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PREFACE

Shotgun proteomics: where do we stand now?

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JIOMICS Editor in Chief

As a general trend, the pipeline for shotgun proteomics can be outlined in four flashes. First, the complex proteome is treated to digest the sample; second the peptides are separated using Liquid Chromatography (LC); third the peptides are sequenced using tandem-mass spectrometry, and finally, the data sets recorded with the mass spectrometer are interrogated using computational biology and bioinformatics.

Making a sample ready for shotgun proteomics analysis has always been a time-consuming operation. Many little steps involving protein extraction, total protein quantification, protein reduction with dithiothreitol, protein alkylation with iodoacetamide, protein digestion with an appropriate enzyme and clean-up to remove salts used to be a processing workflow taking as long as 24 h. To overcome this problem the proteomics community developed a number of approaches intended to simplify the handling and to shorten the number of steps and the time consumed in each one of them. Of the many technological developments, the application of ultrasonic energy meets the requirements of simplicity, low cost, high sample throughput and easy implementation for online applications [1]. Currently, 96 samples can be prepared in just two hours, matching perfectly clinical applications for treating samples in large-scale.

Liquid Chromatography – MS has also evolved dramatically to answer the challenge to analyze complex proteomes such as whole cell lysates, plasma or serum. Thus, from the outset,

the mainstream was to analyze few samples as deep as possible to find out differences that could enable the discovering of the so-called "Biomarkers." This mainstream had a complexity reflected in approaches that described online systems using dual column chromatography [2] or extensive protein prefractionation[3] as the ways to overcome the large dynamic range of protein abundances. Such approaches resulted in time-consuming, expensive and low throughput workflows. The result has been a reduced number of approved biomarkers, as low as 22 during the period comprised between 1995 and 2010 [4]. However, new technological advances in chromatography are called to change the panorama mentioned above dramatically. Thus, the introduction of the Evosep [5] chromatograph concept has taken biomedical research a level forward as it has allowed high throughput and robustness for the first time in proteomics history. As much as 200 samples can be treated per day, rendering approximately 1500 proteins identified in chromatographic runs as short as 15 min.

The advent of the "Evosep" concept as mentioned above has been possible because the advances in mass spectrometry and computation have allowed the changeover from long chromatographic runs (90-120 min) to short ones (5-15 min) with a relatively high number of features differentiated, about 1000 to 2000 proteins, enough to efficiently profile the proteome of one patient and thus allowing to classify large cohort of patients. But, what kind of advances have prompted mass spectrometry to convert the Evosep concept in a disrupting moment in proteomics?

The introduction of the orbitrap in 2005 [6] was a breakpoint in the history of mass spectrometry and has dominated the market during the last ten years. However, it seems the tide to turn over the reign of the orbitraps after the introduction by the Bruker company [7] of the new trapped ion mobility spectrometry (TIMS) technology for higher-speed, higher-sensitivity and robust shotgun proteomics with outstanding single-shot peptide and protein identification performance. The ion mobility has added a new dimension of separation to shotgun proteomics, which join it to the Evosep chromatography concept has allowed to reach an unprecedented level of throughput to proteomics, almost 200 samples per day.

This new technology needs to be complement with computation and here is where the Parallel Accumulation Serial Fragmentation (PASEF) acquisition method enters to play [8].

(PASEF) enables hundreds of MS/MS events per second at full sensitivity. Also, this is due to the combination of unprecedented MS/MS acquisition speed (> 100 Hz), increased sensitivity (X50), uncompromised resolution performances and the benefit of an orthogonal ion mobility separation.

The advent of the proteomics era in the late 90´s held the promise of a new era in diagnosis and prognosis. Such promise was the perspective of some visionaries. A vision that was established too early and that led some people to believe the promise would never be completed. Now, almost two decades later, the promise seems closer than ever. Calculations done, 200 samples can be now handled in just five working days, from sample treatment to mass spectrometry analyses. The only bottleneck that remains is computation, but no for data acquisition but for data interpretation. Moreover, when the computer scientists had resolved this last piece of the puzzle, then, proteomics will go where no other human knowledge has gone before. Such is the endeavor, we the proteomics, have ahead.

References

[1] S. Jorge, J.E. Araujo, F.M. Pimentel-Santos, J.C. Branco, H.M. Santos, C. Lodeiro, J.L. Capelo, Talanta, 178 (2018) 1067-1076. doi: 10.1016/j.talanta.2017.07.079

[2] J.N. Adkins, S.M. Varnum, K.J. Auberry, R.J. Moore, N.H. Angell, R.D. Smith, D.L. Springer, J.G. Pounds, Molecular & Cellular Proteomics, 1 (2002) 947-955. doi: 10.1074/mcp.M200066-MCP200

[3] H. Keshishian, M.W. Burgess, M.A. Gillette, P. Mertins, K.R. Clauser, D.R. Mani, E.W. Kuhn, L.A. Farrell, R.E. Gerszten, S.A. Carr, Mol. Cell. Proteomics, 14 (2015) 2375–2393. doi: 10.1074/mcp.M114.046813

[4] N.L. Anderson, Clin. Chem., 56 (2010) 177-185. doi: 10.1373/clinchem.2009.126706

[5] N. Bache, P.E. Geyer, D.B. Bekker-Jensen, O. Hoerning, L. Falkenby, P.V. Treit, S. Doll, I. Paron, F. Meier, J.V. Olsen, O. Vorm, M. Mann, (2018). doi: 10.1101/323048

[6] A. Makarov, E. Denisov, A. Kholomeev, W. Balschun, O. Lange, K. Strupat, S. Horning, Anal Chem, 78 (2006) 2113-2120. doi: 10.1021/ac0518811

[7] B. Corporation, Bruker Launches the timsTOF™ Pro Mass Spectrometer to Enable the Revolutionary PASEF Method for Next-Generation Proteomics; in: https://ir.bruker.com/investors/press-releases/2017/default.aspx (June 2018), Bruker Corporation, 2017.

[8] F. Meier, S. Beck, N. Grassl, M. Lubeck, M.A. Park, O. Raether, M. Mann, J Proteome Res, 14 (2015) 5378-5387. doi: 10.1021/acs.jproteome.5b00932

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Assessment of occupational exposures to multiple metals with urinary porphyrin profiles

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ABSTRACT

Chronic occupational exposure to low levels of metal mixtures necessitates biomonitoring of exposed workers. However, a single biomarker is rarely sufficient to ascertain the exposure of an individual to a complex mixture, with multiparameter analysis of the same sample considered recently as a preferred approach. Porphyrins are formed as intermediates of heme biosynthesis and different metals can exert their effects at different points of this metabolic pathway, leading to changed urinary porphyrins excretion profiles. The aim of this work was to develop a model that could serve to identify, on an individual basis, multiple metal exposure resulting from mining work, by using urinary porphyrin profiles. Urine samples of workers were obtained from a Portuguese mining company and a non-occupationally exposed group was used as control. The levels of uro-, hepta-, hexa-, penta-, copro- and protoporphyrins were determined by HPLC. It was observed that only heptaporphyrin levels in miners were significantly ($p<0.05$) different from controls. However, when the concentrations of all porphyrins were combined by binary logistic regression, their ability to discriminate between miners and controls was higher than each one of the porphyrins alone, as indicated by a greater curve area under a Receiver Operating Characteristics (ROC) curve. Moreover, when the combined porphyrins were used to calculate the probability of each subject fit in the occupationally exposed group, 83% of 47 individuals were correctly identified with respect to their type of exposure. These results suggest that the integration of the urinary porphyrin profile is a promising tool for the detection of subjects exhibiting biochemical modifications due to occupational exposure to metals in mines.

Keywords: metal mixture, occupational exposure, urine porphyrin profiles, biomarkers

Abbreviations: BM:biomarkers; HPLC: High Performance Liquid Chromatography; ROC: Receiver Operating Characteristics; uro-: uroporphyrins; hepta-: heptaporphyrins; hexa-: hexaporphyrins; penta-: pentaporphyrins; copro-: coproporphyrins; proto-: protoporphyrins; *oem*: occupationally exposed in a mine

1. Introduction

Mining represents one of the most hazardous occupations [1], and has been long recognized as arduous and liable to injury and disease [2]. Biomonitoring of chronic occupational exposure to low levels of metal mixtures in miners as well as other occupations is indispensable for workers' health, with molecular biomarkers (BM) representing critical tools in achieving this task [3, 4]. However a single BM is rarely sufficient to ascertain exposure in a single person to a complex mixture. Accordingly, information from multivariate analysis of multiple parameters, measured simultaneously in the same sample is being considered as a promising approach [5]. Porphyrin analysis represents one such approach. Porphyrins are compounds formed as intermediates of heme biosynthesis [6], and it well established that various metals can exert their effects at different points of the porphyrin metabolic pathway, leading to changed urinary porphyrin excretion profiles. Thus, porphyrins have been recognized as promising BMs of metal mixture-induced toxicity [7]. Indeed, previous work in our laboratory revealed that changes in rat urinary porphyrin profiles could predict the magnitude of effects induced by a metal mixture in individual animals [8, 9]. Our recent work has focused on the translation of these

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observations to human populations exposed to metal mixtures, such as mine workers [3]. The aim of this study was to develop a model that could serve to identify multiple metal exposures resulting from mining work taking advantage of urinary porphyrin profile analysis on an individual basis.

2. Material and Methods

Urine samples from workers in a mining company in Portugal were obtained from the Occupational Health Department under the supervision of a nurse and doctor; The working group (n=29) was composed of male miners aged between 24 and 56 years. All of them were working in the mine for more than one year, A non-occupationally metal exposed group composed of workers from the same company (also male individuals aged between 22 and 60) was used as a control group. Individuals in the control group were not exposed to metals in their work environment (n=20). All the biological samples were collected on the last day of the week and stored at –80°C until the analysis.

Chromatographic porphyrin analysis was performed by HPLC after sample preparation according to Woods et al, 2009 [8] to obtain porphyrin profiles by determining uro-, hepta-, hexa-, penta-, copro- and protoporphyrins concentrations.

Statistical analysis was performed with the SPSS 16.0 statistical package for Windows (SPSS, Inc., Chicago, IL, USA). Data are expressed as means ± standard deviations (SD). 1) The occupational and non-occupational exposed groups were compared by Mann-Whitney U tests, respecting to their levels of each porphyrin. 2) The ability of urinary porphyrins to detect occupational exposures in mines was evaluated by ROC analysis, which is a statistical tool that can be used to evaluate the diagnostic accuracy of BMs, alone or in combination [10]. In this analysis, BMs levels are plotted in a curve under a 1- Specificity (x) vs Sensitivity (y) axis, being the area under the curve directly proportional to the BM(s) diagnostic accuracy. Actually, these plots of 1-Specifity (false positives or 1-true negatives) versus Sensitivity (true positives) is an effective measure of BM(s) accuracy [11]. Thus, an area=100% indicates that all subjects are true positives and true negatives revealing a maximum accuracy of the BM(s); by its turn an area that lies close or under 50% have no information content and indicate that the BM(s) do not have diagnostic utility (Warnock and Peck, 2010)[12]. Here, the levels of each porphyrin alone and the combination all the porphyrins by binary logistic regression, were tested by ROC analysis; the obtained areas were compared to evaluate which BM(s) exhibited the highest diagnostic accuracy. 3) Posteriorly we created a mathematical expression that could serve to determine the odds of a new subject under study being exposed to metals in his work environment [13]; The odd values was calculated using levels of urinary porphyrins. This expression was generated by binary logistic regression. Notably, combining porphyrins profiles had a significantly better diagnostic accuracy under the ROC curves for metal exposure.

3. Results

3.1 Urinary porphyrin profiles

Figure 1 shows that a significant ($p<0.05$) difference and lower urinary levels of heptaporphyrins were noted in miners when compared to controls.

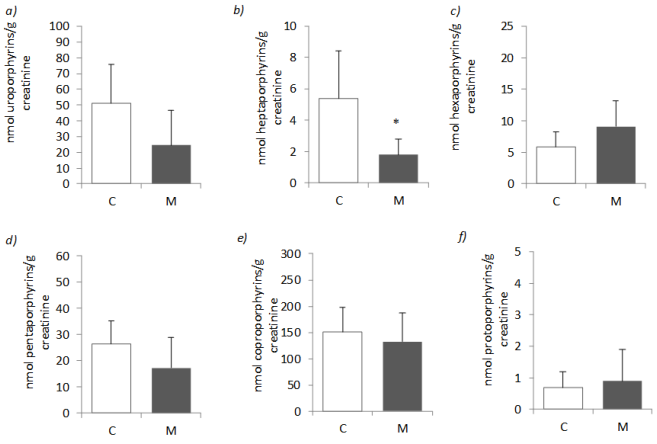


Figure 1. Uroporphyrin (a), heptaporphyrin (b), hexaporphyrin (c), pentaporphyrin (d), coproporphyrin (e) and protoporphyrin (f) urinary levels in controls (C) and miner (M) population. Data represent the mean ± SD and n=20 and 29 in C and M groups, respectively. All groups were compared by Mann-Whitney U tests. * represents $p < 0.05$ versus C.

3.2 Capability of urinary porphyrins to detect occupational exposures in mines

The diagnostic accuracy of each porphyrin alone was tested for their ability to detect subjects occupationally exposed in mines (true positives). This approach was deemed unsatisfactory as the areas under the 1- Specificity (x) vs Sensitivity (y) axis were lower than 50%, attesting to high false positives and false negatives, and indicating lack of diagnostic utility of each of the BMs alone (Figure 2a). Alternatively, when all the porphyrins were combined by binary logistic regression, the number of true positives and true negatives increased substantially as reflected by the increase in the area under the ROC curve (area= 0.743; $p<0.05$) (Figure 2b).

3.3 Detection of mining work related exposure in an individual basis

To detect the mining work related exposure in each subject, we developed a mathematical expression. The expression below represents the odds of a subject to be considered as exposed in a mine (oem), by depicting the concentrations of their urinary porphyrins (uro-, hepta-,

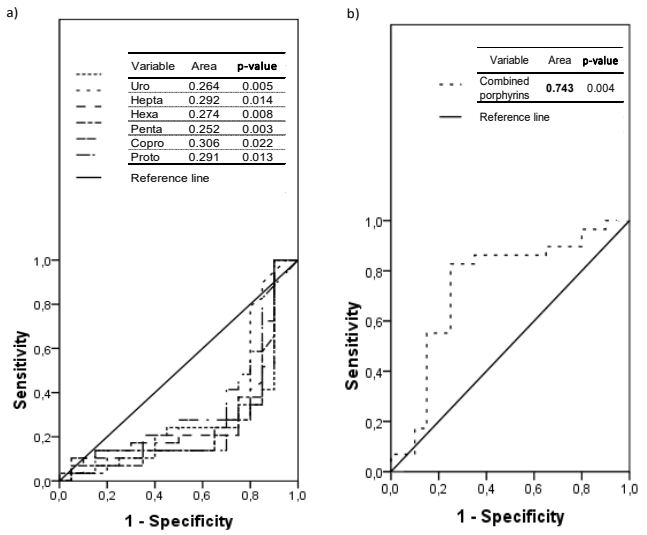


Figure 2. The ability of the urinary levels of uroporphyrins, hepta-porphyrins, hexaporphyrins, pentaporphyrins, coproporphyrins and protoporphyrins individually (a) and their combination by binary logistic regression (b) to identify subjects with mining related exposures. The curve's area under the 1- Specificity (x) vs Sensitivity (y) axis are plotted by ROC analysis: areas are directly proportional to the BMs discriminant capabilities. All represented areas reached statistical significance ($p<0.05$). N=49.

hexa-, penta-, copro- and protoporphyrins), by binary logistic regression:

$$ODDs\ (oem) = 26.6Uro - 176.3Hepta + 484.9Hexa$$
$$+111.3Penta + 5.9Copro + 790.2Proto + 1.4$$

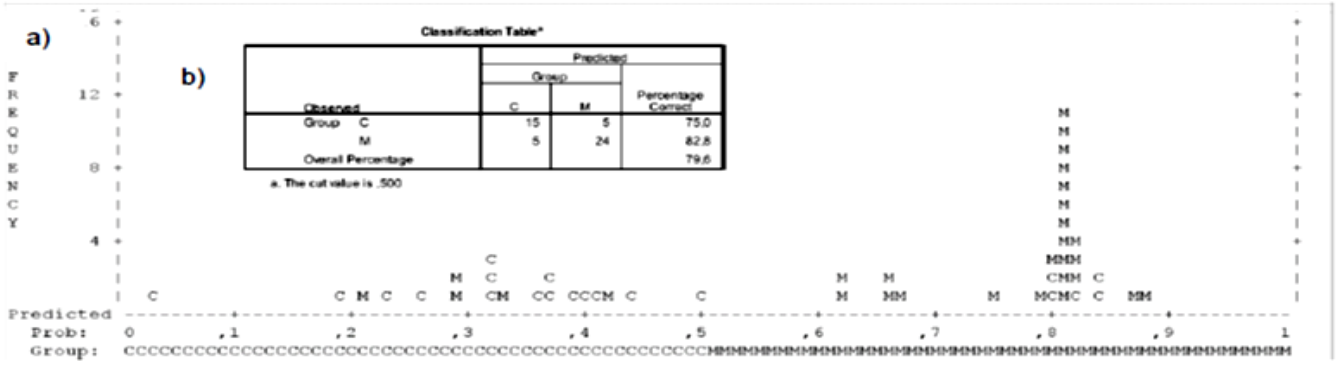
The use of combined urinary porphyrin levels leads to 83% of correct detections of occupational exposure in miners (Figure 3b). More precisely, 15 of 20 subjects included in the control group were correctly identified as non-occupational exposed exhibiting only a 0.15 to 0.45 range odd of being a miner (Figure 3b). Additionally, 4 of the 5 controls were erroneously classified as miners showing a 0.80 to 0.85 range odd of being a miner, while one person had an odd of only 0.5 (Figure 3a). With regard to the miners, 24 of the 29 workers were correctly identified as miners with odds that ranged from 0.6 to 0.9 with a higher frequency at the odd of 0.8 (Figure 3a). Concerning to the false negatives, 5 miners were incorrectly included in the control group and a 0.20 to 0.45 range odd of being a miner was attributed to these persons (Figure 3a).

4. Discussion

Low chronic exposure to metals may result in insidious poisoning that may manifest clinically at a very late stage. Thus it is critical to monitor mine workers so that subclinical toxicity can be identified at the onset of disease or even at earlier stages [3, 14]. While molecular BMs represent key tools in occupational medicine [15], urinary porphyrin profiles may serve as BMs of exposure to toxic metals in humans [16, 17]. Since various metals can exert their effects at different points of the heme synthesis pathway, we hypothesized that the urinary porphyrin profile might serve as a "fingerprint" that would permit a satisfactory identification of individuals exposed to metals due to their occupation in mines.

Our results showed that each of the porphyrins alone poorly detected occupational exposures to metals, even on a group basis, with miners and controls exhibiting differences that lacked statistical significance ($p>0.05$) (Figure1). Miners are exposed to several metals [3], such as lead (Pb), arsenic (As), manganese (Mn), mercury (Hg) and aluminum (Al), which interfere at common, and also at different points, in the heme synthesis sequential pathway [18, 19, 20, 21, 22]. These events may lead to distinct fingerprint in urinary excretion of each of the porphyrins, explaining their poor diagnostic performance when used alone. These results were confirmed by the observation of areas <0.5 under a 1-Specificity (x) vs Sensitivity (y) axis plotted by ROC analysis (Figure 2a). Indeed, BMs with a curve that lies close or under the diagonal reference line (area = 0.5) had no information content, and therefore no diagnostic utility [12]. In contrast,

Figure 3. Predicted probability of each subject belong to the mining group (M) after application of Expression 1 to each studied individual. Each symbol (C or M) represents 1 subject (a) and the ODDs cut value for miners group membership is ≥ 0.5. The table (b) represents the counts of subjects included in each group as predicted by the model vs the real group (observed). N=20 and 29 in C and M groups, respectively.



when the urinary porphyrins profile was applied as a whole with integration of all the porphyrins by binary logistic regression, the area under a ROC curve increased the diagnostic utility (area=0.743) (Figure 2b) [12]. Therefore, the concentrations of all the porphyrins were used to generate an equation to determine the odds of a new subject under analysis to be occupationally exposed in a mine (previously displayed expression). When this equation was tested in the studied subjects, a correct detection of the occupational exposure in the mines was obtained for 83% of the cases (Figure 3b). We suggest that the called "erroneous classification" is not directly related to the model, but rather with the type of environmental exposure of all the populations; the control as well as the mining populations are not homogeneous in terms of work, housing location, social habits and genetic susceptibility. Our results are satisfactory for a preliminary study with N=49, obtaining 25% of false positives and 17% of false negatives. These findings also lead us to proceed with additional future work, expanding on the sample size, to further establish the proof-of-concept of these analyses as predicitive tools of metal mixture exposures.

5. Concluding Remarks

Overall, the integration of the urinary porphyrin profile showed superiority in discriminating miners or subjects non -occupationally exposed, than any of the porphyrin concentrations alone. The proposed multiparameter approach is promising for the detection of mining work related to multiple metal exposures, and should be further validated in future studies. Although our study was focused on porphyrin profile and metals, we may add that metals as well as metal mixtures may increase oxidative stress and originate adverse effects including the neurotoxic ones [23]. In fact, Pb, Hg, Fe and Mn are typical examples of metal transport and toxicity at barriers, like the blood brain barrier, inducing neurotoxicity [24]. Moreover, toxic metals can promote the disruption of essential metal homeostasis, and minor alteration in quantity, form or place of these vital elements may lead to essential metal imbalances, associated with several diseases [25].

Our studies highlight the need for additional time course analyses to better understand gene-omics relationships and adverse outcome pathways. This is important if transcriptomics, proteomics, metabolomics and other omics approaches are to be used together to investigate neurotoxicity or as bio-monitoring tools for exposure.

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References

1] P. Coelho, S. Costa, S. Silva, A. Walter, J. Ranville, A.C.A. Sousa, C. Costa, M. Coelho, J. García-Lestón, M. Ramiro Pastorinho, B., Laffon, E. Pásaro, C. Harrington, A. Taylor, J.P. Teixeira, J Toxicol Environ Health 75 (2012) 893-908. DOI: 10.1080/15287394.2012.690705

2] A.M. Donoghue, Occup Med. 54 (2004) 283-289. DOI: 10.1093/occmed/kqh072

3] S.V. Dhatrak, S.S. Nandi, Indian J Occup Environ Med 13 (2009) 60-64. DOI: 10.4103/0019-5278.55121

4] P.A. Schulte, J.E. Hauser, Toxicol Lett 213 (2011) 91-99. DOI: 10.1016/j.toxlet.2011.03.027

5] J.M. Christensen, Sci Total Environ 166 (1995) 89-135. DOI: 10.1016/0048-9697(95)04478-J

6] B. Quintanilla-Vega, A. Hernandez, T. Mendoza-Figueroa, Toxicol in Vitro 10 (1996) 675-683. PMID: 20650251.

7] G. Wang, B.A. Fowler, Toxicol Appl Pharmacol 233 (2008) 92–99. DOI: 10.1016/j.taap.2008.01.017

8] J.S. Woods, M.D. Martin, B.G. Leroux, T.A. DeRouen, M.F. Bernardo, H.S. Luis, J.G. Leitão, P. L. Simmonds, T.C. Rue, Clin Chim Acta 405 (2009) 104-109. DOI: 10.1016/j.cca.2009.04.014

9] V. Andrade, M.L. Mateus, M.C. Batoréu, M. Aschner, A.P. Marreilha dos Santos, Neurotoxicology 45 (2014) 168-177. DOI: 10.1016/j.neuro.2014.10.009

10] D. Liu, X.-H. Zhou, Acad Radiol 20 (2013) 874–882. DOI: 10.1016/j.acra.2013.03.009

11] K. Hajian-Tilaki, Caspian J Intern Med 4 (2013): 627–635. PMCID: PMC3755824.

12] D. Warnock, C. Peck, Nature Biotechnology 28 (2010) 444–445. DOI: 10.1038/nbt0510-444

13] R. Brant, Biometrics 46 (1990) 1171-1178. DOI: 10.2307/2532457

14] P. Kakkar, F.N. Jaffery, Environ Toxicol Pharmacol 19 (2005) 335-349. DOI: 10.1016/j.etap.2004.09.003

15] G. Scherer, Exp Toxicol Pathol 57 (2005) 75–110. DOI: 10.1016/j.etp.2005.05.007

16] J. Li, Z. Cai, S. Xu, C. Liao, X. Song, L. Chen, J Liq Chromatogr R T 34 (2011) 1578-1593. DOI: 10.1080/10826076.2011.575981

17] J.C. Ng, J.P. Wang, B. Zheng, C. Zhai, R. Maddalena, F. Liu, Toxicol Appl Pharmacol 206 (2005) 176–184. DOI: 10.1016/j.taap.2004.09.021

18] M. Ahamed, M.K.J. Siddiqui, Clin Chim Acta 383 (2007) 57-64. DOI: 10.1016/j.cca.2007.04.024

19] K.M. Kadish, K.M., Smith, R. Guilard (Ed.) The Porphyrin Handbook: Medical aspects of porphyrins, Volume 14, Academic Press, Elsevier Science, 2012

20] M. Krishnamohan, L. Qi, P.K.S. Lam, M.R. Moore, J.C. Ng, Toxicol Appl Pharmacol 224 (2007) 89–97. DOI: 10.1016/j.taap.2007.04.020

21] M.D. Maines, Biochem J 190 (1980) 315-321. DOI: 10.1042/bj1900315

22] J.S. Woods, M.D. Martin, C.A. Naleway, D. Echeverria, J Toxicol Environ Health 40 (1993) 235-246. DOI: 10.1080/15287399309531791

23] M. Farina, D.S. Avila, J.B. da Rocha, M. Aschner, Neurochem Int 62 (2013) 575-94. DOI: 10.1016/j.neuint.2012.12.006

24] W. Zhang, M. Aschner, J.F. Ghersi-Egea, Toxicol Appl Pharmacol 192 (2003) 1-11. PMID: 14554098

25] S. DoKer, S. Manicou, M. Dogan, R. Lobinski, Metallomics 2 (2010) 549-555. DOI: 10.1039/c004508j

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Use of Optical Coherence Tomography on detection of postmortem Ocular findings: pilot data from two cases

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ABSTRACT

PURPOSE: The aim of this study was to describe by means of a portable Optical Coherence Tomography (OCT) the postmortem ocular findings in two cases of forensic interest.

CASE 1. A 41-year-old Caucasian man, dead from a gunshot in the head, was found inside his car. Time of death was precisely assessed from the testimony of eye witnesses. The body was transported at the Morgue of Medico-Legal Institute at the University of Cagliari for autopsy. OCT scans of cornea, anterior chamber and retina were performed at the 6th, 12th and 24th hour postmortem without change eyelid opening status. Corneal examination showed a progressive tendency of tissue to thickening. From a morphological point of view, we also observed a progressive formation of waves in the posterior stroma and in the endothelium [we decided to call this phenomenon, described by our group for first time, Nioi-Napoli sign (NN sign)]. An ongoing modification in reflectivity between anterior (hyperreflective) and posterior (hypo-reflective) segment of the corneal stroma was detecetd and a progressive decrease in amplitude of anterior chamber, mainly related to changes in tissue curvature. The retinal tissue showed since the first scan an increasing trend of retinal oedema togheter with a diffuse vasal depletion. It was also detachable the formation of a scleral tache noir.

CASE 2. A 42-year-old woman, dead from myocardial infarction, underwent an autopsy at the Medico-Legal Institute at University of Cagliari (as suspected case of medical malpractice). Exact time of death was deduced by certificate of death drawn up by the emergency medical team. Scans of cornea, retina and anterior chamber were executed at the 24th, 36th and 48th hour postmortem. From the first scan an enhancement of corneal thickness, if compared to the physiological in vivo ranges, was detected; a change in corneal curvature was observed while no images form the retinal tissue was achieved. Late scans evidenced a progressive corneal endothelial exfoliation, and an enhancement of posterior stroma waving.

CONCLUSION Portable OCT may be an useful device to observe and to record postmortem ocular changes.1,2,3 Its use could represent an important tool to study the early and the late modification of ocular tissues, with a special interest in the forensic scenario as post mortem interval estimation (PMI estimation) and in ophthalmology (viability of cornea for transplantation purposes) .

Keywords: Postmortem Ocular Findings, Optical Coherence Tomography, NN sign, PMI estimation, corneal transplantation

Abbreviations: NN sign—Nioi-Napoli sign, OCT— Optical Coherence Tomography, PMI—post mortem interval .

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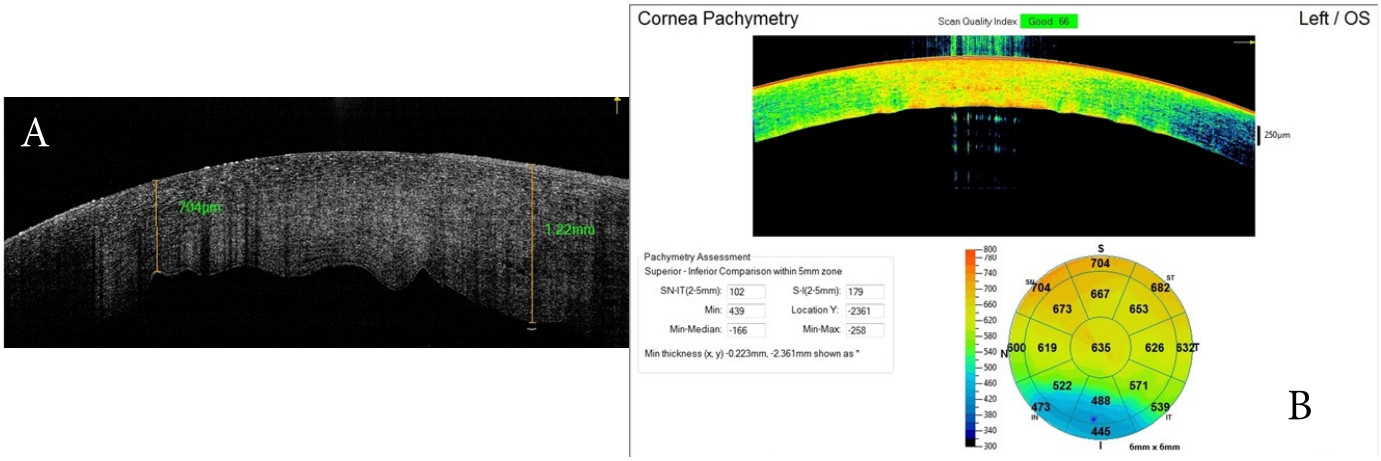

Figure 1. Corneal postmortem pachymetry **(A)** and Postmortem corneal vawing 'NN sign' **(B)**: IT is possible to detect the imaging since five hours after death;. The sign is attributable to corneal oedema and consequent endothelium deformation.

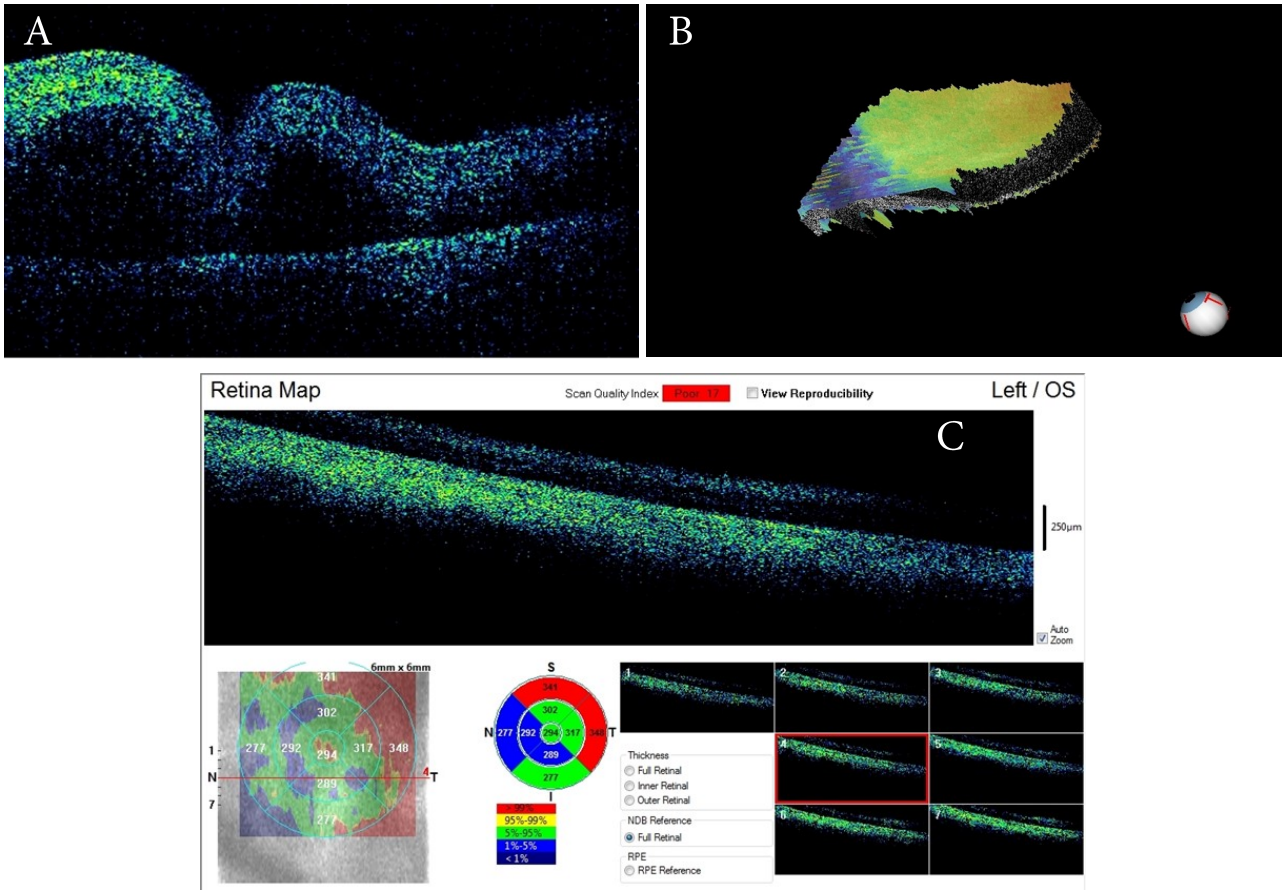

Figure 2. **(A)** OCT retinal postmortem findings. **(B)** OCT image of the fovea 3D image of the fovea. **(C)** 2D image of the retinal tissue.

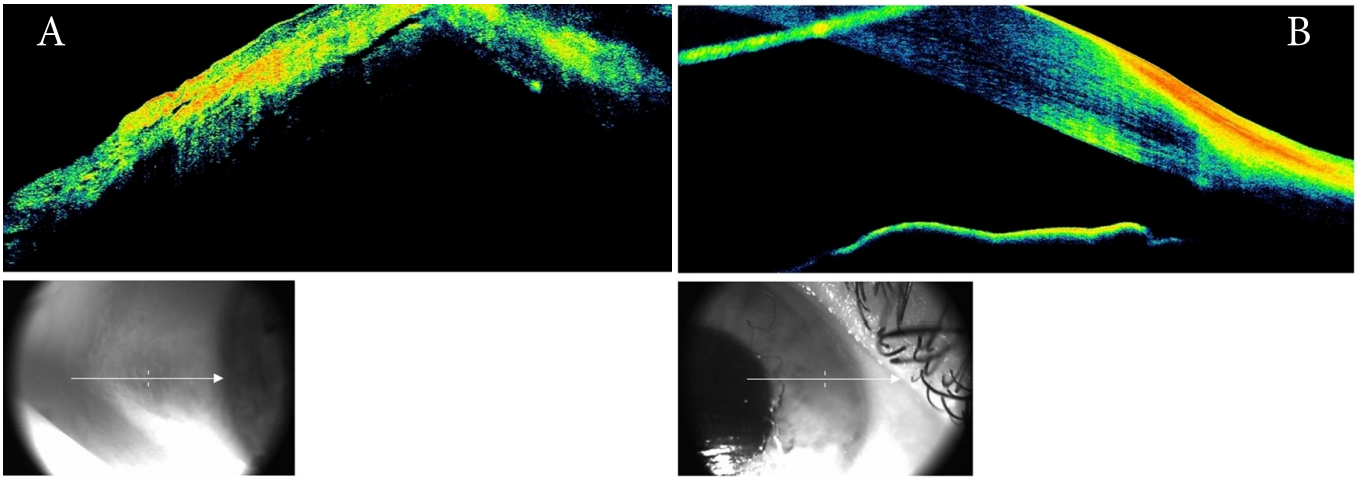

Figure 3. OCT scleral and iridocorneal postmortem findings **(A)** OCT image of the 'tache noir' **(B)** Iridocorneal angle.

References

1] P.E. Napoli, M. Nioi, E. d'Aloja, & M. Fossarello (2016). Post-mortem corneal thickness measurements with a portable optical coherence tomography system: a reliability study. Scientific reports, 6.

2] M. Nioi, P.E. Napoli, C. Iovino, F. Paribello, M. Fossarell, & E. d'Aloja (2017). Corneal findings after death: a preliminary OCT study on an animal model. Investigative Ophthalmology & Visual Science, 58(8), 4873-4873.

3] C. Iovino, P.E. Napoli, M. Nioi, R. Sanna, F. Paribello, E. d'Aloja, & M. Fossarello (2017). Intraobserver variability of postmortem corneal thickness measurements by using a portable OCT system. Investigative Ophthalmology & Visual Science, 58(8), 3516- 3516.

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Quality Improvement for Criminal Investigations: Lessons from Science?

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ABSTRACT

Criminal investigations generally aim at discovering previously unknown facts. The same is true for scientific (or academic) research. Both follow a rather tight framework of rules – most importantly, the principles of objectivity, reliability and validity. However, some of the intentions differ. Science generally attempts to discover and/or explain new principles, while criminal inquiries are instead usually bound to past, often singular, events. For example, the methods used in forensic investigations are required to be well established, standardised and undisputed inasmuch as possible. In contrast, the exploration of new methods is an important feature of the advancement of science. Consequently, both tendencies – similarities and opposites – can be discerned when comparing criminal and academic examinations.

The 'Pareto principle' indicates that the vast majority of all criminal investigations run rather unproblematically. Nevertheless, the highest quality criteria must be guaranteed for these and the remaining, more challenging cases as well – based on the 'fair trial' principle. Acknowledging that mistakes are inevitable (Murphy's law), methodical approaches for error identification, handling, management and reduction are essential.

Error correction mechanisms that are typical for forensic statements normally include a second source of expertise and/or an appeals procedure. In academic science, however, the peer review system has long been established as the most important quality control and error correction system. Furthermore, possible mistakes can usually be corrected in later, more detailed studies. However, the central position of forensic experts and criminal investigators in a legal procedure and the severe personal consequences of incorrect statements emphasize the high importance of continuous improvement of both the qualifications of the investigators and the quality of their methods.

Nevertheless, error reduction provisions should not be restricted to technical measures such as quality management and accreditations. Furthermore, a systemic/organisational approach towards error management seems promising. This involves, among other measures, a systematic examination of mistakes and the recognition of the human factors that underlie them. Nevertheless, an indispensable component for quality enhancement is intense cooperation from both sides – the criminalistic and forensic practice as well as the scientific (basic) research .

Keywords: Forensic, Science, Quality, Expert, Testimony, Error

1. Introduction

According to the general humanitarian principles and the European convention on human rights [1], every suspect is entitled to a fair trial and an investigation that is based on objectivity. The latter, however, is a target that is often difficult to meet, since human decisions are often biased by motives that are not evidence-based. Thus, in a tribunal scenario, objectivity is often anticipated from scientific investigations, usually in the form of forensic science reports and/or testimony from expert witnesses. While this input certainly increases the objectivity, reliability and validity of decisions, the significance of scientific results is frequently misjudged (i.e., overestimated) by laypersons. Therefore, it may be useful for judges, jury members, criminal investigators as well as for the experts, to recall a few principles from the theories of science to gain a better perspective for scientific statements.

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2. Scientific Framework

From the numerous philosophical concepts that interpret the process of human cognition, critical rationalism [2] seems most appropriate as an initial point for scientific investigations. It accepts the world as a factual entity (i.e., things exist independently from the observer) and acknowledges the limitations of the human mind (and instruments) to fully perceive all features of reality. Furthermore, this system proposes a method to gain knowledge from errors, the latter being accepted as an inevitable component of all heuristic explanation efforts.

Thus, the scientific process generally involves the formulation of hypotheses that are checked with experiments that are designed to find errors in the proposed model. Eventually, the theory is refined to circumvent the contradictory findings and an improved postulate is then further tested (Figure 1). Note that the explanation is modified and not the experimental results – the latter are regarded as valid unless proven otherwise.

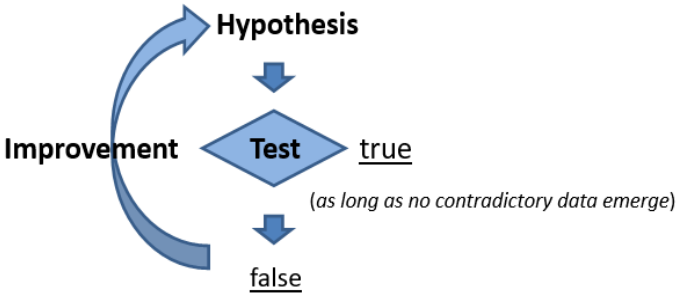

Figure 1. Schematic flow of decision making according to the critical realism concept.

Successively, the refined theories from this process can eventually be regarded as "true" and may be absorbed in the corpus of formal scientific knowledge – unless they are falsified by a later experiment.

It is obvious that this procedure is not appropriate for forensic/criminalistic purposes. It would be rather questionable to proceed until future studies confirm or dismiss a finding that is the basis for a verdict. However, this may be the best chance for revealing misjudgments and exoneration [3] of the accused.

There are two possible types of reasoning in the interpretation of results. The more convenient way for law enforcement (and in many other situations) is reductionist (linear or deterministic) reasoning. Thus, the answer of a questioned statement can only have two possible outcomes – yes or no, true or wrong, guilty or innocent, etc. An example would be a testimony such as: 'Person X was not at the crime scene as he or she was in custody at the specified time'. This applies (only) to one single, specific event. Unfortunately, questions that can be answered with a reductionist approach are rather rare.

Frequently, the answer to a question or the outcome of an investigation, measurement, etc., is a stochastic value – the product of a probability distribution. Such statements normally meet the criterion of a defined standard error (see below). However, in order to minimize that error, a large (or even infinite) number of coherent observations would be ideal. The latter is obviously problematic with forensic samples that are often small and/or cannot be collected in a standardized way. An example for this type of result would be: 'The DNA sample from the crime scene is from person X with an error rate of $p < 0.001\%$'.

By convention, for describing the results, the zero-hypothesis is applied, e.g., causality does not exist, an event did not happen, two actions are not related, a person is not guilty, etc. The value 'p' gives the probability of this assumption being wrong, where $p = 0$ means that a result is certain, $p = 1$ indicates that the statement is false. The values $p < 5\%$ (often also given as $p < 0.05$) is 'significant', $p < 1\%$ (or $p < 0.01$) is 'very significant' and $p < 0.1\%$ ($p < 0.001$) would be 'highly significant'. Accounts of this type are highly appreciated by juries; however, several caveats apply (and should be explained by the expert): Statistical data are prone to a number of methodological errors, e.g. clustering effects, sampling bias, etc. These effects can be avoided by taking a sufficient number of homogenous samples. However, forensic traces are seldom homogenous and usually limited in number. Furthermore, an answer based on statistical results is valid for its statistical basis and not necessarily for each single item (outlier). Also note, that a probability of 1 : 10 000 000 ($p= 0.0000001$) – e.g., in a DNA assessment – still implies that there are at least fifty persons in the EU (out of approx. 511 M citizens) with that combination of features. Thus, relative numbers can provide a wrong impression (compare also [4]).

Furthermore, statistics can only prove correlations and not causality. Thus '*cum/post hoc ergo propter hoc*' (together with 'X'/following 'X', therefore 'Y') errors are likely to appear. The display of statistical data may also lead to an incorrect impression of 'scientific certainty', since all cognitive and methodical errors that might have happened before are masked by the perceived assertiveness of the result.

In addition to the desirable outcomes of a measurement – i.e., an assumption being confirmed or rejected – there is also the possibility that a result may be erroneous (Fig. 2). This can happen for a large number of reasons – e.g. methodical deficiencies, human flaws, or even intentional deception (fabricated evidence).

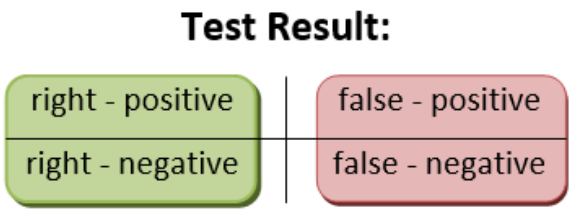

Figure 2. Diagram of the theoretically expectable results of an analysis.

Thus, beside a correct result – a searched feature is present or not – the possibility that a test may be inadequate and thus unable to detect the questioned feature even if it is present must be considered. Vice versa, a positive result may also be derived from a number of sources of error such as oversensitive test systems, contamination, high background "noise", etc. The human factor obviously influences both types of false outcomes. Reduction of these errors is the subject of quality assurance programs, but a complete prevention of all mistakes is theoretically impossible and practically unfeasible.

It should be noted, however, that the rather tight set of rules previously outlined is primarily followed in natural sciences and also in medical research. Humanities, such as psychology, sociology, criminology, etc., are often less strict in their interpretation of epistemological principles

3. Methodological Aspects

Minimum requirements were established attempting to insure a certain degree of relevance and reliability of investigation results in the USA. The Frye standard [5] stated that a method for investigation should be 'accepted by practitioners' – a concept that is problematic for several reasons (it should not be forgotten, that e.g. medical 'practitioners' insisted that e.g., bloodletting was a useful therapy for a long time). Consequently, the Frye standard was replaced by the Daubert standards [6] in 1993. The latter expect that, in addition to being accepted by the 'scientific community', a valid investigation method should be testable, published in a peer reviewed journal and give due regard to the standard error of the results (see also [7]).

Scientific and criminalistic investigations are not equal in their general objectives. While science basically aims to identify general principles, specific questions about single events, i.e. individual crimes, are usually targeted by the various forensic disciplines. For example, the scientific principle of reliability (and reproducibility) demands that a result should be repeatable, obtained under controlled conditions and statistically secured. Consequently, all parameters should be under the control of the experimenter (at least theoretically), a condition that is impossible to achieve in criminal investigations. In forensic practice, traces are scarce, often poorly preserved, sometimes contaminated and therefore not 'standard' in many respects. However, some academic disciplines like palaeontology or pathology may also have similar problems – being restricted to material that is limited or even unique.

Furthermore, while it is generally the aim of scientific research to find new, previously unknown principles – forensic findings should not primarily be innovative, but be widely acknowledged instead. This is a main difference between academic science and forensic investigations. Additionally, science aims usually at discovering and explaining general principles, while forensic work is essentially connected with the clarification of past, normally singular events (i.e., crimes).

The potential consequences of expert testimony imply the demand for the highest professional standards for the quality of an investigation as well as for the competence of the examiners. However, errors inevitably occur in spite of all efforts to avoid them.

The traditional error correction mechanism in academic science is the peer review process (and thus is requested by the Daubert standards). However, reviewed studies may also be wrong [8] (compare also the discussions following the publications of Sokal [9] or Lindsay and Boyle [10]). A second level of validation in academic science is derived from follow-up studies that should be able to replicate the previous results (at least in theory).

This system established in the scientific process is clearly inappropriate for criminalistic/forensic/juridical statements, since it requires an abundance of time and a generalizing approach to a question that is often not adequate for single cases. Instead, the established system involves appeal and obtaining a second expert opinion. Note however that these steps also require time and often a certain financial capacity of the culprit.

Numerous sources of mistakes exist in addition to the potentially false test-results mentioned above. Quality management systems (e.g. ISO/IEC 17025:2005) attempt to insure adequate technical standards and analytical procedures. These rules propose uniform solutions for essentially identical tasks, thus eliminating variability inasmuch as possible. Nevertheless, it is essential to check first if the questioned issues indeed are identical and if all preconditions for a valid analysis are met. Both are rather difficult to assess for the defendant or lawyer. It should also be noted that an implemented SOP (standard operating procedure) or GLP (good laboratory practice) can only assure quality on a formal basis – by minimizing deviations from an 'ideal' prototype/standard – thus eliminating all variations, experimentation and creative processes. Consequently, these frameworks are primarily useful in routine production or analysis sequences (Fig. 3), but may be inherently obstructive for innovative scientific discovery. Nevertheless, in several forensic fields (e.g., in drug- or DNA -analysis) standardisation and quality management routines are certainly indispensable. Alternatively, investigations in exceptional or complex criminal cases can demand flexible and inventive approaches that are not formally standardized or even require the use of (yet) unpublished methods.

4. The Human Factor

Not surprisingly, a main source of flaws originates from human imperfection. Several factors contribute to wrong conclusions, ranging from inadequate crime scene work to basal human cognitive mechanisms (e.g., [11]). Human perception is neither a camera nor a computer and thus prone to flaws in perceiving and drawing conclusions. Other shortcomings, also often resulting from limited personal,

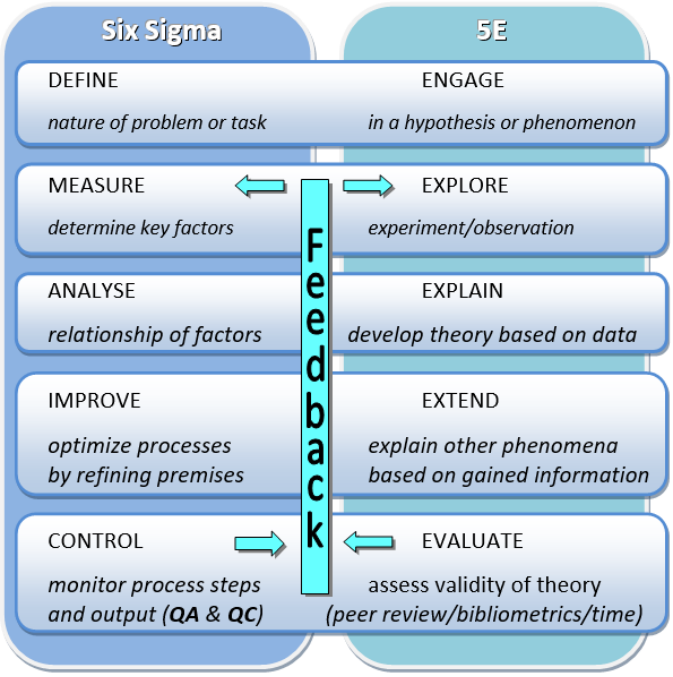

Figure 3. Comparison of quality improvement programs for production/services (Six Sigma) and academy/education (Five E). Note that specific quality assurance (QA) and control (QC) are the last steps in the production schedule while analogous steps in the scientific procedure are less explicit.

equipment or time may adversely influence investigations. However, the latter difficulties may at least partly be countered by organisational and training measures (e.g., [12]).

Encouragingly, the main source of erroneous verdicts is probably not expert statements but wrong witness reports. About 60% of wrong convictions in review of US-courts verdicts [13] result from false testimony (mainly misidentifications – see also [14]). Additionally, mistakes by authorities (police, prosecutor, jury), false accusations (e.g., by a snitch) and mistakes in defence (lawyer) as well as false confessions were found to be major contributors, besides wrong results from forensic investigations [15].

A further problem that is not restricted to formally controlled or accredited processes but rather refers to many types of reporting of results is connected with interpretation. The narrative element, i.e., communication between expert and jury, either in a written report or in testimony at court is an often neglected factor. The inevitable differing conceptions and sometimes even language of practitioners and tribunal must actively be countered by both sides. Formalized schemes of responses [16, 17] such as: '… is (very) likely', '… cannot be approved or denied', '… is (very) unlikely', etc., which are sometimes linked with probability values in reports, may be facilitating conventions but do not support a deeper understanding of a statement (compare e.g., [18, 19]. Instead they attempt to fill a gap between something 'is' or 'is not' (taking into account methodological limits and error margins). If such arbitrary scales are secondarily related to numbers, the results become even more indefinite. For example, is a result of 2.5 in the scale mentioned before (between … is likely and … cannot be approved or denied) really 'better' than a 4 (… is unlikely)? The frequently demanded comparability of testimony from different experts is only seemingly provided by a choice of pre-defined diagnoses, especially when the results strongly depend on the opinion of the expert.

Investigations that are heavily dependent on interpretation by a practitioner like e.g. fingerprints (dermatoglyphics), handwriting, forgery, etc., but also many other kinds of expert assessments (medical, psychiatric, etc.) are principally based on the individual competence of the respective specialist. Formal accreditation procedures or controlling schedules cannot fully assure the correctness of testimonies in such fields (e.g., [20]). Investigation routines based on the four-eyes principle and eventually a second, independent expertise can at least partly overcome this problem (compare also [21]). However, as long as there are expert witness reports used in court they will remain open to criticism. Ensuring, improving and maintaining the qualification of experts and their reports is an essential responsibility also for academia.

Several academic disciplines are less based on observation and rely more on theories. This is typically the case for human and social sciences, psychology, economics, art, etc. However, both systems – data driven and more intuition-based – are commonly applied in these fields. Accordingly, such reports can be applied to cases of law, although with special caution as e.g., the Daubert criteria might not be fulfilled.

Even if the highest professional standards can be maintained, certain errors rooted in the human cognitive system are difficult to control. Cognitive dissonance gives a subconscious bias towards the explanation that causes the least emotional conflict, thus clearing the mind from strong antagonistic sensations. An example for this phenomenon is the 'neutralisation' that criminals exhibit with respect to their victims, (i.e. the victims provoked the attack, have only self to blame, etc.). However, the interpretation of evidence by an investigator underlies similar cognitive mechanisms. This can result even in the inability to perceive adverse details once a theory has formed (i.e. after a few seconds). Precognition of seemingly or factually unrelated details can strongly influence the collection and subsequent evaluation of evidence (e.g., [22, 23, 24, 11]) and external influences (including the judge – compare [25]) can severely affect the outcome of a lawsuit.

Cognitive bias affects everyone in daily life. However, in criminal investigations it may lead to serious adverse effects. Experienced criminal investigators considered in a survey selective perception/expectation/confirmation bias, anchoring/'pars pro toto' errors and 'onus probandi' infringements (shifting the burden of proof to the suspect) as those cognitive factors most likely to negatively affect criminal investigations [12].

Acknowledging the fact that 100% error free conditions are very difficult to attain and impossible to maintain, poses the problem of error handling strategies. As mentioned already, in individual cases an appeal and a second expert opinion can correct a wrong expert assessment. Quality management systems can generally reduce the error rate in routine processes. Nevertheless, errors will occur.

Mistakes that happen in spite of these efforts should strictly be investigated (instead of the understandable tendency to cover them up). In several fields where errors can have dramatic consequences, like e.g., in air transportation or also in clinical medicine, painstaking investigations are often carried out after a disastrous event. The aim of these studies is not only to investigate the specific incident, but also to refine the rules and procedures in order to prevent future adversities. Institutional boards of inquiry with external experts could identify 'hot spots' in forensic investigations by systematic analysis of errors with the aim of suggesting ways to avoid or improve such pitfalls [12].

The demand for objectivity in juridical procedures is often symbolized by the blindfolding of the Roman godess 'Justitia'. Organizational measures, e.g., anonymizing samples, double blind tests, four eyes principle, etc. are important steps for supporting this principle but most important are the appropriate attitude and ethics of the investigator (among many others, see also [26]). Unfortunately, these assets are difficult to teach and to surveil. The integrity of officers is strongly dependent on both the individual values and also on the organizational culture.

5. Conclusions

As indicated before, the importance of forensic expert testimony in the legal system implies the need for highest standards for both the qualification of an expert and the level of technical/methodological quality. The best available practices must be applied to every criminal case – a request that is based on the 'fair trial' principle. The general 'scientific' rules of objectivity, reliability and validity form a common basic framework for academic science as well as forensic investigations (while also maintaining ethical and economical standards). Requirements to fulfil these demands are the continuous professional training of the experts, application and further improvement of technical standards (best practice, GLP, SOP, etc.) and the development and implementation of a system for error management.

While extensive formal education is an essential prerequisite for forensic work, it is evidently the everyday challenge of practical operation that provides the demand as well as the criteria for applicability and validity for all forensic methods. It is common knowledge that practical application is the ultimate test bench for academic results. While it is probably rare that the same person is active equally in scientific research as well as in practical criminalistic work it is essential to provide an environment of information exchange and close cooperation between scientific (basic) researchers and investigating officers.

Summarizing, it may be stated that criminal investigations and scientific research share many features but also show fundamental differences. Nevertheless, both disciplines may complement each other. While science can improve established techniques and procedures or develop new ones (among other virtues), the criminal investigator delivers demand and impetus for such advancements. The practical experience of criminalists often initiates new innovations or improvements in techniques. Also, the ultimate test for any (academic) theory is (forensic) practice. Successful implementation and general recognition of a technique is therefore an acknowledgement of quality – unless a refined process is employed – thus analogous to the peer review system. Consequently, a close collaboration between academic research and practical demand is essential.

In addition, both theoretical knowledge and practical experience are indispensable for the education and training of scientists as well as officers working in this field. The crucial position of the expert in the legal system requires a sufficient number of undisputed experts for the wide field of forensic questions. It is generally known that the chances of solving a case decrease drastically after about 48 hours. It is therefore desirable to have highly qualified investigators at a crime scene as soon as possible.

A strong empirical component is certainly present (and essential) in forensic work. Nevertheless, a sound basis in 'framework' sciences like statistics, physics, chemistry, psychology, etc. – is required before specializing in a particular field. Several proposals aim at improving the qualification of examiners (e.g. [27]). However, the need for improvement has also been emphasized for the scientific basis of forensic approaches (e.g. [28]). Therefore, every initiative to advance scientific standards in forensic investigations must be appreciated. An intense and continuous interchange between academic research and forensic-criminalist practice seems the most advantageous strategy to ensure optimum forensic performance.

References

1] European Convention on Human Rights. (1950) European Court of Human Rights, Council of Europe, Strasbourg. 1950

2] K. Popper, Objektive Erkenntnis. Ein evolutionärer Entwurf. Hoffmann & Campe, Hamburg, 1993.

3] S.R. Gross, M. Schaffner, Exonerations in the United States, 1989 –2012, Report by the National Registry of Exonerations, University of Michigan Public Law Working Paper 277, 2012, pp. 1–103.

4] W.C. Thompson, (2009) Painting the target around the matching profile: the Texas sharpshooter fallacy in forensic DNA interpretation. Law, Probability and Risk 8 (2009) 257–276.

5] Frye vs.United States. D.C. 293 F. 1013 (1923).

6] Daubert vs. Merrell Dow Pharmaceuticals, 509 U.S. 579. (1993) p92-102.
7] M. Du, Analysis of errors in forensic science. J Forensic Sci Med 3 (2017) 139-43.
8] J. Ioannidis, Why most published research findings are false. PLoS Medicine 2 (2005) 697-701.
9] A. Sokal, Transgressing the boundaries: Towards a transformative hermeneutics of quantum gravity. Social Text 46/47 (1996) 217-252.
10] J. Lindsay, P. Boyle, The conceptual penis as a social construct. Cogent Social Sciences 3 (2017) 1-7.
11] I. Dror, R. Morgan, C. Rando, S. Nakhaeizadeh, The bias snowball and the bias cascade effects: Two distinct biases that may impact forensic decision making. J. Forensic Sci. 62 (2017) 832-833.
12] H. Ditrich, Cognitive fallacies and criminal investigations. Science and Justice 55 (2015) 155-159.
13] R. Huff, A. Rattner, E. Sagarin, Guilty until proved innocent: Wrongful conviction and public policy. Crime and Delinquency 32 (1986) 518-544.
14] D Canter, L. Hammond, D. Youngs, Cognitive bias in line-up identifications: The impact of administrator knowledge. Science and Justice 53 (2013) 83-88.
15] B. Kamins, ed., Preliminary Report of the New York State Bar Association's Task Force on Wrongful Convictions. New York Bar Foundation, New York, 2009.
16] H. Katterwe, G. Brandes, R. Eisgruber, W. Grimmer, W. Küppers, W. Marquard, K.H. Pohl, Harmonisierte Befundbewertungsskala für kriminaltechnische Untersuchungen. Kriminalistik 12/2007 (2007) 745-750.
17] S. Bunch, G. Wevers, Application of likelihood ratios for firearm and toolmark analysis. Science and Justice 53 (2013) 223-229.
18] C. Mullen, D. Spence, L. Moxey, A. Jamieson, Perception problems of the verbal scale. Science and Justice 54 (2014) 154-158.
19] R. Marquis, A. Biedermann, L. Cadola, C. Champod, L. Gueissaz, G. Massonnet, W.D. Mazzella, F. Taroni, T. Hicks, Discussion on how to implement a verbal scale in a forensic laboratory: Benefits, pitfalls and suggestions to avoid misunderstandings. Science and Justice 56 (2016) 364-370.
20] C. Roux, B. Talbot-Wright, J. Robertson, F. Crispino, O. Ribaux, The end of the (forensic science) world as we know it? The example of trace evidence. Phil. Trans. R. Soc. B 370 (2015) 1-8.
21] R. Julian, S.F. Kelty, Forensic science as "risky business": identifying key risk factors in the forensic process from crime scene to court. Journal of Criminological Research, Policy and Practice 1 (2015) 195 – 206.
22] T.A. Busey, G. R. Loftus. Cognitive science and the law. Trends in Cognitive Sciences 11 (2007) 111-117.
23] I. Dror, D. Charlton, Why experts make errors. Journal of Forensic Identification 56 (2006) 600-616.
24] P.A. Fraser-Mackenzie, I. Dror, Selective information sampling: Cognitive coherence in evaluation of a novel item. Judgment and Decision Making 4 (2009) 307-316.
25] G. Edmont, D. Hamer, A little ignorance is a dangerous thing: engaging with exogenous knowledge not adduced by parties. Griffith Law Review 25 (2016) 282-413.
26] I. Dror, Cognitive neuroscience in forensic science: understanding and utilizing the human element. Phil. Trans. R. Soc. B 370 (2015) 1-8.
27] G. Sapir, Qualifying the expert witness: A practical Voir dire. Forensic Mag. 2/3 (2007). https://www.forensicmag.com/article/2007/01/qualifying-expert-witness-practical-voir-dire (access 7.11. 2017).
28] National Research Council, Strengthening forensic science in the United States: A path forward. National Academies Press, Washington. 2009. http://www.nap.edu/catalog/12589.html (access 21. 4. 2011).

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Salivary proteomics in ingestive behaviour research: advances, potentialities and limitations

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ABSTRACT

Human saliva proteomics gained interest in the last two decades, mostly due to the non-invasive nature of this fluid collection and to its potential for the diagnosis of different oral and systemic pathologies. Curiously, despite saliva being the fluid that first contacts with food, the interest in the relationship between its composition and ingestive behavior increased recently. The relevance of saliva protein composition in food acceptance and preferences is evidenced by the observation that individuals who differ in oral food perception present particularities in salivary proteome: individuals with different sensitivities for astringency diverge in the levels of several salivary proteins; the same is true concerning the perception of basic tastes, namely bitter and sweet. Even aroma perception depends on saliva protein composition. Interestingly, some of the proteins observed to differ according to oral food perception are proteins that present variations with Body Mass Index (BMI). Besides this potential role of saliva in driving food choices, this fluid may have also potential as a source of biomarkers of ingestion. Although less explored, until now, there are evidences of changes in saliva protein composition related with the type of diet: diets rich in polyphenols induce modifications in saliva composition, in animal models; high-fat diets consumption by rats were observed to change their levels of salivary alpha-amylase. These different points will be reviewed in the present article.

Keywords: Biomarkers of ingestion; Oral food perception; Salivary proteomics

Abbreviations: HPLC – High-performance liquid chromatography; PROP – 6-n-propylthiouracil; CA-VI – carbonic anhydrase VI; PRPs – Proline-rich proteins.

1. Introduction

Saliva has been considerably studied for its use in diagnosis, because this is a fluid that contains many of the molecules present in blood, in amounts proportional to the ones present in this last, but with the advantage, over plasma, of allowing non-invasive and simple collection. As such, repeated sampling is possible, without the need of special trained people or expensive equipment.

Salivary proteins constitute one of the main groups of salivary molecules with potential as biomarkers. For that reason the interest in salivary proteomics emerged, with more than 2400 non-redundant salivary proteins [1]. Generally, saliva proteomics studies rely on methodologies for separation and identification of proteins such as 2-D electrophoresis, capillary electrophoresis and high-performance liquid chromatography (HPLC), in combination with mass spectrometry. Several reviews report the main methodological approaches used in the area of salivary proteomics (e.g. [2–4]).

A considerable number of studies has been made with the aim of comparing heathy individuals with individuals suffering from different oral and systemic diseases. For example: i) in the search for breast (reviewed in [5]), gastric [6], or oral (e.g. [7]) cancer salivary biomarkers; ii) in the understanding and diagnosis of periodontal disease (e.g. [8]); iii) in neurology and psychiatry [9], among other clinical

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areas. But saliva is the fluid that bathes oral cavity tissues and that first contacts with food and, as such, it is somehow expected that saliva can be related with food sensorial perception, ingestion and digestion. However, the use of proteomics to investigate and understand ingestion has been few explored.

This article reviews the research carried out in the field of saliva and ingestive behavior, namely: i) the relationship between salivary proteome and oral food perception (astringency and basic tastes); ii) the way saliva can reflect ingestive strategies and dietary behavior; iii) the potential of saliva as an objective and non-invasive source of biomarkers of ingestion.

2. Salivary proteomics and oral food perception

2.1. Astringency perception

The relevance of salivary proteome in oral food perception started to be studied in the context of the involvement of salivary proteins in astringency development. Tannins are a group of polyphenols with different structural characteristics, but with the common feature of complexing and precipitating proteins what is responsible for the sensation of astringency perceived in foods containing these compounds. In 1954, Bate-Smith [10] proposed that astringency results from the interaction of tannins, present in foods and beverages, with salivary proteins. Since then, and despite some controversy about the exact mechanisms involved, it is accepted that salivary proteins have a major role in astringency development and intensity. Different authors presented evidences that the compounds perceived as more astringent are the ones that precipitate salivary proteins to a higher degree [11,12]. Moreover, differences in astringency perception among different individuals were reported to be linked to different salivary protein profiles. Dinnella and colleagues [13] observed that saliva composition is related to the intensity with which astringency is perceived and that there are differences in salivary responses between individuals high- and low-sensitive to this oral sensation. Recently, we confirmed that individuals presenting different tannic acid detection thresholds differ from each other in their salivary response to stimulation with this compound, and that the proteins whose levels change are salivary proteins with affinity to form insoluble complexes with these polyphenols [14].

2.2. Basic tastes perception

Salivary proteomics has been also related with basic taste perception. Bitter taste responsiveness, evaluated through 6-n-propylthiouracil (PROP) test (i.e. evaluation of the relationship between the intensity perceived of three solutions of PROP and the intensity of three solutions of NaCl) has been related with salivary proteome. S-type cystatins, prolactin-inducible protein, carbonic anhydrase VI (CA-VI) [15,16] and some proline-rich proteins (PRPs) [17] have been observed in different levels in tasters and non-tasters. Moreover, in tasters and non-tasters the changes in saliva composition induced by stimulation with bitter compounds are different: in tasters both S-type cystatins and CA-VI decrease after stimulation, whereas in non-tasters S-type cystatins levels increase [16].

Recently we provided evidences that sweet taste sensitivity is also related with salivary proteome [18]. Salivary amylase is one of the proteins whose levels and enzymatic activity are negatively correlated with sensitivity to this taste, i.e., there is a tendency for the individuals with higher amounts of this protein to have lower sensitivity for sucrose sweet taste. Besides that, some salivary proteins that are related with bitter taste perception appeared to be also related with sweet taste sensitivity, namely cystatins, CA-VI and prolactin-inducible protein [18].

Differences in the salivary proteome of individuals with different sensitivities for salty taste have also been reported. The variations were particularly at level of salivary proteases and protease inhibitors, suggesting that the action of proteases may influence transepithelial sodium transport mediated by epithelial sodium channels [19].

Concerning sour taste, although the relationship between salivary proteome and sensitivity for this basic taste appears to be less explored, changes in protein composition induced by acid stimulation has been reported [20,21]. It would be interesting to investigate how these changes are associated with salivary pH changes and/or with oral perception of this basic taste.

2.3. Retronasal aroma perception

The retronasal perception of the volatiles released during food mastication greatly influences global food perception and may constitute, together with olfaction, one of the main drivers of food preference and acceptance. Aroma release can be affected by factors that are inherent to food products, such as the chemical nature of the volatiles or the physicochemical characteristics of the food, or factors external to food products, such as the physiological characteristics of individuals. Among these last, saliva composition appears to have potential in modulating aroma perception (reviewed in [22]). Despite that the relation between the salivary proteome and aroma perception has been little studied, compared to the relation between this fluid and taste perception, it has been suggested that salivary proteins may affect the release of volatile molecules and their access to receptors: one study reported that the differences in wine aroma perception, between normal weight and obese individuals, are probably related with their different salivary protein composition [23]. Moreover, saliva was also observed to affect olive oil aroma release [24]. From the salivary proteins potentially involved in aroma perception, α -amylase and mucins have been reported to decrease the release of ketones and esters [25].

The effect of saliva in aroma perception appears to be dependent on the characteristics of food matrix. Whereas in foods like gelatine, the addition of saliva resulted in the enhancement of hydrophobic volatiles diffusion and in the decrease in hydrophilic compounds release [26], in cheese this fluid had an opposite effect, with an increase in the release of the hydrophilic compound ethyl propanoate [27].

Another interesting example of how salivary proteins may influence food aroma release is what happens in products rich in polyphenols. Phenolic compounds have been observed to interact with volatiles, modulating their release and the consequent aroma of olive oils [24] or wines [28]. Since salivary PRPs have the ability of binding food polyphenols, it has been suggested that the changes in aroma release from these products, when saliva is added, are the result of polyophenol-salivary protein interaction [24,28].

3. Saliva and food intake related diseases

The interest in studying salivary proteomics under the theme of ingestive behavior is highlighted by the observation that saliva composition differs in individuals with some nutrition-related diseases. If, on one hand, the knowledge of these changes may be of interest for the identification of non -invasive biomarkers of some of these diseases, on the other hand, it may be relevant to better understand metabolism and disease development. By linking some of the potential differences in salivary proteome with the knowledge about salivary proteins influence in oral food perception, as described in earlier section, it is possible to speculate about the factors involved in the unhealthy nutritional choices that result in the diseases.

Diabetes has been one of the metabolic diseases main studied in terms of salivary changes, with several authors reporting changes both at salivary gland level [29] and in salivary proteome [30]. Undernutrition in children has also been reported to associate with changes in salivary proteome [31]. Less well studied are eating diseases, but for anorexia changes in salivary biochemistry has been also found [32].

Previous studies have supplied us with evidence that salivary proteome from obese individuals differ from normal -weight ones [16,33]. Some of the differences are in the amounts of proteins associated with bitter and sweet tastes sensitivities, namely CA-VI, cystatins and salivary amylase. The simultaneous observation that some of the differences in obese salivary proteome are no longer observed in individuals that loose weight after bariatric surgery lead us to hypothesize that weight loss may induce changes oral medium composition, which affect taste perception and, consequently influence food acceptance. This is somehow understandable, since bariatric surgery results in alterations in the levels of hormones, such as leptin, and for this last, an association between blood [34] or salivary levels [35] and sweet taste perception is known.

Maybe the changes in saliva composition observed under nutrition-related diseases are one of the more puzzling issues, where it is necessary to investigate if the salivary differences are responsible for changes in food choices that promotes disease or if are the disease that results in salivary changes. A long-term follow-up of the biochemistry of individuals, from healthy to disease conditions, would be valuable to elucidate this question. Saliva biobanks are not common, but start to emerge [36] and this appears to be a promising way to investigate saliva relationship with ingestive behavior.

4. Can saliva provide non-invasive biomarkers of food intake?

One of the main challenges in nutritional studies is to find objective biomarkers of ingestion. The assessment of food intake is a complicated task for several reasons, including the type of instruments available, which greatly rely on memory and/or subjective assessment. Currently, the most used dietary biomarkers are sodium, nitrogen, sucrose and fructose in 24h urine samples or the doubly water technique [37].

Studies in animals present evidence that salivary proteomics differs among species according to feeding strategies [38]. For a particular type of polyphenols, namely tannins, it is known that consumption elicits changes in the levels of some salivary proteins, among which salivary PRPs have been considerably studied (reviewed in [39]). Even closely related species, such as the ruminant species sheep and goats, present dissimilarities that can be related to their different feeding strategies [40] and change their salivary proteome in a different way when subjected to variations in diet composition [41].

In previous studies, with rodents we did observe variations in salivary glands histology and salivary protein composition by the increase in food's tannin levels [42,43] . Interestingly, the variations were not the same when different structural types of tannins were consumed [44]: although salivary amylase increased after both hydrolysable (tannic acid) and condensed (quebracho) tannin consumption, the protein aldehyde reductase was only identified in saliva of animals supplemented with the condensed tannin [44]. This may lead to the hypothesis of salivary responses that are specific of diet composition.

In children, Morzel and colleagues [45] observed an association between dietary habits and saliva composition. Although the preliminary nature of this study, it reinforces the potential that salivary proteins may have as biological non-invasive markers of food intake. From our point of view, this is an area deserving attention, where more research needs to be done. However, this may be not an easy task, due to the plasticity of saliva, whose composition can be changed both due to short-term and long-term factors. The fluctuations in saliva composition induced by hygiene aspects or pathological conditions can even increase the difficulties, with the need of carefully controlled experiments.

5. Concluding Remarks

Despite the direct contact of saliva with oral structures and food constituents, the focus of salivary proteomics has been mainly in the search for disease biomarkers . However, the potential of saliva in the area of ingestive behavior deserves attention, since it may increase the understanding about food perception and choices and can be even a good source of ingestion biomarkers. By integration of several omics approaches it would be possible to increase the knowledge about this fluid, in the future, helping to understand its importance and its role in food ingestion.

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References

1] W. Yan, R. Apweiler, B.M. Balgley, P. Boontheung, J.L. Bundy, B.J. Cargile, S. Cole, X. Fang, M. Gonzalez-Begne, T.J. Griffin, F. Hagen, S. Hu, L.E. Wolinsky, C.S. Lee, D. Malamud, J.E. Melvin, R. Menon, M. Mueller, R. Qiao, N.L. Rhodus, J.R. Sevinsky, D. States, J.L. Stephenson, S. Than, J.R. Yates, W. Yu, H. Xie, Y. Xie, G.S. Omenn, J.A. Loo, D.T. Wong, Proteomics.Clin.Appl. (2009). DOI: 10.1002/prca.200800140

2] F.M.L. Amado, R.P. Ferreira, R. Vitorino, Clin. Biochem. 46 (2013) 506–17. DOI: 10.1016/j.clinbiochem.2012.10.024

3] Q. Wang, Q. Yu, Q. Lin, Y. Duan, Clin. Chim. Acta (2014). DOI: 10.1016/j.cca.2014.08.037

4] M. Castagnola, E. Scarano, G.C. Passali, I. Messana, T. Cabras, F. Iavarone, G. Di Cintio, A. Fiorita, E. De Corso, G. Paludetti, Acta Otorhinolaryngol. Ital. (2017). DOI: 10.14639/0392-100X-1598

5] E.C. Porto-Mascarenhas, D.X. Assad, H. Chardin, D. Gozal, G. De Luca Canto, A.C. Acevedo, E.N.S. Guerra, Crit. Rev. Oncol. Hematol. (2017). DOI: 10.1016/j.critrevonc.2016.12.009

6] H. Xiao, Y. Zhang, Y. Kim, S. Kim, J.J. Kim, K.M. Kim, J. Yoshizawa, L.Y. Fan, C.X. Cao, D.T. Wong, Sci Rep (2016). DOI: 10.1038/srep22165

7] R. Kawahara, J.G. Bollinger, C. Rivera, A.C.P. Ribeiro, T.B. Brandão, A.F.P. Leme, M.J. Maccoss, Proteomics (2016). DOI: 10.1002/pmic.201500224

8] M.G. Salazar, N. Jehmlich, A. Murr, V.M. Dhople, B. Holtfreter, E. Hammer, U. Völker, T. Kocher, J. Clin. Periodontol. (2013). DOI: 10.1111/jcpe.12130

9] K.L. Wormwood, R. Aslebagh, D. Channaveerappa, E.J. Dupree, M.M. Borland, J.P. Ryan, C.C. Darie, A.G. Woods, Proteomics - Clin. Appl. (2015). DOI: 10.1002/prca.201400153

10] E.C. Bate-Smith, Sect. Title Foods (1954).

11] F. Canon, F. Paté, V. Cheynier, P. Sarni-Manchado, A. Giuliani, J. Pérez, D. Durand, J. Li, B. Cabane, Langmuir (2013). DOI: 10.1021/la3041715

12] B. Sun, M. De Sá, C. Leandro, I. Caldeira, F.L. Duarte, I. Spranger, J. Agric. Food Chem. (2013). DOI: 10.1021/jf303704u

13] C. Dinnella, A. Recchia, S. Vincenzi, H. Tuorila, E. Monteleone, Chem. Senses 35 (2010) 75–85. DOI: 10.1093/chemse/bjp084

14] F. Lamy, Elsa; Mowe, Marco; Pinheiro, Cristina; Rodrigues, Lénia; Lopes, Orlando; Capela e Silva, J. Int. Soc. Antioxidants 3 (2016). DOI: DOI 10.18143/JISANH_v3i4_1338

15] M. Dsamou, O. Palicki, C. Septier, C. Chabanet, G. Lucchi, P. Ducoroy, M.-C. Chagnon, M. Morzel, Chem. Senses 37 (2012) 87–95. DOI: 10.1093/chemse/bjr070

16] L. Rodrigues, G. da Costa, C. Cordeiro, C.C. Pinheiro, F. Amado, E. Lamy, J. Sens. Stud. (2017) e12275. DOI: 10.1111/joss.12275

17] T. Cabras, M. Melis, M. Castagnola, A. Padiglia, B.J. Tepper, I. Messana, I. Tomassini Barbarossa, PLoS One 7 (2012) e30962. DOI: 10.1371/journal.pone.0030962

18] L. Rodrigues, G. Costa, C. Cordeiro, C. Pinheiro, F. Amado, E. Lamy, Food Nutr. Res. 61 (2017) 1389208. DOI: 10.1080/16546628.2017.1389208

19] T. Stolle, F. Grondinger, A. Dunkel, C. Meng, G. Médard, B. Kuster, T. Hofmann, J. Agric. Food Chem. (2017). DOI: 10.1021/acs.jafc.7b03862

20] E. Neyraud, T. Sayd, M. Morzel, E. Dransfield, J. Proteome Res. 5 (2006) 2474–80. DOI: 10.1021/pr060189z

21] K. Lorenz, M. Bader, A. Klaus, W. Weiss, A. Görg, T. Hofmann, J. Agric. Food Chem. 59 (2011) 10219–31. DOI: 10.1021/jf2024352

22] S. Ployon, M. Morzel, F. Canon, Food Chem. (2017). DOI: 10.1016/j.foodchem.2017.01.055

23] P. Piombino, A. Genovese, S. Esposito, L. Moio, P.P. Cutolo, A. Chambery, V. Severino, E. Moneta, D.P. Smith, S.M. Owens, J.A. Gilbert, D. Ercolini, PLoS One 9 (2014) e85611. DOI: 10.1371/journal.pone.0085611

24] A. Genovese, N. Caporaso, L. De Luca, A. Paduano, R. Sacchi, J. Agric. Food Chem. (2015). DOI: 10.1021/acs.jafc.5b00148

25] S. Pagès-Hélary, I. Andriot, E. Guichard, F. Canon, Food Res. Int. (2014). DOI: 10.1016/j.foodres.2014.07.013

26] A.B. Boland, K. Buhr, P. Giannouli, S.M. Van Ruth, Food Chem. (2004). DOI: 10.1016/j.foodchem.2003.09.015

27] M. Doyennette, C. De Loubens, I. Déléris, I. Souchon, I.C. Trelea, Food Chem. (2011). DOI: 10.1016/j.foodchem.2011.03.039

28] J. Aronson, S.E. Ebeler, Am. J. Enol. Vitic. (2004).

29] J. Ittichaicharoen, N. Chattipakorn, S.C. Chattipakorn, Arch. Oral Biol. (2016). DOI: 10.1016/j.archoralbio.2016.01.002

30] A. Caseiro, R. Ferreira, A. Padrão, C. Quintaneiro, A. Pereira, R. Marinheiro, R. Vitorino, F. Amado, J. Proteome Res. (2013). DOI: 10.1021/pr3010343

31] C.S.R. Fonteles, C.F. dos Santos, K.S. da Silva Alves, A.C. de Miranda Mota, J.X. Damasceno, M.C. Fonteles, Nutrition (2012). DOI: 10.1016/j.nut.2011.10.005

32] A.K. Johansson, C. Norring, L. Unell, A. Johansson, Eur. J. Oral Sci. (2015). DOI: 10.1111/eos.12179

33] E. Lamy, C. Simões, L. Rodrigues, A.R. Costa, R. Vitorino, F. Amado, C. Antunes, I. do Carmo, J. Physiol. Biochem. 71 (2015) 691–702. DOI: 10.1007/s13105-015-0434-8

34] R. Yoshida, K. Noguchi, N. Shigemura, M. Jyotaki, I. Takahashi, R.F. Margolskee, Y. Ninomiya, Diabetes 64 (2015) 3751–62. DOI: 10.2337/db14-1462

35] L. Rodrigues, R. Espanca, A.R. Costa, C.M. Antunes, C. Pomar, F. Capela-Silva, C.C. Pinheiro, F. Amado, E. Lamy, J. Nutr. Metab. (2017). DOI: 10.1155/2017/7260169

36] P. Elliott, T.C. Peakman, UK Biobank, Int. J. Epidemiol. (2008). DOI: 10.1093/ije/dym276

37] S.A. Bingham, Public Health Nutr. (2002). DOI: 10.1079/PHN2002368

38] E. Lamy, M. Mau, J. Proteomics 75 (2012) 4251–8. DOI: 10.1016/j.jprot.2012.05.007

39] T. Shimada, J. Chem. Ecol. 32 (2006) 1149–63. DOI: 10.1007/s10886-006-9077-0

40] E. Lamy, G. da Costa, R. Santos, F. Capela E Silva, J. Potes, A. Pereira, A. V Coelho, E. Sales Baptista, Physiol. Behav. 98 (2009) 393–401. DOI: 10.1016/j.physbeh.2009.07.002

41] E. Lamy, G. da Costa, R. Santos, F. Capela e Silva, J. Potes, A. Pereira, A. V Coelho, E.S. Baptista, J. Anim. Physiol. Anim. Nutr. (Berl). 95 (2011) 304–12. DOI: 10.1111/j.1439-0396.2010.01055.x

42] E. Lamy, E.S. Baptista, A.V. Coelho, F.C. e Silva, Arq. Bras. Med. Veterinária E Zootec. 62 (2010) 837–844. DOI: 10.1590/S0102-09352010000400012

43] E. Lamy, G. Graça, G. da Costa, C. Franco, F.C. E Silva, E.S. Baptista, A.V. Coelho, Proteome Sci. 8 (2010) 65. DOI: 10.1186/1477-5956-8-65

44] G. da Costa, E. Lamy, F. Capela e Silva, J. Andersen, E. Sales Baptista, A. V Coelho, J. Chem. Ecol. 34 (2008) 376–87. DOI: 10.1007/s10886-007-9413-z

45] M. Morzel, C. Truntzer, E. Neyraud, H. Brignot, P. Ducoroy, G. Lucchi, C. Canlet, S. Gaillard, F. Nicod, S. Nicklaus, N. Peretti, G. Feron, Physiol. Behav. (2017). DOI: 10.1016/j.physbeh.2017.02.005

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Quantitative proteomic analysis of the *Bacillus thuringiensis* BGSC-4AW1 strain (serovar *andalousiensis*)

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ABSTRACT

Analysis of the proteome of any *Bacillus thuringiensis* (*Bt*) strain should provide important information about mechanisms of infection, about interactions with the host organisms, and about molecular mechanisms allowing the bacterium to survive detrimental conditions. To address these important issues, we analyzed the proteome of the crystal-forming *Bacillus thuringiensis* strain BGSC-4AW1 (var. a*ndalousiensis*). This Quantitative Proteomics-based analysis revealed the presence of important proteins for cell survival and cell proliferation associated with exosporium, coat, and crystal complexes. Currently, it is not possible to discriminate among the specific sub-proteomes associated with the *Bt*'s developmental stages; however, the information provided by this proteomic analysis is potentially useful for mapping the cellular mechanisms involved in cell survival and adaptation to deleterious environmental conditions. The presence of an insecticidal toxin, as well as a cancer cell-killing Cry protein add to the spectrum of biotechnological applications of *Bacillus thuringiensis.*

Keywords: *Bacillus thuringiensis*, proteomics, systems biology, biotechnology, cancer cell-killing Cry protein

Abbreviations: *Bt*: *Bacillus thuringiensis*; ser: serovar; Cry: crystal; MS: Mass spectrometry; CC-KCP: Cancer cell-killing Cry protein; PI-PLC: phosphatidylinositol-specific phospholipase C.

1. Introduction

The Bacillus cereus group consists of six species: *B. anthracis (Ba), B. cereus (Bc), B. mycoides (Bm), B. pseudomycoides (Bp), B. thuringiensis (Bt)*, and *B. weihenstephanensis (Bw)* [1]. Of these, *Bt* has been used for a long time as a bio-pesticide for insect control [2]. The insecticidal capacity of *Bt* resides in the inclusion bodies (parasporal crystalline inclusions) which are usually formed during the sporulation stage [2, 3]. These inclusion bodies contain proteins of various forms, many of which are toxic to several insect species. Many of these toxins are known as *Bt* δ -endotoxins or Cry toxins; other *Bt* crystal proteins have cytolytic activity and are known as Cyt toxins [2].

Another group of *Bt* toxins has been reported as having specific toxicity against human cancer cells without being hemolytic [4]; these proteins have been named "Parasporins" [5]. Six parasporin types, PS1 to PS6, have being described ([6], see http://parasporin.fitc.pref.fukuoka.jp/, for nomenclature of parasporin proteins). The mode of action of *Bt* Cry toxins has been a matter of intensive research for more than three decades [7-15]. The study of the mode of action of parasporins is a nascent field, in which pore formation [6, 16] and calcium-regulated cell death [17] have been proposed as potential mechanisms for their mode of action.

Here we report the Quantitative Proteomics-based characterization of the *Bt* BGSC-4AW1 (var. *andalousiensis*) strain, including the crystal inclusions. There is a great variety of proteins enclosed in these structures, particularly three toxins in the isolated crystals, i.e., the insecticidal toxin

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Cry8Ca, the Cancer Cell-Killing Cry Protein (CC-KCP), and the phosphatidylinositol-specific phospholipase C (PI-PLC). Many of the proteins identified have specific functions associated with spore formation, coat stability, trans-membrane transport, chaperoning activity, and cell homeostasis.

2. Materials and Methods

2.1. Cell cultures and purification of crystals

Bacillus thuringiensis strain 4AW1 was obtained from the Bacillus Genetic Stock Center (BGSC, Biochemistry Department - The Ohio State University, Columbus, OH). The original code is: *Bacillus thuringiensis* subspecies *andalousiensis* T37001 (=EA10192); genotype: wild type isolate (Note: Serotype 37; isolated in Spain; antisera standard. Shotgun whole genome sequence available from http://www.ebi.ac.uk/ena/data/view/ACNG01000080.1; [18]). The filter disk obtained from the BGSC was placed in a 1.5 mL Eppendorf tube and 1.0 mL of LB medium was added and vortexed vigorously. Four hundred µL of this solution was dispersed on an LB agar plate and left to grow overnight at 28 °C in a dry incubator.

The next day, a single colony from the agar plate was transferred to a flask containing 20 mL LB medium, which, after a 12-hr growth period, was transferred into 500 mL of G-tris medium for growing and sporulation. G-tris medium (10 mM Tris-HCl, pH 7.6; $FeSO_4{\cdot}7H_2O$, 0.00005%; $CuSO_4{\cdot}5H_2O$, 0.0005%; $ZnSO_4{\cdot}7H_2O$, 0.0005%; $MnSO_4{\cdot}H_2O$, 0.005%; $MgSO_4$, 0.02%; $CaCl_2{\cdot}2H_2O$, 0.008%; K_2HPO_4, 0.05%, $(NH_4)_2$ SO4, 0.2%; Glucose, 0.1%) was prepared as previously described [19]. After five days of growth in a shaker/incubator at 28 °C and 250 rpm, the cell/spore/crystal mix was collected as described below.

The bacterial culture was centrifuged at 4,500 rpm for 10 min at 4 °C. The resulting pellet containing spores and crystals was washed once with 1M NaCl containing 1% SDS, followed by centrifugation. The resulting pellet was washed twice in 1M NaCl and then with distilled water until no foam was observed, followed by sonication for 5 min at 4 °C in a Fisher sonic dismembrator model 100 (sonication for 30 s followed by 30 s of cooling). The concentrated solution of the spore-crystal pellet was separated into crystals and spores by sedimentation using a discontinuous sucrose gradient (5 ml of 60%, 3ml of 40%, 5 ml of 30% and 5 ml of 10% in $d_{ii}H_2O$) as described [19]. The sucrose-containing tubes were centrifuged at 4,500 rpm for 30 min at 4 °C. As previously described [19], the 10 – 30% sucrose interphase was collected to obtain the inclusion crystal bodies.

The particulate contents of the 10 – 30% interphase fraction was pelleted by centrifugation and the pellet was washed with $d_{ii}H_2O$, followed by washing with 1M NaCl containing 1% SDS, followed by two washes of 1M NaCl, and with $d_{ii}H_2O$ until no foam was observed. The resulting pellet was divided into two fractions, and one of the fractions was taken for imaging by electron microscopy. The other fraction was sonicated as before, and then washed with a solution of 50:50 acetone:ethanol to remove any remaining lipid material. A fraction of this pellet was also used for imaging by electron microscopy. The final pellet was resuspended in 200 µL $d_{ii}H_2O$ and stored at 4 °C for quantitative proteomic analysis.

2.2. Electron microscopy

Ten µL of the pellet from fraction one and 10 µL of the pellet from fraction two were stained for electron microscopy as previously described [20]. Briefly, carbon-coated copper grids were glow-discharged at 300 mesh in a Harrick plasma cleaner, model PDC 32G for 90 seconds at medium power. The sample was applied to the grid and allowed to sit for 2 min, then washed with 5 drops of 2 % aqueous uranyl acetate and allowed to stain for 1 min. The stain was wicked-off with filter paper and the sample was allowed to dry for several minutes. Images were collected with a Tecnai G2 Transmission Electron Microscope (FEI, Hillsboro, OR). Digital images were taken with a Gatan Multiscan 794 camera using Gatan DM3 Software (Gatan Inc., Pleasanton, CA).

2.3. Protein solubilization for proteomic analysis

The solubilization of the crystal-containing pellets was achieved by mixing 5 µL of pellet (in $d_{ii}H_2O$) with an equal amount of Laemmli loading buffer (100 mM Tris-HCl, pH 7.0, 20 % glycerol, 4 % SDS, 0.02 % bromophenol blue; 0.05 % β-mercaptoethanol), followed by boiling for 5 min in a water bath at 95 °C. The mixture was centrifuged at maximum speed on a table-top centrifuge, and the supernatant was used to determine the presence of protein bands using sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-PAGE) with pre-cast gels (Bio-Rad, Hercules, CA). The apparent protein molecular weight was determined with "Kaleidoscope" pre-stained protein standards (Bio-Rad).

2.4. Manual trypsin "in-gel" protein digestion for protein identification by mass spectrometry

Manual "in-gel" protein digestion was done in four steps: i) destaining, ii) reduction of disulfide bridges and alkylation of resulting sulfhydryl groups, iii) proteolytic cleavage of the protein (trypsin digestion), and iv) extraction of resulting peptides. The bands of interest were excised from the Coomassie-stained gel and each gel band was cut into 1.0 x 1.0 cm gel pieces and transferred into Axygen 1.7 mL tubes. The gel pieces were washed with 100 µL HPLC-grade water (Sigma, St. Louis, MO) for 5 min at 25 °C. The water was removed and replaced with ~100 µL of 50:50 acetonitrile (ACN):25 mM ammonium bicarbonate (ABC), followed by shaking and centrifugation and removal of the destaining

solution. These steps were repeated twice. The solution was replaced with 75:25 (ACN:25 mM ABC), vortexed, centrifuged, and washed with HPLC-grade water. These two steps were repeated one more time. After washing, the solution was replaced with 100 % 25 mM ABC, shaken, and removed; 100 % ACN was added and shaken for 5 –10 min, and then removed. The last step was repeated until the gel pieces were small and white. Destained gel plugs were lyophilized for 30 min (with a hole punched in each tube cap).

Reduction was performed with DTT as follows: 25 µL of 50 mM ABC was added to each lyophilized gel piece, then 50 µL of 10 mM freshly-prepared DTT was added to each tube (the DTT stock was prepared in 100 mM ABC). The mixture was incubated at 50 °C for 30 min after which the DTT mixture was removed. For alkylation, 25 µL of 50 mM ABC was added to each gel piece, followed by 50 µL of 50 mM iodoacetamide (freshly-prepared in 100 mM ABC, and kept in the dark). The mixture was incubated in the dark for 20 min, after which the iodoacetamide solution was removed.

In-gel digestion was performed by first washing the gel plugs with 100 µL of 50 mM ABC for 5 min. The ABC was removed, and the gel plugs were washed with 100 µL ACN for five minutes. The washing step was repeated twice, the ACN was then removed, and the gel plugs were dried on a speedvac. Then 100 – 200 µL trypsin (from stock 20 µg/µL, Promega, in HCl) was added (enough to cover the gel plugs), and the tubes were kept on ice for ~50 min. Excess trypsin solution was removed, and the plugs were washed with 25 mM ABC, and finally enough 25 mM ABC was added to cover the gel pieces. The digestion was allowed to continue overnight at 37 °C on a shaker.

The peptides were extracted as follows: 50 µl ACN was added to each gel plug and shaken for 10 min at 25 °C. The combined solution was removed and the plug was transferred to a different Axygen tube. Approximately 100 µL of HPLC-grade water was added to each gel plug, and shaken in a thermomixer for 10 min at 25 °C. The solution was removed and combined with the previous extract. Then an extra 30 µL of HPLC-grade water and 50 µL of ACN were added to each gel plug, and shaken in a thermomixer for 10 min at 25 °C. The last solution was removed and combined with the two previous extracts. The last step was repeated with 100% ACN, and all the solutions were combined and dried in a speedVac.

2.5. Identification of proteins by mass spectrometry (MS)

Extracted peptides were desalted using PepClean C18 spin columns (Pierce, Rockford, IL), according to the manufacturer's instructions, and re-suspended in an aqueous solution of 0.1 % formic acid. Identification of proteins was done using reversed-phase LC-MS/MS on a 2D -nanoLC Ultra system (Eksigent Inc, Dublin, CA) coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific, San Jose, CA). The Eksigent system was configured to trap and elute peptides via an injection of ~250 fmol of sample. The trapping was performed on a 3 cm-long 100 µm i.d. C18 column while elution was performed on a 15 cm-long 75 µm i.d., 300 Å particle ProteoPep II integraFrit C18 column (New Objective Inc, Woburn, MA). The analytical separation of the tryptic peptides was achieved with a 70-min linear gradient of 2 – 10% buffer B at a 200 nL/min, where buffer A is an aqueous solution of 0.1 % formic acid and buffer B is a solution of 0.1 % formic acid in acetonitrile.

Mass spectrometric data acquisition was performed in a data-dependent manner on a hybrid LTQ-Orbitrap mass spectrometer. A full scan mass analysis on an Orbitrap (externally calibrated to a mass accuracy of < 1 ppm, and a resolution of 60,000 at m/z 400) was followed by intensity-dependent MS/MS of the 10 most abundant peptide ions. Collision induced dissociation (CID)-MS/MS was used to dissociate peptides with normalized collision energy of 35 eV, in the presence of He bath gas atoms at a pressure of 1 mTorr. The MS/MS acquisition of each precursor m/z was repeated for 30 s and subsequently excluded for 60 s. Monoisotopic precursor ion selection (MIPS) and charge state screening were enabled for triggering data-dependent MS/MS scans.

Mass spectra were processed, and peptide identification was performed using Mascot ver. 2.3 (Matrix Science Inc.) implemented on Proteome Discoverer Ver 1.3 software (Thermo-Fisher Scientific). All searches were performed against a curated *Bt* serovar *andalousiensis* data base (GSC-4AW1; downloaded from http://patricbrc.org/portal/portal/patric/Downloads?cType=taxon&cId=, selecting *Bacillus thuringiensis* serovar *andalousiensis* BGSC 4AW1 [21]). Peptide-based protein identification was done using a target-decoy approach with a false discovery rate (FDR) of 1 % [22]. A precursor ion mass tolerance of 200 ppm and a product ion mass tolerance 0.5 Da were used, with a maximum of two missed tryptic cleavages [23]. Methionine oxidation was selected as a variable modification.

2.6. Spectral counting-based quantitative proteomics

Spectral counting was performed on the Mascot DAT files using ProteoIQ: ver 2.3.02 (NuSep Inc., Athens, GA). Proteins, identified as explained above, were subjected to "probability-based" confidence measurements using an independent implementation of the statistical models Peptide and Protein Prophet deployed in Proteo IQ [24, 25]. Protein hits were filtered with a probability of 0.5 and a Mascot identity with a significant score cut-off greater than 26.

3. Results

Separation of the 10 – 30% sucrose gradient interphase produced mostly crystals Fig. 1 (B, C). The proteome of the *Bacillus thuringiensis* serovar *andalousiensis* BGSC-4AW1

strain was obtained by first identifying the proteome of the more complex structures shown in Figure 1A, and then the proteome of the purified crystals shown in Figure 1B. The complex structures were not affected by washing first with 1 M NaCl and then with $d_{ii}H_2O$. As shown in Figure 1A, these washing steps resulted in a combination of crystal-, and spore-containing structures surrounded by a membrane. These complex structures were dissolved in Laemmli loading buffer with β-mercaptoethanol, boiled for five minutes, and the proteins were separated on a 1D gel by electrophoresis (Fig. 2). As indicated in Figure 2A, the gel was cut into 10 equal bands, and each band was submitted to in-gel tryptic digestion, followed by protein identification as described under methods. Three hundred and forty-two unique proteins were identified. These proteins are presented in Table 1 (a detailed and extended table containing all the peptides and protein parameters is included in Supplemental Material S1).

The crystal inclusions were cleaned with NaCl, SDS, diiH2O, acetone, and ethanol, producing the crystals shown in Figure 1B (and inset in Fig. 1). These crystals were dissolved in Laemmli buffer with β-mercaptoethanol, boiled for five minutes and separated on a 1D PAGE gel (Fig. 2B).

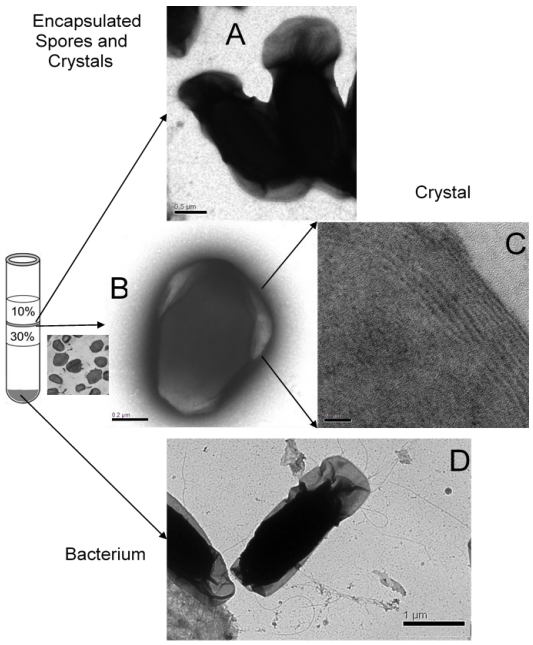

Figure 1. Purification of *Bt* ser. *andalousiesis crystals*. The diagram on the left represents a centrifugation tube with a sucrose gradient from which the 10 – 30 % interphase and the cell pellet were collected as previously described [19]. The interphase contains the structures shown in panels A and B. Panel A shows spores and crystals encapsulated in membranes that were obtained by washing these pellets with 1M NaCl; washing further with 1M NaCl, SDS, and acetone/ethanol produced the clean crystals shown in panel B (several crystals are shown in the inset next to the centrifugation tube); magnification indicates that these crystals are deposited in layers (Panel C). The pellet contained bacterium as indicated in panel D. Notice the sizes of each structure as indicated from the measuring bars at the bottom of each figure.

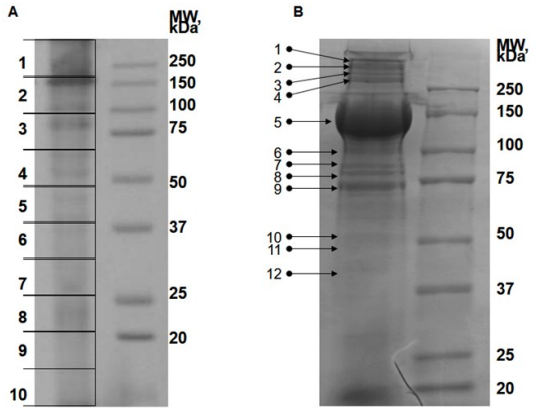

Figure 2. The fraction obtained by washing the 10 – 30 % interphase with 1.0M NaCl, and shown in Figure 1A, was mixed with 10 µL SDS loading buffer, boiled, and the proteins were separated by electrophoresis (A). This gel was cut in 10 equal bands, and each band was subjected to in-gel digestion. The crystals (Figure 1B), obtained by removing the membrane from the structure shown in Figure 1A, were dissolved in 10 µL SDS loading buffer, and boiled for five minutes. The proteins resulting from this separation were removed by cutting the bands as shown (B) and subjected to in-gel protein digestion. Resulting peptides were used to identify proteins.

As indicated in Figure 2B, the separation resulted in 12 major bands, which were excised from the gel and digested with trypsin. The peptides extracted from the gel bands were submitted to MS for protein identification, resulting in twenty-five proteins positively identified in these crystals (Table 2). Seven of these proteins have been reported as structural components of the exosporium on *Bacillus subtilis* preparations [26]. By far the most abundant proteins in these crystalline formations (as calculated from the intensity of the bands in the SDS-PAGE gel (Fig. 2B), and from peptide counting (Table 2), were the pesticidal crystal protein Cry8Ca (26 unique), the phosphatidylinositol-specific phospholipase C (PIPLC, 30 peptides), and the Cancer Cell-Killing Cry Protein (CC-KCP, 22 peptides). The characteristic mass spectra for these three proteins are shown in Figure 3 (a more detailed table with signal intensities and peptides is included in Supplemental Material S2) .

4. Discussion

Knowing the proteome of the crystal inclusions of any of the multiple *Bt* strains is of great importance because this proteome determines the specificity of the toxins, the interactions with the host organism, the virulence of the bacterium, and its capacity to survive deleterious environmental conditions. Many *Bt* strains produce crystal inclusions during their sporulation stage. Presumably, these crystals are responsible of triggering mechanisms that elicit survival responses to detrimental environmental conditions [3, 27-30].

The cellular proteome is dynamic, and there is not a simple way to describe "the proteome" per se; the proteomic

Table 1. *Bacillus thuringiensis* ser. *andalousiensis* (BGSC-4AW1) proteome.

Accession # *	Protein Name †	Coverage	Uniprot Accession Number ‡	Unique peptides
26130616	Heat shock protein 60 family chaperone GroEL	44.67	C3FXI3_BACTU	18
26130562	Alanine racemase	36.76	C3FXG9_BACTU	15
26137067	Spore cortex-lytic enzyme, N-acetylglucosaminidase SleL	37.21	C3G3U6_BACTU	15
26135399	Phage lysin, glycosyl hydrolase, family 25	20.54	*Q637L8_BACCZ	14
26137609	ATP-dependent hsl protease ATP-binding subunit HslU	27.65	*C3I4U8_BACTU	12
26137169	Aldehyde dehydrogenase	35.63	C3G012_BACTU	11
26138644	Chaperone protein DnaK	28.97	C3G8C2_BACTU	11
26130540	4-hydroxyphenylpyruvate dioxygenase	29.30	C3FXF9_BACTU	10
26139154	Acetyl-coenzyme A synthetase	20.24	C3G997_BACTU	9
26135459	Acetoin dehydrogenase E1 component beta-subunit	31.40	*D5TMV5_BACT1	8
26140541	ATP synthase alpha chain	20.12	C3GB10_BACTU	7
26137175	Inosine-uridine preferring nucleoside hydrolase	32.91	C3G468_BACTU	7
26141141	N-acetylmuramoyl-L-alanine amidase	25.90	C3G524_BACTU	7
26130266	Translation elongation factor G	15.03	C3FX39_BACTU	7
26135461	Dihydrolipoamide acetyltransferase component (E2) of acetoin dehydrogenase complex	17.00	**Q97QN9_STRPN	6
26138664	forespore-specific protein, putative	34.11	*Q6HD99_BACHK	6
26137107	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)	17.08	C3FXF4_BACTU	6
26132353	Oligopeptide transport ATP-binding protein OppF (TC 3.A.1.5.1)	28.94	C3FXB7_BACTU	6
26137723	Pyruvate dehydrogenase E1 component alpha subunit	23.99	C3G7D7_BACTU	6
26137925	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	9.95	*C3CND0_BACTU	5
26140537	ATP synthase beta chain	21.96	C3GB10_BACTU	5
26141005	Dehydrogenase	15.37	*C3DYK1_BACTU	5
26134329	Methylmalonate-semialdehyde dehydrogenase	13.79	C3G373_BACTU	5
26140559	Serine hydroxymethyltransferase	16.71	C3GB19_BACTU	5
26130268	Translation elongation factor Tu	16.96	C3FX40_BACTU	5
26137565	3-oxoacyl-[acyl-carrier protein] reductase	28.86	C3G634_BACTU	4
26140715	alternate gene name: ipa-62r	28.28		4
26131779	Long-chain-fatty-acid--CoA ligase	12.55	C3FZH6_BACTU	4
26130278	LSU ribosomal protein L2p (L8e)	21.01	*C3FX36_BACTU	4
26132905	N-acetylmuramoyl-L-alanine amidase, family 2	34.09	C3FYZ3_BACTU	4
26140201	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase	17.96	C3FYX5_BACTU	4
26131657	Polypeptide composition of the spore coat protein CotJC	33.86	*D5TQU6_BACT1	4
26136306	Possible response regulator aspartate phosphatase	13.48	A0RGR9_BACAH	4
26137725	Pyruvate dehydrogenase E1 component beta subunit	16.62	C3G7D6_BACTU	4
26132289	response regulator aspartate phosphatase	13.81	C3G382_BACTU	4
26130284	SSU ribosomal protein S3p (S3e)	22.83	*Q3EJF8_BACTI	4
26136897	Transketolase	8.68	C3G6G6_BACTU	4
26134341	2-methylcitrate synthase	12.87	*Q3EM07_BACTI	3
26138258	6,7-dimethyl-8-ribityllumazine synthase	26.14	C3G7S7_BACTU	3
26131175	Alanine dehydrogenase	12.20	C3FY87_BACTU	3
26140539	ATP synthase gamma chain	17.13	C3GB09_BACTU	3
26138646	Chaperone protein DnaJ	10.51	C3G8C1_BACTU	3
26139305	Citrate synthase (si)	9.42	C3G2R2_BACTU	3
26134974	Cysteine dioxygenase	29.69	C3G3I8_BACTU	3
26138156	Dihydrolipoamide dehydrogenase of branched-chain alpha-keto acid dehydrogenase	8.25		3
26139637	DUF124 domain-containing protein	17.69	C3G9U8_BACTU	3
26132379	Enoyl-[acyl-carrier-protein] reductase [NADH]	19.53	C3FZV0_BACTU	3

26138156	Dihydrolipoamide dehydrogenase of branched-chain alpha-keto acid dehydrogenase	8.25		3
26139637	DUF124 domain-containing protein	17.69	C3G9U8_BACTU	3
26132379	Enoyl-[acyl-carrier-protein] reductase [NADH]	19.53	C3FZV0_BACTU	3
26138854	Enoyl-CoA hydratase	16.67	C3G2S0_BACTU	3
26137611	GTP-sensing transcriptional pleiotropic repressor codY	13.90	C3G743_BACTU	3
26139551	hydrolase, alpha/beta fold family	14.87	A0RK75_BACAH	3
26139899	Iron-sulfur cluster assembly protein SufD	11.86	*F2H0D8_BACTU	3
26130246	LSU ribosomal protein L11p (L12e)	23.40	**Q3EK30_BACTI	3
26130282	LSU ribosomal protein L22p (L17e)	40.71	*A0R8I8_BACAH	3
26132911	NAD-specific glutamate dehydrogenase	7.71	*D5TUX8_BACT1	3
26133615	Peptidoglycan N-acetylglucosamine deacetylase	26.55	C3G1S2_BACTU	3
26140199	Phosphoglycerate kinase	12.18	C3GAJ4_BACTU	3
26130214	Putative ATP:guanido phosphotransferase YacI	10.45	C3FX13_BACTU	3
26133329	Putative symporter YjcG	7.17		3
26137249	response regulator, putative	10.99	*F0PW98_BACT0	3
26140713	Spore cortex-lytic enzyme CwlJ	32.86	*Q3F0G3_BACTI	3
26137163	Squalene--hopene cyclase	8.27	C3G636_BACTU	3
26130300	SSU ribosomal protein S8p (S15Ae)	30.30	**A0RBV3_BACAH	3
26140947	Tellurium resistance protein TerD	19.59	C3GBK3_BACTU	3
26131185	transcriptional regulator/TPR domain protein	7.11	*F0PUF9_BACT0	3
26133927	Tryptophan 2-monooxygenase	7.35	***	3
26139104	UDP-N-acetylmuramate--alanine ligase	9.40	C3G9C2_BACTU	3
26137481	Zinc protease	9.43	C3G6T9_BACTU	3
26138061	2,3-diketo-5-methylthiopentyl-1-phosphate enolase	6.04	C3G7K1_BACTU	2
26132843	2-isopropylmalate synthase	7.11	C3G0C8_BACTU	2
26137345	2-oxoglutarate oxidoreductase, beta subunit	8.68	*D5TUR1_BACT1	2
26134331	3-hydroxyisobutyrate dehydrogenase	12.16	C3G2R7_BACTU	2
26134325	3-hydroxyisobutyryl-CoA hydrolase	6.84	*Q3EKW7_BACTI	2
26137991	3-ketoacyl-CoA thiolase @ Acetyl-CoA acetyltransferase	9.21	*Q6HBP8_BACHK	2
26135847	5-Enolpyruvylshikimate-3-phosphate synthase	6.06	C3G4C0_BACTU	2
26139295	6-phosphofructokinase	9.09	C3G931_BACTU	2
26132847	Acetolactate synthase small subunit	13.61	C3G0C6_BACTU	2
26139961	acyl-CoA dehydrogenase	5.89	C3G2R6_BACTU	2
26130796	Alkyl hydroperoxide reductase protein C	15.51	C3FXT6_BACTU	2
26140225	ATP-dependent Clp protease proteolytic subunit	7.77	C3GAK6_BACTU	2
26133091	Biosynthetic Aromatic amino acid aminotransferase beta @ Histidinol-phosphate aminotransferase	7.03	*Q3EU27_BACTI	2
26135839	Chorismate mutase I / 2-keto-3-deoxy-D-arabino-heptulosonate-7-phosphate synthase I beta	11.73	C3G4C4_BACTU	2
26139749	conserved domain protein	11.34	**Q630Y0_BACCZ	2
26136258	D-3-phosphoglycerate dehydrogenase	5.90	C3G515_BACTU	2
26130868	Delta-1-pyrroline-5-carboxylate dehydrogenase	5.44	*Q3EJ13_BACTI	2
26137729	Dihydrolipoamide dehydrogenase of pyruvate dehydrogenase complex	6.38	*Q3ESR0_BACTI	2
26140193	Enolase	8.82	C3GAJ1_BACTU	2
26136157	FOG: TPR repeat	16.67	C3G8K3_BACTU	2
26135195	Glucose dehydrogenase [pyrroloquinoline-quinone]	7.35	*Q3EMX4_BACTI	2
26130070	Inosine-5'-monophosphate dehydrogenase	7.19	C3FWU7_BACTU	2
26135959	Inosine-uridine preferring nucleoside hydrolase in exosporium	11.92	C3G468_BACTU	2
26139307	Isocitrate dehydrogenase [NADP]	5.81	C3G924_BACTU	2
26139937	lipoprotein, putative	13.71	C3FXA6_BACTU	2
26133679	L-lactate dehydrogenase	6.69	C3GA71_BACTU	2

26130338	LSU ribosomal protein L13p (L13Ae)	15.86	*Q3EK30_BACTI	2
26130248	LSU ribosomal protein L1p (L10Ae)	13.48	**F9ZM33_9GAMM	2
26139347	LSU ribosomal protein L20p	17.80	**F9ZSH6_9GAMM	2
26139002	LSU ribosomal protein L27p	27.08	***F9XYU2_BIFBR	2
26130296	LSU ribosomal protein L5p (L11e)	13.97	**F9ZM55_9GAMM	2
26130302	LSU ribosomal protein L6p (L9e)	13.97	***G0AGC3_9BURK	2
26137959	Maltose/maltodextrin transport ATP-binding protein MalK	6.56	***G0DAV7_ECOLX	2
26138788	MaoC family protein	17.60	*A0RIX7_BACAH	2
26139651	Naphthoate synthase	8.46	C3G9U1_BACTU	2
26140743	O-acetylhomoserine sulfhydrylase / O-succinylhomoserine sulfhydrylase	6.94	C3GBA8_BACTU	2
26132333	Oligoendopeptidase F	4.61	C3FZS6_BACTU	2
26132355	Oligopeptide transport ATP-binding protein OppD (TC 3.A.1.5.1)	7.49	C3FXB7_BACTU	2
26137351	Outer spore coat protein E	13.89	**E5W466_9BACI	2
26130462	oxidoreductase of aldo/keto reductase family, subgroup 1	7.94	**F7SFD7_LACJH	2
26139351	Peptidase, M42 family	5.26	**F0PL34_BACT0	2
26136256	Phosphoserine aminotransferase	6.39	C3G516_BACTU	2
26137655	Polyribonucleotide nucleotidyltransferase	3.93	C3G723_BACTU	2
26137767	Predicted ATPase related to phosphate starvation-inducible protein PhoH	5.66	***D6D447_9BACE	2
26139214	Protein ecsC	10.83	*Q3EZS0_BACTI	2
26137743	Protein-glutamine gamma-glutamyltransferase	10.87	C3G7C8_BACTU	2
26134986	putative cytochrome P450 hydroxylase	7.06	***A4FDF8_SACEN	2
26130074	Pyridoxine biosynthesis glutamine amidotransferase, synthase subunit	8.14	***G0V494_9CLOT	2
26137775	Pyruvate carboxyl transferase	2.09	**F5VEX4_9LACO	2
26138658	Quinolinate phosphoribosyltransferase [decarboxylating]	10.11	C3G9C3_BACTU	2
26140875	Single-stranded DNA-binding protein	11.56	C3GBG8_BACTU	2
26130774	Spore coat protein B	18.12	C3FXU6_BACTU	2
26135521	Spore coat protein F	17.50	C3G208_BACTU	2
26130132	Sporulation-specific protease YabG	8.36	C3FWX6_BACTU	2
26130270	SSU ribosomal protein S10p (S20e)	31.37	**F9ZM42_9GAMM	2
26130324	SSU ribosomal protein S11p (S14e)	21.71	**F9ZMC9_9GAMM	2
26138628	SSU ribosomal protein S20p	34.12	*Q3EJD4_BACTI	2
26130306	SSU ribosomal protein S5p (S2e)	14.46	**F9ZM59_9GAMM	2
26132873	Stage IV sporulation protein A	7.85	C3G0N6_BACTU	2
26137595	Succinyl-CoA ligase [ADP-forming] beta chain	7.51	C3G6V7_BACTU	2
26130210	Transcriptional regulator CtsR	13.73	*F0PQ24_BACT0	2
26137473	Unspecified monosaccharide ABC transport system, substrate-	7.04	***F3Y8A0_MELPT	2
26137713	Zn-dependent hydrolase, RNA-metabolising, CPSF 73 kDa analog	5.41	C3G129_BACTU	2
26134339	2-methylcitrate dehydratase	2.51	C3G2R3_BACTU	1
26132617	3-hydroxybutyryl-CoA dehydratase	8.16	C3FZ01_BACTU	1
26140615	3-hydroxybutyryl-CoA dehydrogenase	6.36	C3GB46_BACTU	1
26132841	3-isopropylmalate dehydrogenase	3.67	C3G0C9_BACTU	1
26140617	3-ketoacyl-CoA thiolase [isoleucine degradation]	3.82	*A0RKJ8_BACAH	1
26136135	3-Oxoadipate enol-lactonase, alpha/beta hydrolase fold family	4.33	*F0PJ99_BACT0	1
26138874	4-hydroxybenzoyl-CoA thioesterase family active site	5.41	***C6X3K5_FLAB3	1
26130956	A/G-specific adenine glycosylase	1.64	*D5TNJ4_BACT1	1
26130932	ABC transporter, permease protein, putative	4.21	C3G2K1_BACTU	1
26139208	Acetate kinase	2.77	C3G971_BACTU	1
26134726	Acetoacetyl-CoA synthetase [leucine]	1.86	***D4YLV6_9MICO	1
26136545	acetyltransferase, GNAT family	11.84	C3G3Q3_BACTU	1

26139192	Acyl-coenzyme A synthetases/AMP-(fatty) acid ligases, YtcI homolog	3.03	***A4VZ54_STRS2	1
26140863	Adenylosuccinate synthetase	1.40	C3GBG2_BACTU	1
26134287	alcohol dehydrogenase, iron-containing	3.00	C3G8H9_BACTU	1
26138176	Amino acid ABC transporter, amino acid-binding protein	4.63	*D5TZE9_BACT1	1
26134834	Aminoacyl-histidine dipeptidase (Peptidase D)	3.27	C3G359_BACTU	1
26138127	Arginine pathway regulatory protein ArgR, repressor of arg regulon	7.38	***F1W4T9_9BURK	1
26139226	Argininosuccinate lyase	4.33	C3G963_BACTU	1
26130844	Aspartyl-tRNA(Asn) amidotransferase subunit A @ Glutamyl-tRNA(Gln) amidotransferase subunit A	3.30	C3FXR2_BACTU	1
26132833	ATP phosphoribosyltransferase catalytic subunit	9.48	C3G0D3_BACTU	1
26138944	ATP-dependent Clp protease ATP-binding subunit ClpX	2.15	C3G8S7_BACTU	1
26130216	ATP-dependent Clp protease, ATP-binding subunit ClpC / Negative regulator of genetic competence clcC/mecB	1.60	***D3QG46_STALH	1
26134176	ATP-dependent RNA helicase YfmL	3.08	**YFML_BACSU	1
26136015	Bacillus cereus group-specific protein, uncharacterized	10.34	**Q6HQP9_BACAN	1
26130636	Beta-galactosidase	4.17	*C3HT03_BACTU	1
26136410	bifunctional P-450:NADPH-P450 reductase 1	1.03	*F0PJ37_BACT0	1
26138109	Biotin carboxylase of acetyl-CoA carboxylase	3.11	C3G932_BACTU	1
26133669	Branched-chain amino acid transport ATP-binding protein LivF (TC 3.A.1.4.1)	6.87	*Q3EWR4_BACTI	1
26132059	Catalase	3.07	C3FZM4_BACTU	1
26137519	cation-transporting ATPase, E1-E2 family	1.77	C3FXW8_BACTU	1
26130970	Cell division inhibitor	4.98	*D5TNI9_BACT1	1
26137857	Cell division protein FtsZ	3.65	*D5TVH6_BACT1	1
26138942	Cell division trigger factor	5.41	C3G8S8_BACTU	1
26132299	CMP-binding-factor 1	5.10	*C3GT26_BACTU	1
26139006	COG0536: GTP-binding protein Obg	3.50	C3G8P6_BACTU	1
26137185	conserved repeat domain protein	0.36	A0RH48_BACAH	1
26137435	Copper-translocating P-type ATPase	0.99	*A0RH48_BACAH	1
26138770	Cystathionine gamma-lyase	5.31	*A0RIY5_BACAH	1
26130946	Cysteinyl-tRNA synthetase related protein	6.82	*SYC_BACHK	1
26130746	Cystine-binding periplasmic protein precursor	8.02	**D3QWU1_ECOCB	1
26137785	Cytochrome c oxidase polypeptide III	4.35	*D5TVL2_BACT1	1
26138898	Cytosine deaminase	3.22	*D5TJA1_BACT1	1
26132697	D-alanine--poly(phosphoribitol) ligase subunit 1	3.17	*DLTA_BACAH	1
26131761	Deblocking aminopeptidase	3.15	*D5TRT4_BACT1	1
26135759	Delta5 acyl-lipid desaturase	1.75	C3G4G3_BACTU	1
26137491	Dihydrodipicolinate synthase	5.14	C3G6U9_BACTU	1
26137727	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	3.50	Q3ESR0_BACTI	1
26138840	DinB family protein	13.77	**A7GTJ0_BACCN	1
26137665	Dipicolinate synthase subunit B	9.59	C3G6V2_BACTU	1
26130220	DNA integrity scanning protein disA	3.36	C3FX16_BACTU	1
26130056	DNA polymerase III beta subunit	2.90	*RPOB_BACHK	1
26134553	DNA-binding protein HBsu	12.22	**D3QCS2_STALH	1
26130326	DNA-directed RNA polymerase alpha subunit	6.37	*RPOA_BACAH	1
26139763	Dolichol-phosphate mannosyltransferase MtrA	2.09	**C7UHF8_ENTFA	1
26132381	dTDP-4-dehydrorhamnose reductase	4.23	C3FZU9_BACTU	1
26135215	DUF1696 domain-containing protein	12.12	**F7RKW6_9GAMM	1
26139967	Enoyl-CoA hydratase	2.14	*A0RLC8_BACAH	1
26134868	Exosporium protein F	5.99	*Q81TN4_BACAN	1
26140593	Fructose-1,6-bisphosphatase, GlpX type	5.30	*D5TQJ5_BACT2	1
26133385	Fumarate hydratase class II	3.25	C3G187_BACTU	1

26139120	General stress protein	8.24	*D5TKV6_BACT1	1
26138121	Geranyltranstransferase (farnesyldiphosphate synthase)	2.03	C3G7Z3_BACTU	1
26130388	Glucosamine--fructose-6-phosphate aminotransferase [isomerizing]	2.00	C3FX89_BACTU	1
26139711	Glucose-6-phosphate isomerase	4.44	C3G9W1_BACTU	1
26138966	Glutamate-1-semialdehyde aminotransferase	5.13	C3G8R6_BACTU	1
26131267	Glutamine ABC transporter, periplasmic glutamine-binding protein	5.80	**Q98FF6_RHILO	1
26139088	Glutamyl aminopeptidase; Deblocking aminopeptidase	4.48	C3G9D0_BACTU	1
26138470	Glycine dehydrogenase [decarboxylating] (glycine cleavage system	2.68	*C3CNZ8_BACTU	1
26139745	Glycyl-tRNA synthetase	2.40	C3G9X8_BACTU	1
26132979	GTP cyclohydrolase I type 1	5.29	C3G0N8_BACTU	1
26132885	GTP-binding protein EngA	2.75	C3G0N0_BACTU	1
26138520	GTP-binding protein Era	3.32	C3G8A9_BACTU	1
26138640	Heat-inducible transcription repressor HrcA	6.21	C3G8C4_BACTU	1
26132235	Histidine triad (HIT) nucleotide-binding protein, similarity with	8.33	*HINT_CAEEL	1
26132831	Histidinol dehydrogenase	2.80	*HISX_BACHK	1
26132817	Histidinol-phosphatase	3.25	C3G2F7_BACTU	1
26138708	Histidyl-tRNA synthetase	2.52	*SYH1_BACHK	1
26140739	Homoserine dehydrogenase	3.02	*D5TRH2_BACT1	1
26133603	Homoserine kinase	6.40	C3G1S8_BACTU	1
26139467	Homoserine O-acetyltransferase	4.28	C3G9H6_BACTU	1
26130180	Hypoxanthine-guanine phosphoribosyltransferase	6.11	C3FWZ8_BACTU	1
26140901	Inner membrane protein translocase component YidC, short form OxaI-like	4.31	**C1AJ61_MYCBT	1
26131102	Inner membrane protein YqiK	3.44		1
26134738	Isovaleryl-CoA dehydrogenase	2.89	**C3VMZ0_BACPU	1
26138234	Lipase/Acylhydrolase with GDSL-like motif	5.79	C3GBA3_BACTU	1
26137939	lipoprotein, putative	6.36	C3FXQ7_BACTU	1
26132303	Macrocin O-methyltransferase	4.63	D5TTE7_BACT1	1
26141217	Mannose-6-phosphate isomerase	9.52	C3GAX9_BACTU	1
26133083	Menaquinone-cytochrome C reductase iron-sulfur subunit	7.65	C3G0P9_BACTU	1
26133081	Menaquinone-cytochrome c reductase, cytochrome B subunit	7.59	C3G0Q0_BACTU	1
26130972	Metallo-dependent hydrolases, subgroup C	5.32		1
26134728	Methylcrotonyl-CoA carboxylase carboxyl transferase subunit	2.34		1
26134337	Methylisocitrate lyase	3.31	A0RDV8_BACAH	1
26140235	Mutator mutT protein (7,8-dihydro-8-oxoguanine-triphosphatase)	7.24	C3FY36_BACTU	1
26136436	N-acyl-L-amino acid amidohydrolase	3.24	C3G5A7_BACTU	1
26139198	NAD kinase	6.37	Q3EUG8_BACTI	1
26140051	NADH dehydrogenase	4.59	C3FXT5_BACTU	1
26137453	NADH-dependent butanol dehydrogenase A	4.39	A0RH94_BACAH	1
26131183	Nicotinate phosphoribosyltransferase	2.46	C3G9C3_BACTU	1
26134116	Nitrite reductase [NAD(P)H] large subunit	1.37	*D5TY66_BACT1	1
26132567	oligoendopeptidase F, putative	2.66	C3FZS6_BACTU	1
26132359	Oligopeptide transport system permease protein OppB (TC 3.A.1.5.1)	4.21	C3FYF1_BACTU	1
26132357	Oligopeptide transport system permease protein OppC (TC 3.A.1.5.1)	3.55	C3G2Y2_BACTU	1
26138318	outer membrane protein CC2294	7.62		1
26135311	Penicillin-binding protein	5.00	C3G2H1_BACTU	1
26137317	peptidase, M16 family	17.14	Q3ELP4_BACTI	1
26132119	peptidase, M48 family	2.61	C3DCG3_BACTU	1
26132615	phaP protein	5.75	Q3EJ17_BACTI	1
26138798	Phenylalanine-4-hydroxylase - Long	2.57	A0RIX2_BACAH	1

26132825	Phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	7.11	HIS4_BACAH	1
26134918	Possible caffeoyl-CoA O-methyltransferase	8.25	*A0RE47_BACAH	1
26132773	Protein LiaH, similar to phage shock protein A	6.36	‡C3G9P8_BACTU	1
26130998	PTS system, N-acetylglucosamine-specific IIB component / PTS	2.62	C3FXZ7_BACTU	1
26139297	Pyruvate kinase	2.39	C3G930_BACTU	1
26130148	Ribose-phosphate pyrophosphokinase	4.10	C3FWY4_BACTU	1
26140307	Ribosomal subunit interface protein	6.11	Q6HB98_BACHK	1
26141003	RNA polymerase sigma-70 factor, ECF subfamily	4.15	C3GBN1_BACTU	1
26137861	RNA polymerase sporulation specific sigma factor SigE	6.28	C3G8E8_BACTU	1
26138982	Rod shape-determining protein MreB	5.31	C3G8Q8_BACTU	1
26139481	S-adenosylmethionine synthetase	3.76	C3G9L2_BACTU	1
26139190	Small acid-soluble spore protein, alpha/beta family, SASP_5	21.54	*F0PTE1_BACT0	1
26135122	Spore cortex-lytic enzyme, lytic transglycosylase SleB	7.11	C3G3U6_BACTU	1
26141029	Spore germination protein GerKC	2.81	*F0PIP2_BACT0	1
26130322	SSU ribosomal protein S13p (S18e) [Bacillus thuringiensis serovar andalousiensis BGSC 4AW1] - [locus|VBIBacThu67491_3297|]	9.09	*Q3EJF8_BACTI	1
26132897	SSU ribosomal protein S1p	3.40	*Q3ENL3_BACTI	1
26138508	Stage IV sporulation protein	1.75	C3G8Q3_BACTU	1
26137339	Stage V sporulation protein required for dehydratation of the spore core and assembly of the coat (SpoVS)	19.77	*Q6HJH0_BACHK	1
26138870	Succinate dehydrogenase iron-sulfur protein	6.32	*Q6HD09_BACHK	1
26130832	Succinate-semialdehyde dehydrogenase [NADP+]	2.48	C3G2M0_BACTU	1
26137083	Succinyl-CoA synthetase, alpha subunit-related enzymes	9.49	*D5TTV7_BACT1	1
26132957	Superoxide dismutase [Fe] @ Exosporium SOD	4.61	‡C3G885_BACTU	1
26135303	ThiJ/PfpI family protein	8.42	A0RA98_BACAH	1
26139204	Thiol peroxidase, Tpx-type	7.83	C3G973_BACTU	1
26137055	Thiol:disulfide oxidoreductase related to ResA	5.24	*RESA_BACHK	1
26139793	Thioredoxin reductase	5.78	C3GAL3_BACTU	1
26139341	Threonyl-tRNA synthetase	1.40	C3G907_BACTU	1
26140591	Transcription termination factor Rho	2.60	C3GB35_BACTU	1
26135155	Transcriptional regulator, AraC family	10.69	C3G9S1_BACTU	1
26138402	Transcriptional regulator, ArsR family	11.46	C3G4F2_BACTU	1
26137615	Translation elongation factor Ts	3.73	C3G741_BACTU	1
26141297	Transposase	8.33	C3GBR5_BACTU	1
26140197	Triosephosphate isomerase	5.18	C3GAJ3_BACTU	1
26136541	Ubiquinone/menaquinone biosynthesis methyltransferase UBIE	2.39	C3FYB2_BACTU	1
26138546	VrrA protein	6.80	C3G896_BACTU	1

*: Accession number from http://patricbrc.org
†: Protein name from http://patricbrc.org
‡: Accession number from http://www.uniprot.org

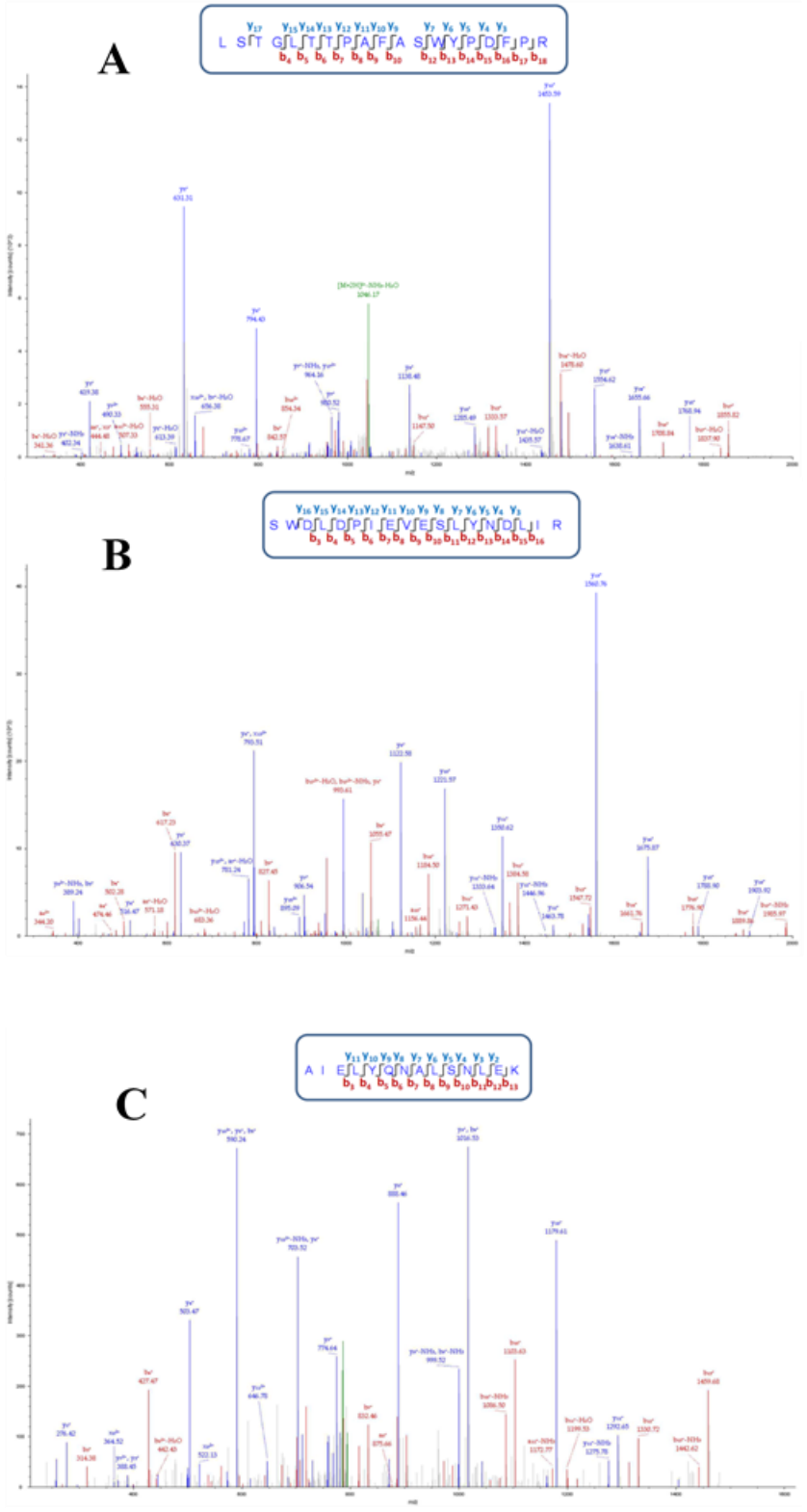

Figure 3. A doubly-charged peptide representing (A) Phosphatidylinositol-specific phospholipase C (PI-PLC); (B) Cry8Ca insecticidal protein; and (C) Cancer cell-killing Cry protein (CC-KCP). b- and y-types fragment ions for each sequence are shown in the sequence map above each MS/MS spectrum. All of the identified peptides along with the protein IDs and the protein parameters are included in Supplemental data S1.

Table 2. The proteome of purified crystal inclusions from *Bt* ser. *andalousiensis* (BGSC-4AW1) .

Accession Number	Protein name	Peptide Counts	Coverage	Number of Unique peptides	MW [kDa]	Calc. pI
26141719	Phosphatidylinositol-specific phospholipase C	334	32.38	30	142.9	5.85
26141725	Pesticidal crystal protein cry8Ca	384	43.80	26	87.8	5.97
26141099	cancer cell-killing Cry protein	254	34.21	22	95.5	5.54
26141097	hypothetical protein	89	27.56	9	68.5	5.20
26138644	Chaperone protein DnaK	30	20.79	8	65.7	4.74
26137169	Aldehyde dehydrogenase	10	19.23	8	53.7	5.45
26137067	Spore cortex-lytic enzyme, N-acetylglucosaminidase SleL	19	19.77	7	48.1	9.20
26130562	Alanine racemase	24	21.59	7	57.4	4.84
26130616	Heat shock protein 60 family chaperone GroEL		9.56	4	43.6	5.73
26138942	Cell division trigger factor		8.47	3	47.2	4.58
26137105	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA (TC 3.A.1.5.1)		2.28	2	63.9	8.31
26140541	ATP synthase alpha chain		3.39	2	54.6	5.44
26140537	ATP synthase beta chain		5.33	2	51.2	5.05
26141301	hypothetical protein		7.94	2	29.2	4.45
26130268	Translation elongation factor Tu		7.34	2	43.5	6.02
26135461	Dihydrolipoamide acetyltransferase component (E2) of acetoin dehydrogenase complex		4.50	2	42.9	5.01
26137435	Copper-translocating P-type ATPase		0.99	1	86.7	5.74
26130266	Translation elongation factor G		1.88	1	76.3	4.98
26130904	hypothetical protein		1.35	1	51.3	9.07
26137611	GTP-sensing transcriptional pleiotropic repressor codY		3.09	1	28.8	5.12
26130296	LSU ribosomal protein L5p (L11e)		4.47	1	20.2	9.70
26135447	hypothetical protein		4.76	1	14.8	9.17
26134587	Exosporium protein D		9.68	1	40.3	8.32
26132369	Exosporium protein F		5.99	1	17.6	5.77
26138646	Chaperone protein DnaJ		3.23	1	17.4	4.49

content of a cell is time- and environment-dependent, therefore we are limited to describing the proteome under specific conditions. In the case of *Bt*, it should be extremely useful knowing the proteome under life-sustaining conditions, and how this proteome changes when the bacterium has to adapt to food-depleted, or extreme environmental conditions, which ultimately result in spore development, and/or formation of crystalline inclusion bodies.

The *Bt* ser. *andalousiensis* proteome presented here points to sets of proteins that may be involved in multiple functions associated with cell growth, including spore formation, spore, coat and exosporium functions, and crystal formation. These protein sets may also be part of the protein mechanisms associated with cellular adaptation to nutrient depletion and adaptation to changes in environmental conditions. The proteomic analysis resulted in the confident identification of 342 distinct proteins (130 of these proteins were identified with two or more peptides, Table 1. See supplemental data for detailed peptide information). Many of these proteins are involved in exosporium, spore, and coat functions, as well as cellular function (Tables 1 and 2; and Fig. 4).

Some proteins presented in Table 1 deserve special attention. Using annotated databases (http://www.uniprot.org/; NCBI, and ExPaSy), we classified 18 proteins as belonging to the spore/coat/exosporium complex (Table 2 and Figure 4A; extended results in Supplemental Material S2). Twenty-nine proteins (Fig. 4 and Supplemental Material S1) are involved in cell division; these proteins must have functions associated with normal cellular growth and development, as well as re-adaptation following a period of deleterious conditions. Two groups of proteins, the Large and Small Ribosomal Subunits (LSU, and SSU, with 14 and 12 proteins, respectively) were found in this study, suggesting that their high numbers should provide the bacterium with the ability to trigger cell growth and cell division when the conditions become favorable.

Identification of all the proteins in this study, and analysis of the protein interaction networks were performed by interrogating NCBI and the "Patric" database (http://patricbrc.org/). Proteins were mapped to the identical proteins or to their closest match in the NCBI database via UNIPROT (http://www.uniprot.org). UNIPROT accession

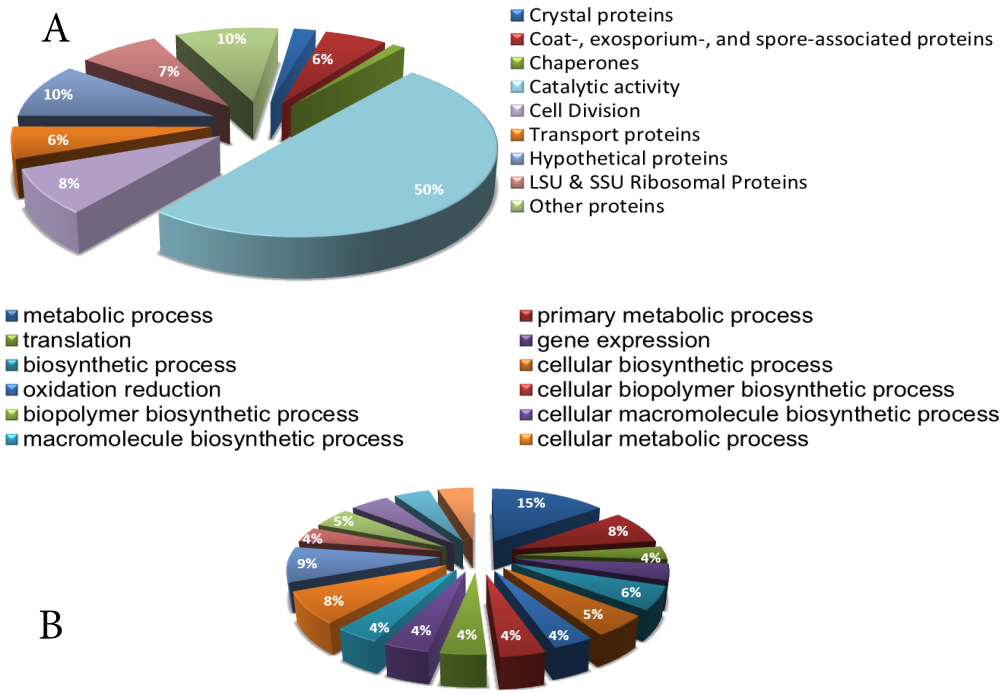

Figure 4. Pie distribution of *Bt* ser. *andalousiensis* proteins.

numbers for most of the proteins found are indicated in Table 1, column 3. Many of these proteins are still not described in this database. In Table 1, one asterisk (*) indicates that the protein was not found in *andalousiensis*; however, it has been identified in another *Bt* strain; two asterisks (**) indicate that the protein was not found in *Bt*, but it was found in other bacilli species, such as *Bacillus cereus*; and three asterisks (***) indicate a protein found in a non-*Bt*-related organism. Two proteins were not found in any database.

The proteins found in the exosporium/crystal complex were classified according to the information available in UNIPROT. The exact number of proteins that are specifically associated with the crystals may be difficult to elucidate because many non-specific proteins become entrapped either during the inclusion-body formation process, or during the crystal purification steps. We therefore counted the peptides identified by MS, and considered only those proteins with 7 or more peptides identified in two independent experiments as being uniquely associated with the crystals. Most of the proteins in the complexes possess catalytic function (50%, Fig. 4A), while 5 (1.5 %) are chaperones. A functional classification of proteins was performed using the online server "Pandora" (http://www.pandora.cs.huji.ac.il/) [31], Fig. 4B.

A large portion of the crystal proteome consists mainly of (See Figure 2B, Table 2; and extended information in Supplemental Material S2 and S3) pesticidal crystal protein Cry8Ca (Accession # from http://patricbrc.org, 261417259; the UNIPROT accession numbers are included in Table 1), phosphatidylinositol-specific phospholipase C (PI-PLC, 26141719), and cancer cell-killing Cry protein (CC-KCP, 26141099). These three proteins represented the majority of the protein content in the crystal and were consistently found in several independent crystal preparations. The remaining significant (>7 peptides in two independent MS runs) proteins in the crystal included hypothetical protein (26141097), chaperone protein DnaK (26138644), aldehyde dehydrogenase (26137169), and spore cortex-lytic enzyme, N-acetylglucosaminidase SleL (26137067). Five of the proteins found in our crystal preparations were previously found as constitutive proteins of the *Bacillus subtilis* exosporium [26] (Supplemental Material S1, and S2).

The presence of three toxins with a wide spectrum of action such as PI-PLC, Cry8Ca, and CC-KCP confers this bacterium the possibility of poisoning a large variety of target organisms. Specifically, Cry8Ca has shown toxicity against cupreous chafer (*Anomala cuprea, coleoptera Scarabaeidae* [32] and *Anomala corpulenta* [33]), *Bt* ser. *andalouisiensis* has shown toxic activity against the coleopteran *Tenebrio molitor* [34], and our preliminary studies on glioblastoma cells (data not included here) shows that purified lysates of BGSC-4AW1 are able to dissolve these cells in culture .

5. Concluding Remarks

The proteome of the *Bt* ser. *andalousiensis* presented here provides data that should be useful for determining the specificity of the bacterium towards target organisms, for understanding the capacity of the spore to survive for long periods of time under non-favorable conditions, as well as

for its ability to "revive" when the conditions turn favorable. Even though *Bt* has been used in biotechnology applications for several decades, the data presented here, and in other publications referenced above, demonstrate further that this is a rich mine of molecules with a wide functional spectrum that could offer endless opportunities for biotechnological applications.

6. Supplementary Material

1. Supplemental Material S1: Mass spectrometry data, protein IDs, identification parameters for complex *Bt* ser. *andalousiensis mixtures*. 2. Supplemental Material S2: Mass spectrometry data, protein IDs, identification parameters for complex Bt. *ser. andalousiensis* crystals. 3. Supplemental Material S3: Complementary mass spectrometry data, protein IDs, identification parameters for *Bt* ser. *andalousiensis*.

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References

1] T.R. Schmidt, E.J. Scott, 2nd, D.W. Dyer, BMC Genomics, 12 (2011) 430. 10.1186/1471-2164-12-430.
2] E. Schnepf, N. Crickmore, J. Van Rie, D. Lereclus, J. Baum, J. Feitelson, D.R. Zeigler, D.H. Dean, Microbiol Mol Biol Rev, 62 (1998) 775-806.
3] A. Aronson, Cell Mol Life Sci, 59 (2002) 417-425.
4] E. Mizuki, M. Ohba, T. Akao, S. Yamashita, H. Saitoh, Y.S. Park, J Appl Microbiol, 86 (1999) 477-486.
5] E. Mizuki, Y.S. Park, H. Saitoh, S. Yamashita, T. Akao, K. Higuchi, M. Ohba, Clin Diagn Lab Immunol, 7 (2000) 625-634.
6] S. Okumura, H. Saitoh, T. Ishikawa, K. Inouye, E. Mizuki, Biochim Biophys Acta, 1808 (2011) 1476-1482. 10.1016/j.bbamem.2010.11.003.
7] E.P. Alcantara, O. Alzate, M.K. Lee, A. Curtiss, D.H. Dean, Biochemistry, 40 (2001) 2540-2547.
8] O. Alzate, C.F. Hemann, C. Osorio, R. Hille, D.H. Dean, BMC Biochem, 10 (2009) 25. 1471-2091-10-25.
9] O. Alzate, C. Osorio, A.M. Florez, D.H. Dean, Appl Environ Microbiol, 76 (2010) 7878-7880.
10] O. Alzate, T. You, M. Claybon, C. Osorio, A. Curtiss, D.H. Dean, Biochemistry, 45 (2006) 13597-13605.
11] S. Arnold, A. Curtiss, D.H. Dean, O. Alzate, FEBS Lett, 490 (2001) 70-74.
12] A. Bravo, I. Gomez, J. Conde, C. Munoz-Garay, J. Sanchez, R. Miranda, M. Zhuang, S.S. Gill, M. Soberon, Biochim Biophys Acta, 1667 (2004) 38-46. 10.1016/j.bbamem.2004.08.013.
13] A. Bravo, S. Likitvivatanavong, S.S. Gill, M. Soberon, Insect Biochem Mol Biol, 41 (2011) 423-431. 10.1016/j.ibmb.2011.02.006.
14] L. Masson, B.E. Tabashnik, Y.B. Liu, R. Brousseau, J.L. Schwartz, J Biol Chem, 274 (1999) 31996-32000.
15] D. Wu, A.I. Aronson, J. Biol. Chem., 267 (1992) 2311-2317.
16] S. Kitada, Y. Abe, H. Shimada, Y. Kusaka, Y. Matsuo, H. Katayama, S. Okumura, T. Akao, E. Mizuki, O. Kuge, Y. Sasaguri, M. Ohba, A. Ito, J Biol Chem, 281 (2006) 26350-26360. 10.1074/jbc.M602589200.
17] H. Katayama, Y. Kusaka, H. Yokota, T. Akao, M. Kojima, O. Nakamura, E. Mekada, E. Mizuki, J Biol Chem, 282 (2007) 7742-7752. 10.1074/jbc.M611382200.
18] T.D. Read, A. Akmal, K. Bishop-Lilly, P.E. Chen, C. Cook, M.P. Kiley, S. Lentz, A. Mateczun, N. Nagarajan, N. Nolan, B.I. Osborne, M. Pop, S. Sozhamannan, A.C. Stewart, A. Sulakvelidze, B. Thomason, K. Willner, M.E. Zwick, http://www.ebi.ac.uk/ena/data/view/ACNG00000000.
19] L. Debro, P.C. Fitz-James, A. Aronson, J. Bacteriol., 165 (1986) 258-268.
20] J.J. Bozzola, L.D. Russell, Electron Microscopy, 2 ed., Jones and Bartlett, Boston, 1999.
21] E.E. Snyder, N. Kampanya, J. Lu, E.K. Nordberg, H.R. Karur, M. Shukla, J. Soneja, Y. Tian, T. Xue, H. Yoo, F. Zhang, C. Dharmanolla, N.V. Dongre, J.J. Gillespie, J. Hamelius, M. Hance, K.I. Huntington, D. Jukneliene, J. Koziski, L. Mackasmiel, S.P. Mane, V. Nguyen, A. Purkayastha, J. Shallom, G. Yu, Y. Guo, J. Gabbard, D. Hix, A.F. Azad, S.C. Baker, S.M. Boyle, Y. Khudyakov, X.J. Meng, C. Rupprecht, J. Vinje, O.R. Crasta, M.J. Czar, A. Dickerman, J.D. Eckart, R. Kenyon, R. Will, J.C. Setubal, B.W. Sobral, Nucleic Acids Res, 35 (2007) D401-406. 10.1093/nar/gkl858.
22] J.E. Elias, S.P. Gygi, Nat Methods, 4 (2007) 207-214. 10.1038/nmeth1019.
23] E.J. Hsieh, M.R. Hoopmann, B. MacLean, M.J. MacCoss, J Proteome Res, 9 (2010) 1138-1143. 10.1021/pr900816a.
24] A.I. Nesvizhskii, A. Keller, E. Kolker, R. Aebersold, Anal Chem, 75 (2003) 4646-4658.
25] A. Keller, A.I. Nesvizhskii, E. Kolker, R. Aebersold, Anal Chem, 74 (2002) 5383-5392.
26] W. Abhyankar, A.T. Beek, H. Dekker, R. Kort, S. Brul, C.G. de Koster, Proteomics, 11 (2011) 4541-4550. 10.1002/pmic.201100003.
27] A. Driks, Cell Mol Life Sci, 59 (2002) 389-391.
28] A. Moir, B.M. Corfe, J. Behravan, Cell Mol Life Sci, 59 (2002) 403-409.
29] W.L. Nicholson, Cell Mol Life Sci, 59 (2002) 410-416.
30] Z.E. Phillips, M.A. Strauch, Cell Mol Life Sci, 59 (2002) 392-402.
31] N. Kaplan, A. Vaaknin, M. Linial, Nucleic Acids Res, 31 (2003) 5617-5626.
32] R. Sato, K. Takeuchi, K. Ogiwara, M. Minami, Y. Kaji, N. Suzuki, H. Hori, S. Asano, M. Ohba, H. Iwahana, Curr. Microbiol., 28 (1994) 15-19.
33] C. Shu, R. Liu, R. Wang, J. Zhang, S. Feng, D. Huang, F. Song, Curr Microbiol, 55 (2007) 492-496. 10.1007/s00284-007-9018-3.
34] E. Quesada-Moraga, E. Garcia-Tovar, P. Valverde-Garcia, C. Santiago-Alvarez, Microbiol Res, 159 (2004) 59-71. 10.1016/j.micres.2004.01.011.

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Comparison of protein precipitation methods for two-dimensional electrophoresis of dog salivary proteins

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ABSTRACT

Despite saliva being one fluid with growing interest as a source of biomarkers, both in humans and animal models, few studies have been reported that use proteomic approaches for canine saliva analyses. Two-dimensional electrophoresis (2-DE) is considerably used in biomarker research and its use for dog saliva study may add relevant knowledge about pathology/physiology. The quality of the results obtained using 2-DE greatly depends on sample preparation. Different protein precipitation methods are frequently used for removing interfering compounds and concentrating samples, but their efficiency varies according to sample characteristics. For dog saliva samples no information was found about the best precipitant and precipitation method for electrophoretic protein profiling.

In this study, six different protein precipitation methods were compared. Precipitation of dog salivary proteins with 20% (w/v) trichloroacetic acid (TCA) resulted in lower protein recovery rate than other methods tested, but allowed protein profiles highly correlated with the ones from original samples. Moreover, this protocol resulted in good protein separation in 2-DE, with the visualization of spots from salivary proteins not observed when samples were treated using other methods. Based on this, we propose the use of TCA for dog saliva whenever precipitation is needed for protein profile analysis.

Keywords: dog, saliva, proteins precipitation, two-dimensional electrophoresis

1. Introduction

Saliva is a clear fluid mainly composed of electrolytes, immunoglobulins, proteins and enzymes [1]. Whole saliva is mainly a mixture of the secretions from the major and minor salivary glands, and oral mucosa, periodontium and oral microflora, which also contribute to its final content. Therefore, whole saliva represents a complex balance among local and systemic sources. Mostly in humans, this has been allowing for the application of saliva in the diagnosis not only for salivary gland disorders but also for oral diseases and systemic conditions such as, among others [2-7]: periodontitis and dental caries; type-2-diabetes mellitus; obesity; several infections; cancer in a number of tissues; human immunodeficiency virus (HIV).

The interest in the characterization of biological fluids protein composition has increased in the last few years for both scientific and veterinary routine advancement [8]. The non-invasive and simple nature of saliva collection allows for repetition and multiple collection of biological material with minimally trained personnel and in a stress-free, painless, and economically viable manner [1,9]. However, saliva of different animal species, including the dog, is still little studied. Thus, protocol optimization for saliva analysis is firstly required.

Two-dimensional gel electrophoresis (2-DE) is one of the

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most popular techniques for the global analysis and initial profiling of salivary proteome for both humans and animals. 2-DE simultaneously separates proteins according to their isoelectric points (pI) and molecular masses, enabling the visualisation and identification of several thousand proteins on a single gel. One of the greatest advantages of 2-DE in salivary protein study relates to its capacity of separating proteins with different post-translational modifications (PTMs), allowing their separate quantification.

One of the constraints of using 2-DE in animal saliva samples is the difficulty to obtain saliva volumes sufficient to achieve the relatively high amount of proteins needed for performance of this technique. In dogs, in order to obtain high volumes of saliva may be not easy: thus different authors reported methods for stimulating saliva production, based on use of citric acid [10,11], soaking cotton rolls [12], or use of beef-flavoured cotton ropes [13]. However, these techniques could interfere with the salivary proteome and result in erroneous results and conclusions.

In 2-DE, sample preparation is an essential step for success. A high amount of charged molecules in solution will affect isoelectric focusing (IEF). In certain animal species, such as ruminants, this may be particularly relevant, since these animals have a high content of bicarbonate and phosphate ions in their saliva [14]. Moreover, charged detergents, lipids, phenolics and nucleic acids also need to be removed prior to IEF. Precipitation is frequently used to concentrate proteins from diluted samples and also to remove compounds that interfere with IEF [15,16]. Various methods for protein precipitation are applied, which rely on different chemical principles, and have different effectiveness according to the characteristics of samples. For human saliva, there are studies evaluating the efficiency and reliability of different protein precipitation protocols [17]. However, for dog saliva, which is different in composition from human saliva as based on biochemical studies [18-20], no such information was reported by now.

The objective of the present study was to compare and select the protein precipitant and precipitation method which will allow protein profiles with the highest similarity to original sample protein profile and, at the same time, efficient and reliable 2-DE analysis. For this, six of the protein precipitation methods already tested in human saliva [17] were compared using canine saliva samples.

2. Materials and Methods

2.1. Saliva collection

Saliva from seven healthy intact adult dogs (age range 2-8 years) of different breeds was collected: Portuguese Podengo (n=2), Border Collie (n=1), Bull Terrier (n=1), Boxer (n=1), Australian Cattle Dog (n=1) and mixed breed (n=1). Animals from different breeds and ages were used to constitute a pool representative of "general" dog saliva, instead of using saliva from only a particular breed and/or age. Saliva samples were taken by the same examiners using a cotton cylinder (Salivette®) [14]. The salivettes were inserted into the oral cavity of the dogs, under the tongue and for chew, until completely soaked with saliva [14]. Dogs did not eat for at least 12 hours prior to the salivary sampling but water were provided ad libitum. After collection, the salivettes were immediately placed in test tubes on ice, until laboratory arrival. The saliva was extracted from the cotton roll by centrifugation at 4°C, at 2650 *g*, for 5 min. Samples were mixed in one pool, which was aliquoted and stored at -20°C until further analysis, less than two months in all cases.

2.2. Determination of total protein concentration

The total protein concentration of the dog saliva pool was determined using Bradford method protein assay [17] with BSA as the standard protein (Pierce Biotechnology, Rockford, IL, USA). Standards and samples were run in triplicate, in 96 wells microplates. Absorbance was read at 600 nm in a microplate reader (Glomax, Promega).

2.3. Protein precipitation

Five different precipitant solutions and six precipitation methods were employed, which were already tested in human saliva [17]: method A (trichloroacetic acid-TCA only), method B (TCA/acetone), method C (TCA/acetone/dithiothreitol-DTT), method D (acetone only), method E (ethanol only) and method F (TCA/acetone fast method). Each of the methods was performed in triplicate.

TCA precipitation (method A)

For precipitation with TCA saliva sample was mixed with 20% w/v TCA (1:1). The mixture was vortexed to mix thoroughly and allowed to precipitate overnight at -20°C. This was followed by centrifugation at 23876 *g*, at 4°C for 30 min. The supernatant was discarded and the pellet obtained was washed twice with 200 µL of cold acetone. For each wash, the pellet suspended was sonicated for 5 min or more until the whole pellet was fully broken to form a suspension (maximum 20 min). This suspension was then incubated at -20°C for 20 min and subsequently centrifuged at 4°C for 5 min at 23876 *g* . This wash procedure was repeated twice. At the end, the acetone-containing supernatant was decanted and the pellet obtained was dried at air to remove any residual acetone. The pellets prepared were stored at -20°C until further use.

TCA/Acetone precipitation (method B)

The procedures were performed as described for method A, except that the solution used for protein precipitation was 1 ml of TCA 20% (w/v) plus 9 mL of acetone (90% v/v).

TCA/Acetone/DTT precipitation (method C)

The procedures were performed as described for method A, except that the solution was constituted by TCA 20% (v/v), acetone 90% (v/v) and 20 mM DTT (2.5 mL of TCA+acetone and 0.0075 g of DTT); and that the first wash was done using acetone 90% (v/v) and 20 mM DTT and the second using acetone 80% (v/v) and 10 mM DTT.

Acetone precipitation (method D)

In this method, saliva was mixed with 90% (v/v) acetone at a proportion 1:3. The mixture was incubated overnight at -20ºC followed by centrifugation at 4ºC, for 30 min at 23876 *g* . The supernatant was discarded and the precipitated was dried at air and frozen until further analysis.

Ethanol precipitation (method E)

The method was carried out similar to method D except that precipitation was done with absolute ethanol.

TCA/Acetone precipitation (fast assay) (method F)

The method was carried out as described for method B, but instead incubation being overnight, it was performed during 1h at 4º C. The rest of procedures were similar.

2.4. Determination of the protein concentration after protein precipitation methods

A volume of saliva corresponding to 25 µg of total protein was precipitated following the methods described above. The precipitates obtained were re-suspended in 25 µL 2-DE rehydration buffer [7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 60 mM DTT], since this is the buffer used in 2-DE. In order to avoid that compounds interfere with Bradford, the solution was diluted with 400 µL ultrapure water. Total protein concentration was determined following Bradford method as described in the section 2.2.

2.5. SDS PAGE and two-dimensional electrophoresis

Proteins were separated by SDS-PAGE electrophoresis in 14% acrylamide gels in a mini-protean apparatus (BioRad) [21]. A volume corresponding to 12 µg of total protein was precipitated by each of the methods described before. The precipitates were re-suspended in sample buffer [Tris–HCl 0.125 M pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoetanol, 20% (v/v) glycerol traces of bromophenol blue], heated at 95ºC for 5 minutes and run at a constant voltage of 140V until the dye front reach the end of the gel. A volume of saliva correspondent to 12µg of total protein that was not previously precipitated was run for control. Gels were fixed in 40% methanol, 20% acetic acid, for one hour, stained with Coomassie Brilliant Blue (CBB) G-250 (0.125% CBB G-250, 20% ethanol) for two hours and destained in several washes with distilled water. The procedures were done in triplicate. Analysis was performed with GelAnalyzer software (http://www.gelanalyzer.com/).

For 2-DE, volumes of saliva correspondent to 150 µg of total protein were precipitated by the methods evaluated in this study, with exception of method F. The precipitates were mixed with 125 µL rehydration buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (v/v) IPG buffer, 60 mM DTT and traces of bromophenol blue], sonicated until total re-suspension and incubated during 1h at room temperature, being subsequently centrifuged for 5 min at 9390 g. IPG strips (7 cm, pH 3-10 NL; GE, Healthcare) were passively re-hydrated overnight with this solution. Focusing was performed in a Multiphor II (GE, Healthcare) for approximately 16kVh, at 20ºC. Focused strips were equilibrated in two steps of 15 min each with equilibration buffer [50 mM Tris–HCl, pH 8.8; 6 M urea; 30% (v/v) glycerol and 2 % (w/v) SDS], with the addition of 1% (w/v) DTT and 65 mM iodoacetamide in the first and second steps, respectively. After equilibration the strips were applied in the top of a SDS-PAGE gel 14% acrylamide and run at 150V constant voltage in a mini-protean system (BioRad). Staining with CBB-G250 and destaining were done through the same protocol described for SDS-PAGE gels. Gel images were acquired using a scanning Molecular Dynamics densitometer with internal calibration and LabScan software (GE, Healthcare), and images were analysed using ImageMaster 2D Platinum v7 software. Spot editing was performed manually and the match was done automatically and corrected manually.

2.6. Protein identification

Spots of interest were manually excised from gels and digested with trypsin following the protocol described before [21]. MALDI TOF-TOF mass spectrometry was used for protein identifications and this technique was performed by an external laboratory (MS Lab, ITQB-UNL; Portugal). Samples were diluted and concentrated using a reversal phase column (R2 pores-Applied Biosystems) and eluted with matrix α-cyano-4-hydroxycinnamic acid (CHCA; Fluka) 5mg/mL in 50% (v/v) CAN and 5% (v/v) formic acid. MS and MS/MS data were acquired in positive reflector mode through MALDI TOF/TOF (4800 Plus AB SCIEX) and through software 4000 Series Explorer, version 3.5.3.3 (Applied Biosystems). The mass of monoisotopic peptides was determined using the algorithm SNAP 2 in the analysis software (Bruker Daltonics) version 3.4. The external calibration was executed using CalMix5 (Protea). The 30 precursor ions most intense of the MS spectra were selected for analysis by MS/MS.

The monoisotopic masses of the peptides were used to search for the protein identification through the use of Protein Pilot v 4.5 (ABSciex) software with the Mascot search engine (MOWSE algorithm). Swiss Prot database,

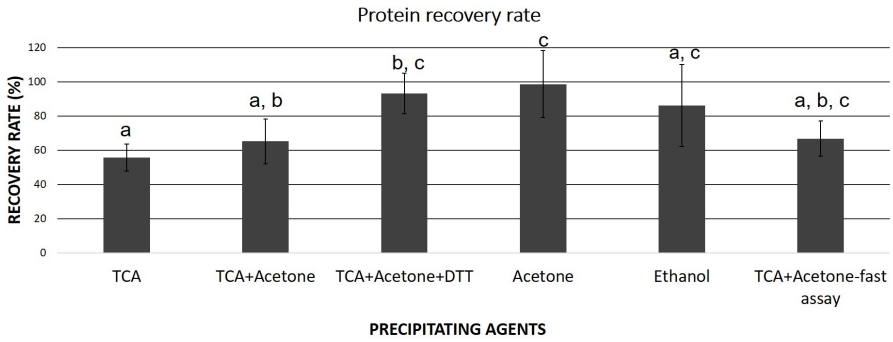

Figure 1. Protein recovery rates obtained for each of the precipitation methods studied. Different letters represent statistically significant differences among methods for $p<0.05$.

restricted to Canidae canidae (32092 sequences; 16404078 residues), was used for all the searches. A minimum mass accuracy of 50 ppm and a mass tolerance of 0.3 Da, two missed cleavages in peptide masses, carbamidomethylation of Cys and oxidation of Met, as fixed and variable amino acid modifications, respectively, were considered. Criteria used to accept the identification were homology scores higher than 58 and, at least, one fragmented peptide with individual significant score ($P<0.05$), in Mascot.

2.7. Statistical analysis

Data for protein recovery rate, percentage volume of SDS-PAGE bands and 2-DE spots were tested for normality and homocedasticity using Kolmogorov-Smirnov and Levene tests, respectively. One-way ANOVA was used for protein recovery rate, with Tukey test used for comparison of the different methods. The relationship between SDS-PAGE profiles (% volume of bands) from each precipitation method and the original sample was evaluated by calculating Pearson coefficient. Statistical significance was considered for $p<0.05$. All statistical analysis procedures were achieved using the SPSS 21.0 software package (SPSS Inc., Chicago, USA).

3. Results

3.1. Protein recovery rate

The different precipitation methods resulted in statistically significant different rates of total protein recovery. The methods with higher losses of protein were TCA (method A) and TCA+acetone (methods B and F). On the other hand, the precipitation with acetone (method D) was the one for which higher protein recovery was achieved (Figure 1).

3.2. SDS-PAGE protein profiles

By relating the percentage of volume of the different profiles, strong positive correlations were obtained between original sample and methods A ($r=0.717$; $p=0.003$), B and F ($r= 0.751$; $p=0.001$, for overnight and $r=0.715$; $p=0.003$ for 1h incubation) and C ($r=0.711$; $p=0.002$). On the other hand, the precipitation methods with acetone (D) and with ethanol (E) resulted in profiles not correlated with control ($r=0.389$; $p=0.151$ and $r=0.339$; $p=0.216$, respectively) (Figures 2 and 3).

When comparing the different 16 protein bands consistently present in SDS-PAGE gels, the profile obtained without sample protein precipitation differs from profiles using protein precipitation methods for 3 protein bands: B, H and N. The differences are mainly in relation to the profiles obtained through precipitation with acetone or ethanol (Table 1).

3.3. Two-dimensional electrophoretic protein profiles

2-DE was run only for precipitated samples, since the volume of original sample needed exceeded the maximum of 25 µL allowed for the dry-gel strips used. Figure 4 shows the

Table 1. Expression levels (% volume) of the protein bands mainly affected by protein precipitation protocols. A – TCA; B – TCA/acetone; C - TCA/acetone/DTT; D – acetone; E – ethanol; F – TCA + acetone fast assay.

Band	TCA (method A)	TCA+acet (method B)	TCA+acet+DTT (method C)	Acetone (method D)	Ethanol (method E)	TCA+acet fast assay (method F)	Control	P value
B	4.54 ± 1.17	6.54 ± 0.32	9.62 ± 1.05	2.40 ± 0.00*	3.57 ± 0.86*	6.79 ± 0.17	6.90 ± 0.65	0.010
H	7.50 ± 0.45*	5.62 ± 0.31*	1.97 ± 0.00	5.39 ± 0.61*	3.96 ± 1.13	5.31 ± 0.70*	1.97 ± 0.00	0.014
N	8.19 ± 0.82	8.50 ± 0.38	2.43 ± 0.00	12.04 ± 3.25*	12.04 ± 1.26*	9.49 ± 2.09*	3.24 ± 0.82	0.016

* Differences comparatively to control group ($P<0.05$)

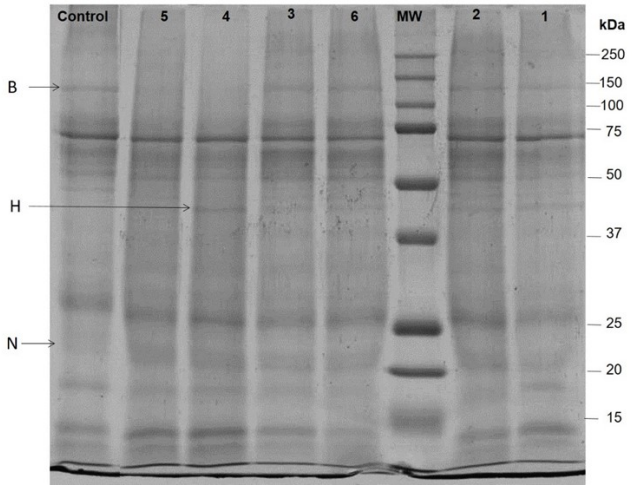

Figure 2. Representative SDS-PAGE profiles for each of the precipitation methods tested. Numbers in each lane indicate the precipitation method used (1 – TCA; 2 – TCA/acetone; 3- TCA/acetone/DTT; 4 – acetone; 5 – ethanol; 6 – TCA + acetone fast assay); MW – molecular mass marker. Precipitation methods 5 and 4 were the ones presenting higher differences from control protein profiles.

representative 2-DE of dog saliva, after each of the precipitation methods under study.

The highest number of resolved spots were obtained for the precipitation with TCA (83 spots), decreasing for the methods acetone (69 spots), ethanol (64 spots), TCA+acetone+DTT (59 spots) and TCA+acetone (55 spots).

The profile in which protein spots were less well resolved was the one obtained for precipitation with absolute ethanol (method E). On the other hand, when precipitations were performed with methods A and D, the profiles did appear enriched in proteins from the alkaline range of the pH gradient. Even in the other pH gradient regions, these two profiles were the ones allowing better focusing of the proteins, with well resolved protein spots. It is particularly interesting the group of protein spots from the alkaline region of the gel that were only observed in the gels obtained following these two precipitation methods (methods A and D - Figure 4).

The six spots observed only in the precipitation methods TCA (A) and acetone (D) were identified by mass spectrometry (Table 2).

These spots correspond to proteins of keratin family or to serine-type endopeptidase inhibitor family. Although some of them are present in databases as uncharacterized proteins, one has been already identified as a submandibular gland secreted protein.

4. Discussion

The present study compared six different protein precipitation methods for the gel-based proteomic analysis of dog saliva. It characterizes the precipitation efficiency on the amount of total protein that can be loosed at the end, as well as on a protein-specific level. Although there are studies in which different protocols for preparation of human saliva samples for 2-DE were compared [17], the protein composition of dog saliva is not well known. As such, there are no guarantees that the most appropriate protocols for preparing human saliva samples are also the ideal ones for the preparation of dog saliva samples.

The proteome is a dynamic structure, so the sample itself and the conditions of its storage for research directly affect the result of proteomic analysis [22]. Moreover, the ideal sample preparation procedure should reproducibly capture the most comprehensive repertoire of proteins without any artefactual modification, proteins loss, degradation or non-proteinaceous contamination [14,15,20-26]. Besides the need for removing sample components that may interfere with isoelectric focusing, saliva analysis by electrophoresis requires a concentration step, due to the dilute nature of these samples, where protein precipitation methods can be useful.

Precipitation methods based in acetone and/or TCA were used by several authors in several animal tissues like ovaries, pericardium, brain, muscle and animal fluids like saliva, cerebrospinal fluid and blood [14,15,20-26]. One of the limitations of precipitation protocols is the protein loss, usually resulting from a poor re-suspension of the precipitates [27]. Among the protein precipitation methods tested in the present work, it was the acetone precipitation

Table 2. MS Identification of differentially expressed salivary proteins.

Spot	Protein	Uniprot entry reference	ID score	Estimated/ Theoretical MW (kDa)	Estimated/ Theoretical pI	Function/Family
1	Keratin, type I cytoskeletal 10	Q6EIZ0	207	14.0/57.7	8.1/5.1	Structural molecular activity
2	Keratin, type II cytoskeletal 8	F1PW98	111	16.0/55.0	8.1/5.7	Structural molecular activity/ Keratin family
3	Double-headed protease inhibitor, submandibular gland	P01002	96	16.0/12.8	7.9/8.3	Serine-type endopeptidase inhibitor
4	Uncharacterized protein	L7N097	168	15.5/51.9	6.0/6.0	Structural molecular activity/Keratin family
5	Uncharacterized protein	F1PR78	121	14.0/12.7	5.0/8.4	serine-type endopeptidase inhibitor
6	Keratin, type I cytoskeletal 10	Q6EIZ0	192	14.0/57.7	7.9/5.0	Structural molecular activity

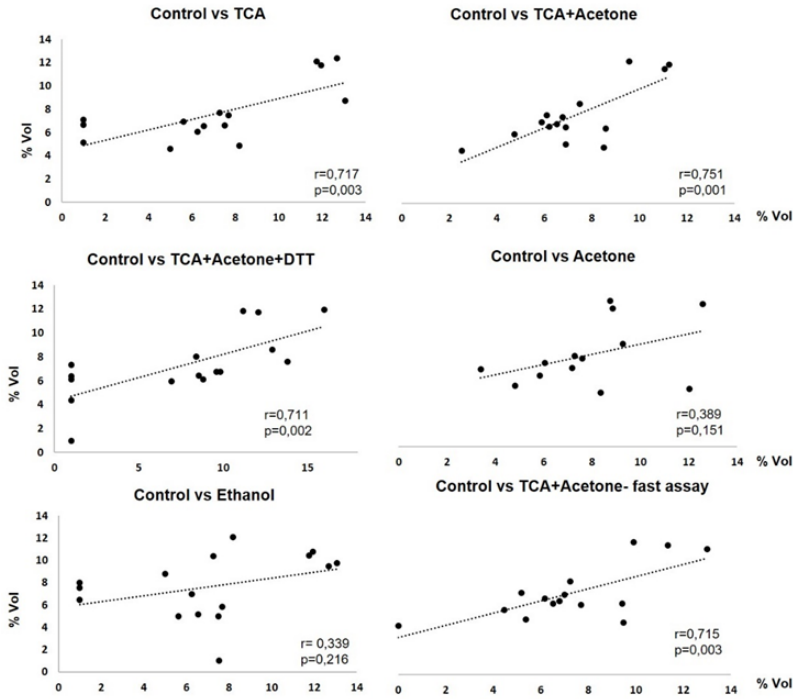

Figure 3. Correlation between the SDS-PAGE profiles of each of the precipitation methods tested and control. Correlations are significant for $p<0.05$.

the one that presented almost no losses, with protein recovery rates near 99%. For human saliva and rat brain samples, protein recovery rates of about 70% and 52.2% with acetone precipitation were reported [17,27], respectively, what suggests that the efficacy of this method depends on the type of biological material. In addition to the reduced losses in total protein, the other great advantage of this protocol is its simple execution, although requiring a high volume of organic solvent.

In our study, TCA-containing precipitation protocols resulted in lower rates of protein recovery (56%), similar to that already observed for human saliva by Jessie et al. (2008) [17], where TCA was observed as one of the precipitating agents leading to higher total protein losses (46.5%). The recovery of the protein depends on the re-solubilisation of the sample, being observed that after protein precipitation with TCA the recovery rate is considerably higher if the samples are sonicated (24% and 77%, without and with sonication, respectively) [27]. In the present work, besides the use of vortex, for resuspension of the precipitate, sonication of the samples was also carried out but even so, no high recovery rates were obtained. However, when acetone and DTT was added to the mixture (method C) higher recovery rates were observed, similarly to what has been reported to human saliva samples [17].

Besides the interest in having a precipitation protocol that allows a high protein recovery rate, it is important that it imposes the minimum of modifications in the protein content of samples, namely in the relative abundance of these proteins. In order to control this aspect, the protein profiles obtained after each precipitation method were compared with the one from the original sample (without precipitation). This was possible to be done only for SDS-PAGE, since in 2-DE the volume of sample necessary to have the amount of protein needed for spot visualization is too high to be run without concentration. Moreover, the use of Centricon devices for protein concentration did not work in these dog saliva samples, with the filters clogged during centrifugation step (data not shown), with the need of further research to identify the dog saliva components responsible for that. Despite the low protein recovery rate, referred above, a high correlation between the protein profiles from TCA precipitation method and the protein profiles from original sample was observed.

Interestingly, 20% (w/v) TCA solution, representing a concentration of 10% of this acid in sample, induces no major changes in dog saliva. Some previous studies in rats reported that salivary proline rich proteins present the particular characteristic of being soluble in 10% TCA solutions [28], suggesting that this precipitation method would lead to a selective loss of these proteins. In the present work, the high correlation found between the profiles obtained from TCA protocols and the original sample profile suggest that the protein losses resulting from the precipitation occur in the same proportion for the different proteins, with no evidence of some proteins being more affected relatively to others. One hypothesis for explaining these results is that dog saliva may not contain these types of proteins. In fact, some authors report the absence of these proteins in animal species' saliva such as dogs and cats due to their eating patterns [30].

The 90% (v/v) acetone and absolute ethanol protocols

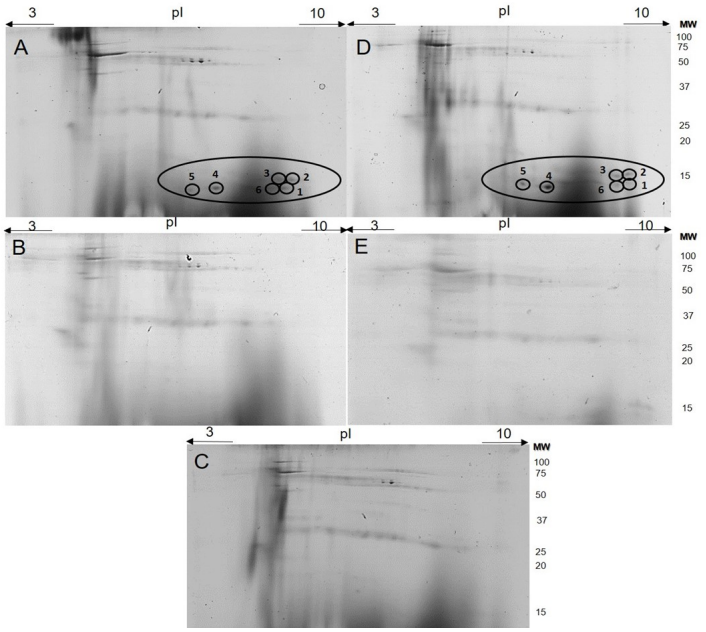

Figure 4. 2-DE protein profiles from dog saliva after various precipitation methods. (A) TCA; (B) TCA+acetone; (C) TCA+acetone+DTT; (D) acetone; (E) ethanol. Gels were stained with CBB-G250 .

were those that gave profiles less correlated with the control profiles. However, in these protocols protein recovery rates were relatively higher. The low correlation between these protocols and the control in SDS-PAGE profile may be due to higher losses of some types of proteins relative to others. Although not identified in the present study (since each band can be constituted by several proteins), proteins with molecular masses around 150 kDa are less intense in acetone and ethanol precipitation methods, whereas proteins with molecular masses around 50 kDa and 25 kDa are highly expressed in acetone and ethanol methods, respectively. The further identification of these proteins are of interest to understand the main changes induced by these protocols.

In 2-DE profiles, precipitations of proteins with TCA or acetone were the methods that allowed the observation of the higher number of spots, with better resolution. It was also only in the gels obtained using these two protocols that spots in the alkaline region were observed. The enrichment in alkaline proteins was already reported for precipitation with TCA [27].

The protein spots observed in the gels obtained following the two methods referred were identified as belonging to two families of dog proteins: keratins and serine-type endopeptidases inhibitors. In the case of keratin 1, which is a constituents of the intermediate filament cytoskeleton in epithelial cells, its presence in saliva from different mammalian species, including dogs, has been reported [31]. It was previously reported, for humans, that the amounts of keratins present in saliva may be a marker of gingival damage [32]. Since, in this study, dog saliva has been collected through cotton rolls, some level of gingival damage may occur, resulting in the identification of this protein in saliva.

Other protein spots, differently expressed among the precipitation methods tested, were identified as protease inhibitors and, interestingly, one of them was identified as a protein secreted by submandibular glands. The exact role of this protease inhibitor in saliva is not known and further studies would be needed to explore and explain why this protein behaves differentially with different precipitation methods.

5. Concluding Remarks

2-DE is a popular technique for the global analysis and initial profiling of saliva prior to further fractionation and identification with other high throughput techniques such as mass spectrometry. This technique needs a sample preparation that, at the same time, allows cleaning of interfering compounds and protein concentration. Among the several precipitation methods studied in the present work, precipitation using 20% (v/v) TCA (method A) showed best results with dog saliva under studied conditions. Although this method results in considerable amount of total protein loss, the protein profiles are highly correlated with the ones from the original samples, with the advantage of allowing good resolution of spots in 2-DE and the visualization of spots from proteins that may be of interest.

6. Supplementary material

Ethical statement: The study did not involve the manipulation of experimental animals. Saliva was collected from healthy, normal weight, adult dogs of different breeds belonging to staff members of the University of Évora and to two kennels, who gave their informed consent for its collection. The saliva collection and all animal procedures

were carried out by a researcher accredited by the Federation of European Animal Science Associations (FELASA), and conformed to Portuguese law (Decreto-lei nº 113/2013, 7 August), which transposed the directive 2010/63/EU of the European Parliament of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The protocols were also reviewed by the Évora University animal welfare commission.

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References

1] E. Scarano, A. Fiorita, P.M. Picciotti, G.C. Passali, L. Calò, T. Cabras, R. Inzitari, C. Fanali, I. Messana, M. Castagnola, G., Paludetti Proteomics of saliva: personal experience, Acta Otorhinolaryngol Italica 30 (2010) 125-130.

2] S. Al Kawas, Z.H. Rahim, D.B. Ferguson, Potential uses of human salivary protein and peptide analysis in the diagnosis of disease, Arch Oral Biol 57 (2012)1-9.

3] S.J. Farnaud, O. Kosti, S.J. Getting, D. Renshaw, Saliva: physiology and diagnostic potential in health and disease, Scientific World Journal 10 (2010) 434-456. DOI: 10.1100/tsw.2010.38

4] D. Belstrøm, R.R. Jersie-Christensen, D. Lyon, C. Damgaard, L.J. Jensen, P. Holmstrup, J.V. Olsen, Metaproteomics of saliva identifies human protein markers specific for individuals with periodontitis and dental caries compared to orally healthy controls, PeerJ 4 (2016) e2433. DOI:10.7717/peerj.2433

5] L. Delmonico, M. Bravo, R.T. Silvestre, M.H. Ornellas, C.M. De Azevedo, G.Alves, Proteomic profile of saliva and plasma from women with impalpable breast lesions, Oncol Lett 12 (2016) 2145-2152. DOI: 10.3892/ol.2016.4828

6] N. Nowroozilarki, S. Jamshidi, T.Z. Salehi, S. Kolahian, Identification of Helicobacter and Wolinella spp. in oral cavity of Toy breed dogs with periodontal disease, Topics in Compan An Med (2017). DOI: 10.1053/j.tcam.2017.07.004

7] A. Tvarijonaviciute, C. Castillo, J.J. Ceron, S. Martinez-Subiela, F. Tecles, P.López-Jornet, Leptin and NGF in saliva of patients with diabetes mellitus type 2: A pilot study, J Oral Pathol Med 46 2017 853-855. DOI.org/10.1111/jop.12587

8] I. Miller, A. Preßlmayer-Hartler, R. Wait, K. Hummel, C. Sensi, I. Eberini, E. Razzazi-Fazeli, E. Gianazza, In between - Proteomics of dog biological fluids, J Proteomics 106 (2014) 30-45. DOI: 10.1016/j.jprot.2014.04.016

9] E. Lamy, M. Mau, Saliva proteomics as an emerging, non-invasive tool to study livestock physiology, nutrition and diseases, J Proteomics 75 (2012) 4251-4258. DOI: 10.1016/j.jprot.2012.05.007

10] R. Bergeron, S.L. Scott, J.P. Emond, F. Mercier, N.J. Cook, A.L., Schaefer Physiology and behavior of dogs during air transport, Can J Vet Res 66 (2002) 211-216.

11] B. Beerda, M.B.H. Schilder, J. van Hooff, H.W. de Vries, J.A. Mol, Behavioural, saliva cortisol and heart rate responses to different types of stimuli in dogs, Appl Anim Behav Sci 58 (1998) 365-381.

12] A.J. Kobelt, P.H. Hemsworth, J.L. Barnett, K.L.Butler, Sources of sampling variation in saliva cortisol in dogs, Res Vet Sci 75 (2003) 157-161. DOI: 10.1016/S0034-5288(03)00080-8

13] N.A. Dreschel, D.A. Granger, Methods of collection for salivary cortisol measurement in dogs, Horm Behav 55 (2009) 163-168. DOI: 10.1016/j.yhbeh.2008.09.010

14] E. Lamy, G. da Costa, R. Santos, F. Capela E Silva, J. Potes, A. Pereira, A.V. Coelho, E. Sales Baptista, Sheep and goat saliva proteome analysis: A useful tool for ingestive behavior research?, Physiol Behav, 98 (2009) 393-401. DOI: 10.1016/j.physbeh.2009.07.002

15] R.L. Chen, E.A. Sage, M.J. Dunn, R. Wait, J.E. Preston, Optimising ovine cerebrospinal fluid preparation for two-dimensional gel electrophoresis, Proteomics 6 (2006) 3170-3175. DOI: 10.1002/pmic.200500623

16] U. Jankowska, A. Latosinska, B. Skupien-Rabian, B. Swiderska, M. Dziedzicka-Wasylewska, S. Kedracka-Krok, Optimized procedure of extraction, purification and proteomic analysis of nuclear proteins from mouse brain, J Neurosci Methods 261 (2016) 1-9. DOI: 10.1016/j.jneumeth.2015.12.002

17] K. Jessie, O.H. Hashim, Z.H. Rahim, Protein precipitation method for salivary proteins and rehydration buffer for two-dimensional electrophoresis, Biotechnology 7 (2008) 686-693. DOI: 10.3923/biotech.2008.686.693

18] H. Nakanishi, T. Ohmori, M. Hara, K. Yoneyama, A. Takada, K. Saito, Identification of canine saliva using mRNA-based assay, Int J Legal Med 131 (2017) 39-43. DOI: 10.1007/s00414-016-1391-7

19] C. Boehlke, O. Zierau, C. Hannig, Salivary amylase – The enzyme of unspecialized euryphagous animals, Arch Oral Biol 60 (2015) 1162-1176. DOI: 10.4239%2Fwjd.v7.i6.112

20] E. Lavy, D. Goldberger, M. Friedman, D. Steinberg, pH values and mineral content of saliva in different breeds of dogs, Israel J Vet Med 67 (2012) 244-248.

21] E. Lamy, C. Simões, L. Rodrigues, A.R. Costa, R. Vitorino, F. Amado, C. Antunes, I. do Carmo, Changes in the salivary protein profile of morbidly obese women either previously subjected to bariatric surgery or not, J Physiol Biochem 71 (2015) 691-702. DOI 10.1007/s13105-015-0434-8

22] P. Pomastowski, B. Buszewski, Two-dimensional gel electrophoresis in the light of new developments, Trends Analyt Chem 53 (2014) 167-177. DOI: 10.1016%2Fj.trac.2013.09.010

23] M. Sandri, A. Colussi, M.G. Perrotta, B. Stefanon, Salivary cortisol concentration in healthy dogs is affected by size, sex, and housing context, J Vet Behav 10 (2015) 302-306. DOI: 10.1016/j.jveb.2015.03.011

24] F. Panchout, J. Letendre, F. Bultelle, X. Denier, B. Rocher, P. Chan, D. Vaudry, F. Durand, Comparison of protein-extraction methods for gills of the shore crab, Carcinus maenas (L.), and application to 2DE, J Biomol Tech 24 (2013) 218-223. DOI: 10.7171/jbt.13-2404-002

25] R. Hao, C. Adoligbe, B. Jiang, X. Zhao, L. Gui, K. Qu, S. Wu, L. Zan, An Optimized Trichloroacetic Acid/Acetone Precipitation Method for Two-Dimensional Gel Electrophoresis Analysis of Qinchuan Cattle Longissimus Dorsi Muscle Containing High Proportion of Marbling, PLoS ONE 10(4) (2015) e0124723. DOI:10.1371/journal.pone.0124723

26] P. Yiou, A. Shaoli, L. Kebin, W. Tao, F. Kui, Z. Hua, S. Yu, Y. Xun, X. Jinghui, Evaluation of extraction procedures for 2-DE analysis of aphid proteins, J Sep Sci 36 (2013) 532-539. DOI: 10.1002/jssc.201200642

27] E. Fic, S. Kedracka-Krok, U. Jankowska, A. Pirog, M. Dziedzicka-Wasylewska, Comparison of protein precipitation methods for various rat brain structures prior to proteomic analysis, Electrophoresis 31 (2010) 3573-3579. DOI: 10.1002/elps.201000197

28] H. Mehansho, A. Hagerman, S. Clements, L. Butler, J. Rogler, D.M. Carlson, Modulation of proline-rich protein biosynthesis in rat parotid glands by sorghums with high tannin levels, Proc Natl Acad Sci 80 (1983) 3948-3952.

29] J.C. Suares, D.K. Shori, G.B., Proctor Expression of proline-rich proteins in the parotid & submandibular glands of the untreated rat, Biochem Soc Trans 25 (1997) 31S.

30] J.B. Harborne, in: Plant Ecology (Ed.), Crawley MJ, Oxford, UK, 1996, pp.132-155.

31] P. de Sousa-Pereira, M. Cova, J. Abrantes, R. Ferreira, F. Trindade, A. Barros, P. Gomes, B. Colaço, F. Amado, P.J. Esteves, R. Vitorino, Cross‐species comparison of mammalian saliva using an LC-MALDI based proteomic approach, Proteomics 15 (2015) 1598-1607. DOI: 10.1002/pmic.201400083

32] W.S. McLaughlin, J. Kirkham, M.J. Kowolik, C. Robinson, Human gingival crevicular fluid keratin at healthy, chronic gingivitis and chronic adult periodontitis sites, J Clin Periodontol 23 (1996) 331-135.

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The use of Kalashnikov (AK-47) in 'Ndrangheta murders: The firearm of the clan.

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ABSTRACT

'Ndrangheta is a mafia criminal organization, hailing from Calabria, Italy. This organization is able to use any kind of weapon and the choice depends on the type of murder to commit. So, even bazooka have been used when the victims, judges or rival mafia clan boss, travelled by armored cars. Kalashnikov is not only used "normally" to commit mafia ambushes, but often it has been found carbonized with the car used by killers. This act confirm that mafia clan have available vast arsenals of weapons and it is a demonstration of what this organization is able to do. Gunshot wounds cause significant mortality and morbidity. The analysis of the features of injuries makes it possible to establish which kind of weapon has been used. The AK-47 is a selective-fire, gas-operated assault rifle and it uses a long stroke gas system. In order to shoot, who uses a AK-47, inserts a loaded magazine, pulls back and releases the charging handle, and then pulls the trigger. It can be semi-automatic, when the firearm fires only once, or full-automatic, if the rifle continues to fire automatically and cyclically fresh rounds into the chamber. AK-47 rifle bullet injuries present with uncharacteristically large entry wounds and cause complex structural injuries. The consequent trajectory is difficult to predict making regional examination and radiological investigations. Bullets may be retained, leaving no exit wound. We report a case of an AK-47 murder. An autopsy was performed and documented the external lesions. Terminal ballistic reconstructions were carried out. The results of the forensic investigations revealed a mafia matrix in the genesis of the homicide. Kalashnikov is not a frequent weapon, so the wounds are not so common to see in the forensic practice. But, in 'ndrangheta homicides, this firearm is preferred for its high harmful power that ensure a murder "without mistakes" and with devastating consequences on the shot body .

Keywords: Forensic sciences, Kalashnikov, Multidisciplinary Approach, autopsy, firearm

Abbreviations: MSCT (Multi-Slice Computed Tomography), CT (Computed Tomography).

1. Introduction

The 'Ndrangheta, along with Cosa Nostra, Camorra and Sacra Corona Unita falls among the criminal organizations, with a mafia connotation. It originates from Calabria, one of the southern regions of Italy. It has ramifications all over the world. It is considered one of the most dangerous criminal organizations in the world and also among the richest; in fact, it boasts a turnover between 50 and 60 billion euros. Its most "profitable" activity is, of course, drug trafficking. Other illegal activities include the participation in tenders, the conditioning of the electoral vote, kidnappings, usury, extortion, arms trafficking, the disposal of toxic waste. Already known in the late 1700s, under the Bourbon kingdom, it has developed and rooted more and more throughout the Calabrian territory. Peculiar characteristics of this organization are the existence of family ties between the affiliates and the low number of repentants, a direct consequence of the first characteristic mentioned and also of the oath made before entering the organization.

The 'Ndrangheta is organized according to a well-structured hierarchy. A person becomes "'ndranghetista", that is affiliated to this organization in two ways: 1) by birth, as already belonging to a mafia family or 2) by "baptism", that

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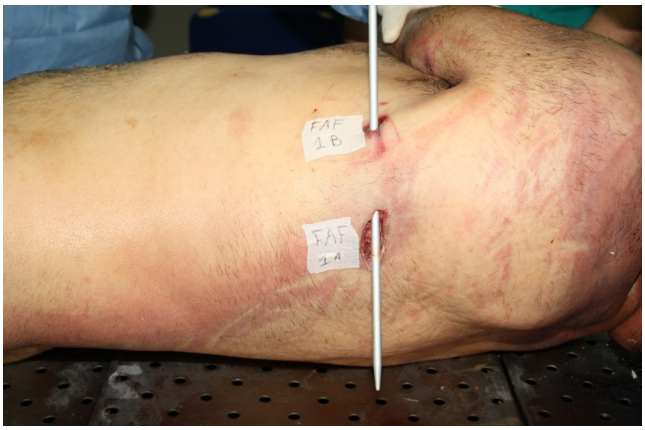

Figure 1.

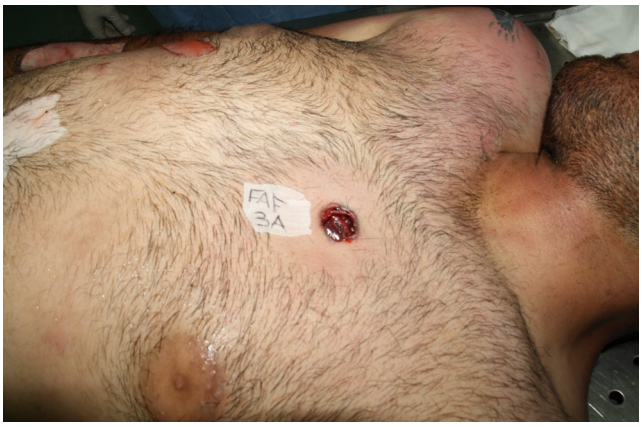

Figure 2.

is through the affiliation rite that binds him to the organization up to death. Throughout its history, the 'Ndrangheta has seen the explosion of various clan wars, some of these characterized by episodes of extreme violence; not all the wars have seen the Calabria region as a place of confrontation, but sometimes, especially because of the widespread diffusion of the 'Ndrangheta, violence has occurred in other Italian regions and even abroad. The clans are equipped with real arsenals and among the most used weapons there is the AK-47 or Kalashnikov. The choice falls on this type of weapon both for its devastating damaging effect and for reliability, in fact it rarely gets into jeopardy.

The AK-47 is a gas-fired selective assault rifle, chambered for the 7.62 × 39 mm projectile. It was developed in the Soviet Union by Michail Timofeevič Kalašnikov. In 1949 the weapon was adopted by the Soviet army with the denomination 7.62 mm AK-47.It is considered the most widespread and manageable assault rifle in the world. It is durable and reliable. To shoot, the operator inserts the magazine, pulls and releases the cocking lever and then pulls the trigger. In semi-automatic, the weapon fires a single shot and the trigger must be released in order to fire again. In automatic mode, the firearm shoots cyclically, charging, triggering and expelling the blows one after the other until the magazine is exhausted or the trigger is released. The gases generated by the shot are partially conveyed through a hole in the upper part of the barrel towards the recoil system, where they push back the piston, which in the retrograde movement pushes the shutter, causing the obturator to withdraw, which in turn expels the cartridge case and takes a new shot back into position thanks to the recovery spring. The system used in the weapon is referred to as "long stroke", as the piston moves backwards for a rather long stroke, physically pushing the shutter-holder back. The distance between the rear sight and the viewfinder in the AK is 378 mm. The sight is adjustable from 100 to 800 meters. The shooter can hit targets at short distances (usually up to 100 m) without any necessary adjustment. The standard version of the weapon uses 7,62 × 39 mm projectiles, with a sprint speed of 715 m / s. The weight of the ammunition is 16.3 g, with a 7.9 g ball. The AK-47 has always been considered a fairly precise weapon. The loader for the AK-47 has a very pronounced curvature that allows easy access to the chamber for the projectiles. The one in use weighs 250 grams (discharge). The weapon can also mount 40 or 75 (drum) rifles commonly used in the RPK light machine gun. There are also variations of 10, 20 or 100 shots. All AK-47 specimens can be fitted with various 40mm hang-up grenade launchers. The standard grenade is the VOG-25 (or VOG-25M) fragmentation, with a lethal radius ranging from 6 to 9 m. In addition to the Soviet Union, the AK-47 (with all its variants and copies) are produced in at least another dozen countries.

2. Case report

We reported a case of young man killed by a Kalashnikov. The corpse was in the prone position. Six holes of a firearm on the body were observed; particularly to the back, chest and left arm. The lesion situated on the chest was the size of 3x2 cm (Figures. 1-2). At the time of inspection the plan metric for ballistic calculations was performed in the following manner: shooting cameras, integrated with the satellite images of the crime scene extrapolated from the website http://www.bing.com/maps/; identification of the location of the discovery of the corpse throughout the use of cadastral maps; measurement of the building and the land in front of the building in the crime scene; pads planimetric and 3D views of the crime scene; shoe-pads and biological agents; evidence collection of shells and cartridges found on the ground; location of shell casings and measurement of distances from the site of the discovery of the corpse. The measurements were used by the ballistic engineer to make the vector calculations. On the scene the ballistic elements were collected. An external examination of the body was carried out, and subsequently a MSCT (Multi-Slice Computed Tomography) and autopsy were performed. On the corpse was found a single fatal blow whose trajectory was: 1-rupture of the dorsal vertebrae, 2-laceration of the left

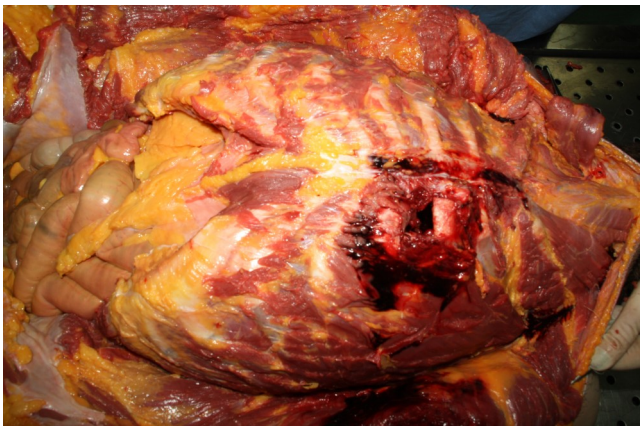

Figure 3.

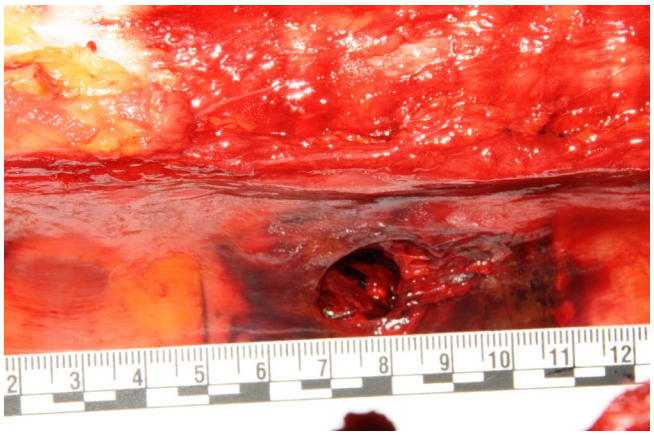

Figure 4.

lung with hemorrhage, 3-outbreak of the heart and pericardium and massive hemothorax (Figures.3-4). The ballistic calculations have allowed us to establish that the subject was facing the shooter at first and then, in an attempt to escape, he was back than killer. In the case presented the speed and violence of this firearms have caused an explosion of the heart. These data show the potentially devastating effects of this weapon. Often, these harmful effects make the shooting dynamics reconstruction difficult. For this reason, we underline the importance of multidisciplinary approach in assessment of the murders in these cases. Only a careful evaluation within the inspection and the performance of vector calculations on the crime scene and the corpse allows a reconstruction of the murder and becomes a scientific evidence in the court for obtaining a correct reconstruction of the events.

3. Discussion

From the review of the literature of the last 20 years, the few articles describing the injuries caused by this rifle concern war scenarios; the lesions described above all concern the cranio-facial district and the management that must be put in place in case the subjects hit by the AK-47 projectiles manage to survive. In general, gunshot injuries are responsible for high morbidity and mortality. The extent of the damage depends on many factors and, generally, the highest energy possessed by the blows exploded at close range causes greater damage. The extent of tissue damage depends on internal tearing, tissue compression, and the presence of cavitations along the projectile path. Moreover, the severity of the lesions depends on the possible impact of the projectile with the bones, since it determines the formation of bone fragments which are also responsible for other injuries independent of the insults caused primitively by the projectile. The injuries caused by the Kalashnikov and involving the head and neck are devastating and can even be fatal, causing an instant death. These regions, in fact, contain vital organs in a confined space. The main cause of death, in these cases, is the conspicuous hemorrhage due to the injury of the large vessels. The damage to the tissues is either direct, due to the passage of the projectile, or connected to the kinetic energy transferred to the tissues themselves. Of course, for the evaluation of cases like these, CT (Computed Tomography) becomes essential. In fact, it facilitates the characterization of the vascular lesions of the neck that may include: partial or complete occlusions, pseudo-aneurysms, intimate flaps, dissections and arterio-venous fistulas. It also provides additional information regarding cervical soft tissue conditions, respiratory and digestive tract conditions, the vertebral canal and spinal cord. Above all, it provides clarifications on the trajectory followed by the projectile. This last function of the CT is also very important from a medico-legal point of view. The forensic pathologists are, in fact, required to search and find every fragment of the projectile, as well as to establish the possible routes. The AK-47 characteristic is therefore at its capacity to cause direct, but also indirect, injuries due to the great kinetic energy that is transferred to the tissues. Peter A Ongom et al., in their article, report precisely the case of a young African woman hit by a bullet exploded by an AK-47 rifle, which had an atypical entry hole and the bullet was placed at the level of the carotid sheath of the neck [1].

As for the lesions involving the brain, a certain tendency for fragments of the projectiles to migrate into or out of the ventricles, inside the cistern, in the hypsense-lateral cerebral hemispheres and also in the cerebellum is to be found. Besides causing death, the presence of these foreign bodies in the brain can cause abscesses, cerebral-spinal fistulas, post -traumatic epilepsies, hematomas and infections. The mechanisms underlying this healthy phenomenon vary: the softness of the brain tissue, the greater weight of the projectile than the brain tissue and the penetrating dynamics coupled with the pulsatile nature of the cerebral ventricles. Moreover, we must consider the force of gravity that, in the movements of the subject, determines the migration of these fragments in certain sites rather than in others, as evidenced in the report by Richard A. Rammo et al. [2].

Sabri T. Shuker et al., in their work, analyze a series of 12

cases of people hit by stray bullets, exploded in the air to express joy or anger. The analysis concerns the physics, the clinic and the management of these wounds. The lesions affected the cranial-facial district and they are a further demonstration of the devastating force of the AK-47, compared with other weapons used for the same purpose. In fact, it has the highest mortality and morbidity rate probably due to the speed of the projectile, its shape and its center of gravity. The wounds have a vertical or very high incidence angle, even if the kinetic energy is much lower compared to that deriving from a directly exploded projectile. In these cases, the possibility of fatal wounds is very high. In the cases examined in this article, the entrance hole through the skin of the cranial vault and the maxilla-facial region is small; usually it is circular or oblique. In adults, it is much more evident, probably due to the incomplete ossification present at the level of the cranial vault of the children. In cases where there were two holes, the entrance was the level of the skull vault, the bullet then came out at the level of the anterior fossa of the skull, or from the orbit, or from the petrous bone. In the only case of a surviving patient, the projectile was in the maxillo-facial region. These wounds are characterized by less tissue damage, without the classical cavitations, moreover the kinetic energy can be more quickly dissipated in the sinus cavities. When the entry is directly at the level of the middle third of the skeleton of the face, the projectile crosses the skull entirely and exits from the other side. The exit hole resembles that of entry, because the projectile encounters less resistance in the passage through the paranasal cavities and the bone is thinner. In the mandibular wounds comminuted fractures of the cortical bone were found, different from those seen in the entry hole or that affected the bones of the sinus cavities. The projectile can be found at the level of the floor of the mouth or the tissues of the neck [3].

Denise A. Whitfield and Steven J. Portouw report a retinal detachment following the injury caused by an AK-47 rifle. A rebounded projectile had hit the young man, thus it endowed with a lower speed and kinetic energy and the capacity to damage the maxillofacial district severely. The bullet entered at the level of the ethmoid sinus, there were no injuries to the ocular globe, nor was there a direct scleral impact. However, the impact force absorbed by the ocular globe and responsible for the detachment can be derived from the force of the shock wave transmitted. The bullet was indeed found in the near breast [4].

Finally, it must be pointed out that even the savage shots can cause serious and even fatal injuries. Miodrag Zdravkovic et al. they report three cases involving lethal injuries from blank cartridges. These are special ammunitions, used for exercises or even in the theater. 0.76 g of freely poured nitrocellulose gunpowder and of the initial powder charge primer of fulminant. Basically, they are used to simulate gunshot noise. In the manuals the safety distance shown is 20 meters. The injuries are due to the explosion wave of the shot and, if the projectiles are exploded at close range or in direct contact, they can have the same consequences as normal ammunition. The entry holes are identical. The difference is that the lesions on the skin are exclusively due to the tension generated by the firing gases and not by the power of the bullet. The characteristics of these holes are: shape of the relatively irregular defect, with frequent presence of laceration of the skin that originates from the edge of the wound; burns; the edges and the initial part of the wound canal are dark; there are particles of dust and soot impressed and stratified in the initial part of the wound canal; besides there is the absence of the bruise ring. Even in histology there are no specific characteristics that make it possible to differentiate the two lesions in a clear way. Moreover, again in the case of blows exploded at absolute distance with conventional ammunition, the wound channel is wider towards the inlet hole and described as having a "truncated cone" shape. The specificity of the wound canal caused by the blank cartridge is that the wound channel also has a truncated cone shape, but its narrowest part is towards the entry hole. In addition, there is no exit hole for the loss of power of the exploding gases [5].

4. Concluding Remarks

In conclusion, the choice of AK-47 rifle depends on the will to ensure a crime free of hitches and with fatal outcome for the victim. Gunshots do not always lead to death; this, in fact, depends on the type of weapon chosen. The mafia clans, like 'Ndrangheta, choose this type of weapons to successfully define the homicidal purpose, as well as to have devastating effects on the victims, in order to disfigure them for revenge.

References

1] Ongom P. A., Kijjambu S. C., Jombwe J. Atypical gunshot injury to the right side of the face with the bullet lodged in the carotid sheath: a case report. J Med Case Rep. 2014 Jan 27;8:29.

2] Rammo R. A., DeFazio M. V., Bullock M. R. Management of migrating intracranial bullets: lessons learned from surviving an AK-47 bullet through the lateral brainstem. World Neurosurg. 2012 Mar-Apr;77(3-4):591.e19-24.

3] Shuker S. T., Sadda R. Craniomaxillofacial falling bullet injuries and management. J Oral Maxillofac Surg. 2012 Jul;68(7):1593 -601.

4] Whitfield D. A., Portouw S. J. Retinal detachment due to facial gunshot wound: the utility of ultrasonography in a medically austere environment. J Emerg Med. 2012 Jun;42(6):678-81.

5] Zdravkovic M., Milic M., Stojanovic M., Kostov M. Three cases of death caused by shots from blank cartridge. Am J Forensic Med Pathol. 2009 Dec;30(4):403-6.

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Proteome Analysis Implicates Adaptive Changes in Metabolism and Body Wall Musculature of *Caenorhabditis elegans* Dauer Larva

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ABSTRACT

Dauer larva is an alternative developmental stage of *Caenorhabditis elegans* (*C. elegans*) that occurs when the environmental condition is unfavorable for growth. Little is known regarding how the proteome of dauer larvae respond to poor environmental growth conditions. Such knowledge is expected to help understand the survival mechanism(s) of dauer larvae. In order to uncover the proteome differences between dauer larvae and normally developed third stage larvae (L3), an L2 stage larvae was starved to create the dauer larvae and this proteome was compared with that of the L3 larvae. Results showed that proteins involved in muscle assembly and fatty acid oxidation are increased in dauer larvae, while proteins involved in maintaining regular organismic activity such as reproduction, translation and apoptotic processes are decreased. The protein expression profile also suggested that the glyoxylate cycle is preferentially utilized during dauer arrest over the tricarboxylic acid (TCA) cycle and significant structural rearrangement occurs on the hypodermis, body wall musculature, and pharynx .

Keywords: larval development, dauer larva, aging, metabolism, proteomics, SILAC

Abbreviations: *C. elegans: Caenorhabditis elegans*; LC-MS: liquid chromatography mass spectrometry; PFOA: perfluorooctanoic acid; SILAC: stable-isotope labeling by amino acids in cell culture; 2D-PAGE: two dimensional polyacrylamide gel electrophoresis.

1. Introduction

Under adequate nutritional and environmental conditions of growth, *Caenorhabditis elegans* (*C. elegans*) rapidly progress from an embryonic stage, through four larval stages (L1 to L4), and to a reproductive adult stage within ~3-4 days at 20 °C. When the L2 larvae encounter unfavorable conditions after progression to the L2 stage (e.g. insufficient food, high temperature, and high-population density), they develop into an alternative L2 stage (L2d) larvae which molt and arrest as dauer larvae until the conditions become favorable [1]. Dauer larvae are thin and surrounded by a specialized cuticle [2]. Their locomotion and metabolic rate are significantly reduced [1,3] and they can survive for several months in this developmentally arrested state without feeding. Once environmental conditions become favorable for growth, the dauer larvae re-enter the life cycle within an hour, and become an L4 stage larvae in approximately 10 hours [4].

Extensive investigation has been done to understand the signaling pathway of dauer formation using molecular genetics and gene expression analysis [3,5,6]. These studies identified many dauer larva formation (daf) genes and revealed that insulin/IGF-1, TGF-β and cGMP signaling pathways have critical roles in dauer formation. However, relatively few proteomic studies have been reported [7–9]. Previous proteomic studies revealed that proteins involved in fatty acid degradation (e.g., alcohol dehydrogenase and aldehyde dehydrogenase) [7] and stress responses (e.g., heat

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shock proteins and antioxidative enzymes) [8,9] are highly expressed in dauer larvae. Although these preceding studies using 2D-PAGE or label-free LC-MS based approaches implied several underlying strategies that dauer larvae take to cope with environmental stress, less than 30 proteins were found to be altered in these studies. Therefore, our knowledge regarding proteome responses to environmental stress remain limited.

In this study, we compared the expression levels of individual proteins in dauer and L3 larvae by a quantitative shotgun approach employing a stable-isotope labeling by amino acids in cell culture (SILAC) [10]. The SILAC approach provided accurate quantitative results on more than 1300 proteins from dauer and L3 larvae. We found that the expressions of many of these proteins were altered in dauer larvae. The observed proteome changes appear to make sense with the alternations in the energy utilization pathways and morphological changes that are known to occur in dauer larvae.

2. Material and Methods

2.1. Materials

$^{12}C_6$- and $^{13}C_6$-lysine (Lys) were purchased from Sigma-Aldrich (St. Louis, MO) and Cambridge Isotope Laboratories (Tewksbury, MA), respectively. Lys-C was purchased from Wako USA (Richmond, VA). All other commercially available chemicals were reagent grade or higher.

2.2. Labeling Bacteria with Light ($^{12}C_6$)-Lys and Heavy ($^{13}C_6$)-Lys

Arginine and lysine auxotrophic *Escherichia coli* (*E. coli*) strain AT713 were obtained from the E. coli Genetic Stock Center at Yale University, and they were labeled with either light ($^{12}C_6$)- or heavy ($^{13}C_6$, 99 atom %)-Lys as described previously [11].

2.3. C. elegans: Strain, Maintenance, and Age Synchronization

WT Bristol N2 strain nematodes were used in this study [12]. Worms were cultured on peptone-free nematode growth media (NGM) plates (51 mM NaCl, 25 mm K_3PO_4, 5 μg/mL cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$) seeded with the light ($^{12}C_6$)- or heavy ($^{13}C_6$)-Lys labeled *E. coli*. Detailed protocols for the culture have been described [11]. To synchronize the worms' age, gravid nematodes were treated with a bleach solution (5% sodium hypochlorite solution, 0.25M NaOH) for 3 to 9 minutes, with occasional vortexing. After bleaching, the surviving eggs (embryos) were incubated in liquid M9 media without food and were allowed to hatch as age-synchronized nematodes [11]. The post-hatched period of the egg was counted as the L1 larva.*2.4. Preparation of L3 and dauer larvae*

L3 larvae were prepared by culturing age-synchronized worms on peptone-free NGM plates seeded with light ($^{12}C_6$)-Lys *E. coli* [11] and grown into the L3 larval stage. The larval stage was verified by visual inspection under a microscope. Dauer larvae were prepared by transferring age-synchronized L2 larvae that had been fed with light ($^{12}C_6$)-Lys *E. coli* onto a *E. coli*-free 2% agar plate and culturing them at 20 °C for 48 h. Three plates, each with approximately 500 worms, were prepared for both L3 and dauer larvae. The worms from each plate were pooled and subjected to the following proteomic study.

2.5. Preparation of $^{13}C_6$-Lys labeled reference L3 and dauer larvae

Heavy ($^{13}C_6$)-Lys labeled L3 and dauer larvae were prepared from the eggs laid by the heavy ($^{13}C_6$)-Lys labeled animals and heavy ($^{13}C_6$)-Lys *E. coli* was given as a food source instead of light ($^{12}C_6$)-Lys *E. coli*.

2.6. Sample preparation for proteomic analysis

Light ($^{12}C_6$)-Lys labeled L3 or dauer larvae were resuspended in 250 μL of 100 mM ammonium bicarbonate containing 4% perfluorooctanoic acid (PFOA) (w/v) [13], protease inhibitor mixture (Sigma-Aldrich), and phosphatase inhibitor mixture 3 (Sigma-Aldrich). Proteins were then extracted by ultrasonication (4.5 kHz three times for 9 s with a 3-min pause on ice between the strokes) using a Virsonic 100 ultrasonic cell disrupter (SP Scientific, Warminster, PA) as described previously [10]. The extracted proteins were reduced with 10 mM dithiothreitol (DTT) at 37 °C for 30 min, then *S*-alkylated by 25 mM iodoacetamide at 25 °C for 45 min. Subsequently, the amount was determined with a DC protein assay kit (Bio-Rad, Hercules, CA). A total of 25 μg of protein was digested by Lys-C at a Lys-C: protein ratio of 1:25 (w/w) at 37 °C for 18 hrs. The digest was then mixed with the equal amount of Lys-C digest of heavy ($^{13}C_6$)-Lys labeled L3 and dauer larvae 1:1 mixture, which served as a reference digest, and analyzed by LC-MS/MS as described below.

2.7. LC-MS/MS analysis

LC-MS/MS analysis was carried out using a NanoAcquity Ultrahigh-pressure liquid chromatography system (Waters, Milford, M) interfaced to Velos Pro Ion Trap/Orbitrap Elite Hybrid Mass Spectrometer (Thermo Scientific, Bremen, Germany). Lys-C digests (typically 2 μg) were chromatographed on a reversed-phase 0.075 × 150-mm C18 Acclaim PepMap 100 column (Dionex Inc.) using a linear gradient of acetonitrile from 2% to 37% over 202 min in aqueous 0.1% formic acid at a flow rate of 300 nL/min [14]. The eluent was directly introduced into the mass spectrometer operated in a data-dependent MS to MS/MS

switching mode, with the 25 most intense ions in each MS scan subjected to MS/MS analysis. The full MS scan was performed at a resolution of 120,000 (full width at half-maximum) in the Orbitrap detector, and the MS/MS scans were performed in the ion trap detector in collision-induced dissociation mode. The fragmentation was carried out using the collision-induced dissociation mode with a normalized collision energy of 35 eV. The data were entirely collected in the profile mode for the full MS scan and the centroid mode for the MS/MS scans. The dynamic exclusion function was applied for previously selected precursor ions with the following parameters: repeat count of 1, repeat duration of 40 s, exclusion duration of 90 s, and exclusion size list of 500. Xcalibur software (Version 2.2 SP1 build 48, Thermo-Finnigan Inc., San Jose, CA) was used for instrument control, data acquisition, and data processing.

2.8. Identification and quantification of peptides and proteins

Proteins were identified by comparing all of the experimental peptide MS/MS spectra against the Wormpep database (ftp://ftp.wormbase.org/pub/wormbase/releases) released on May 7 in 2015 using Mascot database search software (Version 2.2.0, Matrix Science, London, UK). Carbamidomethylation of cysteine was set as a fixed modification, whereas variable modifications included oxidation of methionine to methionine sulfoxide, acetylation of N-terminal amino groups, and replacement of C-terminal Lys with heavy Lys. The mass tolerance was set at 10 ppm for precursor ions and 0.8 Da for product ions. Strict Lys-C specificity was applied, and missed cleavages were not allowed. Protein isoforms and proteins that could not be distinguished based on the peptides identified were grouped and reported as a single protein group. The SILAC Quantification Suite in ProteomicsTools software (version 3.9.9, https://github.com/shengqh/RCPA.Tools/releases) [15], was used to determine the abundance of light ($^{12}C_6$)-Lys and heavy ($^{13}C_6$)-Lys labeled proteins, from which the ratio of light protein to heavy protein was calculated.

2.9. Data analysis

All data analyses were performed using RStudio (version 1.0.136) [16]. For each sample, the output of ProteomicsTools software, and the light ($^{12}C_6$)- to heavy ($^{13}C_6$)-Lys labeled protein ratios for individual proteins were transformed to log2 ratios to normalize the data distributions from which the mean log2 ratio of all the proteins were subtracted to reduce the technical variability associated with the experiment. This data processing was done without imputing missing values.

Student's two-sample *t*-test was used to estimate the significance levels for the difference in protein expressions between the dauer and L3 larva groups. A *P*-value of <0.05 with a *q*-value of <0.05 was considered significant. Gene ontology analysis was performed on significantly altered proteins in dauer larvae compared to L3 larvae using DAVID version 6.8 (https://david.ncifcrf.gov/) [17,18].

3. Results

3.1. The expression levels of many proteins are reduced in dauer larvae

Each of the three biological replicates samples from dauer and L3 larvae were analyzed once by LC-MS/MS. All the LC-MS/MS raw data are available from the corresponding author on request. Supplementary Tables S1-6 list all peptides identified and quantified in the data on the dauer and L3 larvae samples. Supplementary Table S7 summarizes all the proteins that were identified and quantified in the study. We were able to identify and quantify 801 proteins from the dauer larvae and 1321 proteins from the L3 larvae, of which 732 proteins were common to both the larval stages, which corresponds to 91% and 55% of proteins identified from the dauer and L3 larva samples, respectively (Fig 1a). The fact that a significantly lesser number of proteins were identified from the dauer larvae may suggest that a substantially smaller number of protein species is required for dauer larvae than for normally developing L3 larvae to maintain their viability.

Since a constant amount of the $^{13}C_6$-Lys reference digest was added to all the digests of the experimental samples prior to the LC-MS/MS, we were able to obtain the light ($^{12}C_6$)-Lys to heavy ($^{13}C_6$)-Lys protein ratios for all the proteins identified. The distributions of log2 Light/Heavy protein ratios for the proteins quantified in each of the triplicate dauer and L3 larvae samples are shown in Fig 1b. The boxplot demonstrates the high reproducibility of the biological replicates. The interquartile ranges (IQR) for the dauer samples (mean IQR on the triplicate samples = 1.18) is

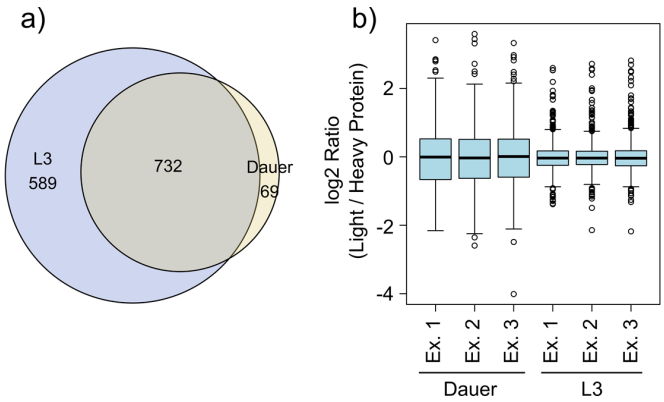

Figure 1. Overview of the proteomics data. (a) Venn diagram depicting the number of proteins identified from the dauer larvae, L3 larvae, and both. (b) Box plot showing the distribution of log2 Light/Heavy protein ratios for the triplicate samples of dauer and L3 larvae. The 95% confidence intervals for the median values on the plots were as follows: dauer Ex. 1 (-0.084 ~ 0.070, n = 0 598), dauer Ex. 2 (-0.105 ~ 0.040, n = 0 610), dauer Ex. 3 (-0.062 ~ 0.080, n = 0 614), L3 Ex. 1 (-0.058 ~ -0.016, n = 0 1039), L3 Ex. 2 (-0.054 ~ -0.017, n = 0 1092), L3 Ex. 3 (-0.062 ~ -0.020, n = 0 1072).

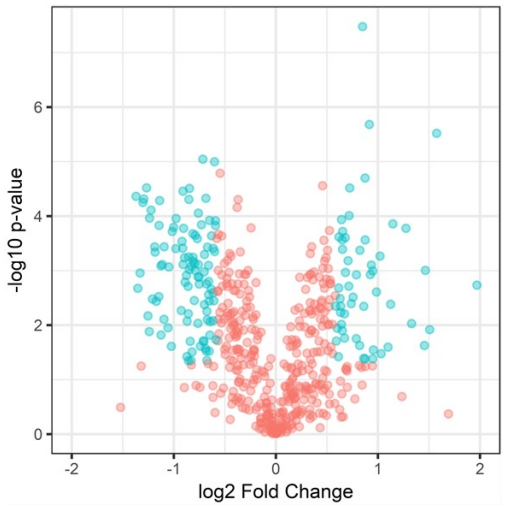

Figure 2. Expression profiles of dauer and L3 larvae proteins. A total of 539 proteins that were quantified in at least two biological replicates in both the dauer and L3 larvae triplicate samples were analyzed. The log2 fold changes (dauer/L3) of proteins are plotted against their negative log10 *p*-values in a volcano plot. Proteins whose expression levels were significantly altered ($p < 0.05$) greater or lesser than 1.5-fold are marked with blue color.

much larger than the L3 larva samples (mean IQR on the triplicate samples = 0.42), indicating that the range of expression levels for individual proteins in dauer larvae is wider than that for proteins in L3 larvae. This suggests that there are higher number of proteins in dauer larvae that are highly up- or down-regulated from those in L3 larvae.

Next we selected 539 proteins that were quantified in at least two biological replicates in both the dauer and L3 larvae triplicate samples. We then plotted the log2 fold changes (dauer/L3) of the 539 proteins against their −log10 *p*-values to visualize the significantly altered proteins (blue dots) (Fig 2). The volcano plot shows that there are 155 proteins (blue spots) that were significantly altered (*p*-value < 0.05) greater or lesser than 1.5-fold in dauer larvae compared to L3 larvae. 54 out of the 155 proteins were expressed in dauer larvae in greater amounts than in L3 larvae while the remaining 101 proteins were expressed in lesser amounts. This result indicates that roughly a quarter of the proteins (155/539) were altered in the dauer larvae. Note that since the same protein amounts from the dauer and L3 larvae samples were analyzed and compared, the log2 dauer to L3 larva fold changes provide relative representation of individual proteins in the total proteome of dauer to L3 larvae.

3.2. Proteins involved in maintaining regular organismic activity are decreased in dauer larvae, while proteins involved in muscle assembly are increased

Proteins that were quantified in at least two biological samples either in the dauer or L3 larvae triplicate samples, which includes the 539 proteins described above, were used in subsequent bioinformatic analyses. The dataset contained a total of 1019 proteins, of which 715 proteins were found in both the dauer and L3 larvae samples. Among the 1019 proteins, 54 proteins were expressed 1.5-fold (both *p*- and *q*-value <0.05) greater in the dauer larvae compared to the L3 larvae (log2 ratio > 0.5849). An additional 19 proteins were uniquely found in the dauer larvae (Supplementary Table S8). We assumed that the unique 19 proteins found only in the dauer larvae were expressed in significantly higher levels compared to the L3 larvae, therefore included in the subsequent bioinformatic analyses. On the other hand, 101 proteins were expressed 1.5-fold (both *p*- and q-value <0.05) lesser in the dauer larvae compared to the L3 larvae (log2 ratio < −0.5849). An additional 375 proteins were found only in the L3 larvae (Supplementary Table S9) and were considered to be expressed significantly higher level in the L3 larvae (lesser levels in the dauer larvae). Note that in addition to *p*- and *q*-value, we applied the threshold value of 1.5 to further filter the dataset to minimize false positives. The threshold value was determined by inspecting the fractions of proteins having *q*-values greater than 0.05 at different threshold values. At the threshold value of 1.5, only 4.9% of proteins had the *q*-values greater than 0.05, indicating that the threshold is not overly assertive and appropriate.

The proteins in Supplementary Tables S8 and S9 were subjected to GO term analysis to predict the physiological changes that might have occurred in the dauer larvae to adapt to starvation conditions. As shown in Fig 3a, proteins increased in the dauer larvae were found to be enriched in six GO-biological processes (BP) terms: 1) locomotion, 2) hermaphrodite genitalia development, 3) lipid storage, 4) striated muscle contraction involved in embryonic body morphogenesis, 5) muscle thin filament assembly, and 6) fatty acid beta-oxidation. On the other hand, many proteins decreased in the dauer larvae were enriched in GO- BP terms that represent normal cellular activities (e.g., reproduction, translation, and apoptotic process). These results suggest that in dauer larvae, metabolic activity is significantly reduced, larval development is halted, muscle related functions are altered, and energy utilization is switched to fatty acids. Similarly, the number of GO-molecular function (MF) terms for proteins that are involved in normal cellular activities such as nucleotide binding, protein binding, and oxidoreductase activity were decreased in the dauer larvae (Fig 3b). Only three GO-MF terms were enriched for proteins increased in the dauer larvae. These were 1) actin binding, 2) motor activity, and 3) actin filament binding, thus implying alterations of muscle assembly in dauer larvae. GO-cellular component (CC) terms enriched for proteins that were increased in the dauer larvae were also mostly related to muscle assembly and contraction. This suggests a significant change in muscle assembly, perhaps to protect them against environmental stress for prolonged survival.

Possible protein-protein interactions between proteins increased in the dauer larvae were examined using STRING web-based software [19]. As shown in Fig 4, the analysis revealed three major networks. The first network involved 11

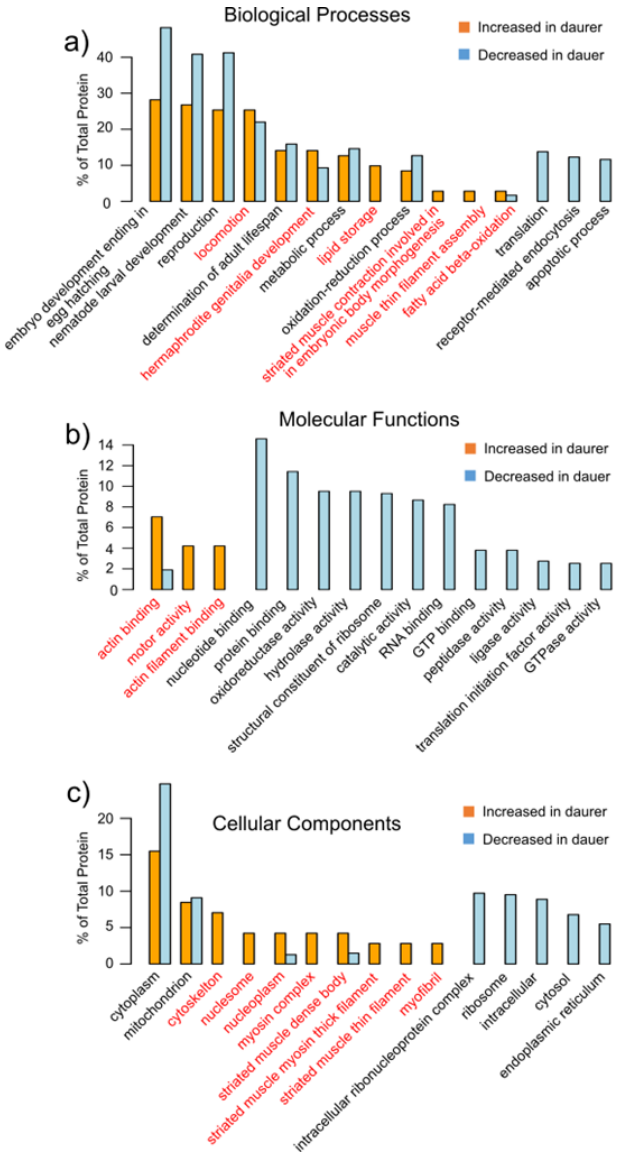

Figure 3. Gene ontology (GO) analysis. The bar plots showing the categorization of the significantly altered proteins (fold-change > 1.5; p-value < 0.05) according to their roles in the major biological processes (a), molecular functions (b) and cellular components (c). The orange and blue bars represent the percentages of the significantly increased and decreased proteins in the dauer larvae from a total of 71 and 473 proteins, respectively. GO terms are ordered from the greatest to least percentages for the significantly increased proteins in the dauer larvae. These are significantly more enriched in dauer larvae than L3 larvae are highlighted in red.

revealed three major networks. The first network involved 11 muscle related proteins, suggesting once again the significant alterations in the muscle assembly of dauer larvae. The second network contained enzymes involved mostly in fatty acid catabolism. The result suggests that the energy utilization in dauer larvae is switched to fatty acids. The third network was comprised of three histone proteins. Increased expression of the histone variants in dauer larvae may suggest regulation of gene expression, cell growth, and proliferation by these histones via regulating DNA accessibility.

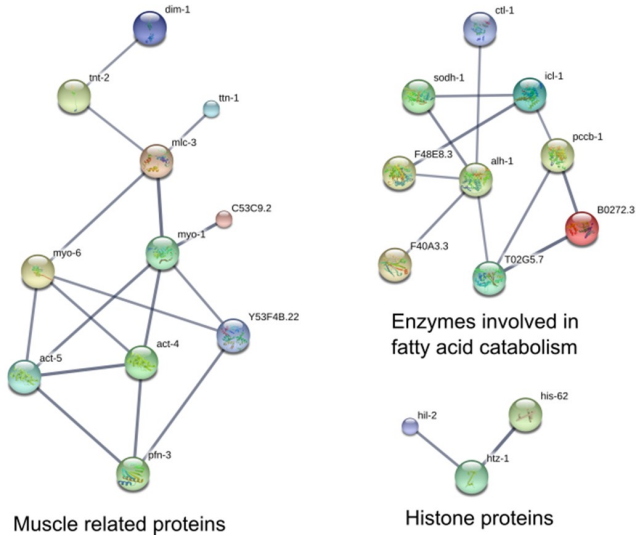

Figure 4. Interaction diagram for the proteins elevated in dauer compared to L3 larvae. The image was created by STRING web software 10.0 with high confidence score > 0.7. Line thickness between nodes indicates the strength of data support. myo-1: myosin-1; myo-6: myosin heavy chain structural genes; mlc-3: myosin essential light chain; pfn-3: profilin-3; dim-1: disorganized muscle protein 1; C53C9.2: uncharacterized protein C53C9.2; tnt-2: troponin T; act-4: actin-4; act-5: actin-5; ttn-1: titin homolog; Y53F4B.22: actin-related proteins; pccb-1: propionyl-CoA carboxylase beta subunit; T02G5.7: uncharacterized protein (possesses acetyl-CoA acetyltransferase activity); B0272.3: probable 3-hydroxyacyl-CoA dehydrogenase; alh-1: aldehyde dehydrogenase; F40A3.3: phosphatidylethanolamine-binding protein homolog; F48E8.3: uncharacterized protein (possesses succinate dehydrogenase activity); icl-1: bifunctional glyoxylate cycle protein; sodh-1: alcohol dehydrogenase 1; ctl-1: catalase-2, htz-1: histone H2A.V; hil-2: histone H1.2; his-62: probable histone H2B 4.

4. Discussion

It is known that the glyoxylate cycle is preferentially utilized in dauer larvae over the tricarboxylic acid (TCA) cycle to convert fat to carbohydrate [20]. There are two glyoxylate cycle specific enzymes, isocitrate lyase and malate synthase (in *C. elegans* the two enzymes are encoded by a single gene and expressed as a bifunctional protein ICL-1), and one TCA cycle specific enzyme, isocitrate dehydrogenase. In our data, the expression level of ICL-1 *(icl-1)* was 1.6-fold higher (p-value < 0.001) and the expression level of NADP-isocitrate dehydrogenase (*idh-1*), inhibition of which was recently shown to promote dauer development [21], were 1.3-fold lower (p-value = 0.014) in dauer larvae compared to L3 larvae. Thus, the results are consistent with the preferential usage of the glyoxylate cycle over TCA cycle in dauer larvae.

Dauer larvae possess a thick protective cuticle that covers the entire body and protects the animal from environmental insults [22,23]. The cuticle is secreted by the epithelial cell hypodermis covering the body. Therefore, it is reasonable to assume that the expression levels of proteins present in the hypodermis are altered. As we predicted, 11 of 73 proteins

Table1. Localization of proteins expressed highly in dauer larvae.

WormBase protein ID	Gene name	UniProt accession number	Protein name	*Expressed location
CE12358	act-4	P10986	Actin-interacting protein 1	body wall musculature, pharynx
CE16463	act-5	O45815	Myosin, essential light chain	pharynx
CE17755	cey-1	O62213	C. elegans y-box	pharynx
CE27706	dim-1	Q18066	Disorganized muscle protein 1	body wall musculature
CE00788	dlc-1	Q22799	Neuronal calcium sensor 2	body wall musculature, pharynx
CE00788	dlc-1	Q22799	Dynein light chain 1, cytoplasmic	body wall musculature, pharynx
CE25238	glrx-10	Q9N456	Glutaredoxin	body wall musculature, pharynx
CE01613	gst-5	Q09596	Major sperm protein 10\ 36\ 56\ 76	pharynx
CE01613	gst-5	Q09596	Probable glutathione S-transferase 5	pharynx
CE16405	hip-1	G5EE04	Uncharacterized protein F08B12.4	hypodermis
CE04890	hmg-11	G5EEL9	HMG	body wall musculature, hypodermis, pharynx
CE23521	icl-1	Q10663	Bifunctional glyoxylate cycle protein	hypodermis, pharynx
CE01341	inf-1	P27639	Eukaryotic initiation factor 4A	pharynx
CE47024	lea-1	H2FLL1	Barrier-to-autointegration factor 1	body wall musculature
CE46912	lea-1	H2FLK7	Probable 3-hydroxyacyl-CoA dehydrogenase B0272.3	body wall musculature
CE50292	lev-11	NA	Tropomyosin isoforms a/b/d/f	body wall musculature
CE28372	mca-3	Q95XP6	Calcium-transporting ATPase	body wall musculature
CE01236	mlc-3	P53014	Myosin, essential light chain	body wall musculature, pharynx
CE01236	mlc-3	P53014	Myosin, essential light chain	body wall musculature, pharynx
CE06253	myo-1	P02567	Muscle M-line assembly protein unc-89	pharynx
CE23736	NA	Q9N5T2	Uncharacterized protein	hypodermis
CE00852	NA	P41938	Probable 3-hydroxyacyl-CoA dehydrogenase B0272.3	body wall musculature, pharynx
CE08610	NA	Q8MXD9	Uncharacterized protein	body wall musculature, hypodermis, pharynx
CE00852	NA	P41938	Probable 3-hydroxyacyl-CoA dehydrogenase B0272.3	body wall musculature, pharynx
CE31529	NA	Q20626	Uncharacterized protein	hypodermis
CE21614	oig-2	Q9XWM1	One IG domain	hypodermis, pharynx
CE00194	pat-2	P34446	Integrin alpha pat-2	body wall musculature, hypodermis
CE00194	pat-2	P34446	Integrin alpha pat-2	body wall musculature, hypodermis
CE32473	paxt-1	Q21738	Partner of xrn-2 protein 1	hypodermis
CE07269	pccb-1	Q20676	Propionyl coenzyme A carboxylase beta subunit	body wall musculature
CE07332	pfn-3	Q21193	Profilin-3	body wall musculature
CE04813	sgt-1	Q21746	Bifunctional glyoxylate cycle protein	body wall musculature, pharynx
CE12212	sodh-1	Q17334	EGF-like domain-containing protein C02B10.3	hypodermis
CE20253	sup-1	Q9XWU2	Protein SUP-1	body wall musculature
CE34313	tnt-2	Q7Z072	TropoNin T	body wall musculature
CE36725	ttn-1	G5EFF0	Titin homolog	body wall musculature
CE03924	unc-78	Q11176	Actin-interacting protein 1	body wall musculature, pharynx
CE30426	unc-89	O01761	Muscle M-line assembly protein unc-89	body wall musculature, pharynx
*The information on the location of the expression was obtained from WormBase (http://www.wormbase.org)				

that were increased in dauer larvae were expressed in the hypodermis (Table 1) suggesting that a significant physiological change occurs in the hypodermis of dauer larvae, presumably to facilitate the production of cuticles. It is also known that dauer larvae are extremely thin [23] and they tend to be motionless [24], thus expected to have significant changes in the body wall musculature. Furthermore, we found that 25 of the 73 proteins that were increased in dauer larvae are expressed in the body wall musculature (Table 1), reflecting the significant structural and physiological rearrangement that occurs on the body wall musculature of dauer larvae. Additionally, the feeding organ pharynx undergoes radial shrinkage and cease pharyngeal pumping during dauer arrest [23]. Among the proteins that were increased in dauer larvae (Table 1), the proteins expressed in the pharynx were also found to be enriched (21 of the 73 proteins). Thus, these results seem to reflect significant morphological changes that occur in dauer larvae.

Several proteomic studies have been done on dauer larvae. Mádi and coworkers prepared dauer larvae by the same way we employed and found that the expressions of four proteins are altered in dauer larvae [7]. They were aldehyde dehydrogenase (*alh-1*), alcohol dehydrogenase-1 (*sodh-1*), phosphatidylethanolamine-binding protein (*F40A3.3*), and pyrophosphatase (*pyp-1*). We found that three of the four proteins (aldehyde dehydrogenase, alcohol dehydrogenase, and phosphatidylethanolamine-binding protein) were also identified in our study. Consistent with the previous study, these three proteins were also increased in dauer larvae in our study. Other two proteomic studies on dauer larvae reported by Jones and coworkers [8] and Erkut and coworkers [9] found that proteins involved in the environmental stress response such as heat shock proteins and superoxide dismutase are increased in dauer larvae. We observed many stress response proteins including eight heat shock proteins (*hsp-12.2*, *hsp-3*, *hsp-110*, *hsp-6*, *hsp-1*, *hsp-4*, *hsp-17*, and *hsp-60*), three superoxide dismutases (*sod-2*, *sod-1*, *sod-1*), two glutathione peroxidases (*gpx-5*, *gpx-2*), and five glutathione S-transferases (*gst-7*, *gst-4*, *gst-5*, *gst-36*, *gst-10*). Contrary to the previous proteomic studies, these proteins were not increased in dauer larvae in our study except glutathione S-transferase 5 (*gst-5*) and catalase (*ctl-1*). The discrepancy between ours and other groups' data could be due to the differences in the protocol employed to generate dauer larvae. Jones and coworkers generated dauer larvae by limiting the available food as we did, but the conditions were different from ours [8]. Erkut and coworkers generated dauer larvae by desiccating nematode at a higher temperature (25 °C). The different results observed by different groups suggest that the proteome of dauer larvae could be dependent on the dauer generation procedure despite all showing similar morphological appearances. Lastly, a global gene expression analysis on dauer larvae has been reported [25]. The study found that genes coding anti-stress proteins such as superoxide dismutase, catalase and heat shock proteins are highly expressed in dauer larvae. This is contrary to our proteomic results. The majority of these anti-stress proteins were not altered in our results. The study also found that the expressions of several histone variants are altered in dauer larvae, suggesting the structural changes of dauer chromatin. This observation is consistent with our results. Thus, it appears that the gene expression data and our proteomic data agree to some extent but not completely. Reasons for this inconsistency include 1) differences in the dauer larvae preparation method between the studies, 2) differences in the extent of transcriptome and proteome coverages (>10,000 transcripts vs ~1,400 proteins), and 3) the fact that mRNA levels do not necessarily reflect the levels of protein expressions [26].

5. Concluding Remarks

The proteome changes we observed in dauer larvae are consistent with the metabolic and morphological changes that occur on *C. elegans* during dauer arrest. Our study provides a foundation for a complete understanding of the adoptive mechanism utilized by this important model organism during unfavorable growth conditions.

6. Supplementary material

Table S1. List of peptides quantified in the dauer larvae experiment 1

Table S2. List of peptides quantified in the dauer larvae experiment 2

Table S3. List of peptides quantified in the dauer larvae experiment 3

Table S4. List of peptides quantified in the L3 larvae experiment 1

Table S5. List of peptides quantified in the L3 larvae experiment 2

Table S6. List of peptides quantified in the L3 larvae experiment 3

Table S7. Proteins identified

Table S8. Proteins highly expressed in dauer larvae compared to L3 larvae and proteins found only in dauer larvae

Table S9. Proteins highly expressed in L3 larvae compared to dauer larvae and proteins found only in L3 larvae

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References

1] C. Erkut, T. V. Kurzchalia, Planta 242 (2015) 389–396. doi: 10.1007/s00425-015-2300-x

2] P.J. Hu, WormBook (2007). doi: 10.1895/wormbook.1.144.1

3] A. Calixto, Life without Food and the Implications for Neurodegeneration, Elsevier Ltd, 2015. doi: 10.1016/bs.adgen.2015.09.004

4] M. Klass, D. Hirsh, Nature 260 (1976) 523–5.

5] D.L. Riddle, M.M. Swanson, P.S. Albert, Nature 290 (1981) 668–671. doi: 10.1038/290668a0

6] P.S. Albert, D.L. Riddle, Dev. Biol. 126 (1988) 270–293. doi: 10.1016/0012-1606(88)90138-8

7] A. Madi, S. Mikkat, C. Koy, B. Ringel, H.J. Thiesen, M.O. Glocker, Biochim Biophys Acta 1784 (2008) 1763–1770. doi: 10.1016/j.bbapap.2008.05.017

8] L.M. Jones, K. Staffa, S. Perally, E.J. LaCourse, P.M. Brophy, J. V. Hamilton, J. Proteome Res. 9 (2010) 2871–2881. doi: 10.1021/pr9009639

9] C. Erkut, A. Vasilj, S. Boland, B. Habermann, A. Shevchenko, T. V Kurzchalia, PLoS One 8 (2013) e82473. doi: 10.1371/journal.pone.0082473

10] Y. Yuan, C.S. Kadiyala, T.T. Ching, P. Hakimi, S. Saha, H. Xu, C. Yuan, V. Mullangi, L. Wang, E. Fivenson, R.W. Hanson, R. Ewing, A.L. Hsu, M. Miyagi, Z. Feng, J Biol Chem 287 (2012) 31414–31426. doi: 10.1074/jbc.M112.377275

11] M. Miyagi, in: A.K. Shukla (Ed.), Methods Enzym., 2017, pp. 77–89. doi: 10.1016/bs.mie.2016.09.015

12] C. elegans S. Consortium, Science (80-.). 282 (1998) 2012–2018.

13] C.S. Kadiyala, S.E. Tomechko, M. Miyagi, PLoS One 5 (2010) e15332. doi: 10.1371/journal.pone.0015332

14] K. Vukoti, X. Yu, Q. Sheng, S. Saha, Z. Feng, A.L. Hsu, M. Miyagi, J Proteome Res 14 (2015) 1483–1494. doi: 10.1021/acs.jproteome.5b00021

15] Y. Guo, M. Miyagi, R. Zeng, Q. Sheng, Biomed Res Int 2014 (2014) 971857. doi: 10.1155/2014/971857

16] R Core Team, (2016).

17] D.W. Huang, B.T. Sherman, R.A. Lempicki, Nucleic Acids Res. 37 (2009) 1–13. doi: 10.1093/nar/gkn923

18] D.W. Huang, R. a Lempicki, B.T. Sherman, Nat. Protoc. 4 (2009) 44–57. doi: 10.1038/nprot.2008.211

19] D. Szklarczyk, A. Franceschini, S. Wyder, K. Forslund, D. Heller, J. Huerta-Cepas, M. Simonovic, A. Roth, A. Santos, K.P. Tsafou, M. Kuhn, P. Bork, L.J. Jensen, C. Von Mering, Nucleic Acids Res. 43 (2015) D447–D452. doi: 10.1093/nar/gku1003

20] W.G. Wadsworth, D.L. Riddle, Dev Biol 132 (1989) 167–173. doi: 0012-1606(89)90214-5 [pii]

21] S. Penkov, D. Kaptan, C. Erkut, M. Sarov, F. Mende, T. V. Kurzchalia, Nat. Commun. 6 (2015) 1–10. doi: 10.1038/ncomms9060

22] P.S. Albert, D.L. Riddle, J. Comp. Neurol. 219 (1983) 461–81. doi: 10.1002/cne.902190407

23] R.C. Cassada, R.L. Russell, Dev. Biol. 46 (1975) 326–42.

24] G.N. Cox, S. Staprans, R.S. Edgar, Dev. Biol. 86 (1981) 456–70.

25] S.J.M. Jones, D.L. Riddle, A.T. Pouzyrev, V.E. Velculescu, L. Hillier, S.R. Eddy, S.L. Stricklin, D.L. Baillie, R. Waterston, M. a Marra, Genome Res. 11 (2001) 1346–1352. doi: 10.1101/gr.184401.tions.

26] N. Nagaraj, J.R. Wisniewski, T. Geiger, J. Cox, M. Kircher, J. Kelso, S. Paabo, M. Mann, Mol Syst Biol 7 (2011) 548. doi: 10.1038/msb.2011.81

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Indicative of Violence in Homicidal Women

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ABSTRACT

The purpose of the multidisciplinary investigation was to analyze the participation of violence in homicidal women (Ferro, 2012:121-129) [6], from the criminological aspects; through field work done at the Preventive and Female Rehabilitation Center of Big Bridge, Jalisco, Mexico (2008-2014). Methods, criminological, sociological and law research methods were handled; with interviews with female inmates accused of homicide; including their life history, using a sample of 25%, and analyzing the psychobiological and socio-legal variables. In the discussion and results, there is influence of psychobiological and social aspects in the violent behavior of women, and who have had a life history impregnated with violence, mainly by the family, patron (Lima, 1991) [11] who repeat with children and/or against those who come to affect them, feeling threatened in their integrity. In this complex analysis there are several indicators and external and internal agents that encourage women to apply violence. This entails an interfactorial and multifactorial relationship between related causalities, as in the study of criminal Victimology (Marchiori, 2000) [13]. It is concluded that violent acts carried out by homicides come from a combination of multifactorial aspects, in addition to the change of role from victim to victim, in 90% of the cases, which brings with it psychological and social problems in the behavior of the homicide, being added in some of them the mental illnesses and with a high degree of aggressiveness.

Keywords: Violence, homicides, victimology, life history, indicatives

1. Introduction

This research describes the multicausality of psychobiological and social aspects of female criminal behavior, analyzing the participation of violence in homicidal women, as victims and victimizers. In this sense, homicide is defined as "depriving a person from life by one or several subjects" (Hikal, 2015: 305) [10].

In order to know the problem, a diagnosis was made of it and the antecedents are described, where it was identified how women from history have experienced violence since childhood, with a high percentage from the primary family, later becoming victimizers. Defining violence as "direct or indirect action, destined to harm a person or to destroy their integrity or psychic, their possessions or their symbolic participations" (Hikal, 2015: 589) [10].

Regarding the methodology, fieldwork was carried out at the Preventive and Rehabilitation Center for Women in Big Bridge, Jalisco, Mexico (2008-2014), with a mixed qualitative and quantitative approach; design of non-experimental, transversal or transectional, correlational / causal research. Also, handling other general and specific methods. An instrument was applied to the female prison population imprisoned for the crime of homicide, in 25% (10 internal) voluntarily from 18 to 42 years, including case studies and life histories.

Among the results of the investigation it was identified that the homicidal being of poor sectors, and being a victim of violence, has very little credibility, therefore there is a lack of complaints, in addition to feelings of guilt, psychological imbalances, lack of adaptation to the environment and its surroundings; Due to all this context where it accumulates aggressiveness, it triggers a victim profile of various crimes such as homicide, which is usually carried out in the impulsive and depressive phases against family members, with a high level of cruelty. Therefore, it is important to consider exogenous and endogenous factors that influence this behavior, together with the consumption of drugs and alcohol, considering the life histories of these women with

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continuous abuse, where there is a preponderant role of the perpetrator's power against the victim, starting a discriminatory and marginalizing process, where the violence to which it has been subjected is minimized.

Regarding the crime of rape, in practically the whole world, the Non-Governmental Organizations (NGOs), those in charge of administering justice, the official censuses, the civil support centers, etc. coincide in the enormous difficulty that exists to obtain precise figures of different types of crimes. One of them, perhaps the most representative of this difficulty, is related to the exercise of sexual violence; However, in different parts of the planet, from countries considered "First World" and with a broad democratic tradition to the supposed underdeveloped countries, the figures that we have, although inaccurate, allow us to observe a complex social phenomenon of still unknown dimensions (Trujano, 1992; Silva, 2008: 67) [20, 19].

Statistics show that "in Mexico, up until 1989 a woman was thought to be raped every nine minutes" (according to data from CAMVAC - Support Center for Raped Women, AC-, 1985, in Trujano, 1991, Silva, 2008: 68) [20, 19].

Walker (1987, Trujano, 1992, Silva, 2008: 73) [22, 20, 19] concluded that the same author comments that victims of upper or upper middle class generally do not report the case due to fear of being exposed to public opinion, so they seek private medical attention and sometimes they do justice with their own hand. Another problem is that the attackers are given very short sentences, for example, in Colorado, United States, at least until 1987, the average was two and a half years in prison.

2. Background. Diagnosis of the problem

Among the experiences of women who commit crimes, there is the violence that started from their childhood, caused mainly by the family. The above is demonstrated in an investigation on violence, which was compiled from May 1982 to March 1983, a total of 834 complaints of which 45.60% involved a girl as a victim. The main aggressor was the mother by 44% (Lima, 1991:133) [11].

Regarding child abuse (1983-1986, Mexico) (Systematic Analysis of the data recorded of abused children in the DIF Program, 1983-1986 Preman, National System for the Integral Development of the Family). It is an investigation that extracts the complaints, proving that 33.93% of children are mistreated. Of the 2,150 abused children were 49.67% of the female sex with an average of 7 years. It was found that the most abused was the first child of the family; being the mother the most aggressor with an average of 28 years of age (Lima, 1991:135) [11].

About battered women in Mexico (This research was carried out with the assistance of Dr. Luis Rodríguez Manzanera and under the direction of the M.C. Alvaro Martinez Solano Redilla and Elsa Delgadillo); the Professional Training Institute of the Attorney General of the Federal District, headed by Dr. Roberto Jocavén García, carried out an investigation to find out the size and characteristics of the problem of physical mistreatment of women in the home (Lima, 1991:139) [11].

Data was collected on 502 cases of women who filed a complaint with the Public Prosecutor's Office, 333 with medical reports of injuries, and 83.16% (out of 333) of the victims received minor injuries or simple blows, which confirms that more than hurting to the victim, the intention was to demonstrate superiority or dominance (Lima, 1991:139) [11].

The 68.53% dedicated to domestic chores in their home. The victimizer 40% of 26-33 years; 50.6% with a grade lower than secondary and 70% were employees, drivers and artisans. And the most frequent hours of mistreatment are the nocturnal ones and the days in which they are accentuated are Saturdays and Sundays. The place of the commission is in 96.81% of the cases the own house and not always in private. One out of every 3 victims has no independent home but lives with his or her family. According to court rulings, women are victims in 78% of cases, of domestic violence (Lima, 1991:139, 141) [11].

This problem has serious consequences, such as the attempted suicide of sexually abused women (Van Egmon, Garnestski, Jonker, 1990, Lima, 1991: 141) [21, 11]. Of the 158 women aged over 20 who were studied, the results showed that people who are sexually assaulted have more severe problems in their history like more suicidal behavior.

Violence is accentuated even more in young people, Juan José Medianero Peña (2006) [14] comments that, we are in a time of technological revolution of great advances in technology and of achievements before only possible in dreams; we can go to the moon or make cloning possible, but all this growth at a scientific and technological level is contrasted with poor human growth at a spiritual and moral level.

One of the manifestations of this indigence is violence, which is present more strongly in young people today, for which reason it is valid to ask ourselves why young violence. To answer our question, a great series of answers need to be investigated; which could be summarized in the violent crimes committed by young people and especially by adolescents is due to the confluence of psychological, family, economic and socio-cultural factors of this time (Medianero, 2006) [14].

Violence is not only a certain type of act, but also a certain potentiality. It does not refer only to a way of "doing", but also of "not doing". One way to identify a violent act is to recognize that violence is a perverse or malignant form of aggressiveness, which an individual exercises against another of the same species and that is characterized by its lack of justification, offensive tendency, illegitimacy and/or illegality.

Adolescence is a stage of identity formation and in the struggle to shape her personality, the adolescent is exposed to the anguish that causes her to obtain his independence and to define his aspirations to develop as an adult,

provoked by having to deal with an environment that he does not know or dominate, and that he often considers threatening. A bad process from adolescence to adulthood can result in a social maladaptation expressible many times through violence.

Unfortunately, some adolescents do not manage to assume that responsibility of the control of their moral behavior, nor a learning according to the rules, habits and customs of the group (socialization). This has as a consequence adolescents grouped in gangs carrying out acts where violence is exercised, committing mischief constantly and, even worse, committing illicit acts and in some cases becoming delinquent adolescents.

According Medianero (2006) [14], when we associate simpliefied young people with violence, we see them as future delinquent adults. Which are like that, because they have parents who neglected them, who are violent; and therefore "violence breeds more violence". It is important to reflect on that, the criminal option does not appear suddenly in the life of a child or transmitted in the genes. Statistics show that before there was a life plagued by abandonment, abuse and deprivation. There was also in general a family marked by poverty, violence and marginality. There was an early contact with the world of the street and an absolute lack of social inclusion spaces (neighborhood, school, club, parish, etc.), there were no institutions that could contain it.

There is also the influence of the media as an incentive for violence. In the world, an hour of television contains on average five to ten violent scenes, presented mostly as pleasant and good. There are also games that directly affect the personal rights of the human being, such as the right to live, spread massively on the Internet or distributed by large videogame stores; and if we remember that the human being learns through games we can realize the damage that these games do in adolescents when creating fixed conditioned responses in their subconscious, it would suffice to mention only some cases that happened in the United States to realize the terrible impact of these on young people (Medianero, 2006) [14].

Today there is another factor that is living with the adolescent and many times being part of his life. This monster is the drug that leads the adolescent sooner or later to act violently with her own or with herself. It can be concluded that the causes of juvenile violence are multiple, being able to highlight the following: (Medianero, 2006) [14]

-Abandonment, abuse and violence within the family (dysfunctional families), as the most important,;
-The lack of social inclusion spaces (such as the school, parish, etc.);
-Poverty and lack of employment;
-The use of alcohol and drugs.

As mentioned above, violence of any kind, as well as sexual violence, are factors that significantly affect female behavior. All the experiences that women have had, some more significant and important than others, mark them definitively in their future life; such is the case of the beginning of their sexuality, with or without the will of the female. From the above, of course, it is the sexual violation that, in many cases, defines the way of acting of the woman, whether in a self-destructive way or not.

As a self-destructive way in which the woman acts, prostitution is found, which was mostly chosen by the women who were raped, depending on the degree of damage (physical, emotional) in which this fact affected them, being the victimizer a relative or another person (s); even worse, if they did not receive help in the form of therapy, many times the guilt that grows in them progressively leads them to commit, in addition to prostitution, crimes such as the ingestion of drugs, robbery, homicide, prostituting daughters, among others. Thus, regarding the affectation of the sexual violation towards women, Marchiori [13] comments on the sexual delinquent behavior of the aggressor and its consequences.

Sexual conduct

In the dynamics of sexual behavior are two elements of importance: the particular sexuality that constitutes a serious alteration in the perpetrator of the crime and the behavior of the victim. There are many classifications (Karpman, 1974; Marchiori, 2001: 29) [13] on sexual crimes, such as rape, incest, impudence with children, exhibitionism and prostitution.

Define Marchiori (2001: 29) [13] that rape is the sexual relationship imposed and consummated with violence, in which the victim is forced to perform it. The modalities of the conduct of rape vary according to the aggressor and the circumstances. There are particularly sadistic violations, in these cases the study of behavior reveals that the satisfaction has been produced by the experience of violent and sadistic aggression on the body of the victim that by the genital significance of the behavior.

The behavior of rape "always supposes a great aggression and it is considered that the pathology of the individual is higher on a sexual level based on the characteristics of the victim, for example, children, the elderly, physically handicapped, victims with mental retardation" (Marchiori, 2001: 30) [13].

Within the consequences of the damage received by raped women, prostitution is found, this is the most common form of female crime. It is a behavior with a great self-destructive meaning. Prostitution conditions and favors the individual who exploits the young prostitute or facilitates the clients (Marchiori, 2001: 31) [13].

Many criminologists believe that "if the number of prostituted women is statistically compared with male crime, there would be a total equilibrium, that is, the rate of prostitution that represents the same percentage as male delinquency is so high". The most common form of female crime is that of antisocial sexual behavior. But what leads the young woman to perform this behavior? "There is a tendency towards self-destruction, which is present in the

psychological patterns of all prostitutes" (Marchiori, 2001: 184) [13].

The young woman, explains Marchiori (2001: 184) [13], may be in opposition to her parents or her environment, feels unfairly treated, has had traumatic experiences and now "comes" through prostitution. Prostitution may also have its origin in a form of neurotic child expression, in which the prostitute transfers her affective needs to the corporal. The socioeconomic situation must also be taken into account, but the essential determinants are psychological, since prostitution is a form of self-destruction and its sexual way of life is a defense mechanism against the disintegration of the self.

According to the "self-destructive tendency of female prostitutes, it is observed once again in the tattooing behaviors and the meaning that it acquires for them, especially in relation to the father figure" (Marchiori, 2001: 185) [13].

Other behaviors that can lead to being destructive in the woman who has been raped, is drug addiction. In the women interviewed in the Preventive and Female Readaptation Center of Big Bridge, Jalisco, the consumption of alcohol and drugs was detected. In the case of those held for the crime of homicide, four (66.7%) of the inmates have used drugs, such as stone; for example: "the interviewee likes to drink and dance, in terms of drugs, she took them before committing the crime to calm herself down"; "Yes, drugs. She was drugged with her partner (victim). She does not drink, she has tried drugs like stone, the first time they offered it and she liked it, they gave it to her to try several, she was a companion. Then I felt like increasing the dose, when I had no money I worked to get it. "

- As for alcohol, four (66.7%) of them consume what they consume.
- Only one (16.7%) of the delinquents reported that they smoke cigarettes.

The following was observed in the criminal and antisocial behavior of these women imprisoned for the crime of homicide: (Source: Interviews with inmates held at the Women's Rehabilitation and Preventive Center of Big Bridge, Jalisco)

For the crime of homicide,

- All (100%) these inmates have a criminal behavior; for example: "Tendency to the inevitable self-destruction (prostitution, drugs) without wanting to leave it"; "Due to the same disease (schizophrenia), she becomes a victim of herself, and a marked tendency to self-destruction, due to the total lack of affection and incomprehension that she suffered as a child"; "She is a very castrated person, not very communicative, with an attitude of not much confidence, with remorse, very conscious. For wanting to reach or keep something or someone does what it is, and for this reason is that she committed the crime of theft, which came out more and became robbery-homicide; she alleges that she commited the first one (to feed her wife and children), but that the second one was commited by her stepson, who accompanied them"; "Too much aggressiveness that leads her to be violent, uncontrolled.

Tendency to self-destruction (prostitution, destructive relationships, lack of orientation). Emotional instability that leads her to commit crime, in this case was the homicide of her partner "; "Too much aggression, resentment, violence, pain; tendency to self-destruction (prostitution, drugs, suicide attempt). This aggressiveness comes from being the daughter of an unknown father, by abandonment of all kinds by the mother; for the violations she has suffered; for the lack of an inhibitory brake that stops her in her self-destructive behavior; and it is added to that the mother of the inmate took her children away, due to her drug addiction behavior; all her problems have been reflected in her way of acting and self-attacking, such as the suicide attempt".

In this way, all types of violence that suffer and end up affecting women, of any social class and socioeconomic level, necessarily have consequences on their behavior, whether positive or negative; of which, the latter are the most common and those that lead the female to commit crimes such as robbery, homicide, among others.

At the beginning of the 21st century, violence was present in almost all human practices, and we can observe it among individuals as between collectives, countries, ethnic groups, creeds, internally between the different political regimes, in the effects produced by socioeconomic models, or in the various social sectors that inhabit the planet. Among the many types of violence recognized today, one of the most studied in the last 30 years, perhaps, directed against women and, more recently, against children (Trujano, 1992, Silva, 2008: 65) [20, 19].

Authors such as Corsi, Dohmen and Sotés (1995) [3] comment that the etymological root of the term violence refers to the concept of force and corresponds to words such as violate, violate or force, which implies the use of force to cause harm. Among the wide range of violent manifestations, the one referring to sexuality seems to involve very particular dyes. In sexual violence, we recognize sexual harassment, sexual abuse and rape. Among these, rape most likely represents the modality that has generated the most studies (Trujano, 1992, Silva, 2008: 66) [20, 19].

This may be due to the gap opened by feminist movements, thanks to which research focused on women's issues, including the violence to which they were subjected, rebounded, exposing the socially inherited nature of the myths and beliefs that, in a stereotyped way, they had dominated until that time, for example: there was the idea that women provoked their victimization and even enjoyed it, or that the aggressors were mentally ill or, at least, subjects with irrepressible impulses (Trujano, 1992, Silva, 2008: 66) [20, 19].

This feminism allowed to fracture the dominant discourses of the time, which normalized and translated the biological differences into generic ones; man-force, woman-weakness. Male violence ceased to be something natural, innate or biological, to become the unacceptable expression of the

power of man over women, and rape stopped being conceived as an act of sexual satisfaction (genital) to be placed in a crime against freedom, which was coerced by the power of the strong over the weak (Trujano, 1992, Silva, 2008: 66) [20, 19].

In 1990, (Trujano, 1992, Silva, 2008: 68) [20, 19], Dr. Ruíz Harrel, director and investigator of forensic services in sexual crimes of the Attorney General's Office of the Federal District, mentioned that only 5% of sexual crimes were denounced and that there was a violation every three minutes (Federal District Attorney General's Office, 1990). In August of 1998 there was talk of one rape every seven minutes (Gutiérrez, 1998) [8], while other sources cited the data of a rape every nine minutes in that year (Sherer, 1998) [18] (Zozaya, 1999, pp. 6-7, Trujano, 1992, Silva, 2008: 68) [24. 20, 19].

For its part, the Center for Therapy in Support of Victims of Sexual Offenses reported, from January to September 1997, 3, 186 cases, of which 85.7% corresponded to women and the rest to men. In 1993 the World Bank estimated that women between 15 and 44 years of age lose healthier years of life due to rape and domestic violence, than due to cancer of the uterus, obstructed labor, cardiovascular diseases, AIDS, respiratory tract infection, car accidents or war (Zozaya, 1999, pp. 6-7, Trujano, 1992, Silva, 2008: 68) [24, 20, 19].

In Mexico, the crime of rape for the Federal District is typified in the Penal Code with the fifteenth title, denominated "crimes against the freedom and the normal psychosexual development", chapter 1. This includes the crimes of sexual harassment, sexual abuse, rape and violation. In the latter case, if the victim chooses to draw up a record, it is important to know that the police interrogation usually takes place within a framework of skepticism and disbelief on the part of the police and, although this situation has changed over the years and exists in the currently specialized agencies run by women, usually represents a difficult situation to face (McCahill, Meyer and Fischman, 1979, Trujano, 1992, Silva, 2008: 69-73) [12, 20, 19].

In addition, there are few victims determined to denounce and, for this, there are two basic reasons to explain this phenomenon: the citizen aware of having been a victim does not want to report, or, on the other hand, some victims are not aware of it (especially if the event occurs within the family sphere) or the rights that assist them. The victims do not report in part for the humiliating and humiliating treatment they receive from the police (Beckmann, 1990) and for the doubts and skepticism with which they receive their demand (Norris and Feldman-Summers, 1981); in fact, cases of black victims or poor women receive less attention from the police (McCahill, Meyer and Fischman, 1979, Trujano, 1992, Silva, 2008: 69-73) [12, 20, 19].

3. Methodology

In methodology, fieldwork was done at the Preventive and Rehabilitation Center for women in Big Bridge, Jalisco, Mexico (2008-2014).

The research approach is mixed, being mostly qualitative than quantitative. The Research Design is non-experimental and transversal or transactional, correlational / causal, because existing situations and events that occurred previously are observed.

The criminological, sociological and legal research methods were managed, such as those of direct, indirect and systematic observation; also using the historical, inductive-deductive, non-rationalist, sociological, statistical paradigm. As well as the use of an instrument that was applied in the interview, where case studies and life stories were made; which was created by several specialists, based on two aspects to analyze, the psychobiological and the social.

The interviews were done in a personal and anonymous way, with closed and open questions. The application of the instrument was done in two stages, the first from 2008 to 2009, and the second from 2013 to 2014. The way to carry out these measurement instruments, is the application of the same, coding of answers, emptying of information, realization of graphs and research report.

The female prison population that participated in the field study, which were the inmates imprisoned for the crime of homicide, who accepted to participate in the investigation and had the willingness voluntarily, were 10, who represent 25% of the total population (penitentiary universe).

The interviewees who participated are between 27 and 42 years old, since the youngest ones refuse to be interviewed; although the ages between 18 and 35 years, are those of the beginning and maximum culmination in which women commit crimes, according to theorists.

Similarly, in order to protect the rights of the interviewees, and our intervention in the application of interviews, the inmates signed an informed consent letter, in which they described what the research is about, the methodology and the authorization of part of the participants, this based on the national bioethics commission

4. Results and Discussion

In the discussion and results, there is influence of psychobiological and social aspects in the violent behavior of women, and who have had a life history impregnated with violence, mainly by the family patron (Lima, 1991)[11] who repeat with children and / or against those who come to affect them, feeling threatened in their integrity. In this complex analysis there are several indicators with external and internal agents that encourage women to apply violence. This entails an interfactorial and multifactorial relationship between related causalities, as in the study of criminal Victimology (Marchiori, 2000) [13].

Regarding the credibility of the victim, this plays a very important role in the sociocultural aspects that surround her, because apparently many people still have numerous myths (Burt, 1980) [2], stereotypes and preconceptions

about rape that adversely affect perceptions on the credibility of the victim in court (Heilbrun, 1980, and Feild and Bienen, 1980, cited by Brekke and Borgida, 1988, Trujano, 1992, Silva, 2008: 74) [1, 20, 19].

Regarding the victim, studies have shown that the victim can be of any sex, age, ethnicity, creed, socioeconomic, professional or intellectual level, marital status, lifestyle, customs or physical attractiveness. In addition, the most common characteristics among victims of rape include their membership in socially less privileged sectors by sex or age (women and girls), socioeconomic level (low), ethnicity (black), youth, with a history of psychiatric care, which they are under the influence of alcohol and/or drugs, or have a low level of psychosocial affectivity (Myers, Templer and Brown, 1984, Trujano, 1992, Silva, 2008: 76-77) [15, 20, 19].

Trujano (1992; Silva, 2008: 77) [20, 19] explains that some women report feeling nothing at the time of the attack, as if the possibility of survival based on decoupling "the mind of the body", as if that was not happening. It seems that, for some victims, the only way to face the fact of being totally subject to the other person and with a high risk of being harmed or dying off, is to "disconnect", to block oneself. Unfortunately, this type of response can be interpreted legally as acceptance of the sexual act.

The psychological damage caused to a rape victim is very great. Trujano (1992; Silva, 2008: 81) [20, 19] comments that some psychologists who have investigated this field have observed that its duration and severity depend on particular characteristics. The first psychological reactions of the victim usually include fright, worry, fear, confusion, helplessness, anger, shame, humiliation, uncontrolled crying, avoidance of feared situations, social isolation, nightmares, retrospections and feelings of guilt and loss of self-esteem. There may also be effects of anxiety, depression, and feelings of exhaustion, obsessive thoughts and others (Citterio, Gualdi and Dall'Asta, 1980, Kilpatrick and Veronen, 1983, Trujano, 1992, Silva, 2008: 83) [20, 19].

Another interesting study "compared victims of attempted rape with victims of consummated violations; where the attack was completed the results were worse, with measures such as "nervous breakdowns", suicidal ideas and suicide attempts "(Kilpatrick, Best, Veronen, Amick, Villeponteaux and Ruff, 1985, Trujano, 1992, Silva, 2008: 83) [20, 19].

Trujano (1992; Silva, 2008: 90) [20, 19] explains that, unfortunately, in opposition to the needs of the victim, relatives and friends often try to silence their speech, their complaint and their demand for justice. Faced with suffering, violence, pain and the incomprehensible, many human beings can develop a response of flight and denial, preferring silence, and assuming that we should not distress the victim more by talking about what happened. They do not know that the opposite is normally just and that their avoidance can be interpreted (although sometimes this is the case), as a shame for what happened, increasing the victim's sense of isolation and "filth", who may feel stained and guilty time for staining their loved ones.

The feeling of guilt has been since ancient times as something universal and implicit in the human being, especially for women; González (2005: 28) [7] mentions that the feeling of guilt is self-reproach for the commission or omission of a behavior, from which one acquires moral conscience, for having learned its prohibition or for having the commitment to carry it out. The prohibition or commitment derive from the order that it gives, that subject of whom is taught in the ideology, constitutes the "superior being" constituted by God or the Father and to whom it is endowed with greater dignity, authority and government. In the dynamics of acceptance of the existence of a "higher being" is located to those who accept it, in conditions of inferiority that predispose it to guilt.

So the above, applied to the victim of rape, even when not guilty of anything, the woman feels a great guilt, which offended everyone and that is why she must pay. This feeling often leads to self-destruction, like prostitution, committing suicide or at least trying it, trying to perform negative behaviors, such as deviant and/or criminal, so that they can "atone" for their guilt of having been raped; and this, joining psychological imbalances that can cause rape in the victim, such as psychosis, schizophrenia, as is the case of one of the women interviewed in the Preventive and Female Readaptation Center of Big Bridge, Jalisco; Most of the time a promising life is destroyed towards the future, with an almost total mismatch to the environment that surrounds it and, sometimes, falling into serious crimes, such as robbery and homicide, as did the interviewee described above, with the murder of her own son. But of course, they involved many factors, both exogenous and endogenous, so that she could commit this type of crime.

In the interrogation, there is a feeling of guilt for having been raped when she was ten years old, by three relatives of her stepmother. In this way, she is a victim of the violation as she is also of his own fault (without being guilty); but also it is of the father who never defended her from the stepmother, it is of the psychological, emotional, physical and sexual mistreatment that she received from her childhood; she is also a victim of her own organism, that is to say, of the schizophrenia that she suffers, and that, in its most critical degree, led her to commit the murder of her son. Thus, as a synonym of all this victimization, there is "the power" "the domination" exercised at a given moment by the victimizer towards the victim; in submission and submission and, as a chain, the victim learns to exercise it, in a moment of liberation, in his (or her) victim (s).

In this way, to understand human behavior, González (2005: 32) [7] refers to the fact that, for many centuries, social science theorists such as politics, law and psychology have been slow to establish legal ideological discourse as an instrument of social power, all the interdisciplinary keys must be tested, to open the doors that allow the understanding of the individual and collective behaviors of men. Psychoanalytic theory, when dealing with power, also requires to know its discourse in order to access the social

institution and contribute to the description of the multifaceted relations between those who rule and those who obey, disengaging from men the explanation of social behaviors considered criminal, and linking them to concealments structured by power in the ideological discourse (Entelman, 1982, pp. 16-19, González, 2005: 32) [4, 7].

For this reason, González (2005: 33) [7] suggests that it is necessary to put an end to the concept of a natural "delinquent man", constructed as a sinful being, sick of evil and that therefore transgresses the norm of the Lord, in turn conceived as good and made for the benefit of all. It is required, on the other hand, to build another man, the one of "Unique Reason", that involves the arbitrariness and the interest of the dominant individual or collective that defines and applies the norm, as well as his responsibility in the construction of what he calls delinquent.

This clarifies, the opposite of the proverb, "who does not pay" and explains the process by which a person, even if innocent, must be sentenced and sacrificed to wash the guilt of other transgressors who do not they were repressed and left unpunished (as an example, the case of the woman interviewed in the Preventive and Female Rehabilitation Center of Big Bridge, Jalisco, held for the murder of her son). That is the "expiatory subject, the one who is built as a delinquent even if he is not, who dies for the forgiveness of sins committed by others" to free them from guilt and which is presented in history as constant to this day (González, 2005: 33) [7].

Lima (1991: 277; Roberts: 207) [11, 17] believes that due to the underestimation that drowns the feminine potential and the lack of belief in their own capacity, a discriminatory and marginalizing process of society towards women is generated. The minimization and social disqualification generates a victimizing mechanism that goes from the asymmetric relationships that are lived in the home every day, to physical aggressions that constitute crimes.

Consequently, the "victimized woman accumulates aggressiveness, temporarily repressed, slowly spilled as a conscious or unconscious revenge that possibly leads to criminality". The patterns observed in the social context, produce consequences that in turn cause suffering to the detriment of women or female groups, due to economic, political, social, etc; women suffer injustices that sometimes lead to the chain victimization-crime (Lima, 1991: 277-278) [11].

Lima expresses that, very often, there is an inverse relationship between crime and victimhood. That is, certain forms of victimization result in the emergence of aggressive behaviors that can lead to antisocial and criminal acts as defense mechanisms, converted into an adaptation disease. There is a chain of crime and victimization phenomena that in certain cases is easy to follow and check; such is the case of prostitutes. In follow-up studies of incest victims, it has been proven that there are women with a tendency to prostitution, to the use of drugs or alcohol, as a consequence of their victimization (Roberts: 255, Lima, 1991: 290) [17, 11].

In the relationship between victimhood and crime, the fundamental aspect is that, in the case of women who are victimized by physical aggression, and as a reaction, they victimize their children or ascendants. And on the other hand, it is discriminated against in many aspects by the same criminology that has not paid attention to female criminality and that does not know the substance of the problem, because the black figure is still very high. For example, Wilson [23] studied 17 textbooks on criminology, published in a period of 12 years to analyze the importance they give to the problem of female crime. Only five of them contained a special chapter called Female Offenders. Three mentioned a subtitle of the subject (Wilson and Rigsby, 1975: 131 et seq, Lima, 1991: 291) [23, 11].

In the interviews for the crime of homicide, the accused have a serious emotional and psychological problem. The reason that led them to commit this type of crime, was very complex and varied, two of them that have a history of conflicting life, they refer that it was due to schizophrenic illness for which they committed infanticide, two others mention that they did not commit it and the another two was in self-defense.

The murderer is the one that causes the death of a person, but who is the individual and why does he do it? Homicide as an individual behavior usually occurs to solve an interpersonal conflict. The subject is faced with the fact that he must face a new problem, that accumulated circumstance to others, can discharge in him an intense aggressiveness, a very impulsive uncontrolle (Marchiori, 2001: 22) [13].

In the individual homicide we distinguish homicide by alcoholism. Criminal behavior in a drunken state is very common in our environment. Alcohol addiction produces disinhibition that leads to aggressive and violent behavior. But this behavior is related to personality disorders, paranoid feelings, insecurity and a marked sense of inferiority. That is, alcohol acts as a trigger in criminal behavior (Marchiori, 2001: 22) [13].

Homicide in psychotic state. "Criminal behaviors can develop without any overt reason, such as cases of homicide committed by schizophrenic psychotics". These are impulsive behaviors, crimes apparently committed without hesitation, unforeseen unleashing is typical of psychotic impulsiveness that can manifest itself in aggressive hatred with respect to a family member or an unknown person (Marchiori, 2001: 23) [13].

The criminal behavior is characterized by being extremely violent until it becomes totally sadistic. Hallucinations and paranoid ideas cause the aggression to be projected in an unexpected way because the individual feels that he must kill the person who is persecuting him (in the case of paranoid schizophrenia). It is an anticipatory behavior at the level of the persecutory processes and for that reason they are unexpected, although in practice the crime and the hallucinations, and they are structured from before, as well

as the disorganization of the personality. Here, as in many cases, aggressive behavior translates the true dimension of mental illness (Marchiori, 2001: 23) [13].

Criminal in psychotic state can be triggered in the pictures of schizophrenia (in simple types, hebephrenic, catatonic, paranoid), in alcoholic psychosis (also related to a deep deterioration and to systematized delusions of persecutory type), in degenerative psychoses (the various senile dementia cases), in infectious psychoses, psychosis due to physical traumas, brain injuries due to accidents or falls that may involve serious changes in behavior and uncontrolled activity. There are also homicides in manic-depressive psychosis, in both phases violent behaviors can be produced in acute delirious mania, the subject is intensely active, with delusions, hallucinations, psychomotricity is intense and their impulses are uncontrolled, they become violent (Marchiori, 2001: 24) [13].

In the depressive phase, homicides occur so that the "family does not suffer in the future", are the crimes in which the mother or father kills the children and then commits suicide. Ey expresses that a whole series of advanced pathological homicidal acts can be described, from the most automatic to the most conscious. Ey distinguishes: (Marchiori, 2001: 24-25) [5, 13]:

a) Homicides that are carried out in a state of total or almost total unconsciousness, this is the case of murders in the course of confusional states;
b) At a level of less profound disorders, it points out the homicidal acts perpetrated by demented or confused patients, whose aggressive impulses escape the control of their intelligence;
c) The homicide may be determined by a delusional motivation, then depends on feelings or ideas of persecution, jealousy, poisoning;
d) Like suicide, homicide can appear as an obsession, drive to which the subject resists to the limit of the possible.

Rasko (Lima, 1991: 120-121) [11, 16] states that two thirds of women victimized dependents (children) or adults between 26 and 60 years (spouses or lovers) in 40% of the cases, proving that They provoked the inner one by causing her to be involved in forms of alcoholism, brutality or humiliation. And most of the homicides were motivated by some emotional conflict.

Marchiori (2001: 194-195) [13] comments that, especially in women, passional homicide is observed. The behavior of homicide always implies a disintegration of the personality since only in a great psychological stress can a person kill another. This behavior of attacking in a destructive way such as homicide behavior, is only projected by a person with a serious psychic problem, because the psychological conflict that allows the projection of primitive and destructive impulses are structured through complex circumstances, but where they predominate confusional and psychotic psychopathological elements (Ey) [5].

In homicidal behavior the aspects of lack of control, marked sensibility and sadism that are projected with enormous symbolic meanings are surprising (Von Hentino, 1960). Among the different types of homicide in women, homicide by emotional identification (jealousy) predominates. In addition the affective element predominates and the aggression is handled not as in the man impulsively, but the aggression is prepared many times meticulously and sadistically (Marchiori, 2001: 194-195) [13].

Homicide occurs in women to solve an interpersonal conflict, this conflict erupts, and it is triggered after a slow process in which the woman feels despised, marginalized and humiliated. Usually the alcoholic husband who beats her, who assaults her physically and morally, is the beginning of feelings of revenge manifested in a very slow way. They are the crimes in which the woman waits for a circumstance in which the victim lies on his back, asleep to assault him until he is killed; in other cases poisoning in small doses (Marchiori, 2001: 25) [13].

In most of the crimes of women there is an affective relationship between the perpetrator and the crime victim, that is, there is an emotional process that triggers the crime. It is also observed that sometimes the woman is not the author of the crime who is going to kill the victim, but the instigator who convinces the husband or another family member to commit the murder (Hesnard, 1980; Marchiori, 2001: 196) [9, 13].

The states of anguish and depression as well as feelings of guilt can lead to crimes in which children are killed so that in the future they do not suffer; the mother kills the children and then commits suicide, that is, the woman comes to the conviction that it is better for the children not to continue living. It happens in this crime in an unexplained way and the violent behavior is not understood with the aspects of the personality of the author, whose trait of non-aggressive person adapted to the environment, with social and moral values, without a criminal record contrasts paradoxically with behavior destructive of the family nucleus. But, the depressive and confusional aspects of the woman had already been formed before the crime, as well as a gradual disorganization of the personality that is projected in the crime (Marchiori, 2001: 196) [13].

In infanticide, there are alternatives of confusion, loneliness and insecurity of the young woman, which will progressively increase her fear of the family and the situation of pregnancy (Marchiori, 2001: 197) [13]. The reaction of the woman accused of the crime of homicide and confined in the Preventive and Female Readaptation Center of Big Bridge, Jalisco; three (50%) of the inmates revealed that they are very impulsive; one (16.7%) of them mentioned that it is only 60%; two are very controlled.

As for the aggressiveness of the interviewees, one mentioned that she is not irritated; another says "I keep very stable, I only get angry when they say high-sounding words"; two more say they are very irritable and aggressive; one of these comments that it is due to "everything since my husband began to treat me badly and cheated me with my

homosexual brother; but I have tried to calm down, "and the other because" I left the house, because I could not stand being there, they forbade me many things; then my partners mistreated me; I took out all the aggressiveness through the use of drugs, because I did not know how to face my problems. Once, when my son said he was selling drugs, he gave me despair, he wanted to kill me and run (Source: Interviews with inmates held at the Women's Rehabilitation and Preventive Center, Big Bridge, Jalisco, Mexico).

Regarding the mental and psychological problems of those accused of the crime of homicide, six (100%) expressed yes having this type of problems, some more than others: for example: "she suffers from schizophrenia, tried to commit suicide as a child (10 years) because they raped her and her stepmother did not believe her; she cut her wrists"; "I do not like being segregated, we live several in a single room. Neutral attitude; Many psychological problems, because of my husband's bad treatment, because he cheated on me with my brother who was gay, that's why I divorced, I do not believe in men.

She found them together (brother and husband) in her own house, I got very angry, then my brother died of HIV. I have trauma from men; Many psychological problems, I tend to get away from people, sometimes I'm neurotic, I get depressed a lot; I can not openly take my emotions; Yes, many psychological problems. I am very impulsive. I left home to get married, I got into drugs. Much irritability and aggressiveness, that's why I left the house, because I could not stand being there, they forbade me many things; then my partners mistreated me; I took out all the aggressiveness through the use of drugs, because I did not know how to face my problems.

Regarding the cruelty of the interviewees, one (16.67%) mentions that she has not been cruel; five (83.33%) say that they have had cruelty, especially against themselves; for example: "Once I cut my veins with a mirror, being drugged; against others, he fought with blows; against herself and who can harm her; It was drugged, taken and prostituted"(Source: Interviews with internal inmates in the Preventive and Female Readaptation Center, Big Bridge, Jalisco, Mexico).

Regarding the lack of control, only one (16.67%) inmate mentions that she controls her emotions; the other five (83.33%) do have a lack of control; for example: "for jealousy; I'm out of control, I'm very impulsive and aggressive; but I am already changing; I am very impulsive, aggressive, I am out of control; The older son was hit once and she does not know where she is now. I have become so uncontrolled by drugs and problems that once I cut my veins with a mirror, being drugged. When arriving here Preventive and Female Readaptation Center, Big Bridge, Jalisco, Mexico, my behavior at the beginning was not good, it was very out of control; He has a lack of control because of the many psychological problems. I am very impulsive.

I left home to get married, I got into drugs. Much irritability and aggressiveness, that's why I left the house, because I could not stand being there, they forbade me many things; then my partners mistreated me; I took out all the aggressiveness through the use of drugs, because I did not know how to face my problems. The six interviewed for the crime of homicide are very anxious (Source: Interviews with inmates in the Preventive and Female Readaptation Center, Big Bridge, Jalisco, Mexico).

Regarding vices (alcohol and drugs), four (66.7%) of the inmates have used drugs, such as stone; for example: "the interviewee likes to drink and dance, in terms of drugs, she took them before committing the crime to calm herself down"; "Yes, drugs. He was drugged with his partner (victim). He does not drink, he has tried drugs like stone, the first time they offered it and he liked it, they gave it to him to try several, he was a companion. Then I felt like increasing the dose, when I had no money I worked to get it". With regard to alcohol, four (66.7%) of them consume what they consume (Source: Interviews with internal inmates in the Women's Rehabilitation and Preventive Center, Big Bridge, Jalisco, Mexico).

The type of antisocial behavior that was perceived in the criminals imprisoned for the crime of homicide, is that all (100%) these inmates have a criminal behavior; for example: "Tendency to the inevitable self-destruction (prostitution, drugs) without wanting to leave it"; "Due to the same disease (schizophrenia), she becomes a victim of herself, and a marked tendency to self-destruction, due to the total lack of affection and incomprehension that she suffered as a child"; "He is a very castrated person, not very communicative, with an attitude of not much confidence, with remorse, very conscious.

For wanting to reach or keep something or someone does what it is, and for this reason is that he committed the crime of theft, which came out more and became robbery-homicide; he alleges that the first one did commit it (to feed his wife and children), but that the second one did the stepson who accompanied them"; "Too much aggressiveness that leads her to be violent, uncontrolled. Tendency to self-destruction (prostitution, destructive relationships, lack of orientation). Emotional instability that leads her to commit crime, in this case was the homicide of her partner"; "Too much aggression, resentment, violence, pain; tendency to self-destruction (prostitution, drugs, suicide attempt).

This aggressiveness comes from being the daughter of an unknown father, by abandonment of all kinds by the mother; for the violations he has suffered; for the lack of an inhibitory brake that stops her in her self-destructive behavior; and it is added to that the mother of the inmate took her children away, due to her drug addiction behavior; all his problems have been reflected in his way of acting and self-attacking, such as the suicide attempt"(Source: Interviews with inmates held at the Women's Rehabilitation and Preventive Center, Big Bridge, Jalisco, Mexico).

Is dangerousness perceived in these criminals? Yes, in all (100%) dangerousness is perceived, in some more than in others; for example: "especially for herself, although she does not want to recognize it. She is very aggressive because of the

environment in which she develops, and that leads her to have impulses that are going to be dangerous at the first provocation"; "Precisely because of her own illness, she has harmed herself and her own child (victim), even if the disease is controlled, the people around her may be in danger"; "In appearance he has a variable aggressiveness, with little tolerance to frustration, is positive, adapted, normal, verbal. However, because he wants to achieve his goals, he can commit more crimes"; "Very dangerous, since she does not know how to control her anger against men, blames her for her misfortune, starting with her ex-husband".

As long as it does not overcome this trauma, it will not stop being dangerous, because it expands its frustration everywhere"; "Very dangerous, precisely because of the way she responds to any stimulus, whether external or internal, she says she does not know how she will respond, she can attack herself and/or attack others; it is her way of blaming others for what she has had to live, for the negative experiences she has had throughout her life" (Source: Interviews with inmates held at the Women's Rehabilitation and Preventive Center, Big Bridge, Jalisco, Mexico).

The problems of the inmates confined in the Women's Preventive and Rehabilitation Center, Big Bridge, Jalisco, Mexico for the crime of homicide are aggravated by the consumption of drugs, of which two (33.33%) said they consumed glass; two (33.33%) cocaine; one (16.7%) nicotine; five (83.33%) consumed alcohol; two (33.33%) were taking tranquilizers.

The antisocial behaviors that the interviewees had had, only one (16.67%) of the inmates had been a member of gangs; three (50%) have practiced vagrancy; for example: "... in vagrancy, yes, and yes she has met with people with criminal behavior, and has done prostitution with drugs. She participated in fights, fought with two men with knives and injured them for threats of rape, and only stole her cell phone; I also fought with clients (in prostitution) for not wanting to pay, to blows. When I worked in security, I only gave macanazos"; the six (100%) inmates have left their homes; five (83.33%) have been related to people who have antisocial or criminal behavior; two (33.33%) have participated in fights; for example: "she fought two men with knives and injured them for threats of rape, and only stole her cell phone"; "She fought with blows"; two (33.33%) have used a weapon; for example: "Only with the mirror, knife and gun (of a friend)"; one (16.67%) has scars from a fight; for example: "She participated in fights, she fought two men with knives and I injured them for threats of rape"; two have tattoos (Source: Interviews with internal prisoners in the Preventive and Female Readaptation Center, Big Bridge, Jalisco, Mexico).

The inheritance-family antecedents of conducts for and antisocial of the interviewed ones were, Prostitution, mental illness (uncle); lesbianism, (two sisters) dancers, alcoholism (maternal family and mother); alcoholism (father and brother) types of conduct that the inmates have carried out: Prostitution, vagrancy, erotic dance, drug addiction, aggression Antecedents of illnesses in the families of the inmates: alcoholism, mental illness, medical and psychiatric diagnosis for the inmates: a for schizophrenia two (33.33%) of the inmates do not know how to control their impulses, and three (50%) do not tolerate frustration. The sexual preference of the inmates is: Four (66.7%) prefer men, one (16.7%) is lesbian and another (16.7%) bisexual (Source:

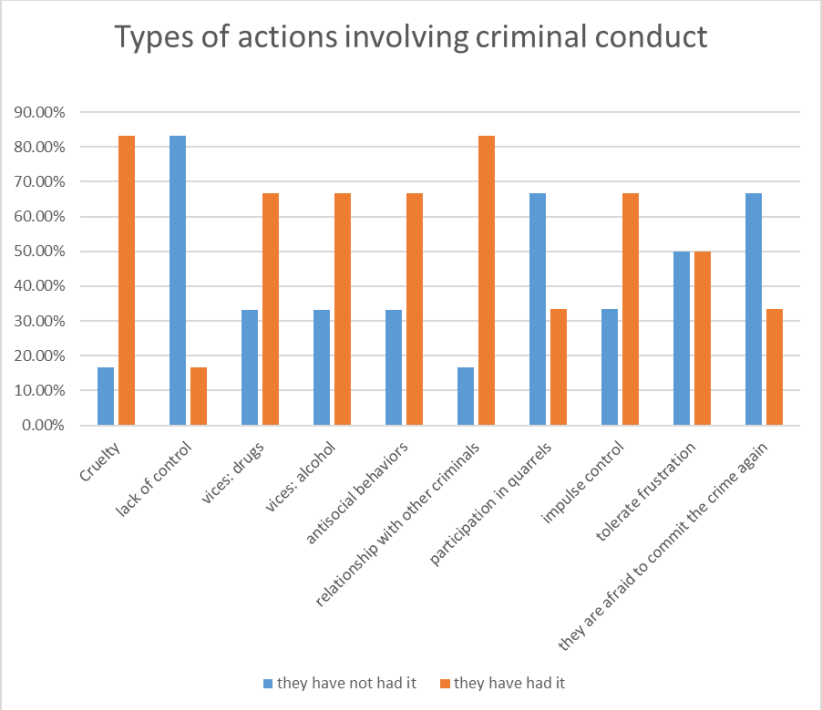

Figure 1. Source - Interviews with internal detainees in the preventive Centre and women's rehabilitation, Big Bridge, Jalisco, Mexico.

Interviews with internal inmates in the Preventive and Female Readaptation Center, Big Bridge, Jalisco, Mexico).

The effects that the criminal behavior of the inmates has caused are: "Absence in school/Change of character/Bad habits/Incommunication"; "Loss of freedom"; "Incommunication"; "Change of character"; "Lack of personal hygiene/Lack of responsibility/Bad habits/ Incommunication (with dads)" (Source: Interviews with internal inmates in the Women's Rehabilitation and Preventive Center, Big Bridge, Jalisco, Mexico).

Types of violence suffered by the inmates: physical; mistreatment by stepmother since childhood, rape; physical abuse by police; aggression of couples in the form of beatings, jealousy, prostitution and money; Intrafamily; sexual, psychological, physical, emotional and social; intrafamily, sexual, psychological (on behalf of mom) compared with the neighbor (Source: Interviews with internal inmates in the Preventive and Female Readaptation Center, Big Bridge, Jalisco, Mexico).

Two (33.33%) of the inmates are afraid of committing another crime; for example: "she is afraid of committing another crime, since she is exposed because of the work she does (prostitution)"; "Because I do not know how I'm going to react". Four of the inmates easily change their mood (Source: Interviews with inmates in the Women's Rehabilitation and Preventive Center, Big Bridge, Jalisco, Mexico).

The external influences that intervened to commit the crime, according to the internal: "The environment where I live, crime. Consider that the cause of their problems is the absence of their parents, lack of communication, boredom. Her criminal behavior did not change her character"; "My sickness; the pressure she felt with her son when he wanted to live with her and the inmate did not want to, because she was afraid of hurting him. The lack of early attention to my disease. The lack of love since childhood, the abuse I suffered, the rape that I suffered".

The interviewee mentions that, she felt rejected by her first child (the victim), it would be due to lack of a partner, she felt that she was not fit; "On the other hand, with her current partner she has never felt rejection or of her young children"; "Unemployment"; "For defense, fights with a partner because I exploited"; "Drugs, a friend"; "Drugs, bad examples, being an unwanted child" (Source: Interviews with internal inmates in the Women's Rehabilitation and Preventive Center, Big Bridge, Jalisco, Mexico).

The type of weapon used by the inmates imprisoned for the crime of murder: (1) tube, because he entered her house to steal; (2) injected medicine for dogs; (3) Ride to the ravine; (4) knife; (5) knife; (6) gun; (7) Razor And for the crime of theft: (1) Physical means, threats; (2) Physical means, I blamed myself for taking things, for helping a friend; (3) There was no such, but according to the alleged victim, we took money from my friend and me; (4) I allegedly stole a trailer from a driver, but did not even drive; (5) Supposedly in complicity I stole things from where I worked, but denies having done it; (6) Supposedly in complicity I stole things from where I worked, but denies having done so; (7) By physical means to the employer, denies having done it, was the secretary; (8) Physical means, it was clothing, and I presented a false check; (9) Physical means, I entered the house that was open, denies having done it; (10) Physical means, supposedly the client they killed, with whom I prostituted myself; (11) Physical means, I took tools; (12) Physical means, I took things from pharmacy (Source: Interviews with internal inmates in the Preventive and Female Readaptation Center, Big Bridge, Jalisco, Mexico).

5. Conclusions

In the right to personal integrity of the murdered woman, society's reactions to it are the high percentage of rejection, violence, abandonment and labeling, denying them the right to equality and freedom, which leads to the deterioration of their dignity.

Since this behavior comes from a violent, intolerant society, with a sexist culture and gender discrimination, and which consequently continuously violates the fundamental human rights of women, the problem must be seen from the social point of view with programs of continuous improvement to eradicate or diminish the violations to the human rights of women from girls, including those who are deprived of their liberty, safeguarding the physical and psychological integrity of these, prevailing the equality of conditions and rights, avoiding abuses, this with the purpose of preserving and defending the physical, mental and moral integrity of the human person.

Similarly, in order to achieve social reinsertion and re-adaptation of prisoners, it is necessary to enforce their rights and generate verifiable mechanisms and strategies for their compliance, as well as follow up on the process of rehabilitation and reintegration of the person who served her sentence outside the prison.

References

1] Brekke, N., and E. Borgida, Expert Psychological Testimony in Rape Trials: a Social-Cognitive Analysis, Journal of Personality and Social Psychology, vol.55, núm. 3, (1988), p. 372-386.

2] Burt, M.R., Cultural Myths and Supports for Rape, Journal of Personality and Social Psychology, vol. 38, núm. 2, (1980), p. 217-230.

3] Corsi, J., M.L., Dohmen and M.A. Sotés, Male violence in couples. An approach to the diagnosis and models of intervention, Editorial Paidós, Mexico, (1995).

4] Entelman, R. et. al., The Legal Discourse, "Psychoanalytical Perspective and Other Epistemological Approaches", Introduction, Buenos Aires, Hachette, (1982), pp. 16-19.

5] Ey, Henry and others, Treaty of psychiatry, Editorial Torray Masson, Barcelona (1961).

6] Ferro Veiga, José Manuel, Criminal Profiles Interfects, Editorial Formación Alcalá, Alcalá La Real (2012) 121-129.

7] González Vidaurri, Alicia, and Sánchez Sandoval, Augusto, Criminology, Editorial Porrúa, Mexico (2005).
8] Gutiérrez, H., Every seven minutes there is a rape in the city of Mexico, newspaper La Crónica (21 August 1998), Mexico, p. 4-B.
9] Hesnard, A., Psychology of crime, Editorial Reus, Barcelona, (1963).
10] Hikal Carreón, Wael Sarwat, Glossary of Criminology, Criminalistics and Criminal Victimology, Flores Publisher and Distributor, Mexico (2015)
11] Lima Malvido, María de la Luz, Women's Crime, Second Edition, Editorial Porrúa, S.A., Mexico, (1991).
12] McCahill, T.W., L.C. Meyer and A.M. Fischman, The Aftermath of Rape, Lexington Books, EUA, (1979), 252 págs.
13] Marchiori, Hilda, Criminal psychology, Editorial Porrúa, S.A. de C.V., Mexico, (2000); Personality of the Delinquent, Editorial Porrúa, Mexico, D.F. (2000); The Study of the Offender, Study and Analysis of Crime, Mexico, D.F. (2001)
14] Medianero Peña, Juan José, Juvenile Violence, Published in General on March 2, 2006 by, HURLOK, psychology of adolescence, 2nd edition of Paidós Publishing House, Spain 1980. Microsoft Encarta 2000 Encyclopedia, Juvenile Delinquency. Found in: http://www.monografias.com/trabajos/laviolencia/laviolencia.shtml http://members.tripod.com/~Daniel_E_Cantoni/home2.htm
15] Myers, M.B.I.D., Templer and R. Brown, Coping Ability of Women who Become Victims of Rape, Journal of Consulting and Clinical Psychology, col. 52, núm. 1, (1984), pp. 73-78.
16] Rasko, G., Victim of the Female Killer, Victimology, Vol. I, núm. 3, Fall, USA (1976).
17] Roberts Chapman, Jane, The Economics of Women´s, Victimization, Published in "The Victimization of Women´s", Sage Yearbooks in Women´s Policy Studies, Vol. 3, USA (1978). pp. 207.
18] Sherer, I.M., Being a woman in the city of Mexico: visuals, devious compliments, as high, rape, assaults weekly information and analysis Process, (March 15, 1998), num 1115, pp. 35-38.
19] Silva Rodríguez, Arturo, Antisocial Conduct: a psychological approach, Editorial Pax Mexico, Mexico, 2008.
20] Trujano, R.P., Rape and attribution of guilt", doctoral thesis, Autonomous University of de Barcelona, Spain, (1992).
21] Van Egmon, D.M., Garnestski, Jonker, D.J.L., Attempted suicide of sexually abused women, published in "Final Program abstracts", Traumatic Stress, reactions, Prevention, coping and treatment. September. Netherlands (1990).
22] Walker, L., The Impact of Forensic Issues on Women´s Rights, present in Human Sexual Aggression: Current Perspectives, conference sponsored by New York Academy of sciences, Nueva York, January 7-9 (1987), pages 22.
23] Wilson, N.K, and Rigsby, C.M., Is Crime a Man´s World?, Issues in the Exploration of Criminality, Journal of Criminal Justice, Vol. 3, no. 2, Summer, USA (1975); pp. 131.
24] Zozaya, M., Domestic violence: problem of public health and human rights, The Jornada newspaper, supplement letter S: health, sexuality and AIDS, (1st. July 1999).

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Molecular analysis of oncogene expressions in different grades of gliomas

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ABSTRACT

The aggressiveness of brain tumors is attributed to the expression of multiple oncogenes involved in proliferation, metabolism and therapeutic resistance whose potential correlation with tumor progression has not been well-studied. In this study, we aimed to investigate the relationship of oncotargets involved in pathogenesis with respect to glioma grades. Gliomas (n=40) were analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and sequencing for the detection of epidermal growth factor receptor (*EGFR*) mutants. Expressions levels of *EGFR*, *EGFR variant III* (*EGFRvIII*), Lck/Yes novel tyrosine kinase (*Lyn*), Spleen tyrosine kinase (*SYK*), insulin receptor substrate 1 (*IRS1*), phosphatidylinositol 3-kinase (*PI3K*), Src homology 2 domain-containing inositol 5-phosphatase 1 (*SHIP1*) and glucose transporter 3 (*GLUT3*) were studied using real-time PCR and compared against glioma grades via statistical methods. Protein expressions were analyzed using immunohistochemistry and western blotting. *EGFRvIII* was detected in 53% and exon 4 deletion (*de4 EGFR*) in 20% of gliomas. Importantly, the expressions levels of candidate oncogenes were significantly upregulated (P<0.05) and positively correlated with the glioma grades. Hence, these oncotargets require high surveillance during tumor progression and further investigations on larger patient cohorts can affirm their role as potential markers in the pathology of glioma, thereby aiding in the development of patient-specific multi-targeted therapy.

Keywords: Glioblastoma; EGFRvIII; exon 4 deletion variant; oncogenes, metabolism

1. Introduction

Glioblastomas are the most common, highly invasive and neurologically destructive tumors with the worst prognosis [1,2]. The standard of care consists of maximum safe surgical resection and radio-/chemotherapy, but these tumors are highly resistant to therapy owing to their diffuse infiltrative nature [3]. In order to develop efficacious treatments, the molecular pathology of glioma involving genetic abnormalities and aberrant signaling mechanisms activated by oncogenes involved in metabolism, proliferation and therapeutic resistance have to be investigated [4,5].

Epidermal growth factor receptor variant III (*EGFRvIII*), a genomic variant of *EGFR*, which is absent in normal tissues, is a marker signature often characterized in glioblastoma [6]. *EGFRvIII* exhibits constitutive signaling property as a result of an in-frame deletion of 801 base pairs (bp) or exons 2-7 loss from the N-terminal extracellular domain of *EGFR* [7]. But the role of *EGFRvIII* in gliomagenesis and the precise molecular mechanism by which it acquires constitutive activity remains complex and unresolved [8]. Previous reports have shown that *EGFRvIII* expression is rapidly lost in primary cell cultures; furthermore, glioblastoma cell lines provide a limited understanding of *EGFRvIII*-driven signaling networks operating in tumors [9]. Nevertheless, some independent studies have shown direct or indirect involvement of *EGFRvIII* with specific downstream signaling molecules involved in metabolism, proliferation and resistance [10-19].

Based on the following collective evidences, molecular targets such as *EGFR, EGFRvIII* and their associated signaling partners, *Lyn, SYK, IRS1, PI3K, SHIP1* and *GLUT3* involved

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in metabolism, proliferation and therapeutic resistance associated with glioma progression were chosen for our analysis. EGFRvIII has been found to be associated with non-receptor tyrosine kinases like Lck/Yes novel tyrosine kinase (Lyn) involved in enhancing proliferation and migration in head and neck cancer [10]. Lyn is indeed an essential factor for cancer cells that rely on EGFR signaling [11]. It was also shown to activate insulin-independent glucose transport in adipocytes [12]. It phosphorylates the downstream effector, spleen tyrosine kinase (Syk) leading to the activation of receptor-associated adaptor proteins like insulin receptor substrate 1 (IRS1) and lipid kinases like phosphatidylinositol 3-kinases (PI3Ks) which are involved in metabolism and proliferation [13-15]. Activated PI3Ks have been shown to be involved in the translocation of glucose transporters from the cytosol to the plasma membrane [16]. The src homology 2 domain-containing inositol 5-phosphatase 1 (SHIP1) was shown to negatively regulate phosphoinositide 3-kinase effectors, thereby acting as a tumor suppressor [17]. EGFRvIII was also demonstrated to promote constitutive PI3K signaling enhancing glioblastoma cell proliferation [18]. Interestingly, the role of EGFRvIII has also been implicated in metabolic fueling in glioma wherein EGFRvIII mediated upregulation of heterogeneous nuclear ribonucleoprotein (*hnRNP*) A1 splicing factor and delta Max led to the increased expression of glycolytic genes like glucose transporter 3 (GLUT3) [19].

Hence, as an initial step, the glioma cases selected for the analysis were screened for *EGFR* mutants. The incidence of *EGFRvIII* in glial tumors has been reported in previous studies [20,21], but the detection of a novel *EGFR* variant, exon 4 deletion (*de4 EGFR*) mutation possessing enhanced proliferation and invasiveness has been reported only once in glioblastoma [22]. Therefore, the present study was undertaken to determine the prevalence of *EGFRvIII* and *de4 EGFR* mutations in the patient cohort and to analyze the expression levels of *EGFR, EGFRvIII, LYN, SYK, IRS1, PI3K, SHIP1* and *GLUT3* with respect to glioma grades. In this pilot study, correlation analysis was performed to determine the significance of *EGFRvIII* expression with that of these selected molecular targets and evaluated the expression levels against the clinical grades of glioma.

2. Material and Methods

2.1 Tumor Samples

Tumor tissues were collected from glioma patients who underwent surgery at the Amrita Institute of Medical Sciences, India from 2009 to 2013. The study protocol and consent were approved by the Institutional Ethics Committee and were performed in accordance with the ethical standards laid down in the Declaration of Helsinki. All tumors were histologically confirmed by pathologists and graded according to the World Health Organization (WHO) classification of tumors of the central nervous system [23].

2.2 Ribonucleic acid (RNA) Extraction and Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from tumor samples (n=40) were extracted from snap-frozen tissues (< 25mg) according to the trizol method of RNA extraction (Sigma-Aldrich, MO, USA). The concentration and purity (260/280 and 260/230 ratios) were checked using Nanodrop spectrophotometer (NanoDrop 2000c, Thermo Scientific, MA, USA) and quality of the preparation was analyzed on a 1% agarose gel. RNA obtained was reverse transcribed into complementary deoxyribonucleic acid (cDNA) using PrimeScript 1st strand cDNA synthesis kit (Takara, CA, USA) as per manufacturer's protocol. The reaction mixture with a total volume of 20 µl contained template RNA (total RNA < 5 µg), 5x primescript buffer, deoxynucleotide (dNTP) mixture (10 mM), Oligo dT primer (50 µM), primescript RTase (200 U/ µl), RNase inhibitor (40 U/ µl) and RNase-free water. The cDNA synthesis reactions were carried out at 50°C for 50 minutes followed by 70°C for 15 minutes in a G-storm GS4 thermal cycler (Life Science Research, Somerset, UK).

2.3 Conventional PCR and Sequencing analysis

The amplifications of cDNAs were performed in 25 µl reaction volumes containing 1 µl product from the RT reaction according to the manufacturer's protocol (PrimeStar Max DNA Polymerase, Takara, CA, USA). *EGFR* and *EGFRvIII* were amplified using forward and reverse primers (Supplementary Data 1) spanning the beginning of exon 1 and within exon 8 respectively. These RT-PCR primers generate a 180 bp truncated product for *EGFRvIII* whereas 846 bp and 981 bp amplification products for *de4 EGFR* and full-length *EGFR* respectively. PCR amplification products from U87 cell line (American Type Culture Collection - ATCC, VA, USA) and plasmid DNA (Plasmid# 20737- MSCV-XZ066EGFRvIII, Addgene, MA, USA) served as positive controls for *EGFR* and *EGFRvIII* respectively. PCR cycling conditions were: initial denaturation at 98°C for 10 seconds, 35 cycles of 98°C denaturation (10 seconds), 64° C annealing (15 seconds) and 72°C extension (10 seconds) and a final extension at 72°C (5 minutes). PCR reaction products (10 µl) were electrophoresed in 1% agarose gels and stained with ethidium bromide. The *EGFRvIII* amplification product was extracted from the agarose gel (QIAquick gel extraction kit, Qiagen, MD, USA) and verified by Sanger sequencing using the RT-PCR primers [24].

2.4 Real-time PCR

Real-time PCR analysis of *EGFRvIII* positive (n=21) and negative (n=4) glioma cases were done on Real-time PCR instrument (7300 Real-time PCR system, Applied Biosystems, CA, USA) using SYBR method. Normal appearing brain tissues (non-malignant and non-traumatic

brain tissues) were used as control samples for gene expression analysis. The cDNA products obtained from the RT-PCR reaction (2 µl) were used as template in 20 µl PCR reaction containing 10 µl of SYBR green mixture (Power SYBR green PCR master mix, Applied Biosystems, CA, USA), 0.4µM of each primer (Supplementary Data 1) and 6.4 µl of distilled water. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification was used as internal control. All reactions were done in duplicate. Amplification conditions were: 95°C for 10 minutes, 40 cycles of 95°C/15 seconds, 60°C/1 minute and a dissociation stage of 95°C/15 seconds, 60°C/15 seconds and 95°C/15 seconds. The threshold cycle number (C_T) was automatically determined by SDS 2.0 software (Applied Biosystems, CA, USA).

2.5. Data Analysis of Real-time PCR

The comparative C_T method was used to compute the fold change in gene expression. The mean C_T number of duplicate run was used for data analysis and the average value for C_T of GAPDH was used as the reference gene C_T number. The relative expression of each gene compared with the reference gene was calculated as ΔC_T, by subtracting the C_T number of reference gene from that of each target gene. This was further normalized to calculate $\Delta\Delta C_T$, i.e. difference in the ΔC_T values between experimental and control samples. The fold change in expression of each target gene was then determined using the formula: $2^{-\Delta\Delta CT}$.

2.6. Immunohistochemistry (IHC) analysis

Immunohistochemical staining was performed on formalin-fixed paraffin embedded (FFPE) sections of the tumor cases. Briefly, as per manufacturer's protocol (Super sensitive polymer-HRP detection system, Biogenex, CA, USA), the four-µm-thick sections were deparaffinized and rehydrated using varying concentrations of ethanol (100%-70%) and washed in phosphate buffered saline (1X PBS). For heat-induced antigen retrieval, the sections were heated in a microwave oven (700W for 5 minutes) in Tris-Ethylenediaminetetraacetic acid (EDTA) buffer (1X, pH 9). The blocking steps were performed using blocking solutions - power block (15 minutes incubation) and hydrogen peroxide block (15 minutes incubation). The sections were then incubated with a primary antibody targeting EGFR and EGFRvIII [25,26], (monoclonal antibody 528 / mAb 528; immunoglobulin G/ IgG purified from Hybridoma-HB8509, ATCC, VA, USA) at 1:100 dilution for 2 hours at 4°C. For the analysis of GLUT3 expression, the sections were incubated with the anti-GLUT3 antibody (Abcam, MA, USA) at 1:50 dilution for 2 hours at 4°C. On the other hand, the sections incubated with mouse negative control - HK119, non-immune serum or immunoglobulins, (Super sensitive polymer-HRP detection system, Biogenex, CA, USA) served as negative controls. After incubation, the sections were washed in PBS followed by incubation with super-enhancer reagent for 30 minutes and then treatment with a secondary antibody (Polymer-Horse radish peroxide reagent) for 30 minutes. The sections were stained using 3, 3'-diaminobenzidine tetrahydrochloride (DAB) for 1-5 minutes and counter stained using hematoxylin. The sections were mounted and then visualized using a bright-field microscope (Leica DMI3000 B, IL, USA).

2.7. Western blotting

Tissue samples (< 25 mg) were lysed in radioimmunoprecipitation assay buffer (RIPA) buffer (50mM Tris HCl pH 8, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS) containing 1X protease and phosphatase inhibitors (Cell signaling technologies, MA, USA). The lysates were normalized for protein concentrations and resolved using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). β-actin was used as the loading control. The proteins were then transferred from the gel onto the polyvinylidene difluoride (PVDF) membrane (Immobilon - P, Merck Millipore, MA, USA) and blocked for one hour using blocking buffer [5% bovine serum albumin (Sigma-Aldrich, MO, USA) in TBST (tris-buffered saline and 0.2% Tween-20)]. The blots were incubated with primary antibodies at 1:5000 dilution (anti-EGFR and anti-Lyn antibodies, Cell signaling technologies, MA, USA; anti-GLUT3 and anti-β-actin antibodies, Abcam, MA, USA) for three hours at 4oC and then washed using TBST. Followed by probing with horseradish peroxidase-linked secondary IgG for 45 minutes at 1:5000 dilution and washed using TBST. The bound antibodies were detected by enhanced chemiluminescence reaction (Clarity™ Western ECL Blotting Substrate, Bio-Rad, CA, USA) and the resulting chemiluminescent signal was detected on the Bio-Rad ChemiDoc XRS+ System using Image Lab™ Software, version 4.0 (Bio-Rad, CA, USA).

2.8. Statistics

The Statistical Package for the Social Sciences (SPSS version 20, IBM Inc., IL, USA) was used for statistical analysis of the real-time PCR data. The data were summarized using mean, standard deviation, standard error for each group. In order to test the differences in mean, Kruskal-Wallis test was applied. When the differences were significant ($P<0.05$), a Dunn-Bonferroni test was performed for multiple comparison. Spearman's rank correlation (Spearman's Rho) test was used for calculating the correlation of the genes with the different clinical grades of glioma. In addition, Pearson's correlation coefficient (r) was calculated to measure the correlation between the candidate genes. The correlation between oncogenes was also verified by the linear regression (r^2) plot.

3. Results

3.1. Incidence of EGFRvIII and de4 EGFR mutations in glioma patients

The clinical details and summary of the RT-PCR analysis of glioma patients are listed in Table 1. The histological features of the tumors sections of the patients are depicted in the representative Figure 1. A total of 40 glioma cases were used in this study for the detection of *EGFRvIII*, *de4 EGFR* and *EGFR* transcripts. Figure 2A and B are representative agarose gel images depicting the RT-PCR detection of amplicons for *EGFRvIII* (180 bp), *de4 EGFR* (846 bp) and *EGFR* (981 bp) from glioma patients. Amplification products from MSCV-XZ066-EGFRvIII plasmid and U87 cell line served as positive controls for *EGFRvIII* and *EGFR* respectively. Figure 2C depicts the sequence chromatogram of the 180 bp *EGFRvIII* amplicon from a glioblastoma patient. As summarized in Table 1, 21/40 cases (53%) showed the presence of *EGFRvIII* mutation and 8/40 cases (20%) were positive for *de4 EGFR* mutation. Furthermore, the incidence of *EGFRvIII* and *de4 EGFR* mutations was observed to be higher in malignant tumors or high-grade gliomas when compared to low-grade tumors.

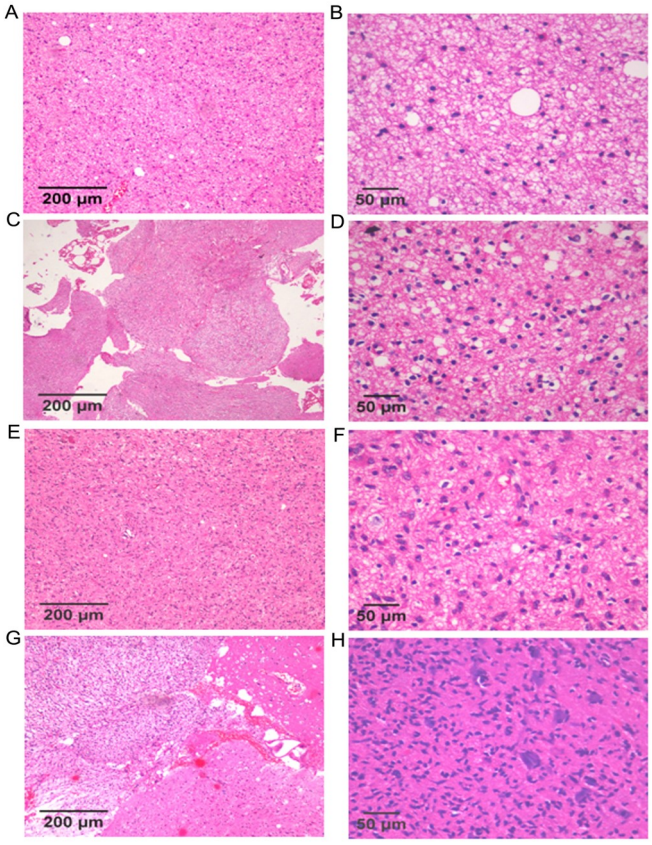

Figure 1. Representative pathological hematoxylin and eosin analysis of tumor sections - A, B) Diffuse fibrillary astrocytoma (IDH-mutant) C, D) Oligodendroglioma (NOS) E, F) Diffuse astrocytoma (IDH-mutant) G, H) Anaplastic astrocytoma (IDH-mutant).

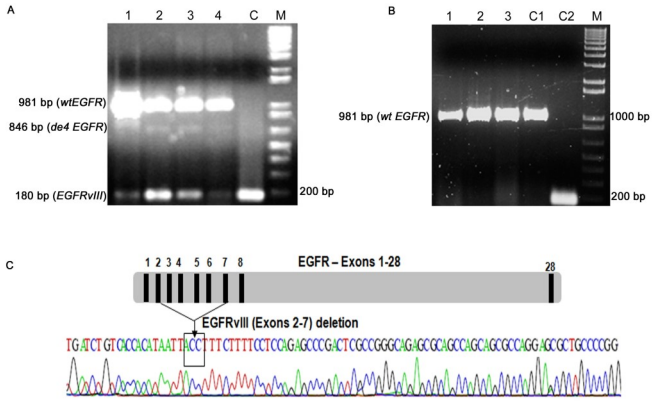

Figure 2. *EGFRvIII* and *de4 EGFR* mutation analysis in glioma patients - (A) RT-PCR products visualized by ethidium bromide staining of a 1% agarose gel: Lanes 1 - 4 - glioblastoma patients with *EGFRvIII* (180 bp), *de4 EGFR* (846 bp) and *EGFR* (981bp), lane C - positive control for *EGFRvIII*, lane M – 1 kb marker. (B) Lanes 1-3 - RT-PCR products from glioma patients with only *EGFR* (981 bp), lanes C1 and C2 - positive controls for *EGFR* and *EGFRvIII* respectively. (C) Sequence chromatogram of a glioblastoma patient depicting *EGFRvIII* deletion.

3.2. Oncogene expressions are significantly upregulated and positively correlated with glioma grades

Using the real-time PCR assay, the fold change in gene expressions of *EGFR, EGFRvIII, Lyn, SYK, IRS1, PI3K, SHIP1* and *GLUT3* in glioma patients were computed by the comparative C_T method of relative quantification and normalized to control. The gene expressions of all candidate oncogenes in the *EGFRvIII* RT-PCR positive glioma cases (n=21) were compared with that of the *EGFRvIII* negative cases (n=4).

Low-grade glioma patients without *EGFRvIII* expression (RT-PCR negative cases), showed decreased expression levels of all selected oncogenes (Figure 3). On the other hand, *EGFRvIII* positive (RT-PCR positive) grade I-IV patients showed a significant increase in gene expression levels of all candidate oncogenes (P<0.05) whereas *SHIP1* levels were consistently downregulated (P< 0.05) when compared to control (Figure 3). The molecular level gene expression profiles of these candidate oncogenes were compared with the clinical grades of glioma using Spearman's correlation test. The gene expressions of *EGFR* (Spearman's coefficient, r = 0.617; P<0.01), *EGFRvIII* (r = 0.838; P<0.01), *LYN* (r = 0.573; P<0.01) and *GLUT3* (r =0.771; P<0.01) showed significant positive correlation with the clinical grades of glioma (Figure 3). The strong positive correlation between expressions of *EGFRvIII* and *GLUT3* in all patients is indicated by the Pearson's coefficient, r = 0.865 (P<0.01) and linear regression value, r^2= 0.75 (Figure 4A and B). The oncogenes analyzed for the expression levels in this study are represented in the schematic Figure 4C.

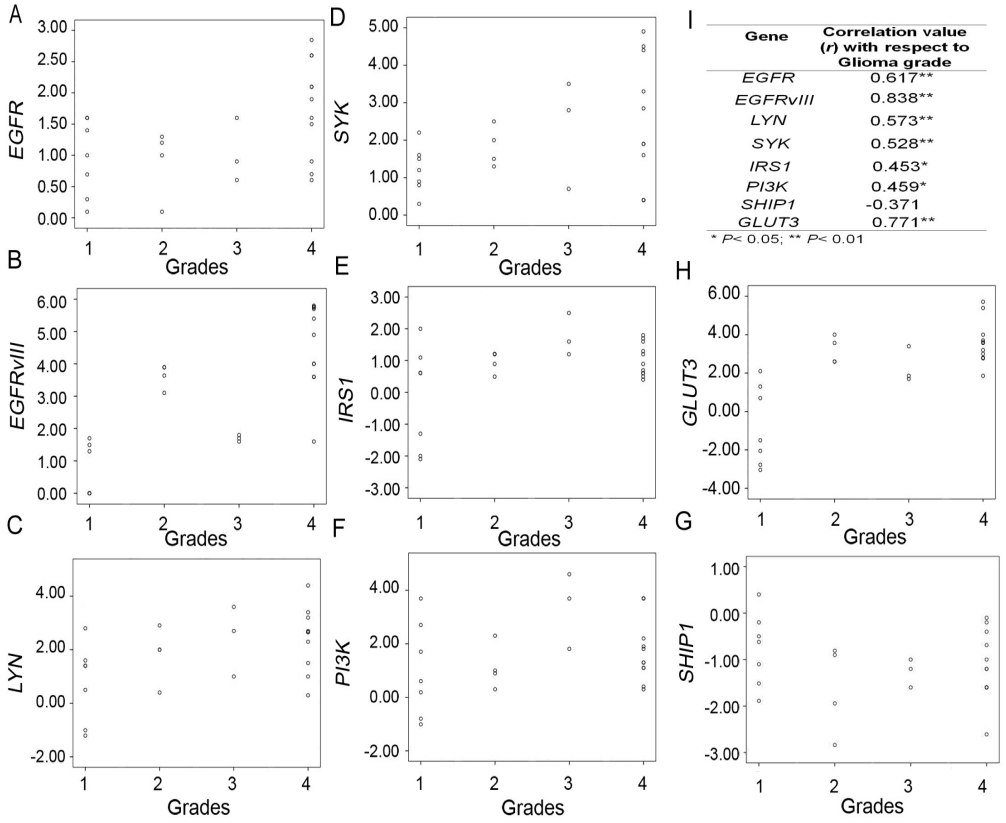

Figure 3. Correlation analysis of gene expressions with respect to glioma grades using Spearman's rank test - A, H) Scatter plots of gene expressions of *EGFR*, *EGFRvIII*, *LYN*, *SYK*, *IRS1*, *PI3K*, *SHIP1* and *GLUT3* with respect to different grades of glioma patients I) Correlation values (*r* values) of all genes with respect to glioma grades analyzed using Spearman's test.

3.3. Expressions of EGFR, EGFRvIII, Lyn and GLUT3 are elevated at the protein level in patient tumors

Immunohistochemical staining was performed to evaluate the presence of EGFR, EGFRvIII and GLUT3 protein expressions in the tumor sections. The regions of receptor expression (EGFR and EGFRvIII) were indicated by the brown staining by mAb 528, on the other hand, there was no staining with the negative control antibody (Figure 5A and B). Similarly, the expressions of GLUT3 protein in the glioblastoma FFPE sections were indicated by the intense brown staining or reactivity with anti-GLUT3 antibody when compared to the negative control antibody treated sections with no reactivity (Figure 5C and D). The protein expressions of EGFR (170 kilodalton or kDa), EGFRvIII (145 kDa), Lyn (56/ 53 kDa isoforms) and GLUT3 (49 kDa) were verified on a western blot (Figure 5E). The levels of protein expression were elevated in the higher grades of the tumors when compared to control. This further confirms the increased expressions of cell surface receptors (EGFR and EGFRvIII), Lyn and GLUT3 at the protein level with respect to the degree or malignant grade of the tumor.

Table 1. Clinical details and summary of RT-PCR analysis of glioma patients for detection of *EGFR*, *EGFRvIII* and *de4 EGFR*.

Glioma grades	Total cases (*n*)	Age Range (years)	Median Age (years)	Treatment			RT-PCR Analysis		
				Tumor resection (*n*)	Radio-Therapy (*n*)	Chemotherapy (Temozolo-mide) treatment (*n*)	Patients with *EGFR* (*n*)	Patients with *EGFR-vIII* (*n*)	Patients with *de4 EGFR* (*n*)
Grade I	15	15-55	32	15	13	None Received	15	3	1
Grade II	11	20-51	33	11	11	None received	11	4	1
Grade III	3	30-52	37	3	3	3	3	3	2
Grade IV	11	26-62	43	11	11	11	11	11	4
Total	40						40	21	8

RT-PCR, Reverse transcriptase polymerase chain reaction; *EGFR*, Epidermal growth factor receptor; *EGFRvIII*, epidermal growth factor receptor variant III; *de4 EGFR*, epidermal growth factor receptor exon 4 deletion variant.

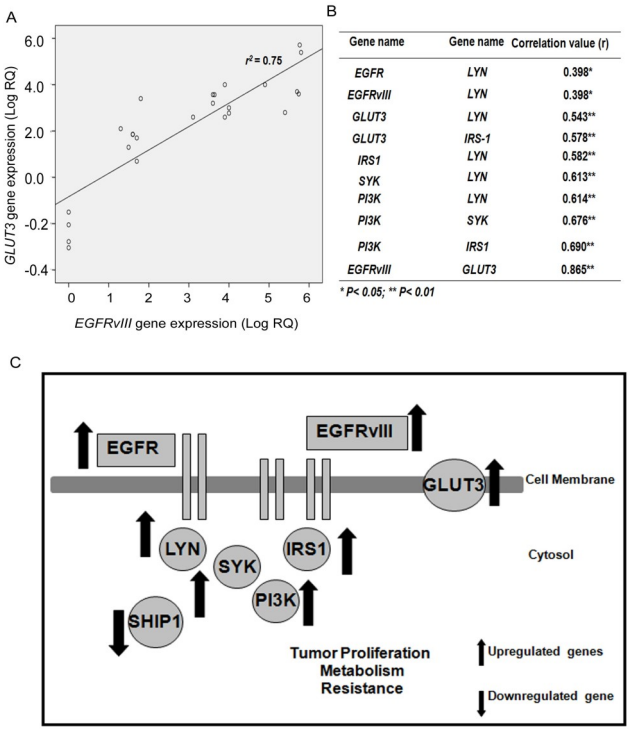

Figure 4. Correlation analysis of EGFRvIII and GLUT3 using Pearson's statistical method - A) Scatter plot depicting correlation between EGFRvIII and GLUT3 gene expressions in all glioma patients using Pearson's test (r^2 - linear regression value) B) Correlation values (r values) of all genes analyzed in this study using Pearson's test C) Schematic representation of the targets analyzed in this study for gene expression levels in glioma patients involved in tumor progression/ pathogenesis [10-19].

4. Discussion

The molecular heterogeneity and complexity of gliomas are governed by multiple oncogenic factors [1,4]. This study was undertaken as a pilot to evaluate the potential correlation of *EGFRvIII* expression with that of the downstream oncogenic targets associated with the progression of glioma. As a first step, we have analyzed the frequency of *EGFRvIII* mutation in glioma subjects (n=40) and detected 21/40 cases (53%) with *EGFRvIII* mutation (Figure 2). The frequency of *EGFRvIII* detection was higher in malignant gliomas when compared to low-grade patients (Table 1). This report is supported by previous evidences on the detection of *EGFRvIII* in high-grade [20,27,28] as well as low-grade gliomas [20]. Interestingly, along with *EGFRvIII*, we have observed a rare variant of *EGFR*, *de4 EGFR* (8/40 cases - 20%) whose frequency also increased with tumor grade (Figure 2, Table 1). But long-term follow-ups on larger patient cohorts are required to decipher the relation of *EGFRvIII* and *de4 EGFR* detection with patient outcomes. Till now, only one group has reported the detection of *de4 EGFR* in glioblastoma and ovarian cancer [22,29]. *De4 EGFR* showed similar properties as that of *EGFRvIII* in promoting metastasis, ligand-independent autophosphorylation and self-dimerization properties by interactions with downstream molecules such as mitogen-activated protein kinase (MAPK)/ extracellular signal–regulated kinase (ERK), protein kinase B (AKT), Jun, Src, E-cadherin, focal adhesion kinase (FAK) and matrix metalloproteinase-9 (MMP9) in glioma and ovarian cancer [22,29]. But the detailed molecular mechanisms of *de4 EGFR* and *EGFRvIII*-mediated capacity for tumor proliferation and invasion require further investigations [22].

In order to better understand the correlation of oncogene expressions with respect to the clinical grades of glioma, we analyzed the gene expression levels of molecules which may play potential roles in metabolism, proliferation and therapeutic resistance [10-19]. We have observed that the increased expression profiles of all candidate oncogenic molecules (*EGFR, EGFRvIII, Lyn, SYK, IRS1, PI3K, and GLUT3*) depicted marked similarities to the clinical grading of glioma and this has not been previously reported (Figures 3 and 4). Importantly, the gene expressions of *EGFRvIII* displayed high levels of positive correlation with that of *GLUT3* in all glioma patients (Figure 4). Further, the protein level expressions of EGFR, EGFRvIII, Lyn and GLUT3 were also elevated in high-grade gliomas as confirmed by immunohistochemical evaluation and western blot analysis (Figure 5). This observation supports the previous findings on the role of EGFRvIII in malignant transformation and enhancing tumorigenicity of glioblastoma [30,31], and that of GLUT3 (but not GLUT1) in neoplastic transformation [32]. Our observations also support the previous evidences of the associations of EGFRvIII with the downstream oncogenic molecules involved in metabolic and proliferative pathways. For instance, the role of EGFRvIII in glucose metabolism has been implicated wherein limiting levels of glucose enhanced Src-induced mitochondrial localization of EGFRvIII promoting survival and proliferation of glioma cells [33]. Earlier reports have shown that EGFR signaling regulates functional GLUT3 and non-receptor Src-family

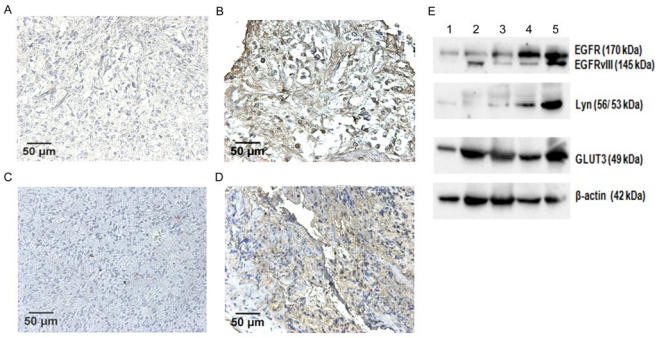

Figure 5. Protein expressions of EGFR, EGFRvIII, Lyn and GLUT3 in glioma patients – Immunohistochemical staining of FFPE tumor sections A) with control antibody B) with monoclonal antibody 528 for EGFR and EGFRvIII expression C) with control antibody D) with anti-GLUT3 antibody E) Western blotting analysis of EGFR, EGFRvIII, Lyn and GLUT3 expressions with respect to β–actin in normal tissues (lane 1) and glioma cases (lanes 2-5: Glioma grades I to IV respectively).

kinase, Lyn [34,35]. Moreover, previous findings have demonstrated that EGFRvIII expressing head and neck cancer displayed enhanced Lyn-mediated proliferation and invasiveness [10]. Lyn was further shown to activate Syk, IRS1 and PI3K through phosphorylation of these downstream effectors [12,13,36]. In contrast to the metabolic and proliferative effects of these oncogenic molecules, the tumor suppressor, SHIP1 regulates or downmodulates the phosphoinositide 3-kinase effectors [18]. Hence, the increased expression profiles of oncogenes observed in the present study in glioblastoma patients is in line with these previous findings with respect to their involvement in metabolism and tumor progression.

Earlier reports have demonstrated that EGFRvIII possessed advantage over EGFR in radioresistant tumors by conferring stronger cytoprotective response to radiation [37]. The importance of EGFR and EGFRvIII inhibition as a therapeutic strategy for radiosensitizing carcinomas has been described [38,39]. Likewise, it was demonstrated that temozolomide resistant astroglioma cells exhibited increased GLUT3 expression and inhibition of selected components of glycolytic pathway (like GLUTs) may represent a promising therapy in order to overcome drug resistance in glioblastoma [40]. Hence, from the data presented here the molecular level expression analysis of the candidate oncogenes could be correlated with the glioma grades and these oncogenes require high surveillance during tumor progression. Therefore, with further studies on larger patient data sets, the analysis of correlations between expression profiles of oncogenes and glioma aggressiveness can serve as a rationale for stratification of patients for EGFRvIII and GLUT3 targeted therapies for sensitizing radio-/ chemoresistant gliomas.

5. Concluding Remarks

In conclusion, our study demonstrated the increased incidence of *EGFRvIII* and *de4 EGFR* mutations in malignant gliomas. The molecular analysis displayed a significant positive correlation of *EGFRvIII, GLUT3* and candidate oncogenic messages with glioma grades which has not been reported before in the clinical setting. This may have potential prognostic significance when expanded to larger patient cohorts. In addition, further investigations on the evaluation of downstream oncogenic targets involved in the pathology of glioma are warranted for the identification of potential molecular markers which would aid in the development of tailored multi-targeted therapies for patients.

6. Supplementary material

Supplementary Data S1 – Primers used in the study

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References

1] F.B. Furnari, T. Fenton, R.M. Bachoo, A. Mukasa, J.M. Stommel, A. Stegh, W.C. Hahn, K.L. Ligon, D.N. Louis, C. Brennan, L. Chin, R.A. DePinho, W.K. Cavenee, Genes Dev. 21 (2007) 2683-2710. DOI: 10.1101/gad.1596707

2] H.K Gan, A.H. Kaye, R.B. Luwor RB, J. Clin. Neurosci. 16 (2009) 748-754. DOI: 10.1016/j.jocn.2008.12.005

3] S.K. Carlsson, S.P. Brothers, C. Wahlestedt, EMBO Mol. Med. 6 (2014) 1359-1370. DOI: 10.15252/emmm.201302627

4] D.W. Parsons, S. Jones, X. Zhang, J.C. Lin, R.J. Leary, P. Angenendt, P. Mankoo, H. Carter, I.M. Siu, G.L. Gallia, A. Olivi, R. McLendon, B.A. Rasheed, S. Keir, T. Nikolskaya, Y. Nikolsky, D.A. Busam, H. Tekleab, L.A. Diaz Jr, J. Hartigan, D.R. Smith, R.L. Strausberg, S.K. Marie, S.M. Shinjo, H. Yan, G.J. Riggins, D.D. Bigner, R. Karchin, N. Papadopoulos, G. Parmigiani, B. Vogelstein, V.E. Velculescu, K.W. Kinzler. Science 321 (2008) 1807-1812. DOI: 10.1126/science.1164382

5] P.S. Ward, C.B. Thompson, Cancer Cell 21 (2012) 297-308. DOI: 10.1016/j.ccr.2012.02.014

6] N. Sugawa, A.J. Ekstrand, C.D. James, V.P. Collins, Proc. Natl. Acad. Sci. USA 87 (1990) 8602–8606. DOI: 10.1073/pnas.87.21.8602

7] A.J. Ekstrand, N. Sugawa, C.D. James, V.P. Collins, Proc. Natl. Acad. Sci. USA 89 (1992) 4309-4313. DOI: 10.1073/pnas.89.10.4309

8] C.T. Kuan, C.J. Wikstrand, D.D. Bigner, Endocr. Relat. Cancer 8 (2001) 83-96. DOI: 10.1677/erc.0.0080083

9] H. Johnson, A.M. Del Rosario, B.D. Bryson, M.A. Schroeder, J.N. Sarkaria, F.M. White, Mol. Cell Proteomics 11 (2012) 1724-1740. DOI: 10.1074/mcp.M112.019984

10] S.E. Wheeler, E.M. Morariu, J.S. Bednash, C.G. Otte, R.R. Seethala, S.I. Chiosea, J.R. Grandis, Clin. Cancer Res. 18 (2012) 2850-2860. DOI: 10.1158/1078-0432.CCR-11-2486

11] T.H. Huang, L. Huo, Y.N. Wang, W. Xia, Y. Wei, S.S. Chang, W.C. Chang, Y.F. Fang, C.T. Chen, J.Y. Lang, C. Tu, Y. Wang, M.C. Hsu, H.P. Kuo, H.W. Ko, J. Shen, H.H. Lee, P.C. Lee, Y. Wu, C.H. Chen, M.C. Hung. Cancer Cell 23 (2013) 796-810. DOI: 10.1016/j.ccr.2013.04.027

12] G. Muller, S. Wied, W. Frick, Mol. Cell Biol. 20 (2000) 4708-4723. DOI: 10.1128/MCB.20.13.4708-4723.2000

13] M.A. Antonyak, D.K. Moscatello, A.J. Wong, J. Biol. Chem. 273 (1998) 2817–2822. DOI: 10.1074/jbc.273.5.2817

14] P. Liu, H. Cheng, T.M. Roberts, J.J. Zhao, Nat. Rev. Drug Discov. 8 (2009) 627-644. DOI: 10.1038/nrd2926

15] T. Kamei, S.R. Jones, B.M. Chapman, K.L. MCGonigle, G. Dai, M.J. Soares, Mol. Endocrinol. 16 (2002) 1469-1481. DOI: 10.1210/mend.16.7.0878

16] H. Makinoshima, M. Takita, K. Saruwatari, S. Umemura, Y. Obata, G.Ishii, S. Matsumoto, E. Sugiyama, A. Ochiai, R. Abe, K. Goto, H. Esumi, K. Tsuchihara, J. Biol. Chem. 290 (2015) 17495-17504. DOI: 10.1074/jbc.M115.660498

17] R.W. Freeburn, K.L. Wright, S.J. Burgess, E. Astoul, D.A. Cantrell, S.G. Ward, J. Immunol. 69 (2002) 5441–5450. DOI: https://doi.org/10.4049/jimmunol.169.10.5441

18] M. Klingler-Hoffmann, P. Bukczynska, T. Tiganis, Int. J. Cancer 105 (2003) 331-339. DOI: 10.1002/ijc.11085

19] I. Babic, E.S. Anderson, K. Tanaka, D. Guo, K. Masui, B. Li, S.

Zhu, Y. Gu, G.R. Villa, D. Akhavan, D. Nathanson, B. Gini, S. Mareninov, R. Li, C.E. Camacho, S.K. Kurdistani, A. Eskin, S.F. Nelson, W.H. Yong, W.K. Cavenee, T.F. Cloughesy, H.R. Christofk, D.L. Black, P.S. Mischel, Cell Metab. 17 (2013) 1000-1008. DOI: 10.1016/j.cmet.2013.04.013

20] D.K. Moscatello, M. Holgado-Madruga, A.K. Godwin, G. Ramirez, G. Gunn, P.W. Zoltick, J.A. Biegel, R.L. Hayes, A.J. Wong, Cancer Res. 55 (1995) 5536-5539.

21] D.A. Bax, N. Gaspar, S.E. Little, L. Marshall, L. Perryman, M. Regairaz, M. Viana-Pereira, R. Vuononvirta, S.Y. Sharp, J.S. Reis-Filho, J.N. Stávale, S. Al-Sarraj, R.M. Reis, G. Vassal, A.D. Pearson, D. Hargrave, D.W. Ellison, P. Workman, C. Jones, Clin. Cancer Res. 15 (2009) 5753-5761. DOI: 10.1158/1078-0432.CCR-08-3210.

22] H. Wang, M. Zhou, B. Shi, Q. Zhang, H. Jiang, Y. Sun, J. Liu, K. Zhou, M. Yao, J. Gu, S. Yang, Y. Mao, Z. Li, Neoplasia 13 (2011) 461–471. DOI 10.1593/neo.101744

23] D.N. Louis, A. Perry, G. Reifenberger, A. Deimling, D.F. Branger, W.K. Cavenee, H. Ohgaki, O.D. Wiestler, P. Kleihues, D. W. Ellison, Acta Neuropathol. 131 (2016) 803-820. DOI: 10.1007/s00401-016-1545-1

24] F. Sanger, S. Nicklen, A.R. Coulson, Proc. Natl. Acad. Sci. USA 74 (1977) 5463-5467. DOI: 10.1073/pnas.74.12.5463.

25] T. Kawamoto, J.D. Sato, A. Le, J. Polikoff, G.H. Sato, J. Mendelsohn, Proc. Natl. Acad. Sci. USA 80 (1983) 1337-1341.

26] A.A. Jungbluth, E. Stockert, H.J. Huang, V.P. Collins, K. Coplan, K. Iversen, D. Kolb, T.J. Johns, A.M. Scott, W.J. Gullick, G. Ritter, L. Cohen, M.J. Scanlan, W.K. Cavenee, L.J. Old, Proc. Natl. Acad. Sci. USA 100 (2003) 639-644. DOI: 10.1073/pnas.232686499

27] A. B. Heimberger, R. Hlatky, D. Suki, D. Yang, J. Weinberg, M. Gilbert, R. Sawaya, K. Aldape, Clin. Cancer Res. 11(2005) 1462-1466. DOI: 10.1158/1078-0432.CCR-04-1737

28] A.J. Wong, J.M. Ruppert, S.H. Bigner, C.H. Grzeschik, P.A. Humphrey, D.S. Bigner, B. Vogelstein,. Proc. Natl. Acad. Sci. USA. 89(1992) 2965-2969.

[29] P. Zhang, P. Zhang, M. Zhou, H. Jiang, H. Zhang, B. Shi, X .Pan, H. Gao, H. Sun, Z. Li, Carcinogenesis 34 (2013) 2639-2646. DOI: 10.1093/carcin/bgt216

30] R. Nishikawa, X.D. Ji, R.C. Harmon, C.S. Lazar, G.N. Gill, W.K. Cavenee, H.J. Huang, Proc. Natl. Acad. Sci. USA 91 (1994) 7727-7731.

31] M. Nagane, F. Coufal, H. Lin, O. Bogler, W.K. Cavenee, H.J. Huang, Cancer Res. 56 (1996) 5079-5086.

32] R.J. Boado, K.L. Black, W.M. Pardridge. Brain Res. Mol. Brain Res. 27(1994) 51-57. DOI: 10.1016/0169-328X(94)90183-X

33] A.N. Cvrljevic, D. Akhavan, M. Wu, et al, P. Martinello, F.B. Furnari, A.J. Johnston, D. Guo, L. Pike, W.K. Cavenee, A.M. Scott, P.S. Mischel, N.J. Hoogenraad, T.G. Johns. J. Cell Sci. 124(2011) 2938-2950. DOI: 10.1242/jcs.083295

34] H. Makinoshima, M. Takita, S. Matsumoto, A.Yagishita, S. Owada, H. Esumi, K. Tsuchihara, J. Biol. Chem. 289(2014) 20813-20823. DOI: 10.1074/jbc.M114.575464

35] P. Sutton, J.A. Borgia, P. Bonomi, J.M. Plate. Mol. Cancer.12 (2013) 76. DOI: 10.1186/1476-4598-12-76

36] M.S. Iqbal, N. Tsuyama, M. Obata, H. Ishikawa, Biochem. Biophys. Res. Commun. 392 (2010) 415-420. DOI: 10.1016/j.bbrc.2010.01.038

37] G. Lammering, T.H. Hewit, K. Valerie, J.N. Contessa, G.P. Amorino, P. Dent, R.K. Schmidt-Ullrich, Oncogene 22 (2003) 5545–5553. DOI: 10.1038/sj.onc.1206788

38] M. Sreeranganathan, S. Uthaman, B. Sarmento, C.G. Mohan, I.K. Park, R. Jayakumar, Int J Nanomedicine 12 (2017) 7165-7182. DOI: 10.2147/IJN.S143529

39] B. Mukherjee, B. McEllin, C.V. Camacho, N. Tomimatsu, S. Sirasanagandala, S. Nannepaga, K.J. Hatanpaa, B. Mickey, C. Madden, E. Maher, D.A. Boothman, F. Furnari, W.K. Cavenee, R.M. Bachoo, S. Burma, Cancer Res. 69 (2009) 4252-4259. DOI: 10.1158/0008-5472.CAN-08-4853

40] B. Le Calve, M. Rynkowski, M. Le Mercier, C. Bruyere, C. Lonez, T. Gras, B. Haibe-Kains, G. Bontempi, C. Decaestecker, J.M. Ruysschaert, R. Kiss, F. Lefranc, Neoplasia 12 (2010) 727-739. DOI: 10.1593/neo.10526

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The Diplomatic and Digital Forensic Science in Born-Digital Records: The Quest for Authenticity

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ABSTRACT

This paper aims to applicate of Digital Diplomatic and Digital Forensics Science in digital-born records, not only to guarantee its authenticity in institutional routines and processes, but also to use as a source of proof in possible legal and juridical scenarios. Inside the discussion about the complexity of the digital records in Archival Science and Information Science (I.S.), have a gap to manage and preserve the digital records, which try to keep them reliable, accurate and authentic in systems that have the same conditions. This gap makes it impossible to preserve records in the long term due, firstly, to the fragility of the systems where they are stored and, secondly, to the constant risks of obsolescence of hardware and software that occur on a day by day. Digital Diplomatic Science assists this process by determining the form and content of the record to evidence its legal-diplomatic authenticity and establish its historical value. Digital Forensic Science, in turn, provides support for the chain of custody to remain intact, regardless of the medium on which the digital record is fixed. It raises the following questions: Is it possible to apply the practice of law and the Digital Forensics in the area of the Archival Science and Information Science, guaranteeing the authenticity of the born-digital record? Which way does the junction between Digital Diplomatic Science and Digital Forensic Science guarantee the conservation and preservation of the born-digital records? To answer such questions, this research proposes to link five areas of knowledge following the scientific experiences conducted at the University of British Columbia in Canada by the InterPares (International Research on Permanent Authentic Records) group. Areas of knowledge that are offering interesting results to preserve not only the record, but also the social memory: 1. Digital Forensics: discipline that joint Forensics Science with Computer Science to analyze the digital evidence. 2. Diplomatics Science: Science to analyze the form and structure of the records. 3. Archival Science: This science involves the whole of the principles, policies, strategies and activities designed to ensure the physical and technological stabilization of records for the purpose of extending indefinitely their life and protecting the accuracy and authenticity of and maintaining the accessibility to their intellectual content. Thus, preservation includes, among many other activities, description, which provides an account of the context, attributes, and relationships of the documents, and the development and maintenance of retrieval systems [1]. 4. Information Science: Science to analyze the information in different contexts. This paper will carried out in a theoretical and qualitative way, taking into account the literature shared in the site Digital Records Forensics Project, coordinated by the author Luciana Duranti of the InterPares group, and the bibliography produced in the areas of knowledge already referenced. The results obtained will contribute to the realization of alternative researches in document conservation within the Archival Science and the Information Science.

Keywords: Digital Diplomatics; Digital Forensics; Born-Digital Record

Abbreviations: I.S. Information Science

1. Introduction

This paper will highlight and broaden the discussion about the contribution of Digital Diplomatic Science, a contemporary methodology of a secular science that analyses the nature, genesis, formal characteristics, structure, transmission and legal consequences of records to prove their authenticity [2]. The contribution provided by Digital Forensic Science, is defined as,

> [...] the application of Computer Science and investigative procedures for legal purposes, linking the analysis of digital evidence, chain of custody, mathematical validation, the appropriate use of tools,

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> reproducibility and possible reports presented by an expert [...][3]

In some studies, the term digital record used to be synonyms of electronic record, but they have differences. Digital record is a document encoded with binary digits, in values of zero and one, and, this research works with it. Electronic record is "[...] any analog or digital document carried by an electrical conductor which requires the use of an electronic equipment to be understood by a person (e.g. the fax) ... "[4]. To clarify the sentence, the author Rondinelli [5] explains that:

> According to the *Cámara Técnica de Documentos Eletrônicos* (2010), electronic record is a document coded in "analogue form or in binary digits, accessible through electronic equipment." In other words, it can be said that every digital record is electronic, but not every electronic record is digital. The cassette, for example, which sound needs an electronic equipment to be heard, is not codified in bits.

The Archival and Diplomatic Sciences, when joint, starts to analyze the record beyond their fixed documentary form (presentation in the same way it was when they were first stored) and stable content (mandatory and unchanged permanence of information). Even in the analogue context, they used to links records ("organicity"), to recognize identifiable context (legal-administrative, provenancial, and procedural) [6], and consider people involved in its creation and participation in its cycle [7]. In the digital environment, these studies developed from Archival and Diplomatic Sciences received more focus.

One of the objectives of Digital Diplomatic Science is to ensure that digital records retain their values of authenticity through two fundamental elements: integrity and identity. Integrity refers to the completeness and solidity of the record and cannot be unlawfully corrupted or tampered with. Identity refers to the attributes of the record that characterizes it and distinguishes it from other records.

The purpose of the study of Digital Forensic Science is to develop methods for verifying this documentary authenticity by preserving the chain of custody both in the legal area of documentary conservation and preservation. The contribution of the two mentioned sciences (see the next section) establishes itself in giving value to those digital records that are often not considered as sources of proof.

The digital record, unlike the conventional record, still raises some doubt as a source of proof. These difficulties arises when the record is generated in a system, and then, to ensure its conservation, it is migrated to another support:

> Every time you migrate a record, you lose the support and just remains the information: it broke the record, broke the source of proof and finally broke its authenticity. Authenticity is only guaranteed through the study of the record and not only of the information. The information has to be fixed in the support and if this support migrates, the fixity has to be documented [8].

With this in mind, the proposal of this research is to analyse the theoretical concepts developed in Digital Diplomatic Science and Digital Forensic Science as a subsidy for digital records to be reliable and authentic from the moment of creation until their access. To accomplish this goal, it will work interdisciplinary with five sciences that will solidify and give a greater understanding to the research problem. 1) Digital Forensic Science, because it is an area that joins the knowledge of Forensic Sciences and Computer Science for the analysis of digital evidence. 2) Diplomatic Science, which analyses the form and content of the record researching its authenticity. 3) Archival Science, been a science that assists in the production and organization of records, without losing sight of the usefulness of new technologies. 4) Information Science, for giving relevant information in its different contexts. 5) Law, because it is a science that establishes legal and juridical issues focused on the documentary context and its privacy. In addition, the InterPARES (International Research on Permanent Authentic Records) surveys, specifically those related to the integrated Digital Records Forensics project, will be addressed, understanding the archival digital record as a potential source of evidence for possible legal and juridical scenarios.

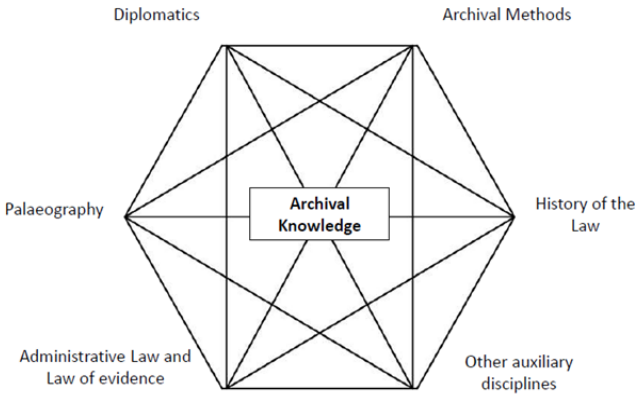

Figure 1. Interdisciplinarity of Archival Science with other sciences like Diplomatics, Archival Methods, Palaeography, Administrative Law and Law of Evidence, History of the Law, and Other Auxiliary Disciplines .

2. Material and Methods

This research is characterized by being a qualitative, theoretical and descriptive-exploratory type. Archival theories and principles will be analysed, starting from the bibliographical research for the delimitation of the study subject and the improvement of the scientific base. Subsequently, the compilation of the data obtained in the bibliographic review will be analysed with the purpose of presenting the results of the theoretical discussion, to answer questions related to the application of Digital Diplomatic Science and Digital Forensic Science in the digital records management. In addition, in the theoretical discussion exists

points of views confrontations, which makes possible not just draw new perspectives for the study of the digital records management, but solve challenges and discuss accurately and consistently the problem. With the delimitation obtained through the theoretical discussion, there is a better viability of reaching the objectives .

3. Results

The Science of Information and Archival Science explores the tools offered by Diplomatic and Digital Forensic Science as an aid to the documentary processes. This allowed the applicability of the mentioned concepts within the processes of the digital record. Initially, a foreign literature was utilized to do a content analysis and determine the progress of Diplomatic and Digital Forensics in this context. The InterPARES (International Research on Permanent Authentic Records) proposals is to analyze the contributions of the Forensic Diplomatic Project. This group was choosen because they are in its fourth phase of the project. Then, it will offer a better understanding of this research field.

4. Discussion

The junction between Diplomatic Science and Digital Forensic Science resulted in the InterPARES research group, the DIGITAL RECORDS FORENSICS PROJECT. It began in April 2008, and involved the Library School of the University of British Columbia, File and Information Studies (SLAIS), the UBC Law School (University of British Columbia) and the Forensic Division Computer Science Department of the Vancouver Police Department. The main challenges analyzed by InterPARES in the areas of Archival Science, Management of Digital Archives and in the legal scope are:

- The identification of records among all the digital objects produced by complex digital systems, and
- The determination of their authenticity:
  - when digital materials are kept outside of the technological environment in which they were produced and/or maintained either by the creating body itself or by third parties like police departments or archival organizations; and
  - when records are of uncertain origin and/or exist in proprietary formats that are hard to maintain over time, thus compromising their long-term research value or their ability to be submitted and/or admitted as evidence in a trial [9].

5. Concluding Remarks

Research of this type will aid in records management and archival processes in several entities, as well as the need to extrapolate its field of investigation to areas not explored in documentary areas, such as Law. Records and systems need to be trusted to carry out the various transactions, and both Digital Diplomatics and Digital Forensic Science are assisting this intention.

References

1] L. Duranti. A. Jansen, in: 5th European Conference on Information Management and Evaluation, ECIME, Como, Italy. 2011, pp.134-139. https://www.bcs.org/content/conEvent/5774

2] L. Duranti, B. Endicott-Popovsky, in: Annual Conference on Digital Forensics, Security and Law, ADFSL, St. Paul, Minnesota, United States, 2010, pp. 109-121. https://commons.erau.edu/adfsl/2010/

3] L. Duranti, B. Endicott-Popovsky, in: Annual Conference on Digital Forensics, Security and Law, ADFSL, St. Paul, Minnesota, United States, 2010, pp. 109-121. https://commons.erau.edu/adfsl/2010/

4] L. Duranti, Archivaria 68 (2009) 39–66. https://archivaria.ca/archivar/index.php/archivaria/article/view/13229/14548

5] R. Rondinelli, O documento arquivístico ante a realidade digital: uma revisão conceitual necessária, first ed., FGV, 2013.

6] L. Duranti. A. Jansen, in: 5th European Conference on Information Management and Evaluation, ECIME, Como, Italy. 2011, pp.134-139. https://www.bcs.org/content/conEvent/5774

7] N. Tognoli. A Construção teórica da diplomática. Em busca da sistematização de seus marcos teóricos como subsídio aos estudos arquivísticos, first ed, UNESP, 2014.

8] D. Flores. 2017. Rede Cariniana. https://www.youtube.com/watch?v=PbGF7wX3Ws8&t=2305s

9] Digital Records Forensics Project. 2011. http://www.digitalrecordsforensics.org/