilibrium
IML-SP	Institute of Legal Medicine of São Paulo
IS	Internal standard
InCor	Heart's Institute, from the Portuguese: *Instituto do Coração*
LENAD	National Survey of Alcohol and Drugs, from the Portuguese: *Levantamento Nacional de Álcool e Drogas*
LLE	Liquid-liquid extraction
LoD	Limit of detection
LoQ	Limit of quantitation
LPME	Liquid phase microextraction
LQC	Low quality control
MAF	Minor allele frequency
MDMA	3,4-Methylenedioxymethamphetamine
MeOH	Methanol
MGB	Minor Groove Binder
MQC	Medium quality control
MRM	Multiple-reaction monitoring
NCOC	Norcocaine
NIDA	National Institute on Drug Abuse
OMIM	Online Mendelian Inheritance in Man
OR	Odds ratio
p	*p*-value
PBS	Phosphate-buffered saline

PCR	Polymerase Chain Reaction
pH	Potential hydrogen
PMR	*Postmortem* redistribution
QL	Qualifier
QT	Quantifier
r^2	Coefficient of determination
RBC	Red blood cells
RT	Retention time
rpm	Rotations per minute
SDRs	Substance-related disorders
SGDs	Sustainable Development Goals
SIM	Mortality Information System, from the Portuguese: *Sistema de Informação sobre Mortalidade*
SoHT	Society of Hair Testing
SNP	Single Nucleotide Polymorphisms
SPE	Solid-phase extraction
SWGTOX	Scientific Working Group for Forensic Toxicology
UPLC-ESI-MS/MS	Ultra-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry
UNIFESP	Federal University of São Paulo, from the Portuguese: *Universidade Federal de São Paulo*
UNODC	*United Nations Office on Drugs and Crime*
VNTR	Variable Number Tandem Repeat
WHO	World Health Organization
WRVH	World Report on Violence and Health
X^2	Chi-square

List of Figures

List of Tables

Index

Introduction

1. Introduction

Crime and violence indexes in Brazil are considered to be high by international standards (EVOY; HIDEG, 2017). The great percentage of crimes, which can ultimately result in death, still remains one of the features most associated to the country and it is showing no signs of decline (MISSE, 2017).The latest World Health Statistics reported a rate of 31.3 intentional homicides per 100,000 inhabitants. This number is 4.9 times higher than that same prevalence in the whole world, currently set at 6.4 homicides per 100,000 inhabitants (WHO, 2018).

In general, violence found in Brazil may be explained due to several factors, including: economic disparity, the availability of firearms and a significant increase in drug use. In fact, several studies around the world have pointed out the close relationship between the consumption of drugs and crime (PINHEIRO et al., 2005; VALVERDE et al., 2006; MURRAY; CERQUEIRA; KAHN, 2013; HOELSCHER, 2015).

Within the most widely used illicit drugs in Brazil, cocaine places itself in the top three with a percentage of 2.3 amongst teenagers and 3.8 for adults regarding its use in the past year (LARANJEIRA et al., 2014). These two features (violence and cocaine abuse) seem to rise in a proportional way, which suggests that they might be linked. However, it remains a challenge to uncover whether drug abuse would be the cause of violence or a violent environment is causing people to take drugs.

In addition, there might be other unidentified reasons behind the abuse of drugs, which could be explained by genetics. That is, the fact that some individuals may have a higher likelihood to use drugs than others (BEVILACQUA; GOLDMAN, 2009; LE STRAT; RAMOZ; GORWOOD, 2016). Some authors have even added that cocaine dependence has a strong genetic component, with an estimated heritability of 72% (KENDLER et al., 2000).

In order to describe each one of these aspects (cocaine, violence and genetics), in a more detailed manner, they will be further explored in this thesis, both individually and in relation to one another, to allow for a much clearer perception on the subject.

Literature Review

2. Literature Review

2.1. Cocaine

Cocaine (benzoylmethylecgonine) is a powerful Central Nervous System (CNS) stimulant (PETKOVSKA et al., 2017). This naturally occurring alkaloid can be found in the leaves of two plant genus: *Erythroxylum novogranatense* and *Erythroxylum coca*, with the second one generally being the most used in terms of illicit production (NOVÁK; SALEMINK; I.KHAN, 1984).

Erytroxylum coca plant, the main source of cocaine, is natural from the Andean region in South America (PLOWMAN, 1982). Historical evidences show that its use dates back to 2500 BC (TREADWELL; ROBINSON, 2007;SILVA et al., 2010). The plant was considered to be sacred and therefore used in religious rituals as well as weddings and funerals (BAHLS, 2002).

At that time, chewing coca leaves was a privilege of the Inca aristocracy only (ZAPATA-ORTIZ, 1970). However, in the beginning of the 16th century with the arrival of Spanish conquers to America, the habit of chewing mixture of toasted coca leaves with other alkaline substances – to facilitate cocaine absorption through the oral mucosa – has spread throughout the Indians who used it to mask feelings of hunger, thirst and fatigue (BLEJER-PRIETO, 1965; ALLEN-GEORGE, 1981; ISENSCHMID, 2003; GOLDSTEIN et al., 2009).

Cocaine has only diffused into Europe when Albert Niemann was able to isolate the alkaloid from the plant, in 1860. After that, the drug was used in several different products around the world, such as the well-known soft drink Coca-Cola®, by John Stith Pemberton. In United States of America, the drink was being sold as a non-alcoholic 'intellectual beverage' capable of bringing a cure for all nervous affections such as headache, neuralgia, hysteria and melancholy (FERREIRA; MARTINI, 2001).

During the same period, two main events have significantly changed the usage pattern of cocaine. The first one was the publication of Sigmund Freud named *Über Coca,* where it was recommended the use of the drug for several psychiatric disorders such as depression, anxiety, alcoholism, morphine addiction as well as different other conditions like digestive problems and asthma (SILVA et al., 2010). The second one was the discovery of the anaesthetic properties of cocaine by Karl

Köller, the first physician who used this substance as a topical anaesthetic in ophthalmological surgery (BLEJER-PRIETO, 1965; ISENSCHMID, 2003).

Cocaine was being sold over-the-counter in different formulations such as a powder, cigarette, tablets as well as an injectable solution (BORTOLOTTI et al., 2012). It was not long until it started being used for recreational purposes, becoming a drug of abuse, which has resulted in several episodes of intoxication, tolerance, dependence and even death (MITTLEMAN; WETLI, 1984; KARCH; STEPHENS; HO, 1998). In fact, it was only after the second decade of the 20th century that regulatory measures have been taken by numerous countries (WILLS, 2005; GOLDSTEIN; DESLAURIERS; BURDA, 2009).

In 1986, another form of cocaine took over – *crack*. This drug has disseminated rather quickly among consumers as it is considered a cheaper version of cocaine and its effects are perceived much quicker (KARCH, 1999).

Regardless of the form, the parent compound of the drug remains the same and it contains a similar metabolic profile in the body. However, the route of administration of cocaine is of extreme importance in order to make a fair comparison between cocaine hydrochloride and freebase as they lead to distinctive use patterns (GREER et al., 2016).

Cocaine hydrochloride is soluble in water, which allows its intravenous administration (VASICA; TENNANT, 2002). This form of cocaine can also be snorted and it has acquired several street names throughout the years such as: ‘blow’, ‘happy dust’, ‘flake’, ‘nose candy’, ‘speedball’ (when mixed with heroin and injected) and ‘white’ (ISENSCHMID, 2003).

As for many other illegal drugs, there are numerous ‘cut substances’ used to adulterate cocaine such as caffeine, ephedrine, phenylpropanolamine, amphetamine, lidocaine, benzocaine, glucose, saccharose, amide and talc (UNODC, 2012; SHANNON, 2016). The main aim of using such undeclared compounds is to add bulk, therefore increasing the dealer’s profit margin; however, such practice can result in additional toxic effects for the user (KUDLACEK et al., 2017).

Cocaine hydrochloride is not suitable for smoking as its melting point is too high and it suffers significant decomposition at temperatures above 198°C (DINIS-OLIVEIRA, 2015). In order to obtain a smoked version of cocaine, the aqueous solution of cocaine hydrochloride must be heated up with basic substances such as sodium bicarbonate to obtain its freebase (BUTLER; REHM; FISCHER, 2017). Once

this gets cooled down, it forms irregular small pellets with a waxy appearance or ‘rocks’, known as *crack*-cocaine (HATSUKAMI; FISCHMAN, 1996).

Crack-cocaine (or simply *crack*) is heat stable and it has a melting point of 98°C (WOOD et al., 1996). As opposed to the hydrochloride salt, this form of cocaine can be easily smoked and its name is connotative to the sound it creates when being heated in the smoking pipes (DINIS-OLIVEIRA, 2015).

2.1.1. Toxicokinetics

The kinetics of cocaine depend greatly not only on the route of administration but also its physical/chemical form, genetics and concurrent consumption of alcohol (DINIS-OLIVEIRA, 2015). Cocaine is typically administered via three routes: snorting, smoking and injecting, with the first two being most prominent (GREER et al., 2016).

Their onset peak varies considerably. When snorting cocaine, the initial feelings of euphoria appear three to five minutes after administration, while for smoking cocaine it takes only six to eight seconds (WARNER, 1993; CHASIN; SILVA; CARVALHO, 2014).

Considering its plasma concentration, it takes the intranasal form about 50 minutes to reach its maximum peak against five minutes for intrapulmonary and intravenous routes (BORTOLOTTI et al., 2012). The vasoconstrictor properties of cocaine, highly limit its absorption via intranasal route, which explains its delayed onset, but at the same time those effects last much longer, around 60 to 120 minutes, when compared to intrapulmonary and intravenous administration, which last 30 to 60 minutes maximum (CONE, 1985; WARNER, 1993; PROSSER; HOFFMAN, 2001; RANG et al., 2012).

The distribution of cocaine is fast and its higher concentrations are found in the brain, spleen, kidneys and lungs followed by blood, heart and muscle (BORTOLOTTI et al., 2012). Cocaine gives origin to several metabolites as it suffers rapid biotransformation due to the hydrolysis of its two ester groups. The main metabolite is benzoylecgonine (BZE), which is formed either by spontaneous hydrolysis or it gets catalysed by carboxylesterases. Ecgonine methyl ester (EME) is originated from the hydrolysis of the benzoate group by carboxylesterase and pseudocholinesterase (MANTOVANI; PEGO; YONAMINE, 2015). Both BZE and EME give origin to ecgonine possibly due to spontaneous hydrolyses (BORTOLOTTI et al., 2012).

Norcocaine (NC) is an active metabolite, which is formed through *N*-demethylation of cocaine through the enzymes of cytochrome P450 and some studies found that it can block noradrenaline uptake more efficiently than cocaine itself (WANG et al., 2001).

Crack undergoes pyrolysis, resulting in a product named anhydroecgonine methyl ester (AEME). Finding this metabolite in biological specimens is exactly what enables to distinguish between cocaine or *crack* use (CONE; HILLSGROVE; DARWIN, 1994). Finally, cocaethylene (CE) results from the concomitant use of cocaine and alcohol. This substance is considered to be more harmful than cocaine itself due to its increased hepatotoxicity and longer half-life (PROSSER; HOFFMAN, 2001).

Cocaine is mainly excreted from the body through the urinary track and up to 9% of the dose is eliminated in its unaltered form with a half-life of 0.8 hours. Nearly 49% is excreted as EME and 54% as BZE with a half-life of 3.1 and 4.5 hours, respectively (AMBRE et al., 1988). **Figure 1** illustrates the main metabolic pathways of cocaine.

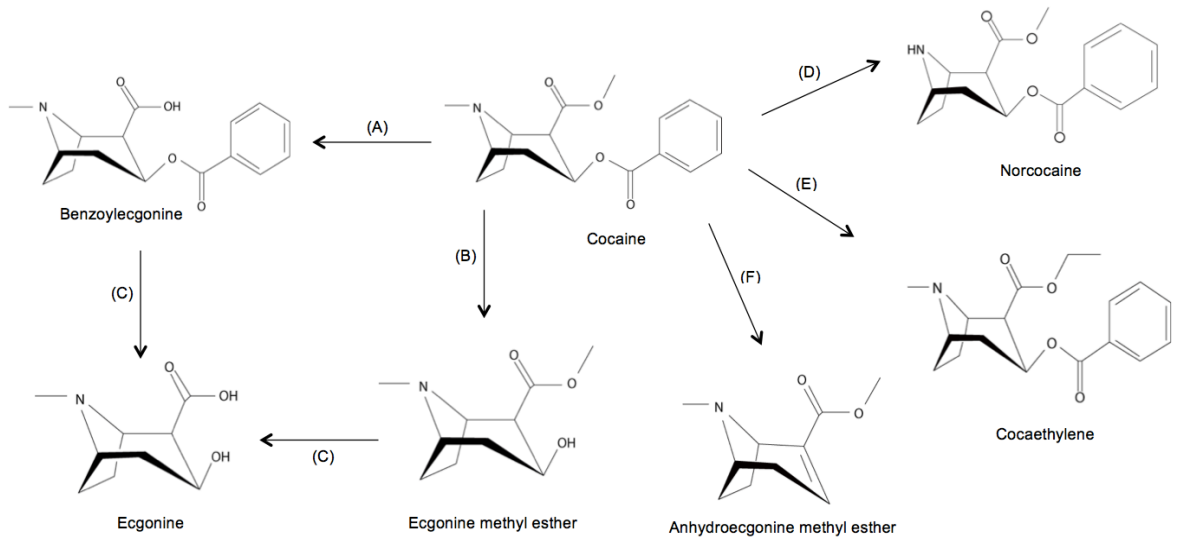

Figure 1. Primary metabolic pathways of cocaine, including its pyrolysis product (anhydroecgonine methyl ester) and the product of transesterification with ethanol (cocaethylene), where, **A**: spontaneous hydrolysis or hydrolysis mediated by carboxylesterases; **B**: carboxylesterases and pseudocholinesterase; **C**: spontaneous hydrolysis; **D**: cytochrome P450 (N-demethylation); **E**: hepatic transesterification and **F**: pyrolysis. Source: adapted from MANTOVANI; PEGO; YONAMINE (2015).

2.1.2. Toxicodynamics

The pleasurable effects of cocaine are due to specific mechanisms in the brain, which cause the so-called 'high'. This CNS stimulant drug is responsible for blocking the re-capture of dopamine due to its binding to dopamine transporter sites (HUANG; GU; ZHAN, 2009). This results in an inhibition of the re-uptake of dopamine into the presynaptic terminal, thereby prolonging its effects in the synapse due to an accumulation of dopamine in the synaptic cleft (UHL; HALL; SORA, 2002; HOWELL; KIMMEL, 2008; DINIS-OLIVEIRA, 2014).

In **Figure 2** it is possible to observe a normal synapse while **Figure 3** illustrates a synapse occurring in the presence of cocaine.

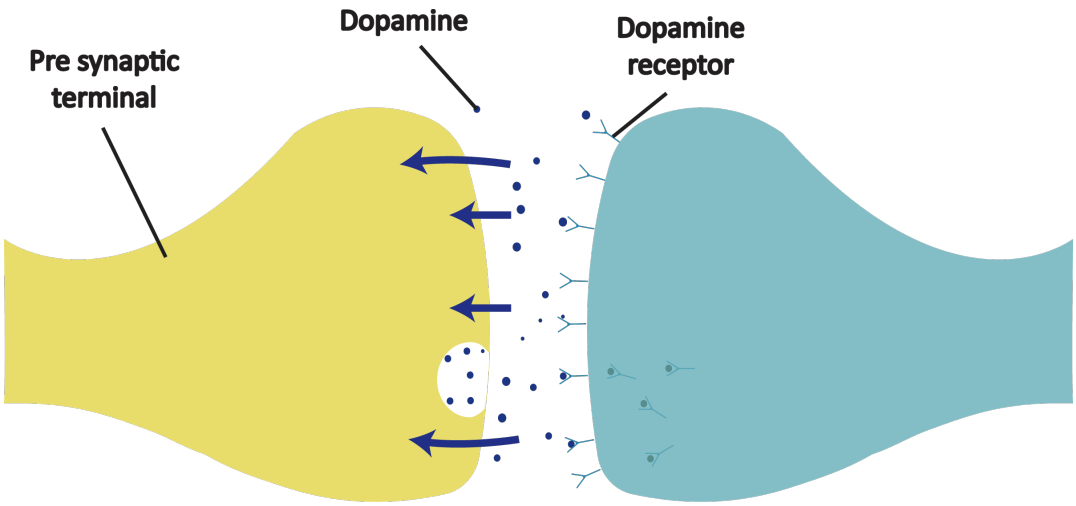

Figure 2. Normal synapse: occurs by releasing dopamine into the synapse, where it can bind to dopamine receptors on neighbouring neurons. Normally, dopamine is then recycled back into the transmitting neuron by a specialized protein called the dopamine transporter. Source: adapted from MORRIS (1998).

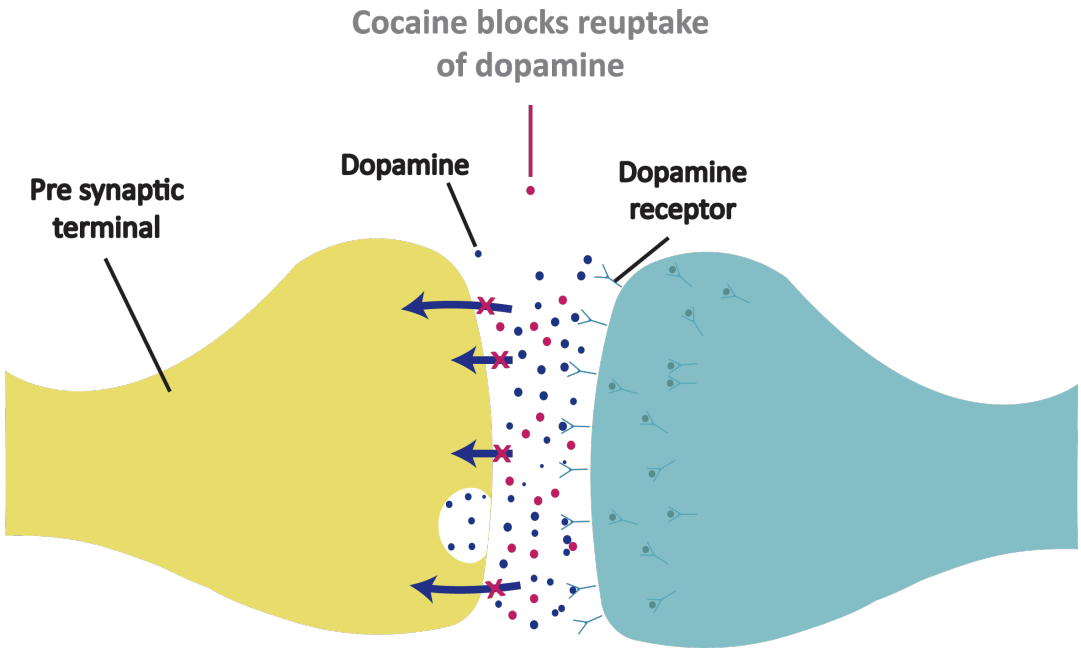

Figure 3. Synapse in the presence of cocaine: cocaine attaches to the dopamine transporter and blocks the normal recycling process, resulting in a build-up of dopamine in the synapse, which contributes to the pleasurable effects of cocaine. Source: adapted from MORRIS (1998).

By doing so, the user experiences a powerful “rush” of euphoria in such an intense way that is well outside the normal range of human experience (DACKIS; O’BRIEN, 2001). It is very common to feel an increase in self-confidence and alertness, which makes individuals believe they have improved mental abilities and that they are now capable of performing certain motor and intellectual tasks. Not only they become alert but also much less tired (SOFUOGLU; SEWELL, 2009; BORTOLOTTI et al., 2012; DINIS-OLIVEIRA, 2015).

However, if on one hand cocaine causes feelings considered to be 'positive' due to intensification of the monoaminergic transmission, on the other hand, prolonged blockade of the uptake caused by chronic use of this drug of abuse, leads to the depletion of monoamines (DINIS-OLIVEIRA, 2014). This substantial decrease in neurotransmitters causes the user to sense a state of dysphoria and starts having feelings of depression, insomnia, anger, loss of sexual desire, development of eating disorders and even suicide (DACKIS; O’BRIEN, 2001; KARILA et al., 2012; MANTOVANI; PEGO; YONAMINE, 2015).

With high doses, there may be state tremors and convulsions, followed by respiratory and vasomotor depression. Sympathomimetic peripheral actions lead to tachycardia, vasoconstriction and increased blood pressure. The temperature of the body may rise due to the boosted motor activity along with the reduction of heat loss (EGRED; DAVIS, 2005; RANG et al., 2012).

2.1.3. Tolerance, addiction and withdrawal

It appears that tolerance occurs to the behavioural effects of cocaine in chronic users after consumption of high doses and comprises of reduction in the euphoric and physiological effects as the ability of cocaine to elevate dopamine levels decreases (SICILIANO; FORDAHL; JONES, 2016). In fact, some users report to usually smoke quantities of *crack* high enough to kill an adult individual. There are also evidences that reverse tolerance, also known as sensitization, may occur (CALIPARI et al., 2013; CHASIN; SILVA; CARVALHO, 2014).

Cocaine has been recognized as one of the substances with the highest potential of abuse in the whole world (HUANG; GU; ZHAN, 2009) and that is due to its great ability of inducing positive reinforcement (WOOLVERTON; JOHNSON, 1992; SMITH et al., 2008). However, some authors believe that even though cocaine causes strong dependence, there is still a debate as to whether its continuous use will, indeed, induce tolerance. According to them, users can increase the dose of the drug, but this may simply reflect the desire for greater effect, and not the development of tolerance (NEGUS; MELLO; CAINE, 2004; RANG et al., 2012).

The mechanism of cocaine addiction is not yet fully understood. Nevertheless, the constant chronic cycle of increase followed by decrease of dopamine levels, definitely contributes to the development of cocaine's addiction, becoming progressively entrenched and uncontrollable (DACKIS; O'BRIEN, 2001).

Also, these mood swings trigger the individual to the so-called 'craving', where they develop a fixation for the drug and will search for any means of administrating it as soon as possible (FILIP et al., 2005; KARILA et al., 2012; CHASIN; SILVA; CARVALHO, 2014).

The withdrawal of cocaine, after administration for a few days, causes sharp deterioration in motor performance and learning behaviour, which is restored by taking the drug dosage. There is thus, a considerable degree of psychological

dependence in which users seek the stimulatory and euphoric effects of the drug (NEGUS; MELLO; CAINE, 2004; RANG et al., 2012).

2.1.4. Toxic effects

Even though cocaine has its most devastating effects in the CNS, its use disrupts various systems within the human body such as the cardiovascular, respiratory, gastrointestinal, renal, skeletal muscle and they can also be spread to the fetus and neonate (WHITE; LAMBE, 2003; KARILA et al., 2012). However, its actions within the CNS are by far the most relevant ones as they can cause tonic-clonic seizures, stroke, psychiatric disorders, schizophrenia, depression, suicide feelings, obsessive-compulsive behaviours and hyperthermia due to a deregulation of the CNS thermoregulatory centre. Death is usually caused by failures in the cardiovascular system as when impaired it can result in arrhythmia, ischemia, myocarditis, aortic rupture, angina pectoris and sudden death (WHITE; LAMBE, 2003; ACKERMAN; RIGGINS; BLACK, 2010; DINIS-OLIVEIRA et al., 2012; ZIMMERMAN, 2012).

2.1.5. Usage patterns

The usage pattern of cocaine is relatively simple to spot by looking at several external injury traces, which can be found in the individual. When snorting cocaine, the user can develop what is known by ‘rat nose’, which is the name given to illustrate a perfusion of the nasal septum. When *crack* is being smoked it is very common to find signs of burns in the lips, tongue and face due to the proximity to the smoking pipe and, finally, when using cocaine intravenously, the needle marks throughout the body are also fairly clear (PASSAGLI, 2008; CHASIN; SILVA; CARVALHO, 2014).

Regardless of the user’s method of choice, cocaine is being highly abused in the country. For this reason, it is important to take a closer look at the statistics, which can give an overview on the use pattern around the world and more specifically in Brazil.

2.1.6. Cocaine use

The United Nations Office on Drugs and Crime (UNODC) estimates that about a quarter of a billion people in the world, representing around five per cent of the global adult population have used an illicit drug at least once in 2015 (UNODC, 2017).

Figure 4 shows just how much of the world population has used the various different drugs (UNODC, 2017).

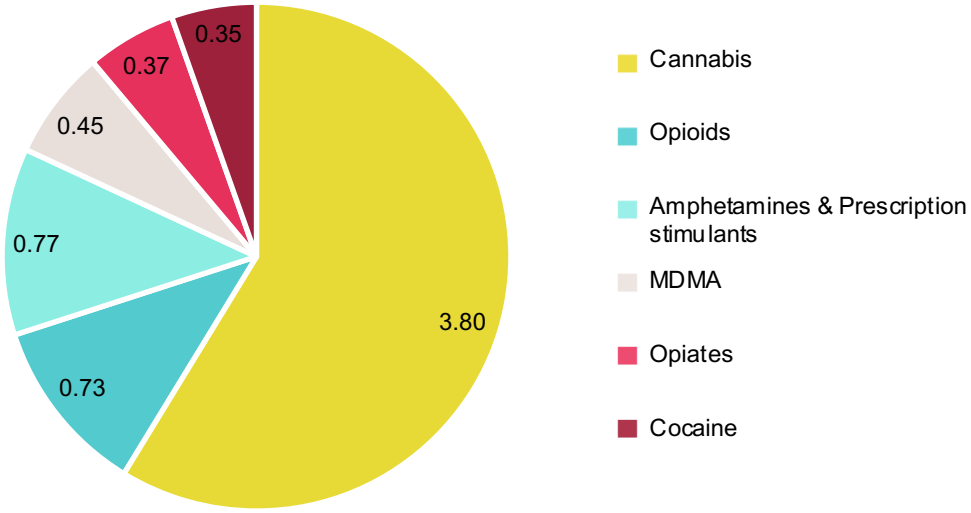

Figure 4. Global estimates of the use of different drugs expressed as percentage values. Evaluations for adults aged 15-64, based on the year of 2015. Source: adapted from UNODC (2017).

Globally, the most consumed illicit drug is *Cannabis* with a worldwide prevalence use of 3.80%, followed by amphetamines and prescription stimulants with a prevalence of 0.77%. Opioids use was found to be at 0.73% and in fourth place comes 3,4-methylenedioxymethamphetamine (MDMA), also known as “ecstasy”, with 0.45% users worldwide. Finally, there are opiates and cocaine with a rather similar pattern of 0.37 and 0.35%, respectively (UNODC, 2017).

When it comes to Europe’s trends, *Cannabis* also remains the most used drug by far with 7.2% of prevalence, followed by new psychoactive substances (3.0% of prevalence) and with cocaine being reported as the third most consumed drug in

Europe, with a prevalence of 1.1%. Finally, MDMA was reported to have a prevalence of 0.8%, followed by amphetamines and opioids (high-risk opioids users) with very close trends of 0.5 and 0.4%, respectively (**Figure 5**) (EMCDDA, 2018).

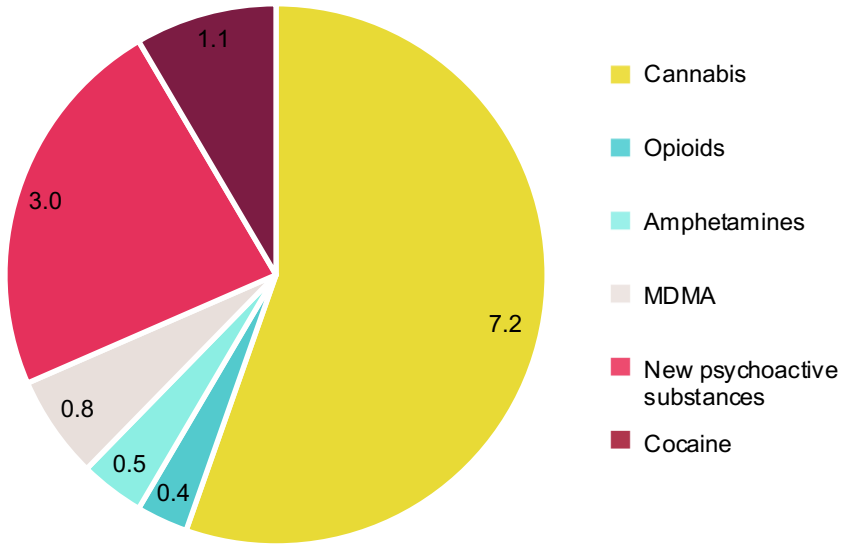

Figure 5. European estimates of the use of different drugs expressed as percentage values. Evaluations for adults ages 15-64, based on the year of 2017. Source: adapted from: EMCDDA (2018).

As for the South American continent it is possible to see that also here, *Cannabis* is the top list drug of choice with a prevalence of 2.90% and, in this case, the second most used illicit drug is cocaine followed by amphetamines with a prevalence of 0.88% and 0.25%, respectively.

Lastly, MDMA and opioids appear to be very similar in its use with values of 0.16 and 0.14%, respectively, followed by opiates with the lowest per cent value of 0.06% (**Figure 6**) (UNODC, 2017).

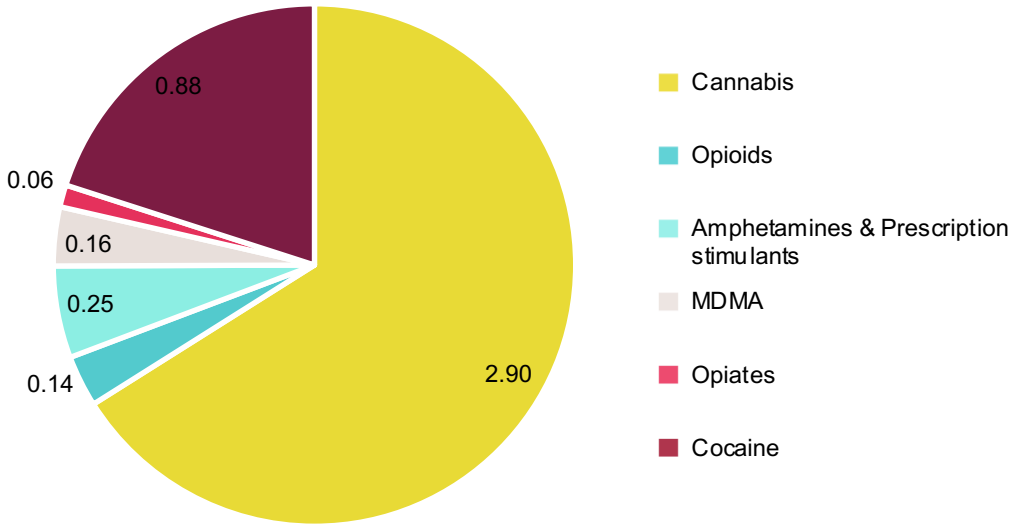

Figure 6. South American estimates of the use of different drugs expressed as percentage values. Evaluations for adults ages 15 - 64, based on the year of 2015. Source: adapted from UNODC (2017).

According to UNODC's World Drug Report 2017, the South American continent shows an annual prevalence of cocaine 2.5 times higher than its global average consumption (UNODC, 2017)

As for Brazil, despite the imminent difficulty in finding more recent data, among the different illicit drugs available, cocaine/*crack* is widely used in the country. It places itself within the top three most used drugs by both young (14 to 17 years old) and adult (18 years old and above) population according to the II Brazilian National Alcohol and Drugs Survey (BNADS) report, elaborated by the National Institute of Science and Technology for Public Policy on Alcohol and Other Drugs (INPAD) together with the Federal University of São Paulo (UNIFESP), as it can be seen in the following **Figure 7** (LARANJEIRA et al., 2014).

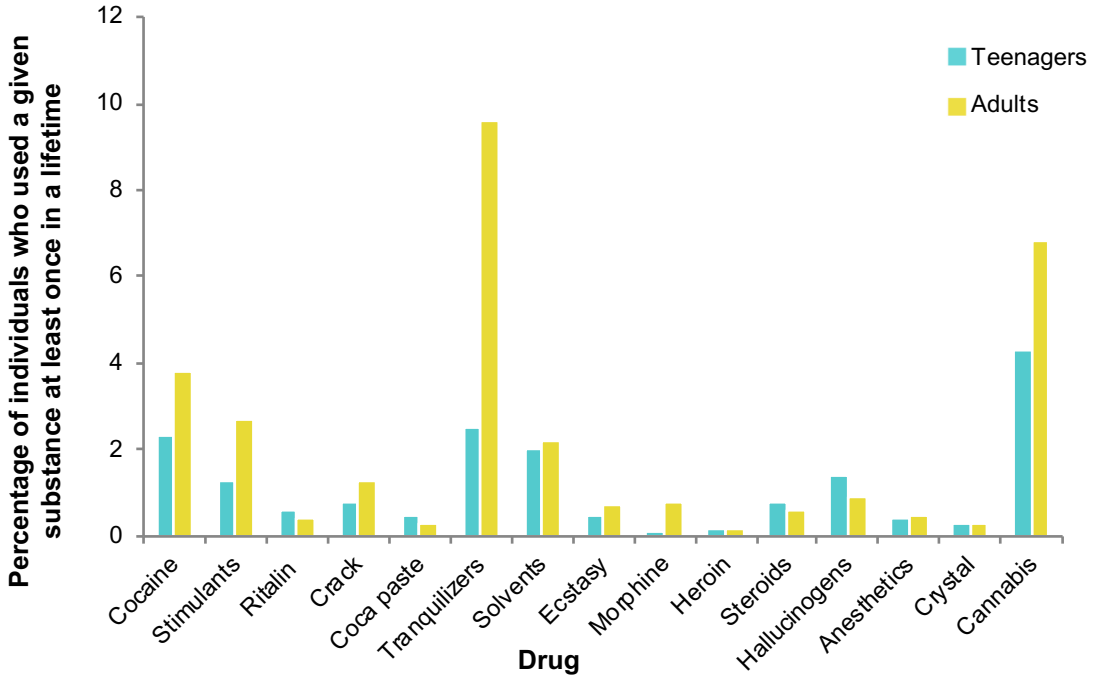

Figure 7. Proportion of individuals who have used a given substance at least once in a lifetime. Data has been gathered in the year of 2012 by INPAD along with UNIFESP. The total number of teenagers in Brazil is of 13,947,197 while the total number of adults is of 134,370,019. Source: adapted from LARANJEIRA et al. (2014).

According to **Figure 7**, the top three most used illicit drugs among teenagers are *Cannabis* with a percentage of 4.3%, followed by tranquilizers with a 2.5% usage and finally cocaine representing 2.3%. As for adulthood, *Cannabis* trades first place with tranquilizers with a 6.8 and 9.6% prevalence use, respectively, and cocaine remains the third most used illicit drug with a percentage value of 3.8 (LARANJEIRA et al., 2014). Data regarding the use in the past 12 months has also been evaluated and this can be seen in **Figure 8**.

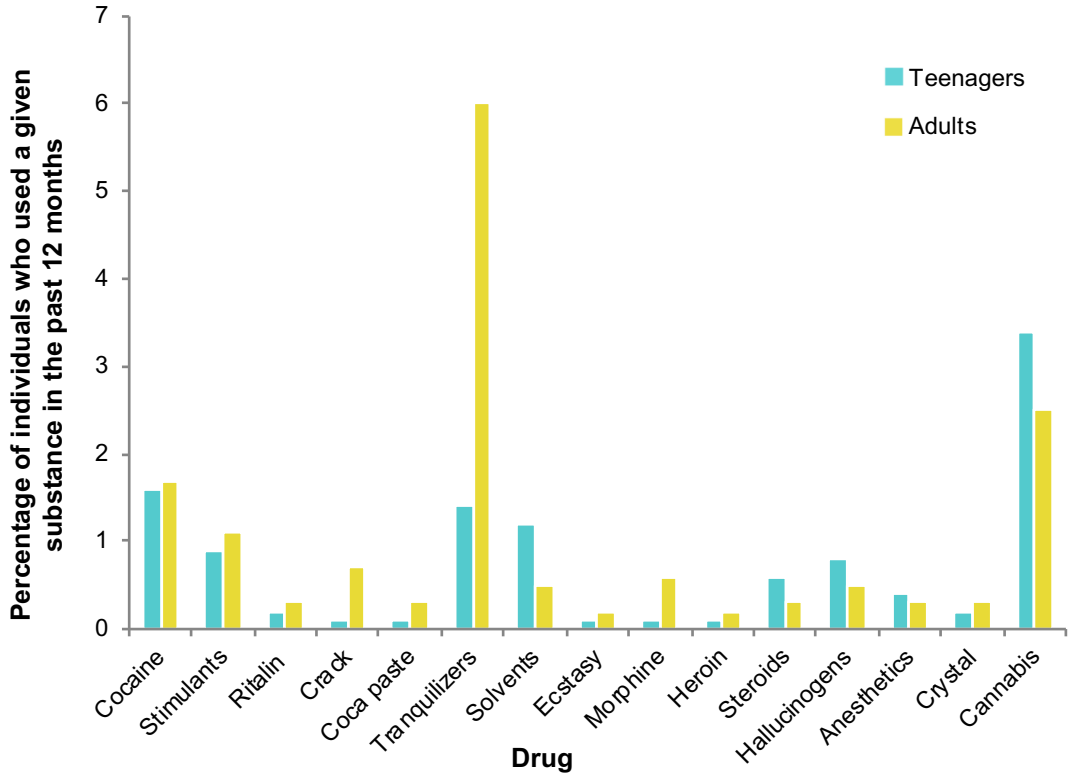

Figure 8. Proportion of individuals who have used a given substance in the past 12 months. Data has been gathered in the year of 2012 by INPAD along with UNIFESP. The total number of teenagers in Brazil is of 13,947,197 while the total number of adults is of 134,370,019. Source: adapted from LARANJEIRA et al. (2014).

In this case, for the year of 2012, while *Cannabis* remains the most used illicit drug in the last 12 months among youngsters, with a percentage of 3.4, cocaine takes second place with values of 1.6% and only then tranquilizers follow with 1.4% usage. As for the adult population, the trend remains similar to that of **Figure 8** with 6.0% using tranquilizers, 2.5% using *Cannabis* and finally 1.7% using cocaine (LARANJEIRA et al., 2014).

Figure 9 illustrates the two most used routes of administration by both teenagers and adults considering the drug use period.

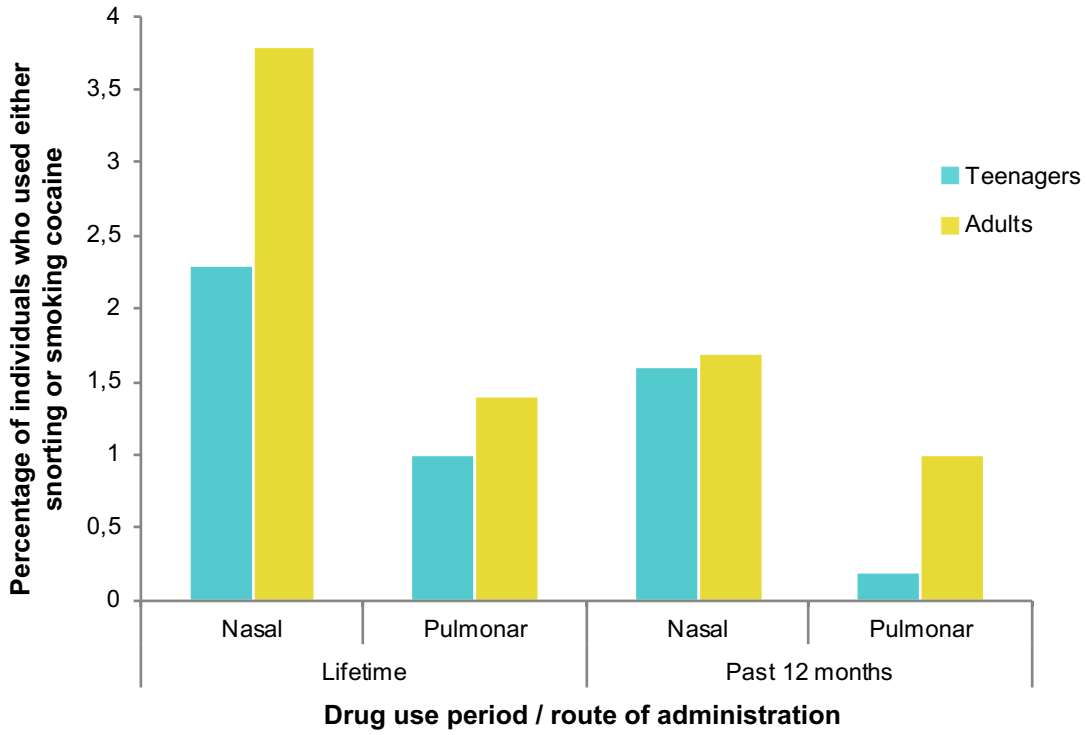

Figure 9. Proportion of young and adult population who have either snorted or smoked cocaine, both in a lifetime and in the past 12 months. Data has been gathered in the year of 2012 by INPAD along with UNIFESP. Source: adapted from LARANJEIRA et al. (2014).

Between the two major routes of administration: nasal (cocaine hydrochloride) and intrapulmonary (*crack* and coca paste), the first one remains the preferred type by both young and adult individuals for both drug use periods (lifetime and past year). In general, it is clear that adults do use more cocaine than adolescents as the percentages are always significantly higher apart from 'snorting cocaine in the past year' where teenagers and adults have quite a similar percentage value of 1.6 and 1.7%, respectively (LARANJEIRA et al., 2014).

The most recent data simply show the percentage of cocaine use in the previous year amongst the general population, which is set at around 1.9% (1.4 – 2.6%). To the best of our knowledge this is the latest data available to represent the use of drugs in Brazil as the most recent report entitled "Association between drug use and urban violence" also discusses the data from the II BNADS (ABDALLA et al., 2018).

2.1.6.1. Cocaine cultivation and trafficking

Coca is strongly cultivated throughout the central and northern Andean Ridge. According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), the most recent data gathered by United Nations (UN) shows quite a significant change in coca cultivation in the year of 2014. While Colombia has witnessed an abrupt rise, Peru on the other hand has been dropping its coca plantation hectares (SCHULTZE-KRAFT, 2016). This is shown in **Figure 10** below.

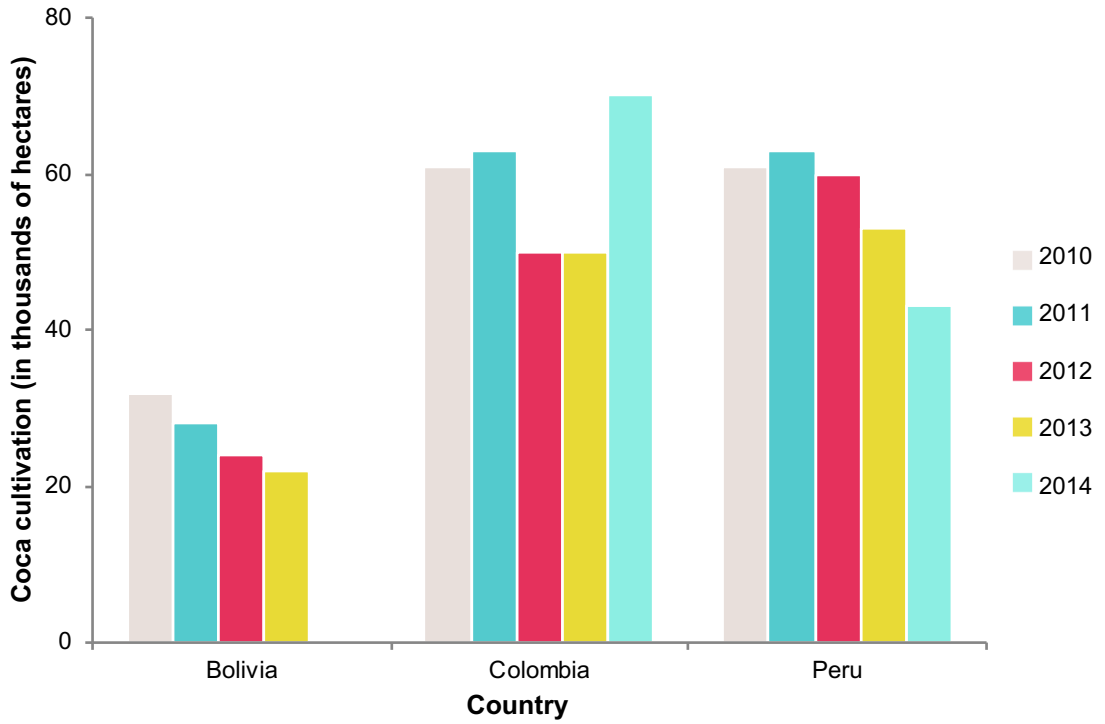

Figure 10. Estimated coca cultivation in Bolivia, Colombia and Peru, 2010-2013/14 (in thousands of hectares). Without taking into consideration Colombia's data for 2014 and with Bolivia's 2014 data still to be published, the overall trend in the period 2010-2013 has been a year-on-year reduction of total combined coca cultivation in the three Andean source countries. Source: adapted from SCHULTZE-KRAFT (2016).

An outstanding increase in cocaine seizures has been observed in the South America continent with a rise of 364 tons in 2010 to 526 tons in 2015 in the South American continent. This is linked to the previously mentioned rise in cocaine production and trafficking in Colombia. Although trafficking in cocaine seems to be a global phenomenon, South America still remains the main departure hub to the rest of the world with 90% of the cocaine intercepted in 2015 (UNODC, 2017).

Brazil's role in this matter has to do with its strategically favourable geographical position, which enables trafficking directly to North America, Western and Central Europe. The trend observed is that cocaine-producing countries (Bolivia, Colombia and Peru), supply the drug to the rest of the region while Brazil (mainly since 2010) and Argentina are the cocaine transit countries most frequently mentioned in major individual drug seizures (UNODC, 2015).

Brazil's seizures have doubled in 2013 to over 40 tons unlike Bolivia, Peru and Venezuela, making it the largest cocaine market in South America (UNODC, 2015). In fact, Brazil was reported as the most frequent departure/transit country for cocaine trafficking into Europe, Africa and Asia in the period of 2010-2015 (UNODC, 2017).

Clearly, this leads to serious public health problems to the country which can compromise several aspects of one's life such as family and community, health, education, environment and will eventually lead up to crime, corruption and dangers for civil society (UNIDCP, 1998). An example of this issue is a place called 'Cracolândia' (*Crackland*), where it is possible to find an enormous gathering of drug users who live in the streets and smoke *crack* for most part of their days.

There are an estimated 2.2 million *crack* users in Brazil (ABDALLA et al., 2014) and *Cracolândias* exist in most metropolitan areas. Of these, São Paulo's is the largest and oldest one as it is the home for more than two thousand addicts since the 1990s, making it the biggest *crack* cocaine gathering in the world (ECONOMIST, 2013).

Today, *Cracolândia* spreads itself for a relatively large perimeter of around 15 blocks (ARRUDA, 2014). Police have unofficially declared *crack* users ill people instead of criminals, leaving them to smoke freely although a surveillance van monitors them 24 hours a day (**Figure 11**) (BANSAL, 2014).

Figure 11. Police cameras from a surveillance van, monitoring a few areas of Cracolândia where users smoke *crack* freely. Source: TORAL (2014).

Although numerous volunteer projects and non-profit organizations have been put into place to try and dismantle *Cracolândia*, they have rarely ever worked. São Paulo's city hall has spent millions of *reais* (Brazilian currency) to deal with the situation and to bring new tactics against the *crack* epidemic, however, it has only resulted in overcrowded prisons which now count with twice as many inmates than a decade ago (ECONOMIST, 2013).

This represents simply a part of the problem. As mentioned previously, having an imminent drug issue in the country leads to a higher number of crimes and violence in general.

In fact, UNODC's 2016 World Drug Report brings up this matter as it shows that being a transit country does bring higher violence rates. However, not every kind of drug raises this problem. The following figure illustrates an example of what is happening in different drug markets (UNODC, 2016).

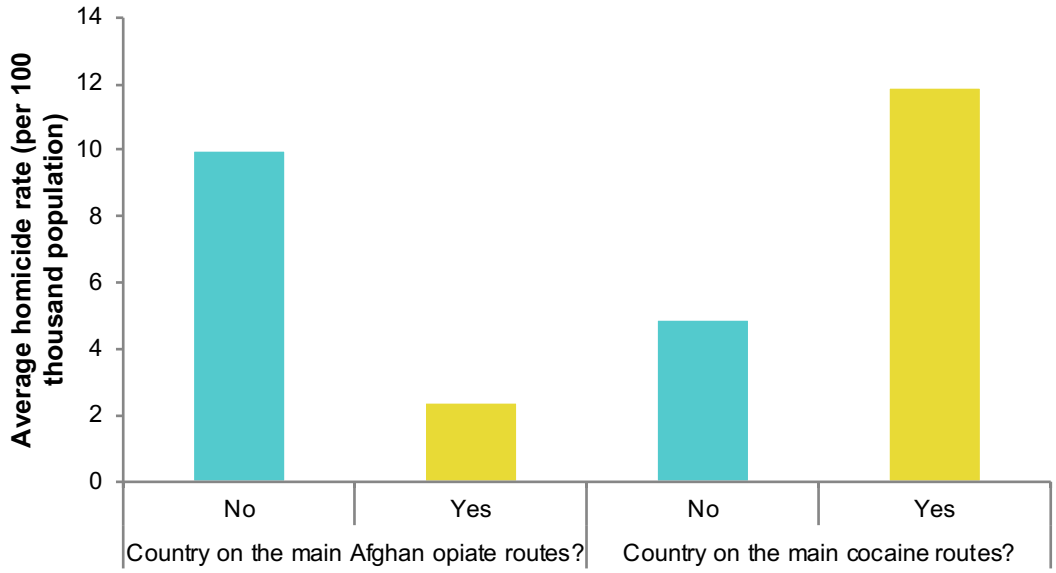

Figure 12. Relationship between drug transit countries and violence for both opiates and cocaine expressed by average homicide rates (per 100,000 inhabitants). Source: adapted from UNODC (2016).

As it can be seen in **Figure 12**, drug trafficking does not necessarily produce violence. Transit countries involved in opiate trafficking do not have higher homicide rates; on the other hand, countries involved in cocaine trafficking routes do have a significantly higher rate of homicides (UNODC, 2016).

The reasons behind this disparity may be the characteristics of cocaine's trafficking world such as competition in the illicit market and differences in the internal structure of trafficking networks. Also, the drug trade is strongly established in countries where opportunities for corruption exist as the power of drug markets offers protection from law enforcement agents; politicians and the business sector (UNODC, 2016).

Apart from that, the actual profits associated with this illicit drug market are somewhat motivational for non-state armed groups to engage in this type of illegal business. In some countries, such as Brazil, there is often an increase in their overall lethality due to extensive armed conflicts among drug market groups (UNODC, 2016).

2.2. Violence

According to WHO's definition of 'violence', an individual or a group of individuals must actually aim to use strength power against another person or a group in order to be categorised as violent. This classification makes it possible to separate this concept from unintended injury or harm and also incidents (RUTHERFORD et al., 2007).

The World Report on Violence and Health (WRVH) has broken down violence into 3 categories referring to the subject of violence: self-directed, interpersonal or collective and into 4 categories according to the nature of violence: physical, sexual, psychological or involving deprivation or neglect. These also may occur simultaneously (RUTHERFORD et al., 2007; WHO, 2014).

The most recent data shows that a minimum of 560,000 people lost their lives violently in the year of 2016, corresponding to a rate of around 7.50 violent deaths per 100,000 inhabitants. On average, interpersonal or collective violence killed at least one person every minute of every day of the year (EVOY; HIDEG, 2017).

From those, about two-thirds (69%) of all victims of lethal violence were intentional homicides, followed by 18% of direct conflict deaths, 10% unintentional homicides and 3% as killings due to legal interventions (EVOY; HIDEG, 2017). As intentional homicides are playing such a worrying role in the global scenario, a further look onto this data shall be in place.

According to the World Health Statistics 2018, monitoring health for the Sustainable Development Goals (SDGs), sourced by WHO, in the year of 2016 there were an estimate of 477,000 murders in the world, which represent an overall rate of 6.4 per 100,000 inhabitants. **Figure 13** shows the mortality rate due to homicide (per 100,000 inhabitants) (WHO, 2018).

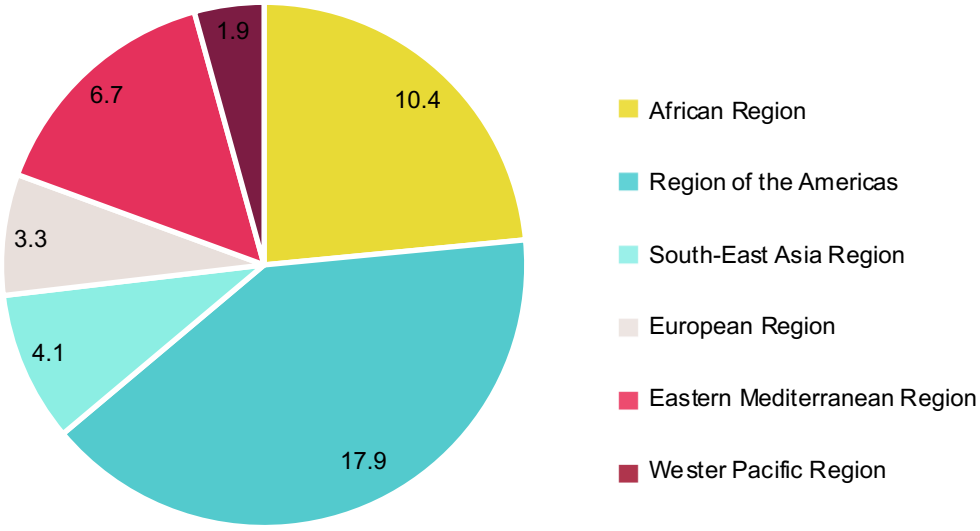

Figure 13. Estimated numbers and rates of homicide per 100,000 people, by WHO region – WHO Member States with a population of less than 90,000 in 2016 were not included in this analysis. Source: adapted from WHO (2018).

This figure shows that the region of Americas holds the highest homicide rate number with a value of 17.9 homicides per 100,000 inhabitants, followed by the African and the Eastern Mediterranean region with values 10.4 and 6.7 homicides, respectively. When unravelling this data, gender wise, men in the WHO Region of the Americas suffered the highest rate of homicide deaths at 31.8 per 100,000 inhabitants, 11 times the rate among men in the WHO Western Pacific Region (WHO, 2018).

These numbers seem to remain stable with time as an earlier WHO report has stated that not only this region of the world was the most violent, back in 2014, but it was also the one with the highest disparity rate regarding the mechanisms used to commit crimes. In that year, it was estimated that 75% of the homicides were committed by making use of firearms, followed by sharp force (16%) and 'other' (9%) (WHO, 2014).

When looking at Brazil, there was an outbreak of the deaths almost exclusively leveraged by firearm homicides, which increased 592.8% from 1980 to 2014 (WAISELFISZ, 2016). As a matter of fact, it is extremely difficult to control the

possession of firearms in Brazil because it is exceptionally heterogeneous across the country, just like so many other socio-economic factors (DREYFUS et al., 2010).

Depending on the state, these firearms possessions manifest themselves in different ways: either from the illegal .38 revolver in the hands of convicts in a favela of São Paulo or Rio de Janeiro, to the unregistered rifle or shotgun in Mato Grosso or Rio Grande do Sul and even the shotgun used to oversee illicit gold mines in Rondônia (DREYFUS et al., 2010).

Recent data corroborate these findings as it was seen that countries that experienced the highest rates of firearm-inflicted deaths in 2016 were in Latin America and the Caribbean. It is even added that firearm violence has increased in the Bahamas, Brazil, Trinidad and Tobago, and Venezuela (EVOY; HIDEG, 2017).

Looking closer at the 20 Latin American countries, according to the latest survey from the Investigation and Analysis of Organized Crime, Brazil was considered the 6th most violent country with a total of 26 homicides per 100,000 inhabitants (GAGNE, 2016). This number seems to have risen, as this year (2018), the homicide rate of Brazil was reported to be at 31.3 per 100,000 inhabitants, 4.9 times the worldwide rate (WHO, 2018).

By examining these numbers from a historical point of view, Brazil's adjusted homicide rate has more than doubled between 1980 and 2002 - 32 homicides per 100,000 inhabitants. Among that 22 year interval, there were about a million homicides in Brazil (MORRISON; BRONKHORST, 2006).

According to the 'Map of Violence: Homicide and Youth in Brazil', a historical evolution of violent mortality in the country represents impressive numbers. In line with records from the Mortality Information System (SIM), between 1980 and 2011, a total of 1,145.908 victims of homicide died in Brazil. **Figure 14** illustrates violent mortality rates by simple age numbers in 2011 (WAISELFISZ, 2014).

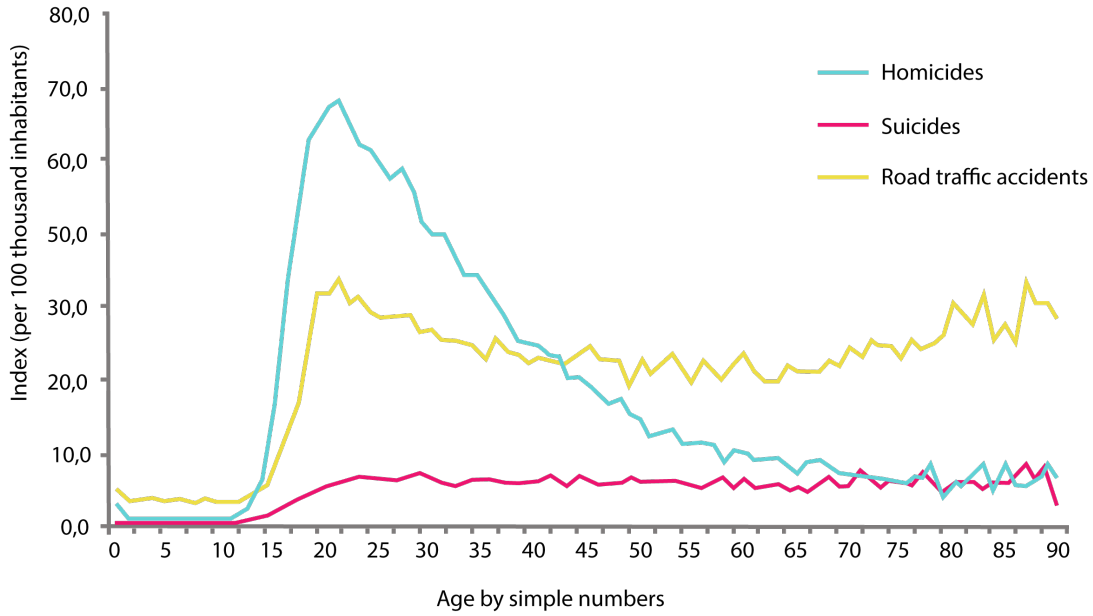

Figure 14. Violent mortality rates by simple ages – Brazil, 2011 with homicides showing the most significant growth between individuals aged between 20 and 24 years old. Source: adapted from WAISELFISZ (2014).

Looking at the critical age at which individuals start to commit crimes, it is possible to confirm that there is a major increase in the homicidal rates at the age of 20/21 years, which is around 70 homicides per 100,000 inhabitants. Suicide, which can be considered violence against oneself, shows an increase at 17/18 years old of around 5 suicides per 100,000 inhabitants. Finally, road traffic accidents also show a significant increase at the age of 20/21, with a number of around 40 deaths from road traffic accidents per 100,000 inhabitants (WAISELFISZ, 2014).

Together, the age group from 19 to 25 years old shows a violent death rate that exceeds 100 deaths per 100,000 inhabitants. The historical evolution of violent mortality in Brazil is remarkably shocking when it comes to its quantitative values.

According to the SIM records, between 1980 and 2011, the absolute number of deaths resulting from violent situations (homicides, suicides and road traffic accidents) occurring in Brazil can be seen in **Figure 15** (WAISELFISZ, 2014).

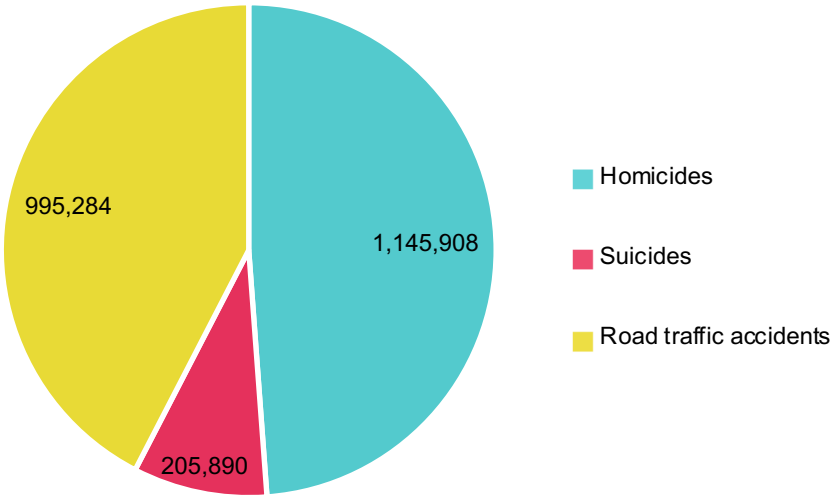

Figure 15. Number of violent deaths in Brazil between 1980 and 2011 consisting of homicides, suicides and road traffic accidents with an absolute number of 1,145,908; 205,890 and 995,284, respectively. Source: adapted from WAISELFISZ (2014).

As a matter of fact, the total growth of deaths in Brazil, between this period in time was of 55.9% (WAISELFISZ, 2014). Moreover, in the year of 2016 the highest numbers of violent death were found to be in Brazil, India, Syria, Nigeria, and Venezuela (EVOY; HIDEG, 2017).

Usually, this magnitude of violence is wrongfully attributed to the gigantic continental dimensions of Brazil. As the following **Figure 16** and **17** show, this argument is not valid as there are various countries with a number of inhabitants quite similar to those of Brazil, like Pakistan, with 170.3 million inhabitants, having much lower homicides rates. Not to mention China or India, which have 1,339.20 and 1,184.60 million people, respectively and their homicide rates are still much lower than those of Brazil (**Figures 16 and 17**) (WAISELFISZ, 2014).

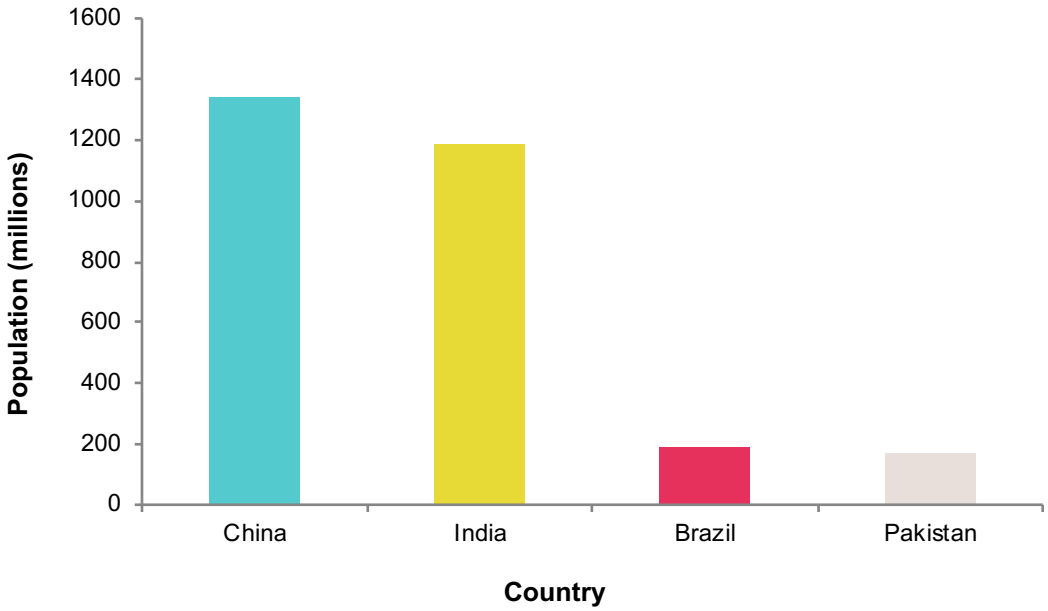

Figure 16. Population, in millions, of China (1,339,20); India (1,184,60); Pakistan (170,3) and Brazil (190,8). Source: adapted from WAISELFISZ (2014).

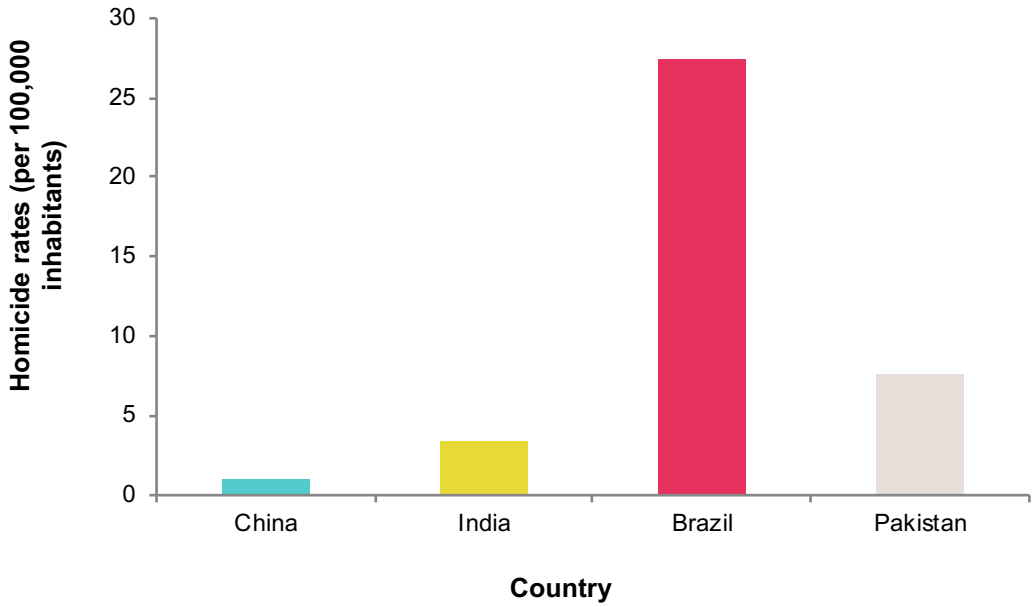

Figure 17. Homicide rates (per 100,000 inhabitants) in China (1.0); India (3.4); Pakistan (7.6) and Brazil (27.4). Source: adapted from WAISELFISZ (2014).

By looking at **Figure 18**, it can be seen a strong growth of homicides since the beginning of the series, in 1980, when the rate was about 11.7 homicides per 100,000 inhabitants by the year of 2003, when the rate reaches 28.9 with a gradient of 4% annual growth. From 2003 onwards, as a result from disarmament campaigns and specific policies in some Federation Units of great demographic weight, homicide rates started falling up until 2007, when violence started escalating once again (WAISELFISZ, 2014).

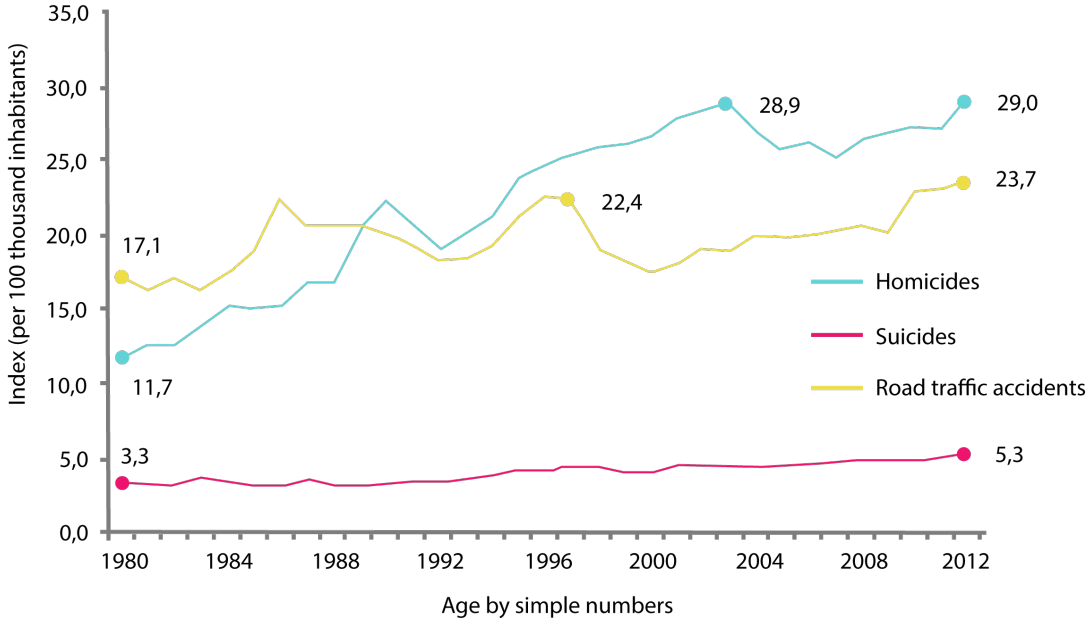

Figure 18. Evolution of violent death rates in Brazil (per 100,000 inhabitants) between 1980 and 2012 where homicides have increased from 11.7 to 29.0 (growth of 17.3 in 32 years); suicides have gone from 3.3 to 5.3 (growth of 2.0 in 32 years) and road traffic accidents from 17.1 to 23.7 (growth 6.6 in 32 years). Source: adapted from WAISELFISZ (2014).

Nowadays, the latest prevalence studies have shown that the current homicide rate is at 28.9 homicides per 100,000 inhabitants, which basically mirrors the last report from 2012 (CERQUEIRA et al., 2017). As it can be seen, there is a strong indication that these values will follow their constant growth as time goes by. This suggests that more efficient measures must be taken to avoid any deplorable consequences.

In addition, tragedies as such, have consequences in health, demographic dynamics and, consequently, the process of economic and social development (CERQUEIRA et al., 2017). It is, indeed, crucial for the country to identify what

exactly is causing this accumulation of violence and Misse has appointed a few reasons, such as: gang and drug-related violence, the excessive use of state force, a corrupt criminal justice system, the militarization of key areas and the cycle of violence generating more violence, underpin these extremely high homicide rates (MISSE, 2017).

Once the different sources of violence are triggered it is necessary to act directly at the root of the problem to try and solve it in the best possible manner. In this work we have focused on the first cause appointed by Misse: drug-related violence, as this issue has been previously addressed, mainly for cocaine use.

2.3. Violence and cocaine

WHO declared that the effects of some drugs, including cocaine/*crack*, amphetamines and benzodiazepines are related to increased aggressive and violent behaviour (ATKINSON; ANDERSON; HUGHES, 2009). So, there are direct and indirect links between violence and drug use.

According to Paul Goldstein, there is a model suggesting three main reasons by which the use of drugs could be correlated with violence. This model consists of the following points: a) psychopharmacological, which attributes the cause of violence to behavioural effects after ingestion of the substance, for example, irritability and restlessness that leads to violent reactions; b) the economic factor, which may result in the commitment of crimes in order to arrange money to buy drugs by addicts and finally; c) systemic, in which violence is seen as endemic in the illegal drug market, leading the participants to commit acts of threat and punishment as a system of alternative dispute resolution (GOLDSTEIN, 1985).

Giannini and colleagues stated that, already by the year of 1993, there were several studies which have looked closer at this relationship, such as: Siegal, 1982; Miller, Gold & Mahler, 1990 and United Stated of America National Institute on Drug Abuse (NIDA), 1985 (GIANNINI et al., 1993).

The first study has explored the effects of cocaine in generating violence, through examining 32 subjects who have inhaled free-based cocaine from whom 9 have exhibited violent behaviours and 13 showed antisocial behaviour. The second study conducted by Miller and colleagues consisted of examining the behaviour of 452 males who called the ‘Cocaine Hotline’. According to the study, 84% of the patients

have reported some sort of paranoid behaviour and 42% expressed some significant increase in anger (GIANNINI et al., 1993).

Finally, NIDA has also accompanied a telephone survey and concluded that from the 500 subjects studied, 83% stated feelings of paranoia as a primary cocaine-related effect. The study conducted by Giannini and colleagues has found a correlation between the level of violence and route of administration of cocaine. For instance, smoking *crack* provides a rapid onset and subsequently a rapid clearance and this route of administration is associated with greater violence than others means of using the drug in situations which do not require sustained action (e.g. violent behaviour against people and objects; child and spouse abuse) (GIANNINI et al., 1993)

Later, in 2002, Chermack and Blow investigated the relationship between alcohol intake and cocaine, simultaneously, with violent behaviour. The results provided evidence that the consumption of both is a risk factor for interpersonal violence and they appeared to be proximal factors related to the severity of violence (CHERMACK; BLOW, 2002).

Additionally, in 2008, another article has supported a connection between alcohol and cocaine use with violence (MACDONALD et al., 2008). In fact, the number of hospital admissions due to mental and behavioural disorders in the year of 2007 in Brazil was of 135,585, given that 68.7% of them were due to alcohol, 5.0% attributed to cocaine and 22.8% to poly-drug abuse (DUARTE; STEMPLIUK; BARROSO, 2009).

Likewise, according to a study that analysed the frequency of drugs detected in material from the necropsy room, between 2006 and 2008, in Brasilia city, the capital of Brazil, it was possible to conclude that both alcohol and cocaine are the mainly identified drugs in a *postmortem* scenario. Alcohol is the most predominant psychoactive substance found, with a percentage of 47.4% followed by cocaine with a percentage of 21.6% (CAMPELO; CALDAS, 2010).

Glebbeek and Koonings have discussed numerous reasons to justify the increasing levels of violence and insecurity in Urban Latin America countries. Among them there is cocaine abuse (GLEBBEEK; KOONINGS, 2016). Moreover, a recent study associating substance abuse to urban violence in Brazil showed that around 9.3% of the Brazilian population has been a victim of at least one form of urban

violence. Yet, this number rises to 19.7% amongst those who used cocaine in the previous year (ABDALLA et al., 2018).

It is also important to consider that the same way the effects of cocaine in the brain can evoke feelings of irritability, fatigue, insomnia, anorexia, misplaced flow of ideas and the potential use of extreme violence, this also works the other way around where a violent environment causes the individual to seek drugs of abuse (MENARD; COVEY; FRANZESE, 2015). That is why it becomes indispensable to identify violence predictors in order to come up with preventive initiatives, which may start by understanding the association between violence and substance abuse (ABDALLA et al., 2018).

When interviewing several drug addict's family members, one of the questions proposed was regarding as to what they thought was the reason behind their addiction. To this query, 46.8% of the interviewed have answered their relative 'has gotten into bad company'; 26.1% attributed it to low self-esteem; 22.7% said it was due to the absence of a parent and finally 10.3% have answered it was because of genetics (LARANJEIRA, 2014).

As it can be seen, people are indeed aware of genetics role in one's behaviour pattern. However, to the general public this information is still very scarce and remote.

2.4. Genetic polymorphisms and cocaine

There are around 3.2 billion base pairs in the human genome. The genetic code is essentially a chain of "letters" arranged in 23 pairs of chromosomes and the majority of this code is identical between any two individuals. Yet, due to historical mutation processes some variations may be found where a portion of the population carry one variant and the other portion another variant (LENCZ; DARVASI, 2017).

Whenever this variant constitutes a single nucleotide change, it is called a single nucleotide polymorphism (SNP) and they are used to study the genetic basis of common diseases and complex traits like height or the metabolic function (LENCZ; DARVASI, 2017). In fact, SNPs are the most common type of polymorphism found in the human genome as they represent 90% of all types of genetic variations among individuals (CHAUDHARY et al., 2015).

The field of pharmacogenomics is on the rise as it is extremely helpful when it comes to giving some answers on how inherited modifications in a single nucleotide represent profound effects on the mobilization and biological action of a drug (CHAUDHARY et al., 2015).

With the growth of technology, both sequencing of the human genome and the large-scale identification of genome polymorphisms have provided essential tools to understand the genetic basis behind the differences in each individual (ORPHANIDES; KIMBER, 2003). The first strong evidence for a genetic contribution to the susceptibility to develop substance-related disorders (SRDs) came from twin and adoption studies (JONES; COMER, 2015).

Several genetic linkage studies (family-based) have been carried out in order to look at individual genes that could play an important role in the development and maintenance of SRDs. To do so, both genome-wide association studies (GWAS) and candidate-gene (CG) approaches have been thoroughly carried out and depending on the drug it was estimated that genetic factors might contribute 40 to 80% of the vulnerability to addiction (JONES; COMER, 2015).

The dopaminergic system has been the subject of several studies regarding the relationship between polymorphisms and cocaine use. This is because, as mentioned previously, the release of dopamine causes feelings of euphoria in the human body. As a direct consequence, such behaviours tend to be repeated over and over again (FERGUSON; BEAVER, 2009).

Food ingestion, sexual intercourse and the use of certain stimulant drugs such as cocaine, are some of the behaviours associated with increasing levels of dopamine. They are highly susceptible to become addictive habits. Hence, dopamine is often outside the normal range of variation and when this occurs its damaging results become evident (FERGUSON; BEAVER, 2009).

Most of the revised literature has focused on dopaminergic system. However, results are somewhat controversial as they show both positive and negative associations between cocaine dependence and genetic polymorphisms.

For instance, in 1993, Noble and colleagues have studied the possible association between dopamine receptor gene *DRD2* and cocaine dependence. Their data showed that there was indeed a strong association of the minor alleles (A1 and B1) of the *DRD2* gene with cocaine dependence, suggesting that the gene represents susceptibility to the drug addiction disorder (NOBLE et al., 1993).

Later, in 1999, Comings and colleagues have carried out a study on 47 Caucasian subjects with cocaine dependence and 305 Caucasian controls to evaluate the role of dopamine receptor gene *DRD3* and they observed a modest role of this receptor gene in susceptibility to cocaine addiction (COMINGS et al., 1999). In the same year, a study by Gelernter, Kranzler and Satel has shown no association between *DRD2* alleles or haplotypes and cocaine dependence, in European and African-American subjects (GELERNTER; KRANZLER; SATEL, 1999).

By 2006, Guindalini and colleagues have looked at genetic variants in the dopamine transporter gene, *DAT1*, associated with cocaine abuse in a Brazilian population. By analysing variable number tandem repeat (VNTR) polymorphisms in 699 dependent subjects against 866 controls they have concluded that there is indeed a significant association between cocaine dependence and a VNTR allele, conferring a small but detectable effect (GUINDALINI et al., 2006). However, Lohoff and colleagues suggested that there is no association between the *DRD2* and *DAT1* polymorphisms and cocaine dependence (LOHOFF et al., 2010).

In fact, some authors consider cocaine misuse as an intricate behaviour, which can be influenced by both genetic and environmental factors together (KOBEISSY et al., 2014). In conclusion, it is considerably notable that overall results can be quite divisive. The need for further toxicogenetic studies is clear, in order to answer an urgent demand for personalized treatments.

This is the main reason why, during the course of this study, it was decided to think rather unconventionally and start looking at SNPs not only from dopamine-related genes but also within genes remotely related to cocaine abuse and its metabolism.

Starting with butyrylcholinesterase (BChE), the main cocaine-metabolizing enzyme in human beings (ZHENG; ZHAN, 2017). In humans, BChE is responsible for metabolizing cocaine to the pharmacologically inactive and water-soluble compounds ecgonine methylester and benzoic acid (MATTES et al., 1996).

BChE is encoded by the *BCHE* gene and it is synthesized in the liver and altered BChE levels – either increased or decreased – may be an indicator of disease or intoxication (NECHAEVA et al., 2018).

Even though its precise physiological function is still unclear, this enzyme is rather important in the field of toxicology, as it is responsible for hydrolysing ester-containing drugs (NECHAEVA et al., 2018). Based on this knowledge, Negrão and

colleagues have looked at the three SNPs from the *BCHE* gene (rs1803274, rs4263329 and rs4680662) in a Brazilian population, as variations in this gene may alter BChE's catalytic activity, resulting in a potential susceptibility to dependence (NEGRÃO et al, 2013).

The proposed hypothesis was based on the fact that users containing polymorphisms in the *BCHE* gene may show distinct addictive behaviours due to differences in effective plasma concentrations of cocaine. In addition, it appears that a decrease in BChE activity may lead to an increase in the amount of cocaine that reaches the reinforcing brain areas, thereby augmenting its propensity to lead to dependence (NEGRÃO et al., 2013).

Negrão and his research group have found a significant association between rs1803274 and *crack*-cocaine (AA genotype) as the preferred route of administration as well as a nominal association between patients (698) and controls (738) for the rs4263329 SNP (GG genotype) (NEGRÃO et al., 2013).

However, the need for further studies to confirm this preliminary result is highly recommended by the authors in order to elucidate the role of *BCHE* and its variants in cocaine dependence (NEGRÃO et al., 2013).

Moving forward to the neuronal approach, Kohno and colleagues have evaluated the links between dopaminergic signalling and prefontal function during risky decision-making. For this purpose, three SNPs were evaluated: rs2283265 from *DRD2* gene, rs6280 from *DRD3* gene and rs4680 from the catechol-O-methyltransferase (*COMT*) gene (KOHNO et al., 2016).

According to the authors, the first SNP (rs2283265) appeared to be playing a role in moderating alternative splicing of exon 6, influencing the proportion of dopamine D2 long (D2L) and D2 short (D2S) receptor expression, as for the second SNP (rs6280), it has shown to be a nonsynonymous SNP which results in serine (ser) to glycine (gly) substitution and enhanced dopamine D3 receptor affinity. Finally, the last SNP (rs4680) from *COMT*, also nonsynonymous, seemed to result in valine (val) to methionine (met) substitution, with reduced thermostability of the enzyme (KOHNO et al., 2016).

In fact, Moyer and colleagues have previously looked at the SNP rs2283265 and its role in D2 alternative splicing. Through the analysis of autopsy tissues in a cohort of cocaine abusers, they were able to find an association with both reduced relative expression of D2S and susceptibility to cocaine abuse (MOYER et al., 2011).

Regarding the *DRD3* gene, previous animal models have shown that cocaine use dynamically affects this D3 dopamine receptor expression and activity in limbic brain regions (BLAYLOCK; NADER, 2012). Based on this knowledge, Verdejo-Garcia and colleagues took a closer look at the relationship between the rs6280 SNP and the history of cocaine use on the structural grey matter in both the ventral striatum and the amygdala – two limbic regions relevant to addiction pathophysiology (VERDEJO-GARCIA et al., 2013).

From this study two major outcomes were found: cocaine dependent individuals who carried the C allele had larger ventral striatal volumes compared to the controls carrying the same genotype and also, those who carried the T/T genotype had smaller amygdala volumes than the controls carrying the same genotype. These results have proven that, indeed, this polymorphism moderates cocaine effects on grey matter structure in limbic regions (VERDEJO-GARCIA et al., 2013).

While cocaine is mainly responsible for blocking the dopamine transporter (*DAT*) and therefore enhancing the postsynaptic effects of dopamine signalling, *COMT* is still a deeply relevant regulatory element in dopamine homeostasis. New evidences are showing that variations found in the *COMT* can influence prefrontal cortex (PFC) dopamine regulation and therefore modulate aspects of cognition, emotions and behaviour (EGAN et al., 2001; TUNBRIDGE; HARRISON; WEINBERGER, 2006).

For this reason, individual differences found in *COMT* may confer a certain vulnerability to cocaine dependence as well as other substance use disorders. This was found to be true by Lohoff and colleagues when, in 2008, they have found an association between the Val^{158}Met polymorphism (rs4680) and cocaine dependence as the results shown an increased frequency of the Met allele (low activity allele) in cocaine users in comparison with the controls. In fact, they have even added that those individuals with the low-activity *COMT* allele may have longer and more effective dopamine release in the brain, resulting in an increase in both duration and intensity of cocaine's reward and, ultimately, might enhance dependence on this drug (LOHOFF et al., 2008).

Even though each research group has focused on different gene families, SNPs and even populations, all have a common outcome: the clear urge for new studies. The idea of looking at the genetic level in order to create revolutionary personalized treatments to prevent susceptible individuals from becoming addicted to such a harmful drug, as cocaine, does seem like the wiser path to take.

Such findings would avoid not only drug misuse, resulting in addiction and even overdose, as well as all the indirect sequels that cocaine abuse triggers, as thoroughly discussed previously.

2.5. *Postmortem* blood and hair specimens

In a *postmortem* scenario there are numerous biological matrices, which can be collected, from liver tissue to vitreous humour almost anything can be used as a toxicological specimen. However, not all of them can actually be used for quantitative purposes and depending on the aim of the analysis it is not worth it to collect a large number of random samples as they may be irrelevant for the case.

The gold standard *postmortem* specimen for quantitative toxicological analysis is whole blood. Since most of the meaningful data from the literature is available for this biological matrix, then it clearly becomes the sample of choice for detecting, quantifying, and interpreting xenobiotic concentrations (DINIS-OLIVEIRA et al., 2015).

The optimum scenario is to collect blood specimens from two different sites: heart and peripheral (KARCH, 2008). Cardiac blood is ideal for screening purposes, as drug levels are generally higher than in femoral blood. For *postmortem* quantitative analyses, blood from the femoral site is preferred as this site is much less affected by *postmortem* redistribution, especially when collected prior to autopsy to avoid contamination with stomach contents (DINIS-OLIVEIRA et al., 2015).

In both cases, the exact source of collection should be clearly identified and a preservative such as sodium fluoride must be used to inhibit certain phenomena such as microorganism conversion of glucose to ethanol and *postmortem* conversion of cocaine to ecgonine methyl ester by cholinesterases. When long-term storage is required, a temperature of -20°C should be in place (KARCH, 2008).

Blood's detection window for cocaine and its major metabolite, benzoylecgonine is of approximately 12 and 48 hours, respectively (VERSTRAETE, 2004). This shows that if the parent compound is found, the individual was probably under the effect of the drug upon death.

As for hair, its collection is relatively much simpler. Cutting a sample of a pencil thickness from the posterior vertex region of the scalp has proven to be the preferred method for collection as this is the area with least variation in growth rates

(COOPER; KRONSTRAND; KINTZ, 2012). It is known that head hair grows from 0.6 to 1.4cm per month (PRAGST; BALIKOVA, 2006).

The hair sample should be tight together and tied previous to cutting in order to correctly label the root end for posterior segmental analysis. Storage simply involves a piece of aluminium foil, that is folded once or twice, with the cut root ends projecting ~15mm beyond the end of the foil (FLANAGAN et al., 2007).

This matrix represents a unique material when it comes to retrospective investigation of chronic exposure since it provides a longer window of detection when compared to blood. In fact, scalp hair may provide retrospective information of the previous 5 to 7 years (DANIEL; PIRACCINI; TOSTI, 2004). Not only this provides a very powerful tool for forensic toxicology but also, in some cases, it may be the only option left. For instance, on exhumed human bodies as this matrix is extremely resistant to decay and it is exempt of any peculiar storage conditions other than room temperature (KINTZ, 2004).

Aims

3. Aims

The key purpose of this study was to investigate whether there is a statistically significant association between certain specific SNPs with one's susceptibility to abuse cocaine. The group of people who constituted the core of this work were violent individuals whose violence has ultimately lead to their death, which means that in the process of looking for cocaine-users and non-users it was also possible to observe the portion of cocaine-related deaths within violent cases arriving at the Institute of Legal Medicine of São Paulo.

Material & Methods

4. Material & Methods

4.1. Material for toxicological analysis

4.1.1. Reagents

All solvents and salts were purchased from Merck (Darmstadt, Germany) and Sigma Aldrich® (St. Louis, USA).

4.1.2. Standards

Drug reference standards of cocaine (COC), benzoylecgonine (BZE), cocaethylene (CE), norcocaine (NCOC) and anhydroecgonine methyl ester (AEME) in the concentration of 1.0 mg/mL and their respective deuterated standards, cocaine-d_3 (COC-d_3), benzoylecgonine-d_3 (BZE-d_3), cocaethylene-d_3 (CE-d_3) and in a concentration of 100 µg/mL in methanol or acetonitrile (all >99% purity), were obtained from Ceriliant Corporation® (Round Rock, USA).

4.1.3. Equipment and accessories

- LC system consisting of an Acquity UPLC coupled to a Quattro Premier XE mass spectrometer, from Waters® (Mildford, USA).
- Acquity UPLC BEH C18 column (2.1 x 100mm, 1.7µm), from Waters® (Mildford, USA).
- MassLynx™ Mass Spectrometry Software, from Waters® (Mildford USA).
- Multi-tube vortex, model VX-2500, from VWR® (Thorofare, USA).
- Centrifuge, model 5702, from Eppendorf (Berzdorf, Germany).
- Thermoblock with a heating function and nitrogen fllow, model Reacti-therm III, from Thermo Fischer Scientific (Waltham, USA).
- Ultrasonic cleaner with five sets of cleaning time, from Cristófoli (Paraná, Brazil).
- Analytical scale, model AT261, from Delta Range (Hayward, USA).
- Excel® 2011, from Microsoft (Redmond, USA).
- Deionised water obtained through a Milli-Q water filter, from Millipore™ (Bedford, USA).

4.1.4. Blood and hair samples

4.1.4.1. Negative samples

Negative blood and hair samples were obtained from volunteers who reported not having used any of the substances under this study. They have been used to carry out all optimization and further validation of the toxicological methods developed.

4.1.4.2. Real case postmortem samples

Blood and hair samples were collected at IML-SP from victims of violent death such as homicides resulting from opposition to police intervention, suicides, drug abuse suspicion and risky behaviours that have resulted in death. Ideally, peripheral blood was collected through puncture of the femoral vein and if not available, then a perforation of the heart took place in order to collect cardiac blood. At least two tubes containing sodium fluoride (NaF) were collected kept at -20°C until analysis.

Hair was collected and stored according to the method proposed by Cooper and colleagues (COOPER; KRONSTRAND; KINTZ, 2012). For both cases, samples are only collected after the respective consent form (Attachment IV) has been filled out and signed by the victim's family member in charge.

4.2. Material for genetic analysis

4.2.1. Reagents

- All solvents and buffers were included in the QIAamp® DNA Mini kits, purchased from Qiagen (Venlo, Netherlands).

4.2.2. SNPs

- rs1803274 (*BCHE*), rs4263329 (*BCHE*), rs2283265 (*DRD2*), rs6280 (*DRD3*) and rs4680 (*COMT*) were all acquired from Thermo Fisher Scientific (Waltham, USA)

- Taqman® Universal PCR Master Mix Kit, from Thermo Fisher Scientific (Waltham, USA).

4.2.3. Equipment and accessories

- Multi-tube vortex, model VX-2500, from VWR (Thorofare, USA)
- Centrifuge, model 5702, from Eppendorf (Berzdorf, Germany)
- Deionised water obtained through a Milli-Q water filter, from Millipore (Bedford, USA)
- TaqMan® SNP Genotyping Assay, from Applied Biosystems (Foster City, USA)
- 7500 SDS – v.2.0.4 software, from Applied Biosystems (Forest City, USA)
- Spectrophotometer NANODROP™, from Thermo Fisher Scientific (Waltham, USA)
- Savant™ Universal SpeedVac™ Vacuum System, from Thermo Fisher Scientific (Waltham, USA)
- Invisorb® DNA CleanUp, from STRATEC Molecular GmbH (Berlin, Germany)
- Rotor Gene 6000®, from Qiagen (Venlo, Netherlands)
- SPSS – 16.0 software, from IBM (Armonk, USA).

4.2.4. Real case postmortem blood samples

Blood samples were collected at IML-SP from victims of violent death such as homicides resulting from opposition to police intervention, suicides, drug abuse suspicion and risky behaviours that have resulted in death. At least two tubes containing ethylenediaminetetraacetic acid (EDTA) were collected kept at -20°C until analysis.

4.3. Methods for toxicological analysis

4.3.1. Determination of COC, BZE, CE, NCOC and AEME in postmortem head hair samples

The current method was based on the procedure proposed by Di Corcia et al. (2012) with few modifications to it and it has been validated in order to confirm the presence of COC, BZE, CE, NCOC and AEME by UPLC-ESI-MS/MS.

4.3.1.1. Extraction procedure

Head hair samples have been separated into 50mg aliquots and decontaminated by washing each aliquot with a mild detergent and water followed by 2.0mL of dichloromethane for 15 minutes (min) at 37°C. Once dried, 2mL of methanol (MeOH) were added to the falcon tubes along with the internal standards (COC-d_3, BZE-d_3 and CE-d_3) at a concentration of 2ng/mg. The tubes were then rigorously sealed with Parafilm M® to incubate the samples at 50°C for 18 hours (h) to enhance drug liberation from the matrix (**Figure 19**). Once cooled, all the volume from the tube has been transferred to a clean Falcon tube and contents were evaporated under nitrogen (N_2) flow at 50°C (PEREIRA DE TOLEDO et al., 2003; DI CORCIA et al., 2012a; ROVERI; PARANHOS; YONAMINE, 2016).

Once dried out, the residue obtained was re-constituted with 50µL of the mobile phase A (1mM of ammonium formate in water with 0.1% formic acid). Finally, an aliquot of 3µL was injected into the UPLC-ESI-MS/MS system (**Figure 20**).

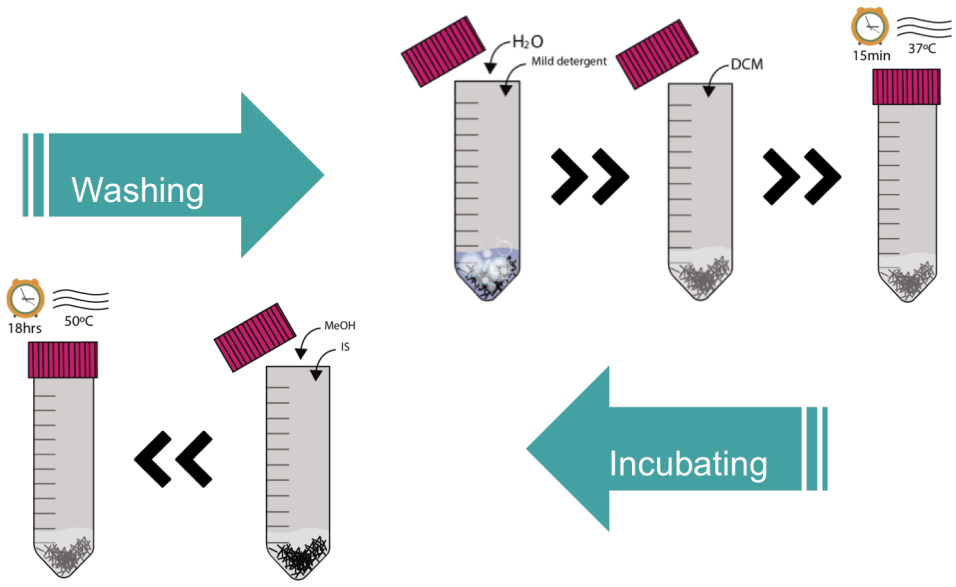

Figure 19. Illustration of the first two steps (washing and incubation) of the extraction procedure from *postmortem* head hair samples to confirm the presence of COC, BZE, CE, NCOC and AEME by UPLC-ESI-MS/MS. Source: personal collection.

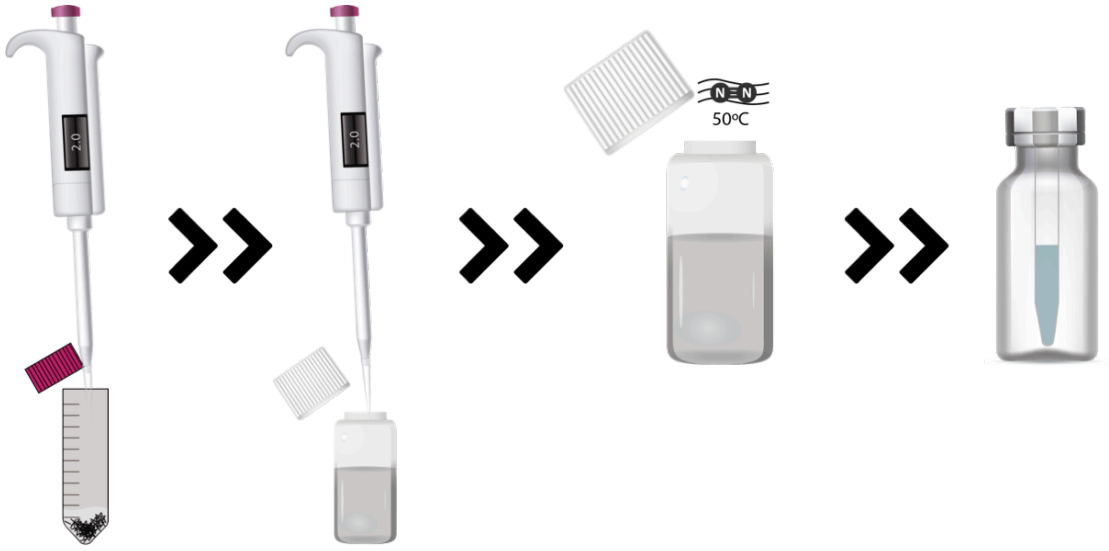

Figure 20. Illustration of the last stage of the procedure, consisting of a dilute-and-shoot step, after methanolic extraction from *postmortem* head hair samples to confirm the presence of COC, BZE, CE, NCOC and AEME by UPLC-ESI-MS/MS. Source: Personal collection.

4.3.2. Determination of COC, BZE, CE, NCOC and AEME in postmortem blood samples

The proposed method was based on the procedures of Kim et al., Hegstad et al., D'Avila et al. and Mohammed, Eissa & Ahmed, with several changes to it and it has been validated in order to confirm the presence of COC, BZE, CE, NCOC and AEME by UPLC-ESI-MS/MS (KIM et al., 2011; HEGSTAD et al., 2014 D'AVILA et al., 2015; MOHAMMED; EISSA; AHMED, 2017).

4.3.2.1. Extraction procedure

Firstly, an aliquot of 100µL of whole blood was pipetted into a 2mL Eppendorf tube followed by the addition of 20µL from a 500ng/mL working solution of the respective internal standards. This mixture was then diluted with 880µL of a cold mixture of acetonitrile(ACN)/MeOH (80:20/v:v) and shaken for 30 seconds (s) using a vortex mixer (KIM et al., 2011; HEGSTAD et al., 2014 D'AVILA et al., 2015; MOHAMMED; EISSA; AHMED, 2017).

Afterwards, the solution was centrifuged at 9000*g* for 6min. To finalize, an aliquot of 3µL was withdrawn from the supernatant and directly injected into the UPLC-ESI-

MS/MS system (**Figure 21**) (KIM et al., 2011; HEGSTAD et al., 2014 D'AVILA et al., 2015; MOHAMMED; EISSA; AHMED, 2017).

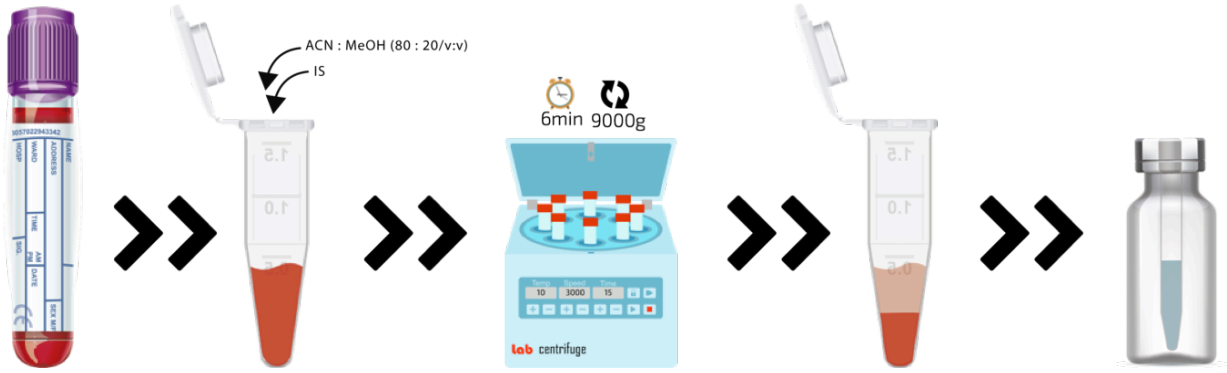

Figure 21. Illustration of dilute-and-shoot procedure, for the extraction of COC, BZE, CE, NCOC and AEME from *postmortem* blood samples to confirm the presence of COC, BZE, CE and NCOC by UPLC-ESI-MS/MS. Source: personal collection.

4.3.3. UPLC-ESI-MS/MS conditions

4.3.3.1. Chromatographic parameters

Analysis were performed using a Waters® UPLC-ESI-MS/MS equipment (see **Figure 22**). The following **Table 1** shows a summary of the main LC parameters used.

Table 1. Chromatographic parameters for both methods developed under this work, including: mobile phases, oven temperature, gas flow and gradient.

4.3.3.2. Mass spectrometry parameters

LC parameters	
Mobile phases	Mobile phase **A**: 1mM of ammonium formate in H_2O + 0.1% formic acid Mobile phase **B**: 1mM of ammonium formate in ACN and H_2O (95:5/v:v) + 0.1% formic acid
Gas flow	0.4mL/min
Oven temperature	40°C
Gradient	Initial: 90% A | 10%B 3.0min: 55%A | 45%B 3.8min: 20%A | 80%B 4.0min: 90%A | 10% B 7.0min: 90%A | 10%B

The mass spectrometer was operated under the multiple-reaction monitoring mode (MRM) using the electrospray ionization technique in positive mode (ESI+; $[M+H]^+$), as follows: desolvation gas, 1100L/h; cone gas, 200L/h; desolvation temperature, 450ºC; source temperature, 120ºC; capillary voltage, 1000V.

The following **Table 2** shows a summary of the main MS parameters used while **Table 3** gives details on all MRM transitions, retention times and experimental conditions for the detection of all compounds and their respective internal standards.

Table 2. Mass spectrometry parameters for both methods developed under this work, including: ionization type, ionization mode and operation mode.

MS parameters	
Ionization type	Electrospray ionization (ESI)
Ionization mode	+
Operation mode	MRM

Table 3. MRM transitions and experimental conditions for the detection of cocaine, cocaine-d_3, benzoylecgonine, benzoylecgonine-d_3, cocaethylene, cocaethylene-d_3, norcocaine and anhydroecgonine methyl ester. Where: RT – retention time; QT – quantifier; QL – qualifier and CLE – collision energy.

Analyte	**RT (min)**	**Percursor ion (m/z)**	**QT (m/z)**	**CLE (V)**	**QL1 (m/z)**	**CLE**	**QL2 (m/z)**	**CLE (V)**
AEME	0.87	182.05	90.74	25	117.89	21	121.86	19
Benzoylecgonine	1.81	290.16	167.90	21	104.78	31	76.80	47
Benzoylecgonine-d_3	1.81	293.23	170.64	19	104.66	29	85.06	31
Cocaethylene	2.79	318.26	196.05	23	81.87	35	104.84	33
Cocaethylene-d_3	2.79	321.32	199.11	19	84.93	33	104.84	41
Cocaine	2.42	303.96	181.81	20	104.68	50	81.79	50

Table 3. *Continuing*

Analyte	RT (min)	Percursor ion (m/z)	QT (m/z)	CLE (V)	QL1 (m/z)	CLE	QL2 (m/z)	CLE (V)
Cocaine-d_3	2.42	307.20	184.91	19	104.76	35	84.78	31
Norcocaine	2.54	290.24	167.91	19	135.83	23	104.79	37

4.3.4. Validation of the method for the determination of cocaine and its metabolites in postmortem head hair samples

The method for the confirmation of cocaine and its metabolites in hair has been validated according to the recommended international parameters. For that matter the following guidelines have been followed: Scientific Working Group for Forensic Toxicology (SWGTOX, 2013): 'Standard Practices for Method Validation in Forensic Toxicology' and Society of Hair Testing (COOPER; KRONSTRAND; KINTZ, 2012): 'Society of Hair Testing guidelines for drug testing in hair'.

In this context, the following parameters have been tested: selectivity; limit of detection (LoD); limit of quantitation (LoQ); linearity; intra-day precision; accuracy and carryover.

4.3.4.1. Selectivity

Selectivity was evaluated through the analysis of six zero samples (only internal standard) and two blank samples (no substance added). The presence or absence of any interfering peaks (endogenous substances) near the analyte's retention time has been assessed.

4.3.4.2. Limit of detection (LoD)

Limit of detection was estimated by using three different blank hair samples, analysed in duplicate over three runs. The mean and standard deviation of all negative samples were calculated. Likewise, spiked hair samples with decreasing concentrations were also evaluated. The lowest concentration of a spiked hair

sample capable of producing a signal greater than the average of the negative signal samples (x) plus 3.3 times the standard deviation (s) was identified as being the LoD:

$$LoD = x + 3.3s$$

4.3.4.3. Limit of quantitation (LoQ)

As for LoQ determination, three samples of a known concentration were analysed over three runs to prove that all the necessary criteria for detection; identification; precision and accuracy have been reached (coefficient of variation (%CV) <15%). Both LoD and LoQ obtained retention times with a maximum variation of ± 2% and mass spectra with the same appearance and ion proportion.

4.3.4.4. Linearity

Linearity was achieved through the analysis of extracted samples, which have been spiked at the desired concentrations in five replicates. The coefficients of determination (r^2) should be ≥ 0.99.

The study of linearity was estimated by the analysis of extracted samples obtained from aliquots of spiked hair, in five replicates, with an internal standard concentration of 2ng/mg. The respective non-deuterated calibrators were added at the following concentrations:

- COC and AEME: 0.5; 1.0; 5.0; 10.0; 15.0 and 20.0ng/mg;

- BZE, CE and NCOC: 0.05; 1.0; 5.0; 10.0; 15.0 and 20.0ng/mg.

4.3.4.5. Intra-day precision

Intra-assay precision has been accessed by the analysis of three quality control (QC) levels in five replicates on a single day.

The results obtained from this experiment are expressed as %CV and were calculated using Microsoft Excel®. The three QC levels studied were: low (LQC); medium (MQC) and high (HQC) as stated in **Table 4**, keeping in mind that LQC concentrations are suggested to be approximately three times the lowest end of the working range of the method and HQC concentrations should be within

approximately 80% of the highest end of the working range of the method, while MQC concentrations may be near the midpoint of the low and high concentrations (SWGTOX, 2013).

Table 4. Low, medium and high QC level values used within the method for the determination of cocaine and its metabolites in *postmortem* head hair samples, all expressed as ng/mg.

Analytes	LQC (ng/mg)	MQC (ng/mg)	HQC (ng/mg)
COC	1.5	7.0	14.0
AEME	1.5	7.0	14.0
BZE	1.5	7.0	14.0
CE	1.5	7.0	14.0
NCOC	1.5	7.0	14.0

The acceptance criteria used was of 20% for low QC and 15% for medium and high QC's.

4.3.4.6. Accuracy

Accuracy assay was performed by the quantification of six replicates for each QC level by using a previous calibration curve. The results obtained from these experiments were expressed as a percentage of the known concentration value:

$$\frac{mean\ concentration\ measured - nominal\ concentration}{nominal\ concentration} \times 100$$

The acceptance criteria used was of 20% for low QC and 15% for medium and high QC's.

4.3.4.7. Carryover

This parameter has been tested through the analysis of three blank samples injected into the UPLC-ESI-MS/MS system immediately after the highest concentrated sample in the calibration curve.

4.3.5. Validation of the method for determination of cocaine and its metabolites in postmortem whole blood samples

The second method developed for the confirmation of cocaine and its metabolites in *postmortem* whole blood has also been validated according to the recommended international parameters. The following guidelines were used: Scientific Working Group for Forensic Toxicology (SWGTOX, 2013): 'Standard Practices for Method Validation in Forensic Toxicology and United Nations Office on Drugs and Crime (UNODC, 2009): 'Guidance for the Validation of Analytical Methodology and Calibration of Equipment used for Testing of Illicit Drugs in Seized Materials and Biological Specimens'.

For this particular method, the tested parameters were: selectivity; limit of detection (LoD); limit of quantitation (LoQ); linearity; intra-day precision; accuracy and carryover.

4.3.5.1. Selectivity

In order to evaluate selectivity, ten different drug-free blood samples were extracted and analysed according to the previously described method to prove the absence of endogenous interfering compounds. In addition, ten blank blood samples have been spiked with concentration of 1000ng/mL of ten different drugs commonly analysed at the laboratory such as: diazepam, phenobarbital, secobarbital, pentobarbital, morphine, codeine, amphetamine, methamphetamine, MDMA and nicotine, to look for potential exogenous interfering substances.

The peaks obtained at the retention time of interest have been compared to those from blood samples spiked with the analytes at the LOQ values.

4.3.5.2. Limit of detection (LoD) and limit of quantitation (LoQ)

Both LoD and LoQ have been determined as described in items **4.3.4.2.** and **4.3.4.3.** under Material & Methods, respectively. Only in this case, blood samples have been used instead of head hear samples.

4.3.5.3. Linearity

To test for linearity of the method, spiked samples at desired concentrations have been extracted in six replicates and further analysed. The linearity was achieved through the analysis of extracted samples, which have been spiked at the desired concentrations in six replicates with an internal standard concentration of 500ng/mL. The r^2 values should be ≥ 0.99.

All the other standards for the analytes of interest have been added at the following concentrations:

- COC, CE and AEME: 1.0; 10.0; 25.0; 50.0; 100.0; 500.0 and 1000.0ng/mL.

- BZE and NCOC: 0.5; 10.0; 25.0; 50.0; 100.0; 500.0 and 1000.0ng/mL.

4.3.5.4. Intra-day precision

This intra-assay precision has been performed through the analysis of three QC levels (low, medium and high) and it is expressed as %CV. The values of these QC's can be found in **Table 5**.

Table 5. Low, medium and high QC level values used within the method for the determination of cocaine and its metabolites in *postmortem* blood samples, all expressed as ng/mg.

Analytes	LQC (ng/mL)	MQC (ng/mL)	HQC (ng/mL)
COC	5.0	100	1000
BZE	5.0	100	1000
CE	5.0	100	1000
NCOC	5.0	100	1000
AEME	5.0	100	1000

The acceptance criteria used was of 20% for low QC and 15% for medium and high QC's.

4.3.5.4. Accuracy and carryover assays

For both accuracy and carryover, the parameters followed have been the same as previously stated in items **4.3.4.6** and **4.3.4.7** under **Material & Methods**, respectively. In this case, blood samples were in place rather than head hear samples.

4.4. Methods for genetic analysis

4.4.1. Determination of relevant genetic polymorphisms

For this purpose, several online database tools have been used such as: PubMed – NCBI (SNP function); Ensembl; Pharmgkb and OMIM. After browsing through the appropriate literature, the relevant polymorphisms that could be related to cocaine metabolism and cocaine addiction were selected and investigated through those databases in order to check for any possible significant information such as: its chromosome location, ancestral allele, minor allele frequency (MAF%) and functional consequence (**Table 6**) and they have been chosen according to their relevance stated in the literature review and also if possessing a MAF > 10%.

Table 6. Synopsis of the relevant genes and their respective SNPs chosen for this study, along with some of their features such as: chromosome location; ancestral allele; MAF; relevant biological function and functional consequence.

SNP	Gene	Chromosome location	Ancestral allele	MAF (%)	Relevant biological function	Functional consequence
rs1803274	*BCHE*	3:165773492	G	15.85	Cocaine metabolism	Missense, transcript variant
rs4263329	*BCHE*	3:165821822	A	16.33	Cocaine metabolism	Intron variant
rs2283265	*DRD2*	11:113414814	G	22.70	Membrane receptor	Intron variant
rs6280	*DRD3*	3:114171968	C	48.64	Membrane receptor	Missense
rs4680	*COMT*	22:19963748	G	36.92	Metabolization of catecholamines	Missense,upstream variant 2KB

4.4.2. SNPs analysis at analysis at the Heart's Institute (InCor)

DNA extraction from whole blood was accomplished by using the kit: QIAamp® DNA Blood Mini, after an initial step of leukocyte cleansing. The analysis took place at the Laboratory of Genetics and Molecular Cardiology, Heart's Institute (InCor), Clinical Hospital – School of Medicine of the University of São Paulo (HCFMUSP).

4.4.2.1. Leucocyte Cleansing for further DNA extraction

The first step for the extraction of genomic DNA from whole blood was the cleansing of leucocytes from the sample. The protocol for this first stage goes as follows:

1. Add all available volume from the blood sample into a conical 15mL tube and fill up to the 1mL mark with red blood cell (RBC) buffer
2. Homogenize the mixture by inversion and then incubate it for 10min at room temperature (RT)
3. Centrifuge for 10min at 3000 rotations per minute (rpm)
4. Discard the supernatant and add 10mL of RBC buffer
5. Homogenize by inversion (if the pellet won't come off, vortex the sample or homogenize it through the use of a Pasteur pipette)
6. Centrifuge for 3min at 1500rpm
7. Discard the supernatant and add 10mL of RBC buffer
8. Homogenize by inversion (if the pellet won't come off, vortex the sample or homogenize it through the use of a Pasteur pipette)
9. Centrifuge for 3min at 1500rpm
10. Discard the supernatant and withdrawn all RBC buffer that is left with the help of a 1mL pipette (if the sample is too haemolysed, perform an addition wash step such as step 9)
11. Add 200µL of phosphate-buffered saline (PBS) and homogenize the mixture
12. Transfer everything into a 1.5mL microcentrifuge tube, previously labelled.

4.4.2.2. DNA Extraction

This next step was performed according to the QIAamp® DNA Blood Mini kit manufacturer's protocol, that goes as follows:

To the 1.5mL microcentrifuge tube already containing the pool of leukocytes from the previous cleansing step:

1. Add 20µL of Rnase A, homogenize it and incubate it for 5min at RT
2. Pipette 20µL of Proteinase K
3. Add 200µL of Buffer AL
4. Mix by pulse-vortexing for 15s
5. Briefly centrifuge the 1.5mL microcentrifuge tube to remove drops from the inside of the lid
6. Incubate at 56 °C for 30min, under agitation (1000rpm) on a dry bath
7. Briefly centrifuge the 1.5mL microcentrifuge tube to remove drops from the inside of the lid
8. Add 200µl of cold ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15s.
9. After mixing, briefly centrifuge the 1.5mL microcentrifuge tube to remove drops from the inside of the lid
10. Carefully apply the mixture from step 5 to the QIAamp Mini spin column (in a 2mL collection tube) without wetting the rim
11. Close the cap and centrifuge at 12,000 for 1min
12. Place the QIAamp Mini spin column in a clean 2mL collection tube (provided), and discard the tube containing the filtrate
13. Carefully open the QIAamp Mini spin column and add 500µL of AW1 Buffer without wetting the rim. Close the cap and centrifuge at 12,000rpm for 3min
14. Place the QIAamp Mini spin column in a clean 2mL collection tube (provided) and discard the collection tube containing the filtrate
15. Carefully open the QIAamp Mini spin column and add 500µL of AW2 Buffer without wetting the rim. Close the cap and centrifuge at 12,000rpm for 3min
16. Place the QIAamp Mini spin column in a new 2mL collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge once again at 12,000rpm for 1min

17. Place the QIAamp Mini spin column in a clean 1.5mL microcentrifuge tube (not provided), and discard the collection tube containing the filtrate

18. Carefully open the QIAamp Mini spin column and add 200µl of MilliQ H_2O (pre-heated at 56°C)

19. Incubate at 56°C for 10min

20. Centrifuge at 12,000rpm for 1min.

4.4.2.3. Evaluation of DNA's concentration and purity

The concentration and purity of the DNA samples was checked using the NANODROP™ 1000 Spectrophotometer.

4.4.2.4. Genetic analysis of polymorphisms

Genotyping was performed by real-time Polymerase Chain Reaction (PCR) employing a TaqMan® SNP Genotyping Assay through a Rotor Gene 6000®, which is designed specifically for each polymorphism allele discrimination.

The genotyping platform must conform to a standard layout offered by the company. Based on the hydrolysis technique of TaqMan® probes, genotyping is performed by assays with two allele-specific Minor Groove Binder (MGB) probes, one probe for the normal sequence, labelled with the VIC® fluorophore, and the other for the mutant sequence, labelled with the FAM® fluorophore and primers, allowing identification of the alleles accurately (**Figure 22**).

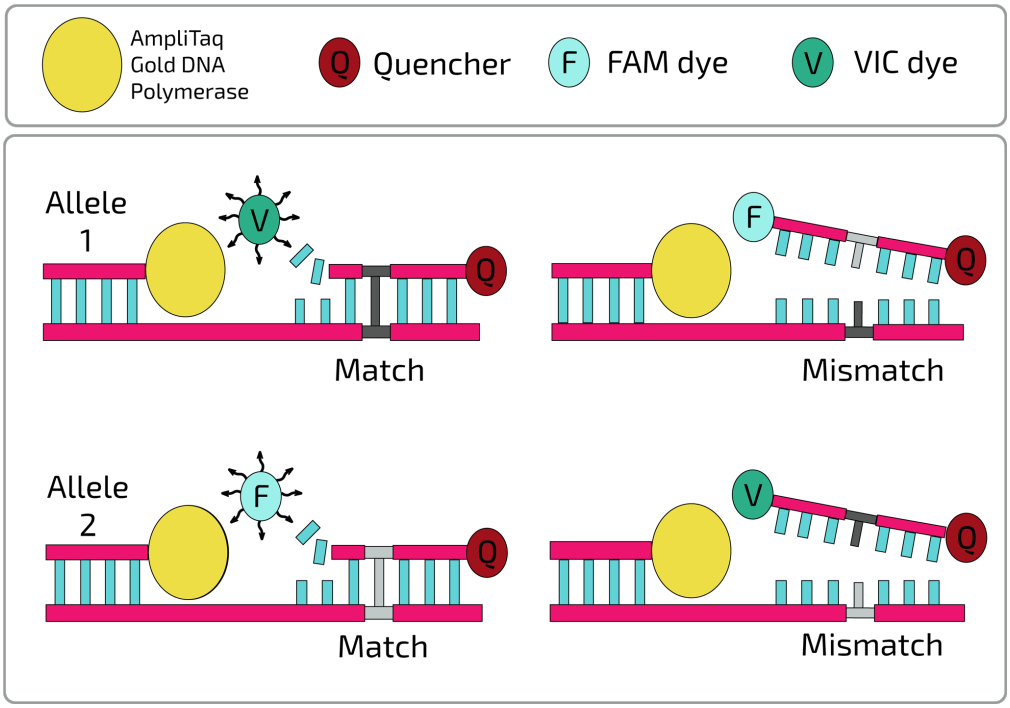

Figure 22. Illustration of the results of matches and mismatches between the target and the probe sequences in the TaqMan® genotyping assays. Allelic discrimination is achieved by the selective annealing of matching probe and template sequences, which generates an allele-specific (fluorescent dye-specific) signal. Source: adapted from DE LA VEGA et al. (2005).

During PCR, each probe hybridizes specifically to its complementary sequence between the forward and reverse primer sites. DNA polymerase is capable of cleaving only probes that hybridize to its specific SNP allele. The cleavage separates the reporter dye from the extinguishing dye, substantially increasing the fluorescence of the reporter dye. Thus, fluorescence signals generated during PCR amplification indicate that the alleles are present in the sample. Then the reading is made by fluorescence uptake.

4.4.2.5. TaqMan® Protocol

The PCR test had a final volume of 12µL, using the TaqMan® Universal PCR Master Mix Kit (6µL), the respective fluorescent probes for each SNP (0.3µL), H_2O (3.7µL) and DNA (2µL).

For PCR assays, the following amplification program was used: (1) a 2min cycle at 60°C; (2) a cycle of 15min at 95°C; (3) 50 cycles of 30s at 95°C and 60s at 60°C and (4) a 60s cycle at 60°C. The equipment ABI 7500 measured the fluorescence emitted by the hydrolysis of the fluorophores VIC and FAM. The 7500 SDS – v.2.0.4 software was used to treat data of the allelic discrimination.

All assays are previously tested, validated and have to go through the company's quality control.

4.4.2.6. Statistical analysis

The continuous variables were presented as mean and standard deviation and categorical variables as frequencies. The Chi-square (X^2) test was performed for comparative analyses of categorical variables such as general characteristics within the case and control groups. Non-parametric Mann-Whitney test has been used for the variables of age, weight and height while for BMI it was used the student's t-test. X^2 and Fisher's exact test were used to calculate the statistical significance difference among the SNPs and user/non-user individuals.

Univariate and multiple logistic regression models were used to assess the odds ratio (OR) for cocaine addiction. Hardy-Weinberg equilibrium analyses were performed using the X^2 test. All statistical analyses were performed using the SPSS software with a significance level of $p < 0.05$.

Results & Discussion

5. Results & Discussion

5.1. *Postmortem* head hair analysis of COC, BZE, CE, NCOC and AEME by UPLC-ESI-MS/MS

5.1.1. Method development

5.1.1.1. Incubation and extraction

Several research groups have defended overnight incubation of hair as being favourable for drug removal from the specimen (ALEKSA et al., 2012; FAVRETTO et al., 2016; SHU et al., 2016).

As for the use of methanol, this practise is significantly well established in the literature for the extraction of various analytes such as cocaine and its metabolites. It is known to be effective in the extraction of drugs from the hair matrix and also, it is readily evaporated, which aids on the steps to follow of the procedure, allowing a simple and rapid re-constitution followed by injection into the instrument (PUJOL et al., 2007; POON et al., 2014; GAMBELUNGHE et al., 2015).

5.1.1.2. Dilute-and-shoot procedure

For years that classical extraction techniques, such as solid-phase extraction (SPE) and liquid-liquid extraction (LLE), have been in place for the determination of drugs of abuse in all sorts of biological matrices. However, modern days require new, simpler procedures that are able to reduce solvent use, not only for the sake of the environment but also the analyst, which can attend to a high demand rapidly, with reduced costing, reproducible and robust.

In fact, hair analysis in particular, is often avoided due to the laborious and costly procedures it involves, especially when looking for a wide range of substances. Hence the proposal of Di Corcia and colleagues of developing a fast, simple and cost-effective extraction procedure dedicated to UPLC-ESI-MS/MS use (DI CORCIA et al., 2012b).

Indeed, this method has shown to be extremely fast, considering the time it took after overnight methanolic extraction, and easy to perform which resulted in a high sample throughput.

5.1.2. Validation results

5.1.2.1. Selectivity

The method showed no interfering peaks at the retention times of interest. The method was selective for the all analytes (see **Figure 23** on item **5.1.2.8.**).

5.1.2.2. LoD

The LoD values obtained in this method were of 0.5 for COC and AEME and 0.05ng/mg for BZE; CE and NCOC, respectively.

5.1.2.3. LoQ

The LoQ values obtained in this method were the same as in item *5.1.2.2*. These are considered to be suitable for this study as they follow the recommendations from the Society of Hair Testing (SoHT) for cut-off values of cocaine (0.5ng/mg) and its metabolites (0.05ng/mg) (COOPER; KRONSTRAND; KINTZ, 2012). As AEME does not have any pre-established cut-off values, considering its usage pattern, the values obtained seemed rather adequate.

5.1.2.4. Linearity

The linear range studied started at the LoQ, up to 20ng/mg for all analytes. **Figure 24** on item **5.1.2.8.** shows a chromatogram of all analytes on their respective LoQ concentrations.

The method has shown to be linear for all analytes with r^2 values equal or above 0.99. Heteroscedasticity phenomenon was observed through the *F-test* and the following table presents the data for the concentration ranges, calibration curve equations, weighing factors and coefficient of determination after proper correction

weights have been placed (**Table 7**). The *x* and *y* letters represent concentration and peak area, respectively.

Table 7. Linearity results from the method for determination of cocaine and its metabolites in *postmortem* head hair samples for all analytes and their respective calibration curves. Values being shown are: concentration range; calibration curve's equations; weighing factors applied and resulting r^2 after adequate correction using the statistical tool *F-test*.

Analytes	Concentration range	Calibration curve equation	Weight factor applied	r^2
COC	LoQ to 20ng/mg	$y = 0.485564x+0.113721$	$1/x^2$	0.998
BZE	LoQ to 20ng/mg	$y = 1.052177x-0.00219$	$1/x$	0.998
CE	LoQ to 20ng/mg	$y = 0.688598x-0.00667$	$1/x$	0.998
NCOC	LoQ to 20ng/mg	$y = 0.247624x-0.0005$	$1/x^2$	0.999
AEME	LoQ to 20ng/mg	$y= 0.078901x+0.002721855$	$1/x^2$	0.998

5.1.2.5. Intra-day precision

Intra-day precision has met the criteria established by international guidelines and the results for these assays can be seen the **Table 8**.

Table 8. Precision results from the method for determination of cocaine and its metabolites in *postmortem* head hair samples for all analyte's quality control levels and their respective intra-day precision values expressed as percentage of relative standard deviation (%CV).

Analytes	Intra-day Precision (%CV)		
	LQC	MQC	HQC
COC	1.6	1.0	1.5
BZE	15.4	3.2	2.2
CE	17.0	1.3	1.6
NCOC	3.6	0.9	4.0
AEME	8.1	12.4	4.9

Values from intra-day precision have ranged from 0.9 to 17.0% for all three levels. As it can be seen, the highest values, 15.4 and 17.0%, are seen within the lowest quality controls and for BZE and CE, respectively. This may be due to the fact that these are some of the analytes with the lowest LoQ values, at 0.05ng/mg, therefore the most susceptible to noise.

5.1.2.6. Accuracy

This parameter has proven to be within the accepted international criteria for method validation and the values are shown in **Table 9**.

Table 9. Accuracy results from the method for determination of cocaine and its metabolites in *postmortem* head hair samples for all analyte's quality control levels and their respective values expressed as a percentage (%).

Analytes	Accuracy (%)		
	LQC	**MQC**	**HQC**
COC	90.5	104.9	96.5
BZE	94.3	99.2	95.1
CE	88.3	95.5	91.7
NCOC	112.8	104.7	102.3
AEME	98.5	99.5	100.0

Overall, the lowest value obtained was of 88.3% (CE's low QC) and the highest 112.88% (NCOC's low QC) which shows that the range is within acceptable values.

5.1.2.7. Carryover

The method has shown no carryover through the absence of peaks at the analyte's retention times on all three consecutive blank samples, injected straight after the highest calibration point.

5.1.2.8. Chromatograms

Each analyte will be presented in order of their retention time.

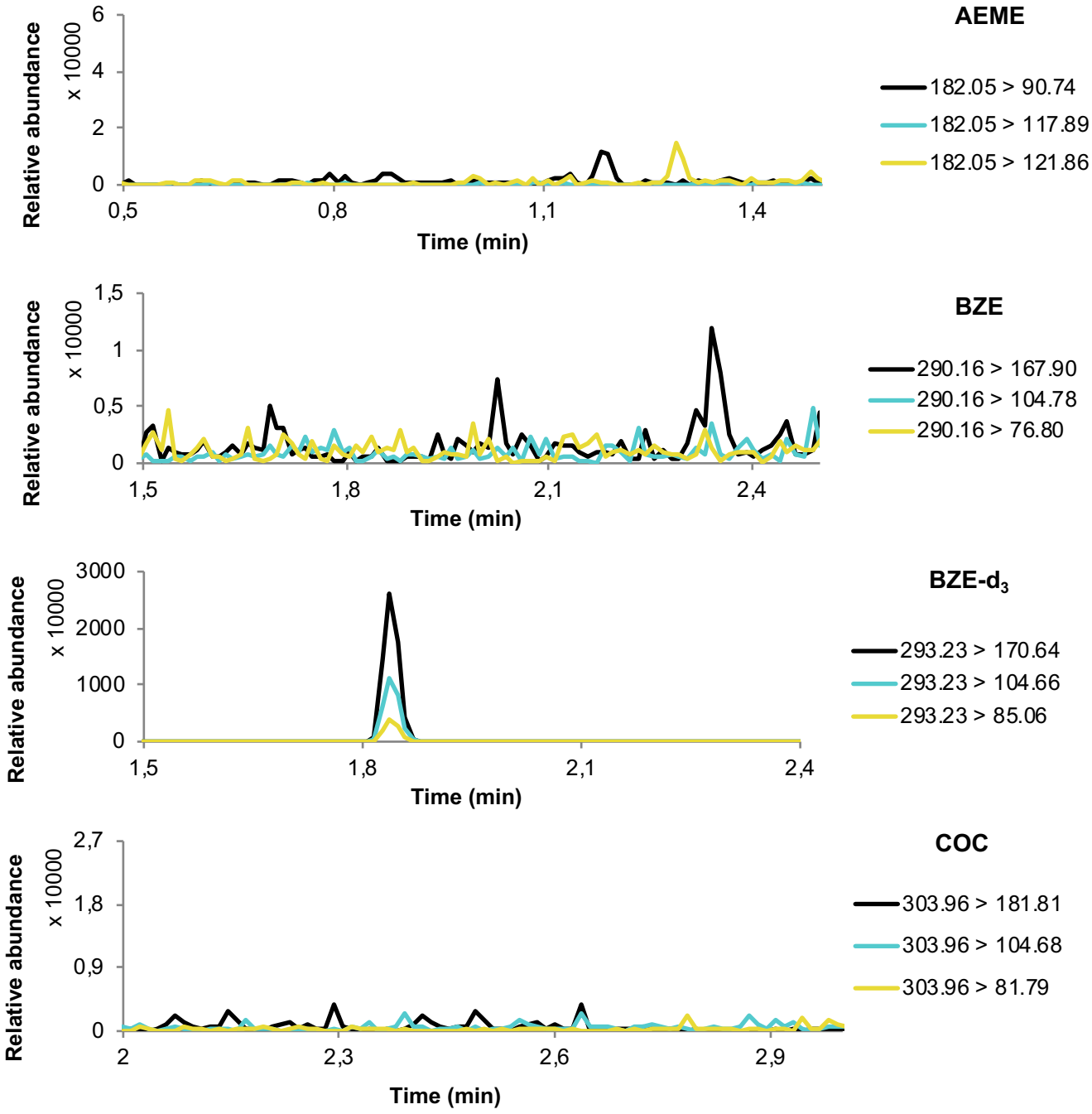

Figure 23. UPLC-ESI-MS/MS separate chromatograms from the analysis of a blank head hair sample spiked with 2ng/mg of the analyte's respective deuterated internal standards, when available. Analysis obtained through application of the method described in item **4.3.1.1.** under **Material & Methods** (total run time is 7min).

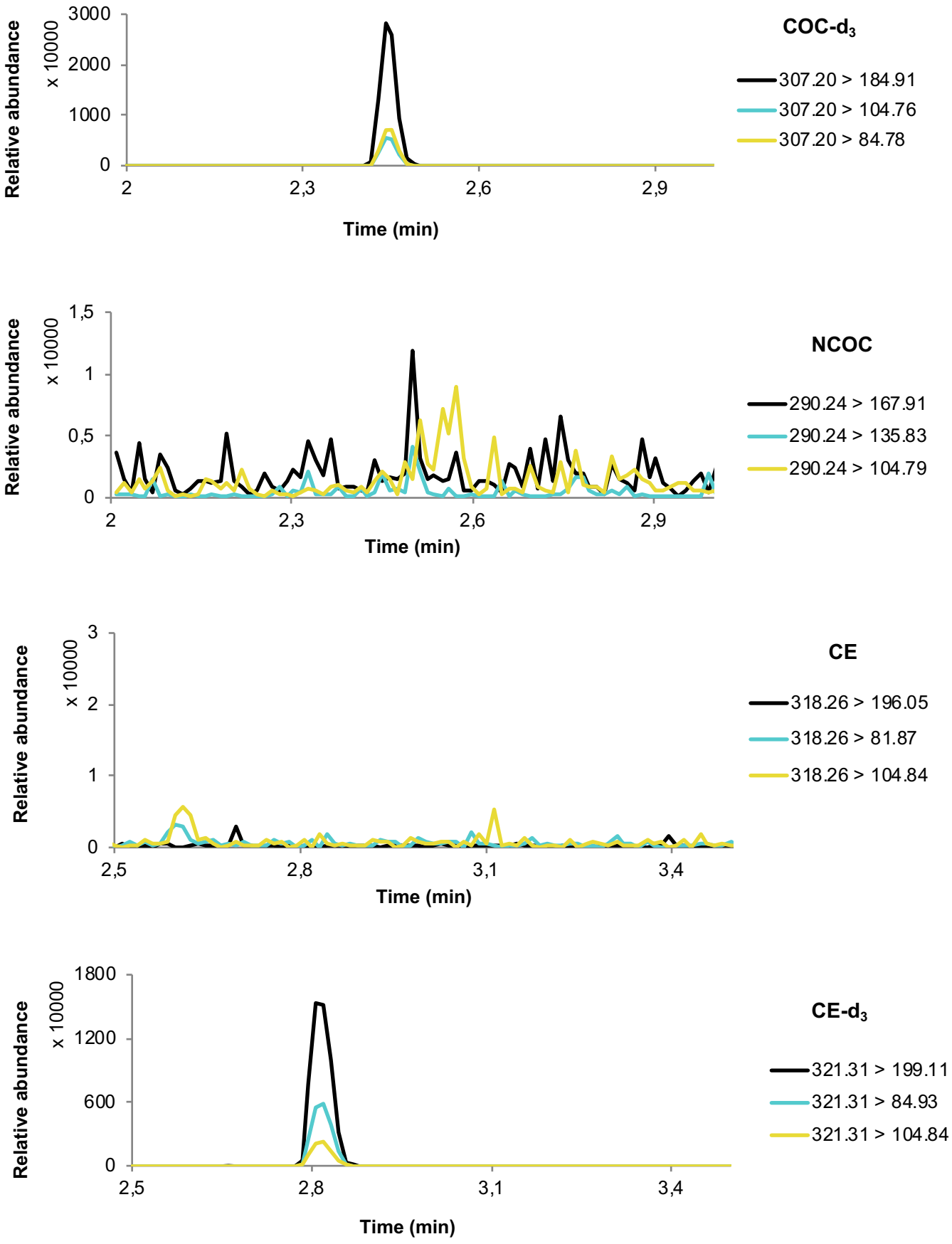

Figure 23. *Continuing*

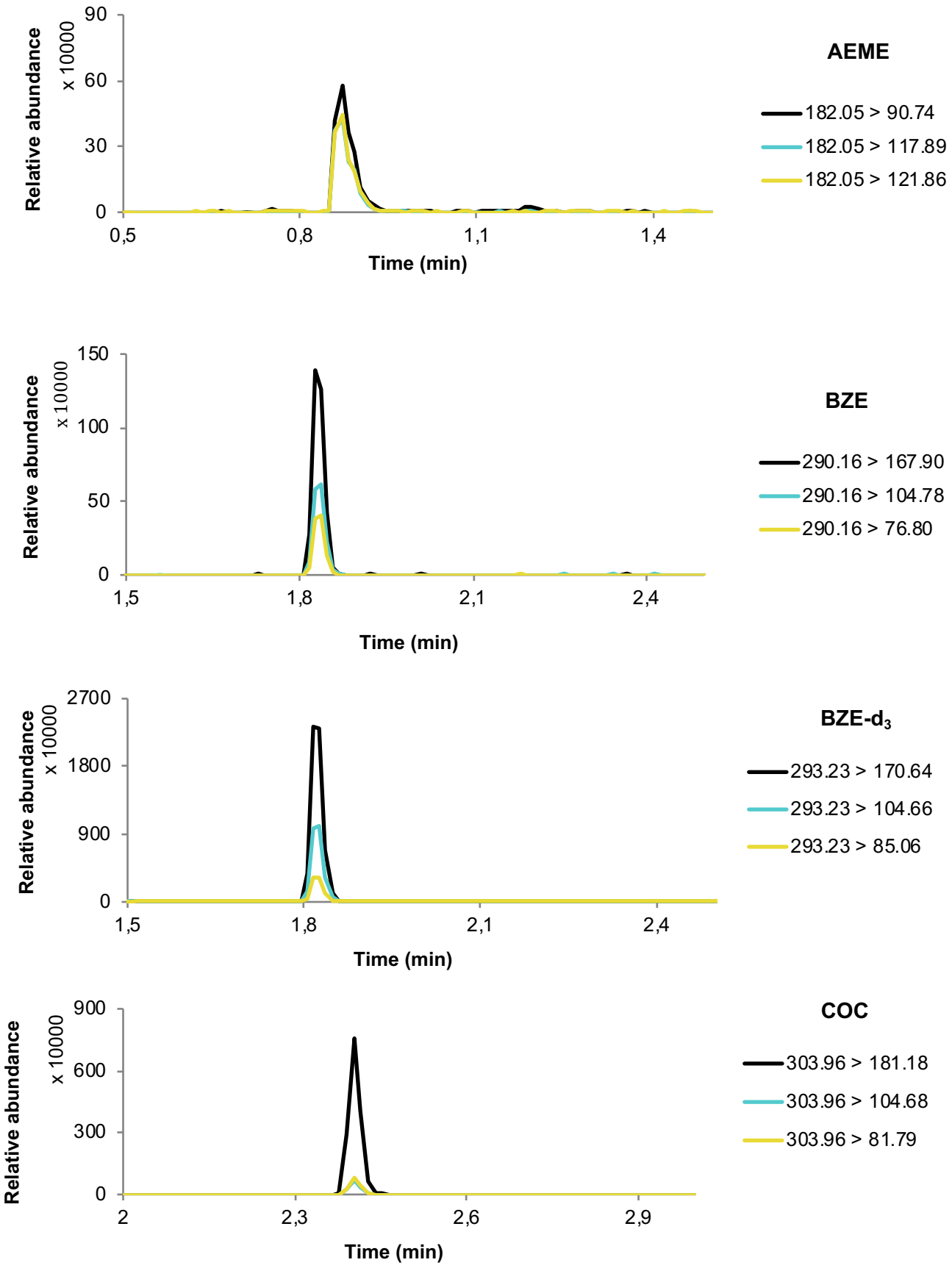

Figure 24. UPLC-ESI-MS/MS chromatogram from the analysis of a spiked head hair sample at the LoQ of the analytes of interest together with 2ng/mg of their respective deuterated internal standards. Analysis obtained through application of the method described in item **4.3.1.1.** under **Material & Methods** (total run time is 7min).

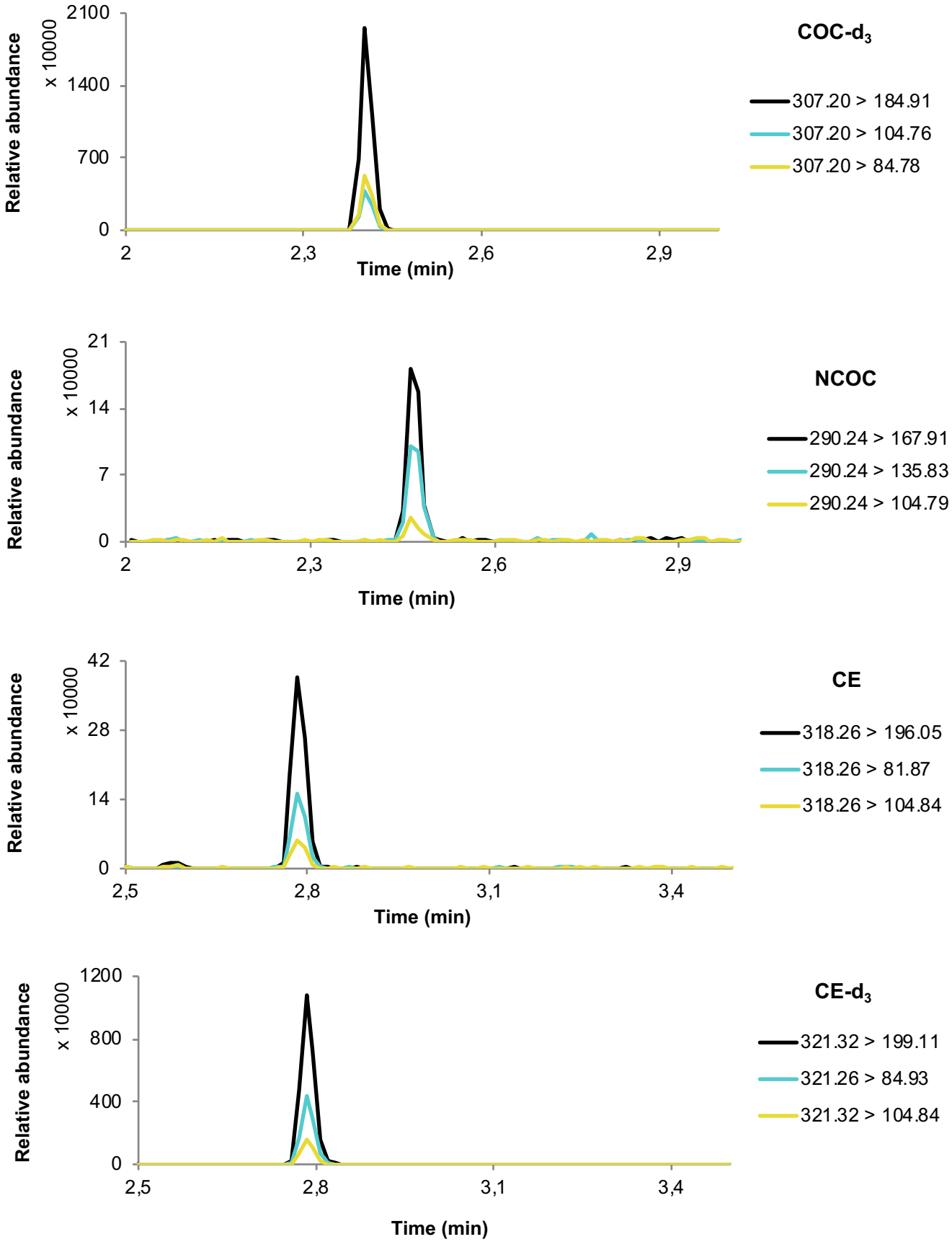

Figure 24. *Continuing*

5.2. *Postmortem* blood analysis of COC, BZE, CE, NCOC and AEME by UPLC-ESI-MS/MS

5.2.1. Method development

5.2.1.1. Protein precipitation

The current procedure was mainly based on the methods proposed by Kim et al., Hegstad et al., D'Avila et al., Mohammed, Eissa and Ahmed and Dziadosz et al. The first part of this procedure involved a protein precipitation step, which is very fast, reliable, and cost effective cleaning procedure (KIM et al., 2011; HEGSTAD et al., 2014 D'AVILA et al., 2015; MOHAMMED; EISSA; AHMED, 2017). Nonetheless, additional caution must be taken as protein precipitation may result in relatively dirty and more complex extracts than those from SPE or LLE (TELVING; HASSELSTRØM; ANDREASEN, 2016).

5.2.1.2. Dilute-and-shoot procedure

As discussed in item **5.1.1.2** under **Results & Discussion**, this is an extremely fast and easy to perform technique. In this case, the final step was simply a direct injection of the withdrawn supernatant into the UPLC-ESI-MS/MS.

The fact that this equipment is extremely versatile and robust, it was possible to avoid time-consuming extraction procedures and the result was a very straightforward, cheap and fast protocol.

5.2.3. Validation results

5.2.3.1. Selectivity

The method has proven to be selective both endogenously and exogenously as no interfering peaks from either the blank samples or the spiked blank samples have been observed (see **Figure 25** on item **5.2.3.8.**).

5.2.3.2. LoD

The LoD values obtained in this method were of 1.0ng/mL for COC, CE and AEME and 0.5ng/mL for both BZE and NCOC. At this point, real case samples were analysed in order to investigate if any of the analytes being validated were actually present in the blood samples and detection of AEME was found to be unsuccessful.

The reason for that is possibly due to its rapid clearance in the blood as according to Scheidweiler et al. (2003), AEME clears quickly from the bloodstream and its half-life is set to be between 18 and 21 minutes (SCHEIDWEILER, 2003). At this point, AEME has been removed from analysis.

5.2.3.3. LoQ

LoQ values of 1.0ng/mL were obtained for both COC and CE and 0.5ng/mL for both BZE and NCOC, as previously discussed in item **5.2.3.2** under **Results & Discussion**. These seem to be reasonable according to the ranges previously reported in the literature (KARCH; STEPHENS; HO, 1998; CHERMACK; BLOW, 2002; JONES; HOLMGREN; KUGELBERG, 2008; ALVES et al., 2016). According to the report from USA's National Highway Traffic Safety Administration entitled: 'Drugs and Human Performance Fact Sheets', after single doses of cocaine, plasma/blood concentrations typically average from 200 to 400ng/mL, while repeated doses of cocaine may result in concentrations greater than 750ng/mL (COUPER; LOGAN, 2014).

5.2.3.4. Linearity

The calibration curves studied ranged from LOQ to 1000ng/mL. The method has proved to be linear for all analytes with r^2 values equal or above 0.99 (see **Figure 26** on item **5.2.3.8.**).

To test for heteroscedasticity phenomenon, the *F-test* has been used and its respective results can be seen in **Table 10**. The *x* and *y* letters represent concentration and peak area, respectively.

Table 10. Linearity results from the method for determination of cocaine and its metabolites in *postmortem* blood samples for all analytes and their respective calibration curves. Values being shown are: concentration range; calibration curve's equations; weighing factors applied and resulting r^2 after adequate correction using the statistical tool '*F-test*'.

Analytes	Concentration range	Calibration curve equation	Weight factor applied	r^2
COC	LoQ to 1000ng/mg	y = 0,00692*x*+0,0027727	$1/x^2$	0.997
BZE	LoQ to 1000ng/mg	y = 0,004457*x*+0,004394	$1/x$	0.999
CE	LoQ to 1000ng/mg	y = 0,0062*x*+0,0041	$1/y^2$	0.992
NCOC	LoQ to 1000ng/mg	y = 0,0018*x*+0,0007247	$1/x$	0.995

5.2.3.5. Intra-day precision

Intra-day precision has met the criteria established by international guidelines (see **Table 11**).

Table 11. Precision results from the method for determination of cocaine and its metabolites in *postmortem* blood samples for all analyte's quality control levels and their respective intra-day precision values expressed as percentage of relative standard deviation (%CV).

Analytes	Intra-day Precision (%CV)		
	LQC	MQC	HQC
COC	11.9	2.3	8.9
BZE	11.5	2.8	5.3
CE	7.3	2.3	3.1
NCOC	5.7	4.5	2.9

Overall, values have ranged from 2.3% (COC and CE's MQC) to 11.9% (COC's LQC) in all quality control levels. This shows concordance with pre-establish precision criteria.

5.2.3.6. Accuracy

Accuracy was found to be within the accepted international criteria. Results can be seen in **Table 12**.

Table 12. Accuracy results from the method for determination of cocaine and its metabolites in *postmortem* blood samples for all analyte's quality control levels and their respective values expressed as a percentage (%).

Analytes	Accuracy (%)		
	LQC	**MQC**	**HQC**
COC	92.1	89.3	97.2
BZE	105.9	92.6	100.0
CE	113.7	90.1	111.3
NCOC	86.4	91.7	97.6

It can be said that the required criteria for this parameter has been met as the lowest accuracy value was of 86.4% for NCOC's LQC and the highest one for 113.7% for CE's LQC.

5.2.3.7. Carryover

No carryover has been found in the present method. No peaks have been found at the analyte's retention times on all three consecutive blank samples, injected straight after the highest calibration point.

5.2.3.8. Chromatograms

Each analyte will be presented in order of their retention time.

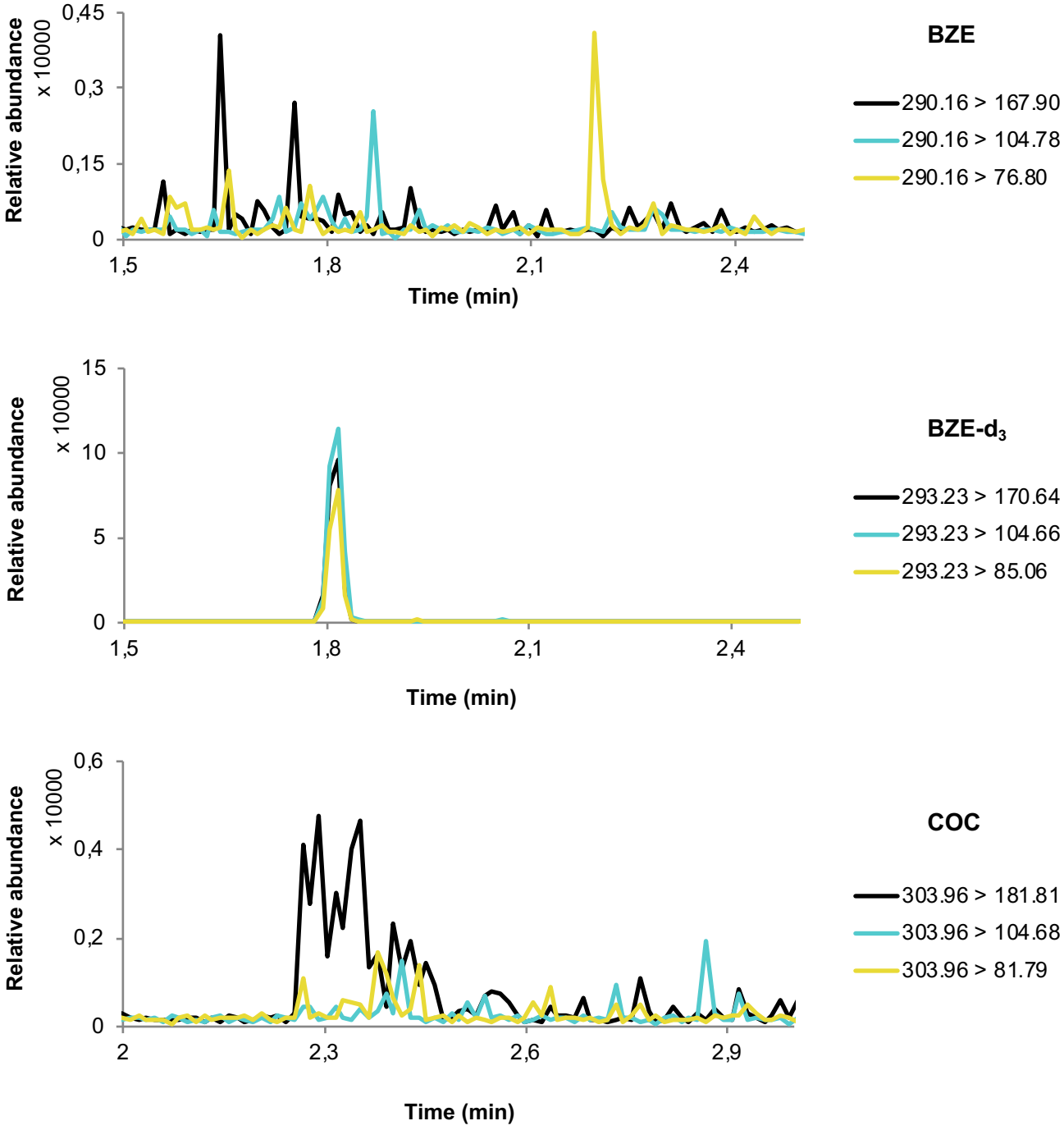

Figure 25. UPLC-ESI-MS/MS separate chromatograms from the analysis of a blank blood sample spiked with 100ng/mL of the analyte's respective deuterated internal standards, when available. Analysis obtained through application of the method described in item **4.3.2.1.** under **Material & Methods** (total run time is 7min).

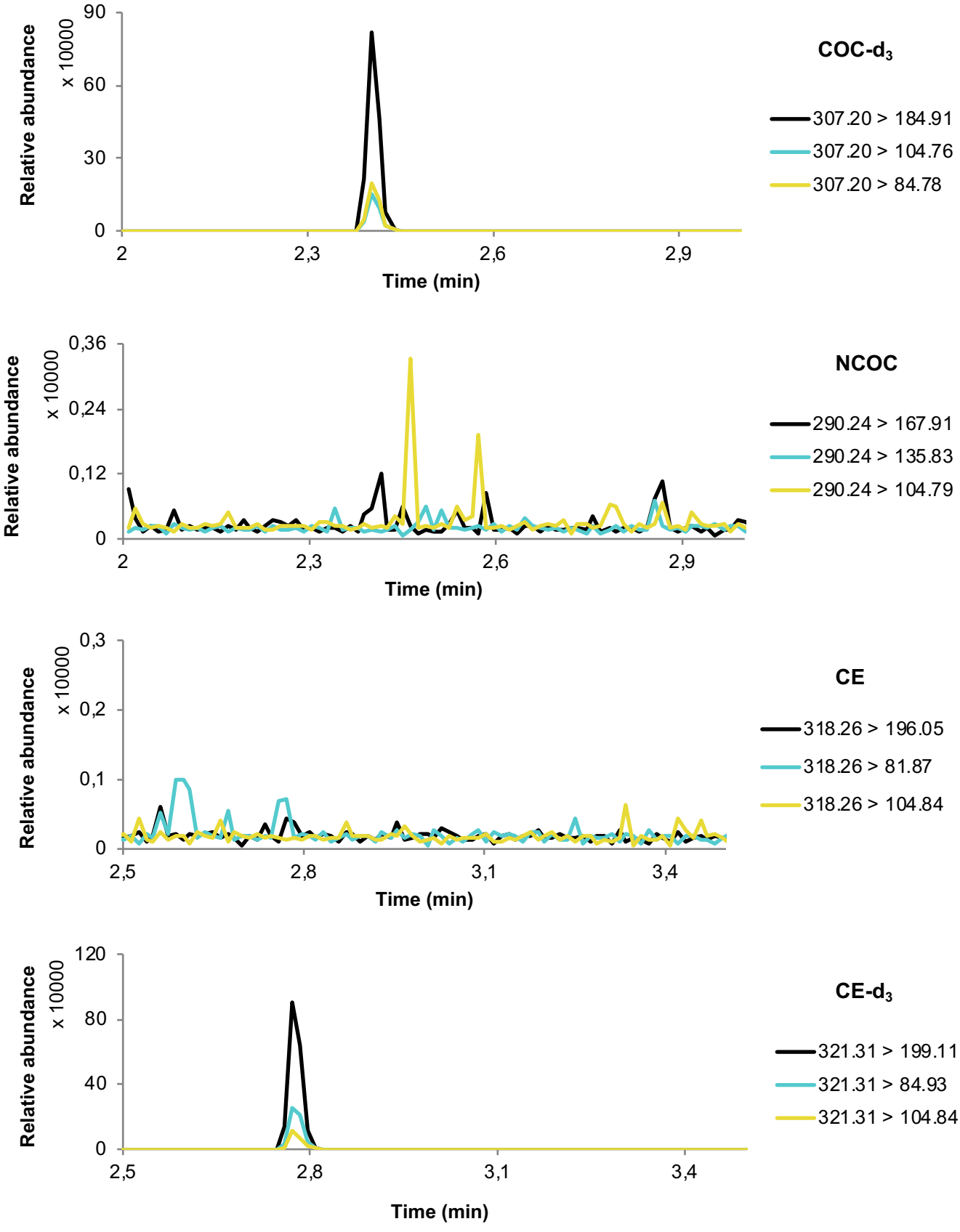

Figure 25. *Continuing*

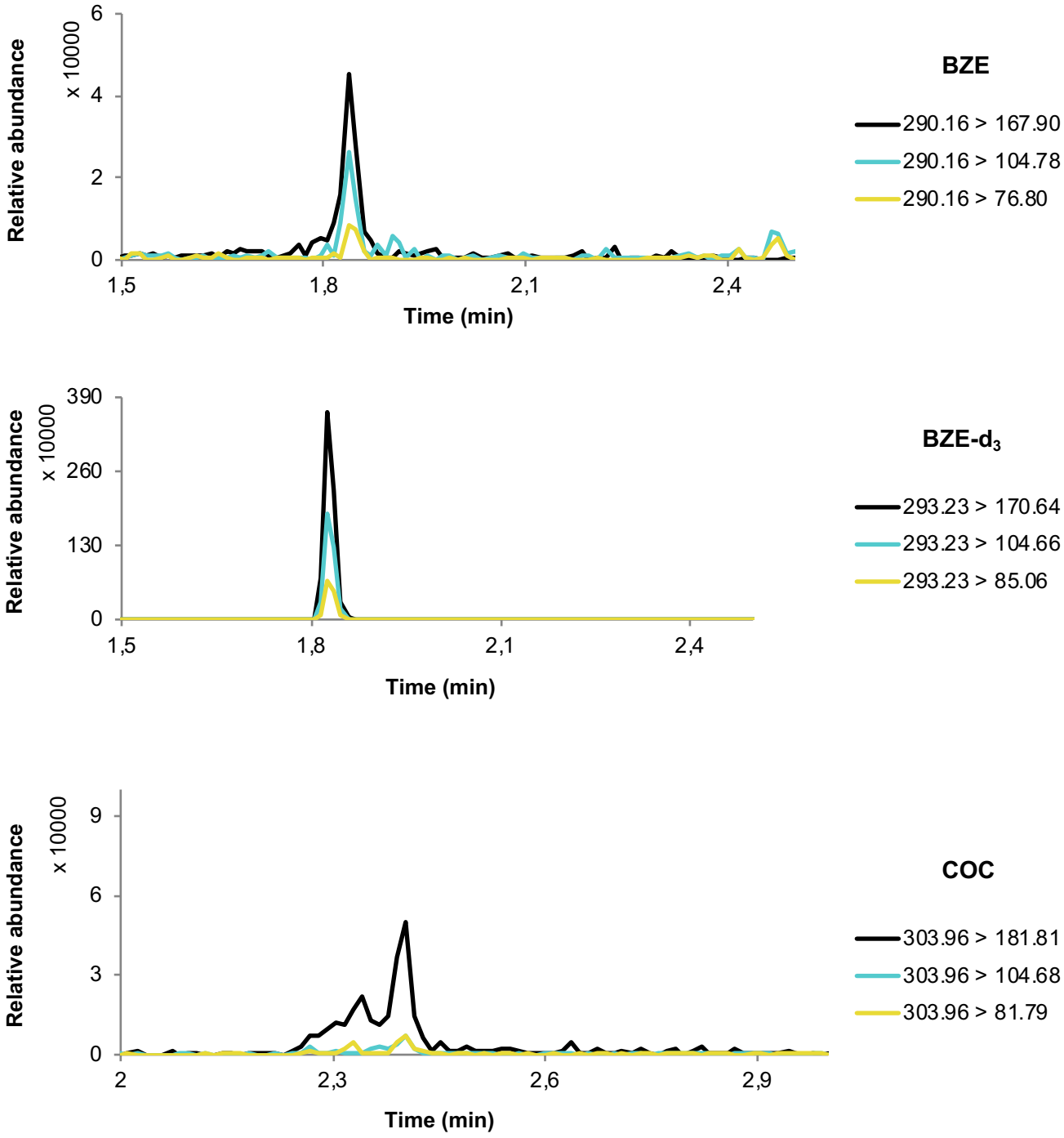

Figure 26. UPLC-ESI-MS/MS chromatogram from the analysis of a spiked blood sample at the LoQ of the analytes of interest together with 100ng/mL of their respective deuterated internal standards. Analysis obtained through application of the method described in item **4.3.2.1.** under **Material & Methods** (total run time is 7min).

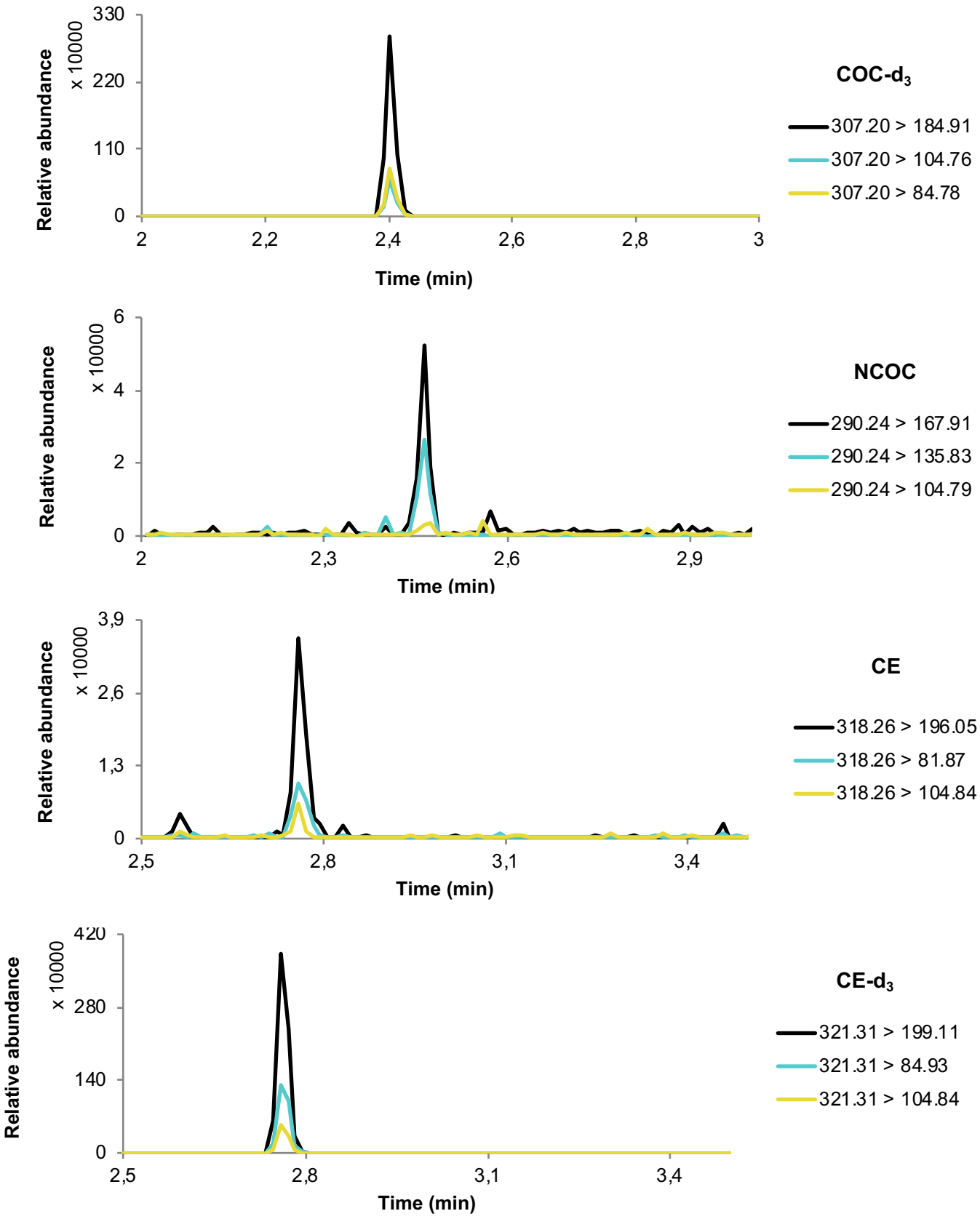

Figure 26. *Continuing*

5.3. Toxicological analysis results

5.3.1. Proportion of cocaine amongst violence deaths arriving at the IML-SP and its relationship to violence

The results from our sampling have shown a rather high positivity rate of cocaine users amongst violent-death cases arriving at the IML-SP.

When looking at hair, considering that in this case the parent-drug is the most preeminent, the positivity rate found for COC was of 53.3%, meaning 56 individuals (46.7% of the hair samples were negative – 49 individuals). The remaining anaytes of BZE, COC, CE, NCOC and AEME were present in 56 (53.3%), 42 (40%), 45 (42.9%) and 14 (13.3%) individuals, respectively, as seen in **Figure 27.**

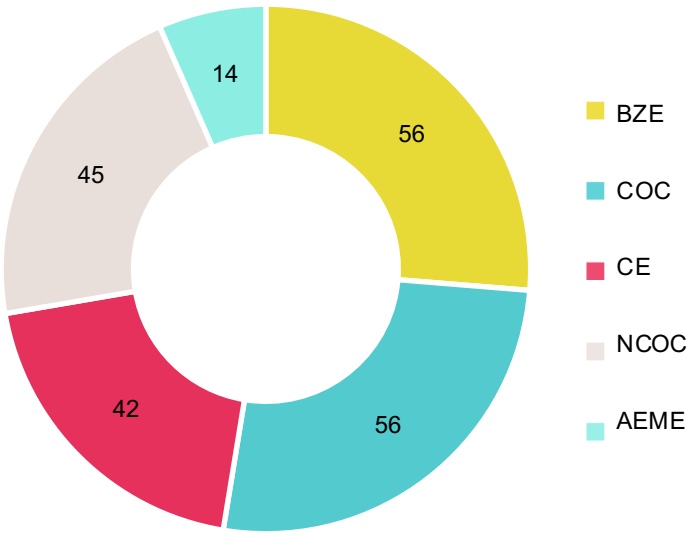

Figure 27. Number of cases that were positive in the hair matrix for all analytes of interest from a total of 105 *postmortem* cases. When applying the method described in item **4.3.1.1.** under **Material & Methods**, positive values for BZE and COC were obtained in 56 individuals, followed by 45, 42 and 14 individuals for NCOC, CE and AEME, respectively.

As for the blood, indicating that the individual was likely under the effect of the drug upon death, the positivity for BZE was found to be of 50.5%, meaning 53 individuals (49.5% of the blood samples were negative – 52 individuals). Taking into account that in this case, BZE is present in higher concentrations and it is more likely to be found than the parent-drug due to metabolism.

In this case, the values for the parent-drug were of 43 (40.9%) COC-positive individuals and the remaining analyse of CE and NCOC present in 24 (22.8%) and 21 (20%) individuals, respectively, as seen in **Figure 28.**

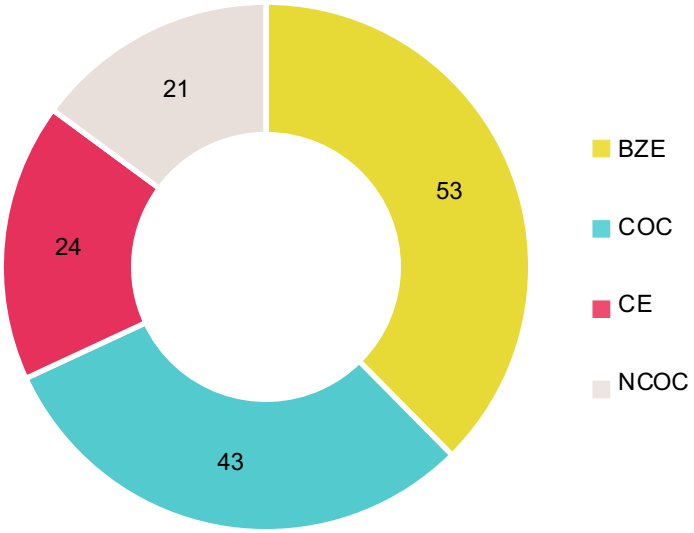

Figure 28. Number of cases that were positive in the blood matrix for all analytes of interest from a total of 105 *postmortem* cases. When applying the method described in item **4.3.2.1.** under **Material & Methods**, positive values for BZE, COC, CE and NCOC were obtained in 53, 43, 24 and 21 individuals, respectively.

Moreover, the rate of individuals who appeared to be positive for both hair and blood was of 41.9% (44 individuals). Regarding each matrix's concentration, as it can be seen in **Figure 28**, it was found that 81.1% (43 individuals) of all blood BZE positive samples showed the presence of the parent drug. This shows us that regardless of having distinctive implications, the results were fairly similar across blood and hair samples as the majority of chronic users were also found to be under the influence of the drug at the time of death.

Considering the relationship between the drug and violent behaviours, it is not possible to infer that cocaine use is playing a role on violent death, as it was not possible to obtain a non-violent control group. Nonetheless, these findings show a reasonably high proportion of the drug within the subjects studied.

This data is in concordance with previous results from Campelo and Caldas who have looked at the frequency of the different drugs of abuse detected in the necropsy room, in Brasilia, from 2006 and 2008 where it was found that both alcohol and cocaine were the most prevalent psychoactive substances found within

postmortem cases with a percentage of 47.4 and 21.6%, respectively within cases of unnatural death (CAMPELO; CALDAS, 2010).

However, the current study aimed to look more deeply into the aggressor's profile rather than the victim's as well as to have a comparison between recent and chronic use by looking at blood and hair samples, respectively.

In a recent study performed by Abdalla and colleagues, it was proven that both cocaine and alcohol use increased the chances of participants engaging in violent behaviours by almost four times. They have also presented some rather astonishing numbers regarding urban violence in Brazil showing that around 9.3% of the Brazilian population has been a victim of at least one form of urban violence. This number rises to 19.7% amongst those who used cocaine in the previous year (ABDALLA et al., 2018).

All of the above aid on proposing that, indeed, cocaine abuse may be one of the risk factors associated to violence.

5.3.1.1. Postmortem head hair concentrations

Postmortem head hair samples were collected at IML-SP and extracted as described in item **4.3.1.1** under **Material & Methods**. Looking at hair concentrations, the mean values found for COC, BZE, CE, NCOC and AEME were of 15.1, 3.1, 0.9, 1.2 and 5.5ng/mg, respectively.

Poon and colleagues (2014) have found very similar values, as within 90% of their cohort (adult hair of a clinical, high risk population), the mean concentration values found for COC, BZE and NCOC were of >16.0, 4.8 and 0.5ng/mg, respectively (POON et al., 2014). Gambelunghe and colleagues (2015), have also found mean concentrations of COC, BZE, CE and NCOC of 9.5, 1.4, 0.7 and 1.2ng/mg respectively, which, again, were significantly similar to the ones in found within this study (GAMBELUNGHE et al., 2015).

The rate of NCOC present in the samples was also accessed and it is in agreement with previous studies as within the lower concentrations (<3ng/mL) very few cases have yielded a positive result for NCOC. However, in higher concentrations (>3ng/mL), users have shown the presence of NCOC in 100% of the cases (POON et al., 2014; GAMBELUNGHE et al., 2015)

In order to demonstrate an example of a real case sample chromatogram, both a low and a high concentration samples were chosen, given that they appeared to be positive for all analytes under study. To illustrate a low concentration *postmortem* head hair sample, positive for all analytes, it was used sample number 19.

The chromatogram can be seen in the following **Figure 29** where the concentrations found were as follows: COC – 5.0ng/mL, BZE – 1.4ng/mL, CE – 1.0ng/mL, NCOC – 0.2ng/mL and finally AEME – 0.6ng/mL.

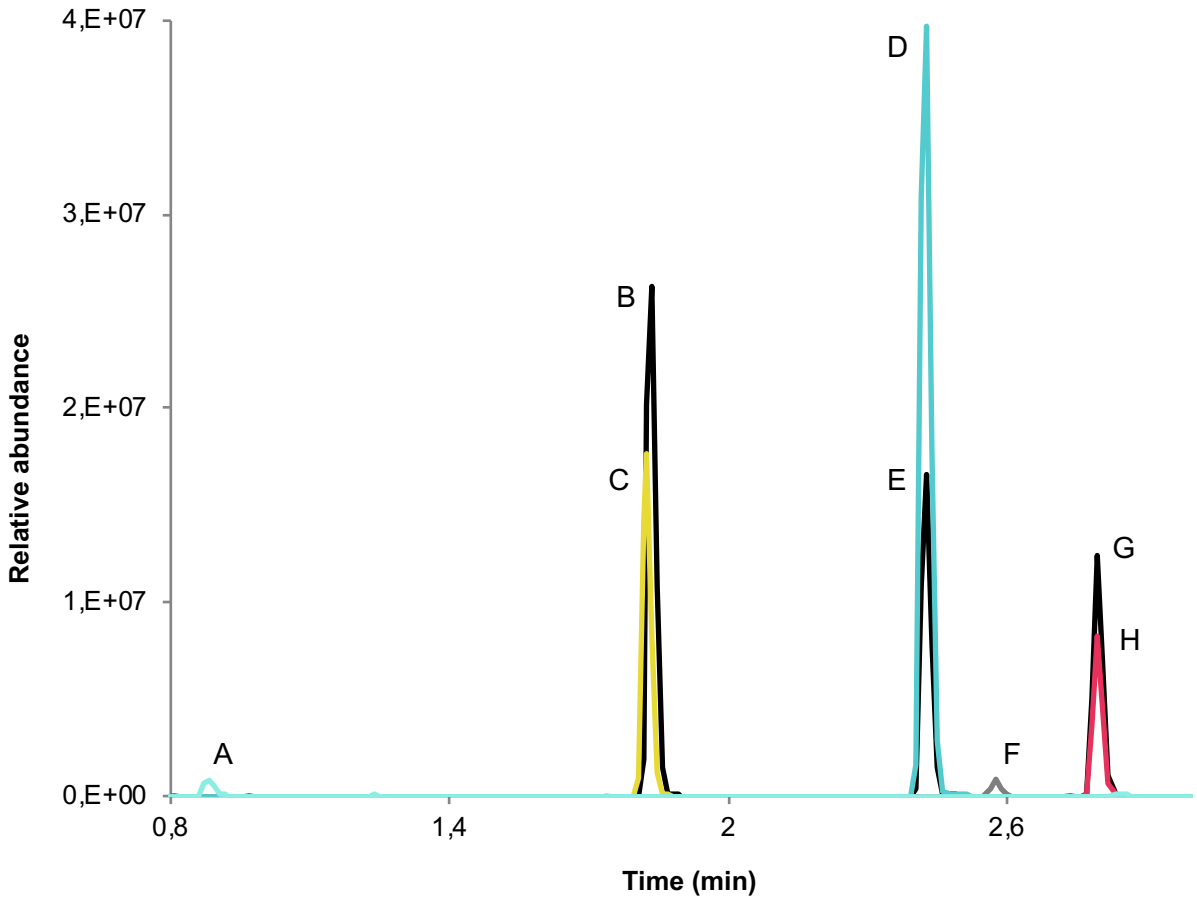

Figure 29. UPLC-ESI-MS/MS chromatogram from the analysis of a real *postmortem* head hair sample from IML-SP along with 2ng/mg of BZE-d3 (**B**); COC-d3 (**E**) and CE-d3 (**G**) as deuterated internal standards. Analysis obtained through application of the method described in item **4.3.1.1.** under **Material & Methods**. The resulting concentrations found for all analytes were: AEME (**A**) – 0.6ng/mg; BZE (**C**) – 1.4ng/mg; COC (**D**) – 5.0ng/mg; NCOC (**F**) – 0.2ng/mg and CE (**H**) – 1.0ng/mg.

As for the high concentration *postmortem* head hair case, sample number 35 was in place. The chromatogram can be seen in the following **Figure 30** where the concentrations found were as follows: COC – 17.8ng/mL, BZE – 3.6ng/mL, CE – 1.1ng/mL, NCOC – 0.4ng/mL and finally AEME – 0.9ng/mL. Note that samples found to be above the calibration curve were not considered.

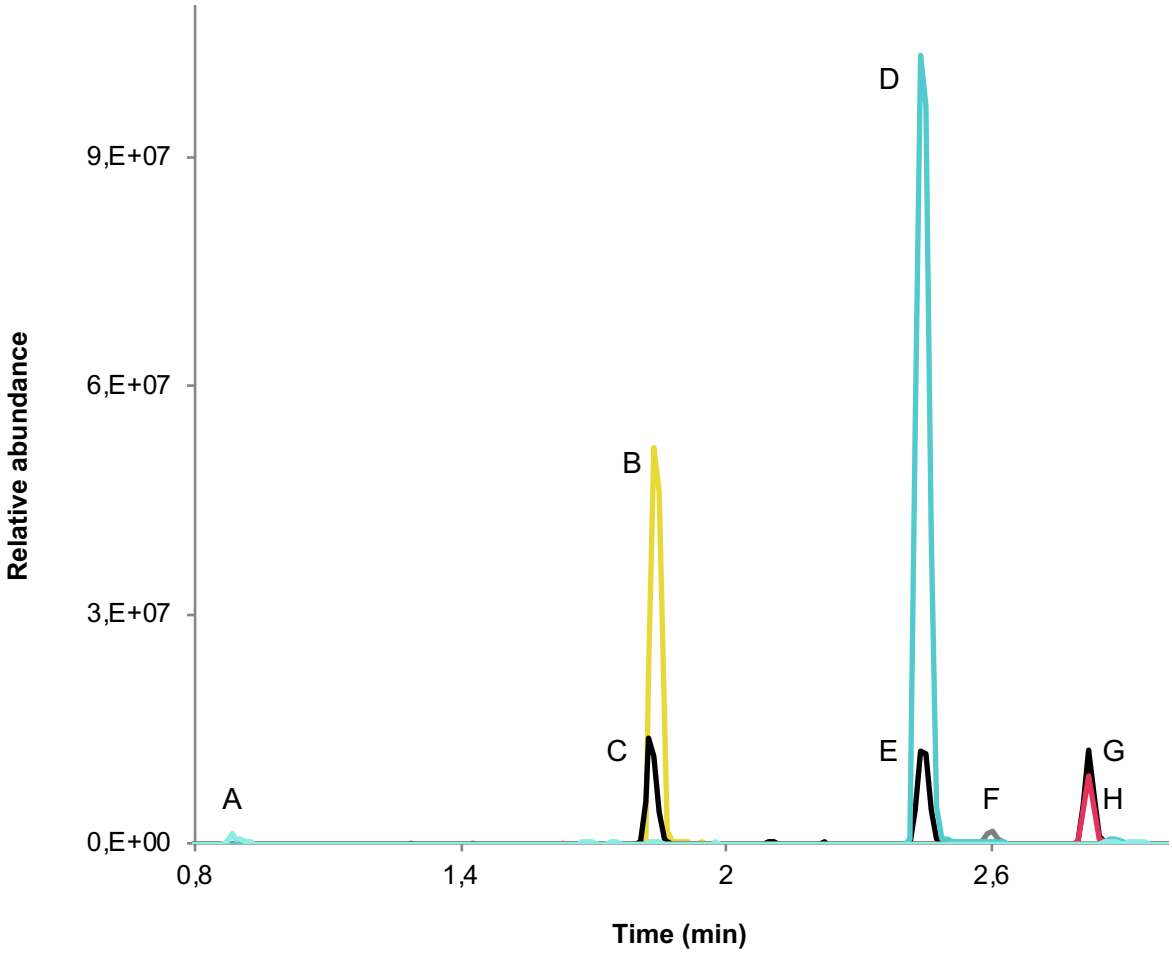

Figure 30. UPLC-ESI-MS/MS chromatogram from the analysis of a real *postmortem* head hair sample from IML-SP along with 2ng/mg of BZE-d_3 (**C**); COC-d_3 (**E**) and CE-d_3 (**G**) as deuterated internal standards. Analysis obtained through application of the method described in item **4.3.1.1.** under **Material & Methods**. The resulting concentrations found for all analytes were: AEME (**A**) – 0.9ng/mg; BZE (**B**) – 3.6ng/mg; COC (**D**) – 17.8ng/mg; NCOC (**F**) – 0.4ng/mg and CE (**H**) – 1.1ng/mg.

5.3.1.2. Postmortem blood concentrations

The corresponding *postmortem* blood samples were also collected at IML-SP and extracted as described in item **4.3.2.1** under **Material & Methods**. In this case, the mean concentrations found for COC and BZE, of 290 and 607ng/mL, respectively, were rather similar to the values reported by Jenkins and colleagues, on the non-

cocaine death cases (homicides, suicides or accidents) with values of 146 and 888ng/mL, respectively (JENKINS et al., 1999).

However, much lower concentrations have been found in other studies in either drug toxicity-related deaths with a COC and BZE mean concentrations of 0.5 and 3.3ng/mL, respectively, or within external injury-related death with values of 0.2 and 2.0ng/mL, respectively (PILGRIM; WOODFORD; DRUMMER, 2013).

In fact, in the study performed by Jenkins and colleagues, the site of collection was not mentioned, while in the study of Pilgrim and colleagues, the majority of the blood source was femoral (JENKINS et al., 1999; PILGRIM; WOODFORD; DRUMMER, 2013). Indeed, this issue recalls that it is important to note the complexity in the interpretation of quantitative blood *postmortem* results.

This is especially true when dealing with COC because of two main reasons: hydrolysis and *postmortem* redistribution (PMR). COC is, in fact, one of the many drugs of abuse that is subject to *postmortem* breakdown to its metabolite, BZE (HEARD; PALMER; ZAHNISER, 2008). Still, its hydrolysis can be slowed down by fluoride preservation (KLINGMANN; SKOPP; ADERJAN, 2001).

Nonetheless, the main issues are site- and time-dependent variations, which constitute the phenomenon of PMR. This is characterized by the redistribution of drugs into blood from solid organs known as “drug reservoirs” such as the lungs, liver, and myocardium. Basic, lipophilic drugs with a large distribution volume are particularly susceptible to PMR (YAREMA; BECKER, 2005).

To avoid this challenging artifact, femoral blood is preferred as even though it is not absent from *postmortem* redistribution, it is still much less affected than cardiac or centrally collected blood (DRUMMER; GEROSTAMOULOS, 2002). Unfortunately, the collection of peripheral blood is not a regular practice at IML-SP where there is an overwhelming amount of routine work.

It is still important to mention that even though this is the preferred site of collection, one must be careful to distinguish between femoral arterial blood (FAB) from femoral venous blood (FVB) as a study performed by Alvear and colleagues showed that the greatest discrepancy found on COC and BZE concentrations were actually within the femoral site itself rather than when compared to the cardiac site (ALVEAR et al., 2014).

In their study, they have found concentrations of COC, in a suicide case, in the right cardiac blood (RCB), left cardiac blood (LCB), FAB and FVB of 1,111.5;

1,635.9; 970.4 and 3,210.6ng/mL, respectively and BZE values of 3,458.9; 3,116.4; 3,031.7 and 19,847.0ng/mL, respectively. These results show that even though the femoral site is of much more value to the *postmortem* scenario than the cardiac site, a wrongful collection may lead to misleading quantitative results (ALVEAR et al., 2014).

Also in this case, two samples were used to demonstrate both low and high concentrations of cocaine and its metabolites. Again, only samples where all analytes were present have been considered.

An example of a real case sample with overall low concentrations obtained was sample number 44. The chromatogram can be seen in the following **Figure 31** where the concentrations found were as follows: COC – 11.9ng/mL, BZE – 285.0ng/mL, CE – 1.7ng/mL and finally NCOC – 0.9ng/mL.

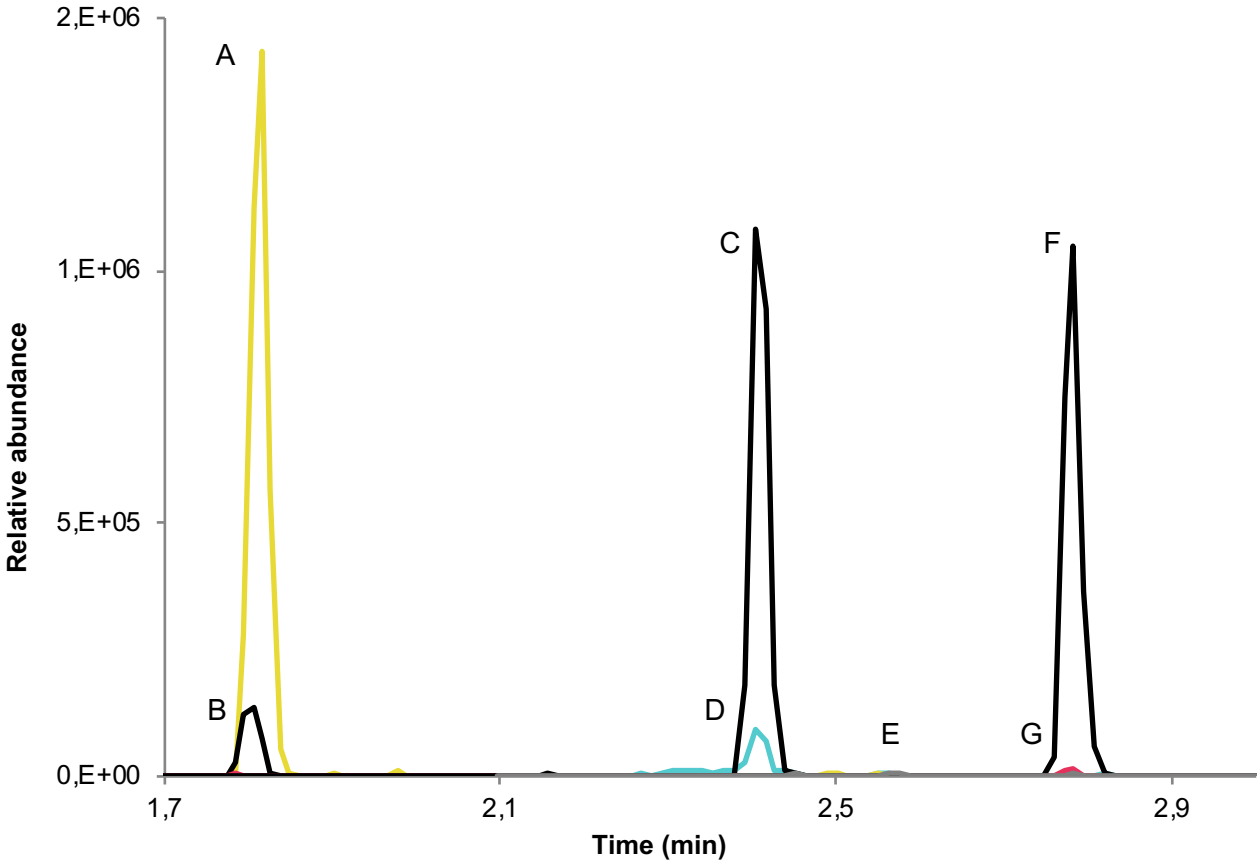

Figure 31. UPLC-ESI-MS/MS chromatogram from the analysis of a real *postmortem* blood sample from IML-SP along with 100ng/mg of BZE-d_3 (**B**); COC-d_3 (**C**) and CE-d_3 (**F**) as deuterated internal standards. Analysis obtained through application of the method described in item **4.3.2.1.** under **Material & Methods**. The resulting concentrations found for all analytes were: BZE (**A**) – 285.0ng/mL; COC (**D**) – 11.9ng/mL; NCOC (**E**) – 0.9ng/mL and CE (**G**) – 1.7ng/mL.

Also for blood, a relatively high concentrated blood sample was chosen: number 83. The chromatogram can be seen in the following **Figure 32** where the concentrations found were as follows: COC – 180.9ng/mL, BZE – 681.2ng/mL, CE – 4.9ng/mL and finally NCOC – 19.2ng/mL. Note that samples found to be above the calibration curve were not considered.

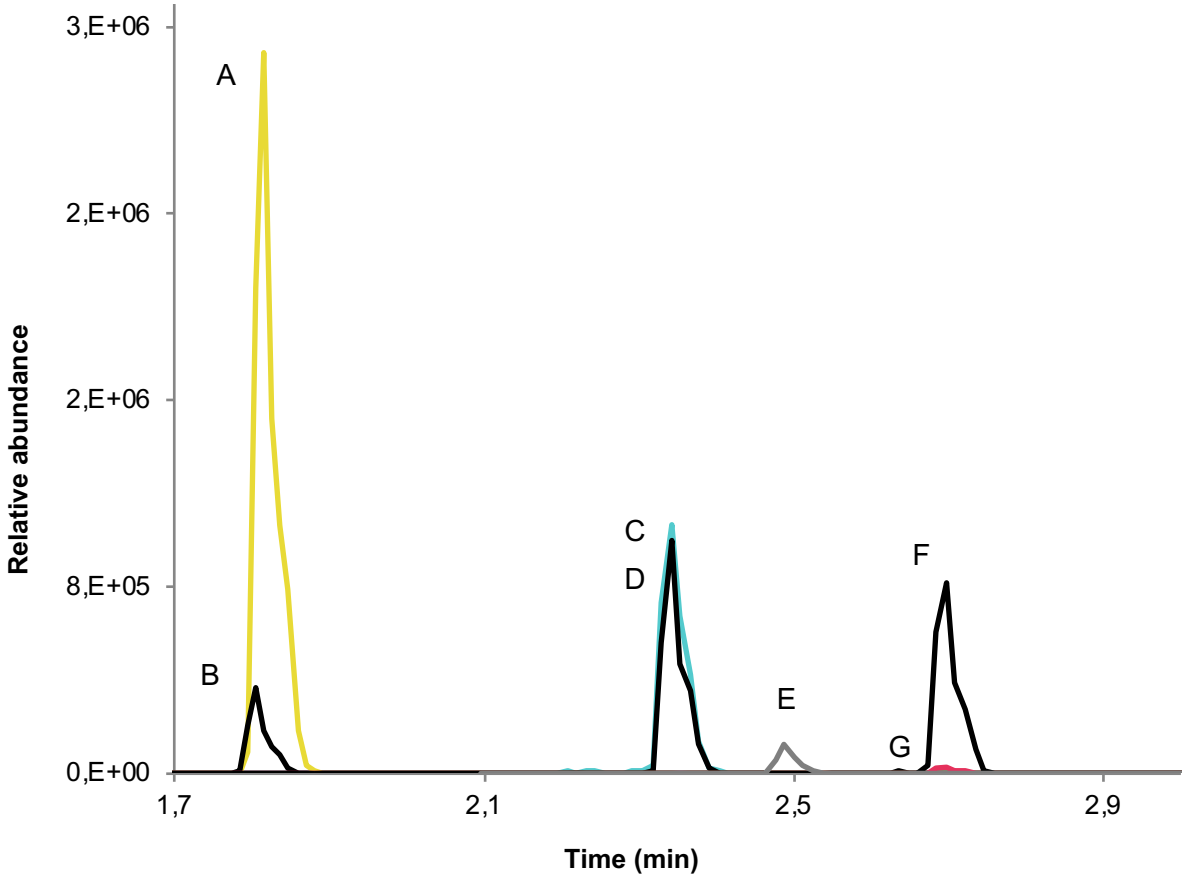

Figure 32. UPLC-ESI-MS/MS chromatogram from the analysis of a real *postmortem* blood sample from IML-SP along with 100ng/mg of BZE-d_3 (**B**); COC-d_3 (**D**) and CE-d_3 (**F**) as deuterated internal standards. Analysis obtained through application of the method described in item **4.3.2.1.** under **Material & Methods**. The resulting concentrations found for all analytes were: BZE (**A**) – 681.2ng/mL; COC (**C**) – 180.9ng/mL; NCOC (**E**) – 19.2ng/mL and CE (**G**) – 4.9ng/mL.

5.3.2. Demographics

Regarding the age of the individuals, 43.8% were between 18 and 25 years-old; 28.6% were 26-36 years-old; 18.1% of the individuals were above 45 years-old and finally 9.5% were in the age range of 37 to 45 (**Figure 33**).

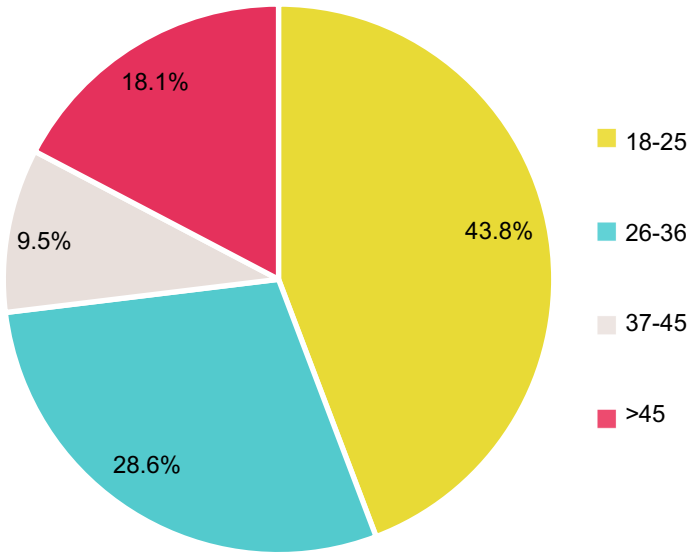

Figure 33. Age ranges of the individuals under study, with the highest value being 18 to 25 years old (46 individuals: 43.8%) followed by 26 to 36 years old (30 individuals: 28.6%); above 45 years old (18 individuals: 18.1%) and finally 37 to 45 years old (10 individuals: 9.5%).

When looking at the gender 92.4% of the collected samples came from men, while the remaining 7.6% were from women (**Figure 34**).

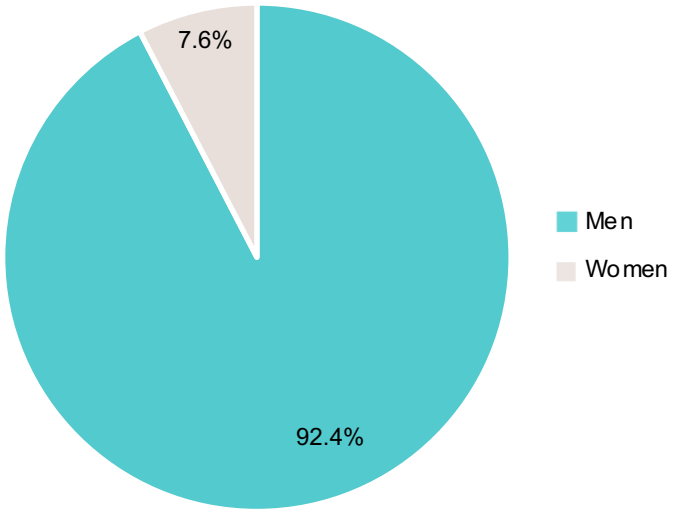

Figure 34. Gender of the subjects collected was divided into men (97 individuals: 92.4%) and women (8 individuals: 7.6%).

By analyzing the demographics within this study, it can be seen that the most critical age range found within the 105 *postmortem* cases was from 18 to 25 years old with a percentage of 43.8%. In fact, the Atlas of Violence in Brazil, has reported that homicide as a cause of mortality of the male youth, 15 to 29 years, is an emblematic fact as it contributes to 47.8% of the total deaths (CERQUEIRA et al., 2017).

It was also mentioned that if they have taken into account only men between 15 and 19 years, this indicator would reach the incredible mark of 53.8%. As it can be seen, the proportions obtained were exceptionally similar, considering that in the Atlas of Violence, a broader age range is applied. (CERQUEIRA et al., 2017).

Gender wise, there is a significant disparity as 92.4% of all violent cases involved men. This finding is consistent with the latest World Health Organization (WHO) report where it is mentioned that in the year of 2015 from about 468,000 murders being reported, four fifths of them were male. It was also stated that men in the WHO Region of the Americas suffered the highest rate of homicide deaths with a rate of 32.9 per 100,000 inhabitants, which is 12 times the rate among men in the WHO Western Pacific Region (WHO, 2017).

5.3.3. Manner of death

The apparent cause of death was described in the Police Incident Record provided with each individual's personal record. Even though it was not possible to achieve the same level of detail in all cases, it was still possible to divide the various manners of death into a 4-category scenario, which included: drug abuse suspicion – when the individual was a known drug user and has likely recklessly abuse the substance, therefore placing himself into a risky situation (possible overdose), homicide resulting from opposition to police intervention – when the individual has gotten into a confrontation with the police and eventually suffered a fatal gunshot wound, general violence and risk behaviour – situations such as bar fights; dangerous motorcycle manoeuvres; robberies; gang rivalry and prison fugitives and finally possible suicide – occasions where suicide was deeply implied and these included suicide by various physical means (hanging, jumping off a bridge, railway fatalities) and situations where the individual has taken drugs specifically as a way of suicide, specifically aiming that outcome and not accidentally.

Overall, general violence and risk behaviours accounted for a total 34 cases (32.4%), followed by drug abuse suspicion with 26 cases (24.8%), homicide resulting from opposition to police intervention with 23 cases (21.9%) and finally possible suicide with 22 cases (20.9%) – as it can be seen represented in the **Figure 35**.

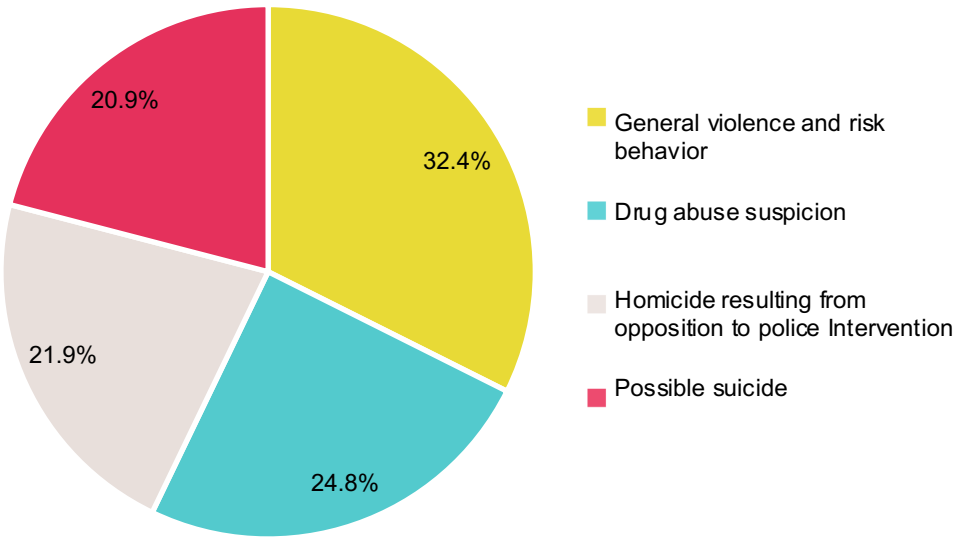

Figure 35. Illustration showing the four categories chosen to represent the various manners of death reported by the Police Incident Record which includes: general violence and risky behaviour (34 cases: 32.4%); drug abuse suspicion (26 cases: 24.8%); homicide resulting from opposition to police intervention (23 cases: 21.9%) and possible suicide (22 cases: 20.9%).

5.3.4. Limitations and strengths

For a starter the lack of a non-violent control group is a drawback when it comes to establishing a straight relationship between the use of cocaine and violence. However, the high proportion seen within the study does give great confidence towards that conclusion.

When it comes to the absence of AEME in the blood samples, a rather efficient indicator for *crack*-cocaine exposure would have been ecgonidine, which must be taken into account for further researches as in fact, this metabolite is infrequently detected in blood and even using *crack*-cocaine smokers within controlled settings did not allow its detection (CONE; HILLSGROVE; DARWIN, 1994; JENKINS; OYLER; CONE, 1995). Nonetheless it was still possible to determine a comparison between recent and/or chronic use and to detect AEME in hair.

The fact that it was only possible to obtain one femoral blood sample against 104 cardiac and centrally collected samples is a major issue as the femoral site is the preferred spot for collection in order to avoid phenomena such as PMR. However, it was possible to simply identify cocaine-users in relation to the frequency of use (chronic or recent) and its probable correlation to violence – as proposed.

5.3.5. Table with all the data

Below it is presented **Table 13**, with the raw data in a more detailed manner for all the 105 *postmortem* cases. It contains valuable information on each case including their given lab number (#individual); their gender (M – male and F – female); their age; their specific circumstance of death according to the Police Incident Report, the category they fall under (where A – drug abuse suspicion, B – homicide resulting from opposition to police intervention, C – general violent and risk behaviour and D – possible suicide), the site from where blood and hair samples where taken and their respective blood and hair toxicological results (where ND – non detected, >1000 – above 1000ng/mL and >20 – above 20ng/mg).

Table 13. Detailed information on the 105 *postmortem* cases including their given lab number (#individual); their gender (M – male and F – female); their age; their specific circumstance of death according to the Police Incident Report, the category they fall under (where A – drug abuse suspicion, B – homicide resulting from opposition to police intervention, C – general violent and risk behavior and D – possible suicide), the site from where blood and hair samples where taken and their respective blood and hair toxicological results (where ND – not detected, >1000 – above 1000ng/mL and >20 – above 20ng/mg).

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#1	F	27	Drug user for 15 years. Found dead on the street.	A	Cardiac	Vertex (close to scalp)	87.3	333.1	5.8	7.3	>20	>20	2.1	1.1	7.4
#2	M	27	Opposition to police intervention - resulted in homicide by firearm.	B	Central	Vertex (close to scalp)	14.9	78.8	ND	ND	2.9	1.2	ND	0.1	ND
#3	M	60	Found dead with report of drug abuse suspicion.	A	Cardiac	Vertex (close to scalp)	21.4	971.8	ND	1.9	>20	12.2	ND	2.5	1.9
#4	M	20	Found dead at home with several bullets. Member of a gang.	C	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#5	M	39	Alcohol user who suffered a fall. Possibly under the influence of the substance.	A	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#6	M	28	Got into a bar fight and suffered several injuries. Got taken to the hospital prior to his death.	C	Central	Vertex (close to scalp)	ND	ND	ND	ND	1.8	0.3	0.1	0.1	ND
#7	M	25	After performing a series of dangerous motorcycle maneuvers. He fell and died on the road.	C	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#8	M	38	Fell off a 6m height while. Supposedly on drugs and stayed for a long time in the hospital before he died.	C	Cardiac	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#9	F	35	HIV positive and homeless individual who suffered a fall with suspicion of drug use.	A	Cardiac	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#10	M	42	Reacted to the robbery and got shot.	C	Cardiac	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#11	M	27	While on a gang fight. He was stabbed several times and taken to the hospital by some friends who quickly disappeared.	C	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#12	M	36	Got into a bar fight.	C	Femoral	Vertex (close to scalp)	29.3	630.1	40.8	0.8	16.2	4.1	0.3	0.4	1.2
#13	M	27	Opposition to police intervention - resulted in homicide by firearm.	B	Central	Vertex (close to scalp)	ND	ND	ND	ND	2.3	0.3	0.3	0.1	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#14	M	24	Opposition to police intervention - resulted in homicide by firearm.	B	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#15	M	20	Prison fugitive who went on a “funk” party, felt sick, eventually passed out and died.	A	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#16	M	25	Opposition to police intervention - resulted in homicide by firearm.	B	Central	Vertex (close to scalp)	10.1	208.0	ND	ND	11.6	0.9	0.2	0.1	ND
#17	F	35	Opposition to police intervention - resulted in homicide by firearm.	B	Central	Vertex (close to scalp)	ND	ND	ND	ND	0.6	0.1	ND	ND	ND
#18	M	48	Jumped of a 10m height, possible suicide.	D	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#19	M	57	Suspect to use drugs. Felt sick and was sent to the hospital where eventually died a week later.	A	Central	Vertex (close to scalp)	ND	ND	ND	ND	5	1.4	1	0.2	0.6
#20	M	21	Drug abuser who supposedly had overdosed.	A	Cardiac	Vertex (close to scalp)	ND	1.3	ND	ND	7.1	0.6	0.4	0.3	ND
#21	F	32	Opposition to police intervention - resulted in homicide by firearm. Lived in *Crackland*.	B	Central	Vertex (close to scalp)	ND	ND	ND	ND	3.5	0.3	0.1	0.1	ND
#22	M	48	Known as a drug user. Felt sick on the street and died immediately.	A	Cardiac	Vertex (close to scalp)	>1000	>1000	3.1	418.8	11.2	1.1	0.2	1.3	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#23	M	18	Opposition to police intervention - resulted in homicide by firearm.	B	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#24	M	20	Opposition to police intervention - resulted in homicide by firearm.	B	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#25	M	19	Opposition to police intervention - resulted in homicide by firearm.	B	Central	Vertex (close to scalp)	ND	2.8	ND	ND	ND	ND	ND	ND	ND
#26	M	32	Possible suicide. (No more data available)	D	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#27	M	28	Got severely injured during a fight and was taken to the hospital where he remained for a week.	C	Cardiac	Vertex (close to scalp)	ND	24.9	ND	ND	14.1	1.6	1.3	0.6	ND
#28	M	68	Killed by his son after the two got into a fight at home.	C	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#29	M	25	Opposition to police intervention - resulted in homicide by firearm.	B	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#30	M	50	Found dead at his home sauna. He was a drug user for 10 years and had tried suicide before.	A	Central	Vertex (close to scalp)	440.9	>1000	1.9	3.1	9.4	1.8	ND	0.1	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#31	M	18	Opposition to police intervention - resulted in homicide by firearm.	B	Central	Vertex (close to scalp)	165.4	>1000	ND	10.9	>20	7.6	ND	2.2	1.8
#32	M	24	During a robbery, the individual suffered a fatal gunshot wound.	C	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#33	M	18	Dangerous motorcycle maneuver. Had been in prison for young offenders several times before.	C	Central	Vertex (close to scalp)	ND	ND	ND	ND	1.4	0.2	ND	ND	ND
#34	F	41	Individual lived in *Crackland* and possibly committed suicide by throwing herself from the bridge.	D	Central	Vertex (close to scalp)	>1000	>1000	>1000	60.9	>20	16.6	8.9	8.6	>20

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#35	M	48	Opposition to police intervention - resulted in homicide by firearm.	B	Central	Vertex (close to scalp)	88.2	288.6	33.8	1.9	17.8	3.6	1.1	0.4	0.9
#36	M	25	Opposition to police intervention - resulted in homicide by firearm.	B	Central	Vertex (close to scalp)	N	347.7	ND	ND	>20	3.9	0.8	4.4	3.7
#37	M	36	Belonged to a gang and while trying to rob a bank it got shot by the police.	C	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#38	M	42	He was a convict who fell off his wheelchair and died.	C	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#39	M	35	The individual had signs of fight and got stabbed to death possibly by a different gang.	C	Central	Vertex (close to scalp)	145.6	>1000	93.1	15.3	>20	11.8	3.4	6.8	3.9
#40	M	25	He was a convict who died after getting involved into a fight with other prisoners.	C	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#41	M	33	Possible suicide. Had tried suicide before.	D	Central	Vertex (close to scalp)	140.8	557.0	95.0	3.1	16.7	1.7	1.7	0.9	ND
#42	M	25	Dangerous maneuver at the dam. He jumped from a really high pier and died at the river.	C	Central	Vertex (close to scalp)	ND	8.8	ND	ND	ND	ND	ND	ND	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#43	M	28	Opposition to police intervention - resulted in homicide by firearm.	B	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#44	M	23	Gang fight, which resulted in several injuries including gunfight wounds.	C	Cardiac	Vertex (close to scalp)	11.9	284.9	1.7	0.9	>20	10.8	1.9	5.6	0.6
#45	M	25	Was using drugs when he jumped on top of a 5m wall. Lost his balance and fell off.	A	Central	Vertex (close to scalp)	25.9	385.3	7.9	0.5	9.8	1.1	0.8	0.2	ND
#46	M	24	Found with a rope hanging from the ceiling. Possible suicide.	D	Central	Vertex (close to scalp)	6.7	130.6	18.1	0.5	1.2	0.1	0.1	ND	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#47	M	31	Found on a wasteland with signs of gunshots. Supposedly a gang rivalry incident.	C	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#48	M	59	Jumped off the 9th floor of a building. Possible suicide.	D	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#49	M	46	Drug user was admitted to the hospital prior to death and with a history of 2 previous strokes. Hospital has declared: possible overdose.	A	Central	Vertex (close to scalp)	ND	ND	ND	ND	8.5	1.1	2.3	0.1	ND
#50	M	48	Alcohol addict. He was depressed and drunk himself to death.	D	Central (decomposed)	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#51	M	27	Drug user. Was found dead at home.	A	Central	Vertex (close to scalp)	79.7	730.3	21.2	3.4	12.1	1.5	0.5	1.4	ND
#52	M	40	Drug user. Felt sick on the street and died.	A	Central	Vertex (close to scalp)	6.1	4.4	ND	ND	5.4	3.4	0.7	0.1	ND
#53	M	20	Dangerous behavior during rush hour. Got killed by a car on a motorway. Supposedly under the influence of drugs.	C	Central	Vertex (close to scalp)	3.5	19.9	5.6	ND	0.7	0.1	0.1	ND	ND
#54	M	33	The individual was incarcerated when he tried to run away and got shot by the police.	B	Central	Vertex (close to scalp)	129.8	61.6	23.3	ND	3.5	2.8	0.2	0.2	ND
#55	M	50	Reacted to the robbery and got shot.	C	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#56	F	22	The individual was previously diagnosed with depression. She was feeling violent and with a desire "to do something". She threw herself on the train line. Possible suicide.	D	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#57	F	31	Found dead at home by the husband. Suspicious death.	C	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#58	M	19	The individual was riding a motorcycle when he got into a fight that eventually led to his death.	C	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#59	M	29	Drug abuse suspicion, possible overdose	A	Cardiac	Vertex (close to scalp)	1.9	12.5	ND	ND	4.0	0.3	ND	0.1	ND
#60	M	30	Found on an abandoned car. Possible suicide due to drug abuse.	A	Cardiac	Vertex (close to scalp)	ND	ND	ND	ND	1.3	0.2	ND	ND	ND
#61	M	28	Possible drug overdose. Had a history of violent behaviors.	A	Central	Vertex (close to scalp)	6.5	39.3	ND	ND	2.1	0.2	0.1	0.1	ND
#62	M	19	Found with an electric wire around his neck and a suicide message on his phone. Had a history of suicidal attempts.	D	Cardiac	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#63	M	23	Drug user. Felt sick on the street and died.	A	Cardiac	Vertex (close to scalp)	222.4	>1000	10.5	12.2	11.7	2.6	0.2	0.3	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#64	M	33	Possible suicide with rope around his neck.	D	Cardiac	Vertex (close to scalp)	16.2	108.4	17.4	ND	>20	1.5	2.0	0.3	0.9
#65	M	18	Opposition to police intervention - resulted in homicide by firearm.	B	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#66	M	24	The individual was seen using solvent-like drugs on the street when he simply fell onto the ground, dead.	A	Central	Vertex (close to scalp)	ND	ND	ND	ND	8.8	0.4	0.5	0.3	ND
#67	M	20	Opposition to police intervention - resulted in homicide by firearm.	B	Central	Vertex (close to scalp)	75.6	>1000	ND	9.9	>20	13.4	ND	6.6	10.6

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#68	M	43	Found dead at home with signs of fighting. Got stabbed several times. Had been in prison before.	C	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#69	M	20	Gang violence. Gunshots on the public highway.	C	Central	Vertex (close to scalp)	ND	ND	ND	ND	1.9	0.1	ND	ND	ND
#70	M	19	Gang violence. Gunshots on the public highway.	C	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#71	M	19	Gang violence. Gunshots on the public highway.	C	Central	Vertex (close to scalp)	ND	5.7	ND	ND	2.7	0.3	ND	ND	ND
#72	M	19	On a robbed car full of guns the individual died due to opposition to police intervention.	B	Central	Vertex (close to scalp)	45.6	>1000	77.1	7.1	7.5	0.9	0.5	0.7	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#73	M	23	Robbed the house of a policeman and hours later he was found dead.	C	Central	Vertex (close to scalp)	13.8	251.0	19.5	ND	13.9	1.5	0.8	0.3	ND
#74	M	25	Possible suicide. Threw himself at the train line.	D	Central	Vertex (close to scalp)	>1000	>1000	ND	29.9	2.0	0.5	ND	0.1	ND
#75	M	27	Reacted to the robbery and got shot. He was admitted to the hospital where he stayed for almost one month prior to his death	C	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#76	M	25	Opposition to police intervention - resulted in homicide by firearm.	B	Central	Vertex (close to scalp)	6.1	59.7	ND	ND	4.5	0.3	0.1	0.1	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#77	M	49	Possible suicide. Threw himself at the train line.	D	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#78	M	62	The individual had his genitalia ripped off. Possibly a known rapist.	C	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#79	M	25	Found dead on his home staircase with signs of fight injuries.	C	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#80	M	35	Known drug user who possibly committed suicide. He was found his bathroom floor with a bucket full of vomit and blood.	D	Central	Vertex (close to scalp)	ND	13.8	ND	ND	0.9	0.2	ND	ND	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#81	M	58	Individual found hanging from his kitchen ceiling. Possible suicide.	D	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#82	M	35	Found dead at home and was known to use drugs constantly.	A	Central	Vertex (close to scalp)	ND	5.1	ND	ND	11.9	0.9	1.8	0.3	ND
#83	M	22	Drug user who spent the weekend using several drugs until he passed out and died. Has been in prison before.	A	Central	Vertex (close to scalp)	180.9	681.2	4.9	19.2	2.8	0.3	0.1	0.1	ND
#84	M	54	Got home with his wife, both drunk. When she woke up he was hanging from the window with a rope around his neck. Possible suicide.	D	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#85	F	26	History of drug abuse. The individual was homeless and sought medical help due to having had a great amount of drug intake acutely with the intention of committing suicide.	D	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#86	M	51	Found at home with everything closed and a bucket of charcoal. Possible suicide.	D	Central	Vertex (close to scalp)	11.9	121.2	ND	ND	ND	ND	ND	ND	ND
#87	M	32	Felt sick and died instantly. History of drug abuse.	A	Central	Vertex (close to scalp)	13.1	85.6	ND	ND	2.1	0.2	0.7	ND	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#88	M	29	Dangerous motorcycle maneuver, which resulted in the death of the individual.	C	Central	Vertex (close to scalp)	10.9	98.6	ND	ND	ND	ND	ND	ND	ND
#89	M	18	Found floating on the river. Possible suicide.	D	Central	Vertex (close to scalp)	14.0	104.1	ND	ND	ND	ND	ND	ND	ND
#90	M	20	Known as a drug user and was seen taking drugs insatiably until he eventually passed out in the middle of the street dead.	A	Central	Vertex (close to scalp)	6.7	82.9	ND	ND	8.2	0.7	0.2	0.3	ND
#91	M	23	Drug user who had used too much. He went to the hospital to seek help but he did not make it and end up dying.	A	Central	Vertex (close to scalp)	14.4	105.4	3.4	ND	4.7	0.8	0.1	0.1	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#92	M	25	Opposition to police intervention - resulted in homicide by firearm.	B	Central	Vertex (close to scalp)	16.3	169.1	1.9	ND	>20	3.9	0.7	1.2	1.5
#93	M	53	He was a drug and alcohol user who had supposedly used drugs and drank so much that he felt over on the sidewalk.	A	Central	Vertex (close to scalp)	10.4	81.6	ND	ND	ND	ND	ND	ND	ND
#94	M	23	Opposition to police intervention - resulted in homicide by firearm.	B	Central	Vertex (close to scalp)	12.1	13.8	ND	ND	1.4	0.1	0.1	0.1	ND
#95	M	25	Opposition to police intervention - resulted in homicide by firearm.	B	Central	Vertex (close to scalp)	10.3	162.5	ND	ND	1.7	0.1	ND	ND	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#96	M	22	Found dead by hanging. Known to be a drug user. Possible suicide.	D	Central	Vertex (close to scalp)	53.6	470.2	61.0	ND	2.0	0.1	0.1	ND	ND
#97	M	25	Opposition to police intervention - resulted in homicide by firearm.	B	Central	Vertex (close to scalp)	17.6	102.6	ND	ND	ND	ND	ND	ND	ND
#98	M	44	Found hanging from a rope and had tried suicide multiple times before. History of having insanity and violent episodes supposedly due to drug abuse.	D	Central	Vertex (close to scalp)	ND	95.0	ND	ND	5.2	0.2	0.9	0.1	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#99	M	53	The individual was previously diagnosed with depression and had a history of alcohol abuse and he cut himself with a stiletto knife. Possible suicide.	D	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND
#100	M	23	Found hanging from the ceiling with an electric wire. Possible suicide.	D	Central	Vertex (close to scalp)	ND	ND	ND	ND	9.8	1.6	0.1	0.4	ND
#101	M	19	The individual went to rob the house of a policeman but he was shot in the chest before.	C	Central	Vertex (close to scalp)	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#102	M	37	Dangerous motorcycle maneuver, which resulted in the death of the individual. Supposedly under the effect of drugs.	C	Central	Vertex (close to scalp)	25.2	640.9	53.4	0.7	ND	ND	ND	ND	ND
#103	M	27	Found in the bathroom of his house using drugs when he was taken to the hospital where remained for a couple of days until he end up dying.	A	Central	Vertex (close to scalp)	ND	ND	ND	ND	6.0	0.2	0.7	0.1	ND

Table 13. *Continuing*

					Site of collection		Blood (ng/mL)				Hair (ng/mg)				
Individual	**Gender**	**Age**	**Circumstances of death**	**Category**	**Blood**	**Hair**	**COC**	**BZE**	**CE**	**NCOC**	**COC**	**BZE**	**CE**	**NCOC**	**AEME**
#104	M	44	Dangerous motorcycle maneuver, which resulted in the death of the individual. Supposedly under the effect of drugs.	C	Central	Vertex (close to scalp)	82.9	452.8	72.7	3.9	ND	ND	ND	ND	ND
#105	M	31	The individual went swimming on the dam under the effect of drugs and alcohol. He was taken to the hospital with several injuries and later came back home where he continued to use drugs until he was found having a convulsion and finally dying.	A	Central	Vertex (close to scalp)	ND	23.9	ND	ND	>20	19.1	2.5	5.4	3.1

5.4. Genetic testing

5.4.1. General demographic characteristics

Table 14 gives details on the general demographic characteristics of the individuals from whom both hair and blood matrices were collected under this study. For all genetic analyses, both chronic and recent users were considered as the general category: cocaine users.

Table 14. General demographic characteristics of the individuals under study, containing details such as: drug use (cocaine user or non-user); age; gender; ethnicity (white or non-white); weight; height and BMI.

General characteristics of the individuals under study	
Cocaine user (%)	61.9
Age (years)	32 ± 12
Gender, male (%)	92.4
Ethnicity, white (%)	42.1
Weight (kg)	73.2 ± 7.6
Height (m)	1.7 ± 0.06
BMI (kg/m)	24.8 ± 2.6

Table 15 also gives general details on the individuals under study, however, separated by groups of cocaine users and non-users.

Table 15. General demographic characteristics of the individuals under study (divided between cocaine users and non-users), containing details such as: age; gender (male or female); ethnicity (white or non-white); weight; height and BMI.

General characteristics of the individuals under study			
	Cocaine user (*n*=65)	Non-user (*n*=40)	*p*-value
Age (years)	30 ± 10	34 ± 14	0.32
Gender, male (%)	93.8	90.0	0.48
Ethnicity, white (%)	36.4	50.0	0.30
Weight (kg)	72.6 ± 6.4	74.3 ± 9.6	0.72
Height (m)	1.7 ± 0.06	1.7 ± 0.07	0.19
BMI (kg/m)	24.2 ± 2.4	25.6 ± 2.8	0.10

According to **Table 15**, it can be seen that the *p*-value for each variable (both numerical and categorical) was not statistically different across both groups (users and non-users) as all *p*-values obtained were above 0.05.

Due to the no-amplification of some samples during genotyping, the sample size (*n*=105) was slightly reduced for all SNPS. The total number of samples accessed was: 97 for rs1803274 and rs4263329 from the *BCHE* gene, followed by 95 for both rs2283265 and rs6280 from the *DRD2* and *DRD3* genes, respectively and finally 90 for rs4680 from the *COMT* gene.

5.4.2. Hardy-Weinberg equilibrium

When performing analysis of SNPs, one of the first parameters to test for is the Hardy-Weinberg equilibrium (HWE). Essentially, this principle is based on the assumption that genetic variation within a population will remain constant from one generation to the next when there are no disturbing factors involved (MEIRMANS, 2018).

However, this equilibrium may be disrupted due to several factors such as mutations, natural selection, non-random mating, genetic drift and gene flow (ROHLFS; WEIR, 2008; MEIRMANS, 2018). To test for HWE, a chi-square (X^2) test was used and if $p < 0.05$ then, the genotype distribution was inconsistent with HWE (see **Table 16**).

Table 16. Statistical results for the Hardy-Weinberg equilibrium with the respective X^2 and *p*-values for all SNPs under study. Significant *p*-values were highlighted in bold.

Hardy-Weinberg equilibrium				
BCHE		***COMT***	***DRD2***	***DRD3***
rs1803274	rs4263329	rs4680	rs2283265	rs6280
$X^2 = 1.09$	$X^2 = 0.05$	$X^2 = 7.96$	$X^2 = 0.97$	$X^2 = 1.25$
$p = 0.30$	$p = 0.82$	$p =$ **0.0048**	$p = 0.32$	$p = 0.26$

According to the results, all SNPs were consistent with HWE (*p*-value > 0.05), except for rs4680 from the *COMT* gene with a *p*-value of 0.0048.

The minor allele frequency (MAF) can be seen in **Table 17** and the genotype distribution frequencies in **Table 18**.

Table 17. Statistical results showing the MAF for all SNPs under study.

MAF (%)				
BCHE		***COMT***	***DRD2***	***DRD3***
rs1803274 Allele T = 21	rs4263329 Allele G = 15	rs4680 Allele A = 37	rs2283265 Allele A = 22	rs6280 Allele C = 45

Table 18. Statistical results showing the genotypic distribution frequencies for all SNPs under study and within the overall population studied.

Genotypic distribution across all individuals (%)				
BCHE		***COMT***	***DRD2***	***DRD3***
rs1803274	rs4263329	rs4680	rs2283265	rs6280
C/C = 64.9 (*n* = 63)	A/A = 71.1 (*n* = 69)	G/G = 46.7 (*n* = 42)	C/C = 63.2 (*n* = 60)	T/T = 27.4 (*n* = 26)
C/T = 28.9 (*n* = 28)	A/G = 26.8 (*n* = 26)	G/A = 32.2 (*n* = 29)	C/A = 30.5 (*n* = 29)	T/C = 54.7 (*n* = 52)
T/T = 6.2 (*n* = 6)	G/G = 2.1 (*n* = 2)	A/A = 21.1 (*n* = 19)	A/A = 6.3 (*n* = 6)	C/C = 17.9 (*n* = 17)

5.4.3. Analysis of the relevant SNPs

For all genes studied and their relevant SNPs, genotypes have been grouped in three different models of dominance (additive; recessive and dominant) according to their allele of lowest frequency. For all dominance models, X^2 tests have been completed and the respective *p*-values obtained.

As for both dominant and recessive models, logistic regression analyses have also been performed in order to account for any influence of given co-variables. In addition, the resulting *p*-values, odds ratio (OR) and 95% confidence intervals (CI) were also acquired.

5.4.3.1. Genotype distribution for BCHE SNPs between cocaine-users and non-users

For the *BCHE* gene, both SNPs rs1803274 and rs4263329 have been genotyped and all three dominance models tested can be seen in **Table 19** and **Table 20**, respectively.

Table 19. Statistical results showing the genotypic distribution frequencies for rs1803274 from the *BCHE* gene in all three dominance models (additive; dominant and recessive).

		Genotypic frequency (%)		
***BCHE* rs1803274**		**Cocaine-users**	**Non-users**	***p*-value**
Additive model	C/C	66.7	62.2	0.82
	C/T	26.7	32.4	
	T/T	6.7	5.4	
Dominant model	C/C	66.7	62.2	0.65
	C/T + T/T	33.3	37.8	
Recessive model	C/C + C/T	93.3	94.6	1.00
	T/T	6.7	5.4	

Table 20. Statistical results showing the genotypic distribution frequencies for rs4263329 from the *BCHE* gene in all three dominance models (additive; dominant and recessive). Significant *p*-values were highlighted in bold.

		Genotypic frequency (%)		
***BCHE* rs4263329**		**Cocaine-users**	**Non-users**	***p*-value**
Additive model	A/A	63.3	83.8	0.06
	A/G	33.3	16.2	
	G/G	3.3	0.0	
Dominant model	A/A	63.3	83.8	**0.03**
	A/G + GG	36.7	16.2	
Recessive model	A/A + A/G	96.7	100.0	0.52
	G/G	3.3	0.0	

As it can be observed in the two previous tables, SNP rs1803274 did not show any statistically significant difference between cocaine users and non-users in either model tested. However, SNP rs4263329 has given a *p*-value of 0.03 within the dominant model where it shows a higher frequency of genotypes A/G + G/G within cases (cocaine users) rather than controls (non-users) with values of 36.7% against 16.2%, respectively.

The same trend has also been observed in the additive model, where there is a higher frequency of both genotypes A/G and G/G in the cases with values of 33.3% and 3.3%, respectively, versus controls where frequencies of 16.2% and 0% have been found, respectively. On the other hand, genotype A/A showed a lower value in cases (63.3%) than in controls (83.3%).

Nevertheless, a significant *p*-value has not been obtained within the additive model, possibly due to the lack of the G/G genotype within non-users. Still, the value obtained may be considered border line ($p = 0.06$).

This finding is actually contrasting with the results found in the study conducted by Negrão and colleagues where they have stated that after adopting a recessive model, the G/G genotype of rs4263329 was less common in cases than in controls (f(G/G) = 1.2% vs. f(G/G) = 2.6%; OR 2.3; 95% CI = 0.99 – 5.32) (NEGRÃO et al., 2013).

5.4.3.2. Genotype distribution for COMT SNP between cocaine-users and non-users

For the *COMT* gene, SNP rs4680 has been genotyped and all three dominance models tested can be seen in **Table 21**.

Table 21. Statistical results showing the genotypic distribution frequencies for rs4680 from the *COMT* gene in all three dominance models (additive; dominant and recessive).

		Genotypic frequency (%)		
***COMT* rs4680**		**Cocaine-users**	**Non-users**	***p*-value**
Additive model	A/A	21.4	20.6	0.62
	A/G	28.6	38.2	
	G/G	50.0	41.2	
Dominant mode	G/G	50.0	41.2	0.42
	G/A+A/A	50.0	58.8	
Recessive model	G/G + G/A	78.6	79.4	0.94
	A/A	21.4	20.6	

According to the results found, the SNP rs4680 did not give any statistical difference when cases were compared to controls. Lohoff and colleagues, on the other hand, did find a significant association within the same SNP (rs4680) where they have reported that a higher frequency of the G allele was seen in cocaine users rather than controls (p = 0.004; corrected p = 0.014) (LOHOFF et al., 2008). This non-concordance between studies may be explained by population differences.

5.4.3.3. Genotype distribution for DRD2 SNP between cocaine-users and non-users

The SNP rs2283265 from the *DRD2* has also been genotyped and all three dominance models tested can be seen in **Table 22**.

Table 22. Statistical results showing the genotypic distribution frequencies for rs2283265 from the *DRD2* gene in all three dominance models (additive; dominant and recessive).

		Genotypic frequency (%)		
***DRD2* rs2283265**		**Cocaine-users**	**Non-users**	***p*-value**
Additive model	A/A	6.7	5.7	0.50
	A/C	26.7	37.1	
	C/C	66.7	57.1	
Dominant mode	C/C	66.7	57.1	0.36
	C/A+A/A	33.3	42.9	
Recessive model	C/C+C/A	93.3	94.3	1.00
	A/A	6.7	5.7	

For all models tested, SNP rs2283265 did not show any statistically significant *p*-value when comparing cases to controls. However, in 2011, Moyer et al. did find an association between both *DRD2* SNPs rs1076560 (SNP1) and rs2283265 (SNP2) with cocaine abuse with MAF values of approximately twofold higher in cocaine abusers than in controls (SNP1 OR = 1.94; 95% CI = 1.07 – 3.50; $p = 0.03$ and SNP2 OR = 2.27; 95% CI = 1.23 – 4.19; $p = 0.01$), however, this has only remained significant in Caucasians once the data has been divided due to the low MAF found in African Americans (MOYER et al., 2011).

Later, in 2013, Spellicy and colleagues have also looked at rs2283265 in order to see whether there was an interaction between the SNP and response to treatment with disulfiram – a potential cocaine addiction pharmacotherapy. They did find a significant association between the G/T and T/T genotypes with a notorious decrease in the number of cocaine-positive urine samples on disulfiram ($p \leq 0.0001$) against those with the GG genotype, which have only shown a marginal decrease ($p = 0.04$). The authors stated that their data may help identify individuals for whom disulfiram maybe an effective pharmacotherapty against cocaine dependence (SPELLICY et al., 2013).

5.4.3.4. Genotype distribution for DRD3 SNP between cocaine-users and non-users

For the *DRD3* gene, SNP rs6280 has been genotyped and all three dominance models tested can be seen in **Table 23**.

Table 23. Statistical results showing the genotypic distribution frequencies for rs2283265 from the *DRD3* gene in all three dominance models (additive; dominant and recessive). Significant *p*-values were highlighted in bold.

		Genotypic frequency (%)		
***DRD3* rs6280**		**Cocaine-users**	**Non-users**	***p*-value**
Additive model	T/T	18.6	41.7	**0.04**
	T/C	62.7	41.7	
	C/C	18.6	16.7	
Dominant mode	T/T	18.6	41.7	**0.01**
	T/C+C/C	81.4	58.3	
Recessive model	T/T+T/C	81.4	83.3	0.81
	C/C	18.6	16.7	

As shown in **Table 23**, the SNP rs6280 has given two significant *p*-values, for both additive and dominant models with values of 0.04 and 0.01, respectively. The same has not been observed for the recessive model (*p*-value of 0.81).

In the additive model, it can be seen that the frequency of genotypes T/C and C/C increase from non-users to cocaine users from 41.7% to 62.7% and 16.7% to 18.6%, respectively. When looking at the dominant mode, a similar trend was observed as the genotypes T/C + C/C present an increase of 58.3% to 81.4% from non-users to cocaine-users.

In contrast, T/T genotype has had a decrease in frequency from controls (41.7%) to cases (18.6%).

5.4.3.5. General demographic characteristics divided by genotype within statistically significant SNPs

Both numerical and categorical characteristics have been plotted against the dominant model for the SNPs that presented statistically significant results: rs4263329 from the *BCHE* gene and rs6280 from the *DRD3* gene. The results can be seen in **Table 24**.

Table 24. General characteristics of the individuals under study (divided according to each statistically significant SNP found in its dominant model), containing details such as: age; gender (male or female); ethnicity (white or non-white); weight; height and BMI. Significant *p*-values were highlighted in bold.

General characteristics of the individuals under study						
	***BCHE* rs4263329**			***DRD3* rs6280**		
	A/A	A/G + G/G	*p*-value	T/T	T/C + C/C	*p*-value
Age (years)	32 ± 13	29 ± 10	0.43	34 ± 14	30 ± 11	0.27
Gender, male (%)	89.9	100.0	0.19	96.2	91.3	0.39
Ethnicity, white (%)	44.4	37.5	0.87	76.9	31.6	**0.008**
Weight (kg)	73.4 ± 8.1	73.5 ± 6.3	1.00	74.6 ± 9.0	72.9 ± 6.9	0.55
Height (m)	1.7 ± 0.07	1.7 ± 0.04	0.42	1.7 ± 0.07	1.7 ± 0.06	0.96
BMI (kg/m)	24.7 ± 2.4	25.1 ± 2.7	0.66	25.3 ± 2.8	24.7 ± 2.3	0.51

As it can be seen in **Table 24**, there was only one characteristic that stood out to be statistically different and that was ethnicity within the genotype T/C + C/C against genotype T/T for SNP rs6280 from the *DRD3*. With a *p*-value of 0.008, ethnicity has shown that when comparing the genotypes within the dominant level for rs6280, there are significantly more Non-white individuals contained within genotypes T/C + C/C.

This result may be showing that a higher proportion of Non-white individuals contain the C allele, which may be conferring a higher risk for cocaine abuse according to the models observed previously where the dominant model has shown that there was a significant higher proportion of T/C + C/C genotypes within cocaine-users (see **Table 23**). Previously, Lohoff et al. have found an association between

the Val^{158}Met polymorphism and cocaine dependence in individuals of African descent (LOHOFF et al., 2008).

In fact, in order to take into account the different variables within the study and their possible influences towards the results, analysis have been adjusted using logistic regression.

5.4.3.6. Logistic regression for all SNPs under study

As mentioned previously, some co-variables within the study may be influencing the results, either where no association was found or where a possible association was observed. For this purpose, the co-variables included were those who could possibly have an impact on the condition being studied: cocaine abuse, and those were: gender, age and ethnicity.

In fact, starting with gender, this parameters does seem to play a role in cocaine abuse as according to the latest UNODC report, men are three times more likely than women to use cannabis, cocaine or amphetamines (UNODC, 2017). Abdalla and colleagues have also reported that in within the whole Brazilian population, which has used any form of cocaine in the prior year, 3.7% were men and only 0.7% were women. The results found for cocaine use in a lifetime were also similar as 7.4% of the individuals were male and only 2.1% were female (ABDALLA et al., 2014).

These finds were then corroborated by Riley, Hempel and Clasen (RILEY; HEMPEL; CLASEN, 2017) and also McHugh and colleagues (MCHUGH et al., 2017). Back in 2008, also Najavits and Lester have found that women, compared to men, had less severe lifetime substance use problems (NAJAVITS; LESTER, 2009).

As for the age parameter, ABDALLA and colleagues described that the mean age of onset for cocaine use within a Brazilian population was of 18.8 years old (ABDALLA et al., 2014). This year (2018), another Brazilian study based on telephone counselling for young cocaine users found that the mean age of the participants was 22.1 years old among young individuals and 17.3 years old among adolescents (BISCH et al., 2018).

Finally, ethnicity wise, a report by the National Institute on Drug Abuse (NIDA) from the United States has reported that data on past-month use of cocaine indicated African Americans and Hispanics have a slightly higher prevalence of use compared with that for whites (NIDA, 1998). Later, in 2010, Wu et al have stated that race/ethnicity is not associated with cocaine dependence (WU et al., 2010).

Bernstein and colleagues have also looked at this association and they have found that Whites had more frequent heroin emergency department (ED) visits than Hispanics and Blacks, while Blacks had more frequent cocaine related ED visits than Whites and Hispanics (BERNSTEIN et al., 2005). The findings are controversial and they simply represent the need to correct for these parameters and therefore using logistic regression.

Starting with the SNPs that did not present any significant association to cocaine abuse, when logistic regression has been applied to both dominant and recessive models in the rs1803274 from the *BCHE* gene, still no association has been found. The C/T + T/T genotypes (OR = 2.21; 95% CI = 0.48 – 10.12; p = 0.31) and the T/T genotype (OR = 0.89; 95% CI = 0.13 – 6.24; p-value = 0.91) remained insignificant.

For the SNP rs4680 from the *COMT* gene, when applying logistic regression to the dominant model with the genotypes G/A + A/A (OR = 0.59; CI 95% = 0.17 – 1.99; p = 0.39) and the recessive model with the genotype A/A (OR = 0.72; 95% CI = 0.27 – 3.83; p = 0.70), no significant association has been found. Finally, for SNP rs2283265 for the *DRD2* gene, both the genotypes C/A + A/A within the dominant model (OR = 0.98; 95% CI = 0.27 – 3.55; p = 0.98) and genotype A/A in the recessive model (OR = $6E^{+08}$; 95% CI = not calculated; p = 0.99) also remained insignificant for the phenotype of cocaine use.

The logistic regression for the significant SNPs can be seen in both **Table 25** and **Table 26** for SNP rs4263329 and rs6280, respectively.

Table 25. Logistic regression for the dominant model of SNP rs4263329 from the *BCHE* gene taking into account the co-variables of gender; age and ethnicity. Significant *p*-values were highlighted in bold.

Logistic Regression			
Variables	**OR**	**95% CI**	***p*-value**
Genotypes A/G + G/G for *BCHE* (rs4263329)	8.91	1.58 - 50.21	**0.01**
Gender (man)	0.98	0.11 - 8.35	0.98
Age	1.06	0.99 - 1.14	0.10
Ethnicity (white)	1.41	0.37 - 5.34	0.61

After logistic regression, the *p*-value for the dominant model (A/G + G/G) remained significant, meaning that the co-variables evaluated did not affect this association and that individuals with the genotypes tested (A/G + G/G) have an estimated increased risk of 8.91 times of using cocaine. In this case, the regressive model has also been tested in order to ensure it persisted with no significance.

For the regressive model tested (G/G genotype), logistic regression was unable to calculate OR; 95% CI and *p*-value for the genotype as there were no non-users containing the G/G genotype individuals. However, the remaining co-variables were found not to be relevant ($p > 0.05$).

Table 26. Logistic regression for the dominant model of SNP rs6280 from the *DRD3* gene taking into account the co-variables of gender; age and ethnicity. Significant *p*-values were highlighted in bold.

Logistic Regression			
Variables	**OR**	**95% CI**	***p*-value**
Genotypes T/C + C/C for *DRD3* (rs6280)	4.96	1.07 - 23.02	**0.04**
Gender (man)	2.39	0.29 - 19.75	0.42
Age	1.05	0.98 - 1.13	0.15
Ethnicity (white)	0.83	0.20 - 3.44	0.79

According to **Table 26**, the dominant model (T/C + C/C) for rs6280 is still statistically significant after correction for the co-variables, meaning that even after logistic regression, the association remains meaningful and its OR is suggesting that individuals with the genotypes tested (T/C + C/C) have an estimated increased risk of 4.96 times of using cocaine (95% CI = 1.07 – 23.02; p = 0.04). Again, also for this case, logistic regression of the recessive model has been tested.

For the T/T genotype (recessive model), the results kept insignificant after correction through logistic regression (OR = 0.99; 95% CI = 0.19 – 5.29; $p = 0.99$).

As it can be seen, both associations found within this study remained significant after adjustment for co-variables. The same was not true for Negrão and colleagues who have also found a nominal difference for rs4263329 between cases and controls

but after adjustment for age and sex, that association was no longer significant (NEGRÃO et al., 2013).

In regards to rs6280, both additive and dominant models (**Table 23**) suggest that the C allele may be linked to a higher risk of cocaine use and even after logistic regression, this relationship remained significant (**Table 26**). This finding is consistent with the results achieved by Verdejo-Garcia et al., who have previously looked at this SNP in relation to its impact on ventral striatal and amygdala volumes and they did find a significant genotype x group interaction ($p < 0.05$) in both the left ventral striatum and right amygdala where cocaine dependent individuals carrying the C allele presented larger ventral striatal volumes than controls (VERDEJO-GARCIA et al., 2013).

In fact, previous studies have also looked at striatal dysmorphology in patients with chronic cocaine dependence and concluded that, indeed, striatal structures are enlarged in cocaine-dependent individuals (JACOBSEN et al., 2001; ERSCHE et al., 2011).

Following additional findings from Negrão and colleagues, the research group has discovered an association between rs1803274 and *crack* cocaine (A/A genotype) as the preferred route of administration ($p < 0.005$) (NEGRÃO et al., 2013). In order to test whether that same SNP would have a similar influence within this study, that relationship has also been looked at.

5.4.3.7. Cocaine HCl vs crack-cocaine use

All SNPs under study have been tested for the relationship between *crack* cocaine and cocaine hydrochloride (HCl) use and only one has shown to be significant: rs4263329 from the *BHCE* gene. In the following **Table 27**, the genotype distribution among *crack*-cocaine, cocaine HCl-users and non-users can be seen.

Table 27. Statistical results showing the genotypic distribution frequencies for rs4263329 from the *BCHE* gene in both additive and dominant models. Significant *p*-values were highlighted in bold.

		Genotypic frequency (%)			
***BCHE* rs4263329**		***Crack*-cocaine users**	**Cocaine HCl-users**	**Non-users**	***p*-value**
Additive model	A/A	92.3	55.3	83.8	**0.01**
	A/G	7.7	40.4	16.2	
	G/G	0.0	4.3	0.0	
Dominant model	A/A	92.3	55.3	83.8	**0.003**
	A/G+G/G	7.7	44.7	16.2	

Both additive and dominant models appear to have shown significant *p*-values of 0.01 and 0.003, respectively. According to the genotypic frequencies, individuals with the G allele, seem to have a preference for cocaine HCl.

According to **Table 27**, individuals who have used cocaine HCl have higher frequencies of the genotypes A/G + G/G (in the dominant model) when compared to *crack*-cocaine users or non-users with values of 44.7%, against 7.7% and 16.2%, respectively, and a *p*-value of 0.003.

5.4.4. Limitations and strengths

The genetic approach applied in this study is known as a CG and it comprises of several drawbacks. While GWAS have been particularly successful in the identification of genetic contributors to a number of complex human traits due to the fact that they comprise of “agnostic” studies with no prior hypothesis being drawn, candidate-gene approaches on the other hand, are completely hypothesis-driven (JONES; COMER, 2015).

For the CG approach to be effective it largely depends upon the correct choice of what genes/pathways to study and that *a priori* hypothesis on the biological functions is highly susceptible to the risk of arbitrariness. In fact, the optimum scenario is to perform a GWAS study followed by the CG approach to identify the actual causative variants within the few convincingly demonstrated associations found. Unfortunately, the GWAS approach is extremely costly (WILKENING et al., 2009).

Nonetheless, CG may represent a few advantages as it offers extra power over GWAS when dealing with smaller studies given the fact that when thousands of samples are analysed, weak genetic effects may not be detected (AMOS; DRISCOLL; HOFFMAN, 2011). In addition, CG studies do allow for a rather focused choice of SNPs, including rare ones and polymorphisms with known function (WILKENING et al., 2009).

The second limitation of this work is its small sample size. Even though CG studies do not require such large sample sizes as in GWAS, a fairly large sample size is still critical to the success of genetic association studies (HONG; PARK, 2012).

Indeed, small sample sizes have previously been indicated as a source of non-replication among studies due to the lack of its statistical power (PATNALA; CLEMENTS; BATRA, 2013).

However, the fact that only violent individuals have been considered for this work, and not the general population, may have contributed in conferring certain homogeneity to the study and perhaps compensating for the small sample size (n=105). In fact, heterogeneity has been cited as a major source of struggles when dealing with genetic studies of complex traits (THOMPSON et al., 2006).

Also, both case and control groups were extremely balanced with 53% cocaine-users *versus* 48% non-users. Indeed, for studies of association, the optimal condition consists of equal numbers of cases and controls (JANES; PEPE, 2006).

Another major concern, which must be taken into account when analysing the data, is population stratification. This phenomenon is an omnipresent threat to the validity of genetic association studies and it arises in the presence of unnoticed population structure where study samples comprise of sets of individuals that vary systematically in both their ancestry and phenotype being investigated. Therefore, false-positive associations may arise because instead of identifying a true association to disease phenotypes, this can be fully explained simply by differences in ancestry (UITTERLINDEN; ZILLIKENS; RIVADENEIRA, 2013).

Additionally, the influence of given external factors such as social economic status (SMART; MURRAY, 1983), location of residence (WARNER et al., 1995), substance availability (MACCOUN; REUTER, 1997), peer groups (LUTHAR et al., 1992) and family factors while growing up (NEEDLE; SU; DOHERTY, 1990) deeply contribute to drug use. In fact, several twin studies have suggested that both genetic and

environmental influences are involved in illicit drug use/dependence (GROVE et al., 1990; PICKENS et al., 1991; TSUANG et al., 1996).

Regarding drug use and its dependence as two separate factors, in the year of 1998, van den Bree et al., discovered that, except for sedatives and opiates, heritability estimates were greater for drug abuse/dependence than for drug use, whereas environmental factors contributed more to drug use (VAN DEN BREE et al., 1998).

Lastly, any findings resulting from CG approaches have to be carefully reported and no definite assumptions must be made. A certain SNP which has been associated to a given phenotypic trait using CG studies, may actually not be involved itself with the trait but simply in linkage disequilibrium with a true functional SNP (YANG, 2012).

While there are some authors that consider CG studies to be out-dated, out of-touch and futile (BORDER; KELLER, 2017), others keep defending its importance and even add that the most popular candidate polymorphisms remain the most commonly studied, yet they do advise that a healthy dose of caution must be given to the assertions being made from CG studies (MOORE, 2017).

Conclusions

6. Conclusions

To conclude, it can be said that a rather high proportion of cocaine-users has been found among cases of violent death arriving at IML-SP and both dilute-and-shoot methods have shown to be very efficient for the detection and quantitation of cocaine and its derivatives in both *postmortem* blood and head hair samples from those same individuals.

Genetic testing has yielded three statistically significant associations. Within rs4263329 from the *BCHE* gene, both genotypes A/G and G/G were frequently higher in cases than controls as well as in cocaine HCl users rather than *crack*-cocaine users and non-users.

Finally, rs6280 from the *DRD3* gene has also shown a statistically significant association in both its additive and dominant model with genotypes T/C and C/C being more frequently present in cocaine users rather than non-users.

These findings can only give a suggestion that these SNPs may be involved in cocaine use. However, at this time, it is not possible to infer any truthful association as further testing such as functional genomic studies are required, together with a larger, more controlled, sample size.

Nonetheless, we believe that every single step towards the field of toxicogenetics is an important milestone and must not be discarded; on the contrary, every attempt contributing to the prevention of drug abuse in the near future is valid.

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Attachments

Attachment I

Approval number 1.613.511 by the Ethics Committee from the School of Pharmaceutical Sciences of the University of São Paulo

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Relação entre morte violenta, uso de cocaína/crack e polimorfismos de genes associados ao sistema monoaminérgico

Pesquisador: Mauricio Yonamine

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);

Versão: 3

CAAE: 45524815.0.0000.0067

Instituição Proponente: Faculdade de Ciências Farmacêuticas da Universidade de São Paulo

Patrocinador Principal: FUND COORD DE APERFEICOAMENTO DE PESSOAL DE NIVEL SUP

DADOS DO PARECER

Número do Parecer: 1.613.511

Apresentação do Projeto:

A Organização Mundial da Saúde (2002) definiu a violência como "o uso intencional da força física ou do poder, real ou em ameaça, contra si mesmo, outra pessoa, ou contra um grupo ou comunidade, que resulte ou tenha possibilidade de resultar em lesão, morte, dano psicológico, mau desenvolvimento ou privação". A violência é ainda um dos grandes problemas presentes no Brasil e dois fatores que poderiam estar associados a tal fato são: o uso de drogas e a predisposição genética. Atualmente, a cocaína apresenta um importante papel nesse cenário. Diversos estudos verificaram que o consumo dessa droga está diretamente correlacionado com a maior incidência de surtos psicóticos e violência. A questão da genética poderia explicar a propensão que certos indivíduos possuem para adotarem uma atitude violenta. A população mundial varie entre si em apenas 1% da sua composição genética. No entanto, isso é suficiente para representar variações entre os indivíduos. Estas pequenas alterações do genoma são conhecidas como polimorfismos. Portanto, uma relação entre a violência e o uso de cocaína, assim como a violência e alguns polimorfismos do genoma humano podem ser correlacionados. O objetivo deste projeto é explorar a possível relação entre a violência, o uso de cocaína e os polimorfismos. Para

Endereço: Av. Prof. Lineu Prestes, 580, Bloco 13A, sala 112
Bairro: Butantã **CEP:** 05.508-000
UF: SP **Município:** SAO PAULO
Telefone: (11)3091-3622 **Fax:** (11)3031-8986 **E-mail:** cepfcf@usp.br

isso, análises serão realizadas em espécimes biológicos postmortem (sangue e cabelo) coletados de vítimas atendidas pelo Instituto Médico Legal de São Paulo (IML-SP), para se verificar a exposição recente ou crônica de cocaína. Os exames genéticos serão realizados através da técnica de reação em cadeia da polimerase em tempo real (qRTPCR) para os olimorfismos que segundo a literatura poderiam ter relação com a violência e/ou propensão ao uso de cocaína: DAT1, DRD2 e DBH. Deste modo, este projeto visa o estudo de três fatores que poderiam estar inter-relacionados: violência, uso de cocaína e polimorfismos de certos genes.

Objetivo da Pesquisa:

O objetivo do projeto é investigar se existe uma predisposição genética associada ao uso de cocaína/crack e uma morte violenta, ou seja, quanto o fator genético de cada indivíduo é capaz de influenciar a violência e o uso de cocaína/crack.

Avaliação dos Riscos e Benefícios:

O texto está adequado indicando riscos mínimos e benefícios relacionados a relação do uso de drogas ilícitas com mortes violentas.

Comentários e Considerações sobre a Pesquisa:

O tema é bastante interessante e importante para relacionar casos de morte por violência e o uso de drogas como crack que estão, infelizmente, disseminadas em nossa sociedade. A pesquisa é bem embasada na literatura científica da área e tem seus objetivos e metodologias bem definidos

Considerações sobre os Termos de apresentação obrigatória:

No TCLE, feito sob forma de convite aos familiares da vítima, é claro e consta que não haverá benefícios diretos, mas o estudo será importante para se tentar estabelecer as causas do uso de drogas e morte precoce. Também foi informado no TCLE o risco mínimo. Todos documentos necessários estão apresentados.

Recomendações:

Não ha recomendações.

Conclusões ou Pendências e Lista de Inadequações:

Aprovado.

Considerações Finais a critério do CEP:

Este CEP entende que a emenda ao projeto de pesquisa pode ser considerada aprovada.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_711896_E2.pdf	05/05/2016 13:21:59		Aceito
Outros	Rodrigo.pdf	05/05/2016 13:20:10	Mauricio Yonamine	Aceito
Outros	Carta.pdf	05/05/2016 13:19:43	Mauricio Yonamine	Aceito
Folha de Rosto	Folha de rosto.pdf	26/05/2015 07:40:03		Aceito
Projeto Detalhado / Brochura Investigador	Projeto_AnaMiguel .pdf	22/05/2015 14:42:20		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.pdf	22/05/2015 14:41:58		Aceito
Outros	Declaração_de_anuência_Ivan.pdf	15/05/2015 18:32:57		Aceito
Outros	Declaração_de_participação_Ivan.pdf	15/05/2015 18:32:49		Aceito
Outros	Declaração_Mario.pdf	15/05/2015 18:32:40		Aceito
Outros	Declaração_Rosario.pdf	15/05/2015 18:32:33		Aceito
Outros	Declaração_AnaMiguel.pdf	15/05/2015 18:32:23		Aceito
Outros	Declaração_de_anuência_Daniel..pdf	15/05/2015 18:32:12		Aceito
Outros	Declaração Sandra.pdf	15/05/2015 18:10:10		Aceito
Outros	Declaração_de_participação_Vilma.pdf	15/05/2015 17:45:33		Aceito
Outros	Descrição_da_equipe.pdf	24/04/2015 15:32:31		Aceito
Outros	Descrição dos itens_orçamento.pdf	24/04/2015 15:31:46		Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

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UF: SP **Município:** SAO PAULO
Telefone: (11)3091-3622 **Fax:** (11)3031-8986 **E-mail:** cepfcf@usp.br

SAO PAULO, 29 de Junho de 2016

Assinado por:
Cristina Northfleet de Albuquerque
(Coordenador)

Endereço: Av. Prof. Lineu Prestes, 580, Bloco 13A, sala 112
Bairro: Butantã **CEP:** 05.508-000
UF: SP **Município:** SAO PAULO
Telefone: (11)3091-3622 **Fax:** (11)3031-8986 **E-mail:** cepfcf@usp.br

Attachment II

Approval number 1.671.423 by the Ethics Committee from the School of Medicine of the University of São Paulo

FACULDADE DE MEDICINA DA UNIVERSIDADE DE SÃO PAULO - FMUSP

PARECER CONSUBSTANCIADO DO CEP

Elaborado pela Instituição Coparticipante

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Relação entre morte violenta, uso de cocaína/crack e polimorfismos de genes associados ao sistema monoaminérgico

Pesquisador: Mauricio Yonamine

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);

Versão: 2

CAAE: 45524815.0.3001.0065

Instituição Proponente: Faculdade de Ciências Farmacêuticas da Universidade de São Paulo

Patrocinador Principal: FUND COORD DE APERFEICOAMENTO DE PESSOAL DE NIVEL SUP

DADOS DO PARECER

Número do Parecer: 1.671.423

Apresentação do Projeto:

A violência é um dos grandes problemas presentes no Brasil fatores como uso de drogas e a predisposição genética podem estar a ela relacionados. Uma relação entre a violência e o uso de cocaína, assim como a violência e alguns polimorfismos do genoma humano podem ser correlacionados. O objetivo deste projeto é explorar a possível relação entre a violência, o uso de cocaína e os polimorfismos. Para isso, análises serão realizadas em espécimes biológicos postmortem (sangue e cabelo) coletados de vítimas atendidas pelo Instituto Médico Legal de São Paulo (IML-SP), para se verificar a exposição recente ou crônica de cocaína. Os exames genéticos serão realizados através da técnica de reação em cadeia da polimerase em tempo real (qRTPCR) para os polimorfismos que segundo a literatura poderiam ter relação com a violência e/ou propensão ao uso de cocaína: DAT1, DRD2 e DBH. Deste modo, este projeto visa o estudo de três fatores que poderiam estar inter-relacionados: violência, uso de cocaína e polimorfismos de certos genes.

Objetivo da Pesquisa:

O objetivo do projeto é investigar se existe uma predisposição genética associada ao uso de

Endereço: DOUTOR ARNALDO 251 21° andar sala 36
Bairro: PACAEMBU **CEP:** 01.246-903
UF: SP **Município:** SAO PAULO
Telefone: (11)3893-4401 **E-mail:** cep.fm@usp.br

cocaína/crack e uma morte violenta, ou seja, quanto o fator genético de cada indivíduo é capaz de influenciar a violência e o uso de cocaína/crack.

Avaliação dos Riscos e Benefícios:

O texto está adequado indicando riscos mínimos e benefícios relacionados a relação do uso de drogas ilícitas com mortes violentas.

Comentários e Considerações sobre a Pesquisa:

O tema é relevante para relacionar casos de morte por violência e o uso de drogas como crack. A pesquisa é bem embasada na literatura cientifica da área e tem seus objetivos e metodologias bem definidos. A pesquisa já foi aprovada pelo CEP, sendo agora submetida emenda ao projeto: inclusão de aluno Rodrigo Yuji Kuninari como colaborador da pesquisa

Considerações sobre os Termos de apresentação obrigatória:

Constam os termos necessários á submissão, incluindo declaração de inclusão do aluno ao projeto

Recomendações:

nada a declarar

Conclusões ou Pendências e Lista de Inadequações:

sem pendências

Considerações Finais a critério do CEP:

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_711896_E2.pdf	05/05/2016 13:21:59		Aceito
Outros	Rodrigo.pdf	05/05/2016 13:20:10	Mauricio Yonamine	Aceito
Outros	Carta.pdf	05/05/2016 13:19:43	Mauricio Yonamine	Aceito
Folha de Rosto	Folha de rosto.pdf	26/05/2015 07:40:03		Aceito
Projeto Detalhado / Brochura Investigador	Projeto_AnaMiguel .pdf	22/05/2015 14:42:20		Aceito

TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.pdf	22/05/2015 14:41:58		Aceito
Outros	Declaração_de_anuência_Ivan.pdf	15/05/2015 18:32:57		Aceito
Outros	Declaração_de_participação_Ivan.pdf	15/05/2015 18:32:49		Aceito
Outros	Declaração_Mario.pdf	15/05/2015 18:32:40		Aceito
Outros	Declaração_Rosario.pdf	15/05/2015 18:32:33		Aceito
Outros	Declaração_AnaMiguel.pdf	15/05/2015 18:32:23		Aceito
Outros	Declaração_de_anuência_Daniel..pdf	15/05/2015 18:32:12		Aceito
Outros	Declaração Sandra.pdf	15/05/2015 18:10:10		Aceito
Outros	Declaração_de_participação_Vilma.pdf	15/05/2015 17:45:33		Aceito
Outros	Descrição_da_equipe.pdf	24/04/2015 15:32:31		Aceito
Outros	Descrição dos itens_orçamento.pdf	24/04/2015 15:31:46		Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

SAO PAULO, 09 de Agosto de 2016

Assinado por:
Maria Aparecida Azevedo Koike Folgueira
(Coordenador)

Endereço: DOUTOR ARNALDO 251 21° andar sala 36
Bairro: PACAEMBU **CEP:** 01.246-903
UF: SP **Município:** SAO PAULO
Telefone: (11)3893-4401 **E-mail:** cep.fm@usp.br

Attachment III

Approval by the Ethics Committee of the Institute of Legal Medicine of São Paulo

SECRETARIA DA SEGURANÇA PÚBLICA
SUPERINTENDÊNCIA DA POLÍCIA TÉCNICO-CIENTÍFICA
Gabinete da Superintendência
Rua Moncorvo Filho, 410 - 4º andar - Ala I - Capital -SP - CEP: 05507-060.
☎ (011) 3811-7000 R 7009 – FAX: (011) 3031-1311 – www.policiacientifica.sp.gov.br

DECLARAÇÃO DE ANUÊNCIA

Declaro estar ciente e de acordo com a realização da pesquisa intitulada "Relação entre morte violenta, uso de cocaína/crack e polimorfismos de genes associados ao sistema monoaminérgico", sob responsabilidade do pesquisador Prof. Dr. Mauricio Yonamine. Declaro conhecer e fazer cumprir as resoluções éticas brasileiras, em especial a Resolução CNS 466/2012.

Declaro que esta instituição está ciente de suas co-responsabilidades como instituição co-participante do presente projeto de pesquisa e de seu compromisso no resguardo da segurança e do bem-estar dos participantes de pesquisa nela recrutados. Declaro, por fim, que esta instituição dispõe da infraestrutura necessária para a garantia de tais condições.

São Paulo, 02 de abril de 2015.

Dr. Ivan Dieb Miziara
Superintendente da Polícia Técnico-Científica

Attachment IV

Consent form

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO – TCLE

1. Informações do Responsável Legal

Nome:			
Documento de Identidade nº:			
Data de Nascimento: / /			
Endereço:		Nº	Complemento:
Bairro:	Cidade:		Estado:
CEP:	Telefones:		

2. Título do Projeto de Pesquisa

Estudo da relação entre morte violenta, uso de cocaína e polimorfismos de nucleotídeo único.

3. Duração da Pesquisa

Quatro anos

4. Pesquisador Responsável

Prof. Dr. Mauricio Yonamine	
Farmacêutico	CRF-SP: 21507
FCF/USP: Análises Clínicas e Toxicológicas	

5. Instituições

Faculdade de Ciências Farmacêuticas da USP, Faculdade de Medicina da USP e Instituto Médico-Legal de São Paulo

Meu nome é Mauricio Yonamine, sou professor da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo e junto com a aluna de doutorado, Ana Miguel Fonseca Pego e Prof. Dr. Ivan Dieb Miziara do Instituto Médico Legal (IML), gostaríamos de pedir a sua colaboração no projeto intitulado "Relação entre morte violenta, uso de cocaína/*crack* e polimorfismos de genes associados ao sistema monoaminérgico" que visa estudar a relação entre a genética, uso de drogas e morte não-natural.

Nesta pesquisa serão incluídas vítimas de morte não-natural, de indivíduos do sexo masculino acima de 18 anos e que as respectivas famílias consintam em participar voluntariamente do estudo. Caso você aceite participar da pesquisa, as amostras necessárias serão sangue e cabelo e serão coletadas durante a necropsia. Para

o estudo serão necessários 20 mL de sangue e uma mecha de aproximadamente a espessura de um lápis, que será coletada em diferentes regiões do couro cabeludo para não causar qualquer dano na aparência estética. Essas amostras serão utilizadas para fazer análises de drogas e análises genéticas relacionadas ao comportamento.

A pesquisa envolve risco mínimo. A identidade da vítima será mantida em sigilo durante todo o processo, inclusive quando os resultados desse estudo forem publicados em artigos de revistas científicas ou forem apresentados em temas de aulas e debates. Contudo, também haverá coleta de informações relativas à vítima como idade, sexo e histórico. Não haverá benefícios diretos, mas o estudo será importante para se tentar estabelecer as causas do uso de drogas e morte precoce.

As amostras de sangue e de cabelo serão serão acondicionadas em embalagens apropriadas e armazenadas no Laboratório de Análises Toxicológicas da FCF/USP, sob minha responsabilidade, e será usado código para identificar a amostra por questões de sigilo. Caso a amostra seja utilizada para pesquisas futuras, novo projeto será submetido ao Comitê de Ética em Pesquisa (CEP).

Você tem garantido o direito de não aceitar participar ou retirar seu consentimento em qualquer fase da pesquisa, sem que isto traga qualquer prejuízo para o andamento médico-legal. Você não receberá pagamento, portanto, a sua participação neste estudo é voluntária. Entretanto, caso haja despesas em virtude da sua participação na pesquisa, esses gastos serão ressarcidos. Da mesma forma, haverá indenização no caso de eventuais danos decorrentes da pesquisa.

Duas vias do termo de consentimento livre e esclarecido serão assinadas pelo pesquisador e o responsável legal, sendo que cada um ficará com uma cópia.

Em caso de dúvidas, entrar em contato com: Ana Miguel Fonseca Pego (fone 3091-2194) e e-mail: anamiguel@usp.br; com o Prof. Dr. Mauricio Yonamine (fone 3091-2194) e e-mail: yonamine@usp.br ou com Prof. Dr. Ivan Dieb Miziara (3031-5063) e e-mail: miz@uol.com.br.

Declaro que, após convenientemente esclarecido pelo pesquisador e ter entendido o que me foi explicado, consinto em participar do presente Protocolo de Pesquisa.

Você autoriza o armazenamento das amostras de sangue e cabelo para pesquisas futuras? ☐ **SIM** ☐ **NÃO**

São Paulo,_____de_____de 20___.

_____ _____

Assinatura do Responsável Legal Assinatura do Pesquisador Responsável

Para qualquer questão, dúvida, esclarecimento ou reclamação sobre aspectos éticos dessa pesquisa, favor entrar em contato com o Comitê de Ética em Pesquisas da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo – Av. Prof. Lineu Prestes, 580 - Bloco 13A – Butantã – São Paulo – CEP 05508-900. Fone: 3091-3622, fone-fax: 3091-3677 – e-mail: cepfcf@usp.br

Attachment V

Student’s record from ‘Janus’ system

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial
FICHA DO ALUNO

9143 - 9117611/1 - Ana Miguel Fonseca Pego

Email:	anamiguel@usp.br
Data de Nascimento:	23/08/1990
Cédula de Identidade:	RNE - G058547-F - DF
Local de Nascimento:	Portugal
Nacionalidade:	Portuguesa
Graduação:	Bachelor of Science in Forensic Investigation - Glasgow Caledonian University - Escócia - 2012

Curso:	Doutorado Direto
Programa:	Farmácia (Fisiopatologia e Toxicologia)
Área:	Toxicologia
Data de Matrícula:	29/08/2014
Início da Contagem de Prazo:	29/08/2014
Data Limite para o Depósito:	29/08/2018
Orientador:	Prof(a). Dr(a). Maurício Yonamine - 07/03/2018 até o presente. Email: yonamine@usp.br
Proficiência em Línguas:	Inglês, Aprovado em 29/08/2014
Data de Aprovação no Exame de Qualificação:	Aprovado em 11/10/2016
Estágio no Exterior:	University of California, Los Angeles, Estados Unidos da América - Período de 22/09/2017 até 13/11/2017
Data do Depósito do Trabalho:	
Título do Trabalho:	
Data Máxima para Aprovação da Banca:	
Data de Aprovação da Banca:	
Data Máxima para Defesa:	
Data da Defesa:	
Resultado da Defesa:	
Histórico de Ocorrências:	Primeira Matrícula em 29/08/2014

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 6542 em vigor a partir de 20/04/2013).
Última ocorrência: Matrícula de Acompanhamento em 05/02/2018
Impresso em: 22/06/2018 05:28:31

Janus - Sistema Administrativo da Pós-Graduação

Universidade de São Paulo
Faculdade de Ciências Farmacêuticas
Documento sem validade oficial
FICHA DO ALUNO

9143 - 9117611/1 - Ana Miguel Fonseca Pego

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
MPT5780-2/2	Genética Forense (Faculdade de Medicina - Universidade de São Paulo)	09/09/2014	13/10/2014	75	5	100	A	N	Concluída
FBC5709-5/2	Biologia Molecular em Análises Clínicas	22/09/2014	27/10/2014	75	5	100	A	N	Concluída
FBF5805-1/3	Delineamento de Experimentos e Ferramentas Estatísticas Aplicadas às Ciências Farmacêuticas	24/09/2014	28/10/2014	45	3	100	A	N	Concluída
RPA5749-3/1	Antropologia Forense, Reconhecimento e Identificação Humana (Faculdade de Medicina de Ribeirão Preto - Universidade de São Paulo)	17/11/2014	07/12/2014	90	6	100	A	N	Concluída
FBC5803-3/5	Sistemas de Garantia da Qualidade em Laboratórios de Ensaio	24/03/2015	06/04/2015	30	2	100	A	N	Concluída
FBA5728-3/11	Aprimoramento Didático	14/04/2015	11/05/2015	60	4	100	A	N	Concluída
FBC5747-2/1	Toxicologia Forense	11/05/2015	14/06/2015	60	4	80	A	N	Concluída
Atividade do Programa	Capítulo do livro Toxicologia Forense, com o trabalho intitulado: "Cocaína", Capítulo 12 da página 217 à 233, Porto - Portugal - 2015 (1)	22/09/2015	22/09/2015	-	1	-	-	-	-
Atividade do Programa	Capítulo do livro Toxicologia Forense, com o trabalho intitulado: "Etanol", Capítulo 14 da página 249 à 271, Porto - Portugal - 2015 (1)	22/09/2015	22/09/2015	-	1	-	-	-	-
Atividade do Programa	Participou da Etapa de Estágio Supervisionado em Docência do Programa de Aperfeiçoamento de Ensino junto à Disciplina FBC0426 Toxicologia Social, ministrada aos alunos de graduação do curso de Farmácia e Bioquímica da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo (2)	01/02/2016	30/06/2016	-	3	-	-	-	-
FBC5802-3/8	Tópicos Avançados em Toxicologia I	08/03/2016	20/06/2016	15	1	75	A	N	Concluída
FBC5784-3/9	Tópicos Avançados em Toxicologia II	02/08/2016	14/11/2016	15	1	100	A	N	Concluída

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito de tese	
Disciplinas:	0	25	36
Estágios:			
Total:	0	25	36

Créditos Atribuídos à Tese: 167

Attachment VI

Curriculum Vitae

Ana Miguel Fonseca Pêgo

Curriculum Vitae

Personal details

Name Ana Miguel Fonseca Pêgo

Filiation Pedro Miguel Vaz Pêgo &
Isabel dos Santos Nunes da Fonseca

Date of birth 23/08/1990 - Porto - Portugal

Professional address Universidade de São Paulo, Faculdade de Ciências Farmacêuticas
Avenida Professor Lineu Prestes 580
Cidade Universitária - Butantã - São Paulo
05508-000, SP - Brasil

Electronic mail address anamiguel14@hotmail.com

Lattes Link http://lattes.cnpq.br/9418993011800159

Academic Career

2014 - 2018 PhD in Toxicology and Toxicological Analysis
University of São Paulo, USP, São Paulo, Brazil
Title: Investigation on the relationship between violent death, cocaine abuse and single nucleotide polymorphisms
Supervisor: Mauricio Yonamine
Scholarship: CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

2012 - 2013 Masters in Forensic Toxicology
University of Glasgow, Glasgow, Scotland
Title: Evaluation of liquid phase microextraction techniques in forensic toxicology
Supervisor: Robert Anderson

2008 - 2012 Graduation in Forensic Investigation
Glasgow Caledonian University, Glasgow, Scotland
Title: Evaluation of the antimicrobial effects of colloidal silver
Supervisor: John Smylie
Scholarship: SAAS – Student Awards Agency For Scotland

2010 - 2010 ERASMUS exchange program in Laboratory Analysis
Tampere University of Applied Sciences, Tampere, Finland

Scholarship: ERASMUS program (6 months)

Additional training

2018 PK/PD of Drugs of Abuse
(Workload: 32h)
Unicamp, Campinas, São Paulo, Brazil

2017 Analysis of Drugs of Abuse in Biological Matrices
(Workload: 9h)
Agilent Technologies, São Paulo, Brazil

2015 Pharmacogenetics and Biomarkers in Psychiatry
(Workload: 12h)
Psychiatry Institute, University of São Paulo, São Paulo, Brazil

2014 Design of experiments and multivariate data analysis
(Workload: 16h)
University of São Paulo, USP, São Paulo, Brazil

2012 - 2013 Diploma of Attendance in the course of Forensic Medicine
(Workload: 30 weeks)
Department of Forensic Medicine and Science, University of Glasgow, Glasgow, Scotland

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Professional career

1. Avans Hogeschool

July 2018 - Now Title: Lecturer & Researcher
Location: Breda, Netherlands

2. Charles River Laboratories

May 2014 - August 2014 Title: Scientist I
Location: Edinburgh, Scotland

3. Scientific Analysis Laboratories

Mar 2014 - May 2014 Title: Laboratory Technician
Location: East Kilbride, Scotland

Awards

2017	Santander Mobility – International exchange grant at Semel Institute for neuroscience and Human Behaviour at UCLA
2016	Developing Countries Fund, The International Association of Forensic Toxicologists
2015	Fellowship Travel Award Winner, The International Union of Toxicology and the Brazilian Society of Toxicology
2008	Fully paid graduation studies, Student Awards Agency For Scotland

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Languages

Portuguese	Native
English	Full proficiency
Spanish	Limited proficiency

Scientific articles published

1. **PEGO, A. M. F.**; FRANCO DE OLIVEIRA, S.C.W.S.E.; FRANCO DE OLIVEIRA,T.; LEYTON, V.; MIZIARA, I.; YONAMINE, M. Cocaine toxicological findings in cases of violent death in Sao Paulo city - Brazil. **Journal of Forensic and Legal Medicine**, v. 60, p. 3–8, 2018.

2. SILVEIRA, G. O.; **PEGO, A. M. F.**; PEREIRA, J.; YONAMINE, M. Green sample preparations for the bioanalysis of drugs of abuse in complex matrices. Bioanalysis, (Accepted manuscript)

3. **PEGO, A. M. F.**; ROVERI, F.; KUNINARE, R.Y.; LEYTON, V.; MIZIARA, I.; YONAMINE, M. Determination of cocaine and its derivatives in hair samples by liquid phase microextraction (LPME) and gas chromatography–mass spectrometry (GC–MS). **Forensic Science International**, v. 274, p. 83–90, 2017.

Work presented in conference meetings

1. **PEGO, A. M. F.**; FRANCO DE OLIVEIRA, S.C.W.S.E.; FRANCO DE OLIVEIRA,T.; LEYTON, V.; MIZIARA, I.; YONAMINE, M. Stability of cocaine, benzoilecgonine, cocaethylene and norcocaine in real case *postmortem* blood samples using dried blood spots technique.
56th Annual Meeting of the International Association of Forensic Toxicologists - TIAFT, 2018, Ghent, Belgium – Poster presentation.

2. **PEGO, A. M. F.**; FRANCO DE OLIVEIRA, S.C.W.S.E.; FRANCO DE OLIVEIRA,T.; LEYTON, V.; MIZIARA, I.; YONAMINE, M. A comparison between *postmortem* blood and hair samples from victims of violent death in Brazil.
55th Annual Meeting of the International Association of Forensic Toxicologists - TIAFT, 2017, Boca Raton, USA – Oral presentation.

3. **PEGO, A. M. F.**; LEYTON, V.; MIZIARA, I.; YONAMINE, M. Determination of cocaine and cocaethylene in *postmortem* blood samples using QuEChERS and GC-MS.
1st interFORENSICS – Brasilia, Brazil– Poster presentation.

4. **PEGO, A. M. F.**; ROVERI, F.; YONAMINE, M. Determination of cocaine, benzoylecgonine, cocaethylene and anhydroecgonine methyl ester in hair samples using liquid phase microextraction (LPME) and gas chromatography-mass spectrometry (GC-MS).
54th Annual Meeting of the International Association of Forensic Toxicologists - TIAFT, 2016, Brisbane, Australia – Oral presentation.

5. **PEGO, A. M. F.**; YONAMINE, M.; ANDERSON, R. Optimization of LPME technique for whole blood in forensic toxicology.
9th Congress of Toxicology in Developing Countries/XIX Congresso Brasileiro de Toxicologia, 2015, Natal, Brazil – Poster presentation.

6. SANCHEZ, C.; **PEGO, A. M. F.**; NASCIMENTO, E.; YONAMINE, M. Quantification of cocaine, cocaethylene and anhydroecgonine methyl ester (AEME) in whole blood using hollow-fibre liquid phase microextraction (HF-LPME) and gas-chromatography

mass-spectrometry (GC-MS).
53rd Annual Meeting of the International Association of Forensic Toxicologists - TIAFT, 2016, Florence, Italy – Poster presentation.

7. **PEGO, A. M. F.;** ANDERSON, R. Evaluation of hollow-fibre microextraction in forensic toxicology. **UK & Ireland Association of Forensic Toxicologists** – UKIAFT, 2013, Dublin, Ireland – Poster presentation.

Book chapters

1. MANTOVANI, C.; **Pego, A. M. F.**; Yonamine, M. **Cocaína**. Toxicologia Forense, 2014.

2. VALLE DE BAIRROS, A.; **Pego, A. M. F.**; Yonamine, M. **Etanol**. Toxicologia Forense. 2014.

Lectures given

1. **Forensic Toxicology's challenges and how to overcome them**. ToxiLatin at Porto Alegre, Rio Grande do Sul, June 2018.

2. **From death to the laboratory through Forensic Toxicology**. II Symposium of toxicological analysis applied to forensic sciences at Teresina, Piauí, May 2018.

3. **Forensic toxicology – the hidden reality**. XV Biomedicine meeting of Minas Gerais at Uberaba, Minas Gerais, May 2018.

4. **Forensic Toxicology.** VI Winter School in Toxicology at São Paulo, São Paulo, July 2017.

5. **Forensic Toxicology – The C.S.I of real life.** I Symposium of the academic League of Forensic Sciences at Uberaba, Minas Gerais, June 2017.

6. **Toxicogenetics.** Part of the graduate subject Forensic Toxicology under the School of Pharmaceutical Sciences at the University of São Paulo in São Paulo, São Paulo, June 2017.

7. **Forensic toxicology – the hidden reality**. I Symposium of Toxicological Analysis Applied to Forensic Sciences at Teresina, Piauí, May 2017.

8. **Forensic Toxicology – The C.S.I of real life.** VI National Symposium of DNA and Forensic Laboratories at Curitiba, Paraná, December 2016.

9. ***Postmortem* toxicology**. I Cycle of courses and lectures from the Brazilian Society of Toxicology at São Paulo, São Paulo, June 2016.

10. **Screening and immunoassay techniques for the detection of stimulants urine** (Theoretical & practical). Part of the subject of Toxicological Analysis under the graduation course in Pharmacy from the Federal University of São Paulo, at São Paulo, São Paulo, March 2016 and March 2017.

11. **Cannabinoids**. I Extension Course in Forensic Toxicology at São Paulo, São Paulo, October 2015.

12. **Truths and myths about THC**. IV Symposium on Biomedicine Einstein at Limeira, São Paulo, September 2015.

13. **Forensic Toxicology**. IV Winter School in Toxicology at São Paulo, São Paulo, June 2015.

Organization of events

1. Member of the Organizing Committee of the "**I Extension Course in Forensic Toxicology**" at the School of Pharmaceutical Sciences, University of São Paulo in October 2015.

Supervisions

1. **Supervised the student Rodrigo Yuji Kuninari**, graduating in Pharmacy at University of São Paulo by carrying out his scientific initiation with the work entitled: “Determination of cocaine, benzoylecgonine, cocaethylene and anhydroecgonine methyl ester in hair through gas chromatography mass spectrometry” from August 2015 to August 2016.

Examinations

1. **Examiner for the title of Graduated in Biomedicine** of the students Gislaine Da Silva Moraes and Meire Cristina De Padua with a final work entitled “Therapeutic Use Of *Cannabis Sativa*” at Integrated College Einstein Limeira, Limeira, São Paulo, December 2015.

2. **Poster examiner** at the 23rd International Symposium on Scientific and Technological Initiation of USP, October 2015.

3. **Poster examiner** at the 22nd International Symposium on Scientific and Technological Initiation of USP, October 2014.

International Exchange

Visiting scholar at UCLA – Los Angeles at the SEMEL Institute for Neuroscience and Human Behaviour, under the supervision of Dr. Edythe London from September to November 2017.

Teaching assistant

1. Assistant to the teaching Professor of the subject of **Physiopathology** (duration: 5

months).

2. Assistant to the teaching Professor of the subject of **Social Toxicology** (duration: 5 months).

Academic responsibilities

Students representative of the Graduate Program in Toxicology and Toxicological Analysis by the Commission Program Coordinator and Scholarship Commission from July 2016 to July 2017 as President.

Extra-curricular activities

1. Students representative of the Graduate Program in Toxicology and Toxicological Analysis by the Commission Program Coordinator and Scholarship Commission since July 2016 as President.

2. Volunteer at Espaço Sementes – Project *Cracolândia* at night.

3. Volunteer at Glasgow Science Festival, Glasgow, Scotland: 2013 – 2014.

4. Volunteer at Lab in a Lorry (all over Scotland): 2012 – 2014.

5. Volunteer at Science, Technology, Engineering and Mathematics Ambassadors – STEM (all over Scotland): 2011 – 2014.