# XJENZA ONLINE

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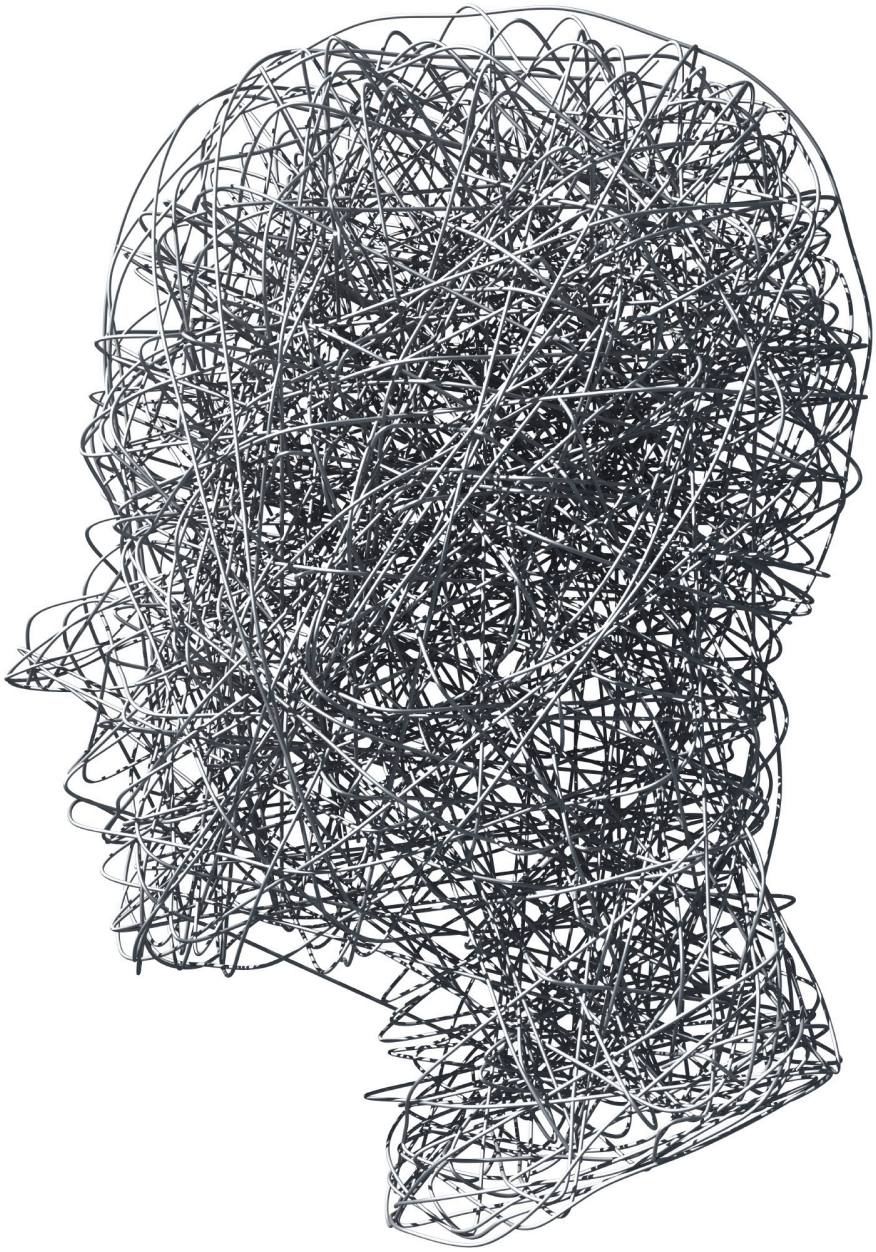

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Malta Chamber of Scientists

The Journal of the Malta Chamber of Scientists

Editor-in-Chief: Giuseppe Di Giovanni

Xjenza Online - Journal of Malta Chamber of Scientists
http://www.mcs.org.mt/

# Editorial Board

# Scope of Journal

Xjenza is the Journal of the Malta Chamber of Scientists and is published in an electronic format. Xjenza is a peer-reviewed, open access international journal. The scope of the journal encompasses research articles, original research reports, reviews, short communications and scientific commentaries in the fields of: mathematics, statistics, geology, engineering, computer science, social sciences, natural and earth sciences, technological sciences, linguistics, industrial, nanotechnology, biology, chemistry, physics, zoology, medical studies, electronics and all other applied and theoretical aspect of science.

The first issue of the journal was published in 1996 and the last (No. 12) in 2007. The new editorial board has been formed with internationally recognised scientists, we are planning to restart publication of Xjenza, with two issues being produced every year. One of the aims of Xjenza, besides highlighting the exciting research being performed nationally and internationally by Maltese scholars, is to provide insight to a wide scope of potential authors, including students and young researchers, into scientific publishing in a peer-reviewed environment.

**Instructions for Authors**
Xjenza is the journal of the Malta Chamber of Scientists and is published by the Chamber in electronic format on the website: http://www.xjenza.com. Xjenza will consider manuscripts for publication on a wide variety of scientific topics in the following categories

- (01) Communications
- (02) Research Articles
- (03) Research Reports
- (04) Reviews
- (05) Notes
- (06) News
- (07) Autobiography

**Communications** are short peer-reviewed research articles (limited to three journal pages) that describe new important results meriting urgent publication. These are often followed by a full Research Article.

**Research Articles** form the main category of scientific papers submitted to Xjenza. The same standards of scientific content and quality that applies to Communications also apply to Research Articles.

**Research Reports** are extended reports describing research carried out in Malta or by Maltese researchers of interest to a wide scientific audience characteristic of Xjenza. Please contact the editor to discuss the suitability of topics for Research Reports.

**Review Articles** describe work of interest to the wide readership characteristic of Xjenza. They should provide an in-depth understanding of significant topics in the sciences and a critical discussion of the existing state of knowledgeon a topic based on primary literature. Review Articles should not normally exceed 6000 words.Authors are strongly advised to contact the Editorial Board before writing a Review.

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**News:** The News section provides a space for articles up to three pages in length describing leading developments in any field of science and technology or for reporting items such as conference reports. The Editor reserves the right to modify or reject articles for consideration as 'news items'.

**Errata:** Xjenza also publishes errata, in which authors correct significant errors of substance in their published manuscripts. The title should read: Erratum: "Original title" by ***, Xjenza, vol. *** (year). Errata should be as short as consistent with clarity.

**Invited Articles and Special Issues:** Xjenza regularly publishes Invited Articles and Special Issues that consist of articles written on invitation by the Editor or member of the editorial board.

# Submission of Manuscripts

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All manuscripts submitted to Xjenza are peer reviewed. Authors are requested to submit with their manuscript the names and addresses of three referees, preferably from overseas. Every effort will be made to use the recommended reviewers; however the editor reserves the right to also consult other competent reviewers.

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Authors are expected to disclose any commercial or other associations that could pose a conflict of interest in connection with the submitted manuscript. All funding sources supporting the work, and institutional or corporate affiliations of the authors, should be acknowledged on the title page or at the end of the article.

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**Submission, Declaration and Verification**
Submission of a manuscript implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis), that it is not under consideration for publication elsewhere, that it has been approved for publication by all authors, and tacitly or explicitly, by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically, without the written consent of the copyright-holder.

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It is important that the file be saved in the native format of the word processor used. The text should be in single-column format and layout of the text to be kept as simple as possible. Most formatting codes will be removed and replaced on processing of the manuscript. The word processor options should not be used in order to justify text or hyphenate words. However, the use of bold face, italics, subscripts, superscripts etc is permitted.

**Article Structure**
A manuscript for publication in Xjenza will ordinarily consist of the following order: Title page with contact information, Abstract, Highlights, Keywords, Abbreviations, Introduction, Materials and Methods, Results, Discussion, Conclusions, Appendices and References.

The manuscript will be divided into clearly defined sections. Each subsection should be given a brief heading. Each heading should appear on its own separate line. Subsections should be used as much as possible when cross-referencing text: refer to the subsection by heading as opposed to simply 'the text'.

**Title page**
- Title should be concise yet informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
- Author names and affiliations. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after each author's name and in front of the appropriate address. Provide full postal address of each affiliation, including the country name and, if available, the e-mail address.
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**Abstract**
A concise and factual abstract is required of up to about 250 words. The abstract should state briefly the background and purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, references and non-standard abbreviations should be avoided. If essential, these must be defined at first mention in the abstract itself.

**Highlights**
Highlights are mandatory for Xjenza. They consist of a short collection of 3-5 bullet points of a minimum of 85 characters (including spaces) each, that convey the core findings of the article and should be submitted in a separate file. Please use 'Highlights' in the file name.Keywords

Immediately after the abstract, provide a maximum of 10 keywords to be used for indexing purposes.

**Abbreviations**
Define abbreviations that are not standard in this field in a footnote to be placed on the first page of the article. Such abbreviations that are unavoidable in the abstract must be defined at their first mention as well as in the footnote and should be used consistenly throughout the text.

**Introduction**
State the objectives of the work and provide an adequate background, avoid a detailed literature survey or a summary of the results.

**Material and Methods**
Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

**Results**
Results should be clear and concise. Numbered/tabulated information and/or figures should also be included.

**Discussion**
This should explore the significance of the results of the work, yet not repeat them. Avoid extensive citations and discussion of published literature. A combined section of Results and Discussion is often appropriate.

**Conclusions**
The main conclusions based on results of the study may be presented in a short Conclusions section. This may stand alone or form a subsection of a Discussion or Results and Discussion section.

**Appendices**
Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

**Acknowledgements**
Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided assistance during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

**Units**
Follow internationally accepted rules and conventions: use the international system of units (SI). If other units are mentioned, please give their equivalent in SI.

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Footnotes should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many word processors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

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TIFF: Color or grayscale photographs (halftones): always use a minimum of 300 dpi.
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Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

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Every reference cited in the text should also be present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

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The full URL should be given and the date the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately or can be included in the reference list.

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**Reference Style**
Text: All citations in the text should refer to:
1. Single author: the name (without initials, unless there is ambiguity) and the year of publication;
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3. Three or more authors: first author's name followed by 'et al.' and the year of publication.
Citations may be made directly (or parenthetically). Groups of references should be listed first alphabetically, then chronologically.
Examples: 'as demonstrated (Allan, 2000a, 2000b, 1999; Allan and Jones, 1999). Kramer et al. (2010) have recently shown ....'
List: References should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by the letters 'a', 'b', 'c', etc., placed after the year of publication.

**Examples:**
**Reference to a Journal Publication:**
Borg J., Papadopoulos P., Georgitsi M., Gutiérrez L., Grech G., Fanis P., Phylactides M., Verkerk A.J., van der Spek P.J., Scerri C.A., Cassar W., Galdies R., van Ijcken W., Ozgür Z., Gillemans N., Hou J., Bugeja M., Grosveld F.G., von Lindern M., Felice A.E., Patrinos G.P., Philipsen S. (2010) Haploinsufficiency for the erythroid transcription factor KLF1 causes hereditary persistence of fetal hemoglobin. Nat. Genet. 42(9), 801-805.
Cope D.W., Di Giovanni G., Orban G., Fyson S.J., Errington A.C., Lorincz M.L., Gould T.M., Carter D.A., Crunelli V. (2009) Enhanced tonic GABAA inhibition is required in typical absence epilepsy. Nat. Med. 15(12), 1392-1398.

**Reference to a Book:**
Di Giovanni G. (2012) Nicotine Addiction: Prevention, Health Effects and Treatment Options. Nova Publishers, New York.
**Reference to a Chapter in an Edited Book:**
Di Giovanni G., Pierucci M., Di Matteo V. (2011). Monitoring Dopamine in the mesocorticolimbic and nigrostriatal systems by microdialysis: relevance for mood disorders and Parkinson's disease. In: Applications of Microdialysis in Pharmaceutical Science. Ed: Tsai T-H. John Wiley & Sons, Inc., Hoboken, NJ, USA.

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-CAS (Chemical Abstracts Service): http://www.cas.org/sent.html.

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**Submission checklist**

The following list will be useful during the final checking of a manuscript prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

- E-mail address
- Full postal address
- Telephone and fax numbers

All necessary files have been sent, and contain:

- Keywords
- All figures including captions
- All tables (including title, description, footnotes)

Further considerations

- Manuscript has been 'spell-checked' and 'grammar-checked'
- References are in the required format
- All references mentioned in the reference list are cited in the text, and vice versa
- Permission has been obtained for use of copyrighted material from other sources (including the Web)

**After Acceptance**

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Formerly *Xjenza*

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# Editorial

**Giuseppe Di Giovanni**
Editor-in-Chief of Xjenza online

This is the second issue of the new Xjenza series, renamed Xjenza Online, the official journal of the Malta Chamber of Scientist (ChoSci). As editor, I am pleased to report that over the past six months there has been an ongoing interest in the journal, from both authors and readers in the ever widening area of science.

We are pleased to show that the journal has a new cover design thanks to Jean Claude Vancell, this issues front image being the logo of the COST Action CM1103. Indeed, the ChoSci and its Physiological Society (MPS) hosted the International Annual meeting of COST Action CM1103 "Interdisciplinary Chemical Approaches for Neuropathology" in association with the "4th Neuroscience Day Malta University" in Valletta at the Old University this October. The University of Malta and the ChoSci organizing committee worked hard for a very successful event, both scientifically and socially. Dr Rona Ramsay, the Chair of the Action from the University of St Andrews, UK, and I put together an exciting program that covered a full range of interests, promoted the exchange of expertise between the various areas of structural-based drug design, synthetic chemistry and neuropathology that can all contribute information to diagnosis and treatment methods. This conference brought together international experts and Maltese scientists, fostering collaborations and dissemination of the work done in Europe. The *'Proceedings of the CM1103 "Interdisciplinary Chemical Approaches for Neuropathology in association with the 4th Neuroscience Day University of Malta" '* can be found at the end of this issue.

With the aim to have as varied perspectives and methodological skills as possible I have increased the number of associate editors. Xjenza's team is now made up of nine editors, with the new inclusion, in alphabetical order, of David Mifsud (Life Science), Ian Cassar (Economics), Ray Fabri (Linguistics) and Ian Thornton (Psychology and Sociology). In addition, I have included an Advisory Board made of international scholars, such as Vincenzo Crunelli (Cardiff University, UK), David Eisner, (Manchester University, UK) Angela A. Xuereb Anastasi ( University of Malta), Frank Vella (University of Saskatchewan, Canada) and Giovanni Romeo (University of Bologna, Italy).

This issue of Xjenza Online continues to be the premier outlet for work carried out in Malta. In addition, we have maintained our promise of taking the journal to an international level. Indeed, our first paper by Giuanluca Valentino and his Swiss colleagues at CERN highlight the use of the Large Hadron Collider together with the cleaning mechanisms used to ensure efficient particle collisions before insertion into beams. It also shows the complex process of beam alignment through a series of algorithms and how this is applied to ensure appropriate functioning of the system. We are also pleased to present to our readers an article by Francesco Crespi, an Italian worldwide expert on serotonin, with insight into neurotransmitter distribution throughout the CNS, its signalling mechanisms and new methods used for measurement of monoamines, in particular voltammetry and NEAR UV.

Briffa and colleagues in a Malta-Spanish collaboration carried out a study into nano-particle silane consolidants in the treatment and restoration of Globigerina limestone. The effectiveness of such treatments and how preventable future damage is, was then evaluated by a number of techniques such as salt crystallisation and capillary tests.

The paper by Victor Grech is concerned on the similarities exhibited in Star Trek to that of the myth of Galatea-Pygmalion. He gives a complex examination into many different scenarios and narratives in the works of Star Trek and their apparent relationships with Greek mythology in relation to science.

Scicluna and her colleagues, in their research note, have presented a range of patient interpretations on whether or not they are experiencing a stroke and how

long these patients took to refer themselves to hospital. This contains specific investigation into the knowledge that patients hold on the symptoms associated with stroke and how this needs improvement.

The ChoSci believes that Xjenza Online might be essential in the scientific development of early-stage researchers in Malta, representing a learning platform on which to write and publish a scientific paper. In this second number of Xjenza Online there is a research article by Claudette Gambin and colleagues, supervised by Dr Mifsud, in which material on Maltese honey and its pollen components, characterising them into specific groups of pollen type and pollen species is produced. Honey from all over the Maltese island was examined and allowed formulation of a pollen spectra as well as examination into incompetent honey production. A review article written under my guidance by Mr Vella, a fourth year medicine student is an in depth overview on how nicotine can administer addiction, with the neuropathophysiology of addiction development, areas of the brain associated with this action and adaptations experienced as a result of this addiction. Vella also gave an explanation of current and novel therapies for smoking cessation treatment. Jurgen Mifsud under the supervision of Kollegger at CERN, Switzerland has described research in which they evaluated the collisions of high-energy nucleons, with respect to the Glauber Model. This model is illustrated through a number of different equations and distribution models. They have also shown the relationships formed when particles are at central collision and off central collision. Mifsud and Attard have described the importance of improved public transport services for Malta's elderly population. Through geographical evaluation, analysis of duration both to and on the buses and lack of available information, it has been shown that there is great difficulty for this particular population.

In this issue we present the first in the series of autobiographies by scholars of distinction. Professor Francis Vella has written an account of the highs and lows and the many achievements as well as challenges he experienced throughout both his academic and scientific career. He gives insight into his research, methods of development in his research and shows great aim to inspire younger individuals.

The news article is by Isabella Borg in which she describes the courses recently put in place on Medical Genetics and different methods of next generation sequencing. It explains how these techniques are of great importance in medical research and how the courses provided have shown researchers the benefits and techniques of these systems.

Once again I would like to pass on my thanks to the authors and associate editors for the high quality of material in this issue of the journal. I would like to single out Mr Jackson Said, without whose help this journal would not have been produced. With that, I thank the authors for their contributions to this issue and Ms Stephanie Chambers, a student from University of Exeter, UK, who is currently attending my laboratory, for her dedication and hard work as Editorial Assistant. As always, we thank our readers for their continued support and interest in our publications.

Giuseppe Di Giovanni,
Editor-in-Chief of Xjenza Online

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Doi: http://dx.medra.org/10.7423/XJENZA.2013.2.01

*Research Article*

# Operational Results with Fast Automatic Beam-Based LHC Collimator Alignment

**Gianluca Valentino[1,2], Ralph W. Assmann[2†], Roderik Bruce[2], Stefano Redaelli[2], Belen Salvachua[2], Nicholas Sammut[1] and Daniel Wollmann[2]**
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**Abstract.** The CERN Large Hadron Collider (LHC) is the largest and highest-energy particle accelerator ever built. It is designed to collide particles at a centre-of-mass energy of 14 TeV to explore the fundamental forces and constituents of matter. Due to the potentially destructive high-energy particle beams, with a total design energy of 362 MJ, the collider is equipped with a series of machine protection systems. The beam cleaning or collimation system is designed to passively intercept and absorb particles at large amplitudes. The cleaning efficiency depends heavily on the accurate positioning of the jaws with respect to the beam trajectory. Beam-based collimator alignment is currently the only feasible technique that can be used to determine the beam centre and beam size at the collimator locations. If the alignment is performed without any automation, it can require up to 30 hours to complete for all collimators. This reduces the beam time available for physics experiments. This article provides a brief recap of the algorithms and software developed to automate and speed up the alignment procedure, and presents the operational results achieved with fast automatic beam-based alignment in the 2011-2013 LHC runs.

**Keywords** Large Hadron Collider - Collimation system - Collimator alignment - Intelligent automation - Operational results.

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## 1 Introduction

The LHC at CERN is a 27 km long, state-of-the-art circular particle collider (Brüning *et al.* 2004). The injector chain accelerates protons or heavy ions from rest to a relativistic energy of 450 GeV, before injecting them in two counter-rotating beams into the LHC. After a further acceleration to the design energy of 7 TeV in the LHC (4 TeV in 2012), the particles in the two beams are brought in collisions at the locations of the four experimental detectors.

A complex beam cleaning system is installed to passively scatter and absorb particles which deviate from the beam core, before they are deposited in the superconducting magnets, thus protecting the LHC against normal and abnormal beam losses (Assmann *et al.* 2002). There are 54 beam-cleaning devices, called collimators, per beam. Each collimator is made up of two blocks or 'jaws' of carbon, tungsten or copper material. The jaws, identified conventionally as 'left' and 'right', are housed in a tank and kept under vacuum. The transverse rotation of the collimators follows a clockwise coordinate system, where the zero angle lies along the x-axis. Hence, for a vertical collimator, the 'left' jaw would be positioned above the beam, and the 'right' jaw would lie below the beam. The four jaw corners can be moved individually using stepping motors, with a precision of 5 µm. The maximum movement speed is 2 mm/s (Masi and Losito, 2008).

The collimators are distributed in the LHC ring as illustrated in Fig.(1). The collider has an eight-fold symmetry. Arcs connect eight long straight sections or insertion regions (IRs), and at the centre of each IR lies an interaction point (IP). Focusing and defocusing quadrupole magnets are used to ensure that particles

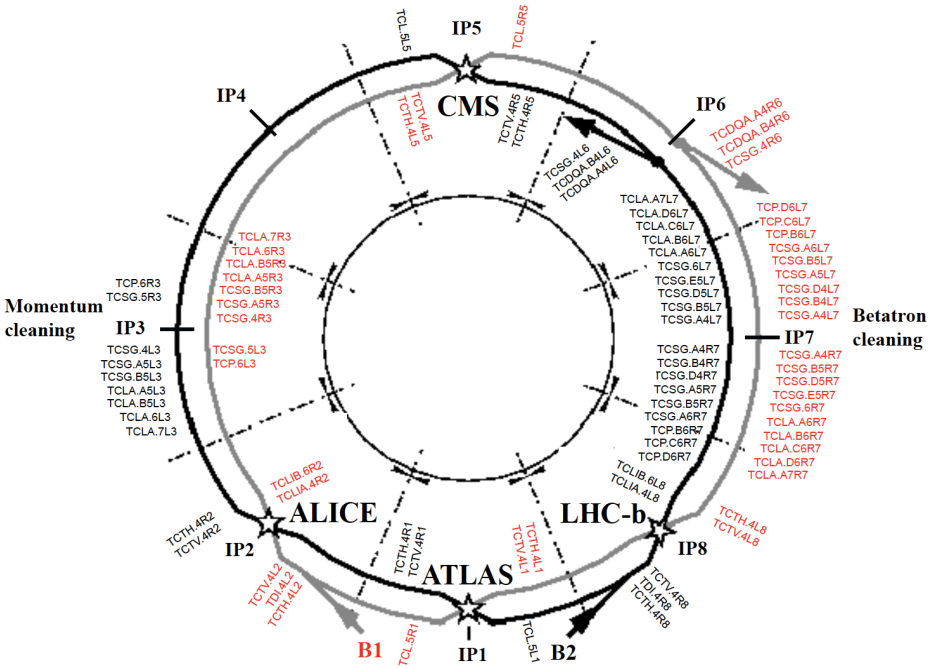



Figure 1: LHC collimation system layout (Bracco, 2009). The majority of the collimators are located in IR3 for momentum offset cleaning, and IR7 for betatron offset cleaning.

remain confined along the design orbit, and this causes them to perform so-called betatron oscillations around the design orbit. In addition, the particles have a certain momentum spread, and hence circulate in different orbits around the design orbit. The majority of the collimators are located in IR3 and IR7 to clean particles with large momentum and betatron offsets respectively. Dedicated collimation insertions are required due to space and radiation constraints, with the remaining IRs being taken up the experimental detectors, RF cavities and beam dump system.

The collimators are arranged in a four-stage hierarchy to reach the required level of cleaning efficiency, defined as the fraction of particles that escapes the collimators and are lost locally at any ring location. A graphical representation of the collimator hierarchy is shown in Fig.(2). The primary collimator (TCP) jaws are placed tightest around the beam, followed by the secondary collimators (TCSG), tertiary collimators (TCT) and absorbers (TCLA). The cleaning is carried out over hundreds of turns, and is hence referred to as multi-turn, multi-stage beam cleaning.

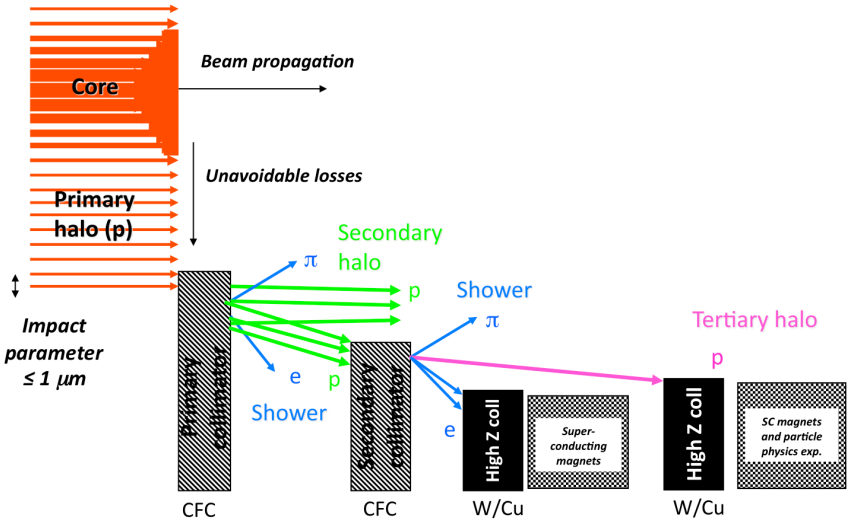



Figure 2: LHC multi-stage beam cleaning (Assmann, 2010). The primary halo is scattered by the TCPs. The secondary shower leaving the primary collimators is then scattered further by secondary TCSG and tertiary TCT collimators, until it is absorbed by TCLAs.

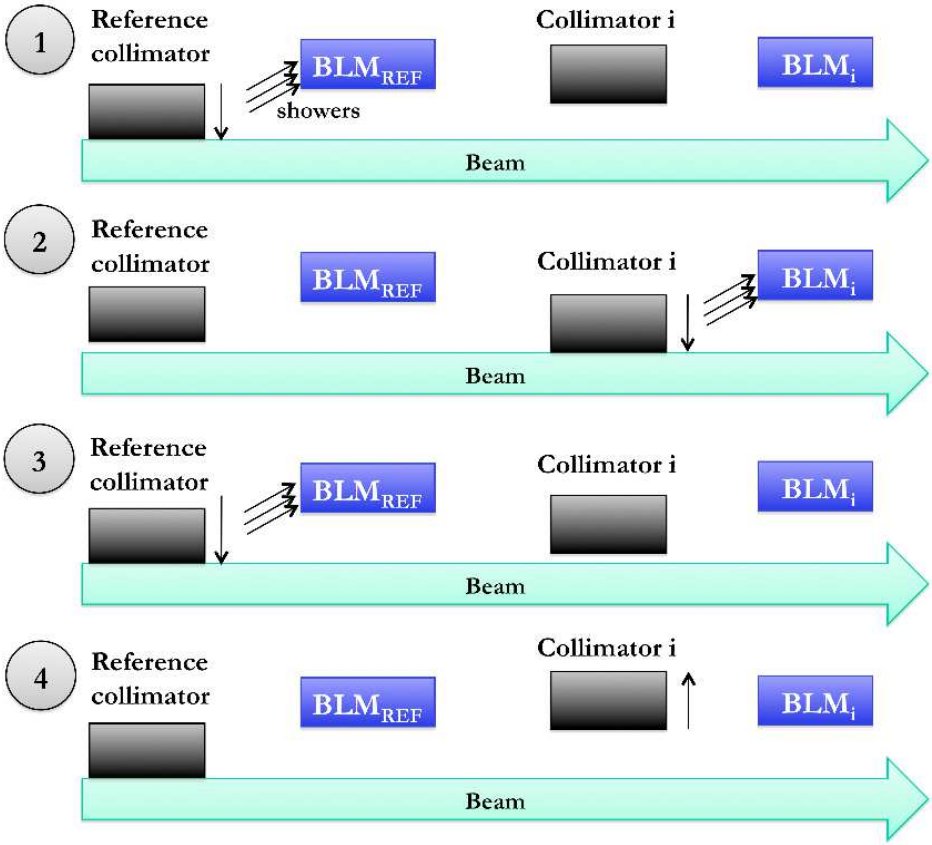



Figure 3: The four-stage beam-based alignment sequence for collimator $i$, using a primary collimator as a reference (Wollmann *et al.* 2010). A single jaw is shown for simplicity.

Beam losses in the LHC are measured using ionization chamber Beam Loss Monitors (BLMs) (Dehning, 2007). The approximately 3600 BLMs are placed at strategic points all around the collider, such as near the superconducting magnets, with some being positioned a few metres downstream of the collimators. A total of 1032 Beam Position Monitors (BPMs) measure the beam orbit in the horizontal and vertical planes at various positions around the ring (Jones, 2007), although often not close to the collimator locations.

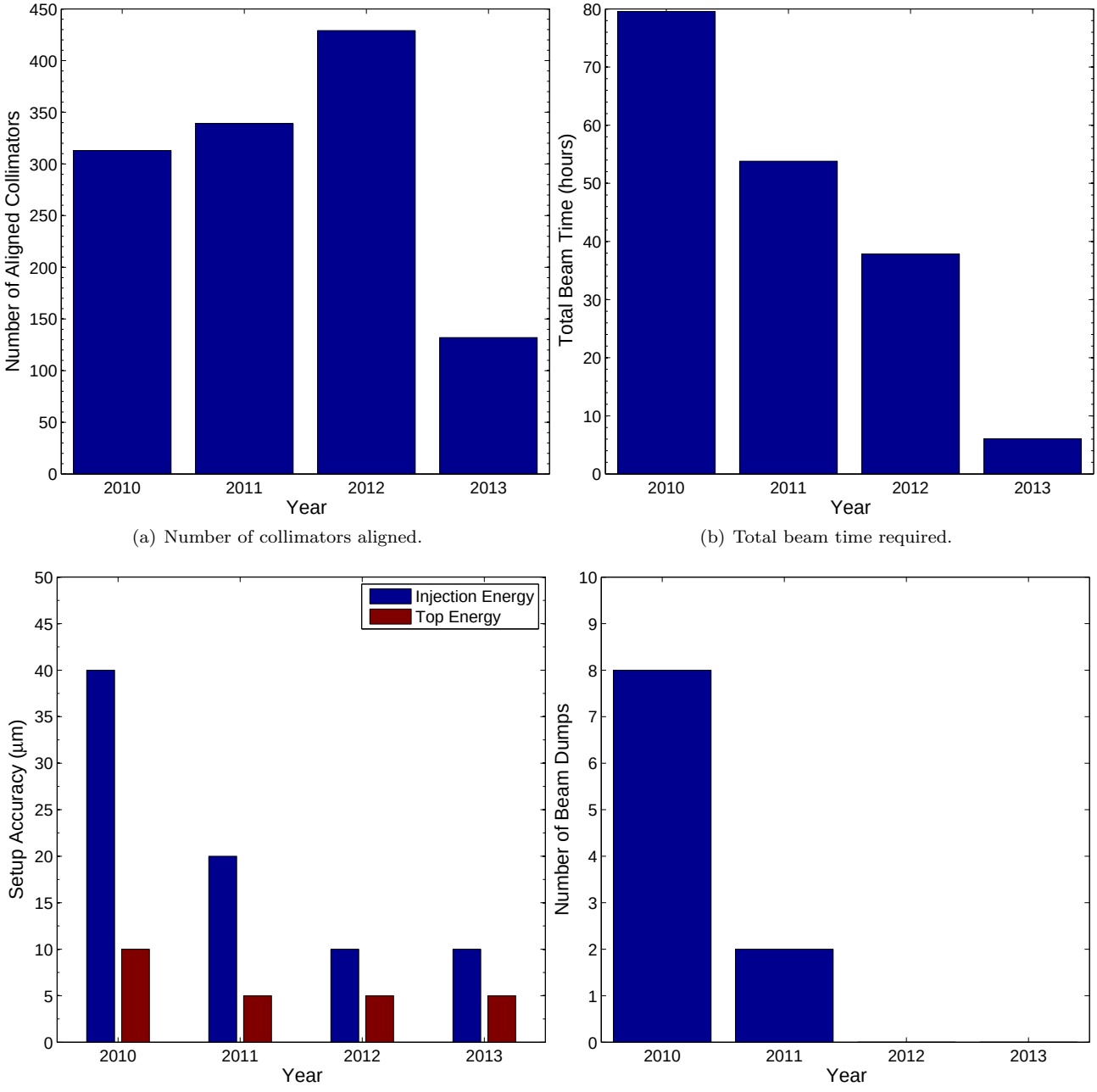
The collimation hierarchy only be set up if the beam centre and beam size at the collimator locations is known. Beam-based collimator alignment is currently the only feasible technique that can be used to determine these parameters. The alignment procedure consists of moving both jaws of a collimator towards the beam, until they touch the beam halo and induce beam losses. This stage is reached when a characteristic spike is observed in the beam losses picked up by a BLM positioned downstream of the collimator.

Collimator alignment was performed 'manually' in the CERN Control Centre during the 2010 LHC run. This means that a collimation expert is required to intervene for each jaw step of a few micrometres, using a software application to set the new jaw position. The expert must also simultaneously observe the BLM signals to ensure that the jaw is correctly aligned to the beam. For these reasons, a software tool was built to automate and speed up the alignment, and was used in the 2011-2013 LHC runs.

This paper is organized as follows. The collimator beam-based alignment procedure and the algorithms and software developed to automate it and speed it up are described in Sections II and III. The operational results achieved in the 2011-2013 LHC run are presented in Section IV, together with a comparison to the alignment results achieved in the previous run.

## 2 Collimator Beam-Based Alignment

Collimator alignments are part-and-parcel of the beam-commissioning period held at the start of each year of LHC operation. They are also performed throughout the year whenever the orbit and optics configuration parameters at the experimental IPs are changed, such as the beam crossing angles and $\beta$-functions at the experimental points (known as the $\beta^*$), as well as for dedicated beam studies and the so-called Van der Meer scans (White *et al.* 2010).




(a) Number of collimators aligned.

(b) Total beam time required.

(c) Setup accuracy at injection and top energy.

(d) Number of beam dumps.

Figure 4: Collimator alignment statistics.

The collimators are aligned in a four-step procedure, which is illustrated in Fig.(3). Only one jaw is shown for simplicity. The jaw of a reference collimator is moved in steps towards the beam to make a reference cut in the beam (step 1). The reference collimator is normally taken to be the TCP in the same plane (horizontal, vertical or skew) as the collimator $i$.

A BLM signal spike can be attributed to a particular jaw movement if only that jaw was moving when the spike occurs. Therefore, the left and right jaws are moved towards the beam separately. After aligning the reference collimator, the same procedure is performed for the collimator $i$ (step 2) and the reference collimator is aligned once again (step 3). The beam centre $\Delta x_i$ can then be determined from the final jaw positions of collimator $i$:

$$\Delta x_i = \frac{x_i^{L,m} + x_i^{R,m}}{2} \tag{1}$$

where $x_i^{L,m}$ and $x_i^{R,m}$ are the measured left and right jaw positions. The measured beam size at collimator $i$,

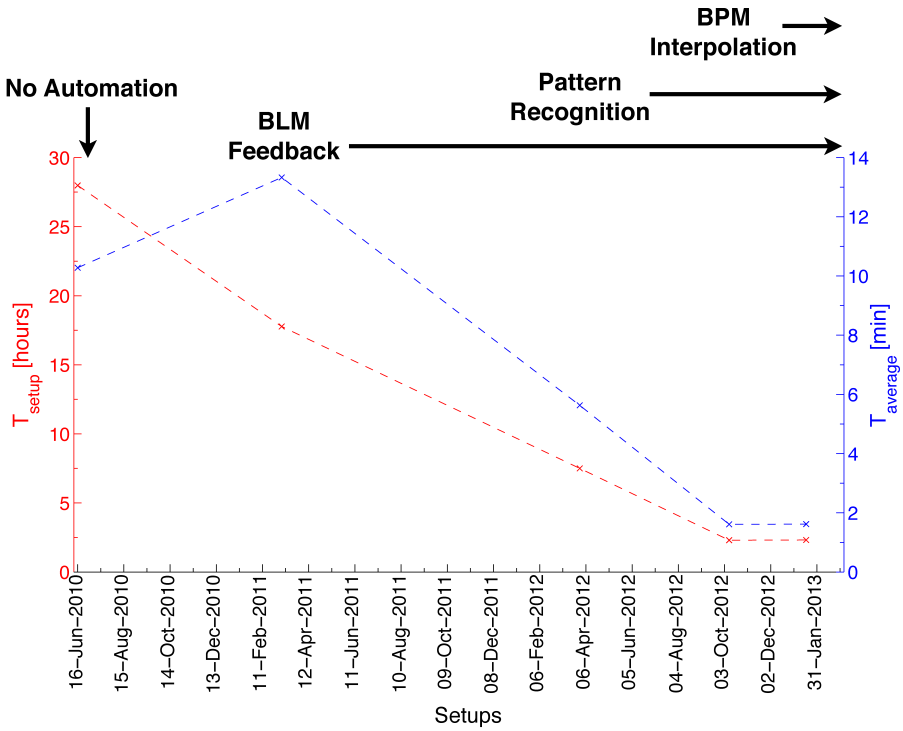



Figure 5: Evolution of the total setup time and average setup time per collimator at flat top in the 2010-2013 LHC runs. A timeline showing the introduction of the various algorithms is super-imposed.

$\sigma_i$, is expressed as a function of the jaw half gap, with $n_1$ being the cut made by the reference collimator in units of nominal beam standard deviations or $\sigma$:

$$\Delta x_i = \frac{x_i^{L,m} - x_i^{R,m}}{2n_1} \tag{2}$$

The nominal 1 $\sigma$ beam size at each collimator is determined from the nominal geometrical emittance $\varepsilon$, the nominal beta functions $\beta_{x,i}$ and $\beta_{y,i}$ at the collimator $i$, and the rotation angle of the collimator jaws $\psi_i$:

$$\sigma_i^{nom} = \sqrt{\beta_{x,i}\varepsilon_x \cos^2\psi_i + \beta_{y,i}\varepsilon_y \sin^2\psi_i} \tag{3}$$

In step 4, the left and right jaws are set to the operational settings, with $N_i$ being the half-gap opening specific to a collimator family:

$$x_i^{L,set} = \Delta x_i + N_i\sigma_i \tag{4}$$
$$x_i^{R,set} = \Delta x_i - N_i\sigma_i \tag{5}$$

# 3 Alignment Software

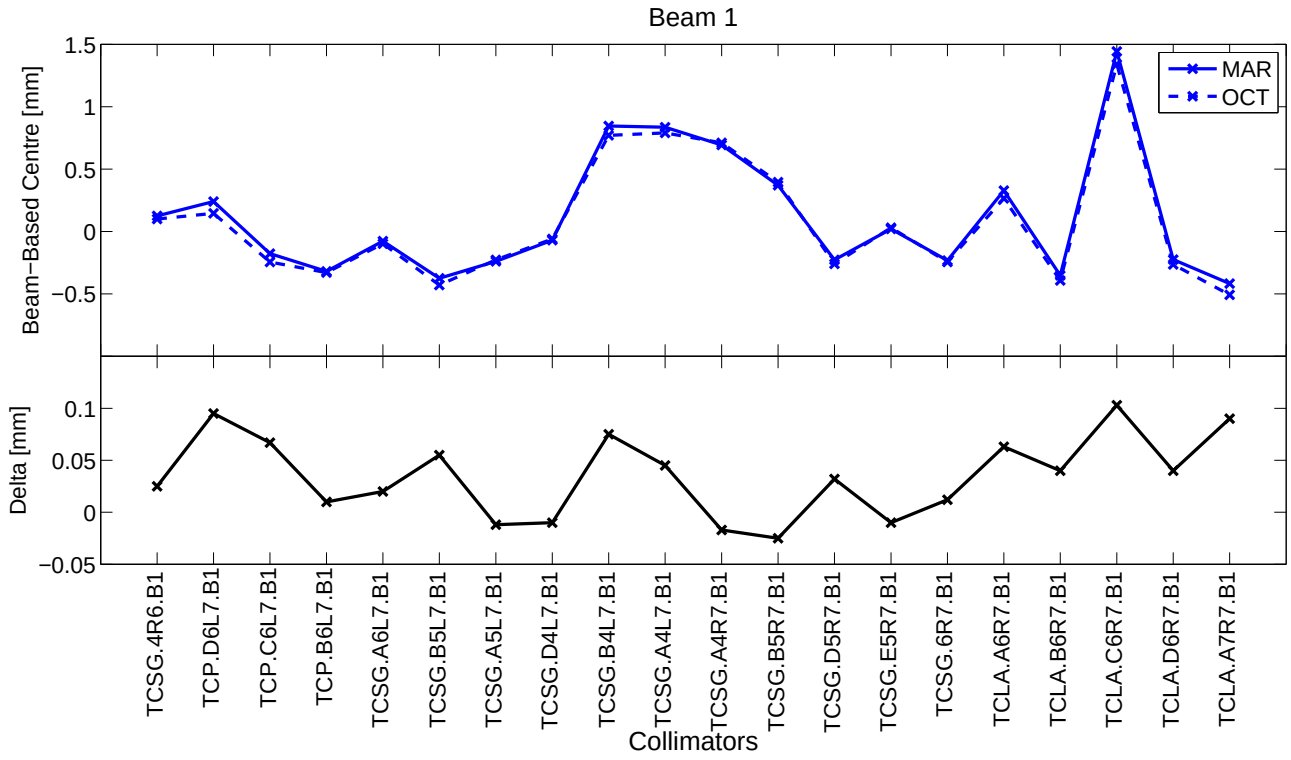
Over the 2010 - 2012 LHC runs, algorithms were developed and introduced in stages to speed up and automate the alignment procedure. The first step in 2011 was the introduction of a BLM feedback loop, that could allow for a single or parallelized movement of collimator jaws in steps towards the beam, until the losses exceeded a pre-defined BLM stopping threshold that was initially set manually (Valentino *et al.* 2012a).

For the start of the 2012 run, other improvements were introduced. Faster BLM data at a rate of 12 Hz allowed for the maximum collimator movement rate of 8 Hz to be used. Previously, the BLM feedback loop was limited by the acquisition of the BLM data at a 1 Hz frequency. Automatic selection of the BLM stopping threshold with every jaw movement reduces the need for expert intervention (Valentino *et al.* 2012b). Classification of the BLM loss signals based on Support Vector Machines (Valentino *et al.* 2012c) is used to determine whether the signal exhibits the typical loss spike and temporal decay characteristics when the threshold is exceeded, indicating that the jaw is aligned to the beam. A tool developed to centre the jaws at a safe and tighter gap around the BPM-interpolated orbit at the collimators at the start of alignment was tested in a dedicated beam study (Valentino *et al.* 2012d). These algorithms were implemented in the existing collimator control Java application (Redaelli *et al.* 2007) in the top-level of the LHC Software Architecture (LSA).

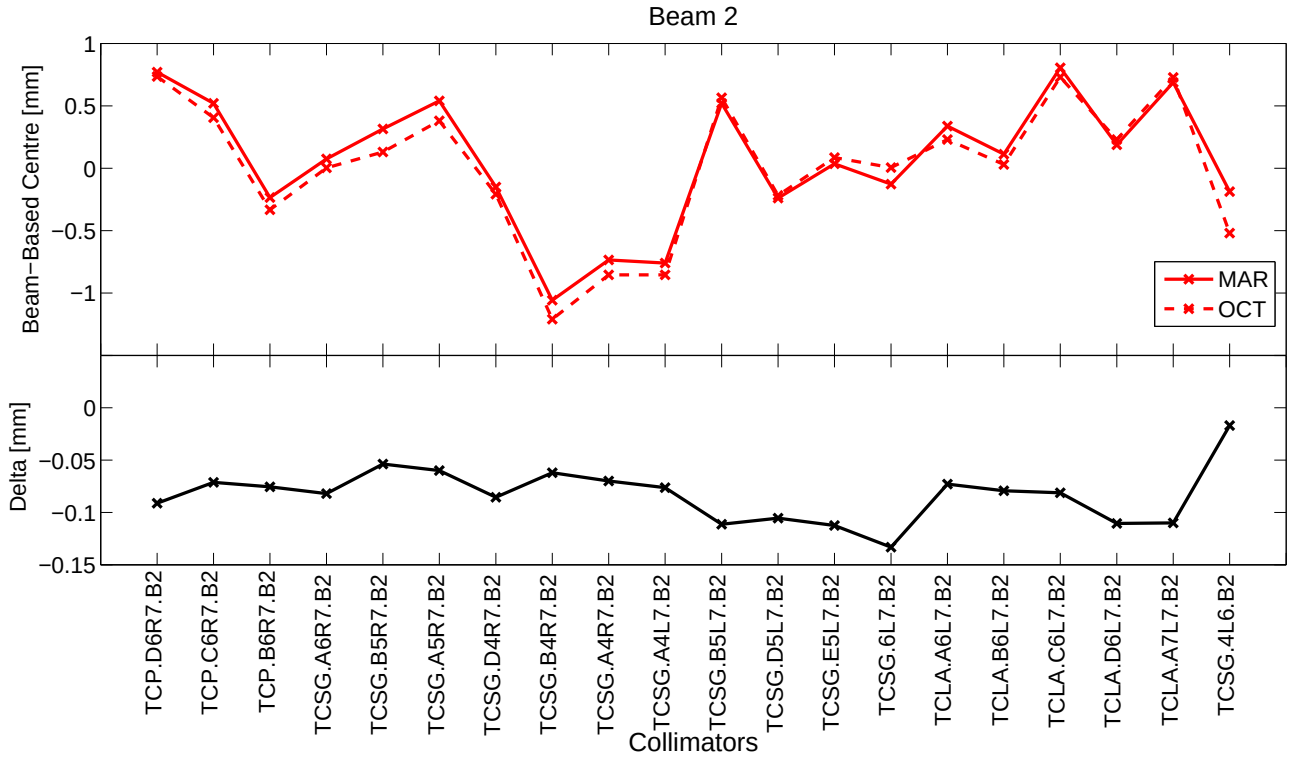
# 4 Operational Results

## 4.1 Alignment Times and Accuracy

Several highlight plots showing the collimator alignment operational results are shown in Fig.(4). The total number of collimators aligned per year has increased (see Fig.(4)(a)), while the total beam time required de-




(a) Measured B1 centres at the collimators.

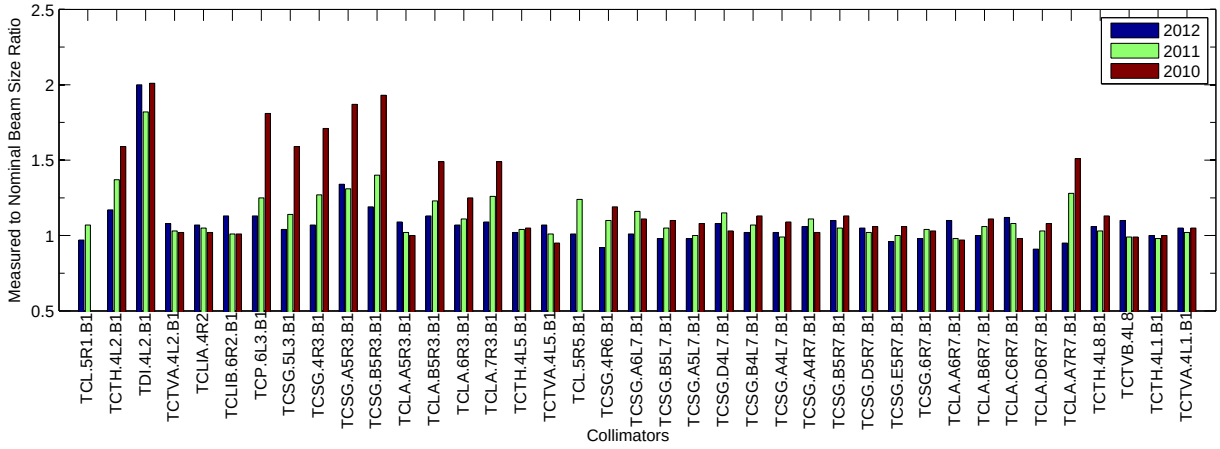



(b) Measured B2 centres at the collimators.

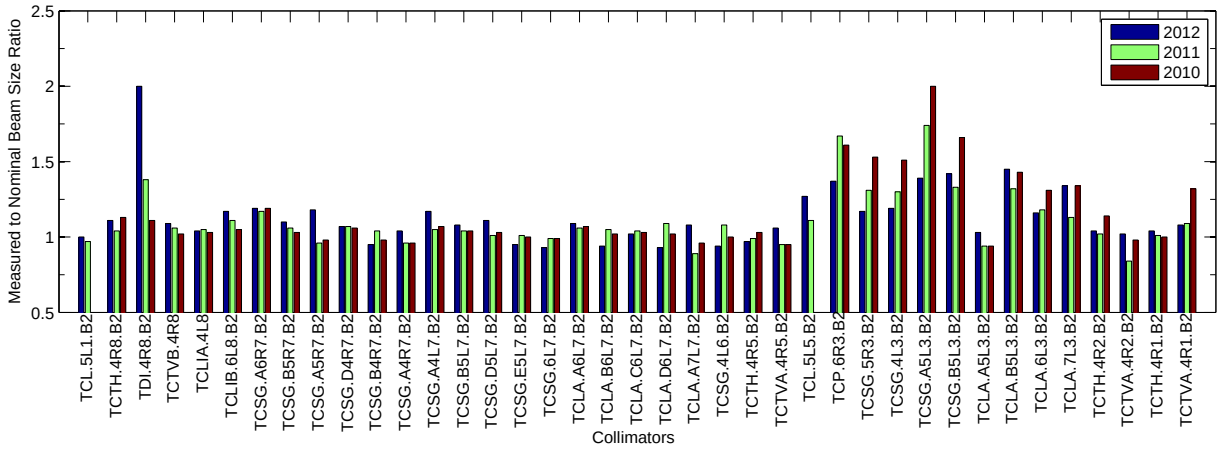
Figure 6: Measured beam centres comparison between March and October 2012.

creased (see Fig.(4)(b)). The reduction in time can be attributed to the phased automation of the alignment procedure described earlier in Section III.

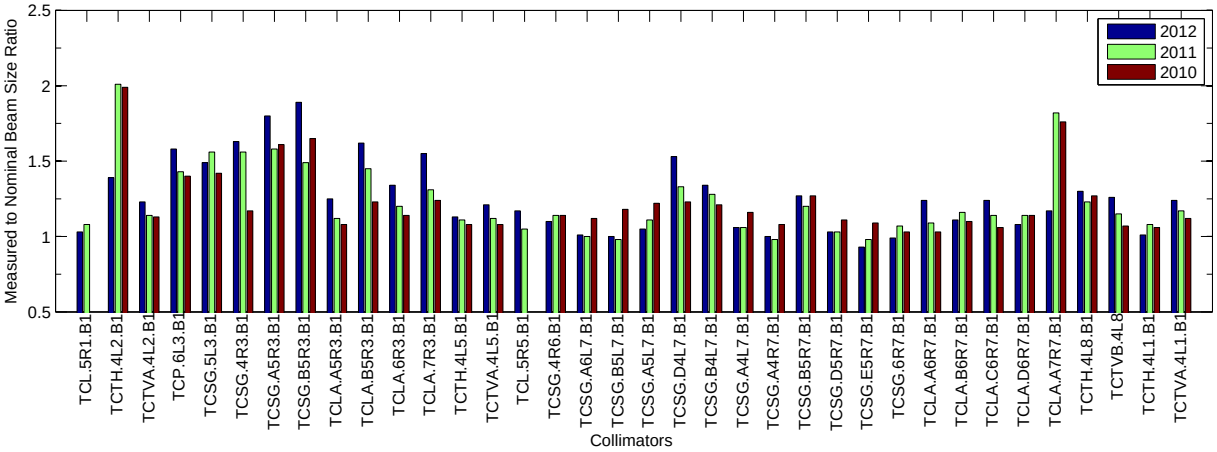
The gains in time could allow for smaller jaw step sizes




(a) B1 collimators, alignments at injection energy.



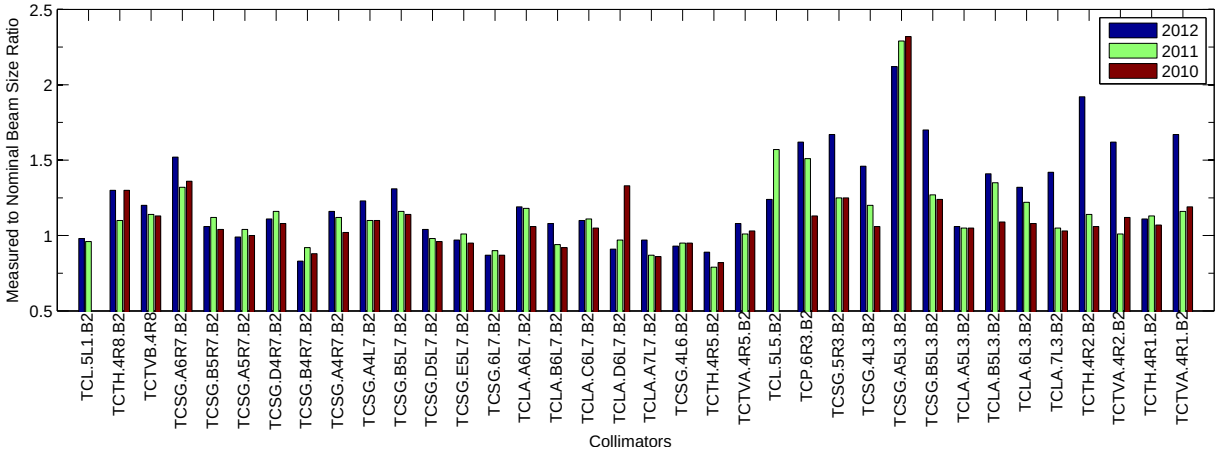

(b) B2 collimators, alignments at injection energy.




(c) B1 collimators, alignments at top energy.

to be used during the alignment (see Fig.(4)(c)). The beam size shrinks as a function of energy, meaning that a smaller step size is required to avoid over-scraping of the beam. For alignments at top energy in 2011-2013, therefore, the minimum jaw step size of 5 μm was used. In addition, no more beam dumps have been triggered due to human error during the alignment, as a result of the alignment automation (see Fig.(4)(d)).

The time required for individual alignments of the full system over the last four LHC runs is given in Fig.(5).




(d) B2 collimators, alignments at top energy.

Figure 7: Nominal to measured beam size ratios for each collimator in the LHC proton runs from 2010 to 2012.

A timeline is superimposed to show the contributions of the different algorithms. Recall also that the BLM data rate was increase from 1 Hz to 12 Hz for the 2012 run onwards. The setup time is observed to decrease from almost 30 hours with manual alignment in May 2010 down to less than 4 hours in a beam test held in January 2013. Similarly, the setup time per collimator decreases from 20 minutes to approximately two minutes.

## 4.2 Comparison of Measured Beam Centres and Beam Sizes

In 2012, the IR6 and IR7 collimators in both beams were aligned on two occasions: at the start of the LHC run in March, and for a beam study in October. The beam centres and beam sizes measured during the beam study were compared to the values achieved in the March alignment. The results are shown in Figs.(6, 7), where the collimator names shown on the x-axis are arranged in order of longitudinal position in the LHC.

The largest change in the beam centre is of 0.185 mm (corresponding to $0.507\sigma$), with the average change being 0.043 mm ($0.146\sigma$) for beam 1 (B1) and 0.089 mm ($0.243\sigma$) for beam 2 (B2). The similarity in the measured values is a reflection of the excellent stability of the LHC, and is the reason why a full collimation system alignment needs to be performed only yearly.

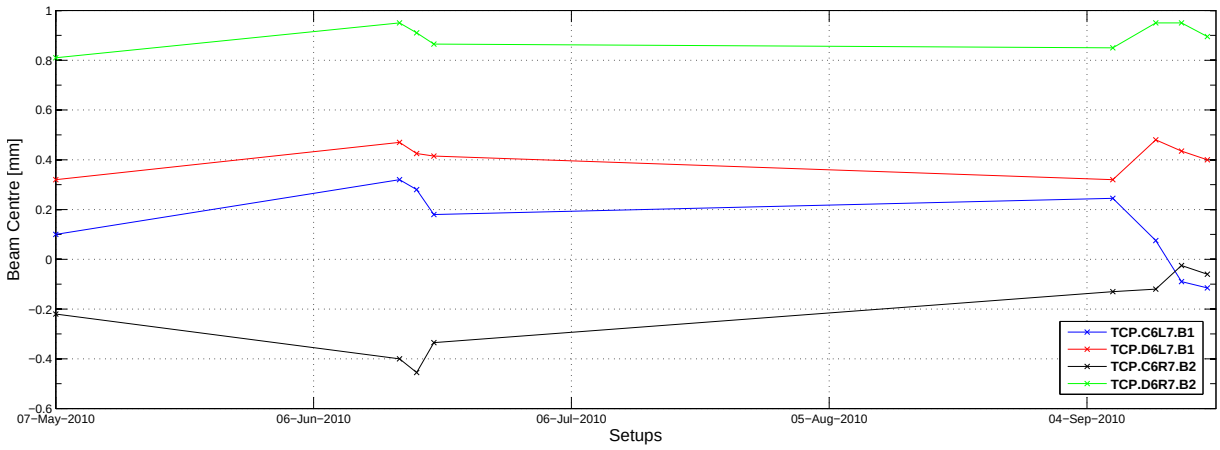
The differences between the nominal and the measured beam sizes can indicate the accuracy of the alignment, quality of the optics correction or misalignment angles of the collimator jaws or the tanks housing the jaws themselves. However, this is true only if certain machine parameters remain constant, such as the $\beta$-beat, which is the error in the optical $\beta$-function with respect to its design value. The proximity of the measured beam size to the nominal beam size can hence be expressed as the ratio of the two parameters. Fig.(7) shows the beam size ratios at each collimator for alignments occurring in the 2010-2012 LHC runs. The alignments were performed at injection energy (450 GeV) and top energy (3.5 TeV in 2011 and 4 TeV in 2012).

The beam size ratios in IR3 are generally larger than 1. This could be due to the fact that there is a high dispersion in IR3, which means that, independent of the alignment, the small energy errors on all particles give a significant contribution to the measured beam size. The tanks of three collimators having a larger beam size ratio than expected (TCLA.A7R7.B1, TCTH.4L2.B1 and TCSG.A5L3.B2) were re-aligned in the tunnel in after the 2011 alignments (Valentino *et al.* 2012a), and the effect on the beam size ratio is visible in the values obtained in 2012.

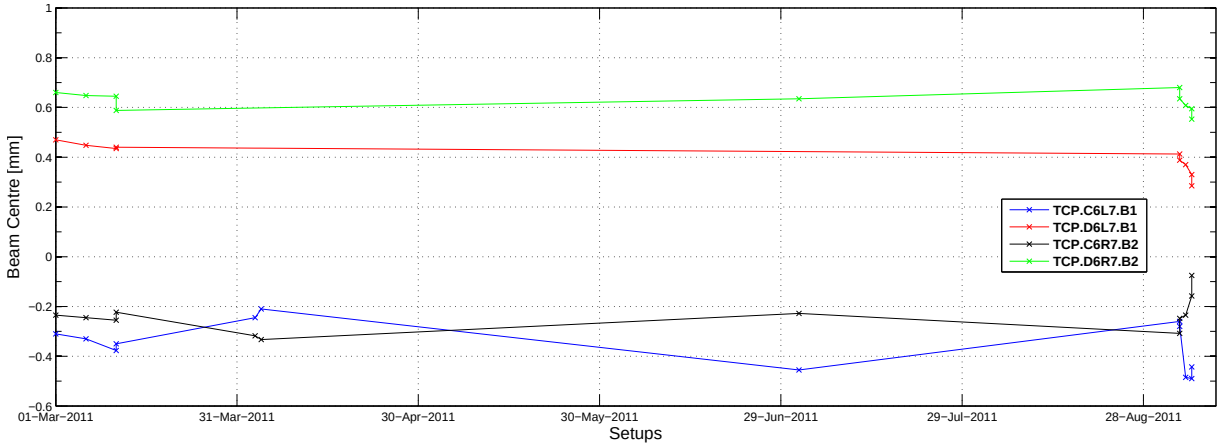
## 4.3 Orbit stability at the TCPs

The horizontal and vertical primary collimators in IR7 are the collimators that are aligned most frequently during the year, being the reference collimators used to align other collimators. The beam centres measured at the TCPs during all alignments held in the 2010-2012 period are shown in Fig.(8). The reference beam orbit at the IR7 TCPs is not changed throughout the year, unlike in the experimental regions, and hence is expected to remain constant. However, orbit drifts can occur due to various effects, including ground motion and the ambient temperature in the tunnel (Steinhagen, 2007).

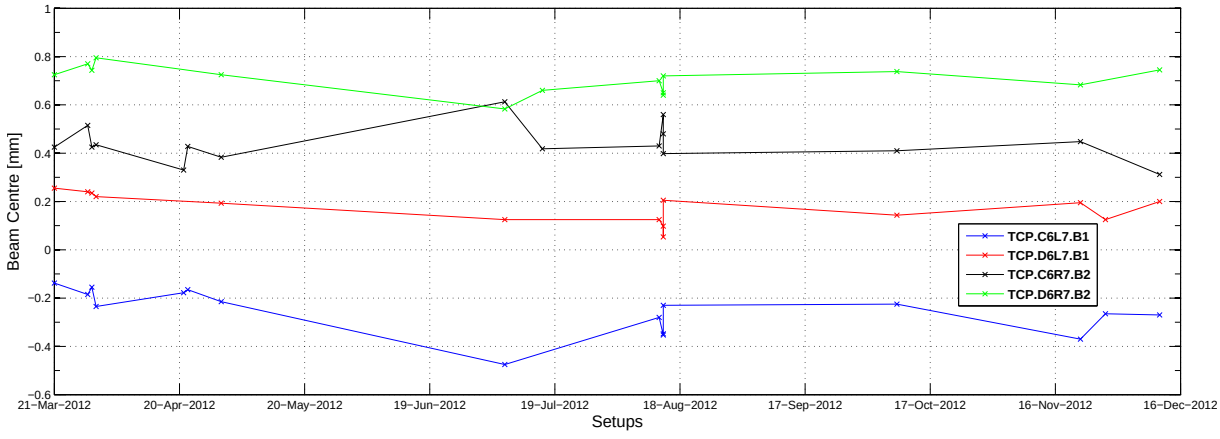
Certain patterns are noticed in the data. For example, there appear to be correlated shifts in the measured centres in one plane or one beam. This could be the effect of systematic misalignments of the quadrupole magnets over time. The variations in the orbit are of the order of a few hundred micrometers, which can be attributed to various effects described above.




(a) TCP measured centres in the 2010 alignments.




(b) TCP measured centres in the 2011 alignments.




(c) TCP measured centres in the 2012 alignments.

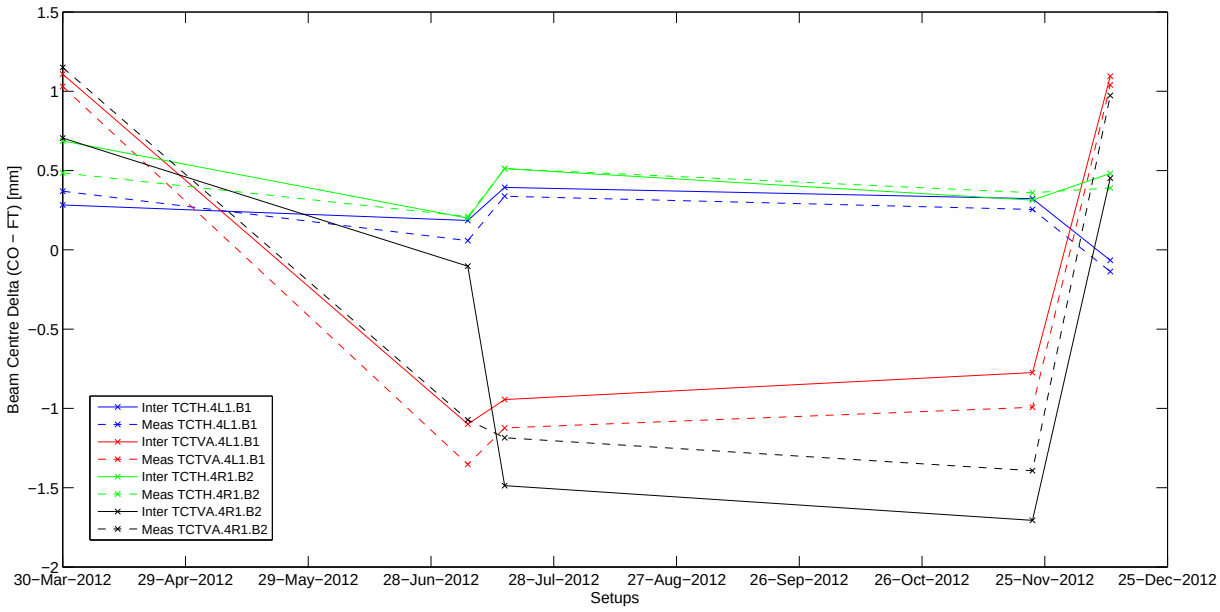
Figure 8: Beam centres measured at the primary collimators in beam-based alignments from 2010 to 2012.

## 4.4 Orbit changes at the TCTs

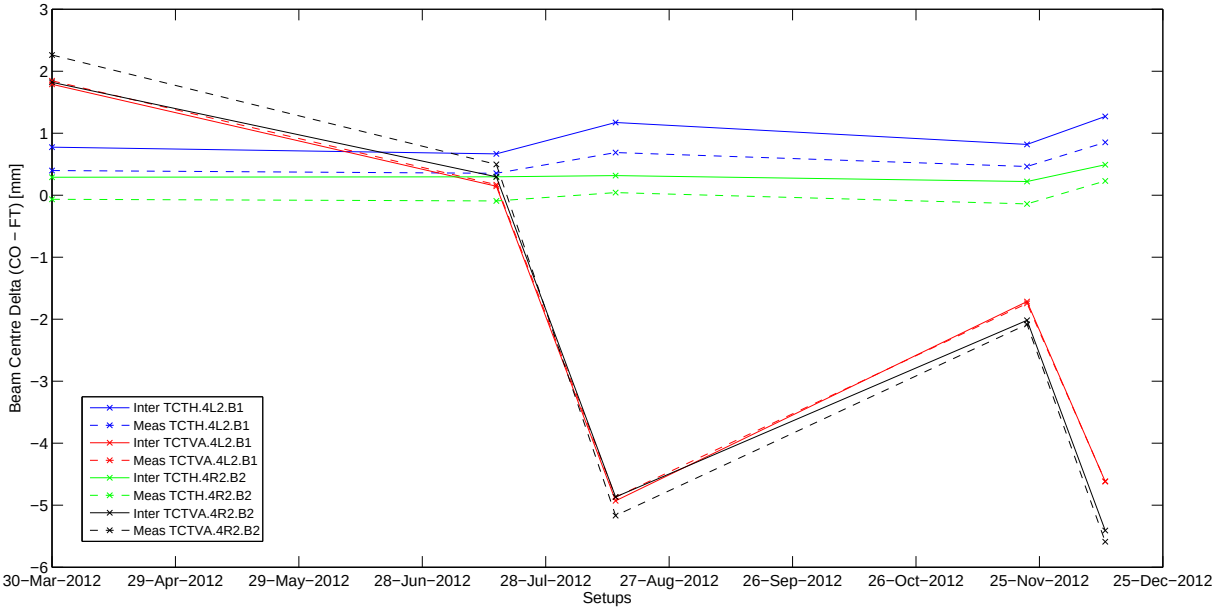
The TCTs need to be re-aligned whenever the orbit or optics configurations at the experimental IPs are changed. Table I lists the configuration changes performed for the collisions beam process in the 2012 LHC run.

**Table 1: Configuration changes performed for the collisions beam process in the 2012 run.**

| **Date** | **Reason for Alignment** | **Crossing angle $[\mu rad]$ in IP1V / 2V / 5H / 8H / 8V** | **Optics $[m]$ in IP1/2/5/8** |
|---|---|---|---|
| 30/03/2012 | Start of run | -145 / -90 / 145 / 0 / 90 | 0.6 / 3.0 / 0.6 / 3.0 |
| 07/07/2012 | $\beta^* = 90m$ | 0 / -90 / 0 / -220 / 0 | 90 / 10 / 90 / 10 |
| 16/07/2012 | Van der Meer scans | 0 / -90 / 0 / 200 / 0 | 11 / 10 / 11 / 10 |
| 22/11/2012 | Van der Meer scans | 0 / 145 / 0 / -220 / 0 | 11 / 10 / 11 / 10 |
| 11/12/2012 | 25 ns bunch spacing | -145 / 145 / 145 / 220 / 0 | 1 / 3 / 1 / 3 |



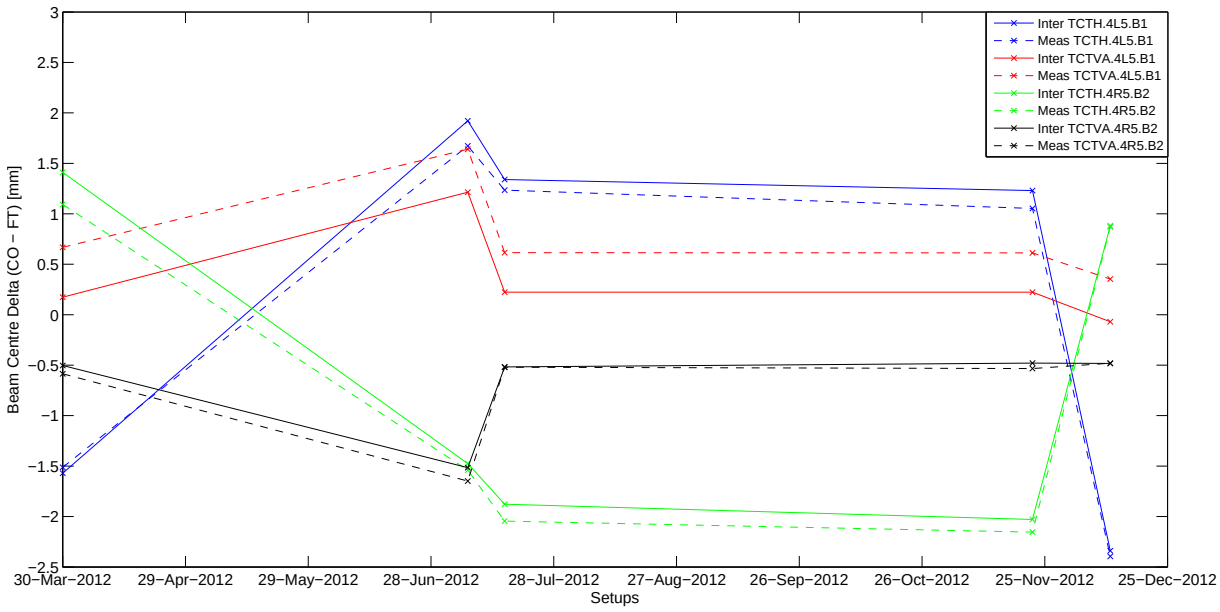

(a) Interpolated and measured beam centre deltas at the TCTs in IR1.



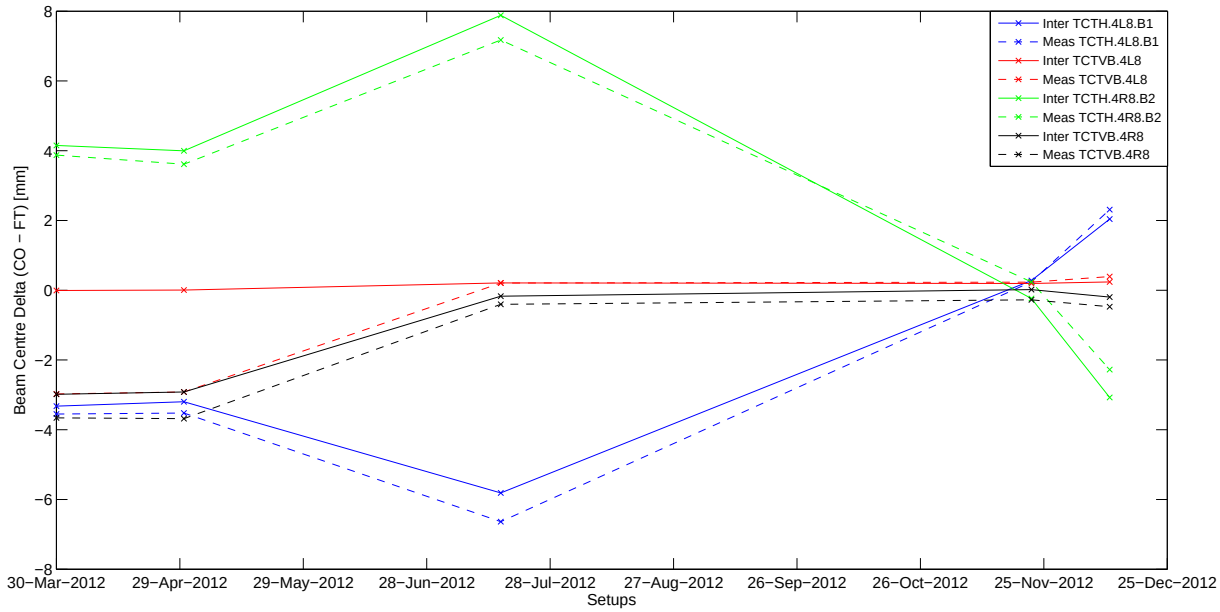

(b) Interpolated and measured beam centre deltas at the TCTs in IR2.

In this analysis, the BPM-interpolated orbit at the TCTs is extracted using the LHC Aperture Meter (Müller *et al.* 2011). As the absolute interpolated orbit is poor at the TCT locations due to errors introduced by BPMs in the IRs (Valentino *et al.* 2012e), the change between the interpolated orbit at flat top (FT) and collisions (CO) were compared with the change in the measured orbit at the same operating points in the machine cycle. The comparison results are shown in Fig.(9), with separate plots for the TCTs in each IR. A very good comparison is observed between the measured and the interpolated orbit changes between FT and CO.

As expected, the beam centres at the TCTs shown in Figs.(9)(a-d) change as a function of the crossing angles in Table I. For instance, van der Meer scans were performed on the 16/07/2012 and on the 22/11/2012.




(c) Interpolated and measured beam centre deltas at the TCTs in IR5.




(d) Interpolated and measured beam centre deltas at the TCTs in IR8.

Figure 9: Comparison of the deltas in the measured and BPM-interpolated orbit at the TCT collimators between collisions and flat top in the 2012 LHC run.

In between these dates, the IP2 vertical crossing angle was changed from -90 µrad to 145 µrad, while the IP8 horizontal crossing angle was changed from 200 µrad to -220 µrad. The beam centres measured on the 22/11/2012 at all TCTs, except the IP2 TCTVs and the IP8 TCTHs, remained the same as the values measured on the 16/07/2012 (within 100 µm). A direct comparison between the measured centres and the crossing angles would require inclusion of other effects such as luminosity orbit bumps, and is beyond the scope of this paper.

# 5 Conclusion

The Large Hadron Collider is passively protected against potentially destructive particle losses by a collimation system. The required jaw positions to establish an efficient four-stage hierarchy are determined via a beam-based alignment procedure. This article presented the operational results achieved with collimator alignment during the first few years of LHC operation. The

various algorithms introduced to automate the alignment procedure have had a significant impact, reducing the beam time required for a full alignment by more than a factor 6. Other alignment statistics, including the number of collimators aligned and the number of beam dumps per year, were presented. The similarity in the beam centres measured at subsets of collimators is an indication of the excellent reproducibility and stability of the LHC.

# 6 Acknowledgements

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*Review Article*

# Serotonin, how to find it...

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**Abstract.** Serotonin or 5-hydroxytryptamine (5-HT) is a monoamine neurotransmitter. Biochemically derived from tryptophan, serotonin is primarily found in the gastrointestinal tract, platelets, and in the central nervous system (CNS) of animals, including humans. Discovered and crystallized over sixty years ago, serotonin operates as a short-range neurotransmitter as well as a long-range signalling modulator, with multiple effects on whole organism functions via plasma, platelet and neuroendocrine, gut, adrenal and other peripheral systems across many species. All of the important functions of serotonin in the brain and body were identified over the ensuing years by neurochemical, physiological and pharmacological investigations. Mainly, all these investigations have been performed via invasive methodologies, particularly in the CNS studies. Here we present a rapid overview of such methodological approaches focussing on voltammetry, one of the most recent technical approaches for serotonin analysis in vivo, in situ and in real time. Furthermore, we introduce a late technical evolution in the attempt to obtain in vivo non invasive measurement of brain serotonin.

*Abbreviations*

Serotonin (5-hydroxytryptamine; 5-HT); central nervous system (CNS); 5-hydroxy-indol acetic acid (5-HIAA); carbon fibre micro electrodes (μCFE); differential pulse voltammetry (DPV); direct current amperometry (DCA); light-induced fluorescence excitation (L.I.F.E.); Near Infrared Spectroscopy (NIRS); ultraviolet (UV); selective serotonin reductase inhibitor (SSRI)

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# 1 Introduction

## 1.1 Serotonin

Serotonin (5-hydroxytryptamine; 5-HT) was initially discovered as a vasoconstrictor substance in blood and later in blood vessel walls, platelets and in enterochromafine cells of the gastrointestinal system, the lungs and the heart (Vialli et al. 1933; Rapport et al. 1948; Peroutka et al. 1994). More than 50 years ago the chemical structure of 5-HT was identified and synthesised (Twarog et al. 1953). Later, the function of 5-HT as a neurotransmitter in the CNS was proposed (Bogdanski et al. 1956) and has been studied intensively since its identification in the pituitary gland (Hyyppa et al. 1973).

In the central nervous system (CNS) serotonin is synthesised in the perikarya of the neuron where the diet amino acid tryptophan is hydroxylated to the 5-HT precursor 5-hydroxytryptophan (5-HTP), which is then decarboxylated to 5-HT (Hamon et al. 1982). To avoid immediate enzymatic oxidation to 5-hydroxy-indol acetic acid (5-HIAA) by monoamine oxidase, 5-HT is contained in neuronal vesicles until it is released into the synaptic cleft. Serotonin then activates either post-synaptic or pre-synaptic receptors or undergoes reuptake via the 5-HT transporter molecule (Sert) into the neuron (Hamon et al. 1982).

Serotonin is involved in regulation of the central neuroendocrine system as well as cognitive functions, mood and basal physiological functions (Van de Kar 1991). Dysfunction of the intra- and interneuronal 5-HT transmitter systems may result in impairment of coping with states of increased stress, cognitive dysfunction and eventually mental diseases (Graeff et al. 1996; Roth et al. 2004). Furthermore, the 5-HT system is involved in regulation of gastrointestinal function and in the development of diseases such as migraine, obesity and nausea

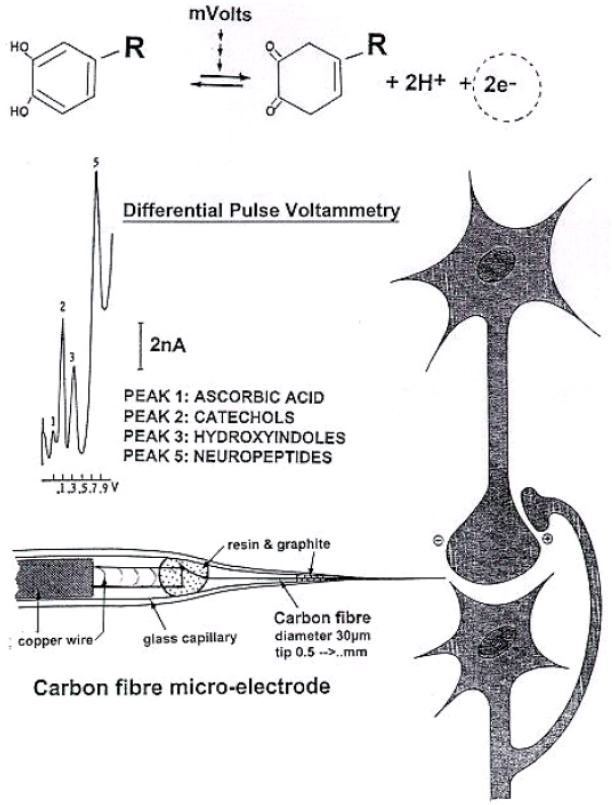


Figure 1: Voltammetry: top: principle, middle: the voltammogram obtained in vivo in the CNS when using DPV together with µCFE; bottom: schematic drawing of the µCFE and its theoretical positioning within the brain tissue [not to scale].

(Meguid et al. 2000; Saxena 1995).

### 1.2 5-HT distribution

5-HT distribution in the brain is diffuse and complex. The location of 5-HT containing cell bodies in the CNS was first shown by Dahlstrom et al. (1964) using the fluorescent histochemical technique introduced by Falk et al. (1962). In 1981, an extremely detailed mapping of 5-HT pathways in the CNS was performed using a specific immunohistochemical technique (Steinbusch 1981).

### 1.3 5-HT cell bodies

The majority of cell bodies lie within the brain stem and mesencephalon in nine groups labelled B1 to B9. Cell bodies B1 to B4 and B6 are located in the medulla. The B1-raphe pallidus, B2-raphe obscurus, B3-raphe magnus and B4/B6-region exsisting under the 4th ventricle. Group B5-cell bodies lie within the raphe dorsalis. Finally, groups B8 and B9 are located in the mesencephalon and lie within the raphe medianus and lemniscus medialis respectively (Steinbusch 1981). 5-HT cell bodies have also been reported by various authors in the locus caeruleus and sub-caeruleus.

### 1.4 5-HT pathways

5-HT nerve terminals extend throughout the brain and spinal cord (Steinbusch 1981). Briefly, the 5-HT innervation of the brain can be divided into two main ascending axon bundles termed the medial and lateral ascending pathways (originating from cell bodies in B-5-6-7-8-9) and descending bulbo-spinal neurones (originating from the raphe nuclei of the medulla oblongata). The main brain areas innervated by 5-HT neurones include the thalamus, hypothalamus, subthalamus, olfactory areas, cortex, basal ganglia, septum, hippocampus and substantia nigra.

## 2 Methods

### 2.1 5-HT release

In vitro studies of 5-HT release are limited by the rapid re-uptake of released 5-HT, necessitating the presence of an uptake inhibitor in the incubation medium (Blackburn et al. 1967) and can only give an approximation of actual in vivo amine levels as the complex cellular and sub-cellular events in tissue cannot be duplicated in vitro.

The first notable in vivo techniques developed for in vivo analysis of 5-HT release are the technique of "push-pull cannulae" (Gaddum, 1961) a perfusion -based technique, as well as "intracranial microdialysis" (Ungerstedt, 1984). These are two similar methods with major limitations, briefly: i] excessive dimension of the probe thus limiting the analysis to large brain areas with tissue damage (Yaksh et al. 1974; Khan et al. 2003); ii] all perfusion techniques make an indirect measurement of release as they measure diffusion and not strictly release, with the chemicals collected being measured "off line". These methods also have quite a slow response time, 10-30 min is needed to collect a volume of perfusate great enough to allow the detection of chemicals within it. Their detection is thus not performed in "real time" with, in consequence, a poor correlation between changes in their extracellular levels, neuronal activity and behaviour.

An alternative method is voltammetry: in 1924 Heyrovsky found that the current at a mercury electrode was not directly proportional to the applied voltage, but that there was the presence of an extra-current determined by the oxidisable chemicals present in the solution. Such extra-current, that is proportional to the concentration of the compound(s) oxidised and/or reduced, is called polarographic current when obtained at a mercury electrode, and voltammetric current when obtained at all other types of electrode (Adams 1969 a,b). Different types of voltammetric techniques are available, the most common of which are chrono-amperometry linear voltammetry, cyclic voltammetry, and pulse voltamme-

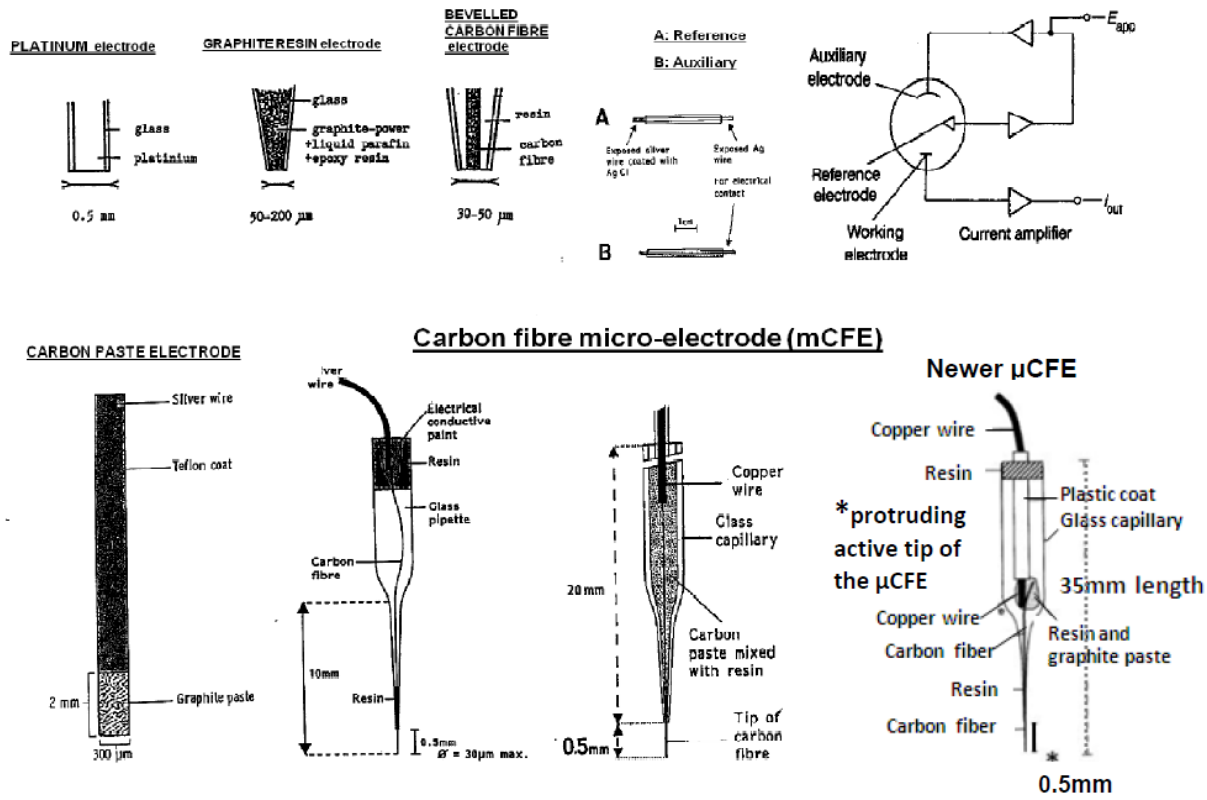


Figure 2: Various types of voltammetric electrodes developed since the 1970s, from the first ones made of platinum wire or teflon coated silver wire to the carbon paste based electrodes and finally to the carbon fibre electrodes (for a review: Stamford et al. 1992).

try. These methodologies are mainly based on the application of a "dynamic" oxidation or oxido-reduction potential and the resulting analysis of electrons "freed" by the chemical(s) under analysis (Fig.(7).

Different types of voltammetric electrodes have been developed since 1969, the most successful type appears to be carbon based electrodes and in particular the carbon fibre micro electrodes (µCFE) see Fig.(1).

## 2.2 In vivo voltammetry

An advanced approach to monitoring changes in monoamine release and their metabolism is the technique of in vivo voltammetry using micro biosensors, mainly carbon-fibre micro electrodes (µCFE) with a 7 to 30 micrometer diameter (see Figs.(1, 2, 3)). The method fulfils many of the criteria required to monitor specific compounds in the extracellular fluid:

I- Measurements can be made in the extracellular fluid of specific large as well as small brain nuclei with minimal damage to the nervous tissue and disturbance to the animal due to the small dimensions of the probe (see Figs.(1, 3)), which can sample an area of approximately 10-6mm$^3$. Thus there is a clear high anatomical resolution of the site of measurement within discrete brain regions of rodents. Furthermore, no signs of the presence of the micro electrode could be observed in the brain tissue when the histological evaluations correct (or incorrect) location of its active tip, within the brain region studied, was performed under light microscopy at the end of each experiment when the brain was rapidly removed and sectioned using a cryostat. This indicates that the sole insertion of µCFE into the brain does not produce tissue lesions detectable by light microscopy. Indeed, a lesion (coagulated brain tissue) was necessary and was obtained at the end of each experiment via application of a direct current through the active tip of the µCFE to verify such position under light microscopy in brain slices stained using the NISSL solution. II- The method allows rapid, repeated measurements with accurate time resolution in vivo, in situ in real time without recourse to perfusion based techniques or radiolabelled transmitter stores, or the need for sample preparation or chromatographic separation. This is the fundamental difference between voltammetry and perfusion techniques (Crespi et al. 1988; Stamford et al. 1992).

III-In vivo voltammetry can be performed in conscious freely moving animals. This, combined with points I and II, avoids the problems associated with anaesthetics and allows correlations within neuronal activity behaviour. Furthermore, recent improvement in the methodology allow voltammetric analysis in telemetric – wireless conditions allowing electrochemical studies in absolute freely moving conditions (Crespi 2010a) (see Fig.(3)). Indeed, the enhanced telemetric system based

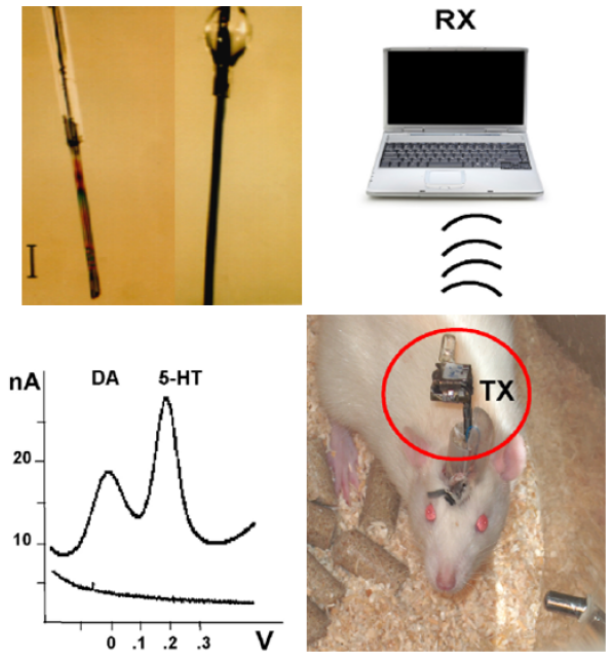


Figure 3: In vivo voltammetry in conscious freely moving conditions
**Top left**: magnification of the electro-active tip of the µCFE before (right) and after Nafion®coating (left). Bar: 100µm.
**Bottom right**: the behaving freely moving rat with the transmitter unit TX placed upon its head transmitting the signal via an infrared channel to the receiving station (RX). The TX is a miniaturised circuit (dimension of about 0.5cm$^3$) of much reduced weight (5–6g). It is mounted on the animal's head and connected to the voltammetric three-electrode system inserted into the brain. A laptop computer, interfaced to the receiving station, performs the required signal acquisition and analysis (top right).
**Bottom left**: representative in vitro DPV curves for dopamine 5µM and serotonin 5µM as measured with Nafion®-µCFE (Crespi et al. 1988). In vivo studies, the tip of the Nafion®-µCFE is inserted in a discrete brain area, i.e. the rat prefrontal cortex as shown by the red arrow in E. [With permission from Biosensors and Bioelectronics].

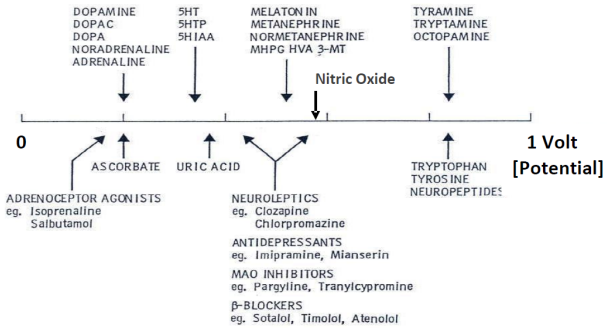


Figure 4: The figure shows different compounds presenting electro-active chemical groups so that they can be measurable with voltammetry.

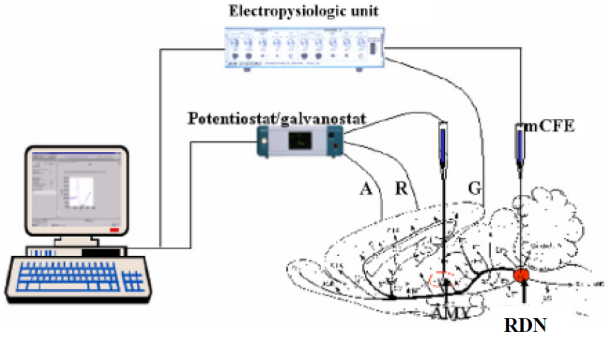


Figure 5: The voltammetric-electrophysiological set up consists of the concomitant use of a potentiostat three-electrode system (A: auxiliary, R: reference and the µ CFE) and an electrophysiological two-electrode unit (G: ground electrode and the µ CFE). Electrodes A, R and G were inserted between the bone and the dura mater through holes (200µ m diameter) that were drilled in the parietal bone. The µ CFEs were inserted in the specific brain regions studied (i.e.amygdale [AMY] and raphe dorsalis nucleus [RDN], respectively) under light microscopy. All electrodes were connected with electrical wires to the polarograph or the electrophysiological apparatus driven by specific softwares (GPES Ecochemie, The Netherlands or MSD Alpha-Omega, Israel, respectively).

on either differential pulse voltammetry (DPV) or direct current amperometry (DCA) "diffused" via a single-way infrared (IR) transmission channel is of much reduced dimension and weight. In particular, parallel in vivo experiments in rats prepared for classical wire-connected DCA or for wireless DCA resulted in superimposed data when the 5-HT system in the frontal cortex was challenged with fluoxetine (Crespi 2010a).

## 2.3 Biogenic amines or metabolites?

As introduced above, voltammetry in association with specifically prepared micro-biosensors detects the cerebral extracellular content of neurotransmitters and respective metabolites.

Pharmacological experiments have indeed demonstrated that ascorbate, noradrenaline and/or dopamine (and its metabolite DOPAC), 5-OH-indoles (i.e. serotonin and its metabolite 5-HIAA) (Crespi 1990; Crespi et al. 1988) uric acid, (Crespi et al. 1983b) homovanillic acid, 3-methoxytyramine (Crespi et al. 1989b) as well as neuropeptides containing electroactive amino acids such as tryptophan, cysteine, tyrosine, (Crespi 1991, Crespi 2011) melatonin and (Crespi 2012; Crespi et al. 1994) nitric oxide (Crespi et al. 2001; Rossetti et al. 2004), can be selectively monitored with this methodology.

At one stage it was thought that in vivo voltammetry could be used to study BIOGENIC AMINES such as DA, 5-HT release directly in situ and in real time (Adams et al. 1978; Marsden et al. 1979). Later on, however, it was determined that the voltammetric signal in vivo was mainly correlated to the oxidation of extracellular metabolites (Crespi et al. 1984) (see Fig.(4)).

The development of a new voltammetric biosensor (the Nafion carbon fibre micro-electrode described in Fig.(3)) made it possible to directly measure the release of 5-HT in vivo (Crespi et al. 1988).

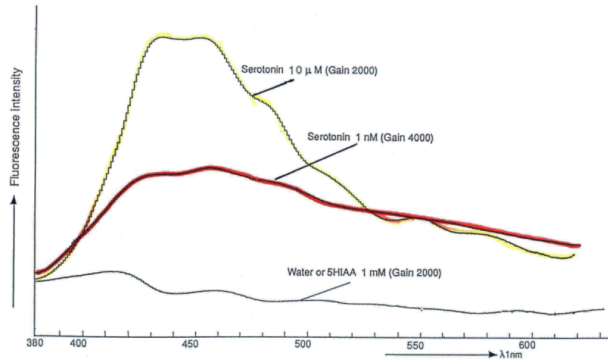


Figure 6: Fluorescence intensity induced by LASER excitation of solutions containing 5-HT nanoM or microM concentrated and 5-HIAA milliM concentrated versus water.

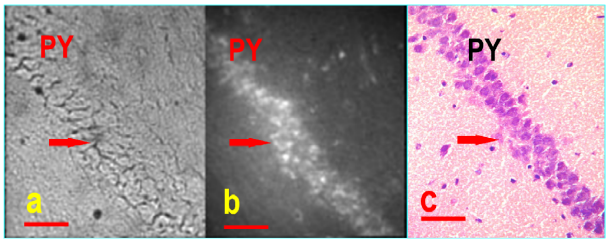


Figure 7: Typical histological pattern of the hippocampus containing the pyramidal cell layer:
(a) Pyramidal 5-HTcells (PY): non stained section without excitation AND
(b) Under excitation at 366nm, resulting in a similar pattern as in (c):
(c) Pyramidal 5-HTcells (PY) stained with hematoxilin eosin, bar=90µm.
Arrows indicate the same histological position.

## 2.4 Parallel electrochemical [voltammetric] and electrophysiological in vivo studies

It has also beenshown that voltammetric analysis of neurotransmitter release and metabolism can be coupled with analysis of vigilance states via coupling EEG, EOG and EMG recordings with electrochemical [voltammetric] measurement. In particular, this approach has been very valuable for studying the putative relationship between sleep-awake circadian rhythm and serotonergic activity in the raphe regions of rodents; i.e., demonstrating a direct relationship between sleep-awake circadian rhythm and release of 5-HT in the raphe nuclei (Crespi and Jouvet 1982), the brain region involved in the regulation of such sleep-awake circadian rhythm (Jacobs and Azmitia 1992; Ursin 2002).

The feasibility of concomitant in vivo recordings of electrophysiological signals such as cell firing and voltammetric measurements of unstimulated levels of extracellular compounds has also been demonstrated either with these two independent techniques combined in vivo at a single electrode (Crespi 2002) or with parallel recordings in cell bodies; i.e., dorsal raphe nucleus [electrophysiology] and voltammetric recordings in related

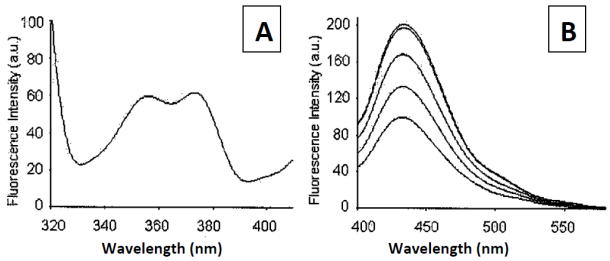


Figure 8: **Left**: Excitation spectrum obtained in the rat hippocampus homogenate following addition of exogenous 5-HT $1 \times 10^{-4}$M and after subtraction of the initial spectrum recorded in the untreated hippocampus homogenate (0.5mg proteins/ml). **Right**: Trend of the emission spectra (excitation 366 nm) monitored in the hippocampus homogenate of untreated rat following addition of exogenous 5-HT at concentrations $5 \times 10^{-6}$M, $5 \times 10^{-5}$M, $5 \times 10^{-4}$M and $1 \times 10^{-3}$M, respectively. Lowest curve refers to the homogenate before 5-HT addition.

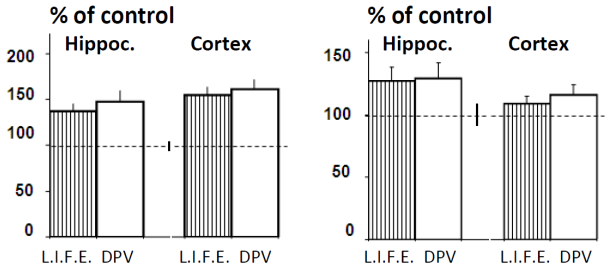


Figure 9: Concomitant fluorescence intensity (L.I.F.E. at 420-470nm range) and DPV levels of endogenous 5-HT measured in homogenates from the hippocampus or cortex obtained from rats submitted to chronic fluoxetine (Left) or chronic stress (Right) treatments. Data are expressed in percent of values from control homogenates. Overall, no statistical differences were obtained when comparing DPV results versus L.I.F.E. data using either paired or unpaired T-Test or univariate test of significance over the parameterised model (for further details see Crespi et al. 2004).

terminal regions or in the amygdala (Crespi 2009)(see Fig.(5)). This verifies the original proposal that a combined treatment with a potassium (SK) channel blocker such as apamin and fluoxetine could overcome the slow onset of the SSRI upon central 5-HT activity that could be related to the slow onset of its therapeutic antidepressant action (Crespi 2010b). Electrochemical and behavioural evidence of a direct relationship between cerebral 5-HT and cytoskeleton in the control of mood was also confirmed (Crespi 2010c). Concomitant behavioural and voltammetric analysis could also be performed, proposing that divergent central serotonergic activities may be responsible for either despair or learning behaviour in intact Wistar or Sprague-Dawley CD rats, respectively (Crespi 2010d)

## 2.5 Serotonin measurements: from invasive to non – invasive approaches

In 1962, Udenfriend showed that serotonin can act as a fluorophore as its light-absorption and emission properties in aqueous solution occur in the near-UV-visible region.

In 1979, Aubin showed the existence of an auto-

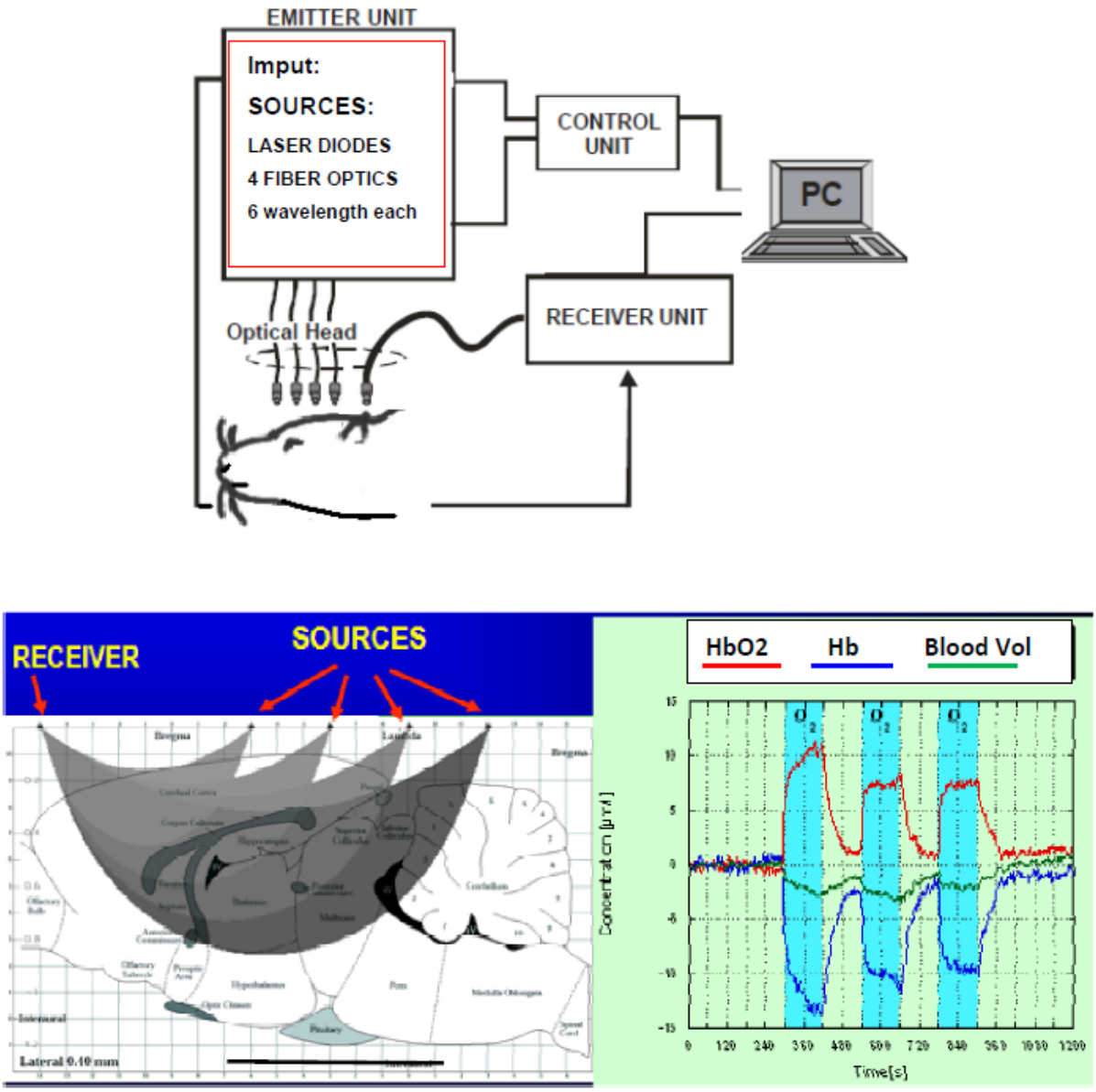


Figure 10: In vivo NIRS: **Top**: block diagram of the NIRS prototype applied to rodents.
**Bottom left**: brain areas monitored using four sources and one receiver: computer simulation of photon paths based on photon migration theory.
**Bottom right**: changes of physiological parameters following pure oxygen administration (for further details see Crespi 2007).

fluorescence emission in the UV-visible interval when biological tissues were submitted to suitable light stimulating conditions.

Based upon this evidence, in 1990 Crespi had proposed the use of light-induced fluorescence excitation (L.I.F.E.) in the attempt to selectively monitor neurotransmitters based upon analysis of their own fluorescence. This approach demonstrated that the autofluorescence properties of a neurotransmitter such as serotonin can be selectively evaluated (see Figs.(6, 7)). Then L.I.F.E. spectroscopy was performed in ex vivo and in vivo experiments: it was observed that serotonin exhibited the well known excitation and emission bands in the UV region (i.e. 250-320nm) and also other minor excitation and emission bands in the near UV region (i.e. 450-580nm). Furthermore, spectrofluorimetric measurements under 366nm excitation performed on solutions supplied with serotonin or on brain homogenates obtained from control or specifically treated rats, were consistent with those obtained in parallel experiments using in vitro or ex vivo voltammetry (see Fig.(8)), therefore proposing the application of in vivo L.I.F.E. Indeed, in vivo L.I.F.E. studies performed in situ and in real time by means of 50µm diameter optic fiber stereotaxically implanted in discrete brain areas of anaesthetised rats, again resulted consistent with

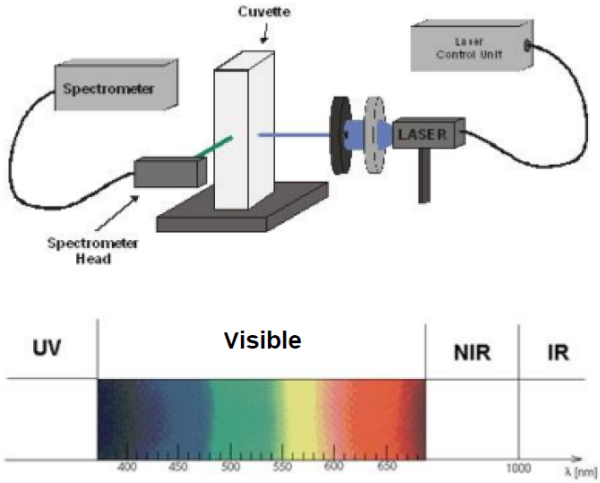


Figure 11: Set-up of instrumentation for in-vitro fluorescence measurements and Laser source spectrum: from near IR to near UV.

parallel in vivo voltammetric experiments performed in rats treated with chemicals selectively affecting the 5-HT system (Crespi et al. 2004) (see Fig.(9)).
However, this methodology as well as in vivo voltammetry, remains invasive.

In the attempt to overcome invasiveness, Near Infrared Spectroscopy (NIRS) methodology was applied: by means of prototype instrumentation for analysis in small rodents (Fig.(10)). This technique allows non invasive in vivo preclinical studies of CNS metabolic functions via direct measurement of oxyhaemoglobin and deoxyhaemoglobin (Crespi 2007). In addition, it permits the assessment of real time brain penetration and efficacy of drug treatments (Crespi et al. 2006).

Therefore NIRS permits (as well as MRI) translational strategy from preclinical to clinical investigations.

## 2.6 Laser source spectrum: from near IR to near ultraviolet (UV)

Based on NIRS (i.e. non-invasive laser based methodology) is the actual attempt to use NON invasive spectroscopy to analyse neurotransmitter in the rat CNS. The technical principle is the same described for the NIRS study of brain metabolism, the main difference related to the source-receiver system needed for detecting natural or induced fluorescence of endogenous chemicals acting as neurotransmitters such as serotonin. For this purpose, other types of sources have been taken into consideration and in particular UV laser sources have been selected such as the Hamamatsu M8903-01 that is a pico-second light pulser with wavelength of 402 nm (near UV wavelength spectrum: 300 to 400nm). It has been combined with the Hamamatsu spectrometer H8353 and a dedicated optical set-up (see Fig.(11)). This source has first been tested using fluoresceine, a compound known to have a spectrum of fluorescence within the green band of the visible wavelength; i.e. 480-560nm.

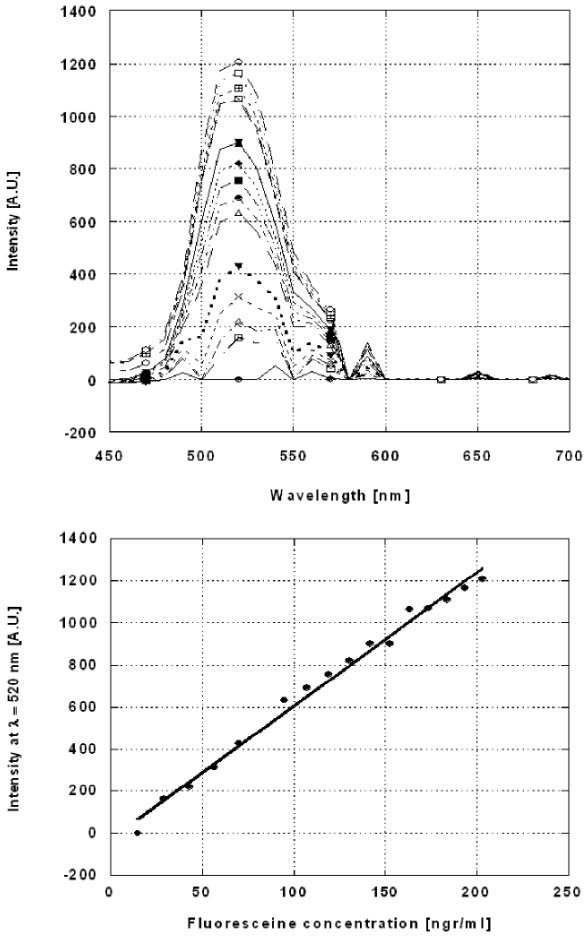


Figure 12: **Top**: Fluoresceine emission spectra obtained with fluoresceine tested at various concentrations: emission intensity peak [arbitrary units, U.A.] is monitored at 520nm;
**Bottom**: Experimental point of revealed intensity at 520nm as a function of fluoresceine concentration.

To do so the Hamamatsu spectrometer H8353 has been completely characterized using the optical set-up shown in Fig.(11), in particular the cuvette contains a solution with different concentrations of fluoresceine: from 10microM up to 100microM in order to test the linearity of the receiving unit. The output beam of the laser source has been collimated by means of a bi-convex lens with focal length f=10 mm. Emission spectra of these fluoresceine concentrations are shown in Fig.(12) TOP, confirming the linearity of the receiving unit. The emission intensity peak was obtained at 520nm and it appeared that the increase in the emission intensity is related to fluoresceine concentration as assessed in Fig.(12) BOTTOM, where the linearity error is 3.19% full-scale.

Based on the L.I.F.E. spectroscopy observations reported above showing that serotonin exhibited also other minor excitation and emission bands in the near UV region, i.e. 450-580nm, experiments were performed in the same conditions described for fluoresceine. The preliminary data gathered, to be confirmed in further

studies, indicate that 5-HT could be also monitored with this set-up within the blue region. Different 5-HT concentrations were tested (1, 10 and 100 microM) and data lead to meaningful differences in the intensity of the fluorescence spectra.

# 3 Concluding Remarks

In conclusion, non-invasive laser based NEAR UV methodology is introducing very promising results on the attempt to analyse auto-fluorescent neurotransmitters, such as serotonin, using optic fibres in vivo and in non-invasive conditions.

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*Review Article*

# The Pygmalion-Galatea myth in relation to simulation scenarios in Star Trek

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**Abstract.** Star Trek has existed since 1966, with a total of 738 hours of viewing time. Like most science fiction, the series frequently alludes to religion or myth but censors such references for the modern world-view by sanitising them with scientific or scientific-sounding explanation. This paper illustrates the depiction of the Galatea-Pygmalion myth in the franchise and relates these to simulation scenarios.

## 1 Introduction

Star Trek (ST) has been with us since 1966, and comprises a vast corpus of works that include twelve films and six series (one of which is animated). This has resulted in a total of 738 hours of viewing time.

Naturally, due to the sheer number of episodes and movies, a tremendous variety of narrative plots have been invoked. These often directly or indirectly allude to religions and myths. However, all such occurrences and references are sanitised with scientific references and explanations, so as to somehow fit in with the modern world view. This viewpoint eschews scenarios that are devoid of rational explanations, such as enchantment and magic.

Darko Suvin defined the science fiction (SF) genre as "the literature of cognitive estrangement", wherein there is the addition of one or more futuristic and scientific elements or premises to narratives. Suvin referred to this as "a strange newness, a novum" (Suvin, 1972). For these reasons, SF is often encouraged by teachers as a means of generating interest in science (Dubeck et al., 2004).

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ST accedes to this notion by attempting to explain future scientific advances leading to the seemingly impossible. Typical examples include travelling faster than light and other technological miracles, thereby eliciting a sense of wonder.

In all of these ways, the franchise provides an interface between literature and other metanarratives, such as the sciences. Indeed, entire books have been written on the physics portrayed in ST (Krauss, 1995). Thus, and to quote just one example, the notion of the instantaneous matter transporter has been explored in extensive depth. This investigation details accidents that ST has documented resulting from the utilisation of such exotic technology, the physics of the reality of what the usage of such a device would entail and the Lockeian concept of physical continuity (Grech, 2011).

John Locke considered personal identity and selfhood to be caused by consciousness and memory and not by the body or the soul. Locke insisted that personal identity is a matter of psychological continuity and that each one of us is born as a blank slate, a *tabula rasa*. We are then shaped by experience, and our identities are defined by our continued consciousness. This is the view adopted by the series, a Lockeian perspective that allows consciousness to be transferred into different vessels (Locke, 1869). Spock, the Enterprise's science officer, directly alludes to this when he discovers that Captain Kirk's consciousness has been displaced into a female body by exotic alien technology: "whatever it is that makes James Kirk a living being special to himself is being held here in this body" (Wallerstein, 1969).

Many other branches of knowledge are invoked in ST, and a few examples including philosophy (Grech, 2013a), metaphysics (Grech, 2013b), psychology (Grech, 2012a) and questions about the very nature of what it is to be human (Grech, 2012b). Like the physics of ST, several of these have also been explored in book length.

For example, philosophy in ST has been reviewed comprehensively, demonstrating that this branch of knowledge shares much with SF in general: the same fundamental purpose of exploration, a relentless roaming of the universe in an attempt to chart and understand our place and purpose within it (Eberl and Decker, 2008).

The related field of ethics in ST has also been scrutinised, establishing the myriad ways in which this franchise (which has become an integral part of popular culture) has engendered sophisticated discussions of ethical theories within episodes. These include virtue ethics (such as ethical reasoning), religion and Christian ethics, Stoicism, and ethics related to notions of duty. Other viewpoints are also outlined, such as logic and utilitarianism, hedonism and existentialism, thereby enlivening episodes while imparting fruit for thought (Barad and Robertson, 2000).

This is arguably one reason why the franchise has endured these many decades, since most of the stories are morality tales, moral fables that have been sanitised by science so as to make them suitable for the contemporary climate.

It has also been shown how ST, despite its attempt to stand on the high ground afforded by science and logic, borrows heavily and continuously from culture. The episodes characters therefore seek artistic expression, the listening to and the actual performance of classical music, and the frequent use of literary quotations and graphic images that directly accede to the humanities (Kreitzer, 1996).

This paper will illustrate the way in which ST renarrates myths, through the artifices of science through one specific example. The method will be the evaluation of the different ways in which the Galatea-Pygmalion (GP) myth has been depicted in the franchise.

This ancient Greek myth was penned by Ovid and describes Pygmalion, a Cypriot sculptor who was unimpressed by his fellow countrywomen. These ladies had denied the divinity of Venus and were punished by being reduced to prostitutes.

Pygmalion therefore created a statue of a woman, an inanimate creation that was more beautiful than any living woman. Pygmalion fell in love with the statue and prayed to Aphrodite for a woman as perfect as his own artifice. The goddess of love eventually pitied him, granted his wish, animated his statue and they went on to have a daughter (Hard, 2004). The trope of animating the inanimate in myth was not new even then. For example Pandora, the first human woman, was made from clay by the gods Hephaestus and Athena at the instructions of Zeus. Daedalus, a latter day scientist and engineer, also used quicksilver to give one of his statues a voice (Hard, 2004). More recently, the myriad roles of artificial sexual partners in SF been explored in more detail (Grech et al., 2012c).

Jones et al. have posited six hypothetical simulation scenarios whereby the universe that we perceive may well be simulations. They conjecture that the observed universe may consist of theoretical alternative constructs of simulated reality: physical presence, intercept, avatar, android, infinite regression, and monism (Jones et al., 2011). These will now be briefly illustrated by using specific episodes in the series.

## 1.1 The Physical Presence

The physical presence scenario is best demonstrated by the holodeck, a device that combines several technologies: replication, transportation and shaped force fields (Grech, 2011). In this way, objects or living creatures and reality itself are simulated, deceiving all five senses "in a virtual environment that is so realistic it cannot be distinguished from the true physical environment" (Jones et al., 2011). Indeed, this technology goes beyond simulation

> Riker: I didn't believe these simulations could be this real. Data: Much of it is real, sir. If the transporters can convert our bodies to an energy beam, then back to the original pattern again (Corey, 1987).

## 1.2 The Intercept Scenario

The intercept scenario proposes a situation wherein although we are in complete control of our consciousness, and the rest, including our bodies, are artificial constructs, existing solely in the mind, a Matrix-type setting. One of the best known examples is Captain Picard's experience when an alien probe paralyses him and dumps his consciousness into an alien setting, living out a life in speed-up/acceleration as a member of an extinct race. He lives out an entire lifetime in twenty-five minutes of objective time, just before being returned to the *Enterprise* (Lauritson, 1992).

## 1.3 The Android Scenario

The android scenario is ubiquitous in Trek since Data and holograms are synthetic creations, simulated individuals.

## 1.4 The Avatar scenario

The avatar scenario is never depicted, possibly because it is the antithesis of humanism, a strongly held belief of Gene Roddenberry, ST's creator (Alexander 1991). This scenario posits us as extremely realistic avatars that are covertly controlled by external beings.

## 1.5 The Infinite Regression Scenario

The Infinite Regression Scenario is inflicted on Riker, the *Enterprise*'s second officer when an alien child buries

him in several Matryoshka-like layers of nested and totally different realities, which he has to individually penetrate. These turn out to be simulations within simulations that have limitless potentials as to the total number of worlds or universes that might be nested within each other.

### 1.6 Monism Scenario just before

Some alien species appear to be able to deliberately alter the perception of the nature of reality by mental means alone, producing the Monism Scenario, such "that although we are in control of our own consciousness, our bodies and the material world that surrounds us are an artificial construction" (Jones et al., 2011). The Original Series bridge crew succumb to aliens in a Monism Scenario when they find themselves in the simulated western town of Tombstone (McEveety, 1968), wherein the almost voodoo-like belief that one has been shot by a pistol can be fatal.

This paper will now describe similar occurrences of the PG myth in ST and will show that the myth accedes to three of these simulation scenarios.

## 2 Narratives

### 2.1 Women (and men) in the holodeck, a Physical Presence Scenario

The holodeck in ST is a device that combines several individual and programmable ST technologies (replication, transportation and shaped force fields) to produce a simulated reality facility (Grech, 2011). In this way, inanimate objects, living creatures and reality itself may be simulated, deceiving all five senses.

Beings may be created or recreated in the holodeck purely for pleasure in "sex programs" (Landau, 1993). However, in one instance the holodeck was utilised by an alien species to distract the Enterprise's captain and second officer while the ship was being stolen (Lynch, 1988). The second officer is smitten by a lovely synthetic woman. He tells her

> "I know you are a computer-generated image, but your smell, your touch, the way you feel. Even the things you say and think seem so real. [...] How far can this relationship go? I mean, how real are you?"

The captain also finds her "astounding" and "very impressive." He also comments that "she's so very different from the images we've experienced on the holodeck, isn't she? She's more intuitive." The second officer affirms,

> "it's as though she's been plugged into my subconscious. She already knows what I want her to say before I'm aware of it myself [...]. It's uncanny. I could develop feelings [...], exactly as I would for any woman."

Attractive women may also be created purely for decorative purposes, such as Ms. Mona Luvsitt (Kolbe, 1995), a valet in a Bond-style holodeck action program who was not only highly attractive and provocatively attired, but also spoke seven languages, possessed degrees in all of the basic sciences, was said to be able to fly any aircraft and made excellent martinis. Her very name is a tribute to the sexually suggestive names customarily given to Bond girls (Erdmann and Block, 2000).

The holodeck has also been used to create synthetic sexual partners for Vulcans, an alien species whose members must mate every seven years shortly upon entering their mating cycle, or die (Grech, 2012a). Such conditions could potentially prove fatal on a starship in the distant reaches of space where members of the opposite sex yet the same species are unavailable. This subterfuge is successful in one instance for a male Vulcan (McNeill, 2000) but fails on another occasion with another (Robinson, 1997).

There are two occasions wherein female Starfleet officers engage in holodeck programs might potentially be construed as leading to sexual encounters and these are health spas (Conway, 1995) and medieval recreations centred around the chivalrous era of the Knights of the Round Table (Conway, 1995).

In another episode, the captain of the starship *Voyager*, is stranded on the other side of the galaxy with the rest of her crew. The female captain is simply uncomfortable fraternising sexually with the crew. Instead, she alters an existing character in a program that simulates a quaint old Irish village in order to suit her personal needs (Kroeker, 2000).

And in the very first aired episode in the original series, a shape-shifting alien appears as an attractive, Swahili-speaking African male in order to entrap the African communications officer, Lt. Uhura (Daniels, 1966).

However, when it comes to these technologies, not all is rosy. Holoaddiction is a fictional psychological disorder in which a person prefers to exist in the simulated world afforded by the holodeck than in the real world (Vejar, 1999). Unwanted complications may also arise from these creations, such as when a male crewperson finds himself becoming attracted to, and indeed, almost falling in love with a holodeck projection of a female scientist that he recreates in order to help him with a problem on the *Enterprise* (Beaumont 1989).

### 2.2 The Nexus and other Monism scenarios

The Nexus is a Monism state and comprises the ultimate solipsistic realm, an extradimensional domain in which

one's every thought, feeling and desire are brought to life, including old lovers. However, the Nexus is an ultimately self-defeating existence, as noted by Captain Kirk who inadvertently finds himself immersed in it. Referring to an old lover, he muses "she isn't real either, is she? Nothing here is. Nothing here matters" (Carson, 1994)

> Some alien species are also shown to be capable of altering the perception of the nature of reality by mental means alone as shown in the very first pilot episode of ST. These aliens produce a perfect illusion. They had us seeing just what we wanted to see [...]. Now let's be sure we understand the danger of this. The inhabitants of this planet can read our minds. They can create illusions out of a person's own thoughts, memories, and experiences, even out of a person's own desires. Illusions just as real and solid as this table top and just as impossible to ignore (Daniels, 1966).

### 2.3 The Android Scenario

This scenario is completely different in that it posits the complete simulation of brain and body, such that consciousness itself becomes a technological construct and it is the individual and not the environment that is simulated.

For example, the *Enterprise* crew discover an immortal human, due to the natural properties of "instant tissue regeneration coupled with some perfect form of biological renewal." He admits that he conceals this and that he lives "some portion of a life", to pretend to age and then move on before my nature was suspected.

"He confess that he "married a hundred times [...]. Selected, loved, cherished. Caressed a smoothness, inhaled a brief fragrance. Then age, death, the taste of dust." He wanted a "perfect, ultimate woman, as brilliant, as immortal as [himself. A] mate for all time [...] physically human but not human. [...] an android." Flint further acknowledges that she was"

> "created here by my hand. Here, the centuries of loneliness were to end [...]. Designed by my heart. I could not love her more [...] I love her. She is my handiwork, my property. She is what I desire" (Golden, 1969).

Similarly, a scientist implants his injured and dying wife's consciousness into an android body that is a perfect simulacrum of her old body. She is not told about the exchange by her husband as "there was no reason for her to know. I wanted her to be happy. I wanted us to be happy" (Scheerer, 1993).

In another episode, alien technology allows a dying scientist to transfer his consciousness into a mechanical android body that is externally indistinguishable from his former body. He also creates an alluring female android companion who falls in love with him (Goldstone, 1966).

In yet another episode, equally alien and sophisticated technology is used to create sentient androids who are female and "lovely [...] programmed to function as human females" (Daniels, 1967).

## 3 Discussion

Despite the frequent occurrences wherein artificial attractive partners manifest themselves in the franchise, in none of the episodes are there covert or overt visual or textual clues that refer directly or indirectly to the PG myth. This is in contrast with the *Voyager* series which is a futuristic reprise of Odysseus's return to Ithaca in *The Odyssey*.

Indeed, in a particular episode, one of the *Voyager* officers finds herself in a setting that is very similar to that in ancient Greece, in an amphitheatre complete with a part in a Greek style play, along with an appropriate declamation at the end of the episode: "*Voyager* will continue on her journey to the gleaming cities of Earth where peace reigns, and hatred has no home" (Livingston 1996). In these ways, ST replaces myth and literally becomes the modern version of myths (Tyrrell, 1977).

The episodes mentioned in this paper were specifically reviewed in order to ascertain whether there are any overt or covert references that allude to the PG myth. There are no verbal or visual clues to be found, so it would appear that the myth is retold in the abovementioned episodes without any direct or indirect references to the original. This is in contrast with the *Voyager* episode "Muse" that directly refers Odysseus and The Odyssey, as already mentioned.

In the PG myth, Galatea is given vital life through divine intervention. This cannot happen in ST, where the role of divinity in providing an *élan vital* is replaced by scientific legerdemain that is equally unexplained, but acceptable as a potentiality that science might one day be able to provide. This is not to say that religion is not integral to ST (Jindra, 1994). While such references are mostly covert, they have been frequent enough to merit book-length analysis, such as the role of ST in the American mythos (Lundeen and Wagner, 1998), and the representation of symbol and archetype in the franchise (Kapell, 2010). More specifically, fans have been shown to adapt specific issues or entire series to their personal needs (Jenkins, 1988), using them as a moral prop and support for general or specific travails that they encounter in life (Geraghty, 2007).

The future fictional world that comprises ST provides fertile ground for the narrative possibility of conjuring

humans or humanoids to specification, including women that completely embody male fantasies (Erdmann and Block, 2000). Such episodes allow the prospect of consciousness to be created outside an organic brain (McCrone, 1993), an actual possibility in the field of artificial intelligence as science races toward the singularity, the critical juncture beyond which, it is surmised that scientific progress will become so rapid that it will outstrip human comprehension (Kurzweil, 2005).

For ST narratives to continue, show after show, intimacy, marriage and settling down to have family simply cannot manifest. Such entanglement would drastically change the nature of the overall scheme of things and prevent characters from acting as free agents, subservient only to their loyalties in relation to quasi-military hierarchies. Indeed, almost as if to demonstrate the perils inherent in marrying a Starfleet officer, two separate episodes specifically demonstrate what happens when the mind of a spouse is taken over by an inimical alien (Grech, 2013c).

Miles O'Brien, a Starfleet engineer, is married to a botanist, Keiko. The spouses' minds are taken over, in different episodes. They are then readily held to ransom with threats to the bodies that were taken over by said aliens (Livingston, 1992; Kroeker, 1996). This can lead to situations that endanger many others, such as when O'Brien is directly threatened with Keiko's death, should he fail to comply with the demands of an alien that has taken over his wife. O'Brien is told by Keiko's body which has been overwhelmed by an alien mind

> "I have taken possession of your wife's body. I will hold it hostage until you do everything I tell you do accurately, and without question ... if you don't do precisely what I ask, I'll kill your wife ... I'll stop her heart forever ... these corporeal bodies of yours. So fragile. Burst even a tiny blood vessel in the brain and every memory, every passionate emotion, gone forever ... you might be able to stop me. But I promise you one thing. If you do, Keiko will die. All I need is a split second to cause a massive brain haemorrhage and she's gone (Kroeker, 1996)."

In both episodes, the original brains are restored to their bodies. Simple friction is also seen when their careers clash, such as when O'Brien is transferred to the space station *Deep Space Nine*, and attempts to remind his wife that "we made the decision together." Kieko rebuts "Not true. That's not true. You decided and asked me to agree with it" (Lynch, 1993). It has therefore been argued that to some extent, the inability or reluctance to enter into matrimony explains the propensity of characters to become attracted to and fall in love with imaginary or synthetic women (Erdmann and Block, 2000). This naturally devolves the role of such creations to that of "the ultimate convenience female," an objectified embodiment of lust with no true consciousness to mollify (Wilcox, 1991).

The dearth of females creating synthetic men for sexual purposes belies recent studies which show that women's desires vary little from the male of the species (Bergner, 2013), hence ST's simulation of so few males for intimate purposes is unrealistic and potentially sexist.

In conclusion, the narratives discussed are ultimately variants of the PG myth, appropriately censored for the modern age through scientific or pseudo-scientific-explanations. These narratives rationalise the myth (which comprises the plot) through a process of cognitive (coherent and rational-appearing) scientific explanation.

To the best of this author's knowledge, the upcoming Star Trek Symposium (10-11 July 2014 – Dolmen Hotel Qawra - http://www.startreksymposium.com) is the first academic event relating solely to Star Trek. This event will present papers similar to this and is open to all.

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*Research Note*

# Stroke patients' interpretation of symptoms and presentation to hospital

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**Abstract. Introduction** The aim of this study was to elucidate patient interpretation of stroke symptoms and to investigate factors which influence timely presentation to hospital. **Methods** All patients admitted to Mater Dei Hospital with a diagnosis of cerebrovascular accident (CVA) or transient ischaemic attack (TIA) between July and September 2011 were recruited prospectively. Data was collected by patient interview and with reference to medical notes in order to determine patient risk factors for stroke, knowledge on stroke, interpretation of stroke symptoms and time interval to presentation to hospital. **Results** The cohort studied ($N = 54$) had an average age of 67.9years ($SD = 10.407$). The risk factors for cerebrovascular disease most frequently found in this group were hypertension (56%), hypercholesterolaemia (56%), family history of stroke (41%) and smoking (39%). Participants interpreted their symptoms as stroke in 33% of cases ($n = 18$), whereas 48% reported that they did not know or suspect any particular cause at the time. The perceived severity of events at symptom onset was reported as 'high' by 41% and 'low' by 57%. Only 31% of participants ($n = 17$) recognised the brain as the organ primarily affected in stroke. Forty five percent of patients sought medical advice within one hour. Fifty-six percent ($n = 30$) first resorted to their family doctor, whilst 28% ($n = 15$) phoned the emergency services. Family doctor as first contact was associated with delayed presentation ($p = 0.007$); conversely, phoning emergency services was associated with earlier presentation to A&E. **Conclusion** The results of this study highlight limited knowledge about stroke in the population involved. It also serves to clarify factors contributing to high rates of late presentation. These findings show the need for an improvement in public awareness in terms of education on stroke and the importance of early presentation to hospital.

**Keywords** Stroke – transient ischaemic attack - symptoms - presentation – interpretation – recognition – awareness - thrombolysis.

## 1 Introduction

Intravenous thrombolytic therapy has been shown to improve outcome at three months in patients with acute ischaemic stroke (Wardlaw, 2001). This service has been available in Mater Dei Hospital since October 2010 and is a key inclusion criterion in administration of treatment within three hours of symptom onset. Late presentation to hospital remains the most frequent reason for exclusion from thrombolysis in Malta, despite the short distances and relatively easy access to medical services (Mallia, 2001).

The aim of the study was to investigate factors that contribute to late presentation, to elucidate patient interpretation of stroke symptoms and to identify potential points of intervention for future reversal of this trend.

## 2 Material and Methods

All patients admitted via the emergency department of Mater Dei Hospital with a provisional diagnosis of cerebrovascular accident (CVA) or transient ischaemic attack (TIA) between July and September 2011 were recruited prospectively.

Inclusion criteria were: the ability to communicate

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sufficiently to participate in the interview, informed consent, an age of above eighteen years and diagnosis of stroke or TIA on discharge. The main exclusion criterion was the inability to communicate and carry out the interview via verbal communication.

Approval for the study was obtained from the University of Malta Research Ethics Committee. Data was collected in a prospective manner from the following sources: patient interview, patient medical and nursing notes of index admission, hospital PACS (Picture Archiving and Communications System), iSOFT Clincial Manager (centralised investigation results) and PAS (Patient Administration System). Patient interview consisted of a structured questionnaire available in Maltese or English according to patient preference and were conducted by one of four researchers within 48 hours of admission.

The structured interview included the following questions: the nature of first symptoms felt, the time of the first symptoms, the participant's interpretation of these symptoms, knowledge on stroke, past personal experience or family history of stroke, the time and nature of medical assistance first sought, mode of transport to hospital and knowledge and recognition of risk factors for cerebrovascular disease. Hospital records were used to report the precise arrival time at the triage bay of the Accident and Emergency department. Patient admission records were used to obtain the patients known risk factors for cerebrovascular disease. Patient demographic data was also collected including age, gender and nationality.

# 3 Results and Discussion

The total number of admissions of acute ischaemic stroke or TIA during the three month period was 105, of which 51 (48%) were excluded. This gave a total population of 54 patients, which has reduced the power of statistical analysis. Therefore, results derived from this study have been used to demonstrate trends which in many instances could not be proven to be statistically significant. Similar publications used for comparison examining this subject were carried out with larger patient populations, therefore, comparison to these studies was also limited. Extension of the data collection period would increase the power of this study.

The reasons for exclusion were: inability to communicate, 59% ($n$ = 30), discharge before interview, 22% ($n$ = 11), a change in diagnosis, 16% ($n$ = 8) and also withheld consent (n = 2). The large proportion of exclusions is a reflection of the high percentage of stroke patients with speech difficulties. But also of a significant proportion of stroke patients being elderly and the increased incidence of cognitive impairment in this age group.

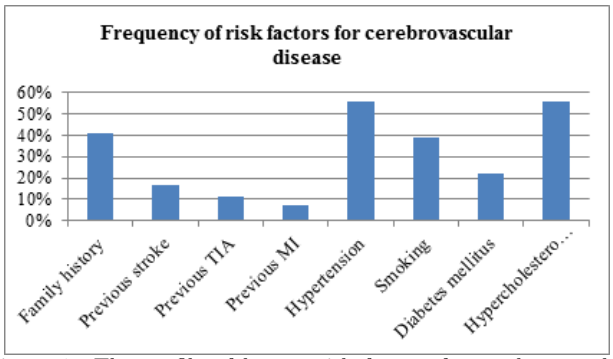


Figure 1: The profile of known risk factors for cerebrovascular disease present in the participant population.

This left a group of 54 participants, of whom 59% were male and 41% female. The average age being 67.9 years (SD = 10.407). The risk factors for cerebrovascular disease most frequently found in this group were: hypertension (56%), hypercholesterolaemia (56%), family history of stroke (41

The only factor that was found to result in a statistically significant earlier presentation were those patients with a family history of stroke (p = 0.016). No statistical significance was found for the following factors: gender, nationality, the presence of three or more cerebrovascular disease risk factors, a past history of stroke or TIA, interpretation of severity, knowledge on stroke and perceived cause of symptoms. This may be due to the relatively low number of participants.

Knowledge of risk factors for stroke was poor, smoking (39%), excess alcohol intake (26%) and hypertension (20%) being those offered most frequently as risk factors known to patients. However, on being asked to choose from a list of lifestyle factors or medical conditions, a much higher proportion of patients correctly identified risk factors for stroke (shown in Fig.(1)).

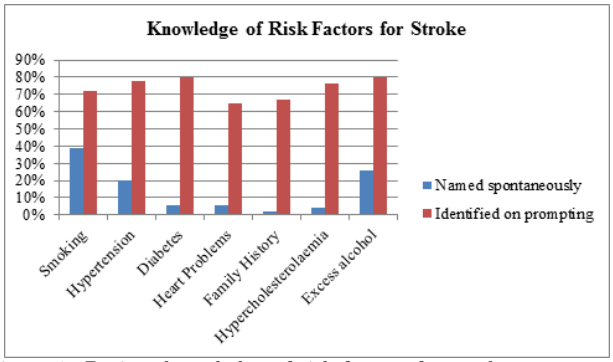


Figure 2: Patient knowledge of risk factors for stroke.

The symptoms most frequently reported by participants were: weakness (70%, $n$ = 38), speech difficulty (44%) and sensory phenomena (30%). The subjective

sensation of 'dizziness' (differentiated by interviewers from vertigo) was reported by 28% ($n = 15$) of the participants. The other 19% reported various other suspected causes, such as a problem with the affected limb. These findings are important when considering which symptoms of stroke to place emphasis on when planning public health awareness projects, such as the 'Act FAST' campaign that has been carried out with success by the United Kingdom National Health Service. In this study, none of these presenting symptoms were found to be significant in influencing earlier presentation to hospital.

The perceived severity of events at symptom onset was reported as 'high' by 41% ($n = 22$) and 'low' by 57% ($n = 31$), assessed by asking patients how worried they were about their symptoms signifying a prominent health problem at the time of which they were experiencing them. Participants correctly identified the brain as the organ damaged by stroke in 31% of cases ($n = 17$). Thirty nine percent blamed the affected limbs as the source of their symptoms, whereas 22% replied that they did not know. These two questions offer the clearest demonstration of a widespread lack of basic understanding of stroke as a disease and its manifestations.

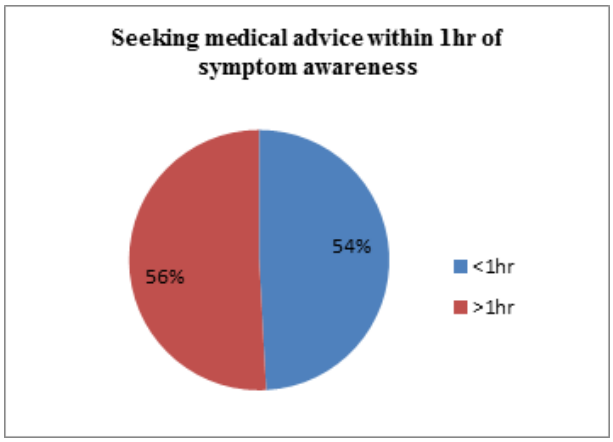


Figure 3: Participants who sought medical advice within 1 hour of symptom onset.

When making initial contact with medical services, 56% ($n = 30$) of participants first resorted to their General Practitioner (GP), 28% phoned the emergency services number 112, 11% made contact with their local health centre and 6% presented directly to Accident and Emergency. Making initial contact with a GP was associated with delayed presentation to hospital ($p = 0.007$), whereas phoning the emergency services number 112 with earlier presentation ($p = 0.009$). This compares well with the findings of two similar studies in the USA (Williams, 1997) (Rosamund, 1998) where use of the emergency services led to earlier presentation. This shows the need to focus on the role of primary care

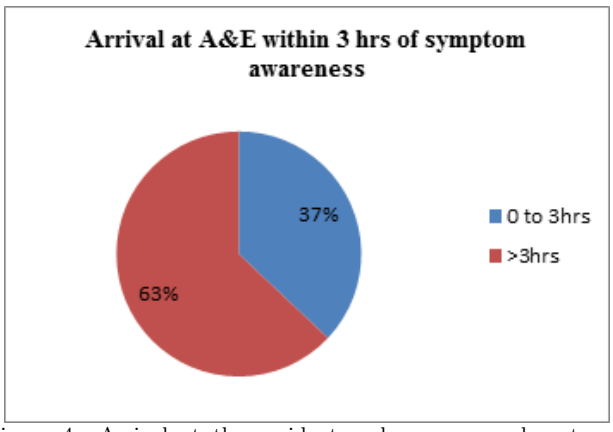


Figure 4: Arrival at the accident and emergency department within 3 hours of symptom onset.

services in Malta, along with public awareness, to drive improvement in management of acute ischaemic stroke, namely by ensuring rapid referral from primary to emergency and tertiary services.

A quarter of participants ($n = 14$) first sought medical advice about their symptoms within thirty minutes of symptom awareness and 45% ($n = 24$) within the first hour. Twenty percent ($n = 11$) only sought medical advice after 24 hours of first awareness of symptoms. One patient reported using the internet to search for information about stroke symptoms and subsequently contacted the emergency services.

Despite 45% of individuals seeking medical advice within the first hour, only 37% arrived at the emergency department within 3 hours of symptom onset. This shows a delay in time between first contact with medical services and arrival at hospital. Fig.(3.3) demonstrates the frequency of patients presenting at the given time periods, in hours, after symptom awareness.

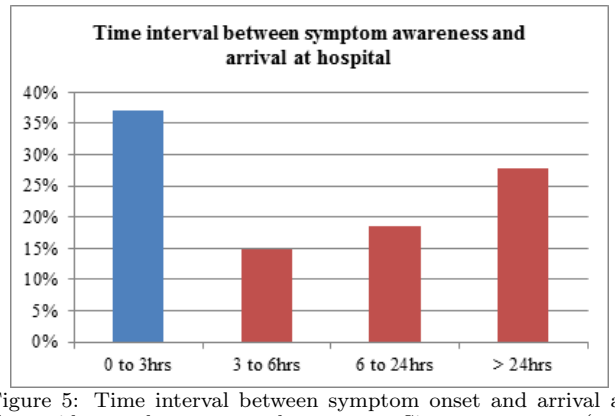


Figure 5: Time interval between symptom onset and arrival at the accident and emergency department. Sixty one percent ($n = 33$) of patients utilized emergency service transport, whereas 39% ($n = 21$) used private means of transport. None of the patients drove themselves.

# 4 Conclusions

The results of this study highlight limited knowledge about stroke in the population involved. This lack of awareness on cerebrovascular disease is associated with delayed presentation to hospital, which has important implications on outcome. The need for improved awareness is not restricted to the general public, but also extends to healthcare providers, notably primary care practitioners and allied healthcare professionals who are in a position to provide advice to the general public. The authors are aware that an initiative aimed at public awareness of stroke symptoms is underway by the Health Promotion Department which is ongoing.

# 5 Acknowledgements

We would like to thank Dr Philip Dingli, Ms Carmen Mallia.

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*Research Article*

# Pollen Characterisation of Maltese Honey

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**Abstract.** In 2004 and 2005, pollen characterisation of 35 samples of honey collected from the islands of Malta and Gozo, was carried out with the aim to identify the botanical origin of honey produced on these islands. Pollen was extracted from the honey samples via centrifugation and identified to pollen type, generic level and where possible, specific level via microscopic analysis. This was done by comparison with available literature and with the aid of prepared reference slides of pollen collected from the plant species commonly present in the Maltese islands. A total of 61 pollen types were identified from 33 families. The best represented families were the Asteraceae, Brassicaceae, Fabaceae and Apiaceae. Thyme (Thymbra capitata (L.) CAV.) pollen was found to be predominant in ten samples, with a percentage frequency that ranged from 10% to 67%. Hedysarum coronarium L. was found to be predominant in five honey samples with percentage frequencies from 48% to 78% while Lotus spp. pollen was found to be predominant in one honey sample with a percentage frequency of 57%. The remaining 14 honey samples possessed pollen spectra which were characterized by a few frequent pollen types that possessed similar percentage frequencies and were thus considered to be multifloral. This is the first work of pollen characterisation of Maltese honey.

**Keywords** Pollen – Honey – Melissopalynology.

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## 1 Introduction

There has never been a study on the pollen of honey produced in the Maltese islands. No work has ever been carried out locally in this field and, so very little is known about which flora characterises local honey except from what is known by the beekeepers themselves. Beekeepers to this day, label their honey according to the season during which it is harvested: spring and autumn honey. Spring honey is known to be polyfloral whereas autumn honey is considered by beekeepers to be mainly produced from nectar collected from eucalyptus and carob trees. A summer honey is also produced but this is labelled by beekeepers as wild thyme honey as wild thyme (Thymbra capitata) is the only bee-important flowering plant species that flowers in abundance during the hot summer months. Little else is in flower during this season.

Maltese honey is highly appreciated and sought after locally, especially thyme honey, which fetches a considerably high price. However, little is known about the microscopic and chemical composition of the honey. With entry to the European Union in May 2004, Malta adopted European legislation on the production and marketing of honey. As a result, Maltese honey must conform to the quality standards defined in EU legislation in order to be placed on the market, both locally and abroad.

This study is a preliminary attempt to gain an insight into the botanical composition of Maltese honey and involves the qualitative analysis of the pollen types found in the honey samples. Qualitative pollen analysis permits the calculation of relative pollen frequencies on the basis of the total count of pollen grains and other honey elements, as well as the identification of

the pollen spectrum that characterises honey produced in a specific geographical area. This type of analysis is therefore important in the identification of the types of honey that are produced locally. Its importance also lies in the added value and improvement of Maltese honey in terms of quality, in such a way as to create a solid niche-market for the product abroad. Quality control of honey relies heavily on melissopalynology, not only for the identification of botanical and geographical origin of honey, but also for the determination of fraudulent activities, such as the blending of Maltese honey with honey originating in other countries.

This study, therefore aims to obtain initial qualitative information on the true botanical identity of Maltese honey and to stimulate interest in a field of study relatively unknown locally.

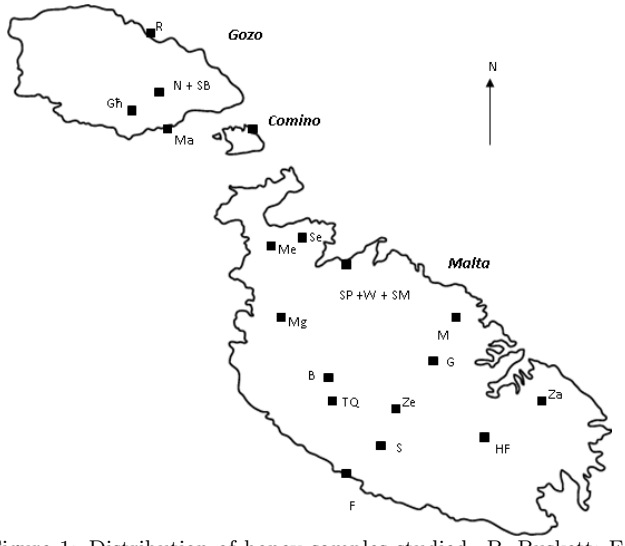


Figure 1: Distribution of honey samples studied. B, Buskett; F, Fawwara; G, Għargħur; Gh, Għajnsielem; HF, ħal Farruġ; M, Magħtab; Ma, Mġarr Gozo; Me, Mellieħa; Mg, Mġarr Malta; N, Nadur; R, Ramla; SB, San Blas; S, Siġġiewi; SE, Selmun; SM, San Martin; SP, St. Paul's; TQ, Ta' Qali; W, Wardija; Za, Zabbar; Ze, Żebbuġ.

# 2 Methods

Of the 35 honey samples that were collected from beekeepers during 2004 and 2005, a total of 28 samples from 17 localities were collected from Malta, 1 sample from Comino and 6 samples from 4 localities from Gozo (Figure 1). Fifteen samples were harvested by the beekeepers in 2004 while sixteen samples were harvested in 2005. The harvest date of four of the samples was unknown (the sample from Magħtab was not labelled with the harvest date while the beekeepers who provided the sample from Għargħur and Mġarr could not remember whether they had harvested the honey sample in 2003 or 2004).

All the honey samples were collected from the beekeepers except for one sample of honey, which was purchased from a retail outlet as the producer was unreachable. This honey was included in the study as it is one of the main honeys that is found on the local market.

At the time of this study honey samples were collected from most of the commercial beekeepers in Malta and Gozo, which due to the islands' small size was limited. Honey samples were collected from the beekeepers themselves as all except two did not sell their produce through retailers.

For the qualitative analysis, 10g of honey was dissolved in 20ml of distilled water at 40°C, to reduce the viscosity of the honey and enable the extraction of the pollen. The honey solution was poured into a 10ml glass centrifuge tube with a pointed tip and then centrifuged at 2500rpm for ten minutes. A Beckman Coulter Allegra X-22R centrifuge was used to centrifuge the honey samples. Honey samples, which were rich in sugar crystals where subjected to another centrifugation step at 2500rpm for ten minutes by redissolving the pellet in another 20ml of distilled water.

Following centrifugation, the centrifuge tube was tilted at a 45° angle to discard the supernatant, and the last drop was blotted dry with a piece of paper tissue. The pellet was then loosened with a disposable plastic Pasteur pipette (volume 1ml) and the lose sediment was drawn up with the pipette and transferred onto a glass slide. The sediment was spread evenly onto the glass slide over an area of $22 \times 22$mm with the aid of the Pasteur pipette as recommended by Von der Ohe et al., (2004). The $22 \times 22$mm square was delineated with a marker pen on the underside of the slides. The glass slide was then placed onto a hot plate for a few minutes to allow the sediment to dry. Where possible, two slides were prepared for each honey sample. However, a few of the honey samples were only sufficient to prepare one microscope slide.

While the sediment was left to dry on the glass slide, a small amount of Kaiser's glycerine jelly mountant was transferred to a cover slip of $22 \times 22$mm. The cover slip was placed on a hot plate at 40°C to dissolve the mountant (which was kept in the refrigerator and thus solidified). The drop of glycerine jelly was applied to the cover slip in the form of a cross diagonally as suggested in Von der Ohe et al., (2004) to ensure that the pollen grains remained in their drying position when the cover slip was lowered onto the glass slide. The cover slip was lowered onto the sediment slowly so as not to trap any air bubbles and the slides were sealed with clear nail varnish after the mountant had cooled and solidified.

## 2.1 Preparation of the glycerine jelly mountant

The mountant that was used in this study was Kaiser's Glycerine Jelly which was prepared from gelatine, dis-

tilled water, glycerine and phenol. 40g of gelatine was heated in 210ml of distilled water until the gelatine dissolved. 250ml of glycerine and 1g of phenol were added to the gelatine and the mixture was heated for 15 minutes and stirred until it became smooth.

The glycerine jelly mountant may be coloured by the addition of 0.5 to 1ml of 0.1% (w/v) basic fuchsin solution which stains the pollen grains pink (Von der Ohe et al., 2004). However, this step was not carried out during the preparation of the slides of the honey samples as the stain tends to make the structural features of the pollen grains less visible and it may thus hinder their identification (Ricciardelli D'Albore by personal communication, 2005). The pollen grains were thus left unstained and appeared in different shades of yellow when viewed under the microscope.

## 2.2 Qualitative Analysis of the Honey Samples

The mounted pollen samples were examined under the microscope at a magnification of ×400. For this analysis a Nikon Eclipse E400 POL microscope with camera attachment was used. The pollen grains were counted in batches of 100, following parallel equidistant lines spaced evenly form one edge of the coverslip to the other.

The percentage frequencies of the pollen types present in the honey can be calculated by counting and identifying between 500 to 1000 pollen grains (Von der Ohe et al., 2004). During this study pollen grains were counted and identified for each honey sample. As microscope slide preparations of the honey samples were observed, the pollen grains were counted in batches of 100 pollen grains. The pollen frequencies of the pollen types identified in each batch were calculated and compared with one another. Results were found to be constant when 700 grains were counted and as a result counting 700 pollen grains was deemed sufficient to obtain reliable pollen frequencies for the pollen types.

Pollen grains were identified to genus and species level only when they could be identified with certainty. Most often this was not possible and they were identified by types. Thus, pollen grains that possessed the characteristics of Cerinthe pollen were classified as Cerinthe type and so on. Wind-pollinated and nectarless species were noted separately. Grains that could not be identified were also counted separately. Pollens grains were not identified because they were not known or else because they were distorted or misshapen. Honeydew elements (fungal hyphae, fungal spores, algae and conidia) were also counted. Complexes of spores and algae were counted as one element.

## 2.3 Interpretation of Results

The pollen frequencies of the pollen types identified in the honey samples were calculated by dividing the number of pollen grains of a pollen type by the total number of pollen grains counted and multiplying by 100. Pollen frequencies of nectar-producing plants were calculated after the number of pollen grains of nectarless species and wind-pollinated species were subtracted from the total. Once the pollen frequencies were calculated, the pollen types were classified as follows, following Louveaux et al. (1978): "Very frequent" for grains constituting more than 45% of the total; "Frequent" for grains constituting 16-45% of the total; "Rare" for grains constituting 3-15% of the total; "Sporadic" for grains constituting less than 3%. Pollen with a frequency of 1% or less is classified as "present".

Honey samples that were rich in pollen of overrepresented species were recounted excluding the overrepresented pollen type. The botanical origin of the honey was determined by calculating the relative frequencies, excluding the pollen from nectarless species. Honey was considered to be produced mainly from one botanical species when the pollen of this species was predominant in the honey. For overrepresented species, such as Eucalyptus, a minimum threshold of 90% was required (as suggested by Louveaux et al., 1978) to define the honey as belonging mainly to that species, while for underrepresented species, such as Thymbra, a minimum threshold of 10% was considered (as suggested by Terrab et al., 2004).

## 2.4 Preparation of Reference Slides for the Pollen Library

A pollen library of all the common plant species found in the areas where the honey was produced was compiled as a reference library for the identification of the pollen extracted from the honey samples.

Pollen was taken from the buds of flowers and allowed to open in a contained environment in order to eliminate contamination by pollen in the environment. The procedure that was followed for the preparation of these slides was that of Louveaux et al. (1978). The anthers or whole flowers were washed in a watch glass containing ether. A ring of pollen formed at the edge of the ether solution, the ether was decanted and the pollen was rinsed with fresh ether and allowed to dry. The pollen grains where then transferred onto a microscope slide, warmed at 40°C and mounted in Kaiser's glycerine jelly.

A digital library of the reference material was also compiled with the aid of a Nikon Eclipse E400 POL microscope with camera attachment and a Nikon Coolpix 995 Digital Camera. The photographs of the pollen grains were taken at a magnification of ×400. The pollen

types extracted from the honey samples were identified with the aid of the reference collection of prepared slides the digital photos, as well as photomicrographs and pollen descriptions from literature. The literature used for the identification of the pollen grains was that of Ricciardelli D'Albore, 1998.

## 2.5 Results

The percentage frequencies of the pollen grains identified in the honey samples, were calculated after subtracting the number of pollen grains of nectarless species and wind-pollinated species from the total number of pollen grains counted.

**Table 1: Honey samples studied in 2004/2005, split by locality and harvest period.**

| | Code | Locality | Harvest Year | Harvest Period |
|---|---|---|---|---|
| Malta | 1 | St. Paul's | 2005 | July |
| | 2 | Siġġiewi | 2005 | Unknown |
| | 3 | Mellieħa | 2004 | July |
| | 4 | Wardija | 2004 | Unknown |
| | 5 | Mġarr | Unknown | Spring |
| | 6 | Mġarr | 2005 | Unknown |
| | 8 | Wied Musa | 2004 | Unknown |
| | 10 | Buskett | 2004 | May |
| | 11 | Għajnsielem | 2004 | May |
| | 12 | Żebbuġ | Unknown | Autumn |
| | 14 | Għargħur | 2004 | Unknown |
| | 15 | Wardija | 2004 | Spring |
| | 16 | Żebbuġ | 2005 | Spring |
| | 17 | Wardija | 2004 | Unknown |
| | 18 | Ta' Qali | 2004 | Unknown |
| | 19 | Mellieħa | 2005 | Unknown |
| | 20 | Mellieħa | 2004 | Unknown |
| | 22 | Selmun | 2005 | Summer |
| | 23 | Zabbar | 2004 | September |
| | 25 | ħal Farruġ | 2005 | Spring |
| | 27 | Fawwara | 2005 | Unknown |
| | 28 | Buskett | 2004 | May |
| | 30 | Mġarr | 2005 | Autumn |
| | 31 | Magħtab | Purchased, undated | Unknown |
| | 32 | Mġarr | Unknown | Unknown |
| | 33 | San Martin | 2004 | Spring |
| | 34 | Fawwara | 2005 | July |
| | 35 | Fawwara | 2005 | April |
| Gozo and Comino | 7 | Comino | 2004 | Summer |
| | 9 | Nadur | 2004 | Unknown |
| | 13 | Ramla | 2005 | Unknown |
| | 21 | Nadur | 2004 | August |
| | 24 | Mġarr | 2005 | Unknown |
| | 26 | Nadur | 2005 | August |
| | 29 | San Blas | 2005 | August |

The results of the qualitative analysis for 30 of the honey samples studied are shown in table 1. Many authors recommend the identification of 500 – 1200 pollen grains for the determination of reliable pollen frequencies to individual pollen types, expressed with an accuracy of ±1% (Louveaux et al., 1978; Ricciardelli D'Albore, 1997; Von der Ohe et al., 2004). An increased error is obtained for counts up to 500 pollen grains (Moar, 1985). Preliminary observation of the honey samples revealed the presence of few botanical species with prevalent in the honey. Thus, consistent results were obtained with counts of 700 grains. In addition, the pollen grains were counted in batches of 100, and when the results were compared they were found to be in general quite consistent with one another.

A total of 61 pollen types were identified from

33 families. The best represented families are the Asteraceae (Compositae), Brassicaceae (Cruciferae), Fabaceae (Leguminosae) and Apiaceae (Umbelliferae). No particular pollen type was present in all the honey samples studied. The pollen types identified in the honey samples were the following: Hedysarum coronarium, 28 samples (<1 – 64%); Diplotaxis spp., 28 samples (<1 – 23%); Lotus spp., 27 samples (<1 – 57%); Vicia type, 26 samples (<1 – 16%); Papaver type, 26 samples (<1 – 38%); Oxalis pes-caprae L., 26 samples (<1 – 11%); Galactites tomentosa (l.) Moench., 25 samples (<1 – 17%); Daucus type, 24 samples (<1 – 21%); Citrus spp., 23 samples (<1 – 10%); Thymbra capitata, 21 samples (<1 – 65%); Reseda type, 21 samples (<1 – 18%); Eucalyptus spp., 19 samples (<1 – 51%); Borago officinalis L., 18 samples (<1 – 4%); Rhamnus spp., 17 samples (<1 – 29%); Limbarda crithmoides (L.) Dumort., 16 samples (<1 – 3%); Plantago spp. and Acacia spp., 14 samples (<1 – 3%); Glebionis coronaria (L.) Cassini ex Spach., 13 samples (<1 – 1%); Ceratonia siliqua L., 12 samples (<1 – 38%); Euphorbia type and Eriobotrya type, 11 samples (<1 - 5%); Medicago type, 10 samples (<1 – 6%); Erica multiflora L., 10 samples (<1 – 4%); Brassica type, 10 samples (<1 – 1%); Asparagus type, 9 samples (<1 – 10%); Malus type, 9 samples (<1 – 7%); Cercis siliquastrum L., 8 samples (<1 – 4%); Smilax aspera L., 8 samples (<1 – 2%); Apiaceae, 6 samples (<1 – 22%); Trifolium type, 6 samples (<1 – 5%); Cerinthe major L., 6 samples (<1 – 7%); Prunus spp., 6 samples (<1 – 3%); Glebionis type, Carthamus spp., Arecaceae (Palmae), 6 samples (<1 – 1%); Ailanthus type, 5 samples (<1 – 4%); Ecballium elaterium (L.) A.Rich., 5 samples (<1 – 3%); Convolvolus type, Allium type and Cucumis spp., 5 samples (<1 – 1%); Olea europea L., 4 samples (<1 – 2%); Capparis type, 4 sample (<1 – 8%); Poaceae, 4 samples, (<1 – 1%); Cucurbita spp. and Lonicera type, 4 samples (<1%); Smyrnium olusatrum L., 3 samples (<1 – 6%); Matricaria type and Teucrium spp., 3 samples (<1%); Aptenia type, 2 samples (2 – 7%); Nicotiana type, 2 samples (<1%); Carpobrotus type, 1 sample (14%); Ferula type, 1 sample (1%); Hedera type (<1 – 1%); Vitis type, Agave type, Eryngium type, Geranium type, Helianthus type, Echium type, Cupressus spp. and Lavandula type, 1 sample (<1%).

## 2.6 Pollen Percentage Frequencies of Thymbra capitata and Uniflorality of Honey

Thyme pollen was found to exhibit a pollen percentage frequency of 10% or higher in 10 samples of the 30 that were studied. The percentage frequency in these samples ranged from 10% to 67%. Thyme pollen tends to be underrepresented in honey (Ricciardelli D'Albore, 1998). Terrab et al. (2004) report a percentage frequency of 8% to be sufficient to characterise a thyme honey as unifloral.

Predominance of a pollen type in honey and determination of uniflorality is not easy to determine for species which are underrepresented. In samples no. 4 from Wardija, no. 7 from Selmun and no. 5 from Mġarr, Thyme pollen exhibited percentage frequencies of 67%, 54% and 49% respectively. The other pollen types exhibited percentage frequencies of < 5%, = 10%, and = 13% . Therefore these three honey samples were clearly considered to be characterised by Thyme.

In samples no. 3 (Mellieħa), 8 (Wied Musa) and 23 (Mellieħa), Thyme exhibited percentage frequencies of 33%, 36% and 34% respectively. Sample no. 3 showed Eucalyptus to possess a percentage frequency of 38% whereas sample no. 23 showed Ceratonia siliqua to possess a percentage frequency of 36%. Eucalyptus is a species which is overrepresented in honey (Ricciardelli D'Albore, 1998) and it requires percentage frequencies of up to 90% to characterise a honey, whereas Ceratonia would require a percentage frequency of 45%. These honey samples were thus considered to be characterised by Thyme.

Sample no. 1 from St. Paul's Bay, was difficult to interpret due to the high percentage of unidentified pollen grains in the sample (25%). However, in an extreme event in which all the unidentified pollen grains belonged to one pollen type, this would not have sufficed to characterise the honey, unless the species was underrepresented.

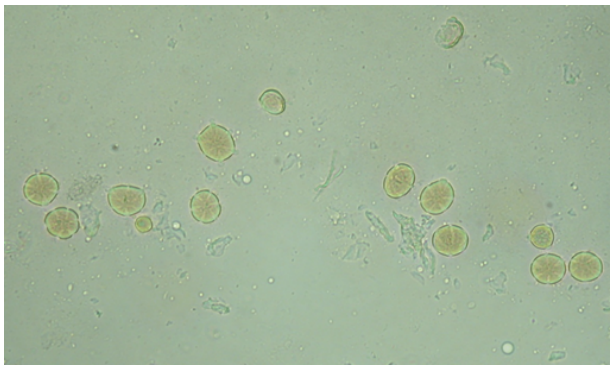

Figure 2: Thyme pollen in honey sample number 1 from St. Paul's Bay.

Honey samples no. 2, 6 and 20 from Siġġiewi, Mġarr and Mellieħa showed Thyme to possess percentage frequencies of 10, 11 and 12% respectively. Further studies would be required to determine whether such honey samples could be considered to be characterised by Thyme when the percentage frequencies of all the other pollen types is low. Lotus in sample 20 possessed a percentage frequency of 32%: a normally represented pollen type which requires a percentage frequency of

45% to characterise a honey. The percentage frequency of unidentified pollen grains in sample no. 6 was rather high (15%) and it was therefore difficult to draw conclusions. Therefore, classification of uniflorality in such cases would require further investigation.

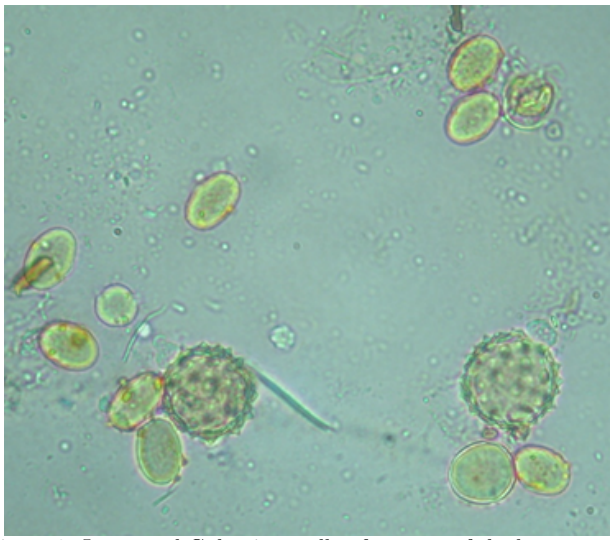

Figure 3: Lotus and Galactites pollen from one of the honey samples studied.

## 2.7 Pollen Percentage Frequencies of Lotus and Hedysarum and Uniflorality of Honey

Hedysarum and Lotus (Fabaceae) are in full flower in spring and are locally considered to be typical of spring blossom honeys. Hedysarum was found to be predominant in five honey samples (samples 9, 10, 11, 13 and 26). Percentage frequencies ranged from 48% to 78%. This is a very good nectariferous species that is normally represented in honey (Ricciardelli D'Albore, 1998). In all five honey samples the other pollen types exhibited frequencies that were very low and as a result, the pollen spectra were easy to interpret. In addition the percentages of unidentified pollen grains were also low (the highest being 7%) and therefore did not pose any difficulty to the interpretation of the results. All five honey samples were thus considered to be characterised by Hedysarum.

Lotus was found to be predominant in honey sample no. 12, in which this species exhibited a percentage frequency of 57%. Once again the pollen spectrum was easy to interpret as the next most abundant species was Hedysarum with a percentage frequency of 26%. This species requires a frequency of 45% to characterise a honey. All other pollen types present were rare, sporadic or just present. The percentage of unidentified pollen grains was 2%. This honey sample was thus also classified as Lotus honey.

## 2.8 Multifloral honeys

The remaining 14 honey samples possessed pollen spectra which were characterised by a few frequent pollen types and many pollen types that classified as rare, sporadic or present. The frequent pollen types were: sample 14 (Lotus, 37%); sample 15 (Hedysarum 36%; Lotus, 28%); sample 16 (Lotus, 16%; Hedysarum. 24%); sample 17 (Vicia, 17%); sample 18 (Rhamnus, 29%); sample 19 (Hedysarum, 24%; Lotus, 28%); sample 21 (Hedysarum, 31%; Lotus, 21%); sample 24 (Daucus, 24%; Hedysarum, 20%); sample 25 (Eucalyptus, 25%); sample 28 (Diplotaxis, 15%; Vicia, 17%); sample 29 (Apiaceae and Lotus, 22%; Daucus, 15%); sample 30 (Ceratonia, 41%; Diplotaxis, 25%). All other pollen types in these samples of honey were classified as rare, sporadic or present.

The results showed that no species was predominant in the honey samples and that they were characterised by more than one botanical species. In sample no. 30 which possessed a percentage frequency of 41%, the percentage of unidentified pollen grains in this sample was 9%. However, Ceratonia pollen grains possess a tetracolporate structure that is easy to identify and therefore this value was not considered to hinder the interpretation of the results. All these honey samples were thus considered to be characterised by more than one botanical species and therefore considered to be multifloral in origin.

## 2.9 Nectarless Species and Wind-Pollinated Species

The nectarless species identified in the honey samples were all present in minor quantities in the honeys that were studied except for Papaver which was present in 26 honey samples and exhibited percentage frequencies of <1% - 38%. This species is nectarless but it is commonly visited by bees for pollen (Ricciardelli D'Albore, 1998). Plantago pollen was identified in 14 honey samples with frequencies of <1 to 3%. This is both a wind-pollinated species (Chambers, 1945) and an insect-pollinated species (Plantago lanceolata has been found to be most effectively pollinated by hoverflies) (Stelleman and Meeuse 1976, Stelleman 1978, 1981) that is visited by bees for pollen. Olea, Vitis, Cupressus, Poaceae and Arecaceae pollen did not occur in frequencies higher than 1% except for Olea which occurred at a frequency of 2% in sample no. 24 from Mġarr.

Pollen from nectarless and wind-pollinated species ends up in honey by contamination processes. Thus, the number of pollen grains from these species is subtracted from the total when the pollen frequencies are calculated, as there is no nectar from these species in the honey.

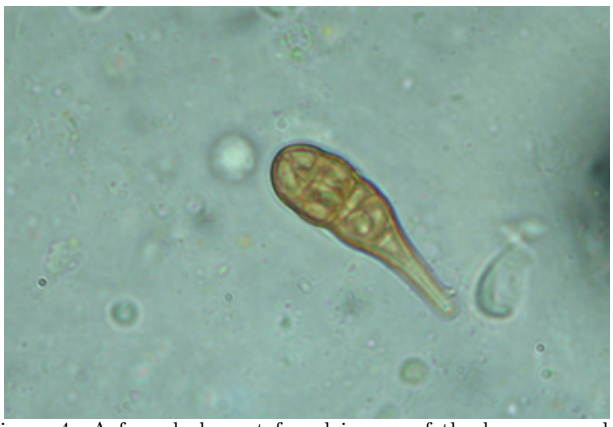

Figure 4: A fungal element found in one of the honey samples studied.

### 2.10 Honeydew Elements and Honeydew Honey

The number of honeydew elements observed in the honey during the pollen counts was also noted. These included fungal hyphae and fungal spores. Numbers were very low and ranged from none to 29 elements counted in honey sample no. 3 from Mellieħa. Honeydew honeys are produced from the excretions of plant-sucking insects, such as aphids, which occur in large population densities and honeys that are produced from these secretions are characterised by the presence of fungal hyphae and fungal spores of sooty mould. Further studies would be required to assess the potential for honeydew honey production locally.

## 3 Discussion

The most important finding in this study is without doubt the high percentage frequency of Thyme pollen observed in Maltese thyme honey. In their studies on the characteristics of thyme honeys from Greece, Tsigouri and Passaloglou-Katrali (2000) obtained an average pollen percentage frequency for Thyme of 42% in the honey samples studied and report that the percentage of Thyme pollen in islands can be as high as 85-90%.

The production of honeys with a high percentage of Thyme pollen is possible in the Maltese islands as Thyme has a wide distribution range, being a typical species of Maltese garigue and phrygana: habitats that are very frequent in the Maltese Islands, especially in the north-west of Malta and the island of Comino. In addition, this plant species is in full bloom during the month of June in which little else is in flower. Thus, if a beekeeper monitors closely the flowering period of this species and places the bee hives in an area that is rich in Thyme, the beekeeper may be able to produce a honey which is purely characterised by Thyme, when the honeycombs are removed from the hives as soon as the Thyme flowers begin to fade.

### 3.1 Pollen Spectra

Pollen spectra comprised 11 to 28 pollen types. The honey samples studied possessed similar spectra in terms of species that occurred in highest abundance except for sample no. 18 from Ta' Qali, which exhibited Rhamnus (29%), Euphorbia (14%), Ceratonia, Citrus and Asparagus as the most abundant pollen types. This honey possessed a spectrum quite unlike the other honey samples studied.

However, the results of the qualitative analysis did not show any honey possessing a particular pollen spectrum that could be traced to a particular geographical area. This is quite understandable as the Maltese islands possess a small surface area and possess a rather homogeneous landscape and honey bees may travel very large distances to locate a food source.

Qualitative analysis not only gives information on the honey spectrum of a honey but can also be employed to observe the variability in the pollen spectrum of a honey from year to year. Honey samples number 10 and 28 were harvested by the same beekeeper from the same location in May 2004 and May 2005 respectively. Sample no. 10 was dominated by Hedysarum (78%) whereas sample no. 28 exhibited Diplotaxis, Galactites, Rhamnus and Vicia as the most abundant pollen types, with frequencies of 11-15%. Therefore, a unifloral Hedysarum honey was harvested in May 2004, while a multifloral honey was harvested in May 2005. This may reflect agricultural practice, since fields may have been planted with Hedysarum one year, but left fallow the next.

Changes in climate from year to year also affect the flowering period and floral abundance and this varies the food availability for the honey bees. As a result, different botanical species are exploited and so different honeys are produced. This highlights the importance of carrying out such microscopic analysis on honey on a yearly basis to determine its botanical origin if the beekeeper is to include this information on the honey label.

### 3.2 Honey label discrepancies

Beekeepers do have a basic idea of the botanical sources of their honey and some beekeepers label their honey with the plant species they believe to be the floral sources of the honey that they produce. However qualitative analysis of these honey samples revealed that several honey samples were incorrectly labelled. Honey sample no.1 from St. Paul's was harvested in July 2005 and the beekeeper labelled it as spring blossom honey. Analysis revealed this honey to be Thyme honey. Likewise, honey samples no.2 from Siġġiewi and no. 8 from Wied Musa were also labelled by the beekeepers as spring blossom honeys and found to be Thyme honeys. Honey sample no. 30 was labelled as honey

produced from Eucalyptus and Carob. Even though this honey did contain both pollen types (Eucalyptus 51% and Carob 36%), organoleptic analysis and physicchemical analysis of the honey would be required in order to further assess the honey and determine whether it is labelled correctly or not as the percentage frequencies alone are insufficient to precisely determine the botanical origin of this honey.

Both the Codex Alimentarius (2001) standards and the European Honey Directive (Directive 2001/110/EC) standards state that the botanical source of a honey may be reported on the label only if the honey has originated from that source and it possesses "the physicalchemical, organoleptic and microscopic characteristics" that are typical of honey that is produced from that source. Thus, beekeepers must be careful when stating the botanical source of their honey as incorrect labelling may lead to the withdrawal of the honey from the market. The botanical source can only be determined by microscopic analysis of the honey together with sensory and physicochemical analysis. If a beekeeper is to label a honey with the botanical origin, the honey must be subjected to these tests in order to verify the floral sources of the honey and thus its botanical origin.

## 3.3 Adulteration

Four honey samples studied (nos. 32 – 35) were found to be so poor in pollen grains that 700 grains could not be counted and identified even when the entire sample preparation was observed under the microscope. Due to the small size of these samples, it was not possible to carry out a second extraction in order to obtain a picture of the pollen spectrum of the honey, except for sample no. 34. Still only 328 pollen grains were counted from the two slide preparations. Such low pollen counts were difficult to explain without the aid of quantitative analysis and physicochemical analysis. Unfortunately, quantitative analysis could not be carried out due to the lack of the required apparatus.

Quantitative analysis makes use of a Millipore vacuum filter pump to extract all the pollen grains found in 10g of honey and 500 pollen grains are counted. The number of visual fields which must be observed in order to count 500 pollen grains depends on their density. If the density of the pollen grains is high, fewer fields will be required. The total number of pollen grains in the sample is then calculated using a mathematical formula that takes count of the area of the microscopic field at the magnification used to count the pollen grains and the surface area of the filter paper containing the sediment (Von der Ohe et al., 2004). According to the number of elements (honeydew elements are also counted), honey is placed into five classes. Class I contains less than or equal to $20 \times 10^3$ elements and includes honeys with under-represented pollen, Class II contains between $21 \times 10^3$ and $100 \times 10^3$ elements and includes multifloral honeys, honeydew honeys and mixtures of both, Class III contains between $101 \times 10^3$ and $500 \times 10^3$ elements and includes unifloral honeys with overrepresented pollen and honeydew honeys, Class IV contains between $501 \times 10^3$ and $10^6$ elements and includes unifloral honeys with strongly overrepresented pollen and some pressed honeys, Class V includes more than $10^6$ pollen grains and includes almost only pressed honey.

The four honey samples, which exhibited an underrepresentation of pollen, could have been fraudulently manipulated by the addition of sugar syrup. This theory, could however, only be confirmed by physicochemical analysis of the honey. Adulteration of honey is the addition of foreign substances to a food product (Sanford, 2003). This is a common practice in which sugar is improperly fed to the honey bees during the honey flow or sweeteners are added to the honey. Sweeteners identified in honey include molasses, corn syrups, maple syrups, sugar cane and sugar beet (Ruoff and Bogdanov, 2004). However, the adulterant that causes greatest concern is the addition of High Sucrose Corn Syrup (HFCS) to honey (Sanford, 2003).

Adulteration of honey with $C_4$ sugars from the addition of sugar cane or corn syrup may be determined by microscopic analysis in which parenchyma cells, single cells from ring vessels, and epidermal cells that originate from sugar cane stems are counted (Kerkvliet and Meijer, 2000). The presence of a high number of plant cells is a good indication of adulteration by cane sugar. However, the official method for analysing adulteration with cane or corn sugar is the measurement of $C_{13}$ present in the honey, expressed as $\delta^{13}C$, which is measured using an isotope ratio mass spectrometer (IRMS) (Kerkvliet and Meijer, 2000). $C_4$ plants such as sugar cane absorb more carbon dioxide than $C_3$ plants, which are the original botanical sources of honey.

Adulteration by the addition of sugar cane and beet is also measured by infrared spectroscopic methods, while addition of high fructose corn syrup is also identified by the analysis for the presence of oligosaccharides, which are not normally present in honey (Ruoff and Bogdanov, 2004).

## 3.4 Filtration

One honey sample from Magħtab (honey sample no. 31) possessed a high density of diatomaceous sand crystals and an extremely low occurrence of pollen grains. Diatomaceous sand crystals are colourless and semi-transparent when observed under the microscope. They are also irregularly shaped. Their high density in the honey sample prevented the correct identification of the few pollen grains that were present.

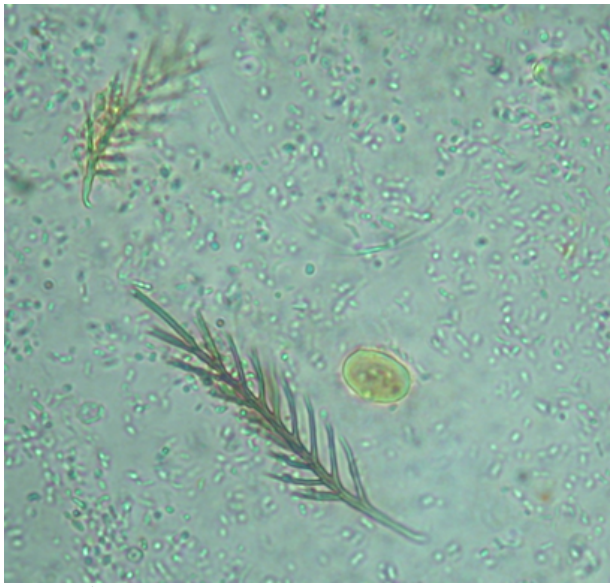

Figure 5: A contaminated honey sample.

Diatomaceous sand is usually employed to filter honey in third countries such as North and South America. The newly revised legislative requirements defined in the Codex Alimentarius, 2001 and the EU Honey Directive (Directive 2001/110/EC) permit the filtration of honey with a mesh size smaller than 0.2 mm with loss of pollen, only if this is unavoidable for the removal of foreign organic and inorganic matter. In any case, honey that has been filtered must be clearly labelled as "filtered" honey if it is to be placed on the market (Ruoff and Bogdanov, 2004). Filtering of honey is not carried out in the Maltese islands and when this honey sample was sent to Professor Giancarlo Ricciardelli D'Albore from the University of Perugia, for a second opinion, he confirmed that the honey most probably was obtained from a Latin American country such as Mexico.

The placement of honey on the market as locally produced honey, even though it was not clearly labelled as honey produced in Malta, is misleading to the consumer and is considered to be a fraudulent practice. It is therefore imperative to carry out routine analyses on honey samples in the Maltese islands to ensure that fraudulent activities are prevented and that the authenticity of these products and their quality is guaranteed to the consumer.

### 3.5 Hygiene

The poor hygiene of many honey samples was observed during the qualitative analysis(figure 5). Many samples possessed more bee hairs than pollen grains. Several honey samples were also observed to be contaminated by bacteria, such as the honey sample from San Martin.

Beekeepers are currently required to pass a hygiene exam in order to obtain a licence to produce honey. However, more work is required to ensure that proper hygienic standards are maintained by local beekeepers.

## 4 Conclusion

Botanical characterisation of honey is a vital tool in identifying the floral sources of Maltese honey and the characterisation of them. It is also an extremely useful tool in identifying fraudulent practices in honey production, such as adulteration.

This paper is based on a dissertation submitted by CG to the Department of Biology, Faculty of Science, University of Malta in part fulfilment of the Degree of Master of Science.

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# Appendix 1

Results of the qualitative analysis of pollen types in Maltese honey, represented as percentages

| **Pollen Type** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Aizooaceae*** | | | | | | | | | | | | | | | |
| *Aptenia* type | - | - | - | - | - | - | 2 | - | - | - | - | - | - | - | - |
| *Carpobrotus* type | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| ***Amaryllidaceae*** | | | | | | | | | | | | | | | |
| *Allium* type | - | - | + | + | - | 1 | - | - | - | - | - | - | - | - | - |
| ***Apiaceae*** | | | | | | | | | | | | | | | |
| *Daucus* type | - | - | - | 3 | 3 | 2 | 7 | 10 | 3 | - | 2 | 1 | 3 | 3 | + |
| *Eryngium* type | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - |
| *Ferula communis* | - | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - |
| *Smyrnium* type | 6 | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| Apiaceae type | 3 | - | - | - | - | - | - | - | + | - | - | - | - | - | - |
| ***Araliaceae*** | | | | | | | | | | | | | | | |
| *Hedera* type | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| ***Arecaceae*** | | | | | | | | | | | | | | | |
| *Arecaceae* type | + | - | + | - | - | - | - | - | - | - | - | - | - | - | + |
| ***Asparagaceae*** | | | | | | | | | | | | | | | |
| *Agave* type | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Asparagus* type | - | 4 | - | - | - | - | - | - | 1 | - | - | 1 | - | 3 | - |
| ***Asteraceae*** | | | | | | | | | | | | | | | |
| *Carthamus* type | - | - | - | - | + | 1 | - | - | 1 | - | 1 | - | - | - | - |
| *Glebionis coronaria* | - | - | + | - | + | + | 1 | + | + | - | - | + | 1 | + | - |
| *Glebionis* type | 1 | - | - | + | - | + | + | + | + | + | + | - | - | + | - |
| *Galactites* type | 1 | 2 | 1 | - | - | 9 | 1 | 1 | 10 | + | 17 | 1 | 2 | 2 | - |
| *Helianthus* type | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Limbarda crithmoides* | 1 | 2 | + | 3 | - | + | 1 | + | - | - | + | - | - | + | - |
| *Matricaria* type | - | - | - | - | + | - | - | - | + | - | - | - | - | - | - |
| ***Boraginaceae*** | | | | | | | | | | | | | | | |
| *Borago officinalis* | - | - | - | - | 2 | 1 | - | - | 1 | - | 1 | 4 | + | 1 | - |
| *Cerinthe* type | - | - | - | - | - | - | + | + | - | - | - | 1 | - | 7 | - |
| *Echium* type | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - |
| ***Brassicaceae*** | | | | | | | | | | | | | | | |
| *Brassica* type | - | - | - | - | - | + | - | - | + | - | 1 | + | - | 1 | + |
| *Diplotaxis* type | 15 | 18 | - | 2 | 5 | 3 | 2 | - | 4 | 3 | 2 | 1 | 5 | 1 | 3 |
| ***Capparidaceae*** | | | | | | | | | | | | | | | |
| *Capparis* type | - | - | - | - | 8 | - | - | - | + | + | - | - | - | - | - |
| ***Caprifoliaceae*** | | | | | | | | | | | | | | | |
| *Lonicera* type | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - |
| ***Convolvulaceae*** | | | | | | | | | | | | | | | |
| *Convolvolus* type | - | - | - | - | - | + | 1 | + | - | - | - | - | + | - | - |
| ***Cucurbitaceae*** | | | | | | | | | | | | | | | |
| *Cucumis* type | - | - | + | - | - | - | + | - | - | - | - | - | + | - | - |
| *Cucurbita* type | - | - | + | - | - | - | - | + | - | - | - | + | - | - | - |
| *Ecballium* type | - | - | - | - | - | - | - | - | - | - | 2 | - | - | - | - |
| ***Cuppressaceae*** | | | | | | | | | | | | | | | |
| *Cupressus* type | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| ***Ericaceae*** | | | | | | | | | | | | | | | |
| *Erica multiflora* | - | - | - | - | - | - | - | - | - | - | - | - | - | + | 1 |
| ***Euphorbiaceae*** | | | | | | | | | | | | | | | |
| *Euphorbia* type | - | 5 | - | - | 2 | + | 1 | - | - | - | - | - | - | - | - |

| **Pollen Type** | **16** | **17** | **18** | **19** | **20** | **21** | **22** | **23** | **24** | **25** | **26** | **27** | **28** | **29** | **30** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Aizooaceae*** | | | | | | | | | | | | | | | |
| *Aptenia* type | - | - | - | - | 7 | - | - | - | - | - | - | - | - | - | - |
| *Carpobrotus* type | - | - | 14 | - | - | - | - | - | - | - | - | - | - | - | - |
| ***Amaryllidaceae*** | | | | | | | | | | | | | | | |
| *Allium* type | - | - | - | - | - | - | - | - | - | - | - | - | - | + | 1 |
| ***Apiaceae*** | | | | | | | | | | | | | | | |
| *Daucus* type | 2 | 2 | - | 2 | 3 | 2 | 4 | + | 21 | 7 | 2 | 2 | - | 7 | 1 |
| *Eryngium* type | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Ferula communis* | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Smyrnium* type | - | - | - | - | - | - | - | - | - | - | - | - | 1 | - | - |
| Apiaceae type | - | - | - | - | - | 1 | - | - | 3 | 1 | 1 | - | - | 22 | - |
| ***Araliaceae*** | | | | | | | | | | | | | | | |
| *Hedera* type | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 | + |
| ***Arecaceae*** | | | | | | | | | | | | | | | |
| *Arecaceae* type | - | - | - | 1 | + | - | - | - | - | + | - | - | - | - | - |
| ***Asparagaceae*** | | | | | | | | | | | | | | | |
| *Agave* type | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| *Asparagus* type | - | - | 10 | 1 | - | - | + | - | + | 5 | - | - | - | - | - |
| ***Asteraceae*** | | | | | | | | | | | | | | | |
| *Carthamus* type | - | - | - | - | + | - | 1 | - | - | - | - | - | - | - | - |
| *Glebionis coronaria* | - | - | - | - | - | + | - | - | + | - | + | - | - | - | + |
| *Glebionis* type | - | - | - | + | 1 | + | + | - | + | + | + | - | - | 1 | + |
| *Galactites* type | 4 | 10 | - | + | 1 | 6 | 1 | - | 6 | 9 | 5 | 3 | 10 | 2 | + |
| *Helianthus* type | - | - | - | - | - | - | - | + | - | - | - | - | - | - | - |
| *Limbarda crithmoides* | - | - | + | + | 1 | - | + | 1 | - | - | - | - | - | 1 | + |
| *Matricaria* type | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| ***Boraginaceae*** | | | | | | | | | | | | | | | |
| *Borago officinalis* | 4 | + | - | + | + | 1 | 1 | - | - | + | 1 | 1 | 2 | - | 1 |
| *Cerinthe* type | 1 | - | - | - | - | - | - | - | - | + | - | - | - | - | - |
| *Echium* type | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| ***Brassicaceae*** | | | | | | | | | | | | | | | |
| *Brassica* type | - | 1 | - | 1 | - | - | - | - | + | - | - | - | - | 1 | - |
| *Diplotaxis* type | 2 | 5 | + | 5 | 2 | 3 | 1 | + | 6 | 9 | 3 | 5 | 14 | 6 | 23 |
| ***Capparidaceae*** | | | | | | | | | | | | | | | |
| *Capparis* type | - | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - |
| ***Caprifoliaceae*** | | | | | | | | | | | | | | | |
| *Lonicera* type | + | - | - | + | - | - | - | + | - | - | - | - | - | - | - |
| ***Convolvulaceae*** | | | | | | | | | | | | | | | |
| *Convolvolus* type | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| ***Cucurbitaceae*** | | | | | | | | | | | | | | | |
| *Cucumis* type | - | - | - | 1 | - | - | - | - | - | - | - | - | - | - | + |
| *Cucurbita* type | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - |
| *Ecballium* type | 3 | + | - | - | - | - | + | - | - | - | - | - | - | - | - |
| ***Cuppressaceae*** | | | | | | | | | | | | | | | |
| *Cupressus* type | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - |
| ***Ericaceae*** | | | | | | | | | | | | | | | |
| *Erica multiflora* | 1 | 1 | - | 4 | - | 1 | 1 | - | - | + | + | - | - | - | - |
| ***Euphorbiaceae*** | | | | | | | | | | | | | | | |
| *Euphorbia* type | - | + | 14 | + | - | 1 | - | - | - | 1 | 1 | - | 7 | - | 1 |

| **Pollen Type** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Fabaceae*** | | | | | | | | | | | | | | | |
| *Acacia* type | + | - | + | - | 1 | + | 1 | 3 | - | - | + | - | - | 1 | - |
| *Ceratonia siliqua* | - | 7 | 3 | - | - | - | - | - | - | 1 | - | - | 1 | - | - |
| *Cercis siliquastrum* | - | - | - | - | - | - | - | - | - | - | - | - | + | + | - |
| *Hedysarum coronarium* | + | - | 1 | 2 | 12 | 1 | 10 | 9 | 50 | 64 | 55 | 26 | 39 | 7 | 33 |
| *Lotus* type | 2 | + | - | 3 | 3 | 2 | 6 | 12 | 2 | - | 4 | 57 | 10 | 33 | 25 |
| *Medicago* type | + | - | - | - | - | - | - | - | 2 | + | - | - | - | + | + |
| *Trifolium* type | + | - | - | - | - | - | - | - | + | - | - | 1 | - | - | - |
| *Vicia* type | 3 | + | 1 | - | 3 | 13 | 1 | 2 | 2 | + | 1 | - | 1 | 7 | + |
| ***Geraniaceae*** | | | | | | | | | | | | | | | |
| *Geranium* type | - | - | - | - | - | - | + | - | - | - | - | - | - | - | - |
| ***Lamiaceae*** | | | | | | | | | | | | | | | |
| *Lavandula* type | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| *Teucrium* type | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - |
| *Thymus capitata* | 28 | 10 | 33 | 65 | 49 | 10 | 54 | 33 | 1 | + | - | - | - | 3 | - |
| ***Myrtaceae*** | | | | | | | | | | | | | | | |
| *Eucalyptus* type | 2 | 23 | 38 | 5 | - | 8 | 1 | - | - | - | + | - | - | 6 | 1 |
| ***Oleaceae*** | | | | | | | | | | | | | | | |
| *Olea* type | - | - | - | - | - | - | - | 1 | - | - | - | - | - | - | + |
| ***Oxalidaceae*** | | | | | | | | | | | | | | | |
| *Oxalis pes-caprae* | 1 | + | + | 1 | 1 | 1 | - | 1 | + | + | 2 | 1 | + | + | - |
| ***Papaveraceae*** | | | | | | | | | | | | | | | |
| *Papaver* type | 1 | 2 | 1 | 2 | + | 13 | - | 4 | 11 | 18 | 4 | - | 23 | 11 | 8 |
| ***Plantaginaceae*** | | | | | | | | | | | | | | | |
| *Plantago* type | 1 | - | - | - | - | - | - | 3 | + | - | - | + | + | - | + |
| ***Poaceae*** | | | | | | | | | | | | | | | |
| *Poaceae* | - | - | - | - | 1 | 1 | - | - | - | - | - | - | - | - | - |
| ***Resedaceae*** | | | | | | | | | | | | | | | |
| *Reseda* type | 2 | 6 | 16 | 1 | - | 18 | 1 | 4 | - | - | - | - | 4 | 2 | - |
| ***Rosaceae*** | | | | | | | | | | | | | | | |
| *Eriobotrya* type | 5 | 2 | - | 5 | - | - | - | - | + | 1 | - | 3 | 2 | 1 | - |
| *Malus* type | - | - | - | - | - | - | 1 | 1 | - | - | 3 | - | - | - | - |
| *Prunus* type | 1 | 3 | - | - | - | - | - | - | - | - | - | - | + | 1 | - |
| ***Rhamnaceae*** | | | | | | | | | | | | | | | |
| *Rhamnus* type | - | - | - | - | 1 | - | 2 | + | - | 2 | 1 | - | - | + | 5 |
| ***Rutaceae*** | | | | | | | | | | | | | | | |
| *Citrus* type | + | - | - | 1 | - | 1 | 2 | - | + | 1 | 2 | + | - | + | 4 |
| ***Simaroubacaceae*** | | | | | | | | | | | | | | | |
| *Ailanthus* type | - | 3 | - | - | - | 1 | - | - | - | - | - | - | - | - | 4 |
| ***Smilacaceae*** | | | | | | | | | | | | | | | |
| *Smilax* type | - | 1 | - | - | - | - | - | - | - | 2 | - | + | - | - | - |
| ***Solanaceae*** | | | | | | | | | | | | | | | |
| *Nicotiana* type | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| ***Vitaceae*** | | | | | | | | | | | | | | | |
| *Vitis* type | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

| **Pollen Type** | **16** | **17** | **18** | **19** | **20** | **21** | **22** | **23** | **24** | **25** | **26** | **27** | **28** | **29** | **30** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***Fabaceae*** | | | | | | | | | | | | | | | |
| *Acacia* type | 1 | - | - | + | 1 | - | - | + | - | + | - | - | + | - | - |
| *Ceratonia siliqua* | - | - | 14 | 2 | + | 1 | - | 35 | - | 10 | + | - | - | - | 38 |
| *Cercis siliquastrum* | - | - | - | + | - | 1 | 1 | 1 | - | 1 | 1 | 4 | - | - | - |
| *Hedysarum coronarium* | 21 | 1 | - | 24 | 14 | 19 | 28 | 1 | 18 | 3 | 32 | 7 | 6 | 15 | 7 |
| *Lotus* type | 14 | 11 | - | 28 | 30 | 13 | 21 | 1 | 12 | 2 | 11 | 3 | 11 | 22 | 2 |
| *Medicago* type | - | - | - | - | - | 2 | 2 | - | - | 2 | 2 | - | 6 | - | - |
| *Trifolium* type | 5 | + | - | + | - | - | - | - | - | - | - | - | - | - | - |
| *Vicia* type | 11 | 16 | - | 3 | 3 | 1 | + | 1 | + | 3 | 1 | 10 | 15 | 2 | - |
| ***Geraniaceae*** | | | | | | | | | | | | | | | |
| *Geranium* type | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| ***Lamiaceae*** | | | | | | | | | | | | | | | |
| *Lavandula* type | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| *Teucrium* type | - | - | + | - | - | - | - | - | - | - | + | - | - | - | - |
| *Thymus capitata* | - | - | - | 5 | 11 | + | 9 | 17 | 2 | 5 | + | 4 | - | 1 | - |
| ***Myrtaceae*** | | | | | | | | | | | | | | | |
| *Eucalyptus* type | 3 | 8 | 10 | 8 | 5 | - | 4 | 51 | 1 | 22 | - | - | - | - | 1 |
| ***Oleaceae*** | | | | | | | | | | | | | | | |
| *Olea* type | - | - | - | - | - | - | - | - | 2 | - | - | + | - | - | - |
| ***Oxalidaceae*** | | | | | | | | | | | | | | | |
| *Oxalis pes-caprae* | 9 | 11 | + | 1 | 1 | 3 | 1 | - | 1 | + | 2 | - | 1 | + | + |
| ***Papaveraceae*** | | | | | | | | | | | | | | | |
| *Papaver* type | 12 | 3 | - | 1 | 6 | 38 | + | - | 5 | 12 | 33 | 17 | 6 | + | 7 |
| ***Plantaginaceae*** | | | | | | | | | | | | | | | |
| *Plantago* type | - | - | + | - | - | + | - | - | 3 | + | + | - | + | 1 | + |
| ***Poaceae*** | | | | | | | | | | | | | | | |
| *Poaceae* | - | - | - | - | 1 | - | - | + | - | - | - | - | - | - | - |
| ***Resedaceae*** | | | | | | | | | | | | | | | |
| *Reseda* type | 2 | 1 | + | 4 | 2 | 1 | 6 | - | 11 | 3 | 1 | - | - | 4 | 2 |
| ***Rosaceae*** | | | | | | | | | | | | | | | |
| *Eriobotrya* type | - | - | - | - | - | - | 1 | + | - | - | - | - | - | 3 | - |
| *Malus* type | 1 | - | - | + | + | - | - | - | 1 | - | - | 7 | 1 | - | - |
| *Prunus* type | - | - | - | 1 | - | - | + | - | - | - | - | - | - | - | - |
| ***Rhamnaceae*** | | | | | | | | | | | | | | | |
| *Rhamnus* type | - | 5 | 29 | + | - | + | 1 | - | 1 | - | + | - | 11 | 1 | 5 |
| ***Rutaceae*** | | | | | | | | | | | | | | | |
| *Citrus* type | 1 | 1 | 10 | 1 | + | + | 1 | - | + | 1 | + | 1 | - | + | - |
| ***Simaroubacaceae*** | | | | | | | | | | | | | | | |
| *Ailanthus* type | - | - | - | - | - | - | 1 | - | - | - | - | - | + | - | - |
| ***Smilacaceae*** | | | | | | | | | | | | | | | |
| *Smilax* type | 1 | - | - | 1 | - | - | - | - | - | + | - | + | - | + | - |
| ***Solanaceae*** | | | | | | | | | | | | | | | |
| *Nicotiana* type | - | - | - | - | - | + | - | - | - | - | + | - | - | - | - |
| ***Vitaceae*** | | | | | | | | | | | | | | | |
| *Vitis* type | - | - | - | - | - | - | - | - | - | - | - | - | - | 1 | - |

+ = values below 1%; - = absence of pollen type in honey sample

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*Research Article*

# The role of Public Transport in addressing Sustainable Mobility for the Elderly Population in Malta

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**Abstract.** Over the past few years, several countries have continued experiencing a growth in their elderly population. Similarly, a number of towns and villages in Malta registered a high elderly population in the last census (NSO, 2012). The elderly people are one of the dominant 'transport disadvantaged' groups in the community. This research aims to analyse whether the current public transport system in Malta is providing effective and efficient mobility for elderly in the town of Luqa. In order to analyse this, the study analysed spatial accessibility, sought to identify barriers encountered by the elderly when using public transport and determine temporal accessibility to medical care. Data was collected using telephone surveys, travel time and bus frequency surveys. Statistical analysis was carried out using IBM SPSS 20 and Geographic Information Systems. The study showed that proximity to bus stops in Luqa does not affect public transport use amongst the elderly. The main barriers that elderly encounter when using public transport are mainly related to long waiting times, lack of comfort on bus stops and inaccessible travel information. Finally, temporal accessibility from Luqa to the State's general hospital, Mater Dei, still requires improvements as it does not meet the desired time budgets of elderly people. By identifying the main concerns this study seeks to encourage policy makers and planners to target future development in public transport taking into consideration the requirements of the growing elderly population.

**Keywords** Elderly population – Public transport – Spatial and temporal accessibility – Barriers in public transport – Luqa.

## 1 Introduction

A main goal of sustainable mobility is to meet the travel demands of present and future population. One major demographic group which has specific mobility needs is the elderly population. Globally there are 800 million people (11% of the global population) that are over 60 years, and this is set to increase to two billion by 2050, representing 22 per cent of the global population (Bloom et al., 2011). The Maltese Islands are following the same trend. In 2011 the number of elderly people over 65 years of age in Malta was 67,841, representing 16.3 per cent of the whole population, compared to 13.7 per cent in 2005 (NSO, 2012). Projections by the National Statistics Office (2011) reveal that the elderly population in the Maltese Islands will increase by 72 per cent in 2060 when compared to this segment of the population in 2010. Therefore, with such a continuous increase in the number of elderly people, analysing their mobility needs is fundamental because such growth will challenge the current transport and urban infrastructure. Different system requirements will be necessary to provide equal travel opportunities that support elderly mobility independence.

Sustainable mobility requires the need to promote travelling through an accessible and reliable public transport system (Gutiérrez et al., 2011). The elderly are one of the "transport disadvantaged groups" in society, that is people that use public transport because they have no other choice due to various factors, mainly age, disability, income and no access to private means of transport (Beimborn et al., 2003). Hence, one main contribution of public transport is to potentially

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minimize social exclusion and increase social justice for those in need (Farrington and Farrington, 2005). Lucas (2012) explains the interrelationship between transport disadvantage and other key issues that can lead to social exclusion (Fig.(1)). Being transport disadvantaged does not necessary mean being social disadvantaged. However these two aspects usually merge directly or indirectly and cause transport poverty. This subsequently results in inaccessibility to fundamental goods and services as well as exclusion from decision-making processes. As a result, social exclusion and further transport inequalities then follow.

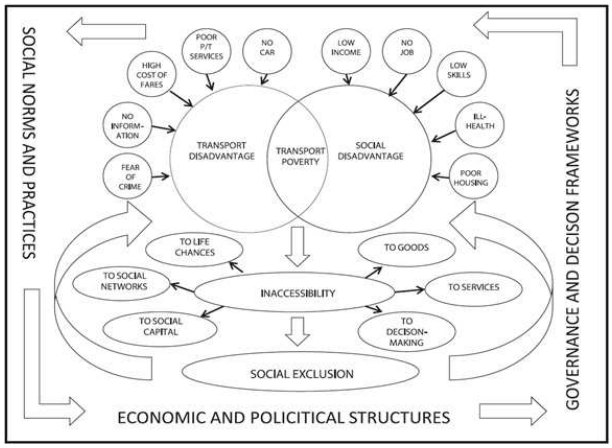


Figure 1: The relationship between transport disadvantage, social disadvantage and social exclusion. Source: Lucas (2012).

Consequently, accessible and reliable public transport is essential to provide the necessary mobility for the transport disadvantaged elderly. Marsden et al. (2007) state that for older people, the journey itself and the feeling of freedom it gives them, are often more important than the actual destination. Consequently, lack of access to transport among older persons could result in social isolation, lower self-esteem, feelings of uselessness, loneliness, unhappiness, low levels of physical activity, reduced independence and depression, which contribute to further poorer health conditions and risks (Victor et al., 2005; Hess, 2009). Shergold and Parkhurst (2010) explain that public transport, in the form of buses, is a key measure to support social sustainability as it helps to avoid social exclusion.

However, public transport is not always a reasonable substitute for private transport for older people (Hess, 2009). The use of public transport by the elderly is negatively affected by several restrictions such as physical limitations, lack of accessibility (e.g. absence of low floor buses, dangerous busy roads and high curbs), fear of falling, safety, bus design, bus driver behaviour, and overall declining quality of public transport systems (Wixey et al., 2005; Marsden et al., 2007). Also, public transport is often oriented towards commuters travelling during peak hours and hardly ever for the off-peak hours when elderly travel the most.

Another important issue to consider when analysing barriers in public transport is travel time. It is an important indicator for both operators and users and significantly impacts transport costs and benefits, particularly in regions trying to foster a modal shift to public transport (Newman and Kenworthy, 1999). Tribby and Zandbergen (2012) explain how determining changes in travel times is one measure of assessing public transport's accessibility equity. This type of accessibility is sometimes more important to study in public transport because frequency and service hours can make some of the necessary, but not temporally fixed obligations unreachable by bus. In actual fact, a crucial obligation, with particular reference to elderly people, is the access to medical care. Although healthier than their European counterparts, in 40 years' time Malta's elderly population will reach the 108,000 mark, of which 20 per cent are expected to require hospitalisation (Times of Malta, 2012). Hence, good and equal access to health care services is crucial for the elderly (Department of Health, 2002).

## 2 Proposed Methodology

The methodology proposed in this work aimed to analyse the relationship between elderly people and public transport accessibility. This was tackled through the analysis of spatial and temporal accessibility together with the analysis of barriers that elderly encounter when using public transport. Spatial and temporal accessibility are two main factors that affect public transport usage (Murray and Wu, 2003) and which fit into the multi-dimensionality of accessibility as a means to measure equity (Tribby and Zandbergen, 2012). However, good accessibility is usually hindered by several barriers. For this reason, the research highlights these obstacles and provides suggestions to minimise their impact. The main data collection methods used were (i) 200 telephone surveys to a sample of elderly people residing in Luqa, (ii) a GPS to locate the elderly residences and, (iii) travel time and bus frequency surveys to analyse temporal accessibility to the State's general hospital in Malta, Mater Dei. The research was carried out throughout a period of one year and three months (November 2011 – February 2013) of which two months (May –June 2012) were utilised for the telephone surveys data collection and another one month (July 2012) for the travel time and bus frequency surveys.

Telephone surveys were chosen because as Conrad and Schober (2000) explain, conversational interviewing ensures a uniform interpretation of the intent of each question. Telephone surveys also have large scale accessibility, particularly for elderly. A high percentage of old

people do not have access to a computer or to the internet so the telephone is the best way of communication for them. Telephone surveys are also more 'safe' compared to a home visit which creates a sense of 'fear'.

Several studies concerning accessibility only dealt with spatial accessibility (proximity). However as emphasised by other researchers, including Cheng (2008), Mavoa et al. (2011) and Tribby and Zandbergen (2012), public transport accessibility should include the walking time to bus stop (spatial accessibility), waiting time, trip journey and walking time from the disembarking stop to destination. In this study, the Closest Facility Function within the Network Analyst Extension in ArcGIS 10 was used to determine the average walking time from the elderly residences to their nearest bus stop. Service frequency is another vital aspect of accessibility and like travel time it can vary markedly between peak and off-peak hours. Bus frequency was analysed by listing all the public transport trips (of different routes) arriving on the respective bus stops.

The Pearson Chi-Square Statistical Test was used to analyse the relationship between various variables and the Kruskal Wallis Statistical Test was used to statistically analyse the effect of proximity to bus stop on public transport use. IBM SPSS 20 was used for the statistical analysis. ESRI's ArcGIS 10 with Network Analyst was used to spatially analyse the geographic data and visualise the results. The Network Analyst extension was specifically used to create buffers around the actual road network and provide a clearer picture of the actual walking distances between the sampled households and the location of bus stops. The Service Area Tool within Network Analyst was used to illustrate the catchment area based on the distance impedance factor. This means that the amount of resistance required to traverse paths in the network to access the bus stop was measured through walking distance. Higher impedance values indicated more resistance to movement.

# 3 The case study – the town of Luqa

Luqa is a town situated in the southeast of Malta, 6.4 kilometres away from the capital city, Valletta (Guillaumier, 2002). Hospital and medical centres are identified as common destinations for elderly people (Fuchs, 1999). The State's general hospital, Mater Dei is located in Msida and was the targeted destination for analysing temporal accessibility to medical care (Fig.(2)).The elderly people in Luqa (60+) amount to 31.65 per cent of the entire locality population (NSO, 2007). Nonetheless it should be highlighted that this population figure, derived from the 2005 Census of Population and Housing, include persons residing in institutions. For this reason, a very important contribution for the high elderly population in Luqa is the state residential home of St Vincent de Paule (the largest elderly residential home in Malta established in 1862). It currently hosts approximately 1,000 residents. Therefore, excluding the number of elderly residing in St Vincent de Paule, the percentage of elderly people in Luqa is approximately 18.5 per cent. Luqa is also identified as one of the localities in Malta that is likely to have an increase in the number of elderly people in the future (MEPA, 2006).

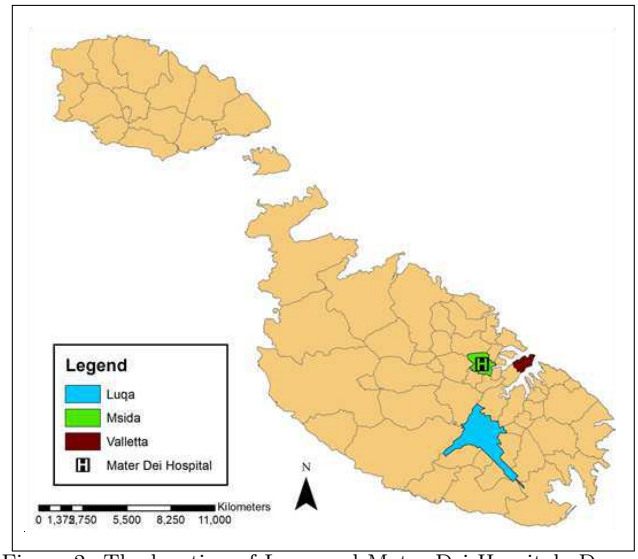



Figure 2: The location of Luqa and Mater Dei Hospital. Drawn by author

Luqa is a residential town which still retains a traditional nucleated morphology. Its historical core is characterized by a narrow organic street pattern. The settlement's shape is organized in concentric streets radiating from the main parish church (MEPA, 2006). Luqa also has an expanding hamlet, Hal Farrug. It was therefore very interesting to analyse the patterns of residence of elderly people in relation to the public transport network (proximity). Despite being a traditional town, very important land uses and landmarks such as the Malta International Airport and St Vincent de Paule Elderly Residence are located in this town (Fig.(3)). These highly influence the transport network as well as the way public transport operates. No transport studies have ever been carried specifically focusing on this town therefore studying and analysing mobility demands of the older population(in particular trips to the general hospital) was essential, especially for future planning and investment in the public transport infrastructure.

# 4 Results and Discussions

The results of the surveys showed that only 35.5 per cent of the sampled elderly population in Luqa were in possession of a driving licence. In addition to this, 12.5 per cent of the males and 35 per cent of the females who held a valid driving licence did not own a car. Sixty-six per cent of the elderly were weekly-bus users. This confirmed what was discussed in Section 1 that the elderly highly depend on public transport for their mobility needs. Nonetheless, this study also showed high car availability because despite the fact that 85.5 per cent of thesample did not own a car, they claimed to have access to one. The main travel purposes identified through the questionnaire were shopping and medical care. The most common modes of transport were walking and by car. The bus was mainly used by the older old, particularly females, for medical purposes.

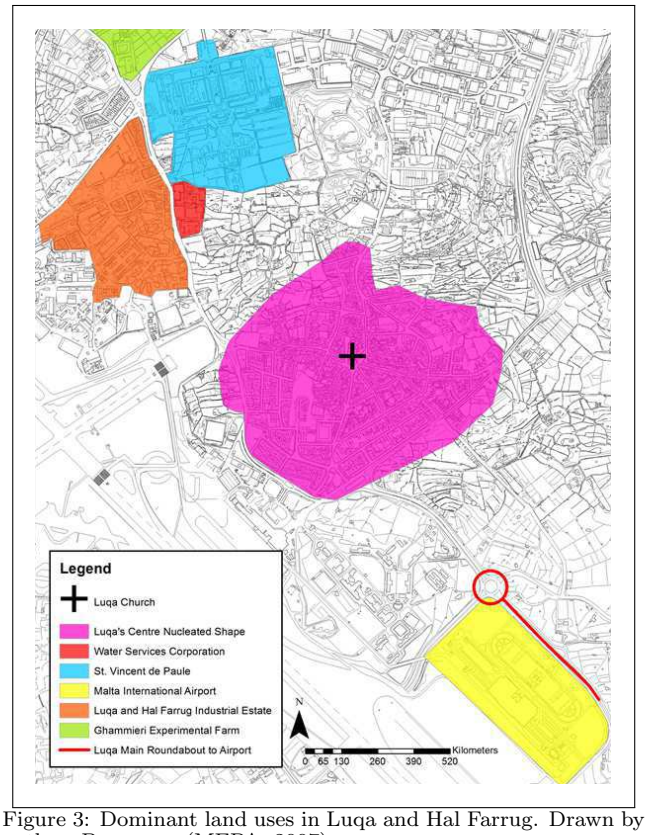



Figure 3: Dominant land uses in Luqa and Hal Farrug. Drawn by author; Base map (MEPA, 2007)

The surveys also revealed that age, health condition and car availability affect mostly public transport usage.It was also evident that health status, car availability and long waiting times at bus stops were the primary reasons for low public transport use amongst the sampled population.

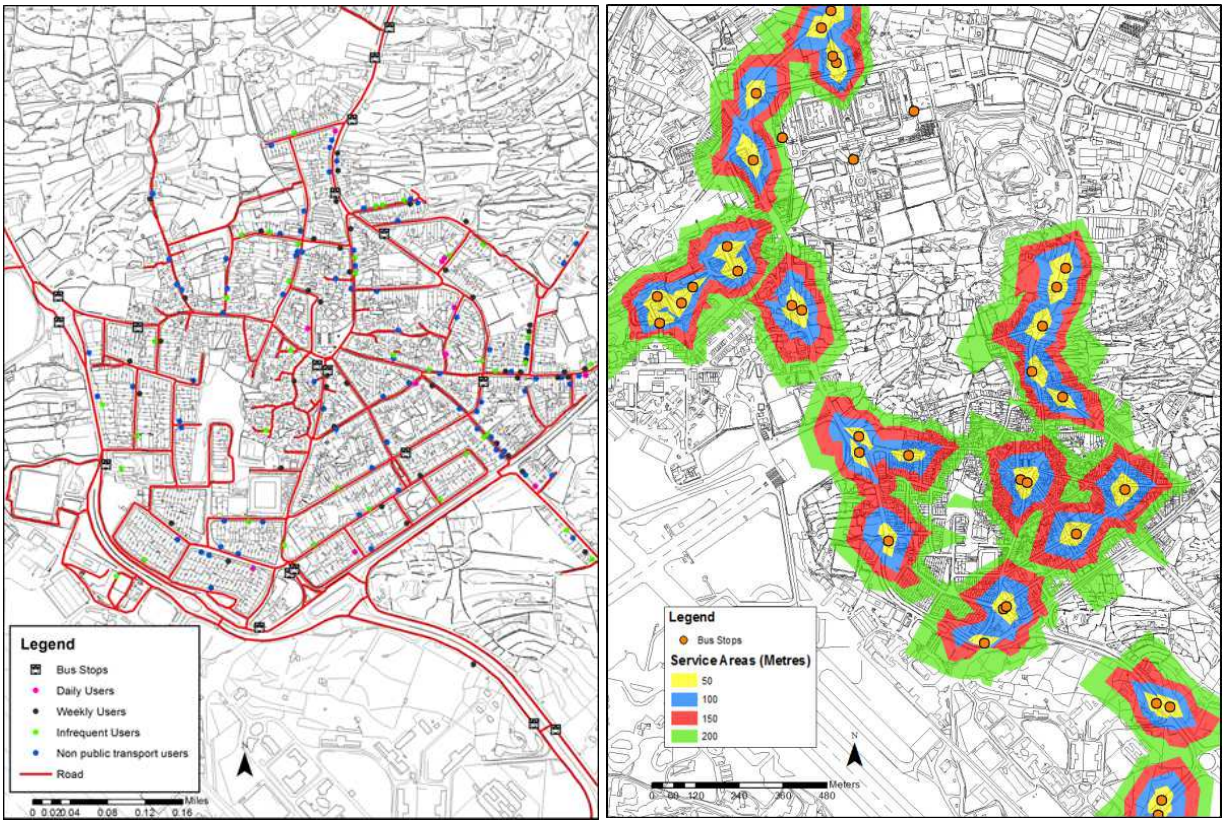
## 4.1 Spatial Accessibility

Spatial accessibility is one of the primary determinants of public transport use and only in the presence of such accessibility will a user consider other factors such as cost, comfort and security (Beimborn et al., 2003). It is nearly universally accepted that a 400 metres walking distance to the nearest bus stop is the maximum distance that people of all ages are willing to comfortably walk to access public transport (Murray and Davis, 2001; Zhao et al., 2003). However, these figures are not applicable to the local context due to the small size of the Maltese Islands. The average service area distance for Malta to access a bus stop is only 150 metres (MEPA, 2003). Obviously, this issue is more critical for the elderly population due to their age and related physical constraints. A Kruskal Wallis p-value of 0.239 indicated that the mean duration (in minutes) to reach the nearest bus stop did not vary much between the elderly people who use public transport daily, weekly, infrequently and never.

In order to analyse this issue spatially, the Network Analyst Extension in ArcGIS10 was used. The pedestrian network was created and eventually the sampled elderly residences (visualised according to public transport usage) and the bus stops were inputted. Their location was determined through the use of a GPS (Fig.(4a)). The impedance factor used in the study was 'metres' representing the distance that the elderly people have to walk through the network in order to reach their nearest bus stop. Ultimately, based on the inputted layers (Fig.(4a)) the creation of service areas determined whether elderly living closest to bus stops were frequent bus users or not (Fig.(4b)). The highest percentage of all elderly people fell within the 200 metres service area, meaning that for most of the sampled population, the 150 metres national threshold was exceeded. Moreover 66 per cent of the frequent bus users were also within the outer buffers which showed that proximity was not a crucial determinant for public transport use. The main conclusion of this analysis showed that proximity to bus stops was not a determinant factor affecting public transport use for sample population of elderly in Luqa.

## 4.2 Barriers encountered by elderly when using public transport

The research showed that 72 per cent of the sampled elderly people in Luqa encountered barriers when using public transport. The two most common barriers were long waiting times followed by a high criticism of the bus stops' infrastructure and comfort. These two issues are highly correlated to each other especially for older adults waiting in different weather conditions. The average walking speed for elderly people is of 1.3 metres




(e) Visualisation of the elderly addresses (divided according to public transport use), bus stops and road network in Luqa. Drawn by author.

(f) Formation of Service Areas (50, 100, 150, 200 metres) around the bus stops in Luqa and Hal Farrug, based on the road and bus stop layers. Drawn by author.

Figure 4

per second whilst that of younger adults is of 1.5 metres/second (Bohannon, 1997; Carey, 2005; Kang and Digwell, 2007). Therefore when dividing the average walking time to reach the nearest bus stop for a younger adult by that of an elderly there was an approximate difference of 86.7 per cent. This highlights the importance of good spatial accessibility for elderly. Although 150 metres are a relatively short distance, for most cases they were exceeded. When neighbourhood barriers were analysed, a considerable percentage (37 per cent) of the frequent users also complained that bus stops are not well distributed to cater for the needs of users from different zones and are also difficult to access due to traffic. This also leads to accumulative time wasted because pedestrians have to wait long to cross the main road. At times, this delays the bus on the stop resulting in more time wasted on route. The issues surrounding the distribution of bus stops and the current infrastructure highlights the need for a better design and location so that different needs of different demographic groups, particularly those of elderly people, could be met. The study also revealed that although proximity did not significantly affect public transport use, it was repeatedly mentioned as an important component of service quality for elderly mobility. Other barriers were related to

- the low frequency of particular services,
- the problems with punctuality which still plague the new bus service (Attard, 2013),
- the lack of accessibility,
- dated and inaccessible travel information,
- lack of safety,
- fear to travel alone and,
- inappropriate driver behaviour.

Informal discussions held with elderly on Luqa bus stops during fieldwork showed that for the majority of cases the elderly were not accurately informed about the correct time schedules and expected bus arrival. This lack of information might compound the amount of time the elderly spent waiting on the bus stop and the overall length of trip. This indicates that although there were actual delays in the public transport system (making the system unreliable) a crucial problem was the elderly population's lack of appropriate knowledge and information about the services.

The surveys also provided for some suggestions to improve the bus service and meet the demands of the el-

derly. These related to better infrastructure (including accessible walkways) and a better distribution of bus stops to minimise the walking distances. Other suggestions included the need for higher service frequency, better route coverage, improved reliability of service, better accessibility, increased safety inside the buses and more accessible travel information.

### 4.3 Temporal accessibility to Mater Dei Hospital

Temporal accessibility is highly interrelated with spatial studies. This work confirmed what was discussed in Polzin et al. (2002): the frequency of the bus service and for how long the users are willing to wait, are indispensable considerations to attract people to use public transport. The research studied temporal accessibility by bus from Luqa to Mater Dei Hospital (which covers a distance of approximately eight kilometres). This was an important issue to consider because as already discussed, medical trips together with shopping were the two most common purposes why elderly people in Luqa travel. Sixty-four per cent of the sampled population stipulated a desired time budget of 20 to 30 minutes. However, all the routes from Luqa to Mater Dei Hospital exceeded this time budget. The shortest route (Route 117) took an average travel time of 36.7 minutes in the peak hours and 35.3 minutes in the off-peak hours. This was followed by Route 118 and 135. The direct routes to hospital provided a shorter travel time than those which involved an interchange. The longest route was route X4/210 (involving the need to change bus at the Marsa Park and Ride Interchange) which exceeded the one hour travel time. Subsequently, when travel time was analysed cumulatively for the X4/210 service, the highest amount of time spent, was waiting on the bus stop particularly at the Marsa Park and Ride Interchange (Fig.(5)). The main reason for long waiting times at this interchange was not just caused by delay, but the lack of coordination between the schedules of the connecting routes arriving and departing from the interchange.

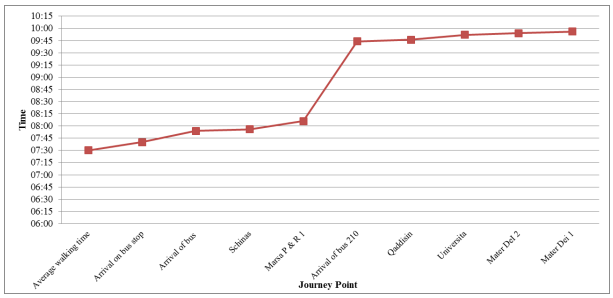



Figure 5: Cumulative travel time graph showing the very long waiting time spent at Marsa Park and Ride Interchange

The bus frequency surveys showed that only few routes operated according to the published time tables (mainly Route 117). Most routes, particularly Route X4 arrived early, delayed or failed to arrive (Fig.(6)). All this indicates that several improvements are required in the services from Luqa to Mater Dei, in an attempt to also improve temporal accessibility of elderly to medical care.

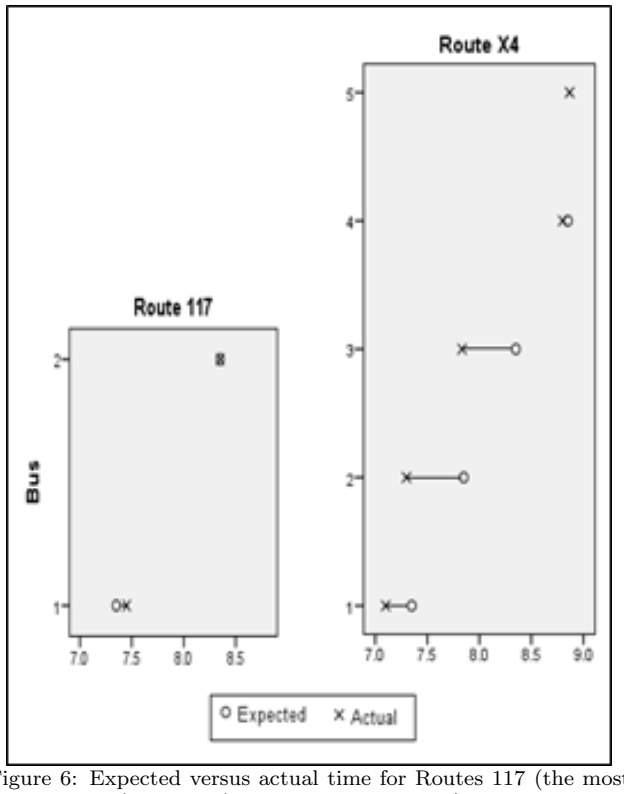



Figure 6: Expected versus actual time for Routes 117 (the most efficient route) and X4 (the least efficient route).

## 5 Relevance and limitations of the study

The chosen field of study has a significant relevance to the islands' socio-economic development and health status of the population. It highlights the importance of an accessible transport system and other indicators that can affect elderly mobility and quality of life. This study was a first to tackle elderly accessibility in public transport in Malta. It is fundamental for policy makers and planners to target future development in public transport, taking into consideration the needs of the elderly. This work also hopes to inform public transport operators to better understand the needs of their clients. Understanding how users perceive the public transport system is a critical issue in delivering accessible transport and subsequently ensuring sustainable mobility.

This research could serve as an incentive for further studies on a local level, that act as a guidance for the development of national policies or programmes target-

ing the mobility needs of an ageing society. The needs of the elderly are not so different from those of the rest of the population. They just become more critical with age. Hence helping old people to meet their needs makes travelling for all sectors ofthe society much easier.

The results could be highly transferable to other demographic groups and other areas in Malta. Particularly, they could be transferable to other 'transport disadvantaged' groups such as disabled people or women with young children. Since in Malta we just have one public transport operator, some of the results could also be easily transferable to other locations. Although the methods used for data collection and analysis (particularly the creation of service areas) were already used by other researchers, they were modified and adapted in a way to give a simple but effective representation of the situation in Luqa. Based on the findings, this study also gives several recommendations for improvements such as the need to improve bus stop design, increase comfort and accessibility, minimise the barriers that elderly encounter and improve the travel information. If elderly are furnished with easier and more understandable information (particularly on the media) they would be more knowledgeable and hence feel safer and more secure when travelling with public transport.

One limitation of the study was that it was based on one case study (Luqa) and not on a national scale. This means that the research results cannot be all transposed to the whole Maltese Islands. Nonetheless this could act as a motivation for future studies focusing on the whole of Malta. Another issue hindering the extension of the study to other areas in the island is the lack of geographic information related to the main and local road network. This study used Geographic Information Systems to analyse spatial and temporal accessibility. Without the reference data set about the road network, the extension of the study to the whole of the islands is not possible. Moreover, the fact that the study was carried out just one year after the public transport reform (started in 3rd July, 2011) could have affected in some manner the perception of the elderly towards the new public transport system.

# 6 Concluding Remarks

This research confirmed that elderly people in Luqa, a town with a high projection of elderly people in the future, has a high percentage of non-drivers. Despite this, it showed a relatively high car availability and high private mobility amongst its elderly. In most of the cases where elderly did not travel, the main reasons were related to age and health conditions and not due to infrastructure provision. The largest percentage of elderly travelled for shopping and medical purposes. This study disagreed with the literature with respect to the relationship between use of public transport and proximity. It is evident from the sampled population in Luqa that mobility by public transport is not affected by proximity to bus stop. In spite of this, as explained in detail in Sections 4.1 and 4.2, an analysis of walking distances to bus stops showed that most elderly lived beyond the national threshold of 150 metres from a bus stop, and informal discussions with elderly using public transport showed that distance impacted their use of the bus. The other barriers that were identified as contributing to difficulties in using public transport were mainly related to waiting times, lack of travel information and uncomfortable bus stop infrastructure. Hence, these barriers show that with reference to Luqa, the current public transport system in Malta is still not providing an efficient and fully accessible public transport service to the elderly. This was particularly the case when the study looked at the accessibility of the main hospital. The study found that average travel time by bus was not within the desired time budget. An efficient public transport system is seen as an important contributor to sustainable mobility. This study has shown that for elderly, a growing population segment in many cities worldwide, public transport systems still suffers weaknesses and offers challenges for their independent mobility.

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*Research Article*

# A Study of Nano-Particle Based Silane Consolidants for Globigerina Limestone

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**Abstract.** This STEPS[1] funded study focuses on the application of hybrid and nanoparticle loaded hybrid silane consolidants for the treatment of 'Franka' Globigerina Limestone. Consolidants act by gluing deteriorated stone material to underlying healthy stone (Dukes, 1972; Gutt, 1973; Garrod, 2001). The consolidants evaluated were a laboratory prepared hybrid silane based on a mixture of tetraethylorthosilicate (TEOS) and 3-(glycidoxypropyl) trimethoxysilane (GPTMS), the same hybrid loaded with silica nanoparticles and loaded with GPTMS-modified silica nanoparticles. In addition, a consolidant based on the hydrolysis product of TEOS was also tested.

Prepared consolidants were applied to test blocks by complete immersion. Untreated stone block were used as benchmarks. Following application, half of the treated samples were subjected to accelerated weathering. All limestone blocks were then characterised by colorimetry and optical and electron microscopy. The pore size distribution was assessed by Mercury Intrusion Porosimetry. A water absorption by capillarity technique was also carried out to assess any changes in water uptake rate. The mechanical properties were assessed by resistance to sodium sulfate crystallisation.

Microscopy observations showed that penetration into the stone occurred to different extents depending on the consolidant. The hybrid consolidant led to yellowing of the limestone but the addition of nanoparticles to the hybrid (modified or not) appeared to help restore the original colour of the stone. The porosity of the limestone was only marginally affected by the different treatments but the somewhat hydrophobic nature of the consolidants led to a disruption in the capillary flow of water into the limestone.

[1]Strategic Educational Pathways Scholarships

**Keywords** Globigerina Limestone - Nanoparticles - Hybrid - Alkoxysilane - Sol-gel.

## 1 Introduction

The stratigraphic setting of the Maltese Islands consists of five main formations. They are listed in chronological order (from older to younger) as follows: Lower Coralline Limestone, Globigerina Limestone, Blue Clay, Greensand and Upper Coralline Limestone (Spratt, 1943; Murray, 1890). Globigerina Limestone is exploited for its good building qualities. It can be described as fine-grained, full of foraminifera shells and visible fossils, and it is primarily composed of calcium carbonate (Cassar, 1999; Gatt, 2006). The microstructure consists of calcite crystallites cemented together by amorphous calcium carbonate which may contain up to 12% clay minerals and 8% quartz (Cassar 1999; Cassar and Vannucci, 2001). Globigerina Limestone is very porous; the volume percent porosity ranges between 32 and 41% with the majority of pores having a size $\leq$ 4 μm (Cassar 1999; Cassar and Vannucci, 2001). Globigerina Limestone may be sub-divided into: 1) franka stone, which exhibits good weathering properties and changes to a pale yellow colour with a resistant surface and 2) soll, which deteriorates rather easily by a process of cavitational weathering producing characteristic honeycomb structured erosional features (Vella et al., 1997). The franka limestone is the one chosen for structural building while the soll is primarily used in building foundations (Vella et al., 1997).

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Natural deterioration and weathering of stone monuments and buildings is inevitable even if there is no human intervention to cause damage. Thus, the need arises for the protection of these structures and the conservation of cultural heritage features in particular.

When it comes to conservation methods several practices can be undertaken, one of which is consolidation. The purpose of an ideal consolidant is to link together the deteriorated stone material with the underlying healthy stone without blocking the pores of the stone while maintaining the aesthetic and physical properties of the stone (Dukes, 1972; Gutt, 1973; Alessandrini et al., 1975; Garrod, 2001). Due to its irreversible nature, consolidation is very often embarked on as a last resort to save the stone when it has reached an advanced stage of deterioration.

Consolidants act by gluing together the deteriorated stone material with the underlying healthy stone (Dukes, 1972; Gutt, 1973; Alessandrini et al., 1975). Depending on the state of the stone, this process can be carried out before or after a cleaning programme. Consolidation is very often an irreversible process. It involves the introduction of organic or inorganic polymeric material within the stone pores making it practically impossible to reverse (Briffa et al., 2012). However, one must appreciate that the consolidation step is the last attempt to save the stone from complete replacement and these drastic measures, in such a situation, are tolerated (Garrod, 2001).


Figure 1: Location of Mqabba (marked in red) [accessed from http://upload.wikimedia.org/wikipedia/commons/thumb/3/32/Mqabba-map.svg/550px-Mqabba-map.svg.png on 24/06/2013]

Due to the increasing popularity of consolidants their performance requirements are constantly being improved. Primary performance requirements stipulate the conditions that a consolidant must fulfil such as physical properties or appearance. Secondary performance requirements include those conditions which are compulsory for a specific use and which are imposed in addition to primary requirements due to specific problems encountered (Clifton, 2008).

Commercial products containing alkoxysilanes, such as tetraethylorthosilicate TEOS, are commonly employed as consolidants for stonework. An advantage of these materials is that they are applied in monomeric form and polymerise by undergoing the sol-gel process involving hydrolysis and condensation reactions forming inorganic silica within the stone structure. Application viscosities are implicitly low and this minimises problems associated with consolidant penetration. Unfortunately, the unreacted monomer may be lost through evaporation (Brinker and Scherer, 1990).

Tetraalkoxysilane consolidants are reported to be very effective at consolidating sandstones (Wheeler, 2005; Wheeler et al., 2000; Weiss et al., 2000). As consolidant precursors polymerise inside the stone, covalent siloxane bonds (=Si-O-Si=) readily form between silanol groups (=Si-OH) present on the surface of sandstone and those on the surface of the growing silicate polymer by condensation reaction. The situation for limestone is reported to be less satisfactory due to silicate based consolidants having little affinity for calcite surfaces (Wheeler, 2005; Wheeler et al., 2000; Weiss et al., 2000). One way to improve this could be through the use of coupling agents.

Another problem that arises with TEOS consolidant films is that they tend to crack on drying (Brinker and Scherer, 1990). As solvent evaporates from a consolidant film (the sol), a point is reached when the gel network is exposed; from this point onwards, further evaporation of solvent occurs from within the pores of the gel structure. As a result, a build-up of capillary forces occurs which translate into tensile stresses and may lead to consolidant fracture (Kim et al., 2008). Yang and co-authors (1998, cited in Scherer and Wheeler, 2009) showed that by adding silica particles to a silicate consolidant, drying shrinkage was observed to decrease while elastic modulus increased. Furthermore, the nanoparticle loaded consolidant material was still able to penetrate the stone despite an increase in viscosity. The dried gel was observed to remain porous (Kim et al., 2008; Yang et al., 1998). Another advantage of particle-modified consolidants (PMCs) is that they seem to perform better in salt-laden environments. Aggelakopoulou et al. (2002) compared the behaviour of Ohio Massilian sandstone treated with PMCs to those treated with a conventional silicate consolidant in a salt crystallisation test. Salt efflorescence in PMC treated stone was enhanced. Aggelakopoulou argues that this is probably due to the fact that the nanoparticles aid capillary flow towards the exterior surface of the stone. Other authors have experimented with PMCs achieving quite promising results (Escalente et al., 2000; Kim et al., 2008; Mosquera and de los Santos, 2008).

In order to incorporate both the concept of the use of the coupling agents and that of nanoparticles, a hybrid sol was prepared based on the Self-assembled Nanophase

Particle (SNAP) surface treatment.

The study aims to compare the consolidation effects of simple silane systems and hybrid silane systems that were doped with silica nanoparticles or modified silica nanoparticles.

# 2 Methodology

## 2.1 Materials

The following chemicals were used as received: tetraethylorthosilicate, TEOS (Aldrich, Reagent Grade), 98% absolute ethanol (Aldrich, Chromasolv), dibutyltin dilaureate, DBTL (Aldrich, Fluka Analytical), 3-(glycidoxypropyl)trimethoxysilane, GPTMS (Aldrich, Reagent Grade), acetic acid (Aldrich, Reagent Grade), Diethylenetriamine, DETA (Aldrich, Reagent Grade) and silica nanoparticles ˜≈ 10nm (Aldrich, Reagent Grade). Franka-type Globigerina Limestone specimens measuring $50 \times 50 \times 50\,\mathrm{mm}^3$ were sourced from a quarry in the limits of Mqabba, a village situated to the south-east of Malta, Fig.(1). The freshly cut surfaces were ground to achieve a flat surface and cleaned of excessive dust using filtered compressed air.

## 2.2 Preparation of Consolidants

The basic silicate consolidant or TEOS consolidant was prepared by mixing TEOS, deionised water and absolute ethanol in a mole ratio of 1:2:5 in a closed glass vessel. The catalyst DBTL (1% v/v) was added to promote the hydrolysis-condensation reactions.

The hybrid sol was a mixture of TEOS and GPTMS in a mole ratio of 1:3 with the addition of 64.8mL of a 0.05M solution of acetic acid. This sol was allowed to mix for 3 days in a sealed container on a magnetic stirrer prior to the addition of 1% v/v DETA. DETA was added to act as the crosslinking agent. Once added the sol was then mixed with 4 volume parts water and immediately applied to the stone.

A mixture of the hybrid sol with the addition of 10% wt/v approximately 10nm silica nanoparticles was also applied as a consolidating system.

The final consolidating system tested was a mixture of the hybrid sol with the addition of 10% wt/v modified silica nanoparticles. The modified silica nanoparticles were prepared by means of the addition of GPTMS in a solution of water and acetic acid to 10nm silica nanoparticles. The quantities of these reactants were mixed in a ratio of 1 : 18.75 : 20 respectively. These values were obtained by calculating the ideal GPTMS ratio needed to react with the silica nanoparticles. The solution was left mixing overnight following which the solution was added to the hybrid sol and then immediately applied to the stone.

In addition to these treatments, a group of stone samples were left untreated to act as controls. This allowed for a comparison of treated stones with original untreated samples.

## 2.3 Mode of application

The consolidating treatments were applied by completely immersing the stone sample for 30 minutes in the consolidant to ensure an even application throughout. Whilst immersed the samples were wedged up from the base of the container using thin non-absorbent supports to act as point contacts. This was done so as to allow for better absorption through exposure of more surface area of the samples.

## 2.4 Drying

Treated samples were left to air dry for a period of 5 weeks prior to undergoing accelerated weathering. The samples that did not undergo accelerated weathering were left to stand and air dry for another 4 weeks until the accelerated weathering cycling was complete. Once this was done the treatment-sample interaction could be characterised and physical property testing could be carried out.

## 2.5 Accelerated weathering

Accelerated weathering was carried out so as to see how the treatment would fare when exposed to rain. This was achieved through repeated 24 hour wet-dry cycles. The cycling involved 8 hours exposure to water vapour at 35°C in an accelerated corrosion test chamber CNS/500 and 16 hours drying at room temperature. During the 16 hours of drying the test chamber was switched off and the samples were left inside the chamber with the lid wide open. The cycling was repeated 28 times for trial period of 4 weeks.

## 2.6 Characterisation

Colour alterations of the limestone specimens before and after treatment were measured with a Minolta CM-508i spectrophotometer. The samples were observed on a microscopic level by means of a Remet SMZ-2T light microscope. An electron microscope (Zeiss-Merlin Field Emission) was used to study the interaction of the consolidant with the interior stone pore surfaces. The interior of the treated stone was exposed by fracturing. The pore size distribution of the samples and the total porosity was determined through Mercury Intrusion Porosimetry. This was carried out using a Quantochrome PoreMaster (PM-60+12) at the Department of Physical Chemistry at the University of Cadiz in Spain.

## 2.7 Testing

A test to determine water absorption by capillarity was carried out according to EN1925:1999 and assessed the

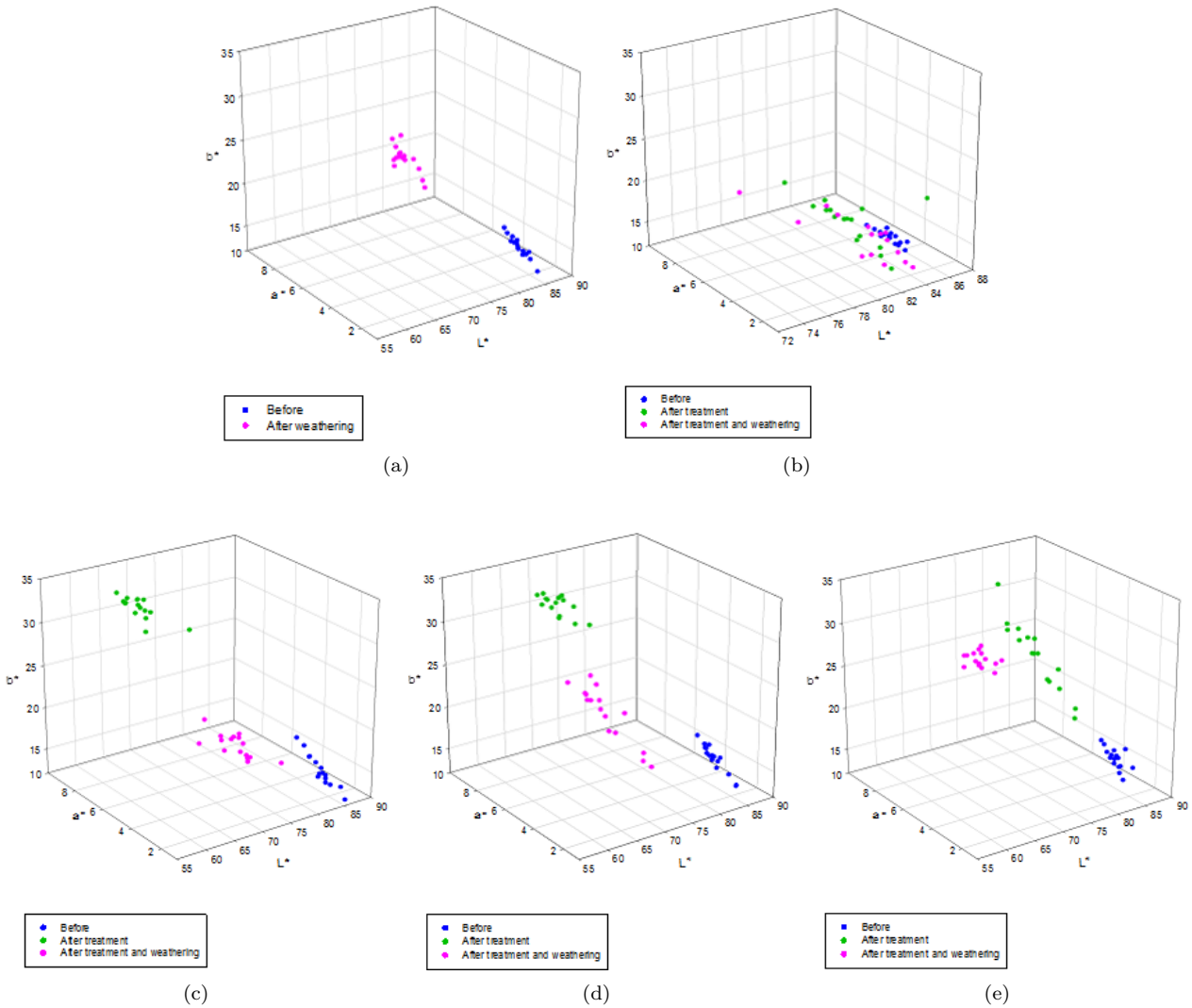



Figure 2: Colour data for before treatment, after treatment and after weathering for stone samples treated with b. TEOS, c. hybrid, d. nanoparticle loaded hybrid by immersion and e. modified nano-particle loaded hybrid by immersion.

flow of water into the stone after applying the different consolidant treatments.

A salt crystallisation test was carried according to EN12370:1999. It allowed for an indirect method of evaluating the mechanical strength of the limestone treated with the different consolidant systems.

# 3 Results and Discussion

## 3.1 Colorimetry

Fig.(2)(a-e) shows stone colour before treatment, after treatment and after treatment and weathering for the different consolidant treatments. The colour data are plotted on 3-d graphs of L* a* b* where L* represents lightness (0% black, 100% white), a* redness-greenness, and b* yellow-blueness. Of more importance to this work are the values of a* > 0 and b* > 0 which represent the colours red and yellow respectively. The colour of untreated Globigerina Limestone falls within the range: L* 77-82%, a* 0.8-1.4 and b* 16-18.5. In general, consolidant treatments led to a darkening of the limestone surfaces. This is in agreement with the results of an earlier study (Briffa et al. (2012) and the results of alkoxysilanes used in the field (Wheeler, 2005).

The largest discrepancy between the before and after L*, a* and b* values was noted for the samples treated with the hybrid by means of immersion (Fig.(2)(c)). The addition of nanoparticles also resulted in a large discrepancy whilst the addition of modified nanoparticles seems to result in a more spread out cluster that is closer to the original colour.

Weathering is seen to have an effect on the colour of the samples as even the untreated weathered sample showed a shift in the before weathering colour data re-

sults and the after weathering colour data results of the graph shown in Fig.(2)(a). The samples became slightly darker, slightly redder and mostly more yellow when compared to those that were not weathered. This is representative of what is reported in published literature regarding the weathering of 'Franka' type Globigerina Limestone. Cassar states that this type of stone withstands exposure well and changes into a honey-coloured stone upon ageing (Cassar, 2002).

## 3.2 Microscopy

### 3.2.1 Light Microscopy

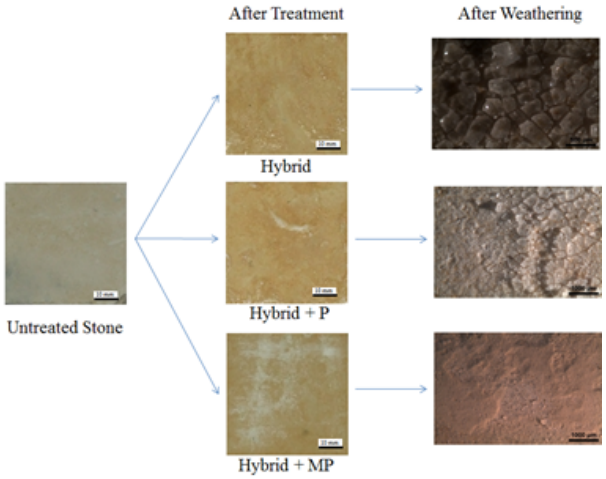



Figure 3: Surface appearance effect of hybrid based treated samples.

Observing the samples under low magnification revealed interesting features, some of which were unfortunately undesirable.

For the untreated sample surface, it is clearly seen that no treatment is present on the surface. The flow chart depicted in Fig.(3) shows the surface of the hybrid linked treatments after treatment and after treatment and weathering. It can be seen that that the addition of the nanoparticles or modified nanoparticles improved the surface colour of the sample when compared to the surface colour of the hybrid.

Although cracking was not seen for the non-weathered samples, the hybrid based treatments all cracked upon weathering. This was not desirable as it is probably interfering with the consolidating effect of the treatment. However, the degree of cracking, that was visually analysed, seemed to vary depending on whether the consolidating system applied contained nanoparticles or modified nanoparticles or neither.

### 3.2.2 Scanning Electron Microscopy

The relatively smooth and often flat surfaces of the foraminifera chambers offered ideal areas for observing the consolidant – limestone interactions. These can be clearly seen in the image of the untreated sample, Fig.(3.1). The distinctive, clear cut stone features observed for the untreated sample are not always seen for the treated samples due to surface cover by the treatment (Fig.(4)(b-e)). For the treated samples a layer of treatment material can clearly be seen on the surface of the stone.

TEOS based consolidants experience cracking during drying as a result of capillary forces. This is reported by a number of researchers (Kim et al., 2009) and was observed by means of the SEM in this work. The degree of cracking seen for the TEOS treated samples was not as extensive as that of an earlier study performed by the author (Briffa et al., 2012). This is possibly due to the difference in ambient temperature when the limestone samples were treated for the different projects.

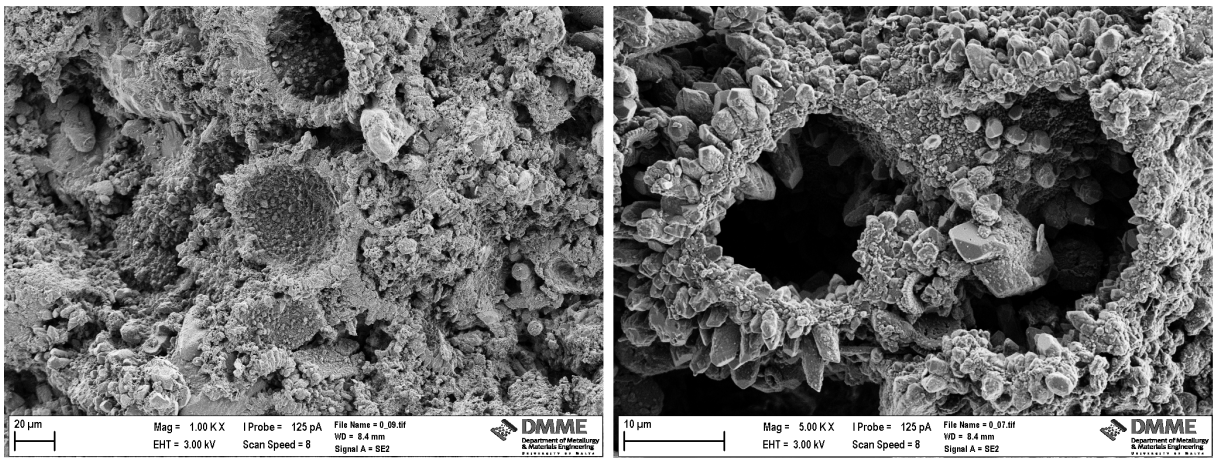
When compared to the TEOS treated micrographs, those of the hybrid based treated samples seem to show that there is more treatment present on the surface of the stone and the treatment layer is thicker.

The main aim of adding the nanoparticles was to reduce the major problem of gel cracking associated with alkoxysilanes. Literature has shown that the addition of particles seems to promote the production of a crack-free gel (Scherer and Wheeler, 2009; Mosquera and de los Santos, 2008).This was also seen with the addition of silica nanoparticles to a TEOS sol in an earlier project (Briffa et al., 2012).However in this case, at a microscopic level, no cracking was noted for the hybrid based treatments and the nanoparticles therefore had little or no effect on this property. The organic groups incorporated into the hybrid material render the consolidant more plastic and less prone to cracking. The addition of the nanoparticles to the hybrid sol led to the deposition of a thick layer of consolidant on the surface of the stone as seen in Figs.(4)(d,e) for the nanoparticle loaded and modified nanoparticle loaded hybrid treated samples respectively.

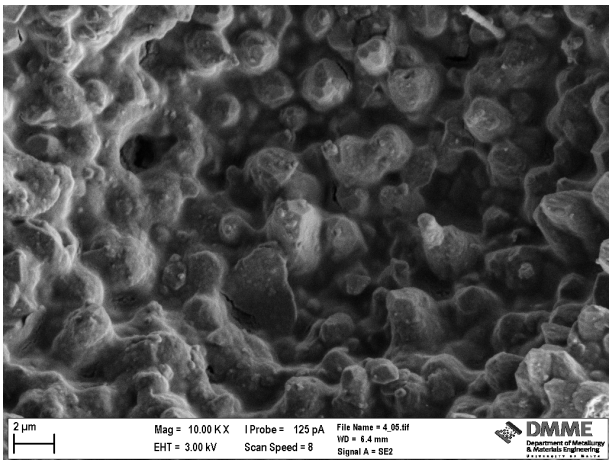
## 3.3 Mercury Intrusion Porosimetry

Mercury Intrusion Porosimetry (MIP) results show the effect of the treatment on the percentage porosity and the pore size distribution of the limestone samples treated by immersion compared to the untreated samples. A minimum of two repeated readings were carried out for each specimen treatment system and the pore size distribution and the percentage porosity for the repeats are very close. Therefore the results can be considered to be repeatable. This also shows that the treatment is evenly applied to each of the samples tested (Mosquera, 2012).

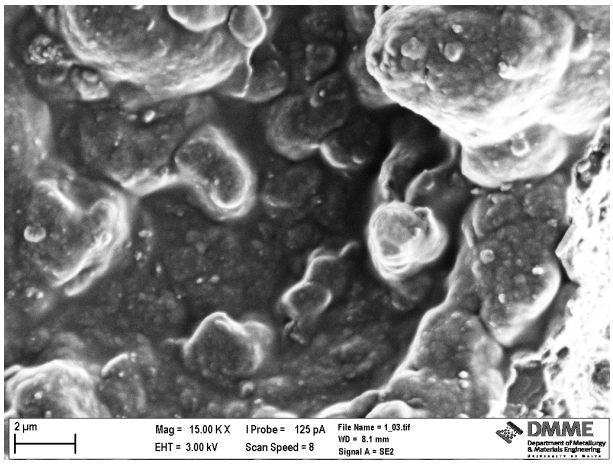
The percentage porosity of the untreated limestone ($\approx$ 39.29%) is in agreement with 32 -41% range values obtained by Cassar (Cassar, 2002). Indeed, even the



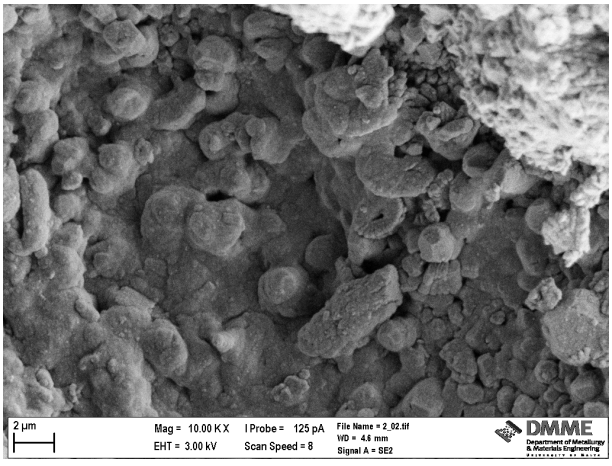

(a) Untreated stone at ×500 and ×5000 magnification.



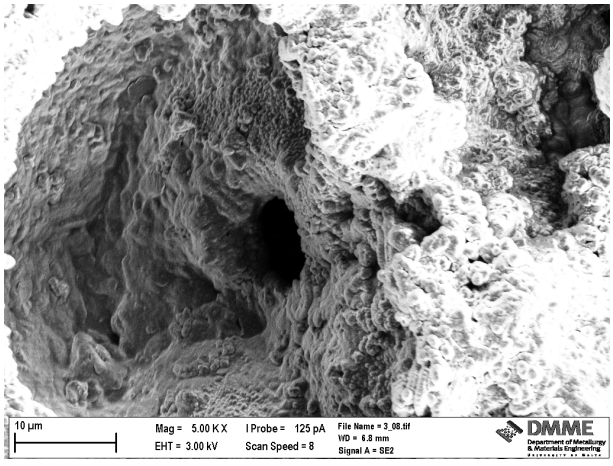

(b) TEOS treated sample at ×10, 000.




(c) Hybrid treated limestone at ×15, 000.




(d) Nano-particle loaded hybrid at ×10, 000.




(e) Modified nano-particle loaded hybrid at ×5000.

Figure 4

porosity results of the treated samples fall within this range. The total porosity values obtained for the different treatments are altered slightly to lower values. This is in agreement with literature (Ksinopolou, 2012, Wheeler, 2005). The slight decrease in porosity indicates that the treatment has penetrated within the samples and supports the electron microscopy observations. The slight decrease in porosity noted could show that

the treatments are lining the stone pores rather than blocking them. This conforms to the SEM observations.

No significantly large changes were observed in the porosity and pore size distribution of the limestone samples following treatment application and weathering. Table 1 lists the values for the total percentage porosity of the limestone samples upon treatment with the different consolidant systems. The percentage porosity after weathering is also given.

**Table 1: Total porosity percentage of non-weathered and weathered samples.**

| Treatment | Total % porosity after consolidation | Total % porosity after weathering |
|---|---|---|
| Untreated | 39.29 | 38.39 |
| TEOS | 35.83 | 42.00 |
| Hybrid | 36.17 | 39.15 |
| Hybrid + Nano-particles | 34.76 | 38.62 |
| Hybrid + Modified nano-particles | 32.87 | 35.94 |

It appears that the presence of the nanoparticles within the hybrid treatment decreases the porosity when compared to the hybrid treatment. This is probably due to the presence of more solid material within the treatment. The addition of the modified nanoparticles further reduces the porosity of the sample. This may be due to the modified particles possibly being larger than the particles that are not modified since the modified nanoparticles are surrounded by functional groups attached to the surface.

In the case of all the treated samples, weathering seems to have increased the total porosity when compared to the non-weathered samples. On the other hand, weathering has little effect on the pore size distribution of the treated limestone samples. A possible reason for the increase in porosity is that the weathering might have broken down the consolidants washing them out of the stone.

Weathering of the untreated sample resulted in a marginal decrease in the total porosity. In addition, it resulted in a decrease in the number of larger pores and an increase in the number of smaller pores. This could be due to calcite dissolution and re-precipitation on the surface of the stone sample during the accelerated weathering.

## 3.4 Water Capillarity

The results for the water absorption by capillarity test carried out according to EN 1925:1999 are shown in Figs.(5.1, 5.2) for non-weathered and weathered samples respectively. Each plotted point is the average of 3 readings obtained from 3 different limestone samples.

In general water absorption in the stone sample increases over time reaching a plateau. The plateau occurs around $500 gm^{-2}$ depending on the treatment. The only samples that fail to reach this plateau during the stipulated time are the TEOS, hybrid and nanoparticle loaded hybrid samples.

The untreated samples absorb the largest amount of water. The hybrid, nanoparticle loaded hybrid and TEOS treated samples all absorb a very little amount of water in the first half of the graph, absorbing slightly more in the second half of the graph but not as much as the untreated or modified nanoparticle loaded hybrid treated samples.

Weathering (Fig.(5.2)) causes the rate and in some cases even the amount of water absorption to increase especially in the case of the hybrid and hybrid and nanoparticles treated samples.

The presence of the silica nanoparticles within the hybrid solution showed an improvement in the water absorption when compared to the hybrid solution on its own, as can be seen particularly for the non-weathered samples. The addition of modified nanoparticles further improves the water absorption properties of the samples.

Yang et al. (1998) showed that, by addition of silica particles to the silicate consolidant, dried gel remained porous. Although in this work the hybrid was also produced from GPTMS, which has a tendency of making the treatment more hydrophobic, the silica particles probably had the same effect on this hybrid treatment as they did on the silicate consolidant.

## 3.5 Salt Crystallisation

The salt used in this test was sodium sulfate decahydrate (Na2SO4.10H20) as recommended in the European Standard EN12370:1999. This test mimics an accelerated real-life situation that involves the deterioration of stone by exposure to repeated sodium sulfate crystallisation. The results of the resistance to salt crystallisation test are presented in Figs.(6)(a, b). Each point in the graphs is the average of 3 readings obtained from 3 different samples. Not all the limestone samples survived the 15 cycles of salt crystallisation.

In both graphs it can be seen that the TEOS treated samples are those that performed best in this test. The untreated samples closely followed. It is interesting to see that the untreated stone behaves so impressively well and hardly seems to be affected by the salt crystallisation.

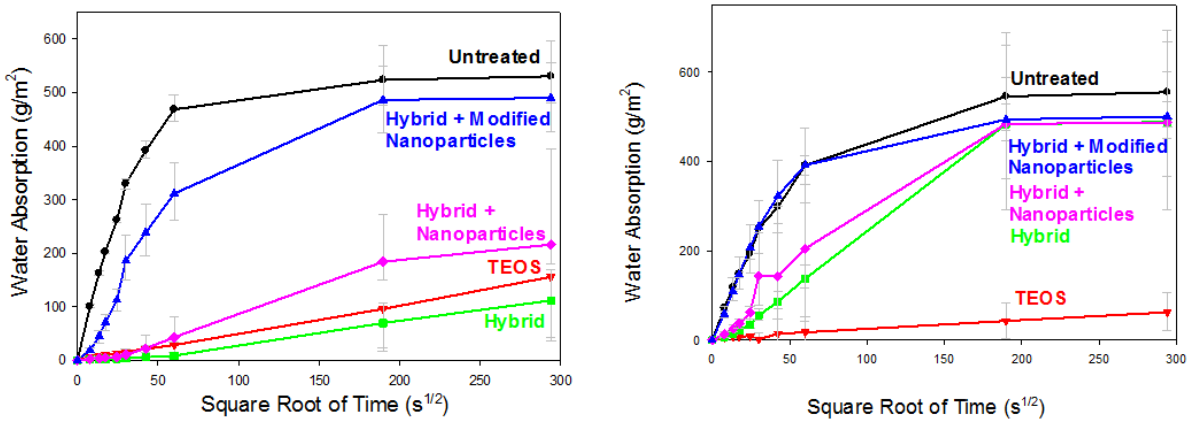



Figure 5: Graph of water absorbed(g/m$^2$) plotted against square root time (s$^{1/2}$) for all of stone samples treated by immersion and for the untreated stone samples and for all of the samples treated by immersed and weathered and for the untreated weathered stone samples.

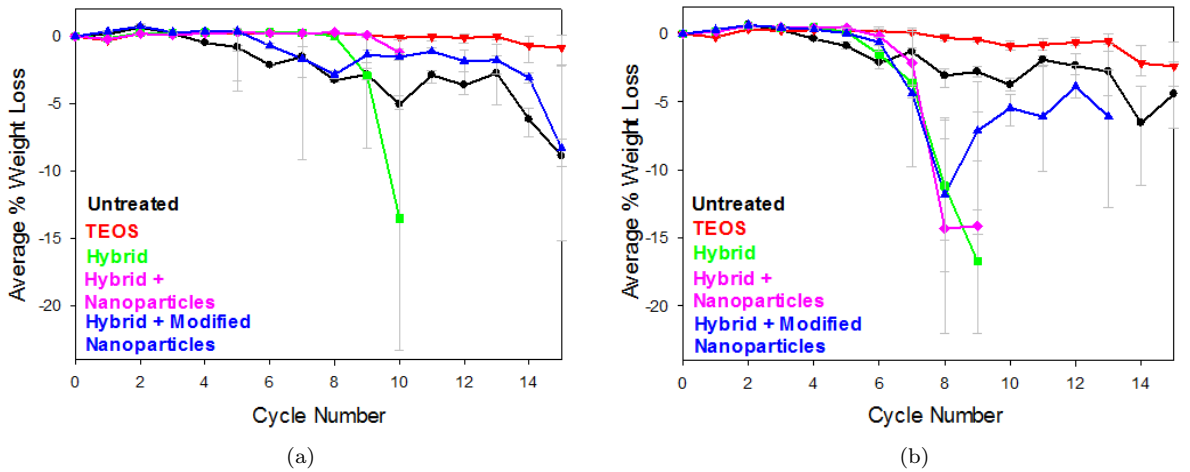



Figure 6: Graphs of salt crystallisation test of immersed (left) and immersed and weathered (right) samples representing average percentage weight loss vs. cycle number.

For the immersed samples shown in Fig.(6)(a) initially the nanoparticle loaded hybrid treated samples looked very promising. These however broke into smaller pieces (spalled) unexpectedly after cycle 10.

When comparing the data for the immersed and immersed and weathered samples, one can see that weathering had a significant effect on the nanoparticle and modified nanoparticle loaded hybrid treated samples and their rate of weight loss. The weathering decreased these samples' resistance to the salt crystallisation test.

Compared to the untreated and TEOS treated samples all the hybrid based samples do not fare so well and large decreases in mass occur. A possible reason for this could be that the hybrid consolidating treatments are efficiently consolidating the limestone hence making it harder for the stone to be deteriorated by the salt and when it is, large pieces are broken off at a time.

# 4 Conclusions

Electron microscopy carried out in this work confirmed that the consolidants are present within the stone. Furthermore the pores of the stone, although altered, are not blocked. The results of the mercury porosimetry, carried out on the immersed non-weathered samples, seemed to confirm this.

The addition of the nanoparticles improved the surface colour of the hybrid treated limestone by better maintaining the original surface colour and the modified particles improved the surface colour even further. The nanoparticles and modified silica nanoparticles also

decreased the amount of cracking of the weathered treatment as evident from the light microscopy work.

The water absorption by capillarity test showed that water flow into the consolidated stone has slowed down compared to untreated limestone. Given that the reduction in water absorption is not caused by a decrease in the porosity of the treated limestone, one possible reason for the drop in water uptake could be an increase in hydrophobicity of the surfaces by the different consolidants. This varies to different extents depending on the consolidating system.

The results obtained for the salt crystallisation test and those obtained for the water test do not follow the same trend. It would have been thought that the results would do so because if water is able to flow through the pores of the stone, so would a solution of salt, and conversely so.

The results obtained from salt crystallisation do not follow those obtained from the mercury intrusion porosimetry. The ability to withstand damage by salt crystallisation is not caused by decreasing the pore size and / or the porosity of the sample similarly to water absorption.

This proves that the salt test does not only depend on the hydrophobicity and the porosity of the stone but is far more complex and is also affected by the mechanical properties of the consolidant.

# 5 Acknowledgements

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*Research Article*

# Optical Glauber Modeling in High-Energy Nuclear Collisions

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**Abstract.** The Optical Glauber Model is used in this study in order to understand the initial conditions in heavy-ion collisions and at the end understand the relationship between the particles produced after the collision. In the first part of this study, the initial geometrical features of the collision as a function of the impact parameter, such as the number of participating nucleons and the number of collisions between nucleons are obtained. Then, after obtaining numerical values for the number of participating nucleons, the study was focused on two distinct particles being produced after the collision and the relationship between them is also determined from the correlation as a function of the impact parameter.

**Keywords** Optical Glauber model - Impact parameter - Number of participants - Number of nucleon-nucleon collisions - Wood-Saxon.

## 1 Introduction

In an ultrarelativistic collision of nuclei, the complexity of the collision event is much higher compared with proton-proton collisions, and it seems to be difficult to study how each event occurred. Given the Glauber model, one can trace back each collision and even get some results of the impact parameter, the number of participating nucleons, and also the number of binary nucleon-nucleon collisions.

Section 2 discusses the basic history of the Glauber model, together with the inputs required in order to carry out a Glauber calculation in a high-energy collision. Section 3 discusses the Optical Glauber model in detail, in which all required formulae are derived, and the respective results are also discussed. Section 4 discusses in one way how the Glauber model is related to experimental data, in which the production of particles after a collision is studied. The relationship between these produced particles is also discussed. Finally in section 5, the current status and future applications of the Glauber model are discussed, with a reference to some results obtained during this study.

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## 2 Theoretical Foundations of Glauber Modeling

### 2.1 A Brief History of the Glauber Model

The Glauber model was developed to resolve the problem of high-energy scattering with composite particles. This idea was of great interest in the fields of both nuclear and particle physics. In 1958, Glauber presented his first collection of various papers and unpublished work from the 1950's. Glauber's work put the quantum theory of collisions of composite objects on a firm basis and provided a consistent description of experimental data for protons colliding with deuterons and larger nuclei. Most striking were the observed dips in elastic peaks, whose position and magnitude were predicted by Glauber's theory, by Czyz and Lesniak in 1967 (Miller et al. 2007).

Bialas et al.'s approach introduced the functions used in this study. For example, they introduced the thickness function and a prototype of the nuclear overlap function. They also introduced the optical limit for analytical and numerical calculations (Miller et al. 2007).

As computational processing increased over the past years, the Glauber Monte Carlo approach has been implemented. This approach was first applied to high-energy heavy ion collisions in the HIJET model (Miller

et al. 2007) and has found its way in practically all A+A simulation codes. This includes HIJING, VENUS and RQMD (Miller et al. 2007).

## 2.2 Inputs to Glauber Calculations

### 2.2.1 Nuclear charge densities

The nucleon density is usually parameterized by a Fermi distribution with three parameters, commonly known as the Wood-Saxon nucleon density;

$$\rho(r) = \rho_0 \frac{1 + w(r/R)^2}{1 + exp\left(\frac{r-R}{a}\right)} \tag{1}$$

where $\rho_0$ corresponds to the nucleon density in the center of the nucleus, $R$ corresponds to the nuclear radius, $a$ to the skin depth, and $w$ characterizes deviations from a spherical shape. This is the model that is going to be used in the calculations that follow, although a reference to the hard sphere model is also made where necessary. The hard sphere model treats the density distribution as a step function, in which the density is constant within the nuclear radius and then it goes down to zero everywhere outside the nuclear radius range. It is represented by the following equation,

$$\rho(r) = \begin{cases} \rho_0 & , r < R \\ 0 & , r \geq R \end{cases} \tag{2}$$

In this analysis, only Lead(Pb) nuclei are considered. Values of the parameters for Pb-207 are given in Table 1 (Alver et al. 2008).

| | |
|---|---|
| R [fm] | 6.62 |
| a [fm] | 0.546 |
| w [fm] | 0 |

Table 1: List of values of the parameters for Pb-207 nuclei.

Using the values defined in Table 1, the density distribution of Pb-207 is plotted (Fig.(1)), where the solid line is representing the density distribution given by the Wood-Saxon nucleon density, and the dashed line represents the distribution given by the hard sphere model.

### 2.2.2 Inelastic nucleon-nucleon cross section

The Glauber model assumes that the nucleons collide inelastically and the number of charged particles produced on each collision to remain the same on an average. As the cross section involves processes with low momentum transfer, it is impossible to calculate this using perturbative quantum chromodynamics. Thus, the measured inelastic nucleon-nucleon cross section ($\sigma^{NN}$) is used as an input and provides the only nontrivial beam-energy

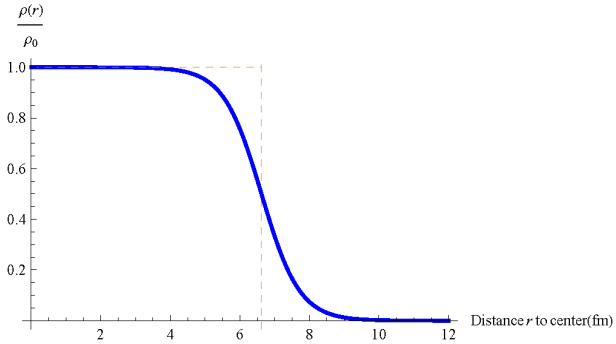

Figure 1: Density distribution for Pb-207 nuclei.

dependence for Glauber calculations. Diffractive and elastic processes are not considered in this analysis, although they are measured experimentally and studied for other research interests (Miller et al. 2007; Esha 2012).

# 3 Optical Glauber Model

In the following formalism of the Optical Glauber model, it is assumed that at sufficiently high energies, the nucleons will carry sufficient momentum that they will be undeflected as the nuclei pass through each other and also that the nucleons move independently in the nucleus and that the size of the nucleus is large compared to the nucleon-nucleon force.

## 3.1 Deriving expressions

The base of the analytical formulae is illustrated diagramatically (Fig.(2)), in which two heavy ions, target A and projectile B, are shown colliding at relativistic speeds with impact parameter $\overrightarrow{b}$ (Miller et al. 2007). In order to derive the equations that follow, one shall consider two flux tubes located at a displacement $\overrightarrow{s}$ with respect to the center of the target nucleus and hence a displacement of $\overrightarrow{s} - \overrightarrow{b}$ from the center of the projectile. During the collision these tubes overlap, and this is what one is interested in, in order to determine the particles being produced during the collision.

The probability per unit transverse area of a given nucleon being located in the target flux tube is given by,

$$\hat{T}_A(\overrightarrow{s}) = \int \hat{\rho}_A(\overrightarrow{s}, z_A) dz_A \tag{3}$$

where $\hat{\rho}_A(\overrightarrow{s}, z_A)$ is the probability per unit volume, normalized to unity, for finding the nucleon at location $(\overrightarrow{s}, z_A)$. Similarly, the equation for the projectile nucleon is simply,

$$\hat{T}_B(\overrightarrow{s}) = \int \hat{\rho}_B(\overrightarrow{s}, z_B) dz_B \tag{4}$$

where $\hat{\rho}_B(\overrightarrow{s}, z_B)$ has a similar meaning to the previous one. The information for both $\hat{\rho}_A(\overrightarrow{s}, z_A)$ and $\hat{\rho}_B(\overrightarrow{s}, z_B)$ is obtained through the nuclear density profile of the respective colliding nuclei. Thus, the product

$$\hat{T}_A(\overrightarrow{s})\hat{T}_B(\overrightarrow{s} - \overrightarrow{b})d^2s \tag{5}$$

then gives the joint probability per unit area of nucleons being located in the respective overlapping target and projectile flux tubes of differential area $d^2s$. One shall define the thickness function $\hat{T}_{AB}(\overrightarrow{b})$, as the integral over the joint probability given by (5), i.e.

$$\hat{T}_{AB}(\overrightarrow{b}) = \int \hat{T}_A(\overrightarrow{s})\hat{T}_B(\overrightarrow{s} - \overrightarrow{b})d^2s \tag{6}$$

One can interpret the thickness function as the effective overlap area for which a specific nucleon in A can interact with a given nucleon in B, indeed it is purely a geometrical factor. The probability of an interaction occurring is then given by

$$\hat{T}_{AB}\sigma_{inel}^{NN} \tag{7}$$

where $\sigma_{inel}^{NN}$ is the inelastic nucleon-nucleon cross section. Elastic processes lead to very little energy loss and are consequently neglected in the calculation. Once the probability of a given nucleon-nucleon interaction has been found, the probability of having $n$ such interactions between nuclei A and B is given by a binomial distribution,

$$P(n, \overrightarrow{b}) = \begin{pmatrix} AB \\ n \end{pmatrix} [\hat{T}_{AB}(\overrightarrow{b})\sigma_{inel}^{NN}]^n [1 - \hat{T}_{AB}(\overrightarrow{b})\sigma_{AB}^{NN}]^{AB-n} \tag{8}$$

where the first term is the number of combinations for finding $n$ collisions out of $AB$ possible nucleon-nucleon interactions, the second term is the probability for having exactly $n$ collisions, and the last term the probability of exactly $AB - n$ misses.

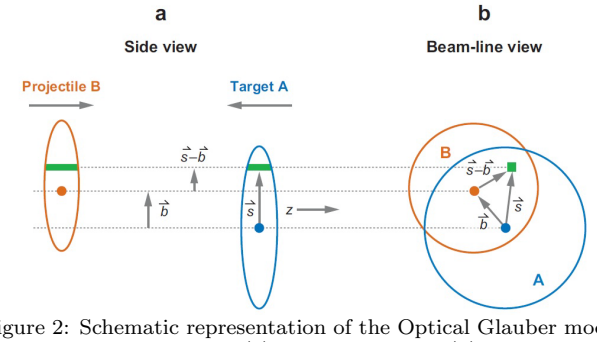


Figure 2: Schematic representation of the Optical Glauber model geometry, with transverse (a) and longitudinal (b) views.

Then the total probability of an interaction between A and B is given by

$$\begin{aligned} \frac{d^2\sigma_{inel}^{AB}}{db^2} &\equiv p_{inel}^{AB}(b) = \textstyle\sum_{n=1}^{AB} P(n, \overrightarrow{b}) \\ &= 1 - [1 - \hat{T}_{AB}(\overrightarrow{b})\sigma_{inel}^{NN}]^{AB} \end{aligned} \tag{9}$$

The total number of nucleon-nucleon collisions as a function of the scalar impact parameter[1] is

$$N_{coll}(b) = \sum_{n=1}^{AB} nP(n, b) = AB\hat{T}_{AB}(b)\sigma_{inel}^{NN} \tag{10}$$

Now the number of nucleons in the target and projectile nuclei that interact is known as either the number of participants or the number of wounded nucleons. The number of participants as a function of impact parameter $\overrightarrow{b}$, is then given by

$$\begin{aligned} N_{part}(\overrightarrow{b}) = A \int \hat{T}_A(\overrightarrow{s})\{1 - [1 - \hat{T}_B(\overrightarrow{s} - \overrightarrow{b})\sigma_{inel}^{NN}]^B\}d^2s \\ + B \int \hat{T}_B(\overrightarrow{s} - \overrightarrow{b})\{1 - [1 - \hat{T}_A(\overrightarrow{s})\sigma_{inel}^{NN}]^A\}d^2s \end{aligned} \tag{11}$$

where the integral over the bracketed terms gives the respective inelastic cross sections for nucleon-nucleon collisions. The number of participants can also be approximated to equation (12), given that $\sigma_{inel}^{NN}\hat{T}_A(\overrightarrow{b})/A \ll 1$

$$\begin{aligned} N_{part}(\overrightarrow{b}) = \int \hat{T}_A(\overrightarrow{s})\{1 - exp[-\hat{T}_B(\overrightarrow{s} - \overrightarrow{b})\sigma_{inel}^{NN}]\}d^2s \\ + \int \hat{T}_B(\overrightarrow{s} - \overrightarrow{b})\{1 - exp[-\hat{T}_A(\overrightarrow{s})\sigma_{inel}^{NN}]\}d^2s \end{aligned} \tag{12}$$

### 3.2 Results

In the equations discussed above, only $N_{part}$ and $N_{coll}$ as a function of the impact parameter, $b$, are illustrated (Fig.(3)), in which the calculations are shown for both the hard sphere model, and also for the Wood-Saxon nucleon distribution model. All calculations were done analytically, and where necessary using numerical integration with the most suitable method for high accuracy. The inelastic nucleon-nucleon cross section was set to 31.5 mb, which averages the cross-section for inelastic interaction within the center of mass energy range of 7 to 60 GeV. Smaller impact parameter in a geometrical picture implies larger overlap, usually termed as central collisions and larger impact parameter collisions have smaller overlap region and termed as peripheral collisions. Hence, $N_{part}$ and $N_{coll}$ decrease with increase in the impact parameter values (Fig.(3)).

## 4 Relating Glauber Model to experiments

Since neither the value of $N_{part}$ nor the value for $N_{coll}$ can be measured directly in an experiment, we employed

[1] Assuming that the nuclei are not polarized, otherwise one cannot replace the vector impact parameter by a scalar distance.

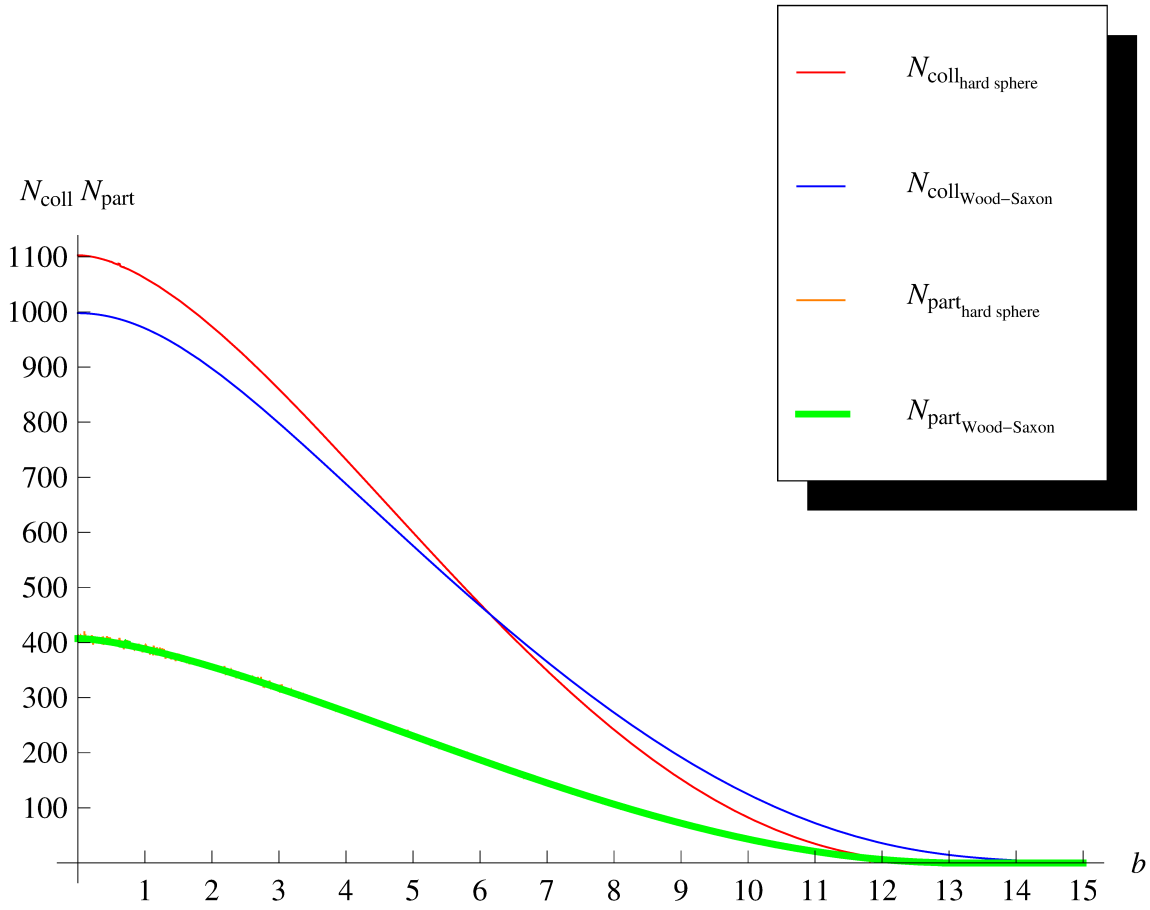


Figure 3: $N_{Part}$ and $N_{Coll}$ as a function of the impact parameter, showing both the hard sphere and the Wood-Saxon model.

a mapping to the number of particles produced in a collision in order to understand the applicability of the Glauber model to experiments. This was done by assuming that a finite amount of particles are being produced in a collision, and a coupling factor was introduced for each particle produced. Indeed, these coupling constants are the measured quantities during an experiment. Then, after assuming that these produced particles follow a Poisson distribution, one could map back to the number of participants in the experiment, with which one can also get the impact parameter by using the Optical Glauber model discussed in the previous section.

## 4.1 Modeling the production of particles after collision

As already mentioned, a mapping from the produced particles back to the number of participants and then to the impact parameter was implemented. In the following calculations, only two distinct particles are considered, since we are only interested in how the number of produced particles correlate with each other. To a first approximation, it was first assumed that the produced particles follow a Poisson distribution, but as the mean number of particles produced is high, statistically, the Poisson distribution, in the limit of large value of the mean, approximates a Gaussian distribution. So, to a second approximation, the produced particles are assumed to follow a Gaussian distribution, instead of a Poisson, but leaving the variance and the mean equal to each other, as is in the case of a Poisson distribution.

We denote the average number of produced particles by $< N_1 >$ and $< N_2 >$, and define their relation with the number of participants by the following equations,

$$< N_1 >= n_1 N_{part} \tag{13}$$

$$< N_2 >= n_2 N_{part} \tag{14}$$

where $n_1$ and $n_2$ are the coupling constants which are measured experimentally. For the purpose of the following calculations, these are set to 10 and 2 respectively, with the values being constrained to experimental results. Also, since the produced particles, $N_1$ and $N_2$, follow a Gaussian distribution with mean having the same value as the variance, their probability distribu-

tion functions can be written as follows

$$P_{N_1} = \frac{1}{\sqrt{2\pi < N_1 >}} \exp\left(-\frac{(N_1 - < N_1 >)^2}{2 < N_1 >}\right) \quad (15)$$

$$P_{N_2} = \frac{1}{\sqrt{2\pi < N_2 >}} \exp\left(-\frac{(N_2 - < N_2 >)^2}{2 < N_2 >}\right) \quad (16)$$

In order to get the correlation between these two particles in a range of values of the impact parameter, a two dimensional distribution constituting of the product of the two Gaussian distribution functions for $N_1$ and $N_2$ was then considered. This is given by the equation that follows

$$P_{N_1,N_2} = \exp\left(-\frac{(N_1 - < N_1 >)^2}{2 < N_1 >} - \frac{(N_2 - < N_2 >)^2}{2 < N_2 >}\right) \quad (17)$$

Different values of the number of participants where taken from the Optical Glauber model, which then resulted in a range of values for the produced particles, $N_1$ and $N_2$,for which a contour plot was then plotted (Fig.(4)). Each contour represents a confidence interval of $1\sigma$ with a given impact parameter (Fig.(4)). In fact, it can trivially be shown from equation (17), that these contours are ellipses, and the region for all possible values of $N_1$ and $N_2$ with a confidence interval of $1\sigma$ was enclosed by the two curves touching the circumference of all produced contours. As the range of the impact parameter is taken infinitesimally small, the whole region will be filled with elliptical contours, and hence a random sample of points (Fig.(4)) was then taken inside this region in order to get a plot on how the correlation between these two produced particles is changing.

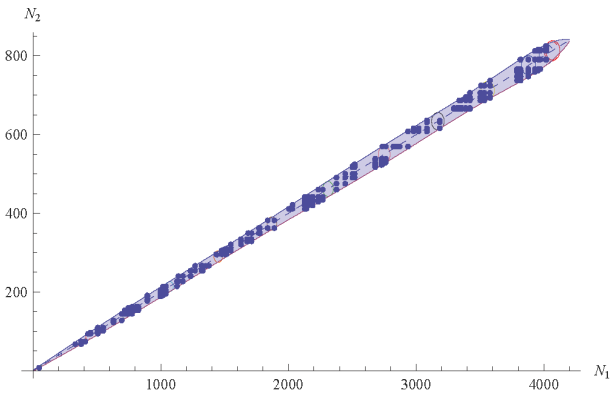


Figure 4: Scatter plot showing the production of two distinct particles, $N_1$ and $N_2$.

The correlation between the two produced particles as a function of the impact parameter, is illustrated, in which the dashed line is the analytical fit, whereas the solid line represents the actual points taken from the sample in the defined region (Fig.(5)).

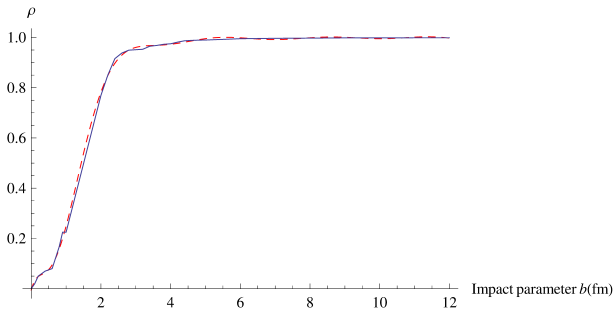


Figure 5: Plot showing the correlation coefficient, $\rho$, between the produced particles $N_1$ and $N_2$ as a function of the impact parameter.

# 5 Conclusion

The Glauber model as used in ultrarelativistic heavy ion physics is based purely on nuclear geometry. It treats the nucleus-nucleus collision as a series of nucleon-nucleon collision processes. The assumption that A+B collisions can be viewed as a sequence of nucleon-nucleon collisions and that individual nucleons travel on straight-line trajectories comes from its origin as a quantum-mechanical multiple-scattering theory. The Glauber model provides us with the number of participating nucleons and the number of binary collisions for a given impact parameter at a given center of mass energy. With the number of participants, $N_{part}$, and the number of binary nucleon-nucleon collisions, $N_{coll}$, the Glauber model introduces quantities that are essentially not measurable. Only the forward energy in fixed-target experiments has a rather direct relation to $N_{part}$.

One main reason for using geometry-related quantities such as $N_{part}$ calculated with the Glauber model is the possibility of comparing centrality-dependent observables measured in different experiments. Basically all experiments calculate $N_{part}$ and $N_{coll}$ in a similar way using Monte Carlo implementation of the Glauber model so that the theoretical bias introduced in the comparisons is typically small. Thus, the Glauber model provides crucial interface between theory and experiment.

The Glauber model comes in two variants, namely the Optical Glauber model and the Monte Carlo Glauber model. While in the Optical Glauber model the nucleus is considered as a smooth matter density, in the Monte Carlo Glauber model, the nucleons are populated stochastically according to the given nuclear density profile. For the Optical Glauber model, the whole procedure is done analytically, whereas in the Monte Carlo version, it is counted. In the case of this study, only the Optical Glauber approach was considered.

The fact that many ultrarelativistic A+B collisions can be understood purely based on geometry led to a widespread use of the Glauber model. A good example is the total charged-particle multiplicity that scales as

$N_{part}$ over a wide centrality and center-of-mass energy range. Another example is the anisotropic momentum distribution of low-$p_T$ ($p_T$ < 2 GeV/c) particles with respect to the reaction plane. Another important application is in the study of hard scattering processes.

One may conclude that for central collisions, the particles produced are not related with each other, but as soon as the collisions happen to be shifted from the central position, the particles produced will be highly correlated with each other (Fig.(5)). In fact the plot for the correlation as a function of the impact parameter shows that the value of the correlation reaches asymptotically unity very fast, hence showing a very strong correlation between the produced particles (Fig.(5)).

# 6 Acknowledgements

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# Appendix 1

Referring to section 4, the particles produced after a collision were assumed to follow a Poisson distribution. One can hence consider a finite amount of produced particles by considering a finite amount of Poisson distributions, not necessarily distinct. Different Poisson distributions, with distinct mean and hence variance, are considered as the produced particles, and the distribution on the right hand side of the plot is the sum of all of these distributions (Fig.(6)). Hence this latter distribution represents the distribution produced by the total amount of particles produced after a collision. We now show that the sum of these Poisson distributions is again a Poisson distribution. We prove further that, since we considered these distributions as Gaussian rather than Poisson, the sum of finite Gaussian distributions is again a Gaussian distribution. Moreover, it can trivially be proved, that the convolution of distinct Gaussian distributions represents actually the sum of Gaussian distributions.

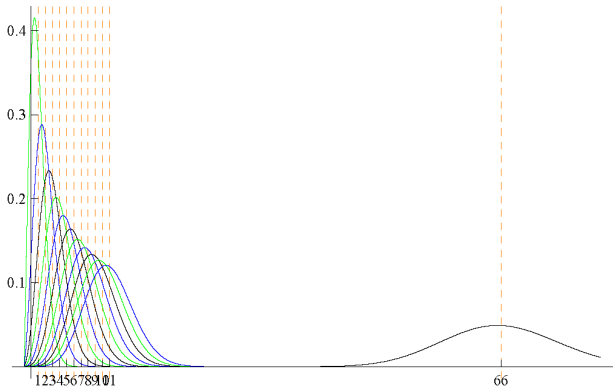


Figure 6: Plot showing a finite number of distinct Poisson distributions on the left hand side, with the sum of these distributions plotted on the right hand side of the plot. The dashed lines represent the mean, and hence the variance of each respective distribution.

We shall prove this result by mathematical induction, although other methods might exist.

We first consider two random variables, $N_1$ and $N_2$, to be independent Poisson random variables with parameters $< N_1 >$ and $< N_2 >$. We show that the random variable $N = N_1 + N_2$ is also a Poisson random variable. Indeed, we consider the moment-generating functions, $M_{N_1}(t)$ and $M_{N_2}(t)$, rather than the probability distribution functions of random variables $N_1$ and $N_2$, and we use the following theorem in order to prove our result.

## Theorem

Let $W_1$, $W_2$,..., $W_n$ be independent random variables with moment-generating functions $M_{W_1}(t)$, $M_{W_2}(t)$,..., $M_{W_n}(t)$. Then for the random variable $W = W_1 + W_2 + ... + W_n$, the moment-generating function is given by

$$M_W(t) = M_{W_1}(t) * M_{W_2}(t) * ... * M_{W_n}(t) \qquad (18)$$

Now for a Poisson distribution

$$P_\lambda(k) = \frac{\exp(-\lambda)\lambda^k}{k!} \quad k = 0, 1, 2, ... \qquad (19)$$

the moment-generating function is given by

$$M_\lambda(t) = \exp(-\lambda + \lambda \exp(t)) \qquad (20)$$

Hence,

$$M_{N_1}(t) = \exp(- < N_1 > + < N_1 > \exp(t)) \qquad (21)$$

$$M_{N_2}(t) = \exp(- < N_2 > + < N_2 > \exp(t)) \qquad (22)$$

Hence for the random variable $N = N_1 + N_2$, the theorem implies that

$$\begin{aligned} M_N(t) &= M_{N_1}(t) * M_{N_2}(t) \\ &= \exp(-(< N_1 > + < N_2 >) \\ &+ (< N_1 > + < N_2 >) \exp(t)) \end{aligned} \qquad (23)$$

But this is the moment-generating function of Poisson random variable $N$, with its parameter being the sum of $< N_1 >$ and $< N_2 >$. Hence, $N$ is a Poisson random variable, being the sum of two independent Poisson random variables.

We now suppose that $N_1, N_2, ...., N_j$ are independent Poisson random variables with parameters $< N_1 >, < N_2 >, ..., < N_j >$ respectively, and that the random variable $N_N = \sum_{k=1}^{j} N_k$ is also a Poisson random variable.

We now show that for $N_1, N_2, ...., N_j, N_{j+1}$ Poisson random variables with parameters $< N_1 >, < N_2 >, ..., < N_j >, < N_{j+1} >$ respectively, the random variable $N_{N+1} = \sum_{k=1}^{j+1} N_k$ is also a Poisson random variable.

Indeed,

$$N_{N+1} = \sum_{k=1}^{j+1} N_k = \sum_{k=1}^{j} N_k + N_{j+1} \tag{24}$$

is a sum of two independent Poisson distributions, which we have already proved that it is again a Poisson distribution. Hence by mathematical induction, we showed that a finite sum of independent Poisson distributions, is again a Poisson distribution.

In a similar way, one can prove by the same argument, that a finite sum of Gaussian distributions, is again a Gaussian distribution. One has to keep in mind that the moment-generating function for a Gaussian distribution is now given by,

$$M_N(t) = \exp\left(\mu t + \frac{1}{2}\sigma^2 t^2\right) \tag{25}$$

where $\mu$ and $\sigma$ are the mean and standard deviation respectively. Moreover, one can also prove that the convolution of Gaussian distributions is the sum of the distributions.

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*Review Article*

# Nicotine Addiction: A Review

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**Abstract.** Nicotine, the major psychoactive compound in tobacco, acts as a potent addictive drug in humans. The addictive nature of nicotine leads to more than 6 million deaths a year. Evidence indicates that nicotine and other drugs of abuse act on central dopaminergic pathways and modulate their neurophysiological mechanisms. Nicotine stimulates dopaminergic pathways and the prefrontal cortex (PFC), inducing enhanced reward perception and increased cognitive function, respectively. These findings are consistent with the fact that nicotine binds to different subtypes of nicotinic acetylcholine receptors present on the neurons found in the PFC and ventral tegmental area of the midbrain. The latter, being the area most involved in addictive behaviour, projects to the limbic system, particularly the nucleus accumbens, and receives afferents from the prefrontal cortex and brainstem. Although dopaminergic pathways and nicotinic acetylcholine receptors are the protagonists of nicotine addiction, several minor pathways and their constituent receptors have been indicated as being either directly or indirectly affected by nicotine. These include serotonergic pathways and central cannabinoid receptors. Despite the scarcity of approved drugs and partial efficacy of approved treatment, insight into nicotine neurophysiological modulation has led to a better appreciation of nicotine-seeking behaviour and subsequent improved design of pharmacological and behavioural approaches to smoking cessation. Tobacco is the single most preventable cause of death in the world today. Better understanding of the neurobiological mechanisms underlying nicotine addiction will ultimately lead to more effective treatments of both nicotine dependence and nicotine rewarding effects.

**Keywords** Nicotine – Addiction – Withdrawal – Nicotinic acetylcholine receptors – Corticolimbic pathways – Smoking cessation.

## 1 Introduction

Tobacco is the single most preventable cause of death in the world today. The World Health Organisation (WHO) estimates that annually, tobacco leads to more than 6 million deaths and causes more than half a trillion American dollars of economic damage (World Health Organisation, 2013). Many types of tobacco products are consumed all over the world but the most popular form of nicotine use is through cigarette smoking. Smoking is a ubiquitous activity: more than 5,550 billion cigarettes are manufactured annually and there are approximately 1.2 billion smokers worldwide– a number expected to increase to 2 billion by 2030 (Mackay and Eriksen, 2012; World Bank, 2003). Tobacco use and its health hazards are therefore a global burden and show how tobacco is a strong motivator, despite the increased awareness of its consequential health hazards. This review will summarise knowledge of the neurophysiology of the addictive behaviour elicited by nicotine, the major psychoactive agent present in all tobacco products.

## 2 Addiction – Theories and Neurobiology

Addiction is a complex phenomenon which is still not completely understood. The traditional view is that addictive substances, such as ethanol, psychostimulants, opioids and nicotine, are all taken for two reasons: either for the pleasure the drugs elicit or to avoid the unpleasant consequences of withdrawal (World Health Organisation, 2004). Addiction is not the mere use of those drugs - it is the inability to ceasing drug intake and a compulsive pattern of drug-seeking and drug-taking behaviour that takes place at the expense of other ac-

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tivities (Robinson and Berridge, 2000). Moreover, the non-addictive, sporadic or occasional patterns of intake of an addictive drug might escalate to a frequent and compulsive one (Wellmann et al., 2004).

# 3 Manifestations of Addiction

The transition from sporadic, intermittent use to compulsive intake is a result of the interaction of the addictive substance with central neurones. This leads to long-lasting neuronal alterations of metabolism and activity, and consequently, the properties of the neuronal circuits that they constitute (Mansvelder and McGehee, 2002). This progressive change in neuronal circuitry leads to a manifestation of complex behaviours such as dependence, tolerance, sensitisation and craving (Kobb and Le Moal, 2008).

As defined in the 2010 version, 10th revision of the International Classification of Disease (ICD-10) classification of mental and behavioural disorders (WHO, 2010), substance use dependence is diagnosed whenever a case is positive on at least three of six criteria (appendix 1). Tolerance and withdrawal are two of them. Tolerance is defined as the idea that increased amounts of drug are required to achieve the same hedonic effect, or, that the same amount produces less effect. Withdrawal is the occurrence of unpleasant physical and physiological symptoms when use of the substance is reduced or discontinued (WHO, 2010). During discontinuation, relapse to substance use is known to be triggered by cues previously paired with substance use, by stress, or by presence of the drug itself (Stewart, 2000). This is elicited by uncontrollable desire for drugs, *craving* – a concept to which there is still no definite definition, due to a lack of applicability of biological models to it (Drummond et al., 2000).

In the 5th revision of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (American Psychiatric Association, 2013), unlike all previous versions, the criteria for substance abuse and substance dependence have been combined into one category, termed *addictions and related disorders*, and specifically expanded for each substance of abuse. Each substance use disorder is then divided into mild, moderate and severe subtypes. Moreover, whereas the DSM-IV substance abuse diagnostic criteria was requiring only 1 symptom, a DSM-V diagnosis now requires at least 2 (American Psychiatric Association, 2013).

# 4 Theories of Addiction

The mere intake of such chemicals is not equivalent to addiction. In fact, it is the properties of the chemical, together with the individual's genomic and behavioural background, which predispose an individual to addiction (Nees et al., 2013; Kendler et al., 2000). Indeed, this multifaceted phenomenon also leads to a variety of theories as to how addiction evolves.

As stated earlier, the traditional view, and the mostly intuitive explanation of addiction, is that addictive substances are taken as a result of either positive or negative reinforcement (Koob and Le Moal, 2008; World Health Organisation, 2004). Both these views have their shortcomings since it is not always the case that drugs produce such effects. For example, psychostimulants do not produce strong withdrawal syndromes, but can be highly addictive. On the contrary, anticholinergics, $\kappa$-opioid agonists and tricyclic antidepressants produce tolerance and withdrawal syndromes, but do not support compulsive patterns of use (Cote et al., 2013). Also, as stated by the pioneer of the reinforcement model (Skinner, 1953), that a stimulus reinforces a particular type of behaviour is merely an observation, and not an explanation of how the former leads to the latter.

A more holistic theory is the incentive-sensitisation theory of addiction (Robinson and Berridge, 1993). The theory states that all addictive drugs share the ability to produce long-lasting sensitisation of neural systems that subserve a subcomponent of reward – incentive salience. Drug-sensitised incentive salience causes drugs to become compulsively and enduringly wanted (which is different from *liking*), independent of drug pleasure, withdrawal, habits or memories. This phenomenon is implicit, as it can guide behaviour without a person necessarily having conscious emotion, desire, or a declarative goal (Robinson and Berridge, 2003, 2000, 1993). Robinson and Berridge (1993) also make it clear that in some cases and individuals, the positive and negative reinforcement models of addiction do apply.

# 5 Neuroanatomy of Addiction

All psychoactive drugs modulate the normal physiology of the central dopaminergic (DAergic) system (Wise, 1998) via different mechanisms. Therefore, an insight into the central DAergic pathways, particularly the nucleus accumbens-related circuitry, is paramount for the understanding of addiction.

Central dopamine (DA) is found mainly in neurons located in the ventral midbrain, especially the substantia nigra pars compacta (SNc) and the nearby ventral tegmental area (VTA). Three projection systems have been described arising from these mesencephalic nuclei: the mesostriatal (nigrostriatal), mesocortical and mesolimbic pathways (Blumenfeld, 2010). The mesostriatal pathway arises from the SNc and projects to the dorsal striatum: the caudate and the putamen. The mesocortical pathway arises mainly from the VTA and projects to the prefrontal cortex (Crossman and Neary, 2010). The roles of the mesocortical projections are to increase working memory and attention span, and the

roles for the mesostriatal pathway are to modulate intentional aspects and magnitude of locomotor activation (Blumenfeld, 2010). These pathways are affected by nicotine, but merely contribute in the reinforcement effect that it has on the human nervous system.

Finally, the mesolimbic pathway arises from the VTA and projects primarily to the nucleus accumbens (NAcc) of the ventral striatum – an area of the limbic system (Blumenfeld, 2010). Most addictive drugs, including nicotine, increase DA levels in the NAcc (Cote et al., 2013; Khalki et al., 2013; Nees et al., 2013). Evidence shows that VTA lesions and DA receptor antagonist microperfusion in the NAcc results in reduced self-administration of many addictive drugs, including nicotine (Mansvelder and McGehee, 2002). Obviously, the NAcc-related circuitry did not evolve to mediate the effects of drugs; its evolutionary intent is to use stimuli beneficial for survival, such as nutrients, water and sexual partners as natural rewards that exert motivational control over behaviour (Kelley and Berridge, 2002).

Despite the established linkage of NAcc DA levels and reward, several studies are now suggesting that this is an indirect causal relationship, since it seems that DA in the NAcc signals novelty or reward expectation, rather than reward itself (Berke and Hymann, 2000; Dani and De Biasi, 2001; DiChiara, 2000; Schultz et al., 1997). Such research correlates with the incentive-sensitisation theory of addiction (Robinson and Berridge, 1993).

# 6 The Addictive Nature of Tobacco

Although tobacco contains substances (such as nornicotine and monoamine oxidase inhibitors) which contribute to tobacco addiction, nicotine, an alkaloid, is the main psychoactive agent (Khalki et al., 2013; Sasaki, 2013). The average human plasma half-life of nicotine is approximately 2 hours, but is about 35% longer in individuals with a particular form of the gene coding for the cytochrome P450 CYP2A6 that is responsible for the primary nicotine metabolic pathway (Ande et al., 2012).

Nicotine acts as an agonist at several populations of both central and peripheral nicotinic acetylcholine receptors (this review tackles *central* receptors only). In humans, acute nicotine administration produces positive effects, including mild euphoria and mildly enhanced cognition; such subjective positive effects support intravenous self-administration behaviour in a variety of mammalian species including mice, rats and non-human primates (Markou and Peterson, 2001; Picciotto and Corrigall, 2002). Persistent nicotine use leads to tolerance that is mediated by neuroadaptation occurring in response to chronic exposure to the alkaloid, thus, within hours upon cessation of nicotine exposure, a nicotine withdrawal syndrome emerges. This syndrome is characterized by depressed mood, mild cognitive deficits and irritability (Shiffman et al., 2004).

In both rats and humans, nicotine withdrawal is characterized by both increases in somatic signs and effective changes such as reward deficits (Jonkman et al., 2007). Jonkman and colleagues (2007) also suggest that, despite no elicitation of an anxiogenic effect itself, nicotine withdrawal potentiates response to anxiogenic stimuli. On the same lines, intracranial self-stimulation (ICSS) studies on rats (Harrison et al., 2002; Johnson et al., 2008) showed that after being administered nicotine, they required lower self-applied current intensities to their reward centres to perceive pleasure. On the other hand, after withdrawal from nicotine, rats required higher intensities to perceive rewarding stimuli. Similarly, other studies showed that rats chronically treated with nicotine required higher current intensities when administered nicotinic receptor antagonists, such as dihydro-β-erythroidine (DHβE) (Watkins et al., 2000) or mecamylamine (MEC) (Hollander and Kenny, 2008). The same antagonist studies also showed that control rats (not treated with nicotine), had the same reward threshold recorded under baseline conditions after being administered DHβE.

Moreover, learning processes also contribute to nicotine dependence. For example, environmental stimuli associated with either the positive subjective effects of nicotine or the induction of nicotine withdrawal motivates nicotine seeking and eventually drug consumption (Kedikian, Faillace and Bernabeu, 2013). Studies on rats (Kenny and Markou, 2006) have shown this phenomenon by pairing flashing light with the effects of DHβE under classical conditioning processing. Those studies showed significant elevations of the reward thresholds once conditioned pairing was successful. An increased threshold was not observed in rats that had equal exposure to nicotine with unpaired light and DHβE. Such studies therefore indicate that nicotine withdrawal can be paired with environmental stimuli which alone can precipitate withdrawal syndrome.

# 7 Central Nicotinic Acetylcholine Receptors

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels comprising five membrane-spanning subunits (Changeux and Taly, 2008; Purves, 2011). Binding of the agonist is transduced into the gating of the receptor ion channel pore that is permeable to multiple cationic species ($Na^+$, $K^+$, $Ca^{2+}$) and large organic cations such as tetraethylammonium (TEA) (Fucile, 2004). 12 genes encode neuronal nAChRs sub-

units: genes CHRNA 2 to 10 encode nine isoforms of the neuronal α-subunit (α2- α10) and genes CHRNB 2 to 4 encode three isoforms of the neuronal β-subunit (β2 - β4) (Elgoyhen et al., 2001; Le Novere et al., 2002). Subunits either combine with different stoichiometries, such as two α- and three β-, or five α7-subunits to form nAChRs with distinct pharmacologic and kinetic properties (Mansvelder and McGehee, 2002).

Such distinct properties include abundancy, $Ca^{2+}$ permeability, and sensitivity to nicotine and desensitisation rates (Wonnacott et al., 2005). The heteromeric α4β2 nAChR (α4β2*) forms the majority of central nAChRs while the homomeric α7 nAChR (α7*) is the second biggest in number (Millar and Gotti, 2009). α7* are mostly permeable to $Ca^{2+}$, having fractional $Ca^{2+}$ currents of 6-12% (Fucile, 2004), comparable to $Ca^{2+}$ currents recorded in N-methyl-D-aspartate (NMDA) receptors and considerably greater than that of heteromeric (α4β2) nAChRs (2-5%) (Haghighi and Cooper, 2000). It follows, as will be later elaborated, that both types lead to increased $Ca^{2+}$ permeation: directly via α7* and indirectly by the activation of voltage-dependent calcium channels (VDCCs) through α4β2*-mediated depolarisation (Beker et al., 2003; Dajas-Bailador et al, 2002).

α4β2* have the highest affinity to nicotine since they are activated even with nicotine concentrations as low as 100-500nM (Dani et al., 2000; Millar and Gotti, 2009). Such high affinity leads to rapid desensitisation once a compatible ligand (such as ACh and nicotine) binds to this subtype (Mansvelder et al., 2002a). On the other hand, α7* are renowned for their multi-gating modes (Papke et al., 2000). α7* manifest relatively rapid desensitisation at relatively high ACh concentrations (100μM or higher) (Papke, 2006). On the other hand, α7* undergo non-desensitising activation at low ACh concentrations of 20μM (Gourlay and Benowitz, 1997; Mansvelder et al., 2002). Such phenomenon suggests that since such concentrations are present in in vivo cerebrospinal fluid (CSF), it is possible that there is a tonic activation of α7* under normal physiological conditions. Such tonicity ceases at high concentrations due to inactivation and desensitisation (Papke, 2006).

# 8 Central Location

Numerous studies show that nicotine stimulates the release of numerous neurotransmitters since nAChRs are situated in different areas of the DAergic pathways and their modulatory circuits. This section will expand on how the expression of the two main subtypes of nAChRs in the corticolimbic structures, mainly the VTA and PFC, lead to the behaviour that nicotine addiction exhibits. Modulation of mesencephalic and PFC output is ultimately due to the balance of excitatory and inhibitory inputs and the intrinsic activity of the neuronal circuits.

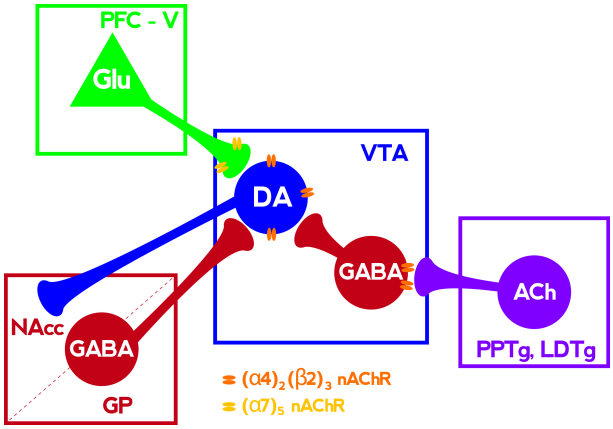


Figure 1: Simplified schematic of the main inputs and outputs of the ventral tegmental area, related nicotinic acetylcholine receptors and neurotransmitters.
The main excitatory inputs to the VTA DAergic neurons are glutamatergic projections that mainly come from layer 5 of the prefrontal cortex (PFC). Conversely, the principal inhibitory inputs to the VTA are γ-aminobutryric acid (GABA)-secreting neurons which are both local (VTA) interneurons and projections from the NAcc and the ventral pallidum. Cholinergic projections to the VTA come from two brainstem nuclei: the pedunculopontine tegmental nucleus (PPTg) and the lateral dorsal tegmental nucleus (LDTg).
Non-α7 nicotinic acetylcholine receptors (nAChRs) can excite dopamine (DA) and γ-aminobutryric acid (GABA) neurons directly, while α7 nAChRs can enhance release from glutamatergic terminals. Endogenous acetylcholine (ACh) release from brainstem cholinergic neurons contributes to the GABAergic input to VTA DA neurons.
In the presence of nicotine concentrations similar to those found in a smoker's blood, the non-α7 nicotinic acetylcholine receptors (nAChRs) desensitise rapidly, effectively inhibiting GABAergic inputs to the dopamine (DA) neurons. The α7 nAChRs will not desensitise as much, which means that glutamatergic inputs will be enhanced as the GABAergic inputs are depressed, thus leading to a net increase in excitation of the DA neurons.
*PFC prefrontal cortex, NAcc nucleus accumbens, GP globus pallidus, VTA ventral tegmental area, PPTg pedunculopontine tegmental nucleus, LDTg lateral dorsal tegmental nucleus Glu glutamate, GABA γ-aminobutryric acid, DA dopamine, ACh acetylcholine, nAChR nicotinic acetylcholine receptor.*

# 9 Ventral Tegmental Area

As shown in Fig.(1), the main excitatory inputs to the VTA DAergic neurons are glutamatergic projections that mainly come from layer 5 of the prefrontal cortex (PFC-V) (Couey et al., 2007). Conversely, the principal inhibitory inputs to the VTA are γ-aminobutryric acid (GABA)-secreting neurons which are both local (VTA) interneurons and projections from the NAcc (medium spiny neurons) and the globus pallidus (GP) (Mansvelder et al., 2002b). Cholinergic projections to the VTA come from two brainstem nuclei: the pedunculopontine tegmental nucleus (PPTg) and the lateral dorsal tegmental nucleus (LDTg) (Mansvelder and McGehee, 2002b).

There are three neuronal components in the VTA that express nAChRs: DA neurons, GABA neurons and glutamatergic presynaptic terminals that synapse on the

DA neurons (Fig.(1)) (Mansvelder and McGehee, 2002). DA neurons express messenger ribonucleic acid (mRNA) for many different nAChR subunits which give rise to at least 3 types of nAChRs, with α4β2* and α7* being the majority (Klink et al., 2001; Pidoplichko et al., 2004). GABA neurons in the VTA express nAChRs that contain α4 and β2 subunits (Mansvelder et al., 2002b) - this has been shown since GABA neurons in the VTA have been blocked by MEC at concentrations that specifically block non-α7*. Moreover, MEC microinfusion in the NAcc did not lead to a decrease in DA, thus showing that NAcc medium spiny neurons themselves express little, if any, nAChRs.

Similar VTA microinfusion studies (Shilstrom et al., 1998), this time with the NMDA receptor antagonist (2R)-amino-5-phosphonovaleric acid (APV), inhibited nicotine-induced increase of DA in the NAcc. This therefore suggests that nicotine modulates glutamate vesicle release at presynaptic glutamatergic terminals. Along the same lines, another study (Mansvelder and McGehee, 2000) showed three phenomena: 1) low-concentration nicotine infusions enhanced VTA glutamatergic transmission (showing the role of nicotine in glutamatergic release), 2) tetrodoxin (TTX), a $Na^+$ voltage-gated channel blocker, did not affect this enhancement (showing a Na+-dependent neurotransmitter release), and that 3) nAChRs are sensitive to metyhyllycaconitine (MLA), a selective α7* antagonist (showing that the $Ca^{2+}$-permeant α7* are involved). Furthermore, Jones and Wonnacott (2004) concluded that α7* are situated on vesicular glutamate transporter (vGluT) positive terminals that were devoid of vesicular cholinesterase transporter (VChat) staining. Such events therefore suggest the presence of only, or a vast majority of, α7 nAChRs on glutamatergic, not cholinergic terminals of VTA DAergic neurons (Couey et al., 2007). In conclusion, secondary to this configuration, glutamatergic projections onto the VTA exert the greatest effect of nAChRs agonists onto VTA DA neurons via axodendritic influences.

# 10 Prefrontal Cortex

The PFC receives glutamatergic inputs from the medial dorsal nucleus of the thalamus (Tmd) (Blumenfeld, 2010). Nicotine excites these thalamocortical projections, leading to an increase in glutamatergic inputs to layer 5 pyramidal neurons as well as to some in layer 6 (Couey et al., 2007). Such effects were blocked by TTX and were elicited with low concentrations of nicotine – both phenomena that indicate the presence of α4β2 nAChRs (Lambe et al., 2003). The same authors also demonstrated that nicotinic modulation of thalamocortical inputs was absent in β2-containing nAChRs knockout (KO) mice. More recently though, studies showed that both α7 and non-α7 nAChRs appear to be important in the PFC synaptosomes (Wallace and Bertrand, 2013). This occurrence is similar to that in the VTA with regards to $Ca^{2+}$ permeation, although different mechanisms are responsible for such ion flux. PFC α7* are primarily found on ryanodine positive terminals and their activation leads to calcium-induced calcium release (CICR). On the other hand, as in the VTA, activation of non- α7* increases $Ca^{2+}$ via recruitment of VDCCs (Mansvelder et al., 2009).

Although no studies have yet pinpointed the specific location of the different nAChRs subtypes, it can still be ascertained that no nAChRs are present on the pyramidal neurons (Couey, 2007). In contrast, specific GABAergic interneuron populations do express mRNA for α4, β2 and α7 subunits; the regular-spiking non-pyramidal (RSNP) interneurons and the low-threshold-spiking (LTS) interneurons (Couey, 2007). nAChRs are also expressed on the medial dorsal thalamic projection terminals (Mansvelder et al., 2009). PFC-V neurons project to various sites, including the ventral striatum (25% circa), hypothalamus (25% circa), amygdala (8%) and the VTA (4%) (Gabbott, 2005).

# 11 Nicotine and Neurophysiological Adaptations

During cigarette smoking, blood nicotine levels reach 300-500nM several minutes after the initiation of smoking and concentrations close to 250nM are sustained for 10 minutes or more (Gourlay and Benowitz, 1997). Such values disrupt the normal activity of central nAChRs which lead to modulation of normal synaptic physiology. Nicotine in the central nervous system activates the high-affinity (α4β2) nAChRs which desensitize within minutes (Dani et al., 2000). However, in vivo biochemical studies showed that a single systemic injection of nicotine enhances DA release in the NAcc for more than an hour (Di Chiara, 2000). This conclusion indicates that it is very likely such changes can be induced even after a person smokes only one cigarette.

These findings lead to the assertion that nicotine has long-lasting neurophysiological effects, which outlast short-term nAChR stimulation and desensitisation (Jiang and Role, 2008; Kawai et al, 2007). This up-regulation has previously been reported to solely involve an increase in the number of nAChR receptors (Wonnacott, 1990; Marks et al., 1992). Recent studies, although not denying an increase in receptor number, suggest that up-regulation is a change in receptor state, rather than a change in receptor number (Mansvelder and McGehee, 2002). Such nicotine-induced up-regulation, by which these long-lasting stimulatory effects ensue, are long-term potentiation (LTP) and depression (LTD) of exci-

tatory glutamatergic inputs of GABAergic transmission in both the PFC and VTA.

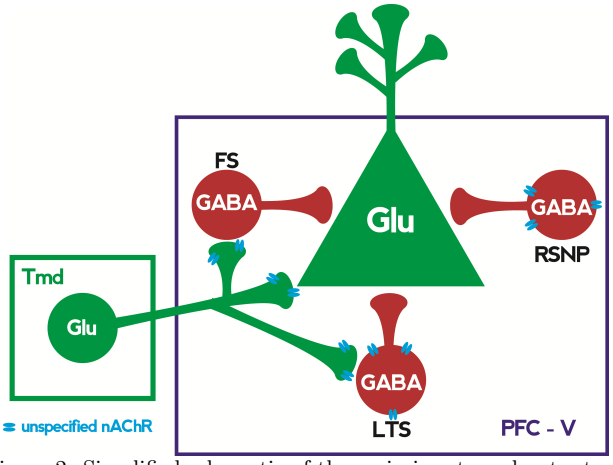


Figure 2: Simplified schematic of the main inputs and outputs of the prefrontal cortex, related nicotinic acetylcholine receptors and neurotransmitters
Nicotinic acetylcholine receptors (nAChRs) are expressed on glutamatergic thalamocortical projections and somata of GABAergic rapid-spiking non-pyramidal cells (RSNP) and low-threshold-spiking interneurons (LTS). GABAergic fast-spiking interneurons (FS) and pyramidal cells do not express nAChRs. Glutamatergic thalamocortical stimulation is increased by nicotine, eliciting an increased excitatory drive to the pyramidal neurons, LTS and FS components. RSNP cells are directly depolarised by nicotine. The net effect is therefore increased inhibition of pyramidal cells activity.
*Tmd medial dorsal nucleus of thalamus, PFC prefrontal cortex, nAChR nicotinic acetylcholine receptor, Glu glutamate, GABA γ-aminobutryric acid, LTS low-threshold-spiking interneurons, RSNP rapid-spiking non-pyramidal cells, FS fast-spiking interneurons, nAChR nicotinic acetylcholine receptor.*

## 12 Prefrontal Cortex Modulation

In normal mouse PFC, nAChRs lead to LTP by pairing the stimulation of the excitatory inputs to layer 5 pyramidal neurons with postsynaptic spikes elicited 5ms after each synaptic response (Couey, 2007). Such synaptic plasticity is brought about by the relative timing of APs in presynaptic and postsynaptic spikes and is hence referred to as spike-timing-dependent plasticity (STDP) (Mansvelder et al., 2009). In STDP, a presynaptic spike preceding a postsynaptic one by a short time window leads to LTP; the reverse order leads to LTD. This coordinated, LTP-inducing stimulus is disrupted following nicotine infusion and a depression is subsequently observed (Couey, 2007). Evidence shows that it is the increase in GABAergic input from interneurons (RSNP, fast-spiking (FS) and LTS) that brings about such depression since the nicotinic modulation of PFC plasticity was abolished by $GABA_A$ receptor antagonists (Mansvelder et al., 2009). As shown in Fig.(2), increase in GABAergic input is elicited by the nicotine-induced increase in glutamatergic inputs from the thalamic projection terminals, which synapse on pyramidal neurons and FS, both of which do not express nAChRs, and on LTS. Moreover, nicotine directly depolarises RSNPs, which do not have connections with thalamic projections, and LTS. Thus, in the PFC the overall effect of nicotine-induced nAChR activation results in a net inhibition of pyramidal cell activity.

Recalling the various PFC projections; such nicotine-induced modulation has widespread effects which to date are still controversial (Mansvelder et al., 2009). For example, Day et al. (2007) report that the nicotine-induced threshold for STDP could reduce cognitive performance. Conversely, the same study suggests that normal PFC-based stimuli during cognitive behaviour increases PFC neuronal activity and therefore makes LTP possible again. Such nicotine-dependent phenomenon could therefore enhance the signal-to-noise ratio (consequently decreasing the unwanted perturbations which all neuronal synapses manifest) and thus leading to improved cognitive performance.

## 13 Ventral Tegmental Area Modulation

4% of the PFC projections in layer 5 extend to the VTA (Gabbott, 2005). As already mentioned, nicotine is able to increase the firing rate of DA neurons. This happens both by direct nicotine stimulation on VTA DA neurons and actions on nAChRs located on GABAergic interneurons and glutamatergic terminals in the VTA (Fig.(1)).

Glutamatergic transmission onto DA neurons is enhanced by activation of presynaptic nAChRs α7*. Interestingly, cholinergic terminals are not in close proximity to glutamatergic terminals and therefore, in normal physiological conditions, ACh stimulates such terminals via a volume mode (Jones and Wonnacott, 2004). This volume mode stimulation is disrupted with nicotine and an increase in glutamatergic secretion occurs. At the same time, the various types of nAChRs on DA neurons are stimulated by the alkaloid, resulting in favourable conditions for the pre- and post-synaptic paired activation leading to LTP of glutamatergic inputs (Mansvelder and McGehee, 2000). LTP is also induced in vivo by an increased AMPA/NMDA receptor ratio (Saal et al., 2003).

Recalling that α7* are tonically active at low neurotransmitter (NT) concentrations, these receptors are not significantly desensitised by low nicotine concentrations associated with tobacco smoking (Gourlay and Benowitz, 1997; Mansvelder et al., 2002a). This ensures that nicotine-induced glutamatergic-DAergic LTP remains unaltered.

In addition to glutamate and dopamine, GABA plays another important role in VTA circuitry. GABAergic interneurons, which predominantly express α4β2 nAChRs,

undergo a transient increase of inhibitory input to the DA neurons. This effect would likely give rise to a short-lived offset of some of the excitatory effects of nicotine, an event that subsides within minutes since the high-affinity α4β2 nAChRs undergo rapid desensitisation. Also, both physiologic (Fiorillo and Williams, 2000) and ultrastructural analyses (Garzón et al., 1999) of VTA cholinergic transmission conclude that the vast majority of brainstem cholinergic projections synapse on GABAergic interneurons while very few synapse on DAergic ones.

An important question is whether VTA GABAergic depression actually contributes to nicotine addiction. In fact, there is evidence (David et al., 1997; Ikemoto et al., 1997) that rats and mice readily self-administer $GABA_A$ receptor antagonists in the VTA. Acetylcholinesterase inhibition also enhanced GABA transmission in the VTA and DA in the NAcc (Mansvelder et al., 2002b) - a phenomenon which complements the results of studies asserting that the majority of cholinergic projections end on GABA interneurons. Such evidence therefore suggests that, as shown in Fig.(2), GABAergic desensitisation will also lead to the cessation of most of the cholinergic effect on DAergic inhibition.

In summary, under normal physiological conditions α4β2* can excite DA and GABA neurons directly, while α7* enhance release from glutamatergic terminals and the somata of DA neurons. Endogenous ACh release from brainstem cholinergic neurons, apart from a scarce effect on DAergic neurons and the far-off glutamatergic terminals, mainly affects GABAergic input to VTA DA neurons. In the presence of nicotine concentrations similar to those found in the blood of a smoker, the α4β2* on GABAergic interneurons desensitize rapidly leading to the cessation of cholinergic influence on their somata and DA neuron disinhibition. α7* do not desensitise as much which means that glutamatergic inputs will be enhanced. The net effect is therefore an increase in excitation of the DA neurons via glutamatergic LTP and GABAergic depression. GABAergic depression might also help in further glutamatergic potentiation as it further favours DAergic neuron depolarisation (Mansvelder and McGehee, 2002). Such effects, which outlast nicotine exposure by hours or more, definitely contribute to our understanding on the long-lasting addictive effects of nicotine.

# 14 Others

Numerous other NTs and neuromodulators influence the activity of the VTA, including serotonin (5-HT) and endogenous opioids (Tzschentke, 2001). Seth et al. (2002), although not able to pinpoint direct evidence for presynaptic nAChRs on cortical serotonergic terminals, showed that 5-HT levels increase on nicotine exposure. Conversely, and more recently, studies conducted on rats reported that nicotine decreased serotonergic cell activity in the dorsal raphe nucleus (dRN) (Touiki et al., 2007).

There is also evidence in humans for a role of endogenous opioids in mediating nicotine dependence (Krishnan-Sarin et al., 1999). Anandamide, an endocannaboid, is indeed implicated in nicotine addiction since its levels in the forebrain and midbrain increase on chronic nicotine administration (Merritt et al., 2008).

# 15 Pharmacological Treatment for Nicotine Addiction

Although it is not the aim of this review to highlight the pharmacological approach to treat nicotine addiction, a brief outline of the current approaches and of promising novel compounds undergoing preclinical testing complement the above evidence of the mechanisms of nicotine addiction, which ultimately has effective smoking cessation as its main objective. The pharmacological treatment of nicotine addiction is divided into two approaches: substitution or eradication (Di Matteo et al., 2007). In addition, pharmacological approaches are more effective when administered during behavioural counselling (Galanti, 2008; Hurt et al., 2009; World Health Organisation, 2004). To date, varenicline is the first-line pharmacotherapy, which demonstrates the greatest efficacy when combined with behavioural support (Carson et al., 2013; West et al., 2008).

# 16 Nicotine Replacement Treatment

Substitutive treatment involves giving nicotine in various formulations in order to substitute tobacco nicotine, hence the term nicotine replacement therapy (NRT) (Hurt et al., 2009). Such treatment is effective since most adverse health effects of tobacco smoking come not from the nicotine itself, but from tars and carbon monoxide, released when tobacco products are ignited (World Health Organisation, 2004). For example, a nicotine transdermal patch provides a relatively stable, fixed dose of nicotine over a period of 16 or 24 hours (Di Matteo et al., 2007). NRT increases the long-term rates of smoking cessation and relieves craving for nicotine and withdrawal syndrome (Rigotti, 2002).

# 17 Specific Non-Nicotine Treatment

The eradicative approach involves the use of non-nicotine compounds. Varenicline, a partial nicotine agonist selectively binds to α4β2 nAChRs (Galanti, 2008). As a partial agonist it partially stimulates receptor-

mediated activity leading to the release of DA and the consequent reduction of cravings and nicotine withdrawal symptoms. Furthermore, it competes with nicotine for the nAChR binding site leading to a decrease in its reinforcing effects (Coe et al., 2005).

Varenicline is considered the best smoking cessation aid to date for long-term abstinence in the general population, with comparison to bupropion (Hurt et al., 2009; West el at., 2008) and NRT preparations (Aubin et al., 2008). Several randomised control trials (RCTs) showed that varenicline seems to be more efficient than bupropion. For example, 2 studies (Gonzales et al., 2006; Jorenby et al., 2006) showed that after a 12-week treatment regime, the drug lead to a 44% abstinence rate, versus 30% for bupropion SR and 18% for placebo. The administration of bupropion, a phenylaminoketone atypical antidepressant, was the first approved drug and is now considered with other first-line pharmacological treatments for nicotine addiction (Sutherland, 2002). The action of bupropion seems to be multifactorial, including (Hurt et al., 2009) inhibition of norepinephrine (NA) and DA reuptake (Ascher et al., 1995), nAChR antagonism (Slemmer et al., 2000). Sustained-release bupropion (bupropion SR) has been shown to be more effective and exhibits a significant dose-response effect (Hurt et al., 1997). Additionally, bupropion SR together with transdermal NRT lead to significantly higher long-term rate of abstinence from smoking (Fiore et al., 2008; Jorenby et al., 1999), since presumably NRT alleviates nicotine withdrawal symptoms and antagonists reduce the rewarding effects of smoking (Di Matteo et al., 2007).

# 18 Non-Specific Treatment

Other non-specific therapies, such as antidepressants, are also used which inhibit NA and 5-HT reuptake (Di Matteo et al., 2007). Smoking cessation has been shown to increase depressive symptoms in many individuals such that antidepressants are widely used to prevent such manifestations (Busch et al., 2011). It is also important to note that nicotine, since it increases central 5-HT levels (Seth et al, 2002), can be a form of self-medication for an underlying depression which might then be unmasked upon smoking cessation (Borelli et al., 1996).

# 19 Promising Treatment under Trial

Electronic nicotine delivery systems (ENDS) are cigarette-shaped electronic devices consisting of a battery-powered heating element to vaporize a solution containing nicotine and thence inhaled as a mist (Choi and Forster, 2013). Both nicotine and smoking-related cues appear to control cigarette craving and withdrawal symptoms, therefore ENDS may be an effective smoking cessation device (Caponnetto, 2012). Current ENDS trials are evaluating smoking reduction and abstinence effects, product preferences, and adverse effects of marketed devices (Polosa, 2011).

Mecamylamine, a non-competitive nicotinic receptor antagonist, has been evaluated for more than a decade (Kirshenbaum et al., 2011; Lundahl et al., 2000). Its non-competitive nature permits nicotine to bind, but not to impose its receptor-mediated effects. This leads to attenuation and eventually extinction of the conditioned addictive behaviour of nicotine, since tobacco consumption would not offer the same degree of motivation and reward. Such phenomenon is in fact shown by a compensatory increase in smoking to make up for the decreased nicotine-induced hedonia (Kirshenbaum et al., 2011).

A Cochrane systematic review showed that opioid antagonists, mainly naloxone, buprenorphine and naltrexone, have the potential to attenuate the rewarding effects of tobacco smoking since the central endogenous circuitry has a role in reinforcing the smoking stimulus (David et al., 2009).

Immunotherapy, also referred to as nicotine vaccination, could also be a promising solution in the near future for the sphere of smoking cessation by injecting a nicotine-like hapten, conjugated with a strong immunogen, with the consequent production of nicotine antibodies (Orson et al., 2008). Anti-nicotine antibodies would then sequestrate intravascular nicotine after tobacco smoking or ingestion (Cornuz et al., 2008). Ongoing phase III trials are expected to give rise to the first nicotine vaccines in the coming years (Aubin et al., 2011; Raupach et al., 2012).

# 20 Conclusion

The levels of consumption of tobacco are declining in developed countries but increasing in developing ones (Rigotti, 2002). Despite this fact, it is still the major preventable cause of death and quit rates remain low despite the availability of contemporary pharmacological treatments aimed at the cessation of tobacco consumption (Haas et al., 2004). Research has definitely provided much more knowledge on the neurophysiology of nicotine addiction on the human species. Despite the fact that promising results have been obtained, one also has to take into account the need for further obstacles in both neuroadaptive mechanisms and treatment to be surmounted. Some data is also obtained from experiments on animal models or in vitro settings and not from human trials. In addition, more research is needed amongst youths, who are ultimately the most vulnerable age group to addictive behaviour (PHS Guideline

Update Panel, 2008; WHO, 2008).

More circumspect research coupled with non-pharmacological approaches, public health awareness and ethico-legal measures are sure to offer better outcomes in the struggle against nicotine addiction and concomitant health hazards.

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# Appendix 1

## Criteria for Substance Dependence in ICD-10

Three or more of the following must have been experienced or exhibited together at some time during the previous year

1. a strong desire or sense of compulsion to take the substance;
2. difficulties in controlling substance-taking behaviour in terms of its onset, termination, or levels of use;
3. a physiological withdrawal state when substance use has ceased or been reduced, as evidenced by: the characteristic withdrawal syndrome for the substance; or use of the same (or a closely related) substance with the intention of relieving or avoiding withdrawal symptoms;
4. evidence of tolerance, such that increased doses of the psychoactive substance are required in order to achieve effects originally produced by lower doses;
5. progressive neglect of alternative pleasures or interests because of psychoactive substance use, increased amount of time necessary to obtain or take the substance or to recover from its effects;
6. persisting with substance use despite clear evidence of overtly harmful consequences, such as harm to the liver through excessive drinking, depressive mood states consequent to heavy substance use, or drug-related impairment of cognitive functioning. Efforts should be made to determine that the user was actually, or could be expected to be, aware of the nature and extent of the harm.

Source: World Health Organisation (1992). The ICD-10 classification of mental and behavioural disorders: clinical description and diagnostic guidelines. Geneva: World Health Organisation. ISBN: 9241544228

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*Biography*

# Lessons from an unplanned scientific and academic life

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## 1 Introduction

It is salutary, before reaching the middle of one's eighties, whilst time is still available and memory is still in good order, to review a long life in its highlights, so as to better appreciate the circumstances that shaped and steered that life through its many days. Besides this appreciation, such a review permits a listing of lessons learned through that life, its joys as well as its woes, in the hope that they may be useful to young readers of your story. Like all other such stories, mine was the story of an individual, who lived under unique circumstances and reacted to them in a unique way. My story is best treated in terms of where it was experienced, that being: Malta (1929 to 1952), Oxford (1952 to 1956), Singapore (1956 to 1960), Khartoum (1960 to 1965), and Saskatoon (1965 to the time of writing). Each transition was necessitated by its own circumstance, brought fresh challenges and sustained a global career with few regrets and much personal and professional satisfaction.

Figure 1: Francis Vella.



## 2 Malta

I was born into a lower middle-class Maltese family in the summer of 1929. At that time the country's economy and government were determined largely from being a British colony and a very important Mediterranean base for the British Navy and garrison. That status continued until 1964 when Malta became an independent republic within the British Commonwealth. By then, due to professional and family reasons, I had become a permanent expatriate.

I was the sixth of eleven children, of whom one died in infancy, and four were girls. Like that of all my siblings, my early education was at the school attached to St Joseph's Convent in Sliema. This was located a short walk from our house and close to the harbour that was frequently home to destroyers of the British fleet. I was considered a good student at that school and can say that my childhood, in those pre-World War II days was a happy, carefree one with lots of swimming in the summer time.

I was successful in my first attempt at the Lyceum entrance exam just before WWII broke out. I spent the four war years at the Sliema Lyceum and the first postwar year in the Valletta Lyceum. Things went relatively well in spite of the heavy bombing by the Luftwaffe and the frequent air raids, but in March 1942 my father died suddenly, early one morning during a day and night spent in an underground air raid shelter. This meant that I became unclear about where I was heading. Going to the Valletta Lyceum was a stroke of luck, as I acquired a different group of teachers. Even with the little contact he had with me in his Maltese language class that year, Mr Paul Zarb (popularly known as Esopu) was instrumental in me being selected to sit the Oxford School Examination, for credits that would get me admission into the Royal University of Malta in October 1945. This was very significant as that was the

year admission was available and if it were missed there would be a three–year wait for the next. Mr Zarb's unexpected, benevolent intrusion (in loco parentis) into my life was a life changer. His six words of judgement ("Even you Vella, if you want") have been with me ever since.

During the summer breaks of those young teen years, I often took a bus to Valletta on a Saturday to borrow novels from The Royal Malta Library. One of these was Ivanhoe by Sir Walter Scott. I was very impressed by a poem I came across there, which ran: "What thou wouldst do, Do when thou canst, For when thou wouldst, Thou canst". It was not just the 'wouldsts' and 'cansts' that had an effect on me, but the unusual expression of practical wisdom incorporated into the adage: "strike while the iron is hot". That quartet has quite often been foremost in my mind and still serves me very well in my everyday life.

The University of Malta in those days, historical as it was, was a small institution with about 300 students, who studied Theology, Law, Medicine or Engineering and Architecture. Admission was every three years and its facilities modest. The professors were capable and respected in their areas and teaching for them was a secondary occupation. In Medicine, most were clinical consultants who had taken higher studies in the UK or Italy. They did their best and were usually well liked.

Before the end of my first year at university I had acquired the necessary number of credits for admission and the required pass marks in Maltese Language and Religion at the Matriculation exam, to be considered a full time student for the M.D. degree. I worked very hard that year and was one of a small handful to pass all examinations at the end of it. I was greatly encouraged by the kind words (the vein of Mr Zarb's) of a Dr Oscar Zammit who taught us laboratory biology. I attribute this success largely to the discipline of studying with a slightly older colleague who had been recruited into the army in the latter part of the war and who lived close to our home. We studied every day, including most weekends, and indulged by going to a late evening movie about once a week. My examination performance was kept up in the following years. Because of my performance in the four pre-clinical years, I was exempted from payment of university fees over the next three. It was during this time that I became very interested in Physiology and the Biochemistry associated with it (as little as that was then). This was largely due to Professor Walter Ganado whose lectures, I found, were always well prepared and very stimulating. He was interested in nutrition and biochemistry as the key to understanding human disease. In my final three years, I maintained this interest. I also felt that the Rhodes Scholarship (of which one was awarded to Malta every year) could be my opportunity for exploring these two subjects further. It was very clear to me by now that I was more interested in the academic aspects of medicine rather than its day-to-day practice.

I was never much of an athlete, but I was a Rover Scout during my university years, loved camping and hiking with my colleagues, was a member of the rather successful rowing team, participated in soiree lyriques and even played a part in a one-act play. These and my consistent performance in examinations played an important part when I applied for the Rhodes Scholarship in my final year at University. My success against two other applicants was my first step in pursuing the rest of my life overseas.

My education in Malta was largely textbook-based. It emphasised memorisation and the authority that came from the position of the author or authors of those books. What was important was the conclusion rather than the results or methods they were derived from. It also largely assumed that understanding and a critical attitude to printed information would come naturally. It is these deficiencies that my teaching career would later on pay great attention to. That education provided me with very little laboratory experience, something I became more aware of with the passage of time. From it all, I came to appreciate what my talents were as well as where I lacked in some aspects, and the need to undertake only what was within my abilities.
This phase in my life taught me these lessons:

1. Do not leave for tomorrow what you can do today.

2. Whether we like it or not we are constantly being assessed, evaluated, weighed up, formally or informally. Often it is to our advantage to know the results of such assessments.

3. Life, with its joys and woes, has to be lived and enjoyed to the full.

4. Make the most of what you have talents for and of what your environment provides to do well, in spite of your deficiencies.

# 3 Oxford

I was accepted into St John's College Oxford to read for honours in Physiology. This would also include some Biochemistry. I adapted to college life rather quickly. My tutor was a recently appointed Fellow who was also medically qualified. Every week, on Tuesday at 8.15 pm I would go to Dr Robert Torrance's college suite and read to him and answer questions on, an essay I had written on a topic he had assigned me. I attended lectures and laboratory sessions that he recommended and spent most of my time at the Science Library chasing

and reading such publications as were relevant to my essay. Textbooks were of no use as they rarely said much about the particular topic. My first few essays were based largely on the conclusions in papers I referred to. However, needless to say, I could not answer questions about methods or results on which those were based. I had to change my style of work. I now had to pay greater attention to methods and analysis of the actual results in each publication. I had to derive my own conclusions and check how they confirmed, or otherwise, those of the author or authors. This was difficult but it was the track that led to me becoming critical and scientific in my reading of other people's work. In effect, it changed me from a memorizer of textbooks dependent on other people for my ideas, into a critical and independent thinker whose thoughts were based solely on the analysis of evidence that was available to me. This was a slow process. I became aware of the changes that had occurred in me in a momentary epiphany that happened one beautiful sunny spring afternoon of my stay in Oxford. I will never forget the moment.

I spent the summer break of 1953 in the Clinical Biochemistry department of the Radcliffe Infirmary, through the good offices of Mr JRP O'Brien (Percy), who was to be my Biochemistry tutor for two terms in my second year. Again I attended the weekly essay reading and tutorial on Tuesday at 8.15 pm at Mr O'Brien's house. Most of the Biochemistry available then was on metabolism. I now met the book on 'Inborn Errors of Metabolism' that was made famous by Sir Archibald Garrod in the early years of the twentieth century. My interest in Garrod would increase several years later. I sat the honours examination at the end of my second year and achieved a second class degree. I was pleased with this result as I felt that it truly reflected my assessment of my own abilities.

It must not be assumed that there was no free time for fun in those years. Meals in college were an occasion for much camaraderie, as were brief coffee meetings with a group of college friends, after lunch or dinner. There were occasional social events at Rhodes House or invitations to dinner at other colleges as a result of friendships formed. Saturday evenings were reserved for a visit to the cinema or to the theatre and when the weather was fine, Sunday afternoon was optimal for long walks as they cleared the mind and gave time for the unconscious to do its work without additional clutter. In the breaks between terms there was travel to other parts of the country and hospitality arranged by the very resourceful and formidable Miss MacDonald of the Isles who generously provided this service to Rhodes Scholars from her London office.

After my exams in 1954, I took a vacation in Malta where I committed to be a witness at the summer wedding of a good friend from my university days there. I recall meeting Professor Ganado one day that summer and hearing from him about the concept of molecular disease. He suggested that I should try to get into that field. I was now 25 years old, time to think of getting married to a young lady I had met in England.

Mr O'Brien had accepted me into his department for my final year. I mentioned molecular disease to him, but he felt that Africa rather than Oxford was the place for that. He suggested that I try to investigate serum peptidase activity, so off to the library I went to review what was known, methods used, results of clinical significance and such like. That took several weeks to achieve. He suggested an untried colorimetric assay for serum peptidases based on a possible change in ninhydrin reactivity of the amino acids released by cleavage of a small peptide substrate. However, the results I obtained by this rather crude method produced little of significance.

I had decided that returning to work in Malta was unlikely and started to think of getting some form of employment so I could get married. I felt that setting up a home in the UK would not suit me or my circumstances and considered joining one of the newer universities in a British colony. I soon discovered that an Assistant Lectureship was available at the medical school of the University of Malaya in Singapore. After taking advice from Mr O'Brien I applied for this position and was offered it. Mr O'Brien arranged for me to be supported by a Mary Goodger Scholarship when my stipend from the Rhodes Scholarship ended and until I got married and left Oxford.

My wedding to Lena took place at the Jesuit Church close to the Radcliffe Infirmary on a very cold Saturday morning early in January 1956. This was the beginning of a new life for Lena and I and one full of new responsibilities. Later that month Lena and I flew to Malta and then to Genoa to board a slow cargo boat, which also carried a dozen passengers, to Singapore. During that voyage my coming teaching duties impressed themselves to me. I spent my free time on that trip reading the recently-published Textbook of Biochemistry by Fruton and Simmonds to expand my knowledge of the subject. I had no experience of lecturing but, with my youthful exuberance, felt confident that I would succeed.
My Oxford experience was a happy one, with its ups and its downs, it taught me that

1. Important decisions entail equally important consequences which, although barely thought about before they present themselves, have to be faced responsibly and usually on their own terms.

2. If your ways of doing things do not produce the desirable results, they must be changed.

3. What you learn, and especially what you learn to

do for yourself, is much more meaningful than much received knowledge.

# 4 Singapore

We arrived in Singapore on a very hot and humid Sunday morning early in April. The weather was a huge contrast to what we had left behind in Europe. I soon discovered that the department in the medical school consisted of a Professor (Australian), a Senior Lecturer (Singaporean), two Lecturers (one English, one Irish), and myself as Assistant Lecturer. It was responsible only for teaching medical students. This meant that my share of the teaching in the first year was small and consisted mainly of demonstrating in the laboratory class (a useful experience it turned out to be). This left me ample time for setting up a research program. The professor suggested that I consider looking for abnormal variants of human haemoglobin (Hb) about which he had read. So there it was, molecular disease, at last. Here was my chance to get into the field. I was assigned a technical assistant and I had to give him some work to do. For this I fell back on an assay for uropepsinogen on which I had information from my Oxford days. The assistant was Stephen Pang, a pleasant young man of about my age, who turned out to be a real treasure.

To begin with, most of my time was spent in the library and consisted of an up-to-date review of publications on the abnormal haemoglobins, which then included the relatively common Hbs S (for sickle cell), C, D, and E. Normal Human Hb was known as A1 ( major adult), A2 (minor adult), and F (foetal). I became deeply involved in reading what was then called Cooley's anaemia (later thalassemia). I quickly found that I was delving into various kinds of anaemia and into haematology and genetics of which I had only what can be best described as a smattering. Here I was learning, completely on my own, what I needed to know because it was integral to the work that I would be doing. There being no photocopying systems available, I made extensive notes on everything that I read. My memory in those days was very good and I retained much useful information from what I read, including who had done or discovered what and when. I was lucky to find a very recent and comprehensive review, published in the New England Journal of Medicine, that described an electrophoresis system in which Whatman filter paper was sandwiched between siliconized glass plates (the 'evaporation – prevented' method). I also found a description of the method for the 'one-minute alkali denaturation' assay for Hb F. I recognized that all this was within my capability. I was ready to go.

I explained to Stephen what I planned to do. He liked it. He stopped work on the assay for uropepsinogen. There was a departmental technician who did glass blowing and other work for the department. He made plastic electrode troughs, power packs and found the glass plates we needed. Because of the hot weather we decided to run the electrophoresis inside a refrigerator in the laboratory, with the power packs sitting on a small table next to it. While this was on, I wrote up my literature review in the form of a paper for possible publication. After checking out the equipment, I felt that the professor was more interested in physicochemical measurements of the electrophoretic mobility of haemoglobin than on anything else, which I considered to be of greater interest. Luckily, he left for a long leave in Australia and very soon thereafter he returned to Australia for reasons of health. I was now on my own and could direct my own project.

The electrophoresis system worked well and reproducibly, with good mobility within 2 to 3 hours. This meant that we could have two or three runs a day, if I went back in the evening to switch off the third run. With nine specimens on each sheet of filter paper, three possible runs per day, four electrophoresis and four power pack units we could scan about 500 specimens per week. Preparation of samples could be effected while electrophoresis was in progress, required several bench top centrifuges and an adequate supply of glassware. I could lend a hand at preparation of specimens or setting up of electrophoresis runs as my other activities permitted. The supply of clean glassware was left to Krishnan (a very loyal and hardworking Indian). Stephen was a well-organised individual and enjoyed the work. We were equal to the task. The method for Hb F was a simple colorimetric one, which was quick, easy and reproducible.

Before long I came across a citrate - agar gel electrophoresis method which could better discriminate several types of haemoglobin. I tried it out and added it to our repertoire. I also found a method based on solubility, specific for Hb S. Since several variant Hbs had a similar electrophoretic mobility to that of Hb S, the test would be useful in excluding this type. Soon after I started this work, new variants had started to be described at a rapid rate, so it was essential that I keep up with the literature and keep a note of any developments.

It was essential for our purposes to have sources of already available blood samples. One good source was the Blood Transfusion Centre, of which Dr (Mrs) MMH Gibson-Hill was in charge. Her blood donors were largely from the British garrison and included significant numbers of Malay, Nepali, Eurasian, Chinese, Indian and European origin. She agreed to provide all 'pilot tubes' that were no longer required for the work in her Centre. All I had to do was send someone to collect them before the Centre closed for the day. This

was easy as Krishnan enjoyed this task and did it very responsibly. We received about 12,000 samples from her in my four years in Singapore. The very first abnormal samples we found came from Malay donors. They were easily categorized as containing Hb A1 (normal adult) and Hb E. This excited everyone in the laboratory, as it showed visible proof that we were not chasing ghosts. Very often, the refrigerator would be opened to inspect progress during a run and a call would go through the lab indicating the number of abnormal specimens that could be seen!

Those blood donor samples formed almost half of the samples examined. The rest came from two other sources in Singapore, Johore Bahru, Malacca, Kuala Lumpur, Penang and Sarawak, the result of having contacted interesting people who were eager to help. In addition, there were some 700 samples from hospital patients (including many children), who were being investigated for severe anaemia, and 2500 cord blood samples. We found numerous instances of heterozygous Hbs D, E, J, K, L, Q, S, and of Hb H disease, Hb E-thalassemia and classical Cooley's anaemia (or thalassemia major). Of these, Hb Q and J Norfolk were new discoveries. A very exciting find was a Chinese man with severe anaemia who turned out to have Hbs Q and H disease. Similarly, the finding of two unrelated cases of Hb H that appeared on one filter paper that contained nine samples. The latter, and others like them, were confirmed by supravital staining of erythrocytes with brilliant cresyl blue which produced characteristic inclusion bodies.

I felt a great need to inform the local medical profession of our findings and so published many results in two local journals (Proceedings of the Alumni Association and Medical Journal of Malaya), but also found publication in peer-review journals. Many of the variants were confirmed in collaboration with Dr Hermann Lehman, then at St Bartholomew's Hospital in London England, a collaboration that enhanced my scientific standing and lasted many years.

We spent part of our first leave in Malta. I recall being asked by paediatrician Dr Tommy Agius Ferrante to help make the diagnosis of Cooley's anaemia in 10-month-old twins in his care. I requested that he produce a blood sample from each. I made arrangements with the microbiologist and friend Dr E Agius and, in his laboratory, prepared the necessary sodium hydroxide and ammonium sulphate solutions and two haemoglobin solutions. I estimated the Hb F in each and found it to be very high, as was expected in Cooley's anaemia, much higher than it should be at that age in normal children. My experience of the previous two years was being put to use in Malta.

The work resumed with vigour after our return. Soon thereafter, I received a copy of a dye-decolourization test for glucose-6-phosphate dehydrogenase (G6PD) deficiency, a defect that underlies primaquine-sensitivity hemolytic anemia, for which I was trying to set up an assay based on determination of glutathione in erythrocytes. The test was simple, worked as expected, and was added to our routine for diagnosis in special anaemic patients. It gave interesting results in young patients with kernicterus of unexplained origin. We later also established a starch block electrophoresis system for assay of Hb A2.

All of the expenses involved in this work were paid for through departmental funds. This was a remarkable attitude of that young university towards support of research by its academic staff.

My reading introduced me to the work of Professor Ezio Silvestroni and Dr Ida Bianco of Rome, who had used very extensively a hemocytometer-pipette-based test for increased erythrocytic osmotic resistance in thalassemia minor (which they called microcythemia). I entered into correspondence with them to arrange a visit to their laboratory when I was next on leave.

Political and other developments in Singapore at that time were a cause for concern and Lena and I decided that it would be best if we made our next leave the final one. During our stay there we had become parents of three young children. It happened that the university encouraged its junior faculty to write up their research work as a thesis to be considered for the award of the PhD degree. I opted to do this and worked very hard over my final six months to produce this thesis, being successful in my efforts.

While waiting for the offer of a suitable position during my final leave from Singapore, I spent two weeks with Professor Silvestroni and Dr Bianco (his wife), very charming people totally dedicated to, and immersed in their work. That proved a very useful experience. On my departure, they provided me with enough Tyrode solution to embark on a small survey (conducted with the help of former colleagues who were school medical officers) in school children in Malta where we were spending part of our leave.

In the short time available I was grateful for an offer as Senior Lecturer (I had been promoted Lecturer after my first year in Singapore) at the medical school in Khartoum starting in July of 1960. This was going to be a big change for us during the tenure of a five-year contract, but it offered new challenges and opportunities and yearly three-month leave, which could be spent in Malta where our children could be registered for a term at the convent school that I had attended. This would provide me with time to pursue my academic interests. This phase of my life taught me

1. Do not waste opportunities that may present themselves to you. Their life may be very short and it is

not likely that they will come again.

2. Professional friendships at home or abroad can be very productive and life-long.

3. The scientific world offers many opportunities which are usually of little or no monetary value but rich in professional rewards.

4. Time is short, the science is hard, but the satisfaction and rewards it provides can be without measure.

# 5 Khartoum

This hot, recently independent, widely-spread city, in the middle of a desert at the junction of the Blue Nile with the White Nile, had a small medical school housed in a new building close to government laboratories and a General Hospital. Its Arab culture and gentle, friendly, people were easy to adapt to and enjoy. The city was by no means a fast one and largely offered the basic essentials and commodities. There was a large European expatriate community. Lena and the children were soon able to fit in and make friends, and the children attended a small play school.

The department was on the third of three floors, cooled by overhead fans, but quite open to sand and rodents. The faculty consisted of a head (British, in the position of Reader, who was very supportive and offered me every possible help), a technician (British) responsible for the students' laboratory, a technical assistant (Egyptian, named Emile, willing but not the equal of Stephen) who was assigned to help me, and a happy young Arab man called Bashir, who ran errands and did odd jobs, who was very interested in helping when he was not otherwise occupied. Secretarial assistance was provided by the dean's office, and Lena would often help me at home with typing. Conditions were primitive compared to those in Singapore.

My teaching duties were not heavy and were shared with the head. My first attendance at a laboratory class showed that the student manual needed to be corrected and upgraded. I did this during my free time at home. In my lectures I tried to give simple explanations, be up-to-date, and on occasion, go beyond the textbook. Most students were very interested and eager and often came to my laboratory for further explanation or information. Within a few weeks, when I had established my laboratory, I started to welcome small groups to demonstrate to them what I was doing, its theoretical background and its significance. The effect that this had was demonstrated when at least four of them took up Biochemistry as a profession, later acquired a Ph.D. in the subject and joined faculties in Khartoum, Saudi Arabia, Kuwait or Oman.

It did not take long for me to acquire enough table top centrifuges, commercial electrophoresis tanks and power packs so that I could start my work. My laboratory could perform electrophoresis on agar gel and starch block, erythrocyte sickle cell preparation, Hb S solubility test, Hb F determination and G6PD testing. All electrophoresis was performed inside a refrigerator or a cold room when this became available. Blood samples that were no longer needed were derived from the government laboratory located just across the road, and blood samples were sent for my attention by clinicians at the General Hospital. Emile slowly learned the techniques from me and very often Bashir was eager to help with preparation of hemoglobin solutions for analysis. One of the problems I faced was to convince clinicians when my results indicated that their patients had sickle cell disease or thalassemia. This arose from the cultural attitude that Arabs are not black people and therefore cannot have the former, and that thalassemia occurred only in countries around the Mediterranean and therefore not in the Sudan. Slowly, my reasoning based on genetic admixture became accepted and my value in the eyes of these clinicians increased appreciably.

It was government policy to sponsor, for specialty training overseas, interested and capable recent medical graduates. One such graduate(Dr Saad Ibrahim) presented himself to the department and became attached to my laboratory. He quickly learned the various techniques and increased the working capacity. He would also help demonstrate in the student laboratory. Arrangements were soon made for him to proceed to London, England where he completed a Ph.D. degree and then returned to head the department. Many years later, we met again when both of us were external examiners at the medical school in Tripoli, Libya.

I had come across, in a Journal of Chemical Education, a very simple assay for blood catalase activity. This involved measurement of the temperature change (with a sensitive thermometer) that occurred quickly, on addition of hydrogen peroxide to a dilute haemolysate. With human blood this could reach 8 or 9 degrees Celsius. I recalled reading about cases of acatalasemia in some Japanese dental patients, reported in the 1940s. I had also read that hamsters could inherit a gene for hypocatalasemia. Dr. Saad quickly located a colony of hamsters at the Veterinary School in Khartoum North and made arrangements for us to test the colony to confirm the findings, which we did. We also measured catalase activity in some other animals. This was an interesting diversion.

I was in frequent demand by young medical graduates to present seminars to them as they prepared themselves for the primary examinations of the Royal College of Surgeons from her London office. These

would be on topics in molecular genetics, inborn errors of metabolism, molecular haematology, chromosome abnormalities, endocrinology, plasma lipoproteins, and other matters of current research interest. They would take place once a week, early in the evening and be attended by 6 to 10, usually male, doctors. They were presented on a voluntary basis and deemed helpful for success of these young doctors in those examinations, an event that was celebrated with great conviviality.

One year, arrangements were made for me and two medical students to go to El Fasher (Western Sudan), during a term break to conduct a sickle cell survey. We arrived with a good supply of glass slides and cover slips, solution of sodium metabisulphite and a microscope, and set out just before daybreak on two mornings to a local clinic run by a governmental medicine assistant where a lineup of patients formed quickly. One student would register the name of a patient after he had been seen by the assistant, the patient would then proceed to the second student who mixed a drop of the patient's blood with one of metabisulphite solution on a slide, covered it with a cover slip and placed it in its proper order on a tray. I would then collect such a tray every 15 to 20 minutes and look for sickle cells on each slide under the microscope. We screened about 200 people per day before the sun was too high and the lineup had disappeared. The students quickly learned to recognize sickle cells and to distinguish them from normal cells. The students (and I) were impressed by how quickly the assistant formed a clinical evaluation of each patient based on his intimate knowledge of the local people and their common ailments. Equally impressive was that he used the same small amount of Benedict's solution to test for glycosuria in 6 to 8 patients, or until a positive result was obtained. The Benedict's was very precious to him as he was provided with an HP Sauce bottle full of the solution once a month! We also tested the patients in the small local hospital. The two young doctors in charge were surprised that I diagnosed sickle cell anaemia in two of them and the sickle trait in several others. We obtained blood samples from these hospital patients, and on our return to Khartoum the two students prepared haemoglobin solutions and submitted them to paper electrophoresis and Hb solubility tests to confirm our results from the microscopy testing.

Each year the annual summer leave from Khartoum was put to good use. In one, I extended the thalassemia survey in Malta, in another I carried out a search for inactive X-chromosomes in young men who fitted a diagnosis of Klinefelter (XXY) syndrome using a simple staining method on buccal epithelial smears, and in another I did library research for an essay on Gregor Mendel. Another required many mornings spent at the Royal Malta Library in Valletta scanning local papers published between 1914 and 1920 for anything that contained the named Archibald Garrod. This arose from my discovery that Garrod was the possessor of an M.D. 'honoris causa' from the University of Malta. My scan revealed a treasure trove. I also found relevant letters and information from the University Archives and interviews of elderly gentlemen who had known him or worked under him during the Great War of 1914-1918. I copied out the relevant items from the papers and made copious notes, which were eventually the basis of publications.

Years later, now in Saskatoon, I was approached to make this material available to Professor Alexander G. Bearn (Rockefeller University, New York) who used them for a chapter on "Malta: The War Years" in his book "Archibald Garrod and the Individuality of Man" (Clarendon Press, Oxford, 1993). The end of this story came in 2004, when I delivered the St Luke Day Lecture organized by the Malta branch of the British Medical Association at the medical school in Malta. My lecture was on the unique graduation ceremony, at which Garrod and three other distinguished British medical specialists who served with him in Malta during the war were awarded honorary M.D. degrees. I took that opportunity to pay my respects to Sir Archibald, by presenting to the University a portrait painted by a local artist from photographs that I supplied. That was the longest gestation period (almost four decades) of one of my lectures.

Early during my final year (now promoted to Reader), I received a letter from a former Khartoum colleague who had taken up a position in Anatomy at the University of Saskatchewan in Saskatoon. He informed me that two positions in Biochemistry were available in the medical school there and that the dean had been told about me and my possible interest. Lena and I decided that this would be a good move for us, so I applied and was offered a position as an Associate Professor that was to be confirmed after my arrival. I accepted the offer and we started proceedings for migration to Canada.

Those five years passed quickly. I had shown local clinicians the occurrence of sickle cell disease, Hb O Arab, thalassemia and G6PD deficiency in their population. I had contributed to the future wellbeing of the Sudan and made other cultural and academic contributions, until it was time for us to find a permanent home. The Sudan had given to me and my family very generously and graciously, despite its poverty and for that we were very grateful.

Lessons learnt from these experiences were:

1. Sometimes family, or other serious considerations, may require adjustments by making temporary changes to how your life proceeds. This is what adventure is about.

2. Be happy when your knowledge and your skills are being used for the benefit of the community.
3. You may never know when and how information you have acquired may become useful to others.
4. Friendships, professional or otherwise, based on mutual esteem and respect are life-long treasures.

# 6 Saskatoon

We arrived in Saskatoon late in June of 1965 after a 3-day train journey from Montreal where we had landed after the overseas journey from Liverpool, England. The addition of a fourth child, a three–month-old daughter, made the whole trip a real adventure. Saskatoon proved to be a very attractive bridge city with huge green trees, neat grassy front lawns, a blaze of flowers, and the university campus with its impressive Greystone buildings which was a delight to walk around, especially in the summertime. Arrangements for schooling had to await the reopening of schools early in September. Meanwhile, we were advised to make the most of the rather short summer in preparation for the cold and snow in the winter.

I went to introduce myself to my new department. I was shown the laboratory space that had been assigned to me, still to be uncluttered so that I could see what useful space there was. This would also be my office space. The faculty consisted of an elderly (very hypertensive) Professor and head, and two Associate Professors who had recently been promoted to that rank in anticipation of my arrival. Any laboratory assistance I needed, including glassware cleaning, was my responsibility to find funds for. What an unexpected civil service attitude and monetary approach compared to that I had experienced over my previous nine years in two less endowed and less developed countries. I had committed myself to settling in Canada and decided to grin, bear it, and hope for the best. I cleared out the laboratory space assigned to me and cleaned it (raising some eyebrows in the process). I looked around and made enquiries to find what equipment there was that I could use in setting up my laboratory. I visited the dean who had been instrumental in my appointment. I explained to him that I was interested in looking for variant human Hbs and was almost told not to waste my time. I replied that there are rare variants to be found and that these could be of the highest interest. He was happy that I would teach the medical students and was prepared to be as supportive as possible.

It did not take long to set up one paper electrophoresis unit. Barely three weeks had passed when I received a blood sample from the Haematology department at the University Hospital from a very recent Jamaican immigrant, who required investigation for complaints he had experienced during his travel. Some technician had noted what looked like sickle cells on a smear of his blood. My diagnosis from an electrophoretic pattern of Hbs S and F (confirmed by a solubility test and a determination of Hb F) was sickle cell disease. This was the first time the diagnosis had been made in the province. This justified me before the dean and raised my profile and the appreciation of my usefulness. It also meant that I would receive blood samples from patients with anaemia of undetermined origin to investigate for abnormalities of haemoglobin and G6PD deficiency. With modest funds provided by the dean, I acquired more electrophoresis chambers and power packs and part-time help for the preparation of haemoglobin solutions and cleanup of glassware (a problem for quite some time).

My first teaching duty was a course on Biochemistry in Medicine. Here my previous experience proved very useful and I was well received by the students. Four years later this was recognized by myself receiving the Pre-Clinical Teacher of the Year award.

Our three older children had been registered in school, were doing well and enjoying themselves. Lena and I had acquired the beginning of a social life, focused mainly on university colleagues and friends we made at church. It seemed that I had faced my worst tribulations in my new country, but there is a price to be paid for everything. I searched for variants in leftover samples from the Haematology department. It was not until about two thousand had been processed that the first variant appeared. This convinced my assistant and those around me that there were interesting findings to be made and that only patience and determination were required. We maintained our efforts and increased the pace of our screening process.

I applied for a modest research grant from the Medical Research Council of Canada. I was granted enough to pay for necessary supplies and for a full-time technical assistant. This position was filled by a recent high school graduate (Albert Labossiere) who appeared at my laboratory seeking a job. He was very interested in science, not afraid of work, eager and capable of learning quickly and on his own. I hired Albert on the spot and also maintained the part- time assistant who had responsibility for preparation of haemoglobin samples. This help expanded our repertoire of analytical techniques which later included fingerprinting of peptide digests of globin. This system was tried out by a medical student as a summer project on globin derived from a Great Horned owl supplied by a clinical colleague who was a dedicated ornithologist, compared with human globin.

Teaching of Biochemistry to science students was still very much in its infancy in the department and consisted essentially of the basic course also taken by the

medical students, and one or two more advanced courses thereafter. I attended the first few laboratory classes and found them not to focus on scientific principles but to consist essentially of repeated colorimetric assays of inorganic phosphate (all in the name of accuracy and reproducibility of results). I therefore made my views known that this needed upgrading. Luckily, soon after, the head decided to vacate his position, the older of the other two faculty members died in a car accident and another (an American) had been recruited from California and was very supportive. An acting head was recruited part-time to administer and prepare the department for the new head, who arrived in time for the next academic year. The new head (a new Canadian, of Scottish origin) set out to bring life to the department, increase the faculty complement, enhance the research capability, and establish an honors program for science students. Change had finally arrived, and the Great Depression, inward-looking, very conservative attitudes were gone. I became involved in planning courses and contributed by teaching the protein section in an advanced two-term course on Biomacromolecules.

Soon after arriving in Saskatoon, I started personal subscriptions to the weekly journals: Science, New England Journal of Medicine and the Annual Reviews of Biochemistry. These I read in my spare time at home and supplemented my brief visits to the medical library. They kept me reasonably abreast of current scientific developments and became essential for my teaching activities. It is fair to say that everything I was teaching in biochemistry, I had taught myself through private reading and study.

The eight years for which I had modest financial support, produced a wide variety of rare haemoglobin variants and several previously unknown ones. The latter included Hb E Saskatoon, Hb Winnipeg, Hb Deer Lodge, Ottawa, G Norfolk, J Broussais, and St Claude. Their characterization, except for Hb Deer Lodge which was achieved largely by Albert, involved collaboration with Professor Herman Lehmann (now at Cambridge, England), Professor Titus Huisman (August, Georgia) and many others largely unknown to me. It had been an exciting search, but new technological developments that were becoming essential for such studies were beyond my technical skills and my capacity to find funding to employ suitable personnel. I had to be satisfied with my work of the previous sixteen years and move on.

In 1971 I was promoted to Professor. Two years later I was awarded a sabbatical year which I spent at The Abnormal Haemoglobins Research Unit in Cambridge, England. This provided a great opportunity for family contacts for my family (including our newest son), for writing up results which I felt deserved publication, for rest and a lot of scientific reading, attendance at lectures, seminars, a Conversazione at the Royal Society in London, several dinners at High Table of Sidney Sussex College, and thinking of my academic future.

On my return to Saskatoon, the head graciously accepted that I now concentrate on teaching, on the condition that I also undertake teaching in the honors program. I cannot assess how much my increased teaching time helped increase research productivity in the department, but suspect that it had no effect. I now became a regular visitor to the library where I focused mainly on looking for reports that I could use in my teaching. As the laboratory component for medical students caused a lot of concern to the department and the students themselves, I suggested that we discontinue a hands-on requirement but use the time available for alternative teaching approaches. I was already recommending a textbook that offered a 'Case-Oriented Approach' to Biochemistry in my teaching to medical students, so I sought published case reports that incorporated significant research on the molecular basis of disease and used them for my alternatives. In this way the case-oriented style had changed largely to a case-based approach.

I developed what I called Structured Learning Experiences (SLEs) for use in an interactive, small-group approach. An SLE was a publication that was rewritten in student-friendly language to provide an introduction, objectives, methods and materials used to meet the objective, and results obtained. Each section was followed by questions that required understanding of the information provided there or obtainable from a textbook. All answers were then written down, which could be individual or derived from the small-group discussions. Answers were to be handed in within two days and assessed by me. This was extra work for me but it was very worthwhile. For further variety I developed 'diagnosis - making games' (that I called Jepsons, after the person who first described the approach) in which information from a case report was provided to small groups, at intervals, and in a sequence that started with the clinical presentation, and went on to results of routine laboratory testing, then to special testing, until a molecular abnormality was identified. Each Jepson ended with my overall review and explanation of the reasoning involved in the particular case. The preparation of Case Reports, SLEs and Jepsons was my responsibility and proved to be an exhilarating yet demanding undertaking. The students attended these sessions and appreciated my efforts in helping to develop a deep interest in a scientific approach to medicine. My American colleague (Dr R.O. Martin), a great supporter and collaborator in these approaches, and I reported our experiences in the journal Biochemical Education (BE). This produced more than a score of requests for samples and for further information from overseas.

During my reading, I developed the habit of keeping notes on words or phrases that were not familiar to me. Later I would send these to the editor of B.E. Many were eventually incorporated into the Oxford Dictionary of Biochemistry and Molecular Biology (First Edition). This experience was put to further use during my retirement (see later).

I always found time to speak to representatives of publishers who had books that could be recommended in departmental courses. This meant that I received copies of many new textbooks. These I would look at closely at home with a focus on new ideas and approaches, errors, style and usefulness. I would then write a brief review of each book. Many of these were also used in B.E. I was now becoming known, outside my university, as an educator.

I received an invitation, through the kindness of one of my former Khartoum students, to be an examiner at the medical school in Tripoli, Libya. As I would be required when I was free of teaching responsibilities, I accepted the invitation. This led to receiving regular invitations for that purpose, and sometimes also for short periods as a visiting professor, in Tripoli. Another of my Khartoum students later invited me on several occasions as examiner to the medical school in Kuwait and in Oman.

I had an invitation from Professor Peter Campbell (London, England) to help him and two others to present a workshop on Biochemical Education in Karachi, Pakistan. Peter was then Chair of the Committee on Education of the International Union of Biochemistry (IUB) and had founded the journal B.E. I was granted permission by the head to accept the invitation. I travelled to London and with Peter proceeded to our destination. Peter explained his plan to me and it appealed to me. It so happened that during our stay in Karachi, Peter developed a condition that made him very dehydrated, in addition to having what looked like a perforated ear drum. He was reluctant to use the local medical facilities, so I took matters in my own hands, bought two dozen fresh oranges, squeezed the juice out of them and managed to get Peter to drink it all. He soon became better. This was my introduction to the international world of biochemistry. When Peter's term as Chair was due to expire, he recommended me as his replacement in that position on the committee. His commendation was approved, which opened up a new world for me.

I was very impressed by Peter's understanding of the problems of biochemical education around the world. I resolved to continue and expand on initiatives he had started. The idea of workshops at the invitation of national Biochemical Societies was pursued and resulted in a score being presented in almost as many countries over the nine years of my tenure. For these I could draw on the help of a group of dedicated and interested professors and in particular that of Professor Alan Mehler (Washington D.C., USA) and Professor Ed Wood (Leeds, England). The former had organized a workshop on Principles of Biochemical Education, to which I was invited, that was held just before the beginning of my term and the IUB congress in Perth (Australia) in 1982. The latter was Editor of B.E., with whom I already had dealings, and met in Perth when it became clear that we had several things in common (including that he had taught biochemistry in Malta several years previously). For some of these workshops I, or those helping me, often sought outside funds which helped fill out the committee's budget and increase its activities. I also encouraged organization of educational activities at international or national meetings and pre-meeting workshops for travel fellows at such meetings. Such events have now become routine. I initiated a small scheme for provision of current textbooks or review literature to departments in need in the developing countries. An important activity was my chairing of a small group responsible for publication of 'Standards for the PhD Degree in Biochemistry and Molecular Biology' (1989), which received a good reception by the international community. Such standards had not been formulated before. At the instigation of the President of IUBMB, I later undertook a revision of these Standards in the form of 'Standards for the PhD Degree in the Bimolecular Biosciences' (2000) and also participated in the third edition of these (2012).

Recognition came with the Master Teacher Award of the University of Saskatchewan, in 1984, the year the award was instituted, and an honorary D.Sc. (for contributions to international biochemical education) from the University of Malta in 1989. These were, naturally, occasions for sincere congratulations, but also for some resentment (I considered the latter to be a rather generous compliment), but life is like that and we have to take the different attitudes of colleagues in our stride.

In 1996, with my 67th birthday pending, I decided to retire from the university, as some circumstances were not to my liking and I had now completed 40 years of professional employment at three universities. Before my retirement took effect, I had accepted an invitation from the organizer of the ASBMB/IUBMB Congress in San Francisco (1997) to organize and conduct a pre-Congress meeting for 150 travel fellows sponsored by those organizations. I had also accepted to speak at an Education Symposium at the annual meeting of Federation of European Biochemical Societies in Barcelona. During the first eighteen months or so of my retirement, I had the freedom to accept several invitations to lecture overseas and to take a couple of travel vacations. Lena had, on occasion, accompanied me on some of my travels and now came more often.

This retirement was changed completely when in mid-February 1998 I received a phone call from Professor W.J. Whelan (Miami, USA), the new president of IUBMB. I had invited Bill to be one of two distinguished speakers at the pre-Congress meeting the previous summer. We were good friends as he had been General Secretary when I was appointed Chair of Education. He told me that the General Secretary (normally elected at a General Assembly) had resigned with immediate effect, almost three years before his term was to end. Would I be prepared to fill the voluntary position in which only expenses were paid by the Union? Astonished at this development, I said I would give him my reply the next day. This I did with encouragement from my family and was told to equip myself quickly with a fax machine, a computer and printer, machines that I had no first-hand knowledge of using. I had to convert a room at home into an office and arrange for part-time secretarial help to do all my typing. One son-in-law bought and set up the machines for me, while a daughter gave me her simple instructions for use of the computer with focus on operations connected with e-mail. Where would I have been without their patience and assiduous help? My learning curve was the steepest one of my life. Suddenly, faxes, e-mails and phone calls became constant arrivals. Patience, not deferring correspondence to the next day, and not wasting time, were essential to adapting to the new regime.

Within a month I had made quick trips to Berlin, Germany (for handing over from my predecessor) and to Miami (to meet the President and the Treasurer and plan for the next annual meeting of the Executive Committee in July 1997). The President (Bill) was a fountain of help. The annual meeting (in Copenhagen, in association with the Federation of European Biochemical Societies meeting) came and went. Then the Treasurer resigned and a replacement was found to complete his term. Bill and I started to produce a quarterly report and also to revise and upgrade the Union's Standing Orders which described procedures and responsibilities within the Union's organization.

By now, ASBMB started inviting me to conduct a one-day program for its travel fellows before its annual meeting. The first time, this coincided with the meeting of the Executive Committee (EC) of IUBMB in San Francisco in 1999. The following year the General Assembly of the Union was to meet in Birmingham at the International Congress. At the Assembly my successor as General Secretary was to be elected to assume duties beginning in 2001. This Assembly meant a lot of extra work for me, but I had learned well how to apportion my time and continued to be well served by the secretarial service that I was using. It was a busy but exhilarating time which brought many new friendships around the world. Appreciation for my contributions was formalised when the E.C. of IUBMB presented me with the Union's Distinguished Service Award and then invited me as its guest when it met in Budapest in 2005. That year the University of Saskatchewan Retirees Association also honored me with its Prime of Life Achievement Award.

I had kept up my interest in keeping notes on words and phrases from biochemical literature. This was rekindled when my term at IUBMB ended and bore fruit when I was invited to be an editor of, and contributor to, the Oxford Dictionary of Biochemistry and Molecular Biology (2nd Edition) and also to contribute a Glossary to Devlin's Textbook of Biochemistry with Clinical Correlations (6th and 7th editions). Both of these brought me appropriate royalties. I also found my assistance to be requested more frequently to assess, and help improve, manuscripts submitted for publication in the journals: Biochemical Education, Hemoglobin, Balkan Journal of Genetics, Turkish Journal of Biochemistry and IUBMB Life. These voluntary activities have kept me involved in my science and gave me the opportunity to assist numerous unknown colleagues, nearly all of whom came from developing countries.

I have contributed, at the request of their authors, to the improvement in manuscripts for at least four textbooks. I have done the same, during my retirement, for three PhD and one MD theses, and for the autobiography of a grateful former student. Such activities have enriched my life.

Lessons I learned from my experience in Saskatoon:

1. "To thine own self be true, and it must follow, as the night the day, thou canst not then be false to any man". Shakespeare (in Hamlet).

2. In the academic life expect to meet envy of, and resentment at, your successes. Take these as a compliment to your skills and achievements.

3. Learn from your mistakes and your weaknesses.

4. The expatriate life presents many challenges but constitutes opportunity for personal growth and often turns out to be a very significant advantage.

5. Go forward in confidence and give the very best of yourself in whatever you undertake.

# 7 Coda

The journey has been long and adventurous, tortuous and strewn with a variety of hurdles along the way. It was supported by the religious faith I had acquired from my parents and place of birth. I have been a scientist, educator, scholar, world traveller and family man, who enjoys what the world and life have to offer. Riches

or ambition have not been high among my priorities. Without Zarb, Zammit, Ganado, Lehmann, Huisman, Campbell, O'Brien, Whelan, Wood, Mehler, Pang or Labossiere, my life would have been very different. It would have been much more so without the constant support of Lena.

My life unfolded in the context of constraints from within me and from my environment, which I had to adapt to as necessary. I may not have lived up to some challenges, indeed I have deliberately avoided a few for some appropriate reason , but I only did what I knew I had it in me to do and could do under the circumstances in which I found myself. I am satisfied with that.

I am grateful to Graham Parslow, Associate Professor, Department of Biochemistry, University of Melbourne, Australia for comments on this essay.

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*News Article*

# Webcast courses in Medical Genetics and Next Generation Sequencing

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The European School of Genetic Medicine organised the 26th Course in Medical Genetics and the 2nd Course in Next Generation Sequencing, between the 12th and 20th May 2013. Both courses were webcast live from the Bologna University Residential Centre, Bertinoro, Italy. Participants in Malta attended these courses at the University's Medical School.

The course in Medical Genetics covered various aspects of this rapidly developing field of Medicine. The different methodologies used in human genome analysis, an introduction to Next Generation Sequencing (NGS), approaches to clinical and molecular genetics, complex genetic disorders, therapy and gene regulation, were covered.

The second course provided a comprehensive insight into NGS technologies, from the basics to the new world of disease gene identification by hand-held devices. It also covered insights into bioinformatics challenges, sample preservation and trans-omic studies, and new frontiers including the investigation of single cells and of the non-coding genome.

Medical Genetics or Genetic Medicine as it is increasingly being referred to nowadays, is a constantly changing clinical specialty. In the past 15 years there has been a massive increase in referrals of conditions regarded as common complex disorders such as breast and bowel cancer, and some cardiac diseases. An increase in the range of medical genetic services has benefited patients with genetic disorders and their families. "Careful clinical observation is at the heart of medical genetic practice" (Donnai). "The new technologies enabling targeted capture and massively parallel sequencing of individual genomes/exomes, have resulted in major discoveries on small clinically well characterised patients" (Donnai). Through the identification of novel genes, new developmental pathways have been discovered; many disorders with overlapping clinical features shown to be due to mutations in functionally related genes, might be responsive to treatment by similar molecules (Donnai).

Prof van Duijn delivered an interesting presentation entitled 'Complex Disease Genetics: GWAS and beyond'. Whole genome association studies using Affymetrix®/ Illumina®arrays, have revealed increased number of variants involved in complex disease. Sometimes it is difficult to predict the genetic risk for conditions such as dyslipidaemia and Alzheimer's disease. Genome tests are currently limited with respect to their predictive ability (van Duijn). "In the near future, personal genome tests would very likely be based on whole genome sequencing, but will these technological advances increase the utility of personal genome testing? The utility of testing depends on the predictive ability of the test, the likelihood of actionable test results, and the options available for the reduction of risks" (van Duijn). The identification of new variants in monogenic diseases could present a challenge due to the number of rare disease variants. The complexity of disease aetiology and disease heritability will affect the prediction of genetic risk for complex diseases. New omic technologies will aid in the discovery of biomarkers which could be used to improve predictions (van Duijn).

Next generation sequencing is a technological revolution in genomics which will have a major impact on the entire field of medicine. "All genomic variation

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Figure 1

that can be linked to disease is detectable in a single experiment!" (Veltman). In the near future it will soon be possible to sequence an entire genome at an affordable price which will lead to personalised medicine. Important characteristics of NGS platforms include: accuracy, ease of mapping / assembly sequence, sequence throughput / coverage, application, robustness, ease of interpretation and costs (Veltman). "The unbiased sequencing of the exome has become an integral part of the toolkit for genetics researchers" (Gilissen). Exome sequencing of trios (mother, father, child) are being offered to families of individuals with unknown disorders. Patients with specific disorders such as retinal dystrophy, cataract and epilepsy are being offered targeted testing using large panels of genes (Donnai). Preliminary results of diagnostic applications of NGS show that there is a much wider phenotypic spectrum associated with mutations in many genes than was previously thought. There have been concerns about the ethical aspects of NGS but as experience increases most centres are finding ways of addressing these in conjunction with patient groups (Donnai).

Handheld diagnostics on nanowires was one of the fascinating presentations during the NGS course. A novel, cheap, portable, handheld DNA sequencing device, is being developed by QuantumMDx for infectious disease applications at point of care testing, as an alternative to slow and relatively expensive capillary electrophoresis DNA sequencing (O'Halloran).

The presentation entitled 'Sample Preservation and Trans-omic Studies to Accelarate Scientific Research' delved into the use, requirements and benefits of biobanking. "Fast development of sequencing technology with decreasing cost has substantially promoted large cohort study, population health screening, and epidemiological prevention study on a genomic level" (Cheng). The 100 million US dollar China National Genebank (Shenzhen), operated by the Beijing Genomics Institute (BGI), aims at combining large bio-specimen collection and transcriptomic data production to accelerate scientific discovery and translation to clinical use (Cheng). "The Genebank has accumulated genetic variation information of more than 2000 monogenetic diseases by collecting suspected pedigree samples countrywide and worldwide" (Cheng). It also

established databases of global human genetic variation, cancer genetic variation and human inhabited microbial composition which are essential for understanding human disease and in the development of personalised medicine.

The faculty members for both webcast courses were internationally renowned experts in the field of Medical Genetics. The cutting edge lectures delivered during both courses, provided a golden opportunity for post-graduate research students studying at the University of Malta, to widen and update their knowledge on the latest developments in Medical Genetics, and also on the technologies used in analysing genetic data.

The courses were organised by Dr Isabella Borg, Director of the European School of Genetic Medicine, Malta Remote Training Centre, based at the University of Malta Medical School, with the assistance of Ms Joanna Vella from the Malta BioBank. Funding was provided by the Malta BioBank, University of Malta, as part of the training initiative.

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# Conference proceedings

# INTERDISCIPLINARY CHEMICAL APPROACHES FOR NEUROPATHOLOGY CM1103

## "4th Neuroscience Day University of Malta"

| 22nd Tuesday | Speaker | Topic // Title |
|---|---|---|
| 14.00-14.30 | UoM - **Richard Muscat**<br>MT COST - **Janet Mifsud** UoM-St Andrews, UK- **Rona Ramsay**<br>UoM - **Giuseppe Di Giovanni** | Welcome //<br>Introduction |
| **Session 1** | **Chair: Rona Ramsay** | |
| 14.30-15.00 | Di Giovanni Giuseppe (MT, WG4) | GPCRs modulation of extrasynaptic GABAARs |
| 15.00-15.30 | Peter Gmeiner (DE, guest speaker) | GPCR ligands probing structure and controlling function |
| 15.30-16.00 | Tipton Keith (CR, WG4) | From magic bullet to scatter-gun; is there a viable alternative? |
| 16.00-16.30 | Mavri Janez (CR, WG4) | How Enzymes Work? QM/MM Simulation of MAO |
| **Session 2** | **Chair: Mück-šeler Dorotea** | |
| 17.00-17.30 | Crespi Francesco (IT, guest speaker) | Non-invasive analysis of brain penetration of chemicals: concomitant Near-Infrared Spectroscopy [NIRS] and pharmacokinetic - pharmacodynamic [PK/PD] study |
| 17.30-18.00 | Magdalena Majekova (SK, WG4) | Bioactivity parameters of indole - type compounds and their possible relevance to treatment of neurological diseases |

| 23rd Wednesday | Speaker | Topic // Title |
|---|---|---|
| **Session 3** | **Chairs: Giuseppe Di Giovanni/Richard Muscat** | 4th Neuroscience @ University of Malta |
| 9.00-9.30 | Valentino Mario (MT, No COST) | Two-photon imaging of cortical microvessels and astrocytic interactions in live mouse brain |
| 9.30-10.00 | Ruben Cauchi (MT, No COST) | Modelling Spinal Muscular Atrophy in Drosophila: a Fruitful Approach? |
| 10.00-10.30 | Neville Vassallo (MT, No COST) | Lipid Membranes - a new target for neurodegeneration |
| 10.30-11.00 | Zammit Christian (MT, No COST) | Immature axons: a new therapeutic target for neonatal white matter ischaemia? |
| **Session 4** | **Chair: Marco-Contelles José** | |
| 11.30-12.00 | Simic Goran (HR, WG4) | The necessity of reliable biomarkers for monitoring potential treatments in Alzheimer's disease |
| 12.00-12.30 | Roberto Di Maio (USA, NoCOST) | Muscarinic stimulation elicits abnormal GABA-ergic differentiation in Mouse-derived stem cells |
| 12.30-13.00 | Philippe De Deurwaerdére (FR, WG3) | 5-HT2C receptors: a G-protein coupled receptor involved in opposite and distributed controls in basal ganglia |
| 13.00-13.30 | Mauro Pessia, (IT, guest speaker) | Potassium channels as target of CNS disorders |
| **Session 5** | **Chair: Di Giovanni/Muscat** | 4th Neuroscience @ University of Malta |
| 14.30-14.50 | Massimo Pierucci (MT, No COST) | Nicotine Addiction and Lateral Habenula |
| 14.50-15.10 | Gabriella Andrina Mifsud(MT, No COST) | Oligodendrocyte pathophysiology and treatment strategies in ischemia |
| 15.10-15.30 | Stephanie Ghio and Michelle Briffa. (MT, No COST) | Amyloid neurodegeneration: from electrophysiology to flies |
| 15.50-16.10 | Frau Robert (IT, WG4) | Positive allosteric modulation of GABA-B receptors: a novel therapeutic approach for schizophrenia |
| 16.10-16.30 | Esteban Gerard (ES, WG1) | 'Effect of new MTDL hybrids based on donepezil, pyridyl and indolyl moieties on Monoaminergic and Cholinergic systems: An HPLC metabolic approach'. |

| 24th Thursday | Speaker | Topic // Title |
|---|---|---|
| **Session 6** | **Chair: Valoti Massimo** | |
| 9.00-9.30 | Musilek Kamil (CZ WG2) | Design, synthesis and evaluation of modulators counteracting ABAD Aβinteraction |
| 9.30-10.00 | Unzeta Mercedes (ES WG3) | In vivo and in vitro biological assessment of ASS234, a novel Donepezil-indolpropargylamine, as a multifunctional molecule with a potential therapeutic profile for Alzheimer's disease |
| 10.00-10.30 | Mück-šeler Dorotea (HR WG4) | Serotonergic receptors, the new targets in the treatment of Alzheimer's disease |

| | | |
|---|---|---|
| 10.30-11.00 | Marco-Contelles José (ES WG2) | The Revisited MAO Inhibition by N-(Furan-2-ylmethyl)-N-prop-2-yn-1-amine Derivatives as Potential Drugs for the Treatment of Alzheimer's Disease |
| **Session 7** | **Chair: Dr Maria Carreiras** | |
| 11.30-12.00 | Najat Aourz (BE, WG3) | Sst2 and sst3 - but not GHS-R1a- receptors are involved in the anticonvulsant effects of cortistatin-14 |
| 12.00-12.30 | Stark Holger (DE, WG1, 2) | Bioisosteric Replacement in Dopamine D2-like Receptor Agonists |
| 12.30-13.00 | Carreiras Maria (PT, WG1,2) | Synthesis, pharmacological assessment, and molecular modeling of AChE/BuChE inhibitors: effect against amyloid-β |
| 13.00-13.30 | Marcello Leopoldo (IT, guest speaker) | Recent Advances in the Study of 5HT7 Receptor Pharmacology: Focus on the Selective Agonist LP-211 |
| **Session 8** | **Chair: K. Tipton** | |
| 14.30-15.00 | Ponimaskin Evgeni (DE, No COST) | Interplay between serotonin receptors 5-HT1A and 5-HT7 in regulation of receptor functions in the brain |
| 15.00-15.30 | Nikolic Katarina (RS WG1) | Pharmacophore Modeling of Novel Non-imidazole Histamine H3 Receptor Ligands with Inhibitory Histamine N-Methyltransferase Activity |
| 15.30-16.00 | Valoti Massimo (IT, WG3) | CYP-dependent metabolism and vascular effects of ASS234, a novel multitarget-directed ligand |
| **Session 10** | **Chair: Simic Goran** | |
| 16.30-17.00 | Vianello Robert (HR WG1) | Recent progress in understanding the catalytic activity of monoamine oxidases |
| 17.00-17.30 | Yelekci Kemal (TR WG1) | In silico design of novel and selective neuronal nitric oxide synthase (nNOS) inhibitors |
| 17.30-18.00 | Butini Stefania (IT, Guest Speaker) | Novel Tools for Disease Modifyng anti-Alzheimer's Drugs: hChEs and b-Amyloid Aggregation Inhibitors |

# ABSTRACTS

## P1.1
## MODULATION OF EXTRASYNAPITIC GABAA RECEPTORS BY G-PROTEIN-COUPLED RECEPTORS

**Giuseppe Di Giovanni**[1,2] **Adam Errington**[1], **Vincenzo Crunelli**[1]
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$GABA_A$ receptors ($GABA_ARs$), the main inhibitory neurotransmitter-gated ion channels in the central nervous system, are finely tuned by other neurotransmitters and endogenous ligands. The regulation of synaptic $GABA_ARs$ ($sGABA_ARs$) by G protein-coupled receptors (GPCRs) has been well characterized and is known to occur either through the conventional activation of second-messenger signalling cascades by G proteins or directly by protein-protein coupling. In contrast, research on the modulation of extrasynaptic $GABA_AR$ (eGABAARs) is still in its infancy and it remains to be determined whether both of the above mechanisms are capable of controlling $eGABA_AR$ function. In this talk, I will summarize the available literature on $eGABA_AR$ modulation by GPCRs, including $GABA_B$, dopamine (DA) and serotonin (5-HT) 2A/2C (5–$HT_{2A/2C}$). Although at present these GPCRs−$eGABA_ARs$ cross-talks have been investigated in a limited number of brain areas (i.e. thalamus, cerebellum, hippocampus, striatum), it is already evident that $eGABA_ARs$ show nucleus and neuronal type-selective regulation by $GPCR_s$ that differs from that of $sGABA_ARs$. This distinct regulation of $eGABA_ARs$ versus $sGABA_ARs$ by GPCRs provides mechanisms for receptor adaptation in response to a variety of physiological stimuli and under different pathophysiological conditions. Further research will advance our understanding of $eGABA_ARs$ and GPCR signalling and offer novel targets for the treatment of many neurological and neuropsychiatric disorders where abnormalities in $eGABA_ARs$ have been suggested to exist.

**KEY WORDS:** Absence epilepsy, metabotropic receptors, monoamines, phosphorylation, tonic $GABA_A$ inhibition.

## P1.2
## GPCR LIGANDS PROBING STRUCTURE AND CONTROLLING FUNCTION

**Peter Gmeiner**[1]
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GPCRs constitute a large superfamily of target proteins (nearly 800 different human genes encode for GPCRs) and each of them can adopt functionally distinct conformations. The first X-ray crystal structures of druggable GPCRs in complex with ligands provide a basis for the investigation of molecular determinants responsible for affinity and selectivity of ligands. Moreover, the structures of different activity states of GPCRs allow us to identify molecular interactions discriminating between inverse agonists, antagonists and agonists. These fundamental results also contribute to the rational discovery of drugs selectively binding to particular conformational states. Thus, there is growing evidence that homo- and heterodimers effect and diversify G-protein coupling. Besides this, the concept of functional selectivity (biased signaling) owing to ligand-specific GPCR conformations has been corroborated. Although GPCR-binding drugs could be evolved for a number of target GPCRs, the rational development of drugs with beneficial selectivity patterns between structurally related GPCRs and functionally relevant GPCR conformations, controlling intrinsic activity profiles, requires a better understanding for GPCR ligand interactions. We have developed GPCR ligands as molecular probes for structural investigations and structure-function relationship studies. Probing the molecular determinants of GPCR function, we designed functionally selective dopamine D2 receptor agonists that are able to differentiate between the activation of two relevant G-proteins, $G_o$ and $G_i$.

**KEY WORDS:** GPCR, molecular probe, functional selectivity.

## P1.3
## FROM MAGIC BULLET TO SCATTERGUN: IS THERE A VIABLE ALTERNATIVE?

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It is over 100 years since the Ehrlich concept of the magic bullet, but, leaving aside monoclonal antibodies,

very few drugs have achieved the selectivity and specificity that he had hoped for. In many cases multi-targeted drugs have proven advantageous. However, the complexities of drug actions and interactions in the tissues make it difficult to envisage likely responses without time-consuming experimentation and testing. Systems biological approaches may help to shorten the time and expense of drug development and assessment. The approach described here involves deconstruction of the putative drug molecule into component structures that can then be used to predict its metabolic fate in the tissues and the metabolic products that might influence its actions. Extensions also allow the possibility of predicting receptor interactions and groups on the molecule that may impede such interactions, which may then assist rational drug design. Finally, *in silico* approaches to investigate tissue and species differences in the metabolism of drugs will be outlined.

**KEY WORDS:** *in silico* drug development, drug metabolism, systems biology.

## P1.4
## HOW ENZYMES WORK? QM/MM STIMULATION OF MAO

**Janez Mavri[1,2], Matej Repič[1], Rok Borštnar[1], Miha Purg[1], Fernanda Duarte[3], S.C. Lynn Kamerlin[3] and Robert Vianello[4]**
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Understanding of biological processes at the molecular level is one of the greatest challenges in biomedical research and the key to understanding how biomolecules, biomolecular systems, cells, and ultimately, living organisms function. Molecular dynamics simulations of hydrated enzymes provide rate constants for enzymatic reactions.

In this talk I will give an overview of this simulation of hydrated enzymes. The choice of initial state, effectively polarized vs. polarizable force fields, proper treatment of long-range electrostatics, protonation states of ionizable residues and associated pKa values, inclusion of explicit water molecules and necessity for hierarchical treatment of enzymes will be discussed. We will touch the ideas behind treatment of chemically reactive systems using QM/MM approach and quantization of the nuclear motion allowing for treatment of tunneling. As a case study I will use monoamine oxidase B (MAO B), an enzyme that catalytically decomposes dopamine and to a lesser extent serotonin. For this enzyme we suggested the mechanism that is consistent with all available experimental data and we performed a series of biomolecular simulations.

**KEY WORDS:** Biomolecular simulation, hydrated enzymes, electrostatics, QM/MM, MAO B, enzyme, dopamine, serotonin.

## P1.5
## NON INVASIVE ANALYSIS OF BRAIN PENETRATION OF CHEMICALS: CONCOMITANT NEAR-INFRARED SPECTROSCOPY [NIRS] AND PHARMACODYNAMIC [PK/PD] STUDY

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Near-infrared spectroscopy (NIRS) selectively monitors non-invasively the absorption spectra of the oxygenation - deoxygenation states of haemoglobin ($HbO_2$/Hb, respectively). These measurements and the total haemoglobin concentration ($HbO_2$ + Hb) considered as total blood volume are indicative of the state of vascular activity, the level of oxygen saturation, and therefore the state of the metabolism in the living tissue. This study proposes that changes in brain metabolism measured by NIRS are a useful index of brain penetration and therefore brain activity of chemical entities. Compounds from different chemical classes were selected on the basis of their known brain penetration and pharmacokinetic profile. In particular, two NK1-SSRI receptor antagonists (GSK135... and GSK189...) having similar molecular characteristics and two glycine-1 transporter inhibitors (GSK270... and GSK267...) were chosen based on in vitro high or low rat brain penetration (B/B) ratio, respectively. It appears that treatment with GSK135 (B/B ratio: 2.70:1) modifies the NIRS parameters while GSK189 (B/B ratio: 0.22:1) does not significantly alter $HbO_2$ - Hb levels when comparing to vehicle treated rats. Similar results are obtained using GSK270 or GSK267 (brain concentration 1hr post treatment: 388 or 13ng/g, respectively).

These results indicate a direct relationship between brain penetration (and possibly efficacy) of drugs and brain metabolism. Thus, they support that *in vivo* non-invasive NIRS contributes to assess brain penetration of chemicals, i.e. significant changes in

NIRS parameters could be related to brain exposure, or vice versa the lack of significant changes in NIRS $HbO_2$/Hb could be indicative of low brain exposure and indeed low efficacy.

**KEY WORDS:** *in vivo* non-invasive NIRS, HbO2/Hb, rat brain, blood brain barrier.

## P1.6
## BIOACTIVITY PARAMETERS OF INDOLE-TYPE COMPOUNDS AND THEIR RELEVANCE TO TREATMENT OF NEUROLOGICAL DISEASES

**Magdalena Majekova[1], Milan Stefek[1], Marta Soltesova Prnova[1], Ivana Milackova[1], Jana Ballekova[1], Zdenka Gasparova[1], Pavol Janega[2] and Pavol Majek[3]**

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Compounds with indolic moiety are known for their manifold potential in biological activities. In the perspective of an intervention against neurological diseases several activities are coming into focus, as the ability to prevent oxidation stress, the preservation of the monoamine neurotransmitter signal (e.g. by the inhibition of MAO enzymes), the anti-inflammation properties, etc. The summary of our recent knowledge in this field is the goal of the presentation.

The hexahydropyridoindoles derived from their parent structure stobadine ((-)-cis-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3-b]indole) exhibited neuroprotective properties manifested in hypoxia-reoxygenation treated brain tissues and slices (in vitro) and ischemia/reperfusion brain (in vivo). From the compounds studied, the derivatives with R8 methoxy substitution exceeded others in the antioxidant and neuroprotective activities. Studies in vivo established anxiolytic effect for the methoxy derivative SMe1EC2 (±)-8-methoxy-1,3,4,4a,5,9b-hexahydro-pyrido[4,3-b]indole-2-carboxylic acid ethyl ester. The derivative SMe1EC2 was found to protect the hippocampus of rats exposed to trimethyltin (a model of Alzheimer-like neurodegenerative disorder) from cell death and damage. For further study, the elaborated model of MAO-B inhibition based on 2v5z complex with safinamide with YAMBER3 force field was used. The key interactions for methoxy substituted derivatives were determined.

The derivatives of 1-indole acetic acid were found to be efficient inhibitors of aldo-keto reductases (AKR). The role of the AKR enzymes in the development of neurodegenerative disorders and a possible intervention via AKR inhibition are brought for discussion.

**KEY WORDS:** Indole-type compounds, MAO-B inhibition, molecular modeling, trimethyltin, neuroprotection.

Acknowledgement: supported by COST-CM1103, VEGA 2/0067/11, VEGA 2/0048/11 and VEGA 2/0030/11.

## P2.1
## TWO- PHOTON IMAGING OF CORTICAL MICROVESSELS AND ASTROCYTIC INTERACTIONS IN LIVE MOUSE BRAIN

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In vivo imaging with two-photon microscopy is becoming an indispensable technique to investigate cellular and subcellular phenomenon in living tissues including the central nervous system. This microscopy enables us to image the dynamics of molecules, morphology, and excitability with minimal invasion to tissues and with unsurpassed spatial and temporal resolution. Two-photon microscopy provides a number of advantages that aid the study of the mechanisms underlying neurovascular coupling and cerebrovascular disease in animal models, including: (i) the resolution needed to visualize single cortical vessels and their surrounding cells; (ii) penetration depths of 250μm through a PoRTS (polished and reinforced thin skull) window and 500μm with dura-removed craniotomies, and even deeper imaging with longer excitation wavelengths; (iii) reduced photodamage and photobleaching; (iv) high-speed user-defined line scans for near-simultaneous measurement of RBC velocity, lumen diameter, and local cellular activity; (v) longitudinal imaging over several months; and (vi) the ability to image vascular dynamics deep in the cortex of awake mice. This two-photon imaging method allows extremely high spatial and temporal resolution for studying pathological mechanisms that underlie ischemic injury.

We will provide examples on how we apply these techniques to the study of local blood flow regulation and vascular pathologies such as small-scale stroke including abnormal changes in calcium cell signalling, vascular dysfunction following photothrombosis, and inflammation.

**KEY WORDS:** Two-photon microscopy, cranial window, neurovascular coupling, cerebrovascular disease, vascular dynamics, photothrombosis, calcium signalling.

## P2.2
## MODELLING SPINAL MUSCULAR ATROPHY IN DROSOPHILA: A FRUITFUL APPROACH?

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Spinal muscular atrophy (SMA) is the most common genetic killer of new-borns. The cause of this devastating neuromuscular disorder has been pinned on very low levels of the survival motor neuron (SMN) protein. SMN partners with the Gemin proteins to form a highly ordered complex. The best-characterised function of the SMN-Gemin complex involves assembly of the basic units that form the spliceosome or the chief editor of RNA messenger molecules that instruct cells how to fabricate proteins. Flies have a minimalistic complex that is amenable to genetic manipulation. We describe the phenotypes resulting from disruption of the *Drosophila* SMN complex. Our findings inform on the molecular pathway that might be negatively impacted in SMA.

**KEY WORDS:** Spinal muscular atrophy, *Drosophila*, survival motor neuron, SMN-Gemin complex, gemins, motor neuron degeneration.

## P2.3
## LIPID MEMBRANES- A NEW TARGET FOR NEURODEGENERATION

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Alzheimer's disease (AD) and Parkinson's disease (PD) are neurodegenerative disorders characterised by the misfolding of proteins into soluble prefibrillar aggregates. In our work, we have demonstrated that amyloid aggregates of recombinant amyloid-β(1-42) peptide, tau-441 and α-synuclein proteins, robustly compromised the membrane integrity of model liposomes. Interestingly, such liposome permeabilisation mimicked that of the pore-forming bacterial peptides gramicidin. Also, we screened 11 natural polyphenolic compounds, 8 synthetic N'-benzylidene-benzohydrazide compounds and black tea extract for protection against membrane damage by the amyloid aggregates. We therefore identified a select group of potent inhibitory compounds which include baicalein, morin, nordihydroguaiaretic acid and black tea extract. Since mitochondria are intimately involved in the pathophysiological cascades of both AD and PD, we further explored the interaction of soluble amyloid aggregates with mitochondrial membranes. Here, we made use of two *in vitro* model systems, namely: (i) lipid vesicles with defined membrane compositions that mimic those of mitochondrial membranes, and (ii) respiring mitochondria isolated from neuronal SH-SY5Y cells. Briefly, it was found that aggregates, but not monomers, induced a robust permeabilisation of mitochondrial-like vesicles, and triggered cytochrome c release from isolated mitochondrial organelles. Importantly, the effect on mitochondria was shown to be dependent upon cardiolipin, an anionic phospholipid unique to mitochondria and a well-known key player in mitochondrial apoptosis. Thus, we propose a generic mechanism of thrilling mitochondria in which soluble amyloid aggregates have the intrinsic capacity to permeabilise mitochondrial membranes, without the need of any other protein.

**KEY WORDS:** Alzheimer disease, Parkinson disease, amyloid aggregate, amyloid-β, tau-441, α-synuclein, mitochondria, baicalein, morin, nordihydroguaiaretic acid.

## P2.4
## IMMATURE AXONS: A NEW THERAPEUTIC TARGET FOR NEONATAL WHITE MATTER ISCHAEMIA?

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Brain injury in the premature infant, especially in very low birth weight infants, is a problem of major importance in our society. Recent advances in neonatal intensive care have dramatically increased the survival rate of such infants. Premature infants are at a great risk of developing cerebral palsy together with cognitive, attentional, behavioural, and socialization deficits that significantly impair their quality of life. Cerebral white matter injury is increasingly recognised as a common form of perinatal brain injury that predisposes to such neurological defects. Extensive studies point to the premyelinating oligodendrocyte to be the key

cellular target involved in neonatal cerebral white matter injury, due to a series of maturation-dependent events. However, the premyelinating oligodendrocyte must not be regarded as the sole target.

By imaging GFP-M expression in neonatal mice optic nerves, we found highly selective injury to ischaemia of the small-diameter fluorescent axons that corresponded to the larger pre-myelinated axons. These axons, after having initiated diameter expansion and expression of functional voltage-gated calcium channels, are exquisitely sensitive to ischemic injury. Moreover, pharmacological treatment with a combination of glutamate receptor blockers and voltage-gated calcium channel blockers offered a high degree of protection following an ischaemic insult. This elevated susceptibility of early maturing axons to ischemic injury may significantly contribute to selective white matter pathology and places these axons alongside pre-oligodendrocytes, previously regarded as the most ischemia-sensitive element within immature white matter. Therefore, future therapeutic strategies must include protection to both of these white matter elements.

**KEY WORDS:** Perinatal brain injury, white matter ischaemia, large pre-myelinated axons, optic nerve, voltage-gated calcium channels.

## P2.5
## THE NEED FOR RELIABLE BIOMARKERS FOR MONITORING POTENTIAL TREATMENTS IN ALZHEIMERS DISEASE

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The search for predictive biomarkers for Alzheimer's disease (AD) is of high priority in neurodegenerative disease research underlined by the lack of significant progress in identifying new treatments for the past 12 years. Despite major efforts and considerable investments, the treatments approved for AD are only palliative. They include cholinesterase inhibitors (donepezil, galantamine, and rivastigmine) that act on the cholinergic deficit, and the NMDA receptor antagonist, memantine, which has neuroprotective effects. These agents are generally considered to have marginal efficacy. As it can be logically assumed that a late therapeutic intervention would be less efficient than an early one, development of biomarkers for AD both to diagnose the disease early and to follow-up its progression, remains a major challenge. Currently, it is comprised of 6 main approaches: 1) behavioral assessment, including measurement of cognitive status using various neuropsychological scales (MMSE, ADAS-Cog, etc.); 2) changes in brain structure (mainly volume of the cerebral cortex, particularly entorhinal cortex and hippocampus); 3) alterations in brain metabolism (most notably within the default mode network) by using FDG-PET; 4) measurement of β-amyloid load within the brain by PIB-PET; 5) cerebrospinal fluid (CSF) biomarker profiles (the three main CSF biomarkers of AD being β-amyloid, total tau, and phosphorylated forms of tau proteins); and 6) post-mortem confirmation of characteristic AD histopathology. In my talk I will attempt to describe new developments within each of these biomarker approaches, analyzing their pathological specificity, early diagnostic sensitivity, and correlation with AD progression. Finally, I will argue that, despite numerous publications and recommendation criteria, the predictive usefulness of these various biomarker approaches, individually or collectively, has yet to be established.

**KEY WORDS:** Alzheimer's disease, biomarkers, default mode network, DTI, fMRI, PET.

## P2.6
## 5-HT2C RECEPTORS: A G-PROTEIN COUPLED RECEPTOR INVOLVED IN OPPOSITE AND DISTRIBUTED CONTROLS IN BASAL GANGLIA

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5-$HT_{2C}$ receptors, one of the seven-transmembrane G-protein coupled receptors for serotonin (5-HT), is a potential therapeutic target of numerous diseases, such as Parkinson's disease and schizophrenia, that involve combined dysfunctions of dopamine (DA) transmission and basal ganglia, a group of subcortical structures involved in motor behaviours. 5-$HT_{2C}$ receptors, present in the whole basal ganglia, would exert tonic, phasic and constitutive controls, the latter being independent of the presence of 5-HT. Using appropriate 5-$HT_{2C}$ receptor pharmacological tools (agonists for phasic, antagonists for tonic, inverse agonists for constitutive control), we have addressed in rats the organisation of these different controls on a motor behaviour, the purposeless orofacial movements, on the expression of the proto-oncogene c-Fos, a marker of change of neuronal activity, and on the electrophysiological

responses of neurons located in the output structures of basal ganglia, namely the entopeduncular nucleus (EPN) or the substantia nigra *pars reticulata*.

Both 5-$HT_{2C}$ agonists and inverse agonists increased abnormal orofacial movements via 5-$HT_{2C}$ receptors. c-Fos imaging studies indicated that different 5-$HT_{2C}$ controls are expressed in the input structures of the basal ganglia, the striatum and the subthalamic nucleus. In addition, agonists and inverse agonists altered neuronal activity in the output structures which could be associated with the emergence of orofacial movements. 5-$HT_{2C}$ controls are influenced by the level of DA transmission. Indeed, DA neurons lesion potentiated behavioural and electrophysiological responses induced by a 5-$HT_{2C}$ agonist by acting in the EPN. The stimulation of D2 receptors enhanced oral dyskinesia and electrophysiological responses of the cortico-subthalamonigral pathway; these effects were suppressed by selective 5-$HT_{2C}$ antagonists. This work illustrates the complexity of the controls exerted by 5-$HT_{2C}$ receptors and their outcome with respect to central DA transmission. A better understanding of the controls in these regions would permit to apprehend possible treatments using $5-HT$ and/or 5-$HT_{2C}$ agents.

**KEY WORDS:** Serotonin 2C receptor, basal ganglia, dopamine, dyskinesia, parkinson's disease, entopeduncular nucleus, subthalamic nucleus, substantia nigra pars reticulata, striatum.

## P2.7
## POTASSIUM CHANNELS AS A TARGET OF CNS DISORDERS

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$K^+$ channels are critical for neuronal excitability and they are essential effectors of neurotransmitter-mediated signaling. They are distinguished by being the largest and most diverse class of ion channels, being encoded by more than 70 genes. In the past decades several types of human diseases have been associated to dysfunction of $K^+$ channels, resulting from mutations in their encoding genes. Indeed, $K^+$ channels defects underlie a number of distinct forms of epilepsies that have been named "$K^+$ *channelepsies*". Also different types of ataxias have been associated with altered $K^+$ channels function. In particular we have shown that episodic ataxia type 1 (EA1), a $K^+$ channelopathy, which manifests with short attacks of cerebellar ataxia, is caused by *loss-of-function* mutations in Kv1.1 (KCNA1) channels. The direct and indirect involvement of $K^+$ channels in a number of psychiatric disorders including autism spectrum disorders (ASDs), schizophrenia, and mental retardation has been reported. ASDs are characterized by impaired ability to properly implement environmental stimuli that are essential to achieve a state of cultural and social inter-relationships. The main features of this disease are marked impairments of verbal and non-verbal communication with restricted and repetitive behaviors. We have performed the genetic analysis of individuals affected by autism and epilepsy and identified new heterozygous point mutations in the KCNJ10 gene that encodes the inwardly-rectifying $K^+$ channel Kir4.1, expressed predominantly, but not exclusively, in astrocytes. Functionally, the mutated channels exhibited a phenotype consistent with *gain-of-function* defects. These new findings highlight the emerging role of inwardly-rectifying $K^+$ channels and astrocyte dysfunction in autism spectrum disorders associated with epilepsy.

**KEY WORDS:** Potassium channels, mutation, epilepsy, ataxia, $K^+$ channelopathy, ASD, astrocyte dysfunction, inward- rectifying $K^+$ channels.

## P2.8
## EFFECT OF ACUTE AND REPEATED NICOTINE ADMINISTRATION ON THE ELECTRICAL ACTIVITY OF THE LATERAL HABENULAR NEURONS IN RATS

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Tobacco smoking represents a well-known risk factor for health that still accounts for a high number of deaths. So far, existing smoking cessation therapies have not been proven very successful at quitting this habit and a better undrstanding of the neurobiology of tobacco dependence is still needed. Nicotine is the neuroactive compound contained in tobacco that is responsible for its rewarding and reinforcing properties by acting on the midbrain dopaminergic system. The lateral habenula (LHb) is an epithalamic structure involved in pain, stress, depression and in encoding aversive stimuli. This structure is known to indirectly

inhibit the DA system through the activation of the RMTg, a GABA-ergic area located at the back of the VTA. The RMTg receives a strong glutamatergic imput from the LHb and is activated by the systemic injection of nicotine in rats. Thus the LHb might represent a possible target for the action of nicotine. Our data shows that systemic administration of nicotine dose-dependently increases the activity of single LHb neurons recorded extracellularly in vivo in rats, particularly at high doses. Following two weeks of nicotine chronic treatment, this response is drastically decreased while after 1 day of withdrawal only low doses of nicotine are again able to significantly increase the firing activity of the LHb neurons compared to the control group. These evidences strongly suggest that the LHb might play an important role in mediating the effects of nicotine on the midbrain DA system thus participating to the mechanism of addiction to this drug.

**KEY WORDS:** Drug of addiction, extracellular recording, serotonin, dopamine.

## P2.9
## OLIGODENDROCYTE PATHOPHYSIOLOGY AND TREATMENT STRATEGIES IN ISCHEMIA

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Oligodendroglia, the myelin-forming cells of the CNS, form a functional unit with axons and play a crucial role in axonal integrity. An episode of hypoxia-ischemia causes rapid and severe damage to these particularly vulnerable cells via the overactivation of glutamate and ATP receptors (excitotoxicity), oxidative stress and mitochondrial disruption. Oligodendrocytes appear to be more vulnerable to HI than other CNS glia, and in certain brain regions and stages of development, more vulnerable than neurons, due to the possession of numerous features, which predispose them to injury. The cardinal effect of oligodendrocyte pathology is demyelination and dysmyelination, and has profound effects on axonal function, transport, structure, metabolism and survival. The oligodendrocyte is a primary ischemic target, in adult-onset stroke and especially in periventricular leukomalacia, and should therefore also be considered a primary therapeutic target. Further emphasis is required on therapeutic strategies targeting oligodendroglia, myelin and their receptors, as these have the potential to significantly attenuate white-matter injury in hypoxia-ischemia.

**KEY WORDS:** Excitotoxicity, hypoxia-ischemia, oligodendrocyte, oxidative stress, stroke.

## P2.10
## AMYLOID NEURODEGENERATION: FROM ELECTROPHYSIOLOGY TO FLIES

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Alzheimer's, Parkinson's and Motor Neuron disease are characterized by the deposition of abnormally aggregated forms of Aβ1-42, α-synuclein and TDP-43, respectively. An intriguing possibility that is being investigated, is the possibility of pore formation in mitochondrial membranes by aggregates of these proteins. Such pores can have deleterious consequences on the electrical physiology of a neuron.
Electrophysiology studies are performed using a lipid bilayer workstation, which allows detailed electrophysiological characterisation upon incubation of amyloid aggregates with mitochondrial membranes. Electrical currents at the level of a single channel are recorded, and changes in membrane permeability can be correlated to toxic channel activity. The potential of natural polyphenols and bioactive extracts to block amyloid pores will be assessed, thereby preventing disruption of neuronal ion homeostasis.
Currently there are no drugs or clear-cut pathogenic mechanisms that do more than improve the symptoms associated with these diseases. Identification of compounds that lead to a marked and consistent recovery, will be a great asset to developing new therapeutic approaches.
Drosophila models of neurodegenerative disease have been successfully used in whole-genome screens aimed at identifying genetic modifiers, which can lead to the discovery of drug targets. The disease fly models are being generated by the overexpression of the respective human transgene in the wild-type fly brain.
A graded dose of a select group of test drugs are being tested and adult flies monitored for survival and climbing ability using well-established protocols. Data will be analysed to determine whether the drug-supplemented diet markedly, and consistently ameliorates the phenotypic defects intrinsic to the disease fly models.

**KEY WORDS:** Amyloid, neurodegeneration, Drosophila, mitochondria, aggregates, drugs.

## P2.11
## POSITIVE ALLOSTERIC MODULATION OF GABA-B RECEPTORS: A NOVEL THERAPEUTIC APPROACH FOR SCHIZOPHRENIA?

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Preclinical and clinical investigations have suggested that Gamma-amino-butyric acid $(GABA)_B$ receptors may play a key role in the pathophysiology of psychiatric disorders. We previously reported that baclofen, the prototypical $GABA_B$ agonist, exerts antipsychotic-like properties in two well-validated rodent models of schizophrenia, the prepulse inhibition (PPI) deficits produced by dizocilpine (MK-801) and the genetically low PPI displayed by DBA/2 mice. However, the adverse side effects elicit by Baclofen, point to develop alternative therapeutic tools for regulating $GABA_B$ in schizophrenia.

Thus, we investigated the impact of a new allosteric enhancers of $GABA_B$, *rac-BHFF* (RAC), on the MK-801 mediated-PPI disruption in Sprague-Dawley (SD) rats and C57/BL mice, two of the most used rodent species in PPI with high baseline of PPI and susceptibility to the NMDA receptor manipulations. Furthermore, we evaluated the properties of RAC in ameliorating the naturally low PPI performance displayed by DBA/2J, in comparison with the positive control antipsychotic, clozapine. RAC did not produce any effects on PPI per se and dose-dependently counteracted the PPI impairments produced by MK-801 in both SD and C57. Notably, dissimilar to Baclofen, these effects were not accompanied with significant alterations of startle parameters. Moreover, RAC was able to restore PPI deficits in DBA/2J, akin to the atypical antipsychotic clozapine.

Our data strengthen previous evidence of $GABA_B$ receptors as an important biological target for the modulation of PPI and suggest a new potential therapeutic application in neuropsychiatric disorders related to sensorimotor gating dysfunctions, without exerting the side effects share by the putative $GABA_B$ agonists.

**KEY WORDS:** $GABA_B$, PAM, pre-pulse inhibition, sensorimotor gating, schizophrenia, NMDA receptors.

## P2.12
## EFFECT OF NEW MULTITARGET-DIRECTED LIGANDS BASED ON DONEPEZIL, PYRIDYL AND INDOLYL HYBRIDS ON MONOAMINERGIC AND CHOLINERGIC SYSTEMS: AN HPLC METABOLIC APPROACH

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The multifactorial nature of Alzheimer's disease (AD) has prompted the search for new Multitarget-Directed Ligands (MTDL) able to simultaneously bind both cholinesterases and monoamine oxidases. We have developed and assessed novel series of MTDLs based on Donepezil-Indolyl hybrids [**MBA98F1** ($IC_{50}$); AChE= 0.19μM; BuChE= 0.83μM; MAO A= 5.5nM; MAO B= 0.15μM], Donepezil-Pyridyl hybrids [**MBA115** ($IC_{50}$); AChE= 1.4nM; BuChE= 0.51μM; MAO A= 53.3μM; MAO B= 10.2μM] or α-Aminonitriles hybrids [DHP6 ($IC_{50}$); AChE= 1.8μM; BuChE= 1.6μM; MAO A= 6.2μM; MAO B= 10.2μM; with metal-chelating properties] for their potential pharmacological use in AD.

The effect of the MAO A-selective inhibitors clorgyline and the multipotent **ASS234** on the monoaminergic system, was also evaluated on human neuroblastoma SHSY-5Y and undifferentiated pC12 cell lines. High activity levels of MAO A were determined in both cell lines; this activity was fully inhibited after treating the cells with 1μM of clorgyline or **ASS234** for 24 hours.

Both inhibitors were able to modulate the levels of monoamines by HPLC after treatments. Levels of serotonin (5-HT) and 3-metoxytryptamine (3-MT) were significantly increased while those of dopamine, DOPAC, 5HIIA and homovanillic acid (HVA) decreased. The levels of noradrenaline and L-DOPA remained unaltered. These results suggest that novel multipotent inhibitors herein presented deserve further investigation for their potential pharmacological treatment of Alzheimer's disease.

**KEY WORDS:** Multipotent drugs, Fe/Cu Chelators, ChE/MAO inhibitors, monoamines, Alzheimer's disease.

## P3.1
## DESIGN, SYNTHESIS AND EVALUATION OF MODULATORS COUNTERACTING ABAD-AβINTERACTION

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Although the aetiology of AD is still unknown, the build-up of amyloid β-peptide (Aβ) is considered to play a central role in the pathogenesis of the disease. It is well established that the intracellular accumulation of Aβis associated with AD and increasing evidence suggests that mitochondria may be an important target for intracellular Aβto exert its neurotoxic effects.

Amyloid-binding alcohol dehydrogenase (ABAD) is to date the most characterized Aβ-binding intracellular protein. Direct interaction of this mitochondrial enzyme with Aβwas confirmed by many different methods. Aβbinding to ABAD triggers a series of events leading to mitochondrial dysfunction characteristic for AD. Thus this interaction may represent a novel target for treatment strategy against AD.

The benzothiazole urea analogues related to known immunosuppressant frentizole was synthesized and in vitro evaluated for its capability to inhibit interaction between ABAD and Aβ. Several prepared compounds showed ability to inhibit ABAD *in vitro*. These promising compounds are going to be further tested on living cells.

**KEY WORDS:** Mitochondria, ABAD, β-amyloid, inhibitor, benzothiazole.

## P3.2
## *In vivo* AND *in vitro* BIOLOGICAL ASSESSMENT OF ASS234, A NOVEL DONEPEZILINDOLPROPARGYLAMINE, AS A MULTIFUNCTIONAL MOLECULE WITH A POTENTIAL THERAPEUTIC PROFILE FOR ALZHEIMER'S DISEASE

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A key pathological hallmark of AD is amyloid beta (A$\beta$ aggregation and deposition. Growing evidence suggest that the neurotoxicity of these peptide is related to the formation of toxic oligomeric aggregates. Thus, a deeply investigated therapeutic strategy comes at present from blocking the formation of these species to non-toxic aggregates. Nevertheless, clinical trials evaluating anti-A$\beta$ drugs are not giving conclusive results and brain penetration of these molecules is also an important challenge to be solved. The multifactorial nature of Alzheimer's disease (AD) supports the most current innovative therapeutic approach, which proposes that single molecules acting on multiple targets might be more suitable for the treatment. Thus, molecules possessing a rich pharmacology are of great interest. In this context, we recently identified ASS234, a new multipotent drug showing an interesting inhibitory profile towards cholinesterase and monoamine oxidase enzymes, possessing also a significant anti-A$\beta$ aggregation activity. In this work, we explore more in detail its anti-A$\beta$ activity and show that ASS234 reduces A$\beta_{1-42}$ aggregation more efficiently than that of A$\beta_{1-42}$, as well as completely blocks the AChE-induced A$\beta_{1-42}$ and A$\beta_{1-42}$ aggregation. We also describe that ASS234 is able to limit the A$\beta_{1-42}$-mediated cytotoxicity, by preventing the activation of the mitochondrial pathway of apoptosis. Moreover, we demonstrate a significant ability of ASS234 to reduce oxidative stress and the finding of its capability to cross the blood-brain barrier. Overall, our results demonstrate that ASS234 is able to bind to multiple targets and suggest that it might be considered for therapeutic development against AD.

**KEY WORDS:** Alzheimer's disease therapy, amyloid cholinesterase inhibitors, multi-target directed ligand, neuroprotection, propargylamines.

## P3.3
## SEROTONERGIC RECEPTORS: THE NEW TARGETS IN THE TREATMENT OF ALZHEIMERS DISEASE

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Serotonergic neurotransmission is implicated in the modulation of many physiological (sleep, sexuality, appetite), behavioural (aggression, mood) and cognitive (learning, memory) functions, which change in aging related disorders. *In vivo* and *in vitro* evidence suggest neuroprotective and pro-cognitive effect of serotonin in Alzheimer's disease (AD). Serotonin also plays a crucial role in the development of behavioural and psychological symptoms of dementia (BPSD) which are present in up to 90% of patients with AD.

Complex functions of the serotonergic system depend on the activity and function of its receptors, classified in seven groups from 5-HT1 to 5-HT7, which differ in terms of structure, action and location. The loss of 5-HT2, 5-HT6 and pre- or post-synaptic 5-HT1A and 5-HT1B receptors were found in patients with AD. It is unclear if these changes are primary or secondary (retrograde), due to the damage of postsynaptic target neurons in regions of the nerve endings. The activation of 5-HT3 and 5-HT4 receptors enhances the acetylcholine release and could induce the pro-cognitive effect. Since preclinical studies have shown that agonists of 5-HT4 and antagonists of 5-HT1A, 5-HT3 and 5-HT6 receptors improve cognitive functions, serotonergic receptors might represent the new pharmacological target for the treatment of AD and BPSD.

**KEY WORDS:** Serotonin, receptor, Alzheimer's disease, medications.

## P3.4
## THE REVISITED MAO INHIBITION BY N-(Furan-2-ylmethyl)-N-prop-2-yn-1-amine DERIVATIVES

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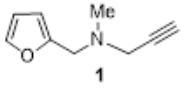

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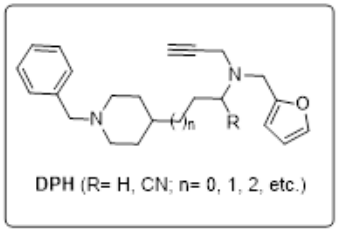


The MAO inhibition analysis of N-(furan-2-ylmethyl)-N-methylprop-2-yn-1-amine (1) has been revisited, showing that this propargylamine is a moderate, but selective, partially reversible and uncompetitive MAO B inhibitor ($IC_{50} = 5.16 \pm 0.86$ μM), whose ADMET properties predict the best profile for acting as CNS drug.

This result paves the way for the projected synthesis and biochemical analysis of new **DPH** ("Donepezil+Propagyl+Hybrid") multipotent molecules, as drugs for the potential treatment of Alzheimer's disease.

**KEY WORDS:** MAO enzymes, inhibitors, propargylamines, kinetics.

## P3.5
## SST2 AND SST3 - BUT NOT GSHR-RECEPTORS ARE INVOLVED IN THE ANTICONVULSANT EFFECTS PF CORTISTATIN-14

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Anticonvulsant and antiepileptic actions of somatostatin-14 have already widely been studied and are thus well known. For the related neuropeptide cortistatin-14 however, only one paper reports on its anticonvulsant effects. Somatostatin-14 and cortistatin-14 are structurally related peptides and have high affinities for the five somatostatin receptor subtypes (sst1-sst5). Despite these homologies, cortistatin-14 seems to act also on other receptors and it has been suggested that the ghrelin receptor (GHSR) may fulfill such a role. Here, we aim to unveil which receptors are involved in the anticonvulsant effects of cortistatin-14 by using in vivo microdialysis and telemetry-based

electrocorticography (ECoG) in rats and mice.
In rats, the involvement of sst2 and sst3 receptors was studied by administering cortistatin-14 (0.1μM - 1μM - 10μM) intrahippocampally, in the presence and absence of sst2 and sst3 receptor antagonists. Seizures were evoked by intrahippocampal pilocarpine perfusion (12mM, 40min) and seizure severity was assessed using a behavioural scoring system and ECoG. Intrahippocampal administration of 1μM and 10μM cortistatin -14 in rats showed clear anticonvulsant actions against pilocarpine-induced seizures. Furthermore, we showed that cortistatin -14 (1μM) - mediated anticonvulsant actions were reversed in the presence of 0.1μM cyanamid, a selective sst2 antagonist or 0.1μM SST3-ODN8, a selective sst3 antagonist. Intrahippocampal perfusion of these antagonists alone did not affect the pilocarpine-induced seizure severity per se.
The involvement of GHSR was tested by administering an anticonvulsant dose (1μM) of cortistatin -14 in both GHSR knock-out (KO) and wild-type (WT) mice. Seizures were evoked by intrahippocampal pilocarpine perfusion (12mM, 40min), and ECoG was used to assess seizure severity, by means of seizure duration. In these mice, both genotype - and treatment dependent alterations in seizure severity were observed by means of two-way ANOVA. Indeed, our results showed that the seizure duration in WT animals was significantly higher, when compared to their KO littermates, and that the seizure duration in the CST- treated animals was significantly lower when compared to the animals receiving only pilocarpine.
In conclusion, our results show that cortistatin -14 prevents seizures in a focal pilocarpine model and that selective sst2 or sst3 receptor antagonism abolishes these anticonvulsant actions in rats. Our findings also demonstrate that the anticonvulsant actions of cortistatin -14 in mice are not mediated via the GHSR receptor.

**KEY WORDS:** Somatostatin, cortistatin, epilepsy, seizures, pilocarpine, ghrelin.

## P3.6
## BIOISOSTERIC REPLACEMENTS FOR OPTIMIZED DOPAMINE RECEPTOR AGONISTS

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LevoDOPA and dopamine agonists have been in therapeutic use for the symptomatic treatment of Parkinson's disease for a long time. Despite the success of this medical approach, numerous unwanted side effects and an unclear receptor-crosstalk raise the need for new and improved therapeutics. Based on the early discovery of Etrabamine and established on the non-ergot dopamine agonist Pramipexole we have developed a series of tetrahydrobenzothiazole derivatives with high receptor affinity, improved receptor subtype selectivity and different efficacy profiles from agonist to antagonist properties. The 2-aminothiazole moiety of Pramipexole has generally been taken as catechol bioisosteric moiety. The replacement of the 2-amino functionality, as well as the modification of the heterocycle, led to novel classes of compounds with moderate to excellent affinity at dopamine D2 and/or D3 receptor subtypes. The synthesis has been performed by reductive amination of cyclohexa-1,4-dione monoketal, followed by deprotection and heterocyclic ring formations by different procedures. Deamination in 2-position could be performed by diazonium formation under reductive conditions.

Some of these derivatives displayed up 400fold binding preference for D3 over D2 receptors, whereas in a functional assay on $[^{35}S]GTP_{\alpha}S$ the binding the preference was less pronounced. Particular compounds showed an impressive biased signaling. The pharmacological *in vivo* profile was assessed for selected compounds in a Parkinsonian model, on 6-OHDA lesioned rats with intraperitoneal (i.p.) and *per os* (p.o.) administration, showing a good potential for further development not only for Parkinson's disease but also for erectile dysfunction.

**KEY WORDS:** Affinity, biased signaling, D2 receptor, D3 receptor, efficacy, pramipexole.

## P3.7
## SYNTHESIS, PHARMACOLOGICAL ASSESSMENT AND MOLECULAR MODELING OF ACETYLCOLINESTERASE/BUTYRYLCHOLINESTERASE INHIBORS: EFFECT AGAINST AMYLOID-BETA - INDUCED NEUROTOXICITY

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Synthesis, molecular modeling, and pharmacological analysis of phenoxyalkylamino-4-phenylnicotinates (**2-7**), phenoxyalkoxybenzylidenemalononitriles (**12-13**), pyridonepezils (**14-18**), quinolinodonepezils (**19-21**), and pyrazolo[3,4-b]quinolines (**35-37**) will be summarized in this talk. The most potent and selective EeAChE inhibitor was ethyl 6-(2-(1-benzylpiperidin-4-yl)ethylamino)-5-cyano-2-methyl-4-phenylnicotinate (**16**) [IC50 (EeAChE)= 0.0167 ± 0.0002 μM], which exhibits the same inhibitory potency as donepezil against hAChE. The most potent and selective hAChE inhibitor was ethyl 6-(4-(1-benzylpiperidin-4-yl)butylamino)-5-cyano-2-methyl-4-phenylnicotinate (**18**) [IC50 (hAChE)= 0.25 ± 0.02 μM]. Pyridonepezils showed to be selective and moderately potent against hAChE inhibition, whereas quinolinodonepezils showed to be poor hAChE inhibitors. Compounds **2, 7, 13, 17, 18, 35** and **36** significantly prevented the decrease in cell viability caused by $A\beta_{1-42}$. All compounds were effective in preventing the enhancement of AChE activity induced by $A\beta_{1-42}$. Compounds **2-7** caused a significant reduction whereas pyridonepezils **16-18** also showed some activity. The pyrazolo[3,4-b]quinolines **36** and **38** also prevented the upregulation of AChE induced by $A\beta_{1-42}$. Compounds **2, 7, 12, 13, 17, 18** and **36** may act as antagonists of VSCC since they significantly prevented the $Ca^{2+}$ influx evoked by KCl depolarization. Docking studies show that compounds **16** and **18** adopted different orientations and conformations inside the active-site gorges of hAChE and hBuChE. The structural and energetic features of the **16**-AChE and **18**-AChE complexes compared to the **16**-BuChE and **18**-BuChE complexes account for a higher affinity of the ligand toward AChE. Compounds **2, 7, 17, 18** and **36** are attractive multipotent molecules acting in different key pharmacological targets. They may accomplish a potential disease-modifying role in the treatment of Alzheimer's disease.

**KEY WORDS:** Alzheimer's disease, pyridonepezils, AChE/BuChE inhibitors, Aβpeptide, neuroprotection, $Ca^{2+}$ dyshomeostasis.

## P3.8
## RECENT ADVANCES IN THE STUDY OF 5-HT7 RECEPTOR PHARMACOLOGY: FOCUS ON THE SELECTIVE AGONIST LP- 211

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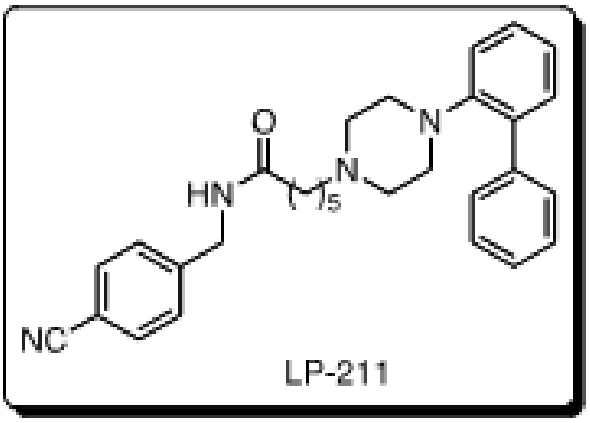
Twenty years after the 5 HT7 receptor was first cloned, there is a large amount of data available in terms of the pathophysiology of this serotonin receptor. Medicinal chemistry efforts have resulted in the identification of 5 $HT_7$ receptor selective agonists and antagonists. While 5 $HT_7$ receptor antagonists have been proposed as antidepressant drugs, the possible therapeutic applications of selective activation of 5 $HT_7$ receptor are emerging in recent years after various selective agonists became available. This lecture will illustrate the process that led to the identification of various selective 5 $HT_7$ receptor agonists in our laboratory, following structure-activity relationship studies on "long-chain" arylpiperazine derivatives. The studies culminated with the discovery of LP-211, a brain-penetrant selective 5 $HT_7$ receptor agonist.




Ki [nM].

| r5-$HT_7$ | h5-$HT_7$ | h5-$HT_{1A}$ | h5-$HT_{1B}$ |
|---|---|---|---|
| 0.58 | 15 | 379 | 215 |
| h5-$HT_{1E}$ | h5-$HT_{2A}$ | h5-$HT_{2B}$ | r5-$HT_{2C}$ |
| > 10000 | 626 | 67 | 91 |
| h5-$HT_3$ | h5-$HT_{5A}$ | h5-$HT_6$ | |
| > 10000 | 178 | 1571 | |

Recent studies conducted with LP-211, have suggested that selective activation of 5 $HT_7$ receptors may represent a novel strategy in the therapy of Fragile-X syndrome; the most common form of inherited intellectual disability and autistic spectrum disorders.

Moreover, treatment of murine striatal and cortical neuronal cultures with LP-211 significantly enhances neurite outgrowth, suggesting the involvement of 5 $HT_7$ receptor in shaping central nervous system connectivity, which may be intimately linked to psychiatric and neurodevelopmental disorders.

**KEY WORDS:** Serotonin 5-$HT_7$ receptor, arylpiperazine, central nervous system.

## P3.9
## ROLE OF HETEROOLIGOMERIZATION BETWEEN SEROTONIN RECEPTORS 5-HT1A AND 5-HT7 IN REGULATION OF RECEPTOR FUNCTIONS

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Serotonin receptors 5-$HT_{1A}$ and 5-$HT_7$ are highly co-expressed in brain regions implicated in depression. However, their functional interaction has not been established. In the present study we show that 5-$HT_{1A}$ and 5-$HT_7$ receptors form heterodimers both in vitro and in vivo. Resonance energy transfer-based assays revealed that, in addition to heterodimers, homodimers composed either by 5-$HT_{1A}$ or 5-$HT_7$ receptors together with monomers co-exist in cells. The highest affinity to form the complex was obtained for the 5-$HT_7$-5-$HT_7$ homodimers, followed by the 5-$HT_7$-5-$HT_{1A}$ heterodimers and 5-$HT_{1A}$-5-$HT_{1A}$ homodimers. Functionally, heterodimerization decreases 5-$HT_{1A}$ receptor-mediated activation of Gi-protein without affecting 5-$HT_7$ receptor-mediated signalling. Moreover, heterodimerization markedly decreases the ability of the 5-$HT_{1A}$ receptor to activate G-protein gated inwardly rectifying potassium channels in a heterologous system. The inhibitory effect on such channels was also preserved in hippocampal neurons, demonstrating a physiological relevance of heteromerization in vivo. In addition, heterodimerization is critically involved in initiation of the serotonin-mediated 5-HT1A receptor internalization and also enhances the ability of the 5-HT1A receptor to activate the mitogen-activated protein kinases. Finally, we found that production of 5-$HT_7$ receptors in hippocampus continuously decreases during postnatal development, indicating that the relative concentration of 5-$HT_{1A}$-5-$HT_7$ heterodimers and, consequently, their functional importance undergoes pronounced developmental changes.

Generally, our data suggest that the regulated and balanced ratio of homo- and heterodimerization on pre- and postsynaptic neurons may be critically involved in both, the onset as well as response to treatment of psychiatric diseases such as depression and anxiety.

**KEY WORDS:** G-protein coupled receptors, serotonin, hetero-oligomerization.

## P3.10
## PHARMACOPHORE MODELING OF NOVEL NONIMIDAZOLE HISTAMINE H3 RECEPTOR LIGANDS WITH INHIBITORY HISTAMINE N-METHYLTRANSFERASE ACTIVITY

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Dual acting compounds able to enhance histaminergic neurotransmission in the central nervous system, are a novel class of nonimidazole histamine $H_3$ receptor ($H_3R$) antagonists, that simultaneously possess strong inhibiting potency on catabolic histamine N-methyltransferase (HMT). The set of thirty-five multipotent $H_3R$/HMT ligands containing a piperidinoalkyl group, are a key structural feature for human H3 receptor ($hH_3R$) antagonism; connected by different spacer lengths to an aminoquinoline moiety, have been studied as a pharmacophoric moiety for HMT inhibiting activity, by the use of 3D-QSAR (Quantitative Structure-Activity Relationship) and pharmacophore study.

In order to better understand the crucial chemical functionalities for combined hH3R/HMT activities, 3D-QSAR pharmacophore models for hH3R antagonistic and HMT inhibiting activities were developed using Pentacle 1.06 program. Created 3D-QSAR models ($hH_3R$: $R^2$ (0.98), $Q^2$ (0.94), RMSE (0.171); and HMT: $R^2$ (0.80), $Q^2$ (0.60), RMSE (0.159)) showed different important DRY, TIP and related variables as essential 3D-pharmacophoric feature for both activities. 3D-Pharmacophoric features for $hH_3R$ antagonistic activity mainly differs from the pharmacophore for HMT inhibiting activity in presence of specific lipophilic/steric components of the $hH_3R$ pharmacophore. The H-bond accepting components of the hH3R pharmacophore, H-bond donating components of the HMT pharmacophore, and a longer optimal distance between H-bond donor and steric

hot spots were observed in the hH3R pharmacophore than in the HMT pharmacophore. Formed 3D-QSAR models were applied for design of novel piperidino-aminoquinoline hybrids, as multitarget hH3R/HMT ligands with a potential therapeutic impact in sleep-wake disorders and cognitive impairment. Designed compounds with 3D-QSAR predicted pKi($hH_3R$)> 9.6 and (pKi($hH_3R$)+$pIC_{50}$(HMT))> 16.8 were selected for further study.

**KEY WORDS:** Histamine H3 receptor, histamine N-methyltransferase, pharmacophore, QSAR, drug design.

## P3.11
## CYP-DEPENDENT METABOLISM AND VASCULAR EFFECTS OF ASS234, A NOVEL MULTITARGET DIRECTED LIGAND

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The determination of the metabolic profiles and the safety of a new drug provides information that might be used to guide further modifications of a chemical, in order to obtain favorable therapeutic properties. In this context, cytochrome P450 (CYP) plays a crucial role in metabolism and toxic action of a drug.
Several monoamine oxidase (MAO) inhibitors present a propargylamino moiety. This chemical group confers properties as irreversible inhibitors towards the MAO and could represent a potential molecular site in the formation of suicide substrates toward CYP, which could be the origin of drug-drug interactions. Furthermore, concerning the safety pharmacology, important aspects that have been highlighted are the interactions with the cardiovascular system. For these reasons the metabolic features of a new series of PF9601N derivatives, characterized by MAO and acetylcholine esterase (AChE) inhibiting properties were studied in human liver microsomes, and the vascular effects were studied in the rat aorta rings.
The compounds presented a concentration-dependent inhibition of CYP(s), however this effect resulted in a fully reversible and a competitive fashion. Furthermore the lead compound ASS234, showed an intrinsic clearance value of CLint= $1.7\mu l x min^{-1} \times mg^{-1}$ and CLint= $129.2\mu l x min^{-1} \times mg^{-1} \times mg^{-1}$ in human and rat respectively, indicating that ASS234 is a poor substrate for human CYPs.
In the vascular studies, ASS234 showed to relax phenylephrine-induced contraction at concentrations, either > 3 μM (endothelium denuded) or > 1 μM (endothelium intact rings). The vasodilating effects exhibited by ASS234, however, were at concentrations two orders of magnitude greater than those effective on AChE, and three orders greater than those effective on MAO. These preliminary *in vitro* results, suggest that ASS234 may have a safety vascular profile.

**KEY WORDS:** Multitarget compounds, metabolic stability, cytochrome P450, liver microsomes, aorta rings.

This work was realized in the framework of COST CMST Action CM1103 and working group D34/0003

## P3.12
## RECENT PROGRESS IN UNDERSTANDING THE CATALYTIC ACTIVITY OF MONOAMINE OXIDASES

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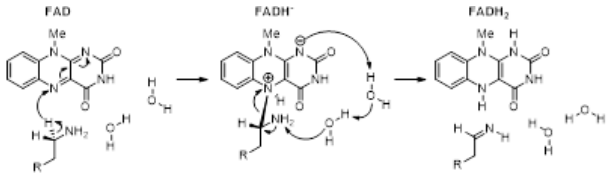
Monoamine oxidase (MAO) is a flavoenzyme responsible for regulating the level of biogenic amines in various parts of brain. Although MAO have been the central pharmacological targets in treating depression and Parkinson's disease for over 60 years, there has been no consensus in the literature about the precise molecular mechanism of its catalytic activity. On the basis of model quantum chemical calculations, we have proposed a new two-step hydride mechanism for the MAO-catalysed oxidative deamination of amines (Scheme 1). In the rate-limiting first step, the flavin N5 atom directly abstracts the hydride anion from the substrate α-carbon atom, and forms a strong covalent adduct intermediate with the thus created cationic substrate. This is subsequently followed by the deprotonation of the substrate amino group to the flavin N1 atom, facilitated with two active-site water molecules, which produces fully reduced flavin, $FADH_2$, and releases neutral imine.

This presentation discusses the significance of the mentioned flavin-substrate adduct formation, since its non-equal feasibility in both MAO isoforms has been suggested, and implied that this feature might play a crucial role in determining differences in catalytic mech-




Scheme 1. Complete two-step mechanism of MAO catalysed amine degradation.

anisms, and substrate selectivities between MAO-A and MAO-B enzymes. Also, we present some results of our preliminary all-atom QM/MM simulations within the Empirical Valence Bond theory that provide further support in favour of the hydride mechanism, particularly in the context of very recent papers by other researchers that do not necessary agree with all aspects of our mechanistic proposal.

**KEY WORDS:** Amine metabolism, computational chemistry, flavoenzymes, monoamine oxidases, neurodegenerative disorders.

## P3.13
## *In silico* DESIGN OF NOVEL AND SELECTIVE NEURONAL NITRIC OXIDE SYNTHASE (nNOS) INHIBTIORS

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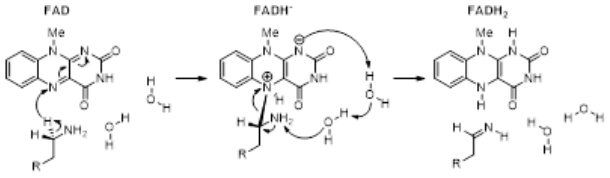
Nitric oxide gaseous free radical molecule (NO) acts as a messenger in various tissues and is responsible for different physiological functions and pathological symptoms. Nitric Oxide synthases (NOS) catalyse the oxidation of L-Arginine to a nitric oxide molecule (NO) and L-citrulline (Figure 1). Mammals contain three different NOS isozymes: Neuronal NOS (nNOS, in the brain), inducible NOS (iNOS, in macrophage cells), endothelial NOS (eNOS, the inner walls of blood vessels). Indeed, NO is a free radical gaseous molecule under normal conditions that is a highly toxic substance to our cells. In our body, it is produced locally at proper concentration and proper time. In endothelial cells, it relaxes smooth muscle causing a decreased blood pressure. Macrophage cells generate NO as an immune defence system to destroy microorganisms and pathogens.

In our brain, after a certain age and under certain pathological conditions, excessive NO is produced, causing tissue damage and oxidative stress. It also reacts with other free radicals to create specific molecular modifications. The overproduction of NO, especially by nNOS (in brain) is implicated in various diseases states such as neurodegeneration, stroke, migraine and chronic headache, Parkinson, Alzheimer, and Huntington diseases, tissue damage, hypotensive crises during septic shock, colitis, arthritis, and various kinds of inflammatory diseases. For this reason, it is important to inhibit nNOS selectively in the brain. Three isozymes show extraordinarily structural similarities hindering the selective inhibitor design. In previous literature there are many outstanding studies, however there has not yet been any drug developed which accomplishes the required affinity and selectivity. In this research, computer-modelling studies were used based on the known crystal structure of three NOS isozymes. The selected scaffolds were used hoping to increase both selectivity and potency toward the nNOS enzyme. Several hundred compounds were screened in *silico* for prioritization of lead candidates. *De novo* design method was used for the modifications of and additions to selected scaffolds within a target-binding site in order to enhance its binding affinity and selectivity to that isozyme. The best candidates showing high activity and selectivity against nNOS over eNOS and iNOS isoforms were determined.




**KEY WORDS:** Nitric oxide synthase, in silico design, selective nNOS inhibitors, de novo design.

## P3.14
## NOVEL TOOLS FOR DISEASE MODIFYING ANTI-ALZHEIMER'S DRUGS; hChEs AND b-AMYLOID INHIBITORS

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Conformational flexibility of AChE active site gorge has been a topic of intense research. We have proposed a thorough structural and bioinformatic analysis of the active site gorge of cholinesterases (ChEs), along with the identification of their fluctuations, which already drew the optimisation of our design strategy to discover extremely potent human Acetylcholinesterase and Butyrylcholinesterase (hAChE and hBuChE) bis-tacrine reversible inhibitors. Starting from these AChE and BuChE ligands, a set of potent multiple binding site homo- and hetero-bivalent inhibitors were designed, aiming to selectively interact with specific protein substructures on the surface of the enzymes

around the peripheral anionic site. Accordingly, functionalised linkers differentially spacing two tricyclic moieties were investigated as molecular yardsticks to probe the finest interactions with specific amino acid residues along ChEs gorge (hot spots). On these molecular supports, and aiming at identifying novel Alzheimer's modifying pharmacological tools, we have more recently developed bis-tacrines functionalized with a specific peptide moiety for interference with the hAChE surface sites which bind amyloid-beta (Aβ) and promote aggregation. These new high molecular weight compounds proved to be inhibitors of hAChE catalytic and non-catalytic functions (binding the catalytic and peripheral sites) can interfere with hAChE-induced Aβaggregation, Aβspontaneous aggregation, and with the Aβself-oligomerization process. Molecular modeling studies for these new ligands in complex with TcAChE confirmed the preliminary results obtained by X-ray and will be presented, highlighting how the bis-tacrine systems span the gorge, while the peptide moieties bulge outside the gorge in proximity of the peripheral site, thus explaining observed activity.

**KEY WORDS:** Cholinesterases, inhibitors, amyloid beta fibrils, amyloid beta oligomers, Alzheimer's disease, anti-Alzheimer's drugs.

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